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Derivation of Matrix Soil Standards for Salt under the British Columbia Contaminated Sites Regulation

Addendum C:

Soil Invertebrate Toxicity Tests: Lessons and Recommendations

February 19, 2002

**Report to the British Columbia
Ministry of Water, Land and Air Protection,
Ministry of Transportation and Highways,
British Columbia Buildings Corporation, and the
Canadian Association of Petroleum Producers**

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1.0 Introduction

The scientific literature contains very little information on the toxicity of sodium chloride (NaCl) to soil invertebrates (Environment Canada, 2000). Consequently much of the soil invertebrate data used in the “*Derivation of Matrix Soil Standards for Salt under the British Columbia Contaminated Sites Regulation*” was generated in-house, as part of the study to produce the draft matrix standards.

The objectives of this part of the study were as follows:

1. Determine the toxicity of sodium chloride to selected soil invertebrates.
2. Investigate the feasibility of using different species of soil invertebrates in toxicity tests, with emphasis on species that occur in Canada.
3. Compare results obtained using the standardised OECD soil with those obtained in natural soils more representative of soil conditions in BC.

For the most part, these results are presented and discussed in section 6.2.2.1 of the main report.

This addendum report explains the rationale behind the choice of test organisms, gives details of the experimental methodology used, and provides additional information on relative sensitivity of different toxicity endpoints. It is intended not only to provide specific information on the toxicity of NaCl to soil invertebrates, but also to provide more general information that may be of use in other soil invertebrate toxicity studies.

2.0 Test species investigated

Information on the distribution and ecology was drawn mainly from Christiansen and Bellinger (1998), Skidmore (1995), Reynolds (1977), Løkke and van Gestel (1998), Lee *et al.* (1998), Hopkin (1997), and from unpublished data.

2.1 Insecta Collembola

2.1.1. *Folsomia candida* Willem

Advantages: This species is one of the most commonly used standard OECD test organisms. It is easy to culture, and since it is parthenogenic, the problem associated with sex ratios in test containers does not arise. It feeds and reproduces readily on a diet of yeast and has a wide tolerance to pH. Thus adding a relatively small amount of yeast to test soils will permit this species to be used in chronic tests in a wide range of different soils, regardless of the natural nutrient status and organic matter content of the soil. It has a faster rate of development than *O. folsomi* or *P. armata* (egg to adult ~ 4-6 weeks)

Disadvantages: It is not widely distributed in nature – not even in Europe – as it tends to be limited to organic substrates (e.g. compost, manure, potting soil). It is also more difficult to handle than species lacking a springing organ. Its parthenogenic mode of reproduction has raised concerns about the rapid development of laboratory-adapted

clones, and questions about whether results obtained using a parthenogenic species adequately represent soil fauna, most of which do not share this mode of reproduction (Hopkin 1997, Chenon *et al.* 2000).

Distribution in Canada: Many records are suspect since it has been confused with other species (especially *F. nivalis* and *F. fimetaria*). However it is known to occur in BC, Ontario and Québec. The culture used in the present study was established using specimens sent to me by Dr. Paul Widden (Loyola University), who collected the organism in litter in a balsam-fir forest in Québec.

2.1.2. *Onychiurus folsomi* Schäffer (Collembola)

Advantages: Preliminary work on the development of a test protocol using this species has already been carried out by Gladys Stevenson (Aquaterra Environmental, Orton, Ontario). The organism is easy to culture, and lacking a furcula, is easy to handle.

Disadvantages: Both sexes are necessary for reproduction, thus raising questions about the influence of the initial sex ratio on reproductive success (see section 5.3). Only ~ half as many juveniles are produced per original test organism than for *F.candida*. It also has a longer development time (egg to adult ~ 2+ months) than *F.candida*.

Distribution in Canada: The culture used in this study was obtained from Gladys Stevenson. My understanding is that it originated in Europe. Although this species is known from several locations in the US, to my knowledge it has not been collected from the field in Canada.

2.1.3. *Protaphorura armata* (Tullberg)

Advantages: This species is widely used in toxicity tests in Europe (especially Sweden) and occurs across Canada. It is a little larger than *O. folsomi*, but like the previous species lacks a springing organ.

Disadvantages: As for *O. folsomi*. In addition, *P. armata* may (or may not) be a species complex (Hopkin 1997).

Distribution in Canada: The culture used in these experiments was developed from individuals collected from leaf litter in a Coastal Douglas-fir forest in BC. It has also been reported from the NWT, Alberta, Québec, Ontario and Nova Scotia.

2.1.4. *Proisotoma minuta* (Tullberg)

Advantages: The chief advantage of using this species lies in its rapid generation time. A 14 - day adult toxicity test unexpectedly produced juveniles in addition to the recovered adults. In addition, smaller amounts of contaminated substrate (10 g/replicate, compared with 30 g/replicate for the previous three species) can be used.

Disadvantages: This species has not been widely used for toxicity testing, consequently no test protocols currently exist. It is extremely small and active, and has springing organ, making it more difficult to handle than species lacking such an organ. Although it appears to survive in culture (Plaster of Paris and charcoal) for several generations on a diet of yeast, my culture has since died out. It appears to survive indefinitely in the medium used to culture *E. fetida* (as does *O. folsomi*).

Distribution in Canada: This species was isolated from compost in BC. It is known from Manitoba, Alberta, Ontario and New Brunswick.

2.1.5. *Parisotoma notabilis* (Schäffer) (Collembola) (= *Isotoma notabilis*)

Advantages: This is one of the most widely distributed species of Collembola in the world. It has been used as a test organism in ecotoxicological studies in Europe. Although males are known from some populations, parthenogenic reproduction also occurs.

Disadvantages: It is relatively small, and possesses a furcula. Its colour (pale grey) means that it is less visible against a soil background than are the white species (e.g. *F. candida*, *P. armata* and *O. folsomi*).

