Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Ecology

Side effects of biological control agents in agriculture

– Does the bacteria *Bacillus amyloliquefaciens* affect the earthworm *Aporrectodea longa*?

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Agriculture Programme - Soil and Plant Sciences
Bachelor thesis
Uppsala 2014

Independent project/Degree project / SLU, Department of Ecology 2014:3

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Credits: 15 hec
Level: G2E
Course title: Degree project in Biology
Course code: EX0689
Programme/education: Agriculture Programme - Soil and Plant Sciences

Place of publication: Uppsala
Year of publication: 2014
Cover picture: Elsa Lagerqvist
Title of series: Independent project/Degree project / SLU, Department of Ecology
Part no: 2014:3
Online publication: http://stud.epsilon.slu.se

Keywords: Biological control, Bacillus amyloliquefaciens, Aporrectodea longa, bioindicators

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Abstract

Pathogenic microorganisms pose a big threat towards food production. Meanwhile, negative impacts on humans and environment are seen by the use of pesticides. Biological control agents are an alternative to the use of chemical pesticides, and plant-growth promoting rhizobacteria, such as *Bacillus amyloliquefaciens* has been shown to have good properties as biocontrol agents. Before these bacteria can be used commercially their impact on other soil organisms has to be evaluated. This thesis looks into what biocontrol bacteria are and how they might affect earthworms. The thesis consists of a literature review and an experiment where earthworms (*Aporrectodea longa*), kept in natural soil, were exposed to the bacteria *B. amyloliquefaciens*. Earthworms were exposed to the bacteria in three ways; contact with skin, gut or both, they were either directly dipped in the bacterial solution or the solution was added to the soil. Earthworm growth, mortality and reproduction were measured to evaluate bacterial impact. Though neither of the treatments affected the earthworms negatively further research to test other means of bacterial exposure is needed before the bacteria can be used in agricultural management. Amongst others it is suggested to investigate how earthworms are affected by eating crop residues treated with the bacteria and to study if earthworms show avoidance behavior when exposed to the bacteria.

Sammanfattning

Sjukdomsframkallande mikroorganismer utgör ett stort hot mot världens matproduktion. Samtidigt uppmärksammas negativa konsekvenser av pesticid-användningen för både människor och miljön. Biologisk kontroll är ett alternativ till användningen av kemiska pesticider. Tillväxtfrämjande bakterier, så som *Bacillus amyloliquefaciens*, har visat bra egenskaper för att utföra biologisk kontroll, men innan dessa bakterier kan användas kommersiellt måste deras inverkan på andra markorganismer bli utredd. Denna uppsats utforskar vad biokontroll bakterier är och hur *B. amyloliquefaciens* skulle kunna påverka daggmaskar. Uppsatsen består av en litteratur genomgång och ett experiment där daggmaskar (*Aporrectodea longa*) hölls i naturlig jord där de exponerades för bakterien *B. amyloliquefaciens*. Dagmaskarna utsattes för bakterien på tre olika sätt; kontakt med skinn, mag-tarmkanal eller båda, genom att de antingen doppades direkt i bakterielösningen eller att bakterielösningen tillsattes i jorden. Tillväxt, mortalitet och reproduktion var de mått som utvärderade bakteriens inverkan på daggmaskarna. Resultatet visar att daggmaskarna inte är negativt påverkade av bakterien men vidare forskning behövs innan bakterien kan användas inom jordbruket. Tidigare studier har föreslagit att vidare forskning skulle kunna ske genom att utvärdera hur daggmaskarna påverkas av att äta rester av grödor behandlade med bakterien och att studera om daggmaskar visar undvikandebeteende när de är exponerade för bakterien.

Introduction

Worldwide, plant pathogenic microorganisms and the diseases they cause pose a big threat to food production and ecosystem stability (Oerke, 2006; Choudhary et al., 2007; Kumar et al., 2011). Humans have tried to manage pests ever since they started cropping and to curb these pathogens, for which farming techniques such as crop rotation, tillage and resistant plants are used (Bennett et al., 2012). However, many authors stress the necessity of using beneficial soil organisms in pest control management for future crop production, as the conventional farming techniques mentioned above are not efficient enough to control diseases in agricultural systems (Pal and McSpadden Gardener, 2006; Choudhary et al., 2007). This has also been acknowledged by the Food and Agricultural organization of the United Nations (FAO) stating; "Governments, […] should encourage and promote research on, and the development of, alternatives posing fewer risks […]"(FAO, 2014). Biological control agents are mentioned as one of these alternatives. Since the 1st of January 2014 Integrated Pest Management (IPM) should be applied in agriculture in Europe (European Commission, 2014). IPM is a working approach in agriculture aiming towards reducing the use of pesticides as much as possible.

Already in 1995 Cook et al. stressed that microbe-induced plant defense should be given much more scientific attention, as this natural method has such great potential to improve health and production of agricultural plants. The authors also stressed the vast, almost unlimited, genetic biodiversity among microorganisms that could serve as biocontrol agents (Cook et al., 1995). In the latest years scientific attention has been given to the effects of microbes as beneficial to plants in many ways: as growth promoting, in enhancing tolerance against drought stress, and as disease suppressing (Leeman et al., 1996; Pieterse et al., 1996; Pieterse et al., 1998; Pieterse et al., 2002; Bakker et al., 2003; Danielsson et al., 2006; Bejai et al., 2009; Kasim et al., 2013). However, the impacts of bacteria on other components of the ecosystem have only been acknowledged to a little extent (Meijer, personal communication) and before bacteria can be available as a bio-pesticide on the market their impact on other soil organisms has to be evaluated.

The aim of this thesis was to investigate the role of biocontrol bacteria in soil, with focus on the *Bacillus amyloliquefaciens* strain UCMB-5113, and what effect they might have on the earthworm species *Aporrectodea longa*. The thesis is divided in to two parts. Firstly a literature review was performed to investigate the general role of biocontrol bacteria. The review also looks into the impacts of earthworms on ecosystems. In a second approach the possible effects of biocontrol bacteria on *Aporrectodea longa* were investigated in a laboratory experiment. The experiment is part of a larger study where the potential negative effects of *B. amyloliquefaciens* on earthworms are evaluated.

Aporrectodea longa was chosen as a study organism because it is one of the most common earthworm species in the soils of Swedish agricultural landscapes (Lagerlöf and Lenoir, 2009). *A. longa* is one of the biggest earthworm species in Sweden (Lagerlöf and Lenoir, 2009), 15-20 cm in length (Andersen, 1997). They are so called 'anecic' earthworms (Brown et al., 2000) which mean that they feed from plant residues on the ground soil surface which they carry down into the soil. Anecic earthworms are important for the mixing of mineral soil and organic matter in the soil profile. Due to the important role of *A. longa* for this mixing it is an ideal bioindicator for testing potential effects of biocontrol bacteria.

