# Evaluating the toxic effects of industrial waste from a historic landfarming site using bioassays

by
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## Declaration

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# Abstract

Landfarming is a widely used method for the disposal of contaminants in the petrochemical industry. It involves ploughing the contaminants into the top soil layer allowing biological breakdown. A historically landfarmed site was identified at a South African petrol refinery. The refinery used to dispose so-called American Petroleum Institute (API) -sludge onto a landfarming site. API-sludge consisted of a mixture of oil and water soluble contaminants originating from a process of separating refinery waste from reusable water and oil. Landfarming on this site was discontinued after excessive quantities of sludge were ploughed into the soil over time and it became obvious that effective biodegradation could not take place. An environmental assessment had to be carried out to assess to what extent the soil has recovered from the contamination and after remediation was done over time.

Bioassays together with chemical analyses were executed to determine the level of pollutants in the soil and to assess the integrated effects of their bioavailable fractions.

The landfarming site of the refinery was divided into two sections namely, a more contaminated north-site and less contaminated south-site. Soil samples were collected from both sites as well as from an off-site (control site). The soils were analysed physically, chemically and used in the bioassays. Two additional control soils were also used, OECD-soil and LUFA2.2 soil.

Chemical analysis of the site soils showed the presence of heavy metals and high levels of diesel range organic hydrocarbons. The north-site had higher levels of contaminants compared to the south-site.

Three species of soil organisms were used in standardised tests: *Eisenia andrei*, *Enchytraeus doerjesi* and *Folsomia candida* were exposed to the respective soils to study their survival, growth, reproduction success and avoidance behavior. Exposures to both site-soils were not acutely toxic to any organisms. *F. candida* had a decrease in juvenile production in both north- and south-site soils (289.42 ± 58.62 and 253.33 ± 122.94 respectively) compared to the control soil (479.89 ± 30.42). *E. doerjesi* showed an increase in produced juveniles exposed to north- and south-site soil (339.75 ± 76.92 and 414.00 ± 17.78 ) compared to control soil (57 ± 34.39). *E. andrei* had similar cocoon production when exposed to south-site soil than in off-site soil (19.00 ± 5.3 and 18.5 ± 9.7 respectively) but significantly less in north-site soil (1.25 ± 0.7). Only *E. doerjesi* showed avoidance of north-site soil.

To determine the sensitivity of the organisms to the API-sludge, they were exposed to concentration series of API-sludge-spiked control soils. The effect concentrations were calculated as the concentration of API-sludge that will decrease the studied endpoints by 50% of the control soil ($EC_{50}$). The $EC_{50}$s varied for each species exposed in the different control soils showing that the toxicity of the API-sludge is to a certain extent dependent on the physical soil properties of the substrate. The reproduction of *F. candida* were most sensitive to the API-sludge in off-site soil ($EC_{50}$ = 90 mg/kg) and the *E. doerjesi* the least sensitive in LUFA2.2 soil ($EC_{50}$ = 36000 mg/kg).

Five plant species were exposed to API-sludge-spiked potting soil and the germination success, early growth rate and biomass were studied. The plants were not as sensitive to API-sludge as the soil animals. Lettuce and grass were affected the most by API-sludge and beans were the most resilient species. With the addition of low levels API-sludge to the substrate, the growth rate of beans was stimulated.

This study showed that the south-site has been successfully remediated and most soil organisms exposed to these soils were not affected by the levels of toxicants present. However, exposures to north-site soil still had negative effects on soil organisms. It is recommended that hydrocarbon contamination should be further remediated in the north-site soil before landfarming should be allowed to continue.

# Opsomming

Ploegverwerking is ‘n algemene remediëringsmetode vir die verwerking van afvalmateriaal in petrochemiese industrieë. Dit behels die inploeg van toksiese afvalmateriaal in die boonstegrondlaag sodat dit biologies afgebreek kan word. ‘n Voorbeeld van ‘n histories ploegverwerkte grondstuk is geidentifiseer by ‘n Suid-Afrikaanse olieraffinadery. Die raffinadery het in die verlede van die grondstuk gebruik gemaak om sogenaamde Amerikaanse Petroleum Instituut-slik (API-slik) daarin te ploeg. Die API-slik bestaan uit ‘n mengsel van olie- en wateroplosbare kontaminante afkomstig van die proses waardeur die raffinadery se afvalprodukte van hernubare water en olie geskei word. Nadat oormatige konsentrasies slik in die grond ingewerk is en bioremediasie nie meer doeltreffend kon voortgaan nie, is die ploegverwerking gestaak. ‘n Omgewingimpakstudie moes uitgevoer word om te bepaal tot watter mate die grond herstel het nadat remediasie oor tyd uitgevoer is.

Toksisiteitstoetse en chemiese analises is uitgevoer om die vlakke van besoedeling sowel as die biobeskikbare fraksie daarvan in die grond te bepaal. Die ploegverwerkte area van die raffinadery is in twee verdeel naamlik, ‘n meer gekontamineerde noordelike area en ‘n minder gekontamineerde suidelike area. Grondmonsters is van die onderskeie areas asook van ‘n ongekontamineerde veld (as kontrole) naby die ploegverwerkte area versamel Die gronde is fisies- en chemies geanaliseer en toksisiteitstoetse is uitgevoer. Twee addisionele kontolegronde is ook tydens die blootstellings gebruik naamlik, OECD- en LUFA2.2-grond.

Die chemiese analises van die ploegverwerkte toetsgronde het getoon dat daar steeds swaarmetale en hoë vlakke van dieselgekoppelde organiese koolwaterstowwe in die gronde teenwoordig is. Kontaminante was in hoër konsentrasies teenwoordig in die grond van die noordelike gebied as in dié van die suidelike gebied.

Drie spesies van grondorganismes is gebruik tydens standaard toksisitetitstoetse. *Eisenia andrei*, *Enchytraeus doerjesi* en *Folsomia candida* is blootgestel aan die onderskeie toets- en kontrolegronde waarna hul oorlewing, groei, voortplantingsukses en vermydingsreaksies bestudeer is. Blootstellings aan die ploegverwerkte toetsgronde het geen akute toksisiteit vir enige van die spesies getoon nie. *F. candida* se juveniele produksie was laer in beide noordelike- en suidelike toetsgronde (289.42 ± 58.62 en 253.33 ± 122.94 onderskeidelik) as in die kontolegrond (479.89 ± 30.42). *E. doerjesi* blootstellings het ‘n toename in juveniele getalle getoon in die noordelike- en suidelike toetsgronde (339.75 ± 76.92 en 414.00 ± 17.78) in vergelyking met die

ongekontamineerde kontolegrond (57 ± 34.39). Kokonproduksie by *E. andrei* was soorgelyk in die suidelike toetsgrond en ongekontamineerde kontrolegronde (19.00 ± 5.3 en 18.5 ± 9.7 onderskeidelik) maar beduidend minder as in noordelike toetsgrond (1.25 ± 0.7). Slegs *E. doerjesi* het ‘n beduidende vermydingsreaksie vir die noordelike toetsgronde getoon.

Om die sensitiwiteit van die organismes aan vars API-slik te bestudeer, is hulle blootgestel aan konsentrasiereekse van API-slik in die onderskeie kontrolegronde. Die effektiewe konsentrasie ($EK_{50}$) is bereken as die konsentrasie van API-slik wat die bestudeerde eindpunte met 50% sal verminder in vergelyking met die kontrolegrond Die $EK_{50}$-waardes vir al die spesies het verskil na blootstelling aan die onderskeie kontrolegronde. Dus, die toksisiteit van die API-slik is tot ‘n sekere mate ook afhanklik van die fisiese grondeienskappe van die blootsellingssubstraat. Die voortplanting van *F. candida* was die gevoeligste eindpunt vir die blootstelling aan API-slik in kontolegrond ($EK_{50}$ = 90 mg/kg) en *E. doerjesi* was die minste gevoelig in LUFA2.2 grond ($EK_{50}$ = 36000 mg/kg).

Vyf plantspesies is ook blootgestel aan API-slikgekontamineerde potgrond en die saadontkiemingssukses, vroeë groeikoers en biomassa is bestudeer. Alhoewel plante nie so sensitief was vir die API-slik soos die gronddiere nie, was blaarslaai en gras die meeste geaffekteer tydens die blootstellings. Boontjies was die ongevoeligste en met die toevoeging van lae konsentrasies API-slik (2.5% API-slik), is hul groeikoers selfs gestimuleer.

Uit die studie was dit duidelik dat die suidelike deel van die grondstuk meer suksesvol as die noordelike geremidieer is en dat meeste grondorganismes wat daaraan bloot gestel is nie geaffekteer is deur die vlakke van kontaminasie wat steeds teenwoordig is in die grond nie. Die toetsgronde uit die noordelike deel het egter steeds negatiewe effekte op die grondorganismes gehad. Dit word voorgestel dat die koolwaterstof kontaminasie verder geremidieër behoort te word in die noordelike deel van die grondstuk voordat verdere ploegverwerking van die afval daar gedoen word.

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# 1. Introduction

## 1.1. Landfarming

During the 1970s, uncontrolled disposal of industrial waste products resulted in pollution of groundwater, soil and air which gave rise to major environmental concerns. To date, petroleum and diesel waste products are being disposed of through various technologies. One such method is landfarming. It is a low-technology method that involves the controlled application of a relatively defined waste to a soil surface, and the incorporation of the waste into the upper soil zone (Genou *et al*. 1994). This method of bioremediation makes use of regular ploughing of the upper soil layer to mix the contaminants with the soil and allow aeration for optimising biological breakdown. The contaminants are degraded, transformed and immobilised by means of biotic and abiotic soil reactions (Rubinos *et al*. 2007). Landfarming is an effective remediation method for the sanitation of soils contaminated with metals, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), wood preservatives (pentachlorophenol or creosote), solvents as well as total petroleum hydrocarbons (TPHs) (Vidali 2001). Soil conditions such as moisture content, aeration, pH (altered by lime addition) and nutrient additives are controlled to optimise the rate of contaminant degradation.

The processes which are stimulated in the soil by landfarming may either be physical (such as volatilisation), chemical (oxidisation, reduction, hydrolysis, precipitation, polymerisation and degradation by means of UV radiation) or microbiological (biodegradation or mineralization) (Rubinos *et al*. 2007). Landfarming gained popularity when environmental concerns associated with uncontrolled disposal became apparent. Environmental regulations were established and applied in North American and Europe that aimed at minimizing the risk of air and groundwater contamination (Environment Canada 2009). Landfarming was widely used because of its simplicity and relative cost effectiveness when compared to other treatment methods (Pearce & Ollermann 1998).

Despite the advantages and benefits, landfarming has physical, chemical and biological ramifications and limitations. One such physical constraint is the mobility of the contaminants in the soil which can not be controlled. Chemical limitations include toxicity to soil organisms, transformation and partitioning of the petroleum and diesel fuel waste products under different

environmental conditions. Landfarming requires a sizeable area for the treatment of contaminated soil which leads to a higher risk of exposing the environment to pollutants (Maila & Cloete 2004).

Recently environmental management systems have attempted to provide waste disposal and treatment in accepted manners for preventing harm of waste products to the environment, inside and outside industrial facilities. This was not always the case and resulted in many areas contaminated with hazardous substances which today can be referred to as 'historically contaminated sites' (Maila & Cloete 2004). Long term studies by Loehr & Webster (1996) confirmed that with the passing of time some chemical concentrations continue to decline after remediation processes have stopped and toxicity is gradually reduced. Even though significant concentrations of residual chemicals may still be present in treated soils, the mobility, toxicity and related risks of these chemicals have been reduced to a large extent (Loehr & Webster 1996). Techniques and methodologies must be developed to assess the extent of contamination and toxic risks involved to establish whether remediation of the contaminated area have rendered levels acceptable for the intended land-use.

A historically landfarmed site was identified at a South African petrol refinery and further investigated during the present study. The landfarming was executed at the site by ploughing petroleum and refinery waste into the soil to a depth of 200mm. The refinery waste is called American Petroleum Institute (API) -sludge because of the API separation technology used for separating the waste from reusable water and oil (Punnaruttanakun *et al*. 2003). It contained approximately 15% hydrocarbons, and unknown levels of heavy metals. When excessive quantities of sludge were ploughed into the soil, the contamination level became too high for effective biodegradation to take place. This led to increased toxic quantities of heavy metals, petroleum hydrocarbons and PAH levels to which vegetation and soil organisms were exposed. Analyses of soils from this site (sampled between October 1993 and June 2000) have showed high levels of total petroleum hydrocarbons (TPHs), heavy metals and PAHs (Personal Communication). In 2000 the soil was treated with activated lime for additional bioremediation purposes. This was done to raise the pH of the soil in order to immobilise the heavy metals and prevent its uptake by soil organisms when the metals are converted to insoluble hydroxides (Personal Communication). In another effort to remediate the landfarming site, fertilizer was ploughed into the soil. This also resulted in aeration to increase biological activity and thus enhance the biodegradation of the toxic organic contaminants (Personal Communication). Khaitan *et al.* (2006) argued that because of petroleum waste's low solubility in water and the presence of a separate hydrocarbon phase, the remediation of these contaminants in soil is still a continuing challenge.

## 1.2. Industrial waste classification and risk limits

Increased industrial development results in an increase in waste that raises various environmental concerns of which contaminated sites are one. Contaminants that exceed regional background levels must be remediated or treated to prevent negative impact or damage to the environment. The idea of remediation is to reduce the contaminated substances and to gain environmental benefits like future land use and cleaner ground and surface water. Remediation processes can also have a negative impact on the environment when potential pollutants are mixed with clean soil or water (Rushton *et al*. 2007). The choice of the remediation techniques to be used have traditionally focused on the extent of cleaning required, the extent and duration of the clean-up period and the economical resources available (Andersson 2003).

In developed countries regulations have been implemented to assess soil quality and establish the environmental and human risks of soil contamination before and after remediation. Various countries in the European Union, Canada and the United States of America (USA) developed soil and groundwater quality guidelines. The guidelines are used to determine if toxic levels of contaminants are present in soil or groundwater. It generally prescribes a maximum allowed concentration for single contaminants or sets of values for specific substances under different conditions in soils or other media (Paton *et al*. 2005). Further, it assists to indicate which methods of remediation should be applied under different conditions (Augulyte *et al*. 2008). However, the assessment approaches for determining contaminated soils differ between guidelines. The United States Environmental Protection Agency (US EPA) determines the level of soil contamination by comparing site-specific contaminant levels to concentrations determined under standardised conditions. The Canadian guidelines suggest adjusting the standardised values according to a specific site's conditions. In The Netherlands the guideline for assessment of soil quality does not only consider physico-chemical variables but also ecotoxicological data (Paton *et al*. 2005).

In South Africa all waste disposals should comply with the standards of the minimum requirements for the handling, classification and disposal of hazardous waste set out by the Department of water affairs and forestry (DWAF) (Department of Water Affairs and Forestry 1998). Thus, waste should be classified before it can be disposed. The objectives of waste classification according to the DWAF requirements are to firstly distinguish between general and hazardous waste by determining the single most hazardous compound in the waste. Secondly, the degree of hazard it

poses to the environment and lastly rating of hazardous waste according to the degree of hazard and set requirements.

### 1.2.1 Hazardous- and General waste

According to the DWAF requirements (Department of Water Affairs and Forestry 1998) waste is divided into two categories considering the risk it poses to humans and the environment, namely general waste and hazardous waste.

General waste can be defined as any waste that does not pose a threat to the public health or the environment when properly managed. Examples include domestic, commercial and certain industrial wastes. General waste can also consist of hazardous substances but at such small quantities that it can be disregarded for being harmful to the environment (Department of Water Affairs and Forestry 1998).

Hazardous waste is identified as waste that has the ability to have significant adverse effects on public health and the environment because of its toxicological, chemical and physical characteristics. Hazardous waste may be organic or inorganic and in some cases, even at low concentrations, give rise to acute in chronic effects in living organisms. Such waste is normally generated during commercial, industrial or agricultural practices.

### 1.2.2. Risk limits

The concentration of contaminants that leaches into can be expressed as the Estimated Environmental Concentration (EEC) factor. The EEC indicates if the contaminant concentrations in the soil are higher than concentrations where it will start to pose risks to the environment and whether it should be further remediated (Department of Water Affairs and Forestry 1998). The DWAF guideline, on handling and disposal of contaminants, includes a list of potential water contaminants and the maximum allowed concentrations for individual compounds, called the acceptable risk limit (ARL). ARLs are determined by multiplying the contaminant concentration, where 50% of specific organisms (aquatic, terrestrial or mammalian organisms) exposed within a given time period are killed (expressed as $LC_{50}$), by a safety factor. In aquatic systems the safety factor is equal to 10. Thus, ARL= 0.1 $\times LC_{50}$. By comparing the concentration of a specific

contaminant (EEC) to the ARL it will indicate the aquatic or terrestrial system is at risk (depending on the $LC_{50}$-value used). If the EEC is lower than the ARL it is considered not to pose a threat to the environment (Department of Water Affairs and Forestry 1998). Previous toxicity studies have shown that organisms exposed in water media are affected more than in soil media. This was due to the decrease in available contaminants in soil as a result of adsorption to the organic matter and mineral clay fractions of the soil (Sverdrup *et al*. 2001; Didden & Römbke 2001). The list of ARL values, of potential toxicants, in the DWAF guideline were calculated using $LC_{50}$-values of mammalian exposures to contaminants in water. However, ARL values for soil organisms exposed to toxicants in a soil media are lacking in the guideline.

## 1.3. Assessment of soil toxicity using bioassays and chemical analyses

By applying only chemical analyses during the assessment of contaminated soils, the information gained are insufficient to determine the risk of pollutants on soil ecology and exposed organisms. Chemical analyses of polluted soils alone do not allow the integration of chemical mixtures, their combined effects or bioavailability on soil systems (Van Gestel *et al*. 2001; Sverdrup *et al*. 2001; Augulyte *et al*. 2008). A proposed method for incorporating bioavailability and direct toxic effects of contaminants on soil biota was to make use of bioassays together with chemical analyses (Henner *et al*. 1997; Lanno *et al*. 2004). The controlled exposure of soil organisms to specific levels of contaminants may elucidate the environmental impact of pollutants and the extent of bioremediation that might be needed to alleviate soil toxicity.

The first step to assess polluted sites and whether remediation was successful is to measure the total concentration of specific contaminants in the soil using chemical extraction methods (Song *et al*. 2002)**.** After chemical analysis biological risk assessments are included to incorporate the physico-chemical properties together with acute or chronic effects of toxicants in the soil to organisms. The toxicity of contaminants may differ between dissimilar soil types that affect their bioavailability. The methodology for assessing toxic risk should be versatile enough to be used generically but should still consider the influence of site specific conditions. Biological assessments are complementary to the chemical analyses and should be optimized so that they are easy to use and sensitive. Standardised methods for various bioassays were developed by the OECD (Organization for Economic Cooperation and Development, Europe) and the ISO (International

Organisation for Standardization). These standardised bioassays are used to evaluate acute and chronic toxicity for various test soil organisms. One of the most important requirements for good risk assessment using bioassays is the selection of representative test species (Plaza *et al*. 2005). The test species must be well studied organisms with regards to their function, taxonomy, life cycle and route of toxicant exposure (Løkke & Van Gestel 1998). The sensitivity of species to soil contaminants often varies. Some soil species are more sensitive to certain chemicals than others. For this reason it is important to use more than one soil organism when doing biological assessments (Davies *et al*. 2003). Previous studies on the toxicity of oil-contaminated soils showed that the survival of the earthworm species, *Eisenia fetida*, was a sensitive endpoint when compared to the oil contaminants' effects on organism in other bioassays, including micro-soil organisms (microtoxicity) and plants (phytotoxicity tests) (Dorn & Salanitro 2000).

*Eisenia*- (earthworms), *Enchytraeus*- (potworms) and *Folsomia*- (springtails) species are some of the standardised soil organisms commonly used in bioassays.

Litter dwelling earthworms and potworms generally inhabit organic matter rich areas (Spurgeon *et al*. 2003)**.** Using these organisms in bioassays can have some limitations. Possible constraints may be that they lack ecological relevance when compared to deeper soil dwelling species that will most likely be more exposed to soil contaminants (Dawson *et al*. 2007). Because both worm species are compost–dwelling organisms (epigeic) rather than soil dwelling, a third test species has to be considered. Although the use a soil dwelling earthworm like *Lumbricus terresteris* would be ideal for soil studies, these organisms have much longer life cycles (Kula & Larink 1997), minimal success in laboratory bred cultures and little to no standard tests are available for them (Hanna & Weaver 2002). Another test organism known to be sensitive to hydrocarbon contamination, found at high concentrations in petroleum and oil waste products, was a springtail spp. *Folsomia candida* (Paumen 2009).

Plant growth in oil polluted soil is generally delayed by the direct toxic effects of the different compounds on the plant tissue (Udo & Fayemi 1975). The hydrocarbons in oil degrade the soil by reducing soil fertility and changing the composition of soil micro-organisms. This has indirect effects on plant growth and other physiological processes (Ogbo 2009). Physiological changes in plant growth and germination success include effects such as the suffocating and dehydration of the plants. The blocking of the stomata which reduces transpiration, a reduction in the rate of photosynthesis and a negative effect on the success of germination and root systems growth (Baker

1970; Sharma *et al*. 1980; Ogbo 2009). Although soils have the ability to adsorb oil and hydrocarbons, it has been shown by previous studies that it still has the ability to have negative effects on plants.

Plants have a higher tolerance when exposed to oil and hydrocarbon contamination than soil invertebrates and microbes (Blankenship & Larson 1978; Dorn *et al*. 1998; Adam & Duncan 1999; Adam & Duncan 2002). The degree at which plants are affected by the contaminants varies greatly depending on the soil type, plant species and the concentration of contaminants in the soil (Ogbo 2009). The concentration at which a plant is significantly affected is called the effective concentration or phytotoxic level and differs among species (Ogbo 2009). Alterations in germination and plant growth are good indicators of the physiological effects of the soil contamination.

The root systems of plants, being in direct contact with the contaminants, play an important role in their ability to tolerate contamination by toxic substances (Adam & Duncan 1999). The roots create a rhizosphere for micro-organisms which are essential in breaking down toxicants. Grass rhizospheres have shown to effectively break down some of these toxicants (Adam & Duncan 1999).

## 1.4. Mixtures of refinery waste products in soil

Petroleum refinery waste products (in the form of sludge) contain a mixture of contaminants such as metals, TPHs, VOCs and PAHs at levels that pose a potential threat to the environment and human health. These threats include carcinogens, endocrine- and metabolic disruptions (Carpenter 2002). Overall, various components in mixtures, like in the case of petroleum refinery waste, have complex interactions and effects on the toxicity of its single components (Gong *et al*. 2001; Augulyte *et al*. 2008) Toxic effects of refinery waste on soil organisms may not necessarily be caused by a single substance in the sludge but rather by a combination of the various components of the mixture. Another fact to consider is that the constituents of a waste mixture are dependent on the materials and methods used in petroleum refining processes and may vary over time.

Although soil properties and bioavailable contaminants are site-specific and single contaminants are not necessarily responsible for the mixed contaminants' toxicity (Paton *et al*. 2005) it is still important to understand the potential toxicity of the different groups of contaminants.

### 1.4.1. Metal toxicity

Metals are accumulated in soils and do not have the ability to degrade but can be immobilized through remediation processes. By immobilising the metals it inhibits their availability for uptake and interaction with soil organisms. Thus, metal polluted site-soils that have been remediated may still have high levels of metals present but at less bioavailable levels (Van Gestel 2008). It is known that metal toxicity is not only dependent its chemical concentration in soil and more factors such as soil pH, organic matter- and mineral clay content influence its bioavailable fraction. When the soil pH is increased the metals have the ability to displace ligands (like organic matter and mineralized clay) bound $H^+$ ions making them less bioavailable. Further, the metal speciation also plays a role in its bioavailability (Spurgeon *et al*. 2006). At a lower pH the metals are present in ionic form and easier for uptake and accumulation in organisms' body tissue. The partitioning of metals between soils and organisms are not only dependent on the pH but also the solubility and speciation of the metals (Janssen *et al*. 1997). Experiments done by Spurgeon *et al.* (2006) displayed that the survival of earthworms (*Lumbricus rubellus*) exposed to metal polluted soils, mainly cadmium, lead and zinc, decreased. However, by altering the soil, when increasing the pH with one unit (pH + 1), the survival success was improved. On the contrary, the metal uptake to the earthworms' body tissue did not significantly differ at different pH's suggesting that it is not the only factor for its bioavailability. Even though the relationship between bioavailability of metals and the physico-chemical conditions of soil is well studied, their direct relationship is far from being understood (Van Gestel 2008).

### 1.4.2. Hydrocarbon toxicity (TPHs and PAHs)

Toxicity tests with hydrocarbons are problematic. Similar to metals, there are no correlation between the hydrocarbon concentrations in soil and its toxic effects. This is due to the fact that they usually occur in complicated mixtures. Thus, the reduction of hydrocarbon concentrations in soil does not correlate with a decrease in ecotoxicity (Salanitro *et al*. 1997; Dorn & Salanitro 2000).

PAHs are a group of organic compounds (Appendix B) that consist of two or more fused aromatic rings rendering them to be stable and resistant towards biodegradation (Haritash & Kaushik 2009). These properties of PAHs cause them to have a greater risk for bioaccumulation and carcinogenic effects to soil animals and plants (Eom *et al*. 2007). Natural sources of PAHs are

volcanoes and forest fires but most industrial processes involving pyrolysis or incomplete combustion of organic materials also emit PAHs (Haritash & Kaushik 2009). PAHs are known to be precursors of mutagenic derivatives or endocrine disruptors (like naphthalene) and are widely occurring in natural media such as soil, sediment, water, air and plants. Since most PAHs are highly hydrophobic, their pathways of transfer through geological and biological media are complex and far from being understood (Henner *et al*. 1997).

Cornelissen *et al.* (2005) suggested that, because of their hydrophobicity, TPHs and PAHs have the ability to bind to organic matter that lowers their bioavailability to soil organisms. By studying the bio-concentration (partitioning process between the chemicals in the soil pore water and the internal phases in organisms) and accumulation of the TPHs and PAHs in the soil organisms are perhaps the first steps towards understanding their environmental risk. Van Brummelen *et al.* (1998) explained that another reason that hydrocarbon toxicity vary is because it may be phototoxic, thus becoming more reactive in sunlight, with the result that soil organisms become more susceptible to the contaminants under natural field exposures compared to exposures under laboratory conditions.

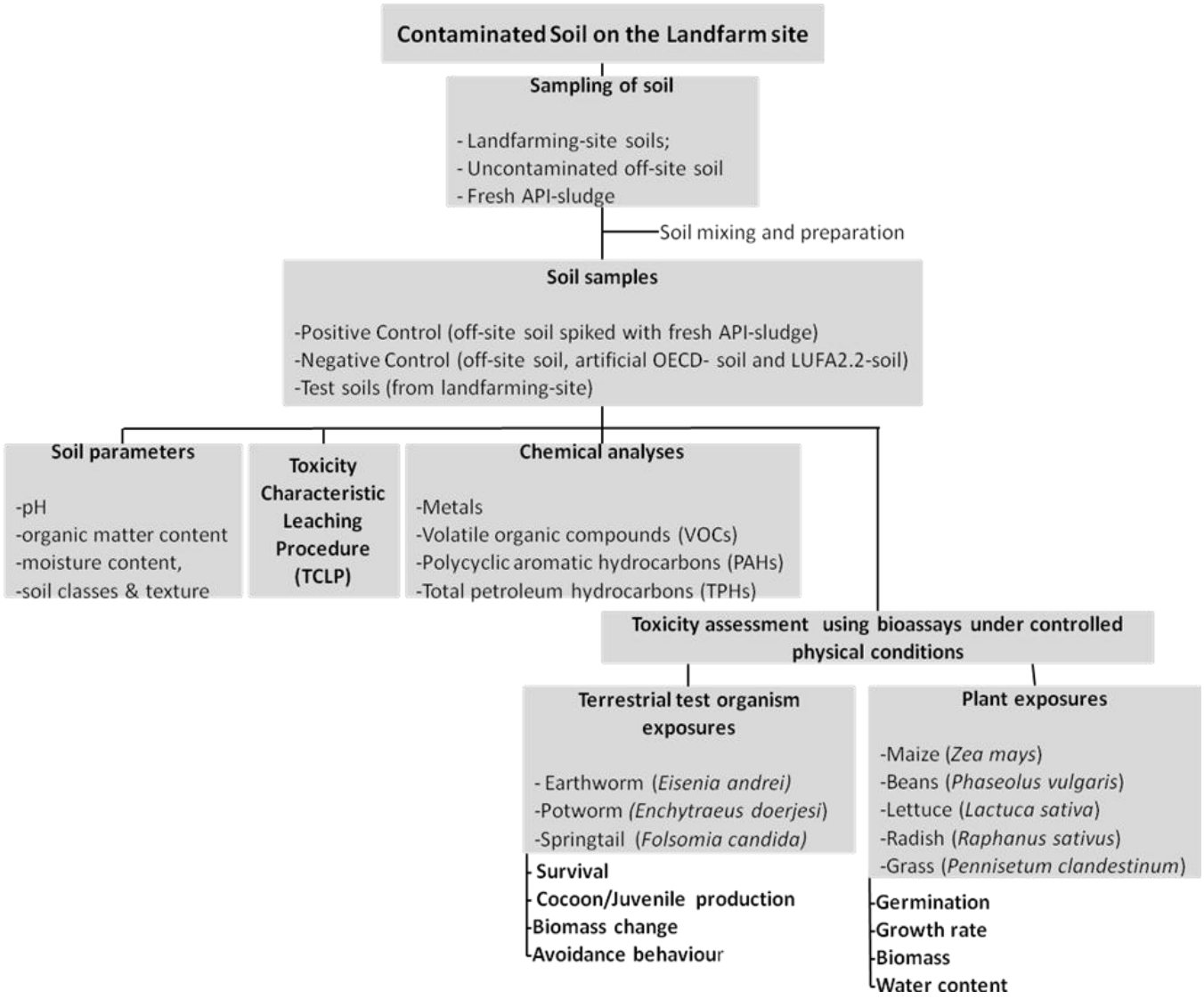
## 1.5. Aims

During this project an environmental assessment of a historically landfarmed site, situated at a South African refinery, was made. Effects of potentially contaminated soil were investigated using bioassays together with chemical analysis to establish whether further remediation techniques were needed and whether further landfarming should be allowed in future. The null hypothesis states that because landfarming was discontinued and remediation methods were applied to the site, the soil will not be toxic to exposed biota.

The specific aims were to:

1. Collect soil samples from the landfarming site as well as from a control site and analysing these soils physically and chemically.
2. Determine the chemical composition of the contaminants still present in the landfarming site soils compared to the API-sludge.
3. Establish effects of contaminants in the landfarming site soil on the survival, growth, reproduction success and avoidance behaviour of three standardized test soil animals (earthworm, potworm and springtail).
4. Establish effects of API-sludge-spiked control soils on the survival, growth, reproduction success and avoidance behaviour of these three soil organisms.
5. Establish effects of API-sludge-spiked control soils on germination success, early growth rate and biomass of five different plant species (beans, maize, lettuce, radish and grass).
6. Predict the contaminant or group of contaminants in the landfarming site soil potentially responsible for the toxicity to biota.
7. Assess whether the concentrations of individual compounds in the landfarming site are within the acceptable risk limits (ARL), according to DWAF requirements, for safe landfarming to be continued in future.

## 1.6. Project design



# 2. Materials & Methods

## 2.1. Field site and sampling design

The sampling site chosen for this study was identified on a historic landfarming site based at an inland oil refinery. It is situated 150km south of Johannesburg in the Sasolburg area, South Africa (Figure 1). The refinery refines up to 90% of its crude oil to petrol, diesel and paraffin products. The waste from the oil refinement process is a mixture that consists of an oil layer and a water soluble fraction that are pumped from the refinery waste outlet during waste/water treatment and the cleaning of oil storage tanks. API-separation is based on the principle of oil floating on water which allows the separation of reusable oil and water that are pumped back to the refinery. A third layer forms between the oil and water, called API-sludge. This layer is inseparable because of compounds present that have both hydrophobic and hydrophilic properties (Shie *et al*. 2000; Punnaruttanakun *et al*. 2003). From the refinery the API-sludge are pumped into a sludge dam with a volume of 4233 $m^3$. The landfarming site of interest is where the refinery disposed of the waste in the form of the API-sludge. Taken from the sludge dams, the API-sludge was worked (ploughed) into the soil of the landfarming site (5.9 hectare) which is approximately three times the volume of the sludge dam. The volume of the landfarm was determined by taking into account that the sludge is worked into the soil to a depth of 200mm. The sampling of the site was designed by using various standardized soil sampling guidelines (Tan 2005; Environment Canada 2nd Draft 2009).

