Ecotoxicological Assessment of Sewage Sludges and Phosphate Recyclates by Standard Tests and New Methods

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Ökotoxikologische Untersuchung von Klärschlämmen und Phosphatrezyklaten mittels Standardtests und neuer Methoden

In der europäischen Union (EU) entstehen bei der Abwasserreinigung große Mengen an Klärschlamm. Behandelter und aufbereiteter Klärschlamm enthält in der Regel beträchtliche Mengen an Nährstoffen. Insbesondere Phosphor, der nicht synthetisch hergestellt werden kann und wesentlich für das Wachstum von Pflanzen ist. Beim Umgang mit Klärschlamm ist die Verwendung in der Landwirtschaft, die durch europäisches und deutsches Recht reguliert wird, die nachhaltigste Möglichkeit der Entsorgung. Doch leider enthält Klärschlamm wegen seiner Funktion als Schadstoffsenke in der Abwasserreinigung auch zahlreiche schädliche Substanzen für die Umwelt. Phosphorrückgewinnung aus Klärschlamm und seine landwirtschaftliche Aufwertung in recycelten Düngern bietet als Alternative zur traditionellen Anwendung Möglichkeiten, die Umweltauswirkungen durch geringere Schadstoffbelastungen zu minimieren. In dieser Arbeit wurden unter Laborbedingungen die potenziellen akuten, ökotoxikologischen Auswirkungen unterschiedlicher Klärschlämme, Phosphatrezyklate (PRs; kristallisiert (Struvit), thermisch behandelt) und eines konventionellen Phosphatdüngers (Tripelsuperphosphat (TSP)) untersucht. Die umfassende Beurteilung der komplexen Gemische soll zu deren sicheren Verwendung in der Landwirtschaft beitragen. Geeignete ökotoxikologische Standardtestmethoden wurden zur Abdeckung der betroffenen Umweltkompartimente (Boden, Wasser, Sediment) ausgewählt und auf Umweltproben angepasst. Folgende Testarten, Hauptparameter und zusätzliche Verhaltensparameter wurden dafür verwendet: Kompostwurm *Eisenia fetida* (Meidungsverhalten), Wasserlinsen *Lemna minor* (Wachstumshemmung, Verfärbung, Kolonieauflösung, Hemmung des Gewichts und der Wurzellänge) und Bachflohkrebs *Gammarus fossarum* (Mortalität, Bewegungsverhalten, Fressverhalten). Bei der Bewertung hatten die Phosphatrezyklate meist eine geringere Wirkung auf die getesteten Organismen als die Klärschlämme (insbesondere der nicht entwässerte Schlamm) und der konventionelle Phosphatdünger TSP. Relevante Konzentrationen der Klärschlämme (außer dem nicht entwässerten Schlamm), der Phosphatrezyklate und von TSP auf dem Feld sollten vermutlich gemäß der berechneten maximalen landwirtschaftlichen Ausbringungsmenge (Worst-Case-Szenario, basierend auf dem Phosphatgehalt) den Invertebraten im Boden (*E. fetida*) nicht schaden. Sollte jedoch ein Teil der Ausbringungsmenge von TSP und des nicht entwässerten Schlammes Oberflächengewässer erreichen, könnte das Überleben des Bachflohkrebses beeinträchtigt werden. Kristallisierte und thermisch behandelte PRs könnten in Oberflächengewässern

geringe Auswirkungen auf das Wachstum der Wasserlinsen verursachen. Im Gegensatz dazu sind zunehmende Effekte auf *L. minor* durch den nicht entwässerten Schlamm zu erwarten. Die ökotoxikologische Bewertung im Vergleich zu den im Worst-Case-Szenario landwirtschaftlich ausgebrachten Mengen, im Bezug zu einer quantitativen und relativen Risikobewertung von einzelnen Schadstoffen der Klärschlämme und Phosphatrezyklate, sprechen dafür, Phosphatrückgewinnung in der Abwasserreinigung und die landwirtschaftliche Wiederverwendung, besonders von Struvit, in Zukunft weiter zu verfolgen. Dadurch lassen sich Umweltrisiken reduzieren und Nährstoffkreisläufe schließen. Des Weiteren wurde festgestellt, dass die Umweltauswirkungen von Klärschlamm und Phosphatrezyklaten nicht allein durch chemische Analysen von einzelnen Schadstoffen bestimmt werden können. Gegenseitige Interaktionen der Schadstoffe in einem derartig vielfältigen, komplexen Gemisch und unterschiedliche Bioverfügbarkeiten der Schadstoffe könnten die Wirkung beeinflussen. Daher empfehlen sich terrestrische, ökotoxikologische Tests im Zielkompartiment Boden für ein potenzielles Standardmonitoring-Konzept für Klärschlamm und wieder gewonnene Phosphatprodukte. Für ein umfangreicheres Monitoring sollte die Wirkungsbewertung im Kompartiment Wasser mit sensitiven aquatischen Organismen (z. B. *G. fossarum*) miteinbezogen werden. Als ein sensitives und einfaches ökotoxikologisches Screening-Hilfsmittel zur Bewertung der Habitatfunktion von kontaminierten Böden und der terrestrischen Toxizität von bestimmten Schadstoffen wurde ein neuer Versuchsaufbau zur ständigen Analyse des Meidungsverhaltens von *E. fetida* entwickelt.

Ecotoxicological assessment of sewage sludges and phosphate recyclates by standard tests and new methods

In the European Union (EU), huge amounts of sewage sludge are generated during the treatment of wastewater. Treated and processed sewage sludge (biosolids) usually contains substantial concentrations of nutrients. Especially phosphorus, which cannot be produced synthetically and is essential for plant growth. For sewage sludge management, agricultural application, regulated by EU and German law, is the most sustainable option of disposal. But unfortunately, sewage sludge also contains, due to its function as pollutant sink in wastewater treatment, a multitudinous amount of harmful substances for the environment. Phosphorus recovery from sewage sludge and its agricultural valorisation in recycling fertilisers as an alternative for traditional application provides opportunities to minimise the environmental effects due to lower pollution loads. In this thesis, the potential acute ecotoxicological effects of different types of sewage sludge, phosphate recyclates (PRs; crystallised (struvite), thermally treated) and a conventional phosphate fertiliser (triple superphosphate (TSP)) were investigated under laboratory conditions. The comprehensive assessment of the complex mixtures shall contribute to their safe use in agriculture. Suitable ecotoxicological standard test methods for covering the affected environmental compartments (soil, water, sediment) were chosen and adjusted for environmental samples. The following test species, main parameters and additional behavioural parameters were applied: earthworm *Eisenia fetida* (avoidance behaviour), duckweed *Lemna minor* (growth inhibition, discolouration, colony break-up, inhibition of weight and root length) and freshwater shrimp *Gammarus fossarum* (mortality, movement behaviour, feeding behaviour). In the assessment, the phosphate recyclates had mostly a smaller effect on the tested organisms than the sewage sludges (especially the non-dewatered sludge) and the conventional phosphate fertiliser TSP. Relevant concentrations of the sewage sludges (except of the non-dewatered sludge), the phosphate recyclates and TSP on the field should probably not affect the soil invertebrates (*E. fetida*) in compliance with the calculated maximum agronomical relevant application amounts (worst-case scenario, based on the phosphate content). But if an amount of the output concentration would reach surface waters, the survival of the freshwater shrimp could be negatively affected by TSP or the non-dewatered sewage sludge. Minor effects on the growth of the duckweed might be caused by crystallised and thermally treated PRs in surface waters. In contrast, increasing effects on *L. minor* are expected by the non-dewatered sewage sludge. The ecotoxicological assessment compared to worst-case application amounts in agriculture with regard to a quantitative and relative risk assessment of single pollutants of the sewage sludges and phosphate recyclates indicate to follow up phosphate recovery in wastewater treatment and recycling in agriculture, especially of struvite, in the future. Thus, environmental risks can be reduced and nutrient cycles can be closed. Furthermore, it was found that the effects of sewage sludge and phosphate recyclates on environment cannot be determined just by chemical analysis of singular substances. Mutual interactions of the pollutants in such varied complex mixtures and different bioavailabilities of the pollutants could influence the effect. Therefore, terrestrial ecotoxicological tests in the target compartment soil can be recommended for a potential standard monitoring concept for sewage sludge and recovered phosphate products. For a more comprehensive monitoring, the assessment of effects on the water compartment by sensitive aquatic organisms (e.g. *G. fossarum)* should be included. As a sensitive and simple ecotoxicological screening tool for the assessment of the habitat function of contaminated soils and the terrestrial toxicity of particular contaminants, a new experimental set-up for analysing the avoidance behaviour of *E. fetida* permanently was developed.

Chapter 1

General Introduction

General Introduction

1.1 Sewage sludge production and disposal

In the EU Member States about 10 million tons of dry matter (DM) of sewage sludge are produced per year. Thereof, 8.7 million tons are recorded by the EU-15 Member States (old members), around 2 million tons by Germany alone and only 1.2 million tons by the new Member States (these statistics do not include Croatia, which has officially joined the EU in 2013) (Milieu Ltd et al. 2010). Over the last decades, the total amount of sewage sludge produced has increased in most of the EU-15 Member States, primarily due to the implementation of the Urban Wastewater Treatment Directive 91/271/EEC (1991a) and will obviously cause a further increase of annual sewage sludge production by the EU-13 Member States, exceeding 13 million tons DM up to 2020 (Milieu Ltd et al. 2010; Leonard 2011). The Directive forced the countries to improve their wastewater management as it prescribes the collection and treatment of municipal wastewater for agglomerations with more than 2000 person equivalents (p.e.) by 31 December 2005 (EEC 1991a). The amount of sludge produced per p.e. differs strongly between the Member States and also between new and old Members of the EU (Milieu Ltd et al. 2010). Variations in percentages of population that are served by centralised wastewater treatment systems as well as variations in wastewater treatment applied in each country and contribution of the industrial sector result in these differences (Kelessidis & Stasinakis 2012). The way to manage these growing amounts of sewage sludge usefully has become a key issue in the European Union. But so far, there is not a clear view concerning sewage sludge handling (treatment and disposal practises) as well as relative legislation across the EU (Kelessidis & Stasinakis 2012). Four different types of disposal make up a considerable amount of the total volume of sewage sludge treated: agricultural use, compost and other applications, landfill and incineration (Figure 1.1). In five of the EU Member States (Portugal, Ireland, the United Kingdom, Luxembourg and Spain) at least three quarters of the total sewage sludge mass was used as fertiliser for agricultural application, which is the most sustainable option for sewage sludge management – while the Netherlands, Belgium, Germany, Slovenia and Austria (as well as Switzerland) reported incineration as their principal form of treatment for disposal (Eurostat 2016).

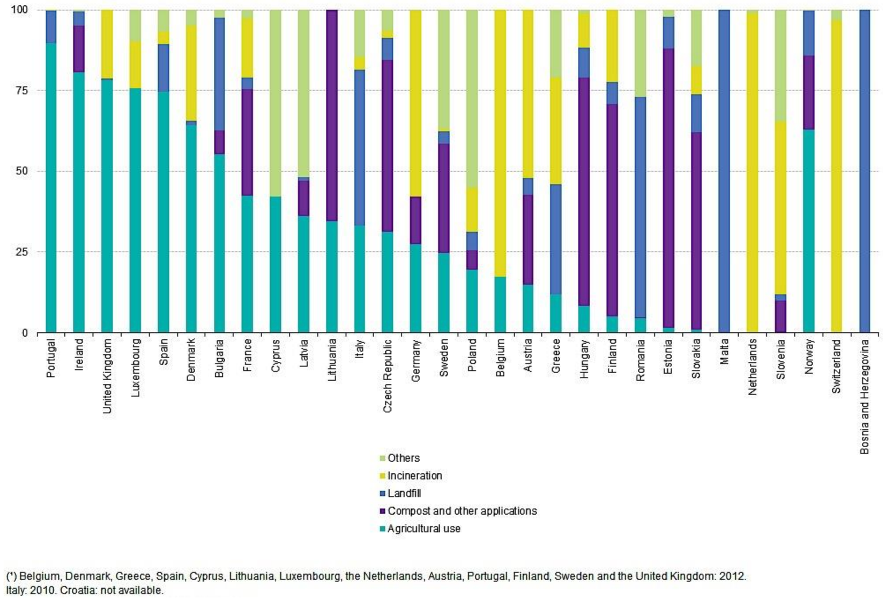

Figure 1.1 Sewage sludge disposal from urban wastewater treatment in Europe in 2013[1], by type of treatment, % of total mass (Eurostat 2016)

1.2 Sustainable sewage sludge management – Phosphorus recovery

Phosphorus (P) is a limited and in its function as a nutrient especially for plant growth an essential and irreplaceable resource which cannot be produced synthetically (Asimov 1959; Filippelli 2008). The huge amount of mineral phosphorus that is annually imported into Europe, mainly mined from phosphorous-rich rocks from Morocco, China and the USA to sustain good harvests, demonstrates its interest as fertiliser nutrient (Vaccari 2009; USGS 2016). Phosphorous-rich rocks are finite and distributed in just a few places on the planet. The geopolitics and economic vulnerability are issues to be taken seriously from the European perspective because they have in fact only one small mine in Finland (Kabbe et al. 2015). So, Europe is highly dependent on phosphorus imports (De Ridder et al. 2012). Therefore, the European Commission has added phosphate rock to the list of 20 Critical Raw Materials for which supply security is at risk and economic importance is high (EC 2014). P recovery and recycling should play an important role in improving resource efficiency and sustainable nutrient management. Although there are various relevant waste streams, carrying huge quantities of phosphorus dissolved in liquids or fixed in solids (e.g. manure or organic waste) (Kabbe et al. 2015), the focus here is on P recovery techniques and recycling from wastewater and sewage sludge. Treated and processed sewage sludge (biosolids) is a nutrient-rich material which represents a relevant phosphorus reserve and has the potential to cover about 20 % of the demand for phosphorus in Europe (RPA et al. 2008). But sewage sludge also contains, due to its function as pollutant sink in wastewater treatment, a multitudinous amount of harmful substances such as pathogens, endocrine disrupters, toxic heavy metals and organic pollutants (Oliva et al. 2009; Wiechmann et al. 2013).

The legislations for sewage sludge management in Germany are based on different European directives followed by German acts and regulations within the thematic fields of waste management and fertilisation in agriculture (Wiechmann et al. 2013). The Recycling Management Act of Germany (KrWG), which was transposed into German law on the basis of the European Waste Framework Directive 2008/98/EC (2008a), governs waste management and thus sewage sludge as well (KrWG 2012). It aims to improve the sustainability of environmental and climatic protection as well as resource efficiency for waste management through optimised waste prevention and recycling by a five-level hierarchy comprising the following elements: (1) prevention or reduction of waste, (2) re-use (without any structural changes), (3) recovery of materials (e.g. recycling and composting), (4) other uses (e.g. energy recovery) and (5) final disposal (landfilling) (KrWG 2012). So, the handling of sewage sludge and the usage of the sludge for recycling or disposal is dependent on its consistency according to the KrWG (2012). Additionally, the Landfill Directive 1999/31/EC prohibits the landfilling of liquid and untreated wastes (EC 1999). In the Sewage Sludge Directive 86/278/EEC, the first steps towards a regulation of the targeted use of sewage sludge in agriculture in the European Union was undertaken. The aim of the directive is to regulate the agricultural use of untreated sewage sludge by avoiding deleterious effects on soil, vegetation, plants, animals and humans while promoting environmentally sound sludge use practices (EEC 1986). For correctly using sewage sludge in agriculture, the sludge should be treated before and mandatory threshold values for heavy metals in the sludge and soil should not be exceeded (Cd, Cu, Ni, Pb, Zn, Hg) (EEC 1986). The current regulation of waste and sewage sludge in Germany (AbfKlärV 1992) implemented the directive with lower threshold values for heavy metals. If the Sewage Sludge Regulation (AbfKlärV) does not apply with regard to the usage of biowaste as fertiliser (e.g. minimal contaminated sewage sludge), the Biological Waste Regulation (BioAbfV) will be effected (BioAbfV 1998). Additionally, the fertiliser regulations controlling the hygienisation (disinfection) of the sludge and the protection of soil and water bodies against overfertilisation (DÜV 2006; DüMV 2012) has to be noted for using sewage sludge as fertiliser. In 2017, the federal cabinet has officially confirmed to aggravate the guidelines for fertilisation in an amended regulation and law for reducing drastically fertiliser application for sustainable agriculture and limiting nitrate loading of ground water in order to finally maintain the European Nitrates Directive 91/676/EEC (EEC 1991b; BMUB 2017b). But in the near future the direct application of sewage sludge shall be mostly ceased in Germany because of the multiple amount of hazardous substances which could have negative effects on the environment and health (CDU et al. 2013; BMUB 2017c). Stricter requirements for pollution loads in compliance with the lower limit values of the Fertiliser Ordinance of Germany (DüMV 2012) already have to be followed since 1 January 2015 (UBA 2015). In the upcoming amended Sewage Sludge Ordinance, expected to come into force in 2018, threshold values for persistent organic pollutants (POPs) will be stated as well (BMUB 2017a, c). The usage of approved sewage sludge as fertiliser will be only allowed for wastewater treatment plants (WWTPs) lower than 50,000 person equivalents. Further, phosphorus recovery will be regularised and mandatory for WWTPs with high treatment capacities (>50,000 p.e.) forcing the recovery of recyclable substances from municipal wastewater and sewage sludge after a transition phase of 12–15 years (BMUB 2017c). The WWTPs will have to recover the phosphorus if the sludge contains more than 2 % phosphorus in the dry matter and/or have to incinerate the sludge in mono-incinerators or use it for energy recovery (BMUB 2017a).

1.3 Phosphorus recovery techniques from wastewater and sewage sludge

Excess quantities of phosphorus cause eutrophication, which can be described as nutrient enrichment of surface waters, leading to an excessive production of algae (partly toxic), and is responsible for turning water green in lakes, reservoirs, rivers, and coastal waters as well as the marine environment in general (Burke et al. 2004). Traditional P removal processes reducing efficiently the phosphorus concentration in wastewater effluents to less than 1 mg l^{-1} (Booker et al. 1999) are based on phosphorus fixation in activated sludge – either by a biological (enhanced biological phosphorus removal) or chemical (precipitation by metal salts) method. But these processes lead to the accumulation of the phosphorus in the liquid or solid sludge phase and an increase in sludge volumes (Le Corre et al. 2009). So, several solutions for technically advanced P recovery and recycling have been developed to provide alternatives to the general direct application of sewage sludge in agriculture. Dissolved phosphorus can be technically recovered from the liquid phase of the sludge prior (2a in Figure 1.2) or subsequent to the sludge dewatering process (2b in Figure 1.2) depending on the infrastructure for wastewater treatment (Le Corre et al. 2009; Egle et al. 2015; Kabbe et al. 2015). If sludge is incinerated undiluted in mono-incineration plants, the resulting ash contains high concentrations of phosphorus (3 in Figure 1.2) which is limited in plant-availability before further treatments (Egle et al. 2015; Herzel et al. 2015; Kabbe et al. 2015).

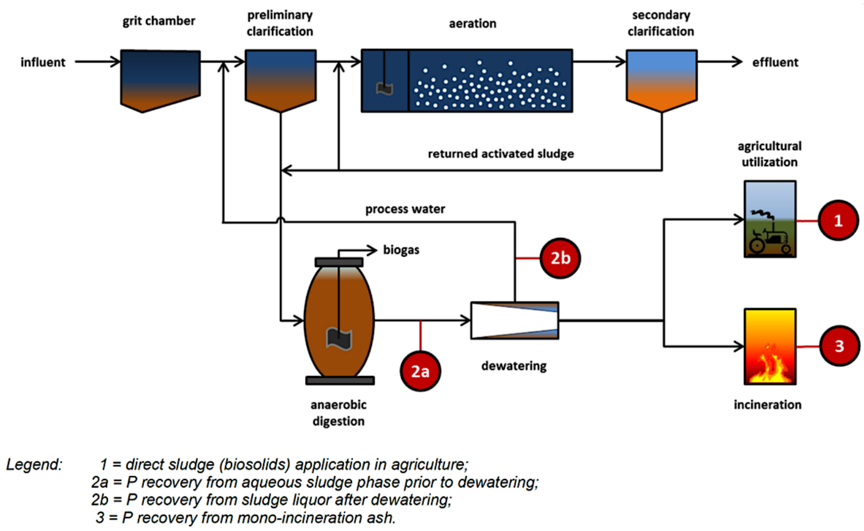

Figure 1.2 Hotspots for P recovery from the wastewater stream (in centralised sanitation systems) (Kabbe 2013, amended from Pinnekamp et al. (2007))

Biologically bound phosphorus which was taken up before by phosphorus accumulating bacteria is released into the liquid phase of the sludge as water soluble ortho-phosphate due to anaerobic conditions during digestion of the sludge. Normally, the concentration of ammonia in the liquid phase is increased as well due to the degradation of the biomass (Stemann et al. 2014). P recovery from the liquid phase is mostly based on precipitation or crystallisation processes occurring directly in the sludge or in the process water after sludge dewatering by mechanical solid-liquid separation like e.g. centrifugation. These techniques are also applicable to industrial wastewater containing significant concentration of dissolved ortho-phosphate (Stemann et al. 2014). All these processes for phosphorus recovery from sludge provide a solid mineral phosphorus product by precipitation or crystallisation as calcium phosphate (CaP) and/or magnesium ammonium phosphate which is most commonly known as struvite ($Mg^{2+}+NH_4^{+}+PO_4^{3-}+6H_2O \rightleftharpoons MgNH_4PO_4{\cdot}6H_2O$). The struvite output can be raised by sufficient magnesium dosing (e.g. $MgCl_2$, $Mg(OH)_2$) and an increasing pH if required in consequence of e.g. CO_2 stripping by aeration or addition of caustic soda (NaOH) in adapted reactors (Le Corre et al. 2009; Stemann et al. 2014; Egle et al. 2015). For the recovery of phosphorus from digested sewage sludge generated by chemical phosphorus elimination an extraction e.g. by acidic treatment of the sludge (addition of e.g. sulphuric acid H_2SO_4 or using carbon dioxide CO_2) has to be applied to mobilise a higher percentage of phosphorus in the liquid phase (Jaffer et al. 2002; Stemann et al. 2014). The challenge of sludge leaching is the separation of the simultaneously remobilised heavy metals from the phosphorus and the post-treatment or disposal of the contaminated and neutralised leaching agents (Kabbe et al. 2015). To prevent the contamination of the recovered P product by heavy metals, citric acid can be added to the filtrate to mask the metal ions (Stemann et al. 2014;

Kabbe et al. 2015) or the metal ions can be separated as sulphides by dosing Na_2S in a subsequent step after the extraction (Kabbe et al. 2015).

The direct application of untreated sewage sludge ash in agriculture is limited due to high heavy metal contents exceeding the limit values of the German Fertiliser Ordinance (DüMV 2012) and the low plant availability of phosphorus after mono-incineration of sewage sludge (Egle et al. 2015; Herzel et al. 2015; Kabbe et al. 2015). Thermo-chemical and wet-chemical treatments are the basis of the technologies for P recovery from sewage sludge ash. In the thermo-chemical processes the phosphorus bound as tricalcium phosphate or aluminium phosphate in the solid phase (or in the slag melt) reacts with additives like $MgCl_2$ or Na_2SO_4, forming new phosphate compounds with a higher plant availability (e.g. $MgCl_2$ forms magnesium phosphates and magnesium calcium phosphates). Further, a separation of heavy metals and phosphorus by volatilisation at high temperatures (addition of auxiliary substances is possible) and/or a phase separation because of density differences of the melts is included in the process type (Donatello & Cheeseman 2013; Herzel et al. 2015; Kabbe et al. 2015). The wet-chemical treatment can be further separated into leaching and extraction. With wet-chemical leaching the ash is washed with an acidic liquid (e.g. addition of sulphuric acid H_2SO_4 or produced by specific microorganisms) which leaches a lot of the heavy metals and phosphorus from the ash. After a required heavy metal removal by a separate precipitation and filtration for the separation of solids from the P-rich leach liquor, the pH of the leachate is subsequently increased with e.g. lime or caustic soda until a high P recovery by precipitation is achieved (Donatello & Cheeseman 2013; Egle et al. 2015; Herzel et al. 2015; Kabbe et al. 2015). The wet-chemical extraction commonly used in the fertiliser industry to produce a commercial fertiliser from raw phosphate forces the transformation of P that is not immediately plant-available to water-soluble and available P. The process is best applicable for ashes with a low heavy metal content due to the lack of a decontamination step (Egle et al. 2015).

1.4 Monitoring of sewage sludge and phosphate recyclates by ecotoxicological test methods

The application of sewage sludge in agriculture can implicate problems of hygiene, toxicity and accumulation of heavy metals and organic pollutants which might have negative effects on biota in soil. If the pollutants end up in surface water or groundwater as the result of an accident or inappropriate handling, run-off (Galdos et al. 2009) or percolation (Luczkiewicz 2006; Alvarenga et al. 2016), aquatic organisms could be negatively affected as well. But as the application of sewage sludge on agricultural land is still a cost-effective possibility of sludge disposal where all valuable substances like nutrients and organic matter can be valorised, it should not be prohibited in general but the monitoring methods should be reviewed to guarantee a safe use of sludge for the environment depending on sludge quality (Wilken et al. 2015). Potential risks linked to the use of recovered phosphate-containing materials (phosphate recyclates) from wastewater treatment as fertiliser cannot be excluded completely either due to harmful residues in the recyclates. Many directives and regulations about the handling of waste and the correct use of sewage sludge and fertiliser exist in the EU and Germany, considering generally chemical analysis of the concentration of certain heavy metals and organic pollutants (target substance monitoring) in the solid material and soil. But the amount and variety of substances in sewage sludge makes overall routine monitoring costly and maybe incomplete as well because of unknown constituents. Therefore, ecotoxicological test methods should be considered to analyse effects of the heterogeneous substance mixture of sewage sludges as well as of recycled fertilisers on the environment more comprehensively. Ecotoxicological information is already required for placing priority substances on the European market through registration and authorisation according to the volumes of manufacture or importation (quantities of 1 tonne or more per year) because these provide an indication of the potential for exposure of man and the environment. For other substances or mixtures in that quantity, incentives should be given to encourage manufacturers and importers to provide this information (EC 2006).

Previously, the ecotoxic effect of wastewater effluents (Gutiérrez et al. 2002) or of heavy metals in the leachate of sewage sludges on water organisms (Fjällborg & Dave 2003; Fjällborg et al. 2005), as well as the direct toxic effect of sewage sludges or their effect over time with bacteria and terrestrial plants (Roig et al. 2012), soil invertebrates and terrestrial plants (Carbonell et al. 2009; Natal-da-Luz et al. 2009a, b) were investigated. Research about phosphate recyclates deals mostly with life cycle assessment (LCA) or risk assessment of phosphorus recovery processes considering contaminant concentrations plus e.g. emissions from transport or chemical manufacture etc. (Bradford-Hartke et al. 2015; Kraus & Seis 2015; Remy & Jossa 2015). The aquatic toxicity of the eluates of raw sewage sludge ash before and after bioleaching on algae, daphnia and bacteria was examined by Zimmermann (2010). But, researchers did not, so far, concentrate on investigating the direct toxic effect of sewage sludge or phosphate recyclates by the use of selected organisms of all potential affected compartments (soil, water, sediment). The test parameters and organisms were chosen due to sensitivity, handling and duration for the mentioned compartments (Wilken et al. 2015): (1) the avoidance behaviour of the earthworm *Eisenia fetida* (Savigny 1826, Oligochaeta, Lumbricidae) in the soil compartment, (2) the growth inhibition and further parameters (discolouration, weight decrease, etc.) of the water plant *Lemna minor* (Linné 1753, Arales, Lemnaceae) in the water compartment and (3) the mortality and behaviour of the freshwater shrimp *Gammarus fossarum* (Koch 1836, Amphipoda, Gammaridae) in the water and sediment compartment. Especially considering the assessment of the habitat function of soil, terrestrial test systems with earthworms as representatives of the soil fauna and indicators for soil quality due to chemoreceptors in the prostomium and anterior segments, as well as sensory cells in the buccal epithelium (Wallwork 1983; Edwards & Bohlen 1996; Csoknya et al. 2005) can be used. The acute avoidance test with a much shorter test duration of 2 days, allowing earthworms to choose a compartment, was evaluated as sensitive as the chronic reproduction test (Hund-Rinke et al. 2003). Gathering comprehensive knowledge, the avoidance behaviour of earthworms was intensely investigated by researchers in the last years. The behaviour was analysed under different conditions of contamination (e.g. Natal-da-Luz et al. 2004; Matos-Moreira et al. 2011; Santos et al. 2012), test conduction and exposure time (e.g. Natal-da-Luz et al. 2008b; Frankenbach et al. 2014; Amaro et al. 2016). Whereas, no research about the test method was concerned with the fact that behaviour patterns like avoidance (the analysed endpoint) could constantly change over time.