In the experiment, individuals of *A. longa* were exposed to the bacteria *B. amyloliquefaciens*. Different exposure treatments were tested: Earthworms were either directly dipped in the bacterial solution or the solution was added to the natural soil where the earthworms were kept. Earthworm growth, mortality and reproduction were measured to evaluate bacterial impact. The null hypotheses assumed that the earthworms would not be affected by the bacteria, which would corroborate the results of an earlier, similar experiment by Lagerlöf (unpublished).

Literature review

Traditional farming techniques to suppress diseases

Crop rotation

Crop rotation is an old, well studied technique. Today it is still an important way to handle pests and diseases (see Peters et al., 2003; Cunfer et al., 2006; Bennett et al., 2012). Peters et al. (2003) argue that if the rotation system is designed for the specific cropping conditions in field, pathogen attack on plants and the survival of saprophytic fungi will decrease. The success of crop rotation is also determined by which pathogen should be suppressed. The pathogen's ability to survive and to spread in the field is species dependent; therefor the rotation system has to be modeled depending on the pathogens traits (Peters et al., 2003).

Tillage

Reduced or non-tillage management can enhance the disease suppressing ability of the soil. This management increases C and N content in the soil, which leads to higher microbial biomass of bacteria and fungi and higher respiration and thereby facilitates antibiosis (Pankhurst et al., 2002; Peters et al., 2003). Similar positive effects on disease resistance have been found in soils with a high content of organic matter as compared to mineral soils, and the associated higher biodiversity in the more organic soils is considered crucial (Ekelund, 1999; Chen et al., 2001; Peters et al., 2003; Bending et al., 2007). However, leaving crop residues on the field is not risk-free as pathogens can be favored by the old crop residues (Pankhurst et al., 2002; Peters et al., 2003). In a review by Bailey and Lazarovits (2003) it was argued that the same pathogen can either be promoted or suppressed by tillage, and that the response of the pathogen to tillage depends on the composition of the microbial community. The quality of the residues also has to be taken into account since residues can e.g. be toxic to the pathogen or serve as a food substrate (Hoitink and Boehm, 1999; Bailey and Lazarovits, 2003).

Resistant breeding

Genes facilitating resistance in crops against pathogens have been known for long (Rudd et al., 2001) and intentional breeding with cultivars showing resistant traits has been going on for many decades (Bai and Shaner, 2004). Resistant traits can be physiological; such as resistance towards infection or spreading of the pathogen within the plant, or morphological; such as a higher and less dense crops which disfavors the pathogens (Masterházy, 1995; Bai and Shaner, 2004). It has been shown that pathogen attack may activate the expression of defense-related genes in the plant, and in more tolerant cultivars this activation is faster than in the less resistant (Bai and Shaner, 2004). Resistant breeding can be a form of conventional plant breeding, where cultivars with good resistance or healthy morphology are mixed with other less resistant cultivars to enhance the resistance in the latter (Masterházy, 1995). Resistance can also be accomplished by genetic techniques where chromosomes are induced or specific gene sequences are transformed into a plant (see Bai and Shaner, 2004, and their references). Breeding for resistance is a complicated task; firstly, resistance is usually due to several traits so focusing on just one will be less successful (Bai and Shaner, 2004). Secondly, the traits for resistance often do not go hand in hand with other favorable agronomic traits such as short straws and early maturity (Masterházy, 1995; Bai and Shaner, 2004).

Pesticides

In the late 19th century farmers begun to use inorganic chemical substances for pest control which led to a strong increase in the production of chemical substances for crop protection in the mid-twenties (Russell, 2005). Today in conventional agriculture, pesticides are commonly used to eliminate weeds, fungi and insects. The use of pesticides has helped many farmers in their struggle against pests, but concerns about the associated risks for ecosystems and humans, such as the toxicity itself and the possible build-up effect of toxic compounds in the soil, have been rising since the 1950’s (Hart and Brookes, 1997; Russell, 2005).

Pesticide effects on soil microorganisms

The effects of pesticides on microorganisms have been studied extensively, both in controlled laboratory experiments and in field experiments. Nevertheless, Gupta et al. (2013) argue that knowledge on the response of soil microorganisms to pesticides at low-level exposure, which reflects the situation in agricultural fields, is still poor, at least in regard to the bacterial community. In addition, results from performed studies are ambiguous. While some experiments have shown that the pesticides can be very toxic to a wide range of soil microorganisms (Ekelund, 1999; Ekelund et al., 2000; Ampofo et al., 2009), other studies have observed no or varying effects, both in field and in laboratory experiments (Hart and Brookes, 1997; Smith et al., 2000; Johnsen et al., 2001; Pal et al., 2005). Johnsen et al. (2001) stress that different soil organisms are affected differently by the application of pesticides. Pesticides have different modes of action in terms of what part of an organism they affect and also how specific they are in which organisms they will suppress. Depending on their modes of action they will be harmful to more or less specific soil organisms (Johnsen et al., 2001; Bending et al., 2007). Pesticides can be directly toxic for non-target microorganisms (Ekelund et al., 2000), or indirectly by killing or reducing the amount of organisms that are food for others (Smith et al., 2000). In some cases both direct and indirect effects are observed (Ekelund, 1999). The more tolerant organisms might use the pesticide or, more probable, the residues from other organisms that have died from the pesticide as food (Ekelund, 1999; Ekelund et al., 2000; Chen et al., 2001; Johnsen et al., 2001; Bending et al., 2007). The sensitivity of microorganisms to a pesticide depends on the species and the pesticide (Ekelund, 1999; Chen et al., 2001; Bending et al., 2007; Ampofo et al., 2009). In addition, the microbial community structure is also considered a major factor when it comes to how severe the effects of the pesticide will be, as microorganisms also contribute to the breakdown of pesticides (Bending et al., 2007).

Pal et al. (2005) argue that microorganisms may have a crucial role in pesticide degradation and also point at the positive effects of soil organic matter on microbial activity and degradation of pesticides. The concentration of a pesticide determines the consequences for the microorganisms. Low concentrations can have an inhibiting impact on microbial processes without killing the organism or the population (Ekelund, 1999; Chen et al., 2001). In a calculation by Ekelund et al. (2000) on the effect of a fungicide on a community of soil flagellates in the field, the fungicide seemed to have quite small effect as it is spread over a large area of soil, and thereby the concentration of the toxic compound is lowered. However, the same authors argue that the possible existence of very sensitive species of microorganisms in natural soils should not be overseen, and the existence of such sensitive species has to be evaluated (Ekelund et al., 2000).

