Universidade de Aveiro

Ano 2022

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A influência do pH do solo no comportamento e toxicidade do Dazomet para promover uma Agricultura sustentável

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Isabel Maira da Cunha Antunes Lopes, Investigadora Principal no Departamento de Biologia da Universidade de Aveiro, do Professor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Professor José Paulo Filipe Afonso de Sousa, Professor Associado na Universidade de Coimbra.

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Há um prazer nos bosques inacessíveis,

Há um enlevamento na costa solitária;

Há uma sociedade onde ninguém penetra,

Junto ao mar profundo, com música no seu rugido:

Não amo menos o homem, mas mais a natureza.

Lord Byron

Palavras-chave Alterações Globais, Agricultura, Sustentabilidade, Análise de Risco, Pesticida fumigant, pH do solo

Resumo

A produção alimentar, como base da subsistência Humana, depende diretamente da agricultura e da sua produtividade. Para a obtenção de elevadas taxas de produção e diminuição de perdas de culturas devido a invasões por pragas, este sector recorre frequentemente e de forma intensiva à utilização de productos protetores de plantas (PPP). Apesar dos benefícios associados à utilização de PPP, estes, ao longo do tempo e juntamente com a sua indevida utilização, culminam em efeitos de diferentes severidades no meio ambiente. Desta forma, acresce-se a necessidade de perceber os efeitos destes compostos no biota em cenários de exposição reais, para se poderem explorar formas mais sustentáveis e benéficas para o ambiente na utilização de PPP. Assim, foi selecionado para este estudo o fumigante Basamid® (composto pela substância activa dazomet) que está em livre comercialização há mais de 10 anos, sendo que foi estendida a sua avaliação pelas autoridades regulamentares (EFSA e Comissão Europeia) por mais 5 anos. Este fumigante atua como fungicida, herbicida, nematocida e esterilizante, e fatores abióticos como a humidade, o pH e matéria orgânica do solo afetam o seu comportamento nas matrizes ambientais, influenciando a velocidade da sua hidrólise, e, desta forma, alterando também o seu tempo de meia vida. Deste modo, o presente trabalho teve como principal objetivo investigar o efeito do pH do solo na toxicidade de Basamid® em organismos não-alvo de diferentes complexidades, representantes de diferentes ecossistemas (terrestre: *Folsomia candida, Enchytraeus crypticus, Hypoaspis aculeifer* e *Eisenia andrei* e aquático: *Lemna minor, Raphidocelis subcapitata, Daphnia magna, Brachionus calyciflorus, Hydra viridissima, Xenopus laevis* e *Danio rerio*) e redes tróficas, bem como na capacidade de recolonização no solo por organismos edáficos após contaminação pelo fumigante. Foram realizados testes com organismos terrestres através da sua exposição a solo contaminado (e pH corrigido a 5.5, 6.5 e 7.5) e com organismos aquáticos por meio de eluatos de solo contaminado com Basamid® (nas mesmas condições de pH). O Basamid® revelou ser muito tóxico para todos os organismos expostos (particularmente *D. magna e F. candida)* identificando-se concentrações letais e sub-letais muito inferiores à dose recomendada. Os resultados das análises quimicas, sugerem que o aumento de pH diminui a concentração de dazomet no solo sendo que para os organismos terrestres o aumento do pH do solo, levou ao aumento da toxicidade de Basamid®, exceto em *E. andrei* onde não se observou a influência do pH. No entanto, os testes de evitamento realizados com *E. andrei*, expostas a solo envelhecido contaminado com Basamid® e nas mesmas condições de pH já referidas, permitiram perceber que ao longo do tempo, a recolonização poderá acontecer 56 dias (a pH 5.5) após aplicação do fumigante e esta ocorrerá de forma mais célere para o pH do solo 6.5 e 7.5 (14 dias). Quanto aos organismos aquáticos não-alvo, os resultados demonstraram não haver um padrão consistente da influência do pH, o que pode estar relacionado com sensibilidade inerente de cada espécie e/ou com condições de exposição, nomeadamente temperatura e composição química dos meios de cultura. Tendencialmente, o padrão mais comum observado para estes organismos foi a diminuição da toxicidade com o aumento do pH do solo. Os resultados obtidos sugerem níveis elevados de toxicidade para organismos terrestres e aquáticos que podem provocar a rotura de cadeias tróficas. Assim, acresce a necessidade urgente de uma nova avaliação do risco ambiental deste fumigante, para a qual o presente trabalho poderá contribuir com informação complementar relativa à exposição em diferentes espécies. Por fim, tendo em vista as características do pH do solo, em conjunto com a aplicação de Basamid®, (maior toxicidade a pH mais elevado para organismos de solo) sugere-se que sejam ser tidos em conta na nova avaliação de risco ambiental de dazomet.

Keywords Global Changes, Agriculture, Sustainability, Risk Assessment, Fumigant pesticida, soil pH.

Abstract The production of food for human subsistence depends directly on agriculture and its productivity. To obtain high production rates and minimum crop losses related to pest and diseases, this sector frequently and intensively resorts to the use of pesticides or plant protection products (PPP). Despite the benefits and need to use PPPs, over time and together with misuse, its effects culminate in different severity scenarios in the environment. Thus, there is a need to understand the effects of these compounds on the biota in real scenarios of exposure of organisms, in order to investigate more sustainable and environmentally friendly ways of using PPP. The fumigant Basamid® (which active substance is dazomet) was selected for this study, being on the market for more than 10 years, with additional extended evaluation by the authorities (EFSA and European Commission) for another 5 years. This fumigant acts as a fungicide, herbicide, nematocide and sterilant. Abiotic factors such as soil moisture, pH and organic matter affect its behaviour in environmental matrices, influencing the rate of reactions such as hydrolysis, altering the fumigant's half-life. Thus, the main objective of this work was to investigate the effect of soil pH on Basamid® toxicity in non-target organisms of different complexities, representing two ecosystems (terrestrial: *Folsomia candida, Enchytraeus crypticus, Hypoaspis aculeifer* and *Eisenia andrei* and aquatic: *Lemna minor, Raphidocelis subcapitata, Daphnia magna, Brachionus calyciflorus, Hydra viridissima, Xenopus laevis* and *Danio rerio*) and trophic web, as well as the ability of soil recolonization by edaphic organisms after contamination by the fumigant. Ecotoxicity assays were performed with soil organisms through exposure to contaminated soil (and pH corrected to 5.5, 6.5 and 7.5) and aquatic organisms through exposure to eluates from soil contaminated with Basamid® (under the same pH conditions). Basamid® proved to be very toxic to all exposed organisms (particularly *D. magna* and *F. candida*) with lethal and sub-lethal concentrations much lower than the recommended dose. From the results of chemical analyses, the increase in soil pH tends to decrease the concentration of dazomet in soil. For terrestrial organisms, the increase of soil pH led to an increase in Basamid® toxicity, except in *E. andrei* where the influence of pH was not observed. However, the avoidance tests carried out with *E. andrei* exposed to aged soil contaminated with Basamid® under the same pH conditions above mentioned, allowed to perceive that over time, recolonization may occur 56 days (pH 5.5) after application of the fumigant and this will occur faster for soil pH 6.5 and 7.5 (14 days). As for non-target aquatic organisms, primary producer, primary and secondary consumer groups were studied. The results were variable, which may be due to the inherent sensitivity of each species and to the exposure conditions, namely different temperature and chemical composition of culture medium. The most common pattern observed for these organisms was the decrease in toxicity with increasing soil pH. The results obtained suggested high levels of toxicity for terrestrial and aquatic organisms what can converge in main trophic chain ruptures. Therefore, there is an urgent need for a new assessment of the environmental risk of this fumigant, to which this work may contribute with complementary information regarding exposure in different species. Finally, in view of the pH characteristics of the soil, together with the application of Basamid®, (greater toxicity at higher pH for soil organisms) these should be taken into account in the new environmental risk assessment of dazomet.

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General Introduction

1. Agriculture and Global Changes

Agriculture is one of the most crucial and demanding activity in the world for Human and planet health, ensuring world food supply (Tilman et al. 2002). Statistically, agriculture represents about 4.8 million ha of area used, worldwide (FAOSTAT 2019) which correspond to around 38% of the land surface use (Borrelli et al. 2020).

There can be distinguished two main different types of agriculture: modern pesticides-dependent agriculture and sustainable soil-function, commonly named as organic agriculture (Bourguet and Guillemaud 2016).

Sustainable agriculture has an important role to biodiversity conservation along with microbial activity and its essential role mechanisms in soil (Datta et al. 2016; Dudley and Alexander 2017). Sustainable agriculture relies in major activity from soil organisms and respective interconnections, management of the soil, selection of resistance species, nutrient availability and organisms engineering (as for example earthworms), capable to improve and enhance fertility of the soil, establishing water systems and enhance microbial activity (Nielsen et al. 2015; Bourguet and Guillemaud 2016; Datta et al. 2016). Furthermore, a sustainable agriculture envisions soil quality by recurring to crops rotation and absence or low rate application of agrochemicals (Muller et al. 2017; Meemken and Qaim 2018). In fact the sustainable/organic agriculture resorts the lowest adverse effects on the entire ecosystems, with less harm to the environment although, so far, it is not productive enough to keep with a fast growing and hungry population (Meemken and Qaim 2018). Therefore, the agriculture dependent on agrochemicals substances is still the most needed to keep providing food to sustain the worldwide population. However, a balance must be kept into attention since advantages towards disadvantages are a fragile separation (Meemken and Qaim 2018). For instance, in intensive agriculture of monocultures leads to the spread of diseases and pests, decreasing resilience to climate change events prompting stress on plants (Agovino et al. 2019). To manage productivity, profit, yield, quantity, and the necessity to protect crops from persistent pests and diseases, the most common practice resource is the application of plant protection products (PPP) (Aktar et al. 2009). The importance of intense agriculture broads a consensus with all scientific, industrial, economic and agricultural community related to the need for feeding the population. Although, it also contributes to climate change, biodiversity losses, habitats destruction, releasing of pollutants and greenhouses gases which altogether interconnect with global changes and *vice versa* (European Environment Agency 2015; Dudley and Alexander 2017; Agovino et al. 2019).

Global changes are the environmental changes that disables life sustainability to occur worldwide, and the interaction of physical, chemical and biological factors involved in the ecosystem management (Zimmerer 2010). Overall these changes, the ecosystem and biodiversity depends mainly in the rate of the occurring changes, the vulnerability and resistance of species and the structure of the ecosystem itself (Nielsen et al. 2015).

There are specific consequences linked to global changes, threatening directly and indirectly the survival of species along with respective ecosystems (Nielsen et al. 2015). Nonetheless, these consequences within global changes are being directly linked to anthropological activities throughout the years, setting a cause-effect of merging events as climate changes, CO_2 increase concentrations, biodiversity losses, hydrological cycles land exploitation affecting in a close way soil structures, soil desertification (Zimmerer 2010; Nielsen et al. 2015).

In consequence of these global changes, soil fertility is endangered and desertification occurs towards an affected irreversible direction that leads to depleting of life in the environment (Dudley and Alexander 2017; Agovino et al. 2019). Then, a vicious circle begins in the way that anthropogenic activity as agriculture, contributes to global and climate changes due to land exploitation and pollutants use, and, these global changes as for water scarcity and soil erosion contributes to poor agriculture productivity and severer effects in the environment (Zimmerer 2010; Dudley and Alexander 2017; Agovino et al. 2019).

1.1 Global Change: Soil Quality and Soil pH

Soils represent the sustainable structure of agriculture, which comprise major ecological functions, sustainability, biodiversity and eco-services (Nielsen et al. 2015). Therefore, soil quality gathers the health of the soil converted in its well provision of agriculture function, effects on organisms and entire ecosystem (Nielsen et al. 2015; Lehmann et al. 2020). Despite the apparent robust character of soils, debilitating and neglecting behaviour due to anthropogenic activities (e.g., intense use of agrochemicals and intensive agriculture, industrial effluents leachates) are being the main reason for soil degradation (Nielsen et al. 2015; Meemken and Qaim 2018; Maia et al. 2018). Soil quality, fertility and biodiversity are being threatened due to the perturbations associated with global changes, climate change, land exploitation, chemical pollution, tillage, deforestation, soil acidification and in consequence, culmination in soil erosion (Maia et al. 2018; Agovino et al. 2019). Soil erosion is one of the major concerns worldwide since it affects drastically food production and productivity, and the entire chain reactions regarding the soil ecosystem (Borrelli et al. 2020).

To improve quality and soil fertility, different soil managements practices can be implemented, as the increase on soil organic matter contents, cultures rotation, non-tillage, improvement of carbon sequestration, microbial activity, management of soil pH (Zimmerer 2010; Tian and Niu 2015; Luetzenburg et al. 2020). For instance, mechanisms as the application of green manure to improve organic matter content, recurring to permaculture and the use of earthworms to increase the processes within soil layers and the use of carbonate compounds to improve soil pH (Datta et al. 2016; Li et al. 2019).

The application of fertilizers with urea, ammonium and sulphur-based in agriculture contributes with soil acidification which is closely related to soil erosion (Tian and Niu 2015; Goulding 2016; Hong et al. 2018). Acidification of the soils occurs through acidic precipitation of hydrogen ions followed by deposition of acidifying gases (e.g., ammonia, hydrochloride and nitric acids, sulphur dioxide), and as already mentioned through fertilizers application (ammonium and sulphur-based)

(Goulding 2016). In severer cases of soil acidification, irreversible clay minerals dissolution, along with cation exchange reduction could be translated into a permanent effect (Tian and Niu 2015; Goulding 2016; Hong et al. 2018). Management of soil pH can be beneficial regarding the improvement of soil quality centred on nutrient availability, microbial activity, biogeochemical reactions, chemicals adsorption, neutralization of pH and increasement of soil fertility (Sheng et al. 2005; Ghimire et al. 2017; Neina 2019). Soil pH measures the acidity and alkalinity from the balance of hydrogen ions concentration, which regarding to soil are directly linked to nutrient cycles (e.g., nitrogen, phosphorus, calcium) (Hong et al. 2018).

To cope with acidification of the soil, neutralization or alkalinization of the soil could be operated through clay management consisting of adding calcium compounds into the soil (Aye et al. 2016; Lawrence et al. 2016; Holland et al. 2018; Li et al. 2019). Different materials can be used, such as ground lime-stone (CaCO3), dolomitic limestone (CaMg(CO3)2, slaked lime ($Ca(OH)_2$), natural shell sands and burnt lime (CaO) among other, (Holland et al. 2018). The methodology of lime application must be adapted to the specific characteristics of the soil. For example, in arable soils the lime material needs to be incorporated whilst for covered grassland it could be applied by spreading pellets of lime. The tillage or no-tillage of the land is also determinant: no-tillage land requires a higher concentration of lime to be apply due to the stagnation of the soil and consequence lower organic content (Holland et al. 2018).

Overall, the benefits of soil liming, other than soil pH increase, are related with soil aggregation improvement, clay flocculation, structural stability, neutralization of excessive hydrogen ions, reduction of lower soluble mineral elements (e.g. aluminium, ferric iron Fe2+), provision substrates for microbial organisms, nutrients availability and cations supply (calcium and magnesium) and therefore, the fertility improvement consequence (Aye et al. 2016; Li et al. 2019).

Sheng et al (2005) performed a study to evaluate the influence of pH in the adsorption of three pesticides (diuron, bromoxynil, and ametryne) to the particles of soil with or without the addition of wheat residue-derived char. The authors observed different effects regarding both pH influence and char amendment, at the end, it was clear the influence of both factors, however, not in a linear response for all three pesticides. While diuron was not influenced by pH, bromoxynil at higher pH (pH of 7) dissociates decreasing its sorptivity by soil and wheat char and ametrine reacted oppositely to bromoxynil, its sorptivity by soil and wheat increased at higher pH. Nevertheless, the impact of soil pH in sorptivity of pesticides is present and can be a feasible approach in soil management. Soil pH is in fact one of the most important factors of the soil quality, agriculture activity and environmental health.

2. Plant Protection Products

Nowadays, the most common practices within agriculture activity, envisioning fast and higher food productivity, depends highly on crops assisted by pesticide's application. Spread worldwide with intense application in China, USA and Brasil, plant protection products (PPP) comprise a complex group of chemicals and non-chemicals (e.g., microorganisms) from different formulations,

mode of action and target goals (EC 2020a). Just in 2019, more than 4 million tonnes of PPP were used worldwide, being the Asian continent the most representative comprising 51.8% of the total pesticide application (FAOSTAT, 2022). The use of these PPP is directly linked to economy growth and profit, industry production, and environment and health care. Many benefits of PPP can be identified namely: productivity and yield increase, crops protection from weeds, diseases management, vector control and pests depletion (Parween et al. 2016; Souza et al. 2020; Sharma et al. 2020). Therefore, PPP are majorly applied to obtain faster production along with greater quantities of food and to deplete pests and weeds to keep responding toa broader need of Human food demand.

Plant protection products or Pesticides gathers different categories, that are based on their main target pests, namely Herbicides, Insecticides, Fungicides, Nematicides, Molluscicide, Rodenticides, Acaricides. Moreover, chemical properties are also used to classify PPP in different groups, namely: Carbamates (organic compounds derived from the carbamic acid), Neonicotinoids (nicotine derived insecticides), Organochlorine (chlorinated compounds belonging to the class of persistent organic pollutants), Organophosphate (consists in phosphate esters), Pyrethrin and Pyrethroids (both derived from Chrysanthemum cinerariifolium) among others (Krieger 2010; Parween et al. 2016; de Souza et al. 2020). In line with different chemical properties, PPPs exhibit different modes of action, that are designed specifically to their target pest's. As an example, is the group of PPPs that inhibit biological process (Ex. Cell division, DNA disfunction and enzyme function), impair the nervous system, inhibit the respiratory chain, among others (Krieger 2010).

Notwithstanding and despite of pesticides advantages, its consequences in the environment can be severely alarming with major consequences for both Human and Environmental Health. The use of pesticides in agriculture to deplete vectors of sever diseases (e.g., Dengue and Malaria) is also a double-sided issue due to the persistent nature of many pesticides in use (Sharma et al. 2020). Hence, major concerns of the PPP application are related to the consequences of direct and indirect application, mis-usage as over-dosage, great persistence in the environment, low safety practices in its application, bioaccumulation in organs and tissue, few data from the real effects in the environment, and the ultimate conjugation with other chemicals (Tilman et al. 2002; Souza et al. 2020; Sharma et al. 2020). For instance, referring to the indirect effects of PPP application, methyl bromide was a PPP fumigant vastly used in agriculture with great effective results in the control of microbial pathogens, as fungi and nematodes. However, severe harms regarding ozone layer depletion were being directly linked to this pesticide application, which led to its banishment from Europe in 2005 (EC 2011a; Qin et al. 2013; Ziedan and Farrag 2016; Oka 2020). On the other spectrum, glyphosate, which is a very well-known herbicide is still in the market. Widely and vastly used since 1971 worldwide due to the great results in weeds elimination, brings higher concerns regarding Human and Environmental health. The continuous application of glyphosate, associated with its high half-life time and solubility, has been causing many effects concerning non-target organisms (including Humans), water contamination and soil erosion (Singh et al. 2020). The half-life time (DT_{50}) of pesticides (which translates the time needed to be degraded) and fate in the

environment can be of major concern since in reaction with external parameters it can be extended in the ecosystem for many years, despite the theoretical DT_{50} assumption for each chemical (Krieger 2010). Thus, due to the application spreads, leachates and diffusion through soil that reaches freshwater systems and underground water, fumes release, higher concentration application, higher half-time life of the chemical and lastly the direct application of PPP, non-target organisms can suffer severed adverse effects, causing cascade effects at higher levels of biological organization, leading to the impairment of both aquatic and terrestrial ecosystems (Aktar et al. 2009; Souza et al. 2020; Sharma et al. 2020).

Until today, synthetic chemical pollution is still persistent and present worldwide. The industry of PPP gathers a large investment in different chemicals, different type of products and mode of action (slow or fast release). However, new technology, new formulation, different mechanisms that can decrease the PPP toxicity to biota, must be investigated along with more biodegradable PPP aiming to lower the persistence in soil and water (Souza et al. 2020; Sharma et al. 2020).

2.1 Fumigants

Within PPP chemicals, fumigants represent a relevant group as they are used in agriculture practices to disinfect and sterilize soils from pests and microbial pathogens, prior plating the crops (Krieger 2010; Qin et al. 2016b; Fang et al. 2020). Fumigants were developed to control pests, weeds, insects microbial pathogens as soil-borne and fungi disinfection, nematodes, and as a pre-plant application for soil sterilization (Chellemi et al. 2013; EFSA 2014; Fang et al. 2020). These PPP are volatile, mobile, gaseous compounds or substances that are converted into gas through the reaction with an abiotic factor (e.g., temperature, pressure, humidity soil pH), and that is applied at a concentration sufficient to be lethal to a given biological target (FAO 2001; Krieger 2010). These biocides are different and distinguish from aerosols, despite of some common properties. An aerosol constitute liquid or solid particles in a gaseous suspension, which act as a dispersion in air and cannot penetrate into the materials, whilst fumigants are the gaseous form itself that diffuse in separated molecules through the soil (FAO 2001). Just in 2016, the six largest agrochemical companies accounted € 32 billion of global agrochemical sales. The use of fumigants, specifically in agriculture, have been recorded worldwide as an alternative to other groups of PPP due to its high efficiency, fast diffusion and reaction in the soil and low cost price (Wang et al. 2006; Ślusarski and Pietr 2009; López-Aranda et al. 2016b; Mao et al. 2017b; Hwang et al. 2017).

Therefore, from the extension use as fumigants, it is possible to discriminate a long list of the most used agrochemicals: chloropicrin, 1,3-dichloropropene, ethylene dibromide, formaldehyde, metam sodium, sodium tetrathiocarbonate, acreylonitrile, ethylene oxide, carbon tetrachloride, tetrachloroethylene, dazomet (MITC), methyl iodide, dimethyl disulfide and methyl bromide (Menge 1982; Krieger 2010; Fang et al. 2018, 2020). Through time, some of these fumigants (e.g., Methyl Bromide) have been abolished due to major harm effects in the environment such as ozone layer depletion, leaching to water systems and contamination, soil erosion and air pollution (EC 2011a). The most common processes associated with the application of fumigants (present in Fig.1) is as

follows: (1) volatilization to the atmosphere and surrounding air, (2) chemical transformation (meaning the degradation into the ecosystem where it was applied) and (3) chemical and biological (by microbial organisms) decomposition in the environment (Guo et al. 2003). The volatilization process is the starting chemical point of the fumigant application along with its conversion into the gas form and consequent diffusion through the soil until the atmosphere.

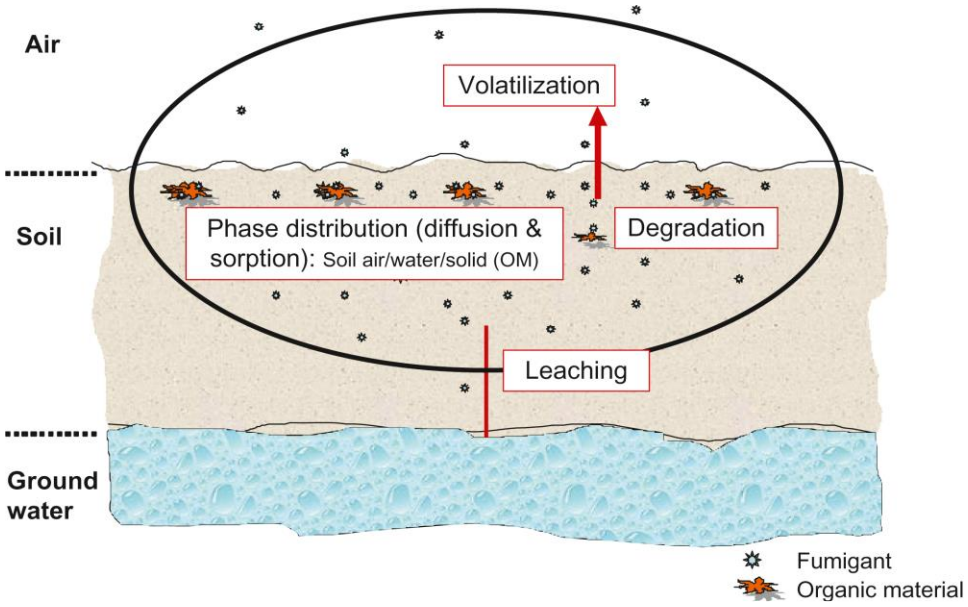

Figure 3- Diffusion and volatilization processes of a fumigant after its application in the soil and respective leaching to groundwaters (adapted from: Krieger 2010).

The degradation by chemical or biological mechanisms can occur in separate or combined. Chemical degradation is the most common pathway occurring in the field, reacting by hydrolysis or functional groups substitution of halogens (nucleophilic group substitution by for e.g., -NH; -OH). The end of fumigation (application of the fumigant process) is defined by the final decomposition performed by microbial organisms that can account for 40-80% degradation of the fumigant depending on the biocide used (Krieger 2010; Qin et al. 2016b). Regarding the chemical properties, these gaseous chemicals have high vapor pressure at low temperatures, low solubility and low boiling point, which induces the chemical reaction and gas partitioning leading to a very toxic and acute effect (Guo et al. 2003; Krieger 2010; Qin et al. 2013). Due to the higher vapor pressure, i.e., higher rate of volatilization and dispersion, there are some studies reporting the conjugation of different fumigants, to obtain more effective results to cope with some volatile losses which leads to a greater toxicity (Mao et al. 2012, 2014; Yan et al. 2017). Nonetheless, despite of their volatility and short period of time for activity in soil, metabolites can be generated, persist in soil and atmosphere, and/or leaching to groundwaters, threatening the environment and non-target species (Guo et al. 2003; Huang et al. 2019b).

On the other note, the efficacy and chemical reaction of fumigants application depends greatly on soil characteristics regarding the abiotic factors and physical properties as: soil texture and content, dryness, moister/humidity, organic matter, temperature, microbial activity and pH (Krieger 2010; Qin et al. 2013; Fang et al. 2017, 2018). Although, fumigants can react and have different degradation rates in soil when exposed to different environmental conditions. For instance, 1,3-dichloropropene degradation can be significantly catalysed with biochar (Qin et al. 2016a) and degrades slower when exposed to higher humidity (Krieger 2010) whilst Dazomet reaction decreases the rate of degradation in the present of higher organic matter (chicken manure) and increases degradation rate at higher humidity of the soil (Fang et al. 2018).

When it comes to fumigation safety two major practices must be followed: (1) fumigant application must be applied in a restrictive area, in order to reduce the toxicity to non-target organisms and (2) the used methods must reduce fumigant emissions to the atmosphere. To cope with the former safety procedure, different approaches can be followed: the higher water saturation of the soil, a method proposed by Qin et al (2013), although there is a higher risk of groundwater contamination, and the use of cover tarp/film after fumigation. The plastic film cover strategy, in the treated area, intends to guarantee the maximum fumigant effect in a contained specific area, and maximum fumigants emissions by prisoning the effects on plagues and pests (Wang et al. 2006; Chellemi et al. 2013; Dangi et al. 2015; Qin et al. 2017). The use of a cover material provide field limitations for the fumigant to spread being target specific by limiting a wider drift on non-target harm and in consequence diminishing concentration losses due to the reduced dissipation of fumigant (Chellemi et al. 2013; Dangi et al. 2015; López-Aranda et al. 2016a). The cover tarp material must not be porous so it can efficiently confine the fumigant to the application area. The second safety procedure is related to the security of the environment where the operators responsible for the fumigation must reduce the emissions to the atmosphere, and thus halting/minimising atmospheric pollution. Therefore it is strictly recommended the use of cover tarps after treating the soil with fumigants (Qin et al. 2016b, 2017). The recommended safety rules of fumigants usage must be followed strictly for the Human and Environment protection (EFSA 2010). Precautions such as safety equipment for the operator to protect the respiratory system, skin and eyes, procedures to facilitate aeration of the places of fumigant application (e.g., open windows and doors in greenhouses), and cover the treated area after the fumigant usage must be pursued. These approaches must be done carefully and insistently not just to directly protect Human Health and the Environment but also to achieve the maximum effect of the fumigant without compromising directly and indirectly the ecosystems. In this way, it is feasible to apply lower amounts of the agrochemical and not over dosage the crops and field production, diminishing higher quantities that further leachate and enter in nearby ecosystems, namely freshwater and atmosphere (FAO 2001; Huang et al. 2019b).

3. Dazomet: 3,5-dimethyl-1,3,5-thiadiazinane-2-thione

As briefly mentioned above, methyl bromide was one of the most extensively used fumigants worldwide due to its high efficiency and low-cost price. Although, due to the sever effects that it caused, regarding for example the ozone layer depletion, other fumigants with formulations expected to be more eco-friendly have been developed and used as a replacement, for example dimethyl disulfid, chloropicrin, and dazomet, the latter was used in the present thesis. Dazomet (DZ) is the active ingredient of the commercial formulation Basamid®, a granular powder fumigant used as a sterilant, nematicide, insecticide, pesticide and fungicide, that targets mostly soil-borne pathogens, root-knot nematodes, weeds and pests (EFSA 2010; Kanesho Soil Treatment SPRL/ BVBA 2014). Furthermore, other than its use as a pesticide, DZ is also used in industrial processes for sterilizing treatments during the production of pulp and paper, material preservation of non-food adhesives, slurries, and high viscous suspensions and wood treatment (USEPA 2009).

Dazomet ($C_5H_{10}N_2S_2$ – represented in Fig.2) is a dithiocarbamate fumigant used worldwide and first registered in Belgium in 1968 as a sterilant (Registation Department Agricultural Division 1992; Szolar 2007; Al-Alam et al. 2017). Although, this fumigant application have been reported since 1631 (Murphy 2011). Dazomet is considered a low persistence chemical with higher vapor pressure and short half-life time (DT_{50}) of about 0.52 days in soil, however it can vary between hours to 7 days in soil, depending on the environmental factors (EFSA 2010).

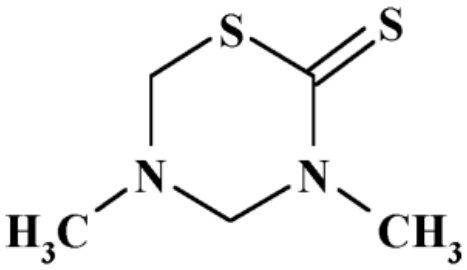

Figure 4- Representation of Dazomet two-dimension chemical structure. (Source: EFSA 2010).

When DZ is applied to the soil, either to the surface or incorporated, it quickly starts to break down into its primary degradation product the methyl isothiocyanate (MITC) gas (which represents 92% of the degradation metabolite). The MITC then reacts with nucleophilic groups such as thiols, that are present in enzymes. This is to be the biological mechanism of action in the biota (USEPA 2008).

The MITC gas is the effective compound responsible for the targeted pest depletion and fumigant treatment. Regarding the reaction of MITC, secondary metabolites (that account for 3% of the total degradation) can also be formed and consist of formaldehyde, monomethylamine, hydrogen sulphide and carbon disulphide (EFSA 2010). Notwithstanding, reported by EFSA (2010) conclusion peer review of the active substance dazomet, the secondary metabolites were negligible and not considered to the purposes of the fumigant environmental risk assessment due to the low toxicity, concentration and persistence. On this work, it will be assumed MITC as the only

effective substance resulting from the degradation of DZ. Established and reported by the Environmental Protection Agency (2009, 2017) the maximum concentration allowed to apply this fumigant, without surpassing the limits for environmental risk and permanent damage in the ecosystems, is about 594 Kg/ha of DZ every three years.

3.1 Dazomet Fumigant Reaction: influence of abiotic factors

From the literature is possible to report that Dazomet reacts faster at higher humidity content and increased alkalinity in solutions (EFSA 2010). Although, scarce information is available in what concerns the effects that environmental abiotic factors may have in the reaction of DZ when applied to soils. The fumigation reaction occurs through hydrolysis, and when applied to the soil the degradation reaction can be catalysed or decreased depending on soil conditions. Therefore, the variation of different environmental factors as temperature, soil water content and soil texture, organic matter and soil pH can be major factors influencing the degradation rate of this fumigant (Krieger 2010; Qin et al. 2013; Fang et al. 2018). So far, only one study reporting soil management of the environmental factors in DZ is published. Fang et al. (2018) performed a study to investigate the effect of different environmental factors: soil texture and water content, soil amendment (chicken manure and fertilizer), temperature and soil pH factor on DZ decomposition. The authors reveal that the decomposition of DZ were strongly dependent on soil texture, temperature and moisture and pH. At higher levels of the former factors (water content, temperature, and soil pH), rates of DZ degradation were increased. On the contrary, the application of chicken manure and urea fertilizer to the soils, caused a decrease in the degradation reaction of DZ. These changes in the DZ reaction when applied to soils may influence its ecotoxicity, both to target and non-target organisms. Therefore, it is important to generate more knowledge on how the different environmental abiotic parameters influence DZ degradation in soil and subsequently its effects on the biota. For example, factors that will determine an increase in DZ hydrolysis may cause a high toxicity in a short period of exposure. Following this, the management of the soil must be taken into account prior the application of this type of chemicals to improve its efficiency in eliminating the target organisms, but also to reduce its environmental effects, and thus promote its more sustainable use.

3.1.1 Dazomet Efficacy and Target Species

Dazomet has been commercialized for an extensive period of more than six decades (Reddy et al. 1964) and is still running in the market under the European Commission and EFSA approval revision (EFSA 2010; EC 2020a). Therefore, its application has been served until today. On this notice, many studies had been reported regarding the effects of this fumigant application in target species that highly affect value crops. A list of some target species can be discriminated, for instance: (i) within the Fungi the following species have been eliminated from agricultural soils with DZ *Verticilium dahliae* (Di Primo et al. 2003; Ślusarski and Pietr 2009; Meszka and Malusà 2014)**,** *Fusarium* spp. (Di Primo et al. 2003; Yücel et al. 2007; Ślusarski and Pietr 2009; Mao et al. 2012,

2014; Hwang et al. 2017) *Phytophthora* spp. (Ślusarski and Pietr 2009; Mao et al. 2012, 2014; Yim et al. 2016) *Pyrenochaeta* sp. (Mao et al. 2014; Lechenet et al. 2017) *Rhizoctonia solani, Pythium* spp. and *Cylindrocarpon* spp. and *Pythium ultimum* (Ślusarski and Pietr 2009; Yim et al. 2016; Hwang et al. 2017) *Sclerotium rolfsii* (Di Primo et al. 2003) *Ilyonectria* sp. and *Mortierella* sp.(Lechenet et al. 2017); (ii) for the Nematoda group target species are *Meloidogyne* spp., *Meloidogyne javanica*, *M. incognita* and *P. penetrans* (Yücel et al. 2007; Mao et al. 2012, 2014; Yim et al. 2016); and (iii) for seed weeds species like *Digitaria sanguinalis*, *Abutilon theophrasti* (Mao et al. 2012)*, Phelipanche mutelii* (Prider and Williams 2014) have been controlled with DZ. Regarding the efficiency of DZ, Nicola et al. (2017) conducted a study where the DZ was applied with the aim of altering soil microbial communities that are associated with apple replant disease. This disease appears as a result of intense monoculture of apple trees, causing a significant growth reduction in trees after their replantation. The application of DZ resulted in lower symptoms of the disease, although, the authors could not directly relate the effect of DZ to the pathogens control or to the used of beneficial plants along the crop. Also, Mao and his colleges (2012, 2014) performed two studies where they evaluated the efficiency of DZ, alone and mixed with 1,3 - dichloropropene or with dimethyl disulfid, on the pest control of cucumber productions. In both studies DZ alone presented a higher efficiency to control *Digitaria sanguinalis* and *Abutilon theophrasti* weeds than to control fungi soil borne *Fusarium* spp. and *Phytophthora* spp. and also the root-knot nematode *Meleidogyne* spp. For the control of fungi and nematode species, the combination of DZ and each of the other two chemicals, showed a higher efficiency than DZ alone. Another study conducted by Ślusarski and Pietr (2009) tested the application of DZ in pepper crops, to control for the soil-borne root rot disease caused by the fungus *Verticillium wilt*. The root rot disease is one of the most common persistent diseases in monoculture crops, here the pepper culture is affected by the *Verticillium dahliae* fungi which leads to diminishing crop production. The authors evaluated the effect of DZ alone and conjugated with biological treatment using *Trichoderma asperellum* isolate B35. The results showed that the conjugation of both chemical and biological treatment (DZ and *T. asperellum)* revealed to be the best combination to treat the disease, although, DZ alone showed once more, to have great results in diminishing the roots disease symptoms.

3.2 Dazomet Ecotoxicity and Environmental Routes of Exposure

The intense use of DZ, alone and conjugated with other agrochemicals, results in high risks to human health and the environment, affecting ecosystems. Dazomet is a non-specific and large spectrum fumigant and in consequence comprise a larger amplitude of effects in non-target organisms from different classes of organisms and ecosystems. There is a major gap concerning the quantification of DZ and respective metabolites in the environment for both surface water, ground-water, atmosphere and soil ecosystem. Reports of DZ presence in such environments are mostly addressed by the regulatory entities in what regards the predicted concentrations and assessment risk (USEPA 2008, 2014; EFSA 2010). Some studies were in fact performed in

organisms as earthworms (Mao et al. 2017b) and in conjugation with abiotic factors (Fang et al. 2017) although investigation of the fumigant presence in the ecosystem is still to be investigated.

Nevertheless, the ecotoxicological risk is predicted based on the DZ and metabolites chemical properties, therefore, it can be defined different pathways of exposure for the non-target organisms.

The presence in the atmosphere, water and soil is a result of its application, hence, despite the volatile property it has been proved to be of high toxicity. Therefore, non-target species inhabiting the different environmental compartments (e.g. birds, invertebrates, fish, mammals and plants) are expected to be exposed to this fumigant (USEPA 2008).

Regarding the environmental contamination of DZ and its metabolites in surface and ground waters represents a great danger to both aquatic organisms, and water quality (EFSA 2010). Due to the higher solubility of DZ and low adsorption in soil, the recommendations for soil moisture at 50% can be essential to reduce the risks for the aquatic ecosystems. When the saturation of the soil is neglected, there is a great potential for runoffs and leaching to the groundwaters (USEPA 2008; EFSA 2010).

