Influence of heavy metals on nematode community structure in deteriorated soil by gold mining activities in Sibutad, southern Philippines

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Abstract

Ore mining is among the most environmentally destructive anthropogenic practices, particularly in developing countries. Correct assessment of its impacts on soil ecosystems requires an understanding of the response of soil food webs. Nematodes, often the most abundant invertebrates in soils, occupy various positions in food webs, and their assemblages are commonly used to reflect soil health. In October 2014, we collected soil samples from five sites of a small-scale mining area in Sibutad, southern Philippines, to assess the influence of mining activities on nematode assemblages. Two sites were considered undisturbed as there were no visible signs of mining, while the other three sites were disturbed. Nematodes were extracted live and identified to genus level using morphology-based identification. We analysed genus composition, genus and trophic diversity, and the life-history based maturity index. We measured soil environmental variables (pH, organic matter, granulometry and several heavy metals), and correlated variation in nematode genus composition to variation in these environmental factors. Small-scale mining activities had variable but generally non-significant impacts on soil properties, altered vegetation and caused increases in concentrations of Hg and Pb, but not consistently so in all impacted sites. The high patchiness in vegetation and heavy metal content were reflected in a high within-site variability of nematode assemblages. Total nematode abundance was significantly lower in two mainly physically disturbed sites, but not so in the most metal-polluted one, suggesting that abundance is not a good indicator of pollution status. Nematode genus composition significantly differed between disturbed and undisturbed sites. By contrast, only few differences between sites were found for diversity or maturity indices, demonstrating that genus composition was a better indicator of mining-related effects than many common indicator indices and highlighting that detailed assemblage analysis is required for a correct interpretation of moderate pollution effects on soil

nematodes. Measured environmental variables together explained 60% of the variation in nematode assemblages in the area; the three 'single best' explanatory variables were the concentrations of Pb, Hg and N, but none of these by itself explained more than 8% of the variation in nematode data, while their combination explained 24%. Some genera of predacious and omnivorous nematodes, which are generally expected to be sensitive to both chemical pollution and physical disturbance (e.g., *Ironus* and *Eudorylaimus*), were most abundant in sites with elevated heavy metal concentrations, which can have repercussions for the interpretation of nematode-based indices such as the MI.

1 Introduction

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3   Ore mining, both large and small-scale, is an important contributor to the economy in many

4 developing countries. For instance, the Philippines is a major exporter of metallic minerals

5 such as gold, copper, nickel and chromium (Hooley, 2005). In Sibutad, a municipality in

6 Mindanao, southern Philippines, gold mining activities have provided livelihood to local

7 communities since the 1980's (Cortes-Maramba et al., 2006). Large-scale mining operations

8 make use of advanced technology in the extraction of mineral deposits, whereas small-scale

9 mining employs manual and fairly rudimentary techniques, which are often environmentally

10 risky (Hinton et al., 2003).

11   Small-scale mining produces about 80% of the Philippines' annual gold supply. However,

12 these substandard routines, aggravated by lack of proper ecological monitoring, can result in

13 deliberate and accidental disposal of wastes (van Straaten, 2000). Despite its economic

14 contribution, it remains a highly polarized issue due to incidences of environmental degradation

15 and health problems among exposed communities (Cortes-Maramba et al., 2006). Mining is

16 associated with the rise of heavy metals in the environment (Getaneh and Alemayehu, 2006).

17 Heavy metals are naturally deposited in rocks and can be released into the environment either

18 by natural weathering or by artificial activities (e.g., digging, ore processing, etc.). They pose

19 a threat because of their potential to bioaccumulate and interfere with various biological

20 processes (Heikens et al., 2001). The gold extraction method by mercury (Hg), also known as

21 amalgamation, is relatively popular among small-scale miners since it is inexpensive.

22 Compared to other mineral extraction methods, amalgamation is easier to perform but

23 potentially risky, and may cause environmental pollution due to improper handling and waste

24 management (Israel and Asirot, 2002; Odumo et al., 2014). Hg is considered to be one of the

25 most toxic elements naturally found in the environment even at very low concentrations

26 (Göthberg and Greger, 2006), and its negative impacts on soil biota (Harris-Hellal et al., 2009)

1 and soil processes are well-studied (Müller et al., 2002). In humans, Hg can induce damaging

2 effects on reproduction, immune system, central nervous system and internal organs (Dietz et

3 al., 2000).

4   Before the 1980's, our sampling area in Sibutad, was predominantly covered with cogon

5 grass (*Imperata cylindrica*), economically unproductive and had only few inhabitants. The

6 discovery of gold deposits in the 1980's caused an influx of miners, with an estimated peak of

7 10,000 in the early 2000's. Although the number of active miners has been gradually

8 decreasing since, a few hundreds are still operating around the mountain sides. Hence,

9 disturbance impact in small-scale mining areas in Sibutad may be caused by past and/or

10 existing mining activities. In practice, small-scale miners use ball mills to grind rocks into fine

11 particles, from which the gold is extracted by amalgamation and blowtorching, which results

12 in the formation of wastes (e.g. Hg and tailings). The lack of proper waste storage can cause

13 Hg and tailings to end up in the soil or river, and finally into Murcielagos Bay, a semi-enclosed

14 bay adjacent to the mined sites. At present, there are approximately 500 small-scale miners in

15 the area of Sibutad who can potentially release 120 to 360 kg of Hg per year (Perez et al.,

16 2007). Previous studies have revealed elevated Hg levels in humans (Cortes-Maramba et al.,

17 2006) as well as in marine organisms from Murcielagos Bay (Lacastesantos, *unpublished*),

18 whereas information on Hg effects on terrestrial animals or plants from the area is lacking. Our

19 initial inspection showed that the river bed of the sampling area was largely composed of thick,

20 dark-brown clay sediments and the water appeared very turbid. Preliminary river water analysis

21 revealed a Hg content of ca. 50 μg L^{-1} (our own unpublished data), which is 5 times higher than

22 the permissible limit for wastewater discharge by EPA, i.e., 10 μg L^{-1} (USEPA, 2014), and 25

23 times higher than the current water quality criterion for the protection of public health by the

24 Philippine government, i.e., 2 μg L^{-1} (www.emb.gov.ph). The high Hg content of the water is

25 most probably caused by the discharges from small-scale mining activities upstream. Mercury

1   concentrations higher than the allowable level proposed by UNEP (2013) are generally

2   expected to be toxic, and in Sibutad where Hg disposal is a problem, Hg levels in soils may

3   have exceeded the 'permissible' limit. Aside from heavy metal pollution, other activities such

4   as burning of vegetation, digging, construction of physical structures (e.g., tunnels, processing

5   plants, etc.) may also affect soil structure, organic matter content and soil pH, which can in

6   turn influence the biological activity of soil biota such as nematodes (Sánchez-Moreno et al.,

7   2006).

8   Nematodes are important biological components in the soil ecosystem due to their

9   functional roles in organic matter decomposition and nutrient cycling (Freckman, 1988; Yeates,

10 2003); their abundance and community composition are widely used as ecological indicators

11 in several different environments (Bongers and Ferris, 1999; Neher, 2001; Shao et al., 2008).

