行政院所屬各機關因公出國人員出國報告書

（出國類別：　研　習　）

環境保育管理研習

Evaluation of the applicability of soil nitrification to assess

服務機關：行政院環境保護署

出國人　職　稱：技士

姓　名：張同婉

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報告日期：九十年十二月十一日

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出國計畫主辦機關審核意見	□1. 依限繳交出國報告 □2. 格式完整 □3. 內容充實完備 □4. 建議具參考價值 □5. 送本機關參考或研辦 □6. 送上級機關參考 □7. 退回補正，原因：□①不符原核定出國計畫 □②以外文撰寫或僅以所蒐集外文資料為內容 □③內容空洞簡略 □④未依行政院所屬各機關出國報告規格辦理 □⑤未於資訊網登錄提要資料及傳送出國報告電子檔 □8. 其他處理意見：
層轉機關審核意見	□同意主辦機關意見 □全部 □部份 （填寫審核意見編號） □退回補正，原因： （填寫審核意見編號） □其他處理意見：

說明：

一、出國計畫主辦機關即層轉機關時，不須填寫「層轉機關審核意見」。

二、各機關可依需要自行增列審核項目內容，出國報告審核完畢本表請自行保存。

三、審核作業應於出國報告提出後二個月內完成。

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報告名稱:

環境保育管理研習 Evaluation of the applicability of soil nitrification to assess pollution

主辦機關:

行政院環境保護署

聯絡人／電話:

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出國人員:

張同婉　行政院環境保護署　綜計處　技士

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關鍵詞: nitrification, bioluminescence, bioavailability

內容摘要: Soil nitrification has been reported to be sensitive to heavy metals and may be a suitable index of soil microbial activities to pollutants. In this study, nitrification measurements and luminescence-based biosensor tests were carried out to assess the pollution impact of metal contaminated soils. Perfusion-based nitrification was the most sensitive method to examine the toxic response of heavy metal. Soil nitrification was inhibited by copper, zinc and nickel. Zinc had strongest influence on nitrification. The incubation-based nitrification method had no significant response to metal toxicity. The bioluminescence-based bacterial sensors respond to acute toxicity, but showed no response to the metal contaminated soils. The aged soils had little evidence of bioavailable metal in the solution phase. The toxicity of metal pollution was associated with various environmental parameters that functional microbial-based tests are valuable techniques in pollution studies.

前言

蘇格蘭風光明媚，優美的自然風景如草原、湖泊為其最大特色，其中歷史悠久的愛丁堡、遠近馳名的海怪－尼斯湖、高爾夫球場起源地－聖安德魯斯更是每年吸引不少旅遊人潮來此一遊，即使英格蘭過慣忙碌城市生活的人們亦視此地為休閒勝地。而亞伯丁（Aberdeen）雖稱為蘇格蘭第三大城市，實際僅是一個寧靜的小城市，因北海（North Sea）石油的開採而成為蘇格蘭石油運輸的樞紐，而建立其重要之地位。

UK 碩士為一年制，分研究（by research）及課程（by course）二種。顧名思義，前者不修學分，但著重論文，後者須修學分，其中課程約佔三分之二，其餘時間則為論文。但即使同校，對於論文、口試的安排，各科系會有不同的處理方式。

我主修的環境科學（Environmental Science）隸屬於亞伯丁大學植物土壤系，亞伯丁大學已有數十年歷史，Department of Plant & Soil 於 1988 年由植物系與土壤系合併而成，除擁有設備精良的各式實驗室，教師皆具備豐富之野外經驗，其師資及研究於 1997 年之評鑑等級為優等（Excellent）。從分子科學到環境科學，所排課程往往不僅限單一領域，而跨越各類學門，經課堂傳授、演講及定期論文發表加強學識交流。

系所安排的課程除本系主辦者，亦有生態系、森林系、農業系及地質學系等主辦課程，故上課也常與生態學、微生物學碩士班，甚至高年級學生同班，少則十數人，人數多時也高達七、八十人。一年的碩士課程，自九月至翌年五月（扣除聖誕假期、復活假期），實際上課 24 週，每 6 週修二門學科，環境科學碩士班須選修 8 門學科，每門 3 學分，共 24 學分，如下所述：

1- 6 週	1. Plant Ecology 2. Data Analysis and Project Planning
7-12 週	3. Environmental Biotechnology 4. Environmental Remote Sensing
13-18 週	5. Soil Microbiology and Microbiology 6. Chemical Analysis
19-24 週	7. Impact Assessment & Remediation of Contaminated Environments 8. Soil Biology and Fertility in Tropical and Temperate Environments

因為必須於一年內修完選修課程並完成論文，為爭取時效，在復活節前，即須提出論文理念或大綱，至五月底課程結束，論文（實驗）即可迅速展開。

由於亞伯丁大學在土壤科學上雄厚的背景及個人對重金屬污染之興趣，我選擇以硝化菌、螢光菌測試土壤重金屬污染

之效應。值得一提的是我所採用的螢光菌是一種經基因轉植的 *E. coli* 及 *Pseusomonos*，其所產生之螢光會受重金屬毒性或營養鹽之影響而有所消長，可以測試土壤受重金屬污染的程度，此法在英國已使用多年，為一種迅速、簡便且有效之檢測方法，論文內容如附。

Applied to Applied Soil Ecology

Evaluation of the applicability of soil nitrification to assess pollution

Tung-Wan Chang

MSc Environmental Science, Department of Plant and Soil Science,

University of Aberdeen

Abstract

Soil nitrification has been reported to be sensitive to heavy metals and may be a suitable index of soil microbial activities to pollutants. In this study, nitrification measurements and luminescence-based biosensor tests were carried out to assess the pollution impact of metal contaminated soils. Perfusion-based nitrification was the most sensitive method to examine the toxic response of heavy metal. Soil nitrification was inhibited by copper, zinc and nickel. Zinc had strongest influence on nitrification. The incubation-based nitrification method had no significant response to metal toxicity. The bioluminescence-based bacterial sensors respond to acute toxicity, but showed no response to the metal contaminated soils. The aged soils had little evidence of bioavailable metal in the solution phase. The toxicity of metal pollution was associated with various environmental parameters that functional microbial-based tests are valuable techniques in pollution studies.

Introduction

Nitrification is one part of the nitrogen cycle that occurs throughout the soil. It involves the oxidation of ammonium, via nitrite, to nitrate. With increasing industrial development, various kinds of waste and toxic elements accumulate in soils, inhibiting essential soil processes including microbial activity. The toxicity of

heavy metals to soil microbial activities depends upon their bioavailability in soils, and this is related to the free metal forms more than to the total soil metal content. Free ion activity is the central element responsible for the interactions of toxicity and uptake between metals and organisms (Hare and Tessier, 1996). Properties such as pH, organic matter and soil texture, determine the metal activity, and the rate of nitrification as well.

Because of its importance in soil and its sensitivity to environmental change, many researchers have used nitrification as an indicator of soil pollution from heavy metals (Bhuiya & Cornfield, 1974; Liang & Tabatabai,1977; Quraishi & Cornfield, 1973). Liang & Tabatabai (1977) identified 19 trace elements that inhibited nitrification in soils and concluded that the accumulation of trace elements in soils will lead to a reduction in the amount of plant available nitrogen. Many heavy metals are toxic to microbial processes when present in excess (Dusek, 1995) .

Recently, in addition to biochemically-based soil tests, bioluminescent bioassays have been developed for detection of soil borne pollutants. The response of luminescence represents the activity of the bacteria responding to an environmental sample. These bioassays are rapid and simple, which leading to a variety of environmental investigation (McGrath et al., 1999 ; Ritchie et al., 2001) . In this paper, biosensors were used to assess the acute toxicity of metals in a Scotish soils. The results were also compared with earthworm assimilation data for the same soil. The objectives of this paper are to discuss (1) to optimize a nitrification bioassay, (2) to assess the effect of heavy metals on nitrification potential under influence of different combination of Zn, Ni and Cu, (3) to use bioluminescence to test the toxicity of these metals, (4) to assess the bioavailability of metals in these soils and the factors influencing their effects.

Materials and methods

Soil samples

The soil used for this study was Insch association, Insch series from Inschfield farm, Aberdeenshire (Table 1)

Table 1. Insch-soil characteristics

Texture	Sandy loam
Sand (%)	57.7
Silt (%)	30.79
Clay (%)	11.51
Organic matter (%)	3.75
pH	6.1
WHC (%)	69

(Tiensing et al., 2001)

Each soil was amended with Zn, Ni and Cu at values related to sewage sludge metal qualities (0, 50%, 100% and 200%). Metal additions were made individually and in combination. There are 36 combination of soils within the range of copper (0, 67.5, 135, 270 mg/kg), zinc(0, 150, 300, 600mg/kg) and nickel(0, 37.5, 75, 150 mg/kg). Soil was spiked in May 2000. These soils were preserved at 4℃, and were taken out 2 days before the experiment was carried out. Each soil was carried out with 3 or 5 replicates.

Nitrification tests

Modified perfusion method

The soil was mixed with acid washed sand in 2:1 ratio in a plastic bag before adding

the ammonium source. An aliquot of 2.0g Sephedex gel, 0.4g Amberlite, and 20g of sand: soil were added to glass leaching tubes(3.0 cm internal diam.) from base to top. Sephedex gel controlled the flow rate of the perfusing solution in order to optimise nitrification.

The amberlite anion exchange resin was used to avoid excess ammonium ions leaching out of the sample. These layers were separated by glass fibre paper.

A 100ml aliquot of perfusing solution, which contained 0.5mM (NH_4) $_2SO_4$ was added into the perfusion tubes, and allow to flow. During perfusion, the solution was added in 4 aliquots (30ml, 30ml, 20ml, and 20ml) to enable perfusion to be maintained for a 9-hour period. A reagent, (0.01M $NaClO_3$) was added to the perfusing solution to block the reaction of nitrite to nitrate, and nitrification potential was assessed by measuring NO_2- N (μg/h/g soil). The leachates were collected in plastic bottles and stored at 4°C until analysis by flow injection analysis.

Incubation method

A 5g aliquot of soil and 50ml 2M KCl were added to a 250ml Erlenmeyer flask. The flasks were stoppered and shaked on a orbital shaker for 1 hour. The soil-KCl suspension was filtered through a Whatman no. 42 filter paper. The filtrate was stored at 4°C until analysis with Flow Injection Analysis. The concentration was determined as initial nitrate (NO_3- N μg /d/g soil) .

A 5g sample of soil was placed in a Universal bottle with 1ml deionized water (50% WHC of Insch-soil) and 0.1ml 25mg/ml $(NH_4)_2SO_4$. The bottles were incubated at 20℃ in the dark. After 3 weeks, the incubated soil was extracted with 50 ml 2M KCl. The soil-KCl solution was put on an orbital shaker and filtered through Whatman 42 filter paper. The filtrate was then analyzed and recorded as final nitrate. The initial concentration of nitrate was substrated from the final for each soil to determine nitrification potential.

Biosensor tests

A 5g aliquot of each soil and 5ml deionized water was added to a 50 ml centrifuge tube, then centrifuged at 1840 G, at 4℃ for 30 minutes. The supernatant was removed from the tube and maintained at 4℃ prior to analysis.

The biosensors (*Pseudomonas fluorescens* pUCD 607 and *Escherichia coli* HB 101) were resuscitated from freeze-dried by mixing with 1M KCl in Universal bottle, and incubating for 1 hour on an orbital shaker. After incubation, a 900μl aliquot of soil solution and 100μl biosensor suspension were added to a 2 ml cuvette, and then mixed thoroughly by a vortex mixer. The bioluminescence was measured by using a Jade luminometer. The bioluminescence effect of *P. fluorescens* was measured after 1 hour of exposure to the biosensor, while *E. coli* was after thirty minutes of exposure.

The test with *P. fluorescens* was carried out with 5 replicates of each soil, while the *E. coli* was 3 replicates in this experiment. The bioluminescence effect was expressed as percentage of luminescence compared to control (non-contaminated) soil.

Statistics

Biosensor tests and perfusion method were analyzed by 2-way ANOVA, and the significant difference ($p \leqq 0.05$) between different treatments was compared at the $p \leqq 0.05$ confidence limit. The nitrifying potential measured by the incubation method was represented as a difference between average initial and final value, thus it was statistically assessed by 2-way ANOVA without replication.

Results and discussion

Nitrification

In the perfusion method, different concentration of metals showed significant effects

on nitrification. Additionally, there were also strong interactions between two metals in each combination ($p<0.001$) . This confirmed the results of previous studies demostrating that nitrification can be affected by heavy metal.

Combinations of metals present in soils may induce complicated responses through physicochemical properties and microbial activities. When zinc, nickel and copper were present individually there was a clear inhibitory tendency of nitrification. The inhibition of nitrification increased with elevated concentration of zinc and copper. Nickel also showed an inhibitory effect, but nitrification potential increased as the concentration rose to 150 mg/kg. In contrast, although the combination of heavy metal had significant effect on nitrification, the inhibition was not clearly explained by concentration (Fig 1).

The perfusion assay was the only technique that yielded sensitive responses to the soils studied. To place this in an ecological context it was compared with data for soil invertebrate studies in the same soil samples using a standard earthworm assimilation toxicity test[Miah pers com]. The concentration of Cu, Ni and Zn accumulated in earthworms was found significantly affected in combination of Ni & Zn and Zn & Cu. Surprisingly, copper and nickel had significant effect within the combination Ni & Zn and Zn & Cu respectively. The concentration of copper and nickel could be varied with other metals, but zinc varied only with zinc itself (Table 2) .

The most clear inhibitory tendency in perfusion-based nitrification occurred in the combination of Ni & Zn except at 150 mg/kg of Ni. The decline is obscure in the combination of Cu & Ni, which zinc was not involved in nitrification inhibition(Fig 1) . Relating to the earthworm data, zinc is the most mobile metal chemically and its behavior is probably independent of nickel and copper.

Table 2. Significance* occurred in each metal within 3 combinations.

	Concentrations of **Zn** mg g-1dried earthworm	Concentrations of **Ni** mg g-1dried earthworm	Concentrations of **Cu** mg g-1dried earthworm
Ni & Zn	Zn	Ni	Zn, Ni**
Zn & Cu	Zn	Zn**	Cu, Zn
Cu & Ni	—	Cu, Ni	Cu

* Significance was tested by ANOVA-2 factor with replication.

** Ni showed significance in the combination of Zn & Cu, and so did Cu in Ni & Zn.

For the incubation method, there was no significant difference in nitrification potential between different concentrations of metal in any combination. It might be due to a reduction of bioavailability of metals or the recovery of microbial activities in Insch-soil. The bioavailability of heavy metal could be influenced through process of precipitation, ion exchange, complexation, or redox potential. Cation exchange capacity and organic matter content could promote adsorption of toxic metals and then reduce their bioavailability (Sauve, 1999). Dissolved organic matter (DOM) enlarges the pool of organic ligands in high pH soils, which significantly decrease the free metal ion activity (Sauve et al., 1998). Formation of stable metal-organic complexes are also result in the reduction of metal bioavailability (Romkens, et al., 1999). In this experiment, these contaminated Insch-soils have been incubated for 1 year. Some physichemical reactions might occur during this period which influence the activities of free ions. In this

experiment, the soils were incubated with nitrogen source (ammonium sulphate) and water content maintained for 3 weeks. It offered nitrifiers a nutrient rich environment, thus nitrification rate exceed the toxic effect from metals. It indicated that nitrification activity may recover effectively after exposure to toxic doses. Compared with perfusion method, incubation is not effective for long-term contaminated soil which metal bioavailability was reduced. Perfusion is proved to be a more sensitive method for measuring nitrification potential in this study.

Biosensor

No significant difference occurred for *E. coli* in each combination of heavy metals. However, when nickel was the sole pollutant, there was a significant deleterious influence on the bacteria, but the influence didn't occur when zinc or copper was present.

On the contrary, there were significant effects due to different concentrations of heavy metals for *P. fluorescens*, especially in the combination of zinc & nickel or the combination zinc & copper. No significant response occurred in copper when it combining with nickel.

The toxicity examination of heavy metals is strongly related to the bioavailability of metals in soil. The bioavailability of metals is thought to be due to the free ion present in the soil. Bioluminescence-based biosensors have been shown to respond to the available ions of metals (McGrath et al., 1999) and was effective indicator of heavy metals (Chaudri et al., 2000). However, the percentages of bioluminescence in *P. fluorescens* were above non-contaminated soil. It suggested that the soils were not toxic to *P. fluorescens* in each combination of metals (Table 3).

Table 3. Mean value of percentage bioluminescence compared to non-metal soil in *P. fluorescens*.

	Ni & Zn					Zn & Cu					Cu & Ni			
	Ni					Zn					Cu			
Zn	**0**	**37.5**	**75**	**150**	**Cu**	**0**	**150**	**300**	**600**	**Ni**	**0**	**67.5**	**135**	**270**
0	100%	115%	159%	99%	**0**	100%	313%	108%	124%	**0**	100%	110%	101%	92%
150	313%	255%	140%	121%	**67.5**	110%	102%	117%	128%	**37.5**	115%	110%	113%	114%
300	108%	129%	125%	125%	**135**	101%	108%	—	139%	**75**	159%	99%	110%	111%
600	124%	132%	136%	122%	**270**	92%	116%	116%	164%	**150**	99%	120%	110%	140%

* No significant effect occurred between different concentrations of Cu in combination of Cu and Ni.

* The unit of concentration of Zn, Ni and Cu is mg/kg.

Significant difference between metals also suggested that there might be some nutrient source present which could enhance the bioluminescence of the bacteria. Yeomans, et al (1999) studied nutrients flow in rhizosphere using *lux*-marked *P. fluorescens*. The result in bioluminescence is above that of control which containing no carbon source. They concluded that carbon source results in increase in bioluminescence that the percentage is higher than 100%. If the increasing light output by *P. fluorescens* in this study is caused by organic carbon source, it might be the reason for the reduction in toxicity of heavy metals in this experiment. Organic matter has been reported as a metal-chelator in many studies, it will decrease the bioavailability of heavy metals, thereby reduce their toxicity. With organic matter in soil, the free ion of metals in Insch-soil might have been

complexed and immobilized by it during previous incubation.

Conclusion

The bioavailability of metals of Insch- soils was reduced within 1-year incubation. One of the reasons might due to the organic carbon present in soils, which was confirmed in the bioluminescence response in *P. fluorescens*. The free metal ions could be chelated and immobilized by organic carbon and then decreased the toxicity of metals dramatically. In this study, *P. fluorescens* and *E. coli* did not give an acute toxic response to Cu, Zn and Ni in each combination. The bioluminescence-based bioassay thereby might not be suitable for detecting toxicity in long-tern incubated soils.

The decrease in activity of heavy metals also occurred in nitrification measuring-incubation method, which had no different toxic response from contaminated soils. Soil nitrification was recovered from previous inhibitory influence of metals under sufficient nutrients. Compared to incubation method, perfusion is the most sensitive method to measure soil nitrification activity even after a period of incubation that bioavailability of heavy metals might have reduced. Nitrification potential was obviously inhibited by copper, zinc and nickel. Zinc had stronger influence on nitrification than nickel and copper, the result was found in assimilation data of earthworms as well. However, the results showed that toxicity response differed with various parameters such as species, organic content, and even the soil pre-treatment. Bioindicator is effective to assess the toxicity responses to pollution in this study.

Acknowledgements

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Department of Plant and Soil Science, University of Aberdeen. Thanks for his prudential instruction and patient during this experiment.

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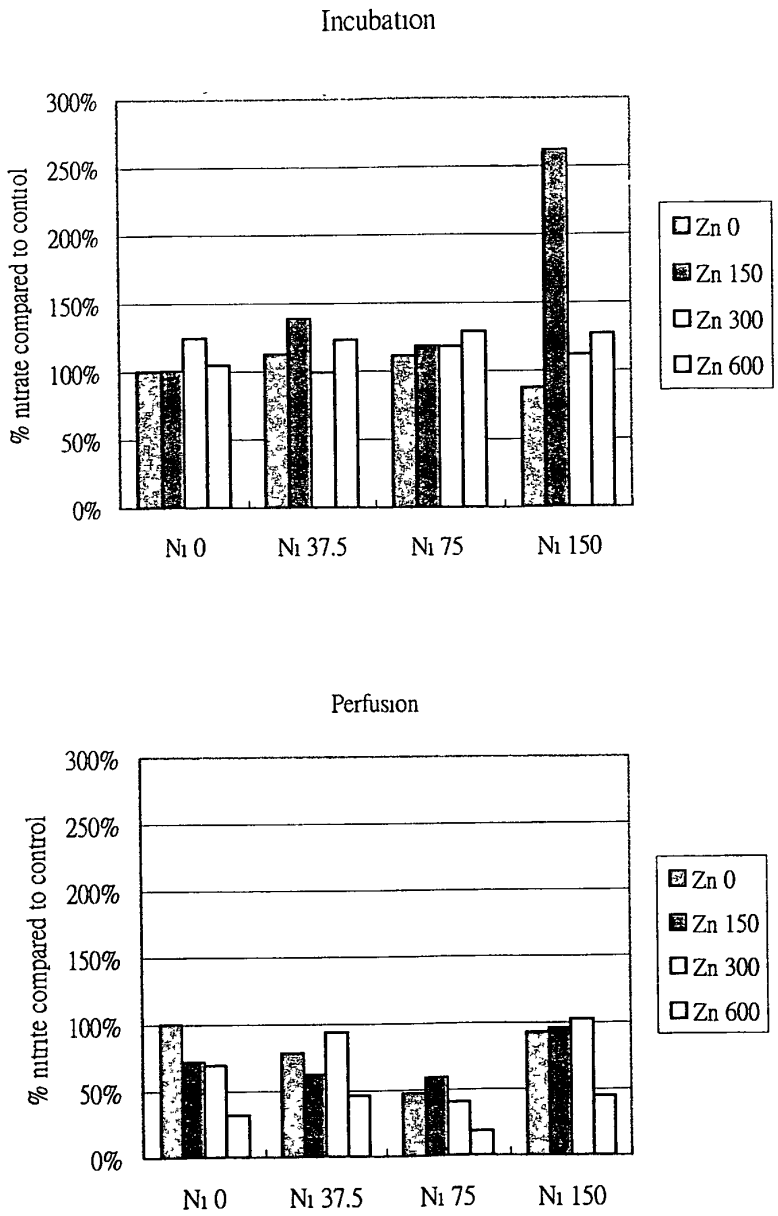


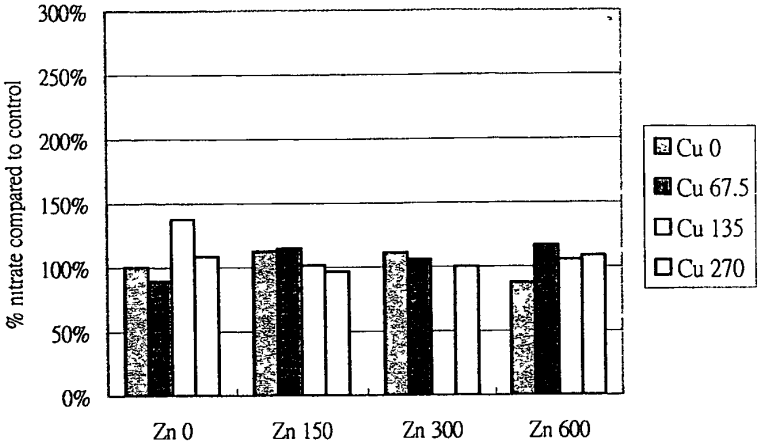
(a) Ni & Zn

Fig 1. Nitrification potential measured using perfusion method and incubation method in combination of (a) Ni & Zn, (b) Zn & Cu, (c) Cu & Ni.

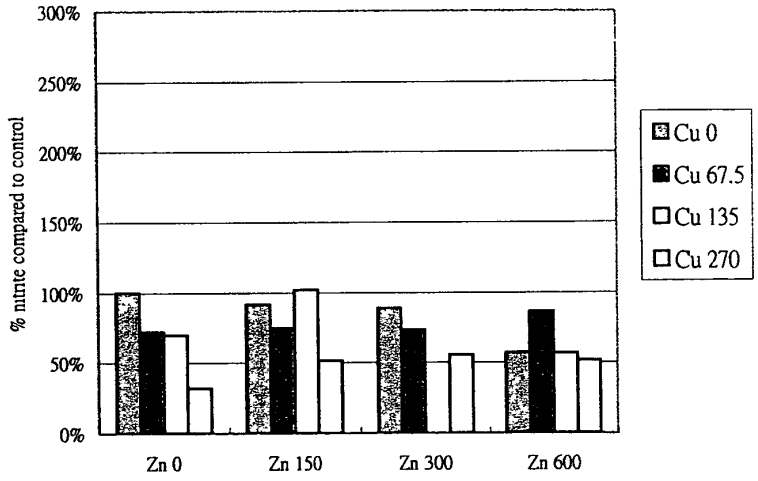
* The concentration of Ni, Zn, Cu are represented as mg/kg.

* Inhibition is obvious in perfusion comparing with incubation.

Incubation

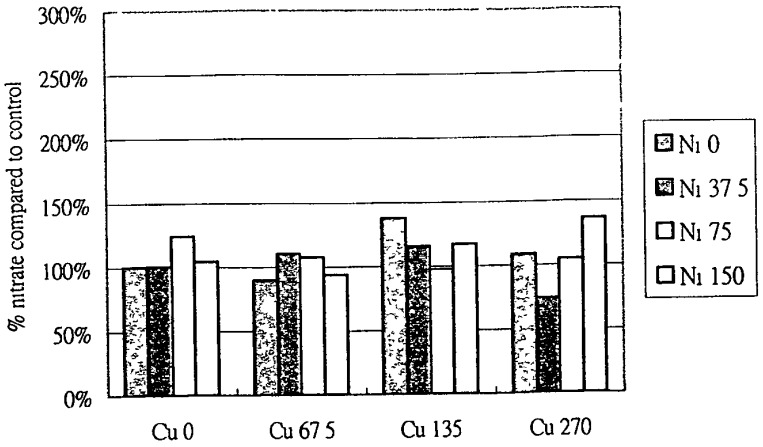


Perfusion



(b) Zn & Cu

Incubation



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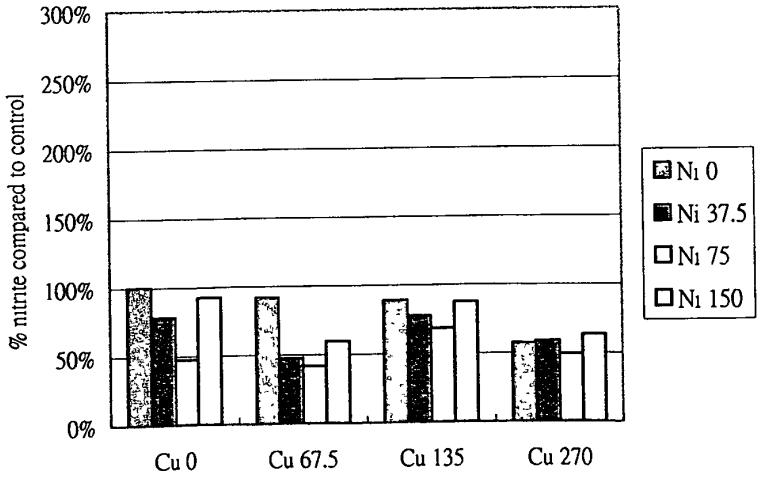

(c) Cu & Ni

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A new perfusion system for the measurement and characterization of potential rates of soil nitrification

K KILLHAM
Department of Soil Science, University of Aberdeen, Aberdeen, AB9 2UE, Scotland, UK

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Summary A new perfusion system for the rapid and simple measurement and characterization of potential rates of soil nitrification is presented. The system involved pumping a buffered NH_4^+ solution, containing chlorate to block oxidation of NO_2^- to NO_3^- (*i e* NO_2^- sole product of nitrification), through a soil column Perfusate was then passed through a cation exchange resin to remove remaining NH_4^+ (preventing further nitrification), and through a sephadex gel to control the perfusion rate. Determinations of NO_2^- eluting from the column enabled rates of nitrification to be calculated. Soil samples were also perfused with C_2H_2 (1%) in the perfusing solution, as a partition of autotrophic and heterotrophic nitrification. In an agricultural soil adjusted to a range of pH values (then maintained at constant pH over 25 years), potential rates of nitrification (using unbuffered perfusate) decreased with increasing soil acidity, while at each pH almost all nitrification was blocked by C_2H_2. This suggested autotrophs dominate nitrification in agricultural soil, regardless of acidity. In an acid coniferous soil, however, 90% of potential nitrification was unaffected by C_2H_2, suggesting heterotrophic nitrification is of particular significance in acid forest soils.

Introduction

Measurement of potential rates of nitrification in soils generally involves perfusion of soil with a NH_4^+-containing solution and subsequent determination of the oxidation products NO_2^- and NO_3^-. Nitrification assays using a soil slurry system have also been used, but these may be more likely to generate anaerobic conditions with the consequent loss, through denitrification, of the nitrification products. Also, both perfusion and slurry systems may overestimate potential rates of nitrification unless nitrification is stopped (*e.g.* by freezing or filtering) in the perfusate and filtrate respectively.

Recent use of specific inhibitors has enabled further characterization of soil nitrification. Sodium chlorate has been used to block chemoautotrophic oxidation of NO_2^- to NO_3^- [1,6]. The chlorate block technique also has great potential in the measurement of net nitrification since only one nitrification product is generated which facilitates a more simple determination of the overall nitrification rate. (Since oxidation of NH_4^+ is the rate limiting step of autotrophic nitrification, the presence of ClO_3^- does not affect the reaction velocity.) Acetylene has been used to inhibit autotrophic oxidation of NH_4^+ to NO_2^- [2,5]. Acetylene has also been

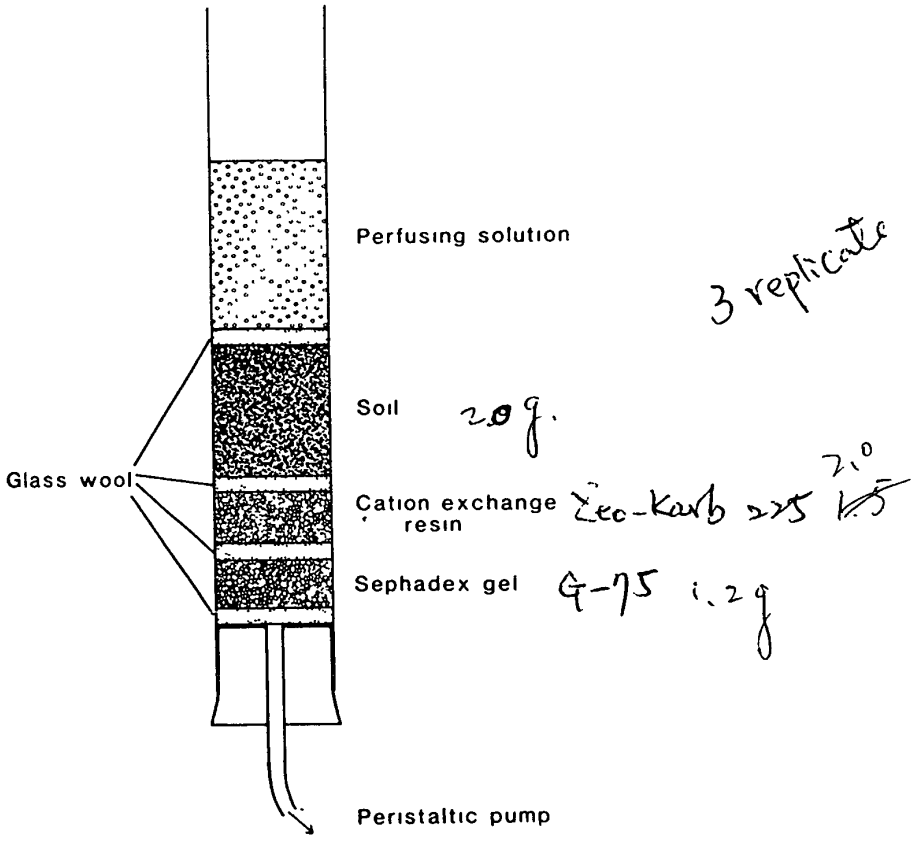

Fig. 1. Perfusion apparatus for measurements and characterization of potentials of soil nitrification.

shown not to inhibit heterotrophic nitrification. This has been demonstrated both for a heterotrophic bacterium[5] and for a fungus[9]. This selective inhibition of nitrification appears to be effective in partitioning autotrophic and heterotrophic nitrification in soil[9].

The aim of this study was to develop, using the recent innovations discussed above, a new and simple NH_4^+ perfusion system for the rapid determination of potential rates of soil nitrification. The aim was to incorporate into the system, therefore, the following features:- a) A chlorate block to facilitate determination of the reaction rate, NO_2^- being assayed as the sole product of nitrification; b) Prevention of further nitrification in perfusate eluting from soil by continuous removal of NH_4^+; c) Partitioning of autotrophic and heterotrophic nitrification by the replacement of ClO_3^- in the perfusing solution with C_2H_2.

Materials and methods

Samples of a freely-drained brown forest earth (total organic carbon, 9%) were collected from the North of Scotland College of Agriculture research station, Craibstone, near Aberdeen, Scotland The site has been sub-divided into plots which have been maintained (using Aluminium sulphate and Calcium carbonate) at constant pH levels ranging from 4.5 to 7.5 over a period of 25 years Samples were collected from the top 10 cm of each of these plots A peaty gley soil (pH 4 4, total organic carbon, 11%, litter layer removed and top 10 cm of soil taken) was sampled from under a stand of 20 y old Sitka spruce at Culloden, near Inverness, Scotland.

The perfusion system (Fig 1) consisted of glass leaching tubes (15 cm long, 1 5 cm internal diam.) slurry packed, from base to top, with sephadex gel (0.3 g, G75, used to slow the flow rate through the column), cation exchange resin (0.5 g, Zeo-Karb 225, Na^+ form) and fresh soil (5 g, sieved to < 2 mm). The layers were separated by thin bands of glass wool. The perfusing solution, buffered to pH 6.5 (2.5 m*M* phosphate buffer), contained an ammonium source (0.5 m*M* $(NH_4)_2SO_4$) and chlorate (0.01 *M* $NaClO_3$). Before NH_4^+ perfusion commenced, the column was first flushed with buffer only.

The bases of the columns were connected via peristaltic tubing to a Watson-Marlow 8-channel peristaltic pump. The pumping rate was maintained so that 50 ml of the perfusing solution was pumped through the column over 6 h. Determination of NO_2^- concentrations in the perfusate were determined colorimetrically with a technicon autoanayzer (model II).

Potential rates of nitrification were also determined with acetylene (1%) dissolved in the perfusing solution (unbuffered 0.5 m*M* $(NH_4)_2SO_4$) as a method of partitioning autotrophic (blocked by C_2H_2) and heterotrophic (unaffected by C_2H_2) activity. Chlorate was not included in these systems. Nitrate in the perfusate was reduced (cadmium reduction) to nitrate prior to colorimetric determination by autoanalyzer.

To test the effectiveness of C_2H_2 in partitioning nitrification, a number of columns were leached with a selective biocide (1 g L^{-1}) in place of chlorate or acetylene in the perfusing solution. The biocides used were Streptomycin sulphate and Cycloheximide.

Nitrification rates, in μmoles NO_2-N (g dry wt. soil)$^{-1}$ h^{-1}, were calculated by taking a linear regression through data points (NO_2^- produced during each 2 h interval) of triplicate columns.

Results and discussion

Nitrification, assayed by the new perfusion system (Fig. 1), occurred in all the soil samples tested (Fig. 2). Chlorate itself did not have any effect on the rate of NH_4^+ oxidation (data not shown). NH_4^+ was not detectable in the perfusate, but eluted in high concentrations from columns where the cation exchange resin was not included (Table 1). Determination of potential nitrification by this method, therefore, was not affected by continued oxidation of NH_4^+ after perfusion. This was confirmed when NO_2^- concentrations in perfusate were found to remain unchanged over several hours following perfusion, but increased markedly in the absence of the cation exchange resin. The Sephadex gel included in the columns to adjust perfusion to rates compatible with nitrification kinetics, was found, when tested independently of the columns, to have no significant effect on the N-ion chemistry of soil extracts passed through it (data not shown).

Sampling from plots of the same agricultural soil, maintained at constant pH over 25 y, nitrification potentials (determined using unbuffered perfusate) were found to be highest at pH 6.5 and 7.0, decreas-

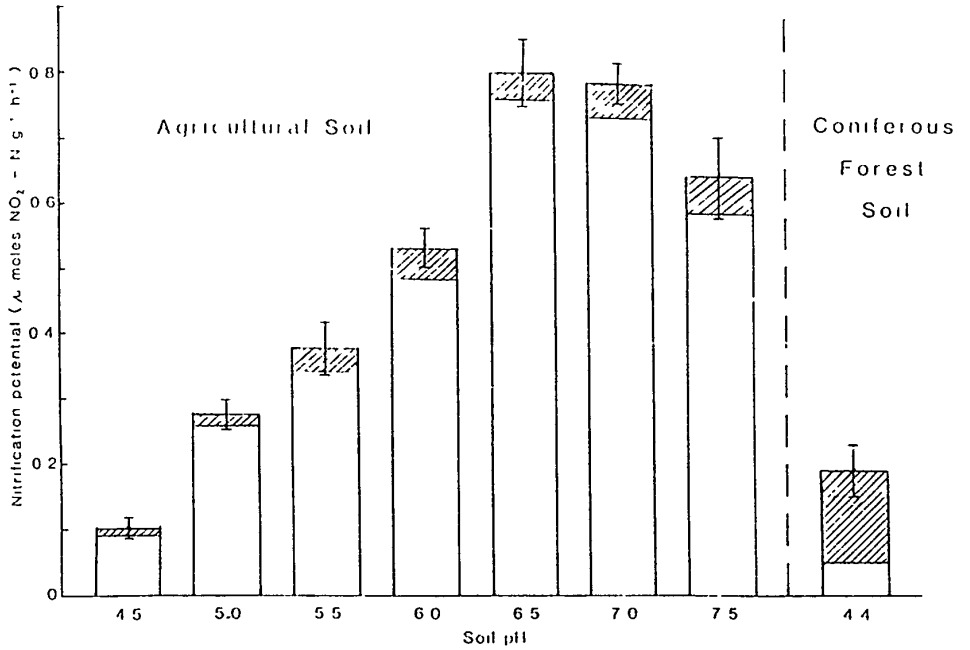

Fig 2. Nitrification potentials* of an agricultural soil and a coniferous forest soil. Shaded areas of histograms represent proportion of nitrification not blocked by C_2H_2.
*unbuffered perfusate

ing sharply with increasing soil acidity. Autotrophic nitrification has traditionally been considered to be particularly acid-sensitive[8]. Nitrification in the more acid plots may be attributable to either acid-adapted autotrophs or perhaps to heterotrophs such as nitrifying fungi. There is now evidence of *in vitro* fungal nitrification where substrate has been both inorganic and organic[3,4,10]. Recent evidence, based on the use of acetylene as a block of autotrophic nitrification (but not significantly affecting heterotrophic nitrification), suggests that there is a greater potential for heterotrophic nitrification than for autotrophic nitrification in acid forest soils[9]. The soils selected to partition nitrification in this study were the agricultural soil from the pH plots, used in the previous experiment, and an acid coniferous soil sampled from under a Sitka

Table 1. NO_2-N and NH_4-N concentrations in total perfusate (50 ml) from an agricultural soil (pH 6 5), with and without cation exchange resin included in the perfusion column

	N-ion concentrations (μM) in total perfusate	
	NO_2-N	NH_4-N
Column + cation exchange resin	308.9 ± 29.6	0 0
Column − cation exchange resin	317.1 ± 36.5	142.7 ± 13 6

Concentrations are means of perfusates from triplicate columns ± standard error

spruce plantation. In the agricultural soil, regardless of the pH at which it had been maintained, potential nitrification rates were almost entirely stopped when C_2H_2 was introduced to the perfusing solution (Fig. 2). The proportion of the potential rate of nitrification blocked by C_2H_2 was always greater than 90%, even for samples from the most acid (pH 4.5) of the pH plots. In an acid (pH 4.4) coniferous soil, however, only about 20% of the potential nitrification was blocked by C_2H_2 (Fig. 2). These results suggest, therefore, that nitrification in agricultural soil is dominantly an autotrophic process (*i.e.* blocked by C_2H_2) regardless of acidity. Autotrophic nitrification under highly acid conditions is thought to be facilitated by the attachment of the nitrifier cells to surfaces. Cells of autotrophic nitrifiers attached to glass beads have been shown to nitrify under acid conditions in which unattached cells did not survive[7]. Autotrophic nitrification in the acid plots of the agricultural (arable) soils, therefore, may well be mediated to some extent by surface-attachment of the nitrifying cells. The results also suggest, however, that nitrification is dominantly heterotrophic (*i.e.* unaffected by C_2H_2) in coniferous forest soil of similar pH to the most acid samples of the agricultural soil.

The above suggestions of the ecological significance of autotrophic and heterotrophic nitrification were largely confirmed by replacing C_2H_2 in the perfusing solution with selective inhibitors of protein synthesis (streptomycin to inhibit bacteria; cycloheximide to inhibit fungi). Although effects were not as pronounced as with C_2H_2 (probably because of the slow action of the inhibitors), nitrification potentials in all samples of the agricultural soil were reduced by streptomycin and largely unaffected by cycloheximide, while potential nitrification in the acid coniferous soil was reduced by cycloheximide and largely unaffected by streptomycin (Table 2).

In conclusion, the new perfusion system offers a simple and reliable

Table 2. Comparison of the effects (% inhibition) of different inhibitors on nitrification potentials (unbuffered perfusate) in an agricultural soil and in an acid coniferous forest soil

Soil	Inhibition		
	Acetylene	Streptomycin	Cycloheximide
Agricultural soil pH 7 5	91.4 ± 10 1	39.4 ± 5 3	5.8 ± 0.8
Agricultural soil pH 7 0	93.5 ± 11 6	45.2 ± 6 5	6 2 ± 0.9
Agricultural soil pH 6 5	95.0 ± 9 0	36 0 ± 5 0	3 9 ± 0 6
Agricultural soil pH 6 0	91 5 ± 13 3	32 5 ± 5 0	8 1 ± 1 3
Agricultural soil pH 5 5	91.7 ± 12 4	34 4 ± 4.9	9 0 ± 1 3
Agricultural soil pH 5 0	93.7 ± 14 5	27 7 ± 3 6	7 2 ± 1 5
Agricultural soil pH 4 5	90.0 ± 8 7	34.6 ± 3 3	7 0 ± 0.8
Acid coniferous soil pH 4 4	26.3 ± 5 2	8.3 ± 1 0	39 0 ± 5 2

Percentages calculated from means of triplicate columns ± standard error

method for measuring potential rates of nitrification (NO_2^- production has been found linear with time for all soils tested) and may also be easily modified to partition these rates into likely autotrophic and heterotrophic components. This type of partitioning approach should enable characterization of nitrification in different soil environments, particularly more acid systems where growing evidence suggests a significant heterotrophic component.

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Chemical Factory Shops

INCUBATION STUDY ON EFFECT OF pH ON NITROGEN MINERALISATION AND NITRIFICATION IN SOILS TREATED WITH 1000 PPM LEAD AND ZINC, AS OXIDES

M. R. H. BHUIYA & A. H. CORNFIELD

Chemistry Department, Imperial College, London, SW7 2AY, Great Britain

ABSTRACT

The effects were studied of adding 1000 ppm Pb and Zn, as oxides, to a sandy soil before adjustment to three pH levels on nitrogen mineralisation and nitrification during subsequent aerobic incubation for 6 weeks at 30°C. At pH 6·0 neither element had any significant effect on either process. At pH 7·0 nitrification was decreased slightly by Pb and Zn, whilst nitrogen mineralisation was decreased slightly only by Zn. At pH 7·7 (soil containing 2% $CaCO_3$) both processes were decreased slightly by Pb and to a fair extent by Zn. Results are discussed in relation to Morgan- and EDTA-extractable levels of Pb and Zn.

INTRODUCTION

High levels of metallic elements may occur in soils through the application of mining, industrial, municipal and sewage wastes, fertilisers and metal-containing pesticides, and through airborne contamination by industrial smoke and fossil fuel combustion products. Variation in soil pH will affect the mode and extent of reaction of metallic compounds added to soils. The reactions will include adsorption on charged surfaces, precipitation in cationic or anionic forms and complexing with organic materials to form soluble or insoluble products. These, in turn, will affect the extent of migration of metals through soils and their sorption by microorganisms.

Swaine (1955) reports that soils usually contain 2–200 ppm total Pb and 10–300 ppm total Zn, although higher levels may occur due to the parent rock or in the vicinity of mines and smelting works. This paper reports on the effects of adding

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1000 ppm Pb and Zn, as oxides, to a soil (containing only moderate concentrations of these elements) on mineral nitrogen changes during incubation after adjustment to three pH levels.

