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ASSESSING ECOTOXICITY OF BIOMINING EFFLUENTS IN STREAM ECOSYSTEMS BY IN SITU INVERTEBRATE BIOASSAYS: A CASE STUDY IN TALVIVAARA, FINLAND

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ASSESSING ECOTOXICITY OF BIOMINING EFFLUENTS IN STREAM ECOSYSTEMS BY IN SITU INVERTEBRATE BIOASSAYS: A CASE STUDY IN TALVIVAARA, FINLAND

Running title: Assessing ecotoxicity of biomining effluents in streams

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Abstract: Mining of sulfide rich pyritic ores produces acid mine drainage waters and has induced major ecological problems in aquatic ecosystems worldwide. Biomining utilizes microbes to extract metals from the ore, and it has been suggested as a new sustainable way to produce metals. However, little is known of the potential ecotoxicological effects of biomining. In the present study, biomining impacts were assessed using survival and behavioral responses of aquatic macroinvertebrates at in situ exposures in streams. The authors used an impedance conversion technique to measure quantitatively in situ behavioral responses of larvae of the regionally common mayfly, *Heptagenia dalecarlica*, to discharges from Talvivaara mine, which applies a biomining technique. Behavioral responses measured in 3 mine-impacted streams were compared to those measured in 3 reference streams. Also a 3-d survival of the mayfly larvae and the oligochaete *Lumbriculus variegatus* was measured in the study sites. Biomining impacts on stream water quality included increased concentrations of sulfur, sulfate and metals, especially manganese, cadmium, zinc, sodium and calcium. Survival of the invertebrates in short-term was not affected by the mine effluents. In contrast, apparent behavioral changes in mayfly larvae were detected, but these responses were not consistent among sites, which may reflect differing natural water chemistry of the study sites. This article is protected by copyright. All rights reserved

Keywords: Behavioral toxicology, Benthic macroinvertebrates, Ecotoxicology, Acid mine drainage, Biomining

INTRODUCTION

Mining of sulfide rich ores has produced major ecological problems in aquatic ecosystems worldwide, often due to excess acid mine drainage waters (AMD) leaking from active or abandoned mines [1]. Both different remediation activities [2, 3] and biomining techniques [4] have been developed to tackle these problems. According to Rawlings [5], biomining is a general term that includes bioleaching and bio-oxidation processes. Bioleaching is defined as a process where insoluble metal sulfides are oxidized by microbes to soluble metal sulfates, and metal is extracted into water. This process can also be called bio-oxidation, but bio-oxidation may also refer to the microbial process of metal recovery without solubilization of the metal. Here, we refer to biomining as a bioleaching process, and the terms are used interchangeably.

Nowadays production of metals such as copper (Cu), cobalt (Co), nickel (Ni), zinc (Zn) and uranium (U) with bioleaching techniques is increasing, especially from ores with relatively low metal content. Biomining is considered more environmentally friendly and cleaner technology with potentially lower risks to the environment than e.g., smelting because of lower energy usage, lower air emissions of SO_2 and CO_2, and controlled collection and treatment of acid metal-loaded solutions [6]. However, potential impacts of biomining activities on freshwater ecosystems remain largely unknown.

Several studies have demonstrated ecological impacts of AMD effluents from open and abandoned mines in stream ecosystems. AMD contains different mixtures of metals and sulfates, often with highly varying pH, and leads to reduced diversity of benthic macroinvertebrate communities with fewer acid sensitive mayfly, stonefly and caddisfly taxa in particular [7, 8]. Metal pollution also reduces especially mayfly larval abundance [9]. Exposure to metals and low pH can also affect behavior of benthic invertebrates [10, 11].

Behavioral stress responses of animals may provide evidence on and early-warning indication of site-specific toxicity and adverse ecosystem impacts [12]. Behavioral changes, like impaired locomotion, can be detected earlier than changes in mortality or development [13, 14]. Behavioral endpoints have gained increased interest in aquatic toxicity studies [e.g. 15], since they are considered integrative measures of toxicity also at low and rapidly fluctuating chemical concentrations.

We assessed quality and potential ecotoxicity of water in streams affected by the present Terrafame mine, formerly at the time of our study and hereafter Talvivaara mine in Sotkamo Northern Finland. This open-pit mine uses a novel bioheapleaching technique where the bacteria, which occur naturally in the ore, are utilized to extract metals. First the ore is mined, crushed, agglomerated and built into heaps, which are irrigated [16]. Iron- and sulfur-oxidizing bacteria and archaea oxidize these metal-containing sulfidic ores. The resulting soluble metals are then recovered from the leaching solution.

Mine effluents with occasional spills, both before and after the present study, with highly elevated concentrations of sulfate and metals have affected the water quality of nearby lakes and rivers. Highly fluctuating pH (from 4.7 to 7.3) has been measured in water bodies near the mine, but during accidental leakages pH fluctuations have been even greater [17].

Our approach was to use in situ bioassay methods in ecotoxicity assessment. In comparison to standard laboratory bioassays, in situ exposure of organisms has a number of advantages: they ensure realistic exposure conditions, reflect potential joined impacts of chemical mixtures and other stressors and may detect rapidly fluctuating exposure conditions [18]. Development of biological sensors has allowed also in situ behavioral measurements of animals [19].

The aims of this study was to assess impacts of biomining activities on water quality and aquatic invertebrates in streams of the Talvivaara mine area by multiple lines of evidences, and test utility of in situ exposure of aquatic invertebrates as ecotoxicity indicators. More specifically, we used the Multispecies Freshwater Biomonitor (MFB) to measure behavioral responses of native mayfly larvae in mine-impacted and reference streams, along with a more traditional toxicity endpoint, survival. In situ survival of laboratory-cultured oligochaete *Lumbriculus variegatus* caged in the same study streams was also measured. We aimed to evaluate if any behavioral stress responses of mayfly larvae to mine-induced pollution could be defined in short-term, and whether they hence might serve as sensitive, early-warning indicators in standard bioassays. Additionally we sampled benthic macroinvertebrates to describe the benthic community structure of the study sites. This study sheds light on the ecotoxicity impacts in the streams before the accidental large-scale spills of the mine's gypsum ponds in November 2012 and spring 2013.

