Soil life under stress

Cover: M.A. Tobor- Kapłon

Printed: Febodruk BV, Enschede

ISBN: 906266267-6

Soil life under stress

Bodemleven onder stress

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 18 september 2006 des ochtends te 10.30 uur

door

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geboren op 11 juni 1976, te Tarnowskie Góry, Polen

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This thesis was accomplished with financial support from TRIAS Program (TRIpartite Approach to Soil system processes) of Delft Cluster, SKB (Foundation for development and transmission of soil knowledge) and NWO (the Netherlands Organisation for Scientific Research), as project 835.80.083.

Moim Kapłonkom

Acknowledgements

Peter de Ruiter and **Jaap Bloem**, without you this thesis would never come to existence; thank you for giving me the opportunity to work with you on this exciting project. You both had always time for me and a lot of patience, not only with all the 'the's' and 'a´s' that were lacking in my manuscripts. Peter and Jaap, you were supervisors every Ph.D. student can dream of! **Ryszard Laskowski** and **Paulina Kramarz,** many thanks for guiding me through my first steps in ecotoxicology. **Erland Bååth** you've showed me that invisible can be also very exciting, I am very grateful for it. **An Vos** you were always there ready to help me, both in lab and in my Dutch life. I do not know how I would solve the problems with ID´s, insurances, soffinummers etc without you. Tank you also for ours looooooong talks! **Ursula Scharler** for your enthusiasm while guiding me through ENA. **Meint Venninga, Paul Römkens, Lucas Bouwman** for all yours advises and help. **Janet Mol, Gertjan Reinds en Rick Wieggers,** ik wil jullie bedanken voor de gezelligheid en volhardig praten tegen mij in het nederlands. **Agnieszka Doroszuk** thanks for being great fellow-AIO and an example of excellent young scientist. **Remko Holtkamp** for superb collaboration and help in understanding complexity of ENA, I really enjoyed it! My all polish friends:
Anias (Pisiek, Pikutek, Michta, Kielak), Aneta, Agnieszka, Ula, Kamila, Tomek, Sylwia, Marysia, Marcin, Monika, Dominika and Sewer your presence here made my life much easier and more fun!
Moi najukochańsi: Mamo, Tato i Ewo, bez Was nie byłabym tym kim jestem. Dziekuję za Wasza bezgraniczną i bezinteresowną miłość. **Piorek,** without your love faith in me and support I would never succeed. Thank you for being with me! **Jagna** you have brought a new light to our life.

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Chapter 1

Overview

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Soils are of vital importance for society because they provide food, fibres, wood and pure water. The capacity of a soil to sustain these functions is called *soil quality*. Soil functioning depends strongly on microbial processes; i.e., organic matter decomposition and nutrient cycling, i.e. carbon (C), nitrogen (N), phosphorus (P) and sulphur (S) – mineralization, N_2-fixation. Any action that causes a negative alteration in these processes leads to decrease of soil quality.

Development of agriculture and industry in the last century has caused serious contamination of soils around the globe. All kinds of pollutants (stresses) have been introduced to soil along with the use of fertilisers, pesticides and sewage sludge as well as dry and wet deposition from atmosphere (Giller et al. 1998). In the Netherlands around 5% of the agricultural land is heavily contaminated by heavy metals (Römkens 2004) and in total 100 000 sites need sanitation. In the EU 16% of land (50 million hectares) requires sanitation (www.milieuloket.nl).

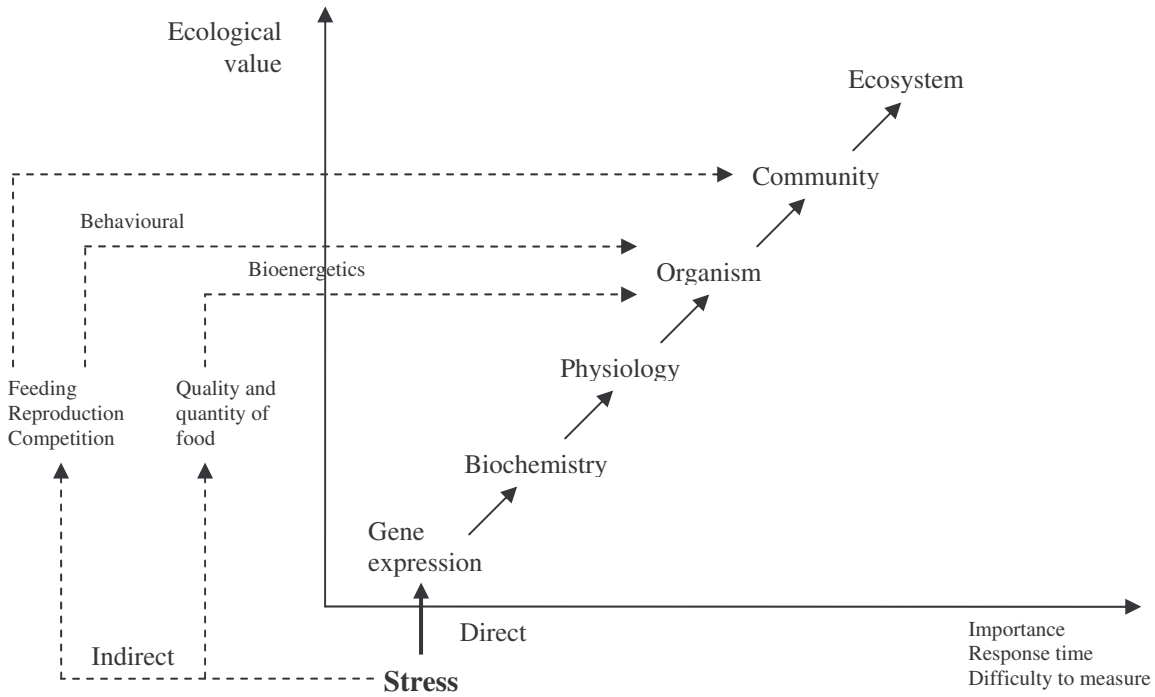

Figure 1. Direct and indirect effects of stress on ecological organization.

Environmental stress can be defined as a factor which is foreign to the system, or which is natural to the system, but applied at an extensive level (Barrett et al. 1976). Stress affects the life history functions (growth, reproduction, lifespan, survival) and behaviour of organisms and, through this, affects all levels of ecological organization, i.e. individuals, populations, communities and ecological processes that are the consequence of biological activity (Fig. 1).

In the first part of this overview I will present some theoretical background regarding the effect of stress on soil organisms, communities, processes, as well as *functional stability* (i.e. the ability of a function to withstand the stress and to return to its normal level after temporary deviation from it (Griffiths et al. 2000)). In the second part I will present and discuss the results of my research as described in this thesis.

Organisms

Stress, when applied, causes that organisms have to activate or develop mechanisms that will counteract or repair damages caused by this stress. Because these processes require energy, an organism has to change its energy allocation according to one of the possible strategies: (i) maintain its original life-span by investing in detoxification (or immobilization) and reparation of damages at the cost of reproduction or (ii) maintain its original production rate at the cost of maintenance and life-span (Fig. 2). Consequently, organisms inhabiting contaminated areas should be smaller, have less offspring or live shorter (Sibly and Calow 1989; Janczur and Kozlowski 2000).

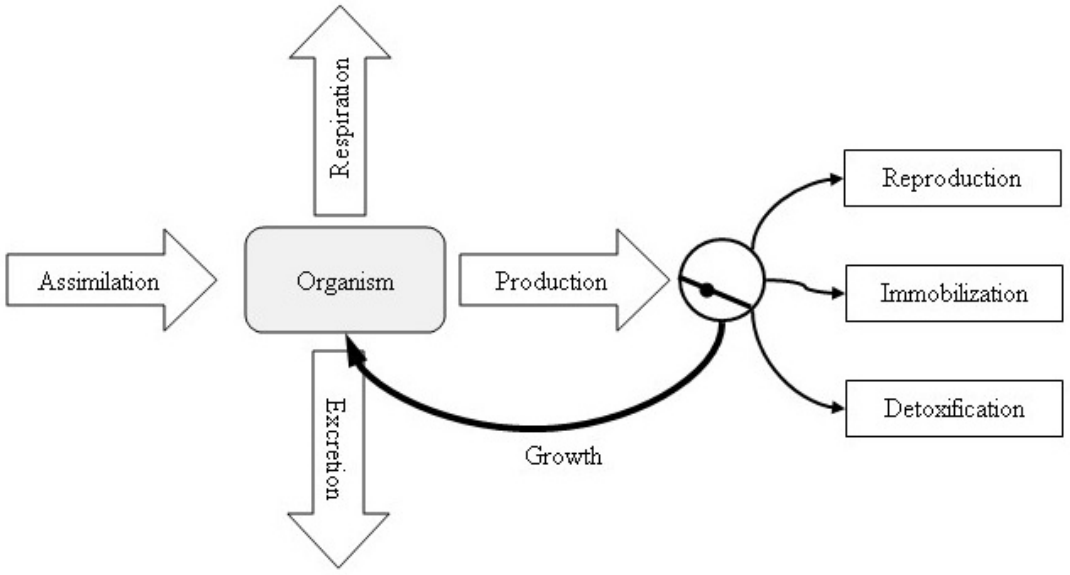

Figure 2. Energy allocation of an organism living in stressed environment (Janczur and Kozlowski 2000).

An organism can also change its behaviour, for example, by avoiding contaminated food (Hopkin 1989) or reacting differently to the presence of a predator (Lefcort et al. 1998). When a particular stress factor is present in the environment long enough, physiological and, in the long-term, genetic adaptation can occur. An example for this phenomenon are populations of the isopod *Porcellio scaber* and the springtail *Orchesella cincta* in the polluted area near a zinc smelter in Budel, the Netherlands, which were genetically adapted to heavy metal contamination (see: Posthuma and Van Straalen 1993).

Communities and processes

As species have different sensitivity, stress factors have different effects on them (Bååth 1989; Tyler et al. 1989). Sensitive species can be killed by stress and therefore the resistant species, due to lowered competition and easier access to resources, can perform better and thus become more abundant. This can lead to shift in community structure (Fig. 3) and towards a higher level of physiological adaptation or community tolerance (Frostegård et al. 1996; Pennanen et al. 1996; Bååth et al. 1998a; 1998b; Kelly et al. 1999; Macnaughton et al. 1999; Witter et al. 2000; Boivin et al. 2002; 2005)

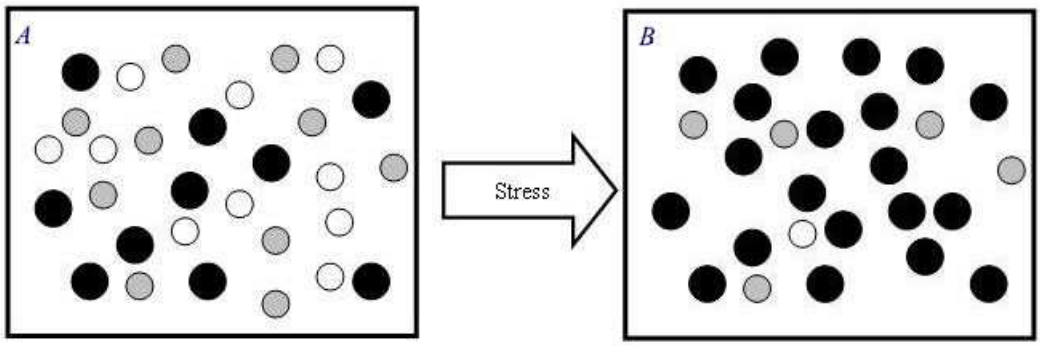

Figure 3. Community structure before (A) and after (B) application of stress. Black bullets represent insensitive species, grey bullets – intermediate sensitive species and open bullets – very sensitive species.

Such changes may cause alterations in ecological processes, in soil e.g. organic matter decomposition, respiration, nitrogen transformation and enzyme activities (Nordgren et al. 1988; Bååth 1989(review); Niklinska et al. 1998; Barajas Aceves et al. 1999). The measured response of an entire community to stress is the sum of the responses of all individuals. However, not all species respond in the same way as each has a different sensitivity to a particular stress and a different manner of managing its energy budget. Similarly like species, processes also have different sensitivity to stress. So-called `broad` processes, i.e. those carried out by many species, e.g. respiration and nitrogen mineralization, are usually not very sensitive. In contrast, `narrow` processes like nitrification and mineralization of specific compounds that are carried out by few, specialized species can be very sensitive to stress (Bååth 1989; Schimel 1995).

The best example of a ‘broad’ process is soil respiration, which appears to be only weakly affected by heavy metal concentrations at around current EU mandatory limits (Brookes 1995; Rajapaksha et al. 2004). Nevertheless, this process is often investigated in contaminated soils as it is seen as a major aspect of soil ecosystem functioning. The effects of soil pollution on respiration may depend on land use. Respiration appears to be relatively

sensitive to metal contamination in forest soils (Bååth 1989), but less so in agricultural soils (Giller et al. 1998).

A systems approach to stress (integrating effects on communities and processes)

Effects of stress on biological communities and ecological processes are inextricably related, as these processes are the direct outcome of the abundance and activity of populations of soil organisms and the way in which these populations influence each others dynamics e.g. through trophic interactions (one eats the other) and competition. Ulanowicz (1986; 1997) developed a method – Ecosystem Network Analysis (ENA) - to study the effect of stress on community structure and ecological processes in an integrated manner. The central hypothesis in his approach is, after Odum (1981), that stress inhibits and even reverses the development of the ecosystem. Therefore the effects of stress can be measured in terms of changes in the ecosystem developmental state. Development of an ecosystem, ecological succession, is a process involving structural changes in the system that are orderly, directional and therefore predictable (Odum 1969). As an ecosystem develops, the amount of energy flowing through the system increases, so does the fraction of this energy flowing through the 'organized' pathways (i.e. species to species transfers). At the same time the fraction transferred through 'unorganized' flows (i.e. respiration, import, export) and the number of parallel trophic pathways decreases. Parallel pathways are the different routes through which carbon reaches one particular species or trophic group. Parallel pathways are related to the level of specialisation; the higher the number of preys per predator, the higher the number of parallel pathways. Along with succession, the diversity of flows, and the feeding specialisation increases, and the community becomes more efficient in processing energy. According to Odum (1981) and Ulanowicz (1986), an effect of stress will cause an inhibition or reversal of these trends. ENA is an analysis that aims to quantify effects of stress in terms of changes in the size and organization of trophic and non-trophic flows of material, energy or nutrients, trophic efficiencies and overall cycling of energy or nutrients (Ulanowicz 1986; 1997).

Stability

The knowledge that stress alters soil ecosystems raises the question: how will stressed ecosystems perform in case of additional stress? Will the responses of already stressed and non-stressed ecosystems be different? In other words – are stressed ecosystems less or more stable (sensitive) to additional environmental stresses and disturbances than not-stressed ones? Stability can refer to different levels of biological organisation, populations, communities, and processes. In this thesis I looked at the stability of processes, i.e.

respiration (carbon mineralization) and microbial growth rate, rather than the stability of population or community structure.

In my approach, a process, or soil ecosystem function, is considered to be stable when it is able to withstand a stress or disturbance and to return to its normal state after temporary deviation from it (Griffiths et al. 2000; 2001a). Stability defined as such has two measurable components, i.e. resistance and resilience. Resistance is the inherent capacity of a function to withstand stress /disturbance, and resilience is the ability to recover after stress/disturbance. Up till now I used the term stress in a relatively broad sense, but in the stability analyses, I made a distinction between a stress and a disturbance. Following Degens *et al.* (2001), stress is an environmental condition (chemical, physical or biological) that has a continuous adverse impact on organisms, while disturbance is an event constrained in time, which may have a short or a lasting impact on organisms.

There are two conflicting hypotheses regarding the effects of stress and/or disturbance on stability; one predicts that ecological functions are less stable in stressed ecosystems than in not stressed ecosystems. This is because organisms living in a stressed ecosystem are burden with costs of tolerance and therefore have limited resources to cope with an additional stress (Stone et al. 2001). The other says that ecological functions are more stable in stressed ecosystems than in not stressed ecosystems, as additional stress can be handled by already developed mechanisms (e.g. through adaptation) that have been used to cope with the first stress (Odum 1981).

It is known that stress negatively affects biodiversity (Giller et al. 1998), and it has been claimed frequently that through the positive relationship between biodiversity and functional stability (Aarts and Nienhuis 1999; Yachi and Loreau 1999; Loreau 2000), stress decreases functional stability as well. Griffiths *et al.* (2000) tested the diversity-stability relationship by first reducing the soil diversity by chloroform fumigation and second applying a further stress in the form of copper, or a further disturbance as a heat shock. They showed that the less diverse soils were less stable to the copper treatment than the not fumigated diverse soils. They found, however, that the reduction in biodiversity caused by fumigation was selective and that only organisms with particular properties could survive it. To overcome this problem Griffiths *et al.* (2001b) performed a similar experiment in which the differences in biodiversity were obtained by a dilution method that was not selective. In this experiment the authors showed that functional stability did not depend on diversity.

Indicators of stress

To evaluate to what extent soil life at contaminated sites is stressed, and whether it may recover, I investigated parameters that could serve as stress indicators. Such indicators should be representative for ecosystem functioning, should respond rapidly, but should also

be able to capture long term effects of contamination. Hence, such indicators can be used to provide information about the quality of soils, as well as to monitor changes in their condition over time (Dale and Beyeler 2001). It is already known that measuring only the concentration of pollutants in not sufficient to indicate whether a given soil is in a 'good' or a 'bad' condition. Soil properties, such as clay and organic matter content and pH cause large differences in metal availability and hence toxicity (Bååth et al. 1998a). Moreover, also microbial properties and processes are affected by the soil properties, which means that these biological parameters are not directly suitable to serve as soil quality indicators (Schloter et al. 2003). A further complicating factor can be the time that passed since application of stressor (Oorts et al. 2006). Over time, heavy metals speciation can change, and so the toxicity. Also, the organisms may adapt to a present level of contamination (Posthuma and Van Straalen 1993; Kelly et al. 1999; Lagisz et al. 2002; Renella et al. 2004). Because in many cases the heavy metal pollution is "old", all the above described processes may have occurred and/or may still occur. So I especially focussed on indicators able to capture the effects of long-term contamination.

Aims and questions central in my thesis

Most cases of soil pollution can be regarded as "old", and this aspect was central in the formulation of my research questions:

1. What are the effects of long-term stress on microbial properties and processes?
2. How will stress affect the size and organization of belowground ecosystems?
3. How stable are processes in long-term stressed soils to additional stress or disturbance?
4. Which parameters can be used as indicators of environmental stress in long-term stressed soils?

The aim of this thesis is to give answers to these questions.

To do so I investigated soils from three locations. The first was the 'Bovenbuurt' - agricultural field, which has been experimentally contaminated with copper at different levels of pH (chapters 2, 4 to 6). The second was the Dutch Wadden-island of 'Schiermonnikoog', which provided different stages of soil ecosystem development (chapter 3). The third was the area near the village of Budel (Noord Brabant, NL) where the soil has been contaminated with zinc and cadmium emitted from a nearby zinc smelter (chapter 7).

Question 1. What are the effects of long-term stress on microbial properties and processes?

This question has been investigated by using the experimentally contaminated soil from the Bovenbuurt site. It was found that more than two decades after application of stress

(copper and pH) negative effects on performance (biomasses, growth rate) of microbial communities and its processes (respiration) were still detectable. Although not all examined microbial parameters revealed effects of contamination, the majority of them did (chapter 2).

Copper had a negative impact on bacterial and fungal biomass. This could result from lower efficiency of the conversion of substrate C to biomass C (Giller et al. 1998) as the organisms in contaminated soils have to invest more energy in detoxification and reparation of damages. A synergistic effect of copper and pH on fungal biomass confirms that copper acts as fungicide. I found that bacteria have bigger cells in soils with low pH than in soils with higher pH. This could result from selective grazing on bigger cells in less acid soils (Hahn and Hofle 1999), where the numbers of bacteria feeding nematodes were higher than in more acidic soils (Korthals et al. 1996). One cannot exclude the possibility that the increase of bacterial cell volume is an adaptive response to low soil pH. By increasing cell volume, bacteria reduce their surface to volume ratio and thus minimize the contact with a harmful environment (Zahran 1997; Sardinha et al. 2003).

The observed decrease of respiration rate in the Cu contaminated soils was not very strong and could have resulted either from metal toxicity, or from the effect of Cu on substrate availability (e.g. binding to organic matter) (Giller et al. 1998). Respiration is generally known to be not sensitive to contamination, as this process is carried out by many species with different sensitivity. Moreover, it can either be increased as the result of increased demand for oxygen for detoxification, or be decreased as the result of overall intoxification. Bacterial growth rate, on the other hand, is known to be very sensitive to stress (Bloem and Breure 2003). I indeed found that the bacterial growth rate was strongly reduced in soils with low pH. The higher growth rate in neutral soils may be related to more intensive grazing by bacterivorous organisms, which can enhance the bacterial growth rate if grazing causes recycling of a growth limiting nutrient (Erler et al. 2004). I did not find an effect of copper on bacterial growth rate. Bloem and Breure (2003) however, showed that the effect of copper on this process was still significant in 1995 – thirteen years after contamination and seven years before our first sampling. This suggests that leaching and ageing have reduced toxicity of copper in this field (Oorts et al. 2006).

The bacterial *specific* growth rate (growth/biomass) is a measure with a so-called "internal control" which allows comparison between soils that differ in total bacterial biomass and soil properties (e.g. organic matter content). In my study the results of specific growth rate supported the results of bacterial growth rate, i.e. both were reduced under stress. Also the bacterial growth efficiency showed a similar trend. Decreased growth efficiencies reflect changes in energy allocation (del Giorgio and Cole 1998), i.e. in stressed soils bacteria respire more carbon (because of increased energy requirement for detoxification) and therefore have less carbon and energy left for growth, whereas in non-stressed soils more

energy is available for growth and reproduction (Chander and Brookes 1991; Pollard and Greenfield 1997; Boon et al. 1998).

Question 2. How will stress affect the size and organization of belowground ecosystems?

As mentioned in the introduction, Ulanowicz (1986; 1997) claims that stress reverses the natural succession and that the effects can be identified and quantified by the Ecosystem Network Analysis (ENA) approach. Nevertheless, up-to-date the ENA information indices have been used to (1) describe a single ecosystem (Abarca-Arenas and Ulanowicz 2002), or (2) to make comparisons of the same ecosystem in two climatically different seasons (Bondavalli and Ulanowicz 1999) and (3) for theoretical ecosystems (Mageau et al. 1998; Latham II and Scully 2004)), but in none of these applications ENA dealt with a gradient of ecological succession.

In order to test whether the ENA information indices indeed can quantify the ecosystem development I calculated them for a gradient of succession on the island of Schiermonnikoog, the Netherlands (chapter 3). I found that the so-called 'absolute' indices, that describe both the size of ecosystems (the amount of carbon flowing through the ecosystem) and the fraction of total flows transferred through organized and unorganized flows, followed trends predicted by the theory (Odum 1969; Ulanowicz 1986; 1997). Nevertheless, according to ENA all soils were still categorized as being in the early phase of succession as the amount of energy transferred through unorganized flows tended to increase. I also showed that the Total System Throughput (TST) was strongly correlated with total biomass. As the other absolute indices are scaled per TST, and are overwhelmed by its contribution, they followed the same pattern. Therefore, it was difficult to judge whether observed trends indeed indicated succession or only the build-up of biomass. However, analysis of relative indices, that are independent of the influence of the TST and describe purely the organization, showed that the ENA indicators changed along with succession that occurred between 0 and 10 years, but not between10 to 100 years. So again ENA indicated that the soils were in the same (early) phase of ecological development. As there was a clear development though between 10 and 100 years, I concluded that the relative information indices were too insensitive to characterise ecosystem development. This insensitivity might have been due to the aggregation of soil organisms into functional groups.

Then I applied ENA on soils experimentally stressed by Cu and pH from the Bovenbuurt site (chapter 4), the same soils as used for the experiments described in chapter 2. The information indices showed that stresses (copper and pH) had impacts on size and organization of the soil ecosystems. The absolute indices revealed an unexpected pattern though, indicating that the more stressed the ecosystem the better it is organized and the more

carbon is flowing through it. In my opinion, this resulted from the model-assumption that stress does not affect the metabolic parameters which are used to calculate flows. This assumption had to be made because data about the effect of copper and pH on these parameters were available only for bacteria, but not for all other functional groups present in the soil food web.

The relative information indices, that are independent of system size, were found to be sensitive to soil contamination, and revealed trends expected in stressed ecosystems (Odum 1981; Ulanowicz 1996). The relative indices showed that the level of specialization of flows increased with a decrease of contamination. I found that diversity and evenness of flows did not have a linear but a quadratic (hump-backed) relationship with contamination. The hump-backed relationship is known in ecology as a response of biodiversity to stress (Connell 1978; Giller et al. 1998). I also found that the less stressed the ecosystem, the more effective it was in processing energy, i.e. the least energy was lost e.g. for detoxification.

Hence, these two ENA analyses showed that the relative information indices can be suitable indicators of stress, but appeared to be not suitable to describe ecological succession.

Question 3. How stable are processes in long-term stressed soils to additional stress or disturbance?

In this thesis the functional stability was measured by “stress (or disturbance) on stress” experimentation (Griffiths et al. 2000). In these experiments a subsequent stress/disturbance is applied on an already stressed soil. The response to the second stress/disturbance is then measured after certain time intervals to assess the resistance (immediate, relative response) and resilience (recovery rate to pre-second-stress level). For this experimental approach I used the soils from the Bovenbuurt site (chapters 5 and 6) and from the Budel area (chapter 7). I applied two kinds of stress, i.e. lead and salt, and two disturbances, i.e. heat and drying-rewetting cycles. After these secondary treatments I measured stability (resistance as well as resilience) of soil respiration, and of growth rates of bacteria and fungi.

Soil respiration and bacterial growth rate were chosen because they represent major ecological processes and population parameters, and are regularly used to show the impact of stress on soil microbial communities (Bååth 1989; Díaz-Raviña et al. 1994; Díaz-Raviña and Bååth 1996; Bååth et al. 1998a; Díaz-Raviña and Bååth 2001; Bloem and Breure 2003).

The results from experiments using the Bovenbuurt soils indicated that stability depends on the type of stress or disturbance applied (chapters 5 and 6). For example, soil respiration was more affected in stressed soils than in not stressed soils by the application of lead, but less by a heat shock. In both cases I did not observe resilience of respiration. These experiments were performed on soils from experimental fields where all parameters except

contamination were kept constant. In chapter 7, I performed stability analysis on soils from a gradient of pollution with zinc and cadmium near the smelter in Budel (NL). In this experiment, the soils varied not only in the level of metal contamination (distance from the smelter) but also in organic matter content and soil pH. In this experiment I found that the respiration in the most polluted soils was the most sensitive to all applied stresses and disturbance; and respiration was resilient in all investigated soils. On the other hand I found that bacterial growth rate was the least stable to heat in the cleanest soils shown by a higher deviation from the control value. The results of this experiment confirmed those presented in chapters 5 and 6. There was no consistent pattern of responses to applied stresses and disturbance.

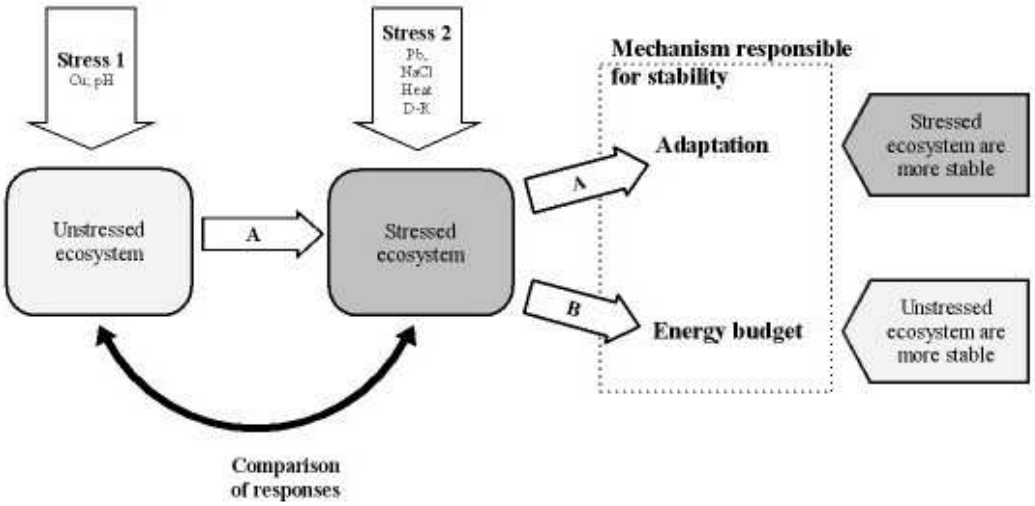

Figure 4. Mechanisms responsible for functional stability in stressed soils. Arrows with letters represent processes induced by stress (e.g. activation or development of mechanisms of detoxification, selection for resistant organisms).

The results of both experiments implied that none of the two hypotheses mentioned in the introduction holds for every situation. In my opinion the stability of soil processes depends on the type of the subsequent stress/disturbance, i.e. whether co-tolerance to the second stress was developed during exposure to the first stress (Fig. 4). If co-tolerance was developed, then more stressed soils are more stable (e.g. higher stability of respiration to heat in more contaminated soils from the Bovenbuurt) (Vinebrooke et al. 2004). In other case, if the second stress requires different detoxification mechanisms than the first stress (no co-tolerance), the processes in clean soils can be more stable as the communities responsible for them are not burden with costs of tolerance to the first stress and therefore have more energy available for detoxification or reparation of damages caused by the second stress (e.g. higher stability of respiration of clean soils to lead in both Bovenbuurt and Budel soils) (Stone et al. 2001).

Question 4. Which parameters can be used as indicators of environmental stress in long-term stressed soils?

In chapters 2 and 4, I evaluated the use of microbial parameters and ENA information indices as indicators of stress in soils. My goal was to assess whether these parameters and indices could give information about the extent to which soil ecosystems are stressed and whether they may recover. For that purpose, I looked for indicators that are

(1) capable to indicate the actual effects of the stressor,

(2) representative for ecosystem structure and functioning,

(3) rapidly responding,

(4) able to capture long-term effects of contamination,

(5) easily measurable,

(6) responding to stress in a predictable manner.

It is very difficult, if not impossible, to find an indicator that fulfils all these requirements.

From the set of microbial parameters I tested, all were relatively easy to measure, however, not all of them fulfilled the other requirements. None of the parameters appeared to be universally applicable to assess the impact of both stresses, and none of the stresses, separately, induced changes in all tested parameters. Some parameters, e.g. protozoan biomass and metabolic quotient did not reveal effects of stress at all, probably due to strong variation. Therefore, I conclude that regardless whether we deal with multiple- or single-stress situations, a set of various indicators is needed to assess the quality of stressed soils.

From the set of ENA information indices, the relative indices appeared to have the properties of a good indicator, though they are not easy to measure. To calculate them, a lot of data about flows within the system have to be gathered, which is laborious and time-consuming task. Nevertheless, the relative indices fulfilled almost all other requirements listed above and, in contrast to microbial parameters, all relative indices revealed effects of both stressors. In contrast, the absolute indices violated the requirement of responding in a predictable manner to stress, which makes them inadequate as indicators.

The set of microbial parameters and information indices I used in this study are suitable to compare the quality of soils in terms of ‘better’ or ‘worse’. They do not, however, allow to set a ‘reference’ value, above or below which soil quality can be considered as good or bad. These indices are applicable for comparative purposes, e.g. to compare soil quality along a gradient of pollution or to evaluate effects of soil management and land use (Bloem et al. 2006).

If the results of “stress on stress” experiments would have shown that functional stability in long-term stressed soils was predicted consistently by only one of the two

alternative hypotheses, then functional stability would have been a useful indicator of soil quality. However, since the requirement of responding in predictable manner was not fulfilled, I have to conclude that functional stability of `broad´ processes (i.e. respiration and growth rate) can not be used to assess soil quality.

Conclusions

In this thesis I showed that despite the stressors were applied decades ago, the effects on life in soil are still detectable. This implies that although a long time has passed, the populations and ecosystem processes have not been able to recover to their original state. I showed that the relative information indices from ENA can be used to assess the effects of stress in terrestrial belowground ecosystems. The results of my study raise new questions such as: do the observed effects on microbial parameters result from physiological or genetic adaptation? How does stress affect other ecosystem properties, such as the cycling of energy, trophic structure and efficiencies in soil ecosystems?