MATERIALS AND METHODS

The soil used was a 'Bagshot' sand (5·5% clay, 12·0% silt) from a cultivated area, air-dried and ground to pass a 2 mm sieve. The soil had pH 6·0 (in water) and contained 0·18% total nitrogen, 2·9 ppm ammonium-nitrogen, 2·1 ppm nitrate-nitrogen, 2·2% organic carbon, 35 ppm total Pb and 74 ppm total Zn. Separate bulk samples of soil were mixed with finely ground (less than 0·25 mm sieve) PbO and ZnO (AR grades) to add 1000 ppm of the metallic elements on the dry soil basis. Each of these samples and a control sample (receiving no metallic oxide addition) was divided into three lots. One of each lot received no further treatment. Another of each lot was mixed with finely ground calcium carbonate (AR grade), based on previous tests, to raise the pH to 7·0. The third sample of each lot received sufficient extra calcium carbonate to give a 2% excess. The treated and control soils at each level of calcium carbonate addition were placed in pots and held at room temperature (18–22°C) for two weeks with addition of water to maintain the moisture content between 40% and 50% saturation. The soils were then air-dried and rubbed through a 2 mm sieve. For incubation 10 g samples in quadruplicate were placed in 10 × 2·5 cm glass tubes and water added to bring the moisture content to 50% saturation. The barium peroxide method (Cornfield, 1961) was used for aeration and absorption of carbon dioxide during incubation of the tubes, which were closed with rubber bungs, for 6 weeks at 30°C. For analysis, 10 ml of water was added to duplicate tubes of each treatment and the pH measured after shaking. Then 10 ml of double-strength Morgan's reagent (N-acetic acid—1·5N—sodium acetate) was added and, after 2 min shaking, the contents were filtered. The filtrates were analysed for ammonium- and nitrate-nitrogen by a microdiffusion method (Bremner & Shaw, 1955) and for added metallic elements by atomic absorption spectroscopy. Two further replicates of each treatment were shaken for 2 min with 20 ml of 0·1N EDTA-Na (pH 7·0), filtered, and the filtrates analysed for the added metallic elements.

RESULTS

Table 1 shows (a) the extent of accumulation of mineral nitrogen (ammonium- plus nitrate-nitrogen) after incubation, in ppm on a dry soil basis, obtained by subtracting initial values from those after incubation and (b) Morgan- and EDTA-extractable levels of Pb and Zn on the dry soil basis. pH values after incubation

TABLE 1

EFFECT OF SOIL pH AND ADDITION OF 1000 PPM LEAD AND ZINC (AS OXIDES) ON MINERAL NITROGEN ACCUMULATION* AND EXTRACTABLE LEAD AND ZINC AFTER SIX WEEKS OF INCUBATION (RESULTS ON DRY SOIL BASIS)

Soil pH	*Treatment*	*Mineral nitrogen* NH_4–*N* ppm	NO_3–*N* ppm	*min–N* ppm	*Morgan-extractable metals* ppm	*EDTA-extractable metals* ppm
	O	2·7	21·7	24·4	—‡	—‡
6·0	Pb	3·2	21·8	25 0	184	712
	Zn	3·1	20 6	23·7	292	669
	O	1·3	34·7	36·0	—	—
7·0	Pb	3·1	30·8	33·9	221	731
	Zn	3·1	30·1	33·2	392	744
	O	5·2	41·3	46·5	—	—
7·-	Pb	3·8	37·8	41·6	312	699
	Zn	3·8	27·6	31·4	341	591
LSD	$P < 0·05$	2·1	2·1	2·3	27	57

* Initial levels subtracted from those after incubation.
† Containing 2% calcium carbonate.
‡ The control soils contained 1, 3 and 4 ppm Morgan-extractable lead, 18, 21 and 20 ppm EDTA-extractable lead, 6, 6 and 5 ppm Morgan-extractable Zn and 19, 19 and 18 ppm EDTA-extractable Zn at pH 6·0, 7·0 and 7·7, respectively.

are not presented since these did not differ by more than 0·2 units from their respective controls. The presence of ammonium-nitrogen in all soils after incubation indicated that nitrification was not being limited by ammonification.

In the control soils (no added metallic oxides) both nitrogen mineralisation and nitrification increased with pH. At pH 6 neither Pb nor Zn had any significant effect on nitrogen mineralisation and nitrification when compared with the control soil of the same pH. At pH 7 nitrification was decreased slightly by both Pb and Zn, whilst nitrogen mineralisation was decreased only by Zn. At pH 7·7 both processes were decreased slightly by Pb and to a fair extent by Zn. Morgan-Pb values increased with, whilst EDTA-Pb values showed no significant differences due to, pH. Morgan- and EDTA-Zn values increased from pH 6 to pH 7 and decreased to pH 7·7.

DISCUSSION

The slightly increasing toxicity of Pb to nitrogen mineralisation and nitrification was roughly correlated with increasing Morgan-Pb, but not with EDTA-Pb. The increasing toxicity of Zn to both processes with increasing pH was poorly correlated with either extractable form of Zn. The possibility of zincate ($Zn(OH)_3^-$) and $Zn(OH)_4^-$) and plumbate ($Pb(OH)_3^-$) species in solution even at the highest pH (7·7) is remote since calculations (Stamm & Morgan, 1970; Lindsay, 1972)

have shown that at this pH their concentrations are negligible compared with other species likely to be present. Norvell (1972) found that the extent of chelation of Zn by several chemical chelating agents usually increased considerably with pH. In the soil of pH 7·7, where the toxicity of added Zn to both processes was particularly high, it may be that a soluble neutral Zn chelate(s) was formed, by reaction with soil organic matter, and diffusion of this to the sites of microorganic activity would not be limited by charged particles in the soil. No information appears to be available on the levels of complexed species of Pb in soils in relation to pH. Since Pb is a more electronegative element than Zn and forms more stable chelates than does Zn (Irving & Williams, 1948) it is likely that Pb forms less soluble complexes than does Zn with soil organic matter. This could explain the relatively low toxicity of Pb compared with that of Zn at pH 7·7.

The generally similar extent of decreases, where these occurred, in nitrogen mineralisation and nitrification due to Pb and Zn additions indicates that neither treatment had any differential effects on the heterotrophic organisms responsible for nitrogen mineralisation and the autotrophs responsible for nitrification.

The results obtained here for Pb and Zn differ from those obtained for Cu (Quraishi & Cornfield, 1973), where it was found that the toxic effects of 1000 ppm added Cu (as sulphate) on both nitrogen mineralisation and nitrification decreased with increasing pH.

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Response of a *Rhizobium*-based luminescence biosensor to Zn and Cu in soil solutions from sewage sludge treated soils

Amar M. Chaudri[a,*], Kirsten Lawlor[a], Sara Preston[b], Graeme I. Paton[b], Ken Killham[b], Steve P. McGrath[a]

[a]*Soil Science Department, IACR-Rothamsted, Harpenden, Herts AL5 2JQ, UK*
[b]*Department of Plant and Soil Sciences, Cruickshank Building, University of Aberdeen, Aberdeen AB24 3UU, UK*

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Abstract

A luminescence based biosensor (Rhizotox-C) was used as an indicator of heavy metal pollution of soils The response of the biosensor to increasing concentrations of total soil Zn, soil solution Zn, soil solution free Zn^{2+}, total soil Cu and total soil solution Cu from soils of a long-term sewage sludge field experiment was investigated The bioluminescence response of the Rhizotox-C biosensor declined as total soil Zn, soil solution Zn and free soil solution Zn^{2+} concentrations increased The EC_{25} values for the biosensor for total soil Zn, soluble soil solution Zn and free soil solution Zn^{2+} were 164 ± 43 mg kg^{-1} soil, 4 ± 0 7 and 2 ± 0 3 mg l^{-1}, respectively. The EC_{50} values were 403 ± 57 mg kg^{-1} soil, 16 ± 3 and 6 ± 1 0 mg l^{-1}, respectively The largest soil solution concentration of Cu was about 620 μg l^{-1}, but this had no significant effect on luminescence This corresponded to a total Cu concentration in bulk soil of about 349 mg kg^{-1}

1. Introduction

Studies looking at the effects of heavy metals on indigenous populations of soil microorganisms or on microbially-mediated soil processes often report short-term acute effects rather than long-term chronic effects These short-term acute effects can probably be attributed to the use of unrealistically high concentrations of pollutants in laboratory tests over relatively short periods of time In the environment, heavy metal pollution can build up over many years through the application of metal-contaminated sewage sludges and pesticides to agricultural soils, and through anthropogenic activities such as mining and smelting Because of this, chronic effects are likely to predominate in the environment However, chronic effects can take many years or even decades to develop (Chaudri et al., 1992,1993, McGrath et al., 1995, Giller et al., 1998), even in highly polluted soils, making early detection of problematic soils difficult On the other hand, acute effects on susceptible microbial populations may be immediate and too short-lived to be noticeable

Current methods of assessing toxicity to soil microorganisms from contaminated soils involve either collective determinations as in the quantification of the soil microbial biomass (Brookes et al., 1986, Chandei and Brookes, 1993) or enumeration of individual species using various culturing techniques (Chaudri et al., 1993, Situla et al., 1999) However, these techniques are slow and labour intensive Hence, there is a need for the development of a soil-based microbial bioassay, which is sensitive enough to rapidly identify potentially contaminated soils For this bioassay to be effective, it must have the ability to rapidly yield information, yet act as a surrogate for chronic changes in soil health, thus giving early warning of potentially toxic soils In this study we used a luminescence based biosensor (Rhizotox-C), with the *lux* cassette for luminescence incorporated into it, as a possible indicator of

* Corresponding author Tel +44-1582-763133 x 2792 fax +44-1582-760981
E-mail address amar chaudri@bbsrc ac uk (A M Chaudri)

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Table 1
Mean soil pH, organic carbon and total metal concentrations

Treatments[a]	Soil pH	Soil % C	Total metal conc (mg kg^{-1})			
			Zn	Cu	Ni	Cd
Control no sludge	6 5	0 99	44	9	7	0 18
Non-metal enriched control sludge	6 3	1 14	53	11	7	0 28
Zn 1	6 3	1 16	141	16	9	0 22
Zn 2	6 2	1 22	278	15	8	0 24
Zn 3	6 0	1 27	291	21	9	0 80
Zn 4	5 9	1 34	441	22	9	0 87
Cu 1	6 1	1 22	75	150	8	0 24
Cu 2	6 3	1 10	57	160	7	0 18
Cu 3	6 0	1 42	69	254	9	0 27
Cu 4	5 7	1 36	73	349	9	0 25
Zn Cu 1	6 2	1 23	94	58	9	0 24
Zn Cu 2	5 8	1 36	189	86	9	0 52
Zn Cu 3	5 7	1 41	230	159	9	0 54
Zn Cu 4	5 7	1 40	328	223	9	0 60

[a] All treatment values are means of two replicate plots, except for the no sludge control treatment where $n = 4$

heavy metal pollution of soil Briefly, prokaryotic bioluminescence is the emission of light as a result of the oxidation of aldehyde (RCHO) and reduced flavin mononucleotides ($FMNH_2$) by molecular oxygen to the corresponding fatty acid (RCOOH), flavin mononucleotide (FMN) and water The reaction is catalysed by the enzyme luciferase and is intrinsically linked to cell metabolism (Hastings et al , 1985) The soils used in this study were from a long-term field experiment to which sludges contaminated with Zn, Cu or Zn plus Cu were added

2. Materials and methods

2 1 Experimental design and treatments

The soils were from a field experiment established in 1982 at ADAS Gleadthorpe in Nottinghamshire, UK, to examine the long-term effects of the application of sewage sludge on soil fertility and productivity The soil is a loamy sand of the Newport association (Typic Quartzipsaments), with 6% clay and 2% organic matter Pressed sludge cake, from a single source, was enriched with either Zn, or Cu, or Zn plus Cu salts and applied to the field experiment to attain a range of soil metal concentrations (Table 1) Control plots received either no sludge or non-metal-enriched sludge of the same type as the contaminated sludges In 1986, metal concentrations in certain plots were found to be lower than the desired target concentrations Hence, further additions of naturally-enriched Zn and Cu sludge cakes were made to selected plots to achieve the desired soil metal concentrations

The experimental design was a randomised block, with two replicate plots of each treatment, except for the no-sludge control treatment, which had four replicate plots All treatments received P and K at recommended rates, when necessary, based on soil analysis, whereas N, at recommended rates, was only added when non-legume crops were grown (MAFF, 1994) Soil pH was maintained from 5 7–6 5 by adding lime or elemental sulphur

2 2 Chemical analysis

Soils were collected from selected treatments (25 cm depth) in November 1996 and sieved moist to < 3 mm, thoroughly mixed and separated into 1 kg (oven dry basis) portions to give triplicate samples for each plot Each portion was placed in a pot with a Rhizon soil moisture sampler (Rhizosphere Research Products, Wageningen, The Netherlands) inserted diagonally across from top to bottom The soils were then gradually brought up to 75% water holding capacity (WHC) over 2 weeks and maintained at this moisture content for a further 2 weeks before extracting 100 ml of soil solution This procedure is described in detail by Knight et al (1998) Two aliquots of this sample were used for determining total concentrations of Zn, Cu and Ni by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Accuris) and Graphite Furnace Atomic Absorption Spectrometry (GF-AAS, Perkin Elmer) for Cd Soil solution pH and dissolved organic carbon (DOC) were determined on other aliquots For 'total' soil metal concentrations, the soils were first digested with aqua regia (HCl HNO_3 4 1 v/v) following the method of McGrath and Cunliffe (1985), before determining by ICP-AES and GF-AAS for Cd

Table 2
Mean soil pore water pH, dissolved organic carbon (DOC) and metal concentrations

Treatments[a]	pH	DOC (mg l^{-1})	Metal concentrations (mg l^{-1})					
			Zn	Free Zn^{2+}	Cd	Free Cd^{2+}	Cu	Ni
No sludge control	6 53	22 14	0 06	0 053	0 0001	0 00003	0 013	0 003
Non-metal enriched control sludge	6 29	14 57	0 08	0 029	0 0002	0 00009	0 010	0 005
Zn 1	6 03	24 75	1 38	0 657	0 0009	0 00067	0 025	0 020
Zn 2	5 72	29 06	5 47	2 627	0 0018	0 00110	0 030	0 025
Zn 3	5 66	29 35	10 91	5 087	0 0093	0 00575	0 045	0 055
Zn 4	5 42	28 60	19 71	9 199	0 0144	0 00868	0 045	0 075
Cu 1	5 95	25 70	0 40	0 208	0 0013	0 00090	0 250	0 040
Cu 2	6 22	22 90	0 26	0 108	0 0006	0 00065	0 240	0 015
Cu 3	6 04	25 81	0 43	0 184	0 0015	0 00095	0 340	0 045
Cu 4	5 54	27 99	1 02	0 399	0 0023	0 00147	0 620	0 065
Zn Cu 1	6 21	31 24	0 68	0 380	0 0012	0 00086	0 120	0 020
Zn Cu 2	5 31	28 43	5 77	2 398	0 0085	0 00459	0 175	0 070
Zn Cu 3	5 28	29 90	10 85	4 755	0 0026	0 00146	0 360	0 115
Zn Cu 4	5 43	36 63	18 69	6 277	0 0042	0 00228	0 600	0 155

[a] All treatment values are means of two replicate plots, except for the no sludge control treatment where $n = 4$

Concentrations of free Zn^{2+} and Cd^{2+} in soil solution were determined using the method of Holm et al (1995) In this method, metal concentrations are determined before and after equilibrium is reached with a calcium-saturated cation exchange resin The proportion of total metal in soil solution present as free metal can be calculated by comparison with a reference experiment However, soil solution free Cu^{2+} concentrations could not be determined using this method, because soil solution Cu concentrations were found to be very small even in the most contaminated soil Only the total Cu in soil and soil solution Cu concentrations are reported

2 3 *Rhizotox-C luminescence bioassay*

The Rhizotox-C biosensor consisted of a lyophilised consortium of *Rhizobium leguminosarum* biovar *trifolii* TA1 *lux*AB and *Escherichia coli* SM10(λ *pir*) *lux*AB The biosensor was resuscitated from freeze dried vials following the procedure of Paton et al (1997) and 50 µl of the cell suspension added to 450 µl of the test solution in a cuvette The luminescence produced was measured in a Bio-Orbit 1253 luminometer, after an exposure time of 37 min The bioassay was done in triplicate and the luminescence expressed as a percentage of the luminescence measured in soil solutions from control treatments, which received non-metal enriched sludge

2 4 *Statistical analyses*

Stepwise multiple linear regression analysis (SMLR) was carried out using Genstat 5 (1987) for Windows (3rd ed), in order to predict the response of the biosensor to the mixture of heavy metals in soil solution All metal concentrations were $\log_{10}$ transformed to normalise the data. Results of the regression analysis are expressed as the proportion of variance accounted for (i e adjusted R^2) Non-linear regression analysis was done, after $\log_{10}$ transformation of the soil solution Zn and free Zn^{2+} data, using the Gompertz model with four parameters (Genstat 5, 1987) The exponential model was used for total soil Zn, as the lower asymptote parameter required by the Gompertz model was not satisfied Curves were fitted to the bioluminescence data, with the Zn data as the independent variable (i e x values)

3. Results and discussion

3 1 *Chemical analysis*

Chemical properties of the soils from plots of the field experiment are shown in Tables 1 and 2 Control plots, which received non-metal enriched sludge, had slightly elevated metal concentrations compared to soils which had received no sludge, because the sludge contained some metals at low concentrations There were no Ni or Cd sludges, and the other sludges applied contained little Ni or Cd Therefore, their concentrations in soils were relatively low, with total soil Ni ranging from 7–9 mg kg^{-1} and Cd ranging from 0 18–0 87 mg kg^{-1} This gave very low soil solution concentrations of Ni, Cd and free Cd^{2+} in all treatments (Table 2) Soil solution pH and dissolved organic carbon (DOC) ranged from 5 28 to 6 53 and

Table 3
SMLR analysis of luminescence from the biosensor against soil pore water chemical properties

Biosensor	Stepwise multiple linear regression variance ratio[a]						
	Soil pore water						
	pH	DOC[c]	free Zn^{2+}	Cu	free Cd^{2+}	Ni	% Variance[b] accounted for
Rhizotox-C	77*	0 7	18*	0 71	2 0	0 03	79

[a] Variance ratio > 5 0 indicates a significant effect, $P < 0.001$ (*)
[c] DOC = Dissolved organic carbon in soil pore water
[b] % variance accounted for by free soil pore water Zn

from 15 to 37 mg l^{-1}, respectively The DOC concentrations were of the same order of magnitude, reflecting the fact that most of the labile sludge organic matter has decomposed since the last sludge addition

3 2 *Effect of heavy metals on bioluminescence*

Stepwise multiple linear regression (SMLR) analysis showed that soil solution pH and free Zn^{2+} were the main predictors of the bioluminescence response from Rhizotox-C (Table 3) The apparent dominant effect of soil solution pH on the decline in luminescence of the biosensor can be explained by the fact that both pH and metal concentration were auto-correlated in the SMLR analysis In tests, the luminescence response from the biosensor was stable across the pH range 4 5 to 7 0 Therefore, the decrease in bioluminescence was due to increased free Zn^{2+} concentration in soil solution, accounting for 79% of the variance and not a simple pH effect The SMLR analysis also confirmed that the variation in soil solution DOC, Cu, Ni and free Cd^{2-} had no significant effect on the luminescence from the Rhizotox-C biosensor (Table 3)

Using non-linear regression analysis, 70% of the variance in bioluminescence of the Rhizotox-C biosensor was explained by total soil Zn, 84% by soil solution Zn and 86% by soil solution free Zn^{2+} (Fig 1) Copper had little effect on luminescence, decreasing it by only 6% compared to control, even at the largest soil Cu concentration, which gave a soil solution concentration of 620 µg l^{-1} (Fig 1) Soil solutions from plots which received Zn plus Cu contaminated sludges caused a similar decline in bioluminescence to that from plots receiving only Zn-contaminated sludges, with comparable concentrations, but with small Cu concentrations (Fig 1) This suggests that Zn, and not Cu, caused the decline in bioluminescence of the biosensor

In our study, it is likely that the concentrations of free ionic Cu were very small, since at the largest soil

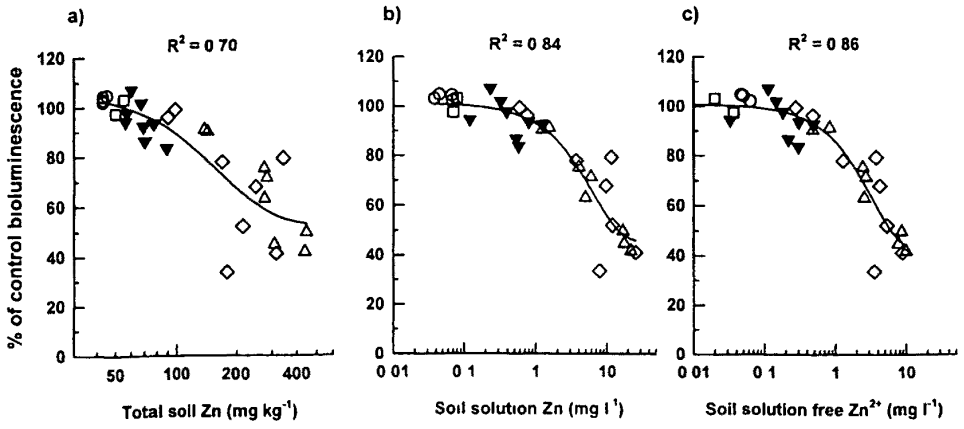

Fig 1 Effect of (a) total soil Zn (b) soil solution Zn and (c) soil solution free Zn^{2+} on % of control bioluminescence from the Rhizotox-C biosensor Treatments ○ No sludge control, □ Non-metal enriched (control) sludge △ Zn contaminated sludge, ◇ Zn plus Cu contaminated sludges, ▼ Cu contaminated sludge

Table 4
Calculated critical Zn values for reduction in bioluminescence of the Rhizotox-C biosensors

Biosensor	Soil pore water (mg l^{-1})		Total in bulk soil (mg kg^{-1})
	Zn[a]	Free[a] Zn^{2+}	Zn[b]
Rhizotox-C			
EC_{25}[c]	4 (0 7)[d]	2 (0 3)	164 (43)
EC_{50}[e]	16 (3)	6 (1)	403 (57)

[a] Calculated using a Gompertz model
[b] Calculated using an exponential model
[c] EC_{25} effective concentration causing 25% reduction
[d] Standard errors of the EC values
[e] EC_{50} effective concentration causing 50% reduction

Cu addition (349 mg kg^{-1}), <0 18% was in soil solution The free Cu^{2+} concentration in soil solution was likely to have decreased further, due to processes which remove Cu, such as chelate formation by binding to soluble organic compounds and microbial adsorption and sorption It is known that metals are generally less toxic when complexed with organic compounds than in the free ionic form (Babich and Stotzky, 1980) In Table 4, using nonlinear regression models, we have calculated total soil Zn, and soil solution Zn and free Zn^{2+} EC_{25} and EC_{50} concentrations that gave a 25 and 50% reduction in luminescence, respectively These values are in the same concentration ranges as those in a previous study using two different biosensors (*lux*-marked *Escherichia coli* and *Pseudomonas fluorescens*), but the same soils (Chaudri et al, 1999)

The relationships between total soil Zn, soil solution Zn and soil solution free Zn^{2+} and luminescence were all well correlated, giving high R^2 values (Fig 1) It would seem that in a single soil type, any could be a reasonable predictor of toxicity However, comparisons between different soil types may be more difficult, as Knight et al (1998) showed that there can be a poor relationship between total and soil solution free ionic concentrations in studies with different soil types Also the free ion is thought to be the toxic species (Sunda et al, 1978, Zevenhuizen et al, 1979), and when comparing different soil types, it may be a more reliable chemical measure Since soil solution is the medium in contact with soil microorganisms, it is important that the free ionic concentration of the metal be used for comparative purposes between different soil types Our study demonstrates the potential for using luminescence-based biosensors in a laboratory bioassay for rapidly identifying contaminated sites In future, these techniques may be extremely useful tools in ecotoxological work and in monitoring for contamination of soils

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The effect of cadmium on the activity of nitrifying populations in two different grassland soils

Ladislav Dušek
Department of Environmental Studies, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

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Abstract

The effect of a laboratory addition of 10, 100 and 500 mg Cd $kg^{-1}_{dry\ soil}$ on ammonification and nitrification was studied using soil samples of two unpolluted grassland soils. Calcareous and non-calcareous soil were selected for this purpose. Various parameters of nitrifying activity were investigated simultaneously: activity during long-term laboratory incubations in the presence and absence of a substrate, mineralization potentials, and potential activity of both ammonium and nitrite oxidizers during short-term incubations in soil slurries. Cadmium was added as aqueous $CdCl_2$.

Additions of both 100 and 500 mg Cd $kg^{-1}_{dry\ soil}$ doses significantly lowered the ability of both soils to nitrify 100 μg added NH_4^+-N $g^{-1}_{dry\ soil}$ as a substrate, which was reflected in a decreased rate of nitrate formation (maximum inhibition reached 60% in the calcareous soil and 45% in the non-calcareous soil). Furthermore, these two concentrations of Cd caused an abnormal accumulation of nitrite immediately after incorporation, particularly in the calcareous soil. The addition of 10 mg Cd $kg^{-1}_{dry\ soil}$ intensified N-mineralization in both soils, probably as a consequence of a higher concentration of readily metabolized substrate originating from killed bacteria or fungi. An excess of nitrate was then formed as a final step. The harmful effect of cadmium was more pronounced in calcareous soil, probably due to the higher sensitivity of nitrite-oxidizers in these soil samples.

Introduction

Cadmium is widely known to be a very hazardous pollutant, toxic to various ecosystems (Nriagu, 1980) and one of the most mobile metallic elements in soils (McBride, 1989). The incorporation of Cd into soil is followed by various reactions with the soil constituents, including adsorption on charged surfaces and precipitation or complexing with organic materials (Sánchez-Martín and Sánchez-Camazano, 1993). All these interactions are highly dependent on soil properties and the fate of Cd after introduction into the soil is not yet clear (Kurek et al., 1982).

Heavy metals may have toxic effects on microbial activity initially after their incorporation into soil if these elements are in soluble, and therefore available form (McBride, 1989). The toxic effect of Cd in soils is believed to be associated with forms of Cd soluble in soil solutions (McBride et al., 1981). Depending on soil parameters, a greater or lesser amount of cadmium is absorbed by the soil. However, this Cd can be, to some extent, subsequently solubilized, e.g. by local variations in soil pH or organic matter content and as a result of microbial activity, e.g. ammonification and subsequent nitrification of ammonium can locally change pH and thus solubilize some amount of Cd (Chanmugathas and Bollag, 1988).

The negative effects of heavy metals like Cd on the activity of soil microflora (decomposition of organic matter, mineralization, nitrification) have already been observed many times as reviewed in Baath (1989). Nitrification in particular deserves investigation because it plays a very important role in soil N-cycling and seems to be sensitive to various types of chemical compounds incorporated into the soil environment: both organic compounds (Karmarkar and Tabatabai, 1991; Ray, 1983, Rice and Pancholy, 1973, 1974) and heavy metals (Baath, 1989; Maliszewska

et al , 1985) In order to reliably assess the possible impact of a pollutant, ammonification and nitrification should be studied simultaneously because ammonification could influence nitrifying activity providing more or less ammonium as a substrate. Both processes appear to be affected by heavy metals in a similar way an increase of the activity at low concentrations of metallic elements and an inhibition at higher concentrations (e.g. Chang and Broadbent, 1982). Generally, nitrification is assumed to be more sensitive to heavy metal toxicity than ammonification (Baath, 1989), but some exceptions were observed: e.g. harmful effect of Cu on ammonification observed in calcareous soil (Liang and Tabatabai, 1977).

The process of nitrification in soils is not only of theoretical interest but has considerable practical importance. Any stimulation of nitrifying activity is undesirable because of possible losses of nitrogen from the soil (Laanbroek and Gerards, 1991). Furthermore, a disturbance in the equilibrium of the two phases of nitrification could lead to an accumulation of its toxic intermediate, nitrite, in the soil (Liang and Tabatabai, 1978). An accumulation of nitrite was observed predominantly in alkaline, calcareous soils, probably due to the higher sensitivity of *Nitrobacter* populations to various stress environmental factors: e.g. organic acids: (Karmarkar and Tabatabai, 1991); heavy metals: (Liang and Tabatabai, 1978); NH_3 formed from ammonium ions: (Flowers and O'Callaghan, 1983).

My experiments were designed to estimate the activity of nitrifying populations immediately after the incorporation of various amounts of Cd using short-term (8 hours) and long-term (33 days) laboratory incubations. The sensitivity and suitability of both experimental approaches were compared. A secondary objective was to compare the sensitivity of ammonification and nitrification to the effect of Cd. Non-calcareous and calcareous grassland soils with comparable pH values were selected for that purpose.

Materials and methods

Site description and soil handling

Both the studied soils were natural grasslands located in the Brno-city region in two areas sufficiently protected against possible sources of pollution, including traffic. Concentrations of heavy metals, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) were found to be lowe background level of these pollutants in the Brno r (Holoubek et al., 1994; data, of project TOCO The concentration of Cd was determined as 0.1 $kg^{-1}_{dry\ soil}$ in Kraví Hora and 0.36 mg $kg^{-1}_{dry\ soil}$ in dovka (September 1993) The soils differed in C content and organic matter content The value and soil texture were quite similar in both the (selected soil parameters are shown in Table 1).

Six 0–10 cm composite samples (each cons of four subsamples) were taken per site in early ber 1993 for determining of field concentratio inorganic nitrogen. Samples were transported t laboratory in cooled boxes at 4°C, immediately s (< 2 mm) and analysed for nitrite, nitrate and am um content (within one hour after sampling) All soil properties were analysed in April 1992 using mixed samples (each consisting of ten random subsamples). These samples were transported t laboratory in polyethylene bags, thoroughly hon nized, sieved (< 2 mm) and then air dried.

For all microbiological experiments, six co ite (0–10 cm) soil samples (each consisting o subsamples) were taken with a spade in early O 1993. The soil was air dried in the laboratory (2 then ground and sieved through a 2 mm sieve ters of soil with roots were carefully removed. prepared samples were stored in the laboratory used for experiments (within two months). Pr any experiment, the soil moisture was adjusted t of WHC and the samples were preincubated (25 days).

Experimental treatments

To study the effect of Cd on ammonification and fication in long-term incubations (33 days), tw ants of these incubations were prepared simultan ly: (1) with $(NH_4)_2SO_4$ substrate in concentrat 100 μg NH_4^+-N $g^{-1}_{dry\ soil}$ and (2) without any add of $(NH_4)_2SO_4$. Distilled water solutions of CdC $(NH_4)_2SO_4$ (if added) were added dropwise to t surface in the amount (approx. 1.5 mL per 10 g of cubated soil) that was necessary to reach the re moisture level (50% WHC) and final concentrat Cd 10, 100, 500 mg $kg^{-1}_{dry\ soil}$

For incubations in soil slurries, $CdCl_2$ was to 50 mL of incubation medium in the amount sponding to the concentrations 10, 100, 500 $kg^{-1}_{dry\ soil}$ that were subsequently added

Table 1 Physicochemical properties of the soils[a]

Soil	pH^c_{water}	pH^c_{KCl}	C^b_{org} (%)	N^b_{total} (%)	NH_4^+-N[c] (mg kg^{-1}_{dw})	NO_3^--N[c] (mg kg^{-1}_{dw})	NO_2^--N[c] ($\mu g\ kg^{-1}_{dw}$)	Ca equivalent[b] %	Soil texture[b,d] (%) Sand	Silt	Clay
Klajdovka (KL)	7 43	6 90	5 39	0 441	8 16	4 5	116 2	4 3	27 63	48 85	23 52
Kraví Hora (KH)	7 59	7 03	2 67	0 267	3 70	1 2	22 3	0 -	35 08	46 25	18 67

[a]Method N_{total} - Page et al (1982), C_{org}, $CaCO_3$ equivalents - Blakemore et al (1987), pH - glass electrode, soil texture - pipette analysis - Kilmer and Alexander (1949)
[b]April 1992
[c]October 1993.
[d]Sand. 50 - 2000 μm; silt· 2 - 50 μm, clay· < 2 μm

The control samples were treated in the same way for each type of experiment with the exception of Cd treatment. Additionally, one series of samples treated with an adequate amount of KCl in water solution was prepared for each type of experiment to control the effect of Cl^- ions.

Chemical analyses

Soil pH was measured by glass electrode in water and 0.1 *N* KCl using 1 : 2 soil : water suspension. Nitrate- and ammonium- nitrogen in extracts (1 *N* K_2SO_4; soil : solution = 1 : 5; 60 min. in end-over-end shaker) were determined using the nitrate and ammonium ion-selective activity electrode (Myers and Paul, 1968). These analyses were calibrated using the steam distillation method (Bremner, 1965). Nitrite concentration in soil extracts was determined photometrically as described in Schmidt and Belser (1982).

All results are expressed on the basis of the oven-dry (105°C/8 hours) weight of the soil (d.w.).

Mineralization potentials

Six replicate samples per treatment (each of 20 g sieved soil) were placed in 300 mL serum bottles and incubated for 29 days at 28°C (Laanbroek and Gerards, 1991). The soil moisture of preincubated samples was adjusted to 30% of WHC (slightly above field moisture at the time of sampling) and corrected to this level daily with distilled water. The bottles were covered by caps made from filter paper to allow gas exchange. The difference in the ammonium and nitrate content between the beginning and the end of the incubation period was defined as mineralization potentials corresponding to the quantity of easily degradable organic-N compounds (Laanbroek and Gerards, 1991).

Potential activity of ammonium oxidizers in buffered soil slurries (short-term incubations)

Preliminary trials were designed to determine the sodium chlorate concentration sufficient for inhibition of nitrite oxidation in accordance with Berg and Rosswall (1985). A dose of 10 m*M* sodium chlorate and 2 m*M* $(NH_4)_2SO_4$ were chosen to reach a maximum rate of potential ammonium oxidation.

Six replicate soil samples (10 g, preincubated) were placed in 50 mL of phosphate buffer (pH = 7.5) with chlorate and substrate. The bottles were then shaken in a water bath (25°C) The amount of accumulated nitrite was determined in hourly samples taken for 6–8 hours. Each of the samples was immediately centrifuged (4,000*g*, 4°C; 30 min.) and the supernatant was directly analysed for nitrite content. Potential activity was expressed as the rate of nitrite accumulation (simple linear regression).

In order to investigate the accumulation of nitrite immediately after the addition of substrate when both phases of nitrification could operate, an experimental variant without chlorate was prepared. This experiment was designed as described above using buffered soil slurry with 2 m*M* $(NH_4)_2SO_4$; but nitrite oxidizers were not blocked by chlorate. The rate of nitrite accumulation was calculated using simple linear regression.

Potential activity of nitrite oxidizers in buffered soil slurries (short-term incubations)

The potential activity of nitrite oxidizers was determined according to Belser and Mays (1982). Six replicate samples (10 g, preincubated) were placed in 50 mL of incubation media: 50 mL phosphate buffer (pH = 7.5) with 0 002% (w:v) nitrapyrin (inhibition

of chemolithotrophic ammonium oxidation) and 0.1 m*M* sodium nitrite.

The suspensions were incubated at 25° C in a rotatory shaker with water bath for 8 hours Samples of 2 mL were taken hourly, immediately centrifuged (4,000*g*, 4°C, 30 min.) and analysed for nitrite content in the supernatant. The rate of nitrite consumption was calculated using simple linear regression.

Activity of nitrifying bacteria in long-term laboratory incubations

The soil samples (20 g, preincubated) were placed in 300 mL serum bottles. Sufficient samples were prepared to give a replication of six for each sampling time during incubation (22°C; 33 days). In the first variant of the incubations, no ammonium sulphate was added and the soil moisture was adjusted to the optimal level of 50% of WHC. In addition to this variant, the activity of nitrifiers was also estimated using 100 μg NH_4^+-N $g_{dry\ soil}^{-1}$ as a substrate. A water solution of $(NH_4)_2SO_4$ was spread (with $CdCl_2$ in treated variants) dropwise as described above. The bottles were covered with caps made from filter paper to allow gas exchange. Soil moisture was controlled every other day and adjusted using distilled water.

Six subsamples were taken regularly in three, two or one-day intervals (depending on the treatment) and immediately extracted by 1 *N* solution of K_2SO_4 (1 : 5; 22°C; one hour in end-over-end shaker). After filtration, the concentration of nitrite, nitrate and ammonium nitrogen were immediately determined in the filtrate. Soil pH was determined simultaneously.

The accumulation of nitrate was studied by plotting NO_3^--N production against the time of incubation. The maximum rate of nitrification and the lag in nitrate production were determined as described in Donaldson and Henderson (1990).

Statistical analyses

The percentage of nitrification inhibition by elements was calculated from [(A - B)]/A.100, where: B = nitrate produced in the treated sample; A = nitrate production in the control sample.

The means of environmental parameters were compared by analysis of variance (after tests for normality and tests for homogeneity of variance). The Newman-Keuls multiple-range test was used for a detailed analysis of differences between the means of experimental variants (Zar, 1974). An $\alpha = 0.05$ was chosen for determining significant differences between experiment variants.

Table 2 Mineralization potential[Y]

Cd (mg kg^{-1})	Mineralization potential[Z] (mg NO_3^--N + NH_4^+-N kg^{-1}) Kraví Hora	Klajdovka
0	62 14 (4 07) a	69 18 (5 12) a
10	73 86 (5 41) b	75 12 (4 80) b
100	61 25 (7 12) a	67 14 (6 18) a
500	66 14 (5 72) a	68 21 (5 21) a

[Y]The numbers are means of six replicates In parentheses S D All concentrations are related to dry soil matter

[Z]Numbers in single column followed by the same letter are not significantly different at $p = 0\ 05$

The consumption and production of nitrite durir short-term incubations in soil slurries were evaluate by simple linear regression. When observed, the sy: moid character of nitrate production during long-ter incubations was estimated using non-linear regre: sion.

Results and discussion

A significantly higher concentration of nitrite w: found in the Klajdovka (KL) site (116.2 ng NO_2^--' $g_{dry\ soil}^{-1}$) than in the Kraví Hora (KH) site (22.3 r NO_2^--N $g_{dry\ soil}^{-1}$) at the time of sampling (Table ! Calcareous soils with higher pH have already be: reported to have higher field concentrations of NO_2^-- (Morrill and Dawson, 1967). Reporting on 116 sc samples, these authors further observed positive si: nificant correlations between the nitrifying capabili of the soil and the pH value and Ca content. Simil- ly, I observed a higher activity of nitrification in sc samples from the calcareous KL site than from the K site.

Nearly the same values of mineralization pote tial (estimated at moisture level slightly higher th. field values· 30% of WHC) that were found in contr samples from both sites (Table 2), reflected a simil quantity of easily degradable organic N in both so (Laanbroek and Gerards, 1991). No adverse effects any added concentrations of Cd on the value of mi eralization potential was found in either of the sc (Table 2). Mineralization potential as a relatively co

ı parameter generally seemed to be less sensitive to .ironmental factors than nitrification; the function ɜ small group of bacteria (Andersch and Anderson, ɔ1).

The N-mineralization of soil samples treated with ımg Cd $kg^{-1}_{dry\ soil}$ appeared to be even enhanced (by proximately 8 6% ın the KL soil and 18.8% ın the ɨ soil) in comparison with control samples (Table

Similarly, the rate of nitrate production during long-m ın- ᵇation was enhanced in the soil treated with) mg ᶜ .. $kg^{-1}_{dry\ soil}$. (Table 3). This effect seemed to be esponse of a higher concentration of readily metabo-ɛed substrate originated from killed bacteria or fungi the variant treated with 10 mg Cd $kg^{-1}_{dry\ soil}$. In both ıls (KH and KL), there was an apparent excess of .trate at the end of the incubation of soil treated with ıs dose of Cd (Table 4), which corresponded to the igher ammonification activity in such treated sam-les. The enhancement of the nitrate production rate y the low dose of Cd was less pronounced in incuba-on with ammonium-N added as a substrate (Tables 3,) ın comparison with the variant treated only with 10 ng Cd $kg^{-1}_{dry\ soil}$. The possible stimulation of ammoni-ication by the low dose of Cd in these samples could ıot influence the nitrifcation rate significantly due to he presence of an excessive amount of substrate (100 ɹg NH_4^+-N $g^{-1}_{dry\ soil}$). It should be emphasized that I ɨtudied mineralization potential as a parameter of soil ɑdjust· ' ᵗo nearly the field moisture level, while all my ɲitrifi...on tests were made using soil samples with ɑn optimal moisture content (50%, WHC). Therefore, the absolute percentage of the observed stimulation of these processes should be compared very carefully.

The stimulation of mineralization and nitrification process by low amounts of various pollutants has already been observed (Andersch and Anderson, 1991; Baath, 1989). However, these data must be interpreted very carefully. Cadmium can have an impact immedi-ately after its incorporation into soil as a biologically active element with a toxic effect on sensitive bacte-ria and fungi. Dead cells of sensitive organisms are a rich source of available nutrients including nitrogen and can be readily metabolized, and consequently an excess of ammonium-N is formed. Nitrifying popula-tions are probably resistant to such low amounts of Cd added to the soil and therefore produce a higher level of nitrate that cannot be removed by normal mechanisms (leaching, denitrification, plant uptake) in laboratory conditions. It seems likely that the stimulation of min-eralization potential in my samples treated with 10 mg Cd $kg^{-1}_{dry\ soil}$ reflected the toxic effect of the low dose of Cd on some sensitive organisms and the subsequent higher production of ammonium-N

The rate of nitrification studied during the 33-day incubation period without substrate addition (optimal moisture level · 50% of WHC) was significantly inhib-ited by 100 and 500 mg Cd $kg^{-1}_{dry\ soil}$ in both soils (Table 3). These two doses of Cd increased the initial accumulation of NO_2^--N in calcareous site KL (Table 3; Figs. 1a–c) The same changes, but quantitatively more apparent, were observed during the long-term incubation with 100 μg NH_4^+-N $g^{-1}_{dry\ soil}$ as a substrate the dose of 500 mg Cd $kg^{-1}_{dry\ soil}$ decreased the rate of nitrate production by nearly 45% in soil KH and by 60% in soil KL comparing with control samples (Table 3). The simultaneous application of NH_4^+-N and Cd caused a significant accumulation of nitrite in the beginning of nitrifying activity in both soils (Figs. 1d–f). As expected, the accumulation of NO_2^--N dur-ing the incubation was higher in the calcareous KL site· 500 mg Cd $kg^{-1}_{dry\ soil}$ increased accumulation of nitrite by approximately 60% comparing with control (Table 3). Final concentrations of accumulated N are reported in Table 4. Lower amounts of NO_3^--N in the soil sam-ples treated with 0, 100 and 500 mg Cd $kg^{-1}_{dry\ soil}$ were associated with a higher ammonium content at the end of the incubation (Table 4), which suggested lower-ing of nitrifying capability in the soils due to the toxic effect of Cd. The concentration of nitrite-nitrogen was increased until the end of the incubation in soil KL that was treated simultaneously with NH_4^+-N and 100 or 500 mg Cd $kg^{-1}_{dry\ soil}$ (Table 4; Figs. 1e–f).

The initial flush of NO_2^--N production that was observed in long-term incubations (Figs. 1a, d) can be regarded as normal and is believed to be mainly caused by a surplus of ammonium nitrogen (soil without sub-strate addition) or by NH_4^+-N added as a substrate. Although the soil samples were preincubated prior to incubation, an initial increase in ammonification activ-ity could be expected in both soils as a consequence of sieving and rewetting of air dried soil. However, the increased accumulation of nitrite in soil samples treat-ed with higher doses of Cd appeared to be associated with a prolongation of lag time in the nitrate produc-tion, especially in the calcareous KL, site (Figs. 1b, c, e, f). The accumulation of nitrite in the early part of long-term incubations caused a temporal decrease in pH value by 0 6–0 95 units, depending on the amount of Cd applied.