The necessity of evaluating the composition of the microbial community, and not just studying microbial activity, has also been stressed by some authors. This is important because microbial activity and biomass can remain constant under pesticide use while there is a change in the specific microbe species in the community. More tolerant organisms may survive the pesticide, and then feed on the dead ones, which could result in total microbial activity remaining at the same level or even increasing (Smith et al., 2000; Girvan et al., 2005). Changes in microbial community composition could have effects on soil function (Jacobsen and Hjelmsø, 2014) as many microbe species have important roles in soil, such as supporting plant growth (see coming sections) or being consumers or predators on other organisms.

Pesticide effects on earthworms

Earthworms are soil organisms that are very exposed to pesticides. They get in contact with these substances via both their skin and gut (Luo et al., 1999; Jager et al., 2003; Gambi et al., 2007; Chakra et al., 2008). Many experiments have been performed to evaluate the toxicity of pesticides (Luo et al., 1999; Zang et al., 2000; Gevao et al., 2001; Jager et al., 2003; Gambi et al., 2007; Chakra et al., 2008) and other soil pollutants (Venkateswara Rao et al., 2003; Lukkari et al., 2004) on earthworms (*Aporrectodea tuberculata, Eisenia andrei* and *E. fetida*). Researchers agree that the physical and chemical characteristics of a pesticide determine how earthworms will be affected. If the pesticide dissolves well in the soil solution it could be absorbed through the skin of the earthworm (Luo et al., 1999; Zang et al., 2000). By feeding of the soil or burrowing in it, earthworms will also be exposed to soil-bound pesticides or pollutants which are otherwise not accessible for soil organisms (Gevao et al., 2001). Hydrophobic chemicals will also more likely be ingested as they do not dissolve in the soil solution (Jager et al., 2003). How an earthworm gets in contact with a toxic compound may affect the speed of intoxication, the responses in the earthworm’s body as well as it can affect the mechanism of action of the compound (Gambi et al., 2007).

Studies of pesticide effects on earthworms have shown different results. In an experiment by Gevao et al. (2001) there was no observation of obvious toxicity when the herbicides Atrazine, Dicamba and Isoproturon were added to the soil;

only a lower weight gain compared to the control was observed, indicating that living conditions for the earthworms were not optimal. In contrast, in a study with two insecticides (Imidacloprid and RH-5849, Luo et al., 1999; Zang et al., 2000) toxic effects on earthworms were observes, in particular sperm deformation; this feature therefore was pointed out as a useful eco-toxicological parameter (Zang et al., 2000). Gambi et al. (2007) studied how the insecticide Zoril 5 affected earthworms both when applied as the pure compound, cabaryl, and as the commercial substance (containing 5% of the pure compound). The exposure to Zoril 5 did not show the same negative effects as the pure compound. Thus, at higher amounts than normally applied in agricultural fields, toxicity was observed but seemed to be dependent on concentration, exposure time or both. Therefore the authors argued that Zoril 5 might cause toxic effects in a longer period of time.

The use of pesticides does not only affect agricultural ecosystems. Chemicals follow rainwater as it runs off, or are transported down to the ground water and finally end up in surrounding lakes, rivers or in the sea. It is outside the frame of this thesis to discuss effects of pesticides in aquatic environments, further useful information regarding this aspect may be found in Keruger (1998), DeLorenzo et al. (2001), Liess and von Der Ohe (2005) and Schäfer et al. (2007).

Soil organisms and disease suppression

Relationships between diversity and functions in ecosystems are complex. Soil biota and productivity impact each other in a feedback cycle; soil biota affects productivity while the cropping system influences the soil biota (Barrios, 2007). Such interactions could be soil organisms predating on each other, plants being toxic to different organisms or soil organisms causing diseases in plants. But interactions with soil microorganisms can also be beneficial for other soil organisms, promote plant growth, suppress diseases and enhance plant stress tolerance (Leeman et al., 1996; Pieterse et al., 1996; Pieterse et al., 1998; Pieterse et al., 2002; Bakker et al., 2003; Danielsson et al., 2006; Koorneef and Pieterse 2008; Bejai et al., 2009; Kasim et al., 2013). The amount of microbial biomass in the soil seems to be a good indicator of the soils' ability to suppress diseases and the type of organic matter determines the composition of the microbial community (Hoitink and Boehm, 1999).

Disease suppression can be facilitated by high levels of organic matter in the soil, as high levels of organic matter increases soil microbial biomass which constitutes a strong competitor towards the pathogens (Hoitink and Boehm, 1999; Bailey and Lazarovits, 2003). Bailey and Lazarovits (2003) argue that application of organic matter has an incremental effect on disease suppression, which, according to Hoitink and Boehm (1999) is at its highest in the middle phase of organic matter degradation. Conditions in fresh or highly humified organic matter seem to be better for the pathogen; therefore the organic amendments need to be managed with knowledge and care (Hoitink and Boehm, 1999).

Kumar et al. (2011) stated high biodiversity in bacterial species as a great advantage for the use of bacteria as biocontrol agents. The possibility to mix different bacterial species and strains and thereby get enhanced growth promotion and suppression against diseases is a promising way to work with these bacteria in the future (Johansson, 2013). *B. subtilis* GB03 and *B. subtilis* MBI 600 are two species available on the market in some countries (Kumar et al., 2011). In Sweden strains of *Pseudomonas* are at the moment the only bacteria available for practical use as biocontrol agents (Meijer, personal communication). However, bacterial biocontrol agents do not show as effective results as chemical pesticides (Kumar et al., 2011), but concerns about human and environmental health, the possible resistance of pathogens towards chemical pesticides and the establishment of an Integrated Pest Management favor alternative ways of disease control more and more.

Bacteria as biological control agents

The term 'biological control' is used in different fields of biology. In plant pathology it is regarded as the ability for disease suppression and weed control by antagonistic microorganisms and pathogens (Pal and McSpadden Gardener, 2006). During the last decade, the strong role of microorganisms in disease suppression has been acknowledged and their ability to suppress diseases has gained more and more interest (Hotink and Boehm, 1999; Peters et al., 2003).

Suppression of diseases by plant-growth promoting rhizobacteria (PGPR) can work via several mechanisms; competition for a specific niche or substrate, release of allelochemicals or antibiotic substances or via induced systemic resistance (ISR, Haas et al., 2002; Bakker et al., 2003; Kumar et al., 2011; Johansson, 2013).

Plant-growth promoting rhizobacteria colonize plant roots and their nearest surroundings (Choudhary et al, 2007). The ability of PGPR to survive on the roots and colonize the plant is important traits for their use as biocontrol agents (Danielsson et al., 2006). Colonization often takes place on young roots (Johansson, 2013). The ability to colonize roots is strain specific (Kumar et al., 2011; Johansson 2013) and has to do with plant- bacteria interactions as the PGPR have to suppress the signaling systems that will defend the plant from unwanted bacteria (Sarosh et al., 2009).