12 Nematode responses to pollution range from sensitive to very tolerant, with substantial

13 differences between species (Kammenga et al., 1994). Therefore, changes in the nematode

14 assemblage structure and function can be used to assess pollution effects or disturbances in

15 soil, and can be measured by diversity and ecological indices, as well as through a detailed

16 analysis of their taxonomic composition (Fiscus and Neher, 2002).

17   The present work was conducted to assess whether nematode assemblage structure reflects

18 the impacts of small-scale mining in the southern Philippines. Specifically, this research aimed

19 to a) determine the extent of pollution, particularly that of Hg, and other disturbances (e.g.,

20 burning of vegetation, digging, etc.) caused by small-scale mining activities in soils in a small-

21 scale gold mining area; b) assess whether the nematode assemblage structure differed between

22 locations with different degrees of mining-related impact; and c) determine whether such

23 mining impacts are better revealed by particular nematode-based (diversity and maturity)

24 indices or by nematode genus composition.

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1 **Materials and Methods**

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3 *Study site and sampling*
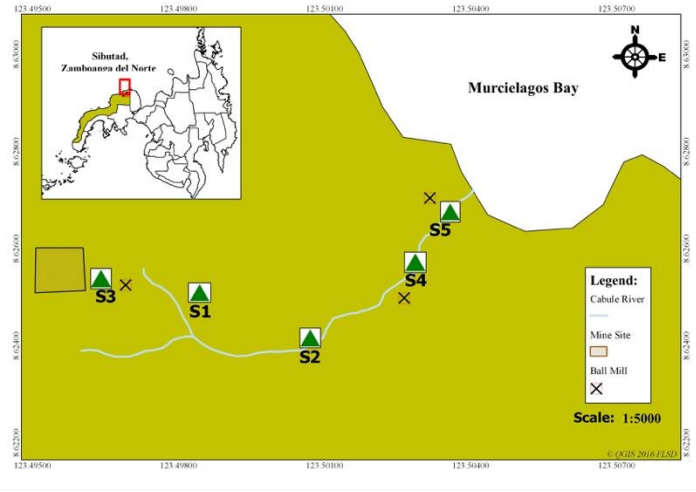
6 Fig. 1. Map of the sampling sites marked by triangles (S1, S2, S3, S4 and S5) in Sibutad, southern Philippines.

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9   The area of Sibutad is situated in the northwestern part of Mindanao, southern Philippines,

10 with an average annual temperature of 27.4 ^{0}C and precipitation of 2310 mm, the latter

11 distributed fairly evenly throughout the year. Our sampling area is situated on a slope of

12 mountain and covers approximately a distance of 1.2 km (between Site 1 and 5) towards

13 Murcielagos Bay (Fig. 1). Some parts of the area have been subjected to 'physical' disturbances

14 such as land clearing, excavation of mountain slopes, open-cast and underground mining,

1 construction of small processing plants and habitation by a few individuals, while other areas

2 have been chemically contaminated owing to mining and ore processing.

3
4 Table 1. Location and brief description of the sampling sites

sampling sites	coordinates	elev. (m)	common vegetation	brief description of the sampling sites
S1 (*undisturbed*)	8° 37' 28.560" N 123° 29' 55.248" W	42	*Imperata cylindrica, Chromolaena odorata, Manihut esculenta, Cocos nucifera, Gmelina arborea, Clitoria* sp., *Cynodon* sp. and ground ferns	no community; no mining activity
S2 (*undisturbed*)	8° 37' 25.176" N 123° 30' 3.384" W	31	*I. cylindrica, M. esculenta, Musa* sp., *C. nucifera* and *Cynodon* sp.	no community; no mining activity
S3 (*disturbed*)	8° 37' 29.676" N 123° 29' 48.300" W	50	*Paspalum conjugatum, Cynodon* sp. and *Musa* sp.	presence of local community (miners and their families); near to the excavated areas on the hill slopes; presence of two ball mill plants
S4 (*disturbed*)	8° 37' 30.864" N 123° 30' 11.196" W	10	*I. cylindrica, C. nucifera, Musa* sp., *G. arborea, C. odorata, Clitoria* sp. and *P. conjugatum*	presence of a local community (non-miners); presence of one ball mill plant
S5 (*disturbed*)	8° 37' 34.608" N 123° 30' 12.996" W	3	*P. conjugatum, C. nucifera* and ground ferns	presence of a few inhabitants (non-miners); presence of one ball mill plant

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6   Soil samples were taken in October, 2014. We divided the study area into five sampling

7 sites – S1, S2, S3, S4 and S5 (Table 1). Five replicate soil samples, each composed of 3

8 composite samples, were randomly collected with approximate interdistances of 8-10 m from

9 each of the sites. S1 and S2, 300 m apart from each other, were characterized by the absence

10 of inhabitants and mining activities, albeit S1 appeared to have a more diverse vegetation than

11 S2. Mining-related activities and/or local communities were manifest in S3, S4 and S5, thus

12 we *a priori* referred to them as 'disturbed' sites as opposed to the 'undisturbed' (reference)

13 sites, S1 and S2. Perennial grass species (e.g., *Paspalum conjugatum*) generally characterized

14 the disturbed sites (S3, S4 and S5) due to their relatively fast colonizing ability after disturbance

15 episodes. S3, the uppermost part (in terms of altitude) of the area, was marked by intense

16 mining activities with the presence of a community of miners (< 30 ind.), two ball mill plants,

17 and the site's close proximity to the excavated areas. S4 had the largest human population (>

18 40 ind.), who were not engaged in mining operations but hosted one ball mill plant. S5 was

19 also inhabited (< 5 ind.) and located about 0.25 km from Murcielagos Bay. An active ball mill

20 plant was found near S5, which was situated at an elevated ground a few meters away (ca. 20

1   m) from this site. Although we cannot rule out the possibility that the undisturbed sites had

2   previously been impacted by mining-related disturbances due to lack of information of the past

3   mining activities, the present Hg and other heavy metal levels were used to assess the impacts

4   of local mining activities since their operation in the 1980's.

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6   *Soil properties*

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8   Five replicate composite samples, each consisting of 500 g (a composite of 3 samples

9   combined), were collected from the upper 5 cm using a hand shovel. Soil samples were placed

10   in ziplocked plastic bags and tightly sealed in a box container until laboratory processing. From

11   each soil sample, 200 g were kept at 4 °C and utilized for the determination of basic soil

12   characteristics, nutrients and heavy metal analyses. Soil pH was determined potentiometrically

13   in the soil suspension of a 1:2.5 soil:water mixture (ISRIC, 1995). Total Organic Carbon was

14   measured by the Walkey-Black method, which involves wet combustion of the organic matter

15   with a mixture of potassium dichromate and sulfuric acid (Walkey and Black, 1934). Total N

16   was determined by the Kjeldahl method (Kjeldahl, 1883) and available P was extracted using

17   acidified ammonium fluoride (Chang and Jackson, 1958). Cu, Zn, Fe, Cd and Pb were extracted

18   by dilute hydrochloric acid procedures (Nelson et al., 1959) and measured by Atomic

19   Absorption Spectrometry, while Hg was measured by Cold Vapor Atomic Absorption

20   Spectrometry (CVAAS). We used empty sample cups treated in exactly the same way as real

21   samples as blanks, and NIST (National Institute of Standards and Technology, Gaithersburg)

22   standard MD 2089 as a reference or 'external standard' for method validation and

23   determination of analytical precision. Detection limits of the heavy metals Cd, Cu, Fe, Pb, Zn

24   and Hg were 0.002, 0.003, 0.006, 0.01, 0.001 and 0.02 mg kg^{-1}, respectively.