The site was divided preliminary into two possible homogeneous strata based on the basis of colour and smell of the soil. Systematic sampling, where the sampling points were taken from evenly spread points on the field, was carried out. Thereafter compositing (mixing of soil samples) of heterogeneous soils were carried out. It enables one to obtain reliable means for a large number of soil samples. Compositing can only be done when soil types are the same for all samples of the strata. The larger the number of sampling points taken from the field and mixed, the better it will represent the whole of the area sampled.

A grid was drawn over the landfarming site and 4kg soil was collected from a depth of 150mm at each of the 34 sampling blocks. Each individual sample was put into plastic Ziploc® bags and taken to the laboratory for further mixing and preparation. Although most sampling protocols suggest that sampling should be done in dry seasons or early autumn, seasonal effects at time of soil

sampling are minimal (Environment Canada 2nd Draft 2009). All sampling were carried out in May 2009 (late autumn and dry season).


**Figure 1:** Map of South Africa showing the relative position of Sasolburg where the sampling site is situated. Retrieved from Aneki (2005).

**A.**


**B.**


**Figure 2: A.** Aerial view of the refinery and landfarm. The landfarming site is indicated by the X**. B.** Close-up of the landfarming site. The area marked with the arrow and cross indicates the site where the off-site soil (control soil) was collected. Retrieved from Google Earth (2010).

## 2.2. Substrates

### 2.2.1. Field soils (site-soils and off-site soil)

While the sampling on the landfarming site was undertaken, observations could be made of the colour, texture and smell of the soil on the smaller northern-site of the landfarm (separated from the southern part by a gravel road). The soil was distinctly darker with a more petrochemical smell than that of the bigger southern-site soil. Soil samples from the north- and south-sites were mixed separately. Bags with samples a, b and c (Figure 3) from each row (A, B, C etc...) were sieved using a 36mm mesh size sieve to get rid of loose rocks, bigger particles as well as excessive plant material. The samples were mixed into buckets labelled A1, A2, B1, B2, C1… etc. Thus 34 samples were taken on the site and mixed into 12 representative samples. The 12 samples were then mixed further by adding A1, A2, B1 and B2 that represent north-site soil and all samples of C, D, E, and F to represent south-site soil. These two samples will be referred to as the 'site-soil samples' in further discussions (Figure 3). Due to the fact that the north-site is only a third of the landfarming site and the south-site two thirds, the 34 samples taken over the whole site were proportionally divided so that the north-site soil were mixed using soil from 12 sampling points and 23 representing the south-site. An 'off-site soil' sampling site was identified close to the landfarm. Soils obtained from this site served as a control soil (reference soil) (Figure 2). Even though the off-site soil sampling site was close to the landfarm it had no signs of recent industrial or agricultural activity, and would potentially have the closest possible relation to the soil properties of the natural soil in the region. The site-soils and control soils should have similar soil properties because texture and organic content could influence the performance of the test species during ecotoxicological evaluation.

Ten litres of API-sludge were also collected from random sampling points in the sludge dams, mixed and used in the laboratory toxicity tests to obtain a positive control in the bioassays.

The API-sludge was used to spike negative control soils so that a positive control could be obtained. According to the OECD protocols (OECD 2004b) it is important to have a positive control where an evident effect could be observed so that the experimental results of the test soils can be compared to it.

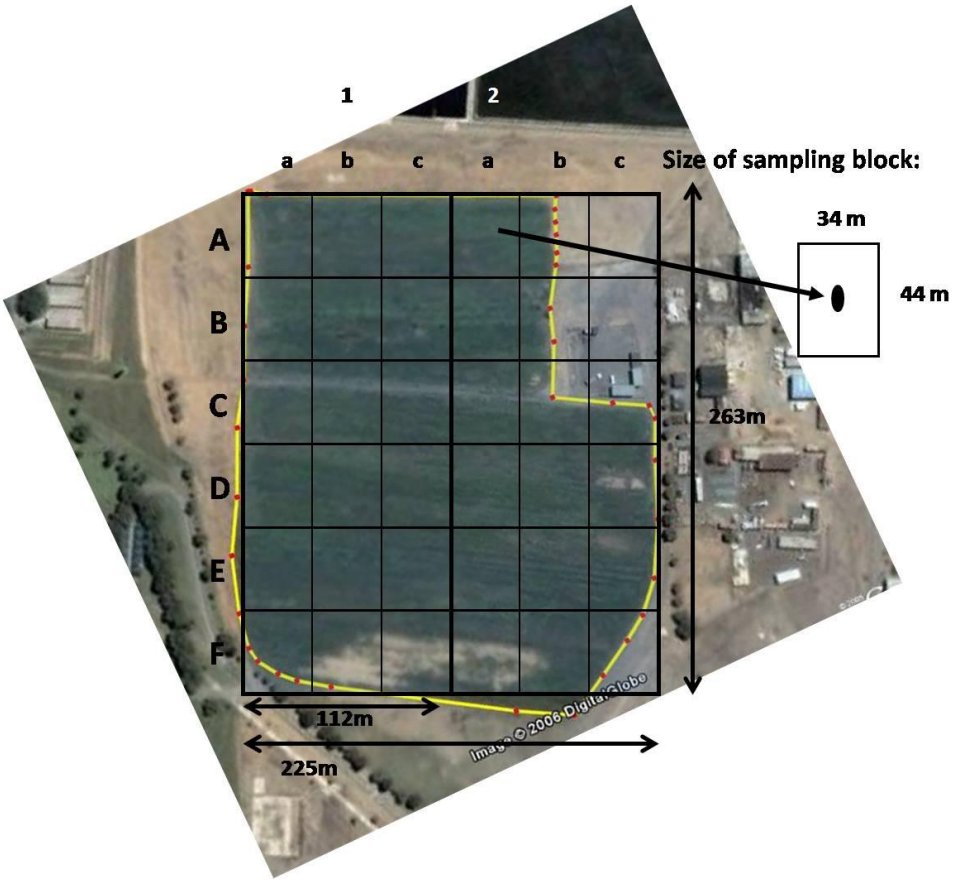



**Figure 3:** Sampling grid drawn over the site indicating the sampling areas. Samples were taken where the grid overlapped the landfarming site. Blocks in row A and B represent the north-site (10 sample sites) and rows C, D, E and F represents the 24 sample site blocks of the south-site. Retrieved from Google Earth (2006).

### 2.2.2. Additional control soils

Because it is known that varying soil components such as mineral clay and organic matter content influence the toxicity and bioavailability of toxicants to soil organisms, two additional control soils were also used for comparison purposes. The two control soils used were OECD-soil and LUFA2.2-soil.

#### *2.2.2.1. OECD-soil*

The OECD control soil was an artificial soil prepared according to the OECD protocol (OECD 2004b). It consists of 35% fine sand, 35% coarse sand (Consol (Pty) Ltd., South Africa), 20% Kaolin clay (Serina Kaolin (Pty) Ltd, South Africa) 10% Sphagnum peat moss (<1mm) (Nirom Peat Moss Inc., Canada).

#### *2.2.2.2. LUFA2.2-soil*

The control soil known LUFA2.2 was imported from Speyer, Germany. It is a commercially available natural soil commonly used as control soil in ecotoxicity tests in Europe. For standardized toxicity tests, guidelines suggest using soils with known characteristics (Römbke *et al*. 2006). For this purpose LUFA soil is a recommended natural and standardized soil type alternative to the OECD-soil with consistent soil properties such as organic matter content, particle size distribution and pH-values (Lufa-Speyer 2010). LUFA2.2 soil is not a mixture of single components (like in the case of the OECD-soil), but natural soils from selected areas of agricultural fields in Germany. This soil should have had no applied pesticides, biocidal fertilizers or organic manure for at least 5 years. The soil was sampled from 0-20 cm depth, prepared and sieved with a 2 mm mesh sieve. LUFA2.2-soil is realistically comparable with field conditions and composition of the site soils for the present study. It is characterised as 'loamy sand' according to the United States Department of Agriculture (USDA) (Appendix A, Figure 1).

Table 1 compares the different properties and characteristics of the artificial OECD soil to the natural occurring LUFA2.2 test soil. All tests soil that will be used in this study is summarised in Table 2.

**Table 1:** Comparison of the soil properties for the two additional control soils.

| | Artificial soil (OECD-soil) | LUFA2.2 soil |
|---|---|---|
| **Maximum water holding capacity (%)** | 60±5.0 | 46.5±6.0 |
| **pH (1M KCl)** | 6.0±0.5 | 5.5±0.1 |
| **Particles sizes and classification (see Appendix A, Figure 21)** | | |
| **Clay (<0.002mm) (%)** | 20±0.0 | 6.4±0.9 |
| **Fine sand (0.002-0.05mm) (%)** | 35±0.0 | 11.6±0.7 |
| **Coarse sand (0.05-2.0mm) (%)** | 35±0.0 | 82.0±0.7 |
| **Organic content (%)** | 10±0.0 | 2.09±0.4 |
| **Soil Type** | Sandy Loam | Loamy Sand |

**Table 2:** Summary of soil samples collected and prepared to be used as substrates in the laboratory experiments.

| | Soil substrates | Description |
|---|---|---|
| **Site-soils Landfarming site samples collected from the sludge disposal landfarming site** | North-site soil | Collected from the northern part of the landfarming site that was visibly more contaminated (darker colour) and with a distinct petrochemical smell |
| | South-site soil | Collected from the larger southern part of the landfarming site that was visibly less contaminated |
| **Negative control soils** | Off-site soil | Collected from a site adjacent to the landfarming site with no history of previous industrial or agricultural activities |
| | OECD-soil | An artificially compiled soil made under strict laboratory conditions according to the OECD guidelines (OECD 2004b) |
| | LUFA2.2-soil | LUFA2.2 soil is commonly used in European toxicity assays collected from a site in Germany. It is a well studied natural soil with optimal soil properties. |
| | Potting soil | Commercially available soil obtained from a local nursery to be used as the substrates for plant exposures. |
| **Positive control soils** | Fresh API-sludge-spiked soils | Negative control soil spiked with API-sludge at such a concentration where a definite effect is visible in the soil organisms. |
| | Concentration series | Negative control soils spiked with different concentrations of fresh API-sludge |

## 2.3. Chemical analyses of PAHs and heavy metal content in the soils

Chemical analyses were carried out on all samples. From the chemical analyses the concentrations 40 tested metals, 16 United States Environmental Protection Agency priority polycyclic aromatic hydrocarbons (16 US EPA priority PAHs) and a range of volatile organic hydrocarbons (VOCs) were quantitatively determined. The chemical soil analyses were analysed at Setpoint (PTY (LTD.) Midrand, Gauteng, South Africa. Different techniques for the analysis of the various compounds were carried out as prescribed by U.S. EPA (U.S.EPA 1996). The metals were detected and quantified using the 32 element scan and inductively coupled plasma mass spectrometry (ICP-MS) analysis. VOCs were determined using GC-MS purge-trap-technique. PAH were determined with the GC-MS solvent extraction technique (acetone/hexane extracts). Where possible, analyses were done in quadruplicate and the mean values were used unless stated otherwise.

## 2.4. Substrate preparation

Possible varying parameters such as pH and moisture content were measured and controlled throughout the whole project. Before any tests the soils were dried at 60 °C for 48 hours (Gallenkamp size one oven BS, model OV-330, Weiss-Gallenkamp Ltd., Loufhborough, U.K.) and sieved (2mm mesh sieves). The pH was measured using the pH-KCl method as stated in the OECD protocols (OECD 2004a). 5 grams of soil from each substrate was weighed and 25ml 1M KCl solution made up in distilled $H_2O$ was added. The samples were then mixed for 5min and left for 2 hours. After a further 5 minutes of mixing the pH was measured (Crison, Micro pH 2001 pH-meter). All pH measurements were done in triplicate and determined before and after each experiment to confirm consistency. Water retention, moisture content and water holding capacity (WHC) were established by using a moisture determination meter (Sartorious electronic moisture analyzer, Model MA45). 5g of test soil were placed in a plastic tube that was covered at one end with wet filter paper. The tubes were submerged vertically into a water bath to a level covering the whole tube but leaving the top of the tube above the water level so that the water can not enter the tube from the top. The tube remained in the water bath for 3 hours thereafter it was placed vertically, with the filter paper covered side at the bottom, on a bed of wet sand to drain for 2 hours. The vessels were covered with a lid at all times to prevent moisture loss through evaporation. The wet samples were

weighed and dried at a constant temperature of 105°C until the sample was completely dry. The WHC was determined as follows:

$$\text{WHC}\,(\%\ \text{of dry mass}) = \frac{\text{Wet mass} - \text{Dry mass}}{\text{Dry mass}} \times 100$$

The total organic matter content in the soil samples was determined by using the Walkley-Black wet combustion method as suggested in (Tan 2005). The clay (µm), silt (µm) and sand (µm) content of all samples were determined using the hydrometer method (Day 1956; Van der Watt 1966). All particle size distribution analyses and organic matter content determinations were carried out at BemLab in Somerset-West, South Africa. See Appendix A for a detailed description of these methods.

## 2.5. Test organisms

Three soil organisms from different taxonomic groups were used in the bioassays namely; *Eisenia andrei* (earthworm), *Enchytraeus doerjesi* (potworm) and *Folsomia candida* (springtail).

### 2.5.1. *Eisenia andrei* – Earthworm

#### *2.5.1.1. Classification*

| | |
|---|---|
| Phylum: | Annelida |
| Subphylum: | Clitellata |
| Class: | Oligochaeta |
| Order: | Haplotaxida |
| Suborder: | Lumbricina |
| Superfamily: | Lumbricoidea |
| Family: | Lumbricidae |
| Subfamily: | Lumbricinae |
| Genus: | *Eisenia* |
| Species: | *Eisenia andrei* Bouché, 1972 |

### *2.5.1.2. Morphology, life cycle and ecology*

Earthworms are the most commonly used animals as a representative of soil fauna because of their high abundance and biodiversity in various soil types (Dawson *et al*. 2007). Earthworms' primary ecological function is to decompose organic matter by incorporating plant litter deeper into the soil and thus mixing the organic and inorganic soil fractions.

*Eisenia andrei* is not a typical soil dweller but rather an epigic species confined to upper soil layers with a high organic matter content (Sims & Gerard 1985). Under natural conditions, these worms live optimally in soils with a pH ranging from 4.5-7.5. They inhabit damp and rotting vegetation in woodlands, wet decaying leaf litter and manure heaps. The earthworms feed on the microorganisms and protozoans in the organic matter content of soil. When culturing these worms in a controlled environment, they can be fed anything from shredded paper, manure and food waste products. Species of the genus *Eisenia* are commonly used in ecotoxicology and the recommended test organisms when establishing soil toxicity according to ecological guidelines (OECD 2004b). Originally *Eisenia* species were used in toxicity tests because of their relatively short life cycles and high reproduction rate that made them ideal test organisms in laboratory studies. Further they proved to act in a similar manner to other test species if the mode of the toxic action was the same, making them good representatives for most earthworms (Kula & Larink 1997).

Two common *Eisenia* species, *Eisenia andrei* and *Eisenia fetida* are generally used in ecotoxicity studies. Mature *Eisenia* species have an average length between 60-120mm, diameter 3-6mm and 80-120 segments. The body is cylindrical, becoming trapezoidal posterior. Colour between individuals varies from light pink to wine red. Mostly the worms are all uniformly pigmented but in some cases the dorsal parts of the worms appear to be striped. A saddle-shaped clitellum covers six to eight segments starting around segment 24 or 25 up to 30 or 33.

*E. andrei* can be biparental (able to reproduce by the exchanging of genetic material with another worm of the same species) and to a lesser extent also uniparental (no sexual fertilization from a second worm) (Edwards & Lofty 1977). In *Eisenia* species cocoons can be produced within four days of mating and be continued for more that 500 days at an average rate of between 3.5-18.5 cocoons every 10 days (Venter & Reinecke 1988). Incubation at 25˚C results in a mean of 2.7 hatchlings per cocoon (Venter & Reinecke 1988). Sexual maturity is attained within 40 to 60 days after hatching under optimised conditions.

#### *2.5.1.3. Culturing*

*Eisenia andrei* was obtained from a synchronized culture, bred from a stock culture at the Ecotoxicology Laboratory, Department of Botany and Zoology, University of Stellenbosch. The original cocoons used to obtain the culture were provided by Professor O. Graff of Braunschweig (Germany). For the present study cocoons were harvested from the stock culture and placed into 24-well plates filled with distilled water and incubated at 20˚C to obtain worms of the same age. Hatched worms were collected on a daily basis and placed in containers with a cattle manure substrate as described by the OECD (OECD 2004b). After reaching the age of 90 days, mature, (clitellate) worms were used in all the acute, chronic and behaviour experiments.

### 2.5.2. *Enchytraeus doerjesi* – Potworm

#### *2.5.2.1. Classification*

| | |
|---|---|
| Phylum: | Annelida |
| Subphylum: | Clitellata |
| Class: | Oligochaeta |
| Order: | Haplotaxida |
| Suborder: | Tubificina |
| Family: | Enchytraeidae |
| Genus: | *Enchytraeus* |
| Species: | *Enchytraeus doerjesi* Westheide & Graefe, 1992 |

#### *2.5.2.2. Morphology, life cycle and ecology*

Enchytraeids live in the upper soil layers and play an important role in decomposition and soil forming processes. They are found in places with high levels of organic material, but can also be present in forest and crop plantation soils where the pH is slightly acidic. (Beylich & Achazi 1999; Memis *et al*. 2004). Enchytraeid populations are often higher in natural acidic soils than in soils with a neutral pH. The content of organic matter and moisture in the soil also influence their occurrence as well as their reaction to stress factors (Didden 1992). Most enchytraeid species are hermaphrodites. Eggs are laid in transparent cocoons. Although they reproduce sexually, asexual

fragmentation is also common. Cocoons take on average 7 days to hatch. The cocoons from potworms produce 10 eggs on average but each individual can produce as many as 1000 eggs over its life span. Potworms take an average of 8 days to mature to adulthood and have an average life span of approximately 90 days. Enchytraeids have the ability to reproduce normally at any temperatures above 8 to 10°C where optimal growth and reproduction occur between 15 to 21°C (Römbke & Moser 2002; Memis *et al*. 2004).

*Enchytraeus doerjesi,* the test species in this study, was first described in 1992 (Westheide & Graefe 1992). They are easy to culture under controlled conditions and fast growing which makes them ideal for ecotoxicological studies using reproduction and the number of juveniles produced as endpoints (Kramarz *et al*. 2005). *E. doerjesi* has a small body size in comparison to some of the other potworms in the genus but share many similarities with another species commonly used in ecotoxicological tests known as *E. buchholzi* Vejdovsky, 1897. The difference between the two species can be easily recognized and thus can be distinguished from each other on the basis of their spermathecal morphology. *E. doerjesi* has long non-glandular ectal ducts together with voluminous ampillae that makes it distinguishable from other species (Westheide & Graefe 1992). Specimens of *E. doerjesi* are small (4-7mm long) whitish worms consisting of between 18 and 37 segments with a pair of chaetae (brushes) on each segment and a clitellum on segment 12 to 13.

#### *2.5.2.3. Culturing*

The potworms used in this study were obtained from a stock culture at the Ecotoxicology Laboratory of the Department of Botany and Zoology at the University of Stellenbosch. The culture was started from adult worms obtained from Professor P. Kramarz of the Jagiellonian University (Poland). The stock culture was kept in commercially available nursery potting soil and fed weekly with rolled oats (Jungle oats™, Tiger brands, South Africa). Adult (clitellate) worms were selected under a microscope to be used in the bioassays.

### 2.5.3. *Folsomia candida* – Springtail

#### *2.5.3.1. Classification*

Phylum: Arthropoda
Subphylum: Entognatha
Class: Collembola
Order: Isotomoidea
Family: Isotomidae
Genus: *Folsomia*
Species: *Folsomia candida* Willem, 1902

#### *2.5.3.2. Morphology, life cycle and ecology*

Collembolans are one of the most abundant classes of arthropods on earth (Løkke & Van Gestel 1998) but because of their small size their contribution to biomass and respiration in a terrestrial ecosystem is very small. Collembolans play an important role in some soil ecosystems and are sensitive to the effects of soil contamination. Their presence and diversity are commonly used to assess the impact of soil pollutants. Collembolans are common test species used in ecotoxicological tests because of their well studied life-cycles; they are easy to culture and have a short life cycle (Fountain & Hopkin 2005). Most collembolans have omnivorous feeding habits that include feeding on fungal hyphae (in soil and leaf litter), bacteria and protozoa.

*F. candida*, and the test species used in this study, is known to be parthenogenetic (reproduce asexually) and most specimens in a population consist of females that can lay up to 1100 eggs in their lifetime (Fountain & Hopkin 2005). Sexual maturity is reached between 21 to 24 days. A mean of 50 eggs are laid at a time and take 7-10 days to hatch. Its lifespan ranges from 111 to 240 days depending of the temperature of its environment. *F. candida* is 1.5 to 3.0mm in length at adulthood and appears white or off-white in colour. They have post-antennal organs behind the base of each antenna that possibly detects airborne chemicals. In general all collembolans have a ventral tube or collophore on the first abdominal segment that allows fluid exchange with the environment. This is also the main exposure path for chemicals that dissolves in soil pore water to the organisms. *F. candida* gets distinguished from other Collembola species by having 16 stout setae on the ventral

side of the manubrium of the furca (Potapov 2001). The optimal pH for *F. candida* cultures is 5.5 and a temperature of 21°C.

#### *2.5.3.3. Culturing*

The springtails species, *Folsomia candida*, was obtained from a laboratory culture in the Ecotoxicology Laboratory, Department of Botany and Zoology, University of Stellenbosch. The culture was obtained from Professor P. Kramarz of the Jagiellonian University (Poland). They were cultured on culture rings with plaster of Paris mixed with activated carbon bottoms. The cultures were fed with baker's yeast (Gold star yeast™, South Africa) and moistened regularly ad libidum. All springtails were age synchronized before any exposures. Synchronization was done by adding 20 adult springtails to culturing rings to lay eggs. After two days the adults were removed from the containers. The eggs started to hatch after 7 days and reached adulthood in 12 to 14 days. All organisms used in this study were at an age of between 20 to 22 days.

## 2.6. *Eisenia andrei* exposures and endpoints

### 2.6.1. Experimental conditions

Earthworm exposures were done according to the protocol set out by the OECD guideline 222. (OECD 2004b) and adjusted as follows. 400g test substrates were placed into 750ml round bottom glass containers and incubated for 48h before the earthworms were added.

As food for the worms, all substrates were supplemented with dried and ground urine-free cattle manure at 0.5g per worm at the start of each experiment. The urine-free cattle manure was prepared by sun-drying for approximately one week thereafter it was ground and sieved (using a 2mm mesh sieve) before adding it to the soil. Eight individual earthworms, weighing between 300 and 500mg, were used in the exposures. Earthworms were placed in control soil (OECD or off-site soil depending on the experiment) two days prior to each experiment for acclimation before being used in the experiments. All exposures were carried out under controlled temperature and moisture conditions in a dark climate room at 20˚C. The soils were moistened to 60% of the maximum water holding capacity (WHC) and the pH was monitored at the start and end of each exposure. For

aeration, small holes were drilled into the lids of the glass containers. Earthworms were fed, and lost moisture was added two weekly until the experiments were terminated.

All soils that were used as substrates in the bioassays are presented in Table 2. The spiked concentration series were obtained by adding fresh sludge to a control soil (either off-site soil or OECD-soil) at the highest concentration in the series and 'diluting' the soil by mixing it thoroughly with clean control soil in the desired ratios.

### 2.6.2. Endpoints

#### *2.6.2.1. Preliminary exposures for optimisation of control soils and API-sludge concentration series*

Earthworms were exposed to control soils (off-site soil and OECD-soils) as well as to contaminated soils at the following concentrations: 0.5%, 1%, 2.5% and 5% API-sludge-spiked soils. Exposures were done for one week to establish at which concentration of API-sludge mortality was observed and a range of API-sludge concentrations series could be determined for further use in the biomass and chronic tests. This was also used as a preliminary study to observe whether any adjustments needed to be made to the variable conditions (pH, water- or organic content) that may influence the tests. It was decided not to spike soils with concentrations higher than 5% API-sludge because it influenced the water content and changed the consistency of the soils.

To determine whether the earthworms will survive and reproduce in the negative control soils, they were exposed to off-site soil and OECD-soil. Further, to establish if the addition of API-sludge will cause effects, and to determine if off-site soil + 1% API-sludge and OECD-soil + 1% API-sludge could serve as positive controls, earthworms were also exposed to these soils. The biomass changes, cocoon production and number of hatchlings of the earthworms exposed to control soils at specific test conditions were compared. All exposures were carried out for 4 weeks in triplicate. To determine the biomass changes the individual earthworms were weighed weekly. The cocoons were collected every 2 weeks until the experiments were terminated. All collected cocoons were incubated in 24-well plates in distilled water at 25˚C. The number of hatchlings from all these controls was compared to confirm whether this was not influenced by the soil types or toxicants (Reinecke *et al*. 2001).

### *2.6.2.2. Survival and chronic tests in the site-soils*

After the optimization of the controls and the test conditions the earthworms were exposed to the different test soils; Off-site soil, north-site soil, south-site soil and a 1% API-sludge-spiked off-site soil (positive control). Every week the earthworms were weighed and cocoons collected as prescribed by the OECD guideline 222. (OECD 2004b). The tests were terminated after 6 weeks. At the end of the test all collected cocoons were counted to determine the cocoon production success. The survival of the adult worms was also noted. Eight replicates for each test soil with 8 worms per replicate were carried out.

### *2.6.2.3. Survival and chronic tests in API-sludge-spiked control soils*

Earthworms were exposed to a concentration series of spiked API-sludge in off-site soil as well as in artificial soil (OECD 2004b). OECD-soils were spiked with fresh API-sludge at concentrations shown in Table 3. Earthworms were weighed weekly to determine biomass chances and exposures were terminated after 4 weeks. All mortalities were noted for the duration of the experiment.

To determine the cocoon production success the earthworms were exposed to a concentration series of API-sludge in off-site soil and in OECD-soil with API-sludge concentrations as shown in Table 3. These exposures were done over 6 weeks. Cocoons were collected every two weeks until the experiment were terminated. All collected cocoons were counted and mortalities of the adult worms were noted. These results were used to determine the effects of fresh API-sludge on the survival and the effects it will have on the earthworm cocoon production. The mortalities of earthworms in a concentration series were used to determine a $LC_{50}$ which is the concentration of API-sludge where 50% of the test group of earthworms will die. The cocoons collected were used to determine the effect concentration ($EC_{50}$) that indicates what concentration of fresh API-sludge will decrease the cocoon production of the test group by 50%.

**Table 3:** All concentration series used in *Eisenia andrei* exposures.

| Soil used | Concentration series of API-sludge (%) | Endpoints monitored |
|---|---|---|
| **OECD-soil** | 0; 0.5; 1; 1.5; 2 and 2.5 | Biomass change, mortalities and cocoon production |
| **OECD-soil** | 0; 0.2; 0.4; 0.5; 0.8; 1; 1.5; 1.6; 2; 2.5 and 3.2 | Mortalities and cocoon production |
| **Off-site soil** | 0; 0.2; 0.4; 0.8; 1.6 and 3.2 | Mortalities and cocoon production |

### *2.6.2.4. Avoidance behaviour*

The test method for these experiments was based on a method described in the International Orgainsation for Standardisation soil quality guideline (ISO 2003). A double chamber test was used to determine movement between test soils and control soils where the endpoint of the test was avoidance of the test soil. The tests were performed in plastic containers (8cm × 11cm × 18cm) divided into two equally sized sections with a plastic divider. Each section was filled with 200g of the specific soil in various combinations namely; a. off-site soil on both sides (negative control), b. off-site soil vs. north-site soil, c. off-site vs. south-site soil, d. off-site vs. off-site soil + 1% API-sludge (positive control see Table 2). All soils were prepared as explained in Section 2.5. The two soil substrates were placed in the different sides of the same container and separated with a divider. The divider was removed and 8 adult earthworms were placed on the middle line on the surface of the soil. The containers were then covered with a perforated lid. Exposures were carried out for 48 hours in a temperature controlled room at 20$^{o}$C in the dark. After the 48 hours the divider was put back into the middle line, the substrates removed, and the number of organisms on each side was determined. If an earthworm were found on the middle line it was counted as 0.5 for both sides of the container and missing worms or worms not found in the soil substrates were assumed to be dead. The number of organism exposed was adjusted by subtracting those that were not found or died before further calculation were carried out. Five replicates were done for every exposure.

## 2.7. *Enchytraeus doerjesi* exposures and endpoints

### 2.7.1. Experimental conditions

The exposures were carried out as described in the OECD guideline 220. (OECD 2004a). 100ml round bottom, glass jars were filled with 30g dry weight test soil each. The jars were covered with perforated lids and left for two days to stabilize. Ten adult (clitellate) specimens were added per container. Food was provided by working in approximately 50 mg of finely ground rolled oats into the top soil layer. Thereafter additional food and water were monitored weekly and amended when needed. The experiments were terminated after 3 weeks. Five replicates were carried out for every exposure unless stated otherwise. On Day 21 the experiments were terminated by adding 10ml absolute ethanol (95%) to each container. After 1 minute 100ml of $H_2O$ (30ml at a time) was added and mixed with the soil by shaking while soil was gradually transferred to 250ml plastic containers. 200µl of a 1% Bengal red solution ($M_r$= 1017.65 g/mol) was added to each container. The containers were closed with lids and shaken until mixed well. The soils were kept at 4°C overnight. After 24h the specimens were washed from the smaller soil particles using a sieve (0.088mm mesh). Specimens left on the sieve were transferred into a white (80cm × 60cm × 30cm) tray with water. The pink stained adult worms were counted and removed from the tray. The smaller juveniles were counted using a light source, counter and magnifying glass.

All exposures were carried out under controlled temperature and moisture conditions in a dark climate room at 20˚C. The soils were moistened to 50% of the maximum water holding capacity. The pH was monitored at the start and end of each experiment. Moisture and food were amended weekly, if needed. Soils that were used in the bioassays are summarised in Table 2. The spiked concentration series were obtained by adding fresh sludge to soil substrates (either off-site soil, OECD-soil or LUFA2.2-soil) and were prepared as explained in Section 2.6.1.

### 2.7.2. Endpoints

#### *2.7.2.1. Preliminary exposures for optimisation of control soils and API-sludge concentration series*

Preliminary testing was done by exposing potworms to the control soils (off-site, OECD- and LUFA2.2-soils) as well as to chosen concentrations (0.5%, 1% 2.5% and 5%) of API-sludge-spiked off-site soil, OECD-soil and LUFA2.2-soil. The exposures were carried out for 2 weeks to establish at which API-sludge concentration mortalities were observed and a range of API-sludge concentration series could be determined for further use in chronic bioassays. This was also used as a preliminary study to observe if any adjustments needed to be made to any of the variable conditions (pH, water- or organic matter content) prescribed by the OECD guidelines. All preliminary exposures were done in duplicate.

#### *2.7.2.2. Survival and chronic tests in the site-soils*

Potworms were exposed to the different test soils: off-site-soil, north-site soil, south-site soil, a 1%-, and 3% API-sludge-spiked artificial soils as positive controls (Table 2). When the exposures were terminated the surviving adults and the number of juveniles produced during the period were used as endpoints.

#### *2.7.2.3. Survival and chronic tests in API-sludge-spiked control soils*

To establish which concentrations of fresh sludge application will have effects on the survival and reproduction of the specimens, they were exposed to three concentration series of API-sludge-spiked in off-site soil, OECD-soil and LUFA2.2-soil. After range finding tests the concentration series were made up as explained in Section 2.6.1. The concentrations of soils spiked with API-sludge in off-site soil, OECD-soil and LUFA2.2 soil are shown in Table 4. This was used to determine the $LC_{50}$- and $EC_{50}$s (for reproduction) (Section 2.6.2.3).

**Table 4:** All concentration series used in *Enchytraeus doerjesi* exposures.

| Soil used | Concentration series of API-sludge (%) | Endpoints monitored |
|---|---|---|
| **Off-site soil** | 0; 0.5; 1; 1.5; 2; 2.5; 4 and 5 | Mortalities and number of juveniles (reproduction) |
| **OECD-soil** | 1; 2; 3; 4 and 5 | Mortalities and number of juveniles (reproduction |
| **LUFA 2.2-soil** | 0; 0.5; 1; 1.5; 2; 2.5; 3; 4 and 5 | Mortalities and number of juveniles (reproduction |

### *2.7.2.4. Avoidance behaviour*

Tests similar to those of the earthworms described in Section 2.6.2.4 were performed. Round bottomed plastic containers (9 cm radius and 7cm height- 1781cm$^2$) were divided into two equally sized sections with a plastic divider. Each section was filled with 30g of a specific test soil. All soils were prepared as explained in Section 2.5. Various combinations with different soils on each side of the container were performed namely; a. Off-site soil on both sides (negative control), b. off-site soil vs. north-site soil, c. off-site vs. south-site soil, d. off-site vs. off-site soil + 3% API-sludge (positive control) and e. off-site soil vs. OECD-soil. The divider was then removed and 20 test organisms were placed on the middle line between the two soil conditions. Containers were closed with perforated lids and kept at 20°C in the dark for 48hours. After 48h the dividers were replaced at the middle line and the numbers of worms counted (using a light and magnifying glass) on each side of the divider. If a specimen were found on the middle line it was counted as 0.5 for both sides of the container and missing worms or worms not found in the soil substrates were assumed to be dead. The number of organisms exposed was adjusted by subtracting those that were not found or died before further calculation were carried out. Five replicates were done for every exposure.