Plant diseases can be suppressed through induced resistance, which in turn can be induced either by a pathogen or by PGPR. When resistance is induced by a pathogen it is called systemic acquired resistance (SAR) and when it is induced by PGPR it is called induced systemic resistance (ISR, Bakker et al., 2003). When the pathway of induced systemic resistance was first observed, ISR and SAR were distinguished on the basis of the phytohormones involved in the signaling chain; salicylic acid (SA) for SAR and jasmonate (JA) and ethylene (ET) for ISR (Leeman et al., 1996; Pieterse et al., 1996; Pieterse et al., 1998). Later on, these chains have been acknowledged to be more complex as they both depend on SA but in different concentrations (Koornneef and Pieterse, 2008; Pieterse et al., 2002; Johansson 2013). Nevertheless, the definitions remain as mentioned above as they, the definitions, are connected to the organism inducing the resistance.

Plants respond to attacks from insects and pathogens by releasing a mixture of the alarm signals SA, JA and ET. The quantity of each, their composition and timing of release varies according to the trigger (Koornneef and Pieterse, 2008; Pieterse et al., 2002). The role of PGPR is to prepare the plant so that the signaling pathway and the subsequent transcription of disease-suppressing genes will go much faster. This disease suppression occurs without any interference between the bacteria and the pathogen. This has been observed by simultaneously inoculating both pathogen and bacteria but in different sites of the plant so that direct contact between the two was impossible (Leeman et al., 196; Pieterse et al., 2002; McSpadden Gardener, 2004; Johansson, 2013). If ISR develops also depends on the combination of host plant and bacteria (Leeman et al., 1996; Pieterse et al., 1996; Pieterse et al., 2002; Danielsson et al., 2006).

Antibiotic production is an important defense mechanism for bacteria, but plays also a significant role for plant protection. In natural ecosystems, defense mechanisms in bacteria are a result of selection pressure, and plants have evolved the ability to interact with bacteria living in the rhizosphere (Cook et al., 1995).

It is difficult to determine the causes of disease suppression because it is the result of several mechanisms, which on the other hand also is a feature that makes the use of PGPR so promising. By combining both ISR and antibiotic substances from PGPR disease suppression can be facilitated; first the pathogen population is weakened by the antibiotics which will make it easier for the plant to cope with the pathogen as the defense capacity is enhanced due to ISR (Bakker et al., 2003).

Research on molecular and physiological mechanisms in regard to IRS has mostly been done on the bacteria *Pseudomonas* (Sarosh et al., 2009). In the recent years, investigations on different *Bacillus* strains have increased (e.g. Wulff et al., 2002; McSpadden Gardener, 2004; Danielsson et al., 2006; Sarosh et al., 2009; Kumar et al., 2011; Johansson, 2013). In the experiment performed for this thesis *B. amyloliquefaciens* strain UCMB-5113 was used and will therefore be focused on in the rest of the review.

Bacillus amyloliquefaciens as biocontrol agent

Bacillus amyloliquefaciens is a rod shaped, Gram-positive, aerobic endospore-forming bacteria that commonly occurs in the soil of agricultural ecosystems where it contributes to crop productivity either directly or indirectly (McSpadden Gardener, 2004; Kumar et al., 2011). For their survival, these bacteria produce a multilayered cell wall structure and endospores that are stress-resistant. They also produce peptide antibiotics, signal molecules and extra cellular enzymes to improve their survival and competitive abilities in the soil (McSpadden Gardener, 2004).

Bacillus amyloliquefaciens has been shown to have good properties as a disease suppressing PGPR. On seeds of oilseed rape, significant protection against four different fungal pathogens has been observed (Danielsson et al., 2006). The authors showed that seeds treated with UCMB-5113 strain had a higher survival rate than control seeds when exposed to fungi in the soil. The interactions between *B. amyloliquefaciens* strain UCMB-5113 and the pathogen *Botrytis cinerea* on oilseed rape have also been investigated by Bejai et al. (2009). The study of gene activity showed presence of JA and ET in plants treated with UCMB-5113, which made the authors suggest that the bacterial treatment of seeds resulted in ISR to *Botrytis*. Increased root biomass was also observed as well as a decrease of disease symptoms of approximately 40% for the plants where *Bacillus* had been applied compared to the control.

The bacteria also enhance plant tolerance towards other forms of stress. Kasim et al. (2013) tested if priming with PGPR would improve wheat growth under conditions of drought stress. The seeds treated with bacteria showed better tolerance to drought stress than non-primed plants, with *B. amyloliquefaciens* strain UCMB-5113 showing the best result. Plant tolerance was seen both as higher fresh weight, dry weight and water content of the plants as well as reduced transcript levels of the genes related to drought stress (Kasim et al., 2013).

Bacillus amyloliquefaciens has been shown to produce several different antibiotics (Wulff et al., 2002; Ongena and Jacques, 2007). In general, bacteria of the genus *Bacillus* produce antibiotics of the group lipopeptides, divided into three families: fengycins, iturins and surfactins. Lipopeptides have the ability to attach to cell membranes, both on plants and soil organisms, and break them down (Ongena and Jacques, 2007). This might be affecting earthworms, and is a mode of action that should be evaluated before using the bacteria commercially (Meijer, personal communication).

The production of antibiotic substances is strain specific (Wulff et al., 2002). However, many bacteria of different species produce the same antibiotic substances (Cook et al., 1995) and the mode of action of the antibiotics studied in more commonly cultivated species can thus be assumed relevant also for other bacteria showing production of the same antibiotics. *B. subtilis* is a well-studied *Bacillus* species and some of its produced antibiotics have also been found for *B. amyloliquefaciens*. Romero et al. (2007) observed high disease suppression by iturin and fengycin lipopeptides in *B. subtilis*. Other studies have shown that surfactin is very important for the ability of *B. subtilis* to colonize plant roots (Bais et al., 2004). The mode of action of antibiotics produced by bacteria is not yet that well-studied. Zhang et al. (2013) evaluated the mode of action of two iturins; bacillomycin L and amphotericin B, produced by *B. amyloliquefaciens* on *Rhizoctonia solani*. They observed that these two iturins had different modes of action on fungal hyphae. Amphotericin B causes membrane permeabilization on fungal hyphae cells while membrane disruption was observed as a crucial role of bacillomycin L in suppression of *R. solani* (Zhang et al., 2013). Despite direct effects of antibiotics on pathogens in the soil, antibiotics have been shown to contribute to the ISR mechanism (Ongena et al., 2007). However, the field of antibiotic mechanisms is not well studied and will not be further explored here.

Chitinase is a chitin degrading enzyme and has also showed antiviral activity (Cheung et al., 2014). Chitinase has been observed as up-regulated in plants when *B. amyloliquefaciens* is present (Sarosh et al., 2009). This enzyme might have negative impact on earthworms as chitin is an important compound of their epidermis (Silverin and Silverin, 2002).