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Nematodes

From each homogenized soil sample, 100 g was taken for nematode collection using a modified tray method (Whitehead and Hemming, 1965). Total nematode abundance was determined and 100 individuals were randomly picked and identified to the genus level according to Andrássy (2005) and assigned 'colonizer-persister' scores according to Bongers (1990, 1999). Nematodes were designated into trophic groups, namely bacterivores, fungivores, omnivores-predators and plant parasites. Assignments to trophic groups used the genus list provided by Yeates et al. (1993).

Nematode assemblages were characterized by a) the absolute abundances per 100 g soil; b) genus richness, expressed as the number of nematode genera (note that we also calculated rarefied richness as expected numbers of genera, which yields a richness estimate that is independent of sample size; however, this resulted in nearly identical richness estimates, hence we prefer to work with the 'pure' richness data here); c) the Shannon-Wiener index (H'), which is a diversity measure encompassing both aspects of richness and evenness [$H' = \sum P_i (\ln P_i)$] (Shannon and Weaver, 1949); d) Simpson's index, calculated as [$1 - D = 1 - \sum P_i^2$], as a measure of evenness (Simpson, 1949); in both indices, P_i is the proportion of individuals of the ith taxon; e) the index of trophic diversity (ITD), a measure of the proportional abundance of each trophic group in the community, was calculated as ITD = [$1 / \sum P_i^2$] where P_i is the proportion of the ith trophic group in the nematode community (Heip et al., 1985); f) the Maturity index (MI), [$MI = \sum v_i p_i$], where v_i is the c-p score of a genus as designated by Bongers (1990, 1999) and p_i is the proportional abundance of that genus in the free-living nematode assemblage. The c-p values reflect the nematode life strategies, and range from 1 (colonizers, tolerant to disturbance) to 5 (persisters, sensitive to disturbance); and g) MI_{2-5} is a modification of MI which excludes nematodes with c-p scores of 1 because they tend to become proportionally more abundant under organic enrichment, and as such, their inclusion in the MI could potentially bias

interpretation of the effects of chemical pollution. The MI and MI_{2-5} reflect the (recent) disturbance history of a soil. In theory, the higher the maturity index values, the more mature and stable and the less disturbed the ecosystem. MI, MI_{2-5}, and other indices such as Structure Index (SI) and Enrichment Index (EI) were also calculated using the NINJA online programme (Sieriebriennikov et al., 2014; https://sieriebriennikov.shinyapps.io/ninja/).

Statistical analyses

Differences between sampling sites in any of the above-mentioned univariate descriptors of nematode assemblages (i.e. abundance, diversity indices, maturity indices) were analyzed using one-way analysis of variance (ANOVA) using the Statistica software package version 7.0. Data were first checked for normality with a Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. In case of a significant ANOVA result, pairwise comparisons between sites were performed using Tukey's HSD test.

Principal coordinates analysis (PCO) of the environmental variables was carried out to determine the differences between sampling sites based on the combination of measured environmental variables. These data included heavy metal concentrations and physico-chemical characteristics of the soil, and were normalized due to the differences in units. Non-metric multi-dimensional scaling (nMDS) was performed to visualize spatial patterns of nematode assemblages. The multivariate Permutational Analysis of Variance (PERMANOVA; Anderson, 2004) within PRIMER was then used to detect differences between nematode assemblages between the different sites, and between our two – admittedly arbitrary – *a priori* groupings of these sites: undisturbed (S1 and S2) and disturbed (S3, S4 and S5). Each term in the analyses was calculated using 999 permutations. Since PERMANOVA is sensitive to multivariate dispersion, PERMDISP was performed to check if observed differences were due to location effects or to heterogeneous variation. Prior to the multivariate analysis, nematode

1   abundances were square root-transformed to downsize the effect of dominant genera. When

2   significant differences were detected, pairwise comparison tests within PERMANOVA[+] were

3   conducted to establish differences between sites.

4   DistLM (Distance-based linear model) routine using a global BEST selection procedure

5   with Bayesian Information Correction (BIC) was carried out to identify the environmental

6   variables that best explained the observed patterns in nematode communities. Distance-based

7   redundancy analysis (dbRDA), a graphical visualization of the DistLM results, was used to

8   show patterns in assemblage composition and environmental variables across samples using

9   Pearson correlation. Similarity percentage (SIMPER) analyses using the untransformed

10   nematode abundance data were used to identify the genera which contributed to the similarities

11   or differences between study sites and between the undisturbed and disturbed sites. The genera

12   were considered 'important' if they contributed at least 5% of the average dissimilarity among

13   the sites (Mirto et al., 2002).

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1   **Results**

2   *Soil properties and heavy metal concentrations*

3   Table 2. Mean concentrations of heavy metals, nutrients and soil properties of the five sampling locations. Values
4   after the mean represent standard deviations (mean ± stdev of five replicates).

|	S1 (undisturbed)	S2 (undisturbed)	S3 (disturbed)	S4 (disturbed)	S5 (disturbed)
Basic soil properties					
OM (%)	7.4 ± 3.93	6.24 ± 1.18	4.53 ± 1.66	4.93 ± 2.86	4.66 ± 3.54
N (mg/kg)	0.32 ± 0.14	0.27 ± 0.08	0.23 ± 0.11	0.26 ± 0.13	0.14 ± 0.04
P (mg/kg)	2.14 ± 1.35	4.15 ± 4.38	3.83 ± 4.66	11.6 ± 9.93	1.5 ± 0.91
pH	5.23 ± 0.49	5.27 ± 0.8	4.58 ± 0.17	5.61 ± 1.02	4.6 ± 0.3
median grain size (µm)	66.3 ± 19.9[a]	24.5 ± 6.78[b]	24 ± 11.02[b]	28.8 ± 6.27[b]	75.5 ± 66.4[a]
clay content (%)	9.01 ± 2.34[a]	17.5 ± 2.92[b]	15.8 ± 3.93[bc]	14.9 ± 2.92[bc]	11.2 ± 3.29[ac]
Heavy metals (mg/kg)					
Cd	1.13 ± 0.82	1.06 ± 0.91	0.87 ± 0.74	1.18 ± 0.86	1.16 ± 1.01
Cu	84.8 ± 113	35.0 ± 11.5	45.5 ± 31.2	85.9 ± 47.2	59.4 ± 35
Fe	2597 ± 703	2346 ± 413	2634 ± 770	2684 ± 861	2098 ± 781
Hg	0.49 ± 0.6[a]	2.00 ± 1.56[a]	1.34 ± 0.83[a]	38.4 ± 43.3[b]	1.51 ± 1.63[a]
Pb	27.6 ± 8.5[a]	32.3 ± 6.95[a]	27.5 ± 13.7[a]	136 ± 78[b]	48.9 ± 11.6[b]
Zn	47.7 ± 73.4	33.3 ± 16.1	24.6 ± 21	65.4 ± 26.9	30 ± 7.8