## 2.8. *Folsomia candida* exposures and endpoints

### 2.8.1. Experimental conditions

The reproduction tests were carried out as described in the OECD guideline 232. (OECD 2009). 20g dry weight of the test soils were placed in 100ml round bottom, glass jars for 2 days before ten synchronized adult springtails were added and the jars closed with lids. All specimens were 20-22 days old at the start of the exposures. Food was provided by adding a pinch of bakers' yeast into the top layer of the soil. During each experiment the food and lost moisture were amended and the containers aerated weekly. All exposures were executed for 4 weeks after which they were terminated. Five replicates per test condition were carried out unless stated otherwise. After four weeks the experiments were terminated by washing the contents of each jar into a 300ml glass beaker using 100ml of water. The contents were gently stirred until all surviving adults and juveniles floated to the surface because of their chitinous exoskeletons. Each container was photographed three times using a Canon EOS 350D Digital single lens reflex camera. Using Adobe® Photoshop® software (Adobe® Photoshop® 2007) the photographs were adjusted and electronically inverted (using the software) to optimise the contrast to be able to count more accurately. The same software program was used to count all collembolans present on each photograph of the soil substrates. An average of the number of organisms counted in each of the three photographs were calculated and taken as the mean number of specimens present in each test container (Figure 4).

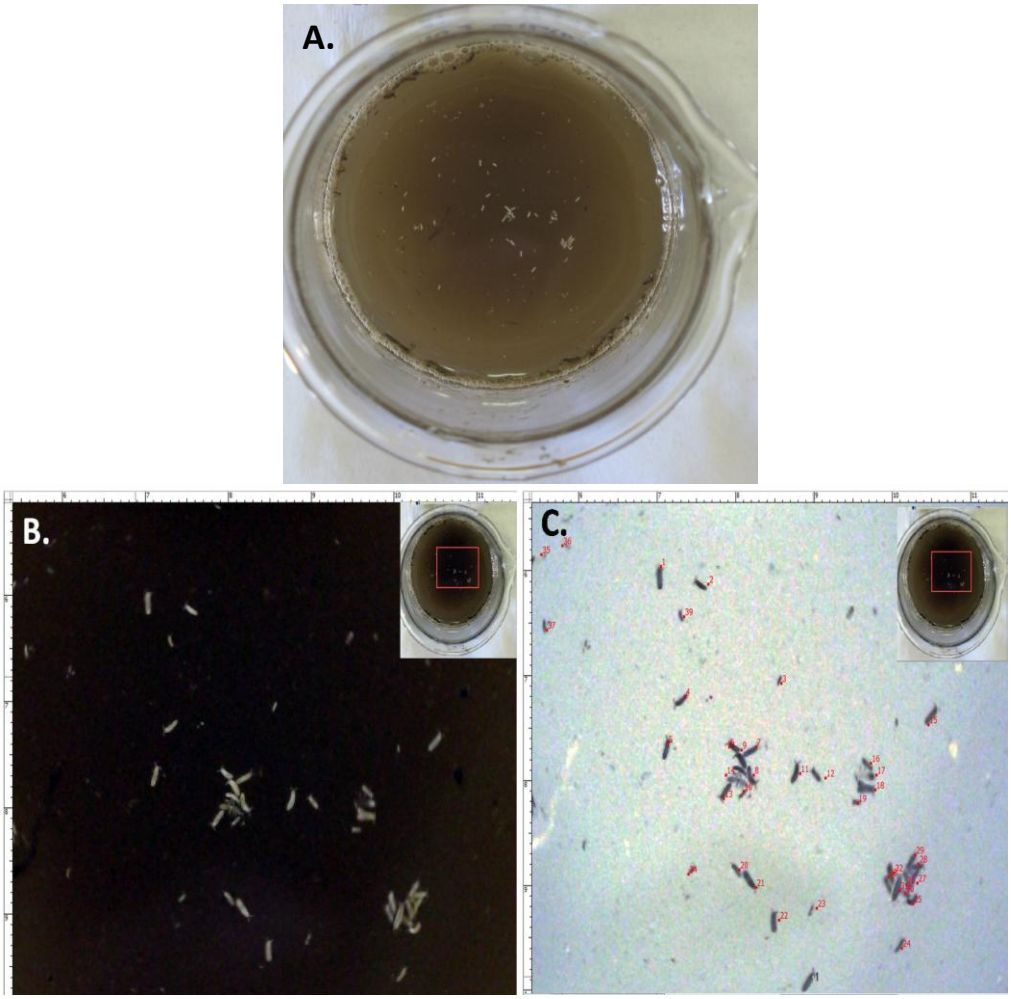



**Figure 4**: **A.** Example of a photograph used to count *Folsomia candida* juveniles at the end of the reproduction test (pictures shown is from a sample of north-site soil exposure). **B.** Enlarged (zoom) view of the same photograph shown in A. **C.** Inverted photograph to increase contrast between the soil and the organisms. Red markings on the photograph indicate the counted organisms.

### 2.8.2. Endpoints

#### *2.8.2.1. Preliminary exposures for optimisation of control soils and API-sludge concentration series*

Preliminary exposures with *F. candida* were executed in the same manner as in Section 2.7.2.1.

### *2.8.2.2. Chronic tests in site-soils*

The test conditions and exposure period were determined and optimized to meet all requirements set out by the OECD guideline for testing of chemicals. 232. (OECD 2009) for the controls. Thereafter the springtails were added to the test soils: off-site-soil, north-site soil, south-site soil, and a 1%, API-sludge-spiked OECD-soil (positive control). At the end of the experiments all organisms were counted. Reproduction of juveniles was used as endpoint in these tests.

### *2.8.2.3. Chronic tests in API-sludge-spiked control soils*

Exposures were carried out in the same manner as in Section 2.7.2.3 except for using different concentration ranges. The concentration series of API-sludge used are shown in Table 5.

**Table 5:** All concentration series used in *Folsomia candida* exposures.

| Soil used | Concentration series of API-sludge (%) | Endpoints monitored |
|---|---|---|
| **Off-site soil** | 0; 0.09; 0.20; 0.21; 0.27; 0.30; 0.33; 0.40 and 0.66 | Number of juveniles (reproduction) |
| **OECD-soil** | 0; 0.2; 0.4; 0.8 and 1.6 | Number of juveniles (reproduction) |
| **LUFA 2.2-soil** | 0; 0.08; 0.15; 0.20; 0.21; 0.27; 0.30 and 0.40 | Number of juveniles (reproduction) |

### *2.8.2.4. Avoidance behaviour*

Tests were carried out in the same manner as for *E. doerjesi* in Section 2.7.2.4. The positive control used were off-site soil vs. 1% spiked API-sludge soil. On completion of the experiment, each side of the container was visualized as explained in Section 2.8.1.

## 2.9. Plant exposures

Additionally to the soil invertebrate bioassays five plant species were exposed to a concentration series of freshly spiked API-sludge in potting soil. Seed germination, early seedling growth rate, biomass change and water uptake were studied. The results were used to establish the phytotoxicity and most sensitive plant species to API-sludge when comparing the test species.

### 2.9.1. Plant species

The seeds of five plant species (obtained from a local nursery in Stellenbosch) were tested for their germination, and the resultant plants for growth rate and gained biomass and water uptake in API-sludge contaminated potting soil. Beans (*Phaseolus vulgaris*) a dicotyledonous legume with root-nodules and was chosen as suggested in a previous study (Eriyamremu *et al*. 2007). Maize (*Zea mays*) is a representative of a fast growing monocotyledonous crop with a adventitious root system not penetrating deep into ground. Lettuce (*Lactuca sativa*) was chosen because it has high water content within its leaves and alternative mechanism for water uptake than the other test species. Radish (*Raphanus sativus*) has a large underground storage organ and relatively little above ground growth (Perez & Gallardolara 1986). Radish was therefore chosen to determine how the API-sludge will affect the growth of its underground storage organ. Grass (*Pennisetum clandestinum*) has a dense root system and is believed act as a good absorbent for toxicants in the soil (Adam & Duncan 1999).

### 2.9.2. Soil preparation

Potting soil was used for the contaminated soil and controls. Water holding capacity (WHC) and pH (1M KCl method) were determined and monitored at the start and end of the experiments to confirm consistency. The concentration series of 0.5%, 2.5%, 5%, 10% and 25% API-sludge and a negative control of uncontaminated soil (0% API-sludge) were used. Spiking was done by adding the highest API-sludge (25%) to unpolluted potting soil and diluted further by adding clean potting soil until the desired concentration ratio was reached. Soil and API-sludge were mixed thoroughly to obtain a homogeneous mixture and left for two days before the seeds were planted.

### 2.9.3. Germination success

For each species a concentration series of 0%, 0.5%, 2.5%, 5% and 10% and 25% API-sludge was prepared, one Petri dish per treatment was used. Ten seeds were planted in each Petri dish, watered manually with distilled water after seeded and further as needed. The 30 Petri dishes were placed in an incubator at 22°C to insure optimal conditions for germination and monitored twice daily for a total of 7 days. Germination was recorded as the emergence of a radicle and every day the number of germinated seeds were recorded.

### 2.9.4. Seedling growth

After germination five seedlings from each plant species were replanted in black trays and transferred to a greenhouse where the environmental conditions were controlled. The plants were watered regularly. Three times a week each individual plant height was measured as the distance from the soil to the tip of the stem, in the case of beans, or to the tip of the top leaf, in the case of maize, radish, lettuce and grass. These measurements were used to determine the growth rate of the plants over the period it has been exposed. The beans and maize exposures were terminated after four weeks. Radish, lettuce and grass exposures were terminated after three weeks. The total growth period of all plant species was divided into a first and second growth period (a period of two weeks for the beans and maize and one and a half weeks for the lettuce, radish and grass). The division allowed one to calculate the initial growth rate and a secondary growth rate when the plants were two weeks (beans and maize) and one and a half weeks (lettuce, radish and grass) old. Any possible effects of the exposures were annotated, such as deformed leaves or death.

### 2.9.5. Biomass determination

At the end of the experiments each individual plant was removed from the tray and the soil was carefully rinsed from the roots. Excess water was dabbed off using paper towels. The plants were cut at the stem base into two sections: the first section included the upper soil parts, stem and leaves, and the second the soil parts including the root system. The aerial parts (stem and leaves) and the roots were weighed individually to determine the wet mass. The plants were placed in labelled

brown bags and oven dried for 48 hours at a temperature of 40˚C. The individual parts were then once again weighed to determine the dry mass. Using the wet and dry mass measurements the water contents was determined and the dry mass used as gained biomass value.

## 2.10. Statistical analysis

### 2.10.1. Soil invertebrate exposures

All data were tested for normality using Statistica® 9 software (StatSoft 2010) by applying the Shapiro-Wilk normality test (if the probability factor was $p \leq 0.05$, the data is non-parametric and $p \geq 0.05$ data is parametric).

All comparison analyses were done as follow: When comparing multiple conditions of parametric data One-way Analysis of Variance (ANOVA) were carried out with the Tukey post test that compared different conditions. Where multiple conditions were compared for non-parametric data Kruskal-Wallis ANOVAs with Dunn's multiple comparison post tests were used. Statistical significance was chosen at a probability of $p \leq 0.05$.

All $EC_{50}$ –values calculated for the chronic tests were done by using sigmoidal dose-response curves (Haanstra *et al*. 1985) with statistical software programs SPSS® 18.0 (PASW 2010) and GraphPad Prism® 5.00 software (GraphPad 2007). A non-linear regression model was used in both software and the best fit model was used. The lethal API-sludge concentration, where 50% of all exposed organisms died after the experimental period, was determined using the trimmed Spearmann-Kärber model (Hamilton *et al*. 1977).

In the avoidance behaviour experiments the number of organisms presents in the test-soils compared to the controls was determined by two-tailed paired t-tests. Test-soil avoidance was considered if organisms avoided the test soils significantly greater than 0.50 (50%). Avoidance behaviour greater than 80% for certain soil type was regarded to be toxic (Yeardley *et al*. 1996; Hund-Rinke *et al*. 2003; Natal-Da-Luz *et al*. 2008). One-way ANOVA's were performed comparing the species avoiding the same test-soil.

### 2.10.2. Plant exposures

All statistical analyses in plant exposures were carried out using Statistica® 9 (StatSoft 2010) and GraphPad Prism® 5.00 (GraphPad 2007) software. Variables were tested for normality with the Shapiro-Wilk normality test. Normality was accepted if the probability factor was greater than 0.05 ($p \geq 0.05$) and non-parametric when $p \leq 0.05$.

The germination success (expressed in percentage germination) was plotted over time to show relationships between the concentrations of API-sludge exposures relative to the negative controls. The growth rate for the two growth periods were determined by determining the difference between the end and the start measured plant lengths and divided by the number of days it was exposed (the days per growth period for the beans and maize were 14 days and 10 days for exposed lettuce, radish and grass). Within each treatment the first and second growth period was compared using the t-tests for dependent soil substrates (for parametric data) or the Mann-Whitney U-test (for non-parametric data). The first and the second period growth rates were compared for each treatment in all exposed plant species. Similarly, the total-, above ground mass and below ground parts mass was compared between treatments. All data comparisons were done using One-way ANOVAs with the Tukey post hoc test (for parametric data) or with the Kruskal-Wallis ANOVAs with Dunn's post hoc test (for non-parametric data). The LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) were calculated using ToxRat Professional (ToxRat®, 2003) based on the protocol described by OECD guideline 208. (OECD 2006). The determined water content of the seedlings was also compared to determine statistical significant differences between the treatments.

# 3. Results

## 3.1. Physical and chemical soil properties

The physical properties of all soils used are summarized in Table 6. The OECD-soil had the highest organic matter content of 10% and the off-site soil contained the least (0.4%). LUFA2.2-soil contained 2.1 % organic matter. North-site and south-site soils had an organic matter of 4.4% and 2.6% respectively. The LUFA2.2-soil and the off-site soil were both characterised as 'loamy sand' where as the site-soils were classified as 'sand' according to the United States Department of Agriculture (USDA) classification system (Appendix A).

**Table 6**: Physical characteristics of all soils used in this study. All data were created according to different standardised protocols as stated in the materials and methods. Values shown as mean ± standard deviation

| | % Clay (<0.002mm) | % Silt (0.002-0.05mm) | % Sand (0.05-2.0mm) | % Organic matter | Soil Type | pH (1M KCl) | Maximum WHC* (%) |
|---|---|---|---|---|---|---|---|
| **OECD-soil** | 20.0 | 0.0 | 70.0 | 10.0 | Sandy Loam | 6.0±0.5 | 60±5.0 |
| **LUFA2.2 soil** | 6.4 | 11.6 | 82.0 | 2.10 ± 0,40 | Loamy Sand | 5.5±0.1 | 46.5±6.0 |
| **Off-site soil** | 9.0 | 2.0 | 89.0 | 0.39 ± 0.05 | Loamy Sand | 6.8±0.5 | 32.7±1.1 |
| **North-site soil** | 3.4 | 2.4 | 94.2 | 4.4 ± 0.21 | Sand | 6.0 ±0.1 | 59.7±1.5 |
| **South-site soil** | 3.0 | 2.0 | 95.0 | 2.57 ± 0.04 | Sand | 5.8±0.4 | 48.7±4.3 |

*WHC = Water Holding Capacity

The chemical composition of all the heavy metals, volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) present in the soils of the landfarming site are summarised in Tables 7, 8, 9 and 10.

**Table 7**: Total concentration of elements detected in site-soils and fresh API-sludge. Values in bold indicate element concentrations above the acceptable risk limit concentration according to DWAF. [1]n=1, [2] mean ± standard deviation where n=4.

| Element detected | Element concentrations (mg/kg) | | | | |
|---|---|---|---|---|---|
| | ARL (DWAF) ° | API-sludge[1] | Off-site soil [1] | North-site soil[2] | South-site soil[2] |
| *Al | **0.39** | **0.44** | 0.06 | **11.63±0.78** | **1.57±0.15** |
| *Mn | **0.3** | **0.65** | 0.05 | **1.67±0.19** | **0.82±0.06** |
| *Pb | **0.1** | 0.05 | 0.05 | **0.29±0.35** | **0.16±0.02** |
| *S | **10** | 6.37 | **12.6** | **59.13±1.24** | **29.71±2.98** |
| *Zn | **0.7** | 0.28 | 0.11 | **3.93±0.07** | **1.81±0.08** |
| *Fe | **9** | **12.6** | <0.04 | 0.09±0.01 | 0.077±0.02 |
| Ba | **7.8** | 0.51 | 0.46 | 0.52±0.03 | 0.65±0.11 |
| Mg | - | 3.26 | 4.62 | 17.23±0.54 | 11.15±0.41 |
| K | - | 0.75 | 2.18 | 7.32±0.1 | 5.04±0.28 |
| Si | - | 0.72 | 3.79 | 141.75±3.77 | 72.58±2.45 |
| Ca | - | 19.9 | 41.1 | 9.91±0.65 | 4.57±0.08 |

°Acceptable risk limit determined by the Department of Water and Forestry of South Africa (in ppm)

*All elements with concentrations higher than that of the acceptable risk limit were detected.

**Table 8:** Total petroleum hydrocarbons (TPHs) present in each of the site-soils. [1]n=1. [2]mean ± standard deviation, n=4.

| | API-sludge[1] | Off-site[1] | North-site soil[2] | South -site soil[2] |
|---|---|---|---|---|
| Gasoline Range Organics (GRO)(µg/Kg) | 120 968 | <25 | <25 | <25 |
| Diesel Range Organics(DRO) (mg/kg) | 104 206 | <150 | 1469.25 ± 125.12 | 675.25 ± 49.22 |

**Table 9**: Total concentrations of volatile organic compounds (VOCs) in fresh API-sludge. Values in bold indicate VOC concentrations above the accepted risk level concentrations set out by DWAF. (All chemical structures are shown in Appendix B).

| Volatile Organic Compound | ARL (DWAF)[1] | API-sludge in (µg/kg) |
|---|---|---|
| Chloroform | **100** | 153 |
| Benzene | **2200** | 4729 |
| Toluene | **1300** | 29 533 |
| Ethylbenzene | **1200** | 10 661 |
| m+p-Xylenes | **1100** | 10 086 |
| o-Xylene | **1100** | 29 963 |
| 1,1,2,2-Tetrachloroethane | - | 7114 |
| Isopropylbenzene | **5000** | 3720 |
| n-Propylbenzene | - | 5441 |
| 1,3,5-Trimethylbenzene | **36000** | 13 827 |
| 1,2,4-Trimethylbenzene | - | 50 793 |
| sec-Butylbenzene | - | 3602 |
| 4-Isopropyltoluene | - | 3864 |
| n-Butylbenzene | - | 3448 |

[1]Acceptable risk limit from the Department of Water and Forestry of South Africa in ppb (µg/L)

**Table 10:** Polycyclic aromatic hydrocarbons (PAHs) concentrations (µg/kg) in site-soils and fresh API-sludge. [1]n=1. [2] mean ± standard deviation, n=4. (The chemical structures of all PAHs are shown in Appendix B).

| PAH detected | PAH Concentration ppb (µg/kg) | | | | |
|---|---|---|---|---|---|
| | CCME[O] (CCME 2001) | API-sludge[1] | Off-site soil[1] | North-site soil[2] | South-site soil[2] |
| Naphthalene | 22,000 | 82.44 | <0.45 | 6.75 ± 0.1 | 6.85 ± 0.15 |
| Acenaphthylene | - | <0.27 | <0.27 | <0.27 | <0.27 |
| Acenaphthene | - | 22.25 | <0.50 | <0.50 | <0.50 |
| Fluorene | - | 60.09 | <0.53 | <0.53 | <0.53 |
| Phenanthrene | 50,000 | 97.52 | <0.54 | 2.85 ± 0.1 | 2.74 ± 0.05 |
| Anthracene | - | 23.85 | <0.59 | 2.95 ± 0.08 | 2.92 ± 0.07 |
| Fluoranthene | - | 34.59 | <0.74 | 6.04 ±0.13 | 6.11 0.12 |
| Pyrene | 100,000 | 55.99 | <0.83 | 6.84 ±0.36 | 6.55 ±0.16 |
| *Benzo(a)anthracene | 10,000 | 46.36 | <1.17 | 8.5 ± 0.70 | 8.34 ± 0.34 |
| *Chrysene | - | 36.81 | <0.81 | 5.4 ± 0.37 | 5.38 ± 0.23 |
| *Benzo(b)fluoranthene | 10,000 | 17.10 | <0.87 | 3.58 ± 0.20 | 3.25 ±0.08 |
| *Benzo(k)fluoranthene | 10,000 | 24.33 | <0.84 | 4.84 ±0.08 | 4.86 ±0.10 |
| *Benzo(a)pyrene | 700 | 42.79 | <0.87 | 8.66 ± 0.40 | 8.23 ± 0.19 |
| *Indeno (1,2,3-cd)pyrene | 10,000 | 50.59 | <1.10 | 10.14 ± 0.21 | 10.15 ± 0.21 |
| *Dibenzo(a,h) anthracene | 10,000 | 58.97 | <1.34 | 11.81 ± 0.21 | 11.82 ± 0.30 |
| Benzo(ghi)perylene | - | 47.57 | <1.07 | 10.79 ± 0.24 | 10.29 ± 0.30 |
| $\sum$ 16 PAHs | - | 701.25 | - | 89.18 ± 3.19 | 87.49± 2.30 |
| $\sum$ 7 carcinogenic PAHs | - | 276.95 | - | 52.96 ± 2.17 | 52.03 ±1.45 |

[O] Standardised maximum allowed PAH concentrations in industrial soils set out by the Canadian Council of Ministers of the Environment (CCME)

* The 7 carcinogenic PAHs

## 3.2. Exposure and endpoints measured- *Eisenia andrei*

### 3.2.1. Survival and chronic tests in the control soils

The mass changes of *Eisenia andrei* specimens exposed to OECD-soil and off-site soil are summarised in Table 11. *E. andrei* specimens exposed to OECD-soils showed the highest increase in biomass over the 4 weeks exposure period whereas worms in the off-site soil showed no significant biomass changes after the same time. In both OECD-soil and off-site soil, spiked with 1% API-sludge, the mean biomass of the organisms decreased. *E. andrei* specimens exposed to 5% API-sludge-spiked OECD- and off-site soils died after 1 week of exposure.

**Table 11:** Mean ± standard deviation growth of *Eisenia andrei* specimens during the 4 weeks of exposure to control soils; OECD-soil, OECD-soil + 1% API-sludge, off-site soil and off-site soil + 1% API-sludge. n=16 (8 per container) in OECD-soil and n = 24 (8 per container) in off-site soil. [a] indicates significant differences ($p \leq 0.05$) when start mass is compared to end mass for each exposure, [b] statistical significant difference ($p \leq 0.05$) of mass changes compared to the mass changes in OECD-soil and [c] show statistically significant differences for mass changes compared to off-site soil ($p \leq 0.05$).

| | Mean biomass (mg) | | Mass change after 4 weeks (%) |
|---|---|---|---|
| | Start | End | |
| **OECD-soil** | 387.57 ± 84.88 | 450.52 ± 101.30 [a] | 33.89 ± 3.44 |
| **OECD-soil + 1% API-sludge** | 372.05 ± 111.75 | 248.7 ± 74.725 [a] | -13.25 ± 12.52 [bc] |
| **Off-site soil** | 366.54 ± 86.41 | 379.68 ± 58.18 | 0.5692 ± 3.46 |
| **Off-site soil + 1% API-sludge** | 345.00 ± 83.13 | 226.79 ± 56.44 [a] | -25.49 ± 3.06 [bc] |

The weekly biomass changes for *E. andrei* specimens exposed to control soils are presented in Figure 5. After one week earthworms at all conditions showed a loss in biomass. The loss in biomass in exposures to the 1% API-sludge-spiked OECD- and off-site soils was significantly different when comparing the mass after 1 week to the start mass. Specimens exposed to both unspiked control soils (off-site and OECD-soil) recovered biomass after week two. The specimens in these exposures showed significant weight differences when their start mass was compared to the end mass after 4 weeks. Specimens exposed to off-site soil gained the lost biomass after the initial

week of exposure. *E. andrei* specimens exposed to the OECD-soil increased in biomass over the last two weeks and a significant difference between the mass at week 2 and the final biomass was observed ($p \leq 0.05$). Earthworms exposed to the spiked soils gained biomass after week 1 but was not able to fully regain all lost biomass in the time period of the experiments. In the case of 1% API-sludge-spiked OECD-soil the initial biomass is significantly different when compared to mass after week 2 and 4. The similarly spiked off-site soil showed only significant differences between the initial biomass and the mass after one week. However the general pattern of biomass change over the experiment is similar for both control soils as well as the two positive control soils (off-site and OECD-soils spiked with 1% API-sludge).

Table 12 summarises the data of cocoon production in the control soils. Earthworms in OECD-soil produced the most cocoons but the earthworms in the off-site soils produced the highest number of hatchlings. No cocoons were produced by worms in the different spiked control soils.

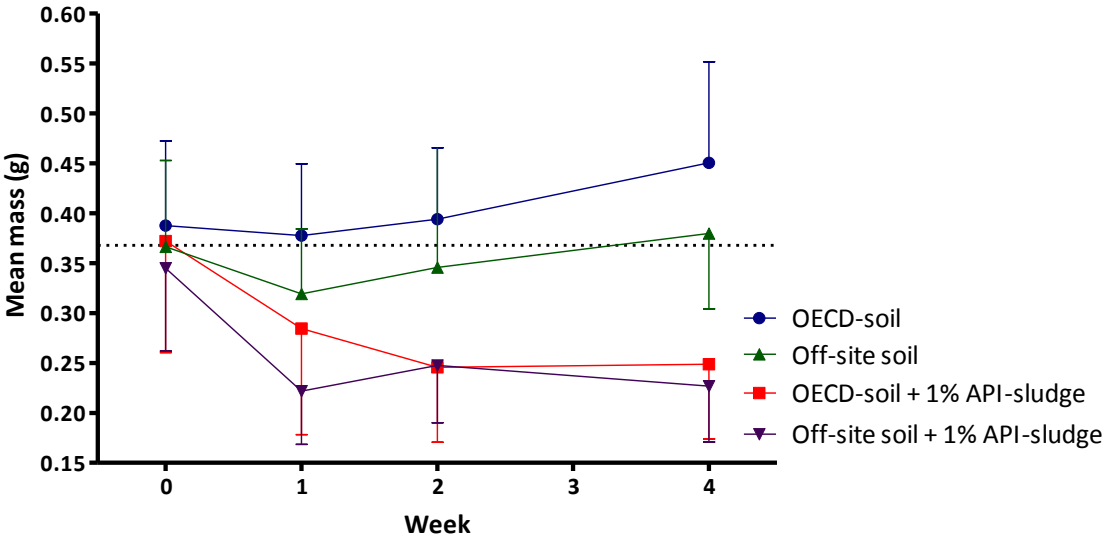



**Figure 5:** Mean ± standard deviation biomass change (in g) of *Eisenia andrei* specimens exposed to control- and API-sludge-spiked control soils for 4 weeks. The dotted line shows the mean of the starting biomass for all organisms and represents a reference line only for comparison.

**Table 12:** Mean ± standard deviation cocoon production and number of hatchlings of *Eisenia andrei* specimens exposed to control soils for 4 weeks of exposure. n=16 (8 per container) in OECD-soil and n = 24 (8 per container) in off-site soil. [a] indicates statistical significance ($p \leq 0.05$) to OECD-soil.

| | All cocoons collected over 4 weeks | Cocoons produced/worm | Total hatchlings |
|---|---|---|---|
| **OECD-soil** | 16.0 ± 5.1 | 1.60 | 20.0 ± 5.6 |
| **OECD-soil + 1% API-sludge** | 0.0[a] | 0.00 | 0.0 |
| **Off-site soil** | 11.0 ± 3.1 | 0.73 | 27.0 ± 5.7 |
| **Off-site soil + 1% API-sludge** | 0.0[a] | 0.00 | 0.0 |

### 3.2.2. Survival and chronic tests in the site-soils

Table 13 compare the data of the initial weight of the earthworms to the end biomass in the experiments of *E. andrei* specimens exposed to site-soils (off-site, north-site and south-site soils) for 6 weeks. No biomass changes for specimens exposed to the site-soils were significantly different although a trend was observed in the mass changes when the weekly data were compared with the off-site soil (control soil). The weekly biomass changes are presented in Figure 6. At the end of the 6 weeks experiment the mean biomass changes in north-site and south-site soils were less than that in the 1% API-sludge-spiked soil. No significant differences for the specimens' mass change in all site-soils were found between the start and end masses.

The reproduction in the test soils over the 6 week experiment is showed in Table 14. The data show *E. andrei* to reproduce similarly in both off-site soil and south-site soils but low to no reproduction were observed in north-site and 1% API-sludge-spiked off-site soils. No significant differences were observed when comparing the cocoon production of specimens exposed to the various test soils.

**Table 13:** Mean ± standard deviation biomass change of *Eisenia andrei* specimens exposed to the test soils; off-site soil, north-site soil, south-site soil and off-site soil + 1% API-sludge after 6 weeks. n=40 (10 per container). No statistical significant differences were observed.

| | Mean biomass (mg) | | Mass change after 6 weeks (%) |
|---|---|---|---|
| | Start | End | |
| **Off-site soil** | 366.94 ± 49.24 | 328.97 ± 18.45 | -5.46 ± 12.63 |
| **North-site soil** | 379.19 ± 10.85 | 278.05 ± 41.80 | -21.35 ± 15.44 |
| **South-site soil** | 394.51 ± 96.56 | 304.30 ± 42.81 | -19.99 ± 12.30 |
| **Off-site soil + 1% API-sludge** | 350.10 ± 60.64 | 285.45 ± 34.20 | -17.01 ± 4.65 |

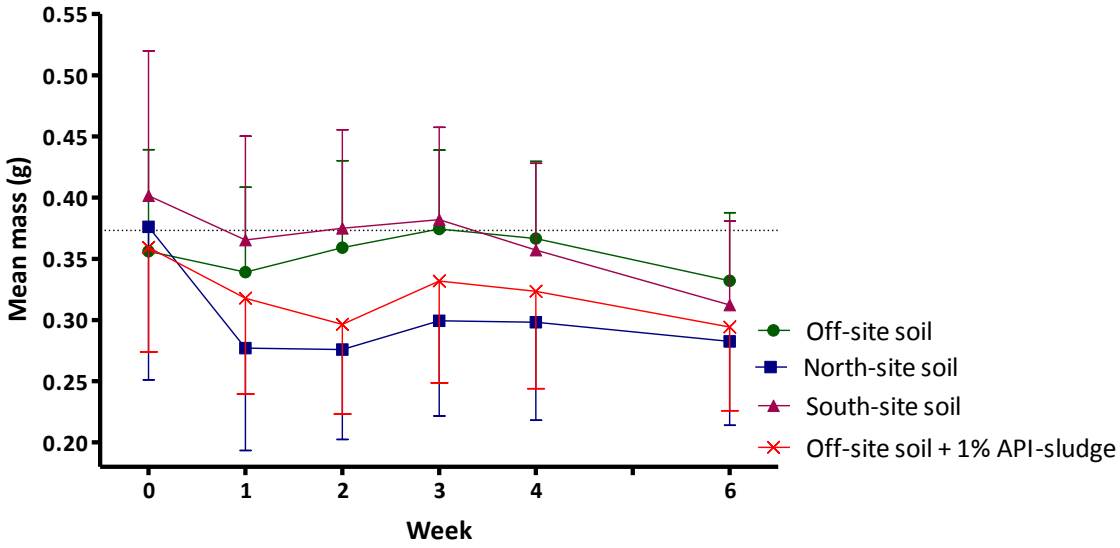



**Figure 6:** Mean ± standard deviation biomass change (in g) of *Eisenia andrei* specimens exposed to test soils for 6 weeks. The dotted line shows the mean of the starting biomass for all organisms and represents a reference line only for comparison.

