

Aus dem Institut für Natur- und Ressourcenschutz  
der Christian-Albrechts-Universität zu Kiel

# **Effects of macrocyclic lactone anthelmintics on seed germination and seedling establishment of grassland species**

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Lars Laber, M.Sc.

aus Groß-Umstadt

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Dekan: Prof. Dr. Georg Thaller

1. Berichterstatter: Prof. Dr. Tobias W. Donath
2. Berichterstatter: Prof. Dr. Tim Diekötter

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## Summary

Macrocyclic lactones (MLs) are used as anthelmintics to treat livestock and pets against infections by endo- and ectoparasites. After treatment, these substances are excreted into the environment via faeces, where they persist and harm a wide range of non-target organisms. So far, research has focussed on the effects of MLs on coprophagous (faeces-feeding) insects, such as dung beetles and dung flies. MLs cause paralysis and subsequently death of these insects when ingested. Even very low concentrations of MLs can have sublethal effects, which reduce fitness, including fecundity, and therefore decrease populations.

While the effects of MLs on insects are well known, effects on plants remain understudied. Plants can come into contact with MLs either when they take these substances up from the soil, in which faecal residues of recently treated livestock have been trampled into or when their seeds germinate in faeces of treated livestock. The spread of seeds via ingestion and later excretion by animals is called endozoochory. This study focussed on the effects of MLs commonly used on livestock, since seeds being excreted in faeces contaminated by MLs are especially at risk. These faeces may contain detectable amounts of MLs for weeks after treatment, depending on dosage and treatment method.

Previous studies have primarily focussed on short term effects of MLs on plant cell conglomerates in cultures, where they caused stress to plant cells and seedlings, which negatively affected growth of certain organs such as roots. A recent study has found effects of moxidectin, a commonly used anthelmintic, on seed germination and seedling emergence from livestock faeces. Moxidectin reduced these parameters, suggesting that MLs could be a factor for the decline of biodiversity of temperate grassland species in Europe. Therefore, this study was conducted to expand the knowledge on the effects of MLs on seeds, seedlings and growing plants. Three experiments focussing on different steps of the development of plants were conducted, focussing on the effects of several MLs on germination, seedling emergence from faeces of treated livestock and growth during repeated exposures to MLs.

The germination experiment included 20 different temperate grassland species and four different MLs (abamectin, doramectin, ivermectin and moxidectin) in three concentrations (0.1; 1; 10 mg l<sup>-1</sup>). The effects of these MLs on germination percentage, mean germination time and synchrony of germination were observed. This was the first large scale germination experiment, documenting the effects of several MLs on a wide variety of species.

For the seedling emergence experiment, seeds of four different temperate grassland species (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae) were ingested by sheep shortly after being treated with an oral formulation containing moxidectin as active ingredient. The feeding of seeds was repeated one and two weeks later to add the effects of decreasing moxidectin concentrations to this experiment.

In this experiment, the number of emerged seedlings, mean emergence time and synchrony of emergence were noted.

For the growth experiment, seedlings of the same four species observed in the previous experiment were exposed to three different concentrations (0.1; 1; 5  $\mu\text{g g}^{-1}$ ) of moxidectin as an oral formulation three times over a period of nine months. In addition, to simulate grassland conditions, competition stress was added as a factor in this experiment. For this experiment, the effects of MLs and competition on aboveground plant biomass, specific leaf area and intact leaf area were observed.

The results of these experiments have shown that effects of MLs on germination parameters were highly species- and substance-specific. In general, formulated substances negatively affected seed germination, while pure substances had a diminished effect. For the first time, evidence was found suggesting that MLs, such as moxidectin, in faeces could have a so called “hormetic effect” on seedling emergence. This means that a large amount of moxidectin residues in faeces had, as initially suspected, a negative effect on seedling emergence and low amounts were actually beneficial for germination success and germination time. This suggests that even very small amounts of MLs still can have significant effects on plants. In addition, results suggested that prolonged contact of plants with moxidectin in soil has negative effect on aboveground plant biomass, specific leaf area and intact leaf biomass, when no competition stress is applied. The addition of competition stress reduced these effects, but had itself significant effects on all observed parameters.

In conclusion, the impacts of MLs on seeds, seedlings and growing plants are more extensive than previously expected. Especially for the regeneration of open landscapes, where the spread of seeds via endozoochory is very important. Faeces can suppress competing plants in their direct vicinity, giving seeds germinating in them an advantage. This advantage could be negated by the effects of moxidectin, reducing the success of seedling establishment and regeneration on pastures. Therefore, regeneration of plant coverage could be delayed or stopped completely, affecting whole populations. This study showed new and unexpected effects of MLs on plants and should be seen as a starting point for additional research concerning this far reaching and understudied topic.

## **Zusammenfassung**

Makrozyklische Laktone (ML) werden als Anthelminthika zur Behandlung von Nutz- und Haustieren gegen Infektionen durch Endo- und Ektoparasiten eingesetzt. Nach der Behandlung werden diese Stoffe über den Kot in die Umwelt ausgeschieden, wo sie ein breites Spektrum von Nichtzielorganismen schädigen. Bisher konzentrierte sich die Forschung auf die Auswirkungen von MLs auf koprophage (kotfressende) Insekten wie Mistkäfer und Dungfliegen. MLs führen bei diesen Insekten zu Lähmungen und anschließend zum Tod, wenn sie aufgenommen werden. Selbst sehr niedrige Konzentrationen von MLs können subletale Wirkungen haben, die die Fitness, einschließlich der Fruchtbarkeit, reduzieren und somit die Populationen verringern.

Während die Auswirkungen von MLs auf Insekten bekannt sind, sind die Auswirkungen auf Pflanzen noch nicht ausreichend untersucht worden. Pflanzen können mit ML in Kontakt kommen, wenn sie diese Stoffe entweder aus dem Boden aufnehmen, in den Kotrückstände von kürzlich behandeltem Vieh hineingetrampelt wurden, oder wenn ihre Samen im Kot behandelten Viehs keimen. Die Verbreitung der Samen durch Aufnahme und spätere Ausscheidung durch Tiere wird als Endozoochorie bezeichnet. Da sich diese Studie auf die Auswirkungen von MLs konzentrierte, die üblicherweise bei Nutztieren eingesetzt werden, sind Samen, die in mit ML kontaminiertem Kot ausgeschieden werden, besonders gefährdet. Je nach Dosierung und Behandlungsmethode können diese Fäkalien noch Wochen nach der Behandlung nachweisbare Mengen von ML enthalten.

Frühere Studien konzentrierten sich in erster Linie auf die kurzfristigen Auswirkungen von MLs auf pflanzliche Zellkonglomerate in Kulturen, wo sie Stress für die Pflanzenzellen verursachten, und auf Sämlinge, wo sie das Wachstum bestimmter Organe wie der Wurzeln negativ beeinflussten. In einer neueren Studie wurden die Auswirkungen von Moxidectin, einem häufig verwendeten Anthelminthikum, auf die Keimung von Samen und das Auflaufen von Sämlingen aus Kot untersucht. Moxidectin verringerte diese Parameter, was darauf hindeutet, dass ML ein Faktor für den Rückgang der biologischen Vielfalt der gemäßigten Graslandarten in Europa sein könnte. Diese Studie wurde durchgeführt, um das Wissen über die Auswirkungen von MLs auf Samen, Sämlinge und wachsende Pflanzen zu erweitern. Es wurden drei Experimente zu verschiedenen Entwicklungsstufen von Pflanzen durchgeführt, wobei der Schwerpunkt auf den Auswirkungen verschiedener MLs auf die Keimung, das Aufgehen von Sämlingen aus dem Kot behandelte Tiere und das Wachstum von Pflanzen bei wiederholter ML-Exposition lag.

Das Keimungsexperiment umfasste 20 verschiedene gemäßigte Graslandarten und vier verschiedene MLs (Abamectin, Doramectin, Ivermectin und Moxidectin) in drei Konzentrationen (0,1; 1; 10 mg l<sup>-1</sup>). Die Auswirkungen dieser MLs auf den Keimungsprozentsatz, die mittlere Keimungszeit und die Synchronität der Keimung wurden beobachtet. Dies war das erste groß angelegte Keimungsexperiment, in dem die Auswirkungen mehrerer MLs auf eine Vielzahl von Arten dokumentiert wurden.

Für das Sämlingsexperiment wurden Samen von vier verschiedenen gemäßigten Graslandarten (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae) von Schafen kurz nach der Behandlung mit einer oralen Formulierung mit Moxidectin als Wirkstoff aufgenommen.

Die Fütterung wurde eine und zwei Wochen später wiederholt, um auch die Auswirkungen abnehmender Moxidectin-Konzentrationen in diesem Versuch mit in die Ergebnisse einzubeziehen. In diesem Versuch wurden die Anzahl der aufgeschlagenen Keimlinge, die mittlere Auflaufzeit und die Synchronität des Auflaufens festgestellt.

Für das Wachstumsexperiment wurden Sämlinge derselben vier Arten, die im vorherigen Experiment beobachtet wurden, dreimal über einen Zeitraum von neun Monaten drei verschiedenen Konzentrationen (0,1; 1; 5  $\mu\text{g g}^{-1}$ ) von Moxidectin als orale Formulierung ausgesetzt. Um die Bedingungen auf dem Grünland zu simulieren, wurde in diesem Versuch zusätzlich Konkurrenzstress als Faktor hinzugefügt. Bei diesem Versuch wurden die Auswirkungen von MLs und Konkurrenz auf die oberirdische Pflanzenbiomasse, die spezifische Blattfläche und die intakte Blattfläche beobachtet.

Die Ergebnisse dieser Studie haben gezeigt, dass die Auswirkungen von ML auf die Keimungsparameter in hohem Maße art- und substanzspezifisch waren. Im Allgemeinen wirkten sich formulierte Substanzen negativ auf die Keimung von Samen aus, während reine Substanzen eine geringere Wirkung hatten. Zum ersten Mal wurden Hinweise darauf gefunden, dass MLs wie Moxidectin im Kot eine so genannte "hormetische Wirkung" auf den Keimlingsaufgang haben könnten. Das bedeutet, dass eine große Menge an Moxidectin-Rückständen im Kot, wie ursprünglich vermutet, einen negativen Effekt auf das Auflaufen der Keimlinge hatte, während geringe Mengen sich tatsächlich positiv auf den Keimerfolg und die Keimzeit auswirkten. Dies deutet darauf hin, dass selbst sehr geringe Mengen von MLs noch erhebliche Auswirkungen auf Pflanzen haben können. Darüber hinaus deuteten die Ergebnisse darauf hin, dass sich ein längerer Kontakt der Pflanzen mit Moxidectin im Boden negativ auf die oberirdische Pflanzenbiomasse, die spezifische Blattfläche und die Biomasse der intakten Blätter auswirkt, wenn kein Konkurrenzstress herrscht. Konkurrenzstress selbst reduzierte die durch Moxidectin verursachten Effekte und führte selbst zu signifikanten Veränderungen in allen untersuchten Parametern.

Insgesamt zeigte sich, dass die Auswirkungen der ML auf Samen, Sämlinge und wachsende Pflanzen weitaus größer sind als bisher angenommen. Dies gilt insbesondere für die Regeneration offener Landschaften, wo die Verbreitung von Samen über die Endozoochorie sehr wichtig ist. Kot kann Pflanzen in seiner unmittelbaren Umgebung unterdrücken, was den darin keimenden Samen einen Vorteil verschafft. Dieser Vorteil könnte durch die Wirkung von Moxidectin zunichtegemacht werden, was den Erfolg der Ansiedlung von Sämlingen auf Weideflächen verringern würde und somit auch negativ auf die Etablierung der Sämlinge und Verjüngung der Weiden wirken würde. Daher könnte die Regeneration der Pflanzendecke verzögert oder ganz gestoppt werden, was sich auf ganze Populationen auswirken würde. Diese Studie zeigte neue und unerwartete Auswirkungen von ML auf Pflanzen und sollte als Ausgangspunkt für weitere Forschungen zu diesem weitreichenden und wenig erforschten Thema dienen.

## General introduction

Anthelmintics are veterinary products used to treat mammals, primarily livestock but also pets and sometimes humans for internal and external parasites (G. Geary & Moreno, 2012; Beynon, 2012). A prominent group of active compounds in this category are macrocyclic lactones, which have been in use in this capacity for more than 40 years now (Campbell & Benz, 1984; Campbell, 2012). Since their widespread introduction as commercially available anthelmintics in the late 1980s, macrocyclic lactones have been an important research topic (Campbell & Benz, 1984; Strong, 1993). This class of molecules is derived of metabolic products of soil living bacteria (*Streptomyces* spp.), which were first discovered in the late 1970s in Japan (Molyneux & Taylor, 2015). Two distinct groups of molecules were defined, milbemycins and the avermectins (Strong, 1993; Prichard *et al.*, 2012). Both groups share a lactone ring as their base structure to which several varying side chains are bonded. They also share the same mode of action but in general milbemycines have a longer half-life in treated animals (Takiguchi *et al.*, 1980; Merola & Eubig, 2018). In this study, four commonly used macrocyclic lactones were studied, the milbemycine moxidectin and the avermectins abamectin, doramectin and ivermectin. Macrocyclic lactones bind to invertebrate-specific glutamate-gated chloride channels of nerve and muscle cells, leading to the hyperpolarization of cells, and ultimately causing paralysis and death of the target organism (Turner & Schaeffer, 1989). Due to their different structure, MLs cannot bind to the chloride channels of vertebrates, which makes them safe for the use on livestock, but also pets and humans. (Campbell & Benz, 1984; Kiki-Mvouaka *et al.*, 2010).

Since MLs can affect a variety of different target species, several different ways of application have been developed to enhance their efficiency. Nearly all methods of application focus on internal application, mostly via the digestive tract, where the majority of parasites are located (Sommer *et al.*, 1992; Lloberas *et al.*, 2013). For oral application, they can be used as a liquid formulation, often applied by a syringe (Sommer *et al.*, 1992). Another option is the use of a bolus, which is a large pill which slowly dissolves and releases the active ingredient in a small dosage over a longer period of time than liquid application (Alvinerie *et al.*, 1999). A widely used option is the subcutaneous injection of a concentrated liquid formulation (Barber *et al.*, 2003; Alvinerie *et al.*). Another common treatment method is the so called “pour-on” solutions. These liquid solutions are applied typically to the back of the animals where they pass through the skin, enter the bloodstream and then spread through the whole body (Herd *et al.*, 1996; Gokbulut *et al.*, 2009).

Despite their ubiquitous use, ML anthelmintics have high potential to spread into the environment and affect non-target organisms (Floate *et al.*, 2005; Lumaret *et al.*, 2012). Their mode of action is not specific to parasites of livestock, but can affect any invertebrate. This is especially relevant on pastures where it affects coprophagous (faeces-feeding) insects (Lumaret *et al.*, 1993; Jacobs & Scholtz, 2015). When faeces are contaminated with anthelmintics, they can have both lethal and sublethal effects on these organisms (Lumaret *et al.*, 1993; Jacobs & Scholtz, 2015; Verdú *et al.*, 2015). Furthermore, the half-life of MLs in the field can vary greatly, depending on temperature, lighting and rainfall.

If the conditions are right, the half-life can be up to 240 days or even completely stop for a period of time (Halley *et al.*, 1989b). As such, the potential to impact not-target organisms is high.

Studies suggest that the detrimental effects of contaminated cow dung pats to non-target organisms can continue for weeks after anthelmintic treatment, resulting in direct death of individuals as well as reduced fitness, the effects of which can be observed over multiple generations and across populations (Förster *et al.*, 2011; Jacobs & Scholtz, 2015). When populations of coprophagous insects decline, the breakdown of faeces is slowed down considerably or, depending on their toxicity, even halted completely (Lumaret *et al.*, 1993; Verdú *et al.*, 2018; Ambrožová *et al.*, 2021). This would affect the plants growing underneath and inside the faeces, which are dependent on faeces breaking down to reach the sunlight or soil (Gokbulak & Call, 2004), reducing the amount of available food for grazing on pastures.

Recent studies have shown that MLs not only affect animals, but plants too (Lumaret *et al.*, 1993; Verdú *et al.*, 2015; Vokřál *et al.*, 2019; Navrátilová *et al.*, 2020a). These effects could be an important factor for the loss of biodiversity in temperate grassland species when plants and seeds are affected by these substances in the field. Plants can take up MLs from the soil after faeces contaminated by it have been trampled into it. Seeds can be affected by MLs when they are ingested and later excreted via faeces of recently treated livestock. Many plant species rely on their seeds being spread via faeces, which is called endozoochory (Anne Bråthen *et al.*, 2007; Soltani *et al.*, 2018). This process can not only transport seeds over large distances but also gives them several advantages when germinating from faeces. Faeces can provide a large amount of resources and displace competitors from the direct vicinity. Species reliant on endozoochorous spread are adapted to survive the various stressors of digestion, such as physical stress by mastication, heightened temperatures inside animals and chemical stress by digestion fluids (Milotić & Hoffmann, 2016). Without these adaptations, it would take far longer to repopulate the gaps created by faeces on pastures (Malo & Suárez, 1995). Because endozoochorous spread often involves grazing livestock (Kuiters & Huiskes, 2010; Xue-yong *et al.*, 2013), seeds could be at risk of being ingested by livestock recently treated with MLs and therefore coming into contact with the MLs during digestion and in faeces. Since many species rely at least partially on this method of endozoochorous spread of seeds, this could potentially affect a wide range of plants (Traveset *et al.*, 2007; Kuiters & Huiskes, 2010).

The mode-of-action of MLs affecting seeds, especially in regards to germination parameters, has not been conclusively discovered yet. Therefore, only theories based on previous research can be presented. Analysis of plants showed a rise in the concentration of stress induced hormones, suggesting at least that contact with MLs is stressful for the plants (Navrátilová *et al.*, 2020b; Langhansová *et al.*, 2021). In addition, the mode-of-action for MLs in invertebrates has been researched extensively (Turner & Schaeffer, 1989; Arena *et al.*, 1995) and studies have shown that analogous structures to those chloride channels affected in invertebrates exist in plants too (Elter *et al.*, 2007). So it can be assumed, that MLs could have similar effects in plants as in invertebrates, affecting similar structures.

Besides, studies have shown that ivermectin can inhibit protein formation by docking with major binding sites (Lehrer & Rheinstein, 2020; Arévalo *et al.*, 2021; Bello, 2021), which could have significant effects on key functions in the seeds or plants development and metabolism.

When the aim of a study is to assess the effects of specific factors, such as stress induced by MLs, on plant populations, general parameters that can be compared across species in their effect size have to be observed. To assess effects on germination, multiple parameters need to be analysed to get an overview of the complete effects. These parameters include germination percentage (GP), mean germination time (MGT) and synchrony of germination (Z) (Ranal & Santana, 2006). All of these parameters have direct effects on the success of seedling establishment and therefore the survival of entire populations (Ranal & Santana, 2006).

These parameters describe the most important factors of seedling germination and establishment. The germination percentage describes the percentage of seeds of a given sample which have germinated (Ranal & Santana, 2006). In general, having more seeds germinate is positive, since this means that more seedlings have the chance to develop into mature plants and produce seeds themselves, ensuring survival of their respective species. Some seeds may stay dormant in soil, ensuring the survival of the species in case of environmental hazards, so having all seeds germinate at the same time is not always positive (Maleki *et al.*, 2023).

Mean germination time is an essential factor for successful seedling establishment, where changes of only a few days can have detrimental effects on growth and reproductive success of plants (Rühl *et al.*, 2016a). For certain seeds to germinate at all, dormancy factors need to be broken (Rees, 1994, 1996). These can include contact to water, previous cold period below 5° C, temperatures and physical or chemical stress (e.g. by mastication) (Baskin & Baskin, 2004). If the germination process is slowed down, the conditions for the emerging seedling may not be as beneficial as they would have been at the ideal, unchanged time of germination (Ranal & Santana, 2006; Rühl *et al.*, 2016a). Important resources could have disappeared, e.g. water after a rainfall or sunlight, when other, faster germinating seedlings are now blocking the seed from light (Craine & Dybzinski, 2013; Trinder *et al.*, 2013).

The synchrony of germination states the degree of germination overlapping in a given sample (Ranal & Santana, 2006). Both extremes have advantages and disadvantages: While germinating on the same close timeframe means that all seeds get the same starting resources and opportunities, which might not be available when they would emerge sooner or later, they are also in competition with one another for the same resources (Rühl *et al.*, 2016a). In addition, if a sudden event threatens the population, like a drought, it could wipe out all seedlings at once, leaving none to grow and renew the population (Rees, 1994; Rühl *et al.*, 2016a). If synchrony is low, the risk of losing all seedlings is low, but most of them might also not benefit from ideal starting conditions and resources (Rühl *et al.*, 2016b). While these parameters can be applied for effects on germination and emergence, for growing plants, different parameters have to be applied.

Here, the amount of biomass measured by weight can be a good indicator when comparing individuals which have been subjected to different treatments (Zobel & Zobel, 2002; Weigelt *et al.*, 2002; Graß *et al.*, 2020). Biomass is positively correlated with higher seed production and therefore reproductive success. In addition, more biomass is also correlated with competition success, simply by being able to reach more resources, e.g. water by a larger root network and sunlight by outgrowing the competition, and taking these resources away from potential competitors (Reader *et al.*, 1994; Graß *et al.*, 2020).

A less specific indicator of stress for the plants is the specific leaf area (SLA). This parameter describes the ratio of leaf area to dry mass and is indicative of the amount of resources a plant put into growing the leaves described (Gong & Gao, 2019). The SLA is often used to describe differences between individuals of the same species in regions with different environmental conditions, such as temperature or water availability, or even differences between leaves of the same individual at different stages of development (Hoffmann *et al.*, 2005; Hassan *et al.*, 2007; Carrascosa *et al.*, 2023). Differences in SLA are common for shadow- and sun-leaves, or older and newly regrown leaves (Cerasoli *et al.*, 2004; Paż-Dyderska *et al.*, 2020). A higher SLA is indicative of fast growth without investing too many resources in the individual leaf, while a lower SLA shows slower growth and heavy investment of resources per individual leaf. Higher investment per leaf yield more resources in result, as the photosynthetic activity in these leaves is higher (Poorter & Evans, 1998), but since they also grow slower, they can be overtaken by competing faster growing plants, reducing the photosynthetic activity if the leaves are then overshadowed (Newbery & Newman, 1978; Wang *et al.*, 2020).

The amount of intact leaf area (ILA) was the most direct visual indicator of stress observed for this study. Damage to leaves can have various reasons, from a lack of water, specific nutrients, and sunlight to damage by herbivores, bacterial, viral or fungal infections (Green & Capizzi, 2019). These damages do not have to be directly related to the leaves themselves but can also be caused by damage to other parts of the plant, e.g. the roots. Damage to leaves can manifest in different ways, depending on the reason for the damage. Typical forms of damage are chlorosis, meaning the loss of chloroplasts and therefore photosynthetically active tissue, turning the afflicted areas of the leaves colourless, or leaf-tip dieback, where the leaves progressively die, starting from the tip (Green & Capizzi, 2019). Other forms of damage manifest by spots all over the leaf, changes in colour or malformed growth (Green & Capizzi, 2019).

In addition to stress caused by damage, stress by competition with other plants is very common and the most applied stressor in natural landscapes (Craine & Dyzinski, 2013; Trinder *et al.*, 2013). In contrast to such stressors as herbivory or chemical damage, competition is a passive stressor, affecting plants indirectly by a lack of water, nutrients or sunlight (Grace, 2012). Hence there is no real indicator of direct competition, but those for the lack of resources caused by other plants (Novoplansky, 2009; Violle *et al.*, 2009).

The effects of competition stress are often overlooked in greenhouse and even common garden experiments, where plants grow also in pots and are therefore not under any competition stress.

Results gathered in this way may therefore not be applicable in nature, since growth without any limits besides the size of pots and the nutrients in the provided soil is very unlikely ever to occur to any plant in nature. Competition is especially relevant in temperate grasslands, since these environments are often grazed by a variety of species (Newbery & Newman, 1978; Graß *et al.*, 2020).

This causes repeated damage to plants, favouring species able to handle this constant stress and regrow fast (Ba & Facelli, 2022). In this highly competitive environment, any species-specific stressors, such as MLs (Chapter 1) could have significant effects on entire populations, which would then be severely disadvantaged compared to other species (Christou *et al.*, 2018).

The combination of biotic (e.g. MLs) and abiotic (e.g. competition, drought) stressors can enhance the damage done to plants (Rizhsky *et al.*, 2004; Suzuki *et al.*, 2016). In some cases, stressors can also cancel each other out, therefore leading to a positive effect of the affected plants (Puckette *et al.*, 2007; Pandey *et al.*, 2017). While most experiments only focus on one specific factor, e.g. macrocyclic lactones or drought, in nature, more than one factor would apply at all times, such a competition stress being nearly omnipresent (Novoplansky, 2009; Atkinson & Urwin, 2012; Grace, 2012; Suzuki *et al.*, 2014). To disregard a second stressor simply by also doing an experiment and checking for both factors individually is therefore not enough to grasp their effects in total (Atkinson & Urwin, 2012; Pandey *et al.*, 2017). This is often overlooked in studies (Ramegowda & Senthil-Kumar, 2015), causing the results not to reflect on the implications these stressors would have when combined in the field.

Although the contamination of the temperate grasslands by MLs and their negative effects on plants in addition to other organisms have been described before, little to nothing is known about these effects on a wide range of species and these effects in combination with other stressors such as competition. To gain insight into this understudied field, this thesis addresses the following questions:

Do macrocyclic lactones affect the germination of temperate grassland species  
(**Chapter 1**)?

Does the treatment of sheep with macrocyclic lactone anthelmintics affect seed spread via endozoochory (**Chapter 2**)?

Does prolonged exposure of temperate grassland species to moxidectin affect their growth and overall vigour (**Chapter 3**)?

**Chapter 1** describes the effects of multiple commonly used macrocyclic lactones on seedling parameters of temperate grassland species. An in vitro germination experiment with 20 temperate grassland species from five different families (Asteraceae, Caryophyllaceae, Fabaceae, Plantaginaceae, Poaceae) was conducted, which exposed seeds to three concentrations (0.1; 1; 10 mg l<sup>-1</sup>) of the MLs abamectin, doramectin, ivermectin and moxidectin and oral formulations of ivermectin and moxidectin.

The parameters germination percentage, mean germination time and synchrony of germination were observed.

**Chapter 2** looked into the effects that treatment of sheep with formulated moxidectin for oral application has on the emergence of seeds from faeces. Sheep were treated with formulated moxidectin (Cydectin®) and then fed a seed mixture of four different species (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae). Their faeces were collected and seedling emergence per g dung, mean emergence time and synchrony of emergence were recorded over a period of 5 months.

**Chapter 3** consisted of a study to assess the effects of prolonged exposure to moxidectin and competition stress on plants. For this experiment, plants of four species (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae) were exposed to three different concentrations of formulated moxidectin (0.1; 1; 5  $\mu\text{g g}^{-1}$ ) in combination with competition stress over a period of 9 months. In this experiment, the effects of these parameters on aboveground plant biomass, specific leaf area and intact leaf area were observed.

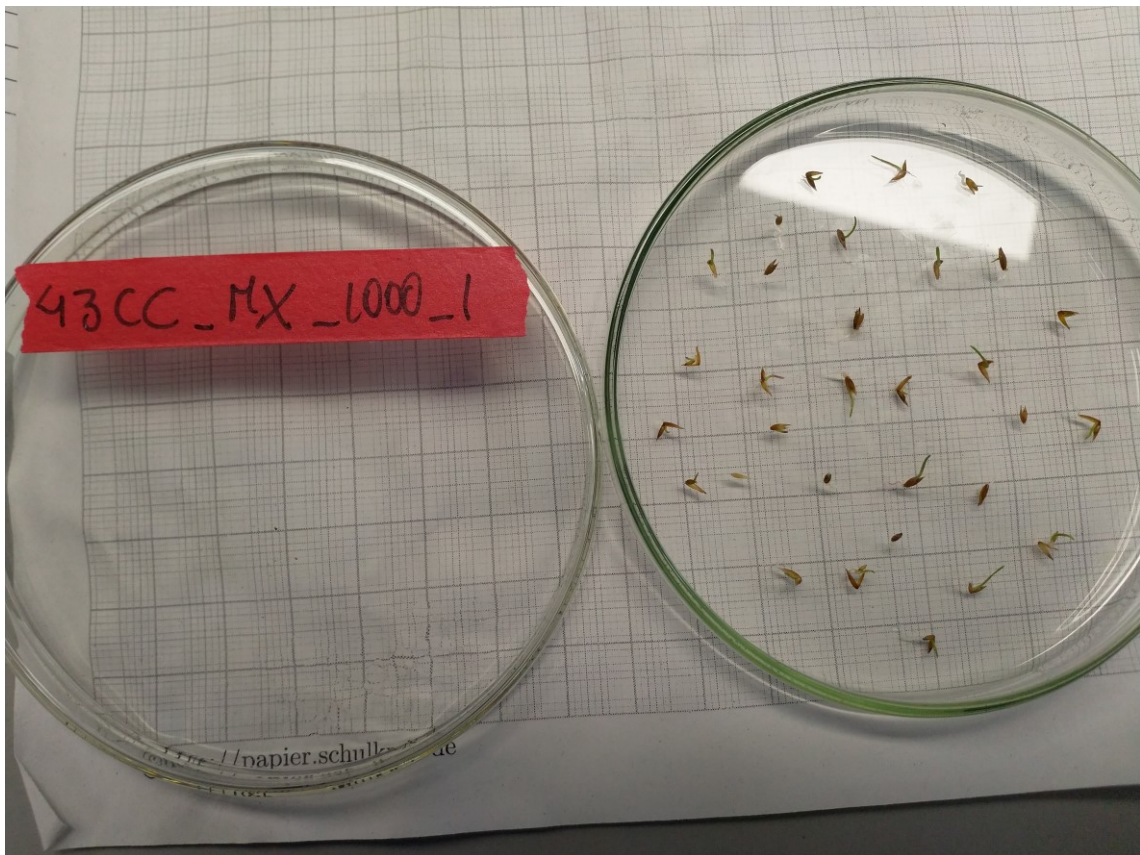
In a comprehensive conclusion, the main findings of all three chapters are discussed and an outlook for improvements of the studies and further experiments is given.

# Chapter 1

## Effects of macrocyclic lactone anthelmintics on seed germination of temperate grassland species

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Lars Laber, Carsten Eichberg, Anne Zimmerbeutel, Rolf-Alexander Düring &  
Tobias W. Donath



## Abstract

- Macrocyclic lactone anthelmintics are widely used to control invertebrate pests in livestock such as sheep. While anthelmintic effects on non-target animals, such as dung-dwelling insects, are well studied, effects on seed germination are largely unknown. Seeds can come into contact with anthelmintics either during passage through the gastro-intestinal tract of grazing animals or when anthelmintics are excreted with their dung into the environment, which may result in changed germination patterns.
- We used four commonly applied macrocyclic lactones to assess their effects on germination: moxidectin, ivermectin, abamectin and doramectin as pure substances; moxidectin and ivermectin also in formulated form. We tested these pharmaceuticals on 17 different temperate grassland species from five plant families. Seeds were exposed to three concentrations of macrocyclic lactones (0.1, 1 and 10 mg l<sup>-1</sup>) under controlled conditions and germination was assessed over a six-week period. From this data, we calculated germination percentage, mean germination time and germination synchrony.
- Most of the tested species were significantly affected in germination percentage and/or mean germination time by at least one of the tested pharmaceuticals, with formulated moxidectin having the largest impact. In general, the effects we found were species- and pharmaceutical-specific. While formulated substances generally reduced germination percentage and increased mean germination time, pure substances increased germination percentage. Synchrony showed less clear patterns in all pharmaceuticals.
- Although effect size and sign varied between species, our study shows that non-target effects of macrocyclic lactones commonly occur in terrestrial plants. This may impede successful seed exchange between habitats via sheep and even translate into profound changes of grazed ecosystems.

## 1. Introduction

Anthelmintics are widely used around the world to rid livestock of parasites, which are a main cause of reduced livestock productivity and welfare. A particular potent and common group of anthelmintics are macrocyclic lactones (MLs), including commonly used representatives such as moxidectin (MOX), ivermectin (IVM), abamectin (ABA) and doramectin (DOR) (Vercruysse & Rew, 2001). Target organisms of MLs are common parasites that live in animals (e.g., their intestines), such as nematodes and tapeworms (Gokbulut *et al.*, 2009). From a medical point of view, MLs are ideal anthelmintics, since their mechanism affects glutamate-gated chloride channels which are absent from almost all vertebrates, making them insensitive to these substances (Vercruysse & Rew, 2001). Since MLs are only weakly metabolised, weakly absorbed in the body and bind preferentially to solid particles due to their high lipophilicity, a large part of the administered active substance is excreted via dung (Lifschitz *et al.*, 2000; Junco *et al.*, 2021).

Many studies have shown that the concentrations of ML anthelmintics in livestock dung are sufficient to kill non-target organisms, in particular dung-decomposing arthropods, such as dung beetles (Lumaret *et al.*, 2012; Blanckenhorn *et al.*, 2013). However, the effects of ML anthelmintics on other invertebrate groups (e.g. earthworms, springtails), on the soil microbiome and on terrestrial vascular plants are largely unknown. Organisms can also be indirectly affected by ML anthelmintics. For instance, a lack of dung-decomposing arthropods can reduce germination success of excreted and viable seeds or nutrient return into the soil (Bang *et al.*, 2005; Milotić *et al.*, 2019; deCastro-Arrazola *et al.*, 2020). While the uptake of MLs from soil by plants has been shown by several studies (Iglesias *et al.*, 2018; Navrátilová *et al.*, 2020a,b), the effects of MLs on plant reproduction are not well studied.

Our research focuses on seed germination since this development stage is a critical stage in the life of plant individuals and determines survival and persistence of plant populations (Grubb, 1977; Fenner, 2000). Most species require specific environmental conditions for successful germination (Baskin & Baskin, 2014). First evidence that ML anthelmintics can influence seed germination was found by Eichberg *et al.* (2016). They tested three temperate grassland species and showed that formulated as well as pure MOX can decrease germination success and alter the timing of germination with the formulation revealing stronger effects compared to the pure substance. Vokřál *et al.* (2019) found IVM solutions reduced *Sinapis alba* seedling root length. To our knowledge, no study has yet assessed effects of ABA or DOR on seed germination parameters.

Seeds potentially come into contact with MLs as they pass through the gastro-intestinal tract of treated domestic ungulates and may remain in contact with MLs when collectively excreted and embedded in dung heaps. Concentrations of MLs are much higher in dung than in digestive contents. As such, ingested seeds are in contact with the highest ML concentration for the longest time (Chiu *et al.*, 1990; Fernandez *et al.*, 2009).

In general, in grasslands, internal seed dispersal (endozoochory) by ungulate species plays an important role for plant population dynamics (Bartuszevige & Endress, 2008; Albert *et al.*, 2015b) and also ensures establishment of new and maintenance of already existing plant populations in grazed grasslands (Rosenthal *et al.*, 2012).

Sheep, for example, are not only effective seed dispersers, but they also recruit seedlings (Rosenthal *et al.*, 2012; Eichberg & Donath, 2018). Especially in fragmented cultural landscapes, where dispersal limitation is a major hindrance to the restoration of species-rich grasslands, and sheep are often moved between habitat islands of the same vegetation type, this coupled function is important for maintenance of a high phytodiversity (Rosenthal *et al.*, 2012). Considering the abundance of livestock regularly treated with MLs, the large numbers of seeds consumed by livestock (Kuiters & Huiskes, 2010) and long elimination half-life of MLs in the gastro-intestinal tract (IVM two days, MOX 15 days; Molento *et al.*, 2004), a considerable number of seeds could come into contact with anthelmintic substances. Therefore, any effects on the effectiveness of seed germination after ingestion by livestock will be relevant for nature conservation. If ML anthelmintics influence germination, this could lead to changes in plant population sizes and, ultimately, to shifts in plant species compositions of grazed habitats.

Besides a reduction of germination percentage, a shift in the timing of germination can also hamper successful establishment of seedlings, if germination occurs at inopportune times (Ross & Harper, 1972; Dyer *et al.*, 2000; Rühl *et al.*, 2016a). Therefore, the number of seeds germinating is not sufficient to assess the effects of environmental conditions on seed germination (Ranal & Santana, 2006; Ranal *et al.*, 2009). Additional parameters of germination behaviour, such as mean germination time or synchrony of germination allow for a more specific evaluation of effects on seed germination (Ranal & Santana, 2006; Ranal *et al.*, 2009). Perennial plant species may rely less on temporally spreading emergence risk because they are less dependent on environmental variation in time than annuals, due to their iteroparous reproduction (Rees, 1996). However, in herbaceous perennials, early emergence is often related to higher vigour and fecundity in terms of seedling survival, seedling height/biomass and number of flowers (Verdú & Traveset, 2005).

