REVIEW OF BIOLOGICAL INDICATORS FOR METAL MINING EFFLUENTS: A PROPOSED PROTOCOL USING EARTHWORMS

Maria Claudia Sandoval, Marcello Veiga, Jennifer Hinton, Bern Klein
Dept. Mining & Mineral Process Engineering. University of British Columbia.
6350 Stores Rd., V6T 1Z4, Vancouver, BC, Canada

ABSTRACT

There is growing concern about the need for unified criteria on bioindicators used to evaluate the impacts of mine effluents or accidental spills. Toxicological tests based on lethal concentrations (e.g. LC50) have been extensively used to assess effluents, although the biological impact of mining activities cannot always be attributed to acute lethality alone. Geochemical methods, such as sequential or selective chemical extraction, have also been widely used to provide indirect evidence of the availability of metals to organisms. However, the relationship between geochemical parameters, metal uptake, and biological effects is frequently not clear due to complicating interactions between variables. In order to comprehensively characterize risks from mining related discharges, concerns with existing, commonly employed methodologies must be resolved and protocols to assess the effects of sub-lethal or chronic exposure must be established.

This paper reviews current understanding of bioaccumulation and bioavailability of heavy metals associated with mining effluents and it explores the concept of the bioaccumulation factor (BAF) and its applicability to mining impacted sites. Existing protocols for assessment of mining related discharges are also compared and a simple, low-cost methodology using earthworms for the evaluation of metal bioavailability in tailings and effluents is proposed. Earthworms are particularly suitable for the assessment of contaminant bioavailability as they are proven metal accumulators and are in full contact with the substrate they consume. As well, they are well studied, easily bred and participate in many food chains and, unlike fish, can be used to assess a variety of media.

INTRODUCTION

In response to escalating public concerns about the increased frequency and magnitude of mining operations throughout the globe, several methodologies have been developed to assess the risks to ecological or human health from exposure to mining-related discharges. Although protocols for acute lethality are the most common current practice, it is becoming increasingly apparent that exposure to small doses of contaminants at key stages of development or for extended periods of time may also have serious repercussions. As these adverse effects may not be overtly measurable, or may affect only subsequent generations, assessing risks from "low-level" exposure is extremely difficult but will undoubtedly be necessary in the next stage of biological impacts research. The objective of this review is to expose deficiencies and limitations of the existing protocols used to assess metal bioavailability and propose a simple, low-cost alternative that can be applied to various media (i.e. sediments, tailings, water).

Bioavailability and Bioaccumulation

Metals can be released into the environment from mining activities through atmospheric emissions, accidental spills, or with acidic or neutral drainages from waste rock or tailings. Metals can be transported with run-off or groundwater in the form of soluble species or associated with colloids or suspended particles. In aquatic systems in particular, metals can undergo biological or chemical transformations into more (or less) bioavailable forms, i.e. species that can be assimilated by organisms (Fig. 1). Mercury (Hg) transformations are a good example of how metals can become more bioavailable in the environment. While in metallic form in the aquatic system, Hg has a limited capacity to be taken up by organisms. However in the presence of organic acids from sediments or darkwaters, metallic Hg is not very stable and soluble Hg-organic complexes can be formed (Meech et al., 1998). These organic complexes either can be accumulated by biota or transformed into the most toxic form of mercury, which is methylmercury. It is apparent that understanding the physio-chemical conditions in receiving waters is vital to predicting metal bioavailability.

A primary indication of bioavailability is bioaccumulation, which occurs when a portion of a substance is retained by an organism. Organisms can bioaccumulate most metals to some degree, although the extent of bioaccumulation varies depending on the bioavailability of the metal, the organism under consideration and concentrations to which they are exposed. Bioaccumulation of a trace metal can occur through exposure to a contaminated medium (water or air) or by ingestion. Depending on the metal and species of concern, certain exposure pathways may be more significant. For instance, exposure through ingestion of food sources is typically more consequential for mercury and selenium than from the surrounding media.