Distribution in Canada: It occurs all over North America and Europe, in a wide variety of habitats from agricultural systems to forests to the arctic tundra. It has been reported from the Yukon, the NWT, BC, Alberta, Manitoba, Ontario, Québec, and Nova Scotia.

A culture of this species was established from litter collected in a Coastal Douglas-fir forest in the present study, but time constraints meant that the potential for using this species in toxicity tests was not investigated.

2.1.6. *Folsomia nivalis* Packard

Advantages: This species is widely distributed in Canada and is one of the most abundant species in northern areas of Canada, including boreal forests and mixedwoods, and in the aspen parkland areas of Alberta. It has been used successfully in tests to determine the toxicity of pesticides (Addison 1996). Both sexes are present in field populations. This species survives and reproduces well in laboratory cultures at room temperatures.

Disadvantages: No protocol presently exists for this species. In previous studies the cultures were maintained in leaf litter rather than the Plaster of Paris and charcoal substrate employed for most collembolan cultures.

Distribution in Canada: The NWT, BC, Alberta, Manitoba, Ontario, Québec, and Nova Scotia.

A culture was established from soil collected in Alberta, but by the time sufficient numbers were obtained, lack of time prevented any testing.

2.2. Oligochaeta: Lumbricidae

2.2.1. *Eisenia fetida/andrei*

Advantages: This is probably the most widely used soil invertebrate species in toxicity testing. There are recognised international protocols for acute (lethal) and reproductive (draft guideline) endpoints. It breeds readily in culture, and compared with most other soil invertebrates, is relatively large and easy to handle.

Disadvantages: It requires a pH of 6.0-7.6 for reproduction. In chronic studies, several grams of food must be added to the test soil to allow the worms to survive and reproduce,

potentially affecting soil organic matter content of the soil, thus changing the bioavailability of the test compound in the soil. Since 500 g of soil are required for every replicate, large quantities of potentially toxic soil are generated. The taxonomic status of the species is also controversial. According to Walter Diehl of the Mississippi State University (quoted in Moul 2001), most individuals identified as *E. fetida* in North America are actually *E. andrei*. However, these two species cannot be separated by morphological characteristics, and frequently co-occur as mixed populations. Attempts to use this species in toxicity tests using forest soil LFH material were not successful (Addison and Holmes 1995).

Distribution in Canada. This species is limited to compost and manure heaps.

2.2.2. *Lumbricus rubellus* Hoffsmeister

Advantages: This species is widely distributed in Canada, inhabiting the upper layers of soil. It is easily to collect and identify and has a wide pH tolerance (3.5-8.0).

Disadvantages: It reproduces slowly under laboratory conditions (at least in my experience). Since the species is widely distributed in Canada sufficient numbers could be collected for toxicity tests on adults. However, unless it will breed readily under laboratory conditions, it cannot be used in the more sensitive reproductive tests.

Distribution in Canada: BC, Alberta, Ontario, Québec, New Brunswick, Nova Scotia, PEI.

Although a culture of *L. rubellus* was established during the present study, low rates of reproduction prevented its use in toxicity studies.

2.2.3 *Dendrobaena octaedra* (Savigny)

Advantages: This species breeds readily and continuously in culture under laboratory conditions. It is tolerant of low pH (3.0-7.7) and its cocoons are resistant to freezing, making it one of the most widely distributed earthworm species in Canada. It typically inhabits upper horizon of soil (litter or LFH), and is common in deciduous and coniferous forests in addition to more disturbed habitats. As it is smaller than *E. fetida* smaller volumes of test materials are required.

Disadvantages: No test protocols currently exist for this species.

Distribution in Canada: BC, Alberta, Ontario, Québec, New Brunswick, Newfoundland, PEI, Nova Scotia.

Unfortunately this species is not common in Vancouver Island, and given the timelines of the salt project, it was not possible to include this species in the present study. It has been successfully used in safety testing pesticides (Addison 1996, Addison and Homes 1996).

2.2.4. *Aporrectodea* species

Advantages: Given the preoccupation with testing mineral soil, the *Apporectodea* species show great potential as test organisms since several species live in mineral soil under natural conditions (endogeic and anecique species). At least three of the species that are common in Canada have been cultured in earthworm beds for fish bait.

Disadvantages: Unfortunately non-experts (and even some who should know better) have been known to confuse the different species. At least one species (*A. turgida*) is known to aestivate – a potential problem for laboratory based experiments at room temperature. To my knowledge, there is no information on the performance of Canadian representatives of *Apporectodea* spp. in reproductive tests under laboratory conditions.

Distribution in Canada: Widely distributed.

No testing of these species was attempted in this study.

2.3. Crustacea: Isopoda

2.3.1. Armadillium vulgare

Advantages: This species of woodlouse breeds readily in culture and has been proposed as a test species in Europe. Males and females can be readily separated using external morphological characteristics. The chief advantage of using this species is that it is a crustacean, and thus increases the taxonomic scope of toxicity studies.

Disadvantages: An artificial diet is generally not adequate for growth and reproduction. Thus for chronic tests, natural leaf litter is incorporated into the test medium.

Distribution in Canada: Although it is widely distributed in urban areas of Canada, it is not considered to be a native species.

3.0 Test soils

Four different soils were included in this study; the standard OECD soil (OECD 1984) and three field-collected soils (Scotch Creek, Clinton and Saanichton). The OECD soil is an artificial, soil used in standardised tests to determine toxicity of contaminants to soil invertebrates. However, since it is an artificial construct, there are serious reservations concerning the relevance of results obtained in this medium to field situations. Thus the present study also included three soils obtained from different areas in BC. The Clinton and Scotch Creek soils were provided by BC-MOTH as examples of soils that were subject to contamination from road salt storage sites in central BC (an area that was expected to be more susceptible to road salt damage than many other areas in BC). The Saanichton soil was used as an example of a soil that was highly productive, with a much higher CEC, but with the lower natural pH characteristic of many soils in BC. Unfortunately it was beyond the scope of this study to include more than a limited number of soils.