Table 3 Long-term laboratory incubations[Y]

Substrate ($\mu g\ NH_4^+$-N g^{-1})	Cd (mg kg^{-1})	Kraví Hora			Klajdovka		
		Nitrite	Nitrate production		Nitrite	Nitrate production	
		Max. conc. (ng N g^{-1}) during incubation	Rate[Z] (mg N kg^{-1} d^{-1})	% Inhibition (stimulation)	Max. conc. (ng N g^{-1}) during incubation	Rate[Z] (mg N kg^{-1} d^{-1})	% Inhibitic (stimulatic
0	0	88 6	2.99 (0 16) a	0	195 2	2.62 (0.16) a	0
	10	74 3	3.43 (0.14) b	+14 7	174.6	3.04 (0.15) b	+16 0
	100	92.4	2.75 (0.17) c	−12 5	263.1	2.11 (0.18) c	−19 5
	500	102.6	2.23 (0.18) d	−25.4	385.4	1.64 (0.11) d	−37 4
100	0	702.6	6.27 (0.57) e	0	4 472.6	718 (0.52) e	0
	10	678.5	6.34 (0.40) e	+1.1	3 768.5	6 84 (0.48) e	-4.7
	100	834.2	5.88 (0.37) e	-6 6	5 159.6	6.24 (0.63) f	-13 1
	500	1223.2	3.45 (0.17) b	-44 9	7 289.3	2.88 (0 19) b	-59 9

[Y]All incubations were repeated six times. In parentheses. S.D. All concentrations are related to dry soil matter
[Z]Numbers in the column followed by the same letter are not significantly different ($p = 0.05$).

Table 4. Accumulation of nitrogen at the end of laboratory incubations[a]

Substrate ($\mu g\ NH_4^+$-N g^{-1})	Cd (mg kg^{-1})	Kraví Hora			Klajdovka		
		Nitrite[b] (ng N g^{-1})	Nitrate[b] (mg N kg^{-1})	Ammonium[b] (mg N kg^{-1})	Nitrite[b] (ng N g^{-1})	Nitrate[b] (mg N kg^{-1})	Ammonium[b] (mg N kg^{-1}
0	0	0	59.6	0.8	0	68 8	1.2
	10	0	66.1	3.2	0	75 3	1.4
	100	0	55.4	6.5	0	57.7	13.8
	500	0	50.1	12.9	0	52.7	21.0
100	0	0	103.1	0.6	0	106.4	0
	10	0	101.6	1.2	0	105.3	2.5
	100	0	102.6	10.2	102.6	97.2	7.4
	500	0	75.12	21.8	213.0	85.1	19 5

[a]All incubations were repeated six times. All concentrations are related to dry soil matter.
[b]Initial concentrations (at the time 0) was the same for all Cd variants.

Nitrate formation invariably lagged behind nitrite formation (Bhargava and Datar, 1989) and many factors have been reported to regulate the lag-time of nitrate formation (Donaldson and Henderson, 1990). There is a danger of toxic effects of NH_3 formed in alkaline soil (pH > 7.5) (Broadbent et al., 1957). Particularly *Nitrobacter* populations has been reported to be sensitive to ammonium in alkaline and especially calcareous soils (Flowers and O'Callaghan, 1983; Wetselaar et al., 1972). Therefore, the negative influence of ammonium could have contributed to the toxic effect of Cd in my samples treated with $(NH_4)_2S$, particularly in the case the of calcareous KL soil.

The potential activity of ammonium oxidizers was not significantly influenced by the addition of 10 Cd $kg^{-1}_{dry\ soil}$ (measured in buffered soil slurries, in presence of chlorate, Table 5). The dose of 100 Cd $kg^{-1}_{dry\ soil}$ significantly inhibited the rate of ammonium oxidation only in soil KL and the dose of 500 Cd $kg^{-1}_{dry\ soil}$ significantly reduced the rate of ammonium oxidation by approximately 16% as compared control samples in both soils (Table 5).

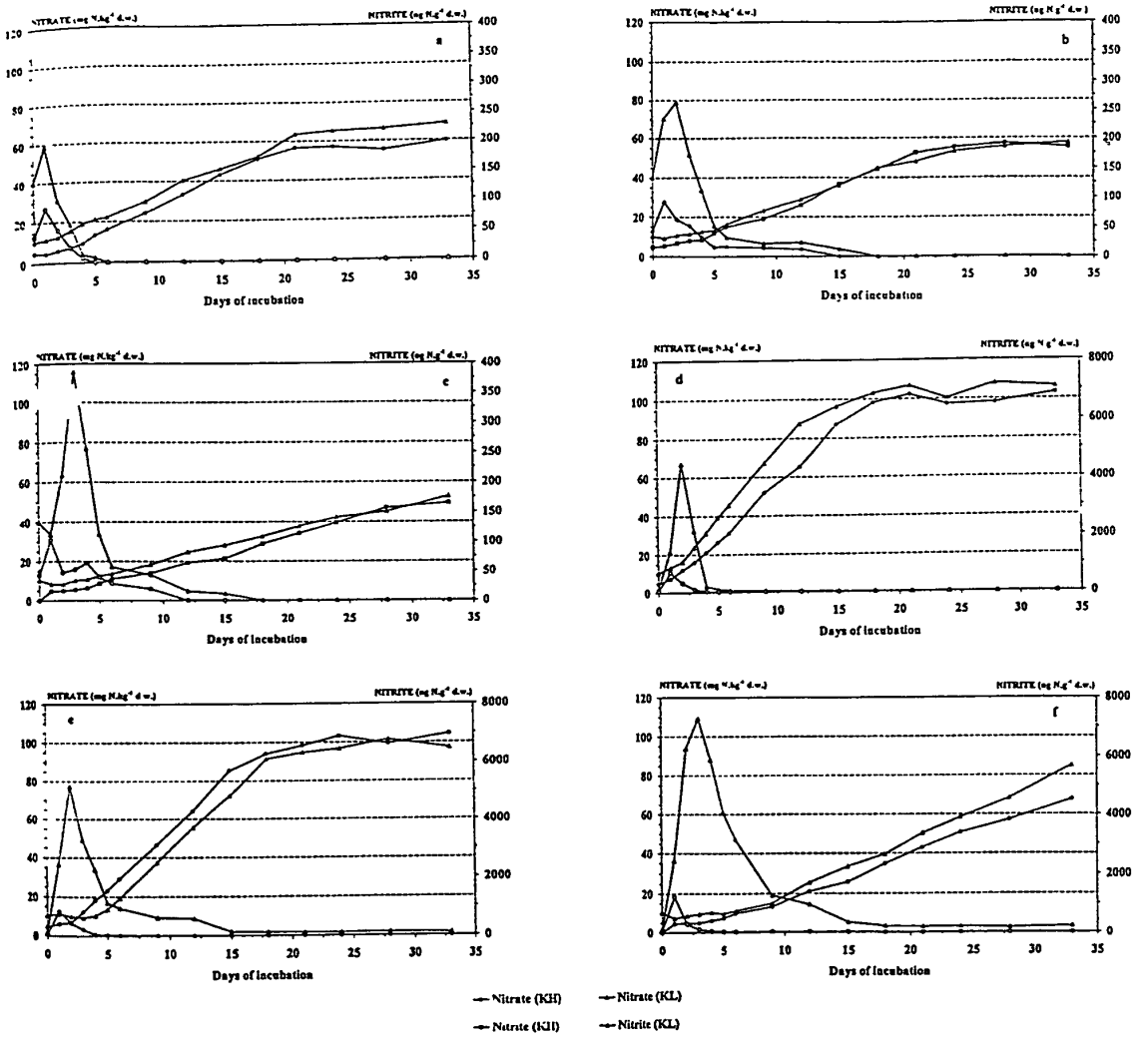

Fig 1 Activity of nitrifiers during 33-days laboratory incubation without substrate addition: (a) Control; (b) 100 mg Cd $kg_{d\ w}^{-1}$; (c) 500 mg Cd $kg_{d\ w.}^{-1}$, and with the addition of 100 μg NH_4^+-N $g_{d.w}^{-1}$: (d) Control; (e) 100 mg Cd $kg_{d.w}^{-1}$, (f)500 mg Cd $kg_{d\ w}^{-1}$.

When applied in the soil slurry without chlorate (i.e. both phases of nitrification could operate), the higher Cd concentrations significantly increased the rate of nitrite accumulation, which strongly suggested the inhibition of nitrite oxidation (Table 5). Compared to the control samples, the rate of nitrite accumulation increased by approximately 22.5% in soil KH and by approximately 34.6% in soil KL in the samples treated with 500 mg Cd $kg_{dry\ soil}^{-1}$ (Table 5, non-chlorate variants).

Assuming no adverse effect of 10 m*M* chlorate on ammonium-oxidizing populations, the comparison of the accumulation of nitrite between chlorate and non-chlorate variants in soil slurries supplemented by NH_4^+-N as a substrate (Table 5) allowed us to estimate approximately the influence of chemicals on populations of NO_2^--N - oxidizers. In the case of control samples (no Cd treatment), the samples without chlorate accumulated a significantly lower amount of nitrite than variants with chlorate: by 62.3% in soil KH and by 40.4% in soil KL (Table 5). These data suggested that nitrite oxidation lagged after ammonium oxidation in both soils However, the calcareous KL soil appeared to be less efficient in oxidizing of accumulated nitrite

Table 5 Potential activity (PA) of ammonium oxidizers - incubations in soil slurries[z]

Cd (mg kg^{-1})	Kraví Hora (KH)		Klajdovka (KL)	
	10 m*M* Chlorate Nitrite production (ng NO_2^--N g^{-1} h^{-1})	Without chlorate Nitrite production (ng NO_2^--N g^{-1} h^{-1})	10 m*M* Chlorate Nitrite production (ng NO_2^--N g^{-1} h^{-1})	Without chlorate Nitrite production (ng NO_2^--N g^{-1} h^{-1})
0	1333 5 (112 7) a	501 9 (28 5) a	1521 6 (120 8) a	907 6 (99.6) a
10	1368 6 (104 9) a	514 2 (33.7) a	1484 7 (102 1) a	961.7 (72.4) a
100	1312 8 (95 4) a	547 8 (44 5) a	1361 3 (98 2) b	1221 6 (94.1) b
500	1114.9 (73 1) b	614 6 (42 8) c	1276 2 (85 4) b	1221.6 (94.1) b

[z]All numbers are means from three replications In parentheses· S D
All concentrations are related to dry soil matter.
2 m*M* $(NH_4)_2SO_4$ was used as a substrate.
Numbers in single column followed by the same letter are not significantly different ($p = 0.05$)

than soil KH, which could have led to the accumulation of a relatively high amount of NO_2^--N in this soil, even in the several hours following the incorporation of substrate (Table 5, control variant). The lower ability of soil KL to oxidize produced nitrite could indicate that (1) nitrite oxidizers in soil KL were more affected by previous laboratory procedures: sieving, air drying, incubation in soil slurries, (2) ammonium oxidizers in soil KL, were less affected by the system of measurement and the stimulation of their activity in the presence of 2 m*M* $(NH_4)_2SO_4$ as a substrate could exceed the capability of the nitrite oxidizers in this soil, (3) although the soil slurries were buffered, the toxic influence of NH_3 on nitrite oxidizers of the calcareous KL soil could not be completely excluded.

Furthermore, a comparison of chlorate and non-chlorate samples revealed a greater toxic influence of Cd on nitrite oxidation in soil KL than in soil KH. For the dose of 500 mg Cd $kg^{-1}_{dry\ soil}$, the nitrite oxidizers in soil KL, consumed only 4.3% of produced nitrite (Table 5, comparison of chlorate and non-chlorate variant) that was produced in soil slurries with chlorate while the nitrite populations in soil KH consumed 44.9% of nitrite that was produced by ammonium oxidizers (Table 5). Comparing the consumption of nitrite within single soil, a higher sensitivity of nitrite oxidation to Cd added to soil KL was obvious (Table 5 - chlorate vs. non chlorate slurries).

The disturbance of nitrite oxidation in the early time after the incorporation of Cd was also apparent from a direct measurement in soil slurries (Table 6). Comparing with control samples the nitrite-oxidizing activity in samples treated with 500 mg Cd $kg^{-1}_{dry\ soil}$ decreased by 18 6% in soil KH and by 30.5% in soil KL. Again, the nitrite oxidizing populations in the calcareous KL soil appeared to be more sensitive to stress factor than nitrite oxidizers in soil KH, which could explain the enormous accumulation of nitrite that was observed during long-term incubations of these soil samples (Fig. 1a–f).

Table 6. Potential activity (PA) of nitrite oxidizers - incubations in soil slurries[z]

Cd (mg kg^{-1})	Kraví Hora Nitrite consumption (ng NO_2^--N g^{-1} h^{-1})	Klajdovka Nitrite consumption (ng NO_2^--N g^{-1}h^{-1})
0	521.8 (33 4) a	743.8 (52.1) a
10	492.6 (27.5) ab	770.4 (38 4) a
100	481 3 (24.9) b	666 3 (42 0) b
500	424.7 (25.1) c	517.0 (31.2) c

[z]All numbers are means from three replications In parentheses S.D.
All concentrations are related to dry soil matter.
2m*M* $(NH_4)_2SO_4$ was used as a substrate.
Numbers in single column followed by the same letter are not significantly different ($p = 0.05$)

It should be emphasized that Cd was incorporated simultaneously with 2 m*M* $(NH_4)_2SO_4$ as a substrate in all measurements in soil slurries (Table 6). The concentration of 4 m*M* NH_4^+-N used as a substrate in soil slurries was a higher dose than the dose of 100 μg NH_4^+-N $g^{-1}_{dry\ soil}$ that was applied in long-term incubations (Table 3). Consequently, the harmful effect of NH_3 evolved from ammonium ion on the activity of nitrite - oxidizers could be worse in the experiments in soil slurries, particularly in the case of calcareous soil KL.

I can summarize that the nitrite oxidizers were more sitive to the toxic effect of incorporated Cd, partic-ly in the calcareous KL, soil. Karkamarkar and atabai (1991) observed an accumulation of nitrite calcareous soil (pH = 7.6; C_{inorg} = 12 g kg^{-1}) r the incorporation of formic, acetic and fumaric ds. Similarly, Liang and Tabatabai (1978) found an umulation of nitrite in calcareous soil (pH = 7. 8; CO_3 equivalent = 5.1) treated with some heavy met-

The higher sensitivity of *Nitrobacter* populations ollutants in calcareous soils may be responsible for accumulation of toxic concentrations of nitrite and sequently for the disturbance of the whole nitrifica-n process. Accumulated nitrite can be toxic for both monium- and nitrite- oxidation process. Further-re, t e can be partially fixed by organic matter l thus removed from the nitrification sequence for a atively long time (Boon and Laudelout, 1962; Nel-n and Bremner, 1969; Woldendorp and Laanbroek, 39).

No change in the activity was found in the sam-s treated with KCl in the amount corresponding to ncentrations of Cl^- added with $CdCl_2$. The maxi-um concentration of this ion added to the soils in this dy was approximately 0.019% (dry weight basis). is amount is unlikely to affect nitrification in my periments. The same conclusion was also made in experiments of Sindhu and Cornfield (1967) and ng and Tabatabai (1978). The addition of $CdCl_2$ to buffered soil slurries did not change the pH val-. The maximum change of pH in the soil prepared long-term incubation caused by the incorporation $CdCl_2$ with NH_4^+-N was 0.17 units. This effect is lieved not to affect the nitrificaton process signifi-ntly.

The simultaneous application of various experi-ental approaches appeared to be very suitable for reliable assessment of the toxic influence of Cd in y study. The disturbance of the whole nitrification ocess as it was observed in long-term incubations soil samples treated with high amount of Cd was parently a consequence of the toxic effect on both monium-and nitrite- oxidizers. The long-term incu-tion without ammonium substrate reflected more N-ineralization than the nitrification process. The addi-n of ammonium sulphate as a substrate intensified e nitrification and provided information concerning e ability of nitrifying populations to nitrify higher nounts of ammonium. Moreover, the disturbance of e whole process could be more pronounced when a eater part of the populations is metabolically active at the time of incorporation of the pollutant. However, the possible toxic effect of NH_3 that can be formed from ammonium substrate in alkaline soils should be taken into account in the assessment of toxicity of a tested pollutant and any observed effect should be discussed with respect to pH value and other soil properties. Short-term incubations in soil slurries appeared to be very suitable for a detailed study of the behaviour of nitrifying populations immediately after the incorporation of Cd.

The estimation of mineralization potential had the advantage that ammonification and nitrification could be studied simultaneously. However, the broad spectrum of micro-organisms involved in the process of ammonification makes this process relatively insensitive to toxic chemicals (van Dijk, 1987; Vonk, 1991). Apart from this drawback, mineralization can be recommended as a test criterion for the testing of toxic chemicals, if studied simultaneously with the nitrification process. Nitrifiers are very sensitive population, so they are useful for tests on the toxic influence of various compounds (Vonk, 1991). Nitrifying bacteria are also regarded as the most sensitive micro-organisms within the biological wastewater treatment process (Nowak and Svardal, 1993).

The soils used in this study were unpolluted, natural grasslands. The ammonification and nitrification in these soils can be expected to be more sensitive to heavy metals than in polluted soils (Rother et al., 1982). The laboratory addition of higher amounts of metal as a single pulse does not correspond to the slow continuous pollution of soil in reality, which could affect the microbial community more severely (Doelman, 1986). It is therefore very difficult to make a reliable prognosis for field conditions from my laboratory results. Furthermore, there is a problem with the different types of soils, since the behaviour of heavy metals or other pollutants will change in different soil matrices. The actual effective concentration of metal is unknown (Baath, 1992) due to the great variability of results, which makes the interpretation of the results and a comparison of the different soils even more difficult.

Comparing the two soils used in this study, a different speciation of Cd after incorporation should be expected mainly because of the different Ca content, structure of humus and slightly different pH values (McBride and Blasiak, 1979; O'Connor et al., 1984; Sánchez-Martín and Sánchez-Camazano, 1993). The time for short-term incubations in soil slurries (up to eight hours) could be regarded as much short-

er than required for the adsorption equilibrium to be reached (Sánchez-Martín and Sánchez-Camazano, 1993). Experiments with soil slurries then probably tested the effect of soluble forms of Cd on sensitive populations immediately after incorporation. Similarly, the effects observed during long-term incubation were probably caused by soluble forms of Cd. In the field, cadmium can be mobilized by local changes in pH value, for example as a result of microbial activity (García Miragaya and Page, 1978) or by the local forming of some organic ligands that can form soluble complexes with Cd thereby diminishing its retention by the soil (Sánchez-Martín and Sánchez-Camazano, 1993). If a relatively high amount of Cd is mobilized in local microsites of the real soil environment, the nitrification process could be affected in the way similar to which I observed in my trials.

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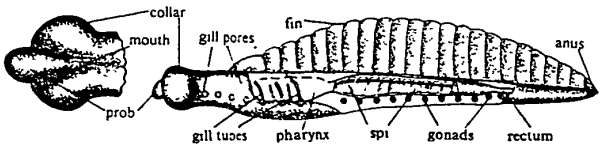

FIG. 4 Reconstruction of *Yunnanozoon lividum*, showing tripartite body plan proboscis (prob), collar and trunk Also shown gill tubes, gill pores pharynx, spiral intestine, rectum, anus, gonads, mouth and segmented fin

with the well-developed dorsal fin, it might have been an active benthic swimmer. It would have taken water with food particles into the mouth. and the food particles would have been separated from the water in the pharynx. and transported directly to the gut The filtered water passed through the supposed inner gill slits in the pharynx, which may not be preserved in fossils, and into the branchial tubes for oxygen exchange, before being removed through the gill pores.

Chen *et al* assigned *Yunnanozoon* to the chordates on the basis of five features a notochord. segmented musculature. an expanded pharynx. branchial arches, and gonads. They gave five reasons for supporting the presence of a notochord in *Yunnanozoon*. but none of them is valid. (1) They claimed that the alleged 'notochord' is positioned dorsal to the pharynx". but it actually passes through the pharynx anteriorly as they described (ref 21. p. 721), which should not be the case in a chordate. (2) They stated that the 'notochord' "is often straight, indicating stiffness'. However. most of the 'notochord' was curved. as shown in our Figs 1*a*, *b h*, *i* and 3*d* and their Figs 1*a* and 2*e*. and its anterior can even be angularly bent (Fig. 1*b*) or folded backwards (Figs 1*c* and 2*c*) (3) There seems to be no evidence for the 'notochord' to resist longitudinal compression more than other elements above and below it. (4) Without a clear shape of its anterior end being ever defined in all specimens. there is no reason for claiming that it "tapers at both ends". (5) Their 'notochord' can be interpreted as the gut. as discussed before. In addition, they described in the Fig 2 legend that, posterior to the pharynx, "the wide. dark bands crossing the notochord . their nature is unknown" However, they can be easily interpreted as the spiral contents or spiral valves in the anterior gut. as shown in our Figs 1*h*, 2*e* and 3*d*. *e*, *f*, *g* Accordingly, their alleged 'notochord' actually represents the upper pharynx and the gut following the pharynx. Furthermore, their claim that their 'notochord' is "the rod" obviously itself conflicts with their description that the "notochord is just as flattened as other soft tissues". because, according to the decay experiment[25], the notochord of cephalochordates is the most resistant of the soft parts to decay Therefore, if a notochord had been present in *Yunnanozoon*, it would have been preserved as a rod-like, dark structure with conspicuous relief (rather than a pale spiral or faeces-like structure), and the trunk could have not been twisted as it was in specimen NWU93-1407 A (Fig. 1*i*).

A comparison of the dorsal segmented unit in *Yunnanozoon* to the myomeres in cephalochordates may also be invalid. because (1) it is not chevron-shaped as in living cephalochordates. or sigmoidal, as in the Middle Cambrian *Pikaia*[26,27]; (2) it is completely dorsally positioned. and never embraces visceral organs. as those in cephalochordates do; and (3) it is blade-like in shape suggesting sclerotization or at least half-sclerotization, and may not be recognized as musculature, especially from the detached appearance (Fig 1*f*, *h*)

It should be stressed that although the pharynx, the branchial tubes ('arches') and the gonads could be present in chordates, the first two are crucial features in hemichordates, and the last can be present in various phyla including the Hemichordata[15,23] All of these facts lead to a conclusion that all of the five features, plus the important tripartite body plan, strongly support the affinities of *Yunnanozoon* with the enteropneust hemichordates rather . the chordates

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Predicting animal cadmium concentrations in lakes

Landis Hare & André Tessier

INRS-Eau, Université du Quebec, Sainte-Foy, Québec G1V 4C7 Can.

Human activities have greatly increased the flux of many potentially toxic metals to aquatic ecosystems[1]. The development and implementation of effective remedial measures depend on our ability to predict the fate and effects of metals in these systems Models based on sound physical-chemical and biological principles, such as the free-ion activity model[2-5], have shown great potential as predictive tools. This model has been effectively explaining the central role of the free-ion concentration activity) as a regulator of interactions (uptake, toxicity) between metals and aquatic organisms[2,3]. It postulates that the biological effects of metals are best predicted by the activity of the free metal ion, rather than by total metal concentration. Because this model was developed in the laboratory under unnatural experimental conditions, it must be validated in field situations before being generally used in nature[3]. We report here that Cd concentrations in an indigenous aquatic insect larva, *Chaoborus punctipennis* best described by the free-ion activity model, provided competition for biological uptake sites between hydrogen and free cadmium ions, as well as cadmium complexation natural organic matter, are explicitly taken into account. results suggest that the free-ion model would provide an effective theoretical framework for the use of animals as indicators metal contamination in nature.

Water samples and larvae of *C punctipennis* (Say). a spread nocturnal predator of lake plankton[6], were collected

23 lakes in the provinces of Ontario and Quebec, Canada (Fig. 1 and Table 1). If Cd complexation with dissolved humic substances is ignored, chemical speciation computer programs predict that >94% of the aqueous Cd in the study lakes would be present as the free Cd ion (complexation by inorganic ligands such as Cl^- and SO_4^{2-} is small in these lakes). These estimates of free Cd ion concentrations ($[Cd^{2+}]^*$) were not strongly related to Cd concentrations in *C. punctipennis* larvae ($[Cd]_{C.\,punc}$; Fig. 2a, $r^2 = 0.13$, $P > 0.1$). The most striking aspect of the weak relationship between $[Cd^{2+}]^*$ and $[Cd]_{Chaob}$ is the position of the three most acidic lakes (pH < 5; filled symbols in Fig. 2a), in which Cd concentrations in larvae are low despite relatively high $[Cd^{2+}]^*$.

Considering the tenets of the free-ion model, we considered that in the more acidic lakes hydrogen ions would effectively compete with cadmium ions at biological uptake sites. It has been shown in the laboratory that, for a given free metal ion concentration, hydrogen ions reduce both the toxicity of Cd to fish[7] and the uptake of Cu, Pb and Zn by algae[8] and bacteria[9]. A simple representation of Cd–proton competition for a biological uptake site (≡X) is

$$Cd^{2+} + \equiv X = \equiv XCd \qquad K = [\equiv XCd]/[Cd^{2+}][\equiv X] \quad (1)$$

$$\equiv XH = \equiv X + H^+ \qquad K_a = [\equiv X][H^+]/[\equiv XH] \quad (2)$$

where charges on the sites are omitted for simplicity, and K and K_a are equilibrium constants. The total concentration of uptake sites is given by:

$$[X]_T = [\equiv XH] + [\equiv X] + [\equiv XCd] \quad (3)$$

which, if combined with the expressions for the equilibrium constants in equations (1) and (2) and assuming that only a small fraction of the sites is occupied by Cd, gives

$$[\equiv XCd] = \frac{KK_a[X]_T}{[H^+] + K_a}[Cd^{2+}] \quad (4)$$

If we assume that Cd accumulated by *C. punctipennis* is proportional to $[\equiv XCd]$, that is $[Cd]_{Chaob} = k[\equiv XCd]$, combining this relation with equation (4) gives

$$[Cd]_{Chaob} = F\frac{[Cd^{2+}]}{[H^+] + K_a} \quad (5)$$

where $F (= kKK_a[X]_T)$ is a constant specific to *C. punctipennis*. As expected, prediction of Cd in larvae is substantially improved when H^+ competition with Cd^{2+} for biological uptake sites is taken into account (Fig. 2b, $r^2 = 0.66$, $P < 0.001$). The values of K_a and F, as defined in equation (5), were determined by least-squares optimization.

Although our predictive capacity is now much greater, 34% of the variability between Cd concentrations in the animals and in their environment remains unexplained. Some of this variability could be due to the influence of dissolved organic matter on Cd speciation, an interaction that has been very difficult to quantify and thus has tended to be ignored. We used the Windermere humic aqueous model (WHAM[10]) to address this problem. Concentrations of fulvic and humic acids required as input data to the WHAM 1.0 computer code were estimated from our measure-

TABLE 1 Concentrations of dissolved constituents and Cd in *Chaoborus*

Lake	Region	$[Cd]_{Chaob}$ ($\mu g\,g^{-1}$)	[Cd] (nM)	[Zn] (nM)	[Ca] (μM)	[Mg] (μM)	$[C_{org}]$ ($mg\,l^{-1}$ C)	pH
Adeline	R.-Noranda	2.88	0.29	9	197	94	5.57	7.26
Bird	Muskoka	0.24	0.19	44	107	35	5.70	6.37
Bousquet	R.-Noranda	6.27	2.37	117	106	50	14.6	6.38
Bigwind	Muskoka	0.94	0.38	–	94	40	6.50	6.04
Caron	R.-Noranda	8.89	3.14	228	245	100	13.3	7.11
Chub	Muskoka	1.94	0.32	104	47	24	7.42	5.50
Clearwater	Sudbury	3.99	4.90	346	137	57	3.40	4.79
Crooked	Sudbury	1.43	7.12	372	77	44	2.52	4.58
Crowley	Sudbury	13.3	2.18	152	69	38	1.29	5.86
Dufresnoy	R.-Noranda	1.11	0.31	10	233	107	5.50	7.24
Dufay	R.-Noranda	1.76	0.32	16	89	71	10.6	6.55
Heva	R.-Noranda	1.88	0.67	45	52	39	12.0	6.18
Hélene	R.-Noranda	0.24	0.15	8	500	239	6.01	7.18
Joannès	R.-Noranda	2.60	1.13	42	188	80	9.26	7.27
Marlon	R.-Noranda	4.80	1.43	57	49	300	9.06	7.10
Plastic	Muskoka	1.34	0.54	123	52	21	3.74	5.94
Ril	Muskoka	0.32	0.20	89	113	35	5.68	6.02
St Joseph	Quebec	0.65	0.15	46	97	19	2.94	6.63
Sunken	Muskoka	0.72	0.25	71	52	21	6.26	6.12
Tantare	Quebec	0.35	0.40	138	47	24	4.02	5.65
Tilton	Sudbury	7.77	1.93	168	97	46	6.17	5.81
Vaudray	R.-Noranda	5.72	1.19	69	89	52	6.31	6.58
Wavy	Sudbury	2.47	2.23	223	52	30	3.07	4.62

Mean concentrations of cadmium in larvae of *Chaoborus punctipennis*, $[Cd]_{C.\,punc}$, and dissolved in water from the 23 study lakes. Also indicated are total dissolved concentrations of the trace metal Zn, the major ions Ca and Mg, and organic carbon, $[C_{org}]$, as well as pH. *Chaoborus punctipennis* larvae were collected during the daytime from littoral lake sediments. Live final instar larvae were held in lake water to defecate their gut contents, then pooled into 3–5 replicate samples (most lakes) of 10–20 individuals each and treated for Cd analysis[17]. Water was collected in dialysis samplers[18], placed near the collection site for larvae, for the measurement of pH, inorganic and organic carbon, major ions (Ca, Mg, Na, K, Cl, SO_4), and trace metals (Cd, Cu, Ni, Pb, Zn), values for each lake are generally the mean of 15 measurements that is, five samples at 1-cm intervals above the sediment–water interface from each of three dialysis samplers. Cadmium in water and animal samples was measured by flameless atomic-absorption spectrophotometry.

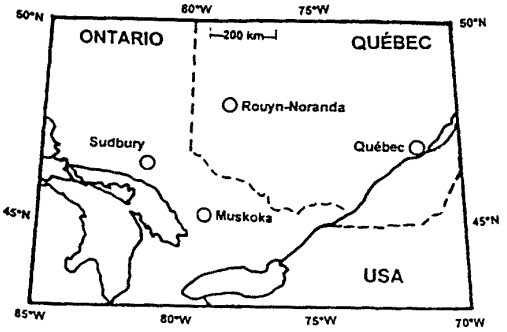

FIG. 1 Locations (○) of 23 eastern Canadian lakes in which measurements were made of cadmium in water and in larvae of the dipteran insect *Chaoborus punctipennis*. Lakes were chosen to encompass a wide range of chemical conditions (Table 1). Study lakes in the mining areas of Sudbury and Rouyn-Noranda are subject to relatively high atmospheric metal deposition from nearby smelters, whereas those in the Muskoka and Quebec City areas are far from mining activities. Insect larvae of the same developmental stage (~10 months old) were sampled from all lakes at the same time of year (springtime 1987–94) to minimize possible differences in the age and Cd-exposure history of larvae from the various lakes. The results of an intensive temporal study on [Cd] in *C. punctipennis* from one of our study lakes[19] suggest that $[Cd]_{Chaob}$ is fairly stable during the 6 months before our spring sampling time.

ments of dissolved organic carbon ([C_{org}]), by assuming that humic substances contain 50% carbon[11], the ratio of humic to fulvic acids is 1.9[12], and all dissolved organic carbon is present as humic substances (such as humic and fulvic acids). The WHAM model predicts that Cd complexation is dominated by humic substances in our study lakes and that the free Cd ion ([Cd^{2+}]) represents from 17 to 88% of the total dissolved Cd present. Support for the free-ion model comes from the fact that unexplained variability in $[Cd]_{Chaob}$ among lakes is reduced by more than half when Cd complexation by dissolved organic matter is taken into account that is, using [Cd^{2+}] ($r^2 = 0.86$, $P < 0.001$, Fig. 2*c*) rather than [Cd^{2+}]* ($r^2 = 0.66$, $P < 0.001$, Fig. 2*b*).

In the WHAM model calculations we assumed that all dissolved organic carbon was present as humic substances. However in some lakes humic substances represent from 60 to 80% of C_{org}[13–15]. If we assume a value of 60% humic substances the percentage of the explained variance of the regression shown in Fig. 2*c* is not altered and the values of the constants F and K_a change only slightly. Note that the relationship between [Cd^{2+}] and $[Cd]_{Chaob}$ is not statistically significant ($r^2 = 0.03$, $P > 0.4$, regression not shown) unless Cd–proton competition is taken into account. Mention should also be made that the data point for the lake with the highest $[Cd]_{Chaob}$ (Crowley Lake, Table 1) has a strong influence on the percentage of explained variability, that is, with this data point removed the r^2 values for the regressions in Fig. 2*b* and *c* change from 0.66 to 0.79 (Fig. 2*b*) and 0.86 to 0.80 (Fig. 2*c*). There is, however, no justification for ignoring the data point corresponding to this low dissolved organic carbon lake. The influence of calcium ions as potential competitors with H^+ and Cd^{2+} for uptake sites on *C. punctipennis*[16] was marginal (explained variability increased by only 2% with the inclusion of calcium terms in equation (5), modified equation not shown). A similar type of correction for possible competition between Cd^{2+}, H^+ and Zn^{2+} for Cd-uptake sites also provided only a small increase in predictive power (4%) at the cost of increased complexity (two additional adjustable parameters in equation (5)).

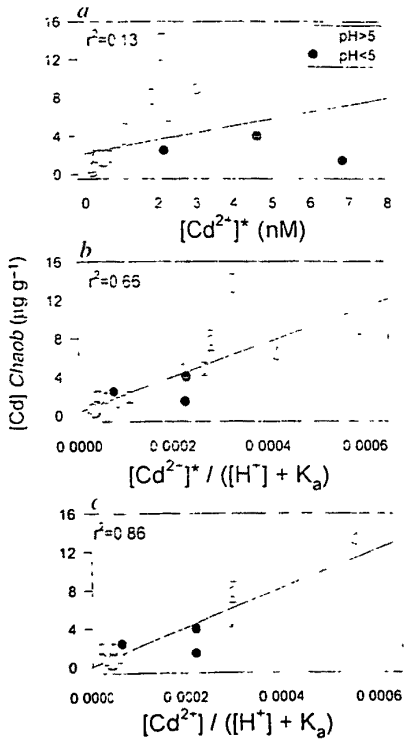

FIG. 2 Relationships between cadmium concentrations in water and in larvae of the insect *Chaoborus punctipennis* ($[Cd]_{Chaob}$) collected from 23 eastern Canadian lakes. Closed symbols, pH < 5.0, open symbols, pH > 5.0. Values for $[Cd]_{Chaob}$ are means ± s.d. (μg g^{-1} dry weight). *a*, Insect Cd versus computed free Cd ion concentrations if Cd complexation with humic substances is not taken into account ([Cd^{2+}]). *b*, Insect Cd versus [Cd^{2+}]*, normalized for hypothesized competition between hydrogen and free Cd ions for biological uptake sites (see equations (1–5)). *c*, As in *b* except that complexation by dissolved organic substances was considered in the calculation of the free Cd ion ([Cd^{2+}]). The chemical speciation model WHAM 1.0, used to account for complexation with humic substances, has been validated using measurements from a range of different environments and investigators[20], the database (equilibrium constants, diffuse layer parameters, molecular masses of fulvic and humic acids) was not changed for our calculations of [Cd^{2+}]. The values (± s.e.) of the slope ($= F$ in equation (5)) and the y intercept of the regression in *c* are 20 500 ± 1 800 μg g^{-1} and 0.04 ± 1.3 μg g^{-1}, respectively, whereas $K_a = 1.9 \times 10^{-6}$ mol l^{-1}. Our estimate of K_a is close to the value (4×10^{-6} mol l^{-1}) reported for fish gill binding sites in a laboratory study of H^+–Cd^{2+} competition[17].

Our data suggest that cadmium concentrations in an aquatic insect are modulated by both Cd complexation with organic matter as well as by competition between free cadmium and hydrogen ions for biological uptake sites. These results support the laboratory-based free-ion model as an effective tool in predicting metal concentrations in aquatic organisms in nature. A practical consequence of our study is the demonstration that larvae of *C. punctipennis* could be used as a sentinel for biologically relevant cadmium concentrations in lake water such as [Cd^{2+}]. Use of this species and other congeners as biomonitors would be helped by the fact that the genus has a global distribution and is often common in lakes covering a wide range of pH, organic matter and metal concentrations (as in our study). The deterministic nature of the free-ion model suggests that it should be effective in predicting the concentrations of trace metals in both freshwater and marine organisms.

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EFFECTS OF TRACE ELEMENTS ON NITROGEN MINERALISATION IN SOILS

C N LIANG & M. A. TABATABAI

Department of Agronomy, Iowa State University, Ames, Iowa 50011, USA

ABSTRACT

Studies to evaluate the effects on nitrogen mineralisation of 19 trace elements showed that all 19 inhibit mineral nitrogen production in soils. Results showed that the relative effectiveness of the trace elements in inhibition of nitrogen mineralisation depends on the soil. When the trace elements were compared by using 5 μmole/g of soil, Ag(I) and Hg(II) were the most effective [with Ag(I) ≥ Hg(II)] and Co(II), As(III), Se(IV), As(V), and W(VI) the least effective in inhibition of nitrogen mineralisation. Other trace elements that inhibited nitrogen mineralisation in soils were: Cu(II), Cd(II), Pb(II), Mn(II), Fe(II), Zn(II), Ni(II), Sn(II), Cr(III), Fe(III), Al(III) B(III), V(IV) and Mo(VI); their degrees of effectiveness varied in the four soils studied. Ag(I), Hg(II), Cd(II), Ni(II), Cr(III), Fe(III), Al(III), B(III), and Se(IV) inhibited nitrification, causing accumulation of ammonium-N in some of the soils used.

INTRODUCTION

Trace element content of soils may be substantially increased by the application of sewage sludge and industrial and mining wastes. Many such materials contain large quantities of various trace elements that could be retained and thus accumulated in soils (Berrow & Webber, 1972; Dean & Smith, 1973; Lindsay, 1973). Also, soil pollution by heavy metals is one of the major environmental problems associated with industries involved in processing such metals. In a review of the literature on the fate and effects of trace elements in sewage sludge applied to agricultural lands, Page (1974) concluded that surface horizons of sludge-amended soils are enriched in trace elements. The extent of this enrichment depends upon the amount of sludge applied and the trace element composition of sewage sludge. Page also concluded that applications of any sludge equal to 400 tonnes/ha, if mixed uniformly through the surface 15 cm, will add more Cd, Cu, Hg, and Zn to this zone than is normally present in natural soils.

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Research dealing with the chemistry of trace elements in soils and the toxicity of the various elements to biological systems, including accumulation in plants, is extensive (Kubota & Allaway, 1972; Lagerwerff, 1972; Chaney, 1973; Page, 1974), but little is known about the relative effects of trace elements on the biochemical processes in soils. Addition of trace elements to soils may affect microbial proliferation and enzymatic activities, possibly leading to a decrease in the rates of the biochemical processes in the soil environment. In the few studies reporting on the effects of selected trace elements on nitrogen mineralisation in, or carbon dioxide evolution from, soils (Turk, 1939; Javillier, 1941; Quraishi & Cornfield, 1971; Bhuiya & Cornfield, 1972), the trace elements were added to soils on a ppm basis, rather than on an equimolar basis, which makes the comparison of their effectiveness in inhibition of nitrogen mineralisation difficult. The effect of any trace element on biochemical reactions in soils may vary with soil pH, organic matter content, and texture. We studied the effects of 19 trace elements commonly found in sludge samples on nitrogen mineralisation in soils. In this study, the amounts of ammonium-N, nitrate-N, and nitrite-N produced were determined in four soils incubated with either a trace element solution (5 μmole of trace element/g of soil) or water.

MATERIALS AND METHODS

The soils used were surface soils (0–15 cm) selected to obtain a range in pH (5·8–7·8), organic matter content (2·58–5·45% organic C), and texture (23–45% clay, 39–54% silt, and 1–38% sand). Before use, each soil was air-dried and crushed to pass a 2-mm screen. In the analyses reported in Table 1, pH was determined with a glass electrode (soil/water ratio, 1:2·5), organic C by the method of Mebius (1960), total N by a semimicro-Kjeldahl procedure (Bremner, 1965), ammonium-N and nitrate-N by a steam distillation method (Bremner & Keeney, 1966), and particle-size distribution by the pipette analysis described by Kilmer & Alexander (1949).

The analyses for organic C and total N were performed on subsamples that had been ground to pass a 100-mesh sieve. The other analyses were performed on the coarser, <2 mm, soil samples. All soil analyses reported are on an oven-dry basis, moisture being determined from loss in weight after drying at 105°C for 24 h.

TABLE 1
PROPERTIES OF SOILS USED

Soil	*pH*	*Organic C* %	*Total N* %	*Inorganic N, ppm* NH_4-N	NO_3-N	*Clay* %	*Silt* %
Webster	5·8	2 58	0 210	3	5	23	39
Judson	6 6	2 95	0·245	3	1	45	54
Harps	7 8	3 74	0 305	2	1	30	44
Okoboji	7 4	5 45	0 463	4	2	34	50

The trace elements used were Fisher certified reagent-grade chemicals. Of these, Ag(I), Cu(II), Cd(II), Zn(II), Fe(II), Co(II), and V(IV) were added as the sulphate; Hg(II), Sn(II), Mn(II), Ni(II), Fe(III), Cr(III), and Al(III) as the chloride; Pb(II) as the acetate; and As(III), B(III), Se(IV), As(V), Mo(VI), and W(VI) as $NaAsO_2$, $Na_2B_4O_7$, H_2SeO_3, Na_2HAsO_4, H_2MoO_4, and Na_2WO_4, respectively.

The effects of trace elements on nitrogen mineralisation in soils were studied by comparing the amounts of nitrogen mineralised (under aerobic conditions) by trace elem[illegible]-amended soils and by unamended soils. In this work, a 10-g sample of soil in an [illegible]oz (250 ml) French square bottle was treated with 3 ml of a solution containing 50 μmole of trace element (or, in the control, 3 ml of water), added dropwise to moisten the whole soil (*ca.* 60% of WHC). The bottle was stoppered and incubated at 30°C. The stopper was removed and the bottle aerated every three days. After 20 days, the ammonium-, nitrate-, and nitrite-N produced were extracted with 50 ml of 2M KCl. Ammonium-N and (nitrate + nitrite)-N were determined by a steam distillation method (Bremner & Keeney, 1966), and nitrite-N by the diazotisation and coupling reaction method described by Barnes & Folkard (1951). Percentage inhibition of nitrogen mineralisation by each trace element was calculated from (A-B/A)100, where A is the amount of N mineralised in unamended soil and B is the amount of N mineralised in trace element-amended soil. All results reported are averages of duplicate determinations.

RESULTS AND DISCUSSION

Table 2 shows the amounts of NH_4-N and NO_3-N produced in unamended and trace element treated soils. The results are those obtained after 20 days of incubation minus those present initially. Nitrite was not detected in any of the soils studied. The amounts of mineral N produced in unamended soils ranged from 46 ppm in Harps soil to 71 ppm in Okoboji soil. With the exception of Judson soil, virtually all the mineral N produced in the untreated soils studied was nitrified. Judson soil evidently did not have enough nitrifying microorganisms, which resulted in accumulation of NH_4-N; 40% of the mineral N produced in this soil during 20 days of incubation remained in the NH_4-N form (this soil was stored under air-dry conditions for several weeks longer than the other soils).

The effects of the trace elements on nitrogen mineralisation varied considerably, however, Ag(I) and Hg(II) were the most effective [with Ag(I) ≥ Hg(II)] and Co(II), Cr(III), Se(IV), As(V), and W(VI) the least effective inhibitors of nitrogen mineralization in the four soils studied (Table 3). Other trace elements that inhibited nitrogen mineralisation were Cu(II), Cd(II), Pb(II), Mn(II), Fe(II), Zn(II), Ni(II), Sn(II), Cr(III), Fe(III), Al(III), B(III), V(IV), and Mo(VI); their degrees of effectiveness varied among the four soils. It seems that the chemical and physical properties of the soils used, and perhaps the nature of N in these soils, have a marked effect

TABLE 2
EFFECTS OF TRACE ELEMENTS ON THE AMOUNT OF NITROGEN MINERALISED BY FOUR SOILS DURING A 20-DAY INCUBATION

Trace element		Webster		Judson		Harps		Okoboji	
Element	Oxidation state	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N	NH_4-N	NO_3-N
		Nitrogen mineralised, μg of N/g of soil							
None		2	57	27	32	2	44	3	68
Ag	I	6	10	29	6	5	14	11	23
Hg	II	11	5	33	3	26	4	44	4
Cu		1	46	23	36	1	42	2	11
Cd		16	33	29	14	11	17	5	53
Pb		0	49	24	24	2	31	1	63
Mn		1	51	20	30	1	33	1	57
Fe		1	53	28	21	1	36	1	59
Zn		1	50	21	31	1	38	1	60
Ni		3	46	25	30	2	36	1	60
Sn		1	53	27	26	2	35	1	62
Co		1	51	25	33	1	42	2	65
Cr	III	19	28	28	22	0	40	1	53
Fe		19	34	28	21	1	35	1	56
Al		7	47	25	24	1	36	1	58
B		3	48	31	24	1	42	1	60
As		0	58	23	33	1	43	2	68
V	IV	1	53	16	35	1	40	1	60
Se		35	23	47	10	2	40	1	64
As	V	1	56	21	34	1	43	2	64
Mo	VI	1	52	5	41	1	35	2	31
W		1	53	7	50	1	41	1	64
LSD	($P < 0.05$)	2.0	1.1	0.8	3.6	0.8	1.2	1.0	1.2
	($P < 0.01$)	2.8	1.5	1.0	4.9	1.0	1.6	1.4	1.7

on inhibition of nitrogen mineralisation by trace elements. However, the average percentage inhibition by the mono- and di-valent ions of the four soils studied in decreasing order were Ag(I)> Hg(II) > Cu(II) > Cd(II) > Pb(II) ≥ Mn(II) > Fe(II) > Zn(II) = Ni(II) > Sn(II) > Co(II), and by the tri-valent ions were Cr(III) > Fe(III) > Al(III) > B(III) > As(III). Results obtained with Judson soil (Table 2) suggest that Mo(VI) and W(VI) enhanced nitrification, as is evident from the amounts of NH_4-N and NO_3-N produced in Mo- and W-treated samples compared with those obtained for the controls.