I also showed that processes in contaminated soils do not respond to additional stress or disturbance in predictable manner. Thus, stability analysis by “stress on stress” experiments appeared to be not a suitable tool for assessment of soil quality. The “stress on stress” experiments were carried using ‘broad’ processes that appear to be relatively insensitive to changes in biodiversity and community structure (Schimel 1995). In contrast, ‘narrow’ processes (not investigated in this thesis) that are carried out by a limited number of species may be more sensitive to such changes. It is well documented that stress causes shifts in community structure (Frostegård et al. 1996; Kelly et al. 1999; Witter et al. 2000; Chander et al. 2001; Sandaa et al. 2001) and affects biodiversity (Giller et al. 1998; Griffiths et al. 2000; Degens et al. 2001; Stoate et al. 2001). Therefore it would be interesting to examine whether the ‘narrow’ processes respond in more predictable manner to additional stress or disturbance than the ‘broad’ processes. That might also open a way to develop better soil quality indicators.

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Chapter 2

Microbial indicators of stress

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Submitted to Applied and Environmental Microbiology

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In this study long-lasting effects of contamination with copper at different levels of pH on microbial properties/ processes have been established. The aim of the study was to evaluate whether these microbial properties are useful as indicators of soil quality. We have used arable soils that have been exposed to copper at different levels of acidity since 1982. The treatments consisted of soil with no or high copper load (0 or 750 kg Cu ha^{-1}) at three pH levels (4.0, 4.7 and 6.1). This setup allowed us to assess effects of long-term contamination and overcome environmental variation (differences in soil properties) and to assess the effects purely of the level of pollution.

Copper had a negative impact on bacterial and fungal biomass and respiration. The respiration alone did not show to be a sensitive indicator of stress as it was reduced by only 10% in the Cu 750 soils. Biomass appeared to be a better indicator of stress as it was affected by almost 20%. At the same time the bacterial growth rate, specific growth rate and growth efficiency tended to increase with pH, showing that low pH has a negative impact on microbial performance. The changes in microbial biomasses might be caused not only directly by copper and acid toxicity but could also result from indirect effects of toxicants e.g. impaired grazing by microbivores. On the other hand the eco-physiological parameters, i.e. specific growth rate and growth efficiency may reveal directly the toxic effect of pollution, because they depicture the changes in channelling energy from growth to maintenance as a result of increased costs of detoxification.

Because none of the tested indicators was universally applicable for both stresses we conclude that a set of indicators is needed to assess the impact of contamination on soil quality.

***Keywords**:* soil, microbial communities, contamination, eco-physiological parameters, microbial indicators

Introduction

Throughout many years the deposition of toxic substances like heavy metals caused soil contamination in many places all over the world. For example, nowadays in the Netherlands approximately 5% of all agricultural land is heavily contaminated with heavy metals (Römkens 2004). Most contamination took place in the last few decades but some have been present already for centuries.

It is known that heavy metals can hamper organic matter decomposition (Chander and Brookes 1991a), soil respiration (Niklinska *et al.* 1998; Rajapaksha *et al.* 2004) and bacterial growth rate (Bloem and Breure 2003) which causes a decrease of soil quality in terms of soil ecosystem functioning.

To evaluate to which extent soil ecosystems are stressed and whether they may recover we need indicators capable to indicate the actual effects of the stressor. Such indicators should be representative for ecosystem functioning and could comprise for instance processes like organic matter decomposition, respiration, nitrogen mineralization, bacterial growth rate etc. Moreover, such indicators should respond rapidly but also be able to capture long term effects of contamination.

In soil ecosystems, microbes are the largest group of soil organisms in terms of biomass and species richness. They can serve as indicators of soil quality (Bloem and Breure 2003) as they play an important role in ecosystem functioning and respond rapidly to stress by adjusting their activity rates (Rajapaksha *et al.* 2004), biomass (Chander and Brookes 1991a) and community structure (Frostegård *et al.* 1996).

There are many indicators of soil quality used in monitoring systems in different countries. Some examples are given by Bloem and Breure (2003) and Bloem *et al.* (2006) e.g. basal respiration, microbial biomass, potential N-mineralization, ammonium oxidation, and bacterial growth rate and diversity. Beside these, also eco-physiological parameters like the metabolic quotient qCO_2 (i.e. respiration/biomass or specific respiration), biomass as percentage of organic matter (C_{mic}:C_{org} ratio) (Anderson 2003), bacterial specific growth rate (SGR) (Pollard and Greenfield 1997) and growth efficiency (GE) (del Giorgio and Cole 1998) are sensitive to changes of environmental conditions. Not all of them are equally sensitive, e.g. basal respiration has been proven to be far less responsive to heavy metal pollution than e.g. bacterial growth rate (Bloem and Breure 2003; Rajapaksha *et al.* 2004).

There are still major problems related to indicator systems. The first problem is that of the strong temporal and spatial variability in soil properties. Some soil properties, such as e.g. clay and organic matter content and soil pH are known to cause differences in metal availability and hence toxicity (Bååth et al. 1998; Frostegård et al. 1993b), but also to affect the microbial properties and processes directly (Schloter *et al.* 2003). This variability causes problems with defining a reference value for a given indicator which makes it difficult to

compare the quality of soils that differ in their properties. To overcome this problem, Brookes (1995) proposed the use of parameters that have an "internal control" like the metabolic quotient, since it normalises effects of different soil properties e.g. differences in organic matter content.

Another important factor is the effect of time. Over time heavy metals can be washed out deeper into the soil profile and their speciation can change so the toxicity. Also, the organisms may adapt to a present level of contamination (Kelly *et al.* 1999; Lagisz *et al.* 2002; Posthuma and Van Straalen 1993; Renella *et al.* 2004).

Because in many cases the heavy metal pollution is "old", and all above described processes could occur, the present study is designed to establish effects of long term soil contamination by heavy metals and acid. We have analysed soils from experimental fields that were contaminated two decades earlier (in 1982) with different loads of copper combined with different levels of pH in a factorial design. This setup allowed to overcome short-lasting effects of contamination and environmental variation (differences in soil properties) and to assess the effects purely of the level of pollution in combination with pH. The analyses consisted of microbial properties and processes, such as fungal and bacterial biomass, overall and specific bacterial growth rate, overall and specific respiration rate and amounts of protozoa. The primary aim of the study was to evaluate which of these microbial properties and processes can be useful in soil quality monitoring systems.

Materials and Methods

Experimental area

Soil samples were taken from an experimental arable field known as 'Bovenbuurt' or 'Wildekamp', located in Bennekom near Wageningen, the Netherlands. The soil has been artificially contaminated with copper and acid in 1982. Three-year crop rotation of potatoes, maize and oat is being practiced. Copper and pH treatments were applied in 1982 as a powdered salt of $CuSO_4$ and lime or sulfur. The copper loading was 0, 250, 500 and 750 kg Cu ha^{-1}, while pH was adjusted to values 6.1, 5.4, 4.7 and 4.0. This resulted in 16 combinations of Cu × pH that were randomly distributed in eight blocks. Each plot is 6 × 11 m (Korthals et al. 1996).

The soil is a fimic anthrosol with a texture of 3% clay, 10% silt, 87% sand, an organic carbon content of 21 g C_{org} kg^{-1} soil and a CEC of 5.6 $cmol_c$ / kg (ammonium-acetate) (Boon et al. 1998; Korthals et al. 1996).

Six treatments were chosen – combinations of two extreme copper loads: 0 and 750 kg Cu ha^{-1} with three pH levels: 4.0; 4.7 and 6.1 (further in the text we will refer to the target values). Samples were taken in 2002 and 2003. In either year samplings were performed in April, June and September.

Samples were taken from the first four blocks. From each replicate plot 15 soil cores (ø 4 cm and 0-10 cm depth) were taken and bulked. Soils were sieved (0.5 cm mesh) and prepared for further analysis.

Chemical analysis

Two extraction methods were used to estimate copper concentrations in soil. To determine soil pH and the actual bioavailable copper, extraction in 0.01M $CaCl_2$ was done. The potentially available copper fraction was measured after extraction with 0.43M HNO_3 was used (Houba et al. 1996; Novozamsky et al. 1993). Soil pH was also measured in 1 M KCl solution.

Measurement of pH and the bioavailable fraction of heavy metals were performed as follows: 10g of field-moist soil were placed into 50 ml plastic tubes. Samples were shaken (30 rev min^{-1}) with 40 ml of 0.01 M $CaCl_2$ for 48 hours. After shaking, pH was measured with a pH-meter (Φ 34 Beckman). Next, tubes with soil slurry were centrifuged at 3000 rev min^{-1} for 10 min, filtered through 0.45 µm filter (Total Organic Carbon free) and used for measuring metal concentrations on an ICP-AES (Parkin Elmer Optima 3300 DV).

For HNO_3 extraction: 4g aliquots were shaken for 4 hours with 40 ml of 0.43M HNO_3; afterwards were filtered through paper filter and analyzed for Cu on ICP-AES (Parkin Elmer Optima 3300 DV).

Dry mass of soil was measured as the loss of mass during drying in oven for 24 hours at 105^{o}C.

Microbial biomass

For microscopic measurement of microbial biomass 20 g aliquots of sieved, field moist soil were mixed with 190 ml of mineral salt solution (P&J medium, (Prescott and James 1955)) in a blender for 1 minute. Samples for bacteria and fungi enumeration (9 ml) were fixed with 1 ml of 37% formalin whereas samples for protozoa counting (9 ml) were not fixed. For bacterial and fungal counting 10 µl of suspension was applied on a glass slide prior cleaned with ethanol and soap. For both measurements slides (soil smears) were prepared in duplicate (Bloem and Vos 2004).

Bacteria were stained with 50 µl of DTAF (5-(4,6-dichlorotriazin-2-yl) aminofluorescein, 0.2 mg per ml of phosphate buffer) for 30 min in the dark on wet tissue and then rinsed three times for 20 min with phosphate buffer (pH 9.0) and one time shortly with demi-water (Bloem and Vos 2004).

Dye for fungi was prepared by dissolving 0.5 mg of Fluorescent brightener ($C_{40}H_{42}N_{12}O_{10}S_2$ Na_2, FW 960.9, Fluostain I, cat. no. F0386, Sigma Chemical Co., St Louis MD, USA) in 10 ml of demi- water and 34.8 mg of Europium Chelate (Kodak cat no.

1305515, Eastman Fine Chemicals, Rochester NY, USA) in 10 ml of 96% ethanol. Both solutions were mixed and filtered through a 0.22 µm syringe filter. For staining of fungi 50µl of staining solution was applied and then left for one hour (in the dark) on wet tissue. After that slides were rinsed three times for 5 min with 50% ethanol and one time with demi– water (Bloem and Vos 2004).

Counting of bacterial biomass was done automatically as described by Bloem et al. (1995) and the counting of fungi manually with an epifluorescence microscope. Biovolumes of bacteria were calculated from cell length and width. The amount of carbon was calculated from biovolume using a specific carbon content of $3.1 \cdot 10^{-13}$ g C μm^{-3}. The fungal biomass was calculated assuming a hyphal diameter of 2.5 µm, a cylindrical shape and a biovolume-to-carbon conversion factor of $1.3 \cdot 10^{-13}$ g C μm^{3}.

Protozoa were enumerated by the most probable number method (MPN) after Darbyshire et al. (1974). Series of 4-fold dilution in P&J medium in microtiter plates were used. *Pseudomonas fluorescens* was used as food bacterium. Presence of flagellates, ciliates and amoebas was checked after one and two weeks of incubation at 18°C.

Soil respiration rate

Carbon dioxide production was measured over a 6-week period of incubation. Results of the first week were not used to avoid short-term artefacts. 200g aliquots of sieved, field moist soil were placed into ±600 ml glass bottles and incubated in darkness at 20°C. To determine the carbon dioxide content in the head space we used a gas chromatograph (Carlo ERBA Instruments, Milan, Italy).

Bacterial growth rate

Bacterial growth rate was determined as the incorporation of [^{3}H]thymidine and [^{14}C]leucine into bacterial DNA and proteins (Bloem and Bolhuis 2006). [Methyl-^{3}H] Thymidine (925 GBq/mmol) and L-[U-^{14}C]leucine (11.5 GBq/mmol) were purchased from Amersham Ltd., Amersham, U.K. Per sample (13 ml screw cap tube) we used 1.5 µl ^{14}C leucine, 2.0 µl ^{3}H thymidine and 16.5 µl unlabelled thymidine (2.35 mg/l). This corresponds with 2 µM and 2.78 kBq ^{14}C leucine and 2 µM and 74 kBq ^{3}H thymidine per tube. Twenty g of soil and 95 ml Prescott and James's mineral salt solution (P&J medium, Prescott & James 1955) were shaken by hand in a bottle for 30 sec. After 1 min of settling, 100 µl of soil suspension was added to 20 µl labelled thymidine and leucine. After 1 h incubation the incorporation was stopped by adding 5 ml of 0.3 N NaOH, 25 mM EDTA and 0.1% SDS. Blanks were prepared by adding the extraction mixture immediately after the start of the incubation. Macromolecules (DNA and proteins) were extracted at 30°C for 18-20 h (overnight). The suspension was mixed and centrifuged for 40 min at 5000 × g. The

supernatant was aspired and cooled on ice. After 5 min 1.3 ml ice-cold 1 N HCl and 1.3 ml ice-cold 29% TCA (w/v) were added. After at least 15 min the precipitated macromolecules (DNA and proteins) were collected on a 0.2 µm pore size cellulose nitrate filter (BA 83, Schleicher & Schuell). The filters were pre-washed with 1 ml 5 mM (unlabelled) thymidine and 1 ml 5 mM (unlabelled) leucine (both ice-cold). The filters were washed 3 times with 5 ml ice-cold 5% TCA, transferred to glass scintillation vials and 1 ml 0.1 N NaOH was added. Then the filter was dissolved by adding 1 ml ethylacetate. Scintillation cocktail was added and radioactivity was counted in Beckman liquid scintillation spectrometer. The bacterial growth rate was calculated using the conversion factors from Michel and Bloem (1993).

Specific parameters

The Specific Respiration Rate (Metabolic Quotient) qCO_2 was computed as the amount of carbon respired (produced as CO_2) per unit of microbial biomass. The Specific Growth Rate (SGR) was calculated as the amount of carbon incorporated into bacterial cells per unit of bacterial biomass. The Growth Efficiency is calculated as the proportion between carbon incorporated into bacterial cells and carbon respired.

Statistical analysis

For the analyses of biological data General Linear Modelling (GLM) was used with pH and Cu as fixed factors and time as within-subject factor (repeated measurements analysis). For analysis of chemical results GLM, with full factorial design with pH and Cu as fixed factors, was used. When necessary, data were transformed to obtain normal distribution. For analysis of respiration rate, soil moisture content was used as a covariate. All analyses were performed on SPSS 11.5 for Windows statistical software (SPSS for Windows, SPSS, Chicago, IL, USA).

Results

Chemical analyses

The copper concentrations in the examined soils are given in table 1. The $CaCl_2$ extractable fraction of copper increased with copper load ($P < 0.0001$) and decreased with increasing pH ($P < 0.0001$).

When extracted with 0.43M HNO_3, copper concentration was significantly affected only by the initial copper treatment ($P < 0.0001$). Nevertheless it can be seen that concentrations tend to increase with soil pH. The pH(KCl) of the investigated soils ranged between 4.1 – 5.2. This was lower than the nominal range of pH values (table 1) due to the

buffering capacity of the soil; after application of lime or sulphur the soil pH changes towards its natural pH (4.5).

Table 1. Actual values of pH (1M KCl and 0.01M CaCl2) and copper concentrations (mean ± SEM).

Cu	pH		pH-KCl		Cu-$CaCl_2$ mg Cu kg soil^{-1}		Cu-HNO_3 mg Cu kg soil^{-1}	
kg ha^{-1}	(nominal)	N	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
0	**4**	4	4.1	0.12	0.49	0.151	27.99	5.177
	4.7	4	4.5	0.12	0.17	0.026	31.09	4.517
	6.1	4	5.1	0.20	0.07	0.025	33.61	9.551
750	**4**	4	4.1	0.10	2.40	0.223	85.69	4.424
	4.7	4	4.5	0.52	1.04	0.229	91.46	8.216
	6.1	4	5.3	0.48	0.31	0.076	100.81	10.649

Microbial biomass

In the investigated soils bacteria were, with respect to biomass, the dominating group of microorganisms (~79% of total microbial biomass); their average biomass was on the level of 36.0 ± 1.73 µg C/g soil (mean ± st error; calculated over all Cu and pH treatments). The biomass of bacteria was lower in the Cu 750 than in the Cu 0 soils (P=0.07) (Fig. 1A). The volume of bacterial cells was affected by soil pH (P = 0.03), it decreased from 0.32 μm^3 in pH 4.0 to 0.29 μm^3 in pH 6.1 soils (st error 0.007) (table 2).

The second group, with respect to biomass (~17%), were fungi with an average biomass of 7.9 ± 0.32 µg C/g soil. The interaction between copper and pH shows that in the Cu 0 soils the biomass decreases strongly with increase in pH, while in the Cu 750 soils fungal biomass is nearly the same in all pH treatments (P = 0.005) (Fig. 1B).

Protozoa were the group with the lowest average biomass (~4% of total microbial biomass): 1.65 ± 0.141 µg C/g soil. Within this group the most abundant were amoebae with an average biomass of 1.4 ± 0.14 µg C/ g soil, followed by flagellates (0.14 ± 0.009 µg C/ g soil) and ciliates (0.07 ± 0.008 µg C/ g soil). Protozoa were not significantly affected by any of the stresses (table 2).

Fungi to bacteria ratio

The fungi to bacteria ratio calculated as the proportion of biomasses was affected neither by copper nor by pH. The interaction between these two factors, however, was significant (P = 0.04) and showed in the Cu 0 soils a decrease with increasing pH, whereas in the Cu 750 soils there was an increase with increasing pH (Fig. 1C).

Table 2. Effects of copper and pH on microbial parameters (means and p-values of main effects and interaction).

		0 kg Cu ha $^{-1}$			*750 kg Cu ha $^{-1}$*			*Main effect (p-value)*		
Indicator	***Unit***	***pH 4***	***pH 4.7***	***pH 6.1***	***pH 4***	***pH 4.7***	***pH 6.1***	***Cu***	***pH***	***Interaction***
Bacteria	µg C/ g soil	44.6	35.4	37.9	35.6	31.2	31.4	0.07	0.27	0.86
Bacterial volume	μm^3	0.32	0.28	0.29	0.31	0.31	0.31	0.20	0.03	0.13
Fungi	µg C/ g soil	12.25	7.78	5.80	6.52	8.62	6.66	0.07	0.04	0.05
Fungi to bact. ratio	C/C	0.34	0.29	0.19	0.23	0.32	0.32	0.91	0.39	0.04
Protozoa	µg C/ g soil	1.52	1.54	1.28	1.51	2.70	1.36	0.11	0.18	0.49
Amoebae	µg C/ g soil	1.28	1.35	1.03	1.37	2.47	1.14	0.13	0.09	0.43
Flagellate	µg C/ g soil	0.17	0.11	0.17	0.11	0.18	0.12	0.68	0.91	0.08
Ciliate	µg C/ g soil	0.08	0.08	0.08	0.04	0.05	0.10	0.76	0.64	0.81
Respiration	µg C g^{-1} soil h^{-1}	0.10	0.11	0.12	0.10	0.10	0.10	0.04	0.40	0.41
qCO_2	µg C g^{-1} C_{mic} h^{-1}	0.003	0.005	0.004	0.004	0.004	0.005	0.26	0.08	0.89
Bact. Growth Rate (TdR)	µg C g^{-1}soil h^{-1}	0.41	0.38	0.86	0.27	0.52	0.66	0.45	0.01	0.13
Bact. Growth Rate (Leu)	µg C g^{-1}soil h^{-1}	0.62	0.54	0.78	0.58	0.74	0.70	0.89	0.06	0.35
TdR to Leu ratio	g C_{TdR} $g^{-1}C_{Leu}$	0.56	0.73	1.19	0.50	0.75	0.84	0.32	0.05	0.47
Bact. Growth Efficiency (TdR)	g C_{prod} g^{-1} C-CO_2	3.03	2.81	6.27	1.96	3.79	4.85	0.45	0.01	0.13
Bact. Specific Growth Rate (TdR)	g C_{prod} g^{-1} C_{bac} h^{-1}	0.01	0.01	0.04	0.01	0.02	0.03	0.81	0.05	0.16

Respiration rate and specific respiration rate

Analysis of covariance showed that the soil respiration varied with the soil moisture content (P = 0.02). The copper contamination reduced the soil respiration (P = 0.04) (table 2).

In contrast to the respiration rate, the respiration quotient qCO_2, i.e. respiration rate per unit of microbial biomass, was neither affected by copper treatment nor by soil pH (table 2).

Bacterial growth rate

The bacterial growth rates increased with pH in case of thymidine (P = 0.013) and leucine (P = 0.06) incorporation. The increase of thymidine incorporation with pH was stronger than that of leucine (table 2).

The ratio between thymidine and leucine incorporation was positively affected by soil pH ($P < 0.05$) (Fig. 2A).

Specific growth rate

Using conversion factors based on previous experiments (Michel and Bloem, 1993) thymidine and leucine incorporation rates can be converted to growth rates in terms of bacterial cells and biomass carbon produced per gram soil per hour. Consequently specific growth rate (SGR) in terms of gram C produced per gram bacterial biomass C per hour can be calculated. Specific growth rate increased with pH (Fig. 2B) ($P < 0.05$ for thymidine and P = 0.04 for leucine). It can be noted that both methods yielded the same estimates at neutral pH but different estimates at acid pH because thymidine incorporation was more reduced at low pH than leucine incorporation (table 2).

Bacterial Growth Efficiency

Bacterial growth efficiency (GE) based on thymidine incorporation increased with pH (P = 0.014) (Fig. 2C). The effect of copper was not significant.

Discussion

The aim of this study was to evaluate microbial properties / processes that would be indicative for long-lasting effects of soil contamination. Such indicators would help us to find out whether or not soil systems are under stress and whether they are able to recover from heavy metal pollution. This study was carried out using soils that endured contamination with copper and acid for more than two decades.

Microbial biomass and respiration

We found that copper negatively affected both bacterial and fungal biomasses, although the effects were limited (20 and 18% reduction in bacterial and fungal biomass, respectively) and marginally significant (p=0.07). This suggests that long term exposure to this metal is harmful for both groups, which is consistent with other observations (Bååth et al. 1998; Frostegård et al. 1993a; Frostegård et al. 1996). Reduction of biomasses in contaminated soils can be a consequence of lower efficiency of the conversion of substrate C to biomass C (Giller *et al.* 1998) as a result of changes in energy allocation from production (growth) to cell maintenance functions like detoxification (Killham 1985).

One should be aware that effects of copper on microbial biomass can be obscured by other factors like grazing which can be more intensive in clean soils. Korthals *et al.* (1996) found in 1992 that the biomasses of bacterial feeding nematodes as well as omnivorous nematodes were strongly reduced by increased copper load in the same soils. We did not find significantly higher numbers of protozoa at higher pH. This may be due to the relatively high variance of the MPN technique for counting protozoa.

The interaction between copper and pH shows a synergistic effect of these two factors. Fungal biomasses in soils with pH 4.7 and 6.1 were similar regardless the copper treatment, whereas in soils with lowest pH and high copper load the biomasses of fungi were lower than in copper-free soils. This indicates that in low pH soils the bioavailability and thus the toxicity of copper increases (Korthals *et al.* 1996).
It has been argued that fungi are more resistant to environmental stresses than bacteria (Chander *et al.* 2001; Cooke and Whipps 1993; Frostegård *et al.* 1996; Rajapaksha *et al.* 2004; Tobor-Kaplon *et al.* 2005). Gong *et al.* (2001) have found increasing fungi to microbial biomass ratio with decreasing pH which shows higher resistance of fungi. In the Cu 0 soils the fungi to bacteria ratio decreases with increasing pH, which confirms that fungi are more resistant to low pH than bacteria and thus are better able to compete with bacteria. In the Cu 750 soils, however, the situation is reversed; fungi are less resistant to the combined effect of copper and low pH than bacteria. Copper is known to be a fungicide; the available fraction of this metal is higher in the pH 4.0 than in the pH 6.1 soils, which could explain this pattern. Copper caused a 10% reduction in basal respiration rate in the Cu 750 soils, which suggests a toxic effect of this metal. It is difficult to judge whether this effect is a result of metal toxicity or rather the effect of metal addition on substrate availability. According to Giller *et al.* (1998) some metals may decrease the amount of substrate available for respiration through the formation of complexes and thus decrease respiration. This result confirms that respiration is not very sensitive to environmental pollution and as such is not useful as indicator of stress.

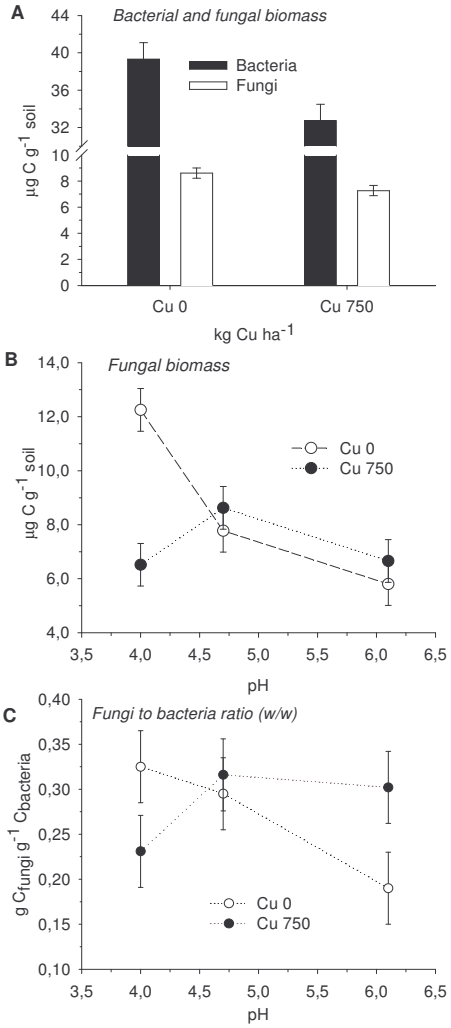

Figure 1. Effect of stress on: (A) bacterial (black bars) and fungal (white bars) biomass (mean ± SE) (average per Cu level); (B) fungal biomass – interaction between copper and pH and (C) fungi to bacteria ratio (w/w). For graph B and C the open circles represent the soils with 0 kg Cu ha^{-1} and the solid circles those with 750 kg Cu ha^{-1}.

The Specific Respiration Rate - qCO_2 (basal respiration expressed per unit of microbial biomass) is expected to increase with stress as a result of lower microbial efficiency (Anderson 2003). In this study we did not find any significant trend of specific respiration rate in relation to stressors. This result is not easy to interpret. On the one hand it may mean that this parameter could be indicative only for a short-lasting effect. On the other hand the fact that in our studies microbial biomass was assessed by direct counting, whereas in other works it was usually done by substrate induced respiration (SIR), could affect the results. SIR is based on the response of (only) the active fraction of the microbial biomass to addition of glucose, whereas direct counts may include also inactive and dead cells.

Bacterial growth rate and efficiency

The bacterial growth rate was found to be two-fold higher in the pH 6.1 than in the pH 4.0 soils. This result supports the use of growth rate as an indicator of environmental

stress as proposed by Bloem and Breure (2003). These authors found that bacterial growth rate is more sensitive to environmental stress than biomass or respiration. This is so because stress constitutes a burden for the energy budget and increases maintenance costs, resources allocated to detoxification and reparation of damages have to be withdrawn from the energy pool devoted to production (Sibly and Calow 1989). The higher growth rate in neutral soils can be also, partially, due to more intensive grazing by bacterivorous organisms which enhance the bacterial growth (Erler *et al.* 2004). The bacterial specific growth rate is a measure with a so-called "internal control" which allows comparison between soils that differ in total bacterial biomass. In our study the results of SGR support the results of bacterial growth rate. Also the bacterial growth efficiency showed a similar trend as the two former indices. Changes in the growth efficiency reflect changes in energy allocation (del Giorgio and Cole 1998). The higher values were found in the pH 6.1 soils compared to the pH 4.0 soils. This implies that in stressed soils bacteria have to invest more carbon into the maintenance (as a result of increased costs of detoxification) than in the production, whereas in non-stressed soils this is reversed (Boon et al. 1998; Chander and Brookes 1991b; Pollard and Greenfield 1997).

We found that the ratio between incorporation of thymidine and leucine increases with soil pH. Changes in this ratio, when studied in aquatic ecosystems, has been interpreted as evidence of unbalanced growth (Díaz-Raviña and Bååth 1996). In our soils, however, the stress has been present for more than twenty years and it is unlikely that unbalanced growth would occur after such a long time. Díaz-Raviña and Bååth (1996) suggest that changes in the Leu/Thy incorporation ratio gives some indication of an altered bacterial community structure. Only bacteria incorporate thymidine, but not all species are able to incorporate thymidine, whereas all bacteria incorporate leucine (Michel and Bloem 1993). Thus, our results may show increased participation of bacteria able to incorporate thymidine in more neutral soils. It is also possible that DNA synthesis, reflected by thymidine incorporation, is more sensitive to stress than protein synthesis, reflected by leucine incorporation (Bloem and Breure, 2003).

Long term effects of copper pollution

Generally, copper affected only respiration rate and biomasses of bacteria and fungi, whereas pH was found to affect more parameters. Though, copper was applied only once on the investigated fields, whereas pH was several times readjusted. The last readjustment was done in 2001 i.e. one year before the first sampling. It may be plausible that copper was washed out deeper into soil profile and thus is not so harmful anymore for organisms inhabiting the top 10 cm layer of soil. We can not exclude the possibility that present bioavailable concentrations of copper are below the toxic level for the examined groups of

organisms or that the organisms could have increased their tolerance to this metal. On the other hand, Tobor-Kapłon *et al*. (2005) showed that the copper factor still appears to affect the functional stability of the microbial populations living in these soils. They found copper-polluted soils to be less resistant and/or resilient to additional stress. Copper, undoubtedly, is a burden for the energy budget, nevertheless some of the organisms can deal with it without decreasing their functioning e.g. growth rate. Additional stress, however, will increase this burden and cause alterations in the functioning of an organism.

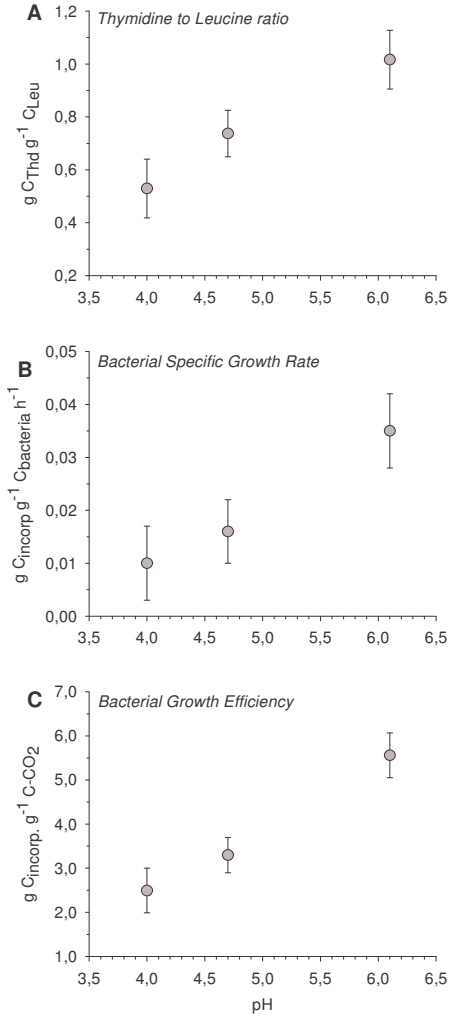

Figure 2. Effect of stress (pH) on: (A) Thymidine to Leucine incorporation ratio, (B) Bacterial Specific Growth Rate and (C) Bacterial Growth Efficiency (mean ± SE) (average per pH level).

Conclusions

From the parameters we tested none appeared to be universally applicable to assess the impact of both stresses, and none of the stresses, separately, induced changes in all tested parameters. Therefore, we conclude that regardless whether we deal with multiple- or single-

stress situations a set of various indicators is needed to assess the quality of stressed soils. The set of microbial parameters we used in this study was suitable to compare the quality of soils in terms of 'better' or 'worse'. It did not, however, allow to set a 'reference' value, above or below which soil quality can be considered as good or bad. Such a set is applicable for comparative purposes, e.g. to compare soil quality along a gradient of pollution or to evaluate effects of soil management and land use (Bloem and Bolhuis 2006)

Acknowledgements

We thank M.R. Veninga, A. Vos, P.R. Bolhuis, W. Schuurmans and I. Smirnova for technical assistance.

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Chapter 3

Information indices as a tool for quantifying development of below-ground terrestrial ecosystems

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Accepted in Ecological Modeling

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Information indices from Ecosystem Network Analyses (ENA) describe the size and organization of an ecosystem and are claimed to quantify ecosystem development [R.E. Ulanowicz, 1986, Growth and Development, Springler-Verslag, New York]. To date, these indices were not used to describe a gradient of ecosystem development in a field situation. Here we used information indices to quantify soil succession with soils of different age on the island Schiermonnikoog, the Netherlands. We evaluated whether information indices describe ecosystem development as predicted by ENA.

