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Nematodes as bio-indicators of environmental impacts of mining activities in the Philippines: a study using field and laboratory approaches

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Summary

The Philippines is known for its natural wealth of metallic minerals (e.g., gold, copper, nickel, aluminum and chromite), with an estimated $840 billion worth of untapped mineral resources. Despite its economic potential, mining remains a highly polarized issue due to its longstanding record of environmental disasters (**chapter 1**). In 2016, out of the 41 active mine sites, 23 mining sites were abruptly closed due to their close proximity to functional watersheds, which are potential source of potable water. This was followed by a total mining ban on new large-scale mining operations in 2017, and currently, attention is now drawn on the rehabilitation of the existing 32 abandoned mining sites. Major mining disasters can be traced back to large-scale mining activities, whereas impacts of small-scale mining activities have received much less attention. This is primarily because small-scale mining operations are less regulated and less monitored compared to large-scale mining. Small-scale miners often resort to rudimentary techniques which can be harmful to the environment, such as the indiscriminate use of mercury (Hg) to facilitate gold extraction. This was evidenced in **chapter 2** where high levels of Hg (127 times higher than the permissible limit of UNEP, 2013), elevated levels of other heavy metals (e.g., Cd, Pb), and other more subtly contrasting soil properties (e.g., OM, N, pH and granulometry) as well as vegetation differences distinguished three sites disturbed by active small-scale mining activity from two undisturbed or less disturbed (no current mining activity) sites. Although traditional diversity and maturity indices did not significantly differ between sites, effects of small-scale mining activities on nematode communities were reflected by the total nematode abundance and by clear differences in genus composition; for instance, *Iotonchus* and *Mesodorylaimus* were indicator taxa in undisturbed sites, whereas *Cephalobus* were indicators for disturbed ones. Hg, Pb and N were main drivers of the nematode assemblage structure. Positive association were found between *Ironus* to Hg and Pb, and *Eudorylaimus* to Cd, both with cp4 scores, counter to the common expectation, which suggests that the assumption of the maturity and related indices that large-bodied predacious or omnivorous nematodes are more sensitive and are therefore more easily eliminated from a system after a strong disturbance, does not always hold.

The lack of a negative impact in terms of ecological indices, particularly in the site with the highest Hg level was rather surprising. Vegetation and other soil parameters such as a more neutral pH and a higher clay content in the site may have played a role in reducing the bioavailability of Hg and other heavy metals. We performed a microcosm experiment (**chapter 3**) using an 'undisturbed' soil amended with Hg levels similar to concentrations commonly found

in the field in the small-scale mining area (i.e. 2.5, 5 and 10 ppm Hg), to assess whether these concentrations would affect nematode assemblages, thus contrasting with our field observations (**chapter 2**). The results were indeed discrepant from the field data; Hg concentrations from 2.5 onwards were already detrimental to nematode abundance while Hg concentration of 5 ppm onwards strongly affected most nematode assemblage descriptors (e.g., abundance, number of genera, Shannon-Wiener index), suggesting that total abundance was the most sensitive nematode-based response variable. The strong discrepancy between the microcosm experiment and field data may be related to pronounced differences in the physico-chemical properties of the soils and the presence/absence of vegetation, factors which can all substantially affect Hg availability. This suggests that nematode-based environmental assessment should be interpreted in a context-dependent manner. Our microcosm set-up also demonstrated 'bottling effects' caused by incubation of nematodes, resulting in a decrease of abundance by 37% after 45 days; however, no significant alterations in diversity and nematode assemblage composition were recorded. The decrease in abundance may be attributed to the artificial nature of the experiment, particularly in the complete absence of vegetation.

With the ecological risks associated with mining, the total ban on open-pit mining by the current Philippine government signifies its strong commitment to protect the environment. Despite the immediate closure of several large-scale mining sites, actions have yet to be taken for the rehabilitation of the 32 abandoned mined-out areas. A traditional approach of rehabilitating these areas involves the addition of organic waste material (mostly agricultural wastes) into the overburden soil used to cover stripped-out areas, followed by forestation by tolerant plant species such as *Acacia* sp.. Visual inspection based on tree survival and vegetation growth has been used as a criterion to assess rehabilitation success; however, this may not be scientifically sound since plants such as *Acacia* sp. are highly tolerant to ecological disturbances, and hence their presence may not always be a good indicator of improving soil condition. We conducted a sampling (**chapter 4**) to examine the soil biota, i.e., nematode communities, present in different rehabilitated subareas of an abandoned gold mine pit, where rehabilitation had been initiated from 13 to 8 years prior to the first sampling period in 2012. We revisited the same sites two years later, in 2014, and compared nematode assemblage structure in the rehabilitated soils between the two time periods (2012 and 2014) under the assumption that any improvement in soil condition after 2 years can be reflected in nematode-based ecological indices and nematode genus composition. We also compared results from both years with those of a reference site just outside the mined area. Our results showed unexpected low abundance in all of the sites, including the reference area in 2012, probably caused by the extremely acidic soils in the area (all

sites had pH < 4.3), whereas low abundance in the same area in 2014 suffered the impacts from vegetation burning. Despite the presence of vegetation in all rehabilitated areas, the low nematode abundance and diversity, already impacted by low pH, may have been exacerbated by the high Pb level and lack of OM in soil. Aside from the relatively low heavy metal levels (except Pb which substantially exceeded the range of standard limits prescribed in most developed countries (Teh et al., 2016)), hints of partial soil recovery were, however, manifested through increase in genus richness and the appearance of more presumedly sensitive genera in Site B (e.g., *Judonchulus*, *Mononchus*, *Oriverutus*, *Labronemella* and *Ecumenicus*), increase in abundance and the number of genera in Site C and increase in abundance in Site D after two additional years of rehabilitation.

Apart from soil rehabilitation efforts, large volumes of wastewater have undergone treatment, which would be recycled for agricultural use of the local community. Heavy metal analysis of wastewater in 2014 revealed that several heavy metals were within the set limits set by the Philippine government through the Department of Environment and Natural Resources (DENR) for such purpose, except for Cd and Cu (**chapter 1**). An experiment was performed to examine the effects of the present level of Cu on community composition using a simple two-species system, and on how shifts in interaction could be induced due to differential tolerance to a toxicant, which in turn, could impact soil ecosystem functioning (here: organic matter decomposition) (**chapter 5**). Our results showed that concentrations similarly found in wastewater differentially impaired the fitness of the soil nematodes, *Acrobeloides nanus* and *Plectus acuminatus* (but *A. nanus* were more tolerant than *P. acuminatus*) and thereby affected the interspecific interactions between them with their differential sensitivity to Cu. Although Cu was linked to a decreased decomposition rate of the leaf litter, but in the absence of microbial data, it is not possible to assign these to direct effects of Cu on the bacteria or indirect effects through the Cu impacts on nematodes and their interactions. Nevertheless, our results were in congruent with previous study that show that low toxicant levels can be still be detrimental to ecosystem functioning by altering the outcome of species interactions (Bontje et. al., 2011).

Chapter 6 discusses the suitability of nematodes as bio-indicators of the impacts of small-scale mining activity and soil recovery in a rehabilitated mining site. We also presented some conceptual challenges and identified current gaps in the present work. Promising research aspects were also identified especially on bioremediation aspects such as the phytoremediation in abandoned mining areas, both large-scale and small-scale mining, where soil improvements can be assessed using nematodes as bio-indicators.

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CHAPTER 1

General Introduction

One of the many environmental challenges in many countries is to find a balance between economical growth and ecological protection, in order to avoid or reduce the all-too-common implication that **rapid economic growth** is closely associated with the **decline of environmental quality** (Shafik, 1994). In the early 21st century, the human population has utilized approximately 68 Gigatonnes (Gt) of materials, including metallic and non-metallic, each year with a resource use continuing to grow at a rate of 7.4% per year (Krausmann et al., 2009). With this increasing global material consumption, mineral mining offers a huge potential as a significant contributor to developing economies. However, mining is often associated with increasing pressure on the environment due to the toxic materials it can generate (Johnson, 2003; Mishra et al., 2008).

In a mineral-rich country like the Philippines, mining has a huge economic potential and has been an important source of livelihood among marginalized communities. However, mining activities, both small-scale and large-scale, have been causing serious ecological problems, both during and (often long) after the exploitation period. Since studies on the impact of mining areas and their rehabilitation once exploitation has stopped remain scarce in the Philippines, generous financial support – both from the private sector and the government – for research in these impacted areas are available to promote 'sustainable' mining in the country. Hence, this PhD study was undertaken to assess pollution impacts caused by small-scale mining activities, and to evaluate soil recovery in rehabilitated sites of a large-scale mining area in Sibutad, Mindanao, southern Philippines, utilizing nematode assemblages as ecological indicators using a combination of field and laboratory approaches.

1.1 Mineral mining: a catalyst of the economy

Since the advent of the industrial age, there has been a continuous demand for raw materials, e.g. metallic and non-metallic minerals, due to the huge number of applications in agriculture, medicine, military or industries. Minerals are naturally abundant in the earth's crust, and their natural distribution is governed by biogeochemical cycles (Singh et al., 2011). Anthropogenic activities, however, can alter such natural cycles leading to a high surplus of minerals in the environment. Although several heavy metals have essential biological functions, they are toxic when in excess. Heavy metals can **alter population and community structure** (Yeates et al., 1995; Sanchez-Moreno and Navas 2007) and **disrupt ecosystem processes** (Nahmani and Lavelle 2002). Despite ecological risks, there is still a continuous demand for several metals to respond to the exponential need due to industrial, technological and economic developments (Skirrow et al., 2013) Hence, more new environments are being explored for mineral deposits,

putting serious pressures even those areas considered as 'protected'. Global protected areas play a key role in biodiversity, but are threatened when 6% of the total terrestrial coverage has been penetrated by metal mining activities (Durán et al., 2013). Other potential areas which are also threatened due to mining exploration include the ocean floor, also known as deep-sea mining, which poses a threat to deep-sea diversity (Miljutin *et al.*, 2011; Ramirez-Llodra *et al.*, 2012).

1.2. Mining in the Philippines: a brief history

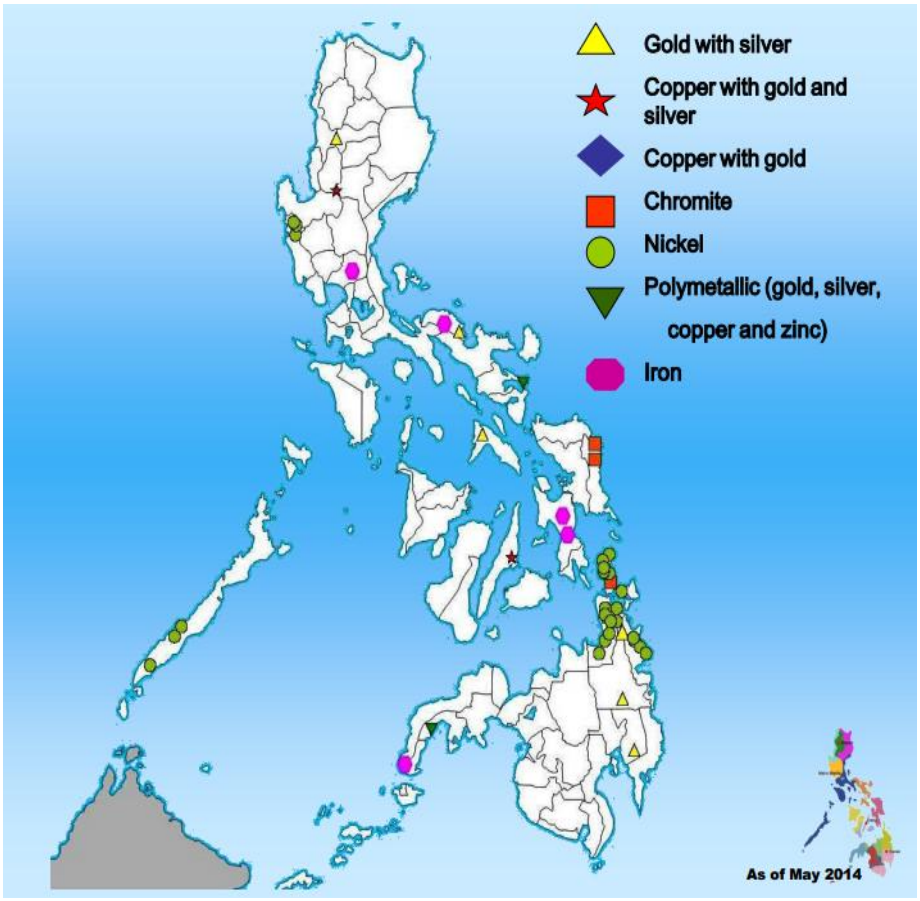

Fig. 1.1. Locations of the large-scale metallic mining sites in the Philippines as of May 2014. (Source: Mines and Geosciences Bureau)

The Philippines is a country endowed with mineral resources, particularly metallic minerals, such as copper, gold, lead, nickel, silver, and zinc (Jimenez *et al.,* 2002). The earliest known records of mining in the Philippines can be traced back to the 3rd century, when traders from China would refer to the northern part of the Philippines, Luzon, as the island of gold (Rovillos et al., 2003). Industrialized mining began during the American colonial periods in the 1940's. After four decades, mining made a significant contribution to export revenues (Rovillos *et al.,* 2003).

Presently, the Philippines has exported minerals and mineral products to several countries such as Japan, Australia, Canada and China making the country the top mineral exporter in the world (Mines and Geosciences Bureau, 2014). Mineral extraction is carried out by l**arge-scale or small-scale mining**; as of 2014, the Mines and Geosciences Bureau (MGB) has recorded 41 large-scale miners (metallic mines) (Fig. 1.1), whereas the number of small-scale miners remains poorly documented.

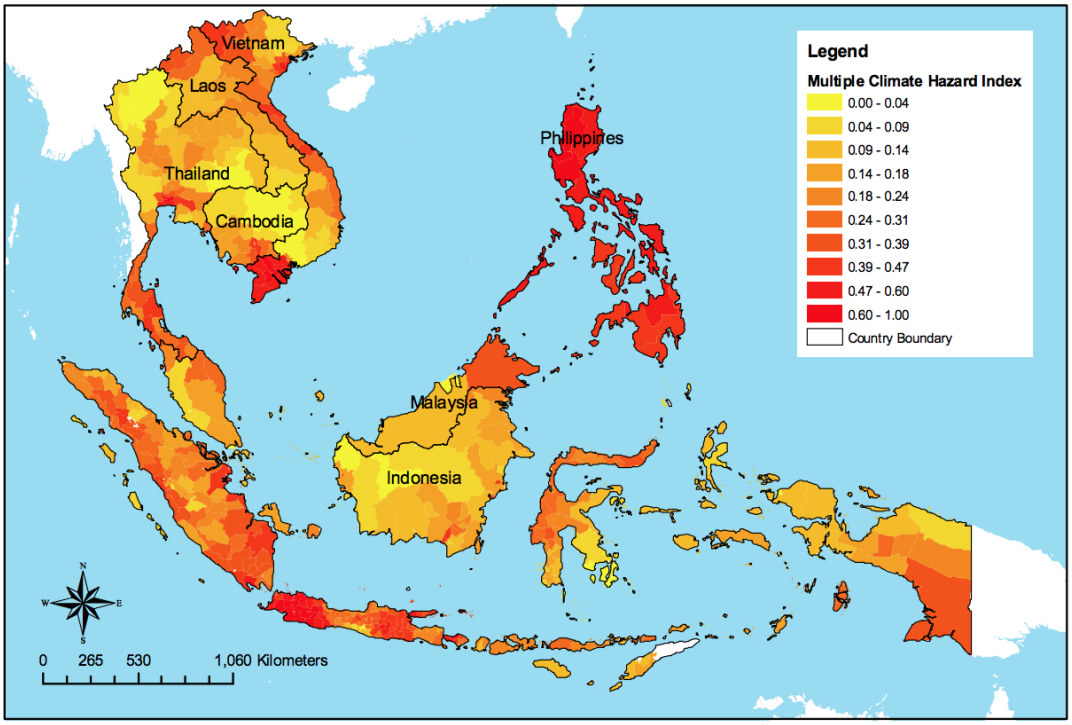

Fig. 1.2. Climate hazard map of Southeast Asia (*adapted from Yusuf and Francisco, 2009*).

Among the Southeast Asian countries, Philippines is one of the most vulnerable (one of the highest multiple climate hazard index) to ecological disasters, such as cyclones, landslides, due to climate change (Yusuf and Francisco, 2009) (Fig. 1.2), thus increasing the risk of chemical contamination during calamities not only from active mining sites but also mined-out areas which have been abandoned. More than 30 large-scale mining companies prematurely terminated their operations and abandoned the mining areas from the 1950's onwards. None of the mining companies was compelled to rehabilitate their mined areas due to lack of pertinent provisions in the previous mining laws. It was only in 1996, through the **Philippine Mining Act of 1995**, when large-scale mining companies were obliged to include rehabilitation in their post-operational plans. Till present, out of the numerous abandoned areas from the 1950's to 1995,

only one mining site in Hinabangan, Samar started its rehabilitation in 2014 (www.ptvnews.ph/denr-raises-urgency-mine-rehabilitation/).

1.3. Mining and its biological and social impacts

Mining can be categorized as large-scale or small-scale mining depending on the nature and extent of mining activities. Large-scale mining is often carried out by big companies and has a huge labor force which utilize advanced technology in the mineral extraction processes. Large-scale employs open-pit mining technique which requires the removal of topsoil, clearing of hundreds of hectares of rainforests, agricultural lands, and utilization of chemicals and huge volume of water. By contrast, small-scale miners refer to informal mining activities (operating without government license) composed of few individuals or family groups. They mostly rely on physical labor using fairly rudimentary techniques, which can be harmful to the environment (Hinton et al., 2003). It has been estimated that 70-80% of gold in the Philippines comes from small-scale gold mining (www.hrw.org; Mines and Geosciences Bureau, 2012).

Despite its economic potential, mining remains a highly polarized issue in the Philippines due to several serious mining-related incidents (Table 1.1). Mining is often associated with habitat destruction due to the removal of vegetation which serves as shelter and food source to wildlife. Habitat destruction is thought to be a potential driver of species extinction worldwide (Pimm and Raven, 2000). Other mining-related impacts such as pollution effects can **decrease the abundance** and **diversity of species** (Ramirez *et al.,* 2005), **decrease the efficiency of nutrient recycling** and **impair ecosystem functioning** (Baath 1989; Nahmani and Lavelle 2002). Proliferation of heavy metals in mining sites can also be deleterious to human health (Jomova and Valko 2011; Tokar *et al.,* 2011); for instance, more frequent haematology-related illnesses have been recorded near mining areas in the Philippines (Castillo *et al.,* 2003). In 2014, several human lives were lost to landslides caused by mining (www.bbc.com/news/world-asia-16420800). Apart from biological and health impacts, mining is also associated with the rise of social problems, such as abuses among women and children in small-scale mining communities in developing countries (Machipisa 1997; Anon 2001; Hilson 2002), and encroachment of ancestral domains of indigenous peoples (Molintas, 2004). In Sibutad, the influx of migrants in the 1980-1990's led to increased incidence of crimes, human rights abuses, child labor and prostitution (Goodland *et al.,* 2009).

Table 1.1. Major mining disasters in the Philippines.

Name of mining company	Location and year	Description
Philex mining company (Padcal mine)	Benguet, 2012	approximately 20 million cubic tons of 'non-toxic' sediments spilled
TVI Pacific Inc	Zamboanga del Norte, 2007	spillage of contaminated water containing cyanide and Hg which flowed to Canatuan and Siocon rivers
Lafayette mining company	Albay, 2005	cyanide contamination from two mine spills which caused several fish kills in 2005
Atlas Consolidated Mining and Development Corporation	Cebu, 1999	discharged approximately 5.7 million m^3 to Subangdako river leading to increase in water acidity resulting in fish kills
Philex mining company (Sibutad mine)	Zamboanga del Norte, 1997, 1998	contamination of rice fields and marine ecosystem due to flashflood after torrential rain resulting in fish kills
Marcopper mining corporation	Marinduque, 1996	4,400 people affected due to the cracking of a 2.6 km long drainage tunnel, spilling a total of 1.6 million m^3 of toxic mine tailings into Makalupnit and Boac rivers

1.4. Rehabilitation of mining sites in the Philippines

Soil rehabilitation aims to revive at least some of the basic ecological services lost after soil degradation (Chazdon, 2008; Boyer and Wratten, 2010). However, the high cost often associated with the clean-up of contaminated soils can burden mining companies (Raskin et *al.*, 1997; Garbisu *et al.*, 2007). In developed countries, recent approaches for soil remediation make use of activated carbon (Brändli, 2008) and biochars (Fellet *et al.*, 2011); both are known for their strong adsorbing capacity for organic contaminants and heavy metals, respectively (Gong *et al.*, 2007; Karami *et al.*, 2011). Activated carbons are sorbing carbonaceous charcoal material produced from the incomplete combustion of organic materials (e.g. coal or coconut shells), followed by activation to increase surface area (Brändli et al., 2008), whereas biochars, precursors of activated carbon, are biological residues combusted under low oxygen condition resulting in porous, low density carbon rich material (Beesley *et al.*, 2011). Despite their efficacy in soil remediation, activated carbon and biochars are still considered expensive, which makes them less attractive in developing countries. Apart from removal of contaminants in soil, soil rehabilitation should also aim that the newly-added topsoil, where biological activity is largely concentrated (Nielsen and Winding, 2002), can support soil communities. Open-pit mining where vegetation is eliminated and topsoil is completely removed and stored for a long period of time, may lead to soil

deterioration, such as loss of organic matter and soil nutrients (Davies et al., 1995; Harris et al., 1993; Mummey et al., 2002b), and in turn, can deleteriously impact soil communities.

To ensure environmental protection, rehabilitation of abandoned mining areas has recently become one of the top priority programs of the Philippine government (www.ptvnews.ph/denr-raises-urgency-mine-rehabilitation/). Soil rehabilitation in mining sites, as mandated by the **Philippine Mining Act of 1995**, develops the sense of responsibility and accountability among mining companies, as well as improve the negative perception of people towards mining.

Mining companies in Mindanao employ fairly similar rehabilitation strategies. For instance, in the rehabilitation of Hinatuan Mining Corporation, a large-scale mining for nickel situated in Surigao del Norte, aside from the usual practice of adding amendments to the soil, the roots of plant seedlings were treated with the fungi mycorrhiza prior to planting in mined-out areas. A similar strategy was also used in Sibutad mining site, except for the mycorrhiza addition. So far, rehabilitation strategies of the two mining companies were claimed to be 'successful' based on the survival rates of plants. Compared to the more advanced approaches involving activated carbons and biochars, this approach incurs a much lower cost. The tree species used in the rehabilitation of Sibutad mining sites was *Acacia auriculiformis*, which is a fast-growing, drought-resistant and nitrogen-fixing tree species. It is widely utilized to prevent soil erosion and to sequester heavy metals (Cadiz et al., 2006) due to its extensive root system. Its ability to survive in poor soil conditions renders *A. auriculiformis* suitable for the rehabilitation of degraded sites (Lamb and Tomlinson, 1994).

Since rehabilitation primarily aims to revive the integrity of ecosystems, it is imperative to establish criteria that are ecologically relevant and scientifically sound. Previous works have relied on the above-ground diversity, biomass and vegetation structure to judge rehabilitation success (Koch, 2007; Norman *et al.*, 2006). Similarly, at the local level, the Mines and Geosciences Bureau (MGB), the agency tasked to monitor the Philippines' mineral deposits, assesses rehabilitation success largely based on the survival rate of plants or their ability to grow, i.e. *Acacia auriculiformis* in the case of Sibutad mines (Mines and Geosciences Bureau, 2014). For instance, high survival rate (> 90%) of trees in the abandoned Sibutad large-scale mining sites was reported in 2014, after more than 10 years of planting of *A. auriculiformis* (www.philstar.com). Utilizing *A. auriculiformis* survival or growth rate as a sole or principal measure of rehabilitation success, however, poses limitations due to these trees' high tolerance to pollution. The present local approach, and all approaches which only focus on aboveground variables, ignore valuable information on the essential functions of soil biota, which are known to play important role in

organic matter decomposition, nutrient cycling, bioturbation, etc. (Brussard, 1997; Coleman et al., 2004).

1.5. Sibutad: host to small-scale mining operations and a rehabilitated mining area

Fig. 1.3. Maps showing the Philippine archipelago and the location of Sibutad (in red; inset), the study areas (A = small-scale mining area and B = rehabilitated mining area) and Sibutad town proper (C).

The municipality of Sibutad (8.6000° N, 123.4667° E), situated on the western part of Mindanao Island (Fig. 1.3), hosts a few small-scale mining sites and a rehabilitated mining site (ca. 38 ha), where rehabilitation started at different periods in time for different parts of the affected area; this makes Sibutad area suitable for biological impact studies in environments with varying degrees of mining-related disturbance and various durations of the rehabilitation trajectory. According to the Philippine Statistics Authority, the population of Sibutad in 2015 was approximately 17,645 which largely depends on farming, fishing and mining for livelihood (Census of Population, 2015). Active small-scale mining is being operated by the local community, whereas the large-scale mine site is owned by Philex Mining Corporation (PMC), the largest producer of gold and copper in the Philippines. Adjacent to Sibutad is Murcielagos Bay, an important source of marine food products such as fish and bivalves, which receives the water effluent particularly from small-scale mining areas through its tributaries.

Mining-related activities in Sibutad have impacted both terrestrial and aquatic environments. Particularly in small-scale mining, improper waste disposal is not uncommon due to lack of strict government monitoring. This was clearly reflected by the Hg levels taken from soil and water samples, where Hg concentrations exceeded those of the EU, US (Teh et al., 2016) and that of the Philippine standard, respectively (www.emb.gov.ph). Elevated levels of Hg in Murcielagos Bay (Lacastesantos *unpublished*) are assumed to have originated from such small-scale mining activities. On the other hand, large-scale mining by Philex Mining Company (PMC) has also been reported to have been involved in various ecological disturbances such as cyanide contamination and mudslides (Goodland et al., 2008). Philex Mining Corporation ventured into an open pit mining in Sibutad in 1996. Out of the 3,515 hectares of land approved by the Mineral Production Sharing Agreement (MPSA) which specifies the total land area to be mined, only roughly 1% or 38 ha. was utilized for mine-related activities from 1997 to 1999. This was far smaller compared to other large-scale mining areas in Mindanao. For instance, the two large-scale mining based in the province of Surigao del Norte, Hinatuan Mining Company and Taganito Mining Company, have a total land area of 773 ha. and 4, 863 ha., respectively. Both mining are still active at present, with only a small portion undergoing rehabilitation within the last 5 years. Sibutad large scale mining ceased its activities in 1999 when the price of gold had fallen on the global market. After its brief mining stint, PMC started rehabilitating several mined areas through forestation as of 1999. A total of 509,011 *A. auriculiformis* trees had been grown in more than 150 ha. by 2012, including those areas not affected by mining-related activities which were barren or covered by the common grass species, *Imperata cylindrica.* Aside from soil rehabilitation, large volumes of wastewater contained in storage ponds also underwent wastewater treatment for recycling for agricultural uses (Table 1.2). However, heavy metal analysis of water samples in 2014 showed that Cd and Cu concentrations in most ponds, except Storm pond 2, exceeded the limits for agricultural and aquaculture purposes, respectively per DENR Administration Order no. 34 Series of 1990 (http://www.emb.gov.ph/wp-content/uploads/2016/04/DAO-1990-34.pdf, Table 1.2).

Table 1.2. Heavy metal concentrations of the wastewater samples taken from the ponds of the rehabilitated large-scale mining area in 2014.

	Heavy metal concentration (ppm)					
Reservoir/Pond	As	Hg	Cd	Cu	Zn	Pb
heap leach pad	<0.005	<0.004	0.11	5.06	7.4	0.04
pregnant pond	<0.005	<0.004	0.18	4.88	15.3	0.04
barren pond	<0.005	<0.004	0.16	4.06	13.0	0.03
storm pond 1	<0.005	<0.004	0.12	3.29	10.8	0.02
storm pond 2	<0.005	<0.004	<0.003	0.04	0.03	<0.003
north slit dam	<0.005	<0.004	0.14	3.8	4.3	0.16
*accepted standard concentrations						
Class C	0.05	0.002	0.01	0.05	-	0.05
Class D	0.01	0.002	0.05	-	-	-

Source: Philex Mining Corporation, Sibutad Project

*Water classification per DENR Administration Order no. 34 Series of 1990

Class C for the propagation of fish and other aquatic organisms and Class D for agriculture, irrigation and livestock watering

1.6. Heavy metals and their transport pathways in the environment

Heavy metals are naturally present in the Earth's crust. These include, among others, Copper (Cu), Lead (Pb), Cadmium (Cd), Mercury (Hg) and Zinc (Zn). Some of these heavy metals have biological importance such as Cu and Zn. Cu affects enzyme activity as it plays a role as a co-factor in oxidative and reductase enzymes (Uauy et al., 1998) while Zn participates in the regulation of cell proliferation (Macdonald, 2000). Other heavy metals such as Cd and Hg have apparently no direct biological function in mammals (Shore and Douben 1993) and can be toxic when present in small amounts. At the cellular level, Cd affects cell differentiation, apoptosis, DNA repair and methylation, gene transcription and translation (Waisberg et al., 2003). Hg, on the other hand, is thought to cause genotoxicity with the formation of reactive oxygen species (ROS), which may react directly with DNA and cause conformational changes in protein responsible for DNA formation and maintenance (Crespo-López et al., 2009). Despite being non-essential elements, Cd and Hg possess several essential practical applications. Cd is often used as an anti-corrosion agent in PVC products, in several alloys and nickel-cadmium batteries; it is discharged into the environment by sewage sludge disposal and metal smelting (Järup, 2003), and offshore oil and gas drilling activities (Lira et al., 2011). Hg is used in thermometers, blood-pressure cuffs, commercial batteries and fluorescent bulbs. Since it is a cheap common ingredient used in the extraction of gold, excessive use of Hg in small-scale areas poses risks to human health and environment. Hg enters the environment by vaporization or it can be accidentally or deliberately released to the environment during mining processes (Israel and Asirot, 2002).

Ore mining also has contributed to the increase of heavy metal levels in the environment through physical and chemical weathering (Dang et al., 2002; Ogola et al., 2002; Li et al., 2009), which alter the natural geochemical cycles, thus affecting the balance of heavy metal distribution. Other anthropogenic sources of heavy metals include industrial effluents, sewage and inadequate agricultural and forest management. Heavy metals can be distributed into the atmosphere (Zereini et al., 2005; Lee et al., 2007), and may affect humans through respiration, soil and aquatic ecosystems through precipitation (Adhikari et al., 2004; Sochova et al., 2005; Lohmann et al., 2006). Heavy metals pose a threat to the environment due to their tendency to enter the food web (Frieberg et al., 1973; Piscator 1980). For example, excessive amounts of heavy metals in a grassland community near a mining site may lead to metal uptake via plant roots (Ali et al., 2013). This results in the bioaccumulation in the tissue of living organisms which can be transferred from one trophic level to another via herbivory, predation, etc. (Van Driel and Smilde 1990; Heikens et al., 2001).

1.7. Triad: establishing the cause-effect relationships between pollutants and organisms

Measurements of heavy metal contents in water and soil samples in Sibutad are mere total concentrations (Table 1.2), and utilizing such information to determine a) bioavailable fractions of pollutants and b) responses of biota (both at species and community level) to extant pollutant concentrations, may provide more ecologically relevant information. The bioavailable fraction of pollutants, which is actually responsible for the toxic effects on organisms (Alloway, 1995), is dependent on several factors, such as organic matter, clay content and soil acidity (Rieuwerts et al., 1998). Increased OM can increase heavy metal adsorption in soil, thus decreasing their bioavailability (Antoniadis et al., 2008). For instance, complexes of Cu^{2+} and Pb^{2+} associated with humic substances are characterized by a high stability (Stevenson, 1976); the more stable, the less it will be biologically available. Soil pH can also affect the bioavailability of heavy metals; decreased pH means a higher bioavailable fraction of the heavy metals for organisms (Plette et al., 1999; Appel and Ma 2002; Riba et al., 2003), but often also more losses through leaching (Tyler, 1978) . Since the toxicity of a chemical varies with its concentration and different factors (e.g., OM, pH and grain size), the significance of a chemical cannot be solely determined by chemical concentrations, but rather on the response of biological organisms, as described in the **triad approach** proposed by Chapman (1990) (Fig. 1.4).

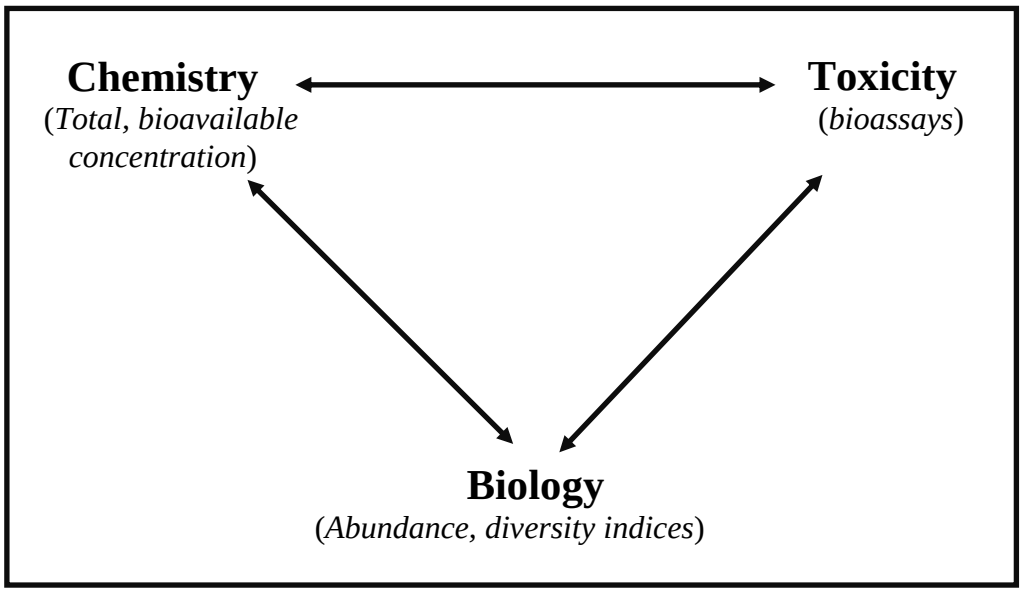

Fig. 1.4. Sediment Triad Approach as proposed by Chapman (1990).