**Table 14:** Mean± standard deviation cocoon production, cocoons produced per worm and survivors of *Eisenia andrei* specimens exposed to site-soils for 6 weeks. n=64 (8 per container). No statistical significant differences were observed.

| Site-soil | All cocoons collected over 6 weeks | Mean cocoons produced/worm | Mean survivors per test container (n=8) |
|---|---|---|---|
| **Off-site soil** | 18.5 ± 9.7 | 0.46 | 8.0 ± 0.0 |
| **North-site soil** | 1.25 ± 0.7 | 0.03 ± 0.04 | 7 ± 0.5 |
| **South-site soil** | 19.00 ± 5.3 | 0.48 ± 0.07 | 8 ±0.0 |

### 3.2.3. Survival and chronic tests in API-sludge-spiked control soils

The biomass changes are shown in Table 15 and the mass changes per week are displayed in Figure 7. Worms exposed to 0%, 0.5% and 1% API-sludge-spiked OECD-soil had an increase in biomass when start mass was compared to end mass. At concentrations 2% and 2.5% spiked API-sludge a loss of biomass was significant when compared to their initial biomass at day 0. From Figure 7 it is apparent that the initial weight loss was the biggest from concentrations of 1%- sludge and higher. The 1% API-sludge soil exposures show significant differences when the biomass at week 1 is compared to biomass at week 3 and 4. After 4 weeks the mass change was smaller for 1%- and 1.5%-sludge concentration exposures but the 2%- and 2.5%-sludge-spiked soils did not regain biomass at the end of the exposure time. The biomass at 2% API-sludge concentration soils was significantly different from the initial biomass when compared to the biomass at weeks 1, 2, 3 and 4. The 2.5% API-sludge concentration exposures were significant different when comparing the initial biomass to the final biomass.

**Table 15:** Mean ± standard deviation biomass of *Eisenia andrei* specimens exposed to a concentration series of API-sludge-spiked OECD-soil for 4 week. n=24 (8 per container). [a] indicates significant differences of $p \leq 0.05$ when start mass is compared to end mass for each concentration, [b] shows $p \leq 0.05$ where mass change is different compared to 2% spiked API-sludge and [c] shows $p \leq 0.05$ in mass change differences compared to 2.5% spiked API-sludge [d] indicates significant differences of $p \leq 0.05$ when concentrations are compared to the control 0% added API-sludge.

| Concentration API-sludge (%) in OECD-soil | Mean biomass (mg) | | Mass change after 4 weeks (%) |
|---|---|---|---|
| | Start | End | |
| **0.0** | 387.53 ± 23.36 | 421.76 ± 30.30 | 9.67 ± 3.34 [b c] |
| **0.5** | 379.18 ± 45.04 | 396.45 ± 37.58 | 3.23 ± 10.20 [b c] |
| **1.0** | 404.91 ± 30.99 | 412.825 ± 19.61 | 10.01 ± 0.01 [b c] |
| **1.5** | 354.51 ± 60.71 | 321.95 ± 63.32 | -12.23 ± 4.26 [c d] |
| **2.0** | 382.78 ± 2.79 | 269.30 ± 38.69 [a] | -30.86 ± 10.24 [d] |
| **2.5** | 392.65 ± 20.07 | 201.60 ± 12.52 [a] | -47.19 ± 1.33 [d] |

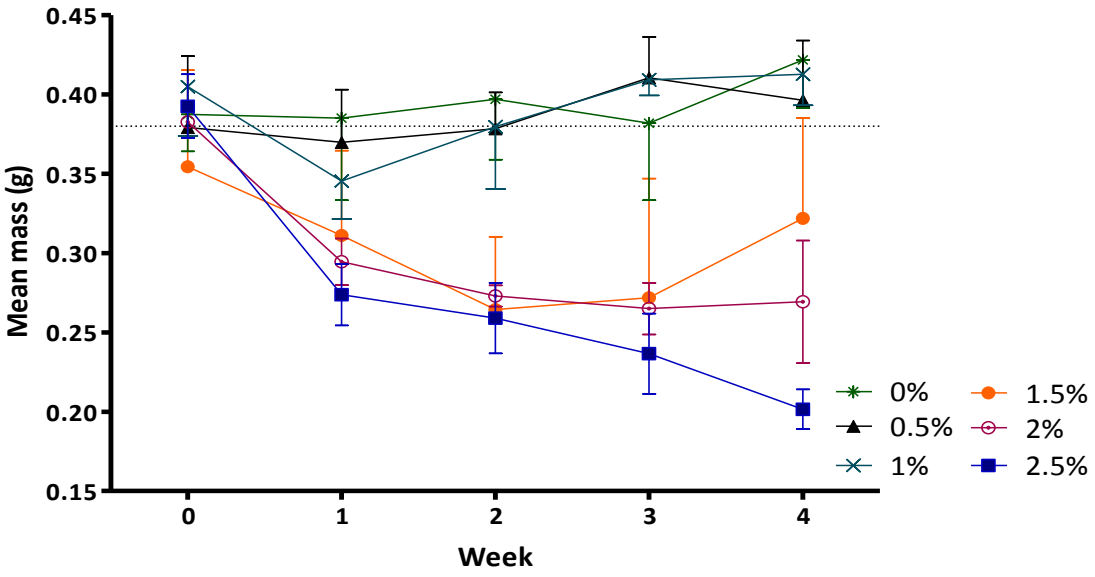



**Figure 7:** Mean ± standard deviation in biomass change (in g) of *Eisenia andrei* specimens exposed to a concentration series of API-sludge in OECD-soil for 4 weeks. The dotted line shows the mean of the starting biomass for all organisms and represents a reference line only for comparison.

Survival and cocoon production for the series of API-sludge-spiked off-site soil are shown in Table 16. In exposures to concentrations with API-sludge higher than 0.8% a significant decrease in cocoon production as well as survival were evident.

The $LC_{50}$ of the earthworm survival in off-site soil spiked with API-sludge was established at 1.15% API-sludge (95% confidence levels: lower = 0.96% upper = 1.37%) (11 500 mg/kg). The $EC_{50}$ was calculated as 1.47% (14,731 mg/kg) API-sludge for cocoon production after the 6 week exposure (Figure 8B). Reproduction decreased with the increase of sludge addition until no reproduction was observed in concentrations higher than 1.6%-sludge.

**Table 16:** Cocoon production and survival of *Eisenia andrei* specimens exposed to a concentration series of API-sludge-spiked off-site soil for 6 weeks (Mean ± standard deviation). n= 48 (8 per container) for 0% API-sludge-spiked soils and all other soils in the series. n=24 (8 per container). [a] indicates statistical differences in survival ($p \leq 0.05$) when compared to reference 0% API-sludge-spiked soil. [b] shows statistical significant differences ($p \leq 0.05$) for cocoon production when compared to 3.2% API-sludge soil exposures. [c] shows statistical significant differences ($p \leq 0.05$) in cocoon production when compared to 1.6% API-sludge-spiked soil exposures.

| Concentration API-sludge (%) in Off-site soil | All cocoons collected over 6 weeks | cocoons produced /worm | Mean survival per test container (n=8) |
|---|---|---|---|
| **0.0** | 51.00 ± 9.00 [b c] | 6.38 ± 1.13 | 8 ± 0.0 |
| **0.2** | 35.67 ± 17.56 [b c] | 4.92 ± 1.79 | 7 ±0.33 |
| **0.4** | 36.33 ± 1.53 [b c] | 4.54 ± 0.19 | 8 ±0.0 |
| **0.8** | 28.00 ± 6.56 [b] | 4.52 ± 2.11 | 6.67 ± 0.5 |
| **1.6** | 4.33 ± 0.58 | 3.44 ± 1.90 | 1.67 ± 0.4 [a] |
| **3.2** | 0.00 ± 0.00 | 0.00 ± 0.00 | 0 ±0.0 [a] |

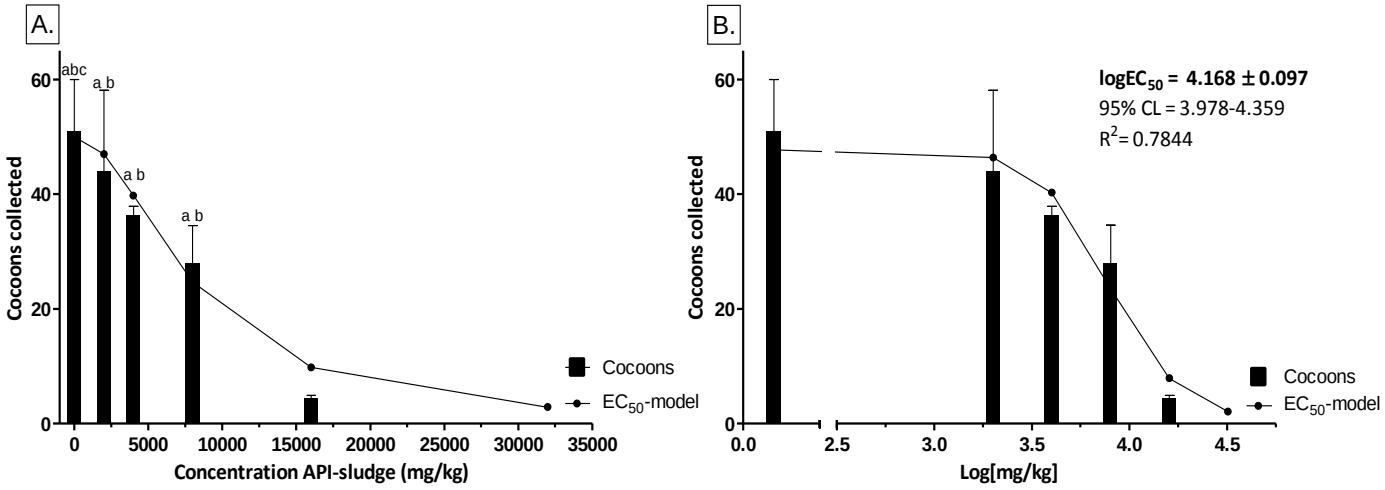



**Figure 8:** Cocoon production of *Eisenia andrei* specimens exposed to an API-sludge-spiked concentration series of off-site soil for 6 weeks. **A.** Mean (± standard deviation) number of cocoons collected at each of the concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated where the concentrations are compared to [a] 32 000 mg/kg API-sludge, [b] 16 000 mg/kg API-sludge and [c] 8 000 mg/kg added API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale that was used for the calculation of the $EC_{50}$ (effect concentration at 50%) for cocoon production.

Survival and cocoon production for the series of API-sludge-spiked off-site soil are shown in Table 17. Cocoons production decreased and significant differences were observed when compared to the control (0%-API-sludge) at spiked soils with API-sludge concentrations higher than 0.8%. After the addition of 1% API-sludge the cocoon production decreased until eventually no cocoons were produced higher than 1% API-sludge.

The $EC_{50}$ for the API- sludge-spiked OECD-soils was determined as 0.59% (5905 mg/kg) for the reproduction of the earthworms after a 6 week exposure time (Figure 9B*)*. The $LC_{50}$ of the earthworm survival in spiked API-sludge was established to be at 3.2% API-sludge.

**Table 17:** Cocoon production and survival of *Eisenia andrei* specimens exposed to a concentration series of API-sludge in OECD-soil for 6 weeks (Mean ± standard deviation). n=48 (8 per container) for 0% API-sludge soils. For all other soils in the series n=24 (8 per container). [a] indicates significant differences ($p \leq 0.05$) when compared to the control 0% API- sludge-spiked soil. For earthworm survival [b] indicates a significant difference ($p \leq 0.05$) of survival in all other API-sludge-spiked soils.

| Concentration API-sludge (%) in OECD-soil | All cocoons collected over 6 weeks | cocoons produced /worm | Earthworm survival (max. 8 exposed per replicate concentration) |
|---|---|---|---|
| **0.0** | 12.50 ± 3.39 | 1.56 ± 0.42 | 8.00 ± 0.00 |
| **0.2** | 6.67 ± 2.52 | 0.83 ± 0.32 | 8.00 ± 0.00 |
| **0.4** | 4.67 ± 3.22 | 0.67 ± 0.46 | 7.00 ± 0.00 |
| **0.5** | 6.67 ± 4.16 [a] | 0.83 ± 0.52 | 8.00 ± 0.00 |
| **0.8** | 5.33 ± 2.31 | 0.71 ± 0.37 | 7.67 ± 0.58 |
| **1.0** | 0.33 ± 0.58 [a] | 0.04 ± 0.07 | 8.00 ± 0.00 |
| **1.5** | 0.00 ± 0.00 [a] | 0.00 ± 0.00 | 8.00 ± 0.00 |
| **1.6** | 0.00 ± 3.61 [a] | 0.00 ± 0.00 | 7.33 ± 1.15 |
| **2.0** | 0.00 ± 0.00 [a] | 0.00 ± 0.00 | 7.67 ± 0.58 |
| **2.5** | 0.00 ± 0.00 [a] | 0.00 ± 0.00 | 8.00 ± 0.00 |
| **3.2** | 0.00 ± 0.00 [a] | 0.00 ± 0.00 | 4.00 ± 1.00 [b] |

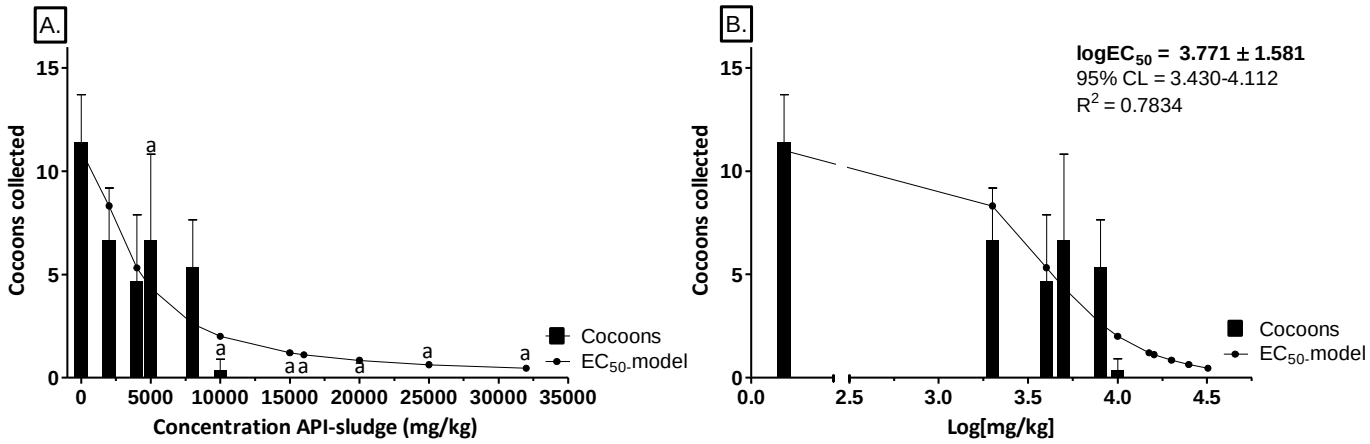



**Figure 9:** Cocoon production of *Eisenia andrei* specimens exposed to an API- sludge-spiked concentration series in OECD-soil for 6 weeks. **A.** Mean ± standard deviation number of cocoons collected at each of the concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated by $^{a}$ where the concentrations are compared to 0 mg/kg API-sludge (control soil). **B.** Graph showing the API-sludge concentration series on a logarithmic scale used for the calculation of the $EC_{50}$ (effect concentration at 50%) for cocoon production.

## 3.3. Exposure and endpoints measured- *Enchytraeus doerjesi*

### 3.3.1. Survival and chronic tests in the site-soils

From the preliminary range finding tests it was established that after 3 weeks there were sufficient reproduction in the control soils and all other tests were also terminated accordingly.

The mean survival and juveniles produced by *E. doerjesi* exposed to the test soils for 3 weeks are summarized in Table 18. Although there were large variation between replicate data for juveniles it still showed that the *E. doerjesi* in north- and south-site soil produced large numbers of juveniles and significantly more than specimens in the OECD- and off-site soils. Organisms in the off-site soil and API-sludge-spiked off-site soils showed similar reproduction ability and significantly less in the case of 1% API-spiked off-site soil when compared to the worms exposed to OECD-soil. No significant differences were observed for survival.

**Table 18:** Survival (mean ± standard deviation) and number of juveniles produced (mean ± standard deviation) of *Enchytraeus doerjesi* specimens exposed to test soils after 3 weeks. n=50 (10 per container). [a] shows the statistically significant differences ($p \leq 0.05$) when compared to the off-site soil, [b] compared to OECD-soil ($p \leq 0.05$) and [c] compared to 1% spiked off-site soil ($p \leq 0.05$) exposures.

| Test soils | Survival after 3 weeks | Juveniles after 3 weeks |
|---|---|---|
| **Off- site** | 8.4 ± 1.5 | 57 ± 34.39 |
| **OECD-soil** | 10 ± 0 | 175.75 ± 56.03 [a] |
| **North-site** | 9.2 ± 1.3 | 339.75 ± 76.92 [a b c] |
| **South-site** | 8.6 ± 3.1 | 414.00 ± 17.78 [b c] |
| **1% Spiked Off-site soil** | 7.8 ± 2.5 | 40.33 ± 25.54 [b] |

### 3.3.2. Survival and chronic tests in API-sludge-spiked control soils

Data for *E. doerjesi* exposed to a concentration series of API- sludge-spiked off-site soils are summarized in Table 19 and illustrated in Figure 10. The reproduction decreased at high concentrations of API-sludge (4% and 5%). No significant mortalities were observed over the duration of the experiments. No $LC_{50}$ could be calculated thus it was assumed to be $LC_{50} \geq 5\%$ API-sludge-spiked off-site soils. The $EC_{50}$ for reproduction was calculated as 26 550 mg/kg (2.66%) (Figure 10B). Worms exposed to soils with more than 2.5% API-sludge produced significantly less juveniles than specimens in the control soil after 3 weeks of exposure.

**Table 19:** Survival (mean ± standard deviation) of *Enchytraeus doerjesi* specimens exposed a concentration series of API-sludge-spiked off-site soil after 3 weeks. n=50 (10 per container). No statistical significant differences were observed.

| Test soils | Survival after 3 weeks |
|---|---|
| **0% off-site soil** | 10.0 ± 0.0 |
| **0.5% off-site soil** | 10.0 ± 0.0 |
| **1% off-site soil** | 10.0 ± 0.0 |
| **1.5% off-site soil** | 10.0 ± 0.0 |
| **2% off-site soil** | 9.5 ± 0.0 |
| **2.5% off-site soil** | 8.4 ± 0.6 |
| **4% off-site soil** | 8.0 ± 1.8 |
| **5% off-site soil** | 10 ± 4.0 |

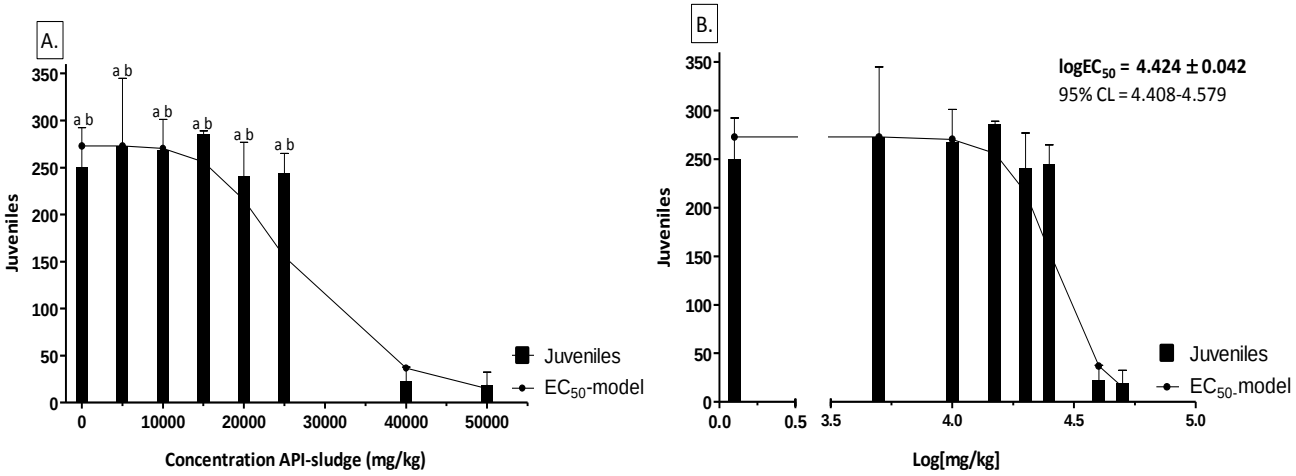



**Figure 10:** Mean ± standard deviation number of juveniles produced by *Enchytraeus doerjesi* specimens in an API- sludge-spiked concentration series of off-site soil for 3 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) showed when all concentrations were compared to [a] 50 000 mg/kg and [b] 40 000 mg/kg API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale used for the calculation of the $EC_{50}$ (effect concentration at 50%) for juvenile production.

In the case of *E. doerjesi* specimens exposed to a concentration series of API- sludge-spiked in OECD-soils the $LC_{50}$ was greater than 5% API-sludge. At concentrations of 3% API-sludge and higher a significant decrease in the number of juveniles produced were present at the end of the experiments. The $EC_{50}$ for reproduction was calculated as 24 874 mg/kg (2.5%) API-sludge (Table 20 and Figure 11B).

**Table 20:** Survival (mean ± standard deviation) of *Enchytraeus doerjesi* exposed to a concentration series of API-sludge-spiked OECD-soil after 3 weeks. n=50. No statistical significant differences were observed.

| Test soils | Survival after 3 weeks |
|---|---|
| **0% OECD-soil** | 10.0 ± 0.0 |
| **1% OECD-soil** | 9.2 ± 1.8 |
| **2% OECD-soil** | 10.0 ± 0.0 |
| **3% OECD-soil** | 9.4 ± 0.9 |
| **4% OECD-soil** | 8.8 ± 2.2 |
| **5% OECD-soil** | 9.2 ± 0.8 |

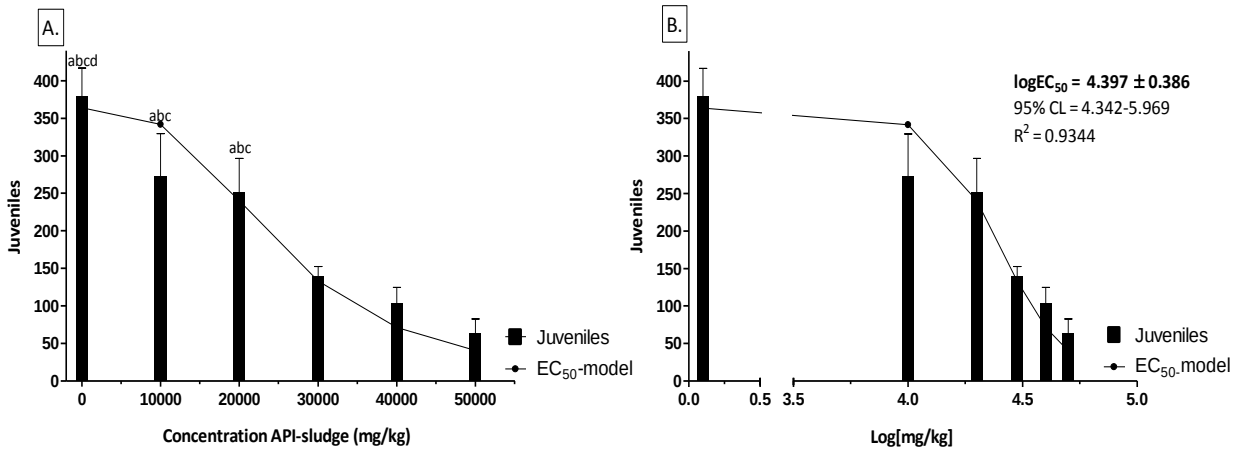



**Figure 11**: Mean ± standard deviation number of juveniles produced *Enchytraeus doerjesi* specimens in an API- sludge-spiked concentration series of OECD-soil for 3 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences where $p \leq 0.05$ are indicated where the concentrations are compared to [a] 50 000 mg/kg [b] 40 000 mg/kg [c] 30 000 mg/kg [d] 20 000 mg/kg API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale used for the calculation of the $EC_{50}$ (effect concentration at 50%) for reproduction.

Data for *E. doerjesi* specimens exposed to a concentration series of API-sludge-spiked in LUFA2.2-soils are shown in Table 21 and illustrated in Figure 12. Only organisms exposed to 5% API-sludge-spiked soils showed a significant decrease in survival (Table 21). An $LC_{50}$ was calculated at an API-sludge concentration of 53 500 mg/kg or 5.35% (95% confidence levels: lower 4.71% upper 6.07%). The $EC_{50}$ for reproduction was calculated as 35 705 mg/kg (3.6%) API-sludge (Figure 12B).

**Table 21:** Survival (mean ± standard deviation) of *Enchytraeus doerjesi* specimens exposed to a concentration series of API-sludge addition in LUFA2.2-soil for 3 weeks. n=50 (10 per replicate). [a] shows statistical significant differences ($p \leq 0.05$) when compared to exposures to 5% LUFA2.2-soil.

| Test soils | Survival after 3 weeks |
|---|---|
| **0% LUFA Soil** | 9.0 ± 1.3[a] |
| **0.5% LUFA Soil** | 10.0 ± 0.0[a] |
| **1% LUFA soil** | 9.3 ± 1.3[a] |
| **1.5% LUFA soil** | 10.0 ± 0.0[a] |
| **2% LUFA soil** | 9.2 ± 1.6[a] |
| **2.5% LUFA soil** | 9.5 ± 2.1[a] |
| **3% LUFA soil** | 9.0 ± 1.0[a] |
| **4% LUFA soil** | 8.0 ± 1.4 |
| **5% LUFA soil** | 5.6 ± 0.9 |

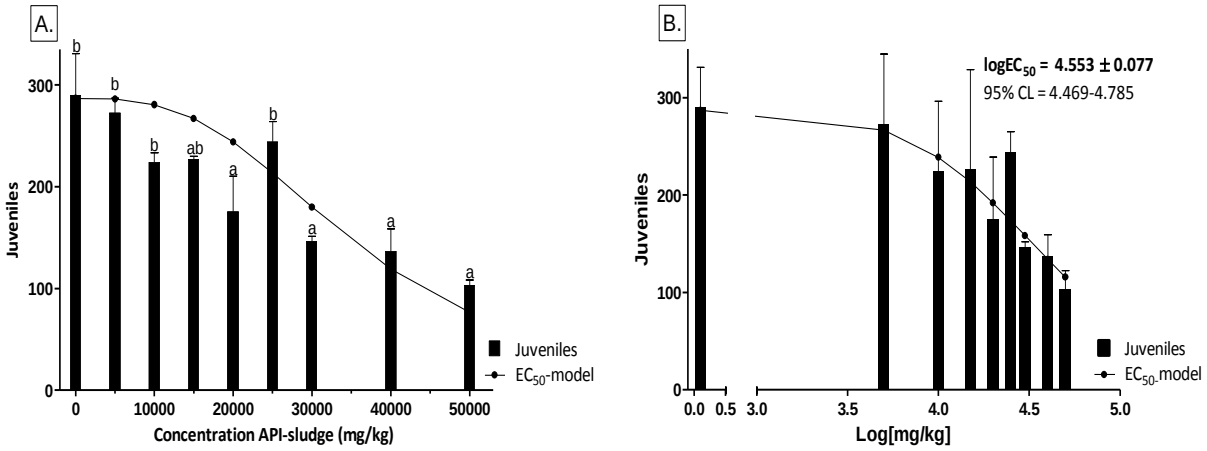



**Figure 12:** Mean ± standard deviation number of juveniles produced by *Enchytraeus doerjesi* specimens in an API-sludge-spiked concentration series of LUFA2.2-soil for 3 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated when the concentrations are compared to [a] 0 mg/kg (control soil) and [b] 50 000 mg/kg API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale used for the calculation of the $EC_{50}$ (effect concentration at 50%) for juvenile production.

## 3.4. Exposure and endpoints measured- *Folsomia candida*

### 3.4.1. Chronic tests in the site-soils

Table 22 displays the results of *F. candida* exposed to the various test soils. No significant differences between the off-site soil and OECD-soil exposures were observed although the total number of juveniles in off-site soils was the highest. North-site and south-site soil exposures showed a statistical significant decrease in juveniles when compared to off-site soil exposures. No organisms have survived exposure greater than 1% spiked API-sludge in off-site soil.

**Table 22:** Juveniles (mean ± standard deviation) produced by *Folsomia candida* specimens exposed to test soils after 4 weeks. n=50 per condition and 10 organisms per replicate. [a] shows the statistically significant differences ($p \leq 0.05$) when compared to the off-site soil, [b] compared to OECD-soil ($p \leq 0.05$) and [c] compared to 1% spiked off-site soil ($p \leq 0.05$).

| Test soils | Mean juveniles after 4 weeks |
|---|---|
| **Off- site** | 479.89 ± 30.42 |
| **OECD-soil** | 388.33 ± 17.86 |
| **North-site** | 289.42 ± 58.62 [a c] |
| **South-site** | 253.33 ± 122.94 [a b c] |
| **1% Spiked off-site soil** | 1.0 ± 1.0 [a b] |

### 3.4.2. Chronic tests in API-sludge-spiked control soils

In a concentration series of API-sludge-spiked off-site soils the $EC_{50}$ for reproduction of the *Folsomia candida* was shown to be 210.44 mg/kg (0.021%) API-sludge (Figure 13B). Only single organisms were counted after 4 weeks in replicates with the addition of 0.01% and 0.02% API-sludge to the off-site soil.

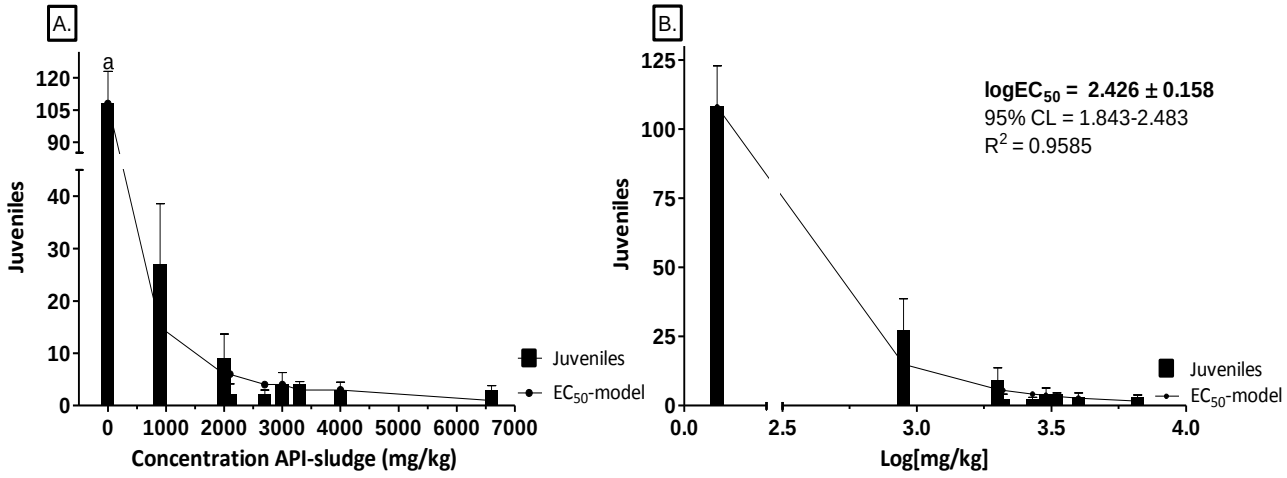



**Figure 13: M**ean ± standard deviation number of juveniles produced by *Folsomia candida* specimens in an API- sludge-spiked concentration series of off-site soil for 4 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated when the concentrations are compared to [a] 0 mg/kg (control soil). **B.** Graph showing the API-sludge concentration series on a logarithmic scale for the calculation of the $EC_{50}$ (effect concentration at 50%) for juvenile production.

Data for *F. candida* exposed to concentration series of API-sludge-spiked OECD-soils are shown in Figure 14. The $EC_{50}$ for reproduction is shown to be 3260.39 mg/kg (0.33%) API-sludge (Figure 14B).

