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Nematodes as indicators of the effect of soil fumigants on soil functioning

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Marina Carrascosa Cotera

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Autora:

Marina Carrascosa Cotera

Ingeniero Agrónomo

Directores:

José Luis Alonso Prados

Ingeniero Agrónomo

Doctor Ingeniero Agrónomo

Sara Sánchez Moreno

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“Lo importante es no dejar de hacerse preguntas”
Albert Einstein

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Resumen

Debido al futuro incierto de la mayor parte de los fumigantes edáficos usados actualmente en la Unión Europea, que pueden implicar riesgos para la salud humana/animal y el medio ambiente, es necesario desarrollar programas de manejo integrado para el control de plagas de cultivos. Estos programas se incluyen como obligatorios en el Reglamento (EC) No. 1107/2009. De acuerdo con este Reglamento, es obligatoria la evaluación del riesgo asociado al uso de productos fitosanitarios sobre los organismos edáficos no diana y sus funciones, además de llevar a cabo ensayos con diferentes especies indicadoras para obtener datos de toxicidad que puedan ser usados posteriormente en la evaluación de riesgo. Sin embargo, la baja representatividad de algunas de estas especies indicadoras en el área Mediterránea supone una gran limitación. En esta situación, el Panel Científico de Productos Fitosanitarios y sus Residuos de la Autoridad Europea en Seguridad Alimentaria (EFSA), ha señalado la necesidad de modificar los datos ecotoxicológicos requeridos para evaluar los efectos adversos de los productos fitosanitarios de una manera más integrada, incluyendo criterios funcionales y estructurales mediante organismos como bacterias, hongos, protozoos y nematodos. De este modo, la EFSA ha recomendado el uso de los nematodos en la evaluación de la funcionalidad y estructura del suelo. Los nematodos están globalmente distribuidos y son morfológicamente diversos; esto junto con su gran abundancia y diversidad de respuestas a las perturbaciones edáficas, los convierte en indicadores adecuados del estado del suelo. Puesto que los nematodos interaccionan con muchos otros organismos que participan en diferentes eslabones de la red trófica edáfica, jugando papeles importantes en procesos edáficos esenciales en los agroescosistemas, la diversidad de nematodos es, a menudo, usada como indicador biológico de los efectos de las prácticas agrícolas en el estado del suelo. En los últimos años, diferentes índices basados en la comunidad nematológica han facilitado la interpretación de datos complejos sobre la ecología del suelo. Los índices de la red trófica edáfica, basados en la abundancia de grupos funcionales definidos como grupos C-P y grupos tróficos, permiten la evaluación de la funcionalidad de la red trófica edáfica. Por otra parte, la dificultad en la identificación taxonómica de nematodos para explicar su uso limitado como indicadores ecológicos, es ampliamente discutida, y existe cierta controversia en cuanto a la eficacia de los diferentes métodos de identificación de nematodos. Se argumenta que la identificación morfológica es difícil y puede llevar mucho tiempo debido a la falta de expertos especializados, y se afirma que las técnicas moleculares pueden resolver algunas limitaciones de las técnicas morfológicas como la identificación de juveniles. Sin embargo, los métodos de identificación molecular tienen también limitaciones; la mayoría de las bases de datos de

secuencias de ADN están fuertemente orientadas hacia los nematodos fitoparásitos, los cuales representan sólo una parte de la comunidad edáfica de nematodos, mientras que hay poca información disponible de nematodos de vida libre a pesar de representar la mayoría de los nematodos edáficos. Este trabajo se centra en el estudio de los efectos de fumigantes edáficos en la funcionalidad del suelo a través del uso de diferentes indicadores basados en la comunidad de nematodos, como los índices de la red trófica, índices de diversidad, abundancia de los taxones más relevantes etc. También se han analizado otros indicadores funcionales relacionados con la supresividad edáfica, el ciclo de nutrientes o la actividad de la microfauna del suelo. En el capítulo 1, la diversidad de nematodos estudiada en una explotación comercial de fresa y sus alrededores durante dos campañas consecutivas en el suroeste español, fue baja en los suelos fumigados con fumigantes químicos ambas campañas y, aunque se observó una recuperación a lo largo de la campaña en la zona tratada, los suelos fumigados mostraron una condición perturbada permanente. La comunidad de nematodos estuvo más asociada al ciclo de nutrientes en la zona sin cultivar que en los suelos cultivados, y se observó poca relación entre la biomasa de las plantas y la estructura de la comunidad de nematodos. Los surcos sin tratar dentro de la zona de cultivo funcionaron como reservorio tanto de nematodos fitoparásitos como beneficiosos; sin embargo estas diferencias entre los surcos y los lomos de cultivo no fueron suficientes para mantener la supresividad edáfica en los surcos. Los suelos tratados fueron menos supresivos que los suelos sin tratar, y se observaron correlaciones positivas entre la supresividad edáfica y la estructura de la red trófica edáfica y la diversidad de nematodos. En el capítulo 2, se evaluaron los efectos de dos pesticidas orgánicos con efecto nematicida y dos nematicidas convencionales sobre las propiedades físico químicas del suelo, la diversidad de nematodos y la biomasa de las plantas en condiciones experimentales en dos tipos de suelo: suelos agrícolas poco diversos y suelos provenientes de una zona de vegetación natural muy diversos. El mayor efecto se observó en el tratamiento con neem, el cual indujo un gran incremento en el número de dauerlarvas en los suelos pobres en nutrientes, mientras que el mismo tratamiento indujo un incremento de poblaciones de nematodos bacterívoros, más estables y menos oportunistas, en los suelos del pinar ricos en materia orgánica. En el capítulo 3, se comparó la eficacia de métodos moleculares (TRFLP, Terminal Restriction Fragment Length Polymorphism) y morfológicos (microscopía de alta resolución) para la identificación de diferentes comunidades denematodos de España e Irlanda. Se compararon estadísticamente las diferencias y similitudes en la diversidad de nematodos, otros indicadores ecológicos y de la red trófica edáfica. Las identificaciones mediante el uso de TRFLP sólo detectó un porcentaje de los taxones presentes en las muestras de suelo identificadas morfológicamente, y los nematodos omnívoros y predadores no fueron detectados

molecularmente en nuestro estudio. Los índices calculados en base a los nematodos micróboros mostraron más similitud cuando se identificaron morfológica y molecularmente que los índices basados en grupos tróficos más altos. Nuestros resultados muestran que, al menos con la técnica usada en este estudio, la identificación morfológica de nematodos es una herramienta fiable y más precisa que la identificación molecular, puesto que en general se obtiene una mayor resolución en la identificación de nematodos. En el capítulo 4, se estudiaron también los efectos de los nematicidas químicos sobre la comunidad de nematodos y la biomasa de las plantas en condiciones experimentales de campo, donde se aplicaron en una rotación de cultivo judía-col durante un ciclo de cultivo. Se aplicaron dos tipos de enmiendas orgánicas con el objetivo de mitigar el efecto negativo de los productos fitosanitarios sobre la diversidad edáfica. El efecto de los nematicidas sobre las propiedades del suelo y sobre la comunidad de nematodos fue más agudo que el efecto de las enmiendas. La incorporación de los restos de cosecha al final del ciclo de cultivo de la judía tuvo un gran efecto sobre la comunidad de nematodos, y aunque el número total de nematodos incrementó al final del experimento, se observó una condición perturbada permanente de la red trófica edáfica a lo largo del experimento.

Abstract

Due to the uncertain future of the soil fumigants most commonly used in the EU, that might involve risks for human/animal health and the environment, there is a need to develop new integrated pest management programs, included as mandatory in the Regulation (EC) No. 1107/2009, to control crop diseases. According to this Regulation, evaluating the risk associated to the use of the plant production products (PPP) on non-target soil fauna and their function, and developing assays with different indicator species to obtain toxicity data to be used in the risk evaluation is mandatory. However, the low representativeness of some of these indicator species in the Mediterranean area is a relevant limitation. In this situation, the Scientific Panel of Plant Protection Products and their Residues of the European Food Safety Authority (EFSA) has pointed out the necessity of modifying the ecotoxicological data set required to evaluate non-target effects of PPP in a more integrated way, including structural and functional endpoints with organism such as bacteria, fungi, protists and nematodes. Thus, EFSA has recommended the use of nematodes in the assessment of the functional and structural features of the soil. Nematodes are globally distributed and morphologically diverse, and due to their high abundance and diversity of responses to soil disturbance, they are suitable indicators of the soil condition. Since nematodes interact with many other organisms as participants in several links of the soil food web, playing important roles in essential soil processes in agroecosystems, nematode diversity is often used as a biological indicator of the effects of agricultural practices on soil condition. In the last years, various indices based on soil nematode assemblages, have facilitated the interpretation of complex soil ecological data. Soil food web indices based on the abundances of functional guilds defined by C-P groups and trophic groups, permit evaluating soil food web functioning. On the other hand, the difficulty of nematode taxonomical identification is commonly argued to explain their limited used as ecological indicators, and there is a certain controversy in terms of the efficacy of various nematode identification methods. It is argued that the morphological identification is difficult and time consuming due to the lack of specialist knowledge, and it is claimed that molecular techniques can solve some limitations of morphological techniques such as the identification of juveniles. Nevertheless, molecular identification methods are limited too, since most of the DNA-based databases are strongly oriented towards plant-parasitic nematodes that represent only a fraction of the soil nematode community, while there is little information available on free-living nematodes, which represent most soil nematodes. This work focuses on the study of the effects of soil fumigants on soil functioning through the use of different indicators based on soil nematode community as soil food web indices, diversity indices, the abundance of

more relevant taxa etc. Other functional indicators related to soil suppressiveness, nutrient cycling, or the activity of soil microfauna have been also studied. In chapter 1, nematode diversity assessed in a commercial strawberry farm and its surroundings for two consecutive growing seasons in southern Spain, was low in fumigated soils with chemical pesticides throughout both seasons and, although yearly recovery occurred within the treated fields, fumigated soils showed a permanent perturbed condition. The nematode community was more closely associated to nutrient cycling in the non-cropped than in the cropped soils, and the link between plant biomass and nematode community structure was weak. Non-treated furrows within the treated fields were a reservoir of both beneficial and plant-parasitic nematodes, but such difference between furrows and beds was not enough to maintain more suppressive soil assemblages in the furrows. Treated soils were less suppressive than unmanaged soils, and there was a positive and significant correlation between soil suppressiveness and soil food web structure and diversity. In chapter 2, the effects of two organic pesticides with nematicide effect and two chemical nematicides on soil physical-chemical properties, soil nematode diversity and plant biomass in experimental conditions were assessed in two types of soils: low diversity soils from an agricultural farm, and high diversity soils from a natural vegetation area. The larger effect was observed on the neem treatment, which induced a large boost of dauer juveniles in the nutrient-depleted soil, while the same treatment induced the increase of more stable, less opportunistic, populations of generalist bacterivore nematodes in the pine forest soil, rich in organic matter. In chapter 3, comparison of the efficiency of molecular (TRFLP, Terminal Restriction Fragment Length Polymorphism) and morphological (microscopy at high magnification) identification methods was carried out in different nematode communities from five sites of different land uses in Spain and Ireland. Differences and similarities on nematode diversity and other ecological and soil food web indices assessed by both methods, were statistically compared. Molecular identification with TRFLP only detected a percentage of the taxa present in the soil samples identified morphologically, and omnivores and predators were not detected molecularly in our study. Indices involving microbial feeding nematodes were more similar between identification methods than indices involving higher trophic links. Our results show that, at least with the technique used in this study, identifying nematodes morphologically is a reliable and more precise identification tool than molecular identification, since a higher taxonomic resolution is in general obtained compared to TRFLP. In chapter 4, the effect of chemical nematicides on nematode community descriptors and plant biomass was also studied in field conditions in an experimental area in which dazomet and dimethyl disulfide was applied in a bean-cabbage rotation system for a single season. Organic amendments were incorporated into the soil with

the aim of mitigate the negative effect of the pesticides on soil diversity. The effect of the nematicides was much more noticeable than the effect of the amendments on soil properties and nematode community descriptors. The incorporation of bean crop residues into the soil at the end of bean crop cycle affected soil nematode community descriptors to a great extent, and although total number of nematodes increased at the end of the experiment, a permanent perturbed soil food web condition was observed along the experiment.

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GENERAL INTRODUCTION

AGRICULTURE IN SPAIN

Spain has been traditionally, an agricultural country, in which agriculture has been one of the fundamental economical supports along ages, and continues being in the present. Currently, around 5% of the Spanish active population works in agriculture (INE, 2013). Spain possesses different agroecosystems especially relevant economically, socially, and environmentally, presenting biotic and physical components, which interact as a system. These systems must be sustainable, maintaining the production in the long term, stables, being permanent according to the agricultural management, environmental conditions and economic pressures, and equitable, promoting equal conditions between producers and intermediaries.

Spain possesses the largest olive surface in the world (E.C., 2012); it is one of the most important cereal producers (MARM, 2011), and the second producer of horticultural crops within the European Union (EU), reaching more than 13 million tons of annual production and a total surface around 350.000 ha, of which 280.000 correspond to outdoor cultivation and 66.000 to protected growing crops (MARM, 2013). In addition, Spain possess the largest surface of vineyard within the EU, reaching 1 million hectares (data from 2012, last data available, EUROSTAT, 2015), and the largest surface of protected crops, with more than 60.000 hectares (in 2009, last data available) (EUROSTAT, 2015). Other minor crops, as the cultivation of cut flowers, are increasing in the last years (MARM, 2013). Spain is the main strawberry producer within the EU, being Huelva one of the principal areas around the world reaching 275.400 tons of strawberry production, with a yield of 36.400 kg $(ha)^{-1}$ and a cultivated surface of around 7.600 hectares, of which 6.800 hectares correspond to outdoor cultivation and 800 to protected growing crops in 2010 (MARM, 2011). In 2011, Spain reached a total production of 262.900 tons, with a yield of 38.100 kg $(ha)^{-1}$ and a cultivated surface of 6.900 hectares (MARM, 2012).

PLANT PROTECTION PRODUCTS

The use of plant protection products (PPP) in agriculture has involved an increase in the quantity and quality of yields, and a better control of pests, diseases and weeds with a positive effect on crop production. The use of PPP in Spain reached 39.000 tons of active substances in 2010 (MARM, 2011). Among PPP, soil fumigants are essential to maintain under the threshold for crop damage, fungi populations (such as *Phytophtora* spp*., Fusarium* spp.), plant-parasitic

nematodes (such as *Meloidogyne* spp. o *Pratylenchus* spp.) and weeds (such as *Cyperus rotundus* L.). Methyl bromide (MeBr) was widely used to control numerous diseases and pests, not only as soil fumigant and sterilizing but as insecticide, acaricide, herbicide and disinfectant until its prohibition by the Montreal Protocol (UNEP, 1987), implemented in Europe by the Regulation CE 2037/2000 (E.C., 2000). After the phasing out of MeBr, due to its negative effects on the stratospheric ozone, much effort has been invested in the development of alternatives to the use of soil fumigants (Fennimore *et al.*, 2008; López-Aranda *et al.*, 2009b; Mao *et al.*, 2012). Two of the alternatives considered have been the use of 1,3-dichloropropen (1,3-D) and chloropicrin (Pic), which were widely used in horticultural crops, and became essential substances on the maintenance, e.g, of the strawberry commercial production in southern Spain (Medina *et al.*, 2006; Porter *et al.*, 2006; López-Aranda *et al.*, 2009a). However, these fumigants present uncertain effects on the environment (Yates *et al.*, 2011) and their commercialization has not been approved by the EU (E.C., 2011a, d). The use of other soil fumigants as dazomet, metam (Na and K) are currently permitted in the European Union (EU) (E.C., 2011b) and they are efficient alternatives to the use of methyl bromide in different crops (López-Aranda *et al.*, 2009a; López-Aranda *et al.*, 2009b; Ceustermans *et al.*, 2010; Pizano *et al.*, 2010). Besides, the use of other active substances with nematicide, fungicide and/or insecticide effect such as *Bacillus firmus* I-1582, etoprophos, fenamiphos, fosthiazate, iprodione, oxamyl and *Paecilomyces lilacinus* cepa 251 (E.C., 2011b) is now possible.

One of the main objectives of the common agricultural policy is the reduction of the negative impacts of agricultural activity on the environment, including the effects of the plant production products. In the EU, there was not a harmonized framework, for all EU Member States, for the authorization and placing on the market of plant protection products until the publication and implementation of the Directive 91/414/CEE, relative to the commercialization of plant protection products (E.C., 1991). According to this Directive, assessment and decision-making criteria for the authorization of the commercialization and placing on the market of plant protection products and active substances were established in the EU. This Directive established the necessity of protect human and animal health and the environment, and considered the use of plant protection products one of the most important methods to protect crops and improve agricultural production. This Directive established, for the first time, the Uniform Principles to be applied in the EU Member States for the evaluation and authorization of plant protection products (Directive 97/57/CEE and Directive 2005/25) (E.C., 1997, E.C., 2005). It forces the European Commission to start a revision program of active substances that

were commercialized in Europe before 1993 according to the previous principles, established by the article 8 of the Directive 91/414/CEE. As a result of this program, 67% of the substances were removed from the market due to the lack of interest of producing companies or because these companies presented uncompleted dossiers. 26% were approved and 7% were not approved after their evaluation, as they did not meet the uniform principles established, and their use was not considered safe for human health and/or environment.

The current Regulation 1107/2009 (E.C., 2009a), which derogates the Directive 91/414/CEE, increases the requirements for the adoption of active substances in the European Union, through the establishment of the "cut-off criteria". These criteria, indicated in the points 3.6.2 a 3.6.4 y 3.7 of the Annex II of the Regulation, are based on the hazard of the active substances. The Regulation demands, for the authorization of a plant protection product, that both the product and the residues derived from an application according to good agricultural practices not having negative effects on human or animal health, and the environment. To evaluate their effects on the environment it is necessary to know their impact on the environment and the ecosystem, such as the destination and distribution of the active substances and their degradation products, and the breakdown products and their effects on non-target fauna. In addition, products must be efficient enough, not having unacceptable effects on vegetables and do not have to cause any suffering on target vertebrates. All these requirements must be evaluated according to the uniform principles established by the Regulation 546/2011 (E.C., 2011c). This Regulation requires evaluating the risk of the use of plant protection products according to good agricultural practices, on non-target fauna and their function, such as soil fauna. The objective of this evaluation is to check that soil functioning to agricultural production remains after the use of PPP, without jeopardize future uses. To carry out the evaluation, various assays carried with the American red earthworm (*Eisenia fetida*), the collembolan *Folsomia candida,* the mite *Hypoaspis acueleifer* and the assessment of C and N mineralization, are required to obtain toxicity data that will be used in the risk assessment. However, the low representativeness of these organisms in the Mediterranean soil fauna is a fundamental limitation when carrying out these assays. The Scientific Panel of Plant protection products and the European Food Safety Authority (EFSA) has pointed out the necessity of modifying the ecotoxicological data set required to evaluate non-target effects of PPP in a more integrated way, including structural and functional endpoints with organism such as bacteria, fungi, protists a and nematodes (SCTEE, 2000). Thus,

the EFSA has recommended the use of nematodes in the assessment of the functional and structural features of the soil (EFSA, 2007).

According to the European Directive on sustainable use of PPP (E.C, 2009b), the massive consume of pesticides must be reduced in order to build agricultural systems more sustainable environmentally. To reach the objectives set by this Directive, all EU Member States are forced to develop the National Action Plan (NAP) (MAGRAMA, 2012). Through the elaboration of such plan to accomplish under the Spanish conditions the objectives of this Directive, the implementation of integrated agricultural pest management programs in Spanish agricultural systems is promoted as an alternative to the intensive use of PPP. Integrated pest management includes the implementation of different physical, mechanical, chemical, biological and genetic techniques, including the use of organic amendments, crop rotation or the use of tolerant or resistant varieties (E.C., 2009b), with the objective of reducing the use of PPP and minimizing their impact on the environment, maximizing at the same time crop productivity. In the last years, in the strawberry production area in southern Spain (Huelva), non-chemical alternatives to the use of some PPP as solarization have been developed (Medina *et al.*, 2009; Medina-Mínguez *et al.*, 2011).

SOIL BIODIVERSITY AND NEMATODES AS INDICATORS OF SOIL FUNCTIONING

Soil biodiversity

Since all ecosystems, including agroecosystems, are subjected to heavy anthropic pressures their study should be focused holistically. Soil plays a fundamental role in ecosystems, hosting thousands of species of organisms that develop fundamental functions involved in ecosystem sustainability. In agricultural production systems, sustainability involves environmentally friendly technology and agricultural practices, accessible and profitable for farmers, and increase agricultural production. In addition, both agricultural yield and the sustainable use of natural resources should be understood together in order to maintain and protect biological diversity, to conserve ecosystems and to promote the use of renewable raw materials (Pretty, 2008). Soil organisms are responsible of most of the soil services and ecological functions, such as carbon sequestration, organic matter decomposition, soil pest control (suppressiveness) and the availability of nutrients to plants (Minns *et al.*, 2001). Soils are not only a no renewable resource, but dynamic systems fundamental to control pest species, and one of the principal responsible of carbon immobilization in the world (Batjes, 1996).

Soil fauna is involved in nutrient cycling mineralizing organic molecules to inorganic bioavailable compounds, increasing its availability to plants and enhancing their growth (Wardle, 1999). An adequate agricultural management, such as reduced tillage or the use of mulching (Costa *et al.*, 2013; Sanderson *et al.*, 2013), could compensate, at least partially, soil carbon loss induced by agricultural activity (Vaccari *et al.*, 2012), due fundamentally to the loss of biomass as a consequence of harvesting. With such techniques, that enhance populations of soil organisms involved in carbon storage, soil loss, nutrient leaching, and the excessive use of agricultural inputs is reduced.

There are numerous hypothesis regarding the relationships between species diversity and ecosystem functioning. Some evidence suggests that soil biodiversity confers stability against stress and disturbance. However, the mechanism that explains how it occurs is not well understood, and such relationship may depend on the type of stress or perturbation to which the system is submitted (Fitter *et al.*, 2005; Brussaard *et al.*, 2007). Some authors consider that high species diversity is important to increase soil functioning, since all species living in the soil significantly contributes to ecosystem functioning (Fitter *et al.*, 2005; Smuckler *et al.*, 2010). However, other authors consider that species can be functionally redundant, developing the same function in the ecosystems, so ecosystem functioning would not be affected by changes on specie biodiversity (Loreau, 2004).

One of the most relevant ecosystem services is the ability of the soil to control pest species and diseases. Both human-induce and intrinsic, soil suppressiveness has been used to fight numerous disease-causing agents such as *Fusarium oxysporum* and *Meloidogyne* spp. (Kloepper *et al.*, 1980; Rodríguez-Kabana *et al.*, 1987). Several organisms are involved in soil suppressiveness (Meyer & Roberts, 2002; Borneman & Becker, 2007), such as arthropods (Moerkens *et al.*, 2012), tardigrades (Sánchez-Moreno *et al.*, 2008a) or nematodes, such as entomopathogenic nematodes that belong to the Rhabditidae family (Campos-Herrera & Guitiérrez, 2009).

Nematodes as indicators of soil functioning

Soil nematodes (phylum Nematoda) are ubiquitous and diverse, and are present in terrestrial and marine systems (Bongers & Ferris, 1999). According to some estimates, four out of every five soil metazoans are nematodes. In agricultural soils, nematode community is usually composed by up to 50 genera belonging to different trophic groups, as they can feed on bacteria, fungi, protists, algae, and other nematodes that can be migratory or sedentary parasites of plants (plant-parasitic nematodes) (Yeates & Bongers, 1999). In addition, they are very abundant and may reach densities of more than a million of individuals by square meter of soil. Nematodes live inside the roots and in the water films around soil particles, permitting them to be in direct contact with soil solutes including contaminants through their cuticle (Fig. GI.1).

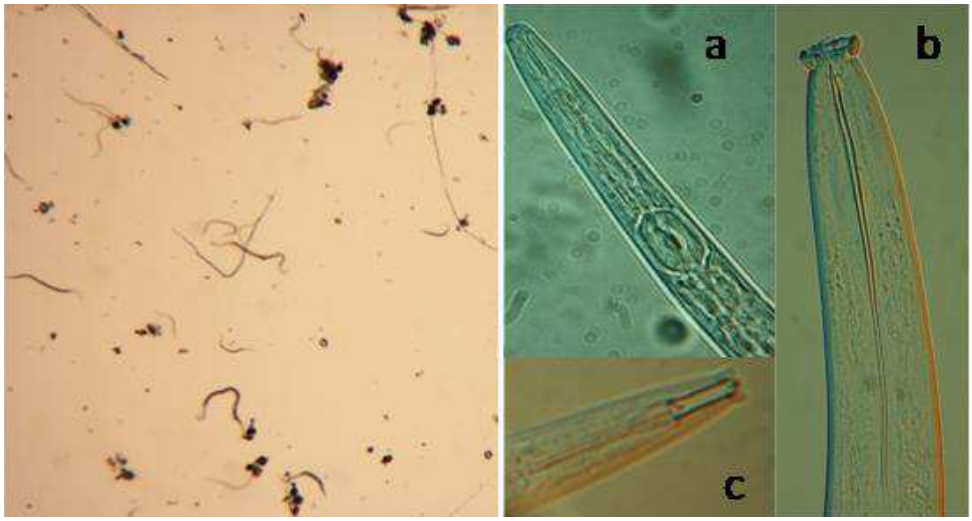


Fig. GI.1. Nematode suspension extracted from soil, under a binocular microscope (20x) (left) and head region of three nematodes (400x) (right)***:*** **a**, fungivores nematode (*Aphelenchus* spp.),**b,** predatory nematode (*Discolaimus* spp.), and **c**, bacterivore nematode (*Mesorhabditis* spp.*)*.

Different indices based on the soil nematode community that have been developed in the last years have facilitated the interpretation of complex data sets difficult to analyze. Nematodes are characterized by different feeding habits and a wide variety of life cycles strategies, and are classified according to the colonizer-persister (C-P value) scale (Bongers & Bongers, 1998). Nematodes with the same C-P value (values between 1, enrichment opportunistic and r strategists-species characterized by high reproduction rates, high mortality rates and acute population decreases in adverse conditions- and 5- predators and omnivores very sensitive to disturbance) share similar life cycles and number and size of eggs, and alike responses to

disturbance. Soil food web indices (Ferris *et al.*, 2001) are based on the abundances of functional groups defined by C-P and trophic groups, and permit to evaluate the functionality of the soil food web. The Structure Index (SI) is an indicator of the connectivity of the web and its suppressiveness capacity; the Enrichment Index (EI) indicates the degree of soil organic enrichment and it is based on the prevalence of enrichment opportunistic bacterivore nematodes; the Channel Index (CI) is based on the ratio fungivores:bacterivores that are involved in organic matter decomposition; recalcitrant organic matter is decomposed by fungi, increasing the accumulation of organic matter rich in humic acids that permit to resist to a posterior degradation, while bacteria are responsible of labile organic matter decomposition, such as simple carbon compounds as root exudates and fresh plant residues. The Basal Index (BI) is based on the abundance of general opportunistic as indicators of soil functional perturbation (Fig. GI.2). Therefore, nematodes are excellent indicators of soil health (Neher, 2001), and they are considered an useful tool to evaluate the responses of taxonomic diversity and agroecosystem functioning to disturbance, as consequence of agricultural management, such as the use of pesticides (Bongers, 1990; Ekschmitt *et al.*, 2001; Ferris & Bongers, 2006; Sánchez-Moreno *et al.*, 2010; Ugarte *et al.*, 2013).

Ecosystem biodiversity and the services it provides should be incorporated in the risk assessment of plant protection products, in order to carry out an evaluation in an integrated way, selecting reliable and informative *endpoints* about ecosystem functions (Schafer, 2012). The effect of plant protection products on soil organisms, such as earthworms, has been widely studied. The relevance of the earthworm responses to the use of plant protection products have been studied individually and on gene expression and physiology, evaluating different indices as their density and biomass (Pelosi *et al.*, 2014). EFSA has recommended the use of the indices previously described based on the nematode community in the assessment of the functional and structural features of the soil. With their inclusion as structural and functional *endpoints*, ecotoxicological data sets required to evaluate non-target effects of PPP in a more integrated way will be modified.

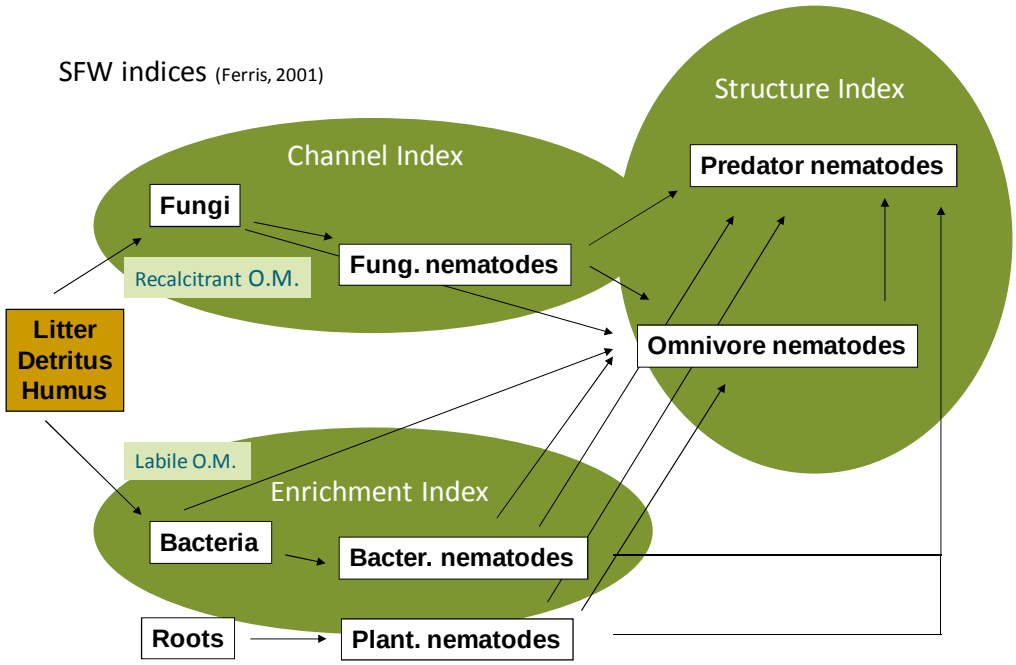


Fig. GI.2. Diagram of soil food web indices (Ferris *et al*., 2001), in which trophic connections are indicated by arrows (arrows are pointed towards the consumers) O.M: organic matter.

There is a certain controversy in terms of the efficacy of the various soil nematode identification methods available. Some authors argue that morphological identification through microscopic analysis to the species level is difficult and time consuming due to the lack of specialist knowledge and the necessity of taxonomically competent people (Coomans, 2002; Floyd *et al.*, 2002; Griffiths *et al.*, 2006; Chen *et al.*, 2010). However, if nematodes are conserved alive in water (at 4ºC), their anatomical structures are clear and morphological identifications are reliable. In addition, morphological identification methods permit to obtain results over a relatively short period of time with affordability. Identifying nematodes to species level is time consuming, but in nematode ecology studies a community level analysis is required, as opposed to the identification of single nematode taxa. The identification to genus or family level is commonly enough to infer the global behaviour of the soil nematode community. In the case of requiring nematode identifications to species level, morphological identification presents, indeed, some limitations. For instance, identification of species is only possible from adult specimens who do not represent the entire nematode assemblage in a soil sample (Griffiths *et al.*, 2002), a limitation overcame by molecular methods. Thus, many authors consider that molecular identification methods such as DNA barcoding or sequencing, PCR-DGGE, PCR-TRFLP and Real-Time PCR (Chen *et al*., 2010) are a more effective, quick and cheap than morphological identification methods identifying nematode communities. There is a wide knowledge of plant-parasitic nematodes, due to the crop economical losses, compared

to free living nematodes, in spite of being the principal components of the soil nematode community (Neher, 2010). Therefore, molecular databases are composed mainly by genetic sequences of plant-parasitic nematodes, which make difficult to identify free living nematodes being a limitation when using molecular techniques to soil nematode identification.

OBJECTIVES

GENERAL OBJECTIVE

The objective of this thesis is the study of the effects of soil fumigants on soil functioning and the identification of soil indicators of these effects based on the nematode community.

SPECIFIC OBJECTIVES

- To study the relationships between nematode diversity and plant growth, nutrient cycling, soil suppressiveness and microfauna activity in agricultural soils fumigated with different products in two crops under field conditions and experimental controlled conditions in laboratory. **Chapters 1 and 2**.

- To study the degree of similarity and difference among nematode communities identified with morphological and molecular methods, and to evaluate the degree of accuracy of each identification method. **Chapter 3.**

- To study the effects of soil fumigants on soil diversity and functioning, and plant biomass and the mitigating effects of organic amendments on the undesirable effects of the use of fumigants in experimental field conditions. **Chapter 4**.

CHAPTER 1. RELATIONSHIPS BETWEEN NEMATODE DIVERSITY, PLANT BIOMASS, NUTRIENT CYCLING AND SOIL SUPPRESSIVENESS IN FUMIGATED SOILS

1.1. INTRODUCTION

Soil is a very important factor in cropping systems; it constitutes a complex aggregate of physical, chemical, and biological components where specialized biota can survive (Fitter *et al*., 2005), developing important and precise ecological roles (Mulder *et al*., 2011). Among soil organisms, nematodes are diverse, abundant and widespread, being present in virtually all habitats across the world, interacting with many other organisms as participants in several links of the soil food web and playing important roles in essential soil processes (Griffiths *et al*., 2004). Their permeable cuticle permits them to be in contact with dissolved compounds in the soil, and their anatomical features provide information about their feeding roles, making them useful indicators of soil diversity and functioning (Giller, 1996; Ettema, 1998; Neher *et al*., 2005). Moreover, nematode classification into ecological groups such as trophic links and the development of soil food web indices (Ferris *et al*., 2001) have produced a significant advance in the interpretation of the relationships between soil diversity and soil functioning (Neher, 2001; Liu *et al*., 2012). Soil fauna-based indicators must reflect changes in soil web condition as a result of land management practices, and consequently reflect ecological processes (Neher, 2001). The Scientific Panel of Plant Protection Products and their residues from the European Food Safety Authority (EFSA) has pointed out the necessity of modifying the ecotoxicological data requirements for plant protection products in order to evaluate them in an integrated way, including structural and functional endpoints with organisms such as bacteria, fungi, protists and nematodes (SCTEE, 2000). The EFSA recommends the use of nematodes during the assessment of the functional and structural features of the soil (EFSA, 2007).

Soil fauna and soil functioning are closely linked, e.g., the composition and abundance of nematode trophic groups and functional guilds can be related to carbon and nitrogen soil content (Bastow, 2011). Soil fauna primarily affects nutrient cycling by grazing on fungi and bacteria and by excreting N-rich compounds that enrich the soil with nutrients (De Ruiter *et al*., 1993). However, in intensive agricultural systems, such relationships are often missing, since plant nutrient availability does not rely heavily on nutrient mineralization, but instead on mineral fertilizers and organic amendments. Fertilization has a direct positive effect on plant growth, but beneficial fauna such as certain soil nematodes may be sensitive to the disturbance produced by both nutrient enrichment (Bongers, 1990; Thoden *et al*., 2011) and mineral fertilizers (Tenuta & Ferris, 2004). Since different groups of nematodes present different sensitivities to agricultural management and soil disturbance (Neher, 1999), nematodes can be used as indicators of soil

food web condition (Ferris *et al*., 2001), and nematode-based indicators can be used to study the relationships between soil diversity and soil physical-chemical properties, essential to understand soil functioning processes. Nematodes may serve as food source to other soil mesofauna as tardigrades, which occupy a wide range of niches in freshwater and terrestrial environments, some of them possessing a worldwide occurrence. Their ability to undergo cryptobiosis permits them to enter into a latent state in response to desiccation (anhydrobiosis), temperature (cryobiosis), low oxygen (anoxybiosis), and salinity changes (osmobiosis) (Nelson, 2002).

Thus soil food webs play essential roles in different ecosystem processes such as plant growth (Wardle, 1999), which occurs within a complex environment where both above and below ground interactions among different organisms take place (Van der Putten *et al*., 2001). Previous studies have found significant relationships between plant growth, soil organisms and soil management (De Deyn *et al*., 2004; Bonkowski *et al*., 2009). At the same time, plant identity and diversity closely affect soil biota at different trophic levels (Wardle *et al*., 2003). Other soil food web functions, such as soil suppressiveness, may also be important to plant development. Both intrinsic and man-induced soil suppressiveness against a number of disease-causing agents and pest organisms has been repeatedly described in the last 30 years (Kloepper *et al*., 1980; Rodríguez-Kabana *et al*., 1987). Such suppression of pest organisms can be developed by a number of microorganisms (Borneman & Becker, 2007), and other organisms such as arthropods (Moerkens *et al*., 2012), tardigrades (Sánchez-Moreno *et al*., 2008a), and nematodes, such as the entomopathogenic Rhabditidae (Campos-Herrera & Guitiérrez, 2009). In agricultural systems, soil management, and especially soil fumigation, may reduce natural soil suppressiveness, turning a suppressive soil into a non-suppressive one (Westphal & Becker, 1999). Detrimental agricultural management practices have been associated with unstructured and unhealthy soils with low suppressiveness ability (Sánchez-Moreno & Ferris, 2007), but, in the absence of specific biocontrol agents, the extent to which soil suppressiveness relies on the complexity and diversity of soil food webs has not been completely disentangled.

Spain is the main strawberry (*Fragaria ananassa* Duch.) producer within the European Union (EUROSTAT, 2015), with 6.400 ha of cultivation surface, concentrated mostly in southern Spain (Huelva) (MARM, 2012). Conventional strawberry cropping is one of the most input-intensive production systems in agriculture, characterized by a stationary production. The intensive strawberry production is sensitive to a series of diseases and pests that may affect crop yield, as soil fungi (*Phytophtora* and *Fusarium*), plant-parasitic nematodes (*Meloidogyne*, *Pratylenchus*)

and weeds (De Cal *et al.*, 2005; Schneider *et al.*, 2008; García-Méndez *et al.*, 2009). Although the integrated pest management has increased and the use of plant protection products has decreased in the last years (EUROSTAT, 2015; Zanón *et al.*, 2014), the intensive production continues being an environmental concern in the strawberry production areas. Since the phase out of methyl bromide, producing strawberries has become increasingly challenging. Various alternatives to methyl bromide soil disinfection less aggressive to the environment have been used (Mao *et al.*, 2012); The use of 1,3- dichloropropene (1,3-D) and chloropicrin (Pic) has increased in strawberry cropping systems in the last years, becoming essential to maintain strawberry commercial production in southern Spain (Porter *et al.*, 2006; López-Aranda *et al.*, 2009b). However, these fumigants present uncertain effects on the environment (Yates *et al.*, 2011) and in 2011 their use were not approved in the European Union (E.C., 2011a, 2011d). Currently, their use in Spain is limited to some exceptional situations for periods shorter than 120 days according to the article 53 of the Regulation (EC) No. 1107/2009, which accepts exceptional uses when phytophatological danger cannot be contained by any other reasonable mean (E.C., 2009). The use of other soil fumigants as dazomet, metam-sodium and metam-potassium is permitted in the EU (E.C., 2011b) and have demonstrated their efficacy as alternatives to methyl bromide in strawberry crops (López-Aranda *et al.*, 2009a; López-Aranda *et al.*, 2009b; Pizano *et al.*, 2010). The use of other substances with nematicide, fungicide and insecticide effects as *Bacillus firmus* I-1582, etoprophos, fenamiphos, fosthiazate, iprodione, oxamil and *Paecilomyces lilacinus* strain 251 is also possible (E.C., 2011b).

In previous studies the effects of soil fumigation, sampling time, habitat, and soil management on soil nematode diversity were studied (Sánchez-Moreno *et al.*, 2010). In this study we aim to infer if the detected effects on soil diversity have further consequences on soil functioning in an intensive agricultural system affecting agricultural sustainable production. Specifically, the objectives of this study were to study the relationships between soil nematode diversity and plant growth, nutrient cycling and soil suppressiveness in fumigated soils.

1.2. MATERIALS AND METHODS

1.2.1. Study site

The study area located in Cartaya (Huelva, southern Spain) (Fig 1.1), was a commercial strawberry farm and its surroundings (Fig 1.2.) (including field margins and an adjacent pine forest). The soil in the study area presented a sandy texture in the field and the pine forest, and a clay-loam texture in the field margins. The climate of this area is oceanic with subtropical influence (Köppen, 1900), with a mean annual precipitation of 490 mm and a mean annual air temperature of 18.1° C (AEMET, 2014a).

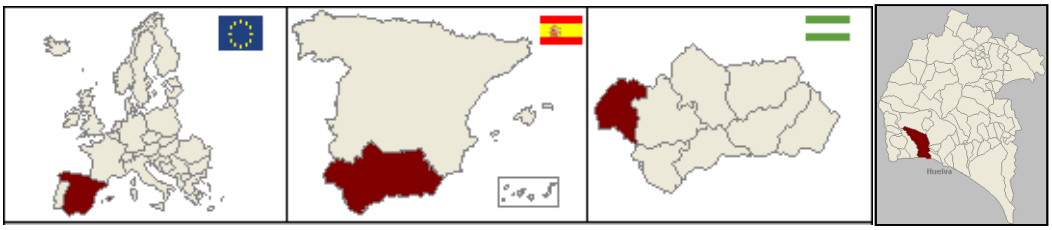


Figure 1.1. Location of the commercial strawberry farm in Cartaya (Huelva, southern Spain).

