THE BIO-REACTOR PROJECT, 1993/1994:

ECOTOXICOLOGICAL TESTING OF HYDROCARBON AND SALT-CONTAMINATED WASTES (WASTES 1 AND 2) FOLLOWING BIOREMEDIATION IN A BIO-REACTOR

by

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A report prepared for:

Gulf Canada Resources

Federal-Provincial DESRT Program

Alberta Environmental Research Trust (AERT)

Canadian Association of Petroleum Producers (CAPP)

National Groundwater and Soil Remediation Program (GASReP)

Environment Canada (resources provided by the Federal Panel on Energy Research and Development - PERD)

DECEMBER 1994

THE UNIVERSITY OF
CALGARY

FACULTY OF SCIENCE
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January 27, 1995

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re: **DRAFT REPORT ON ECOTOXICOLOGICAL TESTING OF HYDROCARBON AND SALT-CONTAMINATED WASTES**

This is just a reminder that your comments and criticisms of the aforementioned report were due on January 15. To date, only one response has been received and I would very much like to get feedback on this research from the rest of the technical committee, if possible. Therefore, could you please forward your reviews to me by the end of next week, Feb. 3; after that date I will proceed with the revisions necessary and assume the report is acceptable to all. Thanks for your co-operation in this matter.

Sincerely,

Suzanne

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Post-it™ Fax Note 7671E	Date Jan. 27/95	# of pages ▸ 1
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Dear Suzanne,

I just received your fax and thought that I had already sent my comments to you, but alas they were sitting here in my computer's memory waiting to be "finalized". Sorry for the delay!

Aside from the usual "typos"- [page i, para. 3, line 16 - "whch"][page iii, item **6.**, line 1 - "ofthe"] in the executive summary - (you'll no doubt run "spell check" before the final version is printed?!), the report was excellent; well-written / easy to read, comprehensive in content, and, on a personal note, quite interesting and relevant to some of our (the Wastewater Technology Centre) current research.

A typo that you may not catch is on page iii, item **4.** - "acenaphtylene" should be "acenaphthylene" I believe. Also, I personally prefer to see references in the text cited as (Symons and Sims, 1989), although I realize some journals omit the apostrophe. I also like to follow the federal government's style manual and the Metric Code of Practice and use "**mL**", although I noticed that the Journal of Water Research (Pergammon Press, U.K.) requests that contributors use "ml" - in contradiction to the official European metric committee's position).

In the **Conclusions**; first item, line 5, "was neither non-toxic **nor** inhibitory" (please see dictionary definition of "nor" for confirmation). Also, under item **4.**, that "acenaphthylene" typo shows up again (and probably elsewhere?).

Once again, congratulations on your fine experimental work and a superb report!

EXECUTIVE SUMMARY

Treatment of industrial wastes and contaminated soil by processes such as bioremediation is aimed primarily at decontamination and detoxification of constituents which threaten biological health. The effectiveness of bioremediation technologies is usually determined by chemical analysis of the treated material; however, ecotoxicological testing may provide a more sensitive and reliable approach for evaluating toxicity potential.

In Alberta, two hydrocarbon, salt-contaminated wastes produced by the oil and gas industry have undergone bioremedial treatment in a solid phase Bio-Reactor located at Nevis, Alberta (see The BIO-REACTOR PROJECT Newsletter, Issues 1 and 2). The two wastes are Waste 1, a crude oil spill agricultural soil (initial TPH = 4.3%; EC = 27 dS m^{-1}) and Waste 2, a diesel invert mud residue (DIMR) (initial TPH = 10.8%; EC = 24 dS m^{-1}). Waste 1 had undergone 16 months bioremediation in the Nevis Bio-Reactor and Bio-Pile while Waste 2 had been treated for 4 months in the Bio-Reactor when the current investigation was initiated. Following bioremedial treatment (aggregation, fertilization, irrigation, heating and aeration), the TPH and EC in Waste 1 were 2.2% and 0.6 dS m^{-1}, and in Waste 2 they were 2.5% and 3.5 dS m^{-1}, respectively.

It was the purpose of this study to develop and evaluate an ecotoxicological protocol for monitoring the success of the bioremediation procedures used the Bio-Reactor in detoxifying the hydrocarbon wastes. This was achieved by testing the acute (short-term) and chronic (long-term) toxicological potential of the two wastes using a battery of techniques including single species (organism) bioassays, which often measure acute response; soil process (decomposition/nutrient cycling) assays which provide a measure of chronic effects; and plant life-cycle assessments which integrate the effects of soil chemical, physical and biological factors as expressed in plant productivity and reproduction.

The single species bioassays included seedling emergence and root elongation of buttercrunch lettuce, barley and canola; earthworm (*Eisenia foetida*) survival; Microtox; and algal (*Selenastrum*) growth inhibition. Soil process assays addressed decomposition (mass loss of alfalfa stems) and mineralization (ammonification and nitrification) potentials of the two

wastes. A greenhouse pot study using barley was utilized for the plant life-cycle assessment and was conducted only if the single species and soil process assays indicated slight or no toxicity. With the exception of the Microtox and algal bioassays, all tests were performed on the solid phase. An extract [1 part waste (dwt equivalent)/4 parts water] was used for the Microtox and algal assays with the intention that these tests would provide insight into the toxicity of possible leachates from the contaminated materials. Where possible, standard protocols summarized in Keddy et al. (CCME Subcommittee on Environmental Quality Criteria for Contaminated Sites, 1992) and/or published by Environment Canada (Environmental Protection Series) were followed.

Decomposition potentials were determined by measuring mass loss of alfalfa stems buried in undiluted waste or soil for a 3 month period while nitrogen mineralization was based on the production of ammonium and nitrate nitrogen in undiluted waste or reference soil over 2 months. The reference soil used in all the tests was a Chernozemic agricultural topsoil obtained from the vicinity of the Bio-Reactor (Erskine, Alberta) (pH = 7.3; EC = 0.7 dS m^{-1}; total C = 3%).

CONCLUSIONS

1. Based on the single species bioassays and chronic soil process assays, Waste 1, an oil contaminated soil, was neither non toxic nor inhibitory after 16 months bioremediation, while Waste 2, a DIMR, was very to extremely toxic after 4 months bioremediation. The barley life-cycle assay indicated Waste 1 reduced reproductive capacity; however, this may be explained by a reduction in soil wettability caused by oil coatings on the soil particles (aggregates), rather than by the presence of toxic compounds. Thus, it appears that the bioremedial treatments applied to Waste 1, i.e., aggregation, fertilization, irrigation, heating and aeration, successfully eliminated the salinity and toxicity associated with this waste.

2. According to Alberta Tier 1 Criteria for Contaminated Soil Assessment and Remediation, the acceptable level of TPH (mineral oil and grease) in hydrocarbon contaminated soils is 0.1%. Although the TPH concentration of Waste 1 (2.2%) was well above the Tier 1 guideline, all the acute bioassays and chronic soil process assays indicated this waste was not toxic. Also, the non toxic Microtox reading on a 1:4 extract of this

waste indicated potential leachates from this material would be non toxic. These results suggest that, for hydrocarbon contaminated wastes such as those tested in this study, bioassays should accompany chemical criteria in order to predict more precisely their ecotoxicological potential.

3. Waste 1 does not require further bioremediation and is ready to be landfilled, landspread or landfarmed. Although Waste 2 has a TPH (2.5%) similar to that in Waste 1, it requires further bioremediation and toxicity testing.

4. The factors causing the toxicity of Waste 2 could not be identified. However, PAH compounds including naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene and anthracene, have been implicated in causing toxic effects on *Photobacterium phospohoreum* (Symons and Sims, 1988). These same compounds, in addition to the aliphatic/aromatic fraction which was much greater in Waste 2 than in Waste 1, may explain the extreme toxicity of Waste 2.

5. Of the single species bioassays, the Microtox and earthworm survival tests were the most sensitive while the algal growth inhibition test was the least sensitive. Although coefficients of variation were generally less than 20%, indicating a high degree of precision and reproducibility for most assays, variability tended to increase with increased toxicity. This may be a result of more erratic behaviour by the organisms as they deal with the stress and cell damage caused by toxic chemicals.

6. With the exception of the algal growth inhibition assay which was insensitive to the toxicity of Waste 2, both the single species (organism) assays and the soil process assays were sensitive to the toxicity of Waste 2 and results from both approaches were in agreement with each other. The plant life cycle assay may be a reliable method for evaluating not only toxicity, but also potential physical problems in the soil or waste which may interfere with productivity. The plant life-cycle bioassay would also be valuable for determining the potential of a bioremediated (non toxic) waste for supporting plant growth if disposal options include landspreading or landfarming.

7. The ecotoxicological protocol tested in this study provided reliable results with regards to evaluating the toxicity of an oil-contaminated soil and a diesel invert mud residue and may be applicable to monitoring detoxification of other hydrocarbon-contaminated wastes undergoing bioremediation.

ACKNOWLEDGEMENTS

This research is a component of The Bio-Reactor Project and funding by Alberta Environmental Research Trust (AERT), Environment Canada through the Groundwater and Soil Remediation Program (GASReP), the Development and Demonstration of Site Remediation Technology program (DESRT), the Federal Panel on Energy Research and Development (PERD), the Canadian Association of Petroleum Producers (CAPP) and Gulf Canada Resources is gratefully acknowledged. We appreciate the helpful comments and guidance provided by Lin Callow, Gulf Canada Resources. Also, critical discussions and recommendations provided by members of the Technical Committee were invaluable in the execution of this research. We are indebted to Richard Johnson and his team of scientists at the Alberta Environmental Centre for sharing baseline information on Wastes 1 and 2 and for their help in collection and transport of the material needed for toxicity testing. S. Goudey of Hydroqual Laboratories Ltd. kindly supplied a culture of *Selenastrum capricornutum*. This research would not have been possible without the skillful and efficient technical assistance provided by Ms. B. Mottle.

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1. INTRODUCTION

When establishing criteria for assessment and remediation of contaminated soils, regulatory agencies have generally opted for non-biological, chemical-specific methods to (predict) evaluate ecological risk. For example, within the framework of the "National Guidelines for Decommissioning Industrial Sites" (CCME, 1991), Alberta Environmental Protection has recently proposed a two-tiered approach to contaminated soil assessment in which Tier 1 is based on acceptable concentrations of chemical contaminants (Province of Alberta, 1994).

Hydrocarbon wastes produced by the Canadian oil and gas industry are notoriously complex chemical mixtures of volatiles, aliphatics, monoaromatics, polycyclic aromatic hydrocarbons, polyaromatic sulphur heterocyclics, polyaromatic nitrogen heterocyclics, hydroxylated polycyclic aromatic hydrocarbons and asphaltenes. Little is known of the ecotoxicity of individual compounds in a hydrocarbon waste, let alone the interactive effects of a multitude of chemical constituents. Thus biological effects of a hydrocarbon contaminant may be extremely difficult to assess from chemical concentration data and, for this reason, bioassays have been proposed as the only reliable method for evaluating ecotoxicity potential (Cairns and Pratt, 1989).

A bioassay is defined by Cairns and Pratt (1989) as "a procedure that uses living material to estimate chemical effects", which should, ideally, address toxicological effects at the organism, population, community and ecosystem levels. Toxicological testing of soil or soil-like materials is still in its infancy, particularly in regard to testing of whole field soils (Sheppard et al., 1992). Single species bioassays, representing a range of trophic levels, are the most commonly employed tests, and standardization of some of these tests plus development of new assays using species more relevant to soil systems are currently in progress (Keddy et al., 1992). More long-term life-cycle bioassays, which measure growth and reproduction, have been recommended since they may be more sensitive than acute bioassays which concentrate on short-term survival and growth (Sheppard et al., 1992, 1993).

Functional aspects of soil biological communities such as microbially-mediated C and N mineralization processes should also be considered for toxicological testing because of the importance of these processes in soil fertility and productivity. Based on a review of the development of assessment and remediation guidelines for contaminated soils by Sheppard et al. (1992), there is, as yet, no

standardized battery of ecotoxicity tests for soils, but development of such a battery should include "tests of lethality, mutagenicity, growth impairment and life-cycle impairment; using plants, decomposers and components of key nutrient cycles". In the present study, the approach recommended by Sheppard et al. (1992) (except for mutagenicity) was used to determine the ecotoxicological potential of two hydrocarbon-contaminated wastes which had undergone bioremediation in the Nevis Bio-Reactor for variable lengths of time.

2. OBJECTIVES OF STUDY

The objectives of the first phase of ecotoxicity testing of the hydrocarbon-contaminated wastes being treated in the Nevis Bio-Reactor were:

(i) to evaluate the acute (short-term) and chronic (long-term) toxicological potential of a crude oil spill agricultural soil (Waste 1) and a diesel invert mud residue (Waste 2) following bioremediation in the Bio-Reactor for 16 and 4 months, respectively.

(ii) to develop and evaluate an ecotoxicological protocol for monitoring temporal detoxification of a soil or waste subjected to various bioremedial procedures. The battery of techniques tested included single species (organism) bioassays which often measure acute response, decomposition and nutrient cycling (soil process) assays which provide a measure of chronic effects, and plant life-cycle assessments which integrate the effects of soil chemical, physical and biological factors as expressed in plant productivity and reproduction.

(iii) to determine the success of various bioremediation technologies in detoxifying hydrocarbon and salt-contaminated wastes using ecotoxicological, rather than chemical, criteria.

3. CHARACTERIZATION OF TEST MATERIALS, REFERENCE SOIL AND ARTIFICIAL SOIL

3.1 Treatment history of Wastes 1 and 2

The hydrocarbon contaminated wastes chosen for the first toxicity trial (November, 1993 to May 1994) were designated as Waste 1 and Waste 2.

Waste 1

Waste 1 was a Chernozemic topsoil from Erskine, Alberta which had been contaminated with crude oil and brine as a result of a pipeline break (AEC Draft Report on Waste 1 1992/93). Prior to treatment in the Nevis Bio-reactor, the contaminated soil contained approximately 6% oil, was highly saline with an electrical conductivity (EC) of 24 mS cm^{-1} and and sodium adsorption ratio (SAR) >20, and was severely water repellent as indicated by the molarity ethanol droplet (MED) test (see Table 4).

Prior to being placed in the Bio-reactor, Waste 1 was treated with 1% each of ground straw, hydrated lime and commercial starch and was then aggregated to yield aggregates primarily in the 1 - 9.5 mm range. The material was then placed in the bio-reactor, leached to remove salts, fertilized, and subjected to heating (35°C) and/or forced aeration (see Table 1 and AEC Draft Report on Waste 1 1992/93 for details of treatments). After 11 months in the Bio-Reactor, all of the Waste 1 treatments were removed from the Bio-Reactor, mixed and placed in a secondary treatment unit, termed a Bio-Pile. Following 5 months in the Bio-Pile, where the material received additional treatment (irrigation, heating and aeration), samples were removed for ecotoxicity testing.

Waste 2

Waste 2 is a Diesel Invert Mud Residue (DIMR) in subsoil (clay loam). Initial characteristics of this material included a high salt content (36 dS m^{-1}), Total Petroleum Hydrocarbon (TPH) content of 10.8%, a pH of 7.5 and less severe water repellancy than that exhibited by Waste 1 (MED = 3.6; see Table 4).

Prior to being placed in the Bio-Reactor, some of Waste 2 was treated with 1% lime and aggregated to obtain aggregates mainly in the 1 - 5 mm size class. Aggregated and non-aggregated material was then placed in the Bio-Reactor where it was leached to remove salts, fertilized and irrigated. Bioremediation treatments tested on Waste 2 in the Bio-Reactor included aggregation, cultivation and aeration. The treatments applied to the various cells in the Bio-Reactor are summarized in Table 1 and further information can be found in

Table 1. Treatments tested on Waste 1 and Waste 2 in the Bio-Reactor. Toxicity testing of Waste 1 initiated following 11 months residence in the Bio-Reactor and 5 months residence in the Bio-Pile. Material from cell treatment 7 was subjected to toxicity testing following 4 months residence in the Bio-Reactor.

WASTE	CELL TREATMENTS	WASTE MANIPULATIONS					
		Aggre-gation	Fertili-zation	Irriga-tion	Heat	Cultiv-ation	Aera-tion
Waste 1	1	+	+	+	+	-	-
(Oil spill)	2	+	+	+	-	-	+
	3	+	+	+	+	-	+
	4	+	+	+	-	-	-
	Bio-Pile[1]	+	-	+	+	-	+
Waste 2	1	+	+	+	+	-	-
(DIMR)	2	-	+	+	+	-	-
	3	-	+	+	+	+	+
	4	+	+	+	+	-	+
	5	-	+	+	+	+	-
	6	-	+	+	+	-	+
	7	+	+	+	+	+	+
	8	+	+	+	+	+	-

[1] Following 12 months of bioremediation, all Waste 1 treatments were combined and placed in the Bio-Pile.

the AEC Annual Report 1993/94 for DIMR.