Bioaccumulation is commonly expressed as a Bioaccumulation Factor (BAF), a unitless value calculated by dividing the steady state tissue concentration of a substance by the steady state concentration in food plus the surrounding living environment (e.g. water for aquatic organisms, air for humans). Alternately, the Bioconcentration Factor (BCF) is calculated by dividing the steady state tissue concentration of a substance by the steady state environmental concentration alone (i.e. water or air). BAF and BCF have been extensively used to evaluate parameters linked to sublethal effects of heavy metals in biota (Parametrix, 1995). For instance, Popovic and Perovic (1999) found that the tendency of chemicals to bioconcentrate is strongly related to its lipophilicity (i.e. hydrophobicity). In this study, BCF differences observed in various trophic levels were correlated to lipid contents, which varied inversely with chemical elimination deficiency of an organism. Bioaccumulation of cadmium (Cd) in squid organs was also evaluated using the BCF, where Koyama (2000) found that, after a 14 day test, the liver exhibited the greatest Cd accumulation. Even though several variations of the BAF have been derived (Blight and

Dyer, cited by Geyer *et al,* 2000), these factors primarily address lipophilic organic compounds and their applicability to metals have not been evaluated extensively.

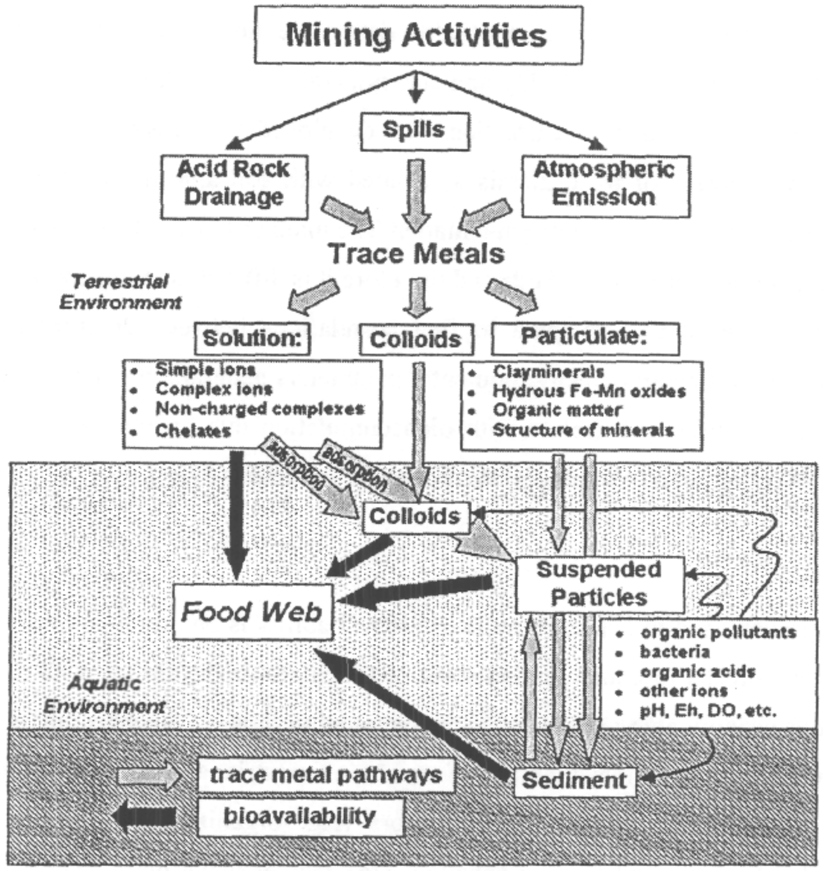

Fig. 1 – Environmental Fate of Trace Elements Released by Mining

Assessing Unavailability

There are two primary approaches to assessing *non-lethal* effects of heavy metal discharges from mining activities. First, geochemical techniques are widely used to provide indirect evidence of the availability of metals to organisms. Second, laboratory or field methods using bioindicators are becoming more prevalent for the site-specific assessment of contaminant bioavailability. Both approaches can provide valuable information about contaminant behaviour in specific environments, but fundamental problems with associated methodologies must be resolved prior to widespread acceptance of any protocol.