To determine the extent to which ML effects on seed germination of plant species of temperate grasslands can be generalised, we carried out a climate cabinet experiment. We tested the effects of four common MLs as pure substances (MOX, IVM, ABA, DOR) at three concentration levels, as well as MOX and IVM as pharmaceutical formulations (Cydectin®, MOX; Oramec®, IVM) on 17 grassland plant species belonging to five families. All active ingredients are used in sheep husbandry.

We addressed the following questions:

- How general are responses of seeds of grassland species to macrocyclic lactone anthelmintics in terms of germination?
- Do higher concentrations of the active ingredients (pure or formulated) lead to stronger effects on seed germination?
- Are there differences in the effects caused by the pure active ingredient or its formulation?

## 2. Materials and methods

### 2.1. Test species and seed material

We tested seed germination of 17 species with predominantly perennial life strategies belonging to five plant families (Asteraceae, Caryophyllaceae, Fabaceae, Plantaginaceae and Poaceae; Table 1). The test species are typical representatives of temperate grasslands in Central Europe and cover a broad spectrum of soil conditions (Ellenberg indicator values for moisture: 3–7, soil reaction: 3–8 and nitrogen: 2–7; Ellenberg, 1988). For most of the test species references exist for natural spread of their seeds by sheep via passage through the gastro-intestinal tract in viable condition (Mitlacher *et al.*, 2002; Albert *et al.*, 2015b; Benthien *et al.*, 2016). A meta-analysis showed that the ecological filtering effect of endozoochorous seed transport is comparatively low, suggesting that more species are dispersed endozoochorously than have been recorded so far (Albert *et al.*, 2015a,b). Seeds were obtained from a commercial supplier (Rieger-Hofmann GmbH, Blaufelden-Raboldshausen, Germany). Nomenclature of plant species follows Müller *et al.* (2021).

### 2.2. Test substances

We tested the four active ingredients moxidectin (MOX,  $C_{37}H_{53}NO_8$ , 640 g mol<sup>-1</sup>), ivermectin (IVM,  $C_{48}H_{74}O_{14}$ , 875 g mol<sup>-1</sup>), abamectin (ABA,  $C_{49}H_{75}O_{14}$ , 873 g mol<sup>-1</sup>) and doramectin (DOR,  $C_{50}H_{74}O_{14}$ , 899 g mol<sup>-1</sup>), which were obtained from SigmaAldrich (Taufkirchen, Germany; purities of all substances  $\geq 95\%$ ). For MOX and IVM, we also used common formulated veterinary products for the application on sheep: Cydectin® 0.1 % oral drench (MOX, Zoetis Deutschland GmbH, Berlin, Germany) and Oramec® 0.08 % oral drench (IVM, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany). Oral administration is the typical application route of macrocyclic lactone (ML) formulations in small ruminants (Prichard *et al.*, 2012).

As MLs, the tested active ingredients have a three-part pharmacophore in common consisting of a 16-membered macrocycle with fused benzofuran and spirochaetal units (McKellar & Gokbulut, 2012). There are two ML sub-classes differing structurally mainly by presence (avermectins) or absence (milbemycins) of a disaccharide substituent at C-13 (McKellar & Gokbulut, 2012). We studied three avermectins (IVM, ABA, and DOR) and one milbemycin (MOX). Besides absence of a sugar moiety, MOX differs structurally from avermectins by the presence of a methoxime moiety (contains N) at C-23 and an olefinic group at C-25 (Prichard *et al.*, 2012). In addition, it has a much lower molecular weight.

The studied avermectins differ only slightly structurally from each other; specifically, in the bonds at C-22, 23 and/or substituents at C-25 (Vercruysse & Rew, 2001). MLs essentially exert the same mode of action: In invertebrates, they bind to glutamate-gated chloride channels leading to an irreversible influx of chloride ions followed by membrane hyperpolarisation and muscle paralysis (Köhler, 2001). However, the precise mode of action has not yet been fully clarified and the structural differences, especially in MOX vs. avermectins, lead to different interactions of MLs with chloride channels and pharmacodynamic behaviours (Prichard *et al.*, 2012).

Anthelmintic formulations contain further ingredients in addition to the respective active ingredient (these are to be summarized here under the term “co-formulants”; Supplementary Table S1). Nevertheless, in order to improve readability, we also refer to the concentration of the active ingredient when it came to effects of formulations.

We studied active ingredient concentrations in the range of 0.1–10 mg l<sup>-1</sup> for all six test drugs. These values are within the range of concentrations of MLs reported for substrates (digestive contents, dung) that are relevant for a seed endozoochorously dispersed by sheep. The comparability of values of ML concentrations in environmental matrices related to sheep is complicated by the fact that there are only few published values and methods and units vary. After intraruminal administration of single formulated MLs (MOX, IVM, ABA) to lambs, Lloberas *et al.* (2013) found mean concentrations for digestive contents in the range of 452 ng g<sup>-1</sup> (0.5 d post treatment; equivalent to 0.45 mg l<sup>-1</sup>) to 32 ng g<sup>-1</sup> (2 d p.t.; 0.03 mg l<sup>-1</sup>). In their study, the mean concentrations of the three tested MLs were similar at 0.5 d p.t. Generally, dung showed much higher ML concentrations than digestive contents, but reported values cover a broad range between 23 ng g<sup>-1</sup> (MOX, 40 h p.t., subcutaneous administration, (Hentz *et al.*, 2019), equivalent to 0.02 mg l<sup>-1</sup>) and 13,615 ng g<sup>-1</sup> (IVM, 1 d p.t., oral administration, Lang, 1996; 13.62 mg l<sup>-1</sup>). The following values for sheep dung were found within this range by other studies: 0.93 µg g<sup>-1</sup> (IVM, 2 d p.t., subcutaneous administration; Vokřál *et al.*, 2019), 1277 ng g<sup>-1</sup> (ABA, 3 d p.t., subcutaneous administration; Kolar *et al.*, 2006), 2186 ng g<sup>-1</sup> (DOR, 2 d p.t., subcutaneous administration; Kolar *et al.*, 2006) and 3390 ppb (MOX, 1 d p.t., oral administration; Steel, 1998).

### 2.3. Experimental design

We prepared aqueous solutions of the six anthelmintic drugs in three concentrations of the active ingredient: 0.1, 1 and 10 mg l<sup>-1</sup>. In a first step, we prepared stock solutions of 0.04, 0.4 and 4 mg ml<sup>-1</sup> in ethanol (purity: 99 %, Carl Roth), since MLs are highly lipophilic (Hennessy, 1997). From each of these stock solutions, 0.5 ml were taken and added to 200 ml of ddH<sub>2</sub>O to create the treatment solutions. In case of the formulated MLs, we added the appropriate amount of the anthelmintic drug directly to ddH<sub>2</sub>O to achieve the same anthelmintic concentrations as used with the pure chemical.

In addition, two types of controls were established: a blank control (C<sub>W</sub>) with ddH<sub>2</sub>O only and an alcohol control with an ethanol concentration of 2.5 µl ml<sup>-1</sup> (C<sub>E</sub>, solvent: ddH<sub>2</sub>O). C<sub>E</sub> was used as a control for the pure substances, since they were dissolved in the same amount of alcohol. C<sub>W</sub> was used as a control for the formulated anthelmintics, since these formulations are aqueous-based solutions.

Throughout the lab work, we used glass materials for the preparation of the solutions as MLs are known to adhere to plastics surfaces (Cerkvenik *et al.*, 2002).

We used glass Petri dishes with a diameter of 9 cm and fitted them with two filter papers each, so that the whole dish and a part of the rim was covered to prevent seeds from slipping under the papers. Each dish was equipped with 50 seeds of one plant species and 5 ml of the respective treatment solution. Seven replicates were generated for each treatment combination (fixed factors: *anthelmintic*, *concentration* and *species*) and for the two control types, resulting in a total number of 2,800 experimental units. To reduce evaporation, five Petri dishes were stacked up in one transparent plastic bag.

Each bag contained only dishes with the same anthelmintic drug and concentration, but different species to avoid cross-treatment effects and ensure randomisation. Dishes were kept in three identical climate cabinets.

Prior to the germination census period, all dishes were exposed to a phase of stratification for one week in which cabinets were set to 4 °C and complete darkness to improve seed germination (Baskin & Baskin, 2014). After this initial cold-wet stratification phase, the dishes were exposed to a day/night cycle of 16/8 hours and 15/5 °C. Twice per week and at equal intervals germinated seeds (appearance of radicle) were counted and removed from the dishes. The positions of the bags in the cabinets and the position of the dishes in the bags were randomly changed after every census. The experiment was terminated after six weeks.

#### 2.4. Data analysis

Three response variables were calculated from the data obtained. Firstly, we calculated final germination percentage (GP, %) for each replicate, i.e. the percentage of germinated seeds from the initial number of seeds (Ranal *et al.*, 2009).

Secondly, mean germination time (MGT) was calculated by the formula:

$$MGT [d] = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i},$$

where  $i$  is the day number,  $t_i$  is the time from day 1 to day  $i$ ,  $n_i$  is the number of germinated seeds at day  $i$  and  $k$  is the last time of germination (Ranal *et al.*, 2009). MGT indicates the number of days the seeds of one replicate needed to germinate on average.

Thirdly, the synchrony of germination ( $Z$ , dimensionless) was calculated by the formulae:

$$Z = \frac{\sum_{i=1}^k C_{n_i,2}}{C_{\sum n_i,2}}, \quad C_{n_i,2} = \frac{n_i(n_i-1)}{2},$$

where  $C_{n_i}$  is the two-by-two combination of the seeds that germinated at day  $i$  and  $n_i$  is the number of germinated seeds at day  $i$ .  $Z$  represents how synchronous a species germinated during the study period and ranges from 0 to 1. Increasing values indicate increasing synchrony of germination (Ranal *et al.*, 2009).

We started our experiment with 20 plant species (four species from each of the five plant families), but three species (*Arenaria serpyllifolia*, Caryophyllaceae; *Serratula tinctoria*, Asteraceae; *Veronica teucrium*, Plantaginaceae) did not show sufficient germination success for statistical testing and were excluded from analyses.

Univariate three-way ANOVA was applied to analyse effects of *species*, *family* and *anthelmintic concentration* on the dependent variables GP, MGT and  $Z$ . For this analysis, *species* was nested in *family*. Although we have chosen only four species per family, which are too few species to assess phylogenetic effects, we included *family* in our model, since we expected it to explain some of the variance. This analysis will be referred to as “across species” from this point onward. One-way ANOVA was used for the analyses of the effects of concentration at the species level, which will be referred to as “on species level”.

Post-hoc Tukey HSD test was done, if significant effects were revealed. Before employing inferential statistics, data was visually checked by QQ plots and scatterplots of observed values against non-standardized residuals whether necessary requirements were met and we employed Box-Cox transformation to meet normality and homogeneity of variance in every case.

Statistical analysis was done with Statistica™ 13.3 (StatSoft Inc., Tulsa, Oklahoma, USA) and graphs were created using ORIGIN® (ORIGINLab Corporation, Northampton, Massachusetts, USA).

### 3. Results

ANOVAs across species revealed that the general response patterns of germination parameters caused by the pure or formulated anthelmintics showed large variation (Table 2). Only the factors *species(family)* and *family* were significant in all germination parameters and anthelmintics. By contrast, the significance patterns of the factor *concentration* and its interactions with other factors, as the main interest of our study, were often quite inconsistent. However, with the exception of *Medicago lupulina*, the germination behaviours of all plant species were influenced by at least one pure macrocyclic lactone (ML) substance or formulation. Interestingly, the comparison of germination in the two controls ( $C_E$  vs.  $C_W$ ) revealed that the solubilizer ethanol, used to dissolve pure MLs in water, also itself had impact on germination of some species with decreasing sign in germination percentage (GP) and increasing sign in mean germination time (MGT) (Table 1, Supplementary Table S2).

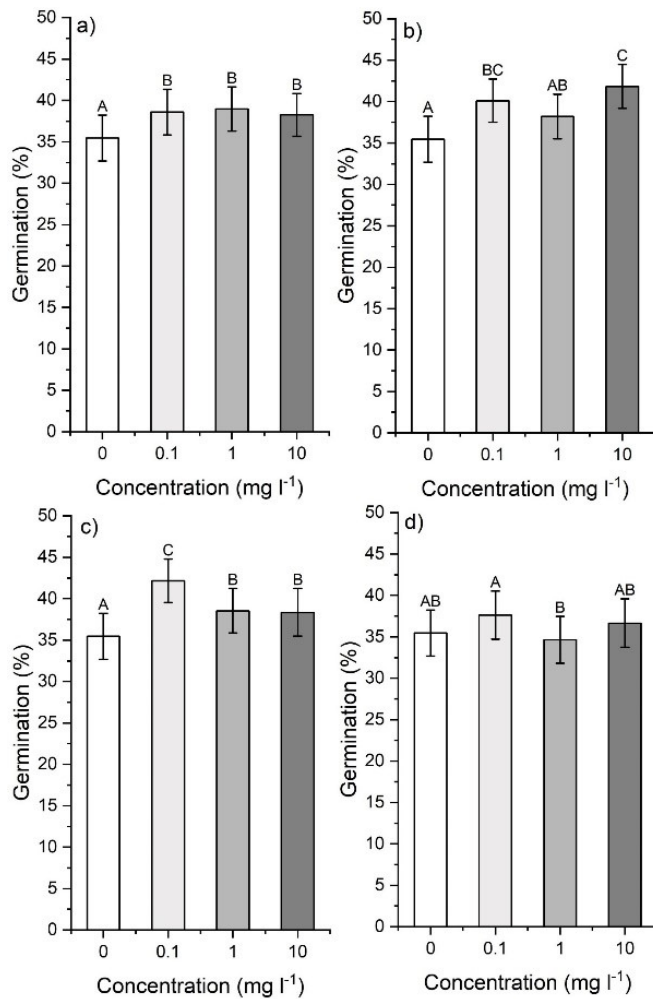
**Table 1.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of all tested macrocyclic lactone drugs on germination percentage (GP), mean germination time (MGT) and synchrony of germination (Z) ( $p > 0.05 = -$ ,  $0.05 \geq p > 0.01 = *$ ,  $0.01 \geq p > 0.001 = **$ ,  $p \leq 0.001 = ***$ ). For detailed information, see Supplementary Tables S21-S26.

Germination parameter	Moxidectin			Cydectin			Ivermectin			Oramec			Abamectin			Doramectin		
	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z	GP	MGT	Z
Species(Family) [S(F)]	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Family [F]	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Concentration [C]	***	-	-	***	***	*	***	-	-	***	***	-	***	***	-	*	-	**
S(F) x C	-	-	**	***	***	***	-	***	-	***	***	***	***	***	-	**	-	-
F x C	*	-	-	***	***	***	-	***	-	*	**	***	***	***	-	-	-	*

#### 3.1. Effects of pure and formulated moxidectin on germination

ANOVA across species for pure MOX treatments revealed a significant main effect for *concentration* and a *family*  $\times$  *concentration* interaction in GP as well as a significant *species(family)*  $\times$  *concentration* interaction in synchrony (Z) (Table 2). Across species, GP was lowest in the control (35.4 %; ethanol control:  $C_E$ ), which differed significantly from all concentration levels (38.3-38.6 %) (Fig. 1a). Analysis on species level showed that for GP only in three species the control was significantly different from at least one concentration level (Supplementary Table S3). In all three species, GP was enhanced for most concentration levels compared to the control. GP of *Centaurea jacea* was enhanced up to 81.5 % (10 mg l<sup>-1</sup>), GP of *Hypochaeris radicata* up to 5.8 % (1 mg l<sup>-1</sup>) and GP of *Plantago lanceolata* up to 84.0 % (10 mg l<sup>-1</sup>) compared to control ( $C_E$ ). For MGT, in contrast to the across-species analysis (Table 2), analysis on species level did reveal concentration effects.

In three species (*H. radicata*, *Hippocrepis comosa* and *Agrostis capillaris*), MGT of MOX-treated seeds differed significantly from the control ( $C_E$ ) under at least one concentration level, while for one species (*Lolium perenne*), we found significant differences between concentration levels, but not compared to the control (Supplementary Table S4). In *H. radicata*, MGT was significantly increased at the 0.1 mg l<sup>-1</sup> concentration level (+ 2.4 d) and 1 mg l<sup>-1</sup> (+ 2.3 d) concentration level compared to control ( $C_E$ ) while in the other two species, MGT was significantly lowered (-2.4 d, *A. capillaris*, 1 mg l<sup>-1</sup>; -4.1 d, *H. comosa*, 0.1 mg l<sup>-1</sup>). For Z, analysis on species level revealed no significant differences in the post-hoc tests in any species (Supplementary Table S5).

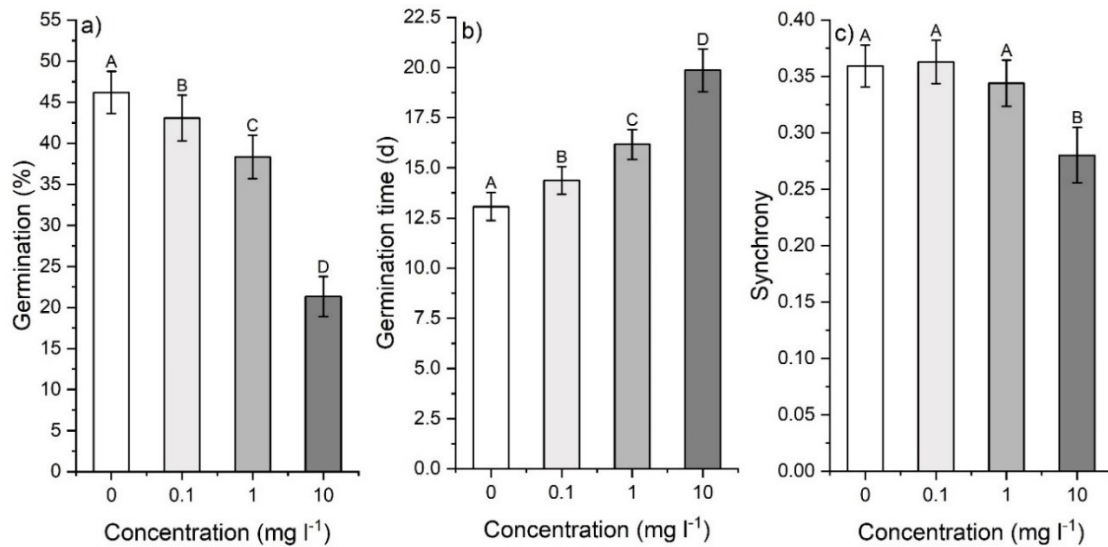


**Figure 1.** Mean germination percentage  $\pm$  SE across species at different concentrations of the pure macrocyclic lactones moxidectin (a), ivermectin (b), abamectin (c) and doramectin (d). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ( $p \leq 0.05$ ; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

In contrast to pure MOX, in formulated MOX (Cydectin) analysis across species showed significant main effects for all factors and their interactions on all response variables (Table 2). Across species, blank control ( $C_W$ ) showed the highest GP and differed significantly from all concentration levels which decreased significantly with increasing concentration (Fig. 2a). In terms of the number of species significantly affected,

formulated MOX showed more effects than pure MOX: GP of 12 species was changed by formulated MOX compared to the control ( $C_W$ ) (Supplementary Table S6).

Eight of these species showed reductions of GP > 80 % at the highest MOX concentration (10 mg l<sup>-1</sup>). In contrast, effects of lower concentrations were less pronounced (Fig. 2a). In nine species, significant differences between concentration levels within the same species were found (Supplementary Table S6).



**Figure 2.** Effects of different Cydectin (formulated moxidectin) concentrations on mean germination percentage  $\pm$  SE (a), mean germination time  $\pm$  SE (b) and mean synchrony of germination  $\pm$  SE (c) (across-species analysis). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ( $p \leq 0.05$ ; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

While in pure MOX treatments, analysis across species revealed no significant differences between concentration levels for MGT compared to the control ( $C_E$ ) (not shown), in formulated MOX treatments, analysis across species revealed significant differences between all concentration levels (including control  $C_W$ ), with MGT rising significantly with concentration (Fig. 2b). In 12 species, MGT increased significantly with formulated MOX concentration compared to the control ( $C_W$ ) (Supplementary Table S7). In formulated MOX, mean increase of MGT across species and concentration levels was around 3.7 days and the highest increase for a single species and concentration level was 27.3 d (*Dianthus deltooides* at 10 mg l<sup>-1</sup>). *H. comosa* (+ 3.8 d, 10 mg l<sup>-1</sup>), *Trifolium pratense* (+5.3 d, 10 mg l<sup>-1</sup>) and *L. perenne* (+7.7 d, 10 mg l<sup>-1</sup>), in which GP was not significantly affected, differed significantly from the control ( $C_W$ ) in regards to MGT. In nine species, significant differences between concentration levels were revealed by analysis on species level (Supplementary Table S7).

Across species, Z was significantly lowered in the highest concentration level of formulated MOX (10 mg l<sup>-1</sup>; Fig. 2c). Only in four test species, significant differences between at least one concentration level and control ( $C_W$ ) were found in Z (Supplementary Table S8).

To sum up, we found contrary response patterns between pure and formulated MOX: Compared to the respective controls ( $C_W$  resp.  $C_E$ ) and across all other factors, formulated MOX significantly decreased GP (-25.9 %) and significantly increased MGT (+26.5 %), whereas pure MOX significantly increased GP (+8.9 %) and decreased MGT

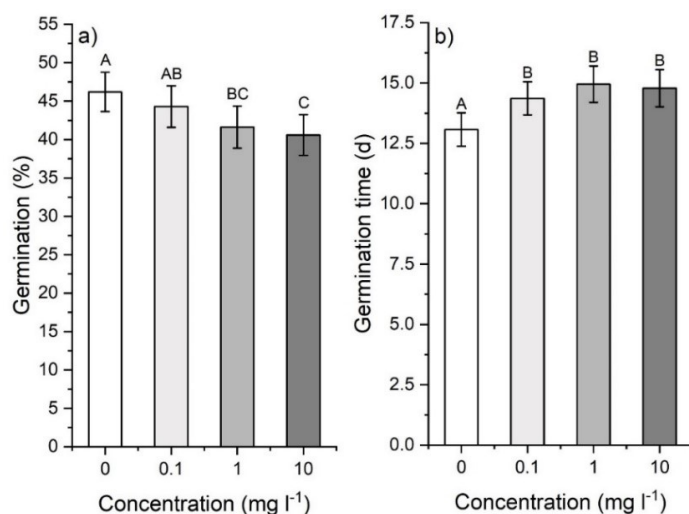
by trend (-0.4 %). Both ML drugs significantly but weakly affected Z (Cydectin: -0.6 %, MOX: +0.4 %).

### 3.2. Effects of pure and formulated ivermectin on germination

Across species, pure IVM caused effects related to *concentration* in GP (main effect) and MGT (interactions), whereas Z was not affected (Table 2). Analysis across species showed that GP was enhanced with the control ( $C_E$ ) differing significantly from the 0.1 and the 10 mg l<sup>-1</sup> level (Fig. 1b). Among concentration levels, the 10 mg l<sup>-1</sup> level differed significantly from the 1 mg l<sup>-1</sup> level (Fig. 1b). Analysis on species level revealed GP to be significantly raised by pure IVM under at least one concentration level compared to the control ( $C_E$ ) only in two species (*C. jacea*, +79.6 %, 10 mg l<sup>-1</sup>; *P. lanceolata*, +68.0 %, 10 mg l<sup>-1</sup>) (Supplementary Table S9).

While analysis across species for MGT revealed no significant differences in-between concentration levels and compared to control ( $C_E$ ) (not shown), analysis on species level revealed that four species were significantly affected by pure IVM at least at one concentration level compared to control ( $C_E$ ) but these effects were inconsistent in sign and size (Supplementary Table S10). The four species significantly affected were *H. radicata* (+3.1 d, 0.1 mg l<sup>-1</sup>), *H. comosa* (-4.1 d, 1 mg l<sup>-1</sup>), *T. pratense* (-2.2 d, 0.1 mg l<sup>-1</sup>) and *Cynosurus cristatus* (+3.6 d, 0.1 mg l<sup>-1</sup>). For Z, only in *T. pratense* (-38.5 %, 10 mg l<sup>-1</sup>), significant differences between concentration levels and control ( $C_E$ ) were revealed (Supplementary Table S11).

Analysis across species on formulated IVM revealed significant effects of almost all main factors and their interactions on GP, MGT and Z (Table 2; exception: no main effect for *concentration* in Z). For GP, the 1 mg l<sup>-1</sup> (41.6 %) and the 10 mg l<sup>-1</sup> (40.5 %) treatment differed significantly from control (46.2 %;  $C_W$ ), while the 0.1 mg l<sup>-1</sup> level did not differ from control and the 1 mg l<sup>-1</sup> level, which also did not differ from the 10 mg l<sup>-1</sup> level (Fig. 3a). Analysis on species level showed significant decreasing effects on GP in three species: *Cerastium holosteoides* (-45.5 %, 10 mg l<sup>-1</sup>), *Lychnis flos-cuculi* (up to -49.7 %, 0.1 mg l<sup>-1</sup>) and *Plantago media* (up to -76.6 %, 1 and 10 mg l<sup>-1</sup>) (Supplementary Table S12).



**Figure 3.** Effects of different Oramec (formulated ivermectin) concentrations on mean germination percentage  $\pm$  SE (a) and mean germination time  $\pm$  SE (b) (across-species analysis). Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ( $p \leq 0.05$ ; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

Analysis across species in MGT revealed a small yet significant increasing effect of *concentration* for formulated IVM, with all concentration levels differing from the control ( $C_W$ ), but not from each other (Fig. 3b). Analysis of *concentration* effects for formulated IVM on species level revealed a significant difference from control in five species: *C. jacea* up to -6.0 d ( $10 \text{ mg l}^{-1}$ ), *H. radicata* up to -2.7 d ( $0.1 \text{ mg l}^{-1}$ ), *C. holosteoides* up to +10.1 d ( $1 \text{ mg l}^{-1}$ ), *T. pratense* up to +1.9 d ( $10 \text{ mg l}^{-1}$ ) and *L. perenne* up to +1.0 d ( $1 \text{ mg l}^{-1}$ ) (Supplementary Table S13).

Despite the strong main effects and interactions (excluding *concentration*) for Z revealed by analysis across species (Table 2), only few species were affected by formulated IVM, i.e. only in *A. capillaris* (up to -35.2 %, 1 and  $10 \text{ mg l}^{-1}$ ) and *D. deltooides* (up to -46.3 %,  $1 \text{ mg l}^{-1}$ ) a significant deviation from the control ( $C_W$ ) was proven (Supplementary Table S14).

Comparing the differences to the respective controls ( $C_W$  resp.  $C_E$ ) across all species and concentrations, formulated IVM significantly reduced GP (-8.7 %), while pure IVM significantly enhanced GP (+12.9 %) (Figs. 1b, 3a). MGT was, compared to the control ( $C_W$ ), only significantly affected by formulated IVM (+1.6 d; Fig. 3b). Only in Z, both pure and formulated IVM had no significant effects compared to control ( $C_W$  resp.  $C_E$ ) across all species (Table 2).

### 3.3. Comparison of effects of formulated moxidectin and formulated ivermectin

Across species, both ML formulations led to similar response patterns in GP (regular decrease with concentration) and MGT (regular increase), with stronger effects in formulated MOX than formulated IVM (GP across the three concentration levels: MOX -25.9 %, IVM -8.7 %; MGT across the three concentration levels: MOX +26.5 %, IVM +12.5 %; Figs. 2a, b and 3a, b).

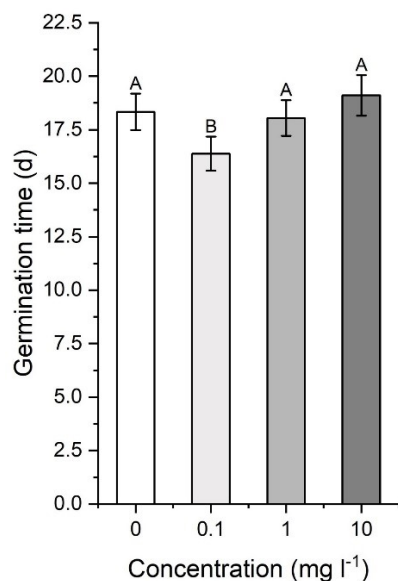
Formulated MOX (16 species) also significantly influenced more species than formulated IVM (11 sp.) taken all germination parameters into account (Supplementary Tables S6-S8, S12-S14).

### 3.4. Effects of pure abamectin on germination

Analysis across species revealed significant interaction and main effects of pure ABA for all fixed factors in GP and MGT, while Z only showed significant general effects for *species(family)* and *family* (Table 2). Across species, GP was highest at  $0.1 \text{ mg l}^{-1}$  (42.2 %) differing significantly from control (35.4 %;  $C_E$ ) and the other concentration levels (38.4 and 38.5 %, 1 and  $10 \text{ mg l}^{-1}$ , respectively; Fig. 1c). Analysis on species level regarding GP revealed significant increases compared to the control ( $C_E$ ) for five species with maxima for each species at the lowest concentration level ( $0.1 \text{ mg l}^{-1}$ ; *C. jacea* +105.5 %, *L. flos-cuculi* +304.5 %, *P. lanceolata* +60.0 %, *P. media* +137.2 %, *Veronica officinalis* +587.8 %; Supplementary Table S15).

While in MGT seven species showed a significant difference compared to the control ( $C_E$ ), effects were more inconsistent than in GP (Supplementary Table S16). Across all species, MGT was highest at  $10 \text{ mg l}^{-1}$  (19.1 d), differing significantly from the  $0.1 \text{ mg l}^{-1}$  level (16.4 d), but not from control (18.3 d) or the  $1 \text{ mg l}^{-1}$  level (18.0 d) (Fig. 4). Only the  $0.1 \text{ mg l}^{-1}$  level differed significantly from control ( $C_E$ ).

For Z, no species showed significant differences between control ( $C_E$ ) and concentration levels or the levels among each other (Supplementary Table S17).



**Figure 4.** Mean germination time  $\pm$  SE across species at different concentrations of pure abamectin. Means and errors were calculated with untransformed data. Box-Cox transformed data were used to test for significance ( $p \leq 0.05$ ; Tukey HSD test). Different letters above bars indicate significant differences between concentration levels.

### 3.5. Effects of pure doramectin on germination

Analysis across species for pure DOR showed that GP was highest at the 0.1 mg l<sup>-1</sup> level (37.6 %), which differed significantly from the 1 mg l<sup>-1</sup> level (34.6 %) (Fig. 1d). By contrast, MGT and Z showed no significant differences between concentration levels for DOR across species (not shown). Analysis on species level revealed that only *P. lanceolata* (+52.0 %, 0.1 mg l<sup>-1</sup>) was significantly affected by DOR compared to control ( $C_E$ ; Supplementary Table S18). MGT was significantly affected in three species (*D. deltoides*, *A. capillaris* and *C. cristatus*) compared to control ( $C_E$ ), but effects were mostly weak and inconsistent with respect to concentration (Supplementary Table S19). In GP and Z, the analysis across species revealed a main and interaction *concentration* effect [Table 2, *species(family) × concentration* for GP, *family × concentration* for Z], but analysed on species level, no significant effects were found for Z (Supplementary Table S20).

### 3.6. Comparison of effects of pure anthelmintics

For GP, the number of species in which the control ( $C_E$ ) was significantly different from at least one concentration level of a pure ML decreases in the following order: ABA (5 species) > MOX (4) > IVM (2) > DOR (1) (Supplementary Tables S3, S9, S15, S18). *P. lanceolata* was the only species in which all four pure substances affected GP (in all cases, an increase in GP was found). The same species did not respond significantly to any pure ML in its timing variables (MGT, Z). For MGT, the following ranking in the number of species with significant differences between control ( $C_E$ ) and treatment was found: ABA (6) > IVM (4) > MOX (3), DOR (3) (Supplementary Tables S4, S10, S16, S19). Of all pure MLs used in this experiment, only DOR increased MGT across species and concentrations, while all others decreased it compared to control ( $C_E$ ). In Z, with the exception of *T. pratense* in pure IVM, no species was affected by any of the pure substances used (Supplementary Tables S5, S11, S17, S20).

## 4. Discussion

### 4.1. How general are responses of seeds of grassland species to macrocyclic lactone anthelmintics in terms of germination?

Our study is the first multi-species study which is concerned with effects of macrocyclic lactones (MLs) on seed germination and showed that, with the exception of *Medicago lupulina*, all test species were influenced in their germination behaviour by at least one ML drug. However, we found large variations in ML effects on seed germination and inconsistent patterns within and among species. We suggest two possible mechanisms in which MLs could affect seed germination. One possibility would be MLs seeping into the seed through the testa and then changing seed dormancy and germination behaviour before it becomes visible in the form of an emerged radicle. Thus, the variations we observed could be the result of differing seed coat traits, such as thickness, chemical composition and special structural elements, like micropores. In addition, the specific factors to break seed dormancy vary between species, so the seeds could be affected on different stages of development, meaning the MLs could maybe not affect all species to the same extent or on the same molecular process (Baskin & Baskin, 2004; Soltani *et al.*, 2018).

A second possibility would be that the tested MLs cannot pass the testa and first come into contact with embryo tissue after the testa has been cracked by the growing radicle. Not only did the effects of a specific ML on different species vary, effects of different MLs on the same species also varied. Since MLs are very similar in their molecular structure and biological activity, this result is surprising. Moxidectin (MOX) and the avermectins show the largest structural differences (Shoop *et al.*, 1995). Differences in molecular structure can have various effects (Prichard *et al.*, 2012), e.g. improving or hindering entrance into cells via protein pathways, impeding or enhancing export of ML molecules, which has an impact on retention time (Shoop *et al.*, 1995). Possibly, the slight differences of MLs in molecular structure impact interactions with other molecules, e.g. the interaction with P-glycoproteins, to which ivermectin (IVM) and MOX have vastly different affinities (Shoop *et al.*, 1995; Griffin *et al.*, 2005; McKellar & Gokbulut, 2012; Lloberas *et al.*, 2013). In animals, this leads to differing excretion rates, since P-glycoproteins are responsible for transporting MLs out of cells back into the intestinal lumen (Griffin *et al.*, 2005; McKellar & Gokbulut, 2012; Lloberas *et al.*, 2013).

Another possible binding site of MLs in seed embryos are glutamate-gated chloride channels, which are their site of action in invertebrates. Plant cells have similar channels, so MLs could have an effect on those (Elter *et al.*, 2007; Ward *et al.*, 2009). Also the disruption of proteins inside plant cells by ML attachment to protein binding sites might be a relevant process. Studies have shown the capabilities of IVM to bind to several viral proteins, rendering them inert and stopping reproduction, as well as binding to the transport proteins IMP  $\alpha/\beta$ 1, stopping transport into the nucleus (Caly *et al.*, 2020; Heidary & Gharebaghi, 2020; Lehrer & Rheinstein, 2020; Bello, 2021). Most likely the substance- and species-specific responses observed result from a combination of the factors mentioned before: Differences in the testa structure, the number and structure of chloride channels and P-glycoproteins and the differences in molecular structure of the MLs probably affecting their ability to pass through the testa and cell membranes and to bind to cell structures. In addition, still unknown mechanisms might play a role.

#### 4.2. Do higher concentrations of the active ingredients respectively formulations lead to stronger effects on seed germination?

In formulated MOX (Cydectin), we found the effects on seed germination, especially on germination percentage (GP) and mean germination time (MGT), to increase in strength with higher concentrations for most species, which is in accordance with previous studies (Eichberg *et al.*, 2016, 2022). Across all species, this pattern was similarly observed for formulated IVM (Oramec), but not for the four pure substances, where we found no regularly increasing or decreasing concentration-specific response patterns. On species level, in all six tested ML pharmaceuticals, non-linear and species-specific response patterns were found, which is also in accordance with previous studies (Eichberg *et al.*, 2016, 2022). We found the highest concentration level of MLs (10 mg l<sup>-1</sup>) to affect the seeds of most test species, whereas the lower concentrations levels (0.1, 1 mg l<sup>-1</sup>) showed effects less frequently and less consistent (exception: pure abamectin).