Strawberries have been cultivated in the farm for the past 20 years. Although in 1995 the use of methyl bromide was restricted by the Montreal Protocol owing to its ozone-depleting properties (UNEP, 1995), this fumigant continued to be used in the farm until 2006 when the prohibition became effective. Since other soil fumigants such as 1,3-dichloropropene (1,3-D; $C_3H_4Cl_2$) and chloropicrin (Pic; CCl_3NO_2) have been proved to be effective MeBr alternatives (Porter et al., 2006) a commercial mixture of 1,3-dichloropropene and chloropicrin or only chloropicrin, were further used in the farm.

Figure 1.2. Commercial strawberry farm and its surroundings**.**

The farm was managed following conventional standard practices for the area. The farm soil was amended with horse manure at a rate of 20.000 kg ha^{-1} at the beginning of the season, prior to any soil treatment. A commercial mixture of 1,3-D and Pic (Telopic, 1,3-dichloropropene 81.9% p/v, chloropicrin 46.5% p/v) was applied at 400 kg ha^{-1} by injection at 20 cm depth in 2010-2011, and a commercial Pic formulation (Tripicrin, chloropicrin 99%) was applied at 400 kg ha^{-1} in 2011-2012, partially due to the restrictions of 1,3-D use imposed by the European Union (E.C., 2011a, 2011d).

Simultaneously with fumigant application, beds were formed and covered with black polyethylene plastic mulch (0.09 µm thick), and plastic irrigation tubing was placed under the cover. The dimensions of the beds and furrows were 42 cm and 45 cm wide respectively. *Honor* strawberry plants were planted at 78.000 plants ha^{-1}, 28 days after fumigation to avoid phytotoxicity. Fertilization occurred through nitrogen-phosphorous-potassium fertigation during the whole length of both cropping seasons. For comparative purposes, field margins and an adjacent pine forest, representing non-cropped soils, were included in the study.

1.2.2. Soil sampling

Soil sampling took place in a 1.7 ha section of the commercial strawberry farm along two cropping seasons and from its field margins and an adjacent pine forest (Fig. 1.3.). Field margins were approximately 3 m wide, and surrounded the 1.7 ha area on three of the field sides. The total length was 450 m. The adjacent pine forest area occupied a surface of 0.25 ha.

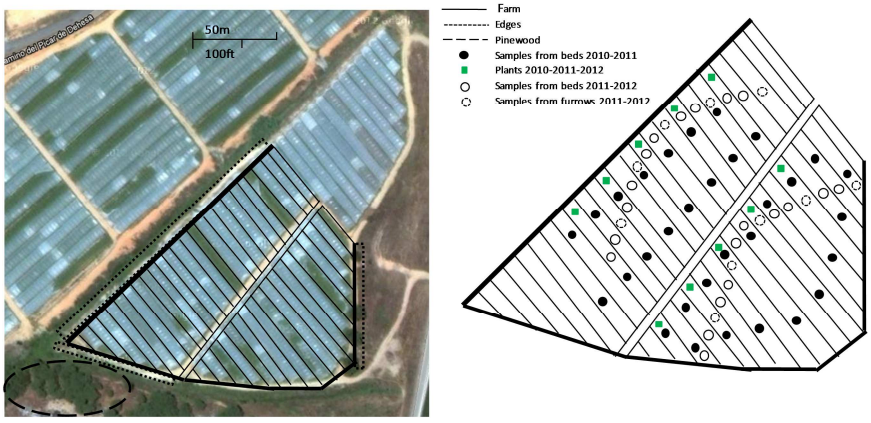


Figure 1.3. Scheme of the study area.

Soil samples were collected during two cropping seasons (Sep 2010- June 2011 and Sep 2011-June 2012). Soil samples were collected a few days before soil fumigation, after transplantation, at two times during the mid-season, and before the final harvest, corresponding to 5, 17, 28, and 35 weeks after soil treatment (WAT) (September and November 2010, February, April and June 2011) during the growing season 2010-2011, and before soil fumigation and 5, 19, 30, and 39 WAT (September and October 2011, January, April and June 2012) during the season 2011-2012.

Samples were taken with a 5 cm diameter soil corer at 0-20 cm depth along the sampling area. Thirty soil samples were taken within the field, five samples were collected from the field margins, and another five samples were collected from the pine forest at each of the five samplings in 2010-2011 (in total 200 soil samples), except 35 WAT that 14 samples were taken from the field. The three habitats (field, field margins, and pine forest soils) were sampled at the same sampling dates. Sampling design was modified in the second cropping season, and soil samples from non-treated furrows between cropping beds were also collected and included in the study. In 2011-2012, 21 samples were collected before soil fumigation, and 20 samples from the beds plus 10 samples from the furrows were collected at each of the following sampling dates after treatment. Five samples from the field margins and five from the pine forest were taken as in the previous year (in total 190 samples) (Table 1.1.). Samples from the adjacent pine forest and field margins were taken for comparative purposes within the study, representing non-cropped soils. All samples were stored at 4°C until processing.

1.2.3. Soil residues

Soil pesticide residues were analyzed using an analytical method developed and validated in the Plant Protection Products Unit (UPF-DTEVPF) of the INIA (Spain) (Sandín-España *et al.*, 2011) based on gas chromatography with detector electron capture at a level of quantification of 0.025 ppm for Pic and 0.015 ppm for 1,3-D. The level of detection was 0.0064 ppm for Pic and 0.00164 ppm and 0.0681 ppm for cis-1, 3-D and trans-1, 3-D respectively. In 2010-2011 residues from 1,3-D and Pic were analyzed for all samples before treatment and 5WAT. Since fumigant residues were almost non-existent 5 WAT, further analyses took place in a reduced number of samples for control purposes. At 17 and 28 WAT residues were measured in 20 samples from the three habitats, and at 35 WAT, analyses were carried in 15 samples. In 2011-2012 Pic residues were analyzed in 12 samples from the three habitats before soil fumigation, and all samples were analyzed 5 WAT. Since residues were, as the previous year, almost absent, reduced number of

samples was analyzed in subsequent samplings for monitoring purposes (20 samples were analyzed, including beds and furrows and the non-cropped habitats, at each sampling date). Besides analyses described above, fumigant residues were analyzed in all rhizosphere samples included in the plant biomass study and these samples were considered from the farm for the calculation of the mean values.

1.2.4. Plant biomass

10 entire plants (roots and shoots), and the soil composing the rhizosphere, were collected at the end of each bed during the last three sampling dates on both seasons. Samples were collected from the end of the beds in order to damage as little as possible to the growing crop at this commercial farm. On the last sampling date (35 and 39 WAT in 2010-2011 and 2011-2012 respectively), when damaging the crop was no longer a concern, 16 and 10 plants were collected respectively at the soil sampling points described in the section 1.2.2. Rhizosphere soil samples collected with the plants were processed as described in sections 1.2.5.- 1.2.7 (Table 1.1.). All biomass samples were used to study plant growth along both seasons. Roots and shoots were weighted, dried at 60 °C in an oven for 48 h, and dry biomass was recorded. Fruits were excluded from the biomass analyses.

Rhizosphere soil collected on the last sampling date, when plants had reached their maximum size, was used for the analysis to relate plant biomass to soil nematode diversity and physical-chemical properties. Since the end section of the beds are not considered to be representative of the condition of the crop they were deliberately excluded from the main study, and such samples were only used for analyses related to plant biomass.

Table 1.1. Number of samples collected in 2010-2011 and in 2011-2012 (0-20 cm depth), before soil treatment (CO-) and 5, 17, 28 and 35 weeks after treatment (WAT) and before soil treatment (CO-) and 5, 19, 30 and 39 WAT (F: field, FM : field margins, P: pine forest, Fu: furrows, Pl: plants).

2010-2011		F*	Pl	FM	P	Total	2011-2012		Fu		Pl	FM	P	Total
									bed	*furrow*				
Sept 2010	CO-	30	-	5	5	40	Sept 2011	CO-	21	-	-	5	5	31
Nov 2010	5WAT	30	-	5	5	40	Oct 2011	5WAT	20	10	-	5	5	40
Feb 2011	17WAT	30	10	5	5	50	Jan 2012	19WAT	20	10	10	5	5	50
April 2011	28WAT	30	10	5	5	50	April 2012	30WAT	20	10	10	5	5	50
Jun 2011	35WAT	14	16	5	5	40	June 2012	39WAT	20	10	10	5	5	50
						220								**221**

* from the beds

1.2.5. Soil analyses

Each soil sample was divided into sub-samples. One subsample of about 500 g was air-dried and fresh and dry soil weights were recorded. Contents of macro and microelements (N, K, P, Mg, Na, and Ca), organic matter (OM), soil texture, electrical conductivity (EC) and soil pH were analyzed from dry soil in an external accredited laboratory. Ca and P were extracted with NH_4Ac and analyzed by atomic absorption spectroscopy, while Na and K were extracted with NH_4Ac and analyzed by atomic emission spectroscopy. N was analyzed by Kjeldahl method (Radojevic & Bashkin, 1999), OM by Walkley-Black method (Walkley & Black, 1934), and pH and EC by potentiometry. Texture was determined using a Bouyoucos densimeter.

A second subsample of 10 g of fresh soil was used to extract NO_3^- and NH_4^+ within two days after soil sampling. NO_3^- and NH_4^+were extracted using a 2M KCl extraction solution, and stored frozen at -20ºC. NO_3^- and NH_4^+were analyzed at the Nutrient Analyses Laboratory of the Rey Juan Carlos University with an automated continuous flow analyzer. Two more subsamples were used for nematode faunal analysis (400 g fresh soil, section 1.2.6) and for soil suppressiveness experiments (100 cm^3 fresh soil, section 1.2.7). Soil fumigant residues were analyzed throughout the cropping seasons, but were always under the level of quantification (Sandín-España, P., personal communication), and will be published elsewhere.

1.2.6. Nematode extraction and identification

Nematodes and tardigrades were extracted from 400 g of fresh soil using the sieving and Baermann funnel method (Barker *et al*., 1985). All nematodes and tardigrades extracted from each sample were counted directly under a dissecting microscope, and at least 100 nematodes from each sample were identified to genus or family under the microscope (Bongers, 1994). Depending on their food source, nematodes were classified as bacterial feeders, fungal feeders, plant parasites/herbivores, omnivores and predators (Yeates *et al*., 1993). Nematodes were also classified along the colonizer-persister (C-P) scale, which classifies nematode families into five groups, from microbial feeders with short life cycles and high reproduction rates (cp 1 and cp 2), to predators and omnivores with long life cycles, low reproduction rates and which are very sensitive to environmental perturbations (cp 4 and 5) (Bongers & Bongers, 1998). Since nematodes belonging to Tylenchidae might be herbivores or fungal feeders (Yeates *et al*., 1993) half of the nematodes identified as Tylenchidae were considered as fungal feeders and half as herbivores in indices calculations. Taxa Richness (S) was calculated as the average number of taxa in each sample, and the total number of nematodes and tardigrades were expressed as the number of individuals per 100 g of dry soil. The Plant Parasitic Index (PPI) (Bongers, 1990) was used as an indicator of the condition of the plant-parasitic nematode assemblages.

Soil food web indices, based on the abundance of functional guilds (Bongers & Bongers, 1998), were calculated to assess soil food web condition. The Structure Index (SI), a weighted measure of the proportion of sensitive predator and omnivore nematodes, is an indicator of soil food web complexity, and is very responsive to soil disturbance. The Channel Index (CI), based on the ratio of fungal to bacterial-feeding nematodes, is an indicator of the prevalence of organic matter decomposition mediated by fungi. The Basal Index (BI), based on the abundance of general opportunistic nematodes, is an indicator of basal, perturbed soil food web condition. The Enrichment Index (EI) is based on the abundance of enrichment opportunistic nematodes and is an indicator of rapid, bacterial-mediated organic matter decomposition (Ferris *et al*., 2001). The SI and the EI permit the diagnosis of the soil food web as disturbed, maturing, structured, or degraded (Ferris *et al*., 2001).

1.2.7. Soil suppressiveness

The lepidopter *Galleria mellonella* L. (Lepidoptera: Pyralidae) is a model organism used to assess the virulence of entomopathogenic nematodes (Millar & Barbercheck, 2001) and fungi (Renwick *et al*., 2006) against arthropod pests. Using *G. mellonella*, soil suppressiveness was studied through the insect bating technique (Bedding & Akhurst, 1975) in the 2011-2012 season. Assays were established within 48h after soil sampling at each sampling date. In each assay, one subsample of 100 cm^3 from each sample collected in the field was placed on a 14 cm diameter plastic petri dish. 5 last instars larvae of *G. mellonella* were set on the surface of each soil dish, covered with its lid, inverted, and sealed with parafilm to avoid desiccation (Fig. 1.4 A, B and C). Dishes were incubated in the dark under controlled conditions in a growth chamber with a temperature setting of 26°C/20°C (day/night). All petri dishes were checked every third day, when larval mortality was recorded, and dead larvae and pupae were removed when present from the dishes. Each assay finished when all larvae had died or became pupa. On average, each dish was checked 6 times (total length of each experiment ≈21 days). The assay took place 5 times, once after each soil sampling, and soil suppressiveness was measured in all samples collected during the growing season 2011-2012 (N= 190).

Mortality rate of *G. mellonella* in each sample was registered as the cumulative mortality across checking days. Assays lasted 21, 24, 24, 18, and 21 days (so dishes were checked 7, 8, 8, 6, and 7 times before all larvae were dead or had become pupae) in the samples collected before treatment, 5, 19, 30 and 39 WAT respectively. Mean cumulative mortality values per sampling date and habitat were calculated to study the relationship between soil suppressiveness and soil food web condition and soil diversity. Dead larvae removed from each dish were incubated for 10 days on White traps (Fig. 1.4D) at room temperature (White, 1927) to check if they were infected by entomopathogenic nematodes. Nematodes emerging from *G. mellonella* cadavers incubated in White traps were identified morphologically under the microscope on the first three sampling dates.

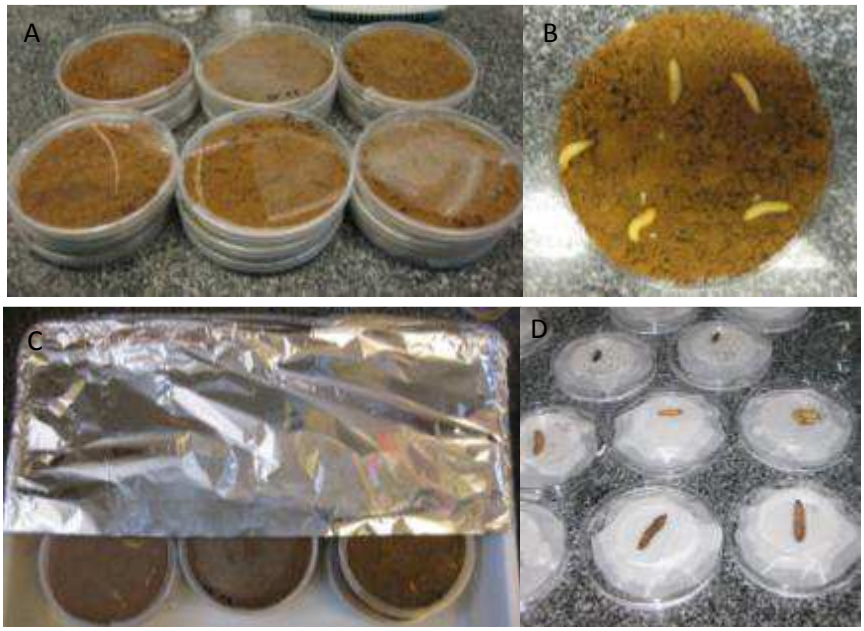


Figure 1.4. A-C, subsamples placed on plastic petri dishes with 5 last instars larvae of *G. mellonella* set on the surface of each soil dish sealed with parafilm to avoid desiccation and incubated in the dark. **D,** dead larvae incubated in White traps.

1.2.8. Statistical analysis

Due to lack of normality, Kruskal-Wallis ANOVAs were used to infer if sampling date significantly affected nematode community descriptors and tardigrade abundance in each habitat. Canonical Analysis (CA) was performed to examine and summarize relationships between soil physical-chemical properties, type of habitat and nematode community descriptors. Both categorical variables (habitats), included in the analyses as categorical (presence/absence) predictors, and soil properties were used to seek for their influence on nematode diversity indicators. To avoid complete correlation among categorical variables within each category, N-1 categories were used in each classification (being N the total number of categories). Soil properties and nematode community descriptors were included as continuous variables. The ordination resulting from the analysis of the relationships among categorical and continuous variables is shown in a canonical bi-plot in which variables that score together are more closely related.

To study the relationships between nematode community composition and nutrient cycling, a Principal Component Analysis (PCA) was performed on nematode taxa abundance's data. The first four axes extracted using PCA were used as descriptors of the nematode community and used as predictors of the amount of organic matter, N, NO_3^-, and NH_4^+ in the soil, and of the C:N

ratio, in multiple regression models performed using the General Linear Models module of the STATISTICA software package (StatSoft, 2013). The partial eta-squared (η^2) values associated with each PCA axis in the models were checked to look for the relationships between nematode community composition and nutrient cycling in each habitat. The p-values of the partial effect of each PCA axis on the five dependent variables indicated the significance of each axis determining nutrient dynamics.

To study the relationships between plant biomass and soil physical-chemical properties, Pearson's correlation analysis was used. ANOVAs were used to check the effect of sampling date on plant biomass. To analyze soil suppressiveness, Kruskal-Wallis ANOVAs were used to check the effect of sampling date (before treatment, 5, 19, 30, and 39 WAT) on the mortality rate of *G. mellonella* in soil from the different habitats. Spearman rank order correlation coefficients were used to relate mean cumulative mortality rates measured for each habitat at each sampling date and ecological indices. All variables were log-transformed before analysis.

1.3. RESULTS

1.3.1. Residues of soil fumigants

All residues of chloropicrin were below the quantification level for all the samples analyzed at each sampling date both cropping seasons (Table 1.2). Most 1,3-D residues values were below the quantification level for the samples collected at each sampling date both cropping seasons. Although in the second cropping season only Pic was applied, residues of 1,3-D also were found in some samples collected from the four different habitats but these values were very low.

Table 1.2. Mean values of pesticide residues before soil treatment (CO-) and 5, 17, 28 and 35 weeks after treatment (WAT) in 2010-2011 and 5,19,30 and 39 WAT in 2011-2012 in four different habitats (F: field, FM: field margins, P: pine forest, Fu: furrows). CP: chloropicrin. 1,3-D: 1,3 – dichloropropene. X: no data. All values in mg/kg (ppm). Level of quantification of 0.025 ppm for Pic and 0.015 for 1,3-D.

	2010-2011							**2011-2012**							
	F		**FM**		**P**			**F**		**FM**		**P**		**Fu**	
	CP	**1,3-D**	**CP**	**1,3-D**	**CP**	**1,3-D**		**CP**	**1,3-D**	**CP**	**1,3-D**	**CP**	**1,3-D**	**CP**	**1,3-D**
CO-	<LQ	<LQ	<LQ	0.016	<LQ	0.016	**CO-**	<LQ	0.033	<LQ	<LQ	<LD	0.283	x	x
5WAT	<LQ	0.025	<LQ	<LQ	<LQ	<LQ	**5WAT**	<LQ	<LQ	<LQ	0.03	<LQ	0.161	<LQ	<LQ
17WAT	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	**19WAT**	<LQ	0.062	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
28WAT	<LQ	0.039	<LQ	<LQ	<LQ	<LQ	**30WAT**	<LQ	0.034	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ
35WAT	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	**39WAT**	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ

1.3.2. Nematode community composition

Fifty four and forty one nematode taxa belonging to the five main trophic groups were identified in 2010-2011 and 2011-2012 respectively (Supplementary material, Table 1.S1.). During the 2010-2011 growing season 23 taxa were bacterial feeders, 5 fungal feeders, 4 omnivores, 10 predators, and 10 plant parasites or herbivores. In 2011-2012 19 taxa were bacterial feeders, 5 fungal feeders, 3 omnivores, 6 predators and 8 herbivores. *Panagrolaimus*, *Acrobeloides*, *Prismatolaimus*, *Aphelenchoides*, Dorylaimida, *Meloydogine*, *Trichodorus*, *Pratylenchus*, *Rotylenchus* and Tylenchidae, together with *Rhabditidae* (dauer juveniles), were the most abundant taxa. Most of the taxa were more abundant in 2011-2012 than in 2010-2011 except in the case of some plant parasites (*Trichodorus*, *Pratylenchus*, *Tylenchorynchus* and *Rotylenchus*) and *Tylenchidae*, which were more abundant in 2010-2011 (Table S1.1.). Kruskal-Wallis ANOVAs

detected a significant effect of sampling date on 26 and 21 taxa during both cropping seasons (Table S1.1).

1.3.3. Soil food web indices and nematode community descriptors

Total number of nematodes progressively increased along the cropping season within the field in 2010-2011, reaching a maximum of 1470.35 nematodes (100 g dry soil)$^{-1}$ in the field margins at 28 WAT (Fig. 1.5). In 2011-2012 nematode abundance also increased along the cropping season in the field, where nematode densities were greater than the previous season, and the acute effect of fumigation 5WAT on nematode abundance was more noticeable. Total number of nematodes (number of nematodes (100 g dry soil)$^{-1}$) was reduced 5WAT from 494.25 (before treatment) to 14.54 in the field soils in 2011-2012 (Fig. 1.6). Nematode abundances did not follow clear patterns in margin and pine forest soils (Fig. 1.6). Maximum tardigrade abundances (number of tardigrades (100 g dry soil)$^{-1}$) were 8.2, 72.5, 86.6 and 118.8 in the fumigated soil, furrows, field margins, and pine forest respectively. Tardigrades were almost absent in the fumigated field during both seasons, and showed variable abundances throughout the seasons in the other habitats (Figs. 1.5 and 1.6).

Taxa Richness significantly varied along the season in field and pine forest soils throughout both seasons, yet increased in the pine forests but decreased in the field the second year (Figs. 1.4 and 1.5). The Plant Parasitic Index during both seasons was strongly reduced after fumigation, a pattern not found in the other habitats (Figs. 1.5 and 1.6). The Plant Parasitic Index had high values in the furrows the second year. The Channel Index, the Basal Index, and the Structure Index also decreased after fumigation in 2010-2011, while the Enrichment Index increased (Fig. 1.5). Out of the eight indicators used, 5, 2, and 2 were significantly affected by sampling date in the fumigated, margin, and pine forest soils respectively in 2010-2011.

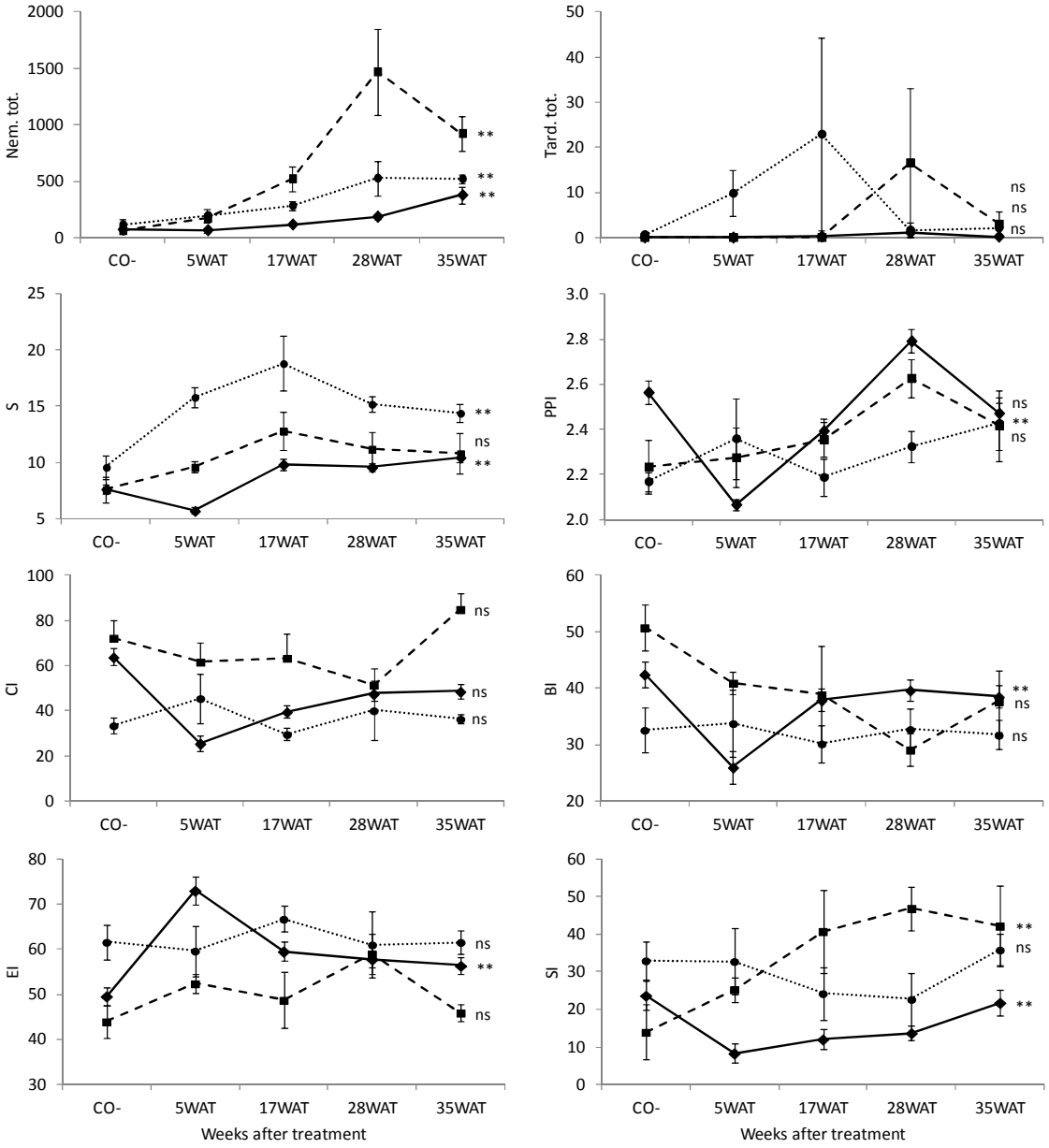


Figure 1.5. Mean values of total number of nematodes (Nem. tot.) and tardigrades (100 g of dry soil)$^{-1}$ (Tard. tot.) , Taxa Richness (S), Plant Parasitic Index (PPI), Channel Index (CI), Basal Index (BI), Enrichment Index (EI), and Structure Index (SI) measured before soil treatment (CO-) and 5, 17, 28, and 35 weeks after treatment (WAT) in three different habitats (solid line: field soil, dashed line: field margins, dotted line: forest soil) during the season 2010-2011. Level of significance of the effect of sampling time on parameters is shown in each chart (** $P < 0.05$, ns: not significant). Error bars show ±SE.

In 2011-2012 temporal patterns of soil food web indices were more variable, and the Enrichment Index and the Structure Index presented at higher reduction at 19 WAT (Fig. 1.6). The Channel Index decreased progressively from the first sampling to 30 WAT to increase slightly at the last sampling in field and field margins soils. In contrast the Channel Index increased constantly from the first sampling date until the end of the season in the pine forest soil (Fig. 1.6). The Structure

Index showed a decrease at the beginning of the season in 2011-2012, to increase afterward in all habitats (Fig. 1.6). Along the second season 7, 0, and 4 indicators were significantly affected by sampling date in the fumigated, margin, and pine forest soils respectively.

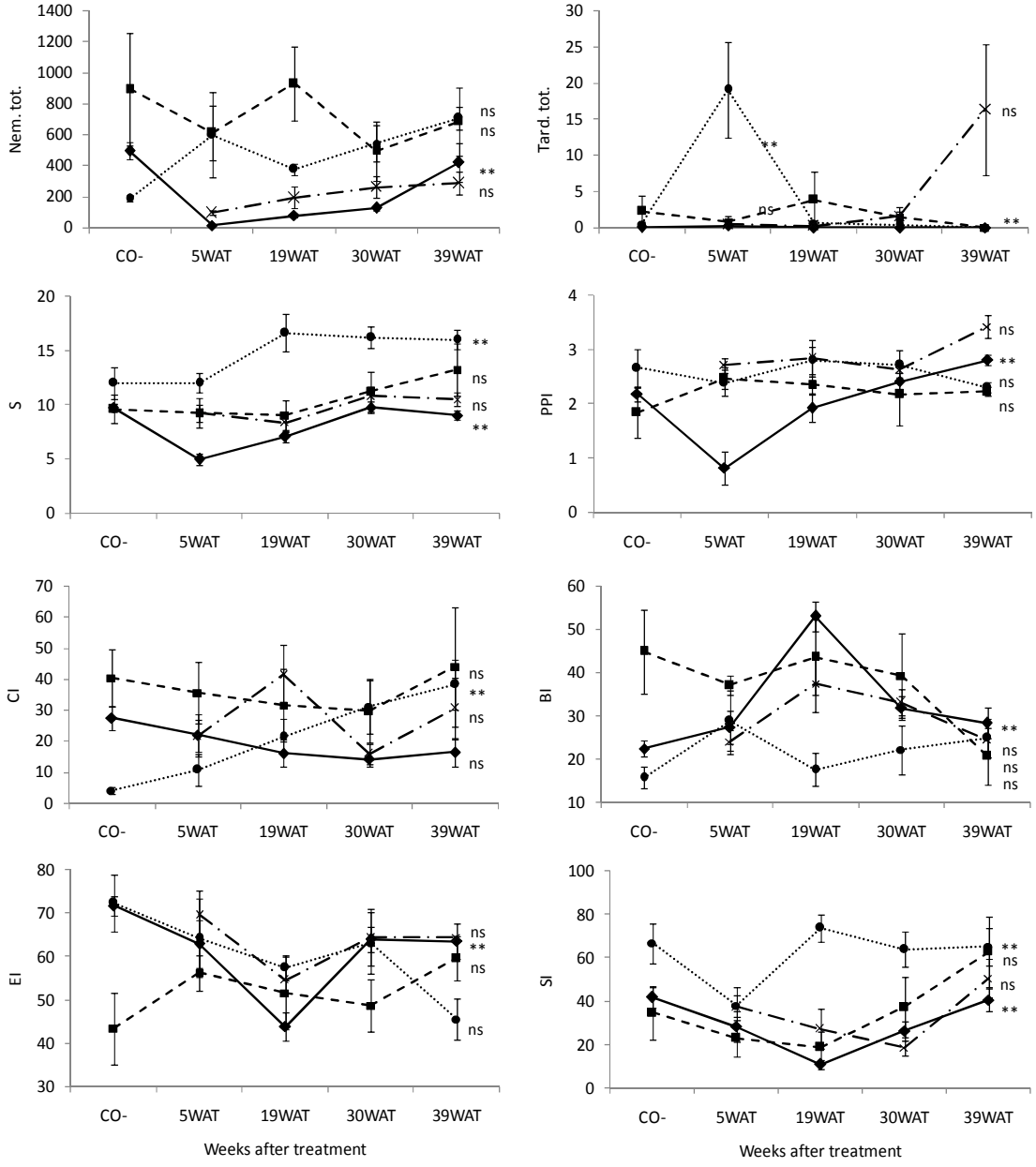


Figure 1.6. Mean values of total number of nematodes (Nem. tot.) and tardigrades (Tard. tot.) (100 g of dry soil)$^{-1}$, Taxa Richness (S), Plant Parasitic Index (PPI), Channel Index (CI), Basal Index (BI), Enrichment Index (EI), and Structure Index (SI) measured before soil treatment (CO-) and 5, 19, 30, and 39 weeks after treatment (WAT) in four different habitats (solid line: field soil, dashed/dotted line: furrow soils, dashed line: field margins, dotted line: forest soil) during the season 2011-2012. Level of significance of the effect of sampling time on parameters is shown in each chart (** $P < 0.05$, ns: not significant). Error bars show ±SE.

1.3.4. Nutrient cycling and plant biomass

Results of the Canonical Correspondence Analysis (CA) are presented in a bi-dimensional plot that shows the association between soil physical-chemical properties, habitat type, and nematode community descriptors (Fig. 1.7). Of the roots extracted during the analysis, root 1 explained 18.9 % and 22.4 % of the variability of independent (soil properties and type of habitat) and dependent (ecological indices) variables respectively, while root 2 explained 8.0 % and 11.9 % of the variability of both sets of variables (Fig. 1.7). High values of NO_3^-, pH, and electrical conductivity (EC), associated with field soils, were related to high values of the Basal Index, while nematode Taxa Richness, the Plant Parasitic Index, total carbon, and high nematode and tardigrade abundances were associated to pine forest soils (Fig. 1.7). Nematode abundance, the Structure Index, and soil Ca, Mg, N, and K scored oppositely to the field. Furrows were situated in an intermediate position between field and pine forest soils, and scored oppositely to the Channel Index, the C:N ratio, and to NH_4^+. Besides NO_3^- and NH_4^+, only P was associated to field soils, while Ca, N, Mg, and K were associated more closely to pine forest soils (Fig. 1.7). Although the Plant Parasitic Index was high in furrow soils (data not shown), its score was determined by its positive association to Taxa Richness while the Channel Index score was determined by its negative association with NO_3^- and NH_4^+, and its low values in field soils.

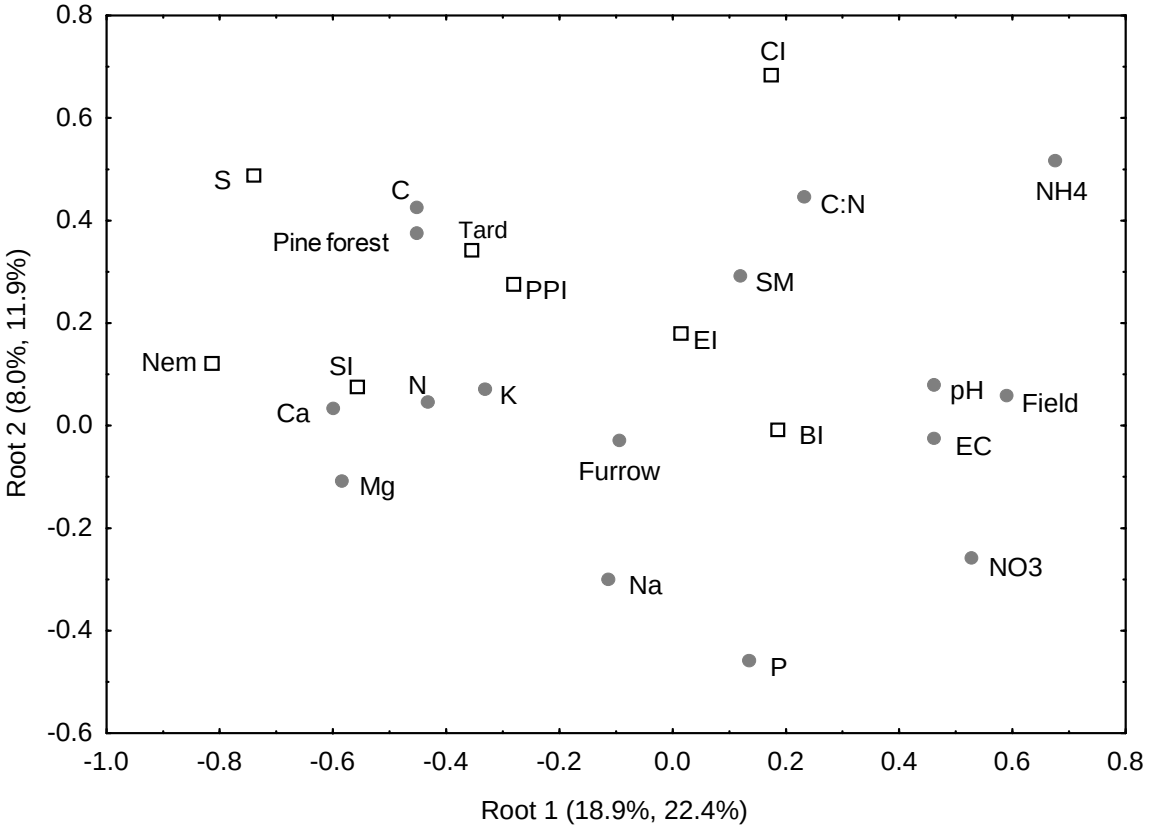


Figure 1.7. Canonical Analysis bi-plot, showing the association between independent variables (dark circles: soil physical-chemical properties (soil moisture (SM), nitrate (NO_3^-), ammonium (NH_4^+), electrical conductivity (EC), total carbon (C), total nitrogen (N), carbon: nitrogen ratio (C:N), potassium (K), phosphorous (P), sodium (Na), calcium (Ca), magnesium (Mg)), and habitat type (farm and pine forest) and dependent variables (nematode community descriptors; white squares (see Figure 1.5. for abbreviations), averaged across two cropping seasons. Percentage of variance explained by each axis is indicated.

To infer to what extent nematode taxa were associated to nutrient cycling, a Principal Component Analysis (PCA) followed by a Multiple Regression Model Analysis was performed. The PCA was performed on nematode taxa to reduce the number of variables, and then the resulting PCA axes were used as predictors of the amount of organic matter (OM), N, NH_4^+, NO_3^-, and the C:N ratio, as indicators of nutrient cycling and soil fertility. The first four axes extracted by the Principal Component Analysis explained 26.7% of the variation of the taxa (Table 1.3.). Nematodes with higher weights in the first axis were enrichment opportunistic (*Mesorhabditis* and *Panagrolaimus*), general opportunistic (*Acrobeloides*, *Acrobeles*, *Wilsonema*, *Eumonhystera*, and *Aphelenchoides*), and nematode taxa indicators of high food web structure (*Teratocephalus*, *Prismatolaimus*, *Achromadora*, *Tripyla*, and Dorylaimida). Low abundances of three enrichment-opportunistic nematodes and the basal fungivores *Aphelenchoides*, together with abundant *Wilsonema*, *Eumonhystera*, *Tylencholaimus*, Tylenchidae, *Trichodorus* and *Discolaimus* defined

the second axis. The third PCA axis was defined by low abundances of *Tylolaimophorus* and *Helicotylenchus* while low abundances of most nematodes and high abundances of *Aphelenchus* defined axis 4 (Table 1.3). When such four PCA axes were used to explain C and N dynamics (OM%, N%, C:N, NO_3^-, NH_4^+) in each of the three habitats studied, they were able to explain lower fractions of their variability (as shown for partial eta-squares, Table 1.4) in field soils than in pine forest and margin soils (Table 1.5).

Table 1.3. Factor coordinates of nematode taxa on the first four axis extracted by Principal Component Analysis. TG: trophic group. C-P: colonizer-persister scale. Only taxa with at least one value higher than 0.3 in any of the factors are shown. Last rows show eigenvalues of each axis and cumulative percentage of variance explained. Taxa with weights higher than ±0.5 in the first PCA axis and than ±0.3 in the other PCA axes are marked in bold numbers.

	TG	C-P	**PCA1**	**PCA2**	**PCA3**	**PCA4**
Mesorhabditis	Ba	1	**0.600**	-0.021	-0.122	0.062
Rhabditis	Ba	1	0.390	**-0.467**	-0.104	0.046
Cruznema	Ba	1	0.182	**-0.421**	0.020	-0.038
Panagrolaimus	Ba	1	**0.691**	**-0.357**	-0.225	0.097
Rhabdolaimus	Ba	1	0.010	0.130	**-0.324**	**-0.642**
Eucephalobus	Ba	2	0.001	0.134	**-0.317**	**-0.633**
Chiloplacus	Ba	2	0.393	0.141	0.248	0.010
Acrobeles	Ba	2	**0.650**	-0.164	-0.147	-0.017
Acrobeloides	Ba	2	**0.666**	-0.390	-0.205	0.072
Plectus	Ba	2	0.398	0.046	-0.064	0.204
Wilsonema	Ba	2	**0.605**	**0.478**	**0.389**	-0.213
Eumonhystera	Ba	2	**0.588**	**0.336**	0.276	-0.107
Teratocephalus	Ba	3	**0.566**	0.261	**0.305**	0.115
Prismatolaimus	Ba	3	**0.620**	0.089	-0.108	-0.035
Achromadora	Ba	3	**0.587**	**0.363**	0.239	-0.058
Leptolaimus	Ba	3	0.219	0.218	**-0.309**	-0.283
Aphelenchus	Fu	2	0.411	-0.031	-0.259	**0.383**
Aphelenchoides	Fu	2	**0.700**	**-0.467**	-0.092	0.040
Tylolaimophorus	Fu	3	0.153	0.137	**-0.550**	-0.245
Tylencholaimus	Fu	4	0.445	**0.427**	-0.120	0.090
Dorylaimidae	O	4	**0.805**	-0.266	-0.003	0.022
Tripyla	P	3	**0.647**	**0.412**	**0.316**	-0.199
Clarkus	P	4	0.329	0.234	-0.073	-0.122
Discolaimus	P	5	0.469	**0.363**	0.001	-0.131
Pratylenchus	Pp	2	0.425	-0.257	-0.259	0.064
Helicotylenchus	Pp	3	0.216	0.041	**-0.451**	-0.092
Criconematidae	Pp	3	-0.211	**0.374**	-0.186	**0.263**
Trichodorus	Pp	4	-0.137	**0.514**	**-0.305**	**0.286**
Tylenchidae	Pp/Fu	2	-0.030	**0.591**	**-0.342**	**0.294**
Eigenvalue			**7.00**	**3.55**	**2.33**	**2.06**
Cumulative % exp.			**12.51**	**18.86**	**23.02**	**26.70**

Table 1.4. Partial eta-squared values of the four PCA axis in the models developed to explain nutrient cycling (OM%, N%, C:N, NO_3^-, NH_4^+) by the composition and abundance of the nematode community at each of the three habitats studied.