Earthworms' impacts on ecosystems

Ecosystem engineers

Earthworms are one of the most important ecosystem engineers in soil. 'Ecosystem engineers' are defined as organisms that create, maintain or modify the structure and function of the ecosystems they live in (Jones et al., 1997; Jouquet et al., 2006). In excess of providing habitats to other organisms in the ecosystem,

ecosystem engineers control the flows of energy, materials and food between different trophic levels. An ecosystem engineer does not necessarily benefit itself from the engineering activity; those engineers are called “accidental” ecosystem engineers (Jones et al., 1997). Via their feeding activities earthworms, that in many ecosystems are accidental ecosystem engineers, change the physical state of the materials they eat. Other organisms can then feed on earthworm feces and the excreted mucus. The movement of earthworms in the soil creates both burrows - where other microorganisms and roots can interact- and macro aggregates and thereby strongly influences soil structure (Jones et al., 1997; Giller et al., 1997; Jouquet et al., 2006).

Different earthworm species produce different sizes of aggregates and thereby have different impact on the compactness of the soil (Derouard et al., 1996). Derouard et al. (1996) distinguished between two major functional earthworm groups in regard to soil aggregation; species producing small aggregates and those producing bigger aggregates. The large aggregates are >5 mm in size, compact and round and while tending to increase soil compaction they also seem to benefit plant growth. These aggregates are made by large earthworm species. These large aggregates are broken down by other species to smaller aggregates; 0.25-2 mm, which decrease the compactness of the soil. These aggregates have very low effect on plant growth. The best soil structure is created when earthworms from both functional groups are present (Jouquet et al., 2006) and together they will probably create the most stable environment for both soil organisms and plants. This point to the importance to look on how different earthworms species are affected by different farming techniques and toxic compounds used in agriculture. Where an earthworm species live and how it moves in the soil determine how exposed it will be to tillage or pesticide use. Negative effects even on only one earthworm species can result in a more unfavorable environment for plants and other organisms.

Earthworms can also affect plant production in many other positive ways. Together with other soil organisms they support plants with nutrients via digestion of organic material; an important step in mineralization (Blouin et al., 2005). Earthworms also have a hormone-like effect on plants, which probably is due to metabolites derived from the microorganism in the soil taken up by the earthworm (Blouin et al., 2005; Tomati et al., 1988). Their feeding patterns contribute to dispersal of microorganisms (Blouin et al., 2005), both growth stimulating and antagonistic microorganisms affecting root pathogens. Microbial activity is stimulated by earthworms, as well as the metabolism and population dynamics of microorganisms (Blouin et al., 2005). This leads to more nutrients and microbial metabolites available in the soil. Earthworms also affect plant photosynthesis positively, e.g. some of the nutrients that are necessary to produce chlorophyll in plants can be derived from ammonium-rich earthworm casts (Blouin et al., 2005).

Earthworms as biocontrol agents

Earthworms can function as biocontrol agents. Blouin et al., (2005) showed that an inhibitory effect by earthworms on plant parasitic nematodes. In their study the average number of nematodes was significantly lower in the presence of earthworms compared to the treatment with only nematodes. This was the first experiment showing that earthworms suppress nematodes and that negative impact of plant pathogenic nematodes on plants is reduced. Earthworms also produces enzymes, such as chitinase, and antifungal and -microbial compounds and thereby suppress pathogens in soil (Meghvansi et al., 2011).

Earthworms as bioindicators

Bioindicators are an important tool when evaluating soil health. Interactions between different species within an ecosystem are controlled by so-called keystone species or higher taxonomic species; these therefore serve as good bioindicators of soil health. In agro-ecosystems earthworms are important keystone species (van Bruggen and Semenov, 2000), and because they are extremely exposed to chemicals and pollutions in soil (Luo et al., 1999; Jager et al., 2003; Gambi et al., 2007; Chakra et al., 2008) they are good indicator organisms for toxins in the environment. This is commonly tested using standard toxixity and reproduction tests following the guidelined of the Organisation for Economic Co-operation and Development (OECD). Acute toxicity tests can be performed by a paper contact toxicity test where chemical solutions of different concentrations are added to filter paper in a glass bottle. The worms are kept in the bottle for 48 to 72 hours. After this period, mortality, body injury or morphologic abnormalities are studied. Acute toxicity tests can also be performed as an artificial soil test where earthworms are kept in artificial soil to which the tested chemical is added with different concentrations. The worms are assumed to die in 7 to 14 days after application. In the controls, mortality should not be higher than 10 per cent when the test is finished (OECD, 1984).

In the reproduction tests the numbers of offspring (cocoons or juveniles) are counted. This test is performed in test containers filled with soil. For a valid test each replicate, containing 10 adults of the species *Eisenia foetida* should have produced ≥ 30 juveniles by the end of the test (OECD, 2004).

OECD recommends using the species *E. foetida*, a compost earthworm, for toxicological experiments as it has a short life cycle; the cocoons hatches after 3- 4 weeks and the earthworms are mature after seven to eight weeks when they are kept in 20°C. They respond to chemicals in the same way as earthworms that are more common in soils (OECD, 1984).

Management practices and earthworms

Management practices influence the presence of earthworms in agricultural landscapes. In an experiment by Suthar (2009) the presence of earthworms in integrated, organic and conventional managed fields were observed. The highest species richness was observed in the organically managed fields (six species found, compared to three in the others), while the species index was highest were integrated management was used. In another experiment Pelosi et al. (2009) saw no significant difference in total density of earthworms between the different systems; conventional, organic and direct seeding with living mulch. However, organic matter is mentioned by both Pelosi et al. and Suthar as strongly determine earthworm abundance in agro-ecosystems (Pelosi et al., 2009; Suthar, 2009).

Suthar (2009) argues that earthworm species that have adapted to disturbance, low content of organic matter and little surface litter are the ones generally dominating agricultural soils. This will reduce the number of some earthworm species and increase others, leading to reduced biodiversity when compared to less disturbed sites. This reduction in biodiversity might have negative impact on soil structure by compacting or de-compacting the soil as argued above in terms of aggregates.

Picture 1. *Aporrectodea longa*. Photo: Sara Söderlund

Laboratory experiment

The experiment aimed to investigate if the earthworm *Aporrectodea longa* is affected by biocontrol bacteria *Bacillus amyloliquefaciens,* and was performed as a microcosm experiment, constructed of 30 cm cylinders referred to as experimental vessels (Picture 2). The vessels contained a mix of soil and manure and the earthworms were exposed to the bacteria in three exposure levels; via skin, gut or both. This experiment is part of a larger study where potential negative effects of *B. amyloliquefaciens* on earthworms are evaluated.

