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Metal Speciation and Toxicity in Sewage Sludge

Annika Ekvall

Department of Sanitary Engineering
Chalmers University of Technology

Chalmers University of Technology
Department of Sanitary Engineering

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Address:
Department of Sanitary Engineering
Chalmers University of Technology
S-412 96 Göteborg, Sweden

Some directions before you read this thesis

Certain readers may wish to only get a grasp of the contents of this thesis and can do so by following the chapter partitions, marked with a blue corner, and reading the précis. The more ambitious reader will look to the published work, marked with a green corner, for more depth.

This thesis contains three published articles and one, as yet, unpublished article as well as a comprehensive literature review. These articles are listed below:

1. Carlson-Ekvall, C. E. Annika and Morrison, Gregory M., Metals in sewage sludge: Species, fractions and biological relevance, *in preparation* (chapter 2).

2. Carlson-Ekvall, C. E. Annika and Morrison, Gregory M., Contact toxicity of metals in sewage sludge: Evaluation of alternatives to sodium chloride in the Microtox assay, *Environmental Toxicology and Chemistry*, **14**, 17-22, 1995 (chapter 3).

3. Carlson, C. E. Annika and Morrison, Gregory M., Fractionation and toxicity of metals in sewage sludge, *Environmental Technology*, **13**, 751-759, 1992 (chapter 4).

4. Carlson-Ekvall, C. E. Annika and Morrison, Gregory M., Metal partitioning and toxicity in sewage sludge, *in preparation* (chapter 4).

5. Carlson-Ekvall, C. E. Annika and Morrison, Gregory M., Toxicity of copper in the presence of organic substances in sewage sludge, *Environmental Technology*, **16**, 243-251, 1995 (chapter 4).

Article 1 can be considered as a complete background to this study and is therefore presented with a précis as chapter 2. Chapter 3 is the experimental section and incorporates article 2 with an extended summary. The bulk of the research is reported in chapter 4 with a focus on three aspects of metal speciation and toxicity in sewage sludge and covered in articles 3, 4 and 5 together with a short summary of the main findings.

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Chapter 1. Introduction

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Sewage sludge is a useful soil improver which contains valuable nutrients, such as phosphorus, nitrogen and organic material. It would be preferable if these nutrients could be recirculated to farmland. However, a limiting factor in the agricultural application of sewage sludge from urban areas is elevated concentrations of metals. Special attention has been paid to the presence of heavy metals in sewage sludge, partly because of their toxicity and partly because of their persistence in the environment. Many countries have therefore presented criteria for permissible concentrations of selected metals in sludge destined for spreading on farmland (Table 1.1). The Swedish directives are significantly more stringent than other countries, particularly with regard to Cd, and to some extent Cu and Pb.

Table 1.1. National and international directives for maximum allowed metal concentrations in sewage sludge applied to agricultural soils.

	Maximum allowed metal concentrations in µg g^{-1} TS*			
	Cd	Cu	Pb	Zn
SNV**, Sweden	2	600	100	800
EU	20-40	1000-1750	750-1200	2500-4000
EPA**, US	39	1500	300	2800
NSW**, Australia	8	1200	300	1800

* TS = Total Solids
** SNV = Swedish National Environmental Protection Board
EPA = Environmental Protection Agency
NSW = New South Wales

Lately, an awareness of the importance of the total metal loading on farmland has reached authorities. Many countries now have additional regulations for maximum metal loadings per hectare, including both the metal added with sludge or other fertilisers and the metal already present in the soil (Table 1.2). In practice, the metal loading is often limited by phosphorus content in the sludge; application of the maximum permitted phosphorus level in the form of a sewage sludge that meets the requirements in Table 1.1 usually gives metal loadings well below the limits stipulated in Table 1.2.

At present, directives are only concerned with total metal concentrations. This is largely because of the difficulty in relating the uptake of metals by crops with specific fractions of metals bound to sludge or sludge-amended soil. This dissertation considers the speciation of metals in sewage sludge and attempts to provide an understanding of the relevance of speciation to potential mobility and bioavailability. The final objective is that this research will be useful in setting guidelines for sludge application to agricultural soil that provides long-term protection on a scientific basis.

Table 1.2. Maximum permitted metal loadings on farmland, including background levels.

	Maximum permitted annual metal loadings in kg / ha			
	Cd	Cu	Pb	Zn
SNV, Sweden, 1995-2000	1.75	600	100	800
SNV, Sweden, after 2000	0.75	300	25	600
EPA, US	39	1500	300	2800
Germany	6	200	200	600
Netherlands	10	200	300	1000
Ontario, Canada	1.6	150	90	330

The scope of this dissertation is to determine the

1. fractionation of metals, especially Cd, Cu, Pb and Zn, in sewage sludge by single extraction and relate the results to toxicity,

2. partitioning of metals into dissolved, colloidal and solid phases and their relevance to mobility and potential bioavailability,

3. synergistic/antagonistic effects of the different sludge components on sludge metal toxicity.

Chapter 2. Consideration of the literature

Chapter 2. Consideration of the literature

Undoubtedly, measurement of the total metal content of a sludge or a sludge-amended soil gives a poor prediction of metal bioavailability and/or toxicity to plants, animals and microorganisms. Many researchers have attempted to find an extraction method, either a single extractant or a complete sequential extraction scheme, that can mimic the natural processes that render metals bioavailable. Also of interest has been the correlation between uptake into a plant, often a vegetable, and the concentration of metal extracted from sludge-amended soil with a chemical extractant. There are results that show correlations between metal uptake by certain crops and extraction with some extractants, the most common being EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid) and $CaCl_2$. However, these correlations are often tenuous.

Sequential extraction methods are based on the assumption that the analysed material, *e.g.* sewage sludge, consists of a few defined fractions, usually termed exchangeable, bound to carbonates, bound to Fe/Mn hydrous oxides, bound to organic matter and residual. Metals are operationally separated into physico-chemical fractions, each having a geochemical or biological meaning. The main problems with these extractions are lack of selectivity, incomplete extraction and readsorption. The assumption that physico-chemical fractions are selectively isolated is a simplified, and sometimes incorrect, description for a sludge sample and this has led to some criticism of extraction methods. In reality, sewage sludge consists of many different fractions which are not very well defined and sometimes not even completely known with respect to structure and composition, which explains the severe selectivity problems that are often found for these extractions.

The duration of extraction can be critical as extraction methods generally assume that equilibrium or total extraction is reached within the contact time. It has been shown that the usual contact time is not sufficient to reach either equilibrium or complete extraction; repeated treatment with the same extractant simply results in continued extraction of metal. The importance of the volume/mass ratio between the extractant and the sample should not be neglected as this determines whether an equilibrium concentration will be reached during the timescale of the extraction.

A further problem in chemical extraction is that most metals are often present in low concentrations compared to the number of organic or inorganic binding sites available. This means that the surfaces of the particles are not saturated with metals. If one fraction is dissolved, free metal ions enter solution. These metal ions can then readsorb onto other fractions. The readsorbed metals are not detected in the analysis of the extracted solution which can lead to a considerable analytical error. Pretreatment of the sample, for example through drying, can alter the binding sites on the particle surface and thereby also cause errors. All these sources of error mean that these extractions will necessarily be sample specific which prohibits the free application of sequential extractions without preliminary trials.

A simple five-step sequential extraction scheme can provide useful information on metal fractionation and can be deciphered within the limits of selectivity and precision for each extraction. Further differentiation, although attractive, becomes difficult to interpret because the compounded error in selectivity and precision provides an unacceptably low accuracy. Nevertheless, some workers have combined a whole range of extractants to present complicated sequential extraction procedures of their own.

Even if problems with specificity, selectivity and readsorption are solved, there is still the question of what the different fractionation results really mean in terms of bioavailability. A good analytical reproducibility (precision) does not necessarily imply meaningful results (accuracy). Perhaps the definitive assessment of the relevance of extraction is through a comparison with biological experiments. Many investigations have reported the impact of elevated metal concentrations on living organisms, and some of them have compared different extractants with either uptake by plants or concentrations in animal tissue.

On the whole, the many comparisons between chemical extractions of metals in sludge/soil and plant uptake have been variable and generally disappointing. Cadmium and Zn give the best correlations because these metals are both relatively weakly surface bound and in a bioavailable form. It does not therefore really matter which of the more commonly selected extractants, $CaCl_2$, EDTA or DTPA, are chosen because all will give a reasonable correlation. Variations in correlation will be dependent on the properties of Cd or Zn binding to the components of the sludge rather than on the selected extractant. Metals such as Cu and Pb preferentially bind to sludge components (organic/sulphide and Fe/Mn hydrous oxide fractions, respectively) and here the selection of extractants that remove surface bound metals becomes irrelevant and therefore gives the poor correlations commonly reported.

Comparison between sludge/soil concentration and plant uptake have proved to be highly site specific. A number of sludge and soil parameters are involved in both the bioavailability and the chemical extractability of metals. Studies have shown that soil parameters such as pH, clay content and organic content, as well as soil treatment such as liming, application of fertiliser or tilling, are important for both bioavailability and extractability. The uptake of metals also differs significantly from one plant species to another. Another problem is that parameters such as pH or organic content can change with time, which means that a metal that was not bioavailable due to complexing by an organic substance or precipitation can become bioavailable during environmental timescales. The fact that all these parameters affect the results of an extraction/bioavailability experiment explains why it has not been possible to successfully predict the uptake of a metal by an organism from results obtained at different sites.

It is unlikely that one single extractant will unequivocably identify the bioavailable fraction. There are too many factors that must be controlled to allow a direct application of a result obtained at one experimental site to another. It may be possible to find one or more extractants that can be used to predict the uptake of one or a few metals to a small group of organisms under very specified conditions. However, the practical use of such an extractant is limited. Chemical extraction of metal fractions can, correctly used, be useful for the investigation of soil properties in order to better understand how metals are distributed in a sludge or soil sample and under what circumstances they may be released from the solid phase into the aqueous phase.

A more promising approach presented in chapter 4 in this thesis involves the operational identification and separation of sludge metals into the mobile colloidal phase and a potentially bioavailable (electrochemically available) phase. Meanwhile synergism/antagonism between metals and the formation of highly toxic lipid-soluble metal species with sludge components should not be overlooked.

A sensible strategy for sludge application to agricultural soils will base loading rates on speciation in terms of mobility and potential bioaccumulation by crops and toxicity to soil organisms. This strategy will be essential in providing for sustainability and a long-term balance between the sanitary system and agricultural soils.

Metals in sewage sludge: Species, fractions and biological relevance

Metals in sewage sludge: Species, fractions and biological relevance

Carlson-Ekvall, C. E. Annika and Morrison, Gregory M.
Department of Sanitary Engineering, Chalmers University of Technology,
S-412 96 Göteborg, Sweden.

Abstract

Sewage sludge is a useful soil improver for agricultural soils but the extent of application can be limited by elevated metal concentrations. Since total metal concentration is a poor measure of sludge metal bioavailability, many studies have attempted to find a simple means to identify the bioavailable metal fraction. A comprehensive review of the literature concerning extraction methods, uptake of metals into organisms and the relation between the two is presented. Single and sequential chemical extraction methods are discussed. The limitations and relevance of metal concentrations in chemical extractants are presented together with the validity of the assumptions the chemical extraction methods are based upon. Results from attempts to correlate single extractions with metal uptake into organisms, mostly plants, are presented and discussed and it is shown that no chemical extractant completely and exclusively extracts the bioavailable metal from any sample. Good correlations can be found between extraction and plant uptake in some cases, but since many uncontrolled parameters influence the binding of metals to sludge-amended soils, the results are site-specific and it is difficult to predict uptake from one sludge-amended soil through extraction results from another. Any single extractant that removes the exchangeable metal will give similar results and will give a reasonable prediction of Cd and Zn uptake, but great care should be taken when comparing results between different soils. For Cu and Pb, no chemical extractant gives acceptable results, partly because of the comparably low percentage of these metals that are found in the exchangeable fraction. Chemical extraction methods do not considerate kinetics of metal-sludge binding and do not discriminate between mobile and potentially bioavailable metals.

Introduction

"Raw sludge is a putrescible, microbiologically hazardous material with an objectionable odour" (1). However, sewage sludge is also a valuable soil improver, partly because of its enrichment with nutrients such as nitrogen and phosphorus and partly because of its high organic and structural content; this makes sludge especially suitable for the improvement of agricultural soils (2). Sewage sludge has previously been considered a waste product from urban areas, but with the realisation of sustainable development the recycling and re-use of valuable nutrients contained in sludge will become a key issue. Sustainability should be considered within the context of a global annual production of sewage sludge which is estimated to 30 million tonnes (3) with approximately 1.8 million tonnes of associated nitrogen and phosphorus, and which is estimated to double by 2000 (4). As such, an emphasis on sludge treatment, utilisation and/or disposal is inevitable (4, 5). Phosphorus in sewage sludge represents a valuable non-renewable source whose recycling into agriculture seems to be a reasonable course of action, especially since the cost of sludge treatment and disposal sometimes represents as much as 50% of the overall cost of the wastewater treatment plant (6, 7). However, the single most limiting factor in the agricultural application of sewage sludge from urban areas is elevated concentrations of heavy metals.

Special attention has been paid to the presence of heavy metals in sewage sludge, partly because of their toxicity and partly because of their persistence in the environment (8-10). A significant proportion of metals entering sewage works concentrate in sewage sludge (11-13), except for Ni (14), and therefore sludge-amendment generally increases the total metal content of agricultural soils (15-20). This leads to some concern about the potential for increased metal concentrations in crops grown on sludge-amended soil (1, 21) as well as soil irrigated with municipal wastewater (22). A study of agricultural soils where sludge composts have been used for as long as 40 years reveals that heavy metal toxicity is regulated by acidification and the decomposition of organic matter (23). There are also other risks to be considered; inhalation of dust by farmers, ingestion of sludge-amended soil by children (24) and grazing cattle and, indeed, twelve pathways for pollutants from sewage sludge applied to land to waterways, crops and humans have been identified (25). A significant correlation has been found between the toxicity of a metal and its abundance in the biosphere, which makes even a small elevation of metal concentration above background levels a concern (10). However, time and again it has been shown that no simple connection exists between total metal content in soil and the concentrations found in plants growing on the same soil (17, 20, 26-28) and consequently it is not possible to model the bioavailability of metals as a function of total metal content (7, 29, 30). Studies have shown that, under certain conditions, and certainly in the short term, sludge application actually decreases the available metal content of soil by increasing the organic binding sites for metals (20, 26, 31-33) or that the elevated nutrient levels increase the yield, thus diluting metals in the produced biomass (25). Only a fraction of the metals in sludge-amended soils are in a chemical form that can be considered available to plants and animals; undoubtedly, the extent of fractionation between available and non-available metal depends on many factors, such as soil type and physico-chemical properties of the soil (34-38). These soil parameters can change due to application of fertiliser or manure which in turn affects the distribution (39) and bioavailability of metals (40, 41). Uptake also varies between different plant species. However, the single most important regulator of plant uptake is the chemical speciation of the metal (42).

The aim of this review is to discuss approaches to the determination of metal speciation in sewage sludge and sludge-amended soils and to assess the biological relevance of these approaches. This is important in moving towards an assessment and understanding of the ecological implications of metals in sewage sludge during and after their application to agricultural soils. The review is also relevant and timely in view of increasingly stringent controls on permissible concentrations of metals in sludge destined for agriculture and in view of an increasing emphasis on recycling phosphorus in sewage sludge.

Sources of metals

The primary source for most metals to sewage sludge is through the sewer system, although certain metals, such as Fe, may be added during wastewater treatment. A sewer system receives domestic wastewater, urban runoff and industrial discharge in varying proportions. Industrial sources have been important for all metals, but increasing control has lowered the concentrations of most metals discharged to the sewer system. However, for some of the metals that enter the sewage system today, industrial sources are still significant. The extent to which industrial discharge and stormwater enter the domestic wastewater system is critical for metal concentrations, particularly for metals such as Cr and Ni which mostly come from industrial sources and Pb, which usually enters the sewer system through urban runoff (43). However, domestic wastewater itself is also a significant source of certain metals (44), for example Cu and Zn. The extensive control of industrial discharges has resulted in a decrease in the contribution of metals from point sources and, consequently, more attention has to be paid to diffuse sources. The main sources of the toxic metals found in sewage sludge will now be dealt with individually and this discussion is followed by an analysis of metals which are usually less important or have been overlooked.

Cadmium

One important source of Cd is phosphate fertiliser (45). The production and use of batteries, paints and plastics are other important sources of Cd (46-48). The industrial discharge of Cd is decreasing due to an increasing awareness of this metal as an environmental hazard and, in many countries, the use of Cd in paints and plastics is no longer permitted. Still, the Cd containing products of yesterday can cause problems with water leaching from waste disposal sites and, in the case of incineration, atmospheric fallout. On the other hand, mining and refining of Zn ores is considered to be the major anthropogenic source of Cd emissions worldwide (49). The circulation of Cd from solid waste through air and back to land through atmospheric deposition is discussed in a comprehensive review by Jonnalagadda and Prasada Rao (50).

Chromium

The largest consumption of Cr is in industry (46), mainly the metallurgical and chemical industries (51). The metallurgical industry uses Cr in stainless steel production and electroplating. Chromium is used in a variety of chemical products, such as pigments, catalysts, wood preservatives and tanning agents (15). Chromium is reintroduced into the environment through the use of Cr-containing phosphate fertilisers, discharge from industries and atmospheric emissions (51).

Copper

The Cu content of sewage sludge originates from a variety of sources, some of which are diffuse and very difficult to control. Some Cu comes from industrial sources (46) but with an increasing control of industrial effluents the importance of the contribution from urban runoff has been revealed. Car brake linings release Cu and the corrosion of roofs and drain pipes contribute to the Cu content of urban runoff (52, 53). Another important source of Cu is drinking water pipes. Many houses have Cu pipes for household connections which, under favourable conditions for corrosion, provide a significant contribution to the sewage Cu content. In Stockholm, 75% of the sludge Cu content originates from corrosion of drinking water pipes (54). This corrosion is dependent on pH and hardness/alkalinity ratio in drinking water (55, 56). Also, the combination of materials in the mains system is important (57).

Nickel

Industry is the dominant source of anthropogenic Ni, mostly through the manufacture of dry cell batteries (15, 48) and through the metallurgical industry (46). Ni in sewage sludge can also originate from the coagulant used in wastewater treatment, where Ni can be found as a contaminant (58).

Lead

The correlation between Pb in urban soils and Pb in the atmosphere is good, and although mining, smelting and refining processes, as for many other metals, all contribute to atmospheric deposition (59), most of the atmospheric Pb originates directly from traffic (60). An important source of Pb in sludge is urban runoff which contains particles from car exhausts and indeed, soil Pb concentrations in cities has been directly correlated to city size (61), and thus to traffic intensity. This Pb is added to the fuel as tetraethyl lead and tetramethyl lead, which are both highly hydrophobic componds. These compounds both degrade, both chemically and microbially, to water soluble forms within a few weeks (62). However, the introduction of catalytic converters on automobiles has created an increasing demand for unleaded petrol. This has resulted in a decrease of lead in the fine fractions of road dust (63), but since it can take some years before the larger fractions of road dust (which contains the greater proportion of surface Pb loading) is washed down into the sewer system, this decrease of Pb concentration might not become evident in sewage sludge concentrations in the immediate future. Nevertheless, a decrease of Pb in soil has been observed in Finland, which has been attributed to decreasing emissions from traffic (64). Another important source is car battery manufacturers (15, 46). Before the introduction of DDT (1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)-ethane), a considerable contribution of Pb to the environment was a direct result of the use of Pb-containing pesticides (65) and inside houses, paints may still contribute to Pb concentrations in dust (66, 67). Plastic rain gutters, which contain small amounts of Pb added in the moulding process, have been observed to contribute to elevated Pb concentrations in roof runoff (53).

Zinc

Zinc is used in galvanized steel and is released into the environment by weathering and corrosion (46). As a consequence of the widespread use of galvanized steel, Zn is ubiquitous and enters the sewer system through urban runoff, household wastewater and industrial effluents. Elevated Zn levels in roof runoff can be attributed to both atmospheric deposition and leaching from galvanized roof surfaces (53).

Other metals

Apart from the above mentioned metals, which are considered the most important, and thus the most investigated, a number of other metals are found in sewage sludge. Some of these less frequently reported metals originate from one or a few sources, and therefore concentrations vary within a wide range depending on the presence or absence of a point source. Examples of this are: Ag, which is used in photographic processing (15, 46); As, which is a mining by-product (68, 69) is used in pesticides and as an impregnating agent for wood (46, 65, 69); Mo, which is used as an additive to stainless steel and is discharged from the shale oil industry (70) and Tl, which is a by-product in mining (71) and is used as a rat poison. Other metals are mostly a result of atmospheric fallout: V from combustion and refining of oil (72) and Cs from nuclear reactions including atomic bombs and nuclear power plants (73, 74). Platinum is used in catalytic converters on automobiles and this has lead to increasing concentrations of Pt in road dust (63) which eventually may lead to an increase of concentration in sludge. Urban runoff also contains Ti. Titanium oxide is a white pigment used in road paints and a considerable amount of Ti wears off the road surface every year (75).

Discussion

The reduction or removal of industrial discharges to the sewer system is a guaranteed measure for restricting metals in sewage sludge to a presently acceptable level. As an example in Göteborg sludge concentrations for cadmium and lead have now been reduced to 3.3 and 73 µg/g dry sludge respectively (1993), compared to 10.1 and 222 µg/g dry sludge in 1973, as a result of industrial discharge restrictions (76).

Further reductions to meet increasingly stringent controls would require a consideration of stormwater and household wastewater. As automobiles represent a significant source for many metals, appropriate stormwater control would include re-routing of stormwater to detention facilities, infiltration or percolation basins and wetlands. The separation and separate treatment of highway runoff in urban areas would also greatly reduce metal concentrations in sewage sludge.

Source separation of sewage from other household activities (dishwater, shower/bathing water, washing water) would reduce metals in sewage, but leave a considerable amount of wastewater to be treated separately. Another, and perhaps preferable option would be to attempt source reduction by identifying metal concentrations in household products and restricting the use of products containing high concentrations.

Metal speciation analysis

Measuring total metal content is the first step in estimating the effect of metals in sludge-amended soil, but it is not sufficient for the determination of biological effects (17, 26, 77). The uptake of metals by microbes, plants and animals is closely related to the chemical species of the metals. One way of investigating which metal species are present in sewage sludge and sludge-amended soil is by selective extraction through the dissolution of a specific fraction of the sample, this procedure renders the metals in that fraction available for analysis. In sequential extraction, extractants of increasing strength are applied to the same sample (78). In single extraction, the sample is divided into sub-samples and each of them is treated with one extractant (79). In simultaneous extraction the extractants are applied in combinations and more than one fraction can be extracted simultaneously; the concentration of metals in each fraction is then calculated through difference (80, 81). A number of sequential extraction schemes have been developed, although the most frequently (and often over-) used is that of Tessier et al (78). A list of some of the most commonly used extraction reagents has been collated by Lake *et al* (19) and the extractants are listed in table 1.

Table 1 Extractants used for metal speciation in sewage sludge (and sediments)

Fraction interpretation	Extractant	Experimental conditions	Reference
Exchangeable	$MgCl_2$, 1 M	pH 7.0, 1 h, agitation	17, 58, 78, 92, 94, 96,103
	$CaCl_2$, 0.1 M	16 h, agitation	86, 87, 98
	$NaCH_3COO$,, 1 M	pH 8.2, 1 h, agitation	78
	$NaCH_3COO$,, 1 M	pH 7.0, 1 h, agitation	17
	$BaCl_2$, 1 M	pH 8.1, 1 or 2 h	17, 104
	NH_4CH_3COO, 1 M	pH 7.0, 1 h	17, 88, 90, 93, 97, 101
	$Mg(NO_3)_2$	not reported	84
	KNO_3	pH 6.9, 16 h	80, 82, 83, 95, 100, 102
Unspecific adsorption	NH_4NO_3	not reported	84
Specific adsorpion	NH_4CH_3COO	not reported	84
Adsorbed	KF, 0.5 M	pH 5.8, 16 h	80, 82, 83
Sorbed components	NaOH	not reported	84

continued

Table 1 continued

Fraction interpretation	Extractant	Experimental conditions	Reference
Carbonate	NH_4NO_3, 1 M	pH 7.0, 1 h	17
	NH_4Cl, 1 M	pH 7.0, 1 h	17
	NH_4ClO_4, 1 M	pH 7.0, 1 h	17
	CH_3COOH, 5 mM	pH 3,5	96
	$NaCH_3COO$,, 1 M	pH 5.0, 5 h, agitation	17,18, 58, 78, 94, 97,101-104
	EDTA (ethylenediamine-tetraacetic acid)	pH 7.1, 16 h	80, 82, 83, 86, 87, 95,100
Fe/Mn-oxides	$NH_2OH.HCl$, 0.1 M in 0.01 M HNO_3	pH 2.0, 12 h	17, 97
	$(COONH_4)_2$, 0.2 M + $(COOH)_2$, 0.2 M	pH 3.0, 2 h	17, 58
	Dithionite	not reported	99
	$NH_2OH.HCl$, 0.05 M in 2 M H_3PO_4	not reported	96
	$NH_2OH.HCl$, 0.04 M in 25% HAc	96°C, 6 or 9 h, occasional agitation	17, 78, 94,101-104
Fe-oxides (amorphous)	$(NH_4)_2C_2H_4$	not reported	84, 88, 89
Fe-oxides (crystalline)	Ascorbic acid/oxalate-buffer	not reported	84, 88, 89
Soluble organics (solvent extractables)	Benzene/methanol	not reported	84
Soluble organics (humic/fulvic acids)	Benzene/methanol/KOH	not reported	84
Organic	NaOH, 0.5 M	16 h, agitation	83, 86, 95, 96, 100
	NaOCl, 25%	pH 9.5	83, 84
	$Na_4P_2O_7$,0.1 M	pH 8.8, 16 or 24 h	58, 80, 82, 83, 88,91
	$K_4P_2O_7$,0.1 M	not reported	98
	HNO_3 or HCl, H_2O_2 and NH_4CH_3COO	85°C, pH 2, 2+3 h, intermittent agitation	78, 87, 94, 97, 101-104
	H_2O_2 + $MgCl_2$	3 h in steam bath	92
	H_2O_2 , bromoethanol	not reported	99
	N,N-dimethylformamide	Subsequent fractionation	85
Sulphide	HNO_3, 6.0 M	pH 3.8, 16 h	80, 83, 95

The use of the extraction procedures summarised in table 1 is based on the assumption that the analysed material, *eg* sewage sludge, consists of a few well-defined fractions, usually termed exchangeable, bound to carbonates, bound to Fe/Mn oxides, bound to organic matter and residual. These fractions are operationally defined into physico-chemical fractions and they are often assumed to have a geochemical or biological meaning. Ure (105) has discussed sequential and single extractions and their relation to speciation in a comprehensive review .

The exchangeable metals are very loosely bound and are extracted by the addition of another positive ion in excess, *eg* Mg^{2+}, Ba^{2+} or NH_4^+. This extraction provides the metals that are exchanged from the surface of clays, organic matter and Fe/Mn oxides by an excess of alkali or alkali earth metals. This means that these metals are relatively weakly bound to a sludge or soil surface and will become available to associated water and, ultimately, plants under relatively small changes in environmental conditions. This fraction has therefore often been considered as a bioavailable fraction. The carbonate fraction is dissolved by changing the pH to 5 or adding chemicals which dissolve carbonates, *eg* ammonium chloride or ammonium nitrate. This fraction is not considered to be immediately bioavailable, but under acidic conditions the carbonates can dissolve and the metals may be released. To dissolve the Fe/Mn oxides and extract the metals co-precipitated with these, the oxides have to be reduced under acidic conditions. The metals in the Fe/Mn oxide and the residual fraction are not normally considered to be bioavailable except under reducing conditions such as those found in river sediments. The organic matter is oxidised at a pH of around 2 and very often H_2O_2 is used in this oxidation. The organic fraction is not normally considered available to plants in the short term, but can be potentially available when organic matter is degraded. However, there are organisms that ingest soil, for example earthworms, and metals in the organic fraction may be taken up through the alimentary canal. The fraction termed residual contains the metals that are quite simply not extracted by the previous extractants. The determination of the metal content in the residual phase is usually through digestion with a concentrated acid oxidation mixture (usually a combination of HNO_3, $HClO_4$, HCl), with HF added where crystalline metal is to be analysed. The residual phase is generally considered completely non-bioavailable under an environmental timescale.

The assumption that physico-chemical fractions are selectively isolated is a simplified, and sometimes even incorrect, description for a sludge sample and this has led to some criticism of extraction methods (106, 107). In reality, sewage sludge consists of many different fractions which are not very well defined, and sometimes not even completely known with respect to structure and composition (13, 82). The term organic matter, for instance, covers a very wide range of compounds which have different complexation capacities for metals, different molecular structure and are oxidised to varying degrees (83). The metals in this fraction may be adsorbed onto an organic particle or incorporated into a cell protein. Naturally, there is no extractant which completely extracts organically bound metals and nothing else (17, 78, 107, 108). The use of H_2O_2 has been somewhat a compromise.