Geochemical Methods

Geochemical methods, such as sequential or selective chemical extraction, and more recently ultrasonic (Perez *et al,* 1998) and microwave single extraction methods (Perez. B *et al,* 2001), have been used to provide indirect indications of the bioavailability of mineral associated metals (e.g. adsorbed to mineral surfaces, within crystal structure, etc). Selective extraction can be used to extract metals from just one mineral phase. A common protocol that provides an indication of the bioavailability of trace elements is the extraction of the labile portion of elements weakly adsorbed on mineral surfaces. Chlorides such as $CaCl_2$ or MgCl have frequently been applied to determine the exchangeable portion of copper adsorbed on soils (McLaren and Crawford, 1973), *and* clay minerals in particular. The most classical and frequently employed method of sequential extraction was developed by Tessier *et al.* (1979), which provides information about forms of trace metals associated with soil and sediment components, such as clay minerals, hydrous ferric oxides or organic matter. The main criticism of selective extraction procedures is the lack of uniformity between methods and therefore it is difficult to compare results (Quevauviller *et al,* 1997). Many studies have attempted to find correlations between environmental factors and metal bioaccumulation, particularly as environmental parameters generally dictate speciation. Frequently these parameters do not directly correlate with bioaccumulation due to internal interactions between them, which result in synergistic and antagonistic effects (Parametrix, 1995).

Bioindicators

A second approach used to express results of bioavailability is based on tissue concentration determined by exposing organisms to contaminated soils, sediments or effluents for an established period of time. Concentrations accumulated in the tissue are compared to those in the substrate or solution in which they reside or consume. As the bioavailability of metals in terrestrial and aquatic systems is dependent upon a number of geochemical *and* biological factors (e.g. organism physiology, internal solubilization capabilities, food quality and feeding behavior, etc), studies using bioindicators may be more revealing than geochemical methods alone. A number of standard protocols using a variety of plants and animals have been developed to assess metals pollution and a few methods applicable to mining related discharges are outlined herein.

<u>Aquatic Organisms:</u> Comparatively few aquatic bioindicators have been developed to assess of sub-lethal toxicity. Established protocols for the evaluation of toxicity of anthropogenic organic compounds using fresh water aquatic biota include methods for amphipods *(Hyalella azteca)* (EPA, 1994-2) and inland silversides *(Menidia beryllina)* (EPA, 1994), which use survival, growth and reproductive capacity as endpoints. African clawed frogs/FETAX *(Xenopm laevis)* (ASTM, 1997b) are also used, but apply evaluation of developmental early stages as an endpoint.

Considerably fewer protocols use bioaccumulation as an endpoint. Species like polychaetes *(Nereis Virens)* (Chapman *etal,* 1995) and oligeochaeta *(Lumbriculus variegates)* (EPA, 1994) are well known bioindicators that can be easily cultured, but are not native to most environments. Established sub-lethal protocols have been developed for aquatic organisms including brook trout *(Salvelinusfontinalis),* fathead and sheepshead minnow full life cycle, as well as *Daphnia magna, Ceriodaphnia dubia,* zebrafish *(Brachydanio rerio),* and mysid shrimp. Tests on algae are often reported as acute tests, although as algae reproduce so quickly within a typical study period (4-5 days), these tests can also be classified as chronic studies (Adams, 1993). Another protocol developed by Environment Canada suggests the use of early life stage salmonid fish to assess sub-acute toxicity (Protocol l/RM/28). Because of its importance for human consumption and the relative ease with which they can be studied, many studies have used fish as bioindicators of acute and sub-acute toxicity.