For the Island of Schiermonnikoog the biomasses of soil organisms and roots were measured on four stages of succession (0, 10, 25 and 100 years old fields). Organisms were grouped based on their feeding characteristics. With these data consumption, respiration, excretion, external input and output flows to, from and between groups were calculated. These flows, in turn, were used to calculate the information indices. Relative information indices describe the organization of an ecosystem; i.e. level of organisation (specialization of flows), diversity and evenness of flows and disorganisation. Absolute indices describe both size, (in terms of energy flow) and organisation of the system. System size is used to scale the absolute indices and will be analysed separately as well.

We found that the absolute indices increased when succession processed, as predicted by theory. This pattern could have been due to the build up of biomass, which apparently did not level off. Because the succession gradient deals mostly with young soils (0, 10 and 25 years old) and only one older field (100 years old), the gradient should include more soils of around 100 years old and older to exclude this possibility. Relative indices, on the other hand, increased initially, but then levelled off. We think that this was due to the strong aggregation of functional groups of especially lower trophic levels, because feeding patterning per functional groups showed (expected) trends for some groups.

Our results suggest that the absolute indices are able to describe changes in succession of terrestrial belowground ecosystems. The relative indices, in contrast, appeared to be insensitive to subtle changes in succession.

Keywords: Ecosystem Network Analysis, information indices, ecological succession, soil, Schiermonnikoog,

Introduction

Ulanowicz (1986; 1997) states that ecosystem development can be quantified with information indices from Ecosystem Network analysis (ENA). These information indices describes characteristics of ecosystem development as hypothesized by Odum (1969), for example the level of specialization, diversity and evenness of flows. Although the expected patterns of the indices are defined for ecological succession, until now they have not been used to quantify a gradient of succession.

Information indices can be divided into two groups: absolute and relative. Absolute indices describe the amount of energy flowing through the system by organized (species to species) flows (Ascendency - A), its capacity (Development Capacity – C) and fraction realized by unorganized flows (respiration, import, export; Overhead - L). These indices are scaled by the total amount of energy proceeded by the system (Total System Throughput – TST), which is known to overwhelm the contribution of the organisation of flows (Mageau et al. 1998). The relative indices describe specialization (Average Mutual Information - AMI), diversity and evenness (Flow Diversity - H) and unspecialised flows (Relative Overhead -RL) of flows (Ulanowicz 1986; Ulanowicz 1997).

The size of the system (TST) is expected to grow with ecosystem development, especially in the early stages. It is expected that the organization (A and AMI) in the system grows as system matures (Ulanowicz 1986; Ulanowicz 1997). The capacity of the system (maximum specialisation with one prey per predator; C and H) is expected to grow with system development. However, it will level off reaching maturity due to a stabilizing number of biota, meaning that the disorganisation (L and RL) will decrease with maturity (Ulanowicz 1997).

Information indices are used to describe a single ecosystem (Abarca-Arenas and Ulanowicz 2002), the same ecosystem in two climatically different seasons (Bondavalli and Ulanowicz 1999) and with different levels of nutrients (Patricio et al. 2006), different ecosystems (Christensen 1995; Dalsgaard et al. 1995; Ulanowicz 1996; Heymans et al. 2002) and theoretical ecosystems (Mageau et al. 1998; Latham II and Scully 2004). Although these studies describe changes in ecosystem properties due to different environmental conditions, none of them deal with a gradient of ecological succession.

A study of an ecological soil succession on the island of Schiermonnikoog, the Netherlands, is presented by Neutel et al. (2001) and Van de Koppel et al. (1997). Plant characteristics and biomass of soil organisms are measured on four fields with different age. Along this succession, the plant productivity increases from bare sandy soil to woody vegetation (van de Koppel et al. 1997) and food chain length and complexity also increase (Neutel et al. 2001). This indicates that this ecosystem development follows Odum's (1969) characteristics and assumptions.

Here we analysed soil succession on the island of Schiermonnikoog using information indices. We expected that information indices calculated for this gradient of development will follow the patterns as hypothesized by Ulanowicz (1986; 1997), where size and organisation of the system should increase with succession and system capacity should increase and eventually level off. We hypothesize that information indices are an adequate tool to quantify the Schiermonnikoog succession.

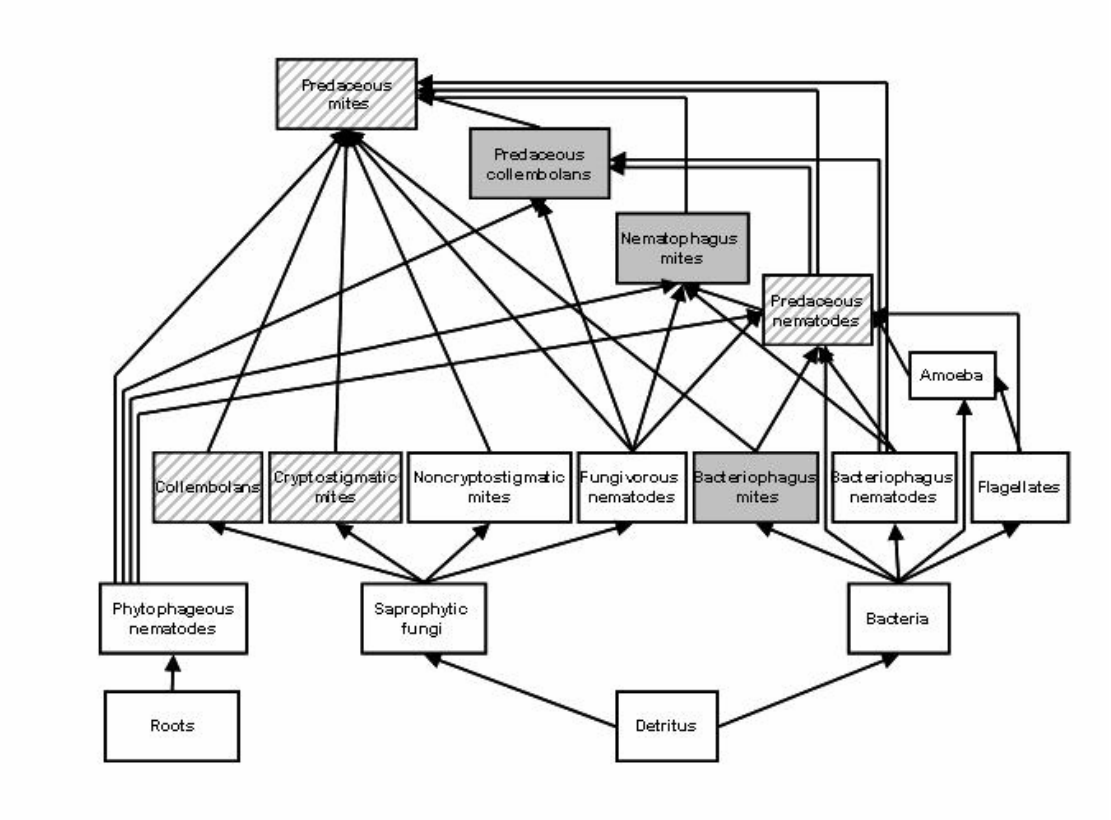

Figure 1. Food web structure of the Island Schiermonnikoog, the Netherlands. Open boxes – groups present in all soils. In the 0 years old soils roots and phytophagous nematodes were present in only one replicate. Hatched boxes –groups present in soils of 10, 25 and 100 years old. In the 25 year old soils the predaceous mites were present only in one replicate plots. Grey solid boxes – groups present in soils of 100 years.

Material and Methods

Study area and measurements

The Island of Schiermonnikoog expands gradually to the east over more than 100 years, which give the opportunity to analyse development from a bare sandy soil to a woody vegetation (van de Koppel et al. 1997). On four stages of this development, 0, 10, 25 and 100 years old fields, the soil ecosystem is analysed (van de Koppel et al. 1997; Neutel et al. 2001). Seventeen functional groups of soil organisms were distinguished if present. In the first stage on average 9 groups were present, 13.8 in the second, 14.3 in the third and 15.5 in the last stage (Neutel et al. 2001). Functional groups were grouped based on prey preference and metabolic characteristics, such as production and assimilation efficiency and natural death rate. Based on the prey preferences of the groups a food web diagram was constructed

for all stages (Figure 1). The biomass of each functional group was measured in the topsoil layer once in 1993 and two times in 1994; Here we used the average per group. Root biomass was: 0.18 (0 years old), 33.58 (10 years), 56.63 (25 years) and 107.79 (100 years; all in kg/ha/cm (depth)) (Johan van der Koppel, unpublished data).

Input data

To calculate all eight indices, for every functional group all incoming and outcoming fluxes must be known. We assumed that incoming fluxes are consumption rate, immigration and output fluxes are excretion, emigration, respiration. With the assumed steady state situation, we calculated consumption, excretion (due to assimilation inefficiency) and respiration (due to production inefficiency) with the model and model parameters from Hunt et al. (1987) and de Ruiter et al.(1993). Because this method uses a top-down approach, mineralization for the lowest trophic level, i.e. roots and detritus can not be calculated. Roots, however, have a direct contribution to respiration. Therefore, we estimated root respiration with data on carbon translocation of *Lolium perenne* (Kuzyakov 2001) and applied it to the measured root biomass in our study. Net input to detritus and roots was calculated parallel with root respiration based on translocation data of *Lolium perenne* (Kuzyakov 2001). Besides these inputs, we assumed that there was no import to (immigration) or export from (emigration) all other functional groups.

Information indices

Although it is not necessary to assume a steady state to calculate the information indices (Ulanowicz 2004), we did so for two reasons. First, the steady state assumption enabled us to estimate feeding rates between all functional groups (see paragraph Input Data). Second, the steady state assumption is a prerequisite in other network analyses, e.g. input-output analysis (Ulanowicz 1986). Therefore, the food webs were brought in steady state before information indices were calculated with the average balancing procedure as in Allesina and Bondavalli (2003). This procedure balances the networks in three steps. First, the inputs (imports) are kept constant while outputs (respirations and exports) and flows are manipulated (input-based approach). Next, the outputs are kept constant while imports and flows are manipulated (output-based approach). On the end an average coefficients are calculated using the corresponding values obtained with input-based and output-based approach.

Here we evaluated information indices as defined by Ulanowicz (1986; 1997), namely A, C, R (all in kg C bits ha^{-1} cm^{-1} (depth) y^{-1}), AMI, H, RL (all in bits) and relative ascendency (RA; dimensionless). The RA is a measure of how close the system organization

is to its optimal theoretical organization (Heymans and Baird 2000). We also evaluated the behavior of TST (kg C ha^{-1} cm^{-1} (depth) y^{-1}).

The actual organization of the system is described by AMI, a measure of specialization in the system. It includes the number of feeding links per group and the ratio of the amount transferred via these links (Ulanowicz 1986) (see the appendix for equations). The optimal organization in the system given the number of functional groups present is described by H, with optimal theoretical organization when a predator feeds from only one prey. The disorganization of the system is described by RL and is thus the difference between H and AMI. Further, RA is a measure of how tight the system is organized. The size of the system is defined as TST and represents the sum of all carbon flow in the system (consumption, excretion, input (immigration), output (emigration) and respiration). The actual size and organization of the system is described by A and is the product of AMI (organization) and TST (size). The optimal organization given the current size is described by C and, hence, is the product of H and TST. Finally, parallel to RL, L is the difference between C and A.

Organization (AMI) and size (TST) should increase along the gradient of succession (Ulanowicz 1997). With early development H will increase, but levels off when maturity is reached. The increase of H is faster than that of AMI during early development and therefore RL will increase, but when H levels of and AMI continue to increase, RL will decrease (Ulanowicz and Kay 1991; Mageau et al. 1998). These changes occur as generalists species that dominate food webs in early stages of development are replaced by efficient specialists. The absolute indices follow the same pattern and thus the pattern of A is like that of AMI, C like H and L like RL. The tightness of the system, RA, is hypothesized to increase with ecosystem development.

Sensitivity analysis

The influence of the parameters input to roots, input to detritus and root respiration on the information indices was tested by conducting a sensitivity analysis. Sensitivity (S; dimensionless) is defined as the normalized change in an information indices fractional to the normalized change in the parameter value (Haefner 1996):

$$S = \frac{\frac{Out_i - Out_o}{Out_o}}{\frac{P_i - P_o}{P_o}} \qquad \text{Equation (1)}$$

With Out_i model output with parameter choice i, Out_0 model output with original parameter value, P_i parameter choice i and P_0 original parameter value. For Example: a

sensitivity of 2 means that the increase of 100% of estimated parameter causes 200% increase in model outcome (information index).

Statistics

Multivariate ANOVA was used to test differences between the stages for all information indices. Linear regression was used to test relationships between absolute information indices and biomass. The software package SPSS version 11.5 was used for above purposes.

Results

Information indices

The total system throughput (TST) increased strongly along the gradient of succession (Fig. 2). Other absolute indices (i.e. development capacity (C), ascendency (A) and overhead (L)) showed the same trend as TST. The total system biomass increased strongly with soil age too ($P < 0.0001$) (Table 1) and there was a positive relationship between all absolute indices and system biomass ($R^2 \sim 1.0$ for all; Fig. 3). Also when the root biomass was excluded we found a positive relationship between biomass and the absolute indices (R^2 0.68-0.74).

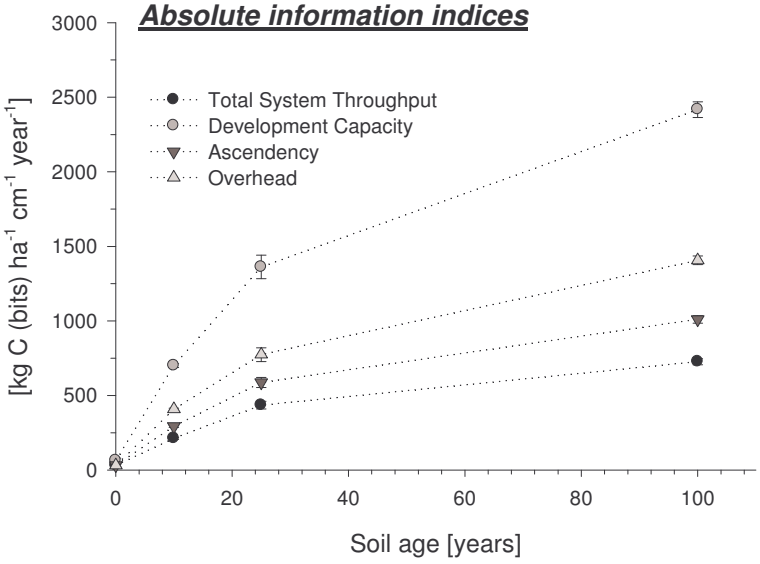

Figure 2. Changes in absolute indices along the gradient of succession (mean) on the island of Schiermonnikoog. Standard error: 17.31 (for TST), 48.77 (C), 21.87 (A) and 27.49 (L). The unit for TST is kg C $ha^{-1}cm^{-1}$ y^{-1}, whereas for other indices kg C bits $ha^{-1}cm^{-1}$ y^{-1}.

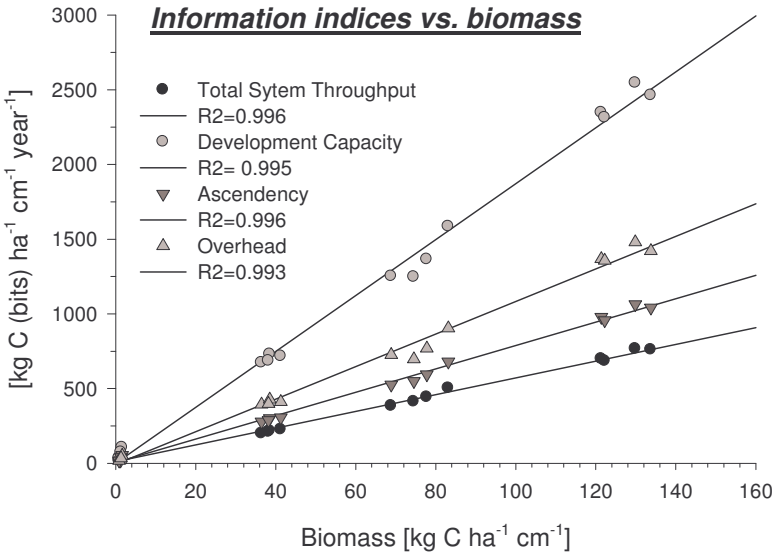

Figure 3. Relationship between absolute indices and total system biomass (without detritus) (n=4).

Relative indices, i.e. average mutual information (AMI), flow diversity (H) and relative overhead (RL), changed differently over succession than the absolute indices (Fig. 4). All of them had the lowest value in the youngest soils and did not increase from stage two to four. The relative ascendancy (RA) showed an opposite pattern as it was significantly higher in the youngest soils than in the other ones.

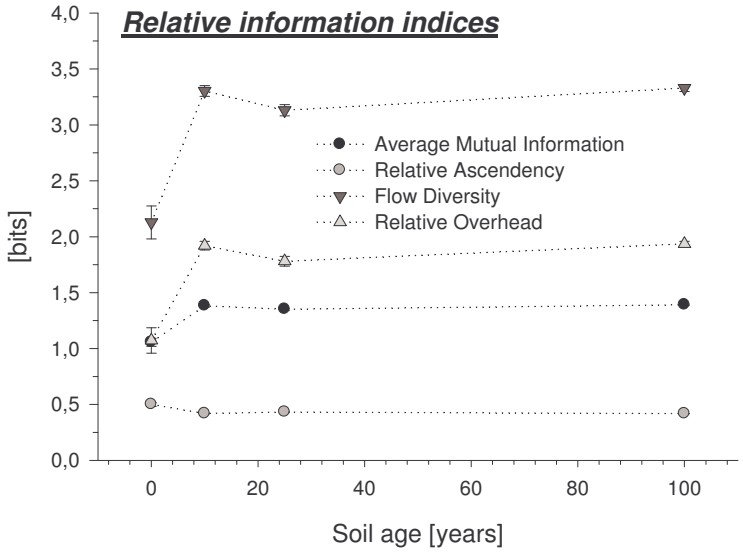

Figure 4. Changes in relative indices along the gradient of ecosystem development on the island of Schiermonnikoog (mean ± SEM). H, AMI and RA are expressed in bits of information, whereas RA is dimensionless.

Sensitivity analysis

We tested the sensitivity of investigated indices to changes in values of import to roots, import to detritus and root respiration, i.e. those input data which had to be estimated.

Generally, the absolute information indices were more sensitive to changes in estimated parameters than the relative indices (Table 2).

All absolute indices were the most sensitive to changes in the import to detritus (0.47 (TST), 0.50 (C), 0.47 (A) and 0.53 (L)) (Table 2). Sensitivity to import to roots was lower: 0.30 for TST, C, L and A. Absolute indices were the least sensitive to changes in respiration of roots (0.11 (TST), 0.08 (C), 0.13 (A) and 0.09 (L)).

The relative information indices showed different sensitivity to estimated parameters than the absolute indices. All of them were the most rigid to variation in import to roots (0.011 (for H), 0.008 (AMI) and 0.011 (RA)) and more sensitive to variation in input to detritus (0.04 (H), 0.11 (AMI) and 0.14 (RA). Improving the parameter values would thus affect the absolute indices rather than the relative indices.

Discussion

Neutel et al. (2001) and van de Koppel et al. (1997) have shown that there was an ecological succession (*sensu* Odum *(*1969)) of belowground ecosystems on the Island of Schiermonnikoog (NL) because they found a development from bare sandy soil to a woody vegetation and an increase in soil food web complexity and plant productivity. Here we analysed the succession on this island with information indices.

The absolute indices revealed that with an increase of soil age the amount of energy processed by the ecosystem increased (an increase of TST) and so did the amount of flow through organized flows (an increase of A; Fig. 2). Together with a rise of C, these factors imply that older soils are more developed than the younger ones (Ulanowicz and Kay 1991; Mageau et al. 1998). The parallel increase of L suggests that the succession was still in early (growth-) phase. As it is theorized (Ulanowicz 1997) and shown in our study, in this phase C grows faster than A and thus L has the opportunity to increase.

We found that the biomass increased with soil age and that the absolute indices are strongly correlated with biomass (Fig. 3). One can argue that this correlation means that observed trends simply depictured the build-up of system biomass rather than the succession. Yet, hypothetically, the biomass during ecosystem development should initially increase until it reaches its maximum and then should level of (Odum 1969). Our results, however, did not show that biomass had already stopped to accumulate. This implies, and therefore confirms the earlier reasoning, that, although the oldest soils are approximately 100 years old, the system was still in the phase of growth and had not yet reached the phase of maturation.

Table 1. Information indices of Schiermonnikoog gradient of ecosystem development (means and p-values).

		Soil age [years]								
Information Indices	***Unit***	***0***		***10***		***25***		***100***		***p-value***
Biomass (without detritus)	kg C ha^{-1} cm^{-1}	1.23	A*	38.59	B	76.07	C	126.81	D	<0.0001
Biomass (without detritus and roots)	kg C ha^{-1} cm^{-1}	1.19	A	5.01	A	19.44	B	19.02	B	<0.001
Diversity		9.00	A	13.75	B	14.25	B	16.00	B	<0.0001
Total System Throughput (TST)	kg C ha^{-1} cm^{-1} y^{-1}	31.30	A	212.4	B	435.2	C	726.6	D	<0.001
Development Capacity (C)	kg C bits ha^{-1} cm^{-1} y^{-1}	63.61	A	700.7	B	1361.5	C	2416.6	D	<0.001
Ascendency (A)	kg C bits ha^{-1} cm^{-1} y^{-1}	32.44	A	293.3	B	587.4	C	1010.3	D	<0.001
Overhead (L)	kg C bits ha^{-1} cm^{-1} y^{-1}	31.17	A	407.4	B	774.1	C	1406.3	D	<0.001
Average Mutual Information (AMI)	bits	1.056	A	1.382	B	1.350	B	1.391	B	<0.001
Flow diversity (H)	bits	2.128	A	3.302	B	3.130	B	3.328	B	<0.001
Relative overhead (RL)	bits	1.072	A	1.921	B	1.780	B	1.937	B	<0.001
Relative ascendency (RA)	-	0.500	A	0.419	B	0.431	B	0.418	B	<0.001

* values with different letters are significantly different

Table 2. Sensitivity of information indices to changes in the estimated parameters. In brackets the values excluding replicates without roots (three replicates of first stage) are reported. TST - total system throughput, C – development capacity, A – ascendancy, L – overhead, H – flow diversity, AMI – average mutual information and RA – relative ascendency.

		TST		DC		A		L		H		AMI		RA	
IR	minimum	0.000	(-0,181)	0.000	(0,190)	0.000	(0,192)	0.000	(0,188)	0.000		-0.006		-0.008	
IR	mean	0.257	(0,316)	0.259	(0,319)	0.255	(0,314)	0.262	(0,323)	0.002	(0,003)	-0.002	(-0,003)	-0.004	(-0,005)
IR	maximum	0.335		0.335		0.329		0.340		0.008		0.010		0.002	
ID	minimum	-0.126	(0,180)	-0.146	(0,156)	-0.014	(0,163)	-0.279	(0,150)	-0.041	(-0,023)	-0.016		-0.036	
ID	mean	0.356	(0,433)	0.370	(0,457)	0.373	(0,430)	0.358	(0,477)	0.014	(0,024)	0.017	(-0,004)	0.003	(-0,028)
ID	maximum	0.467		0.504		0.468		0.529		0.038		0.113	(0,004)	0.142	(0,007)
RR	minimum	0.000	(0,060)	0.000	(0,052)	0.000	(0,060)	0.000	(0,003)	-0.058		0.000		-0.015	
RR	mean	0.085	(0,105)	0.050	(0,062)	0.095	(0,117)	0.018	(0,021)	-0.035	(-0,043)	0.010	(0,012)	0.044	(0,055)
RR	maximum	0.111		0.076		0.128		0.088		0.016		0.020		0.068	

IR - import to roots, ID - import to detritus, RR - root respiration

Because here we have mostly young soils (0, 10 and 25 years old), it could be speculated that if we would have had data of fields close to 100 years old and older we could have shown the expected slow-down of biomass accumulation and, as a consequence, a weaker correlation of indices with biomass.

It is known that in absolute indices the size component (TST) dominates the contributions of the organization component (i.e. AMI in A and H in C) (Mageau et al. 1998). Therefore, a study of changes in the organization of the Schiermonnikoog ecosystem was done by analysing the relative indices. The relative indices, except for RA, increased from the first to the second stage and then levelled of (Fig. 4). According to theory the AMI and H should increase with ecological succession as their absolute analogues (i.e. A and C) and the relative overhead (RL) should follow the same pattern as L (Costanza and Gottlieb 1998). We found that a change in system organization (ecological succession) only occurred between stage one and two. This implies that the ecosystems in soils II, III and IV are in a similar phase of succession, although the differed in number of groups and complexity (Neutel et al. 2001). Though RA showed an opposite pattern than the other indices they are not contradictive. The RA is a dimensionless ratio that excludes the influence of TST. It is therefore a suitable index for comparing ecosystems of different size. In our case, the opposite trend of RA shows that AMI does not increase to the same extent as H during succession, and therefore reiterates the pattern of AMI and H shown in Fig. 4, and that of A and C shown in Fig. 2. Theory says that in the first phase of succession (growth phase) C should increase more rapidly than A (Ulanowicz 1997), so in this phase the values of RA should rather decrease than increase (Mageau et al. 1998). The highest value of the youngest soils implies that these fields were in the early stage of development, whereas the other soils (age 10 to 100 years) were somewhat more advanced in development but were not statistically different.

Based on the trend in the relative information indices along the Schiermonnikoog gradient, we conclude that either (i) all soils except the youngest are in the same phase of development or (ii) the indices are not able to reveal the changes in ecological succession in these soils.

The first option is not in agreement with the analyses of the absolute indices and of Neutel et al. (2001) and Van de Koppel et al. (1997) as they show a development of this ecosystem in terms of complexity and productivity. These analyses were consistent with Elton's (1927) and Odum's (1971) ideas of ecological succession. This would imply that these parameters are more sensitive to changes in ecological succession than the relative information indices.

There are two other factors that may have caused that in our study the relative information indices did not increased along the gradient of ecosystem development as

hypothesized by Ulanowicz (1996; 1997). First, the assumed values of import to detritus and roots and root respiration could have affected the outcome of analysis. The results of sensitivity analysis showed, however, that the influence of these parameters on the relative indices was low (Table 2). Second, the level of aggregation into functional groups (functional species) used in this study could be too strong to reveal the changes in organization. Here we deal with a maximum of 17 groups, whereas in previous ENA studies usually more groups are studied, e.g. 28 (Patricio et al. 2006), 25 (Scharler and Baird 2005). Abarca-Arenas and Ulanowicz (2002) showed that taxonomic aggregation can have strong impact on the values of information indices (particularly of ascendency). Also aggregation of the non-living compartments can have high impact on the indices (Allesina et al. 2005). In theory, during succession diversity of flows increases and so should H. Further, generalists species should be replaced by more specialized ones, what should be visible as an increase in AMI (Mageau et al. 1998). The phenomenon that the relative indices do not change from stage two to four suggest that the aggregation used in our study prevented the changes in specialization to be visible. However, specialisation for individual groups occurred if studied separately. These results suggest that the sensitivity of AMI of functional group increased with trophic level. The low sensitivity of total AMI to succession then resulted from the overwhelming contribution of lower trophic levels that were strongly aggregated. In contrast, the higher trophic levels were less aggregated.

If this last argument is valid, it would have strong impact on the use of information indices to analyse belowground terrestrial ecosystems. Currently it is almost impossible to increase the resolution of the food web, especially for lower trophic levels, because it would require more detailed information about the diet of every species, which is difficult to obtain.

Conclusions

The results of our ENA analysis are equivocal. The absolute information indices showed the expected development of the size and organization of ecosystem along the gradient of succession. The increase in TST resembles the increase in productivity found by van de Koppel et al. (1997). The increase of absolute indices, however, was strongly correlated to system biomass. To exclude the possibility that an increase in biomass caused the changes in absolute indices, one need to analyse a gradient that contains more soil of around 100 years old and older than the one field of 100 years old we used in our study, in which the build up of biomass does not occur. The relative information indices appeared to be less sensitive to ecosystem development than the absolute indices. This might have been due to the strong aggregation to functional groups. The lowest trophic levels had the highest aggregation, the highest biomass and the lowest specialisation over the gradient. Therefore,

the contribution to total AMI of low trophic levels overwhelmed the contribution of AMI of high trophic level, making it insensitive to subtle changes in succession.

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Acknowledgements

We thank Anje-Margriet Neutel and Johan van de Koppel for the data set they provided. We thank S.C. Dekker, P.C. de Ruiter, U. Scharler and R.E. Ulanowicz for comments on previous versions of the manuscript.

Appendix

All indices were calculated with a flow matrix (Fig. A.1) in which flows between compartments, input, output and mineralization was included. Total system throughput (TST) is the sum of all carbon flows in the system and thus the sum of all matrix elements (equation A.1) (Ulanowicz 1986).

$$TST = \sum_{i=1}^{n+3} \sum_{j=1}^{n+3} T_{ij} \qquad \text{Equation A.1}$$

with n the number of functional groups in the system and T_{ij} the flow in element ij. The structure of matrix with size i and j, which includes all flows, is explained in figure A.1.

Flow diversity (H; bits) is a measure for an ecosystem with the optimal organization, which is defined as preys having only a single predator and thus each functional group having only one link to a functional group in a higher trophic level (Ulanowicz 1986) (see equation A.2).

$$H = \sum_{j=1}^{n} Q_j \log Q_j \qquad \text{Equation A.2}$$

with Q_j the probability that species j is the host, calculated as the sum of flows in the jth row divided by TST.

The average mutual information (AMI) is the actual information in the system and is calculated as the sum of the information per element (equation A.3)

$$AMI = \sum_{i=0}^{n+2} \sum_{j=0}^{n+2} f_{ji} Q_j \log \frac{f_{ji}}{Q_i'} \qquad \text{Equation A.3}$$

with f_{ji} the fraction of flow in element *ji* fractional to *T* and Q_i the probability that *i* is the predator, calculated as the sum of flows in the *i*th column divided by *TST*.

The relative overhead (*RL*) is the difference between *C* and *AMI* (equation A.4). Further, relative ascendancy (*RA*) is a measure for how tight the system is organised (equation A.5).

$$RL = H - AMI \qquad \text{Equation A.4}$$

$$RA = \frac{AMI}{H} \qquad \text{Equation A.5}$$

Development capacity (*C*) is *H* given the current size (equation A.6).

$$C = TST * H \qquad \text{Equation A.6}$$

Parallel to C, Ascendency (*A*) is *AMI* given *TST* (equation A.7) and Overhead (*L*) is *RL* given the current *TST* (A.8).

$$A = AMI * TST \qquad \text{Equation A.7}$$

$$L = RL * TST \qquad \text{Equation A.8}$$

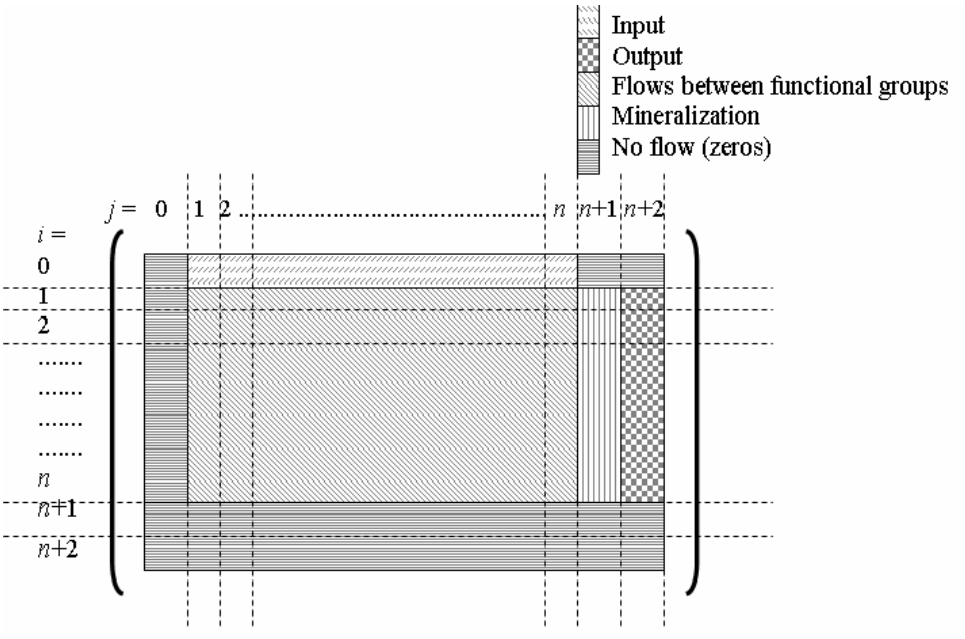

Figure A.1. Flow matrix structure of a food web. Where *i* represent row number, *j* represents column number and n is the number of species in the system. See legend for meaning of parts of the matrix.