Following the triad approach, the present study incorporates 3 components: soil chemistry, which measures contamination; soil bioassay, which measures toxicity and the biological parameters, which measure using either single species tests or a community analysis. Toxicity tests with single-species can provide a rapid evaluation of the effect of pollutants on species under controlled conditions that mostly involves determination of species mortality at a given period of time (e.g. LC_{50}). Many of the threshold concentrations imposed are based upon information from single-species tests (OECD, 1998). For chronic exposures, biological parameters such as body growth, reproduction, life span, behavior, food-finding capacity have been used (ISO/DIS 10872; Roh et al., 2006; Harada et al., 2007; Franzen et al., 2012; Monteiro et al., 2014). Chronic tests allow assessment of toxic effects of low concentrations of heavy metals at a longer period of time. Despite the valuable information, single-species assays pose **limitations** (Sprague 1969; Heckman et al., 2009). For instance, the pattern of toxic effects of chemicals and the endpoint of interest can **vary between species** (Álvarez et al., 2006), e.g., the EC_{50} for the water flea *Daphnia magna* reproduction test differs from that of the EC_{50} for algal population growth. Also, inherent in single-species tests is the use of a **standardized exposure time**. Since toxic effects depend on exposure duration, Heckmann et al. (2010) suggested that toxicity-time curves should be constructed to calculate the **incipient LC50**. Incipient LC50 is defined as the concentration at which 50% of the population can survive for an indefinite period of time. Baas et al. (2007) demonstrated the decrease of LC_{50} values of the collembolan *Folsomia candida* through time upon exposure to cadmium. Hence, extending the test duration for survival as an endpoint till the incipient LC_{50} is achieved, has long been suggested (Sprague, 1969).

Another important limitation with single species tests is that they do not account the effect of a toxicant on species interactions (often not only shifting competitive equilibria, but potentially cascading through to other species in the community).

Scientists have also resorted to experiments using multiple species by constructing small 'communities' in micro and mesocosms. Laboratory-based experiments involving multiple species offer an advantage over single-species tests as they are able to examine the effects of pollutants on species interactions and their role in ecosystem functions and in shaping community structures (Leblanc 1985; Fleeger et al., 2003; Bontje et al., 2011; Martinez et al., 2012), whereas those of artificial 'community' can provide a more realistic assessment of the impact of pollutants at the community level. This PhD work fits into the triad approach framework, but with greater emphasis on the biology (response of nematodes as ecological indicators) and toxicity or bioassay (laboratory or field-based experiments) rather than on pollutant concentration measurements per se.

1.8. Nematodes as indicators of pollution

Ecological indicators can serve as early warning mechanisms of the ecological impact of stressors. Many species used as indicators have been utilized in standardized tests and are used as basis **to establish allowable pollution levels** (OECD, 1995). Some of the standard test organisms include green algae, the earthworm *Eisenia foetida*, the water flea *Daphnia magna*, the collembolan *Folsomia candida* and the nematode *Caenorhabditis elegans*. Nematodes are one of the most commonly used ecological indicators due to their important roles in key soil ecological processes such as the **decomposition of organic matter** and **nutrient recycling** (Griffiths, 1989; Postma-Blaauw et al., 2005; De Mesel et al., 2006). Nematodes possess several features which render them very suitable as ecological indicators (Freckman, 1998; Höss et al., 2006). They are ubiquitous, have relatively short generation times, occupy different trophic levels, are incapable of migrating over long distances, and some soil nematode species are easy to maintain in the laboratory (Höss et al., 2006). Nematode responses to pollution range from sensitive to very tolerant, with substantial differences between even very closely related species (Monteiro et al., 2018 and subm.). Thus, changes in nematode assemblages can reflect pollution or other disturbance events, and can be measured using various ecological indices based on diversity, life-history information, etc. (Simpson 1949; Bongers 1990; Yeates et al., 1994; Ferris et al., 1999), as well as through a detailed analysis of their taxonomic composition (Fiscus and Neher 2002; Georgieva et al., 2002). Ecological diversity indices such as Shannon-Wiener index, Simpson index and trophic diversity index (Table 1.3) have been used to examine nematode communities

in various ecological conditions, disturbed vs. non-disturbed, where the disturbed areas are generally expected to yield lower values of diversity indices than non-disturbed areas (Heip, 1998; Ferris et al., 2001; Ferris and Matute 2003; Sanchez-Moreno and Navas 2007, Navas et al., 2010).

In terms of their life history, Bongers (1990) categorized nematodes with colonizer-persister (cp) values and proposed the use of the Maturity Index (MI). MI is the weighted mean of the cp scores for the individuals in a particular sample. Nematodes with cp value 1 are known as r-strategists (Pianka, 1970) or extremely good colonizers, which thrive particularly well under conditions of organic/nutrient enrichment. They are thus referred to as enrichment opportunists (Bongers and Ferris 1999). They are characterized by high reproduction rates and are tolerant to ecological disturbances, while nematodes with cp value 5 are K-strategists (Pianka, 1970) or persisters. They have low fecundity and are sensitive to ecological perturbations, hence they only become abundant in 'persistently' suitable and undisturbed soils. MI_{2-5} is a modification of MI, which is specifically designed to discriminate disturbance caused by chemical pollution from that caused by organic enrichment (Popovici 1992; Bongers and Ferris 1999). MI_{2-5} excludes nematodes with cp 1 scores from its calculation because they rapidly increase during **organic enrichment** while at the same time being tolerant to chemical pollution; hence, they may not reflect pollution-induced changes in soil ecological conditions. The relationship between the MI and heavy-metal stress can be masked by such high abundance of cp-1 nematodes, which may lead to erroneous interpretation of the MI. Aside from the life history of the nematodes, the use of their functional or feeding groups (Yeates et al., 2003) has also been applied in the assessment of soil condition (Šalamún et al., 2011). For instance, some bacterial-feeding genera such as *Eucephalobus*, *Acrobeloides* and the fungal-feeding *Aphelenchoides* have shown a positive correlation with heavy metals in the soil (Georgieva et al., 2002; Tomar et al., 2009).

As more progress on the conceptual framework on nematodes has been developed, additional and more refined indices have been proposed to increase our understanding on soil conditions and of the structural and functional aspects of the soil food web (Bongers and Ferris, 1999; Neher, 2001). The nematode faunal analysis, which integrates the nematode feeding groups and cp scaling, recognizes the enrichment and structure trajectories, calculated as EI and SI, respectively (Ferris et al., 2001). Structure index reflects the degree of trophic connections in food webs as the system matures, thus it is an indicator of ecosystem stability. Enrichment index (EI), on the other hand, reflects the abundance and activity of primary detrital consumers in response to available resources. During enrichment when microbial activity is intense, opportunistic microbial feeding nematodes can respond by exploiting new resources. The decomposition pathway of the soil food web is represented by the channel index (CI), where a

lower CI suggests a bacterial-dominated (fast) pathway, whereas higher CI suggests a fungal-dominated (slow) pathway. (Ferris et al., 2001).

Despite the applicability of MI in terrestrial systems (Korthals et al., 1996b; 1998; Nagy et al., 2004), the use of Maturity indices in pollution impact studies also poses some limitations. The current scheme of assigning cp values at higher taxonomic levels, such as family or genus level may not be adequate due to the growing evidence showing differences in sensitivity to toxicants at the species level (Monteiro et al., 2018 and submitted); this probably calls for careful re-evaluation of the cp values of nematodes. Furthermore, the allocation of nematodes to different trophic groups has raised doubts. For instance, some *Tylenchus* sp., often considered as fungivores in ecological studies, were found to feed and reproduce on roots (Neher, 2001); 'predaceous' *Mesodorylaimus* sp. feed on bacteria (Russel, 1986); *Filenchus* sp. were initially thought to be plant-feeders (Yeates et al., 1993), but were later found to feed on fungi (Brzeski, 1998; Okada et al., 2002; 2005). Unless a detailed examination is performed to establish nematode food preferences (Moens et al., 1999; Ruess et al., 2010; Weber and Traunspurger, 2013), assignment of their feeding habit would remain ambiguous.

Table 1.3. Overview of ecological indices used in this study

Ecological indices	Equation
absolute abundance	
number of genera = richness	
Pielou's evenness (J)	
Shannon-Wiener index (*H'*)	$H = \sum pi \ln pi$
Simpson index (1-D)	$1\text{-}D = 1 - \sum (pi^2)$
Maturity index (Bongers, 1990)	$MI = \sum (vi.pi)$
$MI_{(2\text{-}5)}$ (Bongers 1990)	$MI = \sum (vi.pi)$ (excluding cp-1 taxa)
index of trophic diversity (ITD)	$ITD = 1 / \sum pi^2$
Enrichment index (EI)	$EI = (e / (e+b)) \times 100$
Structure index (SI)	$SI = (s / (s+b)) \times 100$

where: *vi* = the c-p scores designated by Bongers (1990)
pi = the proportion of the genus in the free-living nematode community
n = the total number of organisms of a particular species
N = the total number of organisms of all species
e = is calculated as $\sum k_e n_e$, where k_e are the weighting assignment to guild that indicate enrichment characteristics of the food web (Ba_1, Fu_1) and n_e are the abundances of nematodes in those guilds
b= is calculated as $\sum k_b n_b$ where k_b are the weighting assignment to guild that indicate basal characteristics of the food web (Ba_2, Fu_2) and n_b are the abundances of nematodes in those guilds
s = is calculated as $\sum k_s n_s$ where k_s are the weighting assignment to guild that indicate structure characteristics of the food web (Ba_3–Ba_5, Fu_3–Fu_5, Om_3–Om_5, Ca_2–Ca_5) and n_s are the abundances of nematodes in those guilds

1.9. Outline of the thesis

The General Introduction (**chapter 1**) encompasses the current issues, challenges and status of mining, with a main emphasis on the Philippines in general and on the area of Sibutad in specific. In general, mining activities – both small-scale mining and large-scale mining areas (including abandoned areas) have been linked to serious ecological disturbances (Dudka and Adriano, 1997). As a result of a stringent legislation recently, large open-pit mining activities have been banned and some mining companies were ordered for closure. The debate now is mostly dealing with the rehabilitation of such mining-impacted sites. Small-scale mining activities (mainly for gold), on the other hand, have proliferated in Sibutad since the 1990's and have continued to remain a threat due to the lack of stringent monitoring from the government. Since most of the small-scale mining areas in Sibutad are connected to Murcielagos Bay through its tributaries, their activities have reportedly led to an increase in Hg in the coastal marine environment where local populations heavily rely on fisheries for food. Aside from its recreational value, Murcielagos Bay is thus the major source of livelihood of the community through fishing. Although elevated Hg levels, both in water and in marine organisms (accumulation), have been documented in Murcielagos Bay (Lacastesantos, unpublished), information on the distribution of Hg and other heavy metals within the vicinity of small-scale mining areas and their impact on soil biota remain unknown. In **chapter 2,** we examined the extent of heavy metal pollution, but also of other mining-related disturbances (for instance fining of sediment, removal of vegetation, soil acidification…) from small-scale mining activities and how it affects **nematode community structure**. The paper was submitted to Ecological Indicators entitled 'Influence of heavy metals on nematode community structure in deteriorated soil by gold mining activities in Sibutad, Southern Philippines'.

Results of chapter 2 indeed confirmed the indiscriminate use of Hg in small-scale gold mining operations where the current Hg levels reached patchily elevated concentrations of up to 38.4 ppm Hg, which was up to 127-fold higher than the permissible level set by the UNEP (2013; 0.3 ppm). Despite this high Hg level, no significant impacts of Hg were observed on nematode assemblages in terms of abundance and genus composition. Using microcosms with natural soil, we applied similar Hg concentrations commonly found in these field sites (2.5, 5 and 10 ppm Hg) to confirm their effect on nematode communities under more controlled conditions. We performed **chapter 3**, a microcosm experiment using relatively 'undisturbed' garden soil amended with Hg concentrations similar to those found in the field and found pronounced effects of Hg on total nematode abundances at concentrations from 2.5 ppm onwards, and on assemblage structure from 5 ppm Hg onwards. The paper entitled 'Effects of mercury (Hg) on

soil nematodes: a microcosm approach' was submitted for publication to the journal Ecotoxicology and Environmental Safety.

Due to the magnitude and lasting effects of risks involved in large-scale mining sites (open-pit mining), including abandoned mined-out areas, the Philippine government mandated in 1999 that rehabilitation of these areas must be implemented. Apart from the 41 active large-scale mining sites (open-pit mining), 32 abandoned mining sites required immediate attention. While there exist traditional approaches for rehabilitation of degraded areas at the local level, visual inspection based on the survivability of metal-tolerant plant species, *Acacia* sp. (here *Acacia auriculiformis*), is often used as a current criterion to judge rehabilitation success. This can be problematic since *Acacia* sp. can tolerate high pollution levels. In **chapter 4**, we examined the response of soil inhabitants, i.e., nematodes, to the rehabilitation strategy of the abandoned rehabilitated sites. Soil recovery was assessed by comparing the nematode assemblage structure in different soils between two time periods under the assumption that soil recovery within sites would be reflected by nematode-based ecological indices, nematode abundance and nematode genus composition. We also identified the physico-chemical parameters of soil which may aid in the optimization of rehabilitation of mined-out areas. This chapter has been prepared for submission to a peer-reviewed journal.

Aside from soil rehabilitation efforts at the large-scale mining area, huge volumes of waste water are stored to be recycled for agricultural purposes. Analysis in 2014 by an independent laboratory showed that several heavy metals such as As, Hg, Zn and Pb from pond waters passed the required 'safe' levels, but **Cd and Cu exceeded** the standard limits prescribed by the Philippine government for agricultural and aquaculture purposes. In addition, having observed clear pollution-induced changes in nematode assemblage composition in chapters 2, 3 and 4, we wanted to demonstrate that mechanisms underlying such shifts can be addressed in detail in dedicated lab experiments. More specifically, here we wanted to test whether a differential sensitivity to heavy metals would shift the outcome of the interaction between two competing nematode species. Taking it one important step further, we also wanted to assess whether such shifts could influence soil ecosystem functioning. In **chapter 5**, we therefore performed a microcosm experiment using soil nematodes and a common leaf grass, *Urochloa mutica*, to demonstrate the effect of copper on nematodes and their interaction, and its possible impact on an ecosystem function, i.e. organic matter (leaf litter) decomposition. The Cu concentrations used in the experiment were well within the concentration range measured from the water samples of the large-scale mining site. This paper has been published as "Copper effects on nematodes and its possible impact on leaf litter decomposition: a microcosm approach",

(Martinez, J. G., Paran, G. P., Rizon, R., De Meester, N. and Moens, T. (2016) European Journal of Soil Biology, 73, 1-7).

Chapter 6 discusses the main findings and methodological challenges on the use of nematodes as bio-indicators in mining impacted sites in the Philippines, using both field and laboratory-based approaches. Current gaps in the present work and potential research aspects were identified for future research undertaking.

CHAPTER 2

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

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This chapter is adapted from:

Martinez, J.G., Torres, M.A., dos Santos, G. and Moens, T. Influence of heavy metals on nematode community structure in deteriorated soil by gold mining activities in Sibutad, southern Philippines. *Ecological Indicators* (under revision).

2.1. Abstract

Ore mining can be one of the most environmentally destructive anthropogenic practices, particularly in many developing countries. In October 2014, soil samples were taken from five different sites of a small-scale mining area in Sibutad, southern Philippines to assess the influence of mining-related activities on nematode communities. Nematodes, often the most abundant invertebrates in soils, play a critical role in soil processes (e.g., decomposition and nutrient cycling) and their assemblages are commonly used to reflect soil health. Nematodes were extracted with a modified tray method and identified to the genus level using morphology-based identification technique. Diversity and maturity indices were determined. Physico-chemical variables of soil, such as OM, N, P, pH, particle size, clay content (%) and heavy metals concentrations (Cd, Cu, Fe, Hg, Pb and Zn) were also measured. Our results show that small-scale mining activities have deteriorated soil properties, altered vegetation and caused slight increases in concentrations of several heavy metals, and a large increase in the concentrations of Hg. The mining-related activities also caused a high patchiness in vegetation and heavy metal contents, which were reflected in a high within-site variability of nematode assemblage composition and of nematode-based indices. Our results also show that nematode genus composition of the different sampling sites was a better indicator of mining-related effects than different commonly used indicator indices (e.g., Shannon index, MI, MI_{2-5}, etc.) which suggests that detailed assemblage analysis is needed for a correct interpretation of moderate pollution effects on soil nematodes. 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 slightly elevated heavy metal concentrations. Such positive responses can have repercussions for the interpretation of nematode-based indices such as the maturity index.

Keywords: bio-indicators, mercury, moderate pollution, heavy metals, nematode assemblages

2.2. Introduction

Ore mining, both large and small-scale, is an important contributor to the economy in many developing countries. For instance, the Philippines is a major exporter of metallic minerals such as gold, copper, nickel and chromium (Hooley, 2005). In Sibutad, a municipality in Mindanao, southern Philippines, gold mining activities have provided livelihood to local communities since the 1980's (Cortes-Maramba et al., 2006). Large-scale mining operations make use of advanced technology in the extraction of mineral deposits, whereas small-scale mining employs manual and fairly rudimentary techniques, which are environmentally risky (Hinton et al., 2003).

Small-scale mining sectors produce about 80% of the Philippines' annual gold supply. However, these substandard routines, aggravated by lack of proper ecological monitoring, can result in deliberate and accidental disposal of wastes (van Straaten, 2000). Despite its economic contribution, it remains a highly polarized issue due to incidences of environmental degradation and health problems among exposed communities (Cortes-Maramba et al., 2006). Mining is associated with the rise of heavy metals in the environment (Getaneh and Alemayehu, 2006). Heavy metals are naturally deposited in rocks and can be released into the environment either by natural weathering or by artificial activities (e.g., digging, ore processing, etc.). They pose a threat because of their potential to bioaccumulate and interfere with various biological processes (Heikens et al., 2001). The gold extraction method by mercury (Hg), also known as amalgamation, is relatively popular among small-scale miners since it is inexpensive. Compared to other mineral extraction methods, amalgamation is easier to perform but potentially risky, and may cause environmental pollution due to improper handling and waste management (Israel and Asirot, 2002). Hg is considered to be one of the most toxic elements naturally found in the environment even at very low concentrations (Göthberg and Greger, 2006), and their negative impacts on soil biota (Harris-Hellal et al., 2009) and soil processes are well-studied also (Müller et al., 2002). In humans, Hg can induce damaging effects on reproduction, immune system, central nervous system and internal organs (Dietz et al., 2000).

At present, there are approximately 500 small-scale miners in the area of Sibutad who can potentially release 120 to 360 kg of Hg per year (Perez et al., 2007). Previous studies have revealed elevated Hg levels in humans (Cortes-Maramba et al., 2006) as well as in marine organisms from Murcielagos Bay (Lacastesantos, *unpublished*), a semi-enclosed bay adjacent to the mined sites, whereas information on Hg effects on terrestrial animals or plants from the area is lacking. Initial inspection showed that the river bed of the sampling area was largely composed

of thick, dark-brown clay sediments and the water appeared very turbid. Preliminary river water analysis revealed a Hg content of ca. 50 μg L^{-1} (our own unpublished data), which is 5 times higher than the permissible limit for wastewater discharge by EPA, i.e., 10 μg L^{-1} (USEPA, 2014) and 25 times higher than the current water quality criterion for the protection of public health by the Philippine government, i.e., 2 μg L^{-1} (emb.gov.ph). The high Hg content of the water is most probably caused by the discharges from small-scale mining activities upstream. Mercury concentrations higher than the allowable level proposed by UNEP (2013) are generally expected to be toxic, and in Sibutad where Hg disposal is a problem, Hg levels in soils may have exceeded the 'permissible' limit. Aside from heavy metal pollution, other activities such as burning of vegetation, digging, construction of physical structures (e.g., tunnels, processing plants, etc.) may also affect soil structure, organic matter content and soil pH, which can also influence the biological activity of soil biota such as nematodes (Sánchez-Moreno et al., 2006).

Nematodes are important biological components in the soil ecosystem due to their functional roles in organic matter decomposition and nutrient cycling (Freckman, 1988; Yeates, 2003); their abundance and community composition are widely used as ecological indicators in several different environments (Bongers and Ferris, 1999; Neher, 2001; Shao et al., 2008). Nematode responses to pollution range from sensitive to very tolerant, with substantial differences between species (Kammenga et al., 1994). Therefore, changes in the nematode assemblage structure and function can be used to assess pollution effects or disturbances in soil, and can be measured by diversity and ecological indices, as well as through a detailed analysis of their taxonomic composition (Fiscus and Neher, 2002).

The present work was conducted to assess whether nematode assemblage structure reflects the impacts of small-scale mining in the southern Philippines. Specifically, this research aimed to a) determine the extent of pollution, particularly that of Hg, and other disturbances (e.g., burning of vegetation, digging, etc.) caused by small-scale mining activities in soils in a small-scale gold mining area; b) assess whether the nematode assemblage structure differed between locations with different degrees of mining-related impact; and c) determine whether such mining impacts are better revealed by particular nematode-based (diversity and maturity) indices or by nematode genus composition.

2.3 Materials and Methods

2.3.1. Study site

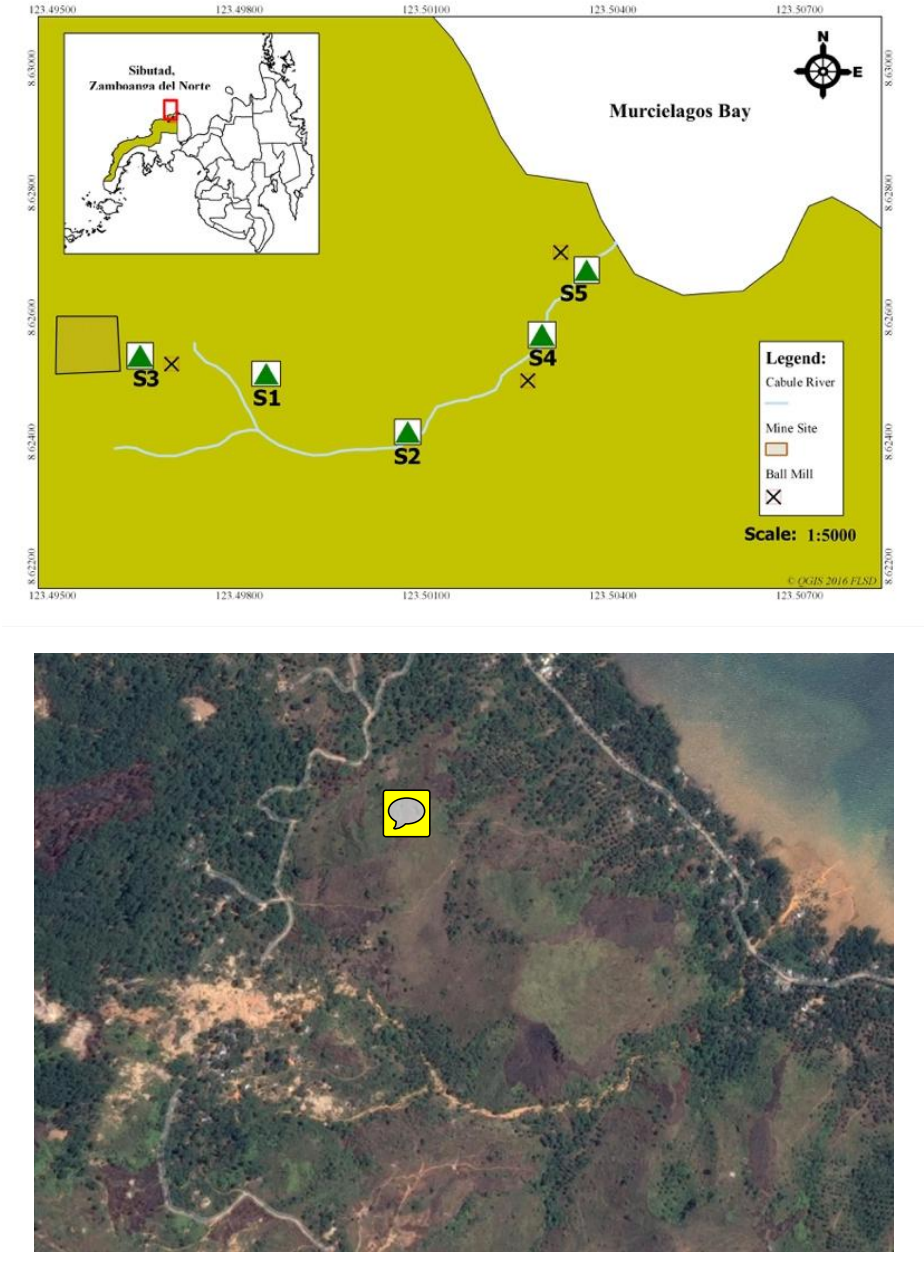

Fig. 2.1. Map of the sampling sites marked by triangles (S1, S2, S3, S4 and S5) in Sibutad, southern Philippines.

The area of Sibutad is situated in the northwestern part of Mindanao, southern Philippines, with an average annual temperature of 27.4 ^{0}C and precipitation of 2310 mm, the latter distributed fairly evenly throughout the year (Fig. 2.1). Before the 1980's, Sibutad sampling area was

predominantly covered with cogon grass (*Imperata cylindrica*), economically unproductive and had only few inhabitants. The discovery of gold deposits in the 1980's caused an influx of miners until the early 2000's; however, the number of active miners has been gradually decreasing since.

Our sampling area is situated on a slope of mountain and covers approximately a distance of 1.2 km (between Site 1 and 5) towards Murcielagos Bay (Fig. 2.1). Some parts of the area have been subjected to 'physical' disturbances such as land clearing, excavation of mountain slopes, open-cast and underground mining, construction of small processing plants and habitation by a few individuals, while other areas have been chemically contaminated owing to mining and ore processing. In practice, small-scale mining workers used ball mills to grind rocks into fine particles, from which the gold is extracted by amalgamation and blowtorching, which results in the formation of wastes (e.g., Hg and tailings).

Table 2.1. Location and brief description of the sampling sites.

sampling sites	coordinates	elev. (m)	common vegetation	description
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 family); near to the excavated areas on the hill slopes; presence of two ball mills
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
S5 (*disturbed*)	8° 37' 34.608" N 123° 30' 12.996" W	3	*P. conjugatum*, *C. nucifera* and ground ferns	presence of few inhabitants (non-miners); near to a ball mill

Soil samples were taken in October, 2014. We divided the study area into five sampling sites – S1, S2, S3, S4 and S5 (Table 2.1). Mining-related activities and/or local communities were manifest in S3, S4 and S5, thus we *a priori* referred to them as 'disturbed' sites as opposed to the 'undisturbed' (reference) sites, S1 and S2. Although we cannot rule out the possibility that the undisturbed sites had previously been impacted by mining-related disturbances due to lack of

information of the past mining activities, the present Hg and other heavy metal levels were used to assess the impacts of local mining activities since their operation in the 1980's.

Five replicate soil samples, each composed of 3 composite samples, were randomly collected with approximate interdistances of 8-10 m from each of the sites. S1 and S2, 300 m apart from each other, were characterized by the absence of inhabitants and mining activities, albeit S1 appeared to have a more diverse vegetation than S2. Perennial grass species (e.g., *Paspalum conjugatum*) generally characterized the disturbed sites (S3, S4 and S5) due to their relatively fast colonizing ability after disturbance episodes. S3, the uppermost part (in terms of altitude) of the area, was marked by intense mining activities with the presence of a community of miners (< 30 ind.), two ball mills, and the site's close proximity to the excavated areas. S4 had the largest human population (> 40 ind.), who were not engaged in mining operations but hosted one ball mill. S5 was also inhabited (< 5 ind.) and located about 0.25 km from Murcielagos Bay. An active ball mill was found near S5, which was situated at an elevated ground a few meters away (ca. 20 m) from this site.

2.3.2. Soil properties

Five replicate composite samples, each consisting of 500 g (a composite of 3 samples combined), were collected from the upper 5 cm using a hand shovel. Soil samples were placed in ziplocked plastic bags and tightly sealed in a box container until laboratory processing. From each soil sample, 200 g were kept at 4 °C and utilized for the determination of basic soil characteristics, nutrients and heavy metal analyses.

Soil pH was determined potentiometrically in the soil suspension of a 1:2.5 soil : water mixture (ISRIC, 1995). Total Organic Carbon was measured by the Walkey-Black method, which involves wet combustion of the organic matter with a mixture of potassium dichromate and sulfuric acid (Walkey and Black, 1934). Total N was determined by the Kjeldahl method (Kjeldahl, 1883) and available P was extracted using acidified ammonium fluoride (Chang and Jackson, 1958). Cu, Zn, Fe, Cd and Pb were extracted by dilute hydrochloric acid procedures (Nelson et al., 1959) and measured by Atomic Absorption Spectrometry, while Hg was measured by Cold Vapor Atomic Absorption Spectrometry (CVAAS). Detection limits of the heavy metals Cd, Cu, Fe, Pb, Zn and Hg were 0.002, 0.003, 0.006, 0.01, 0.001 and 0.02 ppm, respectively.

2.3.3. Nematodes

From each soil sample, 100 g was used 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-Weaver index (H'), which is a diversity measure encompassing both aspects of richness and evenness [$H' = \sum Pi\ (\ln Pi)$] (Shannon and Weaver, 1949); d) Simpson's index, calculated as [$1\text{-}D = 1\text{-}\sum Pi^2$], as a measure of evenness (Simpson, 1949); in both indices, P_i is the proportion of individuals of the i^{th} 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 = [$1 / \sum Pi^2$] where Pi is the proportion of the i^{th} 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; 1995) 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) $MI_{2\text{-}5}$ 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 $MI_{2\text{-}5}$ reflect the (recent) disturbance history of a soil. In theory, the higher the maturity index values, the more mature and stable the ecosystem. MI, $MI_{2\text{-}5}$, and other indices such as Structure Index (SI), Enrichment Index (EI) and metabolic footprint were also calculated using the NINJA online programme (Sieriebriennikov et al., 2014; https://sieriebriennikov.shinyapps.io/ninja/).

2.3.4. 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 abundances were square root-transformed to downsize the effect of dominant genera. When significant differences were detected, pairwise comparison tests within PERMANOVA^{+} were conducted to establish differences between sites.

DistLM (Distance-based linear model) routine using a global BEST selection procedure with Bayesian Information Correction (BIC) was carried out to identify the environmental variables that best explain the observed patterns in nematode communities. Distance-based redundancy analysis (dbRDA), a graphical visualization of the DistLM results, was used to show patterns in assemblage composition and environmental variables across samples using Pearson correlation. Similarity percentage (SIMPER) analyses using the untransformed nematode abundance data were used to identify the genera which contributed to the similarities or differences between study sites and between the undisturbed and disturbed sites. The genera are considered 'important' if they contribute at least 5% of the average dissimilarity among the sites (Mirto et al., 2002). Note that in the present study, we considered roughly 5% (e.g., > 4.5%) of the average dissimilarity between sites.

2.4. Results

2.4.1. Soil properties and heavy metal concentrations

Table 2.2. Mean concentrations of heavy metals, nutrients and soil properties of the five sampling locations. Values 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 (ppm)	0.32 ± 0.14	0.27 ± 0.08	0.23 ± 0.11	0.23 ± 0.13	0.14 ± 0.04
P (ppm)	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	28.8 ± 6.27^{b}	75.5 ± 66.4^{ac}
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 (ppm)					
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

Mean values followed by different letters on the same row indicate significant differences according to a post-hoc Tukey HSD test ($P < 0.05$).

Several basic soil variables such as OM, N, P and pH did not show any significant difference between sites; however, a decreasing trend in OM, N and pH (except S4) were observed in the disturbed sites compared to the undisturbed sites. Other soil properties such as median grain sizes in S2, S3 and S4 were significantly smaller (all $P < 0.05$) compared to S1 and S5 (Table 2.2). In terms of grain size, disturbed sites with ball mill plants, S3 and S4, had significantly finer grain sizes and higher percent of clay content compared to those without ball mill plant, except to S2. Heavy metal concentrations in the disturbed areas were not significantly increased except for Hg and Pb; Hg, which was highest in S4 ($P < 0.05$) while Pb were significantly higher in both S4 and S5. Although S5 had a ball mill plant nearby, the lower Hg content in this area compared to S4 suggests that the tailings were most probably disposed off elsewhere and not on the sampling site.

In a principal coordinates analysis (PCO) of the soil properties (Fig. 2.2), PCO1, explaining 29.6% of the observed variation, shows that Site 4 were associated with increasing metal concentrations including Zn (r = 0.7), Pb (r = 0.67), Cd (r = 0.57), Cu (r = 0.56) and Hg (r = 0.50), with increasing pH (r = 0.66), N (r = 0.48) and P (r =0.83) (ESM 2.1). PCO2 accounted for 28.4% of the observed variation and positioned 2 replicates of S4 and 1 replicate of S1 apart from other sampling sites; it was positively associated with increasing Hg (r = 0.72), Fe (r = 0.68), Pb (r = 0.46) and Zn (r = 0.45) and Cu (r = 0.54). Samples of S4 were rather scattered in the ordination plane (Fig. 2.2).

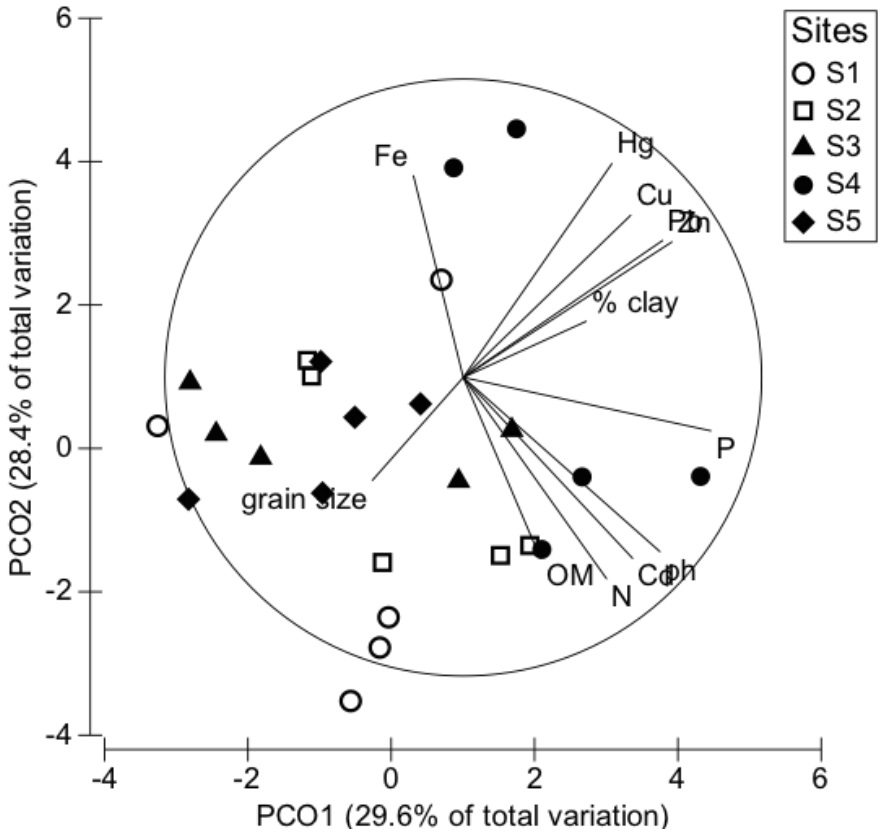

Fig. 2.2. Principal coordinates analysis (PCO) of the environmental variables (5 replicates) from the different sampling sites in the Sibutad small-scale mining area. See table 2.2 for an overview of environmental variables included in the analysis.