When it concerns the atmosphere, the emissions and release of gaseous compounds of DZ and its conversion in metabolites after fumigation are still present, however, the persistence and presence of residues in the atmosphere has been not reported (USEPA 2008). The ecotoxicity of long-term exposure for the birds, bees and insects is mostly devalued due to the low adsorption and fast dissipation, nevertheless, there is an higher potential for acute exposure to the mentioned organisms (USEPA 2008; EFSA 2010)

The terrestrial compartment, and namely the soils, is considered the environmental compartments most affected by DZ due to the direct application in soil (USEPA 2008, 2014; EFSA 2010). Despite the scarce data it is possible to highlight different studies regarding the microbial community (Fang et al. 2020), invertebrates as earthworms (Mao et al. 2017b) and vertebrates as mice (Lam et al. 1993; Peluso et al. 1998).

Mechanisms to effectively remediate fumigants as DZ and metabolites from the ecosystem, and more specific from the soil must be investigated since the risk to the non-target organism is of higher level. The permanent threat of contamination in the environment, as long as a Fumigant is used, is still occurring (Nicola et al. 2017; Huang et al. 2019b).

3.2.1 Non-Target Organisms and Biological Effects

In consequence of DZ fate in the environment, non-target organisms are inevitably threatened and harmed. One of the most sever effect is the depletion of soil beneficial microorganisms involved in soil nutrient transformation, soil quality and fertility (Eo and Park 2014; Nicola et al. 2017). Fang et al (2020) investigated the effect of five fumigants including DZ in the abundance of microbial denitrifying bacteria. Denitrifying bacteria play an important role in the conversion of NO_3 in soil to N_2. The authors observed a significant reduction in the abundance of population of these specific denitrifying bacteria, for instance DZ in an application range of 7.73 and 46.5 mg/Kg decreased in 40% the presence of Proteobacteria group. Another major group of organisms with

essential functions in the soil with higher potential of exposure to the toxic effects of DZ are the earthworms. Mao et al (2017) performed a study where natural and artificial soil were contaminated with DZ *prior* exposure of *Eisenia andrei*. The authors reported an LC_{50} of 5.41 mg a.i. kg^{-1} soil, in artificial soil and an LC_{50} of 0.98 a.i. kg^{-1} soil.

To the best of our knowledge, to date, the few data available on organisms are mostly reported in EFSA reports and it regards only the group of birds, mammals and aquatic species. Regardless, considering the mechanisms of exposure, *via* of diffusion and leaching to water systems, it is expected and implied a greater toxicity to the aquatic organisms (EFSA 2010).

The non-species specificity of DZ, jointly with its multiple chemical functions, leads to crossed effects on the organisms (e.g.in the respiratory, urinary, hepatic and dermal systems), (Krieger 2010). Moreover, the aggravation and extension of the effects can be a consequence of both direct and indirect exposure. As mentioned previously, the primary metabolite of DZ, MITC gas affects primary and mostly the respiratory system. The mode of action of MITC is related to the non-specifically and permanently reaction with proteins, amines, and thiol groups in cells (EC 2010).

The absorption of MITC through the lungs occurs due to the releasing vapours and gases that enters via nasal-pharyngeal with alveolar absorption and consequent mucosal ingestion. Therefore, in mammals the common pathway of fumigant pesticides excretion from the organism is through respiration (exhale process) eliminated by the lungs (NRA 1997; Beach and Whalen 2006; Krieger 2010). Regarding insects, MITC enters in the organism (at larvae stage, pupae and adult stage) through the spiracles structures present on the lateral surfaces of the body (Bond 1989; Krieger 2010). The control of the spiracles in the respiratory activity depends on muscular control. When it comes to the egg stage, the entrance of the gases occurs through the shell (chorion) or in some cases through specialized respiratory channels (Bond 1989). It is known that the poisoning of an insect by a fumigant is influenced by the rate of respiration of that insect; any factor that increases the rate of respiration tends to make the insect more susceptible to the fumigant (Krieger 2010).

Dermal exposure could be classified as first or second routes of exposure to DZ. Skin (and exoskeleton in case of insects) is an essential organ covering the entire body and is responsible for protection of the surrounding. As the first barrier it comprehends one of the main entrances of DZ. Advised by EFSA (2010) and the company of production (BVBA/SPRL 2014), DZ is irritant to the skin, willing to provoke burn and trigger other sever dermal diseases (Krieger 2010).

On a more internal effects and metabolization of the fumigant, it is believed that DZ is retained in urine, kidney and liver (Lam et al. 1993; NRA 1997). Lam et al. (1993) used biomarkers by isotopic labelling of metham ($^{18}CH_3$ and $^{14}CH_3$) to follow the absorption of DZ in rats and mice, the highest percentages were found in the urine (58-80% in 48h). Levels of the biomarkers were reported in the faeces (2-6% for each dose), thus DZ were not considered genotoxic (EC 2010). On this note it is of highly the recommendation of restrictive protective measures, as for the operator, recurring to a full protection equipment, as for the environment by restringing the area of fumigation and recurring to methods to constrain the fumigant, as film tarp cover or managing abiotic factors as applying the DZ at lower temperatures (Fang et al. 2017).

4. Dazomet: Regulatory in European Union

The European commission (EU) in pair with European Food Safety Agency (EFSA) and Members States are the regulatory entities in Europe responsible for pesticides risk assessment and approval to its commercialization and application. Hence, pesticides approved to be in the market by the mentioned entities are regulated under two main regulations: the (EC) No 1107/20093, which comprise the PPP regulation and the (EC) No 396/20054 which regulated the Maximum Residue Level (MRL). The complete process follows three steps: the approval of the active substance and authorization (under good agricultural practices), the so called sustainable use which regards the application process and last, the MRL regulation, which evaluates the levels of PPP in food and in the environment (EC 2020a).

Within the European Union, the regulation of active substances of PPP became applicable only since 2011 (EC 2020a). The PPP risk assessment envisions an environmental safety management of the used pesticides, based on ecotoxicologic effects. The environmental risk assessment follows a strict evaluation distributed from different TIERs with different impacts in the environment. However, different ecological processes as run-off, leaching, spreads of the PPP among others, can also influence the PPP effects in the environment. Moreover, adding the neglected handling by the operators and farmers, higher collateral damages can be observed in the environment (Schmolke et al. 2010; Damalas and Eleftherohorinos 2011). Thus, higher levels of assessment, crossing higher levels of community, ecological modelling, and accounting for drifts in the environment, should be a required cross-design regarding pesticides risk assessment (Schmolke et al. 2010). Currently established, the time gap from the approval of an active substance until new revision and risk assessment runs for 10 years which translates in 10 years of commercialization and application in the environment (EC 2020a). Although, the risk assessment of some PPP does not follows as expected and postpone revisions, low quality data assessed, or even bureaucratic issues are addressed and delays the process of revision. For instance, the risk assessment and revision for the active substance dazomet has been delayed, which consequently triggers some major environmental concerns. Dazomet is included in the Group 4 of the renewable revision submission substances to EU, which due date for new revision should have expired in December 2021, however, it was postpone for more 2 years, until 2023 (EC 2020b). In addition, DZ was classified as one of the 13 active substances responsible for 90% of the registered human health impact, and, one of the three that still runs in the market, as already mentioned (EC 2020a). Despite the higher regulation, regarding PPP, and in specific DZ, the 10 years of used with the addition of two more years until new revision, contributes to the accumulation of effects in the environment across time until the new assessment. Moreover, the changing complex mechanisms evolved in the environment, along with multiple and intensive agriculture practices, may not correspond to the same predicted by the risk assessment required by the regulatory entities at the previous assessment risk.

On the other hand, efforts must be applied towards DZ management, limitation of use and more measures to reduce its impact in the environment. The depuration of DZ from the soil, water and the atmosphere should be of major investigation in the present times, mostly due to the consequences of chemicals accumulation and the new formulation. Within agriculture, the goal must be focus on reduction of chemicals within the agriculture practice, recurring to more natural alternatives and management of crops rotation in order to maintain soil fertility and an healthier environment (Meszka and Malusà 2014).

5. Objectives

Considering the mentioned above and envisioning a more sustainable use of Basamid® in agriculture the major goal of the present work is to assess the influence of soil pH in the behaviour and toxicity of the fumigant Basamid® to ultimately purpose recommendations for its sustainable and soil-specific use in agriculture. This fumigant was selected because is widely used all around the world to sterilize soils pre-planting. To attain the main goal, four specific objectives were delineated:

1. Determine the toxicity of Basamid® to non-target biota across different values of soil pH. Toxicity will be monitored both for terrestrial and freshwater organisms, since dazomet and secondary compounds resulting from its decomposition may reach aquatic systems through leaching and runoff. This will allow identifying the least environmentally toxic combination of Basamid® concentration x soil pH value.
2. Investigate the capacity of soil organisms to recover from contaminated soils across time and soil pH. Avoidances tests with aged-contaminated soil at different soil pH and Basamid® levels will be performed with earthworms to understand if across time, the recovery of soil communities will be possible to occur and how long would it take to observe such recovery.
3. Establish tipping points for the applications of dazomet in soils with different pH values. Recommendations on the use of this fumigant will be established for soils-specific pH, i.e., categories of soil pH will be established with the corresponding recommended dose of dazomet.

6. Thesis structure

This thesis is composed by seven chapters. The Chapter II to VI are presented as scientific papers format. The Chapter IV was already accepted for publication in Journal Science of the Total Environment. The other four papers are in preparation to be submitted. As follow, the description summary of each chapter is reported below:

Chapter I- General Introduction

In this chapter is contextualized an overview about the agriculture practice, the role and influence of global changes, the intense use of PPP and the importance of soil pH. It is also described the impact of the fumigant Basamid® to the environment and non-target organisms.

Chapter II – The influence of soil pH in the toxicity of a dazomet fumigant (Basamid®) to non-target soil organisms

In this study, four terrestrial species were exposed to different soil pH and Basamid® concentration gradient. The endpoints assessed were reproduction and mortality. Results showed a significant influence of soil pH, most specifically the alkalinity of the soil, to all organisms exposed to the fumigant. Higher alkalinity of the soil induced higher toxicity to the organisms. From the four species *Folsomia candida* and *Enchytraeus crypticus* were the most sensitive while *Hypoaspis aculeifer* and *Eisenia andrei* the most tolerant to the exposure.

Chapter III – Avoidance tests as a tool to evaluate the influence of soil pH in earthworm's re-colonization after soil fumigation

Avoidance tests with aged, contaminated soil with Basamid® and at the same established soil pH were performed on the earthworm *Eisenia andrei*. Across 56 days different sets of avoidance tests were performed. Results showed that soil pH influences significantly the time of Basamid® dissipation. At higher alkalinity of the soil faster response of "no-avoidance" behaviour was observed from *E. andrei*. Therefore, soil pH crossed with Basamid® can be manage in order to keep its target functions but allowing the recovery of the soil from the organisms.

Chapter IV – Soil pH matters in the ecotoxicity of Basamid® to freshwater microalgae and macrophytes

Eluates of soil contaminated with Basamid® and pH corrected were obtained to expose a microalga and a macrophyte. Endpoints as growth, biomass, yield and pigments were assessed. The results revealed that these organisms were soil pH dependent, meaning that the toxicity were higher in more alkaline conditions to the *Lemna minor* macrophyte than the *R. subcapitata* microalga. Either way, the microalgae was less sensitive to the effect of Basamid® than the macrophyte. According to the chemical analysis, the alkalinity of the soil accelerates the degradation of dazomet in the medium.

Chapter V – Soil pH influences the toxicity of Basamid® eluates to non-target species of primary consumers

On a similar scenario than in Chapter V eluates of soil contaminated with Basamid® were used to expose *D. magna* and *B. calyciflorus*. Reproduction, feeding, and body size was evaluated. *Daphnia magna* were the most sensitive organism and once more, the organisms were soil pH

dependent. While the *D. magna* were more sensitive to the alkalinity exposure, the *B. calyciflorus* was less sensitive, although, both species were highly sensitive to the soil pH 6.5.

Chapter VI – Ecotoxicity of eluates obtained from Basamid® contaminated soils is pH dependent: a study with *Hydra viridissima*, *Xenopus laevis* and *Danio rerio*

The three species *H. viridissima*, *X. laevis* and *D. rerio* were exposed to eluates of soil contaminated with Basamid® and different soil pH. Across the tests effects as mortality, growth, malformations were assessed. From all organisms, *D. rerio* and *X. laevis* presented similar sensitivity to Basamid® while *H. viridissima* was clearly less sensitive. Soil pH was also a major factor regarding the fumigant toxicity. While *H. viridissima* was most sensitive to acid conditions, both *X. laevis* and *D. rerio* were most sensitive to alkalinity exposure.

Chapter VII – General Discussion and Conclusion

In this final chapter the final remarks of the complete work along with discussion of all gathered results are presented. Difficulties and further perspectives along with good practices to diminish the impact of Basamid® in the environment are also pointed out.

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Chapter II

The influence of soil pH in the toxicity of a dazomet fumigant (Basamid®) to non-target soil organisms.

The influence of soil pH in the toxicity of a dazomet fumigant (Basamid®) to non-target soil organisms.

Authors

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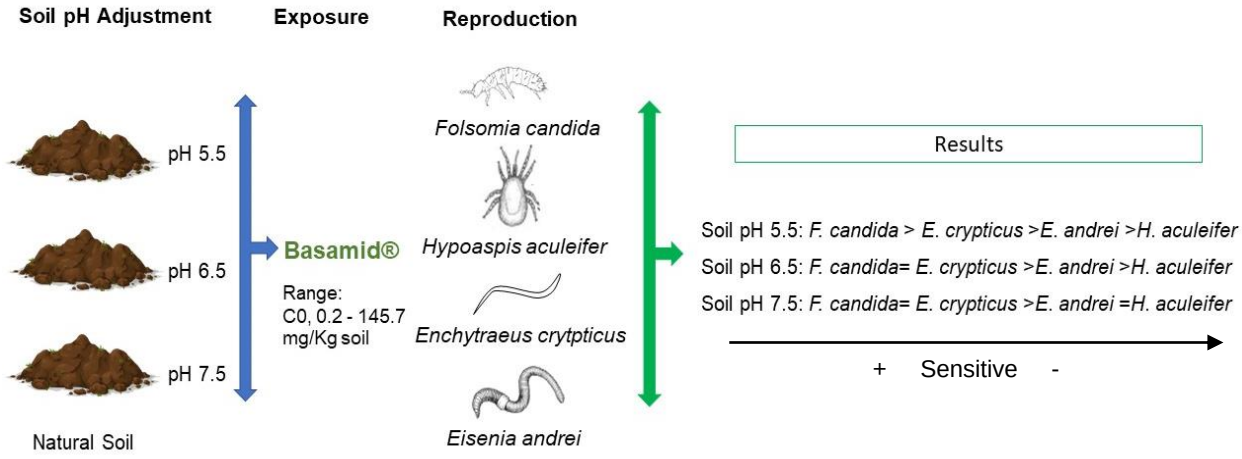
Graphical Abstract



Highlights

- Basamid® is highly toxic to soil-organisms in concentrations below the recommended dose.
- *Folsomia candida* and *Enchytraeus crypticus* were the most sensitive species to Basamid®.
- Higher soil pH enhanced Basamid® toxicity to some organisms' reproduction.
- Soil pH was indifferent in Basamid® toxicity to *E. andrei* (mortality and reproduction).

Abstract

Intense agriculture is mainly dependent on plant protection products (PPPs). Today, PPPs are developed in a wider range of target cultures to treat specifically, target pathogens, however, collateral damages may occur to non-target species. The aim of this work was to evaluate the influence of soil pH in the toxicity of the fumigant Basamid® (97% dazomet, active substance) to non-target soil organisms. Thus, individuals of the species *F. candida*, *H. aculeifer*, *E. crypticus* and

E. andrei were exposed to a natural soil with the pH manipulated (soil pH pf 5.5, 6.5 and 7.5 corresponding to N5.5, N6.5 and N7.5 soils, respectively), contaminated with increasing concentrations of Basamid® attending to respective guidelines (ISO 11267, 2014; OECD 226, 2008; ISO 16387, 2014 and ISO 11268-2, 2011; respectively). Mortality and reproduction endpoints were assessed for all organisms exposed to similar conditions of soil pH and Basamid®. The recommended dose of 145.7 mg dazomet/kg soil (also the highest concentration tested) was highly toxic to organisms. The highest reported LC_{50} was 48.6 mg dazomet/Kg soil for *E. andrei,* for all soil pH. Among the four species, *F. candida* presented the most level of sensitivity for mortality (lowest LC_{50} values of 9.17, 5.26 and 9.75 mg a.i./Kg in N5.5, N6.5 and N7.5 soils, respectively) and reproduction (EC_{50} values of 5.82, 2.71 and 1.12 mg a.i./Kg in N5.5, N6.5 and N7.5 soils, respectively). Soil pH increase, increased Basamid® toxicity to all species except for the earthworms, where no effects were observed regarding soil pH.
Keywords: Dazomet, soil pH, non-target organisms, reproduction, Ecotoxicity.

Introduction

According to the United Nations projections the world population will reach 9.7 billion people by the year of 2050 (United Nations 2019). Alongside with population increase is the intensive agriculture, pointing in the direction of productivity intensification (Tilman et al. 2002; Agovino et al. 2019). Aiming to avoid productivity decreases caused by pathogenic plagues and pests and to achieve higher yield rates, the use of plant protection products (PPPs) worldwide has increased over the last decades (Aktar et al. 2009; Alavanja 2009; Agovino et al. 2019). New PPPs have been developed, based on more aggressive and target-specific active ingredients, fact which have contributed to increase the diversity of chemical mode of actions of PPPs available in the market (Socorro et al. 2016). The PPPs authorizations in the EU market are reviewed every 10 years under the Directive 91/414/EEC and are based on its risk to the environment and public health. In these reviews these substances can be banished or approved to be used for more 10 years in the EU market. However, the current ecological risk assessment scheme followed for PPPs does not consider some factors like soil properties that may influence the behaviour of PPPs (thus influencing its risk) as have been shown when varying soil pH (Fang et al. 2018a).

Basamid® is a PPP with dazomet as active ingredient, developed to substitute Methyl Bromide (MB), a higher ozone layer depletion substance and highly toxic to Human health (Xie et al. 2015; Ziedan and Farrag 2016).

Dazomet (tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione) is a dithiocarbamate soil disinfectant fumigant with a broad spectrum of effects gathering functions as pesticide, herbicide, nematicide and fungicide (EFSA 2010). Dazomet is applied thoroughly and evenly incorporated to a depth of 15 cm mainly by rotary cultivator or rototiller equipment in moisturized soils as a pre-plant PPP (Kanesho Soil Treatment SPRL/ BVBA 2014). This fumigant is mainly advised for multiple crops to fight against pests infections of *Verticillium* sp., *Meloidogyne* spp. and *Rastonia solanacearum* (Harris and Yang 1996; Mao et al. 2014, 2017a) at a recommended dose of 145.7

mg a.i./Kg soil (EFSA 2010; Fang et al. 2018a). The degradation of dazomet is influenced by abiotic factors such as soil structure and texture, humidity, organic matter, temperature and soil pH (EFSA 2010; Fang et al. 2018a). These abiotic factors are responsible for the time rate of the chemical reaction, setting dazomet to hydrolysis degradation (Fang et al. 2018a; Consolazio et al. 2019). The results from degradation are the metabolites formaldehyde, carbon disulphide (CS_2), hydrogen sulphide (H_2S), monoethylamine and methyl isothiocyanate (MITC) that is the releasing gas responsible for the fumigation through which the product act. Fang and colleagues (2018a) conducted a study to test the effect of environmental factors in dazomet chemical reaction using different types of soil. According to their study, dazomet degradation rates increases with the increase of temperature, humidity and soil pH. Despite the known evidences towards dazomet chemical degradation, namely faster reaction with alkalinity, the effect of dazomet toxicity to non-target organisms in different soil pHs was never investigated until date (Morrell et al. 1988; EFSA 2010; Fang et al. 2018a).

Soil pH has a relevant role in nutrients availability, regulates microbial community diversity, manages enzyme activity by balancing the concentration of inhibitors, activators and concentration of substrates in the soil (Dick et al. 2000) and pesticides adsorption (Sheng et al. 2005). Agricultural soils tend to be acidic through time due to natural and anthropogenic activities (Goulding 2016; Zou et al. 2018). Based on Tian and Niu (2015) who did a meta-analysis of 106 studies, soils with N-fertilizers addition had its pHs significantly decreased of 0.26 on average globally. One of the major impacts in the soil pH is the intense use of fertilizers that acidify the soil. The process of liming is the most commonly used method to fertilize the soil. The application of Ca salts promotes the increase of soil pH and, by that way, stabilizes the soil and improves soil properties and nutrients availability (Holland et al. 2018).

The extensive use of PPPs and fertilizers in agriculture in combination with soil properties, may have severe impacts in non-target species (Tilman et al. 2002; Yan et al. 2017). Most of these non-target species have an important role in soil systems contributing to decomposition, energy and matter cycle, biocontrol of pests, soil structure and nutrient and water management activity (Jänsch et al. 2005). Due to the high relevance of these groups in soil systems, and to prevent toxic effects resulting from the use of PPPs to these non-target species, the approval of PPPs in the EU market requires ecotoxicological data for a list of standard species (representative of key-groups; EC, 2013). The species of in-soil organisms included in that list are the collembolans *Folsomia candida*, the mites *Hypoaspis aculeifer* and the earthworms *Eisenia andrei* (EC, 2013). The pot worms of the species *Enchytraeus crypticus* have been widely used in soil ecotoxicology studies as well, not only due to their important role in in the organic matter decomposition, but also due to its relatively high tolerance to a wide range of soil pHs, (pH above 4.0, Jänsch et al. 2005).

The present work aims to evaluate the influence of soil pH in the toxicity of dazomet to non-target soil organisms. To reach this purpose, individuals of the species *F. candida*, *H. aculeifer*, *E. crypticus* and *E. andrei* were exposed to a natural soil with the pH manipulated, contaminated with increasing concentrations of dazomet. The following working hypotheses were assumed: (1) the

recommended dose of the fumigant affects severely the survival and reproduction of non-target soil organisms independently of soil pH, as the mode of action of the chemical is based on the effect of a very toxic gas generated immediately after its application and (2) soil alkalinity increases the toxicity of Basamid® to non-target soil organisms due to the rate time of the fumigant chemical reaction releasing the effective toxic gas.

Materials and Methods

Test soil and test substance

A natural soil collected in an agro-silvo-pastoral woodland submitted to sustainable management in Herdade de Freixo-do-Meio in Alentejo region, Portugal (38°41'44.9''N 8°18'33.7''W) was used in laboratory experiments. After collected, the soil was sieved at 5 mm and defaunated by heating it at 80 °C for 48 h. Physical and chemical properties of soil are presented in Table 1. Soil parameters were measured in three replicates by the following procedures: soil pH (KCl - 1 M KCl 1:5 w:v; H_2O - 1:5, soil:water, w:v), organic matter content (ISO 1995), extractable phosphorous and potassium (extracted by Egner-Riehm solution and determining P through molecular absorption spectrophotometry and K through atomic absorption spectrophotometry by flame emission), cation exchange capacity (ISO 2018), water holding capacity (WHC; ISO, 2014) and texture (ISO 2020).

Table 1- Physical and chemical parameters (average ± standard deviation) of the natural soil used in the laboratory experiments.

Parameters	Natural soil
pH (KCl)	5.40 ± 0.10
pH (H_2O)	5.70 ± 0.00
OM (%)	4.91 ± 0.67
C/N	11.47 ± 0.21
Phosphorous (P2O5) (mg/kg)	32.67 ± 5.13
Potassium (K2O) (mg/kg)	4441 ± 12
CEC (cmol/kg)	21.96 ± 3.93
WHC (%)	68.07 ± 0.87
Texture	Sandy loam
Sand (%)	78.33 ± 1.73
Silt (%)	8.67 ± 2.08
Clay (%)	13.00 ± 0.00

For laboratory experiments, soil pH was manipulated in order to obtain three batches of soil with different pH values. The soil without manipulation of pH: 5.4 ± 0.2 (hereinafter referred as the N5.5 soil) and two batches where different amounts of $CaCO_3$ were added to obtain two pH values: 6.5 ± 0.2 and 7.5 ±0.2 (hereinafter referred as the N6.5 and N7.5 soils, respectively). Water holding capacity was accessed for the three soil batches. The fungicide fumigant Basamid® (97% dazomet; w:w) was used as test substance in the laboratory experiments.

Test Organisms

Four soil invertebrate species were used as test organisms in the laboratory experiments: the collembolans *Folsomia candida* Willem 1902, the predatory mites *Hypoaspis (Geolaelaps) aculeifer* Canestrini 1883, the potworms *Enchytraeus crypticus* Westheide and Graefe 1992 and the earthworms *Eisenia andrei* Bouché 1972. Cultures were maintained in the Soil Ecology and Ecotoxicology laboratory of the Centre for Functional Ecology of the Department of Life Sciences of the University of Coimbra. All cultures were kept at 20 ± 2 °C under a photoperiod of 16:8 h light:dark. *Folsomia candida* were cultured in cylindrical plastic boxes with the bottom filled with a mixture of 11:1 (w:w) of plaster of Paris and activated charcoal. Organisms were fed twice a week with dry granulated yeast in a sufficient amount to avoid fungal contamination. *Hypoaspis aculeifer* were cultured in similar conditions of *F. candida* but fed two to three times a week with a tip of a spatula of cheese mites *Tyrophagus putrescentiae* Schrank, 1781. *Enchytraeus crypticus* were cultured in Petri dishes in bacti-agar medium 1:1 (v:v) (Oxoid, Agar N0. 1) supplemented with a four salt solution of calcium chloride, magnesium sulfate, potassium chloride (0.01 M) and sodium bicarbonate (0.1 M), dissolved in distilled water. The solution was previously autoclaved at 121 °C for 30 min and kept at 4 °C until use. Culture medium was renewed every 2 months. Organisms were fed once a week with 3-5 mg of rolled dry oats previously autoclaved. The earthworms *E. andrei* were cultured in plastic containers with substrate made of a mixture of 1:1 (w:w) of *Sphagnum* peat and cow manure. Substrate moisture was maintained at 60 to 70% of the water holding capacity, pH was adjusted to c.a. 7.00 by the addition of $CaCO_3$. Cultures were fed once a week with cow manure previously defaunated by two freeze and thaw cycles (each cycle was composed of 48h at -20ºC followed by 48h at 25ºC).

Reproduction tests

Laboratory reproduction tests were performed with the four species mentioned above and following the procedures advised in ISO and OECD standard guidelines. For security reasons and to avoid gas dispersion among different tests, each test experiment was performed in individual acclimatized chambers. Immediately before the beginning of each test, a stock mixture composed of dazomet (as powder of Basamid®) and dry soil was prepared and mixed with specific amounts of the test soil to reach the desired concentrations. Afterwards, distilled water was mixed with the test treatments to reach 50% of the maximum water holding capacity of soil. Lastly, each replicate was filled with soil mixture and closed, and the organisms were introduced in the replicates only after all replicates were filled with soil. Replicates were kept under 400-800 lx during the illumination period under a 16:8h light:dark photoperiod. Specific details of the laboratory tests of each species are presented in Table 2.

Table 2- Details of procedures adopted in reproduction tests with *Folsomia candida*, *Hypoaspis aculeifer, Enchytraeus crypticus* and *Eisenia andrei*.

	F. candida	*H. aculeifer*	*E. crypticus*	*E. andrei*
Guideline followed	ISO 11267, 2014	OECD 226, 2008	ISO 16387, 2014	ISO 11268-2, 2011
Synchronization (days)	10-12	28-35	>90	4-12 months
Test period (days)	28	14	28	56
Test containers (cm)	5×9 (D×H)	5×9 (D×H)	5×9 (D×H)	11×12 (D×H)
Nº replicates/treatment	5+1 [a]	5+1 [a b]	5+1 [a b]	4
Nº organisms/replicate	10	10[c]	10	10
Food per container (g)	0.002 (dry yeast)	0.05 (cheese mites)	0.05 (rolled oats)	10 (horse dank)
Days of food supply	0, 14th	0, every other day	0, 14th	0, 7th, 14th, 28th
Soil per container (g DW)	30	20	20	500
Concentrations tested (mg a.i. kg/soil)	0, 0.2, 0.6, 1.8, 5.4, 16.2, 48.6, 145.8	0, 1.8, 5.4, 16.2, 48.6, 72.9, 109.3, 131.2	0, 0.2, 0.6, 1.8, 5.4, 16.2, 48.6, 145.8	0, 1.8, 5.4, 16.2, 48.6, 72.9, 109.3, 131.2

D – diameter, *H* – height.

[a] - Additional replicate without organisms to measure soil moisture and pH at the end of the test.

[b] - Three additional replicates with organisms were used in controls.

[c] - Only female organisms were selected for the test.

Different range of concentrations were traced for the exposure due to different sensitivities and the need to derive the effective lethal and sublethal concentrations.

In *F. candida* reproduction tests, at the end of the test period, the content of each replicate was transferred to a larger vessel that was filled with tap water. After the addition of few drops of blue ink, the soil was gently stirred with a spatula, to let the collembolans float. Lastly, the surviving adults were counted and the collembolans in the water surface were photographed (NIKON). Juveniles were counted through the digital photographs using the open source software Image J (Rasband, 1997-2018).

In mites' tests, at the end of the test period the *H. aculeifer* extraction from replicates was performed using a MacFadyen extractor, using a temperature cycle composed of 25 ºC for 12 h, 35 ºC for 12 h and 45 ºC for 24h. Mites were collected directly to falcon tubes with a 70% ethanol solution and counted using a stereomicroscope (40x of magnification) to record the number of adult female survivors and juveniles.

At the end of enchytraeids tests, *E. crypticus* were killed, fixated and coloured by the addition of a solution composed of 5 mL of a 96% ethanol solution, few drops of a 1% Bengal Rose solution and distilled water in an amount sufficient to fill the replicate until half of the vessel. After 48h, the replicates were sieved in a 20 µm mesh size and organisms were recovered to a Petri dish and counted using a stereomicroscope (40x of magnification). All organisms were counted as juveniles since it is very difficult to distinguish the adults from juveniles due to the proximity of individual sizes.

In earthworm reproduction tests, adults were individually weighted at the beginning of the test and, 4 weeks after the beginning of the test, all surviving adults were removed from each replicate

and weighted to determine the % of the initial biomass in each replicate. The cocoons were maintained in the soil and incubated at the same conditions for an additional period of 28 days. To finalize the tests (after a total test period of 56 days), replicates were placed in a water bath at 50 to 60 ºC to force the movement of the juveniles to the soil surface, where the juveniles were counted manually.

Statistical Analysis

Normality and homogeneity of data were evaluated through Kolmogorov-Smirnov's and Bartlett's test, respectively, before statistical analysis. Significant differences in reproduction (based on total number of juveniles per replicate) and percentage of initial earthworm biomass between test treatments were evaluated by one-way ANOVA analysis. When differences were detected, a Dunnett's post hoc test was used to test for significant differences of treatments compared to the respective control (natural soil without the test substance).

Lethal concentration causing 50% of mortality (LC_{50}) were calculated by probit analysis through Priprobit 1.63 version software (Sakuma, 1998). Concentrations provoking 10 and 50% of reduction in reproduction compared to control (EC_{10} and EC_{50} values) were estimated through non-linear regressions and using the logistic model (EC, 2007). Model assumptions were verified via residue analysis and Q–Q plots. This statistical analysis was assessed in StatSoft, Inc. (2007), STATISTICA (data analysis software system), version 8.0.

One-tailed Behrens-Fisher test was used to pairwise comparisons of EC_{10} and EC_{50} for reproduction between test species within each soil pH, and between soil pH within each species tested, considering α=0.05. These comparisons were performed in Excel Office 2013 software.

Results

Soil pH measurements at the beginning and at the end of each set of tests for the four species confirmed the difference of pH values between the three soil batches: pH values (on average) of 5.5 ± 0.2, 6.5 ± 0.2 and 7.5 ± 0.2 (on average) and water holding capacity (on average) of 101.5; 107.7 and 90.8%, respectively for N5.5, N6.5 and N7.5 soils (individual data for each test are presented as supplementary data, Table 1S).

The validity criteria defined by the ISO and OECD guidelines were always fulfilled for all tests performed and soils. Percentage of survivors in tests with *F. candida*, *H. aculeifer* and *E. andrei* for the three test soils are also presented as supplementary data (Fig. 1S-4S of supplementary data). *Folsomia candida* reproduction was significantly affected in all tested concentrations of N5.5 and N7.5 soils and in concentrations higher or equal to 1.8 mg a.i./Kg in the N6.5 soil (Figure 1). The EC_{10} (compared to the respective controls) in the reproduction of collembolans estimated in N5.5 and N6.5 soils were not significantly different. The comparison of these EC_{10} values could not be performed with the EC_{10} in the N7.5 soil because the dose-response curve in this soil did not allow the estimation of the EC_{10}. Considering the EC_{50} values for collembolans reproduction, Basamid® toxicity significantly increased with the increase of soil pH (Table 3).

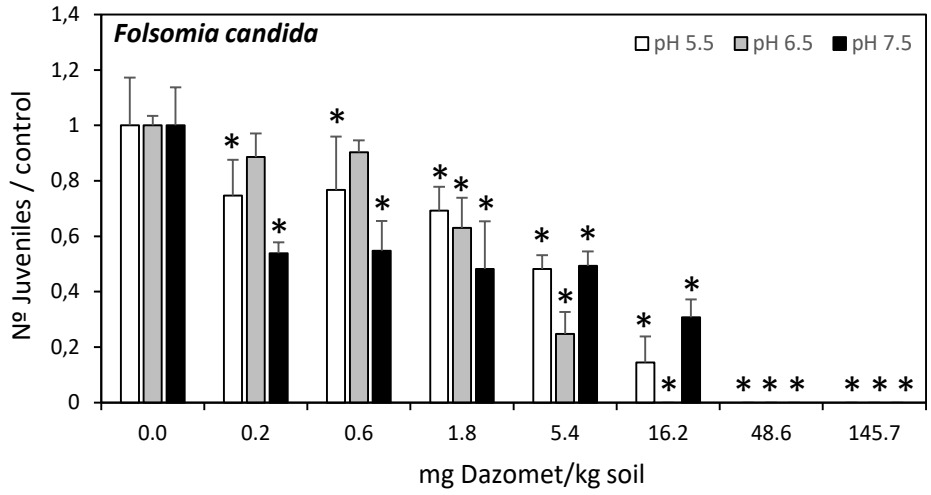


Figure 1- Effect of Basamid® (dazomet a.i.) in *Folsomia candida* reproduction at three different soil pHs. Values are expressed through the average of the ratio between the number of juveniles in each replicate divided by the average of the number of juveniles in the replicates of control (0 mg/kg treatment; average ± standard deviation; *n*=5). White bars correspond to the N5.5 soil, grey bars to the N6.5 soil and black bars to the N7.5 soil. *Number of juveniles significantly lower than that of control (one-way ANOVA, Dunnett's test, p≤0.05).

Hypoaspis aculeifer reproduction presented the Lowest Observed Effect Concentration (LOEC) at 5.4 mg a.i. /Kg concentration for Basamid® (dazomet a.i.) in the N5.5 and N7.5 soils (Fig.2) and at 48.6 mg a.i./kg in the N6.5 soil. As observed for collembolans, Basamid® toxicity was greater as the higher alkalinity of the soils. Based on the EC_{10} for reproduction, differences were not observed for the comparisons of soil pH 5.5 and 6.5 and for the 6.5 and 7.5 (Table 3). Significant differences were observed between the soil pH 5.5 and 7.5. Regarding the EC_{50} no significant differences were observed between soil pH of 5.5 and 6.5 but, significant differences were observed between the N5.5 and the N7.5 soils and between the N6.5 and N7.5 soils.

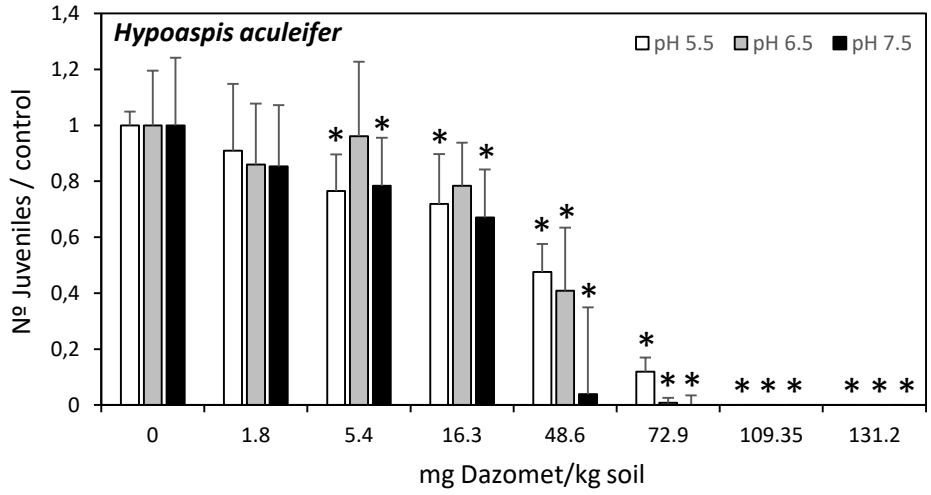


Figure 2- Effect of Basamid® (dazomet a.i.) in *Hypoaspis aculeifer* reproduction at three different soil pH. Values are the ratio (average ± standard deviation) of the number of *Hypoaspis aculeifer* offspring, all in relation to the average number of offspring in the control. White bars correspond to the soil pH of 5.5, grey bars to soil pH of 6.5 and black bars to soil pH of 7.5. *Number of juveniles significantly lower than the control (one-way ANOVA, Dunnett's test, $p<0.05$).

Reproduction of the pot worms *E. crypticus* was significantly affected in all concentrations at the three soil pHs. As observed for collembolans and mites, Basamid® toxicity increased for reproduction with soil alkalinity (Fig. 3). Based on the EC_{10} and EC_{50} values, Basamid® was more toxic in N6.5 and N7.5 soils than in the N5.5 soil (Table 3).