	Field	Field margins	Pine forest
PCA 1	0.10	0.40	0.61
PCA2	0.10	0.34	0.49
PCA3	0.07	0.12	0.18
PCA4	0.04	0.26	0.12

Results of the multiple regression model showed that the four PCA axes were significantly associated with nutrient dynamics (Table 1.5). Soil organic matter, NO_3^-, and NH_4^+ presented the best fit to the developed model. Partial significations of the relationships between each PCA axis and each dependent variable showed that the first axis, which explained most of the nematode taxa variation, was positively associated with organic matter content and total N, while the second axis was positively related to all variables except NO_3^- (Table 1.5). The third axis was negatively associated with organic matter and total N, and positively with the C:N ratio, NO_3^-, and NH_4^+. The fourth axis was only associated with total N and NO_3^- (Table 1.5).

Table 1.5. Univariate results showing the semi-partial effect of each PCA axis on nutrient dynamics inferred by multiple regression. Last row shows the p value associated to the effect of each PCA axis on the whole model. All partial correlations shown are significant ($P < 0.05$). Last row shows the adjusted R^2 values of each dependent variable, indicator of the fit of the variable to the model. ns: not significant.

	OM (%)	N (%)	C:N	NO_3^-	NH_4^+	*P*
PCA 1	0.31	0.32	-0.12	-0.12	-0.45	<0.001
PCA2	0.44	0.18	0.33	-0.40	0.24	<0.001
PCA3	-0.08	-0.13	0.10	0.23	0.14	<0.001
PCA4	ns	-0.09	Ns	-0.13	Ns	<0.001
R^2	0.29	0.15	0.13	0.25	0.26	
P	<0.001	<0.001	<0.001	<0.001	<0.001	

As expected, shoot biomass significantly increased along the growing seasons (Fig. 1.8). Shoot biomass was 12 and 10 times higher than root biomass at the end of the crop in 2010-2011 and 2011-2012 respectively (Fig. 1.8). Spearman rank-order correlation coefficients between dry plant biomass and soil properties measured in rhizosphere soil showed that the amount of organic matter was positively correlated to both shoot (r=0.54, $P< 0.05$), and root (r=0.50, $P< 0.05$) biomass in at the last sampling in 2010-2011, while no significant correlations were found

between plant biomass and other biological indicators. In 2011-2012, the Enrichment Index was positively correlated to shoot biomass (r=0.45, $P < 0.05$), while soil moisture (r=-0.46, $P< 0.05$), the C:N ratio (r=-0.55, $P< 0.05$), Ca (r=-0.53, $P< 0.05$), and NO_3^- (r=-0.44, $P< 0.05$), were negatively associated. Soil N was positively (r=0.72, $P< 0.05$) correlated to dry shoot biomass. In the second season, root biomass was only negatively related to soil pH (r=0.45, $P< 0.05$).

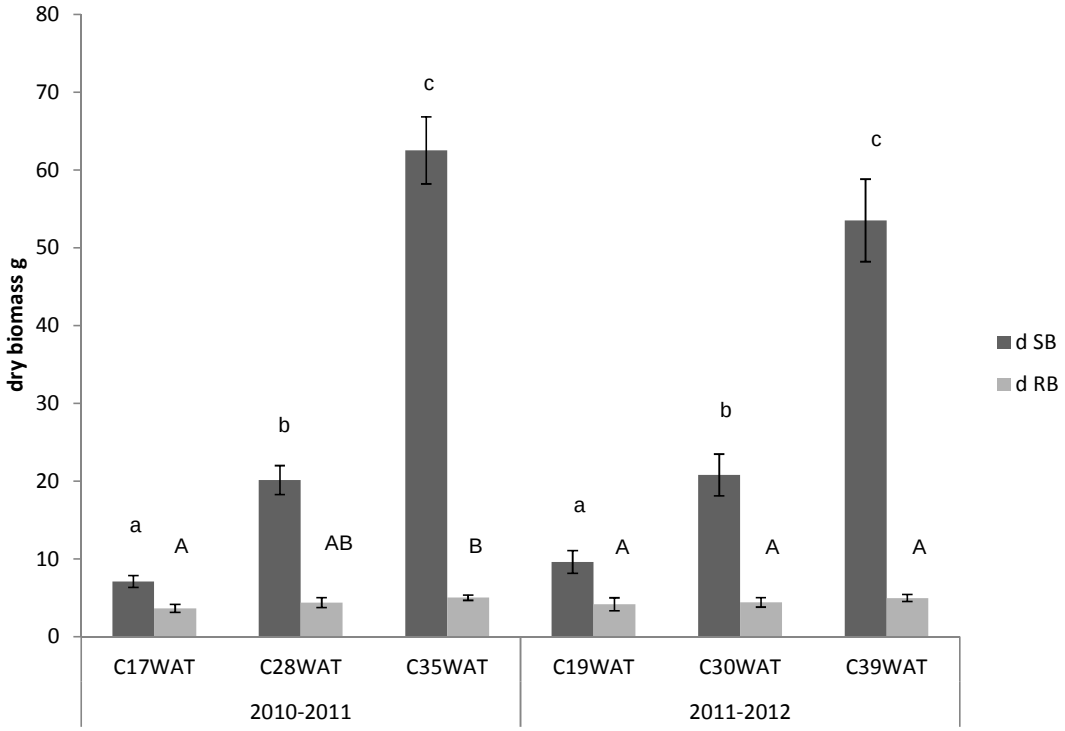


Figure 1.8. Average values of plant biomass (d SB: dry shoot biomass, d RB: dry root biomass) 17, 28 and 35 weeks after treatment (WAT) in 2010-2011 and 19, 30 and 39 WAT in 2011-2012. Different letters mean significant differences at $P < 0.05$. Lower-case and upper-case letters indicate differences for shoot and root biomass respectively. Comparisons are done within each cropping season.

1.3.5. Soil suppressiveness

Results of the soil suppressiveness showed that mortality rates of *G. mellonella* tended to be higher when incubated in pine forest and field margin soils than in field soils. No significant differences were found on mortality rates of *G. mellonella* incubated in bed and furrow soils ($P< 0.05$, data not shown), so "field" soil included samples from beds and furrows in subsequent analyses. When larvae were incubated in close contact to the soil, mean mortality rates of *G. mellonella* were significantly affected by the type of soil at the second and fourth checking days before soil treatment and 19 WAT ($P< 0.05$) (Fig. 1.9A and 1.9C). Differences were greater 5 WAT

(Fig. 1.9B), when mean mortality rates were affected soil type soil during the last five checking dates (P< 0.05). Averaged across all samplings, mortality rates were affected by the type of soil at the fourth, fifth, and sixth checking days (P< 0.05, data not shown). Mean cumulated mortality, averaged across habitat x sampling date, was significantly and positively correlated to the Structure Index (r=0.52, P< 0.05) and nematode Taxa Richness (r=0.61, P< 0.05), and was negatively related to the Basal Index (r=-051, P< 0.05) (Fig. 1.9D-F).

Large number of nematodes emerged from *G. mellonella* cadavers incubated in White traps in 36-47.6% of the samples, depending on the sampling date. Morphological identifications revealed that nematodes emerging from the cadavers were *Panagrolaimus* and Rhabditidae spp. No entomopathogenic nematodes were detected in any sample.

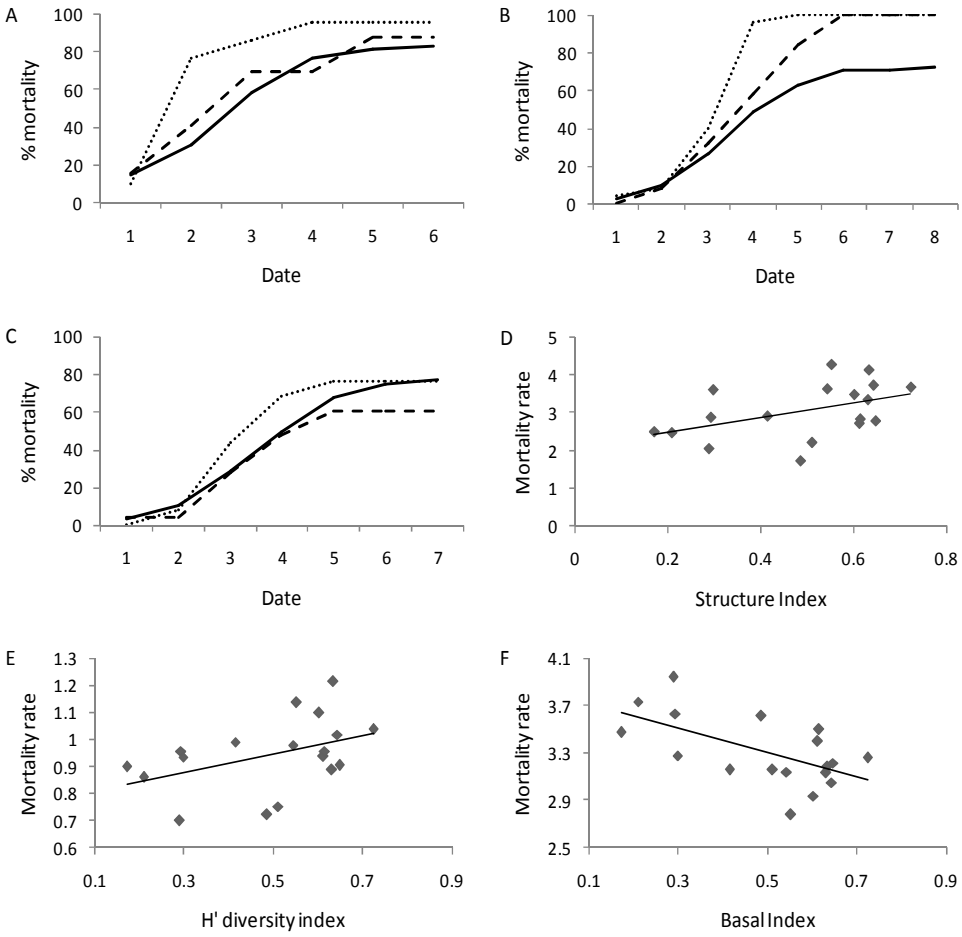


Figure 1.9. Mean cumulative mortality rate of *G. mellonella* measured before soil treatment (A), 5 WAT (B), and 19 WAT (C). Mean cumulative mortality is shown for field (solid line),field margin (dashed line) and pine forest (dotted line) soils, and relationships between rates and the Structure Index (D), Taxa Richness €, and the Basal Index (F).

1.4. DISCUSSION

1.4.1. Nematode community composition, soil food web indices and nematode community descriptors

Pesticides and fertilizers may have drastic effects on soil nematode populations, altering nematode density and diversity, with associated consequences on community structure. Beneficial nematodes, together with other groups of soil fauna, are essential to soil quality, on which agricultural productivity relies to a large extent. Previous work has shown that the use of 1,3-D and Pic deeply affects beneficial nematodes (Sánchez-Moreno *et al*., 2010) which represent between 60% and 80% of the soil nematode community (Neher, 2010). Our study shows that 1,3-D and Pic residues were almost absent 5 WAT during two consecutive seasons. However, the results showed that the number of nematodes in the soil slightly decreased after fumigation during the first season, when 1,3-D + Pic were applied, while an acute decrease was observed in the second season, when only Pic was used. The sequential recovery of nematode abundances along the cropping season, which has been previously reported (Sánchez-Moreno *et al*., 2010), might indicate that soil functioning had recovered, at least to a certain extent, by the end of each season. Annual soil fumigation with 1,3-D and Pic might have led to a community in which only fumigation-resistant populations develop successfully. Previous work has shown that continuous exposure to contamination may lead to the adaptation of the soil nematode assemblage (Van der Wurff *et al*., 2007).

Although nematode abundances varied significantly among sampling dates much more in bed soils than in other habitats, the significant effect of sampling date on nematode abundances in field margins and pine forest soils indicate that other factors different than fumigation are affecting community dynamics, which might be responding to seasonal fluctuations as temperature and rainfall. Bacterial feeding nematodes as *Rhabditis* were very abundant, as previously shown in similar experiments (Renco *et al*., 2010). Other sources of natural variation may also differentially affect nematode taxa, e.g., coarser soils may favor the presence of large nematodes, since texture is directly related to soil particle size (Moore & Lawrence, 2013). A finer soil texture present in the field margins compared to field and pine forest soils could not be related, however, with specific changes on the nematode community in our results. Since the reduction of nematode populations, especially that of microbial-feeding nematodes, can be alleviated by the addition of organic amendments into the soil (Treonis *et al*., 2010), annual

manure incorporation in strawberry crops may be crucial to maintain active soil food webs and their associated functions in such intensive cropping systems.

The reduction of the PPI in treated soils reflects the efficacy of the fumigants, but can be also interpreted as an indicator of high soil disturbance (Palomares-Rius *et al*., 2012). Its subsequent recovery might be accelerated by the continuous increase of root biomass, stimulated by continuous nutrient supply, which supports increasing densities of root feeding nematodes (De Deyn *et al*., 2004). PPI reduction after fumigation varied among seasons (19.1% in 2010-2011, and 62.3% in 2011-2012). In agreement to previous experiments Pic reduced nematode abundances to a larger extent that 1,3-D (Sánchez-Moreno *et al*., 2010).

The presence of a well-developed community of parasitic nematodes, and high nematode abundances and richness characterized furrow soils in comparison to treated beds during 2011-2012, although these differences partially disappeared by the end of the season. Previous studies have shown that tarping beds results in effective containment of fumigant vapors within the covered surface, with minimum fumigant movement towards uncovered furrows (Papiernik *et al*., 2004). However, impermeable films favor lateral gas movement, which can increase fumigant emissions to the uncovered furrows, while permeable films, such as standard polyethylene, do not induce gas movement towards the uncovered surface (Ashworth *et al*., 2010). In our study, the use of standard polyethylene may explain why furrows were a reservoir of both beneficial and plant-parasitic nematodes.

As expected, nematodes belonging to higher trophic links were more abundant in the untreated areas, which in general presented mature and complex soil food webs (high nematode richness values) while treated soils presented a disturbed and enriched soil food web condition (sensu Ferris *et al*., 2001). Contrary to our expectations, chemical fertilizers and organic amendments added to the field did not induce a significant increase of the Enrichment Index in cropped soils in 2011-2012, while an increase of the Basal Index was actually observed. The Enrichment Index, an indicator of soil fertility, increases when enrichment-opportunistic nematodes boost after the microbial amplification that follows fertilization. In 2010-2011 and 2011-2012 the Enrichment Index presented almost opposite patterns in treated soils; increasing after treatment the first season but decreasing in the second, when a basal soil condition was observed. Indeed, the Enrichment Index presented quite similar values across all habitats under study. The Channel Index, an indicator of organic matter decomposition predominantly mediated by fungi, tended to

be higher in field margins, where bacterial decomposition was low, which may be related to low levels of available mineralized nitrogen (Ferris & Matute, 2003). Low values of the Channel Index in the pine forest suggested a low participation of fungi on organic matter decomposition, and the occurrence of organic enrichment processes in this area. These data confirm that, although unmanaged, remaining forest patches in the surroundings of this strawberry cultivation area are not well preserved, and active conservation management would be necessary to maintain and improve their putative function as a reservoir of diversity and ecosystem services.

Tardigrades often present patchy physical (Guil *et al*., 2009) and temporal (Hohberg *et al*., 2011) distributions. Since changes in the abundances of tardigrades have been related to soil organic matter contents (Uhia & Briones, 2002), high tardigrade abundances in the pine forest soils may be related to high carbon contents present in this area. Peak tardigrades abundances in the furrows at the end of the second season showed that low efficiency of pesticides within this zone was probably enough to maintain tardigrade populations in the soil, in spite of tardigrade sensitivity to agricultural management. Sudden increases of tardigrade abundances following a flush of resources availability have been previously reported for some algal-feeding tardigrades (Hohberg *et al*., 2011). However, temporal dynamics or tardigrade abundances might be obscured through their ability to enter into cryptobiosis, a resistant state which is able to tolerate exposure to various chemical and physical extremes (Welnicz *et al*., 2011). Since tardigrades have seldom been studied in agricultural-related systems, their possible use as indicators of the effects of management on soil diversity should be further investigated.

1.4.2. Nutrient cycling and plant biomass

Soil fauna significantly affect carbon and nitrogen cycles, primarily due to their interaction with soil microbes. Bacterial and fungal feeding nematodes are often positively related to the amount of NH_4^+ excreted to the soil (Neher *et al*., 2012), which regulates microbial activity by modifying nitrogen availability to plants (Ekschmitt *et al*., 1999). The populations of general opportunistic nematodes increase under conditions of chemical and nutrient enrichment (Fiscus & Neher, 2002) and the use of different nematode taxa as indicators of organic matter decomposition and nitrogen plant availability have been previously proposed (Neher *et al*., 2012). However, negative effects of toxic N solutions on nematodes, especially of higher c-p values, might be important in cropping systems in which fertilizers are used (Tenuta & Ferris, 2004). Our results show that total organic matter and nitrogen were positively associated, while NH_4^+, NO_3^- and the C:N ratio were

negatively associated with the most abundant taxa, including opportunistic nematodes and the most resistant omnivores and predators such as *Dorylaimida* and *Trypila*. Plant-parasitic nematodes such as Criconematidae (ring nematodes), *Trichodorus* (stubby-root nematode) and Tylenchidae, non-opportunistic fungivores such as *Tylencholaimus*, and sensitive predators such as *Discolaimus*, were positively associated with the ratio C:N and NH_4^+. In contrast, previous studies have shown that plant parasites might be more sensitive to NH_4^+ than enrichment opportunistic nematodes (Tenuta & Ferris, 2004). High NO_3^- soil content was more closely associated with treated soils than NH_4^+, where conversion to NO_3^- is facilitated when soil water content is high. Since only the cropped area was watered, soil water content was restricted to pine forest and field margin soils. The continuous supply of N solutions by fertigation might explain why the composition of the nematode community was more closely associated to nutrient dynamics in pine forest and field margin soils than in farm soils, in which nutrient dynamics rely heavily on chemical inputs.

Plant growth has been reported as being more responsive to the abundance of the lower trophic links of the soil food web (mainly microbes, bacterial and fungal-feeding organisms) than to higher trophic levels (omnivores and predators), which may influence plant growth only under certain conditions (Sackett *et al*., 2010). Relationships between soil fauna and plant growth were mediated by the effects of soil fauna on C and N dynamics independently of the spp. present (Wardle, 1999). In intensive, annual crops with high-input management, root growth is less extensive than in perennial, low-input systems, which, together with the use of highly perturbing techniques, make it difficult to find positive associations between soil food web dynamics and root biomass. Root biomass is, indeed, minimized in commercial strawberry varieties, which maximize shoot (and fruit) biomass. Although roots are relevant for soil functioning (Bonkowski *et al*., 2009), agricultural production promotes varietal selections for higher yields, generating an increase of the root-to-shoot ratio and reducing nutrients in the soil (Neher, 2010), which might also contribute to the weak associations found between nematode community structure and plant biomass. Only the Enrichment Index, an indicator of soil fertility, could be related to plant biomass in this study. Previous studies (DuPont *et al*., 2010) have shown plant production and crop biomass positively correlated to bacterial-feeding nematode abundance. Although the extent to which high nematode abundance might favor plant growth cannot be inferred in this study, the positive correlation observed between the Enrichment Index and plant biomass supports the assumption of the usefulness of the Enrichment Index as indicator of soil fertility (Ferris *et al*., 2001).

1.4.3. Soil suppressiveness

Among the myriad of soil organisms which makes up soil suppressiveness, predatory tardigrades might play an important role (Sánchez-Moreno *et al*., 2008a) and may be considered an indicator of the complexity of the soil food web, which is ultimately responsible of soil suppressiveness (Sánchez-Moreno & Ferris, 2007). In our study, however high numbers of tardigrades in the furrows compared to bed soils was not enough to significantly increase soil suppressiveness in furrow soils. Although slightly less disturbed than bed soils and, to a certain extent, a reservoir of diversity, furrows did not function as a reservoir of suppressive soil organisms. Food web structure (sensu Ferris *et al*., 2001) and soil diversity were positively correlated to soil suppressiveness inferred as larvae mortality. Complex soil food webs with abundant trophic links and possessing high capacity to suppress plant-parasitic nematodes are typical from natural soils (Sánchez-Moreno & Ferris, 2007). The purported ability of soil food web indices as indicators of soil functioning is corroborated by significant correlations found between soil food web indices and soil suppressiveness.

Entomopathogenic nematodes (EPNs) are characteristically present in natural and agricultural areas and have been used as biological control agents of soil pest in several agricultural crops (Campos-Herrera *et al*., 2012). EPNs were not found in the soil in the different habitats included in this study, similarly to previous reports in which conventional management practices severely decreased EPNs occurrence (Campos-Herrera *et al*., 2008). The absence of EPNs in pine forest soils indicates that, besides being unmanaged, such soils are also disturbed. In spite of the absence of EPNs, mortality rates of *G. mellonella* tended to be higher in pine forest compared to field and margin soils. The large number of bacterial feeding nematodes that emerged from some of the *G. mellonella* cadavers in the absence of EPNs had been previously reported (Duncan *et al*., 2003). In field and laboratory studies, Duncan and collaborators found a strong correlation of bacterial-feeding nematode abundances and EPNs, and demonstrated that the joint inoculation of free-living nematodes and EPNs into soils containing bait organisms significantly increased bait mortality rates, while the amount of EPNs emerging from the cadavers decreased. Competition of both functional groups of nematodes has been further demonstrated by molecular techniques (Campos-Herrera *et al*., 2012). Since it was also shown (Duncan *et al*., 2003) that the presence of a bacterial-feeding nematode might decrease by 84% the number of EPNs emerging from the bait organism, our results cannot be considered definitive. EPNs might have been present in the

larvae and remained undetected in morphologically-identified nematode samples emerging from the cadavers.

CHAPTER 2. EFFECTS OF ORGANIC AND CHEMICAL PESTICIDES ON PLANT BIOMASS, NEMATODE DIVERSITY AND THE STRUCTURE OF THE SOIL FOOD WEB

2.1. INTRODUCTION

The use of plant protection products (PPP) is essential to produce healthy and economically viable crops. Pesticides are extensively used in intensive, conventionally-managed horticultural crops, due to their high sensitivity of pests to pesticides and because pesticide activity is often favored by high moisture and warm temperatures typical in horticultural production systems (Miles *et al*., 2013). Nematodes, which are estimated to produce large yield losses, are some of the most common pests affecting horticultural crops, e.g. inducing losses of about 30% of total vegetable production in Almería (southern Spain), the largest horticultural growing area of covered crops in Europe (Talavera *et al*., 2012). Worldwide, pesticides with nematicide activity, as well as cultural practices such as crop rotation and the use of resistant varieties, are used to control numerous species of plant-parasitic nematodes (Qiao *et al.*, 2010; Zasada *et al.*, 2010). Soil fumigants, including 1,3-dichloropropene and chloropicrin, which are commonly used to control nematode pests and soil borne diseases, have not been approved for use in the European Union (E.C., 2011a, d). Given this situation, where the application of soil fumigants is being restricted, there is a need to evaluate both the efficacy and the non-target effects of alternative active substances, and to develop integrated pest management programs to help farmers control nematode-induced diseases in horticultural crops and resistant cultivars. Concern about the effects of conventional PPP on the environment has promoted the use of alternative pest control techniques, *e.g.*, biosolarization (Medina *et al.*, 2009), solarization in combination with cover crops (Wang *et al.*, 2006), organic nematicides (Anver & Alam, 2000; de Almeida *et al.*, 2012), the use of nematophagous fungi (Kim & Riggs, 1995; Singh *et al.*, 2007; Singh *et al.*, 2012), or the use of egg-parasitic fungi and obligate parasitic bacteria (Wesemael *et al.*, 2011), which have emerged in the last years as alternative control mechanisms to the use of chemical nematicides.

Numerous organic soil treatments have been proposed as effective tools to control plant-parasitic nematodes, *e.g*., the combination of neem (*Azadirachta indica* L.) seed powder and leaves of chrysanthemum (*Chrysanthemum coronarium* L.) against *Meloidogyne* spp. (Moosavi, 2012). The use of organic amendments has also been proposed, since, besides giving a direct positive effect on soil fertility, often reduces plant-parasitic nematodes (Oka, 2010). Some authors suggest that the purported improvement of crop health after the addition of such amendments might be due to the positive effects of beneficial nematodes, commonly neglected in this type of studies (Thoden *et al.*, 2011). One of the aims of integrated pest

management programs is to combine different plant protection products, in order to reduce the use of chemical pesticides, and improve crop yield. In previous studies, integrated pest management programs have included the effect of the application of neem seed extract on pea crops to reduce the use of chemical nematicides (Rao *et al.,* 2011).

Soil nematode taxa have different sensitivity or response to specific types of disturbance, treatment, or management (Zhao & Neher, 2013). In agroecosystems, nematode diversity can be used as a biological indicator to estimate the effects of agricultural practices on the soil biological system, and to evaluate soil condition (Neher, 1999; Sánchez-Moreno *et al.*, 2006). Bacterial- and fungal-feeding nematodes may affect organic matter decomposition (Ferris & Bongers, 2006), and are involved in nutrient cycling, releasing nutrients for plant uptake (Ingham *et al.*, 1985). Predators and omnivores may prey upon other nematodes regulating their populations, which have been proposed as a technique for pest control (Khan & Kim, 2007). Organic amendments applied to agricultural crops affect the nematode community promoting the increase of nematodes such as bacterial feeders, which might induce changes on soil bacteria that affect plant growth (Malusa *et al.*, 2012). The interaction of microbial grazers, including soil nematodes, and their prey affects nutrient cycling, increasing nitrogen availability to plants. The development of specific nematological indicators, such as the soil food web indices (Ferris *et al.*, 2001) and other ecological indices such as the maturity indices and the metabolic footprints (Bongers, 1990; Ferris, 2010) has been essential to reach a better understanding of the relationship between nematode community structure and ecosystem functioning (Porazinska *et al.*, 1999; Ferris *et al.*, 2001).

According to the Regulation 1107/2009 (E.C., 2009a), which that replaces the Directive 91/414/EEC, it is compulsory to evaluate the risk of the use of plant production products applied according good agricultural practices, on non-target soil fauna, assessing the effects of these products on soil fauna and their function. The objective of this evaluation is to maintain soil functioning to agricultural production in the long term. To carry out the evaluation, several assays with indicator species are necessary to obtain toxicity data that furtherly will be used in the risk evaluation. However, the fundamental limitation of these assays is the low representativeness of some indicator species in the Mediterranean conditions. The Scientific Panel of Plant Protection Products and their Residues of the EFSA (European Food Safety Authority) has pointed out the necessity of modifying the ecotoxicological data set required to evaluate non-target effects of PPP in a more integrated way, including structural and

functional endpoints with organism such as bacteria, fungi, protists and nematodes (SCTEE, 2000). Thus, EFSA has recommended the use of nematodes in the assessment of the functional and structural features of the soil (EFSA, 2007).

Agricultural soil management may determine soil physical-chemical properties and the availability of micro and macronutrients, affecting soil processes and influencing in various ways the different components of soil nematode fauna and other soil organisms. In previous papers the effect of different agricultural management on the soil nematode community has been established, as well as the relationships that exist between soil management, soil properties, and soil nematodes (Sánchez-Moreno *et al.*, 2006, 2008c; Carrascosa *et al.*, 2014). Different soil management techniques present different efficacy levels in reducing pests and diseases, as well as different undesired effects on soil diversity and functioning (Van Elsas *et al.*, 2002; Sánchez-Moreno *et al.*, 2006; McFadyen *et al.*, 2009). Soil and plant type may model the soil microbial community, determining soil disease suppressiveness (Garbeva *et al.*, 2004). Pesticides together with other components of soil management, may reduce such intrinsic soil suppressiveness (Borneman & Becker, 2007; Sánchez-Moreno & Ferris, 2007) and previous studies have shown how natural soils are more suppressive than agricultural ones (Carrascosa *et al.*, 2014). The effects that agricultural management on soil biota do not depend exclusively on the perturbation induced, but also on the intrinsic characteristics of the soil biota subjected to perturbation. Ecological resilience, or the ability of communities to persist after a perturbation (Holling, 1973), depends on their heterogeneity and diversity (Bengtsson, 2002), so more diverse communities are supposed to maintain their functionality in a better way than poor communities.

In this study, our objective was to determine the effects of organic and chemical pesticides on soil physical-chemical properties, soil biodiversity and plant biomass in experimental conditions. Since applying these products in the field was not possible, the aim was to know their effects on experimental conditions studied in two types of soils; low diversity soils from an agricultural farm and high diversity soils from a natural pine forest area. We hypothesized that organic soil treatments and biological nematicides applied to the soil affect to a lesser extent soil biota and functioning than chemical nematicides, and that rich communities are more resilient to the perturbation induced by nematicides than low diversity ones. According to the objectives of integrated pest management programs, in this study, we aim to know the

effects of both types of pesticides in order to combine their use in agricultural crops and not to substitute chemical by organic products.

2.2. MATERIALS AND METHODS

2.2.1. Soil sampling

Two different types of soil were used in this experiment. The low diversity soil was an agricultural, managed soil from a commercial strawberry farm located in Cartaya (Huelva, southern Spain), whilst the high diversity soil was collected from an unmanaged, natural pine forest (*Pinus pinea* L.) located in the surroundings of the farm. In total, 40 composite samples were collected in March 2012; twenty from raised beds within the strawberry field and 20 from the adjacent pine forest. Both types of soil presented a sandy texture. Farm soil was amended at a rate of 20.000 kg ha^{-1} at the beginning of the cropping season in September 2012, 5 months before collecting the samples for the experiment. Farm soil had been subjected to conventional management, which included yearly soil fumigation with methyl bromide, 1,3-dicloropropene (1,3-D) and chloropicrin (Pic), for the last 15 years, but was not fumigated the year of the experiment. Soil management was the standard for this crop in the area, with a continuous influx of nutrients through nitrogen-phosphorous-potassium fertigation during the whole cropping season. There were 5-month-old cv. Honor strawberry plants (*Fragaria x ananassa*) growing in the beds at the date of sampling. Samples were taken with a soil corer at 20 cm depth, transported to the laboratory in less than 24 hours, and stored for less than a week at 4°C until processing.

2.2.2. Soil treatments

The experiment was performed in microcosms using clay pots (12 cm dia., 1500 cm^3) maintained in a growth chamber under controlled conditions, in a 16/8h light/dark photoperiod, with day and night temperatures of 26ºC/20ºC, respectively. The experimental design included 5 treatments, 4 replicates, 4 sampling dates and two types of soil (N=160). In the laboratory, each soil sample collected from the field was divided into four subsamples of 1 kg of fresh soil (henceforth referred to as samples), corresponding to the replicates of each treatment at the four different sampling dates, when samples were processed, for each habitat. The experimental design included five treatments, four replicates, four samplings and two type of soils (N=160). Forty samples corresponding to first sampling time before the application of the treatments (0), were processed directly, whilst the other 120 samples (5

treatments x 4 replicates x 2 soils x 3 sampling dates) were set into pots and soil treatments were applied (Fig 2.1.).

Soil treatments included two chemical and two organic pesticides, all applied at the recommended field dose by the manufacturer. Chemical pesticides were fenamiphos (emulsionable concentrate, 40% (w/v)), applied at 121 l ha^{-1}, and oxamyl (granules, 10% (w/w)), applied at 40 kg ha^{-1}. Both have an insecticide-nematicide activity. The first organic treatment was a microbial formulation composed of a mixture of *Metarhizium anisopliae*, an insect parasitoid fungus, *Trichoderma viride*, with fungicide action, and *Purpureocillium lilacinus (*formerly *Paecilomyces lilacinus)*, a nematophagous fungus, in a suspension of 10^9 CFU ml^{-1} applied at 2 ml l^{-1} (all active substances approved by the EU). The second one was a commercial pulp of neem (*Azadirachta indica* L.) seeds, applied at 1 g l^{-1}. A non-treated control was included in the experiment. Soil treatments were applied before transplanting the seedlings into the pots (one tomato plant per plot). Tomato (*Solanum* l*ycopersicum*) seedlings were transplanted after the waiting period established for each treatment to avoid phytotoxicity. All plants were watered daily and fertilized with a commercial NPK (6:4:6) fertilizer every two weeks during the experiment. The effects of soil treatments were assessed at 4 different sampling dates during 17 weeks before application of treatments, and 5, 11, and 17 weeks after treatment (WAT).

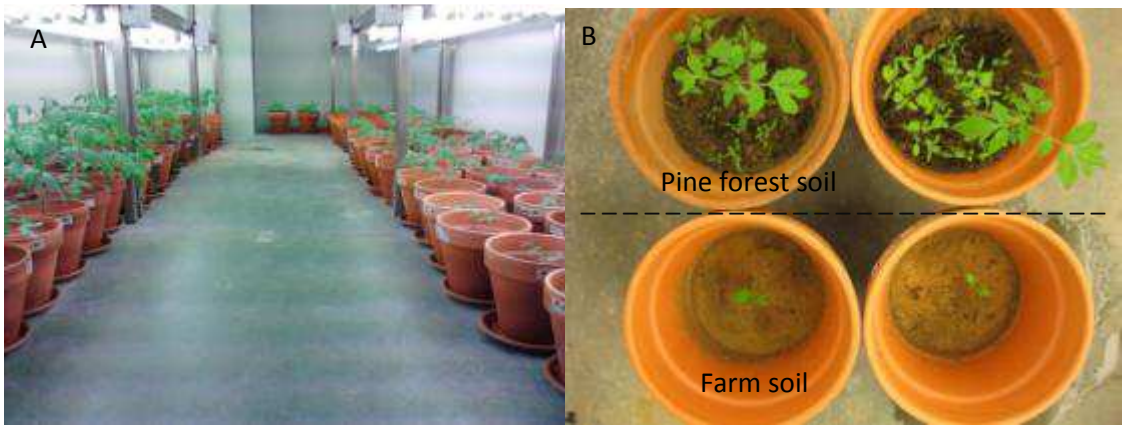


Figure 2.1. A, Clay pots in the growth chamber. **B**, The two different types of soil used in the experiment, from the strawberry farm and from a natural pine forest showing a large difference in plant growth within the same treatment.

2.2.3. Soil analyses

Samplings were destructive and the whole sample within each pot was processed at each sampling date. Each soil sample extracted from each pot was divided into a number of sub-samples. One subsample of about 150 g was air-dried and fresh and dry soil weights were recorded. Dry soil was sent to an external accredited laboratory where contents of macro and microelements (N, K, P, Mg, Na, Ca), organic matter content (OM), soil texture, electrical conductivity (EC) and soil pH were analyzed. Ca, P, Na, and K were extracted with $NH_4C_2H_3O_2$ and analyzed by atomic absorption spectroscopy (Ca, P) and by atomic emission spectroscopy (Na, K). Total soil nitrogen content was determined by the Kjeldahl (Radojevic & Bashkin, 1999) method, total organic matter content by the Walkley-Black method (Walkley & Black, 1934), and pH and electrical conductivity by potentiometry. Soil texture was determined for characterization purposes in six samples (three from each habitat) using a Bouyouco*s* densimeter.

A second subsample of 10 g was used to extract NO_3^- and NH_4^+ within 2 days after each destructive sampling. $NO_3^-$and NH_4^+ were extracted in the laboratory using a 2M KCl extraction solution, and stored frozen at -20ºC. Extractions were sent to the Nutrient Analyses Laboratory of the Rey Juan Carlos University, Spain, and NO_3^- and NH_4^+ were analyzed in a nutrient autoanalyzer. Two more subsamples were used for nematode faunal analysis (400 g fresh soil, section 2.2.5.).

2.2.4. Plant biomass

Root and shoot biomass of each plant was recorded at the last three sampling dates (Fig 2.2.). Entire plants (roots and shoots), and the soil composing the rhizosphere were processed as described in sections 2.2.3.-2.2.6., and used to relate plant biomass, soil nematode diversity, and soil physical-chemical properties. Roots and shoots were weighted, dried at 60ºC in an oven for 48 h, and dry weight was recorded. Since fruits were almost absent, they were excluded from the plant biomass analyses.

Figure 2.2. Dry root and shoot biomass from two different samples.

2.2.5. Nematode extraction and identification

Nematodes were extracted from 400 g of soil using the sieving and Baermann funnel method (Barker *et al.*, 1985). All nematodes were counted under the dissecting microscope, and at least 100 nematodes from each sample were identified to family or genus (Bongers, 1994) under the microscope, classified by trophic habit (Yeates *et al.*, 1993) and defined on the colonizer-persister (CP) scale (Bongers & Bongers, 1998). Depending on their source of food, nematodes were classified as bacterial feeders, fungal feeders, plant parasites/herbivores, omnivores, and predators (Yeates *et al.*, 1993). Since nematodes belonging to Tylenchidae might be herbivores or fungal feeders (Yeates *et al.*, 1993) half of the nematodes identified as Tylenchidae were considered as fungal feeders and half as herbivores in calculations of indices. Taxa Richness (S) was calculated as the average number of taxa in each sample, and total number of nematodes was expressed as number of individuals 100 (g of dry soil^{-1}). The Shannon Diversity Index H' (Shannon, 1949) was calculated to assess nematode diversity.

Soil food web indices (Ferris *et al.*, 2001), based on the abundance of functional guilds (Bongers & Bongers, 1998), were calculated to assess soil food web condition. The Structure Index (SI) indicates the level of food web complexity and responds rapidly to disturbance. The Channel Index (CI) is an indicator of predominant decomposition pathways mediated by fungi. The Basal Index (BI), based on the abundance of general opportunistic nematodes, is an indicator of basal, perturbed soil food web condition. The Enrichment Index (EI) is based on the abundance of enrichment opportunistic that arise from decomposition of organic matter

content (Ferris *et al*., 2001). In addition, the Maturity Index (MI), calculated as a weighted mean of the frequency of different c-p groups in the community, and the Plant Parasitic Index (PPI), indicator of the maturity of the plant-parasitic nematode community, were calculated (Bongers, 1990). The representation of the Structure Index and the Enrichment Index in a bidimensional plot permits classifying soil food web condition into four states: disturbed, maturing, structured, and degraded (Ferris *et al.*, 2001).

2.2.6. Statistical analysis

Due to lack of normality, all variables were to log (x+1) transformed before analyses. ANOVAs were used to detect significant influences of the type of soil, treatment and sampling date on nematode taxa, nematode community descriptors, soil food web indices and plant biomass. Differences between groups were analyzed by a *post-hoc*, Tukey test. Analyses were performed using STATISTICA software package (StatSoft, 2013). Pearson correlation analyses were used to examine associations between root and shoot biomass and soil food web indices, nematode community descriptors and soil physical-chemical properties.

2.3. RESULTS

2.3.1. Nematode community composition

Thirty two nematode taxa belonging to the five main trophic groups were identified, 23 occurring in the farm soil and 30 in the pine forest soil. Of the 32 nematode taxa, 15 were bacterial feeders, four fungal feeders, two omnivores, five predators, and six were plant-parasites or herbivores (Table 2.1). The bacterial-feeding nematodes *Rhabditis, Panagrolaimus*, *Acrobeles*, and *Acrobeloides*, and the fungal feeding-nematodes, *Aphelenchus* and *Aphelenchoides,* were the most abundant, together with dauer juveniles of Rhabditidae (inactive larvae that appear under unfavorable environmental conditions (Cassada and Russell, 1975)). Four taxa (*Rhabditis*, *Cruznema*, *Plectus* and *Aphelenchoides*) were more abundant in the farm than in the pine forest soils in at least one treatment, and 13 taxa (*Mesorhabditis*, *Panagrolaimus, Acrobeles, Acrobeloides, Eumonhystera, Prismatolaimus, Achromadora, Aphelenchus*, *Tylencholaimus*, Dorylaimidae, *Tripyla*, *Trichodorus* and Tylenchidae) were more abundant in pine forest than in farm soils in at least one treatment (Table 2.1). Neodiplogasteridae, *Teratocephalus*, *Tylolaimophorus*, *Paraxonchium*, *Mononchus, Paratylenchus* and *Pratylenchus* were not detected in the farm soil, while *Tylenchorynchus and Rotylenchus* were not detected in the pine forest soil. Averaged across all sampling dates, abundances of 21 nematode taxa, mostly microbivore, were significantly affected by soil type (Table 2.1.).

In general, the highest abundances of bacterial-feeding nematode taxa were found in the pots amended with neem (Table 2.1). Average numbers of *Rhabditis*, *Cruznema* and *Panagrolaimus* were significantly higher in the pots treated with neem than in the other treatments, while average number of nematodes belonging to *Plectus* showed the highest values in the pots treated with the microorganisms mixture ($P< 0.05$, data not shown). Averaged across all sampling dates, the abundances of ten nematode taxa were significantly affected by treatments while the interaction soil x treatment was significant for 18 taxa (both $P< 0.05$). Most of the affected taxa were microbivores (Table 2.1). *Aphelenchus*, one of the most abundant fungal-feeders, presented significantly higher abundances in the pine forest than in the farm soil in all treatments (Table 2.1).