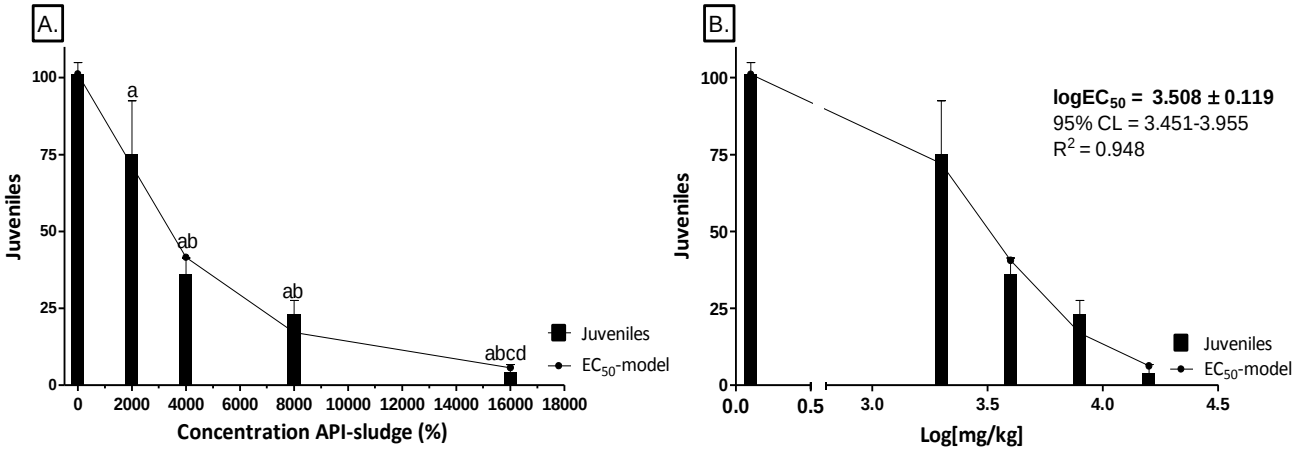



**Figure 14:** Mean ± standard deviation number of juveniles produced by *Folsomia candida* specimens in an API- sludge-spiked concentration series of OECD-soil for 4 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated when the concentrations are compared to [a] 0 mg/kg, [b] 2000 mg/kg, [c] 4000 mg/kg and [d] 8000 mg/kg API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale for the calculation of the $EC_{50}$ (effect concentration at 50%) for juvenile production.

An $EC_{50}$ of 880.254 mg/kg API-sludge (0.09%) for reproduction (Figure 15B) was calculated for *F. candida* exposed to a concentration series of API-sludge-spiked in LUFA2.2-soil. All conditions where API-sludge was added to the soils showed significant differences when compared to the control soils (0% API-sludge).

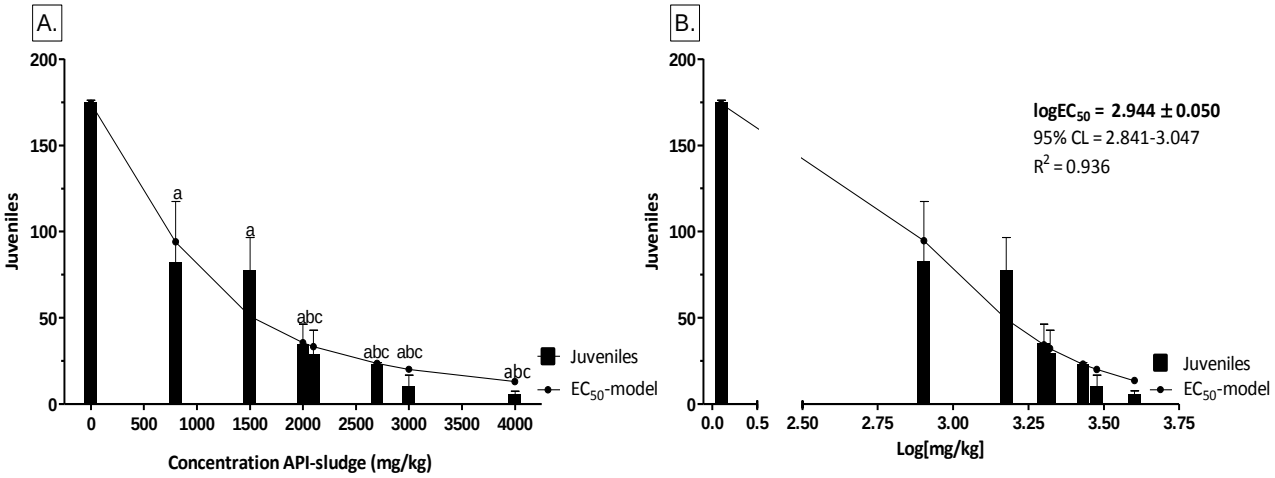



**Figure 15:** Mean ± standard deviation number of juveniles produced by *Folsomia candida* specimens in an API-sludge-spiked concentration series of LUFA2.2-soil for 4 weeks. **A.** Number of juveniles counted at each of the different concentrations of applied API-sludge. Statistical significant differences ($p \leq 0.05$) are indicated when the concentrations are compared to [a] 0 mg/kg, [b] 800 mg/kg and [c] 1500 mg/kg API-sludge. **B.** Graph showing the API-sludge concentration series on a logarithmic scale for the calculation of the $EC_{50}$ (effect concentration at 50%) for juvenile production.

## 3.5. Avoidance behaviour of *Eisenia andrei*, *Folsomia candida* and *Enchytraeus doerjesi* exposed to test soils

Results for the avoidance response experiments for all three different species of organisms tested are summarised in Table 23 and presented in Figure 16.

**Table 23:** Summary of avoidance behaviour for the three different soil organisms exposed to off-site soil and test soils (mean ± standard deviation).

| Treatment 1 | Treatment 2 | Percentage (Mean ± Standard deviation) of organisms present in the test soils (treatment 2) | | |
|---|---|---|---|---|
| | | *Eisenia andrei* n= 32 | *Enchytraeus doerjesi* n= 100 | *Folsomia candida* n= 50 |
| Off-site soil vs. | Off-site soil | 51.56 ± 17.95 | 62.67 ± 24.88 | 49.06 ± 18.61 |
| Off-site soil vs. | North-site soil | 42.19 ± 31.20 | 35.00 ± 20.54 | 60.91 ± 11.58 |
| Off-site soil vs. | South-site soil | 45.31 ± 20.01 | 68.19 ± 14.42 | 60.29 ± 15.57 |
| Off-site soil vs. | Off-site soil + 1% API-sludge | 12.50 ± 17.68 | 11.50 ± 7.20 | 15.93 ± 12.71 |
| Off-site soil vs. | OECD-soil | - | 62.00 ± 17.89 | 78.62 ± 9.08 |

No significant avoidance was observed in any of the three species when exposed to the control with off-site soils on both sides of the containers (control soils). The north-site soil was more clearly avoided by the *E. andrei* and *E. doerjesi* species than by the *F. candida* and statistically different in the case of *E. doerjesi* when compared to the control soil. No significant avoidance was observed when earthworms were exposed to south-site soil versus off-site soil although *E. doerjesi* and *F. candida* showed preference for the south-site soil above that of the off-site soils. All organisms showed more than 80% avoidance behaviour to the 1% API-sludge-spiked off-site soils. Both *E. doerjesi* and *F. candida* test species preferred OECD-soil rather than off-site soils. Avoidance behaviour of *E. doerjesi* and *F. candida* in the OECD-soils resulted in *F. candida* avoiding the off-site soil (treatment 1, Table 23) significantly more than in the case of *E. doerjesi* specimens.

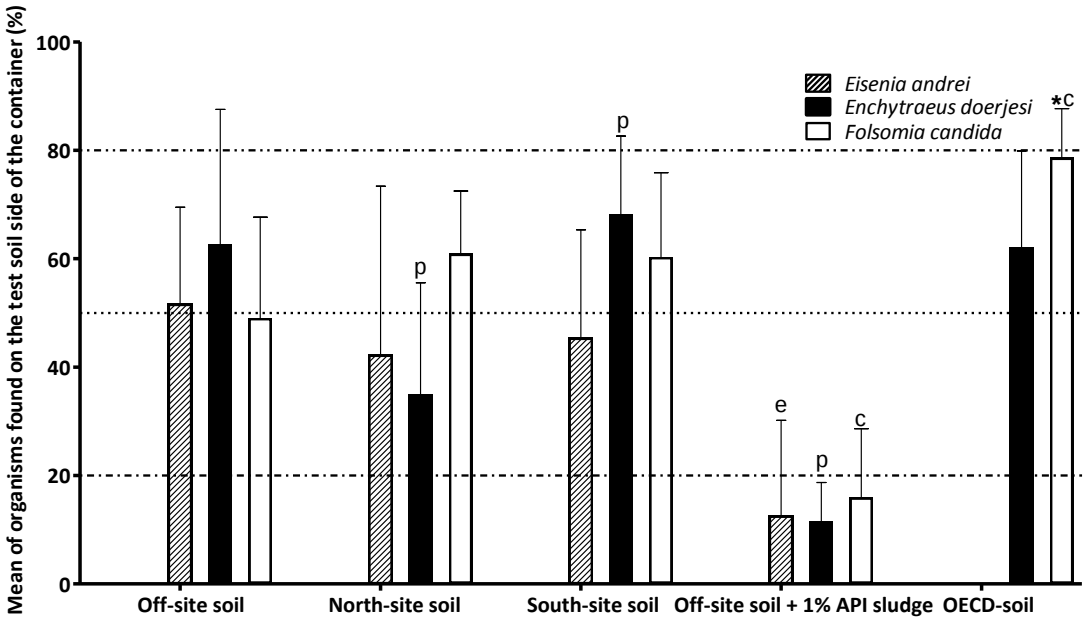



**Figure 16:** Avoidance behavior of *Eisenia andrei*, *Enchytraeus doerjesi* and *Folsomia candida* in test soils. Values show the percentage (mean ± standard deviation) for one side of the test containers where the other side of the container was in every case filled with off-site soil. For *Eisenia andrei* n= 32, *Enchytraeus doerjesi* n= 50 and *Folsomia candida* n= 100. * indicates statistical significant differences ($p \leq 0.05$) when comparing the species in each conditions. Statistical significant differences for a single organisms exposed to the various test soils ($p \leq 0.05$) compared to the off-site soil on both sides of the container are shown as $^{e}$ for *Eisenia andrei*, $^{p}$ for *Enchytraeus doerjesi* and $^{c}$ for *Folsomia candida.*

## 3.6. Plant exposures

### 3.6.1. Germination success

Beans (Figure 17A) displayed germination success higher than 80% within all concentrations of added API-sludge, after 7 days, except at the 10% API-sludge addition. At 10% API-sludge addition the first germination was only seen after 4 days and not 3 like in the case of the other exposures and a lower germination success was observed in comparison to soils with 0% API-sludge. Figure 17B indicates maize seeds have a higher than 80% germination success at all soil with concentrations of API-sludge higher than 0%. Lettuce (Figure 17C) displayed an overall low germination success as only 50% of the seeds in soils with 0% API-sludge germinated. In soils with added API-sludge, the presence of 0.5%- and 5% API-sludge had the lowest germination success. Radish (Figure 17D) displayed a higher germination success within contaminated soil spiked with 0.5%, 2.5% and 10% API-sludge as germination within these concentrations were higher (>70% germination success) than soil with 0% API-sludge (40% germination success). The germination success in soils spiked with 5% API-sludge was only 20%. Grass (Figure 17E) displayed seeds to geminate after only 2 days of exposure in 10% API-sludge-spiked soil but the final germination success was only 20%. In all other treatment exposures the germination success was not higher than 40% including in soil with 0% API-sludge. The highest germination success (40%) was observed in the 2.5% API-sludge-spiked soil.

No seeds (of any species) germinated in the 25% API-sludge contaminated soil.

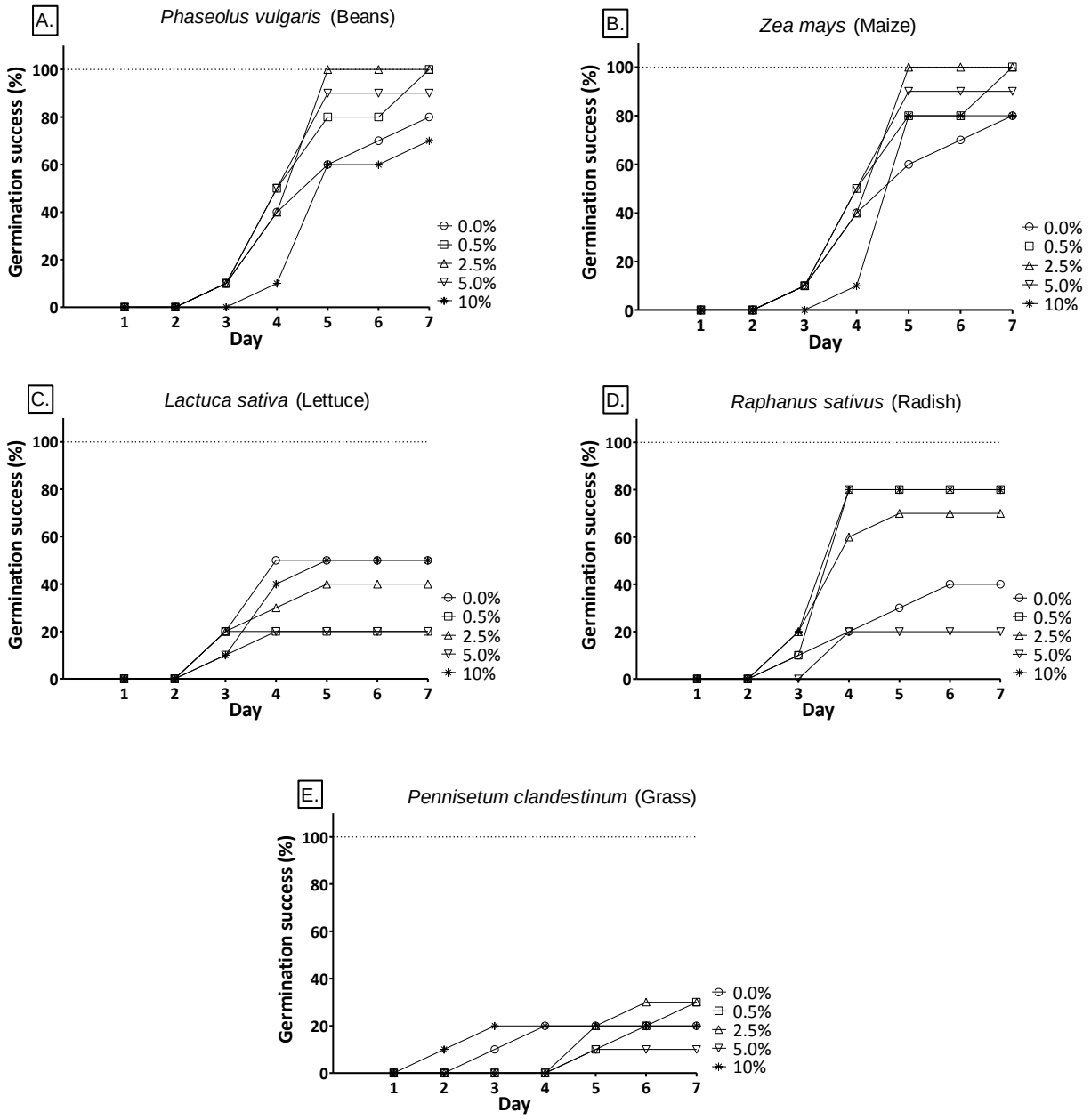



**Figure 17:** Germination success (%) in five plant species exposed to a concentration series of API-sludge in potting soil for 7 days. The various graphs represent the germination of **A.** Beans **B.** Maize **C.** Lettuce **D.** Radish and **E**. Grass.

### 3.6.2. Growth rate

The growth rate of beans-, maize- lettuce-, radish- and grass seedlings exposed to a concentration series of API-sludge are shown in Figure 18. The first growth period of beans exposed to 2.5% API-sludge-spiked soil had a significantly higher growth rate than the 0% and 0.5% API-sludge soil exposures (Figure 18A). In the second growth period the growth rates in 0% and 0.5% API-sludge concentrations were statistically different from 10% API-sludge soil (Figure 18A). When the first and second growth periods at each concentration of API-sludge were compared, the 0% and 2.5% API- sludge-spiked soil exposures showed significant differences from each other. No maize seedling growth rates were significantly different comparing the first and second growth rates (Figure 18B). 10% API-sludge-spiked soil exposures were statistically significant from soils with 0% API-sludge in the first growth period. In the second growth period only the 5% API-sludge soil exposures were statistically less than in the 0% API-sludge soil exposures. In lettuce exposures no significant differences were observed between API-sludge concentrations (Figure 18C). Comparing the growth rates in the concentration series between the first growth period and the second growth period it showed statistical significance in 0%, 0.5% and 10% API-sludge soil treatments (Figure 18C).

In the 0% API-sludge soil exposures the growth rate was significantly higher than the seedlings exposed to 0.5%, 2.5% and the 10% API-sludge soils for the first growth period of radish (Figure 18D). For the second growth period, the growth rate in 0% API-sludge soil was significantly higher than all other concentrations of API-sludge exposures. Comparing the growth rates in the concentration series between the first growth period and the second growth period it was statistically different in 0% and 5% API-sludge-spiked soil exposures (Figure 18D). The first growth period of grass the growth rate in 0% API-sludge soil was significantly higher than all other concentrations of API-sludge exposures. In the second growth period, the growth rate in 0% API-sludge soil was only significantly higher than the 5% API-sludge-spiked soil exposures (Figure 18D).

Grass displayed a higher growth rate in the first growth period than in the second for all concentrations of API-sludge but only significantly so at 0% and 10% API-sludge-spiked soil exposures (Figure 18E). A few individual lettuce seedlings died in the 0.5%, 2.5% and 5% groups and all the individuals in the 10% had died after 15 days.

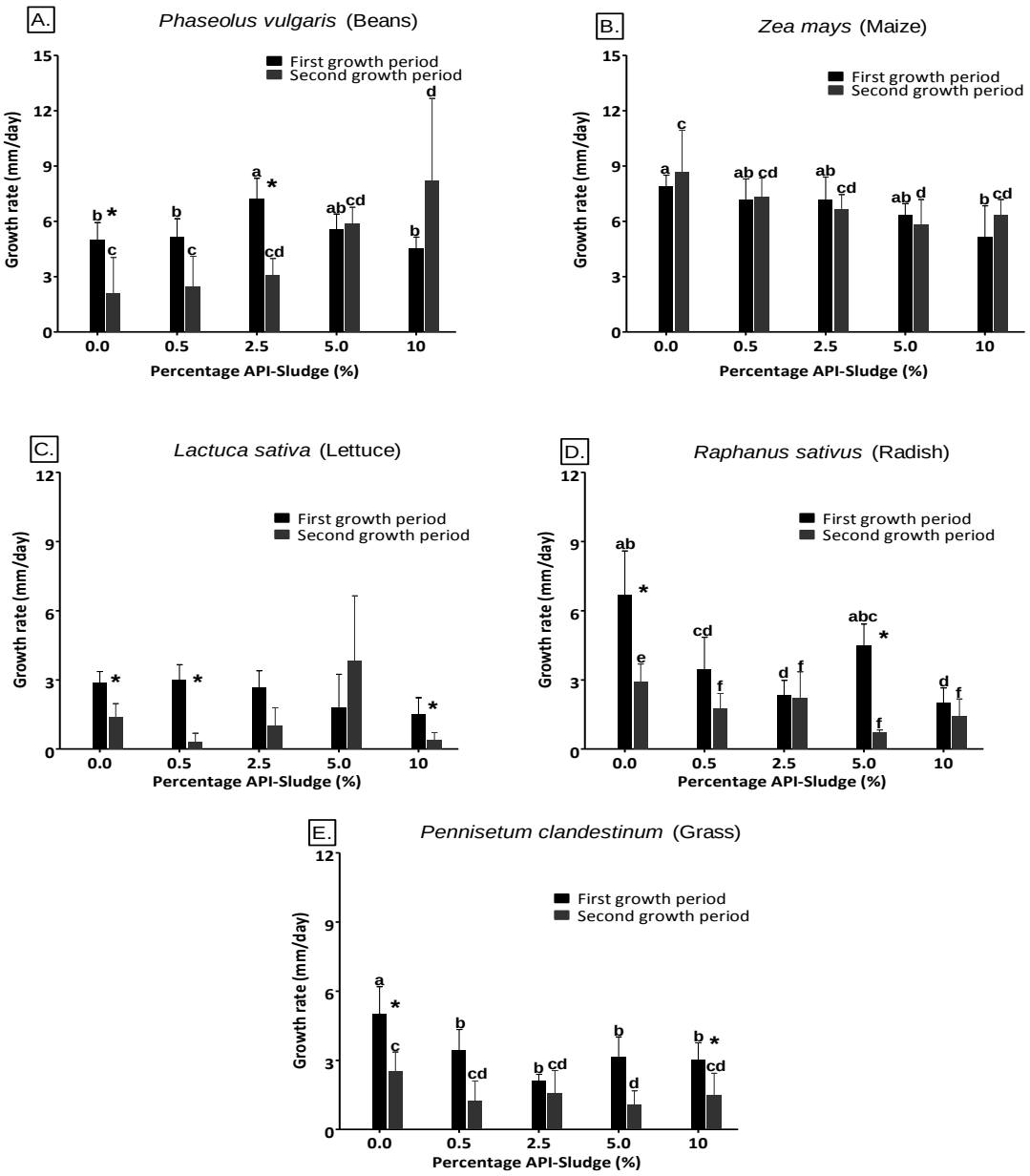



**Figure 18:** Mean growth rate (millimeters per day) of seedlings for five plant species exposed to a concentration series of API-sludge in potting soil for 4 weeks (beans and maize) and 3 weeks (lettuce, radish and grass). Each growth period represents 2 weeks (beans and maize) and 1½ weeks (lettuce, radish and grass). The various graphs represent the germination of **A.** Beans **B.** Maize **C.** Lettuce **D.** Radish and **E**. Grass. n= 5; different letters represent the mean values significantly different among treatments ($p \leq 0.05$) ***** indicates exposures to the concentrations of added API-sludge where the first growth period were statistically different ($p \leq 0.05$) from the growth rate in the second growth period.

### 3.6.3. Biomass

Figure 19 displays the total biomass, above ground mass (stems and leaves) and below ground mass (roots) gained in beans-, maize- lettuce-, radish- and grass seedlings exposed to a concentration series of API-sludge-spiked soil. The total dry mass of bean seedlings in 0% and 0.5% API-sludge-spiked soils had a significantly lower mass than in the 2.5% API-sludge soil (Figure 19A). The LOEC for beans was determined to be greater than 10% API-sludge. Figure 19B displays seedlings at all concentrations of API-sludge soil to have significantly lower total dry mass compared to the seedlings exposed to 0% API-sludge. The LOEC was calculated to be lower than 0.5% API-sludge. A decrease in total biomass was displayed in lettuce exposed to 0.5% API-sludge soil when compared to exposures to 0% API-sludge soil. Only lettuce exposed to 10% API-sludge-spiked soil had significantly lower total dry mass than in the 0% API-sludge soil exposures (Figure 19C). As a result of the decrease in biomass already observed at 0.5% API-sludge soils the LOEC was calculated to be less than 0.5%. The total dry mass and upper ground dry mass of radish seedlings exposed to soil with 0% API-sludge were significantly higher compared to seedlings exposed to any other concentrations of API-sludge (Figure 19D). Low below ground dry mass was recorded in exposures to soil at all concentrations of API-sludge when compared to the total dry mass and upper dry mass of the seedlings. The LOEC for radish (based on the total dry mass) was calculated to be lower than 0.5% API-sludge. Only Grass (Figure 19E) seedlings exposed to soil with 0% API-sludge displayed to have significantly higher upper ground dry mass when compared to seedlings exposed to all other API-sludge added soils. The LOEC was calculated to be at 10% added API-sludge.

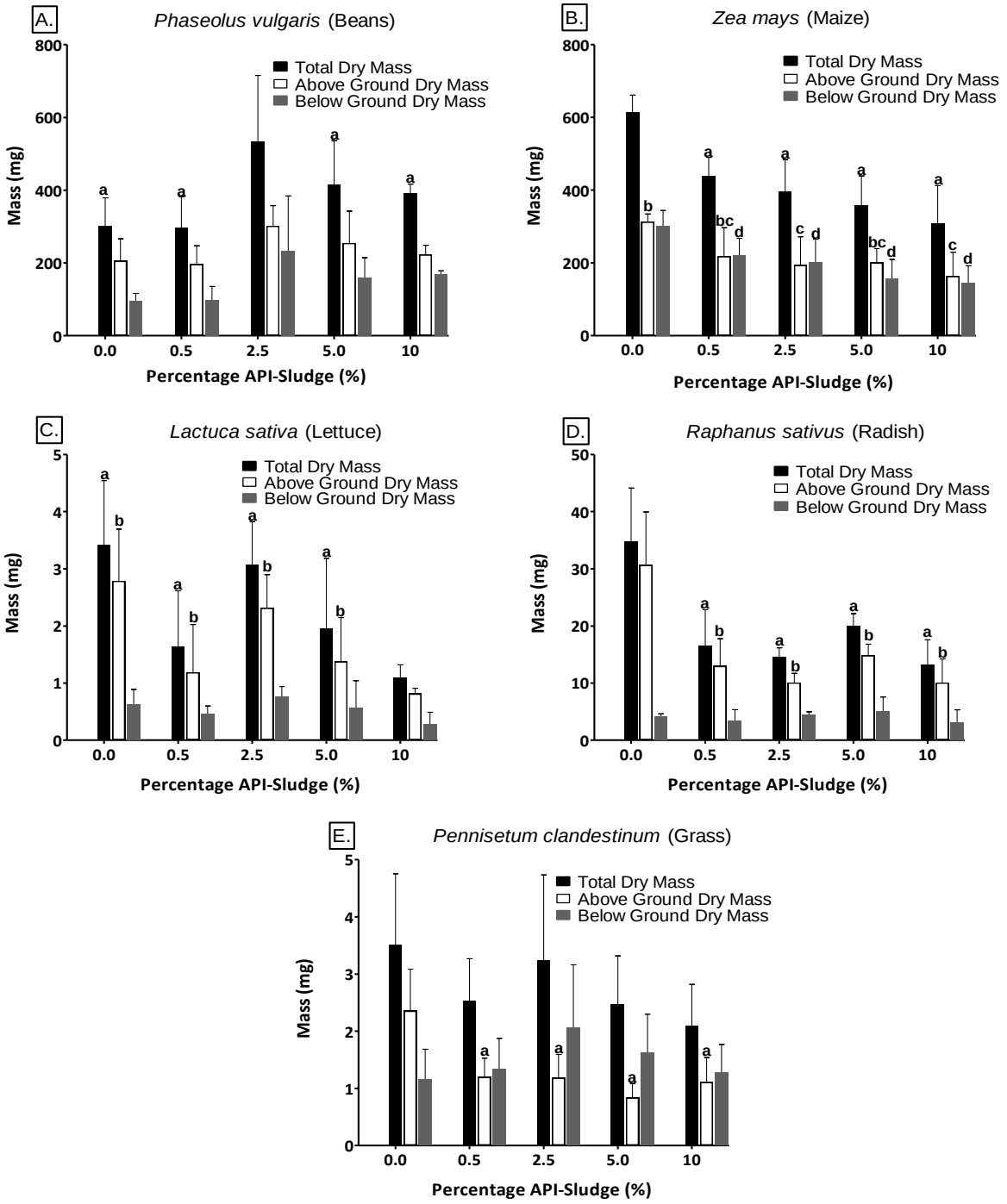



**Figure 19**: Mean ± standard deviation dry weight (mg) of the 5 plant species seedlings exposed to a concentration series of API-sludge in potting soil for 4 weeks (beans and maize) and 3 weeks (lettuce, radish and grass). The various graphs represent the germination of **A.** Beans **B.** Maize **C.** Lettuce **D.** Radish and **E**. Grass. n= 5; different letters represent the mean values significantly different among treatments ($p \leq 0.05$).

### 3.6.4. Water uptake

Table 24 displays the mean water contents (in mg) in seedlings of the different plant species after being exposed to the concentration series of soils spiked with API-sludge.

No linear increasing or decreasing patterns were observed when plants were exposed to an increasing concentration series of API-sludge thus no $EC_{50}$s could be calculated.

**Table 24:** Mean ± standard deviation of water content in seedlings from all five plant species exposed to a concentration series of spiked API-sludge in potting soil.

| | Concentration API-sludge (%) | | | | |
|---|---|---|---|---|---|
| | **0** | **0.5** | **2.5** | **5.0** | **10** |
| | **Water content (mg)** | | | | |
| **Beans**[1] | 2236.54 ± 625.74$^{ab}$ | 2214.98 ± 720.52$^{cd}$ | 4018.12 ± 728.09 $^{ac}$ | 3677.36 ± 878.13$^{bd}$ | 3291.10 ± 429.49 |
| **Maize**[1] | 3809.58 ± 302.09$^{abc}$ | 2523.99 ± 352.76$^{a}$ | 3147.22 ± 455.18$^{d}$ | 2649.76 ± 432.02$^{b}$ | 2216.82 ± 514.54$^{cd}$ |
| **Lettuce**[2] | 50.19 ± 15.62$^{ab}$ | 28.31 ± 1.95$^{ac}$ | 41.09 ± 8.17$^{d}$ | 44.45 ± 11.91$^{e}$ | 5.38 ± 2.38$^{bcde}$ |
| **Radish**[2] | 410.22 ± 85.74$^{abcd}$ | 198.40 ± 21.99 $^{a}$ | 235.06 ± 15.26 $^{b}$ | 190.86 ± 25.06 $^{c}$ | 186.61 ± 30.93$^{d}$ |
| **Grass**[2] | 25.61 ± 3.0$^{abc}$ | 17.03 ± 7.42$^{ade}$ | 24.74 ± 4.88$^{fg}$ | 5.62 ± 3.17$^{bdf}$ | 6.21 ± 1.47$^{ceg}$ |

Similar letters indicate significant difference ($p \leq 0.05$) between treatments within a species
[1]Specimens exposure period of 4 weeks
[2]Specimens exposure period of 3 weeks

# 4. Discussion

This study investigated a historically landfarmed site to establish whether remediation practices on the site were successful. Physical soil properties were studied and contaminants, still present in the soil, were quantitatively determined. Bioassays and physico-chemical analyses were carried out to incorporate the level of contaminants, their bioavailability, as well as their effects on the soil biota. Results from this study indicated that the remediated landfarming site soil were less toxic to soil organisms that were experimentally exposed to it, compared to API-sludge-spiked control soils. However, some of the studied endpoints were still negatively affected by the remediated soils.

## 4.1. Physical and chemical composition of soils

The physical composition of the landfarming-site soils (north- and south-site soils) was similar but differed from the off-site soil. The site-soils contained high organic matter and less clay than the natural off-site soil even though the off-site soil was obtained in close proximity to the landfarming site (Table 6). This may be due to the fact that the landfarming site had undergone extensive mechanical breakdown during the sludge application and bioremediation processes that caused changes in soil characteristics. From the physical characteristics in Table 6 it is clear that the artificial OECD-soil composition was substantially different from other soils used during this study. The clay and organic content were much higher than that of the off-site and natural LUFA2.2 soils which made it less ideal for comparison to the site-soils. The soil properties of the LUFA2.2-soil and off-site soil had a similar composition and both are classified as ‘loamy sand’ according to the United Stated Department of Agriculture classification system (Soil Survey Staff 2010). The pH values of the different soils did not vary extensively and were all found to be between the range prescribed by the OECD (OECD 2004a) for ecotoxicological tests (between 5.5 and 6.8).

The concentration of chemicals in the north-site soil was higher than in the south-site soil. The reason for the higher contaminants in the north-site soil could be explained by the fact that it was closer to the API-sludge dams. According to personal correspondence with staff members on the site it is assumed that during the earlier landfarming process the API-sludge application was higher on the smaller north-site than on the south-site because it was easier to access and the API-sludge application could be performed faster.

From the chemical analyses of the soils (Tables 7-10) it was clear that the concentration of heavy metals, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbons (TPHs) contamination were not present in the off-site soil (control soil) at high enough levels to have been from API-sludge contamination. The only element that was present at high levels in the off-site soil was sulphur (12.6 mg/kg). The site-soils also contained high sulphur levels, however, the concentration of sulphur in the fresh API-sludge was much lower (6.37mg/kg) and probably not the origin of the sulphur contamination in the soils. The industrial development and petrol refinery in the area is known to emit gaseous sulphur dioxide ($SO_2$) into the environment. The $SO_2$ is then likely transported into the atmosphere and potentially spread over a larger area. The main source of S deposition on soil is through acid rain (Äyriis *et al*. 1997). It is known that the combustion of fuels emit $SO_2$ and $NO_2$ which in turn deposits sulphuric acid and nitric acid during the process of acid rain giving a possible explanation for the high levels of S in the soils (Äyriis *et al*. 1997; Department of Water Affairs and Forestry 1998).

High levels of aluminium (Al), manganese (Mn), lead (Pb) and zinc (Zn) were present in both site-soils and API-sludge and the concentrations of Al and Mn were above the acceptable risk limit (ARL) concentrations stated in the DWAF waste disposal guideline (Department of Water Affairs and Forestry 1998) (Table 7). Naturally abundant elements like potassium (K), magnesium (Mg), silicon (Si), barium (Ba) and calcium (Ca) were also present in site soils and off-site soil at levels higher than what were present the API-sludge (Table 7). Similarly to the naturally abundant elements there were higher concentrations of Pb and S present in the off-site and site soils but only low levels in the API-sludge suggesting the Pb and S to have originated from another source. The concentration of iron (Fe) in the API-sludge was high but its concentration in the site soils was as low as levels close to the minimum detectable limit.