With each concentration level tested, the number of molecules increased tenfold, but the effect did not, e.g. the differences from the control in affected species were not increasing in the same level as the ML concentration did (Supplementary Tables S3-20). This might indicate a threshold to exist in at least some species, below which MLs have close to no effect, and above which they can affect germination. Studies have shown that ML overdoses can negatively affect vertebrates, because they are known to then cross the blood-brain barrier and affect neural cells (Martin *et al.*, 2021). This could be attributed to the MLs overwhelming P-glycoproteins, since dogs with genetically caused deficiencies of these proteins are known to be susceptible to IVM side effects (Mealey *et al.*, 2002; Griffin *et al.*, 2005). Since plants have homologous P-glycoproteins, we suggest that a high enough number of ML molecules would overwhelm those as well and then affect intracellular processes and therefore germination (Terasaka *et al.*, 2005; Blakeslee *et al.*, 2007). These thresholds could vary between species, even from the same family, which would also explain why the effects are so inconsistent between species. Higher concentrations of MLs could then negate any effects the lower concentrations caused. These effects occurred only in pure substances and predominantly on MGT.

Interestingly, in contrast to Eichberg *et al.* (2016), where effects of pure MOX on GP were neutral, in the current study, GP of most species was enhanced by the pure ML solutions, including MOX (Fig. 1). In general, the increase in GP may be attributed to the ML molecules leading to intracellular stress and as a response to a higher metabolic activity, as was shown by multiple studies in regards to other typically phytotoxic substances applied in small doses (Migliore *et al.*, 2000, 2003, 2010a; Christou *et al.*, 2018). This assumption is supported by studies showing that MLs, when taken up by plants, trigger stress response mechanisms (Navrátilová *et al.*, 2020b; Langhansová *et al.*, 2021).

#### 4.3. Are there differences between the pure active pharmaceutical ingredient and its formulation?

Formulated MOX affected far more species and those to a much bigger extent than any of the other five MLs tested. Since we were not able to produce controls with all other co-formulants of formulated MOX or IVM, which differ in their ingredients, we cannot rule out the possibility that the larger effect was caused by single co-formulants or their combinations. Until now, ecotoxicological research has focused strongly on the analysis of the active ingredients of agrochemical formulations (Backhaus *et al.*, 2008). However, many active ingredients of veterinary drugs are applied in formulated form. This is because these substances need to be protected from chemical degradation, e.g. by oxidation or hydrolysis, and/or facilitated by co-formulants, e.g. by improving solubility or permeability, in order to be fully effective (Awasthi *et al.*, 2013).

Still, despite their omnipresence the ecotoxicological study of co-formulants has been largely neglected and there are only a few studies on the effects of these substances on living organisms, especially on terrestrial vascular plants. The study of effects of chemical substances occurring in mixtures, e.g. formulations, was also neglected (Backhaus *et al.*, 2008; Christou *et al.*, 2018). Consequently, the scientific as well as the political community is increasingly pointing out that toxic substances in the environment occur in mixtures and not individually and that a risk assessment of agrochemical formulations is only realistic if ingredient mixtures are studied (Nagy *et al.*, 2020; Authority (EFSA), 2022).

There is evidence that synergistic or additive effects occur when mixtures rather than single substances act on plants (Christou *et al.*, 2018). One example would be the stabilization of ML molecules by co-formulants, e.g. protection from degradation by UV-light or temperature. If ML molecules in formulation remain stable for longer than their pure counterparts, they can affect seeds or processes in seeds far longer and by that to a far larger degree, which would explain the differences. It is reasonable to assume that pure MLs degraded relatively quickly in our experiment, having a half-life of around seven days under day-light conditions and moderate temperatures (Halley *et al.*, 1989a, b; Bloom & Matheson, 1993; Adler *et al.*, 2016).

In water, the degradation could be even faster, up to only 12h, according to Halley *et al.* (1993). This would reduce the contact the MLs have with the seeds to potentially far less than the formulated MLs could have, assuming co-formulants stabilize the active ingredients in these conditions.

The solubility-enhancing effect of some co-formulants on lipophilic MLs in aqueous formulations (Shoop *et al.*, 1995) could allow MLs to seep into seeds, which might not have been possible without assistance. This would explain why some species were affected only by formulations, but not by pure MLs. Furthermore, there is also evidence that co-formulants can have an independent toxicity.

For instance, for benzyl alcohol (a solvating and conservative substance) and butylated hydroxytoluene (an antioxidant) which are both particularly frequent and abundant in Cydectin (Supplementary Table S1), it has been reported that they directly affect seed germination (Taylorson, 1988; Zamyatnina *et al.*, 2003; Tang *et al.*, 2013; Lu *et al.*, 2013; Zhao *et al.*, 2019).

Similar to co-formulants, ethanol, which was used as a solubilizer to dissolve the lipophilic pure MLs in water in the present study, affected some test species. GP tended to be reduced and MGT increased in the C<sub>E</sub> compared to the blank control (C<sub>W</sub>). This is not a surprising finding, since species-specific effects of ethanol on germination were already shown. While at concentrations similar to the ethanol concentration used in the present study (0.25 %) no effects on germination percentage were found in previous studies (Salehi *et al.*, 2008; Foley & Chao, 2008; Eichberg *et al.*, 2016), effect size increased significantly with ethanol concentration ( $\geq 0.60$  %) (Salehi *et al.*, 2008; Foley & Chao, 2008; Afzal *et al.*, 2013). Still, we deem this alcohol effect on germination not to question the general validity of our findings, since the controls, i.e. C<sub>E</sub> and C<sub>W</sub>, were systematically compared only to the pure and formulated macrocyclic lactones, respectively. In general, from a methodological point of view it remains unsolved which might be the optimal solubilizer for non-polar chemicals in germination experiments.

#### 4.4. Ecological relevance

While the ecological consequences of decreased GP are rather straight forward, i.e. lower germination directly translates into lower seedling establishment and subsequently lower number of adult plants (Grubb, 1977), changes on MGT and synchrony (Z) might be more ambivalent. In general, seeds of a certain species usually germinate when environmental conditions are ideal; thus, both accelerated and delayed germination can have negative effects on post-germination fate. While the two ML formulations used in our experiment led to increased MGT, pure MLs generally decreased MGT (with the exception of doramectin). A decreased MGT could cause the plant to germinate and grow before the optimal climatic conditions, like a longer rainy period. Conversely, an increase in MGT is usually detrimental to growth, because the plant is then substantially disadvantaged in the race for resources, particularly sunlight and water (Ross & Harper, 1972; Dyer *et al.*, 2000). This can cause a significant reduction of fitness and plant biomass (Rühl *et al.*, 2016a).

Similarly, while a less synchronous germination, i.e. lower Z, indicates a spread of germination into periods potentially less suitable, a more synchronous germination, i.e. higher Z, incapacitates spread of germination over a certain time period as a bet-hedging strategy against environmental stochasticity (Ludewig *et al.*, 2014).

Studies show that, depending on substance and application, MLs are excreted by livestock for weeks or even months in measureable amounts (Chiu *et al.*, 1990; Kolar *et al.*, 2006; Fernandez *et al.*, 2009; Vokřál *et al.*, 2019) and therefore could influence plant germination for a long time throughout the year. Reports suggest IVM persistence in soil varies between 7 and 240 days in extremes (Halley *et al.*, 1993; Alvinerie *et al.*, 1999; Mougin *et al.*, 2003). These timeframes are highly dependent on soil composition and temperature; Bloom and Matheson (1993) measuring the half-life in winter conditions to up to 52 weeks.

Within this timeframe, it is reasonable to assume that animals would undergo a second anthelmintic treatment, leading to the accumulating of MLs in soil over time (Wohde *et al.*, 2016). Accumulation of MLs on or in pastures would then become a serious problem for fauna and flora alike, as our study has shown the effects these substances can have on multiple germination factors.

Although, the mode of action of MLs in plants and the ecological significance of our findings need to be further explored in future studies, our results support the notion that, in general, the germination of most species is affected by MLs. Despite the variability in sign and size of the response of the addressed germination parameters, ecological relevance is very likely since MLs are excreted into the environment largely unchanged and in comparatively large amounts after administration to livestock (Suárez *et al.*, 2009; Beynon, 2012). In addition, large numbers of seeds potentially come into contact with MLs (and co-formulants) in the course of phytomass consumption and passage of eaten seeds through the gastro-intestinal tract of freshly dewormed sheep or other herbivores (Rosenthal *et al.*, 2012). In the long run, effects on the biodiversity of larger areas might occur, especially when plant species rely on endozoochory to spread and bridge larger distances (Albert *et al.*, 2015a,b) and plant populations are exposed to ML influences over a longer period of time (Mougin *et al.*, 2003).

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## Chapter 2

### Dose-dependent in vivo effects of formulated moxidectin on seedling emergence of temperate grassland species

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Lars Laber, Anabell Jandowsky, Kai Frölich, Andre P. Heinrich,  
Rolf-Alexander Düring, Tobias W. Donath & Carsten Eichberg



## Abstract

Sheep function as effective endozoochorous seed vectors in grasslands. Recent laboratory-based studies showed that this important function can be impaired by anthelmintics, which are used to control parasites and enter into the environment via faeces; however, there is a lack of *in vivo* studies. We conducted a seed-feeding experiment with sheep that included four temperate grassland species. A series of three feeding trials was carried out after one of two groups of sheep received a single administration of a common moxidectin-based anthelmintic formulation. Faeces were collected to determine seedling emergence rate and emergence timing as well as moxidectin concentration via HPLC. Seedling emergence differed significantly between the anthelmintic-treated sheep and the control group. This impact depended on time of seed uptake after anthelmintic administration. Number of emerging seedlings was significantly reduced (27.1 %) when faeces moxidectin concentrations were high (on average 3,153 ng g<sup>-1</sup>; 1 d post treatment) and significantly increased (up to 68.8 %) when moxidectin concentrations were low ( $\leq 86$  ng g<sup>-1</sup>; 7, 14 d pt). Mean emergence time was significantly lowered at low moxidectin concentrations. These results demonstrate dose-related effects of deworming on seedling emergence which might affect endozoochory and eventually plant population dynamics in grasslands.

## 1. Introduction

Endo- and ectoparasites such as nematodes and mites are a major factor for illness and poor productivity of livestock (Sutherland & Scott, 2010). To control these pests and guarantee animal welfare, anthelmintic macrocyclic lactones (MLs), such as moxidectin (MOX), are used on a large scale worldwide (Lanusse *et al.*, 1997; Prichard *et al.*, 2012). They kill internal and external invertebrate parasites of target animals by affecting invertebrate-specific chloride channels localised in membranes of nerve and muscle cells (Lumaret *et al.*, 2012; Adler *et al.*, 2016). MLs enter the environment mainly via the faeces of the treated animals and effect non-target organisms such as coprophagous beetles and flies, with detrimental impact on ecosystem services, such as dung removal and nutrient cycling (Afzal *et al.*, 1994; Gokbulut *et al.*, 2001; Kolar *et al.*, 2006; Suárez *et al.*, 2009; Baena-Díaz *et al.*, 2018; Ambrožová *et al.*, 2021). While the detrimental effects on arthropods are demonstrated in a large body of publications (reviewed in Floate *et al.*, 2005; Lumaret *et al.*, 2012; Junco *et al.*, 2021), the impact on other non-target organisms, such as plants, have only recently drawn attention (Bártíková *et al.*, 2016; Eichberg *et al.*, 2016, 2022).

In the life cycle of plants, successful germination and seedling establishment are decisive stages for later growth and reproduction success; thus, any changes in these stages might have significant effects on persistence of plant populations (Grubb, 1977; Kitajima & Fenner, 2000). In this context, an increase in the number of emergent seedlings, as can be caused by a range of environmental factors, almost always indicates higher fitness. In contrast, environmental-related changes in temporal response of a seed cohort, measured as mean time of emergence or synchrony of emergence, are not monotonically linked to recruitment success and the benefits of a changed emergence time (i.e., lower or higher emergence time) or altered temporal spread of emergence (i.e., higher or lower synchrony) may vary with environmental context.

The few studies that addressed effects of MLs on seed germination or seedling development are under laboratory conditions and predominantly found suppressing, but also some stimulating effects, depending on plant species, ML drug and concentration of ML solution (Chapter 1; Eichberg *et al.* 2016, 2022; Vokřál *et al.* 2019). Among tested MLs, formulated MOX appeared to have the strongest effects on seeds and generally led to reductions in germination percentage, increases in mean germination time and reductions in synchrony of germination (Eichberg *et al.*, 2016). To our knowledge, only one study has so far tested ML impacts on seeds *in vivo*, conducting a sheep-feeding experiment that included *Plantago lanceolata* seeds, a key grassland species, and formulated MOX (Eichberg *et al.*, 2016). This study found a strong reduction in the number of seedlings emerging out of sheep faeces. However, in this initial experiment, concentrations of the active ingredient in faeces were not determined and only one time point of seed uptake after deworming was tested. Results of a multi-species laboratory experiment showed that pure MLs enhance germination rates across species compared to controls, including low concentrations (0.1 mg l<sup>-1</sup>; Chapter 1).

At the species level, both increasing and decreasing effects along a series of ML concentrations have been observed in this experiment (Chapter 1). In concert, the initial results on ML effects on seed germination suggest that MLs may also exert non-linear effects, which are commonly occurring in living organisms (Coenders & González, 2014). A particularly widespread non-linear relationship in living organisms is the hormetic relationship which is given when a chemical substance induces suppressing effects to an organism at higher concentrations, but stimulating effects at lower concentrations (Mattson & Calabrese, 2010; Agathokleous *et al.*, 2018).

There is a pronounced time course of faecal ML elimination (Prichard *et al.*, 2012) indicating that seeds eaten are prone to varying ML concentrations depending on the time point of ingestion after deworming. After subcutaneous administration of a single standard dose ( $0.2 \text{ mg kg}^{-1}$  bodyweight) of a ML drug, peak residue levels occurred 2–3 days post treatment in sheep faeces and reached values in the  $\text{ng g}^{-1}$  to  $\mu\text{g g}^{-1}$  [dw] range (Kolar *et al.*, 2006; Vokřál *et al.*, 2019; Hentz *et al.*, 2019). In a study of Hentz *et al.* (2019), MOX excretion of subcutaneously-dosed lambs continues in the low  $\text{ng g}^{-1}$  range over the whole measurement period of 42 days. Generally, MOX shows much longer elimination half-life in ungulate tissues than the closely related ivermectin. For instance, in blood plasma of orally treated sheep, Molento *et al.* (2004) found a 7-fold higher elimination half-life in MOX (15.4 d) than ivermectin (2.1 d). MLs are lipophilic ( $\log K_{OW}$  MOX: 4.8; (Mooney *et al.*, 2021) and oscillate between fat tissues and the lumen of the gastro-intestinal tract that explains their long-lasting efficacy (Coop *et al.*, 2002). The particularly high persistence of MOX activity in host plasma and tissues is related to its higher *in vivo* stability and lower affinity to P-glycoprotein efflux transporters as compared to other MLs (Cobb & Boeckh, 2009; Prichard *et al.*, 2012; Ballent *et al.*, 2013). In contrast to MOX, its main co-formulants in oral formulations (e.g. benzyl alcohol, butylated hydroxytoluene) do not accumulate in tissues and are rapidly metabolised in the digestive tract of mammals and the major fraction of metabolites is excreted via urine within hours (Witschi *et al.*, 1989; Johnson *et al.*, 2017). Because of the very different residence times of MOX and its co-formulants in the host body, MOX probably plays the most important influencing role in the interaction of seeds with orally applied MOX formulations.

Here, we focus on sheep because of their unique role in maintaining or generating a high phytodiversity in grasslands, if kept in large migratory flocks (Plachter & Hampicke, 2010; Rosenthal *et al.*, 2012). Especially responsible for this is their potential to foster long-distance seed dispersal and post-dispersal establishment success. It has been shown that roaming sheep flocks can transport viable seeds of >70 species via their faeces (process of endozoochory; Kuiters & Huiskes, 2010) and functionally connect plant populations of fragmented grasslands via seed dispersal (Honnay *et al.*, 2006; Wagner *et al.*, 2013; Rico *et al.*, 2014). Grassland fragmentation is widespread in the European landscape and coupled with isolation of plant populations due to low rates of seed exchange (Auffret *et al.*, 2017). Since grassland seeds can come into contact with MLs in the digestive tract of recently dewormed domestic ungulates, endozoochory is potentially impaired by ML use.

Against this background, we set up a seed-feeding experiment with sheep that includes four endozoochorously dispersed plant species native to temperate grasslands in central Europe and a series of three seed-feeding trials after a single oral administration of formulated MOX and aimed at answering the following questions:

- Does formulated moxidectin administered orally to sheep affect seedling emergence of ingested seeds?
  - We expected that the anthelmintic drug tested leads to a decrease of seedling emergence, an increase in mean emergence time and a lower synchrony of emergence of ingested seeds.
- Do the effects of formulated moxidectin depend on when the seeds are ingested by the treated animal after deworming?
  - We expected a clear impact of timing of seed ingestion on the tested emergence parameters, which coincides with concentration of moxidectin in sheep faeces.

## 2. Materials and methods

### 2.1. Test species and seed material

We tested seedling emergence of four species with perennial life strategies belonging to four plant families: *Achillea ptarmica* (Asteraceae), *Agrostis capillaris* (Poaceae), *Dianthus deltoides* (Caryophyllaceae) and *Plantago lanceolata* (Plantaginaceae). Their seeds have shown sensitivity to macrocyclic lactone (ML) anthelmintics in previous experiments (Chapter 1; Eichberg *et al.* 2016, 2022). While in *A. capillaris*, *D. deltoides* and *P. lanceolata* the survival of the passage through the gastrointestinal tract of sheep is well documented (Kuiters & Huiskes, 2010; Benthien *et al.*, 2016), in case of *A. ptarmica* survival of the passage can be assumed from findings of the close relative *Achillea millefolium* (Kuiters & Huiskes, 2010). Seeds were purchased from Rieger-Hofmann GmbH, Blaufelden, Germany.

### 2.2. Test substance

We tested the anthelmintic formulation Cydectin® 0.1 % oral drench for the application in sheep (Zoetis Deutschland GmbH, Berlin, Germany; ingredient list: see Supplementary Table S1). The active ingredient of Cydectin is moxidectin (MOX), a globally available broad-spectrum anthelmintic from the milbemycin sub-class of MLs (Cobb & Boeckh, 2009). As in other MLs, the mode of action of MOX is binding to glutamate-gated chloride channels and thereby triggering an irreversible increase of membrane conductance that leads to paralysis and death of parasites, such as nematodes (Shoop *et al.*, 1995; Prichard *et al.*, 2012). Since vertebrates do not possess such type of channels, they are insensitive to this mode of action (Cotureau *et al.*, 2003).

### 2.3. Experimental design

To test the effects of formulated MOX on plant regeneration by seed under *in vivo* conditions, a sheep feeding experiment was carried out comprising two animal groups and four plant species. The two sheep groups received one of either treatment, 1. A single administration of the anthelmintic drug (treatment group) or 2. No deworming treatment (control group). Then, in a series of trials, sheep of both groups were fed with a mix of seeds of the four study species a day, a week and two weeks after administration of the anthelmintics. Faeces were collected for two days after each feeding trial.

Each animal group consisted of eleven sheep, which were provided by the animal park Arche Warder (Warder, Schleswig-Holstein, Germany). All animals in our study were healthy, adult males of different land sheep breeds. Within the same breed, the experimental animals had roughly the same age and bodyweight and both tested sheep groups comprised the same number of animals per breed. Sheep groups were kept separated in two areas of a stable for the whole duration of the experiment. To minimise seed contamination, all sheep were brought from a pasture into the stable five days before first faeces sampling. For the whole experimental phase, sheep were provided food (hay and common sheep pellet food) and water *ad libitum*.

On June 7<sup>th</sup> 2021, Cydectin oral drench was administered to the animals of the treatment group in accordance to the regular treatment cycles and dosed as recommended by the producer (0.2 mg MOX per kg bodyweight). Following the deworming of the treatment group, in a series of three trials, mixtures containing 15,000 seeds of each of the four test species were individually administered to the experimental animals of both sheep groups. The trials were conducted one day (June 8<sup>th</sup> 2021), seven days (June 14<sup>th</sup> 2021) and 14 days (June 21<sup>st</sup> 2021), respectively, after drug administration by means of opened syringes to ensure complete consumption of freshly moistened seed mixtures. At three collection times (24, 30 and 48 h) after each feeding trial, faeces samples were taken directly from the rectum of each individual animal of both groups by a veterinarian. Animal treatment was in accordance with the German Animal Welfare Legislation Act (TierSchG, TierSchVersV). The present feeding experiment did not need approval by an ethical committee or by the government since this type of investigations did not induce pain, suffering or harm to the animals.

Each faeces sample was weighed. For later chemical analysis, we separated approximately 5 g faeces (fresh weight) from each of the two largest faeces samples per collection time and froze (-32 °C) these sub-samples immediately. The remaining material of each faeces sample was mixed with seed-free potting soil in a 1:1 ratio, and spread as a 0.3 cm thin layer on top of a 4 cm thick layer of potting soil in Styrofoam boxes (29.0 cm x 19.5 cm x 5.5 cm). The prepared boxes were exposed outdoors on a large, open-spaced area completely paved with flagstones at Kiel University (Bremerskamp 133, 24118, Kiel, Germany). They were placed in randomized groups directly on the flagstones and covered with exclusion cages.

Constructions consisted of a wooden frame with a hinged lid, which was covered with a layer of gauze to ensure sufficient sunlight exposure and ventilation as well as protection against airborne seeds. In boxes with soil only (contamination control), no seedlings of the test species emerged. Emerging seedlings [appearance of cotyledons (dicots) or first leaf (*A. capillaris*)] were identified, counted and removed twice a week. All boxes were watered regularly to keep the samples continuously moist. The experiment was terminated on November 1<sup>st</sup> 2021, when no seeds had emerged for more than two weeks resulting in a total sample exposure time of five months.

#### 2.4. Determination of moxidectin residues in sheep faeces

From the sheep faeces sub-samples described above, 3–4 g (fresh weight) were used to extract MOX residues. The extraction procedure and MOX quantification were based on a protocol by Wohde et al. (2016). To determine faeces water content, homogenized aliquots of each sample were dried for 24 h at 105 °C. Mean  $\pm$  SD water content was  $75.1 \pm 2.1$  % ( $n = 19$ ). Before extraction, fresh samples were fortified with an internal surrogate standard (ivermectin, LGC-Standards, DRE-CA14488000) and mean  $\pm$  SD ivermectin recovery was  $94.0 \pm 7.8$  % ( $n = 22$ ). A stock solution with 2000 ng ml<sup>-1</sup> ivermectin and MOX (Sigma-Aldrich, PHR1827) was prepared in acetonitrile (gradient grade for HPLC, 20060.320, VWR International) and diluted to the following calibration series: 1, 5, 10, 50, 100, and 2000  $\mu\text{g l}^{-1}$ . To facilitate moxidectin extraction, fresh samples were fortified with purified water in a 15 ml polypropylene vial to increase the water content to 85–89 %. Samples were left to sit overnight, before the internal standard was spiked. For extraction, 25 ml acetonitrile were added. Before each following step, vials were manually shaken for 5 s. Vials were then sonicated for 20 min, horizontally shaken (350 rpm) for 40 min, and sonicated for a further 20 min. After this, phases were separated by centrifugation at 1970  $g$  for 30 min at 20 °C. For clean-up and concentration, a solid phase extraction step was performed as reported by Nieman et al. (2018); the routine for quantification with fluorescence detection on an Agilent 1200 HPLC system was also adopted from this protocol. Derivatization before quantification was performed as described by Wohde et al. (2016). Excitation wavelength of the fluorescence detector was 364 nm and the emission wavelength 463 nm. Column temperature was 30 °C on a Dionex Acclaim PolarAdvantage II C18-Column.

#### 2.5. Data analysis

For each replicate, we calculated final number of emerged seedlings standardised to 1 g faeces [fw] (NES), mean emergence time (MET; days) and synchrony of emergence (Z; dimensionless) as dependent variables. MET and Z were calculated as proposed by Ranal et al. (2009) with regard to the process of seed germination. The three collection times after each trial were merged. To standardise the results across plant species, which varied in germination capacity and in order to meet the assumptions of the applied inferential statistics, we calculated the natural-logarithm response ratio (LnRR) for all dependent variables. This is defined as  $\text{LnRR} = \ln (x_{\text{trt}} / \bar{x}_c)$ , where  $x_{\text{trt}}$  is the value of treated seeds and  $\bar{x}_c$  is the mean value of the control.

An effect of an independent variable was considered significant, when the 95 % confidence interval did not overlap with zero (Koorem *et al.*, 2011).

The overall effects of the between-subject factor (species identity) and the within-subject factor (trial) on NES, MET and Z were subjected to repeated measurement analyses. These consisted of a factorial ANOVA for the between-subject factors and a MANOVA based on Wilks lambda for the within-subject factor since the assumptions of ‘circularity’ and ‘compound symmetry’ were not fulfilled (Ende, 1998). This procedure was employed across species including the independent variables plant species ( $k = 4$ ; *A. ptarmica*, *A. capillaris*, *D. deltoides*, *P. lanceolata*) and seed-feeding trial ( $k = 3$ ; 1, 7, 14 d) for the three dependent variables (NES, MET, Z). On species level, differences between trials per species were calculated by means of Tukey HSD test.

Statistical analysis was conducted using Statistica™ 13.3 (StatSoft Inc., Tulsa, Oklahoma, USA) and graphs were created using ORIGIN® (ORIGINLab Corporation, Northampton, Massachusetts, USA).

### 3. Results

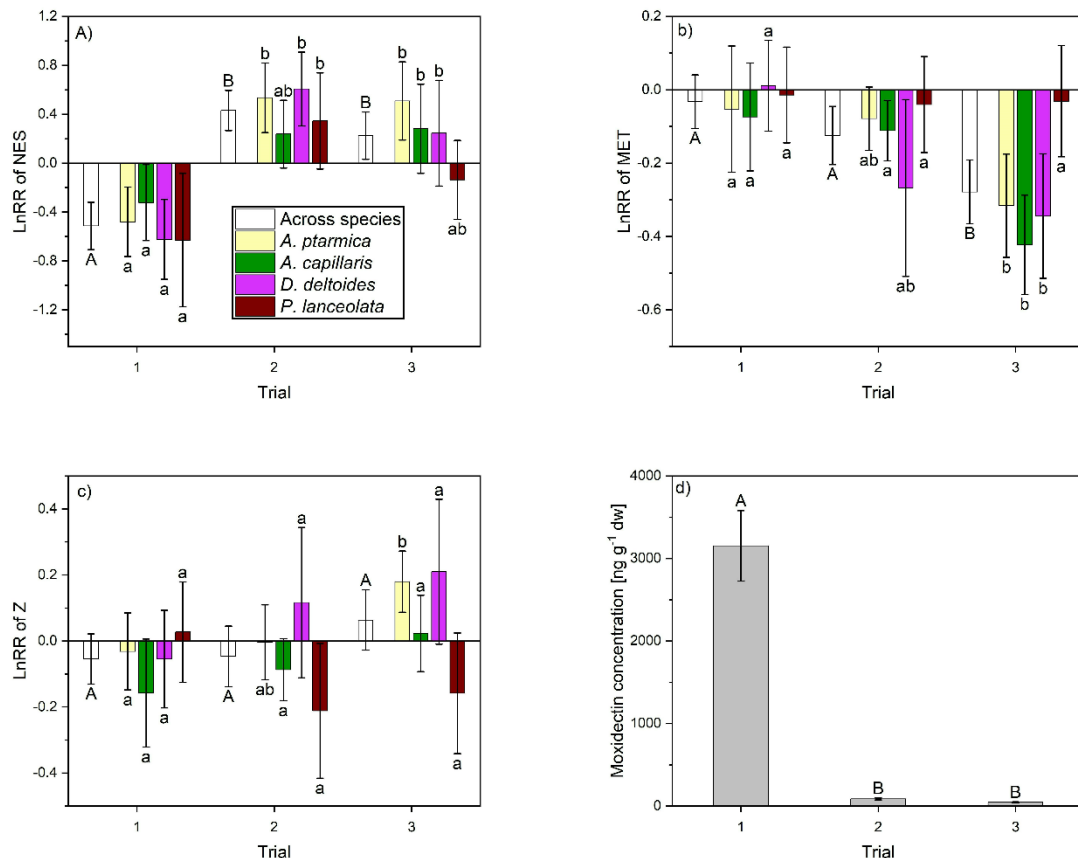
Analysis across species revealed significant main effects of trial on all response variables (NES, MET, Z; Table 1). Only in Z, analysis revealed additionally a significant main effect of species as well as a significant species  $\times$  trial interaction (Table 1).

**Table 1.** Effects of between-subject (BS) and within-subject (WS) factors on natural-logarithm response ratio of seedling emergence (NES), mean emergence time (MET) and synchrony of emergence (Z).

				NES			MET			Z		
BS	Source of variation	DF		MQ	F	p	MQ	F	p	MQ	F	p
	Species (S)	3		0.62	0.83	0.484	0.22	2.39	0.083	<b>0.31</b>	<b>3.97</b>	<b>0.014</b>
	Error	40		0.74			0.09			0.08		
WS	Source of variation	DF <sub>num</sub>	DF <sub>den</sub>	Wilks	F	p	Wilks	F	p	Wilks	F	p
	Trial (T)	2	39	<b>0.32</b>	<b>42.36</b>	<b>≤ 0.001</b>	<b>0.62</b>	<b>11.82</b>	<b>≤ 0.001</b>	<b>0.85</b>	<b>3.39</b>	<b>0.044</b>
	S × T	6	78	0.80	1.55	0.173	0.75	2.02	0.073	<b>0.72</b>	<b>2.28</b>	<b>0.044</b>

Between-subject effects were determined by ANOVA, while within-subject effects were determined by MANOVA. DF: degrees of freedom, DF<sub>num</sub>: degrees of freedom numerator, DF<sub>den</sub>: degrees of freedom denominator, Wilks = Wilks  $\lambda$ , MQ = mean sum of squares, F = F value, p = p value. Significant results ( $p \leq 0.05$ ) are shown in bold.

Across species, NES of treated sheep differed significantly from the control in all three trials (Fig. 1a). Interestingly, there was a change in direction of the effect between trials, i.e. while NES was lowered by 27.1 % compared to the control at trial 1, it was increased by 68.8 % and 45.2 % compared to the control in trial 2 and trial 3, respectively (Fig. 1a, Suppl. Table S2). This general pattern of change in effect direction also held true at the species level with two exceptions: NES of *Agrostis capillaris* (trial 2) and *Plantago lanceolata* (trial 3) were not significantly different from NES in trial 1. In addition, only in *Achillea ptarmica* and *Dianthus deltoides* NES was significantly different from the control in trial 2 and 3 or only in trial 2, respectively (Fig. 1a).



**Fig. 1** Mean ( $\pm$  95 % confidence interval) natural-logarithm response ratios (LnRR) of (a) number of emerged seedlings (NES), (b) mean emergence time (MET) and (c) synchrony of emergence (Z) for the three consecutive seed-feeding trials carried out 1, 7 and 14 d, respectively, after the experimental sheep have been treated with the anthelmintic formulation Cydectin (moxidectin 0.1 %). Effect of formulated moxidectin was considered significant, i.e. different from the control, when the 95 % confidence interval did not overlap with zero. Different letters indicate significant differences between trials on across-species level (capitals) and on species level (lower case), respectively ( $p \leq 0.05$ , Tukey HSD test). (d) Mean ( $\pm$  SE) moxidectin concentrations in sheep faeces of the three trials ( $n = 6$  per trial). Different letters indicate significant differences between trials ( $p \leq 0.05$ , Tukey HSD test).

With respect to MET, there were no significant differences of treated and non-treated sheep in trial 1, both across species and on species level (Fig. 1b). In trial 2, MET was significantly lowered across species (-1.7 d/8.2 %), but only the response of *A. capillaris* (-2.4 d/10.7 %) and *D. deltoidea* (-2.9 d/20.4 %) showed a congruent significant pattern (Suppl. Table S3). In contrast to NES, no significant differences occurred between trial 1 and 2. This changed in trial 3, where MET across species was significantly lowered by 22.0 % (3.6 d) compared to the control, which was caused by a significant reduction of MET in all species, except *P. lanceolata*. While the latter was also the only species with no significant different MET between trials, the other species had significantly lower MET in trial 3 compared to trial 1, but only in *A. capillaris* MET was also significantly lowered in trial 3 compared to trial 2 (Fig. 1b).

Z showed a less clear pattern than NES and MET due to high variation within and between species. In the across-species analysis, there was no significant difference between the treatment and the control at any trial, and the trials did not differ from one another (Fig. 1c; Suppl. Table S4).

Only in two cases, species  $\times$  trial combinations differed significantly from the control, i.e. *P. lanceolata* showed a significant decrease of Z (-7.2 %) in trial 2 and *A. ptarmica* a significant increase of Z (+17.5 %) in trial 3. And in only one case was there a significant difference between trials at the species level, i.e. in *A. ptarmica*, Z was significantly higher in trial 3 than trial 1 (Fig. 1c; Appendix A, Table A.4).

Mean  $\pm$  SE MOX concentration in sheep faeces decreased strongly over time with significant difference between trial 1 ( $3,153.2 \pm 417.5 \text{ ng g}^{-1} [\text{dw}]$ ) and trial 2 ( $86.4 \pm 10.8 \text{ ng g}^{-1} [\text{dw}]$ ), but no further significant decrease from trial 2 to trial 3 ( $45.5 \pm 4.9 \text{ ng g}^{-1} [\text{dw}]$ , Fig. 1d). From trial 1 to trial 3, concentration of MOX dropped by 99.0 %. In faeces from the control group, no MOX was detected ( $0.0 \pm 0.0 \text{ ng g}^{-1} [\text{dw}]$ ).

## 4. Discussion

### 4.1. Formulated moxidectin administered orally affected seedling emergence

In our *in vivo* experiment, we found a clear impact of formulated moxidectin (MOX) on the quantity and timing of seedlings that emerged from seeds ingested by sheep and released viable embedded in faeces substrate. This means, that individual or all components of the tested anthelmintic formulation interacted with the seeds in the sheep digestive tract and/or with the emergent seedlings in the faeces substrate. On its way through a mammalian digestive tract, pharmaceutical mixtures are de-formulated, i.e. they are broken down into its components and these components are metabolised to varying degrees (Backhaus, 2016). Thus, only those seeds that have been eaten by treated animals immediately before or after deworming are exposed to the original formulation. Seeds eaten later will be exposed to diverse mixtures of residues. As stated before, we expect the active ingredient MOX to play the central role in causing the observed effects, because it is less readily metabolised in mammalian digestive tracts than its co-formulants (Witschi *et al.*, 1989; Molento *et al.*, 2004; Johnson *et al.*, 2017) and mainly excreted via the faeces pathway (Afzal *et al.*, 1994).

Ecologically, in our experiment we focused on the endozoochorous seed transport including the first stage of seedling recruitment. This multi-step section of the life cycle of seed plants includes the passage of a seed through the digestive tract of an animal, its excretion together with faeces substrate, and its germination and seedling emergence out of faeces. At each step, macrocyclic lactone (ML) drugs may affect seed and seedling fate. There are different potential processes how MLs might affect seeds and/or seedlings. If the seed coat is penetrable for MLs, which to our knowledge has not yet been tested, embryos could be affected by MOX already at the quiescent or dormant seed stage in the chyme and/or faeces substrate.

As eaten seeds remain in the digestive tract of sheep for approximately two days before they are excreted (Cosyns *et al.*, 2005), the uptake of water (and chemical substances dissolved in it), the first step in the germination process, already starts during passage of the digestive tract (Baskin & Baskin, 2014).

However, after being ingested by a recently dewormed sheep, seeds are not only influenced by anthelmintic substances but in the course of ruminating also regularly and repeatedly by physical (mastication) and chemical (stomach acid, digestive enzymes) factors (Manzano *et al.*, 2005; Ramos *et al.*, 2006; D'hondt & Hoffmann, 2011; Mancilla-Leytón *et al.*, 2011). These factors could potentially scarify the seed coat so that MOX would more readily reach the embryo.