The results showed that Ag(I), Hg(II), and Cd(II) inhibited nitrification in the four soils studied, causing accumulation of NH_4-N (Table 2). It is interesting to note that while Ni(II), Cr(III), Fe(III), Al(III), B(III), and Se(IV) inhibited nitrification in the most acid (Weller) soil, only B(III) and Se(IV) inhibited nitrification in the Judson soil. No such inhibition by these elements was observed in the two calcareous soils (Harps and Okoboji).

TABLE 3
PERCENTAGE INHIBITION OF NITROGEN MINERALISATION IN SOILS BY TRACE ELEMENTS

Trace element		*Percentage inhibition of nitrogen mineralisation in soil specified*				
Element	*Oxidation state*	*Webster*	*Judson*	*Harps* % *Inhibition*	*Okoboji*	*Average*
Ag	I	73	41	59	52	56 3
Hg	II	73	39	35	32	44 8
Cu		20	0	7	82	27·3
Cd		17	27	39	18	25·3
Pb		17	19	28	10	18 5
Mn		12	15	26	18	17 8
Fe		8	17	20	15	15 0
Zn		14	12	15	14	13·8
Ni		17	7	17	14	13·8
Sn		8	10	20	11	12 3
Co		12	2	7	6	6 8
Cr	III	20	15	13	24	18 0
Fe		10	17	22	20	17·3
Al		8	17	20	17	15 5
B		14	7	7	14	10 5
As		2	5	4	1	3 0
V	IV	8	14	11	14	11 8
Se		2	3	9	8	5·5
As	V	3	7	4	7	5·3
Mo	VI	10	22	22	54	27·0
W		8	3	9	8	7·0

Quraishi & Cornfield (1971) studied the effects of 100 and 1000 ppm Cu(II) as CuO or $CuHPO_4$ on nitrogen mineralisation in a calcareous soil treated with 200 ppm N as dried blood and showed that Cu(II) caused 30% and 100% increases, respe ' 'ely, in the amount of nitrogen mineralised during 21 days at 30°C. Our result.. ndicated (Table 3) that the addition of 5 μmole Cu(II)/g of soil (300 ppm on a soil basis) as $CuSO_4$ inhibited nitrogen mineralisation in amounts ranging from 0% in the acid Judson soil to 82% in the calcareous Okoboji soil. None of the 19 trace elements studied stimulated nitrogen mineralisation in any of the four soils used.

Previous studies on the toxicity of arsenate and arsenite on nitrification in soils enriched with nitrifying organisms and perfused with NH_4Cl showed that $2{\cdot}5 \times 10^{-3}$M of arsenate is less effective than the same amount of arsenite (Quastel & Scholefield, 1951). Results obtained in the present work (Table 3) indicated that both As(III) and As(V) have very similar effects on nitrogen mineralisation, presumably because arsenite is oxidised to arsenate within the first few days of incubation.

The toxicity of Ag(I), Hg(II), Cu(II), and Cd(III) could be due to reaction of these ions with sulphydryl groups of the enzyme systems of the microorganisms

involved in nitrogen mineralisation of soils. Because no nitrite-N could be de in any of the soils after 20 days of incubation, it seems that none of the elements studied inhibited *Nitrobacter* (the organism responsible for convers. NO_2^- to NO_3^-), under the conditions of this experiment.

The small amounts of chloride and sulphate ions associated with the elements are unlikely to have stimulated or depressed nitrogen mineralisation i experiments. Sindhu & Cornfield (1967) studied the effects of chlorides an phates of Na, K, Ca, and Mg, added to soils in amounts ranging from 0·1 t (NaCl-equivalent), on nitrogen mineralisation in soils during three wee incubation at 30°C. They found that mineral N production was not affected soils were treated with up to 1 % of the different salts. The amounts of these a added to the soils in the present study ranged from 0·018 % for chloride to 0 C for sulphate.

Our results indicate that accumulation of trace elements, such as the one tested, in soils could lead to a reduction in the amount of plant-available nitr derived from soil organic-matter.

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ASSESSMENT OF THE TOXICITY OF METALS IN SOILS AMENDED WITH SEWAGE SLUDGE USING A CHEMICAL SPECIATION TECHNIQUE AND A *lux*-BASED BIOSENSOR

STEVE P. MCGRATH,*† BRUCE KNIGHT,† KEN KILLHAM,‡ SARA PRESTON,‡ and GRAEME I. PATON‡
†Department of Soil Science, IACR-Rothamsted, Harpenden, Herts AL5 2JQ, United Kingdom
‡Department of Plant and Soil Science, Cruickshank Building, University of Aberdeen AB24 3UU, United Kingdom

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Abstract—Currently, regulations regarding the maximum permitted concentrations of metals in soils are based on measurements of the total concentration However, a range of chemical and biological techniques are being developed to predict the bioavailable component of these pollutants A *lux*-based biosensor was tested in soil solutions extracted from two field experiments at Braunschweig, Germany, that had the same metal inputs, but differed in pH The bioluminescence response was found to decline as the free Zn^{2+} increased, and both soils fitted the same relationship with soil solution metal concentrations The EC25 and EC50 derived from this curve were 1 9 and 6 1 mg/L, respectively In contrast, the response to total Zn concentrations in the bulk soil showed distinct curves for each soil, further highlighting the appropriateness of free Zn^{2+} as a toxicity indicator Other metals were present in the soil, but were unlikely to be toxic, because the observed concentrations were less than their individual toxic threshold values in solution Bioluminescence-based biosensors were concluded to possibly offer an inexpensive and rapid technique to evaluate the bioavailability of metals in soil systems The response of these biosensors can be related to soil solution speciation measurements, and this gives a common basis for expression of toxic thresholds in different soils

Keywords—Pollution Zinc Copper Cadmium Nickel

INTRODUCTION

The disposal of sewage sludge is a growing problem worldwide However, the recycling of waste sludges to agricultural land is a cost-effective disposal route and can also be a valuable source of organic matter and inorganic nutrients such as phosphate and trace elements The metal concentrations in sewage sludges in the United Kingdom have been declining for several years [1], but the concentrations in most sludges are still greater than those of any uncontaminated agricultural soils to which they are applied Some metals are potentially toxic to organisms if their concentrations exceed thresholds for toxicity Many studies have shown that the buildup of potentially toxic metals in agricultural soils can lead to negative effects such as reduced microbial biomass [2], the loss or decline in numbers of specific beneficial microorganisms [3,4], and the reduction in enzyme activity and substrate utilization ability normally associated with a healthy, active soil microbial population [5–7] Because of the potential negative effects of some metals in sewage sludge, the Commission of the European Communities (CEC) issued a directive detailing limits for the application rates of sewage sludge and the total concentrations of several metals permitted in agricultural soils amended with sludges [8] Like many other guidelines, these are based on the total metal concentrations in soil measured on a dry weight basis In recent years, the scientific validity for using total metal concentrations as a basis for metal limits in soils has been questioned Much of the metal present in soil is in insoluble chemical forms, rendering it nonavailable to biological processes The biologically reactive pool is not a constant fraction of the total metal concentration in the soil Therefore, because of differences in the amounts that are potentially reactive in different soils, soils with the same total metal concentration can give very different toxicity [9]

Techniques utilized to compare the bioavailability of metals in soils have relied on arbitrary extractants such as 1 M NH_4NO_3 [10] or biological indicator species such as rye grass [11] These approaches are useful in comparing specific cases, but no one technique has found complete scientific acceptance The direct chemical analysis of a soil solution to measure total soluble metal concentrations and also the proportion of the total soluble metal present as the free ion species is the only method that offers an absolute measurement of metal solubility in the medium that bathes soil organisms and plant roots The toxic action of metals is thought to be due to the free ionic species present in the solution phase [12–18] In vitro toxicity studies have shown that it is the free Zn^{2+} and Cd^{2+} ions that exert the toxic effect on the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* [19] Bioluminescence-based biosensors have been shown to respond to the available fractions of pollutants in freshwater systems [20–23], in artificial matrices [24], and in complex environmental systems [25]

The Microtox® assay is the most widely used of these biosensors and it utilizes the marine bacterium *Vibrio fischeri* This test combines low cost with high reproducibility Although this organism has been shown to be sensitive to a wide range of organic and inorganic pollutants in freshwater, soil, and sediment samples, it performs across a restricted pH range, and requires the addition of a saline buffer [26] However, altering the pH and adding salts to the soil solutions will have significant effects on the speciation of the pollutants present The insertion of *lux* genes (responsible for bioluminescence)

* To whom correspondence may be addressed
(steve.mcgrath@bbsrc.ac.uk)

Table 1 Mean soil organic carbon pH and total metal concentrations[a]

Treatment (ha/year)	Organic carbon (%)	pH	Total metal concentration (mg/kg) Zn	Cd	Cu	Ni
Old arable site soils						
No sludge amendment (180 kg N)	0 9A	7 1A	56 1A	0 4A	11 9A	8 7A
100 m^3 unamended sludge	1 1B	6 8AB	102 2B	0 6A	20 9B	11 5B
100 m^3 amended sludge	1 1B	6 9A	162 7C	0 8B	42 9C	16 1C
300 m^3 unamended sludge	1 5C	6 6B	216 3D	1 1C	36 9C	15 6C
300 m^3 amended sludge	1 5C	6 1C	358 9E	2 7D	93 7D	32 0D
Ex-woodland site soils						
No sludge amendment (180 kg N)	1 5A	6 0A	43 0A	0 3A	9 8A	8 6A
100 m^3 unamended sludge	1 6A	5 9A	87 8B	0 6A	16 5A	8 3A
100 m^3 amended sludge	1 7B	6 0A	162 5C	1 4B	37 1B	16 1B
300 m^3 unamended sludge	2 0C	5 5B	184 7C	0 9AC	32 3B	12 3C
300 m^3 amended sludge	2 3D	5 3C	294 6D	2 8D	91 6C	29 0D

[a] Values within columns followed by the same uppercase letter are not significantly different at $p < 0.05$ according to the least significant difference test in ANOVA

into soil bacteria has enabled the development of rapid biosensors that combine the benefits attributed to Microtox with ecological relevance to soils [21,23,25]

In this study, soils from two long-term field trials at Braunschweig, Germany, that had been amended with sewage sludge were sampled and a cation exchange resin system was used to calculate the free ion concentrations of Zn^{2+} and Cd^{2+} in soil solution The total metal concentrations in the bulk soil were also determined. This assessment of chemical availability was then compared with a bioluminescence-based screening assay. By combining a chemical and biological approach to interpreting soil ecotoxicological responses, our understanding of the chemical species likely to induce toxic effects was enhanced

MATERIALS AND METHODS

Soils used

Moist soil was collected in 1996 from four replicate plots of five treatments on each of the two sewage sludge experiment sites (Table 1) These received the same treatments of either metal-amended or unamended sewage sludges annually from 1980 to 1990, no applications had been made since 1990 [27] One part of the experimental field has been used for arable production for most of this century (termed old arable here), and the other was cleared from forest in approximately 1950 (ex-woodland) Sampling sites were in a 2-m square in the center of each plot to avoid edge effects Thirty samples were taken to 20-cm depth with a screw auger, then bulked for each plot The soils were sieved to <2 mm and 1 0 kg (dry weight equivalent) was put into 12 5-cm plastic pots The soils were brought to 50% of their water holding capacity and equilibrated for 4 weeks

Soil pH was determined using a mixture of 1 2 5 soil to deionized water [28] Total metal concentrations [29] were determined by inductively coupled plasma–atomic emission spectrometry (ICP-AES, ARL Accuris, Accuris, Dublin, Ireland), except for Cd, which was measured by graphite furnace atomic absorption spectrometry (GF-AAS, Perkin Elmer ZL 410, Perkin Elmer, Norwalk, CT, USA)

Extraction and analysis of soil solution

Rhizon soil moisture samplers (SMSs) of the type designed for metals research were obtained from Rhizosphere Research Products (Wageningen, Holland) These samplers consist of a length of porous plastic capped with nylon at one end and attached to a 5-cm length of polyethylene tubing at the other end A nylon strengthening rod is present inside the porous tube and the polyethylene tube is joined to a female Luer lock

All the samplers were washed by forcing 60 ml of 5% HNO_3 through the probe, followed by 60 ml of deionized water and then dried at 30°C before use One sampler was placed into each of three replicate pots A glass rod was used to make a small primary hole in the soil diagonally from the lip of the pot to the base The sampler was first wetted using a slurry of the soil from the pot (1 5 soil to water) before being gently pushed into the soil. The soil was then made up to 50% water holding capacity (WHC) by adding deionized water to the saucer of the pot Two weeks before extraction, the soils were made up to 75% WHC Acid-washed disposable syringes were used to extract soil solution directly from the soil through the SMS

Two aliquots of soil solution were acidified with either 5% HCl or 0 3% HNO_3 and used to determine total Zn and Cd concentrations by ICP-AES and GF-AAS, respectively Other aliquots of soil solution were used for determining dissolved organic carbon (DOC) and pH and for the speciation analysis A cation-exchange resin method was used to determine the concentrations of free Zn^{2+} and Cd^{2+} ions in soil solution The method is based on the measurement of the metal concentration before and after equilibrium is reached with a Ca-saturated cation-exchange resin By comparison with a reference experiment, the proportion of the total metal in the environmental sample present as free metal ions can be calculated This method has been described in detail elsewhere [30]

Pseudomonas fluorescens lux-*based biosensor*

The contents of an ampoule of freeze-dried *P fluorescens* 10586s pUCD607 with the *lux* insertion on a plasmid [31] were resuscitated in 10 ml of Luria Bertani media (containing [g/L] tryptone, 10, NaCl, 5, yeast extract, 5, and glucose, 1) for 90 min The cell suspension was centrifuged at 7,550 *g*, washed twice, resuspended in 0 1 M KCl, and transferred to a sterile 25-ml sample bottle Triplicate 900-µl aliquots of each soil solution sample were transferred to luminometer cuvettes (Clinicon, Petworth, West Sussex, United Kingdom) and equilibrated to 23°C A 100-µl aliquot of the washed cell suspension

Table 2 Mean soil solution concentrations (mg/L) of metal dissolved organic carbon (DOC) and solution pH[a]

Treatment (ha/year)	pH	DOC	Metal concentrations[b] Zn	Free Zn^{2+}	Cd	Free Cd^{2+}	Cu	Ni
Old arable soil								
No sludge amendment (180 kg N/ha)	6 9A	21 5A	0 024A	0 019A	0 0002A	0 0001A	0 018A	<0 005A
100 m^3 unamended sludge	6 8A	23 5A	0 084B	0 031A	0 0004B	0 0002B	0 039B	0 008B
100 m^3 amended sludge	6 5B	25 3A	0 167C	0 093B	0 0012C	0 0009B	0 070C	0 023C
300 m^3 unamended sludge	5 4C	33 3B	0 859D	0 830C	0 0018C	0 0011C	0 079C	0 054D
300 m^3 amended sludge	5 2C	32 1B	4 081E	3 990D	0 0162D	0 0123D	0 200D	0 460E
Ex-woodland soil								
No sludge amendment (180 kg N/ha)	5 1A	20 6A	0 168A	0 105A	0 0019A	0 0015A	0 011A	0 006A
100 m^3 unamended sludge	5 0A	26 6A	0 987B	0 835B	0 0037B	0 0016A	0 033B	0 050B
100 m^3 amended sludge	5 0A	23 4A	2 086B	1 940B	0 0128C	0 0093B	0 055B	0 184BC
300 m^3 unamended sludge	4 6B	45 2B	7 437C	6 780C	0 0211D	0 0162B	0 108C	0 313C
300 m^3 amended sludge	4 4C	49 0B	25 114D	22 700D	0 1135E	0 0896C	0 368D	2 347D

[a] Values within columns followed by the same uppercase letter are not significantly different at $p < 0.05$ according to the least significant difference test in ANOVA

[b] All metal concentrations were normalized by log 10 transformation for statistical analysis, but arithmetic means are presented

was added sequentially to each sample as described by Paton et al [21] Bioluminescence of the samples was measured after a 45-min exposure to the test samples in a Bio-Orbit 1253 luminometer (Scientific Laboratory Supplies, Nottingham, United Kingdom) The measurements of bioluminescence for individual samples were normalized by expressing measured bioluminescence as a percentage of the bioluminescence of the control (no sludge) plots of each experiment

Statistical analyses

For each of the chemical analyses, means and significant differences between treatments were calculated using least significant difference from analysis of variance Mean bioluminescence results from the individual plots were expressed as percentages of the average light output of the replicates from the non-sludge-treated plots on each experiment Linear or Gompertz functions were fitted and EC25s, EC50s, and their standard errors were calculated using Genstat 5 Release 3 22 (Numerical Algorithms Group, Downers Grove, IL, USA) The data were normalized before analysis by log transformation, but back transformed for presentation

RESULTS AND DISCUSSION

The metal concentrations in the two soils studied are a direct result of the incorporation of sewage sludge, which contained several potentially toxic metals (Table 1) Incorporation of sludge also led to significant increases in the total metal and organic carbon concentrations in the topsoil of the experimental plots In both experiments the total concentrations of Cd, Cu, and Zn in the plots that received the largest dose of metal-amended sewage sludge were more than six times the concentrations in the control plots

Considerable increases occurred in the concentrations of metals, especially Zn, in soil solutions from the sludge-treated plots (Table 2) The control plots of the old arable soil had a total Zn concentration in soil solution of only 0 024 mg/L compared to more than 4 0 mg/L in the plots that received the largest amendments of sewage sludge The ex-woodland soil, which has a lower pH, had an even greater difference in Zn concentration in soil solution between the plots, rising from Zn at 0 168 mg/L to more than 25 mg/L This increase in total metal concentration in soil solution was accompanied by an increase in the free Zn^{2+} ion concentration measured in solution (Table 2)

The response of the bioluminescence-based biosensor *P fluorescens* has been shown to be stable across the pH range of the soil solutions used in this study [21] This was confirmed by altering the pH of aliquots of the control sample (to a range of values between 4 5 and 7 0) and measuring bioluminescence response (data not shown)

The mean bioluminescence measurement for each field plot was plotted against the total Zn concentration in the soil (Fig 1) A 50% reduction in bioluminescence was never achieved in the old arable soils In contrast, the ex-woodland soils, which have a lower soil pH, showed a 50% reduction in bioluminescence at Zn concentrations of approximately 175 mg/kg, and a >80% reduction in bioluminescence at soil concentrations of Zn greater than 200 mg/kg (Fig 1)

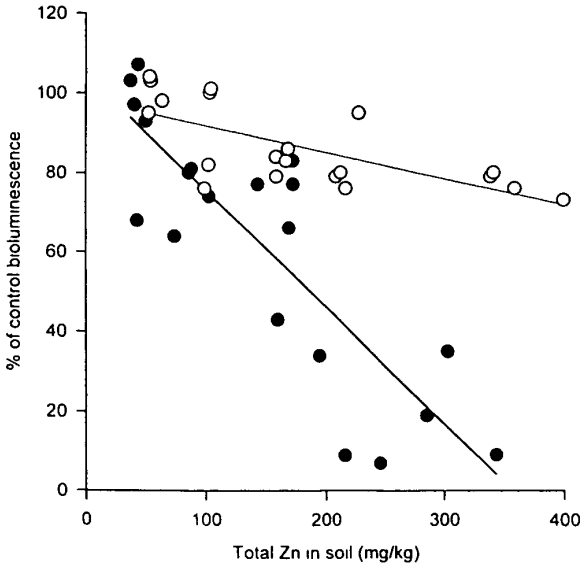

Fig 1 Mean bioluminescence of the *Pseudomonas fluorescens* 10586s pUCD607 biosensor in relation to the total concentration of Zn in soils (● ex-woodland soils ○ old arable soils)

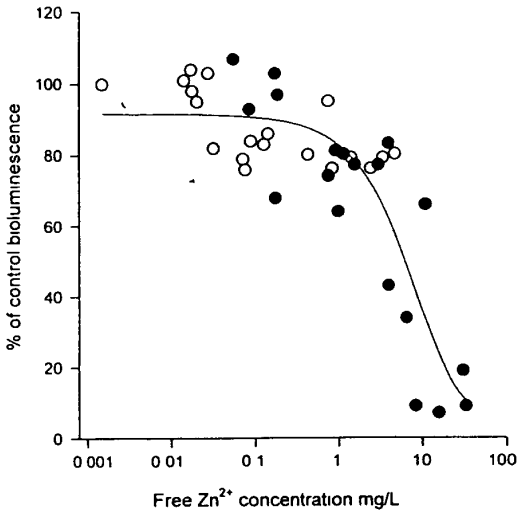

Fig 2 Mean bioluminescence of the *Pseudomonas fluorescens* 10586s pUCD607 biosensor in relation to the concentration of free ionic Zn^{2+} in soil solutions from (●) ex-woodland soils and (○) old arable soils

The effect of the increased total metal concentrations at these experimental sites on *R. leguminosarum* biovar *trifolii* was assessed by Chaudri et al. [3] In their study the authors concluded that the decline in rhizobial numbers in the most contaminated plots, and in several other long-term sewage sludge and metal amendment experiments, was due to the increase in Zn concentration in the soil The authors also found a slight increase in the toxicity of the metal amendments in the ex-woodland soils of lower pH

The relationship between bioluminescence and free Zn^{2+} measurements in the soil solution (Fig 2) explained more than 72% of the variance in the data set The analysis also showed that the curves fitted to the data from either the ex-woodland or old arable soils were not significantly different ($p \leq 0\ 001$), so a single curve is shown The calculated EC25 and EC50 values and their standard errors for Zn^{2+} were 1 9 (±0 6) and 6 1 (±1 2) mg/L, respectively Algal growth rates in aquatic systems also have been shown to depend on free Zn^{2+} and Cu^{2+} activities in solution [14] The fact that the relationship between free ion activity and toxicity has been demonstrated to date predominantly in either laboratory media or in aquatic systems [12–18] presumably relates to the difficulty in quantifying metal speciation in soil systems [30] Examples of toxicity to bacteria in relation to changes in metal speciation in soils, as shown in Figure 2, do not exist in the literature

The effect of soil pH on the availability of metals is well documented Sanders [32] and Adams et al [33] found that the total concentrations of Zn, Cu, and Ni in soil solution increased and the proportion of soluble Zn, Cu, and Ni present as free ions also increased in soils with lower pH values

Other metals were present in the soil solutions tested (Table 2) Because all the metals were derived from the same sources, they were all intercorrelated ($r > 0\ 99$), making multiple or stepwise regression analysis impossible However, a previous solution toxicity study using the same construct showed that the EC50s for Zn and Cu were 0 09 mg/L and the EC50s for Cd and Ni were 0 17 and 0 28 mg/L, respectively [21] Toxicity in pure metal solutions is known to be greater than toxicity in soil solutions, but this shows that Zn and Cu are the most toxic of these metals However, the Cu^{2+} concentrations in the soil solutions were likely to have been much lower than the total soluble Cu values shown in Table 2, because much of the Cu would have been complexed with DOC at these pHs [33] Similarly, about 20% of the Ni was also likely to be complexed in soil solution [33] and the free Cd^{2+} concentrations (Table 2) were never as great as the toxic concentration in pure solution (0 17 mg/L) Therefore, the toxic response in these experiments was likely due to Zn^{2+}

This difference in the effect of metals in the two soils raises the question of how legislation defines contamination of soil with metals The CEC guidelines [8] adopted throughout Europe use the total metal concentration in the soil as an indication of the extent of pollution in soil that is acceptable for agricultural use The results in this study show that a total measurement of metal in soil does not reflect the bioavailability of the metal The same total metal concentration in two soils can result in very different free metal ion concentrations in soil solution (Figs 1 and 2) The response of the biosensor demonstrated that it is the free ionic form in solution that induces an acute ecotoxicity response in soil microorganisms

Even so, a crucial gap exists in current understanding of metal toxicity to soil microbes The two sets of soils used in this study both show a decrease in the number of indigenous *R leguminosarum* biovar *trifolii* present with increasing metal concentrations imposed by the treatments [3] The *lux*-based biosensor measures acute toxicity in response to the free ionic species of metal pollutants in soils But a reduction in the population size of *R leguminosarum* biovar *trifolii* also occurred in the soil with the higher pH (old arable soil) However, in the latter case the acute toxicity assay gave only a small response to the increase in soil metal concentration The chronic exposure of the soil microbial population to increased metal concentrations seems to cause a decline in the number of indigenous *R leguminosarum* biovar *trifolii* at concentrations of bioavailable Zn that do not give an acute toxic response Assessment is needed of the chronic exposure of soil microbial populations to small concentrations of metals, but it should be realized that toxic effects are subtle and take place in nature over long time periods, possibly on the order of years [34] Although acute tests may give an indication of the likely toxicity of metals in different soils, we need to realize that chronic exposure may give effects that cannot be predicted from short-term assays This is presumably because of the long time involved and the many other ecological forces that act in soils under field conditions [35]

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INCUBATION STUDY OF NITROGEN MINERALISATION AND NITRIFICATION IN RELATION TO SOIL pH AND LEVEL OF COPPER(II) ADDITION

M. S. I. QURAISHI & A. H. CORNFIELD

Chemistry Department, Imperial College, London, SW7 2AZ, Great Britain

ABSTRACT

The effects of adding 100 *and* 1000 *ppm Cu, as sulphate, to a sandy loam previously adjusted to three pH levels on nitrogen mineralisation and nitrification during aerobic incubation for three weeks at* 30°C *were studied.* 100 *ppm Cu had no effect on nitrogen mineralisation at pH* 5·1 *and* 5·9, *but decreased it slightly at pH* 7·3. 1000 *ppm Cu stopped nitrogen mineralisation at pH* 5·1, *but was less toxic with increasing pH. At pH* 5·1 *and* 5·9 *nitrification was not affected by* 100 *ppm Cu, but was stopped by* 1000 *ppm Cu. At pH* 7·3 *the accumulation of mineralised nitrogen entirely as nitrate with both added Cu levels suggested that neither level affected nitrification. Results are discussed in relation to exchangeable and complexed forms of Cu present in the soils.*

INTRODUCTION

The copper content of soils may be increased by the application of mining, industrial and sewage wastes or by the use of copper-containing pesticides. Variable results have been reported in incubation studies on the effects of addition of copper compounds to soils on nitrogen mineralisation and nitrification. Gyulakhmedov (1960) found that addition of 0·5 ppm Cu(II) to calcareous chestnut soils enhanced both processes, but that higher levels were toxic. Turk (1939) found that 40 ppm Cu(II) had no effect on, or inhibited both processes in, acid or limed muck soils. Quraishi & Cornfield (1971) found that 100 and 1000 ppm copper (as CuO or $CuHPO_4$) added to a slightly calcareous sandy loam receiving dried blood stimulated both processes, and even 10,000 ppm Cu was not toxic. Variations among soils in their responses to copper additions are probably due to differences in properties such as pH, organic matter content and texture. The present paper reports

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on the effects of adding two levels of copper, as sulphate, on nitrogen mineralisation and nitrification during incubation of a soil previously adjusted to three pH levels.

MATERIALS AND METHODS

The soil used was an alluvial sandy loam (16% clay, 24% silt) from a cultivated area, which after air-drying and sieving (2 mm) had a pH (in water) of 5·1 and contained 0·16% total nitrogen and 2·0% organic carbon. The soil contained 33 ppm total copper, determined by boiling with 6*N*-hydrochloric acid followed by analysis of the extract by atomic absorption spectroscopy (Premi & Cornfield, 1968). On the basis of previous lime requirement determinations, 500 g portions of soil were mixed with 0·3% and 0·7% finely-ground calcium carbonate. These soils, as well as 500 g of original soil, were wetted to 50% saturation in glass pots and held at room temperature for two weeks, water being added, when necessary, to maintain moisture content between 40% and 50% saturation. After air-drying and rubbing through a 2 mm sieve the soils had pH values of 5.1, 5.9 and 7.3 and no free carbonate could be detected in any of them. 150 g samples of soil of each pH were mixed with finely-ground copper sulphate pentahydrate to supply 100 and 1000 ppm added copper on the dry soil basis. For incubation 10 g samples of soils from each of the three treatments (0, 100 and 1000 ppm Cu) were weighed into 10 × 2·5 cm tubes and water was added to bring the moisture content to 50% saturation. This moisture content was known to be optimum for both nitrogen mineralisation and nitrification in this soil. Sufficient replicates of each treatment at each pH were prepared to allow for determination, in duplicate, of pH and analysis for mineral nitrogen and extractable forms of Cu initially and after incubation. The barium peroxide method (Cornfield, 1961) was used for aeration and absorption of carbon dioxide during incubation of the tubes, which were closed with rubber bungs, for three weeks at 30°C. For analysis, 10 ml of water was added to each tube and the pH measured after shaking. Then 10 ml of *N*-sodium acetate was added, and after 2 min shaking the tubes were centrifuged. Suitable aliquots of the supernatant liquid were analysed for ammonium- and nitrate-nitrogen by a microdiffusion method (Bremner & Shaw, 1955), for nitrite-nitrogen by a diazotisation and coupling method (Barnes & Folkard, 1951), and for Cu by atomic absorption spectroscopy. Other replicates were shaken with 20 ml of 0·05 M EDTA (pH 4·0) and analysed for Cu after filtration.

RESULTS AND DISCUSSION

Table 1 shows the extent of accumulation of mineral-nitrogen (ammonium- and nitrate-nitrogen) after incubation, in ppm on dry soil basis, obtained by subtracting initial values from those after incubation. Nitrite could not be detected initially or after incubation in any of the samples. The table also shows the levels of Cu

extracted by the sodium acetate and EDTA reagents. Incubation and the copper sulphate treatments tended to decrease pH, but not by more than 0·3 units.

In the control soils (no Cu treatment) the increasing nitrogen mineralisation and nitrification with pH followed the normal trend.

Addition of 100 ppm Cu had no significant effect on either process in any soil except at pH 7·3, where nitrogen mineralisation was decreased slightly. 1000 ppm Cu stopped nitrification at pH 5·1 and 5·9 and apparently decreased it to a moderate

TABLE 1

EFFECT OF SOIL pH AND LEVEL OF COPPER ADDITION ON MINERAL NITROGEN ACCUMULATION* AND EXTRACTABLE COPPER AFTER THREE WEEKS OF INCUBATION
(Results on dry soil basis)

Soil pH	Cu added†	Mineral nitrogen NH_4-N	NO_3-N	$min-N$	0·5N sodium acetate extr. Cu	0·05M EDTA extr Cu
	ppm	ppm	ppm	ppm	ppm	ppm
	0	11·0	3·1	14·1	<0·1‡	32
5·1	100	9·3	1·9	11·2	0·8	131
	1000	0·0	0·0	0·0	60·0	907
	0	7·2	15·8	23·0	<0·1	26
5·9	100	7·4	19·0	26·4	<0·1	130
	1000	5·7	0·0	5·7	2·5	718
	0	3·7	41·6	45·3	<0·1	28
7·3	100	0·0	38·5	38·5 √	<0·1	130
	1000	0·0	27·6	27·6 √	3·6	749
L.S.S	P < 0·05	3·1	3·3	4·1	2·1	23

* initial levels subtracted from those after incubation
† as copper sulphate
‡ lower limit of detection.

extent at pH 7·3. However, since mineralised nitrogen accumulated entirely as nitrate at pH 7·3 with both added levels of Cu, whilst some ammonium-nitrogen accumulated in the control soil, this suggests that not only was nitrification limited by nitrogen mineralisation, but that both Cu treatments stimulated nitrification. This is confirmed by a previous study (Quraishi & Cornfield, 1971) with this soil, pretreated with sufficient calcium carbonate to make it slightly calcareous and given dried blood before incubation, to ensure that nitrification was not limited by nitrogen mineralisation, where 100 and 1000 ppm Cu stimulated nitrification.

The toxic effect of 1000 ppm Cu on nitrogen mineralisation decreased with increasing pH, but was still apparent even at pH 7·3. The absence of any accumulation of nitrite indicates that where Cu decreased nitrification its effect was no greater on the organism (*Nitrobacter*) responsible for the second stage than that (*Nitrosomonas*) responsible for the first stage of the two-stage chemoautotrophic nitrifying process.

Decreased nitrogen mineralisation and nitrification could not have been due to the sulphate residue of even the 1000 ppm Cu treatment, since it had been shown

for this soil (Sindhu & Cornfield, 1967) that addition of sulphate, as calcium sulphate, at five times the level used in this study had no effect on either process. It is also known for this soil (Ekpete, 1963) that even the maximum reduction (0·3 units) in pH due to the sulphate residue of the added Cu salt could not have significantly affected either process. Thus, any changes in nitrogen mineralisation and nitrification due to the addition of copper sulphate can be ascribed to the effect of the added Cu.

0·5 *N*-sodium acetate extracts exchangeable, including water-soluble, Cu, whilst EDTA extracts these forms as well as complexed forms of Cu. Cu levels obtained by each extractant before incubation were very similar to those obtained after incubation, indicating little change from one form to another due to incubation. Except for the soil of pH 5·1 receiving 1000 ppm Cu, only a very small proportion of the added Cu was found in exchangeable form in any of the soils. On the other hand, most of the Cu, including that originally present in the soil, was recovered by EDTA extraction. Where 1000 ppm Cu was added there was a critical pH between 5·1 and 5·9 where a considerable decrease in exchangeable Cu and a moderate decrease in complexed Cu occurred. Between pH 5·9 and 7·3 there were negligible differences in both extractable forms of Cu at any level of added Cu.

An EDTA extractant similar to that used here was shown by Pizer *et al.* (1966) to be a suitable indicator for the availability of Cu to cereals. In plateau sands Cu deficiency was apparent when EDTA-extractable soil Cu was less than 2 ppm. No information is available on the toxic effects of high Cu levels in soils of varying pH on nitrogen biodynamics in relation to extractable forms of Cu. In the present study the complete inhibition of nitrogen mineralisation by 1000 ppm Cu at pH 5·1 is probably due to the relatively high level of exchangeable Cu. At the two higher pH levels there were no consistent relationships between the extent of toxicity of 1000 ppm Cu and the Cu levels extracted by either reagent. Both processes were stopped or greatly decreased at pH 5·9, whilst nitrogen mineralisation was decreased to a lesser extent at pH 7·1 than at pH 5·9, yet each extractable form of Cu was very similar at both pH levels.

The high proportion of added Cu present in complexed form suggests that, where nitrogen mineralisation and nitrification were inhibited, this was probably due to the ability of Cu to compete with other essential mineral elements for the cation-complexing sites of the enzymes involved in both processes. The results obtained in this study suggest that the toxicity of high levels of Cu to nitrogen mineralisation and nitrification in acid soils may be eliminated or decreased by raising soil pH.

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Toxicological response of a bioluminescent microbial assay to Zn, Pb and Cu in an artificial soil solution: relationship with total metal concentrations and free ion activities

J.M. Ritchie [a,1], M. Cresser [b], J. Cotter-Howells [a,*]

[a] *Department of Plant and Soil Science, Aberdeen University, Cruickshank Building, St Machar Drive, Aberdeen AB24 3UU, UK*
[b] *Environment Department, University of York, Heslington, York YO10 5DD, UK*

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"Capsule": *A* lux*-marked bacterial bioassay shows a toxicological response to metals introduced into a model soil solution*

Abstract

The relationship between toxicological response and both total concentrations and free ion activities of Pb, Cu and Zn in an artificial soil solution has been investigated using *lux*-marked *Escherichia coli* HB101 (pUCD607) as a bioassay SO_4^{2-} (as K_2SO_4) was added as an inorganic complexing agent up to 0 01 M representing the range of ionic strengths found in soil solutions and giving a wide range of free metal ion activities EC_{50} values expressed in terms of concentration, varied significantly with K_2SO_4 molarity for all metals However, when EC_{50} values were expressed in terms of free ion activity they were not significantly different for Pb and Zn, supporting the free ion activity model Conversely, EC_{50} values expressed as free Cu activity did vary significantly with K_2SO_4 molarity, possibly due to a greater degree of adsorption of Cu onto inactive sites on the cell surfaces than for Zn and Pb Linear regression analysis of bioluminescence on free ion activity revealed significant correlations for each metal above the toxicity threshold In conclusion, *lux*-marked *E coli* is suitable for investigating the toxicity of metal ions and complexes in non saline systems although cell surface adsorption effects could be important for some metals, e g Cu

Keywords Heavy metal, Free ion activity model, Bioluminescence, Soil solution, *lux*

1. Introduction

It is known that contamination of the natural environment with toxic metals, e g Pb, Cu and Zn, can result in toxicities to plant, microbial and animal life. The extent to which a toxic metal is available for uptake, i.e its bioavailablity, is affected by the physico-chemical form of the metal in the environment from which it is taken up. For soils, organisms such as plants predominantly take up metals from the soil solution Aqueous complexation plays a vital role in determining the bioavailability of toxic metals present in solution as it determines the size and charge of metal-ligand complexes, and hence can exert a control on the movement of metals across the outer membrane of organisms (Simkiss and Taylor, 1995). Therefore, a knowledge of the chemical speciation of a toxic metal in a soil solution could be used as a predictor of bioavailability to a particular organism *via* uptake from the soil solution.

The free ion activity model (FIAM, Morel, 1983) states that there is a correlation between the toxicity of a metal to an organism and the thermodynamic activity of the free (solvated) metal ion in solution. Although there are exceptions to the model (Campbell, 1995), several studies have broadly confirmed this correlation (see Campbell, 1995, for a review) and it is considered a good indicator of bioavailability as a first approximation

* Corresponding author Present address Department of Biological Sciences, Greenpeace Research Laboratories, University of Exeter, Prince of Wales Road, Exeter EX4 4PS, UK Tel +44-1392-263-917, fax +44-1392-423-635

[1] Present address Division of Geographic Medicine and Infectious Diseases, Box 041, New England Medical Centre, 750 Washington Street, Boston, MA 02111, USA

E-mail address jcotterh@exeter ac uk (J Cotter-Howells)

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(Parker et al , 1995) However, many of these studies are time consuming, typically requiring an exposure period of a few days A number of bioluminescence-based whole cell microbial bioassays have been developed for the detection of pollutants (e g Paton et al , 1995, 1997, Tothill and Turner, 1996), of which the most well known is Microtox™ (Bulich et al , 1981) The advantage of these bioassays over uptake or mortality-based toxicity studies is that toxicity assessment is direct, rapid (15–30 min) and simple. This permits a large number of observations to be made within a reasonable timescale As a consequence, large data sets may be generated, facilitating meaningful statistical analysis While Microtox™ is a robust and rapid test for contaminated water, it is based upon the response of a marine bacterium (*Photobacterium phosphoreum*, now known as *Vibrio fischeri*) requiring pH and salinity of the sample to be adjusted to that required by *V fischeri*. For pollutants in terrestrial and freshwater systems, the potential ramifications of these adjustments in pH and salinity are changes in metal ion speciation and hence toxicity. This has led to the ecological relevance of Microtox™ to pollutants in terrestrial and freshwater systems to be questioned

A new generation of *lux*-marked bioassays have been constructed, in which the *lux* genes from *V fischeri* have been cloned into ubiquitous soil and water bacteria (Rattray et al , 1990) The *lux* genes are used to report upon the metabolic activity of the organism Therefore, measurement of light (bioluminescence) can be used to detect changes in cellular metabolic activity The response of the *lux*-marked organisms to pollutants has been compared to Microtox™ and other microbial activity measurements, e g respiration (Brown et al , 1996, Paton et al , 1997), with greater sensitivity to metals being reported with the new *lux* bioassays

1.1. Aqueous complexation

For a given solution, the total concentration of a dissolved metal, $[Zn_T]$, is the sum of the concentrations of solvated free ($[Zn^{2+}]$) and complexed (e.g. $[ZnSO_4^0]$) forms of Zn

$$[Zn_T] = [Zn^{2+}] + [ZnOH^+] + [Zn(OH)_2^0] + [ZnSO_4^0] + [ZnCl^+] + [ZnHCO_3^-], \text{ etc.}$$

where square brackets denote concentration The distribution of the metal between free and complexed aqueous forms depends on several factors, including Eh, pH and ligand concentration An increase in the concentration of a complexing ligand with a high affinity for metals, e g. SO_4^{2-}, will reduce the concentration of the free metal ion through greater complexation

The thermodynamic *activities* of chemical species, denoted by parentheses (rather than square brackets), can be calculated from species concentration using activity coefficients, e.g $(Zn^{2+}) = \gamma_{Zn^{2+}}[Zn^{2+}]$, where $\gamma_{Zn^{2+}}$ is the activity coefficient In fresh waters, activity coefficients can be calculated using the Davies equation (Lindsay, 1979) for an ion or complex of a given charge at a given ionic strength (I) Here, the activity of an ion or complex is more relevant than concentration as it provides a measure of its *effective* chemical concentration The thermodynamic activity of a free metal ion is considered the best indicator of its availability for uptake by organisms (Campbell, 1995)

Ion-selective electrodes can measure the activity of the free metal ion directly However, their detection limits are often too high and their selectivity is often too poor for the concentrations used in a typical toxicity test (Moto and Dos Santos, 1995) The activities of free ions and complexes can be calculated from a knowledge of the total concentrations of all metals and ligands in solution. This calculation involves many simultaneous equations being solved and is usually performed using a geochemical equilibrium speciation computer program such as that used in this study, GEOCHEM-PC v.2 0 (Parker et al , 1995)

This study examines the relationships between the toxicological response of *lux*-marked *Escherichia coli* and both total concentrations and free ion activityies (as calculated by GEOCHEM-PC) of the toxic metals Pb, Cu and Zn in an artificial soil solution over a range of SO_4^{2-} concentrations Sulphate was added as K_2SO_4 to simulate the range of ionic strengths commonly found in soil solutions

2. Materials and methods

2 1. Test organism

The test organism used was *E. coli* HB101 (pUCD607) which had been genetically modified to contain the plasmid pUCD607 (Rattray et al., 1990) which encodes the *lux* CDABE genes from *V. fischeri* under the control of the tetracycline resistance promoter (Shaw and Kado, 1986). *E. coli* was selected because it is metabolically active over a wide range of environmental conditions. The *lux*-marked bioassay was routinely grown in L-broth [1% (w/v) tryptone, 0 5% (w/v) yeast extract, 0 5% (w/v) NaCl, 0 1% (w/v) glucose] containing kanamycin (50 mg l^{-1}) to maintain the plasmid For the purpose of this study, the bioassay was prepared and stored as freeze-dried cells. Batch-grown cultures were harvested in late exponential phase, and lyophilised using standard procedures (Rudge, 1990) When required, freeze-dried cells were resuscitated for 2 h in 10 ml L-broth at 25°C The cell suspension was then

centrifuged (4°C, 3 min, 10 860*g*) to form a cell pellet, the supernatant discarded and the cells washed by resuspension in 10 ml 0 1 M KNO_3 The washing procedure was repeated, and the cells finally resuspended in 10 ml of 0 1 M KNO_3 giving a cell concentration of approximately 10^8 cfu ml^{-1}.

2 2 *Solution composition*

Known concentrations of Zn, Pb and Cu were added to an artificial soil solution prepared at four different strengths of K_2SO_4 up to 0 01 M The composition of the artificial soil solution (Table 1) used in the experiments was simplified from the recipe given by Quist (1995) by omitting trace nutrients which are not required for acute assays Analytical grade reagents and double deionised water (DDIW) were used throughout the experiment For each metal at each ionic strength, the test solution was made up fresh from stock solutions (×100 fold) stored at 4°C, with the exception of NH_4NO_3 which was made up as a fresh stock solution (×100 fold) daily.

Standard metal stock solutions (approximately 5 mM for Pb, 15 mM for Zn and Cu) were prepared from nitrate salts Test solutions of the artificial soil solution containing known metal concentration (range 15–120 μM for Zn, 1–10 μM for Pb, and 3–25 μM for Cu) were prepared fresh each day K_2SO_4 (prepared as a 0 5 M stock solution) was added to give 0, 0.003, 0 006 and 0 01 M K_2SO_4. Bioassays using *lux*-marked *E. coli* were performed over a range of metal concentrations at each ionic strength The pH of each test solution was recorded prior to conducting the bioassay using a standard pH meter.