5   Mean values followed by different letters on the same row indicate significant differences according to a post-hoc
6   Tukey HSD test ($P < 0.05$).
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9   Several soil properties such as OM, N, P and pH did not show any significant difference

10   between sites; however, OM, N and pH (except for S4) tended to be lower in the disturbed

11   compared to the undisturbed sites. Median grain sizes in S2, S3 and S4 were significantly

12   smaller (all $P < 0.05$) compared to S1 and S5 (Table 2). In terms of grain size, in general,

13   disturbed sites, S3 and S4, had significantly finer grain size and a higher clay content compared

14   to S1, but not S2. Heavy metal concentrations in the disturbed areas were not significantly

15   increased except for Hg, which was highest in S4 ($P < 0.05$), and Pb, which was significantly

16   higher in S4 and S5 than the rest of the sites. Although S3 and S5 had ball mill plants, the lower

17   Hg content in these areas compared to S4 suggest that the tailings were most probably disposed

18   off elsewhere and not on the sampling site.

19   In a principal coordinates analysis (PCO) of the soil properties (Fig. 2), PCO1, explaining

20   29.6% of the observed variation, showed that Site 4 was associated with higher metal

21   concentrations including Zn ($r = 0.7$), Pb ($r = 0.67$), Cd ($r = 0.57$), Cu ($r = 0.56$) and Hg ($r =$

22   0.50), with higher pH ($r = 0.66$) and with higher concentrations of N ($r = 0.48$) and P ($r = 0.83$)

23   (Supplemental Material - ESM 1). PCO2 accounted for 28.4% of the observed variation and

1   positioned 2 replicates of S4 and 1 replicate of S1 apart from other sampling sites; this axis

2   was positively associated with increasing Hg ($r = 0.72$), Fe ($r = 0.68$), Cu ($r = 0.54$), Pb ($r =$

3   0.46) and Zn ($r = 0.45$), while negatively associated with N ($r = -0.67$), OM ($r = -0.61$) and pH

4   ($r = -0.58$) (Supplemental Material - ESM 1). Samples for all sites were rather scattered in the

5   ordination plane in general, except that it was more pronounced for S4 and S1 (Fig. 2).

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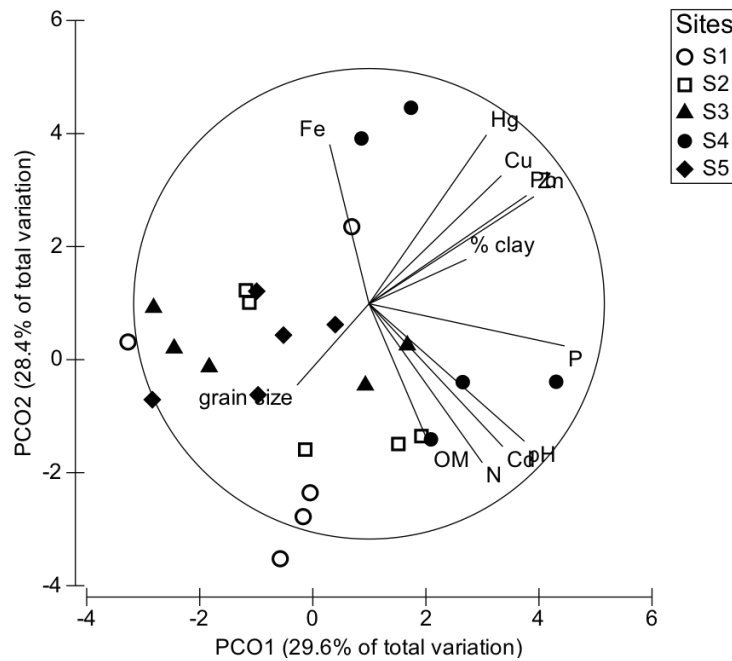
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8   Fig. 2. Principal coordinates analysis (PCO) of the environmental variables from the
     different sampling sites in the Sibutad small-scale mining area. See table 2 for an
9   overview of environmental variables included in the analysis.

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12   *Nematode abundance, genera, diversity and maturity indices*
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15   Total nematode abundance showed significant differences between locations (df = 4; F =

16   3.65; *P < 0.05*); highest density (412 ± 160 ind/100 g soil) was found in S1, whereas S3 had

17   the lowest abundance (204 ± 59 ind/100 g soil) (Fig. 3A). Nematodes belonged to 49 genera,

18   12 of which were bacterial feeders, 5 fungal feeders, 20 omnivores/predators and 12 plant

19   feeders (Supplemental Material - ESM 2). Index of trophic diversity did not show any

1 significant differences (df = 4; F = 2.01; *P > 0.05*) between sites (data not shown), but genus

2 richness did (df = 4; F = 3.61; *P < 0.05*): S1 and S2 had significantly higher number of genera

3 than S5 (Fig. 3B). Shannon diversity and evenness (Simpson index) did not differ significantly

4 among sites (df = 4; F = 2.82 and F = 4.87 for Shannon diversity and evenness, respectively; *P*

5 *= 0.054* and *P = 0.091* respectively; Fig 3C). Nevertheless, there was a trend indicating a higher

6 diversity in undisturbed compared to disturbed sites. Finally, S5 had the highest MI and MI_{2-5},