Once contact has been made with the quiescent, dormant or germinating embryo, MOX could affect chloride channels in cells, leading to a change in membrane conductance (McKellar & Gokbulut, 2012). Even though this mode of action has been proven in animal cells, it is theoretically also conceivable in plant cells because they have homologous channel genes (Elter *et al.*, 2007; Ward *et al.*, 2009). Another possibility might be the hindrance of protein synthesis, where MOX molecules could dock on multiple active sites of protein-substructures/subunits, preventing them from forming correctly and therefore functioning correctly or at all. This process was reported for the ML ivermectin and viral proteins, which were then not able to connect with each other thus stopping viral reproduction (Lehrer & Rheinstein, 2020; Bello, 2021).

#### 4.2. Effects of formulated moxidectin depended on timing of seed ingestion

In line with our expectations, the feeding experiment revealed a strong time-dependency of effects of formulated MOX on ingested seeds. We found a hormetic-like response pattern for the quantity of emergence and for the average time of emergence, but not for the distribution of emergence over time, i.e. synchrony, which showed no general pattern.

Related to the number of emerging seedlings (NES), only the first seed-application trial showed a lowering effect coinciding with high MOX concentrations in the faeces, while the other two trials showed enhancing effects that coincide with low MOX concentrations. On the species level, this general pattern holds true for two test species (*Achillea ptarmica*, *Dianthus deltoides*). However, in all test species, NES was significantly reduced in trial 1 compared to the control and all species showed at least trends of enhancements of NES in later trials. The result for trial 1 is comparable to and consistent with a previous feeding experiment, where sheep received formulated moxidectin immediately after administration of *Plantago lanceolata* seeds (Eichberg *et al.*, 2016). Although mean emergence time (MET) was not affected in the first trial, the significant reduction of MET in the second and/or third trial in three species, corroborated the patterns found in NES, which again indicated a stimulating effect on emergence at low MOX concentrations.

An explanation for the widespread phenomenon of stimulation in the lower concentration range of a substance is upregulation of adaptive cellular stress response pathways (ACSRPs; Mattson & Cheng, 2006; Mattson, 2008). These enhance the expression of genes encoding cytoprotective proteins and growth factors when specific receptors in the cell membrane are triggered (Mattson, 2008; Mattson & Calabrese, 2010). Receptors could be triggered by the effects of reactive oxygen species (ROS; Mattson & Calabrese, 2010) on the cell.

ROS are present in higher concentrations in plants when in prolonged contact with MLs, such as ivermectin (Navrátilová *et al.*, 2020). In plant species, hormetic effects are well known and have been described for several plant parts, including seeds, with research on organic environmental contaminants so far focusing mainly on herbicides (Cedergreen, 2008; Belz *et al.*, 2011; Belz & Duke, 2014) and antibiotics (Migliore *et al.*, 2010b; Xie *et al.*, 2011; Pan & Chu, 2016; Agathokleous *et al.*, 2018). To our knowledge, the present study is the first to find evidence to suggest hormetic effects of anthelmintics on non-target organisms, i.e. plants.

Even when low MOX concentrations were present in the faeces analysed here (14 d after deworming), all emergence variables were affected at least in some species. This finding is in line with other studies that have highlighted the importance of low concentrations of organic environmental contaminants in affecting wild plant populations (Minden *et al.*, 2017). Reported values for ML contents in faeces cover a broad range with the highest reported value for MOX concentration after oral administration to sheep being 3390 ng g<sup>-1</sup> 1 d pt (Steel, 1998), which is in line with our measurements. The route of administration greatly influences the amount of ML excreted and the time period an ML is excreted in relevant/detectable amounts (Suárez *et al.*, 2009). After a single subcutaneous MOX administration to sheep, Hentz *et al.* (2019) found a maximum MOX concentration of 22.7 ng g<sup>-1</sup> in dry faeces at 1.7 d post treatment which is lower than the minimum of 45.5 ng g<sup>-1</sup> that we measured in the present study 14 days after oral MOX administration in sheep faeces.

#### 4.3. Formulated moxidectin has larger ecological relevance than previously assumed

Our study shows that the treatment of sheep with anthelmintics can change emergence patterns of seedlings. Since sheep are important seed dispersers in fragmented landscapes (Eichberg *et al.*, 2007; Kuiters & Huiskes, 2010; Albert *et al.*, 2015a), this impact might have consequences for assembly and diversity of grazed plant communities. Sheep influence not only the spatial distribution of seeds but also their post-dispersal fate (Rosenthal *et al.*, 2012). Without the influence of MLs, emergence of seedlings from sheep faeces is enhanced by sheep trampling because cracking of faeces pellets and mixing with soil material promotes seedling recruitment (Faust *et al.*, 2011). However, trampling on ML-contaminated faeces might lead to a quicker and more complete incorporation of MLs into the soil, potentially harming even seeds in the soil seed bank.

We conclude that the risk MLs pose to grazed habitats concerns not only coprophilous arthropods (Lumaret *et al.*, 2012) but also vascular plants. Also, due to their long elimination half-lives, not only single habitats but habitat networks are affected (i.e., metapopulations), if dewormed sheep are kept in large roaming flocks.

Changes in number and timing of seedling emergence can have opposite effects on a plant population. While more seedlings emerging earlier might give species an interspecific competitive advantage leading to a higher abundance of a species in a plant community, higher seedling densities might induce increased seedling mortality due to higher intraspecific competition (Fenner & Thompson, 2005).

Several studies showed that a reduced emergence time fosters plant growth faster and flower production per plant (Verdú & Traveset, 2005; Luis *et al.*, 2008; Orrock & Christopher, 2010). Still, in the long run, stimulation of emergence through changes in the seed's environment may lead to a depletion of the soil seed bank that species developed as a bet-hedging strategy against short- and long-term environmental stochasticities (Hölzel & Otte, 2004), which are particularly given with grazing (Fenner & Thompson, 2005).

We still know too little about the effects of MLs on seeds and need more research in this field. But it is not too early to recommend that domestic ungulates freshly treated with ML anthelmintics should not be placed on pasture grassland to avoid unwanted side effects on plant populations. This is in line with risk avoidance recommendations for animal non-target organisms, such as dung beetles, that have been given for some time (Floate *et al.*, 2005).

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## Chapter 3

# The effect of formulated moxidectin on the growth and vigour of temperate grassland species

Manuscript in preparation

Lars Laber, Carsten Eichberg, Johannes Junck, Rolf-Alexander Düring &  
Tobias W. Donath



## Abstract

Macrocyclic lactone anthelmintics are used to rid livestock of pests like nematodes and mites. When given as an oral solution, large amounts of its parent drug moxidectin are excreted with faeces. These residues can either contaminate pastures and meadows via direct contact or when spread as manure. This study explores the effects of prolonged contact of growing plants with moxidectin residues. To this end, we added formulated moxidectin in three concentrations (0.1; 1; 5  $\mu\text{g g}^{-1}$ ) concentrations to pots containing one of four grassland species (*Achillea ptarmica*, Asteraceae; *Agrostis capillaris*, Poaceae; *Dianthus deltoides*, Caryophyllaceae; *Plantago lanceolata*, Plantaginaceae) common in central Europe. As an additional stress factor in grasslands, we added a grass (*Poa pratensis*) as a competitor to half of our pots. We measured vigour parameters such as aboveground plant biomass, specific leaf area and intact leaf area to determine which effects moxidectin contamination of grassland areas and added competition stress might have on the development of plants over a full growth period. We found significant effects of the highest concentration (5  $\mu\text{g g}^{-1}$ ) of formulated moxidectin on all observed parameters when plants were not under competition stress. This includes reducing aboveground plant biomass by 21 %, while competition stress alone had significant effects on all measured parameters, reducing aboveground plant biomass by 89.4 %. These results show that contamination of soil by moxidectin residues can affect vigour of grassland species especially when applied over a longer period of time in areas without competition. This is especially relevant for areas dependent on primary succession, such as grazed pastures and dung pats.

## 1. Introduction

Moxidectin is a macrocyclic lactone (ML) used worldwide as an anthelmintic on livestock and pets to kill nematodes and other pests. After treatment, up to 52 % of the original concentration is excreted into the environment via faeces (Afzal *et al.*, 1994). There, it is a threat to non-target organisms, especially coprophageous insects such as dung beetles and dung flies. Several studies have shown the effects of macrocyclic lactone anthelmintics on coprophageous insects, especially dung beetles and what effects this might have on biodiversity and resource availability in soil (Steel, 1998; Baena-Díaz *et al.*, 2018). Only recently, effects on seed germination, seedling emergence (Chapter 1; Eichberg *et al.*, 2022, 2016) seedling growth (Vokřál *et al.*, 2019) and fully developed plants (Navrátilová *et al.*, 2020a; Langhansová *et al.*, 2021) were also described in several studies, but the effects on plant growth beyond the seedling stage are largely unknown.

MLs in general are lipophilic (log K<sub>OW</sub>[water-octanol coefficient] MOX: 4.8; Mooney *et al.*, 2021), basically immobile in soil and degrade easily when they are subjected to sunlight or higher temperatures in general, as there is a distinct difference between degradation in summer and winter (Bloom & Matheson, 1993; El-Saber Batiha *et al.*, 2020). Interestingly, in the direct vicinity of dung pats, ML residues were found in both soil and plants (Iglesias *et al.*, 2018). These dung pats contained traceable amounts of MLs, even weeks after excretion, so the degradation inside the pats is much slower than on the outside in contact with direct sunlight (Mougin *et al.*, 2003). Studies have shown that, depending on environmental conditions, such as temperature and rainfall, the half-life of MLs in dung can range from a few days to several months and degradation could even stop completely in winter (Sommer *et al.*, 1992; Mougin *et al.*, 2003). This suggests, that if regular treatments are applied to livestock in a certain area, MLs could accumulate in the soil (Wohde *et al.*, 2016), enhancing their effects on both fauna and flora. These effects can range from changes in metabolic products, e.g. higher concentrations of radical oxygen species, to actual inhibition of root growth by up to 24 % (Vokřál *et al.*, 2019; Syslová *et al.*, 2019; Navrátilová *et al.*, 2020a,b). In addition, a reduction in yield of soybeans (*Glycine max*) was found by Navrátilová *et al.* (2020a).

These findings make it reasonable to assume that MLs could also affect grassland species, limiting reproductive success and therefore adversely affecting biodiversity. This would be especially concerning in regards to biodiversity in grasslands since many species in these ecosystems are spread via endozoochory by animals and later being excreted with faeces somewhere else (Benthien *et al.*, 2016). This process can be harmful to these seeds, even though it is necessary for some to be able to germinate at all. Through various stressors including mastication and digestion but also higher temperatures in the animal bodies, many seeds are destroyed before being excreted, so a lower seed production can reduce the number of seeds able to germinate even further, risking overall reproductive success (Manzano *et al.*, 2005; Wang *et al.*, 2017; Oveisi *et al.*, 2021).

In this vulnerable stage, additional stress due to contact with MLs could seriously affect germinability of seeds. This has been recently shown by Eichberg *et al.* (2022, 2016). In addition, several studies have shown effects on seedlings after germination (Vokřál *et al.*, 2019; Navrátilová *et al.*, 2020b). In all of those studies, MLs have been the only stressor, which puts their applicability to outside conditions, into question.

Other stressors which are common in pastures and other grassland ecosystems, have been neglected. Competition stress was included in our study to provide a more comprehensive look on how plants are affected by MLs in nature. Competition is an important stressor for plants in almost all landscapes and influences almost all plants (Newman, 1973; Aschehoug *et al.*, 2016). Plants compete for various resources, including sunlight, water and nutrients (Craine & Dybzinski, 2013). Lack of any of those resources can significantly reduce growth and overall vigour of plants (Newman, 1973; Grace, 2012). In contrast to stress by competition, MLs cause stress directly, affecting cell organelles or possibly even protein functions (Elter *et al.*, 2007; Kiki-Mvouaka *et al.*, 2010; Arévalo *et al.*, 2021).

The combination of two stressors such as competition and MLs could multiply their effects on seedlings and growing plants (Zandalinas & Mittler, 2022). While studies have shown that MLs can have significant effects on seedling germination and emergence (Chapter 1; Chapter 2), competition stress was found to have no significant impact on germination and emergence (Loydi *et al.*, 2015). Studies have shown that, after emergence, competition can significantly affect the successful establishment of a seedling (Rorison, 1960; Sagar & Harper, 1961; Newman, 1973), while the effects of ML on plants in later stages of development are scarcely researched. Since both effects can coincide on a regular basis on pastures and other grassland ecosystems, knowing their combined effects on seedling establishment and plant growth would expand our knowledge on the effects of MLs on non-target organisms.

To measure the effects both MLs and competition in general have on plants, several parameters should be taken into consideration. This includes the biomass as the most obvious parameter, since a lack of resources directly influences the ability of plants to grow and accumulate biomass. A high relative biomass is also a good indicator for the overall vigour and possible reproductive success of plants, since these plants have access to more resources and can therefore produce more seeds (Trader *et al.*, 2006). In addition, the specific leaf area (SLA) can indicate if plants are stressed out. Studies have shown that plants under various kinds of stress, such as drought, temperature or competition for sunlight, show changes in their SLA (Poorter & De Jong, 1999; Fernández & Reynolds, 2000; Rosbakh *et al.*, 2015). Another indicator for stress is the intact leaf area. A lack of resources, facilitated by competition can cause damage to leaf, e.g. by leaf-tip dieback (Bal *et al.*, 2015). A comprehensive analysis of all three of these parameters can yield valuable information on how MLs in conjunction with naturally occurring stressors such as competition, affect plants in different ways, expanding our knowledge about this understudied field of ML-related research.

To generate new insight into this scarcely researched topic, we set up a greenhouse experiment, which included four temperate grassland species, three concentrations of formulated moxidectin and the addition of competition plants as an additional stressor to simulate field conditions.

In this experiment, we address the following questions:

- Does formulated moxidectin affect growth of temperate grassland species?
  - We expect formulated moxidectin to have a negative effect on the growth of temperate grassland species.
- How does competition stress affect the reaction of temperate grassland species to formulated moxidectin?
  - We expect competition to make plants even more vulnerable to effects of formulated moxidectin.

## 2. Materials and Methods

### 2.1. Study Species

We used four temperate grassland species with perennial life strategies belonging to four plant families as our focus plants: *Achillea ptarmica* (Asteraceae), *Agrostis capillaris* (Poaceae), *Dianthus deltoides* (Caryophyllaceae) and *Plantago lanceolata* (Plantaginaceae). All species are typical for central Europe and their seeds have shown sensitivity to macrocyclic lactones in previous experiments (Chapter 1; Chapter 2; Eichberg et al., 2022, 2016). In addition, all species are proven or suspected to survive gut passage in livestock and can therefore be spread via endozoochory. Seeds were purchased from Rieger-Hofmann GmbH (Blaufelden, Germany).

### 2.2. Test substance

Oral formulations are one of the most commonly used administration ways of moxidectin, since their application is relatively simple (Corba *et al.*, 1995; Fort Dodge Animal Health, 2001). Since moxidectin is poorly soluble in water, oral formulations use additional chemicals to adjust solubility of the anthelmintic and enhance absorption of the substance into the bloodstream from where it spreads into the whole body (Suppl. Table S1). Due to its chemical properties, moxidectin accumulates in fat tissue, where significant concentrations can be measured much longer than in the rest of the body (Lanusse *et al.*, 1997; Lifschitz *et al.*, 2000). While most of the absorbed moxidectin is excreted via faeces, only less than 1 % leaves the body via urine (Afzal *et al.*, 1994). Moxidectin works similar to other macrocyclic lactones by opening specific chloride channels only found in invertebrates, which causes paralysis and then death (Prichard *et al.*, 2012).

Cydectin® is an oral formulation of moxidectin for sheep. Its active substance is moxidectin, but it also contains several co-formulants such as benzyl alcohol and butylated hydroxytoluene (Suppl. Table S1). We purchased formulated moxidectin (Cydectin: Oral solution for Sheep, 0.1 %) from Zoetis® (Zoetis Deutschland GmbH, Berlin, Germany).

### 2.3. Experimental setup

We carried out a pot experiment in a greenhouse at Kiel University (Kiel, Germany) including the factors species, concentration and competition. We used four temperate grassland species, four different concentrations of formulated moxidectin (0; 0.1; 1 and 5 µg g<sup>-1</sup>) and added a competition species (*Poa pratensis*) to half of our pots.

For each combination of factors, we used 10 individuals in our experiment, adding up to a total number of 320 pots (20 cm × 18 cm × 18 cm). Each pot was filled with a 2:1 ratio of low nutrition soil (Type-0-soil (FE-TYPE)), purchased from GEM Gärtnereinkauf (Kornthal-Münchingen, Germany) and coarse sand. When filled with the 2:1 soil-sand mixture, the mixture had a dry-weight of approx. 3 kg per pot. The same soil mixture was used throughout the entire experiment.

Seedlings were grown in Styrofoam boxes (29.0 cm × 19.5 cm × 5.5 cm) and then moved to pots, when individuals of all species had grown their first true leaf beyond their cotyledons. Pot placement in the greenhouse was chosen at random during the setup and was not changed during the experiment.

Formulated moxidectin was mixed with tap water to dilutions, which at a volume of 100 ml were equivalent to a concentration of 0.1, 1 and 5 µg g<sup>-1</sup> of moxidectin in the soil per pot. These values are within the range of concentrations of macrocyclic lactone residues reported for substrates (digestive contents, dung) excreted by sheep. The aim was for these values to simulate the condition in soil, which has been contaminated by faeces of sheep recently treated with formulated moxidectin and simultaneously use concentrations comparable with previous experiments (Chapter 1). The 10 µg g<sup>-1</sup> concentration was replaced with a 5 µg g<sup>-1</sup> concentration, because a preliminary experiment showed detrimental effects to growing plants, ending the experiment prematurely, otherwise adhering to the concentrations which had already shown significant effects on seeds. The comparability of values of ML concentrations measured in different experiments, even when related to sheep, is difficult since there are only few published values and methods and units vary. Generally, dung showed much higher ML concentrations than digestive contents, but reported values cover a broad range between 23 ng g<sup>-1</sup> (MOX, 40 h p.t., subcutaneous administration, (Hentz *et al.*, 2019), equivalent to 0.02 µg g<sup>-1</sup>) and 13,615 ng g<sup>-1</sup> (IVM, 1 d p.t., oral administration, (Lang, 1996); 13.62 µg g<sup>-1</sup>). These values include the range of the concentrations used in this experiment.

The solution was added to pots at three time points: in the beginning of February, May and August of 2022. Each time, 100 ml per solution were injected as four injections of 25 ml with glass pipettes in premade holes (1 cm diameter, 5 cm deep) into the soil around the plants (Suppl. Fig. S2) to ensure a wide spread of the solution in the soil.

Up until first injection with formulated moxidectin solution, pots were watered from the ground up. After first injection in February, plants were watered from above to prevent mixing of different concentrations of moxidectin in water. In addition, wooden slats topped with a steel mesh were placed on the tables to prevent run off water of different pots mixing and seeping back up into the soil. Liquid fertilizer (Substral® Pflanzennahrung; 7.0 % N, 3.0 % P<sub>2</sub>O<sub>5</sub>, 5.0 % K<sub>2</sub>O, additional micronutrients; recommended dosage: 7 ml l<sup>-1</sup>) was added regularly while watering.

Lamps (SON-K 400 high-pressure sodium discharge-luminaire) provided additional light and warmth in a day/night cycle of 11/13h. Automated fans activated when temperature in the greenhouse rose over 24 °C.

## 2.4. Data collection

At the end of the experiment in mid-September, six weeks after the last injection of formulated moxidectin, specific leaf area (SLA) and aboveground plant biomass (APB) of focus plants were measured. For SLA, three of the youngest fully developed leaves per individual were collected, scanned and the leaf area calculated using the software FIJI (Schindelin *et al.*, 2012). After scanning, these leaves were dried at 105 °C for 48 h and leaf biomass was determined. SLA was calculated as leaf area divided by leaf dry biomass ( $\text{mm}^2 \text{mg}^{-1}$ ). For aboveground plant biomass, all focus plants were cut 2 cm above ground and dried for 48 h before weighing. The biomass of leaves collected for SLA determination and the remaining aboveground biomass were summed up to obtain the total aboveground biomass. During the full duration of the experiment, from February to September at the beginning of every month, ILA (intact leaf area) of plants was determined by visual inspection using five classes, representing the percentage of intact leaf area, shown in Table 1. Examples for damaged areas of leaves are given in Supp. Fig. 3. The data gathered during the experiment was then summed up for each individual focus plant and used for statistical analysis.

**Table 1** Classes to determine intact leaf area (ILA). Percentage of ILA was measured by visual observation once per month.

Percentage of intact leaf area	ILA class
100-76 %	4
75-51 %	3
50-26 %	2
25-1 %	1
0 % (Dead)	0

## 2.5. Statistical analysis

Univariate three-way ANOVA was applied to analyse effects of *species*, *concentration* and *competition* on the dependent variables APB (aboveground plant biomass), SLA (specific leaf area) and ILA (intact leaf area) (Table 1). One-way ANOVA was used for the analyses of the effects of *concentration* at the species level, which will be referred to as “on species level”. Post-hoc Tukey HSD test was done, if significant effects were revealed. Before employing inferential statistics, data was visually checked by QQ plots and scatterplots of observed values against non-standardized residuals. Normality and homogeneity of variance were met in every case, therefore no transformation was necessary.

Statistical analysis was done with Statistica™ 13.3 (StatSoft Inc., Tulsa, Oklahoma, USA) and graphs were created using ORIGIN® (ORIGINLab Corporation, Northampton, Massachusetts, USA).

### 3. Results

During our experiment, the treatments had visible effects on our test plants. A few days after treatment, damage was clearly visible on the plants, indicated by leaves losing colour and becoming brittle. 19 individuals (6 %) did not survive the treatment, although this effect was limited to only one species and the highest concentration and only occurred early in the trials. Most of the damaged plants were able to recover from the damage sustained in the experiment, growing the lost areas back after a few weeks (Suppl. Fig. 3).

#### 3.1. Effects of formulated moxidectin and competition stress on aboveground plant biomass

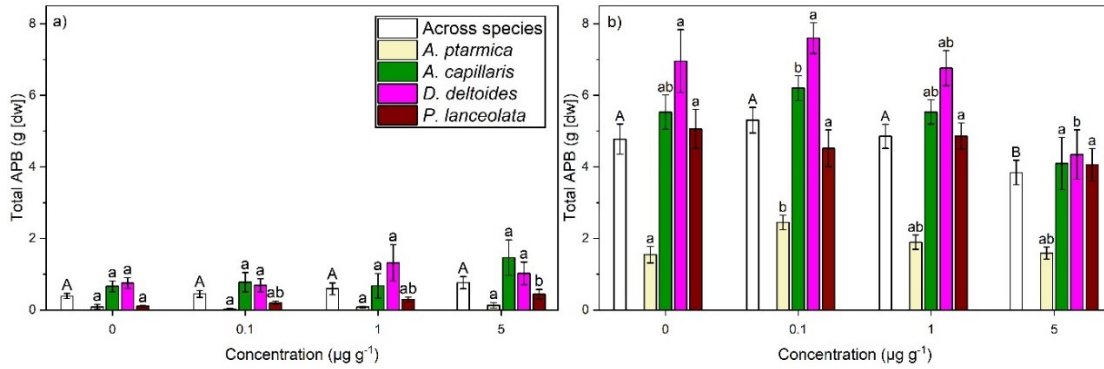
Three-way ANOVA revealed significant *Species*, *Concentration* and *Competition* effects, with the interactions of *Species*  $\times$  *Competition* and *Concentration*  $\times$  *Competition* (Table 2).

**Table 2** Results of ANOVA of all main plant species of the effects of *Species*, *Concentration* and *Competition* on aboveground plant biomass (APB), specific leaf area (SLA) and intact leaf area (ILA) of main plants.

	APB				SLA				ILA			
	df.	MQ	F	p	df.	MQ	F	p	df.	MQ	F	P
Species (S)	3	92.93	65.62	$\leq 0.001$	3	1500.40	24.35	$\leq 0.001$	3	283.4	18.72	$\leq 0.001$
Concentration (C)	3	5.46	3.86	0.01	3	263.0	4.27	0.006	3	494.2	32.64	$\leq 0.001$
Competition (Co)	1	1175.76	830.18	$\leq 0.001$	1	335.40	5.44	0.02	1	89.9	5.94	$\leq 0.015$
S $\times$ C	9	1.60	1.13	0.34	9	117.70	1.91	0.051	9	10.9	0.72	0.69
S $\times$ Co	3	41.23	29.11	$\leq 0.001$	3	770.80	12.51	$\leq 0.001$	3	14.0	0.92	0.43
C $\times$ Co	3	12.53	8.85	$\leq 0.001$	3	38.70	0.63	0.6	3	21.9	1.45	0.23
S $\times$ C $\times$ Co	9	1.98	1.40	0.19	9	29.0	0.47	0.89	9	22.2	1.47	0.16
Error	269	1.42			239	61.60			286	15.1		

Our results show that, across all species, including both competition and non-competition stressed individuals, concentration only affected APB at the  $5 \mu\text{g g}^{-1}$  concentration ( $2.3 \pm 0.3 \text{ g}$ ) compared to the  $0.1 \mu\text{g g}^{-1}$  concentration ( $2.9 \pm 0.3 \text{ g}$ ). Both control ( $2.6 \pm 0.3 \text{ g}$ ) and the  $1 \mu\text{g g}^{-1}$  concentration ( $2.7 \pm 0.3$ ) did not differ significantly from either of the two. Competition in general reduced APB of the focus plants across all species and concentrations of formulated moxidectin and control (Fig. 1A) when compared to plants grown without competition (Fig. 1B). The interaction of concentration  $\times$  competition showed that APB across all species and concentrations, grown with competition ( $0.5 \pm 0.06 \text{ g}$ ) was significantly different from their non-competition counterparts ( $4.7 \pm 0.18 \text{ g}$ , Table 2). Only the highest moxidectin concentration ( $5 \mu\text{g g}^{-1}$ ,  $3.8 \pm 0.3 \text{ g}$ ) reduced APB significantly compared to control ( $4.8 \pm 0.4 \text{ g}$ ) and the two lower concentrations ( $0.1 \mu\text{g g}^{-1}$ ,  $5.3 \pm 0.3 \text{ g}$ ;  $1 \mu\text{g g}^{-1}$ ,  $4.9 \pm 0.3 \text{ g}$ ) and only when no competition was present (Fig. 1B). Since the species used in our experiment were from different families and differed in size and shape, significant Species effects on APB can be attributed to differences between the species, such as growth speed and size and are therefore not discussed in detail.

When looking at the results on species level, the only significant difference found for plants grown with competition, was for *P. lanceolata* between control ( $0.1 \pm 0.02 \text{ g}$ ) and the  $5 \mu\text{g g}^{-1}$  concentration ( $0.44 \pm 0.13 \text{ g}$ ), with the two other concentrations differing from neither the higher concentration nor control.

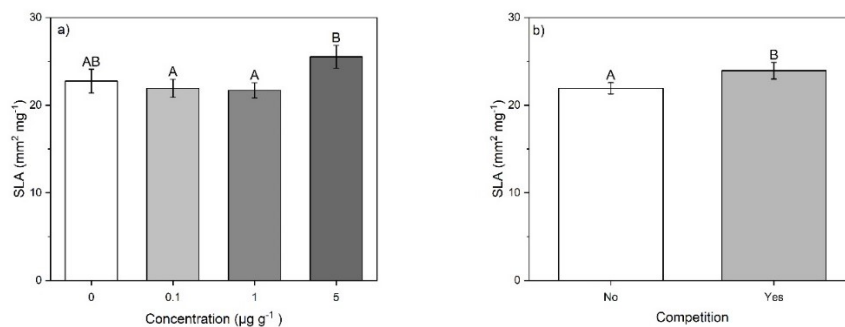


**Fig. 1** Effects of formulated moxidectin on aboveground plant biomass (APB [dry weight [dw], mean  $\pm$  SE]) of all species and each individual species for plants grown with (a) and without competition (b) along a series of concentration levels. Different letters indicate significant differences between concentration levels on across-species level (capitals) and on species level (lower case), respectively ( $p \leq 0.05$ , Tukey HSD test).

For plants grown without competition, significant differences were found in *A. ptarmica*, *A. capillaris* and *D. deltoidea*. For *A. ptarmica*, the  $0.1 \mu\text{g g}^{-1}$  concentration ( $2.5 \pm 0.2 \text{ g}$ ) was significantly higher than control ( $1.5 \pm 0.2 \text{ g}$ ), while both did not differ from the other two concentrations. For *A. capillaris*, dw at the  $5 \mu\text{g g}^{-1}$  concentration ( $4.1 \pm 0.7 \text{ g}$ ) was significantly reduced compared to the  $0.1 \mu\text{g g}^{-1}$  concentration ( $6.2 \pm 0.4 \text{ g}$ ), while both did not differ from control and the  $1 \mu\text{g g}^{-1}$ . For *D. deltoidea*, the  $5 \mu\text{g g}^{-1}$  concentration ( $4.4 \pm 0.7 \text{ g}$ ) was significantly lower than control and the  $0.1 \mu\text{g g}^{-1}$  concentration ( $7.6 \pm 0.4 \text{ g}$ ), while the  $1 \mu\text{g g}^{-1}$  did not differ from any other concentration. For *P. lanceolata* no significant differences between concentrations or control were found (Fig. 1B).

### 3.2. Effects of formulated moxidectin and competition stress on specific leaf area

ANOVA revealed significant effects for *Species*, *Concentration*, *Competition* and the interaction of *Species*  $\times$  *Competition* (Table 2). The  $5 \mu\text{g g}^{-1}$  moxidectin concentration ( $25.5 \pm 1.3 \text{ mm}^2 \text{ mg}^{-1}$ ) enhanced SLA by 12.2 % compared to control ( $22.7 \pm 1.3 \text{ mm}^2 \text{ mg}^{-1}$ ), although this was not a significant difference. Compared to the other two concentrations ( $0.1 \mu\text{g g}^{-1}$ ,  $21.9 \pm 1.0 \text{ mm}^2 \text{ mg}^{-1}$ ;  $1 \mu\text{g g}^{-1}$ ,  $21.7 \pm 0.9 \text{ mm}^2 \text{ mg}^{-1}$ ), the effect was significant (Fig 2A). Competition stress ( $23.9 \pm 0.9 \text{ mm}^2 \text{ mg}^{-1}$ ) significantly enhanced SLA by 9.14 % when compared to non-competition plants ( $21.9 \pm 0.7 \text{ mm}^2 \text{ mg}^{-1}$ ; Fig 2B).

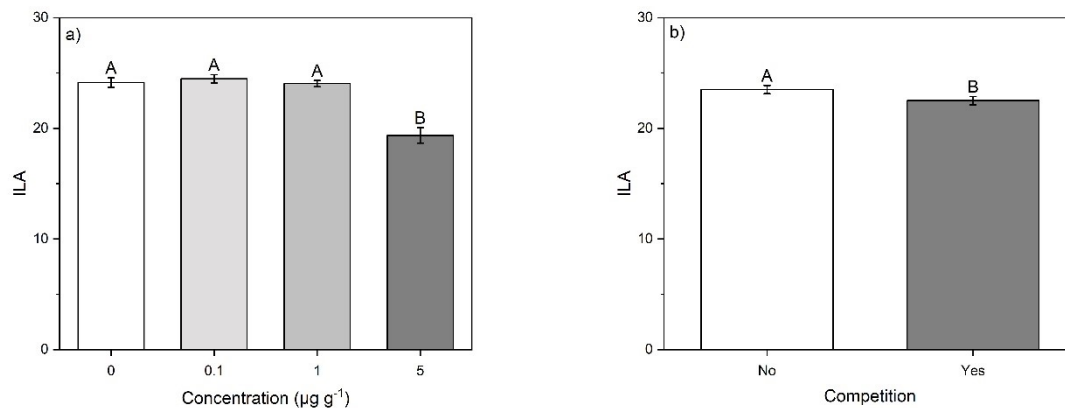


**Fig. 2** Effects of formulated moxidectin concentrations (a) and competition stress (b) on Specific Leaf Area (SLA,  $\text{mm}^2 \text{ mg}^{-1}$ , mean  $\pm$  SE) across species. Different letters indicate significant differences between trials on across-species level (capitals) and on species level (lower case), respectively ( $p \leq 0.05$ , Tukey HSD test).

On a species level, when competition stress was applied, no significant differences were found between any concentrations (Suppl. Fig. S4A). For the plants grown without competition stress, both in *A. capillaris* and *D. deltoides*, significant differences were found. For *A. capillaris*, only the  $5 \mu\text{g g}^{-1}$  concentration ( $35.8 \pm 3.6 \text{ mm}^2 \text{ mg}^{-1}$ ) differed significantly from other concentrations ( $0.1 \mu\text{g g}^{-1}$ ,  $26.3 \pm 1.3 \text{ mm}^2 \text{ mg}^{-1}$ ;  $1 \mu\text{g g}^{-1}$ ,  $26.3 \pm 1.4 \text{ mm}^2 \text{ mg}^{-1}$ ) and control ( $25.5 \pm 1.9 \text{ mm}^2 \text{ mg}^{-1}$ ). *D. deltoides* did significantly differ between the 5 ( $19.6 \pm 1.0 \text{ mm}^2 \text{ mg}^{-1}$ ) and  $1 \mu\text{g g}^{-1}$  ( $15.7 \pm 1.1 \text{ mm}^2 \text{ mg}^{-1}$ ) concentrations, both of them did not differ from control ( $16.1 \pm 0.9 \text{ mm}^2 \text{ mg}^{-1}$ ) and the  $0.1 \mu\text{g g}^{-1}$  ( $16.8 \pm 1.1 \text{ mm}^2 \text{ mg}^{-1}$ ) concentration. Both *A. ptarmica* and *P. lanceolata* showed no significant differences between concentrations and control (Suppl. Fig S4B).

### 3.3. Effects of formulated moxidectin and competition stress on intact leaf area

ANOVA revealed significant main effects of *Species*, *Concentration* and *Competition* but no significant interactions on ILA (Table 2). When comparing the effects of different concentrations of moxidectin across all species and both competition and no competition plants, the  $5 \mu\text{g g}^{-1}$  concentration ( $19.4 \pm 0.7$ ) of moxidectin had a significant impact, reducing the ILA by 19.8 % compared to control ( $24.2 \pm 0.4$ ). The other concentrations ( $0.1 \mu\text{g g}^{-1}$ ,  $24.5 \pm 0.4$ ;  $1 \mu\text{g g}^{-1}$ ,  $24.1 \pm 0.3$ ) differed significantly only from the highest concentration (Fig. 3A). The effects of competition stress ( $22.5 \pm 0.4$ ) across all species and concentrations were also significant, reducing ILA by 4.2 % compared to the non-competition plants ( $23.5 \pm 0.4$ , Fig 3B).



**Fig. 3** Effects of formulated moxidectin concentrations (a) and competition stress (b) across species on intact leaf area (ILA, summed up over the duration of the experiment  $\pm$  SE). Measured during the observation period with the following classes values: 100-76 % = 4; 75-51 % = 3; 50-26 % = 2; 25-1 % = 1; 0 % (Dead) = 0. Different letters indicate significant differences between trials across species ( $p \leq 0.05$ , Tukey HSD test).

For plants grown with competition, on species level significant differences were found between the  $5 \mu\text{g g}^{-1}$  values for *D. deltoides* and *P. lanceolata*. The  $5 \mu\text{g g}^{-1}$  ( $21.1 \pm 1.7$ ) concentration differed from other concentrations ( $0.1 \mu\text{g g}^{-1}$ ,  $25.8 \pm 0.6$ ;  $1 \mu\text{g g}^{-1}$ ,  $25.3 \pm 0.7$ ) and the control ( $25.6 \pm 0.5$ ) for *D. deltoides*. For *P. lanceolata*, the  $5 \mu\text{g g}^{-1}$  concentration ( $19.4 \pm 1.8$ ) differed significantly from control ( $24.0 \pm 0.8$ ) and the  $0.1 \mu\text{g g}^{-1}$  ( $24.9 \pm 0.6$ ) concentration, but not from the  $1 \mu\text{g g}^{-1}$  concentration ( $23.0 \pm 0.5$ ), which did not differ from any concentration or control (Suppl. Fig. S5A). Although values were slightly different, the pattern of significant differences was the same when competition stress was applied (Suppl. Fig. S5B).

## 4. Discussion

Effects of macrocyclic lactones on plants haven been shown in experiments before, where they negatively affected root growth and affected enzyme production (Vokřál *et al.*, 2019; Navrátilová *et al.*, 2020a,b). These studies were done only on seedlings or cell conglomerates in short term experiments. Our study explored the long term effects of plants being repeatedly exposed to moxidectin in soil.