The null hypothesis tested was that the earthworms would not be affected by the bacteria.

Picture 2. Experimental vessels in climate room during the experiment. Photo: Elsa Lagerqvist

Material and method

Experimental design

This experiment is a repetition of an earlier experiment performed by Lagerlöf during the winter 2013-2014 (Lagerlöf, unpublished). Four treatments were tested in order to evaluate the impacts of *Bacillus amyloliquefaciens* strain UCMB-5113 on the earthworm *Aporrectodea longa* (Table 1). A bacterial solution of 10^7 bacteria per ml of water was used. In two of the treatments (Table 1) earthworms were dipped in the bacterial solution (referred to as Dip) for 10 seconds. This was the strongest exposure as the earthworms got in close contact with the bacteria and was aimed to evaluate if there were any negative interactions between the bacteria antibiotics and the skin or cuticula of the earthworms. In two treatments bacterial solution (B.s) were added to the soil. This was aimed to investigate how the earthworms reacted to the bacteria when it is in their close environment where the worms are burrowing and feeding. When solution was added it was carefully poured all over the soil after the worms had crawled down into it. Treatments with no dipping are referred to as N.d. and treatments where water was added instead of bacterial solution are referred to as W. All the treatments were replicated six times.

Treatment “Dip. B.s.” was assumed to be the strongest of the treatments as the worms were exposed to the bacteria both through skin and gut.

Before the earthworms were put into the soil they were washed, weighted and treated with the bacterial solution (see Table 1). Two adult or sub adult individuals were added to each vessel. A developed clitellum was used as an indicator of adulthood. The cylinders were put into a dark climate room (17°C) where they stood for two months. During that time 50 ml water was added every fifth day to prevent the soil from drying out. More manure (0.1 l) was added after one month when the worms were weighted again. Every second week the vessels changed place. This was done in order to not let the result depend on unfavorable conditions in one part of the room. The worms were weighted three times; at the beginning of the experiment and again after one and two months, referred to as ‘weighing occasions’. After the second month the experiment was over and the produced cocoons were counted as well.

Table 1. The four different treatments, each treatment had six replications (6 vessles). Concentration of bacterial solution (10^7 b/ml).

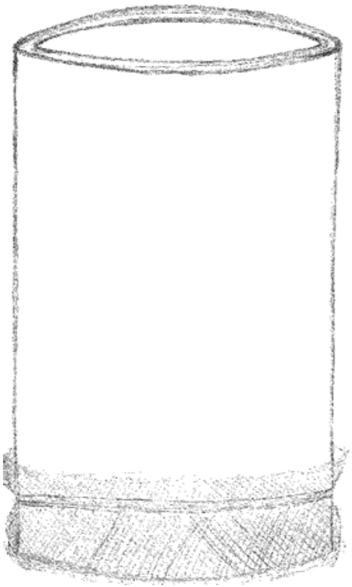
	Treatment	Abbreviations
1	Dipping earthworms in bacterial solution + adding 150 ml bacterial solution to soil	Dip. + B.s.
2	Dipping earthworms in bacterial solution + adding 150 ml water to soil	Dip. + W.
3	No dipping + adding 150 ml bacterial solution to soil	N.d. + B.s.
4	No dipping + adding 150 ml water to soil (control)	N.d. + W.

Preparation of the experiment

The experimental vessels were cylindrical sewage pipes (14.5 cm diameter and 30 cm in height, Picture 3). In the bottom, mosquito net was set with rubber band. To reduce water loss through evaporation plastic bags were put over each vessel. 24 experiment vessels were prepared. 1.5 kg soil and 0.2 l cow manure (*Weibulls; concentrated, dried, organic cow manure*) were carefully mixed together and put into the experiment vessels, trying not to break the clay aggregates of the soil. The water content of the soil was calculated as 12-22 % by weight (proximally 17 %) and for the manure as 52 %. Two days after the vessels were filled with soil, the earthworms were added.

Earthworms and soil sampling

In the present experiment, earthworms of the species *Aporrectodea longa* were used. The earthworms were collected in autumn 2013 and had been stored in a cool room. All earthworms were collected in the region of Uppsala, Sweden. The soil used to fill the experimental vessels was a clay loam with 36.5 % clay, and had been classified as Eutric cambisol. Total carbon content is ca 1.5 % and pH is 6.6



Picture 3. Experimental vessel. Elsa Lagerqvist.

(Kirchmann et al., 1994). The soil was collected at Ultuna in Uppsala, Sweden, and cleared from roots, big plant residues, earthworms, beetles and other insects and then put into a freeze room at -20°C to reduce the amount of soil living organisms.

Bacteria

The bacteria investigated in the experiment were *Bacillus amyloliquefaciens* strain UCMB- 5113. The bacteria originated from a culture managed by Johan Meijer (SLU, Dep. of Plant Biology) and the solution that was used in this experiment contained 10^7 bacteria per ml of water.

Statistical analysis and presentation of data

After the experiment had been performed, ANOVA GLM and Tukey's test, in "Minitab 16 Statistical Software", was used for the statistic evaluation of the results and comparison of individual and between treatments. Differences were considered significant when $p<0.05$. Graphs were done in Excel.

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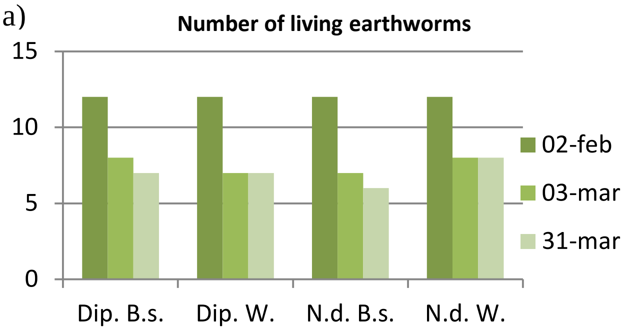
Results

Though data shows that earthworms have died (Fig 1 a)), there was no difference in mortality between the treatments (p= 0,660 for 3/3 and p=0,903 for 31/3). Only earthworms that were still alive when weight was controlled were used for the calculations presented in Figure 2 and 3.

a)



b

Number of living earthworms:
Treat. 1 (Dip. B.s.) n=8 & 7
Treat. 2 (Dip. W.) n=7
Treat. 3 (N.d. B.s.) n= 7 & 6
Treat. 4 (N.d. W.) n =8

Fig. 1 a) and b). 1 a) Number of living earthworms within the different treatment during the experiment. 1 b) n= number of living earthworms within each treatment during the experiment for calculation of mean weight (Fig. 2 a)). Abbr.: Dip. = dipping, of earthworms in bacterial solution N.d. = no dipping, B.s. = bacterial solution to the soil, W. = water to the soil. P-vaules were 0,660 for 3/3 and 0,903 for 31/3.