7 while S3 had the lowest (Fig. 3D), but these differences were not statistically significant.
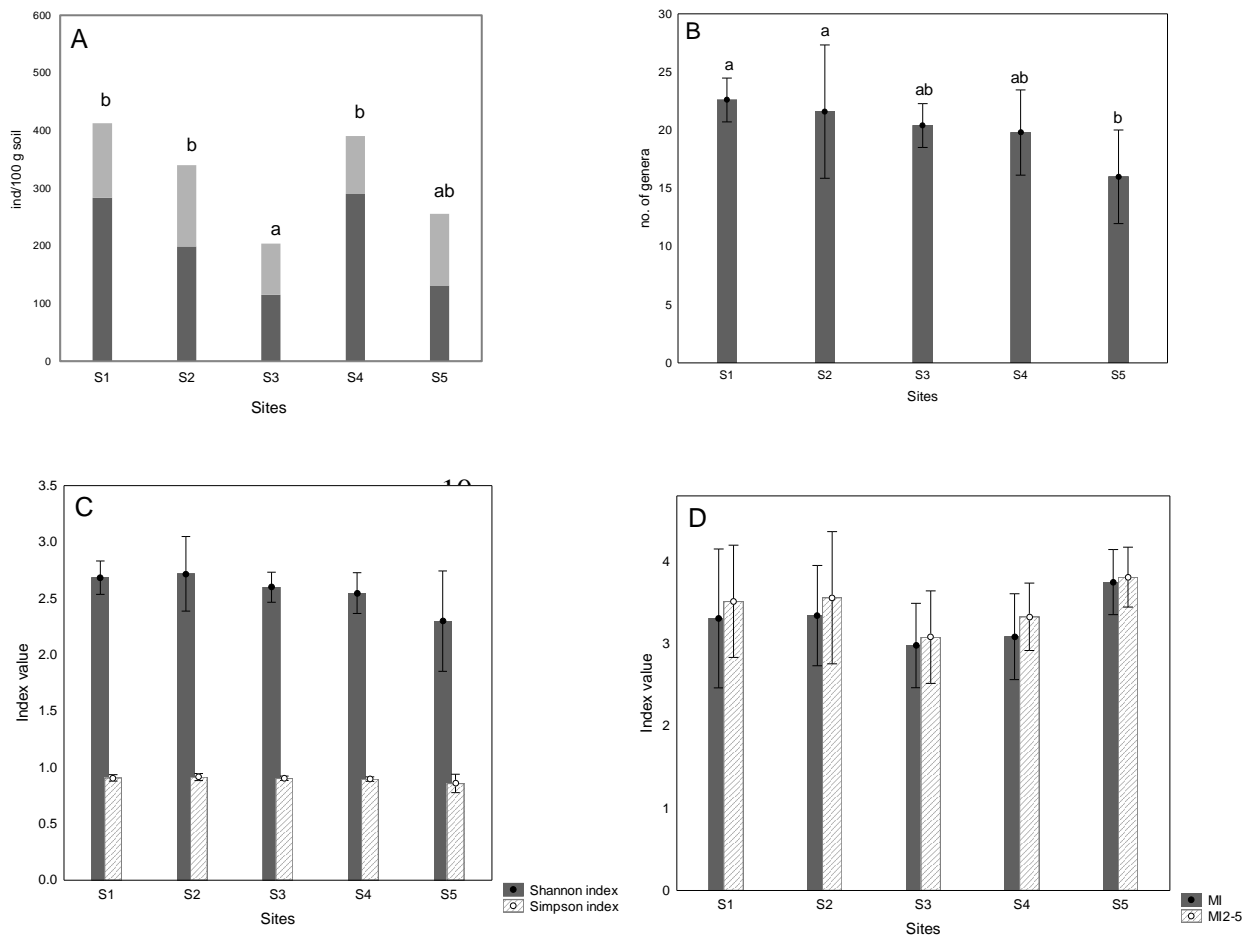
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Fig. 3 (A-D). Summed abundances of plant-parasitic (light-colored bars) and free-living nematodes (dark-colored bars) (A), species richness (B), Shannon and Simpson indices (C), and MI and MI_{2-5} (D). Different letters indicate significant pairwise differences between sites according to a post-hoc Tukey HSD test (*P* < 0.05).

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1   *Nematode assemblage composition*

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3   PERMANOVA revealed highly significant differences in nematode composition between

4   locations (df = 4; F = 3.53*; pseudo-P = 0.001*), with a non-significant PERMDISP (*PERMDISP*

5   *= 0.66*). Pairwise comparisons detected significant differences between all pairs of sites, except

6   the two undisturbed sites, S1 and S2 (Table 3).

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8   Table 3. Pairwise comparisons of nematode assemblage composition (PERMANOVA) between different sites.

Sites	S1 (*undisturbed*)	S2 (*undisturbed*)	S3 (*disturbed*)	S4 (*disturbed*)	S5 (*disturbed*)
S1 (*undisturbed*)	-	0.273	0.006[*]	0.028[*]	0.012[*]
S2 (*undisturbed*)	0.273	-	0.023[*]	0.007[**]	0.011[*]
S3 (*disturbed*)	0.006[**]	0.023[*]	-	0.005[**]	0.005[**]
S4 (*disturbed*)	0.028[*]	0.007[**]	0.005[**]	-	0.007[**]
S5 (*disturbed*)	0.012[*]	0.011[*]	0.005[**]	0.007[**]	-

9   Asterisks (*) and (**) indicate significant differences at *P < 0.05* and *P <0.01*, respectively.
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12   Table 4. Results of the SIMPER (Similarity Percentages) analysis of the nematode data between the undisturbed
13   (S1 and S2) and disturbed sites (S3, S4 and S5). Multiple genera contributed to the site differences. Listed below
14   are all the genera contributing up to a cumulative contribution (Cum. cont. %) of ≥ 75% to such differences.
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Genera	Average abundance		Cum. cont. (%)
Average dissimilarity = 70.03%	undisturbed (S1, S2)	disturbed (S3, S4, S5)	
Iotonchus	31.91	7.35	5.66
Axonchium	29.67	9.29	11.02
Rotylenchulus	21.40	12.25	16.36
Dorylaimellus	8.36	20.22	21.44
Cephalobus	17.55	21.52	26.33
Mesodorylaimus	24.77	21.79	31.28
Helicotylenchus	24.42	5.46	35.96
Eudorylaimus	14.91	22.99	40.42
Mesocriconema	11.00	17.27	44.56
Aphelenchus	20.76	8.05	48.69
Bursilla	11.38	11.64	52.63
Heterocephalobus	17.90	8.15	56.28
Xiphinema	13.97	6.77	59.53
Pratylenchus	4.48	13.45	62.62
Metaporcelaimus	16.13	5.19	65.63
Oxydirus	10.55	9.68	68.60
Ironus	1.62	14.63	71.46
Ecumenicus	7.05	11.51	74.14
Rotylenchus	11.50	2.23	76.74

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28   SIMPER analysis showed that all site pairs had high levels of dissimilarity in nematode

29   assemblages (Supplemental Material - ESM 3). The largest dissimilarity was between S3 and

30   S5 (75.57%), while S1 and S2 were the least dissimilar (63.03%), but only slightly less so than

1   the other site pairs, even though PERMANOVA did not detect significant differences between

2   both undisturbed locations. Several genera were identified to be responsible for the 70.03%

3   dissimilarity between the undisturbed (S1 and S2) and disturbed sites (S3, S4 and S5) (Table

4   4). Particularly, the 'important' genera (i.e. genera contributing roughly 5% to the dissimilarity

5   between the undisturbed and disturbed sites) included *Iotonchus*, *Mesodorylaimus*, *Axonchium*,

6   *Rotylenchulus* and *Helicotylenchus*, all of which were more abundant in the undisturbed sites,

7   and *Dorylaimellus* and *Cephalobus* which were more abundant in the disturbed sites. Six

8   genera were exclusively found either in undisturbed or disturbed sites, but only contributed <

9   1% to the difference between sites: *Opisthodorylaimus*, *Granonchulus* and *Chronogaster* in

10   undisturbed sites, while *Coslenchus*, *Oriverutus* and *Mononchulus* in disturbed sites.

11   dbRDA1 explained 17.7% of the total variation in the nematode data and generally

12   distinguished S5 from the other four sites (Fig. 4). dbRDA1 was positively associated with the

13   relative abundances of *Helicotylenchus* ($r = 0.61$), *Rotylenchulus* ($r = 0.56$), *Aphelenchus* ($r =$

14   0.54), *Alaimus* ($r = 0.49$) and *Paractinolaimus* ($r = 0.47$), while negatively with *Eudorylaimus*

15   ($r = - 0.49$) and *Dorylaimellus* ($r = - 0.83$) (Fig. 4A). dbRDA1 also had a strong negative

16   correlation with Cd ($r = - 0.87$) (Fig. 4B). On the other hand, dbRDA2 generally 'separated'

17   S4 and a few replicates of S3 and S5 from the undisturbed sites, S1 and S2, while explaining

18   14.9% of variation. dbRDA2 was correlated with Pb ($r = 0.68$) and Hg ($r = 0.63$), and the

19   genera positively correlated with it included *Acrobeloides* ($r = 0.60$), *Cephalobus* ($r = 0.53$),

20   *Pratylenchus* ($r = 0.49$), *Bursilla* ($r = 0.48$), and *Ironus* ($r = 0.48$).