2.4.2. Nematode abundance, genera, diversity and maturity indices

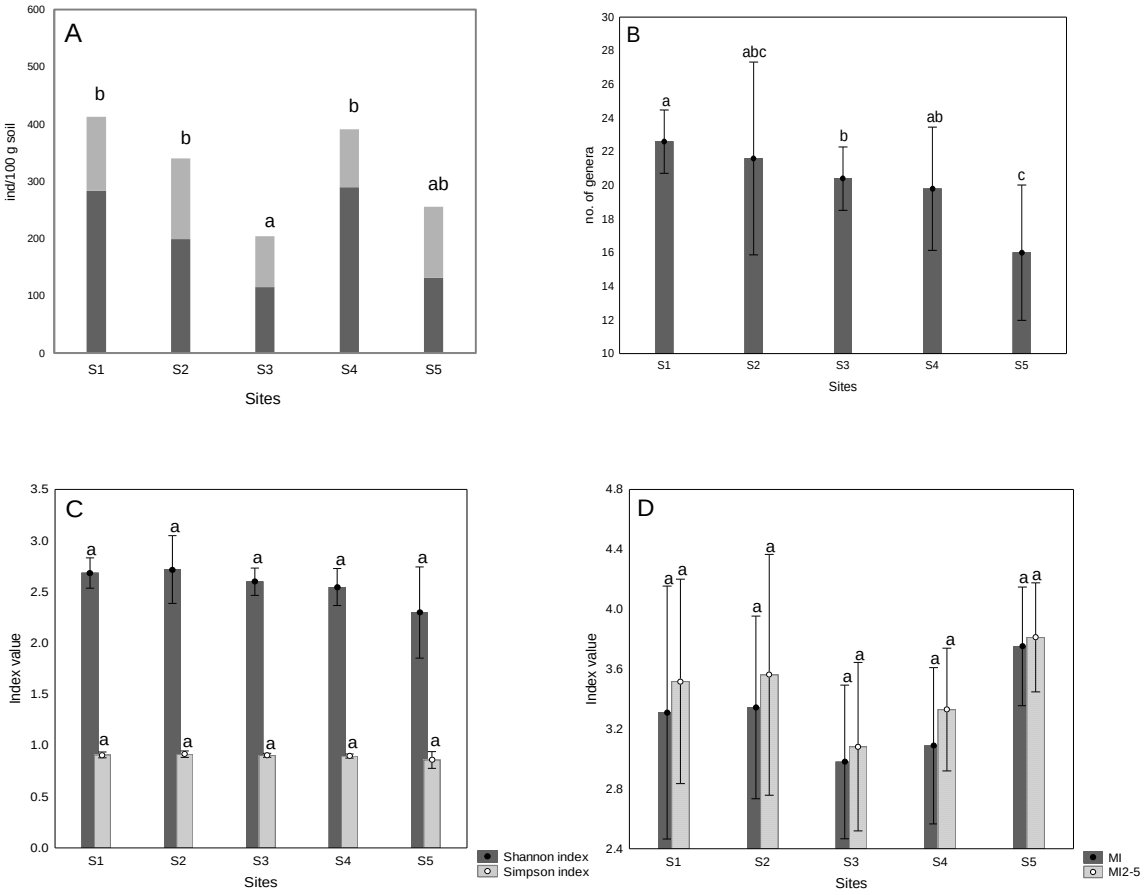

Fig. 2.3 (A-D). Summed abundances of plant-parasitic (light-colored bars) and free-living nematodes (dark-colored bars) (A), species richness (B), Shannon and Simpson indices (C), and MI and MI_{2-5} (D). Different letters indicate significant pairwise differences between sites according to a post-hoc Tukey HSD test ($P < 0.05$).

Total nematode abundance showed significant differences between locations (df = 4; F = 3.65; *P < 0.05*); highest density (412 ± 160 ind/100 g soil) was found in S1, whereas S3 had the lowest (204 ± 59 ind/100 g soil) (Fig. 2.3A). Nematodes belonged to 49 genera, 12 of which were bacterial feeders, 5 fungal feeders, 20 omnivores/predators and 12 plant feeders (ESM 2.4). Index of trophic diversity did not show any significant differences (df = 4; F = 2.01; *P > 0.05*) between sites (data not shown), but genus richness did (df = 4; F = 3.61; *P < 0.05*): S1 had a significantly higher number of genera than S3 and S5 (Fig. 2.3B). Shannon diversity and evenness (Simpson index) did not differ significantly among sites (df = 4; F = 2.82 and F = 4.87 for Shannon diversity and evenness, respectively; *P = 0.054* and *P = 0.091* respectively; Fig. 2.3C). Nevertheless, there was a trend indicating higher diversity in undisturbed compared to disturbed sites. Finally, S5 had the highest MI and MI_{2-5}, while S3 had the lowest (Fig. 2.3D), but these differences were not statistically significant.

2.4.3. Nematode assemblage composition

PERMANOVA revealed highly significant differences in nematode composition between locations (df = 4; F = 3.53*; pseudo-P = 0.001*), with a non-significant PERMDISP (*PERMDISP = 0.66*). Pairwise comparisons detected significant differences between all pairs of sites, except the two undisturbed sites, S1 and S2 (Table 2.3). PERMANOVA also detected highly significant differences in nematode assemblage composition between undisturbed and disturbed sites (df = 1; F = 2.81*; pseudo-P = 0.005*), which are also nicely illustrated by non-metric multi-dimensional scaling (nMDS) ordination with Bray-Curtis similarity (Fig. 2.4); however, this requires careful interpretation since multivariate dispersion (PERMDISP) showed that variances were significantly heterogeneous (*PERMDISP = 0.002*).

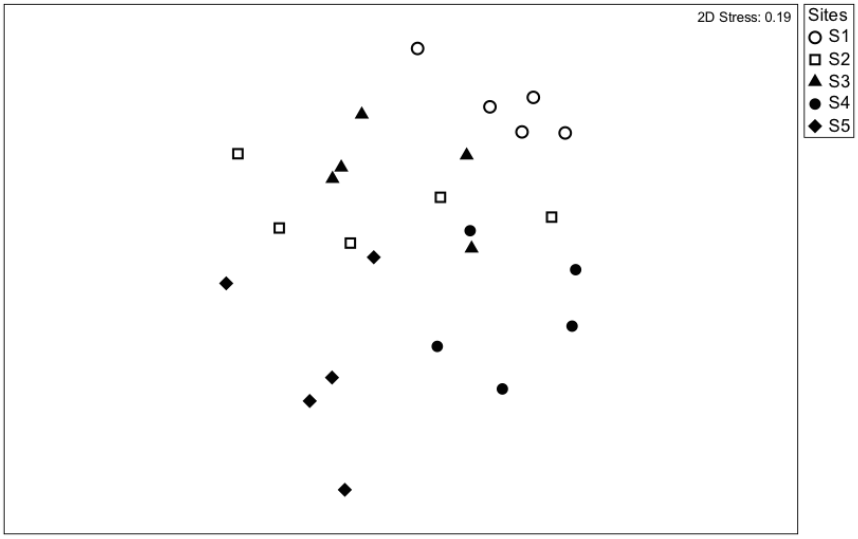

Fig. 2.4. Non-metric multi-dimensional scaling ordination (nMDS) of the nematode genera composition in different sites. Undisturbed sites (S1 and S2) are represented by light-colored symbols while dark-colored symbols represent the disturbed sites (S3, S4 and S5).

Table 2.3. 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**	-

Asterisks (*) and (**) indicate significant differences at *P < 0.05* and *P <0.01*, respectively.

Table 2.4. Results of the SIMPER (Similarity Percentages) analysis of the nematode data between the undisturbed (S1 and S2) and disturbed sites (S3, S4 and S5). Multiple genera contributed to the site differences. Listed below are all the genera contributing up to a cumulative total (Cum. cont. %) of ≥75 % to such differences.

Genera	Average abundance		Cum. cont. %
Average dissimilarity = 70.03%	Undisturbed (S1, S2)	Disturbed (S3, S2, 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

SIMPER analysis showed that all site pairs had high levels of dissimilarity in nematode assemblages (ESM 2.3). The largest dissimilarity was between S3 and S5 (75.57%), while S1 and S2 were the least dissimilar (63.03%), but only slightly less so than the other site pairs, even though PERMANOVA did not detect significant differences between both undisturbed locations. Several genera were identified to be responsible for the 70.03% dissimilarity between the undisturbed (S1 and S2) and disturbed sites (S3, S4 and S5) (Table 2.4). Particularly, the 'important' genera (i.e. genera contributing roughly 5% to the dissimilarity between the undisturbed and disturbed sites) included *Iotonchus*, *Mesodorylaimus*, *Axonchium*, *Rotylenchulus* and *Helicotylenchus* of the undisturbed sites, and *Dorylaimellus* and *Cephalobus* of the disturbed sites. Six genera were exclusively found either in undisturbed or disturbed sites, but only contributing < 1% to the difference between sites: *Opisthodorylaimus*, *Granonchulus* and *Chronogaster* in undisturbed sites, while *Coslenchus*, *Oriverutus* and *Mononchulus* in disturbed sites.

dbRDA1 explained 17.7% of the total variation in the nematode data and generally distinguished S1, S2, S3 and S4 from S5 (Fig. 2.5). dbRDA1 was positively associated with the relative abundances of *Helicotylenchus* ($r = 0.61$), *Rotylenchulus* ($r = 0.56$), *Aphelenchus* ($r = 0.54$), *Alaimus* ($r =$

0.49) and *Paractinolaimus* (r = 0.47) while negatively associated with *Eudorylaimus* (r = - 0.49) and *Dorylaimellus* (r = - 0.83) (Fig. 2.5A). dbRDA1 also had a strong negative correlation with Cd (r = - 0.87) (Fig. 2.5B). On the other hand, dbRDA2 generally 'separated' S4 and a few replicates of S3 and S5 from the undisturbed sites, S1 and S2, from, while explaining 14.9% of variation. dbRDA2 was correlated with Pb (r = 0.68) and Hg (r = 0.63), and the genera positively correlated with it included *Acrobeloides* (r = 0.60), *Cephalobus* (r = 0.53), *Pratylenchus* (r = 0.49), *Bursilla* (r = 0.48), and *Ironus* (r = 0.48).

The best DISTLM with no more than three variables, N, Pb and Hg, explained 24.5% of the fitted variation of nematodes in the area, which indicated that Pb, Hg and N were the drivers of nematode assemblage structure (ESM 2.2). A distance-based linear model including all the measured environmental variables explained 60.4% of the fitted variation (i.e. 32.6% of the total variation at the two first dbRDA axes) in the nematode data (Fig. 2.5 A and B), which suggests that non-measured variables (e.g., vegetation, species interactions) are also important drivers of nematode assemblage structure.

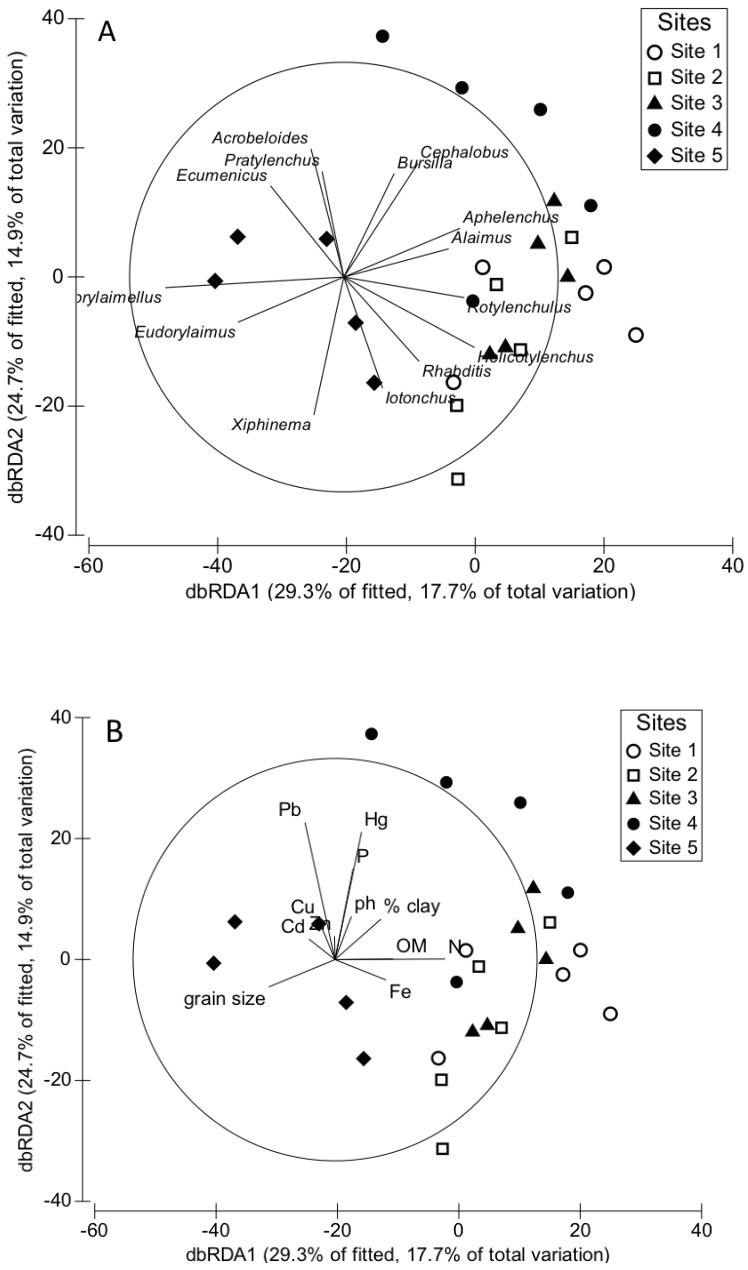

Fig. 2.5 (A and B). Distance-based Redundancy Analysis (dbRDA) plots based on the nematode assemblages and the fitted environmental variables as vectors.

2.5 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.

2.5.1. Basic soil properties and heavy metals

The impacts of small-scale mining activities were reflected by the higher levels of heavy metals (e.g., Hg, Cd, Pb) and other subtly contrasting soil properties (e.g., OM, particle size and % clay content), and vegetation 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, all of which were partly observed in our disturbed locations. In addition to edaphic differences, the disturbed sites can also be distinguished by several fast-growing grass species (i.e. *Paspalum conjugatum*, most common in the study area), which can easily establish and dominate during post-mining succession (Groninger et al., 2007). Although not significant, there was a trend of a slightly higher OM concentrations in the undisturbed sites, S1 and S2, compared to the disturbed sites, S3 and S5 which may be linked to the vegetation cover: the undisturbed sites, S1 and S2, had a more abundant and more diverse vegetation than S3 and S5, thus increasing the input of organic material. OM can increase heavy-metal adsorption in soil, thus decreasing their bioavailability (Antoniadis et al., 2008).

The disturbed sites, S3 and S4, had significantly finer grain sizes (mean of 24 μm and 28.8 μm, respectively) with a higher contribution of clay (15.8% and 14.9%, respectively) compared to the rest of the sites (except to S2) which may be caused by the disposal of fine soil residues from the ball mill plants. Unexpectedly, S2 had a relatively fine grain size similar to that of S3, possibly due to past mining-related disturbances, which is further supported by the relatively high Hg concentrations at this location. Mining activities can also cause soil acidification (4.5 to 5.0) due to the oxidation of iron pyrite (FeS_2) and other sulphidic materials (Johnson and Hallberg, 2005), a process that can lead to the formation of acid mine drainage (AMD). Furthermore, acidic soils can induce adverse impacts on soil health, microbial activity, nutrient availability and ecosystem functioning. Low pH can also increase the solubility and hence the bioavailability of metals (Kim

et al., 2009), which can cause an increased toxicity to soil organisms (Alloway and Alloway, 1990). In the present study, disturbed sites, S3 and S4, tended to have slightly lower pH < 5.0, compared to the undisturbed sites, except to S4.

Due to a lack of established allowable ranges of heavy metals in the Philippines, we compared our data to existing literatures. However, caution is needed when extrapolating since metal effects in soils are influenced by pH, clay and organic matter content (Rieuwerts et al., 1998). Heavy-metal levels of the present study were lower than the allowable concentrations imposed by regulatory bodies from developed countries (Teh et al., 2016), except Cd and Hg when compared to the US and UNEP limits, respectively. While the world average Hg levels in soil ranges from 0.01 ppm to 0.2 ppm (Adriano, 2001), UNEP (2013) recommended an acceptable range from 0.07 ppm to 0.3 ppm. In the present study, all Hg concentrations, except those of S1, exceeded acceptable levels as defined by UNEP (2013).

2.5.2. Nematode abundance, diversity and maturity indices

Nematode abundances in Sibutad were in the abundance range of some heavy metal pollution-impacted sites in, e.g., China and Israel (Shao et al., 2008; Pen-Mouratov et al., 2008), which suggests that the whole area was impacted at least to some extent. A general trend of low nematode abundances in some of the locations (S2 and S3) may be attributed to the fine grain size, probably caused by frequent disposal of very fine soil residues or tailings, especially in S3, during the mineral extraction processes. This suggests that S2, although currently undisturbed, has also been exposed to previous mining activity, which is also reflected in the concentrations of some heavy metals (see above). Grain size can affect nematode communities; often, lower densities are observed in finer textured soils compared to coarser soils (Anderson et al., 1979; Sánchez-Moreno and Navas, 2007). Clayey soils, which contain a substantial fraction of very fine particles, are characterized by reduced soil pores and a high water content. Since nematodes move along soil spaces, clayey soils can impede their movement and the associated high water content can result in oxygen deprivation (Glazer, 2002). Also, the significantly lowest nematode abundance in S3 may be exacerbated, directly or indirectly, by the acidity of the soil (Korthals et al., 1996a; Park et al., 2011). Aside from the fact that low pH can enhance heavy metal toxicity (Korthals et al., 1996a; Kim et al., 2009), negative effects of a low pH on bacterial abundance (Räty and Huhta, 2003) could explain the low proportional abundance of bacterial-feeding nematodes in S5 and to a lesser extent in S3 (both had a pH < 5), though not significantly lower compared to S4 which had a nearly neutral soil pH. No clear nematode abundance trends were, however, observed between nematode densities and our a priori classification of the different

sites. S3 and S4, for instance, were both impacted, yet they differed in the type of disturbance related to exploratory mining activities ('physical' vs 'chemical' disturbance), among other things in a quite different vegetation cover (S4 being more diverse than S3). Plants can affect the soil biota (e.g., nematodes) in several ways – e.g., root exudates and the high inputs of dead OM can cause high abundances of bacteria which can serve as food to bacterial-feeding nematodes (Bongers and Ferris, 1999; Bais et al., 2006). S3 had the lowest and S4 the second-highest mean nematode abundances, suggesting that nematode abundance is a useful indicator of 'physical' disturbance in this area (Neher, 2001; Fiscus and Neher, 2002; Schratzberger and Jennings, 2002), rather than of heavy metal pollution *per se* (Bongers, 1990; Korthals et al., 1996a).

Diversity indices have been used by soil ecologists to assess the impacts caused by heavy-metal pollution, although Pen-Mouratov et al. (2010) found that nematode diversity indices were more affected by soil properties, whereas 'ecological indices' such as the maturity index were more sensitive to disturbance. In many cases, impacted areas are characterized by low nematode diversity compared to non-impacted areas due to the elimination of sensitive taxa (Yeates et al., 1995; Sánchez-Moreno and Navas, 2007; Park et al., 2011) and the increased dominance of tolerant taxa (Lambshead, 1986). This was partly confirmed in the present study where S1 had the highest genus richness, while S5 had the lowest, despite the fact that S4 was the most contaminated site. This is probably due to the higher plant diversity in S4 compared to S5 (Šalamún et al., 2017). Other diversity indices such as Shannon-Weaver and Simpson, however, did not show any significant differences between locations, although they tended to decrease from undisturbed to disturbed sites: S2 ≥ S1 ≥ S3 ≥ S4 ≥ S5, and this trend was only borderline non-significant ($P = 0.054$) for Shannon-Weaver (H') diversity.

In a similar study on metal-pollution impact by Chen et al. (2009), H' index values in less disturbed areas (from 2.24 to 2.69) were fairly comparable to the results from the Sibutad undisturbed sites (2.68 and 2.72), while H' in our disturbed sites (2.30 being the lowest) overlapped with those of the 'undisturbed' areas from that study. This suggests that diversity indices should not merely be compared with those of other studies on the basis of their absolute values, but interpreted in a context-dependent manner (e.g., vegetation, soil type, pollution levels, history…). Aside from soil pH, other factors such as root architecture, root exudates, and soil type also need to be taken into account since they can influence the bioavailability of heavy metals in soil (Rieuwerts et al., 1998; Mench and Martin, 1999).

Maturity indices of nematodes have also been used extensively to assess the status of soil health. In principle, higher MI values (MI and MI_{2-5}) suggest a more stable and less disturbed

environment (Bongers and Ferris, 1999; Neher, 2001). For instance, a negative impact of heavy metal (such as Cu, Ni) concentrations exceeding 100 ppm on the MI was observed in terrestrial systems (Korthals et al., 1996b). However, this cannot be easily translated to our 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., 1996a; Nagy et al., 2004) does not always hold. For instance, in our study, nematodes with cp3-5 scores do 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 (ESM 2. 4).

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 values and assemblage composition in our study could be taken as evidence of the importance of physical disturbance as a driver of nematode assemblage structure and diversity (Fonseca and Gallucci, 2016).

2.5.3. Nematode genera associated with heavy-metal pollution

Previous studies showed that nematode community composition can be sensitive to soil management practices or disturbances (Fiscus and Neher, 2002; Sánchez-Moreno et al., 2006). While the nematode-based indices did not reflect the mining-related disturbances, significant differences in nematode genus composition between undisturbed (S1 and S2) and disturbed sites (S3, S4 and S5), and between all pairs of sites except S1 and S2, were strong indications of the impact of ongoing or recent small-scale mining activities which altered the physico-chemical attributes of the soil, and in turn, differentially impacted nematode genera (Fiscus and Neher, 2002).

Important genera characteristic of the undisturbed sites included the free-living nematodes *Iotonchus* and *Mesodorylaimus*, and the plant-feeding nematodes *Axonchium*, *Rotylenchulus* and *Helicotylenchus* while *Cephalobus* (free-living) and *Dorylaimellus* (plant-feeding) were characteristic of the disturbed sites (Table 2.4). Our results thus confirm those of Šalamún et al. (2012) concerning the near-absence of *Iotonchus* and the high sensitivity of *Mesodorylaimus*, a cp4 nematode, to chemical disturbance (Bongers, 1990; Chen et al., 2009). Thus, the two free-living genera may be considered indicator taxa in relation to mining-related disturbance because based in a community analysis, they contributed most to the dissimilarity between disturbed and undisturbed soils. Good indicators should reflect the structure and/or function of ecological communities and respond to changes in soil condition (Neher, 2001). Often, the focus is on abundant taxa when trying to identify indicators of disturbance (Bongers and Ferris, 1999; Fiscus and Neher, 2002). However, our results demonstrate that a detailed community analysis may also reveal good indicators among the many taxa with low abundances. Other genera such as *Opisthodorylaimus* (cp5), *Granonchulus* (cp4) and *Chronogaster* (cp3) were also found to be sensitive to environmental disturbance in view of their complete absence from disturbed sites. By contrast, the prominence of bacterial-feeding *Cephalobus* (cp2) in disturbed areas agrees well with assumptions of the MI and related indices about the pollution and disturbance-tolerance of bacterivores with cp2 (Bongers and Ferris, 1999; Bert et al., 2009). Other genera such as *Coslenchus* (cp2), *Oriverutus* (cp5) and *Mononchulus* (cp4) were limited to disturbed areas, which is counterintuitive for the latter two genera since both are expected to be sensitive to disturbance (Ferris et al., 2001). Many plant-feeding nematodes, on the other hand, were reported to be tolerant to heavy-metal pollutants (Pen-Mouratov et al., 2008; Šalamún et al., 2012; Gutiérrez et al., 2016), hence the high abundances of *Dorylaimellus* in the disturbed sites suggest that their distribution was more influenced by their host plants, rather than by metal effects.

Aside from the dissimilarity in nematode assemblages between the disturbed and undisturbed sites, significant differences in nematode assemblages also occurred between nearly all pairs of sites, except S1 and S2. Nitrogen and the heavy metals Pb and Hg were identified as drivers of nematode assemblage structure in the mining sites. Nitrogen plays an important role as a main source for primary production and can increase soil microbial biomass (Alon and Steinberger, 1999). Although N content in soils did not differ between sites, a trend of slightly lower N in the disturbed sites compared to S1 was observed. The majority of the metal pollutants, with the exception of Hg, were below the concentrations known to impact soil nematodes in many field studies (Sánchez-Moreno et al., 2006; Sánchez-Moreno and Navas, 2007; Shao et al., 2008; Chen et al., 2009; Gutiérrez et al., 2016). Our results suggest that single heavy metals (e.g., Pb and Hg) may not importantly affect nematode assemblage structure in our study area, but that their combination can; additive effects of metals, such as the Cu-Zn mixture reduced abundance of nematode taxa and trophic groups were (Korthals et al., 2000) and the combination of Cu, Zn and Pb showed negative effects on the nematode community structure, e.g., MI and *H'* (Sánchez-Moreno and Navas, 2007). On the other hand, our results indicate that the free-living *Acrobeloides, Cephalobus*, *Bursilla, Ironus* and the plant-feeding *Pratylenchus* were more abundant under moderately elevated concentration of Pb and high concentration of Hg. While the tolerance of *Acrobeloides* (cp2), *Cephalobus* (cp2), *Bursilla* (cp1) and *Pratylenchus* (pp3) to metal stressors were in accordance with the general MI theory (Bongers, 1990) and previous studies (Georgieva et al., 2002; Šalamún et al., 2012), the positive association of presumedly 'sensitive' genera *Ironus* (cp4) to Pb and Hg, and *Eudorylaimus* (cp4) to Cd, respectively, were unexpected (Fig. 2.5). Care is due when interpreting such relationships given the limited number of sites included in our study; as a result, such a relationship can be largely driven by the fact that these genera had their highest abundance in the one site with the highest concentrations of particular heavy metals (e.g., Pb and Hg in S4). Nevertheless, such positive relationships of nematodes with high cp values (sensitive taxa) to relatively low levels of metal pollution have also been reported (Heininger et al., 2007; Šalamún et al., 2011), and this may have repercussions for the interpretation of Maturity and related indices. In a recent mesocosm study, Šalamún et al. (2015) demonstrated a positive influence of Cd and Cu, both at 40 ppm, on sensitive nematodes (cp5) and on several nematode indices (Structure Index, MI_{2-5} and Shannon diversity) respectively, but values of these indices declined at higher metal concentrations. Stimulatory effects on reproduction and growth by low concentrations of Cd (0.5 ppm for *Plectus parvus* and 1.0 ppm for *Acrobeloides nanus*) and Pb (0.01, 0.05, 0.1 ppm for *Caenorhabditis elegans*) have also been recorded in microcosms (agar plates) (Martinez et al., 2012; Monteiro et al., 2014), and similar

effects can also occur under exposure to combined low concentrations of different metals, as suggested by the present study. However, the positive effect of high Hg combined with low Pb on sensitive taxa, which was evident in S4, w[illegible]ather surprising, given the fact that Hg concentration in this site was 127-fold higher than the permissible level set by UNEP (2013) at 0.3 ppm. Such positive association may be due to the fact that S4 had the particular combination of a more neutral soil pH and the presence of a diverse vegetation, which may have decreased metal bioavailability and favored the growth of bacterivorous nematodes (ESM 2.4) and other smaller invertebrates, which in turn can be prey to some of the larger omnivorous and predacious nematodes, which typically have high cp values. Another potential explanation for this positive effect is interference among different metal ions which can affect metal entry into the cell; e.g., a negative effect of Hg on *Caenorhabditis elegans* was reduced in the presence of Fe (Anbalagan et al., 2005).

2.6 Conclusions

The small-scale mining activities in Sibutad have caused physical (e.g., deteriorated soil properties, altered vegetation) and chemical (strongly increased Hg levels especially 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-Weaver 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 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.

Electronic Supplemental Materials (ESM)

ESM 2.1. PC scores of the Principal Coordinates Analysis (PCO) of the environmental variables from the small-scale mining areas.

Variables	PCO1	PCO2	PCO3	PC04	PC05
OM	0.26	-0.61	-0.02	-0.66	0.05
N	0.48	-0.67	0.13	-0.41	0.07
P	0.83	-0.12	-0.03	-0.03	-0.22
pH	0.66	-0.58	-0.10	-0.06	-0.08
median grain size	-0.31	-0.34	0.87	-0.05	-0.01
clay content	0.41	0.19	-0.84	0.15	0.05
Cd	0.57	-0.61	-0.05	0.45	0.13
Cu	0.56	0.54	0.27	0.13	0.50
Fe	-0.17	0.68	-0.17	-0.59	0.09
Hg	0.50	0.72	0.16	-0.21	-0.37
Pb	0.67	0.46	0.38	0.18	-0.30
Zn	0.70	0.45	0.24	-0.12	0.31

ESM 2.2. Results of the DistLM marginal test and model selection.

No.	Variable	SS (trace)	Pseudo-F	*P*	Prop.
Marginal DistLM test					
1	Fe	6060.2	4.0105	0.001	0.14848
2	Zn	3541.7	2.1854	0.012	$8.6774E^{-2}$
3	Cd	5874.3	3.8668	0.001	0.14393
4	Pb	4387.5	2.7702	0.004	0.1075
5	Cu	4676.1	2.976	0.002	0.11457
6	Hg	4188.2	2.63	0.007	0.10261
7	ph	4422.5	2.795	0.001	0.10835
8	OM	4449.3	2.814	0.001	0.10901
9	N	4945.9	3.1714	0.001	0.12118
10	P	4155.6	2.6072	0.002	0.10182
11	grain size	3147.2	1.9217	0.028	$7.711E^{-2}$
12	clay (%)	2605.2	1.5682	0.092	$6.3831E^{-2}$

Best results for each number of variables

Var. no.	BIC	R^2	No. of var.	Selections
DistLM models				
1	187.37	0.08439	1	4
2	186.96	0.16304	2	6, 9
3	186.44	0.24472	3	4, 6, 9
4	187.84	0.29774	4	2, 4, 6, 9
5	188.19	0.3581	5	3, 6, 8, 9, 11
6	188.19	0.41948	6	2-4, 8, 9, 12
7	188.19	0.45458	7	2-4, 8-10, 12
8	188.19	0.49208	8	1, 2, 4, 6-9, 11
9	188.19	0.52504	9	1, 2, 4-9, 12
10	188.19	0.55444	10	1-9, 12

11	188.19	0.5784	11	1-6,8-12
12	190.53	0.60439	12	All

BIC	R^2	RSS	No. of var.	Selections
Overall best solutions				
187.89	0.08439	35484	1	4
188.03	0.07906	35691	1	9
188.07	0.07780	35740	1	6
188.53	0.06054	36409	1	11
188.72	0.05320	36693	1	8
188.76	0.05172	36750	1	12
188.83	0.04912	36851	1	10
188.86	0.16304	32436	2	6, 9
188.86	0.16304	32437	2	4, 9
189.15	0.03703	37320	1	1

ESM 2.3. Results of the SIMPER analysis of the nematode data between sampling sites. Multiple genera contributed to the site differences. Listed below are all the genera contributing up to a cumulative (Cum. cont. %) total of ≥ 64% to such differences.

Genera	Average abundance		Cum. cont. (%)
Average dissimilarity = 63.03%	S1	S2	
Rotylenchulus	2.28	40.53	8.83
Iotonchus	53.98	9.85	17.63
Axonchium	42.45	16.89	24.42
Mesodorylaimus	27.97	21.58	29.11
Cephalobus	18.11	16.99	33.79
Aphelenchus	23.63	17.89	38.45
Xiphinema	23.02	4.93	42.97
Bursilla	14.32	8.44	47.12
Helicotylenchus	18.31	30.53	51.07
Heterocephalobus	20.08	15.73	54.76
Rotylenchus	13.66	9.35	58.02
Oxydirus	16.94	4.15	61.19
Metaporcelaimus	15.02	17.25	64.22
Average dissimilarity = 72.74%	S2	S3	47.12
Iotonchus	53.98	3.26	10.02
Axonchium	42.45	0.00	19.19
Mesocriconema	13.29	36.02	25.82
Rotylenchulus	2.28	23.68	30.84
Aphelenchus	23.63	10.66	35.62
Xiphinema	23.02	2.71	40.35
Mesodorylaimus	27.97	20.80	44.82
Bursilla	14.32	2.94	49.12
Cephalobus	18.11	17.59	52.99
Rotylenchus	13.66	1.03	56.43
Heterocephalobus	20.08	2.46	59.85
Helicotylenchus	18.31	10.54	63.01
Oxydirus	16.94	0.82	66.14
Metaporcelaimus	15.02	6.78	69.05
Eudorylaimus	12.71	11.74	71.78
Paraphelenchus	3.42	8.90	73.94
Average dissimilarity = 69.93 %	S1	S4	
Iotonchus	53.98	15.07	7.39
Ironus	3.24	41.89	14.37
Axonchium	42.45	11.43	20.67
Cephalobus	18.11	40.39	26.50
Bursilla	14.32	29.58	32.32
Mesodorylaimus	27.97	31.59	37.45
Pratylenchus	0.45	24.48	42.14
Oxydirus	16.94	15.35	46.29
Ecumenicus	7.89	24.85	50.18
Xiphinema	23.02	5.37	54.03
Aphelenchus	23.63	12.64	57.84
Heterocephalobus	20.08	17.91	61.37
Meloidogyne	0.00	17.19	64.78
Helicotylenchus	18.31	4.98	67.74
Rotylenchus	13.66	5.66	70.44
Average dissimilarity = 74.1%	S1	S5	
Dorylaimellus	4.78	51.35	9.92
Iotonchus	53.98	3.72	19.16
Eudorylaimus	12.71	43.46	26.39
Axonchium	42.45	16.45	32.88
Aphelenchus	23.63	0.85	38.15
Xiphinema	23.02	12.24	42.42
Mesodorylaimus	27.97	12.98	46.53
Helicotylenchus	18.31	0.85	50.55

Oxydirus	16.94	12.88	54.40
Bursilla	14.32	2.39	58.19
Cephalobus	18.11	6.58	61.71
Mesocriconema	13.29	13.38	65.15
Hemicriconemoides	10.33	15.30	68.49
Rotylenchus	13.66	0.00	71.67
Heterocephalobus	20.08	4.08	74.67
Average dissimilarity = 63.22%	S2	S3	
Rotylenchulus	40.53	23.68	7.92
Mesocriconema	8.72	36.02	15.66
Helicotylenchus	30.53	10.54	21.75
Cephalobus	16.99	17.59	27.69
Mesodorylaimus	21.58	20.80	32.84
Axonchium	16.89	0.00	37.71
Heterocephalobus	1 5.73	2.46	42.27
Metaporcelaimus	17.25	6.78	46.26
Eudorylaimus	17.11	11.74	50.03
Aphelenchus	17.89	10.66	53.74
Dorylaimellus	11.93	2.06	56.83
Pratylenchus	8.51	8.74	59.84
Hoplolaimus	8.71	0.41	62.76
Tylencholaimus	10.92	0.34	65.59
Average dissimilarity = 68.70%	S2	S4	
Ironus	0.00	41.89	8.36
Rotylenchulus	40.53	11.80	15.46
Cephalobus	16.99	40.39	22.28
Mesodorylaimus	21.58	31.59	27.71
Bursilla	8.44	29.58	33.01
Helicotylenchus	30.53	4.98	38.17
Pratylenchus	8.51	24.48	43.0
Ecumenicus	6.20	24.85	47.50
Heterocephalobus	15.73	17.91	51.5
Meloidogyne	0.82	17.19	55.27
Aphelenchus	17.89	12.64	58.54
Metaporcelaimus	17.25	1.75	61.77
Oxydirus	4.15	15.35	64.94
Dorylaimellus	11.93	7.24	67.81
Eudorylaimus	17.11	13.76	70.58
Average dissimilarity = 71.48 %	S2	S5	
Dorylaimellus	11.93	51.35	11.11
Rotylenchulus	40.53	1.28	20.81
Eudorylaimus	17.11	43.46	28.61
Helicotylenchus	30.53	0.85	35.53
Cephalobus	16.99	6.58	39.92
Mesodorylaimus	21.58	12.98	44.18
Aphelenchus	17.89	0.85	47.96
Hemicriconemoides	0.00	15.30	51.51
Heterocephalobus	15.73	4.08	55.01
Mesocriconema	8.72	13.38	58.41
Xiphinema	4.93	12.24	61.46
Metaporcelaimus	17.25	7.03	64.50
Axonchium	16.89	16.45	67.08
Pratylenchus	8.51	7.12	69.44
Ecumenicus	6.20	9.35	71.80
Average dissimilarity = 72.93%	S3	S4	
Ironus	0.00	41.89	9.56
Mesocriconema	36.02	2.41	17.34
Bursilla	2.94	29.58	23.95
Ecumenicus	0.34	24.85	30.24
Mesodorylaimus	20.80	31.59	36.07
Pratylenchus	8.74	24.48	41.75
Cephalobus	17.59	40.39	47.26

Rotylenchulus	23.68	11.80	51.97
Meloidogyne	0.40	17.19	56.38
Heterocephalobus	2.46	17.91	59.89
Oxydirus	0.82	15.35	63.27
Iotonchus	3.26	15.07	66.24
Eudorylaimus	11.74	13.76	69.17
Aphelenchus	10.66	12.64	72.04
Axonchium	0.00	11.43	74.87
Average dissimilarity = 75.57%	S3	S5	
Dorylaimellus	2.06	51.35	13.89
Eudorylaimus	11.74	43.46	24.08
Mesocriconema	36.02	13.38	32.44
Rotylenchulus	23.68	1.28	38.71
Axonchium	0.00	16.45	43.55
Mesodorylaimus	20.80	12.98	48.00
Cephalobus	17.59	6.58	52.42
Hemicriconemoides	3.15	15.30	56.55
Oxydirus	0.82	12.88	59.94
Xiphinema	2.71	12.24	63.22
Aphelenchus	10.66	0.85	66.33
Pratylenchus	8.74	7.12	69.39
Ecumenicus	0.34	9.35	72.30
Helicotylenchus	10.54	0.85	75.19
Paraphelenchus	8.90	0.00	77.88
Average dissimilarity = 74.7%	S4	S5	
Dorylaimellus	7.24	51.35	10.08
Ironus	41.89	1.70	18.27
Eudorylaimus	13.76	43.46	25.54
Cephalobus	40.39	6.58	32.16
Bursilla	29.58	2.39	38.48
Mesodorylaimus	31.59	12.98	43.84
Pratylenchus	24.48	7.12	49.03
Ecumenicus	24.85	9.35	53.34
Oxydirus	15.35	12.88	57.47
Meloidogyne	17.19	1.63	61.35
Hemicriconemoides	0.00	15.3	64.49
Axonchium	11.43	16.45	67.62
Heterocephalobus	17.91	4.08	70.33
Iotonchus	15.07	3.72	73.58
Xiphinema	5.37	12.24	76.33

ESM 2.4. Mean abundances of nematode genera, percentage composition of trophic and cp groups of nematodes of the sampling sites in Sibutad. Values after the mean represent standard deviations (mean ± stdev).