A simple five-step sequential extraction scheme can provide useful information on metal fractionation and can be deciphered within the limits of selectivity and precision for each extraction. Further differentiation, although attractive, becomes difficult to interpret because the compounded error in selectivity and precision gives unacceptable accuracy. Nevertheless, some workers have combined a whole range of extractants to present complicated sequential extraction procedures of their own. One example is Iwasaki et al 1993 (88), who define 8 fractions: Exchangeable, Pb-displaceable, acid soluble, Mn oxide-occluded, organically bound, amorphous Fe oxide-occluded, crystalline Fe oxide-occluded and residual. Mehlich has designed three extractants of his own, called Mehlich 1, 2 and 3, where especially Mehlich 3, a combination of dilute acids and salts with EDTA (109) has been used in other studies (110-112).

The main problems with these extractions are lack of selectivity, incomplete extraction and readsorption (17, 83, 106, 107, 113-118). An extractant with good selectivity should extract only the desired fraction and leave the other fractions unaffected. As an example, all of the exchangeable metal ions would be extracted by sodium acetate at pH 5 (carbonate fraction) and therefore exchangeable metal has to be removed first. Bermond and Eustace (119) compared extraction by $NH_2OH.HCl$, which is performed at a pH ~2, with extraction by nitric or acetic acid alone, to discriminate between the effect of $NH_2OH.HCl$ and the pH. The results show that the selectivity of $NH_2OH.HCl$ for Fe/Mn oxides is very poor, due to extraction by the acid from other fractions of the sample. However, no readsorption of extracted metals was found (119). As this shows, it is extremely difficult, if at all possible, to completely dissolve one, perhaps not very clearly defined, fraction without affecting the others. Other studies tend to confirm this (107, 120). As oxides and carbonates often have an important structural function, the dissolution of these fractions may expose areas of the particle, usually hidden in the interior, thus providing more exchangeable metal ions (107). This also makes it difficult to dissolve one fraction completely. To do this, all of this fraction has to be available for contact with the extractant. However, there are examples of organic particles completely covered by Fe or Mn hydrous oxides, which have to be removed first (107). Nevertheless, Hirner (84) concluded that selectivity for a particular sample could be greatly improved by choosing the correct extraction sequences and by repeating some extraction steps to avoid problems with mutual inclusion effects between organic and inorganic phases. Washing solutions, which might otherwise be discarded between extraction steps should also be analysed (84). The duration of the extraction can be critical as extraction methods generally assume that equilibrium or total extraction is reached within the contact time. Bermond and Sommer (121) compared repeated extraction of a sewage sludge with thermodynamic calculations of the theoretical metal content in the different fractions. It was found that repeated extraction, performed four times on the same sample and with the same extractant, still extracted the same amount of Cu the third time and a little less Cu the fourth time compared to the first extraction. This demonstrates that the extraction time and the sludge/extractant ratio is critical and that preliminary trials of the extractants should be used for samples before analysis and interpretation can be considered. Additionally, during dissolution of the organic fraction, metals are readsorbed onto remaining solids and, indeed, these results are in accordance with theoretical modelling (121). Similar results were obtained by Rauret et al (122), who carried out Tessier´s scheme (78), but repeated the extractions in step 1 (exchangeable), 2 (carbonate bound), 3 (Fe/Mn-oxides) and 4 (organic), five, six, three and three times, respectively. The five extractions of the exchangeable Cu resulted in sequential concentrations of 2.9, 0.9, 1.2, 0.9 and 1.1 mg/kg, *i.e.*only 41% of finaly extracted metal was retained through the first extraction. In many cases, the first extraction yielded only a small fraction of the total metal finally extracted from that fraction. This shows that one extraction is not enough. When extracting the different fractions only once, the metals from the exchangeable fraction that are not extracted by one extraction will instead be found in the next extraction step, thus giving an incorrect result for that fraction (122). The importance of the volume/mass ratio between the extractant and the sample should not be neglected because this determines whether an equilibrium concentration will be reached during the timescale of the extraction (123). Perhaps of even more concern is that these extractions will necessarily be sample specific which prohibits the free application of sequential extractions without preliminary trials.

Usually, most metals are present in very low concentrations compared to the number of binding sites available. This means that the surfaces of the particles are not saturated with metals. If one fraction is dissolved, free metal ions enter the solution. These metal ions can then readsorb onto the other fractions (113). The readsorbed metals are not detected in the analysis of the solution which leads to a considerable analytical error.

Pretreatment of the sample can also cause errors. It has been shown that drying the sample can affect metal distribution (82, 83, 120, 124). For example, anaerobically digested sludge can contain sulphides which are oxidised if the samples are air dried (125). Rapin et al (115) concluded that none of four storage methods tested (freeze-drying and storage in a freezer at -30°C, storage at 4°C, freeze-drying and storage in a desiccator at room temperature, drying in a convection oven at 105°C and storage in a desiccator) completely preserved the initial physical and chemical characteristics of a sediment. All drying procedures, freeze-drying as well as oven-drying, should be avoided, especially for anoxic sediments and sludges (115). Similar conclusions were made through a round-table discussion, where sampling, storage, separation and extraction were critically considered by 14 research scientists (126). Sampling has also been discussed by Rubio and Ure (127). Weighing, temperature, calibration and other potential sources of error must be considered (128), including the problems that arise in analysing the low metal concentrations obtained by the weaker reagents (129).

Analytical problems such as reproducibility and contamination have to be taken into consideration (77). Experiments with spiked sludges or model sediments have gone some way in helping to solve these problems (83, 113, 130). López-Sánchez et al (131) tested the same five samples with two different extraction schemes and found very little agreement between the two methods. The results for Cd, Cr, Cu, Ni, Pb and Zn partitioning show that the metal distribution obtained through both procedures are significantly different. Accomaso et al (132) performed Tessier´s speciation scheme on four subsamples of the same sediment and found good reproducibility for carbonates and Fe/Mn oxides, but less satisfactory results for organic and exchangeable metal. However, interlaboratory comparison where two soil samples were extracted with EDTA by six laboratories, resulted in widely varying results, especially for Mn, but also for Cu, Pb and Zn (133). The Community Bureau of Reference, BCR, concluded that there is a need for the harmonisation of extraction procedures used for soils and sediments (134). An interlaboratory comparison of three extraction schemes applied to subsamples of the same sediment and five single extractants applied to subsamples of a soil were performed. Based on these experiments, two protocols were developed, one for single extraction of soil samples and one for sequential extraction of sediments, which could be used across and within the EC member nations (135). These procedures have allowed BCR to commence the process of preparing a reference soil sample. A reference sludge sample is available and has been analysed for metal fractionation (79).

No completely satisfactory method has been found for the speciation of metals in sewage sludge. Still, at the present time there is no better method than extraction for evaluating the metal distribution and thus the potential bioavailability of metals.

Extraction results

Sequential or single extraction procedures have been well tested for sediments and soils (87, 136, 137) and to a lesser extent sewage sludge (7, 13, 18, 58, 79, 80, 82, 83, 95, 130, 138, 139). McGrath and Cegarra (86) compared metal speciation in sludge-amended soil with the sludge used for amendment and found some differences. The sludge contained more $CaCl_2$-extractable Cd, more NaOH-extractable Cd, Ni, Pb and Zn and more residual Pb and Cu than in the soils after amendment. Mahler and Ryan (137) compared metal speciation in sludge-amended soil with reference soil samples taken from adjacent fields. Very small differences in metal speciation were found between sludge-amended soils and the reference soils for Cd. Sludge-amendment increased the percentage of Zn in the carbonate fraction and decreased the sulphide fraction (137).

Table 2 shows results for three digested sewage sludge obtained by Stovers scheme (7, 82). Total metal was extracted with $HNO_3/HClO_4$ and the residual metal was calculated by subtraction, which explains the negative value for Pb. Rudd et al (82) extracted both dried and liquid samples with Stovers scheme. It is interesting to note that sludge shows a different fractionation pattern before and after drying. The liquid samples displayed more variation between replicates than the corresponding dried samples. The dried samples gave more reproducible results, but it can be discussed whether it is really desirable to obtain results which, although reproducible, might not reflect the true fractionation of the sewage sludge. Metals were not evenly distributed in the sludge. A particle size fractionation showed that different metals were associated with different particle sizes in the same sludge, and the results are different for different sludges (139). Cd and Cu in raw sludge were mostly associated with particles > 20 µm, whereas Pb was mostly associated with the larger particle sizes. The fractions that contained the highest amounts of metals were the largest particles, >100 µm, and the small particle size range 8-20 µm.

In the following, speciation results for a number of the more commonly analysed metals in sewage sludge are summarised and discussed.

Table 2. Distribution of metal forms in digested sludge by sequential extraction (7, 82).

Sludge type	Metal	Extractant* % of total metal					
		KNO_3	KF	NaP_2O_7	EDTA	HNO_3	residual
Digested sludge (7)	Cd	0	0	14.8	48.8	17.5	18.9
	Cu	6.4	10.4	10.4	22.5	35.1	15.2
	Ni	13.9	8.3	14.3	32.2	6.8	24.5
	Pb	0	8.8	29.1	61.4	4.4	-3.7
	Zn	0.3	0.4	50.3	18.2	9.3	21.5
Digested sludge, liquid (82)	Cd	3.0	3.4	9.1	52.8	31.0	0.7
	Cu	1.0	0.2	7.1	13.3	75.6	2.8
	Ni	9.8	7.7	14.6	50.1	13.2	4.6
	Pb	2.4	0	24.9	54.5	17.1	1.1
	Zn	4.0	3.7	41.3	28.1	21.5	1.4
Digested sludge, air dried (82)	Cd	0.2	1.1	3.8	60.2	34.3	0.4
	Cu	2.5	4.6	5.5	13.5	70.3	3.6
	Ni	9.6	7.5	14.5	54.9	13.4	0.1
	Pb	0.2	0.3	36.1	52.8	9.7	0.9
	Zn	0.4	2.7	50.1	22.3	22.9	1.6

* KNO_3 - exchangeable, KF - adsorbed, NaP_2O_7 - organically bound, EDTA - carbonate, HNO_3 - sulphide.

Cadmium

Most investigations have reported the largest part of Cd in the carbonate fraction, extracted by EDTA (82, 83, 86, 95, 104, 137) or $NaCH_3COO$ (58, 103) or in the organic fraction, extracted by $Na_4P_2O_7$ (58, 80). Some Cd can also be found in the exchangeable (extracted by KNO_3) and adsorbed (extracted by KF) fractions (80). However, elsewhere results disagree with these findings. In one study, 60% of Cd was found in the organic and sulphide fraction (138) which agreed with earlier results by the same authors (13). Another study showed that 73% of the Cd was found in the Fe/Mn oxide fraction (18) while Brennan (97) found 95% of the Cd in the organic/sulphidic phase. Cadmium is usually not found in the residual fraction, which reflects the low natural concentration of Cd. However, one study found the same amount of Cd in the residual phase as in the EDTA-extractable phase, around 40% in each fraction (86). These widely diverging results underline the difficulties in interpreting speciation studies carried out under different and possibly poorly controlled conditions.

A separation into a range of particle sizes showed that Cd was mainly associated with the low molecular weight fraction (140). These results are confirmed by another study (139). After centrifugation, 3-25% of the Cd was found in the supernatant (138).

Spiking the sewage sludge with Cd results in higher concentrations of Cd in the carbonate, organic and sulphide fractions, thus indicating that the additional Cd may be in a more tightly bound, and thus less available, form (80).

A study of mobility of heavy metals in soil profiles showed that Cd occurred mostly as free hydrated ions in composted sewage sludge. The Cd content of the soil solution was independent of organic content, but increased with acidification, which is in accordance with speciation results achieved by chemical extraction (37).

Considerable differences between individual sludges have been reported. An extraction of 17 different sludges with $Ca(NO_3)_2$ and $Ca(NO_3)_2$/EDTA showed very different results. A calculation with GEOCHEM showed that the dissolved fraction consisted mainly of Cd bound to organic material, mostly fulvic acid (23.4-69.1% and 0.2-69.1%, respectively) and free Cd^{2+} (19.8-74.4% and 3.7-99.4%, respectively). These results indicate that general predictions of metal speciation in sludge are difficult (141).

The overall conclusion is that Cd is generally weakly bound, although in anaerobically digested sludge Cd is present in a reduced sulphidic form. Cadmium is also the only metal which is present in significant amounts in the exchangeable or water soluble phases. This indicates that Cd could be mobile in soil water and probably also comparably available for uptake by organisms. Brennan (97) found that even though 95% of the Cd from sludge dumped in the sea was found in the organic/sulphidic phase, agitation with seawater for two hours mobilised Cd to a significant extent: 56% of the total Cd left the organic/sulphidic phase and was found in the seawater soluble phase (97), something that can be explained by the high affinity of Cd for chloride ions (142, 143).

Chromium

Studies have shown that 64% of Cr in sewage sludge is found in the oxidisable phase, extracted by H_2O_2 (13, 138) and is confirmed by another investigation where 71% of the Cr was found in the organic fraction extracted by H_2O_2 (18). Another study found 38% in the Fe/Mn oxide fraction and 49% in the residual fraction (58). McGrath and Cegarra (86) found that the residual phase dominated with 45-95% of the Cr while 10-45% was found in the EDTA-extractable fraction. Similar results were found by Chang et al (95), who reported 77-83% of the Cr in the residual/sulphide phase and Legret (104), who found 44% in the residual phase and 33% as Fe/Mn oxides. No studies have reported more than a few percent of Cr in the adsorbed or exchangeable fraction (13, 18, 58, 86, 95, 104, 138). Only a few studies have focussed on Cr fractionation in sewage sludge which makes it difficult to draw any general conclusions. However, it seems that Cr would be relatively strongly bound in sludge applied to soils and thus it would be unlikely to leak out into soil water or be directly available for plant uptake.

Copper

Copper requires strongly oxidising reagents to be extracted from sewage sludge. Around 75% of Cu was found in the organic, sulphidic and carbonate fractions in one study (130). Brennan found 86% in the organic/sulphidic phase (97). Other studies found more than 90% of the Cu in the oxidisable phase extracted by H_2O_2 (13, 138). These trends are confirmed by a number of further investigations (80, 86, 103). On the other hand, one study reported 32% of the Cu in the Fe/Mn oxide and 55% in the residual phase (58). Legret (104) found an even distribution of Cu between four fractions: carbonate fraction 33%, organic/sulphidic 26%, Fe/Mn oxides 21% and residual 20%. One study has shown that 70% of the Cu is found in the sulphide phase (82). Chang et al (95) found 25-44% in the NaOH extractable phase, and 25-29% of the Cu in the residual/-sulphide phase in sludge-amended soils, but 58% in unamended soil. However, another study could not confirm this and instead demonstrated that most of the Cu was extractable with $MgCl_2$ (83) which can be due to the formation of a chloro complex. On the other hand, agitation of sludge with seawater did not dissolve any Cu into the seawater. However, a change in partitioning was observed; after agitation with seawater, 4% of the residual Cu was found in the moderately reducible phase (97). Certainly, most studies have found that Cu is strongly bound to sludge, mostly in the organic phase. This means that Cu is not likely to be available, unless the organic phase is degraded. Domergue and Védy (37) confirmed that Cu is strongly bound to the organic matter in a lysimetric study of composted sludge. Determination of Cu-binding with an ion-selective electrode tended to confirm that Cu was almost completely organically bound (144), although this speciation method tends to be limited in application through unreliability at low ($< 10^{-7}$ M) concentrations.

Nickel

A number of studies have found that Ni is mostly in the EDTA-extractable, carbonate fraction which contains 50% of the Ni, while the rest of the Ni is quite evenly distributed between other fractions (82) and this was confirmed through a study of model compounds (83). However, other workers have found results which totally disagree. Legret (104) found 56% in the organic/sulphide fraction, while in another study, 55% of the Ni was found in the residual fraction, 32% in the Fe/Mn oxide fraction and only 12% in the carbonate (acid soluble) fraction (58). Similar results were found by Chang et al (95), who found 37-68% in the residual/sulphide fraction. This may be a result of addition of residual Ni with the coagulant used in wastewater treatment which has been proved to contain significant concentrations of Ni. The same dominance of the residual fraction was found by McGrath and Cegarra (86) who found 40-80% of the Ni in this fraction and most of the rest in the NaOH and EDTA extractable fractions. Another study showed that 43% of the Ni is found in the Fe/Mn oxide fraction and 20% in the carbonate, organic and residual fractions, respectively (18). However, too few results are yet available to draw any reliable conclusions concerning which fraction Ni usually partitions into. A conclusion that perhaps can be drawn is that Ni is not expected to be found in the exchangeable fraction. Ni is naturally present in many soils and Ni in the residual phase of soils may be the result of the weathering of igneous rock. A thorough literature survey of the forms, reactions and availability of Ni was made by Uren (146).

Lead

The oxidisable fraction has been shown to contain 85% of the Pb and the rest is found in the residual fraction (13, 138). Another study points to the residual fraction as the most important fraction for Pb, containing 72% (58) or 68% (104). Others have found 53% of the Pb in the carbonate, EDTA-extractable, fraction and 36% in the organic fraction, extracted by $Na_4P_2O_7$ (82) and similar results have been found elsewhere (86, 95, 103). The adsorbed or exchangeable fraction contains less than 1% (13, 82, 95) or around 4% (58). A study on model compounds showed that 70% of the Pb in a liquid sample and 86% of the Pb in a dried sample were extracted by H_2O_2. The percentage for $NaCH_3COO$ was 76% and 47% respectively and for $Na_4P_2O_7$ 22% and 47% (83). Similar results were found by Brennan (97), who found 70% of the Pb in the organic/sulphide phase. For a sequential extraction procedure the percentage was 70% and 72% for $NaCH_3COO$ and less than 15% for all other extractions, which shows the difficulty of comparing sequential extractions to single extractions or even to other sequential extractions when applying the extractants in a different order (83). The overall conclusion is that most of the Pb is found in the fractions that are not easily extracted, which means that Pb would be expected to be comparably unavailable to organisms and to have a low mobility in sludge-amended soils.

Zinc

Zinc was found to be readily extracted from sewage sludge. An extraction with Tessier's scheme (78) found 72% of the Zn in the Fe/Mn oxide fraction, 15% and 17% in the carbonate and organic fractions, 3% in the exchangeable fraction and only 2% in the residual fraction (18). Another study confirmed that Fe/Mn oxides are an important fraction containing 32% of the Zn, but on the other hand this study found as much as 42% of the Zn in the residual phase (58). A study by Chang et al (95) found 22-39% of sludge-applied Zn in the residual/sulphide fraction, 36-47% in the EDTA extractable phase and 22-29% in the organic phase, whereas 83% of the Zn originally present in the unamended soil was found in the residual/sulphide phase. Legret (104) found 49% associated with Fe/Mn oxides and 34% in the carbonate fraction. An extraction of a sewage sludge dumped at the sea demonstrated 26% of the Zn in the organic/sulphidic phase and 60% in the moderately reducible phase, extracted by ammonium oxalate (97). An extraction procedure applied to a model compound showed that H_2O_2 extracted 95% of the Zn in liquid sludge and 100% in dried sludge, $NaCH_3COO$ extracted 83% and 58% respectively and NaOH, which was employed to extract the organic fraction, extracted 73% and 45% (83). This is confirmed in other studies where 85% of the Zn was found in the organic fraction of digested sewage sludge (13, 138) while other studies reported 50% of the Zn in the organic fraction and the rest in the carbonate and sulphide fractions (82). However, the almost complete extraction of Zn by H_2O_2 does not necessarily mean that all the Zn is to be found in the organic fraction but rather is an illustration of the lack of selectivity of some extractants (83). Yet another study showed that the NaOH-extractable, EDTA-extractable and residual fraction contained around 30% each (86). A comparison to a sequential extraction showed that $MgCl_2$, which is assumed to extract the exchangeable fraction, is the most effective extractant for Zn and extracts 37% of the Zn in liquid sludge and 59% in dried sludge. This can be compared to the 44% and 30% extracted by $NaCH_3COO$ which is supposed to extract the organic fraction (83). This study clearly indicates that it is almost impossible to determine the distribution of a metal in a sewage sludge without a very thorough description of the extraction method and that it is necessary to repeat the same extractants in the same order if a comparison between two experiments is to be performed. In this study, the impact of drying is also clearly shown; $Na_4P_2O_7$ extracted 8% of the Zn in the liquid sludge, but as much as 60% in the same sludge after drying (83).

A study of metal speciation in spiked sludge showed that 50% of Zn is organically bound in both primary, secondary and digested sludges and that the additional Zn in the spiked sludge redistributes itself similarly (130). The same authors have also investigated the effects of a thousandfold increase of Zn and found minor changes in the fractionation pattern, mostly an increase in the EDTA-extractable fraction (147). However, one study confirmed that most of the Zn, 66%, is found in the organic fraction, but the additional Zn was found almost entirely in the carbonate and the sulphide fractions, whereas the Zn content in the organic fraction did not significantly increase (80).

The results of Zn speciation and fractionation show that Zn is most likely to be found in the organic phase. However, the results are not clear and there are considerable differences between different sludges and different extraction methods. For example, a study of the mobility of heavy metals in sewage sludge by measuring the flow of metals from composted sewage sludge in a lysimeter to vegetation, mineral subsoil and water, showed that Zn was mostly found as free hydrated ions (37).

Interpretation of speciation results

A comparison between studies of metal distribution in sewage sludge is difficult, particularly where, as is usual, sequential chemical extractions have been employed. Sewage sludge from different sewage treatment plants gives different results. Also, the different metals have different distribution patterns. For example, Cd is the only metal that is found to any extent in the exchangeable fraction and very little Cd is found in the residual fraction, while extraction of Pb requires dissolution of the organic and Fe/Mn oxides, or in some sludges even the residual fraction.

The same sludge can give different results depending on the treatment of the sludge both at the sewage works and in the laboratory. Dewatering, digestion, composting and liming changes the metal speciation and drying of the sludge prior to analysis can also alter the metal distribution in the sludge.

A significant difference in metal speciation has been observed before and after sludge-amendment of agricultural soils. In untreated soils, most of the metals are found in the residual/sulphide fraction (Cr, 83%; Cu, 58%; Ni, 68% and Zn, 83%) or the EDTA extractable fraction (Cd, 49% and Pb, 71%). The fractionation pattern changed after sludge application to more available fractions, especially for Cu, where the percentage of Cu extracted by EDTA and NaOH doubled, and for Zn, which increased from 2% to 22-29% in the organic fraction (95).

Further difficulties are encountered in interpreting sewage sludge fractions because individual sludge fractions may have the same name and interpretation, while incorporating quite different chemical extractants for the same purpose. There can be no doubt that metal speciation analysis in sewage sludge suffers from a doubtful nomenclature. For example, table 1 shows that the carbonate fraction can be extracted by six different extractants, each of them giving different results. Some studies have also clearly shown that sequential, simultaneous or single extractions give different results. In the case of sequential extraction, the order of application is also important.

It is concluded here that it is of utmost importance to report all parameters including the exact extraction procedure when reporting results from fractionation experiments. Satisfactory conclusions can only be obtained if sufficient attention is paid to clearly defining the conditions for extraction. Additionally, metals have to be studied individually, since different metals behave differently in the sludge, both because of their different chemical properties and because of their different origin.

Metal speciation in sludge-amended soils

The behaviour of heavy metals in sludge and sludge-amended soil depends on a range of parameters, including the complexing capacity and the pH of the sludge and the soil. These parameters can change with time which means that to determine the bioavailability of metals some years after sludge application, a new study of the soil parameters and metal speciation is required.

Keefer and Singh (85) extracted sludge-amended soil with *N,N*-dimethylformamide and fractionated the extracts into hydrophobic acids, neutrals and bases and hydrophilic acids, neutrals and bases. Cadmium, Cr, Cu, Ni, Pb and Zn were analysed in the different fractions and correlated to soil properties such as pH, CEC (Cation Exchange Capacity), and clay percentage. For one sludge, a significant correlation was found between Cd, Cr, Ni, Zn and pH and the amounts of different minerals in the soil. However, for the two other sludges, no consistent and significant correlations were found. This demonstrates the difficulty of predicting properties of one sludge-amended soil from results based upon analysis of a different sludge.

Complexing agents and stability constants in sewage sludge.

Sewage sludge contains a variety of complexing agents, mostly organic. Some enter the sludge with the influent sewage, while some originate in the process, for example chemicals added in the waterworks and bacterial extracellular polymers (11, 12). The presence of these complexing agents is one of the most important factors affecting the distribution of metal between the different fractions (32). However, the effect of complex formation varies between metals. It has been shown that most of the Cu in sludge-amended soil solution is in the form of organic complexes, whereas complexes of Cd account for only a small proportion of the total metal in soil solution (32). However, sludge leachate with a high organic content can bind, and thus transport, enough Cd to pose a risk to groundwater quality (148). When all the fractions of a sludge-amended soil are taken into account, a considerable part of Cd, Ni and Zn is complex bound to organic matter. Humic substances appear to be the most important complexant for Cd in natural systems (47), however, other organic components such as bacterial extracellular polymers may dominate in sewage sludge. Sludge addition seems to increase the complexation capacity of soil (20), but at the same time, complex stability decreases (32). However, the overall effect is that sludge application restricts heavy metal mobility, and thus toxicity, in soil (15, 26, 32, 149). A considerable portion of soil organic matter is humic acids which have a large binding capacity for metals. These binding sites are usually far from saturated and sludge-amendment can change the soil conditions in a way that favours complexation of the added metals. This provides one explanation to the fact that sludge-amendment can result in lower levels of mobile metals, even though total metal concentrations may be increased (20, 31). There are also investigations that have demonstrated an increased mobility of metals after sludge-amendment (150).

There are a whole range of potential types of binding sites for metals in sludge, but it has been shown that most of them are carboxyl, phenol and hydroxyl functional groups and also amide and thiol groups (7). The binding capacity for Cd, Cu and Zn is decreased if the carboxyl and phenol groups are methylated, but Ni remains unaffected (7). Fletcher and Beckett (144,145) used ion-selective electrodes to investigate the chemistry of metals in sewage sludge and found that the organic material in sludge contained two distinguishable groups of binding sites, one that can bind most metals and protons, and another that can bind only Cu, Pb and protons. The first site can bind ions such as Ca, Fe and Mg, all of which are common in sewage sludge. This means that they compete with other metal ions and often completely exclude ions such as Co, Ni and Zn. In a sludge with high concentrations of for example Ca or Fe, perhaps from coagulants added during sewage treatment, only metals bound to a second binding site, namely Cu and Pb, will be strongly bound. The structure of these binding sites are altered during anaerobic digestion at the sewage treatment plant which means that the binding capacity for metals is different in raw and in digested sludge (13). Also, the binding capacity and the strength of the complex depends on factors such as pH and the presence of competing metal ions, for example Ca in limed sludge. Composting of sewage sludge changes the speciation significantly. Most total metal concentrations increase, but Cu and Ni concentrations decrease (151). This has the important implication that a sludge that meets the requirements for spreading on soil before composting may not meet these requirements after composting. This is especially true for Cd which can show an increase of total metal up to 58% (151). There is also competition between heavy metals for the same binding sites. Basta and Tabatabai (152) reported an affinity scale for metals to two soils where Pb > Cu > Ni = Cd = Zn (the metals were applied together to the same soil). It was concluded that Cu and Pb, which were the metals with the highest affinity to the soil, were bound as $CuOH^+$ and $PbOH^+$, whereas the other three metals were adsorbed to the surface through replacement of indigenous exchangeable ions such as Ca and Mg ions (152). Elsewhere it has been shown that Ca competes with Cd on the surface of Fe-oxides (153).

Persistence of metals in sludge-amended soil

Metals in sludge-amended soil can leave the soil in two ways: they can be taken up by plants and animals or they can migrate to a lower layer where they can leach out into adjacent waterways or groundwater. A study of the migration of metals in soil amended with sewage sludge containing large amounts of Cd and Ni showed that Cd is the metal which migrates most readily in soil, 60-80 cm in 7 years. Cd was found mostly in the exchangeable phase. Ni, which was primarily associated with the oxidisable phase, migrated 40-60 cm, Pb, found in the acid-soluble and reducible phases, migrated 20-40 cm and Cr, which was mainly in the residual phase, remained in the upper layer (48). The actual depth of metal penetration is dependent on the soil type. In this case (48) the soil had a very coarse texture which can faciliate the movement of the metals. However, these results indicate a connection between speciation and migration. In other types of soils, it has been shown that the metals associated with sewage sludge remain in the topsoil for many years, even if the area is regularly ploughed (150). A study of a crop field which had received sewage sludge twice a year for 12 years showed that Zn had moved down only 20 cm, whereas all the Cu remained in the topsoil (154). Other studies have investigated the migration of metals in soil and found that most metals remain in the upper 5 cm, but movement to a depth of 10 cm has been observed for seven studied metals, Cd, Cr, Cu, Mo, Ni, Pb and Zn (155).

It should be remembered in all studies of sludge-amended soils which cover a longer time period, migration is not the only potential pathway for metal redistribution. Three additional major redistribution mechanisms for sludge-borne metals have been identified, namely, harvest removal, erosion and tillage practice (156). Harvest removal has been thoroughly investigated, although this is usually negligible in terms of the fraction of total metal removed (157, 158). Erosion and tillage on the other hand, have attracted little attention.Yingming and Corey (156) found that 15-20% of Cd, Cu and Zn had moved from the site of application to an adjacent field in 11 years.

It must also be remembered that the structure of the binding sites, and thus the speciation, can change significantly with time after application to soil. This may lead to remobilisation of metals, thus rendering formerly immobile metals bioavailable (7). However, it has been shown that this process can be very slow (8) and metals persist in soils for many years with less than 0.05% of the sludge metals added annually removed in crops (8, 15). A comprehensive review of metal binding to soil components has been made by Mestek and Volka (159, in czech).