2 3 Lux-*based microbial bioassay*

The assay procedure involves the comparison of bacterial light output in the presence of increasing concentrations of individual toxic metals to the light output of the corresponding control solution (artificial soil solution) at the same K_2SO_4 concentration Thus, bioluminescence is expressed as a percentage of a matrix-matched control Triplicate 900-μl test solution samples were pipetted into luminometer cuvettes and 100 μl of cell suspension added, with mixing, to each cuvette (Paton et al , 1995) Bioluminescence was recorded at 22°C after 15 min contact time using a portable luminometer (Jade Luminometer, Labtech International) The response of the organism in the absence of any sample solution was tested over the pH range 4–7 using DDIW adjusted with dilute NaOH or HNO_3 In addition, the response of the organism to the addition of K_2SO_4 (up to 0 1 M) was determined in the absence of any metals. Four replicate vials of test organism (each with triplicate bioluminescence determinations) were used for each treatment.

Table 1
Composition of artificial soil solution after Quist (1995)

Compound	mg per litre	Concentrations in resulting solution (μM)	
		Cations	Anions
$CaCl_2\,2H_2O$	36 76	250 Ca^{2+}	500 Cl^-
KNO_3	15 15	150 K^+	150 NO_3^-[a]
Na_2SO_4	8 522	120 Na^+	60 SO_4^{2-}[b]
NH_4NO_3	26 41	330 NH_4^+	330 NO_3^-[a]
$MgSO_4\,7H_2O$	24 65	100 Mg^{2+}	100 SO_4^{2-}[b]

[a] Total $NO_3 = 480$ μM
[b] Total $SO_4 = 160$ μM

2 4 *Geochemical modelling*

For each metal concentration at each molarity of K_2SO_4, the concentrations of the free ion and complexes were calculated at the pH of the test solutions using GEOCHEM-PC v 2 0 (Parker et al., 1995) after consulting current NIST (1998) values for the stability constants of aqueous Pb, Zn and Cu sulphate, nitrate and chloride complexes All stability constants were identical except those for $PbSO_4^0$ and $PbCl_2^0$ which were updated to NIST values, i e $PbSO_4$, log $K = 2\,69$ and $PbCl_2^0$, log $K = 2\,2$ (GEOCHEM PC values = 2 60 and 1 8, respectively) GEOCHEM-PC includes a stability constant for $Pb(SO_4)_2^{2-}$ but not for the disulphate complexes of Zn or Cu. No values for the disulphate complexes are listed in the NIST (1998) compilation so the GEOCHEM-PC value for $Pb(SO_4)_2^{2-}$ was left unchanged. Activities of free Zn, Pb and Cu were calculated by GEOCHEM-PC using the Davies equation (Lindsay, 1979) The input data were corrected for the addition of KNO_3 in the cell suspension, although the increase in NO_3^- concentration from addition of small amounts of NO_3^- from the metal salts was found to be negligible Redox equilibrium between N species was not considered because of the short timescale of the experiments. Correction of stability constants for the small difference in temperature between the measurement of bioluminescence (22°C) and standard temperature (25°C) were negligible In all computations, precipitation of solids was allowed but did not occur for any metals at any of the molarities of K_2SO_4.

2 5. *Statistical analysis*

The standard method for expressing toxicity utilises EC_{50} values which correspond to the concentration or free ion activity that gives a 50% reduction in light output compared to a matrix-matched control sample For calculation of the EC_{50} values, bioluminescence (expressed as percentage of a control in the absence of toxic metals at the relevant molarity of K_2SO_4) was

plotted against log metal concentration or log free ion activity The concentration or activity corresponding to 50% bioluminescence (i e the EC_{50} value) was determined using the linear regression equation for each of the four replicate vials These values were used to obtain mean and standard deviation values for each metal at each ionic strength One-way analysis of variance (ANOVA) was used to determine if bioluminescence response varied with pH or K_2SO_4 in the absence of metals and whether addition of K_2SO_4 affected mean EC_{50} values for each metal Replicate vials were also used to obtain a mean and standard deviation for each toxicity measurement Linear regression analysis of bioluminescence on free ion activity was performed for each metal by pooling the data from all molarities of K_2SO_4 above the toxicity threshold

3. Results

Ionic strength increased with increasing molarity of K_2SO_4 added and minor changes in I were caused by increases in metal concentration (Tables 2–4) All three metals complexed strongly with SO_4^{2-} which gave rise to a large variation in free metal ion activity, from >90% to 50% of the total metal concentration (Tables 2–4) Other significant complexes (accounting for greater than 0 5% of the total concentration) were chloride (for Pb only) and nitrate, but these did not exceed 10% of the total concentration The adjustment of ionic strength and addition of metals caused only slight variations in pH, from 5 3 to 5 6 for Zn, 5 4 to 5 7 for Pb and 5 25 to 5 6 for Cu The formation of hydroxide complexes was not significant for any of the metals at these pH values

The bioluminescence response of the bacteria did not vary significantly ($P>0$ 05) over the pH range (5 25–5.6) of the experiment and therefore bioluminescence values were not corrected for changes in pH Similarly, the addition of K_2SO_4 by itself in the absence of any metals did not significantly ($P>0.05$) affect bioluminescence (Fig 1) For each metal, as the concentration of K_2SO_4 increased, EC_{50} values for *E coli* expressed as total concentration also increased. For all metals, the

Table 2
EC_{50} values for Zn at four different ionic strengths expressed in terms of both total metal concentration and free ion activity (standard deviation in parentheses)[a]

K_2SO_4 added (M)	I range (mmol l^{-1})[b]	EC_{50} expressed as total concentration (μM)	EC_{50} expressed as free ion activity ($\times 10^6$)	% metal as the free ion[b]	% of metal as complexes[b,c]	
					$ZnSO_4^0$	$ZnNO_3^+$
0	13 18–13 38	51 2 d (5 3)	29 3 (2 9)	96 8	1 2	1 6
0 003	21 57–21 71	61 8 d,e (4 9)	27 1 (3 2)	83 2	15 2	1 2
0 006	29 84–29 96	74 8 e (12)	28 1 (4 7)	75 9	22 7	1 1
0 01	40 73–40 83	95 0 f (10)	30 4 (1 1)	69 9	28 9	0 9
LSD ($P=0$ 05)		13 37	ns			

[a] The distribution of Zn between the free ion and complexed forms is expressed as per cent of total aqueous concentration. Values followed by the same letter in the same column are not significantly different ($P>0.05$) according to Fisher's LSD test ns, not significantly different
[b] Computed by GEOCHEM-PC
[c] Only complexes accounting for >0 5% of the total aqueous concentration are included

Table 3
EC_{50} values for Pb at four different ionic strengths expressed in terms of both total metal concentration and free ion activity (standard deviation in parentheses)[a]

K_2SO_4 added (M)	I range (mmol l^{-1})[b]	EC_{50} expressed as total concentration (μM)	EC_{50} expressed as free ion activity ($\times 10^6$)	% metal as the free ion[b]	% of metal as complexes[b c]				
					$PbSO_4^0$	$Pb(SO_4)_2^{2-}$	$PbNO_3^+$	$Pb(NO_3)_2^0$	$PbCl^+$
0	13 13–13 15	2 90 d (0 16)	1 42 (0 18)	86 8	2 5	–	7 9	1 3	1 1
0 003	20 18–20 19	5 07 e (0 39)	1 85 (0 16)	62 9	29 5	0 6	4 9	1 2	0 7
0 006	28 47–28 48	5 84 e (0 35)	1 71 (0 81)	52 7	40 5	1 4	3 4	1 2	0 6
0 01	39 32–39 32	6 23 e (0 11)	1 69 (0 39)	45 1	47 8	2 7	2 5	1 2	0 5
LSD ($P=0$ 05)		0 201	ns						

[a] The distribution of Pb between the free ion and complexed forms is expressed as per cent of total aqueous concentration Values followed by the same letter in the same column are not significantly different ($P>0$ 05) according to Fisher's LSD test ns, not significantly different
[b] Computed by GEOCHEM-PC
[c] Only complexes accounting for >0 5% of the total aqueous concentration are included

Table 4
EC_{50} values for Cu at four different ionic strengths expressed in terms of both total metal concentration and free ion activity (standard deviation in parentheses)[a]

K_2SO_4 added (M)	I range (mmol l^{-1})[a]	EC_{50} expressed as total concentration (μM)	EC_{50} expressed as free ion activity ($\times 10^6$)	% metal as the free ion[b]	% of metal as complexes[b,c]	
					$CuSO_4^0$	$CuNO_3^+$
0	11 79–11 83	6 61 d (1 3)	5 16 d (0 51)	96 1	1 5	2 1
0 003	20 19–20 21	7 24 d,e (2 0)	3 48 e (0 73)	79 3	18 7	1 5
0 006	28 48–28 49	9 28 e (2 0)	3 07 e (0 59)	70 9	27 3	1 2
0 01	39 32–39 34	21 1 f (1 1)	7 19 f (0 42)	64 4	34 2	1 1
LSD ($P=0$ 05)		2 53	0 904			

[a] The distribution of Cu between the free ion and complexed forms is expressed as per cent of total aqueous concentration Values followed by the same letter in the same column are not significantly different ($P>0$ 05) according to Fisher's LSD test ns, not significantly different
[b] Computed by GEOCHEM-PC
[c] Only complexes accounting for >0 5% of the total aqueous concentration are included

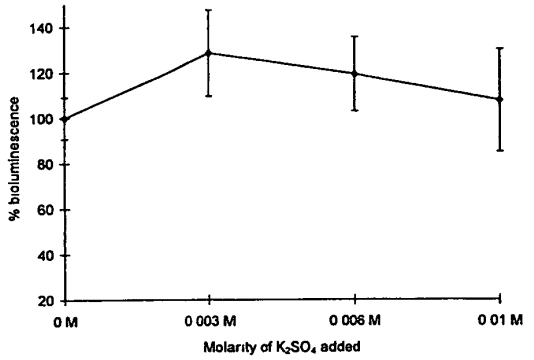

Fig 1 Variation in bioluminescent response of *lux*-marked *Escherichia coli* with addition of K_2SO_4 in the absence of metals Error bars are ±1S D

EC_{50} values were significantly different ($P<0$ 05) between the lowest and highest K_2SO_4 molarities. EC_{50} values increased from 51.2 to 95 0 μM for Zn; from 2 90 to 6 23 μM for Pb and from 6 61 to 21.1 μM for Cu (Tables 2–4) However, when toxicity was expressed as free ion activity, EC_{50} values did not vary significantly ($P>0.05$) for Zn and Pb, giving average EC_{50} values of 28.7 μM (S.D = 1 4) and 1.67 μM (S.D = 0.18), respectively (Tables 2 and 3). In contrast, for Cu, EC_{50} values expressed as free ion activities initially decreased but then increased significantly with K_2SO_4 (Table 4).

The variation in bioluminescence response with K_2SO_4 molarity for total concentrations is clearly shown in Figs 2a, 3a and 4a Metal concentrations and activities are expressed as logarithms as bioluminescence follows a typical dose-response relationship, i e logarithmic Generally, the graphs show the expected trend of toxicity decreasing with increasing K_2SO_4 However, for Cu at concentrations greater than approximately $10^{-5\,2}$ M, only the highest ionic strength (0 01 M K_2SO_4 added) appeared to cause a reduction in toxicity It is clear from the plots of bioluminescence against free ion activity (Figs 2b, 3b and 4b) that, in general, the impact of increasing K_2SO_4 is reduced to such an extent that the data can be considered to fit a single line for each toxic metal.

For Zn (Fig 2b), the shape of the graph of bioluminescence upon free Zn activity indicates a plateau region (up to $10^{-5\,1}$) below the toxicity response threshold Above the toxicity threshold, as (Zn^{2+}) increases, biological response (as measured by a change in bioluminescence) is logarithmically proportional to free ion activity ($r^2=0.96$, $P<10^{-16}$), and follows the FIAM as described by Campbell (1995). This clearly demonstrates that toxicity to the test organism expressed as free ion activity is independent of K_2SO_4 added.

For Pb (Fig 3b) the plateau region beneath the toxicity threshold is not so clearly defined due to stimulation in bioluminescence at low (Pb^{2+}) for some replicates, giving rise to large standard deviations Variations in the carbon content of the resuscitated test organism may have affected toxicity response, despite careful washing. The C content of the supernatant during the washing process changes from >100 to 4–8 mM during the washing process After resuspension in KNO_3 and dilution, C concentration in the test solution will be <40 μM This C is predominantly composed of the sugar, glucose, which does not dissociate into acid and base components under the test conditions and therefore has negligible complexation reactions with cations However, glucose can act as a stimulant to the test organism, causing variability in bioluminescence response, giving rise to these large standard deviations (Ritchie, unpublished data). The threshold toxicity is approximately $10^{-6\,1}$ (Pb^{2+}) and is followed by a linear decline in bioluminescence ($r=0.83$, $P<10^{-10}$). This is a lower correlation coefficient than for Zn but still demonstrates that toxicity to a test organism, expressed as free ion activity, is independent of K_2SO_4 added

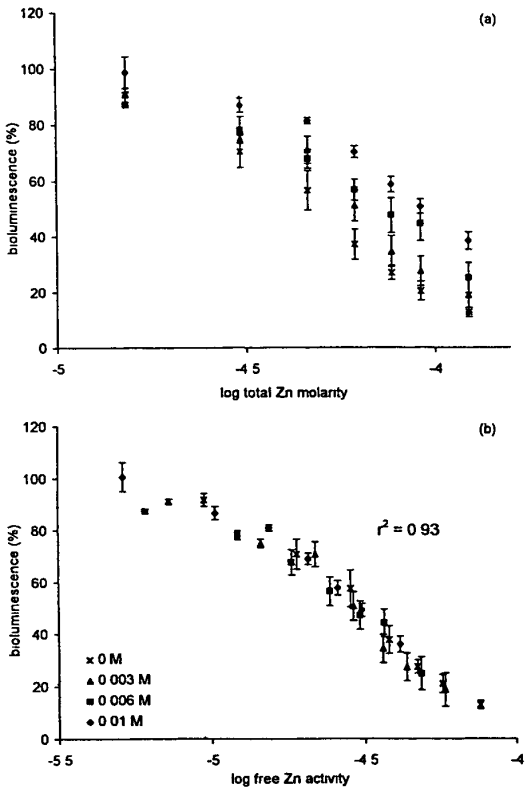

Fig 2 Bioluminescent response (% of matrix-matched control) for the artificial soil solution at four different molarities of K_2SO_4 against (a) log total Zn molarity and (b) log free Zn^{2+} activity Error bars are ±1S D The dashed line represents the threshold toxicity for log (Zn^{2+})

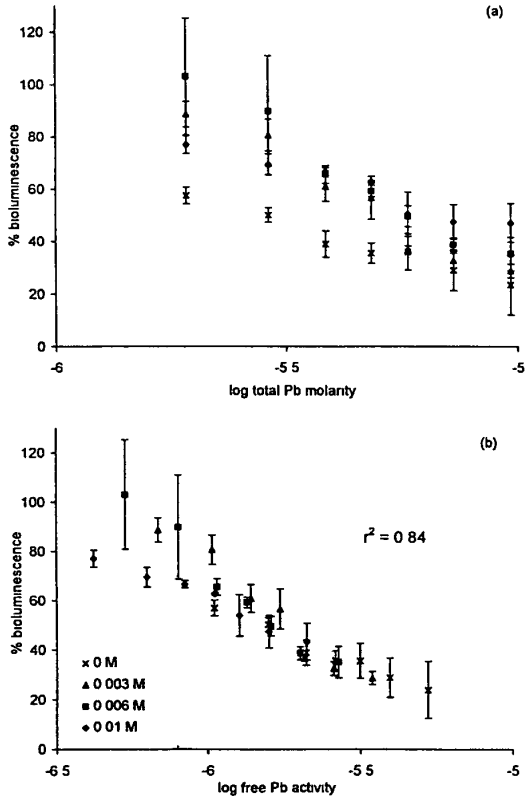

Fig. 3 Bioluminescent response (% of matrix-matched control) for the artificial soil solution at four different molarities of K_2SO_4 against (a) log total Pb molarity and (b) log free Pb^{2+} activity Error bars are ±1S D The dashed line represents the threshold toxicity for log (Pb^{2+})

For Cu, however, adding K_2SO_4 does affect the relationship between bioluminescence and $\log_{10}$ free ion activity (Fig. 4b, Table 4) as the data have a greater spread than those for (Pb^{2+}) or (Zn^{2+}). The plateau region of the graph (Fig 4b) extends up to $10^{-5.7}$ (Cu^{2+}) Above the toxicity threshold there is a significant ($P < 10^{-9}$) relationship ($r^2 = 0.78$) with toxicity and (Cu^{2+}).

4. Discussion

For Pb and Zn, expression of EC_{50} values in terms of free ion activity gives values independent of the anion, SO_4^{2-} This is in agreement with previous work on Al, which demonstrated the non rhizotoxicity of Al sulphate complexes (Kinraide, 1997) and supports the conclusions of other workers (Rainbow, 1995, Hare and Tessier, 1996) that toxicity is proportional to free ion activity This work confirms the suitability of (Zn^{2+}) as a toxicological indicator identified by other workers (McGrath et al., 1999; Chaudri et al., 1999, 2000). The EC_{50} value determined here for toxicity of (Zn^{2+}) to *E coli*, is very close to that obtained by Chaudri et al. (1999) of 38 2 μM (±3.1), also for the toxicity of (Zn^{2+}) (determined using a cation exchange resin technique) to *E. coli*. The current study also suggests that (Pb^{2+}) might also be a good indicator of Pb toxicity, although further experiments using ligands other than SO_4^{2-} would be necessary before a conclusion could be reached

For Cu, it is clear there is at least one other factor affecting toxicity besides (Cu^{2+}) Residual C may affect toxicity interpretation as demonstrated by some of the relatively large standard deviations but close investigation of the data suggests that this is not the primary cause of the inconsistent response At any single metal concentration, pH changes by < 0 2 over the range of added K_2SO_4 making it unlikely that inconsistent response is due to competition between H^+ and Cu^{2+} for binding sites at cell wall surfaces (Campbell and

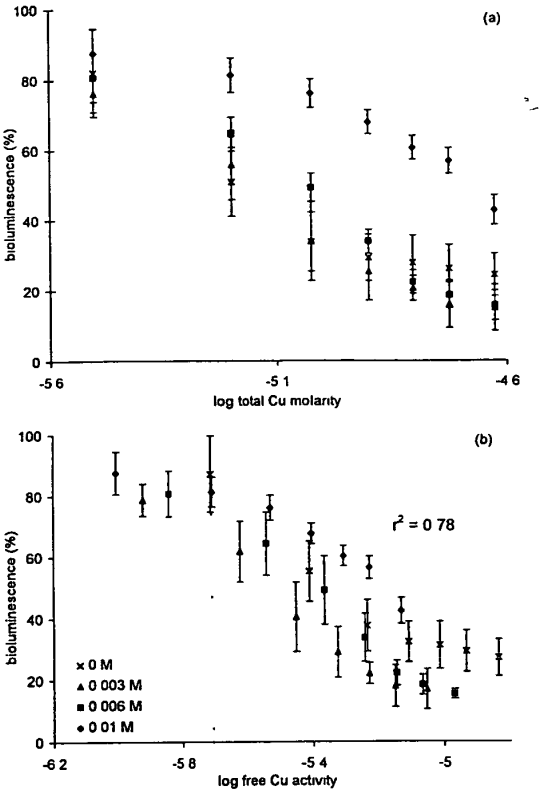

Fig 4 Bioluminescent response (% of matrix-matched control) for the artificial soil solution at four different molarities of K_2SO_4 against (a) log total Cu molarity and (b) log free Cu^{2+} activity Error bars are ±1S D The dashed line represents the threshold toxicity for log (Cu^{2+})

Stokes, 1985). The EC_{50} value for (Cu^{2+}) initially decreases but then increases as percentage of $CuSO_4^0$ increases (Table 4), demonstrating that $CuSO_4^0$ does not contribute to toxicity. Nor is it possible that $CuNO_3^+$ contributes to toxicity as the large difference in EC_{50} values between 0.006 and 0.01 M K_2SO_4 is accompanied by only a very minor change in the percentage of $CuNO_3^+$ (Table 4). In summary, Cu speciation alone cannot explain bioluminescence response

Chemical characteristics of the individual metal ions can govern the biological response of the test organism The classification of metal ions has been attempted by several authors (Nieboer and Richardson, 1980, Stumm and Morgan, 1996) where the placement of metals into groups may be based on a number of factors involving ion characteristics, ligand preferences and extent of polarisation (Gadd, 1992) For example, Pb^{2+}, Zn^{2+} and Cu^{2+} are all classified as borderline in the hard and soft acid scheme (Stumm and Morgan, 1996) Given that the category divisions correspond in some cases to a relative scale of change, it is not surprising that organism response to metal ion species in the same category class varies, and the absolute factor(s) that control toxicity remain(s) unclear Other workers (McGrath et al , 1999, Chaudri et al , 1999, 2000) investigating natural soil solutions have also found results of Cu toxicity difficult to explain, although these workers primarily suggest that Cu is not in the free state in these solutions

The test organism may also respond differently to the individual metal ions tested in this study The cell surface represents the primary site for organism–metal ion interaction and, in general, phosphoryl groups associated with cell wall components represent sites for metal binding (Ford and Ryan, 1995) In Campbell's critique of FIAM (Campbell, 1995), a biological response is considered to occur when the metal species binds to either a physiologically active or a transportation site on the cell wall However, Ledin et al (1997) concluded that the cell surface of a Gram-negative bacterium (such as *E coli*) consists of a number of different metal binding sites, which exhibit varying affinity and capacity for metal ions, depending upon the surrounding solution composition Not all these sites will be active and it is possible that binding to inactive sites on *E coli* occurs to a greater extent for Cu than for Pb or Zn. Such adsorption would be particularly important at low I because at high I, competition for sites from other cations present in the solution may be greater (e g. K^+) This could result in the effect seen in Fig 4a and Table 4 where there are little differences in toxicity response between the 0, 0 003 and 0 006 M K_2SO_4 (lower ionic strengths) but a marked reduction in toxicity for the 0 01 M K_2SO_4 (highest I) Thus, adsorption of Cu to inactive sites on the cell surfaces of *E coli* at low ionic strengths is a plausible explanation for the bioluminescence response to Cu

5. Conclusions

The rapid response and sensitivity to toxic metals of *lux*-marked *E. coli* makes it an attractive test organism for the investigation of the relationship between aqueous complexation and metal toxicity For Pb and Zn, expression of EC_{50} values in terms of free ion activity gives values independent of the anion, SO_4^{2-} in agreement with the FIAM However, Cu does not clearly follow the FIAM, possibly due to adsorption onto cell surfaces at low ionic strengths

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Effect of plant growth on copper solubility and speciation in soil solution samples

P.F.A.M. Römkens [a,*], L.A. Bouwman [a], G.T. Boon [b]

[a] Research Institute for Agrobiology and Soil Fertility (AB-DLO), PO Box 14, NL-6700 AA Wageningen, The Netherlands
[b] University of Groningen, PO Box 800, NL-9700 AV Groningen, The Netherlands

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Abstract

The effect of plant growth on copper solubility and speciation was studied in a 10-week pot experiment. A copper-tolerant grass variety (*Agrostis capillaris* L. var. Parys Mountain) was grown in pots that contained either clean (copper-total approx. 30 mg kg^{-1}) or copper contaminated soil (copper-total approx 170 mg kg^{-1}) at two pH levels (4.7 and 5.5). Also, similar pots without vegetation were included in the study. Due to the addition of NH_4NO_3 fertilizer and subsequent nitrification of ammonia to nitrate, soil pH decreased from 4 7 to 3.5 and from 5.5 to 4, respectively. In the planted pots, soil pH recovered faster after depletion of NH_4^+. This resulted in a decrease in the calcium solution concentrations and an increase in the dissolved organic carbon (DOC) concentrations in the planted pots. However, this was only observed in the clean soil, in the contaminated soil no difference in DOC levels between bare and planted pots was observed. Copper solubility in the contaminated soil was lower in the presence of plants, in the clean soil no differences were observed between the bare and planted pots. In the planted pots, copper activities in solution in both clean and contaminated soils were two orders of magnitude lower than in the bare pots. Copper activities in the non-planted contaminated soil reached potentially toxic levels ($[Cu] \pm 10^{-5}$ to 10^{-6} M) in contrast to the lower levels in the planted pots ($[Cu] \pm 10^{-7}$ to 10^{-10} M) Data and model results show that plant growth improves pH, DOC and calcium in solution to such an extent that both the total dissolved copper concentration and the free metal activity in soils can be reduced. This stresses the potential beneficial role of plants for the immobilization and detoxification of metals in contaminated soils.

Keywords: Fytoremediation, Copper; Speciation, Soil solution, Dissolved organic carbon, DOC

1. Introduction

As a result of increased input from industry, traffic and agriculture, the average trace metal content in soils in industrialized countries has increased considerably (Salomons and Stigliani, 1995). This has become a major point of concern during the last few decades since trace metal levels have reached potentially toxic levels in urban and agricultural soils in the vicinity of mining and metallurgical industries (Helios-Rybicka, 1996). Metal leaching, toxicity and uptake by organisms depends on soil chemical and physical properties as well as physiological properties of organisms present in, or growing on, the soil (Hopkin et al., 1993). However, risk assessment is still entirely based on the total soil metal content. Recent ecotoxicological studies revealed, however, that metal speciation in the solution phase, is one of the key factors that regulates metal uptake by plants as well as toxicity for soil and aquatic organisms (Marinussen, 1997; Renner, 1997). Metal (bio)availability and uptake appear to be related closely to the free metal *activity* in solution instead of the total soil metal content in the solid phase (Hare and Tessier, 1996; Moffett and Brand, 1996). Consequently, changes in soil chemical conditions that control the concentration and free metal activity [e.g. pH, organic matter content and dissolved organic matter (DOC)] induce changes in the availability and uptake without apparent changes in the total metal content (Spurgeon and Hopkin, 1996).

Soil parameters that affect metal solubility and speciation in the soil solution include pH, organic matter, DOC and clay content. With an increase in soil pH,

* Corresponding author Tel +31-317-474782, fax. +31-317-424812.
E-mail address: p f a m.romkens@ab dlo nl (P F A.M. Römkens)

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organic matter and clay content, the solution concentration of most cationic trace metals decreases due to adsorption (McBride, 1994). An increase in DOC and pH usually reduces the ion activity in solution due to complexation. Especially metals like copper and lead are known to form very stable metal–organic complexes (Stevenson, 1994). The presence of plants also influences the chemical conditions in the soil. Root growth causes an increase of the soil organic matter content which stimulates bacterial growth and contributes to the cation exchange capacity (CEC) of the soil as well (Schulin et al., 1995). Furthermore, root exudates can promote (phytosiderophores) or reduce trace metal uptake (Schulin et al., 1995; Alexander, 1977; Maywald and Weigel, 1997) Nutrient uptake (e.g. NO_3^- or Ca^{2+}) by plants also affects rizosphere pH which causes changes in the solubility and speciation of trace metals in soils (Alexander, 1977).

The impact of metallophytes on soil chemical conditions (i.e. DOC, pH) and the ability to accumulate certain trace metals, therefore, renders a potential tool for remediation of contaminated soils (Ebbs et al., 1997; Robinson et al., 1997). Although it may take too long to effectively clean the soil by 'extracting' the metals from the soil by plants (Felix, 1997), the presence of plants 'improves' soil conditions to such an extent that the acute metal toxicity for various soil organisms can be reduced (e g. by increasing the amount of soil organic matter or DOC in solution).

In order to understand the various ways by which plant growth affects soil chemical conditions and the functioning of various soil organisms in contaminated soils, it is necessary to simultaneously measure both chemical and biological parameters. In the experiment presented here, we will describe and evaluate changes in soil chemical conditions, trace metal solubility and speciation that are induced by plant growth. In a separate paper the impact of plant growth and copper contamination on the dynamics of various important soil organisms (bacteria, protozoa, nematodes) in the same experiment is described (Boon et al., 1998).

2. Materials and methods

2.1 Soils used in the experiment

The experiments were performed with soil material from an experimental site at the Wageningen Agricultural University, the Netherlands. On the site, a 3-year crop rotation is performed which includes potatoes, maize and sugar beet In 1982 the field was treated with $CuSO_4 \cdot 5H_20$ to obtain a range in the total copper content of the soil. The field experimental set-up is based on a randomised block design with four copper treatments. 0, 250, 500 and 750 kg Cu ha^{-1} were added to plots of 6×11 m. Within each copper treatment, four pH levels were established by addition of lime or elemental S, which resulted in a pH (pH–KCl) range from 4.1 to 6.1. Each treatment has been repeated eight-fold which resulted in 128 plots. The soil used in this study is a loamy sand with strong human influence (fimic Anthrosol; FAO, 1990). General soil characteristics are shown in Table 1. Analytical data of the soil, field site characteristics and management practices have been described in detail elsewhere (Korthals et al., 1996).

In April 1995, soil samples were taken from four randomly chosen replicate plots for each treatment and stored at 4°C until 22 May 1995. Polyethylene pots (11×9×9.5 cm) were filled with field-moist soil and seeded with a copper-tolerant grass variety, *Agrostis capillaris* L. var. Parys Mountain (Barenbrug Research Wolfheze, the Netherlands). All pots were initially fertilized with NH_4NO_3 (0.15 g pot^{-1}), $MgSO_4$ (0.36 g pot^{-1}), K_2O (0.15 g pot^{-1}) and P_2O_5 (0.11 g pot^{-1}).

After preparation, all pots were placed at random in a greenhouse at ambient temperature (15±2°C). Demineralised water was added via a dish under the pot at regular intervals. In this study a non-contaminated soil (copper-total of ±30 mg kg^{-1}) and a contaminated soil (copper-total of ±170 mg kg^{-1}) are used, at two pH levels (4.7 and 5.5). Of each pH–copper combination, 12 pots were prepared. At $t=0$ (23 May) and after 3, 5 and 10 weeks (termination of the experiment), three replicates

Table 1
General soil characteristics of the soils used in the plant growth experiment (0–20 cm)

Target pH[a]	Cu added in 1982 kg ($kg\ CuSO_4\ ha^{-1}$)	pH[b]	Cu-total (soil) ($mmol\ kg^{-1}$)	CEC_{NH4-Ac}[c] pH 7 ($cmol\ kg^{-1}$)	SOM[d] (%)	Texture (%)		
						>50μm	2–50μm	<2μm
4 7	0	4 8±0 2	0 47±0 20	5 6	3 2±0 3	86	12	2
4.7	750	4.6±0.3	2.42±0 39	n a	3 5±0 4	85	13	2
6 1	0	5 4±0 3	0 48±0 31	n a	3 6±0 1	84	13	3
6 1	750	5 6±0 4	2.94±0 33	n a	3 8±0 1	84	13	3

[a] pH values are adjusted to the target values every 6 years by S application (pH 4 7 plots) or $CaCO_3$ application (pH 6 1 plots)
[b] Measured in the centrifuged soil extracts
[c] CEC, cation exchange capacity
[d] SOM, soil organic matter

from each pH–copper combination were randomly chosen and sampled destructively

2.2 Solid phase analysis

The total copper content of the four treatments was determined by a hot acid extraction using concentrated HNO_3 and $HClO_4$ (Römkens and Dolfing, 1998) The soil organic matter content was determined in air-dried soil (<2 mm) by loss on ignition at 800°C during 6 h after drying at 105°C for 12 h The mineral N-content of the soil ($NH4^+$–N, $NO3^-$–N) at $t=0$ and after 3, 5 and 10 weeks was measured in filtered (0 45 μm) 1 M KCl soil extracts (80 g of soil: 200 ml 1M KCl) using a Technicon segmented flow analyser TRAACS 800 spectrophotometer

2 3. Soil solution analysis

After destructive sampling of the selected pots, soil solution samples were obtained by centrifugation of 200 μg field-moist soil (Del Castilho et al , 1993). Solution samples were filtered through a 0.45 μm HTTP filter (Millipore) by vacuum filtration after centrifugation. The calcium and copper concentration in the filtrate was determined on a Perkin Elmer ICP 2000. Total (TC) and inorganic carbon content (IC) in solution were determined on a Shimadzu TC500 total carbon analyser. The DOC content was calculated as the difference between total and inorganic carbon (TC–IC). Solution pH was measured in the final, filtered solution using a Beckmann pH electrode.

2.4. Calculation of Cu^{2+} activity in the soil solution samples

Copper activities in solution were estimated using a recently developed and calibrated thermodynamic equilibrium model that calculates the free metal activity based on the solution composition (Römkens, 1998). In the model, both pH-dependent deprotonation of humic substances and competition with calcium are taken into account as is shown in Eqs. (1) to (4)

$$H_2HUM \Longleftrightarrow HHUM^- + H^+ \log K_{H1} \quad (1)$$

$$HHUM^- \Longleftrightarrow HUM^{2-} + H^+ \log K_{H2} \quad (2)$$

$$HHUM^- + Me^{2+} \Longleftrightarrow MeHHUM^+ \log K_{Me1} \quad (3)$$

$$HUM^{2-} + Me^{2+} \Longleftrightarrow MeHUM^0 \log K_{Me2} \quad (4)$$

where HUM = organic compound; and Me^{2+} = metal ion (Cu^{2+}, Ca^{2+})

Apart from the interaction with DOC, complexation with inorganic ligands (e.g NO_3^- and OH^- is also taken into account. Eqs. (1) to (4) were implemented in the chemical equilibrium model CHARON (de Rooij and Kroot, 1991) for the calculation of the copper activity in the soil solution samples

3. Results and discussion

3.1. Nitrogen dynamics and soil pH

As a result of NH_4NO_3 application, strong acidification occurred during the first 5 weeks of the experiment due to the microbially stimulated aerobic conversion of NH_4^+ to NO_3^- (nitrification)

$$NH_4^+ + 2O_2 \Longleftrightarrow NO_3^- + H_2O + 2H^+ + \text{energy} \quad (5)$$

Soil acidification stopped after the conversion of NH_4^+ to NO_3^- (Table 2). Nitrification in the bare soils was faster in the high pH soils compared to the low pH soils; almost all ammonium was converted to NO_3^- after 5 weeks, whereas significant amounts of NH_4^+ remained present in the low pH soils after 5 weeks. Plant growth also affected the NH_4^+ conversion (and thus soil pH); in the low pH pots with plants, NH_4^+ depletion was faster compared to the non-planted pots (Table 2). This effect became significant after plant germination (between 2 and 4 weeks). Aside from direct uptake of NH_4^+ by plants, plant uptake of NO_3^- promoted the conversion of NH_4^+ to NO_3^-. In the high pH soils, this effect was negligible (data not shown here). The presence of copper did not affect the NH_4^+/NO_3^- distribution in either the low pH or high pH soils. No differences were observed in the NO_3^- concentrations between weeks 0 and 5. Unfortunately, no NO_3^- data are available from the last sampling date in the planted pots; enhanced nitrate uptake by the plants could potentially affect soil pH as well as a result of OH^- excretion during uptake of soil nitrate. The combination of OH^- release and Ca^{2+} uptake (see below) largely accounted for the observed increase in soil pH that occurred in the planted pots between weeks 5 and 10 (Table 2). This contrasted sharply to the soil pH in the bare pots, which showed a minor increase only after acidification. During the course of the experiment, both bare and planted pots remained well aerated, which prevented soil reduction processes from occuring.

3.2. Effect of plant growth and soil pH on calcium and DOC solution concentrations

As a result of the observed acidification, a release of calcium was observed in all pots, including the planted

Table 2
Effect of plant growth on soil NH_4/NO_3, pH, calcium in solution and dissolved organic carbon (DOC) (average of low and high copper soil)

	Time (weeks)	n	NH_4 (mg kg^{-1})	NO_3 (mg kg^{-1})	pH	Ca (mM)	DOC (mM C)
Bare Pots							
pH_0=4 7	0	6	117±5	125±2	4 7±0 2	9 9±2 4	4 8±0 7
	3	5	76±15	182±33	3 8±0 3	20 3±8 3	6 2±2 7
	5	6	60±34	244±159	3 4±0 2	11 5±7 3	8 0±6 9
	10	6	8±9	225±24	3 7±0 1	16 9±2 4	12 4±2 6
pH_0=6 1	0	6	117±2	128±2	5 3±0 2	13 4±1 7	4 1±0 3
	3	5	2±11	269±77	4 2±0 2	19 6±9 4	4 3±1 0
	5	6	8±5	205±81	3 8±0.2	19 5±11 0	5 9±1 6
	10	6	0±0	242±45	4 5±0 1	28 5±6 5	10 9±2 7
Planted pots							
pH_0=4.7	0	6	117±5	125±2	4 7±0 2	9 9±2 4	4 8±0 7
	3	5	76±11	157±18	4 0±0 2	16 4±6 8	5 3±2 4
	5	6	18±7	108±20	3 5±0 1	13 0±8 0	10 1±1 8
	10	12	–	–	4 7±0 3	4 2±2 3	13 9±5 0
pH_0=6 1	0	6	117±2	128±2	5 3±0 2	13 4±1 7	4 1±0 3
	3	5	22±5	227±26	4 0±0 3	21 9±10 3	5 7±1 6
	5	6	5±3	214±97	4 2±0 4	28 6±19 5	10 4±5 5
	10	12	–	–	6 1±0 4	4 6±0 6	11 8±3 1

pots (Table 2). Calcium-proton exchange was insufficient to buffer the release of protons, which resulted in a strong net acidification of the soil. Until week 5 only minor differences in soil pH were observed between bare and planted pots. After week 5, plant growth became significant and resulted in significant differences in soil pH, calcium solution concentration and DOC between planted and bare pots. As a result of calcium uptake by plants, the calcium solution concentration decreased from 28.5 mM (bare pots, pH 6.1) to 4.6 mM (planted pots, pH 6.1). The observed increase in soil pH in the planted pots is probably related to the change in the calcium concentration. This initiates a further desorption of calcium from the solid phase and promotes H^+ sorption which resulted in a higher soil pH in the planted pots.

In the Cu-0 soils, DOC levels were higher in the planted pots than in the bare pots in weeks 5 and 10 (data not shown). However, DOC levels in the Cu-750 soils were similar in both bare and planted pots. The DOC release, therefore, was not related to plant production only, since plant growth was not significantly different between low and high copper soils. In all soils, an increase in DOC was observed in week 10 that correlates with the increase in soil pH and is most likely due to pH-dependent desorption of DOC (Jardine et al., 1989). The final DOC solution concentration, therefore, will be controlled by a combination of soil chemical conditions, with DOC levels being reduced at high calcium levels [as a result of flocculation of calcium-humates, and formation of ternary complexes at the soil surface (Römkens et al., 1996), and low pH values (adsorption)].

3.3. Effect of plant growth on copper solubility and speciation in solution

The total copper concentration in solution increased with time (Fig. 1) in both the Cu-0 and Cu-750 soils. The strongest increase was observed in the Cu-750 soil at pH 4.7. Especially in the bare pots, copper levels reached excessively high levels, around 125 μM In the Cu-750 soils differences between the planted and the bare soils were significant: copper solution concentrations in the planted Cu-750 soil were significantly lower than in the bare soil. In the Cu-0 soil, no significant differences in the total dissolved copper concentrations were observed between the planted and the bare pots. In the high copper soils, a close correlation exists between total copper concentrations and soil pH (data not shown here); this relation was not observed in the low copper soil. This difference could be a result of the chemical form of copper in both systems In the low copper soil, no additional copper was added. In the high copper soil, more than 90% of the total copper content was added as $CuSO_4$. The copper thus added has been sorbed onto the soil; a large part remained exchangeable and will desorb at low pH values: the slope of the pH–logCu concentration is close to 0 5 which indicates that two protons are needed to replace one copper on the soil surface. In the low copper soil, however, a large part of the total copper content consists of non-exchangeable copper. This copper is incorporated in the structure of soil minerals and organic matter and is released only slowly upon acidification and the relation between soil pH and copper solution concentration, therefore, is not as clear as was the case in the high copper soil

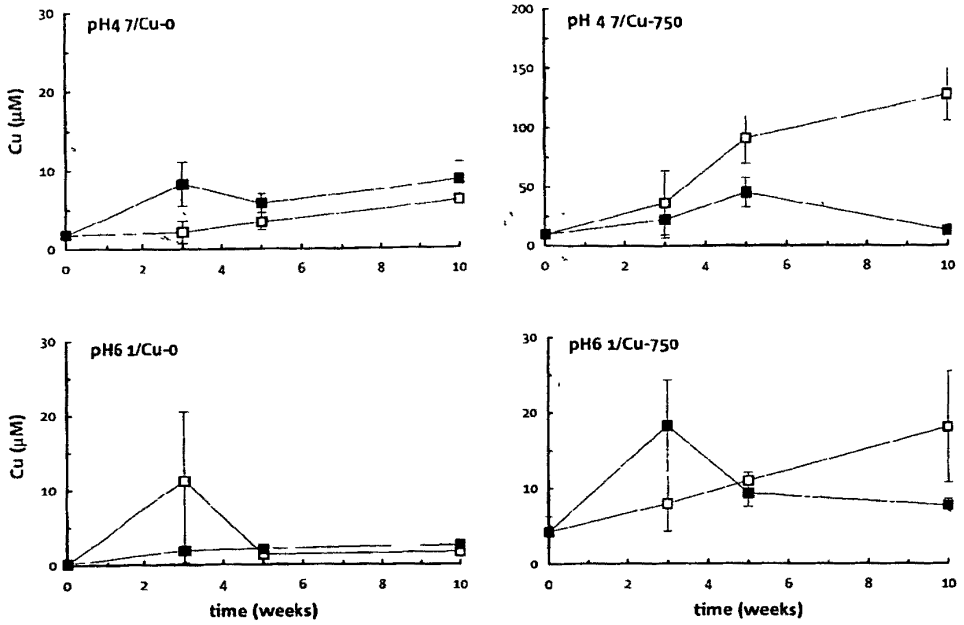

Fig 1 Effect of pH, copper-total and plant growth on dissolved copper concentration (closed symbols planted pots; open symbols bare pots)

Ecotoxicological research revealed that copper uptake and toxicity are closely related to the speciation in solution and especially the 'free' metal ions are considered important species that can be taken up by organisms and plants (Hare and Tessier, 1996; Moffett and Brand, 1996). Therefore, the dynamics of the copper activity were estimated for both the planted and bare soils. In Fig. 2 the results are shown for all soils. Until week 5, no significant differences were observed between the planted and bare pots. However, copper activities decreased sharply in all planted pots after week 5. In the bare soils, however, copper activities remained relatively constant after 5 weeks.

Soil chemical factors that contributed to the decrease in the copper activity are: (1) a higher soil pH in the planted pots (Table 2), (2) lower Ca^{2+} solution concentrations

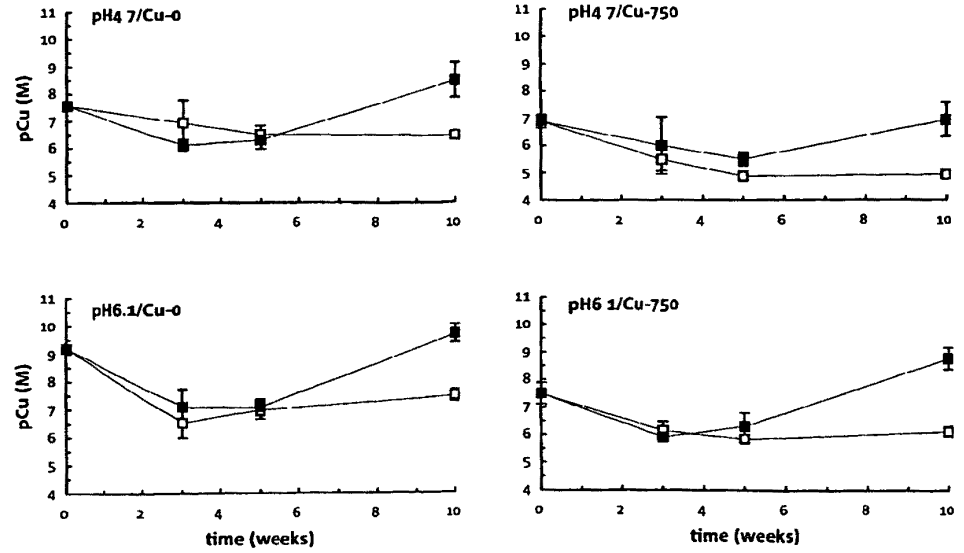

Fig 2 Effect of pH, plant growth and total copper content on copper activity in solution (closed symbols planted pots, open symbols bare pots)

as a result of plant uptake; and (3) higher DOC levels in the planted pots (in the Cu-0 soils)

The higher soil pH in the planted pots caused an increase in copper sorption due to the increased net negative surface charge on organic matter and soil oxides at high pH levels (McBride, 1994). Furthermore, the decrease in the calcium solution concentration in the planted pots also enhances copper adsorption onto the solid phase, thereby reducing the total dissolved concentration

Previous research also showed that calcium suppresses the solubility of DOC at calcium concentrations ranging from 5 to 10 mM (Römkens and Dolfing, 1998). In the experiment performed here, calcium solution concentrations ranged from 4.2 to 28.6 mM and a decrease in the calcium solution concentration, therefore, also enhances the solubility of DOC. The elevated DOC levels in the planted pots (clean soils) directly reduces copper activity levels due to complexation of copper with DOC:

$$Cu^{2+} + H_xHUM \Longleftrightarrow CuH_{(x-n)}HUM + nH^+ \qquad (6)$$

Copper forms stable complexes with DOC in solution and higher DOC levels will, therefore, reduce the free ionic Cu^{2+} activity. At pH levels higher than 5, usually more than 99% of the total dissolved copper concentration is bound to DOC (Römkens, 1998; Temminghoff, 1998). In the planted pots, all three parameters (i.e. a higher pH, higher DOC concentration, and lower calcium concentrations) favor a higher degree of complexation. In both planted and bare pots, copper activity strongly depends on soil pH with copper activity increasing at low pH (Fig. 3). From this it can be concluded that the presence of plants does not change the response of Cu^{2+} as a function of pH: both the bare and planted pots in either the low or high copper soil respond similarly to a change in soil pH. The main reason for the lower copper activities in the planted pots, therefore, is that the presence of plants changed other soil parameters that affect copper complexation in solution (i e. DOC, calcium).