The OECD soil is composed (by weight) of 70 % sand (Lane Mountain silica sand LM#70; Target Products Ltd.), 20% kaolin clay (Lloyd-El Cermanics and Crafts Ltd.) and 10% peat (Marigold Nurseries Ltd.). The peat was rubbed through a 4mm sieve before use. The ingredients were mixed dry, and water was added at the rate of 2 L of

distilled water /10 kg dry ingredients. The pH of the soil was then adjusted to between 6-7 using calcium carbonate (Fisher Scientific).

A brief summary of the physico-chemical properties of the soils is provided (Table 1).

Since measures of salinity were of critical importance for this study, most of the chemical analyses were carried out on saturated paste extracts. The study also included a comparison of saturated paste vs. soil water extract measurements of soil anions and cations. The relationship between Electrical Conductivity (EC), salt ion concentrations and soil toxicity was a particular focus of the study, but details are given elsewhere (Section 5 in the main document)

Experiments in the OECD soil were carried out at 30% wet wt. This approximated the moisture conditions used by Gladys Stevenson in her experiments with *O. folsomi* (Aquaterra 1998), and by David Moul (Pacific Environmental Sciences Centre, Environment Canada, Vancouver, BC) for the determination of mortality of *E. andrei* (Moul 2001) The water holding capacity (WHC) of the OECD soil (determined according to Annex C of ISO 11267) was 115% dry weight. Thus the moisture level used in these experiments corresponded to 37% WHC. For the other three test soils, moisture was adjusted to 60% WHC, corresponding to the upper recommended moisture limit. (ISO 11267). For the Clinton soil, the experiment was repeated using the lower suggested moisture level (40% WHC).

Table 1: Physico-chemical characteristics of the test soils. All concentrations expressed per /kg oven dry wt. soil

Parameter	OECD #3	OECD #4	Saanichton	Scotch Creek	Clinton
% C	2.89	3.75	5.7	0.76	1.51
% Nitrogen	0.06	0.08	0.32	0.04	0.06
% Saturation	84	96	64	22	19
pH	6.1	5.8	4.9	7.6	7.8
EC (dS/m)	0.35	0.32	0.43	0.43	0.99
SAR	0.4	0.3	0.4	0.4	3.6
Cation Exchange Capacity (CEC) (Cmol+/kg)	10.69	15.39	17.28	5.55	8.03
Exchangeable sodium (Cmol+/kg)	0.05	0.08	0.11	0.05	0.36
Sodium (mg/kg)	9.66	8.45	6.85	2.42	22.25
Potassium (mg/kg)	2.81	3.20	9.15	0.61	2.95
Magnesium (mg/kg)	5.63	6.07	5.90	0.83	2.45
Calcium (mg/kg)	36.71	37.82	24.70	14.04	10.96
Chloride (mg/kg)	12.94	14.11	13.44	3.85	2.60
Sulphate (mg/kg)	66.65	68.91	7.70	1.79	1.98
Bicarbonate (mg/kg)	11.17	13.06	11.14	8.21	12.31
Nitrate (mg/kg)	30.91	34.56	85.70	10.71	32.22
Soil Texture	NA	NA	Silt Loam	Sandy Loam	Sandy Loam
% sand			23.2	65.5	57.4
% silt			60	29.1	34.8
% clay			16.8	5.4	7.6

4.0 Methods

4.1 Preparations of test soils

Prior to the start of any experiment, the moisture content of the soil was determined gravimetrically, and the amount of distilled water required to achieve the desired water content was calculated. The test salt (NaCl, KCl (both obtained from VWR, Edmonton) or road salt (provided by MoTH from Ladore Pit #2; imported from Chile)) was then dissolved in the water and added to the soil in a stainless steel bowl. The soils were mixed by hand for 10 minutes and stored in Ziploc® bags in a refrigerator at 4°C. Before being used in experiments, the soils were allowed to warm up to room temperature.

4.2 Chemical Analyses

Chemical analyses on the test soils were carried out by Norwest Labs (Edmonton) and the BC MOF Research Branch (Victoria). Details of the standard methodologies used by Norwest Labs can be obtained from their web site. Soil samples sent to the MoF lab were analysed for exchangeable cations and effective CEC using the methods of Hendershot et al. (1993). Total carbon and nitrogen were measured on finely ground subsamples using a Fisons NA-1500 combustion elemental analyser. Anions were determined using suppressed and non-suppressed ion chromatography on a Waters HPLC/ion chromatograph with an Alltech suppressor. Soluble metallic elements were determined using an ARL 3560 simultaneous ICP spectrometer using both ultra sonic and pneumatic sample nebulization.

4.3 Collembola Experiments

For tests involving *P. minuta*, 10 g of prepared soil were placed in a glass vial (Ø2.8 x 6.4 mm). Ten adult individuals and one ball of Baker's yeast were added to each vial, and the mouth of the vial was closed with parafilm. There were five replicates/ treatment. For all other collembolan tests, 30 g (wet wt.) of prepared substrate was placed in a 125-ml mason jar (Bernadin®), and ten adult test organisms and four balls of yeast were added to each replicate which was then closed with snap lid. There were five replicates/ treatment. The experimental containers were opened twice a week, any uneaten food was removed and replaced with new yeast. Once juveniles were observed in any container, the amount of food offered was increased to five balls, twice a week. Food was never limiting.

4.2 Earthworm reproductive test (*E. fetida/andrei*)

The test was carried out in according to the draft (January 2000) OECD Guideline for assessing the effects of chemicals on the reproductive output (and other sub-lethal end points) of the earthworm species *Eisenia fetida fetida* (Savigny, 1826) or *Eisenia fetida andrei* (André, 1963). However, we used only five individuals/replicate instead of the ten individuals recommended in the guideline. There were three replicates/treatment. Five grams of dry oatmeal pablum was added to each jar at the start of the experiment, and a further two grams of oatmeal was added after three weeks. After four weeks, the surviving adults were removed and weighed. The jars were left for a further 28 days, and

then the juveniles and cocoons were separated from the soil by wet sieving, using the method described by van Gestel *et al.* 1988.