Genus	Family	CP/PP	S1 (undisturbed)	S2 (undisturbed)	S3 (disturbed)	S4 (disturbed)	S5 (disturbed)
Bacteriovores							
Acrobeloides	Cephalobidae	2	4.32 ± 7.41	2.64 ± 5.9	1.20 ± 2.50	11.3 ± 6.91	5.60 ± 8.38
Alaimus	Alaimidae	4	3.19 ± 4.99	4.26 ± 4.56	1.40 ± 3.39	3.24 ± 3.78	0
Bursilla	Mesorhabditidae	1	14.3 ± 23.7	8.44 ± 8.82	1.00 ± 6.58	29.5 ± 39.6	2.39 ± 3.73
Cephalobus	Cephalobidae	2	18.1 ± 25.3	17.0 ± 26.2	8.2 ± 9.7	40.3 ± 25.3	6.58 ± 12.0
Chronogaster	Chronogasteridae	3	1.17 ± 1.66	0	0	0	0
Heterocephalobus	Cephalobidae	2	20.1 ± 16.9	15.7 ± 16.1	1.40 ± 1.81	17.9 ± 23.5	4.08 ± 3.18
Monhystera	Monhysteridae	2	1.14 ± 2.55	0.98 ± 1.99	0.80 ± 1.99	0	1.06 ± 1.55
Panagrolaimus	Panagrolaimidae	1	0.45 ± 1.02	1.80 ± 2.48	0.40 ± 0.91	0	0
Paramphidelus	Amphidelidae	4	2.61 ± 5.84	0.98 ± 2.19	0.60 ± 2.55	1.79 ± 4.00	0
Plectus	Plectidae	2	1.16 ± 2.59	0	1.0 ± 3.62	0	0
Prismatolaimus	Prismatolaimidae	3	1.17 ± 1.66	3.27 ± 4.62	0.40 ± 1.0	6.16 ± 4.30	7.58 ± 7.19
Rhabditis	Rhabditidae	1	7.68 ± 7.34	5.97 ± 10.4	1.0 ± 2.11	0.88 ± 1.96	0
Fungivores							
Aphelenchoides	Aphelenchidae	2	0	1.96 ± 4.37	1.60 ± 2.90	4.18 ± 4.76	0
Aphelenchus	Aphelenchidae	2	23.6 ± 19.6	17.9 ± 18.7	5.80 ± 6.47	12.6 ± 14.6	0.85 ± 1.91
Filenchus	Tylenchidae	2	5.47 ± 9.89	1.32 ± 2.95	1.61 ± 2.21	0.88 ± 1.96	0
Paraphelenchus	Aphelenchidae	2	3.42 ± 7.65	1.32 ± 2.95	4.60 ± 8.81	3.28 ± 3.16	0
Tylencholaimus	Tylencholaimidae	4	10.3 ± 11.5	10.9 ± 9.12	0.20 ± 0.77	0.90 ± 2.00	5.58 ± 6.84s
Omnivores/Predators							
Aporcelaimellus	Aporcelaimidae	5	2.52 ± 2.50	6.27 ± 6.28	0.40 ± 2.63	2.63 ± 5.88	1.90 ± 3.0
Ecumenicus	Qudsianematidae	4	7.89 ± 7.66	6.20 ± 8.01	0.20 ± 0.77	24.8 ± 14.7	9.35 ± 11.5
Eudorylaimus	Dorylaimidae	4	12.7 ± 9.62	17.1 ± 14.6	4.80 ± 14.9	13.8 ± 13.7	43.5 ± 23.4
Granonchulus	Mononchidae	4	2.72 ± 3.75	0	0	0	0
Iotonchus	Iotonchidae	4	53.9 ± 43.9	9.85 ± 8.77	1.60 ± 2.37	15.1 ± 16.8	3.72 ± 5.73
Ironus	Ironidae	4	3.24 ± 3.26	0	0	41.9 ± 37.7	1.70 ± 3.81
Judonchulus	Mononchidae	4	1.16 ± 2.59	0.42 ± 0.94	0	0	0
Labronema	Qudsianematidae	4	0.45 ± 1.02	0.85 ± 1.90	0.20 ± 0.91	1.32 ± 2.94	0
Labronemella	Qudsianematidae	4	8.60 ± 7.82	10.5 ± 12.9	0.40 ± 0.89	0	0
Mesodorylaimus	Dorylaimidae	4	28.0 ± 25.3	21.6 ± 18.5	10.4 ± 15.5	31.6 ± 31.9	12.9 ± 13.5
Metaporcelaimus	Aporcelaimidae	5	15.0 ± 12.8	17.5 ± 13.3	2.80 ± 7.87	1.75 ± 3.92	7.03 ± 6.99
Mononchulus	Mononchidae	4	0	0	0.40 ±2.63	0	0
Mononchus	Mononchidae	4	4.57 ± 10.2	0	1.40 ± 2.79	0	0.69 ± 1.53
Mylonchulus	Mylonchulidae	4	5.71 ± 4.31	7.10 ± 5.77	0.59 ± 1.32	5.79 ± 4.08	2.13 ± 4.76
Opisthodorylaimus	Dorylaimidae	5	0	0.66 ± 1.47	0	0	0
Oriverutus	Dorylaimidae	4	0	0	1.0 ± 5.11	0	1.91 ± 2.65
Oxydirus	Nordiidae	5	16.9 ± 32.8	5.97 ± 10.43	0.40 ± 1.83	15.4 ± 30.8	12.9 ± 5.95
Paractinolaimus	Paractinolaimidae	5	1.17 ± 1.66	0	0.6 ± 2.56	1.95 ± 4.37	0
Parahadronchus	Mononchidae	4	0	1.17 ± 1.66	0	0	0
Prionchulus	Mononchidae	4	0.45 ± 1.02	0.98 ± 2.19	0.4 ± 1.4	0.9 ± 2.00	0
Plant-feeders							
Axonchium	Dorylaimidae	5	42.4 ± 29.4	16.7 ± 5.93	0	11.4 ± 14.4	16.4 ± 12.2
Coslenchus	Tylenchidae	2	0	0	0	4.49 ± 4.43	3.84 ± 7.20
Dorylaimellus	Dorylaimidae	5	3.61 ± 5.28	12.5 ± 18.8	2.06 ± 4.60	7.16 ± 16.0	51.3 ± 47.6
Helicotylenchus	Hoplolaimidae	3	18.3 ± 14.2	30.5 ± 18.8	5.4 ± 8.06	4.98 ± 5.61	0.85 ± 1.91
Hemicriconemoides	Criconematidae	3	10.3 ± 14.2	0	1.80 ± 2.70	0	15.3 ± 19.0
Hoplolaimus	Hoplolaimidae	3	0.65 ± 1.46	8.71 ± 12.4	0.20 ± 0.91	0	0.69 ± 1.53
Meloidogyne	Heterodoridae	3	0	0.82 ± 1.84	0.20 ± 0.89	17.2 ± 22.2	1.63 ± 2.46
Mesocriconema	Pratylenchidae	3	13.3 ± 16.9	8.72 ± 8.39	36.0 ± 23.0	2.41 ±3.83	12.5 ± 18.3
Pratylenchus	Pratylenchidae	3	0.45 ± 1.02	8.51 ± 8.60	5.0 ± 10.4	24.5 ± 30.8	7.12 ± 10.3
Rotylenchulus	Rotylenchulidae	3	2.28 ± 5.10	40.5 ± 22.1	11.0 ± 12.6	11.8 ± 17.6	1.28 ± 2.86
Rotylenchus	Hoplolaimidae	3	13.7 ± 15.9	9.4 ± 11.8	0.60 ± 2.30	5.66 ± 8.64	0
Xiphinema	Longidoridae	5	23.0 ± 26.2	4.93 ± 11.0	1.2 ± 3.01	5.37 ± 12.0	12.2 ± 15.1
Trophic groups %							
bacteriovores			19.7 ± 13.5^{a}	17.7 ± 11.9^{a}	17.4 ± 8.39^{a}	28.1 ± 11.2^{a}	11.4 ± 6.4^{a}
fungivores			11.8 ± 10.6^{ab}	8.9 ± 6.55^{ab}	13.3 ± 9.0^{a}	6.1 ± 5.33^{ab}	2.8 ± 3.62^{b}
omnivores/predators			37.7 ± 14.52^{a}	30.1 ± 9.44^{a}	25.3 ± 12.5^{a}	39.6 ± 12.6^{a}	38.5 ± 13.9^{a}
plant feeders			30.9 ± 12.8^{a}	43.1 ± 17.2^{a}	44.0 ± 17.2^{a}	26.3 ± 13.6^{a}	47.2 ± 10.4^{a}
cp groups %							
cp1			11.0 ± 12.1^{a}	7.7 ± 4.89^{a}	4.3 ± 3.87^{a}	10.8 ± 9.56^{a}	1.9 ± 3.94^{a}
cp2			27.5 ± 18.0^{a}	31.0 ± 24.7^{a}	46.3 ± 19.0^{a}	31.7 ± 11.2^{a}	14.8 ± 9.33^{a}
cp3			1.2 ± 1.72^{a}	1.4 ± 1.61^{a}	0.7 ± 1.15^{a}	1.9 ± 1.10^{a}	5.7 ± 5.36^{a}
cp4			48.5 ± 18.5^{a}	45.2 ± 10.7^{a}	41.2 ± 14.3^{a}	48.9 ± 3.93^{a}	61.6 ± 10.4^{a}
cp5			11.8 ± 9.30^{a}	14.6 ± 13.6^{a}	7.5 ± 7.48^{a}	6.8 ± 11.4^{a}	16.0 ± 6.66^{a}
cp(3-5)			61.5 ± 25.2^{a}	61.2 ± 20.2^{a}	49.4 ± 17.8^{a}	57.6 ± 12.8^{a}	83.3 ± 10.4^{a}

Mean values followed by different letters on the same row indicate significant differences according to post-hoc Tukey HSD test ($P<0.05$).

CHAPTER 3

Effects of mercury (Hg) on soil nematodes: a microcosm approach

This chapter is adapted from:

Martinez, J.G., Quiobe, S. and Moens, T. Effects of mercury (Hg) on soil nematodes: a microcosm approach. *Ecotoxicology and Environmental Safety* (submitted).

3.1. Abstract

Mercury (Hg), one of the most toxic heavy metals, is commonly used in the gold extraction process in many countries. Our previous field work on the impact of Hg on a small-scale mining area in Sibutad revealed no significant negative effects on nematode-based indices despite Hg concentrations up to 127-fold higher than the permissible level set by UNEP (2013). Using a microcosm approach, we now applied similar Hg concentrations as commonly found in these field sites (2.5, 5 and 10 ppm Hg) and determined their impact on nematode communities from a different soil with different physico-chemical soil attributes under controlled conditions. Our results showed (a) limited 'bottling' effects (incubation effects) after a 45-day incubation period: a nematode abundance decrease of up to 37%, but absence of significant differences in diversity and nematode assemblage composition; (b) Hg concentrations of 2.5 ppm significantly impacted total nematode abundance but not the other nematode assemblage descriptors, which were, however, significantly impacted from Hg levels of 5 ppm onwards. Our results demonstrate that total nematode abundance was the most sensitive descriptor to Hg pollution, whereas diversity and assemblage composition were impacted only at higher Hg concentrations. The discrepancy between our microcosm and previous field-based results are probably related to differences in physico-chemical soil attributes.

Keywords: gold mining; mercury pollution; microcosm; soil nematodes

3.2. Introduction

Terrestrial soils are potential reservoirs of a wide range of pollutants, including heavy metals. Heavy metals enter the environment naturally (e.g. volcanic activity, geological weathering, etc.) or through anthropogenic activities (e.g., smelting, mining, fossil fuel combustion) (Appleton et al., 2006; Donkor et al., 2006) and can lead to the deterioration of soil ecosystems (Nwuche and Ugoji, 2008). In the Philippines, mining (both small and large-scale mining) is an essential player of the economy, but poses ecological threats due to the lack of proper waste management and monitoring.

Small-scale gold mining activities provide socio-economic benefits among communities in the Philippines, and perhaps in many developing countries as well (Donkor et al., 2006; Hilson, 2009). Approximately 80% of the country's annual gold supply comes from small-scale mining sectors (Ban Toxics, 2010). Mercury (Hg) is commonly used during an inexpensive gold extraction process known as amalgamation, which is the addition of Hg into the crushed ore to retrieve gold; the Hg residue is often either accidentally or intentionally released into the environment (Perez et al., 2007). The annual anthropogenic release of Hg on a global scale is estimated to be 3,500 tons per year (Nriagu and Pacyna, 1988), and in the municipality of Sibutad, southern Philippines, it was estimated that a 'typical' small-scale gold processor uses ca. 1 kg of Hg per week, or an average of 52 kg per year, which can be potentially disposed of directly into the soil or bodies of water (Cortes-Maramba, 2006). It is therefore no surprise that high Hg concentrations in soils, rivers and marine systems in Sibutad have been revealed (Lacastentantos, unpublished; Martinez et al., unpublished); e.g., terrestrial soils had mean Hg concentrations up to 38.4 ppm Hg, which was ca. 127 times higher than the acceptable level of 0.3 ppm Hg set by UNEP (2013).

Mercury is one of the most toxic heavy metals, even at very low concentrations (Göthberg and Greger, 2006), and is highly persistent; it can readily enter the food web and bioaccumulate (Burton et al., 2006; Kidd et al., 2012). Being at the top of the food chain, humans are exposed to risks leading to defects in reproduction, immune response, nervous system and vital organs (Akagi et al., 2000; Maramba et al., 2006). In terrestrial ecosystems, Hg has been reported to impact soil microflora and ecosystem functioning such as soil respiration (Müller, 2002; Harris-Hellal et al., 2009).

Nematodes, the most abundant and one of the most species-rich metazoan phyla in soil ecosystems, play critical roles in soil processes such as decomposition (Yeates and Coleman, 1982; Freckman 1988) and nutrient cycling (Coleman et al., 1984). Nematodes possess features

which make them ideal candidates as bioindicators either in laboratory or field-based conditions (Bongers and Ferris, 1999; Ferris et al., 2001) and using single-species (Peredney and Williams, 2000; Monteiro et al., 2014) or community analyses (Fiscus and Neher, 2003; Pen-Mouratov et al., 2011). They can easily be sampled and cultured, and their limited mobility prohibits their escape from impacted soils; their permeable cuticles permit the assimilation of xenobiotics from the soil even under food-deprived conditions, thereby providing useful information about soil health. Nematodes respond to different environmental conditions, e.g. pollution, and such changes can be measured through various ecological indices, both structural (e.g. different diversity indices) and 'functional' (e.g. based on feeding guilds, life-history groups, etc.) (Bongers and Ferris, 1999; Ferris et al., 2001).

Although field-based studies have been proven useful in assessing the impacts of different kinds of pollutants on soil biota (Sánchez-Moreno and Navas, 2007; Pen-Mouratov et al., 2008; Park et al., 2011), their interpretation may be hampered due to the complex interplay of various abiotic and biotic factors, including intricate interactions among organisms. For instance, stimulation of 'sensitive' nematodes has been observed on few occasions at low to moderate pollution levels (Heininger et al., 2007; Salamun et al., 2015), contrary to the general expectation that these sensitive species should be among the first to suffer the effects of pollutants (Bongers, 1990; Yeates et al., 1995; Sánchez-Moreno and Navas, 2007). In particular, high mean Hg concentrations combined with slightly elevated concentrations of other heavy metals, showed a positive association with sensitive nematode taxa in a small-scale mining area in Sibutad where Hg spills were prominent (Martinez et al., unpublished); such a result was incongruent with the finding that even much lower Hg concentrations induce negative effects on nematodes (Hermi et al., 2009). Although the apparent stimulation of sensitive soil nematodes by pollutants may have resulted from a combination of factors which can all affect Hg bioavailability, such as organic matter content, more neutral soil pH and presence of vegetation, (Martinez et al., unpublished), supplementing field-based results with microcosm experiments where other variables can be controlled, may provide a better understanding of the impact of Hg on soil biota (Ababio and Baird, 2005).

In the present study, we exposed a natural assemblage of soil nematodes to various Hg concentrations in the range of those obtained from a small-scale mining area in Sibutad, southern Philippines. Since such concentrations are much higher than the permissible range set by UNEP (2013), we would like to assess their impact on soil biota (i.e., nematodes) in a close-to-natural condition using microcosms and determine if differences in soil parameters (e.g., OM, clay content, pH, etc.) can influence Hg effects on nematode communities.

3.3. Materials and Methods

3.3.1. Soil collection

Soil samples were collected from an 'undisturbed' soil in Iligan, Philippines. 'Undisturbed' means no (recent) history of organic and heavy metal pollution. 10 kg of soil were collected from the upper soil layer to a depth of 10 cm, where nematodes are normally found in highest abundances. Upon return to the laboratory, soil samples were immediately homogenized through gentle hand stirring, while large debris fragments were manually removed. Soil pH was determined potentiometrically in a 1:2.5 vol:vol soil:water suspension (ISRIC, 1995). Total N was determined by the Kjeldahl method (Kjeldahl, 1883) and total organic carbon was measured by the Walkey-Black method (Walkey and Black, 1934). The heavy metals Cd, Cu and Pb were measured by Atomic Absorption Spectrometry while Hg was measured by Cold Vapor Atomic Absorption Spectrometry (CVAAS) at FAST (First Analytical Services and Technical Cooperative) Laboratory.

3.3.2. Experimental design

A total of 16 glass microcosms (200 cm x 135 cm x 150 cm), composed of one control and three treatments with four replicates each, were assembled. Each mesocosm was filled with 500 g of homogenized soils. The soils were then spiked with 100-ml aliquots of three Hg concentrations to obtain the final concentrations of 2.5, 5 and 10 ppm Hg in the soil. Hg stock solutions were prepared from $HgCl_2$ (Sigma-Aldrich, ≥ 99.9% purity). The soils were again thoroughly homogenized. The Hg concentrations used were based on the concentrations obtained from the small-scale mining area in Sibutad, southern Philippines, which ranged between 0.49 and 38.4 ppm (Martinez et al., unpublished). The glass mesocosms were placed in a garden under a tree canopy to avoid direct exposure to the sun, thus preventing drying out of soils, and covered with a mesh net (mesh size is approx.1 mm) to prevent entrance of foreign materials except rainwater. The mesocosm set-ups were regularly checked to ascertain that the soils did not dry out, and if necessary, filtered tap water was sprinkled to each of the mesocosms. The experiment was terminated after 45 days.

3.3.3. Nematode collection and processing

To determine the nematode assemblage of the sampling area, nematodes were collected from 100 g samples of homogenized soil immediately after field sampling, which was designated as T0. In the experiment, nematodes exposed to different Hg concentrations (0, 2.5, 5 and 10 ppm)

were collected after 45 days of incubation. All set-ups at T0 and T45 were performed in 4 replicates ((T0 = 4 replicates; T45 = 16 (4 treatments x 4 replicates)). Nematode extraction was performed using the modified tray method (Whitehead and Hemming, 1956) which only collects living and active nematodes for 3 days. Live nematodes were counted under a stereomicroscope (Olympus BX-41). 100 nematodes were randomly hand-picked with a copper needle and mounted in permanent glycerin slides for identification to genus level under a binocular Olympus CX22 microscope (400-1000x magnification). Nematodes were identified based on the identification keys of Bongers (1990) and Andrassy (2008). They were assigned 'colonizer-persister' scores (Bongers, 1990, 1995) and categorized into trophic groups: bacterial-feeders, fungal-feeders, omnivores, predators and plant parasites (Pen-Mouratov et al., 2008).

3.3.4. Nematode assemblage analysis

The following nematode descriptors were determined to describe nematode assemblage structure in response to experimental incubation and Hg contamination: the absolute abundances per 100 g of soil; genus richness, which is the actual number of genera; Pielou's evenness; Shannon-Wiener index (*H'*), which integrates aspects of richness as well as evenness. Although other related indices such MI, MI_{2-5} and SI (Structure index), which can be calculated through the NINJA online programme (Sieriebriennikov et al., 2014), may also be informative, determination of such indices is only relevant when a sufficiently high number of individuals can be assigned cp-scores (Shao et al., 2008); in this study, this was not consistently possible due to low nematode abundances (< 50 ind.) at the two highest Hg concentrations (i.e., 5 and 10 ppm Hg). Univariate analyses were performed on nematode assemblage descriptors using ANOVA (Statistica software package version 7.0.) to a) assess the experimental incubation effect, by comparing the controls at T0 vs T45 (0 ppm), and b) compare the effects of different Hg treatments (0, 2.5, 5, and 10 ppm Hg) at T45. Data were first checked for normality with a Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test; these assumptions were met even without transformation of data. Pairwise comparisons between treatments were performed using Tukey's HSD test if a significant ANOVA result was detected.

Non-metric multi-dimensional scaling (nMDS) was used to visualize trends in nematode assemblage composition between T0 and T45 for the unpolluted control, and between the different Hg treatments at T45. The significance of such trends was subsequently tested using multivariate Permutational Analysis of Variance (PERMANOVA; Anderson, 2004) within PRIMER+. 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 treatment effects or could be the result of heterogeneous variances. Prior to the multivariate analysis, nematode abundances were fourth-root-transformed to downsize the effect of dominant genera. When significant differences were detected, pairwise comparison tests within PERMANOVA$^+$ were conducted to pinpoint differences between treatments. Similarity percentages (SIMPER) analyses using the fourth-root-transformed nematode abundance data were used to identify the genera which contributed to the dissimilarity or similarity between T0 and T45 (0 ppm), and between the different Hg treatments (0, 2.5, 5, and 10 ppm Hg) at T45. We listed all genera up to a cumulative contribution of more than 50% to the average dissimilarity between (or similarity within) treatments (Hermi et al., 2009).

3.4. Results

Table 3.1. Physico-chemical parameters of the garden soil used in our experiment. Data are means followed by the standard deviation (mean ± stdev) of three replicates. % OM is after removal of detritus fragments.

Physico-chemical variables	Values
OM (%)	2.65 ±1.06
N (%)	0.17 ± 0.2
pH	7.77 ± 0.15
Cd (ppm)	< 0.06*
Cu (ppm)	21.8 ± 0.81
Pb (ppm)	9.89 ± 0.39
Hg (ppm)	0.22 ± 0.07
median grain size (µm)	10.7 ± 1.23
clay (%)	10.6 ± 0.45

*concentration below the detection limit

Physico-chemical properties of the soil

The soil used in this experiment had a clay content of 10.6% and a median grain size of 10.7 µm. OM and N were 2.65% and 0.17%, respectively (Table 3.1). Soil pH was neutral at 7.77. The heavy metals Cd, Cu and Pb in soil had much lower concentrations than their 'allowable limits' (Teh et al., 2016). The mean mercury (Hg) level was 0.22 ppm, which is also within the recommended range of 0.07 to 0.3 ppm in soil (UNEP, 2013).

3.4.2. 'Bottling' effects on nematode assemblages

The genera found at the start of the experiment (T0) but not at T45 (0 ppm) included *Aphelenchoides*, *Filenchus*, *Aporcelaimellus*, *Mesodorylaimus*, *Nygolaimus*, *Oxydirus*, *Prionchulus* and *Xiphinema*. By contrast, several genera such as *Acrobeles*, *Labronemella* and *Ironus* were only found at T45 (0 ppm). Nematode density (ind./100 g soil) significantly decreased from T0 to T45 ($P <$

0.05); after 45 days in the unpolluted control, 63% of the initial density at T0 was recovered. Despite this decrease in abundance, the number of genera and Shannon-Wiener diversity did not differ significantly between T0 and T45, while evenness was marginally but significantly higher at T45 than at T0 (Table 3.2). Although there was no overlap between replicates of T0 and T45 in an nMDS based on fourth root-transformed nematode genus composition data (Fig. 3.1), nematode genus composition did not differ significantly between T0 and T45 (df = 1; pseudo-F = 2.55*; P* = 0.116, with a non-significant PERMDISP (*P = 0.126*)). The average dissimilarity between T0 and T45 (0 ppm) was nevertheless 42.3% (Table 3.3). The plant-feeding *Coslenchus*, *Hoplolaimus*, *Xiphinema* and *Rotylenchulus* and the free-living *Oxydirus*, *Mesodorylaimus* and *Filenchus* all contributed importantly to this dissimilarity, each of these genera being more abundant at T0. By contrast, the plant feeding *Tylenchorhynchus* and *Helicotylenchus*, and the free-living *Ironus*, *Heterocephalobus* and *Acrobeloides* were more abundant at T45 (0 ppm).

Table 3.2. Comparison of nematode indices between T0 and T45 of the control treatment (0 ppm). Data are means followed by the standard deviation (mean ± stdev) of three replicates.

	T0	T45
ind./100 g soil	296.3 ± 3.51	187.7 ± 10.3**
number of genera	20.3 ± 1.15	18.6 ± 2.30
Pielou's evenness	0.78 ± 0.02	0.88 ± 0.02**
Shannon-Wiener index	2.35 ± 0.11	2.55 ± 0.08

Asterisk (*) indicates *P-value < 0.05*; (**) indicates *P-value < 0.01*

Table 3.3. Species responsible for differences between groups, T0 (initial nematode community) and T45 (0 ppm) based on similarity percentages (SIMPER) analysis of fourth-root-transformed abundances.

Genera	T45 (0 ppm)	Cum. cont. (%)
Average dissimilarity between T0 and T45	42.3%	
Ironus	(+)	5.6
Heterocephalobus	(+)	11.1
Coslenchus	(-)	15.7
Tylenchorhynchus	(+)	20.2
Hoplolaimus	(-)	24.4
Oxydirus	(x)	28.7
Xiphinema	(x)	32.6
Acrobeloides	(+)	36.3
Rotylenchulus	(-)	39.8
Mesodorylaimus	(x)	43.2
Helicotylenchus	(+)	46.7
Filenchus	(x)	50.1

Note: Species contributing up to a cumulative 50% (Cum. %) of average dissimilarity between treatments are ranked in order of importance of their contribution to such dissimilarity. Note: (+) more abundant after 45 days than at T0; (-) less abundant after 45 days than at T0; (x) present at T0 but not at T45.

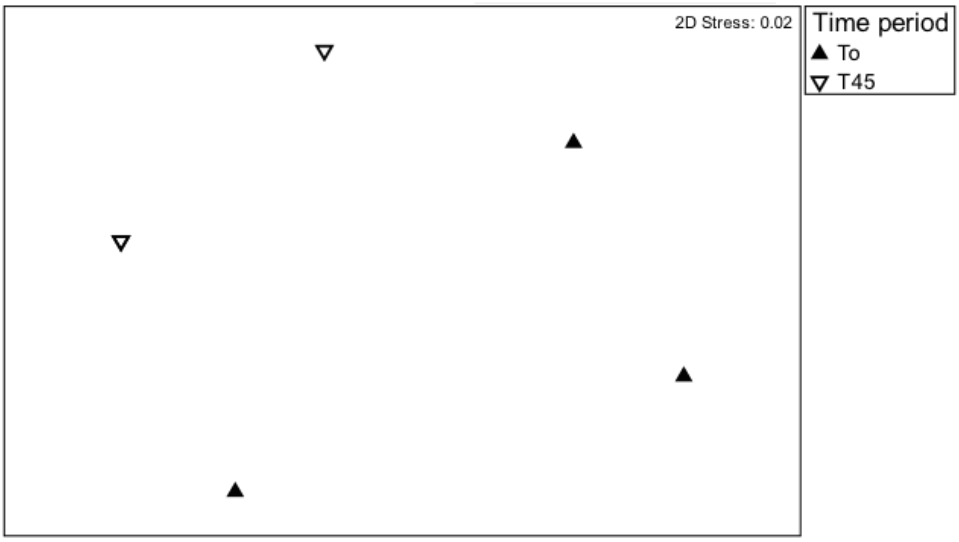

Fig. 3.1. Non-metric multi-dimensional scaling ordination (nMDS) of the nematode genus composition of unpolluted controls at T0 and T45.

3.4.3. Hg effects on nematode assemblages

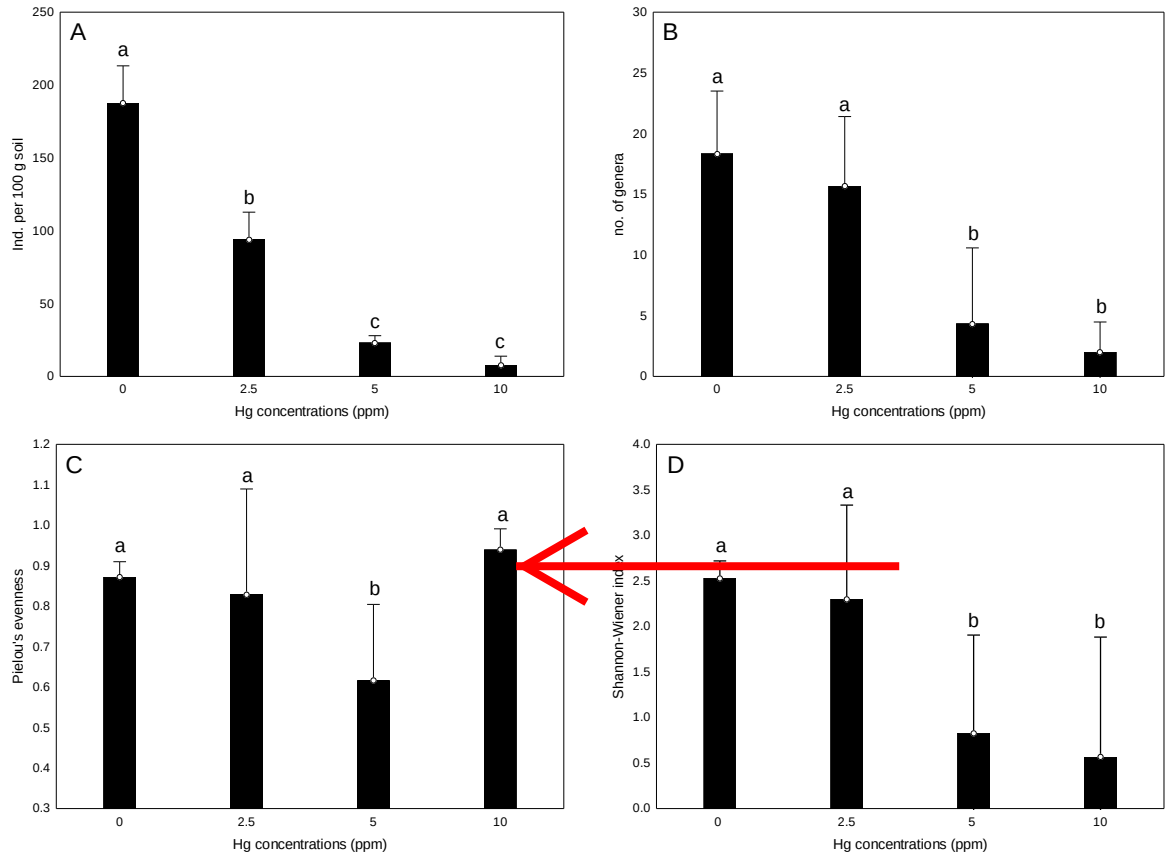

Fig. 3.2 (A-D). Mean nematode abundance per 100 g soil (A), richness expressed as number of genera (B), Pielou's evenness (C) and Shannon-Wiener index (D). Different letters indicate significant pairwise differences between treatments according to a post-hoc Tukey HSD test ($P < 0.05$). Data are means ± 1 SE of three replicates.