A long-term study of sludge-amended soils has been performed by McGrath and Cegarra (86). Sequential extractions were performed on soils which had received sewage sludge from 1942 to 1961. Samples were taken sporadicallyand on seven occations between 1942 and 1983. Speciation analysis showed that large increases of Cd, Cu, Ni, Pb and Zn in the fractions extracted by $CaCl_2$, NaOH and EDTA occurred during ten years after the first sludge addition. After that, very little happened concerning the percentage of metals extracted with the different extractants (86).

The biological relevance of metal speciation in sewage sludge and sludge-amended soils

All metal speciation schemes suffer from severe problems with specificity, selectivity and readsorption. Even if all these problems are solved, there is still the question of what the different fractionation results really mean in terms of bioavailability (113). A good analytical reproducibility (precision) does not necessarily imply meaningful results (accuracy). Perhaps the definite assessment of extraction relevance is through comparison with biological experiments. Many investigations have reported the impact of elevated metal concentrations on living organisms, and some of them have compared different extractants to uptake by plants and concentrations in animal tissue.

Uptake and effects of metals on different organisms

Heavy metals can enter the metabolism of, and thus have toxic effects on most life-forms. However, since metabolism can differ between microbes, plants and animals, these groups will be considered separately.

Microbial effects

If sewage sludge is spread on soil, metals may have a direct toxic effect on soil microorganisms. Some investigations have focussed on the influence of heavy metals on soil microorganisms (160). Fungi and actinomycetes were the most resistant soil microorganisms and *Azotobacter* and nitrifiers appeared to be the most sensitive. This study also showed that different metal species have different toxicity. Zinc and Cu were shown to suppress enzyme activity and respiration. Small doses of these metals had a beneficial effect on microbial growth which perhaps could be explained by the soil being deficient in these metals. However, small doses of As and Pb gave a similar beneficial effect, although these metals have no known biological function. All examined compounds of Hg were very harmful to soil bacteria and stimulated actinomycetes. This means that adding toxic metals to soil does not necessarily inhibit all forms of life to the same extent, but may instead change the population structure of the microbial community. Other studies have reported that Cd applied at concentrations higher than 25 μg Cd/g soil significantly decrease C and N mineralisation. No effect was observed for sludge containing 10 μg Cd/g (161). The total amount of biomass was measured in a sandy loam amended with sewage sludge (162), and it was found that Cu and Zn at concentrations 4.9 and 2.3 times the permitted EC limit decreased the total biomass significantly when present singly. However, a combination of Cu and Zn decreased the biomass by up to 53% when present in concentrations only 1.2-1.8 times the permitted concentration. An experiment with metal-spiked sewage sludge showed negative effects on dehydrogenase activity of the microflora at high metal concentrations (163).

The toxicity of Cd, Cu and Zn and the detoxifying role of Ca was studied on the brown alga *Cystoseira barbata* by complete factorial design 2^k . The results show that Cu was the most toxic metal. Both synergistic and antagonistic effects were observed between Cd and Zn depending on concentration. These metals affected growth as well as chlorophyll and carotenoid synthesis. Ca showed a protective effect for all metals (164).

A very important groups of microorganisms is the nitrogen-fixing bacteria, especially where legumes are to be grown. It has been shown that the application of metal contaminated sewage sludge negatively affects the nodulation of *Rhizobium leguminosarum* bv *trifolii*. In one of the experiments on plots receiving the highest rate of contaminated sludge, three out of four plots had no rhizobia at all. The metal concentrations in these plots were well below the UK and EC limits for Cd, Cu and Ni, but above for Zn. The authors drew the conclusion that Zn toxicity is the main reason for the poor survival of Rhizobium nodules (165). Similar results were found in another study (166). Heavy metals from sewage sludge has also been found to decrease the genetic diversity of *Rhizobium leguminosarum* bv *trifolii* (167).

Metals are often involved in the metabolism of the microorganism. One way to metabolise metals is through biomethylation. This is a process which makes the metals more hydrophobic and therefore more easily incorporated in the biosphere. Methylation has been reported for Pb (12).

Another important group of microorganisms is the anaerobic bacteria used to digest sewage sludge. Heavy metal toxicity is a common cause of anaerobic digester failure (168).

However, investigation of these problems is difficult because of the complexity of both the sludge matrix and the microbial population. The short generation time of the organisms makes it necessary to take into account phenomena such as adaption of bacteria and changes in the community composition (169). For example, bacteria of the *Flavobacteria* species are naturally Cd resistant and *Pseudomonas* bacteria can develop resistance in sludge-amended soils (15), while Angle et al (170) concluded that many soil bacteria are naturally resistant to metals. However, in assessing concentration limits, it cannot be assumed that organisms will evolve an increased resistance (169). It is also difficult to measure uptake in microorganisms because of the difficulty in separating cellular uptake in living cells from adsorption onto the cellular surface. It has been shown that cellular constituents of lysed bacterial cells can complex heavy metals almost as efficiently as growing cells (171). Extracellular exopolymers contribute significantly to metal removal in biological wastewater treatment (172).

Metal concentrations in plants

All plants have means to take up essential metals from soil. These mechanisms are designed to provide the plants with the metals necessary for plant growth and function. Most dicotyledonous plants absorb more heavy metals than monocotyledonous (150). Cereals and legumes accumulate less metals than leafy plants such as lettuce and spinach (20, 26) while carrots and tomatoes accumulate several metals to a significant degree (7). The distribution of the metal in the plant is important in determining whether the metals may enter the food chain. Fortunately, it seems that peas and beans have lower levels of metals in the edible parts of the plant compared to the leaves, while cereals generally do not accumulate metals in the grain (7, 150). Similar results were found by Cimino and Toscano (2) who found that although germination of pea and broad bean was reduced by high concentrations of Cd, Cu and Pb in sludge-amended soils, the metal concentrations in the edible parts were not elevated. Dowdy and Larson (173) studied carrots, lettuce, peas, potatoes, radishes, sweet corn and tomatoes grown in sludge-amended soils. In the cases where elevated concentrations were found, these were negligible and almost exclusively present in the parts of the vegetables that are not used as food. Cadmium, taken up by lettuce, giant endive and curly endive was found mostly in the roots, less in older leaves and the lowest concentration was found in the youngest leaves (174). The uptake of metals can differ considerably within the same species. Florijn and van Beusichem (175) found that Cd uptake in different inbred lines of sweet corn (*Zea mays*, L.) varied by a factor of 10-20.

The uptake mechanism functions in different ways for different metals. Some plants seem to have the ability to discriminate against heavy metals by either excluding them or retaining them in the roots (150). One study showed that Cd, Cu, Ni and Zn can be retained in the roots, whereas root concentrations of Cr and Pb were even lower than the soil concentrations. The authors state that this is not the result of some barrier function, but rather depends on the particularly strong binding of Cr and Pb in the soil (150).

Other workers have found that Zn and Cd is taken up by roots and transported to the aerial parts of the plant, Cu and Pb is mostly retained in the roots and Ni seems to be evenly distributed in the plants (7). The roots themselves can excrete an exudate, composed of polysaccharides, organic acids, amino acids and proteins, into the soil. This exudate complexes metals, and this mechanism is believed to play an important role in protecting the roots from extensive metal uptake, especially for Pb and Cu which both form strong complexes with these substances (176).

The uptake of metals for 12 food crops was investigated (15). The order of accumulation for Cd was, tobacco > lettuce > spinach > celery > cabbage; for Pb was, kale > ryegrass > celery; for Cu was, sugar beet > some varieties of barley; for Ni was, sugar beet > ryegrass > mangold > turnip and for Zn was, sugar beet > mangold > turnip. The Cd, Cu, Pb and Zn contents were generally highest in the roots. Cadmium was accumulated by spinach and lettuce which had a relatively low Ni content, whereas ryegrass which accumulates Ni, had low Cd concentrations (150).

Chang et al (177) investigated the uptake of Cd, Cr, Cu, Ni, Pb and Zn in Swiss chard and radish grown on sludge-amended soil. The concentrations of Cr, Cu, Ni, and Pb did not change significantly, but the uptake of Cd and Zn increased. However, the extent of the concentration increase was not directly proportional to the sludge loading (177). Swiss chard accumulated more metals than radish, which is in accordance with other findings that showed a higher uptake in leafy vegetables compared to other crops (26). Chang et al (95) also investigated the uptake of six metals (Cd, Cr, Cu, Ni, Pb and Zn) to winter barley (*Hordeum vulgare* L.) before and after sludge amendment. Only Cd and Zn increased in the plants (95). This is in accordance with results found by Chlopecka (178), who found elevated concentrations of Cd and Zn in barley grown on soil irrigated with carbonates of Cd, Cu, Pb and Zn.

Another investigation considered the uptake of the Cd, Cu, Pb, Mn and Zn in sweet corn (*Zea mays*) and bromegrass (*Bromus inermis* Leyess). Sewage sludge application increased Zn, Cd and Ni concentrations in bromegrass and sweet corn stover, and Zn and Ni in sweet corn grain, but the concentrations of Cr and Pb were not significantly increased, while Cu concentrations were more associated to the increase in N content rather than the sludge Cu concentration (179). Similar results were found by Hemphill et al (180), who found that leaf tissue Cd and Zn content in sweet corn (*Zea mays*) increased with sludge or commercial N application, whereas the kernel metal content varied less.

Tobacco has been recognised as an effective accumulator of heavy metals such as Cd. Considering that smoking of tobacco products has been identified as a significant contribution to the human Cd load, it has been suggested that tobacco should not be grown on sludge-amended soil (181, 182). Experiments have shown that tobacco plant concentrations of Cd, Cu, Mn, Ni and Zn increase with increased sludge rates, Fe concentration decreases and Pb concentration is unaffected.

Metals taken up by plants have different effects. An increase in wheat plant concentration of Cr, Cu, Mn and Zn, caused by irrigation with sewage and sludge amendment was observed to give decreased activity of measured enzymes including aspartate amino transferase, alanine amino transferase and peroxidase (183). Swiss chard grown on a soil amended with fertiliser containing 5%, 10% or 15% tannery waste showed impaired growth for both the 10% and 15% treatments along with significantly increased Cr levels (184).

Nitrogen-containing fertilisers can enhance Cd uptake (41). This can be explained in terms of ion exchange processes. The added base cations not only displace part of the exchangeable Cd, but also lower the soil pH by displacing H^+ ions. This shows the importance of taking into account not only the properties of the sewage sludge before applying it to soil, but also the characteristics of the soil itself. This is confirmed by Kancheva et al (185) who treated two different soil types with Ni. The barley grown on the carbonate chernozem soil was not negatively affected by Ni, whereas dramatic effects were seen for barley grown on grey forest soil (185). Also, the combination of sludge-amendment and other fertilisers or lime can affect metal uptake (186). A correlation has also been found between DOC (Dissolved Organic Carbon) and metal solubility, which can affect bioavailability (39, 117). Other important factors are OC (Organic Carbon) (187), CEC (Cation exchange capacity)(92, 150, 187, 188), liming (93, 189), organic matter (92, 190, 191) and clay content (150, 187). The bioavailability of sludge Cu increases in the presence of organic matter from sludge (192) and the mobility of Cd increases considerably in sludge-amended soil as a result of elevated DOC (191) .

The uptake of one metal can also be affected by other metals. McKenna et al (193) investigated interactions between Cd and Zn in lettuce and spinach and found a very complex pattern of interaction which differed between different parts of the plant. However, it is clear that uptake from a mixture of both metals cannot be predicted from experiments with only a single metal. Experiments with rice (*Oryza sativa* L.) showed that elevated levels of Cu cause a decrease in Zn uptake (194).

There is also a possibility of synergistic effects in a plant that is exposed to several metals simultaneously. Wallace have found reduced yield and depressed growth in bush beans exposed to combinations of five or six metals in concentrations that, separately, would cause little harm, but when applied simultaneously cause severe effects (195,196). This effect has been shown for a range of plant species (197).

When considering metal loading limits it must be remembered that experiments with soils spiked with metal salts give effects that differ from those observed under natural conditions, and these experiments tend to overestimate the hazards of metals (20). Phytotoxic effects of metals from sludge are very rarely reported, even at very high application rates (25). However, Cd can pose a risk to humans in concentrations lower than the concentrations that give phytotoxic effects (141, 174). The risk of soil toxicity to humans depends on the crop grown, elements which accompany Cd into the edible crop tissues and the level of other essential metals (Zn, Fe and Ca) in the diet (50), but for some individuals with a certain diet, *e.g.* rice with high Cd and low Fe content, the risk for kidney damage is elevated even though no damage can be observed in the crop.

Plant uptake is highly dependent on soil parameters. As mentioned earlier, sludge amendment can decrease the bioavailability of metals because of the additional binding sites that are provided in the sludge (15, 20, 31, 32, 198). Sludge-bound metals are more easily taken up by wheat in sandy soil than plants growing on silty clay loam soils (16). Liming is a very important factor which decreases metal uptake because metal binding to soil, increases with pH (143, 199). Soil pH is therefore a crucial parameter (20, 35, 38, 143, 188, 200-202) especially for soils with a low organic content (203). A low pH enhances metal uptake. An experiment with progressive acidification of dried and liquid sludge revealed that significant quantities of Zn were mobilised at pH 4. Cadmium and Pb were not mobilised until pH 2, but Ni was easily mobilised at all pH values from 7 and downwards. All metals showed increasing mobility with decreasing pH, except Cu, which was hardly mobile at all above pH 0.5 (82). Another study confirmed that bioavailable Zn decreased with increasing pH (204). Similar results were found for Cu uptake in Swingle Citrumelo seedlings (201), whereas Sanders et al (38) found that pH only affected Cu uptake when soil pH was below 5.5. However, Zn and Ni uptake increased with decreasing pH over the range 4.7 to 7.3 (38). The addition of phosphate has been shown to reduce bioavailability of Pb (24). An experiment with soil treatment with several different additives showed that the addition of hydrous Mn oxides decreased the uptake of Cd and Pb by ryegrass and tobacco, and additionally decreased extractability in acetic acid for both metals (205).

Effects on animals

Lead uptake by earthworms has been studied (206, 207). Calcium in the soil seems to affect Pb uptake; Ca in the diet suppresses lead accumulation by earthworms and earthworm species that have active calcium secreting glands have been found to contain lower Pb concentrations than species with less active Ca excretion living in the same soil. Similar reduction of Cd toxicity by Ca is found for the fish mummichog, *Fundulus heteroclitus* (208). Measurement of Cd, Cu, Pb and Zn in earthworms revealed that Cd concentrations were generally higher in earthworms than in the soil, whereas Cu and Pb concentrations were lower (209). The results suggest that Cu and Zn accumulation may be physiologically regulated in earthworms. Other investigations confirm these results (190, 210) and suggest that the earthworm *Lumbricus rubellus* can be used as an indicator species for investigating the rates of heavy metal uptake within terrestrial ecosystems (211). At low soil Cd concentrations, the concentrations of Cd inside the earthworm can be 5-200 times higher than in the soil. However, no adverse effects of this biomagnification has been observed (190). The levels of Cd, Cu, Pb and Zn in meadow voles that inhabitated a field which had received sewage sludge for 10 consecutive years were not elevated, in spite of the elevated contents in earthworms in the same field. However, the voles are active in the field for only 24 weeks a year, which means that transfer from earthworms to higher trophic levels cannot be completely ruled out (210).

The effect of heavy metals on development, reproductive capacity and viability of the grasshopper *Aiolopus thalassinus* (212) has demonstrated that Cd, Hg and Pb had adverse effects on both lifespan and weight of the adult grasshoppers and the hatchability of the nymphs was also affected. A thorough review of the impact of heavy metals (Cd, Cu, Fe, Pb and Zn) on a number of invertebrates was made by Gerhardt (213).

However, the most interesting question is how metals affect animals which are used for food. Pharmacokinetic analyses have been conducted on published experimental data to estimate biotransfer factors for bovine milk (214). Some metals, especially Pb and As, can be transferred to milk when cows are fed with food containing metal salts, but metals from sewage sludge were not found to produce significant elevations of metals in the milk. These findings suggest that metals in sludge are not as bioavailable as metal salts. On the other hand, synergistic effects must be considered. Rats fed with zineb and aldicarb, two chemicals used in agriculture, together with Cu, showed pronounced synergistic effects between the metal and the pesticides (215).

Prediction models

Since heavy metals have adverse effects on organisms and thus are a potential threat to crops grown on sludge-amended soils, methods to predict metal uptake, particularly in plants, are interesting.

Computer modeling

The United States Environmental Protection Agency (US EPA) has developed a model for predicting heavy metal uptake by plants. This model is meant to be used for deciding whether a sludge can be permitted to use for land application. However, it has been shown that the model does not give the expected prediction (29). The US EPA model predicts that plant tissue metal concentration will be linearly related to metal loading rates in the soil, independent of soil structure, pH and other soil parameters. A comparison with field experiments shows that the model underpredicts Cd concentrations in leaves from soils at pH < 7 and overestimates leaf Cu for all fields and sweet corn grain Cd and Zn concentrations. At one site, almost all the plant tissue metal concentrations were overestimated with this model. These results show the difficulty of making general predictions of metal uptake without thorough analysis of the local soil characteristics. For example, studies comparing limed soil to unlimed soil have shown that soil pH is crucial for the metal uptake of plants (199).

GEOCHEM is a multi-purpose computer program for calculating the equilibrium speciation of the chemical elements in a soil solution. This program has been partially successful in the correlation of plant uptake to simple parameters (26, 30). Sweet corn uptake of Cd was strongly correlated with the concentration of $CdCl^+$ in soil, but not to the Cd^{2+} concentration. One drawback of this model is that an extensive input data is needed which demands a thorough investigation of the soil and sludge characteristics.

Another simpler approach is to calculate transfer coefficients (TC) for metals. This coefficient is often defined as metal concentration in plant / metal concentration in soil (150). A TC above 1 means that the metal is accumulated in the plants and a TC below 1 means that the plant takes up less than the average soil concentration. Cd and Zn have TC, 1-10; Ni and Cu have TC, 0.1-1 and Pb, Cr and Hg have TC, 0.01-0.1 (150).

Relation between metal speciation and biological relevance of metals

A useful aim for extraction methods is to find a correlation between biological uptake and an extraction method, preferably a single extractant. As previously mentioned, this is exceedingly difficult due to differences between different sludges and different species.

In 1971, Lakanen and Erviö (216) tried to correlate micronutrient uptake by oats to 8 extractants, mostly ammonium acetate and EDTA at different pH values. A good correlation was found for Mn, Co and Zn uptake but not for Cu, Mo and Fe

The uptake of Cd by four vegetables grown on soils contaminated from a variety of sources was investigated in a study in which 23 different soil parameters were compared with Cd concentration in the vegetables (26). Out of these 23 variables, only 8 were significantly related to Cd accumulation. The most frequently occurring parameter was total Cd, which was inversely related to plant Cd. This illustrates that just because a sewage sludge has a high metal content it is not necessarily unsuitable for agricultural application. Of course, the opposite is equally true; a low metal concentration is no guarantee for "safe" plant concentrations. Some studies (217) have concluded that the plant availability of heavy metals is not always predicted by extraction with DTPA (diethylenetriaminepentaacetic acid).

The uptake of six metals (Cd, Cr, Cu, Ni, Pb and Zn) to winter barley (*Hordeum vulgare* L.) was measured in a soil before and after sludge amendment. Only Cd and Zn increased in the plants, but these changes could not be correlated to the metal extracted by any of the five extractants applied to the soil (95).

However, there are some workers who have managed to find a significant correlation between a soil test and plant heavy metal concentrations. Their results identify DTPA and $CaCl_2$ as the most suitable extractants to mimic plant uptake, at least for metals such as Cd, Cu and Zn (15). Investigations of the transfer of Cd to food crops also demonstrated that DTPA extraction predicts Cd uptake for potatoes, lettuce and cabbage better than $CaCl_2$, EDTA and NH_4NO_3 (199). Other investigations confirm the suitability of $CaCl_2$ for predicting metal concentrations in lettuce, spinach, endive and sweet corn (218) as well as clover, barley and red beet (219). The same authors found a significant correlation between EDTA and DTPA extractions and the uptake of Cu, Ni and Zn in barley (38).

Banjoko and McGrath studied the uptake and tissue concentration of Zn in sweet corn (*Zea mays*) and found significant correlations to different extractants. Plant uptake was closely related to the H_2O_2 and EDTA extracted fractions, while tissue concentrations were related to $CaCl_2$ and H_2O_2 extracted fractions (87). Similar results were found by Soon et al (179), who found a significant correlation between NTA (nitrilotriacetic acid) extraction and Zn and Cd concentrations in sweet corn stover (*Zea mays*)(179).

Harmens et al (220) investigated Zn uptake in Zn-tolerant and Zn-sensitive catchfly, *Silene vulgaris*. $CaCl_2$ extractable Zn correlated well with extracellular Zn, although the correlation with Zn bound to the cell wall was poor. A major difference between the sensitive and the tolerant plants was that the sensitive plants showed higher Zn concentrations in the shoots. The restricted transport of Zn from the root in tolerant *Silene vulgaris* does not, however, seem to explain tolerance (220).

Davies investigated metal uptake by radish, (*Raphanus sativus L*), grown in soil contaminated from lead mining (204). Uptake of Cd was satisfactorily predicted by extraction with acetic acid. This method also predicted the uptake of Pb, but the most satisfactory correlation was found between uptake and total soil Pb. Including other soil properties, such as soil CEC, did not improve the significance of a regression analysis between plant uptake and acetic acid extraction for these two metals. Zinc uptake was best modelled using exchangeable Zn, extracted by NH_4NO_3, although the prediction was improved by including pH. The investigated parameters were soluble Mg, K and P, total soil metal, extraction with EDTA-diammonium, acetic acid and NH_4NO_3, soil pH, organic content and CEC. None of these parameters predicted Cu uptake satisfactorily. However, the soils investigated in this study exhibited a range of metal concentrations which was much wider than usual, which means that the prediction model calculated in this study might not be applicable to other, less contaminated soils. Also, the speciation of Pb contaminated soil from a lead mine may not be representative for Pb speciation in non-contaminated soil.

An investigation of the Cd uptake of sudax (*Sorghum bicolor*) from 17 different sludges showed a correlation between metal uptake and a range of soil extraction parameters; total Cd extracted with $Ca(NO_3)_2$ and $Ca(NO_3)_2$/EDTA, Chelex-100 resin extractable Cd and combinations of these parameters (141). The same investigation demonstrated a good correlation between Cd uptake in sudax and Cd extracted by the chelating resin, Chelex-100 (221).

Birke and Werner (222) investigated two different statistical methods to correlate $CaCl_2$-extracted Cd with Cd uptake in 9 crops; wheat, oat, beans, peas, spinach, lettuce, onion, radish and carrot. The crops were classified in two groups, above or below the German Cd uptake limit. A good correlation was found for all of them when using the Chi squared method, 70% of all samples were correctly classified, and for all except radish when using regression analysis (222). The same was found in a study by Sauerbeck and Styperek (223).

A solution of equilibrated soil suspensions was developed by Jopony (224). This was used to predict uptake of Pb in radish (*Raphanus sativus*) and red fescue (*Festuca rubra*). A good correlation between Pb content in the equilibrated soil suspension and plant uptake was found, whereas the correlation between plant uptake and total soil Pb was poor (28).

Pierzynski and Schwab (225) investigated Zn uptake in soybean (*Glycine max*, L.) after various soil amendments and compared Zn in soybean with different extractants. A good correlation was found, with the extractants containing KNO_3 having the best correlation with plant uptake; R^2 was 0.92 for KNO_3 extraction - plant Zn and 0.88 for extraction with KNO_3 + NaOH - plant Zn. A somewhat weaker correlation was also found for DTPA - plant Zn (225).

Gupta and Aten (226) have developed a mathematical model for predicting metal uptake based on extraction with different extractants. They conclude that $NaNO_3$ extraction correlates well with the uptake of Cu, Cd and Zn in lettuce and rye grass in all 13 soils tested. Other extractants, $CaCl_2$, KNO_3 and NH_4NO_3, also correlated well with plant metal uptake in some of the soil samples. In the same paper Gupta and Aten refer to unpublished results where they also found a good correlation between $NaNO_3$ extraction and Cd and Zn in fodder corn and wheat. Gupta has several publications considering extraction with $NaNO_3$ (227, 228, 229). Sanka and Dolezal (230) found similar results for sweet corn (*Zea mays*), where the uptake of Cd was successfully predicted by extraction with $NaNO_3$, as well as the uptake of Zn in the roots. Zinc uptake in the upper parts of the plant was, however, better predicted by NH_4OAc.

Mulchi et al (112) extracted soil samples with DTPA, Mehlich 1 and Mehlich 3, and correlated the results to metal uptake in tobacco. A good correlation was found between plant uptake for Cd, Cu, Mn and Zn. The correlation for Ni varied between the two sites tested, and the correlation for Pb and Fe was very low.

Addition of a chelating agent such as EDTA or DNP (2,4-Dinitrophenol) to soil can result in lower uptake of heavy metals in plants. Tomatoes showed a reduced accumulation of Cd from soil to which EDTA and DNP was added (231). This may be caused by a decrease in the concentration of free Cd ions. On the other hand, EDTA and DNP addition enhanced the transport of Cd from the roots into the above-ground plant parts. These results suggest that Cd-complexes are formed in the roots, which indicates that EDTA can be taken up by the roots, perhaps as a Cd-EDTA complex. This means that if the interest is mainly focused on the metal concentrations in the edible parts of the plant, addition of EDTA (and perhaps also DNP) can have both beneficial and adverse effects. EDTA and NTA have also been reported to decrease the toxicity of Pb to okra (*Abelmoschus esculentus*) plants (232, 233).

Sewage sludge has proved to be an excellent medium for the green alga *Chlorella pyrenoidosa*, but growth is inhibited by heavy metals. Addition of EDTA favours the growth of algal cells in activated sludge, thus implying that the toxicity of the various heavy metals is removed by this action. However, excessive EDTA addition might lead to nutrient deficiency due to chelation of essential elements (234).

The toxicity of sewage sludge to the marine bacterium *Photobacterium phosphoreum* decreases considerably after extraction with EDTA (79), which would indicate that EDTA extracts the bioavailable fraction of metals. However, in another investigation 50% of the total metal content of sludge-amended soil was extracted with EDTA which is equivalent to thousands of years of uptake by crops, thus indicating that extraction with EDTA may overestimate the bioavailable fraction on an environmental timescale (235).

The concentration of Cd found in the earthworms *Lumbricus rubellus* and *Dendrodilus rubidus*) shows a relation to the fraction extracted by 0.5% acetic acid that approaches direct proportionality (209). Extraction of similar sediments with EDTA or dilute HCl was correlated to the accumulation of Cd, Pb and Zn in the gastropod *Velacumantis australis*, and Pb in the polychaete worm *Phyllodoce novaehollandiae*, but when using a range of sediments with different chemical and physical properties, only Pb bioavailability to *Phyllodoce novaehollandiae* could be predicted with the two extractants (236).

Conclusions

Some general conclusions can be drawn from this study of the literature:

1. The most studied metals in sewage sludge are Cd, Cr, Cu, Ni, Pb and Zn. The main sources are industrial discharge (especially for Cr and Ni), urban runoff and domestic wastewater. Corrosion of drinking water pipes is an important source of Cu. Contribution from industry is decreasing, which highlights urban runoff as a source.

2. There are a number of methods to fractionate sludge metals with different chemical extractants. The most commonly used methods divide the sludge into the following fractions: exchangeable, carbonate, Fe/Mn oxides, organic and residual. However, the selectivity and specificity of most of these methods is poor and it is difficult to

compare the results from different methods.

3. Fractionation results show that Cd is probably the most mobile metal in sewage sludge, followed by Zn. These two metals might be expected to be bioavailable to a significant extent. Copper is mostly bound in the organic fraction and is not likely to be taken up by organisms. The most tightly bound metal is Pb, which means that it would be comparably unavailable to organisms.

4. The plant uptake of metals from sludge-amended soil depends not only on metal speciation in the sludge, but also on soil properties, such as pH and the presence of complexing agents. Plant uptake also varies between different plants and different metals. Tobacco, for instance, accumulates Cd very efficiently and is therefore not recommended for cultivation on sludge-amended soil. Leafy plants accumulate more metals than cereals and legumes. Fortunately, many plants have low metal concentrations in their edible parts.

5. Extractions with DTPA, $CaCl_2$ and EDTA have been found to correlate positively with plant uptake in some experiments. However, the results are not unambiguous. There seems to be no simple relation between plant uptake and total or fractionated metal concentrations.

Discussion

It would be preferable if an analysis of "biologically available" metals could be used to supplement measurements of total metal content. Determination of the different biologically relevant species of a metal can be achieved by a comparison of metal speciation in sludge with the results from biological experiments (16, 18, 30, 150, 160).

However, in this discussion it must be remembered that sewage sludge is not the only, or even the most important, source of heavy metals in soil. Metals can also enter the soil from atmospheric deposition, fertilisers and agrochemicals (237). Sewage sludge becomes a major source of metals for the soil to which it is applied, but on a global scale, only Hg, Ni and Zn exceed 2% of total soil input (15). An exhaustive search of the scientific literature made by Chang, Granato and Page (25) found very few, if any, toxic effects that could be related to metals from sewage sludge.