Following four months in the Bio-Reactor, samples of Waste 2 (Cell 7) which had received the complete package of treatments, i.e., aggregation, fertilization, irrigation, heating, cultivation and aeration, were removed and tested for toxicity.

3.2 Characterization of organic constituents in Wastes 1 and 2

3.2.1 Methods

Waste 1

Five replicate samples of Waste 1 prior to being placed in the Bio-Reactor (initial) and 1 sample of the same Waste removed from the Bio-Pile 5 months after transfer from the Bio-Reactor (intermediate) were submitted to ETL Enviro-Test Laboratories for the following analyses.

(i) Extractable hydrocarbons using a dichloromethane extraction followed by fractionation on an alumina column and gravimetric analysis for:

- the aliphatics and monoaromatics (Fraction 1);

- the polycyclic aromatic hydrocarbons (PAHs) and polyaromatic sulphur heterocyclics (PASH) (Fraction 2);

- the polyaromatic nitrogen heterocyclics (PANH) (Fraction 3);

- the hydroxylated polycyclic aromatic hydrocarbons (HPAH) and organic acids (Fraction 4).

- oil and grease

(i) Characterization of total extractables by GC/FID and non-target characterization by GC/MS.

(iii) Asphaltenes by toluene extractions and gravimetric analysis.

(iv) Non-target volatiles characterization by GC/MSD.

(v) Benzene, toluene, ethylbenzene and xylene (BTEX) analyses.

The 5 replicate initial samples were composited prior to non-target volatile analysis and the non-target GC/MS characterization of extractable compounds. Metals, arsenic, selenium, antimony and mercury analysis, Dean-Stark for total organic carbon in pH 3, pH 7 and pH 8 leachates were also measured on the initial samples. Methodological details and a listing of the raw data are given in September, 1992 and April, 1994 ETL Enviro-Test chemical analysis reports. A summary of the results of the BTEX, oil and grease, extractable hydrocarbons, and asphaltene analysis follow.

Waste 2

Five replicate samples of DIMR were subjected to the same

detailed analysis as Waste 1 before being aggregated and treated in the Bio-Reactor. In addition, TPH (C4 to C18 hydrocarbons) was measured by AEC using a methylene chloride extraction procedure (AEC Annual Report 1993/94).

3.2.2 Results and Discussion

Waste 1

Results of the BTEX, oil and grease (TPH), extractable hydrocarbons, and asphaltene analysis of Waste 1 at the initial and intermediate sampling times are summarized in Table 2. Similar data for Waste 2 at the initial sample time are given in Table 3.

Initial concentrations of BTEX in Waste 1 were relatively low and there was evidence of substantial volatilization of these hydrocarbons within one week of this Waste being placed in the Bio-Reactor (AEC Draft Report on Waste 1 1992/93).

All four fractions of extractable hydrocarbons in Waste 1 decreased substantially during the first 16 months of bioremediation with the order of decomposition being PANH > aliphatics/aromatics > PAHs/PASH > HPAH. Total petroleum hydrocarbons fell from 52000 $\mu g\ g^{-1}$ prior to bioremediation to 21500 $\mu g\ g^{-1}$ following 11 months in the Bio-Reactor and 5 months in the Bio-Pile - a decrease of 59%. Asphaltenes also demonstrated a significant reduction during the bioremediation period.

Waste 2

BTEX levels in Waste 2 prior to bioremediation were higher than levels measured in Waste 1, particularly for the ethylbenzene and xylene components (Table 3). Extractable hydrocarbon levels were also much higher in Waste 2 than Waste 1 with initial TPH concentrations approximating 84000 - 108000 $\mu g\ g^{-1}$.

Air emission studies by AEC revealed that much of the ethylbenzene and xylene was vented from the Bio-Reactor cells within the first month of bioremediation (AEC Annual Report 1993/1994).

Extractable hydrocarbon fractions in Waste 2 were readily degraded in the Bio-Reactor with TPH falling from 10.8% to 2.5% within the first 4 months of the bioremediation process (AEC, pers. comm.). Thus, at the time of toxicity testing, Wastes 1 and 2 contained 2.2 and 2.5% TPH, respectively.

3.3 Chemical/physical characterization of Wastes 1 and 2, reference and artificial soil used in toxicity trial

3.3.1 Methods

Waste 1, Waste 2 and Chernozemic agricultural topsoil (reference soil) were kindly sampled by staff at AEC and sent to the University of Calgary in November 1993. Artificial soil is often

Table 2. Chemical characterization of organic constituents in Waste 1 before bioremediation in the Bio-Reactor (initial) and following 11 months in the Bio-Reactor and 5 months in the Bio-Pile (intermediate). Toxicity tests conducted on waste with intermediate characteristics.

ORGANIC CHEMICAL CONSTITUENTS	INITIAL[1] ($\mu g\ g^{-1}$)	INTERMEDIATE[2] ($\mu g\ g^{-1}$)	DECREASE (%)
BTEX			
Benzene	0.16	ND	
Toluene	3.5	ND	
Ethylbenzene	1.8	ND	
Xylene	13.1	ND	
EXTRACTABLE HYDROCARBONS[3]			
Aliphatics/Monoaromatics	36192	9100	75
PAHs/PASH	11024	4400	60
PANH	6448	1200	81
HPAH	3640	1950	46
TPH	52000	21500	59
ASPHALTENES	746	490	34

[1] Summarized from ETL Enviro-Test Laboratories report (Sept. 1992)

[2] Summarized from ETL Enviro-Test Laboratories report (April 1994)

[3] Determined by Soxhlet extraction with dichloromethane. PAHs/PASH = polycyclic aromatic hydrocarbons/polyaromatic sulphur heterocyclics; PANH = polyaromatic nitrogen heterocyclics; HPAH = hydroxylated polycyclic aromatic hydrocarbons; TPH = total petroleum hydrocarbons.

ND = not determined

Table 3. Organic chemical characterization of Waste 2 (DIMR) before remediation in the Bio-Reactor[1].

ORGANIC CHEMICAL CONSTITUENT	INITIAL CONCENTRATION ($\mu g\ g^{-1}$)
BTEX	
Benzene	0.017
Toluene	0.98
Ethylbenzene	6.02
Xylene	33.0
EXTRACTABLE HYDROCARBONS[2]	
Aliphatics/Aromatics	68040
PAHs/PASH	6737
PANH	974
HPAH	2268
TPH	84000 (108000)[3]
ASPHALTENES	724

1 Data summarized from ETL Enviro-Test Laboratories report, September 1992.

2 Determined by Soxhlet extraction with dichloromethane. See Table 2 for description of acronyms.

3 Concentration in brackets obtained by AEC using methylene chloride extraction (AEC report, March 1994)

employed in toxicity testing as a negative control and as a diluent. The artificial soil used in the present study consisted of (by weight) 71% fine sand (fine grade #70), 20% kaolinitic clay, 8% sphagnum peat (passed through 2 mm sieve) and 1% $CaCO_3$ to adjust the pH (OECD 1984). The wastes, reference soil and artificial soil were homogenized by passing each material through a 4 mm sieve prior to analysis and testing.

Initial chemical/physical characterization was performed on each of the materials prior to toxicity testing to ensure that there were no extreme pH, salinity and nutrient problems which in themselves could have a significant influence on the outcome of the toxicity tests. Methodological details of each test follow.

Moisture content

The moisture contents of three replicates of each material were determined gravimetrically following drying at 80°C for 24 h. Moisture contents are expressed on a dry weight basis.

pH

Using a soil:deionized water ratio of 1:2, the pH was measured on 3 replicates per test material. The slurry of soil or waste and water was stirred for 1 min and the pH measured electrometrically (Orion Research pH meter equipped with combination electrode) after 1 hour. Readings were made 2 min after immersing the electrode in the slurry, i.e., after the readings had stabilized.

Electrical conductivity (EC)

Immediately after measuring the pH of the materials in deionized water, the supernatent was filtered through a Whatman #2 filter and the electrical conductivity of the filtrate measured using a Markson Model 1052 Digital Conductivity Meter.

Extractable NH_4-N and NO_3-N

A dry weight equivalent of 5 g soil/waste was placed in 40 ml 2N KCl and shaken for 1 hour using a reciprocal shaker at high speed. Each sample was vacuum filtered through a Whatman #42 filter paper and the filtrates analyzed on a Technicon AutoAnalyzer II using the chemistry described in Industrial Method No. 98-70W/A for ammonia and Industrial Method No. 100-70W/B for nitrates (Technicon Industrial Systems, Tarrytown, N.Y.). Three replicates were analyzed per soil or waste and results expressed as NH_4-N and NO_3-N.

Extractable PO_4-P

A dry weight equivalent of 1 g soil/waste was placed in 25 ml "modified Bray extractant", shaken for 2 min and filtered through a Whatman #42 filter paper. The filtrate was analyzed for PO_4-P using a Technicon AutoAnalyzer II and the ammonium molybdate/ascorbic acid

chemistry described in Industrial Method No. 94-70W/B (Technicon Industrial Systems, Tarrytown, N.Y.). Three replicates were analyzed per soil or waste.

Total carbon

Total C was measured in a Leco Carbon Determinator CR12 using 3 to 5 replicates of oven-dried (80°C) soil per sample. Three replicate samples were analyzed for each soil or waste.

3.3.2 Results and Discussion

Moisture

Moisture contents of the various materials were ($\bar{x} \pm SD$): Waste 1 - 14.4±0.4; Waste 2 - 15.2±0.2; Reference - 6.5±0.1; Artificial - 0.8±0. Prior to conducting the toxicity tests the moisture contents of Wastes 1 and 2 and reference and artificial soils were adjusted to 23% (dwt basis) with deionized water.

pH

The pH's of Wastes 1 and 2 and the reference soil were neutral to slightly alkaline while the artificial soil was slightly acidic (Table 4). The pH's of the various soils were not considered inhibitory to biological activity.

Electrical conductivity (EC)

Electrical conductivities were low in all the test materials with the exception of Waste 2 where the salinity was high enough that it could potentially inhibit biological activity (Table 4). However, preliminary trials with this Waste indicated that this salt level did not restrict microbial respiration and biomass essential to the bioremediation process (Danielson, Bio-Reactor Project 1993/1994 report, 1994).

Extractable NH_4-N and NO_3-N

Available N in all the test materials were considered sufficiently high to support the growth and development of the test organisms used in the toxicity trials over the short term (Table 4). Nitrate-N levels were high in the two Wastes, presumably due to fertilization treatment during the bioremediation process.

Extractable PO_4-P

Fertilization also raised PO_4-P levels in the waste material to levels greater than those measured in the reference and artificial soils (Table 4). Phosphorus levels were considered adequate for biological response testing.

Total carbon

Total carbon contents of Wastes 1 and 2 were 5.2 and 4.4%, respectively, of which TPH's comprised 2.2% in Waste 1 and 2.5% in Waste 2 (Table 4). There was sufficient carbon in all the soil/waste materials to support microbial biomass although the

Table 4. Chemical/physical characteristics of Wastes 1 and 2 and reference and artificial soil used in toxicity Trial 1. Data are means (n = 3) ± SD.

	WASTE 1[1]	WASTE 2[2]	REFERENCE[3]	ARTIFICIAL[4]
$pH_{1:2}$	7.5 ± 0	7.1 ± 0.01	7.3 ± 0.2	6.4 ± 0.07
$EC_{1:2}$ (dS m^{-1})	0.6 ± 0.04	3.5 ± 0.2	0.7 ± 0.03	0.4 ± 0.1
Initial MED[5]	9.2	3.6	0	NM
NH_4-N ($\mu g\ g^{-1}$)	0.4 ± 0.7	8.3 ± 0.5	7.9 ± 0.6	15.3 ± 1.0
NO_3-N ($\mu g\ g^{-1}$)	32.6 ± 1.1	29.2 ± 0.6	1.5 ± 0.1	0.4 ± 0.1
PO_4-P ($\mu g\ g^{-1}$)	147 ± 8.6	74.0 ± 8.3	43.8 ± 2.4	6.2 ± 0.4
Total C (%)	5.2 ± 0.2	4.4 ± 0.05	3.0 ± 0.03	3.7 ± 0.4
TPH (%)	2.2	2.5	NA	NA

1 Crude oil spill on topsoil. 11 months in Bio-Reactor; 5 months in Bio-Pile.

2 Diesel invert mud residue (DIMR). 4 months in Cell 7 in Bio-Reactor.

3 Uncontaminated reference soil - chernozemic agricultural soil from Erskine, AB.

4 Artificial soil: 71% fine sand; 20% kaolinite; 8% peat; 1% $CaCO_3$.

5 MED = Wettability determined by Molarity Ethanol Droplet test. Measured prior to incubation in the Bio-Reactor (AEC Draft Reports 1993, 1994).

NA = not applicable; NM = not measured

bioavailability of the C varied depending on the state of decomposition of the organic matter in each soil or waste.

4. BIOASSAYS USED FOR ECOTOXICOLOGICAL TESTING OF WASTES 1 AND 2

4.1 Introduction

As stated previously, one of the aims of this phase of toxicity testing was to develop and test a battery of bioassays which would address not only single species response with respect to lethality, growth and life cycle impairment, but would also consider soil processes, particularly the decomposition and carbon (C) and nitrogen (N) mineralization processes. The tests selected for inclusion in the bioassay protocol are summarized in Table 5 and were conducted in 3 phases beginning with the single species bioassays followed by soil process assays which measure multispecies response and terminating with the plant life-cycle bioassay.

Single species bioassays

Single species bioassays for assessing the quality of soil or soil leachates have been developed for a range of organisms including bacteria (Microtox), algae (*Selenastrum*), various species of terrestrial vascular plants, macroinvertebrates (*Daphnia*), earthworms and springtails (Keddy et al. 1992). These organisms represent a range of trophic levels and many are considered surrogates of the indigenous soil organisms. Single species bioassays are often short-term and usually have only one endpoint, i.e., measurement of acute lethality or acute effects on metabolic processes (e.g. light production) or growth (e.g. *Selenastrum* growth inhibition). They usually do not consider chronic or sub-lethal effects on growth and reproduction.

Single species bioassays are often recommended for toxicity testing, primarily because they have been standardized to a greater degree than other types of tests, demonstrate a high degree of sensitivity and reproducibility, and are relatively rapid and inexpensive to conduct. However, they have been criticized for their ecological relevance and for the limited number of organisms available for testing. Keeping these shortcomings in mind, single species bioassays can, nevertheless, be valuable indicators of possible ecological effects in the field, particularly if performed on a range of species and if conducted in association with soil process assays. In the present study, single species bioassays were performed to determine the degree of acute toxicity in the wastes and to determine if the plant life-cycle bioassay, which measures sub-lethal response, should be conducted.

Soil process assays

During the bioremediation of hydrocarbon-contaminated wastes

Table 5. Battery of ecotoxicity tests used for monitoring Wastes 1 and 2 while undergoing bioremediation in the Bio-Reactor and Bio-Pile (in the case of Waste 1). All tests carried out on the solid phase except for the Microtox and algal tests which were performed in a 1:4 (soil:water) extract.

1.	Single species bioassays (acute response)
	• seedling emergence/root elongation (buttercrunch lettuce, barley, canola)
	• earthworms (*Eisenia foetida*) survival
	• Microtox (reduction in light produced by *Phosphobacterium phosphoreum*)
	• algal (*Selenastrum capricornutum*) growth inhibition
2.	Soil process (decomposition/nutrient cycling) assays (acute or chronic response)
	• C mineralization (respiration; microbial biomass C; metabolic quotients; ratio of microbial C:substrate C)
	• N mineralization (ammonification and nitrification)
	• decomposition (mass loss of alfalfa stems)
3.	Plant life-cycle bioassay; Waste 1 only (chronic response)
	• greenhouse pot experiment with barley
	• productivity (shoot and root production); time to flowering; seed production after 3 to 4 months

and under conditions of non-limiting mineral nutrients, microbial respiration generally exhibits a characteristic pattern consisting of a short period of high activity when microbial respiration is stimulated by high bioavailability of C, followed by a long period of low activity when C bioavailability has decreased and microorganisms are degrading more complex, recalcitrant C compounds (Fig. 1). Since microbial respiration is directly related to the disappearance of C from hydrocarbon-contaminated wastes, it is probably also linked to the detoxification of various components of the hydrocarbon contaminant.