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Chapter 4

Can the information indices (Ecosystem Network Analysis) be used as indicators of environmental stress in terrestrial ecosystems?

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Submitted to Environmental Toxicology and Chemistry

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Information indices from Ecosystem Network Analysis (ENA) can be used to quantify the development of an ecosystem in terms of its size and organization. There are two types of indices, i.e. absolute indices that describe both the size and organization of ecosystem (Total System Throughput (TST) – system size, Ascendency (A) – size of organized flows and Development Capacity (C) – upper limit for A, Overhead (L)- size of unorganized flows) and relative indices that describe only the organization (Average Mutual Information (AMI=A:TST), Flow diversity (H=C:TST), Relative Overhead (RL=L:TST)).

It is theorized that environmental stress impair the ecosystem development and that the effect of stress can be quantified with the ENA information indices. Here we applied ENA on a case of environmental stress in a terrestrial ecosystem, i.e. soils that have endured long-term exposure to elevated copper concentration and lowered pH.

The absolute indices showed an unexpected pattern of response to pollution, suggesting that ecosystems in polluted soils are more active and better organized than these in unpolluted soils. The relative indices, alternatively, responded to pollution as predicted by theory, i.e. with decrease of stress (pollution level) the level of specialization increased (increase of AMI) and losses of energy, e.g. due to respiration, decreased (decrease of Overhead). The diversity and evenness of flows showed hump-backed relationship with stress. Less polluted soils appeared to be less vulnerable to external disturbances and more efficient in processing energy (higher relative ascendancy (RA = A:C)) than polluted soils. The relative information indices were rigid to changes in values of assumed parameters. The relative indices, opposite to absolute indices, appeared to be useful as indicators of environmental stress on the ecosystem level.

Keywords: information indices, Ascendancy, Development Capacity, Average Mutual Information, Flow Diversity, Ecosystem Network Analysis, stress, soil,

Introduction

The effects of environmental stress (e.g. heavy metals) on different levels of ecological organization (i.e. individuals, populations, communities and ecosystems) have frequently been described (Bouwman *et al.* 2001; Kelly *et al.* 1999; Mozdzer *et al.* 2003; Pennanen *et al.* 1996; Posthuma and Van Straalen 1993; Stone *et al.* 2001; Szafraniec *et al.* 2001). The higher the level of organization the more difficult it is to assess the impact of the stressor, especially in case of multiple pollution. To study the effects of pollution on the ecosystem level therefore is a big challenge due to the complex nature of such systems.

Odum (1981) and Ulanowicz (1986) claim that stress inhibits and even reverse the development of the ecosystem and therefore effects of stress can be measured as changes in ecosystem development.

Development of an ecosystem (called ecological succession) is defined as a process involving structural changes in the system that are orderly, directional and therefore predictable (Odum 1969). To be able to compare the successional stages of different ecosystems, Odum (1969) proposes a set of twenty four indices, organized in six groups i.e. community energetics, community structure, life history, nutrient cycling, selection pressure and overall homeostasis.

On the basis of Odum's indices, Ulanowicz (1997; 1986) has developed his own set of indices of ecosystem development (absolute and relative information indices). The absolute indices describe: the amount of energy flowing through the ecosystem (i.e. Total System Throughput (TST)), a fraction of this energy that is realized through organized flows (Ascendancy (A)), the upper limit for A (Development Capacity (C)) and an amount of energy realized by unorganized flows (respiration, import, export, parallel pathways) (Overhead (L=C-A)). Relative indices, on the other hand, give information about the organization of flows, i.e., the level of specialization of flows (Average Mutual Information (AMI)), diversity and evenness of flows – an upper limit for AMI (Flow Diversity (H)), the level of disorganization (Relative Overhead (RL=H-AMI)) and how 'tight' the ecosystem is organized (Relative Ascendancy (RA=A:C)). As the system develops, these indices tend to increase, except for L that decreases as A approaches C (Mageau *et al.* 1998; Ulanowicz 1997). Because stress cause reversal of ecological succession all indices should decrease with an increase of stress, except for L that should increase, and therefore can be used as indicators of environmental stress (Mageau *et al.* 1998; Ulanowicz 1986).

Information indices together with the input-output analysis, trophic status and cycle analysis are combined by Ulanowicz (1997; 1986; 2004) into Ecosystem Network Analysis (ENA), which allows quantification of trends expected in developing ecosystems. Ulanowicz (1986) has applied this analysis on a tidal Juncus marsh creek ecosystem in order to asses the impact of environmental stress (warm water inflow). This work gives support to three

hypotheses of the effects of stress on the network of trophic exchanges: environmental stress (1) negatively impacts system size and development (information indices), (2) lowers trophic efficiencies and (3) degrades the structure of recycle pathways within the system.
A number of studies describes the impact of persistent stressors e.g. a dam (Baird and Heymans 1996), nitrogen overloading (Christian and Thomas 2003), reduced freshwater inflow (Scharler and Baird 2005), and recurring hypoxia (Baird *et al.* 2004) on estuaries. To our knowledge, however, there are hardly any studies that use the ENA to investigate the impact of persistent stressors (e.g. heavy metals) or a combination of stressors on terrestrial ecosystems.

In this study we tested the first hypothesis posed by Ulanowicz (1986) in order to find out whether the information indices can be used as indicators of multiple stress in terrestrial ecosystems. We calculated and analyzed the information indices for an agricultural below-ground ecosystem that endured long-term exposure to increased copper concentration and lowered soil pH. From previous studies we knew that in these soils the pollutants have strong, negative effects on the nematode community (Korthals *et al.* 1996), microbial properties and process rates (Tobor-Kapłon *et al. submitted*), and on the stability of processes (respiration, growth rate) to additional stresses or disturbance (Tobor-Kapłon *et al.* in press; Tobor-Kapłon *et al.* 2005).

Materials and Methods

Experimental area

Soil samples were collected from an experimental arable field known as Bovenbuurt, located 3 km NNE of Wageningen, the Netherlands. In 1982 the field was divided into 128 plots (6 x 11m each) organised in eight blocks. Each block consists of 16 plots on which different treatments were applied. Each treatment is a combination of different copper load and pH level. Copper was applied once as CuSO4 · 5H2O powder at loads of 0, 250, 500 and 750 kg Cu ha^{-1}, pH (KCl) was adjusted to values of 6.1; 5.4; 4.7 and 4.0 by addition of lime or sulphur (Korthals et al. 1996). Present concentrations of heavy metals and pH in the 10 cm topsoil layer are given in Table 1. The actual pH values are lower than the experimentally aimed ‘target’ values. This occurred because the last pH readjustment was done in 2001. For consistency we use the target values to identify treatments.

The soil is a fimic anthrosol with a texture of 3% clay, 10% silt, 87% sand, an organic carbon content of 21 g kg^{-1} soil and a CEC of 5.6 $cmol_c\ kg^{-1}$ (ammonium-acetate) (Boon *et al.* 1998; Korthals *et al.* 1996).

Table 1. Actual values of pH (1M KCl and 0.01M $CaCl_2$) and copper concentrations (mean ± SEM) in the 10 cm topsoil of the experimental field.

Cu	pH		pH-KCl		Cu-$CaCl_2$ [a] mg Cu kg $soil^{-1}$		Cu-HNO_3 [b] mg Cu kg $soil^{-1}$	
kg ha^{-1}	(nominal)	N	Mean	S.E.M	Mean	S.E.M	Mean	S.E.M
0	**4.0**	4	4.1	0.12	0.49	0.15	28.0	5.18
	4.7	4	4.5	0.12	0.17	0.03	31.1	4.52
	6.1	4	5.1	0.20	0.07	0.02	33.6	9.55
750	**4.0**	4	4.1	0.10	2.40	0.22	85.7	4.42
	4.7	4	4.5	0.52	1.04	0.23	91.5	8.22
	6.1	4	5.3	0.48	0.31	0.08	101	10.6

a - actual available Cu fraction (extracted with 0.01M $CaCl_2$)

b - potentially available Cu fraction (upon digestion with 0.43M HNO_3)

Six treatments were chosen – combinations of two extreme copper loads: 0 and 750 kg Cu ha^{-1} with three pH levels: 4.0; 4.7 and 6.1. Samples were taken in April, June and September 2002.

Soil samples were taken from the first four blocks. For microbial and nematode biomass measurements 15 soil cores, 4 cm in diameter and from 0-10 cm depth were taken from each replicate plot, bulked and divided into two subsamples.

Soils for microbial analysis were sieved (0.5 cm mesh) and prepared for further analysis. Dry mass of soil was measured as the loss of mass during drying in oven for 24 hours at 105^{o}C and organic matter content as the loss on ignition (6h at 550°C). The soil for the analysis of nematode biomass was not sieved.

Four cores of 6 cm diameter and 8 cm deep were taken per plot for measurement of enchytraeid biomass. These samples were processed immediately after sampling. For microarthropods five core samples were taken (4 cm in diameter, 0-10 cm depth). These samples were frozen and stored at -18^{o}C until analysis.

Estimation of biomasses

We sampled soil organisms three times in April, June and September 2002. For the calculation of flows and information indices we used averages of the three sampling events.

Microbes

Samples for bacteria and fungal biomass estimations were prepared following Bloem and Vos (2004). Bacterial biomass was counted automatically (Bloem *et al.* 1995) and fungi were counted manually under a fluorescent microscope.

Protozoa were enumerated by the most probable number method (MPN) after Darbyshire et al. (1974). *Pseudomonas fluorescens* was used as a food source. Presence of flagellates, ciliates and amoebae was checked after one and two weeks of incubation at 18^{o}C.

Nematodes

Nematodes were extracted from the soil samples using an Oostenbrink elutriator (Oostenbrink 1960). Next, nematodes were heat-killed and fixed with 4% formalin. Subsequently they were brought on a permanent mass-slide and identified to genus level at 400x-1000x magnification according to Bongers (1988). Biomass was assessed on the basis of the abundance of each genus and body mass calculated for each genus on the basis of average body size according to Bongers (1988). Allocation to feeding groups was according to Yeates et al. (1993).

Enchytraeids

Enchytraeids were extracted in a wet Tullgren extractor. The extracts were studied under a stereo microscope (Zeiss Microscope). All specimens were identified to genus level. Body sizes were converted to biomass according to Abrahamsen (1973).

Microarthropodes

For determination of micoroarthropods biomasses after thawing, the samples (four replicates) were bulked and gently mixed. One subsample of 250 g was taken for extraction. An oil flotation method (Kuenen *et al.* in preparation) was used to extract the microarthropods from the soil. All extracted animals were photographed using a Leica Wild M8 Stereo-microscope equipped with a Leica DC 200 digital camera. Body size was measured with use of digital image software. Biomass was calculated on the basis of body-size.

Roots

The biomass of barley roots, the crop growing on he experimental field in 2002, was estimated on the basis of work of Bolinder et al. (1997). The calculated biomass for June in Cu 0; pH 6.1 soils was set as a reference. We assumed that the root biomass was affected by treatments as was the yield of maize, see Korthals et al. (1996). Another assumption we made was that the root biomass in April was 10% of the biomass in June. In September the barley was already harvested, therefore root biomass was 0% of the June value. For further calculations we used the average of three sampling points. To estimate the amount of carbon

respired by roots, the data on carbon translocation by *Lolium perenne* (Kuzyakov 2001) was used. On the basis on the same work we calculated the carbon import to roots and to detritus.

Soil respiration rate

Carbon dioxide production was measured over a 6-week period of incubation. Results of the first week were not used to avoid short-term artefacts. 200g aliquots of sieved, field moist soil were placed into ±600 ml glass bottles and incubated in darkness at 20^{o}C. To determine the carbon dioxide content in the head space we used a gas chromatograph (Carlo ERBA Instruments, Milan, Italy).

Ecosystem Network Analysis

Information indices

The information indices are calculated as described by Ulanowicz (1986) using the Wand software (Allesina and Bondavalli 2004). The formulas for all indices are given in Table 2.

TST (Total System Throughput) is the sum of all flows within the system, including not only flows between the functional groups, but also import from and export to outside of the system and respiration. It is thus a measure of the size and activity of an ecosystem. The size of organized flows (species to species) within a system is represented by A (Ascendency), whereas C (Development Capacity) represents the maximal organization given the present size and is an upper limit for the A. Ulanowicz (1997; 1986) defined maximal organization as the situation where one predator has only one prey and thus every compartment is linked with only two compartments (with its prey and predator). The size of unorganized flows (respiration, import, export and parallel pathways) within a system is represented by L (Overhead), which is a difference between C and A. All mentioned indices are scaled by the total energy flow present within the system (TST) and therefore called absolute indices. It is known that the size component (TST) overwhelms the contribution of organization components in the absolute indices (Mageau *et al.* 1998). To investigate the organization of the system, the relative indices have to be taken into consideration. Relative indices are independent of the size of a system (TST) and purely describe its organization. The information regarding the network of material exchange within the system is given by AMI (Average Mutual Information). If material from any particular component in the system had an equal chance of flowing to any of the potential recipients then we would have no information regarding the flow network, however, if all material from a particular component

was transferred to only one of the potential recipients, we would have complete information regarding the flow structure (Costanza and Gottlieb 1998).

Table 2. Formulas used for calculations of information indices.

	Index	Unit	Formula
Absolute indices	Total System Throughput (TST)	kg C ha^{-1} 10cm $depth^{-1}$ y^{-1}	$TST = \sum_{i+1}^{n+2}\sum_{j+1}^{n+2} t_{ij}$
	Ascendency (A)	kg C bits ha^{-1} 10cm $depth^{-1}$ y^{-1}	$A = TST\sum_{i=0}^{n+1}\sum_{j=0}^{n+1} f_{ji}Q_j \log\frac{f_{ji}}{Q_i'}$
	Development Capacity (C)	kg C bits ha^{-1} 10cm $depth^{-1}$ y^{-1}	$C = TST\sum_{j=1}^{n} Q_j \log Q_j$
	Overhead (L)	kg C bits ha^{-1} 10cm $depth^{-1}$ y^{-1}	$L = C - A$
Relative indices	Average Mutual Information (AMI)	bits	$AMI = \frac{A}{TST}$
	Flow Diversity (H)	bits	$H = \frac{C}{TST}$
	Relative Overhead (RL)	bits	$R = H - AMI$
	Relative Ascendency (RA)	-	$RA = \frac{A}{C} = \frac{AMI}{H}$

n is a number of functional species (compartments), *0* stands for imports, *n+1* for exports and *n+2* for respiration
Q_j – is the probability that species *j* is the host, calculated as the sum of flows in the *j*th row divided by TST
f_{ji} - is the fraction of flow in element *ji* in comparison to the TST
Q_i - is the probability that *i* is the predator, calculated as the sum of flows in the ith column divided by TST

Analogue to the absolute indices, H (Flow Diversity) is an upper limit of AMI ($H = AMI_{max}$). This index gives information about diversity and evenness of flows between compartments (Baird *et al.* 1998). RL (Relative Overhead) is the unorganized part. The information about the 'tightness' of organization is given by RA (Relative Ascendency), which can ranges between zero, when L equals C, and one when A equals C. The dimension for the relative indices, except for RA, are bits of information. These units are taken from the information theory and represent the measure of information inherent in a single binary decision (Ulanowicz 1997).

During the ecosystem development both absolute indices and their relative analogues should perform as follows: with an increase in development the TST, C and A should increase, whereas L should initially increase as C increases faster than A but later will start to decrease as A starts to approach C (Ulanowicz 1997). Stress should reverse these trends.

Input data

For the calculation of information indices, for each group data on the inflow (consumption), flow to detritus (natural death rate and excretion), respiration, outflow (part of

biomass consumed by higher trophic level), import (immigration) and export (emigration) is needed for each functional group in the food web. Here we assumed that there was no import or export to and from all functional groups except for import to roots and detritus. We used a food web structure of the Lovinkhoeve system (de Ruiter *et al.* 1993) with minor modifications (Fig. 1). By assuming a steady state situation the inflow, flow to detritus, respiration and outflow can be calculated on the basis of a Food Web model (de Ruiter *et al.* 1993; Hunt *et al.* 1987). This model, however, due to its top-down approach, does not calculate the respiration of roots and therefore we used values calculated as described in paragraph 'Root'. The sum of respiration of all groups as calculated from Food Web model did not equalled the measured respiration. Therefore, we adjusted the calculated values to reach the measured value. The respiration of root was not part of the adjustment. The import to detritus and root were calculated as described in paragraph 'Root'.

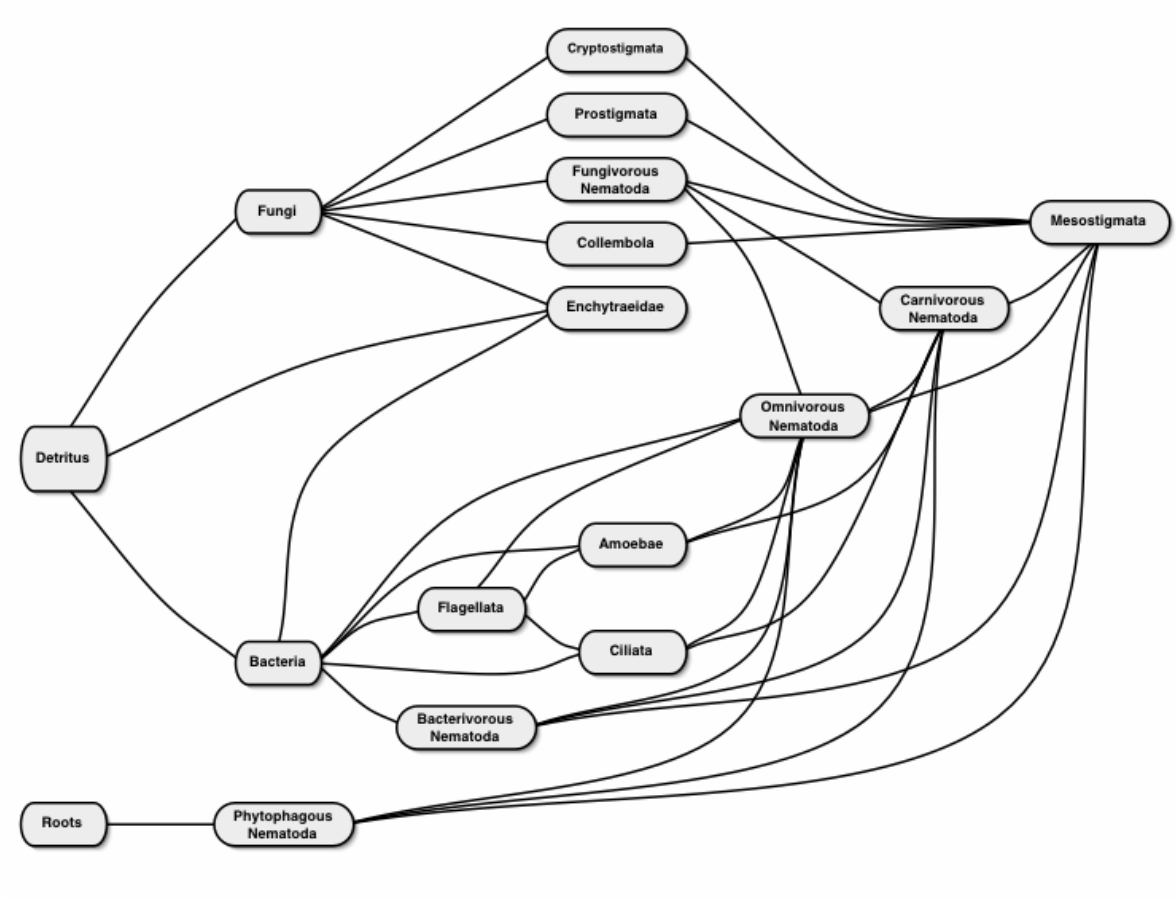

Figure 1. Diagram of the potential food web structure in the soils of the experimental field.

Balancing

The Food Web model calculates flows among the functional groups assuming that the food web is in steady state. The steady state conditions imply that flows of matter and energy

entering and leaving a given compartment are equal (the same is for whole ecosystem), so that there is no increase or decrease in mass in the ecosystem (Allesina and Bondavalli 2003). However, import to roots and detritus and root respiration were estimated without a steady state assumption disrupting the system's steady state. Also the adjustment of respiration increased the magnitude of deviation from steady state. Therefore, to have the whole ecosystem in steady state we have balanced the system. Allesina and Bondavalli (2003) developed a mathematical procedure that can balance networks. This procedure is called balancing as it balances the incoming and outgoing flows. Note that the steady state conditions are not required for the computation of the information indices (Ulanowicz 2004), we decided to bring the networks to the steady state. This will allow us to use the same data sets for other analyses of ENA (i.e. input-output analysis and description of trophic efficiencies) in which the steady state conditions are required

All networks were balanced with the average procedure (Allesina and Bondavalli 2003) using the WandBalance software (http://www.dsa.unipr.it/netanalysis/). The average procedure balances the networks in three steps. First, the inputs (imports) are kept constant while outputs (respirations and exports) and flows are manipulated (input-based approach). Second, the outputs are kept constant while imports and flows are manipulated (output-based approach). Third, the flows calculated with the input-based and output-based approach are averaged.

Sensitivity analysis

To test the influence of the estimated carbon input to roots and to detritus and of root respiration, we performed a sensitivity analysis. Sensitivity was calculated as the normalized change in a given index (outcome) scaled by the normalized change in a value of tested parameter (Haefner 1996):

$$S = \frac{\frac{Out_i - Out_o}{Out_o}}{\frac{P_i - P_o}{P_o}}$$

Where: S is sensitivity (-), Out_i model output (index) with parameter choice i, Out_0 model output with original parameter value, P_i parameter choice i and P_0 original parameter value.

Statistical analysis

When necessary, data were log-transformed to obtain normal distribution. For analyses we used Multivariate ANOVA with pH and Cu as the fixed factors. The relationship between copper concentration and H was analysed by regression analysis (curve estimation).

Analyses were performed using the SPSS statistical software package (SPSS 11.5.0; LEAD technologies, Inc.).

Results

Data about the biomasses of functional groups are given in table 2.

System size and organization

All four absolute information indices (i.e. TST (Total System Throughput), C (Development Capacity), A (Ascendency) and L (Overhead)) showed an interaction effect of copper and pH (table 3). In the Cu 0 soils there was no change of values of all indices over the range of soil pH. In the Cu 750 soils, however, indices tended to decrease with an increase of soil pH. The highest values of all indices were found in the most polluted soils i.e. Cu 750; pH 4.0 and Cu 750; pH 4.7.

The relative indices (analogues of the absolute indices) (i.e. H (Flow Diversity), AMI (Average Mutual Information) and RL (Relative Overhead)) also showed the effects of copper and pH treatment but different than the absolute indices. In the Cu 0 soils H (Fig. 2A) decreased with an increase of soil pH, but increased with pH in the Cu 750 soils. There was a significant (quadratic) relationship between H and 0.01M $CaCl_2$-extractable copper concentration ($p = 0.004$) (Fig. 3). The AMI (Fig. 2B) was affected by the synergistic effect of copper and pH. In both Cu treatments AMI increased with soil pH but this trend was stronger in the Cu 750 soils, where AMI was strongly reduced in the pH 4.0 soils than in the Cu 0 soils. In the pH 6.1 soils was no difference in AMI between copper treatments. In the Cu 750 soils RL (Fig. 2C) was constant over the range of pH, whereas in the Cu 0 soils decreased with an increase of soil pH. The RA (Relative Ascendency) (Fig. 2D) was higher in the Cu 0 than in the Cu 750 soils, and in both copper treatments it tended to increase with soil pH.

Sensitivity analysis

We tested the sensitivity of investigated indices to changes in values of carbon import to roots and to detritus and of root respiration. Generally, the absolute information indices were more sensitive to changes in estimated parameters than the relative indices (table 4).

All absolute indices were most sensitive to changes in the carbon import to detritus (IR) (approx. -0.2 for all) and least sensitive to changes in respiration of roots (RR) (0.01, 0.02, -0.001 and 0.03 for TST, C, A and L, respectively); sensitivity to carbon import to roots (IR) was approx. -0.09.

Table 3. Biomasses (in kg C ha^{-1} 10cm $depth^{-1}$ $year^{-1}$ (mean ± standard deviation)) of the different functional groups of organisms in the belowground food web of the experimental field at different soil Cu and pH levels.

Species	0 kg Cu ha^{-1}						750 kg Cu ha^{-1}					
	pH 4.0		pH 4.7		pH 6.1		pH 4.0		pH 4.7		pH 6.1	
	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev
Mesostigmatic mites	0.007	0.006	0.019	0.034	0.021	0.021	0.010	0.004	0.034	0.052	0.018	0.012
Carnivorous nematodes	0.110	0.040	0.209	0.146	0.162	0.202	0.065	0.033	0.118	0.129	0.129	0.077
Omnivorous nematodes	0.361	0.051	0.201	0.076	0.158	0.113	0.080	0.046	0.140	0.167	0.285	0.183
Cilliates	0.103	0.086	0.114	0.121	0.069	0.084	0.051	0.035	0.041	0.034	0.085	0.067
Amoebae	2.664	2.044	2.737	1.274	1.889	1.019	2.587	0.739	4.714	2.637	2.153	1.507
Cryptostigmatic mites	0.019	0.016	0.017	0.015	0.014	0.007	0.032	0.024	0.020	0.017	0.015	0.013
Prostigmatic mites	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.001	0.001	0.001	0.001	0.001
Fungivorous nematodes	0.036	0.029	0.038	0.020	0.026	0.006	0.072	0.022	0.068	0.025	0.038	0.010
Collembolans	0.052	0.026	0.024	0.016	0.039	0.019	0.030	0.012	0.027	0.018	0.044	0.014
Enchytraeids	0.138	0.153	0.060	0.027	0.092	0.132	0.052	0.082	0.025	0.023	0.251	0.275
Flagellates	0.226	0.147	0.163	0.053	0.246	0.231	0.128	0.013	0.216	0.050	0.190	0.083
Bacteriovorous nematodes	0.511	0.056	0.476	0.051	0.815	0.145	0.665	0.082	0.627	0.156	0.662	0.259
Fungi	15.73	4.211	11.79	3.020	8.639	1.960	9.853	0.780	13.94	1.822	10.32	1.862
Bacteria	58.14	11.41	39.56	11.16	53.74	10.68	36.73	6.625	33.88	4.687	34.80	19.93
Plant parasitic nematodes	0.112	0.044	0.246	0.029	0.266	0.041	0.089	0.057	0.158	0.093	0.241	0.039

Table 4. Effects of copper and pH on information indices (means and *p*-values of main effects and interaction).

Index	Unit	0 kg Cu ha^{-1}			750 kg Cu ha^{-1}			p-value		
		pH 4.0	pH 4.7	pH 6.1	pH 4.0	pH 4.7	pH 6.1	Cu	pH	Cu x pH
Absolute indices										
Total System Troughput (TST)	kg C ha^{-1} 10 cm^{-1} depth y^{-1}	2260	2225	2740	5950	3618	2508	0.002	0.030	0.006
Development Capacity (C)	kg C bits ha^{-1} 10 cm^{-1} depth y^{-1}	7773	7530	9008	19825	12875	8810	0.001	0.027	0.008
Ascendency (A)	kg C bits ha^{-1} 10 cm^{-1} depth y^{-1}	3023	3108	3768	7223	4850	3498	0.001	0.061	0.006
Overhead (L)	kg C bits ha^{-1} 10 cm^{-1} depth y^{-1}	4748	4425	5243	12585	8035	5310	0.000	0.018	0.009
Relative indices										
Flow Diversity (H)	bits	3.44	3.39	3.29	3.35	3.56	3.52	0.013	0.251	0.009
Average Mutual Information (AMI)	bits	1.34	1.39	1.38	1.23	1.34	1.40	0.007	0.000	0.010
Relative Overhead (RL)	bits	2.10	1.99	1.91	2.12	2.22	2.12	0.000	0.041	0.024
Relative Ascendency (RA)	-	0.39	0.41	0.42	0.37	0.38	0.40	0.000	0.000	0.254

The relative information indices showed a different sensitivity to estimated parameters than the absolute indices; H and AMI were most sensitive to changes in carbon import to detritus and least to import to roots (H: -0.019, 0.0001 and 0.007; AMI: 0.024, 0.006 and -0.012 for ID, IR and RR; respectively), whereas RA was more sensitive to changes in root respiration than to changes in the two other parameters (-0.005, 0.005 and -0.019 for ID, IR and RR respectively).

Table 5. Sensitivity of information indices to changes in estimated parameters (i.e. import to roots (IR), import to detritus (ID) and respiration of roots (RR)). TST - Total System Throughput, C - capacity, A – ascendancy, L – Overhead, H – Flow Diversity, AMI – Average Mutual Information and RA – Relative Ascendency.

Parameter	kg Cu ha^{-1}	pH	TST	C	A	L	H	AMI	RA
ID	0	4.0	-0.102	-0.133	-0.128	-0.137	-0.032	-0.026	0.005
ID		4.7	-0.003	-0.037	-0.026	-0.046	-0.035	-0.023	0.012
ID		6.1	-0.034	-0.070	-0.054	-0.082	-0.037	-0.020	0.016
ID	750	4.0	-0.503	-0.481	-0.520	-0.458	0.022	-0.017	-0.040
ID		4.7	-0.329	-0.325	-0.362	-0.302	0.004	-0.033	-0.037
ID		6.1	-0.077	-0.116	-0.104	-0.124	-0.039	-0.027	0.012
IR	0	4.0	0.108	0.110	0.119	0.103	0.001	0.011	0.010
IR		4.7	0.166	0.163	0.173	0.156	-0.003	0.007	0.010
IR		6.1	0.138	0.138	0.144	0.133	0.000	0.006	0.006
IR	750	4.0	0.004	0.007	0.007	0.007	0.003	0.003	0.000
IR		4.7	0.016	0.020	0.019	0.020	0.004	0.004	0.000
IR		6.1	0.107	0.106	0.112	0.103	-0.001	0.004	0.005
RR	0	4.0	0.012	0.022	0.001	0.035	0.010	-0.011	-0.021
RR		4.7	0.019	0.026	-0.002	0.045	0.006	-0.021	-0.027
RR		6.1	0.017	0.028	-0.004	0.051	0.011	-0.021	-0.032
RR	750	4.0	0.001	0.002	0.001	0.003	0.002	0.000	-0.002
RR		4.7	0.002	0.006	0.000	0.009	0.004	-0.002	-0.006
RR		6.1	0.013	0.022	-0.005	0.039	0.008	-0.018	-0.026

Balancing

The balancing procedure, that was meant to bring the systems to steady state, caused an increase in carbon flows in all systems (see Table 5). The more the system was contaminated with copper and pH the more unbalanced were the flows. In the most polluted soils (Cu 750; pH 4.0), the TST increased after balancing on average almost by a factor of five, whereas other absolute indices increased by a factor of six. At the same time, these indices were only slightly affected by balancing in the clean soils.

The effect of balancing on the relative indices was not that strong; nevertheless, the AMI, H and RL increased by approx. 20% in the two most polluted soils and remained almost unchanged in other soils. This is as expected since relative indices are calculated as organisation of flows and the size of the flows is not included directly. The RA was the least affected by the balancing procedure.

Table 6. Relative changes (X_{AB}/X_{BB}) in the values of information indices after balancing (mean); X_{AB} - value after balancing, X_{BB} - value before balancing.

Index	0 kg Cu ha^{-1}			750 kg Cu ha^{-1}		
	pH 4.0	pH 4.7	pH 6.1	pH 4.0	pH 4.7	pH 6.1
	Mean	Mean	Mean	Mean	Mean	Mean
Total System Troughput (TST)	1.28	1.19	1.30	4.86	2.41	1.39
Development Capacity (C)	1.40	1.22	1.38	6.12	3.01	1.52
Ascendency (A)	1.34	1.25	1.41	5.71	2.81	1.56
Overhead (L)	1.43	1.21	1.36	6.39	3.15	1.49
Flow Diversity (H)	1.09	1.03	1.06	1.26	1.25	1.09
Average Mutual Information (AMI)	1.04	1.05	1.09	1.18	1.17	1.12
Relative Overhead (RL)	1.12	1.01	1.04	1.32	1.31	1.07
Relative ascendency (RA)	0.96	1.02	1.02	0.93	0.93	1.03

Discussion

As stated in introduction, the complex nature of an ecosystem makes the study of the effect of stress on ecosystem level a difficult task. Here we study the long-term effect of exposure to elevated copper concentration and altered soil pH on belowground ecosystem using the approach developed by Ulanowicz (1997; 1986; 1996). We examine the size and organization of this ecosystem to evaluate whether stress inhibits or even reverse the ecological succession as predicted by Odum (1981). We also try to evaluate whether these indices can be used as indicators of environmental stress.