21   A distance-based linear model including all the measured environmental variables

22   explained 60.4 % of the fitted variation (i.e. 32.6 % of the total variation at the two first dbRDA

23   axes) in the nematode data, which suggests that non-measured variables (e.g. vegetation,

24   species interactions) are more important drivers of nematode assemblage structure in our

25   sampling area. The best DISTLM with no more than three variables, Pb, Hg and N, explained

1 24.5 % of the fitted variation of nematodes in the area. These three variables also yielded the

2 three best DISTLM models with a single variable, and dominated the best model solutions with

3 two environmental variables (Supplemental Material - SM 4).
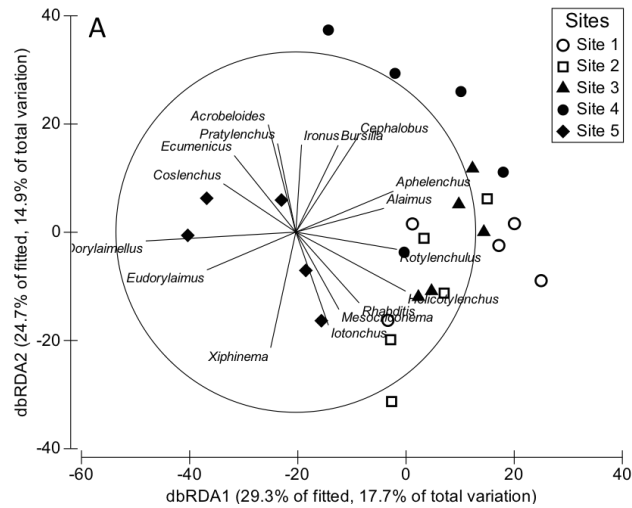
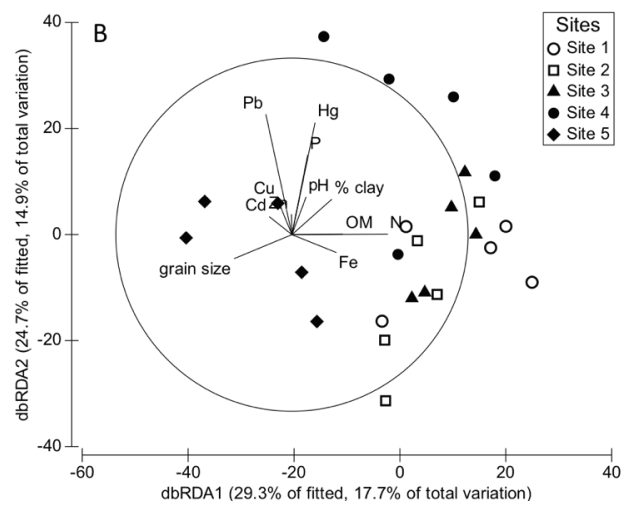4

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Fig. 4 (A and B). Distance-based Redundancy Analysis (dbRDA) plots based on
the nematode assemblages and the fitted environmental variables as vectors.

17

Discussion

Several studies have been conducted in large-scale mining areas (Pen-Mouratov et al., 2008; Shao et al., 2008) but researches dealing with the direct impact of small-scale mining activities on soils and their soil fauna assemblages have hitherto been more scanty (Harris-Hellal et al., 2009; Odumo et al., 2014). This is probably due to the fact that large-scale mining operations can result in more obvious and drastic ecological disturbances, which may require immediate intervention. Small-scale mining activities may also cause indirect impacts by changing the basic soil characteristics, vegetation and distribution of heavy metals, which in turn affect soil organisms. The proliferation of small-scale mining activities in the Philippines remains a threat because they are not properly regulated, and the extent and severity of their ecological impacts are not well studied.

Basic soil properties and heavy metals

The impacts of small-scale mining activities were reflected by the higher levels of heavy metals (mainly Hg and Pb), but not consistently so: Hg was only strongly elevated at S4, while Pb at S4 and S5. There were hints of subtle differences in other soil properties (e.g., OM, N, pH and granulometry) between undisturbed and disturbed sites, but apart from granulometry, these were never statistically significant. Vegetation thus provided the only obvious differences between undisturbed (S1 and S2) and disturbed (S3, S4 and S5) sites. These disturbances by mining activities may be 'physical' due to the location being close to the excavated areas, as in the case of S3, or 'chemical' due to the locally higher concentrations of heavy metals, such as in S4 and S5.

Mining sites are usually characterized by more acidic soils with low OM concentrations (Johnson and Hallberg, 2005; Banning et al., 2008; Šalamún et al., 2014) and fine soil particles due to the ball milling process, characteristics which we observed only partly, i.e. finer

1   sediments in some disturbed locations. In addition to edaphic differences, the disturbed sites

2   can also be distinguished by several fast-growing grass species (i.e. *Paspalum conjugatum*,

3   most common in the study area), which can easily establish and dominate during post-mining

4   succession (Groninger et al., 2007). The disturbed sites, S3 and S4, had significantly finer grain

5   sizes (mean of 24 µm and 28.8 µm, respectively) with a higher contribution of clay (15.8% and

6   14.9%, respectively) compared to the rest of the sites (except to S2), which may be caused by

7   the disposal of fine soil residues from the ball mill plants. Unexpectedly, S2 had a similarly

8   fine grain size similar as S3, possibly due to past mining-related disturbances, which is further

9   supported by the relatively high Hg concentrations at this location. This may also explain why

10   the nematode genus composition at S2 was closer to that of the disturbed mining sites than S1

11   in a nMDS plot (data not shown).

12   Due to a lack of established allowable ranges of heavy metals in the Philippines, we

13   compared our data to existing literatures from elsewhere. However, caution is needed when

14   extrapolating since metal effects in soils are influenced by pH, clay and organic matter content

15   (Rieuwerts et al., 1998). Heavy-metal levels of the present study were lower than the allowable

16   concentrations imposed by regulatory bodies from developed countries (Teh et al., 2016),

17   except Hg when compared to UNEP limits. While the world average Hg levels in soil ranges

18   from 0.01 mg kg^{-1} to 0.2 mg kg^{-1} (Adriano, 2001), UNEP (2013) recommends an acceptable

19   range from 0.07 mg kg^{-1} to 0.3 mg kg^{-1}. In the present study, all Hg concentrations, except

20   those of S1, exceeded acceptable levels as defined by UNEP (2013), indicating a mining history

21   in all sites except S1.