Nitrogen is one of the key elements required to maintain the productivity of both plants and the soil microbial biomass. The fertility of a soil is often related to its ability to cycle nitrogen. Cycling of nitrogen in the soil consists of the mineralization of organic N in plant residues and organic matter to ammonium N and subsequent nitrification of ammonium N to nitrite and nitrate.

Organic N $\rightarrow$ NH_4^+ $\rightarrow$ NO_2^- $\rightarrow$ NO_3^-

Both NH_4-N and NO_3-N can be taken up by plants and microbes which, when they die, return organic N to the soil.

The transformation from organic N to NH_4 can be performed by a wide range of microorganisms, both bacteria and fungi and, therefore, this process may be relatively insensitive to the presence of toxic materials (Vonk 1991). In contrast, nitrification (the transformation of NH_4-N to NO_3-N) requires a highly specialized group of autotrophic bacteria belonging primarily to the *Nitrosomonas* and *Nitrobacter* genera. In order for the nitrification to proceed, a source of NH_4-N must be available. Due to the highly specialized nature of the nitrification process, this component of the nitrogen cycle is believed to be extremely sensitive to soil perturbation including the introduction of toxic chemicals.

Because of the importance of the nitrogen cycle in maintaining soil productivity, both ammonification and nitrification assays, with or without the addition of an organic or inorganic source of N, have been recommended for testing side-effects of pesticides in soil in Europe (Andersch and Anderson 1991; Vonk 1991).

Although the decay of various hydrocarbon fractions in a contaminated waste may proceed at a rapid pace by a highly active microbial biomass, this biomass may not be adapted to decomposing plant residues. In a situation where a bioremediated soil is landspread or landfarmed and planted with a crop species, sustainability of the crop would be dependent to a large degree on decomposition and nutrient mineralization processes proceeding at a rate similar to that in uncontaminated agricultural soil.

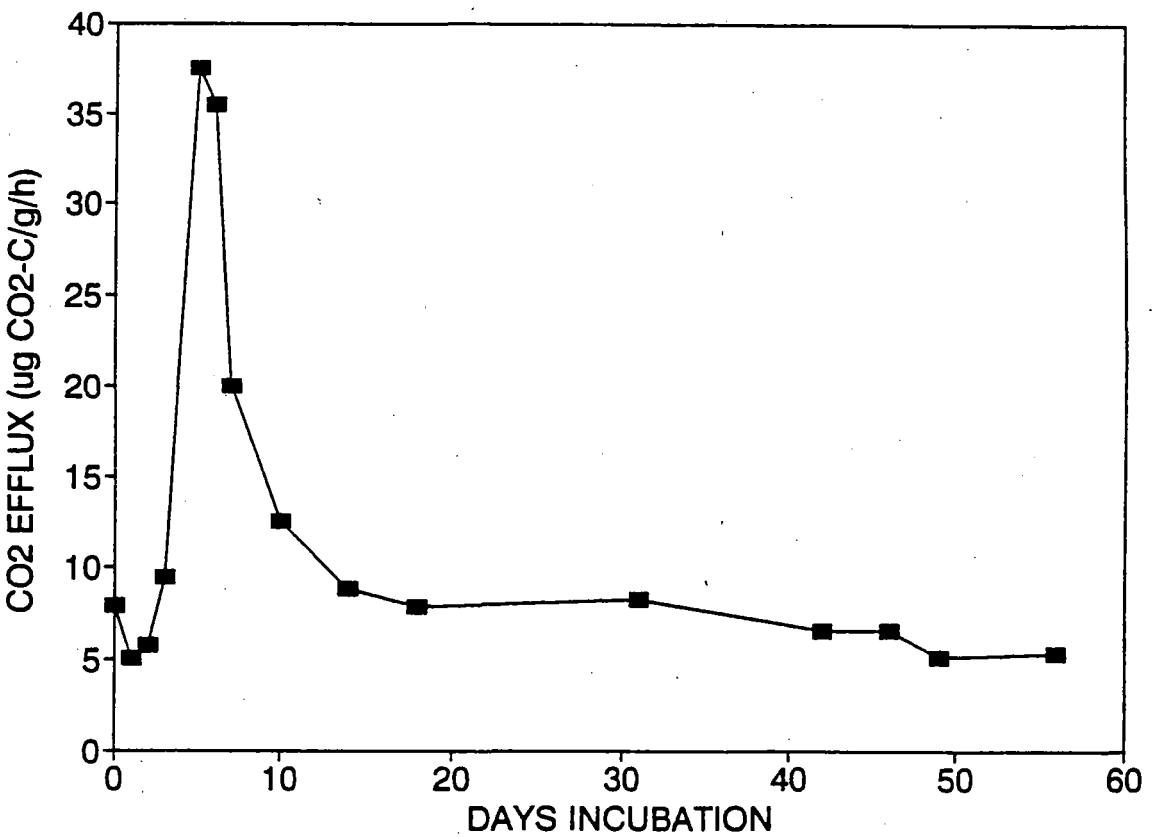

Figure 1. Microbial respiration (CO_2 efflux) of DIMR amended with 400 μg N g^{-1}. (Fig. 3.1-1 in Danielson 1994). Note initial phase (duration approx. 10 days) of high respiration followed by long period of relatively low activity.

Plant life-cycle assay

Plant life-cycle bioassays are valuable for ecotoxicity testing in that they integrate the effects of soil chemical, physical and biological factors as expressed in plant productivity and reproduction over the long-term (time from seed to seed). Most plant bioassays focus on only one or two responses, primarily seed germination or root elongation. Plant life-cycle assays allow the measurement of multiple endpoints including germination, shoot production, root production, plant height, time to flowering and seed production (Sheppard et al. 1993).

Because plant life-cycle assays are usually long-term they are more a measure of chronic response to soil contaminants than are single species bioasssays which tend to measure acute response over the short-term.

Life-cycle bioassays require a large time investment and, thus, are recommended only if previously conducted acute bioassays indicate slight to no toxicity. In the present study a plant life-cycle bioassay was conducted on Waste 1 using barley as a test plant species. Barley was used since this is an important crop species in this region and has been used successfully in landfarming trials (Danielson et al. 1990).

4.2 Methods

4.2.1 Single species bioassays

Seedling emergence and root elongation - acute response

Three plant species (lettuce plus two crop species) were tested including buttercrunch lettuce, barley (Diamond variety obtained from Alberta Wheat Pool) and canola (mixture Polish and Argentina varieties obtained from Alberta Wheat Pool). All seed was obtained from the same seedlot and was untreated.

Seedling emergence and root elongation were measured in the following treatments:

1. control (100% reference soil)
2. 5% (5% waste by volume; 95% reference soil by volume)
3. 25% (25% waste; 75% reference soil)
4. 50% (50% waste; 50% reference soil)
5. 75% (75% waste; 25% reference soil)
6. 100% (100% waste)

A 100% concentration of artificial soil was also tested as a comparison with the 100% reference soil. Artificial soil was not used as a negative control or diluent because the Microtox test suggested slight toxicity in this material (see Table 9).

The various treatments were achieved by mixing sieved, remoistened (23% dwt) reference with Waste 1 or 2 soil, by volume, in the appropriate dilutions. Disposable, plastic containers (100 mm wide; 30 mm deep) were filled with a 2.5 cm deep layer of waste/soil mixture with each container receiving the same volume and weight of test material in each treatment. Containers with artificial soil were treated in the same way. There were 3 replicate containers /treatment /waste /plant species.

For lettuce and canola, 30 seeds were spread evenly over the surface of the soil/waste in each container, then covered with a thin layer of sand and sprayed with deionized water. Due to the larger size of the barley seed, only 20 seeds of this species were planted in each container. Seed was stored at 5°C between trials.

Once planted, each container was placed in a plastic bag, incubated in the dark for 48 h and then placed in a growth chamber with a photoperiod of 16 h light followed by 8 hours dark. Temperature in the growth chamber was 24 ± 2°C and the light conditions were in the vicinity of 3200 lux. Growing conditions were similar to those recommended by Greene et al. (1989) as outlined in Keddy et al. (1992).

Seedling emergence was recorded after 4 days for barley, 5 days for canola and 7 days for lettuce. Time of measurement corresponded with the amount of time required by each species to exhibit maximum germination in the control treatment. Seedling emergence was determined by counting the number of seedlings above the soil surface.

After measuring emergence, all plants were washed free of soil/waste and blotted dry. Root elongation was determined on 10 randomly chosen plants by measuring the length from the root/shoot interface to the tip of the longest root.

Mean seedling emergence and root elongation ± SD were determined for each species grown in each soil/waste concentration. Seedling emergence and root elongation as a percent of the control (100% reference soil) were then plotted against waste concentration and LC50 or IC50's determined graphically pending further investigation of appropriate statistical analyses.

Earthworm survival

Survival of *Eisenia foetida* Sav., the red composting worm, was determined in the same treatments as those listed for the seedling emergence/root elongation assays, i.e., 100% reference soil; 5%, 25%, 50%, 75% and 100% Waste 1 or 2 mixed with reference soil; and 100% artificial soil. Each soil or waste was adjusted to 23% moisture with deionized water (dwt basis) and the appropriate dilutions mixed by volume. Small, disposable

plastic containers (6 cm dia.; 3 cm deep) were filled with a 2 cm deep layer of each soil/waste mixture. Care was taken to ensure that each container in each treatment contained the same volume and mass of soil.

Redworms were obtained from a bait supplier immediately prior to setting up the test. Large worms (minimum weight = 200 mg) possessing clitellums were selected and sprayed with deionized water to remove composting material. Only one worm was placed in each container to avoid the problem of cascade deaths as recommended by Sheppard and Evenden (1992). Each container was capped with a perforated lid and incubated at room temperature (21°C) under constant light. Four replicates consisting of 10 worms per replicate were set up for each treatment.

Survival of the worms was determined after 7 and 14 days by emptying each container and checking for live, mobile worms. Dead and dying worms were usually located at the soil surface and in the toxic Waste 2 treatments most died within 24 hours after being introduced.

Mean worm survival ± SD was calculated for each treatment and LC50's estimated graphically.

Microtox

The Microtox assay was conducted on an aqueous extract of the reference and artificial soils and the two wastes. The aqueous extract was obtained using the method outlined in Matthews and Hastings (1987). Briefly, the equivalent of 25 g dwt material was placed in 100 ml deionized water in a 250 ml flask and shaken for 24 hours on a mechanical shaker. The soil/waste/water slurries were then allowed to settle for 1 hour and the supernatent decanted into centrifuge tubes. The samples were not adjusted osmotically with 2% NaCl at this stage since osmotic adjustments are made during the Microtox assay. The supernatent was centrifuged at 15000 rpm for 10 min and then subsampled for the Microtox assay. The pH of the supernatent was checked to ensure it was in the 6.0 to 8.5 range prior to conducting the assay.

Measurements of light production by *Phosphobacterium phosphoreum* in each of the soil or waste extracts were made using the Microtox kit and procedures produced by Microbics Corp. The Microtox procedures are summarized also in Report EPS 1/RM/24 published by Environment Canada (1992). Three replicate extracts were assayed for each of the reference, artificial and waste materials. Light level readings were determined 5 and 15 minutes after addition of the reagent blank and light loss values were plotted against extract concentration (2.8, 5.6, 11.2, 22.5, 45, 54.1, 60.2% for Waste 2; 11.2, 22.5, 45 and 90% for the soils and Waste 1) on log paper. IC20's and IC50's were

determined using graphical and linear regression procedures of log transformed data.

4.2.2 Soil process-based assays

Microbial respiration, biomass, metabolic quotients and C_{mic}/C_{org} ratios

Soil microbial activity is determined to a large extent by moisture, temperature and availability of carbon. Standardizing these variables, where possible, can greatly reduce sample variation, and allow for more precise comparisons amongst soil treatments. In the present study, soil/waste temperature and moisture conditions were standardized at 23°C and 23% (48% for artificial soil), respectively.

Five replicates, each equivalent to 100 g dwt, were tested for each waste, reference and artificial soil. Each sample was placed in a plastic bag, hydrated to 23% moisture and allowed to equilibrate at 23°C for 3 days. Samples were then placed in glass tubes and attached to an ADC 225 Mk3 infrared gas analyzer (IRGA) where CO_2 efflux was measured hourly for 24 h. Basal respiration was calculated for the 24th hour of measurement and expressed as μg C evolved g^{-1} soil h^{-1}.

After measuring basal respiration, an optimum dosage of glucose (predetermined for each soil or waste to be 2000 μg g^{-1}) was mixed thoroughly into each sample and substrate-induced respiration (SIR) and microbial biomass C were determined using a modification of the method described by Anderson and Domsch (1978). This technique involves the addition of glucose to a soil sample and measuring the microbial response that occurs immediately prior to the onset of microbial growth. The initial respiratory response can be related to the microbial biomass C by using the regression equation, $x = 40.04y + 0.37$, where y is the substrate (glucose) induced respiration in ml CO_2 h^{-1} 100 g^{-1} soil and x is mg biomass C 100 g^{-1} soil [Anderson and Domsch (1978)].

Measurement of microbial respiration and biomass using the SIR method allowed the determination of microbial metabolic quotients (qCO_2) (Anderson and Domsch 1985). The qCO_2, which is the basal respiration C per unit of microbial biomass C h^{-1}, gives insight into the energetics of the microbial biomass C and it, plus the microbial C:soil/waste C ratios (C_{mic}:C_{org}), may be useful indicators of the bioavailability and stability of C in the waste material. Therefore, these parameters may be indicative of the state of decay of the hydrocarbon fractions in a waste material which in turn may be related to the degree of toxicity of the material.

Decomposition of alfalfa stems

Decomposition studies to determine the effects of pesticides in Europe have used ground C^{14}-

labelled straw, cellulose filter paper or straw culms. In the present study, the substrate used to determine the decomposition potential of Wastes 1 and 2 relative to the reference and artificial soils was alfalfa stems. Alfalfa was used because it has a relatively low C:N ratio (approx. 13); thus the potential for N to limit decomposition of this substrate is less likely.

Air-dried alfalfa stems obtained from AEC were cut into 5 cm pieces and placed in 2 mm mesh bags, the equivalent of 1 g stems per bag. Plastic containers, identical to those used in the seedling emergence/root elongation studies, were filled with hydrated Waste 1, Waste 2, reference or artificial soil (23% for the wastes and reference soil; 48% for the artificial soil). There were 5 replicate containers per waste or soil and the containers in each treatment were filled with the same weight of waste/soil. During the filling process, one bag containing alfalfa stems was buried in each container. The containers were sealed with a perforated lid, incubated at room temperature (21 - 23°C) and waste/soil moisture contents were adjusted weekly by weight with deionized water.

Following 3 months incubation, the alfalfa stems were cleaned, dried at 80°C and weighed to determine mass loss. Treatment effects on mass loss were determined by a one-way analysis of variance and Scheffé's Multiple Contrasts ($p<0.05$).

Nitrogen mineralization

Net nitrogen mineralization (ammonification and nitrification) potentials of the two wastes, reference and artificial soil were assessed by determining the difference between extractable N levels initially and following 8 weeks incubation. Prior to setting up the incubation study, 5 replicate subsamples from each of the wastes, reference and artificial soil were analyzed for NH_4-N and NO_3-N using the extraction and analytical procedures described previously.

For the incubation study, 5 replicate, 50 g dwt equivalent aliquots of Waste 1, Waste 2, reference and artificial soil were placed in plastic bags and each sample was amended with 135.1 mg ground alfalfa. The alfalfa served as a source of organic nitrogen for the N mineralizing microorganisms and was added at a rate equivalent to adding 5 mg N per replicate. The moisture contents of Wastes 1 and 2 and the reference soil were adjusted to 23% with deionized water; the moisture content of the artificial soil was adjusted to 48%. Each alfalfa-amended, hydrated sample was placed in a glass tube and secured with plastic foam stoppers. Samples were incubated at room temperature for 8 weeks and soil/waste moisture contents adjusted weekly by weight with deionized water.

Following the incubation period, each sample was again

analysed for NH_4-N and NO_3-N and the difference between initial and final concentrations calculated as the N flux over an 8 week period. No net increase in the amount of N mineralized was considered indicative of inhibitory effects on the ammonification and nitrification processes.

4.2.3 Plant life-cycle assay

Since the organism and process-based assays revealed that Waste 2 was either very toxic or inhibitory, the plant life-cycle bioassay was not conducted on this material. For Waste 1 which exhibited little to no toxicity in the acute bioassays, a measure of potential chronic toxicity based on a plant life-cycle assay was considered essential.