Table 2.1. Average number of nematodes (100g dry soil)$^{-1}$ ±SE and total number of nematodes (100g dry soil)-^{1} ±SE in each treatment averaged across five sampling dates in each type of soil. Trophic group (TG) (Ba= bacterial feeders, Fu= fungal feeders, Pp= plant parasites and herbivores, P= predators, O= omnivores) and colonizer-persister group (CP) of nematode taxa are indicated. F: farm soil, P: pine forest soil. S, T, S*T: effect of the type of soil, treatment and the interaction soil x treatment on nematode taxa. ** P< 0.05; ns: not significant. Different letters indicate significant differences at *P*< 0.05 between pine forest and farm soil in each treatment.

			Fenamiphos		**Oxamyl**		**Microorganisms**		**Neem**		**Control**		**S**	**T**	**S*T**
	TG	**CP**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**			
Dauer juveniles	Ba	1	4.06	16.31	13.57**b**	3.97**a**	4.72	6.58	51.16**a**	1035.39**b**	1.77	10.42	**	**	**
			±3.53	±16.31	±9.27	±2.39	±4.72	±4.64	±33.74	±327.53	±1.31	±5.32			
Mesorhabditis	Ba	1	12.02**b**	0.16**a**	7.48**b**	1.12**a**	5.71**b**	1.23**a**	26.41	36.55	6.11	4.6	**	**	**
			±2.84	±0.12	±1.93	±0.55	±2.01	±0.62	±10.70	±21.86	± 2.24	±2.62			
Rhabditis	Ba	1	0.12	1.81	0.98	2.06	2.14	0.64	2.24**a**	89.97**b**	4.66	1.48	**	**	**
			±0.12	±1.10	±0.88	±1.24	±2.14	±0.35	±1.95	±31.59	±2 .71	±0.65			
Cruznema	Ba	1	0.00	0.00	0.05	0.25	0.00	0.16	0.23**a**	18.96**b**	0.59	3.28	**	**	**
			±0.00	±0.00	±0.05	±0.19	±0.00	±0.11	±0.23	±7.21	±0. 48	±2.67			
Panagrolaimus	Ba	1	185.31**b**	16.56**a**	103.85**b**	28.58**a**	153.71**b**	43.08**a**	599.44**b**	70.21**a**	161.79**b**	28.13**a**	**	**	**
			±74.92	±3.68	±19.97	±5.96	±18.04	±19.14	±202.64	±22 .64	±33.95	±8.59			
Neodiplogasteridae	Ba	1	0.79	0.00	0.00	0.00	0.00	0.00	2.03	0.00	0.00	0.00	**	ns	**
			±0.79	±0.00	±0.00	±0.00	±0.00	±0.00	±1.13	±0.00	±0. 00	±0.00			
Cervidellus	Ba	2	0.00	0.00	0.00	0.13	0.00	0.09	0.77	0.00	0.9	0.08	ns	ns	ns
			±0.00	±0.00	±0.00	±0.13	±0.00	±0.09	±0.77	±0.00	±0. 62	±0.08			
Acrobeles	Ba	2	54.94**b**	0.88**a**	16.35**b**	0.45**a**	27.57**b**	0.33**a**	49.08**b**	2.96**a**	27.45**b**	0.17**a**	**	**	**
			±23.74	±0.51	±5.06	±0.23	±4.85	±0.24	±15.38	±2.20	± 7.35	±0.17			
Acrobeloides	Ba	2	594.75	85.55	276.19	106.49	173.33	119.85	285.55**b**	108.60**a**	224.35**b**	63.81**a**	**	ns	**
			±498.46	±17.77	±120.36	±22.54	±41.91	±32.07	±86.49	±35.16	±53.12	±11.84			
Plectus	Ba	2	3.06	0.5	0.27	0.49	1.24**a**	13.42**b**	1.02	0.77	2.52	8.45	ns	**	**
			±1.58	±0.24	±0.27	±0.49	±0.92	±6.12	±0.70	±0.58	±1. 44	±4.24			
Wilsonema	Ba	2	0.37	0.07	0.83	0.00	1.40	0.77	1.58**b**	0.00**a**	1.56	0.00	**	ns	ns
			±0.28	±0.07	±0.58	±0.00	±0.59	±0.77	±0.84	±0.00	±0. 94	±0.00			

Table 2.1.Continued.

			Fenamiphos		Oxamyl		Microorganisms		Neem		Control		S	T	S*T
	TG	CP	P	F	P	F	P	F	P	F	P	F			
Eumonhystera	Ba	2	2.48**b** ±1.12	0.12**a** ±0.12	2.23**b** ±1.24	0.08**a** ±0.08	2.1 ±1.23	0.09 ±0.09	8.30**b** ±3.90	0.08**a** ±0.08	3.99**b** ±1. 83	0.14**a** ±0.12	**	ns	**
Prismatolaimus	Ba	3	12.87**b** ±8.06	0.25**a** ±0.21	2.77**b** ±1.21	0.00**a** ±0.00	11.91**b** ±2.82	0.02**a** ±0.02	4.91**b** ±2.67	0.00**a** ±0.00	7.95**b** ±1. 68	0.05**a** ±0.05	**	**	**
Achromadora	Ba	3	5.75**b** ±1.77	0.45**a** ±0.26	6.65**b** ±2.59	0.22**a** ±0.15	14.52**b** ±8.22	0.08**a** ±0.08	5.76**b** ±3.20	0.22**a** ±0.22	6.63**b** ±4. 68	0.16**a** ±0.16	**	ns	**
Teratocephalus	Ba	3	0.24 ±0.24	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.24 ±0.24	0.00 ±0.00	2.60 ±2.60	0.00 ±0.00	0.18 ±0. 18	0.00 ±0.00	ns	ns	ns
Metateratocephalus	Ba	3	0.00 ±0.00	0.00 ±0.00	0.89 ±0.89	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.43 ±0.43	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Aphelenchus	Fu	2	18.40**b** ±6.82	0.68**a** ±0.38	12.27**b** ±3.90	0.92**a** ±0.49	71.95**b** ±19.99	4.73**a** ±4.37	97.15**b** ±29.71	2.29**a** ±0.99	50.80**b** ± 14.94	2.04**a** ±1.07	**	**	**
Aphelenchoides	Fu	2	55.83 ±31.06	4.78 ±2.11	9.78 ±5.34	12.54 ±2.67	11.07 ±2.56	239.08 ±138.81	13.06**a** ±3.95	110.97**b** ±40.35	26.32 ±7.91	209.59 ±101.36	**	**	**
Tylolaimophorus	Fu	3	0.00 ±0.00	0.00 ±0.00	0.72 ±0.72	0.00 ±0.00	0.42 ±0.42	0.00 ±0.00	1.54 ±1.54	0.00 ±0.00	0.22 ±0. 22	0.00 ±0.00	ns	ns	ns
Tylencholaimus	Fu	4	3.46**b** ±1.08	0.04**a** ±0.04	2.14 ±0.94	0.42 ±0.30	1.19 ±0.57	0.23 ±0.23	11.45**b** ±6.11	0.00**a** ±0.00	2.19 ±1. 75	0.00 ±0.00	**	ns	**
Dorylaimidae	O	4	16.25**b** ±6.24	0.41**a** ±0.19	20.16**b** ±5.44	0.84**a** ±0.47	10.92**b** ±2.88	0.63**a** ±0.38	15.40**b** ±8.15	0.88**a** ±0.46	15.26**b** ±5. 43	1.13**a** ±0.63	**	ns	**
Paraxonchium	O	5	0.12 ±0.12	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.67 ±0.46	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.47 ±0. 47	0.00 ±0.00	**	ns	ns
Tripyla	P	3	2.60**b** ±1.07	0.00**a** ±0.00	1.64**b** ±0.66	0.14**a** ±0.10	1.76 ±1.07	0.09 ±0.09	7.97**b** ±3.56	0.00**a** ±0.00	1.38**b** ±0. 69	0.08**a** ±0.08	**	ns	**

Table 2.1.Continued.

			Fenamiphos		Oxamyl		Microorganisms		Neem		Control		S	T	S*T
	TG	**CP**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**			
Mononchus	P	4	0.00 ±0.00	0.00 ±0.00	0.09 ±0.09	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Clarkus	P	4	0.39 ±0.39	0.12 ±0.12	0.53 ±0.36	0.21 ±0.21	0 ±0.00	0.38 ±0.31	0 ±0.00	0.08 ±0.08	0.36 ±0. 36	0 ±0.00	ns	ns	ns
Anatonchus	P	4	0.5. ±0.50	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Discolaimus	P	5	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.28 ±0.28	0.08 ±0.08	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Paratylenchus	Pp	2	0.00 ±0.00	0.00 ±0.00	0.05 ±0.05	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Pratylenchus	Pp	3	0.00 ±0.00	0.00 ±0.00	1.15 ±0.88	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.60 ±0.41	0.00 ±0.00	0.47 ±0. 47	0.00 ±0.00	**	ns	ns
Tylenchorhynchus	Pp	3	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.34 ±0.34	ns	ns	ns
Rotylenchus	Pp	3	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.06 ±0.06	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0. 00	0.00 ±0.00	ns	ns	ns
Trichodorus	Pp	4	1.12 ±0.95	0.00 ±0.00	0.78 ±0.56	0.35 ±0.22	1.64**b** ±0.79	0.00**a** ±0.00	0.32 ±0.32	0.00 ±0.00	0.00 ±0. 00	0.08 ±0.08	**	ns	ns
Tylenchidae	Pp/Fu	2	7.60**b** ±3.27	0.18**a** ±0.12	5.14**b** ±1.67	0.91**a** ±0.32	7.86**b** ±2.35	0.98**a** ±0.48	8.30**b** ±1.99	1.56**a** ±0.74	11.01**b** ±3.71	1.70**a** ±0.57	**	ns	**
Total			410.71**b** ±63.55	118.62**a** ±31.74	545.03**b** ±145.08	150.85**a** ±26.33	462.67**b** ±54.04	412.55**a** ±150.63	1193.6 ±345.70	1421.9 ±373.55	534.29**b** ±78.37	318.54**a** ±101.45			

2.3.2. Soil physical-chemical properties

Organic matter content, soil N, the C:N ratio, and total contents of Ca, Mg, Na and K were significantly higher in pine forest than in farm soils, while P content was higher in the farm than in the pine forest soils (Table 2.2). The effect of treatment on soil physical-chemical properties was not significant ($P< 0.05$) for any of the variables while the effect of sampling date was significant ($P< 0.05$) for EC, pH, soil N, the C:N ratio, Ca, Na, NO_3^- and NH_4^+ (Table 2.2).

Table 2.2. Mean values ±SE of physical-chemical properties (EC: Electrical Conductivity, OM: Organic Matter, C:N: carbon to nitrogen ratio). Different letters mean significant differences at $P< 0.05$. Levels of significance between habitats are shown. The significance of the effect of treatment (T) and sampling date (SD) is indicated (ns: no significant, ** $P< 0.05$).

	F	P	T	SD
EC (ds/m)	0.07 ±0.01	0.07 ±0.00	ns	**
pH	6.06 ±0.03	6.08 ±0.04	ns	**
OM (%)	0.48**a** ±0.01	4.18**b** ±0.13	ns	ns
N (%)	0.15**a** ±0.01	0.32**b** ±0.02	ns	**
C:N	3.08**a** ±0.19	8.55**b** ±0.37	ns	**
P (ppm)	150.96**b** ±4.09	83.43**a** ±15.61	ns	ns
Ca (ppm)	1038.38**a** ±39.14	2028.13**b** ±105.37	ns	**
Mg (ppm)	176.08**a** ±4.23	649.43**b** ±19.22	ns	ns
Na (ppm)	119.04**a** ±15.36	193.60**b** ±18.72	ns	**
K (ppm)	160.36**a** ±11.37	311.58**b** ±14.69	ns	ns
NO_3^- (ppm)	0.60 ±0.09	0.41 ±0.04	ns	**
NH_4^+ (ppm)	0.92 ±0.06	0.90 ±0.05	ns	**

2.3.3. Nematode community descriptors and soil food web condition

In the farm soil, total number of nematodes decreased markedly 5 WAT in all treatments except in the pots amended with neem, increasing thereafter and remaining high throughout the whole experiment (Table 2.3). In the neem-amended pots, a large significant increase ($P < 0.05$) in nematode abundances was observed 5 WAT and, although abundances decreased thereafter, they were still high at the end of the experiment in farm soils (Table 2.3). Taxa Richness was highest before treatments were applied to the farm soils in three of the five treatments and did not recover the initial values by the last sampling date (Table 2.3). The Shannon Diversity Index was significantly different ($P< 0.05$) 5 and 17 WAT in the neem-amended pots. In the pots treated with oxamyl and neem the Structure Index decreased after soil treatment, without recovering the initial value at the end of the experiment (Table 2.3). The Channel Index decreased 5 and 11 WAT in the microorganism treatment and the control respectively, increase thereafter to give the highest values at the end of the experiment (Table 2.3).

In pine forest soils, nematode abundances decreased after treatment in the oxamyl and fenamiphos treatments (Table 2.4). Final nematode abundances surpassed initial values in all treatments except in the pots treated with fenamiphos and neem (Table 2.4). Taxa Richness did not decrease significantly after soil treatment, but tended to decrease towards the end of the experiment, as did *The Shannon* Diversity Index. The Enrichment Index increased in the pots treated with oxamyl, microorganisms and the non-treated control after treatments, recovering the initial values at the end of the experiment, when the Structure Index decreased in pots treated with fenamiphos and neem (Table 2.4). The Channel Index significantly decreased 11 WAT in the pots treated with oxamyl, increasing thereafter and recovering the initial values at the end of the experiment. The Basal Index showed the highest values at the last sampling date in the pots treated with fenamiphos, oxamyl and microorganisms. In the non-treated control, the Basal Index decreased 5 WAT, recovering the initial values at the end of the experiment. The Maturity Index decreased after treatment but recovered the initial values at the end of the experiment in the pots treated with microorganisms (Table 2.4).

Table 2.3.Mean values ±SE of nematode community descriptors (No.: total number of nematodes per (100 g dry soil)$^{-1}$; S: Taxa Richness, H': The Shannon's diversity index), soil food web indices (EI: Enrichment Index, SI: Structure Index, CI: Channel Index, BI: Basal Index), MI: Maturity Index and PPI: Plant Parasitic Index at different sampling dates ((0 (before soil treatment), 5, 11 and 17 weeks after treatment (WAT)) for each soil treatment (fenamiphos, oxamyl, microorganisms, neem) and control with no treatment in the samples with farm soil. Different letters indicate significant differences at $P< 0.05$.

	Fenamiphos				Oxamyl				Microorganisms				Neem				Control			
	0	**5WAT**	**11WAT**	**17WAT**	**0**	**5WAT**	**11WAT**	**17WAT**	**0**	**5WAT**	**11WAT**	**17WAT**	**0**	**5WAT**	**11WAT**	**17WAT**	**0**	**5WAT**	**11WAT**	**17WAT**
No.	123.14**b**	14.86**a**	77.01**b**	259.46**b**	132.89**b**	32.84**a**	199.16**b**	238.52**b**	114.52**ab**	66.21**a**	926.06**b**	543.40**b**	179.35**a**	2750.76**b**	1945.52**b**	811.98**ab**	155.05**b**	37.74**a**	236.62**b**	844.77**c**
	±23.76	±3.52	±21.34	±91.26	±17.09	±12.18	±61.06	±40.09	±13.37	±13.30	±472.48	±258.90	±62.12	±948.94	±691.50	±299.30	±37.30	±7.06	±109.05	±250.53
S	7.50**c**	2.75**a**	3.25**ab**	5.00**bc**	7.50	4.75	5.00	6.00	8.75	5.00	4.00	5.25	8.50**b**	5.50**a**	5.75**ab**	5.25**a**	8.25**b**	5.25**a**	5.75**ab**	5.50**ab**
	±0.29	±0.75	±0.25	±0.41	±1.19	±0.25	±1.08	±1.00	±0.75	±1.73	±0.41	±1.03	±0.50	±0.29	±0.48	±0.85	±0.63	±0.75	±0.25	±0.65
H'	1.08	0.57	0.40	0.69	1.04	1.10	0.89	1.01	1.22	0.88	0.73	1.03	1.21**ab**	1.44**b**	1.19**ab**	0.84**a**	1.07	0.85	1.15	0.85
	±0.10	±0.22	±0.12	±0.13	±0.14	±0.07	±0.29	±0.12	±0.16	±0.13	±0.20	±0.17	±0.10	±0.09	±0.11	±0.24	±0.10	±0.15	±0.14	±0.29
EI	53.64	29.11	31.96	41.02	51.81	56.69	49.54	61.40	52.38	37.83	52.41	54.07	60.23	87.76	79.77	70.75	58.22	43.95	57.33	58.11
	±2.66	±16.82	±12.70	±8.28	±5.52	±2.56	±13.12	±2.84	±7.50	±13.34	±13.51	±3.54	±5.80	±4.57	±7.85	±9.57	±5.82	±7.38	±4.00	±4.48
SI	7.78	6.92	3.83	1.99	9.36**c**	0.00**a**	0.78**ab**	7.00**bc**	17.08	10.42	0.00	1.00	13.90**b**	0.00**a**	0.00**a**	2.71**a**	4.17	11.16	3.16	0.00
	±2.51	±4.72	±2.26	±1.15	±2.86	±0.00	±0.78	±3.36	±11.20	±10.42	±0.00	±1.00	±4.96	±0.00	±0.00	±2.71	±2.41	±11.16	±1.83	±0.00
CI	9.01	2.14	6.19	9.40	11.04	32.37	6.38	7.55	12.89**ab**	5.57**a**	30.91**ab**	57.35**b**	7.28	1.96	21.43	43.71	5.94**a**	2.43**a**	40.94**b**	61.78**b**
	±0.76	±2.14	±4.74	±6.79	±1.78	±8.51	±2.89	±3.58	±2.84	±2.88	±22.01	±10.21	±1.89	±0.29	±9.16	±19.69	±2.01	±1.25	±11.40	±17.34
BI	44.72	69.60	67.03	58.03	46.06	43.31	50.28	37.59	44.89	57.73	47.59	45.71	37.80	12.24	20.23	29.11	40.93	53.21	42.13	41.89
	±2.94	±17.59	±12.97	±7.82	±5.51	±2.56	±13.14	±3.04	±8.50	±14.34	±13.51	±3.53	±5.89	±4.57	±7.85	±9.61	±5.41	±9.28	±4.07	±4.48
MI	1.83	1.91	1.89	1.86	1.84	1.82	1.78	1.76	1.89	1.92	1.80	1.89	1.79	1.34	1.50	1.67	1.76	1.90	1.84	1.87
	±0.01	±0.05	±0.06	±0.05	±0.03	±0.01	±0.08	±0.01	±0.02	±0.10	±0.10	±0.03	±0.03	±0.08	±0.17	±0.18	±0.05	±0.06	±0.04	±0.06
PPI	0.00	0.00	0.00	0.00	0.75	2.00	0.83	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.75	0.00	0.00
	±0.00	±0.00	±0.00	±0.00	±0.75	±1.15	±0.83	±0.00	±0.50	±0.00	±0.00	±0.00	±0.00	±0.00	±0.00	±0.00	±1.00	±0.75	±0.00	±0.00

Table 2.4. Mean values ±SE of nematode community descriptors (No.: total number of nematodes per (100 g dry soil)$^{-1}$; S: Taxa Richness, H': The Shannon's diversity index), soil food web indices (EI: Enrichment Index, SI: Structure Index, CI: Channel Index, BI: Basal Index), MI: Maturity Index and PPI: Plant Parasitic Index) at different sampling dates ((0 (before soil treatment), 5, 11 and 17 weeks after treatment (WAT)) for each soil treatment (fenamiphos, oxamyl, microorganisms, neem) and control with no treatment in the samples with pine forest soil. Different letters indicate significant differences at $P< 0.05$.

	Fenamiphos				Oxamyl				Microorganisms				Neem				Control			
	0	5WAT	11WAT	17WAT	0	5WAT	11WAT	17WAT	0	5WAT	11WAT	17WAT	0	5WAT	11WAT	17WAT	0	5WAT	11WAT	17WAT
No.	397.65**ab**	393.27**ab**	165.45**a**	649.41**b**	384.79**b**	89.77**a**	258.87**b**	1446.70**c**	343.44**a**	353.71**a**	377.07**a**	776.46**b**	360.40	1213.71	2025.60	1174.70	238.41**a**	769.20**b**	317.20**a**	812.37**b**
	±65.60	±77.70	±62.81	±137.95	±86.80	±17.74	±72.31	±178.68	±68.15	±82.01	±31.01	±46.09	±75.04	±227.16	±1349.06	±224.74	±14.48	±95.49	±90.55	±127.46
S	12.75**b**	12.00**ab**	7.25**a**	8.50**ab**	14.00**b**	10.75**ab**	7.00**a**	8.75**ab**	12.50**b**	11.00**b**	7.50**a**	8.00**a**	11.50	10.75	7.75	8.25	12.00**b**	11.75**b**	6.75**a**	7.75**a**
	±0.63	±1.53	±0.85	±1.32	±0.41	±1.31	±1.22	±1.31	±0.65	±0.41	±0.65	±0.91	±0.29	±0.85	±0.75	±1.38	±0.71	±0.95	±0.48	±0.85
H'	2.01	1.58	1.46	1.32	2.00**c**	1.64**bc**	1.18**ab**	0.92**a**	2.05**b**	1.67**ab**	1.35**a**	1.35**a**	1.91**b**	1.59**ab**	1.32**ab**	1.04**a**	1.95**b**	1.71**ab**	1.29**a**	1.29**a**
	±0.09	±0.18	±0.18	±0.20	±0.08	±0.17	±0.20	±0.14	±0.09	±0.07	±0.04	±0.19	±0.02	±0.15	±0.09	±0.21	±0.10	±0.16	±0.03	±0.17
EI	77.49	80.09	70.30	61.67	75.62**ab**	81.79**b**	78.16**ab**	45.49**a**	66.32**ab**	81.01**b**	79.53**ab**	62.80**a**	70.10	87.63	82.02	82.36	65.40**ab**	81.08**b**	71.68**b**	55.73**a**
	±0.83	±1.79	±3.70	±8.21	±4.13	±2.51	±12.38	±8.49	±4.55	±0.90	±3.35	±4.93	±1.71	±3.12	±4.09	±6.88	±2.25	±1.09	±3.34	±5.79
SI	50.75**b**	39.04**ab**	25.96**ab**	10.12**a**	52.35	47.72	48.28	12.57	29.54	48.55	20.25	18.50	14.83**ab**	47.95**b**	22.00**ab**	9.90**a**	24.46	39.06	21.17	12.09
	±6.64	±17.40	±3.47	±5.44	±3.81	±17.04	±12.57	±2.20	±6.87	±8.84	±7.19	±8.89	±2.70	±9.50	±11.84	±2.47	±8.23	±11.04	±5.92	±5.89
CI	7.93	9.73	5.50	7.80	7.23**b**	4.61**b**	0.28**a**	7.27**b**	15.64	6.28	6.27	17.17	10.33	4.40	8.04	4.46	13.64	6.31	9.09	20.82
	±1.16	±6.28	±3.21	±1.32	±0.76	±1.75	±0.16	±1.87	±1.94	±1.31	±2.04	±4.81	±2.20	±2.34	±3.58	±2.39	±2.98	±1.72	±4.83	±8.71
BI	17.85**a**	17.39**a**	26.85**ab**	36.92**b**	18.72**ab**	14.44**a**	17.80**a**	50.10**b**	29.83**ab**	15.74**a**	19.36**ab**	34.76**b**	28.35	10.94	17.31	17.33	31.12**b**	16.27**a**	26.58**ab**	41.74**b**
	±0.49	±2.51	±3.28	±8.17	±2.70	±2.11	±9.62	±7.45	±4.79	±1.29	±3.12	±5.68	±1.42	±2.85	±4.19	±6.79	±2.89	±0.98	±3.56	±5.83
MI	1.88	1.76	1.76	1.75	1.90	1.83	1.72	1.88	1.86**b**	1.79**ab**	1.60**a**	1.83**ab**	1.72	1.59	1.57	1.45	1.84	1.72	1.72	1.85
	±0.08	±0.08	±0.04	±0.06	±0.11	±0.16	±0.16	±0.07	±0.04	±0.09	±0.06	±0.04	±0.03	±0.11	±0.06	±0.13	±0.04	±0.12	±0.03	±0.05
PPI	1.31	0.00	0.00	0.00	2.14	1.74	0.00	0.75	2.05	1.40	1.00	0.00	0.00	0.88	0.00	0.63	0.00	0.63	0.00	0.00
	±0.80	±0.00	±0.00	±0.00	±0.72	±0.60	±0.00	±0.75	±0.71	±0.87	±1.00	±0.00	±0.00	±0.88	±0.00	±0.63	±0.00	±0.63	±0.00	±0.00

Soil food web condition evolved differently in farm and pine forest soils (Fig.2.1). In farm soils, the structure component of the soil food web declined after soil treatment and slightly improved in the non-treated control (Fig. 2.3), presenting very low values at last sampling date. By contrast, in pine forest soils the Structure Index, that indicates the level of food web complexity and responds rapidly to disturbance, initially increased in the pots treated with microorganisms, neem, and in the non-treated control, and decreased in the chemical treatments (Fig. 2.3). In farm soils, the enrichment component of the soil food web, based on the abundance of enrichment opportunistic that arise from decomposition of organic matter content web, decreased in soils treated with fenamiphos, microorganisms, and in the non-treated control, recovering the initial values at the last sampling date (Fig. 2.3). In pine forest soils, the Enrichment Index increased in all treatments 5 WAT, and decreased thereafter below the initial levels in the chemical treatments and the non-treated control (Fig. 2.3).

Irrespectively of the sampling date, comparisons among treatments show that the Channel Index values were highest ($P< 0.05$) in the pots treated with microorganisms in the farm soil, and in the microorganisms and the non-treated control in the pine forest soil. The lowest values of the Channel Index were found in the chemical treatments, oxamyl and fenamiphos, in pine forest and farm soils respectively (Fig. 2.4). The Basal Index showed the lowest value in the pots amended with neem in both types of soil. The Maturity Index was significantly lower in the pots amended with neem than in the other treatments in the pine forest and farm soils, while the Shannon Diversity Index was higher in the neem treatment than in the fenamiphos and control treatments in the farm soils. The Plant Parasitic Index and Taxa Richness did not show significant differences between treatments in any of the type of soils (Fig. 2.4).

The soil source significantly affected ($P< 0.05$) the Basal Index, which was lower in pine forest than in farm soils ($P< 0.001$), and Taxa Richness and the Shannon Diversity Index, which were higher in pine forest than in farm soils ($P< 0.001$) (data not shown).

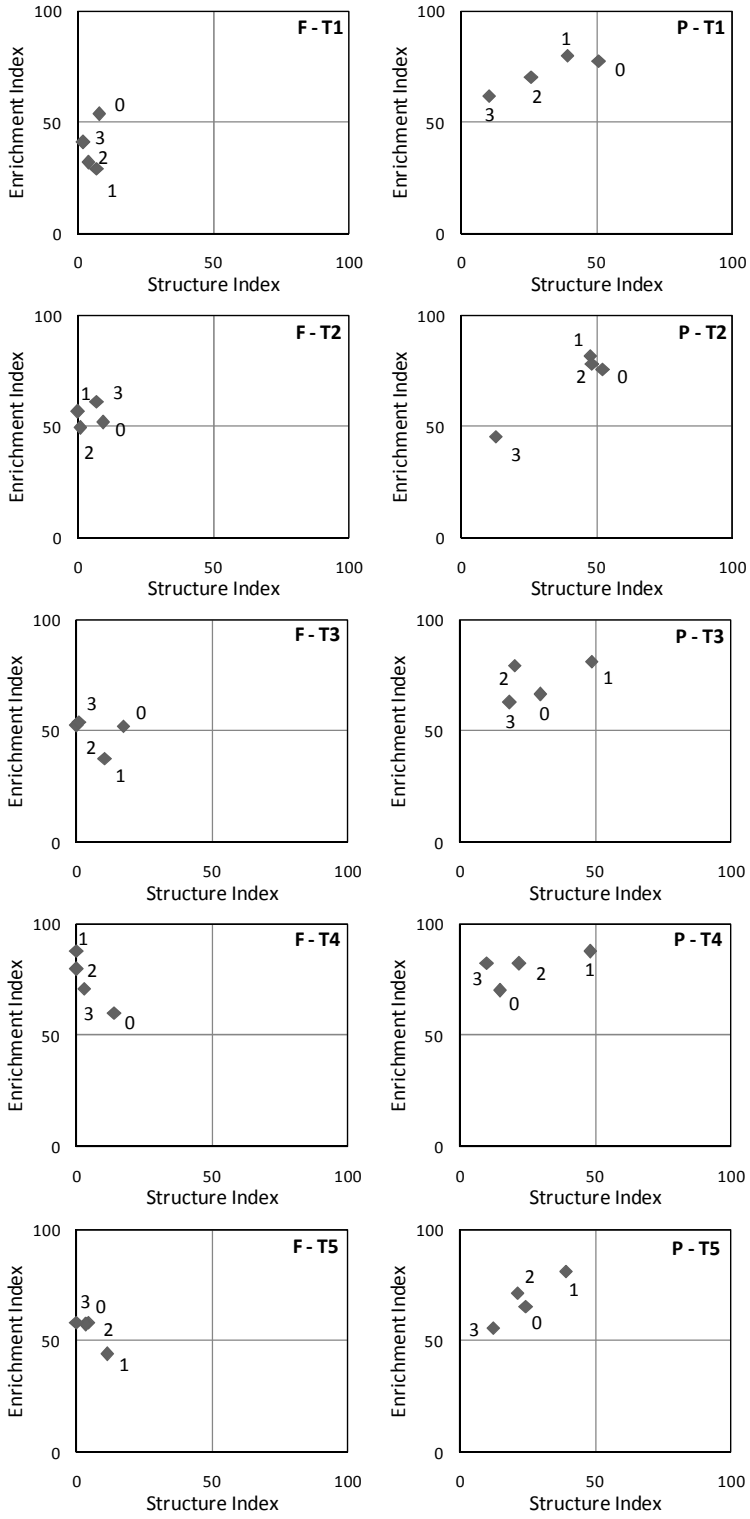


Figure 2.3. Evolution of nematode food web condition in farm (F) and pine forest (P) soils. Dots represent the Enrichment and Structure Indices scores at four sampling dates (0, 1, 2, 3: before treatment and 5, 11, and 17 weeks after treatment respectively; T1: fenamiphos, T2: oxamyl, T3: microorganisms, T4: neem, T5: non-treated control).

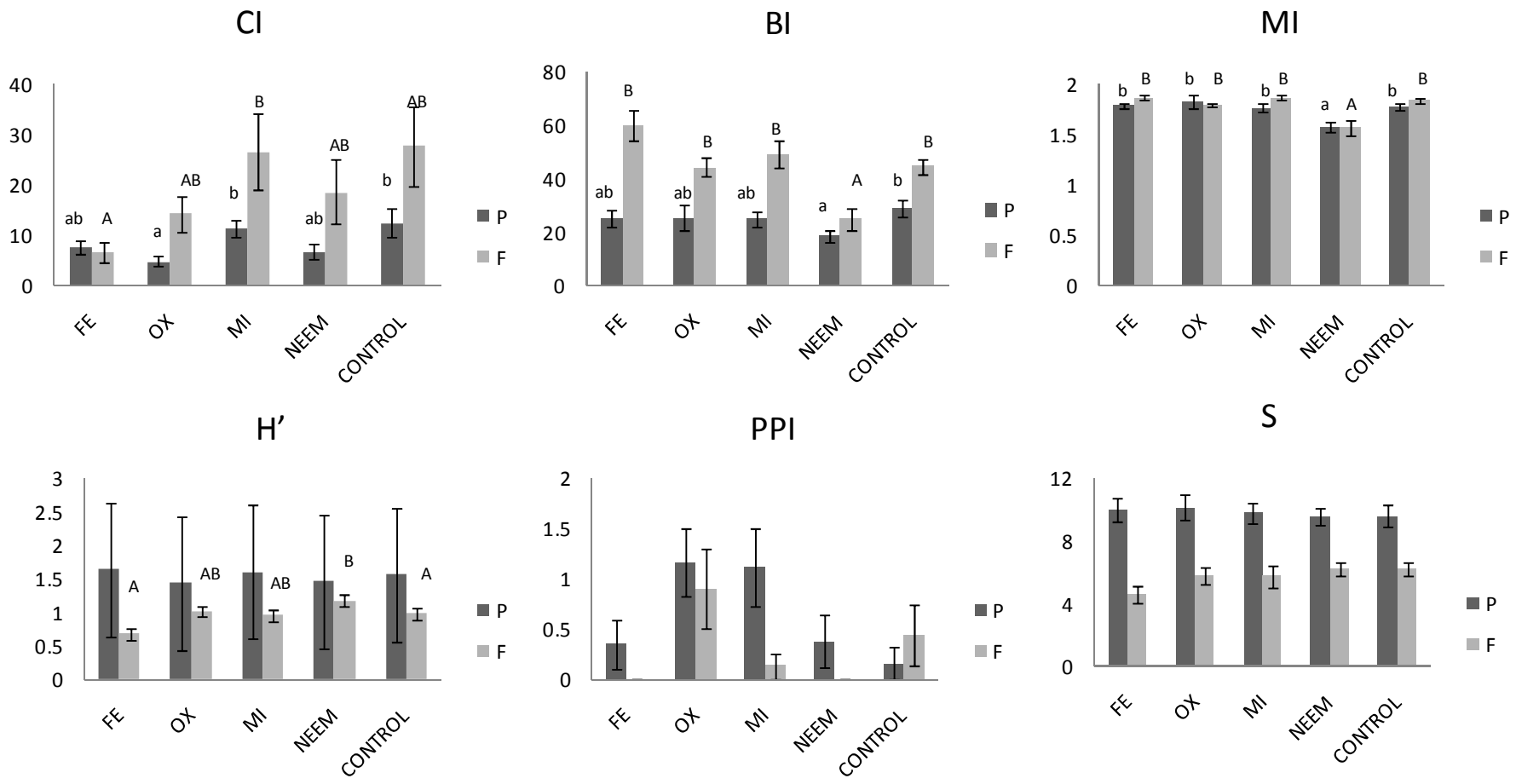


Figure 2.4. Mean values of the Channel Index (CI), Basal Index (BI),Maturity Index (MI), The Shannon's diversity index (H'), Plant Parasitic Index (PPI) and Taxa Richness (S) in pine forest (P) and farm soils (F). Different letters mean significant differences ($P < 0.05$) among treatments in pine forest (lower-case) and farm (upper-case) soils. FE: fenamiphos, OX: oxamyl, MI: microorganisms, NEEM: neem, CONTROL: non-treated control. Error bars show ±SE.

Remarkable changes in the bacterial-feeding nematode populations were observed along sampling dates in the neem treatment (Fig. 2.5). Total number of bacterial-feeding nematodes with CP values 2-5 increased after soil treatment in both types of soils. Afterwards, they continued increasing, reaching the highest values 11 WAT in pine forest soils, but decreased in farm soils giving the lowest values at the end of the experiment. Final abundances of these nematodes were more than two-fold higher in the pine forest than farm soils. *Dauer* juveniles increased markedly after treatment in the farm soil, reaching abundances of more than 2000 nematodes 100 (g dry soil)$^{-1}$ 5 WAT and decrease thereafter, while a slight increase was observed after neem addition in pine forest soils. The mean number of dauer juveniles was more than ten-fold higher in the farm than in the pine forest soils 5 WAT. The number of Rhabditidae also increased after treatment and decreased thereafter in both types of soils (Fig. 2.5).

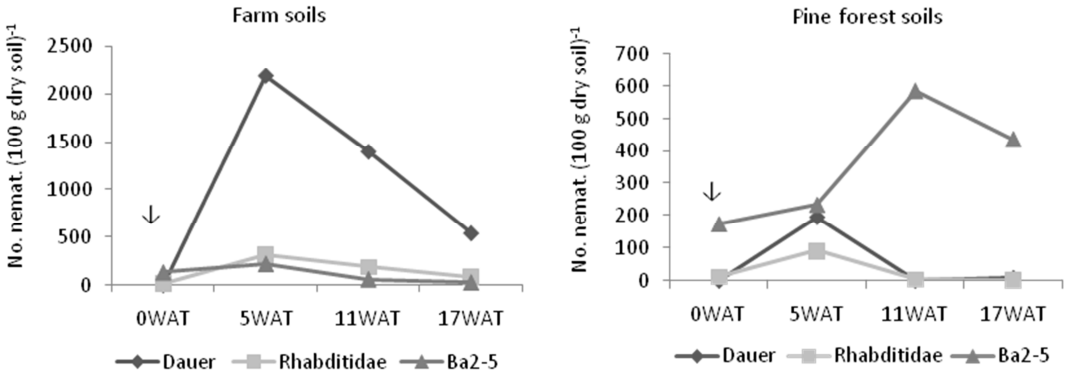


Figure 2.5. Average number of bacterial feeding nematodes (CP 2-5), dauer juveniles and Rhabditidae (No. nemat. (100 g dry soil)-^{1}) at different sampling dates (0: before treatment; 5, 11 and 17 weeks after treatment (WAT)) in farm and pine forest soils treated with neem. Arrows indicate time of treatment application.

2.3.4. Relationship between physical-chemical properties, soil food web condition and plant biomass

In both types of soil, shoot and root biomass significantly differed among treatments 5 WAT and 5 and 11 WAT, respectively (Fig. 2.6 A-D). Shoot and root biomass were significantly higher (P < 0.05) in the neem than in the other treatments (P< 0.05, data not shown). Average shoot biomass was 2-fold higher than root biomass at the last sampling time in the pots with both type of soil (Fig. 2.6 A-D). Both root and shoot biomass increased along sampling dates, although root biomass stabilized at 11 WAT in farm soils (Fig. 2.6 B). At the end of the experiment, shoot biomass was significantly lower in the farm than in the pine forest soil in pots treated with fenamiphos, oxamyl and in the non-treated control, while no significant differences (P< 0.05) were observed between habitats in shoot biomass in the microorganisms and neem treatments (Fig. 2.6E). Root biomass was significantly lower in farm than in pine forest soils in pots treated with fenamiphos, neem and in the non-treated control, while no significant differences (P< 0.05) were observed between habitats in the oxamyl and microorganisms treatments (Fig. 2.6 F).

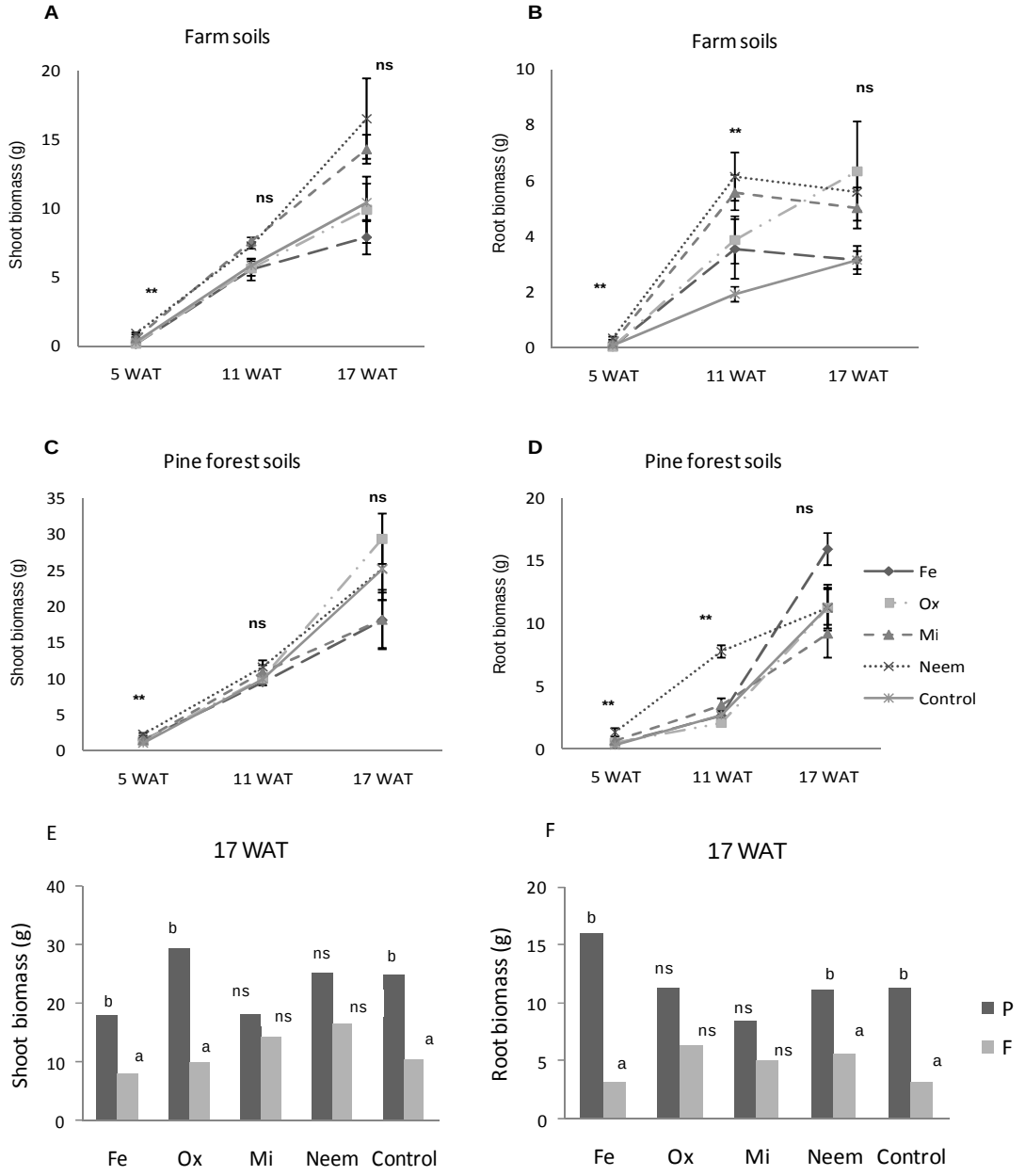


Figure 2.6. A-D: Average shoot and root dry biomass at three different sampling dates (5, 11, and 17 weeks after treatment (WAT)) in pots with farm and pine forest soils. **E, F**: Average shoot and root dry biomass in each treatment in pots with farm and pine forest soils 17 WAT. Fe: fenamiphos, Ox: oxamyl, Mi: microorganism, Neem: neem, Control: non-treated control. ** Significant differences (P< 0.05) among treatments at each sampling date; ns: no significant differences. Different letters indicate significantly different (P> 0.05) biomasses in farm and pine forest soils at each treatment at the end of the experiment. Error bars show ±SE.