### 4.1. Formulated Moxidectin has negative effects on growth of temperate grassland species

Our results show that formulated moxidectin has a detrimental effect on the growth of temperate grassland species, affecting aboveground plant biomass (APB), specific leaf area (SLA) and intact leaf area (ILA) of individual plants. Results regarding APB suggest that high moxidectin concentrations in soil can have a negative effect when no competition stress is applied. Adding competition stress significantly reduced APB but also reduced moxidectin-related effects. Studies have shown that the effects of moxidectin cause stress reactions in plants, hindering their growth (Vokřál *et al.*, 2019; Navrátilová *et al.*, 2020a). Stress reactions after direct contact with macrocyclic lactones were previously described and affected the production of stress-related phytohormones, indicating a severe reaction to MLs inside plants (Navrátilová *et al.*, 2020b). A significant reduction of plant biomass across species can have detrimental effects for entire plant populations. Seed production is correlated with biomass (Trader *et al.*, 2006), therefore a reduction of biomass results in reduced reproductive success. Especially in areas where plants are affected by MLs, it can be assumed, that seeds would also be affected, reducing germination success even more (Eichberg *et al.*, 2016, 2022).

The results of our study indicated that the ILA of plants was reduced significantly both by the highest moxidectin concentration (-19.8 % compared to control) as well as by competition stress (-4.3 % compared to plants without competition). From this we can conclude that moxidectin affected the plants ILA more than the added stress by competition. In addition, damage to the leaves of all four focus plant species was only seen for plants grown without competition and only when formulated moxidectin was applied (Suppl. Fig. 2). Since formulated moxidectin affected the plants nearly instantly after application, these plants had no time to adapt to this kind of stress. In contrast, competition stress is an indirect stressor, indicated by other factors such as lack of water or nutrients for plants suffering from competition (Trinder *et al.*, 2013). These factors build up over time and give plants time to adapt, e.g. by changing how many resources they invest in individual leaves, resulting in changes to the SLA (Cheplick, 1998).

Results have shown that SLA was significantly enhanced by both the highest moxidectin concentration (+12.2 % compared to control) and competition stress (+ 9.1 % compared to plants without competition). This suggests that these changes in SLA are related to the effects of competition for sunlight when competition stress was applied or the negative effects of formulated moxidectin on ILA, when no competition stress was applied. This corresponds with our results, as the leaves of our focus plants suffering from competition stress would be mostly shadow leaves, because the competition plants grew faster and higher than our focus plants, thus limiting their access to sunlight.

These shadow leaves are generally associated with a higher SLA than sun leaves (Paž-Dyderska *et al.*, 2020). Reduced access to sunlight could also be one of the reasons why those plants did not grow as much as their not competition-stressed counterparts. To compensate for the loss of ILA due to the effects of high formulated moxidectin concentrations, affected plants would try to regrow their leaves fast, which can cause a higher SLA in those regrown leaves, as was shown by Cerasoli *et al.* (2004) on partially defoliated oak saplings.

Significant damage to leaves was not reported in other publications dealing with the effects of macrocyclic lactones on plants (Navrátilová *et al.*, 2020a; Langhansová *et al.*, 2021). The damages reported in our study were seen a few days after injection and most plants were able to recover from the damage after every injection (Suppl. Fig. 2). The overall appearance of damaged leaves suggests that either water, nutrients or both are not able to reach the tip of the leaf and then gradually recede from there. This could be caused by a variety of things: Primarily damage to the roots, so that water and nutrients cannot be taken up properly from the soil, or the uptake of certain specific nutrients is hindered because specific channels might be affected by the macrocyclic lactones. This kind of uniform damage is indicative for effects of factors such as toxic chemicals used as soil-applied herbicides (Green & Capizzi, 2019). This is consistent with the experimental setup, where formulated moxidectin was injected into the soil. This formulation contains both benzyl alcohol and butylated hydroxytoluene (Suppl. Table S1), both of which have shown phytotoxic properties (Babich, 1982; Vercruysse & Rew, 2001). Therefore, we conclude that the apparent direct effects on leaves in our experiment could at least partially have been caused by the co-formulants in the formulated moxidectin solution. This is consistent with previous experiments (Chapter 1), where formulated moxidectin solutions had a stronger effect on germination parameters than pure moxidectin solutions. Since pure moxidectin solutions also affected germination parameters it cannot be ruled out that moxidectin is at least partially responsible for effects on leaves and that the combination of both moxidectin and the co-formulants could enhance their effects even more.

In previous studies (Chapter 1; Chapter 2), the idea that MLs in plants could have the same or similar effects as they would have in animals was discussed, since plants possess analogous structures to those which are affected by the MLs in animals (Chapter 1; Chapter 2; Eichberg *et al.*, 2022, 2016). This could cause the permanent opening of specific chloride channels, causing them to be inoperable, leaking Cl<sup>-</sup> (Elter *et al.*, 2007; Martin *et al.*, 2021). This in turn could then affect plant organelles such as stomata, closing them and thus affecting the whole plant (Geilfus *et al.*, 2015). Another theory is that MLs can affect proteins, docking with their active sites and therefore making them unable to perform their normal functions, which was shown for ivermectin (Lehrer & Rheinstein, 2020; Bello, 2021). Moxidectin, which has a similar structure to ivermectin, could have similar effects (Lanusse *et al.*, 1997).

#### 4.2. Competition stress reduces the effects of moxidectin for grassland species

Our results show that the effects of formulated moxidectin are stronger in plants not affected by competition than in such that were affected by competition plants in their pots. This suggests that competition is a much larger stressor for plants than moxidectin, since the effects of moxidectin are only relevant when no competition is present. We assume that this is linked to their reduced growth. In our experiment, plants suffering from competition stress were generally smaller and therefore would also possess smaller roots, which would limit exposure to moxidectin in the soil (Hodge *et al.*, 1999; Craine & Dybzinski, 2013). The potential uptake of moxidectin would be reduced compared to the plants without competition. In addition, the competition plants were usually outgrowing the experiment plants, so these would have taken up most of the available moxidectin since their root system was far larger and therefore the area of exposure was far larger than for the small experiment plants (Grace, 2012).

Our research suggests that competition stress, which happens nearly everywhere in grasslands (Boyer, 1982), negates the effects of moxidectin on aboveground biomass and these effects can therefore be regarded as an irrelevant threat to grassland species. But it has to be taken into account where most of the regeneration takes place: Since MLs are mostly spread into the environment via faeces, that's where most of the effects would occur. Faeces are suppressing established vegetation, giving the seeds that survived the gut passage a chance to germinate with less or completely without competition (Auman *et al.*, 1998; Gokbulak & Call, 2004). Effects on these areas with reduced competition have been covered in this study by our pots with no added competition plants. In addition, these areas could have been grazed by livestock before, which would be an additional factor reducing competition (Couvreur *et al.*, 2005; Cosyns *et al.*, 2006; Tjelele *et al.*, 2015). This important step of regeneration could therefore still be affected by formulated moxidectin residues, since here a "low competition" situation can be assumed, which adds to the ecological relevance of the findings of this study, especially for the effects found in the "non-competition" groups.

#### 4.3. Conclusion

In summary, moxidectin can have detrimental effects on growth and vigour of certain temperate grassland species. Effects are more pronounced when no competition stress is applied. All parameters were significantly affected by concentration levels which can be found in the environment when livestock is treated with ML anthelmintics. Since MLs are excreted with faeces, they would most likely influence grazed environments, where livestock would leave gaps for re-emergence of plants (Malo & Suárez, 1995; Gokbulak & Call, 2004). Here, effects not only on germination and emergence of parameters of seeds (Chapter 1; Chapter 2) but also establishment and growth as shown in this experiment, can have significant effects on plant populations and biodiversity of grassland ecosystems. This is especially true for fragmented ecosystems dependent on spreading of seeds via endozoochory, which are increasingly threatened by extinction due to moving herds of livestock becoming less and less common in the landscape. In light of this and previous studies regarding the effects of MLs on various organisms (Chapter 1; Chapter 2; Förster *et al.*, 2011; Mougin *et al.*, 2003), regulations should be put in place to limit the spread of MLs into the environment.

This could be done by regulating the time treated animals should be in stables after treatment and if that's not possible to at least prevent accumulation of MLs in soil by changing grazing areas during treatment.

### **Acknowledgements**

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## Discussion and synthesis

The aim of this thesis was to elaborate on the effects that macrocyclic lactones have on plants in critical stages of development, from seeds to seedlings to mature plants. Species- and substance-specific effects on seed germination across 17 tested species were found, in which formulated macrocyclic lactones (MLs) were reducing germination percentage (GP) and enhancing mean germination time (MGT), while for pure MLs, GP was enhanced and MGT reduced (Chapter 1). Furthermore, when seeds of four species were spread via endozoochory by sheep treated with formulated moxidectin, the number of emerged seedlings (NES) was reduced on a high moxidectin concentration but enhanced on lower ones. All moxidectin concentrations reduced the mean emergence time (MET), lower concentrations leading to more reduction (Chapter 2). In addition, both competition stress and continued contact of high concentrations of formulated moxidectin with growing plants reduced aboveground plant biomass (APB), enhanced specific leaf area (SLA) and reduced intact leaf area (ILA) across all tested species. When competition stress was applied, formulated moxidectin had no significant impact on any of the observed parameters (Chapter 3).

### *Macrocyclic lactones have positive and negative effects on seeds and seedlings*

In all three chapters of this thesis, MLs had significant effects on germination, emergence, growth and vigour. While pure MLs showed species and substance-specific effects (Chapter 1), the effects of formulated substances, especially formulated moxidectin showed a clear trend for all observed parameters (Chapter 1; Chapter 2; Chapter 3). While in Chapter 1 and 3, these effects were seen as negative in an ecological context (lower GP, higher MGT, lower APB, higher SLA, lower ILA), in Chapter 2, formulated moxidectin had positive effects on NES and MET at lower concentrations. These positive effects could be described as a “hormetic-like”-response pattern, since they fit the general pattern described by Hormesis, but more information, e.g. the range of the hormetic vs the toxic doses, would be needed to ensure that this would be the correct term used. Hormesis is a phenomenon which describes the positive effects small doses of a toxic agent have on an organism (Agathokleous *et al.*, 2019; Agathokleous & Calabrese, 2022). This kind of effect is often attributed to an overcompensation after being affected by a stressor (Calabrese, 1999, 2001). While the effects of a low dosage of a stressor can lead to overcompensation against the effects of this stressor by the organism, resulting in an overall positive reaction, a high dosage cannot be compensated, therefore resulting in a negative reaction (Calabrese, 2001).

While a higher number of germinated seedlings and faster germination can be seen as positive, e.g. by allowing seeds to outperform their competition by germinating faster and in higher numbers (Orrock & Christopher, 2010), studies have shown that every change to homeostasis can be detrimental to organisms. A higher number of germinating or emerging seeds in a given timeframe caused by biotic or abiotic effects, could also have negative effects on plant populations, since this lowers the number of seeds in the soil seed bank (Mulugeta & Stoltenberg, 1997). When environmental effects then kill off all emerged seedlings, e.g. by a sudden drought or flood, even fewer seeds would remain to restore the population in the next season or when conditions are favourable again, endangering the survival of the population.

A lower MGT or MET can also have negative effects on the emerging seedlings, since conditions needed for their successful establishment may not be in place yet, making it harder for these seeds to thrive (Orrock & Christopher, 2010; Leverett *et al.*, 2018; Torres *et al.*, 2020). This shows that the effects of MLs on seeds are far more complex and the ecological effects on plant populations are harder to assess than previously anticipated and that more research on the long term effects of MLs exposure to plant populations and ecosystems is necessary to determine the complex consequences brought on by these substances.

#### *Co-formulants add to the effects of formulated moxidectin on seeds and plants*

In all three chapters, formulated moxidectin had significant effects on all observed parameters. While in Chapter 1 and 3, formulated moxidectin directly affected seeds and growing plants, this was not the case for Chapter 2. In Chapter 2, formulated moxidectin was administered to the sheep one day before the administration of the seeds. Therefore, and because the co-formulants are soluble in water and thus are absorbed differently by the body (Babich, 1982; Vercruysse & Rew, 2001), which moxidectin is not, direct contact of seeds and co-formulants within the gastrointestinal tract is very unlikely. Contact in faeces is also not a realistic option, since co-formulants are excreted fast via urine and not faeces (Witschi *et al.*, 1989; Shearer *et al.*, 1993; Vercruysse & Rew, 2001; Yehye *et al.*, 2015).

So it can be assumed that only for Chapter 2, the effects on emergence characteristics were caused solely by moxidectin residues when formulated moxidectin was used. For Chapters 1 and 3, the added effects of co-formulants, which have also been described as phytotoxic, such as butylated hydroxytoluene (BHT) and benzyl alcohol (BA) could be at least partially responsible for the effects (Taylorson, 1988, 1989; Zamyatnina *et al.*, 2003; Tang *et al.*, 2013; Zhao *et al.*, 2019). In Chapter 1, the effects of pure MLs, dissolved in a small amount of ethanol (0.25 % of final solution) and then water, were also investigated. When comparing the results for all MLs used in Chapter 1, it becomes clear that formulated MLs had stronger effects on germination than pure MLs.

Since these experiments were not performed with “true” controls for formulated MLs, meaning the same formulation but without the ML, it cannot be ascertained, how much of the effects observed in formulated moxidectin were a results of the co-formulants or the ML alone and how much of the effects were caused by the combined effects of the co-formulants and the ML. The co-formulants might have stabilized the ML, facilitated easier passing of the testa or had effects themselves (Shoop *et al.*, 1995; Awasthi *et al.*, 2013). In Chapter 3 it can be estimated that the co-formulants were at least partially responsible for the effects caused by formulated moxidectin. Although it is not clear if this was due to the phytotoxic effects of the co-formulants themselves or linked to these substances stabilizing the moxidectin and enabling it to enter plants.

While the co-formulants might be one explanation for the effects found in this study, the active ingredient could also be responsible. In both Chapter 1 and Chapter 2, the modes-of-action with which MLs in general and especially moxidectin could have affected seeds and seedlings, could only be speculated on, since not much research has been done in this field.

Possible theories include the opening of chloride channels, which leads to depolarization of cells (Elter *et al.*, 2007) or affecting protein synthesis and function by docking on active sites, which renders them useless (Lehrer & Rheinstein, 2020; Arévalo *et al.*, 2021). Both modes-of-action could cause stress reactions, measurable via expression of reactive oxygen species (ROS) and the enzymes plants use to defend themselves from those ROS, which were described in previous studies related to the effects of MLs on grown plants (Gechev *et al.*, 2006; Suzuki *et al.*, 2014; Navrátilová *et al.*, 2020b; Langhansová *et al.*, 2021). Both of these also give an explanation as to why only the highest concentration is significantly affected: Usually, special carrier proteins are able to remove MLs from cells, but they can be overwhelmed under certain conditions (Edwards, 2003; Kiki-Mvouaka *et al.*, 2010). This could happen when their function is hindered in some way, e.g. when by genetic disposition, not enough of these proteins are produced or the number of molecules is much higher than could be dealt with (Geyer *et al.*, 2009; Kiki-Mvouaka *et al.*, 2010). In the case of this study, it is reasonable to assume that a threshold for significant ML-related effects for these plants is somewhere above the  $1 \mu\text{g g}^{-1}$  but lower than the  $5 \mu\text{g g}^{-1}$  moxidectin-concentrations used in the experiments. The thresholds of individual species could also be much higher or lower, since Chapter 1 showed, that reactions to MLs are highly species specific.

While in Chapter 1, the effects of pure moxidectin were also studied to compare the effects of MLs with and without co-formulants, this was not possible for Chapter 3, since dissolving moxidectin in an aqueous solution was essential to ensure a homogenous spread of the solutions in soil (Awasthi *et al.*, 2013, p. 7; Huang & Xu, 2022). When large amounts of MLs, dissolved in ethanol are then mixed in water, the MLs tend to flocculate and therefore are not able to move in the soil anymore. This was seen in a preliminary experiment to Chapter 1, where the goal was to establish a safe method of dissolving the pure MLs in an aqueous solution. The solutions used in Chapter 1 had low concentrations of MLs in ethanol, diluted in water, so the MLs could not flocculate and were dispersed in the solution. This would not have been possible in Chapter 3, as the amount of water needed per pot would not have been possible to inject without risking run-off of most of the solution.

Still, it is possible that the co-formulants had some effect on the gastrointestinal tract, changing the microbiome and therefore possibly the characteristics of faeces, the seeds would be excreted with for a longer period of time. Studies have shown significant changes to the gut microbiome of mammals when treated with ivermectin, which is another ML similar to moxidectin (He *et al.*, 2018; Dicks *et al.*, 2022; Belo *et al.*, 2023). Changed faecal characteristics such as a different nutrient composition (Hammer *et al.*, 2016) could alter the effects of endozoochorous spread on seeds, e.g. by differing effects of bacteria on seeds during digestion (Traveset *et al.*, 2007; Brochet *et al.*, 2010; Egea *et al.*, 2022).

If co-formulants are indeed responsible for at least part of the effects found in all Chapters, the assessment of their effects on plants are even harder to determine than previously anticipated.

Formulated MLs are used all over the world in different variations, differing in the active ingredients and their concentrations, the amount and variety of co-formulants and the way of application to animals (Vercruysse & Rew, 2002; Coop *et al.*, 2002; Campbell, 2012; McKellar & Gokbulut, 2012).

All of these could vary significantly, as even the comparison between two formulated products for the use in sheep (Cydectin® and Oramec®) has shown in Chapter 1. Therefore, more research on various application forms and active substances is needed to get more information and to verify the results obtained in the presented studies.

*Competition stress reduces the effects of formulated moxidectin on growth and vigour of temperate grassland species*

In Chapters 1 and 2, the effects of varying moxidectin concentrations on seeds were observed, while in Chapter 3, competition was added as an additional stressor. In Chapter 1 and 2, the effects of MLs on germination and emergence and their implications for whole populations in nature were discussed. Chapter 3 has shown that, although MLs might significantly affect plants, these effects are not found when competition is present. This puts the implications of Chapter 1 and 2 in question. While germination percentage most likely was affected, studies suggest that the close proximity of seeds in the experimental setup could have affected the mean germination time (Turkington *et al.*, 2005; Orrock & Christopher, 2010). After seedling emergence (Loydi *et al.*, 2015), competition is one of the most ubiquitous stressors in nature, affecting nearly all species, especially in temperate grasslands (Newbery & Newman, 1978; Novoplansky, 2009; Grace, 2012).

Studies have shown that the combination of two stressors can either enhance their effects, increasing their negative impact on the plant, or negate them, causing the stressors to cancel each other out, leading to a positive impact on the plant (Suzuki *et al.*, 2014; Ramegowda & Senthil-Kumar, 2015). Previous studies have described a variety of combinations of biotic and abiotic factors with varying results (Rizhsky *et al.*, 2004; Puckette *et al.*, 2007; Suzuki *et al.*, 2014; Pandey *et al.*, 2017; Zandalinas & Mittler, 2022). While Chapter 3 has shown that competition reduces the effects of MLs, in the other two studies, no particular stressor besides the MLs was added. For a comprehensive view on the effects of MLs on plants in the field, the effects of other stressors should be included in experiments if possible. This could include different temperatures, as MLs, including moxidectin and ivermectin are known to degrade faster at higher temperatures, possibly changing their effects on plants (Halley *et al.*, 1989a). Eichberg *et al.* (2022) found that the effects of formulated moxidectin changed depending on temperature, but this change was also dependent on species, so no clear trend as higher temperatures being beneficial or detrimental could be established. This is consistent with the results of Chapter 1, showing a high species-specificity in regards to the effects of MLs on plant. Another interesting stressor would be drought, as this is clearly detrimental to plants, but might also reduce the mobility of MLs in soil, preventing the uptake by plants in the first place (Rath *et al.*, 2016; Iglesias *et al.*, 2018).

In conclusion, these results show that experiments reliant on only one stressor might not be representative of the effects encountered in the field, where multiple stressors might affect plants and each other to a point where the actual effects could differ significantly from those observed in any kind of lab, greenhouse or common-garden experiment.

Although this thesis shed light on several previously unknown aspects of the interactions of MLs and plants, there are still open questions which could be resolved with further experiments. Suggestions for further research related to the topics in this study will be addressed in the following chapter.

## Outlook

Chapter 1 showed that there are significant differences between the effects of pure substances dissolved in ethanol and the effects of formulated substances. While the effects ethanol had on the germination parameters were not consistent across species (e.g. some species were affected by when comparing the blank and ethanol control, while others were not) the differences between formulation and pure substance were. This means that the possible effects of co-formulants cannot be overlooked. Especially for chapter 3, where formulated moxidectin was used on growing plants, it was not possible to assess how much of the damage caused in the experiment was due to the ML used (moxidectin) and how much was done by the co-formulants. The formulation was used to distribute the ML in the soil, since using the pure substance dissolved in ethanol and water (as was done in Chapter 1) risked the moxidectin molecules clinging to surface areas and organic material (Gruber *et al.* 1990). This would prevent the moxidectin molecules from reaching the roots of the plants and create pockets of very high moxidectin concentrations, while most of the soil would remain moxidectin free. These could potentially falsify the results of this study, since it was expected that MLs seeping into the soil would do so at least in a wider area, resembling livestock faeces trampled into the soil. Further research into the effects of these substances alone and in combination with MLs is necessary to determine the impact of the study conducted in Chapter 3. Studies on the effects of formulated MLs should include the effects of the individual substances, the substances in combination with one another and the substances including the ML, on plants. With sufficient means, e.g. the exact ingredient lists and concentrations, to replicate the exact contents and mixture of used formulated products, the creation of a formulation blank control would be possible. Such a control would include all co-formulants in the same concentration as in the product but would exclude the ML itself. This is necessary, since the effects of multiple chemicals could enhance each other, so testing them individually on seeds or plants would not give insight in the complete effects of formulated MLs. Experiments with a formulation blank control would determine the effects of the co-formulants and how these affect the ML. These formulations could not only help in keeping the MLs stable as a molecule but also affect their movement into seeds and plant organelles. This changes what effects they could have on plants in general.

Chapter 2 showed that, two weeks after the sheep had been treated with formulated moxidectin, both the number of emerging seedlings and the mean emergence time of said seedlings was still affected by the substance.

Chemical analysis showed that when the third trial was conducted (two weeks after initial treatment) concentration of moxidectin residues was reduced by over 98 % compared to the concentration at the first trial three days after treatment. Therefore, it can be assumed, that even this very low concentration still significantly affected the seedlings. Further research should be done to assess at which concentration and point in time the effects are not significant and the germination parameters are back on their control level.

In addition, the experiment used oral formulations, so this experiment should be repeated with other common ML application forms, such as bolus, pour-on treatment or subcutaneous injection. Using different kinds of treatments would lead to different co-formulants and MLs passing the gastrointestinal tract in different concentrations for

differing periods of time (Fort Dodge Animal Health, 2001; Cotreau *et al.*, 2003; Cobb & Boeckh, 2009; Perez). All these factors could give more insight in the validity of the results obtained in Chapter 1 and 2, widening the scope of the effects of MLs on temperate grassland species. Further research on how these different forms of ML application would affect germination and emergence parameters would give valuable information to deepen the understanding of how MLs actually influence seeds and how populations of grassland species react to different kinds of treatments.

In Chapter 3, effects of prolonged exposure to moxidectin residues on plants were found. Across all factors employed in this study, only the highest concentration ( $5 \mu\text{g g}^{-1}$ ) of formulated moxidectin led to significant effects, which all show that the exposure is detrimental to overall growth and vigour of the plants involved. So it would be important for further research and risk assessment to know, where between the 1 and  $5 \mu\text{g g}^{-1}$  concentrations the effects start to become significant or when a trend becomes visible. Studies have shown that ML-related effects could be bound to a threshold (Schinkel, 1999; Edwards, 2003; Geyer *et al.*, 2009), so to know where this possible threshold would be could give more insight into the mechanisms with which MLs actually affect plants.

Since previous studies have shown that MLs can be taken up by plants in the roots and then be transported to the leaves (Navrátilová *et al.*, 2020b; Langhansová *et al.*, 2021), it would be interesting to know, if ML residues can also be found in other organs, particularly in flowers. This could potentially mean that ML residues could be found in nectar and pollen, as was shown with neonicotinoids, a group of insecticides (Stoner & Eitzer, 2012; Zhang *et al.*, 2023), affecting pollinators and also in the seeds produced by affected plants. Since the negative effects of MLs on seeds were shown in Chapter 1 and 2, this could disadvantage those seeds before they are even fully developed. In addition, from the results gathered in Chapter 3, it can be assumed that plants that were exposed to ML residues are already negatively affected, so they would not produce as many seeds, which also could be of lower quality, reducing their germination and establishment success (Trader *et al.*, 2006; Ierna *et al.*, 2020). This could mean that exposure of plants to MLs not only reduces their vigour and fitness directly but could also be detrimental to future generations and therefore affect whole populations in the long run.

### *Conclusion*

In total, this study shows that MLs affect temperate grassland species in all stages of development to a degree which could be significant for their growth and reproductive success. In addition, the experiments conducted suggest that far more non-target organisms are affected by MLs and by more parameters than previously anticipated. Therefore more research should be conducted in this field and the scope of the experiments should be widened to include not only multiple stages of development of a single type of organism but also multiple trophic stages should be included to fully grasp the effects these substances could have on whole ecosystems.

To contain potential spreading of MLs into the environment, recommendations should be updated: Animals should only be treated when necessary, and if so, be kept indoors for longer to prevent spread of MLs by faeces. Also, when possible, treated animals should not be kept on the same pasture every time to keep the contamination on each pasture as low as possible.



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## Supplementary information

### Chapter 1

**Supplementary Table 1.** Ingredients of Oramec® and Cydectin® according to the producers' leaflets. Contents marked with “?” are mentioned on leaflet, but no concentration is given.

Ingredient (mg ml <sup>-1</sup> )	Cydectin	Oramec	Function
Moxidectin	1	--	Anthelmintic
Ivermectin	--	0.8	Anthelmintic
Benzyl alcohol [BA]	40	30	Preservative/solvent
Butylated hydroxytoluene [BHT]	2.5	--	Antioxidant/emulsifier
Ethylenediaminetetraacetic acid [EDTA]	0.27	--	Preservative
Polysorbate 80	?	--	Emulsifier
Propylene glycol	?	200	Solvent/emulsifier
Monosodium phosphate dihydrate	?	--	Buffer
Monosodium phosphate dodecahydrate	?	--	Buffer
Sodium hydroxide	?	--	Buffer
Trisodium phosphate	--	12	Acidity regulator
Phosphoric acid	?	--	Preservative
Non hazardous substances	--	757.2	Solvent

**Supplementary Table S2.** Tested plant species and information on plant family and germination percentage (GP; mean  $\pm$  SE) for the ethanol control treatment (C<sub>E</sub>).

Family	Species	Ethanol control (C <sub>E</sub> ) GP [%]
Asteraceae	<i>Achillea ptarmica</i> L.	15.4 $\pm$ 4.5
	<i>Centaurea jacea</i> L.	15.4 $\pm$ 1.7
	<i>Hypochaeris radicata</i> L.	93.4 $\pm$ 1.4
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	35.1 $\pm$ 3.9
	<i>Dianthus deltoides</i> L.	18.9 $\pm$ 1.9
	<i>Lychnis flos-cuculi</i> L.	12.3 $\pm$ 2.6
Fabaceae	<i>Hippocrepis comosa</i> L.	17.4 $\pm$ 2.2
	<i>Medicago lupulina</i> L.	18.6 $\pm$ 3.0
	<i>Trifolium aureum</i> POLLICH	70.6 $\pm$ 3.1
	<i>Trifolium pratense</i> L.	81.4 $\pm$ 1.9
Plantaginaceae	<i>Plantago lanceolata</i> L.	21.4 $\pm$ 4.1
	<i>Plantago media</i> L.	7.7 $\pm$ 2.2
	<i>Veronica officinalis</i> L.	4.9 $\pm$ 1.7
Poaceae	<i>Agrostis capillaris</i> L.	59.7 $\pm$ 4.0
	<i>Cynosurus cristatus</i> L.	75.4 $\pm$ 2.8
	<i>Lolium perenne</i> L.	53.1 $\pm$ 5.6
	<i>Phleum phleoides</i> (L.) H. KARST	1.7 $\pm$ 0.7

**Supplementary Table S3.** Mean germination percentage (GP) for the test species under the influence of three concentrations of pure moxidectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	15.4 $\pm$ 4.5	-	-	0.971	-
		0.1	13.7 $\pm$ 4.4	-1.7	-11.2		-
		1	15.1 $\pm$ 2.3	-0.3	-1.9		-
		10	14.6 $\pm$ 1.6	-0.9	-5.6		-
	<i>Centaurea jacea</i> L.	0	15.4 $\pm$ 1.7	-	-	< 0.0001	A
		0.1	27.1 $\pm$ 1.6	+11.7	+75.9		B
		1	26.6 $\pm$ 2.1	+11.1	+72.2		B
		10	28.0 $\pm$ 1.6	+12.6	+81.5		B
	<i>Hypochaeris radicata</i> L.	0	93.4 $\pm$ 1.4	-	-	0.011	A
		0.1	94.0 $\pm$ 1.8	+0.6	+0.6		AB
		1	98.9 $\pm$ 0.4	+5.4	+5.8		B
		10	92.0 $\pm$ 1.5	-1.4	-1.5		A
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	35.1 $\pm$ 3.9	-	-	0.472	-
		0.1	40.3 $\pm$ 2.6	+5.2	+14.7		-
		1	34.6 $\pm$ 5.5	-0.6	-1.6		-
		10	41.7 $\pm$ 3.4	+6.6	+18.7		-
	<i>Dianthus deltoides</i> L.	0	18.9 $\pm$ 1.9	-	-	0.163	-
		0.1	16.0 $\pm$ 3.2	-2.9	-15.2		-
		1	24.0 $\pm$ 2.3	+5.1	+27.3		-
		10	17.7 $\pm$ 2.6	-1.2	-6.1		-
	<i>Lychnis flos-cuculi</i> L.	0	12.3 $\pm$ 2.6	-	-	0.293	-
		0.1	20.9 $\pm$ 3.1	+8.6	+69.7		-
		1	17.4 $\pm$ 3.5	+5.1	+41.8		-
		10	19.1 $\pm$ 3.8	+6.9	+55.7		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	17.4 $\pm$ 2.2	-	-	0.099	-
		0.1	21.1 $\pm$ 1.4	+3.7	+21.3		-
		1	22.0 $\pm$ 2.1	+4.6	+26.2		-
		10	15.4 $\pm$ 2.2	-2.0	-11.5		-
	<i>Medicago lupulina</i> L.	0	18.6 $\pm$ 3.0	-	-	0.938	-
		0.1	16.3 $\pm$ 2.0	-2.3	-12.3		-
		1	18.0 $\pm$ 2.8	-0.6	-3.1		-
		10	17.4 $\pm$ 1.9	-1.1	-6.1		-
	<i>Trifolium aureum</i> POLLICH	0	70.6 $\pm$ 3.1	-	-	0.675	-
		0.1	72.3 $\pm$ 2.7	+1.7	+2.4		-
		1	68.9 $\pm$ 3.0	-1.7	-2.4		-
		10	67.7 $\pm$ 2.3	-2.9	-4.1		-
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 1.9	-	-	0.748	-
		0.1	85.4 $\pm$ 1.7	+4.0	+4.9		-
		1	81.7 $\pm$ 2.6	+0.3	+0.3		-
		10	82.6 $\pm$ 4.5	+1.1	+1.4		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.4 $\pm$ 4.1	-	-	0.043	A
		0.1	31.4 $\pm$ 5.7	+10.0	+46.7		AB
		1	33.7 $\pm$ 3.4	+12.3	+57.3		AB
		10	39.4 $\pm$ 3.1	+18.0	+84.0		B
	<i>Plantago media</i> L.	0	7.7 $\pm$ 2.2	-	-	0.035	A
		0.1	10.0 $\pm$ 2.3	+2.3	+29.7		AB
		1	12.9 $\pm$ 2.7	+5.2	+66.8		AB
		10	17.1 $\pm$ 1.6	+9.4	+122.3		B
	<i>Veronica officinalis</i> L.	0	4.9 $\pm$ 1.7	-	-	0.215	-
		0.1	7.7 $\pm$ 2.8	+2.9	+58.7		-
		1	16.9 $\pm$ 6.8	+12.0	+246.9		-
		10	7.1 $\pm$ 2.3	+2.3	+47.0		-
Poaceae	<i>Agrostis capillaris</i> L.	0	59.7 $\pm$ 4.0	-	-	0.439	-
		0.1	63.1 $\pm$ 3.4	+3.4	+5.7		-
		1	54.3 $\pm$ 4.8	-5.4	-9.1		-
		10	58.3 $\pm$ 2.6	-1.4	-2.4		-
	<i>Cynosurus cristatus</i> L.	0	75.4 $\pm$ 2.8	-	-	0.466	-
		0.1	78.9 $\pm$ 1.2	+3.4	+4.6		-
		1	75.4 $\pm$ 2.6	0	0.0		-
		10	73.4 $\pm$ 2.7	-2.0	-2.7		-
	<i>Lolium perenne</i> L.	0	53.1 $\pm$ 5.6	-	-	0.701	-
		0.1	55.4 $\pm$ 4.0	+2.3	+4.3		-
		1	60.0 $\pm$ 3.8	+6.9	+12.9		-
		10	55.1 $\pm$ 3.3	+2.0	+3.8		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	1.7 $\pm$ 0.7	-	-	0.496	-
		0.1	2.3 $\pm$ 0.8	+0.6	+33.9		-
		1	2.0 $\pm$ 0.6	+0.3	+17.0		-
		10	3.4 $\pm$ 1.0	+1.7	+100.6		-