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Chapter 3. Experimental: Development of an osmotic alternative to NaCl in Microtox

Chapter 3. Experimental: Development of an osmotic alternative to NaCl in Microtox

Two methods of measurement were central to the research reported here, namely Anodic Stripping Voltammetry (ASV) and Microtox. An important consideration in studies of metal speciation is the development of analytical methods for separating individual species or defined fractions. Electrochemical analysis, such as ASV has been used to provide a separation of ionic metal from other metal species. The results have been evaluated with a toxicity test, namely Microtox.

Anodic Stripping Voltammetry was used to determine the electrochemically available fraction in studies of the partitioning of sludge bound metals into the aqueous phase. This fraction includes the chemically dissolved metals and some weak metal complexes, but not strongly bound complexes, such as metals bound to humic acid, or metals present in the colloid phase. In theory at least, the flux of metal across the diffusion layer in ASV is comparable to metal transport to a biological membrane and correlations have been found between ASV-lability and metal toxicity. ASV gives an indication of potential bioavailability under defined conditions and provides an important separation of relatively unavailable colloidally bound metals from potentially available free and weakly complexed metals. ASV was therefore used in this research unchanged from previous reports on the basis that minor improvements to the method would not significantly affect the findings reported here.

On the other hand, the lack of a suitable and rapid, comparative toxicity test in non-saline solutions was seen as a limitation for studies of metal toxicity in sewage sludge and indeed, the separation of sludge metals into different chemical fractions is of little practical interest unless the comparison to a definitive measure of potential bioavailability or toxicity can be made. To do this, some means of measurement of potential bioavailability is required. The method chosen in this study was Microtox, which measures the acute toxicity of a sample, solid or in solution, to a luminescent marine bacterium, *Photobacterium phosphoreum*. The Microtox concept consists of a photometer which measures light output from the bacteria, combined with a cooling element which incubates the bacteria at 15°C. The bacteria are bought freeze-dried and are reconstituted into a 2% NaCl solution just prior to measurement. The result is reported as an EC50 for 5, 15 or 30 minutes and the whole measurement is complete within the hour. The correlation between toxicity to Microtox and to other test species has been reported in the literature as being generally good. However, in this study Microtox is used to compare toxicity between similar samples to determine which is the most toxic, and to compare the toxicity of the same sample before and after a chemical extraction. Microtox is therefore not used here to determine the ultimate toxicity of sewage sludge.

One major drawback of Microtox for metal speciation studies is that the test is performed in a 2% NaCl solution due to the osmotic requirements of the bacteria. Chloride ions are known to interfere with the sample, *i.e.* by forming complexes with metal ions or extracting metals from solid samples. This means that the test shows the toxicity of the sample immersed or dissolved in 2% NaCl, which may be quite different from the true toxicity of the sample. In my research, I was interested in comparing sewage sludge toxicity to metal speciation, but since the concentration of NaCl in sewage sludge or agricultural soil is much less than 2%, the toxicity results indicated by the Microtox standard method may be misleading. Much of the preparatory work was therefore concerned with the search for an alternative osmotic agent.

Four criteria were formulated and considered essential for an alternative osmotic regulator:
Firstly, it must not affect the bacteria in any way; it should not improve their viability by acting as a nutrient or adversely affect their viability by being toxic to the bacteria.
Secondly, it must not affect dissolved toxicants by forming complexes or precipitate with the toxicant of interest.
Thirdly, it must not affect the sample, *i.e.* it should not dissolve a solid sample or change the speciation of any of the sample components.
Finally, for solid samples and metals, which both require a longer test time than 15 minutes, sodium must be present in a sufficient concentration to provide optimum luminescence.

Sodium chloride fails on criteria two and three for the assessment of metal toxicity, both in sewage sludge and in freshwater samples. To avoid the unwanted effects of NaCl, another osmotic agent which does not affect the sample or the bacteria had to be found. The results of my research point to sodium perchlorate ($NaClO_4$), which additionally is used by electrochemists as an electrolyte for metals and therefore has the added benefit of being suitable for electrochemical speciation studies. My research shows that it is also non-toxic to *Photobacterium phosphoreum*. $NaClO_4$ is soluble in water in high concentrations, does not extract metals from solid samples and does not form complexes with any common metal or other cation, with one important exception; $KClO_4$ is precipitated even at very low concentrations of K. However, K is seldom the main source of toxicity in an environmental sample, which means that the only effect of $KClO_4$ precipitation is that the concentration of perchlorate is slightly lowered. The selection of $NaClO_4$ involved testing a whole range of potential alternative solutions and some examples of typical results are presented in Table 3.1. In this case, osmotic pressure (S) was held constant and both EC50 (concentration at which 50% of light emission was inhibited) and viability of the blank (30 min / 0 min light emission) were calculated.

Both of the magnesium salts tested gave EC50 values for Cu which were ten times higher than the value obtained with NaCl (Table 3.1). It can be concluded that Mg^{2+} ion has a protective effect against ionic Cu toxicity. The results also indicate that Ca^{2+} might exert a similar, although less pronounced, effect. These effects in freshwater samples would normally be attributed to toxicity variation with water hardness.

To ensure good reproducibility and precision it is important that the luminescence of the blank is sufficient to fall within the range of the photometer and that it is stable during the 30 minutes that are required for measuring metal toxicity. Most of the surrogates tested did not meet this requirement (Table 3.1). The decreasing blank luminescence can then be explained by a gradually decreasing Na^+ concentration due to consumption of the NaCl that accompanies the bacterial inoculum. The NaCl present in the pellet of the freeze-dried bacteria is sufficient for measuring EC50 values after 5 or 15 minutes, but for measurement of solid phase (and particularly metal) toxicity a test period of 30 minutes is required and extra Na^+ must be added.

Table 3.1. Copper toxicity to *Photobacterium phosphoreum* in NaCl and a selection of the osmotic surrogates tested.

Surrogate substance	S mol l^{-1}	Surrogate concentration g l^{-1}	Blank(30min)/ Blank(0min)	EC50(30 min) Cu, µg l^{-1}
NaCl	0.541	31	1.00	200
Sucrose	0.551	240	0.60	-
Glycerol	0.530	80	0	-
$NaHCO_3$	0.555	50	1.13	654
NH_4Cl	0.559	30	0	-
$MgSO_4.7H_2O$	0.541	205	0.30	1955
$CaCl_2.2H_2O$	0.531	53	0.36	618
KCl	0.540	40	0.70	147
KNO_3	0.554	65	0.57	17

It was suspected that Na^+ severely limited luminescence and therefore a selection of different salts containing Na^+ was tested. The stable blank values confirmed our suspicion that Na^+ plays an important role in the light emission process. Of the osmotic surrogates tested, Na_2SO_4, $NaHCO_3$ and $NaClO_4$ were the most promising and were selected for further studies with other metals.

A complex wastewater or a solid sample, for example sewage sludge, usually contains a range of metals at potentially toxic concentrations. Therefore, the toxicities of Cd, Pb and Zn were tested in NaCl, $NaHCO_3$, Na_2SO_4 and $NaClO_4$ (Table 3.2).

The results in Table 3.2 demonstrate that the toxicity of metals can be highly variable depending on the choice of osmotic solution. In contrast to NaCl, neither Na_2SO_4 nor $NaClO_4$ are expected to form complexes with metal ions (except for the possible formation of $PbSO_4$ (s) in Na_2SO_4) and consequently, the EC50 values for Cd, Cu and Zn are similar in Na_2SO_4 and $NaClO_4$. The fact that EC50 values for NaCl differ from the surrogates is in accordance with the formation of chloride complexes with the metal ions which have a different toxicity from ionic metal. This also indicates that the use of Na_2SO_4 and/or $NaClO_4$ might give toxicity data that have a closer resemblance to reality for samples which do not have a naturally high chloride concentration.

Table 3.2. Toxicity of metals to *Photobacterium phosphoreum* in sodium containing osmotic surrogates.

	EC50(30 min), µg l^{-1}		
	$NaCl$	Na_2SO_4	$NaClO_4$
Cd	12 000	1 060	690
Cu	170	70	74
Pb	210	-	54
Zn	340	2 300	1 800

Contact toxicity for sewage sludge confirmed that osmotic surrogates that contain sodium, but not chloride, give EC50 values both for raw and for reference sludge that are very similar and significantly different from the EC50 values found when NaCl is used (Table 3.3).

Table 3.3. Contact toxicity of sewage sludge in osmotic surrogates.

Surrogate substance	Toxicity, g l^{-1}		
	Raw sludge	Digested sludge	Reference sludge
KCl	3.5	0.61	0.13
$NaCl$	0.89	0.42	0.36
$NaNO_3$	0.48	0.17	0.15
$NaHCO_3$	0.27	0.26	0.036
Na_2SO_4	0.49	1.0	0.15
$NaClO_4$	0.42	0.068	0.10
Mannitol	1.1	0.45	1.4
Sucrose	1.1	0.27	0.67

Results of the solid phase experiments show that the use of any of the osmotic surrogates $NaClO_4$, Na_2SO_4 or $NaNO_3$ give similar EC50 values both for raw and for reference sludge. These EC50 values differ significantly from the results obtained with

NaCl. Most environmentally interesting solid samples (sediments, soils or sludges) can be suspected of containing Pb, and because the use of Na_2SO_4 leads to $PbSO_4$ precipitation, the use of $NaClO_4$ may be the best choice for such samples. This phenomenon could be useful as parallel toxicity tests of solid samples in Na_2SO_4 and $NaClO_4$ might differentiate the toxicity of Pb from other metals.

CONTACT TOXICITY OF METALS IN SEWAGE SLUDGE: EVALUATION OF ALTERNATIVES TO SODIUM CHLORIDE IN THE MICROTOX® ASSAY

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CONTACT TOXICITY OF METALS IN SEWAGE SLUDGE: EVALUATION OF ALTERNATIVES TO SODIUM CHLORIDE IN THE MICROTOX® ASSAY

C.E. ANNIKA CARLSON-EKVALL* and GREGORY M. MORRISON
Department of Sanitary Engineering, Chalmers University of Technology, S-412 96 Göteborg, Sweden

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Abstract—The presence of chloride ions in the Microtox® test can cause problems when testing metal toxicity, both due to extraction of metals from solid samples and formation of chloro complexes of metals in the liquid phase. To investigate alternatives to NaCl in the Microtox test, the toxicity of Cu, Cd, Pb, and Zn to *Photobacterium phosphoreum* was tested in 28 osmotic surrogates for NaCl. It was found that Na^+ must be present to keep the blank luminescence stable for 30 min. The results point to $NaClO_4$ as the most satisfactory surrogate solution as it has an inert behaviour and does not form complexes with any metal of environmental interest. Raw, digested, and reference sewage sludges were tested in the osmotic surrogates. The EC50 values for sludges were lower in solutions of $NaNO_3$, Na_2SO_4, and $NaClO_4$, and higher in sucrose, mannitol, and KCl, compared to NaCl. $NaClO_4$ can be recommended as an osmotic surrogate for sewage sludge testing. Another problem with the Microtox assay is the lack of pH control in the cuvette. Copper toxicity tests were carried out in Tris buffer and KH_2PO_4 at two different concentrations and at pH 7 and 8. The results show that 1 mM phosphate buffer, pH 7.0, can be recommended for solid samples. However, owing to formation of $KClO_4$ a buffer containing potassium is not recommended in combination with $NaClO_4$.

Keywords—Sewage sludge Toxicity Microtox® Metals Osmotic surrogates

INTRODUCTION

Screening of water sample toxicity with the marine bacterium *Photobacterium phosphoreum*, especially in combination with toxicity identification evaluation procedures, is now accepted as a realistic alternative to more conventional toxicity tests [1,2]. The *Photobacterium* test has recently been applied to contact-toxicity studies of solid samples such as sediments [3] and sewage sludge [4]. Although hydrophobic organic compounds inhibit light emission in the presence of urban river sediments [5], contact toxicity in sewage sludge can, to a great extent, be attributed to metals [4].

It should be stressed that the 2% (w/v) osmotic adjustment solution, used during the contact of *Photobacterium* with the solid phase being tested, can markedly increase metal concentration in the solution owing to direct extraction of metals as chloro complexes. There is also a risk that an excess of chloride ions converts other metal species into chloro complexes, thus altering the toxicity of the sample [6]. This may give misleading results for samples such as sewage sludge [4] and natural soil solutions, where most of the Cd, Mn, Ni, and Zn is found as hydrated free ions and Cu mostly as organic complexes [7]. Sludge/water partitioning will also be affected by the pH of the sample under investigation, and, as the osmotic adjuster contains no buffer, unreliable results may be obtained. A sample with a pH outside the range where *Photobacterium* can perform may also give an apparent toxicity as the luminescence may be inhibited by pH effects rather than true toxicity.

For studies of metal toxicity, a nonextracting osmotic surrogate should be used. The most used surrogate is sucrose [8,9], although this may act as a nutrient. The presence of a nutrient may affect luminescence in two ways. First, *Photobacterium* light emission starts when the logarithmic growth phase is already far advanced [10,11] and a nutrient addition may start a new growth phase, during which the luminescence initially decreases. Second, many metals are transported into the bacterial cell through energy-dependent transport systems. The latter is much slower because of transport-limited conditions, which means that metals taken up by an energy-driven transport may be less inhibiting under conditions of starvation. A study by Brynhildsen et al. [12] showed that the toxicity of Cd, Cu, and Zn to a *Klebsiella* species increased considerably when glucose was added, and, indeed, glucose has been shown to stimulate the light-producing system [1]. On the other hand, inhibition of luminescence by catabolic repression has been observed in *Vibrio fischeri* [13], which shows the difficulty of predicting exactly what will happen in the presence of a nutrient.

To assess the influence of varying ionic strength, Hinwood and McCormick [8] studied the toxicity of Cd, Cu, and Zn in solutions with salinities ranging from 1 to 7% (w/v) and in 20.4% (w/v) sucrose. They found that EC50 values for Zn and Cd varied by two orders of magnitude, the highest salinities giving the highest values. This would be in accordance with the theory that metals form chloro complexes that have a lower toxicity than the hydrated cation. However, experiments with Cu showed similar results for different salinities that, if this theory is correct, can only occur if the toxicity of copper chloride is the same as for the free copper ion. This

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*To whom correspondence may be addressed.

is somewhat contrary to commonly held views. Hinwood and McCormick [8] suggested that the sensitivity of *Photobacterium phosphoreum* decreases with increasing ionic strength. This is confirmed by experiments showing that the measured toxicity of ammonia, which is not expected to form chloride complexes, decreases with increasing salinity. Ankley et al. [3] found sediment elutriate toxicity to be less in seawater (3.5% w/v NaCl) than in distilled water, osmotically adjusted with 2% (w/v) NaCl. A comparison of six single toxicants tested in 20.4% (w/v) sucrose, which has a very low ionic strength, and in 2.0% (w/v) NaCl, showed that Zn and Ni are much more toxic to *Photobacterium phosphoreum* in sucrose than in NaCl [9]. The bacteria may be stressed by the less than optimum osmotic pressure, and such stress could affect the toxicity measurement [8], which would explain why low salinities lead to an increase in sensitivity. However, Hinwood and McCormick [8] measured the 5-min EC50 value for phenol over a range of salinities from 1 to 7% (w/v) and found no significant difference between high and low salinities; these results tend to contradict the theory that ionic strength influences toxicity. Ankley et al. [9] also point out that even though some metals are more toxic in sucrose than in NaCl, chlorine showed almost no toxicity in sucrose, indicating that the theory that different ionic strengths can explain differences in toxicity between different media is perhaps not the whole truth, at least for nonmetallic toxicants, and other parameters have to be taken into consideration.

This work approaches the problems inherent in the measurement of contact toxicity with respect to metals in sewage sludge. Particular emphasis is placed on the provision of osmotic surrogates that do not complex with or, indeed, extract metals in sewage sludge.

MATERIALS AND METHODS

The Microtox® experiments were performed with a Microtox 500 incubator block (Microbics, Carlsbad, CA). The bacteria used were Microtox Reagent, a freeze-dried culture of *Photobacterium phosphoreum*, which was reconstituted with Microtox Recon solution. The solid-phase tests were performed with Microtox solid-phase test tubes and microsieves. The standard Microtox diluent and osmotic adjustment solution were substituted for the solutions under investigation. All chemicals were of *pro analysi* quality.

Both raw and digested sludges were sampled at the Ryaverket wastewater treatment plant in Göteborg, Sweden. Ryaverket receives sewage from 550,000 individuals as well as urban runoff and some industrial effluents. The reference sludge was "sewage sludge of domestic origin," reference material no. 144, from the Community Bureau of Reference (BCR).

RESULTS

Variation of pH, buffer, and ionic strength

One of the drawbacks of the standard Microtox method is the complete lack of pH control in the test cuvette. This is especially important in the solid-phase test; for example, digested sewage sludge is slightly acid and is difficult to compare to limed sludge, which is basic. Luminescent bacteria are not affected by pH within the range 4.5 to 9.5 [6], and certainly limed sludge can have a pH close to this upper limit [4]. Experiments with simultaneous variations of pH (pH 7, pH 8), buffer (Tris, KH_2PO_4), buffer strength (1 mM, 10 mM), osmotic agent (NaCl, sucrose), and NaCl concentration (2%, 3.1% w/v) were carried out. A total of 24 samples was needed. The experimental plan is shown in Table 1, and the results were evaluated by an ANOVA analysis performed with a statistical program (Statgraphics).

In the standard Microtox test, 2% NaCl is used. However, Hinwood and McCormick [8] suggested that 2% NaCl may result in an osmotic pressure less than optimal and lead to osmotic stress; therefore, a 3.1% NaCl solution was included in these tests. Watanabe and Hastings [11] tested the light output of *Photobacterium phosphoreum* in salinities between 1 and 8% and concluded that 3% gave maximum luminescence. Hinwood and McCormick [8] evaluated sucrose as an alternative to NaCl, and their research showed that 20.4% sucrose gave the highest light output; the same sucrose concentration was also used by Ankley et al. [9]. The 5-min EC50 value was chosen in these experiments because of the very weak luminescence of the samples containing sucrose after 15 and 30 min. This agrees with the findings presented later in this report and by Watanabe and Hastings [11] that Na is essential for luminescence.

The ANOVA analysis shows that buffer concentration is the most important factor (Table 2) and has an effect on the EC50 values; this is statistically significant at the 1% level, whereas the choice of buffer and osmotic pressure has a less significant influence. The F value for the choice of buffer is 4.599, which is only slightly above $F = 4.49$, which corresponds to the 5% significance level, whereas the osmotic pressure has an F value just below the 5% significance level. The effect of choice of buffer can be explained by an increase in cell permeability to chemicals through partial release of outer membrane components; such an effect has been reported for Tris buffer [14]. Experiments with outer membrane-defective mutants of *Photobacterium phosphoreum* have shown that the outer membrane is involved in the regulation of bacterial bioluminescence [15]. In this study, the average EC50 value for Cu in Tris buffer for all experiments was 5,150 ± 2,640 μg/L compared to 15,300 ± 6,020 μg/L in phosphate buffer. No significant difference was found between pH 7.0 and pH 8.0, which accords with previously reported results [6]. Because the results for 1 mM phosphate buffer, pH 7.0, were very similar to the results from the standard procedure, the osmotic surrogates investigated in the following experiment were dissolved in this buffer. The use of a 1-mM buffer can therefore be recommended where pH control is desired. This is particularly important where metal toxicity is to be investigated.

Test of osmotic surrogates

Copper toxicity in the liquid-phase test. Using the Microtox standard procedure for measuring Cu toxicity in a solution, 20 different substances (in phosphate buffer) were tested as substitutes for the 2% (w/v) NaCl that is normally used (Table 3). Test concentration was chosen on the basis of osmotic pressure exerted. The EC50 values for Cu at 5, 15, and 30 min were measured as well as the luminescence of the

Table 1. Five-min EC50 values for Cu in different solutes with four parameters varied simultaneously

	Varied parameters				
Osmotic agent	Osmotic pressure (mol/L)	Buffer	Buffer concn. (mmol/L)	pH	5-min EC50 (μg/L)
NaCl, 2%	0.35	Tris	10	7.0	–*
	0.35	Tris	10	8.0	5,800
	0.35	Tris	1	7.0	800
	0.35	Tris	1	8.0	1,700
	0.35	Phosphate	10	7.0	31,000
	0.35	Phosphate	10	8.0	25,000
	0.35	Phosphate	1	7.0	1,600
	0.35	Phosphate	1	8.0	3,300
NaCl, 3.1%	0.54	Tris	10	7.0	4,100
	0.54	Tris	10	8.0	26,000
	0.54	Tris	1	7.0	1,600
	0.54	Tris	1	8.0	800
	0.54	Phosphate	10	7.0	35,000
	0.54	Phosphate	10	8.0	68,000
	0.54	Phosphate	1	7.0	3,500
	0.54	Phosphate	1	8.0	1,400
Sucrose, 20.4%	0.44	Tris	10	7.0	1,200
	0.44	Tris	10	8.0	8,700
	0.44	Tris	1	7.0	–
	0.44	Tris	1	8.0	850
	0.44	Phosphate	10	7.0	11,000
	0.44	Phosphate	10	8.0	1,600
	0.44	Phosphate	1	7.0	880
	0.44	Phosphate	1	8.0	990

*–, no luminescence detected.

blank after 30 min compared to the initial blank value. One of the demands that the surrogate substance has to fulfill is that it must not adversely affect bacterial viability. Some of the osmotic surrogates – for example, the alcohols – were apparently toxic to the bacteria, and the sample showed no luminescence at all after only a few minutes. Some of the sugars, namely fructose, sucrose, and maltose, showed results with a very weak correlation between luminescence and concentration. This was probably due to complexation of the Cu and the very high viscosity of the solution. This made it very difficult to calculate reliable EC50 values. When using these sugars, concentrations between 15 and 24% (w/v) are needed to obtain sufficient osmotic pressure.

Both of the magnesium salts tested gave EC50 values for Cu that were 10 times higher than the value obtained with NaCl. It can be concluded that the Mg^{2+} ion has a protective effect against Cu^{2+} ion toxicity. It has been shown that Mg^{2+} has a stabilising effect on the outer membrane of gram-negative bacteria, such as *Photobacterium phospho-*

Table 2. ANOVA analysis of copper EC50 values in the varied parameters

Variation	Sum of squares	*d.f.*	Mean sum of squares	*F* value
Main effects	3,464	5	693	4.563
Buffer	698	1	698	4.599
Buffer concentration	1,959	1	1,959	12.906
pH	128	1	128	0.843
Osmotic pressure	1,001	2	501	3.297
Residual	2,429	16		
Total	5,892	21		

Table 3. Copper toxicity to *Photobacterium phosphoreum* in NaCl and a range of osmotic surrogates

Surrogate substance	*S* (mol/L)	Concn. (g/L)	Blank(30 min)/ blank(0 min)	30-min EC50 (μg/L)
NaCl	0.541	31	1.00	200
Sucrose	0.551	240	0.60	–
D-Fructose	0.543	150	0.32	–
D-Glucose·H_2O	0.550	165	0.53	193
Maltose·H_2O	0.540	253	0.51	180
Mannitol	0.537	150	0.77	287
Ethylene glycol	0.560	60	0	–
Glycerol	0.530	80	0	–
Propylene glycol	0.547	70	0	–
Citric acid·H_2O	0.570	175	0	–
Na citrate·$2H_2O$	0.572	114	0.99	–
$NaNO_3$	0.568	50	0	–
$NaHCO_3$	0.555	50	1.13	654
NH_4Cl	0.559	30	0	–
$(NH_4)_2SO_4$	0.558	65	0	–
$MgCl_2 \cdot 6H_2O$	0.577	75	0.39	1,792
$MgSO_4 \cdot 7H_2O$	0.541	205	0.30	1,955
$CaCl_2 \cdot 2H_2O$	0.531	53	0.36	618
KCl	0.540	40	0.70	147
KNO_3	0.554	65	0.57	17
$KHCO_3$	0.559	60	0	–

reum [14]. The results also indicate that Ca^{2+} might exert a similar, although less pronounced, effect. These effects in freshwater samples would normally be attributed to toxicity variation with water hardness. There are results that suggest that K^+ has a destabilising effect on the bacterial outer membrane [14], which may explain the very low EC50 for KNO_3 and the absence of luminescence in the presence of $KHCO_3$. The EC50 of Cu in KCl is close to the EC50 obtained when NaCl is used, but this may be because of the effect of chloride. Hinwood and McCormick [8] suggest that changing ionic strength affects EC50 values for metals, but these results were not confirmed in our study.

Sodium-containing osmotic surrogates. To ensure good reproducibility and precision it is important that the luminescence of the blank is sufficient to fall within the range of the photometer and that it is stable during the 30 min that are required for measuring metal toxicity. Most of the surrogates did not meet this requirement (Table 3). It was thought that the presence of Na^+ might be necessary for light emission, as suggested by Watanabe and Hastings [11]. The decreasing blank luminescence can then be explained by a gradually decreasing Na^+ concentration due to consumption of the NaCl that accompanies the bacterial inoculum. The NaCl present in the pellet of freeze-dried bacteria is sufficient for measuring EC50 values after 5 or 15 min [6], but for measurement of solid-phase (and particularly metal) toxicity a test period of 30 min is required and extra Na^+ must be added.

Watanabe and Hastings [11] showed that the ionic requirement for growth of *Photobacterium phosphoreum* could be satisfied by either Na^+ or K^+, but the development of luminescence is highly specific for Na^+ [11]. Luminescence is very weak in 1% (w/v) NaCl and peaks at 3% (w/v) NaCl, where the luminescence is 10,000 times stronger. Clearly, sodium ion plays an important role in the regulation of luminescence. It is likely that a high intracellular K^+ concentration is important for the transcription of luminescence genes [16]. This increased concentration of K^+ can be achieved by a high extracellular concentration of Na^+ [17] through the Na^+/K^+ pump.

A selection of different salts containing Na^+ was tested (Table 4). Instead of keeping the osmotic pressure constant, the concentration of Na^+ was adjusted to 0.53 M, which corresponds to 3.1% (w/v) NaCl. This concentration is recommended by Hinwood and McCormick [8] to minimise osmotic stress, although the results obtained by Watanabe and Hastings [11] indicate that the positive results with this concentration may be due more to a suitable sodium concentration than to osmotic stress. The salts were dissolved in distilled water and the pH was adjusted to a neutral pH with H_2SO_4 or NaOH where required (the amount of extra Na^+ added with the NaOH is negligible). Results are shown in Table 4. The stable blank values suggest that Na^+ plays an important role in the light-emission process. Some of these salts—for example, Na-acetate, $Na_2S_2O_3$, and Na-salicylate—gave little or no luminescence, probably because the osmotic surrogate is in itself more toxic than the Cu dissolved in it. Some salts (Na_2-β-glycerophosphate, Na-lactate, and NaH_2PO_4) gave EC50 values that were considerably higher than those measured in NaCl, probably due to the formation of nontoxic complexes. Of the osmotic surrogates tested, Na_2SO_4, $NaHCO_3$, and $NaClO_4$ were the most promising and were selected for further studies with other metals.

Table 4. Copper toxicity to *Photobacterium phosphoreum* in osmotic surrogates containing sodium

Surrogate substance	Concn. (g/L)	Cu 30-min EC50 (μg/L)	Blank(30 min)/ blank(0 min)
NaCl	31.0	170	0.94
$NaCH_3COO$	43.5	–	0.62
Na_2SO_4	37.7	70	0.83
$NaHCO_3$	50.0	360	0.68
$NaClO_4$	81.3	74	0.52
Na lactate	59.4	1,200	0.70
$Na_2HPO_4 \cdot 7H_2O$	71.1	>100,000	0.93
Na salicylate	84.9	–	0
$Na_2S_2O_3$	65.8	–	0.72
Na_2-β-glycerophosphate	81.1	14,300	0.83

Toxicity of Cd, Pb, and Zn in the liquid-phase test. A complex wastewater or a solid sample (for example, sewage sludge) usually contains a range of metals at potentially toxic concentrations. Therefore, the toxicities of Cd, Pb, and Zn were tested in NaCl, $NaHCO_3$, Na_2SO_4, and $NaClO_4$ (Table 5). It was very difficult to correlate the luminescence found in the $NaHCO_3$ solutions with the concentration of toxicant. The complete absence of a trend showing lower toxicity at higher toxicant concentration made it impossible to calculate EC50 values, and this is the reason that $NaHCO_3$ is not included in Table 5. This effect may be due to formation of gaseous CO_2 in the cuvette, which disturbs the measurement of the luminescence as well as the stability of the chemical environment of the bacteria. When choosing an osmotic surrogate, the solubility of the anion in combination with the tested metal is of importance.

For example, in the case of Pb toxicity in the presence of Na_2SO_4, $PbSO_4(s)$ precipitates before the toxic concentration of Pb is reached and, consequently, no toxicity is found for Pb in Na_2SO_4 (Table 5). This was the reason for testing $NaClO_4$, in which most metal ions are very soluble, in the experiment. Neither Cu, Cd, Pb, Zn, nor any other metal of environmental concern forms a complex with the ClO_4^- ion, which effectively makes $NaClO_4$ the most suitable osmotic surrogate for metal toxicity testing. Also, $NaClO_4$ showed the highest sensitivity to metals, with the exception of Zn.