4.3 Armadillium vulgare tests

This species typically inhabits and feeds on decomposing litter. Two types of preliminary experiments were carried out using decomposing bigleaf maple (*Acer macrophyllum*) leaves which were soaked in known concentrations of NaCl or distilled water for 24 hours, allowed to drain for 1 h, and were then fed to the test organisms.

4.3.1 Feeding inhibition tests (adults)

The tests were carried out in large petri dishes (Ø 14 cm). OECD soil (40 g, 30% wet wt) was spread evenly on the bottom of each dish, and 4 g (wet wt.) of leaves were placed on top of the soil. One adult woodlouse was added to each test container, which was then closed with a lid. There were five replicates /treatment. After seven days, the woodlice were removed from the dishes and re-weighed, and the number of fecal pellets produced during the experiment was counted.

4.3.2 Growth experiments (juveniles)

For each replicate, five juvenile woodlice were weighed and then placed in a 125mL Bernadin jars with 3 g (wet wt.) treated leaves. There were 5 replicates/treatment and four treatments (including control). After 14 days, the animals were re-weighed and the increase in weight was expressed as a percentage of the original weight.

4.5 Statistical Analyses

Most of the results were analysed using non-linear regression analysis as described in Stephenson *et al.* 2000, using Systat[®] 6.0. For some of the acute (lethal) tests, linear regression provided the best fit. Analysis of Variance (ANOVA) with or without covariance was also used (Minitab[®] 12).

5.0 Results and Discussion

5.1 Extraction of Collembola from soil

Recovery of adults from the test soils using floatation (as described in Aquaterra, 1998) was excellent (80-100%) for all test species. For *O. folsomi*, floatation was also an effective means of recovering juveniles (95% recovery). However, especially in the case of the OECD soil, the smaller, more fragile juveniles of *F. candida* and *P. minuta* tended to become trapped in the floating organic matter, and were consequently more difficult to see. Recovery of the juveniles for these species using floatation was low (20% for *P.minuta*; 70% for *F. candida*). In addition, considerable time (up to 12 hours), and several episodes of gentle stirring of the soil slurry were required for all the animals to float to the surface. At the same time, the cuticles of individuals left floating in the slurry

tended to become waterlogged, with the result that they floated just below the surface and eventually sank to the bottom. Flootation was also difficult to implement when large numbers of samples were due to be processed on the same day.

In view of the difficulties encountered, an alternative dry method of extraction was used for all species. The containers and sample holders from a high gradient extractor were used on the bench top with two desk lamps (40W) providing the heat and light stimulus for the test animals to exit the sample. The Collembola were collected over a period of two days into distilled water. They were then either counted immediately, or were filtered from the water, stored in 70% alcohol, and then counted at a later date. This method of extraction proved to be as effective for *O. folsomi* as the floatation method, but in addition collected 75% of the juvenile *P. minuta* and 90-100% of the juvenile *F.candida*. All counting was done under a binocular microscope.

This method of extraction was less labour intensive than the floatation method, allowed a large number of samples to be processed at once, and in addition, meant that the dry soil samples could be saved and used for chemical analyses.

5.2 Toxicity of NaCl and Road Salt to Soil Invertebrates

Summaries of the results of experiments to determine the toxicity of sodium chloride (road salt) to soil invertebrates are shown in Table 2 (EC₅₀ endpoints) and Table 3 (LC₂₀ endpoints). Comparisons with other studies and derivation of toxicity thresholds are presented in the main report (section 6.2.2.1) and will not be repeated here.

For the EC₅₀ endpoints there was a good fit between the data and the response curves generated by the nonlinear logistic model. In the OECD soil the most sensitive endpoint for determining salt toxicity was earthworm reproduction, as measured by cocoon production in *E. fetida/andrei*. Of the collembolan species tested, *F. candida* was the most sensitive to NaCl. *Onychiurus folsomi* and *P. armata* showed very similar sensitivities to salt.

The soil type had a profound influence on toxicity. For example, the amount of NaCl necessary to produce a 50% reduction in reproductive success in *F. candida* in the Clinton soil was only 15% of the concentration that produced the same effect in the OECD soil.

The LC₂₀ endpoints were far more variable. Over the short-term, soil invertebrates were extremely tolerant to NaCl. In fact, for one of the test species (*P. armata*) there was no measurable mortality over seven days, even at 15 g NaCl /kg soil.

Table 2: Toxicity of sodium chloride and road salt to soil invertebrates – EC50 endpoints.

Species	End-point	Salt	Soil	Moisture % WHC	Model	EC ₅₀ mg/kg	95% CL		R ²
							lower	upper	
<i>F. candida</i>	Rep ²	NaCl	Clinton	40%	NLR ¹ -Logistic	487	243	732	0.646
<i>F. candida</i>	Rep	NaCl	Clinton	60%	NLR-Logistic	913	743	1084	0.889
<i>F. candida</i>	Rep	NaCl	Scotch Creek	60%	NLR-Logistic	935	807	1062	0.910
<i>E. fetida/ andrei</i>	Coc ³	NaCl	OECD	37%	NLR-Logistic	1884	1484	2283	0.776
<i>P. armata</i>	Rep	NaCl	Scotch Creek	60%	NLR-Logistic	2151	1768	2534	0.864
<i>F. candida</i>	Rep	NaCl	OECD	37%	NLR-Logistic	2765	1897	3635	0.762
<i>F. candida</i>	Rep	Road salt	OECD	37%	NLR-Logistic	3338	2955	3722	0.842
<i>F. candida</i>	Rep	NaCl	Saanichton	60%	NLR-Logistic*	3713	3515	3910	0.990
<i>E. fetida/ andrei</i>	Growth	NaCl	OECD	37%	NLR-Logistic*	4681	1975	4387	0.968
<i>O. folsomi</i>	Rep	Road salt	OECD	37%	NLR-Logistic*	6061	4855	7268	0.682
<i>P. minuta</i>	Rep	NaCl	OECD	37%	NLR-Logistic*	6415	5219	7611	0.903
<i>O. folsomi</i>	Rep	NaCl	OECD	37%	NLR-Logistic*	6521	5522	7520	0.935