**Supplementary Table S4.** Mean germination time (MGT) for the test species under the influence of three concentrations of pure moxidectin in comparison to the ethanol control ( $C_E$ ; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	25.2 $\pm$ 1.4	-	-	0.962	-
		0.1	26.1 $\pm$ 1.9	+0.9	+3.3		-
		1	26.4 $\pm$ 1.9	+1.2	+4.6		-
		10	25.3 $\pm$ 1.6	+0.1	+0.4		-
	<i>Centaurea jacea</i> L.	0	21.3 $\pm$ 1.2	-	-	0.357	-
		0.1	20.8 $\pm$ 0.8	-0.5	-2.6		-
		1	19.1 $\pm$ 0.5	-2.2	-10.3		-
		10	20.6 $\pm$ 0.8	-0.7	-3.6		-
	<i>Hypochaeris radicata</i> L.	0	7.1 $\pm$ 0.5	-	-	<b>0.015</b>	A
		0.1	9.6 $\pm$ 0.6	+2.4	+34.1		B
		1	9.5 $\pm$ 0.8	+2.3	+33.8		B
		10	8.9 $\pm$ 0.4	+1.8	+24.3		AB
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	26.8 $\pm$ 1.4	-	-	0.784	-
		0.1	26.8 $\pm$ 1.2	0.0	+0.2		-
		1	25.8 $\pm$ 1.4	-1.0	-3.7		-
		10	25.2 $\pm$ 1.5	-1.6	-6.0		-
	<i>Dianthus deltoides</i> L.	0	26.3 $\pm$ 1.2	-	-	0.128	-
		0.1	25.9 $\pm$ 1.6	-0.4	-1.5		-
		1	27.1 $\pm$ 1.3	+0.8	+3.0		-
		10	30.0 $\pm$ 0.8	+3.7	+14.0		-
	<i>Lychnis flos-cuculi</i> L.	0	31.6 $\pm$ 1.0	-	-	0.883	-
		0.1	31.7 $\pm$ 1.3	+0.1	+0.1		-
		1	32.6 $\pm$ 1.1	+1.0	+2.9		-
		10	31.2 $\pm$ 1.5	-0.4	-1.2		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	13.0 $\pm$ 0.9	-	-	<b>0.014</b>	A
		0.1	8.9 $\pm$ 0.5	-4.1	-31.6		B
		1	10.9 $\pm$ 1.2	-2.1	-16.4		AB
		10	11.9 $\pm$ 0.8	-1.1	-9.1		AB
	<i>Medicago lupulina</i> L.	0	10.0 $\pm$ 0.5	-	-	0.356	-
		0.1	8.1 $\pm$ 1.0	-1.9	-18.6		-
		1	9.8 $\pm$ 0.6	-0.2	-1.5		-
		10	9.5 $\pm$ 1.3	-0.5	-4.5		-
	<i>Trifolium aureum</i> POLLICH	0	4.9 $\pm$ 0.7	-	-	0.577	-
		0.1	5.3 $\pm$ 0.1	+0.4	+6.9		-
		1	5.7 $\pm$ 0.4	+0.8	+16.2		-
		10	5.6 $\pm$ 0.5	+0.7	+13.8		-
	<i>Trifolium pratense</i> L.	0	6.5 $\pm$ 0.6	-	-	0.232	-
		0.1	6.3 $\pm$ 0.4	-0.2	-3.3		-
		1	5.2 $\pm$ 0.4	-1.3	-20.4		-
		10	5.6 $\pm$ 0.5	-0.9	-13.4		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.5 $\pm$ 1.4	-	-	0.707	-
		0.1	22.2 $\pm$ 0.6	+0.7	+3.5		-
		1	22.9 $\pm$ 0.6	+1.4	+6.5		-
		10	21.8 $\pm$ 0.9	+0.3	+1.5		-
	<i>Plantago media</i> L.	0	24.3 $\pm$ 1.8	-	-	0.564	-
		0.1	25.0 $\pm$ 2.7	+0.7	+2.8		-
		1	24.0 $\pm$ 1.4	-0.3	-1.3		-
		10	21.5 $\pm$ 0.4	-2.8	-11.7		-
	<i>Veronica officinalis</i> L.	0	29.4 $\pm$ 1.1	-	-	0.265	-
		0.1	31.6 $\pm$ 1.3	+2.3	+7.7		-
		1	32.2 $\pm$ 1.6	+2.8	+9.5		-
		10	29.2 $\pm$ 0.7	-0.2	-0.7		-
Poaceae	<i>Agrostis capillaris</i> L.	0	13.6 $\pm$ 0.2	-	-	<b>0.009</b>	A
		0.1	13.3 $\pm$ 0.5	-0.3	-2.7		A
		1	11.2 $\pm$ 0.7	-2.4	-18.1		B
		10	12.5 $\pm$ 0.6	-1.1	-8.3		AB
	<i>Cynosurus cristatus</i> L.	0	17.9 $\pm$ 0.5	-	-	0.729	-
		0.1	19.4 $\pm$ 1.4	+1.5	+8.2		-
		1	17.9 $\pm$ 0.7	0.0	0.0		-
		10	19.5 $\pm$ 1.8	+1.6	+8.6		-
	<i>Lolium perenne</i> L.	0	10.7 $\pm$ 0.3	-	-	<b>0.037</b>	AB
		0.1	11.5 $\pm$ 0.5	+0.8	+8.0		A
		1	11.1 $\pm$ 0.6	+0.4	+3.5		AB
		10	9.6 $\pm$ 0.3	-1.1	-10.1		B
	<i>Phleum phleoides</i> (L.) H. KARST.	0	30.8 $\pm$ 1.8	-	-	0.707	-
		0.1	30.5 $\pm$ 2.1	-0.3	-0.7		-
		1	27.6 $\pm$ 2.6	-3.2	-10.2		-
		10	30.1 $\pm$ 2.2	-0.7	-2.1		-

**Supplementary Table S5.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of pure moxidectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.28 ± 0.13	-	-	0.309	-
		0.1	0.24 ± 0.06	-0.04	-14.3		-
		1	0.32 ± 0.09	+0.04	+14.3		-
		10	0.12 ± 0.03	-0.16	-57.1		-
	<i>Centaurea jacea</i> L.	0	0.18 ± 0.02	-	-	0.604	-
		0.1	0.14 ± 0.01	-0.04	-22.2		-
		1	0.16 ± 0.04	-0.02	-11.1		-
		10	0.18 ± 0.02	0	0.0		-
	<i>Hypochaeris radicata</i> L.	0	0.47 ± 0.06	-	-	0.831	-
		0.1	0.44 ± 0.06	-0.03	-6.4		-
		1	0.44 ± 0.04	-0.03	-6.4		-
		10	0.54 ± 0.09	+0.07	+15.0		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.17 ± 0.02	-	-	0.772	-
		0.1	0.21 ± 0.03	+0.04	+23.5		-
		1	0.22 ± 0.05	+0.05	+29.4		-
		10	0.24 ± 0.05	+0.07	+41.2		-
	<i>Dianthus deltoides</i> L.	0	0.23 ± 0.07	-	-	0.699	-
		0.1	0.15 ± 0.02	-0.08	-34.8		-
		1	0.19 ± 0.05	-0.04	-17.4		-
		10	0.19 ± 0.02	-0.04	-17.4		-
	<i>Lychnis flos-cuculi</i> L.	0	0.23 ± 0.07	-	-	0.483	-
		0.1	0.26 ± 0.04	+0.03	+13.0		-
		1	0.37 ± 0.11	+0.14	+60.9		-
		10	0.24 ± 0.08	+0.01	+4.4		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.25 ± 0.07	-	-	0.338	-
		0.1	0.36 ± 0.03	+0.11	+44.0		-
		1	0.23 ± 0.04	-0.02	-8.0		-
		10	0.27 ± 0.07	+0.02	+8.0		-
	<i>Medicago lupulina</i> L.	0	0.22 ± 0.04	-	-	0.401	-
		0.1	0.22 ± 0.01	0	0.0		-
		1	0.16 ± 0.04	-0.06	-27.3		-
		10	0.24 ± 0.05	+0.02	+9.1		-
	<i>Trifolium aureum</i> POLLICH	0	0.37 ± 0.03	-	-	<b>0.045</b>	A
		0.1	0.28 ± 0.01	-0.09	-24.3		A
		1	0.31 ± 0.02	-0.06	-16.2		A
		10	0.36 ± 0.02	-0.01	-2.7		A
	<i>Trifolium pratense</i> L.	0	0.52 ± 0.06	-	-	0.167	-
		0.1	0.39 ± 0.02	-0.13	-25.0		-
		1	0.42 ± 0.02	-0.1	-19.2		-
		10	0.4 ± 0.06	-0.12	-23.1		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.14 ± 0.02	-	-	0.096	-
		0.1	0.2 ± 0.03	+0.06	+42.9		-
		1	0.14 ± 0.01	0	0.0		-
		10	0.15 ± 0.02	+0.01	+7.1		-
	<i>Plantago media</i> L.	0	0.17 ± 0.07	-	-	0.194	-
		0.1	0.16 ± 0.07	-0.01	-5.9		-
		1	0.25 ± 0.06	+0.08	+47.1		-
		10	0.33 ± 0.07	+0.16	+94.1		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	0.957	-
		0.1	0.27 ± 0.15	+0.14	+104.0		-
		1	0.34 ± 0.14	+0.21	+158.8		-
		10	0.31 ± 0.16	+0.18	+137.6		-
Poaceae	<i>Agrostis capillaris</i> L.	0	0.34 ± 0.05	-	-	0.315	-
		0.1	0.29 ± 0.02	-0.05	-14.7		-
		1	0.36 ± 0.05	+0.02	+5.9		-
		10	0.27 ± 0.02	-0.07	-20.6		-
	<i>Cynosurus cristatus</i> L.	0	0.33 ± 0.04	-	-	0.576	-
		0.1	0.36 ± 0.04	+0.03	+9.1		-
		1	0.34 ± 0.03	+0.01	+3.0		-
		10	0.29 ± 0.03	-0.04	-12.1		-
	<i>Lolium perenne</i> L.	0	0.45 ± 0.06	-	-	0.059	-
		0.1	0.4 ± 0.05	-0.05	-11.1		-
		1	0.39 ± 0.05	-0.06	-13.3		-
		10	0.59 ± 0.06	+0.14	+31.1		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.5 ± 0.5	-	-	0.283	-
		0.1	0.17 ± 0.17	-0.33	-66.0		-
		1	1	+0.5	+100.0		-
		10	0.11 ± 0.11	-0.39	-78.0		-

**Supplementary Table S6.** Mean germination percentage (GP) for the test species under the influence of three concentrations of formulated moxidectin (Cydectin) in comparison to the blank control ( $C_w$ ; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	29.1 $\pm$ 9.1	-	-	<b>0.0002</b>	A
		0.1	19.1 $\pm$ 5.1	-10.0	-34.3		AB
		1	9.1 $\pm$ 3.1	-20.0	-68.6		BC
		10	0.6 $\pm$ 0.4	-28.6	-98.0		C
	<i>Centaurea jacea</i> L.	0	25.7 $\pm$ 2.5	-	-	< <b>0.0001</b>	A
		0.1	28.9 $\pm$ 3.5	+3.2	+12.3		A
		1	25.1 $\pm$ 2.7	-0.6	-2.2		A
		10	10.9 $\pm$ 0.9	-14.9	-57.8		B
	<i>Hypochaeris radicata</i> L.	0	92.0 $\pm$ 2.0	-	-	< <b>0.0001</b>	A
		0.1	95.1 $\pm$ 1.7	+3.1	+3.4		A
		1	86.0 $\pm$ 3.1	-6.0	-6.5		A
		10	70.0 $\pm$ 4.4	-22.0	-23.9		B
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	47.1 $\pm$ 2.7	-	-	< <b>0.0001</b>	A
		0.1	36.3 $\pm$ 3.5	-10.9	-23.0		AB
		1	32.9 $\pm$ 3.2	-14.3	-30.3		B
		10	0.6 $\pm$ 0.4	-46.6	-98.8		C
	<i>Dianthus deltoides</i> L.	0	63.4 $\pm$ 4.8	-	-	< <b>0.0001</b>	A
		0.1	59.1 $\pm$ 4.6	-4.3	-6.8		AB
		1	38.9 $\pm$ 7.5	-24.6	-38.7		B
		10	2.0 $\pm$ 1.1	-61.4	-96.8		C
	<i>Lychnis flos-cuculi</i> L.	0	55.7 $\pm$ 3.9	-	-	< <b>0.0001</b>	A
		0.1	35.7 $\pm$ 4.7	-20.0	-35.9		B
		1	33.1 $\pm$ 4.9	-22.6	-40.5		B
		10	0.9 $\pm$ 0.4	-54.9	-98.5		C
Fabaceae	<i>Hippocrepis comosa</i> L.	0	22.3 $\pm$ 2.5	-	-	0.217	-
		0.1	18.0 $\pm$ 2.2	-4.3	-19.2		-
		1	15.7 $\pm$ 1.4	-6.6	-29.5		-
		10	20.0 $\pm$ 2.4	-2.3	-10.3		-
	<i>Medicago lupulina</i> L.	0	25.4 $\pm$ 4.7	-	-	0.613	-
		0.1	24.6 $\pm$ 1.6	-0.9	-3.4		-
		1	21.7 $\pm$ 3.2	-3.7	-14.6		-
		10	20.0 $\pm$ 3.4	-5.4	-21.4		-
	<i>Trifolium aureum</i> POLLICH	0	71.4 $\pm$ 3.3	-	-	<b>0.030</b>	A
		0.1	68.9 $\pm$ 1.8	-2.6	-3.6		AB
		1	68.3 $\pm$ 2.6	-3.1	-4.4		AB
		10	49.4 $\pm$ 8.1	-22.0	-30.8		B
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 2.6	-	-	0.341	-
		0.1	86.3 $\pm$ 3.3	+4.9	+6.0		-
		1	81.7 $\pm$ 2.9	+0.3	+0.3		-
		10	76.9 $\pm$ 4.9	-4.6	-5.6		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	34.0 $\pm$ 3.1	-	-	<b>0.014</b>	A
		0.1	32.6 $\pm$ 3.82	-1.4	-4.2		A
		1	29.4 $\pm$ 3.0	-4.6	-13.4		AB
		10	18.9 $\pm$ 3.8	-15.1	-44.5		B
	<i>Plantago media</i> L.	0	22.0 $\pm$ 3.2	-	-	< <b>0.0001</b>	A
		0.1	3.4 $\pm$ 0.7	-18.6	-84.4		BC
		1	5.1 $\pm$ 0.7	-16.9	-76.6		B
		10	1.7 $\pm$ 0.5	-20.3	-92.2		C
	<i>Veronica officinalis</i> L.	0	5.4 $\pm$ 0.9	-	-	0.129	-
		0.1	10.6 $\pm$ 5.1	+5.2	+95.0		-
		1	4.6 $\pm$ 1.8	+0.9	-15.7		-
		10	0.9 $\pm$ 0.9	-4.6	-84.2		-
Poaceae	<i>Agrostis capillaris</i> L.	0	70.6 $\pm$ 4.3	-	-	< <b>0.0001</b>	A
		0.1	65.1 $\pm$ 3.1	-5.4	-7.7		AB
		1	53.7 $\pm$ 3.2	-16.9	-23.9		B
		10	4.9 $\pm$ 1.1	-65.7	-93.1		C
	<i>Cynosurus cristatus</i> L.	0	78.3 $\pm$ 4.1	-	-	< <b>0.0001</b>	A
		0.1	83.7 $\pm$ 1.1	+5.4	+6.9		B
		1	78.9 $\pm$ 1.6	+0.6	+0.7		B
		10	32.9 $\pm$ 5.0	-45.4	-58.0		B
	<i>Lolium perenne</i> L.	0	53.7 $\pm$ 5.5	-	-	0.255	-
		0.1	62.9 $\pm$ 1.6	+9.2	+17.0		-
		1	62.9 $\pm$ 5.4	+9.2	+17.0		-
		10	52.3 $\pm$ 5.2	-1.4	-2.6		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	7.4 $\pm$ 0.8	-	-	< <b>0.0001</b>	A
		0.1	2.0 $\pm$ 1.2	-5.4	-73.1		B
		1	4.6 $\pm$ 0.8	-2.9	-38.5		A
		10	0	-7.4	-100.0		B

**Supplementary Table S7.** Mean germination time (MGT) for the test species under the influence of three concentrations of formulated moxidectin (Cydectin) in comparison to the blank control ( $C_w$ ; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	14.5 $\pm$ 2.3	-	-	<b>0.034</b>	A
		0.1	17.0 $\pm$ 2.9	+2.5	+17.3		AB
		1	26.0 $\pm$ 1.7	+11.5	+78.7		B
		10	25.5 $\pm$ 10.5	+11.0	+75.5		AB
	<i>Centaurea jacea</i> L.	0	22.1 $\pm$ 0.9	-	-	0.190	-
		0.1	19.4 $\pm$ 1.9	-2.7	-12.3		-
		1	21.9 $\pm$ 1.1	-0.2	-1.0		-
		10	24.6 $\pm$ 2.4	+2.5	+11.5		-
	<i>Hypochaeris radicata</i> L.	0	8.5 $\pm$ 0.3	-	-	< 0.0001	A
		0.1	6.4 $\pm$ 0.6	-2.1	-24.7		A
		1	7.5 $\pm$ 0.6	-1.0	-12.2		A
		10	11.7 $\pm$ 1.0	+3.2	+36.9		B
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	10.4 $\pm$ 0.3	-	-	< 0.0001	A
		0.1	18.9 $\pm$ 1.6	+8.5	+82.1		B
		1	22.7 $\pm$ 1.9	+12.3	+119.2		BC
		10	30.5 $\pm$ 1.5	+20.1	+194.3		C
	<i>Dianthus deltoides</i> L.	0	9.8 $\pm$ 0.3	-	-	< 0.0001	A
		0.1	11.6 $\pm$ 0.8	+1.8	+18.5		AB
		1	14.8 $\pm$ 2.5	+5.0	+51.1		B
		10	37.1 $\pm$ 1.7	+27.3	+279.4		C
	<i>Lychnis flos-cuculi</i> L.	0	19.3 $\pm$ 2.1	-	-	0.893	-
		0.1	19.0 $\pm$ 2.0	-0.3	-1.5		-
		1	20.1 $\pm$ 1.8	+0.8	+4.5		-
		10	24.0 $\pm$ 8.1	+4.7	+24.5		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	9.6 $\pm$ 0.8	-	-	<b>0.008</b>	A
		0.1	10.6 $\pm$ 0.6	+1.0	+11.2		AB
		1	10.8 $\pm$ 0.9	+1.2	+13.0		AB
		10	13.4 $\pm$ 0.5	+3.8	+39.8		B
	<i>Medicago lupulina</i> L.	0	6.5 $\pm$ 1.0	-	-	0.247	-
		0.1	8.9 $\pm$ 0.8	+2.4	+38.4		-
		1	8.2 $\pm$ 1.6	+1.7	+26.4		-
		10	7.4 $\pm$ 0.5	+0.9	+14.2		-
	<i>Trifolium aureum</i> POLLICH	0	5.1 $\pm$ 0.3	-	-	< 0.0001	A
		0.1	5.3 $\pm$ 0.4	+0.2	+3.5		A
		1	5.8 $\pm$ 0.4	+0.7	+13.6		A
		10	13.4 $\pm$ 1.5	+8.3	+162.7		B
	<i>Trifolium pratense</i> L.	0	3.0 $\pm$ 0.4	-	-	< 0.0001	A
		0.1	5.7 $\pm$ 0.6	+2.7	+91.2		B
		1	6.4 $\pm$ 0.4	+3.4	+115.7		BC
		10	8.3 $\pm$ 0.5	+5.3	+178.7		C
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	15.1 $\pm$ 0.6	-	-	< 0.0001	A
		0.1	18.3 $\pm$ 1.3	+3.2	+21.6		AB
		1	21.1 $\pm$ 1.0	+6.0	+39.9		B
		10	27.1 $\pm$ 1.8	+12.0	+79.7		C
	<i>Plantago media</i> L.	0	15.0 $\pm$ 1.3	-	-	<b>0.006</b>	A
		0.1	21.0 $\pm$ 1.3	+6.0	+40.0		B
		1	22.9 $\pm$ 1.4	+7.9	+53.1		B
		10	22.0 $\pm$ 3.1	+7.0	+46.8		B
	<i>Veronica officinalis</i> L.	0	31.4 $\pm$ 2.3	-	-	0.627	-
		0.1	29.0 $\pm$ 1.0	-2.4	-7.7		-
		1	27.6 $\pm$ 1.8	-3.8	-11.9		-
		10	29.0	-2.4	-7.6		-
Poaceae	<i>Agrostis capillaris</i> L.	0	9.7 $\pm$ 0.4	-	-	< 0.0001	A
		0.1	12.6 $\pm$ 0.6	+2.9	+29.7		B
		1	13.0 $\pm$ 0.7	+3.3	+33.4		B
		10	25.0 $\pm$ 2.0	+15.3	+156.8		C
	<i>Cynosurus cristatus</i> L.	0	13.2 $\pm$ 0.4	-	-	< 0.0001	A
		0.1	14.3 $\pm$ 0.3	+1.1	+8.5		A
		1	14.5 $\pm$ 0.6	+1.3	+10.5		A
		10	33.3 $\pm$ 0.9	+20.1	+152.9		B
	<i>Lolium perenne</i> L.	0	8.4 $\pm$ 0.3	-	-	< 0.0001	A
		0.1	9.7 $\pm$ 0.9	+1.3	+15.3		A
		1	9.5 $\pm$ 0.4	+1.1	+13.2		A
		10	16.1 $\pm$ 0.8	+7.7	+92.5		B
	<i>Phleum phleoides</i> (L.) H. KARST.	0	23.5 $\pm$ 1.3	-	-	0.227	-
		0.1	29.4 $\pm$ 5.0	+5.9	+25.2		-
		1	26.8 $\pm$ 1.6	+3.3	+13.9		-
		10	-	-	-		-

**Supplementary Table S8.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of formulated moxidectin (Cydectin) in comparison to the blank control (C<sub>w</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ). \* indicates species for which no ANOVA could be calculated because germination at the highest concentration level was too low to calculate Z

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.19 ± 0.05	-	-	*	-
		0.1	0.36 ± 0.05	+0.17	+89.5		-
		1	0.35 ± 0.17	+0.16	+84.2		-
		10	-	-	-		-
	<i>Centaurea jacea</i> L.	0	0.16 ± 0.02	-	-	<b>0.028</b>	AB
		0.1	0.18 ± 0.02	+0.02	+12.5		B
		1	0.14 ± 0.02	-0.02	-12.5		AB
		10	0.08 ± 0.03	-0.08	-50.0		A
	<i>Hypochaeris radicata</i> L.	0	0.51 ± 0.05	-	-	<b>0.028</b>	AB
		0.1	0.53 ± 0.06	+0.02	+3.9		B
		1	0.42 ± 0.05	-0.09	-17.7		AB
		10	0.33 ± 0.03	-0.18	-35.3		A
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.31 ± 0.03	-	-	*	-
		0.1	0.2 ± 0.03	-0.11	-35.5		-
		1	0.2 ± 0.02	-0.11	-35.5		-
		10	-	-	-		-
	<i>Dianthus deltoides</i> L.	0	0.54 ± 0.08	-	-	0.592	-
		0.1	0.41 ± 0.04	-0.13	-24.1		-
		1	0.46 ± 0.11	-0.08	-14.8		-
		10	0.5	-0.04	-7.4		-
	<i>Lychnis flos-cuculi</i> L.	0	0.16 ± 0.02	-	-	*	-
		0.1	0.24 ± 0.02	+0.08	+50.0		-
		1	0.29 ± 0.05	+0.13	+81.3		-
		10	-	-	-		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.34 ± 0.03	-	-	0.268	-
		0.1	0.32 ± 0.05	-0.02	-5.9		-
		1	0.4 ± 0.06	+0.06	+17.7		-
		10	0.28 ± 0.04	-0.06	-17.7		-
	<i>Medicago lupulina</i> L.	0	0.42 ± 0.07	-	-	0.232	-
		0.1	0.24 ± 0.03	-0.18	-42.9		-
		1	0.42 ± 0.06	0	0		-
		10	0.39 ± 0.1	-0.03	-7.1		-
	<i>Trifolium aureum</i> POLLICH	0	0.38 ± 0.04	-	-	0.409	-
		0.1	0.36 ± 0.03	-0.02	-5.3		-
		1	0.3 ± 0.02	-0.08	-21.1		-
		10	0.43 ± 0.1	+0.05	+13.2		-
	<i>Trifolium pratense</i> L.	0	0.55 ± 0.03	-	-	0.137	-
		0.1	0.51 ± 0.06	-0.04	-7.3		-
		1	0.43 ± 0.05	-0.12	-21.8		-
		10	0.41 ± 0.04	-0.14	-25.5		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.15 ± 0.01	-	-	0.636	-
		0.1	0.23 ± 0.06	+0.08	+53.3		-
		1	0.19 ± 0.02	+0.04	+26.7		-
		10	0.21 ± 0.06	+0.06	+40.0		-
	<i>Plantago media</i> L.	0	0.24 ± 0.05	-	-	0.711	-
		0.1	0.44 ± 0.29	+0.2	+83.3		-
		1	0.39 ± 0.2	+0.15	+62.5		-
		10	-	-0.24	-100.0		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	<b>0.018</b>	A
		0.1	0.38 ± 0.05	+0.25	+189.1		AB
		1	0.62 ± 0.19	+0.49	+378.6		B
		10	1.00	+0.87	+669.2		B
Poaceae	<i>Agrostis capillaris</i> L.	0	0.54 ± 0.05	-	-	< 0.0001	A
		0.1	0.41 ± 0.06	-0.13	-24.1		A
		1	0.36 ± 0.04	-0.18	-33.3		A
		10	0.08 ± 0.08	-0.46	-85.2		B
	<i>Cynosurus cristatus</i> L.	0	0.37 ± 0.02	-	-	<b>0.0004</b>	A
		0.1	0.33 ± 0.02	-0.04	-10.8		AB
		1	0.39 ± 0.03	+0.02	+5.4		A
		10	0.25 ± 0.02	-0.12	-32.4		B
	<i>Lolium perenne</i> L.	0	0.59 ± 0.05	-	-	< 0.0001	A
		0.1	0.65 ± 0.09	+0.06	+10.2		A
		1	0.48 ± 0.06	-0.11	-18.6		A
		10	0.19 ± 0.02	-0.4	-67.8		B
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.38 ± 0.12	-	-	*	-
		0.1	0.58 ± 0.42	+0.2	+52.6		-
		1	0.1 ± 0.07	0.28	-73.7		-
		10	-	-	-		-

**Supplementary Table S9.** Mean germination percentage (GP), for the test species under the influence of three concentrations of pure ivermectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	15.4 $\pm$ 4.5	-	-	0.580	-
		0.1	20.3 $\pm$ 4.9	+4.9	+31.5		-
		1	20.6 $\pm$ 4.7	+5.1	+33.3		-
		10	28.6 $\pm$ 8.8	+13.1	+85.2		-
	<i>Centaurea jacea</i> L.	0	15.4 $\pm$ 1.7	-	-	<b>0.004</b>	A
		0.1	27.4 $\pm$ 2.1	+12.0	+77.8		B
		1	26.3 $\pm$ 2.8	+10.9	+70.4		B
		10	27.7 $\pm$ 3.3	+12.3	+79.6		B
	<i>Hypochaeris radicata</i> L.	0	93.4 $\pm$ 1.4	-	-	0.996	-
		0.1	94.0 $\pm$ 1.5	+0.6	+0.6		-
		1	93.7 $\pm$ 2.5	+0.3	+0.3		-
		10	93.4 $\pm$ 2.4	0.0	+0.0		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	35.1 $\pm$ 3.9	-	-	0.423	-
		0.1	42.9 $\pm$ 3.6	+7.7	+22.0		-
		1	40.3 $\pm$ 3.7	+5.2	+14.7		-
		10	42.3 $\pm$ 3.0	+7.2	+20.4		-
	<i>Dianthus deltoides</i> L.	0	18.9 $\pm$ 1.9	-	-	0.569	-
		0.1	29.4 $\pm$ 8.2	+10.5	+56.0		-
		1	24.0 $\pm$ 2.3	+5.1	+27.3		-
		10	24.9 $\pm$ 4.8	+6.0	+31.8		-
	<i>Lychnis flos-cuculi</i> L.	0	12.3 $\pm$ 2.6	-	-	0.102	-
		0.1	22.6 $\pm$ 3.7	+10.3	+83.7		-
		1	21.1 $\pm$ 3.4	+8.9	+72.0		-
		10	26.0 $\pm$ 5.8	+13.7	+111.6		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	17.4 $\pm$ 2.2	-	-	0.083	-
		0.1	16.6 $\pm$ 1.8	-0.9	-4.9		-
		1	22.3 $\pm$ 2.0	+4.9	+27.9		-
		10	27.4 $\pm$ 5.5	+10.0	+57.4		-
	<i>Medicago lupulina</i> L.	0	18.6 $\pm$ 3.0	-	-	0.135	-
		0.1	24.0 $\pm$ 5.4	+5.4	+29.2		-
		1	11.4 $\pm$ 2.8	-7.1	-38.5		-
		10	18.3 $\pm$ 2.7	-0.3	-1.5		-
	<i>Trifolium aureum</i> POLLICH	0	70.6 $\pm$ 3.1	-	-	0.371	-
		0.1	71.1 $\pm$ 3.0	+0.6	+0.8		-
		1	64.3 $\pm$ 5.6	-6.3	-8.9		-
		10	73.1 $\pm$ 2.1	+2.6	+3.6		-
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 1.9	-	-	0.669	-
		0.1	83.1 $\pm$ 1.6	+1.7	+2.1		-
		1	85.4 $\pm$ 2.6	+4.0	+4.9		-
		10	82.3 $\pm$ 3.0	+0.9	+1.1		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.4 $\pm$ 4.1	-	-	<b>0.048</b>	A
		0.1	33.1 $\pm$ 1.0	+11.7	+54.6		AB
		1	29.1 $\pm$ 3.9	+7.7	+36.0		AB
		10	36.0 $\pm$ 4.4	+14.6	+68.0		B
	<i>Plantago media</i> L.	0	7.7 $\pm$ 2.2	-	-	0.105	-
		0.1	15.1 $\pm$ 1.9	+7.4	+96.4		-
		1	14.3 $\pm$ 1.7	+6.6	+85.3		-
		10	16.0 $\pm$ 4.0	+8.3	+107.5		-
	<i>Veronica officinalis</i> L.	0	4.9 $\pm$ 1.7	-	-	0.120	-
		0.1	8.6 $\pm$ 2.8	+3.7	+76.4		-
		1	2.3 $\pm$ 0.8	-2.6	-53.0		-
		10	9.4 $\pm$ 3.1	+4.6	+94.0		-
Poaceae	<i>Agrostis capillaris</i> L.	0	59.7 $\pm$ 4.0	-	-	0.623	-
		0.1	56.6 $\pm$ 4.8	-3.1	-5.3		-
		1	61.7 $\pm$ 2.5	+2.0	+3.4		-
		10	62.3 $\pm$ 1.9	+2.6	+4.3		-
	<i>Cynosurus cristatus</i> L.	0	75.4 $\pm$ 2.8	-	-	0.970	-
		0.1	76.3 $\pm$ 3.5	+0.9	+1.1		-
		1	76.9 $\pm$ 2.2	+1.4	+1.9		-
		10	74.9 $\pm$ 4.3	-0.6	-0.8		-
	<i>Lolium perenne</i> L.	0	53.1 $\pm$ 5.6	-	-	0.161	-
		0.1	56.9 $\pm$ 4.3	+3.7	+7.0		-
		1	54.0 $\pm$ 3.8	+0.9	+1.6		-
		10	65.7 $\pm$ 2.3	+12.6	+23.7		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	1.7 $\pm$ 0.7	-	-	0.404	-
		0.1	3.7 $\pm$ 1.4	+2.0	+117.0		-
		1	1.7 $\pm$ 0.5	0.0	+0.0		-
		10	2.9 $\pm$ 0.9	+1.2	+67.3		-

**Supplementary Table S10.** Mean germination time (MGT) for the test species under the influence of three concentrations of pure ivermectin in comparison to the ethanol control ( $C_E$ ; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	25.2 $\pm$ 1.4	-	-	0.054	-
		0.1	28.9 $\pm$ 2.6	+3.6	+14.8		-
		1	20.8 $\pm$ 1.1	-4.4	-17.3		-
		10	27.8 $\pm$ 2.6	+2.6	+10.0		-
	<i>Centaurea jacea</i> L.	0	21.3 $\pm$ 1.2	-	-	0.657	-
		0.1	22.5 $\pm$ 1.2	+1.2	+5.6		-
		1	23.3 $\pm$ 1.006	+2.0	+8.9		-
		10	23.4 $\pm$ 1.1	+2.1	+9.6		-
	<i>Hypochaeris radicata</i> L.	0	7.1 $\pm$ 0.5	-	-	<b>0.004</b>	A
		0.1	10.2 $\pm$ 0.7	+3.1	+43.0		B
		1	8.8 $\pm$ 0.7	+1.7	+24.1		AB
		10	9.0 $\pm$ 0.3	+1.9	+26.5		AB
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	26.8 $\pm$ 1.4	-	-	0.203	-
		0.1	23.2 $\pm$ 0.9	-3.6	-13.3		-
		1	23.6 $\pm$ 1.5	-3.2	-11.7		-
		10	25.4 $\pm$ 1.1	-1.4	-5.3		-
	<i>Dianthus deltoides</i> L.	0	26.3 $\pm$ 1.2	-	-	<b>0.024</b>	AB
		0.1	20.7 $\pm$ 1.2	-5.6	-21.2		A
		1	27.1 $\pm$ 1.3	+0.8	+3.0		B
		10	26.1 $\pm$ 2.3	-0.2	-0.8		AB
	<i>Lychnis flos-cuculi</i> L.	0	31.6 $\pm$ 1.0	-	-	0.185	-
		0.1	33.7 $\pm$ 1.3	+2.1	+6.6		-
		1	29.8 $\pm$ 1.2	-1.8	-5.8		-
		10	32.3 $\pm$ 1.3	+0.7	+2.0		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	13.0 $\pm$ 0.9	-	-	<b>0.006</b>	A
		0.1	11.0 $\pm$ 0.7	-2.0	-16.0		AB
		1	8.9 $\pm$ 0.4	-4.1	-31.4		B
		10	11.2 $\pm$ 0.8	-1.8	-14.0		AB
	<i>Medicago lupulina</i> L.	0	10.0 $\pm$ 0.5	-	-	0.066	-
		0.1	8.8 $\pm$ 2.3	-1.2	-11.6		-
		1	16.5 $\pm$ 3.2	+6.5	+65.7		-
		10	10.6 $\pm$ 1.4	+0.6	+5.8		-
	<i>Trifolium aureum</i> POLLICH	0	4.9 $\pm$ 0.7	-	-	<b>0.026</b>	A
		0.1	4.8 $\pm$ 0.5	-0.1	-2.9		A
		1	6.7 $\pm$ 0.4	+1.8	+35.7		A
		10	6.1 $\pm$ 0.3	+1.2	+23.1		A
	<i>Trifolium pratense</i> L.	0	6.5 $\pm$ 0.6	-	-	<b>0.022</b>	A
		0.1	4.3 $\pm$ 0.4	-2.2	-33.9		B
		1	5.6 $\pm$ 0.6	-0.9	-13.3		AB
		10	4.6 $\pm$ 0.4	-1.9	-29.4		AB
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.5 $\pm$ 1.4	-	-	0.354	-
		0.1	20.8 $\pm$ 0.7	-0.7	-3.1		-
		1	18.7 $\pm$ 1.2	-2.8	-12.9		-
		10	20.5 $\pm$ 1	-1.0	-4.7		-
	<i>Plantago media</i> L.	0	24.3 $\pm$ 1.8	-	-	0.641	-
		0.1	22.0 $\pm$ 1.2	-2.3	-9.8		-
		1	22.4 $\pm$ 1.5	-1.9	-8.0		-
		10	21.9 $\pm$ 1	-2.4	-10.1		-
	<i>Veronica officinalis</i> L.	0	29.4 $\pm$ 1.1	-	-	0.164	-
		0.1	33.5 $\pm$ 1.9	+4.1	+14.0		-
		1	24.9 $\pm$ 4.4	-4.5	-15.2		-
		10	28.2 $\pm$ 1.2	-1.2	-4.1		-
Poaceae	<i>Agrostis capillaris</i> L.	0	13.6 $\pm$ 0.2	-	-	0.204	-
		0.1	11.9 $\pm$ 1.1	-1.7	-12.7		-
		1	13.1 $\pm$ 0.3	-0.5	-4.2		-
		10	13.2 $\pm$ 0.5	-0.4	-3.0		-
	<i>Cynosurus cristatus</i> L.	0	17.9 $\pm$ 0.5	-	-	< <b>0.0001</b>	A
		0.1	21.5 $\pm$ 0.8	+3.6	+20.0		B
		1	17.6 $\pm$ 0.4	-0.3	-1.8		A
		10	16 $\pm$ 0.7	-1.9	-11.0		A
	<i>Lolium perenne</i> L.	0	10.7 $\pm$ 0.3	-	-	0.486	-
		0.1	9.8 $\pm$ 0.5	-0.9	-8.6		-
		1	10.2 $\pm$ 0.6	-0.5	-4.8		-
		10	10.7 $\pm$ 0.5	0.0	0.0		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	30.8 $\pm$ 1.8	-	-	0.233	-
		0.1	22.9 $\pm$ 2.4	-7.9	-25.6		-
		1	27.8 $\pm$ 2.8	-3.0	-9.6		-
		10	28.9 $\pm$ 3	-1.9	-5.9		-