Plants: Plants are frequently used as bioindicators. Environment Canada suggests the use of macrophytes *(Lemna minor)* (Report EPS l/RM/37- 1999) as bioindicators of aquatic toxicity from effluents. This test method measures growth inhibition by evaluating the effluents using frond *(i.e.* an individual leaf-like structure) dry weight over a 7-day period. This test is limited in that results are difficult to correlate with impacts to other ecosystem components. Laboratory and field tests using lettuce *(Lactuca Sativa)* (EPA 1996; Folsom et al, 1991; WES, 1989) have also been used, but this is not naturally occurring in most locations. Marsh Grass *(Echinochloa Crusgalli)* (Walsh et al, 1991) and Rye Grass *(Lolium Perenne)* (ASTM E1598-94; ASTM, 1997) have been also used as toxicity monitors using survival and growth as endpoints, but salinity of the tested soils can significantly influence the results and similarities to certain weeds can complicate field applications.

Invertebrates: Terrestrial and aquatic invertebrates have also been widely used as indicators of lethal and sub-lethal toxicity. A methodology developed by Silva *et al* (2000) was used to evaluate bioavailability of metals in tailings from three separate mines. Results show that although appreciable quantities of metals accumulated in worms, the Bioaccumulation Factor (BAF), or ratio of metal concentration in earthworms to that in the substrate, was below unity for all metals, with the exception of arsenic from a single mine. Even though the initial concentration of arsenic in earthworms from one mine (2 ppm) was considerably lower than from the two others evaluated (531 ppm and 212 ppm, respectively), the BAF from the first mine samples (4.4) was considerably higher. The low calculated BAF suggested that there is not a greater potential for bioaccumulation of metals from mines with high background levels, despite the amount accumulated in tissues. For example, worms incorporated 144 ppm of Pb from one mine tailings although the BAF is far below unity (0.12). In another test, Hg concentrations in worm tissues exposed to contaminated soils from a chlor-alkali plant was as high as 263 ppm. Even with very high Hg concentration in worms, due to high substrate concentrations (25,000 ppm) the BAF (0.02) does not suggest appreciable bioaccumulation. It is apparent that a simple correlation between metals in organism tissue divided by metals in the substrate does not sufficiently reflect metal incorporation into the organism when the substrate is extremely contaminated. The bioaccumulation threshold, or ability of an organism to assimilate metals, is a consideration that should be incorporated into any representative factor used.

Acute Toxicity

The classical approach to evaluating acute toxicity is through the determination of the concentration of a specific substance at which 50% of the test-organism population dies (LC50). Although most protocols address metals and other substances in various aquatic systems, very few methods, with the exception of mining effluents guidelines for an LC50 bioassay of rainbow trout developed by Environment Canada, have been specifically devised for mining related discharges. All methodologies, however, recognize that the toxicity of metals in aquatic systems is inherently linked with bioavailability, a factor that is controlled by metal speciation. Parameters such as physico-chemical characteristics (pH, Eh, DO, etc), and the presence and abundance of competing ions and methylating agents significantly influence the speciation, and thus, bioavailability of metals in natural waters, sediments and soils (Parametrix, 1995).

In Canada, monitoring of effluent quality is required by the Metal Mining Liquid Effluent Regulations (MMLER) but aquatic monitoring in receiving waters is not. The Assessment of the Aquatic Effects of Mining in Canada report (AQUAMIN - *1996)* outlined the gaps and research needs for the proper assessment of the aquatic effects of mining. The classical approach of evaluating acute toxic levels does not necessarily provide an indication of long-term impacts in the life cycle of the species. In 1995, 21 effluents from different Canadian mines were evaluated and 62% of them passed the trout lethality test. After reviewing 700 reports related to 95 Canadian mine sites and conducting detailed studies in 18 sites, the AQUAMIN (1996) Steering Group agreed in the *need for information on biological availability of metals, their bioaccumulation and their ecological effects as well as the development of appropriate quality assurance/quality control procedures.* The recommendations of the AQUAMIN Steering Group include that the revised MMLER should require periodic acute lethality tests for all mines. Despite the concerns expressed in the conclusion of the AQUAMIN steering committee report, the evaluation of chronic effects was not considered in the recommendations.