¹NLR is Non-linear regression ² Rep is Reproduction ³ Coc is Cocoon production

*analyses using log₁₀ (n+1) transformation of original counts (weights)

Table 3: Toxicity of sodium chloride and road salt to soil invertebrates – LC₂₀ endpoints

Species	Duration (days)	Salt	Soil	Moisture % WHC	Model	LC ₂₀ mg/kg	95% CL		R ²
							lower	upper	
<i>F. candida</i>	28 d	NaCl	Clinton	40%	NLR ¹ -logistic	3098	2310	3885	0.744
<i>F. candida</i>	28 d	Road salt	OECD	37%	LR ²	4313	na	na	0.788
<i>P. armata</i>	28 d	NaCl	Scotch Creek	60%	NLR-logistic	5503	4339	6667	0.808
<i>E. fetida/ andrei</i>	28 d	NaCl	OECD	37%	NLR-logistic	5534	na	na	0.990
<i>O. folsomi</i>	28 d	NaCl	OECD	37%	LR	5524	na	na	0.666
<i>F. candida</i>	7 d	NaCl	OECD	37%	NLR-logistic	9586	8830	10340	0.886
<i>F. candida</i>	7d	NaCl	Saanichton	60%	NLR-logistic	9507	8716	10299	0.892
<i>O. folsomi</i>	28 d	Road salt	OECD	37%	LR	11227	na	na	0.571
<i>P. armata</i>	7 d	NaCl	OECD	37%	LR	16117	na	na	0.703
<i>O. folsomi</i>	7 d	NaCl	OECD	37%	LR	16450	na	na	0.726

Other results

<i>P. armata</i>	7 d	NaCl	Saanichton	60%	no mortality up to 15 g NaCl/kg
<i>F. candida</i>	28 d	NaCl	Saanichton	60%	no mortality up to 10 g NaCl/kg
<i>F. candida</i>	28 d	KCl	OECD	37%	No convergence of model
<i>F. candida</i>	28 d	NaCl	Scotch Creek	60%	No convergence of model
<i>F. candida</i>	28 d	NaCl	Clinton	60%	No convergence of model

¹NLR is Non-linear regression

²LR is Linear regression

Soil invertebrates have a number of adaptations that allow them to survive periods of inhospitable conditions in the soil. In the experiments carried out as part of this study the following were observed in response to non-lethal salt concentrations. Individuals

- curled up in a ball,
- became inactive,
- ceased moulting,
- ceased feeding, and finally
- ceased reproducing

But they were still alive.

From an ecological point of view, once a species stops reproducing, that species is dead.

At the salt concentrations that produced a 20% reduction in adult survival all reproduction had already ceased (Table 4). The only exception was the in the Clinton soil, where the LC₂₀ value was approximately equal to the level of salt contamination at which no reproduction was observed.

Table 4: Comparison of estimated LC₂₀ values for soil invertebrates in salt-contaminated soil, with actual test salt (NaCl) concentrations in which there was zero reproduction.

Species	Soil	Duration of LC test (days)	Calculated LC ₂₀ (mg/kg)	NaCl concentration at which all reproduction had ceased (mg/kg) actual
<i>F.candida</i>	OECD	7 d	9580	5600
<i>F.candida</i>	Saanichton	7 d	9507	5600
<i>F.candida</i>	Clinton	28 d	3098	3200
<i>O.folsomi</i>	OECD	7 d	16450	10000
<i>P.armata</i>	Scotch Creek	28 d	5503	3200
<i>E.fetida/andrei</i>	OECD	28 d	5534	3200

In several tests there was no statistically significant relationship between salt concentration and survival. In fact, the numbers of adults surviving at higher concentrations of NaCl were sometimes higher than at lower concentrations. Based on simple observation of the behaviour of individuals in the experiments, a possible explanation is as follows. When conditions are near the threshold that allows reproduction to occur, then the physiological cost of laying eggs is high. In the present experiment, where osmotic stress was a factor, deposition of eggs would represent a considerable water loss to the individual. Thus egg production would diminish the probability that the adult individual would survive. Once reproduction was no longer attempted, the animals were better able to conserve moisture, and increased their chances

of survival. A similar argument can be made with respect to the cessation of moulting. Hence LC_{20} data tended to be more variable and more difficult to analyse than the EC_{50} data based on reproductive endpoints. They also underestimate the true ecological consequences of soil salt concentrations.

5.3 Use of *O. folsomi* as a test organism

O. folsomi was used in two experiments to investigate the toxicity of NaCl and road salt (containing ferrocyanide) in the standard OECD soil. In one of the replicates only three juveniles were recovered, while the mean for this treatment was 92 juveniles/replicate. Examination of the surviving adults showed that all eight of the survivors were females. Assuming that the sex ratio at the experiment was representative of the ratio during the experiment, the absence of males in the replicate provided a good explanation for the lack of juveniles, and that replicate was consequently excluded from statistical analyses. In order to investigate the effect of sex ratio on reproduction, the sex of all the surviving adults in this experiment was determined (Figure 1). In order to minimise the chance of including a confounding effect of gender-related NaCl mortality, only data from the controls and treatments up to and including 3200 mg NaCl/kg soil were included in the analysis. ANOVA confirmed that for these data, salt concentration was not a significant factor ($p=0.469$) in determining juvenile production. On the other hand, the covariate sex ratio had a highly significant impact on resulting juveniles numbers ($p=0.014$).