Nematode density at 2.5 ppm Hg was only half that in the control ($P < 0.05$), but was in turn much higher than at 5 and 10 ppm Hg (Fig. 3.2A). By contrast, none of the nematode diversity indices differed significantly between the control and 2.5 ppm Hg, hence Hg effects on diversity only became manifest at the two higher Hg concentrations (Fig. 3.2B, C and D). One exception was evenness, which was significantly lower at 5 but not at 10 ppm Hg.

PERMANOVA revealed highly significant differences in nematode genus composition between treatments (df = 3; pseudo-F = 7.34; $P = 0.001$), with a non-significant PERMDISP ($P = 0.301$) (Fig. 3.3). As for most diversity indices, pairwise tests showed that nematode assemblages were not significantly different between 0 and 2.5 ppm ($P > 0.05$), while assemblages at 5 and 10 ppm were significantly different from that of the control (both $P < 0.01$) (ESM 3.1). SIMPER analysis further showed that dissimilarity in nematode assemblages increased with increasing Hg concentration: 37.2% between 0 and 2.5 ppm Hg, 73.0% between 0 and 5 ppm Hg and 85.3% between 0 and 10 ppm Hg (ESM 3.2). The genera *Rotylenchus*, *Acrobeloides*, *Helicotylenchus*, *Heterocephalobus*, *Panagrolaimus*, *Tylenchorhynchus*, *Plectus* and *Eudorylaimus* contributed most to the dissimilarity between the unpolluted control and 2.5 ppm Hg, being less abundant or eliminated (i.e., *Acrobeloides* and *Plectus*) at 2.5 ppm Hg (Table 3.6). Between the control and 5 ppm Hg, *Monhystera* were least abundant, whereas *Rotylenchulus*, *Rotylenchus*, *Helicotylenchus*, *Tylenchorhynchus*, *Heterocephalobus*, *Ironus* and *Prismatolaimus* were eliminated at 5 ppm. Between the control and 10 ppm Hg, a majority of the nematode genera were completely eliminated except for *Acrobeloides*, *Cephalobus*, *Aphelenchus* and *Eudorylaimus*.

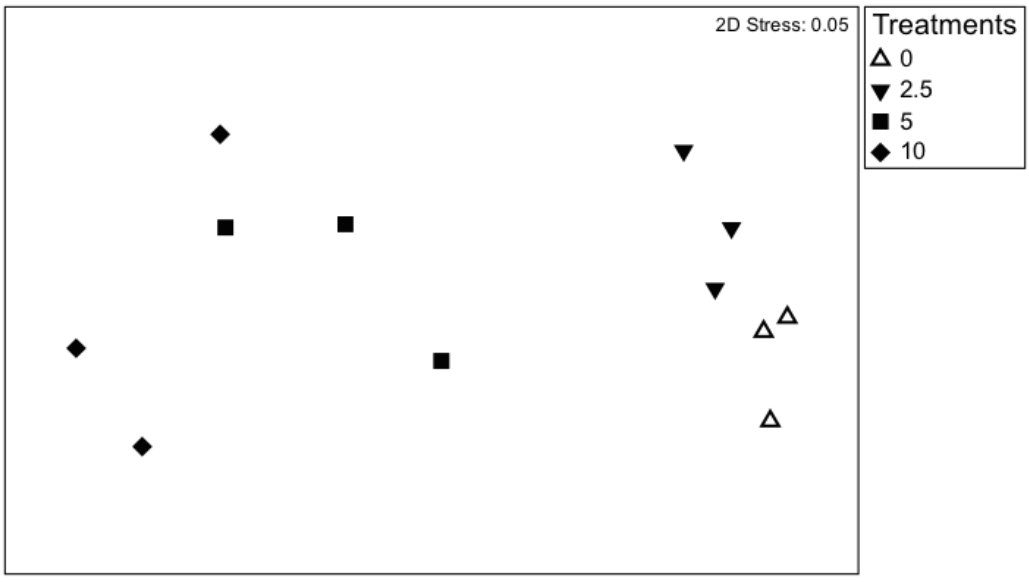

Fig. 3.3. Non-metric multi-dimensional scaling ordination (nMDS) of the nematode genus composition between different Hg concentrations (0, 2.5, 5 and 10 ppm).

Table 3.4. Species responsible for differences between control and Hg-treated mesocosms based on similarity percentages (SIMPER) analysis of fourth-root transformed nematode abundances.

2.5 ppm	Cum. (%)	5 ppm	Cum. (%)	10 ppm	Cum. (%)
Rotylenchus (-)	7.4	*Rotylenchulus* (x)	8.0	*Rotylenchulus* (x)	7.5
Acrobeloides (x)	14.7	*Rotylenchus* (x)	15.5	*Rotylenchus* (x)	14.4
Helicotylenchus (-)	19.4	*Helicotylenchus* (x)	22.8	*Helicotylenchus* (x)	21.2
Coslenchus (+)	23.9	*Tylenchorynchus* (x)	29.0	*Tylenchorhynchus* (x)	27.0
Heterocephalobus (-)	28.2	*Heterocephalobus* (x)	35.2	*Achromadora* (x)	32.8
Panagrolaimus (-)	32.5	*Ironus* (x)	41.3	*Heterocephalobus* (x)	38.5
Tylenchorynchus (-)	36.8	*Prismatolaimus* (x)	47.3	*Pratylenchus* (x)	44.3
Tylenchus (+)	40.8	*Monhystera* (-)	52.5	*Ironus* (x)	50.0
Plectus (x)	44.8			*Prismatolaimus* (x)	55.6
Aphelenchus (+)	48.7				
Eudorylaimus (-)	52.5				

Species contributing to a cumulative 50% (Cum. %) of average dissimilarity between treatments.
Note: (+) more abundant than in the unpolluted control; (-) less abundant compared to the control; (x) present in control but absent from Hg treatment.

3.5. Discussion

3.5.1. '*Bottling' effects on nematode assemblages*

Micro/mesocosm studies have been useful in increasing our understanding of the impact of various kinds of pollutants on nematode communities (Šalamún et al., 2015). They are more ecologically relevant than single-species tests (Cairns and Pratt et al., 1993) because they tend to maintain both biotic and abiotic parameters, while partly reducing the complexity of the ecosystem (Rohr et al., 2016). Nevertheless, the 'artificial' nature of micro/mesocosms poses limitations with their tendency to alter the natural assemblages of nematodes after a period of time. For instance, a substantial decrease in nematode density by up to 70% has been recorded in aquatic micro- and mesocosm experiments (Gwyther et al., 2009; Gingold et al., 2013;), while a decrease of up to 11% has been observed in a terrestrial microcosm experiment (Martikainen et al., 1998). Although the present work showed a higher decline in the density of terrestrial nematodes by 37%, it retained almost 90% of species richness and did not alter the Shannon-Wiener diversity value. Nematode community composition between the two time periods, T0 vs T45, also did not change significantly. The decline in total abundance, due to the reduction of plant-feeders (e.g., *Coslenchus*, *Hoplolaimus* and *Rotylenchulus*), and the elimination of the plant-feeder *Xiphinema* and of some free-living nematodes, i.e., *Oxydirus*, *Mesodorylaimus* and *Filenchus* (Tables 3.3 and 3.7), was probably caused by the exclusion of plants in the experimental set-up. Plants can influence the soil biota such as nematodes through the structure they provide as well as by their root exudates and detrital matter, which can increase bacterial abundances, which in

turn serve as food to bacterivorous nematodes (Bongers and Ferris, 1999; Bais et al., 2006). Manual mixing of soils at the start of the experiment may also lead to mechanical stress, as some genera can be sensitive to tillage, e.g., *Aphelenchoides* and *Aporcelaimellus* (Fiscus and Neher, 2002); both genera were no longer found in any of the treatments at T45. Despite the experimental 'incubation' effects, the relatively high recovery in terms of number of genera, Shannon-Wiener index and nematode community composition after a 45-day incubation period suggest the suitability of soil microcosms for community level bioassays for pollution-impact studies (Martikainen et al., 1998; Šalamún et al., 2015).

3.5.2. Hg effects on nematode assemblages

Despite Hg concentrations several times higher than the acceptable limit by UNEP (2013), our previous work on a small-scale mining in Sibutad revealed no significant impacts on total nematode abundance, Shannon-Wiener diversity index, genus richness and evenness of the most Hg polluted soils (Martinez et al. unpubl.), nor was any of these variables correlated with Hg concentration; moreover, presumably sensitive nematode taxa (mostly belonging to cp-groups 4 and 5 (Bongers 1990)) thrived in Hg-polluted soils. The results of the present study, on the other hand, show a very different pattern: nematodes were clearly affected by Hg in a concentration-dependent manner; a drastic decrease in total nematode abundance occurred at 2.5 ppm Hg and became even more pronounced at higher concentrations. Negative effects on nematode diversity, both in terms of genus richness, evenness and Shannon-Wiener diversity, became significant from a concentration of 5 ppm onwards, and this was also the lowest Hg concentration which yielded significant impacts on nematode genus composition, albeit that the average dissimilarity between soil with 2.5 pm Hg and unpolluted control soil was already 37.2%. Hence, among the various descriptors of the nematode assemblages used here, total nematode abundance appeared to be the most sensitive one. This is only partly congruent with the results of Hermi et al. (2009) who found significant declines in abundance but also in species richness of nematodes in a Tunisian lagoon at increasing Hg concentrations (0.084, 0.167 and 0.334 ppm), while evenness increased in polluted microcosms as a result of decreasing abundances of the most dominant species. As a consequence of the opposing trends in richness and evenness, Shannon-Wiener diversity remained unaffected (Hermi et al., 2009). Our results, by contrast, suggest a broad multispecies impact of Hg, negatively impacting abundances of many species to a similar degree (hence the absence of a decline in evenness), but eliminating only few (hence insignificant decreases in richness and Shannon-Wiener diversity) at a concentration of 2.5 ppm. Furthermore, it is striking that Hermi et al. (2009) found significant effects on the marine

nematodes in their study at Hg concentrations more than 20-fold lower than in the present study (0.084 vs 2.5 ppm). Obviously, many factors differed among our terrestrial soil and their marine lagoonal sediment. Increasing salinity typically reduces heavy-metal (including Hg) toxicity (Hall and Anderson, 1995; Verslycke et al., 2003). Furthermore, the organic matter concentration in our soil was twice as high as in the lagoon sediments studied by Hermi et al. (2009). Such differences may explain part of the discrepancy in observed lowest effect concentrations (LOEC). In addition, our data do not allow to assess a real LOEC for those descriptors that were already affected at 2.5 ppm, as no Hg concentrations without a significant effect were included. Nevertheless, we may also expect pronounced differences in sensitivity between species. As an example, in agar-based experiments, reproductive capacity of *Caenorhabditis elegans* was only impaired at 15 ppm Hg (Wu et al., 2011) and mortality of juvenile *Diplolaimella* sp. occurred only at 10 ppm Hg (Vranken and Heip, 1986).

Toxic effects on nematodes are caused by the uptake of heavy metals by feeding, as contaminants (e.g., Cd) can bind readily with, or be taken up by, bacteria (Höss et al., 2011), which can lead to the accumulation of metals in the animal gut (Samoiloff, 1973; Howell, 1983), or through adsorption by their metabolically active cuticle (Bird, 1980). Similar uptake mechanisms may also occur with the heavy metal Hg, a powerful neurotoxin, resulting in the disruption of neuronal functions (Dufault et al., 2009) and oxidative stress, which can lead to membrane peroxidation and formation of reactive oxygen species (ROS) (Shanker et al., 2005; Pinheiro et al., 2008). Apart from the direct effects of Hg on nematodes, indirect effects through modifications of species interactions (e.g., facilitation, competition, etc.) can lead to shifts in species composition. For instance, our previous work showed mutual facilitation between two nematodes species (*Acrobeloides nanus* and *Plectus parvus*) under unpolluted conditions, while cadmium pollution not only led to a decrease of the less tolerant *P. parvus*, but also to an increase in *A. nanus* because of a reduced competition with *P. parvus* (Martinez et al., 2012). Both direct and indirect effects can result in the stimulation, reduction or elimination of several nematode genera.

The strong discrepancy between the microcosm experiment and field data from the small-scale mining area in Sibutad probably relates to pronounced differences in the physico-chemical properties of the soils and the presence/absence of vegetation, factors which can all substantially affect Hg availability (Rieuwerts et al., 1998). Pronounced differences in organic matter (2.7% in the microcosm vs a range of 4.5% -7.4% in the field), clay content (10.6% in the microcosm vs 15.3% - 24.4% in the field), soil pH (7.7 in the microcosm vs 4.6 - 5.4 in the field) and vegetation (absent in the microcosm vs present in the field) probably explain the lack of negative impacts in

the field. Higher % OM and % clay in the field may have reduced metal bioavailability as both OM and clay can bind with heavy metals through their negatively charged surfaces (Stevenson 1976; Sandrin and Maier, 2003; Antoniadis et al., 2008), making them less available to organisms other than those which (deposit-)feed on dead OM in the soil. By contrast, acidic soils can enhance the bioavailability of metals, thus increasing their toxicity effects (Kim et al., 2009). Finally, the presence of vegetation in the field may have also reduced heavy metal effects on nematode assemblages (Korthals et al., 1998; Šalamún et al., 2017), as the plant roots sequester heavy metals from soil.

Apart from the traditional community descriptors (e.g. abundance, richness, evenness, Shannon-Wiener index), nematode genus composition can also be helpful in the assessment of the impact of soil pollution (Fiscus and Neher, 2002; Salamún et al., 2011). As shown in the present study, nematode community composition was impacted by increasing Hg concentration; it differed significantly at 5 ppm Hg compared to the control with the elimination of several plant-feeding nematodes (e.g., *Rotylenchulus*, *Rotylenchus*, *Helicotylenchus* and *Tylenchorynchus*) as well as the free-living genera, e.g., *Ironus* and *Prismatolaimus*, and with the elimination of all genera except *Cephalobus*, *Acrobeloides*, *Aphelenchus* and *Eudorylaimus* at 10 ppm Hg (Table 3.4 and ESM 3.3). The tolerance of the cp-2 nematodes *Cephalobus*, *Acrobeloides* and *Aphelenchus* to heavy metals in soil is consistent with the 'colonizer-persister' concept of Bongers (1990), whereas the presence of *Eudorylaimus* (cp-4) at the highest Hg concentration is counter to the idea that dorylaimid nematodes (with cp-scores of 4 and 5) tend to be sensitive to chemical pollution (Bongers, 1999; Fiscus and Neher, 2002; Höss, et al., 2011), but in line with our observation of high abundances of this genus in contaminated sites at the Sibutad small-scale mining area (Martinez et al., unpublished). These results call for caution when interpreting the presence of persister nematodes as a sign of limited pollution impacts.

3.6 Conclusions

Mercury concentrations obtained from small-scale mining areas in Sibutad, previously shown to have no pronounced effects on nematode assemblages in the field, were found to be detrimental to nematode communities in experimental microcosms. Such discrepancy can be related to differences in physico-chemical characteristics of soil such as OM, pH, % clay and the presence/absence of vegetation, which suggests that nematode-based environmental assessment should be interpreted in a context-dependent manner. The present study also demonstrates the importance of supplementing field-based data with microcosm experiments to increase

understanding on the impact of heavy metals (i.e., Hg) on soil biota, and confirms that differences in soil properties can influence Hg toxicity (or Hg bioavailability).

Electronic Supplemental Materials (ESM)

ESM 3.1. Pairwise comparisons of nematode assemblage composition (PERMANOVA) between different sites.

Treatments	0 ppm Hg	2.5 ppm Hg	5 ppm Hg	10 ppm Hg
0 ppm	-	0.12	0.008**	0.005**
2.5 ppm Hg	0.12	-	0.02*	0.07**
5 ppm Hg	0.008**	0.02*	-	0.118
10 ppm Hg	0.004**	0.01*	0.118	-

Asterisks (*) and (**) indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

ESM 3.2. Average dissimilarity (%) of nematode assemblages between Hg treatments.

Average Dissimilarity (%)	0 ppm Hg	2.5 ppm Hg	5 ppm Hg	10 ppm Hg
0 ppm	-			
2.5 ppm Hg	37.2	-		
5 ppm Hg	73.0	67.8	-	
10 ppm Hg	85.3	83.6	51.85	-

ESM 3.3. Absolute abundances of nematode genera at T0 (initial nematode assemblage) and the different Hg concentrations (0, 2.5, 5 and 10 ppm) at T45. Values after the mean represent standard deviations of three replicates.

Genus	CP	T0	T45			
			0 ppm	2.5 ppm	5 ppm	10 ppm
Bacterivores						
Acrobeles	2	0	1.54 ± 2.67	0.45 ± 0.78	0	0
Acrobeloides	2	1.02 ± 1.76	4.68 ± 2.42	0	0.40 ± 0.70	1.11 ± 1.92
Alaimus	4	0	0	0.45 ±0.79	0.40 ± 0.70	0
Bursilla	1	1.02 ± 1.72	2.84 ± 4.92	0	0	0
Cephalobus	2	16.2 ± 9.33	16.1 ± 14.6	4.89 ± 6.04	2.01 ± 1.29	1.07 ± 1.84
Heterocephalobus	2	18.4 ± 31.8	8.59 ± 7.10	1.14 ± 1.02	0	0
Monhystera	2	14.2 ± 11.4	3.85 ± 1.38	5.16 ± 4.55	0	0
Panagrolaimus	1	3.00 ± 3.06	3.14 ± 3.51	0.45 ± 0.78	0	0
Plectus	2	2.02 ± 1.76	1.54 ± 1.34	0	0	0
Prismatolaimus	3	16.2 ± 10.7	7.05 ± 2.84	9.05 ± 5.98	0	0
Rhabditis	1	0	0	0.97 ± 1.68	0	0
Fungivores						
Aphelenchoides	2	2.02 ± 1.75	0	0	0	0
Aphelenchus	2	5.09 ± 4.67	1.66 ± 2.87	1.56 ± 1.40	0	0.53 ± 0.92
Filenchus	2	3.05 ± 3.05	0	0	0	0
Omnivores						
Achromadora	3	4.06 ± 1.78	7.76 ± 1.92	24.2 ± 18.9	0.84 ± 0.73	0
Aporcelaimellus	5	2..01± 3.48	0	0	0	0
Eudorylaimus	4	24.0 ± 13.3	36.4 ± 7.57	9.03 ± 5.31	17.2 ± 2.61	4.96 ± 1.73
Labronemella	4	0	0.83 ± 1.43	0	0	0
Mesodorylaimus		3.04 ± 3.05	0	0	0	0
Predators						
Clavicaudoides	4	1.02 ± 1.77	0.83 ± 1.44	1.32 ± 2.28	0	0
Discolaimus	4	3.06 ± 5.30	0.83 ± 1.44	0	0	0
Iotonchus	5	3.04 ± 3.05	2.84 ± 4.92	0	0	0
Ironus	4	0	7.58 ± 3.01	8.25 ± 0.44	0	0
Miconchus	4	1.02 ± 1.76	0.71 ± 1.23	0	0	0
Mylonchulus	4	2.03 ± 3.52	0.71 ± 1.23	0	0	0
Nygolaimus	5	1.02 ± 1.77	0	0.66 ± 1.14	0	0
Oxydirus	5	10.2 ± 15.0	0	0.45 ± 0.78	0	0
Prionchulus		1.00 ± 1.74	0	0	0	0
Plant-feeders						
Coslenchus	2	9.14 ± 10.2	0.71 ± 1.23	3.98 ± 3.95	0.87 ± 1.51	0
Helicotylenchus	3	3.03 ± 3.02	16.4 ± 11.9	3.66 ± 4.09	0	0
Hoplolaimus	3	26.4 ± 9.92	3.97 ± 3.58	2.04 ± 0.62	0.81 ± 1.40	0
Longidorus	5	2.02 ± 1.75	1.45 ± 2.52	0	0	0
Pratylenchus	3	12.1 ± 15.9	8.17 ± 5.84	2.70 ± 1.25	0.40 ± 0.70	0
Rotylenchulus	3	94.3 ± 20.8	21.8 ± 10.3	7.54 ± 6.59	0	0
Rotylenchus	3	5.06 ± 4.62	16.1 ± 4.16	2.63 ± 4.57	0	0
Tylenchorhynchus	3	1.02 ± 1.77	9.60 ± 7.64	1.63 ± 1.49	0	0
Tylenchus	2	0	0	1.77 ± 2.01	0	0
Xiphinema	5	5.09 ± 4.66	0	0	0	0

CHAPTER 4

Nematode community structure of rehabilitated surface mining sites in Sibutad, southern Philippines

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4.1. Abstract

In several developing countries with abandoned and active mining sites, rehabilitation is a crucial post-mining activity to assist in the recovery of damaged ecosystems. In the Philippines, rehabilitation is often carried out through addition of organic amendments (e.g. agricultural wastes) and development of aboveground vegetation. Rehabilitation success is often judged based on the survival rate of plants or their ability to grow. However, this may not be sufficient enough since many of the tree species used in rehabilitated areas, e.g. *Acacia* sp., can tolerate harsh soil conditions. In the present study, we used soil organisms, i.e., nematodes, to asses soil recovery in 'rehabilitated' areas by comparing nematode assemblage descriptors (e.g., abundance and diversity indices) and genus composition between sites: Site A (reference site) and Sites B, C and D which were rehabilitated in 2004, 2003 and 1999, respectively, and between the two time periods (2012 vs. 2014). Nematodes were collected in 2012 and 2014 and identified to the genus level using traditional approach (morphology-based identification). Soil variables such as N, P, OM, pH, particle size and heavy metals (e.g., As, Ba, Cd, Co, Cu, Pb, Sr and Zn) were quantified. Results show that there was an overall low abundance and diversity in all of the sampling sites in Sibutad, probably due to naturally low pH in the area (highest mean pH was 4.2). Despite 'successful' forestation, nematode abundance and diversity remained very low in the rehabilitated areas, which was most probably related to high Pb level and lack of OM in soils. Hints of partial soil recovery were, however, manifested by the occurrence of 'sensitive' nematodes in rehabilitated areas and increase in nematode descriptors, such as abundance and genus richness, after two additional years of rehabilitation.

Keywords: soil recovery, organic amendments, mining, nematodes, *Acacia auriculiformis*

4.2. Introduction

The rapid growth of the human population has led to an increasing demand worldwide for raw materials, both metallic and non-metallic. Despite their economic potential and their contribution to meeting the increased material demands, mining activities remain among the most destructive anthropogenic practices impacting humans and the environment (Kitula, 2006; Zhang et al., 2012). In a mineral-rich country like the Philippines, with an estimated $840 billion worth of untapped mineral resources (Mines and Geosciences Bureau of the Department of Environment and Natural Resources, 2012), mining activities have been prominent in many parts of the country: 41 are presently active large-scale minings covering a total land area of 0.8 million hectares as of 2016 (Mines and Geosciences Bureau, 2017), while another 32 have been abandoned since the 1950's until the 1990's, posing risks to local communities. An exact figure of small-scale mining operations remains unaccounted for, but the number of workers in such small-scale operations has been estimated at ca. 200,000 (Bugnosen, 2001).

Mining activities can cause several ecological disturbances (e.g., physical disruption, alteration of soil properties, proliferation of heavy metals and loss of biodiversity), and in abandoned and untreated mining areas, such disturbances can persist for several years (Fernández-Caliani, 2009). Since 1996, the Philippine government has been imposing that mining companies should include rehabilitation plans as part of post-mining activities to minimize ecological degradation. Soil rehabilitation includes the restoration of damaged ecosystems to improve ecosystem productivity and services (Mansourian, 2005), but often remains less attractive due to its high cost (Berti and Cunningham, 2000) and technical complexity (Poschenrieder and Coll, 2003; Garbisu *et al.*, 2007). Recent techniques in soil remediation include the utilization of activated carbon and biochars to adsorb organic and heavy metals (Brändli et al., 2008; Beesley at al., 2011), while a much simpler and inexpensive approach has been adopted in the southern Philippines (i.e. Sibutad); this involves putting the stripped soil with organic amendments (e.g. plant materials and other agricultural wastes) and other topsoil sourced from nearby areas back to the mined areas, followed by the establishment of vegetation.

Rehabilitation primarily aims to revive the integrity of ecosystems, thus it is imperative to establish evaluation criteria that are scientifically sound and ecologically relevant. Several studies have relied on the aboveground biomass, vegetation structure and diversity to assess rehabilitation success (Norman *et al.*, 2006; Koch, 2007). At the local level, rehabilitation success is commonly judged from the survival of specific plant species or their ability to grow (Mines and Geosciences Bureau, 2014). Focus has been on *Acacia auriculiformis*, a species which has the

potential to sequester heavy metals (Cadiz et al., 2006) and consolidate soil to prevent soil erosion. The efficacy of *A. auriculiformis* in rehabilitating degraded areas has been demonstrated (Lamb and Tomlinson, 1994; McNamara et al., 2006). However, utilizing *A. auriculiformis* survival or growth rate as the sole or principal measure of rehabilitation success may be biased because of this plant's high tolerance to pollution. Moreover, our present local approach, and all approaches which only focus on aboveground variables, ignore valuable information on the soil microbiota, e.g., nematodes which are known to respond more quickly to changing soil conditions (e.g. soil pollution) than plants. Nematodes are among the most abundant taxa in terrestrial soils. They play important roles in soil functioning and are used to assess either the impacts of pollution in terrestrial soils (Pen-Mouratov et al., 2008) or the success of reclamation initiatives (Wu et al., 2005) with the use of various ecological indices, both structural (e.g. diversity and related indices) and 'functional' (e.g. based on feeding guilds, life-history groups, etc.), and with assemblage composition (Fiscus and Neher, 2002; Pen-Mouratov et al., 2008; Martinez et al., unpublished).
The Sibutad mining area is a privately owned, open-pit large-scale mining, which employed modern technology in the extraction of ore deposits, in contrast to the rudimentary and manual operations in artisanal, small-scale mining activities. Open-pit mining, also known as surface mining, is a mining technique which extracts valuable minerals deposited near the earth's surface, and standardly requires the removal and stocking of the top soil until completion of the mining activities (Ghose, 1989). In the case of Sibutad, stockpiling of soil lasted for ca. 3 to 6 years, which may well have caused deterioration of soil quality and accompanying impacts on its soil biota. After mine closure in 1999, impacted subsystems were rehabilitated; for instance, a total of 509,011 *A. auriculiformis* trees were planted covering 157 hectares from 1999 till 2004 (www.philstar.com).

The high survival rate of *A. auriculiformis* in reclaimed sites in Sibutad can be interpreted as rehabilitation 'success', where the development of aboveground vegetation is hypothesized to reflect improving soil conditions. To test this, we examined nematode assemblage structure and physico-chemical variables of rehabilitated soils and compared them with those from a nearby reference (unmined) site; we then identified which soil environmental variables could explain any observed differences in nematode assemblages. Rehabilitation of the three impacted sites was conducted at different periods of time: 13, 11 and 8 years prior to the first sampling period in 2012, and this was followed by a second sampling in 2014. We compared nematode assemblage structure in the rehabilitated soils between the two time periods (2012 and 2014) under the assumption that any improvement in soil condition after 2 years within the site may be reflected in nematode-based ecological indices and nematode genus composition.

4.3. Materials and Methods

4.3.1. Study site

Fig. 4.1 Satellite map showing the location of the different sampling sites (Sites A, B, C and D), mining ponds (a, b, c and d) and a leach pad (*) in the rehabilitated mining area in Sibutad.

The study was conducted in the municipality of Sibutad in the Zamboanga peninsula, southern Philippines (Fig. 4.1). Sibutad is characterized by an average annual temperature of 27.4 ^{0}C and precipitation of 2,310 mm, the latter distributed fairly evenly throughout the year. The discovery of mineral deposits in the 1980's led to an influx of migrants who engaged in both large-scale and small-scale mining activities. Aside from the several small-scale mining activities, Sibutad was also host to a large-scale mining, a 3,515 ha. open-pit mining area of copper and gold (but only 38 ha. was utilized for mining-related activities), which started its operations in 1997. However, it was terminated two years later due to the declining price of gold on the global market (Querubin and Yumul, 2001). Open-pit mining involves stripping of the topsoil to extract precious minerals

during the mining process. The soil is then stockpiled over unmined land forming chains of external dumps until the end of the mining operation, and is used afterwards to refill the stripped areas. During its brief mining stint, the Sibutad mining project was reportedly linked to several ecological disturbances such as mercury and cyanide contamination and siltation (Goodland and Wicks, 2008). Common mining activities (e.g. digging, extraction of minerals, etc.) can release heavy metals to the environment, while stripping and temporary storage of soils can alter its physical, biological and chemical attributes (Sheoran et al., 2010), which in turn affect soil biota such as nematodes and microbes.

Site A, with the highest elevation (269 m) among the sites, was situated 0.39 km, 0.74 km and 0.9 km from Sites B, C and D, respectively (Fig. 1). Like any other unmined areas in Sibutad, Site A was characterized by the presence of a native cogon grass species (*Imperata cylindrica*), patches of ground ferns (*Pteris* sp.) and absence of any mining-related activities, as reflected by the lower heavy metal concentrations compared to the permissible limits (Teh et al., 2016) and those of the rehabilitated sites; hence, it was chosen as the reference site. Several sites in the mining area were rehabilitated, yet only three (Sites B, C and D) were considered due to ease of accessibility. Sites B, C and D were located within the secured perimeter of the mining company and were free of any human interference since rehabilitation in 2004, 2001 and 1999, respectively, while Site A experienced burning of vegetation in 2014, a common phenomenon among unmined areas, either by natural causes (e.g., summer drought, combustibility of vegetation, etc.) or by human intervention. Sites B and C were actual open mine sites and are presently situated near to a pond (water storage for treatment) and a leach pad (used to separate specific minerals), respectively (Fig. 4.1). Site D, on the other hand, served as the 'dumping' or 'storage' site of the stripped soil from Sites B and C. All reclaimed sites were forested with the non-native tree species *A. auriculiformis* during the initial stages of rehabilitation, but different pollution histories and different sources of borrowed soil, used to cover mined-out areas, may have led to differences in soil characteristics (Tables 4.1 and 4.2) which favored certain plant species (e.g., shrubs, herbaceous plants) to flourish as understory: Sites B and C were generally vegetated with ground ferns (*Pteris* sp.) and herbaceous plants, respectively, as understory, whereas a significant understory was lacking in Site D (Fig. 4.2).

Site A

Site C

Site B

Site D

Fig. 4.2. Pictures of the different sampling sites (A, B, C and D) in the Sibutad area: Site A is the reference area while Sites B, C and D are the rehabilitated areas. Other information can be found in Table 4.1.

Table 4.1. Information on the coordinates, altitude, year when rehabilitation started, soil type and vegetation of the rehabilitated sites in the Sibutad mining area.

sites	coordinates	alt. (m)	yr. rehabilitated	soil type (%)			vegetation
				clay	*silt*	*sand*	
Site A	N 08°37'38.45" E 123°29'21.70"	269	-	14.4	65.0	20.6	*Imperata cylindrica* (cogon grass), *Pteris* sp.
Site B	N 08°37'49.19" E 123°29'22.77"	176	2004	13.9	53.8	32.2	*Acacia auriculiformis*, *Pteris* sp.
Site C	N 8°38'01.41" E 123°29'11.50"	116	2001	13.0	46.9	40.1	*A. auriculiformis*, herbaceous plants
Site D	N 08°37'54.94" E 123°28'56.37"	148	1999	19.7	53.3	27.9	*A. auriculiformis*

4.3.2. Nematode sampling

Field samplings were conducted in October 2012 and 2014. Soil samples were taken from the upper 5 cm of the soil of four different sites: 1 non-mined area (reference) and 3 reclaimed areas. There were five sampled plots per site with an approximate interdistance of 15-20 m. Each sample plot was a composite of 3 samples (interdistance of 2 m), combined and homogenized to obtain 500 g of soil. Each soil sample was tightly sealed in zip-lock plastic bags and placed in an insulated container. Soil samples were kept at 14 ^{0}C until laboratory processing. They were sieved through a 2-mm mesh sieve to separate larger debris before biological and chemical analyses. 100 g of soils were collected for the extraction of nematodes using an Oostenbrink elutriator. The sample obtained from the Oostenbrink elutriator was then centrifuged with Ludox TM to further remove the organic debris content, which otherwise hampered nematode counting and collection. Total nematode abundance was determined and 100 nematodes were randomly collected for identification, or all specimens in samples containing fewer than 100 nematodes. Nematodes were identified to the genus level according based on the identification keys of Andrassy (2005) and Bongers (1988), and assigned 'colonizer-persister' scores according to Bongers (1990, 1999) and Bongers et al. (1998). Nematodes were further assigned to trophic groups, namely bacterivores, fungivores, omnivores-predators and plant-parasites (Pen-Mouratov et al., 2008).

4.3.3. Analysis of basic soil properties and heavy metals

Soils collected for nutrient and heavy metal analyses were placed in an insulated box maintained at a temperature below 14 ^{0}C, and were transported to the laboratory for immediate processing. Soil samples for physico-chemical analyses were stored in the freezer. 200 g of soil was used to measure soil pH, nutrient, organic matter and heavy metal contents. Soil pH was determined potentiometrically in a 1:2.5 soil : water suspension (ISRIC, 1995). Total Organic Carbon was measured by the Walkey-Black method, which involves wet combustion of the organic matter with a mixture of potassium dichromate and sulfuric acid (Walkey and Black, 1934). Total N was determined by the Kjeldahl method (Kjeldahl, 1983), and available P was extracted using acidified ammonium fluoride (Jackson, 1958). Major and trace elements were characterized by total element analysis of bulk soil samples following fusion with 2 g lithium metaborate powder in a platinum crucible for 15 min at 950 °C in a preheated muffle furnace, and then dissolved in 100 ml of 4% HNO_3 (ISO 14869). Contents of major and trace elements were then measured with a Varian 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). Loss on ignition (LOI) was determined by heating the samples at 1000 °C.

4.3.4. Data analysis

Nematode assemblages were characterized by determining a) the absolute abundances per 100 g soil; b) the genus richness determined as the actual number of genera; c) Pielou's J as an evenness index; d) the Shannon-Wiener Index (*H'*), which is a diversity measure encompassing aspects of both richness and evenness [$H' = \sum Pi\ (\ln Pi)$], where *Pi* is the proportion of individuals of the i^{th} taxon. All diversity indices (e.g., abundance, no. of genera, evenness and Shannon) were calculated using the PRIMER package (Anderson, 2004). Other nematode related indices such as MI, MI_{2-5}, SI (Structure index) and EI (Enrichment index) may be informative as well; however, they were not calculated due to the the very low nematode abundances (i.e. < 100) which put their reliability in doubt (Shao et al., 2008).

Univariate analyses of the physico-chemical properties of soil were performed to determine differences between sites in 2012. Nematode diversity indices of the different sites in 2012 and 2014 were analyzed with two-way ANOVA using the PRIMER package. Data were first checked for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's test, respectively, and log-transformed when necessary. In case of a significant ANOVA result, pairwise comparisons between sites were performed using Tukey's HSD test.