MATERIAL AND METHODS

Study sites

The study sites included 3 streams receiving runoff from Talvivaara multimetal mine: R. Kalliojoki (IM1), R. Tuhkajoki (IM2) and R. Lumijoki (IM3), and 3 reference streams not receiving mine effluents: R. Aittopuro (R1), R. Kohisevanpuro (R2) and R. Joutenjoki (R3) (Figure 1). R3 and IM3 are situated at Vuoksi watershed, and other rivers at the Oulujoki watershed. R1 and IM1 differ from the other streams in having catchment areas in the metalliferous black shales region with high background metal concentrations in the bedrock [20, 21]. These black shales are rich in Ni, Zn and Cu with naturally acidic and metal-rich surface water runoff, especially if the bedrock is exposed and in contact with water. For example, median background Ni concentration in streams in black shales area has been 8 μg/L, and in streams in other geochemical areas in the region 2 μg/L [21]. All study streams have brown water color (175–300 mg Pt/L) and hence high dissolved organic carbon (DOC) concentrations, as indicated by the measured DOC available from IM1 (17 mg/L), IM2 (16 mg/L), IM3 (25 mg/L) and R3 (17 mg/L). Talvivaara Mining Company Plc situated in Sotkamo, Finland started biomining activities in 2008 with the main products being Ni and Zn. In 2008 and 2009 the construction of the mine continued and metal production was intermittent due to the technical problems [22]. In 2010 and 2011 the production was running throughout the year. The mine has applied the bioheapleaching technique to extract Ni, Zn, Cu, Co and U from the low-grade ore [16]. Pentlandite, pyrrhotite, chalcopyrite, sphalerite and pyrite are the main sulfide minerals of this black schist ore [16].

Stream water quality

Water samples (n=2) for metal (total and dissolved), phosphorus, selenium, sulfur and sulfate analyses were taken in September 2012 at the beginning and at the end of the behavioral measurements each day to describe the exposure conditions in the study sites. Also temperature, specific conductance and oxygen concentration were measured in situ with YSI 6600 V2-4 Multi-Parameter Water Quality Sonde. pH was measured afterwards in the laboratory from the water samples. Samples to represent the dissolved metal fraction were filtered (Whatman 25 mm GD/XP syringe filter, pore size 0.45 µm) in the field. Samples were preserved with 65% suprapur nitric acid (HNO_3), and stored +4°C until elementary analysis with ICP-OES (Al, Ba, Fe, Mn, Sr, Ti, Zn, Ca, K, Mg, Na, S, P), ICP-MS (As, Cd, Co, Cr, Cu, Ni, Pb, Se, U,V, Zn), or Ion Chromatography (SO_4^{2-}) in accredited (FINAS T003, K054; EN ISO/IEC 17025) Environmental Measurement and Testing Laboratory of the Finnish Environment Institute.

Mean dissolved metal concentrations were compared to the national environmental quality standards (EQS) for Cd, Ni and Pb [23, 24]. National annual average, AA-EQS, is defined for all these 3 metals, but maximum short time permissible level, MAC-EQS, only for Cd. Because EU allows its member states to take into account the site-specific background concentrations in implementing EQSs [23], these EQSs are adjusted in the black shales geological region by adding background concentrations to the EQSs as following the practice in Kauppi et al. [17]. In the stream waters in the black shales region, median background concentrations for Cd is 0.18 µg/L (range <0.02–0.54 µg/L), for Ni 13 µg/L (0.5–314 µg/L) and for Pb 0.17 µg/L (0.07–1.59 µg/L) [20] and adjusted AA-EQSs for Cd 0.8 µg/L, Ni 22-35 µg/L and Pb 8 µg/L [17]. Some uncertainties were detected in the metal analysis results indicating sample contamination, which had happened probably during filtering, so the estimate of the total metal concentration was used in subsequent correlative analysis instead of the dissolved fraction. The total metal concentrations with a correction factor of 0.97 for Cd, and 0.96 for Ni [25] were used in the comparisons with EQSs.

Test species

Mayfly larvae, *Heptagenia dalecarlica* Bengtsson 1912 (Ephemeroptera, Heptageniidae) were used in tests with behavior and survival as the endpoints. Larvae of *H. dalecarlica* were sampled from submerged stone surfaces from R. Mustinjoki, which is located near the study sites, but unaffected by mine effluents (Figure 1).

Oligochaeta, *Lumbriculus variegatus* (Müller, 1774), which was used in the survival tests, were from a laboratory-reared population from the culture sustained in standardized conditions in the Finnish Environment Institute. Culture conditions were as follows: 1.0 mM artificial freshwater, pH 7, temperature 20±2°C, continuous aeration, soft unbleached paper tissue substrate, and 16:8 h light:dark cycle. Oligochaeta were transported to the study sites in containers filled with their culture medium.

Behavioral measurements

Behavior of the mayfly larvae was measured in situ at each 6 study sites in September 2012 using Multispecies Freshwater Biomonitor, MFB. MFB quantitatively records the behavioral patterns of an individual by non-visual impedance conversion technique [19]. The behavioral signals within the frequency range of 0.5–8.5 Hz are analyzed by a discrete FFT (Fast Fourier Transform) yielding a percentage of time an animal spends on certain mode of movements with specific signal frequencies. A calibration of the system to link a frequency range to each typical mode of behavior was done beforehand in the laboratory by simultaneous recording in the MFB and visual observation of larval locomotion like crawling and swimming, and gill ventilation. In the field, the MFB was operated with a 12 V battery. Recordings with empty chambers in situ proved that there were no disturbances from e.g. turbulence in the water. Measurements were done in 6 subsequent days, one site per day. Larvae of *H. dalecarlica* were collected each morning, placed carefully into aerated plastic containers filled with stream water (5 L), and transported to the test site. Aeration was provided during transportation. Only viable, actively moving and uninjured animals were used in MFB-measurements. Animals were placed individually into test chambers and acclimated 30 min before the measurements started in stream water. Locomotory behavior (<2 Hz) and ventilation (≥2.0 Hz) of each larva (n=8 per site) was measured for 4 min at 10 min intervals, so altogether 18 repeated measurements were obtained from each individual during the 3 h exposure. Behavior of all 8 individuals was measured simultaneously in 8 individual test chambers. MFB-measurements were done between 9.30 AM and 5.30 PM on each day to avoid possible effects of changing light conditions on behavior. MFB-measurement sites were selected so that water velocity was on average 0.01–0.02 m/s next to the submerged MFB-test chambers in all streams ensuring similar conditions for the larvae. Animals were not fed during the measurements.