Ten plastic plant pots, each 12.5 cm dia. by 12 cm deep, were each filled with the same mass of 4 mm sieved Waste 1. Another 10 pots were each filled with the same mass of reference soil. Ten barley seeds (same seed lot as that used in the seedling emergence/root elongation assay) were placed in each pot and covered with a thin layer of fine sand. Each pot was watered to saturation with deionized water, covered with a plastic bag and placed in a growth chamber under light and temperature conditions identical to those described in 4.2.1.

Three to four days following germination, the barley seedlings were thinned to 5 per pot by eliminating the smallest seedlings. Pots were watered with deionized water as necessary; overwatering was avoided. Three weeks after seeding, the pots were moved to the greenhouse where a fertilization regime was initiated. Once weekly, following watering, each pot received a dose of Plant Prod 28-14-14 soluble fertilizer at a rate of 100 μg N g^{-1} dwt waste or soil. Plants were grown in the greenhouse until they flowered and the seed heads had begun to dry (12 weeks). At this point the seed heads, foliage and roots in each pot were harvested separately, dried at 80°C for 24 h and weighed. Significant differences in time to flowering, seed, foliage and root production by barley between Waste 1 and the reference soil were determined by two sample t-tests.

4.3 Results and Discussion

Seedling emergence and root elongation

Lettuce, barley and canola seedling emergence and root elongation all exhibited the same pattern of response to Wastes 1 and 2 although sensitivity to Waste 2, in particular, varied amongst the species. Waste 1 with a TPH of 2.2% was not toxic to seedling emergence and root elongation by lettuce and barley (Table 6, 7; Figures 2 to 7). Canola was slightly more sensitive to Waste 1 than the other two species since both seedling emergence and root elongation by this species were slightly inhibited in the 100%

Table 6. Seedling emergence of lettuce, barley and canola in Wastes 1 and 2. Data are means (n = 3) ± SD. Response in artificial soil included as a comparison to response in the reference agricultural soil (0 waste concentration).

WASTE or SOIL	PLANT SPECIES	SEEDLING EMERGENCE (%)					
		WASTE CONCENTRATION (%)					
		0	5	25	50	75	100
Waste 1	Lettuce	90 ± 9	100	89 ± 7	88 ± 12	93 ± 7	88 ± 2
	Barley	100	100	98 ± 3	98 ± 3	100	98 ± 3
	Canola	90 ± 0	88 ± 8	90 ± 9	91 ± 7	83 ± 12	66 ± 4
Waste 2	Lettuce	90 ± 9	88 ± 4	67 ± 6	12 ± 7	0	0
	Barley	100	100	63 ± 3	52 ± 3	3 ± 3	0
	Canola	90 ± 0	87 ± 6	41 ± 7	11 ± 11	0	0
Artificial	Lettuce	83 ± 3					
	Barley	100					
	Canola	91 ± 10					

Test Duration: Lettuce - 7 days; Barley - 4 days; Canola - 5 days.

Table 7. Root elongation of lettuce, barley and canola in Wastes 1 and 2. Data are means (n = 3) ± SD. Response in artificial soil included as a comparison to response in the reference agricultural soil (0 waste concentration).

WASTE or SOIL	PLANT SPECIES	ROOT ELONGATION (CM)					
		WASTE CONCENTRATION (%)					
		0	5	25	50	75	100
Waste 1	Lettuce	6.2±0.3	7.6±0.2	9.9±0.3	10.2±0.5	11.0±0.1	11.0±0.7
	Barley	10.0±0.5	10.7±0.2	10.2±0.2	9.6±0.6	9.2±0.1	9.0±0.1
	Canola	6.2±1.0	6.8±0.1	6.9±0.1	7.4±0.7	6.7±0.8	5.6±0.8
Waste 2	Lettuce	6.2±0.3	7.5±0.7	7.5±0.6	4.0±1.0	0	0
	Barley	10.0±0.5	9.4±0.8	5.9±0.5	2.9±0.5	1.1±0.1	0.3±0.1
	Canola	6.2±1.0	5.8±0.4	3.4±0.4	1.9±0.6	0	0
Artificial	Lettuce	5.9±0.3					
	Barley	10.4±0.2					
	Canola	5.7±0.3					

Test Duration: Lettuce - 7 days; Barley - 4 days; Canola - 5 days.

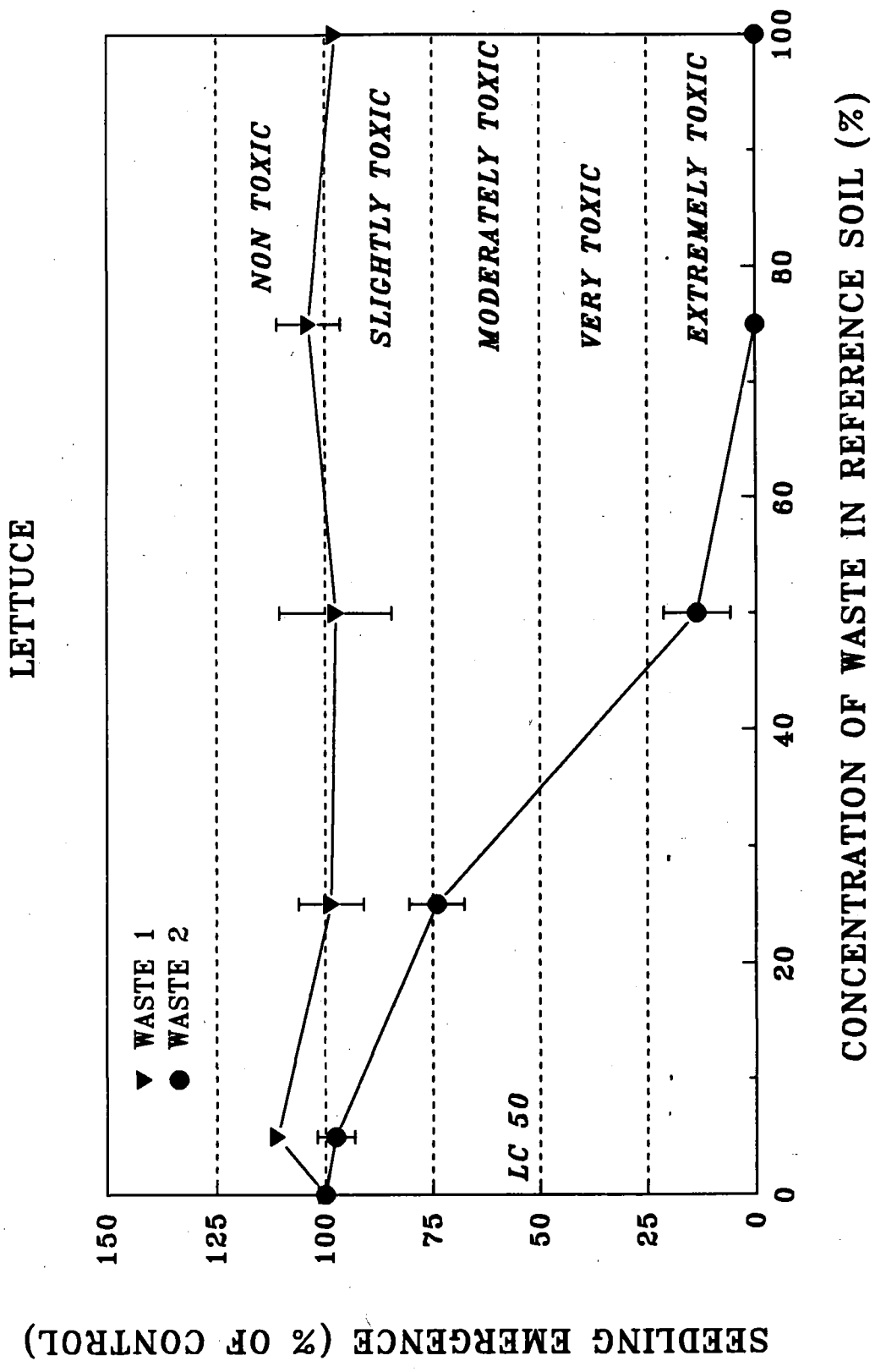

Figure 2. Emergence of lettuce seedlings in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

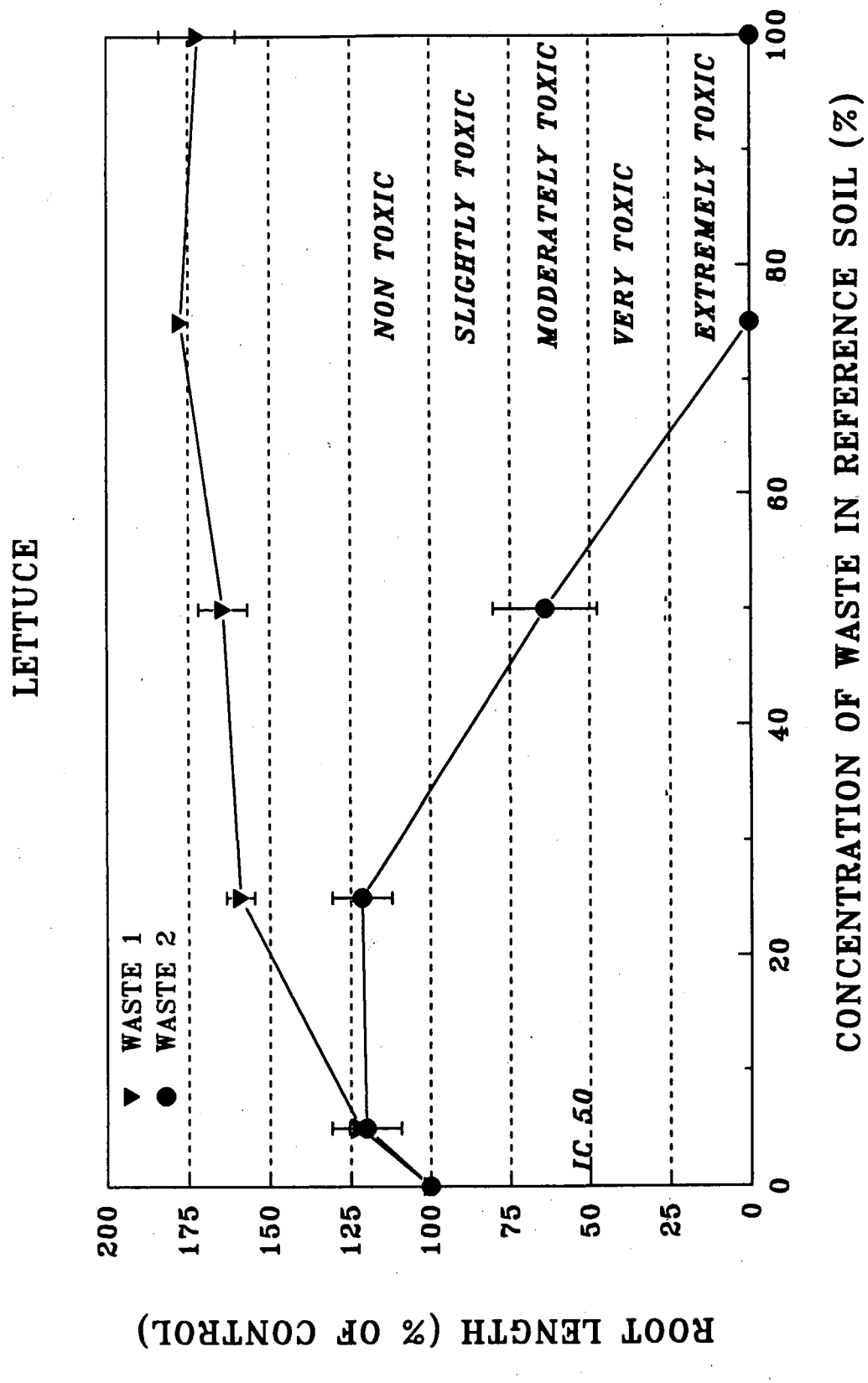

Figure 3. Root elongation by lettuce in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

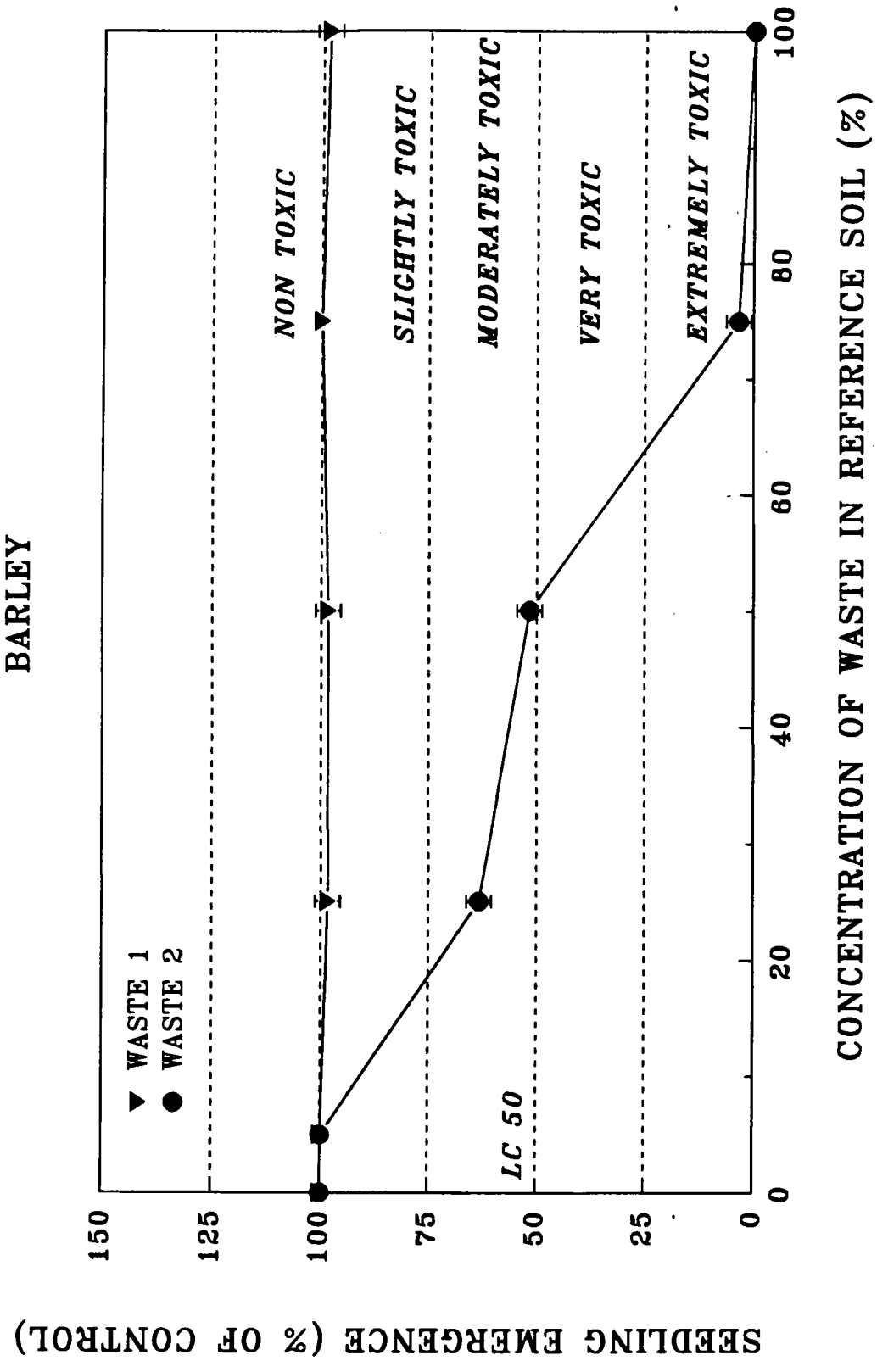

Figure 4. Emergence of barley seedlings in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