Principal coordinates analysis (PCO) was carried out to determine the differences between sampling sites based on the combination of measured (in 2012 only) environmental variables, which were normalized due to the differences in units. Non-metric multi-dimensional scaling (nMDS) was also used to visualize spatial distribution of nematode communities in 2012 and 2014. Two-way multivariate analysis was performed using the PRIMER package to detect significant differences in the distribution of nematode communities between different sites and times (Clarke and Warwick, 1994). When significant overall differences were found, pairwise comparisons within PERMANOVA$^+$ were conducted to establish differences between sites and/or sites x year. Each term in the analysis was calculated using 999 permutations. Since PERMANOVA is sensitive to multivariate dispersion, analysis of multivariate dispersion (PERMDISP) was performed to check if the differences were due to 'location' and 'time' effects or possibly to heterogeneous variation (Anderson, 2004).

DistLM (Distance-based linear model) routine using a global BEST selection procedure with Bayesian Information Correction (BIC) was performed to identify the environmental variables that best explain the observed patterns in nematode communities. Collinear variables were checked with Draftman's plot correlation. This was followed by a distance-based redundancy analysis (dbRDA), a graphical visualization of the DistLM results used to show patterns in

assemblage composition and environmental variables across samples using Pearson correlation. Similarity percentage (SIMPER) analyses using the square-root-transformed nematode abundance data were used to identify the genera contributing most to the differences between sites and between years. Genera were considered 'important' if they contributed at least 5% of the average dissimilarity between the sites (Mirto et al., 2002).

4.4. Results and Discussion

Soil rehabilitation in the Sibutad large-scale mining area mainly involved the addition of organic amendment and development of plant cover (forestation). Organic amendments help improve soil quality through stimulation of microbial activity, enhancement of nutrient formation (de Mora et al., 2005; Sheoran et al., 2010) and reduction of heavy metal bioavailability (Bolan and Duraisamy, 2003). Seeding of *A. auriculiformis* in turn helps to stabilize soils and to reduce their metal content through sequestration (Cadiz et al., 2006). Although complete restoration or re-establishment of pre-impacted ecosystems in all their structural and functional aspects is practically not feasible (Bradshaw, 1987), rehabilitation efforts should ideally increase similarities in soil properties and biological parameters between rehabilitated and reference sites, as a consequence of rehabilitation efforts, which can be useful indicators of soil recovery (de Mora et al., 2005; Banning et al., 2008).

4.4.1. Basic soil properties and heavy metal concentrations

Differences in soil properties, heavy metal levels and vegetation cover may have been greatly influenced by the topsoils, taken from various areas, which were used to cover mine-out sites. The soils in the large-scale mining area were very fine in all sites, but not as fine as those found in a small-scale mining site with regular ball-milling activities, situated ca. 1 km from Site A. Acidic soils are typical in mining sites due to the formation of acid mine drainage (AMD), a product of oxidation between iron pyrite and other sulphidic materials (Johnson and Hallberg, 2005; Banning et al., 2008); however, in the present study, Sibutad soils appeared to be naturally acidic (pH < 4.5), as confirmed by the low pH range of 4.6 to 5.6 of the nearby small-scale mining site. Naturally low soil pH in Sibutad (i.e., Site A) could be the result of the combination of high precipitation in a tropical climate and topography (high elevation) which can accelerate leaching of base-forming cations, e.g. Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} (McCauley et al., 2009) and other nutrients such as N and P. Acidic soils can substantially reduce microbial activity (Rousk et al., 2010), cause a shift from bacterial to fungal-driven pathways (Ruess, 2003) and increase metal bioavailability, which in turn increases metal toxicity (Kim et al., 2009). In the longer run, as

acidic soils tend to enhance leaching, they may result in decreasing soil heavy-metal concentrations (Tyler, 1978; Voegelin et al., 2003).

The first axis of a principal coordinates analysis (PCO1) of the environmental variables explained 34.5% of the observed variation, clearly separating Site A (reference site) and Site B (impacted, rehabilitated in 2004) from Sites C and D (impacted, rehabilitated in 2001 and 1999, respectively); PCO2 accounted for 20.1% of the variation and clearly delineated Site C from Site D and partly separated sites A and B (Fig. 4.3). PCO1 was positively correlated with soil OM and N content and with the concentration of Cr. PCO2 was positively correlated with the concentration of Zn, with pH and with median grain size, while negatively with Co and Cu concentration (Fig. 4.3, ESM4.1).

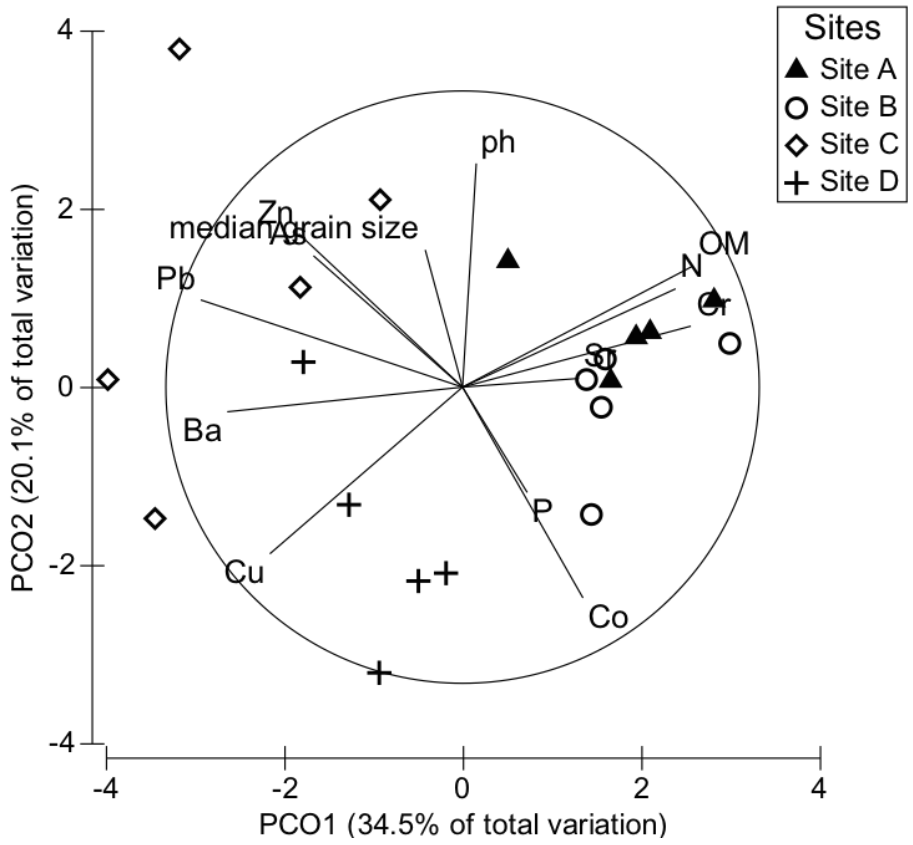

Fig. 4.3. Principal coordinates analysis (PCO) of the environmental variables from the different sampling sites. See Table 2 for an overview of environmental variables included in the analysis.

Table 4.2. Mean concentrations of heavy metals, nutrients and soil properties of the four sampling locations. Values after the mean represent standard errors (mean ± stdev) of five replicates.

	Site A	Site B	Site C	Site D
Basic soil properties				
OM (%)	7.06 ± 0.75	6.36 ± 1.46	2.75 ± 0.96**	1.36 ± 1.04**
N (ppm)	0.22 ± 0.02	0.28 ± 0.06	0.14 ± 0.04**	0.08 ± 0.04*
P (ppm)	0.73 ± 0.07	9.98 ± 9.2*	2.93 ± 2.43*	4.6 ± 8.37
pH	3.98 ± 0.27	3.54 ± 0.42	4.24 ± 1.46	2.86 ± 0.09**
grain size (µm)	21.8 ± 2.26	28.2 ± 6.24	28.6 ± 5.43*	18.1 ± 3.04
Heavy metals (ppm)				
As	17 ± 28.6	1 ± 0*	26.4 ± 1.07	7.8 ± 9.73
Ba	245 ± 86.1	301.8 ± 169.2	800 ± 421.4**	433 ± 119.4*
Co	9.2 ± 1.48	12 ± 4.18	6.2 ± 3.56	10.4 ± 3.05
Cr	35.8 ± 16.4	40.2 ± 12.5	13 ± 2.0**	15.6 ± 9.76*
Cu	59.4 ± 23.7	87 ± 40.7	118.6 ± 65.2	118 ± 14.3*
Pb	25.4 ± 9.37	53.6 ± 21.8*	851.8 ± 223.1*	206.4 ± 220
Sr	2,227 ± 654	347.2 ± 196**	196.6 ± 47.8**	903.4 ± 1 255*
Zn	17.8 ± 6.22	51.8 ± 31.2*	220.2 ± 233.9**	103 ± 104.3*

(*) Asterisks indicate significant differences at $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the reference site A.

The basic soil properties of the reference site (Site A) and of the impacted Site B were remarkably similar: site B had significantly higher P; no other parameters differed significantly between Sites A and B. By contrast, Sites C and D differed in multiple parameters from the reference site: OM and N contents were significantly lower in the impacted Sites C and D (Table 4.2). Organic matter (%) was also significantly lower in Sites C and D than in the reference site and Site B, despite the fact that soils in the rehabilitated areas were amended with organic materials (e.g. agricultural wastes) prior to spreading. This is probably caused by stockpiling or temporary storage of soils (particularly in Site D), a common practice when rehabilitating large-scale mining areas that could last for years, which can affect the biological, chemical and physical properties of soils (Davies et al., 1995). Stockpiling can be detrimental to the organic pools due to the absence of plant OM input (Harris et al., 1993; Mummey et al., 2002b); it can also lead to gradual decomposition of OM or leaching of dissolved OM especially in tropical countries with high rainfall (Sheoran, 2010). Since OM content importantly affects a soil's ability to retain and cycle nutrients, low OM, in combination with low pH, could also explain the low N concentrations in Sites C and D.

Several heavy metals such as Ba, Co, Cu, Pb and Zn remained elevated in the rehabilitated Sites C and D compared to the reference site, but Cr and Sr showed an opposite pattern, both being significantly higher in Site A (Table 4.2). Zn concentrations were well within the allowable limits set by the US and EU standards, while Pb and Cu concentrations in Sites C and D, respectively, only slightly exceeded these limits (Teh et al., 2016). Barium concentrations were shown to be

elevated in land mining areas (Shock et al., 2007), which was also confirmed by the present study; however, Ba levels remained well below the concentrations known to affect earthworms and enchytraeids (NOEC of 1,348 ppm and 1,800 ppm, respectively), but exceeded the NOEC of collembolans (211 ppm) (Kuperman et al., 2006) and of the marine nematode *Litoditis marina* (< 302 ppm (Lira et al., 2011)). Among the rehabilitated sites, Site B was the least polluted with only Pb being significantly higher than in the reference site ($P < 0.05$), but such concentration was still well within the permissible level according to international regulatory bodies (Teh et al., 2016). The relatively low metal concentrations in Site B may be due to the distinct presence of a common ground fern, *Pteris* sp., a known 'hyperaccumulator' of arsenic (Wang et al., 2002; Xie et al., 2009) and probably of other heavy metals as well. Overall, heavy metal concentrations in the rehabilitated sites were not high, which indicates that rehabilitation strategy may be partly efficient as it helped to reduce metal concentrations in the soil.

4.4.2. Nematode abundance, nematode-based indices and genus composition

A total of 38 distinct nematode genera were collected during the entire duration of the study on the large-scale mining area in Sibutad: 31 nematode genera were collected in 2012 (7 bacterial feeders, 2 fungal feeders, 6 omnivores, 5 predators and 11 plant feeders), whereas 37 genera were collected in 2014 (8 bacterial feeders, 4 fungal feeders, 7 omnivores, 8 predators and 10 plant feeders). The plant-feeding *Dorylaimellus* was exclusively found in 2012, while several free-living genera such as *Acrobeloides*, *Aphelenchoides*, *Judonchulus*, *Labronemella*, *Mononchus*, *Panagrolaimus* and *Paractinolaimus* were only present in 2014. Except for the factor year, site and its interaction with year (site x year) significantly impacted total nematode abundance (PERMANOVA, all *pseudo-P* < 0.01, Table 4.3). The factors site and year and its interaction significantly affected genus richness (PERMANOVA, all *pseudo-P* ≤ 0.01), whereas site and year, but not their interaction, significantly affected Shannon-Wiener diversity. Evenness did not differ among years, sites or their interaction.

Table 4.3. Result of the two-way PERMANOVA of nematode assemblage descriptors between sites in 2012 and 2014.

Effect	df	Pseudo-F	P (perm)
nematode abundance			
site	3	10.271	0.001
year	1	0.0767	0.794
site x year	3	5.4004	0.003
genus richness			
site	3	21.645	0.001
year	1	11.666	0.011
site x year	3	4.1854	0.012
Shannon-Wiener index			
site	3	14.493	0.001
year	1	6.0544	0.018
site x year	3	1.3939	0.269
evenness			
site	3	2.4939	0.063
year	1	1.0181	0.343
site x year	3	1.4743	0.234

Table 4.4. Result of the pairwise comparison of nematode assemblage descriptors between sites in 2012 and 2014.

	A	B	C	D
nematode abundance				
sites				
2012				
A	-	0.531	0.009	0.009
B	0.531	-	0.009	0.005
C	0.009	0.009	-	0.633
D	0.009	0.005	0.633	-
2014				
A	-	0.01	0.276	0.208
B	0.01	-	0.044	0.006
C	0.276	0.044	-	0.32
D	0.208	0.006	0.32	-
years				
2012, 2014	0.034	0.584	0.022	0.127
genus richness				
sites				
2012				
A	-	0.041	0.001	0.001
B	0.041	-	0.001	0.054
C	0.001	0.001	-	-
D	0.001	0.054	-	-
2014				
A	-	0.074	0.344	0.001
B	0.074	-	0.043	0.001
C	0.344	0.043	-	0.216
D	0.001	0.001	0.216	-
years				

2012, 2014	0.1	0.029	0.04	0.091
Shannon-Wiener index				
sites				
2012				
A	-	0.022	0.008	0.003
B	0.022	-	0.04	0.044
C	0.008	0.04	-	0.634
D	0.003	0.044	0.634	-
2014				
A	-	0.315	0.013	0.016
B	0.315	-	0.138	0.118
C	0.013	0.138	-	0.813
D	0.016	0.118	0.813	-
years				
2012, 2014	0.49	0.33	0.24	0.083
evenness				
sites				
2012				
A	-	0.024	0.139	0.086
B	0.024	-	0.753	0.352
C	0.139	0.753	-	0.265
D	0.086	0.352	0.265	-
2014				
A	-	0.005	0.143	0.137
B	0.005	-	0.844	0.327
C	0.143	0.844	-	0.414
D	0.137	0.327	0.414	-
years				
2012, 2014	0.257	0.948	0.677	0.174

In 2012, Sites A and B had significantly higher nematode abundances than Sites C and D (all $P < 0.01$, Table 4.4, Fig. 4.4). Genus richness was significantly highest in Site A ($p < 0.05$), and Site B was higher than Sites C ($P < 0.01$) and D ($P \leq 0.05$); the spatial pattern of 2014 differed from that of 2012. In 2014, the reference site (Site A) had similarly low abundances as Sites C and D; its genus richness was higher than Site D ($P < 0.01$), while not different from Sites B and C. Shannon-Wiener diversity was significantly higher in Sites A and B than in Sites C and D, and overall in 2012 compared to 2014 (Table 4.4). Thus, the highest density (165 ind. per 100 g of soil) and number of genera (16) were found in the reference site in 2012 (Fig. 4.4); these values were much lower than at a nearby reference site of a small-scale mining area in Sibutad, where the mean density reached 412 ind. per 100 g soil from 20 genera (Martinez et al., *unpublished*). The naturally (more) acidic soils in the large-scale mining area in Sibutad may be principally responsible for the low nematode abundances and diversity in the reference area (Háněl, 2001; Pen-Mouratov et al., 2008). Low soil pH can affect microbes (Räty and Huhta, 2003), which are an important food source to several nematode genera: many bacteria are sensitive to acidic soils,

while fungi are more tolerant (Rousk et al., 2010). It is therefore somewhat unexpected that only very few fungal-feeding nematodes were observed in this study. The lower nematode abundances and diversity in Site A in 2014 compared to 2012 were undoubtedly caused by the burning of cogon grass (*I. cylindrica*) in Site A in 2014 (pers. observ.), a phenomenon which frequently occurs in unmined areas in Sibutad. Burning of vegetation disrupts supply of organic material in the soil and influences the composition of soil fauna (Lavelle et al., 1997). This can lead to a reduction in microbial biomass which may take 4 to 13 years to recover (Prieto-Fernández et al.,1998; Villar et al., 2004), and in turn, may also affect nematode communities. This may have resulted in the loss (e.g., *Axonchium*, *Dorylaimellus* and *Tylenchorhynchus*) or decrease (e.g., *Xiphinema*, *Rotylenchus*, *Longidorus*, *Oxydirus*) in abundance of several plant-feeding and free-living nematodes (e.g., *Eudorylaimus*, *Bursilla*, *Prionchulus*, *Iotonchus*, etc.) in 2014 (Table 4.8).

Apart from the mean values of the different diversity indices, their variances were considerably larger in the disturbed Sites B and particularly C and D than in Site A (see Fig. 4.4B, C, D), at least in 2012. This may be indicative of lack of stability (Tilman, 1996) or incomplete community recovery where rather unstable assemblages have so far established or are still establishing in specific sites. High variability within sites probably further related to a) more increased patchiness in relation to the vegetation (trees and their understory) in the rehabilitated sites than in the grass and ground fern vegetation of Site A; and b) high variability in soil parameters in all sites (de Goede and Bongers, 1994) may have lead to high abundances of certain nematode genera in some replicate soil samples (e.g. *Mesodorylaimus*, *Dorylaimellus*), while they were rare or absent in others.

Aside from the traditional ecological indices, nematode genus composition can also be helpful in the assessment of the impact of soil disturbances (Fiscus and Neher, 2002; Šalamún et al., 2011). In the present study, nematode genus composition differed significantly between sites, years as well the site x year interaction (PERMANOVA, all *pseudo-P* < *0.01*, Table 4.6), which suggests that effects of previous mining activities on nematode communities still persisted despite rehabilitation efforts. Although multivariate dispersion was significant for site (site and year *PERMDISP* = *0.001* and *0.065*, respectively), non-metric multidimensional scaling (nMDS) showed that Sites A, B, C and D were very distinct to one another other despite relatively large variability between replicates (Fig. 4.5). Pairwise comparisons showed highly significant differences between the control and all the rehabilitated sites (all $P \leq 0.01$), as well as between rehabilitated Site B on the one hand and Sites C and D on the other (both $P \leq 0.01$) in both years (Table 4.7); Sites C and D also differed in 2012 and 2014 (both $P < 0.01$).

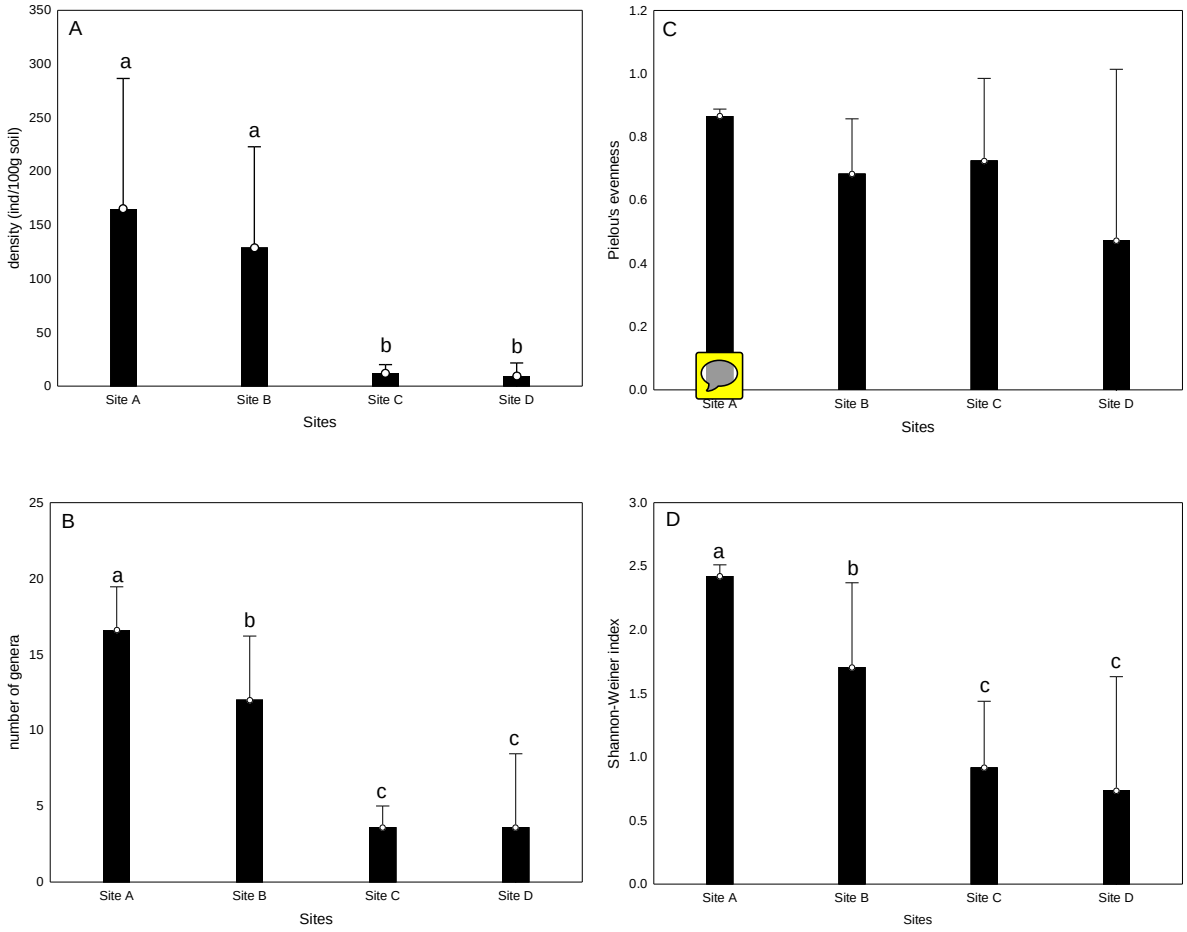

Fig. 4.4 (A-D). Nematode abundance (A), number of genera (B), Pielou's evenness (C) and Shannon-Wiener index (D) in 2012. Data represent mean ± SE of five replicates. Different letters indicate significant differences at $P < 0.05$.

In 2012, Sites A, B, C and D had an average similarity within sites of 60.4%, 50.5%, 45.3% and 19.7% respectively. The least dissimilar (most similar) nematode community assemblage of 60.4% occurred between the reference site (Site A) and Site B, while the most dissimilar (94%) was between Site A and Site C (Table 4.5). Nematode genera contributing (> 5%) to the difference between Sites A and B included *Axonchium*, *Xiphinema* and *Eudorylaimus*, which were more abundant in Site A, while *Bursilla* and *Hoplolaimus* were more abundant in Site B. *Axonchium* and *Eudorylaimus* were less abundant in Site C compared to Sites A and B. Several genera commonly found in Sites A and B, such as *Xiphinema*, *Iotonchus*, *Longidorus*, *Metaporcelaimus* and *Bursilla* were absent from Site C, while the first four, and *Axonchium* and *Eudorylaimus*, were absent from Site D.

Several free-living genera, e.g., *Eudorylaimus*, *Iotonchus*, *Metaporcelaimus* and *Prionchulus*, all relatively abundant in the reference site, are large-bodied omnivorous or predacious nematodes with cp scores of 4 or 5 which are generally considered sensitive to strong disturbances (Korthals et al.,

1996a; Nagy et al., 2004). The absence of these trophic groups (omnivorous or predacious), as in Sites C and D, demonstrates that mining-related effects on nematode communities last even after 11 and 13 years of rehabilitation, respectively. By contrast, some presumedly 'sensitive' genera, such as *Prismatolaimus* (cp3) and *Labronema* (cp4), were found exclusively in Site B, albeit in very low abundance, which could hint at improving soil conditions at this site, as evidenced by the high resemblance of soil variables with those of the reference site (Fig 4.3; Table 4.2). While free-living nematodes can respond negatively to metal pollution, this is often less the case for plant-feeding nematodes (Pen-Mouratov et al., 2008; Šalamún et al., 2011; Gutiérrez et al., 2016); hence, the distribution of plant-feeding nematodes in this study may have been more influenced by the presence of different host plants in the different sampling sites than by the pollution history. For instance, *Hoplolaimus* and *Rotylenchulus*, common in all rehabilitated (impacted) areas, are known as parasites of *A. auriculiformis* (Marais et al., 1993; Duponnois et al., 2000), whereas *Mesocriconema* and *Longidorus*, only found in the reference site, are known to be associated with cogon grass, *Imperata cylindrica* (Horst, 2013).

Table 4.5. Results of SIMPER analysis indicating the genera contributing to the overall dissimilarity between the reference and rehabilitated sites in 2012 as indicated by square-root-transformed abundances, average dissimilarity and individual contributions (Contrib. %) to dissimilarity.

Genera	Average Abundance		Contrib. (%)
Average dissimilarity = 60.4%	Site A	Site B	
Bursilla	2.6	7.0	11.4
Hoplolaimus	0	2.7	6.2
Axonchium	5.6	3.2	6.2
Xiphinema	4.4	1.8	6.0
Longidorus	2.6	0	5.7
Prionchulus	2.4	0	5.2
Eudorylaimus	3.5	1.1	5.2
Average dissimilarity = 94.0%	Site A	Site C	
Axonchium	5.6	0.2	11.1
Xiphinema	4.4	0	8.7
Hoplolaimus	0	2.7	6.1
Iotonchus	2.8	0	6.0
Eudorylaimus	3.5	0.6	5.7
Longidorus	2.5	0	5.7
Metaporcelaimus	2.5	0	5.6
Bursilla	2.6	0	5.4
Prionchulus	2.4	0	5.1
Average dissimilarity = 93.6%	Site A	Site D	
Axonchium	5.6	0	11.7
Xiphinema	4.4	0	8.7
Eudorylaimus	3.5	0	7.2
Iotonchus	2.8	0	6.1
Longidorus	2.5	0	5.7

Metaporcelaimus	2.5	0	5.7
Bursilla	2.6	0.2	5.4
Prionchulus	2.2	0	5.1
Average dissimilarity = 82.2%	Site B	Site C	
Bursilla	7.0	0	24.0
Axonchium	3.2	0.2	9.8
Oxydirus	2.5	0	8.2
Hoplolaimus	2.7	2.7	7.5
Xiphinema	1.8	0	6.5
Mesodorylaimus	1.8	0.4	5.2
Cephalobus	1.5	0.3	5.1
Average dissimilarity = 89.2%	Site B	Site D	
Bursilla	7.0	0.2	21.7
Axonchium	3.3	0	9.8
Hoplolaimus	2.7	0.8	8.8
Oxydirus	2.5	0	7.5
Xiphinema	1.8	0	6.0
Average dissimilarity = 89.0%	Site C	Site D	
Hoplolaimus	2.7	0.8	32.5
Cephalobus	0.3	1.0	10.7
Tylencholaimus	0	0.7	9.6
Mesodorylaimus	0.5	0.5	6.8
Eudorylaimus	0.6	0	6.6
Oriverutus	0.0	0.6	5.5

4.4.3. Temporal evolution in nematode assemblage composition: indications for recovery?

Comparison of nematode assemblages within sites over a period of time may provide insights into the trajectory and possible success of rehabilitation efforts (Bongers and Ferris, 1999; Neher, 2001; Fiscus and Neher, 2002). In Site C, total nematode abundance significantly increased from 2012 to 2014 ($P < 0.05$), and so did the different diversity indices ($P < 0.05$), consistent with Háněl (2001), even if many of these increases were not statistically significant (Table 9). Genus composition at these two sites (Sites C and D) also differed significantly between 2012 and 2014 (PERMANOVA, $P \leq 0.001$; Fig. 4.5): Site C had a large increase of the enrichment opportunist *Bursilla* (contributing almost 50% to the difference between years) in 2014, which suggests higher organic matter and nutrient concentrations, supporting a more active microbial food web. Site D, by contrast, showed increased abundances of several nematode genera (e.g., *Cephalobus*, *Oriverutus*, *Heterocephalobus*, , etc.) in 2014 (Table 4.8), whereas Site B had a significantly higher genus richness in 2014 ($P < 0.05$) due to the appearance of several presumedly 'sensitive' nematodes (with high cp values), such as *Judonchulus*, *Mononchus*, *Oriverutus*, *Labronemella* and *Ecumenicus*. While signs of a partial soil recovery were thus detected in the rehabilitated sites, there were marked decreases in nematode abundance and diversity, and a significant change in genus composition in the reference site in 2014 ($P < 0.05$) (Tables 4.7 and

4.9); all these changes were most probably caused by the burning of Imperata cylindrica grass in Site A in 2014 (see above).

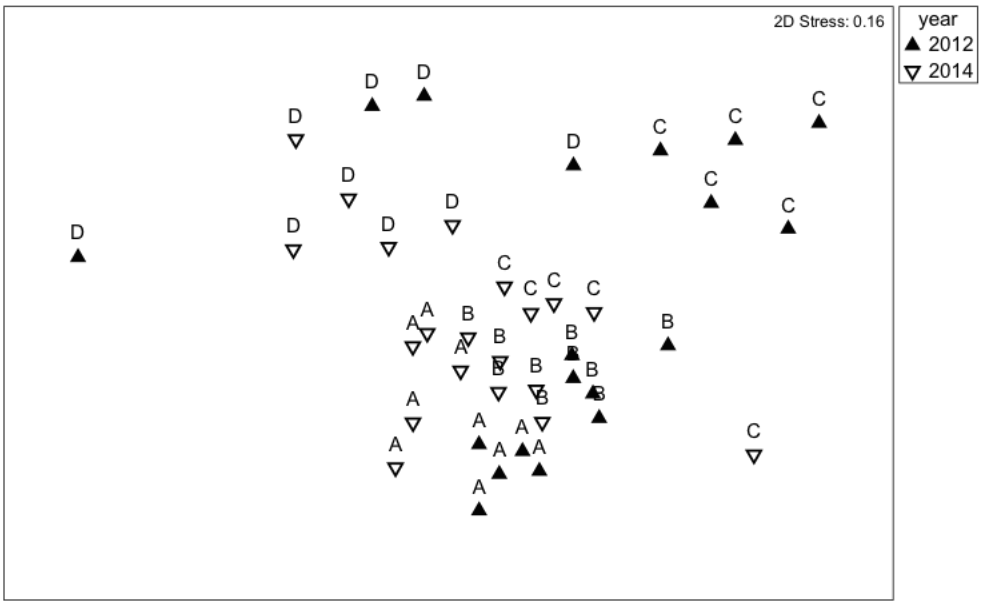

Fig. 4.5. Non-metric Multidimensional scaling (nMDS) of nematode assemblages of the different sites (A, B, C and D) between 2012 and 2014.

Table 4.6. Result of the two-way PERMANOVA of nematode genus composition between sites in 2012 and 2014.

Effect	df	Pseudo-F	P-value
site	3	7.402	0.001
year	1	5.256	0.001
site x year	3	3.828	0.001

Table 4.7. Result of the pairwise comparison of nematode genus composition between sites in 2012 and 2014.

	A	B	C	D
sites				
2012				
A	-	0.01	0.012	0.009
B	0.01	-	0.006	0.01
C	0.012	0.006	-	0.008
D	0.009	0.01	0.008	-
2014				
A	-	0.01	0.009	0.009
B	0.01	-	0.009	0.009
C	0.009	0.009	-	0.009
D	0.009	0.009	0.009	-
year				
2012, 2014	0.005	0.007	0.012	0.177

Table 4.8. Result of the SIMPER analysis indicating the genera contributing to the overall dissimilarity within each sites after 2 years (2012 vs 2014) as indicated by square-root-transformed abundances, average dissimilarity and individual contributions (Contrib. %) towards dissimilarity.

Genera	Average Abundance		Contrib. (%)
Sampling sites	2012	2014	
Site A (average dissimilarity of 62.7%)			
Axonchium	5.6	0	12.2
Xiphinema	4.4	1.2	7.0
Eudorylaimus	3.6	0.5	6.5
Bursilla	2.6	1.2	5.2
Site B (average dissimilarity of 61.9%)			
Bursilla	7.0	5.8	7.7
Hoplolaimus	2.7	0.5	6.0
Oxydirus	2.5	0	5.7
Mesodorylaimus	1.8	2.8	5.3
Axonchium	3.3	1.8	5.1
Site C (average dissimilarity of 86.2%)			
Bursilla	0	6.0	26.7
Hoplolaimus	2.7	1.0	11.6
Iotonchus	0	2.28	11.4
Mylonchulus	0.4	1.8	6.8
Heterocephalobus	0	1.5	5.2
Site D (average dissimilarity of 82.0%)			
Cephalobus	0.5	2.8	35.0
Oriverutus	0	2.6	20.0
Heterocephalobus	1.0	2.3	19.1
Tylencholaimus	0.7	0.5	8.1
Acrobeloides	0	1.1	5.2

Table 4.9. Mean values of total nematode abundance and the different nematode-based indices between different sites in 2012 and 2014. Values after the mean represent standard errors (mean ± stdev) of five replicates.

Ecological indices	Site A (reference site)		Site B		Site C		Site D	
	2012	*2014*	*2012*	*2014*	*2012*	*2014*	*2012*	*2014*
abundance	165 ± 97.9	58 ± 7.6*	128 ± 75.8	126 ± 35.9	12 ± 2.89	70.4 ± 43.2*	9.4 ± 4.4	43.2 ± 32.1
no. of genera	16.6 ± 2.30	14 ± 2.55	12.0 ± 1.52	19.2 ± 4.76*	3.6 ± 1.14	11 ± 6.16*	3.6 ± 3.91	7.2 ± 1.92
Pielou's evenness	0.85 ± 0.02	0.89 ± 0.03	0.68 ± 0.14	0.69 ± 0.13	0.72 ± 0.21	0.66 ± 0.28	0.79 ± 0.10	0.78 ± 0.13
Shannon index	2.42 ± 0.03	2.33 ± 0.21	1.71 ± 0.24	2.05 ± 0.54	0.91 ± 0.18	1.42 ± 0.68	0.74 ± 0.32	1.53 ± 0.43

(*) Asterisks indicate significant differences at $^{*}P < 0.05$ compared to the control (2012)

4.4.4. Relationship between nematodes and soil properties

One of the goals of soil rehabilitation is the improvement of soil quality and recovery of soil biota. Hence, identification of soil variables that are positively correlated with high abundances and/or with the taxonomic and functional composition of soil communities may be helpful in formulating advice for the optimization of rehabilitation measures for disturbed soils. Our present work showed that the measured environmental variables together explained 76.8% of the fitted variation in the nematode data (i.e., 37.6% of the total variation at the two first dbRDA axes). The soil variables OM and Pb together explained more than 37% of the variation in

nematode assemblages (ESM4.3); OM (r = 0.88) showed strong positive correlations, while Pb was negatively correlated (r = -0.84) with dbRDA1; the two variables clearly 'separated' Sites A and B from Sites C and D (Fig. 6B). Other soil variables which also showed high positive correlation to dbRDA1 include N (r = 0.78), while most of the heavy metals showed high correlation such as Ba (r = -0.72), Cr (r = 0.67), Cu (r = -0.62) and Zn (r = -0.51) (Fig. 4.6B). Among the heavy metals, only Pb substantially exceeded the range (150 ppm to 600 ppm Pb) of standard limits prescribed in most developed countries (Teh et al., 2016); Sites C and D had concentrations > 800 ppm and > 200 ppm, respectively; concentrations > 300 ppm have been shown to be detrimental to soil nematodes, particularly those belonging to life-history groups cp 4 and cp 5 (Zullini and Peretti, 1986).