Survival -tests

Survival as an end point was measured in the 3-d exposures at sites IM3 and R3 for the *H. dalecarlica* (Ephemeroptera) and at all 6 sites for the *L. variegatus* (Oligochaeta). The mayfly larvae were collected from R. Mustinjoki and exposed in groups of 3–6 individuals per test chamber with 7 or 8 replicates per site. *L. variegatus* was exposed in groups of 20 individuals per test chamber with 4–6 replicates per site. The in situ chambers (20 x 8 cm) used were made of transparent PVC plastic tubing and had three rectangular mesh (150 μm) windows (10 x 5 cm) on sides and thus, followed the main construction in Burton et al. [26]. Chambers were placed in water with the aid of wire baskets and zip ties. Baskets were anchored using bricks and iron rods and placed on the same water current conditions as the behavioral chambers.

Benthic macroinvertebrate community

Benthic macroinvertebrate were sampled from all sites using a standardized 30 second kick net sampling method with 2 replicates from 3 river sections with a differing water velocity and sediment grain size (soft, gravel and stony bottom). This resulted in 6 samples per site, all preserved in 70% ethanol on site. In the laboratory, macroinvertebrates were sorted from the samples, identified (to species or genus, family for Diptera) and counted per taxon. The reference condition approach is used in ecological status assessment of the member states of European Union [27]. The macroinvertebrate metrics that are used in the Finnish national system to assess the ecological status of streams, the number of type-specific taxa, type-specific EPT families (Ephemeroptera, Plecoptera, Trichoptera) [28] and percent model affinity (PMA) [29] were calculated using species data from gravel and stony bottom samples. Type-specific taxa and EPT families are characteristic for each river type in that they have been observed at ≥40% of reference sites. Hence, their expected probability of capturing is 0.4 or greater and their total expected number the sum of the estimated probabilities, in the absence of human disturbance [28, 30]. The observed community metrics were compared to type specific reference values obtained from Aroviita et al. [28]. The national typology of rivers is based on the size and geology (e.g. proportion of peatland /natural water color) of their catchment area, and according to that typology system R1 and R2 belong to ‘very small (catchment area < 10 km^2) humic (natural color > 90 mg Pt/L) rivers, IM3 and R3 to small (10–100 km^2) humic rivers, and IM1 and IM2 to middle sized (100–1000 km^2) humic rivers (the Finnish Environment Institute’s Water Quality Database). For each stream and metric we compared the observed value with the type-specific expected (reference) value [28], and the metrics were assigned to status class (high/good meeting the quality standard or moderate/poor/bad failing the standard and indicating deterioration) according to the national classification scheme [28].

Statistical analysis

The normality of behavioral data was tested by Shapiro-Wilk test and equality of variances by Levene test. Arcsine transformation was applied, but it failed to fit the data to the assumptions of parametric tests, so non-parametric Mann-Whitney was used to test the differences in the average time (%) larvae spent on locomotion or ventilation between the mine-impacted sites and the pooled data from 3 reference sites, which represents the natural variation in larval behavior in streams that do not receive mine effluents. Spearman or Pearson correlation was used to test association of larval behavior (average time % in locomotion and ventilation) in the first and last measurement hour with the water quality variables (metals, SO_4^{2-}, temperature, O_2, conductivity, pH) across all sites. Oligochaete survival data with arcsine transformation were tested for equality of variances by Levene test followed with randomized blocks ANOVA. Reference streams and contaminated streams formed the treatments and stream pairs were the blocks. Mayfly survival was tested with t-test.

RESULTS

Stream water quality

Concentrations of some trace metals and major ions were higher in mine-impacted streams (Table 1). In particular, average concentrations of the trace elements Cd, Zn, Ni, Mn, Sr and U were elevated in mining-influenced sites by factors of about 3 to 5 greater than the reference streams. The major ions Ca, Na, Mg and sulfate were elevated by factors of about 3.7 to 20 greater than reference sites, and total sulfur species (S) by a factor of 18. The environmental quality standards for inland surface water were exceeded for Cd (national annual average AA-EQS 0.1 µg/L) in 2 mine-impacted streams and in 1 reference stream (Table 1). Cd was highest in IM1, although it did not exceed the AA-EQS adjusted for black shales catchment areas (0.8 µg/L). Maximum short time permissible level, MAC-EQS for Cd (0.45 µg/L) was not exceeded (Supplemental Data, Table S1). AA-EQSs for Ni (21 µg/L, or 22-35 µg/L in black shales area) or Pb (7.5 µg/L, or 7.7 µg/L for rivers with a catchment > 25% peatland, and 8 µg/L in black shales area) were not exceeded in any of the sites, although Pb in IM1 was elevated compared to the other sites. Fe and Al concentrations were equal or higher in the reference streams than in mine-impacted streams. On average, temperature was slightly higher and oxygen levels lower (probably reflecting higher temperature) in mine-impacted streams than in the reference streams. However, the most pronounced difference in water quality was the much higher specific conductance in mine-impacted sites (average 360 µS/cm, range 180-598), relative to the reference sites, (average 37 µS/cm, range 23-64) (Table 2). During 2012, the sulfate, sulfur and metal concentrations in all 3 mine-impacted streams fluctuated considerably (Supplemental Data, Table S2).

Larval behavior

Larval *H. dalecarlica* had 3 distinct behavioral patterns detectable by MFB: (i) high amplitude, low frequency signals of locomotory activity like crawling and swimming behavior, (ii) low amplitude and higher frequency signals of gill ventilation and (iii) inactivity (Figure 2).

In the reference sites, the mayfly larvae were in locomotory activity approximately 12.7 % of the measured time, and ventilated 1.4 % of the time. The mayfly larvae were more active (U=36.0, $p<0.01$) in the site IM1 near the mine, than in the reference streams (Figure 3).