1.5 Objectives of this thesis

The aim of this study was to investigate and compare potential acute ecotoxicological effects of different types of sewage sludge, recovered phosphate-containing materials (phosphate recyclates) from sewage sludge and a conventional phosphate fertiliser under laboratory conditions. For this purpose, suitable ecotoxicological standard test methods for covering the affected environmental compartments and additional behavioural parameters were chosen and adjusted for environmental samples. Further, a permanent, automated and non-optical monitoring method for analysing the avoidance behaviour of earthworms over time instead of a fixed point in time should be developed for the usage as a simple ecotoxicological screening tool for the assessment of contaminated soils. This study aims to contribute to the correct use of sewage sludge and recycled fertilisers in agriculture by the assessment of their ecotoxicological effects. The study conducted by the project partner LimCo International GmbH was part of the EU Project P-REX "Sustainable sewage sludge management fostering phosphorus recovery and energy efficiency (Contract No 308645)" dealing with the documentation and description of all relevant aspects of alternative technologies and options that exist for recovery and recycling from wastewater, thus enabling informed decisions and accelerating the transition to a circular economy of phosphorus.

Chapter 2

Toxic potential of different types of sewage sludge as fertiliser in agriculture: ecotoxicological effects on aquatic, sediment and soil indicator species

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Toxic potential of different types of sewage sludge as fertiliser in agriculture: ecotoxicological effects on aquatic, sediment and soil indicator species

2.1 Abstract

Purpose Treated and processed sewage sludge (biosolids) generated during the treatment of wastewater usually contains substantial concentrations of nutrients, especially phosphorus which is essential for plant growth. Sewage sludge therefore can be used as an alternative fertiliser in agriculture. But since sewage sludge could also contain pollutants, analysis and ecotoxicological tests on affected soil and stream water organisms are necessary in order to guarantee its harmless use.

Materials and methods Three test species were chosen to cover the environmental compartments water, sediment and soil. The following test species and parameters were applied to evaluate the acute effects of three sewage sludge samples: *Lemna minor* (growth inhibition, discolouration and colony break-up), *Gammarus fossarum* (mortality, behaviour), *Eisenia fetida* (avoidance behaviour). Chemical assessment included nutrients, organic pollutants and heavy metals.

Results and discussion The assessment of a non-dewatered sludge (S1) sample resulted in an inhibition of growth of *L. minor* starting from 0.6 g total solid (TS) l^{-1} after 7 days (EC_{50} 1.2 g TS l^{-1}). *G. fossarum* displayed significantly decreased movement activity at 0.5 and 1.2 g TS l^{-1} sludge concentration during an exposure time of 2 days, leading to decreased survival after 4 days of exposure in 0.5 g TS l^{-1} (LC_{50} 0.5 g TS l^{-1}). After 2 days, *E. fetida* exhibited an increased avoidance behaviour of contaminated soil from 0.2 g TS kg^{-1} sewage sludge (EC_{50} 0.4 g TS kg^{-1}). The dewatered sludge samples (S2 & S3) had a lower toxic effect on the test organisms. *G. fossarum* was the most sensitive test species in the applied test set-ups. The realistic application amounts of the tested sewage sludge samples of approximately 6 g TS kg^{-1} (maximum allowed application amount of sewage sludge) and approximately 3 g TS kg^{-1} (maximum agronomical relevant application amount) in worst-case studies are higher than the analysed EC_{50}/LC_{50} values of S1 and of the LC_{50} (*G. fossarum*) of S2 and S3.

Conclusions All three tested sewage sludge samples have to be classified as toxic at high concentration levels under laboratory conditions. Realistic output quantities of S1 will negatively influence soil invertebrates and freshwater organisms (plants and crustacean), whereas the dewatered sludge samples will most likely not have any acute toxic effect on the

test organisms in the field. Test with environmental samples should be conducted in order to support this hypothesis.

Keywords Phosphorus fertiliser • Potential toxic effects • Sewage sludge • Toxicity tests

2.2 Introduction

Since the technology of waste systems has improved and the expansion of wastewater treatment plants (WWTP) were undertaken, the pollution of our water ways has been reduced. During the cleaning process of wastewater in WWTPs, a high amount of sewage sludge is produced. Treated and processed sewage sludge (biosolids) is a nutrient-rich material which can be used as landfill, for energy production or as a fertiliser in agriculture, which is the most sustainable option for sewage sludge management. Sewage sludge represents a relevant phosphorus reserve and has the potential to cover about 20 % of the demand for phosphorus in Europe (RPA et al. 2008). Phosphorus is a non-renewable, essential but limited resource for plant growth which cannot be produced synthetically or substituted by any other substance. The huge amount of mineral phosphorus that is annually imported into Europe, mainly from the US, Morocco and China to sustain good harvests, demonstrates its interest as fertiliser nutrient (USGS 2009; Vaccari 2009) and the necessity for using sewage sludge as a fertiliser in agriculture. However, the application of sewage sludge in agriculture underlines the problems of hygiene and toxicity. The toxicity and accumulation of the polyacrylamide-based polymers used in sludge treatment, as well as heavy metals and organic pollutants, might have a negative effect on biota. Thus, the risks linked to the use of sewage sludge as a fertiliser need to be investigated further to protect environment and human health.

The first step towards a regulation of the use of sewage sludge in agriculture in the European Union was undertaken in the "European Council Directive in 1986 on the protection of the environment". The aim of the directive is to regulate the use of untreated sewage sludge in agriculture in such a way as to prevent harmful effects on soil, vegetation, animals and humans, whilst encouraging its correct use (EEC 1986). In order to ensure the correct use, mandatory threshold values for heavy metals in sewage sludge and soil were determined (Cd, Cu, Ni, Pb, Zn, Hg) (EEC 1986). Furthermore, the sludge should be treated before being used in agriculture (EEC 1986). Germany implemented the directive with lower threshold values for heavy metals and is planning to add threshold values for persistent organic pollutants (POPs) to their regulation of waste and sewage sludge (AbfKlärV 1992). Additionally, for the use of sewage sludge as a fertiliser, it has to meet the requirements of the fertiliser regulations in Germany. Hereby, these regulations control the hygienisation (disinfection) of the sludge and the protection of soil and water bodies against overfertilisation (DÜV 2006; DüMV 2012). Moreover, the Recycling Management Act of Germany (KrWG) contains regulations for the handling of sewage sludge as waste and the usage of the sludge for recycling or disposal based on its consistency (KrWG 2012). Finally, several federal states in Germany maintain a negative attitude towards the use of sewage sludge as fertiliser and have achieved a wide pullout from soil-oriented sewage sludge exploitation, e.g. Baden-Württemberg (UM 2007), whereas other federal states are still using it, e.g. Niedersachsen (pullout is planned). Additionally, the "European Directive in 2008 on waste" states that (1) necessary measures ensuring waste management without harming the environment (e.g. no risk to water, air, soil, plants or animals) shall be taken, and (2) ecotoxic properties of the waste which render it hazardous will arise if it presents or may present immediate or delayed risks for one or more sectors of the environment (EC 2008a).

Although many directives and regulations about the handling of waste and the correct use of sewage sludge exist in the EU and Germany, there is still a lack of declarations about detailed ecotoxicological analysis methods of sewage sludge. Mandatory chemical analyses only verify the concentration of certain heavy metals and organic pollutants in sewage sludge. The various effects of the whole toxic mixture on the environment can only be revealed by ecotoxicity tests. Previous research investigated either for example the toxic effect of wastewater effluents through a Microtox® test of *Vibrio fischeri* (Gutiérrez et al. 2002) or, e.g. the toxic effect of heavy metals in the leachate of sewage sludges on water organisms (Fjällborg & Dave 2003; Fjällborg et al. 2005). Other researchers examined the direct toxic effect of sewage sludges or their effect over time with bacteria and terrestrial plants (Roig et al. 2012), soil invertebrates and terrestrial plants (Carbonell et al. 2009; Natal-da-Luz et al. 2009a, b) or developed a novel assay that measures denitrification inhibition in a model denitrifier (Holzem et al. 2014). So far, researchers did not concentrate on investigating the direct toxic effect of sewage sludge by the use of selected organisms of all affected compartments and additional behavioural studies. Therefore, the aim of this study was to detect the potential toxic effects of sewage sludges considering the complex substance mixture with the aid of (1) the avoidance behaviour of the earthworm *Eisenia fetida* (Savigny 1826, Oligochaeta, Lumbricidae) in the soil compartment, (2) the behaviour and mortality of the freshwater shrimp *Gammarus fossarum* (Koch 1836, Amphipoda, Gammaridae) in the water and sediment compartment and (3) the growth inhibition of the water plant *Lemna minor* (Linné 1753, Arales, Lemnaceae) in the water compartment. As this is one of the few first studies to evaluate the ecotoxic risk of sewage sludge on both invertebrates and aquatic plants, we generate new knowledge as a basis for future legislative decisions concerning the use of sewage sludge as a fertiliser in agriculture.

2.3 Materials and methods

2.3.1 Samples

The three sewage sludge samples were taken at two different wastewater treatment plants in Germany, sewage sludge 1 and 2 being taken in the same WWTP. Both were treated with a biological phosphorus removal. Additionally, sludge 2 was dewatered with polyacrylamide. Sludge 3 was a dewatered sludge sample from another WWTP with chemical phosphorus removal by precipitation of phosphates as ferric phosphates. The sludge samples were stored in 5-l canisters (PE) at 4 °C prior to and between the toxicity tests. After 1 min of shaking, each test sludge was mixed with the appropriate reference medium (Table 2.1). For the ecotoxicological tests no further pre-treatments of the sludges were necessary. The ecotoxicological tests were conducted with the fresh matter of the sewage sludge samples for a direct and unaltered analysis and to avoid chemical modifications. For a better comparison only, the fresh matter (FM) of the sludge samples was converted into the total solid (TS) content by multiplication with the percentage amount of TS of each sewage sludge sample (Table 2.3).

Table 2.1 Summary of test parameters of ecotoxicity tests for sewage sludge samples (*Lemna* sp. growth inhibition test, gammarid acute toxicity test, earthworm avoidance test)

Test organisms	*L. minor*	*G. fossarum*	*E. fetida*
Acclimation	Equal to test parameters (at least for 4 days)	Equal to test parameters, with one alder leaf (for 4 days)	Darkened box filled with a peat/ LUFA 2.3/compost mixture (humid conditions/room temperature/ at least for 4 days)
Test parameters			
Exposure medium	Onefold Steinberg medium (mixed with fresh stream water)	Fresh stream water (sand as sediment, permanent aeration)	50 % LUFA standard soil 2.3/50 % compost
Exposure length	7 days (168 h)	4 days (96 h)	2 days (48 h)
Number of organisms	13 fronds	8	5
Replicates	3	3	3
Temperature	24±2 °C	18±3 °C	20±3 °C
Photoperiod/intension	24 h day 6 500–10 000 lx	16 h day/8 h night 400–800 lx	16 h day/8 h night 400–800 lx
Endpoints	Growth inhibition Discolouration Colony break-up	Mortality Movement behaviour (measured after 2 and 4 days) Stress ventilation (measured after 2 days)	Avoidance behaviour
Acceptability criteria	Control growth rates >0.2 per day	Control mortality <10 %	Number of dead or missing worms <10 %

2.3.2 Chemical analysis

In the manually stirred, freeze-dried and ground sludge samples, the following parameters were analysed: percentage of nutrients and dry matter content, concentrations of organic pollutants and heavy metals. The analyses were performed by IASP (Institute of Agricultural and Urban

Ecological Projects, Berlin, Germany), LUFA Nord-West (Agricultural Analysis and Research Institute, Hameln, Germany) and Institute of Ecopreneurship, FHNW (University of Applied Science and Arts Northwestern Switzerland, Basel). The water samples of sewage sludge 1–3 of the toxicity tests after 4 and 7 days of exposure were analysed for dissolved organic pollutants and heavy metals by the Institute for Sanitary Engineering, Water Quality and Solid Waste Management (ISWA, University of Stuttgart, Germany). The references of the analysis can be seen in Table 2.2.

Table 2.2 Methods and references of the analysed substances in the sewage sludge samples and in the water samples

Substances	Methods/reference	Institute
Sludge samples		
Nutrients		
Total solid (TS)	DIN 38414-2 (1985)	IASP, Berlin,
Organic total solid (OTS)	Calculated	Germany/
Ash	VDLUFA II, 10.1 (1995)	LUFA, Hameln,
P_2O_5, K_2O, MgO, CaO, S	ISO 11885-E22 (2007)	Germany
N_{total}	VDLUFA II, 3.5.2.7 (1995)	
Pollutants		
Heavy metals	Aqua regia extraction with ICP-OES measurement	FHNW, Basel,
Benzotriazole	VDLUFA VII, 3.3.3 (1996)	Switzerland
Pharmaceuticals, herbizides, etc.	Pressurised liquid extraction (Pamreddy et al. 2013) + solid-phase extraction with LC-MS/MS measurement	
Water samples		
Heavy metals	ISO 11885-E22 (ICP-OES measurement) (2007)	ISWA,
Organic micropollutants	ISWA-internal method (liquid/liquid extraction, GC/MS measurement)	Stuttgart, Germany

ICP-OES inductively coupled plasma optical emission spectrometry, *LC-MS* liquid chromatography–mass spectrometry, *GC/MS* gas chromatography/mass spectrometry

2.3.3 Ecotoxicological tests

Chronic growth inhibition test with L. minor

In this test, chronic effects on the growth of the duckweed *L. minor* were assessed according to ISO 20079 (2005). An overview of the test parameters can be seen in Table 2.1.

The acclimation, control and test performance were implemented with a onefold modified Steinberg medium (ISO 2005), a medium with all essential macro and micro nutrients for duckweed growth diluted in fresh stream water (Hockgraben, Konstanz, Germany; 47° 40' 02.3" N, 9° 12' 04.2" E). Test plants were obtained from a small natural pond in the region of Lake Constance. The reference was a copper chloride ($CuCl_2$) dilution in Steinberg medium. The used copper concentration (1 mg l^{-1}) was chosen based on literature data (OECD toolbox) and pre-trials. Five different sewage sludge concentrations (0.1, 1, 2.5, 5, 10 % of volume or weight of the fresh matter) were assessed to cover the whole toxicity range from 0 to

100 % growth inhibition. Further triplicates of 2 and 5 % FM were mixed for water chemical analysis. The samples were added into the test vessels with onefold modified Steinberg medium and stirred (∼1 min). After sedimentation for each treatment at three replicates, 13 fronds of acclimated plants of *L. minor* (three colonies of 3 fronds, one colony of 4 fronds with similar frond size) were placed into the test vessels (250 ml, *h* 9.5 cm, *d* 7.5 cm) with an entire liquid volume of 150 ml. All tests were carried out at 24±2 °C in a climate chamber with an intense, permanent illumination ranging from 6500–10,000 lx. To minimise evaporation and accidental contamination, the test vessels were covered by translucent foil. Cultivation and acclimation (at least for 4 days) were conducted under the same experimental conditions.

After 7 days, the total number of leaves was counted. Additionally, the number of single leaves and the number of leaves with any discolouration (brown, yellow or bleached) were determined for evaluating the percentage of colony break-up and discolouration. Water samples of the triplicates of 2 and 5 % FM were taken at the test end and a composite sample was used for chemical analysis. Significant modifications of the test medium such as the occurrence of precipitated substances or algae growth were also noted. According to the equations of ISO 20079 (2005), the growth rate and percent inhibition for each sample concentration were calculated. Growth rates >0.2 per day of the control were acceptable. The growth inhibition was assessed through a linear regression analysis. The effect concentration of 50 % effect (EC_{50}) was consulted for evaluation. Moreover, sample concentrations causing >80 % discoloured leaves or >50 % colony break-up were regarded as toxic.

Acute toxicity test and behaviour measurements with G. fossarum

The acute toxicity test was conducted as described in the Ecological Effects Test Guidelines by the United States Environmental Protection Agency (Gammarid Acute Toxicity Test; EPA 1996) with adjustments for environmental samples and in order to perform a realistic test set-up under laboratory conditions (overview of test parameters in Table 2.1).

Test organisms, the freshwater shrimps *G. fossarum*, were obtained from a small stream in the region of Lake Constance and were cultivated at 15 °C in a climate chamber (feeding with leached, steeped alder leaves and red chironomid larvae). The cultivation, acclimation (4 days under test conditions with one alder leaf) and test performance were conducted with fresh stream water (Hockgraben, Konstanz, Germany; 47° 40' 02.3" N, 9° 12' 04.2" E). Five different concentrations of each sludge sample were chosen and assessed covering the whole acute toxicity range of mortality (S1 1, 2, 3, 4, 5 %; S2 0.1, 1, 2, 3, 5 %; S3 0.1, 0.5, 1, 2, 3 %; in percentage of volume or weight of the fresh matter). Further treatments of 2 and 5 % FM were mixed for water chemical analysis. The control was tested in fresh stream water. As toxin reference samples (positive controls), a copper chloride dilution ($CuCl_2$) in fresh stream water and a sample with a pH of 4.5 obtained by the addition of nitric acid (96%) in fresh stream water were used. A test of copper chloride was conducted during the sludge tests and a dose-response curve was fitted (linear regression/probit method, LC_{50} 0.59 mg l^{-1}, LC_{50} lethal concentration of 50 % lethality). The low pH of 4.5 was chosen to ensure behavioural effects but not 100 % mortality after 2 days (48 h) of the experiment (based on Felten et al. (2008); *G. pulex* pH 4.1, average mortality 71 % after 38 h). For acclimation and the test performance, a whole liquid volume of 1 l in 5-l test vessels (33 × 19 × 11 cm) of polypropylene (PP; no accumulation of heavy metals because of no existent reactive groups; Martienssen & Warlimont 2005) with 150 g incinerated commercial sand (1 h, 500 °C) were applied. Contrary to the guideline, sand was added to each treatment to provide a realistic experimental set-up to allow for the natural behaviour of gammarids to hide and to search for food in sediment. Moreover, the addition of sand provided substrate particles in all treatments. Each treatment was run in triplicates and contained eight gammarids of sizes 3 and 4 (size class of gammarids; neonate <2 mm (1), 2–4 mm (2), 4–7 mm (3), >7 mm (4)). All tests including the acclimation were carried out at 18±3 °C in a test shelf with an alternate illumination (16 h day/8 h night; 400–800 lx) and permanent air supply, which is an addition to the guideline in order to balance out the oxygen consumptive effect of microbes in sewage sludge. Furthermore, the number of testing animals was reduced from 20 to 8 to provide sufficient space and avoid stress, which might result in aggressiveness and cannibalism. After 4 days of acclimation, the treatments were prepared by removing the alder leaf and the gammarids. Afterwards, the sludge samples were added to the test vessels (freshwater and sand) and stirred (∼1 min). After sedimentation, the gammarids were relocated, and for each living gammarid, a thawed chironomid was added as food. The survival and feeding behaviour of gammarids were visually measured after 4 days of exposure. Water samples of the triplicates of 2 and 5 % FM were taken at the test end and a composite sample was used for chemical analysis. The test was valid if the control mortality was <10 %. Moreover, the movement activity and trends of stress ventilation of the gammarids were measured with the Multispecies Freshwater Biomonitor® (MFB) after 1-, 2- (depending on the impact of the sludge) and 4-day exposure time. The MFB is based on an electric four-polar impedance conversion, a non-optical method able to measure different behaviour parameters simultaneously in water and sediment (Gerhardt et al. 1994; Gerhardt et al. 1998). Locomotion results in irregular low signal frequencies with high amplitudes (between 0 and 2 Hz, summarised in band 1), whilst ventilation reveals regular high signal frequencies with small amplitudes (between 2.5 and 5 Hz, band 2) (Gerhardt et al. 1994). For behaviour measurements, eight individual amphipods of the three replicates of one concentration were gently transferred to measuring chambers (one gammarid per chamber). The measurement chambers (*l* 5 cm, *d* 2 cm), covered by lids with nylon mesh (mesh size 1 mm), were placed horizontally in an appropriate PP box filled with stream water. The movement activity of animals was recorded for 50 min (six measurement periods at 4 min). After recording, the gammarids were returned to the test vessels containing the respective concentration of sludge. Through the obtained data, the percentage of mortality was calculated and evaluated by linear regression analysis as a basis to generate the LC_{50} value. The movement frequencies were analysed with the Fourier frequency transformation (FFT) (Gerhardt et al. 1998).

Avoidance test with the earthworm E. fetida

The avoidance test was performed according to ISO 17512-1: avoidance test for determining the quality of soils and effects of chemicals on behaviour of earthworms (ISO 2008). To improve the test, set-up adjustments for better evaluation of our samples were taken. An overview of the test parameters can be seen in Table 2.1.

First, *E. fetida* cultures were obtained from an earthworm breeder in Germany (Wormfarm Nassenheide). Then, the earthworms were held and raised in two darkened boxes. One box was only used for reproduction with 100 % peat substrate, and the other box was filled with peat, LUFA 2.3 soil (standard soil, LUFA Speyer, Germany) and compost of unloaded green waste (VDLUFA quality seal certification, composting plant, Singen, Germany) for acclimation purposes. The worms were fed with horse manure, held under humid conditions and at room temperature during cultivation and acclimation. The sludge samples were mixed manually in five different concentrations (0.1, 1, 2.5, 5, 10 % of volume or weight of the fresh matter) with a 50/50 mixture of LUFA 2.3 soil and compost to reach a whole weight of 1 kg (including the water of about 30 % of the water-holding capacity of the soil mixture; ISO 2009). At the beginning of the test, the test vessels (5-l plastic box of PP, 33 × 19 × 11 cm) were divided into two equal sections by means of a vertically introduced divider for each treatment at three replicates. One half of the vessel was filled with 1 kg of humid test soil, and the other half was filled with 1-kg humid control soil (50/50 mixture of LUFA 2.3 and compost). In the control treatment boxes, both sections were filled with control soil. As references, copper chloride ($CuCl_2$ ~100 mg kg^{-1}) and diclofenac sodium ($C_{14}H_{10}Cl_2NNaO_2$ ~200 mg kg^{-1}) were used. The used reference concentrations were chosen according to literature data (OECD toolbox) and pre-trials. After the addition of soil, the separator was removed and five subadult/adult earthworms (Ø 35/45 mm) were placed on the separating line of each test vessel. The plastic boxes were covered with appropriate lids with nets to enable a permanent air exchange and to prevent the worms from escaping. All tests were carried out at about 20±3 °C in a test shelf simulating a natural photoperiod (16 h day/8 h night; 400–800 lx). To avoid lateral light effects, plastic boxes were wrapped with light-tight foil. Further, no feeding was performed during the test.

We chose larger test vessels (wider vessels with a lower filling level) as proposed in the standard procedures for reasons of (1) reduction of contact area of uncontaminated and contaminated soils to avoid a potential contamination of control soil, (2) reduced risk to harm worms by inserting the divider, and (3) space for the worms to distinguish clearly between "worm in control soil" and "worm in contaminated soil". Moreover, avoidance is seen as a directed movement away from the contamination and requires a certain amount of space which should at least be two to three times the body size of the worm. In order to improve the possibility of distribution and to reduce bundle building of the worms (worm aggregation), which could lead to less movement and a smaller contact area with the surrounding soil, the quantity of worms was reduced from 10 to 5. Building of bundles was a behaviour of the worms which had been observed in the culture boxes. The addition of compost to LUFA 2.3 was conducted to obtain loosened soil with more hollows.

At the end of the test period of 48 h, the control and test soils in each vessel were separated by inserting the dividers. The number of worms was determined in both sections of the boxes (n_c number of worms in control soil, n_t number of worms in test soil (contaminated), N total number of worms). Worms located at the dividing line were counted as 0.5 to each section. Dead worms were also registered but were not included to the assessment. The test was considered valid if the number of dead or missing worms was <10 %.

For calculation of the percentage effect (x avoidance) of a sample concentration, the mean number of worms in the test soil was compared to the mean number of worms in the control soil according to the equation ($x = ((n_c - n_t)/N) \times 100$) of ISO 17512-1 (2008). A linear regression analysis was prepared with values of an increased avoidance behaviour and positive responses (=the worms prefer the control soil). The EC_{50} value was calculated according to ISO 17512-1 (2008). Moreover, the percentage of worms x_c in the control section was calculated ($x_c = (n_c/N) \times 100$) and regarded as toxic if the control soil contained over 80 % of the worms as described by Hund-Rinke & Wiechering (2001).

2.3.4 Data analysis

The results were stated as LC_{50}/EC_{50} values where a dose–response curve could be fitted using a linear regression analysis. The LC_{50}/EC_{50} values were calculated after probit transformation. Values for effect concentrations were reported with 95 % confidence intervals (IC_{95}).

Normally distributed data of behaviour measurements of *G. fossarum* were analysed with a one-way analysis of variance (ANOVA), non-normally distributed data with a nonparametric Kruskal-Wallis test. The behaviour data were analysed according to effects of concentration levels and time. Pairwise testing was followed by Holm-Sidak test (normally distributed data) or Tukey's test for nonparametric data. For all statistics and graphs, we were using Sigma Plot (Systat Inc.) and ToxRat® Professional (ToxRat Solutions GmbH). Significances were marked in the graphs by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For comparison of the LC_{50}/EC_{50} values with literature, we used the OECD QSAR Toolbox for Grouping Chemicals in Categories and filling gaps in (eco)toxicity data, the Wikipharma database which contains publicly available ecotoxicity data for pharmaceutical substances and the Web of Science Search Platform.

2.4 Results

2.4.1 Chemical analysis

The FM of the samples of sludge 2 and sludge 3 (S2 & S3) had a similar content of nutrients (N_{total} 1.5 %, total P_2O_5 ~2.5 %, CaO ~1.4 %), whereas sludge 1 (S1) had lower nutrient contents due to the low amount of total solid (N_{total} 0.2 %, total P_2O_5 0.2 %, CaO 0.1 %). The concentration of MgO in S2 (0.84 % of FM) was higher than in S1 and S3. In all three sludge samples, K_2O occurred in a similar content (~0.05 % of FM) (Table 2.3). The dry matter of S1 had the highest amounts of organic pollutants in total (carbamazepine 2106 ng g^{-1} TS, benzotriazole 6122 ng g^{-1} TS, diclofenac 1935 ng g^{-1} TS). However, S3 had the highest amount of benzotriazole (12,767 ng g^{-1} TS). All of the sludges contained the analysed heavy metals in a nearly similar µg g^{-1} range (As, Cr, Cu, Hg, Ni, Pb, U, Zn, Cd) with the exception of the copper concentration of S3 (832 µg g^{-1} TS), which was nearly fourfold higher than in S1 and S2 and the iron concentration of S3 (121 mg g^{-1} TS, sixfold higher). The highest content of heavy metal was Fe in S1–S3 (19–121 mg g^{-1} TS), followed by zinc (886–950 µg g^{-1} TS). Arsenic, quicksilver and cadmium occurred in the lowest amounts in a range between 0.7 and 4.7 µg g^{-1} TS in all three sludge samples (Table 2.3).