Previous reports on the bioremediation of the site mentioned application of lime ($CaCO_3$) to the landfarming site in 2000 to increase the soil pH (Personal Communication). It is known that pH is one of the most important factors influencing the mobility of heavy metals in soils (Spurgeon *et al*. 2006; Van Gestel 2008). Lime addition effectively reduced the mobility of copper, lead and other metals in contaminated soils by raising the soil pH. When the elements are immobilised by the addition of lime their leaching ability and bioavailability were expected to decrease (Kumpiene *et al*. 2008). Previous reports on the site (in the year 2006 and 2007, not published) showed that the concerning metals with concentrations higher than the ARL (determined for contamination in water and not soil) were vanadium (V), Pb and Mn with concentrations of 1.32 mg/kg, 0.107 mg/kg and 0.04 mg/kg respectively (the concentrations are

means calculated from measured concentrations in samples taken from the whole site). During the present study no traces of V higher than the detectable limits was observed but the Pb, Mn and Zn concentrations were higher ( ± 0.23 mg/kg, ± 1.25 mg/kg and ± 2.87 mg/kg respectively) that those of the ARL (Table 7). The reason for differences in concentrations of contaminants in this study compared to previous analyses might be due to a difference in sampling techniques used. Also, the first samplings were done 3 years before the sampling of the present study and the level of effective bioremediation and soil pH may have varied. The toxicity of metals is dependent on the tolerance of soil organisms to the bioavailable concentration of these metals. Zn is known to be an essential micronutrient required to ensure normal growth, reproduction and development in soil organisms as opposed to Pb that does not have a known physiological function (Kumpiene *et al*. 2008). It can be argued that by decreasing the mobility of metals in soil, both the essential and non-essential metals will be compromised for soil organism uptake. Thus, when soil organisms are unable to obtain essential metals from the soil their survival may again be compromised.

High concentrations of diesel range organics (DROs) were present in the site-soils and are possibly the most concerning contaminants with regard to the toxicity of the soil to organisms. The levels of DROs in the north-site soil were more than double that of the concentration found in the south-site soil (1469.25 ± 125 mg/kg and 675.25 ± 50 mg/kg respectively) (Table 8). Although there were still high levels of DROs present in the site soils after remediation, it was less compared to the DRO concentration in the API-sludge (104 206 mg/kg). High levels of total petroleum hydrocarbons (TPHs), comprising of longer aliphatic chain DROs and shorter aliphatic chain gasoline range organics (GROs), were present in the API-sludge (Table 8). Comparison of the levels of TPHs in the API-sludge to the concentrations found in the site-soils suggests that remediation successfully reduced the shorter aliphatic chained GROs however it was not as effective for the DROs' remediation.

No traces of the volatile organic compounds (VOCs) were found in the site soils although high levels of VOCs were present in the API-sludge (Table 9). The degradation of VOCs in soil has been well studied. According to Insam & Seewald (2010) soil acts as an ideal natural biofilter for the purpose of degradation. It provides a variety of microbes (for biological degradation), specific environmental conditions (optimal pH, temperature and oxygen levels) and adsorbants like water, organic matter and clay minerals all contributing to conditions for optimal degradation. VOC adsorption increases with higher organic carbon content but decreases in acidic soils (Insam & Seewald 2010). Thus, when the organic contents in the site-soils increased during landfarming and later when the pH was raised with lime addition, it may have

optimised the breakdown of VOCs present in the soil. Insam & Seewald (2010) further suggested that VOCs breakdown by soil microorganisms is only possible when the VOCs are changed from gas phase to liquid phase or when adsorbed to organic matter or clay mineral surfaces with polar and apolar interactions. However, since VOCs are highly volatile, most of the lighter VOCs would rather evaporate and disperse in the air than be adsorbed to the soil matrix. Thus, it can be assumed that the VOCs in the site-soils have either evaporated during the landfarming of the API-sludge or have been successfully bioremediated.

Analyses of PAHs, during the present study, showed that PAHs were present in the bioremediated landfarming site-soil as well as in the API-sludge (Table 10). The United States of America Environmental Protection Agency (US EPA) designated 16 PAHs as priority pollutants that can be harmful to humans. Further, the International Agency for Research of Cancer (IARC) established 7 of these to be possible human carcinogens. These carcinogenic PAHs are: Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indeno (1,2,3-cd)pyrene and Dibenzo(a,h) anthracene (Nadal *et al*. 2004). All of the 16 priority PAHs were found to be present, at different concentrations, in the API-sludge except the substance acenaphtylene. Although acenaphthene and fluorene were also present in the API-sludge these PAHs were not present in the site-soils at detectible concentrations. The total concentrations of the 16 priority PAHs and 7 carcinogenic PAHs in both site-soils were present in similar levels and significantly less than in the fresh API-sludge (701.25 µg/kg). The total concentration of the 16 priority PAHs in north-site soil was 89.18 ± 3.19 µg/kg and 87.49 ± 2.30 µg/kg in the south-site soil. When comparing the levels of PAHs in the site-soils to other studies where the PAH concentrations were established in sites close to oil refineries, the concentrations in the present study sites were much lower. Bakker *et al.* (2000) have found levels as high as 3000-14 000 mg/kg measured for the 7 carcinogenic PAHs 1.3-4.2km from a refinery in Zelzate (Belgium). Another study by Škrbic *et al.*(2005) that focussed on a refinery in Novi Sad, Montenegro showed levels of the priority PAHs up to 47 870 mg/kg. In the present study the total priority PAH concentrations in the site soils were only ± 89 µg/kg and ± 52 µg/kg for the 7 carcinogenic PAHs. These low values of PAHs found in the site-soils may be because these compounds had undergone degradation during the remediation process in comparison to other refinery site studies where remediation technologies were not applied. A proposed explanation for the breakdown of PAHs by Mohan *et al.* (2006) suggested that PAHs undergo degradation in the atmosphere through photolysis as well as through biotic and abiotic degradation when exposed in the soil substrate over long periods. Thus, the PAHs originally present in the API-sludge could have degraded during the application process when landfarming was executed and further degraded when exposed in the soil media after the landfarming was discontinued.

## 4.2. Bioassays

The bioassay results elucidated the potentially harmful fraction of contaminants in the landfarming site soils and whether the remediation was successful in decreasing its toxicity to soil organisms. From the results, obtained from API-sludge-spiked exposures, the most sensitive endpoints to the API-sludge were established as well as the lowest concentration of API-sludge at which the endpoints are affected.

### 4.2.1. Exposures of soil organisms to control soils

It is known that the earthworm test species used, grow optimally in substrates with high organic content (Sims & Gerard 1985). However, comparing the results of earthworm exposures in off-site to OECD-soil it can be concluded that the experimental conditions in these control soils were optimal for earthworm exposures (Table 11, 12 and Figure 5). Even though the off-site soil had a low organic matter content (± 0.4%) in comparison to its high fraction in OECD-soil (10%) the earthworms still survived, did not lose weight after 4 weeks of exposure and produced cocoons and hatchlings. This indicated that both the off-site soil and OECD-soil were suitable control soils and that the hatching success was not significantly influenced by exposure to the different soil types.

To examine potential positive controls where one could expect the earthworms to be affected, OECD- and off-site soils were spiked with 1% API-sludge. The exposed specimens showed significant weight loss (Table 11 and Figure 5) and no reproduction after 4 weeks (Table 12) in both the spiked OECD- and off-site soils confirming that the 1% API-sludge-spiked soils could serve as suitable positive controls.

Survival of the potworm species, *E. doerjesi*, exposed to the control soils (Table 14) was lower in off-site soil than in the OECD-soil and a significantly lower number of juveniles were found in the off-site soil compared to the OECD-soil. Thus, it was clear that soil type influenced the survival and reproduction of exposed specimens and that the off-site soil was not an ideal control soil for *E. doerjesi* exposures. It is perhaps possible that the lower organic matter content in the off-site soil influenced the results. Potworms are known to be living in soils with organic rich matter (Beylich & Achazi 1999; Memis *et al*. 2004). Thus, the lower reproduction in the off-site soil compared to OECD-soils in general may be explained by the fact that the off-site soil type is so extremely different from the potworms' natural environment. In the positive control soil (1% API-sludge-spiked off-site soil) exposures the survival and juvenile production were

lower compared to OECD-soil exposures. However, exposures to the positive control did not show significantly different results from off-site soil without spiked 1% API-sludge. This indicated that the potworms were not sensitive to an API-sludge addition of 1%. Higher concentrations of API-sludge-spiked soils would potentially be a more accurate positive control where potworms would be affected by the sludge addition. From these results it, can be deduced that the OECD-soil acted as a good control soil because the survival and reproduction were not negatively affected by it. The off-site soil and 1% API-sludge-spiked soil were not a good control soil for this species, since low juvenile production was found in the off-site soil and no significant effects of the specimens were observed in the positive control soil exposures.

During the concentration series exposures of *E. doerjesi*, the number of juveniles present in all the control soils, which have not been spiked in the concentration series of API-sludge, differed between the soils after the 3 week exposures (first bar of each graph in Figures 10A and 11A). When comparing these values to those for juveniles obtained from to the same soil type exposures during the site-soil experiments (Table 18) they were found to be higher. Although one would expect not to see great variance when comparing these soil exposures (because the experiments were carried out in the same manner with specimens obtained from the same culture) it was still the case. Thus, by only carrying out these experiments at different times, it already showed great variability in the results.

A comparison of *F. candida* exposed to the three different control soils where no API-sludge was added showed the different soil types influenced the springtail reproduction. The highest number of juveniles was obtained in the off-site control soil (479.89 ± 30.52) and not in the OECD-soil (388.33 ±17.86) which was the opposite from what was found for the potworms but these differences were not significant (Table 18 and 22). It is not apparent which soil characteristics, in the different control soils, could have been responsible for the changes in juvenile numbers. However, *F. candida* exposures to the control soils were appropriate since high numbers of juveniles were found in the off-site soil and OECD-soil and no juveniles were found in the positive control soil.

During the concentration series exposures of *F. candida*, the number of juveniles present in all the control soils, which have not been spiked in the concentration series of API-sludge, was similar after the 4 week exposures (first bar of each graph in Figures 13A and 14A). However, these numbers were lower when compared to exposures in the same control soil types during the site-soil experiments (Table 22). These experiments were not executed simultaneously and springtail cultures used were not synchronised at the same time. Thus, even though all the soil

conditions for the different experiments were consistent, the same species were used and obtained from the same stock culture there was still great variability.

### 4.2.2. Exposures of soil organisms to site-soils

*Eisenia andrei* exposed to the landfarming site-soils (north- or south-site soils) all survived after 6 weeks. Even though no significant biomass changes were observed for the earthworms exposed to the control soil (off-site soil) and site-soils a difference was noticed (Figure 6). In both off-site and south-site soils the exposed earthworms showed decreased biomass after one week thereafter the biomass recovered during the remaining experimental time. The biomass changes in the control soil and south-site soil suggest that the specimens in the south-site soil were not affected by the contaminants still present in this soil. In the case of the 1% API-sludge-spiked soil and north-site soil exposures, earthworms lost biomass after the first week and were not able to regain the biomass as successfully as in the off-site soil and south-site soil, implying that *E. andrei* biomass was affected negatively by the contaminants present in these soils. *E. andrei* specimens exposed to the north-site soil showed the greatest biomass loss; even greater than what was found for the positive control soil. Earthworms exposed to the south-site soil produced a similar number of cocoons than those exposed to the off-site soil (19.0 ± 5.3 and 18.5 ± 9.7 respectively). In north-site soil the cocoon production was less (1.25 ± 0.7). Thus, the higher concentrations of contaminants still present in the north-site soil may be more bioavailable to soil organisms and could explain why the exposed specimens' biomass and reproduction were negatively affected.

The specimens exposed to all four test soils (off-site soil, site-soils and off-site soil + 1% API-sludge) lost biomass during the first week of exposure to the particular soils (Figure 6). This observation may be explained by the fact that the earthworms still needed time to adjust to the test soil substrate. Even though all earthworms were acclimised to the changing substrate, by exposing them to control soils prior to the experiments, it is clear that this exposure time was not sufficient for the worms to fully adjust to their new environment before the exposures to the test soils were carried out.

More than double the number of juveniles were found for *E. doerjesi* in the north- and south-site soils (339.75 ± 76.92 and 414.00 ± 17.78 respectively) compared to the number of juveniles in the off-site and OECD-soils (only 57 ± 34.39 juveniles in off-site soil and 175.75 ± 56.03 juveniles in OECD-soil) after 3 weeks of exposure (Table 14). The reason for the higher number of juveniles in the site soils compared to the off-site soil may be because of the higher

organic matter content of the site-soils (4.4% and 2.6% respectively) compared to the organic matter (0.4%) in the off-site soil. The juvenile production of *E. doerjesi* in the site-soils was even higher than in the OECD-soil even though the OECD-soil had a higher organic matter content than the site soils. This suggests that the organic matter content in the soil is not the only factor that influenced the toxicity of the contaminants. The species was less sensitive to the contamination and thus not sensitive to the API-sludge contamination. During the bioremediation processes, applied to the landfarming site, the site-soils were aerated and soil characteristics were altered to such an extent that it may have increased its microbial activity which was probably lacking in the artificial soils. Furthermore, *E. doerjesi* feeds on decomposed plant matter and microorganisms whose numbers are most likely higher in naturally obtained soils allowing it to be an additional food source that might not be available in artificial OECD-soil (Didden 1992).

*F. candida* exposures to site-soil showed significantly less juveniles than what was found in the control soils. The juveniles found in the north-site soil were significantly lower than both off-site and OECD-soil exposures whereas the juveniles in the south-site soil exposures were only significantly less than the juveniles found in the off-site soil. These results suggested that the contaminants present in the site soils (at these concentrations) had an inhibitory effect on the reproduction of *F. candida* and that this species was sensitive to the API-sludge contamination (north-site soil more than the south-site soil).

### 4.2.3. Avoidance behaviour tests

The three test species were all exposed to two soil types in one container: site-soils on one side and off-site soil (control soil) on the other. This was done to establish whether exposed soil organisms had the ability to avoid contamination. This provided an additional endpoint when the sensitivity of the soil organisms to the contaminants was investigated.

Specimens of all three test species exposed to off-site soil on both sides of a container, were equally distributed between the two sides. However, when the three test species' avoidance behaviour was monitored in the site-soils, different observations were made. Only *E. andrei* avoided both site-soils (north- and south-site soil) in contrast to the *F. candida* specimens that strongly preferred these soils above the off-site soil. In the case of *E. doejesi*, the north-site soil was avoided and the south-site soil was preferred compared to off-site soil. When correlating this data to the sensitivity of other studied endpoints (survival, reproduction etc.) for the test organisms it is interesting to note that even though *F. candida* were most affected by the site-soil during chronic tests, they did not avoid these soils. The reason perhaps being that they were not

able to 'sense' the contaminants present in the soil. It is clear that *F. candida* preferred to move to soils with the higher organic matter content in all exposures as has been confirmed by similar studies by Paton *et* al.(2005), Lors *et al.* (2006) and Natal-Da-Luz *et al.* (2008). Thus, another reason for the observed avoidance pattern may be that *F. candida* specimens would rather prefer to move towards site-soil with higher organic matter content than avoiding the contaminants present in these soils.

*E. andrei* was able to avoid (however not very strongly) both site-soils despite the fact that during the longer exposures (in the chronic tests) to the site-soils detrimental effects were only seen in north-site soil exposures.

*E. doerjesi* avoidance of the north-site soil and preference for the south-site soil were both statistically significant even though this species was the least sensitive to the site-soils during in the long term exposures. Previous studies by Amorim *et al.* (2005) and Achazi *et al.* (1999) that did similar avoidance behaviour tests with *E. albidus* and *E. crypticus* (similar species to *E. doerjesi* and used in standardised tests) towards organic pest- and herbicides found that the avoidance test was a more sensitive endpoint compared to their reproduction. However, it is difficult to establish whether the specimens were not avoiding the south-site soil because it was not toxic and successfully bioremediated, or because of its preference to a specific physical soil type.

Hund-Rinke *et al.* (2003) suggested that one can assume contaminants to be toxic if the exposed organisms show avoidance to the soils greater than 80%. Thus, the 1% API-sludge-spiked soil sample can be assumed to be toxic to all three test organisms when these criteria are used as guideline since all three species showed avoidance of more than 80%.

These avoidance behaviour experiments may be used as an additional assessment for quick and simple verification of the longer term chronic exposures and can potentially be utilized as an early screening tool when doing future assessments on the landfarming site soil (Yeardley *et al*. 1996; Hund-Rinke & Wiechering 2001; Natal-Da-Luz *et al*. 2004).

### 4.2.4. Exposures of soil organisms to API-sludge-spiked soils

#### *Eisenia andrei* exposures

Biomass changes seen for *Eisenia andrei* specimens exposed to OECD-soil confirm their ability to regain lost biomass over 4 weeks even with the addition of API-sludge concentrations up to 1% (Table 15 and Figure 7). However, at API-sludge-spiked concentrations higher than 1% the earthworms were unable to regain lost biomass.

The survival and cocoon production in two concentration series, one in off-site soil and another in OECD-soil did not result in similar lethal concentrations ($LC_{50}$) and effect concentrations ($EC_{50}$- for cocoon production). Survival success after 6 weeks of exposure showed the $LC_{50}$ in API-sludge-spiked OECD-soil to be almost double that found in the off-site soil (3.2%- and 1.15% API-sludge respectively). Hanna & Weaver (2002) and Kula & Larink (1997) did studies on the effects of contaminants in OECD-soil compared to the same contaminants toxicity in natural soils (when using LUFA2.2-soil as natural soil). They found that biomass change was not a sensitive endpoint compared to earthworm survival. They further argued that the $LC_{50}$ in LUFA2.2-soil with less organic matter contents were lower than in artificial OECD-soil when exposed to mixtures of metals and hydrocarbons. These were similar to the effects observed in this study when comparing survival in OECD-soil with high levels of organic carbon content to the off-site soil that had a lower organic hydrocarbon content (Table 25). The higher sensitivity of earthworm survival to the API-sludge in the off-site soil can be explained when considering that the bioavailability of contaminants to soil organisms is higher in soils with lower organic matter contents (Lanno *et al*. 2004). Another study of oil contaminated soils by Dorn & Salanitro (2000) explained that oil waste products with a high aromatic carbon contents (and similar contaminants to what was present in the API-sludge) are initially acutely toxic to *E. fetida* (their survival) but loses toxicity after bioremediation has been applied. Also, that crude oil exposures in bioremediated silty soils with low organic matter content was more acutely toxic than in high organic matter soils (Dorn *et al*. 1998)**.**

When considering cocoon production as endpoint the cocoon production in OECD-soil, without additional API-sludge, was significantly lower than in the case of the off-site soil (12.50 ± 3.39 cocoons in 0% API-sludge OECD-soil and 51.00 ± 9.00 cocoons in 0% API-sludge off-site soil). Further, the $EC_{50}$ for cocoon production in the OECD-soil was 0.59% API-sludge. This was more sensitive than the $EC_{50}$ of 1.47% API-sludge in the off-site soil. Thus, the API-sludge negatively affected earthworm cocoon production; more if exposed in OECD-soil as opposed to in off-site soil. A reason for these observations may be explained by considering the degradation of the high levels of organic hydrocarbons still present in the API-sludge over time. Hydrocarbon

degradation in natural soil is more optimal because of its higher microbial activity as opposed to artificial soil (Althoff *et al*. 2009). Its breakdown is more efficient, making it less detrimental to exposed specimens. However, the microbial communities may also be affected by the API-sludge addition. Previous studies on oil and hydrocarbon contaminated soils indicated that microbial communities in soil are stressed by the contamination which may also be the case for the API-sludge contamination (Brohon *et al*. 2001). Anigboro & Tonukari (2008) suggested a possible way in which oil substances affect soil organisms. They explained that the oil (or API-sludge in this study) coats the soil particles and surface resulting in anaerobic soil conditions that stress the soil environment due to oxygen constraints.

### *Enchytraeus doerjesi* exposures

The survival success of this species in the concentration series of API-sludge-spiked control soils (off-site, OECD- and LUFA2.2-soil) was between 90-100% and the calculated no observed effect concentrations (NOEC) were in all instances higher than the spiked concentrations used in the series range. The $EC_{50}$s for reproduction were similar in off-site soil and OECD-soil and were at an API-sludge concentration of 2.50% and 2.66% respectively (Table 25). However it was less sensitive (with a higher $EC_{50}$ of 3.6% API-sludge) when exposed in LUFA2.2-soil. Studies by Giere (1979) and Giere & Hauschildt (1979) have shown some enchytraeid species to be strongly resistant to oil pollution and they may be capable of excreting or accumulating oil derivatives in their body tissue. The toxicity of individual PAHs to potworms was studied by Achazi *et al.* (1999). They reported no direct toxic effects of low levels of Benzo(a)pyrene (BaP) and Fluoranthene (Fla) to *E. crypticus*. Even though the LOEC values for BaP (1 mg/kg) and Fla (10 mg/kg) were low for reproduction in agar medium, it increased to higher than 100 mg/kg when the medium was changed to soil (Didden & Römbke 2001) making the organisms less sensitive to the toxicants when exposed in soil media as was the case in the present study. For many chemical contaminants like oil, hydrocarbons and other industrial chemicals the known toxicity to potworms are limited (Didden & Römbke 2001). Also, great interspecies differences towards contaminant sensitivity limit the ability to accurately compare the effects of contaminants when using only enchytraeid test species.

***Folsomia candida* exposures**

The *Folsomia candida* specimens exposed to the concentration series in control soils confirmed the sensitivity of this species to the API-sludge even at low concentrations (Table 25). The $EC_{50}$ for reproduction was the lowest in off-site then LUFA2.2- and highest in OECD-soil (0.021%-, 0.09% and 0.33% API-sludge respectively). It is known that metal and hydrocarbon toxicity decreases with the increase of organic matter content and mineral clay in soil (Styrishave *et al*. 2008). Thus, the bioavailable fraction of contaminants in the API-sludge, responsible for its toxicity to *F. candida*, differs between soils because it was probably adsorbed to the different organic matter and mineral clay contents of the soils.

**Table 25:** Summary of bioassay results. Comparison of the three test species and endpoints monitored in various test soils.

| | | *Eisenia andrei* | *Enchytraeus doerjesi* | *Folsomia candida* |
|---|---|---|---|---|
| **Off-site soil** | | | | |
| | Survival (%) | 100 | 100 | - |
| | Survival ($LC_{50}$ - % API-sludge) | 1.15 | ≥ 5 | - |
| | Cocoon production ($EC_{50}$- % API-sludge) | 1.47 | 2.66 | 0.021 |
| | Showed significant soil avoidance? | × | × | ✓ |
| **OECD-soil** | | | | |
| | Survival (%) | 100 | 100 | - |
| | Survival ($LC_{50}$- % API-sludge) | 3.2 | ≥ 5 | - |
| | Cocoon production ($EC_{50}$ -% API-sludge) | 0.59 | 2.5 | 0.33 |
| | Showed significant soil avoidance? | - | × | × |
| **LUFA2.2-soil** | | | | |
| | Survival (%) | - | 90 | - |
| | Survival ($LC_{50}$- % API-sludge) | - | 5.35 | - |
| | Cocoon production ($EC_{50}$ - % API-sludge) | - | 3.6 | 0.09 |
| | Showed significant soil avoidance? | - | - | - |
| **North-site soil** | | | | |
| | Survival (%) | 88 | 86 | - |
| | Reproduction different from control soils? | ✓ [b] | ✓ [a] | ✓ [b] |
| | Showed significant soil avoidance? | × | ✓ | × |
| **South-site soil** | | | | |
| | Survival (%) | 100 | 78 | - |
| | Reproduction different from control soils? | × | ✓ [a] | ✓ [c] |
| | Showed significant soil avoidance? | × | × | × |

[a] had significantly **more** juveniles than the off-site and the OECD-soil ($p \leq 0.05$)

[b] significantly **less** cocoons/juveniles present when compared to both off-site soil and OECD-soil ($p \leq 0.05$)

[c] significantly **less** juveniles present when compared to only off-site soil ($p \leq 0.05$)

### 4.2.5. Species sensitivity

In an attempt to compare the soil organism sensitivity to remediated soils with exposure to the spiked API-sludge soils, it was important to consider the spiked control soil which had the most similar composition to the site-soils. Although none of the control soils had a comparable relation (because of their different soil properties) to the site-soils the focus was on the off-site soil because it represents the natural soil in the region and is assumed to be the original soil composition of the site-soils before it was landfarmed. The sensitivity of the terrestrial animals and the determined endpoints in response to fresh API-sludge, according to $LC_{50}$s- and $EC_{50}$s in off-site soil, can be ranked from most to least sensitive: *F. candida* reproduction > *E.andrei* cocoon production > *E. andrei* survival > *E. doejesi* reproduction > *E. doejesi* survival (Figure 20). Numerous studies have shown similar sensitivity patterns than those found in this study when earthworms (*E. fetida*), springtails (*F. candida* and *F. fimetaria*) and a range of crop plants and microorganisms were exposed to metal and hydrocarbon mixtures or oils (Dorn *et al*. 1998; Sverdrup *et al*. 2001; Eom *et al*. 2007; Sverdrup *et al*. 2007; Wilke *et al*. 2008) which confirmed the sensitivity of *F. candida* reproduction to hydrocarbon contaminated soils. Thus, when choosing species for a test battery *F. candida* would be a good indicator species to use because of its sensitivity to hydrocarbon and oil contaminants.

Punnaruttanakun *et al.* (2003) attempted to establish the concentrations of crude oil that will lead to the mortality of earthworms in laboratory studies and suggested an allowable level not higher than 1% oil contamination in soils for earthworm survival which was similar to the results obtained in this study. When comparing the $EC_{50}$s for reproduction in different soil types, the three species of soil organisms were not equally sensitive to the applied API-sludge in the different soils (Figure 20). This stresses the importance to use more than one test species when applying bioassays as a soil quality assessment tool.

It is known that the bioavailability and toxicity of most contaminants are highly dependent on the composition of the soils as well as the organic matter content (Dorn *et al*. 1998). If this was the case one could predict that with the increase of organic matter and mineral clay content the bioavailability and indirectly also toxicity would decrease to soil organisms. However, this was not the case: The reproduction of *F. candida* was distinctly more sensitive in off-site soil, *E. andrei* in OECD-soil and *E. doerjesi* showed almost equal sensitivity towards the API-sludge exposures in OECD- and off-site soil. From these results it is clear that although the bioavailability of a contaminant may commonly be related to its hazardous fraction it seems to not be the only determining factor when considering the toxicity of contaminants in different

soils. Organic contaminants (like hydrocarbons) are generally hydrophobic and will rather be adsorbed to soil mineral clay particles and organic matter. This means that only a fraction of these contaminants are available to organisms via their water uptake and main exposure pathway for soil organisms according to Paton *et al.* (2005). However, the bioavailability of metals to the soil organisms is more dependent on the pH of the soil (Van Gestel 2008). Geissen *et al.* (2008) proposed that species sensitivity comparisons are possible if the routes of uptake are similar but because the contaminants in this study consisted of a mixture of toxicants, at different concentrations, multiple routes of uptake would be apparent.

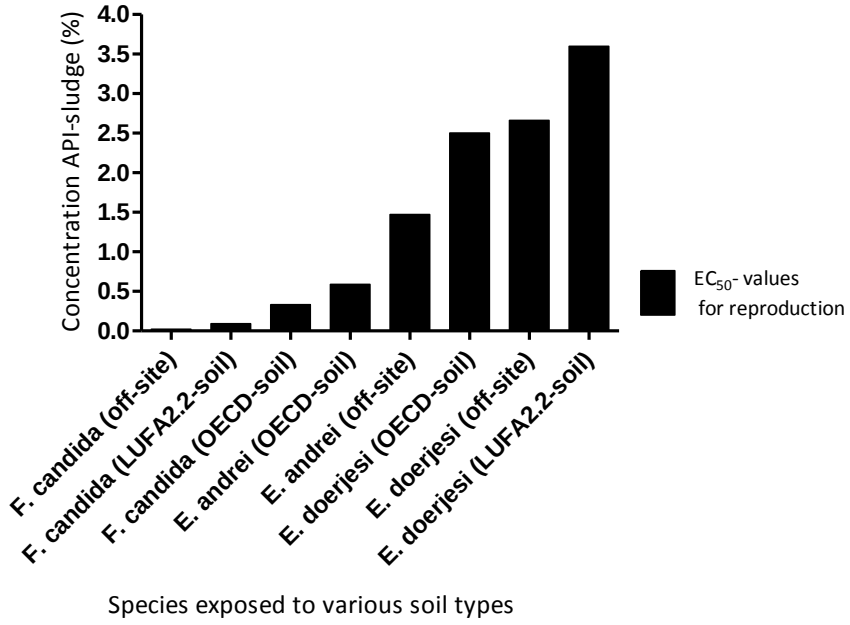



**Figure 20:** Effect concentrations where 50% of the test organisms' reproduction is affected when exposed to a concentration series of API-sludge in various soil types.

## 4.3. API-sludge toxicity

To predict the contaminant or group of contaminants in the landfarming site soil potentially responsible for the toxicity to biota, the concentrations of the contaminants were investigated. The DROs (a fraction of the TPHs) present in the soils were at such high levels in comparison to the other constituents in the soil and should be considered as the most concerning soil contaminant. In the fresh API-sludge, high levels of additional VOCs and the GROs were present in relatively large quantities that are absent in the site-soil. These observations confirm that remediation did decrease the toxicity of the API-sludge in the site-soils and was similar to what was found by DiToro *et al.* (2007). DiToro *et al.* (2007) explained that the smaller organic

hydrocarbon components in oil weather faster than the bigger molecules and by doing that decreases the toxicity in the soil. The varying levels of these contaminants may have different effects on the sensitivity of the bioassays which will therefore differ when comparing the remediated soils to soils with fresh added API-sludge.

The single most toxic contaminants still present in the site-soils are difficult to determine. By integrating the biological assay data with the chemical results an attempt was made to identify which contaminants may be responsible for the toxicity in the landfarming site soils. To determine the potentially toxic fraction of the single contaminants in the soil, the following calculation was done: An $EC_{50}$-fraction was determined by dividing the concentration of the single contaminants in the API-sludge and soils by their $EC_{50}$ obtained from literature (Table 27). Two of the most sensitive endpoints obtained from this study were used for these comparisons (*F. candida* reproduction in off-site soil and *E. andrei* cocoon production in off-site soil). Where the literature values for the $EC_{50}$ for reproduction were not available, the $LC_{50}$s/$LD_{50}$s for the species were used. The $EC_{50}$-fraction for metals and PAHs in the soils were always lower when compared to the $EC_{50}$s of the single contaminants obtained from literature, except for the DROs (Table 27). Thus, it could be assumed that the DROs have the greatest impact on the reproduction of the organisms. When all the $EC_{50}$-fractions were added, the concentrations of contaminants in the fresh API-sludge predicted to have an $EC_{50}$ 5 times higher than what was determined in the off-site soil for the *F. candida*. In the case of *E. andrei* specimens, the total fractions did not add up to the toxicity observed in the fresh API-sludge-spiked concentration series. This indicated that when comparing the toxicity of the contaminants from literature to those of the contaminants in the API-sludge and soil, the springtail reproduction was predicted to be higher than that found in this study. As for the earthworms, $EC_{50}$-fractions of the single contaminants did not add up to the toxicity found for API-sludge. The reason was that only contaminants that were present in the site-soils or contaminants with known $EC_{50}$s in the literature were considered in the calculations (Tables 26 and 27). However, the API-sludge had an additional range of VOCs and GROs also present that was not considered when adding the toxic fractions. Thus, the reason why the toxicity of the fresh API-sludge to *F. candida* was much higher than the experimentally determined $EC_{50}$ may be due to this species sensitivity for the additional VOCs and DROs in the fresh API-sludge which were not incorporated in the calculation.

In the case of the site-soils, the toxicity of the added single contaminant fractions divided by the $EC_{50}$s determined in this study were much lower than the $EC_{50}$s determined with fresh sludge. That indicates that the remediation did decrease the toxicity on the landfarming site for

*F. candida*. This was also the case for *E. andrei*. It is important to note that when considering the single contaminants in a mixture, the assumptions are not entirely accurate because of the varying in the contaminants' potential to interact with each other and their different bioavailability in soil types that may influence the toxicity of mixtures (Jonker *et al*. 2005; Eom *et al*. 2007; De Laender *et al*. 2009).