Differences in larval behavior were not statistically significant between the mine-impacted site IM2 and the reference sites (locomotion U=67.5, $p=0.22$ and ventilation U=67.0, $p=0.21$), even though also in IM2 larvae tended to be more active than in the reference sites (Figure 3). Larvae were active on average 17 % of the time in the IM2, which is situated in the same watercourse but further downstream from the more polluted IM1, where larvae were active approximately 39 % of the time. In the most saline, mine-impacted IM3 the larvae ventilated less than in the reference sites (U=53.5, p=0.06) and also showed apparently lower (U=81.0, p=0.51) locomotory activity (Figure 3). Larval locomotory activity was associated moderately to water concentrations of Zn (r_s=0.61, p<0.05), and strongly to Cd (r_s=0.73, p=0.01) and Co (r=0.78, p<0.01). Larval ventilation was associated to Cd (r_s=0.61, p<0.05) and K (r= –0.61, p<0.05).

Survival of L. variegatus and H. dalecarlica

The survival of oligochaetes after the 3 day exposures was high (Table 3) with no difference between the treatments (p=0.998) or the stream pairs (blocks, p=0.383) and there was no interaction either (p=0.168). The only indication of stress was pale coloration and slime excretion in worms exposed at IM3. There was no difference in survival of *H. dalecarlica* larvae (t-test, p=0.6), but the number of exuviae (shed exocuticle) was higher in mine-impacted IM3 (5) compared to the reference site R3 (0) after exposure, suggesting higher molting rate in the mine-impacted stream.

Benthic invertebrate community structure

Benthic invertebrate fauna was typical of humic streams and also in reference sites consisted of taxa tolerant to acidic conditions and metals (Supplemetary Data, Table S3). For instance, mayflies were few and the test species *H. delecarlica* was missing from all the streams. The metrics considered (number of taxa, number of type-specific taxa, number of EPT-families, PMA) did not obviously indicate impairment of invertebrate fauna in mining-influenced streams relative to the reference streams (Table 2). In IM2 and R3 all metrics had values sufficiently close to the expected (type specific reference) values meeting the criteria of ‘good’ status at least, whereas in the other streams at least one metric (most often PMA) deviated from the expected value to the extent indicating ‘moderate’ status and suggesting human impact.

DISCUSSION

Biomining effects observed in the present study indicated metal pollution and salinization of stream waters, but inconsistent biological responses few years after the onset of the biomining operations. The mine-impacted streams had elevated concentrations of most metals, sulfur and sulfate compared to the reference sites. Median sulfate concentration in Finnish streams, 3.8 mg/L, (in black shale regions 8.3 mg/L) [20, 31] was clearly exceeded in all mine-impacted sites, most notably in IM3. Sulfate is formed when sulfide minerals are oxidized [5]. The elevated sulfate in mine effluents originates from the used sulfur compounds to control the bioheapleaching process (e.g. sulfuric acid H_2SO_4 to adjust pH of the irrigation solution) and neutralization of H_2SO_4 with lime and sodium hydroxide, the ore itself at low pH, and from raffinate solution containing heavy metals and iron placed into the gypsum ponds in summer 2012. The gypsum ponds are used to recover the excess metals prior discharging the effluents into the receiving water systems. Elevated Ca observed in the present study in mine-impacted sites is attributed to the use of lime (CaO and $Ca(OH)_2$) to precipitate the metals in alkali conditions. High Na concentrations in mine-impacted streams results from the use of NaOH to control the odor emissions (airborne hydrogen sulfides) and to regulate pH. Metals such as Cd, Co, Cu, Mn, Zn, U and presumably also Ba and Sr originate from the ore, and end up into mine effluents despite the precipitation processes.

The increase of major ions has been associated with anthropogenic disturbances including mining operations, and recognized harmful to invertebrates [32, 33]. Clements and Kotalik [33] found that the field-based specific conductance benchmark of 300 µS/cm, developed by the US Environmental Protection Agency, is protective of aquatic insect communities. Ephemeroptera were found to be particularly sensitive to increased major ion content of water, and e.g., mayfly drift was increased and abundance decreased at specific conductance near or <300 µS/cm [33]. This suggested benchmark of 300 µS/cm was exceeded in mine-impacted sites IM1 and IM3 of the present study.

Fe and Al concentrations were high in all sites, likely due to the peatland catchment of the streams. Fe and Al have been shown to occur naturally with highest concentrations in stream waters in peatland areas in Finland where acid conditions increase solubility, and transportation with dissolved and colloidic humic substances is suggested to occur [34]. Peatland catchment is typical in northern Finland [35], also in the study region, which contributes to the water quality also in the reference sites. Peatland drainage results in highly humic water with brown water color, low pH and elevated metals, especially Cr [35]. High water color is related to high DOC concentrations [36] but also Fe concentration has shown to affect water color [37]. DOC reduces metal bioavailability and thus toxicity by complexing metal ions [38]. At the present study Cr occurred at approximately equal concentrations in the mine-impacted and the reference sites. Water quality of the sites is thus a mixture of natural chemical stressors and chemical stressors originating from the biomining.

In the present study we found that behavioral response of larval *H. dalecarlica* to mine effluents was diverse and site-specific, which might reflect variable water chemistry in mine-impacted sites with different mixture of chemicals, and with contrasting natural water chemistry. Larval activity was increased in the metal-contaminated site IM1, but the opposite effect, although not statistically significant, was detected in another mine-impacted site IM3 with also a high sulfate concentration.

The observed variation in behavioral responses of mayflies is presumable related to different modes of toxicity in variable exposure conditions. Mayfly larvae breathe oxygen dissolved in the water by diffusion through body surface [39]. The larvae can enhance oxygen and carbon dioxide exchange by beating gills, and thus moving water across these respiratory surfaces. This gill beating was detectable by MFB. Gills are also important in osmoregulation. In hypotonic freshwater environment the larvae need to actively uptake ions from water to compensate for diffusive loss [40]. Chloride epithelia are specialized sites for ion uptake located in tracheal gills and in sides of the body in mayfly larvae, and are actively ventilated by gills. It has been shown that when salinity decreases, ventilation and eventually the density of chloride cells over subsequent moltings in mayfly larvae increase [39, 40]. In contrast, when salinity increases, both ventilation rate and the density of chloride cells decrease. We measured decreased gill ventilation in the mine-impacted site IM3 with very high water salinity compared to the other sites. Additionally, the larvae were molted clearly more often than in reference R3, which also may suggest a higher ionoregulatory and/or metal stress. Vuori & Kukkonen [41] found higher absorbed metal concentrations in recently molted trichopteran larvae than in unmolted larvae, and suggested that metals may either enhance the molting or that molting enhances metal accumulation. In contrast, the opposite has been observed with mayflies which reduced molting in response to increasing Pb concentrations [42]. Molting has been shown to be a primary mode of elimination for both adsorbed and absorbed Mn in aquatic insects [43].