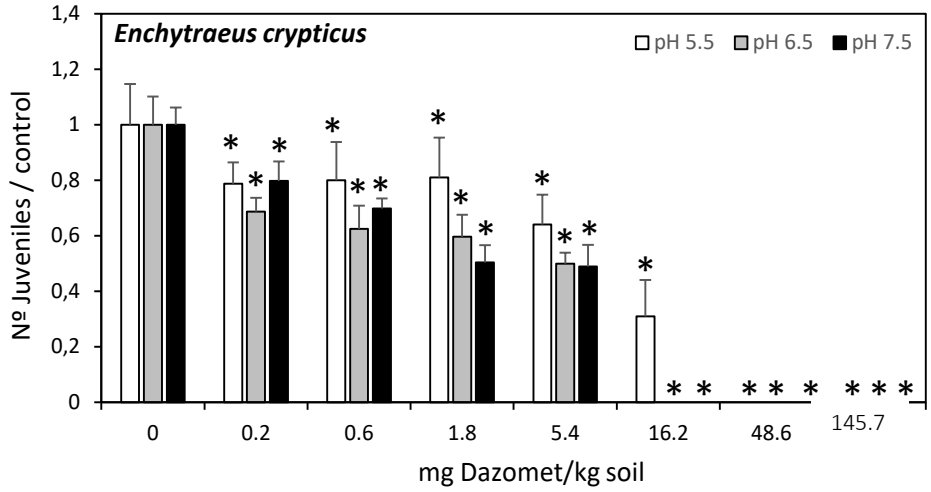


Figure 3- Effect of Basamid® (dazomet a.i.) in *Enchytraeus crypticus* reproduction at three different soil pH. Values are the ratio (average ± standard deviation) of the number of *Enchytraeus crypticus* offspring, all in relation to the average number of offspring in the control. White bars correspond to the soil pH of 5.5, grey bars to soil pH of 6.5 and black bars to soil pH of 7.5. *Number of juveniles significantly lower than the control (one-way ANOVA, Dunnett's test, $p<0.05$).

Reproduction of the earthworms *E. andrei* presented a LOEC of 1.8 mg a.i./Kg in the N5.5 soil, of 16.2 mg a.i./Kg in the N6.5 soil and of 5.4 mg a.i./Kg in the N7.5 soil. Contrary to the other species, Basamid® toxicity did not change with soil pH as both EC_{10} and EC_{50} values of test did not have statistical differences between the three soils (Fig.4 and Table 3).

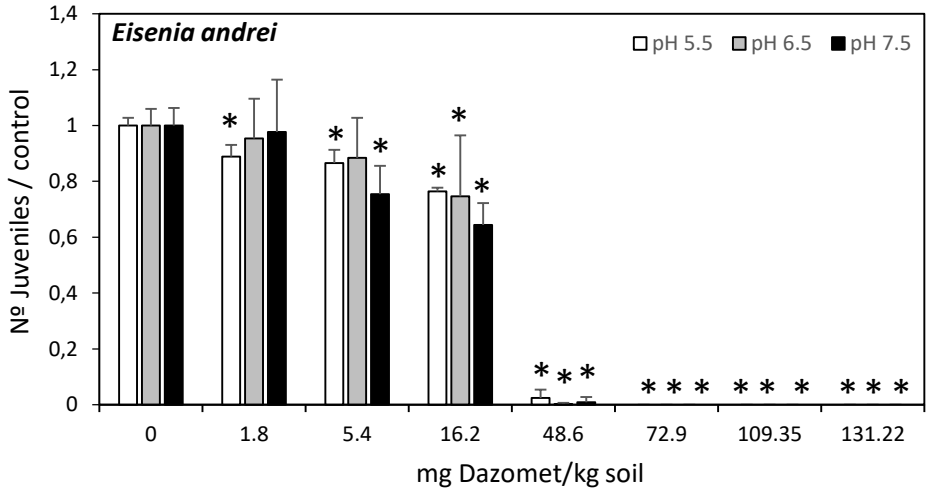


Figure 4- Effect of Basamid® (dazomet a.i.) in *Eisenia andrei* reproduction at three different soil pH. Values are the ratio (average ± standard deviation) of the number of *Eisenia andrei* offspring, all in relation to the average number of offspring in the control. White bars correspond to the soil pH of 5.5, grey bars to soil pH of 6.5 and black bars to soil pH of 7.5. *Number of juveniles significantly lower than the control (one-way ANOVA, Dunnett's test, $p<0.05$).

Table 3- Reproduction EC_{10} and EC_{50} values (and respective 95% confidence intervals), LC_{50} and NOEC and LOEC values for all tested species at 3 soil pHs spiked with Basamid® (dazomet as a.i.).

		EC_{10}	**EC_{50}**	**LC_{50}**	**NOEC**	**LOEC**
Species	**pH**	mg a.i./kg soil				
F. candida	**5.5**	1.19 ^{Aa} (0.22-2.16)	5.82 ^{Aa} (3.79-7.85)	9.17 (2.42-53.5)	>0.2	0.2
	6.5	0.74 ^{Aa} (0.42-1.06)	2.71 ^{Ba} (2.19-3.23)	5.26 (0.82-79.87)	0.6	1.8
	7.5	n.e.	1.12 ^{Ca} (0.10-2.13)	9.75 (2.35-70.0)	>0.2	0.2
H. aculeifer	**5.5**	32.60 ^{Ab} (21.97-43.23)	50.77 ^{Ab} (45.09-56.45)	44.9 (n.e)	1.8	5.4
	6.5	39.06 ^{ABb} (3.15-74.98)	47.64 ^{Ab} (42.63-52.65)	19.7 (n.e)	16.3	48.6
	7.5	11.69 ^{Ba} (7.73-15.66)	21.74^{Bb} (16.56-26.92)	18.0 (5.74-44.5)	1.8	5.4
E. crypticus	**5.5**	2.66 ^{Aa} (0.79-4.53)	10.23 ^{Ac} (7.03-13.42)	29.0 (21.9-39.8)	0.0	0.2
	6.5	0.10^{Bc} (0.0-0.22)	2.12 ^{Ba} (1.07-3.17)	18.5 (4.06-57.0)	0.0	0.2
	7.5	0.15 ^{Bb}(0.03-0.28)	2.14 ^{Ba} (1.38-2.89)	21.17 (n.e)	0.0	0.2
E. andrei	**5.5**	14.21 ^{Ac} (12.73-15.70)	22.79 ^{Ad} (19.82-25.75)	>48.6	<1.8	1.8
	6.5	13.88 ^{Ad} (8.21-19.54)	20.42 ^{Ac} (8.73-32.10)	>48.6	5.4	16.2
	7.5	10.96 ^{Aa} (7.14-14.78)	20.25 ^{Ab} (16.23-24.27)	>48.6	1.8	5.4

n.e. – Data did not allow the estimation of the toxic value. Lower-case letters mean significant differences among different species and within the same soil pH, upper-case letters mean significant differences among soil pH within the same species.

On a general note, the relative sensitivity of the four tested organisms respecting the reproduction endpoint (based on EC_{50} derived values) for the three soil pH can be expressed as (from the most sensitive to the less sensitive species):

Soil pH 5.5: *F. candida* > *E*. *crypticus* >*E*. *andrei* >*H*. *aculeifer*

Soil pH 6.5: *F. candida*= *E. crypticus* >*E*. *andrei* >*H*. *aculeifer*

Soil pH 7.5: *F. candida*= *E. crypticus* >*E*. *andrei* =*H*. *aculeifer*

Discussion

The exposure of soil organisms to Basamid® was critically toxic to all tested organisms. Additionally, reproduction was significantly affected at concentrations considerably lower than the recommended dose of 145.7 mg a.i/ Kg soil advised by the supplier company. The toxicity of Basamid® was significantly influenced by soil pH for all tested organisms except for *E. andrei.* The effect of soil pH itself, independently of Basamid® exposure was not considered an influent factor to the organism's sensitivity. As it is possible to observe, differences within the controls for the three soil pHs were not statistically significant.

In general, for all tested organisms, toxicity of Basamid® increased with the soil pH increase (i.e. increase of soil alkalinity) except for earthworms, where toxicity of Basamid® did not differed significantly among the tested soils.

Two different sensitivity groups can be identified, the *F. candida* and *E. crypticus* which presented the most sensitivity species to Basamid® across the alkalinity of the soil and the *E. andrei* and *H. aculeifer* which presented the least sensitivity species group exposed to the same conditions of pH values.

Previous studies have reported that soil pH may affect the interactions of pesticides adsorption to soil particles (depending on its mode of action), also interfering on the pesticides degradation rates (Nicholls 1988; Sheng et al. 2005). Concerning the a.i. dazomet, it is known that the increase of soil water content and pH, promotes hydrolysis and accelerates the MITC (methylisothiocyanate) gas release, that is the metabolite responsible for the fumigant effect (Nicholls 1988; FAO 2001; EFSA 2010). On the other hand, according to EFSA 2010, the half-life of dazomet can vary with soil structure and properties, varying from 4 h to 7 days. Therefore, at soils with higher pH a faster release of MITC gas is expected, which might result in a greater effect in a shorter time frame compared to soils with lower pH values. This fact supports our data respecting the occurrence, on a general note, of higher toxicity in the organisms exposed to soils with higher pH. Additionally, these results agrees with previous studies in which the effects and chemical reaction of dazomet-based fumigants (as degradation and conversion to MITC) were reported to be pH factor dependent (Morrell et al. 1988; Fang et al. 2018a). Morrell and colleagues (1988) conducted a study using another commercial formulation of dazomet, Mylone® to evaluate the effect of pH in the decomposition of the major metabolite MITC and in the growth of the fungi *P. carbonica*. The authors performed different buffers at different pHs containing dazomet to apply in wood inoculated with the fungi *P. carbonica.* The results reported that the alkalinity improved the formation of MITC

conversion in solution, which inhibited the fungal growth. This study demonstrated the effective control of target pathogens at higher pH. Therefore, this relation of MITC effects on target organisms at higher pH can conduct and supports the importance to investigate the effects of dazomet and respective major metabolite MITC to non-target organisms at the same conditions. Notwithstanding of the different *vias* of exposure, it could be expected a similar result of higher toxicity effects, in the same conditions, to non-target species which resemblance to the present study results. Another study performed by Fang and colleagues (2018b) aimed to investigate the influence of dazomet (using Basamid® formulation) in the diversity of microbial communities (more specifically in microbes of the natural communities with functional genes responsible for nitrification) in two natural soils with pH values of 4.3 and 7.2. The authors reported that, after treating both soils with dazomet (with a dose corresponding to 58 mg/Kg soil), microbial communities were significantly affected by dazomet. This effect was evidenced by the reduction of the abundance of 16S rRNA and N-cycling functional genes in the most alkaline soil where dazomet appeared to be less persistent or more rapidly degraded. These data seem to support our findings suggesting that the degradation of dazomet is faster in soils with higher pH. However, it should be taken into account that, contrarily with what happen in the present study, where the soil pH was manipulated within the same natural soil, in the study conducted by Fang et al. (2018a) two different natural soils were used, which means that both soils differed not only in pH but also in other soil properties that may also interfere in the dazomet degradation/persistence.

Other studies have been conducted to evaluate the effect of dazomet toxicity to non-target organisms (i.e. *Eisenia fetida*, soil microorganisms, Mao et al. (2017b), Yim et al. (2016, 2017)), however, those studies have not considered the pH factor influence. It has been reported that soil pH along with other soil properties such as moisture content and temperature, can promote a faster hydrolysis reaction in dazomet fumigation (Fang et al. 2018a). Increasing dazomet diffusing in soil might have consequences of higher availability in the soil, therefore, higher extension of leachates targeting a wider range of non-target organisms (Nicholls, 1988; Fang et al. 2018a).

As reported in the present work, the effects of dazomet to non-target organisms were very severe, affecting the reproduction of all organisms in a natural soil with a pH corrected to 5.5, 6.5 and 7.5 with $CaCO_3$. Among the four species tested, *F. candida* presented the most level of sensitivity (with the lowest LC_{50} values of 9.17, 5.26 and 9.75 mg a.i./Kg in N5.5, N6.5 and N7.5 soils, respectively).

Despite of the lack of data in the literature regarding the effects of dazomet in collembola, these results are in agreement with other different studies previously performed where *F. candida* presented higher sensitivity to chemicals than the oligochaetes and the predatory mite *H. aculeifer* (Owojori et al. 2014; Chelinho et al. 2017; Kamoun et al. 2018; Renaud et al. 2018). On the other hand, regarding mortality, *E. andrei* presented the higher tolerance to dazomet from all four organisms tested, independetly of soil pH with LC_{50} >48.6 mg a.i/Kg soil. This occurence can be related to the complexicity of the organisms and different mechanisms influenced by the earthworm movement within the soil. As mentioned above, dazomet tends to dissipate fast influenced by

abiotic parameters, and has a lower adsorbance to soil particles (log P_{OW} = 0.63 at 20°C, pH 5.8, (99.9%), EFSA, 2010). Therefore, the organism's movement in the soil can be also leading to faster dissipation of dazomet from the soil. Thus, due to earthworm bigger size in comparison to the other organisms it can be occurring a higher effect on dazomet dissipation and lower adsorption to the soil, leading to lower effects on *E. andrei*. Furthermore, it must be considered the effect of the additional organic matter as food provision (horse dank) to the earthworms, whereas the other three organisms were fed with oats, yeast or fungi. Reported by Fang and colleagues (2018a), the authors also observed that increasing organic matter in the soil increases the DT_{50} of dazomet, which leads to slower degradation rate. On this note, the higher content of organic matter in the earthworm's soil compared to the other three organisms could explain the lack of soil pH influence in Basamid® toxicity regarding mortality. By comparison, the earthworms mixing the organic matter with the spiked soil (leading to a slower reaction of dazomet), can induce an overlap effect of the organic matter towards the effect of soil pH. Regarding the influence of soil pH on dazomet effects to the other three organisms' reproduction, on a general approach, at soil pH 5.5 less sever effects were observed for all organisms, based on EC_{10} and EC_{50}. The higher toxicity to *F. candida*, *E. crypticus* and *H. aculeifer* reproduction with higher alkalinity of the soil could be related to the fast chemical reaction of dazomet in soil at higher pH as it was observed already by Fang and colleagues (2018a). In this case, when comparing to the earthworms where no influence of soil pH was observed, the organisms were exposed to a lower organic content. The influence of soil pH was highly represented towards the organic matter, in opposition of what might be occurring to the earthworm's exposure. Furthermore, another factor that might be influencing the reproduction on *F. candida*, *E. crypticus* and *H. aculeifer* towards increasing concentrations crossed with soil pH could be the availability of Ca^{2+} elements and the respective individual needs of each organism to complete the reproduction cycle. Regarding *E. andrei*, the lack of soil pH effect in reproduction when exposed to Basamid® could be also due to the longer cycle and exposure time test. Contrary to the other organisms, the earthworms are set in the test for 4 weeks to be able to lay the cocoons and then are removed from the test. These 4 weeks seems to be more than enough time for dazomet, which has a short DT_{50} in soil to dissipate and no related-effects occur (EFSA 2010). Therefore, at the moment of the hatching, it could be possible that dazomet isn´t present in great concentrations, along with its metabolites.

Derived from the present study, our data gives strength to the need of using natural soils with different abiotic parameters in the environmental risk assessment of PPPs as these factors may interfere in the extension of the risk estimated. Our data also suggest that the application of fumigants with Basamid® composition, should take into account the soil pH in order to be able to reduce the adverse effects in non-target organisms. As observed, the soil pH influenced significantly dazomet's mode of action in all four soil non-target organisms. To give response to the hypothesis of this study, it was verified that the recommended dose of 145.7 mg a.i./kg soil is highly toxic to organisms, since the higher LC_{50} were observed for *E. andrei* (less sensitive organism) at 48.6 mg a.i./ kg soil. Regarding the second hypothesis, it is clear the effect of soil pH in Basamid®

effects concerning the organism's reproduction, therefore, Basamid® mode of action is pH dependent.

Given the high toxicity of Basamid® to non-target species (even at concentrations lower than the recommended dose) and taking into consideration literature data, that support that dazomet is more toxic in the highest soil pHs but also less persistent in soil, further research is needed to evaluate the time frame needed to have a recolonization of dazomet impacted soils and at which point soil pH interfere in such period.

Conclusions

In conclusion, the results obtained suggest that applying Basamid® at the recommended doses significantly affects mortality and reproduction of the studied non-target soil invertebrates. Moreover, soil pH can extent dazomet's chemical reaction which supports the need to reduce the recommended dose effects in the conditions mentioned above or the need for mitigation or restrictions protective measures. Nevertheless, more specific studies regarding the mode of action and the ability for species recovery after dazomet application in such different soil pH conditions must be investigated.

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Supplementary Data

Tables

Table 1S- Mean values with standard error for soil humidity and soil pH soil at the beginning and at the end of each set of tests for the four species in test.

Species	pH	pH $_{beginning}$	pH $_{end}$	H $_{beginning}$	H $_{end}$
F. candida	**5.5**	5.79 ± 0.02	5.42 ± 0.02	48.50 ± 0.7	42.85 ± 0.7
	6.5	6.40 ± 0.02	6.57 ± 0.03	50.15 ± 1	41.39 ± 0.84
	7.5	7.76 ± 0.01	7.44 ± 0.003	53.10 ± 0.5	47.51 ± 0.5
H. aculeifer	**5.5**	5.59 ± 0.008	5.71 ± 0.001	50.15 ± 0.3	43.68 ± 0.6
	6.5	6.52 ± 0.04	6.45 ± 0.01	49.34 ± 0.4	49.74 ± 0.53
	7.5	7.62 ± 0.02	7.41 ± 0.002	53.48 ± 0.31	54.6 ± 1.2
E. crypticus	**5.5**	5.81 ± 0.02	5.52 ± 0.06	49.86 ± 1.3	45.30 ± 1.3
	6.5	6.31 ± 0.02	6.57 ± 0.03	46.86 ± 0.9	44.69 ± 0.9
	7.5	7.63 ± 0.03	7.47 ± 0.004	51.59 ± 0.3	48.92 ± 1.3
E. andrei	**5.5**	5.49 ± 0.02	5.51 ± 0.02	44.77 ± 0.9	45.54 ± 1.1
	6.5	6.45 ± 0.01	6.46 ± 0.02	50.69 ± 1.1	50.08 ± 0.6
	7.5	7.50 ± 0.02	7.49 ± 0.01	50.59 ± 0.15	50.98 ± 0.8

Figures

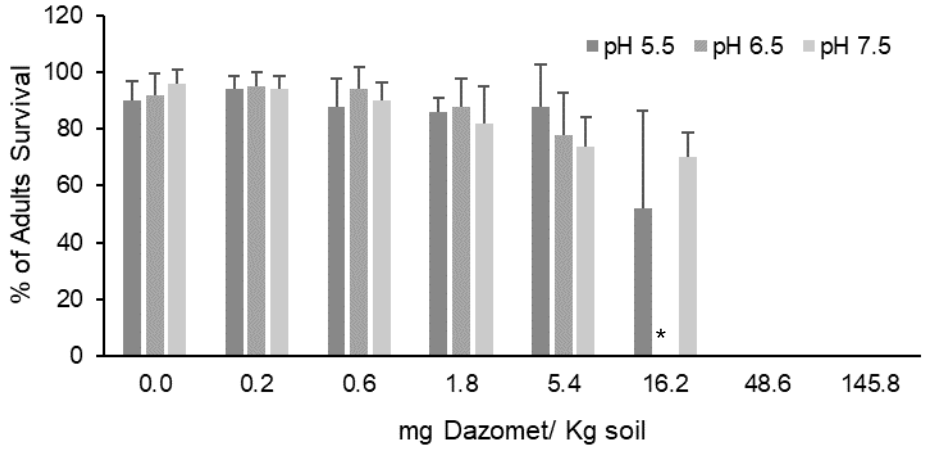

Figure 1S- *Folsomia candida* adults survival rate exposed to dazomet in different soil pH 5.5,6.5 and 7.5. *- Significant differences between each soil pH and respective control, bars represent standard deviation (Dunnet's test $p<0.05$).

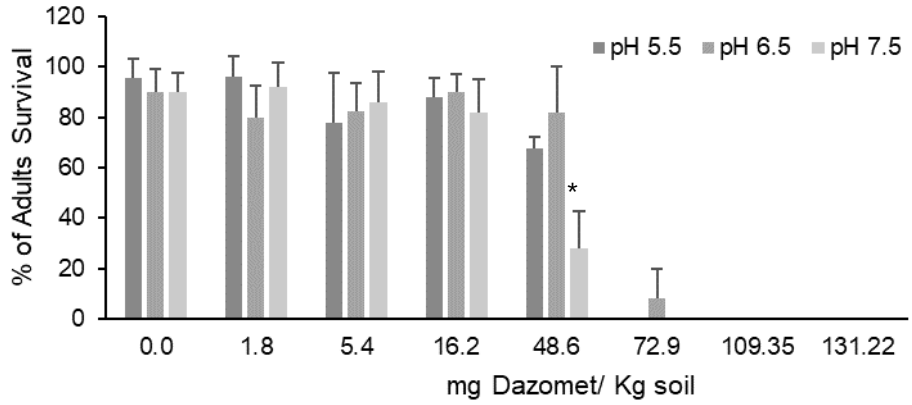

Figure 2S- *Hypoaspis aculeifer* adults survival rate exposed to dazomet in different soil pH 5.5,6.5 and 7.5. *- Significant differences between each soil pH and respective control, bars represent standard deviation (Dunnet's test p<0.05).

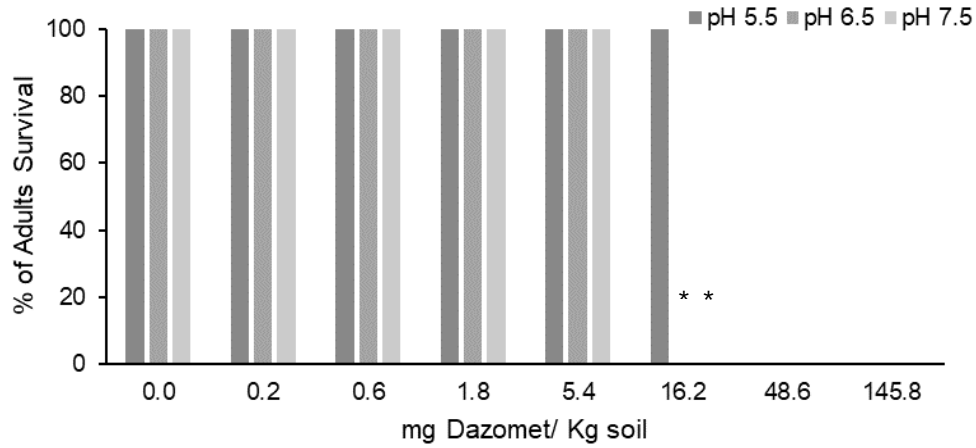

Figure 3S- *Enchytraeus crypticus* adults survival rate exposed to dazomet in different soil pH 5.5,6.5 and 7.5. *- Significant differences between each soil pH and respective control, bars represent standard deviation (Dunnet's test p<0.05).

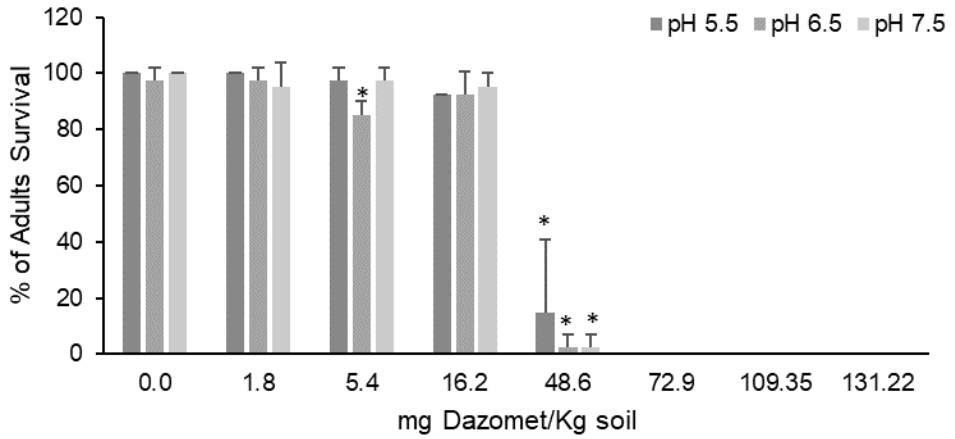

Figure 4S- *Eisenia andrei* adults survival rate exposed to dazomet in different soil pH 5.5,6.5 and 7.5. *- Significant differences between each soil pH and respective control, bars represent standard deviation (Dunnet's test $p<0.05$)

Chapter III

Avoidance tests as a tool to evaluate the influence of soil pH in earthworm's re-colonization after soil fumigation.

Avoidance tests as a tool to evaluate the influence of soil pH in earthworm's re-colonization after soil fumigation.

Authors

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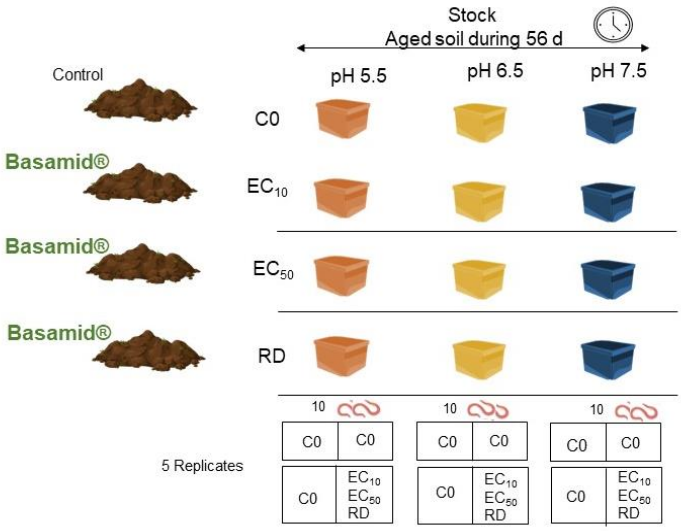
Graphical Abstract



Results

pH	5.5 ± 0.2	6.5 ± 0.2	7.5 ± 0.2
	Time to No Avoidance behaviour (days)		
EC_{10}	4	4	0
EC_{50}	21	14	5
RD	56	14	14

Highlights

- Avoidance tests with earthworms revealed high sensitivity to Basamid® exposure.
- Soil pH influenced earthworms' avoidance behaviour when exposed to Basamid®.
- Higher alkalinity of the soil accelerates Basamid® gas release from the soil.
- Avoidance tests are suitable to estimate the severity of Basamid® damage across time.
- Avoidance tests can be included in ERA to estimate the minimum time needed for recolonization.

Abstract

To encompass the consequent negative effects that occur in the environment to non-target organisms due to the intense application of plant protection products, it is important to assess the possibility of impacted sites recovery. In this line, this work aimed at understanding the effects of

soil pH in Basamid® long-term exposure along with soil organisms' ability to recolonize the impacted soil. Multiple avoidance tests of 24 h across different times during 56 d with *Eisenia andrei* were performed with aged soils at pH of 5.5, 6.5 and 7.5. Three concentrations were assessed based on *E. andrei* reproduction EC_{10} and EC_{50} and the recommended dose of Basamid®. As a fumigant and with higher volatility and lower DT_{50} in soil (of 0.52 d at 20ºC), it should be expected a faster dissipation from the soil. However, environmental factors such as soil pH can vary the velocity of such dissipation reaction. From the overall avoidance tests, the organisms needed about 56 d at soil pH 5.5 to start to not avoid the spiked soil while in the soil pH 6.5 and 7.5, only 14 d were needed to observe the same response. A no avoidance response, through time, was interpreted as good conditions in the soil and availability to start a recolonization process. These results allowed to observe a variable minimal time needed for the earthworms to start not avoiding the soil spiked with Basamid® under the influence of soil pH.

In addition, this approach of avoidance tests across time demonstrated to be an important complement tool regarding environmental risk assessment in the way that recovery of an impacted site can be assessed and with a time frame estimation.

Keywords: *Eisenia andrei,* ageing soil*,* soil alkalinity, annelids, Basamid®.

Introduction

Intensification of agriculture due to the increasing population on earth have promoted the intensive use of plant protection products (PPPs) and soil has been one of the most affected systems due its direct exposure to PPPs (Lehmann et al., 2020). Soil system has multiple key ecological functions ensuring the provision of relevant ecosystem services like nutrient cycling, carbon storage, water quality maintenance, and support of biodiversity (Nielsen et al., 2015; Thiele-Bruhn et al., 2012). The provision of some of these services depends not only on the edaphic communities but also on soil properties like pH. Soil pH is an important abiotic parameter, influencing soil structure and quality, being also responsible for nutrient availability, biochemical reactions, enzymatic activity and soil fertility (Dick et al., 2000; Sheng et al., 2005). Soil pH can also influence the behaviour of PPPs, interfering on its chemical reactions in the soil, and consequently on its availability to edaphic organisms (Fang et al., 2018). This is particularly evident for fumigants as the mode of action of these type of PPPs is usually based on a chemical reaction chain that depending on soil structure and composition or different variations of environmental factors, can react chemically, converting into gas form at different rates. For instance, Fang and colleagues (2018) performed a study to evaluate the influence of environmental factors in the fumigant dazomet decomposition rate and observed that higher humidity and alkalinity of the soil accelerates the fumigant gas release in opposition of higher content of chicken manure or urea fertilizers. More recent studies (Gabriel et al., *in prep.*) have reported that increasing alkalinity of the soil increases toxicity of dazomet (sourced by the commercial formulation Basamid®) to soil invertebrates like *Folsomia candida*, *Hypoaspis aculeifer* and *Enchytraeus crypticus*, at concentrations much lower than the recommended dose. The fumigant formulation Basamid® were also selected to this study

based on its major composition of dazomet, an active substance with postponed revision for environmental evaluation to 2023 (EC 2020).

The active ingredient of Basamid® dazomet (active substance present in 97%) breaks in methyl isothiocyanate gas (MITC) after its application when in contact with humidity conditions. Dazomet is weakly persistent in soil (DT_{50} of 0.52 d) but has a large spectrum target, being used as herbicide, fungicide, nematicide and sterilant in agriculture (EC, 2011; EFSA, 2010; Kanesho Soil Treatment SPRL/ BVBA, 2014). With a large spectrum target function, causing mostly lethal effects in short period, it should be expected sever effects in the ecosystem. Acute effects in the environment drives to severer threats to the ecosystems of extreme or even permanent effects such as species depletion (Rani et al., 2021). However, a recovery of the ecosystem after treatment, over time, may also be expected. Based on the sever effects of Basamid® in soil organisms, even with a lower DT_{50} allied to the need of its use in pests' depletion, there is the need to understand the effects across time envisioning further recovering and recolonization of the soil after fumigation. To do so, avoidance test can be used to investigate the minimum time needed to achieve a recovery of a previous impacted site as already investigated in different studies (Natal-da-luz et al., 2008a; Renaud et al., 2022).

Avoidance soil tests are cost-effective assays used to evaluate in short time the quality of soil or water and investigate the presence of toxic substances at low concentrations recuring to organisms behavioural response (ISO 17512-1, 2008; Lopes et al., 2004; Moreira-Santos et al., 2008; Natal-da-Luz et al., 2008a, 2008b). The principle of an avoidance test is to observe the organism behaviour when exposed at once to dual or multiple condition -scenarios as different types of contamination, different soils with different physico-chemical composition, contaminated versus non-contaminated soils (Garcia et al., 2008; ISO 17512-1, 2008). The most common used standard organisms are the earthworms, springtails and acari from soil ecosystem (Loureiro et al., 2005; Natal-da-Luz et al., 2008a; Renaud et al., 2022), although studies with daphnids and zebrafish have been also reported (Lopes et al., 2004; Moreira-Santos et al., 2008). The performance of avoidance tests can be used as a complement to the requested ecotoxicological tests, at first screening TIERs in risk assessment due to its reproducibility and type of response from the organism in short time (Natal-da-Luz et al., 2008a; Natal-Da-Luz et al., 2009; Renaud et al., 2022). Furthermore, it must be brought to the attention the potential use of avoidance tests to investigate the possibility of sites recovery through time (Natal-da-Luz et al., 2008a) or the possibility to translate a minimum time needed for site recolonization (Renaud et al., 2022). The PPP risk assessment leans over to the "closed" effects in the environment in a segment of assessment TIERs but the follow up on recovery communities along time is mostly discarded (Renaud et al., 2022). To this, avoidance tests can be an important tool to address the impacts in the environment across communities' recovery from impacted sites through time.

In this work, avoidance tests with earthworms *Eisenia andrei* were performed using soils mixed with different amounts of CaCO3 (for pH manipulation), spiked with Basamid® in laboratory in different concentrations and submitted to different periods of ageing. Once observed that, without

ageing, all spiked soils were avoided when combined with clean soil, the spiked soils were aged over different time periods and used in additional avoidance tests. The shortest ageing period after which the spiked soil no longer provoked avoidance behaviour to the test organisms (when combined with clean soil) was considered the minimum time period needed to start the soil recolonization (Clements and Rohr, 2009; Renaud et al., 2022). This new approach brings novelty and higher relevance towards environmental risk assessment and environmental protection tools regarding the community's recovery potential of impacted sites. As it can be already considered, in EFSA (2016) the acceptability of the effects magnitude of a product in the environment, depends on the recolonization time that must be assured in a relevant time frame (EFSA, 2016).

This work aimed to assess the influence of soil pH in the time needed to start the re-colonization of the soil after the application of Basamid® in different concentrations. The earthworms *Eisenia andrei* were used as a model species representative of soil fauna and the tool proposed for this evaluation were the two-chamber avoidance tests.

Material and Methods

Test soil and test substance

In the laboratory experiments, a natural soil was used, collected in Herdade de Freixo-do-Meio in Alentejo region, Portugal (38°41'44.9''N 8°18'33.7''W) that is an agro-silvo-pastoral woodland under sustainable management. Soil physical and chemical parameters are presented in Table 1 (for information about the methods used for the measurement of soil physical and chemical parameters see Ferreira et al. 2022). The soil was collected from the top 20-cm layer and was free of fertilizers and pesticide applications for more than five years. Once collected, the soil was sieved at 5 mm and defaunated through heating at 80 °C for one or more periods of 48h followed by similar periods at room temperature. The fumigant Basamid® (97% of Dazomet; w:w) produced by Certis Europe was used as test substance in the study and was obtained from a local supplier. Physical and chemical properties of dazomet are presented in Table 2.

Table 1- Chemical and physical characterization (average ± standard deviation; n=3) of the natural soil of Herdade do Freixo-do-Meio (Portugal). *Data taken from Ferreira et al. (2022).*

Parameter	
pH (KCl)	5.10 ± 0.10
Organic Matter Content (%)	4.91 ± 0.67
C/N	11.47 ± 0.21
Total Nitrogen (g/kg)	2.49 ± 0.29
Inorganic Nitrogen (mg/kg)	182.77 ± 15.50
Phosphorous (P_2O_5) (mg/kg)	32.67 ± 5.13
Potassium (K_2O) (mg/kg)	441.00 ± 12.12
Cation Exchange Capacity (cmol/kg)	21.96 ± 3.93
Water Holding Capacity (%)	68.07 ± 0.87
Texture	Sandy loam
Sand (%)	78.33 ± 1.73
Silt (%)	8.67 ± 2.08
Clay (%)	13.00 ± 0.00

Table 2- Physical and chemical properties of dazomet. Chemical properties and DT_{50} values were collected from EFSA Conclusion, (2010).

Properties	Dazomet
IUPAC name	3,5-dimethyl-1,3,5-thiadiazinane-2-thione
Empirical formula	C5H10N2S2
Molecular mass	162.3
Solubility (pH 7)	3.5 g/L at 20 °C
Log Po/w	0.63 at 20°C (pH 5.8)
Henry's Law constant	5.10 x 10-5 Pa m³/mol at 20 °C
Degradation/dissipation (DT_{50})	Very low to low: 0.07d – 5.4 d (20 ºC)
Soil (lab at 20 °C)	0.52 d
Soil (field at 20 °C)	1.2 d

Test organisms

The earthworms *E. andrei* were used in the laboratory experiments. The individuals were cultured in plastic containers with a mixture of *Sphagnum* peat and cow manure in a ratio of 1:1 (w:w) as substrate. The water content of the substrate was kept at 60 – 70 % of its maximum water holding capacity and the pH of 6-7 was adjusted with the addition of $CaCO_3$. Cultures were fed once a week with horse manure previously defaunated through two freeze-thawing cycles (each cycle composed of 48h at -20ºC followed by a period of 48h at 25ºC) and were maintained at 20 ± 2 °C under a photoperiod of 16:8 h light: dark.

Avoidance tests

Avoidance tests with earthworms were performed following the methods described in the ISO standard nº 17512-1 (ISO, 2008) with some adaptations.

The natural soil collected was used for all tests but mixed with different amounts of $CaCO_3$ to obtain three different soil pHs: 5.5 ± 0.2, 6.5 ± 0.2 and 7.5 ± 0.2. Once prepared the three portions of soil with different pHs, each soil was spiked by mixing it thoroughly with a previously prepared concentrated mixture of the same soil and Basamid® in the correct proportion to obtain the desired dazomet concentration.

For the avoidance tests, the water content of each soil was adjusted to 40-60% of its maximum water holding capacity immediately before the beginning of the ageing period or before being introduced in the test containers (for tests with freshly spiked soil, i.e., day 0). The ageing of the soil was performed for each test concentration and soil pH and a clean soil was aged in the same conditions of the spiked soils to be used in the dual-control tests to control the behavioural measurements in each ageing period. Soil ageing was performed in plastic closed containers (one container per test concentration and soil pH) at 20 ± 2 ºC and under a photoperiod of 16:8h (light:dark) . Over the ageing period, the containers were frequently aerated and the moisture of the three batches of soil was maintained by compensating the weight losses with the addition of distilled water every five days.