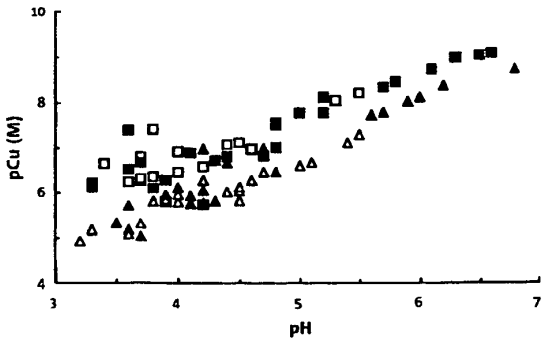

Fig. 3 Effect of pH on copper activity in solution (squares Cu-0 soil, triangle Cu-750 soil, closed symbols: planted pots, open symbols bare plots)

4. Conclusions

The presence of a copper-tolerant crop significantly altered soil chemical conditions in the plant growth experiment described here. In the planted pots, the conversion of ammonia to nitrate was faster than in the non-planted pots, which resulted in a more pronounced recovery of the soil pH. Plant uptake of calcium and increased adsorption onto the solid phase also resulted in a higher soil pH and, in the clean soils, also in higher DOC levels The differences between the planted and bare soils became significant after plant germination (±2–4 weeks). Especially in the copper contaminated soil, the dissolved copper concentration in the planted pots was significantly lower than in the bare pots due to the higher soil pH and lower calcium solution concentration. This illustrates the potential beneficial effect of plant growth for copper leaching from contaminated soils. It should be kept in mind, though, that the results obtained here are valid only for copper-contaminated non-calcareous sandy soils. Well-buffered (calcareous) clay soils, for example, are much less sensitive to acid inputs which reduces the beneficial effect of plants on soil pH. However, a large part of the soils in the Netherlands with intensive animal husbandry (i.e. with long-term manure application and elevated copper levels) are sandy soils like the ones included in this study. In those soils, copper is added in a highly labile form and it can be expected that the behavior of copper in those soils is quite close to that observed in the experiments described here.

Cu^{2+} activities in solution were also sigificantly ($p<0.001$) lower in the planted pots in both copper-contaminated and non-contaminated soils. Without plant cover, potentially toxic Cu^{2+} levels were observed in the contaminated soil at low pH; plant growth, however, reduced these to acceptable levels. The results presented here also suggest that the impact of plant growth on copper chemistry is an indirect one: by changing several important 'macro-chemical' parameters, such as calcium, pH and DOC, both copper activity and solubility are reduced. In order to understand the impact of plant growth and trace metal speciation it is, therefore, necessary to take into account macro-chemical parameters, such as pH, DOC, and calcium, as well.

The results from the 'chemo-dynamics' presented here support the observations on the dynamics of the microorganisms in the same experiment (Boon et al , 1998). In the planted pots, bacterial growth was more rapid than in bare pots. Also, the total number of protozoa and

bacterivorous nematodes were higher in the planted pots compared to the bare pots. These effects are most likely a result of the higher DOC levels (higher food supply) and the strongly reduced free copper activity in solution in the planted pots. Although the use of metal-tolerant plants may not be suitable for a rapid remediation, i e. metal extraction, of the soil, the results presented here suggest that plant growth can be used effectively to stimulate the biological activity in contaminated soils as well as to reduce the actual toxicity of metals. With time, this could lead to a more favorable soil-biological 'climate', thereby enhancing the potential for re-growth of other, less tolerant species as well as immobilization of the contaminants by newly incorporated soil organic matter.

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Nitrification potential in field-collected soils contaminated with Pb or Cu

Sébastien Sauvé[a,d,*], Alain Dumestre[a,b], Murray McBride[a], James W. Gillett[c], Jacques Berthelin[b], William Hendershot[d]

[a]*SCAS Department, Cornell University, Bradfield Hall 908, Ithaca, NY 14853, USA*
[b]*Centre de Pédologie Biologique, CNRS (UPR no 6831), F-54501, Vandoeuvre-lès-Nancy, France*
[c]*Cornell University, Superfund Basic Research & Education Program, Rice Hall, Ithaca, NY 14853, USA*
[d]*Department of Natural Resources Sciences, McGill University-Macdonald Campus, Ste-Anne-de-Bellevue, QC, Canada H9X 3V9*

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Abstract

The influence of soil properties on nitrification potential was studied using a simple 4 h incubation test applied to 56 field-collected soils contaminated mainly with Pb or Cu The soils were analyzed for total and dissolved metal and the soil solution was speciated for free Pb^{2+} or Cu^{2+} ions. The rates of nitrite production (NH_4^+ oxidation to NO_2^-) after the addition of a 1.0 mM NH_4^+ substrate and 2.0 mM $NaClO_3$ varied between 25 and 4200 μmol NO_2^- kg^{-1} of soil d^{-1}. The rates of nitrate production (NH_4^+ oxidation to NO_2^- minus NO_2^- oxidation to NO_3^-) for the same experimental conditions without chlorate varied between 2 and 2420 μmol NO_2^- kg^{-1} of soil d^{-1} The results show that the nitrification potential relationships with soil properties varied between sites. Soil pH and organic matter are the most influential parameters, but solution free metal activity and total metal content are also significant. It is not clear if soil pH and organic matter directly affect the nitrification potential or if they indirectly modify the chemical properties affecting metal speciation However, given the high sensitivity of this microbial process towards many environmental soil parameters, the nitrification potential does not appear to be a straightforward specific bioindicator for the evaluation of soil metal contamination

Keywords. Nitrification, Pb, Cu, Free metal, Speciation, pH, Soil organic matter

1. Introduction

1.1. Nitrification

Nitrification is the soil microbial process responsible for the transformation of ammonium (NH_4^+) into nitrite (NO_2^-) and then to nitrate (NO_3^-) (Eq. (1)). It is a critical part of the nitrogen cycle and contributes to the availability of soil N to plants and other soil organisms (Paul and Clark, 1989). Because of the high specificity of the implicated bacteria (mainly *Nitrosomonas* spp. for the first step (R_1) and *Nitrobacter* spp. for the second step (R_2)), nitrification is reputed to be a sensitive indicator of soil pollution from heavy metals (Bhuiya and Cornfield, 1974; Liang and Tabatabai, 1977, Ross and Kaye, 1994). Some studies suggest that the nitrite oxidation step

*Corresponding author Present address Department of Natural Resources Sciences, McGill University-Macdonald Campus, Ste-Anne-de-Bellevue, QC, Canada H9X 3V9 Tel : +1-514-398-7890, fax +1-514-398-7990, e-mail· sfs2@cornell edu

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(R_2) might be more sensitive than ammonium oxidation (R_1), which could potentially lead to accumulation of toxic nitrites in aquatic systems (Smith et al., 1997a) or soils (Wilke, 1989, Smith et al., 1997b; Dušek, 1995).

$$NH_4^+ \xrightarrow{R_1} NO_2^- \xrightarrow{R_2} NO_3^- \quad (1)$$

Soil nitrification potential is dependent on many factors. availability and chemical form of N-sources, CO_2 status, organic carbon availability, redox potential conditions, pH, moisture, soil texture, and tillage practices (Staley et al., 1990; Paul and Clark, 1989). Nitrification will also be affected by the presence of any inhibitory toxic compounds, hence sensitive to cation exchange capacity and organic matter content which tend to promote adsorption of toxic materials and reduce their bioavailability.

The standard means of quantifying nitrification is through measurements of the oxidation products of NH_4^+ (mainly NO_2^- and NO_3^-) in a perfusion system. Chlorate can also be added to the NH_4^+ substrate to prevent autotrophic oxidation of NO_2^- to NO_3^- and incidentally prevent potential gaseous-N losses due to denitrification of nitrates (Belser and Mays, 1980; Berg and Rosswall, 1985; Killham, 1987). In the presence of chlorate, nitrite becomes the sole transformation product of NH_4^+ oxidation and hence the first step of the nitrification process (R_1) can be quantified by a simple colorimetric measurement of NO_2^- formation. The comparison of the rates of NO_2^- produced with and without chlorate allows the determination of the rates of NO_2^- oxidation (i.e., in the absence of chlorate the measured concentration of NO_2^- corresponds to R_1–R_2; R_2 (NO_2^- oxidation to NO_3^-) can be obtained by difference).

1.2. Chemical speciation of soil metal

The major portion of total soil Pb or total soil Cu is insoluble, precipitated or adsorbed on the solid phase. Only about 1% to <0.01% of the total soil Pb or Cu is dissolved in the soil solution (Sauvé et al., 1997a, b) and from 40% to 99% of the dissolved Pb is complexed to dissolved organic matter (Sauvé et al., 1998c). Usually more than 98% of dissolved Cu is complexed (Lebourg et al., 1998; Sauvé et al , 1997a). Thus, only a very small fraction of the soil total metal burden is present as the free metal species in the soil solution Heavy metal bioavailability is linked to the free metal activity in both aquatic systems (Allen and Hansen, 1996, Campbell, 1995) and in soils (Sauvé et al., 1996, 1998a). Because of the influence of chemical speciation and soil chemical properties, we have chosen to use an inventory of field-collected soils to which no metal salts were added and which were not exposed to soil amendments in the laboratory (lime, clay, organic amendment, etc.).

Few field-collected soil studies are available for the evaluation of metal toxicity to indigenous soil microbial processes. Oftentimes, metal toxicity studies are made using soils spiked with a single metal salt. This is often done because it is very difficult to isolate the relative influence of various soil parameters and multiple organic and metal toxicants commonly found in contaminated sites. Nevertheless, the initial toxicity of metal salts added to soils is usually much higher than metals resident in soils for long periods of time. In field-collected soils, the metal speciation reflects a relative steady-state with respect to chemical adsorption and precipitation onto the soil's solid phase. Since the kinetics of certain organic-matter mediated reactions are reputed to be very slow (Hering and Morel, 1990), tests made with soils newly spiked with metal salts are not representative of the trace metal speciation in natural systems.

1.3. Objectives

The objectives of this study were to measure the nitrification potential of field-collected soils contaminated with Pb or Cu and evaluate the relative importance of soil parameters such as pH, organic matter, total metal content and soil solution free metal speciation to a sensitive microbial process like nitrification.

2. Material and methods

2.1. Soil samples

We have purposefully chosen to use only soils in which the main contamination was either Pb or Cu to simplify the interpretation of interactions of metal speciation and soil chemical properties. Other con-

taminants with potential impact were below recognized soil quality criteria (CCME Subcommittee on Environmental Quality Criteria for Contaminated Sites, 1991). The soils originated from various sites where the contamination occurred at least 10 years ago. Such mono-metal contaminated sites are difficult to find and although we would have preferred to use a larger dataset, we had no other suitable sites available On each site, various soil samples were taken to assess the variability arising from differences in soil properties and contamination levels.

Orchard soils were collected from the Cornell University apple orchard (Ithaca, NY, USA) as well as from the Macdonald Campus of McGill University apple orchard (Ste-Anne-de-Bellevue, QC, Canada). Both sites had accumulated Pb from long-term use of Pb arsenate until about 1970. A former car-battery recycling/Pb smelting site was also sampled from the Longue-Pointe garrison (Montréal, QC, Canada). A Danish site contaminated by Cu wood treatment spills was also sampled (Hygum, Denmark). A group of uncontaminated acidic forest soils was also included: one soil from Ste-Anne-de-Bellevue (QC, Canada), four from Sutton (QC, Canada) and another from Ithaca (NY, USA). Some of the soil samples were collected in the spring of 1996; they were sieved moist to <6 mm and stored in plastic bags at room temperature (samples 1–6, 16–22 and 28–43 in Table 1). The other soils had been collected two years before, air-dried, sieved to <2 mm and stored in plastic bags. The total soil metal analyses were determined using hot digestion in concentrated HNO_3 and analysis by flame atomic absorption spectrometry (AAS). Dissolved metal was determined using 1:2 soil:0.01 M KNO_3 extractions for Pb (Sauvé et al., 1997a) and 1:2 soil:0.01 M $CaCl_2$ extractions for Cu (Sauvé et al., 1997b), and both were analyzed with graphite furnace AAS. The chemical speciation of Pb^{2+} was carried out using differential pulse anodic stripping voltammetry (see Sauvé et al., 1998b for analytical details). Soil solution free Cu^{2+} was speciated using an ion-selective electrode method (see Sauvé et al., 1995 for analytical details). The free metal activity in solution is reported as pPb^{2+} or pCu^{2+}, which by analogy to pH is the negative log(M) ion activity. Hence pPb^{2+} of 8 is equivalent to 10^{-8} M of free Pb^{2+} ions. Important chemical properties of the contaminated soils are given in Tables 1 and 2, further sampling and soil chemistry details as well as complete soil data can be found in (Sauvé et al., 1997a, b).

2.2 Nitrification tests

The NH_4^+ oxidation (nitrification) potential was measured by adapting a perfusion method (Killham, 1987). We added ~5 ml deionized H_2O (corrected to account for the soil moisture content) to 5 g (dry weight equivalent) of moist soil in a 250 ml erlenmeyer flask. After a rehumidification period of 16 h, 5 ml of a substrate solution containing 2.0 mM NH_4^+ as $(NH_4)_2SO_4$ and 4.0 mM $NaClO_3$ was added. After correcting for dilution, the final concentration of $NaClO_3$ used in the substrate was 2 mM. This level was chosen because a preliminary experiment aimed at optimizing chlorate rates for discrimination of NH_4^+ and NO_2^- oxidation showed that excessively high chlorate concentrations (20 mM or above) were inhibiting not only the oxidation of NO_2^- to NO_3^- (reaction step R_2 in Eq. (1)) but also the oxidation of NH_4^+ to NO_2^- (reaction step R_1 in Eq. (1)).

The final soil:solution ratio was 1:2 (w:w). The samples were then incubated for 4 h at 24±1°C on a rotating shaker (120 rpm) which maintained aerobic conditions. The soil suspensions were then centrifuged at 15 000 g for 10 min and filtered through 0.22 μm cellulosic membranes. The concentration of nitrite was determined immediately with a sulfanilamide colorimetric procedure, measuring the absorbance at λ=520 nm (Rider and Mallon, 1946). Incubation of the 1 mM NH_4^+ substrate solution without soil produced no nitrite above the detection limit of ~0.5 μM NO_2^-. This assay was repeated in triplicate for each soil. The NO_2^- oxidation step was determined using an identical procedure without chlorate in the substrate solution. One soil sample (no. 22) was run as control with each batch of eight samples and consistently showed 10–15% variability (with chlorate= 297±34 μM kg^{-1} d^{-1} and without chlorate=164± 24 μM kg^{-1} d^{-1}).

2.3. Statistics

Statistical analyses and graphics were done using the SYSTAT software (Wilkinson, 1992). The level of statistical significance was reported using: NS when

Table 1
The soil chemical properties for the Pb dataset

No	Sample	Group	Total Pb (mg kg^{-1})	Dissolved Pb (µg l^{-1})	pPb^{2+}	pH	OM (%C)	Nitrite accumulation (with $NaClO_3$) (µmol kg^{-1} d^{-1})	Nitrite accumulation (without $NaClO_3$) (µmol kg^{-1} d^{-1})
1	Mac 01	Orchard	72	3 5	8 4	5 20	2 52	131±15	29±10
2	Mac 02	Orchard	93	1 8	8 8	5 60	3 03	242±9	53±10
3	Mac 03	Orchard	56	2 6	8 8	5 06	2 43	169±8	37±4
4	Mac 04	Orchard	118	4 2	8 9	5 68	3 62	724±14	173±6
5	Mac 05	Orchard	123	1 7	9 0	6 42	5 84	2025±487	959±97
6	Mac 06	Orchard	98	2 6	9 0	6 14	3 45	595±131	169±13
7	Cor 01	Orchard	441	52 1	7 2	4 54	2 91	25±2	13±1
8	Cor 02	Orchard	529	36 0	7 4	4 96	4 14	43±14	29±9
9	Cor 03	Orchard	86	0 7	9 2	5 65	2 15	208±29	134±29
10	Cor 04	Orchard	400	18 4	8 5	5 78	2 65	140±21	183±32
11	Cor 05	Orchard	325	12 0	10 0	7 12	2 40	104±10	100±8
12	Cor 06	Orchard	475	21 5	8 7	6 15	3 99	115±22	157±24
13	Cor 15	Orchard	15	4 1	9 7	6 38	1 44	446±30	274±28
14	Cor 16	Orchard	30	15 0	10 3	6 65	1 12	304±27	274±44
15	Cor 17	Orchard	31	3 7	9 4	5 95	1 65	662±69	459±72
16	Cor 31	Orchard	273	14 0	8 5	5 36	2 73	264±9	144±5
17	Cor 32	Orchard	284	6 7	9 1	7 35	2 86	1906±88	1235±145
18	Cor 33	Orchard	194	9 2	9 9	7 21	1 51	1478±256	1156±434
19	Cor 34	Orchard	358	7 5	8 2	5 27	2 78	185±9	56±0
20	Cor 35	Orchard	419	75 7	6 8	4 55	2 15	35±6	24±2
21	Cor 36	Orchard	710	124 1	6 8	4 33	3 83	70±1	53±7
22	Ber 03	Forest	20	1 0	8 6	5 11	5 46	296±33	156±29
23	Mar 01	Forest	27	8 9	7 5	3 80	1 83	30±7	25±9
24	Sut 01	Forest	11	3 4	8 1	4 08	3 63	26±4	22±1
25	Sut 02	Forest	10	2 0	8 0	4 07	3 16	26±3	23±0
26	Sut 03	Forest	11	6 9	7 8	3 49	4 30	25±3	21±3
27	Sut 04	Forest	12	12 7	7 8	3 67	4 06	32±8	20±2
28	L-P 02	Industrial	1422	5 7	9 3	7 68	1 94	290±18	259±23
29	L-P 07	Industrial	6698	5 6	9 0	7 72	2 04	305±49	271±24
30	L-P 08	Industrial	6680	34 0	7 8	7 74	1 44	241±39	187±25
31	L-P 09	Industrial	978	4 9	8 2	7 60	2 87	867±6	540±58
32	L-P 10	Industrial	3369	21 6	7.8	7 45	2 69	735±49	587±29
33	L-P 11	Industrial	1117	2 9	8 2	7 57	2 38	983±20	498±95
34	L-P 12	Industrial	621	1 1	8 6	7 59	2 40	506±27	581±154
35	L-P 13	Industrial	673	2 2	8 7	7 50	3 19	4201±535	1707±425
36	L-P 15	Industrial	523	14 7	9 4	7 59	1 72	698±584	314±146
37	L-P 18	Industrial	804	1 9	9 6	7 84	2 55	303±19	202±46
38	L-P 20	Industrial	905	10 7	8 9	7 71	2 91	600±44	360±29
39	L-P 32	Industrial	72	1 0	9 4	7 76	0 58	140±65	106±26
40	L-P 41	Industrial	1567	6 8	9 0	7 62	2 19	214±27	188±28
41	L-P 42	Industrial	520	1 6	8 9	7 67	1 27	114±24	93±8
42	L-P 45	Industrial	14861	56 8	7 7	7 52	1 57	196±64	168±14
43	L-P 46	Industrial	637	1 0	8 3	7 66	1 76	1010±695	797±147

Total Pb (HNO_3 digestions), dissolved Pb (0 01 M KNO_3 extracts), pPb^{2+} (free Pb^{2+} activity), soil organic matter (Nelson and Sommers, 1982) and nitrite accumulation in 4 h incubation of NH_4^+ with and without ClO_3

not significant ($p>0.1$), * when $p<0.1$, ** for $p<0.01$, and *** when $p<0$ 001. The effects of soil drying on the measured nitrification potential was tested. Nine of the moist soils were air-dried and after six months of dry storage the nitrification potential was re-measured. The nitrification rates were not significantly different

Table 2
The soil chemical properties for the Cu dataset

No.	Sample	Group	Total Cu (mg kg^{-1})	Dissolved Cu (µg l^{-1})	pCu^{2+}	pH	OM (%C)	Nitrite accumulation (with $NaClO_3$) (µmol kg^{-1} d^{-1})	Nitrite accumulation (without $NaClO_3$) (µmol kg^{-1} d^{-1})
1	Hygum 1	Denmark	26	11	8 82	6 26	2 19	1005±137	182±82
2	Hygum 4	Denmark	35	20	8 51	6 23	2 14	872±84	234±134
3	Hygum 5	Denmark	46	19	8 64	6 21	2 15	748±163	393±279
4	Hygum 6	Denmark	135	43	8 38	6 39	2 18	1105±16	67±1
5	Hygum 7	Denmark	191	54	7 70	6 04	2 32	723±556	157±42
6	Hygum 8	Denmark	375	99	8 23	7 01	2 65	275±133	118±8
7	Hygum 9	Denmark	331	86	7 70	6 51	2 54	271±140	117±3
8	Hygum 10	Denmark	332	88	7 06	6 00	2 60	1011±8	107±13
9	Hygum 15	Denmark	346	75	8 37	6 93	2 73	1592±80	233±27
10	Hygum 16	Denmark	470	136	6 91	5 91	2 94	616±54	128±4
11	Hygum 17	Denmark	577	162	8 23	7 11	2 84	161±8	89±18
12	Hygum 18	Denmark	1136	285	6 75	6 42	2 93	113±10	110±4
13	Hygum 19	Denmark	2277	545	6 22	6 61	3 38	138±27	99±31

Total Cu (HNO_3 digestions), dissolved Cu (0 01 M $CaCl_2$ extracts), pCu^{2+} (free Cu^{2+} activity) and soil organic matter (Nelson and Sommers, 1982) and nitrite accumulation in 4 h incubation of NH_4^+ with and without ClO_3

before and after drying for eight out of the nine samples tested (pairwise t-tests, $p<0.05$).

3. Results and discussion

3.1. Site variability

The measured nitrification potentials for NH_4^+ oxidation varied between 25 and 4200 µmol NO_2^- produced kg^{-1} dry soil d^{-1} in the presence of 2 mM $NaClO_3$ in the substrate and between 13 and 1800 µmol NO_2^- produced kg^{-1} dry soil d^{-1} in the absence of $NaClO_3$ (Tables 1 and 2). The chemical properties of the soils varied between pH values of 3.49–7.84, soil organic matter from 0.58% to 5.84% C, total soil Pb from 10 to 14 900 mg Pb kg^{-1} dry soil (Table 1) and total Cu from 14 to 3100 mg Cu kg^{-1} dry soil (Table 2). The statistical analyses were performed on $\log_{10}$-transformed values of nitrification rates to allow normalization of the data distribution.

The rates of the two nitrification steps (R_1 and R_2) were correlated and as shown in Figs. 1–4, in most cases the rates for NO_2^- oxidation followed a trend similar to that of NH_4^+ oxidation, but at a lower absolute level. The effect of the various soil properties on nitrification varied among the sites, making generalizations difficult. To elucidate the relationships, each measured soil property was graphed and compared individually to nitrification (Figs. 1–4) The data are presented for the Mac and Cor orchard samples, for the L-P site and for the Danish site (DK). The For group represents mostly acidic forest soils and is not as interesting; the nitrification rates are very low, and most correlations had low or no significance (data not presented). The orchard samples and the L-P samples have markedly different soil properties (L-P soils have the highest pH (calcareous) and clay content, lower soil organic matter and highest levels of total Pb contamination). The relationship of dissolved Pb or Cu to nitrification was not shown because of a general lack of statistical significance of total dissolved metal in explaining nitrification rates in different soils.

3.2. Total soil metal

The relationship between nitrification rates and total soil metal is depicted in Fig. 1. Although the Cor vs. total Pb and DK vs. total Cu relations suggest that increasing total metal decreases nitrification rates, the relationship was not significant in the case of L-P vs. total Pb. Conversely, for the Mac soils, which have a low level of total soil Pb contamination (note the different unit scales displayed in Fig. 1), increasing soil Pb induced an increase in the nitrification rates

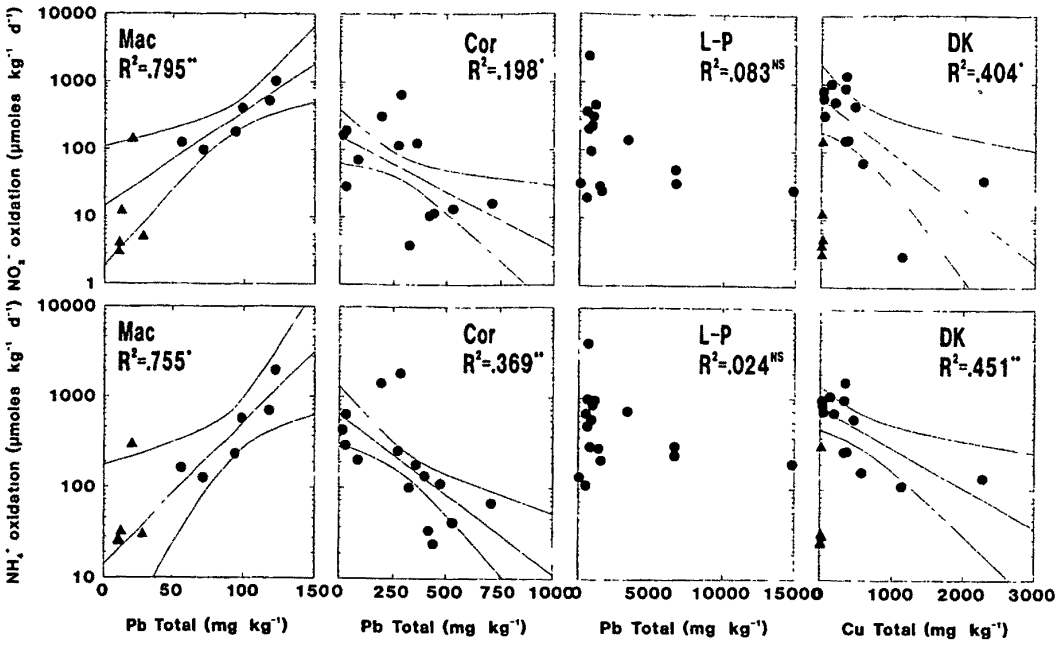

Fig 1. The nitrification rates as a function of total soil Pb for Mac, Cor, L-P and DK soils The For soils (▲) are also included for comparison, but were not computed in the regressions The regression lines represents the statistically significant regressions with the 90% confidence intervals

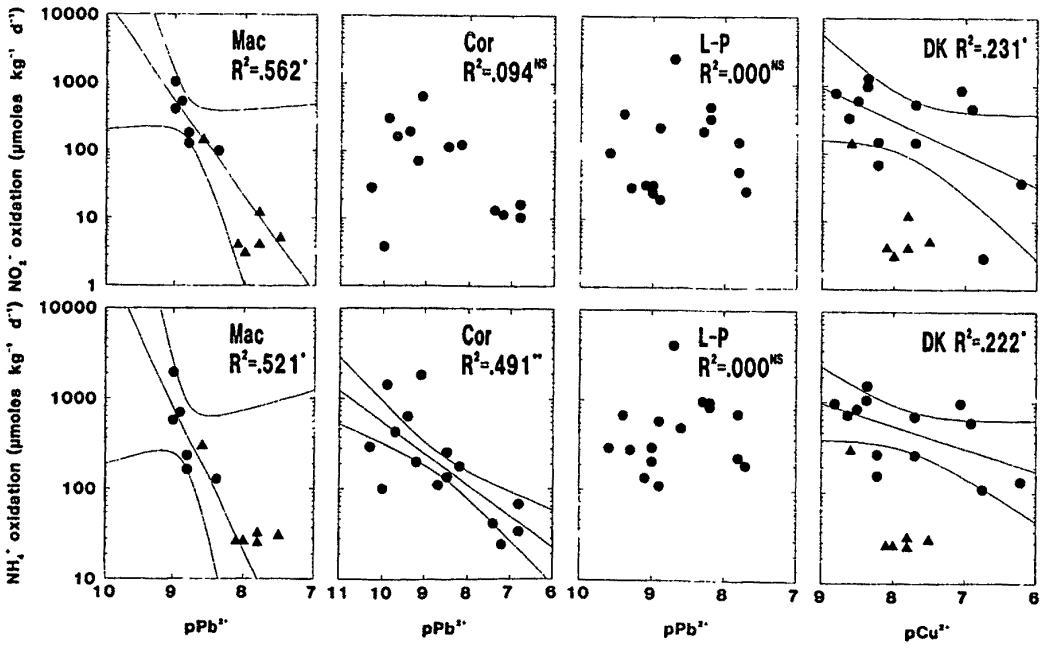

Fig 2. The nitrification rates as a function of free solution Pb^{2+} activity, for Mac, Cor, L-P and DK soils The For soils (▲) are also included for comparison, but were not computed in the regressions The regression lines represents the statistically significant regressions with the 90% confidence intervals

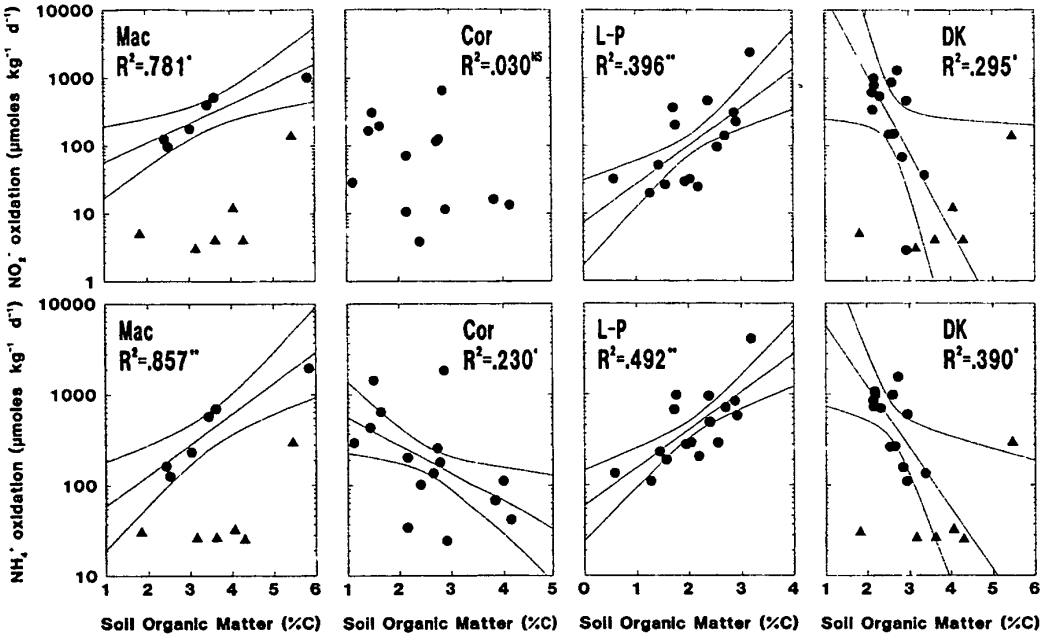

Fig. 3. The nitrification rate as a function of soil organic matter for Mac, Cor, L-P and DK soils. The For soils (▲) are also included for comparison, but were not computed in the regressions. The regression lines represents the statistically significant regressions with the 90% confidence intervals

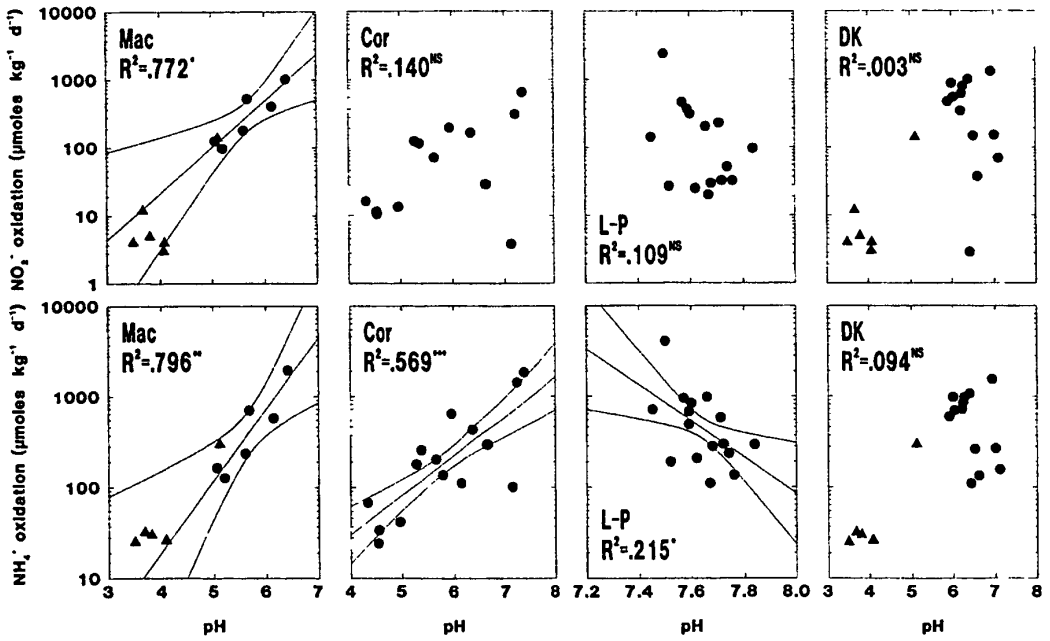

Fig 4 The nitrification rates as a function of soil pH Mac, Cor, L-P and DK soils The For soils (▲) are also included for comparison, but were not computed in the regressions The regression lines represents the statistically significant regressions with the 90% confidence intervals

(although this was possibly due in part to concomitant pH and OM variations) Similar stimulation of microbial assays under low contamination levels have been reported before (Dušek, 1995; Díaz-Raviña et al., 1994) and can be attributed to the death of sensitive microbes which feed the less sensitive populations, hence the nutrient-induced stimulation. It is clear that the total metal content of the soil was not sufficient to explain the variations in nitrification rates This could be due to a combination of direct and indirect effects. Nitrification could be influenced by total metal, but the bioavailability of total soil metals are affected by soil properties determining speciation Thus soil properties may affect nitrification directly or indirectly through effects on metal speciation

3 3. Free metal activity

The trends in nitrification rates relative to free metal activity and total soil metal were somewhat similar. The relation of L-P soils vs. free Pb^{2+} or total metal generated a cluster of points (Figs. 1 and 2), whereas the orchard soils (Mac and Cor vs. free Pb^{2+}) and the DK vs. free Cu^{2+} relations seemed to show a trend of decreasing nitrification with higher free metal activities (Fig. 2). But, as was the case for total metal, the variability explained by free metal activity was low and the slope differed among the sites and soils groups

In contrast to total soil Pb, free Pb^{2+} integrates the effects of chemical speciation. In this case, we could not attribute the observed variability in the nitrification-pPb^{2+} relationship to indirect effects of other soil properties upon speciation. This suggests that the nitrification potential is directly influenced by soil properties like pH and organic matter (Paul and Clark, 1989; Curtin et al., 1998) independently of the effects of those properties on metal speciation.

3.4. Soil organic matter

In the case of soil organic matter, for the Mac and L-P groups the regressions were relatively tight and show a positive impact of higher soil organic matter levels upon nitrification (Fig. 3). The positive influence of organic matter may have been due to concomitant increase in available organic carbon, to the existence of organic microsites with more favorable chemical properties, or to changes in the metal speciation or fractionation (although the latter should have been evident in the total metal vs. free metal activity comparison) Conversely, the Cor and DK sites showed more variable, inverse relationships between soil organic matter and nitrification The apparent negative impact of soil OM may be due to a correlation with total soil metal levels, since earlier studies have shown that soil metal contamination may reduce OM decomposition, promoting OM accumulation and higher soil OM content (Wuertz and Mergeay, 1997; Sauvé et al , 1997b). Incidentally, the quality of the soil organic matter and not only its total amount is important and as such some of the site differences may have been due to differences in the properties of the soil organic matter.

3.5. Soil pH

It is important to note that the soil groups in Fig. 4 do not cover the same pH range, the orchard soils are all below pH 7.4, whereas in L-P the pHs are all above 7.4 and in the DK site, the soil pH values are between 5.9 and 7.1.

The nitrification process is inhibited at pH below 6 for sites Mac and Cor, which is in accordance with many data reported previously (Liang and Tabatabai, 1977, Killham, 1987, Rother et al., 1982; Curtin et al., 1998). But within the high pH group (L-P), pH has a negative effect on nitrification. This would suggest that the optimum pH for nitrification is between 6.5 and 7.5. Although it is not clear why nitrification is inhibited at higher pH, it may be due to NH_4^+ toxicity (Malhi and McGill, 1982) or from increased buffering of free Pb^{2+} activity in solution due to higher concentrations of dissolved Pb (resulting from higher levels of dissolved organic matter and hydroxy and carbonate ion-pairs (Bruemmer et al., 1986; Sauvé et al., 1998b, c)). DK is the only site without a significant influence of pH on nitrification. The small pH effects observed in L-P and DK are probably due to the rather narrow pH range at the two sites.

3.6. Multiple regressions

Multiple stepwise linear regressions show that in using the orchard soils (Mac and Cor) the $\log_{10}$ of nitrification potential (NH_4^+ oxidation) yields a sig-

nificant empirical regression (R^2=0.671*** n=21) dependent on pH***, total Pb** and soil OM*. A similar regression for L-P (R^2=0.492** n=16) depends only on soil OM***. Using the complete Pb dataset yields a different regression dependent on pH***, soil OM** and pPb^{2+}* (R^2=0.597*** n=43). The resulting relationship for the DK site is dependent simply on total soil Cu (R^2=0.451** n=13).

Similarly, for the second nitrification step (NO_2^- oxidation), multiple stepwise linear regressions for the orchard soils yield a regression dependent on total Pb*** and soil OM* (R^2=0.475** n=19). For L-P, it is also dependent on soil OM** (R^2=0.396** n=16) For the complete Pb dataset, the regression (R^2=0.385***, n=43) depends on pH***, soil OM* and total Pb*. In the DK site, the resulting best-fit regression is dependent on dissolved Cu or total Cu (R^2=0.430** or 0.404** n=13).

It is obvious that such empirical regressions, although statistically significant, have limited usefulness, since the predictive parameters are different for Pb and Cu and vary from site to site. The regressions mostly serve to demonstrate the complexity of nitrification and the strong influence of soil pH and soil OM, which are difficult to dissociate from the effects of toxic metal levels. The strong influence of pH and soil OM is illustrated in Fig. 5. Thus, identifying the actual toxic effects of metals is rendered much more difficult by the nitrification* pH* OM relationship which already explains 43% of the variability in the NH_4^+ oxidation and 20% for the NO_2^- oxidation. Using only the Pb dataset the same relationship explains 59% of NH_4^+ oxidation and 40% of NO_2^- oxidation. With the Cu dataset, the most significant relationship is with total Cu (Fig. 1). This shows that for the Pb dataset, metal levels are not the most significant parameters influencing nitrification and for the DK site, total Cu explains only 40–45% of the measured nitrification rates. It is clear that a large part of the variability is simply a function of soil pH and soil OM. The dependence of nitrification on pH and soil organic matter is a well-known phenomenon (e.g., Curtin et al., 1998); these results further suggest that the relationships vary among sites and that the impact of metal contamination up to 15 000 mg Pb kg^{-1} and 3000 mg Cu kg^{-1} is still subordinate to the influence of other soil properties. Of course, these are field-collected, long-term contaminated soils where a certain microbial adaptation to the high metal levels has presumably occurred. Ideally, a test of metal toxicity on nitrification would have to be conducted on soils with the same pH and same level of organic matter and only metal as a varying parameter. Unfortunately, this would only be feasible with metal-spiked soils with the resulting less realistic chemical speciation. It would also fail to address the critical influence those soil parameters have on metal toxicity to soil microbial processes.

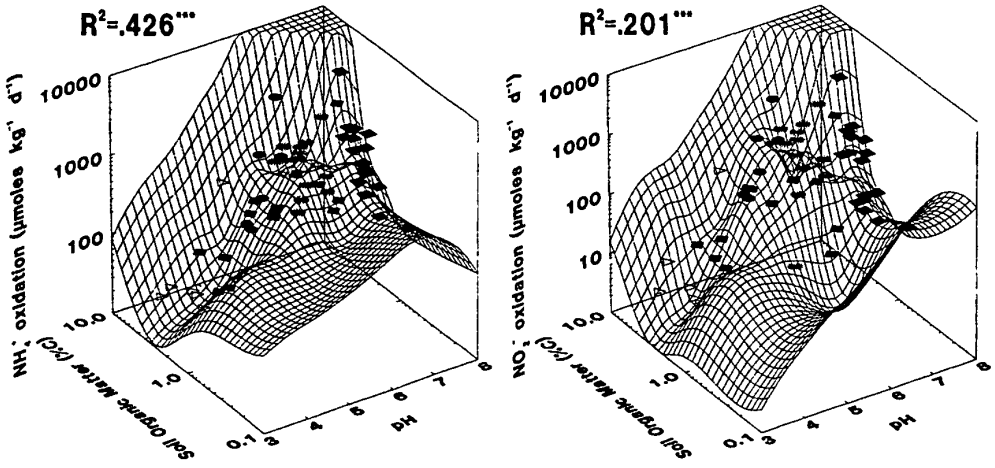

Fig. 5. The nitrification rates as a function of soil pH and soil organic matter, the symbols are represented as Mac (●), Cor (◆), For (▲), L-P (■) and DK (*) The graph surfaces are obtained using distance-weighted least-square smoothing (Wilkinson, 1992)

4. Conclusions

The measured nitrification potentials cannot be explained using a single soil parameter (total soil metal, dissolved metal and free metal activity, soil pH or soil organic matter) The results show that nitrification is sensitive mostly to soil pH and soil OM but also to metal levels It is not clear if soil pH and organic matter affect the nitrification potential directly or if they act indirectly by affecting the chemical speciation of the metals. Elucidating this would require further study using more soils of diverse origins and covering an even wider range of soil properties. However, given the high sensitivity of this microbial process towards environmental parameters, measuring nitrification potential is not a simple, straight-forward bioindicator test for the impact of soil metal contamination.

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Lead Phosphate Solubility in Water and Soil Suspensions

SÉBASTIEN SAUVÉ* AND MURRAY MCBRIDE
Cornell University, SCAS Department, Bradfield Hall 908, Ithaca, New York 14853

WILLIAM HENDERSHOT
McGill University-Macdonald Campus, Department of Natural Resources Sciences, Ste-Anne-de-Bellevue, H9X 3V9, QC, Canada

The widespread occurrence of soil Pb contamination requires a good understanding of the factors controlling Pb solubility. Pb solubility will often be controlled by phosphates, which are used to reduce environmental risks of Pb-contaminated soils. The solubility equilibria of two different synthetic lead phosphate minerals were determined electrochemically across a wide range of pH and Pb and phosphate concentrations. The resulting chemical equilibrium data could not be explained by standard solubility equations, although a simple empirical regression explained the data very well. The same lead phosphate mineral was then added to two different soils which were equilibrated across a pH range 4–8. Differential pulse anodic stripping voltammetry was used to determine the ASV-labile Pb in the extracted soil solutions, and the free Pb^{2+} ion activity was calculated. The free Pb^{2+} activity in the soil solutions followed the expected pH relationship, highest solubility at low pH and gradual decrease with increasing pH. In contrast, total dissolved Pb was high at low pH, decreased until pH of ~6, and then increased again at higher pH. This behavior is attributed to the strong Pb-complexing capacity of the soil organic matter which is solubilized at neutral pH and above.