Table 5. Toxicity of metals to *Photobacterium phosphoreum* in sodium-containing osmotic surrogates

	30-min EC50 (μg/L)		
Metal	NaCl	Na_2SO_4	$NaClO_4$
Cd	12,000	1,060	690
Cu	170	70	74
Pb	210	–	54
Zn	340	2,300	1,800

The results in Table 5 demonstrate that the toxicity of metals can be highly variable depending on the choice of osmotic solution. Cadmium is 17 times more toxic in $NaClO_4$ than in NaCl, and the three other metals also show great variability of the EC50 value. In NaCl, the bacteria tolerate Cd concentrations many times higher than the tolerable Zn concentration; the EC50 for Cd is 35 times the Zn EC50. On the other hand, Cd is twice as toxic as Zn in the other two osmotic surrogates (Table 5). In contrast to NaCl, neither Na_2SO_4 nor $NaClO_4$ is expected to form complexes with metal ions (except for the formation of $PbSO_4(s)$) and, notably, the EC50 values for Cd, Cu, and Zn are very similar in Na_2SO_4 and $NaClO_4$. The fact that EC50 values for NaCl differ from the surrogates accords with the theory that Cl^- forms complexes with the metal ions. The results in Table 5 indicate that chloro complexes of Cd, Cu, and Pb have a lowered toxicity compared to the free ion, although for Zn the opposite is true. This also indicates that the use of Na_2SO_4 and/or $NaClO_4$ might give toxicity data that have a closer resemblance to reality for samples that do not have a naturally high Cl^- concentration.

Solid-phase test. To study whether NaCl could be substituted in the solid-phase test, the contact toxicities of three solid samples, raw sludge, digested sludge, and reference sludge, were tested in eight different osmotic surrogates. Four of the osmotic surrogates in Table 6 are in concentrations corresponding to the same osmotic pressure as a 3.1% (w/v) NaCl solution: 20.5% (w/v) sucrose; 15% (w/v) mannitol; 5% (w/v) $NaNO_3$; and 4% (w/v) KCl. The remaining three osmotic surrogates were used in concentrations that resulted in a final sodium concentration of 0.53 M, corresponding to a concentration of 3.1% (w/v) NaCl: 3.8% (w/v) Na_2SO_4; 5.0% (w/v) $NaHCO_3$; and 8.1% (w/v) $NaClO_4$ (Table 6).

Tests of subsamples of each sludge type showed that the EC50 values for raw and digested sludge had a standard deviation of 15%. For reference sludge a higher standard deviation, 48%, was found. The results in Table 6 show that raw sludge is less toxic in sucrose and mannitol than in NaCl. The same applies to the reference sludge, but digested sludge does not show the same trend. The EC50 value of digested sludge in sucrose is lower than in NaCl, which indicates an increased toxicity, but for mannitol the difference is not significant. Bacteria dissolved in osmotic surrogates containing sodium but not chloride show a significantly higher sensitivity for raw sludge and reference sludge than bacteria in NaCl. With the exception of Na_2SO_4, this is also true for digested sludge. Digested sludge is more toxic than raw sludge in most of the osmotic surrogates. This can be explained by the fact that heavy metals are present in higher concentrations in digested sludge than in raw sludge owing to the loss of organic material in the digestion procedure [4]. Reference sludge is the most toxic sludge type except when the sugars are used. The reference sludge is ground to a much smaller grain size, and this could possibly affect the result of the toxicity test [18,19]. The bacteria can adhere to the sample surface and thus fail to pass the microsieve used in the solid-phase test to separate bacteria and sample before measurement. However, as the main purpose of this study was not a comparison of different sludges but a comparison of the effects of different osmotic surrogates on the same sludge, the possible effects of grain size and structure were not considered here.

Table 6. Contact toxicity of sewage sludge in osmotic surrogates

Surrogate substance	Toxicity (g/L)		
	Raw sludge	Digested sludge	Reference sludge
KCl	3.5	0.61	0.13
NaCl	0.89	0.42	0.36
$NaNO_3$	0.48	0.17	0.15
$NaHCO_3$	0.27	0.26	0.036
Na_2SO_4	0.49	1.0	0.15
$NaClO_4$	0.42	0.068	0.10
Mannitol	1.1	0.45	1.4
Sucrose	1.1	0.27	0.67

The results show that the osmotic surrogates that contain sodium but not chloride give EC50 values both for raw and for reference sludge that are very similar and significantly different from the EC50 values found when NaCl is used. The only exception is $NaHCO_3$, which as shown earlier in this report gives unpredictable results.

DISCUSSION

When using the Microtox test, some kind of osmotic regulation is required. The addition of this osmotic regulator must lead to reproducible conditions and must not affect the relevance of the results. Three criteria are important when choosing this osmotic regulator.

First, it must not affect the bacteria—that is, it should not be toxic to the bacteria or act as a substrate for bacterial growth. Second, it must not affect dissolved toxicants—that is, it should not form complexes or precipitate with the toxicant. Third, it must not affect the sample—that is, it should not dissolve a solid sample or change the speciation of any of the sample components.

For solid samples and metals, which both require a longer test time than 15 min, a fourth criterion could be formulated, namely that sodium must be present in a sufficient concentration to provide optimum luminescence.

Chloride ions are suspected of both forming complexes with dissolved metal ions and changing the speciation of metals in sewage sludge, and this is why an effort has been made to find an osmotic surrogate for NaCl. Because the presence of Na^+ is necessary for maintaining the luminescence reasonably stable for the 30-min contact time required to assess metal toxicity, the choice of osmotic surrogates is limited. When investigating different Na-containing salts, many of them were found to be toxic to the bacteria. Of the remaining salts, some did not meet the second criterion, but formed nontoxic complexes with metals or contained anions that formed insoluble salts with the tested metals.

The results of this study point to $NaClO_4$ as the most suitable and promising osmotic surrogate. It is nontoxic, dissolves readily in water, and has a very inert behaviour in water solution. Very few metals form complexes with $NaClO_4$, with the important exception of K^+. This makes it difficult to use $NaClO_4$ in combination with a phosphate buffer containing K^+. In most cases when single metals are studied, or when complex samples such as wastewater or sewage sludge are studied, no buffer is required and $NaClO_4$ can be used. Na_2SO_4 has the advantage of a more stable blank value, but because the solubility of $PbSO_4$ is lower than the toxic dose of Pb, Na_2SO_4 is unsuitable for any sample where Pb as a source of toxicity cannot be completely excluded. For testing single chemicals or samples that do not contain Pb, Na_2SO_4 can be used.

Results of the solid-phase experiments show that use of any of the osmotic surrogates $NaClO_4$, Na_2SO_4, or $NaNO_3$ gives almost the same EC50 values both for raw and for reference sludge. These EC50 values differ significantly from the results obtained with NaCl. This may be because the error introduced in the standard toxicity test by the addition of chloride ions to a sample naturally low in chloride is eliminated. However, earlier experiments with Cu in $NaNO_3$ (Table 3) gave no luminescence, and therefore this osmotic surrogate cannot be recommended without further tests. Most environmentally interesting solid samples (sediments, soils, or sludges) can be suspected of containing Pb, and because the use of Na_2SO_4 leads to $PbSO_4$ precipitation, the use of $NaClO_4$ may be the best choice for such samples. Parallel toxicity tests of solid samples in Na_2SO_4 and $NaClO_4$ might differentiate toxicity of Pb from other metals.

In the case of solid samples with pH values outside the optimum range for the bacteria, a buffer is preferred. A suggestion is 1 mM KH_2PO_4, pH 7, and a solution of 3.8% (w/v) Na_2SO_4, which works for most samples as long as Pb is not responsible for the toxicity. Otherwise, $NaClO_4$ can be used with another buffer that does not contain K^+. Both these suggestions will give more accurate results for samples—for example, sewage sludges—which usually have a chloride content significantly lower than the 2 to 3.1% (w/v) present in the standard Microtox test procedure.

It is not possible at present to exclude Cl^- completely from the Microtox test procedure as some NaCl is present in the freeze-dried bacteria, and a very small amount will be added with the bacteria in each test cuvette. However, this small concentration of chloride is negligible compared to the concentration of the osmotic surrogate.

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Chapter 4. Results and discussion: Metal fractionation, sludge components and toxicity

Chapter 4. Results and discussion: Metal fractionation, sludge components and toxicity

Since the total metal content of a sludge-amended soil can represent a poor measure of metal bioavailability and toxicity, other methods should be developed. It is widely known that sediments are composed of different fractions with varying physico-chemical properties. Sewage sludge is also composed of different fractions such as organic matter coming from wastewater and inorganic precipitates of coagulants added in the wastewater treatment process. These fractions can contain metals in varying concentrations. This common property for sediments and sludge opens up the possibility of using chemical extraction schemes, originally developed for sediments, on sludge samples, to separate metals from the different sludge fractions. Here, a variation on a commonly used sequential extraction scheme was applied to sludge samples from five different stages of the processing of sewage sludge at a wastewater treatment plant.

Four metals (Cd, Cu, Pb and Zn) were fractionated into five fractions of sludge, namely exchangeable, carbonates, complexed, organic and Fe/Mn hydrous oxides (Table 4.1 and Table 4.2). Subsamples of sludge were treated with the following chemical extractants; ammonium acetate (exchangeable), sodium acetate (carbonate), EDTA (complexed), hydrogen peroxide (organic) and hydroxylamine hydrochloride (Fe/Mn hydrous oxides). Contrary to most other extraction schemes, where extractants are applied sequentially, the extractants were applied singly. The sludges were sampled at the Ryaverket wastewater treatment plant, Göteborg. Samples were raw, raw dewatered, limed, digested and digested dewatered sludge.

The results showed that Cd is readily extracted from all sludge types. Both EDTA and hydroxylamine hydrochloride extracted 100% of the total Cd in two sludges, and in three of the samples, 55-75% of total Cd was extracted by hydrogen peroxide. Three of the samples had more than 15% in the exchangeable fraction. Zn was also found to be easily extracted by hydroxylamine hydrochloride (Table 4.1). Hydrogen peroxide extracted 65-82% of total Zn in all sludges except the limed sludge.

Table 4.1. Single extraction results for Zn in sewage sludge.

	% of total Zn				
	Ammonium acetate	Sodium acetate	EDTA	Hydroxylamine hydrochloride	Hydrogen peroxide
Raw sludge	0.8	7.1	34	82	75
Raw dewatered	1.1	19	42	90	82
Limed	19	0	0	90	23
Digested	1.2	0	33	96	70
Digested dewatered	0.04	23	40	79	65

Table 4.2. Single extraction results for Pb in sewage sludge.

	% of total Pb				
	Ammonium acetate	Sodium acetate	EDTA	Hydroxylamine hydrochloride	Hydrogen peroxide
Raw sludge	0	1.6	22	25	11
Raw dewatered	17	11	36	24	33
Limed	0	0	0	16	0
Digested	0	14	24	14	9.9
Digested dewatered	0	21	18	4.9	0

No extractant removed more than 55% of total Cu. Hydrogen peroxide was the most effective extractant (35-55%), followed by EDTA (20-30%). The results for Pb were even lower (Table 4.2); no extractant removed more than 25% of total Pb except in the raw dewatered sludge where EDTA extracted 36% and hydrogen peroxide extracted 33%.

There is no marked difference between sludge samples before and after dewatering or between digested and raw sludge. However, limed sludge is very different from the other sludges. Both Zn and Pb show lower concentrations in most extractants in the limed samples.

Generally, Cd and Zn were readily extracted by the weaker extractants. Copper was more difficult to extract due to its affinity to the organic/sulphide phase and Pb was mostly bound to the residual fraction.

However, the relevance of these extractions can be discussed. These extractants indicate the geochemical partitioning of metals within the sludge particle, but provide little information on surface binding or partitioning into soil-water solution when applied to agricultural soil. It was therefore decided to investigate the partitioning of metals into the aqueous phase under controlled conditions to allow interpretation in terms of both mobility and potential bioavailability.

The partition coefficient, K_D, is strictly defined as a volume/solid partitioning between adsorbed metal (mg kg^{-1}) and the equilibrium dissolved concentration (mg l^{-1}), although in practice K_D is an operational partitioning of particulate and aqueous metal after a specified contact time. The aqueous phase can be divided into two analytically defined phases, colloidal and electrochemically available. Colloidal phase partitioning allows a consideration of metal mobility, while the electrochemically available phase indicates potential bioavailability. The partitioning of the four metals Cd, Cu, Pb and Zn was studied by slurrying sewage sludge samples in a solution of sodium perchlorate ($NaClO_4$) at varying pH, sludge/water ratio, ionic strength and contact time. $NaClO_4$ was added to provide the suitable electrochemical conditions for ASV measurements and to allow comparisons with toxicity. At pH 3.0, the concentration of all metals in the electrochemically available phase was greater than at higher pH values; at pH 4.5 the concentrations in the electrochemically available phase were close to the detection limit for all metals except Zn, especially in the filtered samples.

Three K_D values were calculated (Table 4.3); one for the two-phase system, K_{D2} (particulate/aqueous), and two for the three-phase system, $K_{D3p/c}$ (particulate/colloid) and $K_{D3c/f}$ (colloid/electrochemically available, filtered). However, as the colloidal fraction is treated as pseudo-solid in one case ($K_{D3c/f}$) and as pseudo-dissolved in the other ($K_{D3p/c}$), the measurements had to be related to organic carbon (Table 4.3). Results could also be related to colloidal solids concentration by drying, but this can lead to a considerable analytical error as the colloidal concentration is often under the measurable limit of 50 mg l^{-1}.

Table 4.3. K_D values for a sludge/water solution of 0.7 g l^{-1} in 0.5 M $NaClO_4$ at pH 3.0.

	K_D, l g^{-1}			
	Cd	Cu	Pb	Zn
Two-phase system:				
K_{D2}	2.6	13.3	11.3	1.6
K_{D2OC}	0.83	4.2	3.6	0.49
Three-phase system:				
$K_{D3p/c}$	3.6	14.1	13.8	1.8
$K_{D3p/cOC}$	1.1	4.4	4.4	0.55
$K_{D3c/fOC}$	0.18	1.1	0.28	0.52
$K_{D3c/uOC}$	-	0.82	0.76	0.52

The K_{D2} and $K_{D3p/c}$values are similar for all metals, which means that the aqueous phase in the two-phase system is a fairly good approximation of partitioning into the colloid phase. Both K_{D2} and $K_{D3p/c}$ are lower for Cd and Zn than for Cu and Pb, which shows that a greater fraction of Cd and Zn leave the particulate phase and enter the colloid or electrochemically available phase than Cu and Pb, which mostly remain in the particulate phase. This is interpreted as Cd and Zn being more bioavailable than the other two metals. The $K_{D3c/fOC}$, which describes the relation between the electrochemically available metal and the colloid phase (where colloidal bound metals are related to organic carbon), is higher for Cu than for the other metals. This shows that Cu has a greater tendency to partition into the colloid phase and indeed, very little Cu is found in an electrochemically available form.

Ionic strength had little influence on the K_D values. However, the sludge/water ratio affected the K_D values. At low sludge/water ratios, K_{D2} increased for both Zn and Cu, as did $K_{D3p/c}$ for Zn. The $K_{D3p/c}$ for Zn decreased with decreasing pH, but the $K_{D3c/f}$ remained rather constant, which means that at a low pH, more Zn is found in the colloid phase, which is considered mobile, whereas the relation between the colloid phase and the electrochemically available phase, which is considered bioavailable, is not altered. The consequence for sludge application to agricultural soils might be that loading rates affect metal mobility into soil solution, while bioavailability remains unaffected.

Generally, partitioning results (Table 4.3) are in accordance with the earlier fractionation results, which showed that Cd and Zn are more loosely bound to the sludge particle surface than Cu and Pb. Insight into the mechanisms of metal/sludge interactions provides an explanation for partitioning. Exchangeable metal, like K_D, represents an operational interpretation of surface site equilibrium. Not surprisingly, the amount of exchangeable metal depends on the sludge/water ratio, as can also be seen for K_D values. However, partitioning goes further in identifying metal interactions between colloids and solution.

The problem with single extractants, and to some extent also partitioning coefficients, K_D, is that they ignore reaction kinetics. It is simply assumed that contact times are sufficient to reach equilibrium. In the single extraction experiments, great care was taken to keep the exact contact times that have been reported elsewhere, to allow a comparison. The partitioning into the electrochemically available phase in filtered and unfiltered samples was measured as a function of time, and it was concluded that the usual 1 h contact time is not enough to reach steady state, but the reactions are slow enough to allow a good reproducibility even if the contact time varies by a few minutes. Both Zn and Cu enter the electrochemically available phase rapidly during the first few minutes, after which leaching continues at a slow rate. Lead also dissolves rapidly in small amounts, but is then slowly readsorbed and the concentration in the electrochemically available phase decreases with time.

The results from the single extractions and the partitioning experiments were compared to toxicity in order to identify the toxic fraction. The toxicity of the sludge samples as well as the toxicities of the metals themselves were measured with Microtox. A comparison was made between the toxicity of a sludge sample and the metal content of the sludge and it was found that most of the sludge toxicity can be directly attributed to the Cu content. Comparisons between metal concentrations in an extractant and toxicity involved a comparison of the toxicity of sludge before and after extraction. The results show that extraction with EDTA removes the toxic fraction, which means that EDTA is the most suitable extractant for prediction of toxicity of the extractants tested.

One limitation of this method is that it is difficult to attribute toxicity to a single metal when testing a complex sample. It may well be that an extractant that removes the toxicity from a sample only removes the one metal that happens to be responsible for the toxicity of this specific sample. This means that an extractant that extracts the readily removed metals such as Cd or Zn will extract the toxic metal fraction in samples that have concentrations of these metals that are high enough in the available fractions to be responsible for the toxicity. The same extractant may be useless for a sample where for example Pb is responsible for toxicity. This approach is, however, only valid within a number of constraints and assumptions, for example that all metals have only one toxic species, usually assumed to be the uncomplexed metal ion.

The electrochemically available metal is often considered to be a good approximation of the toxic fraction in aqueous samples. An adsorption experiment was performed where known amounts of Pb and Zn were added to sludge samples. K_D values were calculated and the metal content in the electrochemically available phase was compared with the response from Microtox standardised to an ionic metal concentration. The sludge samples with added Zn showed a toxicity that indicated a much higher Zn concentration than was actually present in the electrochemically available and colloidal phase combined, indicating that ionic Zn cannot account for total Zn toxicity. This is interesting because the toxicity of all combinations of the metals Cu, Pb and Zn to Microtox was tested and an antagonistic effect was found between Cu and Zn. Therefore, increased toxicity of Zn in sludge is the result of interaction with sludge components other than these metals. In chemical extraction and partition experiments, it is assumed that there is only one toxic species of each metal. Another assumption is that all metals exert their toxicity completely independent of the other chemical compounds present in the sample. Neither modelling of the sludge/water system by fractionation nor by partition coefficients (K_D) can give the complete picture, which would explain the unexpectedly high toxicity found here for Zn added to sludge.

Directives for metal concentrations in sewage sludge are based on maximum allowed concentration of metals in sewage sludge regardless of the presence of other components in the sludge. It is, however, well known that metals can interact with other substances through many different mechanisms, resulting in both increasing and decreasing bioavailability and toxicity.

Metals are only a small component of sludge. Sewage sludge consists of a multitude of different substances, most of them organic. The discussion about organic sludge components has mostly concerned the trace amounts of anthropogenic substances discharged into the sewer system such as detergents, plasticisers and pesticides. Disconnecting industrial wastewater pipes or banning the use of dangerous substances are more or less effective means of removing these from the sewage sludge. However, the bulk of the sludge consists of other substances that are naturally present in sewage and has its origin in our own bodies, or are the products of our daily activities including food preparation, laundry and bathing. Examples of these substances are fatty acids, proteins, cholesterol, caffeine and cellulose. These substances are impossible to remove from sludge, since they are bulk, or major, components of the sludge. The effects of these substances on each other and on metals in sludge has attracted very little attention and yet they are always present together with the sludge metals.

In this study, the toxicity of Cu in combination with some substances representing different groups of sludge components was tested. Some model substances with known reactions with metals were included for comparison. The toxicity to Microtox of the sludge components and the model substances was tested. An excess of the test substance in solution was then used instead of the usual diluent when testing Cu toxicity. In the case of no interaction, the EC50 of the Cu should be the same as in the usual diluent, since the test substance is present both in the cuvettes together with the Cu and in the blank. If there are synergistic effects, the EC50 for Cu will be lower, while antagonistic effects give a higher EC50 (Table 4.4). The results show that some of the natural components of sludge, including myristic and palmitic acid, as well as some industrially produced substances, such as nonylphenol, increase the toxicity of Cu two or even three times. Other substances, such as NTA or EDTA which are complexing agents present in certain washing powders and benzoic acid which is a common food preservative, detoxify Cu and very little toxicity is found until the concentrations exceed the complexing capacity of the tested substance. The combined effect of all sludge components is an elevation of Cu toxicity in sludge and the presence of synergistic interactions between Cu and organic substances is clearly indicated.

Table 4.4. Toxicity of Cu in the presence of organic substances.

Organic substance	Tested substance concentration, (μmol l^{-1})	EC50(30min) Cu (μg l^{-1})	Toxicity gradient Cu (%/ (μg l^{-1}))
NTA	10	589	0.42
Oxine sulphonate	10	354	0.48
Ethylenediamine	10	346	0.32
Benzoic acid	10	163	0.32
No organic substance added	-	136	1.43
Caffeine	10	84	1.11
Palmitic acid	2	63	1.30
Nonylphenol	2	47	1.88
Oxine	10	14	6.31

The EC-50 is not the only measure of toxicity and additional information is obtained from the toxicity gradient (Table 4.4). This tells us whether toxicity begins to show suddenly just below the EC50, or if the presence of some substances makes even a low concentration of Cu toxic. The toxicity gradient is defined as the increase of toxicity, expressed as % light reduction (between the EC20 and EC50 values) with increasing concentration. Almost all tested substances result in a steeper toxicity gradient than Cu alone. Some of the tested substances are known to form lipid soluble complexes with Cu and these complexes enter the cell directly through the cell membrane. Other substances are not known to form a complex with the metal. However, they can enhance metal uptake, and thereby toxicity, by affecting the cell membrane or the active metal transport pathways through the cell membrane.

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FRACTIONATION AND TOXICITY OF METALS IN SEWAGE SLUDGE

C. E. ANNIKA CARLSON* AND GREGORY M. MORRISON

Department of Sanitary Engineering, Chalmers University of Technology, S-412 96 Göteborg, Sweden

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ABSTRACT

Single extractions of metal fractions in sewage sludge are compared with toxicity to the marine bacterium *Photobacterium phosphoreum*. Copper, and to a lesser extent Pb and Zn, were in concentrations sufficiently high to account for extracted toxicity. The results point to ammonium acetate and EDTA (ethylenediaminetetraacetic acid) as the most suitable single extractants for extracting the toxic fraction, ammonium acetate for limed sludge and EDTA for the raw and digested sludges. Zinc was found to have antagonistic effects on Cu toxicity at low Zn/Cu ratios. This may be an important mechanism in alleviating Cu toxicity in sludge-amended soils.

INTRODUCTION

Sewage sludge is a useful soil improver in agriculture and there is concern over elevated metal concentrations in the applied sludge and potential bioaccumulation (1-4). There is considerable evidence that, once applied, the sludge-bound metals are persistent in the soil environment (1) and there is a shortage of methods to rapidly assess the potential environmental and health risks associated with sludge application in agriculture. Most countries have limits for total heavy metal concentrations in sewage sludge for agricultural application. However, measuring only the total metal content in the sludge does not necessarily provide enough information for estimating the biologically available metal. For example, an investigation by Alloway *et al.* (5) of Cd uptake by crops showed that uptake by vegetation decreased with increasing soil Cd content. Soil amendment by sludge may increase the total Cd content, but also increases the amount of metal binding sites in the soil which decreases the amount of metal available for plant uptake. Research has therefore been directed to fractionating biologically available heavy metals in sewage sludge. The availability of trace metals for metabolic processes is closely related to their chemical speciation (6,7). Tessier *et al.* (8) developed a scheme for sequential extractions which has been altered and improved by others (9, 10,11). Applying this scheme to sewage sludge requires that some attention is paid to a few problems. First of all, there is a problem with readsorption; the metals leached out from one fraction of the sludge can be adsorbed on to the surface of the remaining parts of the particle (12,13,14). This can be a particular problem in sewage sludge which has a high organic content. Secondly, the different extractants have a poor selectivity (2). There are also some difficulties deducing what the different metal fractions mean in terms of bioavailability, mobility and toxicity (6,15,16). The correlation of extracted metal fractions in sewage sludge, determined by sequential extraction, with availability to organisms is usually poor (5,16,17).

In this paper, single extractions of metal fractions in sewage sludge are compared with direct sludge toxicity to the bacterium *Photobacterium phosphoreum* in order to identify the specific metals and metal fractions providing toxicity in sludge. Toxicity tests have the advantage of good precision and can be used to rapidly assess synergistic/antagonistic effects of the metals tested.

EXPERIMENTAL

Sample collection and preparation

Sewage sludge was collected at the Ryaverket wastewater treatment plant, Göteborg, Sweden, which receives sewage from 550,000 persons. The sludge was taken at 5 different points in the treatment process; raw sludge, raw dewatered sludge, limed sludge, digested sludge and digested dewatered sludge (Table 1). The limed sludge was digested sludge mixed with CaO (20.4% of sludge dry weight is added at the treatment plant). Excess water was first removed under an infrared lamp and the drying was continued to constant weight in a hot air oven at 65°C. Dry sludge was stored in a desiccator and ground before use. All single extractions were performed on at least two sub-samples.

Sludge characteristics

Sludge characteristics are described in Table 1. The pH was directly measured in a slurry of 2 g dried sludge in 20 ml water. Chemical oxygen demand (COD) was measured by dichromate oxidation with spectrophotometric detection. The total metal concentrations are comparable to, or lower than concentrations reported elsewhere (10,17,18,19).

Methods of extraction

Sample digestion for total metal analysis: For the analysis of Cu, Pb and Zn, 0.1 g of the dried sludge was weighed into a Teflon vessel, Parr Microwave Acid Digestion Bomb (4781, 45 ml), together with 4 ml HNO_3 and digested in a microwave oven for 30 s at 400 W. After a cooling period of one hour, the vessel was opened and the contents were diluted to 50 ml. The higher temperature and pressure gives a more effective oxidation and permits the use of solely HNO_3 and avoids $HClO_4$ addition. Cadmium was treated differently due to the possibility of Cd reacting with silica groups in the sludge and forming insoluble complexes (20). To avoid this problem, *aqua regia* (HNO_3:HCl, 1:3) was used instead of nitric acid. 0.2 g dried sludge was heated in the microwave oven for 2 min together with 3 ml HCl and 1 ml HNO_3. The sample was then diluted to 50 ml.

Single extractants: In contrast to other solid fractionation methods the Cd and Pb remaining in the sludge after extraction was analysed and not the metal extracted. This method reduces problems with interference in instrumental analysis either, due to extracted salts, remaining organics or matrix effects from the extracted solution itself. This was found to particularly improve Cd analysis but made no difference to Cu and Zn. The sludge samples were extracted with one of the following extractants: 0.01 M ethylenediaminetetraacetic acid (EDTA) for 16 h, 1 M ammonium acetate for 1 h, 0.04 M hydroxylamine hydrochloride adjusted to pH 2 at 96°C for 5 h, 1 M sodium acetate adjusted to pH 5 for 5 h and 30% hydrogen peroxide, pH 2, for 5 h. The extraction with ammonium acetate removes what is usually termed the exchangeable fraction. The carbonates in the sludge are dissolved by sodium acetate. When used as a single extractant, sodium acetate will also include the exchangeable fraction. Extraction with hydroxylamine hydrochloride dissolves the fraction of the sludge that is composed of oxides, mainly of manganese and iron, and should include the two previously mentioned fractions when used as a single extractant. Hydrogen peroxide oxidises a significant percentage of the organic and sulphidic components in the sludge. Exchangeable and carbonate bound metals will also be extracted at pH 2 but not the oxide fraction. The extractions with ammonium acetate, sodium acetate, hydroxylamine hydrochloride and hydrogen peroxide were all performed according to methods described previously (8). EDTA is a strong complexing agent which will remove all metals that are more weakly bound to sludge than to EDTA itself. This extraction is also free from readsorption problems (12). 1 g of sludge was shaken in a

Table 1. Sludge characteristics.

Sludge types	pH	COD	Total metal content (μg g^{-1} sludge)			
		(mg O_2 g^{-1})	Cu	Cd	Pb	Zn
1. Raw	6.7	940	190	0.41	64	370
2. Raw dewatered	6.7	750	200	2.21	88	380
3. Limed	9.7	680	150	1.92	37	270
4. Digested	7.3	670	240	1.79	71	420
5. Digested dewatered	7.2	520	290	1.79	61	520

50 ml polyethylene centrifuge tube with 10 ml of 0.01 M EDTA for 16 h. The remaining sludge was centrifuged and washed with ultra-pure (double distilled Milli-Q system) water before analysis. Metal concentrations in the remaining sludge were corrected for loss of weight on extraction. This was particularly significant for hydrogen peroxide and hydroxylamine hydrochloride.

Metal analysis

Two different methods were used to analyse metal content. Zinc and Cu were analysed directly in the extracted liquid after suitable dilution. This was done with atomic absorption, flame (Perkin-Elmer 2380) and graphite furnace (Perkin-Elmer 603). Lead and Cd were analysed by dissolving the residual sludge from the centrifugation after extraction in the same way that is described above for the total metal content. Both Pb and Cd were analysed by anodic stripping voltammetry (Metrohm, VA processor 646, VA stand 647) at a hanging mercury drop electrode with 300 s deposition time in the differential pulse mode. The amount of extracted metal was calculated as the difference between the amount in the residual sludge and the total metal content in the unextracted sludge.