On the basis of theory (1997; Ulanowicz 1986) we expected that the information indices would have lower values in the Cu 750 than in the Cu 0 soils and that their values would decrease with a decrease of soil pH. We also expected an interaction-effect between copper and pH. We showed that the absolute information indices indeed revealed the effects of both copper and soil pH but not as expected. The antagonistic effect imposed by copper and pH on the TST, A and C suggests that the ecosystems in the most stressed soils processed more material than those in the less stressed soils and are more organized. Ulanowicz and Kay (1991) claim that systems with high L are potentially more resilient to external perturbations. It would imply that in our study the more polluted soils are potentially more resilient to an external disturbance. Especially the effect on TST was surprising since environmental stressors (like heavy metals) are known to have negative effects on the growth and photosynthesis of primary producers (Ali *et al.* 2004; Vasillev *et al.* 2003) and on the activity of other organisms. (Kramarz and Laskowski 1999; Maryanski *et al.* 2002; Posthuma and Van Straalen 1993; Stone *et al.* 2001; Tobor-Kapłon *et al. submitted*). We think that it was caused by the fact that we balance the food webs in order to bring them to steady state conditions. The comparison of information indices before and after balancing (Table 6) showed that in the unpolluted and weakly polluted soils, all absolute indices were less

affected by balancing than in the most polluted soils. In the most polluted soils balancing procedure caused a strong (500-600%) increase in the values of these indices. This shows that in the more polluted soils, flows were less balanced (the deviation from the steady state was higher) than in the less polluted soils. The balancing procedure is supposed to bring flows to a steady-state situation but this is done in purely mathematical way (Allesina and Bondavalli 2003) that does not take into account ecological authenticity. The deviation from steady state before balancing could result from assumptions we made regarding the metabolic parameters used for calculations of flows. It is known that stress affects the metabolism of an organisms by changing its production (Aceves *et al.* 1999; Jongmans *et al.* 2003) or survival (Stone *et al.* 2001). We know that the P/B ratio of bacteria, which at steady state equals natural death rate, was about 30% lower in the pH 4.0 than in the pH 6.1 soils (Tobor-Kapłon *et al. submitted*). We assumed, however, that the parameters used to calculate flows (i.e. assimilation and production efficiencies and natural death rate) are not affected by the treatments. This had to be done because data about the effect of copper and pH on these parameters for every species present in a food web are not available.

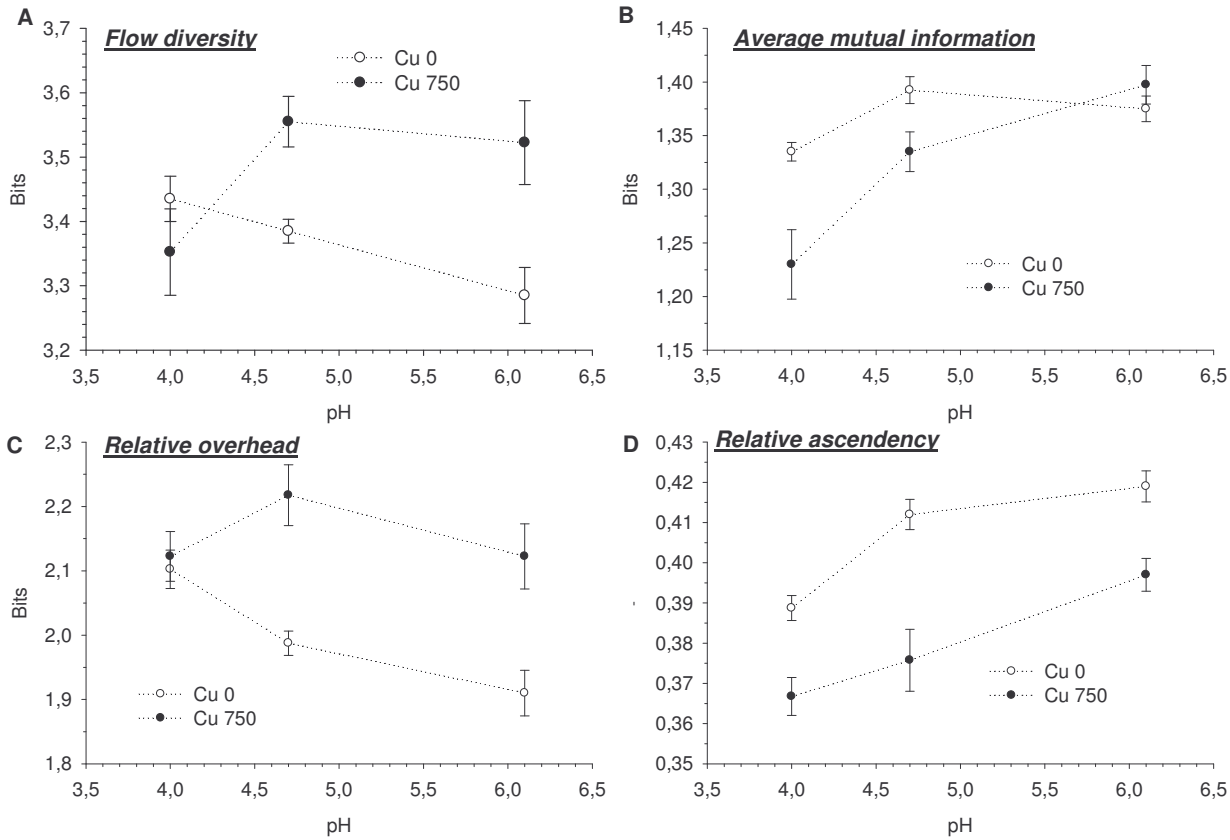

Figure 2. Effect of field treatments on the relative indices: A – Flow Diversity (H), B – Average Mutual Information (AMI), C – Relative Overhead (RL) and D – relative ascendancy (RA). Open circles represents Cu 0, and grey circles Cu 750 soils (means ± SEM).

The relative indices, alternatively, appeared to be less affected by the balancing procedure than the absolute indices. This is probably due to fact that they are independent of TST, which is known to overwhelm the contribution of organization elements in the absolute indices (Mageau *et al.* 1998). We have also shown that the relative indices are more rigid to changes in assumed values of import to root and detritus and root respiration than the absolute indices. These two facts make the relative information indices in this study more suitable for examining the effects of pollution on an ecosystem level than the absolute indices.

The relative indices did show the expected effect of stress on ecosystem organization. We found a hump-backed relationship between H and copper concentration (Fig. 3) that seems to confirm the intermediate stress hypothesis (Connell 1978; Giller *et al.* 1998). AMI revealed a synergistic effect of copper and pH (fig. 2B). Soil acidity has a stronger negative effect on AMI in the Cu 750 than in the Cu 0 soils. It shows that pollution inhibits organization of flows within the ecosystem and that the level of specialization increases with a decrease of stress. This is in line with predictions of Ulanowicz (1986) and means that more polluted soils are populated by generalist whereas specialists inhabit clean soils. The effects on RL supplement these findings i.e. the more contaminated the soils, the higher the fraction of unorganized flows. This indicates that in more polluted soils more energy is lost due to respiration (detoxification) and energy is transferred by parallel pathways (less specialization). Analogically to L, the higher RL the more resilient the ecosystem should be to external perturbations.

Some authors (Heymans and Baird 2000; Heymans *et al.* 2002) claim that the RA is a better index to compare systems than any of the other information indices. It excludes the influence of TST and shows how close the AMI is to its upper limit (H). Ulanowicz (1986) claims that RA will increase with a decrease of stress as stress has a negative impact on organization. In this study, we found that relative ascendancy followed trends predicted by theory. We found that RA increased in both copper treatments with soil pH and was higher in the Cu 0 than in the Cu 750 soils (fig. 2D). The observed pattern means that less stressed soils are more tightly organized, more efficient and resistant to external disturbances than the more stressed soils (Heymans and Baird 2000; Heymans *et al.* 2002).

The results suggest that the less stressed ecosystems should have higher resistance, whereas more stress systems higher resilience to external perturbations. The experimental stability analysis (resistance and resilience to additional stress or disturbance) performed on this soils did not confirmed this results (Tobor-Kapłon *et al.* in press; Tobor-Kapłon *et al.* 2005).

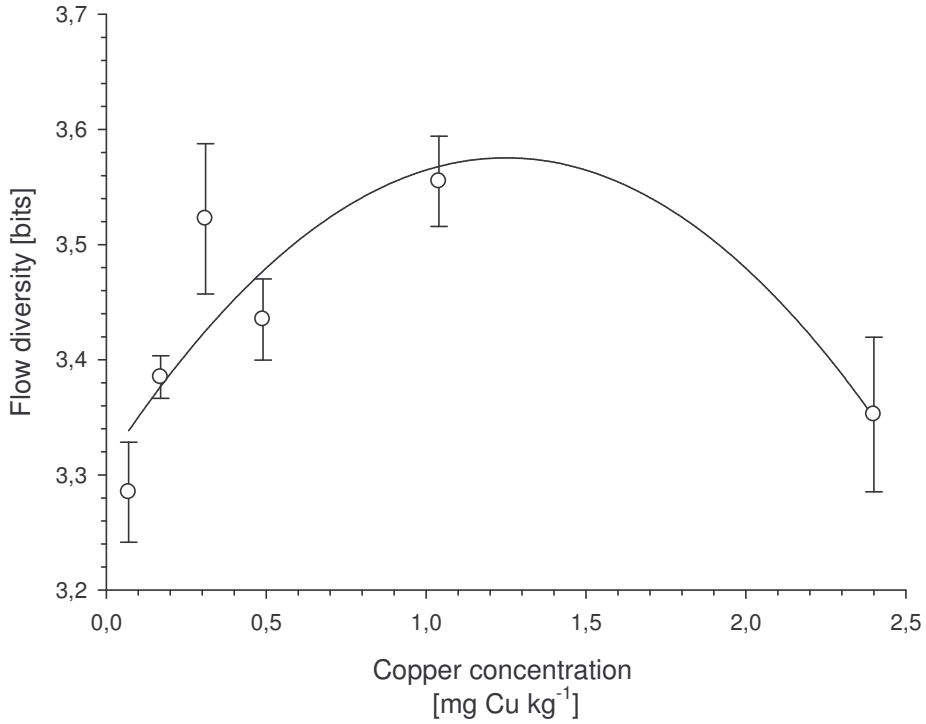

Figure 3. Hump-backed relationship between Flow Diversity (H) and stress expressed as 0.01M $CaCl_2$ exchangeable fraction of copper (mean ± SEM).

The respiration and bacterial growth rate showed a range of patterns of stability to stresses (lead and salt) and disturbances (heat and drying-rewetting). In some cases processes were more stable in non stressed soils, whereas in others in stressed soils.

This study showed that the ENA is a useful tool to study the effect of stress on the organization of a terrestrial ecosystem. Obtained results indicated that the relative information indices can be used as indicators of environmental stress in terrestrial ecosystems. We found them responding to pollution in the way predicted by the theory. They appeared to be rigid to changes in assumed parameters and not affected by the balancing procedure. On the other hand the absolute indices did not responded to stress in predictable manner, with which they violated the requirement of good indicator (Dale and Beyeler 2001). From the set of relative information indices the Relative Ascendency seems to be the most useful indicator due to its dimensionless character that allows comparison between system of different size and organization.

Acknowledgements:

We thank Dr. C. van Gestel for his valuable comments, Dr. K. Ilieva-Makulec for help with identification of nematodes and A. Vos, M.R. Veninga and W. Schuurmans for technical assistance.

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Chapter 5

Functional stability of microbial communities in contaminated soils

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Oikos 111(1):119-129

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Functional stability, measured in terms of resistance and resilience of respiration and growth rate of bacteria and fungi, was studied in soils that have been exposed to copper and low pH for more than twenty years. We used treatments, consisting of soil with no or high copper load (0 or 750 kg ha^{-1}) and low or neutral pH (4.0 or 6.1). Stability was examined by applying an additional stress in the form of lead or salt.

After addition of lead, respiration (decomposition of freshly added lucerne meal) showed lower resistance at low than at neutral pH and at high copper than at low copper. The most acid and contaminated soil was the least resistant. Respiration showed no resilience after addition of lead. Bacterial growth rate (thymidine incorporation) also showed resistance at low pH but only in soils that were not contaminated with copper.

After addition of salt, respiration showed no differences in resistance but the soils without copper contamination showed higher resilience. Bacterial growth rate showed lower resistance at low pH than at neutral pH, the latter in which the growth rate increased by on average 123%. This increase at high pH was faster in soil without copper than in soil with copper contamination in which the growth rate initially decreased and then increased. The effects of secondary stress depended on the nature of the stress (lead or salt) and on the parameter measured (respiration or bacterial growth rate).

In general the highest resistance and/or resilience were found in the least contaminated soils with neutral pH and/or no copper contamination. Thus, the microbial communities in the cleaner soils showed the highest functional stability. The results seem to confirm the notion that environmental stress alters ecosystems such that supplementary stress will have stronger impacts than in an unstressed system. The results may also confirm the insurance-hypothesis that reduced biodiversity due to the first stress negatively affected community stability. As an alternative, we discuss the observed effects in terms of altered energy budget.

***Keywords**:* stress, functional stability, microbial community, respiration, growth rate

Introduction

Intensification of land use and other human activities have caused high input of toxicants such as heavy metals, pesticides, fertilizers into soil environments. These substances have become chronic or acute stresses for soil ecosystems and constitute evolutionary pressure on soil organisms, communities and ecosystem functioning. The most abundant and diverse group of soil organisms are microbes. Bacteria and fungi are primary consumers of detritus and thus these groups have the greatest direct contribution to the functioning of soil systems (e.g. decomposition of organic matter and nutrient cycling) (Bloem *et al.* 1997).

Physiological effects of stress on soils organisms can be expressed in terms of altered energy budgets. Every organism allocates assimilated energy to production (both - growth and reproduction) and maintenance (including detoxification processes). Stress constitutes an additional burden for the energy budget and increases maintenance costs, because resources allocated to detoxification and reparation of damages produced by a stressor are not available any more for production (Sibly and Calow 1989). Therefore growth and reproduction are very sensitive to stress (Bloem and Breure 2003). Each species has a different way of managing its energy budget and thus the response of an entire community to stress is the sum of all individual reactions.

Changes in the energy budgets of soil organisms may lead to changes in ecosystem functioning, e.g. in terms of organic matter decomposition and respiration (Barajas Aceves *et al.* 1999; Niklinska *et al.* 1998; Nordgren *et al.* 1988). The effects are however ambiguous, as soil pollution has been found to increase as well as decrease respiration rate (Bååth 1989). Those two effects may occur simultaneously, because one compound can have various impacts on different soil organisms. Organisms that are more resistant will increase respiration because they demand more oxygen for decontamination processes, while more vulnerable individuals will reduce the rate of that process due to overall intoxication. Also, effects of soil pollution on respiration may depend on land use. Respiration appears to be sensitive to metal contamination in forest soils (Bååth 1989), but for agricultural soils the results are conflicting (Giller *et al.* 1998). Respiration is not a very sensitive indicator because it appears to be unaffected at heavy metal concentrations at around current EU mandatory limits (Brookes 1995; Rajapaksha *et al.* 2004). In general bacterial growth rate has been found to be more sensitive to contamination than biomass and respiration rate both in water and soil (Bååth 1992; Jones *et al.* 1984). Díaz-Raviña and Bååth (1996) investigated the impact of heavy metals on bacterial growth rate during fourteen months of incubation. They showed strong reductions immediately after stress application followed by recovery. Others (Boon *et al.* 1998; Pennanen *et al.* 1996) showed that strong reductions in growth rate can still be found many years after addition of heavy metals. The impact of toxicants on

fungal growth rate is not so well documented. Some authors argue that fungi are less sensitive to additional stress than bacteria (Pennanen *et al.* 1998). A number of publications also show the negative effect of contamination on microbial biomass (Barajas Aceves *et al.* 1999; Giller *et al.* 1998; Pennanen *et al.* 1998) which is thought to be the result of lower microbial substrate utilisation efficiency.

There is evidence that high levels of stress decrease microbial diversity, as a result of species loss due to lack of sufficient tolerance to particular stress factors (Giller *et al.* 1998). Torsvik *et al.* (1998) investigating the impact of heavy metals on the biodiversity of soil bacterial communities found that pollution diminished total genetic diversity. This reduction depended on the level of pollution.

The effects of stressors may, ultimately, lead to changes in community structure. As a result of removal of sensitive species due to stress, some niches become free and can be taken over by more resistant species. Due to lower competition and easier access to resources they can perform better and thus become more abundant. Many authors (Bååth *et al.* 1998a; 1998b; Frostegård *et al.* 1996; Kelly *et al.* 1999; Macnaughton *et al.* 1999; Pennanen *et al.* 1996; Witter *et al.* 2000) found that toxicants like metals or organic compounds lead to changes in community structure and towards a higher level of physiological adaptation or community tolerance. Witter *et al.* (2000) showed increased tolerance to cadmium, zinc and copper in soils to which the last application of sludge amended with various metals was added six years earlier. Increased tolerance to TNT was found by Gong *et al.* (2000) in soils previously treated with that compound.

The knowledge that stress alters soil communities raises the question: how will altered communities perform in case of additional stress? Will the responses of altered and non-changed communities be different? In other words – are stressed communities less or more sensitive to additional environmental stresses and disturbances than not-stressed ones?

According to Griffiths *et al.* (2000) a system is stable when it is able to withstand disturbances and maintain its normal state. Such defined stability has two measurable components: resistance - which is the inherent capacity of the system to withstand disturbance, and resilience - which is the ability to recover after disturbance. Stability increases with increase in these two components.

There are two contrasting hypotheses regarding stability of ecological processes. The first predicts that non-stressed systems are more stable since they dispose large resources that allow them to maintain function in case of stress (Aarts and Nienhuis 1999; Loreau 2000; Naeem and Li 1997). The other predicts that stressed systems are more stable because due to first stress they gained abilities (adaptation, physiological changes) to cope with stress and thus maintain function (Odum 1981).

Some attempts have been done to verify which of the above hypotheses applies to soil systems. Griffiths *et al.* (2001a; 2000) compare functional stability of species-rich and species-poor soils. To reduce biodiversity in their first experiment they used chloroform fumigation (Griffiths *et al.* 2000) whereas in the second one a wide range of environmental impacts like: different plant diversity, contrasting land use and industrial pollution which was assumed to have resulted in diminished species richness in soil (Griffiths *et al.* 2001a). On these soils they applied several types of additional stresses and measured the response in terms of soil respiration after certain time intervals. They showed that despite first treatment did not caused significant differences in respiration rates between soils, the second (Cu) stronger affected that process in more stressed soils. The results also showed that initially more diverse communities recover faster from additional stress (heat) than the less diverse ones. Since the changes in biodiversity after chloroform fumigation were not random Griffiths *et al.* (2001b) have performed similar experiment to check whether stability is related to biodiversity. They used non stressed soils that differed between each other only in biodiversity. To obtain random differences in species richness they used dilution method. The experiment showed that response to stress did not correspond to biodiversity.

Another approach was used by Degens *et al.* (2001). They investigated the effect of various kinds of stresses on catabolic evenness (a component of microbial functional diversity defined as the uniformity of substrate use) of two types of soils. In their experiment, they used soils that differ in catabolic diversity. They assumed that these differences were caused by different types of land use (pasture and arable soils). The results showed that the effects of stress application were stronger in soil with lower catabolic diversity (arable soil).

Three of mentioned above works (Degens *et al.* 2001; Griffiths *et al.* 2001a; 2000) demonstrated that stressed soils (fumigation, environmental stresses) were more sensitive to additional stresses and thus less stable, while Griffiths *et al.* (2001a) demonstrated that biodiversity per se do not affect stability.

In this paper we investigate functional stability of soil microbial communities that have been exposed to different levels of copper and pH for more than 20 years. The primary stress is present in soil long enough to allow evolution towards adaptation to occur. The soils differ in biodiversity, community structure and tolerance (Bloem and Breure 2003). Our aim was to test whether a stressed microbial community (low pH, high copper) was less or more stable than a not stressed community (neutral pH, low copper). Like Griffiths *et al.* (2000; 2001a) we measured the response of respiration rate to additional stress but we also measured the effects on most important population parameters, which are growth rates of bacteria and fungi.

Materials and methods

Experimental area and sampling

Soil samples we collected from an experimental arable field known as Bovenbuurt, located at the Wildekamp in Bennekom, approx. 3 km NNE of Wageningen, The Netherlands. In 1982 the field was divided into 128 plots (6 x 11m each) organised in eight blocks. Each block consists of 16 plots on which different treatments were applied. Since 1982 a 3-year crop rotation of potatoes, maize and sugar beet has been practised. Each treatment is a combination of different copper load and pH level. Copper was applied once as a powdered $CuSO_4 \cdot 5H_2O$ at the load of 0, 250, 500 and 750 kg ha $^{-1}$, pH (KCl) was adjusted to values 6.1; 5.4; 4.7 and 4.0 by addition of lime or sulphur. Every six years the pH was checked and readjusted if necessary.

The soil is fimic anthrosol with a texture of 3% clay, 10% silt, 87% sand, an organic carbon content of 2.1 % by mass and a CEC of 5.6 $cmol_c$ / kg (ammonium-acetate) (Boon et al. 1998).

For our experiments we chose four treatments: combinations of two extreme copper (Cu 0 and Cu 750) and two extreme pH levels (pH 4.0 and 6.1). The present values of total copper concentrations of chosen treatments are for Cu 0, pH 4.0: 37.7; Cu 0, pH 6.1: 40.3; Cu 750, pH 4.0: 113.6 and Cu 750, pH 6.1: 85.0 mg Cu kg^{-1}. For the same treatments pH (KCl) values are: 4.0; 5.2; 3.9 and 5.1. The present values of pH and Cu are different from target values since last adjustment was done in 2001. Samples were taken from first four blocks. From each plot 15 soil cores, 4 cm in diameter and 10 cm depth were taken and then mixed and sieved (0.5 cm mesh).

Chemical analysis

Concentrations of bioavailable metals were measured in samples used for respiration measurements. Directly after each measurement, 10 g samples were taken from each bottle and placed into 50 ml plastic tubes. Samples were shaken (30 rev min-1) with 40 ml of 0.01 M $CaCl_2$ for 48 hours. After shaking, pH was measured with a pH-meter (Φ 34 Beckman). Tubes with soil slurry were centrifuged at 3000 rev min^{-1} for 10 min; samples were filtered through a 0.45 µm syringe-filter (Total Organic Carbon free). After measurement of electroconductivity (Consort K810) the samples were split into two portions. One was used for TC/IC (Total Carbon/ Inorganic Carbon) analysis (TOC 5050A, Shimadzu) and the second was acidified with HNO_3 to C%=1 HNO_3 and used for measuring metal concentrations on an ICP-AES (Perkin Elmer Optima 3300 DV).

In some cases only three replicates were used.

Functional stability

For stability analysis we used the approach of Griffiths et al. (2000). All measurements were done three times: 24 hours, two weeks and two months after stress addition. The average change of all three sampling time points was taken as a measure of resistance, while the change over time we adopted as measure of resilience. Due to absolute differences in investigated parameters (respiration rate, bacterial and fungal growth rates) between soils and sampling dates, resistance was normalised to the control value, i.e.

Resistance = % change from control = $[(x_{treated} - x_{contr.})/ x_{contr.}] \bullet 100\%$

where x is mg CO_2/kg h for respiration and DPM (decays per minute) for bacterial and fungal growth rate measurement.

Respiration

Field moist soil from each plot was divided into 200 g subsamples and each of them was placed in a ± 600 ml glass flask. The samples were divided into three sets. One set was treated with lead nitrate (1000 mg Pb kg^{-1} dw), the second with NaCl (6.67 g kg^{-1} dw) and the third one was a control. Samples were then stored in darkness at 20°C.

Carbon dioxide production was measured with GC apparatus (Carlo ERBA Instruments). 24 hours before each measurement lucerne meal was added to each sample and mixed thoroughly, to offset nutrient limitations (3.2 mg C g^{-1} dw).

Microbial growth rate under stress

Portions of 50 g sieved soil were placed into plastic pots. The same treatments as in case of respiration measurement were applied but without enrichment with lucerne shoot material before measurements. Appropriate portions of soil were taken from the pots directly before analysis.

Bacterial growth rate

Bacterial growth rate we measured as the rate of incorporation of radioactively labelled thymidine into bacterial DNA, according to the microcentrifugation method described by Bååth et al. (2001). Thymidine is incorporated only by bacteria and not by eukaryotes like fungi. Measurement was performed as follow: 5 g of soil was shaken with 40 ml of distilled water in plastic tubes, then centrifuged for 10 min (3000 rev min^{-1}) and filtered thought glass wool. Supernatants (1.5 ml) were placed into eppendorf tubes. Samples were incubated for two hours at 20°C with 3.75 µl radioactively labelled thymidine (diluted with miliQ-water 1:3) (methyl[^{3}H]thymidine, 925 GBq $mmol^{-1}$, Amersham, U.K). Incorporation, washing of

excess tracer, and measurement of radioactivity incorporated by actively growing bacterial cells were described in detail by Bååth et al. (2001).

Fungal growth rate

Fungal growth rate we measured as the incorporation rate of radioactively labelled acetate into ergosterol according to the method described by Bååth (2001). Ergosterol is a fungus specific substance. Soil samples (1 g) were placed into glass tubes and incubated overnight with 1.5 ml water and 0.5 ml acetate solution (0.48 ml of non-radioactive acetate and 0.02 ml of ^{14}C acetate solution ([1.2.-^{14}C] acetic acid, sodium salt, 2.07 GBq mmol $^{-1}$, Ammersham, UK)). To stop the incorporation 1 ml of 5% formalin was added, the test tubes were centrifuged, and the supernatant (with nonincorporated acetate) was discarded. The ergosterol was then extracted and measured using high-performance liquid chromatography. The ergosterol peak was collected into scintillation vials, and the amount of radioactivity incorporated into ergosterol was then measured on a scintillator (Beckman liquid scintillation spectrometer).

Because of lack of well established conversion factors for both analyses the results are expressed in DPM units (decays per minute).

Statistical analysis

Results of chemical analysis were not normally distributed and we used a non-parametric test Spearman rank correlation to analyse correlation of respiration and metal concentrations. For other analyses we used ANOVA with Cu, pH and time as fixed factors. In the statistical analyses the average change (usually reduction) of all three sampling time points (24 h, 2 weeks and 2 months) was taken as the measure of resistance. A significant effect of time indicates resilience. Some of the results of respiration, bacterial and fungal growth rate were Log_{10} transformed in order to obtain a normal distribution.

Results

Primary stress: copper and pH

Before the application of the secondary stressors, the field soil were analysed with respect to respiration and microbial (bacterial and fungal) growth rates.

The respiration rates in the control soils were significantly different between soils with different pH ($P = 0.004$). With neutral pH (6.1) they were 20.6 ± 0.78 while in acid soils they were 17.3 ± 0.85 mg CO2 kg $^{-1}$ h $^{-1}$. There were no statistically significant differences between the copper treatments.

Table 1. Acidity and concentrations of bioavailable fraction of heavy metals in soils without addition of lead.

Factor	Treatment	Day 1	Day 14	Day 60
		Mean (SEM)	Mean (SEM)	Mean (SEM)
pH	Cu 0; pH 6.1	5.60 (0.04)	5.05 (0.03)	4.61 (0.28)
	Cu 0; pH 4.0	4.15 (0.02)	4.07 (0.10)	4.15 (0.10)
	Cu 750; pH 6.1	5.34 (0.06)	5.55 (0.03)	5.59 (0.05)
	Cu 750; pH 4.0	4.53 (0.06)	4.05 (0.09)	4.66 (0.28)
Cu	Cu 0; pH 6.1	0.26 (0.09)	0.31 (0.07)	2.60 (1.49)
	Cu 0; pH 4.0	1.06 (0.05)	n.d.	0.11 (0.01)
	Cu 750; pH 6.1	0.77 (0.09)	n.d.	0.09 (0.01)
	Cu 750; pH 4.0	5.29 (0.44)	5.87 (1.34)	2.23 (1.40)
Pb	Cu 0; pH 6.1	9.29 (0.58)	10.97 (0.95)	52.25 (25.49)
	Cu 0; pH 4.0	197.96 (31.79)	n.d.	8.61 (1.45)
	Cu 750; pH 6.1	6.36 (1.07)	n.d.	0.31 (0.05)
	Cu 750; pH 4.0	120.15 (15.69)	126.00 (23.47)	97.83 (79.56)

n.d. – not determined

The bacterial growth rates were not affected by either of field treatments. Fungal growth rates were significantly higher in acid than in neutral soils ($P < 0.0001$) (407.1 ± 16.73 and 306.0 ± 18.65 DPM for pH 4.0 and pH 6.1 respectively), while no differences between copper treatments were found.

Secondary stress

The effects of the secondary stresses were expressed as the relative (%) change compared with the original value before the secondary stress was applied.

Lead

Respiration

The addition of lead decreased the respiration rate (induced by addition of lucerne meal) in all soils (Fig. 1A). Communities from acid soils were less resistant to lead than those from neutral soils (ANOVA, $P = 0.005$). The respiration rates were reduced by on average 37.4 ± 4.48% in pH 4.0 soils, compared with 20.5 ± 3.27% (mean ± standard error) in pH 6.1 soils. Soils not contaminated with copper were more resistant to lead than those with copper: average reductions were 23.7 ± 4.10% and 34.2 ± 4.61% in Cu 0 and Cu 750, respectively (ANOVA, $P = 0.056$). The most vulnerable, to addition of lead, were communities from the acid soils with high copper level that showed a reduction in respiration of 47.4 ± 5.91%.

There was no sign of resilience (recovery to pre-stressed level) during two months following stress addition in any of the four treatments (ANOVA, P = 0.67).

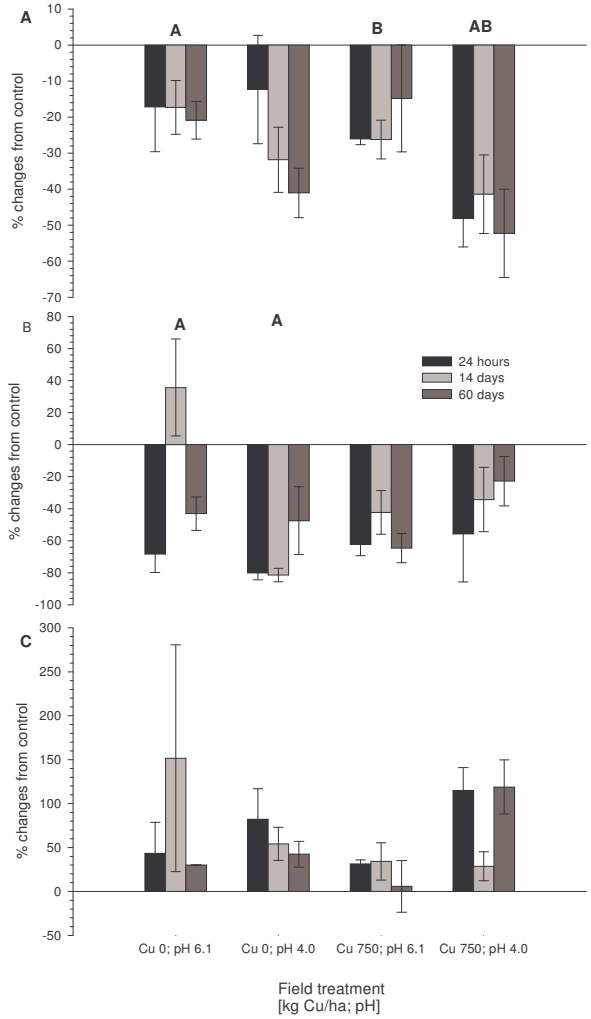

Figure 1. Effect of stress, in the form of lead, at increasing time intervals following application of stress (mean ± SEM) on the ability of microbial communities (inhabiting soils that differ in level of pollution) to decompose lucerne residues (A) and on the growth rates of bacteria (B) and fungi (C). Means of three time points with the same letters are significantly different (Tukey's HSD, $p<0.05$). 0% represents no change from control level.

These results should be treated with caution however. Although the same amount of $Pb(NO_3)_2$ (1000 mg Pb kg^{-1} dw) was added to each soil, the interplay between soil pH and metals caused changes in the concentrations of the bioavailable fraction of lead (table 1 & 2). Thus the doses of applied stress were different among treatments. The lead addition also created an increase in acidity and increased concentrations of bioavailable copper which might also be a confounding stress factor in the examined communities. Note that actual pH values at the time of sampling were lower than the experimentally aimed 'target' values. In this study we will use the target values to identify treatments. Figure 1A shows changes in respiration rate in respect to target values of copper and pH while Figure 2 – shows the

relationship between changes in respiration rates and measured pH as well and as concentrations of copper and lead.