22
23   *Nematode abundance, diversity and maturity indices*

24
25   Nematode abundances in Sibutad were in the range of some heavy metal pollution-impacted

26   sites in, e.g., China and Israel (Shao et al., 2008; Pen-Mouratov et al., 2008), which suggests

1 that the whole area was impacted at least to some extent. A general trend of low nematode

2 abundances in some of the locations (S2 and S3) may be attributed to the finer grain size,

3 probably caused by disposal of very fine soil residues or tailings, especially in S3, during the

4 mineral extraction processes. This suggests that S2, although currently undisturbed, has also

5 been exposed to previous mining activity, which is to some extent reflected in the Hg

6 concentration (see above). Grain size can affect nematode communities; often, lower densities

7 are observed in finer textured compared to coarser soils (Anderson et al., 1979; Sánchez-

8 Moreno and Navas, 2007). Clayey soils, which contain a substantial fraction of very fine

9 particles, are characterized by reduced soil pores and a high water content. Since nematodes

10 move along soil spaces, clayey soils can impede their movement and the associated high water

11 content can result in oxygen deprivation (Glazer, 2002). No clear nematode abundance trends

12 were, however, observed between nematode densities and our a priori classification of the

13 different sites. S3 and S4, for instance, were both impacted, yet they differed in the type of

14 disturbance related to exploratory mining activities ('physical' vs 'chemical' disturbance),

15 among other things in a quite different vegetation cover (S4 being more diverse than S3). Plants

16 can affect the soil biota (e.g., nematodes) in several ways – e.g., root exudates and the high

17 inputs of dead OM can cause high abundances of bacteria which can serve as food to bacterial-

18 feeding nematodes (Bongers and Ferris, 1999; Bais et al., 2006). S3 had the lowest and S4 the

19 second-highest mean nematode abundances, suggesting that nematode abundance is a useful

20 indicator of 'physical' disturbance in this area (Neher, 2001; Fiscus and Neher, 2002;

21 Schratzberger and Jennings, 2002), rather than of heavy metal pollution *per se* (Bongers, 1990;

22 Korthals et al., 1996).

23   Diversity indices have been used by soil ecologists to assess the impacts caused by heavy-

24 metal pollution, although Pen-Mouratov et al. (2010) found that nematode diversity indices

25 were more affected by soil properties, whereas 'ecological indices' such as the maturity index

1   were more sensitive to disturbance. In many cases, impacted areas are characterized by low

2   nematode diversity compared to non-impacted areas due to the elimination of sensitive taxa

3   (Yeates et al., 1995; Sánchez-Moreno and Navas, 2007; Park et al., 2011) and the increased

4   dominance of tolerant taxa (Lambshead, 1986). This was partly confirmed in the present study

5   where S1 had the highest genus richness, while S5 had the lowest, despite the fact that S4 was

6   the most contaminated site. This is probably due to the higher plant diversity in S4 compared

7   to S5 (Šalamún et al., 2017). Other diversity indices such as Shannon-Wiener and Simpson,

8   however, did not show any significant differences between locations, although they tended to

9   decrease from undisturbed to disturbed sites: S2 ≥ S1 ≥ S3 ≥ S4 ≥ S5, and this trend was only

10   borderline non-significant ($P = 0.054$) for Shannon-Wiener (H') diversity.

11   In a similar study on metal-pollution impact by Chen et al. (2009), H' index values in less

12   disturbed areas (from 2.24 to 2.69) were fairly comparable to the results from the Sibutad

13   undisturbed sites (2.68 and 2.72), while H' in our disturbed sites (2.3 being the lowest)

14   overlapped with those of the 'undisturbed' areas from that study. This suggests that diversity

15   indices should not merely be compared with those of other studies on the basis of their absolute

16   values, but interpreted in a context-dependent manner (e.g., vegetation, soil type, pollution

17   levels, history…). Aside from soil pH, other factors such as root architecture, root exudates,

18   and soil type also need to be taken into account since they can influence the bioavailability of

19   heavy metals in soil (Rieuwerts et al., 1998; Mench and Martin, 1999).

20   Maturity indices of nematodes have also been used extensively to assess the status of soil

21   health. In principle, higher MI values (MI and MI$_{2-5}$) suggest a more stable and less disturbed

22   environment (Bongers and Ferris, 1999; Neher, 2001). For instance, a negative impact of heavy

23   metal (such as Cu, Ni) concentrations exceeding 100 mg kg^{-1} on the MI was observed in

24   terrestrial systems (Korthals et al., 1996). However, this cannot be easily translated to our

25   results, where the lowest and highest MI values (MI and MI$_{2-5}$) were both found in a disturbed

site, S3 and S5, respectively, and both with a rather high variability between replicates. Counterintuitively, S5 combined the highest MI values with the lowest Shannon diversity, which was attributed to the high proportional abundance of cp3-5, with a pronounced contribution of *Eudorylaimus* (> 10%). A high MI value in S5 is counter to the overall expectation that disturbance wipes out sensitive taxa and enhances the dominance of tolerant and/or successful colonizer taxa (Yeates et al., 1995; Bongers and Ferris, 1999; Sánchez-Moreno and Navas, 2007). The implicit assumption of the MI and related indices that large-bodied predacious or omnivorous nematodes (with cp scores of 4-5, sometimes 3) are more sensitive and are therefore more easily lost from a system after a strong disturbance (Korthals et al., 1996; Nagy et al., 2004) does not always hold. For instance, in our study, nematodes with cp3-5 scores did not always display such sensitivity under moderate pollutant concentrations, in agreement with other recent studies (Heininger et al., 2007; Šalamún et al., 2011; Gutiérrez et al., 2016). In fact, 40% of the nematode genera, and between 25 and 40% of the abundances in our study were predators/omnivores with a cp score of 4 or 5, and this did not systematically differ between disturbed and undisturbed sites (Supplemental Material - SM 2). It does explain why MI values were generally high in all our study sites.

Diversity, maturity and other related indices (e.g., SI and EI) were not markedly different between sampling sites due to the high variability between replicate samples. For instance, mean differences of maturity index up to ca 0.7 – the variability found here between replicate samples at a single location – are usually considered high; such high within-site variability may be linked to the patchiness of both vegetation and heavy metal content (pers. observation), where vegetation type affects MI directly through inputs of OM, or indirectly through effects on soil type, bacterial abundance, metal bioavailability, etc. (Yeates, 1999). Hg was very patchily distributed on a small scale (a range of 0.4 to 38.4 ppm), resulting in much more localized pollution impacts than we had anticipated. Alternatively, the high dispersion in index

1   values and assemblage composition in our study could be taken as evidence of the importance

2   of physical disturbance as a driver of nematode assemblage structure and diversity (Fonseca

3   and Gallucci, 2016).

4

5   *Nematode genera associated with heavy-metal pollution*

6

7   Previous studies showed that nematode community composition can be sensitive to soil

8   management practices or disturbances (Fiscus and Neher, 2002; Sánchez-Moreno et al., 2006).

9   While the nematode-based indices did not reflect the mining-related disturbances, significant

10 differences in nematode genus composition between undisturbed (S1 and S2) and disturbed

11 sites (S3, S4 and S5), and between all pairs of sites except S1 and S2, were strong indications

12 of the impact of ongoing or recent small-scale mining activities which altered the physico-

13 chemical attributes of the soil, and in turn, differentially impacted nematode genera (Fiscus and

14 Neher, 2002).

15 Important genera characteristic of the undisturbed sites included the free-living nematodes

16 *Iotonchus* and *Mesodorylaimus*, and the plant-feeding nematodes *Axonchium*, *Rotylenchulus*

17 and *Helicotylenchus*, while *Cephalobus* (free-living) and *Dorylaimellus* (plant-feeding) were

18 characteristic of the disturbed sites (Table 4). Our results thus confirm those of Šalamún et al.