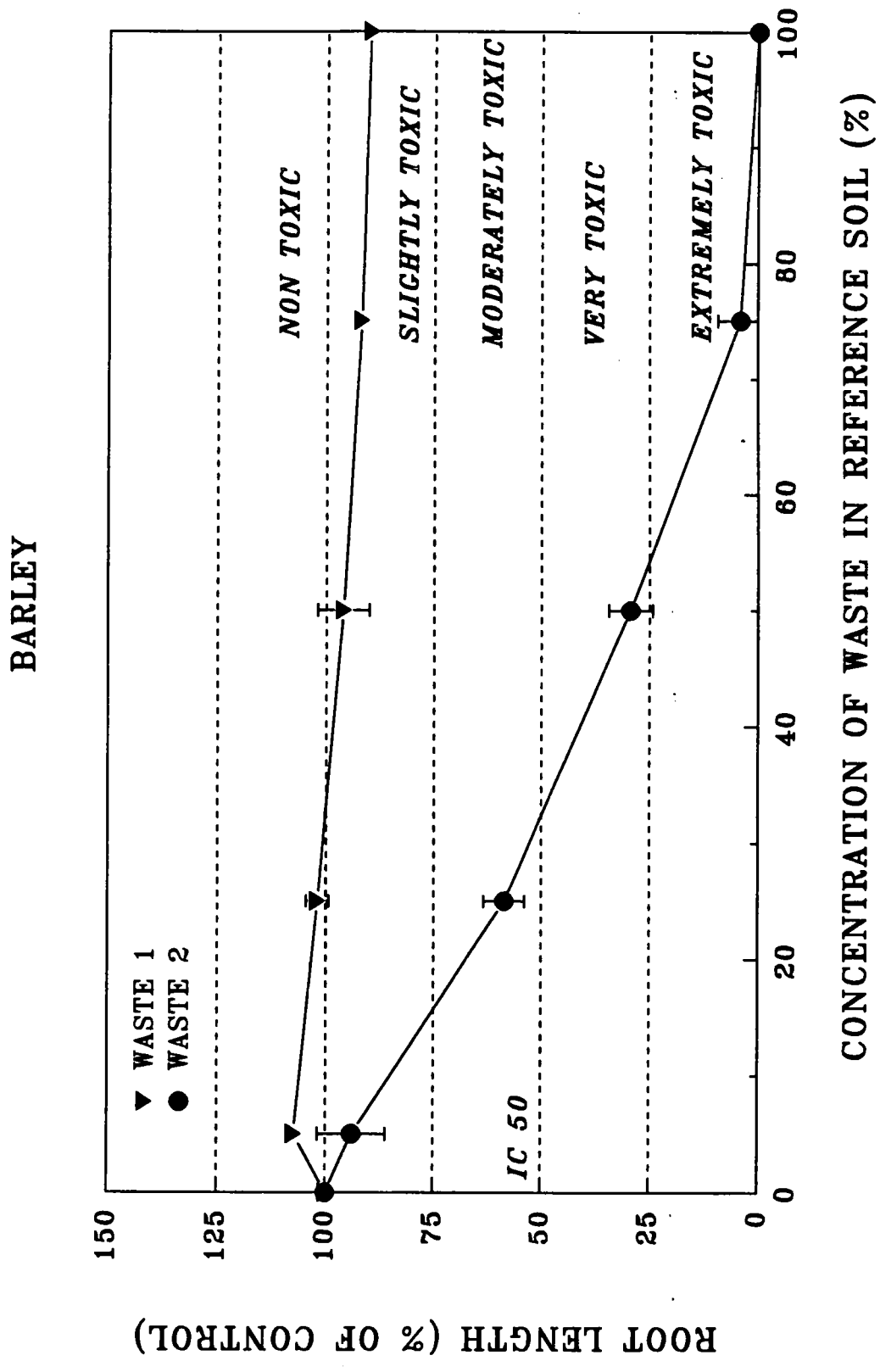

Figure 5. Root elongation by barley in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

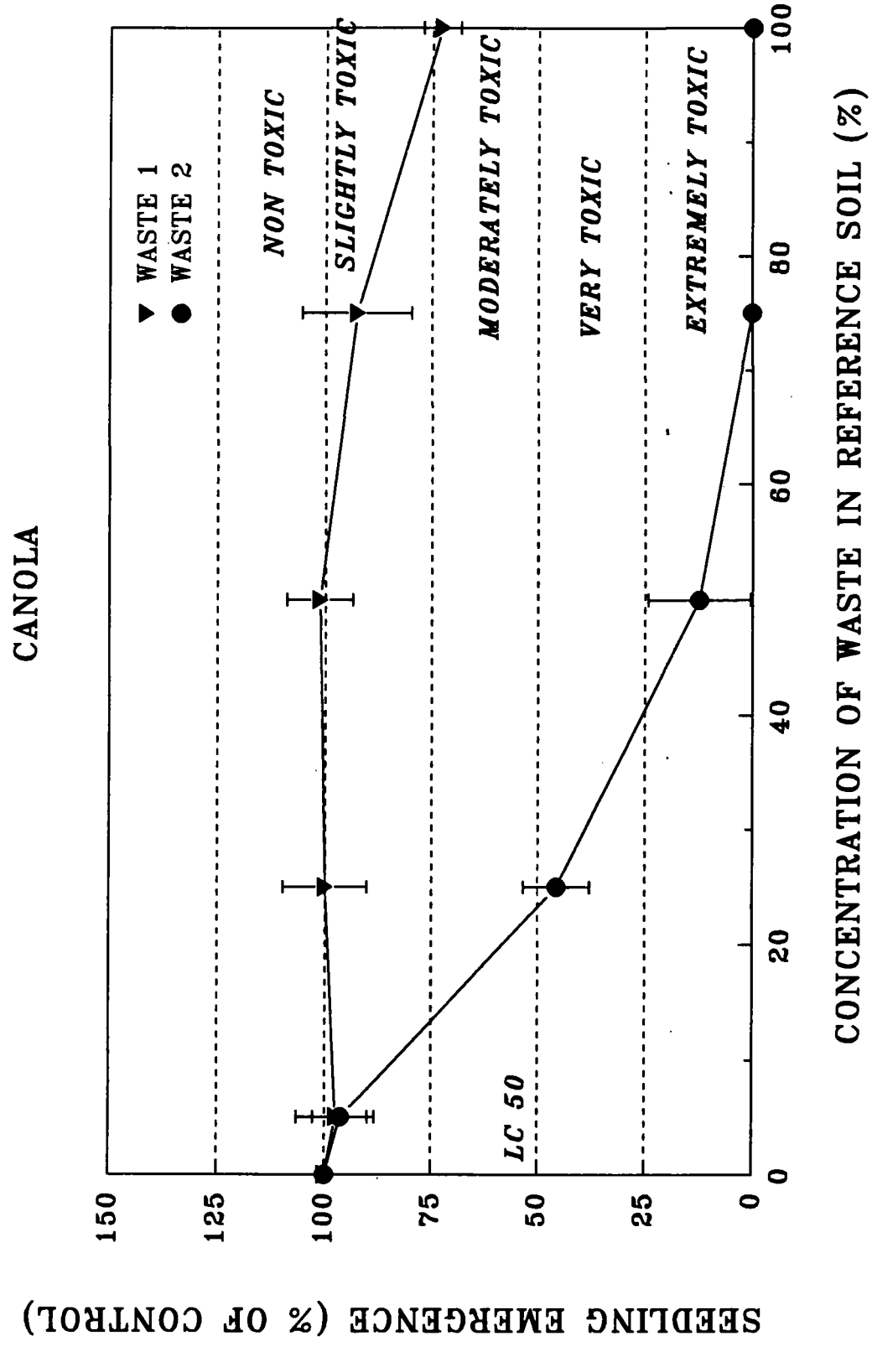

Figure 6. Emergence of canola seedlings in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

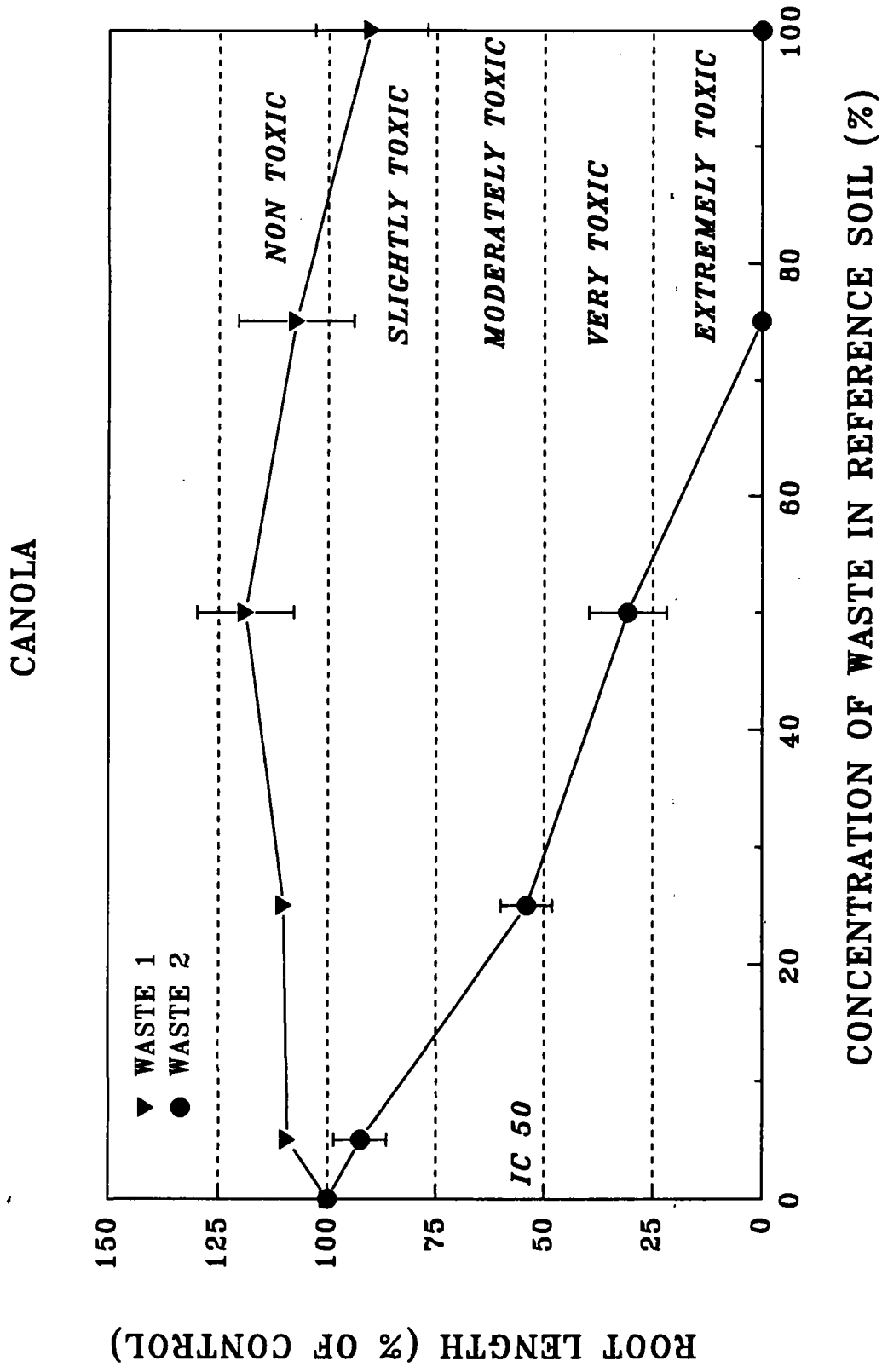

Figure 7. Root elongation by canola in reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 3) ± SD.

concentration of this Waste. Relative to the reference soil, root elongation by lettuce and canola was stimulated by Waste 1 and this may be a reflection of higher nutrient availability (N and P) in Waste 1 than in the reference soil (see Table 4). Coefficients of variation for the emergence and root elongation measurements in Waste 1 were generally less than 10% (Appendix Table 1) indicating that, under the conditions of this study, these tests have a high degree of precision and reproducibility.

In contrast to Waste 1, Waste 2 with 2.5% TPH was highly toxic to seedling emergence and root elongation by all three plant species (Tables 6,7; Figures 2 to 7). Lettuce and canola were particularly sensitive with barley less so. There was virtually no germination of any of the species in concentrations of Waste 2 greater than 50% and both emergence and root growth were substantially reduced in the 50% concentration. Based on LC50 and IC50 estimates for these tests, Waste 2 was rated as very to extremely toxic (Table 10).

Coefficients of variation for seedling emergence and root elongation were higher in Waste 2 than in Waste 1 and were especially high at the higher waste concentrations. Similar observations have been made by EMA Ltd. for lettuce seed emergence in contaminated soils from an sour gas plant site (EMA Ltd. Final Report 1994.) The increased variation with increased toxicity may be due to more erratic behaviour by the organisms as they deal with the stress and cell damage caused by the toxic components in the more concentrated waste material.

Earthworm survival

Results of the earthworm survival assay supported the seedling emergence and root elongation observations. Earthworm survival in all concentrations of Waste 1 was excellent after both 7 and 14 days exposure (Table 8, Fig. 8). Also, there was no mortality in the reference and artificial soils. It was clear from these results that Waste 1 was not toxic to *Eisenia foetida* over the short-term.

However, Waste 2 was observed to be extremely toxic to the earthworms (Table 8, Fig. 8). Although there was little mortality in the 5% waste concentration, raising the waste concentration to 25% resulted in almost complete mortality. No worms survived in Waste 2 at concentrations greater than 25%.

The very steep fall in earthworm survival between the 5 and 25% concentrations of Waste 2 suggests there is a toxic threshold of this Waste beyond which this species does not survive. Worms were observed to be highly sensitive to Waste 2 as indicated by their attempts to escape from the containers once placed in contact with this material. This behaviour suggested that toxicity was due to

Table 8. Earthworm (*Eisenia foetida*) survival in Wastes 1 and 2 after 7 and 14 days. Data are means (n = 4[1]) ± SD. Response in artificial soil included as a comparison to response in the reference agricultural soil (0 waste concentration).

WASTE or SOIL	TEST TIME (DAYS)	EARTHWORM SURVIVAL (%)					
		WASTE CONCENTRATION (%)					
		0	5	25	50	75	100
Waste 1	7	100	100	97.5±5.0	100	100	100
	14	100	100	100	100	100	100
Waste 2	7	100	100	5.0±5.8	0	0	0
	14	100	95.0±5.8	5.0±5.8	0	0	0
Artificial	7	100					
	14	100					

[1] 10 worms per replicate

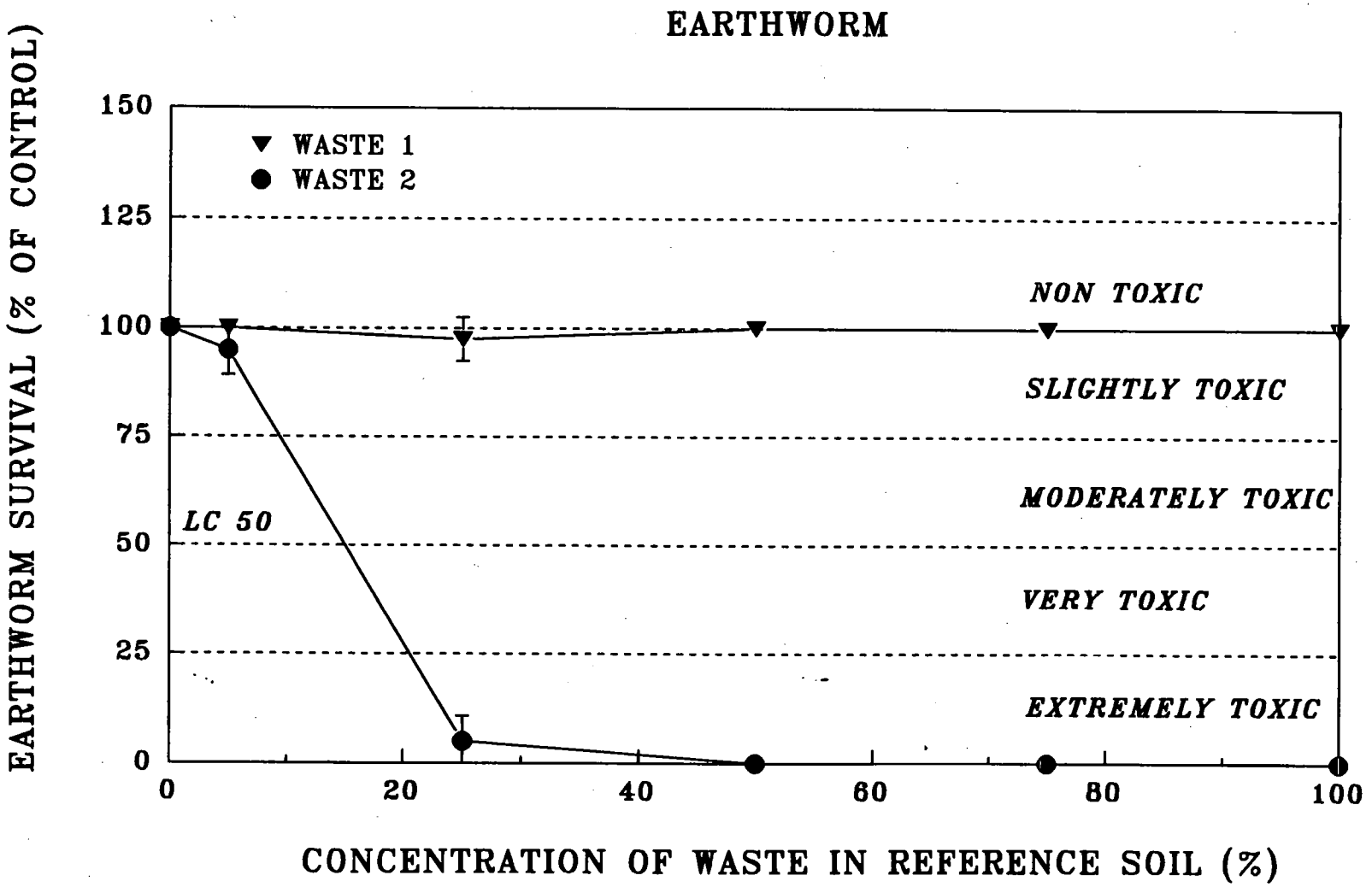

Figure 8. Earthworm survival following 14 days exposure to reference soil and various concentrations of Wastes 1 and 2. Data are means (n = 4) ± SD.

volatiles or body contact with undesirable components of the waste. Most worms in the greater than 5% concentrations of Waste 2 died within the first 24 hours after exposure and dead worms were almost always found on the surface of the waste rather than buried in the material. Coefficients of variation were high (120%) in the 25% concentration of Waste 2 primarily because only 1 worm in each of 2 of the 4 replicates had the stamina to tolerate the toxic conditions in this Waste over a 14 day period.