In the present study we observed increased larval activity in the IM1 situated nearest to the mining area. Also Macedo-Sousa et al. [11] observed such activity increase in macroinvertebrates exposed to AMD, which could be interpreted as an escape response to avoid unsuitable environment. According to Gerhardt et al. [44], locomotion and ventilation of a mayfly larvae *Choroterpes picteti* (Leptophlebiidae) was higher in acid-only exposure compared to acid mine drainage exposure with similar pH, but the pH range was much lower (from 3.3 to 4.5) than in the present study. Behavioral stress responses to acid mine drainage have been observed also in other aquatic organisms, but with much higher metal concentrations than in the present study [44]. In general, mayflies and stoneflies are sensitive to metal contamination [7]. *H. dalecarlica* larvae seem not to inhabit the study sites, as it was not found in the benthic invertebrate samples. This species is known to be sensitive to low pH and metals, like most other Heptageniidae species [7, 45].

All metal and sulfate concentrations were lower in IM2, except for Cu and Mn, indicating dilution of the biomining effluents downstream and lower impacts of the mine, compared to the IM1 which is located nearer the mine within the same watercourse. We also observed the attenuation of differences in the behavioral activity. In both of these mine-impacted streams larval locomotion was higher than in the reference sites, but these differences were smaller and not statistically significant in IM2.

The survival of oligochaetes and mayflies after 3 days of exposure was not affected by the mine effluents although clear stress indications in oligochaetes (color change, slime excretion) were observed in the IM3. Hence, mine effluent risk assessment relying merely on the traditional acute endpoints is not recommended based on the present behavioral data. Chronic endpoints can surely catch the elevated risk more reliably but as those are more laborious and time consuming, early-warning behavioral endpoints can help site management. Aquatic insects are generally considered tolerant to acute metal exposure, but in contrast, they seem to be more sensitive to chronic exposure [46]. The discrepancy between acute and chronic effect concentrations has led to criticism for short-term metal toxicity testing with aquatic insects [47]. For example, Mebane et al. [48] found that the mayfly Rhitrogena sp. (Heptageniidae) was completely absent from a field site with Cu ≤5µg/L and with other potential stressors low, although 4-d LC50 (lethal concentration, 50%) as high as 137 µg/L has been reported for *Rhitrogena*. The lack of response of short-term survival tests in the present study is consistent with these earlier observations. In contrast, behavioral tests in the present study showed altered aquatic insect behavior in one of the mine-impacted sites, which may indicate the greater sensitivity of behavioral endpoints. Behavioral responses of a shrimp (*Atyaephyra desmaresti*) to acid mine drainage occurred more rapidly than effects on survival [10], suggesting also that behavioral endpoints may be more sensitive with shorter response times compared to traditional endpoints like survival. Behavioral changes can be detected rapidly within the first hours of exposure also according to Gerhardt et al. [44] and De Lange et al. [13]. Short response times reduce study length and therefore may reduce costs in risk assessments.

The benthic invertebrate community structure did not show clear effects of mine pollution. However, the reference condition approach, which we used in accordance with the national assessment scheme, suffered from the fact that we could not properly match the local reference sites with the impacted sites. They particularly differed in size, the reference streams R1 and R2 being much smaller than IM1 and IM2. Species richness is generally higher in bigger streams with larger catchment area [7, 49] and also species composition strongly varies with stream size [e.g. 30]. To alleviate this problem, macroinvertebrate metrics were compared with the external type-specific reference values representing expected communities unaffected by human disturbances [see 30]. However, due to various uncertainties, comparing the degree of deviation from reference values and resulting status classification across river types might still be unjustified. Where a fair comparison in this respect was possible, IM3 showed values only slightly smaller than or comparable to R3, suggesting no major effect. Among the impacted sites, IM1 closest to the mine had markedly lower metric values than the more distant and less polluted IM2 representing the same river type within the same watercourse. Whereas this might suggest a pollution effect, the reference site R1 corresponding to IM1 in having black scale-dominated catchment geology, showed the proportionally greatest deviation from reference values. Benthic macroinvertebrate communities are considered sensitive to mine water pollution [7, 8], but in naturally acidic streams, like those of our study, the benthic communities might be naturally impoverished and extant taxa be pre-selected for their resilience to ionic stress. Hence, in these regions in particular, behavioral assays such as those presented here using the MFB might provide a more sensitive early warning of potential adverse effects than classic benthic field surveys.

CONCLUSIONS

We assessed ecotoxicity of biomining effluents in stream ecosystems by *in situ* invertebrate bioassays. Water chemistry showed increased metals concentrations and increased major ion content (salinization) of the receiving water bodies by biomining effluents with some but inconsistent behavioral stress reactions across the mine-impacted sites. Larval ventilation and locomotory behavior of the regionally common mayfly species, *H. dalecarlica*, were distinguishable by Multispecies Freshwater Biomonitor© technique. Hence MFB proved to be technically suitable for measuring behavioral responses of the species for in situ risk assessment. Oligochaete and mayfly larvae survival was not acutely affected by mine effluents, and there were no clear effects on invertebrate communities either. However, behavioral changes in mayfly larvae were observed indicating behavioral responses can be more sensitive than survival or community responses in detecting an effect of water contamination by metals and sulfate. Our results highlight the challenge of defining behavioral stress reactions across sites with contrasting natural water chemistry and with variable mixture concentrations of effluents.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data availability—Readers can access the data and associated metadata and calculation tools by contacting the corresponding author (johanna.k.salmelin@jyu.fi).