In all treatments most earthworms gained weight during the experiment (Fig 2 a). There were no significant difference between the treatments (p=0,888 for 2/2, p=0,252 for 3/3 and p=0,687 for 31/3). There was no significant difference (p=0,974) in percent weight change between the treatments, from the start of the experiment to its end (Fig 2 b). This indicates that the bacteria do not harm to the earthworms.

a)

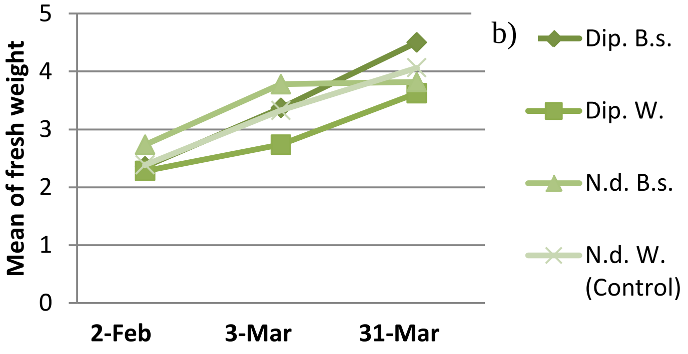
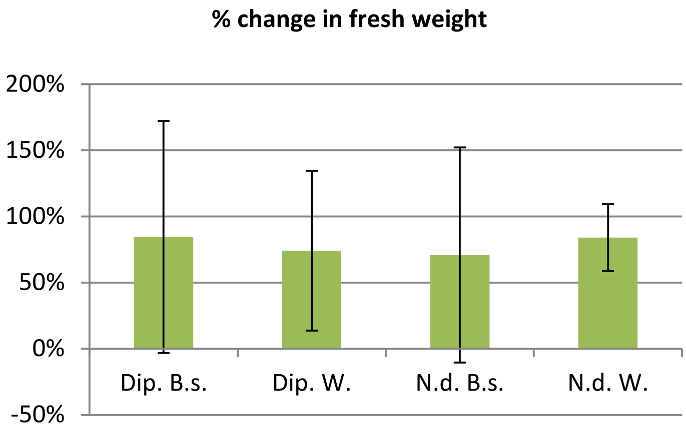


Fig. 2 a) Mean of fresh weight of alive *Aporrectodea longa* for the different treatments during the experiment. P-values were; 0,888 for 2 Feb, 0,252 for 3/3 and 0,687 for 31/3. Abbr.: See fig 2b.



2 b) Percental change in fresh weight of alive *A. longa* from the start to the end of the experiment. P= 0,974. Abbr.: Dip. = dipping, of earthworms in bacterial solution N.d. = no dipping, B.s. = bacterial solution to the soil, W. = water to the soil.

There were no obvious trend among any of the treatments of either being negatively or positively affected by the treatments, and the mean weight of earthworms exposed to the bacteria does not differ significant from the control (p=0,888 for 2 Feb, 0,252 for 3/3 and 0,687 for 31/3, Fig 3).

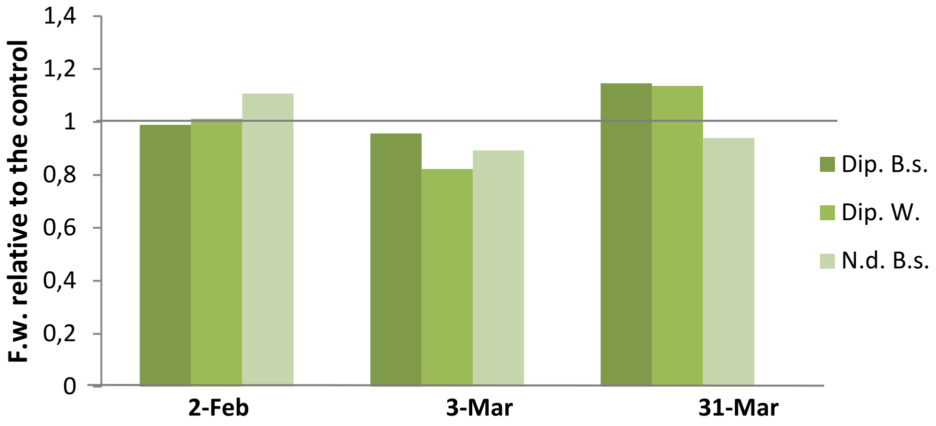


Fig. 3. Mean fresh weights of *Aporrectodea longa* with different treatments during the experiment relative to the weight of *A. longa* in the control (=treatment N.d. W.), visualized as the line. Abbr.: Dip. = dipping of earthworms into bacterial solution, N.d. = no dipping, B.s. = bacterial solution to soil, W. = water to soil. P-values were; 0,888 for 2 Feb, 0,252 for 3/3 and 0,687 for 31/3.

The cocoon production for this experiment was low with 1-2 cocoons per treatment (six vessels. Table 2). That is a strong contrast to the result from the earlier experiment by Lagerlöf (unpublished) where cocoon production ranged from 16 to 78 cocoons per treatment even though the set up were the same.

Table 2. Total production of cocoons from the different treatments collected at the end of the experiment and cocoon production in an earlier experiment by Lagerlöf (unpublished). 1: Dip. B.s., 2: Dip. W., 3: N.d. B.s. 4: N.d. W. Abbr.: Dip. = dipping, N.d. = no dipping, B.s. = bacterial solution,

Treatment	Cocoons	Cocoons earlier experiment
1	1	77
2	2	72
3	2	16
4	2	78

Discussion

The results show no significant difference in weight change among *Aporrectodea longa* treated with *Bacillus amyloliquefaciens* compared to the control. Some earthworms died but there was no significant difference in lethality between the treatments. Amongst the earthworms that lived throughout the experiment almost all gained weight, regardless of treatment.

Causes of death

The majority of worms died during the first month of the experiment. This points to that their physical condition when entering the experiment was of big importance for their ability to survive. Before the experiment, the worms had been stored in a cool room for some months (1-4 months). Even though they were kept for a week in fresh soil and cow manure at room temperature to recover, before entering the experiment, some worms must have been too negatively affected by the longtime storage. Usually adult earthworms are able to handle cold periods by going into a resting-phase (Lagerlöf and Lenoir, 2009), but this seemed not to be the case for some of these worms. This assumption is drawn since there were no significant differences between the treatments.

Cocoon production

The amount of produced cocoons was low, when compared to the cocoons produced in the earlier experiment by Lagerlöf (Table 2). This could again be due to the bad physical conditions of the earthworms or an unfavorable environment before the experiment started, as a low number of cocoons were found in all cylinders irrespective of treatment.