Earthworm survival was one of the most sensitive assays tested in this trial (Table 10) with precision and reproducibility rated as excellent.

Microtox

The Microtox test was conducted on the aqueous phase (1:4 solids:water extract) rather than the solid phase as was the case for the other single species bioassays in this trial. Nevertheless, the response by *Phosphobacterium* to extracts of Wastes 1 and 2 corresponded closely with the responses of the vascular plants and earthworms in waste/soil, i.e., light production by the bacterium was not inhibited by the Waste 1 extract - in fact it was stimulated and therefore rated as not toxic - while the extract from Waste 2 significantly reduced light production by the bacterium and was rated as extremely toxic (Table 9). The Microtox test has not been recommended for soil quality assessments (Keddy et al. 1992) and has been open to criticism for soil or soil leachate toxicity testing because it utilizes a marine bacterium rather than a soil or freshwater bacterium. However, in the present study, it strongly supported the solid phase results and was the most sensitive assay in the test battery having an IC50 of 12% (Table 10). Under the conditions of this study, the Microtox assay was rapid, sensitive and exhibited low variability (coefficients of variation for IC50's of Waste 2 $<10\%$; Appendix Table 2C), suggesting that this test may be more desirable for toxicity testing of soil leachates than the algal growth inhibition assay (see section 3, this report) recommended by Keddy et al. 1992. Eisman et al. (1991) also found that the Microtox system was a reliable method for assessing petroleum hydrocarbon toxicity.

Summary of single species bioassays

Based on LC50 and IC50 estimates for each single species bioassay, Wastes 1 and 2 were given a toxicity rating (Table 10). Using this approach Waste 2 with 2.5% TPH was rated as very to extremely toxic while Waste 1 with 2.2% TPH was not toxic. According to Alberta Tier I criteria for contaminated soil assessment and remediation, the acceptable level of TPH (mineral oil and grease) in hydrocarbon contaminated soils is 0.1%. Although the TPH

Table 9. Inhibitory concentrations (IC) which caused a 20 or 50% reduction in light production by *Photobacterium phosphoreum* (Microtox). Data are means (n = 3) ± SD.

		CONCENTRATION (%)		
	SOIL or WASTE	MEASUREMENT TIME		TOXICITY[1]
		5 min	15 min	
IC20	Reference	>100	>100	None
	Artificial	18.2 ± 8	19.3 ± 2.0	Toxic(?)
	Waste 1	>100	>100	None
	Waste 2	3.7 ± 0.7	3.4 ± 0.6	Toxic
IC50	Reference	>100	>100	None
	Artificial	141 ± 26	139 ± 36	None
	Waste 1	>100	>100	None
	Waste 2	14 ± 1.2	12 ± 0.9	Extreme

[1] IC20 toxicity ratings based on ERCB ratings (0-30% = toxic; 30-50% = slightly toxic; >50 = nontoxic).

IC50 toxicity also based on ERCB ratings (<25% = extremely toxic; 25-50% = very toxic; 51-75% = moderately toxic; 76-100% = slightly toxic; >100% = non toxic.

ERCB Env. Res. Centre Bioreactor ?

Table 10: Toxicity levels in Wastes 1 and 2 based on LC_{50} or IC_{50} values obtained from acute organism assays.

ACUTE TEST	TOXICITY LEVEL (LC50 or IC50)[1]	
	WASTE 1	WASTE 2
Lettuce emergence	Not toxic	Very toxic (34%)
Lettuce root elongation	Not toxic	Moderately toxic (55%)
Barley emergence	Not toxic	Very toxic (42%)
Barley root elongation	Not toxic	Very toxic (32%)
Canola emergence	Not toxic	Extremely toxic (23%)
Canola root elongation	Not toxic	Very toxic (28%)
Microtox	Not toxic	Extremely toxic (12%)
Earthworm survival	Not toxic	Extremely toxic (13%)
Algal growth inhibition	Not toxic	Not toxic

[1] Toxicity rating:

Extremely toxic:	< 25%
Very toxic:	25-50%
Moderately toxic:	51-75%
Slightly - non toxic:	76-100%

concentration of Waste 1 was well above the Alberta Tier I criteria, all the acute bioassays indicated it was not toxic. In addition, the Microtox assay on the 1:4 extract of this Waste indicated potential leachates from this material would also test nontoxic.

Canola emergence and root elongation in undiluted Waste 1 were somewhat inhibited suggesting slight toxicity; however, the toxicity was not great enough to allow estimation of an LC50 or IC50. Toxicity ratings based on the algal growth inhibition assay are included in Table 10 for comparison with the other single species assays and the nontoxic response observed in this assay is discussed in more detail in section 5 of this report. Of the single species assays tested, the Microtox and earthworm survival tests were the most sensitive while lettuce root elongation and barley emergence were the least sensitive (Table 10).

The factors causing the toxicity of Waste 2 could not be identified. However, PAH compounds including naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene and anthracene, have been implicated in causing toxic effects on *Photobacterium phosphoreum* (Symons and Sims 1988). These same compounds, in addition to the aliphatic/aromatic fraction which was much greater in Waste 2 than Waste 1, may explain the high toxicity of Waste 2. Toxicity identification and evaluation (T.I.E.) studies are necessary to understand the linkage between the chemistry and biological responses of these wastes.

4.3.2 Soil process-based assays

Microbial respiration, microbial biomass, metabolic quotients and C_{mic}:C_{org} ratios

The reference soil used in the present study was an agricultural chernozem low in labile C and relatively high in stable, recalcitrant C. A comparison of respiration rates at the time of toxicity testing (following 16 months bioremediation for Waste 1, and 4 months for Waste 2), revealed that respiratory activity was 4.2x higher in Waste 2 than in the reference soil (Fig. 9A; Table 11). Respiration of Waste 1 was similar to that measured in the reference soil. These data indicate that, at the time of toxicity testing, the bioavailability of C in Waste 2 was much greater than that in Waste 2 although TPH contents were similar in the two wastes. The higher bioavailability of C in Waste 2 may have been related to higher concentrations of aliphatics and aromatics in this waste at the time of the toxicity trials.

The pattern observed for microbial respiration was reflected in the microbial biomass which was also much greater (3.3x) in Waste 2 relative to the reference soil (Fig. 9B; Table 11). Microbial biomass in Waste 1 resembled that in the reference soil. This indicates that C in Waste 1 was more highly

Table 11. Carbon mineralization indices for Wastes 1 and 2 relative to reference agriculatural soil.

ACUTE TEST	STATUS RELATIVE TO REFERENCE SOIL	
	WASTE 1	WASTE 2
Soil Respiration	1.3x greater	4.2x greater
Microbial Biomass C	1.3x greater	3.3x greater
Microbial Metabolic Quotient[1]	same	1.3x greater
C mic:C org Ratio[2]	0.24x lower	2.2x greater

[1] Microbial metabolic quotient = respiration per unit amount of microbial biomass C.

[2] C mic: C org ratio = microbial biomass C:soil organic C

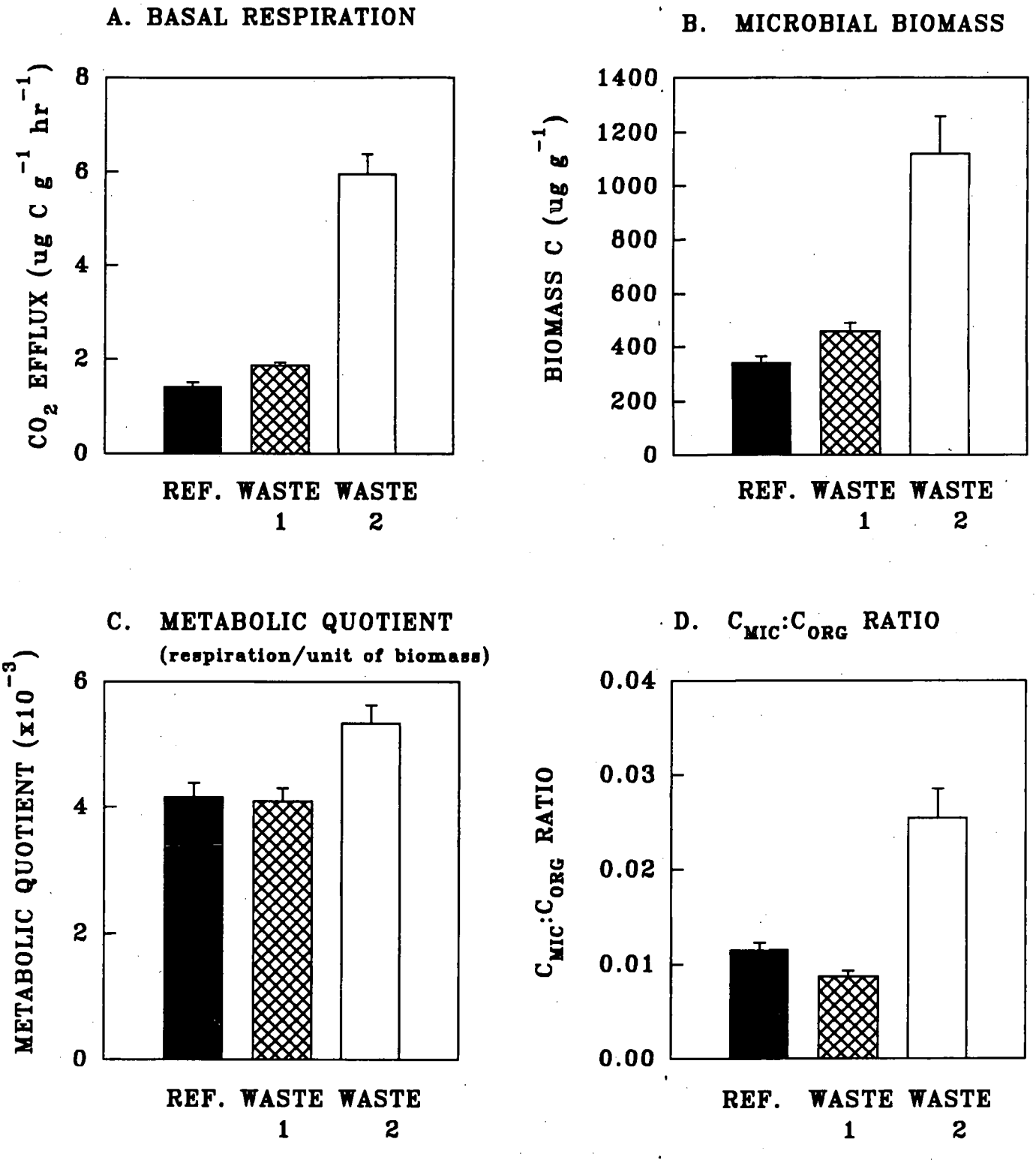

Figure 9. Carbon mineralization indices for reference soil and Wastes 1 and 2 following 16 and 4 months bioremediation, respectively. A. Basal respiration. B. Microbial biomass C. C. Metabolic quotient (respiration per unit of microbial biomass. D. Ratio of microbial biomass C:waste C. Data are means (n = 5) ± SD.

weathered and more recalcitrant (less available; more complex) in Waste 1 than in Waste 2 and, thus, less able to sustain a large microbial biomass as is the case during the initial phase of hydrocarbon decay when there is more C available for microbial tissue production. From these data, it is clear that, at the time of toxicity testing, Waste 2 was not as metabolically stable as Waste 1 and the reference soil, i.e., the C in Waste 1 was more highly degraded than that in Waste 2.

Based on a wide range of studies (see Anderson 1994), it appears that increased stabilization of soil organic matter may be related to an increase in the efficiency of C utilization by microorganisms as more C is put into tissue production (biomass) and less C is respired as CO_2. This change in the energetics of microbial communities is reflected in the metabolic quotient which is a measure of the respiratory rate per unit of microbial biomass (μg CO_2-C μg biomass C^{-1} h^{-1} in the present study). Thus the metabolic quotient integrates respiratory and biomass measurements and may be more useful for monitoring changes in microbial communities as environmental conditions (e.g., pH, moisture, temperature, C availability etc.) change (Anderson 1994). Recently, Hund and Schenk (1994) reported a very close correlation between microbial metabolic quotients and the disappearance of PAH in a contaminated soil (metabolic quotients decreased as the PAH was degraded).

In the present study, the metabolic quotient of Waste 2 was 1.3x higher than those in Waste 1 and the reference soil which were similar (Fig. 9C; Table 11). This suggests that the organisms in Waste 2 were releasing more hydrocarbon-C as CO_2 per unit of microbial tissue than were organisms in Waste 1 or the reference soil. Alterations in metabolic quotients as hydrocarbons are being degraded may be a result of changes in the composition of the microbial community or changes in the physiological status of the organisms. These changes are probably controlled to a large degree by a reduction in the amount and quality of hydrocarbon-C available for microbial growth, respiration and maintenance with increasing degradation of the various hydrocarbon fractions.

The ratio of microbial biomass C to organic matter C, which has been recommended as a useful indicator for following organic matter dynamics under different agricultural management practices, (Sparling 1992) may also be a valuable index for monitoring the status of hydrocarbon disappearance during the bioremediation process. As the bioavailability of hydrocarbon-C decreases, the supply of C for sustaining the microbial biomass decreases. This process continues until all the easily available fractions of hydrocarbon have been degraded and the C in the waste, as measured by the TPH, has stabilized. Once the availability or

supply of C has stabilized, microbial biomass levels will also stabilize indicating an equilibrium between C supply and demand. Thus, during the initial phases of hydrocarbon degradation, it would be expected that C_{mic}:C_{org} would be high as more C is available for biomass production; however, as bioavailability of the hydrocarbon fractions decreases, the supply of C for biomass growth and maintenance also decreases and C_{mic}:C_{org} would be expected to fall. No change in the C_{mic}:C_{org} ratio with time would indicate that the C bioavailability and demand by the microorganisms is in equilibrium - a state typical of the more advanced stages of organic matter decomposition.

At the time of the toxicity trials, Waste 2 exhibited an elevated C_{mic}:C_{org} ratio relative to Waste 1 and the reference soil (Fig. 9D; Table 11). If it is assumed that carbon in the reference soil is of a stable and recalcitrant nature and C supply and demand to the decomposers is in equilibrium, i.e., the C_{mic}:C_{org} ratio is stable, then Waste 1 hydrocarbons had entered a stable phase similar to that in the reference soil at the time of testing, while Waste 2 hydrocarbons were still in an active state of decay and not yet in a stable phase at the time of the toxicity trials. Further monitoring of Waste 2 will determine at what point the C_{mic}:C_{org} ratio stabilizes and if this corresponds to the loss of particular fractions of the hydrocarbon.