Adverse Effects and Endocrine Disruption

Adverse effects can be generated even when sub-clinical symptoms are not evident. As many adverse effects are quite difficult to measure or quantify, very few methodologies or protocols have been established. Within the last decade, disruption to the endocrine system caused by studied and confirmed by several researchers. Endocrine disruption is the ability of certain chemical compounds to mimic and replace some specific proteins in an organism, thereby disturbing its hormonal system. Endocrine disrupting chemicals (EDCs) are substances that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis (i.e. the ability to sustain normal function in response to exposure to certain stressors) and the regulation of reproduction and development. EDCs can affect processes related to: organism growth, immune system function, sexual development, reproduction, and malformation. Moreover, it is becoming apparent that even at relatively low exposure levels, those at which organisms may show no apparent signs of stress or disease, a multitude of effects may develop long after the period of exposure and even in subsequent generations (Moore *et al,* 1997).

Despite the potential magnitude of effects to biota from exposure to relatively low levels of some metals, many issues must be resolved before regulators and industry will respond. According to a review conducted by Brown *et al* (2001), the main criticism of research on endocrine effects of metals is that there is considerable variability in experimental protocols, for instance routes of contaminant administration to organisms are not standardized, and endpoints have not been clearly defined. Research into adverse effects and endocrine disruption by heavy metals is evolving to respond to these methodology issues, as well as variability in site-specific parameters, such as seasonal effects, distance from pollution sources, species present, etc. An in-depth review of metals as EDCs conducted by the International Council on Metals and the Environment and the Environment concluded that metals present at levels found in the environment are unlikely to affect endocrine functions (Moore *et al,* 1997). As knowledge gaps in this field are significant, particularly with respect to heavy metals, and most research has commenced only within the last decade, this conclusion may be premature. Further study of the impacts of metals on endocrine disruption is certainly needed.

PROTOCOL TO DETERMINE METAL BIOAVAILABILITY USING EARTHWORMS

An appropriate bioindicator organism must be reasonably well understood in terms of biological qualities (e.g. physiology) and must be broadly applicable to various external *(e.g.* geochemical) conditions. Earthworms may be a viable alternative to traditionally applied organisms *(e.g.* fish) as they are simple, well-studied creatures that can provide indications of bioavailability in a short time frame at relatively low costs. Substantial evidence indicates that earthworms accumulate heavy metals from polluted soils and other media (Edwards, 1996; Goats and Edwards, 1988; Rhett *et al,* 1988; Neuhauser *et al,* 1985; Ireland, 1983). Earthworms are particularly suitable for the assessment of contaminant bioavailability as they ingest large quantities of soil and are in full contact with the substrate they consume. As well, they constitute up to 92% of the invertebrate biomass of soils and participate in many food chains, acting as a food source for a wide variety of organisms including birds, fish, insects, various mammals, and reptiles (Ireland, 1983; ASTM E1676-95). In addition, they are easily bred, have been extensively studied, and are approved for use in toxicity testing by the US EPA, the European Economic Community and the Organization for Economic Cooperation and Development (ASTM E1676-95). A methodology is presented herein using the earthworm *Eisenia foetida,* commonly known as the barnyard or tiger worm, to evaluate the bioavailability of metals in tailings and aqueous solutions, such as mine effluents. This methodology can also be applied to soils and sediments, as well as a wide range of inorganic and organic contaminants.

This protocol primarily evolved from the American Society for Testing and Materials Standard Guide for Conducting Laboratory Soil Toxicity Tests for the Lumbricid Earthworm *Eisenia foetida* (ASTM-1676-95). Upon review of existing methodologies for earthworms and similar organisms methodology was derived that is applicable to evaluate metals associated with solids and solutions that can be applied under various conditions. Existing methodologies reviewed are summarized in Table 1.