Table 2.3 Measured concentrations of sum parameters, N and P compounds, nutrients related to the fresh matter FM of the sludge samples, organic pollutants and heavy metals of the sludges related to the dry matter of the sludge samples

Substance group	Sludge 1	Sludge 2	Sludge 3
Sum parameters, N and P compounds, nutrients (percentage of FM)			
TS	2.41	23.25	28.68
OTS	1.79	15.84	16.91
Ash	0.62	7.41	11.77
N_{total}	0.16	1.51	1.48
$P_2O_{5\ \text{mineral acid soluble}}$	0.22	2.39	2.69
$P_2O_{5\ \text{neutral ammon citrate acid soluble + water-soluble}}$	0.19	2.16	2.54
$P_2O_{5\ \text{citric acid soluble}}$	0.19	2.05	2.40
$P_2O_{5\ \text{water-soluble}}$	0.05	0.15	0.01
$K_2O_{\ \text{mineral acid soluble}}$	0.05	0.08	0.02
MgO	0.03	0.84	0.10
CaO	0.13	1.29	1.50
S	0.03	0.28	0.67
Organic micropollutants (ng g^{-1} TS)			
Benzotriazole	6 122	697	12 767
Carbamazepine	2 106	546	873
Diclofenac	1 935	254	348
Estrone	<LOD	<LOD	<LOD
Mecoprop	<LOD	<LOD	<LOD
Sulfamethoxazole	<LOD	<LOD	<LOD
Heavy metals (µg g^{-1} TS)			
Arsenic	4.0	3.5	4.7
Cadmium	1.2	1.2	1.0
Chromium	27.9	27.7	17.6
Copper	214.9	220.2	831.7
Iron	18 934.4	18 976.3	120 629.1
Lead	28.7	29.3	35.7
Nickel	25.5	22.6	17.6
Quicksilver	0.7	1.1	1.1
Uranium	13.0	13.8	32.7
Zinc	885.9	902.3	949.6

Source: LUFA Nord-West Hameln, IASP Berlin and FHNW Basel

TS total solid, *OTS* organic total solid

Table 2.4 Measured concentrations of organic pollutants and heavy metals of sludge-treated water samples with different concentrations (percentage of the fresh matter (FM)) after 4- and 7-day exposure

Substance group	Sludge 1	Sludge 2	Sludge 3
Organic micropollutants (µg l^{-1})			
AHTN			
1 % FM 4 days	0.05	0.06	0.12
5 % FM 4 days	0.15	0.27	0.62
1 % FM 7 days	0.03	0.09	0.10
5 % FM 7 days	0.10	0.22	1.64
Carbamazepine			
1 % FM 4 days	0.19	0.60	1.04
5 % FM 4 days	0.92	2.14	3.27
1 % FM 7 days	0.20	0.76	0.99
5 % FM 7 days	0.78	2.73	4.20
Diclofenac			
1 % FM 4 days	0.27	0.68	1.05
5 % FM 4 days	1.11	1.88	3.36
1 % FM 7 days	0.11	0.49	0.77
5 % FM 7 days	0.92	2.18	4.10
HHCB			
1 % FM 4 days	0.24	0.30	0.74
5 % FM 4 days	0.67	1.47	3.43
1 % FM 7 days	0.10	0.49	0.61
5 % FM 7 days	0.48	1.23	9.29
Naproxene			
1 % FM 4 days	0.02	0.03	0.04
5 % FM 4 days	0.03	0.06	0.13
1 % FM 7 days	0.02	0.03	0.03
5 % FM 7 days	0.03	0.05	0.18
TCPP			
1 % FM 4 days	0.17	0.39	0.20
5 % FM 4 days	0.63	2.43	0.81
1 % FM 7 days	0.14	0.73	0.12
5 % FM 7 days	0.42	3.00	2.60
Triclosan			
1 % FM 4 days	0.29	0.34	0.36
5 % FM 4 days	0.60	0.58	0.61
1 % FM 7 days	0.40	0.39	0.50
5 % FM 7 days	0.59	0.73	1.20
4-Nonylphenole			
1 % FM 4 days	0.22	0.39	1.15
5 % FM 4 days	1.54	0.76	5.98
1 % FM 7 days	0.24	0.84	1.57
5 % FM 7 days	1.16	1.18	12.3
4-Tert-octylphenole			
1 % FM 4 days	0.04	0.07	0.49
5 % FM 4 days	0.12	0.14	1.67
1 % FM 7 days	0.02	0.06	0.56
5 % FM 7 days	0.08	0.21	3.20

Heavy metals (µg l^{-1})			
Cadmium			
1 % FM 4 days	<25	<25	<25
5 % FM 4 days	<25	<25	<25
1 % FM 7 days	<25	<25	<25
5 % FM 7 days	<25	<25	<25
Copper			
1 % FM 4 days	<20	<20	<20
5 % FM 4 days	<20	<20	150
1 % FM 7 days	<20	<20	<20
5 % FM 7 days	<20	50	550
Zinc			
1 % FM 4 days	<25	<25	45
5 % FM 4 days	99	162	157
1 % FM 7 days	43	68	40
5 % FM 7 days	<25	137	761

Source: LimCo International GmbH Konstanz and ISWA Stuttgart

AHTN 6-acetyl-1,1,2,4,4,7-hexamethyltetraline, *HHCB* 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran, *TCPP* Tris(2-chlorisopropyl)phosphate)

In the overlaying water of all three sludge treatments, the organic pollutants diclofenac, naproxen, carbamazepine, triclosan, 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclo-penta-γ-2-benzopyran (HHCB), 6-Acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN), Tris(2-chlorisopropyl)phosphate (TCPP), 4-tert-octylphenole and 4-nonylphenole could be found in the range of 0 to 15 µg l^{-1} after 4 and 7 days of exposure (Table 2.4). The analyses of the water samples also showed that the concentrations of carbamazepine and TCPP were threefold to fivefold higher in treatments of S2 than in S1 and that the water sample of 14 g TS l^{-1} (~5 % FM) of S3 had the highest content of the analysed organic pollutants, especially of diclofenac, carbamazepine, HHCB and 4-nonylphenole (4–12 µg l^{-1}). The water samples of S3 had also the highest content of dissolved Cu (4 days 5 % FM, 150 µg l^{-1}; 7 days 5 % FM, 550 µg l^{-1}). In the water samples of S1 and S2, the copper content was below the detection limit in the majority of cases. Zinc occurred in most water samples of all three sludge samples (<25– 200 µg l^{-1}), but the highest amount could be found in 14 g TS l^{-1} (~5 % FM) of S3 after 7 days of exposure (761 µg l^{-1}) (Table 2.4). Summing up the results of the analysed substances of the water samples, it could be determined that the higher the concentration of tested sludge in the water sample and the longer the exposure time were, the higher the concentration of both dissolved organic pollutants and heavy metals were.

2.4.2 Chronic growth inhibition test with *L. minor*

All three sewage sludge samples had a negative effect on growth of *L. minor*. 2.4 g TS l^{-1} of S1, 23.3 g TS l^{-1} of S2 and 28.7 g TS l^{-1} of S3 also resulted in 100±0, 97.8±2.2 and 100±0 % discoloured leaves, and of S1 and S2 in 58.8±7.8 and 65±6.3 % colony break-up (Figure 2.1a, b). The EC_{50} values of the sludge samples were S1 1.2 g TS l^{-1}, S2 7.8 g TS l^{-1} and S3 9.6 g TS l^{-1} (Table 2.5). Concentrations below 0.2 g TS l^{-1} of S1 and 2.3 g TS l^{-1} of S2 even supported growth due to the fertilisation effect of nutrient-rich sewage sludge samples. 0.02 g TS l^{-1} of S1 and 0.2 g TS l^{-1} of S2 caused nearly 15 % more growth in comparison to the control; it further induced visually bigger leaves in diameter and a more intense green colour. Minor till medium green algae growth could be detected in lower concentrations to 0.6 g TS l^{-1} of S1 and ~6.5 g TS l^{-1} of S2 and S3. The reference toxicant, 1 mg l^{-1} Cu^{2+}, also affected growth negatively and caused between 40 and 80 % discolouration as well as colony break-up (10–50 %) in all three treatments (Figure 2.1a, b). Additionally, no algae growth could be noticed in the copper treatments.

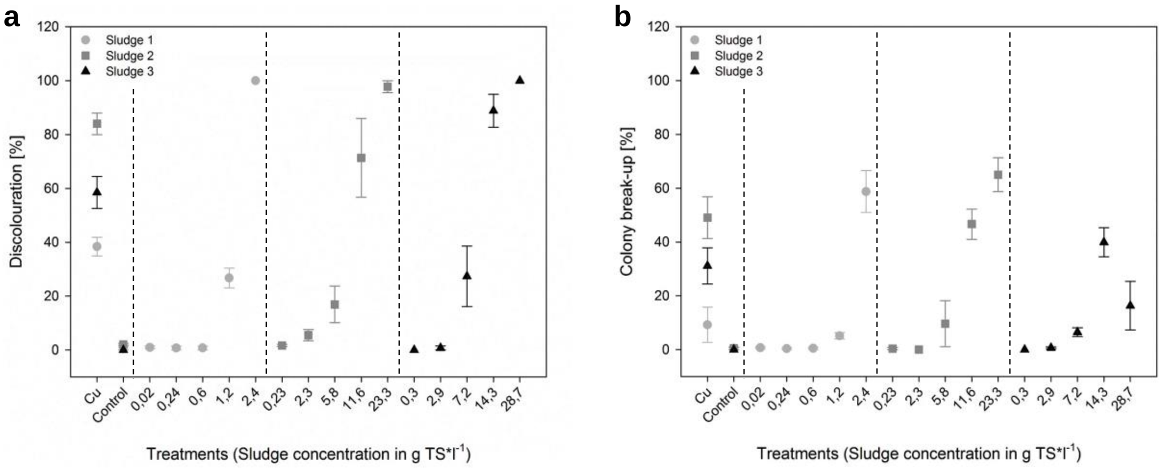

Figure 2.1 **a** Discolouration [%] and **b** Colony break-up [%] of *L. minor* after 7 days; three replicates per treatment; treatments, sludge concentration in grams total solid per litre (corresponding each 0.1–10 % of volume or weight of the fresh matter); reference Cu^{2+} 1 mg l^{-1}

2.4.3 Acute toxicity test and behaviour measurements with *G. fossarum*

All three sludge samples caused high mortality rates of *G. fossarum* at high concentration levels after 4 days. The LC_{50} values of the sludge samples were S1 0.5 g TS l^{-1}, S2 1.3 g TS l^{-1} and S3 1.6 g TS l^{-1} (Table 2.5). However, concentrations below ~0.3 g TS l^{-1} of all three sludge samples resulted in an average of mortality below 20 % after 4 days. At pH values of 4.5, all gammarids died after 1 day resulting in 100 % mortality; therefore, no behavioural measurements (feeding and MFB analysis) could be conducted. After test initiation, the prepared pH of 4.5 probably still decreased and resulted so in a higher effect on the gammarids. Cu^{2+} concentrations of 500 µg l^{-1} and higher negatively influenced the survival of *G. fossarum*. The feeding behaviour decreased on average by 25 % at higher sludge concentrations of S2 and S3. For S1, no feeding behaviour was analysed.

An effect on movement activity of *G. fossarum* at sludge concentrations between 0.5 and 7 g TS l^{-1} was already detected after 1 or 2 days (Figure 2.2a–c). The movement activity even significantly decreased from 40 % to 20 % at low TS concentrations of S1 and at higher TS concentrations of S2 and S3. In addition to that, a trend of increased stress ventilation could be determined for these concentration levels after the same exposure time (Figure 2.3a–c). After 4 days (end of exposure period), no effect on movement activity of the survived gammarids exposed to low concentrations could be noted. Only the survivors in higher concentrations of S2 and S3 (4.7 & 5.7 g TS l^{-1}) showed significantly lower movement activity (Figure 2.4a–c).

Table 2.5 Comparison of the three sludges by the 50 % effect concentration/50 % lethal concentration (EC_{50}/LC_{50}, according to the probit method) including the 95 % confidence interval (IC_{95}) of sludge 1–3 of the *Lemna* sp. growth inhibition test, the gammarid acute toxicity test and the avoidance behaviour of *Eisenia*

	Test organism	Linear regression equation (y axis linear, x axis log)	r^2	Number of values	LC_{50}/EC_{50} ± IC_{95} (g TS l^{-1} or *g TS kg^{-1})
Sludge 1	*G. fossarum*	$f(x) = 142.65x + 96.77$	0.903	5	0.5 (–0.1/+0.1)
	L. minor	$f(x) = 136.09x + 38.24$	0.999	3	1.2 (–0.5/+1.0)
	*E. fetida**	$f(x) = 94.77x + 90.58$	0.690	3	0.4 (–0.1/+0.1)
Sludge 2	*G. fossarum*	$f(x) = 62.12x + 44.27$	0.887	5	1.3 (–0.5/+0.6)
	L. minor	$f(x) = 95.17x - 36.49$	0.983	4	7.8 (–3.2/+5.5)
	*E. fetida**	$f(x) = 199.32x - 174.57$	0.447	3	12.3 (–/–)
Sludge 3	*G. fossarum*	$f(x) = 66.33x + 38.42$	0.870	5	1.6 (–0.5/+0.5)
	L. minor	$f(x) = 97.64x - 46.24$	0.919	4	9.6 (–2.3/+3.0)
	*E. fetida**	$f(x) = 221.46x - 222.80$	0.641	3	15.1 (–/–)

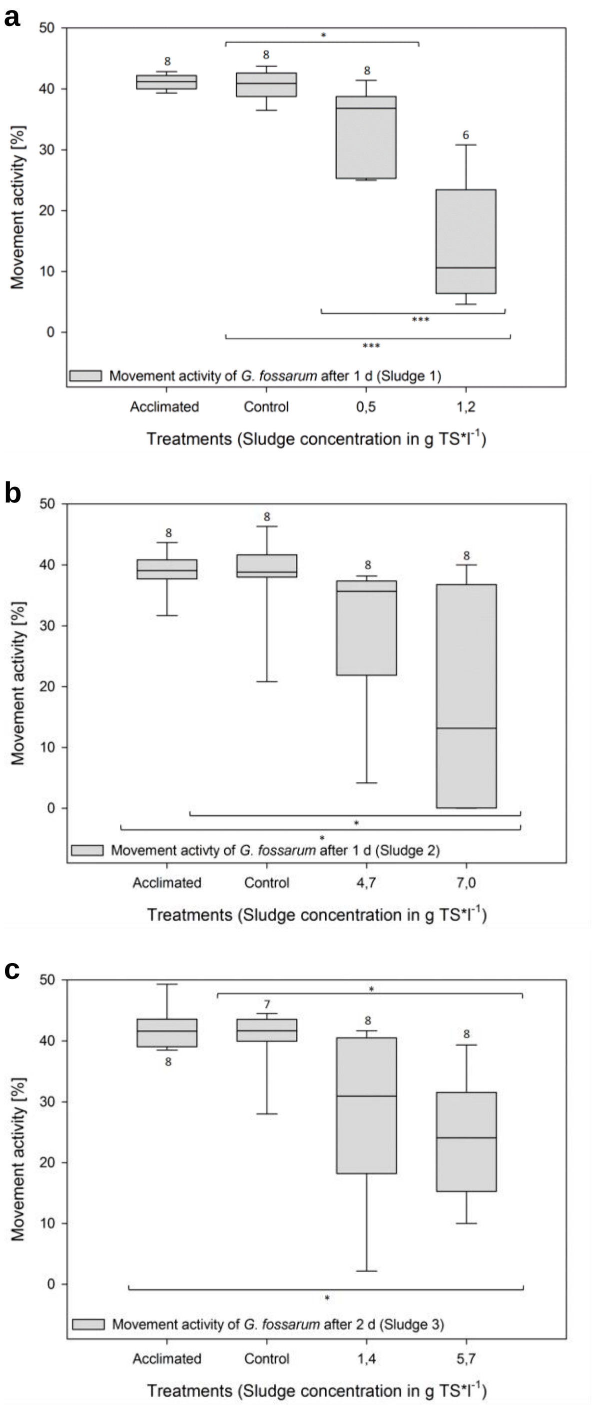

Figure 2.2 a–c Multispecies Freshwater Biomonitor® (MFB) behaviour measurements of movement activity of *G. fossarum* after 1 or 2 days (*numbers above the whiskers* represent the number of gammarids measured for 50 min); movement activity, percentage of frequencies between 0.5 and 2 Hz; treatments, sludge concentration in grams total solid per litre; acclimated, acclimated gammarids (4 days) measured before test start; one-way ANOVA/pairwise Holm-Sidak or Kruskal-Wallis/pairwise Tukey

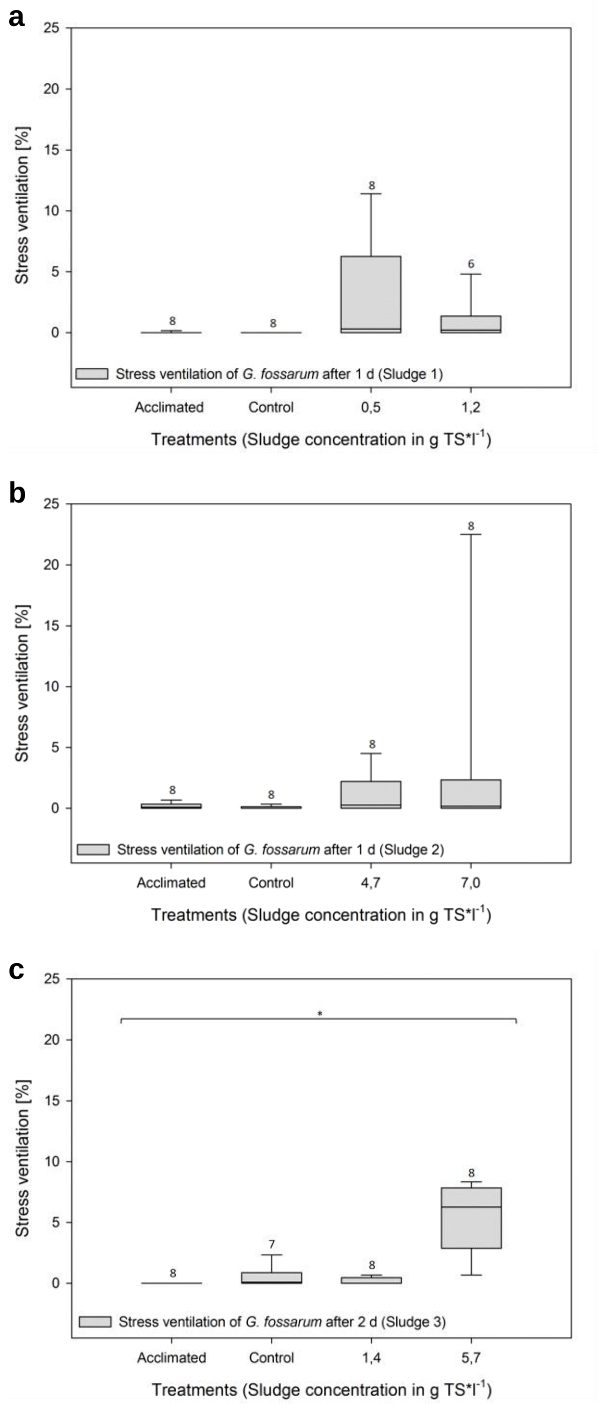

Figure 2.3 a–c Multispecies Freshwater Biomonitor® (MFB) behaviour measurements of stress ventilation of *G. fossarum* after 1 or 2 days (*numbers above the whiskers* represent the number of gammarids measured for 50 min); stress ventilation, percentage of frequencies between 2.5 and 5 Hz; treatments, sludge concentration in grams total solid per litre; acclimated, acclimated gammarids (4 days) measured before test start; one-way ANOVA/pairwise Holm-Sidak or Kruskal-Wallis/pairwise Tukey

2.4.4 Avoidance test with the earthworm *E. fetida*

1.2 g TS kg^{-1} of S1 and 23.3 & 28.7 g TS kg^{-1} of S2 and S3 caused 100 % avoidance behaviour according to ISO 17512-1 (2008), whereas sludge 1 displayed a concentration-dependent linear relationship in avoidance behaviour within the whole range of tested concentrations. Lower concentrations of S2 and S3 did not cause clear avoidance behaviour because *E. fetida* were also located at some concentrations in the contaminated site (Figure 2.5). However, 11.6 and 14.3 g TS kg^{-1} of S2 and S3 resulted in at least 50 % avoidance behaviour (Figure 2.5). The EC_{50} values of the sludges were S1 0.4 g TS kg^{-1}, S2 12.3 g TS kg^{-1} and S3 15.1 g TS kg^{-1} (Table 2.5). Considering the approach of Hund-Rinke & Wiechering (2001) implying that an avoidance behaviour of at least 80 % is regarded as toxic, the sludge samples were toxic at S1 ≥0.6 g TS kg^{-1}, S2 ≥23 g TS kg^{-1} and S3 ≥29 g TS kg^{-1}. The S1 reference toxicant concentrations of diclofenac$^-$ and Cu^{2+} were too low to have a negative effect on *E. fetida*. Concentrations of more than 150 mg kg^{-1} diclofenac$^-$ caused 100 % avoidance behaviour of *E. fetida* (Figure 2.5). Furthermore, one worm died during the tests (in the control soil of 29 g TS kg^{-1} of S3).

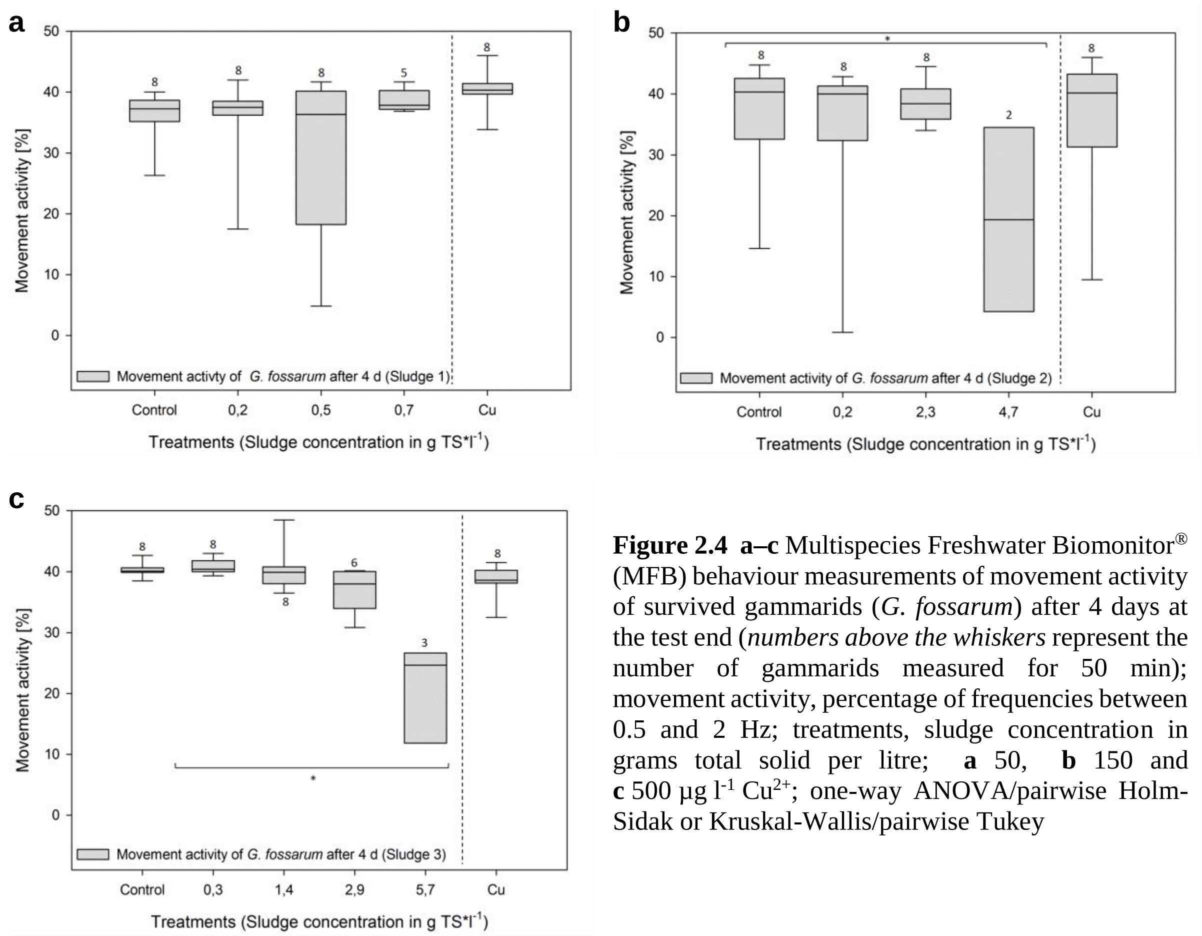

Figure 2.4 a–c Multispecies Freshwater Biomonitor® (MFB) behaviour measurements of movement activity of survived gammarids (*G. fossarum*) after 4 days at the test end (*numbers above the whiskers* represent the number of gammarids measured for 50 min); movement activity, percentage of frequencies between 0.5 and 2 Hz; treatments, sludge concentration in grams total solid per litre; **a** 50, **b** 150 and **c** 500 µg l^{-1} Cu^{2+}; one-way ANOVA/pairwise Holm-Sidak or Kruskal-Wallis/pairwise Tukey

2.5 Discussion

2.5.1 Potential toxicity of sewage sludges in comparison/Correlation of chemical analysis and ecotoxicological results

S1 (non-dewatered bio-P sludge) was more toxic than S2 and S3 considering LC_{50}/EC_{50} values of all three ecotoxicological tests (Table 2.5). The effect concentrations of S1 were 3- to 38-fold lower than of S2 and S3. The higher toxic effect on the test organisms might be supported by the fact that S1 has high concentrations of the organic pollutants benzotriazole (6122 ng g^{-1} TS), carbamazepine (2106 ng g^{-1} TS) and diclofenac (1935 ng g^{-1} TS) and of the heavy metal iron (18.9 mg g^{-1} TS) (Table 2.3). Additionally, it has been shown that the hydroxyl group of benzotriazole is a potential site for a chemical association to organic matter (Beller & Simoneit 1986; Richnow et al. 1994). Therefore, it might be more particle bound and taken up better as food by the particle eater *E. fetida*, resulting in higher toxic effects on the earthworms.

S2 (dewatered bio-P sludge) was more toxic than S3 considering the effect concentrations of the gammarid acute toxicity test, the *Lemna* sp. growth inhibition test and the earthworm avoidance test, while it had 0.3 to 2.8 g TS per litre or kilogram lower LC_{50}/EC_{50} values than S3 (Table 2.5). The analyses of the water samples show that the concentration of TCPP was up to threefold higher in treatments of S2 than in S3 after 4 and 7 days (Table 2.4). However, the higher occurrence of TCPP may not solely cause the higher toxic potential effect of sludge 2 on the duckweed and the amphipod. In comparison to studies about the effect of TCPP on algae and daphnids, the analysed TCPP concentrations (0.39–3.0 µg l^{-1}; Table 2.4) are much lower than the effect concentrations in mg l^{-1} (Griebenow 1998). Furthermore, it should be mentioned that dewatering has a positive effect on reducing the toxicity of the sewage sludge comparing the toxicity of S1 and S2 possibly linked with decreasing concentrations of the organic pollutants (Table 2.3).

S3 (dewatered ferric sludge) had the lowest toxic effect on all three test organisms compared to S1 and S2 considering the LC_{50}/EC_{50} values of the gammarid acute toxicity test, the *Lemna* sp. growth inhibition test and the earthworm avoidance test (Table 2.5). Although the dry matter of S3 contained high concentrations of benzotriazole (12767 ng g^{-1} TS), copper (832 µg g^{-1} TS) and in the water samples, iron (121 mg g^{-1} TS) and nonylphenole only in water samples (not analysed in dried matter of sludges) (Tables 2.3 & 2.4).

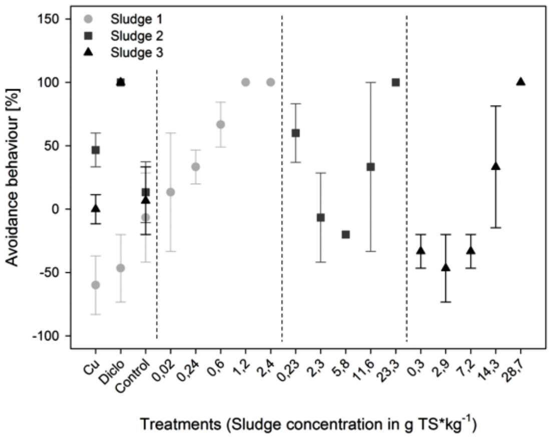

Figure 2.5 Avoidance behaviour of *E. fetida* after 2 days (calculation according to ISO 2008); three replicates per treatment with five earthworms; treatments, sludge concentration in grams total solid per kilogram (corresponding each 0.1–10 % of volume or weight of the fresh matter); references S1 (Cu^{2+} 1 mg kg^{-1}, diclofenac^{-} 1.9 mg kg^{-1}), S2 (Cu^{2+} 95 mg kg^{-1}, diclofenac^{-} 185 mg kg^{-1}), S3 (Cu^{2+} 95 mg kg^{-1}, diclofenac^{-} 280 mg kg^{-1})

Generally, it can be extracted from Table 2.4 that the highest concentrations of the pollutants in the water samples occurred after 7 days in the majority of cases. This leads to the conclusion that it takes time for the pollutants in the sludges to dissolve into the aqueous phase. Therefore, longer exposure times of aquatic tests could result in higher toxic effects of the sludges. The longer exposure time (7 days) of the *Lemna* sp. growth inhibition test might have a lower toxic effect due to the fact that the sludges also contain nutrients which have a positive effect on plant growth. Concentrations below 0.2 g TS l^{-1} of S1 and 2.3 g TS l^{-1} of S2 (1 % FM) even supported growth of *L. minor* compared to the control. Considering the different effects of the sludge samples using various test organisms, *G. fossarum* appears to be the most sensitive organism in this study (Table 2.5).