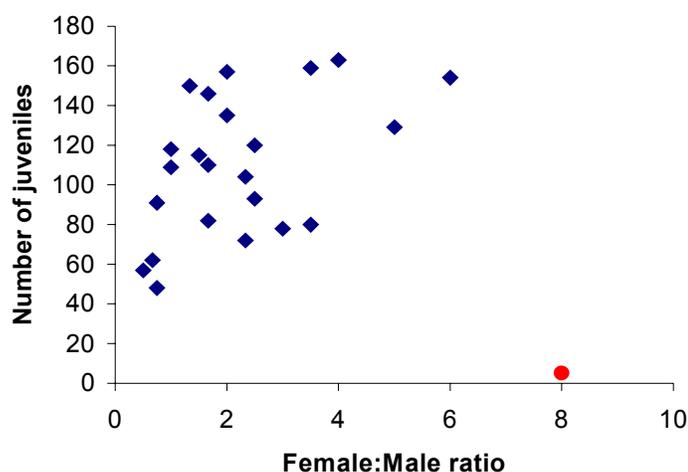


Figure 1: Relationship between sex ratio and reproductive success in *O. folsomi*. (●) replicate with 8 adult females, 0 males.

The analysis showed that, within certain limits, increasing the female: male ratio resulted in higher numbers of offspring. In order to reduce the probability of a skewed sex distribution in the experimental vessels, in the second experiment involving *O. folsomi*, (OECD soil and road salt), the number of original adults was increased to 15 from 10 adults per replicate. However, overall juvenile production was considerably lower in this set of experiments than in the previous set of experiments (Figure 2a).

It is possible that the explanation lies in the different diets that the Collembola were fed prior to the experiments. For the experiments with pure NaCl, the test individuals were collected directly from the culture medium in which they had arrived (organic-rich soil, peat and manure, diet of oatmeal and alfalfa pellets). By the time the road salt experiments were carried out the animals had been kept on a Plaster-of Paris substrate and fed Baker's yeast for six weeks. It is possible that the animals were not fed sufficiently (either quantity or quality) becoming undernourished, and that the residual effects carried on into the experiments – even though an excess of food was provided during the actual experiments.

Since reproduction was so poor during this set of experiments, especially in the controls, it was not deemed useful to analyse the sex ratio of all the replicates. However, as a matter of interest, in three of the control replicates checked at random, there was an average of 0.38 females for every male, while in four of the replicates with 0.56 g road salt/kg soil (with much higher reproductive success), the sex ratio was one female per male. The implication is that sex ratio may be a problem even if larger numbers of individual are used in every replicate.

5.4 Comparison of O. folsomi results with results obtained using other species

Figure 2 shows the results of the experiments to determine the effects of NaCl and road salt on *O. folsomi* and *F. candida* respectively.

In all experiments, *O. folsomi* was less sensitive to salt than *F. candida* (see Table 2 for details). The response of *F. candida* to road salt was not significantly different than the response to pure NaCl (2-way ANOVA; concentration effect, $p < 0.001$, salt effect (road salt vs NaCl), $p = 0.839$). As discussed previously, overall reproduction of *O. folsomi* was lower in the second (road salt) experiment than in the first (NaCl) experiment, regardless of salt concentration. Reproduction in the controls (i.e no added salt) in the second experiment was significantly lower than in the first (NaCl) experiment (ANOVA, $p = 0.001$). However, even though the response curves of *O. folsomi* reproduction differed in the two experiments, the EC_{50} (reproductive) values for *O. folsomi* in road salt versus pure NaCl were very close, with overlapping 95% confidence limits (Table 2). Thus there was no indication that road salt was more (or less) toxic than pure NaCl for either *O. folsomi* or *F. candida*.

The reproductive output of different collembolan species in uncontaminated soils (controls) in 28 day chronic tests, is compared in Table 5. *Folsomia candida* routinely produced 200+ juveniles in the course of the test period, regardless of the soil type. Both species of onychiurids (*P. armata* and *O. folsomi*) produced roughly half as many offspring per test individual as did *F. candida*. Other factors being equal, detection of reproductive impairment is easier if the control values are large.