Summary of respiration/biomass results

Soil respiration, microbial biomass C, the microbial metabolic quotient and the C_{mic}:C_{org} ratio were all significantly higher in Waste 2 than in Waste 1 and the reference soil. All of these indices indicate that at the time of the toxicity trials the hydrocarbon decay process in Waste 2 had not yet stabilized while that in Waste 1 had, relative to the reference soil. Since Waste 2 also exhibited acute toxicity in the single organism bioassay while Waste 1 did not, it is possible the stability of the hydrocarbon-C as indicated by the soil respiration, microbial biomass, metabolic quotient and C_{mic}:C_{org} measurements may be tied to the acute toxicity of hydrocarbon-contaminated, nonhazardous wastes such as those being treated in the Bio-reactor.

Decomposition of alfalfa stems

The decomposition of alfalfa stems was significantly inhibited in Waste 2 even though this waste contained more microbial biomass than the reference, artificial and Waste 1 soils (Fig. 10a). Thus, in hydrocarbon-contaminated soils a high microbial biomass does not necessarily indicate a high decomposition potential for all substrates. In the case of Waste 2, the presence of the hydrocarbon residues and the conditions in the Bio-Reactor have led to selection of a microbial biomass which is more efficient at degrading hydrocarbons than at degrading plant residues.

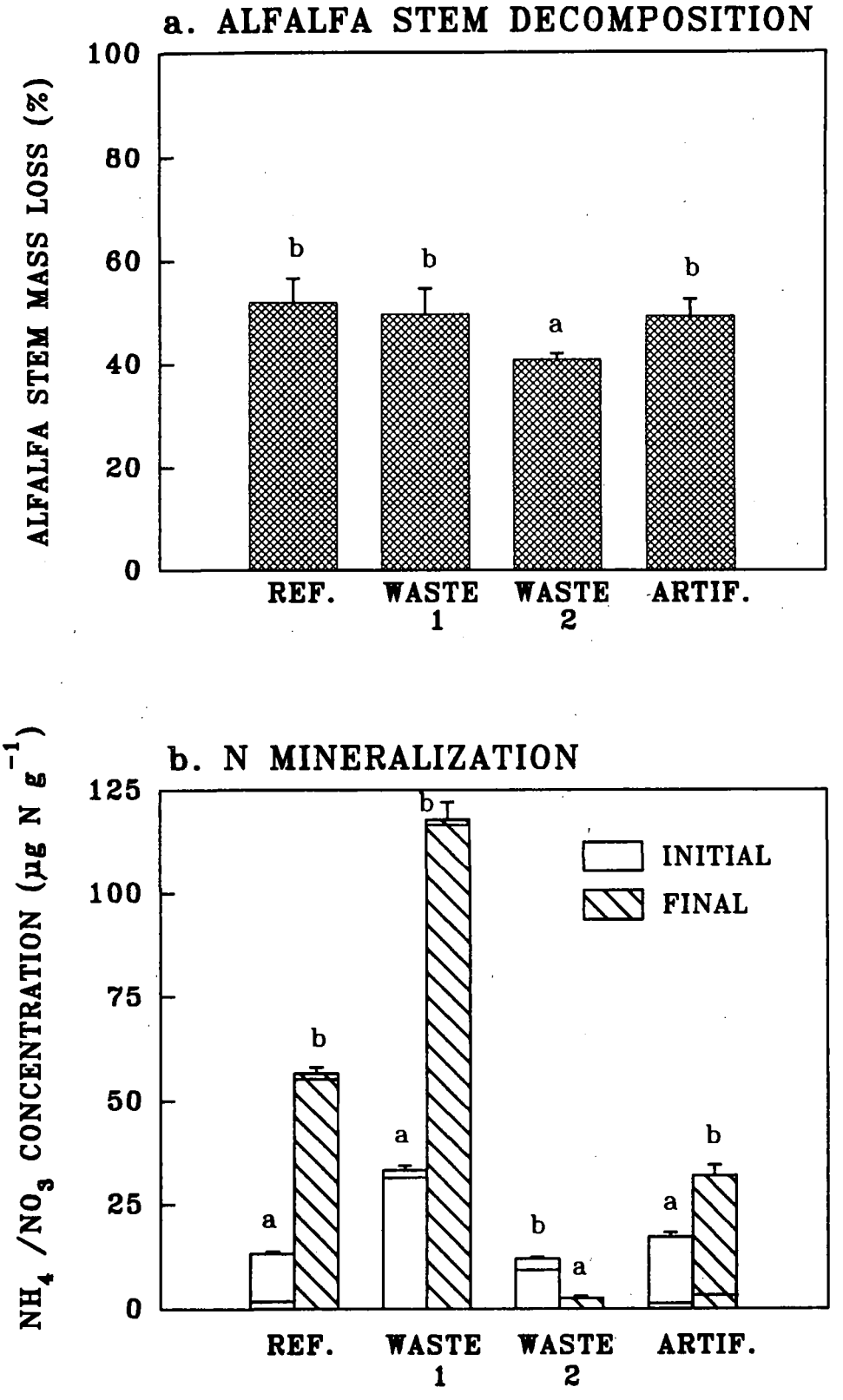

Figure 10. A. Mass loss of alfalfa stems and net N mineralization in reference and artificial soils and Wastes 1 and 2. NH_4-N production above line; NO_3-N production below line. Data are means (n = 5) ± SD. Different letters above bars denote significant difference ($p < 0.05$).

The decomposition potential in Waste 1 was almost identical to that in the uncontaminated reference soil indicating no inhibition of the decay process in this waste.

N mineralization and nitrification

The mineralization and nitrification of organic N from soil/waste N sources and the introduced alfalfa was relatively high in the reference soil and Waste 1 over the 8 week incubation period (Fig. 10b; Table 12). Ammonium-N production was low in these two materials presumably due to rapid nitrification of any NH_4-N released during the decay of the alfalfa. These data suggest that neither mineralization nor nitrification were inhibited in Waste 1 relative to the reference soil.

In contrast, both NH_4-N and NO_3-N in Waste 2 exhibited a net decrease over the 8 week incubation period suggesting that both mineralization and nitrification were either non-existent or inhibited in this material. Also, microbial uptake of mineralized and nitrified N should not be discounted in this waste since this may explain the reduction in extractable N during the incubation period.

The artificial soil exhibited substantial NH_4-N production during the 8 week incubation, but NO_3-N production was limited. Very low rates of nitrification are typical of mature forest soils, and the peat in the artificial soil may be behave similarly to a mature, humified forest soil.

Summary of soil process assays

A summary of the inhibitory status of Wastes 1 and 2 relative to measurements in the reference soil is presented in Table 13 and shows that after 16 months of bioremediation, Waste 1 was not inhibitory to C and N mineralization processes. However, Waste 2 appeared to restrict alfalfa stem decomposition and N mineralization and nitrification even though it was clear the microbial biomass was active and vibrant in this material. The soil process observations are in agreement with the acute toxicity tests in that Waste 1, which was not inhibitory to soil processes, tested not toxic in the single organism bioassays while Waste 2, which restricted some soil processes, tested acutely toxic in the single organism bioassays.

4.3.3 Plant life-cycle bioassay

During the three month greenhouse study, no significant differences in shoot and root production were detected between barley grown in Waste 1 and that grown in the reference soil (Table 14). However, flowering was significantly delayed in Waste 1 while seed production was reduced by 78% relative to measurements made in the reference soil. It is unclear whether the significantly lower reproductive capacity observed in Waste 1 was due to residual toxicity of the hydrocarbon or due to physical alteration of the

Table 12. N mineralization and nitrification potential in reference, Waste 1, Waste 2 and artificial soil amended with ground alfalfa leaves and incubated at 23°C for 8 weeks. Data are means ± SD.

Treatment	NH_4-N ($\mu g\ g^{-1}$)		NO_3-N ($\mu g\ g^{-1}$)		$NH_4 + NO_3$-N ($\mu g\ g^{-1}$)		N flux[1]
	Initial[2]	Final	Initial	Final	Initial	Final	($\mu g\ g^{-1}$)
Reference	11.9 ± 0.5	1.8 ± 0.4	1.3 ± 0.1	54.8 ± 1.2	13.2 ± 0.5	56.5 ± 1.6	43.3 ± 1.7
Waste 1	3.3 ± 0.3	1.7 ± 0.2	29.8 ± 0.9	116.0 ± 4.5	33.2 ± 1.1	117.7 ± 4.3	84.5 ± 5.0
Waste 2	3.1 ± 0.1	2.4 ± 0.5	8.9 ± 0.3	0	12.1 ± 0.3	2.4 ± 0.5	-9.6 ± 0.4
Artificial	15.7 ± 1.0	27.6 ± 3.6	1.4 ± 0.1	4.3 ± 4.4	17.1 ± 1.1	31.9 ± 2.5	14.8 ± 1.7

[1] Flux in NH_4-N and NO_3-N combined over 8 week incubation period.

[2] Initial = N concentration immediately prior to incubation; Final = N concentration after 8 weeks incubation.

Table 13. Inhibition of Wastes 1 and 2 based on soil process assays. Inhibitory status relative to measurements in reference agricultural soil.

SOIL PROCESS	INHIBITORY STATUS	
	WASTE 1	WASTE 2
Carbon mineralization		
Microbial respiration	Not inhibitory	Not inhibitory
Microbial biomass C	Not inhibitory	Not inhibitory
Microbial metabolic quotient	Not inhibitory	Not inhibitory
Alfalfa stem decomposition	Not inhibitory	Inhibitory
Nitrogen mineralization		
Ammonification	Not inhibitory	Inhibitory
Nitrification	Not inhibitory	Inhibitory

Table 14. Time to flowering and foliage, root and seed production by barley in a plant life cycle bioassay of Waste 1 versus a reference agricultural soil. Data are means (n = 10) ± SD[1].

Measurement	Growth Medium		Reduction (%)
	Reference	Waste 1	
Days to flowering	56.2 ± 0.6a	62.0 ± 5.4b	
Plant Production (g pot^{-1})			
Foliage	9.4 ± 2.4a	9.4 ± 3.9a	0
Roots	2.5 ± 0.9a	2.2 ± 1.5a	0
Seed	6.8 ± 2.6b	1.5 ± 2.1a	78
TOTAL	18.8 ± 5.3b	13.1 ± 6.0a	30

[1] Differences between reference soil and Waste 1 for each measurement were determined by a two-sample T-test. Means in each row followed by a different letter are significantly different ($p < 0.05$).

soil caused by the oily characteristics of the contaminant. It is believed that physical properties rather than chemical properties are responsible for the reduced reproductive capacity in Waste 1. This waste exhibited severe water repellancy at the time of testing (MED = 4.5) which may have interferred with water and fertilizer availability, particularly during warm days when the surface soil was prone to dessication. These data indicate that soil physical properties should be taken into account in plant life-cycle bioassays since response to these properties may be confused with a toxic response. This study underscores the importance of assessing reproductive capacity because it may be more sensitive to physical/chemical problems in the soil/waste than plant productivity alone.

5. COMPARISON OF MICROTOX AND *SELENASTRUM* GROWTH INHIBITION ASSAYS FOR LEACHATE TOXICITY TESTING

5.1 Introduction

In a survey of whole organism bioassays for soil quality assessments, Keddy et al. (1992) recommended the algal (*Selenastrumcapricornutum*)growth inhibition assay as a sensitive test for assessing the toxicity of soil elutriates or leachates. Since leachates from landfilled or landspread hydrocarbon-contaminated wastes have the potential of contaminating ground water, it was decided to compare the Microtox and algal growth inhibition tests for determining toxicity of water extracts from Wastes 1 and 2 and the reference soil.

5.2 Methods

Procedures for obtaining water extracts of the wastes and reference soil (1 part solids:4 parts deionized water) and conducting the Microtox assays have been described previously (section 4.1.1). The same extracts were used for both the Microtox and algal growth inhibition assays. Three replicate extracts were prepared from each of the two wastes and the reference soil. The extracts were not pre-filtered since preliminary measurements revealed that filtration reduced the toxicity of the extract. A culture of the freshwater alga, *Selenastrum capricornutum*, was kindly provided by S. Goudey of Hydroqual Laboratories Ltd.

The algal growth inhibition test was conducted using the procedures outlined in Report EPS 1/RM/25 (Environment Canada 1992). Because facilities were not available for electronic cell enumeration, algal cells were counted manually using a Zeiss photomicroscope and a haemocytometer slide. Using this approach, difficulties were encountered in obtaining an adequate density of algal cells which would allow accurate enumeration of the initial density of cells in the stock algal culture. In order to boost the initial cell density, it was necessary to double the nutrient concentration of the growth medium for the stock algal culture and increase the amount of "starter" algal culture used from 1 to 3 mls. After 4 days growth, this culture adjustment resulted in a concentration of 1.36×10^7 cells ml^{-1} which was sufficient to obtain a relatively accurate initial cell count. Using this density of cells, algal growth over a 72 hour period was determined in 3.13, 6.25, 12.5, 25, 50 and 100% concentrations of each of the extracts from Waste 1, Waste 2 and the reference soil. Algal growth in the waste extracts were plotted as a % of the growth in the reference soil extract.

5.3 Results and Discussion

In a review of single species bioassays, Keddy et al. (1992) rated the *Selenastrum* growth inhibition test as one of the most sensitive for assessing soil quality and recommended it for both screening and definitive toxicity testing of soil leachates and elutriates. In many cases, the *Selenastrum* assay was found to be more sensitive than the Microtox test (Keddy et al. 1992). This was not the case in the present study where the water extract from Waste 2 proved to be highly toxic using the Microtox test, but only slightly to not toxic using the *Selenastrum* growth inhibition assay (Table 15; Fig. 11). Very little growth inhibition was detected at concentrations of Waste 2 extract below 25%; above 25% growth was inhibited by approximately 25% resulting in a slightly toxic reading (Fig. 11). Waste 1 extracts did not inhibit *Selenastrum* growth; to the contrary, growth in the Waste 1 extracts was stimulated relative to that measured in extracts of the reference soil (Fig. 11). Of all the single species bioassays tested in the present study, the *Selenastrum* growth inhibition test appeared to be the least sensitive (Table 10); it was the only test which gave a slightly toxic to non toxic reading .

Possible explanations for the insensitivity of the algal growth inhibition test are:

(1) dilution of toxic components in the wastes during the extraction procedure may be so great that the extracts are rendered nontoxic to algal growth. However, the Microtox produced a highly toxic reading for Waste 2 on the same extract as that used in the algal assay suggesting that, in this case, *Photobacterium* was more sensitive than *Selenastrum*. Lower dilutions of extracts, perhaps 1:2 solids:water rather than 1:4 as was used in this study, should be tested to determine if this is the case.

(2) during the process of adjusting the initial algal cell density for microscopic enumeration, it became evident that the stock culture inoculum is grown under starvation conditions (low nutrient levels) to prevent the cells from clumping. Both Wastes 1 and 2 had high concentrations of nitrate and phosphate (see Table 4) which were probably released into the water during the extraction procedure. Higher inorganic nutrient availability in the test extracts, particularly at the lower test concentrations, may stimulate algal growth once starving cells are introduced into the extract and this was very evident for Waste 1 extracts. Enhanced growth as a result of improved nutrient conditions may counteract or mask the response of the algae to toxic elements in the

Table 15. Comparison of algal (*Selenastrum capricornutum*) and Microtox bioassays using Waste 2. Both assays were performed on the same extract (1 part soil/4 parts water) with no filtration.