For a better understanding of the different negative effects of the sludge samples on the test organisms, we compared the pollutant concentrations which appeared significantly in the sewage sludge samples to the toxicity of the single pollutants by literature research of the EC_{50}/LC_{50} values of the *Lemna* sp. growth inhibition test, the gammarid acute toxicity test, the earthworm avoidance test or the most similar tests in comparison. These substances were the heavy metals cadmium, copper, iron and zinc and the organic pollutants benzotriazole, carbamazepine and diclofenac. The single toxicity data of iron and copper can be seen in Table 2.6. No literature was found about the mortality or avoidance behaviour of *E. fetida* after single Fe exposure.

The Fe concentrations and the pH values in the water samples were not analysed, only the total Fe content (Fe(II) and Fe(III)) in the dry matter of the sludges (Table 2.3). So, the iron concentrations of the measured effect concentrations (EC_{50}/LC_{50} values; Table 2.5) on *L. minor* and *G. fossarum* of the three sewage sludge samples (S1–S3) were calculated and lead to the following concentrations: *L. minor* (S1 22.7 mg Fe l^{-1}, S2 148.0 mg Fe l^{-1}, S3 1.2 g Fe l^{-1}) and *G. fossarum* (S1 9.5 mg Fe l^{-1}, S2 24.7 mg Fe l^{-1}, S3 193 mg Fe l^{-1}). The calculated Fe concentrations of the EC_{50} of *L. minor* of the three sludge samples are higher than the iron EC_{50} of *L. minor* (3.7 mg l^{-1}; Table 2.6). The Fe concentrations of the LC_{50} of *G. fossarum* of S2 and S3 are also higher than the iron EC_{50}/LC_{50} of similar freshwater organism (9.6–20 mg l^{-1}; Table 2.6). Therefore, iron contained in the sludge samples could negatively affect the growth of *L. minor* and the mortality of *G. fossarum* in this study. After 7 days, the measured copper concentrations (microgram range) in the water samples of all three sludge samples of 5 % FM (Table 2.4) were at least half as low as the copper EC_{50} of *L. minor* after 4 days (1.1 mg l^{-1}; Table 2.6). After 4 days of exposure, only the water samples of 5 % FM of S3 contained a copper concentration (150 µg l^{-1}; Table 2.4) above the copper LC_{50} value of *G. pulex* (37 µg l^{-1}; Table 2.6). Therefore, copper could be one of the factors which contributed to the higher toxicity of S3 on *G. fossarum*. Also, the significant lower movement activity and the higher stress ventilation of *G. fossarum* (Figures 2.2 & 2.3) in treatments of 5.7 g TS l^{-1} (≙ 2 % FM; Cu^{2+} <20–150 µg l^{-1}; Table 2.4) of S3 could be caused by the high copper concentration. This effect was also investigated by Gerhardt (1995a) in treatments of 35 µg l^{-1} copper. Only the dry matter of S3 (832 mg kg^{-1} TS; Table 2.3) contained more copper than the LC_{50} value of *E. fetida* (Table 2.6), but this value would represent 100 % of the total solid of the sludge sample. However, the highest concentration of the sludge sample which was tested was 10 % FM mixed with soil. Ten percent FM of S3 (28.7 g TS kg^{-1}) resulted in a copper concentration of ~24 mg kg^{-1} soil which is 28-fold lower than the LC_{50} (Table 2.6). Comparing the other investigated heavy metals (Cd, Zn) and organic pollutants with the containing concentration in the sludge samples and the single toxicities, no single negative effects could be detected. The concentrations in the sludge samples were in most cases significantly lower than the single EC_{50}/LC_{50} values.

This comparison implies that the toxic effect of the sewage sludge samples most likely arises from the complex substance mixture in sewage sludges and not from particular contained pollutants. Moreover, there is the possibility that complex mixture toxicity effects among the metals and organic substances might have occurred differently in the three environmental sludge samples, as they tend to be both dose- and ratio-dependent as well as time-dependent (van Gestel et al. 2011). Antagonistic or synergistic effects causing lower or higher toxic potentials of the sludge samples could occur by co-precipitation of heavy metals, competition between the metals at uptake sites and different mode of actions in the organisms (Gerhardt 1995b; Kienle et al. 2009). Due to a high Fe concentration in S3, it might be that more iron oxides or iron hydroxides depending on the pH occurred in the water treatments of the sludge samples, which are capable of removing cations of heavy metals (Gerhardt 1995b; SenGupta 2002). Antagonistic toxicity effects of metals could also be observed by the survival of *Leptophlebia marginata* (mayfly) in a long-term exposure of a metal mixture of Cd and Fe (Gerhardt 1995b). Kraak et al. (1994) has detected that metal mixtures could have a concentration additive effect (Zn+Cd) or an antagonistic effect (Zn+Cu) on the filtration rate of the freshwater mussel *Dreissena polymorpha*. Studies of different binary mixtures of metals and pesticides showed only synergistic lethal effects on the marine microcrustacean *Tigriopus brevicornis* (Forget et al. 1999). Moreover, it could be that a higher amount of the analysed Fe concentration of S3 (ferric sludge) mainly appears as $FePO_4$ (lowly water soluble) emerging during chemical precipitation of phosphates of wastewater (Pöppinghaus et al. 1994) which leads to the assumption of a lower bioavailability of Fe in sludge samples of S3 and a lower toxic effect.

Table 2.6 Toxicity data of the single pollutants by literature research of the EC_{50}/LC_{50} values of the *Lemna* sp. growth inhibition test, the gammarid acute toxicity test, the earthworm avoidance test or the most similar tests

Substance	Organism	Measured endpoint	Concentration	References
Fe	*Lemna minor*	EC_{50} 96 h (pH 7.5) growth inhibition	3.7 mg l^{-1}	Wang 1986
	Daphnia magna	EC_{50} 48 h (Fe^{3+}) immobilisation	16 mg l^{-1}	Sorvari & Sillanpää 1996
		LC_{50} 48 h (pH 7.7)	9.6 mg l^{-1}	Biesinger & Cristensen 1972
	Gammarus roeseli	LC_{50} 108 h (Fe^{2+})	20 mg l^{-1}	Walter 1966
	Leptophlebia marginata	LC_{50} 96 h (pH 7)	106.3 mg l^{-1}	Gerhardt 1994
		LC_{50} 96 h (pH 4.5)	89.5 mg l^{-1}	
		EC_{50} 96 h (pH 7) escape behaviour	70 mg l^{-1}	
		EC_{50} 96 h (pH 4.5) escape behaviour	63.9 mg l^{-1}	
Cu	*Lemna minor*	EC_{50} 96 h growth inhibition	1.1 mg l^{-1}	Wang 1986
	Lemna minor (clone St)	EC_{50} 96 h growth inhibition	0.33 mg l^{-1}	Naumann et al. 2007
	Gammarus pulex	LC_{50} 96 h	0.037 mg l^{-1}	Taylor et al. 1991
		EC 0.5 h behavioural responses	0.035 mg l^{-1}	Gerhardt 1995a
	Chironomus decorus	LC_{50} 48 h	0.74 mg l^{-1}	Kosalwat & Knight 1987
	Eisenia fetida	LC_{50} 14 days	643 mg kg^{-1}	Neuhauser et al. 1985
		LC_{50} 14 days	683 mg kg^{-1}	Spurgeon et al. 1994

2.5.2 Legal mandatory threshold values and realistic application amounts

The mandatory threshold values of seven heavy metals for applied sewage sludge were validated (AbfKlärV 1992). All heavy metals in the three sludges were far below the threshold values excluding the copper concentration of S3 (832 µg g^{-1} TS; Table 2.3). Usually, it is also necessary to include the environmental quality standards (EQS) of priority substances and certain other pollutants in surface waters according to the European Water Framework Directive 2008/105/EC (2008b). But the analysed substances of the sludges did barely correspond with the list of the priority substances and for this reason, we did not elaborate on the EQS in our assessment.

Moreover, two realistic application amounts were calculated for evaluation of the application of the tested sludge samples on the field: (1) the maximum allowed application amount of sewage sludge and (2) the maximum agronomical relevant application amount by the total P_2O_5 content. Both approaches mirror worst-case scenarios.

According to Sect. 6 of the regulation of waste and sewage sludge (AbfKlärV) of Germany, it is not allowed to apply more than 5 t of dry matter of sewage sludge per hectare within 3 years (AbfKlärV 1992). Additionally, the mandatory threshold values of the sewage sludge and of the soil the sludge will be applied on have to be considered (see above). The affected depth (5 cm) of the soil and the area have to be multiplied by the density factor of the soil (1.5 g cm^{-3}) to convert the area value into the weight of soil of the agricultural area (OECD 2000a). One hectare equals 750 t of soil. In conclusion, this means that 5 t of sewage sludge on 750 t of soil result in sludge concentrations of approx. 6.0 g TS kg^{-1}.

Considering the agronomical relevant application amounts of fertilisers by the P_2O_5 content, the maximum amount is 220 kg P_2O_5 ha^{-1} (KTBL 2005). This amount is given in recommendations in Germany for very low P content soil of grassland which has a high P demand. The sewage sludge concentrations in soil resulting from this very high application quantity are S1 3.3 g TS kg^{-1}, S2 2.9 g TS kg^{-1} and S3 3.1 g TS kg^{-1}. The concentrations were calculated by the amount of total solid TS, the total P_2O_5 content of each sludge sample (Table 2.3) and the conversion of the area value into the weight of soil by the OECD guideline (see paragraph above).

The agronomical relevant application amounts are about half of the allowed sewage sludge concentrations in soil. This would imply that the allowed and agronomical applied concentrations of the tested sludge samples of S2 and S3 would probably not affect *E. fetida* (S2 EC_{50} 12.3 g TS kg^{-1}, S3 EC_{50} 15.1 g TS kg^{-1}), considering the acute toxicity with behavioural measurements under laboratory conditions (Table 2.5). The calculated application amount of S1 (3.3 g TS kg^{-1}) would have an effect on the avoidance behaviour of the earthworms (S1 EC_{50} 0.4 g TS kg^{-1}; Table 2.5). The aquatic organisms *L. minor* and *G. fossarum* could also be affected of S1 if an amount of the allowed sludge concentration (6.0 g TS kg^{-1}) or the agronomical applied concentrations (3.3 g TS kg^{-1}) reached surface water by accident, erosion or leakage compared to effect concentrations (Table 2.5). These pathways cannot be excluded in polluting surface waters in general. Amounts of S2 and S3 in the water caused by the above reasons could also have minor negative effects to *G. fossarum*. But S2 and S3 would not affect the aquatic plant *L. minor* because in general, the field concentrations are lower than the EC_{50} values of *L. minor*.

Acute effects in the field might still occur because there is no guarantee of consistent equal distribution of the sewage sludge on agricultural land. Chronic effects of S2 and S3 on the earthworm *E. fetida* or other terrestrial organisms cannot be excluded because of e.g. the possibility of accumulation of persistent heavy metals or organic pollutants (POPs) to toxic levels in the soil by repeated fertiliser application or negative effects of low concentrations over a longer time period. Moreover, two chronic toxic tests have been conducted in the EU Project P-REX by IASP Berlin, Germany; the C and N transformation by microorganisms were tested according to the OECD Guidelines for the Testing of Chemicals Nos. 217 and 216 (2000a, b). The sludge samples were tested in the concentrations of 2, 4, 6, 8 and 10 % FM of soil for 28 days. As sewage sludge also represents a carbon and nitrogen source for soil microorganisms, the C transformation test resulted in a positive effect for all three samples and the N transformation resulted in a positive effect for the samples of sludge 1 and 2. The respiration rate (C transformation) was increased by increasing concentrations of sludge in soil for all three sludge samples compared to the control (P-REX 2014). But, this test is possibly not adequate for organic substances because potential negative effects could be overlaid by the effect of the organic substance and also by microorganisms which were brought into the system with the sludge samples. The N transformation in soil samples treated with sludge 1 and 2 was also increased for all tested concentrations compared to the control. Only the concentrations over 2 % FM of sludge 3 (>5.7 g TS kg^{-1}) led to a significant inhibition of the N transformation (P-REX 2014) which is equal or twofold higher in comparison to the application amounts of the sludges on the field. Therefore, the sludge samples tested in this work might have no chronic effect on the field on soil microorganisms. But, Stoven & Schnug (2009) investigated soil samples of a decennial sewage sludge disposal by the occurrence of earthworms and microbial activities. The sewage sludge-treated soil samples had an increased concentration of heavy metals compared to the mineral fertilised control plots. In this work, sewage sludge addition enhanced soil organic matter concentration and allured more earthworms on the one hand, and on the other hand, the microbial decomposition was retarded by toxic effects of heavy metals due to reduced enzyme activities. The comparison of the two different investigations of sewage sludge-treated soil shows that a longer application time can also lead to positive and negative effects. Different wastewater sources and treatment types result in various compositions of pollutants and nutrients in sewage sludges. Depending on the compositions and concentrations of the tested samples, both positive and negative effects on test organisms can occur. Such coherences influence the assessment and complicate the comparison of toxicity data of sewage sludges.

2.6 Conclusions

All three tested sewage sludge samples can be classified for all three test species as toxic at high concentration levels under laboratory conditions. The usage of the tested dewatered sewage sludges as phosphorus fertilisers in agriculture will most likely not have an acute toxic effect on the test organisms. The non-dewatered sludge (S1) will negatively influence soil invertebrates and freshwater organisms (plants and crustacean) regarding the application amounts, although the heavy metal concentrations are in compliance with the mandatory threshold values. In compliance with the IC_{95} of the EC_{50}/LC_{50} values of the tested sludge samples, sludge 1 has a higher toxic effect on the test organisms compared to S2 and S3. Overall, *G. fossarum* was the most sensitive tested organism.

Before a safe use of sewage sludge can be guaranteed, further studies are necessary in order to (1) study the risks of inappropriate handling or input of sewage sludge-treated soil in surface waters by weather conditions, (2) estimate additive or even synergistic effects of the entire substance mixture in the sewage sludges, (3) study effects of repeated pulses (acute) and chronic toxicity and (4) study of environmental samples after *in situ* application of sewage sludge.

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Chapter 3

Ecotoxicological assessment of phosphate recyclates from sewage sludges

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Ecotoxicological assessment of phosphate recyclates from sewage sludges

3.1 Abstract

Sewage sludge contains valuable plant nutrients, especially phosphorus. But unfortunately, it also contains pollutants which are hazardous for the environment. Phosphorus recovery from sewage sludge and its agricultural valorisation in recycling fertilisers based or containing recovered phosphate provides opportunities to minimise negative environmental effects caused by direct sludge application or conventional fertilisation. For validation, crystallised (struvite) and thermally treated phosphate recyclates (PRs) were chemically analysed, ecotoxicologically assessed and compared with a conventional phosphate fertiliser (triple superphosphate (TSP)). Three test species covering the environmental compartments water, sediment and soil were applied to evaluate the acute toxic effects of the phosphate product samples in laboratory tests (*Lemna minor*, *Gammarus fossarum*, *Eisenia fetida*). The assessment and comparison showed that TSP was more toxic than the phosphate recyclates at the higher tested concentrations, probably due to a higher water solubility and not to chemical composition. Higher concentrations of the crystallised PRs caused mostly a slightly higher negative effect on tested parameters of the duckweed and the freshwater amphipod than the thermally treated PRs. Agronomical relevant application amounts of all phosphate recyclates and TSP (worst-case scenario) might not have an acute toxic effect on the soil invertebrates. The phosphate recyclates might have minor effects on the growth of *L. minor*, and TSP might negatively affect the survival of the freshwater amphipods. Recovered phosphate-containing materials, in particular struvite, proved to be of high quality and low hazard in a relative risk ranking; thus, it could be one of the future alternatives of phosphorus fertilisation in agriculture.

Keywords Ecotoxicity • Phosphate recyclates • *Lemna* • *Gammarus* • *Eisenia*

3.2 Introduction

Sewage sludge from municipal sewage treatment plants contains a host of valuable plant nutrients, especially of phosphorus. But apart from nutrients, sewage sludge also includes toxic heavy metals and organic pollutants (Oliva et al. 2009; Wiechmann et al. 2013). Sewage sludge applied as fertiliser in agriculture can result in accumulation of pollutants in soil. This can be hazardous to groundwater and surface waters if the pollutants end up in such waterbodies as the result of run-off (Galdos et al. 2009) or percolation (Luczkiewicz 2006; Alvarenga et al. 2016). Further, the environmental hazards posed by agricultural usage of sewage sludge are difficult to determine because the interactions and transformation processes of the pollutants mixture are often unknown. Concerning the multitudinous amount of hazardous pollutants to the environment and health, the direct application of sewage sludge shall be mostly ceased in Germany in the future (CDU et al. 2013; BMUB 2017c). Only the usage of approved sewage sludge as fertiliser is allowed. The instructions of the Biological Waste Regulation (BioAbfV 1998), the Sewage Sludge Regulation (AbfKlärV 1992) and since January 1, 2015, also the lower limit values of the Fertiliser Ordinance of Germany (DüMV 2012) have to be followed (UBA 2015). The stricter requirements for pollution loads in sewage sludge and the classification of phosphorus as a critical raw material strengthening the finite nature of this resource, point up that phosphorus recovery from sewage sludge has to be regularised and will be mandatory for sewage treatment plants with high treatment capacities (>50,000 person equivalents) in the amended AbfKlärV (UBA 2015; BMUB 2017c).

In the recent years, several ways of recovering phosphorus from the liquid phase, from sewage sludge or from sewage sludge ash of municipal wastewater treatment plants have been developed (Le Corre et al. 2009; Egle et al. 2015; Kabbe et al. 2015). Recovery from the liquid phase is mostly based on precipitation or crystallisation processes (Jaffer et al. 2002). Phosphorus precipitates or crystallises as calcium phosphate (CaP) and/or magnesium ammonium phosphate which is most commonly known as struvite ($MgNH_4PO_4 \cdot 6H_2O$). By magnesium dosing and an increasing pH, the struvite output can be raised (Le Corre et al. 2009). P recovery from digested sludge can be realised by an acidic extraction of phosphorus at a low pH with a subsequent crystallisation (Jaffer et al. 2002; Stemann et al. 2014). After mono-incineration of sewage sludge, the direct application of the ash in agriculture is limited due to heavy metal contents above the limit values of the DüMV and the low plant availability of phosphorus. Technologies for P recovery from sewage sludge ash are mostly grounded on two different approaches: thermo-chemical and wet-chemical treatment. The thermo-chemical processes include a separation of heavy metals and phosphorus by volatilisation at high temperatures (addition of auxiliary substances is possible) and/or a phase separation because of density differences of the melts. Further, the phosphorus in the solid phase (or in the slag melt) reacts with additives like $MgCl_2$ or Na_2SO_4, forming new phosphate compounds with a higher plant availability (Donatello & Cheeseman 2013; Herzel et al. 2015). Wet-chemical treatment of ash is based on washing the ash with an acidic liquid, which leaches a lot of the heavy metals and phosphorus from the ash. After a heavy metal removal and filtration, the pH of the leachate is subsequently increased until a high P recovery by precipitation is achieved (Donatello & Cheeseman 2013; Herzel et al. 2015).

Despite, the precision removal or masking of heavy metals during precipitation potential risks linked to the use of phosphate recyclates in agriculture might still exist and need to be investigated to protect the environment. The effects of the remaining pollutant mixture of the recycled fertilisers can be evaluated more comprehensively by ecotoxicological tests than by standard chemical analysis. Previous research about the toxicity of conventional phosphate fertilisers which are produced by using phosphate rock that was partly radioactive and contaminated with heavy metals concentrated mainly on the analysis of the occurrence of uranium and cadmium and the transfer to soil, water bodies and groundwater (Saueia et al. 2005; Khater & Al-Sewaidan 2008; Nziguheba & Smolders 2008; Cordell et al. 2009). Further studies investigated potential risks for consumer if pollution is carried upwards the food chain by crop uptake (Chen et al. 2007; Murtaza et al. 2015). Manufacturer of conventional phosphate fertilisers mentioned ecological information about aquatic toxicity in material safety data sheets prepared according to EU standards, but no references about the test conductions were cited (ICL 2012). Abbiramy et al. (2013) performed an acute toxicity test of one superphosphate fertiliser to earthworms using the paper contact method according to OECD No 207 (Organisation for Economic Co-operation and Development). Toxicity research about phosphate recyclates is mostly based on life cycle assessment (LCA) or risk assessment of phosphorus recovery processes considering contaminant concentrations plus e.g. emissions from transport or chemical manufacture, etc. (Bradford-Hartke et al. 2015; Kraus & Seis 2015; Remy & Jossa 2015). Zimmermann (2010) investigated the aquatic toxicity of the eluates of raw sewage sludge ash before and after bioleaching on algae, daphnia and bacteria.

The objective of this study was to assess potential toxic effects of different recovered phosphate-containing materials (phosphate recyclates (PRs)) and compare their toxicity with a conventional phosphate fertiliser (triple superphosphate (TSP)). To cover all affected compartments, the following tests were used for the ecotoxicological assessment: (1) the growth inhibition of the water plant *Lemna minor* (Linné 1753, Arales, Lemnaceae) in the water compartment, (2) the mortality and behaviour of the freshwater shrimp *Gammarus fossarum* (Koch 1836, Amphipoda, Gammaridae) in the water and sediment compartment and (3) the avoidance behaviour of the earthworm *Eisenia fetida* (Savigny 1826, Oligochaeta, Lumbricidae) in the soil compartment.

3.3 Materials and methods

3.3.1 Samples

Three crystallisation products (Cryst 1, Cryst 2, Cryst 4) and five ash products (Therm 1–5) of recovered phosphate-containing materials (PRs), obtained from treated sewage sludges, sludge liquors or sludge ashes from municipal wastewater treatment plants in Europe, were chemically and ecotoxicologically analysed. Additionally, the effects of a reference phosphate fertiliser, TSP, and of a reference heavy metal, copper ($CuCl_2$), were assessed. An overview of the phosphate recyclates, used abbreviations and their production techniques can be found in Table 3.1.

Table 3.1 Overview of tested phosphate recyclates and reference phosphate fertiliser

Phosphate recyclates (abbreviation)	Product	Technique
Crystallisation products		
Cryst 1	Struvite	Recovered from sludge (aqueous phase) after dewatering
Cryst 2	Struvite	Recovered from sludge (aqueous phase) after digestion prior dewatering
Cryst 4	Struvite	Recovered from sludge after enforced redissolution by acidulation with H_2SO_4
Ash products		
Therm 1	Raw ash	Mono-incineration
Therm 2	Treated ash	Calcination with $MgCl_2$
Therm 3	Treated ash	Calcination with Na_2CO_3
Therm 4	P-slag	After metallurgic treatment
Therm 5	CaP/Struvite	After acid leaching of ash
Reference fertiliser		
TSP	Triple superphosphate	Conventional fertiliser production

Source: Herzel et al. (2015), Stemann et al. (2014)

CaP calcium phosphate, H_2SO_4 sulfuric acid, $MgCl_2$ magnesium chloride, Na_2CO_3 sodium carbonate

The dry and ground samples were stored in 2-l bottles (PE) at room temperature prior to and between the toxicity tests. After shaking, each sample was mixed with the appropriate reference medium (Table 3.2). For the ecotoxicological test of the direct assessment of the dry matter of the phosphate recyclates, no further pre-treatments of the samples were necessary. For the assessment of the eluates of TSP and Cryst 4 produced according to DIN 12457-1 (2002), 250 g of the dry matter of each PR was shaken in 500 ml fresh stream water for 24 h at 20 °C (overhead shaker with 10 rpm). The fresh stream water was obtained from the Hockgraben in Konstanz, Germany (47° 40' 02.3" N, 9° 12' 04.2" E). After sedimentation (~10–15 min), the eluates were filtered using folded filters and a vacuum filtration with glass fibre filters (*d* 90 mm, pore size 1.2 µm). Before, no grain size reduction was required because the samples were already finely ground. Furthermore, no pH adjustment of the eluates was performed because of the comparability to the direct assessment of the phosphate recyclates. The eluates were stored at 4 °C. The concentrations of the phosphate recyclates in the direct assessment and of the eluates (see sections below) were chosen to evaluate effects of realistic application amounts of phosphate recyclates in agriculture (Table 3.5) and of environmentally realistic concentrations to be reached in surface waters by accident, erosion or leakage. The aim was to assess realistic potential effects in agriculture under laboratory conditions; therefore, our demand on the test set-up was not to have a concentration range covering the whole toxic effect range.

Table 3.2 Summary of test parameters of ecotoxicity tests for phosphate recyclate samples (*Lemna* sp. growth inhibition test, gammarid acute toxicity test, earthworm avoidance test)

Test organisms	*L. minor*	*G. fossarum*	*E. fetida*
Acclimation	Equal to test parameters (at least for 4 days)	Equal to test parameters, with one alder leaf (for 4 days)	Darkened box filled with a peat/ LUFA 2.3/compost mixture (humid conditions/room temperature/ at least for 4 days)
Test parameters			
Exposure medium	Onefold Steinberg medium (mixed with fresh stream water)	Fresh stream water (sand as sediment, permanent aeration)	50% LUFA standard soil 2.3/50% compost
Exposure length	7 days (168 h)	4 days (96 h)	2 days (48 h)
Number of organisms	13 fronds	10	5
Replicates	3	3	3
Temperature	24±2 °C	18±3 °C	20±3 °C
Photoperiod/intension	24 h day 6 500–10 000 lx	16 h day/8 h night 400–800 lx	16 h day/8 h night 400–800 lx
Endpoints	Growth inhibition Discolouration Colony break-up Weight decrease Root length decrease	Mortality Movement behaviour Feeding behaviour	Avoidance behaviour
Acceptability criteria	Control growth rates >0.2 per day	Control mortality <10 %	Number of dead or missing worms <10 %

3.3.2 Chemical analysis

In the milled and manually mixed phosphate recyclates and TSP, the following parameters were chemically analysed: percentage of nutrients and organic dry matter content, concentrations of organic pollutants and heavy metals. In the ash products and in TSP, no analysis of the organic pollutants was conducted because during incineration (850–950 °C; Wiechmann et al. 2013), the organic pollutants of the sewage sludges were destroyed and conventional phosphate fertilisers do not contain organic pollutants. The analyses were performed by the Institute of Agricultural and Urban Ecological Projects (IASP, Berlin, Germany), LUFA Nord-West (Agricultural Analysis and Research Institute, Hameln, Germany) and Institute of Ecopreneurship, FHNW (University of Applied Science and Arts Northwestern Switzerland, Basel). The references of the analysis can be seen in Chapter 2 (Rastetter & Gerhardt 2017).

3.3.3 Ecotoxicological tests

Chronic growth inhibition test with L. minor

In this test, chronic effects on the growth of the duckweed *L. minor* were assessed according to ISO 20079 (2005). An overview of the test parameters can be seen in Table 3.2. The detailed test procedures can be read in Chapter 2 (Rastetter & Gerhardt 2017).

Five concentrations of the dry matter (DM) of the samples were assessed (0.01, 0.1, 1, 5 and 10 g DM l^{-1}). The reference concentration of copper chloride (0.3 mg Cu^{2+} l^{-1}) was chosen based on our own experimental data in this research (EC_{50} 0.7 mg Cu^{2+} l^{-1}). After 7 days, the total number of leaves was counted and the percent inhibition for each sample concentration was calculated (ISO 2005). Additionally, (1) the number of single leaves, (2) the number of leaves with any discolouration (brown, yellow or bleached), (3) the dry weight after 7 days of air drying and (4) the average measured root length was determined for evaluating (1) the percentage of colony break-up and (2) of discolouration, (3) the decrease of the dry weight and (4) of the root length in comparison to the control after 7-day test duration.

Acute toxicity test and behaviour measurements with G. fossarum

The acute toxicity test was conducted as described in the Ecological Effects Test Guidelines by the US Environmental Protection Agency (Gammarid Acute Toxicity Test; EPA 1996) with adjustments for environmental samples and in order to perform a realistic test set-up under laboratory conditions according to Chapter 2 (Rastetter & Gerhardt 2017) (overview of test parameters is shown in Table 3.2).