Toxicity to *Photobacterium phosphoreum*

Dried sludge, usually 50 mg, was placed in a 5 ml polyethylene test tube together with freeze-dried bacteria (*Photobacterium phosphoreum*) in 2.5 ml of dilution solvent (Microtox solid-phase test diluent, 686092). 0.5 ml of sludge mixture was transferred to a new test tube which already contained 2 ml of bacteria in dilution solvent and a series of dilutions were made. After 20 minutes, a microfilter was pressed to the bottom of the tube, thus separating the solid phase (sludge) from the liquid phase (which contained the bacteria). 0.5 ml of the liquid phase was transferred to a cuvette and luminescence was measured in a Microbics M500 luminescent photometer. EC-50 (wt sludge/vol diluent) was calculated from change in luminescence with dilution of sediment. EC-50 is defined as the toxicant concentration that causes a 50% reduction in light emission.

Data treatment

A large standard deviation seems to be inevitable when measuring very low concentrations of Cd (17,21). Five sub-samples were analysed for each sludge type and the standard deviation for raw, raw dewatered, limed, digested and digested dewatered sludge was 56, 87, 49, 37 and 50% respectively. Corresponding standard deviations were for Cu; 9.0, 4.3, 4.9, 10.5 and 4.9%, for Zn; 1.1, 0.5, 8.1, 6.6 and 1.6% and for Pb; 8.5, 8.5, 5.5, 2.9 and 23.8%. Measuring Cd in the extractants with atomic absorption at these low concentrations was very difficult due to severe interference problems. Some of the main problems are discussed by Robards and Worsfold (21). The low total concentration also makes a comparison with other fractionation results difficult, since some other studied sludges contain significantly higher Cd concentrations (10,17).

RESULTS AND DISCUSSION

Total Metal

Total metal concentrations for Cu, Pb and Zn were on average 10-21% higher after digestion and for Cu and Zn, 3-24% higher after dewatering (Table 1). Digestion results in a loss of organic material, as reflected in COD results, which accounts for the increase in metal concentration. Dewatering results in 3-24% higher concentrations of Cu and Zn, and the results for Pb are 38% higher for dewatered raw sludge and 14% lower for dewatered digested sludge. Since the majority of the metals coming to a sewage treatment plant are concentrated in the sludge (3,4,22-24) dewatering only has a small effect on the metal concentrations. The sludge samples taken from different points in the process also reflect incoming metal concentrations at different times. The variation in Pb concentrations could be caused by temporary peaks due to incoming stormwater. The addition of lime reduces total metal concentration due to a lower metal content in the lime than in the sludge. Cadmium concentrations were more variable than the other metals analysed.

Metal Fractionation

The results of the single extraction are shown in Figure 1. One of the most obvious trends is that the limed sludge has a reaction pattern that differs from all the other sludges. This has three reasons: The total metal content is lower in the limed sludge, pH is higher in the limed sludge (Table 1) and the high content of Ca competes with

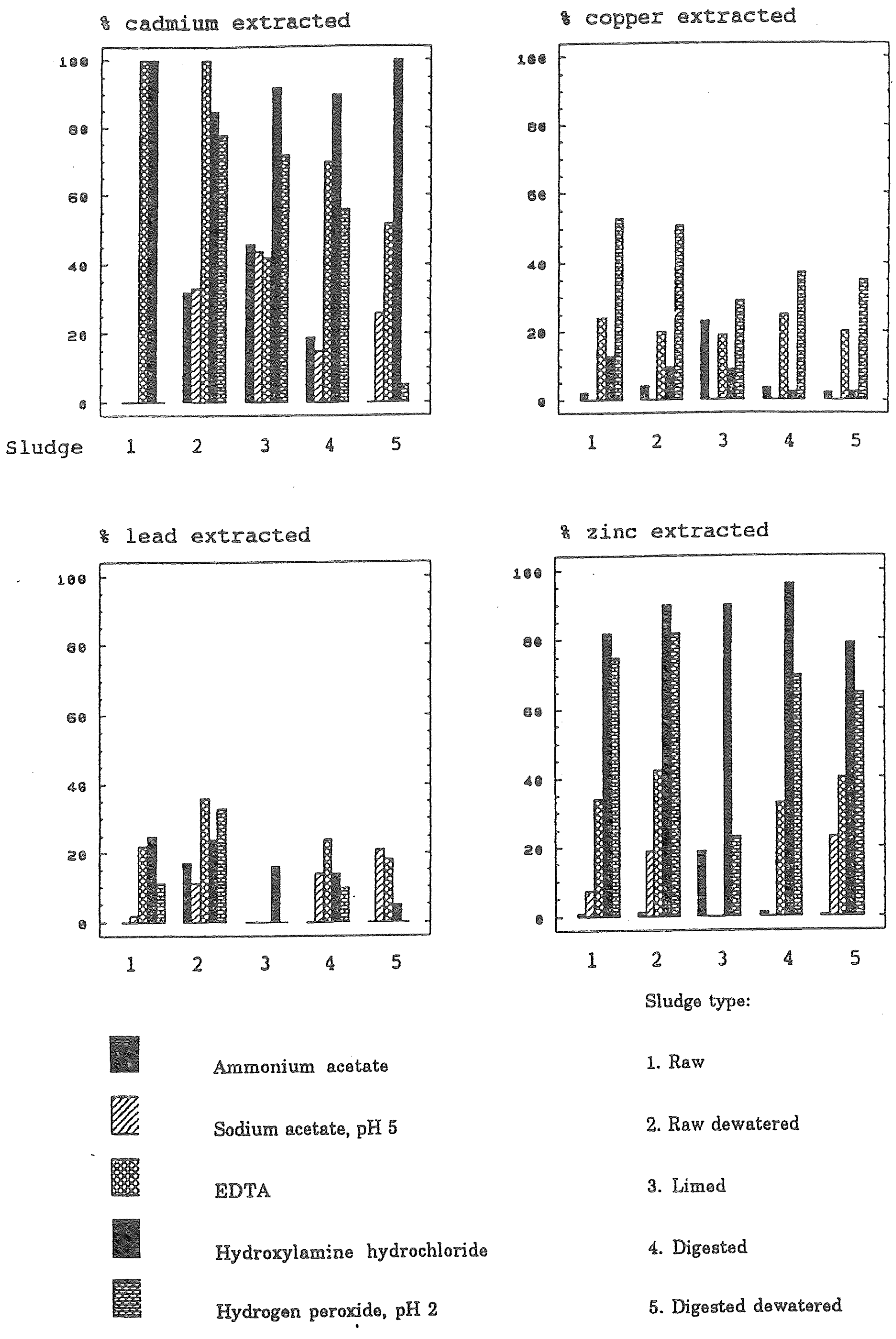

Figure 1. Single extractions of metals in five types of sewage sludge.

the other metal ions. The latter effect is shown very clearly in the results from the EDTA extraction, where almost no Pb and Zn, and very little Cu, are extracted from the limed sludge. An experiment with 0.1 M EDTA showed that EDTA does extract metals from limed sludge provided EDTA is in excess over Ca. The extractability of metals in both sludge and soil has been shown to be very pH dependent (1,17,25-27). Gibbs and Angelidis (22) found a higher concentration of metals in the oxidisable phase in the digested sludge than in the raw sludge, a result that is not confirmed in this study.

For most sludges, less than 25% of the Pb is extracted by the various extractants. In the case of dewatered raw sludge, EDTA extracts 36% and hydrogen peroxide 33% of the Pb. This shows that most of the Pb is very strongly bound in the residual fraction and not likely to be available for either uptake by organisms or leaching into adjacent waterways. There was an unusually high ammonium acetate-extractable Pb peak for raw dewatered sludge. In limed sludge, Pb seems to be completely immobile, except for a small amount of Pb extracted with hydroxylamine hydrochloride. Other studies confirm that a very small part of the Pb content in sludge is readily mobile (22,24), and that most of the metal is found in the residual fraction (18). However, in one study hydrogen peroxide was shown to extract 85% of the Pb (24).

Most previous studies of sewage sludge have shown that Cu is mainly associated with the organic/sulphidic phase and is not readily exchangeable (10,17,19,24). Our study shows that 51-53% of the Cu in the raw sludges and 35-37% in the digested sludges is extracted by hydrogen peroxide, and 20-25% of the Cu in the same sludges is extracted by EDTA. This shows that about one half of the Cu is found in the residual fraction and that the second greatest part of the Cu is found in the organic/sulphidic fraction. The percentage of loosely bound Cu (exchangeable and carbonate bound) is relatively small except in the limed sludge, where 24% of the Cu is extracted by ammonium acetate. The EDTA results for the digested sludges are in accordance with results from Lester *et al* (2).

A previous study on a model compound found that most of the Zn is organically bound (28). Angelidis and Gibbs found similar results (24). In this study, Zn extracted by hydrogen peroxide is 65-80%, by hydroxylamine hydrochloride 80-90% and by EDTA 32-42%. The relatively weak binding of Zn is in accordance with literature results (10,17,19).

Cadmium is the only metal which is found to any extent in the exchangeable phase and this agrees with other researchers who have concluded that Cd is the only metal for which the readily soluble fraction is of any significance (3,18,24,29). The use of model compounds has shown that the carbonate phase, extracted with sodium acetate, is important for Cd (28). Most of the Cd is extracted by hydroxylamine hydrochloride. This shows that Cd is found mostly in the inorganic forms. It is, however, difficult to draw any detailed conclusions since the Cd concentration is very low and the standard deviation too high in our samples making the fractionation results uncertain.

Toxicity results

All sludge samples were tested for toxicity to *Photobacterium phosphoreum* both before and after extraction. The results are shown in Table 2.

The most noticeable result is that extraction with EDTA removes most of the toxicity to *Photobacterium phosphoreum*. This suggests that the toxicity of sludge is derived from the metal

Table 2. EC-50 values for extracted sludge samples, gl^{-1}

sludge type	unextracted sludge	ammonium acetate	sodium acetate pH5	EDTA	hydroxylamine hydrochloride	hydrogen peroxide
1. raw	0.6	0.8	1.3	2.2	0.8	1.6
2. raw d.w	0.6	1.9	0.9	2.7	0.3	1.4
3. limed	0.4	5.6	3.0	0.5	3.1	0.4
4. digested	0.6	1.6	2.1	6.6	1.0	0.5
5. digested d.w	1.1	4.8	1.8	10.5	1.1	1.2

d.w = dewatered

content and not from other components (*i.e.* organic pollutants). The only sample which is not less toxic after EDTA extraction is the limed sludge. Since EDTA does not remove the metals from the limed sludge, this result confirms metals as the cause of toxicity. Table 2 also shows that the toxicity of the limed sludge is very much reduced after extraction with ammonium acetate with the same trend for digested sludges. Ammonium acetate removes only about 20% of the Zn and 24% of the Cu in the limed sludge, and almost nothing in the digested sludges. The exchangeable fraction, *i.e.* the metals removed by ammonium acetate, although it is small, would be expected to contain the most toxic metals. Extraction with hydrogen peroxide has only a minor effect on the toxicity of digested or limed sludges, but for the raw sludge a significant decrease in toxicity is found. Hydrogen peroxide removes the readily oxidised organic substances that can be found in raw sludge and thus removes the toxicity that these substances may exert. During the digestion of the sludge, these readily oxidised substances disappear and do not contribute to the toxicity. The hydroxylamine hydrochloride extraction shows no toxicity difference compared to the unextracted sludge, except for the limed sample which shows less toxicity.

Single extractants and toxicity

The EC-50 values for Cd, Cu, Pb and Zn of the different sewage sludge samples to *Photobacterium phosphoreum* are shown in Table 3. Since the solid phase test allows contact between bacteria and sludge for approximately 25 min, the 30 min EC-50 value was considered to be the appropriate value to compare with the sludge toxicity. The concentration of metals in the test tube that contains the EC-50 concentration of sludge was then calculated and compared with the EC-50 for that metal according to the equation:

$$\frac{\text{EC-50(sludge sample) x (sludge metal conc.)}}{\text{EC-50(metal)}} \text{ x } 100$$

where EC-50(sewage sludge) is gl^{-1}
sludge metal concentration is μgg^{-1}
and EC-50(metal) is μgl^{-1}.

The results are shown in Table 4. This shows that there is enough Cu in many of the samples to explain, and in the case of digested dewatered sludge to exceed, the toxicity. The extractant that removes most of the Cu is hydrogen peroxide, but the extraction with EDTA relates better to toxicity reduction due to removal of a different, more available fraction (Figure 1 and Table 2). Zinc and Pb may also contribute to toxicity, although the low availability of Pb probably means that it is unimportant. A somewhat surprising result is the low toxicity exerted by Cd. The Cd concentrations are well below 0.1% of the toxic concentration. This has two reasons; the low Cd concentrations and the very high tolerance of *Photobacterium phosphoreum* for Cd at low test times. This does not mean that Cd is an unimportant component in sludge. It must be remembered that *Photobacterium phosphoreum* is only one out of

Table 3. EC-50 of *Photobacterium phosphoreum* for metals in aqueous solution.

	EC-50 (5 min) (μgl^{-1})	EC-50 (15 min) (μgl^{-1})	EC-50 (30 min) (μgl^{-1})
Cd	95,000	30,000	12,000
Cu	1,600	1,700	200
Pb	5,200	79	210
Zn	16,000	1,900	1,600

Table 4. Metal concentration in an EC-50 dose of sludge, % of metal EC-50.

Unextracted sludge	Cd	Cu	Pb	Zn
1. Raw	0.0021	57	18	14
2 Raw d.w	0.011	60	25	14
3 Limed	0.0064	30	7.0	6.0
4 Digested	0.0090	72	20	16
5 Digested d.w	0.016	160	32	36

d.w = dewatered

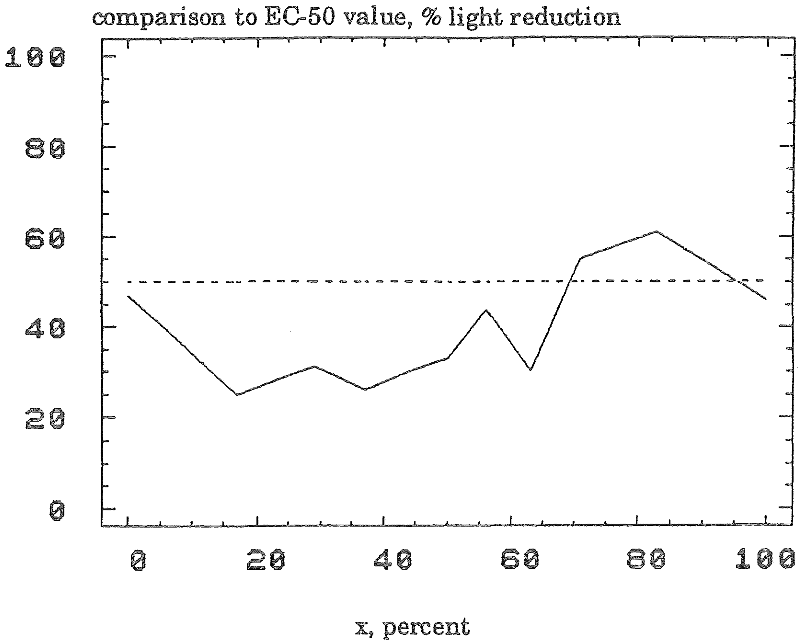

Figure 2. Synergism/antagonism of Zn and Cu to *Photobacterium phosphoreum*. Both Cu and Zn were added in varying proportions to the EC-50.% of Zn is represented as x,% of Cu is (100-x). The dashed line represents the additive EC-50 value for Cu and Zn. Higher values indicate synergism, lower values indicate antagonism.

several possible choices of test organism. There are bacteria that have developed a Cd resistance (1). Another and perhaps more likely reason is that the 30 min incubation is insufficient to exert toxicity. Table 3 shows that the toxicity of Cd to *Photobacterium phosphoreum* increases with time and a longer incubation time might result in a different relation between the metals tested.

Synergism/antagonism

Sludge samples contain several metals, and to understand the combined effects of the metals, toxicity tests were performed on pairs of metals. All combinations of Pb, Cu and Zn were tested but only Cu with Zn showed anything other than an additive effect. Copper combined with Zn showed an antagonistic effect at low Cu/Zn ratios (Figure 2). Below 11% where the concentration of both metals is 2.8 µmol l^{-1} (Cu, 178 µgl^{-1}; Zn, 183 µgl^{-1}), Cu is in excess over Zn but with increasing Zn concentration Zn probably replaces Cu in the enzyme systems that are affected by the metals. Since Zn is less toxic than Cu, Zn has an antagonistic effect on Cu. As Zn concentration increases, the effect becomes additive and at high concentrations of Zn only the toxic effect of Zn is significant. A further investigation of the synergism/antagonism effects could be useful for establishing methods for a rapid estimation of the effects of sludge amendment on soil.

CONCLUSIONS

Toxicity of sewage sludge was accounted for by Cu, and to a lesser extent Pb and Zn. The metal related toxicity was extracted by EDTA in raw and digested sludge, and by ammonium acetate in limed sludge. Zinc was found to have antagonistic effects on Cu toxicity at the concentrations normally found in sewage sludge. Further research is required to identify the antagonistic/synergistic relationships of mixtures of metals in sludge-amended soils.

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Metal partitioning and toxicity in sewage sludge

Metal partitioning and toxicity in sewage sludge

C.E. Annika Carlson-Ekvall* and Gregory M. Morrison

Department of Sanitary Engineering, Chalmers University of Technology,
S-412 96 Göteborg, Sweden

Abstract

Partition coefficients, K_D, for Cd, Cu, Pb and Zn in a sludge/water solution were determined considering the sludge/water solution as a three-phase system (particulate, colloidal and electrochemically available) over a range of pH values, ionic strength, contact time and sludge/water ratios and compared with the K_D values for sludge/water solution as a two-phase system (aqueous phase and particulate phase). Partitioning results were interpreted in terms of metal mobility from sludge to colloids and in terms of potential bioavailability from colloids to electrochemically available. The results show that both mobility and potential bioavailability are high for Zn, while Cu partitions into the mobile colloidal phase.

Introduction

A primary restriction on the use of sewage sludge as a soil improver in agriculture is, in many countries, a concern about the metal content. However, toxic effect or bioaccumulation implies transfer of adsorbed metal from the sludge particle surface into soil solution and conversion to a bioavailable form. This has lead to an interest in attempting to relate the concentration of metal selected by single or sequential chemical extractants to bioaccumulation by crops under field and laboratory conditions (1-15). It is doubtful whether an all encompassing extractant can be found, because these extractants assess geochemical partitioning and potential mobility rather than bioavailability; the relationship between mobility and bioavailability may be relevant for metals such as zinc which is both mobile and available, but it is doubtful for metals such as copper whose availability is related to organic complexation characteristics (16). To understand the mechanism through which metals are released from sludge and into soil solution, this research determines partition coefficients, K_D, for the distribution of the metal between the sludge particle, formed colloids and the surrounding water and relates the partitioning to direct toxicity.

The partition coefficient, K_D, is usually defined as a volume/solid partitioning where K_D ($l\ kg^{-1}$) is the relationship between adsorbed metal ($mg\ kg^{-1}$) and equilibrium dissolved concentration ($mg\ l^{-1}$). K_D has also been defined in terms of desorption and adsorption, depending on whether the experiments are carried out at natural concentration or with added metal (17). In practice, the equilibrium concentration cannot be measured as natural particles rarely approach equilibrium with respect to metals and therefore the determination of K_D usually involves an operational separation of the particulate and aqueous phases through filtration at a specified time of contact. However, Pankow (18) has highlighted the importance of defining a third, colloidal phase. The colloidal phase usually represents a small percentage of the total volume, but it can contain metals in amounts comparable to the water phase. The distinction between phases will always be operationally defined and here, an electrochemically available fraction will be defined as metals measured at a mercury drop electrode (detected by differential pulse anodic stripping voltammetry, DPASV). This has been shown to correspond to metals which are dissolved in the water and completely surrounded by water molecules (19), but since this is difficult to strictly detect or define, the above mentioned operational definition is preferred. The term particulate phase refers to any particle sufficiently large to be separated from a sludge/water solution by filtration through a 0.45 µm cellulose acetate filter. The term colloidal phase is defined as compounds associated with particles small enough to pass a 0.45 µm filter and/or large, partly lipophilic, dissolved molecules such as humic acids. The latter metal/organic complexes are known to be unavailable under certain electrochemical conditions (19). When dealing with a sludge/water solution as a two-phase system, the term aqueous phase is used for the fraction that passes through the 0.45 µm filter. Since there are a multitude of organic substances present in sewage sludge (20, 21) and some of these substances are known to interact with metals to increase their toxicity (22), the metals present in the colloidal phase are relevant in terms of mobility and potential bioaccumulation; a two-phase system is therefore unsatisfactory for interpreting partitioning.

To facilitate the K_D calculations, the metals in the particulate phase will be denoted by PP. Correspondingly, CP, EA and AP will denote colloidal, electrochemically available and aqueous metal. When describing the sludge/water slurry as a two-phase system, the partition coefficient K_{D2} is defined as K_{D2} = PP/AP. The units will then be l g^{-1} organic carbon (OC) in the sludge, since the PP is expressed as µg metal g^{-1} organic carbon in the sludge and AP in µg metal l^{-1}.

If the slurry is seen as a three-phase system, several partition coefficients can be calculated, with the common subscript 3 to denote the three phases. Thus, $K_{D3p/c}$ is defined as PP/CP (l g^{-1} OC). $K_{D3c/u}$ is CP/EA, with CP expressed as µg mg^{-1} OC and EA as µg l^{-1} unfiltered sample. $K_{D3c/f}$ is defined as CP/EA but with the EA measured in the filtered sample. Both $K_{D3c/u}$ and $K_{D3c/f}$ will then have the units l mg^{-1} OC. K_D values are related to OC here, instead of dry weight (18), for three reasons. Firstly, to allow interpretation of the metals in the colloidal phase as both adsorbed (µg g^{-1}) and in equilibrium concentration (µg l^{-1}). Secondly, OC can be precisely and accurately measured in both solid and dissolved phases and finally, metals are known to favourably complex to organic ligands.

A number of assumptions are made in this article which, although acceptable in sewage sludge and sludge-amended soil systems, may not necessarily apply to soil or sediment systems where the organic content is much lower. As dry solids are difficult to measure for the colloidal phase, and because metals are known to partition over organic material, partitioning was related to measured filtered organic carbon for the colloidal phase and particulate organic carbon for sludge. For the electrochemically available fraction a source of error may then be encountered for organic metal complexes which dissociate at the mercury drop electrode and should otherwise be considered colloidal. In this article partition coefficients are reported for solutions of sludge in $NaClO_4$ to allow comparison with the Microtox toxicity test. It can be argued that a perchlorate solution does not represent a natural situation. However, the role of perchlorate is as a non-complexing medium for control of ionic strength under operationally defined conditions. This allows comparison between and within experiments.

We report K_D values for the partitioning of Cd, Cu, Pb and Zn over sewage sludge at different sludge/water ratios, contact times, pH and ionic strength. The sludge/water solution is considered as operationally defined two-phase and three-phase systems through desorption experiments. Relationships between metal partitioning and toxicity are studied through adsorption experiments.

Experimental

Sample collection and preparation

Raw sewage sludge was collected at the Ryaverket wastewater treatment plant, Göteborg, Sweden, which receives domestic sewage from 550 000 persons, together with some industrial discharge and stormwater. Properties of the sludge are shown in table 1. This sludge sample is typical for a densely inhabitated area with a sewer system which is partly separate and partly combined and where most industrial discharge is pre-treated before entering the sewer system. The mean metal concentrations for 1993, calculated from the monthly means, are included in table 1 to demonstrate that the sample was relatively representative for Cu, Pb and Zn, although a slightly lower concentration for Cd was found.

Table 1. Properties of the raw sludge investigated.

	Sludge sample	Mean value 1993
Dry substance (%)	94.5	-*
Total Organic Carbon (% of dry substance)	31.45	-*
COD (mg O_2 g^{-1})	880	-*
Total Cd (µg g^{-1} dry substance)	2.0	3.3
Total Cu (µg g^{-1} dry substance)	420	519
Total Pb (µg g^{-1} dry substance)	58	73
Total Zn (µg g^{-1} dry substance)	1310	1430

* Not measured at the sewage treatment plant.

The sludge was dried in a hot air oven at 65°C and ground before storage in an air-tight container. A fresh slurry of sludge in $NaClO_4$ solution was prepared for each experiment. All the glassware used in the experiments was washed in an acid bath, containing 10% HNO_3, overnight and rinsed in ultrapure water at least three times.

Sludge/water ratio, pH and ionic strength experiments

Sludge concentrations were varied between 0.1 g l^{-1} and 1.0 g l^{-1}, pH values were 3.0, 3.5, 4.0 and 4.5, and $NaClO_4$ concentrations were 0.5 M, 0.05 M and 0.005 M. $NaClO_4$ has a double function in solution: the electrochemical analysis demands a minimum ionic strength and the Microtox bacteria require that the osmotic pressure is kept within certain limits to ensure bacterial performance. $NaClO_4$ was chosen as the ionic adjusting solution due to a non-complexing nature for metals, because of its suitability as an osmotic surrogate in toxicity studies (23) and due to its good electrochemical performance.

After stirring, 20 ml of sludge/water solution was transferred to a voltammetric cell and immediately analysed for Cd, Cu, Pb and Zn. Electrochemically available metal was determined at a hanging mercury drop electrode by DPASV using a Metrohm VA processor 646 and VA stand 647. The sample was deaereated with nitrogen for 5 minutes and preelectrolysed at -1200 mV for 30s to measure Cu and Zn. In order to analyse Cd and Pb, which are present at µg l^{-1} concentrations, the same sample was deaerated for another 30 s and preelectrolysed at -800 mV for 600s. The concentration of electrochemically available metal was calculated by comparison with standards prepared in a matrix containing the same concentration of $NaClO_4$ and at the same pH.

The rest of the sludge/water solution was filtered and 20 ml was analysed as for the unfiltered sample. Another 20 ml of the filtered sample was transferred to two quartz cuvettes, 10 ml in each. After addition of 50 µl concentrated suprapure HNO_3 and 50 µl H_2O_2, organics in the sample were oxidised in a Metrohm UV digester for 2,5 h. After cooling to room temperature, the sample was analysed by DPASV. A further aliquot of the filtered sample, 100 ml, was sampled and stored in a polyethylene container in the freezer for TOC (Total Organic Carbon) analysis. TOC was analysed on a Shimadzu TOC-5000 Analyser.

Time experiments

Sludge (1 g l^{-1}) was stirred in 0.5 M $NaClO_4$ (adjusted to pH 3.0) for one hour, filtered and an aliquot (20 ml) transferred to the voltammetric cell. Measurements were repeated at 715 s intervals to monitor the change of electrochemically available metal in a two-phase system with time. To follow the change of electrochemically available metal in a three-phase system with time, sludge (1 g l^{-1}) was rapidly mixed with 0.5 M $NaClO_4$ (adjusted to pH 3.0) and 20 ml was transferred to the voltammetric cell. Measurement commenced within one minute from sludge contact with the $NaClO_4$ solution and was repeated 9 times every 715 s.

Adsorption and toxicity experiments

A sludge concentration of 0.1 g l^{-1} was mixed with 0.5 M $NaClO_4$. Lead (400, 600 and 1200 µg l^{-1}) or Zn (500, 1000 and 1500 µg l^{-1}) were added to samples of prepared sludge/water solution and stirred for 1 h. Metal concentrations in the different phases were measured. Further experiments used a wider range of concentrations; Cd (1000-6000 µg l^{-1}), Cu (100-1800 µg l^{-1}), Pb (100-1800 µg l^{-1}) or Zn (500-6000 µg l^{-1}) was added and the slurry was stirred for 1 h, after which 2 ml of the sludge/water solution was transferred to a Microtox cuvette for toxicity analysis.

The toxicities of both sludge/water solutions and a range of metal concentrations were measured with the Microtox method. Microtox is an instrument which uses the light-emitting marine bacterium *Photobacterium phosphoreum.* The freeze-dried bacteria were reconstituted with distilled water and mixed with a diluent containing 0.5 M $NaClO_4$, instead of the 2% NaCl used in the standard method (23, 24). A series of dilutions of the test substance were made and the luminescence was measured after 5, 15 and 30 minutes in a Microbics M500 luminescence photometer. A special Microtox Solid Phase Method (24) is available which includes a microsieve to separate solid material from the bacteria. However, bacteria may adhere to the solid phase, thus giving a false toxic response. It was therefore decided not to use the solid phase method. Toxicities of sludge slurries were so high that the suspended solids concentration was between 20 and 400 mg l^{-1}; it was found experimentally that the settled sludge at the bottom of the test cuvette did not interfere with luminescence (22).

Strictly, this test measures inhibition of bacterial luminescence and therefore the results are expressed as a gamma value, Γ, which is defined as $(I(s,0) \cdot I(b,t)/I(b,0) \cdot I(s,t)) - 1$, where I = light intensity at time 0 and t minutes, b = blank, s = sample. The reported value is usually the EC50 value, which is the concentration that corresponds to $\Gamma = 1$ and is the concentration that causes 50% light reduction compared to the blank. However, dose-response curves were made for all four metals, and in those, the Γ values are used directly. All tests were performed at 15°C. Data were analysed according to the manufacturers operating manual (24).