Introduction

Lead contamination is widespread in industrialized countries, most urban soils having total Pb levels above the geochemical "background levels" of 10–20 mg of Pb kg^{-1} (*1*). Even in contaminated soils, most of the Pb is insoluble, precipitated or bound to the soil solids. Since only the metal dissolved in the soil solution is potentially available to plants and soil organisms in the short term, the low solubility of Pb-bearing minerals such as carbonates, phosphates, and sulfides may control Pb bioavailability depending upon soil conditions and the initial form of the Pb contamination. The least soluble Pb minerals in aerobic soils are the lead phosphates. Consequently, in many situations, the soil solution concentration of phosphate may control the solubility and availability of Pb (*2*).

Phosphate amendments have been used to reduce the solubility and bioavailability of Pb in contaminated soils (*3, 4*). In this respect, land application of sewage sludge and manure establish a high soil phosphate availability, which can be expected to reduce Pb solubility.

* Corresponding author e-mail address: sfs2@cornell.edu

The potential effect of increased soil phosphate levels upon the solubility of Pb in contaminated soils can be predicted using theoretical solubility equilibrium data. Unfortunately, experimental data for the solubility of lead phosphates are scarce, and these few studies were made under specific and narrow experimental conditions. Specifically, the existing solubility products for orthophosphates, pyromorphites, and other phosphate phases were derived under conditions of very low pH and very high phosphate and Pb activity (*5, 6*). Thus, the estimated solubility products may not apply satisfactorily over the wider range of pH, phosphate activity, and Pb^{2+} activity relevant to soils.

Various solubility diagrams have been presented to explain the solubility of Pb and the interactions with various other mineral phases (*5, 7*). It should be noted that such diagrams, while a reasonable representation of pure synthetic systems, may be less applicable to soils. In soils, the presence of additional cations and anions, a heterogeneous adsorptive mineral phase, and an ill-defined organic matter component limit theoretical solubility diagrams to no more than a representation of potential trends. Reactive surfaces often control metal solubility in soils with low to moderate contamination, only with severe loadings does mineral precipitation control solubility (*8, 9*). In addition, dissolved organic matter can enhance metal solubility, organic matter in the solid phase contributes to metal adsorption and modifies reactive mineral surfaces.

The objectives of this study were to (1) determine the solubility of two synthetic lead phosphate precipitates over a wide range of pH and Pb and phosphate levels and (2) investigate the solubility equilibrium of the same lead phosphate mineral phase mixed with soil, thus exploring the pH-dependent solubility of Pb in the presence of excess phosphate in soils.

Materials and Methods

Analytical Determinations. Previous solubility studies of lead phosphates were restricted by the detection limit of flame atomic absorption spectrometry (*5, 6*). In this study, the activity of free Pb^{2+} ions is determined electrochemically, using a Pb ion-selective electrode for the synthetic systems and differential pulse anodic stripping voltammetry (DPASV) for the soil solution speciation of Pb.

Lead ion-selective electrodes have been shown to give a Nernstian response to the activity of free Pb^{2+} ions down to ~10^{-10} M (*10*) and ~$10^{-11.8}$ M in Pb activity-buffered solutions (*11*). This sensitivity is sufficient to measure the solubility of lead phosphate minerals over a wide range of conditions. The Pb ion-selective electrode is subject to interference from other metals (Cu, Fe, and Hg) (*11*). This prevents its use with soil solution extracts, particularly because many contain significant quantities of dissolved Fe in solution.

Soil solution extracts were therefore analyzed for Pb using ASV, yielding the concentration of labile Pb (*12, 13*). Using the analysis for total dissolved Pb by graphite furnace atomic absorption spectrometry (GFAAS) and subtracting the ASV-labile Pb, we obtain the concentration of nonlabile Pb in the soil solution

$$\text{(nonlabile Pb)} = \text{(dissolved Pb by GFAAS)} - \text{(ASV-labile Pb)} \quad (1)$$

The ASV-labile Pb pool includes inorganic complexes and excludes organic complexes which are not sufficiently labile to be detected by the ASV Hg electrode (*13*). If we assume that most organically complexed Pb is in electrochemically

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TABLE 1. The Stability Constants for Calculations of Free Pb^{2+} Ion Activity in Soil Solution (*22, 25*)

equation	log *K*
$Pb^{2+} + H_2O \rightleftharpoons PbOH^+ + H^+$	−7 71
$Pb^{2+} + 2H_2O \rightleftharpoons Pb(OH)_2^0 + 2H^+$	−17 12
$Pb^{2+} + 3H_2O \rightleftharpoons Pb(OH)_3^- + 3H^+$	−28 06
$Pb^{2+} + HCO_3^- \rightleftharpoons PbHCO_3^+$	3 45
$Pb^{2+} + CO_3^{2-} \rightleftharpoons PbCO_3^0$	6 27
$Pb^{2+} + 2CO_3^{2-} \rightleftharpoons Pb(CO_3)_2^{2-}$	9 49
$Pb^{2+} + NO_3^- \rightleftharpoons PbNO_3^+$	1 17
$Pb^{2+} + Cl^- \rightleftharpoons PbCl^+$	1 58
$Pb^{2+} + SO_4^{2-} \rightleftharpoons PbSO_4^0$	2 62

nonlabile form, the activity of Pb^{2+} ions can then be calculated using known formation constants to partition the measured ASV-labile inorganic Pb into the various potential inorganic ion pairs or complexes and free ionic Pb^{2+} (eq 2 and Table 1)

$$\text{free } Pb^{2+} = (\text{labile Pb by ASV}) - [PbOH^+ + Pb(OH)_2^0 + Pb(OH)_3^+ + PbHCO_3^- + PbCO_3^0 + Pb(CO_3)_2^{2-} + PbNO_3^+ + PbCl^+ + PbSO_4^0] \quad (2)$$

This calculated free Pb^{2+} activity could overestimate activity because it would not account for weak organic ligands that might form ASV-labile complexes with Pb A similar speciation calculation was performed to calculate the activity of $H_2PO_4^-$ from the concentration of dissolved inorganic phosphate determined by the molybdate blue reaction, the dissociation reaction data given in ref 7 and the appropriate solution chemical properties

Synthetic Lead Phosphate Preparation. Two different solids were prepared according to methods obtained from the literature (*5*) We have chosen solids which were simple to prepare and which contained only Pb and PO_4 without other ions, so as to simplify the comparison between the aqueous and the soil systems Secondary Pb orthophosphate ($PbHPO_4$) was made by slowly adding 500 mL of 0 1 M Na_2HPO_4 to 500 mL of 0 1 M $Pb(NO_3)_2$ at 80 °C and maintaining at low heat on a hot plate for 3 h The slurry was stirred for 24 h and stored in the original solution This precipitate consisted of crystalline platelets identified as $PbHPO_4$ by matching the X-ray diffraction pattern to that of known minerals at the X-ray diffraction facility in the Geology Department of Cornell University The procedure to prepare tertiary Pb orthophosphate [$Pb_3(PO_4)_2$] was similar, using lead acetate instead of $Pb(NO_3)_2$ The resulting precipitate was rinsed with distilled-deionized water (dH_2O) three times after cooling and stored in dH_2O This less crystalline solid was identified as $\beta Pb_9(PO_4)_6$ using X-ray diffraction

Equilibration and Analysis. Both materials were equilibrated in the laboratory at 23 ± 1 °C for 20 and 100 days after adding 20 mL of suspension (~0 3 g of solids) to 30 mL centrifuge tubes and adding aliquots of NaOH (0−0 3 mL of 5 M solution), HNO_3 (0−0 05 mL of 10% solution), $Pb(NO_3)_2$ (0−0 2 mL of 0 01 M solution), and Na_2HPO_4 (0−0 5 mL of 0 001 M solution) in various combinations to obtain a range of phosphate, Pb, and pH To approach equilibrium from over and under saturation, half of the treatment combinations had the original solution replaced with dH_2O before adding the reagent aliquots Finally, the ionic strength of the solutions was adjusted with $NaNO_3$ to 0 1 M (which is necessary to use the ion-selective electrode) The free Pb^{2+} activity was measured in the equilibrated supernatants after centrifugation (10 min at 15000*g*) using a Pb ion-selective electrode (Orion 948200), a double-junction reference electrode (Orion 900200), and an Orion 701A pH meter The pH was measured using a Fisher 805MP meter and a combination

TABLE 2. The Stability Constants Were Taken from ref 25, and References Therein (*26−29*)[a]

equation	log *K*
$H^+ + IDA \rightleftharpoons HIDA^-$	9 34
$2H^+ + IDA \rightleftharpoons H_2IDA$	11 94
$H^+ + H_2IDA \rightleftharpoons H_3IDA^+$	13 74
$Na^+ + IDA \rightleftharpoons NaIDA^-$	0 36
$Pb^{2+} + IDA \rightleftharpoons PbIDA$	7 50
$Pb^{2+} + 2IDA \rightleftharpoons PbIDA_2$	16 9
$Pb^{2+} + H^+ + IDA \rightleftharpoons PbHIDA^+$	10 2
$Pb^{2+} + NO_3^- \rightleftharpoons PbNO_3^+$	1 17

[a] The IDA constants were compiled and interpolated to 0 02 M ionic strength, and for the other constants the values at 0 1 M ionic strength were used

TABLE 3. Basic Soil Properties before Addition of Lead Phosphate

soil	land use	pH_{CaCl_2}	soil O M (% C)	total Pb (mg/kg)	dissolved Pb ($\mu g\ L^{-1}$)	labile Pb (nM)
Macdonald	forest	5 11	5 46	19 5	1 0	4 3
Cornell	orchard	6 19	4 77	151	7 1	15 1

glass electrode (Orion 91-55) The phosphate in solution was determined using the molybdate blue colorimetric method Electrolytic conductivity was measured using a YSI model 31 meter to estimate the ionic strength (IS)

Ion-Selective Electrode Calibration. The ion-selective electrode determination of free Pb^{2+} activity was made using a modification of a method used for copper in soil solutions (*14*) The procedure consists of calibrating the ion-selective electrode using metal buffer solutions of known activity which then allows direct measurement of the electrode potential and, therefore, the metal activity in the samples The inner reference electrode was filled with standard calomel electrode solution (Orion solution 900002) and the outer compartment filled with 10% KNO_3 (Orion 90-00-03) To reduce the drift due to redox reactions on the surface of the electrode, it was calibrated every five samples and polished with 3 μm aluminum oxide polishing strips (Orion 301044) The standard curve relating electrode potential measurements to activity was made using Pb activity buffers containing 10^{-3} M iminodiacetic acid and 10^{-4} M $Pb(NO_3)_2$ in 0 1 M KNO_3 The Pb^{2+} activity in the buffers was pH dependent and calculated using MINEQL$^+$ (*15*), and the constants were given in Table 2 The Pb^{2+} activity in the buffers varied from pPb^{2+} = 4 06 at pH 4 to pPb^{2+} = 11 85 at pH 10 By comparison to the Cu electrode, the Pb electrode gave a similarly linear response, which however, tended to drift between each polishing and calibration while maintaining a similar response slope Frequent renewal of the electrode surface by polishing and recalibration improved the accuracy of the method (for analytical details on Pb ion-selective electrodes, see refs 10 and 11)

Soil and Precipitates. For this experiment, we used two soils, an uncontaminated calcareous forest soil and an orchard soil contaminated with Pb from sewage sludge and Pb arsenate pesticides We have chosen soils with similar organic matter levels because we wanted to study the impact of a moderate level of soil Pb contamination upon the solubility of the added Pb while keeping the effect of organic matter level constant The forest soil is an Inceptisol from the Morgan Arboretum on the Macdonald Campus of McGill University (Ste-Anne-de-Bellevue, QC), the orchard soil is an Alfisol from the Cornell University Orchard (Ithaca, NY) Basic soil properties can be found in Table 3

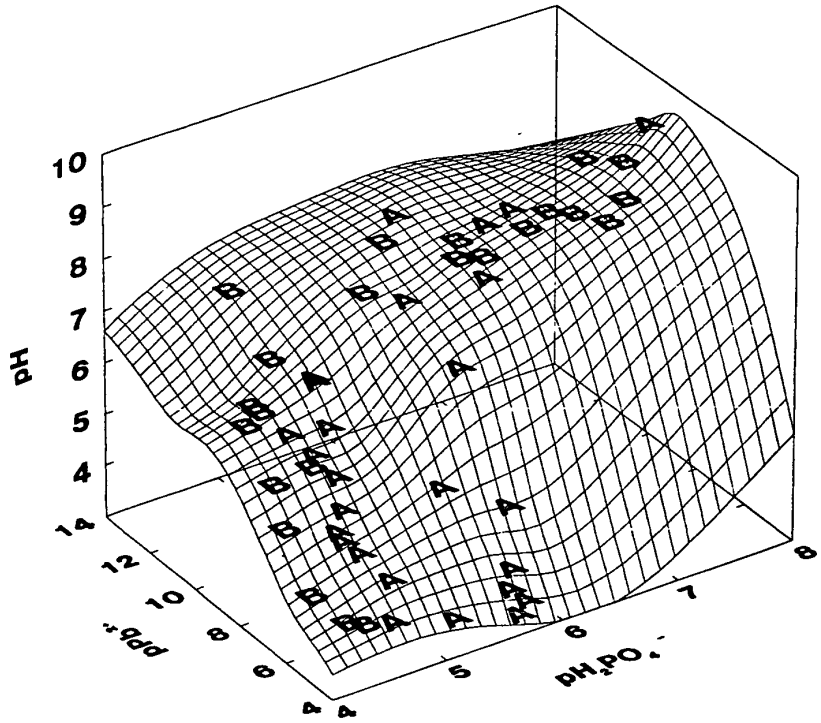

FIGURE 1 The solubility of the two synthetic lead phosphates for 20 and 100 day equilibrations, A represents $PbHPO_4$ and B represents $\beta Pb_9(PO_4)_6$. The plotted surface is a distance-weighted least-square smoothing (*15*).

Ten grams of soil and 20 mL of 0 01 M KNO_3 were added to 30 mL centrifuge tubes The soil was extracted using KNO_3 because neutral salt extractions are often used to evaluate soil metal bioavailability (*9, 14*), and although some $PbNO_3^+$ is formed, this is accounted for by eq 2 and in most cases, it is negligible Fifteen tubes were used for each soil, with up to 3 mL of 0 1 M KOH or HNO_3 added to generate a pH range 4–8 The tubes were shaken and equilibrated for 2 days before a 0 5 mL spike of the $PbHPO_4$ slurry was added to each tube This spike is equivalent to an increase of ~530 mg of Pb kg^{-1} of dry soil, enough to consider the soil markedly contaminated (pristine soils generally contain between 10 and 20 mg of Pb kg^{-1})

The spiked soils were then shaken every other day for a month (10 m at 200 rpm on a reciprocal shaker) The bottles were kept loosely covered (to prevent dust deposition) but not closed, so as to allow for gas exchange and keep the solutions aerobic and in equilibrium with atmospheric CO_2 Then, the tubes were centrifuged at 15000*g* to separate the supernatants The pH and electrolytic conductivity were determined in the supernatants before filtration The solutions were then passed through 0 22 μm cellulosic membranes, and analyzed for labile Pb by ASV and for phosphate by colorimetry The extracts were made 1 mM in Na_4EDTA (ultrapure grade) to preserve the samples before total dissolved Pb was determined by GFAAS with a Varian Zeeman SpectrAA instrument

Results

Synthetic Lead Phosphate. The solubility data for both lead phosphate mineral phases were not statistically different The solubility after equilibration for 20 and 100 days were not different either, suggesting that there is no significant solubility change occurring between 20 and 100 days The results are combined and illustrated in Figure 1 The data are only presented graphically but the complete dataset can be obtained from the authors The solubility relationship involving pPb^{2+}, pH, and $pH_2PO_4^-$ was highly significant However, it did not seem to match that of any of the known Pb-PO_4 mineral phases, including pyromorphite, which is considered the least soluble lead phosphate mineral under these conditions The $PbHPO_4$ system could not be readily distinguished from $\beta Pb_9(PO_4)_6$ on the basis of solubility The free Pb^{2+} activity in the solution was lower in this pure system than would be expected from mineral equilibria in a soil where solubility is controlled by $PbHPO_4$ (*7*)

Calculations of the ion activity product (IAP) for the $PbHPO_4$ mineral phase theoretically controlling Pb solubility ($^{c*}K_{s0}$ based on the reaction $PbHPO_4 + H^+ \Leftrightarrow Pb^{2+} + H_2PO_4^-$, using the Davies equation for activity coefficients) shows a significant pH-dependent effect (Figure 2a) The scatter in the estimates of solubility could be attributed to the presence of more than one mineral phase or perhaps a single phase of variable crystallinity The resulting IAP using the whole data set was $-7\ 53 \pm 1\ 89$ While quite variable, this IAP is clearly lower than the log $^{c*}K_{s0}$ value of -4 25 found by Nriagu (*5*) Much of this discrepancy can probably be attributed to the pH trend of the IAP and the possible presence of multiple phases of different mineralogy and crystallinity (*16, 17*) The data show that the solution concentrations were all below the levels required for saturation with respect to of PbO, $Pb(OH)_2$, or $PbCO_3$ Nriagu (*5*) determined log $^{c*}K_{s0}$ at very acidic pH values (2–4) which Figure 2a indicates are likely to produce less negative estimates of IAP which may not reliably be extrapolated to more typical soil conditions Nriagu (*5*) also discussed the possible transformation of $PbHPO_4$ into $Pb_3(PO_4)_2$ or other lead phosphates as the solution conditions vary

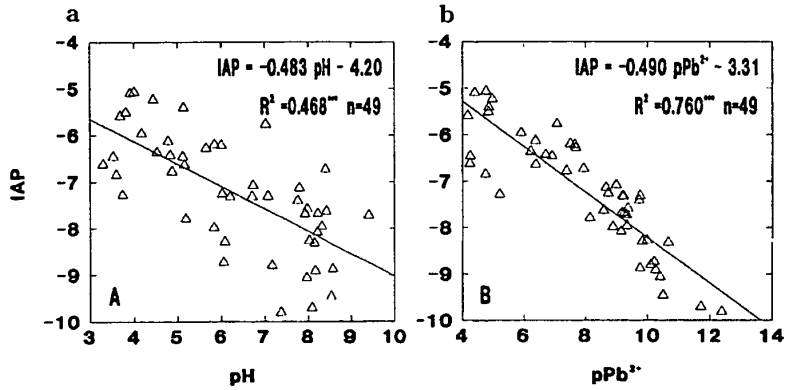

FIGURE 2. The IAP for the synthetic Pb-PO_4 as a function of pH (A) and as a function of pPb^{2+} (B).

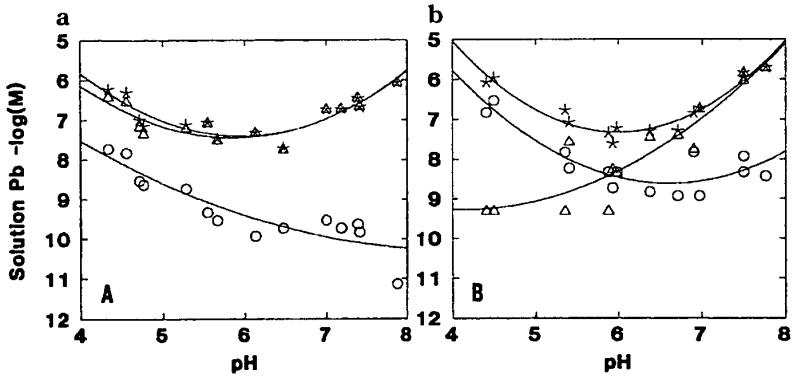

FIGURE 3. The free Pb^{2+} activity (○) (by ASV), the OM-bound Pb in solution (△), and the total dissolved Pb (*) (by GFAAS) in the soil solutions equilibrated with a 530 mg of Pb kg^{-1} spike of $PbHPO_4$. The data are presented as a function of pH and the units are −log(M). The data for the forest soil are in panel A, and the data for the orchard soil are in panel B. The lines are quadratic smoothing.

The effect of pH could also be partly explained by incongruent surface processes such as competitive adsorption of Pb^{2+} and other cations on the surface exchange sites of the precipitated minerals, since some phosphate minerals have substantial adsorption capacity for metals (*18*) If the free Pb^{2+} concentration is partly controlled by adsorption of Pb^{2+} ions onto the surfaces of the minerals, then we might expect a relationship between the observed IAP and pPb^{2+} Indeed, as Figure 2b illustrates, more of the scatter in the IAP of $PbHPO_4$ is explained by the solution pPb^{2+} instead of pH

The chemical equilibrium data described by Figure 1 are most accurately modeled using an empirical linear regression

$$pPb^{2+} = 1.47pH - 0\ 98p(H_2PO_4^-) + 4\ 13$$
$$R^2 = 0.836,\ n = 49 \quad (3)$$

where each variable and the whole regression are significant ($p < 0\ 001$) The addition of a class factor in the regression to distinguish the two mineral phases shows no significant differences ($P > 0\ 8$) between the solubility relationships of $PbHPO_4$ and $\beta Pb_9(PO_4)_6$.

The best-fit relationship described by eq 3 has a coefficient of about 1 5 for pH and 1 for $pH_2PO_4^-$ Although this regression is highly significant and describes the Pb, pH, and $pH_2PO_4^-$ relationships of Figure 1 by a very simple model, this is an empirical oversimplification of limited use, derived from a simple synthetic solid/solution system in the absence of any organic ligands or soil Nevertheless, the solubility results suggest that under realistic pH and Pb and phosphate solubility conditions of soils, solubility functions more complex than eq 3 are unlikely to be justified statistically or conceptually If a simple batch equilibration cannot be explained with straightforward solubility equilibrium relationships, it would be quite surprising if those equilibrium equations were successful in explaining the behavior of Pb in contaminated soils

Soil and Precipitates. The Pb dissolved in the soil solution (measured by GFAAS) includes uncomplexed metal, inorganic ion pairs, and organic complexes The labile Pb pool measured by ASV reflects only the metal in solution that is available for reduction at the electrode surface (mainly free metal and labile complexes). By partitioning the labile Pb pool into those ion pairs calculated to be most prevalent in the solution (namely $PbOH^+$, $Pb(OH)_2^0$, $Pb(OH)_3^+$, $PbHCO_3^-$, $PbCO_3^0$, $Pb(CO_3)_2^{2-}$, $PbNO_3^+$, $PbCl^-$, and $PbSO_4^0$), an estimate of free Pb^{2+} was obtained by difference (eq 2 and Figure 3) At neutral pHs and higher, organic matter solubility increases and may cause the "ASV-labile" Pb to include some labile organic complexes Any ASV-labile organically bound Pb would have been included in the inorganic fraction by default, causing an overestimate of the free Pb^{2+} activity If the short-term bioavailability of Pb is controlled solely by the free metal ions in solution (calculated from the ASV-labile Pb pool), then this study shows that at pH above 6, the presence in soil of more than 500 mg of Pb kg^{-1} as lead phosphate generates a free Pb^{2+} ion activity in the soil solution below 10^{-9} in the pristine calcareous soil and below 10^{-8} in the contaminated orchard soil The Pb^{2+} activities measured in the orchard

soil are higher and more variable than those for the pristine forest soil This may be the result of differences in the soil properties as well as the higher initial Pb content of the orchard soil, which was contaminated by lead arsenate and sewage sludge

In contrast to the estimated free Pb, the dissolved Pb includes nonlabile dissolved organic complexes and is up to 5 orders of magnitude higher than free Pb in these soils (Figure 3) Both soils had a similar relation of dissolved Pb to pH The dissolved Pb diminished from 124–228 μg of Pb L^{-1} at pH 4 3–4 5 in both soils down to 3–12 μg of Pb L^{-1} between pH 5 5 and 6 5 Further pH increase raised the total dissolved Pb, up to 420 μg of Pb L^{-1} in the orchard soil at pH 7 8 and 310 in the forest soil at pH 7 5 (Figure 3) These are very high solubilities relative to various groundwater quality standards [e g , 10–15 μg of Pb L^{-1} (*19*)], and imply that lead phosphates are not sufficiently insoluble to prevent groundwater contamination in moderately contaminated soils at low and high pH (5 > pH > 7) Figure 3a shows that in the forest soil, most of the Pb dissolved in the soil solution is bound to dissolved organic matter In the orchard soil, nonlabile Pb constitutes most of the dissolved Pb at pH 6 and above, only under more acidic conditions does the labile Pb become predominant over the nonlabile forms of Pb At higher pH, the dissolved Pb in nonlabile complexes increases significantly in both soils (Figure 3), and it appears that dissolved organics solubilize Pb from the phosphate minerals, effectively mobilizing the metal Other researchers have noted greater solubility of Pb and other metals in contaminated soils as the pH is raised above 6 (*20–22*)

The bioavailability of Pb complexed with dissolved organic matter is not well-known It may be tightly bound and unavailable for release to the soil solution Furthermore, the increased solubility of Pb at pH above 6 5 raises concerns for metal mobility, irrespective of the actual bioavailability of the Pb in the solution We used KOH (as opposed to lime) to increase the pH in this experiment, which may have generated higher dissolved organic matter concentrations than an equivalent addition of $CaCO_3$ Nevertheless, liming contaminated soils to pH 7 or higher increases the dissolved organic matter (*20, 21*) and correspondingly generate a significant increase in the dissolved Pb in the soil solution Even if this Pb pool is potentially unavailable for biological uptake, it is still in a mobile form and prone to leaching (*23*) We hypothesize that at the higher soil pH, the strong complexation of Pb by dissolved organic matter dissolves a fraction of the lead phosphate, despite the low solubility of this mineral Thus, soil remediation by phosphate addition to reduce the solubility of Pb is not likely to be completely successful in immobilizing this metal, although it may limit its toxicity

In a different experiment, Ma et al (*24*) added rock phosphate to various Pb-contaminated soils and found that, after up to 8 weeks of incubation, the phosphate did not significantly reduce the extractability of Pb by EDTA-NH_4OAc (*24*). The EDTA extraction is considered an estimate of the soil metal pool associated with organic matter, therefore, also suggesting that phosphate amendments have little impact on the retention of Pb by the organic components of contaminated soils

Synthetic Mineral vs Soil Solubility. The solubility of both synthetic lead phosphate phases was described very well using eq 3 In the soil equilibration experiment, the soil pH and solution $pH_2PO_4^-$ were used to attempt to predict Pb^{2+} activity using eq 3, assuming that these lead phosphate phases controlled the solubility The predicted activities are compared to the actual measurements in Figure 4 Although the regression for the whole dataset (full line) shows a close fit to the 1 1 line (dotted line), the actual data points show wide variations between predicted and measured values Even

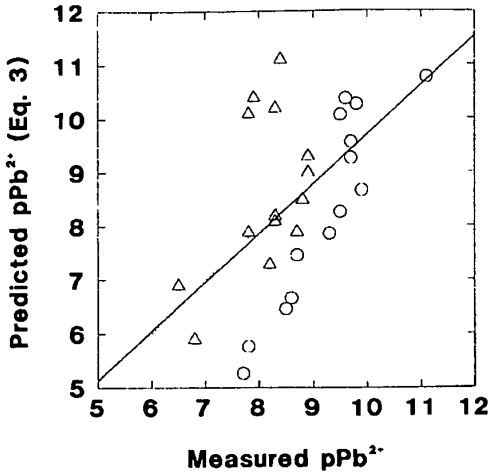

FIGURE 4. The free Pb^{2+} activity predicted using eq 3 as a function of the measured values The ○ represent the forest soil, the △ represent the orchard soil, the full line represents the linear regression for the dataset, and the dotted line is the 1:1 perfect fit. The units are −log(M).

though both soils had similar properties (except total Pb content), free Pb^{2+} activity is overestimated in most cases for the forest soil (circles in Figure 3) and drastically underestimated in four cases in the orchard soil (triangles in Figure 4) The most likely explanation is that the difference in total metal content between the two soils is large enough to modify the free metal activity This suggests that solubility in soils is not controlled by chemical equilibrium with a pure solid phase (which would be independent of the total content) but by surface retention processes which vary with the level of saturation of adsorption sites (*8, 9*)

Conclusions

The solubility of two synthetic Pb-PO_4 phases was measured using a Pb ion-selective electrode in a batch equilibration under a wide range of conditions The resulting chemical equilibria are impossible to explain using a simple mineral solubility equation Furthermore, the highly significant empirical solubility relationship derived from the data for pure lead phosphate systems is not applicable to soil systems, probably because surface reactions and soil organic matter play an important role in determining the solubility of Pb At low pH, adsorption by soil reduces the solution concentration of Pb below what is observed in pure systems At higher pH, dissolved organic matter increases the dissolved Pb in the soil solution Although this may be partly an artefact due to the use of KOH to increase the pH, we can expect a qualitatively similar trend using lime in the field

Although phosphate amendments and lime are beneficial in reducing the solubility, mobility, and bioavailability of Pb, the optimum pH to reduce solubility is between 5 5 and 6 5 Higher pH increases organic matter solubility, which may induce dissolution of lead phosphate by organic complexation reactions

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Soil Solution Speciation of Lead(II): Effects of Organic Matter and pH

Sébastien Sauvé,* Murray McBride, and William Hendershot

ABSTRACT

The effects of adjusting the levels of soil organic matter in a Pb-contaminated soil on the solubility and free Pb^{2+} speciation were studied within the pH range 3 to 8. A contaminated orchard soil containing 284 mg Pb kg^{-1} was treated with leaf compost to increase soil organic matter and with H_2O_2 to decrease it, yielding six soil organic matter levels between 25.6 and 83.7 g C kg^{-1}. The equilibrated solutions were then analyzed for total dissolved Pb by graphite furnace atomic absorption spectrometry and for labile Pb by differential pulse anodic stripping voltammetry (DPASV). The labile Pb values were used to calculate the free Pb^{2+} activity based on the assumption that organo–Pb complexes are not DPASV-labile. The data showed that 30 to 50% of dissolved Pb is present as soluble OM complexes at low pH and up to 80 to 99% at near-neutral pH. The solubility of Pb shows a linear decrease from pH 3 to 6.5 and is independent of soil organic matter in that pH range. From pH 6.5 to 8, higher pH promotes the formation and dissolution of organo–Pb complexes, which increase Pb solubility. In this pH range, higher organic matter content results in higher concentrations of dissolved and labile Pb.

INTRODUCTION

Most of the Pb present in contaminated soils is insoluble and bound to the solid phase, therefore not extractable by water or dilute salt solutions. Unless the soil pH is quite acidic, only the presence of chelating agents can generate significant quantities of Pb in the soil solution

It is well known that free Pb^{2+} activity increases as pH decreases, with most Pb minerals being less soluble at higher pH (Lindsay, 1979, p. 449). Although this relationship is indisputable in pure systems without organic matter, very few studies have evaluated Pb speciation or measured free Pb^{2+} in soil solution extracts (Santillan-Medrano and Jurinak, 1975; Jopony and Young, 1994, Kalbasi et al , 1995; Sauvé et al., 1998). Solution metal speciation can be accomplished using electrochemistry (Mota and Correia dos Santos, 1995) or through a variety of other techniques, such as resins, ligand exchange, spectroscopy, or size separations (Apte and Batley, 1995). Each technique has advantages and disadvantages In the case of Pb, DPASV is one of the most sensitive and simplest techniques (Sauvé et al., 1998). The presence of colloidal organic matter might interfere with the DPASV measurements; conversely, some of the Pb weakly bound to dissolved organic acids might dissociate at the Hg drop, causing an increase in the measured DPASV-labile Pb and overestimating inorganic Pb in solution. Nevertheless, we believe that these analytical problems are manageable and that DPASV is one of the best available techniques for the chemical speciation of Pb^{2+} at the low solubilities typical in soils

S Sauvé and M McBride, Soil, Crop, and Atmospheric Sciences, 908 Bradfield Hall, Cornell Univ , Ithaca, NY 14853, and W Hendershot, Dep of Natural Resources Science, McGill Univ , Macdonald Campus, Ste-Anne-de-Bellevue, QC, H9X 3V9 Canada Received 19 May 1997 *Corresponding author (sfs2@cornell edu)

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The presence of soil organic matter complicates solubility models Although the soil organic matter present in the solid phase acts as an exchanger and chelator, thereby decreasing free metal activity with increasing pH, some organic matter also dissolves in the soil solution The dissolved organic matter (DOM) increases with higher pH and significantly enlarges the pool of organic ligands in the soil solution The release of dissolved organic ligands into solution could decrease the free Pb^{2+} activity in the soil solution even as organo–Pb complexes increase the concentration of dissolved Pb. In real soil systems, organic matter complexation will also be interacting with dissolution–precipitation and chemisorption–desorption reactions.

The objective of this study was to measure the solubility and speciation of Pb in 0 01 *M* KNO_3 soil extracts in response to pH variations and the addition and removal of soil organic matter. It was expected that this would clearly elucidate the role of solid and dissolved organic matter in Pb solubility and speciation

MATERIALS AND METHODS

We used a soil collected from the Cornell University orchard (Ithaca, NY), which had received lead arsenate pesticide applications prior to 1970 The sample was collected in the spring of 1996, sieved moist to 6 mm, and stored moist in sealed plastic bags The soil is a silty clay loam, Hudson series, classified as a fine, illitic, mesic Glossaquic Hapludalf The natural soil pH ($CaCl_2$) was 5 90 and it contained 284 mg Pb kg^{-1} A leaf compost, collected from a rural location near Ithaca, air dried, and ground in a Waring blender, was used as an organic matter amendment The pH ($CaCl_2$) of the leaf compost was 7 33 and it contained 379 g C kg^{-1} and 18 mg Pb kg^{-1}

For each soil and organic matter treatment, 25 g (dry soil equivalent) of moist soil (30 5 g moist) was added to 250-mL polyethylene bottles with 50 mL of a 0 01 *M* KNO_3 solution and the organic matter treatment Soil organic matter was increased through the additions of 5 and 15% (w/w) of leaf compost (1 25 and 3 75 g dry) The organic matter reductions were made by adding 1, 4, and 10 mL of 30% H_2O_2 The bottles were then shaken on a reciprocal shaker for 20 min every 12 h After 48 h, aliquots of HNO_3 and KOH were added to generate a pH range between 3 and 8

The OM-enriched and -depleted soil suspensions were then shaken every other day for 10 min at 200 rpm on a reciprocal shaker When not shaking, the bottles were left covered but not closed, allowing gas exchange After 40 d, the samples were centrifuged at 15 000 × *g* to separate the supernatants The pH and the electrolytic conductivity were determined in the supernatant before filtration The solutions were then passed through 0 22-μm cellulosic membranes, and analyzed for labile Pb by DPASV The extracts were made 1 m*M* Na_4EDTA (ultrapure grade) to preserve the samples before dissolved Pb was determined by graphite furnace atomic absorption spectrometry with a Varian Zeeman SpectrAA instrument (Varian Associates, Sunnyvale, CA) The organic C

Abbreviations: DOM, dissolved organic matter, DPASV, differential pulse anodic stripping voltammetry

Table 1. The stability constants (log K) for calculations of free Pb^{2+} ion activity in soil solution (Lumsdon et al., 1995; Smith and Martell, 1989, p. 643).

Equation	log K
$Pb^{2+} + H_2O \rightleftharpoons PbOH^+ + H^+$	−7.71
$Pb^{2+} + 2H_2O \rightleftharpoons Pb(OH)_2^0 + 2H^+$	−17.12
$Pb^{2+} + 3H_2O \rightleftharpoons Pb(OH)_3^- + 3H^+$	−28.06
$Pb^{2+} + HCO_3^- \rightleftharpoons PbHCO_3^+$	3.45
$Pb^{2+} + CO_3^{2-} \rightleftharpoons PbCO_3^0$	6.27
$Pb^{2+} + 2CO_3^{2-} \rightleftharpoons Pb(CO_3)_2^{2-}$	9.49
$Pb^{2+} + Cl^- \rightleftharpoons PbCl^+$	1.58
$Pb^{2+} + SO_4^{2-} \rightleftharpoons PbSO_4^0$	2.62
$Pb^{2+} + NO_3^- \rightleftharpoons PbNO_3^+$	1.17

content of the treated soils was measured using Walkley–Black titrations (Nelson and Sommers, 1982) and DOM was estimated by colorimetry by calibrating the absorbance of the extracts at λ = 254 nm with soil extracts of known DOM (Moore, 1985)

The DPASV analysis yields an estimate of the concentration of labile Pb in the soil solution (Florence, 1986). Assuming that the DPASV-labile pool represents inorganic species and excludes organic complexes, the activity of Pb^{2+} ions can be calculated using known association constants by partitioning the DPASV-labile inorganic Pb into various potential ion pairs and free ionic Pb^{2+} (Table 1) Carbonate equilibrium is calculated assuming equilibrium with atmospheric CO_2 The free Pb^{2+} activity calculated in this way is probably an overestimate, especially at near-neutral pH and above, because it does not account for weak labile organo–Pb complexes

RESULTS AND DISCUSSION

Soil Organic Matter

The soil organic matter treatments resulted in an increase in soil organic matter to 42 7 and 83.7 g C kg^{-1} after the addition of 5 and 15% (w/w) leaf compost The control contained 29 7 g C kg^{-1}, and the three H_2O_2 treatments (1, 4, and 10 mL) resulted in 28 1, 26 6, and 25.6 g C kg^{-1}, respectively. The highest H_2O_2 treatment resulted in a marginally lower soil organic matter content with highly variable and divergent solution properties. This treatment was therefore not included with the interpretation of the data set. The unusual behavior of the high-H_2O_2 treatment may be due to transformations of mineral components and especially the particle surfaces of the soil (Shuman, 1983). However, the levels of H_2O_2 utilized here are below what is usually used to remove soil organic matter (Lavkulich and Wiens, 1970, Omueti, 1980)

The DOM varied between 7 5 and 88.3 mg C L^{-1} and follows a simple relationship with the soil organic matter content and pH (Fig. 1). The DOM increased with higher soil organic matter and also with pH above ≈7. The highest soil OM treatment also showed an increase in solubility at lower pH (quadratic function with a minimum at pH ≈ 6 5) Overall, the DOM is related to total soil organic matter and pH by the function

$$DOM = -30\,83pH + 2\,54pH^2 + 14\,89SOM + 68\,20$$
$$R^2 = 0\,965^{***},\ n = 54 \qquad [1]$$

where DOM is in milligrams C per liter and SOM is soil organic matter in grams C per kilogram

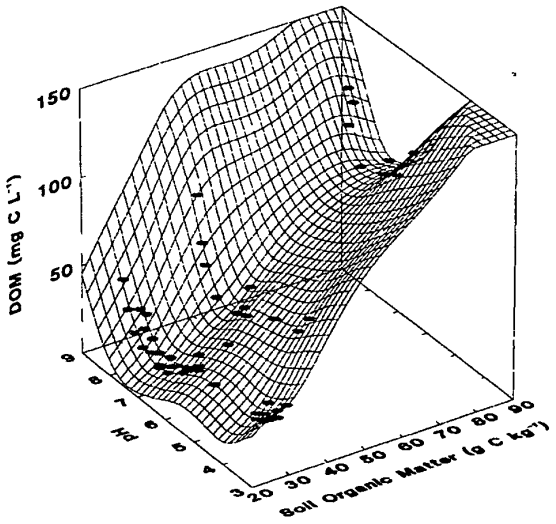

Fig. 1. Dissolved organic matter (DOM) as a function of pH and soil organic matter. The graph surface is the result of a distance-weighted least-square smoothing technique (Wilkinson, 1992, p. 750).

Free Lead(II) Activity

The calculated activity of free Pb^{2+} ions in the soil solutions, after equilibration, varied between 10^{-5} M at pH 3 and 10^{-10} M at pH 7 to 8 (Fig. 2). Figure 2 also shows that DOM had little or no effect on Pb^{2+} activity below pH 6 In contrast, at higher pH it appeared to cause a significant increase in activity. The surface of Fig 2 can be represented by a nonlinear regression (Eq [2]) showing that the free Pb^{2+} activity can be predicted

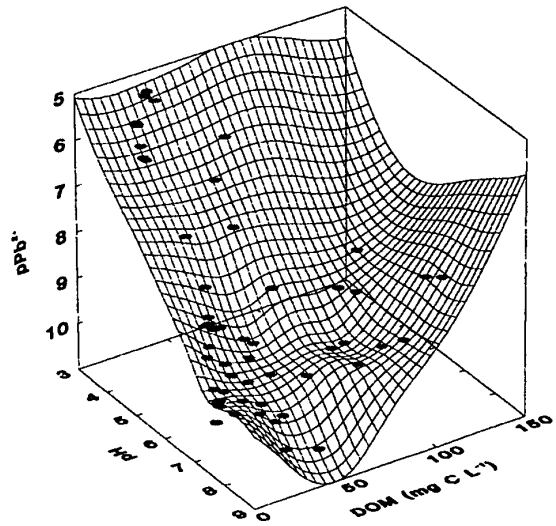

Fig. 2. Free Pb^{2+} activity (pPb^{2+}) as a function of pH and dissolved organic matter (DOM). The graph surface is the result of a distance-weighted least-square smoothing technique (Wilkinson, 1992, p. 750).

using quadratic terms for pH ($P < 0.001$) and a linear term for dissolved organic C ($P < 0.01$)

$$pPb^{2+} = 2.44pH - 0.139pH^2 - 0.058DOM - 0.79$$
$$R^2 = 0.900^{***}, n = 54 \quad [2]$$

where pPb^{2+} represents the negative log activity of free Pb^{2+}. Although the increase in the estimated free Pb^{2+} activity at higher pH is probably an artefact due to the sensitivity of DPASV to increasing concentrations of labile organo–Pb pairs in soil solution, this is nevertheless a significant rise in labile Pb, suggesting a concomitant increased mobility and reactivity of Pb in soil solutions at higher pH.

Dissolved Lead

The pH-dependent solubility of Pb varies from 3.6 μg Pb L^{-1} at neutral pH to 10 400 μg Pb L^{-1} under strongly acidic conditions. For comparison, most drinking water remediation criteria are around 10 μg L^{-1} (Subcommittee on Environmental Quality Criteria for Contaminated Sites, 1991). As with pPb^{2+}, the relationship of the logarithm of the dissolved Pb to pH is nearly linear until pH ≈ 6, but flattens at higher pH (Fig. 3). The resulting nonlinear regression is similar to Eq. [2], with a significant quadratic pH effect ($P < 0.001$) and a linear OM component ($P < 0.01$).

Dissolved Pb =

$$2.18\ pH - 0.14pH^2 - 0.024DOM - 1.05$$
$$R^2 = 0.967^{***}, n = 54 \quad [3]$$

where dissolved Pb is in units of $-\log M$.

In this case, the near-constant or increasing Pb solubility at high pH can be attributed to the increasing proportion of Pb hydroxy and carbonato species and especially Pb–OM complexes in solution. Although the data presented graphically appear to show a small solubility increase at high pH on the logarithmic scale, the absolute solubility increase is quite significant. This trend is attributed to increasing dissolved organic C, which causes an increase in the concentration of soluble Pb (Fig. 3). At pH 8, quadratic regressions for each separate treatment show that the solubility increases with soil organic matter content (data not shown). This suggests that soil organic matter has the potential to increase the solubility of Pb in neutral-pH soils through the formation of organo–Pb complexes. Although Pb complexed with DOM has unknown bioavailability, it is certainly mobile and prone to leaching (Miller and Friedland, 1994).

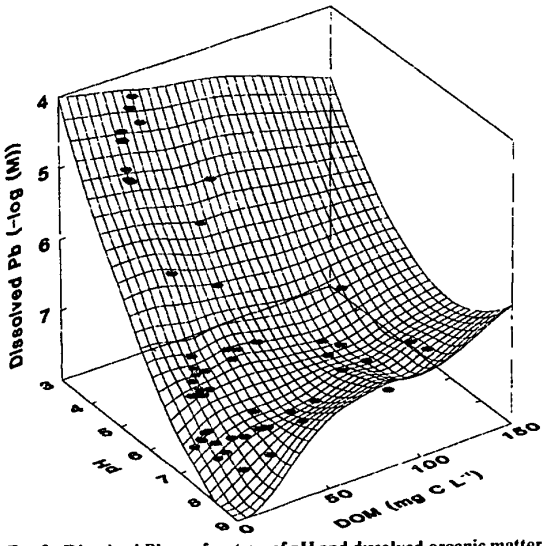

Fig. 3. Dissolved Pb as a function of pH and dissolved organic matter (DOM). The graph surface is the result of a distance-weighted least-square smoothing technique (Wilkinson, 1992, p. 750).

Free Lead(II) vs. Total Dissolved Lead

The relative importance of free Pb^{2+} relative to dissolved Pb can be determined by comparing the organically complexed (calculated from DPASV-labile Pb) to total dissolved Pb. This reveals that 30 to 50% of dissolved Pb is present in DOM complexes at low pH, and typically up to 80 to 99% at near-neutral pH (Fig. 4). No trends or effects from the several treatment levels of OM are apparent, although the high variability in the measurements may have obscured such effects. These results demonstrate the importance of organo–Pb complexes, which comprise >90% of dissolved Pb in most of the soil solutions above pH 6, and as much as 50% in strongly acidic solutions. This means that in most agronomic situations, the bulk of the Pb in solution is likely to be present as organic complexes. This also invalidates some speciation schemes which have assumed that Pb is mostly associated with large particles (>0.45 μm) and that soil solutions subjected to centrifugation and micron filtration only contain inorganic ion-pair species (Jopony and Young, 1994).