Five concentrations of the dry matter of the samples were chosen for the direct assessment of the phosphate recyclates (0.005, 0.01, 0.1, 1 and 5 g DM l^{-1}). Five concentrations of the equivalent volume of the eluates (0.01, 0.02, 0.2, 2 and 10 ml l^{-1}) of the most effective samples in the direct assessment (TSP, Cryst 4) were investigated to show the effects of the samples due to potential elution of the phosphate products in the soil. For the eluate analysis, gammarids were used because the freshwater amphipods were the most sensitive test species in the test set-up (Chapter 2; Rastetter & Gerhardt 2017). As a toxin reference sample (positive control), a copper chloride dilution ($CuCl_2$) was used (0.5 mg Cu^{2+} l^{-1}). A test of copper chloride was conducted during the experiments and a dose-response curve was fitted (linear regression/probit method, lethal concentration of 50 % lethality (LC_{50}) 0.59 mg Cu^{2+} l^{-1}). For acclimation and the test performance in this research, a whole liquid volume of 1 l in 2-l test vessels (17 × 15 × 9 cm) of polypropylene with 50 g incinerated commercial sand (1 h, 500 °C) were applied. Further, ten gammarids instead of eight gammarids were used in each treatment and a fresh leached alder leaf of similar size was added as food as opposed to thawed chironomids. The survival and feeding behaviour of gammarids were visually measured after 4 days of exposure. Moreover, after 4 days of exposure time, the movement activity of the gammarids was measured with the Multispecies Freshwater Biomonitor® (MFB) (Gerhardt et al. 1994; Gerhardt et al. 1998). Eight individual amphipods of the three replicates of the control, 0.1 and 5 g DM l^{-1} and copper were measured for 50 min in the appropriate PP box with the same sample concentration. The movement frequencies were analysed with the Fourier frequency transformation (FFT) (Gerhardt et al. 1998). Through the obtained data, the percentage of mortality and the decrease of feeding and movement activity compared to the control was calculated.

Avoidance test with the earthworm E. fetida

The avoidance test was performed according to ISO 17512-1: avoidance test for determining the quality of soils and effects of chemicals on behaviour of earthworms (ISO 2008). To improve the test, set-up adjustments for a better evaluation of our samples based on Chapter 2 (Rastetter & Gerhardt 2017) were taken. An overview of the test parameters can be seen in Table 3.2.

Five different concentrations of the phosphate recyclates and of TSP in a 50/50 mixture of LUFA 2.3 soil (natural standard soil, LUFA Speyer, Germany) and compost were assessed (0.5, 1, 5, 25 and 50 g DM kg^{-1}). As reference, copper chloride (250 mg Cu^{2+} kg^{-1}) was used. The used reference concentration was chosen according to our own experimental data.

For calculation of the avoidance of a sample concentration after 48 h, the mean number of worms in the test soil was compared to the mean number of worms in the control soil according to the equation ($x = ((n_c - n_t)/N) \times 100$) of ISO 17512-1 (2008).

3.3.4 Data analysis

The results were stated as no observed effect concentration/lowest observed effect concentration (NOEC/LOEC) values in Table 3.5. Normally distributed raw data were analysed with a one-way analysis of variance (ANOVA), non-normally distributed raw data with a nonparametric Kruskal-Wallis test according to the effects of the different concentrations. Pairwise testing was followed by the Tukey test. For the statistics and graphs of the phosphate recyclates and references, we were using Sigma Plot (Systat Inc.). The data of the copper experiments were stated as LC_{50}/EC_{50} values where a dose–response curve could be fitted using a linear regression analysis after probit transformation (ToxRat® Professional (ToxRat Solutions GmbH)). Significances were marked in the graphs by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.4 Results

3.4.1 Chemical analysis

The results of the chemical analysis of the phosphate recyclates and TSP can be seen in Table 3.3. The dry matter of the crystallised PRs (Cryst 1, 2, 4) had a similar content of nutrients (N_{total} 4.7–5.7 %, total P_2O_5 23–30.5 %, K_2O 0.1–0.3 %, MgO 12.5–18.2 %, CaO 0.1–1.6 %). The nutrient contents of the dry matter of the thermally treated PRs (Therm 1–5) were partially in a similar range (N_{total} <0.08 %, K_2O 0.2–0.7 %). Total P_2O_5 was between 9.5 and 15.6 % of the DM of Therm 1–4, excluding Therm 5 was higher with 28.3 %. Therm 2 had a higher amount of magnesium oxide (10.3 %) than the other ash products (2.0–3.2 %). The concentrations of CaO in Therm 4 and 5 (36.2 and 34.7 %) were twice as high as in Therm 1– 3. The crystallisation products had a higher amount of total P_2O_5 and MgO than the ash products, excluding Therm 5 (total P_2O_5) and Therm 2 (MgO), whereas the CaO concentrations of the thermally treated PRs were much higher than in the crystallised PRs. The DM of the reference phosphate fertiliser TSP had the highest amount of total P_2O_5 (51 %) and also a high concentration of CaO (26.2 %). Cryst 4 had the highest amounts of organic pollutants in total (benzotriazole 108 ng g^{-1} DM, carbamazepine 113 ng g^{-1} DM, diclofenac 8.5 ng g^{-1} DM) of the crystallisation products. The organic pollutants were not analysed in the ash products and in TSP. The highest contents of heavy metals were iron (Fe), zinc (Zn) and copper (Cu) in the phosphate recyclates. In general, the thermally treated PRs had the highest concentrations of heavy metals (Cu 97–893 µg g^{-1} DM, Fe 15.7–147.3 mg g^{-1} DM, Zn 69.3–2434.4 µg g^{-1} DM). TSP had the highest amount of uranium (63 µg g^{-1} DM). Arsenic, cadmium and quicksilver occurred in the lowest amounts (≤16 µg g^{-1} DM) or under the limit of detection or quantification in the phosphate recyclates and TSP.

Table 3.3 Measured concentrations of sum parameters, N and P compounds, nutrients, organic pollutants and heavy metals of the phosphate recyclates and TSP related to the dry matter of the samples

Substance group	Cryst 1	Cryst 2	Cryst 4	Therm 1	Therm 2	Therm 3	Therm 4	Therm 5	TSP
Sum parameters, N and P compounds, nutrients (percentage of DM)									
OTS	12,9	18,1	18,7	0,1	0,3	0,0	0,1	10,1	18,1
Ash	45,8	52,0	45,3	99,8	99,5	100,0	99,8	87,4	77,0
N_{total}	5,7	4,7	5,0	<0.08 (LD)	<0.08 (LD)	<0.01 (LD)	<0.08 (LD)	<0.08 (LD)	<0.08 (LD)
P_2O_5 mineral acid soluble	30,5	26,6	23,0	15,6	13,9	12,9	9,5	28,3	51,0
P_2O_5 neutral ammon citrate acid soluble + water-soluble	28,6	25,1	22,0	2,5	3,9	12,8	0,6	26,9	46,9
P_2O_5 citric acid soluble	29,7	26,4	23,0	7,5	12,2	14,1	4,1	27,2	47,5
P_2O_5 water-soluble	0,6	1,1	0,6	<0.01 (LD)	0,1	1,3	0,0	1,2	44,4
K_2O mineral acid soluble	0,1	0,1	0,3	0,6	0,3	0,7	0,7	0,2	1,0
MgO	18,2	15,6	12,5	2,5	10,3	2,3	3,2	2,0	1,3
CaO	0,1	1,0	1,6	19,1	17,4	14,0	36,2	34,7	26,2
S	0,0	0,1	1,3	1,3	1,0	1,1	0,1	8,0	1,1
Organic micropollutants (ng g^{-1} DM)									
Benzotriazole	6,9	11,0	108,0	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Carbamazepine	0,4	39,0	113,0	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Diclofenac	1,1	1,9	8,5	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Estrone	<LD	<LD	<LD	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Mecoprop	<LD	<LD	<LD	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Sulfamethoxazole	<LD	<LD	<LD	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
Heavy metals (µg g^{-1} DM)									
Arsenic	4,9	1,5	1,6	11,2	9,4	7,6	<4.5 (LQ)	16,0	7,5
Cadmium	<0.2 (LQ)	<0.3 (LQ)	0,1	6,9	1,9	1,4	0,1	4,3	4,2
Chromium	3,0	33,2	5,5	96,1	50,4	311,4	103,0	28,0	121,2
Copper	1,5	62,9	58,9	893,0	494,1	835,2	97,0	659,9	13,0
Iron	313,9	4 060.8	19 648.7	147 296.6	119 545.3	108 538.6	21 744.8	15 697.7	3 444.3
Lead	<2.2 (LQ)	19,7	10,9	228,4	50,3	87,4	<3.3 (LD)	8,5	3,1
Nickel	6,1	19,7	6,6	64,9	72,1	342,8	28,1	11,8	41,2
Quicksilver	<0.7 (LQ)	0,5	0,6	<1.1 (LQ)	<1.0 (LD)	<1.2 (LQ)	<1.1 (LD)	0,4	0,2
Uranium	<16.9 (LQ)	<19.4 (LQ)	<21.5 (LQ)	48,6	43,4	37,1	<43.8 (LQ)	24,9	63,0
Zinc	2,4	73,4	87,5	2 434.4	1 133.2	1 930.1	69,3	1 177.2	182,9

Source: LUFA Nord-West Hameln, IASP Berlin and FHNW Basel

DM dry matter, *OTS* organic total solid, *LD* limit of detection, *LQ* limit of quantification, *n.t.* not tested

3.4.2 Chronic growth inhibition test with *L. minor*

The tested concentrations of the crystallised and thermally treated PRs had a similar effect on growth of *L. minor*. The highest tested concentration of 10 g DM l^{-1} resulted in a maximal growth inhibition of 15–34 % with the exception of Therm 3 with 49 % (Figure 3.1b–d). The highest concentration of the copper reference (2 mg Cu^{2+} l^{-1}) and reference phosphate fertiliser

TSP (10 g DM l^{-1}) caused 100 % growth inhibition (Figure 3.1a). Concentrations of the phosphate recyclates and TSP of 0.01 and 0.1 g DM l^{-1} had a low negative effect on growth (mostly <10 %) and supported growth in treatments of Cryst 1, 4 and Therm 3. The reference toxicant, 300 µg Cu^{2+} l^{-1}, in PR and the TSP treatments caused between ∼40 and 60 % growth inhibition, excluding Cu of Cryst 4 (20 %) (Figure 3.1a–d). The crystallisation and ash products had no effect on the additional test parameters, discolouration and colony break-up, whereas high concentrations of copper and TSP caused up to 100 % discoloured and single leaves (Table 3.4). Higher concentrations of the phosphate recyclates (5 and 10 g DM l^{-1}) also resulted mainly in a weight decrease of 20 to 45 % due to a lower amount of leaves or smaller leaves in diameter. The same concentrations had a similar effect on the root lengths of the duckweed, excluding the higher root length decrease of Cryst 4 (60–75 %) and the lower decrease of Therm 1 (0 %). 80 to 100 % decrease of weight and root length were caused of the higher concentrations of copper and TSP (except of root length decrease of TSP 30–40 %) (Table 3.4). The lowest NOEC/LOEC values were 0.1 and 1 g DM l^{-1} of Cryst 1, Therm 1 and 3 for the main parameter, the growth inhibition. The NOEC/LOEC values of the additional test parameters were mostly ≥10 and >10 g DM l^{-1}. The test parameters growth inhibition, discolouration and weight decrease of TSP had a NOEC/LOEC of 1 and 5 g DM l^{-1} (Table 3.5). Additionally, a minor green algae growth could be detected in low concentrations of TSP and the phosphate recyclates. In higher concentrations of copper, TSP and the ash products, no algae growth could be noticed, whereas in higher concentrations of the crystallisation products, a medium algae growth occurred.

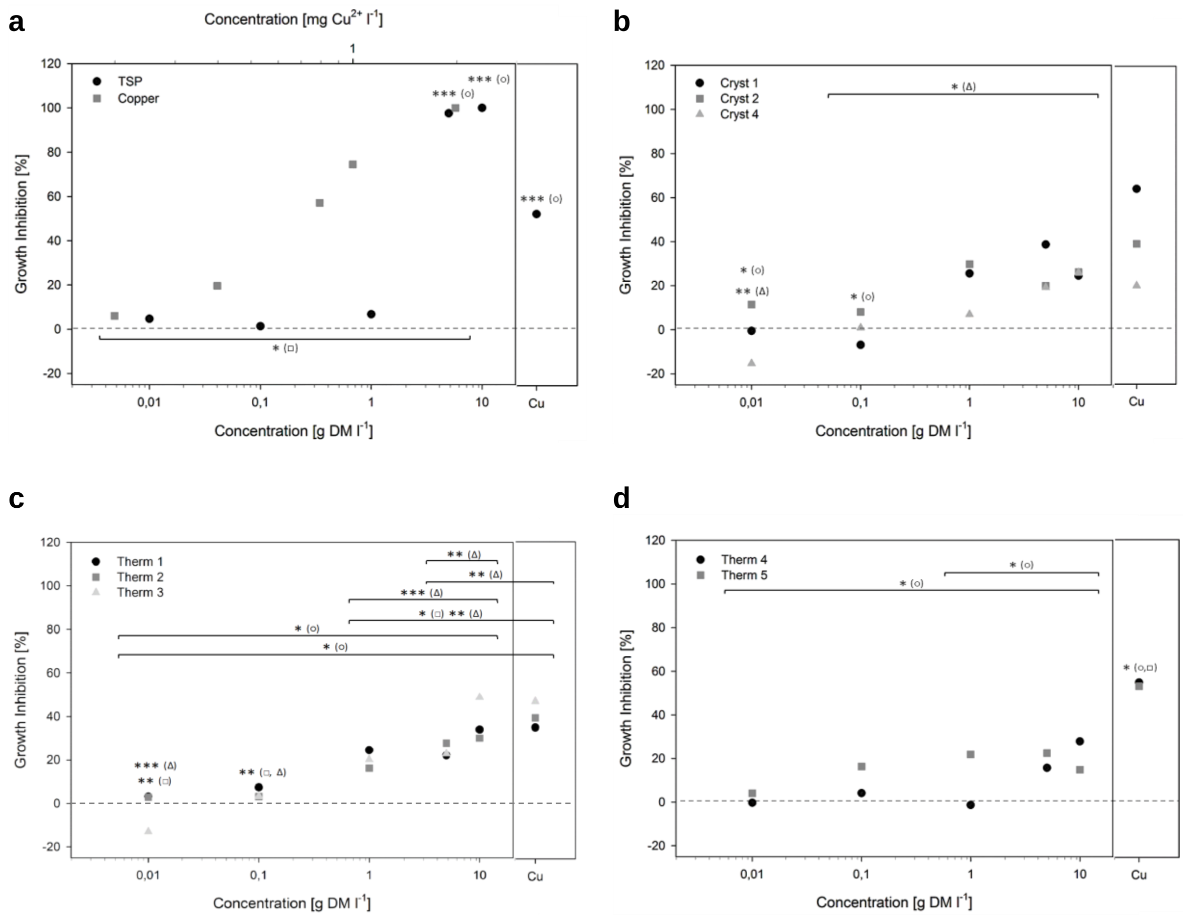

Figure 3.1 **a** Inhibition of growth of *L. minor* after 7 days of TSP and copper; three replicates per treatment of TSP and four replicates per treatment of copper (started each with 13 fronds); Cu^{2+} 300 µg l^{-1}; dry matter (DM). One-way ANOVA/Kruskal-Wallis and pairwise Tukey (TSP (Cu, 5 and 10 g DM l^{-1}differ significantly from the control, 0.01, 0.1 and 1 g DM l^{-1} except themselves)) **b** of Cryst 1, 2 and 4; three replicates per treatment (started each with 13 fronds); Cu^{2+} 300 µg l^{-1}; dry matter (DM). Three data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey (Cryst 1 (0.01 and 0.1 g DM l^{-1}differ significantly from other concentrations except themselves), Cryst 4 (0.01 g DM l^{-1}differs significantly from other concentrations except 0.1 g DM l^{-1})) **c** of Therm 1–3; three replicates per treatment (started each with 13 fronds); Cu^{2+} 300 µg l^{-1}; dry matter (DM). Three data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey (Therm 2 (0.01 and 0.1 g DM l^{-1} differ significantly from 5 and 10 g DM l^{-1} and Cu)) **d** of Therm 4–5; three replicates per treatment (started each with 13 fronds); Cu^{2+} 300 µg l^{-1}; dry matter (DM). Two data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey (Therm 4 (Cu differs significantly from other concentrations except 10 g DM l^{-1}))

Table 3.4 Effects of the additional test parameters of the *Lemna* sp. growth inhibition test and the gammarid acute toxicity test for all measured concentrations of the reference copper, the reference phosphate fertiliser TSP and the phosphate recyclates

Additional test parameter	Cu^{2+} [mg l^{-1}]						TSP [g DM l^{-1}]							Cryst 1 [g DM l^{-1}]						
	0	0.2	0.4	0.8	1	2	0	0.01	0.1	1	5	10	Cu	0	0.01	0.1	1	5	10	Cu
Lemna minor																				
Colony break-up [%]	0	0	1	25	83	100	0	1	0	0	95	0	2	0	1	0	2	4	10	3
Discolouration [%]	2	2	4	73	94	100	1	3	3	2	100	100	26	0	2	0	1	5	6	71
Weight decrease [%]	-	9	27	76	84	87	-	3	-4	-1	88	83	61	-	-26	-44	1	35	16	56
Root length decrease [%]	-	0	27	95	100	100	-	-20	0	34	34	38	92	-	22	8	22	33	44	96
	Cu^{2+} [mg l^{-1}]						TSP [g DM l^{-1}]							Cryst 1 [g DM l^{-1}]						
	0	0.2	0.5	0.75	1	2	0	0.005	0.01	0.1	1	5	Cu	0	0.005	0.01	0.1	1	5	Cu
Gammarus fossarum																				
Movement decrease [%]	-	-	2	-	43	-	-	-	-	1	17 [1]	-	4	-	-	-	-1	-	40	4
Feeding decrease [%]	-	66	93	97	100	100	-	69	15	29	76	97	93	-	80	63	36	56	80	93

Additional test parameter	Cryst 2 [g DM l^{-1}]							Cryst 4 [g DM l^{-1}]							Therm 1 [g DM l^{-1}]						
	0	0.01	0.1	1	5	10	Cu	0	0.01	0.1	1	5	10	Cu	0	0.01	0.1	1	5	10	Cu
Lemna minor																					
Colony break-up [%]	0	0	3	6	0	0	7	2	0	5	2	1	3	0	1	0	0	0	0	0	0
Discolouration [%]	0	0	0	2	0	0	8	2	0	2	2	0	3	4	1	2	2	1	3	3	10
Weight decrease [%]	-	22	13	38	28	33	62	-	-38	0	-4	16	30	37	-	10	19	39	29	45	52
Root length decrease [%]	-	25	38	44	44	50	63	-	-23	38	46	60	75	63	-	-8	15	15	0	0	80
	Cryst 2 [g DM l^{-1}]							Cryst 4 [g DM l^{-1}]							Therm 1 [g DM l^{-1}]						
	0	0.005	0.01	0.1	1	5	Cu	0	0.005	0.01	0.1	1	5	Cu	0	0.005	0.01	0.1	1	5	Cu
Gammarus fossarum																					
Movement decrease [%]	-	-	-	-1	-	3	16	-	-	-	-4	-	44	16	-	-	-	6	-	16	19
Feeding decrease [%]	-	77	37	82	50	86	100	-	28	41	23	91	91	100	-	71	55	87	52	71	68

Additional test parameter	Therm 2 [g DM l^{-1}]							Therm 3 [g DM l^{-1}]							Therm 4 [g DM l^{-1}]						
	0	0.01	0.1	1	5	10	Cu	0	0.01	0.1	1	5	10	Cu	0	0.01	0.1	1	5	10	Cu
Lemna minor																					
Colony break-up [%]	0	1	0	0	0	0	0	0	0	0	1	0	2	1	0	0	0	0	0	0	1
Discolouration [%]	1	1	1	1	2	2	28	3	2	2	6	3	12	49	2	2	3	2	3	5	49
Weight decrease [%]	-	-8	-11	23	30	29	56	-	-32	3	27	30	41	58	-	-1	4	-5	23	38	71
Root length decrease [%]	-	3	-4	0	23	19	88	-	-18	0	9	18	62	95	-	-10	-20	-10	10	34	94
	Therm 2 [g DM l^{-1}]							Therm 3 [g DM l^{-1}]							Therm 4 [g DM l^{-1}]						
	0	0.005	0.01	0.1	1	5	Cu	0	0.005	0.01	0.1	1	5	Cu	0	0.005	0.01	0.1	1	5	Cu
Gammarus fossarum																					
Movement decrease [%]	-	-	-	-3	-	-4	-13	-	-	-	-10	-	8	-13	-	-	-	-4	-	-3	19
Feeding decrease [%]	-	33	60	44	19	-29	62	-	1	6	-8	54	65	62	-	10	36	42	78	46	68

Additional test parameter	Therm 5 [g DM l^{-1}]						
	0	0.01	0.1	1	5	10	Cu
Lemna minor							
Colony break-up [%]	0	0	0	1	1	0	2
Discolouration [%]	0	0	1	1	1	0	25
Weight decrease [%]	-	12	23	35	23	6	73
Root length decrease [%]	-	15	24	29	15	18	92
	Therm 5 [g DM l^{-1}]						
	0	0.005	0.01	0.1	1	5	Cu
Gammarus fossarum							
Movement decrease [%]	-	-	-	-1	-	72	9
Feeding decrease [%]	-	25	-94	21	33	88	40

[1] 1 g DM l^{-1} of TSP (gammarids of 5 g DM l^{-1} of TSP died after 4 d, no movement activity measurements were possible)

3.4.3 Acute toxicity test and behaviour measurements with *G. fossarum*

The highest tested concentration of the phosphate recyclates (5 g DM l^{-1}) had a low or no effect on the survival of *G. fossarum* after 4 days (Figure 3.2b–d). Five grams of DM per litre of the crystallisation products (Cryst 1, 2 and 4) caused on average a mortality of 20–33 %. Lower concentrations of the crystallised PRs (0.005–1 g DM l^{-1}) and TSP (0.005–0.1 g DM l^{-1}) had minor effects on the survival of the gammarids (approximately <20 %). The toxicity test with the eluate of Cryst 4 and TSP resulted in a lower or equal mortality in comparison to the direct assessment (Figure 3.2a, b). The ash products caused ≤10 % mortality in the tested concentration range (0.005–5 g DM l^{-1}) (Figure 3.2c, d). At the end of the test, all gammarids of the highest concentration of the direct assessment of TSP, the eluate of TSP and the copper reference were dead (100 % mortality; Figure 3.2a). Concentrations of 500 µg Cu^{2+} l^{-1} negatively influenced the survival of *G. fossarum* (except Cu of Therm 1 and 5 (~10 % mortality)) (Figure 3.2a–d). The feeding behaviour decreased variably, not continuously, within the concentration range of copper, TSP and the phosphate recyclates. Low concentrations already caused in part high effects on the feeding (up to 80 %; Table 3.4), while higher concentrations resulted in some lower effects. The highest concentration level reached a decrease of 46–100 % with the exception of 5 g DM l^{-1} of Therm 2 with an increase of -29 % (Table 3.4). After 4 days, the movement activity of gammarids exposed to 1 mg l^{-1} copper, 5 g DM l^{-1} Cryst 1, 4 and Therm 5 changed clearly with a decrease between 40 and 72 % (Table 3.4). The reference phosphate fertiliser TSP and its eluate had the lowest NOEC/LOEC values of the tested samples for the investigated mortality (TSP, 0.1 and 1 g DM l^{-1}; TSP eluate, 1 and 5 g DM l^{-1}) and for the decrease of feeding (TSP eluate, 0.01 and 0.1 g DM l^{-1}). The NOEC/LOEC values of all phosphate recyclates were ≥5 and >5 g DM l^{-1} (Table 3.5). No NOEC/LOEC values of the decrease of movement activity were calculated due to limited data (Tables 3.4 & 3.5).

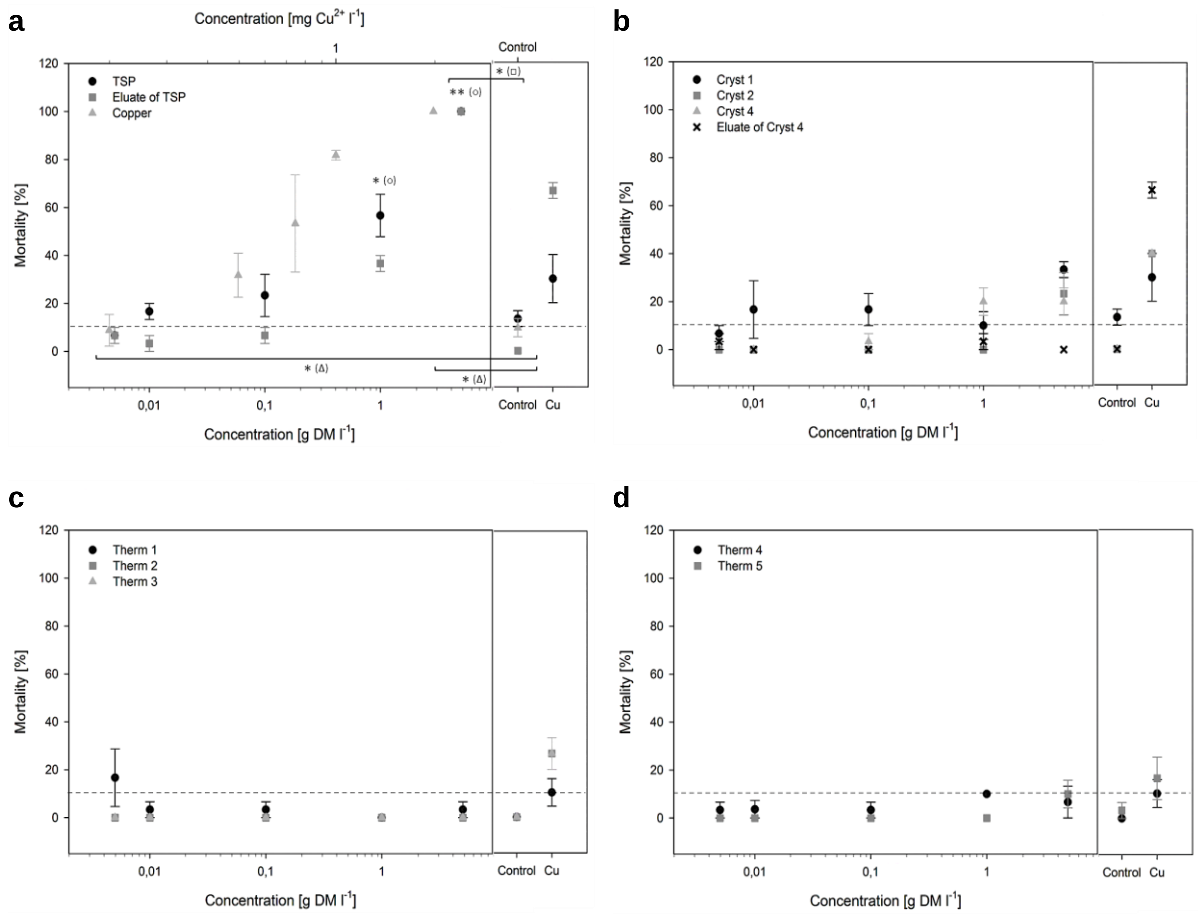

Figure 3.2 **a** Mortality of *G. fossarum* after 4 days of TSP, the eluate of TSP and copper (mean value, standard error); three replicates per treatment with ten gammarids (except copper with six replicates of two test set-ups); Cu^{2+} 500 µg l^{-1}; dry matter (DM). Two data points per each TSP concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey (TSP (1 g DM l^{-1} differs significantly from other concentrations except Cu)) **b** of Cryst 1, 2 and 4 and the eluate of Cryst 4 (mean value, standard error); three replicates per treatment with ten gammarids; Cu^{2+} 500 µg l^{-1}; dry matter (DM). Four data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey **c** of Therm 1–3 (mean value, standard error); three replicates per treatment with ten gammarids; Cu^{2+} 500 µg l^{-1}; dry matter(DM). Three data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey **d** of Therm 4–5 (mean value, standard error); three replicates per treatment with ten gammarids; Cu^{2+} 500 µg l^{-1}; dry matter (DM). Two data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey

Table 3.5 No observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) of the direct assessment of the reference phosphate fertiliser TSP, the phosphate recyclates and the eluates of TSP and Cryst 4 for all measured parameters of the *Lemna* sp. growth inhibition test, the gammarid acute toxicity test and the avoidance behaviour of *Eisenia* (one-way ANOVA/Kruskal-Wallis and pairwise Tukey), and the maximum application amounts of the phosphate products in agriculture (worst-case scenario, calculation can be extracted from discussion section 3.5.2)

Test organism and parameter		NOEC/LOEC (g DM l^{-1}/g DM kg^{-1})										
		TSP	TSP eluate	Cryst 1	Cryst 2	Cryst 4	Cryst 4 eluate	Therm 1	Therm 2	Therm 3	Therm 4	Therm 5
Max. application amount (g DM kg^{-1})		0.6	-	1.0	1.1	1.3	-	1.9	2.1	2.3	3.1	1.0
Lemna minor												
Growth inhibition	NOEC	1	-	0.1	5	5	-	5	0.1	0.1	5	≥10
	LOEC	5	-	1	10	10	-	10	1	1	10	>10
Colony break-up	NOEC	≥10	-	≥10	≥10	≥10	-	≥10	≥10	≥10	≥10	≥10
	LOEC	>10	-	>10	>10	>10	-	>10	>10	>10	>10	>10
Discolouration	NOEC	1	-	≥10	≥10	≥10	-	≥10	≥10	≥10	≥10	≥10
	LOEC	5	-	>10	>10	>10	-	>10	>10	>10	>10	>10
Weight decrease	NOEC	1	-	≥10	≥10	≥10	-	≥10	≥10	≥10	≥10	≥10
	LOEC	5	-	>10	>10	>10	-	>10	>10	>10	>10	>10
Root length decrease	NOEC	≥10	-	5	≥10	≥10	-	≥10	≥10	5	≥10	≥10
	LOEC	>10	-	10	>10	>10	-	>10	>10	10	>10	>10
Gammarus fossarum												
Mortality	NOEC	0.1	1	≥5	≥5	≥5	≥5	≥5	≥5	≥5	≥5	≥5
	LOEC	1	5	>5	>5	>5	>5	>5	>5	>5	>5	>5
Movement decrease	NOEC LOEC	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Feeding decrease	NOEC	≥5	0.01	≥5	≥ 5	≥ 5	≥ 5	≥ 5	≥ 5	≥ 5	≥ 5	≥ 5
	LOEC	>5	0.1	>5	> 5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
Eisenia fetida												
Avoidance behaviour	NOEC	≥50	-	≥50	≥50	≥50	-	≥50	≥50	≥50	≥50	≥50
(g DM kg^{-1})	LOEC	>50	-	>50	>50	>50	-	>50	>50	>50	>50	>50

n.c. not calculated; - not tested

3.4.4 Avoidance test with the earthworm *E. fetida*

Higher concentrations of copper (250 mg Cu^{2+} kg^{-1}), TSP (25 and 50 g DM kg^{-1}), the crystallisation (5, 25 and 50 g DM kg^{-1}) and ash products (50 g DM kg^{-1} of Therm 1, 3 and 5) resulted in an avoidance behaviour above 80 %. Concentrations below or equal to 5 g DM kg^{-1} of TSP and the phosphate recyclates and 100 mg Cu^{2+} kg^{-1} did mostly not cause clear avoidance behaviour because some earthworms were partially also located in the contaminated sites. Therefore, lots of variations in the avoidance behaviour of *E. fetida* occurred (Figure 3.3a–d), and no NOEC/LOEC values could be calculated significantly. The NOEC/LOEC values of the avoidance behaviour of TSP and all phosphate recyclates were ≥50 and >50 g DM kg^{-1} (Table 3.5). The reference toxicant of the different treatments (250 mg Cu^{2+} kg^{-1}) had a negative effect on *E. fetida* and resulted in an avoidance behaviour of 70–100 % (except of Cu of Therm 1 with 60 %) (Figure 3.3a–d).