COD and pH

Chemical oxygen demand (COD) was measured by dichromate oxidation with spectrophotometric detection (Dr Lange). The pH meter was a HI 8424 from Hanna Instruments. Since K^+ is one of the few ions that precipitates with ClO_4^-, the usual pH electrode which contains KCl cannot be used due to precipitation of $KClO_4$ on the electrode membrane. Therefore, a saturated solution of NaCl was used instead of KCl in the electrode, and the pH-meter was recalibrated.

Chemicals

The chemicals used were $NaClO_4.H_2O$, p.a. which was pH-adjusted with 70% $HClO_4$, p.a., both from Merck, or with NaOH, puriss. from Eka Nobel. To the samples digested in the UV digester, 30% H_2O_2, suprapur, from Merck and 65% HNO_3, Scan pure, from Chem Scan AS, was added. The metal standard solutions were diluted from 1 g l^{-1} Titrisol solutions of Cd, Cu, and Pb from Merck and 1 g l^{-1} Fixanal Zn solution from Riedel de Haën. The acid bath was a 10% solution diluted from 65% HNO_3, p.a. from Merck. The filters were 0.45 µm cellulose acetate from Sartorius.

Results

Typical calculated K_D values are presented in table 2.

Table 2. K_D values for a sludge/water solution of 0.7 g l^{-1} in 0.5 M $NaClO_4$ at pH 3.0.

	Cd	Cu	Pb	Zn
Two-phase system:				
K_{D2}, (l g^{-1})	2.6	13.3	11.3	1.6
K_{D2OC}, (l g^{-1})	0.83	4.2	3.6	0.49
Three-phase system:				
$K_{D3p/c}$, (l g^{-1})	3.6	14.1	13.8	1.8
$K_{D3p/cOC}$, (l g^{-1})	1.1	4.4	4.4	0.55
$K_{D3c/fOC}$, (l mg^{-1})	0.18	1.1	0.28	0.52
$K_{D3c/uOC}$, (l mg^{-1})	-	0.82	0.76	0.52

For Zn, and for Cu at some sludge/water ratios, the colloids contain ten times more metal than the electrochemically available phase, which means that $K_{D3p/c}$ can be approximated with K_{D2}. This is however not true for Pb, where the colloid phase and the electrochemically available phase both contain equally small concentrations of Pb, while almost everything remains in the solid phase. This can be interpreted in terms of agricultural application of sewage sludge where the mobilisation of Zn and Cd will be significantly greater than for Pb which shows a preference for the solid phase of the sludge.The fact that only Zn, and to some extent Cu, shows a similar partitioning, interpreted in terms of mobility and bioavailability, for two-phase and three-phase systems explains why, despite a considerable research literature, only poor correlations between sludge fractionation and crop uptake have been found (27). However, the sludge/colloid partitioning, whether expressed as $K_{D3p/c}$ or in terms of organic carbon, $K_{D3p/cOC}$, or even K_{D2}, (Table 2) does not consider potential bioavailability which is better expressed through colloid/electrochemically available partitioning (filtered, $K_{D3c/fOC}$ or unfiltered, $K_{D3c/uOC}$).

The similarity between partition coefficients for sludge solids/water, K_{D2}, and for sludge solids/colloids, $K_{D3p/c}$, illustrates the importance of colloidal mobilisation of metals into solution. This can be interpreted in terms of agricultural application of sewage sludge where the mobilisation of Zn and Cd will be significantly greater than for Cu and Pb which show a preference for the solid phase of the sludge. However, the sludge/colloid partitioning, whether expressed as $K_{D3p/c}$ or in terms of organic carbon, $K_{D3p/cOC}$ (Table 2) does not consider potential bioavailability which is better expressed through colloid/electrochemically available partitioning (filtered, $K_{D3c/fOC}$ or unfiltered, $K_{D3c/uOC}$). The fact that only Zn shows a similar partitioning, interpreted in terms of mobility and bioavailability, for two-phase and three-phase systems explains why, despite a considerable research literature, only poor correlations between sludge fractionation and crop uptake have been found.

Changes with time

Two experiments were performed to study metal partitioning with time in the filtered and unfiltered samples. This is necessary to ensure that the correct mixing time is chosen for at least an operational equilibrium.

In the unfiltered samples, the Cu concentration remained constant with time. Zinc increased 25% and Cd increased 2,5 times the initial value. However, Pb concentration showed a rapid decrease (Figure 1). This result indicates that the Pb is rapidly released from some fraction of the solid phase, but then two thirds of the electrochemically available Pb is readsorbed again onto either the colloidal phase or the solid phase. Changes in concentration of Pb (unfiltered) and Zn (filtered and unfiltered) with time is shown in figure 1.

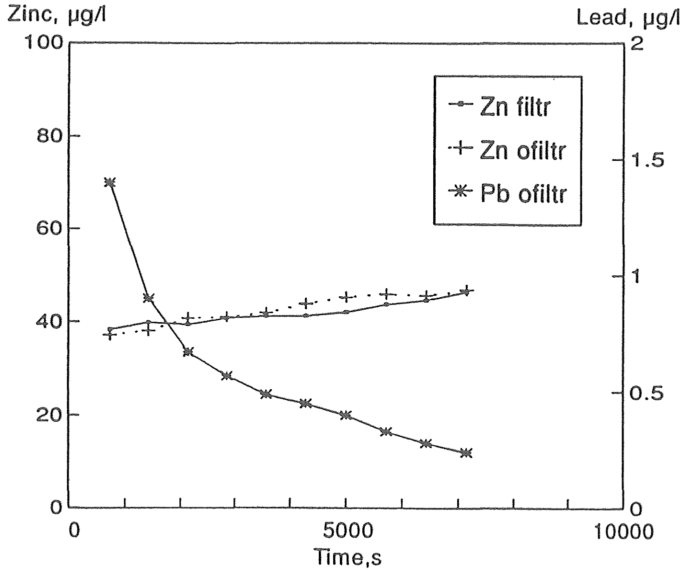

Figure 1. Concentrations of electrochemically available Zn and Pb as a function of time.

After filtration, only the colloidal and the electrochemically available phases are present. There is a possibility that metal will be transported in and out of the colloidal phase, and since only the EA is measured by ASV, this is recorded by changes in the measured concentration of EA metals. Both Zn and Cd concentrations increased with time (20% and 27% respectively), while Cu and Pb varied only marginally. Presumably, Zn and Cd slowly leach out from the colloidal phase, while some kind of equilibrium for both Cu and Pb between the aqueous phase and the colloidal phase is reached within an hour.

A comparison of the concentration of Pb in the presence and absence of a solid phase indicates that readsorption onto the solid phase seems to be the predominant path for the initially released Pb. Readsorption has been reported elsewhere during sequential extraction (25).

It was concluded from these experiments that it is important to measure after exactly 1 h, for unfiltered samples, while the filtered samples are not as sensitive. Although a complete equilibrium over longer time-scales may not be reached within 1 h, this contact time was selected as an operational definition of equilibrium concentration. As it may not in practice be possible to achieve equilibrium for a sludge/water system, this assumption can be considered suitable for comparative purposes, although the K_D values may differ.

Effect of ionic strength

Three ionic strengths were tested, 0.5 M, 0.05 M and 0.005 M $NaClO_4$, at four sludge/water ratios, 0.1 g l^{-1}, 0.4 g l^{-1}, 0.7 g l^{-1} and 1.0 g l^{-1}.In preliminary experiments it was found that a pH of 3.0 is needed to get measurable concentrations of for example Cd. No clear relationship between ionic strength and K_D was found in this study (Table 3). However, there is a tendency for slightly higher K_D values at the highest ionic strength for Cu and Zn, whereas for Pb, the highest K_D values are found at the lowest ionic strength. Some examples of K_D values are presented in table 3.

Table 3. Variation of K_D values with ionic strength for Zn and Cu.

Sludge concentration (g l^{-1})	Concentration of $NaClO_4$ (mol l^{-1})	K_D for Zn			K_D for Cu		
		K_{D2OC} (l g^{-1})	$K_{D3p/cOC}$ (l g^{-1})	$K_{D3c/fOC}$ (l mg^{-1})	K_{D2OC} (l g^{-1})	$K_{D3p/cOC}$ (l g^{-1})	$K_{D3c/fOC}$ (l mg^{-1})
0.4	0.5	0.49	0.56	0.93	3.0	3.6	13.3
	0.05	0.14	0.16	0.61	1.0	1.2	0.46
	0.005	0.38	0.43	0.85	1.9	1.9	-*
0,7	0.5	0.49	0.55	0.52	4.2	4.4	1.1
	0.05	0.38	0.43	0.32	1.8	1.9	9.7
	0.005	0.45	1.2	0.03	2.0	2.0	-*

*) No measurable Cd concentration in the filtered samples

Effects of increasing sludge/water ratio

Variations of sludge/water ratios were studied in 0.5 M $NaClO_4$ at pH 3. K_D values for Zn are shown in figure 2 as a function of sludge concentration.

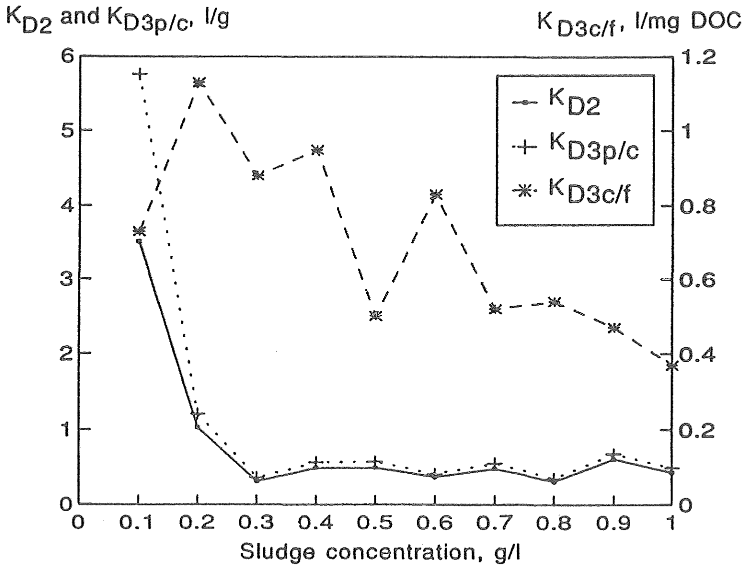

Figure 2. Effect of sludge concentration on K_D values for Zn.

Particulate/aqueous and particulate/colloidal partitioning (K_{D2} and $K_{D3p/c}$) for Zn decrease from a high value at 0.1 g sludge l^{-1} to a much lower value which remains constant from 0.3 g sludge l^{-1} to 1.0 g l^{-1} (Figure 2). Copper shows the same trend, but the differences between the highest and the lowest K_{D2} and $K_{D3p/c}$ are smaller. The results for Cd point in the same direction. On the other hand, neither filtered colloidal/electrochemically available nor unfiltered colloidal/electrochemically available partitioning ($K_{D3c/f}$ nor $K_{D3c/u}$) show the same trend. For Zn, these two K_D values do not vary with sludge concentration, and for Cu, a small increase in both $K_{D3c/f}$ and $K_{D3c/u}$ can be observed. This means that at low sludge/water ratios, 0.1-0.2 g l^{-1}, the greater proportions of the metals Cd, Cu and Zn remain in the particulate phase than at higher sludge/water ratios. However, the relation between colloidal and electrochemically available metal is rather constant. This shows that the partitioning of the metals Cd, Cu and Zn between the colloidal phase and the particulate phase depends on the concentration of sludge, in contrast to the partitioning between colloidal phase and electrochemically available metal, which is more independent of the sludge concentration.

As in earlier experiments, Pb has a partitioning of its own. Particulate/aqueous partitioning (K_{D2}) first decreases with increasing sludge/water ratio and then increases again to a higher value than from the beginning, thus forming a U-shaped curve. Particulate/colloidal partitioning $K_{D3p/c}$ on the other hand, although it varies, does not show any specific trend. Both filtered and unfiltered colloidal/electrochemically available partitioning ($K_{D3c/f}$ and $K_{D3c/u}$) have low values. When looking at the concentration levels for Pb, it can be seen that both colloidal and electrochemically available Pb concentrations are low. It can be concluded that most of the Pb remains in the particulate phase with little tendency to partitioning into the electrochemically available phase.

When looking at pH 3,5-4,5, the trend for K_{D2} and $K_{D3p/c}$ is even more obviuos for Cu, while the trend for Zn is reversed. However, the concentrations of Cu and Zn decrease with increasing pH, both in the electrochemically available phase and, most markedly, in the colloidal phase.

Effects of increasing pH

The most obvious effect of increasing the pH from 3.0 to 4.5 is seen for Zn, where particulate/aqueous and particulate/colloidal partitioning (K_{D2} and $K_{D3p/c}$) values increase with increasing pH (Table 4). This is due to a decrease of Zn in the colloidal phase. The electrochemically available Zn decreases even more, which gives decreasing filtered and unfiltered colloidal/electrochemically available partitioning coefficient ($K_{D3c/f}$ and $K_{D3c/u}$) values for the sludge concentrations 0.1 and 0.4 g l^{-1}. The same is true for $K_{D3c/f}$ for 0.7g l^{-1}.

Values for particulate/aqueous and particulate/colloidal partitioning (K_{D2} and $K_{D3p/c}$) for Cu increase with increasing pH, but the increase is of a smaller magnitude than for Zn. The greatest difference in K_{D2} and $K_{D3p/c}$ is seen when going from pH 3.0 to pH 3.5, whereas a further increase of pH gives small changes in the values for K_{D2} and $K_{D3p/c}$. The results for Pb are similar, but not as clear. The concentrations of Cd in all phases except the particulate were too low for pH-values above 3.0 to calculate K_D with any degree of certainty. Values for K_D for Zn, Pb and Cu are show in table 4.

Table 4. K_D values as a function of pH.

pH	Sludge concentration	Zn			Cu			Pb		
		K_{D2OC} (l g^{-1})	$K_{D3p/cOC}$ (l g^{-1})	$K_{D3c/fOC}$ (l mg^{-1})	K_{D2OC} (l g^{-1})	$K_{D3p/cOC}$ (l g^{-1})	$K_{D3c/fOC}$ (l mg^{-1})	K_{D2OC} (l g^{-1})	$K_{D3p/cOC}$ (l g^{-1})	$K_{D3c/fOC}$ (l mg^{-1})
3.0	0.4	0.49	0.56	21.2	2.97	3.64	13.3	2.38	10.2	0.92
	0.7	0.49	0.55	0.52	4.19	4.41	1.15	3.56	4.35	0.28
3.5	0.4	1.41	1.63	1.02	12.2	12.16	-*	9.85	12.4	0.63
	0.7	2.79	3.11	0.62	6.79	6.79	-*	11.7	24.1	0.07
4.0	0.4	10.7	14.6	0.36	18.2	18.2	-*	25.9	25.9	-*
	0.7	18.8	22.7	0.36	9.51	9.51	-*	5.30	6.59	0.30
4.5	0.4	3.84	5.66	0.30	18.8	18.8	-*	15.1	17.5	0.87
	0.7	37.5	46.2	0.19	8.17	9.28	0.33	10.6	10.6	-*

*) Concentration of metal in the filtered fraction too low for reliable measurements.

Effect of dissolved organic carbon

Dissolved organic carbon (DOC) has a very significant effect on metal partitioning due to complexation and colloidal formation. The latter increases mobility, while the former decreases potential bioavailability. DOC shows no variation with the pH, but an increasing sludge/water ratio gives increasing DOC (Figure 3). This has important implications for the colloidal partitioning of metals where sludge concentration rather than pH determines $K_{D3p/c}$ whereas partitioning into the electrochemically available form ($K_{Dc/u}$ or $K_{Dc/f}$) will be pH controlled.

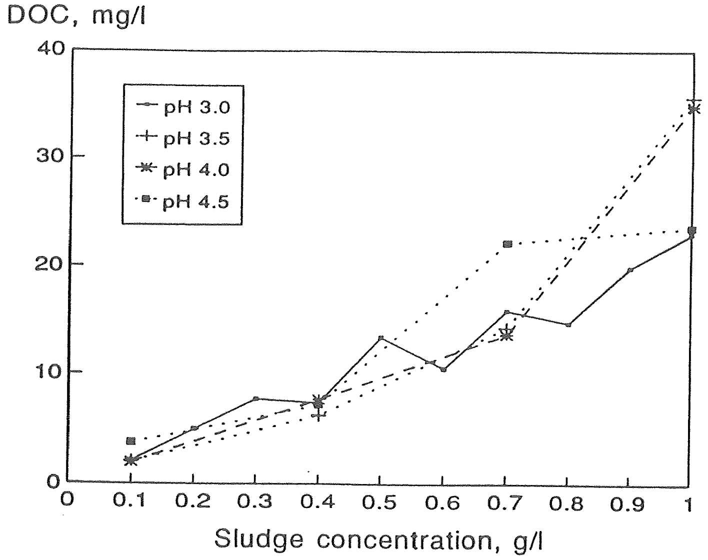

Figure 3. Variations of DOC with sludge/water ratio.

Comparison between toxicity of metal-amended sludge and partition coefficient

Sewage sludge was slurried in 0.5 M $NaClO_4$ (pH ~7, not pH-adjusted) for one hour together with increasing concentrations of Cd, Cu, Pb or Zn. Cadmium was found to have an EC50 value so high that reasonable results could not be obtained, while Cu EC50 values varied from very high to very low, a result of the formation of highly toxic, lipid-soluble complexes (22). It was therefore decided to focus the following experiments on Pb and Zn.The used sludge concentration must be below the sludge EC50 to give a reasonable luminescence. In this experiment, a sludge concentration of 0.1 g l^{-1} was chosen. The metal concentrations added to the sludge were chosen to cover a range of toxicities from no toxicity at all to complete darkness. The toxicity of the metal-sludge mixtures was measured and compared to a dose-response curve for pure metal salts. The toxic metal concentration was calculated and is presented in table 5 as a toxicity K_D (K_{D2tox}) and compared to the directly measured adsorption (K_{D2ads}). The difference between the two K_D values is that in K_{D2ads} aqueous metal is measured electrochemically after UV-digestion, while in K_{D2tox} aqueous metal is replaced by a biological response standardised by dose-response measurements in the absence of sludge. Both aqueous measurements are compared to adsorbed metal concentration on the sludge.

For Pb, values of K_{D2ads} and K_{D2tox} are a similar order of magnitude within the range of the added metal concentrations. This means that Pb partitioning and toxicity when added to sewage sludge follows ionic Pb response. However, although this is also the case for Zn added at a higher concentration (1500µg l^{-1}), a quite different pattern is found for lower Zn concentrations where negative K_{D2tox} values are found. In this case Zn toxicity in the presence of sludge is higher than would be anticipated from concentrations predicted by K_{D2} alone. It has been shown previously that Zn can have an antagonistic effect on Cu toxicity (26), so presumably, the synergistic effect reported here is with another component of sewage sludge. One explanation is the formation of lipid-soluble complexes of Zn with certain organic components. These complexes are known to significantly increase Cu toxicity (22).

Table 5. Real and toxicity related adsorption K_{D2} values for Pb and Zn in the presence of 0.1 g l^{-1} sludge.

Metal	Added metal, concentration µg l^{-1}	K_{D2ads}, l g^{-1}	K_{D2tox}, l g^{-1}
Zn	500	2.4	-0.09
	1000	0.69	-0.08
	1500	1.1	2.7
Pb	400	37	31
	600	25	84
	1200	29	47

Conclusions and discussion

Many workers have tried to find a simple way to predict the concentration of bioavailable metal in a sludge-amended soil, assuming that metals partition between a solid phase which is both unavailable to plants and immobile and an aqueous phase that is both mobile and bioavailable. Many attempts to model the latter with different chemical extractants have been made, some successfully related to crop uptake, some less successful. The results in this paper show that there are three phases that can contain metals, of which two are mobile, the colloid phase and the electrochemically available metal, but only the latter is believed to be bioavailable. This means that an extractant that removes both mobile phases will overestimate the bioavailable metal if the greater part is found in the colloid phase. The results in this paper show that the metal content in the colloid phase is often much greater that in the electrochemically available phase.

The variability of chemical extraction results, usually obtained by sequential extraction, is that the dissolution of metals from the particulate phase into the aqueous phase is a slow process which takes more than 2 h to complete. This makes it important to choose the same extraction time when comparing two experiments, and to be particular about keeping the extraction time exact. The leaching process is probably also dependent on particle size and specific surface, which further complicates interpretation of chemical extraction results. Partitioning of metals through K_D allows a more detailed interpretation of metal speciation in sludge/water solutions, but should be further complimented by studies of the kinetics of metal partitioning between the three defined phases.

The toxicity results for the metal-amended sludge illustrate the difficulty in predicting toxicity from purely chemical experiments. The adsorbed Zn was more toxic than would be expected if all Zn would have been present in ionic form. In the light of our earlier experiments on Cu toxicity in the presence of organic sludge components (22), it can be concluded that there is no guarantee that all metal detected in the electrochemically available phase have the same toxicity, neither can all colloidal metal be considered non-toxic. The presence of metal species with higher toxicity than the hydrated ion, for example a small lipid-soluble complex, must be considered. This demonstrates the inportance of validating all chemical methods through comparison with biological tests, such as for example Microtox.

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TOXICITY OF COPPER IN THE PRESENCE OF ORGANIC SUBSTANCES IN SEWAGE SLUDGE

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TOXICITY OF COPPER IN THE PRESENCE OF ORGANIC SUBSTANCES IN SEWAGE SLUDGE

C. E. ANNIKA CARLSON-EKVALL* AND GREGORY M. MORRISON

Department of Sanitary Engineering, Chalmers University of Technology, S-412 96 Göteborg, Sweden

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ABSTRACT

The toxicity of Cu to *Photobacterium phosphoreum* was studied in the presence of both model substances and organic substances that have been identified as sludge components. Copper toxicity increased in the presence of LAS, caffeine, myristic acid, palmitic acid, nonylphenol, ethyl xanthogenate and oxine, with a steep toxicity gradient for the latter two compounds. Other substances, including complexing agents, ameliorated Cu toxicity. Synergistic/antagonistic effects of organics and Cu in sludge were tested through comparison of the toxicity of Cu added to three sludge types with the concentration of electrochemically available Cu at different sludge/Cu proportions. The toxicity of Cu in raw and digested sludge was much higher than could be explained by the concentration of electrochemically available Cu alone and was attributed to synergism and potentially the formation of lipid-soluble Cu complexes.

Keywords: Toxicity, lipid-soluble Cu, sewage sludge, *Photobacterium phosphoreum*, electrochemical availability

INTRODUCTION

Sewage sludge is a useful soil improver as it contains nitrogen, phosphorus and certain essential metals of value for agriculture. A major drawback is the presence of elevated concentrations of detrimental metals with concern for long-term bioaccumulation and soil toxicity after application. In this respect, sludge quality is usually assessed by measurements of total metal concentration, although it is known that bioavailability and toxicity of metals relates to the speciation of the metal rather than the total concentration (1, 2). However, the influence of bulk components of sewage sludge on metal toxicity is often neglected.

Lipid-soluble complexes of Cu significantly increase Cu toxicity (3) because these complexes can rapidly penetrate a biomembrane by passive diffusion, which is a rapid path of transport compared to the slower active uptake of ionic Cu (1). Dithiocarbamates and xanthates are known to increase the bioavailability of Cd and Pb (4, 5) and certainly, oxine and ethyl xanthogenate increase Cu toxicity (3), although the presence of these complexes in domestic sewage sludge is unlikely. However, organic compounds with similar properties are found in sewage sludge and originate from shampoos, soaps and fats. These compounds can combine in-sewer with copper either transported in stormwater runoff or as corrosion products of household drinking water connections. Copper in soil is usually present as organic complexes (6) and the addition of large quantities of organic matter is known to significantly increase the uptake of Cu through roots (7).

More specifically, sewage sludge components include organic substances derived from domestic sewage and the food industry (fatty acids, benzoic and tannic acid, cholesterol, caffeine), complexing agents (NTA) and detergents used both in the household (LAS, tri-n-butylphosphate) and in industrial cleaners (nonylphenol ethoxylates), plasticisers (phthalates) and chemicals (coagulants and polymers) added during the sewage treatment process (8-11). Some of these components are lipophilic and may well form complexes with sludge-bound copper and thereby increase toxicity and potential bioaccumulation.

This investigation centres on interactions between known components of sewage sludge and copper, in terms of a standardised toxicity test.

EXPERIMENTAL

Sludge

Three samples of sewage sludge were tested. Raw sludge and dewatered digested sludge were sampled at the Rya treatment plant, Göteborg, which receives sewage from 550,000 persons and some industrial discharge. The sludge was dried at 65°C overnight and ground. As a reference, Sewage Sludge of Domestic origin, Reference Material no 144 from the Community Bureau of Reference (BCR) was also used.

Chemicals

Organic substances tested were: myristic acid, palmitic acid, stearic acid and oleic acid. These fatty acids commonly make up at least a few percent of the sludge by dry weight (8). Other food-derived components tested were benzoic acid, caffeine (1,3,7-trimethylxanthine) and tannic acid. Detergent components tested included NTA (nitrilotriacetic acid), LAS (linear alkylbenzene sulphonate), nonylphenol and tri-n-butylphosphate. Diethylphthalate is a plasticiser. Anionic and cationic polymer are added to the sludge in the sewage treatment plant to facilitate dewatering. Model substances tested were PAR (4-(2-pyridylazo)-resorcinol), TAR (4-(2-thiazolylazo)-resorcinol), neocuproin (2,9-dimethyl-1,10-phenanthroline), oxine (8-hydroxyquinoline), oxine sulphonate, ethyl xanthogenate, APDC (ammoniumpyrrolidine-dithiocarbamate) and ethylenediamine.

Benzoic acid, NTA and tri-n-butylphosphate were from Merck; oxine from Riedel de Haan; tannic acid, nonylphenol and oxine sulphonate from Aldrich; PAR, TAR and TAN from Sigma. Myristic acid and palmitic acid were obtained from Tefac, Karlshamn, Sweden. Stearic acid, oleic acid and caffeine were from Apoteksbolaget (National Corporation of Swedish Pharmacies). LAS was obtained directly from Berol, Sweden. Anionic polymer (Duromax AM 45) and cationic polymer (Duromax CA 58) which are copolymers of aminoacrylate-acrylamide and polyacrylate were obtained from the Rya sewage treatment plant, Göteborg.

The copper used was a Titrisol standard solution from Merck, containing 1 g $CuCl_2$ per litre.

Microtox Toxicity Test

Microtox is an instrument which uses the light-emitting bacterium *Photobacterium phosphoreum*. The freeze-dried bacteria were reconstituted with distilled water and mixed with a diluent containing 2% NaCl. A series of dilutions of the test substance were made and the luminescence was measured after 5, 15 and 30 minutes in a Microbics M500 luminescence photometer. Those substances that were not readily soluble in water were first dissolved in a small quantity of ethanol before dilution with ultrapure water. No stock solution contained more that 1% ethanol, which was found to be well below the concentration that is toxic to the Microtox bacteria.

For the sewage sludge contact experiments, the sludge was stirred with water to a slurry and aliquots were transferred to each test cuvette with a pipette. Sludge toxicity was determined with the Microtox solid-phase method. The toxicity of Cu in the presence of sludge was determined with the Microtox standard method for liquid samples. Toxicities of sludge slurries were so high that the suspended solids concentration was between 20 and 400 mg l^{-1}; it was found experimentally that the settled sludge at the bottom of the test cuvette did not interfere with the luminescence.

Strictly, this test measures inhibition of bacterial luminescence and therefore the results are expressed as an effect concentration, an EC_{50} value, which is the concentration that causes 50% light reduction compared to the blank. All tests were performed at 15°C. Data were analysed according to the manufacturers operating manual (12).

Electrochemical Measurements

Electrochemically available Cu was determined at a hanging mercury drop electrode in the differential pulse mode by ASV (Anodic Stripping Voltammetry) using a Metrohm VA processor 646 and VA stand 647. Aliquots of a slurry of raw, digested or reference sludge were added to 50 ml of 2% NaCl in concentrations corresponding to between 10 and 90% of an EC_{50} dose of sludge. Copper was added in increasing concentrations for each sludge concentration. After 30 minutes equilibration, 20 ml of the sample was transferred to the voltammetric cell.

The sample was deaerated with nitrogen for 5 minutes and preelectrolyzed at -500 mV for 120 or 180 s. The concentration of electrochemically available Cu was calculated by comparison with standards prepared in 2% NaCl.

RESULTS AND DISCUSSION

Initial Toxicity Tests of Cu and Organic Substances

Before investigating interactions of Cu with organic substances in toxicity tests it was necessary to establish the toxic concentrations of Cu and the organic substances alone. The EC_{50} (30 min) value for Cu to *Photobacterium phosphoreum* in 2% NaCl was 136 ± 3.4 $\mu g\ l^{-1}$ and the EC_{50} (30 min) values for the organic substances are shown in Table 1 (sludge components) and Table 2 (model substances). For the sludge components, a number of substances gave EC_{50} (30 min) values close to 10 $\mu mol\ l^{-1}$, including myristic acid, palmitic acid, tannic acid and nonylphenol (Table 1). Neocuproin and APDC showed toxicity at concentrations around 2 $\mu mol\ l^{-1}$ or lower (i.e. close to the EC_{50} (30 min) for Cu) and were therefore excluded from further testing (Table 2).