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Figure 1. The study sites. Three mine-impacted streams, IM1 (R. Kalliojoki), IM2 (R. Tuhkajoki) and IM3 (R. Lumijoki), and 3 reference streams, R1 (R. Aittopuro), R2 (R. Kohisevanpuro) and R3 (R. Joutenjoki) are indicated by arrows. R1 does not receive mine effluents, but is located in the metalliferous black shales region like IM1. Mayfly larvae used in behavioral and survival tests were sampled from R. Mustinjoki. Black line indicates a boundary between northern Oulujoki and southern Vuoksi watersheds. Purple hatch shows Talvivaara mining district. Base map © MML.

Figure 2. *H. dalecarlica* larvae had 3 distinct behavioral patterns detectable by MFB: (i) high amplitude, low frequency signals of locomotory activity like crawling and swimming, (ii) low amplitude and higher frequency signals of gill ventilation and (iii) inactivity shown in the graph as amplitude (V) against the time (s). Data acquired from the reference R. Joutenjoki (R3).

Figure 3. Average time (%) (±SE) the mayfly *H.dalecarlica* larvae spent on locomotion or ventilation during 3 h exposure time in 3 reference sites, which data is pooled, and in 3 mine-impacted rivers (IM1=R. Kalliojoki; IM2=R. Tuhkajoki; IM3=R. Lumijoki) Asterisk denotes statistically significantly ($\alpha = 0.05$) differing behavior between the mine-impacted site and the reference sites.

Table 1. Water metal, phosphorus, selenium, sulfur and sulfate concentrations of streams during behavioral measurements as mean (±SD) of total concentration. Dissolved organic carbon (DOC) concentrations were measured in 2010–2015. Highlighted Cd exceeds the national AA-EQS for streams.

	Al	As	Ba	Cd	Co	Cr	Cu	Fe	Mn
Mine-impacted	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	µg/L
R. Kalliojoki (IM1)	340 (0.0)	0.29 (0.0)	18.0 (1.4)	0.37 (0.3)	1.20 (0.0)	0.80 (0.0)	1.45 (0.1)	1.90 (0.0)	185 (7.1)
R. Tuhkajoki (IM2)	310 (0.0)	0.26 (0.0)	16.0 (0.0)	0.20 (0.0)	0.78 (0.0)	0.60 (0.0)	1.70 (0.0)	1.30 (0.0)	370 (0.0)
R. Lumijoki (IM3)	235 (7.1)	0.26 (0.0)	16.5 (0.7)	0.15 (0.0)	0.55 (0.0)	0.65 (0.1)	1.40 (0.0)	1.30 (0.0)	510 (0.0)
Reference									
R. Aittopuro (R1)	410 (0.0)	0.22 (0.0)	12.0 (0.0)	0.02 (0.0)	0.61 (0.0)	0.80 (0.0)	1.10 (0.0)	1.50 (0.0)	88.5 (0.7)
R. Kohisevanpuro (R2)	400 (14.1)	1.30 (0.0)	9.20 (0.1)	0.08 (0.0)	0.97 (0.0)	0.80 (0.0)	1.25 (0.1)	3.55 (0.1)	41.0 (0.0)
R. Joutenjoki (R3)	370[a]	0.29	21.0	0.17	0.57	0.60	1.00	1.80	95.0

	Ni	Pb	Sr	Ti	Zn	U	V	Ca	K
Mine-impacted	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	mg/L
R. Kalliojoki (IM1)	16.0 (0.0)	3.50 (3.1)	32.0 (1.4)	4.95 (0.1)	37.5 (0.7)	0.16 (0.0)	0.83 (0.0)	12.9 (0.7)	0.95 (0.1)
R. Tuhkajoki (IM2)	16.0 (0.0)	1.14 (0.2)	23.0 (0.0)	3.45 (0.1)	39.5 (0.7)	0.14 (0.0)	0.77 (0.0)	7.90 (0.0)	0.70 (0.0)
R. Lumijoki (IM3)	10.0 (0.0)	2.15 (0.2)	75.0 (0.0)	3.85 (0.1)	11.0 (0.0)	0.16 (0.0)	0.79 (0.0)	48.6 (0.4)	1.55 (0.1)
Reference									
R. Aittopuro (R1)	1.60 (0.0)	0.34 (0.0)	12.0 (0.0)	4.95 (0.1)	4.00 (0.0)	0.07 (0.0)	0.86 (0.0)	2.10 (0.0)	0.65 (0.1)
R. Kohisevanpuro (R2)	13.0 (0.0)	0.72 (0.0)	15.0 (0.0)	6.50 (1.3)	14.0 (1.4)	0.04 (0.0)	0.96 (0.1)	3.60 (0.0)	0.90 (0.0)
R. Joutenjoki (R3)	1.40	2.90	14.0	5.70	6.00	0.07	1.10	1.80	0.30

	Mg	Na	S	P	Se	SO_4^{2-}	DOC
Mine-impacted	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
R. Kalliojoki (IM1)	4.35 (0.2)	31.5 (2.3)	34.0 (2.8)	<50	<0,2	95.0 (7.1)	17 (3.9)[b]
R. Tuhkajoki (IM2)	3.50 (0.0)	21.2 (0.1)	23.0 (0.0)	<50	<0,2	66.5 (0.7)	16 (1.4)[c]
R. Lumijoki (IM3)	9.40 (0.0)	56.3 (0.4)	83.5 (0.7)	<50	<0,2	255.0 (7.1)	25 (6.4)[c]
Reference							
R. Aittopuro (R1)	1.00 (0.0)	1.40 (0.0)	0.96 (0.0)	<50	<0,2	2.30 (0.0)	-
R. Kohisevanpuro (R2)	3.00 (0.0)	4.20 (0.0)	6.30 (0.0)	<50	<0,2	18.0 (0.0)	-
R. Joutenjoki (R3)	0.70	1.10	0.54	<50	<0,2	1.05 (0.1)	17 (4.3)[b]

[a] When SD is not provided, the concentration is based on a single measurement.
[b] H. Arola, University of Jyväskylä, Jyväskylä, Finland, unpublished
[c] the Finnish Environment Institute's Water Quality Database

Table 2. Water quality parameters measured in the field during behavioral measurements (mean ±SD), and the number of benthic macroinvertebrate taxa, type-specific taxa (TT), EPT-families (Ephemeroptera, Plecoptera, Trichoptera) and PMA-index describing benthic community structure in the study sites. The highlighted values indicates the cases when the biological quality of the site is lower than good, according to the classification criteria of EU's Water Framework Directive based on benthic macroinvertebrate metrics. The larvae of the mayfly test species, *H. dalecarlica*, were sampled from R. Mustinjoki.