Table 2. Acidity and concentrations of bioavailable fraction of heavy metals in soils to which stress, in the form of lead (1000 mg Pb kg -1) was added.

Factor	Treatment	Day 1	Day 14	Day 60
		Mean (SEM)	Mean (SEM)	Mean (SEM)
pH	Cu 0; pH 6.1	5.83 (0.03)	5.30 (0.02)	4.90 (0.31)
	Cu 0; pH 4.0	4.55 (0.13)	4.45 (0.05)	4.63 (0.16)
	Cu 750; pH 6.1	5.82 (0.12)	5.73 (0.04)	5.61 (0.11)
	Cu 750; pH 4.0	4.83 (0.06)	4.43 (0.14)	4.95 (0.23)
Cu	Cu 0; pH 6.1	0.22 (0.05)	0.31 (0.07)	1.34 (0.66)
	Cu 0; pH 4.0	0.53 (0.07)	n.d.	0.05 (0.00)
	Cu 750; pH 6.1	0.78 (0.08)	n.d.	0.07 (0.01)
	Cu 750; pH 4.0	2.07 (0.28)	2.32 (0.43)	1.06 (0.42)
Pb	Cu 0; pH 6.1	0.20 (0.04)	0.41 (0.05)	0.05 (0.01)
	Cu 0; pH 4.0	0.05 (0.01)	n.d.	0.00 (0.00)
	Cu 750; pH 6.1	0.01 (0.01)	n.d.	0.00 (0.00)
	Cu 750; pH 4.0	0.22 (0.11)	0.36 (0.04)	0.05 (0.01)

n.d. – not determined

To investigate the impact of the two initial stresses, pH and Cu, on respiration rates Spearman Rank Order Correlation analysis was used. It showed that the resistance increased with soil pH ($P = 0.003$) (Fig. 2A) and decreased with concentrations of bioavailable copper ($P = 0.014$) (Fig. 2B). Resistance was also negatively correlated with the bioavailable lead concentrations ($P = 0.05$; Fig. 2C).

Bacterial growth rate

The bacterial growth rates were negatively affected by the addition of lead in all soils (Fig. 1B). A significant interaction between copper and pH indicates a difference in response between soils with and without copper contamination (ANOVA, $P = 0.03$). Without copper, bacterial growth rates were more reduced in the acid soils than in the neutral soils: 69.7 ± 8.44% versus 25.2 ± 18.5%. In copper-contaminated soils the pattern was reversed: smaller reductions in the acid soils (37.6 ± 12.3%) than in neutral soils (56.4 ± 6.25%). The differences in responses to stress were significant only between different pH levels in soils

not polluted with copper (p = 0.048, Tukey's HSD test). On overall, the bacterial growth rates showed a significant resilience (P = 0.02).

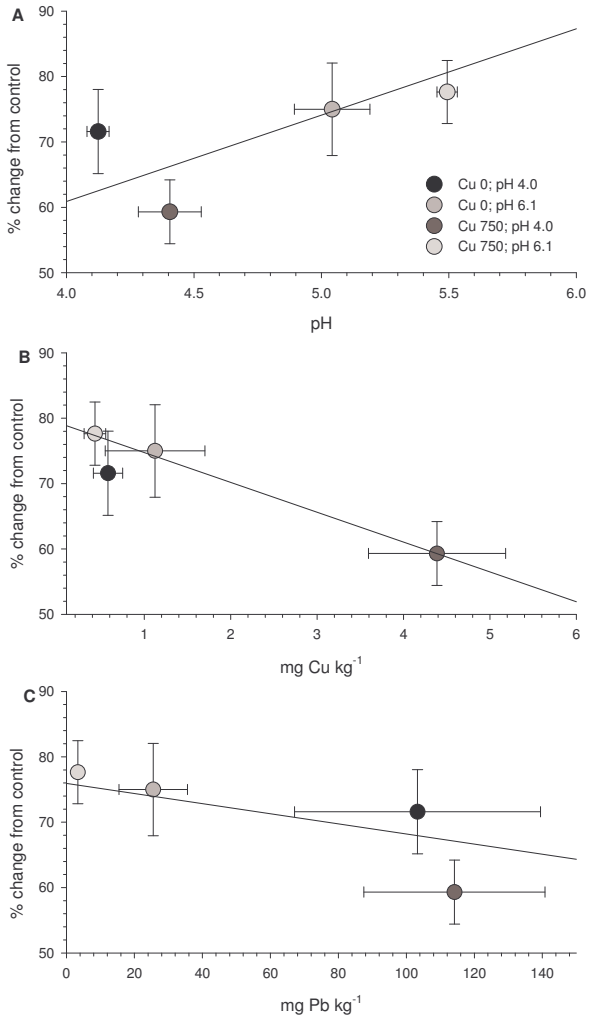

Figure 2. Relationships of changes in respiration rates in soils on which stress, in the form of lead, was applied and measured soil pH (A) and bioavailable metals concentrations copper (B) and lead (C). Points represent means of all replicates and bars standard error. 100% represents no change from control level.

Fungal growth rate

Fungal growth rates increased in all treatments (Fig. 1C). There were no significant differences between treatments (ANOVA Cu: P = 0.67, pH: P = 0.09). There was also no significant resilience in all treatments (ANOVA, P = 0.67).

Salt

Respiration rate

Similarly to lead, addition of salt reduced respiration rates in all soils. Differences in pH and copper levels did not create distinctions in resistance to the applied salt stress.

However, soils not contaminated with copper showed higher resilience than copper-polluted soils (ANOVA, interaction Cu x time, P = 0.02) (Fig. 3A).

Bacterial growth rate

The response of bacterial growth rate to the addition of salt depended on the initial field treatment. Soil acidity had the strongest influence on the resistance of the bacterial growth rate to salt (P < 0.0001). In acid soils (pH 4.0) bacterial growth rates decreased with on average 48.3 ± 8.96% whereas in neutral soils (pH 6.1) growth rates increased with 122.8 ± 34.46% (Fig. 3B). The interaction between copper and pH was on the level of statistical significance P = 0.06, which suggests a difference in response between soils with and without copper contamination. In non contaminated soils (Cu 0) the difference between the increase in neutral soils (136.1 ± 33.92%) and the decrease in acid soils (60.1 ± 9.83%) was stronger than in copper contaminated soils (Cu 750) where the increase was 109.6 ± 62.07% at pH 6.1 and the decrease was 37.8 ± 14.10% at pH 4.0. In the least polluted soils (Cu 0; pH 6.1) growth rates overshoot control values already 24 h after addition of salt. At the same time reduction (50.9 ± 11.00%) occurred in neutral soils with high copper load (Cu 750; pH 6.1), which was followed by strong increase in growth rates (140.3 ± 48.59% and 239.2 ± 148.45% two weeks and two months after stress addition respectively). In all soils there was a strong resilience of bacterial growth rates (P = 0.002).

Fungal growth rate

Effects of salt on fungal growth rate showed a response pattern reverse to that of the bacterial growth rate. The fungal growth rates increased in all soils except the soils non-polluted with copper with neutral pH (Cu 0, pH 6.1), in which there was only a non significant reduction of 1.8 ± 11.0% (Fig. 3C). Increases were stronger in acid soils (200 ± 35.9%) than in neutral soils (22.0 ± 11.0%). The results indicate that fungi performed better in soils where bacterial growth rate was suppressed. This was confirmed by a negative correlation between changes in fungal and bacterial growth rates in soils treated with salt (R = - 0.59, P < 0.001). When lead was applied such a negative correlation was not found (R = 0.04, P = 0.84).

Discussion

The aim of that study was to find whether long term exposure to increased copper level and/or lowered soil pH impair functional stability of soil community.

It has been suggested that environmental stress can alter an ecosystem to such an extend that supplementary stress will have stronger impacts than in an unstressed system (Stone *et al.* 2001). On the other hand adaptation can increase tolerance and thus make

stressed system more resistant to additional stress (Odum 1981). The results of our study are in line with the first statement and show that long term exposure of soil microbial communities to stresses such as copper and low pH reduce the functional stability of the soil ecosystem. Although, the effects of secondary stress depended on the nature of the stress (lead or salt) and on the parameter measured (lucerne decomposition or bacterial growth rate), in general the highest resistance and/or resilience were found in the least contaminated soils with neutral pH and/or no copper load.

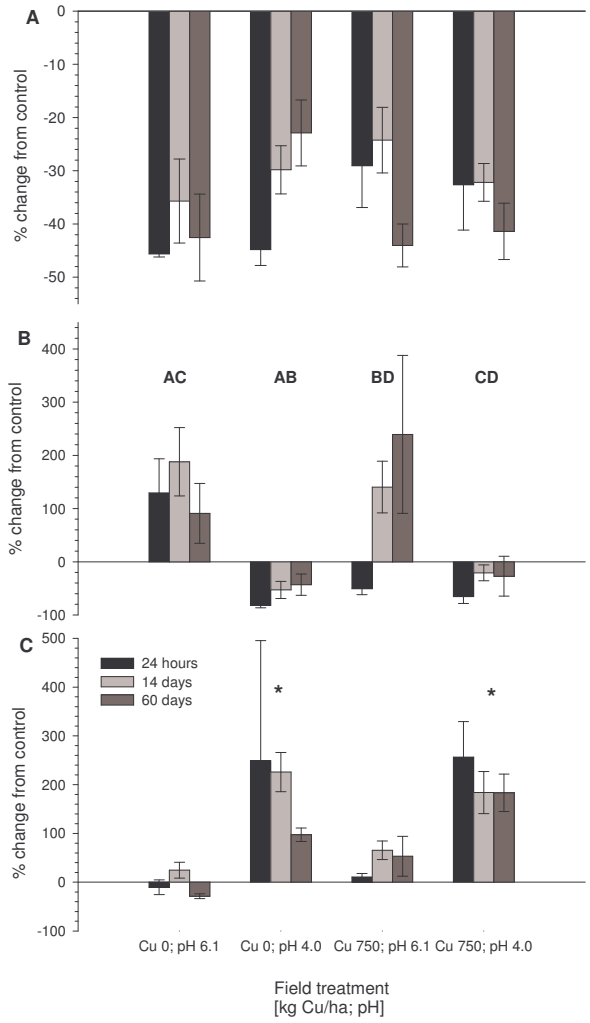

Figure 3 Effect of stress, in the form of salt, at increasing time intervals following application of stress (mean ± SEM) on the ability of microbial communities (inhabiting soils that differ in level of pollution) to decompose lucerne residues (A) and on the growth rates of bacteria (B) and fungi (C). Means of three time points with the same letters are significantly different whereas with the symbol (*) are not significantly different (Tukey's HSD, $p<0.05$).). 0% represents no change from control level.

Effects of stress on microbial respiration

The second stress, lead or salt, caused a reduction in decomposition of lucerne meal in all soils but each of them induced a different pattern of response. Addition of lead caused

stronger reductions in more polluted soils than in clean ones. Our results are comparable with those obtained by Griffiths et al. (2000) despite that the first stress was present in our soils for more than twenty years. These authors found that soils not/ or shortly fumigated (more diverse) were more resistant to addition of copper and more resilient to brief heating. In their experiment as well as in ours, none of the soils showed significant recovery of respiration after application of heavy metals. This indicates that heavy metals constitute a strong persistent stress which prevents resilience (Griffiths et al. 2000). In another experiment et al. (2001a) found greater resilience of polluted industrial soils after addition of copper, compared with non polluted controls. They suggested that these polluted soils were characterised by extreme versatility of inhabiting communities. However, these soils were not polluted by metals but by oil. It is plausible that pollutants like petroleum products do not reduce soil communities to the point where metal tolerant species would have disappeared. Because oil is food for microbes it may increase rather than decrease bacterial numbers and diversity. Van Munckgof et al. (1998) observed in diesel contaminated soil near a fuel station higher bacterial numbers, higher bacterial growth rates, and higher numbers and diversity of nematodes.

Interpretation of the results becomes more complicated when data about metals' bioavailability are taken into consideration. The interplay between metals and pH after lead addition caused enhancement of primary stress (increase in bioavailable copper and drop in pH) and created differences in concentrations of bioavailable lead. The strongest increase in bioavailable copper was noted in the most polluted soil in which inhibition of decomposition was strongest. Therefore, it is difficult to distinguish whether the strongest reduction in respiration in these soils was due to lowest resistance or strongest stress. These difficulties do not apply to salt stressed soil.

When salt was applied, all communities appeared to have similar resistance but different resilience of respiration. Soils without copper contamination showed an average resilience from - 45% to - 32% two weeks after stress addition and it was on the same level after two months. At the same time a progressive reduction in decomposition occurred in copper contaminated soils (from 31% reduction after 24 hours to 43% reduction after two months from the addition of salt). This may indicate that in copper-free soils, killing of salt sensitive species lessened the competition (Sardinha *et al.* 2003) and caused a sudden release of bioavailable carbon from dead bacterial cells. Such rapid food supply could stimulate activity of resistant species that could then compensate for the loss of others. It is likely that salt-resistant species were present at too low quantity to maintain function in copper-contaminated soils. Another explanation can be that detoxification of copper is so energetically costly that prevents recovery from additional stress.

Effects of stress on bacterial growth rate

Bacterial growth rate and total soil respiration in both cases (lead and salt) gave different patterns of response to the second stresses. The growth rates of bacteria were more reduced than the soil respiration. Bacterial growth rate also showed a quite high resilience (recovery) while resilience of respiration was observed only after salt stress and then only in the soils without copper contamination. This difference in response patterns between respiration and bacterial growth rates might be related to differences in treatment of soils used for both measurements. Lucerne meal was added only to the soils in which respiration was measured. This was necessary to ensure sufficient response within 24 h. Because not all organisms can utilise this material with the same efficiency, only the activity of responsive microorganisms is measured. The relatively high amount (3.2 mg C g^{-1} soil) of plant residues added probably caused a selection towards opportunists. In contrast, bacterial growth rate was measured within 2 h after adding a radioactive tracer ([^{3}H]thymidine) to subsamples of the soil. The low concentration of thymidine (0.1 µM) and the short incubation time prevent changes in growth rate during incubation. So the measured growth rate reflects the actual growth rate of the total soil bacterial community.

The bacterial growth rates were strongly reduced by addition of lead in all soils. Bacterial growth rates were more reduced at pH 4.0 (70%) than at pH 6.1 (25%), but the difference was significant only in soils without copper added. Opposite to respiration, bacterial growth rate showed some resilience. It might be because bacterial communities which were already adapted to the primary stress (Cu), developed during the incubation period tolerance to the secondary stress (Pb) (Díaz-Raviña and Bååth 2001, 1996; Díaz-Raviña *et al.* 1996; Leyer and Johnson 1993; Witter *et al.* 2000). This would be in contrast with observations of Díaz-Raviña *et al.* (1994) who showed that communities from copper-contaminated agricultural soils developed (co-)tolerance to copper, cadmium, zinc and nickel but not to lead. They also showed that lead-contamination did not induce tolerance to lead even after six months of incubation. They claimed that development of lead tolerance occurred but that this process was very slow. Frostegård *et al.* (1993) supported the results of Diaz-Ravina *et al.* (1994) by showing that exposition to Cd, Pb, Zn and Ni induced similar changes in the PLFA pattern (microbial community structure), while the effect of Cu addition on PLFA differed from the effects of the other metals. This implies that copper and lead select for organisms with different mechanisms of detoxification and therefore development of co-tolerance is unlikely. Nevertheless the metal concentrations used by Diaz-Ravina *et al.* (1994) are much higher (16-64 mmol/kg) than in our study (5 mmol/kg). The experiments of Frostegård *et al.* (1993) showed that significant differences in PLFA composition occurred only when doses of 32 mmol Pb/kg of soil or higher were applied. So it is not unlikely that our dose was not strong enough to prevent development of lead tolerance.

The effects of salt application on bacterial growth rate were strongly affected by soil acidity. In acid soils, although significant recovery occurred, the primary stress did not allow growth rate to reach pre-stressed level. At the same time salt seemed to stimulate bacterial growth rate in neutral soils rather than reducing it. It has been reported that bacteria exposed to pH 5.8 are able to develop resistance e.g. to osmotic stress (Leyer and Johnson 1993). So it is likely that our soils with pH 6.1 could develop such resistance and thus were able to deal with salt stress. Both salinic and osmotic stresses kill sensitive bacteria and cause release of easily available carbon from their cells. Since growth of soil bacteria is usually carbon limited (Aldén *et al.* 2001) such a sudden increase in food availability is beneficial for organisms that are more resistant and can stimulate their growth. This mechanism may have occurred in all investigated soils, especially when it is assumed that the bacteria from acid soils were more burdened by detoxification of primary stress and thus could not multiply as rapidly as microbes from neutral soils. The decrease in the bacterial growth rate 24 hours after stress addition in neutral, copper-contaminated soil may indicate that the presence of copper constitutes an extra burden for the energy budget of the inhabiting soil organisms. However, it seems that neither copper nor salt was strong enough to prevent recovery and even stimulation of growth.

Thus, after lead addition bacterial growth rate was more reduced at pH 4.0 (70%) than at pH 6.1 (25%), although the difference was significant only in uncontaminated soil. After addition of salt bacterial growth rate showed lower resistance at pH 4.0 (48% decrease) than at pH 6.1 where growth rate increased by on average 123%. The increase at neutral pH was faster in uncontaminated soil (Cu 0) than in contaminated soil (Cu 750) where the growth rate initially decreased and then increased. These results support the hypothesis that already stressed communities are more sensitive to secondary stress.

Effects of stress on fungal growth rate

It has been argued that fungi are more resistant to environmental stresses than bacteria (Cooke and Whipps 1993; Frostegård *et al.* 1996). This was confirmed by results of fungal growth rates in soils with only primary stress where values of incorporation were higher in acid soils in which bacterial growth rates were low. Also results of "stress on stress" experiment gave support for that argument since the fungal growth rates increased rather than decreased by presence of both lead and salt. Nevertheless the stressing factors may have affected fungal growth rate indirectly. The relatively higher resistance of fungi compared with bacteria, can be due to their lower surface to volume ratio which results in lower diffusion of contaminants into cells. They are large enough to detect differences in intensity of attractants/ repellents at different points of their surface. Fungi perform better in direct movement or growth towards desirable sites or away from harmful condition so they can

better avoid contact with toxicant. Damaged hyphae may be evacuated and sealed of and conferring ability for repair and defiance (Cooke and Whipps 1993).

Fungi interact with bacteria in many ways. Since bacteria are a great sink for nutrients in soil they are capable of subjecting fungi to severe competition for substrate (e.g. carbon). Also some bacteria can attach to fungal hyphae and feed on them. Others can suppress fungal performance by producing antibiotics (antagonism). Therefore stress, by killing bacteria, reduces the competition for food as well as antagonisms between those two groups and thus it makes the environment more suitable for fungi. Owing to their ability to feed on death bacterial cells, fungi have then an additional source of food (Cooke and Whipps 1993) and can increase their growth.

After addition of lead and salt, fungi performed better in soils in which bacterial growth rates were inhibited. A significant negative correlation was found after salt addition but not after lead addition. This might be due to stronger differences in responses to addition of salt between treatments; when lead was added, bacterial growth rates were inhibited in all soils, whereas addition of salt strongly stimulated this process in neutral soils and caused strong inhibition in acid ones.

Stability of food web

Field observations and theoretical models have indicated that the structure of soil food webs in terms of energy channels is important to stability (Moore and de Ruiter 1991; Moore and Hunt 1988; Moore *et al.* 2003). Field observations have suggested that the fungal channel has a lower resilience (Moore and de Ruiter 1991) while the theoretical models have predicted that stability is enhanced when the relative importance of the two channels become more in balance (Moore *et al.* 2003). The present results did not confirm these notions. In all soils bacterial were by far the most dominating group of microbes and the response of the fungi seemed importantly influenced by the response of the bacteria. Also, for an adequate test of the importance of energy channels data are required on the biomasses and growth rates of microbes, their consumers and top predators before and after the applications of stress.

Conclusion: The effects of stress on stability

Regardless the technique used to measure stability it was shown that lead and salt induced different changes in examined processes. This is probably due to fact that each of them exerts selection pressure for organisms with specific properties. This could imply that there is no “general” stability to stresses and/ or perturbations but rather stability to particular ones. Nevertheless, changes in functional parameters after lead addition are difficult to interpret when the effects of interactions between metals and pH are taken into consideration. It is not unequivocal whether the stronger responses in more contaminated soils were caused

by lower resistance of inhabiting communities or by stronger stress. It is not unlikely that if lead would be applied in doses that would result in equal bioavailable concentrations, the changes in investigated parameters would induce similar changes like after addition of salt.

Changes in respiration and bacterial growth rates due to both lead and salt addition seem to confirm the hypothesis that less stressed environments constitute better conditions for inhabiting organisms and thus enhance functional stability (Griffiths *et al.* 2001a). Taking into consideration that stress tends to decrease biodiversity (Giller *et al.* 1998) one can also argue that the results support the so-called "insurance hypothesis of biodiversity" which states that more diverse systems are more stable to perturbation (Aarts and Nienhuis 1999; Loreau 2000; Naeem and Li 1997). On the other hand, the results can be interpreted also with respect to energy budget (Congdon *et al.* 2001; Sibly and Calow 1989). Organisms inhabiting non- or mild-stressed environments are not or only weakly loaded by costs of detoxification and this load increases with increase of contamination level (Stone *et al.* 2001). Such costs can be e.g. production of metallothioneins or Heat Shock Proteins (HSPs), exclusion of contaminants etc. (Bruins *et al.* 2000). Both biodiversity and energy budgets may be important for maintaining ecosystem processes. The mechanisms behind a reduced functional stability in contaminated soils, such as reduced biodiversity and changes in adaptations, energy allocation, quality and availability of food etc. are beyond the scope of this study and need further investigation.

Acknowledgements

We thank M.R. Veninga, A. Vos, P.R. Bolhuis and W. Schuurmans for technical assistance.

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Chapter 6

Functional stability of microbial communities from long-term stressed soils to additional disturbance

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Environmental Toxicology and Chemistry 25(8): 1993-1999

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Functional stability, measured in terms of resistance and resilience of soil respiration and bacterial growth rate, was studied in soils from field plots that have been exposed to copper contamination and low pH for more than two decades. We tested whether functional stability follows patterns predicted by either the “low stress – high stability” or the “high stress – high stability” hypothesis. Treatments consisting of soils with no or high copper load (0 or 750 kg Cu/ha) and low or neutral pH (4.0 or 6.1) were used. Stability was examined by applying an additional disturbance by heat (50°C for 18 h) or drying-rewetting cycles. After heating, respiration indicated that the Cu 0 soils were less stable (more affected) than the Cu 750 soils. Bacterial growth rate was more stable (resistant) to heat in the pH 6.1 than in the pH 4.0 soils. Growth rate was stimulated rather than inhibited by heating and was highly resilient in all soils. Respiration was less affected by drying-rewetting cycles in the pH 4.0 soils than the pH 6.1 soils. Bacterial growth rate after drying-rewetting disturbance showed no distinct pattern of stability.

We found that the stability of a particular process could vary significantly depending on the kind of disturbance, and therefore neither of the two hypotheses can adequately predict the response of the microbial community to disturbance.

Keywords: disturbance, functional stability, respiration, bacterial growth rate

Introduction

An ecosystem is considered to be stable when it is able to withstand a stress or disturbance and to return to its normal state after temporary deviation from it (McNaughton 1994; Griffiths *et al.* 2000; Wardle *et al.* 2000). Stability defined as such has two components that can be measured: resistance – the inherent capacity of the system to withstand stress /disturbance; and resilience – the ability to recover after stress/disturbance.

Two contrasting concepts regarding stability of ecological processes exist; the first one predicts that non stressed systems are more stable since they possess a large variety of resources that allow them to maintain function in case of stress (McNaughton 1977; Doak *et al.* 1998; Yachi and Loreau 1999). The other predicts that stressed systems are more stable because due to long-term exposure to a stressor, they gained abilities (adaptation, physiological changes) to cope with stress and thus maintain function (Odum 1981).

Griffiths *et al.* (2001b) found that the functional stability of soil microbial communities, measured in terms of respiration, is not directly related to the biodiversity but rather to the combination of consecutive stressing factors (Griffiths *et al.* 2000; 2001a). In a previous study (Tobor-Kapłon *et al.* 2005), we applied the 'stress on stress' approach as used by Griffiths *et al.* (2000) to test whether long term exposure to an elevated copper concentration or an altered soil pH or both, affects the functional stability of microbial communities to stress. In that study, functional stability is measured by looking at the response of soil respiration and bacterial growth rate to an additional stress, in this case lead or salt. It appeared that polluted soils were less resistant and/or resilient than not- or less-polluted soils, which supports the first theory of stability which says that exposure to stress reduces the functional stability of the soil ecosystem.

The aim of the present study is to investigate the effects of long-term exposure to stress (copper and/ or low pH) on the functional stability of microbial communities to disturbance. Disturbance has been defined as an environmental event that is constrained in time but may have a lasting (positive or negative) impact on microbes. Stress, on the other hand, is a chemical, physical, or biological condition that has a continuous adverse impact (Degens *et al.* 2001). In contrast to stress, whihch impact on stability was tested in our previous study (Tobor-Kapłon *et al.* 2005), disturbance is not present any longer in the soil during measurements of resistance and recovery. Stability to disturbances may rely on other mechanisms than stability to stress.

Soil respiration and bacterial growth rate were chosen because they represent major ecological processes and are regularly used to show the impact of stress on soil microbial communities (Bååth 1989; Díaz-Raviña *et al.* 1994a; 1996; Bååth *et al.* 1998a; Díaz-Raviña and Bååth 2001; Bloem and Breure 2003; Rajapaksha *et al.* 2004). Respiration rate represents the response of the whole soil microbial community, including fungi and bacteria.

Respiration, however, is not very sensitive (Bååth 1989; Díaz-Raviña *et al.* 1994a; Brookes 1995; Díaz-Raviña *et al.* 1996; Bååth *et al.* 1998a; Díaz-Raviña and Bååth 2001; Bloem and Breure 2003; Rajapaksha *et al.* 2004), and it can either increase or decrease in response to stress (Bååth 1989). Bacterial growth rate, on the other hand, is specific for bacteria and very sensitive (Brookes 1995; Bloem and Breure 2003; Rajapaksha *et al.* 2004). Rajapaksha *et al.* (2004) showed that addition of 128 mg Zn/kg soil caused 30% reduction in respiration and 90% in bacterial growth rate. The reduction of bacterial growth rate is measurable instantaneously after application of stress (Díaz-Raviña and Bååth 1996; Rajapaksha *et al.* 2004), as well as after many years (Pennanen *et al.* 1996; Boon *et al.* 1998).

Two types of disturbance, a heat shock and drying-rewetting cycles, were applied. The soils that have been exposed to elevated copper concentrations and altered soil pH for more than two decades. Since in our mild climate temperatures are rarely very high and rain is relatively frequent, we assumed that these soils are not adapted to the applied disturbances.

Materials and Methods

Experimental area and sampling

Soil samples were collected from an experimental arable field known as Bovenbuurt or Wildekamp, located 3 km NNE of Wageningen, the Netherlands. In 1982 the field was divided into 128 plots (6 x 11m each) organized in eight blocks (replicates). Each block consists of 16 plots on which different treatments were applied. Each treatment is a combination of different copper load and pH level. Copper was applied once as $CuSO_4 \cdot 5H_2O$ powder at loads of 0, 250, 500, and 750 kg Cu/ha, pH (KCl) was adjusted to values of 6.1; 5.4; 4.7, and 4.0 by addition of lime or sulphur (Korthals *et al.* 1996). Every six years the pH was checked and readjusted if necessary. Present concentrations of heavy metals and pH are given in Table 1. Except in the most acid plots, the actual pH values are lower than the experimentally aimed 'target' values. This occurred because the last pH readjustment was done in 2001, and since then, the lime used for pH adjustments leached down. Therefore, soil pH returns slowly to the normal value of this soil, i.e., approximately 4.5. For consistency we use the target values to identify the treatments.

The soil is a fimic anthrosol with a texture of 3% clay, 10% silt, 87% sand, an organic carbon content of 21 g/kg soil and a CEC (cation exchange capacity) of 5.6 $cmol_c$ /kg (ammonium-acetate) (Korthals *et al.* 1996; Boon *et al.* 1998).

For our experiments we chose four treatments: combinations of the two extreme copper loads (0 and 750 kg Cu/ha; further called Cu 0 and Cu 750 soils) and the two extreme pH levels (pH 4.0 and 6.1).

For the first experiment (heating) samples were taken in October 2003 and for the second (drying-rewetting) in February 2004. The two sampling dates differed in weather

conditions. The average soil temperature at 10 cm below surface in the week preceding the first sampling was 5.3°C and before the second sampling it was 9.6°C. At 5 cm depth it was 4.5 and 7.1°C before the first and second sampling respectively (www.met.wau.nl). Soil moisture content, measured as loss of weight at 105°C, was 12.2% and 15.5% for the first and the second sampling, respectively.

Samples were collected from the first four blocks. From each plot 15 soil cores, 4 cm in diameter and 10 cm depth were taken. Samples, from one plot, were mixed to obtain one bulk of soil, immediately sieved (0.5 cm mesh) and prepared for analyses.

Table 1. Actual values of pH (1M KCl) and copper concentrations measured after extraction with 0.01M $CaCl_2$ and Aqua Regia (mean ± (SEM)).

Cu	pH		pH (1M KCl)		mg Cu/kg (0.01M $CaCl_2$)		mg Cu/kg (Aqua Regia)	
(kg/ha)	(nominal)	N	Mean	SEM	Mean	SEM	Mean	SEM
0	4	4	4.1	0.12	0.49	0.15	37.7	7.05
	6.1	4	5.1	0.20	0.07	0.02	40.3	8.61
750	4	4	4.1	0.10	2.40	0.22	113.6	5.63
	6.1	4	5.3	0.48	0.31	0.08	85.0	6.56

Preparation of samples

For either experiment field moist soil from each plot was divided into two subsamples (± 800 g) and placed into 2 L glass jars. Moisture was adjusted to 60% of the maximum water holding capacity (WHC) and the soil was stored for two weeks in darkness at 20°C.
In the first experiment one set of subsamples was heated for 18 h at 50°C (experiment 1). In the second experiment (drying-rewetting; experiment 2), one set was treated with eight drying-rewetting cycles (Degens *et al.* 2001). Each cycle consisted of 24 h of drying at 25°C in an oven with constant air flow followed by rewetting (gravimetrically) to 60% water holding capacity (WHC) with deionized water. The second set of subsamples in either experiment served as an undisturbed control. After treatment, samples were stored in darkness at 20°C until measurements were done at different points in time.

Functional stability

For stability analysis we used the approach of Griffiths *et al.* (2000). Due to absolute differences in the investigated parameters (respiration rate and bacterial growth rate) between

soils and sampling dates, the response was normalized to the control value (undisturbed soil), i.e.,

Resistance = % change from control = $[(x_{\text{treated}} - x_{\text{contr.}})/ x_{\text{contr.}}] \bullet 100\%$

where x is mg CO_2/kg h for respiration and DPM (decays per minute) for bacterial growth rate measurement.

In both experiments we used the repeated measurement design. In each experiment four field treatments were used, i.e., Cu 0 and pH 4.0, Cu 0 and pH 6.1, Cu 750 and pH 4.0, Cu 750 and pH 6.1; each treatment was replicated four times. Measurements were repeated over time, i.e., 24 h, two weeks and two months after disturbance. The response after 24 h was used as a measure of resistance and the change over time was adopted as a measure of resilience. The overall effect of a disturbance was the average response over the time of the whole experiment, i.e., the average of all three time points.

Respiration

Twenty-four hours before each measurement, 200g of soil from each jar was placed into ± 600 ml glass bottles and dry Lucerne (alfalfa) meal was added and mixed thoroughly. Lucerne was added in amounts corresponding to 3.2 g C/kg soil, which is sufficient to induce respiration and offset nutrient limitation. Lucerne meal contained 42% of carbon and had C/N ratio of 14 (L. Bouwman, Alterra, Wageningen, the Netherlands; personal communication).

For the first measurement samples were prepared as described above directly after termination of disturbance. Carbon dioxide production was measured with a gas chromatograph (GC) with a thermo conductivity detector (TCD; Carlo ERBA Instruments, Milan, Italy). For calibration three standards were used: air; mixture of O_2 (10%), N_2 (83%) and CO_2 (5%) (Hoek Loos, Schiedam, the Netherlands) and 10% CO_2 in helium (Matheson TRI-GAS, Twinsburg, OH, USA).

Bacterial growth rate

Samples for measurement of the bacterial growth rate were prepared at the same time as these for respiration measurement. We measured the bacterial growth rate as the rate of incorporation of radioactively labeled thymidine into bacterial DNA, according to the micro-centrifugation method described by Bååth *et al.* (2001). Thymidine is incorporated only by bacteria and not by eukaryotes like fungi because the latter lack the enzyme thymidine kinase.