Table 1. Toxicity of organic substances found in sewage sludge.

Sludge components	EC_{50} (30 min) ($\mu mol\ l^{-1}$)
Myristic acid	12
Palmitic acid	16
Stearic acid	>50
Oleic acid	124
Benzoic acid	>50
Tannic acid	5.3
NTA	>50
Nonylphenol	5.6
Caffeine	41
Diethylphthalate	>50
Tri-n-butylphosphate	>50
LAS	38
Anionic polymer*	$>3 \times 10^{-5}\%$
Cationic polymer*	$>3 \times 10^{-5}\%$

* No toxic effect is detected at this concentration. The polymers are diluted to 0.05-0.1% v/v (anionic) and 0.08% v/v (cationic polymer) before use at the sewage treatment plant.

Table 2. Toxicity of model substances.

Model substance	EC_{50} (30 min) ($\mu mol\ l^{-1}$)
PAR	25
TAR	12
Neocuproin*	<1**
Oxine	>50
Oxine sulphonate	>50
Ethyl xanthogenate	16
Ethylenediamine	>50
APDC*	<5

* Not included in further testing due to high toxicity

** EC_{50} (5min) = 4 $\mu mol\ l^{-1}$

EC_{50} values and toxicity gradient of Cu in organic substances

The toxicity of Cu in a solution of organic substance was determined by addition of a constant concentration of each organic substance to the 2% NaCl used as diluent. The organic substances were at a concentration of 10 $\mu mol\ l^{-1}$ when possible, to ensure an excess concentration compared to Cu, while organic substances that showed toxicity around 10 $\mu mol\ l^{-1}$ were tested at 2 $\mu mol\ l^{-1}$, which results in an approximately 1:1 relation between organic substance and Cu at the normal EC_{50} (Cu, 30 min) value.

The presence of cationic polymer increased the toxicity of Cu, but no relationship between Cu concentration added and toxicity increase could be ascertained. The results for TAR and PAR were excluded because interference with measurement of the luminescence was found when Cu was added, probably due to colour change.

The interactions between Cu and organic substances were investigated through changes in EC_{50} (30 min) values and the relationship between the EC_{50} (30 min) and EC_{20} (30 min) values (Table 3). The latter relationship is the toxicity gradient and is defined as the increase of toxicity, expressed as % light reduction (between the EC_{20} (30 min) and EC_{50} (30 min) values) with increasing concentration. The gradient was calculated by dividing 30% by the difference between the EC_{50} (30 min) and EC_{20} (30 min) values in $\mu g\ l^{-1}$. All organic substances tested affected the EC_{50} (30 min) values for Cu and/or the toxicity gradients (Table 3) and the tested substances can be classified into three distinct groups.

1. EC_{50} similar to ionic Cu, but a lower toxicity gradient; ionic Cu is taken up by organisms through active or facilitated transport via channels in the cell membrane. Organic substances which do not form complexes with Cu result in EC_{50} values similar to ionic Cu. These substances can be found around the value for ionic Cu in Table 3 and examples are benzoic acid, oleic acid and stearic acid, diethylphthalate and tri-n-butylphosphate. However, many of these substances have toxicity gradients that are considerably lower than the gradient found for ionic Cu. This means that a small toxic effect appears at lower Cu concentrations when these organic substances are present compared to Cu alone and this can be attributed to synergism between the organic substance and Cu at low Cu concentrations.
2. EC_{50} much higher than for ionic Cu; addition of a substance which forms a non-toxic complex with Cu, and plays no role in Cu toxicity, results in an interval where no toxicity is observed. If the Cu is very strongly bound to the complex, toxicity is not found

Table 3. Toxicity of Cu in the presence of organic substances.

Organic substance	Tested concentration, ($\mu mol\ l^{-1}$)	EC_{50} (30min) ($\mu g\ l^{-1}$)	EC_{20} (30min) ($\mu g\ l^{-1}$)	Toxicity gradient (%/ ($\mu g\ l^{-1}$))
Tannic acid	2	>500	-	-
NTA	10	589	518	0.42
Oxine sulphonate	10	354	291	0.48
Ethylenediamine	10	346	251	0.32
Benzoic acid	10	163	70	0.32
Tri-n-butylphosphate	10	142	90	0.58
No organic substance added	-	136	115	1.43
Oleic acid	10	124	64	0.50
Stearic acid	10	110	54	0.54
Diethylphthalate	10	109	44	0.46
LAS	10	108	75	0.91
Anionic polymer	3×10^{-5}%	100	74	1.15
Caffeine	10	84	57	1.11
Myristic acid	2	66	47	1.58
Palmitic acid	2	63	40	1.30
Nonylphenol	2	47	31	1.88
Oxine	10	14	9.3	6.31
Ethyl xanthogenate	2	<20	-	-

until Cu is added in excess and then the toxicity gradient should be similar to the gradient observed for ionic Cu. However, most of the Cu complexing substances tested had low toxicity gradients which can be attributed to lability of the complex with indications of toxicity before the complexing capacity is reached. NTA, ethylenediamine and oxine sulphonate are examples of substances which combine a high EC_{50} with a low toxicity gradient. The results for NTA, oxine sulphonate and tannic acid are in accordance with results from other studies (3, 13). Clearly, the response of *Photobacterium phosphoreum* to complexed Cu will be affected by the stability constant in 2% NaCl which corresponds to an ionic strength of 0.34. Oxine sulphonate and ethylenediamine can form a 1:2 complex with Cu (log K = 22 and 20 respectively (15)) and this explains the lower EC_{50} values for these compounds compared to NTA, at the same molar concentration. Other substances which also complex Cu include EDTA (1, 2), fulvic acid (1, 3, 13) and humic acid (13, 14). In Figure 1, toxicity, represented by the gamma value, Γ (12), for Cu in 10 µmol/l NTA is plotted against Cu concentration and compared to the steeper curve for ionic Cu. Γ is defined as $(I(s,0)\cdot I(b,t)/I(b,0)\cdot I(s,t)) - 1$, where I = light intensity at time 0 and t minutes, b = blank, s = sample.

3. EC_{50} lower than for ionic Cu; a substance which forms a lipid-soluble complex with Cu can rapidly penetrate a biomembrane and exert its toxicity inside the cell. Complexes of Cu with ethyl xanthogenate and oxine are known to react according to this mechanism (3, 16). These complexes show very high toxicity with EC_{50} values representing only 10-20% of the EC_{50} for ionic Cu and with a high toxicity gradient. This means that the difference between a non-toxic concentration and a concentration sufficient to completely extinguish luminescence is very small and indeed, this makes an exact analysis of the EC_{50} for the Cu-ethyl xanthogenate complex almost impossible. Other workers have found strong synergism between Cu and two metal complex pesticides, zineb and maneb, at the same Cu concentration level as oxine and ethyl xanthogenate for the Microtox test (17). The toxicity of Cu in 10 µM oxine is shown as a function of Cu concentration in Figure 1 and demonstrates a significantly steeper toxicity curve than found for ionic Cu.

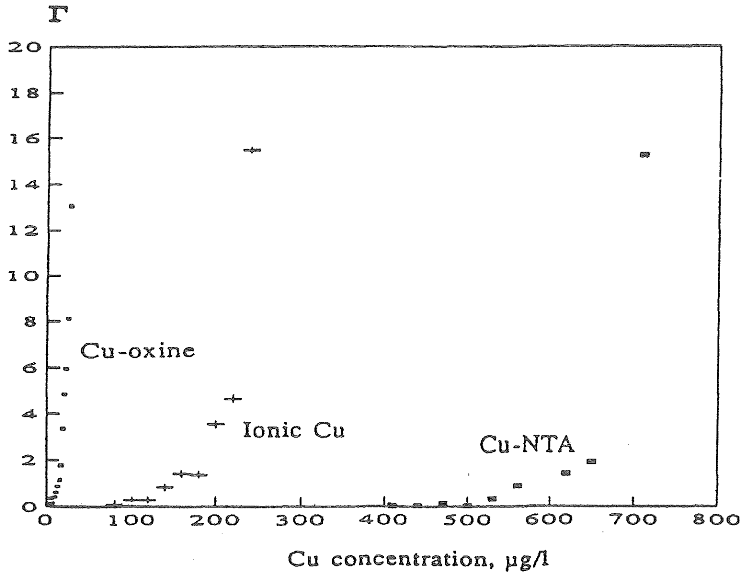

Figure 1. Response of *Photobacterium phosphoreum* to Cu addition in the presence and absence of 10 $\mu mol\ l^{-1}$ oxine and 10 $\mu mol\ l^{-1}$ NTA, expressed as Γ values.

The EC_{50} value for Cu in palmitic acid and myristic acid is much lower than for ionic Cu, but the toxicity gradient is similar (Table 3). The anions of these acids can form complexes with Cu, but these are not as strong as complexes such as NTA and EDTA. However, both these acids, as well as stearic acid and oleic acid, are difficult to dissolve in a medium which is not toxic to the bacteria. This means there is a possibility that the increase in Cu toxicity might have been underestimated in this work. Similarly, linoleic acid has been reported to increase Cu toxicity (3).

A combination of Cu with certain substances yields similar EC_{50} values as those found for known lipid-soluble Cu complexes, but without a dramatically increased toxicity gradient. However, theoretically no actual complex should be formed. These lipid-soluble substances may dissolve in the cell membrane and alter the membrane structure. This destabilisation of the membrane may affect the cell sensitivity to ionic Cu, which results in a lower EC_{50}. Nonylphenol appears to exhibit this effect (Table 3) and detergents, such as LAS, also destabilise a biomembrane.

Copper Titrations with Sludge and Toxicity

The aim of the following experiments was to first determine the amount of added Cu required to give an electrochemically available Cu concentration of 136 µg l^{-1} (the EC_{50} value for Cu alone) in the presence of specified amounts of sludge. After assessing the EC_{50} values for the tested sludges (12) with the Microtox solid phase method (0.22, 0.27 and 0.36 g l^{-1} for raw, digested and reference sludge), the required Cu was determined by titrations of sludge with Cu at sludge concentrations corresponding to 10 to 90% of an EC_{50} sludge concentration. In Figure 2, an example of the resulting curve is shown. Available Cu was measured electrochemically and the concentration of added Cu required to give an EC_{50} dose of available Cu (i.e. 136 µg l^{-1}) was determined. It has been demonstrated previously that electrochemically available metal can be related to the toxic fraction (1, 3, 14, 18).

This experiment was followed by determining the amount of electrochemically available Cu present where a 50% light inhibition was recorded at different sludge concentrations.

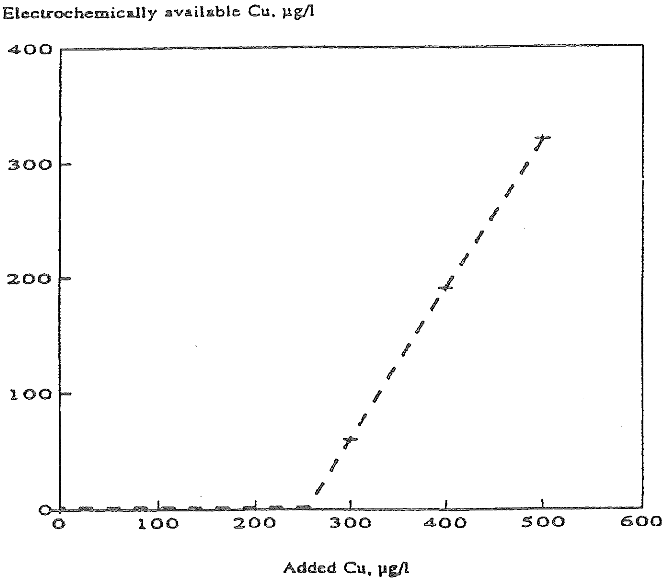

Figure 2. Example of a titration curve for sludge with Cu followed by measurement of electrochemically available Cu. This titration is for a raw sludge solution at 60% of the EC50 value, (EC50 of raw sludge = 0.22 g l^{-1}).

A dilution series of Cu was added to a slurry of raw sludge at a concentration of 0.022 g l^{-1}, which is 10% of the EC_{50} value of raw sludge, in 2% NaCl. A cuvette with sludge, but without Cu, was included as a blank, to eliminate the toxic effect of the sludge itself, and the EC_{50} for Cu in the presence of sludge was determined with the Microtox standard method. All three sludge types were tested for a range of sludge dilutions (representing 10-80% of their indicated EC_{50} values) in 2% NaCl. In this way, the concentration of added Cu giving 50% light inhibition could be determined and with the help of the results from the previous experiment (Figure 2), the concentration of electrochemically available Cu present at 50% light reduction at each dilution could be calculated (Figure 3) and compared to the EC_{50} for Cu alone.

If the toxicity of Cu and sludge is purely additive, the amount of electrochemically available Cu should coincide with the EC_{50} value for Cu alone over the whole range of sludge solutions. When raw or digested sludge are present in a dose corresponding to 50% or more of the sludge EC50 value, which is still a very dilute sludge solution, toxicity occurs even though no measured electrochemically available Cu is present. In the case of 60, 70 and 80% raw or digested sludge, one or more components in the sludge exert a toxic effect which increases with added Cu.

There is no clear difference between raw sludge and digested sludge. The substance which is responsible for the effects with Cu is either not degraded in the digestion process or present in such a large excess that the small percentage of the concentration present in raw sludge that passes the digester unaffected is enough to exert a synergistic effect. Certainly, the Cu-organic complexes responsible exhibit increased toxicity similar to LAS, caffeine, anionic polymer,

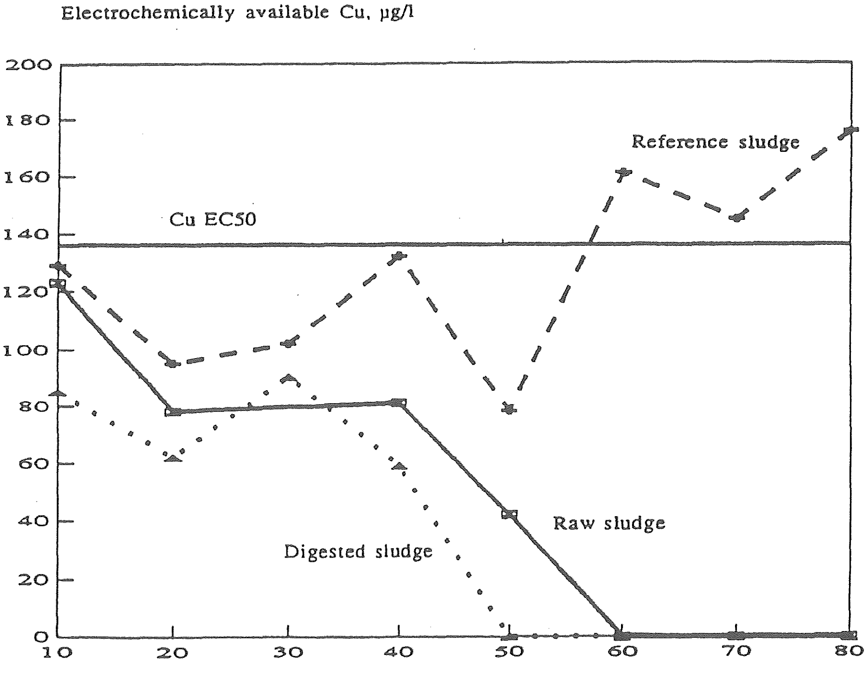

Figure 3. Effect of the presence of varying concentrations of sludge (expressed as% of EC_{50} value) on the response of *Photobacterium phosphoreum* to an EC_{50} dose of Cu measured as electrochemically available Cu.

palmitic acid, myristic acid and nonylphenol (Table 3) and the latter interact with Cu even at low concentrations. It is interesting to note that the reference sludge does not show the same trend (Figure 3). The reference sludge is thoroughly dried and ground and has been stored for an unknown period. This treatment might degrade some of the substances responsible for the effects found in raw and digested sludge. The sludge may also have been altered by drying at a higher temperature than our samples.

CONCLUSIONS

The results presented in this paper show that the toxicity of copper in sewage sludge can increase dramatically in the presence of certain organic substances present in sewage sludge. A significant proportion of organic matter in sludge is degradable, both photochemically (19) and microbially. If the bioavailability of a metal is mediated by lipid-soluble organic substances, the uptake of metals would decrease when the organic substances degrade. Bidwell and Dowdy (20) found a dramatic decrease in Cd uptake during the first 2-3 years after cessation of sludge application, although levels of extractable Cd remained constant. Another implication is that a sludge, even if it contains a low metal concentration, can interact with metals already present in the soil and render them more mobile. It is known that the application of liquid animal manure results in the solubilisation of soil metals due to the high content of dissolved organic matter, a process which ceases when the dissolved organic matter is digested (21).

Based on the results found in this study, it can be concluded that measurement of total metal concentration in sewage sludge is insufficient for decision-making concerning the suitability of sludge for soil amendment. Two factors have to be considered; firstly, metal speciation, including the presence of substances which may form complexes with metals and secondly, the metals already present in the soil must be taken into consideration due to the possibility of interactions between soil metals and substances added with the sludge.

Organic substances showing synergistic effects on Cu toxicity, for example the fatty acids, are common components of domestic sewage. Synergistic effects of the fatty acids are found at concentrations several orders of magnitude lower than the concentration actually found in sludge. Nonylphenol ethoxylates are being replaced by other more biodegradeable detergents in Sweden, but LAS continues to be used in considerable amounts. There are still many substances present in high concentrations in sewage sludge which are not identified and, considering the finding here that all organic substances tested in some way affected Cu toxicity, further work might attempt a separation of sludge fractions which induce Cu toxicity.

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Chapter 5. Conclusions and recommendations

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After a thorough study of the literature concerning the bioavailability of metals from soils contaminated with metals, either from sewage sludge or from other sources such as minespoil, it can be concluded that no chemical extraction method has been found that successfully predicts metal uptake by plants. The correlation of uptake with total metal is usually poor, in some experiments non-existent. The numerous attempts to correlate metal bioavailability to a single extractant has shown a slightly better correlation in some cases, but since metal uptake varies with a number of parameters such as soil pH, organic content and clay content, this approach is of practical use only for soils with similar properties. Attempts to predict the uptake from an unknown soil with other properties will not be successful, and thus, extraction with one single chemical will have limited practical use. The results also show that the correlation between single extraction and metal uptake is better for Cd and Zn than for Cu and Pb.

The results reported here for the fractionation of municipal sewage sludge provide an explanation to the poor correlations between plant uptake of Cu and Pb and results for chemical extraction. It was found that a significant fraction of both Cd and Zn are loosely bound to the sludge, while Cu shows higher affinity for the organic/sulphide fraction and Pb was found to be mostly in the residual fraction. This gives support to the contention that a single extractant that predicts the bioavailability of Cu and Pb cannot be found. If, in spite of these findings, it is decided to use a single extractant, my results point to EDTA as a suitable extractant, because all metals giving a toxic effect are removed, although other findings show that almost any chemical that extracts the surface bound (exchangeable) metal will give similar results. Great care should be taken when interpreting these results.

A deeper understanding of the binding of metals to sludge may lead to a clearer prediction of bioavailable metal. Determination of partition coefficients could be a useful approach. Most earlier experiments model the sludge/water slurry as a two phase system. Metals bound to very small particles and large organic molecules cannot however be strictly considered dissolved. The need for discrimination between the truly dissolved phase and the colloidal phase becomes obvious. A model with three phases is therefore a better approximation of the sludge/water system. The three phase system includes a solid phase which is identical with the solid phase in a two-phase system. The aqueous phase is separated into two phases: the dissolved metals, which are in my research approximated by the electrochemically available metals, and the colloidal phase, which includes the organically bound metals which are considered mobile but not bioavailable. The electrochemically available metals are known to correlate well with the bioavailable metal.

Fractionation, using a sequential extraction scheme, indicates that Pb would stay in the solid phase, whereas both Cd and Zn would be found in the aqueous phase. The results from partitioning experiments confirm this as they show that Cd and Zn are found to a significant extent in the electrochemically available fraction. Copper is found to a significant extent in the organic fraction. A part of the organic fraction is dissolved in the aqueous phase as colloids, and, because Cu binds strongly to organic material, Cu is mostly found in the colloid phase.

The results for Cd and Zn show that these metals are weakly bound to the sludge and explains why there is a good correlation between bioavailability and extraction in some cases. This also explains why a two-phase system can be a good approximation for Cd and Zn. Copper, on the other hand, is to a significant extent found in the colloid phase, which is mobile, and therefore often extracted by single extractants, but in some cases not at all toxic. These results also provide an explanation to why the correlation between Cu in a single extraction and plant uptake is generally poor and why a model of the sludge/water system which does not take colloids into consideration gives poor results for Cu. Pb is mostly found in the solids, which is in accordance with the lack of extraction of Pb with any of the extractants tested in the fractionation experiments.

The results in the partitioning experiments point to Zn and Cd as metals which show a high potential bioavailability, as these metals partition into the electrochemically available phase. A comparison to a toxicity test (Microtox) should then show a good correlation between electrochemically available metals and the toxic fraction. However, adsorption experiments show that when a known toxic concentration corresponding to the EC50 value is added to a sludge sample, the toxicity is higher than expected. Clearly, the toxicity of a metal-containing sludge sample cannot be explained by measuring the concentration of metal ions in the electrochemically available fraction alone. Also, the complexation of Cu with organic material does not necessarily render it non-toxic and unavailable for organisms. The approach that there is only one toxic metal species that can be isolated by simple chemical means is perhaps a little too simplified.

A sludge sample has many components, most of them unidentified or at least unquantified. There is of course a possibility that these different substances can interact, either by forming complexes outside the organism, or by interacting inside the cell. The results from toxicity experiments with mixtures of two metals showed that there is an antagonistic effect between Cu and Zn. This indicates that the total toxicity may be lower than the sum of the individual toxicities in a mixture of metals. However, there are many other substances in a sewage sludge, some of them known to interact with metals. Addition of certain organic substances, for example NTA (nitriloacetic acid) or EDTA which are both complexing agents found in washing powder, decrease the toxicity of Cu considerably by the formation of a strong, non-toxic complex. On the other hand, there are small organic molecules known to form lipid-soluble complexes with metals. My research shows that such complexes can be present in sewage sludge. Myristic acid, palmitic acid and nonylphenol are examples of known sludge-borne substances that considerably increase the toxicity of Cu to Microtox.

An important question is which effect dominates in the sludge; detoxification by NTA and similar complexing agents or increase in toxicity through the formation of lipid-soluble metal complexes. My research shows that the toxicity of Cu in sludge is higher than could be expected if only ionic metal was present, which suggests that the lipid-soluble complexes of Cu provide the overriding contribution to sludge Cu toxicity.

This has two important implications. The first is that it is not possible to classify a sludge as toxic or non-toxic without a toxicity test. There is no simple way to identify the toxic concentration of a metal by a chemical test that measures total content, concentration in an extracted fraction or through partitioning into different fractions. The second important implication is that there is little we can do to avoid these synergistic effects between metals and organic components in the sludge. Substances such as nonylphenol are in many sewage treatment plants monitored together with other priority pollutants. Nonylphenol is a degradation product of an industrial detergent which is nowadays used less and it is, at least theoretically, possible to prevent the discharge of such substances to the sewer system. It is much more difficult to remove naturally occurring substances. Caffeine, cholesterol, myristic acid and palmitic acid are together with other food derived products unavoidable substances in sewage sludge. The whole point of a modern sanitary system is to remove and take care of domestic wastewater, including wastewater from kitchens and toilets. It is of course impossible to remove substances originating from food or our own metabolism from the sludge without missing the whole point of the present sanitary system.

A number of conclusions can be reached. First of all, if we want to lower the toxicity of metal-organic complexes in fresh sludge, it may be a sensible approach to try to remove metals to a critical level. This critical level might be defined in terms of the loading of metals normally found within the sludge-agricultural system (background levels and concentrations). Secondly, if this is not possible, the metal-organic complexes should be degraded before spreading on farmland. There is still a lot to be done in this field. Methods such as composting, anaerobic digestion, UV-degradation and different sludge spreading concepts should be evaluated. Thirdly, and most important for our research, the need for a better method to predict the toxicity and bioavailability of metals in sludge is even more urgent in the light of our findings.

What demands could then be made on such a predictive method? My research, together with earlier findings, shows that it is difficult to create a model of either, sludge/water or sludge-amended soil/water. A number of key parameters are involved in determining how, and in which chemical form, the metal leaves the particle and enters the soil water. Without complete control over these parameters, both single extraction and more complicated extraction schemes will be difficult to interpret. This is of course a problem, because the whole point of all the attempts to correlate bioavailability with these simple extraction methods is to find a standardised method that can be used on any sludge or soil sample.

The reason why this does not work is because the assumption, that metals in sludge are present in only one single species which exerts its toxicity independently of all other substances present, is not true. Neither is the assumption that all extractions result in a complete removal of the metals in a specified fraction of the sample, leaving the other fractions unaffected. Since I have found that metals not only interact with each other, but also with a range of organic substances in sludge, the metal mobility, bioavailability and toxicity must be measured directly. There is also a need for further research concerning the kinetics of metal binding to sludge. There is no point in comparing results from one extraction or partitioning experiment with another experiment with different duration if the influence of time on the results is not known in detail. It is also necessary to evaluate the relevance of laboratory timescales to environmental timescales.

More emphasis should be placed on the discrimination between the terms toxicity, bioavailability and mobility. My conclusion is that Cu, which is often bound to large, hydrophilic complexing agents, both anthropogenic such as NTA and natural, such as humic substances, is mobile but has only limited bioavailability or toxicity. To be toxic, the metal must of course be bioavailable, but for a bioavailable metal to be acutely toxic, exceedance of an effect concentration is necessary. For some relatively non-toxic metals, an organism can take up considerable amounts without toxic effects. It remains to be investigated if a metal can be toxic without being mobile. The organic metal-complexing exudates excreted from the roots of some plants seem to play an interesting part in this, a mechanism which deserves more attention.

In this thesis, the only method used for the assessment of toxicity measurement is Microtox. The Microtox standard method has been criticised in chapter 3 for giving irrelevant results for non-saline samples. This can be avoided by substituting $NaClO_4$ for NaCl. However, this is not the only criticism of the Microtox method. The connection between the response of a marine bacterium and uptake by wheat or radish is doubtful and is not attempted here. The approach in this thesis is to investigate under which circumstances a metal can enter the cell and exert a toxic effect. The effects a metal may have, how the toxicity is expressed inside the cell and what damage it can do to an organism is a field of research that might be investigated elsewhere.

However, one aspect of toxicity testing must be discussed here and that is the connection between acute toxicity and long-term environmental hazards. Microtox is an acute toxicity test which gives results within the hour. When discussing whether to apply sludge to farmland, the risk for immediate crop damage is not a main issue, but rather what happens over many years in terms of potential human exposure. An acute toxicity test can identify which substances show immediate toxic effects, but it might not identify the long-term effects of low doses. Neither can Microtox indicate if new, toxic substances are formed during the degradation of sludge.

Since the process of determining the suitability of sewage sludge as a soil improver is so full of uncertainties, it is tempting to draw the conclusion that the best solution to this problem is simply to stop using sludge for this purpose. However, this strategy creates two new problems. Firstly, the potential environmental hazards connected with the sludge does not disappear just because it is deposited somewhere else. The leaching of toxic metals can be just as harmful to the environment in a park or on a golf course even if we place a greater distance between the sludge metals and an ecosystem. To avoid these problems, the sludge has to either be safely deposited or be destroyed, both often economically unfavourable alternatives.

Secondly, sewage sludge represents a valuable source of phosphorus which should be recycled. Alternatives include phosphate fertiliser or manure. None of these are free from metals and it is important to compare the bioavailable metal content with phosphate concentration in the sludge to alternatives. Also, the mining of phosphate fertiliser introduces more Cd to the biosphere, whereas application of sewage sludge or manure only recirculates the Cd already present in the biosphere.

Most important, however, is that phosphate is a limited resource which will become scarce with time. It is necessary to recirculate phosphorus and we cannot afford to remove large quantities of this essential nutrient by dumping it into the sea or depositing it together with other kinds of wastes that contaminate the sludge with pollutants that may be both hazardous and persistent. In a sustainable society, the recirculation of phosphorus in sewage sludge is an urgent problem that needs a solution.

It has been estimated that the worldwide sewage sludge production is 30 million tonnes, of which 70% is deposited on land. The contribution of metals to soils from sludge is estimated to 20 - 340 tonnes of Cd, 4 900 - 21 000 tonnes of Cu, 2 800-9 700 tonnes of Pb and 18 000 - 57 000 tonnes of Zn every year. This means that, although sewage sludge may not be a particularly important metal source on a global scale, it can locally represent one of the most important sources. The major sources of trace metal pollution to the aquatic ecosystem include domestic wastewater effluents and the dumping of sewage sludge. One way to decrease direct pollution from domestic wastewater effluents is to build sewage treatment plants. This means that action taken to improve water quality in the environment produces more sludge, of which a higher percentage will be deposited on land. In other words, metals do not disappear from the biosphere and since anthropogenic activities are now responsible for a larger contribution of metals in the biosphere than the natural biogeochemical cycling of metals, the problem of increasing concentrations of toxic metals in the biosphere is becoming increasingly urgent every year. We therefore need to be able to move from the assessment of metal speciation and toxicity in sewage sludge presented here towards methods which allow an assessment of the relevance of metal speciation in sewage sludge during and after application to agricultural soils over environmental timescales. This is essential for the protection of future human generations and may involve the development of analytical techniques in metal speciation with a longer time perspective.