Site	Temperature C°	Specific conductance µS/cm	O_2 mg/L	pH min–max	Number of taxa	TT[a]	EPT[b]	PMA[c]
Mine-impacted								
R. Kalliojoki (IM1)	11.3 (±0.1)	303 (±14.5)	9.5 (±0,01)	5.1 - 5.5	24	14 (21.3)	10 (13.1)	0.241 (0.424)
R. Tuhkajoki (IM2)	13.1 (±0.1)	180 (±2.1)	9.5 (±0.1)	5.1 - 5.6	37	21 (21.3)	12 (13.1)	0.443 (0.424)
R. Lumijoki (IM3)	10.7 (±0.05)	598 (±4.0)	9.13 (±0.04)	5.5 - 5.5	20	12 (14.3)	7 (9.5)	0.266 (0.429)
Reference								
R. Aittopuro (R1)	9.3 (±0.1)	24(±0.0)	10.9 (±0.1)	5.8 - 5.9	18	5 (9.1)	4 (7)	0.181 (0.471)
R. Kohisevanpuro (R2)	9.4 (±0.1)	64 (±0.6)	10.5 (±0.02)	5.1 - 5.5	18	6 (9.1)	5 (7)	0.237 (0.471)
R. Joutenjoki (R3)	9.7 (±0.2)	23 (±0.0)	10.7 (±0.9)	4.9 - 5.1	22	11 (14.3)	9 (9.5)	0.343 (0.429)
R. Mustinjoki	10.0 (±0.01)	24 (±0.0)	11.0 (±0.1)	5.8 - 6.0 [d]	-	-	-	-

[a] the reference value for number of type-specific taxa in parenthesis
[b] the reference value for number of type-specific EPT-families in parenthesis
[c] Percent Model Affinity [29], calculated according to Aroviita et al. [28]; the type-specific reference values in parenthesis
[d] acquired from the Finnish Environment Institute's Water Quality Database (measured in August 2011 and 2012).

Table 3. Survival of the oligochaete *L. variegatus* and larvae of the mayfly *H. dalecarlica* after 3-d exposures.

	L. variegatus			*H. dalecarlica*		
	Survival percentage	SD	n	Survival percentage	SD	n
Mine-impacted						
R. Kalliojoki (IM1)	95	7	6			
R. Tuhkajoki (IM2)	96	6	4			
R. Lumijoki(IM3)	80	12	5	97	9	8
Reference						
R. Aittopuro (R1)	89	11	5			
R. Kohisevanpuro (R2)	93	13	5			
R. Joutenjoki (R3)	86	15	6	95	9	7