For the measurement we used 5 g of moist soil. After extraction with 40 ml of Milli-Q® (Waters Chromatography B.V., Etten-Leur, the Netherlands) water, 1.5 ml samples of extracted bacterial suspension were incubated for two hours at 20^{o}C with 3.75 µl radioactively labelled thymidine (diluted with Milli-Q water 1:3) (methyl[^{3}H]thymidine, 925

GBq mmol-1, Amersham Bioscience, Buckinghamshire, UK). Incorporation, washing of excess tracer, and measurement of radioactivity incorporated by actively growing bacterial cells were described in detail by Bååth *et al.* (2001). Due to lack of conversion factor from ^{3}H activity to cells or units of carbon produced, the results are expressed in DPM.

Statistical analysis

For analysis of the resistance to disturbance, as measured after 24 h, we used the Univariate General Linear Model procedure with full factorial design and Cu and pH as fixed factors. For analyses of the resilience and the overall effect of a disturbance (including all three time points) we used the General Linear Model procedure (repeated measures) with Cu and pH as fixed factors and time as the repeated measure. All analyses were done using the SPSS 11.5.0 statistical package (SPSS for Windows, SPSS, Chicago, IL, USA).

Results

Control soils

In the undisturbed control soils, the respiration was significantly higher in the pH 6.1 than in the pH 4.0 soils ($p < 0.01$ in both experiments). No effect of copper on respiration rate was found in either of the experiments. In neither experiment was the interaction between copper and pH found to be significant. The respiration was higher in the soils sampled in October 2003 than in the soils sampled in February 2004 ($p = 0.002$) (Fig. 1A). It should be remembered that all measurements were performed after two weeks pre-incubation at 20°C and 60% WHC.

Similarly to the respiration, the ^{3}H activity (indication of bacterial growth rate) in the control soils was significantly affected by soil pH ($p = 0.035$ and $p < 0.0001$ in experiment 1 and 2 respectively). The bacterial growth rate was higher in the soils sampled in February 2004 than in the soils sampled in October 2003 ($p < 0.001$) (Fig. 1B).

Functional stability

Respiration

The resistance (response 24 h after disturbance) to heat was neither affected by copper nor by pH (Fig. 2A). The overall effect of heat was stronger in the Cu 0 soils (24.7 ± 10.9% decrease; mean ± standard error) than in the Cu 750 soils (7.7 ± 10.9% increase) ($p = 0.057$). Respiration in the Cu 0 soils was inhibited by 30.7 ± 15.2% during the first measurement 24 h after cessation of disturbance, while two months later the inhibition had decreased to 10.2 ± 8.3%. However, due to high variation this trend of recovery was not significant.

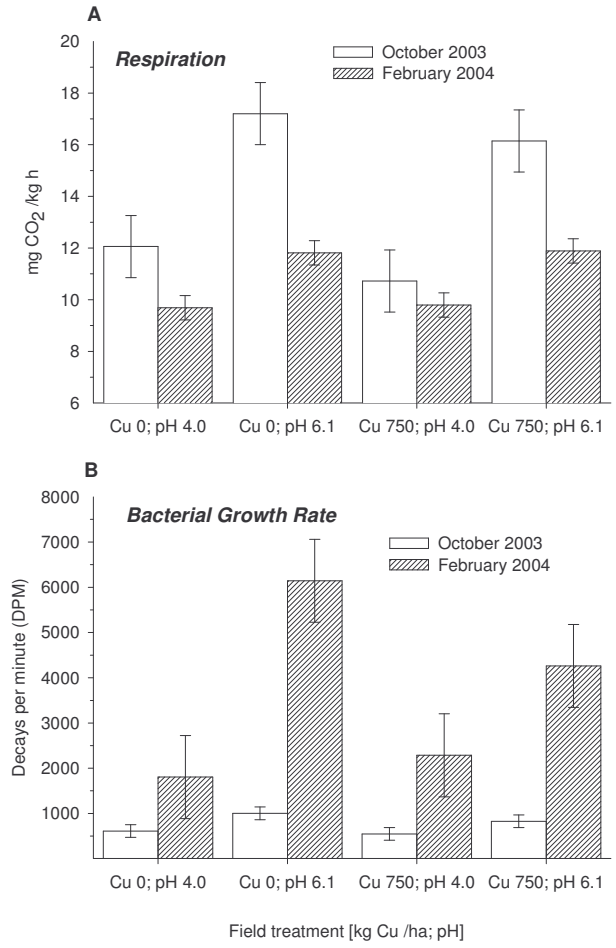

Figure 1. The respiration rate (A) and bacterial growth rate (B) in undisturbed control soils (mean ± standard error, $n = 4$). Open bars represent soils sampled in October 2003 and hatched bars represent soils sampled in February 2004.

The resistance to drying-rewetting cycles was neither affected by Cu nor pH. Respiration, on average during the experiment, was stimulated rather than inhibited by drying-rewetting cycles. Stronger stimulation was observed in the pH 6.1 soils than in the pH 4.0 soils (10.9 ± 2.9 and 0.6 ± 2.9% increase at pH 6.1 and pH 4.0, respectively) ($p = 0.028$) (Fig. 2B).

Bacterial Growth Rate

The resistance of bacterial growth rate to heat was stronger in the pH 6.1 than in the pH 4.0 soils (1.4 ± 11.9 vs 52.5 ± 11.9% reduction; respectively) ($p = 0.01$) (Fig. 3A). After initial reduction an increase of bacterial growth rate occurred in all soils ($p = 0.003$); after 24 h the growth rates were 27.0 ± 8.4% lower than in the control soils, and two weeks later they were 96.2 ± 29.6% higher than in the controls. Two months after disturbance, the average

response in the heated soils was still 57.4 ± 32.5% higher than in the control soils. The overall effect of heating was stimulation rather than inhibition.

The bacterial growth rate measured 24 h after the drying and rewetting cycles was neither affected by Cu nor by pH. The bacterial growth was stimulated in all soils during the course of the experiment. No significant effect of copper or of soil pH on the bacterial growth rate was found. However, the interaction between these two factors was significant (p = 0.04). A significant ($p < 0.01$) decrease of bacterial growth rates occurred over time following the last rewetting (Fig. 3B).

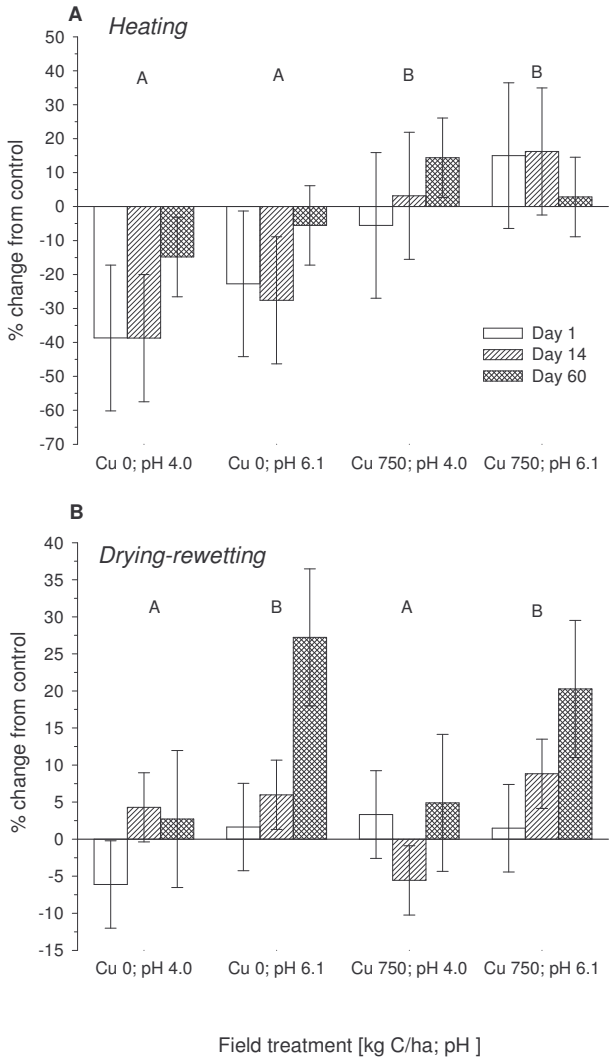

Figure 2. The stability of respiration to disturbance (heat shock (A) and drying-rewetting cycles (B)) in soils that differ in pollution levels (mean ± standard error, n = 4). Means of three time points with different letters are different at the level of significance p = 0.057 (heat) and p = 0.03 (drying-rewetting).

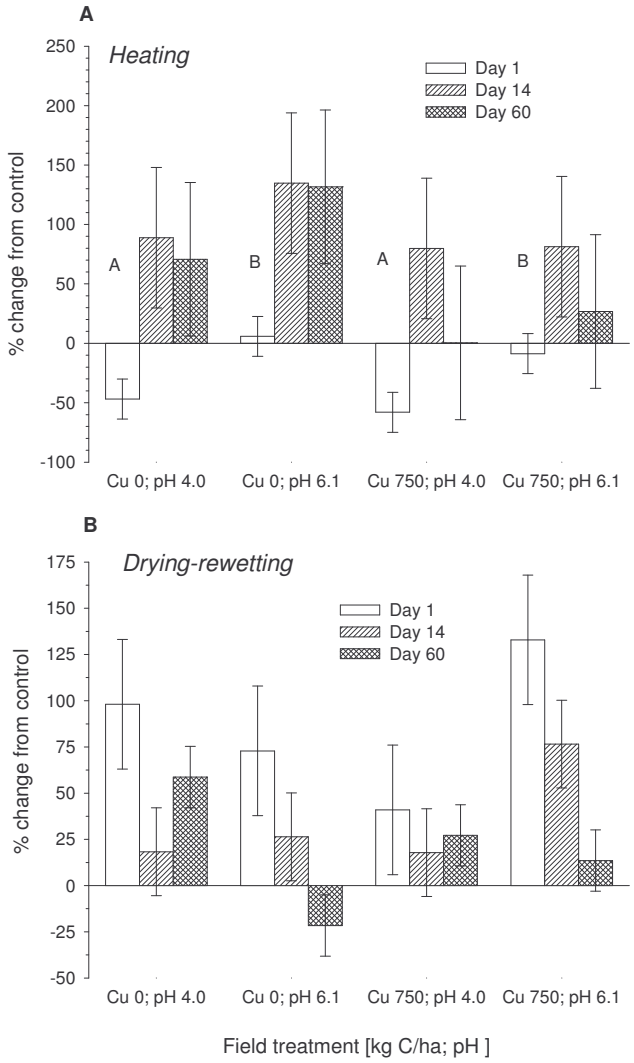

Figure 3. The stability of bacterial growth rate to disturbance (heat shock (A) and drying-rewetting cycles (B)) in soils that differ in pollution levels (mean ± standard error, $n = 4$). Different letters represent significant differences in response 24h after cessation of heat ($p < 0.05$).

Discussion

In this study, we investigated whether long term exposure to increased copper concentration and/or lowered soil pH, defined as stress factors, affects the functional stability (i.e., resistance and resilience) of the soil microbial community to additional disturbances.

The data show a relatively high variability. However, it is only slightly higher than we found in our previous works on functional stability (Tobor-Kapłon *et al.* 2005; Tobor-Kapłon *et al.* 2006). In our opinion the high variability can have three causes. First, we used true replicates, not subreplicates from one bulked sample; each replicate originates from a separate plot that is surrounded by plots on which other treatments were applied. Thus spatial variation from the field is included. The treatments were applied more than twenty years ago. The time between application and sampling was long enough to generate differences between soils with the same target treatment. The variability of bacterial growth rate presented in other articles (Bååth 1992; Díaz-Raviña *et al.* 1994a; 1994b; 1996; 1996; Bååth *et al.* 1998b;

2001; Díaz-Raviña and Bååth 2001; Rajapaksha *et al.* 2004), was much lower than in our experiment. However, these authors generally used soils that were contaminated in the lab shortly before measurements. Second, the effects of the disturbances were not very strong, which could also contribute to variability. In our former research done on the same soils (Tobor-Kapłon *et al.* 2005), we found somewhat lower variation but there the impact of the stressors was much stronger, i.e., respiration was reduced by 50% when lead and 45% when salt was applied, and bacterial growth rate was reduced by 80% in both cases. Third, we used relative values, among which the variability can be higher than among absolute values because there are two sources of variation. We do not prefer, however, to use the absolute values because normalization to the control (giving relative values) has been found useful to compare the results of different experiments (Griffiths *et al.* 2000; 2001a; 2001b; Tobor-Kapłon *et al.* 2005; 2006). We are interested in the relative changes after disturbance, rather than temporal and spatial variation shown by the absolute values.

Soil respiration rate and bacterial growth rate, measured before disturbance was applied, were lower in the pH 4.0 than in the pH 6.1 soils. This indicates that the low soil pH is still a stressful condition for the soil microorganisms. Since no significant effect of copper was found, it could be concluded that the current copper level in the contaminated soils is not stressful any longer for the microbes inhabiting these soils. In this study, we have shown, however, that the copper still has an impact on the functional stability of these soils. This impact, nevertheless, was not as strong as we found in our former study (Tobor-Kapłon *et al.* 2005), where additional (persistent) stress in the form of lead or salt was applied.

The differences in soil respiration and bacterial growth rates between the samplings of October and February could result from seasonal variation, although the samples were pre-incubated for 2 weeks at 20°C and 60% WHC. Nevertheless, these differences should not affect the results of stability analysis because only the relative differences in process rate were compared.

Respiration

Respiration rate was less stable (more affected by heat) in the Cu 0 (25% reduction) than in the Cu 750 soils (8% increase). Griffiths *et al.* (2000) found similar results; soils fumigated for a longer time were more resistant to heat than non-fumigated soils and shortly fumigated soils. The short and non-fumigated soils were also more resilient than the stressed soils. In our experiment, a recovery trend could also be seen in the Cu 0 soils but due to high variation it was not significant. We used a higher temperature than Griffiths *et al.* (2000; 2001a; 2001b) (50 vs 40^{o}C) because in a preliminary experiment the effect of heating at 40^{o}C was negligible.

The observed stability of respiration in Cu 750 soils to the heat cannot be explained in terms of an altered energy budget (Sibly and Calow 1989). The energy budget of organisms inhabiting the Cu 750 soils is burden with the costs of copper detoxification and therefore less energy is available to cope with heat. A possible explanation, however, is that exposure to copper caused selection towards copper-tolerant organisms, which at the same time appeared to be not only tolerant to heat but even stimulated by this treatment (Vinebrooke *et al.* 2004).

As in the case of heat disturbance, the investigated soils did not differ in their resistance to drying-rewetting cycles. The overall effect of this disturbance during the course of the experiment was stronger in the pH 6.1 soils (increase) than in the pH 4.0 soils (no change). An increase of respiration after drying-rewetting cycles has been reported in the literature and is explained as a response to a pulse of easily available carbon released from microbial cells and soil aggregates due to the disturbance (Kieft *et al.* 1987; Pulleman and Tietema 1999; Merckx *et al.* 2001; Fierer and Schimel 2002). Such a release and thus increase of respiration would be expected to occur in all soils, however. Therefore it is difficult to judge in which of the investigated soils the respiration rate is more stable to drying-rewetting cycles. According to the definition mentioned in the introduction, the respiration in the pH 4.0 soils is more stable, as it remained almost unaffected. One could claim that development of tolerance towards low pH involves changes in cell surface properties that make the exposed organisms more resistant to changes in osmotic pressure (Leyer and Johnson 1993; Faleiro *et al.* 2003). Thus, the organisms inhabiting the pH 4.0 soils could be more resistant to rapid changes in water potential than those from the pH 6.1 soils. Another reason for the lack of increase of respiration could be that the consumed carbon was used rather for growth than for respiration. It is known, however, that some environmental stresses can inhibit the rate of decomposition of organic matter (Merckx *et al.* 2001). Therefore, it is likely that an increase in respiration in the pH 4.0 soil was simply inhibited by acidity instead of being stable to the applied disturbance.

Bacterial growth rate

We found the bacterial growth rate to be more stable to heat in the pH 6.1 than in the pH 4.0 soils. Heat inhibited growth of bacteria in the pH 4.0 soils by almost 50%, whereas it hardly affected the growth of bacteria in the pH 6.1 soils. It is known that the cardinal temperatures for bacterial growth rate are not fixed, but depend on other environmental factors such as pH and available nutrients (Prescott *et al.* 1996). This implies that the upper temperature limit for growth of bacteria was lower in the more acidic soils. All soils recovered quickly from the disturbance by heat, and already after two weeks the growth rates were increased rather than decreased. Heat is a transient shock, and once it has stopped it

does not affect organisms directly anymore. The increase of bacterial growth rate may be explained by an increase of the pool of easily available carbon caused by death of sensitive bacteria, and by the possibility that during the time of heating, the growth of more adapted bacteria speeds up and slowly growing organisms are outcompeted (Pettersson and Bååth 2003). This may be related to a change in community structure. Shifts in community structure after application of stress/disturbance are a known phenomenon (Frostegård *et al.* 1996; Pennanen *et al.* 1998; Torsvik *et al.* 1998). Recovery after disturbance is probably associated with changes in community structure. We were, however, interested in the stability of respiration and bacterial growth rate, regardless of whom in the community is responsible for these processes. Information about changes in the community structure is interesting but beyond the scope of our present study. Moreover, it would be hard, if not impossible, to determine the contribution of each bacterial subpopulation (DNA band, genotype, or "species") to the total respiration and growth rate.

Drying-rewetting initially increased the bacterial growth rate in all soils. This was followed by a decrease indicating the recovery of the bacterial community from disturbance. A possible explanation is that a release of organic carbon caused by disruption of soil aggregates and litter fragmentation (Pulleman and Tietema 1999) and a lowered competition for food caused by death of sensitive species (Kieft *et al.* 1987) may have created favorable conditions for growth of surviving species immediately after cessation of the disturbance. The recovery can be associated with a decline of substrate (Fierer and Schimel 2002; Mamilov and Dilly 2002).

Stress, disturbance, and stability

The difference in response patterns between respiration and bacterial growth rate may be related to differences in treatment of the soils used for both measurements. Lucerne meal was added only to the soils in which respiration was measured. The soils were amended to ensure sufficient response to measure differences. Because not all organisms can utilize this material with the same efficiency, only the activity of responsive microorganisms was measured during an incubation of 24 h. The relatively high amount (3.2 mg C/g soil) of plant residues added probably caused a selection towards opportunists. In contrast, the low concentration of thymidine (0.1 μM) and the short incubation time of 2 h prevent changes in the bacterial growth rate during incubation. So the measured growth rate can be assumed to reflect the actual growth rate of the total soil bacterial community. Besides that, the respiration is known to be less sensitive to environmental factors than the bacterial growth rate (Rajapaksha *et al.* 2004).

Both the bacterial growth rate and respiration rate were stimulated rather than inhibited by disturbance. The only exception was the response of respiration in the Cu 0 soils

to heat. The increase instead of decrease of process rates makes the present results different from the results of Griffiths *et al.* (2000) and Tobor-Kapłon *et al.* (2005).

By definition, the stability increases with a decrease of deviation from the control value (resistance), and increases with resilience. It should be recognized that deviation from the control is not necessarily a decrease. Generally, the less affected the process the more stable it is. In this study we found different patterns of stability to the two disturbances. Regarding soil respiration our results support Odum´s concept (Odum 1981), which states that exposure of a system to stress makes it more resistant to another stress or disturbance. However, the strong resistance of bacterial growth rate in the pH 6.1 soils to heat supports rather the alternative hypothesis, which says that unstressed systems are more stable. In spite of the clear difference between pH 4.0 and pH 6.1 soils in the overall response of respiration to drying-rewetting cycles, we cannot say which of the two hypotheses is supported by this result.

Our present results with stability to disturbance supplement the findings in our previous study, with the same soils (Tobor-Kapłon *et al.* 2005), where we measured stability to additional persistent stresses (lead and salt) and found the highest resistance and/or resilience in the least contaminated soils with neutral pH and/or no copper load. Probably there is no 'general' stability to stresses and/ or disturbances, but rather stability of a particular process to a given stress/disturbance. It seems that stability depends on the mechanisms of adaptation, or changes in the energy budget of the organisms, that were developed during the first stress. If the mechanisms of detoxification/ reparation developed due to the first stress can be used to cope with the additional stress/ disturbance, then the stability of originally stressed soils will be stronger than the stability of clean soils. In contrast, when additional stress/ disturbance requires other routes of detoxification/ reparation, the nonstressed communities will be more stable due to bigger energy resources available for detoxification/ reparation processes.

Acknowledgement:

We thank Meint R. Veninga and An Vos for technical assistance.

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Chapter 7

Functional stability of microbial communities in contaminated soils near a zinc smelter (Budel, the Netherlands)

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Ecotoxicology 15(2): 187-197

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Environmental pollution causes adverse effects on many levels of ecosystem organization; it might affect the use efficiency of the available resources which will make the system more sensitive to subsequent stress. Alternatively the development of community tolerance may make the system more resistant to additional stresses.

In this work we are investigating the functional stability, measured in the terms of resistance and resilience, of microbial populations inhabiting contaminated soils near a zinc smelter. We measure the changes in respiration and bacterial growth rates in response to addition of stress (lead, salt) or disturbance (heat). We used soils that differ in the level of pollution with zinc and cadmium originating from an adjacent smelter.

Our results showed, with regard to respiration, the most polluted soils having lowest stability to salt (stress) and heat (disturbance). This confirms the hypothesis that more stressed systems have less energy to cope with additional stress or disturbance.

The bacterial growth rates were affected in the different way than respiration. There was no difference in resistance and resilience between soils to addition of lead. In case of salt treatment, the least polluted soils have highest stability. In contrary, the least polluted soils were the least stable to the increased temperature, which supports the hypothesis that more stressed soils are more stable to additional stress/disturbance due to properties they gained when exposed to first stress.

Stability is described in terms of how the populations/ processes respond to change/stress. These responses, their nature and size, depend on the kinds of stress factors, especially whether a subsequent stress is similar (in terms of the mechanism with which the organisms deal with the stress) as the first stress.

Keywords: stress, disturbance, functional stability, microbial community, respiration, growth rate.

Introduction

Emission of toxic substances from metal smelters not only causes air pollution but also soil contamination. These substances constitute stress for soil ecosystems; they affect the physiology of individual organisms, the community structure and community tolerance (Bååth 1989; Bååth et al. 1998; Calow 1991; Díaz-Raviña and Bååth 2001; Kelly and Tate 1998; Pennanen et al. 1996). Stress affects the functioning of an organism by changing its energy allocation. Detoxification and reparation of damages increase the costs of maintenance. In non stressed environments maintenance represents more than 80% of organisms' energy use (Congdon et al. 2001). An increase of energy required for maintenance will cause a decrease of energy available for growth and reproduction (Sibly and Calow 1989). Therefore growth and reproduction are considered to be very sensitive to stress (Bloem and Breure 2003).

Environmental stress may change the community structure of microbial populations. Stress negatively affects the more sensitive species and decreases their competition ability. Therefore, the more resistant species become more abundant. Many authors showed that environmental stress e.g. metals or organic contaminants can provoke such changes in community structure and successively cause development of community tolerance (Bååth et al. 1998; Bååth et al. 1998; Frostegård et al. 1996; Gong et al. 2000; Kelly et al. 1999; Macnaughton et al. 1999; Pennanen et al. 1996; Witter et al. 2000).

Exposure to one type of stress may cause a development of community tolerance towards this particular stress but may also cause a development of co-tolerance to another type of stress or disturbance (Díaz-Raviña *et al.* 1994; Vinebrooke *et al.* 2004). This happens when detoxification of different stresses relies on similar physiological processes (Bruins *et al.* 2000; Giller *et al.* 1998). However, when another way of detoxification is required an organism will have to develop or generate new mechanisms of detoxification which will demand extra energy. Such additional burden may affect the energy budget and functioning of the organism. Each species has a different way of managing its energy budget and thus the response of an entire community to stress is the sum of all individual reactions. The sum of such alterations, together with effects of stress on interactions between organisms and between organisms and their environment, will bring changes in the community and finally in ecosystem functioning. Such effects can be measured as changes in the rates of processes like growth and respiration.

The knowledge that stress alters soil communities raises the question: how stressed communities, for example in long term polluted ecosystems, will react to a next stress or disturbance? Are stressed communities less or more stable to environmental stress or disturbances than not-stressed ones?

Griffiths et al. (2000) defined stability as the ability of the system to withstand disturbance and maintain its normal state. Such defined stability has two measurable components: resistance - which is the inherent capacity of the system to withstand stress/disturbance, and resilience - which is the ability to recover after stress/disturbance.

To define 'stress' and 'disturbance' we use the classification of Degens et al. (2001). They characterize stress as an environmental condition (chemical, physical or biological) that has a continuous adverse impact on microbial communities. Disturbance they define as an event constrained in time which nevertheless may have a lasting positive or negative impact on microbes.

The effect of stress on functional stability can be measured by "stress on stress" (or "disturbance on stress") experiments (Griffiths et al. 2001; 2000; 2001). In this type of experiments a stressed (polluted) system is subjected to additional stress or disturbance and the systems' response (change in process rate) is measured at different time intervals following the application of stress/ disturbance. To assess the resistance and resilience of the stressed system, its process rates are compared to the rates in a non-stressed control.

There are two contrasting hypotheses regarding the stability of ecological processes. The first predicts that non-stressed systems are more stable because they possess larger energetic resources than stressed communities that allow them to maintain function in case of stress (Aarts and Nienhuis 1999; Loreau 2000; Naeem and Li 1997; Stone et al. 2001). The other hypothesis predicts that stressed systems are more stable since due to the first stress they gained abilities (adaptation, physiological changes) to cope with additional stress and thus maintain function (Odum 1981).

Until now stability measurements were performed mostly on agricultural soils in which the first stress was strongly controlled in experimental set ups. These soils were either stressed by fumigation (Griffiths et al. 2000), underwent reduced biodiversity due to dilution (Griffiths et al. 2001) or were stressed with elevated copper concentration and/ or low pH (Tobor-Kapłon et al. in press; Tobor-Kapłon et al. 2005). In such experiments, beside differences in the applied stresses/ disturbances, soils are treated in the same way e.g. they receive the same external input of nutrients, are usually situated very close to each other so there is no influence of spatial variation in soil characteristics. This is usually not the case in uncontrolled field situations. To our knowledge, there has been hardly any research done on stability of field soils exposed to uncontrolled stress (pollution). Such work could show whether the functional stability of polluted field soils show similar results as experiments performed with experimental soils.

In the present study we investigate functional stability of soil microbial communities from forest soils that have been exposed for a long time to increased concentrations of zinc and cadmium originating from an adjacent zinc smelter. This stress could affect the

functioning of soil microbial communities in terms of their physiology, community structure and tolerance. With functional stability we mean that we look at processes rather than at population dynamics. We measured changes in the soil respiration which is assumed to be mainly due to bacteria. Respiration reflects decomposition of organic matter and is a major ecological process. We also measure the changes in bacterial growth rate, which is a more specific and very sensitive parameter. The microbial community in these soils is dominated by bacteria (Koopmans et al. 1993). To allow comparison between the results of the present study and former ones (Tobor-Kapłon et al. in press; Tobor-Kapłon et al. 2005) we have chosen for stresses and disturbance as used in our previous work i.e. lead, salt and heat.

Materials and methods

Description of area and sampling

The sampling area is situated in the south of the Netherlands in the vicinity of a zinc smelter in Budel. The first factory was established there in 1892. In 1973 for environmental reasons a new cleaner technology was introduced. Nowadays the smelter produces 230,000 tons zinc per year.

Sampling sites were situated along a gradient of pollution going from the smelter to the North-East. All sites were covered by Scotch pine (*Pinus silvestris* L.). Samples were collected from three locations that were situated at intervals of approx. 1, 2 and 6 km from the smelter (further referred to as soils A, B, and C). The locations have a similar soil structure: sandy with approximately 2.5% of clay (Koopmans et al. 1993). Samples were taken from the upper 10 cm of the soil mineral layer (litter was removed). On either location four replicate plots were sampled. From each replicate 15 soil cores, 4 cm in diameter and 10 cm depth were taken and then mixed and sieved (0.5 cm mesh).

Preparation of samples

After sieving, field moist soil from each plot was divided into subsamples of approx. 250 g and each of them was placed in a 300 ml plastic jar. The samples were divided into four sets, each containing four samples. One set was treated with lead nitrate (1000 mg Pb kg^{-1} dw), the second was treated with sodium chloride (6.67 g NaCl kg^{-1} dw), the third was heated for 18 hours at 50°C and the fourth one served as a non-stressed control. After treatment samples were kept in darkness at a temperature of 20°C until measurement. From each jar samples for respiration, bacterial growth rate measurement and chemical analyses were taken.

Respiration

Twenty four hours before each measurement 200g of soil from each jar was placed into ± 600 ml glass bottles and dry lucerne meal (alfalfa (*Medicago sativa*)) was added and mixed thoroughly. Lucerne was added in amounts corresponding to 3.2 g C kg^{-1} soil, which was sufficient to induce respiration and offset nutrient limitation. Without substrate addition the respiration is usually too low to measure significant differences within 24 h. Carbon dioxide production was measured with a GC apparatus (Carlo ERBA Instruments). For the first measurement samples were prepared as described above directly after application of stress and termination of disturbance.

Bacterial growth rate

Bacterial growth rate was measured as the rate of incorporation of radioactively labelled thymidine into bacterial DNA, according to the microcentrifugation method described by Bååth et al. (2001). Thymidine is incorporated only by bacteria and not by eukaryotes like fungi.

Measurement was performed as follows: 5 g of soil was shaken with 40 ml of miliQ-water in plastic tubes, then centrifuged and filtered through glass wool. Supernatants (1.5 ml) were decanted into eppendorf tubes. Samples were incubated for two hours at 20^{o}C with 3.75 µl radioactively labelled thymidine (methyl[^{3}H]thymidine, 925 GBq $mmol^{-1}$, Amersham, U.K) diluted1:3 with miliQ-water. Incorporation, washing of excess tracer, and measurement of radioactivity incorporated by actively growing bacterial cells are described in detail by Bååth *et al.* (2001). Radioactivity was measured using a Beckman liquid scintillation spectrometer. Because a well established conversion factor is lacking for this method, the results are expressed in DPM units (decays per minute). DPM values are proportional to the amount of cells produced during two hours of incubation.

Functional stability

For stability analysis we used the approach of Griffiths et al. (2000). Due to absolute differences in the investigated parameters (respiration rate and bacterial growth rate) between soils and sampling dates, resistance was normalised to the value of the control (soils without addition of stress or disturbance), i.e.

Resistance = % change from control = $[(x_{treated} - x_{contr.})/ x_{contr.}] \cdot 100\%$.

All measurements were done four times: 24 hours, two weeks, one and two months after disturbance. The response after 24 hours was used as a measure of resistance, while the change over time was adopted as a measure of resilience. The overall effect of a stress/disturbance was the average response over the time of the experiment.

Chemical analyses

For each measurement, 10 g samples were placed into 50 ml plastic tubes. Samples were shaken (30 rev min^{-1}) with 40 ml of 0.01 M $CaCl_2$ for 48 hours. After shaking, pH was measured with a pH-meter (Φ 34 Beckman). Tubes with soil slurry were centrifuged at 3000 rev min^{-1} for 10 min; samples were filtered through a 0.45 μm syringe-filter (Total Organic Carbon free). Measurement of heavy metal concentrations was performed using an ICP-AES (Parkin Elmer Optima 3300 DV).

Soil acidity (pH-KCl) was measured only once in non-treated samples. Aliquots of field moist soil (10g) were placed into plastic tubes and 50 ml of 1M KCl was added. Samples were shaken for 3 hours on the shaker at 180 rev min^{-1}. After shaking, pH was measured with a pH-meter (Φ 34 Beckman).

Dry mass was measured as a loss of weight after drying at 105°C for 24 hours, whereas organic matter content was determined as a loss-on ignition (550°C, 6 hours).

Statistical analysis

For the analysis of resistance to stress and disturbance (response 24 h after stress addition) we used the Univariate General Linear Model (SPSS) procedure with site as fixed factor. For analyses of resilience and overall effect, using all four time points (24 hours, two weeks one and two months), we used also the Univariate General Linear Model procedure with full factorial design with site and time as fixed factors. All analyses were performed using SPSS 11.5.0 statistical software. Because the results of respiration of control soils were not normally distributed a log-transformation was applied.

Results

Soil properties

The concentration of zinc was highest in soils from site A (1 km distance from the smelter) and lowest in site C soils (6 km from the smelter) ($p < 0.0001$). Similarly, the concentrations of cadmium were different at each site and were lowest on location C and highest on location A ($P < 0.0001$). The concentration of lead was significantly lower in site C soils than in the soils from the two other sites ($p < 0.0001$) (table 1).