**Table 26:** Summary of the concentrations of metals, PAHs and DROs present in the API-sludge, and site-soils.

| Contaminants | Concentration in soil (mg/kg) | | |
|---|---|---|---|
| | API-sludge | North-site | South-site |
| **Zn** | 0.280 | **3.930± 0.07** | **1.810 ±0.08** |
| **Pb** | 0.050 | **0.290±0.35** | **0.160±0.02** |
| **Mn** | **0.650** | **1.670±0.19** | **0.820±0.06** |
| **Al** | **0.440** | **11.63± 0.78** | **1.570±0.15** |
| **S** | 6.370 | **59.130±1.24** | **29.710±2.98** |
| **∑16 PAHs** | 0.701 | 0.089±0.003 | 0.087±0.002 |
| **Diesel Range Organics** | 104 206 | 1469.250±125.12 | 675.250±49.22 |

Bold values indicate concentrations above ARL (Department of Water Affairs and Forestry 1998)

**Table 27:** Comparison of the API-sludge $EC_{50}$s obtained for *Folsomia candida* and *Eisenia andrei* to single contaminant concentrations and $EC_{50}$s.

| | *Folsomia candida* | | | | | *Eisenia andrei* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $EC_{50}$ fraction = (Chemical cons. in soil (Table 26)/ $EC_{50}$ from literature) | | | | | $EC_{50}$ fraction = (Chemical cons. in soil (Table 26)/ $EC_{50}$ from literature) | | |
| **Contaminant** | **$EC_{50}$ (mg/kg) (survival/ juveniles)** | **Reference** | **API-sludge** | **North-site** | **South-site** | **$EC_{50}$ (mg/kg) (survival/ juveniles)** | **Reference** | **API-sludge** | **North-site** | **South-site** |
| **API-sludge (off-site soil)** | 210.00* | | | | | 14700.00* | | | | |
| **Zn** | 10.400[1] | CCME (2007) | 0.027 | 0.378 | 0.174 | 15.000[1] | CCME (2007) | 0.019 | 0.262 | 0.121 |
| **Pb** | 3.600[1] | CCME (2007) | 0.014 | 0.081 | 0.044 | 5.400[1] | CCME (2007) | 0.009 | 0.054 | 0.030 |
| **Mn** | 1663.000[1] | Didden & Römbke (2001) | 0.000 | 0.001 | 0.000 | 927.000[1] | Didden & Römbke (2001) | 0.001 | 0.002 | 0.001 |
| **Al** | - | | | | | - | | | | |
| **S** | - | | | | | - | | | | |
| **∑16 PAHs** | 79.000[1] | Sverdrup *et al.* (2002) | 0.009 | 0.001 | 0.001 | 118.000[1] | Sverdrup *et al.* (2002) | 0.006 | 0.001 | 0.001 |
| **Total petroleum hydrocarbons** | 23[2] | Verbruggen (2004) | 4530.695 | 63.880 | 29.359 | 72[2] | Verbruggen (2004) | 1447.306 | 20.406 | 9.785 |
| | | **Fraction total** | 4530.745 | 64.341 | 29.578 | | **Fraction total** | 1042.095 | 15.011 | 6.904 |
| | | **Fraction total / $EC_{50}$-API-sludge*** | 21.575 | 0.306 | 0.141 | | **Fraction total / $EC_{50}$-API-sludge*** | 0.098 | 0.001 | 0.001 |

[1]Literature values ($EC_{50}$ for reproduction)
[2]Literature values ($LC_{50}$/$LD_{50}$)
*$EC_{50}$s calculated in off-site soil in the present study

The contaminants in a mixture has the ability to influence each other's toxicity (through addition, or antagonistically) which makes it harder to predict the effects of single contaminants (Lapa *et al*. 2002). The bioavailability and toxicity of contaminants are also site-specific because of the proposed interactions of toxicants with different soil types. Owing to these factors the mixture of toxicants in the API-sludge were rather considered as a single contaminant to determine the critical concentration of API-sludge in the soils. However, the toxicity of mixtures, such as the API-sludge in the landfarming site soils, is difficult to predict. By comparing the levels of specific contaminants to $EC_{50}$ for oil mixtures would be a suggestion. However, as previously explained and seen in Figure 20 the $EC_{50}$s vary between soil types and species. Further, the contaminants mentioned in previous studies with a similar composition of toxicants as was found in the API-sludge, all differed in concentrations and specific toxicants. These varying factors make it difficult to compare $EC_{50}$s from literature to the results of this study. A suggestion was to compare the number of cocoons or juveniles produced in the off-site soil (with no added API-sludge) to those produced in the site-soils and at $EC_{50}$s for the determined endpoints. To be able to compare these values, and prevent incorporating more variables, only

effects from a single control soil at a time was compared. Even though in some cases the organism exposures to the off-site soils were not the most sensitive for the endpoints, they were still used for comparison to see what the reproduction rate would have been if landfarming was not executed in the area. For *E. andrei* the number of cocoons produced in the off-site soil with no added API-sludge (control soil) was determined to be a mean of 51 ± 9.0 cocoons (Table 16). The estimated number of cocoons (extrapolated from Figure 5A) at the $EC_{50}$ was determined to be 25 cocoons. A lower number of cocoons was obtained in both north-site and south-site soil exposures (a mean of 1.25 and 19.0 cocoons respectively) than counted in the control soil and at the $EC_{50}$. From this it is clear that there are still toxicants present in both north- and south-site soils at levels that have the ability to reduce more than 50% of cocoon production in earthworms. The *F. canidida* exposed to 0% API-sludge-spiked off-site soil produced 108 ± 15.0 juveniles (extrapolated from Figure 14A) and 54.0 at the $EC_{50}$ of 0.021% API-sludge. *F. canidida* exposed to the north- and south-site soils produced 289.42 ± 58.62 and 253.33 ± 122.94 juveniles respectively (Table 22). Even though it seemed that the springtails had a successful higher juvenile production, the comparison was not valid because the site-soil exposures and the API-sludge concentration series experiments were carried out by using two different synchronised cultures, and different numbers of juveniles were obtained in similar control soils. However, when comparing the reproduction of specimens from the same synchronised culture exposures to off-site soil 479.89 ± 30.42 juveniles (Table 22) were counted. This indicates that for the organisms exposed to site-soils the reproduction was inhibited to approximately half of those exposed in the control soil although it was still significantly higher than the juveniles produced at the $EC_{50}$. Thus, a suggestion for successfully comparing the concentration series data to the site-soil data would be to first normalise it.

The juveniles counted in *E. doerjesi* exposures to north- and south-site soils were 339.75 ± 76.92 and 414.00 ± 17.78 (Table 18) respectively. A mean of 250 ± 42.5 juveniles were counted in the control samples (with no API-sludge) and 183.5 at the $EC_{50}$ (extrapolated from Figure 10A). Thus, the number of produced juveniles in the site-soils was more than 2 fold the estimated value at the $EC_{50,}$ showing that the site-soils did not negatively affect the potworm reproduction. Although the off-site soil was obtained close to the landfarming site, the physical composition was much different compared to the site-soils. A more accurate comparison would be to compare the number of cocoons or juveniles in the site-soils to the exposures to control soils with a more similar composition. Thus, the same comparison could be done by using the LUFA2.2-soil as a control (rather than the off-site soil) or the control soil where the reproduction of the organisms was the most sensitive to the API-sludge-spiking (in the case of *E. andrei* and

*E. doerjesi* exposures to OECD-soil). Even though useful results can obtained from this approach, it can also be criticized on the grounds that the assumptions are not entirely accurate because of the great variation between the physical soil properties and the variation in results when using animal cultures. When different organisms were exposed to the concentration series in freshly spiked API-sludge it still contained high levels of VOCs and DROs in comparison to the concentrations in the site-soils. For example no VOCs were present in the site-soils but were still present in the API-sludge. It is important to argue that the $EC_{50}$s obtained in this study were obtained for the mixture of toxicants in the fresh API-sludge and do not reflect the toxicity of the contaminants still present in the site-soils.

To considering the API-sludge as a single contaminant and not a mixture was suggested. The ARL of the API-sludge could then be determined. The calculation of the API-sludge ARL, using earthworm survival as endpoint and a sensitivity factor of 10 can be expressed as follows:

$$\text{ARL} = LC_{50} \times 0.1$$

$$= 11500 \text{ mg/kg} \times 0.1$$

$$= 1150 \text{ mg/kg or } 0.115\% \text{ API-sludge}$$

By considering the most sensitive endpoint of *F. candida* reproduction, the ARL is calculated to be only 21mg/kg or 0.0021% API-sludge. At the addition of 0.0021% API-sludge concentrations no effects to the endpoints tested in this study could be predicted. However, this concentration is unrealistically low and not relevant because during landfarming tons of API-sludge was applied to the soil surfaces at a time.

## 4.4. Plant exposures

The concentrations at which the different plant species could tolerate the added API-sludge in the soil differed between species. When increasing the API-sludge application to concentrations higher than 10% all plants started to die. The probable reason may be that the sludge fills all of the soil pores that inhibit the water and oxygen absorption of the plants at high concentrations of API-sludge. The soil can not adsorb the hydrocarbons when the concentrations get too high and it become more bioavailable for plant uptake (Baker 1970). Beans had a higher germination success, growth rate, total dry mass and water content in the 2.5% treatment than in the controls (Figures 17A,18A,19A and Table 24) suggesting that moderate contamination has the ability to improve these properties in beans. It is not clear whether it is a greater water uptake ability that supported the growth or whether the water uptake is a result of the rapid growth. In previous studies soybean growth was shown to be stimulated at even lower concentrations of oil

contamination (Baker 1970). There are three possible reasons for this; the presence of single contaminants in the API-sludge may act as a beneficial and/or hormone mimicking compound (Baker 1970), the API-sludge addition may change the micro-organism composition in the soil favourably (Nicolotti & Egli 1998) or the bean's nitrogen-fixing ability (due to its symbiosis with *Rhizobium* species in their root-nodules) allows it to survive the more strenuous conditions (Van Berkum & Bohlool 1980).

The LOEC of beans, in soil was higher than 10% API-sludge present also indicating that they have a high tolerance towards the API-sludge. In agreement with previous studies (Adam & Duncan 1999; Ogbo 2009; Wyszkowski & Ziólkowska 2009), beans may be proposed to be a good phytoremediator for hydrocarbon contamination.

Figures 18B, 19B and Table 24 respectively shows the decline in growth rate, total dry mass and water content of maize with increasing concentrations of the contaminants. However, the germination success results indicate that seeds may be less sensitive to the contamination (Figure 17B), unlike the reduced seed germination found by other studies with oil contamination (Ogbo 2009). Studies that assessed the sensitivity of maize towards oil contaminants found it to reduce germination success as well as growth rate (Ekundayo *et al*. 2001; Basic *et al*. 2009). On the contrary, (Dorn *et al*. 1998) found maize to be less sensitive when compared to other crop species exposed to crude oil. The low LOEC-value for maize (0.5% API-sludge) suggests that maize is at least sensitive to the API-sludge when considering their ability to gain biomass and thus, can not tolerate the API-sludge contamination.

The lettuce had low germination success in the control soil and when API-sludge was added it decreased even further (Figure 17C). Lettuce seeds are sensitive to high temperatures and during germination the 22˚C of the incubator might not have been optimal for germination. Reynolds & Thompson (1971) suggests the optimal temperature for lettuce seed germination to be between 15˚C and 18˚C. The growth rate for lettuce in the control soil was slow and a lack of growth rate differences between the various API-sludge-spiked soils (Figure 18C) could also be a result of the naturally slow growth rate in the control soil which was further reduced by the presence of API-sludge. Plaza *et al.* (2005) classified lettuce to be very sensitive to oil contamination, observing the worst effects during stem elongation (plant growth) rather than the germination. Figure 19C and Table 24 show the total dry mass and water content at low and high levels of API-sludge to affect lettuce the most, while intermediate levels (2.5% and 5% API-sludge addition) are not as detrimental as it did not differ statistically from the control soil exposures.

The API-sludge application increased the germination success of radish except in the 5% API-sludge contaminated soil (Figure 17D). These results were contradictory compared to the germination success of studies done by Banks & Schultz (2005) that did germination studies in petroleum-contaminated soils with radish. Banks & Schultz (2005) further argued that radish germination is not sensitive for hydrocarbon contamination as significant differences between contaminated and uncontaminated soil exposures were observed. In this present study, even though the germination success where decreased at 5% API-sludge addition the seedling growth rate at the same concentration was not negatively affected (Figure 18D). The exposures to 0% API-sludge soil had a greater growth rate during the whole growth period and thus a much larger final dry mass (Figure 18D and 19D). The exposures to 10% API-sludge-spiked soil had an overall slow growth rate, lowest mass and low water content concluding that radish was started to be affected at the increase in API-sludge concentrations higher than 10%.

Although one could expect that the increase in API-sludge contamination would have caused delayed seed germination and germination success for grasses, it was not the case. With the addition of 10% API-sludge the seeds started to germinate on day 2 in comparison to the first seedlings in the control soil only visible after 3 days of exposure (Figure 17E). Thus, the addition of API-sludge stimulated initial germination. Like all the other species, the grass seeds were relatively resistant to the API-sludge addition in the germination phase even though the germination success for grass was low in the control soil exposures. No significant decreases in growth rates and biomass. The increase in API-sludge addition did not show a decrease in growth rates for the grass (Figure 18E and 19E). However, a study by Adam & Duncan (1999) indicates that the addition of low levels of diesel oil to soil had the ability to improve growth in some grass species (Annual canary grass, *Phalaris canariensis*). The decrease in grass' growth rate observed for the second growth period might be due to the grass growing singly in seedling trays as opposed to their natural dense growth (Figure 18E). Grass was not sensitive to API-sludge contamination showing no significant differences for the measured endpoints and explaining why the LOEC value is so high.

Seeds of all species have shown germination during their exposure to API-sludge-spiked soils. Previous studies showed that the germination of poplar and spruce trees was not sensitive to exposed oil contamination but rather their growth (Nicolotti & Egli 1998). The reason that seeds are more resistant to direct exposure to toxicants may be because seeds contain their own reserves and are less dependent on the soil structure, that are affected by contamination (Wyszkowski & Ziólkowska 2009). However, the fact that not all germinated seeds survived (e.g. lettuce) might mean they are susceptible to the contaminant if they come in direct contact

with the toxicants (Nicolotti & Egli 1998). Lettuce and grass seeds are small and have low levels of nutrients per seed. Thus, these seedlings need to obtain nutrients from the environment early after germination (Taiz & Zeiger 2006). This may explain their low germination success and sensitivity to their exposed soil substrate.

An important function of the roots is to take up water from the soil. With exposures to the increased API-sludge concentrations a decrease in water uptake was observed for all plant types except for beans. Water uptake was negatively affected by contamination in maize, lettuce, radish and grass (Taiz & Zeiger 2006). The water uptake is possibly hindered by the smaller hydrocarbons present in the API-sludge allowing the plants to absorb it into their vascular tissue and further blocking the water transport system (Baker 1970). This would explain the mortalities among the lettuce that has a naturally higher water uptake that is transported to their leaves Reynolds & Thompson (1971).

Investigations into the above and below ground mass produced varying results (Figure 19). In beans, radish and grass, neither the root system nor the above ground growth was significantly affected by the contaminant. The maize grew extensive roots in soil with 0% API-sludge but the rhizosphere was smaller for the seedlings in the contaminated soils. From this one may deduce that the addition of API-sludge inhibited the root system growth and the maize root system is sensitive to the contamination. Although the radishes were not grown long enough to produce visible underground storage organs it still seemed to prioritise its below ground mass in all treatments. On the contrary to the below ground mass, the above ground parts suffered a decrease in biomass when the API-sludge concentrations increased. This is understandable considering that radish produce the underground bulb as survival strategy.

As this study only focused on endpoints; germination, growth rate and biomass, it neglects information of the API-sludge's environmental stress on cellular level in the plants. (Achuba 2006) found increased levels of metabolic elements including free sugar and amino acids and decreased levels of chlorophyll in plants grown in oily soil indicating stress in plants. It is thus possible that the current study's crop plants would have shown more evidence of stress on cellular level than the endpoints examined in this study.

Another important aspect to consider is the role of soil micro-organisms on altering the soil and its interaction with plant roots. The responses observed in the plants could be a result of changes in the composition and abundance of bacteria, fungi and other protozoans species in the soil itself (Nicolotti & Egli 1998). Some micro-organisms survive and possibly proliferate due to the high carbon content in the API-sludge (Nicolotti & Egli 1998). In that case, the micro-organisms can compete with the plants for nitrogen and oxygen (Gudin & Syratt 1975).

Nitrogen-fixing plants, such as legumes, compete better in the presence of additional carbon sources (like hydrocarbons in oil) (Nicolotti & Egli 1998). This may explain the tolerance observed for beans towards the API-sludge contamination an less so for plants like maize and radish (Wyszkowski & Ziólkowska 2009). Li *et al.* (1990) suggested that plant growth increased at low oil concentrations and decreased at high oil concentrations. The study further found a similar pattern in the associated microorganisms (*Spartina alteriflora*) in a salt-marsh. They proposed that the increased plant growth was caused by increased $N_2$-fixation and nitrogen mineralization, most likely connected to the increased microorganism activities that resulted from the increase in carbon available in the environment (from the crude oil). (Achuba 2006) found that at the addition of 0.5% crude oil to soil, cellular and metabolic activities were 'stimulated' in cowpeas (*Vigna unguiculuta*). It is interesting to note that hydrocarbons were used as fertilizers on farms in the past (Basic *et al*. 2009). It is thus possible that at a low API-sludge concentration, the mentioned benefits of hydrocarbons in soil may exceed the disadvantages, including poor aeration (Anigboro & Tonukari 2008) and phytotoxicity (Rowell 1997), but when the concentration increases, the disadvantages can start to exceed the benefits. There is thus a possible threshold below which a plant benefits from hydrocarbon contamination and over the threshold a plant is detrimentally affected although it is not know if crops grown in hydrocarbon contaminated soil are safe for human or animal consumption.

It would be useful to further investigate the effects of API-sludge contamination over the whole life span of the crop plants until reproduction of a second generation plants. The ability of plants to bioaccumulate various contaminants may also show the effects of the contamination on the plants.

# 5. Conclusion

From this study there can be concluded that when incorporating bioassays, with chemical analyses, it provided a more detailed assessment of the remediated landfarming site and its toxicity. The remediated site soils still affected exposed soil organisms and the null hypotheses could not be rejected.

The concentrations of the various metals, and PAHs and DROs were all higher in the north- than in the south-site soil. Thus, the contamination still present in the north-site soil was much higher than in the south-site soil.

In the bioassays, the site-soils were not acutely toxic to the exposed test organisms. Even though this was the case, the north-site exposures still showed to have caused a loss in earthworm biomass over 6 weeks, inhibition of cocoon production, decreased juvenile production of exposed *F. candida* as well as a significant avoidance of *E. doerjesi*. Thus, the north-site soils still negatively affected these soil organisms. The distinct difference in toxicity between the north- and south-site soils was clear although it was not apparent which individual contaminant was responsible for the increased toxicity in the north-site soil.

From the exposures of the test animals to the concentration series of API-sludge-spiked control soils, their sensitivity to the API-sludge could be ranked from most sensitive to least: *F. candida*, *E. andrei* and *E. doerjesi*. It was clear that the physical soil properties influenced the toxicity of the API-sludge to the animals exposed.

The various plants species exposed to API-sludge were affected differently at the endpoints examined in this study. A conclusion can be made that the tolerance of plants towards the API-sludge varies over the time of exposure and species. Although plant growth was stimulated by the addition of low concentrations of API-sludge in some cases, the addition of API-sludge had detrimental effects on plant growth and development when higher levels of API-sludge were added.

The toxicity of mixtures in soils are site and soil specific which made the remediated soil and spiked soil exposures incomparable to standard accepted toxic levels and more difficult to detect which of the individual contaminants posed to be present at hazardous concentrations to the environment. This study suggests that mainly the presence of DROs contributed to its toxicity.

When similar studies will be executed in future a few suggestions can be deduced from this study. Chemical analyses can be included for the soils before and after organism exposures as well as chemical analysis of bio-organisms. This may elucidate the potential bioavailable fraction of the toxins in the soil. Further, a suggestion can be made to include tests with micro-

organisms bioassays that will contribute to the effects of the soil pollution on soil micro-organisms.

A predicted ARL for the API-sludge where no effects on the organisms would be observed was determined as 21 mg/kg for *F. candida* reproduction and 1150 mg/kg for *E. andrei* survival.

The results of this study indicates that the high level of DROs still present in the site-soils need to be remediated before landfarming may be carried out on the site in the future.

# 6. References

ACHAZI, R.K., FRÖCHLICH, E., HENNEKEN, M. & PILZ, C. 1999. The effect of soil from former irrigation felds and of sewage sludge on dispersal activity and colonizing success of the annelid *Enchytraeus crypticus* (Enchytraeidae, Oligochaeta). *Newsletter on Enchytraeidae* **6**: 117-126.

ACHUBA, F.I. 2006. The effect of sublethal concentrations of crude oil on the growth and metabolism of cowpea (*Vigna unguiculata*) seedlings. *The Environmentalist* **26**: 17-20.

ADAM, G. & DUNCAN, H. 2002. Influence of diesel fuel on seed germination. *Environmental Pollution* **120**: 363-370.

ADAM, G. & DUNCAN, H.J. 1999. Effect of diesel fuel on growth of selected plant species. *Environmental Geochemistry and Health* **21**: 353-357.

ADOBE® PHOTOSHOP®. 2007 Adobe Photoshop CS3 extended, version 10.0. www.adobe.com.

ALTHOFF, P.S., TODD, T.C., THIEN, S.J. & CALLAHAM, M.A., JR. 2009. Response of soil microbial and invertebrate communities to tracked vehicle disturbance in tallgrass prairie. *Applied Soil Ecology* **43:** 122-130.

AMORIM, M.J.B., RÖMBKE, J. & SOARES, A.M.V.M. 2005. Avoidance behaviour of *Enchytraeus albidus*: Effects of Benomyl, Carbendazim, phenmedipham and different soil types. *Chemosphere* **59**: 501-510.

ANDERSSON, J. 2003. Environmental Impacts of Contaminated Site Remediation - A Comparison of two Life Cycle Assessments. MSc. dissertation. Linköpings University, Linköpings, Sweden.

ANEKI. 2005. Available from http://www.aneki.com/maps/south_africa-map.gif. [2010, September 11]

ANIGBORO, A.A. & TONUKARI, N.J. 2008. Effect of Crude Oil on invertase and amylase activities in Cassava leaf extract and germinating cowpea seedlings. *Asian Journal of Biological Sciences* **1**: 56-60.

AUGULYTE , L., KLIAUGAITE, D., RACYS, V., JANKUNAITE, D., ZALIAUSKIENE, A., ANDERSSON, P.L. & BERGQVIST, P.A. 2008. Chemical and ecotoxicological assessment of selected biologically activated sorbents for treating wastewater polluted with petroleum products with special emphasis on polycyclic aromatic hydrocarbons. *Water, Air, and Soil Pollution* **195**: 243-256.

ÄYRIIS, M., NISKAVAARA, H., BOGATYREV, I., CHEKUSHIN, V., PAVLOV, V., DE CARITAT, P., HALLERAKER, J.H., FINNE, T.E., KASHULINA, G. & REIMANN, C. 1997. Regional patterns of heavy metals (Co, Cr, Cu, Fe, Ni, Pb, V and Zn) and sulphur in

terrestrial moss samples as indication of airborne pollution in a 188,000 km$^2$ area in northern Finland, Norway and Russia. *Journal of Geochemical Exploration* **58**: 269-281.

BAKER, J.M. 1970. The effects of oil on plants. *Environmental Pollution* **1**: 27-44.

BAKKER, M.I., CASADO, U.B., KOERSELMAN, J.W., TOLLS, J. & KOLLÖFFEL, C. 2000. Polycyclic aromatic hydrocarbons in soil and plant samples from the vicinity of an oil refinery. *The Science of the Total Environment* **263**: 91-100.

BANKS, M.K. & SCHULTZ, K.E. 2005. Comparison of plants for germination toxicity tests in petroleum-contaminated soils. *Water, Air, and Soil Pollution* **167**: 211-219.

BASIC, F., BERTOVIC, L., BRKIC, V., DURN, G., KISIC, I., MESIC, M., MESIC, S. & ZGORELEC, Z. 2009. The effect of drilling fluids and crude oil on some chemical characteristics of soil and crops. *Geoderma* **149**: 209-216.

BEYLICH, A. & ACHAZI, R.K. 1999. Influence of low soil moisture on enchytraeids. *Newsletter on Enchytraeidae* **6**: 49-58.

BLANKENSHIP, D.W. & LARSON, R.A. 1978. Plant growth inhibition by the water extract of a crude oil. *Water, Air and Soil Pollution* **10**: 471-476.

BROHON, B., DELOLME, C. & GOURDON, R. 2001. Complementarity of bioassays and microbial activity measurements for the evaluation of hydrocarbon-contaminated soils quality. *Soil Biology & Biochemistry* **33**: 883-891.

BRUZZONITI, M.C., FUNGI, M. & SARZANINI, C. 2010. Determination of EPA's priority pollutant polycyclic aromatic hydrocarbons in drinking waters by solid phase extraction-HPLC . *Analitical Methods* **2**: 739-745.

CCME. 2007. Council for Canadian Ministers of the Environment. Canadian Environmental Quality Guidelines for the Protection of Environmental and Human Health, 7$^{th}$ edition, Winnipeg, Manitoba, Canada.

CCME. 2001. Council for Canadian Ministers of the Environment. Reference methods for the Canada-wide standard for petroleum hydrocarbons in soil-Tier 1 Method. Winnipeg, Manitoba, Canada

CHEMWIN. 1999. ChemWindow®, version 6.0. Bio-Rad Laboratories, Sadtler division.

CORNELISSEN, G., GUSTAFSSON, O., BUCHELI, T.D., JONKER, M.T.O., KOELMANS, A. & VAN NOORT, P.C.M. 2005. Extensive Sorption of Organic Compounds to Black Carbon, Coal, and Kerogen in Sediments and Soils: Mechanisms and Consequences for Distribution, Bioaccumulation, and Biodegradation. *Environmental Science and Technology* **39**: 6881-6895.

DAVIES, N.A., HODSON, M.E. & BLACK, S. 2003. Is the OECD acute worm toxicity test environmentally relevant? The effect of mineral form on calculated lead toxicity. *Environmental Pollution* **21**: 49-54.

DAWSON, J.J.C., GODSIFFE, E.J., THOMSON, I.P., RALEBITSO-SENIOR, T.K., KILLHAM, K.S. & PATON, G.I. 2007. Application of biological indicators to assess recovery of hydrocarbon impacted soils. *Soil Biology & Biochemistry* **39**: 164-177.

DAY, P.R. 1956. Report of the Committee on Physical Analysis, 1954-1955. *Soil Science Society of America Proceedings* **20**: 167-169.

DE LAENDER, F., JANSSEN, C.R. & DE SCHAMPHELAERE, K.A.C. 2009. Non-simultaneous ecotoxicity testing of single chemicals and their mixture results in erroneous conclusions about the joint action of the mixture. *Chemosphere* **76:** 428-432.

DEPARTMENT OF WATER AFFAIRS AND FORESTRY 1998. *Minimum requirements for the handling, classification and disposal of hazardous waste* (2nd edition). Department of water Affairs and Forestry, Pretoria, South Africa.

DIDDEN, W. & RÖMBKE, J. 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotoxicology and Environmental Safety* **50**: 25-43.

DIDDEN, W.A.M. 1992. Ecology of terrestrial *Enchytraeidae*. *Pedobiologia* **37**: 2-29.

DITORO, D.M., MCGRATH, J.A. & STUBBLEFIELD, W.A. 2007. Predicting the toxicity of neat and weathered crude-oil: Toxic potential and the toxicity of saturated mixtures. *Environmental Toxicology and Chemistry* **26**: 24-36.

DORN, P.B. & SALANITRO, J.P. 2000. Temporal ecological assessment of oil contaminated soils before and after bioremediation. *Chemosphere* **40**: 419-426.

DORN, P.B., VIPOND, T.E., SALANITRO, J.P. & WISNIEWSKI, H.L. 1998. Assessment of the acute toxicity of crude oils in soils using earthworms, microtox and plants. *Chemosphere* **31**: 845-860.

EDWARDS, C.A. & LOFTY, J.R. 1977. *Biology of Earthworms*. Chapman and Hall, London.

EKUNDAYO, E.O., EMEDE, T.O. & OSAYANDE, D.I. 2001. Effects of crude oil spillage on growth and yield of maize (*Zea mays* L.) in soils of midwestern Nigeria. *Plant Foods for Human Nutrition* **56**: 313-324.

ENVIRONMENT CANADA. 2009. Guidance Document on the Sampling and Preparation of Contaminated Soil for Use in the Application of Biological Testing 2nd draft. Ottawa, Canada.

EOM, I.C., RAST, C., VEBER, A.M. & VASSEUR, P. 2007. Ecotoxicity of a polycyclic aromatic hydrocarbon (PAH)-contaminated soil. *Ecotoxicology and Environmental Safety* **67**: 190-205.

ERIYAMREMU, G.E., ASAGBA, S.O., ONYENEKE, E.C. & AGUEBOR-OGIE, B. 2007. Bonny Light Crude oil and its fractions alter radicle galactose dehydrogenase activity of Beans and Maize. *Trends in Applied Sciences Research* **2**: 433-438.

FOUNTAIN, M.T. & HOPKIN, S.P. 2005. *Folsomia candida* (Collembola*):* A "Standard" Soil Arthropod. *Annual Reviews of Entomology* **50**: 201-222.

GEISSEN, V., GOMEZ-RIVERA, P., LWANGA, E.H., MENDOZA, R.B., NARCÍAS, A.T. & MARCÍAS, E.B. 2008. Using earthworms to test the efficiency of remediation of oil-polluted soil in tropical Mexico. *Ecotoxicology and Environmental Safety* **71**: 638-642.

GENOU, G., VAN MEENEN, P., VAN DER WERT, H., DE NIJS, W. & VERSTRAETE, W. 1994. Degradation of oil sludge by landfarming - a case study at Ghent Harbour. *Biodegradation* **5**: 37-46.

GIERE, O. 1979. The impact of oil pollution on intertidal meiofauna. Field studies after the La Coruna-spill, May 1976. *Cahiers de Biologie Marine* **20**: 231-251.

GIERE, O. & HAUSCHILDT, D. 1979. Experimental studies on the life cycle and production of the littoral oligochaete *Lumbricillus lineatus* and its response to oil pollution. In: *Phenomena in Marine Plants and Animals*, (eds) E. Naylorm & R.G. Hartnall, **122.** Pergamon, Oxford.

GONG, P., WILKE, B.M., STROZZI, E. & FLESISCHMANN, S. 2001. Evaluation and refinement of a continuous seed germination and early deedling growth test for the use in the ecotoxicological assessment of soils. *Chemosphere* **44**: 491-500.

GOOGLE INC. 2010. Google Earth software, version 5.2.1.1588. Available from http://www.googleearth.com. [2010, September 1]

GOOGLE INC. 2006. Google Earth software version 5.1.1.1588. Available from http://www.googleearth.com. [2006, April 11]

GRAPHPAD PRISM®. 2007. PRISM, version 5.0. www.grafhpadprism.com

GUDIN, C. & SYRATT, W.J. 1975. Biological aspects of land rehabilitation following hydrocarbon contamination. *Environmental Pollution* **8**: 107-112.

HAANSTRA, L., DOELMAN, P. & OUDE VOSHAAR, J.H. 1985. The use of sigmoidal dose response curves in soil ecotoxicological research. *Plant and Soil* **84**: 293-297.

HAMILTON, M.A., RUSSO, R.C. & THURSTON, R.V. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environmental Science & Technology* **11**: 714-719.

HANNA, S.H.S. & WEAVER, R.W. 2002. Earthworm survival in oil contaminated soil. *Plant and Soil* **240**: 127-132.

HARITASH, A.K. & KAUSHIK, C.P. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Journal of Hazardous Materials* **169:** 1-15.

HENNER, P., SCHIAVON, M., MOREL, J.L. & LICHTFOUSE, E. 1997. Polycyclic aromatic hydrocarbons (PAH) occurrence and remediation methods. *Analusis Magazine* **25**: 56-59.

HUND-RINKE, K. & WIECHERING, H. 2001. Earthworm avoidance test for soil assessments. *Journal for Soils and Sediment* **1**: 15-20.

HUND-RINKE, K., RÖMBKE, J., ACHAZI, R. & WARNEKE, D. 2003. Avoidance test with *E. fetida* as indicator for the habitat function of soils - results of a laboratory comparison test. *Journal for Soils and Sediment* **3**: 7-12.

INSAM, H. & SEEWALD, M.S.A. 2010. Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils* **46**: 199-213.

ISO 2003. International Standardisation Organisation- Soil Quality - Avoidance test for testing the quality of soils and the toxicity of chemicals-Test with earthworms (*Eisenia fetida*). **238.** Paris, France.