Santillan-Medrano and Jurinak (1975) reported Pb^{2+} activities in soil solutions that were orders of magnitude higher than our results — but they loaded the soils with metal salts up to 100 000 mg Pb kg^{-1} dry soil (10% w/w). The discrepancy is therefore not surprising. Kalbasi

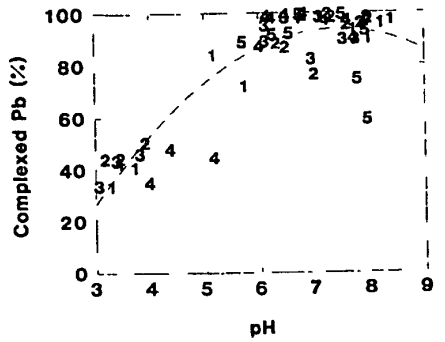

Fig. 4. The fraction of Pb in solution that is complexed with dissolved organic matter as a function of pH. The numbers 1 to 5 represent the soil organic matter levels (26.6, 28.1, 29,7, 42.7, and 83.7 g C kg^{-1}, respectively).

et al (1995), on the other hand, reported Pb^{2+} activities ranging from $10^{-7.21}$ to $10^{-9.37}$ in 14 soils with pH of 7.7 to 8 6 These activities are up to one log unit higher than our results, but their soils generally had a higher level of Pb contamination, from 152 to 2043 mg Pb kg^{-1}, compared with our soil containing 283 mg Pb kg^{-1}.

CONCLUSIONS

The DOM showed a simple relationship to soil pH and soil organic matter content The solubility of Pb in soil solutions was pH dependent, increasing as the pH was adjusted from 6 to 3 We observed no unequivocal effects of manipulating organic matter content in the acidic pH range. At near-neutral pH, the activity of Pb^{2+} showed no clear relationship to pH and a small but significant increase resulting from changing organic matter content. The estimated Pb^{2+} activities at pH > 6 show a linear increase with DOM, which may be an indication that labile Pb–organic matter complexes are creating a positive error in the estimate of free Pb^{2+} In the near-neutral pH range, higher soil organic matter content increases DOM, thereby promoting the formation of organo–Pb complexes and increasing Pb solubility

The speciation results demonstrate that most of the Pb in soil solutions is present as organo–Pb complexes. The results further reveal that increasing soil pH above 6.5 may actually increase the solubility of Pb, raising concerns for metal mobility. The increased lability of Pb at higher pH in a contaminated soil suggests a potential increase in bioavailability, although research is needed to evaluate the toxicity and possible bioaccumulation of Pb–organo complexes relative to free Pb^{2+}.

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Soil solution extraction techniques for microbial ecotoxicity testing: a comparative evaluation†

Tinnakorn Tiensing, Sara Preston, Norval Strachan and Graeme I. Paton*

Department of Plant & Soil Science, University of Aberdeen, Cruickshank Building, St Machar Drive, Aberdeen, UK AB24 3UU E-mail g i paton@abdn ac uk, Fax +44 (0)1224 272703, Tel +44 (0)1224273834

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The suitability of two different techniques (centrifugation and Rhizon sampler) for obtaining the interstitial pore water of soil (soil solution), integral to the ecotoxicity assessment of metal contaminated soil, were investigated by combining chemical analyses and a luminescence-based microbial biosensor Two different techniques, centrifugation and Rhizon sampler, were used to extract the soil solution from Insch (a loamy sand) and Boyndie (a sandy loam) soils, which had been amended with different concentrations of Zn and Cd The concentrations of dissolved organic carbon (DOC), major anions (F^-, Cl^-, NO_3^-, SO_4^{2-}) and major cations (K^+, Mg^{2+}, Ca^{2+}) in the soil solutions varied depending on the extraction technique used Overall, the concentrations of Zn and Cd were significantly higher in the soil solution extracted using the centrifugation technique compared with that extracted using the Rhizon sampler technique Furthermore, the differences observed between the two extraction techniques depended on the type of soil from which the solution was being extracted The luminescence-based biosensor *Escherichia coli* HB101 pUCD607 was shown to respond to the free metal concentrations in the soil solutions and showed that different toxicities were associated with each soil, depending on the technique used to extract the soil solution This study highlights the need to characterise the type of extraction technique used to obtain the soil solution for ecotoxicity testing in order that a representative ecotoxicity assessment can be carried out

Introduction

The assessment of soil quality is not possible based solely on chemical criteria It is now widely accepted that a risk assessment for soils should be based on the total content of pollutants and their mobile and bioavailable fractions.[1,2] It is therefore essential that chemical and ecotoxicological analyses are integrated in order that a more meaningful assessment of soil quality can be obtained

The extraction of the interstitial pore water of soil (soil solution) is an important step in an ecotoxicity assessment of soil. Soil solution chemistry is important for understanding the behaviour and fate of environmental pollutants, the availability of nutrients to plants and many other fundamental soil chemical processes The soil solution may be defined as the aqueous liquid phase of the soil and its solutes Under natural conditions, the soil solution composition varies as a function of time and position in the soil[3] Furthermore, the chemical composition of the soil solution also depends on the soil type, pH, ionic strength, ion complexation by ligands and ion competition[4] Several methods have been used to obtain soil solutions either in the field or in the laboratory[5,6] The methods commonly used to obtain soil solutions are based upon the principles of pressure, vacuum, displacement, and centrifugation, and are summarised in Table 1 However, there is a body of evidence showing that the chemical composition of soil solutions is influenced by the extraction technique used,[20,25,30] although how this may impact on an ecotoxicity assessment of soil has not yet been investigated

Assessing the bioavailable fraction of the pollutant in an ecotoxicity test requires the application of rapid, low cost, novel techniques The bioavailable fraction of metals in soils has been attributed to the free ionic species in the soil solution[31–34] Luminescence-based microbial biosensors have been shown to respond to the bioavailable fraction of metal pollutants in soil solutions,[31,35,36] and are therefore useful tools in a soil ecotoxicity assessment In a luminescence-based assay, bacterial luminescence is negatively correlated with an increase in the toxicity of a pollutant since bacterial luminescence is linked to electron transport[37] and therefore provides a measure of the metabolic activity of the cell

The aims of this study were to evaluate two widely used soil solution extraction techniques (centrifugation and Rhizon sampler) on the ecotoxicity assessment of two soils amended with Zn and Cd at different concentrations

Materials and methods

Soils

Two soils were used for this study, Boyndie, a loamy sand of the Boyndie series (Fragiorthod/Iron Polozol) and Insch, a sandy loam of the Insch series (Dystrochrept/Dystric Cambisol) These soils were sieved moist (< 3 mm), homogenised and then stored at 3–5 °C prior to use The characteristics of these soils are summarised in Table 2 Soil pH was determined using a 1 2 soil to soil water ratio

Metal amendment of soils

Triplicate samples of each soil were amended with $Zn(NO_3)_2$ $6H_2O$ (Sigma, Dorset, UK) and $Cd(NO_3)_2 \cdot 4H_2O$ (Aldrich, Dorset, UK) to obtain total metal concentrations of 75, 150 and 300 mg kg^{-1} dry weight for Zn and 1 5, 3 and 6 mg kg^{-1} dry weight soil for Cd These concentrations represented 50%, 100% and 200% of the maximum metal concentrations currently permitted under the UK sewage

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Table 1 Summary of the techniques commonly used for extracting soil solutions

Method	Summary	Reference
Column displacement/miscible displacement	Requires a large sample volume Slow sample turn-around Not suitable for soils with a sandy and loamy sand texture Special skill is required in packing the column	7, 8
Vacuum displacement	Modification of the column displacement method Involves the use of a vacuum extractor to obtain soil solution Lower time requirement and faster sample turn-around	9, 10
Immiscible displacement	Displacing solution is an organic solvent Alters the chemical characteristics and speciation of compounds in the soil solution Displacing solutions are highly toxic to soil flora and fauna Unsuitable for ecotoxicity testing	11–13
Centrifugation	Requires a sample holding device that enables the solution to be isolated from the soil Reliable and rapid technique Low cost Easy to use and requires no special skill Non-destructive method Can be applied in conjuction with the displacement method	5, 14–20
Suction technique	*Suction cup*— Requires a cup made from a porous material (commonly ceramic, aludum or Teflon) Non-destructive method Preconditioning of the surface of the suction cup is required to avoid strong sorption effects High cost of some items (*e g*, Teflon cups)	6, 21–25
	Porous plastic tube— Inert nature and small pore size (< 0 2 μm) of plastic tubing ensures no sorption problems or microbial and colloidal contamination Non-destructive method Low cost	26–29

sludge guidelines.[38] Control soils were those which were not amended with metals The soils were raised to 50% of their water holding capacity (WHC) with distilled water, placed in plastic pots, and left to equilibrate for 30 days.

Soil solution extraction techniques

All glassware and plastic was washed in 1% HNO_3 and rinsed twice in distilled water prior to use All soils were raised to 80% WHC with distilled water before the soil solution was extracted The following techniques were used for the extractions

Centrifugation. A modification of the centrifugation technique of Adams *et al*[7] was used to collect the soil solution The extraction unit consisted of a sample container cup, a solution collection cup, a filter, and a plastic bag to collect the solution A plastic bag (12 cm × 8 cm) was put inside the container cup A soil sub-sample (150 ± 0.1 g) was placed onto Whatman No 42 filter paper in the sample container cup, which was connected to the collection cup The soil solution was extracted by centrifuging for 1 h at 4 °C and 1818*g* in an MSE Coolspin 2 centrifuge. The soil solution was collected into a plastic bag, transferred to a clean glass bottle and stored at 4 °C prior to analysis

Table 2 Soil characteristics

	Boyndie	Insch
Series	Boyndie	Insch
Texture	Loamy sand	Sandy loam
Sand (%)	79 1	57 7
Silt (%)	14 3	30 79
Clay (%)	6 53	11 51
Organic matter (%)	3 57	3 75
pH	5 5	6 1
WHC[a] (%)	44	69

[a]WHC, water holding capacity

Rhizon sampler. Rhizon soil moisture samplers were obtained from Rhizosphere Research Products (Wageningen, The Netherlands) These samplers consisted of a 10 cm length of an inert porous polymer tube, capped at one end with nylon, and attached to a 10 cm length of a poly(vinyl chloride) (PVC) tube at the other end A 15 cm stainless-steel strengthening wire is present inside the porous polymer tube and the PVC tube is joined to a female Luer lock The Rhizon samplers were washed by forcing 50 ml of 1% HNO_3 through the probe followed by 50 ml of distilled water, and then drying before use A soil sub-sample (225 g dry weight) from each pot was placed into a plastic container and a small glass rod used to make a small primary hole in the soil prior to the Rhizon samplers being gently inserted The soil solution was extracted over a 24 h period by applying a suction pressure using a syringe connected to the Luer lock The soil solution collected in the syringe was transferred to a glass bottle and stored at 4 °C prior to analysis

Chemical analysis and metal speciation

The pH values of the soil solutions were measured immediately using a standard pH electrode (Hanna HI 8424, Norlab Instrument Ltd, Aberdeen, UK) The DOC was measured using an automated Labtoc UV digestion analyser (Pollution and Process Monitoring, Kent, UK) The concentrations of F^-, Cl^-, NO_3^-, SO_4^{2-} were determined by ion chromatography (Dionex Series 4500I, Dionex UK Ltd, Surrey, UK) The separation column used was an Ionpac A54 ASC ion exchange column with a pre-guard column and the mobile phase was 1 7 mmol $NaCO_3^{-1}$ mmol $NaHCO_3$ Sub-samples of

the soil solution were acidified with 1% HNO_3 and used to determine the total concentrations of various metals Total concentrations of Mg, Ca and Zn were determined by flame atomic absorption spectrometry (FAAS, Alpha Models 4, Baird Atomic Ltd, UK), K by flame atomic emission spectrometry (FAES, Alpha Models 4) and Cd by graphite furnace atomic absorption spectrometry (GFAAS, Perkin-Elmer 3300, Perkin-Elmer, Buckinghamshire, UK) The last of these involved the addition of 0 2 mg PO_4^{3-} as a matrix modifier

The concentrations of Zn^{2+} and Cd^{2+} were determined using the method described by Holm *et al*[39] The method involved measuring the total concentration of metal before and after equilibrium with a calcium-saturated cation exchange resin Ground and sieved Amberlite IR120 plus (100–200 mesh) cation exchange resin (Sigma) was used and converted from the Na-form to the Ca-form by the sequential addition of 1000 ml of 1 M $Ca(Ac)_2$, 0 01 M $Ca(Ac)_2$, and 0 01 M $Ca(NO_3)_2$ An aliquot (100 mg) of Amberlite IR120 was weighed into a 50 ml polypropylene centrifuge tube and 10 ml of soil solution added The centrifuge tube was placed on an orbital shaker for 24 h at 25 °C Sub-samples of the supernatant were then acidified with 0 3% HNO_3 and the concentration of Zn and Cd remaining in the soil solution determined A reference solution was prepared with the same final concentrations of Ca, Zn and Cd as the soil solutions and treated in the same way as the soil solutions The proportions of Zn^{2+} and Cd^{2+} were calculated by comparison with the reference solution as described by Holm *et al*[39]

Soil ecotoxicity assessment

The toxicity of the metals was determined using the luminescence-based microbial biosensor *Escherichia coli* HB101 pUCD607 *E coli* HB101 was marked with the *lux* CDABE genes, isolated from *Vibrio fischeri*, using the multicopy plasmid pUCD607.[40] Cultures of *E coli* HB101 pUCD607 were freeze-dried using standard protocols[41] and stored at −20 °C Freeze-dried cultures were resuscitated by resuspending the cells in 10 ml of 0 1 M KCl and incubating at 25 °C for 1 h with shaking Aliquots (900 µL) of each soil solution were transferred to luminometer cuvettes (Clinicon, Petworth, West Sussex, UK) and a 100 µL aliquot of the resuscitated cells added and mixed into each sample at 15 s intervals The luminescence of the samples was measured after a 15 min exposure time using a 1 s measurement on a Bio-Orbit 1253 luminometer (Labtech International, Uckfield, UK) All the assays were carried out in triplicate The luminescence of the samples was expressed as a percentage of the luminescence of the control samples

Statistical analyses

Two-way analyses of variance (ANOVA) were carried out on the data using the statistical package Minitab for Windows, release 12 1 (State College, PA, USA) Significant differences between treatments were elucidated using least significance difference (LSD) values The relationship between the free and the total metal concentrations in the soil solutions was determined using linear regression analyses on log-transformed values The response of the biosensor to the total and free metal concentrations in the soil solutions was determined using non-linear regression analyses Sigmoid functions were fitted to the biosensor data and EC_{25} (effective concentration causing a 25% decrease in luminescence) and EC_{50} (effective concentration causing a 50% decrease in luminescence) values calculated All regression analyses were performed using SigmaPlot for Windows version 5 (Jandel Corporation, CA, USA)

Results and discussion

Chemical analyses

The soil solution extracted from the Boyndie soil using the centrifugation technique had a higher pH than the soil solution extracted using the Rhizon sampler technique (Table 3) The opposite was true for the Insch soil, the pH of the soil solutions extracted using the centrifugation technique being lower than those extracted using the Rhizon sampler technique (Table 3) The higher the metal addition applied the lower the pH of the soil solution compared with control soils, irrespective of the extraction technique employed (Table 3) The concentration of DOC was significantly ($p<0.05$) higher in soil solutions extracted using the centrifugation technique over those extracted using the Rhizon sampler technique, for both Boyndie and Insch soils (Table 3) Overall, there was a significant ($p<0.05$) increase in the concentrations of K, Mg and Ca with an increase in the level of metal added (Table 3) This is not surprising as the Zn and Cd ions in solution would have exchanged with ions such as K, Mg and Ca, which were absorbed onto the surfaces of soil particles. The concentrations of K, Mg and Ca were significantly ($p<0.05$) higher in soil solutions extracted using the centrifugation technique compared with those extracted using the Rhizon sampler technique

The concentration of F^- in the soil solutions was, overall, not significantly different between the two extraction techniques (Table 4) Overall, the concentration of Cl^- was significantly higher in soil solutions extracted using the centrifugation technique compared with those extracted using the Rhizon sampler technique, for both Boyndie and Insch soils (Table 4) Overall there was a significant ($p<0.05$) increase in the concentration of NO_3^- in the solutions extracted from soils which were amended with high levels of metals using the centrifugation technique compared with those extracted using the Rhizon sampler technique (Table 4) However, the concentrations of NO_3^- in the solution extracted from soils amended with no metals were significantly ($p<0.05$) higher using the Rhizon sampler technique compared with those extracted using the centrifugation technique (Table 4)

Table 3 Soil solution pH and concentrations of DOC, K, Mg, and Ca

		Boyndie soil solution conc /mg L^{-1}					Insch soil solution conc./mg L^{-1}				
Extraction technique	% of metal conc limit	pH	DOC	K	Mg	Ca	pH	DOC	K	Mg	Ca
Centrifugation	0	6 49	28 60	0 67	5 60	26 18	5 12	18 15	7 04	7 95	78 84
	50	5.81	17 35	16 46	29 69	89 23	4 85	12 85	17 77	16 40	145 99
	100	5 77	15 15	23 86	43 18	186 81	4 58	13.50	28 64	23 26	202 03
	200	5 50	26 65	36 61	42 43	213 14	4 43	17 53	41 39	36 70	314 35
Rhizon sampler	0	6 26	30 10	1 25	7 14	19 90	5 74	9 53	10 96	4 34	38 50
	50	5 30	10 65	21 68	34 95	112 90	5 48	6 75	24 87	17 33	142 61
	100	5 44	10 50	28 20	42 53	175 70	4 80	8 63	32 12	22 92	193 82
	200	4 48	17 60	40 81	43 52	237 29	4 87	9 35	44 43	36 05	295 99
	LSD[a]	ND[b]	2 38	1 99	6 74	10 38	ND[b]	2 50	1 62	0 93	12 24

[a]LSD, least significant difference ($p=0.05$) [b]ND, not determined

Table 4 Soil solution anion concentration

Extraction technique	% of metal conc limit	Boyndie soil solution conc /mg L^{-1}				Insch soil solution conc /mg L^{-1}			
		F^-	Cl^-	NO_3^-	SO_4^{2-}	F^-	Cl^-	NO_3^-	SO_4^{2-}
Centrifugation	0	0 11	47 46	163 90	5 86	<DL[a]	15 41	182 01	6 15
	50	0 29	41 51	648 08	3 59	<DL[a]	18 61	690 15	2 01
	100	0 29	40 88	1082 97	0 94	<DL[a]	14 61	909 54	1 62
	200	1 15	41 58	1863 32	2 16	0 36	18 83	1319 13	1 64
Rhizon sampler	0	0 17	44 80	636 90	7 66	<DL[a]	12 20	737 33	6 53
	50	0 39	35 94	670 04	3 34	<DL[a]	16 23	760 25	2 21
	100	0 34	33 78	1011 07	1 33	<DL[a]	11 98	903 89	0 71
	200	0 82	39 12	1292 62	5 65	0 37	14 48	1064 50	7 31
	LSD[b]	0 17	3 26	25 20	0 65	ND[c]	1 36	94 13	0 60

[a]DL, detection limit [b]LSD, least significant difference ($p = 0\ 05$) [c]ND, not determined

Table 5 Soil solution concentration of total Zn, Cd, Zn^{2+} and Cd^{2+}

Extraction technique	% of metal conc limit	Boyndie soil solution conc./mg L^{-1}				Insch soil solution conc /mg L^{-1}			
		Zn	Zn^{2+}	Cd	Cd^{2+}	Zn	Zn^{2+}	Cd	Cd^{2+}
Centrifugation	0	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]
	50	13 19	8 61	0 0475	0 0291	3 82	0 78	0 0073	0 0062
	100	26 16	21.21	0 1150	0 0682	9 28	4 68	0 0199	0 0163
	200	74 00	66 42	0 6400	0 4333	45 47	36 37	0 2370	0 1493
Rhizon sampler	0	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]	<DL[a]
	50	3 04	1 01	0 0182	0 0114	1 82	0 51	0 0084	0 0026
	100	17 98	8 36	0 0805	0 0463	3 14	0 88	0 0177	0 0091
	200	66 38	48 05	0 4477	0 3520	11 22	4 35	0 0379	0 0167
	[b]LSD	5 47	7 16	0 0697	0 0651	8 16	9 67	0 0331	0 0179

[a]DL, detection limit [b]LSD, least significant difference ($p = 0\ 05$)

The concentrations of SO_4^{2-} in the soil solutions extracted using the Rhizon sampler technique were generally significantly ($p < 0\ 05$) higher than those extracted using the centrifugation technique (Table 4) The significant increase in the concentration of nitrate in soil solutions with an increase in the level of metal added reflected the fact that the metals were added as nitrate salts (Table 4)

Metals and their speciation

The concentrations of Zn and Zn^{2+} in soil solutions extracted from Boyndie soils using the centrifugation technique were significantly ($p < 0\ 05$) higher than those extracted using the Rhizon sampler technique (Table 5) The concentrations of Zn and Zn^{2+} in soil solutions extracted from Insch soils using the centrifugation technique, compared with those extracted using the Rhizon sampler technique, were only significantly ($p < 0\ 05$) higher in soil amended with 300 mg kg^{-1} Zn (200% of the metal concentration limit) (Table 5) The concentrations of Cd and Cd^{2+} in Insch and Boyndie soil solutions extracted using the centrifugation technique were only significantly higher ($p < 0\ 05$) than those extracted using the Rhizon sampler technique in soils amended with 6 mg kg^{-1} Cd (200% of the metal concentration limit) (Table 5)

There was a linear increase in the concentration of Zn^{2+} in the soil solution with an increase in total Zn in the soil solution, irrespective of the extraction technique employed (Fig 1a) A similar relationship between the Zn^{2+} and total Zn in the soil solutions was observed by Chaudri *et al* [35] The concentration of Cd^{2+} in the soil solution also increased linearly with an increase in the total Cd in the soil solution, irrespective of the extraction technique used (Fig 1b)

Soil ecotoxicity assessment

The toxicity of aqueous solutions of Zn and Cd to the *E coli* HB101 pUCD607 biosensor are of the same order of magnitude (data not shown) The concentrations of Cd in the soil solutions were two orders of magnitude lower than the concentrations of Zn in the soil solutions, therefore the response of the biosensor could be attributed solely to the presence of Zn in the soil solutions (Table 5) Since the biosensor was responding to the concentration of Zn in the soil solutions, irrespective of the extraction technique used, it was possible to combine the toxicity data obtained using both extraction techniques for each soil Single relationships showing the decrease in the luminescence of the biosensor with an increase in the concentrations of total Zn and Zn^{2+} in the soil solutions were therefore obtained for Boyndie and Insch soils (Figs 2 and 3, respectively) The toxicities of the soil solutions extracted using the centrifugation technique were generally greater due to the higher concentrations of Zn present (Figs 2 and 3) The latter was particularly apparent for Insch soil, in which the luminescence of the biosensor did not even drop to half its control level (Fig 3)

The non-linear regression analyses showed that the concentrations of Zn^{2+} and total Zn in Boyndie soil solution accounted for 81 and 85% of the variance in the luminescence response of the biosensor, respectively (Fig 2a and 2b) The concentrations of Zn^{2+} and total Zn in Insch soil solution accounted for 96 and 97% of the variance in the luminescence response of the biosensor, respectively (Fig 3a and 3b) The EC_{25} and EC_{50} values for Zn and Zn^{2+} were of the same order of magnitude as those quoted by Chaudri *et al* [35] (Table 6)

Evaluation of extraction techniques

An ideal soil solution extraction technique would enable the solution phase to be extracted from the solid phase in sufficient quantities for the desired chemical and biological assessment There are a number of extraction techniques available and their advantages and disadvantages have been summarised (Table 1) In this study, it was shown that the composition of the soil solution extracted using the centrifugation technique

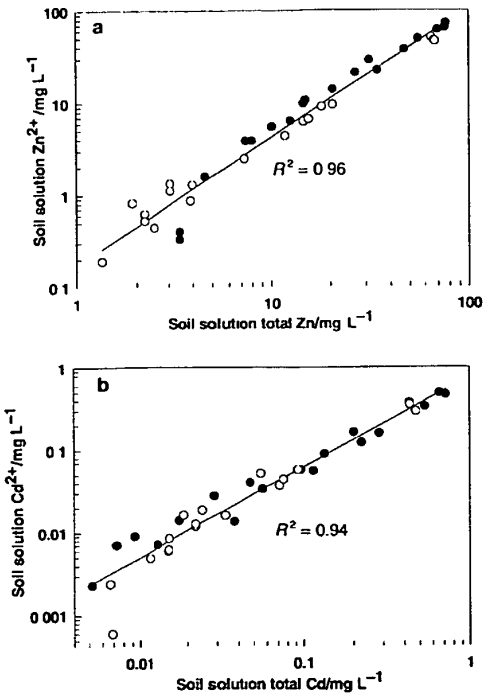

Fig. 1 Relationship between Zn^{2+} and total Zn (a), and Cd^{2+} and total Cd (b) in soil solution extracted using different techniques ●, centrifugation and ○, Rhizon sampler

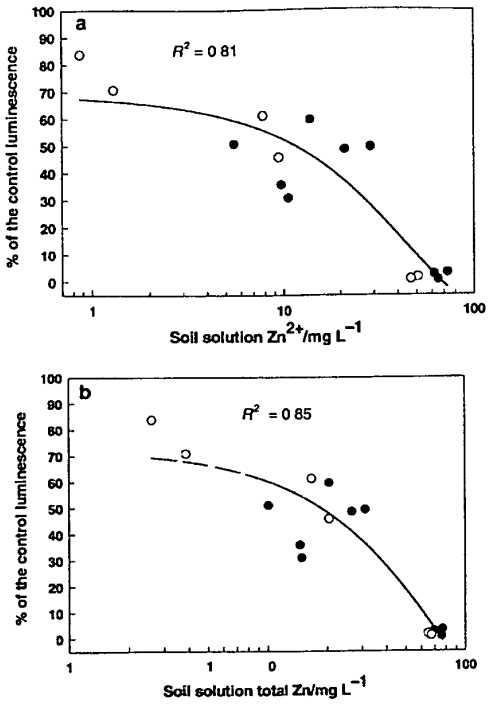

Fig. 2 Relationship between the luminescence of *E coli* HB101 pUCD607 (expressed as a percentage of the control) and Zn^{2+} (a) and total Zn (b) in soil solution extracted from Boyndie soil using different techniques ●, centrifugation and ○, Rhizon sampler

was significantly different from that derived by the Rhizon sampler technique In assessing a suitable extraction technique the following key attributes were considered important (a) the technique should enable the extraction of a representative soil solution, (b) sample preparation should be rapid, reproducible and simple to administer, (c) the technique should be reliable and inexpensive, and (d) there should be minimal adsorption of material onto the surfaces of the equipment

The centrifugation technique resulted in a soil solution with a higher concentration of Zn and Cd than the Rhizon sampler technique These differences are certainly due to the mechanism involved in extracting the soil solution In the centrifugation technique a variable matric potential, which is a function of the force of centrifugation, is imposed on the sample to extract the solution from a broad range of pore sizes However, in the Rhizon sampler technique, a constant matric potential lower than that of centrifugation is used to extract the soil solution Soil water is removed from the pores and the physical structure of the sample remains intact, hence the desorption/sorption characteristics of the matrix are controlled.

One of the advantages of the Rhizon sampler technique, in comparison to centrifugation, is that it can be successfully applied in the field Furthermore, the Rhizon sampler technique has been described as mimicking the physical aspects of plant uptake, with the porous plastic rod inserted into the soil acting like a root The Rhizon sampler technique is particularly applicable where a large number of samples need to be extracted simultaneously since the labour and equipment requirements would be less than for the centrifugation technique On this basis, the Rhizon sampler technique is a more versatile extraction technique and more suitable for determining the bioavailable fraction of metals in soils

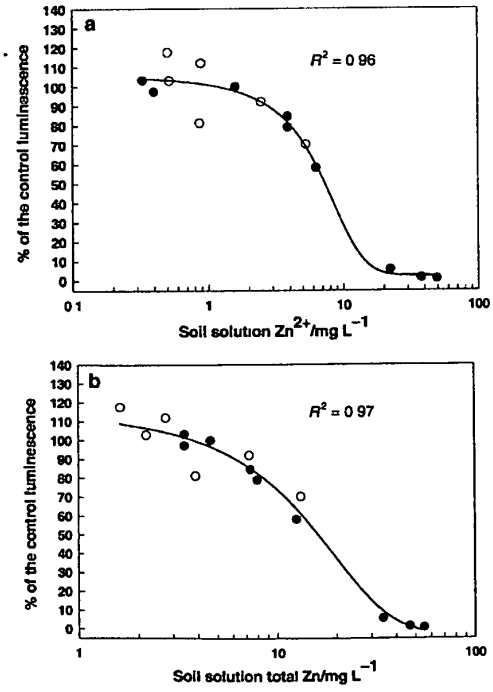

Fig. 3 Relationship between the luminescence of *E coli* HB101 pUCD607 (expressed as a percentage of the control) and Zn^{2+} (a) and total Zn (b) in soil solution extracted from Insch soil using different techniques ●, centrifugation and ○, Rhizon sampler

Table 6 Critical Zn values resulting in a decrease in luminescence of the *E coli* HB101 pUCD607 biosensor

	Zn^{2+}/mg L^{-1}		Total Zn/mg L^{-1})	
Soil solution	EC_{25}	EC_{50}	EC_{25}	EC_{50}
Boyndie	NA[a]	11 52	NA[a]	18 62
Insch	3 87	5 41	6 11	6 62

[a]NA, not available

Conclusion

There is an urgent requirement to assess the efficiency of techniques used to extract interstitial pore waters from soils, both for nutrient and toxicity assessment In this study it was shown that soil solutions extracted using centrifugation had different chemical and ecotoxicological characteristics to soil solutions extracted using a Rhizon sampler Furthermore, the trends observed between the two extraction methods differed between soils It is therefore crucial that the method used to extract the soil solution is carefully considered prior to estimating the level of pollution in soil The application of ecotoxicity tests to soil solutions can only be adequately interpreted after a comprehensive understanding of the benefits and limitations of the extraction techniques commonly used to obtain them has been achieved

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Assessment of *lux*-marked *Pseudomonas fluorescens* for reporting on organic carbon compounds

Catrin V Yeomans [a] *, Fiona Porteous [a], Eric Paterson [b], Andrew A Meharg [c], Kenneth Killham [a]

[a] *Department of Plant and Soil Science University of Aberdeen Aberdeen AB24 3UU UK*
[b] *Macaulay Land Use Research Institute Craigiebuckler Aberdeen AB15 8QH UK*
[c] *Institute of Terrestrial Ecology Monks Wood Abbots Ripton Huntingdon PE17 2LS UK*

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Abstract

Carbon-flow from plant roots to the rhizosphere provides a major source of nutrients for the soil microbial population However, quantification of carbon-flow is problematic due to its complex composition This study investigated the potential of *lux*-marked *Pseudomonas fluorescens* to discriminate between forms of carbon present in the rhizosphere by measuring the light response to a range of carbon compounds Results indicate that bioluminescence of short-term carbon-starved *P fluorescens* is dependent upon the source and concentration of carbon This system, therefore, has the potential to both quantify and qualify organic acids present in rhizodeposits

Keywords Bioluminescence, C-flow *Pseudomonas fluorescens* Rhizosphere Starvation

1. Introduction

The flow of organic compounds from living roots to the rhizosphere supports microbial communities that are typically more active, more abundant and have a distinct species composition, than those in non-rhizosphere soil [1] The wide range of compounds released from roots is termed rhizodeposition and includes exudates, secretions, sloughed cells and gases [2] C-flow is of fundamental importance to microbial activity, ecosystem functioning and agricultural productivity Accurate measures of flow of root derived C through the microbial biomass are, therefore, essential to our understanding of these processes [3]

The quantity and quality of rhizodeposition are affected by below ground conditions such as root impedance [4], nutrient status [5] and the presence of rhizosphere microbial populations [6] Microbial populations transform rhizodeposits, complicating interpretation of the origin of organic compounds Simple measurements of total soil respiration cannot, therefore, distinguish CO_2 due to microbial mineralisation of organic compounds from CO_2 due to root respiration

A number of approaches have been used to quantify rhizodeposition such as isotopic labelling tech-

* Corresponding author Tel +44 (1224) 273725, Fax +44 (1224) 272703, E-mail c yeomans@abdn ac uk

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niques involving ^{13}C and ^{14}C [7,8] which lead to approximations of total organic C inputs from roots [9], and bacterial N_2O production as an indication of bacterial metabolism [10].

Reporter gene technology offers the potential to assess C-flow through microbes. *Lux*-marked bacteria have been used to study root colonisation [11] and to report on the survival and metabolic activity of bacteria in soil [12]. Reporter bacteria which are selected to express their signal in direct proportion to their metabolic activity would be of great value in quantifying C flux through a microbial component associated with roots. After validation, using techniques such as $^{14}CO_2$ labelling, such reporters could provide a basis for partitioning respiratory CO_2 flux of plant fixed C into the contributions made by root respiration and mineralisation of rhizodeposits by rhizosphere microorganisms.

The aim of this study was to investigate the response of a *lux*-marked, ubiquitous, rhizosphere bacterium (*Pseudomonas fluorescens* 10586 pUCD607) to the principal components of rhizodeposition [13], and to whole root material collected from wheat plants. The potential use of such reporter systems to determine the quality and quantity of organic carbon in samples was assessed.

2. Materials and methods

2.1 Bacterial strain and growth conditions

P fluorescens 10586 pUCD607 (*lux*CDABE from *Vibrio fischeri*, *kan*^{r}, *amp*^{r}) [14] was grown in LB broth at 25°C, 200 rpm. LB agar plates contained 1% (w/v) bacteriological agar. Kanamycin was added (50 μg ml^{-1}) to the growth medium and agar plates.

2.2 Starvation conditions

Late exponential phase cells of *P fluorescens* 10586 pUCD607 were harvested by centrifugation (5 min, 10000×*g*), the supernatant decanted and cells resuspended in an equal volume of C-free M9 minimal medium (Difco) containing 50 μg ml^{-1} kanamycin. This process was repeated and the final cell suspension shaken (200 rpm) in C-free M9 containing 50 μg ml^{-1} kanamycin for 4 h at 25°C. The activity of the cells during short-term C starvation was monitored at 30 min intervals by removing samples for luminometry readings and removing samples every 60 min for dilution plate counts of colony forming units.

2.3 Carbon substrates

All solutions were prepared in C-free M9 minimal medium using acid washed glassware. Solutions of glucose, glutamic acid and succinic acid were prepared at concentrations ranging from 0.1–50 mM C. An extract of root material was also prepared from wheat root systems [15] that had been dried at 70°C, ground and stored at 4°C until required for use. A suspension of root material was made in C-free M9 medium (5.0 g l^{-1}), and filtered through 0.2 μm filters.

2.4 Chemical analysis of soluble wheat root carbon

The chemical composition of soluble wheat root C solution was analysed using anthrone to determine total reducing sugars [16], reverse phase HPLC for free amino acids (phenyl thiohydantoin derivatisation and spectrophotometric detection) and free organic acids (refractive index detection). Total organic carbon was measured using a Labtoc analyser (Pollution and Process Monitoring, Sevenoaks, UK).

2.5 Bioassay protocol

One ml aliquots of cells starved for 2 h were added to 9 ml of C solution in a sterile acid washed Universal bottle. The cells and C solution were thoroughly mixed and incubated at room temperature for 30 min. After 30 min, light output from 1 ml samples was recorded. Samples were also removed for serial dilution plate counts of colony forming units. All assays were carried out in triplicate.

2.6 Luminometry

Light output was measured using an automated BioOrbit 1251 luminometer (Labtech International, Uckfield, UK) with a Multiuse software package. Light output from 1 ml samples was integrated over a 10 s period and results recorded as the means

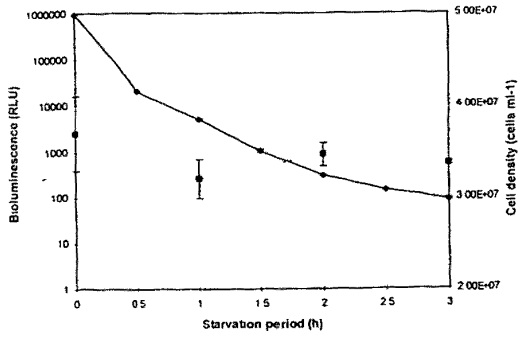

Fig 1 Bioluminescence (♦) and cell density (■) of log phase *P fluorescens* pUCD607 during starvation in carbon-free M9 medium Results are expressed as means of triplicates ± the standard error of the mean

of triplicate samples Luminescence was expressed as relative light units (RLU), 1 relative light unit being equivalent to 1 mV 10 s^{-1}

2 7 *Curve fitting*

Dose-response curves for percentage increase in bioluminescence at different concentrations of C substrate were fitted for each C substrate to the means of three replicates using a Michaelis–Menten rectangular hyperbola equation V_{max} and K_m were calculated for each response by non-linear regression ($y = Ax/K+x$) [17]

3. Results

3 1 *Starvation in M9*

The bioluminescence of *P fluorescens* 10586 pUCD607 decreased rapidly over a 4 h period when starved in C-free M9 medium Bioluminescence fell from an initial value of approximately 95 000 RLU to less than 100 RLU in 3 h (Fig 1) Cell number did not change over this time, indicating that the reduction in light output was due to a decrease in metabolic activity rather than cell death Cells starved for 2 h were used in bioassays

3 2 *Chemical analysis of soluble root material*

Reducing sugars comprised 93 5% of the soluble wheat root C, by mass balance The remaining 6 5% consisted of low molecular mass organic acids

3 3 *Response of P fluorescens 10586 pUCD607 to different carbon sources*

Cell number did not increase over the 30 min incubation period (data not shown), indicating that an increase in bioluminescence was due to increased levels of metabolic activity per cell in the microbial suspension Results are expressed as a percentage increase in bioluminescence above that of a control bioassay containing no added C source. All C sources tested resulted in an increase in bioluminescence of *P fluorescens* 10586 pUCD607 after 30 min incubation at room temperature The results show typical Michaelis–Menten kinetics, apart from those for glutamic acid which at low C concentrations exhibits a sigmoidal response Each C source gave a characteristic response, V_{max} and K_m value (Table 1) The response curve for light output from *P fluorescens* 10586 pUCD607 to the soluble wheat root C was similar, over the same concentration range, to that observed for glucose (Figs 2–5)

4. Discussion

The *lux*-marked *P fluorescens* 10586 pUCD607

Table 1
Light output of *P fluorescens* 10586 pUCD607 in the presence of different carbon sources

Carbon source	V_{max} (RLU)	K_m (mM)
Glucose	2913 ± 147	15 74 ± 1 95
Glutamic acid	2682 ± 3770	0 54 ± 0 22
Succinic acid	713 ± 4	0 26 ± 0 07
Soluble wheat root	2462 ± 277	11 04 ± 3 88

Results are expressed as means of triplicates ± the standard error of the mean

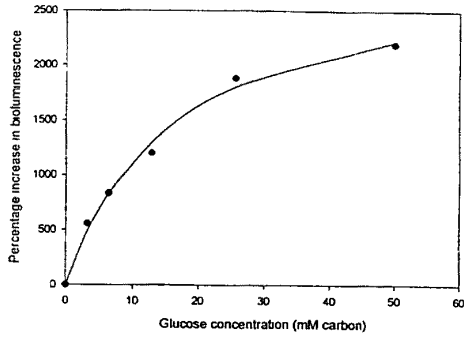

Fig 2 Bioluminescence response of log phase *P fluorescens* 10586 pUCD607 starved in carbon-free M9 minimal medium for 2 h to a 30 min exposure to varying concentrations of glucose at room temperature Results are expressed as means of triplicates (●) and the line shows the Michaelis–Menten equation fitted to these results

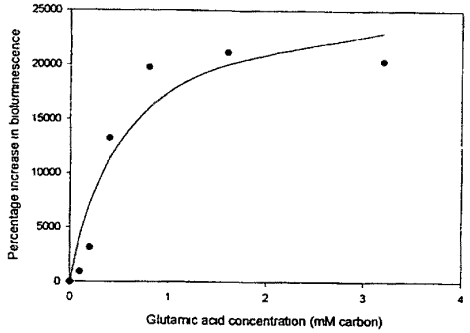

Fig 4 Bioluminescence response of log phase *P fluorescens* 10586 pUCD607 starved in carbon-free M9 minimal medium for 2 h to a 30 min exposure to varying concentrations of glutamic acid at room temperature Results are expressed as means of triplicates (●) and the line shows the Michaelis–Menten equation fitted to these results

provided a characteristic bioluminescence response to a sugar, an amino acid and an organic acid, demonstrating the potential of this system to differentiate between different forms of C Soluble wheat root C used in this experiment was considered to accurately reflect components of rhizodeposition [2], indicating that the system is applicable to actual samples of rhizodeposits. The light response to root C approximated that for glucose, which gave similar responses to those for sucrose and fructose (data not shown), suggesting that the composition of the soluble root C was predominantly reducing sugars This was verified by chemical analysis of the root material, indicating the potential of this system to both quantify and qualify rhizodeposition By comparing the bioluminescence response of *P fluorescens* 10586 pUCD607 obtained from rhizodeposits to calibration curves of a wide range of C compounds, the C composition of rhizodeposits could be elucidated

These preliminary results show that the *lux* rhizobacterial reporter system developed offers potential for investigation of rhizosphere C-flow, although fur-

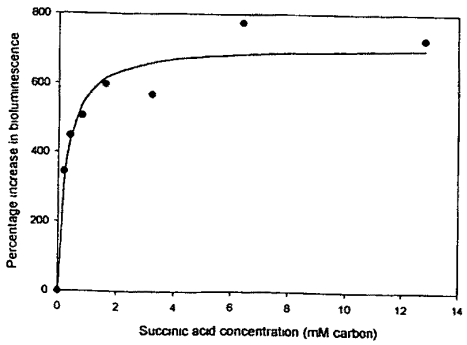

Fig 3 Bioluminescence response of log phase *P fluorescens* 10586 pUCD607 starved in carbon-free M9 minimal medium for 2 h to a 30 min exposure to varying concentrations of succinic acid at room temperature Results are expressed as means of triplicates (●) and the line shows the Michaelis–Menten equation fitted to these results

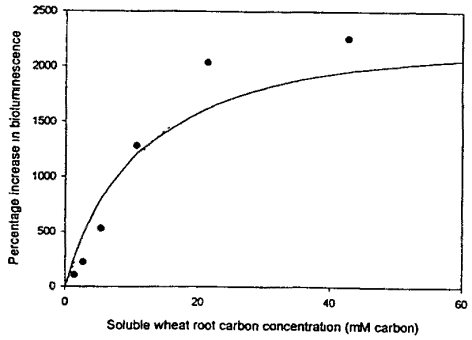

Fig 5 Bioluminescence response of log phase *P fluorescens* 10586 pUCD607 starved in carbon-free M9 minimal medium for 2 h to a 30 min exposure to varying concentrations of soluble wheat root carbon at room temperature Results are expressed as means of triplicates (●) and the line shows the Michaelis–Menten equation fitted to these results The result is compared to the Michaelis–Menten equation fitted to results obtained for glucose (dotted line)

ther in vitro studies are required to examine the effect of a wider range of C compounds and mixed C sources on bioluminescence In addition, the relationship between microbial assimilation of C compounds, respiration and light output should be examined

This novel technique to monitor C-flow through microbes can be exploited in several ways to lead to a greater understanding of C flux within the rhizosphere Changes in both the quantity and quality of rhizosphere C-flow affect microbial activity in soil [18] and although the effects of light intensity and CO_2 level on root exudation [19,20] have been investigated, qualitative changes in the concentration of sugars, amino acids and organic acids have not been measured Root exudates can be collected from plants grown under controlled conditions in microcosms [21], enabling the *P fluorescens* 10586 pUCD607 bioassay to determine the carbon composition of rhizodeposits harvested from plants grown under different environmental conditions The approach described also has the potential to quantify the impact of individual bacterial species on rhizosphere C-flow

Rhizobacteria could, theoretically, be tagged with a variety of genes allowing an even more powerful means of studying rhizosphere C fluxes with different reporters under the control of different promoters, although the genetic burden placed upon cells may, in practice, limit this approach The use of different reporters in conjunction with luciferase systems could produce rhizobacteria that express different, quantifiable, phenotypes in the presence of different forms and concentrations of C which would lead to much greater resolution of C-flow through the rhizosphere

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