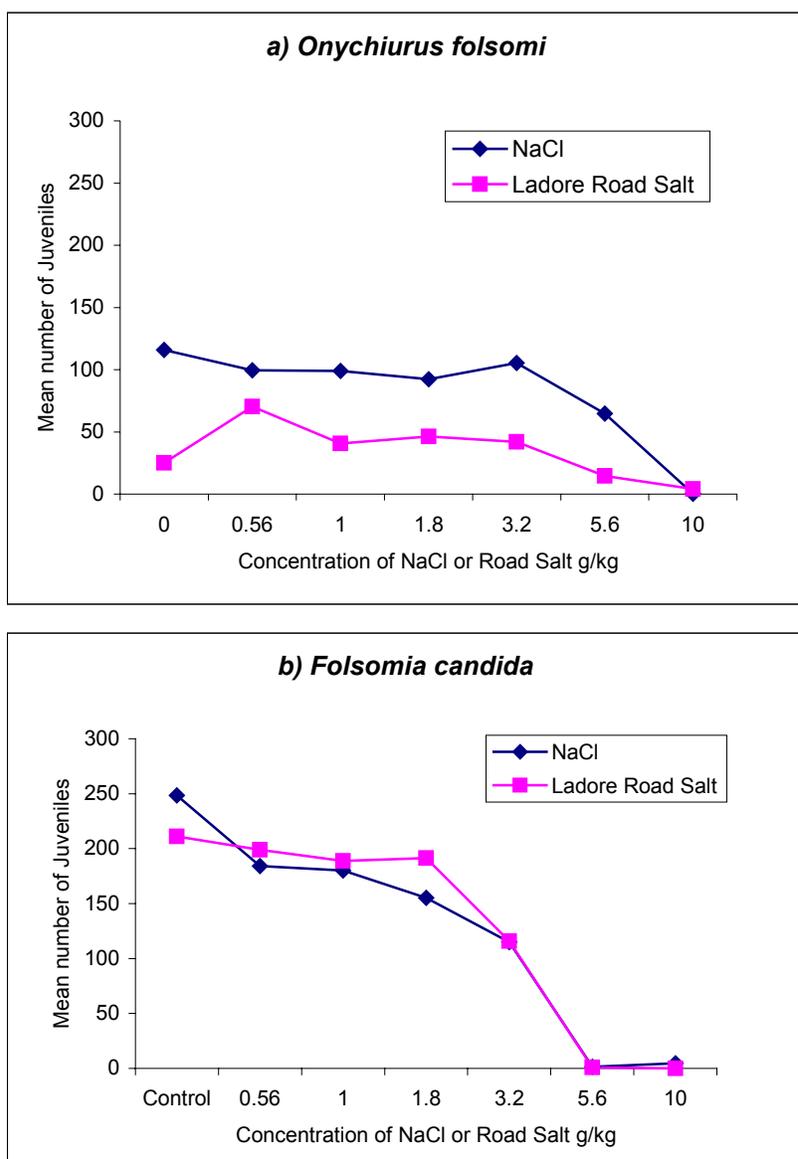


Figure 2: Comparison of the effects of NaCl and commercial road salt on reproduction of a) *O. folsomi*, and b) *F. candida* in OECD soil (28 d).

In the experiment with *P. armata*, one of the replicates unexpectedly produced no offspring whereas the mean for that treatment was 149 juveniles/replicate. This raises the distinct possibility that sex ratio may be as important a consideration for the use of *P. armata* as it is for *O. folsomi* in toxicology experiments. Although all the surviving adults from this experiment have been saved, there has not been an opportunity to determine the sex ratios.

Table 5: Comparison of numbers of juveniles produced in control soils by different species of Collembola over a 28 day period.

Species	No. test organisms/ replicate	Soil	Mean no. juveniles in controls (\pm SEM)
<i>F. candida</i>	10	OECD (Experiment 1)	248 (\pm 24.4)
<i>F. candida</i>	10	OCED (Experiment 2)	211 (\pm 9.6)
<i>F. candida</i>	10	Scotch Creek	236 (\pm 34.9)
<i>O. folsomi</i>	10	OECD (Experiment 1)	116 (\pm 18.9)
<i>O. folsomi</i>	15	OECD (Experiment 2)	25 (\pm 7.2)
<i>P. armata</i>	10	Scotch Creek	141 (\pm 18.9)

5.5 Earthworm results (*E.fetida/andrei*)

The following endpoints were investigated: growth, cocoon production, number of hatched cocoons, and juvenile emergence. Growth and cocoon production can both be measured after 28 days, whereas the other endpoints require an additional 28 days before they can be determined. In the present study, since counting the cocoons after 28 days would have required destructive sampling, the total number of cocoons produced was estimated by adding together the number of hatched (empty) and unhatched cocoons (with and without living contents) found in the sample at the end of the experiment (i.e., 56 days after the start of the experiment, and 28 days after the adults had been removed).

Table 6: Sensitivity of *E. fetida/andrei* to NaCl in OECD soil (28 d)

Endpoint	Model	NaCl (mg/kg)	95% CL		R ²
			lower	upper	
LC ₂₀ Mortality	NLR-Logistic	5534	na	na	0.990
EC ₅₀ Growth	NLR-Logistic*	4681	1975	4387	0.968
EC ₅₀ Total cocoons	NLR-Logistic	1884	1485	2284	0.776
EC ₅₀ Juveniles	NLR-Logistic*	1379	566	2192	0.612
EC ₅₀ Hatched cocoons	NLR-Logistic	906	237	1576	0.553

- analyses using log₁₀ (n+1) transformation of original counts (weights)

In these experiments, mortality, growth and total cocoon all produced clearly measurable responses to increasing salt concentration (Table 6). Data on juveniles and the number of hatched cocoons recovered from the test containers were more variable, but were more sensitive to salt concentrations. Given that the first three endpoints can be measured after 28 day, whereas the final two endpoints require a total test duration of 56 days, it is debatable whether the extra information is worth the cost. However, in the case of NaCl toxicity, the additional data provide insight in the mechanism of toxicity.

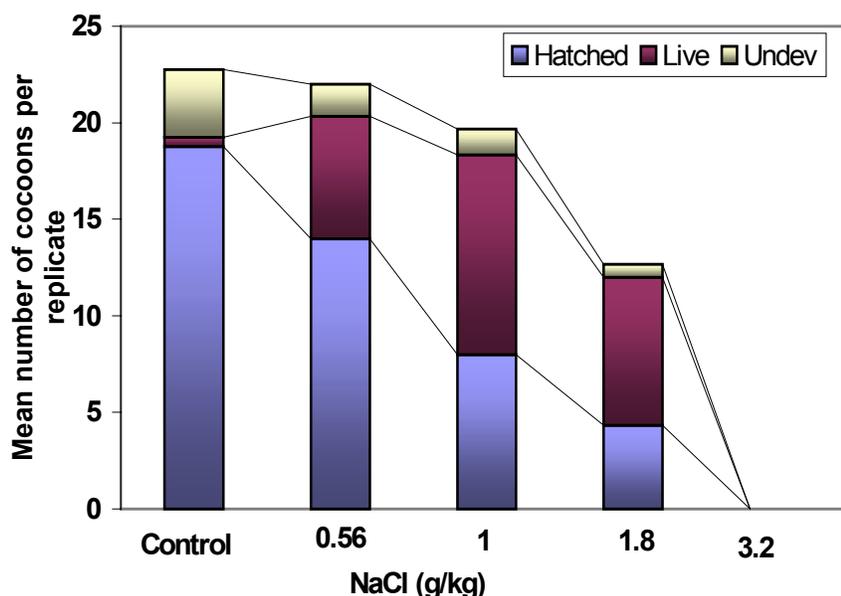


Figure 3. Mean number of hatched, live (with visible juveniles inside) and undeveloped cocoons in OECD soil treated with different amounts of NaCl.

As can be seen in Figure 3, not only did the total number of cocoons produced decline with increasing salt concentration, as salt concentration (and the osmotic potential) increased, the proportion of cocoons that were able to hatch during the experimental period, also declined. Thus the impact of salt was twofold: decreasing the absolute production of cocoons, but also increasing the time taken for the cocoons to hatch.