Shoot and root biomass were, at the beginning and at the end of the experiment, positively related to electrical conductivity (EC), organic matter content, N, ratio C:N, Ca, Mg and Na, as was shoot biomass at 11 WAT (except to EC). pH showed opposite relationships to shoot and root biomass, which were also negatively related to phosphorous 5 and 11 WAT and 5 WAT respectively, and positively related to potassium 17 WAT. Some positive correlations were

observed between shoot and root biomass and NO_3^-, while root biomass was positively related to NH_4^+ 5 WAT (Table 2.5).Positive correlations ($P< 0.05$) were observed between the Enrichment Index, the Structure Index, Taxa Richness, The Shannon diversity Index, and total nematode abundance with shoot and root biomass 5 WAT and with shoot biomass 11 WAT. Such relationships only lasted until the end of the experiment for the Structure Index and nematode richness and abundance, and for root biomass in the case of the Shannon diversity Index. The Basal and the Maturity Index were negatively related to shoot and root biomass at the beginning of the experiment and shoot biomass 11 WAT for the Basal Index (Table 2.5).

Table 2.5. Correlation coefficients between dry plant biomass (SB: shoot biomass; RB: root biomass, both in g), physical-chemical properties (EC: electrical conductivity, OM: organic matter content, C:N: ratio carbon nitrogen), soil food web indices (EI: Enrichment Index, SI: Structure Index, CI: Channel Index, BI: Basal Index), MI: Maturity Index and PPI: Plant Parasitic Index and nematode community descriptors (S: Taxa Richness; H': The Shannon diversity index, No.: total number of nematodes (100 g dry soil)$^{-1}$ at each sampling date 5, 11 and 17 weeks after treatment (WAT). All values given indicate significant relationships (P< 0.05), ns: not significant.

	5 WAT		11 WAT		17 WAT	
	SB	**RB**	**SB**	**RB**	**SB**	**RB**
EC (ds m^{-1})	**0.47**	**0.39**	Ns	ns	**0.42**	**0.69**
pH	**-0.53**	**-0.46**	**-0.35**	ns	**-0.37**	**-0.43**
OM (%)	**0.83**	**0.64**	**0.83**	ns	**0.58**	**0.78**
N (%)	**0.82**	**0.61**	**0.34**	ns	**0.52**	**0.63**
C:N	**0.69**	**0.53**	**0.74**	ns	**0.38**	**0.52**
P (ppm)	**-0.73**	**-0.57**	**-0.44**	ns	ns	Ns
Ca (ppm)	**0.76**	**0.56**	**0.76**	ns	**0.58**	**0.77**
Mg (ppm)	**0.81**	**0.61**	**0.81**	ns	**0.54**	**0.82**
Na (ppm)	**0.70**	**0.47**	**0.50**	ns	**0.56**	**0.73**
K (ppm)	ns	ns	Ns	ns	**0.40**	**0.55**
NO_3^-(ppm)	**0.53**	**0.55**	**0.61**	ns	ns	**0.52**
NH_4^+(ppm)	ns	**0.25**	Ns	ns	ns	Ns
EI	**0.46**	**0.35**	**0.43**	ns	ns	Ns
SI	**0.61**	**0.49**	**0.60**	**-0.32**	**0.34**	**0.48**
CI	ns	ns	Ns	ns	ns	Ns
BI	**-0.78**	**-0.59**	**-0.45**	ns	ns	Ns
MI	**-0.32**	ns	Ns	ns	ns	Ns
PPI	ns	ns	Ns	ns	ns	Ns
S	**0.73**	**0.58**	**0.55**	ns	**0.49**	**0.50**
H'	**0.67**	**0.54**	**0.49**	ns	ns	**0.41**
No.	**0.79**	**0.65**	**0.31**	**0.32**	**0.58**	**0.46**

2.4. DISCUSSION

2.4.1. Nematode community descriptors and soil food web condition

Previous studies on the effects of chemical and non-chemical treatments on the interaction between nematode functional guilds and soil properties, such as biochemical parameters, have shown that chemical disinfection affects such interaction to a larger extent than organic disinfection (Kapagianni *et al.*, 2010). Soil nematicides reduce numbers of nematodes from all trophic groups (Timper *et al.*, 2012) and chemical nematicides, such as fenamiphos and oxamyl, have a marked effect on total nematode abundances compared to other treatments (Nordmeyer *et al.*, 1989; Chabrier *et al.*, 2005). Fenamiphos is reported to be an effective tool for controlling soil pest nematodes, but might reduce as well non-target nematodes, such as beneficial free-living nematodes (Langat *et al.*, 2008). As expected, our results show relevant differences in the nematode response to nematicide use depended not only on the identity of the nematicide used, but also on the initial condition of the nematode community and soil properties. In our experiment, nematode abundances in pots with farm soil decreased significantly both after chemical treatments and in the non-treated control, also showing the detrimental effect of potting in the absence of any soil treatment. By contrast, in pine forest soils only oxamyl reduced nematode abundances, and potting did not have such a detrimental effect. Similarly, Taxa Richness and food web complexity decreased sharply after various treatments or potting in farm, but not in pine forest soils. Soil resilience has been linked to numerous soil attributes, depending on the group of organisms, or functions, subjected to perturbation. For example, microbial resilience has been linked to soil physical-chemical properties (Griffiths & Philippot, 2013), and, more specifically, soil resilience measured as carbon utilization profiles has been recently linked to low soil P (Wakelin et al., 2013). In Mediterranean ecosystems, plant resilience to disturbance has been linked to ecological diversity (Lavorel, 1999). Functional diversity of nematodes, on the other hand, has been previously associated with the ability of nematode communities to persist after perturbation (Ferris *et al*., 2001), and, in soil systems, organic matter may play a role in protecting soil fauna and its functions (Ponge *et al*., 2013). In our experiment, pine forest soil possesses several of these attributes. Differential soil properties including high organic matter and low soil P content, and a more complex soil food web, might have contributed equally to higher soil resilience to both application of pesticides and physical disruption by potting in pine forest soils. In addition, low perturbation in pine forest soils might have supported by some sensitive nematodes such as the plant parasites *Trichodorus*, which did not appear in farm soils.

An active fungal-mediated decomposition channel was observed in the microorganism, neem and control treatments in farm soil, due to high abundances of *Aphelenchoides*. The Channel Index was two-fold higher in the farm soil than in the pine forest soil, suggesting a low participation of fungi on organic matter decomposition in natural soil and the occurrence of organic enrichment processes, which may be related to high levels of available mineralized nitrogen (Ferris & Matute, 2003) in these treatments.

In this study, bacterial and fungal feeding nematodes were the most abundant taxa in all treatments, but especially in the neem-amended pots. The effects of different neem compounds on free living nematodes have been previously studied (Akhtar, 1998; Abbasi *et al.*, 2005), but, depending on the study, its application either reduced (Akhtar, 1998), or enhanced the total number of free-living nematodes (Akhtar & Mahmood, 1996). Large increases of bacterial-feeding nematodes in amended soils are often related to the bloom of populations of bacteria that follows the incorporation of organic matter, which is in turn followed by an increase of bacterial-feeding nematodes (Zelenev *et al.*, 2004). The large increase of bacterial-feeding nematodes in the pots amended with neem, however, might not been explained by organic matter addition, since the amount of organic matter incorporated into each pot (around 0.3 g of organic matter) seemed hardly sufficient to support such bloom, and soil organic matter content did not significantly increase after neem addition, neither was it significantly different from the other treatments. Indeed, the extraordinary increase of nematode numbers in the nutrient-depleted farm soils was mostly due to the appearance of dauer juveniles, which, after neem addition, reached a maximum mean abundance of 2200 nematodes (100g soil)$^{-1}$, a ten-fold higher increase than the one that occurred in pine forest soils. Nematode dauer juveniles, which might be a pre-adaptation to parasitism (Crook, 2014), are resistant, dispersal stages of free-living Rhabditidae that occur when food resources decline (Cassada & Russell, 1975). Dauers are also the infective juveniles of entomopathogenic nematodes, whose emergence from insect cadavers is triggered by high levels on ammonia (San-Blas *et al.*, 2008). By contrast, in the pine forest soil bacterial-feeding nematodes with CP 2-5 increased almost three times more than in the farm soil. The occurrence of dauers is a product of food exhaustion (Green & Harvey, 2012), and they may appear as soon as 2 days after organic matter addition into the soil (Zelenev *et al*., 2004). Incorporation of N-rich plant biomass into the soil induces dauer formation (Van Eekeren *et al.*, 2009). The results of this study confirm that the sudden increase in dauers occurred in the nutrient-depleted soil, while the same treatment in the pine forest soil, rich in organic matter, induced the increase of more stable, less opportunistic, populations of generalist bacterivore

nematodes. In addition to the effect of the neem addition on the population and performance of bacterial-feeding nematodes, the neem treatment also resulted in the highest diversity values, although such positive effects did not include a positive effect on the soil food web maturity, which was significantly lower in the neem-amended pots than in the other treatments.

Soil treatments largely affected the abundances of some fungal-feeding (CP-2) nematodes such as *Aphelenchus* and *Aphelenchoides*, which increased in the pots treated with microorganisms. Although *P. lilacinus,* one of the components of this treatment, is an effective nematophagous fungus (Hashem & Abo-Elyousr, 2011; Pandey *et al.*, 2011; Sances *et al.*, 2012), the other two fungi present in the composition of the product, *T. viride* and *M. anisopliae,* may have served as food resource for these fungal-feeding nematodes. Previous studies (Hasna *et al*., 2007) have shown that fungivore nematodes such as *Aphelenchus avenae* and *Aphelenchoides* spp. are attracted to fungivore fungus such as *T. harzianum*. To our knowledge, previous studies on the ecology of these fungi had focused only on their effects against root-knot nematodes (Jegathambigai *et al.*, 2008), without testing the effects on other nematode taxa as free-living nematodes.

Plant-parasitic nematodes were almost absent in all treatments, especially in the farm soil, and the Plant Parasitic Index gave in general very low values. According to Bongers (1990) and Bongers & Bongers (1998), plant-parasitic and free-living nematodes often give opposite responses to soil organic enrichment, so organic enrichment induces an increase in the Plant Parasitic Index and a decrease in the Maturity Index. Such an opposite relationship was not detected in this study. Although the Plant Parasitic Index varied greatly among treatments, such variation was irregular and not significant ($P > 0.05$).

2.4.2. Relationship between physical-chemical properties, soil food web condition and plant biomass

Soil C, N, and all microelements except P showed higher values in the pine forest than in the farm soil. Despite the continuous influx of nutrients through nitrogen-phosphorous-potassium fertigation into the farm soil before soil collection, it contained lower amounts of micro and macronutrients than pine forest soils. Higher values of total nitrogen in the pine forest than in the farm soil might be partially related to higher micro and mesofauna abundances, since it has been proved that nematode excretion, for example, contributes significantly to soluble soil

nitrogen (Ekschmitt *et al.*, 1999). Moreover, abundances of bacterial- and fungal-feeding nematode are often positively correlated to the amount of NH_4^+ excreted into the soil (De Ruiter *et al.*, 1993; Neher *et al.*, 2012), and were, in this experiment, the most abundant nematode taxa in both types of soil. Also despite the organic matter incorporated into the farm soil at the beginning of the cropping season in the form of manure, both organic matter content and the ratio C:N were lower in the farm than in the pine forest soil, probably due to the low capacity of agricultural soils to retain carbon in complex soil carbon forms. It has been shown that cropped soils around the world are depleted of their soil organic carbon between 25 and 75% depending on the climate, soil type, and management (Lal, 2011).

The neem treatment increased root and shoot biomass, which significantly differed among treatments 5 WAT in both types of soil, and these differences were still noticeable 11 WAT for root biomass. Organic matter content was not significantly different ($P > 0.05$) between sampling dates in the neem-amended pots, so such differences cannot be attributed to this factor. However, since neem compounds have been used to fight against a wide range of pests (Akhtar, 1998; Dwivedi, 2008; Al-Samarrai *et al*., 2012), such pesticide activity might, at least partially, explain the higher plant biomass in the neem than in the other treatments. Bjornlund *et al.* (2012) showed that bacterial feeders might increase plant performance when nutrients are limited; similarly in the present study, the pots treated with neem and microorganisms had the highest numbers of bacterial feeders and plant biomass. However, shoot and root biomass were two-fold higher in the pine forest than in the farm soils; compared to the farm soils, the pine forest soils were characterized by a high nutrient availably with total amount of organic matter was more than four-fold higher, and most of the macro and micronutrients presented higher values.

Previous studies have shown how during the decomposition of some active substances, such as dazomet, the process of nitrification is stimulated and compounds derived from mineralization, such as NH_4^+, NO_3^-, HCO_3^- and SO_4^-, improve plant nutrition. In addition, some beneficial organisms, such as actinomycetes, main responsible of organic matter decomposition, are stimulated, and might generate vitamins and antibiotics, that stimulating plant growth (Zanón-Alonso *et al.*, 2011). In this study, the degradation of neem compounds, might explain the higher plant biomass in the neem than in the other treatments.

CHAPTER 3: COMPARISON OF MOLECULAR AND MORPHOLOGICAL IDENTIFICATION METHODS OF SOIL NEMATODE ASSEMBLAGES AND FOOD WEB DESCRIPTORS FROM DIFFERENT AGROECOSYSTEMS

3.1. INTRODUCTION

Soil nematodes are morphologically diverse, present a global distribution, are able to colonize different environments and survive along wide temperature ranges. Terrestrial nematodes are a key component of the soil food web, interacting with other soil fauna. They are widely recognized as bioindicators of the soil environmental condition (Bongers & Ferris, 1999; Ekschmitt *et al.*, 2001; Fiscus & Neher, 2002; Yeates, 2003) and they are involved in soil functioning and processes (Ferris *et al.*, 2001). They can be classified according to their trophic habit (bacterivores, fungivores, herbivores, omnivores, and predators) (Yeates & Bongers, 1999) based on the characteristics of their feeding apparatus, and along the colonizer-persister scale (a gradient from colonizer, resistant nematodes to persister, sensitive nematodes) (Bongers, 1990). In addition, they have a permeable cuticle that allows them to be in permanent contact with soil solutes. The nematode community, thus, has been proposed as an ecologically relevant biological indicator of soil quality (Ritz & Trudgill, 1999), which might be used in national-scale soil monitoring programs (Ritz *et al.*, 2009).

Among soil management techniques, the use of plant protection products (PPP) might be a global threat to soil diversity (Lo, 2010; Muñoz-Leoz *et al.*, 2011; Scholz-Starke *et al.*, 2013).In recent years, requirements regarding soil monitoring in response to the use of PPP are increasing. The Scientific Panel of Plant Protection Products and their Residues of the European Food Safety Authority (EFSA) has pointed out the necessity of modifying the ecotoxicological data requirements for plant protection product evaluation, including structural and functional endpoints related to organism such as bacteria, fungi, protists and nematodes (SCTEE, 2000). EFSA has recommended the use of nematodes in the assessment of the functional and structural features of the soil (EFSA, 2007).Previously studies showed how pesticides, widely used in agricultural crops, can affect soil nematode biodiversity (Liang *et al.*, 2001; Sánchez-Moreno *et al.*, 2010; Carrascosa *et al.*, 2014). Nematodes are easily extracted from soils (Ritz & Trudgill, 1999), but there is a certain controversy in terms of the efficacy of the various nematode identification methods available. Some authors argue that morphological identification through microscopic analysis to the species level is difficult and time consuming due to the lack of specialist knowledge and the necessity of taxonomically competent people (Coomans, 2002; Floyd *et al.*, 2002; Griffiths *et al.*, 2006; Chen *et al.*, 2010). However, if nematodes are conserved alive in water (at 4ºC), their anatomical structures are clear and morphological identifications are reliable. In addition, morphological identification

methods give results over a relatively short period of time with affordability. Identifying nematodes to species level is time consuming, but in nematode ecology studies a community level analysis is required, as opposed to identifying single nematode taxa. The identification to genus or family level is commonly used to infer the global behaviour of the soil nematode community.

When nematode identification to the species level is required, morphological identification has some limitations. For instance, identification of species is only possible from adult specimens who do not represent the entire nematode assemblage in a soil sample (Griffiths *et al.*, 2002), a limitation overcome by molecular methods. Although most of the DNA-based databases are strongly oriented towards plant-parasitic nematodes (De Ley *et al.*, 2005), free living nematode sequence data is increasing continuously. Different molecular methods to identify nematodes have been proposed (Chen *et al.*, 2010). Terminal-restriction fragment length polymorphism (T-RFLP) is a semi-quantitative PCR-based fingerprinting technique, most frequently used to study bacterial communities (Marsh, 1999), but also applied to archaea (Chin *et al.*, 1999) and arbuscular mycorrhizal fungi (Uibopuu *et al.*, 2009) communities. TRFLP has been used, more limitedly, in ciliates (Marsh, 1999), protists (Countway *et al.*, 2005), mites (Gibb *et al.*, 2008) and nematodes (Donn *et al.*, 2012; Edel-Hermann *et al.*, 2008). One advantage of TRFLP over similar fingerprint methods is the ability to compare data across electrophoresis runs. T-RFLP separates sequences on the basis of restriction site polymorphism and thus, after digestion, base-pair sizes of labeled terminal fragments are detected on an automated sequencer (Marsh, 1999; Nunan *et al.*, 2005). Directed TRFLP analyses can be performed using pre-existing sequence information, which is analyzed in order to select restriction enzymes that separate the taxa under study. These enzymes provide yield diagnosis peaks of interest, so different taxa result in different restriction fragments. T-RFLP is commonly used as a community fingerprinting method, with no inference of the identity of the analyzed taxa.

In this study a comparison of the efficiency of molecular and morphological methods has been carried out in different nematode communities from samples collected in five sites with different land use in Spain and Ireland. Morphological and molecular identification techniques were carried out using microscopy at high magnification and dTRFLP (directed Terminal Restriction Fragment Length Polymorphism) respectively. Differences and similarities on nematode diversity and nematode community descriptors assessed by both methods were statistically compared.

3.2. MATERIALS AND METHODS

3.2.1. Study areas

Most sampling sites selected were treated with plant protection products in order to evaluate their effect on soil nematode communities and these sites include agroecosystems with varying levels of nematode diversity. In Spain, the study areas were: (1) a commercial strawberry (*Fragaria ananassa* L.) farm and its surroundings, including field margins and an adjacent pine forest, located in Cartaya (Huelva, southern Spain), the main strawberry production area within the European Union; (2) an experimental plot with a French bean crop (*Phaseolus vulgaris* L.) and (3) an aubergine (*Solanum melongena* L.) greenhouse crop, both cultivated in the Plant Variety Evaluation Centre (Madrid, Spain) and two olive (*Olea europaea* L.) groves, one conventional (4) and one organically (5) managed, located in Valdepeñas (Ciudad Real, central Spain). In Ireland the study area was (6) a commercial strawberry (*Fragaria annanasa* L.) farm located in Adamstown (Co. Wexford, Ireland), a strawberry production area in south east Ireland, characterized by mild weather during the growing season (June-September).

3.2.2. Soil sampling

In June 2012, 20 samples were collected from the Spanish commercial strawberry farm and its surroundings. The farm was managed following conventional standard practices for the area, including yearly application of chloropicrin (Pic). *Honor* strawberry plants were planted 28 days after fumigation to avoid phytotoxicity. Fertilization occurred through nitrogen-phosphorous-potassium fertigation during the whole length of the cropping season. Five samples from the non-fumigated furrows, 3 from the fumigated beds, 2 from the field margins, 5 from an adjacent pine forest, and 5 from the soil composing the rhizosphere of the plants collected before the final harvest during the growing season 2011-2012 were included in this study. Samples were taken with a 5 cm diameter soil corer at 20 cm depth. In June, at the beginning of the growing season 2012, 6 samples were collected from the Irish commercial organic strawberry farm located in Adamstown. The farm was sampled randomly, and each soil sample was composed by five soil cores collected from the top 10 cm using a 2.3 cm diameter soil corer.

In May 2013 soil samples were collected from three conventional and three organically-managed olive groves in the vicinity of Valdepeñas (Cuidad Real, Central Spain). Nine composed samples from the six groves, 5 from conventional and 4 from organic systems were included in the present study. Each 1-kg sample was composed by three subsamples collected at 0-20 cm depth around a single olive tree with a 5 cm soil corer. In July 2013, soil samples were collected from a conventionally-managed bean crop located in an experimental field station in Colmenar de Oreja (Madrid). Samples were collected with a 5 cm soil corer at 20 cm depth. In August 2013, 14 samples were collected from the aubergine crop, which was conventionally-managed. Aubergine seeds were sown in April 2013, and soil samples were collected in September 2013, at the end of the cropping season with a 5 cm soil corer at 20 cm depth. To compose each soil sample, two individual cores were taken from the central area of each individual plot in order to avoid the edge effect.

In total 72 samples were included in this study, 26 collected in 2012 and 46 collected in 2013 from five different crops in Spain and Ireland. Samples were not representative from each habitat, since they were collected randomly in order to compare the nematode community identified molecularly and morphologically. Comparisons among crops or systems in terms of soil functional structure and soil food web structure were not performed. Comparison of the nematode community composition, trophic group abundances, and nematode community indices were always performed between samples analyzed morphologically and molecularly, to infer the precision of each method charactering nematode communities from different crops.

3.2.3. Nematode extraction

Nematodes from the samples collected in 2012 were extracted in Ireland from 100 g of fresh soil with an Oostenbrink-Elutriator (Nagy, 1996; Verschoor & de Goede, 2000), decanting and sieving through consecutive 250, 150, 75 and 53 µm sieves decreasing in pore size down the stack. It was followed by a Baermann funnel extraction over 48h (Barker *et al.*, 1985). The nematode suspension collected was left overnight to settle, and then reduced to a volume of 4 ml, of which 2 ml were transferred to a micro-centrifuge tube for DNA extraction and immediately stored at -20 °C. Nematode present in the 2 remaining ml were heat-killed at 60°C for 2 min, fixed in 1% formaldehyde and transferred to a counting dish, in which total number of nematodes was counted under a low power microscope. The number of nematodes used

for DNA identification was assumed to be the same as for morphological identification. At least 100 nematodes from each sample were identified to genus or family level (Bongers, 1994) microscopically.

Nematodes from the samples collected during the year 2013, were extracted in Spain from 400 g of fresh soil using the sieving and Baermann funnel method (Barker *et al*., 1985). The nematode suspension collected was left overnight to settle and then reduced to a volume of 20 ml, which was transferred to a counting dish where total number of nematodes was counted under a low power microscope. Samples were centrifuged at 1.500 rpm for 3 min and the supernatant was removed to leave the nematode pellet in 2 ml water to be identified. At least 100 nematodes from each sample were identified alive morphologically to genus or family level (Bongers, 1994) under the microscope. Once nematodes were identified morphologically, slides were washed and nematodes were recovered, transferred to a counting dish and counted again. These nematodes were kept for DNA-based identifications. Aliquots of 2 ml were fixed by adding 1.4 µl of DESS solution (Yoder *et al.*, 2006) for further TRFLP identification.

3.2.4. Morphological identification

Nematodes were classified as bacterial feeders, fungal feeders, plant parasites/herbivores, omnivores and predators (Yeates *et al.*, 1993). Nematodes were also classified along the coloniser-persister (C-P) scale, which classifies nematode families into five groups, from microbial feeders with short life cycles and high reproduction rates (CP 1 and CP 2), to predators and omnivores with long life cycles, low reproduction rates and very sensitive to environmental perturbations (CP 4 and 5) (Bongers & Bongers, 1998). Since nematodes belonging to Tylenchidae might be herbivores or fungal feeders (Yeates *et al.*, 1993) half of the nematodes identified as Tylenchidae were considered as fungal feeders and half as herbivores in indices calculations. Morphological identifications were carried out in the laboratory of the Plant Protection Products Unit (DTEVPF) of the INIA in Madrid (Spain), using microscopy at high magnification (Leica, DM2500).

3.2.5. Molecular identification

3.2.5.1. Nematode DNA extraction

In the samples collected and processed in 2012, DNA was extracted as described by Donn *et al*. (2008), using a bead-beating and subsequent purification procedure through a Purelink PCR purification column (Invitrogen, Paisley, UK). In the samples collected and processed in 2013, DNA was extracted using a bead beating and subsequent purification procedure using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA). The extracted DNA was analyzed by directed-terminal restriction fragment length polymorphism (dTRFLP).

3.2.5.2. PCR amplification

Almost the full length of the SSU rDNA was amplified by PCR using the primers SSU_F74 (AAR CYG CGW AHR GCT CRK TA) and Nem_18S_R (GGG CGG TAT CTR ATC GCC) (Floyd *et al.*, 2005; Donn *et al.*, 2012) PCR was performed in a 25 µl final volume including 1xTaq buffer (60 mM Tris-SO_4, 18 mM NH_2SO_4), 2 mM $MgSO_4$, 0.2 mM dNTPs (Promega, Southampton, UK), 0.5 µM of each primer (unless indicated otherwise, all primers were synthesised by Eurogentec, Southampton, UK) and two units of High Fidelity Platinum Taq DNA polymerase (Invitrogen). Cycling was performed on a Gene Amp® PCR System 9700 thermal cycler (Applied Biosystems, Warrington, UK) with the following run parameters: one initial denaturising cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s; 55 °C for 30 s; 68 °C for 1 min and a final extension step of 68 °C for further 10 min.

3.2.5.3. Directed TRFLP

Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis is a highly reproducible method in which fluorescently labelled products are sized and quantified in an automated DNA sequencer (Chen *et al*., 2010). This technique was applied to the amplified nematode community DNA using the two restriction enzymes *Ple*I and *Bts*CI (New England Biolabs, Hitchin, UK) to digest SSU rDNA with the addition of the fluorophore FAM. Uncut PCR product was run. Any peaks, smaller than the uncut PCR product, found in the uncut profiles were assumed to be PCR artefacts and subtracted from the digested samples. These typically represented 4% of the total fluorescence. PCR products were digested with each enzyme

combination in 20 µl final volume including 6 µl PCR product, 0.7x buffer and 100 µg mL-1 BSA both supplied with the enzymes (New England Biolabs, Ipswich, MA, USA). Two units of *Ple*I were added to the digestion mix, and reactions were incubated at 37°C for 1 h followed by 65°C for 20 min in order to denature the enzyme. Two units of *Bts*Cl were then added to each and the reactions incubated at 50°C for further 1 h. Digestion products were then diluted 1:10 in sterile distilled water. Fragments were analyzed on an ABI 3730 capillary sequencer (Donn *et al*., 2012), at the James Hutton Institute, Dundee, UK. Data from the sequencer were analysed in the laboratory of the Crop and Soil Systems Research Group in the Scottish Rural College (SRUC) in Edinburgh (UK).

3.2.6. Statistical analysis

Peaks in the directed TRFLP profiles were assigned to nematode taxa on the basis of sequencing of nematode assemblages according to Donn *et al*., 2012. From directed TRFLP analysis, and the relative abundance of TRFLP peaks corresponding to nematode taxa (Donn *et al.*, 2011) was calculated. The total fluoresce of a sample is absorbed at different peaks, which corresponds to the different nematode taxa detected. In each sample, the fluorescence absorbed at each peak with respect to the total fluorescence absorbed represents the relative abundance of each taxa expressed in percentage of fluorescence. Although there is a chance of losing rare nematode peaks, any peaks constituting less than 1% of the total fluorescence were excluded from further analyses in order to remove artefactual background peaks.

STATISTICA software package (StatSoft, 2013) was used for all statistical analysis regarding morphological and molecular identifications. Molecular and morphological data were log-transformed (ln (x+1)) before analysis. Scatterplots and correlation analyses were performed to study the similarity on the abundances of the most abundant taxa and trophic group abundances in nematode samples identified with morphological *vs* molecular methods. Total number of nematodes identified with both methods and Taxa Richness were calculated.

Canonical Correspondence Analysis (CA) was performed to examine and summarize relationships between nematode community descriptors (independent variables) and nematode taxa identified morphologically and molecularly (dependent variables). The ordination resulting from the analysis of the relationships among variables is shown in a canonical bi-plot in which variables that score together are more closely related.

3.3. RESULTS

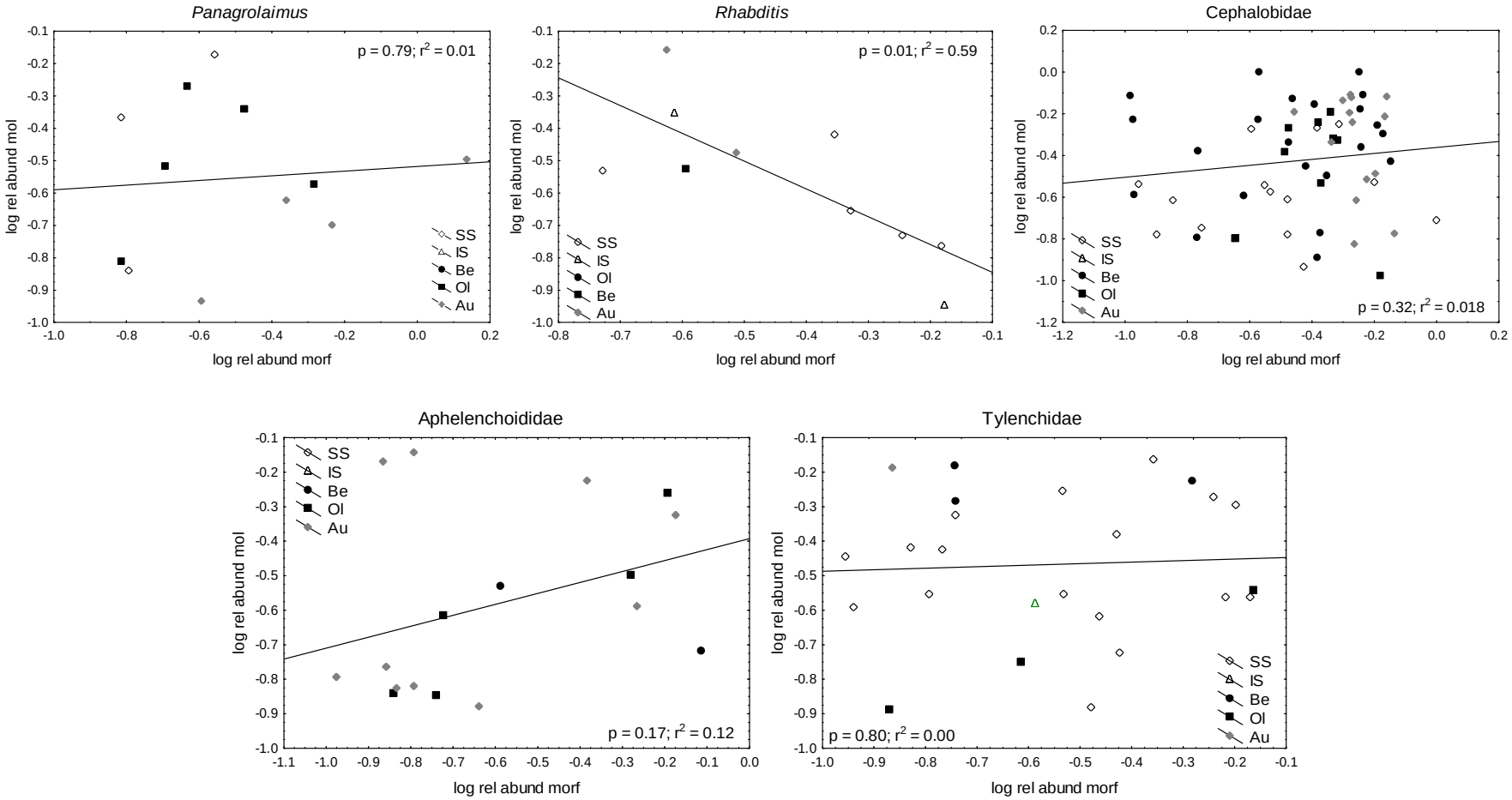
3.3.1. Nematode community composition: nematode taxa

TRFLP peaks were assigned to nematode taxa as previously determined by Donn *et al*., 2012, while morphological identification were carried out according to Bongers, 1994. The total number of nematode taxa identified morphologically was more than three-fold higher than molecularly (Table 3.1). Nematodes identified molecularly were assigned to 1 order, 4 families and 7 genera, while nematodes identified morphologically were assigned to 3 families and 33 genera (Table 3.1). *Mesorhabditis, Rhabditis, Panagrolaimus, Helicotylenchus* and Tylenchidae were identified with both methods. TRFLP identified some nematodes of the family Cephalobidae and also some of its genera such as *Acrobeloides, Cervidellus* and *Chiloplacus*, and of the order Aphelenchida and to two of its families (Aphelenchoidae and Aphelenchoididae) (Table 3.1). The representation of the relationships between the abundances of the most abundant taxa identified morphologically and molecularly show that the relative abundances of *Panagrolaimus*, Cephalobidae, Aphelenchoididae and Tylenchidae identified morphologically and molecularly were positive related ($P< 0.05$) while *Rhabditis* relative abundances were negatively related ($P< 0.05$) (Fig. 3.1).

Table 3.1. Nematode taxa identified with molecular (ML) and morphological methods (MR). TG: Trophic group. C-P: c-p taxon value in the colonizer-persister scale. Ba: bacterial feeders, Fu: fungal feeders; O: omnivores; P: predators; Pp: herbivores and plant parasites. T-RFLP peaks (bp) assigned to nematode taxa according to Donn *et al*., 2007; Donn *et al*., 2012.

TG	C-P	Taxa	Id method	T-RFLP peak	Code	TG	C-P	Taxa	Id method	T-RFLP peak	Code
Ba	1	*Dauer*	MR		—	Fu	2	*Aphelenchus*	MR		Fu1
Ba	1	*Mesorhabditis*	ML, MR	136+578	Ba1	Fu	2	Aphelenchoididae	ML	464	Fu3
Ba	1	*Rhabditis*	ML, MR	598	Ba2	Fu	2	*Aphelenchoides*	MR		Fu2
Ba	1	*Cruznema*	MR		Ba3	Fu	3	*Diphterophora*	MR		Fu4
Ba	1	*Diploscapter*	MR		Ba4	Fu	4	*Tylencholaimus*	MR		Fu5
Ba	1	*Panagrolaimus*	ML, MR	129+132	Ba5	O	4	Dorylaimida	MR		O1
Ba	2	Cephalobidae	ML	761	Ba18	P	3	*Tripyla*	MR		P1
Ba	1	Neodiplogasteridae	MR		Ba6	P	4	*Mononchus*	MR		P2
Ba	2	*Chiloplacus*	MR		Ba7	P	4	*Mylonchulus*	MR		P3
Ba	2	*Cervidellus*	MR		Ba8	P	4	*Clarkus*	MR		P4
Ba	2	*Acrobeles*	MR		Ba9	P	4	*Prionchulus*	MR		P5
Ba	2	*Acrobeloides*	MR		Ba10	P	5	*Discolaimus*	MR		P6
Ba	2	Plectidae	ML	364	—	Pp	2	*Paratylenchus*	MR		Pp1
Ba	2	*Plectus*	MR		Ba12	Pp	3	*Meloidogyne*	MR		Pp2
Ba	2	*Wilsonema*	MR		Ba13	Pp	3	*Pratylenchus*	MR		Pp3
Ba	2	*Eumonhystera*	MR		Ba14	Pp	3	*Tylenchorhynchus*	MR		Pp4
Ba	3	*Teratocephalus*	MR		Ba11	Pp	3	*Rotylenchus*	MR		Pp5
Ba	3	*Prismatolaimus*	MR		Ba15	Pp	4	*Trichodorus*	MR		Pp6
Ba	3	*Achromadora*	MR		Ba16	Pp	3	*Helicotylenchus*	ML, MR	592	Pp7
Ba	4	*Alaimus*	MR		Ba17	Pp	5	*Xiphinema*	MR		Pp8
Fu	2	Aphelenchida	ML	224+229	—	Pp	5	*Longidorus*	ML	604	Pp10
Fu	2	Aphelenchidae	ML	636	Fu4	Pp/Fu	2	Tylenchidae	ML, MR	296+354+359	Pp9

Fig. 3.1. Relationship between relative abundances of the most abundant nematode taxa identified with morphological and molecular methods extracted from samples collected in Spanish strawberry crop (SS), Irish strawberry crop (IS), olive groves (Ol), bean crop (Be) and aubergine crop (Au). Only values > 0 are represented.



3.3.2. Nematode community composition: trophic groups

The nematode community composition, analyzed with molecular and morphological methods were compared. In total 11 and 38 taxa were identified with molecular and morphological methods respectively (Table 3.2). Nematodes from the five main trophic groups were identified morphologically while only bacterial, fungal and plant-feeding nematodes were identified molecularly. Neither omnivores nor predators were detected by TRFLP. Bacterial feeding nematodes were the most abundant taxa, followed by fungivores in both identifications (Table 3.2). On average, more than 70% of the nematodes identified molecularly were bacterivores. Mean Taxa Richness was more than two-fold higher in the morphological than molecular identifications (Table 3.2).

Table 3.2. Number of nematode taxa belonging to each trophic group identified molecularly and morphologically, and mean relative abundances of each trophic group (TG). Ba: bacterial feeders, Fu: fungal feeders; O: omnivores; P: predators; Pp: herbivores and plant parasites; S: average Taxa Richness per sample; No.: average number of nematodes per sample.

TG	Molecular identification		Morphological identification	
	Number of identified taxa	Mean relative abundances (%)	Number of identified taxa	Mean relative abundances (%)
Ba	5	75.2%	18	64.0%
Fu	3	2.5%	4	12.0%
O	0	0.0%	1	5.2%
P	0	0.0%	6	0.7%
Pp	3	22.3%	9	18.1%
Total	**11**	**100%**	**38**	**100%**
Mean No.	436		587	
Mean S	4.61		9.29	

The minimum number of nematodes that had to be present in a sample to be detected molecularly was calculated for all the taxa that were identified molecularly and morphologically simultaneously. In the 8 nematode taxa that were detected both molecularly and morphologically, the minimum number of nematodes that had to be present in a sample to be detected was only 3 for *Panagrolaimus* and Cephalobidae present in the Spanish strawberry samples (Table 3.3). Nematodes were counted before DNA extraction, and the number of nematodes (100g of dry soil)$^{-1}$ present in each sample was registered. In another

two Spanish samples the minimum number of *Mesorhabditis* and *Rhabditis* necessary to be detected molecularly was respectively 4 and 7, while for the other taxa the minimum number of nematodes to be detected molecularly was higher than 10 (Table 3.3).

Table 3.3. Minimum number of nematodes present in a sample to be detected molecularly. Only taxa that were identified molecularly and morphologically where analyzed. Numbers in bold indicate 10 or less nematodes detected in a sample. ID: id sample. No.: total number of nematodes in each sample. F: farm, Fu: furrow, P: pine forest, PL: plant, E: edge; OC: Conventional olive grove; OE: Ecological olive grove, B: Bean; Au: aubergine.

Habitat	ID	*Panagro-laimus*	*Mesor-habditis*	*Rhab-ditis*	Cephalo-bidae	Aphelen-chida	Aphelencho-ididae	Tylenchi-dae	*Helicoty-lenchus*	No.
SS	F2	33	-	175	67	-	-	-	-	372
	F3	**3**	-	-	**8**	-	-	**5**	-	16
	E1	31	-	-	-	-	-	-	-	31
	E2	-	-	-	-	14	-	**9**	-	158
	Fu1	-	-	-	**4**	-	-	**8**	-	31
	Fu2	-	-	83	36	-	-	21	-	189
	Fu3	-	-	-	**3**	-	-	-	-	18
	Fu4	**3**	**4**	-	11	-	-	13	-	37
	Fu5	-	-	**7**	-	-	-	**5**	-	118
	P1	-	-	281	-	-	-	124	-	461
	P2	-	-	-	-	-	-	187	-	301
	P3	-	-	-	58	-	-	92	-	342
	P4	-	-	-	74	-	-	114	-	309
	P5	-	-	-		-	-	179	-	263
	PL1	-	-	-	**10**	-	-	**5**	-	40
	PL2	-	-	-	-	-	-	**7**	-	30
	PL3	-	-	112	177	-	-	242	-	590
	PL4	-	-	-	30	-	-	19	-	49
	PL5	-	-	-	19	-	-	20	-	70
IS	F1A	-	-	84	-	-	-	-	-	126
	E2A	-	-	2038	-	-	-	-	-	2038
	F1A	-	-	294	-	-	-	319	-	1225
OI	O1 C	49	-	-	79	-	-	28	-	154
	O2 E	98	-	-	106	-	22	106	-	364
	O3 C		-	-	**10**	-	-		-	172
	O4 C		-	-	117	-	-	32	-	248
	O5 E		-	-	122	-	-	-	-	298
	O6 C	26	-	-	84	-	15	-	-	172
	07 C	**6**	-	-	102	-	18	-	-	126
	O8 E	-	-	-	289	-	-	-	-	536
	00 E	-	-	-	-	-	187	-	-	1336

Table 3.3. Continued.