Avoidance tests of 24h were performed (instead of the ISO recommended 48h tests) as this time period seemed the most adequate considering the DT_{50} in soil of 0.52 d of the fumigant (EFSA, 2014). The selected concentrations to be used in the avoidance tests were based on the derived effect concentrations (in soil pH of 5.5) from the reproduction tests previously performed by Gabriel et al (*in prep*): 13.82, 20.42 and 145 mg dazomet/kg that correspond to EC_{10}, EC_{50} and application dose recommended by the manufacturer (RD), respectively. Over 56 days of ageing (the time period of an earthworm reproduction test with *E. andrei*), two-chamber avoidance tests with the three soil pH batches were performed. Spiked soils with different ageing periods were combined with clean soil (equally aged and within the same soil pH of the spiked soil) to define the shortest ageing period above which no significant avoidance behaviour is detected for each test concentration (Minimum time period for a re-colonization; MPR). The ageing periods of 0, 1, 2, 4, 5, 14, 21, 28 and 56 days were considered for the soil with a pH of 5.5. Once a combination, at a certain ageing period, did not present significant avoidance behaviour at the end of the 24h of exposure, no further ageing periods were tested for that combination/concentration. This approach was also applied for the other soils (of 6.5 and 7.5 pHs) but not all combinations were tested as, for the selection of the combinations to be tested, it was assumed that the MPR is higher as the higher the soil pH. It was also assumed that the highest dazomet concentrations will correspond to the highest MPR. Following these assumptions, the selectin of the combinations to be tested in avoidance tests with soils of 6.5 and 7.5 pH were based on the avoidance behaviour observed in the avoidance test with the soil of 5.5 pH. Five replicates were used per each avoidance test combination and each replicate consisted of a plastic box (20 cm length, 12 cm width and 5 cm height) divided into two sections of the same size with a plastic card vertically introduced in the middle line of the box. Each section of the replicates was filled with 250 g of soil (dry weight equivalent). A dual-control test was also performed using clean soil in both sections of the replicates for each soil pH to check if, in the absence of dazomet in both sides of the replicate, a random distribution is found over both sections. After filling the boxes with the soil, the divider was removed, and ten earthworms (previously washed and wiped in absorbent paper) were placed exactly in the middle line of the replicates and covered with a perforated lid to reduce water losses by evaporation. All tests were performed at 20 ± 2°C and under a photoperiod of 16:8 h light: dark. At the end of the test period, the card divider was inserted in the middle line of the replicates and the earthworms were counted in each section of the replicates. The organisms found in the middle of the box were counted as 0.5 independently of the part of the body found in each side of the replicate. For this experiment, only earthworms with visible clitellum and weight above 250 mg were used. Earthworms were used in the test only once and discarded at the end of each avoidance test. Soil pH and moisture were measured immediately before the beginning of each avoidance test in all combinations.

Statistical Analysis

An avoidance behaviour was considered when the number of organisms in the treatment section was equal to or higher than the number of organisms in the control section. The significance of avoidance behaviour found in each combination was analysed by Fisher exact test. This statistical analysis is based on a contingency table that compares the observed behaviour in a specific combination with a hypothetical distribution in a non-avoidance response (null hypothesis). For the two-section avoidance tests (i.e., combination composed of clean soil in one section and spiked soil in the other section) a one-tailed test was performed, and the null hypothesis assumed that half of the total number of organisms stay in the soil that is being assessed and no avoiders exist. For the dual-control tests (clean soil in both sections of the replicate) a two-tailed test was performed, and in these cases, the null hypothesis assumed an equal distribution in both sides of the control section meaning that an equal number of organisms stay and leave both sections of the replicate. Null hypotheses were rejected for a probability equal or lower than 0.05 ($p \leq 0.05$).

Results

Dual-control tests showed an even distribution of individuals in both sections of the test containers (i.e., no significant differences were observed between the number of organisms in each section of the replicates) and 100% of survival in all ageing periods tested ($p > 0.05$ for all dual-control tests performed).

Similar in all three soil pH avoidance tests mortality was not observed for any assessed combinations. Earthworms showed significant avoidance behaviour since day zero for all the three concentrations tested ($p < 0.05$) except for EC_{10} in soil pH of 7.5. With increase concentration of Basamid® in all soil pH tested, longer time were needed to observe a significant no avoidance behaviour.

Regarding soil pH of 5.5, avoidance behaviour was absent since day 4^{th} of the EC_{10} concentration test, day 21^{st} of the EC_{50} concentration and day 56^{th} for the higher tested concentration, RD (Table 3 and Fig. 1S of supplementary data).

In soil pH 6.5 the avoidance behaviour was absent since day 4^{th} of the EC_{10} concentration exposure and days 14^{th} the EC_{50} concentration and RD (Table 3 and Fig. 2S of supplementary data).

Regarding soil pH 7.5, the non-avoidance behaviour was observed since day zero of the EC_{10} concentration test, day 5^{th} of the EC_{50} concentration and day 14^{th} for the RD (Table 3 and Fig. 3S of supplementary data). In soil pH of 7.5, at higher concentration, the no avoidance behaviour was observed earlier in time when compared to the other two soil pH.

In Table 3 the minimum period of time needed for a recolonization (MPR) estimated through the earthworm's avoidance tests for each soil pH and each test concentration is presented. Across the different soil pH and time and in crescent order of concentration of avoidance combination, the time needed to no avoidance behaviour observance was shorter as higher it was the alkalinity of the soil.

Table 3- Minimum period of time needed for a recolonization based on the avoidance behaviour observed in earthworm avoidance tests with the species *E. andrei* when exposed to dazomet (sourced from Basamid®) at the concentrations 13.82, 20.42 and 145 mg/kg (corresponding to its reproduction EC_{10} and EC_{50} and to the recommended dose, respectively for the natural soil with the natural pH 5.1) in a natural soil with a pH of 5.5 or with the pH adjusted to 6.5 and 7.5.

pH	**5.5 ± 0.2**	**6.5 ± 0.2**	**7.5 ± 0.2**
	Time to No Avoidance behaviour (days)		
EC_{10}	4	4	0
EC_{50}	21	14	5
RD	56	14	14

Discussion

In the course of this main work, avoidance tests of 24 h were performed with aged-contaminated soils. The adaptation of 24 h avoidance tests instead of the standard 48 h was believed to be fully adequate without jeopardizing the final results. As observed in Natal-da-Luz and colleagues (2008a) an adequacy of a 24 h avoidance tests was confirmed. The authors investigated the avoidance behaviour of earthworms and springtails exposed continuously across a period of 1 to 7 and 1 to 14 days, respectively, to two reference chemicals (benomyl and carbendazim). For the earthworm's avoidance behaviour, two different sets were assessed: 1) during the 7 days only avoidance records were performed at day 2 and 7 (corresponding to the 48 h avoidance tests) and 2) avoidance records were performed at days 1,2,4 and 7 (corresponding to the 24 h proposed avoidance tests). Overall, the authors confirmed, for the substances in study, that different responses between 48 h and 24 h avoidance tests would not occur and the 24 h test would be sufficient to observe a response from the organisms to impacted soils with good consistence of results in both species. In accordance, for the present work, the adaptation to 24 h avoidance tests also relied in Basamid® short DT_{50} that can vary from 4 h to 7 days, depending on environmental soil conditions as moisture, organic matter and soil pH (EFSA, 2010; Fang et al., 2018).

In this current study another variation was performed, the avoidance tests proceeded for an extension of 56 days with aged soil in a multiple independently assessment. The duration of the test for the total 56 days was chosen so it could be comparable with the standard earthworms' reproduction tests (ISO 11268-2, 2011).

Previous studies (Gabriel et al. *in prep.* and (Mao et al., 2017)) have shown that sublethal exposure to Basamid® of soil invertebrates (including *E. andrei*) is significantly toxic in concentrations below the concentrations expected in the soil after one application of the recommended dose. Although, regarding this present study, there was no mortality outcome in the

avoidance tests, even at the higher concentration tested of 145.7 mg dazomet/kg soil (in which Gabriel et al., *in prep* observed to be highly lethal with 100% mortality rate). Specifically for *E. andrei*, soil pH did not significantly influence dazomet toxicity to the earthworms' reproduction (Gabriel et al. *in prep.*) which reported EC_{50} at the soil pH 5.5, 6.5 and 7.5 were similar and varied from 22.79 to 20.25 mg dazomet/kg soil. For this case, the absence of pH influence in Basamid® toxicity to the earthworms could be related to the enclosure and higher contact exposure of the organisms to Basamid® through the soil when comparing to the effects obtained from the avoidance tests. Furthermore, contrary to what occurred in avoidance tests, organic matter (horse manure) was added in reproduction tests to provide as food. Reported by Fang and colleagues (2018), increasing organic content in soil, decreases the rate of dazomet degradation. Therefore, in the case of the reproduction tests in Gabriel et al. (*in prep*) it can have been occurring a slower degradation rate of dazomet and, in consequence, an increasing enclosure time exposure, leading to higher toxicity of the fumigant. Thus, where the effect of soil pH did not influence the reproduction of the earthworms, in the avoidance tests there was a clear response across time and Basamid® concentration towards the influence of soil pH. Since different results regarding the effect of soil pH when the same species is exposed to similar conditions of soil spiked with Basamid® and varying only the type of test, avoidance tests revealed a highly potential to be recommended and used as a complement step to the common standard tests.

In the present study, Basamid® concentration and soil pH showed to be factors that significantly influence the avoidance behaviour of *E. andrei*. Also, the interval of 56 days showed to be relevant and suitable for the three soil pH tested in order to observe a consistent avoidance or no-avoidance response across time.

It was observed that in the soil with the lowest pH (pH of 5.5) the fumigant was persistent in soil for longer time compared to that in soil with higher pH (pHs of 6.5 and 7.5). Because of that, earthworms' avoidance behaviour in combinations with soil pH of 5.5 was performed for combinations with more aged soils compared with that in the same combinations of soils with higher pH. On the other hand, significant effects in the avoidance tests were observed towards higher alkalinity of the soil, which revealed to be needing a shorter time to observe a "no avoidance" response. Moreover, in soil with a pH of 7.5, the EC_{10} was never avoided by the earthworms (even at day 0 of ageing), while the combinations with the EC_{50} spiked soil stopped to be avoided at the 5^{th} day of ageing. In combinations with the RD spiked soil, the earthworms stopped to avoid the spiked soil after 14 days of ageing, which is 42 days less than in soil with a pH of 5.5. Therefore, soil pH showed to be a major influencing factor regarding the time period needed for dazomet to dissipate from the soil.

The shorter period of time needed to obtain a non-avoidance behaviour by the earthworms in soils with the highest pH might be due to the fact that dazomet gas release occurs at faster reactions in alkaline conditions (EFSA, 2010; Fang et al., 2018; Morrell et al., 1988). Since the alkalinity accelerates the reaction of the fumigant, the dissipation of dazomet from the soil occurs also faster. As evidenced by avoidance tests in the present study, when agrochemicals dissipate

faster from the soil, organisms can possibly, be able to recover and recolonize the previous impacted system sooner (Brock et al., 2006; Rico et al., 2016). Moreover, and regarding the present work, a consistent behaviour across time (i.e., non-avoidance response) can emphasize the capacity of the impacted site recolonization. Also, addressing the influence of soil pH, the manipulation of such factor can allow to manage the time frame needed for the recolonization to occur towards decreasing the time needed for the recovery of the soil system.

On this note, with the possibility of soil management through environmental factors to influence the reaction of a PPP in ecosystem, it is possible to envision on a larger scale a community recover from an impacted site. To corroborate these results it can be addressed the "principle of community recover" which lies in the capacity of organism's communities to recolonize a certain habitat, even at some level of pollution, after a disturbance episode (Brock et al., 2006; Clements and Rohr, 2009). The mentioned principle determines the capacity of an ecosystem to restore its services after an impact. The magnitude of the impact must be restricted to control reversible effects in the ecosystem and achieve the possibility of recolonization (Brock et al., 2006; Clements and Rohr, 2009). Moreover, the ecosystem recolonization is dependent on the type of effect in the environment and the time needed for the recolonization which may also depend on soil properties, PPPs reaction and mode of action (Desneux et al., 2006; Eo and Park, 2014).

In fact, on a similar approach with this study, Renaud and colleagues (2022) validated a recolonization concentration concept using soil organisms (earthworms and springtails) and soil contaminated with copper. The authors presented the negative correlation between avoidance (ACx) and recolonization (RCx) responses (ACx=RC100-x). Two approaches were set: avoidance tests (of 48 h) in individual placement (to discard the influence of the placement site in a dual-chamber) and copper gradient avoidance tests in the same chamber to ensure that previous exposures don't influence the organism's behaviour in avoiding/reaching the soil. From the experience, the authors could observe that organisms that avoid a contaminated soil will not recolonize that same soil. At the same time, transitional soil contamination could not be sufficiently severed, enable the organisms to cope with some limited exposure and consequent possibility of recovery can occur. Nevertheless, variable factors (e.g., concentration of the contaminant) can influence such magnitude of the recovery and time to such recover.

The approach of avoidance tests performed with aged-contaminated soils presented in this study evidenced that the evaluation of ecotoxicity of PPPs through laboratory tests (as currently requested for the data requirements of EU) do not provide information about the continuous effects after soil contaminations or the minimum time needed for a recolonization. This aspect is particularly important for fumigants as the threshold limits established for the use of these PPPs is regulated according to the ability of recovery of the systems after its application. Therefore, the risk assessment of a PPP should also address a plan for environmental and biodiversity recovery, and the consideration of DT_{50} of the PPPs is not sufficient. The present study clearly evidence that the avoidance test is a relevant tool to assess the minimum time needed for an environmental recovery. Further experiments following the same test design but using other standard species

(representative of other taxa) are needed to evaluate if the minimum time for a recolonization of other key groups is similar to that found for *E. andrei*.

Conclusion

The present study showed that, even with a higher volatile reaction and lower DT_{50} as the Basamid® fumigant, soil organisms may be able to detect lower concentrations and avoid contaminated soils. Data obtained also shown that soil pH has high influence in dazomet degradation and toxicity to soil organisms. The application of avoidance tests as a complement to standard ecotoxicological tests showed to be also considered as a potential tool in risk assessment. Moreover, avoidance test could also be used as a complement tool in environmental risk assessment to address the capacity of a recovery from impacted soil and the management of the minimum time needed for this recovery to occur towards recolonization of these impacted sites.

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Supplementary data

Figures

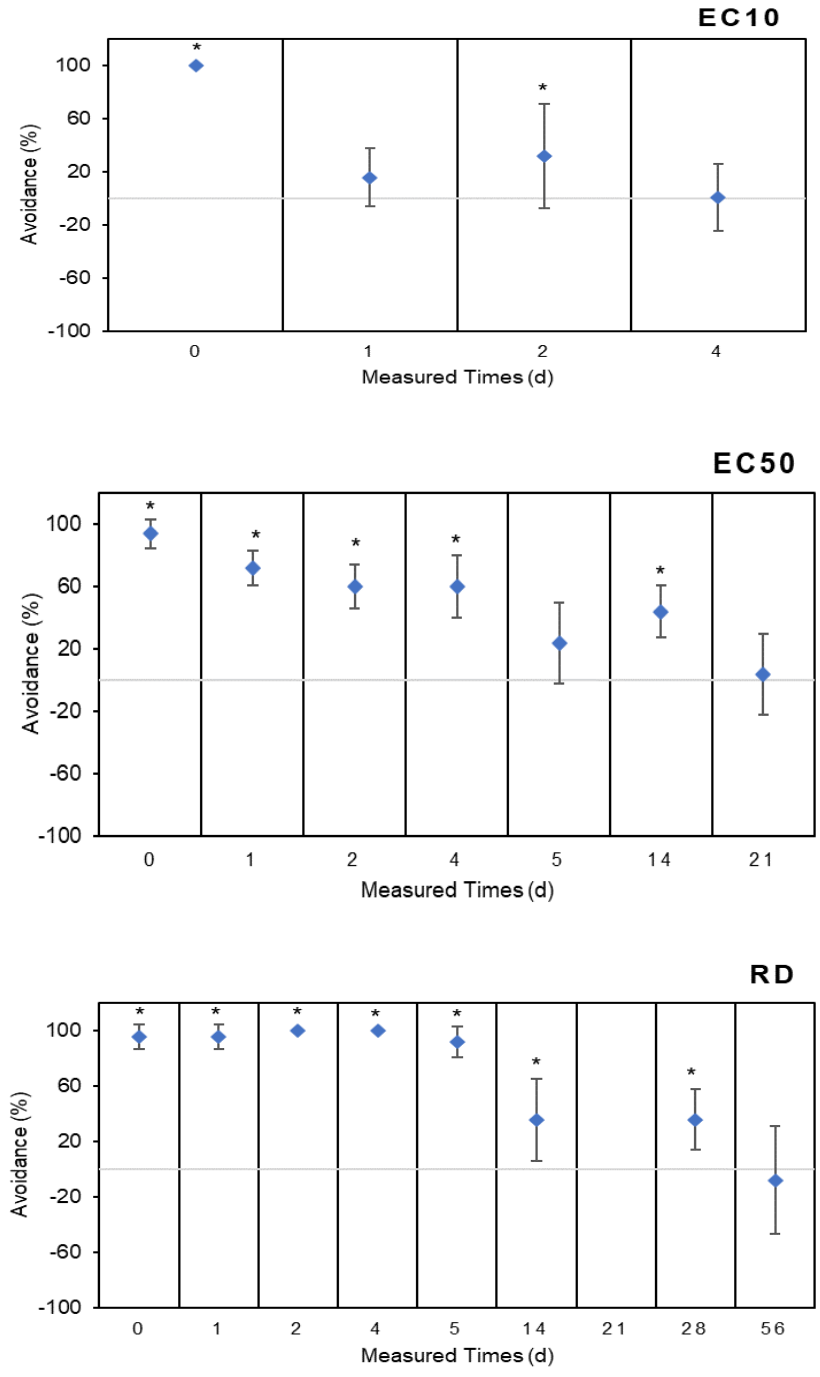

Figure 1S- Avoidance behaviour of *E. andrei* exposed to aged soil pH of 5.5 across 56 days sprayed with dazomet to the *E. andrei* reproduction EC_{10} and EC_{50} and the lowest recommended dose. The * translate the significant differences between the dazomet sprayed side and the control, i. e. significant avoidance behaviour Behrens-Fisher ($p \leq 0.05$).

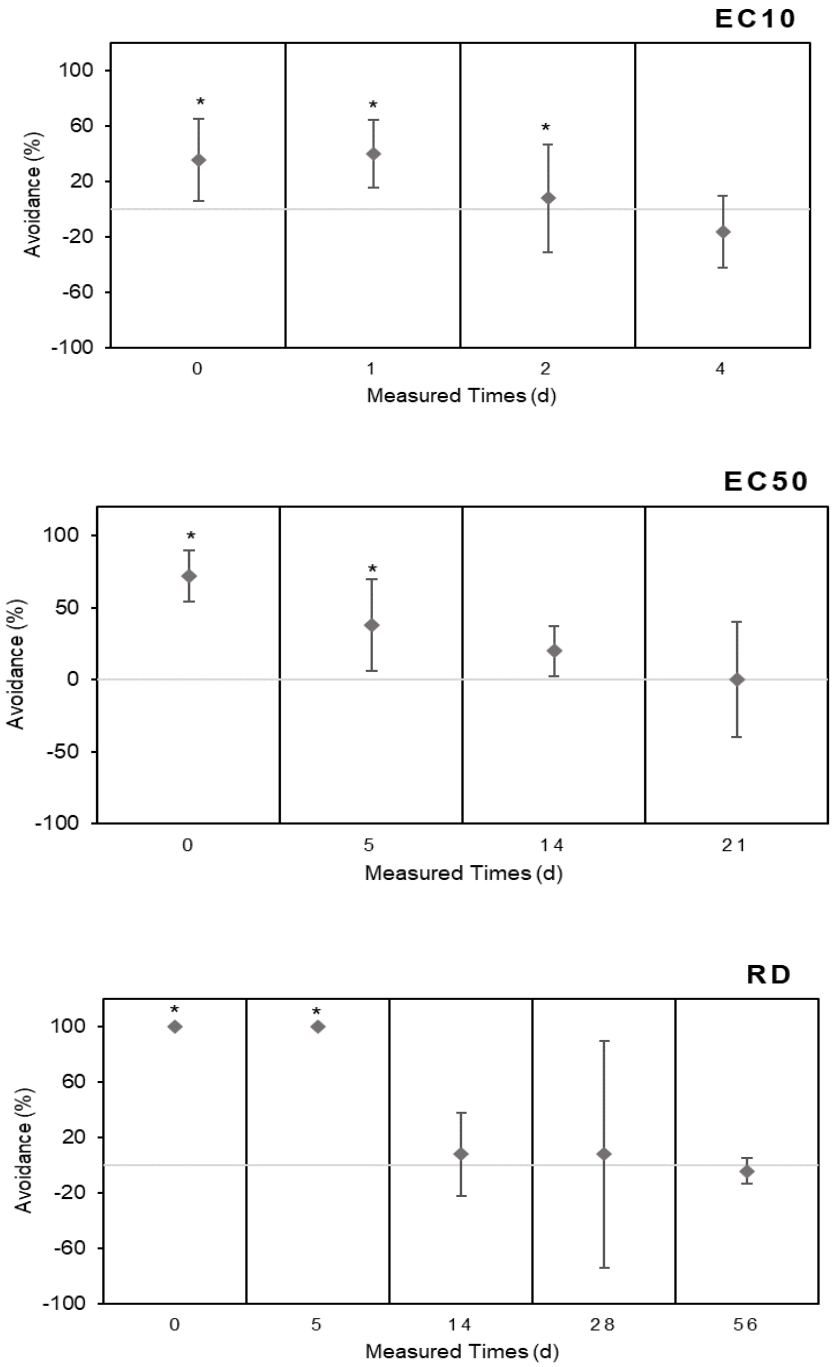

Figure 2S- Avoidance behaviour of *E. andrei* exposed to aged soil pH of 6.5 across 56 days sprayed with dazomet to the *E. andrei* reproduction EC_{10} and EC_{50} and the lowest recommended dose. The * translate the significant differences between the dazomet sprayed side and the control, i. e. significant avoidance behaviour Behrens-Fisher ($p \leq 0.05$).

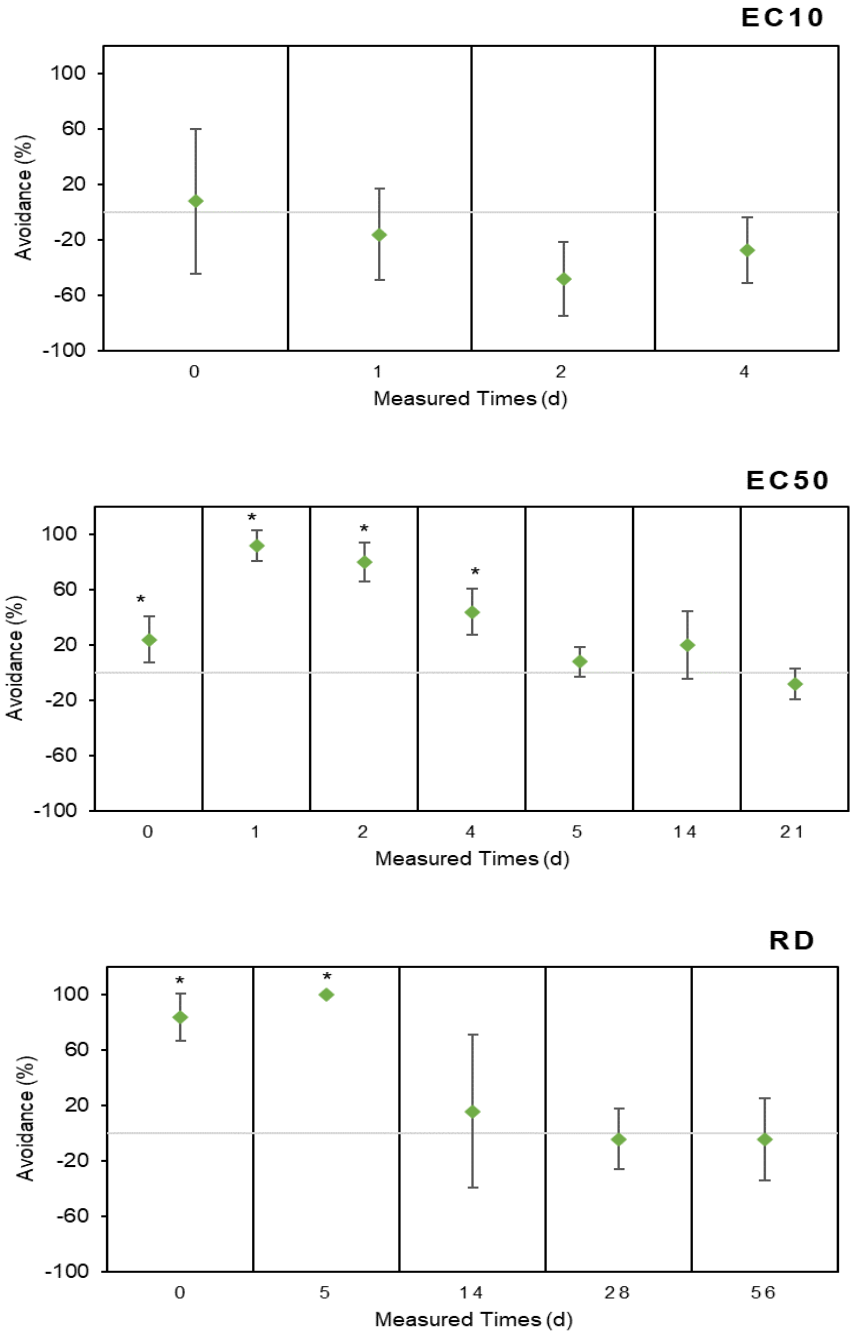

Figure 3S- Avoidance behaviour of *E. andrei* exposed to aged soil pH of 7.5 across 56 days sprayed with dazomet to the *E. andrei* reproduction EC_{10} and EC_{50} and the lowest recommended dose. The * translate the significant differences between the dazomet sprayed side and the control, i. e. significant avoidance behaviour Behrens-Fisher ($p \leq 0.05$).

Chapter IV

Soil pH matters in the ecotoxicity of Basamid® to freshwater microalgae and macrophytes.

Soil pH matters in the ecotoxicity of Basamid® to freshwater microalgae and macrophytes.

Authors

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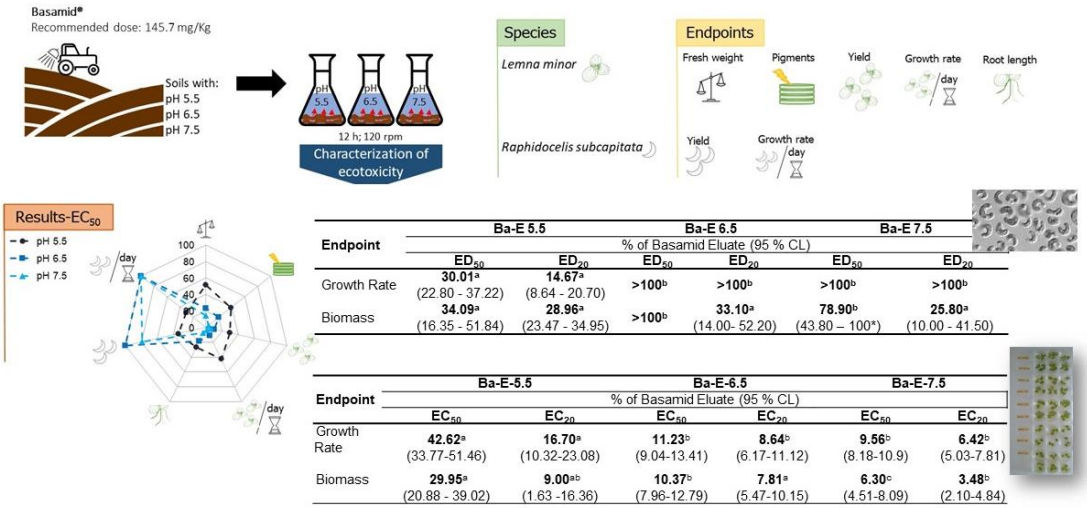
Graphical Abstract



Endpoint	Ba-E 5.5		Ba-E 6.5		Ba-E 7.5	
	% of Basamid Eluate (95 % CL)					
	ED_{50}	ED_{20}	ED_{50}	ED_{20}	ED_{50}	ED_{20}
Growth Rate	**30.01**[a] (22.80 - 37.22)	**14.67**[a] (8.64 - 20.70)	**>100**[b]	**>100**[b]	**>100**[b]	**>100**[b]
Biomass	**34.09**[a] (16.35 - 51.84)	**28.96**[a] (23.47 - 34.95)	**>100**[b]	**33.10**[a] (14.00- 52.20)	**78.90**[b] (43.80 – 100*)	**25.80**[a] (10.00 - 41.50)

Endpoint	Ba-E-5.5		Ba-E-6.5		Ba-E-7.5	
	% of Basamid Eluate (95 % CL)					
	EC_{50}	EC_{20}	EC_{50}	EC_{20}	EC_{50}	EC_{20}
Growth Rate	**42.62**[a] (33.77-51.46)	**16.70**[a] (10.32-23.08)	**11.23**[b] (9.04-13.41)	**8.64**[b] (6.17-11.12)	**9.56**[b] (8.18-10.9)	**6.42**[b] (5.03-7.81)
Biomass	**29.95**[a] (20.88 - 39.02)	**9.00**[ab] (1.63 -16.36)	**10.37**[b] (7.96-12.79)	**7.81**[a] (5.47-10.15)	**6.30**[c] (4.51-8.09)	**3.48**[b] (2.10-4.84)

Highlights

- *Raphidocelis subcapitata* and *Lemna minor* represent aquatic producers with different routes of exposure.
- Recommended doses of Basamid® caused adverse effects in the tested species.
- Soil pH may influence Basamid® toxicity to aquatic biota.
- Soil pH 5.5 caused higher Basamid® toxicity to *R. subcapitata* than to *L. minor*.

Abstract

Intensive agriculture along with the use of agrochemicals has been associated with soil infertility, erosion, and soil acidity. Management of soil pH through liming is a common practice in agriculture to increase soil fertility and nutrient availability. When altering soil pH, different chemical reactions occur depending on soil composition and agrochemicals presence. Basamid® is a fumigant used worldwide targeting soil nematodes, fungi, and weeds in diverse crops, that can reach freshwater ecosystems by leaching through the soil layers. The major goal of this work was to assess the influence of soil pH in the toxicity of Basamid® eluates to the microalgae *Raphidocelis subcapitata* and the duckweed *Lemna minor*. For this, eluates were prepared from soils with different pH (5.5, 6.5 and 7.5), contaminated with the recommended dose of Basamid®

corresponding to 145.7 mg of dazomet/kg soil. Soil was amended with calcium carbonate ($CaCO_3$). *Raphidocelis subcapitata* and *L. minor* were exposed to the eluates during 72 h and 7 days respectively, and multiple endpoints were assessed: growth rate, biomass, pigment as chlorophyl content and cell damage.

Results showed that soil pH can influence the performance of the tested species and also be a major factor in influencing Basamid®'s toxicity. However, a clear pattern of the influence of soil pH on Basamid®'s toxicity was not observed and was species dependent. For *R. subcapitata* lower soil pHs induced higher toxicity of Basamid®'s to the algae [ED_{50} for growth rate: 30% (confidence limits-CL: 22.8-37.2) for soil pH 5.5; >100% for soil pH 6.5 and pH 7.5], while for *L. minor* the opposite was observed [ED_{50} for number of fronds: 27.2 % (CL: 22.8-31.6) for pH 5.5; 20.3% (CL: 10.0-30.6) for pH 6.5 and 10.7 % (CL: 6.3-15.1)].

Overall, these results showed that leachates of Basamid® through soils, at recommended doses, can have a severe impact on aquatic systems, with or without the influence of abiotic factors.

Keywords: Pesticide, dazomet, liming, leaching, duckweed, green algae

Introduction

Freshwater ecosystems may be significantly exposed to pesticides leachates and runoffs after agricultural field application, an activity considered a major source of contaminants (Chen et al. 2019). Agriculture is one of the most important activities worldwide, responsible for food production and soil management (Thiele-Bruhn et al. 2012; Michler et al. 2019; Agovino et al. 2019). Intensive agriculture, traditionally involved a broad and high-level usage of agrochemicals to achieve crops' maximum yield and economic profits (Thorngren et al. 2017; Chen et al. 2019).

Wide spectrum agrochemicals as Basamid® are still being used to cope with different pests and plant diseases. Basamid® is a fumigant from the dithiocarbamate group with sterilant, fungicide, nematicide, and herbicide characteristics (EFSA 2010; Certis USA LLC 2012) and largely used as it depletes a broad spectrum of pests, such as *Phelipanche mutelii, Ralstonia solanacearum*, *Meloidogyne* sp. and related diseases induced in multiple cultivars as tomatoes, strawberries, cucumber, ginger or apple trees, among others (Prider and Williams 2014; Mao et al. 2017a; Nicola et al. 2017). Its granular formulation is advised to be applied in 500 kg/ha on a pre-plant and seed basis (EFSA 2010; Certis USA LLC 2012; BVBA/SPRL 2014). When in contact with humidity this fumigant releases a gas called methyl isothiocyanate (MITC), which is the major metabolite of dazomet (the active ingredient of Basamid®), and the responsible for pest's depletion (EFSA 2010). Notwithstanding, other environmental factors can trigger the formation of MITC gas, namely, soil porosity, organic matter and pH (Fang et al. 2018). Basamid® is soluble in water and prone to soil leaching and runoff after heavy rainfalls reaching superficial and groundwaters (Zhang and Wang 2007; EFSA 2010; USEPA 2017). The Food and Agricultural Organization specifications and evaluations for plant protection products (PPP) has classified it as very toxic to fish, crustaceans, and algae (FAO 2001). Nonetheless, exposure has solely been assessed for the active ingredient

and by direct dilution in water, which is a far less realistic scenario comparatively to leachates or eluates prepared from Basamid® contaminated soils mimicking what happens in the environment.

Despite the continuous application of agrochemicals, which contributes to biodiversity losses, soil erosion increase, soil acidity, and contamination (Thiele-Bruhn et al., 2012; Zimmerer, 2010), efforts are being made to implement more ecological solutions to reduce the application of such substances and potentiate their effects on-source (on the soil itself) and off-source (superficial water bodies through runoff or leachate). Liming is a common practice in agricultural activity used to increase soil pH by applying calcium [$CaCO_3$, $CaMg(CO_3)_2$ and $Ca(OH)_2$] and limestone compounds (Holland et al., 2018; Paradelo et al., 2015). This practice has great benefits, namely for soil fertility, availability of nutrients (for example, P, K and S), abundance of earthworms and consequent decomposition rates, together with indirect effects on biodiversity (i.e., increase in microarthropods due to the increase in soil nutrients) or availability of metals (for example, reducing the solubility of aluminium, magnesium, or iron) (Holland et al., 2018; Li et al., 2019; Paradelo et al., 2015). Soil pH is a chemical characteristic involved in water balance (Slessarev et al. 2016), enzymatic activity, nutrients cycling, and in the diversity of microorganisms (Dick et al., 2000). Still, it should be kept in mind that both the application of agrochemicals and the management of soil pH can be agricultural practices running simultaneously. Moreover, considering that the pH of the soil may be responsible for the magnitude of reactions, adsorption, and dissociation of agrochemicals (Sheng et al., 2005), the ecotoxicity of the leachates or runoffs, contaminated with pesticides, to aquatic biota might be much more serious than previously reported. In fact, dazomet was classified, along with 12 other active compounds, as responsible for 90% of the costs of health damage, based on data from 2003 (EC 2020). In addition, the European Commission, responsible for the regulation of these products [Commission Implementing Regulation (EU) 2018/1266 of 20 September 2018 amending Implementing Regulation (EU) No. 540/2011] has extended the period of dazomet approval until 2023. Hence, a combined assessment of both factors (Basamid® and soil pH) should be a priority to further understand the potential impacts of this type of broad-spectrum and widely used pesticide on the biota and provide additional evidence to support the decision on the extension of authorization for the marketing and use of this compound at the time of the next 2023 revaluation by the European Commission.

Therefore, considering the potential for dazomet to reach the aquatic systems, the main objective of this study was to evaluate the influence of soil pH in the toxicity of eluates obtained from soils contaminated by Basamid® on two freshwater producers species that might be directly exposed to leachates of this agrochemical: the microalgae *Raphidocelis subcapitata* and the duckweed *Lemna minor* (EFSA 2010; Vryzas 2018). Both species are well-known and characterized by rapid growth rates, low maintenance in the lab and can provide multiple endpoints as chlorophyll pigments, cell damage, growth rates, and biomass content to assess adverse effects (Safi et al. 2014).

Material and Methods

Chemicals

The granular commercial formulation of Basamid® (97% of dazomet) was acquired from KST-Kanesho Soil Treatment SRL/BV. Basamid®'s half-life can vary from 4 h to 7 days depending on soil type and properties, decomposing rapidly at temperatures above 25 ºC and slowly at temperatures lower than 10 ºC (EFSA 2010; Prider and Williams 2014). To manage soil pH, soil was corrected by $CaCO_3$ addition (ACS reagent, Merck, Darmstadt, Germany).

Test species

Two model species of producers were selected to assess the influence of soil pH in the toxicity of Basamid® eluates (Ba-E): the green microalgae *R. subcapitata* and the macrophyte *L. minor*. The axenic stock culture of *R. subcapitata* was maintained in continuous light intensity of 8000 lux in MBL medium at 20 ± 2 ºC (OECD 201, 2006b) and was renewed every week. The culture of *L. minor* was maintained under controlled conditions of temperature of 20 ± 2 ºC, photoperiod of 16:8h light: dark and illumination of 8000 lux. Cultures were kept in sterilized Steinberg medium, according to the OECD 221 (2006a) guideline and renewed once to twice a week.