Table 1: Major Earthworm Protocols and Studies

Protocol	Description	Comments
ASTM 1676-95: Standard Guide for Conducting Laborator y Soil Toxicity Tests for the Lumbricid Earthworm *Eisenia foetida*	Using the Earthworm *Eisenia foetida* a 28 day test was devised; involved the creation of a sphagnum based synthetic soil to be mixed with test media.	- acute toxicity test - complexation of synthetic soil/organics with metals possible; also variability of synthetic soils from test-to-test likely.
US EPA 600R94024: Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates ASTM 1383-93a: Standard Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates	Using the aquatic invertebrate *Lumbriculus variegates a* 28 day test for sediments; involved a fully saturated system and continuous flow-through of overlying water	- limited to sediments - requires collection and treatment of water
Lockheed Martin Environmental Restoration Program: Development and Validation of Bioaccumulation Models for Earthworms (1998)	Compared bioaccumulation of selected organics and inorganics by earthworms using 33 studies from 12 countries and developed bioaccumulation models/numerical correlations.	- does not provide specific methodology information on studies reviewed - numerical correlation between worms and substrate derived from various protocols
The Prediction of Field Toxicity of Chemicals to Earthworms by Laboratory Methods (Goats and Edwards, 1988)	Review of available methods for toxicity prediction using earthworms and development of new methods; evaluated various synthetic and natural substrates, modes of exposure and compared field methods for toxicity to the same series of contaminants	- evaluated contaminants in solutions only - acute toxicity test - standardized substrate (using filter paper or silica) deemed most appropriate for solution evaluation
Hazardous Materials Assessment Team (HMAT) 14 day Soil Test Using Earthworms (Callahan, 1988)	Involves addition of contaminant of concern with standardized mixture of sand, clay and peat and exposure of worms for 14 days.	- evaluated contaminants in solutions only - bioaccumulation test - contaminant interactions with prepared soil probable

Proposed Earthworm Protocol

Specific components of the methodology, including exposure time, the composition of the substrate, physiological requirements of the earthworms (pH, humidity, organic carbon) and procedures to void contents of the intestinal tract (so that only tissue is analyzed), were derived from a review of these major studies and other laboratory and field scale studies. The methodology is summarized as follows and described in greater detail below:

- A mixture of 80g of tailings, sediments or clean sand (for solution assessments), 20g of prepared cellulose and 80mL of distilled water (in solids evaluation) or solution are manually homogenized in a 900ml glass jar (45% moisture content);
- Fifteen cleaned, weighed worms *(Eisenia foetidd)* are placed in the mixture for a period of 28 days;
- At the conclusion of the exposure period, worms are removed, counted, cleaned and depurated for 24 hours to void contents of the stomach and intestinal tract;
- Depurated worms are re-cleaned and weighed and pre-digested for full metals scan;
- Jar contents are also sampled and analysed for metals for comparison with worm tissues.

The details of the methodology are described below:

Organism Culturing and Selection. *E. foetida* can be initially acquired from local composting cooperatives and cultured in a dark plastic, ventilated bin. Diets of alfalfa pellets, a mixture of vegetable food waste or horse manure have been compared and it was determined that optimum growth occurs in pure horse manure substrate. Worms are hand-selected for testing on the basis of sexual maturity, as evidenced by the presence of a clitellum (a ~3mm wide ring around the body), size (0.3 - 0.45 g wet weight), and liveliness (actively responds when anterior segments prodded). Prior to use in jar experiments, chosen worms are stored for 24 hours on damp filter paper to void contents of the stomach and intestinal tract.

Cellulose Preparation. Cellulose is prepared in advance by shredding white, kaolin based paper, followed by converting to a pulp by mixing in a blender with distilled water, and subsequently drying at 30° C for 48 hours. Dried paper can be broken down into a softer cellulose mixture using a blender.