The soils differed significantly in pH (KCl) ($p < 0.0001$) and organic matter content ($p < 0.0001$). The most acid were the C soils with pH 3.0 whereas A soils had a pH of 3.9. Highest organic matter content was found in C soils (8.3 ± 0.9%) and lowest in A soils (3.0 ± 0.3%) ($p < 0.0001$).

Table 1. Soil acidity, organic matter content and bioavailable concentrations of heavy metals (expressed in mg kg^{-1} organic matter and in mg l^{-1} of 0.01M $CaCl_2$ extract) in not treated soils. Means marked with different letters are statistically different (Tukey's HSD, $P < 0.05$)**.**

			pH (0.01M $CaCl_2$)			pH (1 M KCl)			Zn			Cd			Pb			Na			Organic matter[a]		
	Site	N	Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.		Mean	S.E.M.	
mg kg^{-1} o.m	A	16							1297	273	A	8,42	0,82	A	90,4	9,14	A	496	203	A	30,0	2,67	A
	B	16							533	35,4	B	6,88	0,86	A	88,9	5,99	A	199	8,36	A	32,5	3,13	A
	C	16							185	10,6	C	2.60	0,14	B	29,5	3,25	B	115	7,27	B	82.5	9,40	B
mg l^{-1}	A	16	3,76	0,03	A	3,86	0,08	A	8,97	2,05	A	0,06	0,01	A	0,57	0,06	A	3,04	1,17	A			
	B	16	3,77	0,01	A	3,82	0,02	A	3,99	0,35	B	0,05	0,01	A	0,62	0,02	A	1,42	0,05	A			
	C	16	3,09	0,02	B	3.04	0,05	B	3,24	0,20	B	0,05	0,00	A	0.48	0,03	B	2,00	0,11	A			

[a] g o.m kg^{-1} soil

Table 2. Acidity and bioavailable concentrations of heavy metals (expressed per liter of 0.01M $CaCl_2$ extract) in soils to which stress, in the form of lead (1000 mg Pb kg^{-1}) or salt (6.67 g NaCl kg^{-1}) was added (averages of all time points). Means marked with different letters are statistically different (Tukey's HSD, $P < 0.05$).

			pH (0.01M $CaCl_2$)			Zn mg l^{-1}			Cd mg l^{-1}			Pb mg l^{-1}			Na mg l^{-1}		
Stress	Site	N	Mean	S.E.M		Mean	S.E.M		Mean	S.E.M		Mean	S.E.M		Mean	S.E.M	
Lead	*A*	16	3,69	0,01	A	8,18	1,48	A	0,07	0,01	A	60,9	5,71	A	1,32	0,09	A
	B	14	3,73	0,02	A	4,55	0,41	B	0,07	0,01	A	56,2	5,68	A	1,33	0,05	A
	C	16	3,05	0,02	B	3,56	0,19	B	0,05	0,00	A	36,7	4,48	B	2,13	0,28	B
Salt	*A*	16	3,82	0,01	A	7,09	1,17	A	0,07	0,01	A	0,83	0,03	AB	588	6,06	A
	B	16	3,90	0,04	A	7,39	3,44	A	0,06	0,01	A	0,72	0,03	AB	572	7,89	AB
	C	16	3,14	0,02	B	3,06	0,17	A	0,05	0,00	A	0,60	0,05	BC	510	35,4	BC

Although the same amount of $Pb(NO_3)_2$ (1000mg Pb kg^{-1} dw) and NaCl (6.67 g kg^{-1}) was added to each soil, the interplay between metals, soil pH and organic matter content caused changes in the concentrations of the bioavailable fraction of lead and sodium (table 1 & 2). Thus the available doses of applied stress were different among treatments. The concentrations of heavy metals (Pb, Zn and Cd) remained on the same level over time following addition of stress.

Primary stress (contamination by the smelter)

The respiration rate was significantly different between the sites ($p = 0.003$; calculated on the basis of log-transformed data) and was higher in the C soils than in the other ones. The original values of soil respiration were: 8.8 ± 3.1 in A soils, 6.2 ± 0.3 in B and 10.1 ± 0.5 mg CO_2 kg^{-1} h^{-1} in C soils.

In contrast to respiration rate, [^{3}H]thymidine incorporation (indication of bacterial growth rate) was not significantly different between the sites ($p = 0.2$). The incorporation rates (DPM incorporated during 2 h of incubation) were: 1.4 ± 0.1 x10^4 in A, 1.3 ± 0.1 x 10^4 in B and 1.1 ± 0.1 x 10^4 DPM in C soils.

Secondary stress (applied in pot experiments)

Effects of lead (stress)

Respiration

The different sites showed no significant difference in the resistance of respiration to lead ($p = 0.12$). ANOVA showed significant ($p = 0.03$) recovery of respiration, from on average 7.4 ± 5.7% reduction one day after stress addition to 23.7 ± 11.1% increase two months later. There was no difference in the recovery between the soils ($p = 0.2$) (Fig. 1A).

Bacterial growth rate

The bacterial growth rate was greatly reduced in all soils after stress addition. No difference in the resistance of bacterial growth between soils was found. There was no effect of time nor interaction between time and site ($p = 0.2$ and $p = 0.7$ respectively). Neither was the effect of site significant ($p = 0.8$). The results of effects of lead on bacterial growth rate should be taken with caution since extremely high variation was found in the measurements of day 35 (Fig. 1B).

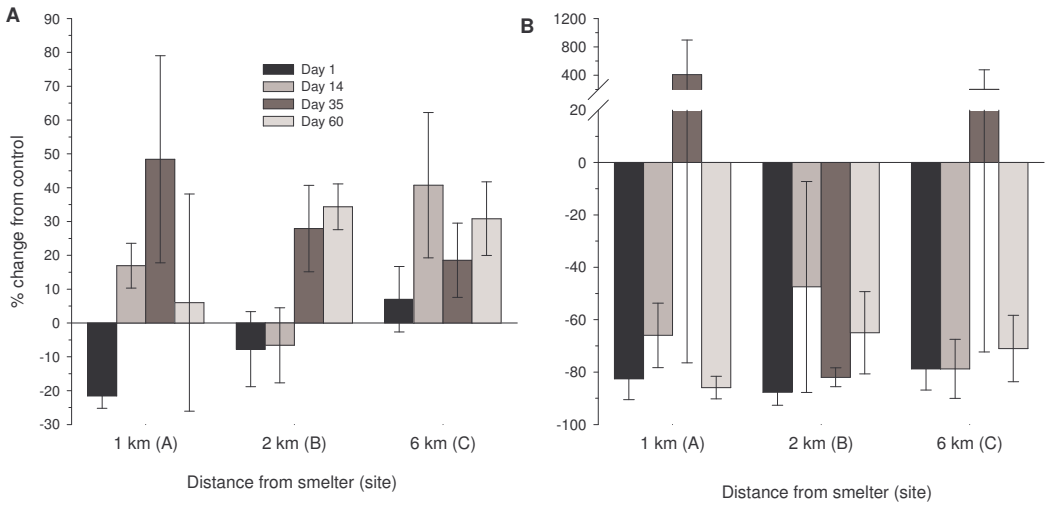

Figure 1. Effect of stress (lead) at increasing time intervals following application of stress (mean ± SEM) on (A) the ability of soil microbial communities to decompose lucerne (alfalfa) residues and (B) on the growth rate of bacteria. The soils at increasing distance from a zinc smelter differ in the level of pollution.

Effects of salt (stress)

Respiration

Salt treatment caused a reduction of respiration in all soils. The soils from the least contaminated site (C) were, initially, significantly more resistant than the soils from the most polluted site (A) (15.8 ± 15.3% versus 57.1 ± 3.5% reduction for sites C and A, respectively) ($p = 0.03$). Soils with different levels of contamination differed in the overall effect of salt on their respiration ($p = 0.002$). The most affected were soils from site A (40.1 ± 7.8% reduction) whereas respiration at the most remote site (C) was hardly affected (on average 1.1 ± 8.2% increase). There was no recovery from the salt stress ($p = 0.1$). The interaction between time and site was also insignificant ($p = 0.9$) (Fig. 2A).

Bacterial growth rate

There were no differences in resistance between soils from different sites ($p = 0.9$). In all cases salt reduced bacterial growth rate by approx. 90%. The statistically significant effect of time ($p = 0.01$) suggests that bacterial growth rates were able to recover from this stress. The interaction of time and site was not significant ($p = 0.3$), which means that the recovery was statistically not different among soils (Fig. 2B).

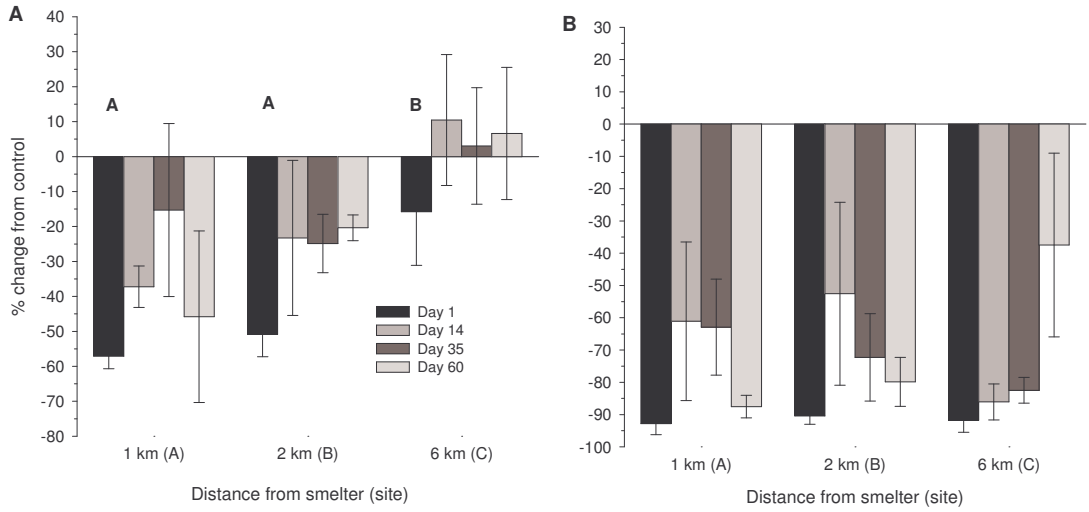

Figure 2. Effect of stress (salt) at increasing time intervals following application of stress (mean ± SEM) on (A) the ability of soil microbial communities to decompose lucerne (alfalfa) residues and (B) on the growth rate of bacteria. The soils at increasing distance from a zinc smelter differ in the level of pollution. Bars representing resistance (day 1) marked with different letters are significantly different (Tukey's HSD, $p < 0.05$).

Effects of heat (disturbance)

Respiration

The resistance of the investigated soils to heat (disturbance) was different between sites ($p = 0.03$); the strongest resistance was in the cleanest soils (C: 48.9 ± 2.4% reduction) and the weakest resistance was in the most polluted one (A: 71.9 ± 20.7% reduction). The highly significant effect of time ($p < 0.0001$) indicates the resilience of respiration going from on average from 62% reduction after 1 day to 61% increase after two months from disturbance) (Fig. 3A).

Bacterial growth rate

The soils did not differ in the resistance of bacterial growth rate to heat ($p = 0.6$). The significant interaction between time and site ($p = 0.02$) indicates different patterns of recovery between the soils; the bacterial growth rates in C soils recovered most rapidly and strongly overshoot the control values (Fig. 3B).

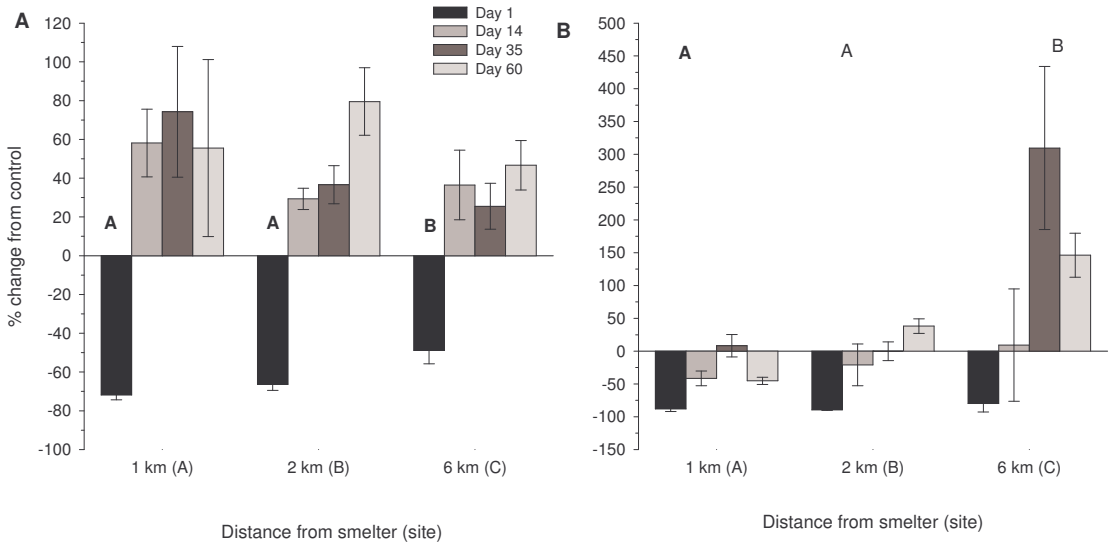

Figure 3. Effect of disturbance (heat) at increasing time intervals following application of stress (mean ± SEM) on (A) the ability of microbial communities to decompose lucerne (alfalfa) residues and (B) on the growth rate of bacteria. The soils at increasing distance from a zinc smelter differ in the level of pollution. Bars representing resistance (day 1) (Fig 3A) marked with different letters are significantly different (Tukey's HSD, $p < 0.05$). Sites marked with different letters (Fig 3B) have a different pattern of recovery (Tukey's HSD, $p < 0.05$)

Comparison of effects of different stresses

Respiration

Analysis of variance showed that there was a significant difference between resistance (effect on day 1) to the different stresses and the disturbance ($p < 0.0001$). Respiration was most resistant to lead and least resistant to heat. Considering the average effect of the whole incubation period, salt was the only treatment that imposed a negative effect on respiration rate. In contrast, the two other treatments despite initial decrease of respiration caused on average an increase of this process. The effect of treatment (lead, salt or heat) was different at each site ($p = 0.04$) and recovery followed different patterns in different treatments ($p < 0.0001$) and at different sites ($p = 0.07$).

Bacterial growth rates

The bacterial growth rate was less resistant to the applied treatments than respiration. Salt had the strongest effect on the average response of bacterial growth ($p < 0.0001$). Caution, however should be taken when comparing the effect of lead with the other treatments. The extremely high variation of the day 35 might obscure the effect of this treatment.

Discussion

The aim of this study was to examine the functional stability of microbial populations inhabiting soils that differ in level of pollution caused by heavy metals. Aerial deposition of Zn, Cd and Pb emitted from the adjacent smelter caused an increase in heavy metal concentrations in the investigated soils. The highest concentrations were observed in the A soils (site closest to the smelter) and the lowest in C soils (the most remote site). Heavy metals cause, usually, an inhibition of decomposition of organic matter, which leads to an increase of the organic matter layer in contaminated areas (Bååth 1989; Giller et al. 1998). Here we have, apparently, a reversed situation; the organic matter content was the highest in the most remote area, which was the least contaminated by the heavy metals. It has to be mentioned that we used soils from the mineral horizon after removal of the litter layer. Increased organic matter content in the mineral layer may indicate that the soil fauna inhabiting this environment is more active and thus able to transfer more organic material into the soil profile. This difference might also be due to the natural spatial variation, partly dependent on (historical) land use and vegetation.

In this study we examined whether increased zinc and cadmium concentrations affect the stability (i.e. resistance and resilience) of respiration and bacterial growth rate to environmental stress or disturbance.

Respiration

Respiration reflects decomposition of organic matter which is a major ecological process, and thus reflects the condition of the system (Giller et al. 1998). In the present study measurements in the untreated control soils showed that the respiration rate was the highest in the most remote (clean) locations. This may indicate that non-stressed environments constitute better conditions for the functioning of soil microorganisms (Giller et al. 1998). However, it may also reflect the higher content of organic matter in the C soils.

Immediately after application of a second stress/disturbance in addition to the long-term contamination, the system undergoes a shock which reveals as a change in process rates. The stronger the change, the less resistant is the process to the particular stress/disturbance. Later, the mechanisms of recovery, responsible for resilience, start to work.

The second stress (lead or salt) and disturbance (heat) caused changes in respiration (decomposition of lucerne meal (alfalfa)) in all soils but each stress/disturbance induced a different pattern of response. In the cases of salt and heat, the cleanest soils (C) were the most resistant and the most polluted soils (A) were the least resistant.

Although the average values of responses measured 24 hours after the stress addition suggest that the C soils were most resistant to lead, the statistical analysis did not reveal significant differences. There was a strong recovery in all soils after the addition of lead. The

initial respiration rate was regained, and even exceeded the control level, in all soils already two weeks after the stress addition. The lack of significant differences in resistance and strong recovery of respiration after lead application is different from the results of Griffiths et al. (2000) and of our former work (Tobor-Kapłon et al. 2005). In both studies no recovery from heavy metal stress was found and the less stressed soils were more stable to additional stress.

In contrast with our former results, one could also argue that the communities close to the smelter (A soils), that have already been exposed to zinc and cadmium for a long time, may have developed co-tolerance to lead (Díaz-Raviña et al. 1994) and therefore may be expected to be more stable than the communities in less polluted (C) soils. Indeed, lead had the weakest effect on respiration compared to salt and heat. However, the relatively weak effect of lead was found also at the least polluted site. These most remote (C) soils are also the richest in organic matter which is known to immobilise heavy metals, and indeed are characterized by the lowest $CaCl_2$-exchangeable metal concentrations. Therefore the addition of lead resulted in smaller increase of bioavailable lead in C soils than in A soils. Thus, the difference in organic matter content between the sites may be the reason that no significant differences in stability were found.

The mechanism behind recovery, observed after application of lead, probably relied on the lessened competition caused by the death of the sensitive organisms (Sardinha et al. 2003) and associated with this the sudden release of bioavailable carbon from dead bacterial cells. Such rapid food supply could stimulate the activity of resistant organisms that could then start to compensate for the loss of others. Such a mechanism may also explain the recovery of respiration and bacterial growth rate after application of the two other treatments.

Also in the case of heat (disturbance), respiration showed recovery in all soils. Although the resilience was similar in all soils, the most polluted ones due to their low resistance were the least stable to heat. The cleanest soils, as the most resistant, showed the highest stability of respiration rate. In this case, changes in the energy budget of organisms inhabiting the examined soils probably are responsible for different levels of stability to additional stress. The organisms from highly polluted soils have lowered resources due to the allocation of energy to detoxification and reparation of damages caused by the first stress, which makes an additional stress more severe for them (Calow 1991; Kuperman and Carreiro 1997).

Heat imposed the strongest changes in respiration when compared with the other treatments, while in our former experiments (Tobor-Kapłon et al. in press; Tobor-Kapłon et al. 2005) we found heat (disturbance) to be less harmful than lead and/or salt. The earlier experiments, however, were performed on agricultural soils which are exposed to higher variation in temperatures. This could increase their tolerance against heat. Soils used for the

present experiment are covered by a litter layer and moreover are shielded by forest which protects them very well against temperature fluctuations.

The respiration showed no resilience after the addition of salt. The most polluted soils remained the most affected, thus the least stable, and the cleanest ones the most stable. In this case the resistance of respiration determined the stability of the soils to the addition of salt.

Griffiths et al. (2000) found a similar pattern of stability of respiration to stress (copper); the weaker stability (resistance) of the more stressed soils was interpreted as a confirmation of the "insurance hypothesis of biodiversity" (Aarts and Nienhuis 1999; Loreau 2000; Naeem and Li 1997). This hypothesis says that more diverse systems are more stable. In later work, however, (Griffiths et al. 2001) proved that reduced biodiversity per se does not reduce stability, but merely the stress applied to reduce biodiversity in the earlier experiments (Griffiths et al. 2000). Also our present results indicate effects on the physiology of the organisms. In our former work (Tobor-Kapłon et al. 2005) we have also found that not contaminated soils were more stable (resilient) to salt than contaminated soils. Though the energy-budget-explanation also holds in the present study, an increased tolerance to an acidic environment can also have an impact on stability to salt. It is known that development of tolerance towards low pH involves changes in cell surface properties that makes the exposed organisms more resistant to changes in osmotic pressure (Faleiro et al. 2003; Leyer and Johnson 1993). The C soils have the lowest pH of all examined soils, which might make them more tolerant to salt.

Bacterial growth rate

Bacterial growth rate was generally more sensitive to the applied stresses and disturbance than respiration. Immediately after treatment bacterial growth rates were reduced by more than 80%.

Lead treatment imposed a very strong stress for bacterial growth rate; in all soils the growth was initially inhibited by approx. 80%. No difference in the resistance, neither in resilience to lead was found. This suggests that the bacterial growth in all these soils has a very low stability to the addition of lead.

There was no difference between the soils in the resistance of bacterial growth to salt. In A and B soils after initial recovery a reduction in growth occurred. The constant recovery of bacterial growth in C soils (cleanest ones) makes them the most stable to this stress.

There was hardly any difference between the soils in their resistance to heat. The bacterial growth rate was resilient in all soils; however, in the C soils the recovery was the strongest. These soils, due to their higher organic matter content, are probably the richest in food sources. Thus, organisms that survived the heat shock were able to strongly increase

their growth rate. The C soils were thus the most affected by heat and therefore the least stable to this disturbance.

The effects on bacterial growth rate can not be directly compared with effects on respiration because of differences in the treatments of the soils used for the two types of measurements. Lucerne meal was added only to the soils in which respiration was measured. This was necessary to ensure a sufficient response within 24 hours. Because not all organisms can utilise this material with the same efficiency, only the activity of responsive microorganisms is measured. The relatively high amount (3.2 mg C g^{-1} soil) of plant residues added may therefore have caused a shift in community composition. Bacterial growth rate was measured within 2 hours after adding a radioactive tracer ([^{3}H]thymidine) to subsamples of the soil. The low concentration of thymidine (0.1 μM) and the short incubation time prevent changes in growth rate during the incubation. So the measured growth rate reflects the actual growth rate of the total soil bacterial community.

Conclusions

We found that soils that differ in the level of pollution have different stability to applied stresses and disturbance. The results give support to both concepts of stability: (1) non-stressed systems are more stable because they possess larger energetic resources, and (2) stressed systems are more stable since they gained abilities (adaptation, physiological changes) to cope with additional stress. This implies that there is no 'general' stability to stresses/disturbance but rather stability of a particular process to a given stress/disturbance. The responses of microbial processes to stress, their nature and size, depend on the kinds of stress factors, especially whether a subsequent stress is similar to the first stress, in terms of the mechanism with which the organisms deal with the stress.

Acknowledgements

We thank M.R. Veninga, A. Vos, S. Racamy and W. Schuurmans for technical assistance.

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Soils are of vital importance for society because they provide food, fibres, wood and pure water. The capacity of a soil to sustain these functions is called *soil quality*. Soil functioning depends strongly on microbial processes. Any action that causes a negative alteration in these processes leads to decrease of soil quality.

In many places around the world soils have been seriously contaminated in the last century. Contamination affects the organisms living in these soils, the processes carried out by the organisms and consequently soil quality. In this thesis I studied how long-term soil contamination affects microbial populations and processes, ecosystem properties and functional stability. I also investigated which parameters are suitable as indicators of soil quality in long-term contaminated soils.

Contamination had a negative impact on many examined microbial parameters, e.g. biomasses, respiration and growth rate. Decades after contamination, microbial populations had not recovered to pre-contamination levels (Chapter 2). Some parameters like protozoan biomass and metabolic quotient (respiration/biomass, qCO_2) did not show any effect of stress probably due to strong variation. In general the effects imposed by copper were weaker than those imposed by soil pH. No single parameter indicated effects of both stressors. Thus, a set of indicators is needed to assess the condition of contaminated soils.

It has been claimed that stress affects ecological succession and that this can be measured by information indices provided by so-called Ecosystem Network Analysis (ENA). However, to date ecological succession has not yet been described in terms of information indices. Therefore I tested these indices as a tool to describe ecological succession in belowground ecosystems (chapter 3). I used data from a primary succession on the island of Schiermonnikoog. The indices that describe both size and organization of ecosystem followed trends predicted by the theory, but at the same time these indices were strongly correlated with total system biomass. Therefore I could not say whether the observed trends reflect ecosystem development or simply the build up of biomass. However, analysis of relative indices that are independent of biomass and describe only the organization of the ecosystem, showed that succession occurred only in soils between 0 and 10 years old, whereas soils ranging from 10 to 100 years old were apparently in the same phase of ecological development. Since other authors clearly have shown that there has been succession in these soils in all stages between 0 and 100 years, I conclude that the relative information indices are not sensitive indicators of ecosystem development.

In contaminated soils, however, relative information indices were sensitive to stress caused by high concentrations of copper and by low pH (chapter 4). Stress affected the organization of belowground ecosystems as predicted by the theory. The level of specialization decreased with stress, whereas the losses of energy, e.g. due to respiration and the number of parallel pathways, increased. Stressed soils were more vulnerable to external

perturbations and less efficient in processing energy than unstressed soils. The diversity and evenness of flows showed a hump-backed relationship with stress. As the relative information indices responded to stress in predictable manner and each of them revealed effects of both stresses I concluded that these indices are useful indicators of environmental stress.

In chapters 5 and 6, I used "stress on stress" experiments to test the functional stability (resistance and resilience) of soil respiration and bacterial growth rate to additional stress (lead or salt) or disturbance (transient heat shock or drying-rewetting cycles) in experimentally contaminated soils. The results described in Chapter 5 seemed to confirm the hypothesis that microbial processes in not-stressed soils are more stable to additional stress, as the microorganisms have larger resources (energy and diversity) available to deal with harsh conditions. In stressed soils, on the contrary, adaptation requires resources, and therefore fewer resources are available to deal with an additional stress. The microbial processes showed different responses to disturbances (Chapter 6) than to stress (Chapter 5); disturbances caused stimulation rather than inhibition. In some cases stressed soils appeared to be more stable to additional disturbance than not-stressed soils, in other cases the opposite was found.

In chapter 7, I tested the functional stability in a real field situation with zinc and cadmium pollution from an adjacent smelter. In this experiment processes responded differently than in the former experiments. For instance, respiration showed recovery after application of a second stress (lead), which was not the case in soils from the experimental fields contaminated with copper at different levels of pH. Probably the response of a process depends on whether a co-tolerance towards a given (subsequent) stress was developed during exposure to the first stress.

I conclude that functional stability in long-term stressed soils does not respond to additional stress in a predictable manner, and therefore is not a useful indicator of soil quality.

De bodem is belangrijk voor een ecosysteem, omdat het zorgt voor water, voedsel, hout en vezels. De capaciteit van een bodem om deze eigenschappen te waarborgen wordt bodemkwaliteit genoemd. Hoe de bodem functioneert, hangt sterk af van microbiële processen. Alles wat een negatieve invloed heeft op deze microbiële processen zorgt daardoor voor een verminderde bodemkwaliteit.

Op veel plaatsen in de wereld zijn bodems de laatste eeuw sterk vervuild. Vervuiling beïnvloedt het voorkomen en functioneren van de levende organismen in deze bodems en daarmee ook de bodemkwaliteit. In dit proefschrift bestudeer ik hoe langdurige vervuiling de microbiële populaties en dus ook processen, ecosysteem eigenschappen en functionele stabiliteit beïnvloedt. Tevens kijk ik of er parameters zijn die als een indicator voor de bodemkwaliteit in vervuilde bodems kunnen dienen.

Vervuiling had een negatieve invloed op veel van de onderzochte microbiële parameters, zoals de biomassa, respiratie en groeisnelheid (Hoofdstuk 2). Tientallen jaren na de vervuiling bleken microbiële populaties niet herstelt tot de waarden van voor de vervuiling. Enkele parameters, zoals protozoa biomassa en het metabolische quotiënt, lieten, waarschijnlijk door de grote variatie, geen reactie zien op stress. Over het algemeen waren de effecten van koper minder sterk dan die veroorzaakt door de pH. Ik concludeer dat een combinatie van parameters noodzakelijk is om de conditie van de vervuilde gronden te kwantificeren, omdat geen van de onderzochte parameters gevoelig bleek voor beide typen vervuiling.

In de literatuur wordt aangenomen dat stress de ecologische ontwikkeling beïnvloedt en dat die ontwikkeling gekwantificeerd kan worden met informatie indices afkomstig van “Ecosystem Network Analysis (ENA)”. Echter, tot nu toe is er geen studie waarin de ecologische ontwikkeling wordt gekwantificeerd met informatie indices. Daarom test ik in hoofdstuk 3 of informatie indices een goed instrument zijn om ecologische ontwikkelingen in bodemecosystemen te beschrijven. Hiervoor zijn gegevens van een primaire successie op het eiland Schiermonnikoog gebruikt met bodems van 0, 10, 25 en 100 jaar oud. Ik laat zien dat de indices die zowel de grootte als de organisatie van het systeem vertegenwoordigen reageerden zoals de theorie voorspelt, maar dat deze indices wel sterk gecorreleerd waren met de totale biomassa. Daarom kan er niet zonder meer gesteld worden dat de gevonden trends van deze indices de ecologische ontwikkeling vertegenwoordigen of dat ze alleen de opbouw van biomassa weergeven. Indices die de organisatie van het systeem beschrijven (zonder de grootte en dus onafhankelijk van absolute biomassa), lieten alleen verschillen zien tussen 0 jaar oude en oudere bodems. De waarden van deze indices verschilden dus niet significant tussen 10 en 100 jaar oude bodems. Andere studies aan dezelfde bodems laten wel een verschil in ontwikkelingsstadium zien tussen 10 en 100 jaar en

daarom concludeer ik dat de indices die alleen organisatie beschrijven, ongevoelig zijn voor ecologische ontwikkeling.

In tegenstelling tot bij de ecologische ontwikkeling bleken de indices die alleen organisatie beschreven wel gevoelig voor veranderingen door koper en pH veroorzaakte stress (Hoofdstuk 4). De organisatie werd daardoor beïnvloedt zoals de theorie voorspelt: de specialisatie nam af en het energieverlies in het systeem nam toe met een toename van stress. Verlies van energie werd onder andere veroorzaakt door een toename van respiratie en disorganisatie in het systeem. Bodems onder stress waren gevoeliger voor verstoringen en minder efficiënt in het verwerken van energie dan bodems zonder stress. De maximale organisatie van het systeem, waarin alle organismen optimaal gespecialiseerd zijn, gaf een parabolisch verband met koper concentratie en dus stress. Indices die zowel grootte als organisatie van het systeem beschrijven, vertoonden onverwachte reacties op stress. Dit kwam doordat het in balans brengen van de ecosystemen, gedaan voor de berekening van de indices, een groot effect op de grootte van het systeem had. Aangezien de indices die alleen organisatie beschrijven wel als verwacht reageerden op stress, concludeer ik dat deze indices een goede indicator zijn voor omgevingsstress.

In hoofdstuk 5 en 6 gebruik ik stress op stress experimenten om de functionele stabiliteit (weerstand en veerkracht) van de bodem respiratie en de bacteriële groeisnelheid door additionele stress (lood of zout) en verstoringen (tijdelijke factor – warmte en droogte en bevochtiging cycli) te onderzoeken in experimenteel bevuilde bodems. De resultaten in hoofdstuk 5 lijken de hypothese te bevestigen dat processen in minder vervuilde bodems stabieler zijn voor extra stress doordat ze grotere hoeveelheden substraat opnemen (energie en diversiteit) en zodoende beter met extreme condities kunnen omgaan. De onderzochte processen lieten een andere reactie zien op verstoringen (hoofdstuk 6) dan door stress (Hoofdstuk 5), waarbij in de meeste gevallen eerder een stimulatie dan remming van de processen plaatsvond. In sommige gevallen leidde extra stress tot stabielere en in andere gevallen tot minder stabielere systemen.

In hoofdstuk 7 heb ik de functionele stabiliteit in een natuurlijke veldsituatie onderzocht voor met zink en cadmium vervuilde bodems. De invloed van deze vervuiling in een natuurlijke veldsituatie had een ander effect op de processen dan de stress bij de veldexperimenten. De respiratie liet bijvoorbeeld een herstel zien na toevoeging van een tweede stress factor (lood) in een natuurlijk veld, in tegenstelling tot de bodems van het experimentele veld. De veranderingen van een proces door additionele stress hangen waarschijnlijk af van of er een co-tolerantie voor deze (extra) stress wordt ontwikkeld tijdens de blootstelling aan de initiële stress. Op basis van bovenstaande experimenten met langdurige stress, concludeer ik dat de functionele stabiliteit niet in een te voorspellen

richting op stress reageert en dat functionele stabiliteit daardoor geen goede indicator is voor de bodemkwaliteit.

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