Eluate's preparation and soil pH adjustment

A natural agro-silvo-pastoral woodland soil under sustainable management was collected from Herdade do Freixo-do-Meio, Alentejo, in Portugal (38°41'44.9''N 8°18'33.7''W) (soil properties can be consulted in Ferreira et al., 2022). Eluates of the soil at three different pH values were prepared with the culture medium of each tested species (MBL for the algae, and Steinberg for the macrophyte). Three soil pH's were considered: the natural soil pH_{KCl} of 5.5 ± 0.2 and two others: 6.5 and 7.5, amended with $CaCO_3$ addition. The addition of $CaCO_3$ quantities necessary to obtain the established soil pH values, followed a calibration curve (*data not shown*). The $CaCO_3$ was added and thoroughly mixed in the soil until achieving a complete homogenisation of the two components. The three pH values were selected based on the optimum soil pH for horticulture (around 5.5 and 7.2) and to have pH values representative of the following soil categories: acidic, neutral and alkaline pH (Penn and Camberato 2019). For the assays, two controls were set, the species respective culture media solely (CTR) and a control eluate (CTR-E). The CTR-E was performed for each of the three soils with different pH (CTR-E 5.5, CTR-E 6.5, and CTR-E 7.5, respectively for soil at pH 5.5, 6.5 and 7.5), and without being contaminated with Basamid®. Basamid® eluates treatments (Ba-E) were obtained by spiking the soils (at the three pHs) with the recommended dose (RD) (EFSA 2010; Certis USA LLC 2012) of Basamid® (corresponding to 145.7 mg of dazomet/Kg of soil), and by using the respective culture medium (for the *R. subcapitata* and for *L. minor*) at a ratio of 1:4 / m:v. Eluates were left shaking for 12 h at 120 rpm followed by 12 h of rest for sedimentation (DIN 38 414 S4, 1984). The obtained suspension was filtered with cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain) for all CTR-E and Basamid® treatments (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, treatments of the soil

spiked with Basamid® at the three soil pH established). The eluates of Basamid®, obtained for each soil pH, were then diluted with the respective culture medium to obtain the dilutions to be tested in the ecotoxicity assays with the microalgae and macrophyte: 0.9 to 100% (using a dilution factor of 1.5x) of eluate from soil spiked with Basamid® (Table 1).

Ecotoxicity assays

The primary producers *R. subcapitata* and *L. minor* were exposed to the following treatments: (i) CTR: consisting of the culture medium solely (MBL or Steinberg, respectively), (ii) CTR-E: consisting of the eluate of each soil (with pH 5.5, 6.5, 7.5) not contaminated with Basamid®, and (iii) seven dilutions of Ba-E, corresponding to the eluate obtained from each soil (with pH 5.5, 6.5, 7.5) contaminated with the recommended dose of Basamid®. A summary of the procedures and methods used to perform these ecotoxicity assays is presented in Table 1. The endpoints assessed for *R. subcapitata* were growth rate and biomass, while for *L. minor* growth rate, cell damage (electrolyte leakage and blue Evans' dye), and photosynthetic pigments were surveyed.

Table 1- Summary of the procedures followed in the ecotoxicity assays with *Raphidocelis subcapitata* and *Lemna minor*. CTR- Control consisting of culture medium; CTR-E –eluate of each soil not contaminated with Basamid®. Ba-E – eluates of each soil spiked with 145 mg/Kg of dazomet.

	Raphidocelis subcapitata	***Lemna minor***
Standard guideline	OECD 201 (2006b)	OECD 221 (2006a)
Exposure period	72 h	7 d
Test vessels	24 wells plate	6 wells plate
No. of replicates per treatment	3	6
No. of organisms per replicate	$5x10^5$ cells/mL	12 fronds
Light intensity	8000 lux	8000 lux
Volume per test container (mL)	1	8
Photoperiod (light:dark h)	24:0	24:0
Temperature (ºC)	23 ± 2	23 ± 2
Treatments (Ba-E dilutions and dilution factor)	CTR, CTR-E Ba-E: 0.9 – 100% (1.5x)	CTR, CTR-E Ba-E: 0.9 – 100% (1.5x)

72-h growth rate assay with Raphidocelis subcapitata

Assays carried out with *R. subcapitata* followed the OECD 201 (2006b) guideline for growth rate inhibition, adjusted for 24-wells plates. Each well consisted of one replicate with 900 µL of each test solution and 100 µL of *R. subcapitata* inoculum at a concentration of $5x10^5$ cells/mL. Three replicates were performed for each control (MBL medium - CTR, and soil eluate control at the three soil pH - CTR-E 5.5, CTR-E 6.5 and CTR-E 7.5) and for each dilution of the eluates obtained from Basamid® contaminated soils (0.94, 1.88, 3.75, 7.5, 15, 30, 60, 100%, Ba-E 5.5 for soil at pH 5.5, Ba-E 6.5 for soil at pH 6.5 and Ba-E 7.5 for soil at pH 7.5). Every peripheral well of the plate was filled with distilled water to prevent evaporation of the test solutions. In addition, blank treatments

(without the addition of algae) were prepared for CTR, CTR-E and for each dilution of the Ba-E. The parameters of pH and conductivity were measured at the beginning of each assay for each treatment (pH330i WTW Weilheim, Germany and WTW440i, Weilheim, Germany portable meters, respectively). At the start of the assay, water samples were taken from each treatment to quantify dazomet and MITC (please see section *Chemical Analysis*, Table 2). The 24-well plates were left in a phytoclimatic chamber at 23 ± 2 ºC in continuous light at 8000 lux for 72 h, medium was resuspended daily to prevent sedimentation. At the end of the test, the absorbances (ABS, used as a surrogate of cell density, CD) were measured in a microplate reader at 440 nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, VT, USA). The ABS were then converted into CD (cells mL^{-1}) according to the following equation (Venâncio et al., 2018):

CD (cells/mL) = -17107.5 +(ABS x 7925350) (equation 1)

The Biomass (*B*, cell mL^{-1}) and the Growth rate (GR, cells day^{-1}), were computed according to equations 2 and 3 as follows:

B=NF-NI (cells mL^{-1}) (equation 2)

where *NF* is the biomass of the algae at the end of the assay and *NI* is the biomass of the algae at the beginning of the assay

GR = (ln NF- ln NI) / t (cells day^{-1}) (equation 3)

where *NF* is the biomass of the algae at the end of the assay (cell/mL), *NI* is the biomass of the algae at the beginning of the assay, and *t* is the time of exposure (days).

7-days Lemna sp. growth inhibition assay

Assays performed with *L. minor* followed the OECD 221 (2006a) guideline for yield and growth inhibition. Six replicates of *L. minor* with 12 fronds in each replicate were exposed to 8 mL of each CTR, and soil pH CTR-E (CTR-E 5.5 CTR-E 6.5 and CTR-E 7.5) and Ba-E treatments (from 0.9-100% with 1.5x dilution factor also at the three soil pH Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, Table 1) and left for 7 days in full light (8000 lux) at 23 ± 2 ºC in a climatic chamber. The parameters of pH and conductivity were measured at the beginning and at the end of each assay for each treatment (pH330i WTW Weilheim, Germany and WTW440i, Weilheim, Germany portable meters, respectively). At the beginning of the assay, water samples were taken from each treatment to proceed with the quantification of dazomet and MITC (please see section *Chemical Analysis*, Table 2). The yield (Y, number of fronds) and growth rate (GR, fronds day^{-1}) were computed through the following equations (4 and 5):

Y= FF-FI (No. fronds) (equation 4)

where FF is the number of fronds at the end of the assay and FI is the number of fronds at the beginning of the test (12 fronds)

GR = (ln FF- ln FI) / t (fronds day^{-1}) (equation 5)

where FF is the number of fronds at the end of the assay and *FI* is the number of fronds at the beginning of the test (12 fronds), and *t* is the time of exposure (7 days).

In addition to the standard endpoints suggested in the OECD guideline, other sub-individual endpoints were evaluated in *L. minor* with the purpose to further understand the effects of Basamid® on cellular membranes and cell death. The quantification of the electrolyte leakage (EL) followed the procedure of Lutts and colleagues (1996), and it provides information on potential damage on cell membranes. Briefly, one frond from each replicate of each treatment was gently picked up and washed with Milli-Q® water, removing the excess water with absorbent paper. The fronds were immediately weighed and placed in glass tubes previously filled with Milli-Q® water. The ratio mass/volume was the same in all tubes. Tubes were left shaking in an orbital shaker for a period of 12 h. At the end of this period the conductivity was measured – $C_{initial}$ (portable probe WTW440i, Weilheim, Germany) and the vials were autoclaved for 10 min at 121 ºC (Uniclave 88, AJC). After cooling, the conductivity was again measured (C_{final}). The membrane permeability was estimated by the ratio of the conductivities ($C_{initial}/C_{final}$) and expressed in %.

The Evan´s blue staining method was performed according to Baker and Mock (1994), with minor adaptations. It may provide further comprehension on possible membrane fragilities and thus, be complementary to EL. Fresh fronds were collected from each replicate of each treatment and immediately weighed. The fronds were incubated with an Evan's blue solution of 0.25% (w/v) for 12 h at 100 rpm in an orbital shaker. Ended the incubation period, the fronds were washed with distilled water to remove any blue solution surrounding the tissues and then, fronds were homogenized in 1.5 mL of a solution containing 50% (v/v) MeOH and 1% (w/v) SDS to release trapped Evan's blue. The homogenate was further incubated in a chamber for 15 min at 50 ºC and centrifuged at 8800 *g* for 15 min. The absorbance of the supernatant was read at 600 nm and expressed in relation to fronds fresh weight. Damaged cells are those able to trap the dye inside and, therefore, higher absorbance values were expected if *L. minor* fronds were stressed by eluates exposure.

The photosynthetic pigments chlorophylls *a* and *b* (Chl *a* and Chl *b*) and total carotenoids were determined by UV-Vis spectrophotometry (Jenway, 6505 UV/Vis) through *L. minor* leaves pigment extract (Lichtenthaler, 1987). Fresh leaves from each replicate of each treatment were stored at -80 ºC and then lyophilize. After lyophilization each sample was weighted (dry weight -DW) and left to hydrate for 10 min, (100 µL distilled water, in microtubes). The pigments extraction was performed by weighting 10-60 mg of leaf tissue in 10 mL of ethanol (96%) and left to incubate for 24 h. Afterwards, 500 µL of ethanol (96%) were added to each sample and left overnight for pigment extraction. The microtubes were wrapped in aluminium foil to avoid degradation by light in an Hotte's chamber at room temperature (20 ± 2 ºC). Chlorophylls and carotenoids were determined by spectrophotometry through absorbance readings at 470, 648 and 664 nm. An additional reading was performed at 750 nm to account for any possible debris of the frond present in the suspension. Absorbance of the samples were constricted within the range of 0.2 and 0.8 following Lichtenthaler (1987) methodology. Pigment endpoints estimations were calculated according to the following equations (Chl *a*, Chl *b* and total chlorophyll - equation 6, 7 and 8, respectively; total carotenoids – equation 9):

$$Chl_a = \frac{(13.36A664.2-5.19A648.6)*8.1}{DW} \quad [mg.\ g^{-1}\ dw] \quad \text{(equation 6)}$$

$$Chl_b = \frac{27.43A648.6-8.12A664.2)*8.1}{DW} \quad [mg.\ g^{-1}\ dw] \quad \text{(equation 7)}$$

$$Chl_{a+b} = \frac{(5.24A664.2-22.24A648.6)*8.1}{DW} \quad [mg.\ g^{-1}\ dw] \quad \text{(equation 8)}$$

$$Chl_b^a = \frac{Chl\ a}{Chl\ b} \quad \text{(equation 9)}$$

where, A648.6 is the absorbance at 648.6 nm, A664.2 is the absorbance at 664.2 nm and DW is the dry weight of plant tissue extracted (mg).

Quantification of dazomet and MITC in test solutions

Blank samples were fortified at different levels to establish the calibration curves and allowed to sit for at least 2 h at room temperature for both dazomet and MITC. Replicates of three, from each eluate (Ctr-E and Ba-E) of the three soil pH tested were filtered through 0.20 µm syringe prior to injection. Measurements were performed by high performance liquid chromatography coupled to UV detection (HPLC-UV), using a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155) and Gilson Unipoint System software. The quality of chemical analyses was checked using dazomet and MITC standards (purity >99%, Sigma-Aldrich, Steinheim, Germany). Scanning individual standard solutions from 191 to 400 nm allowed the selection of the most accurate wavelengths for elution monitoring. Maximum absorption was achieved at 286 nm for dazomet and 299 nm for MITC. The compounds were analysed in isocratic mode with mobile phase consisting of methanol (A) and water (B), at 40:60 proportion. The injection volume was 20 µL, flowing at a rate of 1 mL.min^{-1}, running for 10 min (dazomet) and 15 min (MITC).

Statistical Analysis

Significant differences in the measured endpoints, comparing Ba-E exposed organisms with the respective CTR-E, were performed by one-way analysis of variances (ANOVA), after homogeneity of variances confirmed by Levene's test and normality of data with Shapiro-Wilk test. To compare each treatment with the respective control (CTR-E), after significant differences recorded by ANOVA, a two-tailed Dunnett's test was performed. Differences between dazomet quantification across pH treatments (Ba-E) and media were assessed by a two-way ANOVA (crossed soil pH with Basamid® eluate treatments of both species). Basamid® eluate non-observed effect dilution (NOED) and the highest observed effect dilution (LOED) were recorded. The dilution (presented as % of eluate) provoking 20 and 50% of effect (ED_{20} and ED_{50}, respectively) for each endpoint were computed by fitting a non-linear regression of a logistic model. The comparison between ED_{20} and ED_{50} within each pH level tested were performed with Behrens-Fisher test. This statistical analysis was performed in StatSoft, Inc. (2007), STATISTICA (data analysis software system), version 10.0.

Results

The validity criteria for the controls of both species *Raphidocelis subcapitata* and *Lemna minor* were fulfilled according to the respective OECD guideline (OECD 201, 2006b; OECD 221, 2006a). Physical-chemical parameters of the eluates, as pH and conductivity, are presented in Table 1S and 2S of supplementary data (Sup. Data).

Chemical Analyses

Dazomet and MITC were quantified in the two tested media (MBL and Steinberg) and in the 100 % eluates of the treatments CTR-E (for soils with pH 5.5, 6.5, 7.5) and Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, through HPLC-UV. The two metabolites were not detected in the controls of the test media and in CTR-E. For MITC, concentrations were below the method's detection limit (LOD: 1.32 µg/mL) for all analysed samples. Regarding dazomet, concentrations varied with soil's pH, with a significant reduction in the measured concentrations with increasing values of soil pH (Dunnett's $p<0.05$ Table 2). The measured values (different from the initial concentration used for the eluate treatments) corresponded to a range of 0.394 to 0.106 µg/mL (from Ba-E 5.5 to Ba-E 7.5 in MBL media) and 0.720 to 0.187 µg/mL (from Ba-E 5.5 to Ba-E 7.5 in Steinberg media). As for the influence of test medium in the measured concentrations of dazomet, higher concentrations were measured in eluates from Ba-E 5.5 and Ba-E 6.5 prepared with Steinberg medium. For Ba-E 7.5 no differences were observed between both medium.

Table 2- Measured concentration (average ± standard deviation) of dazomet in eluates of soil contaminated with Basamid® at the recommended dose (145 $mg.kg^{-1}$ corresponding to 100% treatment) at the three soil pH 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, respectively). Letters represent homogenous groups between treatments (Ba-E) and symbols represent homogenous groups between medium eluates (MBL and Steinberg).

	Ba-E 5.5	Ba-E 6.5	Ba-E 7.5
	Dazomet concentration (µg/mL)		
100% Ba-E in MBL	$0.394 \pm 0.023^{a\#}$	$0.148 \pm 0.009^{b\#}$	$0.106 \pm 0.002^{b\#}$
100% Ba-E in Steinberg	$0.720 \pm 0.173^{a\$}$	$0.423 \pm 0.099^{b\$}$	$0.187 \pm 0.009^{c\#}$

72-h growth rate assay with Raphidocelis subcapitata

For *R. subcapitata* there were no significant differences for growth and biomass between both controls (MBL medium - CTR and Eluate control - CTR-E), for each pH level tested. Exposure to the treatments Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5 caused significant reductions on growth rate and biomass starting at dilutions 30, 62.7 and 48.3% (LOEDs) (Dunnett's test $p<0.05$; Fig. 1-4S). The eluate dilutions causing 20 and 50% of effect (ED_{20} and ED_{50}, respectively) on growth rates and biomass, for each soil pH, are listed in Table 3. As for growth rate, a higher toxicity was observed for eluates originated from soil with pH 5.5 (Ba-E 5.5), since the ED_{50} was 30.0%, whilst at Ba-E 6.5 and Ba-E 7.5 the ED_{50} and ED_{20} could not be computed due low effects observed even at

100%. The statistical analysis for the ED_{20} of growth rates showed that the Ba-E 5.5 also constituted a distinct group from Ba-E 6.5 and Ba-E 7.5 (Table 3). The obtained ED_{50} for Ba-E 5.5 (34.1%) was lower than the ED_{50} of Ba-E 6.5 (>100%) and Ba-E 7.5 (78.9%) (Behrens-Fisher' test $p<0.05$; Table 3), thus suggesting soil pH 5.5 to potentiate the toxicity of Basamid® to *R. subcapitata*. However, no statistical differences were found among the ED_{20} values for biomass (Behrens-Fisher' test $p>0.05$).

Table 3 - Summary of the eluate dilutions (here presented as % of eluate) causing 50 and 20% of effect (ED_{50} and ED_{20}, respectively), and respective 95% confidence limits within brackets (95% CL), computed for each endpoint evaluated in the freshwater microalgae *Raphidocelis subcapitata*, after 72 h of exposure to several dilutions of soil eluates contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5, Ba-E 7.5). When less than 20 and 50% of effects were reported at 100%, the ED_x is indicated as >100. For 100*, the superior limit is statistically greater than the exposure of 100% Basamid® treatment tested. Superscript letters (a, b) indicate homogenous groups among the three soil pH's tested within the respective endpoint and ED_x (Behrens-Fisher test, $p<0.05$).

Endpoint	Ba-E 5.5		Ba-E 6.5		Ba-E 7.5	
	ED_{50} 95% CL	**ED_{20}** 95% CL	**ED_{50}** 95% CL	**ED_{20}** 95% CL	**ED_{50}** 95% CL	**ED_{20}** 95% CL
Growth rate (d^{-1})	**30.0^{a}** (22.8 - 37.2)	**14.7^{a}** (8.6 - 20.7)	**>100^{b}**	**>100^{b}**	**>100^{b}**	**>100^{b}**
Biomass ($cells.mL^{-1}$)	**34.1^{a}** (16.4 - 51.8)	**28.9^{a}** (23.5 - 34.9)	**>100^{b}**	**33.1^{a}** (14.0- 52.2)	**78.9^{b}** (43.8 – 100*)	**25.8^{a}** (10.0 - 41.5)

7-d growth inhibition assay with Lemna minor

When comparing the two controls (Steinberg medium - CTR and Eluate control - CTR-E) no statistical differences were found for the analysed endpoints, with the exception for fresh weight of *L. minor* exposed to CTR and CTR-E from soil at pH 7.5. In Figures 1A and 1B and in Table 3S are summarized the ED_{20} and ED_{50} values computed for all endpoints assessed in *L. minor* exposed to Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5. For the freshwater macrophyte and based on the range of the estimated ED_{50}'s, the obtained eluate from Ba-E 6.5 and Ba-E 7.5 induced a higher toxicity than the eluate obtained from Ba-E 5.5 (Fig. 1A, Table 3S). Depending on the assessed endpoint, the ED_{50}'s of eluate treatments Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, ranged from 26.9 to 51.8%, 10.4 to 22.8%, and 1.88 to 14.7%, respectively. The Ba-E 5.5 was significantly different from the two other Ba-E treatments, for all the endpoints, with the exception of number of fronds (comparatively to Ba-E 6.5) and chlorophyll *a* (comparatively to Ba-E 6.5 and Ba-E 7.5). The Ba-E 6.5 and Ba-E 7.5 shared very similar ED_x values, except for yield and root length. Significant differences between the controls and treatments were observed for the assessed endpoints in the three pH levels (Dunnett's test $p<0.05$; Table 3S and Figs. 5S-15S). For the treatment Ba-E 5.5, significant differences were observed for number of fronds and root length (at 0.94%, 1.88% and at ≥ 7.5% of eluate dilution), growth rate (at ≥ 0.94% of eluate dilution), fresh weight, chlorophyll *b,* Evan's blue, electrolyte leakage and total chlorophyll (at 100% of eluate). For the Ba-E 6.5 significant

differences were observed for number of fronds (at ≥ 15% of eluate dilution), root length (at 0.94%, 1.88% and ≥ 15% of eluate dilution), growth rate and fresh weight (at ≥ 15% of eluate dilution), chlorophyll *a*, total chlorophyll and carotenoids (at 30% of eluate dilution), and Evan's blue (at ≥ 15% of eluate dilution). For the Ba-E 7.5 significant differences were observed for number of fronds (at ≥ 15% of dilution), root length (at ≥ 0.94%, 1.88% and ≥ 15% of eluate dilution), growth rate (at ≥ 15% of eluate dilution), fresh weight (at ≥15% of eluate dilution), Evan's blue (at 100% eluate), chlorophyll *a* (at 60% and 100% eluate), total chlorophyll (at 30 and 60% eluate dilution) and carotenoids (at 30 and 100% eluate, Fig. 5S-15S of Sup. Data). Based on ED_{50}, the toxicity of Ba-E 5.5 eluate was significantly lower than Ba-E 6.5 and Ba-E 7.5 for fresh weight, growth rate and roots length. For the number of fronds, the toxicity effects of Ba-E 7.5 eluate were significantly higher than Ba-E 5.5. Significant differences related to the pigment's chlorophyll *b*, and carotenoids were not observed for all dilutions of Ba-E treatments. Regarding the potential toxicity of Basamid® eluates on cellular membranes, no injuries as disruption, disintegration or complete cell death was observed within the tested range of dilutions. Both electrolyte leakage and the Evan's blue staining endpoints did not present significant differences across the tested dilutions of Basamid® eluates for all soil pH tested (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, Table 3S and Fig. 9S-15S of Sup. Data). Significant differences were not observed within the three Ba-E for fresh weight and chlorophyll a. For the growth rate and number of fronds the toxicity of Ba-E 5.5 eluate was significantly lower than Ba-E 6.5 and Ba-E 7.5 and the toxicity of Ba-E 7.5 eluate were significantly higher than Ba-E 5.5 and Ba-E 6.5. Regarding roots length, all soil pH were significant different from each other.

The ED_{20} and ED_{50} along with respective 95% confidence interval, NOED and LOED for all of the endpoints assessed are listed in Table 3S and in Fig. 5S-15S, respectively.

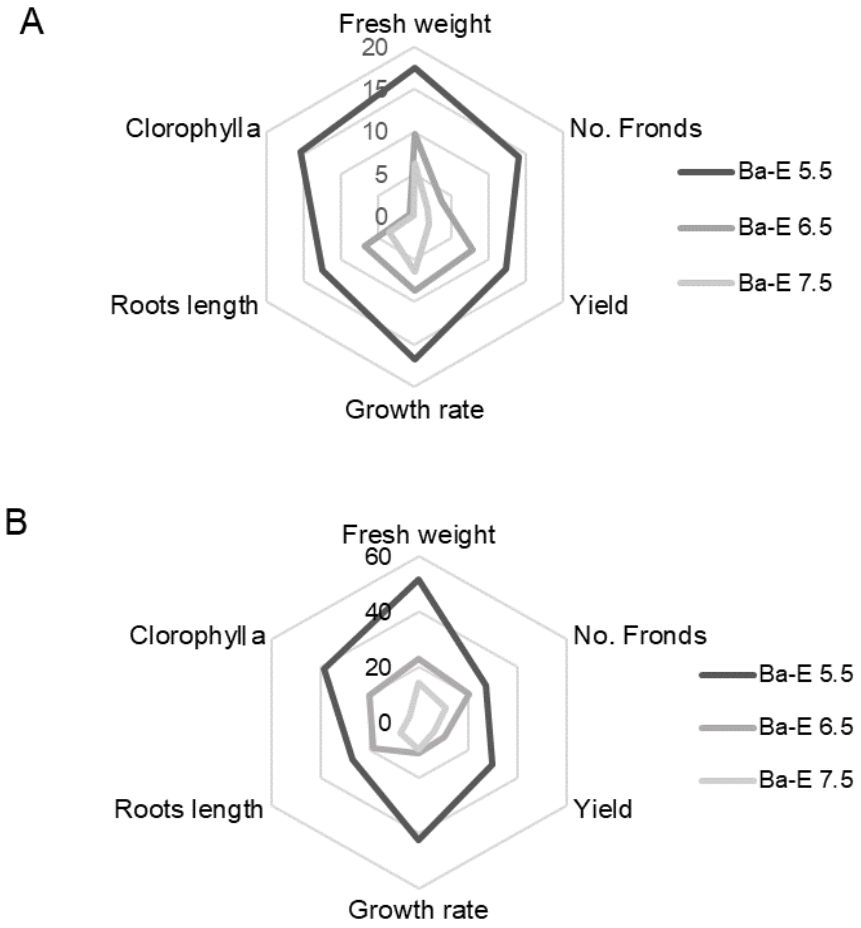


Figure 1 - Radar plot indicating the dilutions of eluates obtained from soils contaminated with Basamid®, with different pH (5.5: Ba-E 5.5; 6.5: Ba-E 6.5 and 7.5: Ba-E 7.5), causing 20% (A) and 50% (B) of effect on *Lemna minor* fresh weight, yield, number of fronds, root length, growth rate and chlorophyll *a*.

Discussion

Leachates and runoffs of dazomet (the active ingredient of Basamid® formulation), widely used in agriculture practices, may constitute a major contaminant threat to the aquatic systems (Qi et al. 2018; Chen et al. 2019). Aquatic organisms such as algae and duckweed can be equally and directly exposed to these runoffs and leachates (EFSA 2010; Vryzas 2018). It has been previously shown by Morrell and colleagues (1988) and Fang and authors (2018) that dazomet chemical reaction is dependent of external factors such as organic matter, soil composition, humidity and pH. Still, there is scarce information related to soil pH factor and dazomet's toxicity to non-target species, specifically in aquatic organisms. In agriculture, soil pH is an important factor determining crop's productivity and soil fertility (Slessarev et al. 2016; Penn and Camberato 2019). Within the climate change context, and subsequent scenarios of acidity and erosion of the lands, farmers find the need to amend soil pH in order to achieve maximum yield of the crop productivity (Paradelo et al. 2015; Goulding 2016; Holland et al. 2018; Li et al. 2019). As Fang and colleagues (2018) and Consolazio and co-authors (2019) observed, soil pH is an important factor to accelerate the

reaction of dazomet in soil, moreover, the half-life (DT_{50}) of dazomet decreases in alkaline medium (EFSA 2010). Here, we tested the effects of soil pH in the toxicity of eluates obtained from soil contaminated with Basamid® to the algae *R. subcapitata* and duckweed *L. minor*. The recommended dose of Basamid® translated in active ingredient is 145.7 mg dazomet/kg soil (EFSA 2010; Certis USA LLC 2012), which in this study was the higher treatment tested soil eluate (100%). The results from the chemical analysis performed to eluates of 100%, showed a low concentration of dazomet ranging from a maximum of 0.720 µg/mL (in Ba-E 5.5 in Steinberg media) to a minimum of 0.106 µg/mL (in Ba-E 7.5 in MBL media). This would be expected due to the high volatile reaction of the fumigant when in contact with water and consequent reaction of dissipation to the atmosphere (EFSA 2010). Additionally, the concentration of dazomet decreased in the eluates obtained from soils with higher pH values, what is in concordance with the literature (EFSA 2010, Fang et al., 2018, Consolazio et al., 2019). The chemical reaction acceleration of dazomet at higher pHs is due to base-catalysis of the hydrolyses. The chemical reaction increases with the effect of alkalinity leading to the hydroxide reaction on the amine group in dazomet composition (Consolazio et al. 2019). Chemical analyses run on the major metabolite, MITC, did not detect it in the eluates. MITC has been reported as the most toxic metabolite resulting from dazomet hydrolysis (EFSA 2010), although, as already mentioned, it was not detected, and the toxicity observed for both *R. subcapitata* and *L. minor* was most probably not related to MITC. Instead, observed toxicity may have been caused by dazomet and other secondary metabolites (e.g., formaldehyde and TDL-S) acting on the organisms exposed, although these metabolites were not analysed.

On the other hand, the concentration of dazomet in the eluates performed with Steinberg medium (used in *L. minor* assays) was superior to the concentrations measured in the eluates performed in MBL medium (used in *R. subcapitata* assays), despite the same initial contamination of the soil. This difference on final concentration in the eluates may be directly related to the different composition of both media. By comparison, Steinberg medium is much richer in metals (e.g., Mo, Zn, Cu, Co, Fe) while MBL only has in common Na, K, Mg, Ca, Mn and Fe, and at lower concentrations. This difference in metals composition can be a major factor regarding the reaction with EDTA solution, which is at higher concentration in Steinberg medium. The EDTA is known for its metal chelating properties, which could explain the higher binding to metal in Steinberg media. Consolazio and colleagues (2019) performed a study to investigate the effect of pH, temperature and pyrite in dazomet kinetics degradation. The authors reported that Fe^{2+} catalysed the dazomet hydrolysis reaction. However, EDTA can act in the soil by breaking some weak bonds of metal elements (Zhang et al. 2010). Therefore, the higher concentration of EDTA in Steinberg medium can be influencing the availability of metals in the soil transferred to the eluate and inversely of what may be occurring in MBL, the hydrolysis reaction can be occurring at lower rate taking longer time to dissipate from the eluates. Nevertheless, the presence of different components passible to react with the soil might be interacting with dazomet and decreasing its hydrolysis reaction in Steinberg media and, therefore, translating in higher concentration in the eluates. Regardless of

different dazomet concentration in both Ba-E media (MBL and Steinberg), the same effect of soil pH was observed, i.e., the concentration of dazomet decreased towards alkalinity of the soil.

Centred on the main goal of this study, results showed the significant effect of different soil pH in the toxicity of Basamid® containing eluates. Respecting to the influence of soil pH alone in the microalgae *R. subcapitata*, differences were not observed in the CTR-E exposure of the three soil pH (5.5, 6.5 and 7.5). Moreover, the effective pH of CTR-E ranged from 7.4-7.9 (close to the pH of MBL medium) what might be insufficient to pose a significant effect. Nevertheless, the effects of pH have been reported for algae growth. Sakarika and authors (2016) performed a study on the green algae *Chlorella vulgaris*, where they tested the influence of pH on algae growth. The experiment consisted of exposing *C. vulgaris* to a range of pH from 3 to 11 and observe the effects on lipid content, biomass, and growth rate. At pH ranging between 7 and 8 (similar range to the eluates pH in this study) the authors reported a greater biomass productivity and at range 6-8 an increasing growth rate. The final conclusions reported the optimum pH of 7.5 and 8 for growth rate and biomass which are in line with this work where the pH of the eluates tested varied from 7.4 and 7.9, even with a different species of algae.

For the *L. minor* exposure assay, the effect of pH alone showed a significant influence in the endpoints assessed for the macrophyte. From all of these, with the exception of the cellular membrane and pigments, *L. minor* showed better performance at CTR-E 5.5 than in CTR-E 6.5 and CTR-E 7.5. A higher bioavailability or mobilization of essential elements involved in *L. minor* growth at soil pH 5.5 may have occurred. Despite the similar effective soil pH in the eluate controls, different concentrations of $CaCO_3$ were added to soil to adjust pH. Therefore, the $CaCO_3$ can be influencing the bioavailability or passage of specific elements in the soil to the eluates, elements that can be important for the *L. minor* growth. This better performance at soil pH 5.5 (CTR-E 5.5) can translate the lower effects when exposed to the Basamid® eluates and soil pH combined since it was possible to relate the pH of 5.5 with lower effects in the macrophyte.

When comparing the effect of soil pH in Ba-E exposure for both species, *R. subcapitata* showed more tolerance to Basamid® than *L. minor*, except for Ba-E 5.5, based on the growth rate. The soil pH of 5.5 induced the higher toxicity of Ba-E to *R. subcapitata* among the three soil pHs (5.5, 6.5 and 7.5) while for *L. minor* this soil pH was the one inducing the lowest toxicity. In this study the influence of pH was considered as the prime factor influencing Basamid® eluates toxicity to the tested species. Furthermore, the differential sensitivity of the two species to Basamid® may also be due to differences in their morphology and physiology. Moreover, for dazomet, an $EC_{50,72h}$ of *Pseudokirchneriella subcapitata* (now known as *R. subcapitata*) of 0.275 µg/mL was already reported (Lewis et al., 2016). This data is in agreement with the range of dazomet concentration measured in the present study (in the Ba-E 5.5 at 100 % eluate the concentration of dazomet reported was 0.394 µg/mL in MBL medium).

In this work, *L. minor* showed to be the most sensitive species to Ba-E exposure in terms of growth rate, with LOED of 0.9, 15 and 15% (for Ba-E 5.5, 6.5 and 7.5, respectively) while for *R. subcapitata* were 30, 62.3 and 48.3% (for Ba-E 5.5, 6.5 and 7.5, respectively). Regarding the

pigments assessed only for *L. minor,* they were less sensitive than the growth rate and number of fronds. Moreover, the mode of action of Basamid® as weed sterilant (EC, 2011; Certis USA LLC, 2012; BVBA/SPRL, 2014) can be one of the main reasons for the higher sensitivity of *L. minor*. When comparing *L. minor* with *R. subcapitata* the duckweed has a bigger area of contact exposure through the leaves and roots which can enhance the entrance and contact exposure to the *L. minor*.

Different studies have shown the influence of pH in the toxicity of pesticides to the macrophyte growth (Rosenkrantz et al. 2013; Suthar et al. 2015). For instance, it was reported by Rosenkrantz and authors (2013) a decrease of pesticides toxicity in *Lemna* sp. with pH increase. The authors observed an increase (up to 10-fold) of EC_{50} in *Lemna gibba*, exposed to four different sulfonylurea herbicides, when increasing the pH from 6 to 9. Nonetheless, our data are not in line with those results. Without considering pH effect, Cedergreen and colleagues (2004; 2005) performed two different experiments: (1) tested 12 herbicides toxicity with different aquatic plant species and (2) evaluated the toxicity of 10 herbicides in *L. minor* and algae *Pseudokirchneriella subcapitata*, respectively. From both studies, *L. minor* was presented as one of the most sensitive species towards pesticides exposure, which is consistent with our data (Cedergreen et al. 2004; Cedergreen and Streibig 2005).

Furthermore, *L. minor* has a much slower intrinsic growth rate than *R. subcapitata*, which for the microalgae may pose an advantageous mechanism to cope with external chemicals. Also, *L. minor* has a great tendency to bioaccumulate compounds such as metals and pesticides, which could be an additional explanation for its great sensitivity in comparison to the algae (Mkandawire et al. 2014). Additionally, the cell wall of *R. subcapitata* can be the main mechanism of defence of algae from the surround medium as it is observed for *C. vulgaris* (Safi et al. 2014) while *Lemna* sp. present a potential higher exposure directly through the leaves and systemically throughout the exposed roots (Mkandawire et al. 2014).

The influence of pH alone in each individual organism and the influence of pH in dazomet Ba-E eluates bioavailability, may have also contributed to the different sensitivity of both *R. subcapitata* and *L. minor*. Thus, the effect of soil pH in the eluates might result in the main aspect of chemical reaction acceleration which decreases the DT_{50} and increases the toxicity response (Fang et al. 2018; Consolazio et al. 2019). Either way, severe changes in the lower trophic levels can be expected due to the reported effects at such lower LOEDs (e.g., 0.94% in *L. minor* for growth rate). These changes can be mirrored in the following trophic levels that depend on these species for food, refuge, or oxygen production.

The leachates and runoffs of Basamid®'s and respective metabolites can cause risks to the aquatic systems. Furthermore, abiotic factors such as soil pH may pose an additional influence factor regarding the toxic effects of agrochemicals as Basamid® to the aquatic biota. Altogether, our data marks the importance of a new and fast revision for Basamid® effects in the environment since the active ingredient dazomet has been freely commercialized since 2010 and its new revision has been postponement to 2023 (EFSA 2010; EC 2020a).

Conclusion

There is scarce information in the literature regarding the influence that soil abiotic parameters may have on the impacts of agrochemicals runoffs in aquatic plants and algae. Thus, in the present study the influence of soil pH in the toxicity of eluates obtained from soils contaminated with Basamid®'s to *R. subcapitata* and *L. minor* was tested. Results showed that soil pH can be a major factor in determining Basamid®'s toxicity on these primary producers. Species differential sensitivity also contributed to the observed toxicity. The duckweed presented more sensitivity to the effect of soil alkalinity on the eluate's toxicity contrarily to the green microalgae. These results showed that leachates and runoffs of recommended doses of Basamid® can have a severe impact on aquatic ecosystems' primary producers. Soil pH can influence the toxicity of Basamid® at different extents by increasing or diminishing its effects, nonetheless, in lower concentrations of the fumigant sever effects can be observed in both tested organisms. Management of the soil with agrochemical use, must have a new approach in modern agriculture, aiming to maintain the balance of ecosystems and biodiversity.

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Supplementary Data

Tables

Table 1S- Values of the physical-chemical parameters measured at the beginning of the 72 h assay with *Raphidocelis subcapitata* exposed to MBL control medium (CTR), MBL control soil eluate (CTR-E) and to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E).

Soil pH	Treatments Ba-E: dilutions (dilution factor)	pH	Conductivity (mS/cm)
5.5	CTR	7.39	0.51
	CTR-E	7.70	0.75
	Ba-E: 0.94 – 60% (1.5x)	7.35 - 7.90	0.51 – 0.55
6.5	CTR	7.86	0.45
	CTR-E	7.48	0.99
	Ba-E: 10 – 100% (1.3x)	7.49 - 7.84	0.89 – 0.88
7.5	CTR	7.89	0.46
	CTR-E	7.91	0.91
	Ba-E: 10 – 100% (1.3x)	8.12 - 8.40	0.51 -0.93

Table 2S- Values of the physical-chemical parameters measured at the beginning and at the end of the 7 days assay with *Lemna minor* exposed to Steinberg control medium (CTR), Steinberg control soil eluate (CTR-E) and to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E).