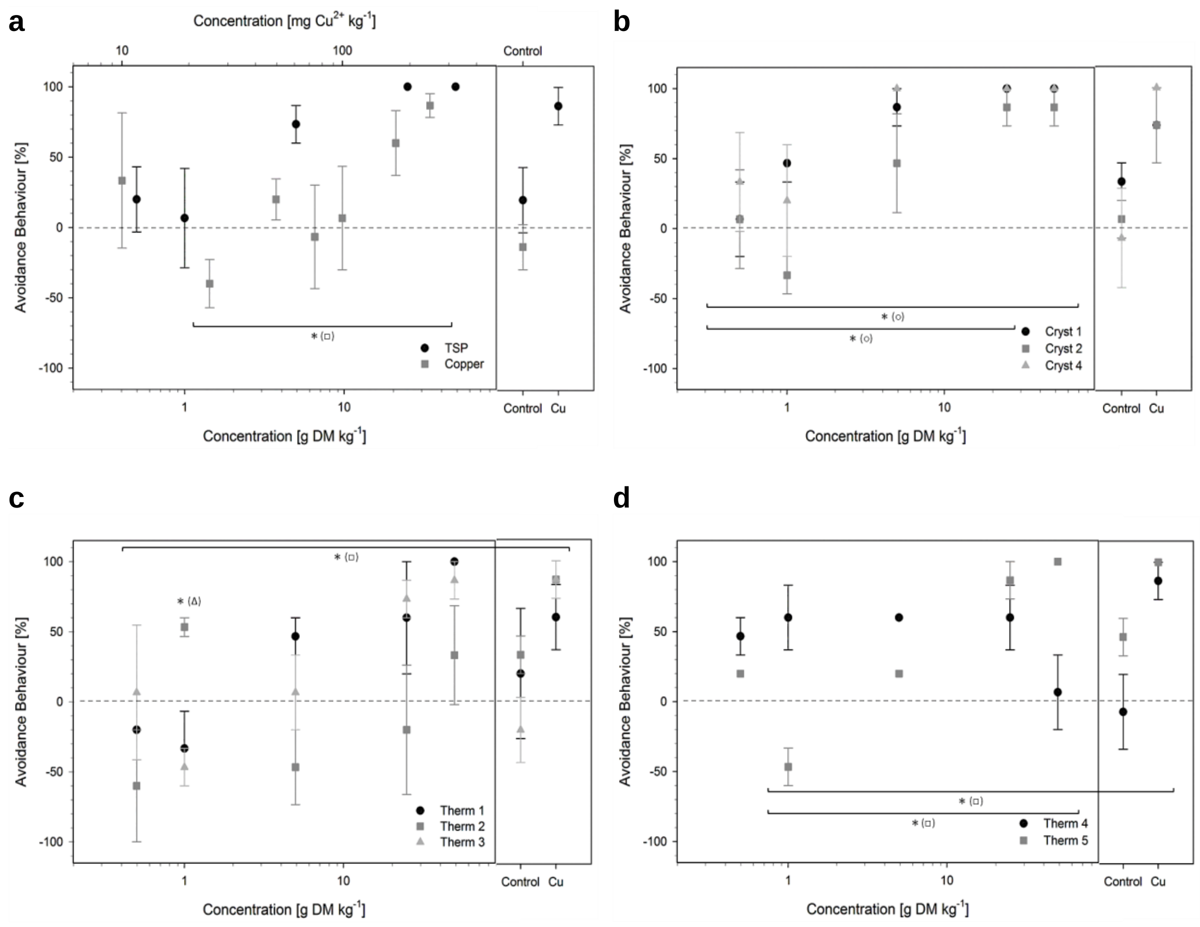

Figure 3.3 **a** Avoidance behaviour of *E. fetida* after 2 days of TSP and copper (mean value, standard error); three replicates per treatment with five earthworms (except copper with six replicates of two test set-ups); Cu^{2+} 250 mg kg^{-1}; dry matter (DM). One-way ANOVA/Kruskal-Wallis and pairwise Tukey **b** of Cryst 1, 2 and 4 (mean value, standard error); three replicates per treatment with five earthworms; Cu^{2+} 250 mg kg^{-1}; dry matter (DM). Three data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey **c** of Therm 1– 3 (mean value, standard error); three replicates per treatment with five earthworms; Cu^{2+} 250 mg kg^{-1}; dry matter (DM). Three data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey (Therm 3 (1 g DM kg^{-1} differs significantly from 25 and 50 g DM kg^{-1} and Cu)) **d** of Therm 4–5 (mean value, standard error); three replicates per treatment with five earthworms; Cu^{2+} 250 mg kg^{-1}; dry matter (DM). Two data points per concentration; one-way ANOVA/Kruskal-Wallis and pairwise Tukey

3.5 Discussion

3.5.1 Comparison of toxicity of reference phosphate fertiliser TSP and phosphate recyclates

The growth inhibition of *L. minor* of 5 and 10 g DM l^{-1} of TSP was twofold to fourfold higher than of the phosphate recyclates, whereas the lower concentrations of TSP had a lower effect on growth than the phosphate recyclates (0.01–1 g DM l^{-1}). Even 10 g DM l^{-1} of the phosphate recyclates caused a maximum growth inhibition of 49 % (Therm 3) (Figure 3.1a–d). The survival of *G. fossarum* in treatments of 1 and 5 g DM l^{-1} TSP was lower (<50 and 0 %) than in treatments of the same concentrations of the phosphate recyclates. The mortality of gammarids exposed to the crystallised PRs was mostly <20 % and to the ash products even <10 % (Figure 3.2a–d). The additionally investigated eluates of TSP and Cryst 4 had a lower or equal effect on the survival of the gammarids compared to the direct assessment of the DM of the two phosphate products (Figure 3.2a, b). The crystallisation products resulted in a higher avoidance behaviour of *E. fetida* at lower concentrations than TSP and the ash products. The crystallised PRs caused already an avoidance behaviour above 80 % from 5 g DM kg^{-1} (except of Cryst 4 ∼50 %). TSP had the same effect from 25 g DM kg^{-1} (but 100 % avoidance) and the thermally treated PRs only from 50 g DM kg^{-1} of Therm 1, 3 and 5 (Figure 3.3a–d). Based on these results, the reference phosphate fertiliser TSP was more toxic than the crystallised and thermally treated PRs at the higher concentrations in the tested concentration ranges of the three conducted ecotoxicological tests. The higher negative effects on the test organisms might be supported by the fact that TSP had higher concentrations of the heavy metals uranium (63 µg g^{-1} DM) and chromium (121 µg g^{-1} DM) than the phosphate recyclates (except of Therm 3 with 311 µg g^{-1} DM of chromium) (Table 3.3). Higher concentrations of the crystallised PRs resulted in a higher negative effect on (1) the root length of the duckweed and (2) the mortality, feeding and movement behaviour of the freshwater amphipod than the thermally treated PRs. Moreover, an obvious avoidance behaviour could already be detected at lower concentrations of the crystallisation products than of TSP and the ash products. The occurrence of organic pollutants (ng g^{-1} DM) could be one of the factors which contributed to the higher toxicity of the crystallised PRs compared to the thermally treated PRs (Table 3.3). The fact that the ash products partly contained the highest concentrations of heavy metals (Table 3.3) leads to the assumption that the existing compounds of pollutants had a lower bioavailability and consequently a lower toxic effect. Further, the higher water solubility of P_2O_5 of TSP (∼40 % higher; Table 3.3) might have caused the higher toxic effect on the survival, growth and behaviour of the test organisms in comparison to the chemical composition of pollutants. Simplício et al. (2017) analysed the toxicity of two different chemical forms of phosphate (KH_2PO_4, superphosphate $Ca(H_2PO_4)_2$) in two organisms from different trophic levels (snail, fish). In the treatments of KH_2PO_4 with a high solubility of P_2O_5, *Biomphalaria glabrata* was more sensitive to phosphate than *Danio rerio*. A toxic effect of superphosphate could only be observed at high concentrations because the phosphate fertiliser tends to precipitate with high calcium concentrations. In another study investigating potential effects of phosphate to aquatic organisms (fish, algae and cladocerans), also no toxicity could be determined because of the low solubility of phosphate in the tested limit assays of 100 mg l^{-1} tricalcium phosphate ($Ca_3(PO_4)_2$) and calcium hydrogenorthophosphate ($CaHPO_4$) (Kim et al. 2013). Maenpaa et al. (2002) could demonstrate a significant reduction in earthworm heavy metal bioavailability due to KH_2PO_4 and TSP phosphorus amendments to soil at the level of 5 g kg^{-1}. Phosphate anions also inhibited arsenic (As) uptake by reducing As bioavailability or by competing for cellular transport carriers of the earthworm *E. fetida* (Lee & Kim 2008) and of the aquatic macrophyte *Spirodela polyrhiza* (Rahman et al. 2008). So, the interaction of phosphate and the pollutants mostly determine the ecotoxicity of the analysed phosphate products.

Within the required ecotoxicological tests for covering all of the phosphate product affected compartments (soil, water and sediment), the main parameters of the *Lemna* test and the *Gammarus* test showed a similar sensitivity by evaluating the toxicity of the phosphate recyclates (growth inhibition, mortality). Admittedly, the growth inhibition of the *Lemna* test resulted partially in 10 to 20 % higher effects at the same concentrations of the phosphate recyclates, but the *Gammarus* test is shorter in time (4 vs. 7 days) (Figures 3.1a–d & 3.2a–d). Growth and the additional parameters root length and biomass of *L. minor* can be generally regarded as equivalent considering the measured effects (Figure 3.1a–d, Table 3.4). Discolouration and colony break-up of *L. minor* caused no obvious results in testing the phosphate recyclates, but the copper reference successfully affected the number of discoloured and single leaves of the duckweed. The feeding behaviour of *G. fossarum* appears to be the most sensitive parameter at all because even the lower tested concentrations caused up to 80 % decrease of feeding. Due to limited data, no clear statement about the movement behaviour of *G. fossarum* can be made in this context (Table 3.4). Further, the earthworm avoidance test (*E. fetida*) provided no obvious results at concentrations below 5 g DM kg^{-1} most likely due to a response effect (behaviour) at a fixed endpoint (after 2 days) (Figure 3.3a–d).

3.5.2 Realistic application amounts and associated risks

The maximum agronomical relevant application amounts of the phosphate recyclates were calculated for evaluation of potential effects of the samples on the field. The calculation was based on the maximum amount of P_2O_5, which is given in recommendations in Germany for very low P content soil of grassland which has a high P demand (220 kg P_2O_5 ha^{-1}, worst-case scenario) (KTBL 2005). For the conversion of the area value into the weight of soil of the agricultural area, the affected depth (5 cm) of the soil and the area has to be multiplied by the density factor of the soil (1.5 g cm^{-3}) (OECD 2000a). One hectare equals 750 t of soil which results then again in 0.3 g P_2O_5 kg^{-1}. The maximum output concentrations (g DM kg^{-1}) in Table 3.5 were calculated by dividing the maximum P_2O_5 concentration in soil by the total P_2O_5 content in the dry matter of each phosphate product (Table 3.3). The lowest calculated maximum agronomical relevant concentration was 0.6 g DM kg^{-1} of TSP, followed by the crystallised PRs (1.0–1.3 g DM kg^{-1}) and the thermally treated PRs (1.9–3.1 g DM kg^{-1}, except of Therm 5 with 1.0 g DM kg^{-1}) (Table 3.5). The direct comparison of the maximum output concentrations and the effects of the main parameters of the ecotoxicological tests revealed the following results. Relevant concentrations of the phosphate recyclates and TSP would probably not result in an obvious avoidance behaviour (>80 %) of the earthworms under laboratory conditions (Figure 3.3a–d). If an amount of the output concentration of TSP (0.6 g DM kg^{-1}) reaches surface waters by accident, erosion or leakage, the survival of *G. fossarum* could be affected negatively (Figure 3.2a), whereas amounts of the crystallisation and ash products would have no effect on the survival of *G. fossarum* (Table 3.5, Figure 3.2b–d) and minor effects on the growth of *L. minor* (<30 %, Figure 3.1b–d). TSP would probably not affect the growth of the duckweed considering the agronomical applied concentration (Figure 3.1a).

Moreover, the mandatory threshold values of heavy metals (As, Pb, Cd, Cr, Ni, Hg) for fertilisers of the DüMV were validated (DüMV 2012). All heavy metals in the crystallisation products were below the threshold values (Table 3.3). Therm 1 (raw ash) exceeded the values of Pb (150 vs. 228 mg kg^{-1} DM) and Cd (1.5 vs. 6.9 mg kg^{-1} DM). The Cd values of Therm 2 & 3 and TSP (1.9, 4.3 and 4.2 mg kg^{-1} DM) did not comply with the threshold value either. Further, the Ni concentration of Therm 3 was above the threshold value (80 vs. 343 mg kg^{-1} DM; Table 3.3) (DüMV 2012). According to the fertiliser ordinance exceeded copper, chromium (total), iron and zinc concentrations need to be labelled and may only be used for special applications (e.g. without soil) (DüMV 2012). In comparison to three different analysed sewage sludges having concentrations below the DüMV thresholds (Chapter 2; Rastetter & Gerhardt 2017), the crystallised PRs had mostly lower and the thermally treated PRs often much higher heavy metal concentrations (Table 3.3). In spite of the different heavy metal contents, concentrations between (1) 1.2–9.6 and (2) 0.5–1.6 g DM l^{-1} of the sewage sludges resulted in (1) 50 % of growth inhibition of *L. minor* and (2) 50 % mortality of *G. fossarum* (Chapter 2; Rastetter & Gerhardt 2017). The highest tested concentrations of the phosphate recyclates (10 and 5 g DM l^{-1}) had a maximal effect of ∼50 % growth inhibition and ∼30% mortality (see result section 3.4). The behaviour of the earthworms was also more affected by the non-dewatered sludge (S1) which caused 50 % effect at a concentration of 0.4 g DM kg^{-1} (Chapter 2; Rastetter & Gerhardt 2017). An obvious avoidance behaviour (>80 %) caused by the phosphate recyclates started first at concentrations of 5 g DM kg^{-1} and higher (Figure 3.3b–d). Regarding the agronomical relevant application amount of the non-dewatered sludge (S1 3.3 g DM kg^{-1}), the tested soil invertebrates (*E. fetida*) and freshwater organisms (*L. minor* and *G. fossarum*) will be negatively influenced (Chapter 2; Rastetter & Gerhardt 2017).

Within the EU Project P-REX, a quantitative risk assessment of the PR pollutants for environment/human and a relative risk ranking for the different pollutants between the phosphate recyclates was performed (Kraus & Seis 2015). Kinetic models according to the Technical Guidance Document on risk assessment (IHCP 2003) and a solute transport model (HYDRUS) were used to estimate the exposure of single pollutants of the phosphate recyclates to topsoil and leachate to quantify potential risks for the endpoints humans, soil organisms and groundwater (predicted environmental concentration (PEC)). Further, the predicted no effect concentrations (PNECs) of the single pollutants for the endpoints were determined. The risk ratios of the phosphate recyclates calculated from quotient PEC to PNEC were in the same magnitude as the reference phosphate fertiliser TSP. Against the background of the made assumptions, there are no unacceptable risks caused by the organic substances, arsenic, chromium, copper, mercury, nickel or lead, whereas cadmium and zinc are hazards which are of concern in the phosphate recyclates (Kraus & Seis 2015). For the relative risk ranking focusing on the quality of the phosphate recyclates, an assessment based on phosphorus specific hazard loads and a comparison to diffuse discharge by atmospheric deposition into the ecosystem was conducted. By comparison, the crystallisation products (struvite) can be considered as high-quality phosphate recyclates. Struvite shows in general the lowest hazard concentrations related to the dry matter and has a high total phosphate content for a recycled fertiliser. For the ash products, an improvement regarding heavy metal depletion is in some cases advisable (Kraus & Seis 2015).

3.6 Conclusions

At the higher concentrations in the tested concentration ranges of the three conducted ecotoxicological tests, the reference phosphate fertiliser TSP was more toxic than the crystallised and thermally treated phosphate recyclates under laboratory conditions. Relevant concentrations of all phosphate recyclates and TSP might not have an acute toxic effect on the soil invertebrates (*E. fetida*), whereas TSP might negatively affect the survival of the freshwater amphipods (*G. fossarum*) and the phosphate recyclates might have minor effects on the growth of *L. minor*.

Considering the comparison with the analysed sewage sludges and the quantification of potential hazards of phosphate recyclates within the project, application of crystallisation products (struvite) instead of sludges and TSP could reduce ecotoxicological effects and risks. So, phosphate recovery and recycling in agriculture, especially of struvite, should be followed up in future.

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Chapter 4

Continuous monitoring of avoidance behaviour with the earthworm *Eisenia fetida*

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Continuous monitoring of avoidance behaviour with the earthworm *Eisenia fetida*

4. 1 Abstract

Purpose The avoidance behaviour of earthworms is a functional ecotoxicological test parameter for the assessment of contaminated soil to examine its habitat function. But, the endpoint analysis of behavioural parameters leaves questions unanswered about changes within the test period and its optimal duration. Therefore, a permanent, automated and non-optical method based on the Multispecies Freshwater Biomonitor® (LimCo International GmbH) for monitoring the avoidance behaviour of earthworms individually was developed.

Materials and methods For an optimal experimental set-up of the biomonitor, the signal pattern of a clear movement of an earthworm (*Eisenia fetida*), the chamber design (paired chambers), potential artefacts, the effect of the electrical field (impedance technology) and the optimal measurement period were determined. To analyse the avoidance behaviour over the time, the movement of the earthworms was monitored in contaminated treatments of a heavy metal (Cu) and a conventional phosphate fertiliser (TSP) in paired chambers with the biomonitor.

Results and discussion Signal pattern with frequencies between 0 and 1 Hz characterising the movement behaviour (locomotion) of *E. fetida* could be detected within the single biomonitor measurements. So, a clear statement about the location of the worm in the experimental set-up (paired chambers) could be made. By comparing different exposure times of contaminated treatments (Cu, TSP) and the control of avoidance measurements with the biomonitor, the test duration could be reduced from 48 to 16 h. The measured avoidance behaviour over time of different concentrations of Cu and TSP resulted in equivalent effects compared to the standard avoidance test. On the basis of the measured data, at least 50 % of the avoidance behaviour over time is proposed to regard a treatment as toxic.

Conclusions The new experimental set-up for analysing the avoidance behaviour of *E. fetida* permanently could be recommended as an ecotoxicological screening tool for the assessment of the habitat function of contaminated soils and for evaluating the terrestrial toxicity of particular contaminants.

Keywords Earthworm • Avoidance behaviour over time • Biomonitoring • Ecotoxicological screening test

4.2 Introduction

To obtain information about the potential effects of contaminants in soil, ecotoxicological test systems are proposed to complement conventional chemical analysis. Considering the assessment of the habitat function of soil, terrestrial test systems with earthworms as representatives of the soil fauna can be used. Existing standardised laboratory test systems with the earthworms *Eisenia fetida*/*E. andrei* are mortality tests (OECD 1984; ISO 2012a) combined with sublethal effects, e.g. biomass weight loss (EPA 2012), a bioaccumulation test (OECD 2010), reproduction tests (ISO 2012b; OECD 2016) and an avoidance test (ISO 2008). Also, Environment Canada (2007) and ASTM International (2009) provide documents with similar ecotoxicological test methods and endpoints. Further, the International Organisation for Standardisation (ISO) specifies methods and techniques for sampling earthworms from field soils as bioindicators and for determining the effects of substances on earthworms in the field (ISO 2006, 2014). The reproduction test was implemented to detect effects resulting from sublethal concentrations of toxicants under laboratory conditions and to obtain information on environmental effects (ISO 2008). However, the reproduction test has a long test period with 56 days and is very labour-intensive by determining the number of juveniles. Later on, the standardised avoidance test based on a laboratory comparison test with eight contaminated soils in three laboratories (Hund-Rinke et al. 2003) has been developed in two different designs: a two-section and a six-section unit (ISO 2008). The earthworms are allowed to choose the compartment, control and a treatment. At the end of the test period, the location and the number of worms in each section are determined (ISO 2008). The avoidance test was evaluated as sensitive as the reproduction test, but within a shorter test period (Hund-Rinke et al. 2003). Since the availability of the earthworm avoidance test method, first performed by Yeardley et al. (1996), many research experiences have been gained: with contaminated soils (Hund-Rinke et al. 2003; Natal-da-Luz et al. 2004) as well as with soils spiked with different single substances (heavy metals, pesticides, etc.) (Marques et al. 2009; Shoults-Wilson et al. 2011; Santos et al. 2012) or substance mixtures (biosolids, fertilisers, etc.) (Artuso et al. 2011; Matos-Moreira et al. 2011) and/or under different test conditions (e.g. tropical conditions (Garcia et al. 2008), in a vertical distribution (Amaro et al. 2016), different soil textures and organic matter content (Natal-da-Luz et al. 2008b) or different exposure times (Natal-da-Luz et al. 2008a; Frankenbach et al. 2014)). On the other hand, no research about the test method was concerned with observations that behaviour patterns like avoidance (the analysed endpoint) could constantly change over time. Previous studies about permanent monitoring of the behaviour of earthworms are generally based on optical methods to analyse food preferences or dispersal on and in soil or mating behaviour (Nuutinen & Butt 1997; Valckx et al. 2010; Caro et al. 2012; Griffith et al. 2013; Rajapaksha et al. 2013). For teaching purposes, Wilson & Johnson (2016) developed a running wheel for continuously analysing the movement of earthworms or as a result of stimuli by video recording or automated detection using infrared sensors. To investigate the locomotory behaviour of aquatic oligochaetes, a non-optical monitoring method (Multispecies Freshwater Biomonitor® (MFB), LimCo International GmbH) was applied (Gerhardt 2007, 2009; Sardo et al. 2007). The objective of this research is to develop a permanent, automated and non-optical monitoring method in soil based on the MFB for analysing the avoidance behaviour of earthworms individually and over time instead of a fixed point in time to obtain more sensitive results on the basis of time-to-effect data.

4.3 Materials and methods

4.3.1 Development of an optimal experimental set-up

Signal pattern of earthworm E. fetida

For measuring the locomotion and avoidance behaviour of the earthworms quantitatively, the Multispecies Freshwater Biomonitor® (MFB, LimCo International GmbH, Germany), an automated biomonitor system based on quadrupole impedance technology (Gerhardt et al. 1994) and cylindrical measurement chambers (*l* 8 cm, *d* 2 cm) with 4 stainless steel electrode plates were used for the first time. Whereby, one pair (plates located opposite) generated the current and the other pair detected the changes in the electrical field due to the resistance of the organism within the field (impedance). Lids with plankton net (mesh size 100 µm) were applied to enable a permanent air exchange and to prevent the worms from escaping. The *E. fetida* (Savigny 1826, Oligochaeta, Lumbricidae) cultures (worm farm Nassenheide, Germany) were held and raised in two darkened boxes used for (1) reproduction filled with 100 % peat substrate and (2) acclimation filled with peat, LUFA 2.3 soil (standard soil, LUFA Speyer, Germany) and compost of unloaded green waste (VDLUFA quality seal certification, composting plant, Singen, Germany). The worms were fed with horse manure (every 2 months with about 1 kg per culture box), held under humid conditions and at room temperature during cultivation and acclimation. Subadult worms (without a clitellum) with a length of about 3 cm were chosen for measuring to enable the worms moving about freely and stretching fully horizontally in the used chambers. For determination of a signal pattern of

E. fetida in the biomonitor, single earthworms (n = 24) were measured for 24 h in measurement chambers loosely filled with 12–14 g (depending on the structural composition of the compost) of a moistened 50/50 mixture of LUFA 2.3 soil and compost of unloaded green waste (∼30 % of the water holding capacity (ISO 2009); T = 20±3 °C). The addition of compost to LUFA 2.3 was conducted to obtain loosened soil with more hollows (Chapter 2; Rastetter & Gerhardt 2017). The movement of the earthworm was recorded in intervals starting every 10 min with 4-min measurements (with system adjustments of a noise level of 150 mV and a threshold of 170 %). During the measurement period, the chambers were in a completely darkened container to avoid an impact of the light on the movement behaviour of the earthworms (negative phototaxis; Smith 1902; Doolittle 1971) and were kept under moist conditions with 100 % relative humidity. After the measurement, the survival of the worms was checked visually. Moreover, the same measurements were conducted in contaminated soil (100 g DM kg^{-1} of triple superphosphate TSP, conventional phosphate fertiliser), manually mixed, to analyse differences of the signal pattern of earthworms under stress in comparison to the control worms (n = 16). The raw monitoring signals were analysed with the Fourier frequency transformation (FFT) (Gerhardt et al. 1998) to obtain the abundance of different frequencies between 0 and 8.5 Hz within the 4-min measurement (17 frequency intervals in steps of 0.5 Hz).

Determination of measurement artefacts

For determination of potential measurement artefacts, single (n = 24) and paired chambers (n = 24) of the biomonitor, which were only filled with the moistened soil mixture, were measured for 24 h (parameters and system adjustments see method section *Signal pattern of earthworm E. fetida*). The paired chambers (experimental set-up, Figure 4.1) were joined together by a connector with a small, closable hole at the top. Between the two chambers, a distance of 0.5 cm was applied to ensure the possibility for inserting an earthworm centrally and to avoid mutual interferences between the chambers. The used filling quantity of the mixed soil in the paired chambers was in total 25–29 g (12–14 g per each chamber and ∼1 g per the space between).