JANSSEN, R.P.T., POSTHUMA, L., BAERSELMAN, R., DEN HOLLANDER, H.A., VAN VEEN, R.P.M. & PEIJNENBURG, J.G.M. 1997. Equilibrium partitioning of heavy metals in Dutch field soils. II. Prediction of metal accumulation in earthworms. *Environmental Toxicology and Chemistry* **16**: 2479-2488.

JONKER, M.J., SVENDSEN, C., BEDAUX, J.J.M., BONGERS, M. & KAMMENGA, J.E. 2005. Significant testing of synergistic/antagonistic, dose-level-dependent, or dose-ratio-dependent effects in mixture dose-response analysis. *Environmental Toxicology and Chemistry* **10:** 2701-2713.

KHAITAN, S., KALAINESAN, S., ERICKSON, L.E., KULAKOW, P., MARTIN, S., KARTHIKEYAN, R., HUTCHINSON, S.L.L., DAVIS, L.C., ILLANGASEKARE, T.H. & NG'OMA, C. 2006. Remediation of Sites Contaminated by Oil Refinery Operations. *American Institute of Chemical Engineers* **25**: 20-31.

KRAMARZ, P.E., ZWOLAK, M. & LASKOWSKII, R. 2005. Effect of interaction between density dependence and toxicant exposure on population growth rate of the potworm *Enchytraeus doerjesi*. *Environmental Toxicology and Chemistry* **24**: 537-540.

KULA, H. & LARINK, O. 1997. Development and standardisation of test methods for the prediction of subletal effects of chemicals on earthworms. *Soil Biology & Biochemistry* **29**: 635-639.

KUMPIENE, J., LAGERKVIST, A. & MAURICE, L. 2008. Stabilization of As, Cr, Cu, Pb and Zn in soil using amendments – A review. *Waste Management* **28**: 215-225.

LANNO, R., WELLS, J., CONDER, J., BRADHAM, K. & BASTA, N. 2004. The bioavailability of chemicals in soil for earthworms. *Ecotoxicology and Environmental* **57**: 39-47.

LAPA, N., BARBOSA, R., MORAIS, J., MENDES, B., MÉHU, J. & SANTOS OLIVEIRA, J.F. 2002. Ecotoxicological assessment of leachates from MSWI bottom ashes. *Waste Management* **22**: 583-593.

LI, Y., MORRIS, J.T. & YOCH, D.C. 1990. Chronic low level hydrocarbon amendments stimulate plant growth and microbial activity in salt-marsh microcosms. *Journal of Applied Ecology* **27**: 159-171.

LOEHR, R.C. & WEBSTER, M.T. 1996. Performance of long-term, field-scale bioremediation processes. *Journal of Hazardous Materials* **50**: 108-128.

LØKKE, H. & VAN GESTEL, C.A.M. 1998. *Handbook of Soil invertebrate toxicity tests*. Wiley, Chichester, England.

LORS, C., ALDAYA, M.M., SALMON, S. & PONGE, J.F. 2006. Use of an avoidance test for the assessment of microbial degradation of PAHs. *Soil Biology & Biochemistry* **38**: 2199-2204.

LUFA-SPEYER 2000. Landwirtschaftilche Untersuchungs- und Forschungs- Anstalt Speyer. Available from http://www.lufa-speyer.de [2010, June 15]

MAILA, M.P. & CLOETE, T.E. 2004. Bioremediation of petroleum hydrocarbons through landfarming: Are simplicity and cost-effectiveness the only advantages? *Environmental Science & Biotechnology* **3**: 349-360.

MEMIS, D., ÇELIKKALE, M.S. & ERCAN, E. 2004. The effect of different diets on the white worm *(Enchytraeus albidus* Henle, 1837) reproduction. *Turkish Journal of Fisheries and Aquatic Sciences* **4**: 5-7.

MOHAN, S.V., KISA, T., OHKUMA, T., KANALY, R.A. & SHIMIZU, Y. 2006. Bioremediation technologies for treatment of PAH-contaminated soil and strategies to enhance process efficiency. *Reviews of Environmental Science Biotechnology* **5**: 347-374.

NADAL, M., SCHUHMACHER, M. & DOMINGO, J.L. 2004. Levels of PAHs in soil and vegetation samples from Tarragona County, Spain. *Environmental Pollution* **132** : 1-11.

NATAL-DA-LUZ, T., RIBEIRO, R. & SOUSA, J.P. 2004. Avoidance tests with Collembola and earthworms as early screening tool for site-specific assessment of polluted soils. *Environmental Toxicology and Chemistry* **23**: 2188-2193.

NATAL-DA-LUZ, T., RÖMBKE, J. & SOUSA, J.P. 2008. Avoidance tests in site-specific risk assessment-Influence of soil properties on the avoidance response of Collembola and earthworms. *Environmental Toxicology and Chemistry* **27**: 1112-1117.

NICOLOTTI, G. & EGLI, S. 1998. Soil contamination by crude oil: impact on the mycorrhizosphere and on the revegetation potential of forest trees. *Environmental Pollution* **99**: 37-43.

OECD 2004a. Organization for Economic Cooperation and Development guideline for testing of chemicals. **220**. *Enchytraeidae* Reproduction Test. Paris, France.

OECD 2004b. Organization for Economic Cooperation and Development guideline for the testing of chemicals. **222**. Earthworm reproduction test *Eisenia fetida andrei*. Paris, France.

OECD 2006. Organization for Economic Cooperation and Development guideline for the testing of chemicals. **208**. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. Paris, France.

OECD 2009. Organization for Economic Co-operation and Development guideline for testing of chemicals. **232**. Collembolan Reproduction Test in Soil. Paris, France.

OGBO, E.M. 2009. Effects of diesel fuel contamination on seed germination of four crop plants - *Arachis hypogaea, Vigna unguiculata, Sorghum bicolor* and *Zea mays*. *African Journal of Biotechnology* **8**: 250-253.

PASW. 2009. SPSS Inc. for Windows, version 18.0.0. www.SPSS.com.

PATON, G.I., KILLHAM, K., WEITZ, H.J. & SEMPLE, K.T. 2005. Biological tools for the assessment of contaminated land: applied soil ecotoxicology. *Soil Use and Management* **21**: 487-499.

PAUMEN, M.L. 2009. Invertebrate life cycle responses to PAC exposure. PhD. thesis, University of Amsterdam, Amsterdam, The Netherlands.

PEARCE, K. & OLLERMANN, R.A. 1998. Status and scope of bioremediation in South Africa. *Bioremediation: Principles and Practice-Bioremediation Technologies* **3**: 155-182.

PEREZ, J.D. & GALLARDOLARA, F. 1986. Effects of wastewater from olive processing on seed germination and early plant growth of different vegetable species. *Journal of Environmental Science and Health* **B21**: 349-357.

PLAZA, G., NALÊCZ-JAWECK, G., ULFIG, K. & BRIGMON, L. 2005. The application of bioassays as indicators of petroleum-contaminated soil remediation. *Chemosphere* **59**: 289-296.

POTAPOV, M. 2001. Synopses on Palaearctic Collembola. Vol. 3. Isotomidae. *Abhandlungen und Berichte des Naturkundemuseums Görlitz* **73**: 1-603.

PUNNARUTTANAKUN, P., MEEYOO, V., KALAMBAHETI, C., RANGSUNVIGIT, P., RIRKSOMBOON, T. & KITIYANAN, B. 2003. Pyrolysis of API separator sludge. *Journal of Annual Applications of Pyrolysis* **68-69**: 547-560.

REINECKE, A.J., REINECKE, S.A. & MABOETA, M.S. 2001. Cocoon production and viability as endpoints in toxicity testing of heavy metals with three earthworm species. *Pedobiologia* **45**: 61-68.

REYNOLDS, T. & THOMPSON, P.A. 1971. Characterisation of the high temperature inhibition of germination of lettuce (*Lactuca sativa*). *Physiologia Plantarum* **24:** 544-547.

RÖMBKE, J., JÄNSCH, S., JUNKER, T., POHL, B., SCHEFFCZYK, A. & SCHLLNAß, H.J. 2006. Improvement of the applicability of ecotoxicological tests with earthworms, springtail, and plants for the assessment of metals in natural soils. *Environmental Toxicology and Chemistry* **25**: 776-787.

RÖMBKE, J. & MOSER, TH. 2002. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. *Chemosphere* **46**: 1117-1140.

ROWELL, M.J. 1997. The effects of crude oil spills on soil: a review of literatures. In: *The reclamation of agricultural soils after oil spills, Part 1*, (ed) J.A. Toogood, University of Alberta Institute of Pedology, Edmonton.

RUBINOS, D.A., VILLASUSO, R., MUNIATEGUI, S., BARRAL, M.T. & DIAZ-FERROS, F. 2007. Using the Landfarming Technique to Remediate Soils Contaminated with Hexachlorocyclohexane Isomers. *Water, Air and Soil Pollution* **181**: 385-399.

RUSHTON, D.G., GHALY, A.E. & MARTINELL, K. 2007. Assessment of Canadian regulations and remediation methods for diesel oil contaminated soils. *Annals of Applied Biology* **4**: 465-478.

SALANITRO , J.P., DORN , P.B., HUESEMANN, M.H., MOORE, K.O., RHODES, I.A., RICE JACKSON, L.M., VIPOND, T.E., WESTERN, M.M. & WISNIEWSKI, H.L. 1997. Crude oil hydrocarbon bioremediation and soil ecotoxicity assessment. *Environmental Science and Technology* **31**: 1769-1776.

SHARMA, G.K., CHANDLER, C. & SALEMI, L. 1980a. Environmental pollution and leaf cuticular variation in Kadzu (*Puereria lobata* Willd). *Annals of Botany* **45**: 77-80.

SHIE, J., CHANG, C., LIN, J., WU, C. & LEE, D. 2000. Resources recovery of oil sludge by pyrolysis: kinetics study. *Journal of Chemical Technology and Biotechnology* **75**: 443-450.

SIMS, R.W. & GERARD, B.M. 1985. *Earthworms*. The Linnean Society of London and The Estuatine and Brackish-Water Sciences Association, London.

ŠKRBIC, B., CVEJANOV, J. & ĐURIŠIC-MLADENOVIC, N. 2005. Polycyclic aromatic hydrocarbons in surface soils of Novi Sad and bank sediment of the Danube river. *Journal of Envinmmental Science and Health* **A40**: 29-42.

SOIL SURVEY STAFF. 2010. Keys to Soil Taxonomy, 11th edition. USDA-Natural Resources Conservation Service, Washington, DC.

SONG, Y.F., JING, X., FLEISCHMANN, S. & WILKE, B.M. 2002. Comparative study of extraction methods for the determination of PAHs from contaminated soils and sediments. *Chemosphere* **48**: 993-1001.

SPURGEON, D.J., LOFTS, S., HANKARD, P.K., TOAL, M., MCLELLAN, D., FISHWICK, S. & SVENDSEN, C. 2006. Effect of pH on metal speciation and resulting metal uptake and toxicity for earthworms. *Environmental Toxicology and Chemistry* **25**: 788-796.

SPURGEON, D.J., WEEKS, J.M. & VAN GESTEL, C.A.M. 2003. A summary of eleven years progress in earthworm ecotoxicology. *Pedobiologia* **47**: 588-606.

STATSOFT. 2010. STATISTICA (data analysis software system), version 9.0. www.statsoft.com.

STYRISHAVE, B., MORTENSEN, M., KROGH, P.H., ANDERSON, O. & JENSEN, J. 2008. Solid-Phase Microextracion (SPME) as a tool to predict the bioavailability and toxicity of pyrene to springtail, *Folsomia candida*, under various soil conditions. *Environmental Science & Technology* **42**: 1332-1336.

SVERDRUP, L.E., HAGEN, S.B., KROGH, P.H. & VAN GESTEL, C.A.M. 2007. Benzo(a)pyrene shows low toxicity to three species of terrestrial plants, two soil invertebrates, and soil-nitrifying bacteria. *Ecotoxicology and Environmental Safety* **66**: 362-368.

SVERDRUP, L.E., JENSEN, J., KELLEY, A.E., KROGH, P.H. & STENERSEN, J. 2002. Effects of eight polycyclic aromatic compounds on the survival and reproduction of *Enchytreaeus crypricus* (Oligochaeta, Clitellata). *Environmental Toxicology and Chemistry* **21**: 109-114.

SVERDRUP, L.E., KELLEY, A.E., KROGH, P.H., NIELSEN, T., JENSEN, J., SCOTT-FORDSMAND, J.J. & STENERSEN, J. 2001. Effects of eight polycyclic aromatic compounds on the survival and reproduction of the springtail *Folsomia Fimetaria* L. (Collembola, Isotomidae). *Environmental Toxicology and Chemistry* **20**: 1332-1338.

TAIZ, L. & ZEIGER, E. 2006. *Plant Physiology* (4th edition). Sunderland, Mass. Sinauer Associates.

TAN, K.H. 2005. *Soil sampling, preparation, and analysis*. Taylor & Francis, Boca Raton, Florida.

TOXRAT 2003. Software for the statistical analysis of biotests. ToxRat Solutions GmbH, Alsdorf. Germany.

U.S.EPA 1996. United States Environmental Protection Agency. *Method 2015B: Nonhalogenated Organics using GC/FID.* U.S. Government printing office, Washington, DC.

UDO, E.J. & FAYEMI, A.A.A. 1975. The effect of oil pollution of soil on germination, growth and nutrient uptake of corn. *Journal of Environmental Quality* **4**: 537-540.

VAN BERKUM, P. & BOHLOOL, B.B. 1980. Evaluation of nitrogen fixation by bacteria in association with roots of tropical grasses. *Microbiological reviews* **44**: 491-517.

VAN BRUMMELEN, T.C., VAN HATTUM, B., CROMMENTUIJN, T., KALF, D.F. 1998. Bioavailability and ecotoxicity of PAHs. In Neilson AH, ed, *The Handbook of Environmental Chemistry,* Vol 3—PAH and Related Compounds. Springer-Verlag, Berlin, Germany 203–263.

VAN DER WATT, V.H. 1966. Improved tables and a simplified procedure for soil particle analysis by hydrometer method. *South African Journal of Agriculture* **9**: 911-916.

VAN GESTEL, C.A.M. 2008. Physico-chemical and biological parameters determine metal bioavailability in soils. *Science of the Total Environment* **406**: 385-395.

VAN GESTEL, C.A.M., VAN DER WAARDE, J.J., DERKSEN, J.G.M., VEUL, M.F.X.W., BOUWENS, S., RUSCH, B., KRONENBURG, R. & STOKMAN, G.N.M. 2001. The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. *Environmental Toxicology and Chemistry* **7**: 1438-1449.

VENTER, J.M. & REINECKE, A.J. 1988. The life-cycle of the compost worm *Eisenia fetida* (Oligochaeta). *South African Journal of Zoology* **23**: 161-165.

VERBRUGGEN, E.M.J. 2004. National Institute for Public Health and the Environment. Environmental Risk Limits for Mineral Oil (Total Petroleum Hydrocarbons). Bilthoven, The Netherlands.

VIDALI, M. 2001. Bioremediation. An overview. *Pure Applied Chemistry* **73**: 1163-1172.

WALKLEY, A. 1935. An examination of methods for determining organic C and nitrogen in soils. *Journal of Agricultural Science* **25**: 598-609.

WESTHEIDE, W. & GRAEFE, U. 1992. Two new terrestrial *Enchytraeus* species (Oligochaeta, Annelida). *Journal of Natural History* **26**: 479-488.

WILKE, B.M., RIEPERT, F., KOCH, C. & KÜHNE, T. 2008. Ecotoxicological characterization of hazardous wastes. *Ecotoxicology and Environmental Safety* **70**: 283-293.

WYSZKOWSKI, M. & ZIÓLKOWSKA, A. 2009. Role of compost, bentonite and calcium oxide in restricting the effect of soil contamination with petrol and diesel oil on plants. *Chemosphere* **74**: 860-865.

YEARDLEY, R.B.JR., LAZPRCHAK, J.M. & GAST, L.C. 1996. The potential of an earthworm avoidance test for the evaluation of hazardous waste sites. *Environmental Toxicology and Chemistry* **15**: 1532-1537.

# Appendix A: Determination of particle size distribution and total organic carbon content.

## Hydrometer Method (Particle size determination)

50 g air dried soil (<2mm) of each soil type were put in a 250ml beaker with 100ml water. 10ml of a 40g/L hexameta-phosphate sodium carbonate solution (dispersing reagent solution) were added, stirred and left for 15 minutes. Thereafter the beaker contents were transferred to a dispersion beaker and dispersed with a mechanical disperser. Soil suspensions were then transferred to a 1l measuring cylinder and filled with water. The suspension was left overnight at room temperature. The next day the suspension was stirred and timed. Table 1 refers to the times the readings were taken for the determination of the clay reading. The hydrometer (ASTM 0-60 g/l, Type 152H) was placed into the suspension and the reading and temperature was noted. The suspension was mixed again and clay plus slit reading was determined (Table 1). The suspension was then sieved with a 0.25mm mesh sieve and washed under running tap water until the water ran clear. The material on the sieve were transferred back into a 250ml glass beaker and dried at 100°C overnight. The dried soil was sieved through a 0.5mm and 0.25mm mesh sieve respectively and the material in each sieve were weighed.

### Calculations

The hydrometer is calibrated at 68°F and temperature measurements in degrees Celsius had to be converted for the calculations (Table 2).

Particle size distribution was calculated using the following formulae:

$$\text{Clay } (\%\ {}^{m}/_{m}) = \frac{\text{Corrected hydrometer reading} \times 100}{m}$$

$$\text{Clay plus Slit } (\%\ {}^{m}/_{m}) = \frac{\text{Corrected hydrometer reading} \times 100}{m}$$

$$\text{Coarse sand (\% m/m)} = \frac{\text{Material remaining n 0.5mm sieve} \times 100}{\text{m}}$$

$$\text{Medium sand (\% m/m)} = \frac{\text{Material remaining n 0.25mm sieve} \times 100}{\text{m}}$$

Fine sand (% m/m) = 100- (Slit +Clay) – Coarse Sand – Medium Sand

Slit (% m/m) = (Clay + Slit) – Clay

m= Soil mass

**Table 1:** Times of hydrometer readings for the various particle size fractions.

| | | Temperature (°C) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 22 | | 24 | | 26 | |
| **Fraction** | **Hydrometer reading** | Min | Sec | Min | Sec | Min | Sec |
| **Silt and Clay** | 0-10 | 6 | 33 | 6 | 13 | 5 | 56 |
| | 10-20 | 5 | 46 | 5 | 20 | 5 | 16 |
| | 20-30 | 5 | 03 | 4 | 49 | 4 | 36 |
| | 30-40 | 4 | 08 | 3 | 56 | 3 | 46 |
| | | Hr | Min | Hr | Min | Hr | Min |
| **Clay** | 1-10 | 10 | 53 | | 24 | 9 | 55 |
| | 10-20 | 9 | 37 | 9 | 11 | 8 | 46 |
| | 20-30 | 8 | 23 | 8 | 1 | 7 | 39 |
| | 30-40 | 7 | 10 | 6 | 49 | 6 | 31 |

**Table 2:** Temperature correction of hydrometer readings.

| Temperature (°C) | Correction | Temperature (°C) | Correction |
|---|---|---|---|
| 18.5-18.7 | -1.5 | 22.7-22.9 | 0.0 |
| 18.8-19.0 | -1.4 | 23.0-23.1 | 0.1 |
| 19.1-19.3 | -1.3 | 23.2-23.4 | 0.2 |
| 19.4-19.5 | -1.2 | 23.5-23.7 | 0.3 |
| 19.6-19.8 | -1.1 | 23.8-24.0 | 0.4 |
| 19.9-20.1 | -1 | 24.1-24.3 | 0.5 |
| 20.2-20.9 | -0.9 | 24.4-24.5 | 0.6 |
| 20.5-20.6 | -0.8 | 24.6-24.8 | 0.7 |
| 20.7-20.9 | -0.7 | 24.9-25.1 | 0.8 |
| 21.0-21.2 | -0.6 | 25.2 -25.4 | 0.9 |
| 21.3-21.5 | -0.5 | 25.5-25.7 | 1 |
| 21.6-21.8 | -0.4 | 25.8-26.0 | 1.1 |
| 21.9-22.0 | -0.3 | 26.1-26.4 | 1.2 |
| 22.1-22.3 | -0.2 | 26.5-26.8 | 1.3 |
| 22.4-22.6 | -0.1 | 26.9-27.1 | 1.4 |

According to the calculated percentage of the different soil fractions in the soil, the soil could be characterised using Table 3 and Figure 1.

**Table 3:** Diameter of the soil particles for classifying soils according to the USDA (Soil Survey Staff 2010).

| Soil type | Diameter (mm) |
|---|---|
| Clay | <0.002 |
| Silt | 0.002-0.05 |
| Sand | 0.05-2.00 |
| Fine pebbles | 2.00-5.00 |
| Medium pebbles | 5.00-20.00 |
| Coarse pebbles | 20.00-75.00 |

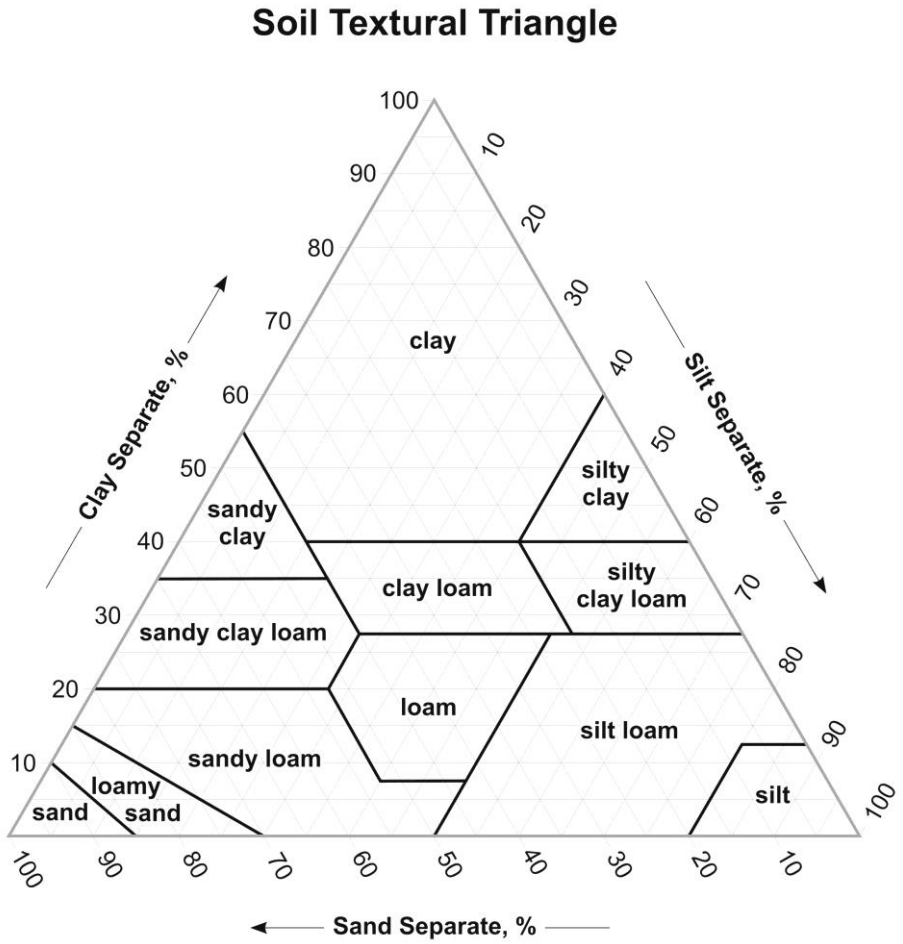



**Figure 1:** United States Department of Agriculture (USDA) soil texture pyramid (Soil Survey Staff 2010).

## Walkley-Black wet combustion method (Total organic carbon content determination)

50g of each soil sample with particle size smaller than 100mm was put in a 500ml Erlenmeyer flask. 10ml of 0.2M $K_2Cr_2O_7$ (49g $K_2Cr_2O_7$ /L) was added and mixed well. 20ml concentrated $H_2SO_4$ was added carefully and mixed thoroughly. The container was left for 20-30 minutes to cool to room temperature, after the acid was, added and a further 20ml of distilled water was added and stirred. 5 drops of ferroin indicator (1, 10- Phenanthroline Ferrous Sulfate Complex) was added. Back titration was done by adding 0.5M $FeSO_4.7H_2O$ (140g $FeSO_4.7H_2O$ /L). The red-brown colour of the sample changed to a clear blue-green endpoint and the amount of added $FeSO_4.7H_2O$ in ml was noted (T ml). The Protocol was repeated with a blank sample and the amount of added $FeSO_4.7H_2O$ was noted (B ml).

### Calculations

% Organic carbon = $(B\text{-}T \times N \times 3 \times 1.14 \times (\frac{100}{500mg}))$

3 = equivalent weight of carbon in mg,

1.14 = oxidation factor (Walkley 1935)

The organic matter content was assumed as the total organic carbon contents in the soil samples.

# Appendix B: Chemical structures for all VOCs and PAHs analysed

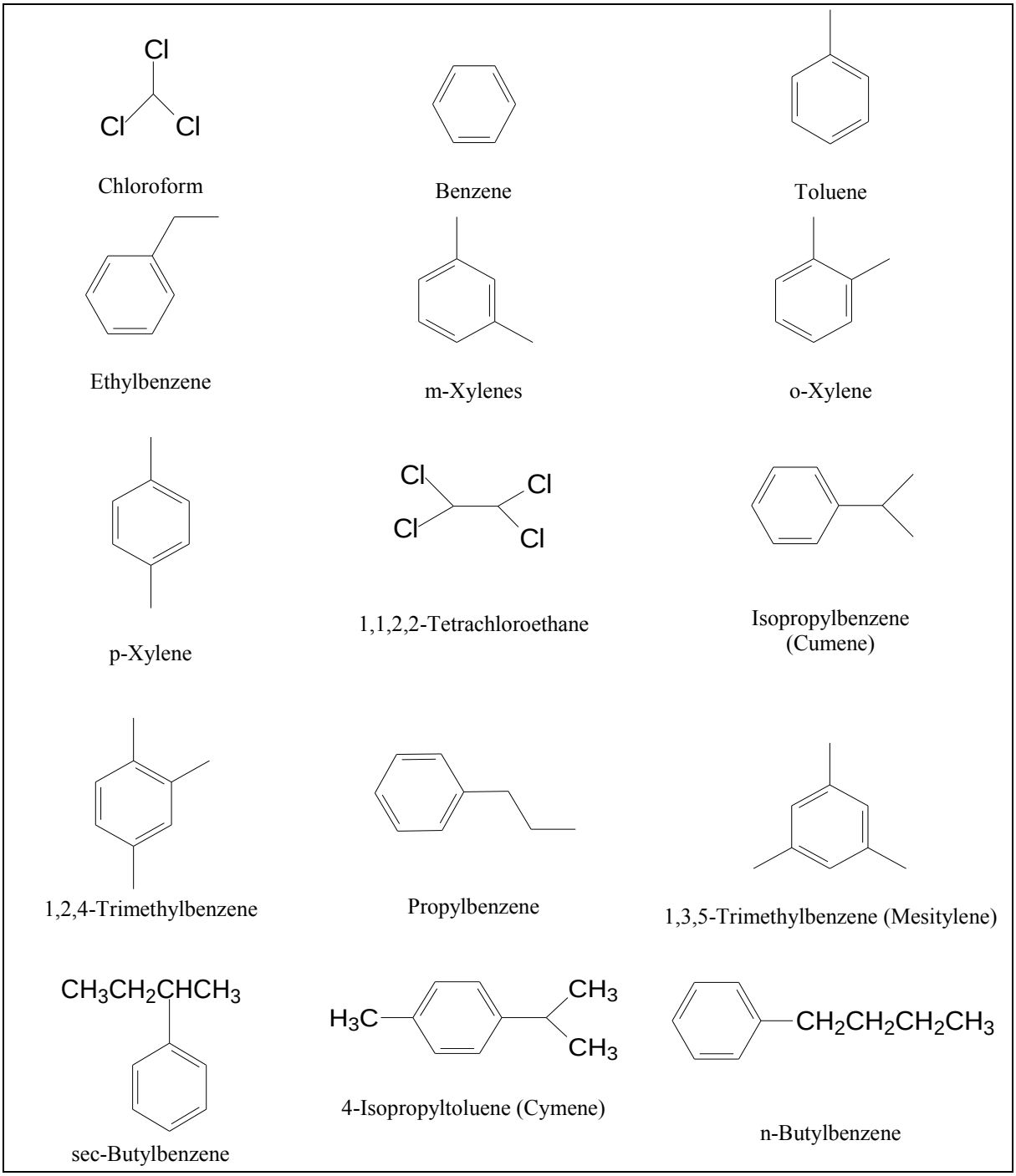



**Figure 2:** All volatile organic compounds (VOCs) detected in the soils (ChemWindow® 6.0).

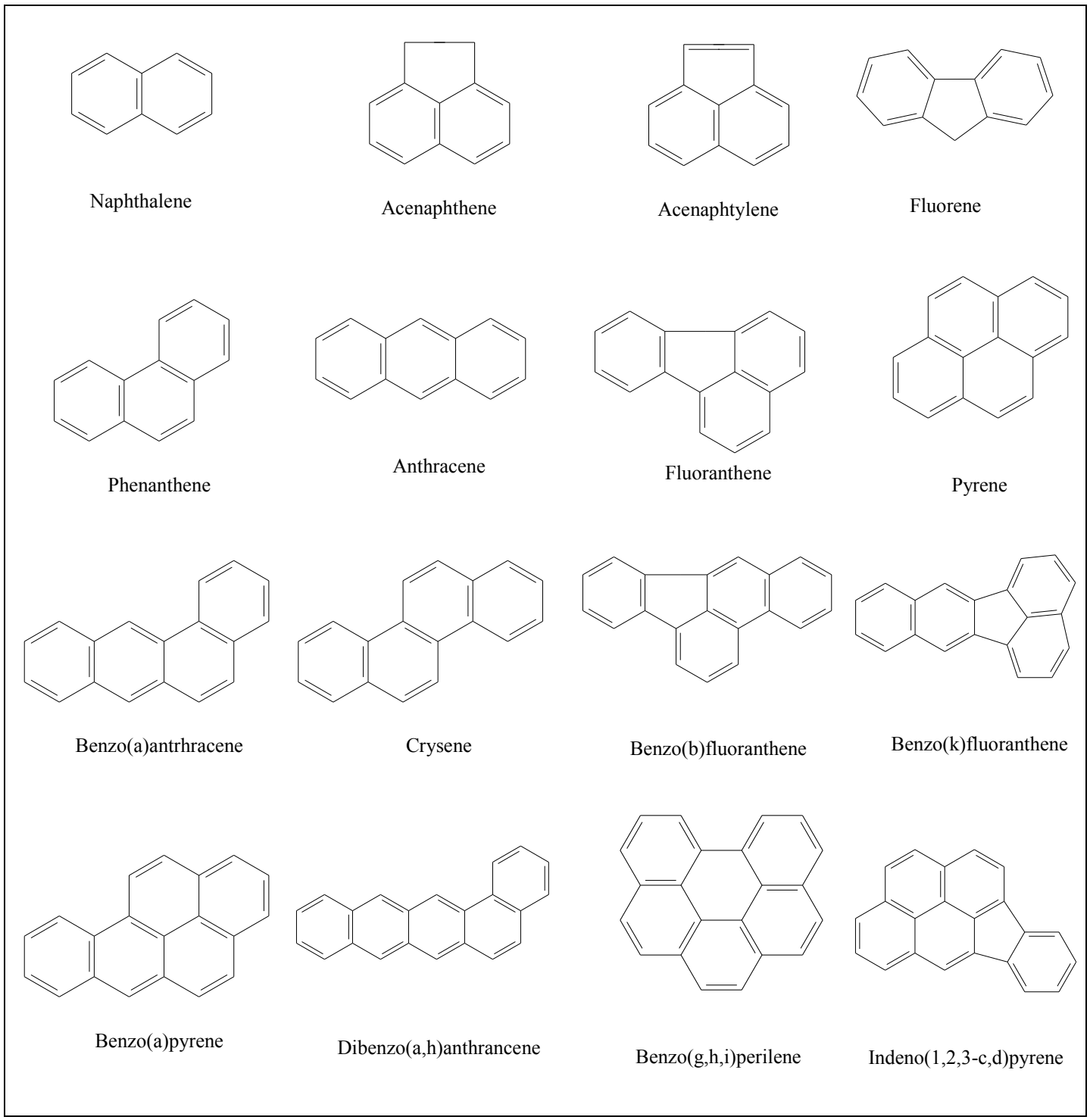



**Figure 3:** Chemical structures of the 16 US EPA priority polycyclic aromatic hydrocarbons (PAHs). Figure adopted from Bruzzoniti *et al.* (2010).