Soil pH	Treatments Ba-E: dilutions (dilution factor)	pH		Conductivity (mS/cm)	
		start	end	start	end
5.5	CTR	6.89	8.22	1.00	0.82
	CTR-E	7.19	8.57	1.05	1.02
	Ba-E: 0.94 – 100 (1.5x)	6.07 - 7.36	8.00 - 8.57	0.84 - 1.07	0.80 -1.09
6.5	CTR	6.36	8.40	0.89	0.75
	CTR-E	7.75	8.58	1.12	1.04
	Ba-E:0.94 – 100 (1.5x)	6.26 - 7.75	8.02 - 8.64	0.89 - 1.17	0.71 - 1.33
7.5	CTR	6.52	7.93	0.85	0.77
	CTR-E	8.55	8.68	0.91	0.89
	Ba-E:0.94 – 100 (1.5x)	6.08 - 8.55	7.46 - 8.68	0.85 -1.19	0.69 - 1.3

Table 3S- Summary of the effective dilutions (here presented as % of eluate) causing 50 and 20 % of effect (ED_{50} and ED_{20}) and respective 95% confidence limits (CL), computed for each of the evaluated endpoints in

the freshwater macrophyte *Lemna minor* after exposure for 7 days to eluates from soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). Significant differences between ED_{50} and ED_{20} within soil pHs are represented for each endpoint with letters. > Indicate the highest % of effect at 100% of the treatment, whenever the ED_{50} and/or ED_{20} could not be computed. n.d. data did not allow estimation of the value.

Endpoint	Ba-E 5.5		Ba-E 6.5		Ba-E 7.5	
	ED_{50} 95% CL	**ED_{20}** 95% CL	**ED_{50}** 95% CL	**ED_{20}** 95% CL	**ED_{50}** 95% CL	**ED_{20}** 95% CL
Fresh Weight (mg)	**51.8**[a] (28.8-74.8)	**17.5**[a] (4.6-30.4)	**22.9**[b] (16.1-29.6)	**9.82**[a] (4.8-4.8)	**14.7**[b] (6.9-22.9)	**6.4**[a] (2.7-10.1)
Yield (%)	**29.9**[a] (20.9 - 39.0)	**9.0**[ab] (1.6 -16.4)	**10.4**[b] (7.9-12.8)	**7.8**[a] (5.5-10.2)	**6.3**[c] (4.5-8.1)	**3.5**[b] (2.1-4.8)
Number of Fronds	**27.2**[a] (22.8-31.6)	**14.0**[a] (11.2-16.9)	**20.3**[a] (10.0-30.6)	**3.7**[b] (0.2-7.3)	**10.7**[ab] (6.3-15.1)	**1.8**[b] (0.5-3.1)
Growth Rate (cells.mL^{-1})	**42.62**[a] (33.77-51.46)	**16.70**[a] (10.32-23.08)	**11.23**[b] (9.04-13.41)	**8.64**[b] (6.17-11.12)	**9.56**[b] (8.18-10.9)	**6.42**[b] (5.03-7.81)
Root Length (mm)	**26.9**[a] (22.12-31.)	**12.5**[a] (9.6-15.4)	**18.4**[b] (11.7 - 25.2)	**6.8**[b] (3.5-10.1)	**7.6**[c] (6.0-9.2)	**3.5**[c] (2.3-4.7)
Chlorophyll *a*	**38.5**[a] (0 - 89.9)	**15.4**[a] (0 - 36.0)	**19.9**[a] (0 - 59.2)	**0.6**[a] (0 - 3.9)	**4.0**[a] (0 -13.4)	**0.2**[a] (0 - 1.3)
Chlorophyll *b*	n.d.	n.d.	n.d.	n.d.	**1.9** (0- 6.1)	**1.5** (0 - 5.0)
Carotenoids (mg/g DW)	n.d.	n.d.	n.d.	n.d.	**9.9** (2.90 - 16.8)	**1.6** (n.d. - 3.7)
Total chlorophyll	n.d.	n.d.	n.d.	n.d.	**4.9** (0 - 13.7)	**3.2** (0 – 9.7)
Even's blue staining (mg)	>100	>100	>100	>100	>100	>100
Electrolyte leakage (%)	>100	>100	>100	>100	>100	>100

Figures

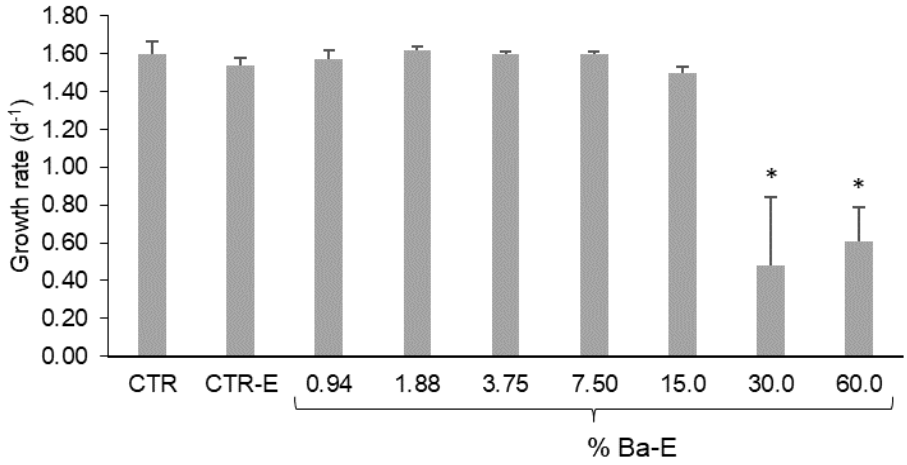

Figure 1S- Average growth rate of *Raphidocelis subcapitata* exposed, for 72 h, to the eluates of soil contaminated with Basamid®, at soil pH of 5.5. The symbol * represents significant differences between Ba-E treatments and CTR-E (control of soil eluate at pH 5.5). Error bars, bars represent the standard deviation.

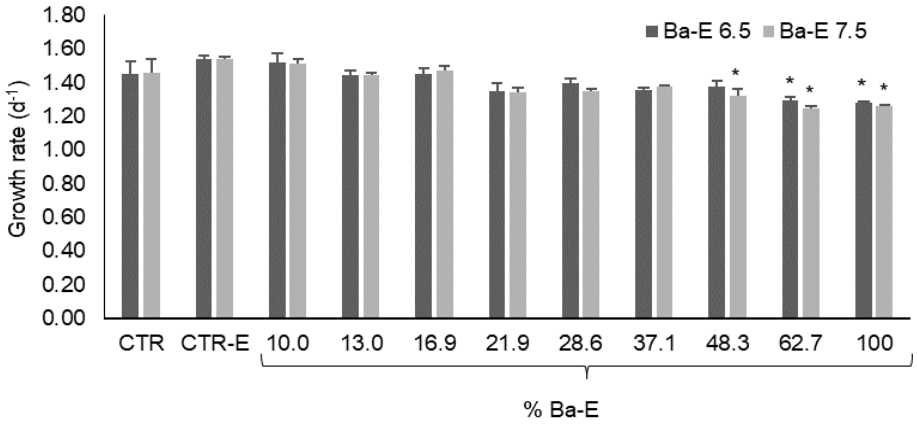

Figure 2S- Average growth rate of *Raphidocelis subcapitata* exposed, for 72 h, to the eluates of soils contaminated with Basamid®, at different soil pH: 6.5 and 7.5 (Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (6.5 and 7.5). Error bars represent the standard deviation.

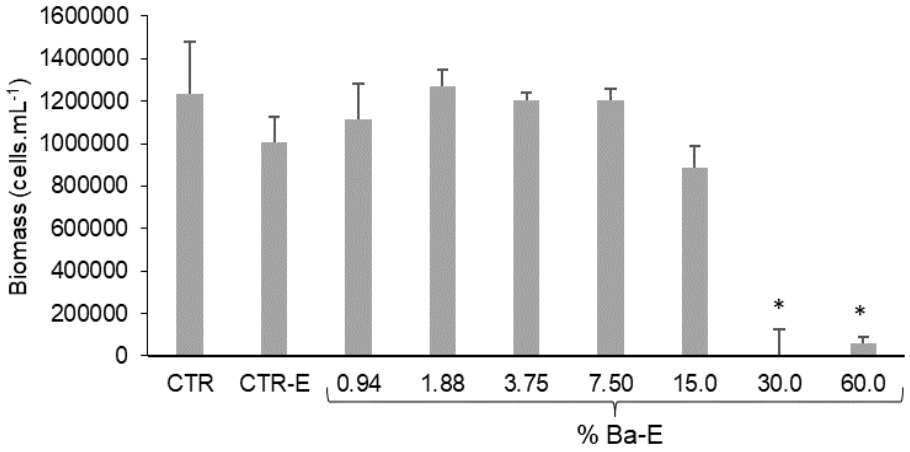

Figure 3S- Average biomass of *Raphidocelis subcapitata* exposed, for 72 h, to the eluates of soil contaminated with Basamid®, at soil pH of 5.5. The symbol * represents differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5). Error bars represent the standard deviation.

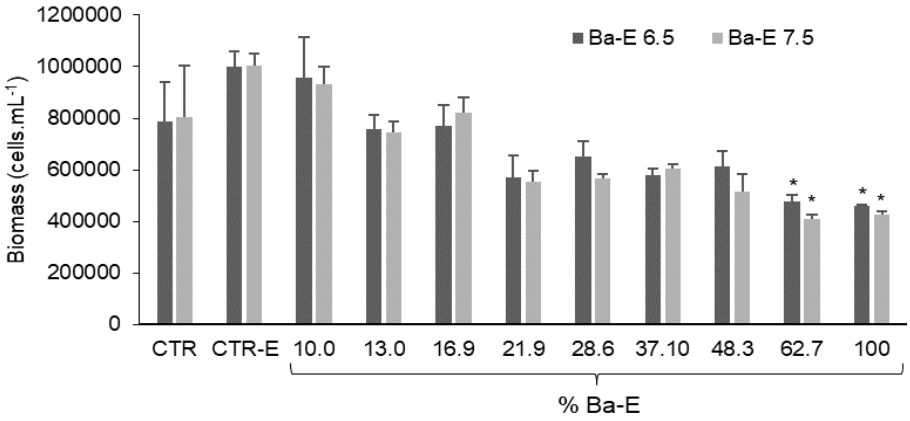

Figure 4S- Average biomass of *Raphidocelis subcapitata* exposed, for 72 h, to the eluates of soils contaminated with Basamid®, at different soil pH: 6.5 and 7.5 (Ba-E 6.5 and Ba-E 7.5). The symbol * represent differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (6.5 and 7.5). Error bars represent the standard deviation.

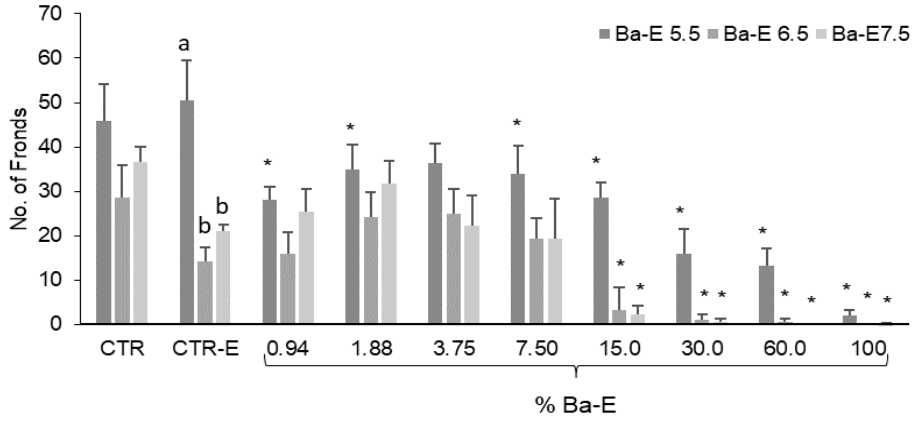

Figure 5S- Average number of fronds of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbols * represent differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

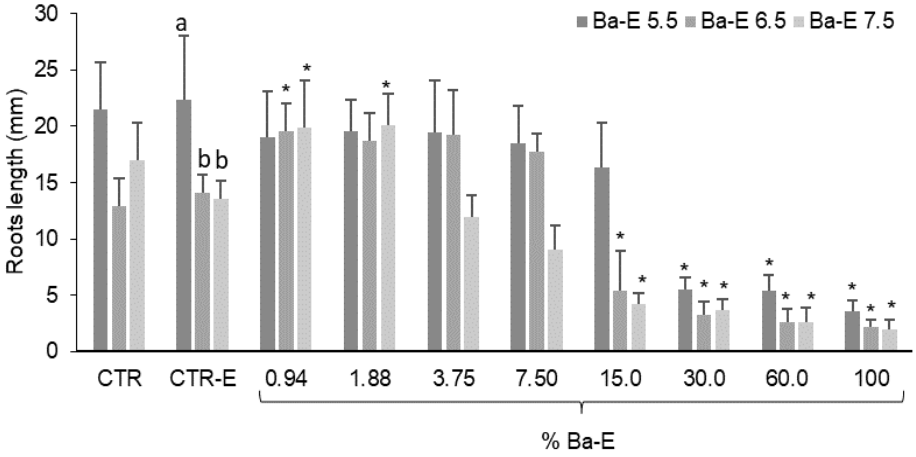

Figure 6S- Average of roots length of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

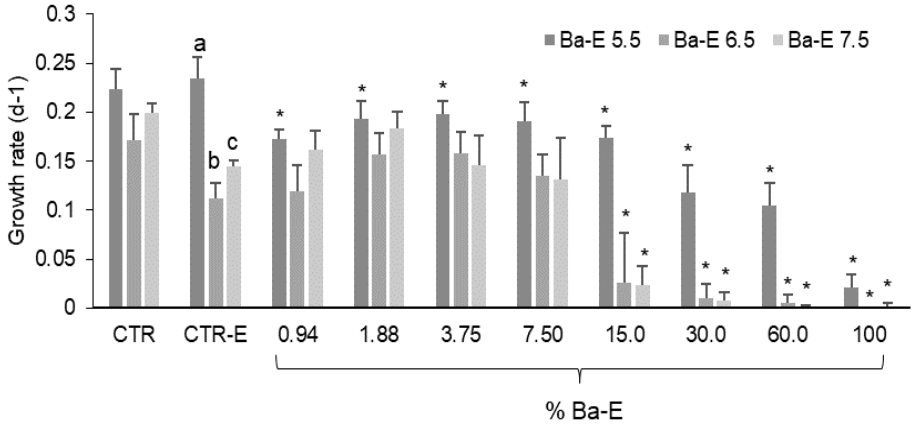

Figure 7S- Average growth rate (d^{-1}) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

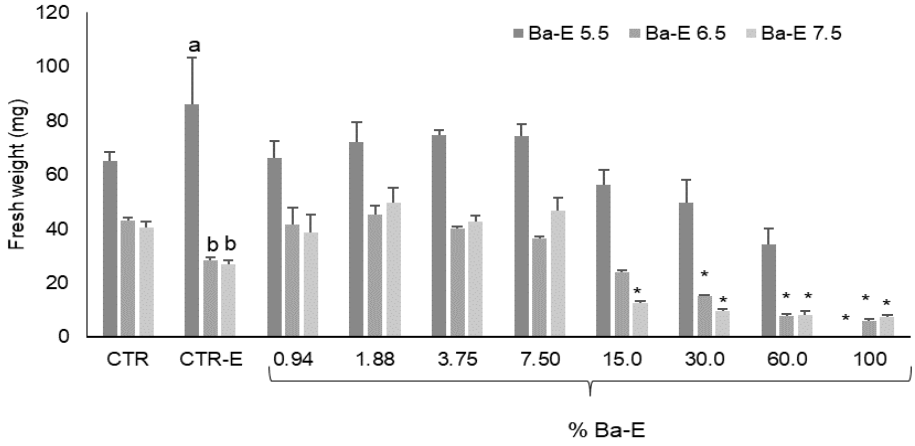

Figure 8S- Average fresh weight (mg) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

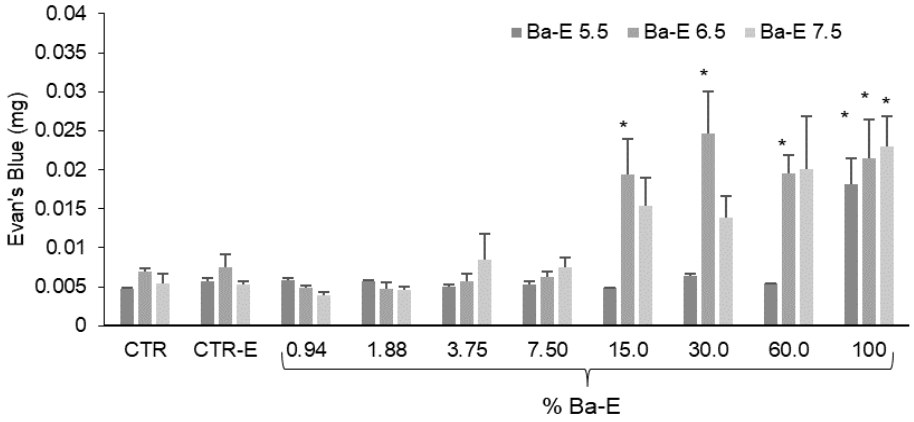

Figure 9S- Average Evan's Blue (mg) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E obtained from the soils with each of the three pH tested (5.5, 6.5 and 7.5). Error bars represent the standard deviation.

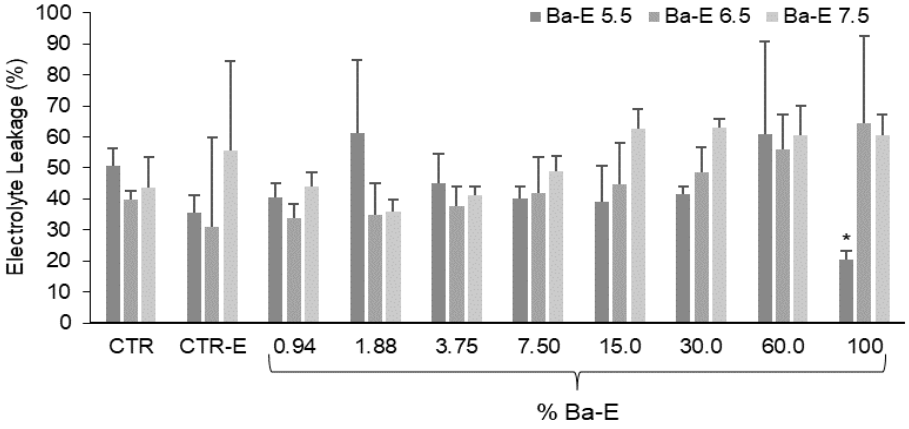

Figure 10S- Average electrolyte leakage (%) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol *, represent significant differences between Ba-E treatments and the respective CTR-E obtained from the soils with each of the three soil pH tested (5.5, 6.5 and 7.5). Error bars represent the standard deviation.

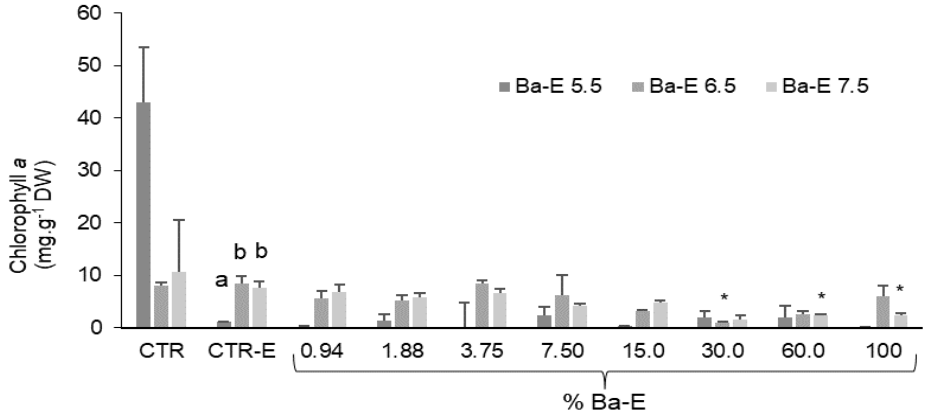

Figure 11S- Average chlorophyll a (mg/g DW) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

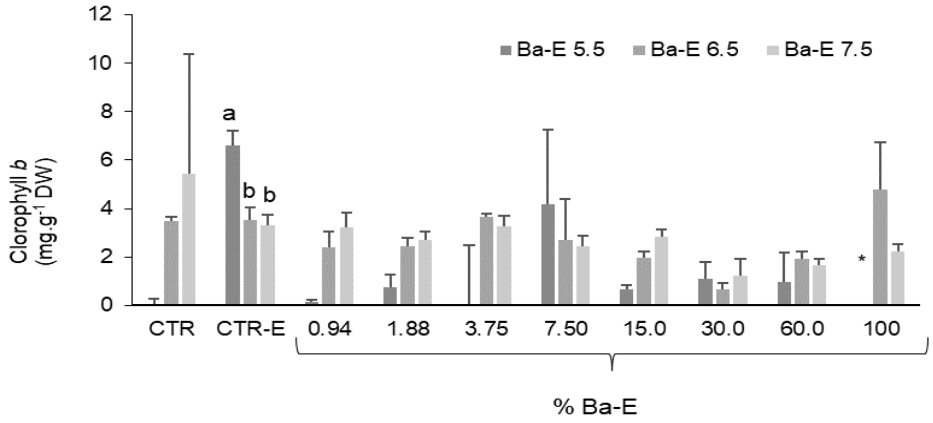

Figure 12S- Average chlorophyll b (mg/g DW) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

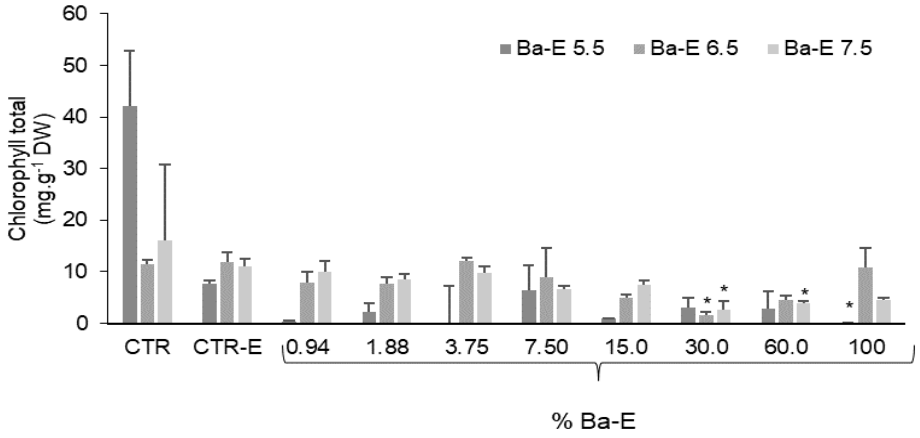

Figure 13S- Average chlorophyll total (mg/g DW) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E obtained from the soils with each of the three respective soil pH tested (5.5, 6.5 and 7.5). Error bars represent the standard deviation.

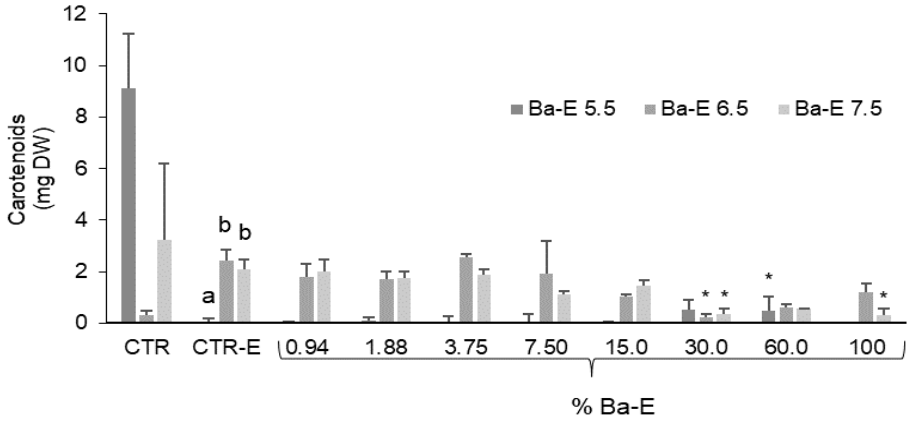

Figure 14S- Average carotenoids (mg/g DW) of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). * Represent significant differences between Ba-E treatments and the respective CTR-E of the respective soil pH tested (5.5, 6.5 and 7.5), letters represent differences among CTR-E obtained from the soils with each of the three pH tested (pH 5.5, 6.5 and 7.5). Error bars represent the standard deviation.

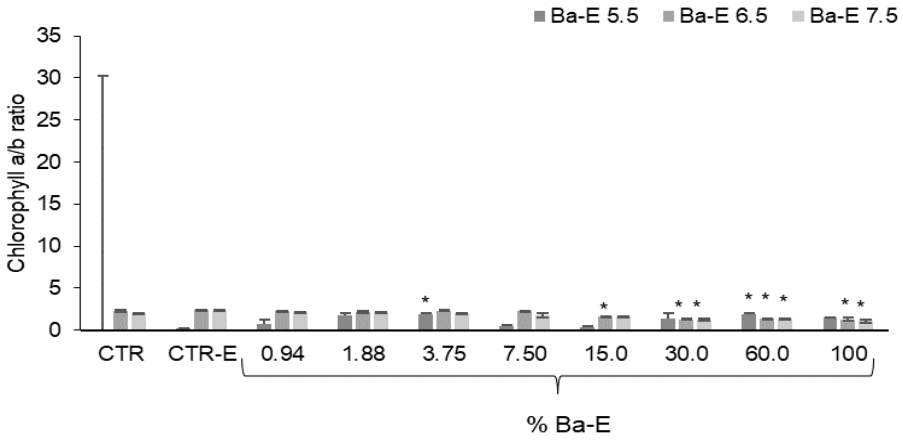

Figure 15S- Chlorophyll a/b ratio of *Lemna minor* exposed, for 7 days, to the eluates of soils contaminated with Basamid®, at three different soil pH: 5.5, 6.5 and 7.5 (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The symbol * represent significant differences between Ba-E treatments and the respective CTR-E obtained from the soils with each of the three respective soil pH tested (5.5, 6.5 and 7.5). Error bars represent the standard deviation.

Chapter V

Soil pH influences the toxicity of Basamid® eluates to non-target species of primary consumers.

Soil pH influences the toxicity of Basamid® eluates to non-target species of primary consumers

Authors

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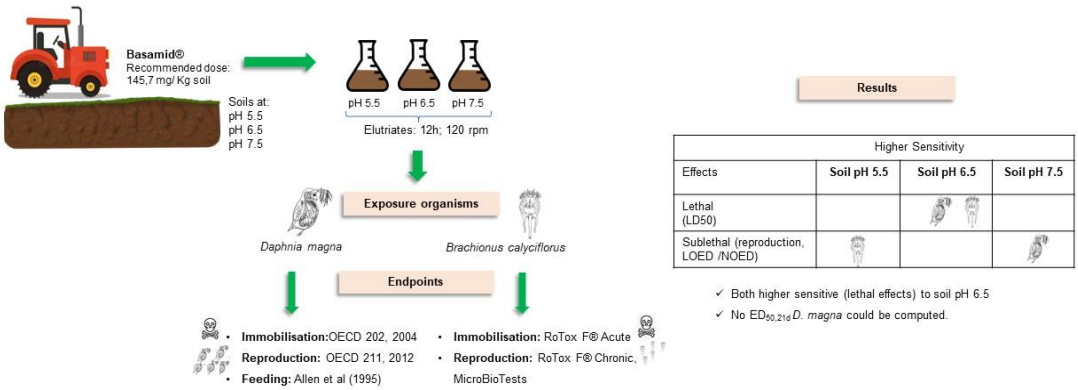
Graphical Abstract



Highlights

- *Daphnia magna* and *Brachionus calyciflorus* were exposed to Basamid® contaminated eluates.
- *D. magna* was more sensitive to Basamid® eluates than *B. calyciflorus*.
- The soil pH 6.5 contaminated with Basamid® were highly lethal to both species.
- The soil pH 5.5 exerted more toxicity of Basamid® eluates to *B. calyciflorus*
- The soil pH 7.5 exerted less toxicity of Basamid® eluates to *D. magna*.

Abstract

Leachates and runoffs of plant protection products constitute a major threat to the aquatic systems that are adjacent to agriculture fields. Basamid® is a fumigant nematicide and fungicide agent known to reach superficial and ground waters. When in contact with water it breaks down forming several volatile compounds, mainly methyl isothiocyanate (MITC). Soil abiotic parameters, such as pH, can influence this breakdown process, and thus, the toxic effects that Basamid® can cause in aquatic biota. Therefore, this study aimed to understand the influence of soil pH (5.5, 6.5 and 7.5) on the toxicity of eluates obtained from soils contaminated with Basamid® on two primary consumers *Daphnia magna* and *Brachionus calyciflorus.* For this, the lethal and sublethal toxicity (reproduction for both species and feeding only for daphnia) of

eluates originated from soils at pH 5.5, 6.5 and 7.5, contaminated with Basamid® (Ba-E 5.5; 6.5 and 7.5, respectively), were assessed. For each Ba-E seven dilutions were tested: from 0.096 to 100%. The LD_{50} of Basamid® eluates for *D. magna* varied from 3.07% to 7.82% (Ba-E 6.5 and Ba-E 5.5 respectively) while for *B. calyciflorus* varied from 18.09% to 84.70% (Ba-E 6.5 and Ba-E 7.5, respectively). Both species were less sensitive to the effect of soil pH 7.5 in Basamid® eluates toxicity and more sensitive to the effect of soil pH 6.5. Regarding the sublethal effects, soil pH negatively influenced the toxicity of Basamid® to *D. magna* reproduction towards the lower pH exposure (LOED: 0.125% Ba-E 5.5) while *B. calyciflorus* was affected negatively towards higher pH exposure (ED_{20}: 7.42% [5.10-9.74] at Ba-E 7.5).

Despite the fumigant property of Basamid®, the possibility of it reaching aquatic systems is present and the recommended dose must be reviewed due to the sever effects at such lower concentrations to the aquatic organisms.

Keywords: Fumigant, alkalinity, leachates, freshwater biota.

Introduction

The need to use plant protection products (PPP) to deplete pests and treat diseases in crops is a commonplace in intensive and modern agriculture. Despite the PPP application being targeted to the soil compartment (directly in the soil, or indirectly on the cultivars through spraying on leaves for e.g.), agrochemicals frequently reach superficial and ground waters across multiple pathways, namely by air drift and spray or through rainfall transport as runoffs and/or leachates (Schriever and Liess 2007; Lefrancq et al. 2017; Zhang et al. 2018; Li et al. 2021). Thus, biota inhabiting the surrounding nearby water systems may become in contact with these compounds, which may cause them adverse effects (Neuwirthová et al. 2019).

The European Commission, the Members States and the European Food Safety Authority (EFSA) are responsible for the regulation of agrochemicals and PPP, building their decision-making in the potential effects over the environment and human health (European Commission, 2020). Decisions on active substances approval runs under the Directive 91/414/EEC and follows a 10-years validation period until new revision and regulation (EC 2020). Despite of the legislation and regulation for the use of PPP in agriculture, the process of revision and the data available are sometimes neglected and delayed, which can lead to decisions less compatible with the environment, its sustainability and integrity. In 2003, a group of 13 active substances were responsible for 90% of health effects, in which dazomet is included and is still authorized for marketing (EC 2020). The process of revision and evaluation of dazomet active ingredient has been postponed until 2023, being currently approved to circulate in the market (EC 2020). Dazomet is the active ingredient of several commercial formulations (e.g., Basamid®, Mylone®, Dazoberg®, Thiazone®), used worldwide in several cultures (e.g., tomatoes, strawberries, cucumber, seed weeds), acting as a fungicide, nematicide, herbicide and insecticide fumigant (EFSA 2010). The target species and diseases gather a broad spectrum of organisms as *Verticilium* wilt, *Sclerotium rolfsii*, *Fusarium oxysporum* (Di Primo et al. 2003), *Ralstonia solanacearum* bacteria wilt (Mao et al. 2017). The most widely used commercial formulation is

Basamid® granular powder, which respective breaking products can leachate and reach superficial and groundwaters (Zhang and Wang 2007; USEPA 2017) affecting the aquatic system biodiversity (Eo and Park 2014; Nicola et al. 2017). This commercial formulation must be applied in the soil under humid conditions (according to the recommendations of EFSA 2010; BVBA/SPRL 2014; USEPA 2017) after which dazomet starts to react with water, releasing its major metabolite methyl isothiocyanate (MITC) gas through hydrolysis, which is responsible for pest control. The recommendation dose (RD) of dazomet is around 145 mg/kg of soil and should be applied in a temperature range from 5-25 ºC in humid soil before plantation (EFSA 2010; BVBA/SPRL 2014). Dazomet is soluble in water and its half-life (DT_{50}) is variable from 4 h to 7 days, depending on soil composition and abiotic factors such as water content, organic matter, soil structure and pH (EFSA 2010; Fang et al. 2018). Under alkaline conditions, dazomet is less stable and hydrolyses faster degrading into the fumigant gas (EFSA 2010).

According to EFSA document *Conclusion on the Pesticide Peer Review* (EFSA 2010), dazomet presents an EC_{50} for acute exposure on *Daphnia magna* (test of 48 h exposure, static) of 0.427 mg dazomet/L although, information for chronic, long-term effects with the commercial formulation are not presented, only for the active substance (EFSA 2010). Moreover, knowledge on the influence of soil physical and chemical parameters, namely pH, on the toxicity of granular Basamid® to aquatic organisms are not presented by the report or scientific literature. It is clear that decisions to consider use of these products for another ten-year period seems excessive when based on a narrow data set for effects characterization on non-target organisms. Thus, empirical evidence on ecotoxicological effects, single or in combination with other abiotic factors, must be produced. The pH is one of the most important factors in the soil and its management seeks several improvement outcomes as water movement, nutrient cycling and availability (e.g., P and K), microbial activity, physical-chemical reactions (e.g., decreasing metallic and mineral elements in soil), fertility (e.g., translated into enhanced food production) (Dick et al. 2000; Paradelo et al. 2015; Tian and Niu 2015; Slessarev et al. 2016; Holland et al. 2018) and sorption of pesticides (e.g., influence on pesticides environmental fate) (Sheng et al. 2005). The management of soil pH in agriculture activity is processed mostly by liming, which consists of increasing soil pH through the addition of calcium compounds (Paradelo et al. 2015; Holland et al. 2019). Liming is a common practice to enhance food production and fertility of soil, increase the availability of nutrients and decrease metallic and mineral elements in the soil (Paradelo et al. 2015; Holland et al. 2019; Li et al. 2019). The present work aims at providing additional information towards an extended ecotoxicological dataset for freshwater biota to support the re-evaluation of dazomet use in agriculture, bringing together the broad-spectrum fumigant effects in the environment across relevant abiotic factors such as soil pH.

Thus, the major goal of this study was to assess how soil pH influences the toxicity of eluates obtained from soils, with different pHs, contaminated with Basamid® on two zooplankton species. For this, the following specific objectives were established: i) to evaluate the lethal and sublethal effects of the eluates in the freshwater non-target organisms *Daphnia magna* and *Brachionus calyciflorus;* and ii) to provide new ecotoxicological data to support future regulatory

frameworks revisions for the marketing of dazomet. As far as we know, this work is the first to provide information on sub-lethal effects in aquatic organisms, regarding the exposure to the commercial formulation of dazomet (Basamid®, more realistic scenario of exposure through soil eluates) under exposure scenarios that involve soils with different pH values.

Materials and Methods

Organism cultures and maintenance

The planktonic crustacean *Daphnia magna* and rotifer *Brachionus calyciflorus* were selected as model species to perform the ecotoxicology assays, as they present many advantages, namely being easy to culture and maintain in the laboratory, present a fast reproduction cycle, and multiple sensitive endpoints that can be assessed to quantify the ecotoxicity of chemicals. Adding to this, both are widely distributed in different freshwater systems and have high ecological relevance with an important role in the trophic net balance (Zhang et al. 2016; Tkaczyk et al. 2021). Also, these species are sensitive to a broad spectrum of chemical compounds being suited for ecotoxicity bioassays (Moreira et al. 2016; Zhang et al. 2016; Bownik and Pawlik-Skowrońska 2019).

Parthenogenic cultures of *Daphnia magna* BEAK were maintained in ASTM hardwater medium (ASTM, 2002) under controlled conditions of temperature (21 ± 2 ºC) and photoperiod (16:8 h light: dark). Every other day, the cultures were changed and fed with green microalgae *Raphidocelis subcapitata* (at a concentration of 3 x 10^5 cell/mL/daphnia) complemented with filtered organic additive Marinure 25 (an extract from the marine algae *Ascophyllum nodosum*; Pann Britannica Industries Ltd., Waltham Abbey, UK according to Baird et al., 1989). The sensitivity and health conditions of the *D. magna* cultures were ensured before each experiment by performing reference assays with potassium dichromate, according to OECD 202 (2004) guideline.

The *B. calyciflorus* neonates used for the assays were obtained from resting egg cysts purchased from MicroBioTests, Ghent, Belgium. Until use, cysts were maintained refrigerated at 4 ºC. The rotifer cysts were left to hatch by the transference from 4 ºC to 25 ± 2 ºC under 8000 lux in freshwater medium (RoTox F® medium) 16 to 18 h before the beginning of each assay.