Effects of the electrical field on the survival of the earthworms

Twenty-four paired chambers (Figure 4.1) were filled with the moistened soil mixture, and per each set-up, an earthworm was inserted centrally through the hole between the two chambers. The worms were measured (parameters and adjustments see method section *Signal pattern of earthworm E. fetida*). and thus exposed to the electrical field for 48 h corresponding to the standard avoidance test duration (ISO 2008). After measuring the earthworms were directly checked visually and put in darkened plastic boxes (PP, 17 × 15 × 9 cm) filled with 500 g of the moistened soil mixture and a small amount of horse manure as food (8 worms per box). After 1 week, the worms were checked visually once more for survival and behavioural changes.

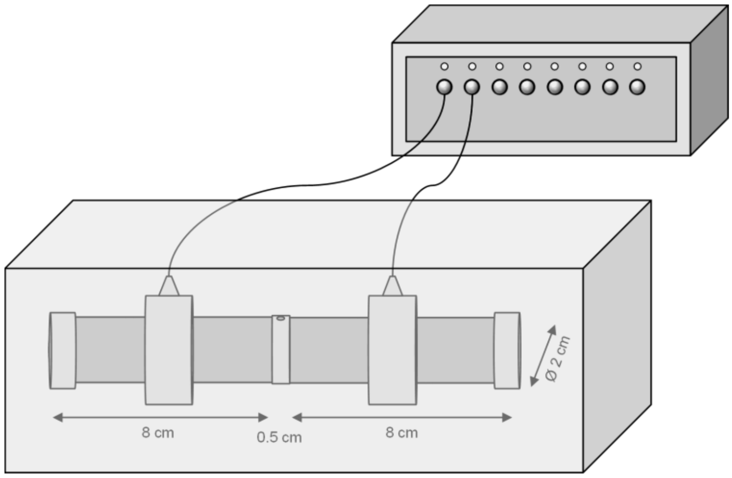

Figure 4.1 Experimental set-up – two MFB measurement chambers, joined together by a connector with a small, closable hole at the top for inserting an earthworm centrally, placed in a completely darkened and kept moistened chamber and connected to the MFB and a computer

Determination of an optimal measurement period

After four different points in time (8, 16, 24 and 44 h), the avoidance behaviour of the earthworms in a control and a contaminated experimental set-up was determined and compared. For the control set-up, both chambers (Figure 4.1) were filled with the humid control soil mixture (n = 20). For the contaminated set-up, high concentrations of copper ($CuCl_2$ 1000 mg Cu^{2+} kg^{-1}, added as water solution) and of the conventional phosphate fertiliser, triple superphosphate (TSP 100 g DM kg^{-1}, added as dry matter DM), were applied. The substances were chosen based on high avoidance behaviour of the earthworms in the regular standard test (Chapter 3; Rastetter et al. 2017). For that, one chamber was charged with the manually mixed, contaminated soil mixture (12– 15 g), and the other chamber was filled with the humid control soil mixture (n = 20). The earthworms were inserted centrally, the hole was closed and the worms were measured for 44 h (considered as equivalent to the standard test duration of 48 h). The same parameters and system adjustments as in the method section *Signal pattern of earthworm E. fetida* were used. After the measurement, three different calculation methods for analysing the avoidance behaviour of the different points in time were conducted and the number of changeovers was analysed by using the biomonitor signals after the FFT (see Sect. 4.3.3).

4.3.2 Monitoring of the avoidance behaviour of *E. fetida*

The avoidance behaviour of single earthworms (n = 15) in a control and contaminated experimental set-up with six different concentrations of the analysed contaminants, Cu and TSP, in the humid soil mixture was assessed after a reduced test duration of 16 h (see result section *Determination of an optimal measurement period*) (Cu 10, 50, 100, 175, 250 and 1000 mg Cu^{2+} kg^{-1}; TSP 0.5, 1, 5, 25, 50 and 100 g DM kg^{-1}). The chambers of the experimental set-up (Figure 4.1) were filled and the worms inserted according to the method section *Determination of an optimal measurement period*. An overview of the applied adjustments and parameters for the final monitoring set-up can be seen in Table 4.1. For analysing the avoidance behaviour at the test end, the same methods as in the method section *Determination of an optimal measurement period* were used. The test was invalid according to the standard test (ISO 2008) if the number of dead (or missing) worms was >10 % per treatment at the test end. Dead worms were not included to the assessment.

Table 4.1 Overview of the applied system adjustments of the biomonitor and the test parameters finally used for the analysis of the avoidance behaviour of *E. fetida*

Test organism	*E. fetida* (subadult, length 3 cm)
System adjustments	
Settings	Noise level 150 mV, Threshold 170 %, Band 1 0–1 Hz
Recording	Every 10 min with 4 min measurement
Experimental set-up	Chambers (*l* 8 cm, *d* 2 cm) joined together by a connector (distance 0.5 cm) with a hole at the top; lids with plankton net (mesh size 100 µm); filling quantity 12–14 g per chamber an ~1 g per space between
Test parameters	
Exposure medium	50 % LUFA standard soil 2.3/50 % compost (moistened)
Exposure period	16 hours
Number of organisms	15 (measured individually)
Temperature	20±3 °C
Light	Without light
Relative humidity	100 %
Endpoints	Avoidance behaviour over the time (Percentage effect (ISO 2008), Worms in control chamber (Hund-Rinke & Wiechering 2001))

4.3.3 Data analysis

For analysing the avoidance behaviour by using the measurement data of the biomonitor, band 1 (average of the frequency interval of 0–0.5 and 0.5–1 Hz, see result section *Signal pattern of earthworm E. fetida*) of each 4-min measurement was subtracted with 30, because only values higher than 30 % of band 1 were determined as a clear movement signal of a worm (see result section *Signal pattern of earthworm E. fetida*). Resulting negative values were set to zero. The measurement datasets were separated into time periods of 4 h with 24 measured values per chamber of an experimental set-up (paired chambers) and were analysed with the nonparametric Wilcoxon signed-rank test (Sigma Plot, Systat Inc.). Significant results ($p < 0.05$) led to the location of the worm, in which chamber the worm was situated in the experimental set-up. Non-significant results could be explained by the fact that either worm moved barely (see result section *Signal pattern of earthworm E. fetida*) or was located equally in both chambers over the 4 h (generally in measurements of the control or low concentrations). For each period of 4 h within the different measurement periods (8, 16, 24 and 44 h) of the method section *Determination of an optimal measurement period* and *Monitoring of the avoidance behaviour of E. fetida*, the significant location of the worm and additionally the duration of avoidance or attraction were determined. If no significant location was measured within the 4 h, the significant location of the previous time period could be used, because no significant changeover of the worm was detected within the time period. Worms were counted as 0.5 to each chamber if there was no obvious assignment (significant location) at all within the analysed time period.

For the calculation of the percentage avoidance behaviour x_t of a single worm over the time, the duration of avoidance/attraction was compared to the total analysed time ($x_t = (t/T) \times 100$). For the percentage effect (x avoidance), the total number of worms in the contaminated chambers n_t was compared to the total number of worms in the control chambers n_c according to the equation ($x = ((n_c - n_t)/N) \times 100$) of ISO 17512–1 (2008). The percentage of worms x_c in the control chamber was calculated ($x_c = (n_c/N) \times 100$) and regarded as toxic if the control soil mixture contained over 80 % of the worms as described by Hund-Rinke & Wiechering (2001). For the analysis of the number of changeovers, significant changes of the location of the worms over the time were counted. The percentage avoidance behaviour over the time of Cu and TSP at the different points in time compared to the control were analysed with the nonparametric Mann-Whitney rank sum test (Sigma Plot, Systat Inc.). The analysis of the percentage avoidance behaviour of the different concentrations of copper and TSP compared to the control was conducted by the nonparametric Kruskal-Wallis test followed by a multiple comparison versus the control group, the Dunnett's method (Sigma Plot, Systat Inc.). Significances were marked in the graphs by asterisks (* p <0.05, ** p <0.01 and *** p <0.001).

4.4 Results

4.4.1 Development of an optimal experimental set-up

Signal pattern of earthworm E. fetida

After FFT analysis, the movement behaviour (locomotion) of *E. fetida* was characterised by frequencies between 0 and 1 Hz and summarised in band 1. The frequency pattern of the intervals of 0–0.5 and 0.5–1 Hz occurred most frequently and with the highest abundance within the measurements. An example of a raw signal of the locomotion of one single worm in a 4-min measurement with the corresponding FFT signal can be seen in Figure 4.2a, b. The movement of an earthworm represented by band 1 in control soil over 24 h and a therein contained potential activity break (locomotion stop, ~3 h) are shown in Figure 4.2c. Values above 30 % of band 1 in the 4-min measurement period were determined as a clear movement signal of one worm in a chamber and occurred during the measurements over 24 h with an averaged abundance of 46 %. Whereby, 54 % of the 4-min measurement periods on average were below 30 % of band 1, which means that some earthworms moved barely in general (e.g. only 1.5 h of clear movement signals during 24 h) or had obvious activity breaks within the measurements (see above). The same set-up with contaminated soil showed an equal signal pattern of frequencies to the control measurements until the worms died. In few cases the percentage of movement (band 1) decreased over a time before the worms died (see exemplary Figure 4.2d). In the control measurements, all of the worms were alive, whereas all the worms in the contaminated soil were dead.

Determination of measurement artefacts

In the measurements of the soil-filled single and paired chambers without earthworms, the signal pattern of artefacts had either a smaller abundance of the relevant frequency intervals (0–0.5 and 0.5–1 Hz; band 1 <30 %) or an abundance of irrelevant frequency intervals (>1 Hz, Figure 4.3a, b) within the 4-min measurements compared to a clear earthworm signal pattern (Figure 4.2b). Values below 30 % of band 1, artefacts or very less active earthworms, within the 4-min measurements were eliminated before data analysis (see method section 4.3.3). Consequently, occurring artefacts could be regarded as negligible.

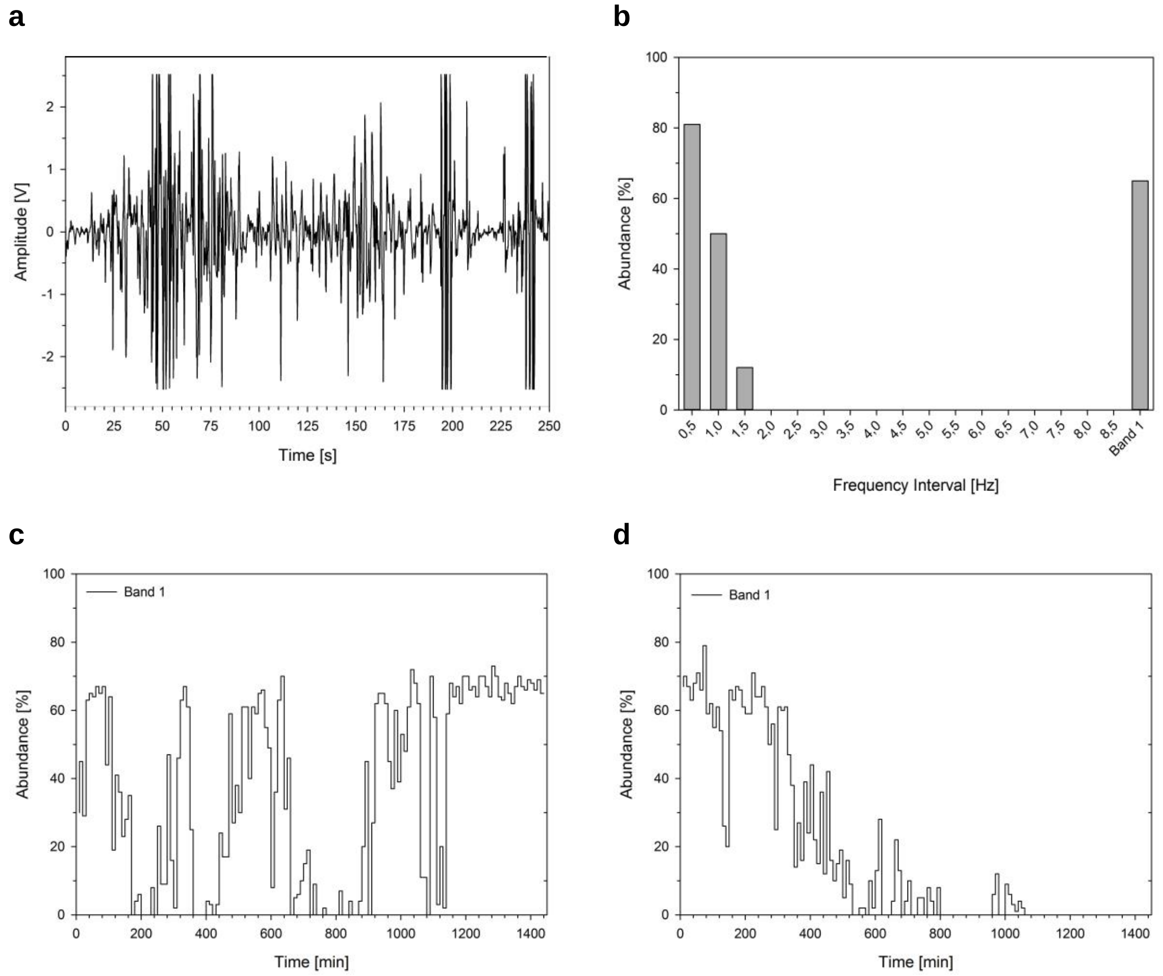

Figure 4.2 **a** Example of a raw signal of *E. fetida* of one measurement interval in a single soil-filled chamber (4 min) **b** Abundance of different frequency intervals of the raw signal (Figure 4.2a) after a Fourier frequency transformation (FFT) and band 1 (mean value of the frequency intervals from 0 to 1 Hz) **c** Example of the movement of *E. fetida* (abundance of band 1, mean value of the frequency intervals from 0 to 1 Hz) in control soil over 24 h **d** Example of the movement of *E. fetida* (abundance of band 1, frequencies from 0 to 1 Hz) in contaminated soil (100 g DM kg^{-1} of triple superphosphate TSP) over 24 h

Effects of the electrical field on the survival of the earthworms

The survival of all worms was not negatively affected by the electrical field of the biomonitor. Directly after the measurement and also after 1 week of recovery, the worms were lively and had no obvious changes in behaviour/movement, nor increased mortality. Moreover, biomonitor measurements of organisms in water (fish, Crustacea) showed that neither the measured behaviour was significantly affected by the electrical field nor preferred the organisms a chamber in an on/off experiment (Craig and Laming 2004; Kirkpatrick et al. 2006; Stewart et al. 2010).

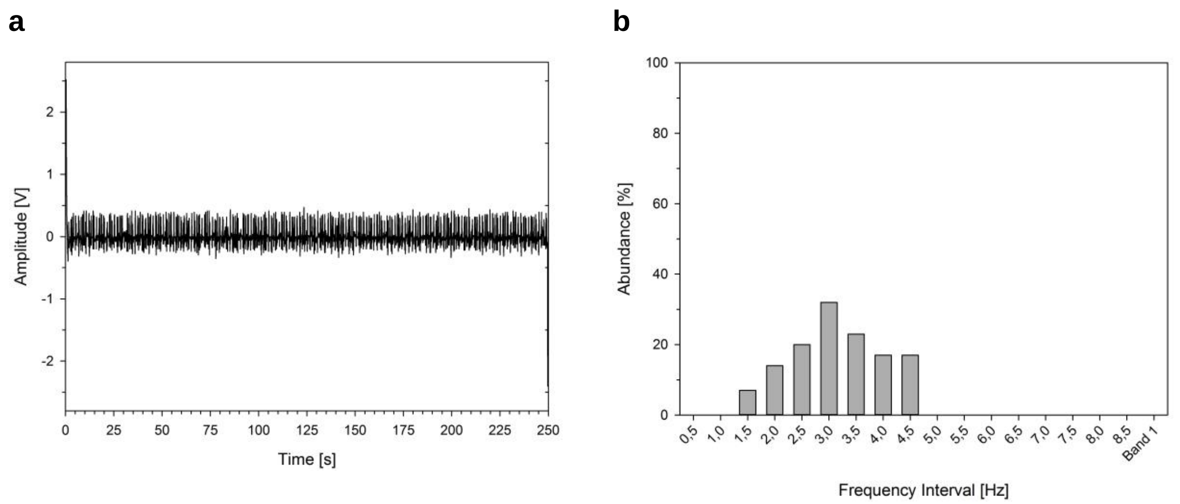

Figure 4.3 a Example of a raw signal of an artefact of one measurement interval in a soil-filled chamber without an earthworm (4 min) **b** Abundance of different frequency intervals of the raw signal (Figure 4.3a) after a Fourier frequency transformation (FFT) and band 1 (mean value of the frequency intervals from 0 to 1 Hz)

Determination of an optimal measurement period

After 8 h of measuring, the earthworms of the contaminated treatments showed a significant higher activity in the control chambers compared to the control treatments, resulting in a higher avoidance behaviour over time (Cu $p = 0.001$, TSP $p = 0.010$; Figure 4.4a). Highly significant differences of the avoidance behaviour over the time could even be measured after 16 h (Cu $p < 0.001$, TSP $p < 0.001$) and continued for the following points in time (24, 44 h) (Figure 4.4b–d). Above 50 % avoidance over the time was analysed for Cu (63 %) and TSP (61 %) after 16 h (Table 4.2). After 16 h, the calculated avoidance behaviour according to ISO (2008) was ≥75 % for copper and 85 % for TSP. In comparison to the following points in time, a maximum increase of 15 % of the ISO avoidance could be reported, whereas after 8 h a difference up to 45 % was analysed (TSP 40 %) (Table 4.2). The analysed percentage of worms in the control chamber of measurements with Cu and TSP exceeded the 80 % limit which was regarded as toxic after 16 h of measuring (Table 4.2). Most of the worms measured in the contaminated set-up already decided after 16 h to enter the control chamber and to stay there (averaged number of changeovers; Cu 0.05, TSP 0.2). After 8 h, almost no significant changeovers were reported in the different measurements (control 0.1, Cu 0, TSP 0.05). A minimal increase of changeovers in the Cu and TSP measurements could be detected after 44 h (Cu 0.35, TSP 0.3), whereas the changeovers in the control increased up to an average of 1.1 (highest analysed number of changeovers 4). Due to the highly significant differences of the contaminants to the control, the high effect of avoidance according to ISO (2008) and

Hund-Rinke & Wiechering (2001) and the significant decision for the control chamber after 16 h, the test duration could be reduced from 48 (standard test duration) to 16 h.

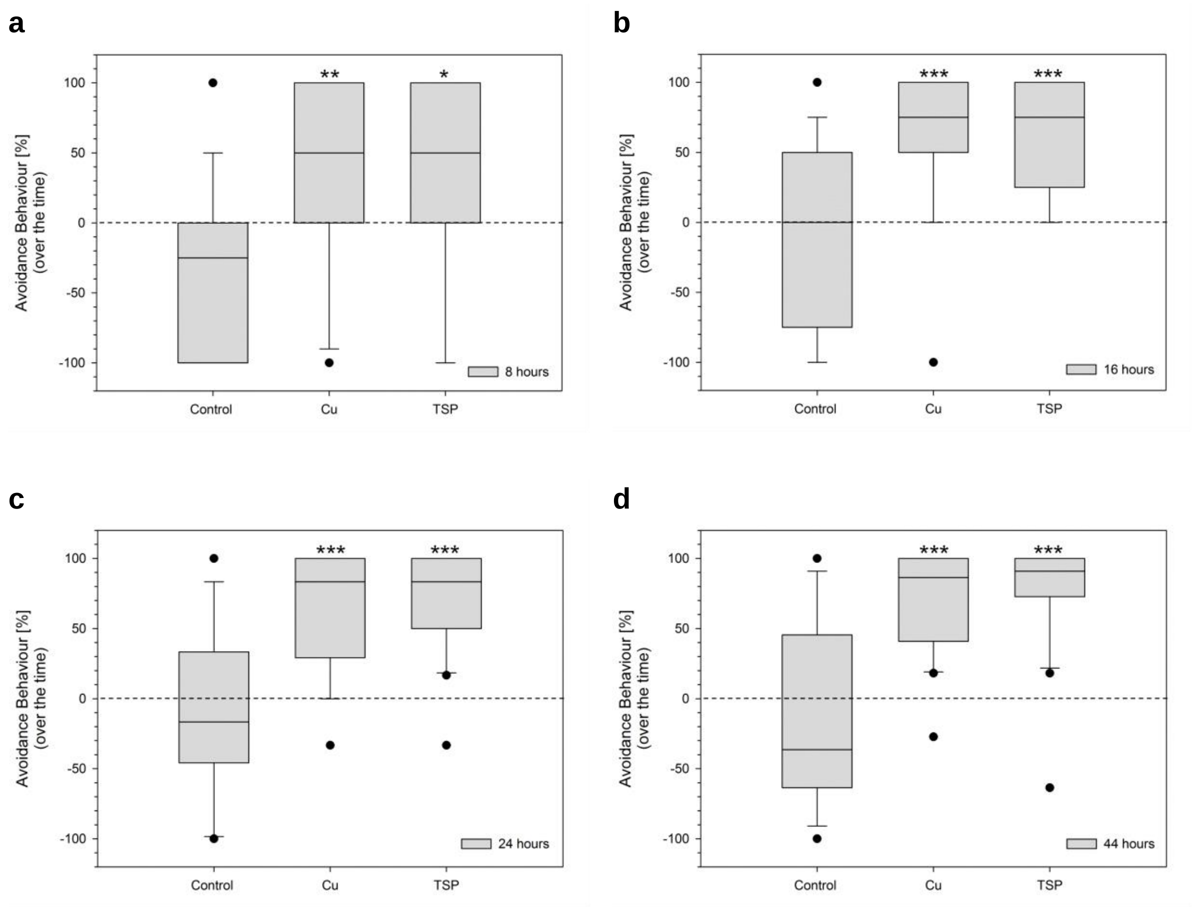

Figure 4.4 Avoidance behaviour over the time [%] of *E. fetida* (n = 20) individually measured in the biomonitor (Cu 1000 mg Cu^{2+} kg^{-1}, TSP 100 g DM kg^{-1}) of the different points in time, Mann-Whitney rank sum test (comparison to the control group), dry matter (DM) **a** 8 h **b** 16 h **c** 24 h **d** 44 h

4.4.2 Monitoring of the avoidance behaviour of *E. fetida*

Starting from 175 mg Cu^{2+} kg^{-1} of copper and 25 g DM kg^{-1} of TSP, the analysed concentrations of the contaminants resulted in a significant increase of the avoidance behaviour over the time compared to the control ($p < 0.05$) (Figure 4.5a, b). The corresponding mean values of the avoidance over the time were above 50 % (except of 50 g DM kg^{-1} of TSP with 47 %) (Table 4.3). Concentrations below 175 mg Cu^{2+} kg^{-1} of copper and 25 g DM kg^{-1} of TSP did not cause an obvious avoidance behaviour over the time, according to ISO (2008) and Hund-Rinke & Wiechering (2001) because some earthworms were partially located in the contaminated chamber of the experimental set-up as well (Figure 4.5a, b and Table 4.3). The calculated percentage effect (avoidance, ISO) of the significant concentrations of Cu and TSP was >70 % (except of 50 g DM kg^{-1} of TSP with 60 %) (Table 4.3). In the same treatments, 80 % and more of the worms were consequently located in the control site of the paired chambers after 16 h (Table 4.3). No worms died during the measurements.

Table 4.2 Mean values of three different calculation methods for analysing the avoidance behaviour of *E. fetida* in the biomonitor (Cu 1000 mg Cu^{2+} kg^{-1}, TSP 100 g DM kg^{-1}) of the different points in time (n = 20); (1) percentage avoidance behaviour over the time, (2) percentage effect (avoidance) according to ISO 17512–1 (2008) and (3) percentage of worms in the control chamber (Hund-Rinke & Wiechering 2001)

	8 hours			16 hours			24 hours			44 hours		
	Control	Cu	TSP	Control	Cu	TSP	Control	Cu	TSP	Control	Cu	TSP
Avoidance behaviour over the time [%]	-28	45	30	-10	63	61	-8	67	70	-13	71	77
Percentage effect (avoidance) [%]	-30	55	40	-5	75	85	-15	80	90	-30	90	90
Worms in control chamber [%]	35	78	70	48	88	93	43	90	95	35	95	95

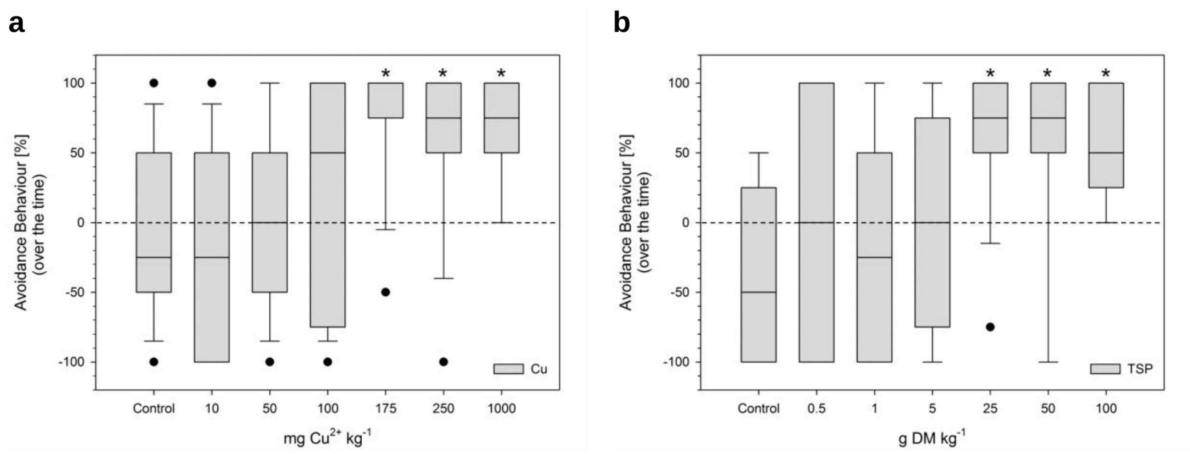

Figure 4.5 Avoidance behaviour over the time [%] of *E. fetida* (n = 15) individually measured in the biomonitor after 16 h of the different concentrations of **a** Cu and **b** TSP; Kruskal-Wallis test followed by a multiple comparison versus the control group (Dunnett's method), dry matter (DM)

Table 4.3 Mean values of three different calculation methods for analysing the avoidance behaviour of *E. fetida* in the biomonitor of different concentrations of copper and TSP after 16 h (n = 15); (1) percentage avoidance behaviour over the time, (2) percentage effect (avoidance) according to ISO 17512–1 (2008) and (3) percentage of worms in the control chamber (Hund-Rinke & Wiechering 2001) compared to the mean values of the standard test after 48 h (TSP, 3 replicates per treatment with 5 earthworms (n = 15); Cu, 6 replicates of 2 test set-ups (n = 30); (Chapter 3; Rastetter et al. 2017))

	Cu [mg Cu^{2+} kg^{-1}]							TSP [g DM kg^{-1}]						
	0	10	50	100	175	250	1000	0	0.5	1	5	25	50	100
Biosensor														
Avoidance behaviour over the time [%]	-3	-22	3	23	70	62	72	-32	-3	-20	-7	63	47	53
Percentage effect (avoidance) [%]	-20	-27	0	7	87	73	87	-20	-5	-15	-7	86	60	80
Worms in control chamber [%]	40	37	50	53	93	87	93	40	47	40	47	93	80	90
Standard test														
Percentage effect (avoidance) [%]	-13	33	20	7	60	87	100	20	20	7	73	100	100	100
Worms in control chamber [%]	43	67	60	53	80	93	100	60	60	53	87	100	100	100

4.5 Discussion

The results according to ISO (2008) and Hund-Rinke & Wiechering (2001) of the measurements in the biomonitor compared to the results of the standard earthworm avoidance test (conducted under the same test conditions (temperature, soil mixture, humidity, test substances); Chapter 3; Rastetter et al. 2017) showed in both experimental set-ups of copper and TSP no clear avoidance behaviour at concentrations below 175 mg Cu^{2+} kg^{-1} and 5 g DM kg^{-1}. From 175 mg Cu^{2+} kg^{-1} and 25 g DM kg^{-1} a clear avoidance effect could be observed in both set-ups with values ≥60 % (percentage effect/avoidance) and ≥80 % (worms in control chamber) (Table 4.3). On the other hand, 5 g DM kg^{-1} of TSP had no avoidance effect in measurements with the biomonitor, but resulted in the standard test in increased avoidance behaviour of the earthworms. Other studies about the avoidance behaviour of earthworms of copper differed by the applied chemical form, test species, soil type or exposure media and test duration. Hund-Rinke et al. (2005) investigated the effect of *E. fetida* of $CuCl_2$ in a sandy field soil after 48 h resulting in a significant avoidance of concentrations ≥50 mg Cu^{2+} kg^{-1}. In a natural loam soil (Kettering) *E. fetida* avoided the treatments with $Cu(NO_3)_2$ and $CuSO_4$ at concentrations ≥110 and ≥600 mg Cu^{2+} kg^{-1} after 24 h (Arnold et al. 2003). In 320 mg Cu^{2+} kg^{-1} of copper sulphate in a natural LUFA 2.2 soil, ≥80 % of the worms (*E. andrei*) were located in the control side after 48 h resulting in a significant avoidance behaviour (Loureiro et al. 2005). Demuynck et al. (2016) used filter papers and water agar gels as exposure media of $CuCl_2$, $Cu(NO_3)_2$ and $CuSO_4$ on the contrary to evaluate the contribution of the dermal and the digestive exposure routes on the avoidance reactions of the different chemical forms of copper. Already 10 mg Cu^{2+} l^{-1} of all tested copper salts were significantly avoided during all the exposure durations (0–8 and 24–32 h, each 2 h), whereas differences were partly noticed between salts but could not be explained directly by the concentration of the anions in these salts. The reported results show that it is hard to compare the concentrations causing significant effects because different test conditions could result in other availabilities of Cu^{2+} to earthworms. The percentage avoidance in the biomonitor was mostly lower than in the standard test which was conducted with the same copper salt ($CuCl_2$) and soil type (Chapter 3; Rastetter et al. 2017) and did not reach 100 % (Table 4.3). For analysing the percentage avoidance (ISO 2008) in the biomonitor, all measurements, also of worms with a low general activity over the entire test period were applied. So, worms were counted as 0.5 to each chamber if there was no obvious assignment after 16 h due to barely locomotion within the 4-h time periods (e.g. two worms each in the treatments of 250 and 1000 mg Cu^{2+} kg^{-1}, three worms in 100 g DM kg^{-1} of TSP). Further, in the conducted standard avoidance test with five earthworms per treatment, a group dynamical-directed effect might arise and so the avoidance effect could be amplified in comparison to the individual measurements with the biomonitor. Petry (1989) observed a group-dynamical behaviour of the sludge worms, *Tubifex tubifex*, whereby the worms jointly released the aggregation form of the worms, which is usually the present form of the worms in clean water because of increased movement activity after a discharge of pollutants. For earthworms, no research about group-dynamical behaviour was found. The measured avoidance behaviour over the time of the individual measured earthworms in the biomonitor resulted admittedly in a lower percentage avoidance than the analysed avoidance according to ISO (2008) and Hund-Rinke & Wiechering (2001). But, the new applied test parameter enables more detailed insights into the avoidance behaviour based on a time-to-effect evaluation by combining the location of the worm and the duration of the effect (avoidance) at any time. More than 40 years ago, Sprague (1969) described the potential of time-to-effect concepts in form of time-to-death assessments in aquatic toxicity. The importance of the factor time for ecological risk assessment was demonstrated in several approaches concentrating on consideration of exposure time in toxicity evaluation (DeFoe & Ankley 2003; Singh & Singh 2015), time-to-event models (Newman & McCloskey 1996; Bonnomet et al. 2002) and extrapolation of toxicity data to any exposure time (Connell et al. 2016). A percentage avoidance behaviour over the time of at least 50 % was reached for treatments with 80 % of the worms and more in the control chamber of the biomonitor measurements. So, for the assessment at least 50 % are proposed to regard a treatment as toxic (toxicity criterion).