TEST	TEST DURATION	IC20	TOXI-CITY	IC50	TOXI-CITY
Microtox	15 min	3.7 ±0.7	Toxic	12.2 ±0.9	Toxic
Algal	72 hours	NM	Not toxic	NM	Not toxic

NM = not measureable

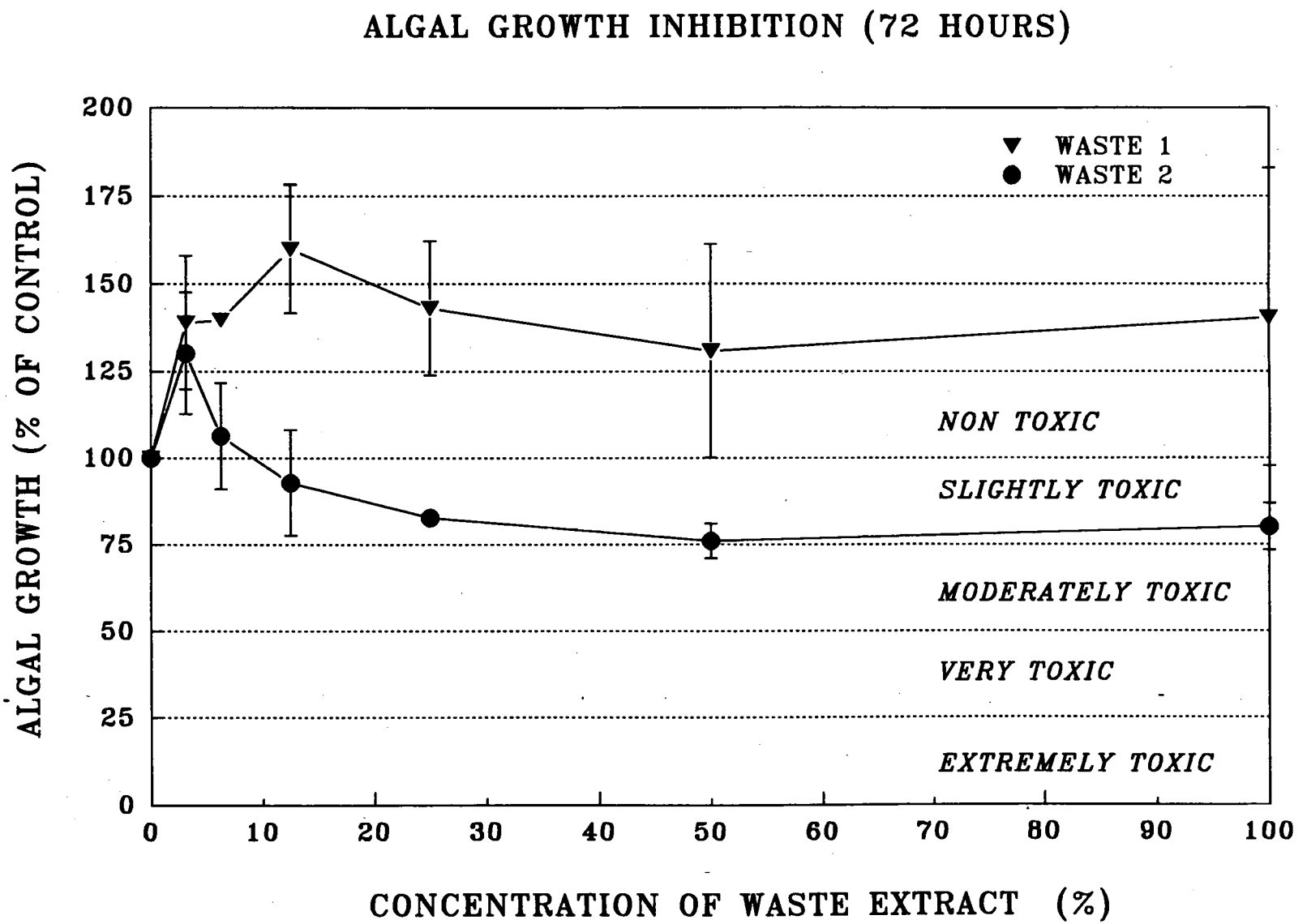

Figure 11. Growth of *Selenastrum* in a 1:4 water extract of reference soil and Wastes 1 and 2. Data are means (n = 3) ± SD.

test extracts.

In addition to the difficulties encountered with obtaining an accurate cell count using microscopic enumeration, the algal assay tended to be more variable than the other acute bioassays. Coefficients of variation for algal cell counts ranged from 2.7 to 16.4% in Waste 1 and 1.2 to 30.4% in Waste 2 (Appendix Table 2). For the hydrocarbon contaminated wastes tested in this study, the algal growth inhibition assay proved to be not as sensitive or precise as the Microtox, seedling emergence, root elongation and earthworm survival assays.

6. CONCLUSIONS

1. According to the single species bioassays and chronic soil process assays, Waste 1, an oil contaminated soil, was neither non toxic or inhibitory after 16 months bioremediation while Waste 2, a DIMR, was very to extremely toxic after 4 months bioremediation. The barley life-cycle assay indicated Waste 1 reduced reproductive capacity; however, this may be explained by a reduction in soil wettability caused by oil coatings on the soil particles (aggregates), rather than the presence of toxic compounds. Thus, it appears that the bioremedial treatments applied to Waste 1, i.e., aggregation, fertilization, irrigation, heating and aeration, successfully eliminated the salinity and toxicity associated with this waste.

2. According to Alberta Tier I Criteria for Contaminated Soil Assessment and Remediation, the acceptable level of TPH (mineral oil and grease) in hydrocarbon contaminated soils is 0.1%. Although the TPH concentration of Waste 1 (2.2%) was well above the Tier I guideline, all the acute bioassays and chronic soil process assays indicated this waste was not toxic. Also, the non toxic Microtox reading on a 1:4 extract of this waste indicated potential leachates from this material would also be non toxic. These results suggest that, for hydrocarbon contaminated wastes such as those tested in this study, bioassays should accompany chemical criteria in order to predict ecotoxicological potential more precisely.

3. Waste 1 does not require further bioremediation and is ready to be landfilled, landspread or landfarmed. Although Waste 2 has a TPH (2.5%) similar to that in Waste 1, it requires further bioremediation and toxicity testing.

4. The factors causing the toxicity of Waste 2 could not be identified. However, PAH compounds including naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene and anthracene, have been implicated in causing toxic effects on *Photobacterium phosphoreum* (Symons and Sims 1988). These same compounds, in addition to the aliphatic/aromatic fraction which was much greater in Waste 2 than in Waste 1, may explain the high toxicity of Waste 2.

5. Of the single species bioassays, the Microtox and earthworm survival tests were the most sensitive while the algal growth inhibition test was the least sensitive. Although coefficients of variation were generally less than 20% indicating a high degree of precision and reproducibility for most assays, variability tended to increase with increased toxicity. This may be a result of more erratic

behaviour by the organisms as they deal with the stress and cell damage caused by toxic chemicals.

6. With the exception of the algal growth inhibition assay which was insensitive to the toxicity of Waste 2, both the single species (organism) bioassays and the soil process assays were sensitive to the toxicity of Waste 2 and results from both approaches were in agreement with each other. The plant life cycle assay may be a reliable method for evaluating not only toxicity, but also potential physical problems in the soil which may interfere with productivity. The plant life-cycle bioassay would also be valuable for determining the potential of a bioremediated (non toxic) waste for supporting plant growth if disposal options include landspreading or landfarming.

7. The ecotoxicological protocol tested in this study provided reliable results with regards to evaluating the toxicity of an oil-contaminated soil and a diesel invert mud residue and may be applicable to monitoring detoxification of other hydrocarbon-contaminated wastes undergoing bioremediation.

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8. APPENDIX TABLES

Appendix Table 1. Means, standard deviations and coefficients of variation for lettuce, barley and canola bioassays. Coefficients of variation are presented in brackets (n = 3; 30 seeds/rep, 20 for barley; barley measured after 4 days, canola after 5 days and lettuce after 7 days.

PARAMETER	WASTE	SPECIES	WASTE CONCENTRATION (%)					
			0	5	25	50	75	100
EMERGENCE (%)	1	LETTUCE	90.0 ± 8.8 (9.8)	100 ± 0 (0)	88.9 ± 7.0 (7.9)	87.8 ± 11.7 (13.3)	93.3 ± 6.7 (7.2)	87.8 ± 1.9 (2.2)
		BARLEY	100 ± 0 (0)	100 ± 0 (0)	98.3 ± 2.9 (3.0)	98.3 ± 2.9 (3.0)	100 ± 0 (0)	98.3 ± 2.9 (3.0)
		CANOLA	90 ± 0 (0)	87.8 ± 8.4 (9.6)	90.0 ± 8.8 (9.8)	91.1 ± 7.0 (7.7)	83.3 ± 11.5 (13.8)	65.5 ± 3.9 (6.0)
	2	LETTUCE	90.0 ± 8.8 (9.8)	87.8 ± 3.9 (4.4)	66.7 ± 5.8 (8.7)	12.2 ± 6.9 (56.6)	0	0
		BARLEY	100 ± 0 (0)	100 ± 0 (0)	63.3 ± 2.9 (4.6)	51.7 ± 2.9 (5.6)	3.3 ± 3.0 (90.9)	0
		CANOLA	90 ± 0 (0)	86.7 ± 5.8 (6.7)	41.1 ± 6.9 (16.8)	11.1 ± 10.7 (96.4)	0	0
ROOT LENGTH (cm)	1	LETTUCE	6.2 ± 0.3 (5.0)	7.6 ± 0.2 (2.9)	9.9 ± 0.3 (3.0)	10.2 ± 0.5 (4.9)	11.0 ± 0.1 (0.9)	10.7 ± 0.7 (6.5)
		BARLEY	10.0 ± 0.5 (5.0)	10.7 ± 0.2 (2.0)	10.2 ± 0.2 (2.0)	9.6 ± 0.6 (6.3)	9.2 ± 0.1 (1.1)	9.0 ± 0.1 (1.1)
		CANOLA	6.2 ± 1.0 (16.1)	6.8 ± 0.06 (0.9)	6.9 ± 0.05 (0.7)	7.4 ± 0.7 (9.5)	6.7 ± 0.8 (11.9)	5.6 ± 0.8 (14.3)
	2	LETTUCE	6.2 ± 0.3 (4.8)	7.5 ± 0.7 (9.3)	7.5 ± 0.6 (8.0)	4.0 ± 1.0 (25.0	0	0
		BARLEY	10.0 ± 0.5 (5.0)	9.4 ± 0.8 (8.5)	5.9 ± 0.5 (8.5)	2.9 ± 0.5 (17.2)	1.0 ± 0.1 (10.0)	0.3 ± 0.1 (33.3)
		CANOLA	6.2 ± 1.0 (16.1)	5.8 ± 0.4 (6.9)	3.4 ± 0.4 (11.8)	1.9 ± 0.6 (31.6)	0	0

Appendix Table 1. Means, standard deviations and coefficients of variation for lettuce, barley and canola bioassays. Coefficients of variation are presented in brackets (n = 3; 30 seeds/rep, 20 for barley; barley measured after 4 days, canola after 5 days and lettuce after 7 days).

PARAMETER	WASTE	SPECIES	WASTE CONCENTRATION (%)					
			0	5	25	50	75	100
EMERGENCE (%)	1	LETTUCE	90.0 ± 8.8 (9.8)	100 ± 0 (0)	88.9 ± 7.0 (7.9)	87.8 ± 11.7 (13.3)	93.3 ± 6.7 (7.2)	87.8 ± 1.9 (2.2)
		BARLEY	100 ± 0 (0)	100 ± 0 (0)	98.3 ± 2.9 (3.0)	98.3 ± 2.9 (3.0)	100 ± 0 (0)	98.3 ± 2.9 (3.0)
		CANOLA	90 ± 0 (0)	87.8 ± 8.4 (9.6)	90.0 ± 8.8 (9.8)	91.1 ± 7.0 (7.7)	83.3 ± 11.5 (13.8)	65.5 ± 3.9 (6.0)
	2	LETTUCE	90.0 ± 8.8 (9.8)	87.8 ± 3.9 (4.4)	66.7 ± 5.8 (8.7)	12.2 ± 6.9 (56.6)	0	0
		BARLEY	100 ± 0 (0)	100 ± 0 (0)	63.3 ± 2.9 (4.6)	51.7 ± 2.9 (5.6)	3.3 ± 3.0 (90.9)	0
		CANOLA	90 ± 0 (0)	86.7 ± 5.8 (6.7)	41.1 ± 6.9 (16.8)	11.1 ± 10.7 (96.4)	0	0
ROOT LENGTH (cm)	1	LETTUCE	6.2 ± 0.3 (5.0)	7.6 ± 0.2 (2.9)	9.9 ± 0.3 (3.0)	10.2 ± 0.5 (4.9)	11.0 ± 0.1 (0.9)	10.7 ± 0.7 (6.5)
		BARLEY	10.0 ± 0.5 (5.0)	10.7 ± 0.2 (2.0)	10.2 ± 0.2 (2.0)	9.6 ± 0.6 (6.3)	9.2 ± 0.1 (1.1)	9.0 ± 0.1 (1.1)
		CANOLA	6.2 ± 1.0 (16.1)	6.8 ± 0.06 (0.9)	6.9 ± 0.05 (0.7)	7.4 ± 0.7 (9.5)	6.7 ± 0.8 (11.9)	5.6 ± 0.8 (14.3)
	2	LETTUCE	6.2 ± 0.3 (4.8)	7.5 ± 0.7 (9.3)	7.5 ± 0.6 (8.0)	4.0 ± 1.0 (25.0	0	0
		BARLEY	10.0 ± 0.5 (5.0)	9.4 ± 0.8 (8.5)	5.9 ± 0.5 (8.5)	2.9 ± 0.5 (17.2)	1.0 ± 0.1 (10.0)	0.3 ± 0.1 (33.3)
		CANOLA	6.2 ± 1.0 (16.1)	5.8 ± 0.4 (6.9)	3.4 ± 0.4 (11.8)	1.9 ± 0.6 (31.6)	0	0

Appendix Table 2. Means, standard deviations and coefficients of variation (CV) for earthworm, algal (*Selenastrum* bioassays) and Microtox bioassays. CV's presented in brackets.

A. EARTHWORM SURVIVAL AFTER 14 DAYS (n = 4; 10 worms/replicate)

WASTE	WASTE CONCENTRATION (%)					
	0	5	25	50	75	100
1	100 ± 0 **(0)**	100 ± 0 **(0)**	98 ± 5 **(5.1)**	100 ± 0 **(0)**	100 ± 0 **(0)**	100 ± 0 **(0)**
2	100 ± 0 **(0)**	95 ± 6 **(6.3)**	5 ± 6 **(120)**	0	0	0

B. ALGAL CELL COUNTS AFTER 72 HOURS (n = 5)

WASTE	WASTE CONCENTRATION (%)						
	0	3.13	6.25	12.5	25	50	100
1	17.8 ± 1.9 **(10.7)**	20.9 ± 3.2 **(15.3)**	18.9 ± 2.7 **(14.3)**	16.5 ± 2.7 **(16.4)**	14.7 ± 0.4 **(2.7)**	13.5 ± 0.9 **(6.7)**	14.3 ± 1.2 **(8.4)**
2	17.8 ± 1.9 **(10.7)**	24.7 ± 0.3 **(1.2)**	24.9 ± 0.3 **(1.2)**	28.5 ± 3.3 **(11.6)**	25.5 ± 3.4 **(13.3)**	23.3 ± 5.4 **(23.2)**	25.0 ± 7.6 **(30.4)**

C. MICROTOX - WASTE 2 ONLY, IC20 or 50 after 5 and 15 min (n = 3)

IC20 5 min	IC20 15 min	IC50 5 min	IC50 15 min
3.7 ± 0.7 **(18.9)**	3.4 ± 0.6 **(17.6)**	13.5 ± 1.2 **(8.9)**	12.2 ± 0.9 **(7.4)**

Appendix Table 3. Means (n = 5), standard deviations and coefficients of variations (CV) for soil process assays. CV's presented in brackets.

MEASUREMENT	TEST MATERIAL	MEAN ± SD	CV(%)
Basal respiration ($\mu g\ C\ g^{-1}\ h^{-1}$)	Reference	1.42 ± 0.09	6.3
	Waste 1	1.87 ± 0.06	3.2
	Waste 2	5.95 ± 0.44	7.4
Microbial Biomass C ($\mu g\ g^{-1}$)	Reference	342 ± 23	6.7
	Waste 1	458 ± 32	7.0
	Waste 2	1120 ± 138	12.3
Metabolic Quotient (CO_2-C/unit biomass C x 10^3)	Reference	4.16 ± 0.22	5.3
	Waste 1	4.09 ± 0.21	5.1
	Waste 2	5.34 ± 0.29	5.4
C_{mic}/C_{org} Ratio	Reference	0.0115 ± 0.0008	7.0
	Waste 1	0.0087 ± 0.0006	6.9
	Waste 2	0.0254 ± 0.0031	12.2
Nitrogen Mineralization	Reference	43.3 ± 1.7	3.9
($\mu g\ NH_4$-N + NO_3-N g^{-1} soil)	Waste 1	84.5 ± 5.0	5.9
	Waste 2	-9.6 ± 0.4	4.2

Appendix Table 4. Coefficients of variation (CV) for barley life cycle measurements (n = 10).

MEASUREMENTS	COEFFICIENT OF VARIATION (%)	
	REFERENCE	WASTE 1
Days to Flowering	1.1	8.7
Foliage mass	25.5	41.5
Root Mass	36.0	68.2
Seed Mass	38.2	140.0
Total Plant Mass	28.2	45.8