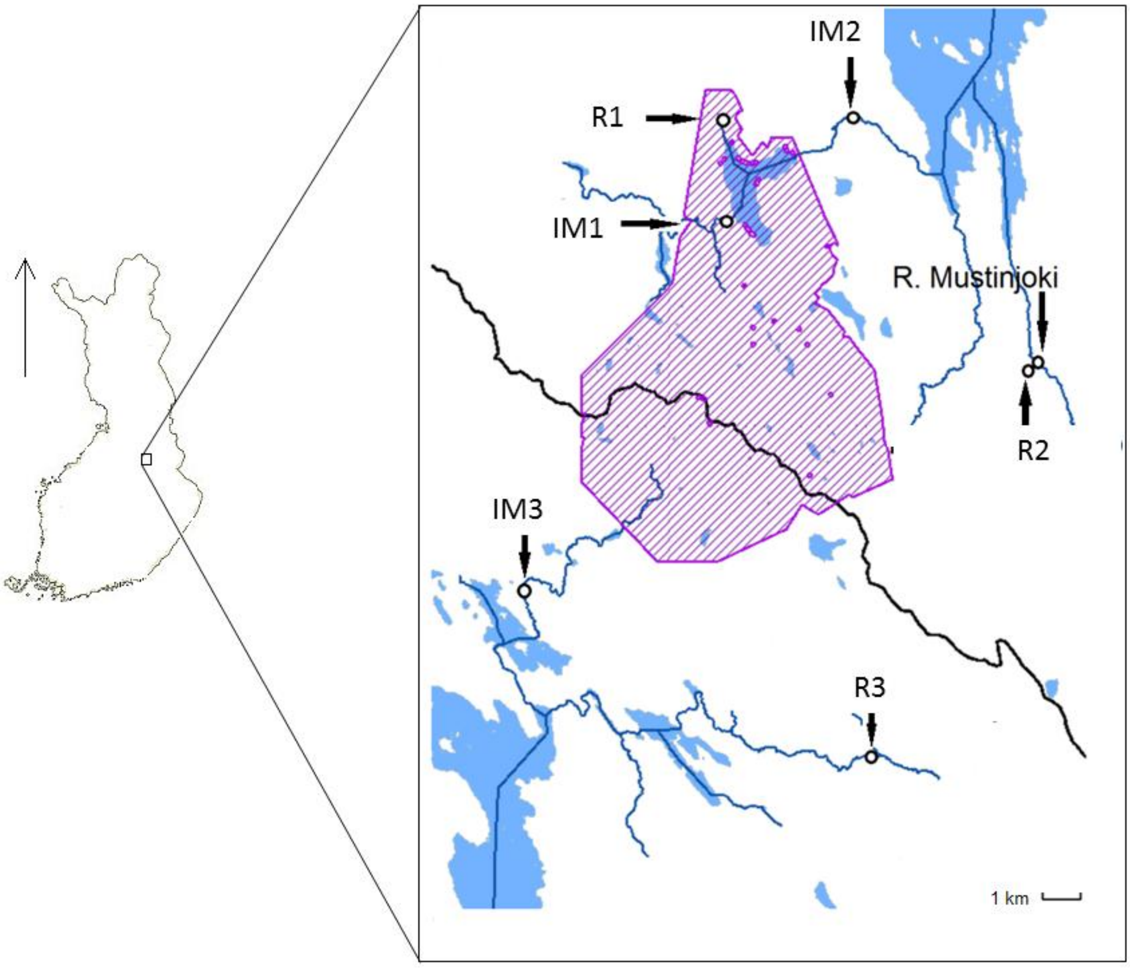


Figure 1

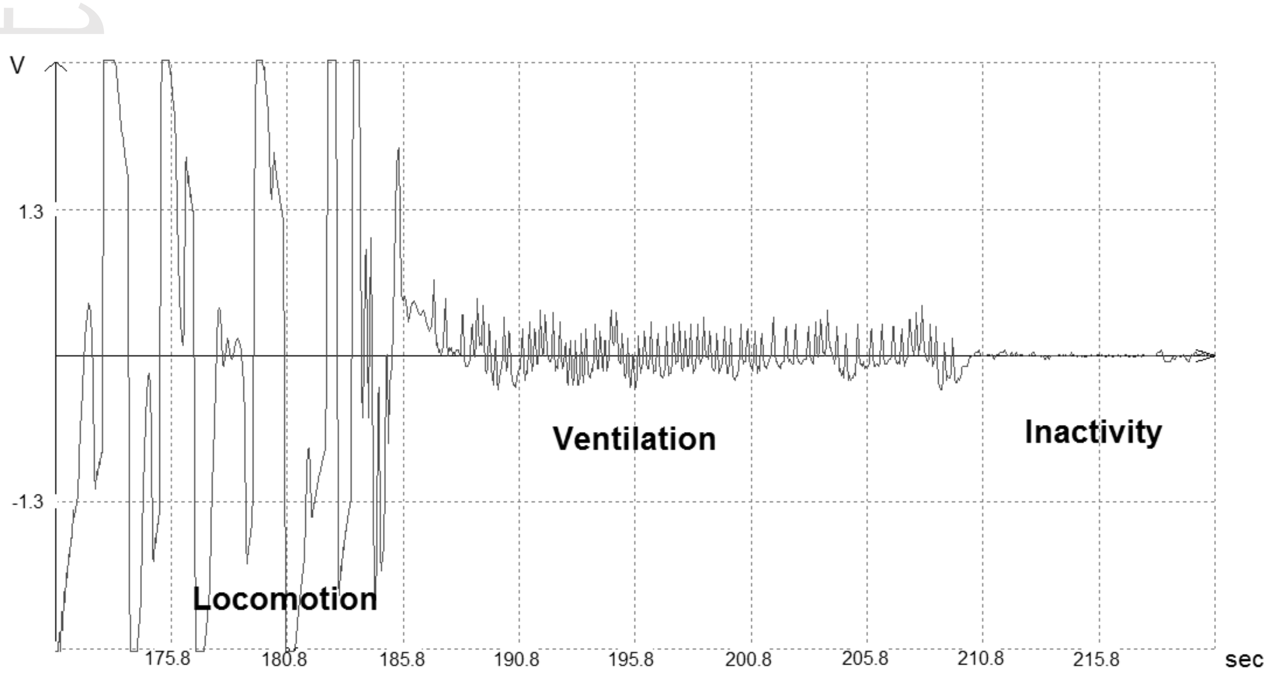
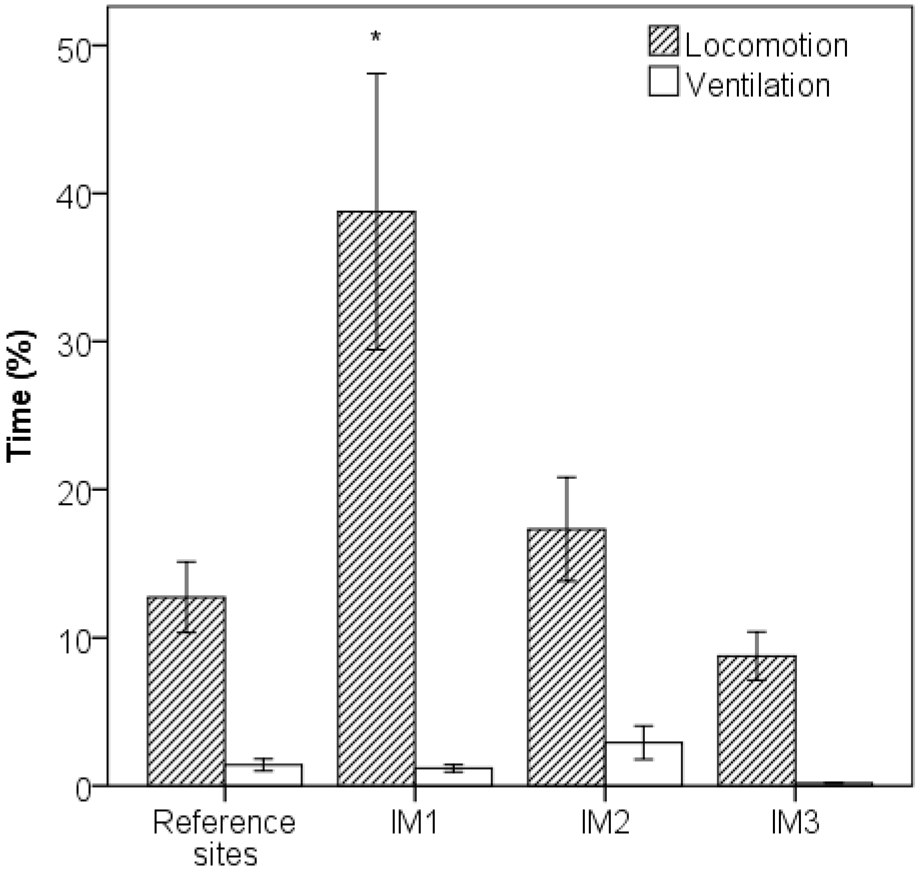

V
1.3
-1.3
Locomotion
Ventilation
Inactivity
175.8
180.8
185.8
190.8
195.8
200.8
205.8
210.8
215.8
sec

Figure 2



Locomotion
Ventilation
*
Time (%)
50
40
30
20
10
0
Reference sites
IM1
IM2
IM3

Figure 3