Tailings/Soil/Sediment Jar Preparation. Tailings or contaminated soil (80g) are combined with cellulose (20g) and manually homogenized with distilled water (80mL) in 900 mL jars. Fifteen mature earthworms are placed in each jar (in triplicate) for a period of 28 days (deemed to be steady-state). A blank containing cellulose, water and clean sand is also prepared in conjunction with the test jars.

Solutions/Effluents Jar Preparation. Clean, fine sand (80g) is combined with cellulose (20g) and manually homogenized with solution to be evaluated (80mL) in acid-washed 900mL jars. Fifteen mature earthworms are placed in each jar (in triplicate) for a period of 28 days. A blank containing cellulose, water and clean sand is also prepared in conjunction with the test jars.

The proportions used in the protocol were derived empirically from previous tests conducted by these researchers, previous studies and knowledge of earthworms' natural habitat. Earthworms thrive in moisture contents between 30 - 45% and at a pH ranging from 5 to 9. For the evaluation of effluents, some pH adjustment (ideally using NaOH) to achieve an amenable pH may be required.

Post-Exposure Period. At the conclusion of the exposure period, worms are removed from each jar, carefully washed, dried, and counted. Observations such as motility, light sensitivity and physical qualities *(e.g.* discolouration) are documented to provide some indication of toxic responses. Following this, worms are depurated (*i.e.* starved) for a period of 24 hours to void contents of the intestinal tract and subsequently re-washed and re-weighed. Both worms and jar contents are analyzed for total metals to determine bioaccumulation following worm digestion.

Worm Digestion and Analysis. Following post-depuration washing and drying, worms are placed in 250ml, acid washed Erlenmeyer flasks and digested in 20 mL of 0.7M nitric acid. After a 24-hour period the solution is slowly reduced to 10mL at low heat. Distilled water is then added up to a volume of 120mL. Samples are split into 60mL volumes, poured into acid-washed polyethylene containers and promptly refrigerated. One of the two samples is kept as a back-up and the other submitted for full metals scan, commonly by ICP-MS.

This methodology is not limited to the evaluation of metal bioavailability in impacted effluents, but can also be used to predict bioavailability of metals from tailings or waste rock. Using the well-established protocol developed by Lawrence (1990), Ieachate can be extracted from standard humidity cells (columns). The bioavailability of this solution can subsequently be evaluated using the earthworm protocol and, in conjunction with geochemical results from the columns, can be used to predict long-term risks from tailings or waste rock.

CONCLUSIONS

Bioindicators of pollution from metals and other substances are essential components of a comprehensive assessment of ecological and human health risks. Impacts to biota from mining related discharges are commonly evaluated on the basis of toxicity, although biological effects are often not limited to acute lethality alone. Metals present in a bioavailable form may be bioaccumulated and thereby detrimentally affect long-term organism health. Metal bioavailability is usually determined using either geochemical methods or bioaccumulation studies. Geochemical techniques provide indirect evidence regarding metal-bearing minerals and the availability of metals to organisms. However, the relationship between geochemical parameters, metal uptake, and biological effects is frequently not clear due to complicating interactions between variables.

Direct analysis of tissues from organisms exposed to specific field or laboratory conditions can also provide evidence of bioavailability. The extent of bioavailability from these tests is often evaluated using a bioconcentration or bioaccumulation factor (BCF or BAF, respectively), which compares metal concentrations in organism tissues with the surrounding substrate. Although bioaccumulation studies can provide valuable information, the BCF or BAF may not be suitable indicators of risk from mining discharges, primarily due to the high metals concentrations encountered in these environments, which yield misleading, low factor levels. As demonstrated in experiments using earthworms, bioaccumulation may still be appreciable, but the resulting BCF or BAF may be low, suggesting comparatively low-risk conditions. Other measures or parameters should therefore be developed for metal-rich conditions, such as those found in mining.

The protocol proposed herein evolved from a detailed review of existing earthworm protocols and laboratory and field studies. Preliminary verification of the methodology using tailings, sediments and solutions has achieved excellent results and this protocol promises to be a highly effective, low-cost and useful tool for the assessment of mining discharges to the environment.

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