Earthworms produce 10-90 cocoons a year (Lagerlöf and Lenoir, 2009) with the larger ones such as *A. longa*, at the lower end of that range (Paoletti, 1999). Some of the earthworms used in this experiment had already been used in the experiment performed a couple of month earlier. In that experiment a lot of cocoons were produced and the worms might not have been able to produce cocoons during this experiment period even if the living conditions were good. Another explanation to the low production of cocoons is the fact that when one earthworm in a cylinder dies the other one will not be able to reproduce. Earthworms are hermaphrodites but need a partner for reproduction (Lagerlöf and Lenoir, 2009). To avoid this problem three earthworms or more should be put into the cylinders, but then the cylinders needs to be bigger.

Furthermore, in temperate regions cocoon production is concentrated to spring, early summer and autumn (Edwards, 2004). The earthworms were collected in autumn 2013 and had been stored for different periods of time in the cool room before the present experiment was performed, therefore it might have been too early in the season for the earthworms to reproduce. Due to these reasons, cocoon production is probably a bad measure of the wellbeing of earthworms in this particular experiment. This should also be considered when planning experimental designs in the future. To relate to the biology and natural lifecycle of the organism used is of great importance and should be kept in mind. However, even if cocoon production might have been a bad measure in this particular experiment, cocoon production has been used before as a measure in toxicology tests. Žaltauskaité and Sodiené (2014) observed inhibited cocoon production when earthworms were exposed to Cd and Pb. However, if the inhibition were due to a direct toxic effect or indirect by late maturity was not clear, as the sexual maturity also was significantly affected by these metals.

Earthworm age

Some of the earthworms used could also have been so old that their ability to reproduce was lowered. As they were collected in the field their exact age was unknown. Large earthworm species as *A. longa* may live for up to three years and sometimes even longer (Lagerlöf and Lenoir, 2009), but it is not too unlikely that some of them can have been in that age by the time for the experiment. It would be beneficial to use cultured earthworms as the age of the earthworms then is known. Old age could be the cause of the low cocoon production.

The effect of toxins may be related to earthworm age. Žaltauskaité and Sodiené (2014) observed young earthworms (juveniles) to be more severely affected by toxins than adults. If juveniles are negatively affected by a toxin it will severely affect the whole earthworm community in a longer time-scale. To evaluate the effect of the bacteria on earthworms of different age is therefore another important area to study.

Experimental design

As mentioned earlier the death of earthworms was probably due to bad physics. In the present experiment the earthworms were acclimatized for one week. This was shown to not be enough for determining which worms that was healthy enough to live through the experiment. A longer acclimatization time would have reduced deaths during the experiment and thereby given more polite data. In an experiment performed by Velki et al. (2014) the earthworms were given 10 weeks to acclimatize in the cylinders before treatments were applied. If that method were used in this experiment the deaths of earthworms because of bad fitness or stress would have been reduced, or more precisely; bad physics would have been detected and those earthworms would not have been used in the experiment. In the experiment performed for this thesis four to six weeks seemed to be enough time for acclimatization.

In the experiment performed by Velki et al. (2014) the cylinders used were higher (100 cm) and wider (~30 cm in diameter) than in the present experiment (30 and 14.5 cm, respectively), which allows the worms to move more naturally. It has been shown that earthworms try to move from sites where toxic compounds are present. To perform avoidance tests have been argued as an important tool in determine the negative impacts of chemicals and metals on many soil organisms (Yeardley et al., 1996; Natal da Luz et al., 2004; Loureiro et al., 2005). Avoidance tests also show response to chemicals or metals at concentrations lower than those needed for evaluating LC50 (Yeardley et al., 1996). This could be of interest as the bacteria did not seem to cause any acute or sub-lethal toxicity to earthworms in this test. However, avoidance behavior by the earthworms might have been a response to antibiotic production if the worms were able to escape.

This experiment was not performed according to OECD’s guidelines neither concerning the method nor the earthworm species. OECD’s guidelines are restricted to the use of the species *Eisenia foetida*, stated to be a good representative for all soil living earthworm species. This statement should be questioned. How earthworms move and feed in soil vary between species which leads to that different species might be affected differently by toxins in soil. Furthermore, as both *B. amyloliquefaciens* and the earthworm *A. longa* are common in Swedish agricultural soil some coevolution has probably occurred which could lead to that they tolerate each other. If another earthworm were used the result might have been different. Therefore it could be of interest to look at different species as their way of living probably will determine how exposed they are to toxins, in particular if avoidance behavior is studied. Today studies of avoidance behavior do not have a guideline, but the ability to study different species should be considered if a guideline for avoidance behavior is established.

Bacteria activity and antibiotic production

As the bacteria did not harm the worms it raises the question; were they really prompted to do that in this experimental environment? In the solution the bacteria are in spores form and in that form they will not do any harm to the earthworms since they are not metabolically active. However, the manure that was added to the soil would have activated the bacteria (Meijer, personal communication). Furthermore, already under conditions of low stress *B. amyloliquefaciens* produces large amounts of antibiotics and thereby they probably would have produced antibiotics in this environment (Meijer, personal communication).

Further research

From this experiment I suggest that further studies should look into how earthworms are affected by eating plants that have been sprayed with the bacteria. This would show if interactions between *B. amyloliquefaciens* and other bacteria in the worm’s gut will have any negative consequences on the earthworm. Another mechanism to study is the occurrence of known produced antibiotic substances or other hormones and enzymes in the earthworms, and if these have any effect on the present bacteria. To look at the effect of the bacteria on earthworms of different age could also be of interest because the sensitivity of earthworms could differ with age, as argued by Žaltauskaité and Sodiené (2014). *A. longa* is an earthworm that moves much in soil; therefore it could also be of interest to look at avoidance behavior due to the bacteria.

Conclusions

On the basis of the present experiment, no harmful effects on earthworms of the species *Aporrectodea longa* were observed by the bacteria *Bacillus amyloliquefaciens* strain UCMB-5113. This confirms the results gained from the earlier experiment performed by Lagerlöf (unpublished data). These results are positive, as the bacteria shows promising traits as PGPR.

Further research thus needs to be done. This research should look more into the effects on earthworms eating crop residues from seeds or crops treated with the bacteria and thus the interactions of *B. amyloliquefaciens* and other bacteria in the gut of the earthworms. To investigate the earthworms´ uptake of different substances released by the bacteria could be another method to evaluate the impact of the bacteria. Effects on earthworms of different age and on their avoidance behavior due to the present of the bacteria are other areas of interest.

Interactions in nature can be tough, and biological treatments towards pests and diseases can be harmful to the ecosystem. To know the impacts on different organisms are of great interest when deciding what kind of pest treatment farmers should use in their fields.

Acknowledgement

I would like to thank my supervisor Jan Lagerlöf for his guiding through this work, Johan Meijer for clearing things out concerning the bacteria and Sara Söderlund for assistance with the experiment.

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