19 (2012) concerning the near-absence of *Iotonchus* and the high sensitivity of *Mesodorylaimus*,

20 a cp4 nematode, to chemical disturbance (Bongers, 1990; Chen et al., 2009). Thus, the two

21 free-living genera may be considered indicator taxa in relation to mining-related disturbance,

22 because based on a community analysis, they contributed most to the dissimilarity between

23 disturbed and undisturbed soils. Good indicators should reflect the structure and/or function of

24 ecological communities and respond to changes in soil condition (Neher, 2001). Often, the

25 focus is on abundant taxa when trying to identify indicators of disturbance (Bongers and Ferris,

26 1999; Fiscus and Neher, 2002). However, our results demonstrate that a detailed community

1 analysis may also reveal good indicators among the many taxa with low abundances. Other

2 genera such as *Opisthodorylaimus* (cp5), *Granonchulus* (cp4) and *Chronogaster* (cp3) were

3 also found to be sensitive to environmental disturbance in view of their complete absence from

4 our disturbed sites. By contrast, the prominence of bacterial-feeding *Cephalobus* (cp2) in

5 disturbed areas agrees well with assumptions of the MI and related indices about the pollution

6 and disturbance-tolerance of bacterivores with cp2 (Bongers and Ferris, 1999; Bert et al.,

7 2009). Other genera such as *Coslenchus* (cp2), *Oriverutus* (cp5) and *Mononchulus* (cp4) were

8 limited to disturbed areas, which is counterintuitive for the latter two genera since both are

9 expected to be sensitive to disturbance (Ferris et al., 2001). Many plant-feeding nematodes, on

10 the other hand, were reported to be tolerant to heavy-metal pollutants (Pen-Mouratov et al.,

11 2008; Šalamún et al., 2012; Gutiérrez et al., 2016), hence the high relative abundances of

12 *Dorylaimellus* in the disturbed sites suggest that their distribution was more influenced by their

13 host plants, rather than by metal effects.

14 Aside from the dissimilarity in nematode assemblages between the disturbed and

15 undisturbed sites, significant differences in nematode assemblages also occurred between

16 nearly all pairs of sites, except S1 and S2. Nitrogen and the heavy metals Pb and Hg were

17 identified as drivers of nematode assemblage structure in the mining sites. Nitrogen plays an

18 important role as a main source for primary production and can increase soil microbial biomass

19 (Alon and Steinberger, 1999). Although N content in soils did not significantly differ between

20 sites, a trend of lower N in the disturbed sites compared to S1 and S2 was observed. By contrast,

21 concentrations of Pb were higher in S4 and S5, while Hg was highest in S4. The majority of

22 the metal pollutants, with the exception of Hg, were below the concentrations known to impact

23 soil nematodes in many field studies (Sánchez-Moreno et al., 2006; Sánchez-Moreno and

24 Navas, 2007; Shao et al., 2008; Chen et al., 2009; Gutiérrez et al., 2016). Our results suggest

25 that not only single heavy metals (e.g., Pb and Hg) may affect nematode assemblage structure

in our study area, but also their combination can as a result of additive effects. Such additive effects, like in Cu-Zn combination, have been shown to reduce the abundance of nematode taxa and trophic groups (Korthals et al., 2000), while the combination of Cu, Zn and Pb showed negative effects on nematode community structure, e.g., MI and H' (Sánchez-Moreno and Navas, 2007). At the individual level, additive and interfering effects of heavy metal mixtures, such as (Hg + Cd) and (Hg + Fe), were observed on stress pathways (heat shock, oxidation stress and metallothionein) of the nematode *Caenorhabditis elegans* (Anbalagan et al., 2012). Our results indicate that the free-living *Acrobeloides, Cephalobus*, *Bursilla, Ironus* and the plant-feeding *Pratylenchus* were more abundant under moderately elevated concentrations of Pb and high concentrations of Hg. The tolerance of *Acrobeloides* (cp2), *Cephalobus* (cp2) and *Bursilla* (cp1) to metal stressors agrees with the general MI theory (Bongers, 1990; Georgieva et al., 2002), while the presence of *Pratylenchus* (pp3) agrees with the idea that plant-feeding nematodes can be tolerant to heavy metal contamination (Pen-Mouratov et al., 2008; Šalamún et al., 2012; Rodríguez Martín et al., 2014). However, the positive associations of presumedly 'sensitive' genera *Ironus* (cp4, a predator) to Pb and Hg, and *Eudorylaimus* (cp4, an omnivore) to Cd, respectively, were unexpected (Fig. 4). Although nematode populations, including those of species with high cp-scores and of entomopathogenic nematodes, have been shown to adapt to historical pollution after long periods of mining (< 2500 years) (Campos-Herrera et al., 2016; Gutiérrez et al., 2016), it seems unlikely that such adaptation would already be prominent at locations with only a very recent mining history (> 35 years in our study area). Therefore, the positive effect of high Hg (127-fold higher than the permissible level set by UNEP (2013)) combined with relatively low but elevated Pb concentrations on sensitive taxa, especially in S4, may be due to a combination of other factors: the more neutral soil pH at this site and the presence of a more diverse vegetation cover may both reduce metal bioavailability and thus buffer, directly and indirectly, the potential impact of contamination on soil communities

(Šalamún et al., 2017). In a mesocosm study, Šalamún et al. (2015) demonstrated a positive influence of Cd and Cu, both at 40 mg kg^{-1}, on sensitive nematodes (cp5) and on several nematode indices (Structure Index, MI_{2-5} and Shannon diversity), but values of these indices declined at still higher metal concentrations. Such positive relationships of nematodes with high cp values (sensitive taxa) to relatively low levels of metal pollution have also been reported in other field studies (Heininger et al., 2007; Šalamún et al., 2011), and this may have repercussions for the interpretation of Maturity and related indices.

Conclusions

The small-scale mining activities in Sibutad have caused physical (e.g., finer soil texture, altered vegetation) and chemical (strongly increased Hg levels in S4 but overall low concentrations of other heavy metals) disturbances. While often-used indices based on nematode assemblage structure (e.g., maturity index, Shannon-Wiener diversity) did not reflect clear patterns between locations with different degrees of mining-related impact, nematode assemblage composition (at genus level) did. This suggests that detailed assemblage analysis, while time-consuming, is required to interpret moderate pollution or disturbance effects on soil nematodes. Moreover, our results demonstrate that a detailed community analysis may reveal good indicators of disturbance among the nematode taxa with low abundances. Given the 'below-effect' concentrations of most individual metals with the exception of Hg, and the fact that combinations of different metals (and N) provided the best explanation for variation in nematode assemblage composition, the present study suggests synergistic effects of some heavy metals on nematode assemblages. Counter to expectation, supposedly sensitive nematode genera, i.e. mainly predacious/omnivorous nematodes with low colonizer abilities, were more abundant at moderate than at low heavy metal concentrations. Such positive responses have repercussions on the interpretation of indices such as the maturity index.

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