Habitat	ID	*Panagro-laimus*	*Mesor-habditis*	*Rhab-ditis*	Cephalo-bidae	Aphelen-chida	Aphelencho-ididae	Tylenchi-dae	*Helicoty-lenchus*	No.
Be	B1	-	-	-	220	-	-	-	-	628
	B2	-	-	-	139	-	-	-	-	160
	B3	-	-	-	109	-	-	11	-	176
	B4	-	-	-	291	-	-	-	-	334
	B5	-	-	-	505	-	-	-	-	526
	B6	-	-	-	152	-	-	-	-	160
	B7	-	-	-	1045	-	-	-	-	1100
	B8	-	-	-	390	-	-	-	-	424
	B9	-	-	-	245	-	-	27	-	302
	B10	-	-	-	81	-	-	-	-	218
	B11	-	-	-	39	-	-	-	-	232
	B12	-	-	-	64	-	-	**10**	-	145
	B13	-	-	-	324	-	-	-	-	324
	B14	-	-	-	885	-	-	-	-	912
	B15	-	-	-	1273	-	-	-	39	1312
	B16	-	-	-	239	-	-	-	-	386
	B17	-	-	-	166	-	-	-	-	166
	B18	-	-	-	360	-	-	-	-	468
	B19	-	-	-	164	-	-	-	-	356
	B20	-	-	-	472	-	-	-	-	472
	B21	-	-	-	338	-	-	-	-	380
	B22	-	-	-	446	-	-	-	-	460
	B23	-	-	-	435	-	-	-	-	444
Au	A1	-	-	-	528	-	-	-	-	556
	A2	-	-	-	223	-	12	-	-	392
	A3	-	-	-	331	-	41	-	-	372
	A4	-	-	-	515	-	77	-	-	592
	A5	-	-	-	356	-	28	-	19	468
	A6	-	-	-	556	-	-	-	-	732
	A7	-	-	-	600	-	-	-	-	652
	A8	-	-	-	410	-	45	-	-	640
	A9	-	-	-	557	-	80	-	-	724
	A10	-	-	-	101	-	-	-	**10**	160
	A11	-	-	-	691	-	42	-	-	832
	A12	-	-	-	498	-	60	36	-	600
	A13	-	-	-	680	-	-	-	-	764
	A14	-	-	-	689	-	66	-	-	944

Percentage contribution of each trophic group to the whole community and Taxa Richness in each sample identified molecularly and morphologically is shown in Table 3.4. From the 72 samples studied, 59, 19, and 52 samples were used in the analyses regarding bacterial, fungal

and plant feeding nematodes respectively, since only the samples with relative abundances >0 were represented (Fig. 3.2). The contribution of bacterial and plant-feeding nematodes to the nematode community identified with molecular and morphological methods were not correlated (P=0.27; P=0.22), while fungal feeding nematode relative abundances were positively correlated (P <0.05) (Fig. 3.2).

Table 3.4. Percentage abundances of nematode taxa of each trophic group identified molecularly and morphologically in samples taken in Spanish and Irish strawberry crops (SS and IS), olive crop (Ol), bean crop (Be), and aubergine crop (Au). ID: id sample. No.: total number of nematodes per sample. S: Taxa Richness per sample. F: farm, Fu: furrow, P: pine forest, PL: plant, E: edge; OC: Conventional olive grove; OE: Ecological olive grove, B: Bean; Au: aubergine.

Habitat	ID	Molecular							Morphological						
		Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S	Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S
SS	F1	87.6	0	0	0	12.4	69	6	71.9	0	0	21.9	6.3	69	8
	F2	100	0	0	0	0	372	3	24.3	0.4	0	1.3	73.9	372	12
	F3	68.8	0	0	0	31.2	16	4	58.3	0	0	0	41.7	16	5
	E1	100	0	0	0	0	31	1	10	70	0	0	20	31	7
	E2	77.1	17.3	0	0	5.6	158	8	6.5	38.2	0	22.8	32.5	158	8
	Fu1	74.4	0	0	0	25.6	31	5	42.3	15.4	0	19.2	23.1	31	11
	Fu2	83.9	0	0	0	16.1	189	6	67.4	7.6	0	14.4	10.6	189	11
	Fu3	100	0	0	0	0	18	3	33.3	11.1	5.6	22.2	27.8	18	5
	Fu4	46.1	0	0	0	53.9	37	5	27.8	11.1	2.8	13.9	44.4	37	11
	Fu5	5.7	0	0	0	94.3	118	5	25	0	1.9	22.2	50.9	118	12
	P1	60.6	0	0	0	39.4	461	5	62.9	18.9	3.4	8.6	6.3	461	14
	P2	38.3	0	0	0	61.7	301	4	67.3	10	2.7	11.8	8.2	301	19
	P3	72.6	0	0	0	27.4	342	5	54.2	12.7	11	14.4	7.6	342	17
	P4	58	0	0	0	42	309	6	44.6	19.6	5.4	18.8	11.6	309	18
	P5	31.7	0	0	0	68.3	263	4	30.1	31.6	6.8	21.1	10.5	263	14
	PL1	69.7	0	0	0	30.3	40	6	45.5	3	6.1	15.2	30.3	40	10
	PL2	0	0	0	0	100	30	3	72.4	3.4	0	10.3	13.8	30	11
	PL3	59.3	0	0	0	40.7	590	5	21.4	0.4	0	1.7	76.5	590	11
	PL4	62	0	0	0	38	49	4	72.3	6.4	0	6.4	14.9	49	8
	PL5	58.3	0	0	0	41.7	70	6	75	2.9	0	17.6	4.4	70	9

Table 3.4. Continued.

Habitat	ID	Molecular							Morphological						
		Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S	Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S
IS	F1A	66.6	0	0	0	33.4	126	4	48.4	1.6	0	19.4	30.6	126	10
	F2A	50.5	0	0	0	49.5	88	4	36.7	13.9	0	6.3	43	88	11
	F3A	43.4	0	0	0	56.6	155	4	43.1	10.3	0	12.9	33.6	155	15
	E1A	100	0	0	0	0	2313	2	21.9	14.9	0	14.9	48.2	2313	9
	E2A	100	0	0	0	0	2038	2	20.8	17.9	1.8	8.3	51.2	2038	13
	Fo1A	24.4	0	0	0	75.6	1225	5	50	8.9	3.6	10.7	26.8	1225	17
Ol	O1 C	82.3	0	0	0	12.4	154	4	51.2	15.5	0	3.1	30.2	612	11
	O2 E	56	6.2	0	0	0	364	7	52.2	35.7	1.7	0.9	9.6	756	10
	O3 C	100	0	0	0	31.2	172	3	45.7	20	0	5.7	28.6	828	8
	O4 C	86.8	0	0	0	0	248	4	57.7	27	0	0	15.3	644	7
	O5 E	80	20	0	0	5.6	298	5	34.4	38.9	0	0	26.7	536	13
	O6 C	91.1	8.9	0	0	25.6	172	5	50.4	12	0	3	34.6	700	11
	07 C	85.7	14.3	0	0	16.1	126	4	70.2	18.2	0	0.8	10.7	324	10
	O8 E	100	0	0	0	0	536	4	34.5	4.4	0	2.7	58.4	748	10
	00 E	86	14	0	0	53.9	1336	5	46	21.2	0	0.7	32.1	1752	11
Be	B1	35.3	0	0	0	94.3	628	4	97.3	2	0	0	0.7	1060	9
	B2	90.7	0	0	0	39.4	160	4	86	8	0	0	6	380	7
	B3	72.2	0	0	0	61.7	176	7	79.1	10.4	0	3.7	6.7	316	10
	B4	87.3	0	0	0	27.4	334	4	95.6	3.5	0	0	0.9	426	7
	B5	96	0	0	0	42	526	4	99.2	0.8	0	0	0	518	4
	B6	94.9	0	0	0	68.3	160	4	98.1	1.3	0	0.6	0	226	8
	B7	94.8	0	0	0	30.3	1100	4	85.5	14.5	0	0	0	1192	8
	B8	91.6	0	0	0	100	424	4	94.6	3.8	0	0	1.5	764	6
	B9	90.6	0	0	0	40.7	302	5	91.6	3.4	0	3.4	1.7	364	6

Table 3.4. Continued.

Habitat	ID	Molecular							Morphological						
		Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S	Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S
Be	B10	37.2	0	0	0	38	218	4	96.7	3.3	0	0	0	248	3
	B11	17	0	0	0	41.7	232	5	86.5	12.1	0	0	1.4	266	7
	B12	45.8	0	0	0	33.4	145	7	86.4	5.5	0	3.6	4.5	380	9
	B13	100	0	0	0	49.5	324	2	93.7	1.6	0	1.6	3.2	756	6
	B14	96.6	0	0	0	56.6	912	4	91.4	6	0	2.6	0	1016	4
	B15	97	0	0	0	0	1312	4	83.6	10.7	0	1.9	3.8	1396	9
	B16	61.7	0	0	0	0	386	4	83.8	16.2	0	0	0	964	9
	B17	100	0	0	0	75.6	166	2	89.5	7.9	0	0	2.6	212	9
	B18	77.1	0	0	0	12.4	468	5	97.4	2.6	0	0	0	592	4
	B19	45.9	0	0	0	0	356	5	98.7	0.6	0	0	0.6	584	7
	B20	100	0	0	0	31.2	472	2	95.3	3.1	0	0.8	0.8	1076	5
	B21	89.2	2.9	0	0	0	380	6	92.9	4.5	0	0.6	1.9	448	7
	B22	97.1	0	0	0	5.6	460	4	91.1	7.1	0	0	1.8	844	6
	B23	98	2	0	0	25.6	444	4	93.1	3.8	0	0	3.1	512	10
Au	A1	100	0	0	0	0	556	3	73.4	10.8	0	0.7	15.1	908	8
	A2	57.3	2.6	0	0	40.1	392	7	93	7	0	0	0	568	6
	A3	89.4	10.6	0	0	0	372	4	67.7	18.5	0	0.8	13.1	584	8
	A4	86.8	13.2	0	0	0	592	4	57.4	31.1	0	0	11.5	728	8
	A5	78.3	6	0	0	15.7	468	8	77.8	9	0	0.7	12.5	640	10
	A6	84.3	15.7	0	0	0	732	4	61	16.1	0	0	22.9	1084	8
	A7	100	0	0	0	0	652	2	53.8	9.8	0	0	36.4	796	8
	A8	81.6	6.8	0	0	11.6	640	7	54.7	13.7	0	0.9	30.8	1328	11
	A9	85	10.7	0	0	4.3	724	6	54	19	0	0	27	624	7
	A10	78.2	0	0	0	21.8	160	7	59.5	4.3	0	0	36.2	560	10

Table 3.4. Continued.

Habitat	ID	Molecular							Morphological						
		Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S	Ba (%)	Fu (%)	Pr (%)	O (%)	Pp (%)	No.	S
Au	A11	90.4	5	0	0	4.6	832	6	84.5	8.8	0	1.4	5.4	1024	9
	A12	83	10.5	0	0	6.5	600	6	60.7	23.8	0	0	15.6	664	10
	A13	90.8	6.1	0	0	3	764	6	74.5	2.9	0	0	22.6	752	10
	A14	83.8	7.2	0	0	9.1	944	8	69.2	9.2	0	0	21.7	1112	9

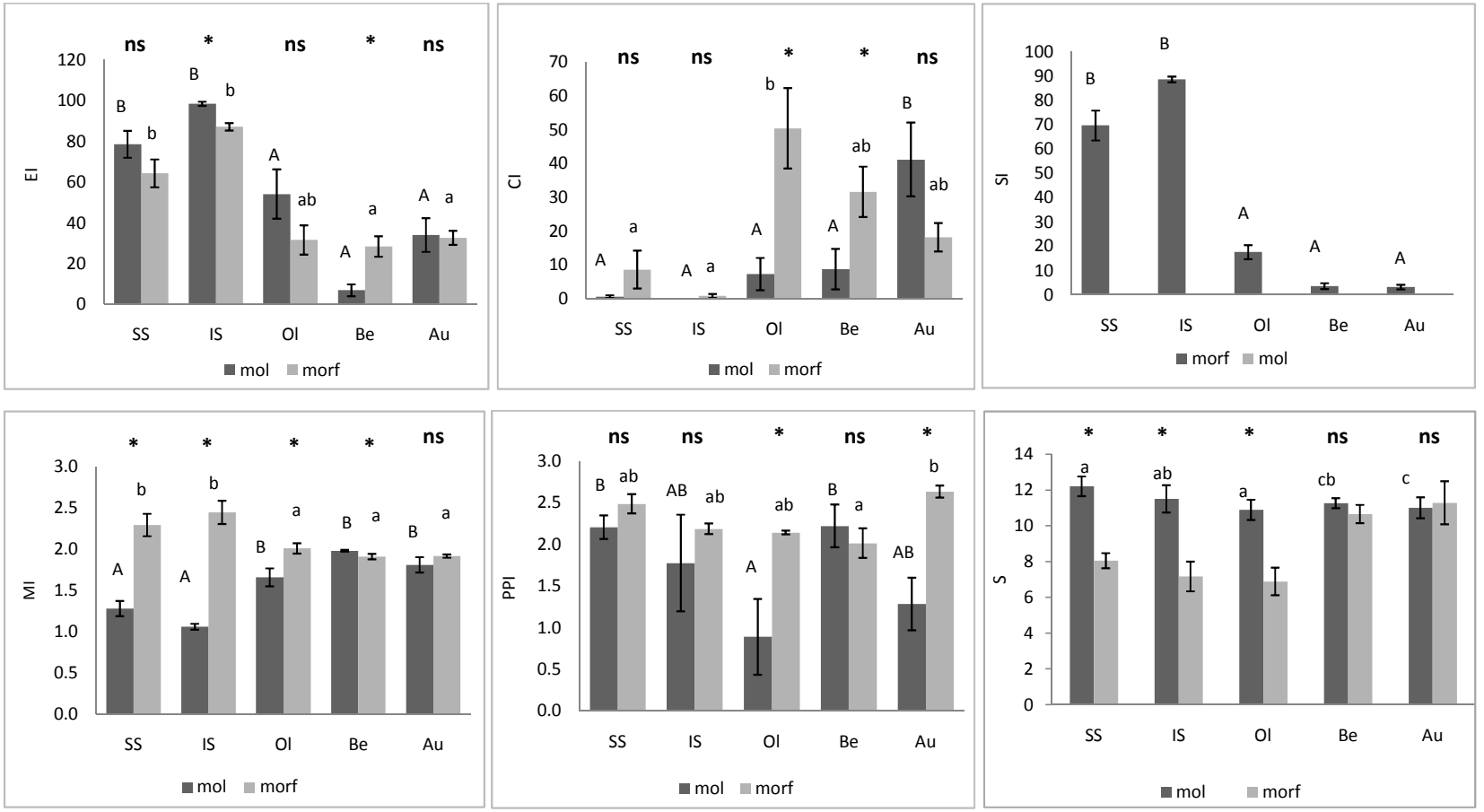
3.3.3. Nematode community descriptors

Mean values of nematode community descriptors calculated from nematode data obtained molecularly and morphologically were compared across the five different crops. The Enrichment Index showed quite similar differences among crops when the nematode community was identified with both techniques; the highest Enrichment Index values were observed in the samples from Spanish and Irish strawberry crops, while the lowest values were observed in the bean and aubergine crops (Fig. 3.3). The Channel Index, on the contrary, showed the highest value in the aubergine crop when samples were identified molecularly and in olive grove samples when they were identified morphologically, and the lowest values were observed in the Spanish and Irish strawberry crops with molecular and morphological identification methods. The Structure Index was > 0 only when samples were identified morphologically, since no nematode indicators of food web structure (bacterivores CP 3-5, fungivores CP 3-5, omnivores and predators), were detected molecularly (Table 3.1). The highest values the Structure Index in the morphological identification were observed in the samples collected from the Spanish and Irish strawberry crops while the samples from the olive, bean and aubergine crops showed the lowest values (Fig. 3.3). The Maturity Index showed an opposite pattern in the samples identified molecularly and morphologically; while higher Maturity Index values were observed in the samples collected in the Spanish and Irish strawberry crops that were identified morphologically, such crops presented the lowest values when samples were identified molecularly. On the contrary, the other three crops presented the lowest and the highest values of the Maturity Index when samples were identified morphologically and molecularly respectively (Fig. 3.3). The Plant Parasitic Index also presented quite different values in each crop when the nematode community was identified with molecular or morphological methods, and differences among crops were not similar in both identification methods (Fig. 3). Differences in Taxa Richness values in samples identified with both methods were large. Highest Taxa Richness values in nematode communities identified morphologically and molecularly were observed in the Irish strawberry and aubergine crops respectively, while lowest values for both characterization methods were observed in the bean and olive crops respectively (Fig. 3.3).

Mean values of nematode community descriptors were compared when nematodes were identified molecularly and morphologically for each crop. Significant differences (P <0.05) were observed for the Enrichment, Channel and Plant Parasitic Indices in two of the five crops

studied when nematodes were identified molecularly and morphologically. The Maturity Index showed significant differences between identification methods in all crops except aubergine, and mean values of the Taxa Richness were significantly different between molecular and morphological methods in three of the five crops. When all samples were analyzed together without grouping them according to their crops, significant differences ($P< 0.05$) were observed between identification methods for all community descriptors except for the Enrichment Index (Channel Index: $P <0.05$, Maturity Index: $P< 0.01$, Plant Parasitic Index: $P <0.05$, Taxa Richness: $P <0.01$) (data not shown).

Figure 3.3. Mean values of soil food web indices (EI: Enrichment Index, CI: Channel Index, SI: Structure Index), MI: Maturity Index and PPI: Plant Parasitic Index and S: Taxa Richness of nematodes communities identified with morphological and molecular methods in each crop. SS: Spanish strawberry, IS: Irish strawberry, Ol: olive, Be: bean, Au: aubergine. Different letters mean significant differences among crops at $P < 0.05$ for each identification method. *: significant differences between identification methods at $P < 0.05$ for each crop. ns: no significant. Significant differences between identification methods (ID) are shown.

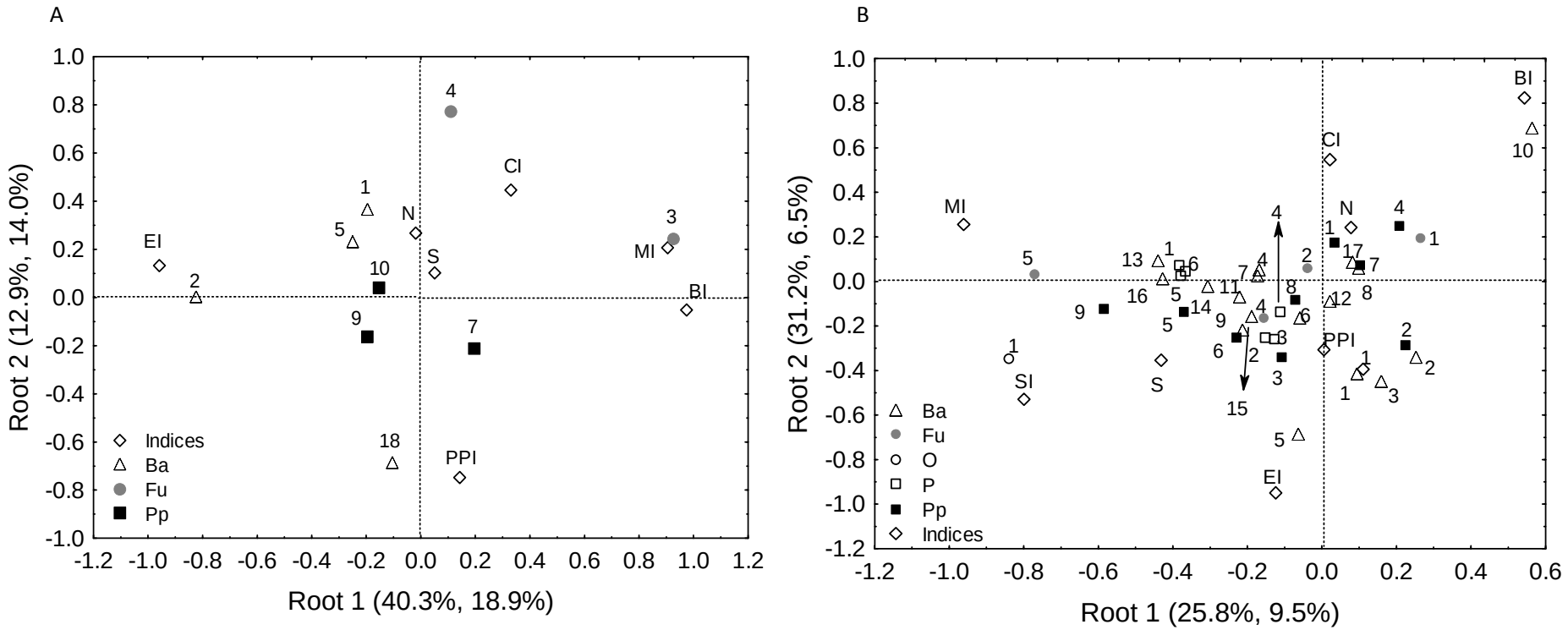


Two Canonical Correspondence Analyses (CCA), represented in a bi-dimensional plot, were performed to show similarities between nematode community descriptors and nematode taxa when nematodes were identified with molecular and morphological methods (Fig. 4A and 4B). When nematodes were identified molecularly, roots extracted by the analysis explained 40.3% and 18.9 % (root 1) of the variability of independent (nematode community descriptors) and dependent (nematode taxa identified molecularly) variables respectively, while root 2 explained 12.9% and 14.0% of their variability (Fig. 3.4A). When nematodes were identified morphologically, root 1 explained 25.8% and 9.5% of the variability of independent (nematode community descriptors) and dependent (nematode taxa identified morphologically) variables respectively, while root 2 explained 31.2% and 6.5 % of the variability (Fig. 3.4B).

In the molecular identifications (Fig. 3.4 A), high values of the Enrichment Index were related to high values of *Rhabditis* and high values of the Channel Index were related to high values of Aphelenchidae. High values of the Plant Parasitic Index were related to Cephalobidae while high values of the Maturity and Basal Index were related to high values of Aphelenchoididae. Total number of nematodes (N) and the Taxa Richness (S) together with two bacterivores and two plant parasitic nematodes (*Panagrolaimus* and Tylenchidae and *Longidorus* and *Mesorhabditis*) were situated in an intermediate position. The Maturity and Basal Indices scored oppositely to the Enrichment Index and the Channel Index to the Plant Parasitic Index. Total number of nematodes and the Taxa Richness were situated in an intermediate position (Fig. 3.4 A).

In the morphological identifications (Fig. 3.4 B), high values of the Maturity Index were related to high values of Tylencholaimus. High values of the Enrichment Index were related to *Panagrolaimus.* High values of the Structure and Basal Indices were related to high values of the omnivore Dorylaimida and *Acrobeloides* respectively. The Basal Index scored oppositely to the Enrichment, Structure and Maturity Index. Most of the nematode taxa identified, belonging to the five trophic groups, and the Plant Parasitic Index, the Channel Index, total number of nematodes and the Taxa Richness were situated in an intermediate position (Fig. 3. 4B).

Fig. 3.4. Canonical Correspondence Analysis (CCA) bi-plots showing the association between independent variables (nematode community descriptors) and dependent variables (relative abundances of nematode taxa identified with molecular **(A)** and morphological methods **(B)**, codes for nematode taxa abbreviations are shown in Table 1). Soil food web indices (EI: Enrichment Index, CI: Channel Index, SI Structure Index), MI: Maturity Index and PPI: Plant Parasitic Index, and S: Taxa Richness. Ba: bacterivores, Fu: fungivores, O: omnivores, P: predators, Pp: herbivores or plant parasites. Percentage of variance explained by each axis is indicated. Plectidae and Aphelenchida did not present enough variability to be included in the analysis of molecular data.



3.4. DISCUSSION

3.4.1. Nematode taxa identified molecularly and morphologically

This study represents a fundamental step in the evaluation of the efficiency of molecular methods assessing soil nematode diversity.Previous studies have mainly compared molecular and morphological characterizations of specific nematode species, using various molecular methods (Zhao *et al.*, 2008; Sirca & Urek, 2009; Meza *et al.*, 2011). To our knowledge, few previous studies have aimed to evaluate the structure and diversity of the soil nematode assemblage using molecular determinations (Griffiths *et al.*, 2006; Edel-Hermann *et al.*, 2008; Griffiths *et al.*, 2012).

In this study, nematodes were extracted from the soil matrix prior to DNA extraction, which allowed the use of a larger and more representative nematode sample (e.g., nematodes extracted from 100 g of fresh soil) than extracting DNA directly from soil (DNA typically extracted from 0.5 g soil) (Foucher *et al.*, 2004; Hamilton *et al.*, 2009). Besides, the use of a clean nematode sample permits the use of general eukaryotic primers in the molecular analyses, rather than requiring specific primers targeting the group of interest in the mixture of soil DNA (Correa *et al.,* 2013; De Weerdt *et al.,* 2011). Identifying nematode assemblages using molecular methods requires a DNA region that demonstrates sufficient divergence to separate sequences of different taxonomical groups, but sufficient constraint to avoid saturation of informative sites (Donn *et al.*, 2011). Different molecular techniques have been used to identify both terrestrial and aquatic nematodes. Aquatic nematodes have been identified using the mithocondrial cytochrome c oxidase subunit I (COI) gene (Velasco-Castrillon & Stevens, 2014), and taxa have been compared to morphological identifications. In total 5 orders were identified. When nematodes were identified molecularly, nematode families and species were identified (e.g. Panagrolaimidae, Criconematidae and *Plectus murrayi),* while *Eudorylaimus* was identified morphologically but not molecularly. A similar resolution was obtain with molecular and morphological identification methods, since nematodes were identified to the specie level with both identification methods. However, according to Morise *et al.*, 2012, the COI gene-based barcoding may provide higher taxonomic resolution than conventional SSU rDNA- barcoding in soil community analyses when nematode communities in unmanaged and agricultural soils were analysed.

In previous studies (Griffiths *et al.*, 2012), TRFLP was used to analyze the effect of tillage on different nematode communities, but limitations in the taxonomical resolution of nematode taxa obscured changes in the community composition. Since nematodes were not identified morphologically, no comparison between the nematode community composition identified with both methods was performed. Using the same methodology, 18 and 11 nematode taxa were identified by Griffiths *et al*., 2012 and in our study respectively. However, only 3 trophic groups were identified in our study, while nematodes belonging to the five main trophic groups were identified by Griffiths *et al*., 2012. Monhysteridae, Aphelenchoides, Dorylaimidae and Mononchidae were identified morphologically in our study but not molecularly, while Griffiths *et al*., 2012 identified them with molecular methods. The total number of nematodes per sample ranged between 200 and 2500 nematodes in previous study (Griffiths *et al*., 2012) and was greater than in our study, where the average number of nematodes across all samples was 436. A greater number of nematodes present in the samples analyzed by Griffiths *et al*., 2012, could explain a larger number of nematode taxa detected.

Comparing nematode communities identified with different methods is sometimes a difficult task, since each technique possesses its own limitations and strengths, and complete nematode recovery and total efficiency is never achieved (Okada & Oba, 2008).To identify nematodes morphologically, being able to see nematode internal structures properly is essential (Bongers, 1994).Since juveniles do not present fully developed internal structures and the characteristics of the adult reproductive apparatus is an essential diagnostic characteristic to identify nematodes to genus level, often juveniles cannot be identified morphologically. Similarly, dauer juveniles are non-growing stages belonging to the family Rhabditidae (Cassada & Russell, 1975), which cannot be identified to genus or species level with morphological techniques and are often abundant, .e.g. in fertilized agricultural soils. We suggest that dauer juveniles could be identified more precisely with molecular methods. However, a good precision of the molecular method used is required to solve the taxonomical determination; in our study, high numbers of dauer juveniles identified morphologically in some Irish strawberry crop and bean crop samples were identified as Rhabditidae with molecular methods, but no further resolution was achieved.

Another limitation of the morphological approach is the necessity of data extrapolation, since often only around 100 individual nematodes are identified per sample, while virtually all nematodes in the community could be identified molecularly. Previous studies that used TRFLP

to identify nematode assemblages found varying percentage composition of feeding guilds depending on soil management type (Donn *et al.* 2012), including nematodes belonging to five main nematode trophic groups. Our results show bacterial and fungal feeders as the most and least abundant taxa respectively when nematodes were identified molecularly. Mean nematode relative abundances of omnivores and predators represented a very low percentage of the total nematode abundances in the samples identified morphologically, representing around the 5% and the 1% respectively of the total nematodes/100 g of dry soil. Total number of nematodes belonging to these trophic groups might be too low to be detected by molecular methods, and omnivores and predators were not identified molecularly in our study. On average, only a percentage from 1% (e.g. Aphlenchida) to 80% (e.g. Cephalobidae) of the taxa identified morphologically were detected molecularly, depending on the taxa. As a consequence, the Structure Index could not be calculated for the samples identified molecularly due to the lack of taxa belonging to some trophic groups.

Large differences were observed between nematode taxa when they were identified molecularly and morphologically, as a higher number of taxa were identified morphologically than molecularly. In some occasions, on the contrary, some nematode taxa were identified with molecular methods but were not identified morphologically. The plant-parasitic nematode *Longidorus* was identified molecularly in 1 sample but was not identified morphologically. The TRFLP peak of 604 pb used to identify *Longidorus* might belong to a close genera, *Xiphinema*, for which no TRFLP peak size has been previously described. Another plant-parasite, *Helicotylenchus,* was identified molecularly in more than 30 samples representing more than 50% of total nematode abundances, but was only present in 11 samples identified morphologically and presented very low abundances. The TRFLP peak assigned to *Helicotylenchus* is 592 bp (Donn *et al.,* 2012), very close to another peak of a similar size, *Rhabditis* (598 bp), that might in our samples correspond to the taxon detected molecularly, since *Rhabditis* abundances were very high in those samples. The peaks identified by Suzanne Donn were from nematodes in Scottish soils and may not be the same peaks as for Irish or Spanish nematodes. This may be a reason for low taxa richness found in the molecular identifications.

Most nematodes (>60%) in the samples collected in the bean and aubergine crops, were identified as Cephalobidae and *Acrobeloides* molecularly and morphologically respectively. TRFLP efficiently detected these taxa, although identification only reached the family level.

Such concordance between the morphological and molecular approach might be due to the high abundance of *Acrobeloides*, which facilitated an accurate detection due to high DNA template concentration. Efficiency of DNA extraction and high DNA yields are important to obtain low detection limits and greater probabilities of success in the PCR amplification (Burgmann *et al.*, 2001; Donn *et al.*, 2008). In our samples, however, only 3 *Panagrolaimu*s and 3 Cephalobidae were necessary in a sample to be detected molecularly.

3.4.2. Soil food web indices and nematode community descriptors

Due to the differences observed in nematode taxa identified molecularly and morphologically, mean values of the Enrichment, Channel, Plant Parasitic and Maturity indices and Taxa Richness obtained by both methods significantly differed. As discussed earlier, these differences might be due to a lower taxonomic resolution obtained with molecular than morphological methods. Indices involving microbial feeding nematodes were more similar between methods than indices involving higher trophic links; while differences in the Enrichment Index among habitats when nematodes were identified molecularly and morphologically were similar (highest values were observed in the strawberry crops), as to a certain extent were the Channel Index values (lowest values were observed in the strawberry crops), the Maturity Index showed opposite patterns when calculated with morphological and molecular data. The results of canonical analysis showed the Maturity Index in the molecular identifications associated to higher abundances of Aphelenchoidae, while *Aphelenchoides,* the most abundant taxa belonging to the Aphelenchoidae family, was very weakly associated to the same Index in the morphological identifications. On the contrary, *Tylencholaimus* and Dorylaimida were the taxa most closely related to the Maturity Index when nematodes were identified morphologically, but were not identified molecularly. High values of the Enrichment Index were related to *Panagrolaimus,* a bacterivore with cp 1 belonging to the same cp group that *Rhabditis* which was highly associated in the molecular identifications with the same index. The absence of higher trophic links in the molecular identifications may explain the strong relationship observed between the Basal and the Maturity Indices, an artefact due to the fact that most nematodes used to calculate the Maturity Index in the molecular identification belonged to cp groups 1 or 2, the same ones used to calculate the Basal Index. Since the Structure Index could not be calculated when nematodes were identified molecularly and no differences were observed for the Taxa Richness between molecular and morphological

identifications in each crop, nothing can be concluded for these nematode community descriptors when we compared results between identification methods.

CHAPTER 4. EFFECT OF SOIL NEMATICIDES ON NEMATODE DIVERSITY AND FUNCTIONING AND THE MITIGATING EFFECT OF ORGANIC AMENDMENTS UNDER EXPERIMENTAL FIELD CONDITIONS

4.1. INTRODUCTION

In agricultural systems, the use of nematicides may reduce plant-parasitic nematodes that exhibit a yield-depressing potential (Giannakou & Karpouzas, 2003; Qiao *et al.*, 2010). Indeed, nematicides reduce all nematodes present in the soil, including non-target nematodes (Boutsis *et al.*, 2011; Meszka *et al.*, 2011) that play critical roles in soil structure and functioning (Ferris *et al.*, 2004; Khan & Kim, 2007). Besides, management intended to improve plant productivity such as tillage and fertilization may also alter the nematode community structure (Porazinska *et al.*, 1999; Okada & Harada, 2007; Sánchez-Moreno & Ferris, 2007; Overstreet *et al.*, 2010). Different soil fumigants with nematicide and herbicide effects have been used for soil disinfection in agricultural crops alone or in combination with other pesticides to control nematodes and soil borne disease complexes (Medina-Mínguez *et al.*, 2011; Mao *et al.*, 2012; Slusarski & Pietr, 2009), which might be an alternative to the use of pesticides not approved by the EC. Among these pesticides, dazomet is approved in the EU as nematicide, herbicide, fungicide and insecticide (E.C., 2010), while dimethyl disulphide (DMDS) is under evaluation in the EU (http://ec.europa.eu/food/plant/pesticides/approvalactivesubstances/ docs/list candidates basic en.pdf) but it is already registered in USA (EPA, Environmental Protection Agency) as herbicide, insecticide, fungicide and nematicide (http://www.epa.gov /pesticides/chem search /reg actions/pending/fs PC-029088 09-Jul 10.pdf)(Gautier *et al.*, 2008). Previous studies have shown that DMDS fumigation impacts both the activity of beneficial and plant parasitic nematodes (Coosemans, 2005; Charles & Heller, 2010; Dangi *et al.*, 2014). Dimethyl disulfide is naturally produced by some plants belonging to the genus *Allium* (Arnault et al., 2013). This molecule, alone or in combination with chloropicrin, might be a viable replacement for existing fumigants in horticultural crops (Heller *et al.*, 2009).

Soil amendments have positive effects on crops, as they are involved in carbon sequestration and may reduce the negative effects of pesticides on soil fauna and soil properties (Windeatt *et al.*, 2014). For example, sorption of organic compounds from organic amendments can affect the behaviour of pesticides in soil, decreasing their mobility (García-Jaramillo *et al.*, 2014). The positive effects of amendments, such as biochar and compost, on soil fertility, have been previously studied (Liu *et al.*, 2012). Biochar, produced by combustion of biomass under oxygen-limited conditions and high temperature (pyrolisis), has received much attention as a soil remediation amendment in recent years. The physical-chemical characteristics of biochar are influenced mainly by the temperature achieved during the combustion process and by the

type of biomass. Higher pyrolisis temperatures result in an increased surface area and carbonized fraction of biochar, leading to high sorption capability of pollutants and pesticides. If the sorption strength of a particular biochar is very high, its use as soil amendment might adversely affect pest control, as pesticides can be retained by the biochar, which might impede its spreading into the soil, necessary to control pests and diseases (Graber *et al.*, 2011). Moreover, biochars derived from various source materials show different properties in relation to surface area and porosity, important attributes in their use in agricultural systems (Sohi *et al.*, 2010; Sparkes & Stoutjesdijk, 2011). Any impact of biochar on soil organisms can potentially translate to changes in plant nutrient availability and crop productivity, possibly affecting crop yield (Domene *et al*., 2014). Biochar has been proved to be effective improving soil physical-chemical properties and increasing plant growth, especially in combination with organic amendments such as compost (Schulz *et al*., 2013). It has also been suggested that it can even enhance crop resistance to diseases (Tang *et al*., 2013).

Nematodes are abundant in virtually all environments, are morphologically diverse and have diverse life strategies and feeding habits (Yeates *et al.*, 1993). Classification of nematodes into functional guilds and trophic groups (Yeates & Bongers, 1999) makes them good candidates for bioindicators of the condition and processes of ecosystems. They can be used as biological indicators of agricultural practices as e.g. tillage, fertilization, use of pesticides, and in the evaluation of soil health (Sánchez-Moreno *et al.*, 2006). Bacterial and fungal-feeding nematodes contribute indirectly to organic matter decomposition and are involved on nutrient cycling, releasing nutrients for plant uptake (Ingham *et al.*, 1985). Predatory and omnivore nematodes prey on other nematodes as plant parasites, regulating their populations and controlling pests (Khan & Kim, 2007), while plant feeding nematodes are commonly associated with economically important agricultural diseases (Neher, 2001). Soil food web indices and other ecological indices have been developed to measure changes in the structure and function of nematode communities in response to disturbance (Porazinska *et al.*, 1999; Ferris *et al.*, 2001). In agriculture, the soil system provides several ecological services, being involved in nutrient cycling, that supports crop yield and thereby farmers' incomes (Barrios, 2007). The value of these services is driven by many essential natural functions of the edaphic community and its biological activity. Besides, the biological activity of soil fauna as a functional parameter is a sensitive ecological indicator of the effects of pesticides on soil fauna communities, related to agricultural management intensities (Burrows & Edwards, 2002; Marinari *et al.*, 2006). Among other functional test, the activity of soil organisms can be experimentally determined

by measuring their feeding rate using a bait lamina test (Von Törne, 1990), which has been previously used to evaluate the effect of pesticides on biological activity of soil microarthropods (Paulus *et al.*, 1999; Scholz-Starke *et al.*, 2011).

In agricultural systems, organic amendments have a direct positive effect on soil fertility and plant growth, but nematode taxa can be sensitive to the disturbance produced by this nutrient enrichment (Bongers, 1990; Thoden *et al.*, 2011). Soil food web indices and their relation to soil physical-chemical properties are potential indicators essential to understand soil functioning and processes (Sánchez-Moreno *et al.*, 2008b; Carrascosa *et al.*, 2014).

Our objective was to determine the effects of different chemical nematicides and soil amendments on soil physical-chemical properties, soil biodiversity, soil food web structure, and plant biomass. We hypothesized that soil amendments mitigate the effect of chemical nematicides, increasing soil biodiversity and functioning and stimulating plant growth.

4.2. MATERIALS AND METHODS

4.2.1. Study site

The study area was an experimental station located in the vicinity of Colmenar de Oreja (Madrid, Central Spain). The soil presented a clay-loam texture. During the period in which the experiment was performed, mean annual precipitation reached 389 mm and a mean annual air temperature was 14.5°C (AEMET, 2014b). The experimental area occupied a total surface of 720 m^2 divided into 12 blocks 4 m wide and 15 m long. Each block was divided into 3 elementary plots 3 m wide and 4 m long separated by a 1 m strip (Fig. 4.1). The whole experimental surface was drip irrigated during the experiment.

C1	DMDS1	DZ1	C2	DMDS2	C3	DZ2	DMDS3	DZ3	C4	DMDS4	DZ4
B	C	Ø	Ø	Ø	B	B	C	B	B	C	Ø
C	B	B	C	B	Ø	C	B	Ø	Ø	Ø	C
Ø	Ø	C	B	C	C	Ø	Ø	C	C	B	B

Figure 4.1. Outline of the experimental area showing the distribution of chemical treatments and amendments. DZ: dazomet, DMDS: dimethyl disulfide, C: non-treated control, B: biochar+compost, C: compost, Ø: non-amended control.

4.2.2. Soil treatments and amendments

The experimental design included two factors; the first factor was the chemical treatment and the second factor was the type of amendment.Chemical treatments included dazomet (in its commercial granular formulation Basamid®, dazomet 99% pure active ingredient), dimethyl disulfide (DMDS, in its commercial formulation Paladin EC, 99.1% pure active ingredient) and a non-treated control. Basamid and Paladin were applied at 500 kg $(ha)^{-1}$ and 300 kg $(ha)^{-1}$ by direct incorporation into the soil at 20 cm depth and through drip irrigation respectively. Soil amendments included compost at 20.000 kg $(ha)^{-1}$, holm oak biochar applied at 25.000 kg $(ha)^{-}$

[1] plus compost at 20.000 kg $(ha)^{-1}$ and a non-amended control. Biochar presented a surface area of 200 m^2 $(g)^{-1}$, a particle size < 6 mm, a density of 370 g $(l)^{-1}$ and a carbon percentage of 80-88%. Soil fumigant application took place in April 26th2013. After soil fumigation, plots were covered with 0.09 µm VIF transparent plastic mulch (Fig 4.2). Plastic cover was removed 21 days after soil fumigation to avoid phytotoxicity and a germination test was performed to discard phytotoxicity.