**Supplementary Table S11.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of pure ivermectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.28 ± 0.13	-	-	0.143	-
		0.1	0.34 ± 0.11	+0.06	+21.4		-
		1	0.12 ± 0.04	-0.16	-57.1		-
		10	0.33 ± 0.07	+0.05	+17.9		-
	<i>Centaurea jacea</i> L.	0	0.18 ± 0.02	-	-	0.912	-
		0.1	0.17 ± 0.03	-0.01	-5.6		-
		1	0.2 ± 0.03	+0.02	+11.1		-
		10	0.2 ± 0.05	+0.02	+11.1		-
	<i>Hypochaeris radicata</i> L.	0	0.47 ± 0.06	-	-	0.759	-
		0.1	0.42 ± 0.09	-0.05	-10.6		-
		1	0.39 ± 0.04	-0.08	-17.0		-
		10	0.47 ± 0.06	0.00	0.0		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.17 ± 0.02	-	-	0.119	-
		0.1	0.22 ± 0.02	+0.05	+29.4		-
		1	0.2 ± 0.03	+0.03	+17.7		-
		10	0.27 ± 0.04	+0.10	+58.8		-
	<i>Dianthus deltoides</i> L.	0	0.23 ± 0.07	-	-	0.687	-
		0.1	0.14 ± 0.03	-0.09	-39.1		-
		1	0.19 ± 0.05	-0.04	-17.4		-
		10	0.22 ± 0.08	-0.01	-4.4		-
	<i>Lychnis flos-cuculi</i> L.	0	0.23 ± 0.07	-	-	0.117	-
		0.1	0.39 ± 0.07	+0.16	+69.6		-
		1	0.19 ± 0.03	-0.04	-17.4		-
		10	0.23 ± 0.05	0.00	0.0		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.25 ± 0.07	-	-	0.129	-
		0.1	0.36 ± 0.11	+0.11	+44.0		-
		1	0.47 ± 0.07	+0.22	+88.0		-
		10	0.34 ± 0.03	+0.09	+36.0		-
	<i>Medicago lupulina</i> L.	0	0.22 ± 0.04	-	-	0.611	-
		0.1	0.33 ± 0.07	+0.11	+50.0		-
		1	0.33 ± 0.08	+0.11	+50.0		-
		10	0.27 ± 0.05	+0.05	+22.7		-
	<i>Trifolium aureum</i> POLLICH	0	0.37 ± 0.03	-	-	0.513	-
		0.1	0.32 ± 0.02	-0.05	-13.5		-
		1	0.41 ± 0.06	+0.04	+10.8		-
		10	0.35 ± 0.03	-0.02	-5.4		-
	<i>Trifolium pratense</i> L.	0	0.52 ± 0.06	-	-	<b>0.035</b>	A
		0.1	0.44 ± 0.03	-0.08	-15.4		AB
		1	0.42 ± 0.05	-0.10	-19.2		AB
		10	0.32 ± 0.03	-0.20	-38.5		B
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.14 ± 0.02	-	-	0.639	-
		0.1	0.16 ± 0.02	+0.02	+14.3		-
		1	0.18 ± 0.03	+0.04	+28.6		-
		10	0.17 ± 0.02	+0.03	+21.4		-
	<i>Plantago media</i> L.	0	0.17 ± 0.07	-	-	0.075	-
		0.1	0.15 ± 0.02	-0.02	-11.8		-
		1	0.35 ± 0.08	+0.18	+105.9		-
		10	0.27 ± 0.04	+0.10	+58.8		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	0.982	-
		0.1	0.24 ± 0.06	+0.11	+85.0		-
		1	0.5 ± 0.5	+0.37	+284.6		-
		10	0.29 ± 0.18	+0.16	+120.8		-
Poaceae	<i>Agrostis capillaris</i> L.	0	0.34 ± 0.05	-	-	0.198	-
		0.1	0.43 ± 0.06	+0.09	+26.5		-
		1	0.29 ± 0.03	-0.05	-14.7		-
		10	0.31 ± 0.03	-0.03	-8.8		-
	<i>Cynosurus cristatus</i> L.	0	0.33 ± 0.04	-	-	0.099	-
		0.1	0.27 ± 0.03	-0.06	-18.2		-
		1	0.37 ± 0.04	+0.04	+12.1		-
		10	0.42 ± 0.07	+0.09	+27.3		-
	<i>Lolium perenne</i> L.	0	0.45 ± 0.06	-	-	0.520	-
		0.1	0.55 ± 0.08	+0.10	+22.2		-
		1	0.42 ± 0.05	-0.03	-6.7		-
		10	0.46 ± 0.03	+0.01	+2.2		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.5 ± 0.5	-	-	0.569	-
		0.1	0.03 ± 0.03	-0.47	-94.0		-
		1	-	-0.50	-100.0		-
		10	0.17 ± 0.17	-0.33	-66.0		-

**Supplementary Table S12.** Mean germination percentage (GP) for the test species under the influence of three concentrations of formulated ivermectin (Oramec) in comparison to the blank control ( $C_w$ ; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	29.1 $\pm$ 9.1	-	-	0.119	-
		0.1	15.4 $\pm$ 5.4	-13.7	-47.1		-
		1	12.0 $\pm$ 3.2	-17.1	-58.8		-
		10	11.4 $\pm$ 3.1	-17.7	-60.8		-
	<i>Centaurea jacea</i> L.	0	25.7 $\pm$ 2.5	-	-	0.358	-
		0.1	29.4 $\pm$ 4.3	+3.7	+14.5		-
		1	21.7 $\pm$ 1.5	-4.0	-15.6		-
		10	26.3 $\pm$ 2.1	+0.6	+2.3		-
	<i>Hypochaeris radicata</i> L.	0	92.0 $\pm$ 2.0	-	-	0.052	-
		0.1	96.0 $\pm$ 1.2	+4.0	+4.4		-
		1	94.0 $\pm$ 3.3	+2.0	+2.2		-
		10	86.0 $\pm$ 3.0	-6.0	-6.5		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	47.1 $\pm$ 2.7	-	-	<b>0.002</b>	A
		0.1	43.7 $\pm$ 1.6	-3.4	-7.3		A
		1	39.4 $\pm$ 4.9	-7.7	-16.4		AB
		10	25.7 $\pm$ 4.5	-21.4	-45.5		B
	<i>Dianthus deltoides</i> L.	0	63.4 $\pm$ 4.8	-	-	0.331	-
		0.1	69.1 $\pm$ 3.9	+5.7	+9.0		-
		1	54.0 $\pm$ 7.2	-9.4	-14.9		-
		10	60.9 $\pm$ 6.4	-2.6	-4.1		-
	<i>Lychnis flos-cuculi</i> L.	0	55.7 $\pm$ 3.9	-	-	<b>0.0002</b>	A
		0.1	28.0 $\pm$ 2.8	-27.7	-49.7		B
		1	38.0 $\pm$ 4.3	-17.7	-31.8		B
		10	30.0 $\pm$ 4.0	-25.7	-46.2		B
Fabaceae	<i>Hippocrepis comosa</i> L.	0	22.3 $\pm$ 2.5	-	-	0.992	-
		0.1	22.3 $\pm$ 2.0	0	0.00		-
		1	21.7 $\pm$ 3.4	-0.6	-2.6		-
		10	22.0 $\pm$ 1.3	-0.3	-1.3		-
	<i>Medicago lupulina</i> L.	0	25.4 $\pm$ 4.7	-	-	0.242	-
		0.1	20.3 $\pm$ 2.0	-5.1	-20.2		-
		1	17.1 $\pm$ 2.1	-8.3	-32.6		-
		10	24.9 $\pm$ 3.2	-0.6	-2.2		-
	<i>Trifolium aureum</i> POLLICH	0	71.4 $\pm$ 3.3	-	-	0.553	-
		0.1	72.6 $\pm$ 3.3	+1.1	+1.6		-
		1	66.6 $\pm$ 3.9	-4.9	-6.8		-
		10	67.4 $\pm$ 3.3	-4.0	-5.6		-
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 2.6	-	-	0.600	-
		0.1	84.9 $\pm$ 1.6	+3.4	+4.2		-
		1	84.9 $\pm$ 1.9	+3.4	+4.2		-
		10	83.4 $\pm$ 1.9	+2.0	+2.5		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	34.0 $\pm$ 3.1	-	-	0.315	-
		0.1	35.7 $\pm$ 2.8	+1.7	+5.0		-
		1	34.0 $\pm$ 2.5	-0	0.0		-
		10	28.9 $\pm$ 2.5	-5.1	-15.1		-
	<i>Plantago media</i> L.	0	22.0 $\pm$ 3.2	-	-	< <b>0.0001</b>	A
		0.1	5.4 $\pm$ 1.4	-16.6	-75.3		B
		1	5.1 $\pm$ 1.4	-16.9	-76.6		B
		10	5.1 $\pm$ 1.7	-16.9	-76.6		B
	<i>Veronica officinalis</i> L.	0	5.4 $\pm$ 0.9	-	-	<b>0.017</b>	A
		0.1	27.1 $\pm$ 4.9	+21.7	+400.8		B
		1	8.0 $\pm$ 2.8	+2.6	+47.6		AB
		10	20.3 $\pm$ 8.4	+14.8	+274.3		AB
Poaceae	<i>Agrostis capillaris</i> L.	0	70.6 $\pm$ 4.3	-	-	0.218	-
		0.1	64.9 $\pm$ 5.5	-5.7	-8.1		-
		1	58.3 $\pm$ 5.9	-12.3	-17.4		-
		10	54.6 $\pm$ 6.5	-16.0	-22.7		-
	<i>Cynosurus cristatus</i> L.	0	78.3 $\pm$ 4.1	-	-	0.899	-
		0.1	78.6 $\pm$ 3.3	+0.3	+0.4		-
		1	81.1 $\pm$ 1.6	+2.9	+3.6		-
		10	79.4 $\pm$ 2.6	+1.1	+1.5		-
	<i>Lolium perenne</i> L.	0	53.7 $\pm$ 5.5	-	-	0.359	-
		0.1	54.6 $\pm$ 7.8	+0.9	+1.6		-
		1	65.4 $\pm$ 3.1	+11.7	+21.8		-
		10	60.9 $\pm$ 3.5	+7.2	+13.3		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	7.4 $\pm$ 0.8	-	-	<b>0.019</b>	A
		0.1	4.9 $\pm$ 1.1	-2.6	-34.6		AB
		1	6.0 $\pm$ 1.2	-1.4	-19.3		AB
		10	2.9 $\pm$ 0.6	-4.6	-61.5		B

**Supplementary Table S13.** Mean germination time (MGT) for the test species under the influence of three concentrations of formulated ivermectin (Oramec) in comparison to the blank control ( $C_w$ ; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	14.5 $\pm$ 2.3	-	-	0.108	-
		0.1	20.9 $\pm$ 2.6	+6.4	+44.2		-
		1	17.6 $\pm$ 2.5	+3.1	+20.8		-
		10	25.9 $\pm$ 4.3	+11.4	+78.2		-
	<i>Centaurea jacea</i> L.	0	22.1 $\pm$ 0.9	-	-	<b>0.023</b>	A
		0.1	16.1 $\pm$ 1.1	-6.0	-27.0		B
		1	19.4 $\pm$ 1.4	-2.7	-12.2		AB
		10	19.2 $\pm$ 1.6	-2.9	-13.3		AB
	<i>Hypochaeris radicata</i> L.	0	8.5 $\pm$ 0.3	-	-	<b>0.004</b>	A
		0.1	5.8 $\pm$ 0.4	-2.7	-32.4		B
		1	6.6 $\pm$ 0.5	-1.9	-23.1		B
		10	6.5 $\pm$ 0.6	-2.0	-23.5		B
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	10.4 $\pm$ 0.3	-	-	<b>0.002</b>	A
		0.1	16.9 $\pm$ 0.8	+6.5	+62.9		B
		1	20.5 $\pm$ 3.5	+10.1	+97.5		B
		10	17.1 $\pm$ 2.7	+6.7	+65.2		B
	<i>Dianthus deltoides</i> L.	0	9.8 $\pm$ 0.3	-	-	0.086	-
		0.1	10.2 $\pm$ 0.2	+0.4	+4.5		-
		1	14.1 $\pm$ 2.2	+4.3	+44.7		-
		10	11.7 $\pm$ 1.9	+1.9	+19.3		-
	<i>Lychnis flos-cuculi</i> L.	0	19.3 $\pm$ 2.1	-	-	0.826	-
		0.1	17.6 $\pm$ 1.1	-1.7	-8.7		-
		1	19.7 $\pm$ 1.6	+0.4	+2.2		-
		10	18.5 $\pm$ 1.1	-0.8	-4.3		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	9.6 $\pm$ 0.8	-	-	0.126	-
		0.1	13.1 $\pm$ 1.8	+3.5	+37.1		-
		1	10.4 $\pm$ 0.3	+0.8	+8.9		-
		10	11.0 $\pm$ 0.9	+1.4	+15.0		-
	<i>Medicago lupulina</i> L.	0	6.5 $\pm$ 1.0	-	-	0.102	-
		0.1	9.5 $\pm$ 1.2	+3.0	+47.2		-
		1	8.9 $\pm$ 0.9	+2.4	+38.1		-
		10	7.7 $\pm$ 0.9	+1.2	+19.2		-
	<i>Trifolium aureum</i> POLLICH	0	5.1 $\pm$ 0.3	-	-	0.428	-
		0.1	5.9 $\pm$ 0.7	+0.8	+14.8		-
		1	5.6 $\pm$ 0.4	+0.5	+10.3		-
		10	6.1 $\pm$ 0.2	+1.0	+20.2		-
	<i>Trifolium pratense</i> L.	0	3.0 $\pm$ 0.4	-	-	<b>0.011</b>	A
		0.1	3.9 $\pm$ 0.2	+0.9	+31.5		AB
		1	4.7 $\pm$ 0.3	+1.7	+58.3		B
		10	4.9 $\pm$ 0.7	+1.9	+66.0		B
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	15.1 $\pm$ 0.6	-	-	0.183	-
		0.1	18.0 $\pm$ 1.3	+2.9	+19.2		-
		1	17.8 $\pm$ 1.3	+2.7	+18.3		-
		10	15.9 $\pm$ 0.9	+0.8	+5.1		-
	<i>Plantago media</i> L.	0	15.0 $\pm$ 1.3	-	-	0.299	-
		0.1	19.0 $\pm$ 2.5	+4.0	+26.8		-
		1	20.2 $\pm$ 1.9	+5.2	+34.8		-
		10	17.1 $\pm$ 2.3	+2.1	+14.1		-
	<i>Veronica officinalis</i> L.	0	31.4 $\pm$ 2.3	-	-	0.790	-
		0.1	29.6 $\pm$ 1.5	-1.8	-5.7		-
		1	30.6 $\pm$ 2.4	-0.8	-2.5		-
		10	28.4 $\pm$ 0.9	-3.0	-9.6		-
Poaceae	<i>Agrostis capillaris</i> L.	0	9.7 $\pm$ 0.4	-	-	0.068	-
		0.1	10.8 $\pm$ 0.4	+1.1	+11.4		-
		1	12.3 $\pm$ 0.7	+2.6	+27.0		-
		10	12.4 $\pm$ 1.6	+2.7	+27.9		-
	<i>Cynosurus cristatus</i> L.	0	13.2 $\pm$ 0.4	-	-	0.280	-
		0.1	13.6 $\pm$ 0.4	+0.4	+3.7		-
		1	13.7 $\pm$ 0.5	+0.5	+3.8		-
		10	14.3 $\pm$ 0.4	+1.1	+8.8		-
	<i>Lolium perenne</i> L.	0	8.4 $\pm$ 0.3	-	-	<b>0.044</b>	A
		0.1	9.1 $\pm$ 0.3	+0.7	+8.0		AB
		1	9.4 $\pm$ 0.2	+1.0	+12.0		B
		10	8.9 $\pm$ 0.2	+0.5	+5.6		AB
	<i>Phleum phleoides</i> (L.) H. KARST.	0	23.5 $\pm$ 1.3	-	-	0.173	-
		0.1	25.8 $\pm$ 1	+2.3	+9.9		-
		1	28.3 $\pm$ 2.1	+4.8	+20.2		-
		10	28.1 $\pm$ 2	+4.6	+19.6		-

**Supplementary Table S14.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of formulated ivermectin (Oramec) in comparison to the blank control (C<sub>w</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.19 ± 0.05	-	-	0.455	-
		0.1	0.26 ± 0.08	+0.07	+36.8		-
		1	0.22 ± 0.11	+0.03	+15.8		-
		10	0.4 ± 0.13	+0.21	+110.5		-
	<i>Centaurea jacea</i> L.	0	0.16 ± 0.02	-	-	0.720	-
		0.1	0.19 ± 0.02	+0.03	+18.8		-
		1	0.22 ± 0.05	+0.06	+37.5		-
		10	0.2 ± 0.04	+0.04	+25.0		-
	<i>Hypochaeris radicata</i> L.	0	0.51 ± 0.05	-	-	0.720	-
		0.1	0.6 ± 0.05	+0.09	+17.7		-
		1	0.58 ± 0.08	+0.07	+13.7		-
		10	0.55 ± 0.06	+0.04	+7.8		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.31 ± 0.03	-	-	0.349	-
		0.1	0.31 ± 0.04	0	+0.0		-
		1	0.23 ± 0.02	-0.08	-25.8		-
		10	0.26 ± 0.05	-0.05	-16.1		-
	<i>Dianthus deltoides</i> L.	0	0.54 ± 0.08	-	-	<b>0.008</b>	A
		0.1	0.47 ± 0.05	-0.07	-12.96		AB
		1	0.29 ± 0.03	-0.25	-46.30		B
		10	0.47 ± 0.05	-0.07	-12.96		A
	<i>Lychnis flos-cuculi</i> L.	0	0.16 ± 0.02	-	-	0.854	-
		0.1	0.18 ± 0.02	+0.02	+12.5		-
		1	0.16 ± 0.02	0	0.0		-
		10	0.15 ± 0.03	-0.01	-6.3		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.34 ± 0.03	-	-	0.269	-
		0.1	0.31 ± 0.03	-0.03	-8.8		-
		1	0.48 ± 0.09	+0.14	+41.2		-
		10	0.35 ± 0.08	+0.01	+2.9		-
	<i>Medicago lupulina</i> L.	0	0.42 ± 0.07	-	-	0.553	-
		0.1	0.36 ± 0.08	-0.06	-14.3		-
		1	0.29 ± 0.04	-0.13	-31.0		-
		10	0.38 ± 0.07	-0.04	-9.5		-
	<i>Trifolium aureum</i> POLLICH	0	0.38 ± 0.04	-	-	0.522	-
		0.1	0.33 ± 0.02	-0.05	-13.2		-
		1	0.39 ± 0.04	+0.01	+2.6		-
		10	0.33 ± 0.02	-0.05	-13.2		-
	<i>Trifolium pratense</i> L.	0	0.55 ± 0.03	-	-	0.824	-
		0.1	0.55 ± 0.05	0	0.0		-
		1	0.53 ± 0.03	-0.02	-3.6		-
		10	0.51 ± 0.03	-0.04	-7.3		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.15 ± 0.01	-	-	0.688	-
		0.1	0.17 ± 0.03	+0.02	+13.3		-
		1	0.14 ± 0.01	-0.01	-6.7		-
		10	0.16 ± 0.02	+0.01	+6.7		-
	<i>Plantago media</i> L.	0	0.24 ± 0.05	-	-	0.060	-
		0.1	0.09 ± 0.07	-0.15	-62.5		-
		1	0.4 ± 0.1	+0.16	+66.7		-
		10	0.18 ± 0.08	-0.06	-25.0		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	0.308	-
		0.1	0.27 ± 0.03	+0.14	+110.3		-
		1	0.29 ± 0.14	+0.16	+121.0		-
		10	0.2 ± 0.08	+0.07	+50.2		-
Poaceae	<i>Agrostis capillaris</i> L.	0	0.54 ± 0.05	-	-	<b>0.014</b>	A
		0.1	0.38 ± 0.01	-0.16	-29.6		AB
		1	0.35 ± 0.07	-0.19	-35.2		B
		10	0.35 ± 0.02	-0.19	-35.2		B
	<i>Cynosurus cristatus</i> L.	0	0.37 ± 0.02	-	-	0.472	-
		0.1	0.4 ± 0.04	+0.03	+8.1		-
		1	0.44 ± 0.04	+0.07	+18.9		-
		10	0.37 ± 0.03	0	0.0		-
	<i>Lolium perenne</i> L.	0	0.59 ± 0.05	-	-	0.298	-
		0.1	0.62 ± 0.07	+0.03	+5.1		-
		1	0.61 ± 0.03	+0.02	+3.4		-
		10	0.72 ± 0.05	+0.13	+22.0		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.38 ± 0.12	-	-	0.107	-
		0.1	0.61 ± 0.18	+0.23	+60.5		-
		1	0.33 ± 0.05	-0.05	-13.1		-
		10	-	-0.38	-100.0		-

**Supplementary Table S15.** Mean germination percentage (GP) for the test species under the influence of three concentrations of pure abamectin in comparison to the ethanol control ( $C_E$ ; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	15.4 $\pm$ 4.5	-	-	0.078	-
		0.1	8.3 $\pm$ 1.2	-7.1	-46.3		-
		1	6.9 $\pm$ 1.4	-8.6	-55.6		-
		10	25.7 $\pm$ 9.5	+10.3	+66.7		-
	<i>Centaurea jacea</i> L.	0	15.4 $\pm$ 1.7	-	-	< 0.0001	A
		0.1	31.7 $\pm$ 2.1	+16.3	+105.5		B
		1	25.1 $\pm$ 1.4	+9.7	+63.0		BC
		10	21.1 $\pm$ 2.4	+5.7	+37.0		AC
	<i>Hypochaeris radicata</i> L.	0	93.4 $\pm$ 1.4	-	-	0.389	-
		0.1	96.6 $\pm$ 1.9	+3.1	+3.4		-
		1	94.3 $\pm$ 1.8	+0.9	+0.9		-
		10	97.1 $\pm$ 1.8	+3.7	+4.0		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	35.1 $\pm$ 3.9	-	-	0.692	-
		0.1	33.1 $\pm$ 3.0	-2.0	-5.7		-
		1	38.0 $\pm$ 4.4	+2.9	+8.1		-
		10	31.7 $\pm$ 3.4	-3.4	-9.8		-
	<i>Dianthus deltoides</i> L.	0	18.9 $\pm$ 1.9	-	-	0.163	-
		0.1	14.9 $\pm$ 5.2	-4.0	-21.2		-
		1	27.7 $\pm$ 4.5	+8.9	+47.0		-
		10	18.0 $\pm$ 4.4	-0.9	-4.6		-
	<i>Lychnis flos-cuculi</i> L.	0	12.3 $\pm$ 2.6	-	-	< 0.0001	A
		0.1	49.7 $\pm$ 3.1	+37.4	+304.5		B
		1	28.6 $\pm$ 5.5	+16.3	+132.5		C
		10	21.4 $\pm$ 3.2	+9.1	+74.4		AC
Fabaceae	<i>Hippocrepis comosa</i> L.	0	17.4 $\pm$ 2.2	-	-	0.815	-
		0.1	20.6 $\pm$ 2.5	+3.1	+18.0		-
		1	18.3 $\pm$ 2.5	+0.9	+4.9		-
		10	19.4 $\pm$ 2.4	+2.0	+11.5		-
	<i>Medicago lupulina</i> L.	0	18.6 $\pm$ 3.0	-	-	0.073	-
		0.1	21.4 $\pm$ 1.6	+2.9	+15.4		-
		1	13.7 $\pm$ 1.2	-4.9	-26.2		-
		10	15.1 $\pm$ 2.3	-3.4	-18.5		-
	<i>Trifolium aureum</i> POLLICH	0	70.6 $\pm$ 3.1	-	-	0.613	-
		0.1	71.4 $\pm$ 5.5	+0.9	+1.2		-
		1	65.1 $\pm$ 1.4	-5.4	-7.7		-
		10	68.0 $\pm$ 2.6	-2.6	-3.6		-
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 1.9	-	-	0.132	-
		0.1	81.7 $\pm$ 2.7	+0.3	+0.4		-
		1	81.7 $\pm$ 2.4	+0.3	+0.4		-
		10	89.7 $\pm$ 3.8	+8.3	+10.2		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.4 $\pm$ 4.1	-	-	0.009	A
		0.1	34.3 $\pm$ 1.0	+12.9	+60.0		B
		1	32.9 $\pm$ 2.5	+11.4	+53.3		AB
		10	21.1 $\pm$ 4.1	-0.3	-1.3		A
	<i>Plantago media</i> L.	0	7.7 $\pm$ 2.2	-	-	0.021	A
		0.1	18.3 $\pm$ 3.6	+10.6	+137.2		B
		1	14.6 $\pm$ 2.4	+6.9	+89.0		AB
		10	10.0 $\pm$ 1.3	+2.3	+29.7		AB
	<i>Veronica officinalis</i> L.	0	4.9 $\pm$ 1.7	-	-	< 0.0001	A
		0.1	33.4 $\pm$ 6.9	+28.6	+587.8		B
		1	4.3 $\pm$ 1.7	-0.6	-11.8		A
		10	7.1 $\pm$ 2.6	+2.3	+47.0		A
Poaceae	<i>Agrostis capillaris</i> L.	0	59.7 $\pm$ 4.0	-	-	0.483	-
		0.1	60.3 $\pm$ 3.5	+0.6	+1.0		-
		1	64.6 $\pm$ 3.5	+4.9	+8.1		-
		10	70.3 $\pm$ 8.3	+10.6	+17.7		-
	<i>Cynosurus cristatus</i> L.	0	75.4 $\pm$ 2.8	-	-	0.212	-
		0.1	82.0 $\pm$ 1.5	+6.6	+8.7		-
		1	75.4 $\pm$ 2.5	0.0	0.0		-
		10	75.1 $\pm$ 3.4	-0.3	-0.4		-
	<i>Lolium perenne</i> L.	0	53.1 $\pm$ 5.6	-	-	0.697	-
		0.1	55.4 $\pm$ 4.0	+2.3	+4.3		-
		1	60.0 $\pm$ 3.8	+6.9	+12.9		-
		10	55.1 $\pm$ 3.3	+2.0	+3.8		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	1.7 $\pm$ 0.7	-	-	0.078	-
		0.1	3.7 $\pm$ 1.3	+2.0	+117.2		-
		1	4.0 $\pm$ 1.2	+2.3	+133.9		-
		10	5.7 $\pm$ 0.8	+4.0	+234.2		-

**Supplementary Table S16.** Mean germination time (MGT) for the test species under the influence of three concentrations of pure abamectin in comparison to the ethanol control ( $C_E$ ; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	25.2 $\pm$ 1.4	-	-	0.159	-
		0.1	26.4 $\pm$ 2.3	+1.2	+4.6		-
		1	21.2 $\pm$ 1.0	-4.0	-16.0		-
		10	27.0 $\pm$ 2.4	+1.8	+7.0		-
	<i>Centaurea jacea</i> L.	0	21.3 $\pm$ 1.2	-	-	<b>0.016</b>	A
		0.1	16.1 $\pm$ 0.9	-5.2	-24.5		B
		1	22.4 $\pm$ 1.6	+1.1	+5.0		A
		10	19.7 $\pm$ 1.6	-1.6	-7.5		AB
	<i>Hypochaeris radicata</i> L.	0	7.1 $\pm$ 0.5	-	-	< <b>0.0001</b>	A
		0.1	6.2 $\pm$ 0.3	-0.9	-13.2		A
		1	10.6 $\pm$ 0.8	+3.5	+48.4		B
		10	9.7 $\pm$ 0.7	+2.6	+36.6		B
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	26.8 $\pm$ 1.4	-	-	0.576	-
		0.1	25.9 $\pm$ 1.4	-0.9	-3.1		-
		1	24.8 $\pm$ 0.8	-2.0	-7.4		-
		10	27.5 $\pm$ 1.6	+0.7	+2.7		-
	<i>Dianthus deltoides</i> L.	0	26.3 $\pm$ 1.2	-	-	0.128	-
		0.1	22.7 $\pm$ 2.3	-3.6	-13.5		-
		1	23.2 $\pm$ 1.1	-3.1	-11.6		-
		10	28.0 $\pm$ 1.8	+1.7	+6.5		-
	<i>Lychnis flos-cuculi</i> L.	0	31.6 $\pm$ 1.0	-	-	< <b>0.0001</b>	A
		0.1	18.7 $\pm$ 2.4	-12.9	-41.0		B
		1	32.5 $\pm$ 1.6	+0.8	+2.6		A
		10	34.3 $\pm$ 0.4	+2.7	+8.4		A
Fabaceae	<i>Hippocrepis comosa</i> L.	0	13.0 $\pm$ 0.9	-	-	<b>0.033</b>	A
		0.1	9.8 $\pm$ 0.9	-3.2	-24.9		B
		1	10.8 $\pm$ 0.3	-2.2	-17.2		AB
		10	10.6 $\pm$ 0.7	-2.5	-19.1		AB
	<i>Medicago lupulina</i> L.	0	10.0 $\pm$ 0.5	-	-	0.396	-
		0.1	10.7 $\pm$ 0.9	+0.7	+7.2		-
		1	9.1 $\pm$ 1.2	-0.9	-9.2		-
		10	8.7 $\pm$ 1.2	-1.3	-12.8		-
	<i>Trifolium aureum</i> POLLICH	0	4.9 $\pm$ 0.7	-	-	0.162	-
		0.1	5.3 $\pm$ 0.3	+0.4	+7.0		-
		1	5.3 $\pm$ 0.3	+0.4	+7.6		-
		10	6.3 $\pm$ 0.4	+1.4	+27.7		-
	<i>Trifolium pratense</i> L.	0	6.5 $\pm$ 0.6	-	-	<b>0.0003</b>	AB
		0.1	5.0 $\pm$ 0.3	-1.5	-23.4		AC
		1	6.6 $\pm$ 0.3	+0.1	+2.3		B
		10	4.1 $\pm$ 0.3	-2.4	-36.5		C
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.5 $\pm$ 1.4	-	-	0.107	-
		0.1	24.2 $\pm$ 0.8	+2.7	+12.4		-
		1	21.1 $\pm$ 0.9	-0.4	-1.7		-
		10	25.1 $\pm$ 1.9	+3.6	+16.8		-
	<i>Plantago media</i> L.	0	24.3 $\pm$ 1.8	-	-	0.169	-
		0.1	22.4 $\pm$ 0.6	-1.9	-7.8		-
		1	21.6 $\pm$ 0.8	-2.7	-11.1		-
		10	26.0 $\pm$ 2.0	+1.7	+6.7		-
	<i>Veronica officinalis</i> L.	0	29.4 $\pm$ 1.1	-	-	<b>0.028</b>	AB
		0.1	26.9 $\pm$ 0.9	-2.5	-8.4		A
		1	33.2 $\pm$ 1.9	+3.8	+13.1		B
		10	28.9 $\pm$ 1.6	-0.5	-1.6		AB
Poaceae	<i>Agrostis capillaris</i> L.	0	13.6 $\pm$ 0.2	-	-	0.116	-
		0.1	13.4 $\pm$ 0.4	-0.2	-2.0		-
		1	12.4 $\pm$ 0.3	-1.2	-8.9		-
		10	13.0 $\pm$ 0.5	-0.6	-4.7		-
	<i>Cynosurus cristatus</i> L.	0	17.9 $\pm$ 0.5	-	-	< <b>0.0001</b>	A
		0.1	13.5 $\pm$ 0.6	-4.4	-24.5		B
		1	17.6 $\pm$ 0.6	-0.3	-1.8		A
		10	19.5 $\pm$ 1.0	+1.6	+8.6		A
	<i>Lolium perenne</i> L.	0	10.7 $\pm$ 0.3	-	-	<b>0.006</b>	A
		0.1	10.2 $\pm$ 0.5	-0.5	-4.5		A
		1	9.7 $\pm$ 0.5	-1.0	-9.0		AB
		10	8.5 $\pm$ 0.3	-2.2	-20.6		B
	<i>Phleum phleoides</i> (L.) H. KARST.	0	30.8 $\pm$ 1.8	-	-	0.288	-
		0.1	23.0 $\pm$ 6.2	-7.8	-25.1		-
		1	30.7 $\pm$ 2.0	-0.1	-0.1		-
		10	30.8 $\pm$ 2.6	0.0	0		-

**Supplementary Table S17.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of pure abamectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.28 ± 0.13	-	-	0.279	-
		0.1	0.18 ± 0.07	-0.10	-34.40		-
		1	0.08 ± 0.08	-0.20	-70.24		-
		10	0.36 ± 0.14	+0.08	+27.44		-
	<i>Centaurea jacea</i> L.	0	0.18 ± 0.02	-	-	0.371	-
		0.1	0.19 ± 0.03	+0.01	+3.43		-
		1	0.21 ± 0.03	+0.03	+16.52		-
		10	0.15 ± 0.01	-0.03	-16.52		-
	<i>Hypochaeris radicata</i> L.	0	0.47 ± 0.06	-	-	0.189	-
		0.1	0.49 ± 0.02	+0.02	+3.76		-
		1	0.37 ± 0.04	-0.10	-20.77		-
		10	0.48 ± 0.05	+0.01	+2.59		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.17 ± 0.02	-	-	0.427	-
		0.1	0.26 ± 0.04	+0.09	+50.69		-
		1	0.23 ± 0.03	+0.06	+34.09		-
		10	0.24 ± 0.05	+0.07	+43.42		-
	<i>Dianthus deltoides</i> L.	0	0.23 ± 0.07	-	-	0.284	-
		0.1	0.3 ± 0.11	+0.07	+28.90		-
		1	0.2 ± 0.02	-0.03	-14.59		-
		10	0.13 ± 0.02	-0.10	-43.11		-
	<i>Lychnis flos-cuculi</i> L.	0	0.23 ± 0.07	-	-	0.756	-
		0.1	0.26 ± 0.04	+0.03	+14.11		-
		1	0.26 ± 0.07	+0.04	+16.19		-
		10	0.28 ± 0.03	+0.05	+23.29		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.25 ± 0.07	-	-	0.327	-
		0.1	0.34 ± 0.08	+0.09	+37.09		-
		1	0.35 ± 0.05	+0.10	+40.81		-
		10	0.38 ± 0.06	+0.14	+55.60		-
	<i>Medicago lupulina</i> L.	0	0.22 ± 0.04	-	-	0.747	-
		0.1	0.24 ± 0.05	+0.02	+7.27		-
		1	0.27 ± 0.03	+0.06	+25.95		-
		10	0.29 ± 0.06	+0.07	+31.43		-
	<i>Trifolium aureum</i> POLLICH	0	0.37 ± 0.03	-	-	0.134	-
		0.1	0.30 ± 0.02	-0.07	-18.54		-
		1	0.30 ± 0.03	-0.07	-18.47		-
		10	0.36 ± 0.02	-0.01	-2.41		-
	<i>Trifolium pratense</i> L.	0	0.52 ± 0.06	-	-	0.405	-
		0.1	0.44 ± 0.04	-0.08	-14.73		-
		1	0.43 ± 0.04	-0.09	-17.99		-
		10	0.5 ± 0.03	-0.02	-3.54		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.14 ± 0.02	-	-	0.120	-
		0.1	0.17 ± 0.01	+0.03	+19.52		-
		1	0.2 ± 0.02	+0.06	+40.78		-
		10	0.15 ± 0.03	+0.01	+3.30		-
	<i>Plantago media</i> L.	0	0.17 ± 0.07	-	-	0.508	-
		0.1	0.28 ± 0.06	+0.11	+64.09		-
		1	0.23 ± 0.04	+0.06	+32.61		-
		10	0.21 ± 0.05	+0.04	+25.25		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	0.975	-
		0.1	0.24 ± 0.02	+0.11	+83.87		-
		1	0.24 ± 0.07	+0.11	+88.03		-
		10	0.27 ± 0.1	+0.14	+109.25		-
Poaceae	<i>Agrostis capillaris</i> L.	0	0.34 ± 0.05	-	-	0.991	-
		0.1	0.32 ± 0.03	-0.02	-4.56		-
		1	0.32 ± 0.02	-0.02	-6.60		-
		10	0.32 ± 0.03	-0.02	-5.04		-
	<i>Cynosurus cristatus</i> L.	0	0.33 ± 0.04	-	-	0.469	-
		0.1	0.35 ± 0.01	+0.02	+5.35		-
		1	0.44 ± 0.07	+0.11	+33.50		-
		10	0.37 ± 0.05	+0.04	+12.61		-
	<i>Lolium perenne</i> L.	0	0.45 ± 0.06	-	-	0.058	-
		0.1	0.4 ± 0.05	-0.05	-11.81		-
		1	0.39 ± 0.05	-0.06	-14.38		-
		10	0.59 ± 0.06	+0.14	31.54		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.5 ± 0.5	-	-	0.851	-
		0.1	0.48 ± 0.21	-0.02	-3.33		-
		1	0.3 ± 0.24	-0.20	-40.00		-
		10	0.38 ± 0.13	-0.11	-22.22		-