Preparation of the soil eluates

A natural soil from an agro-silvo-pastoral woodland under sustainable management was collected from Herdade do Freixo-do-meio, Alentejo, in Portugal (38°41'44.9''N 8°18'33.7''W) (physico-chemical properties of the soil can be consulted in Gabriel et al, *submitted*). The choice of testing eluates instead of direct exposure of the organisms to the compound dissolved in the test media, allows the simulation of a proxy scenario to the events occurring in the environment. Eluates from soils at three different pH values, without (Ctr-E) and with Basamid® addition (Ba-E), were prepared: the natural soil with a pH_{KCl} of 5.5 ±0.2, and with pH adjusted to 6.5 and 7.5. The chosen range of soil pH to be tested represent the most common and optimum range of pH for horticulture (from 5.5 until 7.2) and the acidic, neutral and alkalinity categories

(Penn and Camberato 2019). For the two highest pH levels, the original soil pH was amended with calcium carbonate ($CaCO_3$). To obtain the eluates, the culture medium of each test species (ASTM and RoTox F® medium) was added to the soils with different pH at a 1:4 (m:v) proportion and left stirring for 12 h at 120 rpm followed by 12 h resting period in a refrigerated chamber, to allow particles sedimentation (DIN 38 414 S4, 1984). Overall, 6 eluates were prepared to be tested with each species: (i) eluates of non-contaminated soil at pH 5.5 (Ctr-E-5.5); 6.5 (Ctr-E-6.5) and 7.5 (Ctr-E-7.5), and (ii) eluates of soil contaminated with Basamid® at soil pH of 5.5 (Ba-E-5.5), 6.5 (Ba-E-6.5) and 7.5 (Ba-E-7.5).

Basamid® eluates (Ba-E) were performed by soil spiking with the recommended dose (RD) of Basamid® (97% purity of dazomet) 145 mg of dazomet/Kg of soil (EFSA 2010; Certis 2012). The respective culture medium of each model species was used to obtain the eluates (ASTM hardwater and ASTM moderately hard synthetic medium for *D. magna* and *B. calyciflorus,* respectively; ASTM, 2002 and RoTox F®). Afterwards, the obtained suspension was filtered with cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain) to avoid suspended particles that could clog the organisms filtering apparatus.

Ecotoxicity assays

Physico-chemical parameters as pH and conductivity (mS/cm) were measured at the beginning and end of each assay (pH330i WTW Weilheim, Germany and WTW440i, Weilheim, Germany portable meters, respectively). The conditions of organism's exposure are summarized in Table 1 and described in detail in the sections below.

Assays with Daphnia magna: Immobilisation, Feeding, and Reproduction

The 48 h immobilization assay performed with neonates of *D. magna* followed the standard guideline OECD 202 (2004). For each replicate, five *D. magna* neonates, aged less than 24 h and from third, fourth, or fifth broods were used to start the assay. The daphnids were exposed to the following treatments: (i) control-Ctr, consisting of the respective culture medium (ASTM hardwater), (ii) control of soil eluates-Ctr-E, consisting of eluates obtained from non-contaminated soils at the three pH values; (iii) 7 dilutions (0.096% to 60%) of the eluates obtained from the three soils contaminated with Basamid® - Ba-E (Table 1). For each treatment four replicates were performed, each consisting of a 60 mL glass vessel filled with 30 mL of each test solution. The assay ran for 48 h at 20 ± 2 ºC and a 16:8 h light:dark photoperiod. The immobilisation, as a surrogate for mortality, was assessed after 24 and 48 h of exposure.

The feeding assay was performed according to the methodology described on Allen et al. (1995). In brief, four days old *D. magna* from the third, fourth or fifth broods were exposed to Ctr, Ctr-E and dilutions of the treatments Ba-E eluates. The four days old daphnids were maintained at the same conditions of the parents' cultures until being used in the feeding assays. The dilutions of Ba-E eluates used in this assay were selected based on the $LD_{50,48h}$ computed for the *D. magna* exposed to the same eluate's treatments. Five neonates were randomly selected and exposed to 20 mL in four individual replicates to a control (Ctr), control

eluate (Ctr-E) and 7 dilutions of eluates obtained from Basamid® contaminated soils: Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5 (Table 1). Food was provided for 24 h ($5x10^5$ cells/mL *R. subcapitata*). The test run at 20 ± 2 ºC for 24 h, in dark conditions to prevent algae growth. In the beginning of the assay, four blanks were established to ensure the initial growth of algae and to prevent significant differences between the beginning and end of the test. To assess the algae consumption, cell density was estimated through medium absorbance (ABS) at 440 nm (Jenway, 6505 UV/VIS spectrophotometer, Burlington, VT, USA). The conversion of ABS in cell density (Conc) followed the Equation (1), centred on a previous calibration curve for the *R. subcapitata* growth. To convert cell density in feeding rates (cells ingested/hour/daphnid), the final and initial Ln (Lnf and Lni, respectively) of the number of algae cells were related with time (tf-ti) (Allen et al., 1995; Eq. 2).

(1) Conc = −17. 107.5 + (ABS * 7925.350); (R2 = 0.99)

(2) FR = (Lnf – Lni) / (tf – ti)

The *D. magna* reproduction test followed the OECD guideline 211 (OECD, 2012). One neonate aged less than 24 h at the beginning of the test from third, fourth or fifth broods was placed in 50 mL control medium (Ctr), control eluate (Ctr-E) and of eluates of soils contaminated with Basamid® (Ba-E). Ten replicates were established per treatment: Ctr, Ctr-E and each dilution of Ba-E. Medium was changed every other day and food and algae extract provided every day in the same proportion as for the maintenance of the cultures (3×10^5 cell/mL/daphnia of the green algae *R. subcapitata* complemented with filtered organic additive Marinure 25). The assay was left at 20 ± 2 ºC at 16:8h (light: dark). Daily, for the 21-days assay, mortality, and the number of offspring per parent was assessed. Also, at the end of the assay, all females were measured using a stereomicroscope, whilst the initial body length was assessed by measuring a representative sample of the neonates from the brood used to start the assay. The following endpoints were assessed: the time until the release of the first brood, somatic growth, and the populational growth rate (*r*). The somatic growth (SG, mm day^{-1}) was calculated through the difference between the final and initial lengths (lf and li, respectively) and the time interval (tf-ti, days; Eq 3). The *r*, computed based in Euler-Lotka equation and standard errors, is a demographic parameter that relates the age (x), survival (lx) and fecundity of the females until age x (mx). Calculations were estimated by jack-knifing (Meyer et al., 1986; Eq. 4)

(3) SG = (tf – ti) / (tf – ti)

(4) $1=\sum_{x=0}^{n} e^{-n} \times lx \times mx$

Table 1- Summary of tests exposure conditions and treatments used in the lethal and sublethal assays carried out with *Daphnia magna* and *Brachionus calyciflorus*. Ctr-Control; Ctr-E- control eluate of non-contaminated soils, Ba-E-eluate of Basamid® contaminated soils and respective D.F.-dilution factor, n.a- not applicable.

	Daphnia magna			***Brachionus calyciflorus***	
	Lethal	**Sublethal**		**Lethal**	**Sublethal**
Guideline followed	OECD 202, 2004	OECD 211,2012	Allen et al. (1995)	RoTox F® Acute, MicroBioTests, Belgium	RoTox F® Chronic, MicroBioTests, Belgium
Test period	48 hours	21 days	24 hours	24 hours	48 hours
Test containers	60 mL vessel	60 mL vessel	60 mL vessel	24 wells plate	24 wells plate
Number of replicates	4	10	4	5	5
Number of organisms per replicate	5	1	5	5	1
Food concentrations (algae cells/mL)	n.a.	3×10^5	$5x10^5$	n.a.	2×10^6
Days of food supply	n.a.	Everyday	1^{st}	n.a.	1
Volume per test container (mL)	30	50	20	1	2
Photoperiod (light:dark h)	16:8	16:8	16:8	0:24	0:24
Temperature (ºC)	20 ± 2	20 ± 2	20 ± 2	25 ± 2	25 ± 2
Medium change	n.a.	Every other day	n.a.	n.a.	n.a.
Treatments (%, D.F.)	Ctr, Ctr-E, 0.94-60 (1.5x) of Ba-E	Ctr, Ctr-E, 0.096-0.782 (1.5x) of Ba-E	Ctr, Ctr-E, 1.5-9.4 (1.3x) of Ba-E	Ctr, Ctr-E, 7.02-100 (1.5x) of Ba-E	Ctr,Ctr-E, 7.02-100 (1.4x) of Ba-E
Assessed endpoints	Mortality	Reproduction and body length	Feeding rate	Mortality	Reproduction

Brachionus calyciflorus: Immobilisation and Reproduction assay

The lethal effects caused by Basamid® eluate on the freshwater rotifer *B. calicyflorus* were assessed by following the standard procedure for acute Rotoxkit F® (MicroBioTests, Ghent, Belgium). Assays ran in 24-well plates, where five replicates were assigned to each treatment: control treatment (Ctr- ASTM moderately hard synthetic medium), control eluate (Ctr-E), and 8 dilutions treatments of eluates of Basamid® contaminated soils: Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5. Five organisms were placed in each well filled with 1 mL of the test solutions. The assays were conducted for 24 h at 23 ± 2 °C in total darkness on a climatic chamber. After exposure, the mortality rates were computed through the counting of the total number of dead organisms with the aid of a stereomicroscope. An organism was considered dead if it did not exhibit any movement within 10 seconds after gentle agitation of the medium.

The 48 h *B. calyciflorus* reproduction assay followed the Rotoxkit F® chronic protocol (MicroBioTests, Ghent, Belgium). Immediately after hatching, the organisms were pre-fed as recommended by the guideline for a period of two hours. Then, organisms were transferred individually to 24-well plates, 1 organism per well, previously prepared with the respective 2 mL of each solution per well. Organisms were exposed to the same treatments as described above for the acute Rotoxkit F® assay (Table 1). After 48 h of incubation at 23 ± 2 °C in total darkness on a phytoclimatic chamber, the total number of swimming organisms per well was counted. The populational growth rate (*r*) was calculated integrating the mean number of rotifers after 48 h incubation (N_{final}), mean number of rotifers at the start (N_{start} =1), and time of exposure (T, days) Eq. 5. The validity criteria of the assays followed the threshold value of 0.55 for population growth in the control.

(5) $r = \ln N_{final} - \ln N_{start} / T$

Quantification of dazomet and MITC in test solutions

To define the calibration curves for both dazomet and MITC, blank samples were fortified at different levels and allowed to sit for at least 2 h at room temperature. Three replicates from each control and treatments (Ctr, Ctr-E and Ba-E) of the three soil pH tested were performed and each replicate was filtered through 0.20 µm syringe prior to injection. Measurements were performed by high performance liquid chromatography coupled to UV detection (HPLC-UV). A Gilson modular system (Gilson, Middleton, WI, USA) was used equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155) and Gilson Unipoint System software. For chemical quality analyses standards of dazomet and MITC were used (purity >99%, Sigma-Aldrich, Steinheim, Germany). Scanning individual standard solutions from 191 to 400 nm allowed the selection of the most accurate wavelengths for elution monitoring. Maximum absorption was achieved at 286 nm for dazomet and 299 nm for MITC. The compounds were analysed in isocratic mode with mobile phase consisting of methanol (A) and water (B), at 40:60 proportion. The injection volume was 20 µL, flowing at a rate of 1 mL/min, running for 10 min (dazomet) and 15 min (MITC).

Statistical analysis

Significant differences in the number of offspring, length, population rate (*r*), feeding rate and survival were identified by using a one-way analysis of variances (ANOVA), after verification of homogeneity of variances by Levene's test and normality by Shapiro-Wilk. Comparison of each treatment with the respective control were performed with the multi-comparison Dunnett's test after significant differences recorded by the one-way ANOVA ($p<0.05$) test, to identify the non-observed effect dilution (NOED) and the highest observed effect dilution (LOED). A two-way ANOVA was used to assess the crossed factors of soil pH and Basamid® eluates of the assays. The dilution (presented as % of eluate) inducing 20 and 50% of effect (ED_{20} and ED_{50}, respectively) for each endpoint were computed by fitting a non-linear regression of a logistic model. The comparison between ED_{20} and ED_{50} within each pH level were performed with Behrens–Fisher tests, a nonparametric comparison. Lethal dilutions provoking 20 and 50% of mortality (LD_{20} and LD_{50}, respectively), along with 95% confidence interval, were calculated using probit analysis through the software Priprobit (Sakuma 1998). The statistical analysis was assessed with StatSoft, Inc. (2007), STATISTICA (data analysis software system), version 8.0.

Results

Validity criteria for all assays carried out were fulfilled. For *D. magna*, the results of the reference assay (performed to ensure the test organisms health and sensitivity) using potassium dichromate test (*data not shown*), were in accordance with the respective guideline (OECD 202, 2004) with an estimated $LC_{50,24h}$ for $K_2Cr_2O_7$ within the range 0.6 - 2.1 mg/l. Moreover, during the lethal assays, mortality of the control vessels did not surpass 20%, whilst in the sublethal assays, mortality of the control did not surpass 10% and females released a minimum of 60 neonates during the full duration of the assay (OECD 202, 2004 and OECD 211, 2008, respectively).

For *B. calyciflorus,* in the lethal assays, control mortality did not surpass 10%, whilst in the sublethal assays, the control populational growth rate was always superior to the threshold of 0.55 suggested by the bench protocol (Acute and Chronic RoTox F®, MicroBioTests, Belgium). Physico-chemical properties as pH and conductivity of the performed assays for both species are presented in supplementary data (Sup. Data) for the performed assays (Table 1S-4S).

Chemical Analysis

In the Ctr and Ctr-E treatments no dazomet or MITC were detected. The MITC metabolite was below the detection limit (LOD: 1.32 µg/mL) for all analysed samples. Regarding dazomet quantification, concentrations varied with soil's pH variation. As for ASTM hardwater (medium used for the *D. magna* assays) significant differences were observed between Ba-E-5.5 and both Ba-E-6.5 and Ba-E-7.5, meaning higher concentration of dazomet at Ba-E-5.5 than at Ba-E-6.5 and Ba-E-7.5 (Dunnett's test: $p<0.05$; Table 2); for the latter two pH concentrations of dazomet were similar (Dunnett's test: $p>0.05$, Table 2). For the rotifer medium, all treatments were significantly different, i.e., a decreasing concentration of dazomet with increased soil pH

was observed. When comparing the eluates obtained with both media, for each pH, dazomet concentration was significantly higher at RoTox F® at Ba-E-5.5 and significantly lower at Ba-E-7.5. No differences were observed within both Ba-E-7.5 (Dunnett's test: p> 0.05).

Table 2- Measured concentration (average ± standard deviation) of dazomet in eluates of soil contaminated with Basamid® at the recommended dose (145 mg of dazomet/Kg, corresponding to 100% treatment) at the three soil pH 5.5, 6.5 and 7.5 (Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5, respectively). Letters represent differences between treatments (Ba-E) and symbols represent differences between medium eluates (ASTM and RoTox®)

Medium	**Ba-E-5.5**	**Ba-E-6.5**	**Ba-E-7.5**
100% Treatment		Dazomet µg/mL	
ASTM	0.361±0.009^{b*}	0.143±0.006 ^{a*}	0.157±0.030 ^{a*}
RoTox F®	0.408±0.007$^{a\#}$	0.163±0.008$^{b\#}$	0.115±0.012^{c*}

Lethal assays: Daphnia magna and Brachionus calyciflorus

Table 3 summarizes the computed LD_{50}, LD_{20}, NOED, and LOED of both species for the soil pH of Ba-E, exposed to 24 h, 48 h and 21 d for the different assessed endpoints. Overall, the estimated LDs varied with soil pH for both species, but not in a pH-dependent way, i.e., LDs did not increase or decrease with pH increase. Despite the tendency for higher toxicity at the intermediate pH (6.5) comparatively to the other two pH levels, an increased in the computed LD_{50} at treatment Ba-E-7.5 comparatively to Ba-E-5.5 and Ba-E-6.5 were observed (Table 3).

For *D. magna*, Ba-E-7.5 exerted lower lethal toxicity ($LD_{50,48h}$ = 9.48%), though the 95 % confidence interval could not be computed for the $LD_{50,48h}$, than the other two Ba-E eluates (Ba-E-5.5 and Ba-E-6.5). Whereas Ba-E-6.5 treatment, showed the highest toxicity of the three exposure treatments, with LD_{50}s at 24 h and 48 h of 3.07% and 1.11%, respectively.

Likewise, for *B. calyciflorus* the same trend of soil pH influence on the toxicity of Basamid® treatments were observed, the Ba-E-6.5 presented higher toxicity than the eluates Ba-E-5.5 and Ba-E-7.5. The computed $LD_{50,24h}$ for Ba-E-7.5 was of 84.7% while for Ba-E-5.5 and Ba-E-6.5 exposure was of 41.4% and 18.1%, respectively (Table 3).

Comparing both species (considering only 24 h of exposure since it is the timepoint in common), *D. magna* was always more sensitive to the influence of soil pH in Basamid® eluates than the *B. calyciflorus* by a factor of 3.5, 4.2 and 9.9-fold for the $LD_{20,24h}$ and by a factor of 5.3, 5.9 and 8.6 regarding the $LD_{50,24h}$ in Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5, respectively. To highlight that any LD_x value surpassed 50% of dilution of the 100% eluate (corresponding to the recommended dose) for *B. calyciflorus*, while for *D. magna* all values were below 10% of dilution of the 100% eluate treatment. The survival rates of both species are represented in Fig. 1S and 2S A and B of the Sup.Data.

Table 3- *Daphnia magna* and *Brachionus calyciflorus* lethal dilutions causing 20 and 50% of effect ($L(E)D_{20}$ and LD_{50} with respective 95% confidence interval), non-observed effect dilution (NOED), highest observed effect dilution (LOED) of eluates (%) originated from Basamid® contaminated soil with pH 5.5,6.5 and 7.5 (Ba-E-5.5, Ba-E-6.5, Ba-E-7.5) for the lethal and sublethal endpoints assessed. n.c. – data not computed, no observed tested dilutions provoking 20 or 50% of effect. Superscript letters (a, b) indicate homogenous groups among the three soil pH's tested within the respective endpoint and LD_x (Behrens-Fisher test, $p<0.05$)

		Ba-E-5.5				**Ba-E-6.5**				**Ba-E-7.5**			
	Duration	**NOED**	**LOED**	**$L(E)D_{20}$**	**$L(E)D_{50}$**	**NOED**	**LOED**	**$L(E)D_{20}$**	**$L(E)D_{50}$**	**NOED**	**LOED**	**$L(E)D_{20}$**	**$L(E)D_{50}$**
Daphnia magna													
Lethal													
	24h	**3.75**	**7.5**	**7.13** (6.65 - 7.58)	**7.82** (7.34 - 8.27)	**1.88**	**3.75**	**2.00** (1.47- 2.46)	**3.07** (2.51 -3.80)	**5.63**	**8.44**	**7.58** (n.c.)	**7.50** (0 - 90.1)
	48h	**3.75**	**7.5**	**3.42** (2.53 - 4.06)	**4.63** (3.87 - 5.52)	**<0.94**	**0.94**	**0.45** (0.12- 0.78)	**1.11** (0.58 -1.60)	**5.63**	**8.44**	**5.95** (0 - 25.6)	**9.48** (n.c.)
Sublethal	21d												
No. offspring/adult		**0.096**	**0.125**	n.c.	n.c.	**>0.782**	**>0.782**	n.c.	n.c.	**<0.096**	**0.096**	n.c.	n.c.
Somatic growth		**>0.782**	**>0.782**	n.c.	n.c.	0.**096**	**0.125**	n.c.	n.c.	**<0.096**	**0.096**	n.c.	n.c.
Populational growth (*r*)		**0.162**	**0.211**	n.c.	n.c.	-	**0.096**	n.c.	n.c.	**0.096**	**0.125**	n.c.	n.c.
Feeding		**1.5**	**1.95**	**1.78** (1.51-2.05)	**1.95** (1.76-2.14)	**>1.5**	**1.5**	**0.98** (0.47-1.49)	**1.38** (1.04-1.73)	-	-	n.c.	n.c.
Brachionus calyciflorus													
Lethal	24h	**35.6**	**53.3**	**25.2** (20.4-29.5)	**41.4** (35.6-49.3)	**12.4**	**17.4**	**8.51** (0-18.0)	**18.1** (0-33.7)	**71.4**	**100**	**75.2** (70.1-80.4)	**84.7** (80.1-89.3)
Sublethal	48h	**12.0**	**14.4**	**12.1^{a}** (11.3-12.8)	**12.9^{a}** (11.9-13.6)	**12.0**	**14.4**	**10.5^{ab}** (7.65-13.3)	**15.4^{b}** (12.9-17.9)	**<10.0**	**10.0**	**7.42^{b}** (5.10-9.74)	**12.5^{a}** (10.34-14.6)

Sublethal assays: Daphnia magna and Brachionus calyciflorus

Regarding *D. magna*, within each eluate, i.e., Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5, no significant differences were observed between the two controls (control ASTM and control Eluate) for the total number of neonate and females' somatic growth ($p<0.05$). The ED_{20} and ED_{50} for *D. magna* were not possible to compute for the number of neonates, somatic growth, and population growth rate (r), thus, comparisons among treatments were performed considering the obtained NOEDs and LOEDs (Table 3; Fig. 1 to 3). Regarding the average number of neonates released per female, the soil pH 6.5 was the one that influenced the least Basamid® eluates toxicity with no registered differences between treatments and control conditions. Following, was pH 5.5 with 0.096% and 0.125% as estimated NOED and LOED values, and pH 7.5 with the higher tested dilution (0.096%) as the LOED (Table 3; Fig. 1). Looking at the somatic growth of females during the 21-days period, no significant differences were detected for treatment Ba-E-5.5, whilst the NOED and LOED for Ba-E-6.5 was of 0.096% and 0.125%, respectively. For this same endpoint at Ba-E-7.5, females were significantly bigger than control conditions at dilutions of 0.096, 0.162, 0.274, 0.463, and 0.782% (Fig. 3). The populational growth rate (*r*) decreased significantly at Ba-E-5.5 of eluates dilutions of 0.211, 0.356, and 0.463% comparatively to the control, but at higher dilutions when pH was increased to 7.5, with an estimated NOED and LOED of 0.125 and 0.162% (Table 3; Fig. 3). At Ba-E-6.5, Basamid® eluates toxicity did not seem to influence the overall *r*¸ with a statistically significant increment on *r* at the dilution of 0.096% comparatively to the control (Fig. 3). For the three endpoints (average number of neonates/females, female somatic growth, and (*r*)), the influence of pH 5.5 and pH 6.5 on Basamid® eluates were very similar and within the three pH levels tested these two (pH 5.5 and 6.5) were the ones causing similar influence of lower No. of offspring in comparison to Ba-E-75, with most of the times sharing the same result-effects to the organisms (Fig. 1-3). The Ba-E-7.5 was often a single group and the influencing pH level in the compound toxicity. Regarding the *B. calyciflorus*, the rotifer presented a sublethal higher tolerance to eluates exposure and soil pH influence than *D. magna*. The influence of soil pH 5.5 and 7.5 caused a higher and similar toxicity of Ba-E in the reproduction of *B. calyciflorus* (ED_{50}: 12.9 [12.9-13.6] and ED_{50}:12.5 [10.34-14.6], respectively) followed by soil pH 6.5 (ED_{50}: 15.4 [12.9-17.9]). The reproduction ED_{50} for *B. calyciflorus* shown higher toxicity in the Ba-E-5.5 and Ba-E-7.5 follow by the Ba-E-6.5. The NOED and LOEDs of the rotifer were significantly higher than the respective ones for *D. magna*, specifically for reproduction were over 80-fold higher for the same soil pH tested. The NOED, LOED, ED_{20} and ED_{50} for the assessed endpoints are presented in Table 3 and Fig.4.

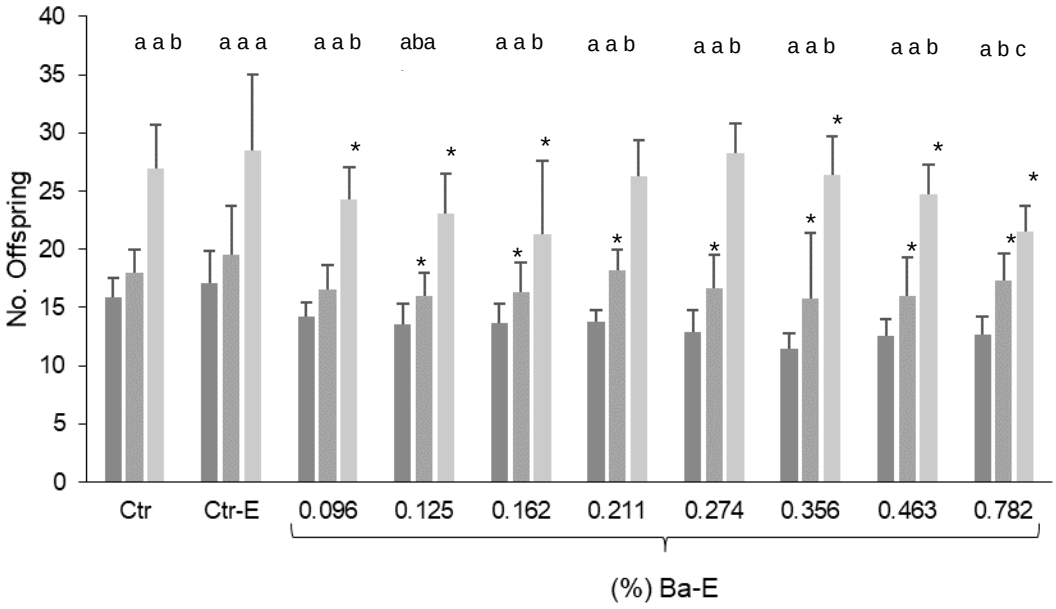


Figure 1- Number of offspring released by *Daphnia magna* exposed, for 21 days, to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (ASTM), Ctr-E- control eluate, letters mean differences between pH factor, * differences between treatments and the respective control. *- significant differences between each respective control, bars correspond to standard deviation.

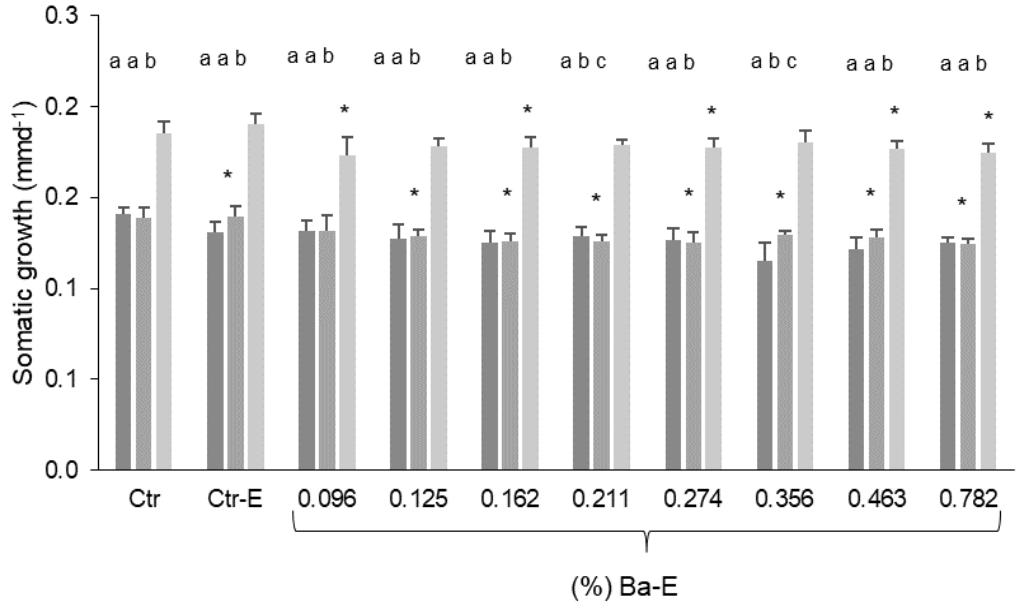


Figure 2- *Daphnia magna* adults' somatic growth exposed, for 21 days, to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (ASTM), Ctr-E- control eluate, letters mean differences between pH factor, * differences between treatments and the respective control. *- significant differences between each respective control, bars correspond to standard deviation.

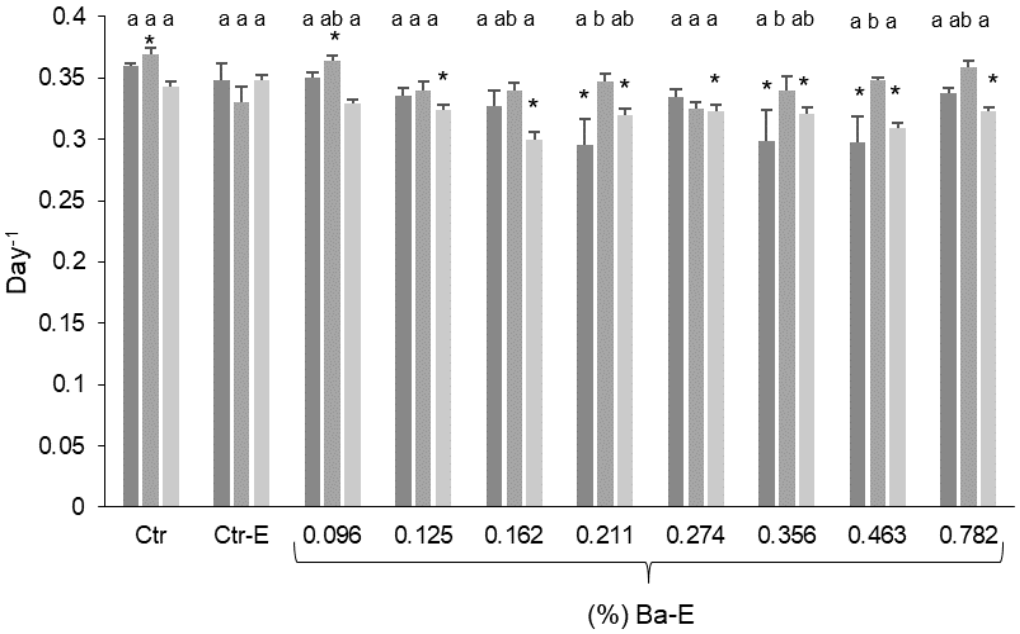


Figure 3- *Daphnia magna* population growth rate (r) exposed, for 21 days, to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (ASTM), Ctr-E- control eluate, letters mean differences between pH factor, * differences between treatments and the respective control. *- significant differences between each respective control, bars correspond to standard deviation.

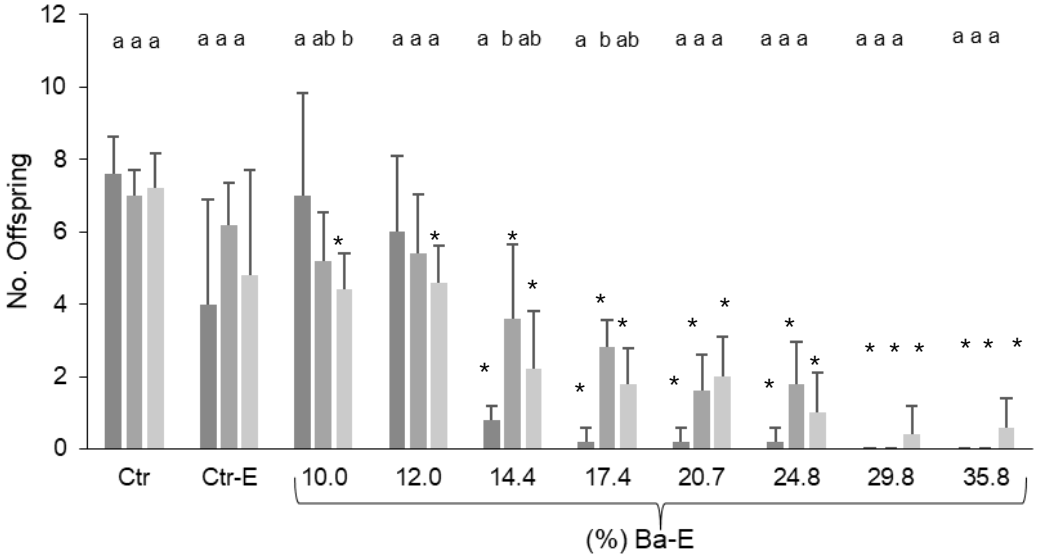


Figure 4- Offspring abundancy of *Brachionus calyciflorus* exposed, for 48h, to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (RoTox®), Ctr-E- control eluate, letters mean differences between pH factor, * differences between treatments and the respective control. *- significant differences between each respective control, bars correspond to standard deviation.

Feeding assay: Daphnia magna

The feeding rate of *D. magna* was assessed when exposed to Ba-E eluates (Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5). Regarding the controls, soil pH itself influenced the feeding rate of *D. magna*, with the non-contaminated soil eluate. In Ctr-E 6.5 and Ctr-E-7.5 it was observed a reduction on algae consumption rate comparatively to the non-contaminated soil control eluate of Ctr-E-5.5 (ANOVA two-way $p<0.05$). In Ba-E-7.5 treatment there was a decreasing rate of algae consumptions starting at the highest dilution, while for Ba-E-5.5 and Ba-E-6.5 the consumption of food was gradually diminishing across lower dilutions of Basamid® eluates. The influence of soil pH 7.5 (EDx was not possible to compute due to the lack of consumption) were more toxic than the effect of soil pH 6.5 (ED_{20}: 0.98% [0.47-1.49] and ED_{50}: 1.38% [1.04-1.73]) and 5.5 (ED_{20}: 1.78% [1.51-2.05] and ED_{50}: 1.95% [1.76-2.14]) with statistical significance (Behrens-Fisher test $p<0.05$). The ED_{20} and ED_{50}, NOEDs and LOEDs are presented in Table 3 and Fig. 3S of Sup. Data.

Discussion

Leachates and runoffs of agrochemicals into aquatic systems can be a major risk and source of pollution threatening biodiversity, posing at risk water quality and the ecosystem functioning (Chen et al. 2019; Wang et al. 2020). Thus, this work aimed at simulating a more realistic scenario of agrochemicals exposure by performing ecotoxicological assays with *D. magna* and *B. calyciflorus* exposed to Basamid® soil eluates considering soil pH alterations.

Results from the chemical analysis confirm what was expected: lower concentrations at higher pHs. Verified by Fang and colleagues (2018), the increase of soil pH increases dazomet rate of hydrolysis which leads to higher dissipation of the fumigant. In ASTM medium the initial concentration of dazomet in Ba-E-6.5 and Ba-E-7.5 were similar although significantly lower than in Ba-E-5.5. In the RoToxF® medium, dazomet's concentration were significantly lower with increasing of soil pH. Regarding both media within the same soil pH, differences were observed. This occurrence could be related with media composition, with exception of Ba-E-7.5, the concentration of dazomet was higher in RoToxF® medium. The ASTM media is much more concentrated than the RoToxF® which can influence some mechanism, together with the soil, facilitating the faster degradation of dazomet and, consequent concentration in the eluate.

The variation of pH from 5.5 (natural soil pH) to 7.5 seemed to somehow soften the lethal effects caused by Basamid® alone, whereas the soil pH 6.5 induced higher toxicity in *D. magna*. This result can be confirmed by the chemical analysis, which relates the faster release reaction in more alkaline conditions caused by a faster hydrolysis of dazomet and respective metabolites (EFSA 2010; Fang et al. 2018). In these conditions, the consequent dissipation from the medium might be the primary reason of the decreasing DT_{50} and effects in the organisms. Also, the influence of pH itself on the organism physiology and mechanism to cope with the fumigant exposure could be a relevant factor to be considered (Yin and Niu 2008; Weber and Pirow 2009; Cox et al. 2018). According to the respective guidelines for acute and chronic tests (mentioned above), the optimal pH of *D. magna* is between 6 and 9 with limited variation of 1.5, these values

are in accordance with the reported pH values of the eluates used in this work. Moreover, the soil pH used for the eluates were amended with calcium, an essential element for crustaceans what might be a significant trigger for the lower effects at higher pH, the availability of such essential elements can allow a better fitness to cope with external factors (Hessen et al. 2000; Cox et al. 2018). The effects of Basamid® eluates exposure varied in a concentration-dependent manner and were influenced by soil pH. Both species presented higher rates of mortality at dilutions much lower than the higher level of Basamid® eluate tested, (herein considered as the 100% Basamid® treatment corresponding to the soil contaminated at the recommended dose – 145 mg dazomet/Kg soil).

Soil pH negatively influenced the toxicity of Ba-E to *D. magna* reproduction towards the Ba-E-5.5 exposure while for the *B. calyciflorus* affected negatively towards Ba-E-7.5 exposure. These results are corroborated by the literature (Hessen et al. 2000; Yin and Niu 2008; Cox et al. 2018) in which is demonstrated a higher reproduction rate in *D. magna* at more alkaline conditions, mostly because of the need of calcium element and lower level of dazomet. Even with similar values of measured pH of the eluates, the availability of calcium in the eluate can be significantly different. The reproduction rate of *D. magna* when exposed to sublethal treatments was higher at Ba-E-7.5 and daphnids also increased body length. The effects in Ba-E-5.5 and Ba-E-6.5 in Basamid® eluates were similar and more sever to *D. magna* regarding reproduction and body length. This occurrence can be related to the lower concentration of dazomet at Ba-E-7.5 in the eluates, leading to lower effects on *D. magna* reproduction. Although, despite the higher reproduction rate in the Ba-E-7.5 exposure, the *r* rate, translating the first reproduction brood, occurred earlier in the most acidic media tested. This occurrence could be a mechanism of defence from the parents by reproducing fast to keep the generations through time. Regarding the feeding capacity of *D. magna*, soil pH was also an influent factor in what concerns the rate of food intake in *D. magna*. Additionally, it was possible to observe significant effects of lower food intake or even suppression of feeding capacity at the Ba-E-7.5 exposure. As well, at Ba-E-7.5 algae (food used in the assay) were present at higher rate compared to the control, instead of being completely consumed by *D. magna* and no higher additional rates of mortality were observed. Therefore, two hypotheses could be occurring (1) lack of mobility of daphnids to be able to feed properly caused by dazomet or respective metabolites (e.g., MITC or formaldehyde), or (2) the eluate from soil pH 7.5 that might have lower concentration of dazomet translates in lower toxicity to algae when exposed to Ba-E and allows the higher rate of algae at the end of the assay. On the other hand, the feeding test was performed with neonates with 4 days old instead of adults, their sensibility to Basamid® eluates and pH factor was not assessed what can be a limitation factor for the poor consumption of the neonates. The 4 days old neonates could have different difficulties to be able to feed during the fragile life stage of development. Regarding the effect of soil pH factor, higher rates of survival were observed for the Ba-E-7.5 which can discard the hypothesis of the consumption of the algae be related to the presence or absent of living organisms. Instead, due to higher survival, it can be occurring some mechanism in the neonates resulting in some inability of daphnids to feed

themselves. On the contrary, at Ba-E-5.5 this inability to feed were not observed even with superior rate of mortality than at Ba-E-7.5 treatments, which relates once more for some alteration effect on ability or metabolism of the *D. magna* to be fed.