It is therefore not surprising that both plant-feeding and free-living nematodes, particularly those with high cp values, had a strong positive correlation with dbRDA1 (Fig. 4.6A). The presence of several supposedly 'sensitive' genera (e.g., *Eudorylaimus*, *Iotonchus*, *Ironus*, *Mesodorylaimus* and *Metaporcelaimus*) in the reference site and to a lesser extent (e.g., *Prismatolaimus* and *Labronema*) in Site B, and their often strong positive correlation to dbRDA1 is likely linked to the relatively low heavy-metal concentrations in these soils. In addition, OM content was deficient in Sites C and D, which may also have contributed to lower total abundances of nematodes and the absence of sensitive species from the latter two sites. Organic amendments can enhance microbial communities (Vázquez et al., 1996; Villar et al., 2004; de Mora et al., 2005) and improve soil structure, water retention capacity, cation exchange capacity, etc. Eventually, this will lead to 'bottom-up' effects in the soil food web, positively affecting soil organisms such as protozoans and nematodes (Treonis et al., 2010). It is likely that during the re-instatement of topsoil in mine-out areas, the newly added soils were characterized by poor OM, sensitivity to erosion and nutrient deficiency (Bradshaw, 1987; Scullion and Malinovszky, 1995; Sheoran et al., 2008). Our results suggest that rehabilitation of the study area should aim to enhance OM contents in soils in order to re-establish mature soil communities. This is in congruence with previous findings where soil properties and functions, i.e., OM content, OM turn-over and mineral nutrient cycling, were essential requirements for a successful rehabilitation strategy (Banning et al., 2008). Although the present results have pointed out the benefits of soil amendment to facilitate soil recovery, nonetheless, such practice should be carried out with caution because long-term use of amendments, e.g., N fertilization, can reduce microbial biomass and respiration rate (Smolander et al., 1994; Ananyeva et al., 1999).

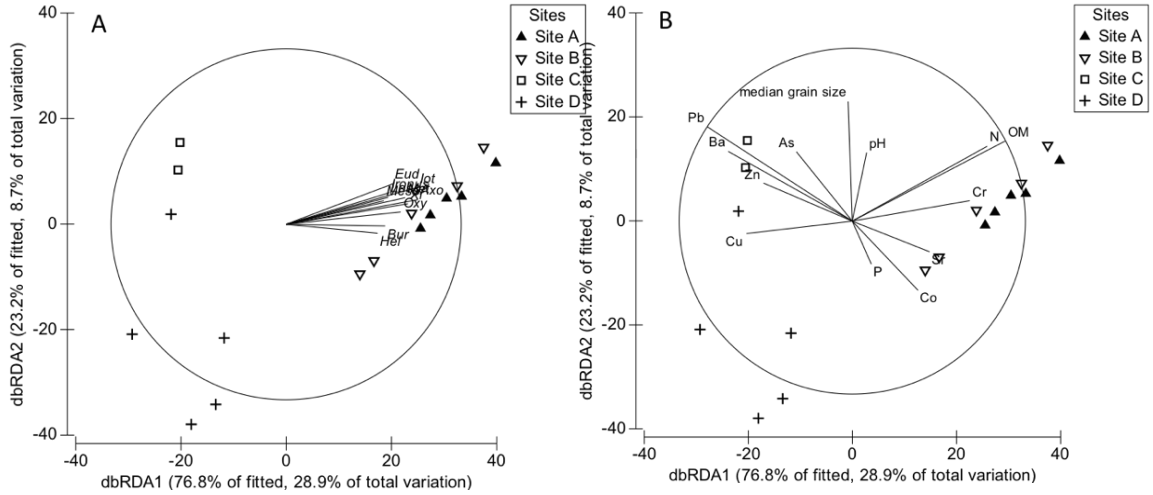

Fig. 4. 6 (A and B). Distance-based Redundancy Analysis (dbRDA) plots based on the nematode assemblages and the fitted environmental variables as vectors. Names of the genera with Pearson correlation $r > 0.5$ are abbreviated: *Axo* = *Axonchium*, *Bur* = *Bursilla*, *Eud* = *Eudorylaimus*, *Hel* = *Helicotylenchus*, *Iot* = *Iotonchus*, *Iro* = *Ironus*, *Long* = *Longidorus*, *Mes* = *Mesodorylaimus*, *Met* = *Metaporcelaimus*, *Oxy* = *Oxydirus* and *Xi* = *Xiphinema*.

4.5. Conclusions

In the Philippines, rehabilitation success is often judged based on the ability of plants to survive. However, plant species, commonly used in the rehabilitation of mining-impacted areas, can thrive in harsh environmental conditions, thus making them poor 'indicators' of soil improvement. This is clearly demonstrated in the present study, where despite successful development of vegetation in all rehabilitated areas, nematode abundance and diversity remained very low up to 15 years after the onset of rehabilitation. This supports our contention that the development of aboveground vegetation does not reflect full improvement of soil communities. Nonetheless, hints of partial recovery of the impacted soils were observed, for instance low heavy metal levels and the appearance of a few presumedly 'sensitive' nematode genera in one or more of the rehabilitated sites (Site B), as well as an increase in total nematode abundance and diversity after two additional years of rehabilitation.

Electronic Supplemental Materials (ESM)

ESM 4.1. PC scores of the Principal Coordinates Analysis (PCO) of the environmental variables from the large-scale mining area of Sibutad.

Ecological variables	PCO1	PCO2	PCO3	PCO4	PCO5
OM	0.78	0.40	0.19	-0.33	0.01
N	0.72	0.34	0.50	-0.22	0.19
P	0.19	-0.31	0.49	0.49	0.39
pH	0.05	0.76	-0.02	0.32	0.28
median grain size	-0.12	0.48	0.72	-0.33	-0.09
As	-0.50	0.44	-0.25	-0.38	0.41
Ba	-0.79	-0.08	0.22	-0.47	-0.03
Co	0.40	-0.71	0.34	-0.11	0.19
Cr	0.77	0.20	0.13	-0.07	-0.42
Cu	-0.65	-0.55	0.30	-0.22	0.15
Pb	-0.88	0.30	0.21	-0.11	-0.09
Sr	0.39	0.01	-0.73	-0.41	0.20
Zn	-0.55	0.52	0.05	0.5	0.00

ESM 4.2. Results of the DistLM marginal test and model selection.

No.	Variable	SS (trace)	Pseudo-F	*P*	Prop.
Marginal DistLM test					
1	OM	15792	5.8516	0.001	0.24533
2	N	11900	4.0821	0.001	0.18486
3	P	4115.4	1.2294	0.237	6.3933E-2
4	pH	3831.4	1.1392	0.300	5.9521E-2
5	median grain size	5200.3	1.582	0.115	8.0787E-2
6	As	3014.3	0.88431	0.527	4.6828E-2
7	Ba	10667	3.5754	0.001	0.16572
8	Co	3269.8	0.96328	0.455	5.0797E-2
9	Cr	11692	3.995	0.001	0.18163
10	Cu	7964.2	2.5415	0.014	0.12372
11	Pb	14727	5.3399	0.001	0.22879
12	Sr	9433.5	3.0909	0.004	0.14655
13	Zn	6738.2	2.1945	0.026	0.10468

Best results for each number of variables

Var. No.	BIC	R^2	RSS	Selections
DistLM models				
1	161.08	0.24533	48578	1
2	161.26	0.37626	40151	1, 11
3	162.68	0.45817	34878	1, 3, 11
4	163.93	0.49934	32228	1, 3, 11, 12
5	165.08	0.54113	29538	1, 3, 4, 7, 12
6	166.17	0.58148	26940	1, 3, 4, 7, 9, 12
7	167.02	0.61949	24493	1-5, 7, 12
8	167.91	0.65828	21997	1-5, 7, 9, 12
9	169.22	0.6924	19801	1-5, 7, 9, 10, 12

10	170.06	0.7173	18198	1-5, 9-13
11	170.06	0.74612	16342	1-11
12	171.25	0.76806	14930	1-12
13	172.81	0.78415	13894	All

BIC	R^2	RSS	No. of var.	Selections
Overall best solutions				
161.08	0.37626	40151	2	1, 11
161.26	0.45817	34878	3	1, 3, 11
161.9	0.24533	48578	1	1
161.96	0.43897	36114	3	1, 2, 11
161.98	0.43833	36155	3	1, 11, 12
162.09	0.34406	42223	2	1, 5
162.09	0.34385	42237	2	2, 11
162.18	0.34095	42423	2	1, 12
162.31	0.42901	36755	3	1
162.33	0.22879	49643	1	1, 2, 11

ESM 4.3. Mean absolute abundances of nematode genera, trophic and cp groups of nematodes of the sampling sites in Sibutad (n=5). Values after the mean represent standard deviations (mean ± stdev).

Genus	CP/PP	Family	Site A	Site B	Site C	Site D
Bacterivores						
Alaimus	4	Alaimidae	0.58 ± 1.16	0.38 ± 0.84	0.4 ± 0.89	0
Amphidelus	4	Amphidelidae	0.89 ± 1.00	0	0	0
Bursilla	1	Mesorhabditidae	12.8 ± 18.2	57.6 ± 41.6	0	0.2 ± 0.44
Cephalobus	2	Cephalobidae	0.4 ± 0.89	4.64 ± 6.06	0.4 ± 0.899	2 ± 2.55
Heterocephalobus	2	Cephalobidae	1.32 ± 1.23	7.48 ± 15.1	0.2 ± 0.44	0
Plectus	2	Plectidae	0.4 ± 0.89	0.66 ± 1.01	0.6 ± 0.89	0
Prismatolaimus	3	Prismatolaimidae	0	0.46 ± 1.03	0	0
Fungivores						
Aphelenchus	2	Aphelenchidae	0.52 ± 1.16	0	0	0.2 ± 0.44
Tylencholaimus	4	Tylencholaimidae	0	0	0	1.4 ± 2.61
Omnivores						
Ecumenicus	4	Qudsianematidae	0.52 ± 1.16	0	0	0.2 ± 0.45
Eudorylaimus	4	Dorylaimidae	14.9 ± 13.3	2.38 ± 2.84	0.6 ± 0.54	0
Labronema	4	Qudsianematidae	0	1.24 ± 0.89	0	0
Mesodorylaimus	4	Dorylaimidae	6.60 ± 3.65	6.58 ± 9.75	1 ± 2.23	0.6 ± 0.89
Metaporcelaimus	5	Aporcelaimidae	6.57 ± 3.77	1.53 ± 2.47	0	0
Oriverutus	4	Dorylaimidae	5.7 ± 7.74	0	0	0.6 ± 0.89
Predators						
Iotonchus	4	Iotonchidae	8.0 ± 3.47	4.82 ± 6.65	0	0
Ironus	4	Ironidae	3.47 ± 4.12	0.6 ± 0.89	0	0
Mylonchulus	4	Mylonchulidae	0.4 ± 0.89	0.6 ± 0.89	0.4 ± 0.55	0
Oxydirus	5	Nordiidae	5.47 ± 5.62	7.74 ± 7.9	0	0
Prionchulus	4	Mononchidae	7.04 ± 7.42	0	0	0
Plant feeders						
Axonchium	5	Dorylaimidae	35.8 ± 25.3	12.7 ± 10.5	0.2 ± 0.45	0
Dorylaimellus	5	Dorylaimidae	5.07 ± 7.9	3.12 ± 3.79	0	0.4 ± 0.89
Helicotylenchus	3	Hoplolaimidae	5.9 ± 5.91	0.78 ± 0.8	0	0.2 ± 0.44
Hoplolaimus	3	Hoplolaimidae	0	11.6 ± 12.7	8 ± 5.43	3.0 ± 6.71
Longidorus	5	Longidoridae	6.74 ± 2.65	0	0	0
Mesocriconema	3	Pratylenchidae	4.46 ± 5.62	0	0	0
Pratylenchus	3	Pratylenchidae	0.92 ± 1.27	0.46 ± 1.03	0	0
Rotylenchulus	3	Rotylenchulidae	0	0	0	0.4 ± 0.55
Rotylenchus	3	Hoplolaimidae	6.44 ± 7.58	0	0	0
Tylenchorhynchus	3	Belonolaimidae	1.04 ± 2.32	0	0	0 .2 ± 0.44
Xiphinema	5	Longidoridae	23.1 ± 16.3	3.42 ± 2.13	0	0

ESM 4.4 Mean absolute abundances of nematode genera, trophic and cp groups of nematodes of the sampling sites in Sibutad (n=5). Values after the mean represent standard deviations (mean ± stdev).

Genus	CP/PP	Family	Site A	Site B	Site C	Site D
Bacterivores						
Acrobeloides	2	Cephalobidae	2 ± 2.82	0	0.6 ± 0.89	2.2 ± 2.6
Alaimus	4	Alaimidae	0.6 ± 0.89	2.2 ± 3.45	0	0
Amphidelus	4	Amphidelidae	0	3.8 ± 7.95	0	0
Bursilla	1	Mesorhabditidae	12.8 ± 18.2	57.6 ± 41.6	0	0.2 ± 0.44
Cephalobus	2	Cephalobidae	4.0 ± 4.06	6.2 ± 7.6	1.8 ± 1.30	6.8 ± 7.5
Heterocephalobus	2	Cephalobidae	5.4 ± 4.51	3.8 ± 5.36	3.8 ± 3.56	9.8 ± 9.6
Plectus	2	Plectidae	0	1.4 ± 1.14	0.6 ± 0.89	0.4 ± 0.89
Prismatolaimus	3	Prismatolaimidae	0	0.8 ± 1.3	0.6 ± 1.34	0
Fungivores						
Aphelenchoides	2	Aphelenchidae	0	0.6 ± 0.55	0.2 ± 0.45	0.2 ± 0.45
Aphelenchus	2	Aphelenchidae	0.4 ± 0.55	1.0 ± 1.22	0.8 ± 0.84	0.2 ± 0.45
Paraphelenchus	2	Aphelenchidae	0.2 ± 0.45	5.0 ± 2.92	0.4 ± 0.89	0.4 ± 0.89
Tylencholaimus	4	Tylencholaimidae	1.4 ± 1.67	0.8 ± 0.84	0	0.8 ± 1.3
Omnivores						
Ecumenicus	4	Qudsianematidae	4.4 ± 2.89	0.2 ± 0.45	0.2 ± 45	0.8 ± 1.3
Eudorylaimus	4	Dorylaimidae	0.6 ± 0.89	1.8 ± 1.3	0.4 ± 0.55	0
Labronema	4	Qudsianematidae	1.2 ± 2.68	0.8 ± 0.45	0.2 ± 0.45	0
Mesodorylaimus	4	Dorylaimidae	4.4 ± 3.43	0.2 ± 11.9	1.0 ± 1.0	0.4 ± 0.55
Metaporcelaimus	5	Aporcelaimidae	2 ± 1.58	4.0 ± 6.82	1.0 ± 1.41	0
Oriverutus	4	Dorylaimidae	0	0.8 ± 0.84	1.6 ± 3.05	14.2 ± 22.6
Predators						
Iotonchus	4	Iotonchidae	5.2 ± 5.26	3.2 ± 3.96	5.6 ± 3.29	0
Ironus	4	Ironidae	0.6 ± 1.34	0.6 ± 0.89	0	0
Judonchulus	4	Mononchidae	0	1.0 ± 1.22	0.4 ± 0.55	0
Mononchus	4	Mononchidae	0.2 ± 0.45	1.0 ± 1.2	0	0.2 ± 0.45
Mylonchulus	4	Mylonchulidae	0.4 ± 0.55	2.2 ± 2.28	3.6 ± 3.21	0.2 ± 0.45
Oxydirus	5	Nordiidae	0.4 ± 0.89	0	0	0
Paractinolaimus	5	Paractinolaimidae	0	0.4 ± 0.55	0	0
Prionchulus	4	Mononchidae	0.2 ± 0.45	0	0	0
Plant-feeders						
Axonchium	5	Dorylaimidae	0	1.8 ± 1.3	0	0
Helicotylenchus	3	Hoplolaimidae	3.6 ± 3.05	1.6 ± 2.61	0.2 ± 0.45	0.4 ± 0.89
Hoplolaimus	3	Hoplolaimidae	0	0.6 ± 0.89	1.6 ± 1.82	0
Longidorus	5	Longidoridae	1.2 ± 1.79	6.6 ± 14.2	0	0.2 ± 0.45
Mesocriconema	3	Pratylenchidae	7.8 ± 4.97	3.2 ± 3.42	0.2 ± 0.45	0.8 ± 1.09
Pratylenchus	3	Pratylenchidae	0.8 ± 1.3	0.2 ± 0.45	0.4 ± 0.89	2.2 ± 4.92
Rotylenchulus	3	Rotylenchulidae	0.4 ± 0.89	0	1.4 ± 1.52	0
Rotylenchus	3	Hoplolaimidae	4.6 ± 7.6	0.6 ± 0.89	0	0
Tylenchorhynchus	3	Belonolaimidae	0	11.2 ± 23.9	0.2 ± 0.45	2.8 ± 4.38
Xiphinema	5	Longidoridae	2 ± 2.34	10.6 ± 10.9	0.2 ± 0.45	0.2 ± 0.45

CHAPTER 5

Copper effects on soil nematodes and their possible impact on leaf litter decomposition: a microcosm approach

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This chapter is adapted from the publication:

Martinez, J.G., Paran, G.P., Rizon, R., De Meester, N. and Moens, T., 2016. Copper effects on soil nematodes and their possible impact on leaf litter decomposition: A microcosm approach. *European Journal of Soil Biology*, *73*, pp.1-7.

5.1. Abstract

Scientists and policy makers have to establish criteria to distinguish 'acceptable' from 'harmful' levels of pollution. Earlier studies have shown that even amounts of heavy metal pollutants well below LC50 or EC50 concentrations, can affect the fitness of individual bacterivorous nematode species, as well as the balance of the horizontal interactions between them. Species interactions are critical in shaping community structure and promoting ecosystem functions like organic matter decomposition - a key process that drives the flow of energy and nutrients in ecosystems. In this paper, we exposed two bacterial feeding soil nematodes, *Plectus acuminatus* and *Acrobeloides nanus*, to different Cu concentrations in monospecific and two-species microcosms containing leaf litter of the common grass species *Urochloa mutica* for a period of 60 days. We demonstrate that toxicant concentrations well below LC50 not only impair the fitness of the nematodes, but may also affect the interspecific interactions between them as a result of their differential sensitivity to Cu. Both *Plectus* and *Acrobeloides* are bacterial feeders and may thus affect the decomposition of leaf litter by impacting on the abundance and composition of bacteria. We observed Cu effects on the decomposition of *Urochloa*, but in the absence of data on the microbial community, it is not possible to assign these to direct effects of Cu on the bacteria or indirect effects through the Cu impacts on nematodes and their interactions.

Keywords: bacterivorous nematode, Cu, sublethal pollution, decomposition, mutual facilitation

5.2. Introduction

Heavy metals have received considerable attention in ecotoxicological studies due to their propensity to persist in the environment. The movement of heavy metals across trophic levels results in bioaccumulation (Heikens et al., 2001) and may trigger biodiversity loss (Hewitt et al., 2010) and disrupt ecosystem functions (Riess et al., 2009), which in turn can lead to a depletion of ecosystem services (Faupel and Transpaurger, 2012; McMahon et al., 2012). In particular, copper (Cu) has pronounced negative impacts not only on natural ecosystems (Korthals et al., 1998; Millward and Grant, 2000), but also in the vicinity of mining sites in developing countries. For instance, in Boac river in Marinduque province, Philippines, Cu was identified as the primary pollutant in a massive acid drainage spill, leading to the 'biological death' and the loss of ecosystem functioning of the river in 1996 (David, 2003). While the deleterious environmental effects of high loads of Cu have clearly been illustrated, heavy metal concentrations below the LC50 or EC50 have also been reported to affect populations, species interactions, and ecosystem functioning (Kammenga et al., 1997; Martinez et al., 2012; Bontje et al., 2011), even though such concentrations are often used as a basis to establish 'acceptable pollutant levels' (OECD, 1984; 1995).

Decomposition is a key process that drives the flow of energy and nutrients in ecosystems. However, decomposition rates may decrease due to heavy metal pollution (Chew et al., 2011), leading to the immobilization of essential nutrients (Parker et al., 2001) which may have reverberating effects from the lower to the upper trophic levels. While the preponderance of studies has hitherto focused on the effect of high pollution levels on ecosystem processes, studies dealing with the impact of sublethal concentrations on population fitness (Brinke et al., 2001) and species interactions (Martinez et al., 2012), and their concomitant effects on decomposition processes, remain scarce.

Nematodes possess several features which render them very suitable test organisms in pollution impact studies (Höss et al., 2006). They are ubiquitous, have relatively short generation times, occupy different trophic levels, and some soil nematode species are easy to maintain in the laboratory. Although decomposition is a largely microbially driven process, nematodes can also play a significant role in organic matter decomposition and nutrient cycling by stimulating microbial activity (Ingham et al., 1985; Bongers, 1990; Alkemade et al., 1993). Previous studies have shown that higher decomposition rates occurred in the presence of nematodes (Abrams and Mitchell, 1980; Ingham et al., 1985; Alkemade et al., 1993). Such decomposition effects of nematodes can be highly species-specific (De Mesel et al., 2003; Postma-Blaauw et al., 2005). The

effects of nematodes on decomposition processes are also affected by interspecific interactions (Postma-Blaauw et al., 2005; De Mesel et al., 2006). Horizontal interactions such as competition, facilitation and inhibition can affect nematode population development and assemblage composition (Sohlenius, 1985; dos Santos et al., 2009). The outcome of such within-trophic group interactions may be affected by pollutants. Therefore, the effects of nematodes on decomposition may also be affected by exposure to sublethal pollutant concentrations, owing to different nematode species exhibiting differential responses to toxicity (Martinez et al., 2012).

Here, we hypothesized that the effect of pollutants at concentrations below EC50/LC50 on nematode population abundances and interactions may also have a concurrent impact on decomposition process. Generally, *Plectus* species exhibit higher sensitivity to heavy metals than *Acrobeloides* sp. (Kammenga et al., 1994). In non-polluted conditions, the interaction of *A. nanus* and *P. parvus* was contramensal (+ , -), and such interaction affected bacterial biomass in soils compared to treatments with single nematode species (Postma-Blaauw et al., 2005). Hence, exposure to metal concentrations could differentially affect nematode species, and the outcome of their individual or combined response may influence leaf litter decomposition. To investigate this, we performed a microcosm experiment with two bacterial-feeding soil nematodes, *Plectus acuminatus* and *Acrobeloides nanus*, and exposed them to different Cu concentrations in monospecific and combination cultures. At the same time, we measured the decomposition rate of phytodetritus which was offered as a substratum in the microcosms.

5.3. Materials and Methods

5.3.1. Nematode cultures

The bacterial-feeding soil nematodes *Plectus acuminatus* and *Acrobeloides nanus* were obtained from the Nematology Laboratory of Ghent University, Belgium. Although males have been reported for both species, they are both generally considered parthenogenetic species (Kammenga et al., 1996; Álvarez et al., 2006), and we never observed any males in our cultures. They are general opportunists with a cp value of 2 (Bongers, 1999), and are widely distributed in many soils. They were reared in the laboratory and fed *Escherichia coli* OP50. Both nematode species can be easily distinguished from each other, even under low magnification, by the shape of their tails (Anderson, 1968; Boström, 1997). *A. nanus* have a generation time of ca. 11 days at 21 ^{0} (Sohlenius, 1973) while *P. acuminatus* develop to reproductive adults in 3.5 weeks at 20 ^{0}C (Kammenga et al., 1996).

5.3.2. Microbial inoculum

We isolated microbes from a grassland in Iligan, Philippines, where the plant material, *Urochloa mutica* was also collected. A microbial inoculum was prepared by rinsing 10 g of *U. mutica* leaves with 90 mL sterile distilled water. A nutrient broth composed of 3.0 g beef extract and 5.0 g peptone dissolved in 1 L of water was used to maintain the microbial culture. We verified the suitability of the microbial inoculum as a food source for both nematodes in a preliminary test by comparing for each species the population abundances with those of the same nematode species fed *E. coli*. After 2 weeks, both nematode species exhibited very similar population development on both microbial food sources, suggesting that the inoculum was a suitable food source. Note that the microbial inoculum is a mixture of several bacterial species and fungi, both of which participate actively in litter decomposition. Unlike bacteria, fungi probably do not function as a food source to *Acrobeloides* and *Plectus*. We measured the microbial densities with a spectrophotometer and standardized the initial abundance of the inoculum by always adding 100 μL and 200 μL of a suspension of 9.0 x 10^7 bacterial cells mL^{-1} to the single-species (SSE) and the combination experiments (CE), respectively.

5.3.3. Main experiment

The main experiment was performed in 3.5 cm and 5.5 cm diameter Petri dishes with 2.5 and 5.0 mL, respectively, of 1.5% Bacto agar (DIFCO). The differences in size of the Petri plates and the volume of the agar were designed to provide (nearly) equal space and resources per capita. Hence, single-species experiments (SSE) were carried out in the smaller plates while the combination experiments (CE) in the larger plates. For single-species experiments (SSE), ten adults of *A. nanus* or *P. acuminatus* were handpicked using a copper wire and added to the smaller plates, and a separate control set-up without nematodes was also prepared. Ten individuals each of both species were inoculated together for the combination experiment (CE). Desired sublethal concentrations of Cu were prepared from $CuSO_4.5H_2O$ (purity = 99.7%, Sigma): 0, 2, 4 and 8 ppm or 0, 31.7, 63.5 and 127.9 μM, respectively. These concentrations were based on LC50 estimates for *P. acuminatus* under Cu exposure by Kammenga and Riksen (1996): LC50 in their study equaled 3.6 ppm or 56.6 μM after 46 days.

Heavy metals were introduced to the plates and the required agar volumes were poured into the Petri plates, which were then gently shaken to homogenize the mixture. Fresh leaves of *U. mutica*, a common grass species, were washed with distilled water and air-dried (De Mesel et al., 2003) at 20 ^{0}C for 24 h. The leaves were cut in pieces approximately 1 cm in length, weighed and distributed to each of the small Petri plates (Alkemade et al., 1993). Two equally sized pieces of

leaves were added to the larger Petri plates. Afterwards, aliquots of the natural bacterial inoculum were added and incubated for 48 h at 25 ^{0}C. Microcosms were replicated four times per treatment and time (15, 35, 60 days) and were sampled destructively (4 replicates x 4 treatments x 4 concentrations x 3 moments in time = 252 plates). For an efficient collection of the nematodes, the agar was immersed in hot water (60-80 °C). Nematodes were extracted with a 10 μm sieve and preserved in 4% formaldehyde. Leaf fragments were thoroughly rinsed in tap water over the same sieve to collect nematodes that were present on the leaves. Since population fitness can be assessed from abundance data (Murray, 1985; Benton, 2000), the abundance of vermiform stages of nematodes (juveniles and adults) was counted, and in the combination cultures, they were identified to species level under a stereomicroscope at 60x magnification after 15, 35 and 60 days. The leaves, mostly intact, were carefully picked and dry-weighed using an analytical balance (Mettler Toledo). Leaf litter weight loss was determined after 15, 35 and 60 days by air-drying of the fragments at 20 ^{0}C for 24 h, using the formula:

$$\Delta \text{ wt.} = \frac{\text{dry weight}_{initial} - \text{dry weight}_{final}}{\text{dry weight}_{initial}} \times 100$$

Note that we tested differences between our air-drying procedure and a more accurate drying procedure for 48 h at 60 °C on 30 leaf fragments, and found only 2.56% (± 0.72%) difference (range from 0.8 to 3.9%).

5.3.4. Data analysis

Analyses of data were performed using PRIMER 6 version 6.1.11 with PERMANOVA+ add-on version 1.0.1 (Anderson, 2001), since the data did not meet the assumptions for parametric variance analysis. The effects of nematode treatment (SSE vs. CE), pollutant concentrations (0, 2, 4, 8 ppm) and time (15, 35 and 60 days), as well as of their first-order and second-order interactions, on population abundance and decomposition were analyzed with a three-factor PERMANOVA. When PERMANOVA indicated significant differences, posterior pairwise comparisons within PERMANOVA were conducted. PERMDISP was performed to test the homogeneity of multivariate dispersions (centroid from the mean). To allow assessment of nematode interactions in the CE, we created a mock community (MC) by summing the abundances of *P. acuminatus* and *A. nanus* from the SSE to represent the hypothesis of no species interactions. A lower or higher observed total abundance of both species in the CE compared to the mock community (MC) implies a negative or positive interaction, respectively.

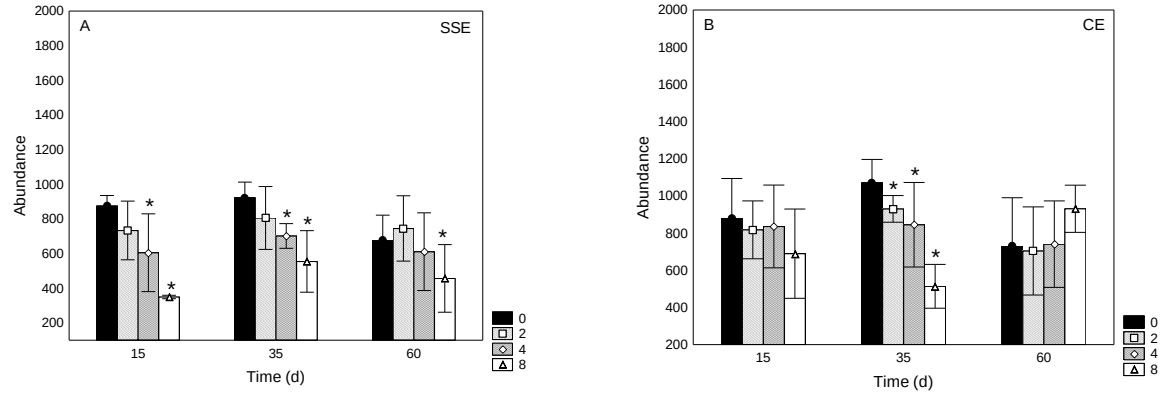
5.4 Results

5.4.1. Population abundance in monoculture and combination culture

Abundances of both nematode species were significantly affected by time, Cu concentrations and nematode treatments (all $P < 0.001$), as well as by their first-order interaction (except time x nematode treatment) and the second-order interaction (time x Cu concentration and nematode treatment). All PERMDISP values for abundances of both species indicated fairly homogenous dispersion of the data ($P > 0.05$). Abundance of *P. acuminatus* was significantly affected by time and Cu concentration (both $P < 0.05$), but not by time x Cu concentration ($P > 0.05$). During the first two sampling periods, *P. acuminatus* abundances in the SSE gradually decreased with increasing Cu concentration, an effect which largely faded at the last sampling event except at 8 ppm Cu (Fig. 5.1A). In the CE, significant negative effects of Cu on *P. acuminatus* were only observed after 35 days at 2, 4 and 8 ppm Cu (Fig. 5.1B). Towards the end of the experiment (60th day), the abundance of *P. acuminatus* in the non-polluted plates in the SSE and CE (Fig. 5.1A and B) started to decline significantly (both $P < 0.05$).

On the other hand, on the first two samplings, the abundance of *A. nanus* showed an increasing trend with increasing Cu concentration in both SSE and CE at 2, 4 and 8 ppm Cu on the 15th day, and at 8 ppm Cu on the 35th day (Fig. 5.1C and D). On the 60th day, a negative effect of Cu on *A. nanus* was observed at 8 ppm in the SSE but not in the CE, where a significantly higher abundance of *A. nanus* was observed at the highest Cu concentration compared to the control (Fig. 5.1D).

P. acuminatus



A. nanus

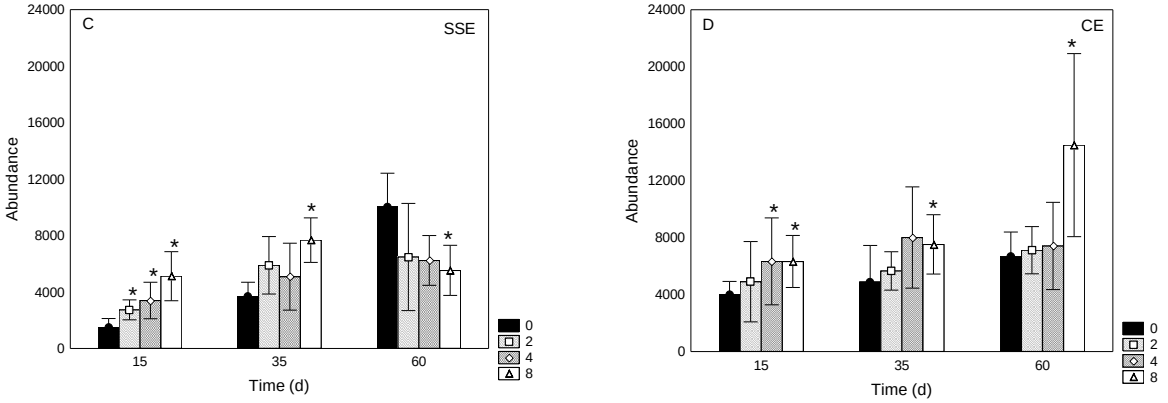


Fig. 5.1 (A-D). Nematode abundances of *Plectus acuminatus* (A and B) and *Acrobeloides nanus* (C and D) in the single-species (SSE) and combination experiments (CE) in different Cu concentrations over time. Data are means ± 1 SE of four replicates. Asterisks (*) indicate significant differences compared to the control (0 ppm) at $P < 0.05$.

Table 5.1. Three-way PERMANOVA results of *Plectus acuminatus* and *Acrobeloides nanus* abundances as a function of time (15, 35, 60 days), Cu concentration (0, 2, 4, 8 ppm) and nematode treatments (SSE, CE).

Effect	Df	*P. acuminatus*		*A. nanus*	
		F	*P*-value	F	*P* (perm)
time (ti)	2	6.03	0.003**	45.2	0.001**
Cu concentration (cu)	3	26.7	0.001**	13.6	0.001**
nematode treatment (ne)	1	36.2	0.001**	27.4	0.001**
ti x cu	6	6.23	0.001**	2.94	0.011*
ti x ne	2	0.94	0.400	1.40	0.277
cu x ne	3	4.26	0.012*	5.17	0.004**
ti x cu x ne	6	4.32	0.002**	9.40	0.001**

$P \leq 0.05^{*}$
$P \leq 0.01^{**}$

5.4.2. Species interactions

Positive interactions between *P. acuminatus* and *A. nanus* were evident on the 15th day at 0, 2 and 4 ppm Cu (all $P < 0.05$) (ESM 5). *A. nanus* benefitted from the presence of *P. acuminatus* while *P. acuminatus* remained unaffected. Such non-reciprocal interaction effect diminished at the later sampling moments and after 60 days, a negative interaction was observed in the unpolluted plates ($P < 0.05$; see ESM 5A): *A. nanus* were now less abundant in the presence of *P. acuminatus,* while *P. acuminatus* remained unaffected. In contrast, at the highest Cu pollution ($P < 0.05$), a mutual positive interaction became evident after 60 days, with the abundances of both species being significantly higher in the CE than in the SSE (ESM 5D).