Figure 4.2. **A-B** Application of chemical treatments dazomet (dazomet 99%, Basamid) and DMDS (DMDS 99.1%. Paladin), and **C-D** soil amendments, holm oak biochar and compost.

Forty days after soil treatment (5th June 2013) and one day after the first soil sampling (5 WAT), organic amendments were applied to the soil. One week later, French dwarf bean (*Phaseolus vulgaris* L.*)* seeds were sowed in 4 rows per plot separated by 60 cm. Plants in each row were separated ±5 cm. Before sowing, the soil was fertilized with N:P:K (15:15:15) at 400 kg $(ha)^{-1}$. Plastic irrigation tubing was set in each plot. Irrigation occurred during the whole length of the experiment. At the end of the bean cropping cycle, by the end of August, crop residues were incorporated into the soil by ploughing. In order to assess the effects of the

pesticides and amendments on soil biota in the medium term, a cabbage crop (*Brassica oleracea* L.) was established in September 2nd. The planting pattern of this crop was 60 x 60 cm (Fig 4.3).

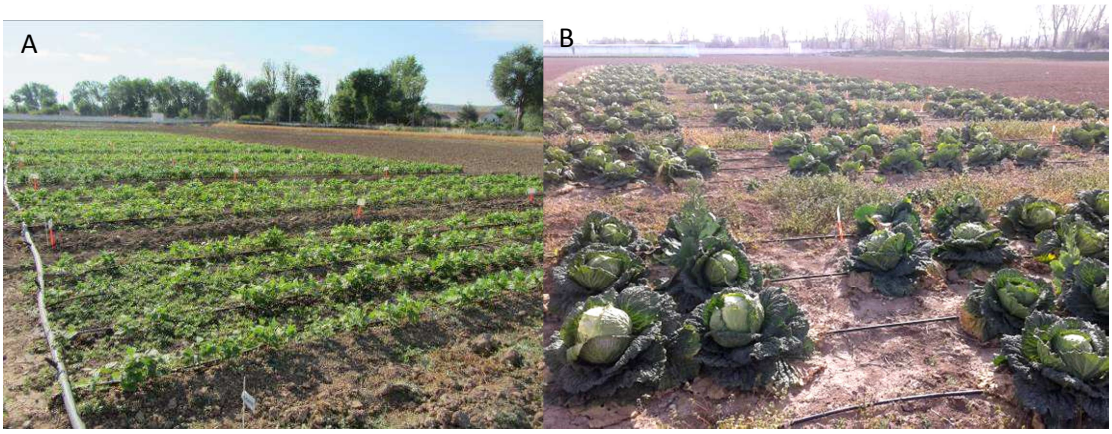


Figure 4.3. A French dwarf bean crop (*Phaseolus vulgaris* L.) and, **B** cabbage crop (*Brassica oleracea* L.).

4.2.3. Soil sampling

From each elementary plot one composite soil sample of around 1 kg of fresh soil was taken by collecting 2 sub-samples from each of the two central crop rows to avoid edge effects. Seven soil samplings were carried out along the experiment, before soil treatment and 5, 9, 12, 16, 30 and 44 weeks after treatment (WAT) (in 24thApril, 4thJune, 2ndJuly, 16th July, 19thAugust, 2nd December 2013 and 11thMarch 2014 respectively). After the initial sampling, the next four samplings corresponded to the bean crop while the last two samplings corresponded to the cabbage crop. Samples were taken with a 5 cm diameter soil corer at 20 cm depth. In total 252 samples were collected during the experiment. All samples were immediately transported to the laboratory and stored at 4°C until processing.

4.2.4. Soil analyses

One subsample of about 250 g was air-dried and fresh and dry soil weight was recorded. Dry soil was sent to an external accredited laboratory where contents of macroelements (N, P, K), organic matter (OM), soil texture, electrical conductivity (EC) and soil pH were analyzed in each sample. P was extracted with NH_4Ac and analyzed by atomic absorption spectroscopy while K was extracted with NH_4Ac and analyzed by atomic emission spectroscopy. N was analyzed by the Kjeldahl method (Radojevic & Bashkin, 1999), organic matter content by the Walkley-Black

method (Walkley & Black, 1934), and pH and EC by potentiometry. Texture was determined using Bouyoucos densimeter.

A second subsample of 10 g was used to extract $NO_3^-$and NH_4^+ within two days after each sampling. $NO_3^-$and NH_4^+ were extracted using a KCl 2M extraction solution, and stored frozen at -20ºC. Extractions were sent to the Nutrient Analyses Laboratory of the Rey Juan Carlos University, and NO_3^- and NH_4^+ were analysed with a nutrient autoanalyzer. One more subsample of 400 g of fresh soil was used for faunal analysis (section 4.2.5).

4.2.5. Nematode extraction and identification

Nematodes were extracted from 400 g approx. of fresh soil using the sieving and Baermann funnel method (Barker *et al.*, 1985). Once extracted, all nematodes were counted under the dissecting microscope, and at least 100 nematodes from each sample were identified to family or genus level under the inverted microscope, and classified by trophic habit (Yeates *et al.*, 1993) and along the coloniser-persister (C-P) scale. Depending on their food source, nematodes were classified as bacterial feeders, fungal feeders, plant parasites/herbivores, omnivores and predators (Yeates *et al.*, 1993). The coloniser-persister (C-P) scale classifies nematode families into five groups, from microbial feeders with short life cycles and high reproduction rates (CP 1 and CP 2), to predators and omnivores with long life cycles, low reproduction rates and very sensitive to environmental perturbations (CP 4 and 5) (Bongers and Bongers, 1998). Taxa Richness (S) was calculated as the average number of taxa in each sample, and the total number of nematodes was expressed as number of individuals (100 g dry soil)$^{-1}$ (No.). The Shannon Diversity Index (H') (Shannon, 1949) was calculated to measure nematode diversity.

Soil food web indices, based on the abundance of functional guilds (Bongers & Bongers, 1998), were calculated to assess soil food web condition. The Structure Index (SI) indicates the level of food web complexity and responds rapidly to disturbance. The Channel Index (CI) is an indicator of the predominant decomposition pathway (fungal *vs* bacterial; high CI values indicate organic matter decomposition mainly mediate by fungi). The Basal Index (BI), based on the abundance of general opportunistic nematodes, is an indicator of basal, perturbed soil food web condition. The Enrichment Index (EI) is based on the abundance of those nematodes that respond most rapidly to microbial food resources arising from decomposition of organic

matter (Ferris *et al*., 2001). The Maturity Index (MI), calculated as a weighted mean frequency of the C-P groups in the community, and the Plant Parasitic Index (PPI), in which only plant-feeding nematodes are included, were calculated (Bongers, 1990).

4.2.6. Plant analyses

Plant biomass in the bean crop was measured in four plants (shoots) collected from the two central crop rows in each plot9, 12 and 16 WAT. Shoots were weighted, dried at 60 °C in an oven for 48 h, and biomass and shoot length was recorded. Fruits were excluded from the biomass analyses.

Differences on leaf colour of the plants in the plots treated with compared to the other treatments were obvious at 9 WAT, and the effective quantum yield was measured. Five plants from the two central crop rows of each plot were randomly chosen, and measures were performed in one leaf of each plant 9, 12 and 16 WAT (Fig. 4.4). To assess the effective quantum yield (ΔF/Fm') of photochemical energy conversion in photosynthesis, a photosynthesis yield analyzer was used (MINI-PAM, Walz). A PAM (pulse amplitude modulated technique) fluorometer applies modulated pulses of measuring light for selective detection of chlorophyll fluorescence yield.

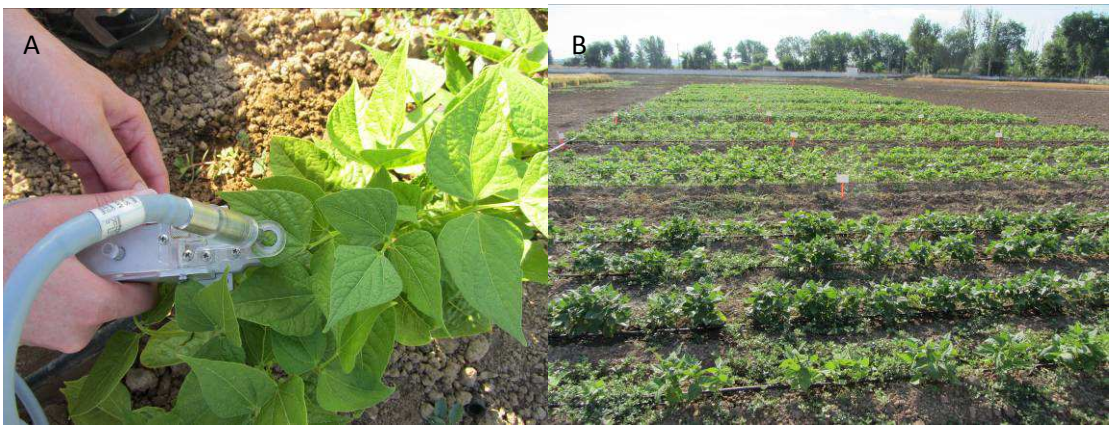


Figure 4.4. A, Measures of the effective quantum yield (ΔF/Fm') performed in the leaves of each plant. **B,** Different colour observed between the plants treated with dazomet (Basamid, dazomet 99%) and the other treatments.

4.2.7. Weed cover

The weeds observed in the plots were mainly *Portulaca oleracea*, but also *Convolvulus arvensis* and *Amarantus* spp. and mainly *Portulaca oleracea.* A quadrat of 50 cm x 50 cm was used to measure mean weed cover in each plot. Three measures were carried out in each plot when weed growth started to be evident9WAT, and was measured again 12 WAT. After the 12 WAT weed cover measure, a manual weed control was carried out to avoid an excessive infestation of the crop (Fig 4.5.).

Figure 4.5. Weed cover and measuresof the percentage of weeds cover in each elementary plot.

4.2.8. Soil fauna feeding activity: Bait lamina test

The test system is based on the visual assessment of feeding on small portions of thin laminated bait substrate exposed to edaphic processes (Von Törne, 1990; Kratz, 1998). Each bait lamina is a PVC strip 1 cm wide and 16 cm long with 16 holes of 2 mm diameter filled with a mixture of cellulose, bran flakes and active coal. Each bait-lamina supplies yes/no answers. In this experiment bait-lamina strips were inserted into the soil along the two central crop rows close to the plants (4 strips in each row; 8 in each plot). After 15 days all bait-lamina strips were removed from the soil, and the total number of empty holes was recorded, and the percentage of bait ingested was calculated (Fig 4.6). It is assumed that disappearance of material is brought about by microbial processes and migrobiogenic metabolism which is mainly influenced by the feeding activity of soil invertebrates (Kratz, 1998).

Figure 4.6. Bait-lamina strips.

4.2.9. Statistical analysis

Due to lack of normality, all variables were transformed to log (x+1) before analyses. ANOVAs were used to detect significant effects of amendments, chemical treatments and sampling date on nematode community descriptors, soil physical-chemical properties, soil food web indices, plant biomass, weed cover, and bait lamina results. Differences among groups were analyzed by a post-hoc, Tukey test. Pearson correlation analyses were used to examine associations between variables. Scatterplot diagrams were performed to show the relationships between plant biomass and effective quantum yield. Analyses were performed using STATISTICA software package (StatSoft, 2013).

4.3. RESULTS

4.3.1. Soil physical-chemical properties

In order to study the overall temporal pattern of soil properties, mean values of physical-chemical properties were compared before (0-) and after (5 WAT) soil treatment, after soil amendment incorporation (9 WAT) and at the end of the winter crop (44 WAT). Comparisons were performed within each fumigation treatment (Table 4.1) and amendment type (Table 4.2). Results show similar patterns in the three soil treatments and in the three amendments for most of the variables studied along sampling dates. Soil moisture (SM) generally increased along the first three dates irrespectively of the treatment or amendment, decreasing thereafter 44 WAT (Tables 4.1 and 4.2). Although similar values of electrical conductivity (EC) were observed along sampling dates, it increased along the experiment showing the highest values at the end of the experiment in all treatments and amendments (Tables 4.1 and 4.2). Soil pH maintained similar values along sampling dates in all treatments and amendments (Tables 4.1 and 4.2). Organic matter soil content showed the highest values at the last sampling date in the three treatments and amendments (Tables 4.1 and 4.2). Total N decreased 9 WAT, and values at the end of the experiment were less than half than the initial values in all treatments and amendments (Tables 4.1 and 4.2), while the ratio C:N in general increased after treatment and showed the highest values 44 WAT in all treatments and amendments (Tables 4.1 and 4.2). Soil P and K showed highest values ($P< 0.05$) 9 WAT, when K contents were extremely high, and decreased thereafter (Tables 4.1 and 4.2). P and K content increased after the application of the amendments and in the non-amended control showing the highest values ($P< 0.05$) 9 WAT and decreasing thereafter, except in the case of the compost+biochar amended plots, in which no significant differences were observed on K values among sampling dates. As in the case of the chemical treatments, very high values of K were observed 9 WAT in all amended plots and the non-amended control. Mean NO_3^-values increased after treatment, decreasing thereafter and showing 44 WAT similar values than before treatment (Table 4.1). In general, NH_4^+ soil content tended to increase after treatment in all treatments and decreased to initial values 44 WAT in the plots treated with dazomet and the non-treated control (Table 4.1). Mean values of NO_3^-increased 5 WAT decreasing thereafter showing 44 WAT the same values than before the application of the amendments in the amended and the control plots, while mean values of NH_4^+ increased 5WAT decreasing slightly at the end of the experiment in the amended and control plots (Table 4.2). Soil

physical-chemical properties in the amended and non-amended plots were compared in each chemical treatment, in order to know the mitigating effect of organic amendments on soil properties. No significant differences ($P > 0.05$) on soil physical-chemical properties were observed between the amended and the non-amended control in each chemical treatment at any sampling date (data not shown).

Table 4.1.Mean values ±SE of physical-chemical properties (SM: soil moisture, EC: electrical conductivity, OM: organic matter) measured on April 24th 2013 (before soil treatment (0)), June 4th 2013 (5 WAT), July 2nd 2013 (9 WAT), and March 11th 2014 (44 WAT) (WAT: Week After Treatment), in plots treated with DMDS, dazomet and the non-treated control. Different letters mean significant differences at $P < 0.05$. Level of significance at $P< 0.05$ between sampling dates in each treatment are shown.

	DMDS				DAZOMET				NON-TREATED CONTROL			
	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)
SM (%)	0.09**a**	0.12**b**	0.15**c**	0.11**b**	0.08**a**	0.12**c**	0.13**d**	0.10**b**	0.10**a**	0.12**bc**	0.15**c**	0.11**ab**
	±0.01	±0.00	±0.00	±0.00	±0.00	±0.00	±0.00	±0.00	±0.01	±0.00	±0.00	±0.00
EC(dsm)$^{-1}$	0.18**a**	0.24**b**	0.26**b**	0.45**c**	0.22**a**	0.24**ab**	0.31**b**	0.37**bc**	0.20**a**	0.16**a**	0.30**b**	0.51**c**
	±0.01	±0.01	±0.01	±0.05	±0.02	±0.01	±0.02	±0.04	±0.01	±0.02	±0.04	±0.05
pH	8.35**ab**	8.25**a**	8.37**ab**	8.40**b**	8.39	8.32	8.31	8.42	8.30a	8.45**b**	8.41**b**	8.49**b**
	±0.03	±0.02	±0.05	±0.03	±0.02	±0.09	±0.03	±0.04	±0.02	±0.02	±0.03	±0.02
OM (%)	1.06**a**	1.08**a**	1.15**a**	1.51**b**	1.03**a**	1.18**b**	1.00**a**	1.63**c**	0.99**a**	1.07**ab**	1.12**b**	1.75**c**
	±0.02	±0.02	±0.04	±0.01	±0.02	±0.04	±0.04	±0.01	±0.03	±0.03	±0.04	±0.01
N (%)	0.23**b**	0.31**c**	0.12**a**	0.12**a**	0.30**c**	0.32**c**	0.22**b**	0.12**a**	0.30**b**	0.33**b**	0.15**a**	0.12**a**
	±0.05	±0.01	±0.01	±0.00	±0.03	±0.03	±0.04	±0.00	±0.04	±0.01	±0.02	±0.00
C:N	3.80**b**	2.07**a**	6.05**c**	7.63**c**	2.22**a**	2.34**a**	3.46**a**	8.11**b**	2.34**a**	1.91**a**	4.81**b**	7.86**c**
	±0.63	±0.11	±0.40	±0.19	±0.27	±0.23	±0.46	±0.19	±0.30	±0.08	±0.41	±0.77
P (ppm)	19.42**a**	116.58**bc**	135.17**c**	77.17**b**	75.08**a**	86.92**ab**	102.08**b**	74.75**a**	88.50**a**	84.00**a**	105.58**b**	66.50**a**
	±4.42	±25.21	±12.59	±2.43	±2.15	±2.55	±5.94	±2.89	±2.52	±2.43	±7.63	±6.94
K (ppm)	571.67**b**	670.00**c**	2191.67**d**	446.67**a**	552.50**b**	713.33**c**	2241.67d	466.67**a**	566.67**b**	706.67**c**	2450.00d	483.33**a**
	±11.40	±13.14	±52.88	±19.48	±12.19	±9.87	±63.32	±19.94	±14.43	±12.45	±33.71	±23.24
NO_3^-(ppm)	0.51**b**	1.54**c**	0.34**a**	0.49**b**	0.67**a**	1.32**b**	1.76**b**	0.59**a**	0.56**a**	0.98**b**	0.45**a**	0.52**a**
	±0.04	±0.10	±0.02	±0.02	±0.08	±0.11	±0.20	±0.02	±0.04	±0.11	±0.05	±0.01
NH_4^+(ppm)	0.36**a**	0.48**b**	0.63**c**	0.59**c**	0.42**a**	2.68**c**	1.15**b**	0.54**a**	0.43**a**	0.58**a**	1.18**b**	0.56**a**
	±0.01	±0.00	±0.02	±0.05	±0.00	±0.26	±0.22	±0.00	±0.01	±0.14	±0.41	±0.01

Table 4.2. Mean values ±SE of physical-chemical properties (SM: soil moisture, EC: electrical conductivity, OM: organic matter), measured on April 24th 2013 (before soil treatment (0)), June 4th 2013 (5 WAT), July 2nd 2013 (9 WAT), and March 11th 2014 (44 WAT) (WAT: Weeks After Treatment), measured in amended plots with compost, compost and biochar and the non-amended control. Level of significance at $P < 0.05$ between sampling dates in each amendment are shown.

	COMPOST				COMPOST+BIOCHAR				NON-AMENDED CONTROL			
	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (0)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)
SM (%)	0.08**a** ±0.00	0.12**c** ±0.00	0.14**c** ±0.00	0.10**b** ±0.00	0.09**a** ±0.01	0.12**bc** ±0.00	0.14**c** ±0.00	0.10**b** ±0.00	0.10**a** ±0.01	0.12**a** ±0.00	0.14**b** ±0.00	0.10**a** ±0.00
EC (dsm)$^{-1}$	0.20**a** ±0.01	0.23**ab** ±0.02	0.26**b** ±0.01	0.46**c** ±0.06	0.23**a** ±0.02	0.20**a** ±0.01	0.33**b** ±0.04	0.45**b** ±0.04	0.18**a** ±0.01	0.22**ab** ±0.02	0.28**ab** ±0.02	0.42**c** ±0.05
pH	8.34**ab** ±0.03	8.31**a** ±0.04	8.33**ab** ±0.05	8.46**b** ±0.03	8.33 ±0.03	8.32 ±0.04	8.37 ±0.03	8.43 ±0.03	8.38 ±0.03	8.39 ±0.08	8.38 ±0.03	8.42 ±0.04
OM (%)	1.01**a** ±0.03	1.13**b** ±0.03	1.09**ab** ±0.03	1.63**c** ±0.03	1.01**a** ±0.02	1.09**ab** ±0.02	1.18**b** ±0.05	1.62**c** ±0.03	1.05**a** ±0.02	1.11**a** ±0.04	1.00**a** ±0.03	1.63**b** ±0.03
N (%)	0.26**b** ±0.05	0.33**b** ±0.01	0.19**ab** ±0.04	0.12**a** ±0.00	0.28**b** ±0.04	0.31**b** ±0.02	0.17**a** ±0.02	0.12**a** ±0.00	0.30**b** ±0.05	0.31**b** ±0.02	0.12**a** ±0.01	0.11**a** ±0.00
C:N	3.39**ab** ±0.69	2.01**a** ±0.09	4.46**b** ±0.56	7.24**c** ±0.69	2.61**a** ±0.35	2.11**a** ±0.15	4.51**b** ±0.43	8.01**c** ±0.20	2.36**a** ±0.22	2.20**a** ±0.22	5.35**b** ±0.55	8.36**c** ±0.30
P (ppm)	59.50**a** ±9.77	116.75**b** ±25.19	110.42**b** ±8.26	72.92**ab** ±6.42	62.92 ±8.74	85.50 ±1.98	127.00 ±8.01	77.00 ±3.01	60.58**a** ±10.10	85.25**b** ±3.00	105.42**b** ±12.50	68.50**ab** ±3.82
K (ppm)	565.00**b** ±15.35	685.83**c** ±13.45	2300.00**d** ±52.22	471.67**a** ±17.70	566.67**a** ±14.05	706.67**b** ±14.37	2441.67**c** ±41.67	519.17**a** ±18.11	559.17**b** ±8.30	697.50**c** ±10.81	2141.67**d** ±52.88	405.83**a** ±12.15
NO_3^-(ppm)	0.58**a** ±0.09	1.37**b** ±0.15	0.84**a** ±0.23	0.54**a** ±0.02	0.57**a** ±0.03	1.22**b** ±0.11	0.93**a** ±0.26	0.56**a** ±0.02	0.58**a** ±0.04	1.24**b** ±0.10	0.78**a** ±0.18	0.51**a** ±0.01
NH_4^+(ppm)	0.41**a** ±0.01	1.46**b** ±0.38	0.81**b** ±0.08	0.60**ab** ±0.05	0.40**a** ±0.01	0.98**b** ±0.29	1.16**b** ±0.41	0.54**ab** ±0.01	0.40**a** ±0.01	1.30**b** ±0.36	0.99**b** ±0.23	0.55**ab** ±0.01

4.3.2. Nematode community composition

Twenty six nematode taxa belonging to the four main trophic groups were identified along the experiment (data not shown). Eighteen, 19 and 15 nematode taxa were identified before treatment (April 2014, 0), at the end of the bean crop (August 2013, 16 WAT) and at the end of the cabbage crop (March 2014, 44 WAT) respectively (Table 3). Thirteen taxa were bacterial feeders, 4 fungal feeders, 2 omnivores, 1 predator, and 6 plant parasites or herbivores. Predators were not identified in this study. *Mesorhabditis*, *Acrobeloides*, *Aphelenchus* and *Aphelenchoides* were the most abundant taxa. Tukey test post-hoc, detected a significant effect of the amendments on only 2 taxa, while the effect of the interaction between sampling date and treatment and sampling date and amendment was significant on 5 and 6 taxa respectively in the three sampling dates showed (Table 4.3).

Table 4.3. Mean number of nematodes (100g dry soil)$^{-1}$ ±SE in April 24th 2013 (before soil treatment (0), June 4th 2013 (5 WAT), July 2nd 2013 (9 WAT), and March 11th 2014 (44 WAT) (WAT: Weeks After chemical Treatment). Trophic group (TG) and C-P value (C-P) is indicated for each taxa. Ba: bacterial feeders, Fu: fungal feeders, O: omnivores, P: predators; Pp: plant parasites and herbivores. S*T, S*A: effect of the interaction sampling date*treatment and sampling date*amendment on each nematode taxon. ns: not significant , **: $P < 0.05$. Only the nematodes present in these sampling dates are shown.

	TG	C-P	24 Apr 13 (0)	19 Aug 13 (16WAT)	11 Mar 14 (44WAT)	A	T	S*T	S*A
Dauer	Ba	1	7.23**b** ±2.25	0.30**a** ±0.15	2.05**ab** ±0.65	ns	ns	ns	**
Mesorhabditis	Ba	1	0.24**a** ±0.19	0.16**a** ±0.08	104.82**b** ±25.26	ns	ns	**	**
Rhabditis	Ba	1	0.88 ±0.35	1.44 ±0.62	1.22 ±0.61	ns	ns	ns	ns
Cruznema	Ba	1	2.12 ±1.58	0.33 ±0.17	17.33 ±17.19	ns	ns	ns	ns
Panagrolaimus	Ba	1	7.72 ±5.21	3.72 ±2.39	2.67 ±1.64	**	ns	ns	ns
Chiloplacus	Ba	2	0.07 ±0.04	0.00 ±0.00	0.00 ±0.00	ns	ns	ns	ns
Cervidellus	Ba	2	0.02 ±0.01	0.03 ±0.02	0.00 ±0.00	ns	ns	ns	ns
Acrobeles	Ba	2	0.09 ±0.05	0.10 ±0.07	0.00 ±0.00	ns	ns	**	**
Acrobeloides	Ba	2	21.28**a** ±5.18	119.05**b** ±10.69	483.70**c** ±45.97	ns	ns	**	**
Eumonhystera	Ba	2	0.02 ±0.02	0.05 ±0.05	0.00 ±0.00	ns	ns	ns	ns
Prismatolaimus	Ba	3	0.00 ±0.00	0.10 ±0.09	0.60 ±0.49	ns	ns	ns	ns
Alaimus	Ba	4	0.00 ±0.00	0.00 ±0.00	0.22 ±0.22	ns	ns	ns	ns
Aphelenchus	Fu	2	2.96**a** ±0.97	5.35**a** ±1.65	30.51**b** ±7.73	**	ns	**	**
Aphelenchoides	Fu	2	11.21**a** ±6.35	0.59**a** ±0.18	17.39**b** ±8.01	ns	ns	**	**
Tylencholaimus	Fu	4	0.10 ±0.07	0.00 ±0.00	0.00 ±0.00	ns	ns	ns	ns
Dorylaimidae	O	4	0.43 ±0.14	0.62 ±0.24	0.58 ±0.27	ns	ns	ns	ns
Paratylenchus	Pp	2	0.02 ±0.02	0.23 ±0.16	0.04 ±0.04	ns	ns	ns	ns
Meloidogyne	Pp	3	0.00 ±0.00	0.14 ±0.07	0.00 ±0.00	ns	ns	ns	ns

Table 4.3 (continued).

	TG	C-P	24 Apr 13 (0)	19 Aug 13 (16WAT)	11 Mar 14 (44WAT)	A	T	S*T	S*A
Pratylenchus	Pp	3	1.21 ±0.51	0.49 ±0.18	0.87 ±0.60	ns	ns	ns	ns
Tylenchorhynchus	Pp	3	0.26 ±0.15	0.20 ±0.12	0.28 ±0.22	ns	ns	ns	ns
Helicotylenchus	Pp	3	0.00 ±0.00	0.10 ±0.10	0.00 ±0.00	ns	ns	ns	ns
Tylenchidae	Pp/Fu	2	1.05 ±0.29	0.33 ±0.21	0.36 ±0.24	ns	ns	ns	ns

4.3.3. Soil food web indices and nematode community descriptors

Mean values of nematode community descriptors were compared among treatments and among sampling dates (Fig. 4.7). The Enrichment Index showed in general highest values in the non-treated plots, and a decrease was observed from the beginning of the experiment onwards in the three treatments, to increase 30 WAT and thereafter, when the cabbage crop had been established for three months (Fig. 4.7). In general, the Basal Index showed lower values in the non-treated control along the experiment, increasing slightly at the end of the bean crop and decreasing thereafter until the end of the experiment (Fig. 4.7). The effect of sampling date on both indices was significant ($P< 0.05$) in the three treatments. The Channel Index showed a similar pattern in the plots treated with DMDS and the non-treated control, increasing at the end of the bean crop and decreasing to reach initial values at the end of the experiment, while no significant differences were observed among sampling dates in the plots treated with dazomet. The Structure Index varied significantly along sampling dates in the two treatments with soil fumigants and showed very low values at the end of the winter crop, while no significant differences were observed among sampling dates in the non-treated control, and only varied among treatments at the end of the summer crop (Fig. 4.7). The Maturity Index showed similar temporal patterns in the three treatments, showing similar values before treatment and at the end of the experiment, while the effect of sampling date on this index was significant ($P<0.05$) only in the plots treated with dazomet (Fig. 4.7). Lower values of the Maturity Index were observed in general in the non-treated control than in the chemical treatments. The Plant Parasitic Index was strongly reduced after fumigation, a pattern not found in the non-treated control, where the lowest values were observed 12 and 30 WAT in the plots treated with DMDS, and values at the end of the winter crop were more

than four-fold lower than before treatment. Plant Parasitic Index significantly varied among sampling dates in the treated plots. Taxa Richness showed the highest values in the non-treated control along the experiment, showing an acute decrease after treatment in the plots treated with dazomet and DMDS, increasing thereafter 30 WAT, reaching similar values as in the non-treated control at the end of the experiment. A similar pattern was observed for the Shannon diversity Index which decreased after treatment and showed the lowest values 12 WAT in the plots treated with DMDS, increasing thereafter in all treatments. Higher values were observed in the non-treated than in the treated plots and the effect of sampling date on this index was significant ($P< 0.05$) in all the treatments. Total nematode abundances increased acutely at the end of the summer crop, when crop residues were incorporated by ploughing into the soil at the end of August and before the cabbage crop was established, reaching a maximum 30 WAT (December 2013) with values 10^3 times higher than before treatment, and maintaining constant values until the end of the experiment (Fig. 4.7). The tendency was similar in the three treatments. No significant differences ($P> 0.05$) were observed among treatments before the application of soil fumigants, and at the last two sampling dates, during the cabbage crop, for any of the indices studied, except for the Plant Parasitic Index, which reached highest and the lowest values 30 WAT in the non-treated control and DMDS respectively (Fig. 4.7).

As in the case of soil chemical properties, no significant differences on nematode community descriptors were observed between amended and non-amended control in each chemical treatment at any sampling date (data not shown). Mean values of nematode community descriptors were compared among four sampling dates, before treatment (0), 5 WAT, 9 WAT (one month after the incorporation of the amendments) and 44 WAT (at the end of the cabbage crop) in each type of amendment (Table 4.4). The Enrichment Index decreased after amendments were applied, showing the lowest values one month after the incorporation, and increasing at the end of the winter crop in amended plots (Table 4.4). The Channel Index decreased 9 WAT in the compost-amended plots, but did not vary in the other plots, and recovered initial values at the end of the experiment. The Basal Index increased after treatment in the non-amended plots reaching the highest values at the end of the experiment. The Maturity Index showed the highest values 9 WAT and decreased at the end of the experiment in the compost-amended plots (Table 4.4). The Plant Parasitic Index showed the lowest values 9 WAT in the compost amended plots and 44 WAT in the compost+biochar and the non-amended plots. Taxa Richness showed the lowest values one month after the

incorporation of the amendments in the amended plots, while in the non-amended plots the highest values were observed before treatment, decreasing thereafter (Table 4.4). The Shannon diversity index decreased after treatment in the non- amended control and showed the lowest values at the end of the experiment. Total number of nematodes significantly increased (P< 0.05) at the end of the experiment irrespectively of the amendment (Table 4.4).

Figure 4.7. Mean values of the Enrichment Index (EI), Basal Index (BI), Channel Index (CI), Structure Index (SI), Maturity Index (MI), Plant Parasitic Index (PPI), Taxa Richness (S), The Shannon diversity Index (H'), and total number of nematodes (100 g of dry soil)$^{-1}$ (No.), measured on April 24th 2013 (before soil treatment (O-)), June 4th 2013 (5WAT) , July 2nd 2013 (9WAT), July 26th 2013 (12WAT), August 19th 2013 (16WAT), December 2nd 2013 (30WAT) and March 11th 2014 (44 WAT) (WAT: Weeks After Treatment) in pots treated with DMDS (dotted line), DZ: dazomet (dashed line) and C: non-treated control (solid line). Significance of the effect of sampling date on the indices and treatment effect at each sampling date are shown in each chart (* P < 0.05, ns = not significant). Error bars show ±SE.

Figure 4.7. See figure legend on the previous page.

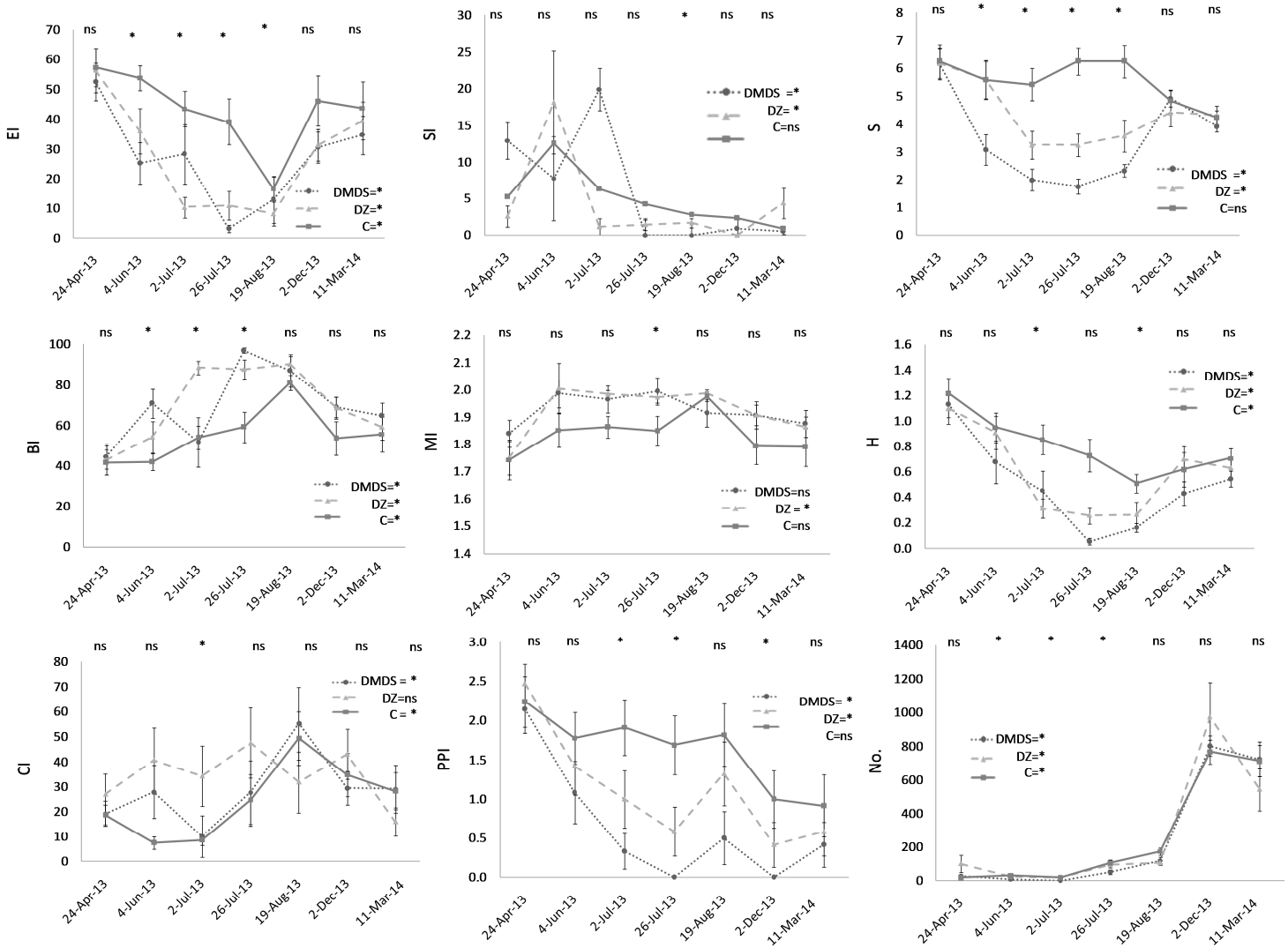


Table 4.4. Mean values ±SE of the Enrichment Index (EI), Basal Index (BI), Channel Index (CI), Structure Index (SI), Maturity Index (MI), Plant Parasitic Index (PPI), Taxa Richness (S), The Shannon diversity Index (H'), and total number of nematodes (100 g of dry soil)$^{-1}$ (No.), measured in April 24th 2013 (before soil treatment (O)), June 4th 2013 (5WAT) , July 2nd 2013 (9WAT), and March 11th 2014 (44WAT) in plots amended with compost, compost and biochar and the non-amended control. Differences (P < 0.05) among sampling dates in each amendment are shown. WAT: weeks after treatment.

	COMPOST				COMPOST+BIOCHAR				NON-AMENDED CONTROL			
	24-Apr-13 (O)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (O)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)	24-Apr-13 (O)	4-Jun-13 (5WAT)	2-Jul-13 (9WAT)	11-Mar-14 (44WAT)
EI	41.22**b**	40.32**ab**	19.54**a**	40.30**b**	51.10**b**	36.34**ab**	19.70**a**	43.16**b**	73.84	38.43	42.93	34.53
	±51.84	±57.21	±32.70	±58.24	±66.69	±51.81	±31.93	±56.99	±81.56	±54.06	±64.71	±50.74
SI	5.13	14.54	17.65	2.86	6.38	15.24	1.45	2.63	9.20	8.68	8.27	0.28
	±2.34	±5.68	±8.75	±1.73	±3.29	±6.62	±1.45	±1.45	±3.87	±5.26	±7.38	±0.28
CI	23.79**b**	24.13**b**	4.20**a**	19.50**b**	28.08	29.99	24.18	15.62	13.01	22.08	24.55	38.16
	±3.07	±10.63	±1.95	±6.48	±8.36	±10.42	±10.71	±5.88	±4.68	±10.68	±10.75	±9.24
BI	56.50	53.76	66.92	58.90	47.76	56.64	71.52	55.57	24.83**a**	56.62**ab**	55.81**ab**	65.38**b**
	±4.50	±7.54	±8.94	±8.13	±7.18	±7.85	±8.50	±5.95	±3.14	±7.17	±10.30	±7.38
MI	1.89**ab**	1.95**ab**	2.16**b**	1.83**a**	1.80	1.97	1.78	1.84	1.63	1.92	1.87	1.87
	±0.03	±0.06	±0.17	±0.06	±0.06	±0.08	±0.16	±0.04	±0.07	±0.07	±0.11	±0.06
PPI	2.21**b**	1.62**ab**	0.81**a**	1.25**ab**	2.15**b**	1.62**b**	1.14**ab**	0.17**a**	2.49**b**	1.04**a**	1.30**ab**	0.50**a**
	±0.32	±0.36	±0.35	±0.39	±0.31	±0.36	±0.35	±0.17	±0.25	±0.38	±0.40	±0.34
S	5.75**b**	5.67**ab**	3.42**a**	4.58**ab**	5.50**b**	4.33**ab**	3.33**a**	3.92**ab**	7.33**b**	4.25**a**	3.92**a**	4.00**a**
	±0.46	±0.86	±0.47	±0.29	±0.51	±0.56	±0.67	±0.29	±0.57	±0.66	±0.77	±0.17
H'	0.94	0.95	0.52	0.63	1.12	0.77	0.42	0.64	1.38**b**	0.82**ab**	0.67**a**	0.60**a**
	±0.10	±0.17	±0.11	±0.09	±0.13	±0.12	±0.11	±0.07	±0.07	±0.14	±0.17	±0.07
No.	55.20**a**	31.20**a**	13.19**a**	509.40**b**	20.19**a**	15.26**a**	10.19**a**	671.99**b**	73.64**b**	20.88**a**	17.45**a**	800.34**c**
	±32.76	±8.60	±4.29	±92.81	±5.50	±3.98	±2.60	±120.06	±43.47	±9.70	±6.89	±99.53

4.3.4. Plant analyses, weed cover, and soil faunal feeding activity

There were no significant differences (P> 0.05) on shoot biomass and shoot length among amendments at any sampling date (Table 4.5). Mean values of shoot biomass were significantly higher (P< 0.05) in the plots treated with dazomet 9 WAT, while mean values of shoot length showed the highest values in the non-treated control compared to the other treatments 12 WAT (Table 4.5). No significant differences were observed among treatments 12 and 16 WAT on shoot biomass and shoot length.

Mean values of weed cover were lower (P< 0.05) 9 and 12 WAT in the plots treated with dazomet than in the other treatments (Fig. 4.8). The effective quantum yield of photosynthesis measured 9 WAT showed the highest and the lowest values (P< 0.05) in the plots treated with dazomet and in the no-treated control respectively, while 16 WAT no significant differences were observed. When faunal activity was measured with bait lamina, mean numbers of ingested baits were significantly higher (P< 0.05) in the non-treated than in treated plots 12 WAT, while no significant differences were observed 44 WAT (Fig. 4.8). No differences were observed among amendments for any of the variables (data not shown).Positive correlations were observed 12 WAT between the Enrichment Index (r=0.51; P< 0.05), the Plant Parasitic Index (r=0.34; P< 0.05), Taxa Richness (r=0.39; P< 0.05), and the Shannon diversity Index (r=0.42; P<0.05), and the percentage of bait lamina ingested, while negative correlations (r=-0.41; P< 0.05) were observed between the Basal Index and percentage of bait lamina ingested (data not shown).