Terrestrial toxicity analysis by the earthworm avoidance test, a choice chamber test, deals with negative responses to contaminated soils. For studying preference-avoidance behaviours and investigating the chemo-responses of aquatic animals, a number of aquatic choice chamber experiments have been conducted (e.g. examining olfaction (Gerlach et al. 2007) or water quality (Kroon & Housefield 2003)). Other existing terrestrial choice chamber tests with earthworms basically consider food (e.g. tree litter (Rajapaksha et al. 2013; Ashwood et al. 2017) or soil preferences (Kim et al. 2015)). In addition to the choice chamber test of Rajapaksha et al. (2013), an infrared webcam recording technique was used to directly observe litter selection behaviour of *Lumbricus terrestris* under cover of darkness at the soil surface. For different earthworm behavioural studies, similar optical techniques have also been used (e.g. for preferential feeding outdoors (Griffith et al. 2013), mating behaviour (Nuutinen & Butt 1997), surface behaviour (Valckx et al. 2010) or dispersal behaviour in soil (X-ray) (Caro et al. 2012)). For a faster evaluation of image/video data of the movement of earthworms, an automated optical analysis was developed by Kodama et al. (2014) tracking two-dimensional positions of earthworm parts with a simultaneous output of the change in its body length.

Due to the permanent and non-optical monitoring of the movement behaviour of earthworms in this study, a reduction of the standard test period from 48 to 16 h could be implemented (see result section *Determination of an optimal measurement period*). Frankenbach et al. (2014) demonstrated a potential reduction from 48 to 24 h performing the standard earthworm avoidance test to identify a test which is comparable to modern methods of chemical analysis whereby results are often available within 24 h (on-site analysis) (BBodSchV 1999). For the new experimental set-up with the biomonitor, a lower volume of soil (less than the half) compared to the predefined test vessels according to the standard test of the ISO guideline (2008) and no light source were required. Further, only the individual behaviour of the earthworms without group-dynamical artefacts was measured. So, permanent monitoring of the avoidance behaviour of earthworms could be recommended as a sensitive and simple tool for the assessment of the habitat function of contaminated soils and for evaluating the terrestrial toxicity of particular contaminants. For further validations of the new experimental set-up, a ring test (inter-laboratory calibration) is suggested.

4.6 Conclusions

The permanent, automated and non-optical monitoring of the earthworms and the associated time-to-effect-based evaluation method as well as the reduced test duration and the potential cost efficiencies are indicative that the new experimental set-up for analysing the avoidance behaviour of *E. fetida* could be used as an ecotoxicological screening tool for the assessment of contaminated soils within an environmental risk assessment.

Acknowledgements

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Chapter 5

General Discussion

General Discussion

5.1 Ecotoxicological assessment of sewage sludges and phosphate recyclates in reference to the risk assessment of contained single pollutants

In the thesis, it was observed that the phosphate recyclates had mostly a smaller effect on the tested organisms under laboratory conditions than the sewage sludges and the conventional phosphate fertiliser TSP (Chapter 3; Rastetter et al. 2017). The sewage sludges resulted in following effects of the main parameters of the ecotoxicological test methods: (1) 50 % of growth inhibition of *L. minor* at concentrations between 1.2 to 9.6 g DM l^{-1}, (2) 50 % mortality of *G. fossarum* between 0.5 to 1.6 g DM l^{-1} and (3) 50 % avoidance of *E. fetida* between 0.4 to 15.1 g DM kg^{-1}. The highest effects on the organisms of the analysed sludges were obtained by the non-dewatered bio-P sludge S1 (Chapter 2; Rastetter & Gerhardt 2017). 100 % of growth inhibition, mortality and avoidance behaviour was caused by the highest tested concentrations of TSP (10 and 5 g DM l^{-1}, 50 g DM kg^{-1}). Whereas, the highest tested concentrations of the PR samples in water (10 and 5 g DM l^{-1}) have reached a maximal effect of about 50 % growth inhibition and 30 % mortality (Chapter 3; Rastetter et al. 2017). Concentrations of 5 g DM kg^{-1} and mostly of higher concentrations of the phosphate recyclates in soil caused an obvious avoidance behaviour of the earthworms (>80 %) similar to the effect of TSP and the dewatered sludges. But 0.4 g DM kg^{-1} of the non-dewatered sludge already resulted in 50 % avoidance of *E. fetida* (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). Though in compliance with the main effect parameters (avoidance, growth and mortality) and the calculated maximum agronomical application amounts (worst-case scenario, based on the phosphate content) of the sludges (2.9–3.3 g DM kg^{-1}), the phosphate recyclates (1.0– 3.1 g DM kg^{-1}) and TSP (0.6 g DM kg^{-1}), relevant concentrations of the dewatered sludges, the phosphate recyclates and TSP on the field should probably not affect the soil invertebrates (*E. fetida*). But the survival of the freshwater amphipod could be affected negatively if an amount of the output concentration of TSP or the non-dewatered sludge which highly influenced the location of *E. fetida* in soil would reach surface water. Minor effects on the growth of the duckweed might be caused by amounts of the crystallised (except of Cryst 4) and thermally treated PRs in surface waters and increasing effects by the non-dewatered sludge (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). In addition, a maximum allowed application amount of 5 tons of dry matter per hectare within 3 years (correspond to 6 g DM kg^{-1} (Chapter 2; Rastetter & Gerhardt 2017)) applies for sewage sludge used for a soil related recycling according to the current and the amended Sewage Sludge Regulation. But additionally, the application amount is usually limited by the phosphorus content according to the demand regulations of nutrients of the Fertiliser Ordinance (AbfKlärV 1992; BMUB 2017a).

The comparison of the analysed ecotoxicological effects and the chemical composition of the sewage sludges and the phosphate products as well as literature data of the toxicity of particular single pollutants showed that the ecotoxicity not necessarily depended on the occurrence of single pollutants but most probably on potential additivity or interactions of substances in the complex mixture and different bioavailabilities of the constituents related to solubility of the samples in general (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). Although the ferric dewatered sludge had mostly high concentrations of pollutants, especially of copper (832 µg g^{-1} DM), iron (121 mg g^{-1} DM) and benzotriazole (13 µg g^{-1} DM), it was just less toxic than the other investigated sewage sludges (Chapter 2; Rastetter & Gerhardt 2017). The same observation has also been made with the thermally treated PRs containing mostly much higher heavy metal concentrations, but no organic compounds, compared to the crystallisation products (struvite), and resulted in lower negative effects on the tested organisms either (Chapter 3; Rastetter et al. 2017). Some ash products partly exceeded the mandatory threshold values for heavy metals (As, Pb, Cd, Cr, Ni, Hg) of lead, cadmium and nickel for the dry matter of fertilisers listed in the regulation (DüMV 2012; Rastetter et al. 2017, Chapter 3); whereby iron, copper and zinc, being essential trace elements, only need to be labelled in compliance with maximum levels (DüMV 2012).

Depending on the dose, ratio and exposure time of the heavy metals and organic pollutants contained in the analysed sludge and phosphate recyclate samples, different toxicity effects of the complex mixture on the organisms might have appeared (van Gestel et al. 2011). Additive effects of toxicants in mixtures occur when chemicals act together to produce an effect, but do not enhance or reduce each other’s action. To predict the joint action of chemicals from information about the responses of an organism to individual mixture components two simplified basic concepts assuming chemicals in a mixture do not influence each other’s toxicity (i.e. they do not interact with each other at the biological target site) are available: the concentration addition (CA) and independent action (IA) (Jonker et al. 2011; Kortenkamp & Altenburger 2011; SCCS et al. 2012). If chemicals in a mixture act with the same or similar modes of action, CA assumes that 1 chemical can be replaced totally or in part by an equal fraction of an equivalent effective concentration of another. If these fractions (toxic units) sum up to a value of 1, the hypothesis of CA holds true (Jonker et al. 2011). The effect of a mixture based on CA is equivalent to the effects of the sum of the potency-corrected concentrations of each compound (SCCS et al. 2012). In case of chemicals with different modes of action the IA known as response addition related to the sum of probabilistic risks is preferred as reference. The proportional effect that the total mixture provokes can be calculated by the proportional effects that the individual components would cause if applied individually at the concentration at which they are present in the mixture (Jonker et al. 2011). If the combined effect of a mixture is different from the effect expected based on the additivity assumption, synergism or antagonism could play a role – causing larger or smaller effects (Kortenkamp & Altenburger 2011). Co-precipitation of heavy metals, competition between metals (and scarcely organic compounds) for the routes of mediated uptake, interactions at the metabolic level and the mode of actions in the organisms could cause antagonistic or synergistic effects (Gerhardt 1995b; Kienle et al. 2009; Svendsen et al. 2011). Synergistic effects of organic chemicals have been observed in experiments by testing e.g. polycyclic aromatic hydrocarbons (PAHs) mixtures acting as receptor agonists and being more toxic than an additive approach on the early life stage of the fish *Fundulus heteroclitus* by increasing deformities (Wassenberg & Di Giulio 2004). Interactions of organic chemicals are generally related to biotransformation processes, whereas the level of uptake and kinetics is more common for metals, partly essential for physiological processes, as a consequence of crossing the biological membranes by ion channels or carrier enzymes, and their active regulation. Inside the body, metal ions can replace each other within functional parts of proteins and biochemical pathways due to their similarity (Svendsen et al. 2011). In binary mixtures of heavy metals, antagonistic effects could be detected e.g. in long-term exposure measurements of Cd and Fe for analysing the survival and behaviour of *Leptophlebia marginata* (mayfly) – probably due to precipitation and competition at the uptake site (Gerhardt 1995b) – and by measuring the filtration rate of the freshwater mussel *Dreissena polymorpha* exposed to Zn and Cu (Kraak et al. 1994). Whereas mixtures of Zn and Cd resulted in an additive concentration effect on the filtration rate of *D. polymorpha*, and Cu and Cd had a synergistic effect (Kraak et al. 1994). Studies of different binary mixtures of metals and pesticides showed synergistic lethal effects in all cases on the marine microcrustacean *Tigriopus brevicornis* and seemed to partly enhance the acetylcholinesterase inhibition at a sublethal level (Forget et al. 1999).

Besides, the bioavailability of chemicals – the fraction that is capable of being used by a living organism – plays also a role by effect assessments of chemical mixtures in the environment. Physical and chemical characteristics of the medium like the organic matter content and pH, and of the chemicals (e.g. ionisation, aqueous solubility) are the major determinants of the bioavailability, which can be affected through the presence of other chemicals as well (e.g. competitive sorption, precipitation, etc.) (Spurgeon et al. 2011). So, the lower toxic effect of the tested ferric dewatered sewage sludge (S3) compared to the other sludges might occur due to the high Fe concentration (Chapter 2; Rastetter & Gerhardt 2017) which partly appears in form of iron oxides or iron hydroxides depending on the pH in the water treatments and pore water, being capable of removing cations of heavy metals (Gerhardt 1995b; SenGupta 2002). Moreover, a higher amount of the Fe concentration of S3 might mainly appear as $FePO_4$ (lowly water soluble) emerging during chemical precipitation of phosphates of wastewater (Pöppinghaus et al. 1994) and could consequently result in a lower effect because of a lower bioavailability of Fe itself. In addition, water-soluble and bioavailable phosphate anions might have affected the ecotoxicity of the analysed phosphate recyclates and the conventional phosphate fertiliser (TSP), which had a higher negative effect on all test organisms and the highest content of water-soluble P_2O_5 in the dry matter (44 %; Chapter 3; Rastetter et al. 2017). But also, antagonistic effects due to phosphate anions might occur in the sewage sludge and phosphate recyclate treatments. A significant reduction in earthworm heavy metal bioavailability due to KH_2PO_4 and TSP phosphorus amendments to soil was demonstrated by Maenpaa et al. (2002). Further, phosphate anions inhibited the arsenic uptake by reducing arsenic (As) bioavailability or by competing for cellular transport carriers of the earthworm *E. fetida* (Lee & Kim 2008) and of the aquatic macrophyte *Spirodela polyrhiza* (Rahman et al. 2008).

In addition to the ecotoxicological assessment, a quantitative risk assessment of single pollutants in the sewage sludges, phosphate recyclates and the conventional phosphate fertilisers (TSP) for the endpoints human and the environment (soil and groundwater), and a relative risk ranking for the different pollutants between the sewage sludges and phosphate products was conducted within the EU Project P-REX (Kraus & Seis 2015). The whole risk management process comprises two parts: the scientific risk assessment and risk management, which is about measures considering issues of acceptability as well as the feasibility of risk reduction. Divided into four steps, the risk assessment deals with hazard identification, exposure assessment and hazard and risk characterisation (van Leeuwen & Vermeire 2007). For estimating the exposure, the predicted environmental concentration (PEC), of the single pollutants contained in the sewage sludges and phosphate recyclates to topsoil and leachate, a kinetic model according to the Technical Guidance Document (TGD) on risk assessment (IHCP 2003) and a solute transport model (HYDRUS) for refinement were used to quantify potential risks for the analysed endpoints (Kraus & Seis 2015). The model of the TGD considers the input of pollutants by realistic fertiliser application to restock the phosphorus storage and atmospheric deposition as well as the outputs for organic substances by volatilisation, biodegradation and leaching (IHCP 2003), and for heavy metals in the modified model only by leaching described by soil hydraulic properties and the retardation of metals (Kraus & Seis 2015). For the hazard characterisation, the predicted no effect concentrations (PNECs) of the single pollutants for the endpoints (1) soil organisms, (2) humans and (3) groundwater were determined by (1) reports of the Institute of Health and Consumer Protection of the European Union, (2) an approach of the tolerable concentration in soil by using the tolerable daily intake (VKM 2009) and bio-concentration factor for the equilibrium between soil and plant and (3) minor threshold values for leachate according to LAWA (2004). The risk ratios were calculated from quotient PEC to PNEC for each endpoint, hazard and product (IHCP 2003). Whereby, risk (RQ) was classified as follows: “risk reduction required” ($RQ > 1$), “risk reduction recommended” ($0.01 < RQ < 1$) and “negligible risk” ($RQ < 0.01$) (Kraus & Seis 2015). The risk ratios of the phosphate recyclates were in the same magnitude as the reference phosphate fertiliser TSP. Against the background of the made assumptions, there are no unacceptable risks requiring urgent reduction caused by the organic substances, arsenic, chromium, copper, mercury, nickel or lead, but nevertheless risk reduction measures are still recommended. Whereas, cadmium and zinc are hazards which are of concern in the analysed products (Kraus & Seis 2015). Focusing on the sludge and phosphate recyclates quality, a relative risk ranking based on phosphorus specific hazard loads compared to diffuse discharge by atmospheric deposition into the ecosystem was conducted. In summary of the risk assessment, the crystallisation products (struvite) can be considered as high quality phosphate recyclates containing in general the lowest hazard concentrations related to the dry matter and a high amount of total phosphate. For the thermally treated PRs having high heavy metal loads, an improvement regarding heavy metal depletion is in some cases advisable (Kraus & Seis 2015). Furthermore, the plant availability of the sewage sludges and phosphate recyclates was investigated within the project measuring the dry matter yields of maize in acidic and neutral soil in pot trails by the Institute of Agricultural and Urban Ecological Projects (IASP) in Berlin, Germany (P-REX 2015). Almost all samples of the sewage sludges and phosphate recyclates increased yields on both soil types compared to the control. The used concentration of the products in the pot tests depended on the P content to reach a phosphor concentration of 125 mg kg^{-1} in soil. The dewatered bio-P sludge (S2), all crystallised PRs (struvite) and two ash products (Therm 3 and 5) reached satisfying results of relative fertiliser efficiency values calculated by determining the yield increase according to the control and the proportion compared to the yield increase of the reference phosphate fertiliser TSP. Yields of the struvite products were comparable to plants fertilised with the conventional phosphate fertiliser TSP (P-REX 2015).

The ecotoxicological assessment in this study compared to worst-case applications in agriculture and the quantification of the potential hazards of single pollutants of the sewage sludges and phosphate recyclates as well as the caused yield increase indicate to follow up phosphate recovery in wastewater treatment and recycling in agriculture, especially of struvite, in the future for reducing environmental risks and closing of nutrient cycles.

5.2 Assessment of ecotoxicological test methods for monitoring of sewage sludge and phosphate recyclates

Within the chosen and partly adjusted ecotoxicological standard test methods, a similar sensitivity of the main parameters, the growth inhibition of the *Lemna* test and the mortality of the *Gammarus* test could be shown by evaluating the acute toxicity of the phosphate recyclates under laboratory conditions. Admittedly, the growth of the *Lemna* test was affected partly 10 to 20 % more at the same concentrations of the phosphate recyclates, but the *Gammarus* test is shorter in time (4 days vs. 7 days) (Chapter 3; Rastetter et al. 2017). Though considering the different effects of the sludge samples and TSP, *G. fossarum* appears to be the most sensitive organism comparing the main parameters of all three test methods (Chapter 2; Rastetter & Gerhardt 2017). It might be that the toxicity of the sewage sludges and TSP with respect to the growth of *L. minor* is weakened by an overlapping fertiliser effect due to the contained nutrients increasing plant growth. Lower concentrations of some sludge samples and phosphate recyclates even supported growth compared to the control group in the experiments (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). The additional parameters of the *Lemna* test, root length and biomass, can be generally regarded as equivalent to the growth determined by evaluating the total number of leaves, whereas discolouration and colony break-up caused no obvious results in testing the phosphate recyclates and TSP (Chapter 3; Rastetter et al. 2017). But the copper reference and some sewage sludge samples successfully affected the number of discoloured and single leaves of the duckweed (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). At all, the most sensitive parameters seem to be the behaviour measurements of *G. fossarum*, feeding of alder leaves and movement. Even lower concentrations of the phosphate recyclates, TSP and Cu caused up to 80 % decrease of feeding of alder leaves at the test end after 4 days. Feeding behaviour measurements with thawed chironomids resulted in lower effects with an average decrease of 25 % at higher sludge concentrations, which highly effected the survival of the gammarids (Chapters 2 & 3;

Rastetter & Gerhardt 2017; Rastetter et al. 2017). In the higher sewage sludge treatments with a high mortality at the test end (4 days), a significant decrease of movement activity (locomotion) and trend of increased stress ventilation could already be detected after 1 or 2 days of exposure (Chapter 2; Rastetter & Gerhardt 2017). With regard to the effects of the main parameters of the dewatered sludges and TSP, the avoidance of the earthworms was less sensitive, whereas a comparison with the effects on *L. minor* and *G. fossarum* of the phosphate recyclates is difficult because of the different concentration ranges due to measuring realistic environmental concentrations under laboratory conditions. The lower observed effects in the soil compartment might occur due to interactions of the pollutants with e.g. the organic matter and consequently a lower bioavailability.

For evaluation of chronic effects of the sewage sludges, the phosphate recyclates and TSP in soil under laboratory conditions, the C and N transformations and the phytotoxicity were analysed by IASP in Berlin following the OECD guidelines for testing chemicals Nos 217 and 216 (2000a, b), and ISO 11269-2 (2012c): Effects of contaminated soil on the emergence and early growth of higher plants (P-REX 2014). The C and N transformation test of the sewage sludges resulted in a positive effect for all the samples (except of the nitrogen test of S3). Potential negative effects could be overlaid by an increasing transformation due to organic substance, nitrogen and microorganisms which were contained in the sludge samples (P-REX 2014). In addition, validity criteria were partly not met by testing the effect on microorganisms of the phosphate products. Consequently, the tests were considered as possibly not suitable for organic substance like sewage sludge and phosphate products (P-REX 2015). In the phytotoxicity test, the emergence inhibition of turnip rape (*Brassica rapa*) was similar or more sensitive than the dry matter inhibition of all tested samples. Regarding the results, the thermally treated PRs showed a lower negative effect on the two terrestrial plant growth parameters than the sewage sludges and the crystallisation products comparing the EC_{50} concentrations in the percentage of fresh matter (except of Therm 3) (P-REX 2015). Even concentrations of 100 g DM kg^{-1} of the ash products (Therm 1, 2, 4) did not result in 50 % inhibition of the dry matter and emergence. The lowest observed EC_{50} concentrations with regard to the dry matter were caused by the non-dewatered sludge (S1), Cryst 4, Therm 3 and TSP in a range of 1 to 5 g DM kg^{-1} (P-REX 2015). Considering the calculated maximum agronomical relevant application amounts (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017), sludge 1 (3.3 g DM kg^{-1}) and Therm 3 (2.3 g DM kg^{-1}) could negatively affect plant growth in environment under worst-case conditions. The lower tested concentrations of the sewage sludges and phosphate recyclates (2 to 4 % of the fresh matter, corresponding to 0.5–10 g DM kg^{-1} of the sludges (depends on the dry matter content) and 20– 40 g DM kg^{-1} of the P-recyclates) partly increased plant growth of *B. rapa* (P-REX 2014). In some cases, this effect was also observed for lower concentrations (below 0.2–2.3 g DM l^{-1} of the sludges and below or equal to 0.1 g DM l^{-1} of the P-recyclates) tested in the experiments with the duckweed *L. minor* (Chapters 2 & 3; Rastetter & Gerhardt 2017; Rastetter et al. 2017). In the chronic terrestrial phytotoxicity test, the reference phosphate fertiliser TSP and the sewage sludges had mostly a higher negative effect on plant growth than the phosphate recyclates (except of Cryst 2 & 4, Therm 3 with regard to emergence inhibition). This effect could also be observed for the aquatic organisms (*L. minor*, *G. fossarum*) considering TSP and for all tested organisms considering the non-dewatered sewage sludge (S1) in this study.

In summary, the results show that the overall toxicity of such a varied complex mixture as sewage sludges and phosphate recyclates cannot be determined just by chemical analysis of singular substances. Besides chemical analysis and risk assessment of single pollutants based on literature data and models without considering mutual interactions of pollutants, ecotoxicological evaluations are reasonable methods to provide a more comprehensive assessment of complex mixtures. However, the conduction of ecotoxicological tests is often labour-intensive and therefore they can be costly, too. But in general, the terrestrial tests with turnip rape and the earthworms evaluating the ecotoxicity in soil, the target compartment of fertilisers, can be recommended for a potential standard monitoring concept. Although, the earthworm avoidance test is assigned to acute toxicity tests in terms of duration, it was evaluated as sensitive as the chronic reproduction test (Hund-Rinke et al. 2003). For a more comprehensive monitoring, the effect on the water compartment should also be evaluated (e.g. by the sensitive organism *G. fossarum*).

To simplify the conduction of the earthworm avoidance test, a new experimental set-up for measuring the locomotion and the avoidance behaviour based on a permanent, automated and non-optical monitoring was developed in this study (Chapter 4). First, different pre-tests were performed to obtain an optimal experimental set-up for measuring earthworms (*E. fetida*) with the biomonitor in soil (Multispecies Freshwater Biomonitor®, LimCo International GmbH, Germany), which was originally developed for aquatic organisms. The signal pattern of a clear movement of *E. fetida* in a single and soil-filled measurement chamber, the chamber design (paired chambers), potential artefacts in chambers only filled with soil during measurements and the effect of the electrical field (impedance technology) on the earthworms were determined (Chapter 4). By comparing avoidance measurements with the biomonitor, analysed as avoidance behaviour over time of contaminated treatments of a reference heavy metal (copper) and a reference phosphate fertiliser (triple superphosphate) after different exposure times, the original test duration could be reduced from 48 to 16 hours (Chapter 4). The measurements of the avoidance behaviour of single earthworms of different concentrations of copper and TSP with the biomonitor showed that the new test set-up provides comparable results of the analysed references to the standard test, but in less time and with potential cost efficiencies (Chapter 4). So, the permanent monitoring of the avoidance behaviour of *E. fetida* could be used as a ecotoxicological screening tool for the assessment, standard monitoring, of the habitat function of contaminated soils or of soils treated with fertiliser within an environmental risk assessment.

In order to further secure the usage of recycled phosphate products and sewage sludge in agriculture by ecotoxicological methods, studies about the effects of repeated pulses on terrestrial and aquatic organisms, and investigations of environmental samples directly after application in the field and at different weather conditions (e.g. after heavy rainfall) should be conducted.

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Author Contribution

I, Nadja Rastetter, have written this thesis on my own using only the resources mentioned in the text and the references section. The thesis was created under the guidance of Dr. Almut Gerhardt, LimCo International GmbH.

Chapter 2 – Toxic potential of different types of sewage sludge as fertiliser in agriculture: ecotoxicological effects on aquatic, sediment and soil indicator species

The experiments were planned and adjusted together with AG. The test organisms were obtained and cultivated by NR. All the methods and data analyses were performed by NR, except of the chemical analyses, which were conducted by project partners (IASP, FHNW), LUFA Nord-West and ISWA. The manuscript was revised by AG.

Chapter 3 – Ecotoxicological assessment of phosphate recyclates from sewage sludges

The experiments were planned and adjusted together with AG. The test organisms were obtained and cultivated by NR. All the methods and data analyses were performed by NR, except of the chemical analyses, which were conducted by project partners (IASP, FHNW) and LUFA Nord-West. Some experiments of the earthworms and the duckweed were conducted by Claudius Leeser under supervision of NR. The manuscript was revised by Prof. Dr. Karl-Otto Rothhaupt and AG.

Chapter 4 – Continuous monitoring of avoidance behaviour with the earthworm *Eisenia fetida*

The experiments were designed and planned together with AG. The experimental set-up for the avoidance tests with the MFB was designed by AG and NR. The test organisms were obtained and cultivated by NR. All the methods and data analyses were performed by NR. The manuscript was revised by AG.

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