**Supplementary Table S18.** Mean germination percentage (GP) for the test species under the influence of three concentrations of pure doramectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in percentage points [pp] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	GP (mean $\pm$ SE) [%]	Deviation of treatment from control [pp]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	15.4 $\pm$ 4.5	-	-	0.132	-
		0.1	8.3 $\pm$ 2.5	-7.1	-46.3		-
		1	5.4 $\pm$ 1.5	-10.0	-64.8		-
		10	10.6 $\pm$ 2.3	-4.9	-31.5		-
	<i>Centaurea jacea</i> L.	0	15.4 $\pm$ 1.7	-	-	0.052	-
		0.1	22.9 $\pm$ 3.7	+7.4	+48.1		-
		1	24.0 $\pm$ 1.6	+8.6	+55.5		-
		10	21.7 $\pm$ 1.5	+6.3	+40.7		-
	<i>Hypochaeris radicata</i> L.	0	93.4 $\pm$ 1.4	-	-	0.159	-
		0.1	96.6 $\pm$ 1.1	+3.1	+3.4		-
		1	96.0 $\pm$ 1.1	+2.6	+2.8		-
		10	96.9 $\pm$ 1.0	+3.4	+3.7		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	35.1 $\pm$ 3.9	-	-	<b>0.049</b>	AB
		0.1	44.6 $\pm$ 3.1	+9.4	+26.8		B
		1	28.6 $\pm$ 4.6	-6.6	-18.7		A
		10	40.9 $\pm$ 4.5	+5.7	+16.3		AB
	<i>Dianthus deltoides</i> L.	0	18.9 $\pm$ 1.9	-	-	<b>0.026</b>	AB
		0.1	21.1 $\pm$ 2.4	+2.3	+12.1		B
		1	14.3 $\pm$ 2.7	-4.6	-24.3		AB
		10	11.7 $\pm$ 1.6	-7.2	-37.9		A
	<i>Lychnis flos-cuculi</i> L.	0	12.3 $\pm$ 2.6	-	-	0.075	-
		0.1	8.0 $\pm$ 2.4	-4.3	-34.9		-
		1	7.1 $\pm$ 1.7	-5.2	-41.9		-
		10	13.4 $\pm$ 1.0	+1.1	+9.3		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	17.4 $\pm$ 2.2	-	-	0.685	-
		0.1	22.0 $\pm$ 6.0	+4.6	+26.2		-
		1	22.3 $\pm$ 4.2	+4.9	+27.9		-
		10	16.6 $\pm$ 2.2	-0.9	-4.9		-
	<i>Medicago lupulina</i> L.	0	18.6 $\pm$ 3.0	-	-	0.492	-
		0.1	14.3 $\pm$ 3.3	-4.3	-23.1		-
		1	12.9 $\pm$ 1.3	-5.7	-30.8		-
		10	16.0 $\pm$ 2.7	-2.6	-13.8		-
	<i>Trifolium aureum</i> POLLICH	0	70.6 $\pm$ 3.1	-	-	0.299	-
		0.1	66.6 $\pm$ 2.5	-4.0	-5.7		-
		1	66.0 $\pm$ 2.4	-4.6	-6.5		-
		10	72.3 $\pm$ 2.6	+1.7	+2.4		-
	<i>Trifolium pratense</i> L.	0	81.4 $\pm$ 1.9	-	-	0.908	-
		0.1	82.0 $\pm$ 6.6	+0.6	+0.7		-
		1	83.1 $\pm$ 2.0	+1.7	+2.1		-
		10	78.6 $\pm$ 6.7	-2.9	-3.5		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.4 $\pm$ 4.1	-	-	<b>0.045</b>	A
		0.1	32.6 $\pm$ 1.8	+11.1	+52.0		B
		1	27.1 $\pm$ 1.6	+5.7	+26.7		AB
		10	22.9 $\pm$ 3.2	+1.4	+6.7		AB
	<i>Plantago media</i> L.	0	7.7 $\pm$ 2.2	-	-	0.922	-
		0.1	8.9 $\pm$ 2.1	+1.2	+14.9		-
		1	8.3 $\pm$ 2.7	+0.6	+7.5		-
		10	6.6 $\pm$ 2.0	-1.1	-14.8		-
	<i>Veronica officinalis</i> L.	0	4.9 $\pm$ 1.7	-	-	0.056	-
		0.1	1.1 $\pm$ 0.6	-3.7	-76.5		-
		1	2.3 $\pm$ 1.1	-2.6	-53.0		-
		10	6.3 $\pm$ 1.9	+1.4	+29.3		-
Poaceae	<i>Agrostis capillaris</i> L.	0	59.7 $\pm$ 4.0	-	-	0.096	-
		0.1	68.0 $\pm$ 2.9	+8.3	+13.9		-
		1	54.9 $\pm$ 2.8	-4.9	-8.1		-
		10	68.3 $\pm$ 6.6	+8.6	+14.4		-
	<i>Cynosurus cristatus</i> L.	0	75.4 $\pm$ 2.8	-	-	0.767	-
		0.1	78.0 $\pm$ 2.1	+2.6	+3.4		-
		1	78.6 $\pm$ 2.2	+3.1	+4.2		-
		10	78.3 $\pm$ 2.4	+2.9	+3.8		-
	<i>Lolium perenne</i> L.	0	53.1 $\pm$ 5.6	-	-	0.377	-
		0.1	61.7 $\pm$ 2.2	+8.6	+16.1		-
		1	54.6 $\pm$ 4.0	+1.4	+2.7		-
		10	60.0 $\pm$ 3.8	+6.9	+12.9		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	1.7 $\pm$ 0.7	-	-	0.366	-
		0.1	2.9 $\pm$ 0.4	+1.2	+67.1		-
		1	3.4 $\pm$ 1.0	+1.7	+100.5		-
		10	2.3 $\pm$ 0.5	+0.6	+33.7		-

**Supplementary Table S19.** Mean germination time (MGT) for the test species under the influence of three concentrations of pure doramectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in days [d] and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	MGT (mean $\pm$ SE) [d]	Deviation of treatment from control [d]	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	25.2 $\pm$ 1.4	-	-	0.649	-
		0.1	23.4 $\pm$ 4.1	-1.8	-7.4		-
		1	24.8 $\pm$ 4.2	-0.4	-1.6		-
		10	27.5 $\pm$ 1.8	+2.3	+9.1		-
	<i>Centaurea jacea</i> L.	0	21.3 $\pm$ 1.2	-	-	0.556	-
		0.1	22.3 $\pm$ 0.7	+1.0	+4.5		-
		1	24.2 $\pm$ 2.1	+2.9	+13.3		-
		10	22.0 $\pm$ 1.2	+0.7	+3.0		-
	<i>Hypochaeris radicata</i> L.	0	7.1 $\pm$ 0.5	-	-	0.319	-
		0.1	7.1 $\pm$ 0.5	0.0	0.0		-
		1	8.4 $\pm$ 0.4	+1.3	+17.6		-
		10	7.6 $\pm$ 0.7	+0.5	+6.5		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	26.8 $\pm$ 1.4	-	-	0.290	-
		0.1	29.3 $\pm$ 1	+2.5	+9.3		-
		1	31.1 $\pm$ 1.5	+4.3	+16.3		-
		10	29.2 $\pm$ 1.9	+2.4	+8.9		-
	<i>Dianthus deltoides</i> L.	0	26.3 $\pm$ 1.2	-	-	<b>0.013</b>	A
		0.1	27.4 $\pm$ 1	+1.1	+4.3		AB
		1	31.6 $\pm$ 1.1	+5.3	+20.3		B
		10	28.0 $\pm$ 0.9	+1.7	+6.5		AB
	<i>Lychnis flos-cuculi</i> L.	0	31.6 $\pm$ 1.0	-	-	0.528	-
		0.1	32.0 $\pm$ 1.3	+0.4	+1.2		-
		1	28.6 $\pm$ 3.8	-3.0	-9.6		-
		10	31.7 $\pm$ 0.8	+0.1	+0.1		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	13.0 $\pm$ 0.9	-	-	0.369	-
		0.1	11.6 $\pm$ 0.5	-1.4	-11.5		-
		1	14.0 $\pm$ 1.6	+1.0	+7.0		-
		10	11.4 $\pm$ 1.0	-1.6	-12.3		-
	<i>Medicago lupulina</i> L.	0	10.0 $\pm$ 0.5	-	-	0.987	-
		0.1	10.0 $\pm$ 0.5	0.0	0.0		-
		1	10.6 $\pm$ 1.1	+0.6	+5.8		-
		10	10.1 $\pm$ 0.7	+0.1	+1.5		-
	<i>Trifolium aureum</i> POLLICH	0	4.9 $\pm$ 0.7	-	-	0.308	-
		0.1	5.5 $\pm$ 0.4	+0.6	+11.6		-
		1	6.1 $\pm$ 0.5	+1.2	+23.9		-
		10	5.7 $\pm$ 0.4	+0.8	+14.9		-
	<i>Trifolium pratense</i> L.	0	6.5 $\pm$ 0.6	-	-	0.798	-
		0.1	7.2 $\pm$ 0.6	+0.7	+11.7		-
		1	6.8 $\pm$ 0.5	+0.3	+5.2		-
		10	6.7 $\pm$ 0.5	+0.2	+2.8		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	21.5 $\pm$ 1.4	-	-	0.183	-
		0.1	25.1 $\pm$ 1.7	+3.6	+17.0		-
		1	25.6 $\pm$ 0.6	+4.1	+19.1		-
		10	24.2 $\pm$ 1.4	+2.7	+12.5		-
	<i>Plantago media</i> L.	0	24.3 $\pm$ 1.8	-	-	0.154	-
		0.1	21.7 $\pm$ 1.0	-2.6	-10.8		-
		1	24.1 $\pm$ 1.2	-0.2	-0.9		-
		10	26.6 $\pm$ 1.6	+2.3	+9.3		-
	<i>Veronica officinalis</i> L.	0	29.4 $\pm$ 1.1	-	-	0.847	-
		0.1	27.5 $\pm$ 2.5	-1.9	-6.4		-
		1	29.9 $\pm$ 2.3	+0.5	+1.9		-
		10	30 $\pm$ 1.9	+0.6	+2.0		-
Poaceae	<i>Agrostis capillaris</i> L.	0	13.6 $\pm$ 0.2	-	-	<b>0.007</b>	A
		0.1	14.1 $\pm$ 0.7	+0.5	+3.3		A
		1	12.9 $\pm$ 0.4	-0.7	-5.8		AB
		10	11.7 $\pm$ 0.5	-1.9	-14.6		B
	<i>Cynosurus cristatus</i> L.	0	17.9 $\pm$ 0.5	-	-	<b>0.0009</b>	A
		0.1	19.9 $\pm$ 0.3	+2.0	+10.8		B
		1	17.9 $\pm$ 0.3	0.0	0.0		A
		10	17.7 $\pm$ 0.3	-0.2	-1.2		A
	<i>Lolium perenne</i> L.	0	10.7 $\pm$ 0.3	-	-	0.495	-
		0.1	10.8 $\pm$ 0.5	+0.1	+0.8		-
		1	9.9 $\pm$ 0.6	-0.8	-7.1		-
		10	10.1 $\pm$ 0.5	-0.6	-5.6		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	30.8 $\pm$ 1.8	-	-	0.656	-
		0.1	29.4 $\pm$ 1.8	-1.4	-4.5		-
		1	30.5 $\pm$ 2.7	-0.3	-0.7		-
		10	33.2 $\pm$ 2.2	+2.4	+7.9		-

**Supplementary Table S20.** Mean synchrony of germination (Z) for the test species under the influence of three concentrations of pure doramectin in comparison to the ethanol control (C<sub>E</sub>; cf. Methods section). Deviations of treatment means from control means are given in absolute numbers and percentages [%]. ANOVA results for *concentration* on species level are given; significant p-values ( $p \leq 0.05$ ) are in bold. In case of significant ANOVA results, Tukey HSD test was employed; different letters indicate significant differences ( $p \leq 0.05$ ).

Family	Species	Concentration of active ingredient [mg l <sup>-1</sup> ]	Z (mean ± SE)	Deviation of treatment from control	Deviation of treatment from control [%]	ANOVA p-value	Tukey HSD significant differences
Asteraceae	<i>Achillea ptarmica</i> L.	0	0.28 ± 0.13	-	-	0.586	-
		0.1	0.26 ± 0.15	-0.02	-8.5		-
		1	0.21 ± 0.06	-0.07	-26.2		-
		10	0.11 ± 0.07	-0.17	-61.5		-
	<i>Centaurea jacea</i> L.	0	0.18 ± 0.02	-	-	0.926	-
		0.1	0.21 ± 0.03	+0.03	+16.9		-
		1	0.22 ± 0.06	+0.04	+22.1		-
		10	0.18 ± 0.02	0.00	+2.6		-
	<i>Hypochaeris radicata</i> L.	0	0.47 ± 0.06	-	-	0.924	-
		0.1	0.48 ± 0.05	+0.01	+1.6		-
		1	0.45 ± 0.03	-0.02	-4.0		-
		10	0.43 ± 0.02	-0.04	-8.6		-
Caryophyllaceae	<i>Cerastium holosteoides</i> Fr.	0	0.17 ± 0.02	-	-	0.295	-
		0.1	0.21 ± 0.02	+0.04	+23.6		-
		1	0.23 ± 0.03	+0.06	+36.0		-
		10	0.19 ± 0.02	+0.02	+9.1		-
	<i>Dianthus deltoides</i> L.	0	0.23 ± 0.07	-	-	0.124	-
		0.1	0.19 ± 0.03	-0.04	-18.4		-
		1	0.38 ± 0.11	+0.15	+66.9		-
		10	0.17 ± 0.05	-0.06	-27.1		-
	<i>Lychnis flos-cuculi</i> L.	0	0.23 ± 0.07	-	-	0.554	-
		0.1	0.39 ± 0.13	+0.16	+68.3		-
		1	0.35 ± 0.12	+0.11	+49.9		-
		10	0.22 ± 0.04	-0.01	-6.0		-
Fabaceae	<i>Hippocrepis comosa</i> L.	0	0.25 ± 0.07	-	-	0.585	-
		0.1	0.25 ± 0.04	0.00	+1.5		-
		1	0.28 ± 0.06	+0.03	+11.9		-
		10	0.42 ± 0.13	+0.17	+69.4		-
	<i>Medicago lupulina</i> L.	0	0.22 ± 0.04	-	-	0.078	-
		0.1	0.13 ± 0.04	-0.09	-41.4		-
		1	0.26 ± 0.06	+0.04	+16.0		-
		10	0.31 ± 0.05	+0.09	+38.8		-
	<i>Trifolium aureum</i> POLLICH	0	0.37 ± 0.03	-	-	0.246	-
		0.1	0.3 ± 0.03	-0.07	-19.1		-
		1	0.35 ± 0.02	-0.02	-6.2		-
		10	0.35 ± 0.02	-0.02	-5.0		-
	<i>Trifolium pratense</i> L.	0	0.52 ± 0.06	-	-	0.191	-
		0.1	0.4 ± 0.04	-0.12	-23.5		-
		1	0.43 ± 0.03	-0.09	-17.7		-
		10	0.50 ± 0.04	-0.02	-3.0		-
Plantaginaceae	<i>Plantago lanceolata</i> L.	0	0.14 ± 0.02	-	-	0.547	-
		0.1	0.19 ± 0.04	+0.05	+33.3		-
		1	0.21 ± 0.04	+0.07	+47.3		-
		10	0.18 ± 0.02	+0.04	+27.0		-
	<i>Plantago media</i> L.	0	0.17 ± 0.07	-	-	0.284	-
		0.1	0.29 ± 0.15	+0.12	+72.7		-
		1	0.40 ± 0.17	+0.23	+133.3		-
		10	0.09 ± 0.06	-0.08	-47.9		-
	<i>Veronica officinalis</i> L.	0	0.13 ± 0.07	-	-	0.534	-
		0.1	0	-0.13	-100.0		-
		1	0.58 ± 0.42	+0.45	+348.7		-
		10	0.52 ± 0.18	+0.38	+293.8		-
Poaceae	<i>Agrostis capillaris</i> L.	0	0.34 ± 0.05	-	-	0.736	-
		0.1	0.39 ± 0.05	+0.05	+14.6		-
		1	0.34 ± 0.02	0.00	-0.8		-
		10	0.38 ± 0.04	+0.04	+11.2		-
	<i>Cynosurus cristatus</i> L.	0	0.33 ± 0.04	-	-	0.656	-
		0.1	0.29 ± 0.04	-0.04	-11.4		-
		1	0.34 ± 0.02	+0.01	+2.0		-
		10	0.33 ± 0.02	0.00	-1.2		-
	<i>Lolium perenne</i> L.	0	0.45 ± 0.06	-	-	0.439	-
		0.1	0.41 ± 0.04	-0.04	-8.6		-
		1	0.5 ± 0.05	+0.05	+10.8		-
		10	0.54 ± 0.07	+0.09	+19.7		-
	<i>Phleum phleoides</i> (L.) H. KARST.	0	0.5 ± 0.5	-	-	0.352	-
		0.1	0	-0.50	-100.0		-
		1	0.22 ± 0.11	-0.28	-55.6		-
		10	0.00	-0.50	-100.0		-

**Supplementary Table S21.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of pure moxidectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>5494.6</b>	<b>≤ 0.001</b>	12	<b>21.422</b>	<b>≤ 0.001</b>	12	<b>0.050</b>	<b>≤ 0.001</b>
Family [F]	4	<b>3906.7</b>	<b>≤ 0.001</b>	4	<b>153.162</b>	<b>≤ 0.001</b>	4	<b>0.081</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>99.1</b>	<b>≤ 0.001</b>	3	0.025	0.925	3	0.010	0.094
S(F) × C	36	18.2	0.236	36	0.208	0.114	36	<b>0.008</b>	<b>0.007</b>
F × C	12	<b>29.0</b>	<b>0.037</b>	12	0.282	<b>0.050</b>	12	0.008	0.094
Error	408	15.4		395	0.159		377	0.005	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

**Supplementary Table S22.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of formulated moxidectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>627.93</b>	<b>≤ 0.001</b>	12	<b>8.759</b>	<b>≤ 0.001</b>	12	<b>0.067</b>	<b>≤ 0.001</b>
Family [F]	4	<b>561.52</b>	<b>≤ 0.001</b>	4	<b>45.858</b>	<b>≤ 0.001</b>	4	<b>0.046</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>712.57</b>	<b>≤ 0.001</b>	3	<b>13.912</b>	<b>≤ 0.001</b>	3	<b>0.017</b>	<b>0.018</b>
S(F) × C	36	<b>17.28</b>	<b>≤ 0.001</b>	35	<b>0.746</b>	<b>≤ 0.001</b>	32	<b>0.012</b>	<b>≤ 0.001</b>
F × C	12	<b>81.15</b>	<b>≤ 0.001</b>	12	<b>0.958</b>	<b>≤ 0.001</b>	12	<b>0.028</b>	<b>≤ 0.001</b>
Error	408	2.95		366	0.145		339	0.005	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

**Supplementary Table S23.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of pure ivermectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>3024.7</b>	<b>≤ 0.001</b>	12	<b>18.482</b>	<b>≤ 0.001</b>	12	<b>0.039</b>	<b>≤ 0.001</b>
Family [F]	4	<b>2303.8</b>	<b>≤ 0.001</b>	4	<b>112.046</b>	<b>≤ 0.001</b>	4	<b>0.071</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>154.7</b>	<b>≤ 0.001</b>	3	0.262	0.256	3	0.002	0.661
S(F) × C	36	11.5	0.587	36	<b>0.503</b>	<b>≤ 0.001</b>	36	0.006	0.051
F × C	12	14.1	0.326	12	<b>0.691</b>	<b>≤ 0.001</b>	12	0.007	0.078
Error	408	12.2		394	0.193		371	0.004	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

**Supplementary Table S24.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of formulated ivermectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>3030.4</b>	<b>≤ 0.001</b>	12	<b>4.727</b>	<b>≤ 0.001</b>	12	<b>0.126</b>	<b>≤ 0.001</b>
Family [F]	4	<b>2076.9</b>	<b>≤ 0.001</b>	4	<b>15.858</b>	<b>≤ 0.001</b>	4	<b>0.181</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>134.1</b>	<b>≤ 0.001</b>	3	<b>0.514</b>	<b>≤ 0.001</b>	3	0.003	0.711
S(F) × C	36	<b>41.9</b>	<b>≤ 0.001</b>	36	<b>0.100</b>	<b>≤ 0.001</b>	36	<b>0.011</b>	<b>≤ 0.001</b>
F × C	12	<b>29.3</b>	<b>0.017</b>	12	<b>0.128</b>	<b>0.002</b>	12	<b>0.016</b>	<b>≤ 0.001</b>
Error	408	13.1		400	0.049		382	0.005	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

**Supplementary Table S25.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of pure abamectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>2732.3</b>	<b>≤ 0.001</b>	12	<b>47.89</b>	<b>≤ 0.001</b>	12	<b>0.053</b>	<b>≤ 0.001</b>
Family [F]	4	<b>1892.5</b>	<b>≤ 0.001</b>	4	<b>330.81</b>	<b>≤ 0.001</b>	4	<b>0.123</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>145.5</b>	<b>≤ 0.001</b>	3	<b>9.22</b>	<b>≤ 0.001</b>	3	0.009	0.178
S(F) × C	36	<b>36.2</b>	<b>≤ 0.001</b>	36	<b>2.05</b>	<b>≤ 0.001</b>	36	0.005	0.601
F × C	12	<b>51.7</b>	<b>≤ 0.001</b>	12	<b>1.63</b>	<b>≤ 0.001</b>	12	0.005	0.513
Error	408	9.5		395	0.58		380	0.006	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

**Supplementary Table S26.** ANOVA results for effects of *species* (nested within *family*), *family* and *concentration* of pure doramectin on germination percentage, mean germination time and synchrony of germination.

Source of variation	Germination Percentage			Mean Germination Time			Synchrony		
	d.f.	MQ	p	d.f.	MQ	p	d.f.	MQ	p
Species(Family) [S(F)]	12	<b>2184.83</b>	<b>≤ 0.001</b>	12	<b>14.511</b>	<b>≤ 0.001</b>	12	<b>0.067</b>	<b>≤ 0.001</b>
Family [F]	4	<b>2017.57</b>	<b>≤ 0.001</b>	4	<b>81.435</b>	<b>≤ 0.001</b>	4	<b>0.051</b>	<b>≤ 0.001</b>
Concentration [C]	3	<b>15.83</b>	<b>0.045</b>	3	0.178	0.236	3	<b>0.028</b>	<b>0.003</b>
S(F) × C	36	<b>10.53</b>	<b>0.004</b>	36	0.130	0.406	36	0.007	0.218
F × C	12	6.79	0.309	12	0.103	0.624	12	<b>0.012</b>	<b>0.019</b>
Error	408	5.68		386	0.125		362	0.006	

d.f. = degrees of freedom; MQ = mean sum of squares. Significant results ( $p \leq 0.05$ ) are shown in bold.

## Chapter 2

**Supplementary Table S1.** Ingredients of Cydectin® 0.1 % oral drench for the application at sheep (Zoetis Deutschland GmbH, Berlin, Germany) according to the producers' leaflet. Contents marked with “?” are mentioned on leaflet, but no concentration is given.

Ingredient	Concentration (mg ml <sup>-1</sup> )	Function
Moxidectin	1.00	Anthelmintic
Benzyl alcohol [BA]	40.00	Preservative/solvent
Butylated hydroxytoluene [BHT]	2.50	Antioxidant/emulsifier
Ethylenediaminetetraacetic acid [EDTA]	0.27	Preservative
Polysorbate 80	?	Emulsifier
Propylene glycol	?	Solvent/emulsifier
Monosodium phosphate dihydrate	?	Buffer
Monosodium phosphate dodecahydrate	?	Buffer
Sodium hydroxide	?	Buffer
Phosphoric acid	?	Preservative

**Supplementary Table S2.** Number of emerged seedlings (NES) for the three consecutive seed-feeding trials carried out 1, 7 and 14 d, respectively, after the experimental sheep have been treated with the anthelmintic formulation Cydectin (moxidectin 0.1 %).

		NES [seedlings per gram of fresh faeces]			
		Anthelmintic	Control	Deviation from control	Deviation percentage [%]
Across species	Trial 1	1.26	1.73	-0.47	-27.15
	Trial 2	1.23	0.73	+0.50	+68.75
	Trial 3	1.37	0.94	+0.43	+45.18
<i>A. capillaris</i>	Trial 1	1.83	2.26	-0.43	-23.21
	Trial 2	1.71	1.20	+0.51	+30.01
	Trial 3	1.83	1.15	+0.68	+37.32
<i>A. ptarmica</i>	Trial 1	0.96	1.40	-0.44	-46.17
	Trial 2	1.15	0.59	+0.56	+48.49
	Trial 3	1.55	0.83	+0.73	+46.85
<i>D. deltoides</i>	Trial 1	0.72	1.18	-0.46	-63.71
	Trial 2	0.68	0.32	+0.36	+53.15
	Trial 3	0.78	0.49	+0.29	+37.53
<i>P. lanceolata</i>	Trial 1	1.54	2.09	-0.55	-36.04
	Trial 2	1.39	0.81	+0.58	+41.44
	Trial 3	1.30	1.31	-0.00	-0.15

**Supplementary Table S3.** Mean emergence time (MET) for the three consecutive seed-feeding trials carried out 1, 7 and 14 d, respectively, after the experimental sheep have been treated with the anthelmintic formulation Cydectin (moxidectin 0.1 %).

		MET [d]			
		Anthelmintic	Control	Deviation from control	Deviation percentage [%]
Across species	Trial 1	19.08	19.12	-0.04	-0.22
	Trial 2	18.86	20.54	-1.68	-8.18
	Trial 3	12.78	16.38	-3.61	-22.01
<i>A. capillaris</i>	Trial 1	20.07	20.98	-0.91	-4.52
	Trial 2	22.39	24.78	-2.40	-10.71
	Trial 3	14.00	20.77	-6.78	-48.42
<i>A. ptarmica</i>	Trial 1	16.13	16.22	-0.09	-0.57
	Trial 2	15.92	17.05	-1.13	-7.08
	Trial 3	9.36	12.48	-3.12	-33.36
<i>D. deltoidea</i>	Trial 1	15.99	15.49	+0.50	+3.16
	Trial 2	14.27	17.18	-2.91	-20.36
	Trial 3	12.72	17.24	-4.52	-35.52
<i>P. lanceolata</i>	Trial 1	24.14	23.81	+0.32	+1.34
	Trial 2	22.86	23.15	-0.29	-1.25
	Trial 3	15.02	15.03	-0.00	-0.02

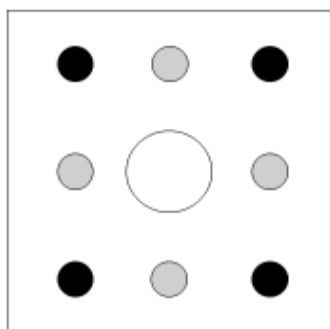
**Supplementary Table S4.** Synchrony of emergence (Z) for the three consecutive seed-feeding trials carried out 1, 7 and 14 d, respectively, after the experimental sheep have been treated with the anthelmintic formulation Cydectin (moxidectin 0.1 %).

		Z			
		Anthelmintic	Control	Deviation from control	Deviation percentage [%]
Across species	Trial 1	0.13	0.14	-0.01	-0.42
	Trial 2	0.12	0.11	+0.01	+4.16
	Trial 3	0.19	0.17	+0.02	+11.70
<i>A. capillaris</i>	Trial 1	0.12	0.14	-0.02	-12.72
	Trial 2	0.11	0.10	+0.01	+7.72
	Trial 3	0.18	0.17	+0.01	+4.12
<i>A. ptarmica</i>	Trial 1	0.15	0.15	-0.00	-1.13
	Trial 2	0.11	0.11	+0.00	+1.49
	Trial 3	0.20	0.16	+0.04	+17.45
<i>D. deltoidea</i>	Trial 1	0.13	0.14	-0.01	-3.85
	Trial 2	0.17	0.13	+0.04	+19.38
	Trial 3	0.23	0.17	+0.06	+24.88
<i>P. lanceolata</i>	Trial 1	0.13	0.12	+0.01	+7.51
	Trial 2	0.10	0.11	-0.01	-7.16
	Trial 3	0.16	0.18	-0.02	-11.61

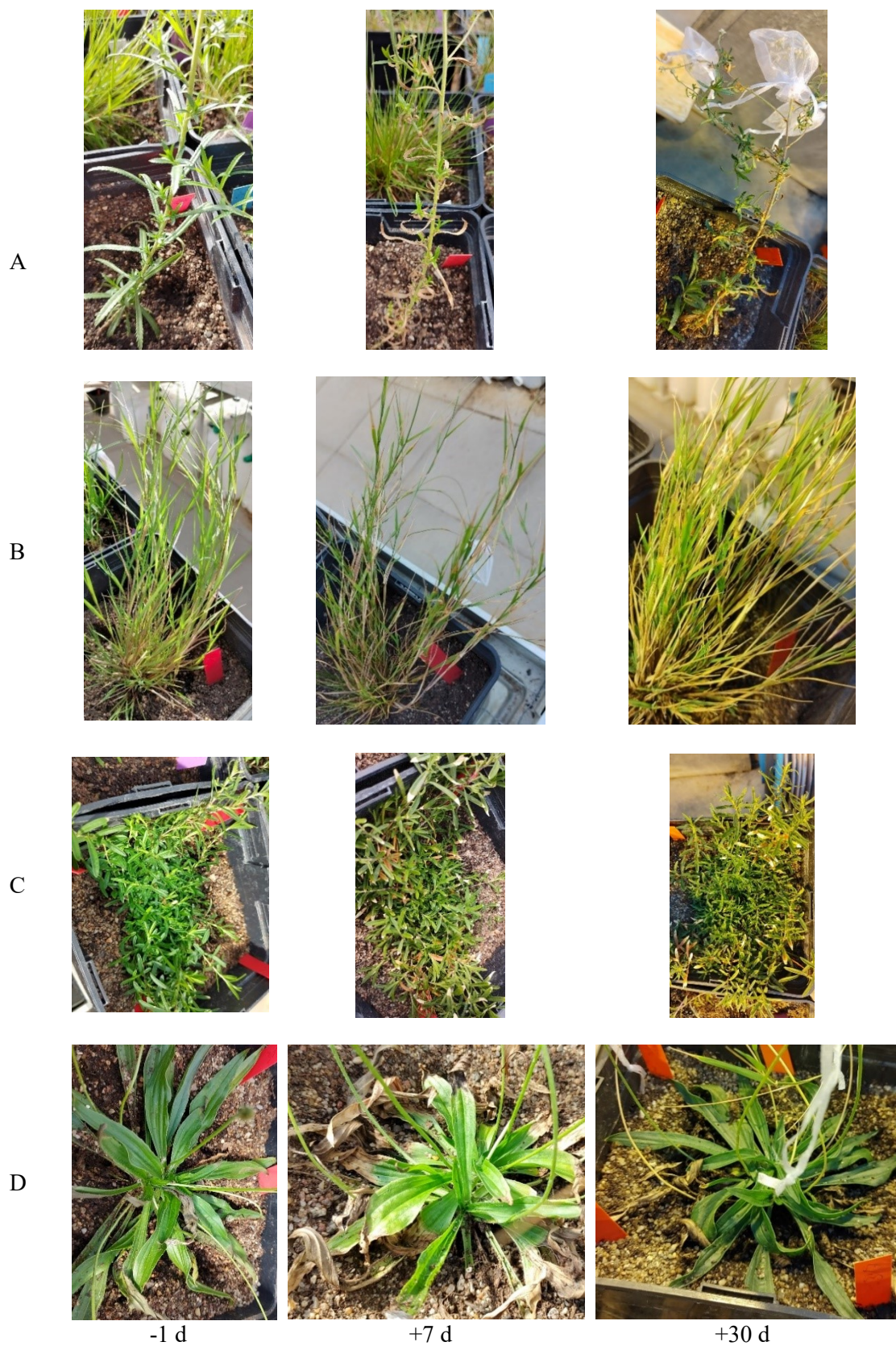
### Chapter 3

**Supplementary Table S1.** Ingredients of Cydectin® 0.1 % oral drench for the application at sheep (Zoetis Deutschland GmbH, Berlin, Germany) according to the producers' leaflet. Contents marked with “?” are mentioned on leaflet, but no concentration is given.

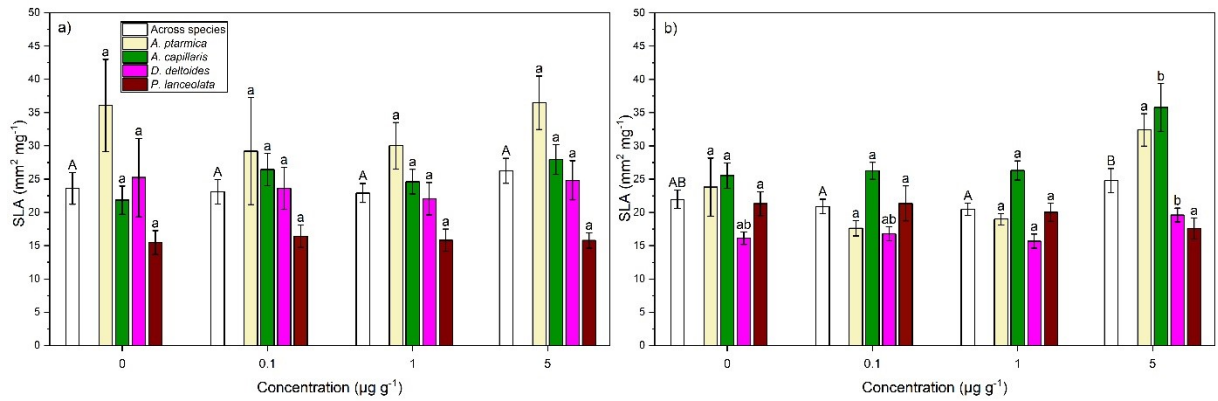
Ingredient	Concentration (mg ml <sup>-1</sup> )	Function
Moxidectin	1.00	Anthelmintic
Benzyl alcohol [BA]	40.00	Preservative/solvent
Butylated hydroxytoluene [BHT]	2.50	Antioxidant/emulsifier
Ethylenediaminetetraacetic acid [EDTA]	0.27	Preservative
Polysorbate 80	?	Emulsifier
Propylene glycol	?	Solvent/emulsifier
Monosodium phosphate dihydrate	?	Buffer
Monosodium phosphate dodecahydrate	?	Buffer
Sodium hydroxide	?	Buffer
Phosphoric acid	?	Preservative



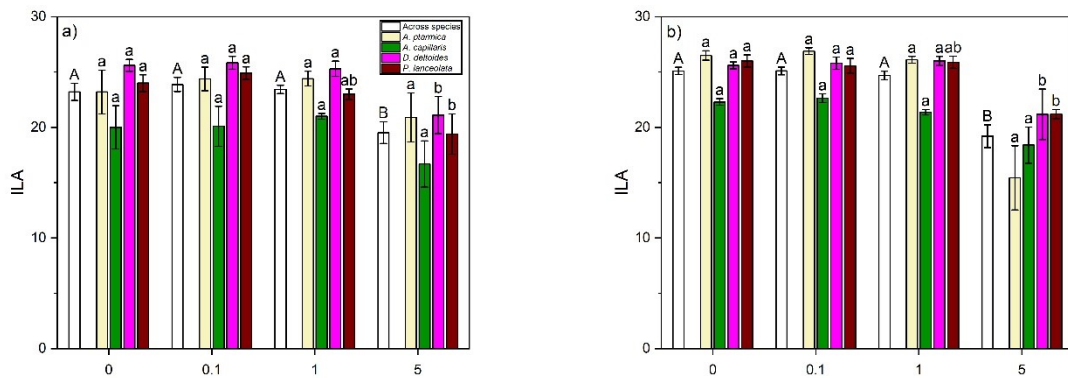
**Supplementary Figure S2** Position of main plant (white), competition plants (grey) and injection spots (black).



**Supplementary Figure S3:** Visible morphological symptoms after application of  $5 \mu\text{g g}^{-1}$  Cydectin (MOX) on plant individuals of A) *Agrostis capillaris*, B) *Achillea ptarmica*, C) *Dianthus deltoides*, D) *Plantago lanceolata* 1 day before last application, 7 and 30 days after last application. Arrows indicate tip dieback.



**Supplementary Figure S4** Effects of moxidectin concentration on Specific Leaf Area (SLA) of all species and each individual species for the plants with competition (A) and without competition (B). Different letters indicate significant differences between trials on across-species level (capitals) and on species level (lower case), respectively ( $p \leq 0.05$ , Tukey HSD test).



**Supplementary Figure S5** Effects of moxidectin concentration on intact leaf area (ILA) of all species and each individual species for the plants with competition (a) and without competition (b) summed up over the duration of the experiment. Measured during the observation period with these values: 100-76 % = 4; 75-51 % = 3; 50-26 % = 2; 25-0 % = 1; 0 % (Dead) = 0. Different letters indicate significant differences between trials on across-species level (capitals) and on species level (lower case), respectively ( $p \leq 0.05$ , Tukey HSD test).