Shoot biomass and shoot length were positively correlated to soil moisture and electrical conductivity (Table 4.6). Shoot biomass was positively correlated to pH in the plots treated with DMDS. Total N, P and K were negatively correlated to shoot biomass and shoot length in the plots treated with dazomet. Negative correlations were observed between shoot biomass and shoot length, and P in the plots treated with and with DMDS and K in all treatments. The ratio C:N was positively related to shoot biomass in the plots treated with dazomet. NO_3^- and NH_4^+ were positively related to both biomass parameters in the plots treated with DMDS. No significant correlations were observed between plant biomass and soil physical-chemical properties in any of the amendments (data not shown). No significant differences on plant biomass were observed between amended and non-amended control in each chemical treatment at any sampling date (data not shown).

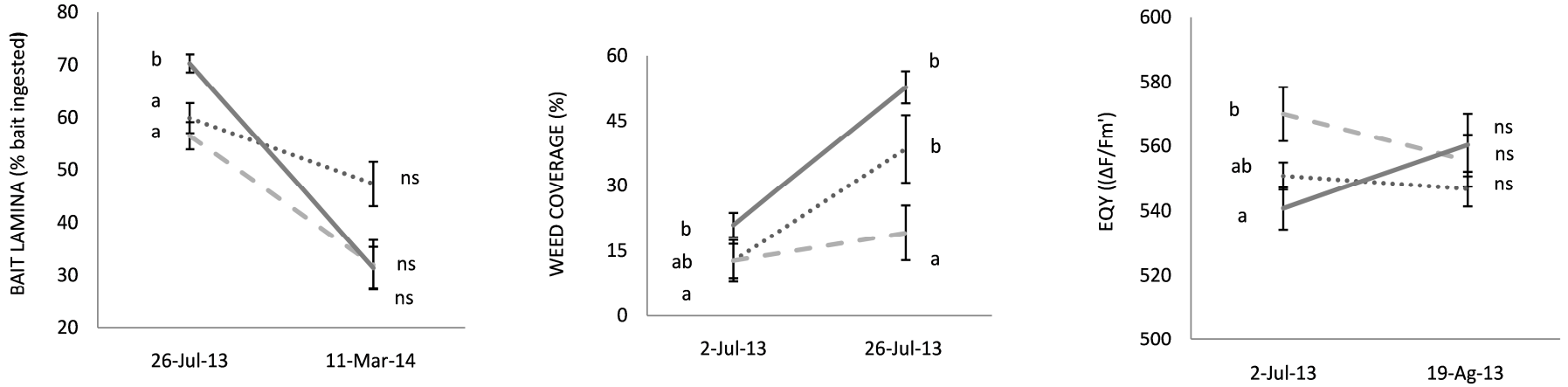
Table 4.5.Mean values ±SE of plant biomass (SB: shoot biomass (g), SL: shoot length (cm)) measured in July 2nd 2013 (9 WAT), July 26th 2013 (12 WAT), and August 19th 2013 (16 WAT). Different letters mean significant differences at $P < 0.05$ among amendments and chemical treatments at each sampling date.

		COMPOST	COMPOST+BIOCHAR	NON-AMENDED CONTROL	DMDS	DAZOMET	NON-TREATED CONTROL
July 2th 2013 (9 WAT)	**SB**	1.16	1.19	1.05	1.04**a**	1.39**b**	0.97**a**
		±0.11	±0.08	±0.08	±0.05	±0.11	±0.04
	SL	19.77	22.64	18.75	21.67	20.29	19.2
		±0.60	±2.33	±0.53	±2.41	±0.56	±0.62
July 26th 2013 (12 WAT)	**SB**	8.48	8.32	7.86	7.8	9.29	7.57
		±0.60	±0.47	±0.63	±0.43	±0.60	±0.54
	SL	46.21	45.21	44.33	45.75**ab**	42.88**a**	47.13**b**
		±1.11	±1.44	±0.75	±1.09	±1.05	±0.94
19th August 2013 (16WAT)	**SB**	19.89	18.64	22.94	22.24	18.39	20.84
		±1.21	±1.03	±2.22	±1.96	±1.28	±1.48
	SL	66.92	67.52	69.48	66.65	69.9	67.38
		±3.60	±4.79	±4.08	±3.12	±3.55	±5.48

Table 4.6. Pearson correlation coefficients between plant biomass (SB: shoot biomass (g), SL: shoot length (cm)), and physical-chemical properties. SM: soil moisture, EC: Electrical Conductivity, OM: Organic Matter, C:N: ratio carbon nitrogen. Bold numbers indicate significant correlation at $P < 0.05$.

		DMDS	DAZOMET	CONTROL
SM (%)	SB	**0.59**	**0.77**	**0.69**
	SL	**0.65**	**0.76**	**0.68**
EC ds $(m)^{-1}$	SB	**0.56**	**0.47**	**0.45**
	SL	**0.72**	**0.55**	**0.46**
pH	SB	**0.44**	0.08	-0.25
	SL	0.25	0.04	-0.25
MO (%)	SB	0.01	0.19	-0.09
	SL	0.21	0.09	0.06
N (%)	SB	0.08	**-0.37**	-0.04
	SL	0.17	**-0.37**	0.13
C:N	SB	-0.13	**0.34**	-0.08
	SL	-0.15	0.29	-0.11
P (ppm)	SB	**-0.56**	**-0.35**	-0.22
	SL	**-0.57**	**-0.36**	-0.14
K (ppm)	SB	**-0.80**	**-0.81**	**-0.90**
	SL	**-0.67**	**-0.79**	**-0.76**
NO_3^-	SB	**0.33**	-0.15	0.28
	SL	**0.41**	-0.08	0.16
NH_4^+	SB	**0.50**	-0.27	-0.17
	SL	**0.53**	-0.27	-0.18

Figure 4.8. Mean values ±SE of bait ingested (%), weed cover (%) and Effective Quantum Yield (ΔF/Fm') measured on July 2nd 2013 (9 WAT), July 26th 2013 (12 WAT), August 19th 2013 (16 WAT), and 11 March 2014 (44 WAT) in pots treated with DMDS (dotted line), dazomet (dashed line) and non-treated control (solid line). Different letters mean significant differences at *P*< 0.05 between treatments. Error bars show ±SE.



4.4. DISCUSSION

4.4.1. Organic amendments, soil properties and nematode community

Previous studies have shown that free-living nematode populations and microbial activity are significantly lower in fumigated soils compared to cover-cropped soils, and that different organic fertilizers and amendments enhance plant growth and induce an increase in the total population of nematodes, by increasing the bacterial- and fungal-feeding species(Collins *et al.*, 2005; Malusa *et al.*, 2012). Various studies in recent years have found biochar as a remediation tool to reduce the negative effects of pesticides on the environment (Kookana *et al.,* 2011; Tang *et al.*, 2013). Moreover, biochar and biochar combined with organic fertilizers as compost, might increase soil total organic carbon content and provide plant-available and mineralizable nutrients, improving soil fertility and plant growth (Schulz *et al.,* 2013). In accordance to previous studies, in which soil disinfection did not affect organic C and N content (Kapagianni *et al.*, 2010), we found organic matter and the C:N ratio significantly increased at the end of crop, probably due to the incorporation of crop residues that induced a large increase of soil organic matter and carbon inputs, and not as a consequence of the pesticide application. In contrast to the expected results, there were not evident effects of the amendments on organic matter content. Previous studies have shown that soil fumigation with different chemical pesticides, including DMDS, increase mineral nitrogen the first weeks after treatment, with a temporary effect, recovering later the basal level observed in the non-treated control (Yan *et al.,* 2012). Fumigants may retard the biological oxidation of ammonia by suppressing the activity of the ammonia-oxidizing bacteria involved in the first step of nitrification. In our study total N content and soil mineral nitrogen content (as the sum of NH_4^+ and NO_3^-) increased along the experiment, and although these effects were observed in the non-treated and non-amended controls too, mean values were significantly lower than in the treated plots. Since soil fumigation increase soil mineral nitrogen content due to the mineralization of microbial biomass killed by fumigation (Shen *et al.*, 1984; De Neve *et al.*, 2004), this process was probably enough to explain such increase. Although nitrification, one of the most important processes among nitrogen transformation in the soil, can be significantly reduced by soil fumigation (Duniway, 2002), causing an accumulation of soil ammonium and reduction of soil nitrate (Zhang *et al.,* 2011; Ebbels, 1971), our results suggest that fumigants did not retard the biological oxidation of ammonia by reducing the activity of nitrifying bacteria, since high values of nitrate and ammonium were observed after soil

treatment. The application of NPK fertilizer when seeds were sowed 6 weeks after treatments, explained the great increase of nitrogen, phosphorous and potassium values at the first sampling after sowing. Although nitrogen helps plant foliage to grow, phosphorous helps root and flowers to develop and potassium is important for overall plant health, too high values of these macronutrients can negatively affect plant growth. The excess of P on the root area can induce a late absorption of other micronutrients as Zn, Fe and Cu, and the excess of K in the soil might inhibit the absorption of Mg and Ca (MAGRAMA, 2009). In this study, negative correlations were observed betweenplant biomass and these macronutrients in the soil at the time of sampling, which might indicate a macronutrient excess in the soil. However, positive correlations observed between plant biomass and NH_4^+ and NO_3^-in the plots treated with DMDS indicate greater nutrient availability in this treatment. High values of electrical conductivity at the end of the crop indicate a high content of dissolved salts, probably due to the incorporation of organic matter with the incorporation of crop residues that induced an increase on soil electrical conductivity. Clark *et al.*, 2007 showed that, although soil electrical conductivity was markedly influenced by different types of amendments, crop residues as lucerne- and wheat-amended soils significantly increased electrical conductivity.

The soil biological condition before soil treatments was very poor in terms of nematode diversity and abundance. *Acrobeloides* and *Aphelenchoides*, two of the most ubiquitous nematode taxa in the soil worldwide, were also the most abundant taxa in our experimental area. Most of the nematode taxa identified in the experiment were microbial feeders with short life cycles and high reproduction rates, enrichment opportunistic (Ba1 and Fu2) and nematodes typically present in soils presenting a perturbed food web condition (Ba2 and Fu2)(Bongers, 1990; Ferris *et al.*, 2001). While the effects of chemical treatments and amendments did not have in general a significant and observable effect on the abundances of singular nematode taxa, the effect of sampling date was significant on the abundances of *Mesorhabditis*, *Acrobeloides*, *Aphelenchus* and *Aphelenchoides*, which increased at the end of the experiment. In contrast to the relatively little effect of chemical treatments on the abundances of singular nematode taxa, the incorporation of organic matter through the incorporation of bean crop residues was very noticeable. Previous studies have shown that in general, any form of organic matter input increases rapidly bacterial and fungal feeding nematode populations (Nahar *et al.*, 2006; Thoden *et al.*, 2011). In our experiment, abundances of other nematode trophic groups, such as omnivores and plant-parasitic nematodes, did not significantly vary along the experiment, probably due to their very low

abundances at the beginning of the experiment. However, the high abundances of some plant-parasitic nematodes observed at the last sampling, as *Paratylenchus* and *Meloydogine* can be explained by the fact that plant-parasitic nematodes often present a patchy distribution (Orui *et al.*, 1996).

4.4.2. Nematode community descriptors and soil food web condition

Soil food web indices have been widely studied to infer soil food web condition in agricultural crops (Bongers & Ferris, 1999; Sánchez-Moreno *et al.*, 2006). Chemical treatments reduced total number of nematodes, and although chemical treatments had a limited effect on the nematode abundance of each taxa, pesticides significantly reduced mostly bacterivores belonging to the organic enrichment opportunistic group as observed by the decrease on the Enrichment Index (Pen-Mouratov & Steinberger, 2005). In this study, both the Basal Index, indicator of depleted food webs, and the Channel Index, indicator of active fungal-mediated decomposition channels, slightly increased after chemical treatments, indicating a moderate increase of Ba2 and Fu2 nematodes, mainly *Acrobeloides*, *Aphelenchus* and *Aphelenchoides*, indicators of a basal and perturbed soil food web condition. When bean crop residues were incorporated into the soil at the end of August, the Enrichment Index increased continuously until the end of the experiment, as a result of the increase of enrichment opportunistic nematodes responding to organic matter inputs. The Maturity Index showed low values and did not fluctuate along the experiment, probably due to the low abundance of nematodes belonging to high trophic levels (Bongers, 1990), and these low values are indicative of a disturbed environment (Freckman & Ettema, 1993). Previous studies have shown that low values of the Structure Index are typical from perturbed soils (Park *et al.,* 2014; Sánchez-Moreno *et al.*, 2010), as in the case of our experiment, in which very low structure values were observed in general along the experiment. In terms of diversity, the effect of the chemical treatments on the nematode community was acute, reducing Taxa Richness and the Shannon diversity Index values. This acute effect of the treatments on nematode community descriptors might have obscured the effects of the incorporation of the amendments on nematode community descriptors, which were barely noticeable.

4.4.3. Plant analyses, weed cover, and soil faunal feeding activity

Bait lamina assays have been previously used to assess soil fauna trophic activity in the soil under various soil conditions e.g. various soil moisture ranges (Simpson *et al*., 2012). In this study, pesticides affected negatively soil fauna activity, since the percentage of baits ingested showed the highest values in the non-treated control 12 WAT. Such differences have disappeared at the end of the experiment probably due to the recovery of the soil fauna activity. Besides, the activity of opportunistic and plant-parasitic nematodes inferred by the Enrichment and Plant Parasitic Indices together with nematode diversity inferred by Taxa Richness and The Shannon diversity Index were positively correlated to the percentage of baits ingested. Indeed, the negative correlation observed between the Basal Index and soil fauna activity, indicates that in soils with basal conditions soil faunal activity is reduced. As demonstrated by Sánchez-Moreno *et al.*, 2009, soil food web indices based on the nematode community reflect the activity of other soil fauna with similar functional roles and environmental sensitivities.

On the other hand, Earlywine *et al.,* 2010 and Rosskopf *et al.*, 2005 demonstrated that DMDS and dazomet had an herbicide effect, reducing the vegetative growth of *Amaranthus caudatus, Trifolium repens* and *Stellaria* spp. in different crops. In this study, greater weed cover was observed in the non-treated control compared to the treated plots in the two sampling dates in which weed cover was measured, and although two different species of *Amaranthus* were observed, *Portulaca oleraceae* was the most abundant weed in all plots, demonstrating the herbicide effect of DMDS and dazomet.

Previous studies have measured the effective quantum yield in plants to infer the effects of different stress sources e.g. the stress caused by grafting (Calatayud *et al.,* 2013) or by drought in different crops(De Dauw *et al.,*2013). However, to our knowledge, no previous studies have aimed to study the effect of dazomet on the effective quantum yield measured in plants. The intense green colour observed in plants growing in the plots treated with dazomet 9 WAT, corresponded in fact effectively with high values of the effective quantum yield measured in such plants compared to the other treatments. Moreover, a positive correlation was observed between shoot biomass and effective quantum yield. Shoot biomass was significantly higher in the plants treated with dazomet than in the other treatments. Dazomet, in contact with soil moisture, releases methyl-isothiocyanate (MITC) gases that spread within soil pores,

inactivating fungi, nematodes, bacteria, and weeds (Zanón-Alonso *et al.*, 2011). Positive effects of dazomet on plant growth have been associated to chemical compounds generated during decomposition of the active substance (Zanón-Alonso *et al.*, 2011). During the decomposition of dazomet, the process of nitrification is stimulated and compounds derived from mineralization, such as NH_4^+ and NO_3^-, improve plant nutrition. Moreover, some beneficial organisms, such as actinomycetes, main responsible of organic matter decomposition, are stimulated, and might generate vitamins and antibiotics, stimulating plant growth (Zanón-Alonso *et al.*, 2011). Regarding the effects of amendments on plant biomass, we suggest that in this study fumigation obscured, as least partially, the effect of the amendments and as a consequence no significant differences were observed on plant biomass among amendments.

CONCLUDING REMARKS

IMPACT OF PESTICIDES AND OTHER TYPES OF SOIL DISTURBANCE ON SOIL NEMATODE COMMUNITIES

Chapters 1, 2, and 4 of this thesis present the results of the impact of different types of soil disturbance on the nematode community, especially of the effects of various pesticides applied in various agricultural crops. In the first chapter, the effects of 1,3-dicloropropene (1,3-D) and chloropicrin (Pic) on the soil nematode community were studied in a commercial strawberry crop and its surroundings (Chapter 1). In the second one, the effects of dazomet, oxamyl, fenamiphos and organic pesticides were studied in microcosms under experimental conditions using tomato plants (Chapter 2), while in the last chapter the effect of dazomet and dimethyl disulphide were studied in a rotation of bean and cabbage crops under experimental field conditions (Chapter 4). During the development of this thesis, some of these active substances, such as 1,3-D and Pic, have not been approved by the European Commission, and new substances have been incorporated into the market as alternatives to fight against agricultural pests and diseases. The current Regulation (EC) 1107/2009 (E.C., 2009), that derogates the Directive 91/414, regulates the placing on the market of plant production products. Such Regulation is more restrictive than the old Directive and the acceptance of new active substances have been seriously limited.

The results of this thesis show that, in general, the soil nematode community was deeply affected by all chemical pesticides and organic pesticides applied in different crops in the three different studies (Chapters 1, 2 and 4). In chapter 1, soil functioning had recovered, at least to a certain extent, by the end of each season in the strawberry crop, as shown by the sequential recovery of nematode abundances along the cropping seasons. Previous studies on the effects of chemical and organic disinfection methods on the interaction between nematode functional guilds and soil properties have shown that chemical disinfection affects such interaction to a larger extent than organic disinfection (Kapagianni *et al.*, 2010). In chapter 2, in which both organic pesticides with nematicide effect and chemical nematicides were applied to two types of soil, not only the nematicides used, but also the initial condition of the nematode community and soil properties, determined the differences on nematode responses to different nematicide applications. Two different types of soil were used in this experiment; the low diversity soil was an agricultural, managed soil from a commercial strawberry farm and the high diversity soil was collected from an unmanaged natural pinewood (*Pinus pinea* L.). In microcosms experiments, a detrimental effect of potting, even in the absence of any soil treatment, was observed in pots filled with farm soil, while pots filled with pinewood soil

showed higher resilience to both the application of pesticides and physical disruption by potting, than farm soils. In Chapter 4, biochar and compost were used as a remediation tool to reduce the negative effects of pesticides to the environment. Previous studies showed that free-living nematode populations were significantly reduced in fumigated soils compared to cover crop-cropped treatments and that different organic fertilizers and amendments affected nematode community (Collins *et al.*, 2005; Malusa *et al.*, 2012). However, our results show that the effect of pesticides was much more noticeable than the effect of amendments on soil properties and nematode community descriptors, and we can conclude that fumigation obscured, at least partially, the effects of the amendments.

In general, bacterial- and fungal-feeding nematodes were the most abundant taxa in all soils, and were thus the taxa most affected by fumigation. In Chapter 1, nematodes belonging to higher trophic links were more abundant in the untreated areas, which in general presented mature and complex soil food webs, while treated soils presented a disturbed and enriched food web condition (*sensu* Ferris *et al*., 2001). In Chapter 2, a large dauer juveniles explosion occurred in the nutrient-depleted soil in the neem-amended pots, while the same treatment applied to pinewood soil, rich in organic matter, induced an increase of more stable, less opportunistic, populations of generalist bacterivore nematodes. In Chapter 4, a permanent basal and perturbed soil food web condition was observed along the field experiment, even before soil treatments were applied, probably due to the intensive management carried out for years in the experimental station in which the experiment was developed. In such experiment, chemical soil treatments and biochar and compost amendments did not have in general a significant effect on the abundances of singular nematode taxa. However, the incorporation of organic matter through the incorporation of the bean crop residues before the establishment of the cabbage crop, induced an acute increase of the most abundant taxa, but not of the populations of most of the plant-parasitic and omnivore nematodes, that presented very low abundances at the beginning of the experiment.

TARDIGRADES AS INDICATORS OF SOIL FOOD WEB STRUCTURE IN TREATED SOILS AND SOIL SUPPRESSIVENESS

Tardigrades, which occupy a wide range of niches in freshwater and terrestrial environments, were studied in Chapter 1. High tardigrade abundances were observed in the pinewood soils, probably related to high soil carbon contents present in this area. Peak tardigrade abundances found in the strawberry field furrows showed that the low efficiency of pesticides within this

field zones was probably enough to maintain tardigrade populations in the soil. Since tardigrades have seldom been studied in agricultural-related systems, their possible use as indicators of the effects of management on soil diversity should be further investigated. Tardigrades are some of the soil organisms involved in soil suppressiveness.

In chapter 1, soil suppressiveness was studied, and our results showed that soil food web structure (sensu Ferris *et al*., 2001) and soil diversity were positively correlated to soil suppressiveness, which was inferred through an experiment in which *Galleria mellonella*, a model lepidopter, was used as a bait organism for soil biota. Higher larvae mortality in natural soils than in the treated soils was recorded, showing that soil management, especially soil fumigation, reduced natural soil suppressiveness, turning a suppressive soil into a non-suppressive one.

RELATIONSHIPS BETWEEN SOIL PHYSICAL-CHEMICAL PROPERTIES AND NUTRIENT CYCLING AFTER PESTICIDE APLLICATION

In Chapter 1, the continuous supply of N solutions by fertigation explains why the composition of the nematode community was more closely associated to nutrient dynamics in pinewood and field margin soils than in farm soils, in which nutrient dynamics rely heavily on chemical inputs. Also, in spite of the organic matter incorporated into the farm soil at the beginning of each cropping season in form of manure, organic matter content and the ratio C:N were lower in the farm than in the pinewood soil, probably due to the low capacity of agricultural soils to retain carbon in complex carbon forms.In Chapter 2, higher values of total nitrogen in the pinewood than in the farm soil might be partially related to higher micro and mesofauna abundances, since it has been proved that, e.g., nematode excretion significantly contributes to soluble soil nitrogen (Ekschmitt *et al.*, 1999).

PLANT BIOMASS AND ITS RELATION WITH NEMATODE COMMUNITY STRUCTURE UNDER PESTICIDE APPLICATION

Although roots are relevant for soil functioning (Bonkowski et al., 2009), agricultural production promotes varietal selections for higher yields, generating an increase of the root-to-shoot ratio and reducing nutrients in the soil (Neher, 2010). Such reduction of the root system might contribute to the weak associations found between nematode community structure and root and shoot biomass in the commercial strawberry field studied in Chapter 1.

However, the positive correlation found between the Enrichment Index and shoot biomass supports the assumption of the usefulness of the Enrichment Index as indicator of soil fertility (Ferris et al., 2001). In Chapter 2, in which organic pesticides were applied to potted soil, pesticide activity of neem compounds (Akhtar, 1998; Dwivedi, 2008; Al-Samarrai *et al.*, 2012), might explain higher plant biomasses in the neem than in the other treatments. Besides, shoot and root biomasses were two-fold higher in plants growing in microcosms with pinewood (characterized by high nutrient availability) than with farm soils. In experimental field conditions (Chapter 4), an excess of N, P, K contents in the soil probably induced a late absorption of other micronutrients, affecting negatively plant growth. The intense green colour observed in the plants treated with dazomet and higher values of shoot biomass in the plants treated with this pesticide than in the other treatments, revealed that during the decomposition of the active substance dazomet, the process of nitrification was stimulated and compounds derived from mineralization improved plant nutrition, as previously described (Zanón-Alonso *et al.*, 2011).

RESPONSES OF SOIL FAUNA ACTIVITY TO PESTICIDES APPLIED UNDER FIELD EXPERIMENTAL CONDITIONS

In field experimental conditions (Chapter 4), *bait lamina* assays were performed to evaluate soil ecosystem services that are driven by functions of the edaphic community and its biological activity. In this study, pesticides affected negatively soil fauna activity, and percentage of baits ingested by microfauna was higher in the non-treated control than in the fumigated plots. Such differences have disappeared at the end of the experiment, indicating that soil fauna activity had recovered. The activity of opportunistic and plant-parasitic nematodes inferred by the Enrichment and Plant Parasitic Indices, together with nematode diversity inferred by Taxa Richness and The Shannon diversity Index, were positively correlated to the percentage of baits ingested. The negative correlations observed between the Basal Index and soil fauna activity, indicated that when the soil presented a basal condition, soil faunal activity was reduced. As demonstrated by Sánchez-Moreno *et al.*, 2009, soil food web indices based on the nematode community reflected the activity of other soil fauna.

COMPARISONS BETWEEN MOLECULAR AND MORPHOLOGICAL IDENTIFICATION METHODS OF NEMATODE ASSEMBLAGES

In chapter 3 differences and similarities on nematode diversity and nematode community descriptors assessed by morphological and molecular techniques (microscopy at high magnification and TRFLP (Terminal Restriction Fragment Length Polymorphism) respectively), from samples collected from different land use areas in Spain and Ireland have been studied.
This chapter represents a fundamental step in the evaluation of the efficiency of molecular methods assessing soil nematode diversity. To our knowledge, few previous studies have aimed to evaluate the structure and diversity of the soil nematode assemblage using molecular determinations (Griffiths *et al.*, 2006; Edel-Hermann *et al.*, 2008; Griffiths *et al.*, 2012).

Results of this chapter showed that TRFLP technique applied to the amplified nematode community DNA only detected a percentage of the taxa present in the samples identified morphologically, and omnivore and predator nematodes were not identified molecularly. TRFLP were very efficient detecting nematodes belonging to the family Cephalobidae, although their identification only reached the family level. Such concordance between the morphological and molecular approach might be due precisely to the high abundance of *Acrobeloides*, which facilitates an accurate detection of the taxon due to high DNA template concentration. Our results show that, at least with the technique used in this study, identifying nematodes morphologically is a reliable and more precise identification tool nowadays, since a higher taxonomic resolution was in general obtained compared to TRFLP. However, since the peaks identified by Donn *et al*., 2008 were from nematodes in Scottish soils this may be a reason for low taxa richness found in the molecular identifications. Further research is needed, and finding a way to link more closely results obtained by molecular and morphological methods might be relevant in the future to get reliable and informative nematode assemblage identifications.

CONCLUSIONS

CHAPTER 1. Relationships between nematode diversity, plant biomass, nutrient cycling and soil suppressiveness in fumigated soils.

- Nematode diversity was very affected by soil fumigation with 1,3-dichloropropene and chloropicrin and, although yearly recovery occurred within the treated field, fumigated soils showed a permanent perturbed condition, as expected in an intensive agricultural system.

- The soil nematode community was more closely associated to nutrient cycling in non-cropped than in cropped soils.

- There was a weak relationship between nematode community structure and plant biomass.

- Non-treated furrows within the fields supported more abundant and diverse beneficial and plant-parasitic nematode assemblages, but this difference was not enough to maintain more suppressive communities.

- Fumigated soils were less suppressive to the lepidopter *G. mellonella* than non-treated ones, and there was a positive and significant correlation between soil suppressiveness and soil food web structure and diversity.

CHAPTER 2. Effects of organic and chemical pesticides on plant biomass, nematode diversity, and the structure of the soil food web.

- The effects of organic and chemical nematicides on the nematode community depended not only on the toxicological attributes of the active substances used but also on the attributes of the nematode community subjected to perturbation.

- Chemical nematicides had a strong effect of the nematode community, which were stronger on low diversity communities than in high diversity ones.

- Diverse nematode communities were more resilient to perturbation.

- Neem application induced greater plant growth than the other treatments and a bloom of bacterial feeding nematode populations, which rapid depletion in nutrient-poor soils induced a large bloom of dauer juveniles in the farm soil, and a more stable bacterivore community in nutrient-rich pine forest soils.

CHAPTER 3. Comparison of molecular and morphological identification methods of soil nematode assemblages and food web descriptors from different agroecosystems.

- A higher number of taxa were identified morphologically than molecularly.

- High numbers of dauer juveniles identified morphologically in the Irish strawberry crop and bean crop samples were identified as Rhabditidae with molecular methods, but no further resolution was achieved.

- Mean relative abundances of omnivore and predator nematodes represented a very low percentage of the total nematode abundances in the samples identified morphologically. Omnivores and predators were not identified molecularly in our study. Total numbers of nematodes belonging to these trophic groups might be too low to be detected by molecular methods.

- Molecular identification with TRFLP only detected, on average, a percentage ranging from 1% (e.g. Aphlenchida) to 80% (e.g. Cephalobidae) of the taxa present in the samples identified morphologically.

- The Structure Index could not be calculated in the samples identified molecularly due to the lack of taxa belonging to some trophic groups.

- Concordance between morphological and molecular identification was achieved for highly abundant taxa, which facilitated an accurate detection of the taxon probably due to high DNA template concentration.

- Due to the differences observed on nematode taxa identified molecularly and morphologically, mean values of the Enrichment, Channel, Plant Parasitic and Maturity Indices and Taxa Richness obtained by both methods significantly differed.

- Indices involving microbial feeding nematodes were more similar across methods than indices involving higher trophic links.

CHAPTER 4. Effect of soil nematicides on nematode diversity and functioning and the mitigating effect of organic amendments under experimental field conditions.

- The soil biological condition before soil treatments was very poor in terms of nematode diversity and abundance and a permanent perturbed soil food web condition was observed along the experiment.

- The incorporation of crop residues into the soil affected soil nematode community descriptors to a great extent, and enhanced soil fertility.

- An excess of NPK inputs applied in the soil through fertilization reduced plant biomass. Effective quantum yield and shoot biomass were significantly higher in the plants treated with dazomet than in the other treatments, probably associated to chemical compounds generated during the decomposition of the active substance.

- Pesticides affected negatively the activity of other groups of soil fauna apart from nematodes.

- Soil food web indices based on the nematode community reflected the activity of other soil fauna.

- Since pesticides had a noticeable negative effect on soil properties and nematode community descriptors, amendments were not enough to mitigate this negative effect.

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SUPLEMENTARY INFORMATION

Table 1. S1. Average number of nematodes ±SE (100g dry soil)$^{-1}$ in each habitat in 2010-2011, 2011-2012 (0-20 cm depth). Trophic group (TG) and C-P value (C-P) is indicated for each taxa. Ba: bacterial feeders, Fu: fungal feeders, O: omnivores, P: predators; Pp: plant parasites or herbivores. F: Field. FM: Field margins, P: Pine forest, FU: furrow. Levels of significance between sampling dates (SD) before treatment (CO-), 5, 17, 28 and 35 and before treatment (CO-), 5, 19, 30 and 39 weeks after treatment (WAT) in 2010-2011 and 2011-2012 respectively are shown. ** $P < 0.05$, ns: not significant.

			2010-2011				2011-2012				
	TG	**C-P**	**F**	**FM**	**P**	**SD**	**F**	**FM**	**P**	**FU**	**SD**
Dauer	Ba	1	0.62	0.15	0.01	ns	3.67	1.87	2.48	6.35	**
			±0.11	±0.13	±0.07	ns	±1.15	±0.86	±1.01	±2.03	ns
Mesorhabditis	Ba	1	0.24	1.13	1.40	**	2.79	11.79	15.03	1.73	ns
			±0.05	±0.51	±0.27	ns	±0.66	±5.2	±5.32	±0.59	ns
Rhabditis	Ba	1	0.12	0.09	0.10	**	2.44	3.61	1.33	5.92	**
			±0.03	±0.03	±0.03	ns	±0.58	±1.58	±0.80	±2.14	ns
Cruznema	Ba	1	1.12	0.11	0.04	**	1.40	5.06	0.76	13.7	**
			±0.25	±0.06	±0.03	ns	±0.34	±3.01	±0.39	±9.68	ns
Diploscapter	Ba	1	0.00	0.00	0.00	ns	0.05	0.00	0.73	0.00	ns
			±0.00	±0.00	±0.00	ns	±0.05	±0.00	±0.73	±0.00	ns
Panagrolaimus	Ba	1	1.70	1.61	6.28	**	38.41	83.99	59.76	33.16	**
			±0.21	±0.27	±1.09	ns	±7.46	±17.94	±14.7	±6.84	ns
Neodiplogasteridae	Ba	1	0.50	0.00	0.13	**	0.74	0.00	0.45	2.10	**
			±0.13	±0.00	±0.09	ns	±0.23	±0.00	±0.27	±1.22	ns
Diplogasteridae	Ba	1	0.11	0.00	0.00	**	0.00	0.00	0.00	0.00	ns
			±0.07	±0.00	±0.00	ns	±0.00	±0.00	±0.00	±0.00	ns
Mononchoides	Ba	1	0.01	0.00	0.06	**	0.00	0.00	0.00	0.00	ns
			±0.00	±0.00	±0.05	ns	±0.00	±0.00	±0.00	±0.00	ns
Rhabdolaimus	Ba	1	0.00	0.00	0.03	ns	0.00	0.00	0.00	0.00	ns
			±0.00	±0.00	±0.03	ns	±0.00	±0.00	±0.00	±0.00	ns

Table 1.S1 (continued)

			2010-2011				2011-2012				
	GT	**C-P**	**B**	**E**	**P**	**SD**	**B**	**E**	**P**	**FU**	**SD**
Diplenteron	Ba	1	0.03	0	0	**	0	0	0	0	ns
			±0.02	±0.00	±0.00	ns	±0.00	±0.00	±0.00	±0.00	ns
Eucephalobus	Ba	2	0.01	0	0.04	ns	0	0	0	0	ns
			±0.01	±0.00	±0.04	ns	±0.00	±0.00	±0.00	±0.00	ns
Chiloplacus	Ba	2	0.01	0	0	ns	0.08	3.01	0.23	0.07	**
			±0.01	±0.00	±0.00	ns	±0.08	±1.77	±0.23	±0.07	ns
Cervidellus	Ba	2	0	0.04	0.08	**	0.31	0.6	0.22	0.14	ns
			±0.00	±0.02	±0.04	ns	±0.16	±0.6	±0.16	±0.14	ns
Acrobeles	Ba	2	0.19	0.12	0.62	**	6.62	18.07	15.39	6.01	**
			±0.05	±0.07	±0.25	ns	±1.58	±7.13	±4.53	±2.31	ns
Acrobeloides	Ba	2	5.69	8.56	9.04	**	48.42	253.91	99.89	59.62	**
			±0.44	±1.05	±0.99	ns	±4.5	±49.55	±25.18	±9.83	ns
Teratocephalus	Ba	3	0	0.03	0.15	ns	0	2.36	11.92	0	ns
			±0.00	±0.03	±0.07	ns	±0.00	±1.44	±7.69	±0.00	ns
Plectus	Ba	2	0.05	0.1	0.52	ns	1.22	3.01	2.55	0.83	**
			±0.01	±0.07	±0.19	ns	±0.55	±1.37	±1.21	±0.59	ns
Wilsonema	Ba	2	0	0.02	0.59	ns	0	8.17	9.96	0	ns
			±0.00	±0.02	±0.29	ns	±0.00	±7.31	±3.58	±0.00	ns
Eumonhystera	Ba	2	0.08	0.05	0.9	**	0.35	2.73	17.34	0.51	ns
			±0.02	±0.04	±0.37	ns	±0.24	±1.07	±7.94	±0.25	ns
Prismatolaimus	Ba	3	0.29	0.86	0.69	**	5.91	21.58	10.85	2.87	**
			±0.10	±0.49	±0.18	ns	±2.08	±7.1	±1.78	±0.92	ns
Achromadora	Ba	3	0.01	0.61	0.17	ns	0.15	9.57	10.97	0.56	ns

Table 1.S1 (continued)

			2010-2011				2011-2012				
	TG	**C-P**	**B**	**E**	**P**	**SD**	**B**	**E**	**P**	**FU**	**SD**
Leptolaimus	Ba	3	0	0	0.02	ns	0	0	0.39	0	ns
			±0.00	±0.00	±0.02	ns	±0.00	±0.00	±0.28	±0.00	ns
Alaimus	Ba	4	0.08	1.09	0.45	**	0	0	0.39	0.04	ns
			±0.02	±0.20	±0.15	ns	±0.00	±0.00	±0.30	±0.04	ns
Aphelenchus	Fu	2	0.47	3.6	1.02	**	2.55	26.29	7.92	1.55	**
			±0.11	±0.88	±0.21	ns	±0.91	±6.04	±2.56	±0.46	ns
Aphelenchoides	Fu	2	0.02	0.02	0	**	17.93	29.36	10.82	17.85	**
			±0.01	±0.01	±0.00	ns	±51.27	±73.10	±44.25	±46.13	ns
Ditylenchus	Fu	2	0.01	0.06	0.1	ns	0	0	0	0	ns
			±0.00	±0.04	±0.07	ns	±0.00	±0.00	±0.00	±0.00	ns
Diphterophora	Fu	3	0	0.01	0.01	ns	0.04	0	0.16	0.1	ns
			±0.00	±0.01	±0.01	ns	±0.04	±0.00	±0.12	±0.08	ns
Tylolaimophorus	Fu	3	0.06	0.25	0.43	ns	0.48	0.31	1.22	0.06	ns
			±0.04	±0.16	±0.19	ns	±0.25	±0.31	±0.99	±0.05	ns
Tylencholaimus	Fu	4	0.46	1.95	1.17	**	0.74	38.35	44.93	0.1	**
			±0.09	±0.56	±0.24	ns	±0.32	±17.13	±20.00	±0.06	ns
Dorylaimidae	O	4	0.01	0	0	ns	12.22	42.3	41.24	11.93	**
			±0.00	±0.00	±0.00	ns	±2.65	±11.01	±7.51	±2.76	ns
Paratylenchus	Pp	2	0.52	0.02	0.08	**	0.12	±0.00	0.08	0	ns
			±0.39	0.01	0.07	ns	±0.12	±0.00	±0.08	±0.00	ns
Malenchus	Pp	2	0	0	0	ns	0	0	0	0	ns
			±0.00	±0.00	±0.00	ns	±0.00	±0.00	±0.00	±0.00	ns
Meloidogyne	Pp	3	0.64	2.27	0.16	**	54.99	1.42	0.24	1.26	**
			±0.19	±0.96	±0.10	ns	±35.60	±1.42	±0.24	±1.12	ns

Table 1.S1 (continued)

			2010-2011				2011-2012				
	TG	**C-P**	**B**	**E**	**P**	**SD**	**B**	**E**	**P**	**FU**	**SD**
Pratylenchus	Pp	3	0.00	0.05	0.00	**	1.17	15.47	2.62	2.42	**
			±0.00	±0.05	±0.00	ns	±0.41	±6.55	±1.41	±0.66	ns
Tylenchorhynchus	Pp	3	0.01	0.00	0.05	**	0.13	11.06	0.08	0.04	ns
			±0.01	±0.00	±0.05	ns	±0.13	±10.61	±0.08	±0.04	ns
Rotylenchus	Pp	3	0.00	0.01	0.00	ns	0.00	17.33	0.14	0.11	**
			±0.00	±0.01	±0.00	ns	±0.00	±16.12	±0.14	±0.08	ns
Helicotylenchus	Pp	3	0.00	0.04	0.03	ns	0.01	0.79	0.24	0.00	**
			±0.00	±0.03	±0.03	ns	±0.01	±0.64	±0.24	±0.00	ns
Criconematidae	Pp	3	0.27	0.36	0.32	**	0.00	0.00	0.00	0.00	ns
			±0.08	±0.27	±0.08	ns	±0.00	±0.00	±0.00	±0.00	ns
Diphterophora	Pp	3	0.00	0.00	0.00	ns	0.00	0.00	0.00	0.00	ns
			±0.00	±0.00	±0.00	ns	±0.00	±0.00	±0.00	±0.00	ns
Trichodorus	Pp	4	3.67	3.65	3.05	**	2.55	1.26	10.05	13.66	**
			±0.43	±0.91	±1.08	ns	±0.77	±0.62	±3.38	±5.67	ns
Xiphinema	Pp	5	0.00	0.00	0.00	ns	0.00	0.00	0.71	0.00	ns
			±0.00	±0.00	±0.00	ns	±0.00	±0.00	±0.43	±0.00	ns
Tylenchidae	Pp/Fu	2	107.79	574.62	269.85	**	13.55	81.28	62.59	11.84	**
			±18.07	±128.02	±44.31	ns	±2.20	±22.79	±16.90	±4.56	

PUBLICATIONS, INTERNATIONAL CONFERENCES AND STAYS ABROAD

PhD publications

Carrascosa, M., Sánchez-Moreno, S. & Prados-Alonso, J.L. (2014). Relationships between nematode diversity, plant biomass, nutrient cycling and soil suppressiveness in fumigated soils. European Journal of Soil Biology. 62:49-59.

Carrascosa, M., Sánchez-Moreno, S. & Prados-Alonso, J.L. (2015). Effects of organic and conventional pesticides onplant biomass, nematode diversity and the structure of the soil food web. Nematology. 17:11-26

Future PhD Publications

M. Carrascosa, S. Sánchez-Moreno, J.L Alonso-Prados, Tim Daniell, Roy Neilson, Dote Stone, Bryan S. Griffiths.Comparison of molecular and morphological identification methods of soil nematode assemblages and food web descriptors from different agroecosystems.

M. Carrascosa, S. Sánchez-Moreno, J.L Alonso-Prados. Effect of soil nematicides on nematode diversity and functioning and the mitigating effect of organic amendments under experimental field conditions.

International conferences

Carrascosa, M., Sánchez-Moreno, S. & Prados-Alonso, J.L. (2011).Poster. Effect of 1,3-dicloropropene and chloropicrin on soil diversity and functioning in a strawberry field and its surroundings in southern Spain. XLIII ONTA Annual Meeting. Coimbra, Portugal 04-09 September 2011.Nematropica. 41(2):138. ISSN: 2220-5616.

Stays in International Research Centers

Teagasc Research Centre. Johnstown Castle, Wexford, Ireland. May-August 2012.

Scottish Rural College (SRUC). University of Edinburgh. Edinburgh, Scotland, UK. October-November 2013.