5.4.3. Leaf litter decomposition

Weight loss of *U. mutica* was significantly affected by time, Cu concentration and nematode treatment (all $P < 0.01$) (Table 5.2). First-order interactions (except time x concentration) and the second-order interaction (time x concentration x treatment) also contributed significantly to the differences in decomposition of *U. mutica* leaves (Table 5.2). All PERMDISP values for decomposition of leaf litter on agar plates with *P. acuminatus*, *A. nanus*, CE (both nematode species together) and NN (no nematodes) indicated fairly homogenous dispersion of the data ($P > 0.05$). In both the *Plectus* and combination treatments, both time and Cu concentration significantly affected decomposition rate, whereas in the *Acrobeloides* and no nematode treatments, only time affected weight loss. In monoculture experiments containing *P. acuminatus*, none of the sublethal Cu concentrations affected the leaf litter decomposition in the first 15 days (Fig. 5.2A). However, prolonged exposure to Cu triggered a decrease in weight loss in many of the Cu treatments in the SSE with *P. acuminatus* compared to the unpolluted control. For instance, on the last sampling period, all polluted treatments (except at 8 ppm) showed a lower decomposition than the unpolluted control ($P < 0.05$). On the other hand, in monocultures of *A. nanus*, decomposition remained unaffected by Cu in all treatments after 15 and 35 days. A decreasing trend in weight loss at increasing Cu concentrations on the 60th day was not statistically significant ($P > 0.05$) (Fig. 2B). In the combination experiments, decomposition occurred at similar rates in the unpolluted control and in the Cu-treated plates in the first 15 days. On the 35th day, decomposition was significantly lower at 8 ppm while on the 60th day, decomposition at 2 ppm and 8 ppm was also significantly lower than in the control (Fig. 5.2C). In nematode-free plates, we generally observed no significant differences in weight loss at different pollution levels, except at 4 ppm on the 35th day (Fig. 5.2D).

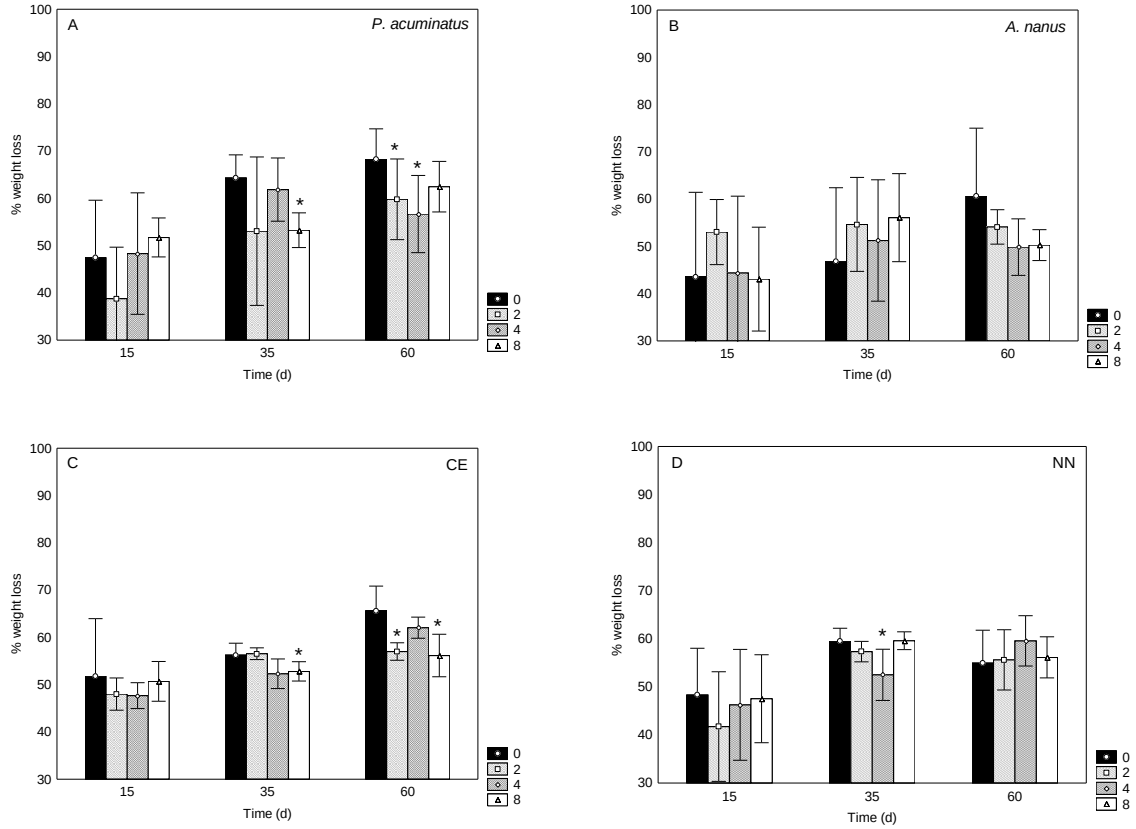


Fig. 5.2 (A-D). Weight loss of *Urochloa mutica* leaves on agar plates with *Plectus acuminatus* (A), *Acrobeloides nanus* (B), CE (combination experiment; C) and NN (no nematodes; D) over time (15, 35, 60 days) in different Cu concentrations (0, 2, 4, 8 ppm). Data are means ± 1 SE of four replicates. Asterisks (*) indicate significant differences compared to the control (0 ppm) at $P < 0.05$.

Table 5.2. Three-way PERMANOVA results of the weight loss of *Urochloa mutica* as a function of time (15, 35, 60 days), Cu concentrations (0, 2, 4, 8 ppm) and nematode treatments (SSE, CE, NN).

Effect	Df	F	*P* (perm)
time (ti)	2	79.6	0.001**
Cu concentration (co)	3	3.65	0.01**
nematode treatment (ne)	3	8.69	0.001**
ti x co	6	1.59	0.157
ti x ne	6	2.99	0.006**
co x ne	9	2.47	0.019*
ti x co x ne	18	2.59	0.002**

$P \leq 0.05^{*}$
$P \leq 0.01^{**}$

Comparison of leaf weight loss across plates with and without nematodes (NN) over time at different Cu concentrations showed the following trends: weight loss in the presence of *P. acuminatus* at the last sampling period was higher than that of the nematode-free plates (NN) at both the highest (8 ppm) and lowest (0 ppm) Cu concentration ($P < 0.05$), whereas decomposition in the presence of *A. nanus* was consistently somewhat lower than that of the

NN, at all Cu concentrations except at 0 ppm on the 60th day, but the difference was only significant at 8 ppm (Fig. 5.2). In the CE, a lower decomposition rate than that of the NN was observed at both the lowest and highest Cu concentration ($P < 0.05$) on the first 35 days. However, towards the end of the experiment, CE showed a significantly higher weight loss than the NN ($P < 0.05$), similar to the rest of the concentrations, although these did not differ significantly from the NN (Fig. 5.2).

5.5 Discussion

5.5.1. Effects of Cu on nematode abundances and interactions

5.5.1.1. Single-species experiments

Plectus acuminatus have a generation time of ca. 3.5 weeks and lay 5 to 7 eggs per day over a period of 8 weeks under conditions similar to the ones of the present experiment (Kammenga et al., 1996). Hence, under pristine condition, we expected that the 10 *P. acuminatus* adults inoculated at the start of the experiment would yield an abundance of ca. 1000 to 1,500 individuals within the first generation (after ca. 21 days). Maximal *P. acuminatus* abundances in our experiment were only slightly in excess of 900 individuals per microcosm. There was a decline in population abundance towards the end of the experiment. These maximal abundances are remarkably congruent with those obtained in previous microcosm experiments involving the congeneric species *P. parvus* and *P. aquatilis* on unpolluted agar and in aquatic sediments, respectively (Martinez et al., 2012; Gaudes et al., 2013). When compared to the expected reproductive potential, however, they are rather low, suggesting that one or more factors in the microcosms are limiting population growth. Martinez et al. (201[illegible]ttributed the decline of *P. parvus* densities after the population maximum to food depletion. This may also have played a role in the present experiment, because the leaf litter quality likely decreases during decomposition, potentially resulting in gradually lower microbial growth. However, in view of the very high abundances attained by *Acrobeloides*, it is doubtful that food limitation was the main responsible for the relatively low population peak of *Plectus* in the present experiment. *Plectus* spp. are also relatively sensitive to a variety of chemical compounds; accumulation of excretory products of nematodes and bacteria, such as ammonia, in our closed microcosms may exert toxic effects on *Plectus* spp., as suggested for monhysterid nematodes in similar microcosm setups (De Mesel et al., 2003). *Acrobeloides nanus* on the other hand, showed a continuous increase in densities throughout the experiment. *A. nanus* abundance in the present experiment was much higher than in a previous experiment (Martinez et al., 2012), and this is probably due to the differences of the experimental

set-up: the food resources (the previous experiment utilized *E. coli* and food was limited) and the heavy metal used. Furthermore, *A. nanus* reached maximal abundances more than tenfold those of *P. acuminatus* (>10,000 individuals after 60 days). In natural soils, *Acrobeloides* were also found to reach considerably higher density than *Plectus* (Wang et al., 2003). *Acrobeloides nanus* have a considerably shorter generation time and higher fecundity than *Plectus* species (Li et al., 2005), and a life span of 50 to 60 days (Álvarez et al., 2006). They are known to better tolerate unfavorable conditions (for instance in case of overcrowding or under pollution) than *Plectus* species (Bongers, 1990; Smit et al., 2002). It is therefore not surprising that, when exposed to Cu, *P. acuminatus* were more negatively affected than *A. nanus*. The higher sensitivity of *P. acuminatus* to heavy metals was consistent with existing reports (Kammenga et al., 1996; Martinez et al., 2012). In contrast, *A. nanus* abundances were positively affected by low Cu concentrations and remained high even at the highest Cu concentrations in the first 35 days (Fig. 5.1C). An initial population increase of nematodes under conditions of low to moderate pollution is not unusual (Benton and Grant, 2000; Álvarez et al., 2006; Brinke et al., 2013). Faupel and Traunspurger (2012) also observed an increase in densities of four species of bacterivorous nematodes under low contamination.

Our results in the polluted set-ups confirmed the importance of time in toxicity tests. For instance, the observed decrease of *A. nanus* in the SSE at the highest concentration after 60 days may imply that toxic effects are magnified after prolonged exposure (Fig. 5.1C). This suggests that in addition to well-known time effects in short-term toxicity assays aiming to determine LC50 or EC50 values (Heckman et al., 2010), effects at longer time scales may also be important in population-level assays with low stressor levels. This is consistent with the findings of Álvarez et al. (2006) who only found a decrease in reproduction of this species under low Cd concentrations (2 to 12 ppm) after more than two months of exposure.

5.5.1.2. Combination experiments

A mutualistic interaction, evidenced by higher abundances of both species on non-polluted CE compared to the monospecific treatments, was observed after 15 days of the non-polluted CE (ESM 5). Nematodes may have stimulated microbial activity (Traunspurger et al., 1997), particularly in the early phases of decomposition of complex organic materials (Alkemade et al., 1992; Riemann and Helmke, 2002). Such effects may be at least partly complementary between different nematode species and as a consequence, interspecific effects would be stronger than the monospecific ones (Postma-Blaauw et al., 2005). Higher microbial activity may imply higher food availability for bacterial-feeding nematodes and hence stimulate their population

development (Findlay and Tenore, 1982), leading to more abundant individuals in the CE than in the SSE on non-polluted plates (ESM 5).

Nematode effects on the microbial assemblages in microcosms may be more complex than a mere stimulation of microbial abundance: shifts in bacterial assemblage composition induced by nematodes have been observed (De Mesel et al., 2004), and a facilitative effect of a rhabditid nematode on Monhysteridae was explained by the hypothesis that overgrazing by the rhabditid reduced bacterial densities to a lower level more favourable for the Monhysteridae (dos Santos et al., 2009). In our non-polluted set-up, the mutualistic interactions soon disappeared. There was a continuous increase of *A. nanus* in the SSE unlike in the CE, where it decreased from the 35th to the 60th day (Fig. 5.1C and D), whereas the abundance of *P. acuminatus* remained the same in both set-ups (Fig. 5.1). This raises the possibility that *A. nanus* in non-polluted condition may suffer competition or inhibition by *P. acuminatus*.

Species interaction may also be affected by the presence of contaminants (Fleeger et al., 2003). Our experiment showed changes in the above-described nematode interactions as Cu concentrations increased: positive interactions on the 15th day were observed at 0, 2 and 4 ppm but faded at 8 ppm (Fig. 5.2). This observation was consistent with our previous study (Martinez et al., 2012) where species in combined cultures appeared less affected than in monocultures. This different response to toxicants between species in monocultures and combined culture may also bear on the applicability of results from single-species tests to real assemblages.

5.5.2. Decomposition

Nematodes tend to have qualitative and temporary effects on decomposition processes rather than long-lasting bulk effects, at least in closed microcosms (De Mesel et al., 2003). Nematodes can play a role in decomposition by stimulating bacterial communities: (a) microbioturbation, for instance, improves O_2 and nutrient distributions in soils and sediments (Alkemade et al., 1992; Aller and Aller, 1992; Bonaglia et al., 2014) and may increase detritus decomposition rates by as much as 30% (Alkemadi et al., 1992), (b) fragmentation of organic matter particles (Coull, 1999), (c) preventing bacteria from rapidly reaching carrying capacity, (d) excretion of nitrogen-rich compounds, which stimulate microbial growth (Ingham et al., 1985; Ferris et al., 1998), (e) influencing the composition of the microbial community (De Mesel et al., 2004; Postma-Blaauw et al., 2005; Zhou et al., 2013) and (f) dispersing microbes throughout the soil and water. Under unpolluted conditions, there is a difficult-to-explain greater positive influence of *P. acuminatus* than of *A. nanus* on decomposition. We can only speculate on the causes. The larger size and higher mobility of the former species likely translate into a greater microbioturbation as

well as transport of microbes and perhaps also into a higher *per capita* consumption and excretion. On the other hand, a higher *per capita* effect of *Plectus* could be expected to be balanced or even overturned by the more than fivefold higher abundances of *Acrobeloides*, which was not the case. It is possible that the very high abundances reached by *Acrobeloides* in the present experiment reduced the abundances of active bacteria and as such negatively impacted decomposition rates.

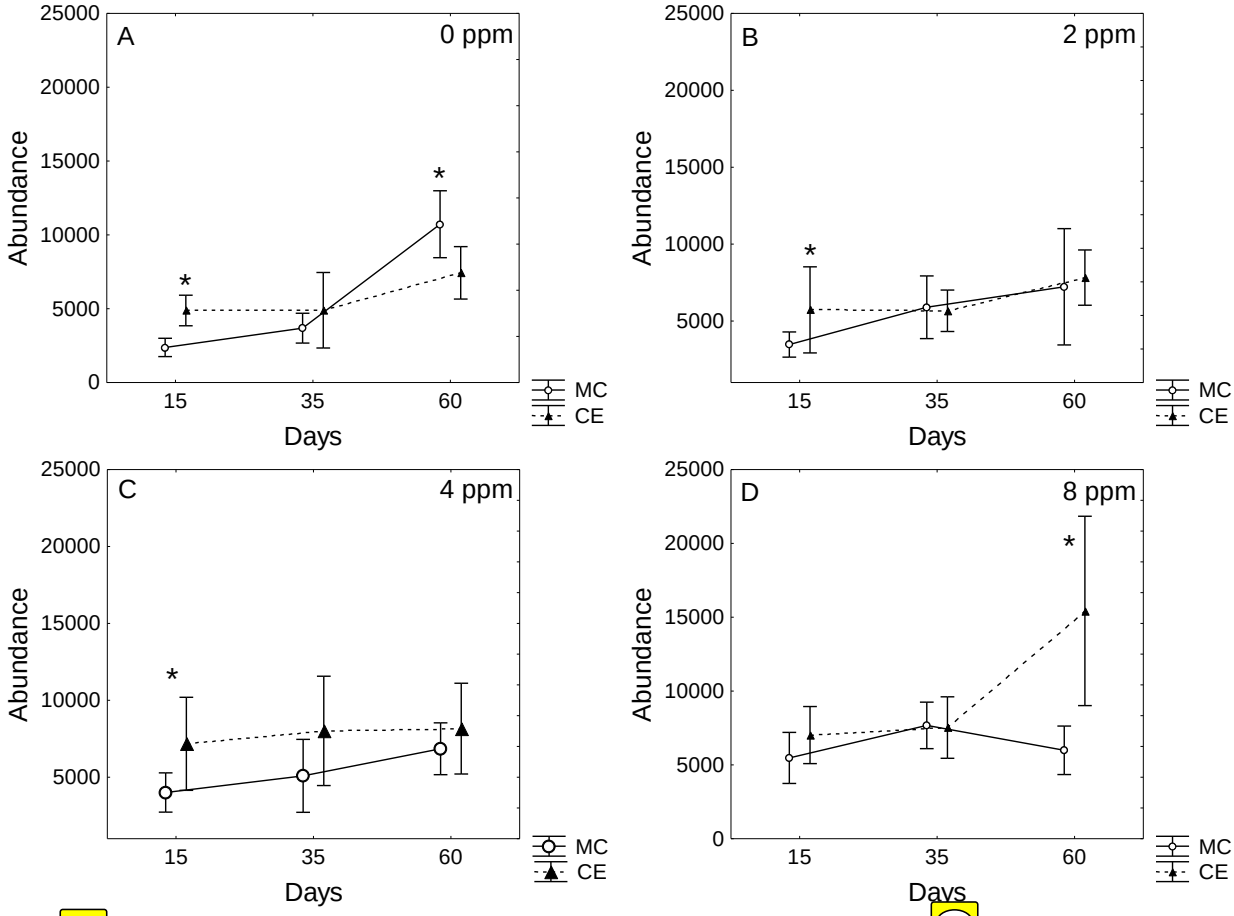
Decomposition rates have been reported to decrease in highly polluted areas (Berg et al., 1991; Nahmani and Lavelle, 2002). Effects of low concentrations of heavy metals on decomposition rates have received less attention. Our results show no significant Cu effects at all Cu concentrations after 15 days. However, effects were visible after 35 and 60 days (Fig. 5.2). Although previous studies have shown a positive correlation between nematode abundance and organic matter decomposition (Alkemade et al., 1993; Lillebo et al., 1999), such a correlation was not observed in the present study. Furthermore, in the absence of microbial information for the present experiment, caution is needed when linking lower nematode abundances due to Cu pollution to decreased decomposition rates. Since decomposition is mainly a microbially driven process, bacterial and fungal responses to Cu may have been equally or even more important than the responses of nematodes (Rajapaksha et al., 2004; Kong et al., 2006). Bacteria are often more sensitive to Cu, whereas fungi are far more tolerant, causing shifts in the balance between both under Cu contamination (Hiraki, 1994; Rajapaksha et al., 2004). According to Bååth (1989), the ultimate effect of heavy metals on microbial activity is a decreased litter decomposition. In the absence of data on microbial abundance and activity in our microcosms, it is difficult to assign any observed treatment effects to a Cu impact on bacteria or/and on nematodes. In addition, the fact that weight loss within the first 15 days of our experiment was very high compared to later on in the experiment may have further obscured more subtle differences between treatments. This fast initial weight loss is undoubtedly the result of intense and explosive microbial activity during the early stages of decomposition as a result of the pretreatment of the leaves, which resulted in a rapid leaching of proteins and carbohydrates from the leaves (Mcclaugherty and Berg, 1987; Berg et al., 1991), in combination with an overall high palatability of the *Urochloa* leaves.

5.6 Conclusions

Our results demonstrate that even relatively low concentrations of Cu can negatively impact nematode abundances, and this can be exacerbated by longer exposure time. Furthermore, the response of nematode species to pollutants in monoculture experiments differed from that of the combination setup, which may have an important implication on the applicability of results from single-species tests to real assemblages. Previous studies have already demonstrated that decomposition processes are negatively affected by severe pollution episodes. Our results suggest that even relatively low pollution levels could affect organic matter decomposition, as a result of Cu effects on nematodes and their interspecific interactions and/or on bacteria.

Electronic Supplemental Materials (ESM)

Species interactions



ESM 3 (A-D). Comparison of nematode abundances between the mock community (MC) and the combination experiment (CE) at different Cu concentrations over time. The mock community, which has no interspecies interaction, consists of the combined abundances of *P. acuminatus* and *A. nanus* from the SSE, while CE is the total nematode abundance in mixed cultures (interacting species). Data are means ± 1 SE of four replicates. Asterisks (*) indicate significant differences between nematode treatments (MC, CE) at $P < 0.05$.

CHAPTER 6

General Discussion

After the immediate closure of the 23 large-scale mining areas in 2017 due to their close proximity to watershed areas (http://newsinfo.inquirer.net/867793/denr-shuts-down-23-mining-areas), there are now approximately 18 active large-scale mining sites left (open-pit mining), 32 abandoned mine-out areas and an unaccounted number of small-scale mining operations all over the Philippines. Recently, large-scale mining activities are being subjected to strict monitoring by the government and various stakeholders, whereas small-scale mining operations often receive less government restrictions and controls. Nevertheless, both large-scale (open-pit) and small-scale mining activities have been linked to various ecological disturbances, such as mercury contamination, siltation, flashfloods and landslides (**chapter 1**). In response to the growing ecological and social problems associated with mining, the Philippine government has implemented a total ban on open-pit mining in 2017 (www.reuters.com). Rehabilitation initiatives for abandoned mining areas, on the other hand, in most cases have yet to be taken. Much as for other kinds of anthropogenic pollution or disturbance, mining related disturbances are often assessed from measurements of mere concentrations of pollutants or other chemical and physical soil parameters. A proper assessment of true impacts on soil ecosystems and their functioning, however, should not solely rely on measurements of abiotic factors, but should also include ecological impacts of disturbances, as described by Chapman (1990) through a triad approach. Along with chemical concentrations, the triad approach entails other essential components such as bioassays, which measures toxicity, and biological responses using ecological indicators, either as single species or a community of species. The ecological impacts of disturbances such as heavy metal pollution, but also the success of rehabilitation measures in restoring 'normal' soil communities and ecosystem functioning, can be assessed with the use of bio-indicators such as nematodes. Nematodes are widely used by many soil ecologists as test organisms in field studies (Bongers and Ferris, 1999; Neher, 2001), due to their ability to respond to different ecological conditions and the fact that nematodes occupy multiple trophic level through their various trophic strategies. They are also widely used in laboratory-based experiments due to their ease of handling, transparent body and relatively short life-span (Williams and Dusenbery, 1990; Höss and Williams, 2009). Nematodes perform important functions in the soil environment, e.g. through their involvement in organic matter decomposition (Yeates and Coleman, 1982; Freckman 1988; De Mesel et al., 2006) and nutrient cycling (Coleman et al., 1984; Bardgett et al., 1999).

6.1. Extracting the most out of nematode assemblages: what information can be used for indicator purposes?

Done

Our results in **chapter 2** show that small-scale mining activities in Sibutad were responsible for the alteration of several physico-chemical parameters of soil (e.g., particle size, pH and organic matter), slight increases in Cu, Pb and Zn concentrations, and a large increase in Hg concentration in soil and river ecosystems. Based on Teh et al. (2016), Hg and Pb clearly exceeded the 'acceptable' limits prescribed by UNEP (2013) and the US (Teh et al., 2016), respectively. Despite the ecological perturbations, traditional diversity and maturity indices did not show strong negative effects, contrary to the general expectation that impacted areas should be characterized by a decreased nematode diversity compared to non-impacted ones with the elimination of sensitive taxa (Yeates et al., 1995; Lee and Correa, 2007; Sánchez-Moreno and Navas, 2007; Park et al., 2011; Gutiérrez et al., 2014) and/or the increased dominance of tolerant taxa (Lambshead, 1986). Statistically, the large variability of ecological index values in **chapter 2** reduced the chance of obtaining 'significant' results between sites, and such variations were not restricted to this area, but also occurred in the nearby rehabilitated mining area (**chapter 4**). This can be the result of patchiness of environmental variables such as vegetation (pers. observation), which are not common in many, particularly non-agricultural, sampling sites. Nevertheless, other nematode community descriptor such as total nematode abundance was found to be sensitive to physical disturbance caused by small-scale mining activities (**chapter 2**) and to Hg pollution (**chapter 3**). Our results also demonstrate that detailed community composition analysis proved to be more powerful than mere indices. This was shown in the small-scale mining area (**chapter 2**) where nematode genus composition differed between the 'undisturbed' (S1 and S2) and 'disturbed' sites (S1, S2 and S3), indicative of the influence of ongoing or recent-small-scale mining activities. Genus composition also proved to be a useful indicator of Hg pollution in microcosms (**chapter 3**), of disturbances in the rehabilitated mining area which persisted for several years after rehabilitation and of soil recovery of the different rehabilitated sites after two additional years of rehabilitation (**chapter 4**).

Detailed community analysis can identify potential bio-indicators which are sensitive or tolerant to disturbance. The use of indicator taxa stems from the observation that nematode genera respond specifically to physical and nutrient/chemical disturbances (Fiscus and Neher, 2002; Georgieva et al., 2002; Heininger et al., 2007). This was substantiated by a more recent study by Zhao and Neher (2013) using data from 20 different studies who found correlations of several nematode genera to specific types of disturbances, such as the addition of synthetic and organic fertilizers and heavy metal contamination. Based on our results in small-scale mining areas, the

two free-living genera, *Iotonchus* and *Mesodorylaimus*, both cp4 nematodes, were identified as good indicator taxa in relation to mining-related disturbance, and in previous findings, *Iotonchus* and *Mesodorylaimus*, showed high sensitivity to chemical disturbance (Bongers, 1990; Chen et al., 2009), whereas *Cephalobus* may indicate high pollution levels due to their broad tolerance, consistent with previous findings (Bongers and Ferris, 1999; Bert et al., 2009).

6.2 Nematodes as bio-indicators of soil recovery in a rehabilitated mining site

A number of environmental disasters were caused by large-scale mining (**chapter 1**). With the current efforts of the present government to protect the environment (hence the immediate closure of the 23 large-scale mining operations, cancellation of permits and a total open-pit mining ban), rehabilitation of abandoned mining areas should be the next top priority (www.ptvnews.ph/denr-raises-urgency-mine-rehabilitation/), thus implementation of ecologically sound rehabilitation strategies has become increasingly important.

Traditional methods to rehabilitate mined-out area have been utilized by large-scale mining companies in Mindanao. For example, apart from the common practice of mixing the soil with organic amendments, nickel mining companies in Suriago (Hinatuan Mining Corporation and Taganito Mining Corporation) have used the fungi mycorrhiza as 'treatment' of plant roots prior to planting in mined-out areas. A similar strategy was also used in Sibutad mining site, except for the mycorrhiza addition. The outcome reflects a 'fully' successful rehabilitation of impacted areas because of the high survival rates of plants, which is the principal criterion to judge rehabilitation success. This indicates that similar strategies and criterion could be used in the rehabilitation of other mined-out areas in the future.

One of the ultimate goals of soil rehabilitation is to revive some basic ecological services lost after soil degradation (Chazdon, 2008; Boyer and Wratten, 2010). Close examination of nematode communities based on community descriptors (i.e., total abundance, diversity indices and genus composition) revealed low abundances and low diversity in the whole sampling area (including the reference site), which may be linked to the natural acidity of the soil (even the reference site had a pH of 4.0). The presence of vegetation in the rehabilitated areas (**chapter 4**) did not support full establishment of nematode communities, particularly in Sites C and D, despite 11 to 13 years of rehabilitation since 2001 and 1999, respectively, contrary to the local impression. This is probably because rehabilitation of mining degraded areas, such as open-pit mining, take some time (Mummey et al., 2002; Banning et al., 2008), depending on the degree of disturbance. Mummey et al. (2002) showed that only 20% of the total microbial biomass (represented as fatty acid methyl esters, FAME) of the undisturbed area was recovered in a

reclaimed area (surface mining) after 20 years of rehabilitation. Soil invertebrates such as collembolans started re-appearing in disturbed soils after 10 to 13 years of soil rehabilitation, whereas microarthropods reached abundance level found in undisturbed areas after 30 to 50 years of rehabilitation (Gardi et al., 2002). Compared to the present study, Site D (the most impacted site) showed roughly 6% and 30% recovery in nematode abundance and Shannon diversity index, respectively, after 13 years of rehabilitation compared to the levels found in the reference site. Nonetheless, in rehabilitated sites, an increase in total abundance, number of genera and the presence of presumedly sensitive genera after an additional two years of rehabilitation indicate that soil recovery is ongoing but slow. Our data (**chapter 4**) also suggest that recovery of nematode communities in these impacted areas may take a considerable extra time, unless relevant remediation measures in soils would be taken to address the current high Pb levels (higher than the acceptable limits by most regulatory bodies according to Teh et al. (2016)) and the deficiency of OM content, which is essential in the re-establishment of soil biota (Villar et al., 2004; de Mora et al., 2005). Although microorganisms have been widely used as indicators of soil recovery in mining sites (Ros et al., 2003; Banning et al., 2011) due to their ability to respond more quickly to changes in environmental condition (Nielsen and Winding, 2002), our study shows that nematodes can also be a promising indicator to soil recovery in surface mining, since they can also respond to changing soil conditions which can be reflected in the community descriptors and distribution of nematode genera.

Apart from field studies, nematodes also offer great potential as test organisms in laboratory-based experiments. In the present work (**chapter 5**), we tested the possible impacts of wastewater stored in a treatment facility (mining ponds) in the large-scale mining site (**chapter 4**, Fig. 4.1) on nematodes, as the private mining company plans to reuse (donate) the water for agricultural purposes to the local community (see **chapter 1**). More specifically, the study would allow us to examine metal effects not only on the fitness of soil nematodes, but on the interaction as well and their subsequent impact on organic matter decomposition. Before reusing, heavy metal contents must be checked to ensure that they are within the acceptable range for aquaculture and agricultural purposes set by the Philippine government (www.emb.gov.ph). Heavy metal analysis of the treated wastewater in 2014 revealed that As, Hg, Pb and Zn were well within the permissible levels, except for Cd and Cu. Cd, with a maximum concentration of 0.18 ppm, was 18-fold and 3.6-fold higher than those of the aquaculture and agricultural permissible limits, respectively. However, this actual concentration of 0.18 ppm was way lower compared to our previous experimental work on agar which showed Cd effects (decrease in abundance of all developmental stages) on the soil nematode *Plectus parvus* from 3

ppm onwards (Martinez et al., 2012) after 48-day exposure. On the other hand, Cu, with a maximum concentration of 5.06 ppm, was 101-fold higher than that of the aquaculture limit (no value was assigned for agricultural use) (chapter 1; Table 1.2). This actual concentration may pose a threat to the soil biota, as suggested by our previous work on agar, showing that 4 ppm Cu reduced the total abundance of the soil nematode, *Plectus acuminatus* after 15-day exposure, and probably microbes as well, which in turn reduced the decomposition rate of the leaf litter.

6.3 Limitations on the use of nematodes in pollution studies

Although nematodes proved to useful in assessing the effects of mining and soil recovery in rehabilitated mining sites in the Philippines, both in the field and under laboratory-based conditions, our study has revealed some potential limitations which should be addressed in future research. Our incomplete understanding and disagreement on the feeding habits of nematodes may seriously hamper our interpretation of nematode indices. For instance, *Tylenchus* sp., often considered as fungivores in ecological studies, may feed and reproduce on roots (Neher, 2001); 'predaceous' *Mesodorylaimus* sp. can also feed on bacteria (Russel, 1986); *Filenchus* sp. were initially thought to be plant-feeders (Yeates *et al.*, 1993), but were later found to be fungal-feeders (Brzeski, 1998; Okada *et al.*, 2002; 2005). With *Mesodorylaimus* and *Filenchus* in our data (chapter 2), this may have substantially affected the calculation of indices, such as the Index Trophic Diversity and Structure index, which rely on the feeding habits of nematodes. Unless a detailed examination is performed to establish nematode food preferences (Moens & Vincx, 1997; Moens *et al.*, 2014; Ruess et al., 2010; Weber and Traunspurger, 2013), assignment of their feeding habits would often remain ambiguous. This suggests that even closely related species may show different feeding preferences (Moens et al., 1999; Vafeiadou, et al., 2014; Deycke et al., 2016).

Aside from the observation that closely related species may differ in their feeding habits, they may also respond differently to stressors. This has an important consequence on the use of Maturity index and its related indices. Note that maturity index and its related indices follow the original scheme of assigning cp values at higher taxonomic level, such as family (Bonger, 1990). This means that all genera belonging to a certain family are assigned a common cp score since they are assumed to share similar attributes morphologically, physiologically, behaviorally or even their response to toxicants. However, this may no longer be adequate since there is now evidence that even close related species may respond differently to toxicants (Monteiro *et al.,* 2018).

6. 4. Suggestions for future research

The number of mining areas that require rehabilitation may increase in the years to come. With great ecological risks involved, rehabilitation of abandoned mining sites requires sense of urgency. Since rehabilitation of mining areas is practically costly (Berti and Cunningham, 2000), other alternative strategies may be explored to help in the soil recovery, such as the use of plants in the sequestration of heavy metals, a process known as phytoremediation (Mendez and Maier, 2008; Tangahu et al., 2011). In the present study, we have found plant species which holds promise as accumulator of arsenic and possibly of other heavy metals as well, i.e., *Pteris* sp., commonly known as ground ferns (Xie et al., 2009) found in the large-scale mining area (**chapter 4**), and also some potential plant species in the small-scale mining area (**chapter 2**). Research studies on the ability of plant to sequester heavy metals have been reportedly promising in the country (Cadiz et al., 2006); however, much focus is given on the quantity of heavy metals absorbed by plants, rather than on the effect on soil organisms after the metals are sequestered from the soil which can assessed using nematodes as indicators of soil improvement. Research work on establishing a standard permissible limit of heavy metals in soils in the Philippines may also be the way forward. While many countries have established their own permissible limits (Teh et al., 2016), Philippines continues to adapt such information from the US and EU regulatory bodies for decision-making and policy-making processes (Appleton et al., 2006). The fact that these values were obtained through routine testing based on the country's environmental conditions (e.g., soil and climatic conditions) makes it problematic. We demonstrated in **chapter 3** that differences in soil factors such as OM, pH, particles size and vegetation may lead to discrepancy between Hg effects in the field and that of the mesocosm.

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Publications

- Martinez, J.G., dos Santos, G., Derycke, S. and Moens, T., 2012. Effects of cadmium on the fitness of, and interactions between, two bacterivorous nematode species. *Applied Soil Ecology*, *56*, pp.10-18.

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- Martinez, J.G., Quiobe, S. and Moens, T. Effects of mercury (Hg) on soil nematodes: a microcosm approach' (*Ecotoxicology and Environmental Safety*, **submitted**).

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