5.6 Armadillium vulgare results

These experiments were designed to emulate a surface spill of saline water, similar to what might be produced by a spill of produced water. Chemical analysis of the leaves exposed to saline water for 48 h showed that the amounts of Na⁺ and Cl⁻ adsorbed and absorbed by the leaf litter were extremely high (Table 7).

Table 7: Comparison of Na⁺ and Cl⁻ in salt contaminated water, and in leaf litter soaked in the water.

NaCl in water (g/L)	Na ⁺ in water (g/L)	Cl ⁻ in water (g/L)	Na ⁺ recovered from litter (g/kg)	Cl ⁻ recovered from litter (g/kg)
0	0	0	0.36	0.47
1	0.39	0.61	2.50	3.28
5	1.97	3.03	7.07	12.65
10	3.93	6.07	10.09	17.66

5.6.1 Feeding inhibition

Feeding activity of *A. vulgare* adults was significantly affected by salt contamination of the litter (ANOVA; $F=12.083$, $p<0.001$). The numbers of fecal pellets produced in leaves treated with 5 or 10 g NaCl/L were both significantly lower than the controls or leaves exposed to water containing 1 g NaCl /L ($p<0.05$).

Table 8: Effect of NaCl contamination on feeding activity in *Armadillium vulgare*

Concentration of NaCl in which leaves were soaked	Mean number of fecal pellets	SEM
0	241	27
1	181	9
5	105*	14
10	133*	13

* indicates means significantly different than control ($p<0.05$, Bonferoni comparison of means)

5.6.2 Growth experiments

There was a dose related effect of salt (NaCl) on the growth rate of juvenile *A. vulgare*. (linear regression, $p=0.029$) (Figure 4). It should be noted that even though a significant regression between NaCl concentration and growth rate was obtained, a 50% reduction in growth rate (i.e. EC_{50}) was not achieved at the tested concentrations over the 14 d period.

Preliminary results of both the feeding inhibition and the growth experiments with *A. vulgare* show promise for development as protocols for detecting and quantifying impacts of contaminants in leaf litter. Although this species appears to be extremely tolerant to high concentrations of NaCl (expressed as mg NaCl/g dry wt. litter), it should be remembered that these high levels of salt contamination arise as a results of soaking leaf litter in water with salt concentrations well below those that would be encountered in a spill of produced water.

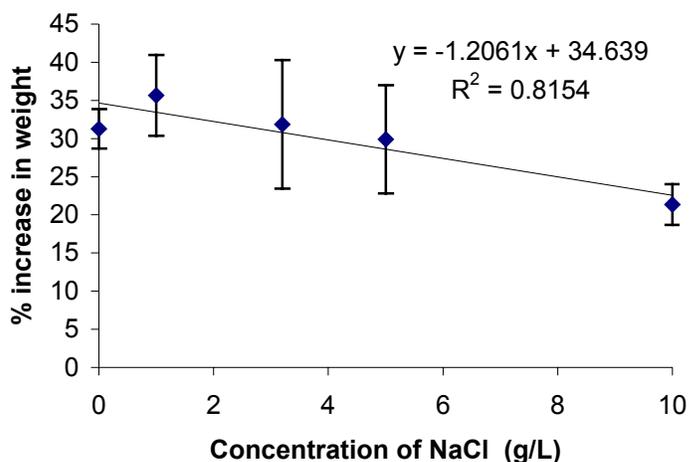


Figure 4. Relationship between weight gain in *A. vulgare*, and NaCl concentration in the water used to re-hydrate leaf litter.

6.0 General Recommendations Regarding Use of Soil Invertebrates in Toxicity Testing

1. Include *Folsomia candida* as a test organism. It survives and reproduces in a wide variety of different soils, large numbers of juveniles are produced in a relatively short period of time, it is parthenogenic and so avoids the sex ratio problem, relatively small amounts of contaminated soils are needed for the tests, and the addition of the tiny quantities of yeast necessary to support the insects has a minimal effect on bioavailability of test substances. Moreover, there is an internationally recognised test protocol for this species (ISO 1999), and it does occur in Canada.
2. Develop tests based soil invertebrates and soils that are representative of Canadian conditions. While tests on *E. fetida/andrei* and *F. candida* in OECD standard soils allow comparison of relative toxicity, we need to know how these values will be expressed in real situations.
3. If possible, avoid the use of LC data. Soil invertebrates are adapted to deal with periods of inhospitable conditions. In the experiments described in this report, by the time the 20% of the adults had died, all reproduction had long since ceased. Consequently unless there is good reason to believe the test substance will break down or be otherwise rapidly removed from the environment, LC₂₀ endpoints will underestimate the ecological impact of the contaminant.

4. Most of the common test organisms, including *E. fetida/andrei* and *F. candida* naturally occur in organic materials (compost, manure, LFH material). However toxicology testing is carried out in mineral soil. Thus, in order to allow these test organisms to survive (and breed) in this alien environment, the soil must be amended. Given the preoccupation with testing mineral soil, the development of protocols that use test organisms that actually live in mineral soil is recommended (e.g. some *Apporectodea* spp.).
5. In relatively undisturbed soils, the vast majority of soil invertebrates inhabit the top few centimetres of the soil profile, and are highly concentrated in the litter or LFH layers. Scraping these aside to measure toxicity in the underlying mineral horizons does not make much sense from an ecological perspective. Canada is mostly forested, and forests have LFH layers that will intercept pollutants. Given the long- range transport of pollutants, and the proliferation of activities such as mining, and oil and gas development in remote areas, the assumption that contaminants are only deposited in agricultural types of soils may not be valid. Other test systems should be developed.
6. This report considered only a limited number of taxa. Other groups besides Collembola and earthworms need to be represented in ecotoxicological tests. Løkke and van Gestel (1998) provide an excellent review on the progress that has been made in Europe in the development of toxicity tests for soil invertebrates. Many of the methods they discuss could be adapted for use with North American species.

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