When considering the effects of pH in the rotifers, the reported optimal pH from the respective guideline (RoTox®) ranges between 6 and 8, which is in accordance with the reported pH of the tested Ba-E treatments. Supported by literature, the pH factor, independently of the fumigant effect, might be a major aspect affecting the rotifer survival and life cycle (Yin and Niu 2008, 2011). As observed for the sublethal effects on reproduction of *B. calyciflorus*, higher alkalinity of the soil induced higher toxicity of Ba-E, however, the Ba-E-6.5 treatment presented less effect on *B. calyciflorus* reproduction. The effect of dilution for Ba-E-7.5 exposure was in fact more severe than on Ba-E-5.5 and Ba-E-6.5, although it was possible to observe an effect of complete mortality at higher dilutions in the two lower soil pH opposing to the soil pH 7.5. This occurrence can be related once more to the higher dissipation rate of Basamid® at more alkaline conditions which agrees with the chemical analysis that report lower concentrations of dazomet in the Ba-E-7.5.

Regarding both species here tested, exposed to the same conditions, *D. magna* was the most sensitive to Basamid® eluates effects. Considering the effect of soil pH, complete mortality was observed in dilutions 6.7-fold lower than the 100% Ba-E for all treatments in *D. magna*, whilst for *B. calyciflorus* at dilution of 1.96-fold lower for the Ba-E-6.5 eluates. Additionally, both organisms are filter-feeders, which may enhance the interaction between the compound and the entire system of the organisms. Although, there is no information regarding Basamid® mode of action towards to effects on zooplankton and consequently its diet consumption, which represents an additional exposure pathway. It might be assumed a similar effect on both cladoceran and rotifer to the one already described for soil organisms, namely: deregulating enzymatic pathways, nervous and respiratory systems (Oka 2020). Regarding the effect of soil pH on both species' survival, *D. magna* was more sensitive to the Ba-E than *B. calyciflorus* however, both species were highly affected by the effects of soil pH 6.5 in treatment Ba-E-6.5. On the other side, even with higher sensitivity of *D. magna* compared to *B. calyciflorus*, both species were highly sensitive to Basamid®.

While some consistence was observed regarding the effects of soil pH 6.5 on lethal effects of both organisms, the influence of pH at the sublethal levels followed different tendencies. For the reproduction endpoint, opposite effects were observed, *D. magna* was less affected by the increasing alkalinity of the soil, probably due to the lower concentration of dazomet and higher level of calcium which is fundamental for these organisms to reproduce. Whilst the *B. calyciflorus* was more sensitive to the increase of soil alkalinity, in this case, the rotifer can be more sensitive to some eventual metabolites resulting from dazomet such as MITC, formaldehyde and TDL-S, despite of not being quantified in this work (EFSA, 2010).

Both rotifer and daphnid species are currently used in ecotoxicological assays not just as a comparison between species with similar responses but also due to their sensitivity and representativeness of the aquatic ecosystem (Moreira et al. 2016). Despite the greater sensitivity of

D. magna compared to *B. calyciflorus* both were highly sensitive to Basamid® in dilutions significantly higher compared to the 100 % Ba-E. Furthermore, on a similar study approach, Gabriel et al. (*submitted*), found out that both primary producers *Raphidocelis subcapitata* (ED_{50}: >100% Ba-E for growth rate in both Ba-E-6.5 and Ba-E-7.5) and *Lemna minor* (ED_{50}: 9.56% [8.18-10.9]) Ba-E for growth rate in Ba-E-7.5) were less sensitive than *D. magna*. These results can be a bottom trigger of trophic web collapses, meaning that, if higher trophic groups (primary consumers) present higher sensitivity to Basamid® than primary producers, it can impulse an advantage to eutrophication to a further ecosystems disruption.

Conclusion

In this study, it was demonstrated that liming and consequently increasing the alkalinity of the soil, can result in a less toxic effect of Basamid® exposure to the aquatic organisms. Nevertheless, the recommended dose authorized from the EC and EFSA, must be reconsider due to its very high toxicity to aquatic organisms, even with indirect exposure through eluates of the contaminated soil. The results presented and discussed here form a solid basis that aims to highlight that PPP review and approval should be done more often, and therefore intends to contribute to more informed regulatory frameworks.

Investigation towards agriculture and the use of PPP must be conducted coping with the Directive 2009/128/EC, 2009 envisioning new solutions for more sustainable agriculture practice, aiming at the reduction of the environmental risks and impacts of intense use of PPPs.

Considering the off-site effects Basamid®, the limiting of its use nearby aquatic systems or at least creation of buffer zones in the vicinity of agricultural areas where this compound is applied might be future measures to take in consideration.

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Supplementary data

Tables

Table 1S- Initial (i) and final (f) pH and conductivity (C, µS/cm) physical-chemical properties of *D. magna* Immobilisation test (48h) exposed to eluates of soil contaminated with Basamid® at three soil pH of 5.5, 6.5 and 7.5. Ctr - ASTM medium, Ctr-E - Control Eluate.

	pH 5.5				pH 6.5				pH 7.5			
Treatments (%)	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf
Ctr	7.87	494	8.35	622	7.45	550	8.18	605	8.06	599	8.23	652
Ctr-E	7.82	869	8.10	324	6.95	549	8.19	584	7.73	871	8.10	458
0.94	7.88	342	8.39	573	7.51	548	8.36	543	8.06	600	8.18	650
1.88	7.86	276	8.39	572	7.54	548	8.39	540	8.13	597	8.24	649
3.75	7.84	281	8.41	569	7.58	549	8.40	545	8.12	597	8.23	649
7.5	7.81	257	8.42	555	7.55	553	8.35	545	8.07	596	8.19	644
15	7.85	241	8.41	553	7.52	555	8.33	551	7.97	598	8.12	646
30	7.86	251	8.41	522	7.41	563	8.29	560	7.82	615	7.97	797
60	7.88	266	8.39	529	7.26	576	8.10	574	7.63	743	8.03	803

Table 2S- Initial (i) and final (f) pH and Conductivity (C, µS/cm) physical-chemical properties of *D. magna* Reproduction test (21d) exposed to eluates of soil contaminated with Basamid® at three soil pH of 5.5, 6.5 and 7.5. Ctr - ASTM medium, Ctr-E - Control Eluate.

	pH 5.5				pH 6.5				pH 7.5			
Treatments (%)	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf
Ctr	7.55	578	7.77	564	7.96	564	8.05	617	7.78	626	7.72	765
Ctr-E	7.54	666	7.79	670	7.58	689	8.17	777	7.23	904	8.46	802
0.096	7.63	576	7.78	570	8.12	566	8.13	620	7.79	631	8.48	648
0.125	7.67	578	7.80	610	8.11	571	8.10	675	8.00	631	8.47	642
0.162	7.65	578	7.81	572	8.09	570	8.11	645	8.04	624	8.46	635
0.211	7.64	577	7.82	571	8.09	576	8.12	622	8.05	624	8.44	639
0.274	7.70	573	7.82	542	8.14	576	8.13	621	8.06	624	8.43	637
0.356	7.72	575	7.84	534	8.13	598	8.14	602	8.08	624	8.43	642
0.463	7.71	574	7.94	531	8.12	606	8.15	627	8.19	630	8.42	653
0.782	7.72	572	7.93	535	8.13	621	8.14	625	8.23	632	8.37	648

Table 3S- Initial (i) and final (f) pH and Conductivity (C, µS/cm) physical-chemical properties of *B. calyciflorus* Immobilisation test (24h) exposed to eluates of soil contaminated with Basamid® at three soil pH of 5.5, 6.5 and 7.5. Ctr- control (ASTM moderately hard synthetic medium), Ctr-E - control of soil eluates from non-contaminated soils eluate.

	pH 5.5					pH 6.5				pH 7.5			
Treatments (%)	pHi	Ci	pHf	Cf	Treatments (%)	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf
Ctr	8.2	253	7.55	407	Ctr	8.20	253	7.55	407	8.20	253	7.55	407
Ctr-E	7.10	313	7.39	314	Ctr-E	7.55	396	7.66	305	8.07	396	7.98	286
7.02	7.78	309	7.54	320	12.39	7.96	291	7.70	203	8.10	291	7.96	273
10.53	8.08	267	7.61	291	17.35	7.94	218	7.73	152	8.17	218	7.94	299
15.8	8.09	236	7.60	225	26.03	7.92	249	7.70	205	8.09	249	7.94	347
23.7	8.06	307	7.59	305	36.44	7.88	266	7.69	303	8.03	266	7.94	239
35.55	8.05	201	7.62	310	51.02	7.82	306	7.68	479	7.93	322	7.92	317
53.33	7.90	363	7.59	380	71.42	7.74	465	7.73	311	7.94	465	7.99	302
80	7.82	309	7.58	290	100	7.96	469	7.84	352	7.70	469	7.92	360

Table 4S- Initial (i) and final (f) pH and Conductivity (C, µS/cm) physical-chemical properties of *B. calyciflorus* Reproduction test (48h) exposed to eluates of soil contaminated with Basamid® at three soil pH of 5.5, 6.5 and 7.5. Ctr- control (ASTM moderately hard synthetic medium), Ctr-E - control of soil eluates from non-contaminated soils eluate.

	pH 5.5				pH 6.5				pH 7.5			
Treatments (%)	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf
Ctr	7.90	260	8.20	407	7.90	260	8.20	407	7.90	315	8.20	407
Ctr-E	7.19	310	7.10	314	7.19	310	7.10	314	7.19	267	7.10	314
10	7.18	257	7.78	320	7.50	352	7.86	389	7.55	260	8.28	283
12	7.68	261	8.08	291	7.71	229	8.43	376	7.61	244	8.85	267
14.4	7.76	248	8.09	225	7.80	265	8.48	367	7.77	264	8.90	287
17.38	7.78	248	8.06	305	7.78	269	8.43	369	7.80	277	8.85	300
20.73	7.79	270	8.05	298	7.90	270	8.42	368	7.82	283	8.84	306
24.8	7.79	275	8.04	380	7.75	283	8.07	371	7.80	316	8.49	339
29.8	7.13	281	8.05	290	7.71	298	8.06	372	7.72	313	8.48	336
35.8	7.68	290	8.06	301	7.65	288	8.06	373	7.70	279	8.48	302

Figures

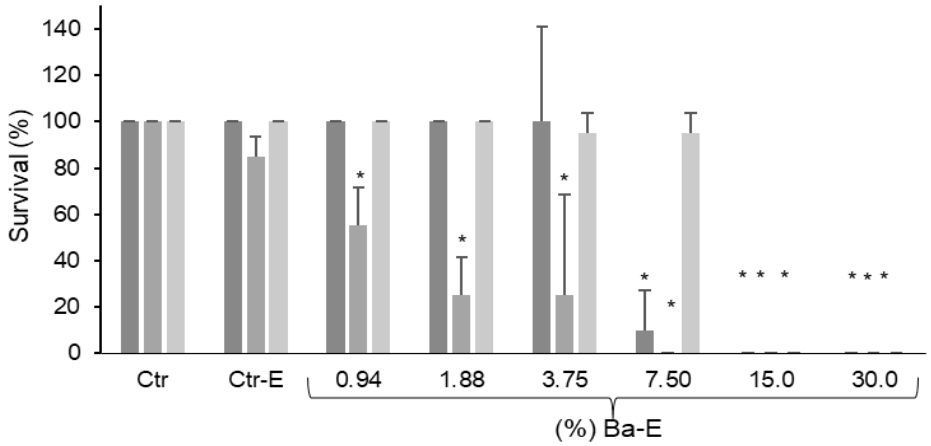

Figure 1S - Average of survival (%) of *Daphnia magna* neonates exposed, for of 48 h, to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (ASTM), Ctr-E- control eluate, *- significant differences between each respective control, bars correspond to standard deviation.

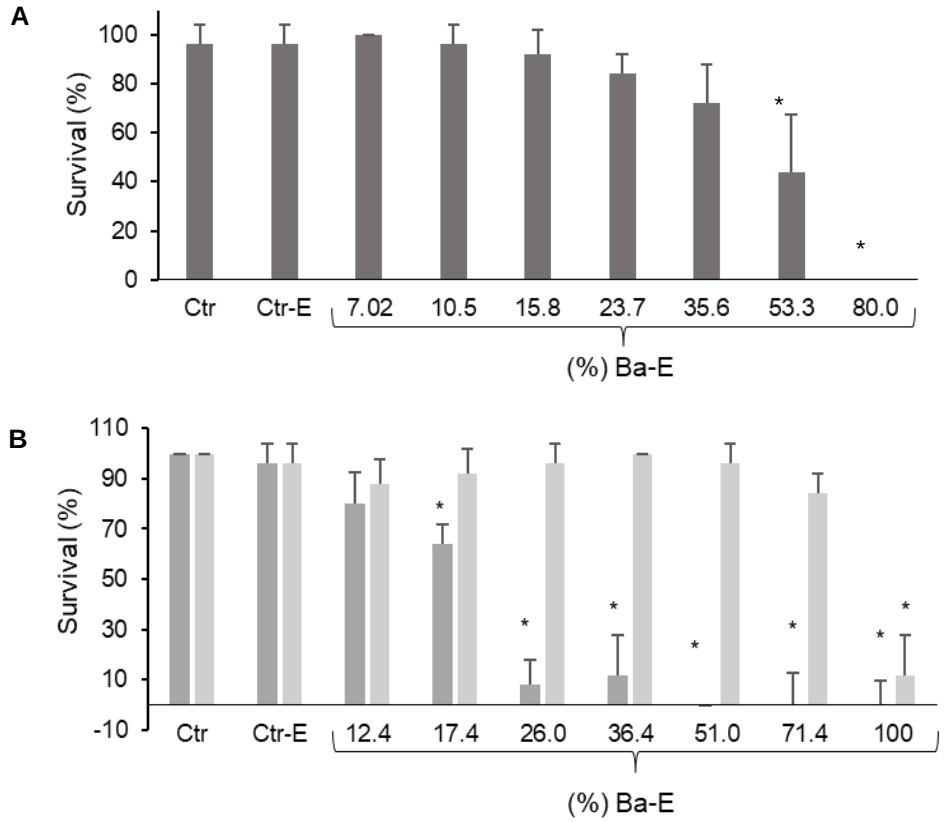

Figure 2S A and B - *Brachionus calyciflorus* acute test for immobilisation, survival rates (in %) of neonates exposed for 24h to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5 (A), 6.5 and 7.5 (B). Ctr- medium control (ASTM moderately hard synthetic medium), Ctr-E- control eluate. *- significant differences between each respective control, bars correspond to standard deviation.

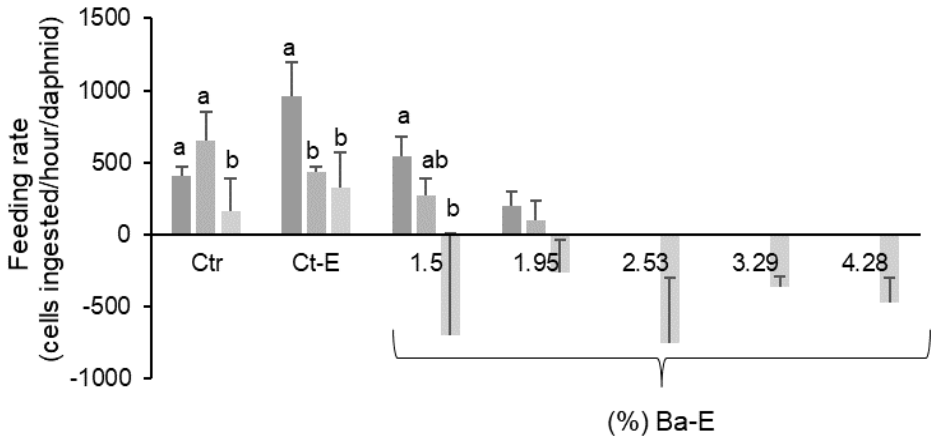

Figure 3S - *Daphnia magna* feeding assay exposure to Basamid® treatments (Ba-E) at different soil pH, from dark to light grey corresponds to soil pH 5.5, 6.5 and 7.5. Ctr- medium control (ASTM), Ctr-E- control eluate, letters mean differences between pH factor, bars correspond to standard deviation.

Chapter VI

Ecotoxicity of eluates obtained from Basamid® contaminated soils is pH dependent: a study with *Hydra viridissima, Xenopus laevis* and *Danio rerio*.

Ecotoxicity of eluates obtained from Basamid® contaminated soils is pH dependent: a study with *Hydra viridissima*, *Xenopus laevis* and *Danio rerio*

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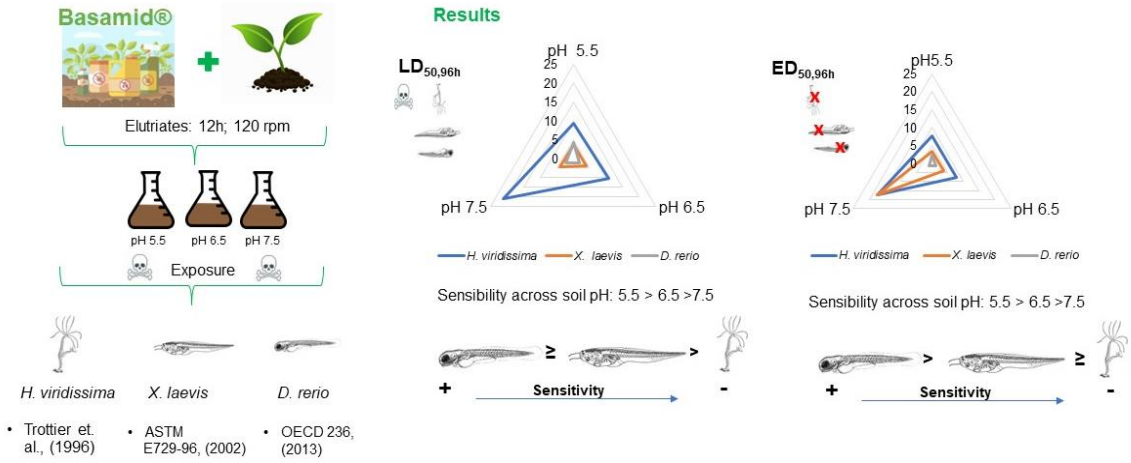
Graphical Abstract



Highlights

- Eluates of Basamid® (Ba)-contaminated soils were highly toxic to aquatic biota.
- The toxicity of Ba eluates to aquatic biota showed dependence on the soil pH.
- *Hydra viridissima* presented higher tolerance to Ba eluates than the vertebrates' species.
- *Xenopus laevis and Danio rerio* were similarly sensitive to Ba exposure within soil pH.

Abstract

Agrochemicals are mostly used to deplete pests and treat diseases in terrestrial agro-ecosystems, although, through leachates of the soil may reach the aquatic systems. Environmental parameters such as soil pH can influence the magnitude of agrochemicals degradation and chemical reaction. The major goal of this work was to investigate the influence of soil pH on the toxicity of eluates obtained from Basamid® contaminated soils to *Hydra viridissima*, *Xenopus laevis* and *Danio rerio*. For this, a natural soil with pH amended to 5.5, 6.5 and 7.5, was spiked with the recommended dose (RD) of Basamid® (145 mg dazomet/kg soil) and eluates (Ba-E) were prepared with the respective species culture medium. Dilutions of the eluates (0.14-100%), obtained from the

three soils (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, corresponding to soil spiked with Basamid® RD at soil pH of 5.5, 6.5 and 7.5, respectively), were used to expose the organisms.

Results showed that for *H. viridissima* increased soil alkalinity provoked less mortality comparatively to lower soil pH [lethal dilution $(LD)_{50,96h}$ of Ba-E 5.5: 10.6 % and $LD_{50,96h}$ of Ba-E 7.5: 21.2 %]. As for *X. laevis* and *D. rerio* Ba-E ecotoxicity was similar across soil pH ($LD_{50,96h}$ varied from 5.7 to 6.9% and from 2.1 to 4.3%, respectively). For malformations, 20% effect dilution (ED) in *H. viridissima* was significantly higher at Ba-E 7.5 ($ED_{20,96h}$: 17.4%), comparatively to the ED_{20} at Ba-E 5.5 and Ba-E 6.5 ($ED_{20,96h}$: 7.9 % (7.3-8.4) and 7.7% (6.9-8.4), respectively). From the three tested organisms and based on both lethal and sublethal effects, *H. viridissima* presented the highest tolerance to Basamid® eluates and soil pH was a major factor determining the fumigant toxicity, with higher soil pH levels inducing, lower toxicity. The eluates obtained from soils contaminated with RD of Basamid® induced severe effects to the three aquatic species.
Keywords: Dazomet, Soil pH, aquatic biota, amphibia, fish, cnidaria.

Introduction

Plant Protection Products (PPP) regulation and approval is under tutelage of the European Commission (EC) along with the Members States and the Environmental Safety Society Agency (EFSA) (EC, 2020a; EFSA, 2014). The risk assessment of approved PPP runs through the market for about ten years until a new revision applies, time period that may be extended without revision, especially in cases where any toxicological data indicates that the substance may cause any harm to the environment. However, as new ecotoxicological evidence on these compounds emerges in the scientific literature, a thorough and new evaluation should be carried in the shortest period possible, aiming at avoiding potential harmful damage to the environment and even to human health. Within the large inventory of PPPs placed in the market, dazomet, the major composition of Basamid® commercial formulation, is a worldwide and commonly used broad spectrum nematicide, fungicide, insecticide, and soil sterilant (EFSA, 2010). Whether for the commercial formulation or the active ingredient there is a paucity of solid biological and chemical evidence of its adverse effects in the environment, reason for which most likely the EC has extended the period of approval for these substances until 2023 (EC, 2020b, 2020a). Namely, it is known that the gas released from the reaction of dazomet with water (methyl isothiocyanate - MITC) is a highly volatile gas, with no-selective biocidal activity, which in theory means that it may kill whatever living organism (Consolazio et al., 2019; Fang et al., 2018; Xie et al., 2015). Actually, the few aquatic toxicity data available for these compounds suggests that since dazomet is quickly degraded into MITC, this secondary metabolite is most likely the main responsible for the observed toxicity, as already evidenced for the freshwater species *Oncorhynchus mykiss*, *Daphnia magna*, and *Pseudokirchneriella subcapitata* (EFSA, 2010). But, according to the regulatory authorities, the degradation product MITC by presenting high volatility is considered of low persistency, and thus not an ecological risk driver, which means low to no potential toxicity to the environment (BVBA/SPRL, 2014; EFSA, 2010; USEPA, 2017). Despite, as mentioned above, it must be

pinpointed that the available toxicity data refers to single species of three trophic levels (one algae, one cladocerans, and one fish) and was driven for acute, short-term exposures. If one considers the effects of other dithiocarbamates, group to which Basamid® belongs (e.g., toxicity to non-target biota, thyroid function disruptive, teratogenicity; reviewed by Lushchak et al., 2018), the precautionary principle should be applied, setting in motion research on the toxic effects that this compound may cause aiming at deriving safer environmental and health guidelines. Moreover, the data available at date, lacks environmental relevance, i.e., do not assume potential changes in the toxicity of the compounds under different abiotic factors. The time range of degradation of Basamid® depends on soil composition and several abiotic factors, such as organic matter, temperature, water content and pH (EFSA, 2010; Fang et al., 2018). Within these, soil pH is one of the most prominent parameters regarding soil quality and sustainability, responsible for nutrient availability, water balance, chemical reaction and microbial diversity (Dick et al., 2000; Sheng et al., 2005; Slessarev et al., 2016; Hong et al., 2018; Borrelli et al., 2020). For instance, erosion of the soils due to scarcity of water, runoffs, salinity increase, extensive farming and agriculture, tillage, overuse and misuse of PPP may alone or in combination lead to soil acidity and infertility (Borrelli et al., 2020; Eswar et al., 2021; Hong et al., 2018; Luetzenburg et al., 2020). Therefore, with such impact on soil processes, soil pH can potentiate or reduce PPPs toxicity. In line with this is that the fumigant Basamid®, when in contact with alkaline soil, reacts by accelerating the release of the gas MITC (EFSA, 2010; Fang et al., 2018; Morrell et al., 1988). Furthermore, it is known that soil pH can influence the absorbance of PPPs into the soil particles' (Huang et al., 2019), and overall influence and facilitate the active ingredient dazomet transportation through the soil into superficial and groundwater systems (EFSA, 2010; Zhang and Wang, 2007).

Regarding the metabolite MITC, taking notice on the characteristics previously mentioned and in accordance with what the literature suggests, only residual MITC values are expected at surface or groundwaters (Santos, 2020). Notwithstanding, it is fallacious to neglect any potential effect since no significant research has been conducted in regard to quantification in aquatic/sediment matrices, under different abiotic stressor scenarios, and/or on other relevant aquatic species. The simulation scenario using FOCUS PEC program of EFSA estimated a MITC surface water concentration of 0.75 µg/L, after a soil application of 192 kg/ha (EFSA, 2010). To say that the report is dated back to 2010, and most probably consumption rates have increased since then as well as concentrations reaching environmental matrices. Considering that concentrations of MITC of 248 ppb (≈248 µg/L) were reported to induce mortality on *Danio rerio* early life stages ($LC_{50,96h}$), whereas concentrations of ≈16 ppb (≈16 µg/L) may already induce severe notochordal distortions on larvae of the same fish species (Haendel et al., 2004; Tilton et al., 2006), highlights here the importance to evaluate its ecotoxicity aiming at contributing to the next revision on its approval and/or derive environmental safety values.

Thus, the main objective of this study is to understand the influence of three different soil' pHs on the toxicity of eluates obtained from soil contaminated with Basamid® at a recommended dose level in three freshwater organisms. Three different taxonomic and functional level representative

species were selected to conduct this ecotoxicological survey on eluates obtained from Basamid®-contaminated soil: *Hydra viridissima* (cnidarian), *Xenopus laevis* (amphibia) and *D. rerio* (fish). To the best of our knowledge, this is the first research providing information on Basamid® toxicity for these three species, thus enriching the appraisal on overall hazard potential of these products to the ecosystems. Additionally, this research also provides information on more realistic scenarios, i.e., exposure to a commercial formulation and through eluates of a natural soil subjected to variable environmental abiotic factors as soil pH.

Methods and Materials

Tested chemical

The commercial formulation Basamid®, purchased to KST- Kanesho Soil Treatment SRL/BV, was used to perform this study. Basamid® is composed with 97 % dazomet (tetrahydro-3,5-dimethyl-2H-1,3,5-thiadiazine-2-thione), a heterocyclic molecule consisting of carbon, nitrogen, sulphur and hydrogen. Dazomet, the primary metabolite of Basamid® formulation degrades on a secondary metabolite, the methyl isothiocyanate (MITC). The fumigant half-life can vary from 4 h to 7 days depending on soil properties, it decomposes rapidly into gas, at high humidity and temperatures above 25 ºC (EFSA, 2010; Prider and Williams, 2014).

Model species

Hydra viridissima, *Xenopus laevis* and *Danio rerio* were chosen as model species to assess the ecotoxicity of eluates originated from Basamid® contaminated soils. These species were chosen because they represent different levels of complexity in biological organization, different taxonomic, trophic, and functional levels that may translate in different pathways of exposure and, thus, differential toxicity. Moreover, for each organism multiple sublethal endpoints (e.g., related both with physiological and teratogenic effects) can be assessed. The specific culture maintenance assets are labelled in Table 1S of the supplementary data (Sup. Data). *Hydra viridissima* is a sessile invertebrate, with reported high sensitivity to a large number of contaminants and visible morphological variations (Trottier et al., 1996), whereas *X. laevis* tadpoles exhibit an epibenthonic behaviour thriving at sediments' surface, and *D. rerio* larvae are pelagic. Regarding the last two species (amphibian and fish), both vertebrate species with external fertilization and embryonic development, it must be highlighted that due to the sharing of similar pathways of exposure to the toxicants in the water-dependent life-stages, the majority of toxicity data for amphibians has come from the extrapolation of data results of fish in general (Ismail et al., 2019; Modra et al., 2011; Shinya et al., 2014). Despite, amphibians' development is substantially different from that of fish due to a highly sensitive morphological and hormonal change at some stage of their lifecycle that cannot be at all predicted by fish embryonic development. Additionally, the amphibian skin is devoid of scales and highly permeable which leads to a higher exposure and uptake of chemicals through the skin. Thus, generating data for early life stages of amphibians is of outmost importance, especially in regard to less explored compounds such as Basamid®.

Preparation of soil eluates

A natural soil from an agro-silvo-pastoral woodland under sustainable management with no history of pesticides application for at least 10 years was collected from Herdade do Freixo-do-meio (Alentejo, Portugal), soil characterization can be consulted at Ferreira et al, (2022). The natural pH of the soil was used pH_{KCl} of 5.5 ± 0.2, along with two more soil pH of 6.5 and 7.5. To raise soil pH, $CaCO_3$ was added according to a calibration curve (data not shown). Eluates of the natural soil at the three pH values were prepared for each species culture medium [*Hydra* medium (Trottier et al., 1996), FETAX medium for *X. laevis* tadpoles (Norton, 1996) and carbon filtered water for embryos of *D. rerio*]. A control eluate (Ctr-E) for each species medium and soil pH (Ctr-E 5.5, Ctr-E 6.5 and Ctr-E 7.5 for the respective soil pH of 5.5, 6.5 and 75), without the addition of Basamid®, were performed. To obtain the Basamid® eluates, soil was previously spiked with the fumigant recommended dose (RD) at about 145 mg dazomet/Kg of soil (BVBA/SPRL, 2014; EFSA, 2010) for each species medium and soil pH (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5 for soil pH 5.5, 6.5 and 7.5, respectively). Eluates were performed at 1:4 (m:v) and left shaken in an orbital agitator for 12 h at 120 rpm followed by 12 h of rest for sedimentation (DIN 38 414 S4, 1984). All of the obtained eluates were filtered with cellulose nitrate membranes of 0.20 µm (ALBET-Hannemuehle S.L., Barcelona, Spain), before being used for the ecotoxicity assays. Dilutions of the Basamid® eluates were performed with the respective test medium at a range of 0.14 – 100 % (soil spiked with the recommended dose of 145 mg dazomet/kg soil corresponded to the 100 % Basamid® eluate treatment).

Ecotoxicity assays

Each species, *H. viridissima*, *X. laevis* and *D. rerio*, was exposed to the range of Basamid® eluates dilutions (starting at 100 % eluates) obtained from soil contaminated with Basamid® at the three pH levels (Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). For each assay, two controls were carried out, one with culture media solely (herein designated as Ctr), and the Ctr-E of each soil pH (corresponding to eluates of the non-contaminated soils, at the three pH values: Ctr-E 5.5, Ctr-E 6.5 and Ctr-E 7.5). In the beginning and end of each assay, parameters as pH and conductivity of all tested solutions were measured (Table 2S-4S of Sup. Data).

96-h lethal and morphological assay with Hydra viridissima

The mortality and morphological changes assays with the cnidarian followed the methodology described by Trottier et al. (1996). For both assays one healthy non-budding hydra was placed in 2 ml of test solution, per well, in a 24-wells plate, completing 6 replicates for each treatment: Ctr, Ctr-E 5.5, Ctr-E 6.5 and Ctr-E 7.5, and dilutions of the Basamid® eluates (0.47, 0.94, 1.88, 3.75, 7.5, 15, 30 and 100 % of Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5). The assays were performed under controlled conditions, at 20 ± 2 ºC and 16:8 h (L:D) photoperiod, for 96 h. Organisms' mortality and morphological alterations were daily checked under a stereomicroscope (Leica MZ6). The scoring

of the morphological changes was based on Wilby's (1988) classification (score of 0 to 10), where 0 corresponds to dead or disintegration of the body of the hydra, scores below 6 correspond to malformations which are irreversible and 10 corresponds to a healthy green organism. Summary of the exposure details of the toxicity assays are described in Table 1.

96-h Xenopus laevis tadpoles' assay

The tadpoles of *X. laevis* tadpoles were exposed to Basamid® eluates by following the E1439-98 (1998) standard guideline. To obtain tadpoles for the exposure test, an adult male and female were injected with human chorionic gonadotropin (hCG 5000 IU, UK) in the dorsal region (with 150 and 500 U hCG, respectively), were placed in a tank and left overnight. After 12-14 h the eggs were collected from the tank to observe for viability and fecundity, and then transferred to a new aquarium to allow their growth until reaching NF stage 45 (Nieuwkoop and Faber, 1994), when were used to perform the toxicity assays. Tadpoles at stage NF 45 were used to perform the ecotoxicity assays, by exposing them to: Ctr, Ctr-E 5.5, Ctr-E 6.5 and Ctr-E 7.5 and to the following dilutions of Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5: 0.47, 0.94, 1.88, 3.75, 7.5, 15 and 30 %. Five replicates were performed per treatment, each with three tadpoles added to 150 mL per test solution and/or control medium. Exposure occurred for a period of 96 h, at 23 ± 1 ºC and 14:10 h L:D photoperiod. Food (0.5 mg Tetramin®) was provided at the beginning and at the 48 h of the test, as well as medium renewal. The mortality, morphological development (NF stage), snout–vent length, malformations and weight were assessed at the end of the assay under a stereomicroscope (Leica MZ6). Summary of the exposure details of the toxicity assays is depicted in Table 1.

96-h Danio rerio embryo toxicity test

For the embryo toxicity assay with *D. rerio* the OECD 236 (2013) guideline was followed. The eggs of zebrafish were collected within 30 min after adults natural mating and then rinsed in carbon-filtered water from the Zebrafish system. Before use, eggs were screened for viability, infertility, and damage, under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). Eggs were exposed to the following treatments: Ctr, Ctr-E 5.5, Ctr-E 6.5 and Ctr-E 7.5 and to the following dilutions of Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5: 0.14, 0.21, 0.31, 0.47, 0.94, 1.88, 3.75, 7.5, 15, and 30 %. For each treatment were carried out 10 replicates that were set in 24-wells plates. Each replicated consisted of 2 ml of test solution and one egg of *D. rerio* with less than 8 h. The mortality, morphological development, and malformations were assessed at the end of test under a stereomicroscope (Leica MZ6). Summary of the exposure details corresponding to the toxicity assays are labelled in Table 1.

The teratogenic index (TI) was calculated, for the three tested species, as the ratio between the LD_{50} and the ED_{50} for malformations (Table 4). A TI value higher than 1.5 means a greater potential for the organism to further develop with malformations (e.g. Williams et al., 2015).

Table 1- Summary of the exposure conditions in the ecotoxicity assays performed with *Hydra viridissima*, tadpoles of *Xenopus laevis* and embryos of *Danio rerio,* exposed to Basamid® eluates (Ba-E), D.F.- dilution factor, Ctr-medium control, Ctr-E- control eluate, - not applicable.

	H. viridissima	*X. laevis*	*D. rerio*
Guideline followed	Trottier et. al., (1996)	ASTM E1439-98 (1998)	OECD 236 (2013)
Test period	96 h	96 h	96 h
Test containers	24-wells plate	200 mL vessel	24-wells plate
Number of replicates per treatment	6	5	10
No. of organisms/ replicate	1	3	1
Food per test container	-	0.5 mg Tetramin®	-
Days of food supply	-	0 and 48h	-
Volume per test container (ml)	2	150	2
Photoperiod (light:dark h)	16:8	14:10	14:10
Temperature (ºC)	21 ± 2	23 ± 2	27 ± 2
Medium change	-	48 h	-
Treatments (Ba-E, D.F)	Ctr, Ctr- E, 0.47-100% (2x)	Ctr, Ctr-E, 0.47-100% (2x)	Ctr, Ctr-E, 0.14 -60% (1.5x)

Quantification of dazomet and MITC in test solutions

Chemical analysis were performed to Ctr and to the 100% of Ctr-E, Ba-E 5.5, Ba-E 6.5 and Ba-E 7.5, by following the same procedure as in Gabriel et al, 2022 (*submitted*). Measurements recurring to an UV-high performance liquid chromatography (HPLC-UV analysis) were performed. To establish the calibration curves blank samples were fortified at different levels and allowed to sit for at least 2 h at room temperature for both dazomet and MITC. Prior to injection, replicates of 3, from each eluate (Ctr-E and Ba-E) of the three soil pH tested were filtered through 0.20 µm syringe. Then, consequent measurements using a Gilson modular system (Gilson, Middleton, WI, USA) equipped with a pump (Gilson 321) and an automatic injector (Gilson 234) coupled to an UV/Vis detector (Gilson 155) and Gilson Unipoint System software were used. Standards of dazomet and MITC were also analysed (purity >99%, Sigma-Aldrich, Steinheim, Germany) to assure the quality of the chemical analyses. Maximum absorption was achieved at 286 nm for dazomet and 299 nm for MITC. The compounds were analysed in isocratic mode with mobile phase consisting of methanol (A) and water (B), at 40:60 proportions. The injection volume was 20 µL, flowing at a rate of 1 mL/min, running for 10 min (dazomet) and 15 min (MITC).

Statistical Analysis

The Basamid® eluate dilution causing 20 and 50 % ($L(E)D_{20}$ and $L(E)D_{50}$, respectively) of effect for the lethal and sublethal endpoints (length, weight, and malformations) were computed by fitting a non-linear regression of a logistic model. Comparisons of $L(E)D_{20}$ and $L(E)D_{50}$ among species, within each pH level, were made by using a nonparametric multiple comparison Behrens-Fisher test.

Normality and homoscedasticity of variances were verified for all the endpoints that were assessed for each species by Shapiro-Wilk test and Levene's test, respectively. Significant differences between the controls and treatments were computed by One-way ANOVA followed by the Dunnett's test. When normality or the homoscedasticity of variances were not verified, non-parametric ANOVA were applied, followed by the Dunn's test. Differences between measured dazomet concentrations across treatments (Ba-E) and media were assessed by a two-way ANOVA (crossed soil pH with Basamid® eluate treatments). Significance was set at $p< 0.05$. The data analysis software StatSoft, Inc. (2007), STATISTICA, version 8.0 was used to run all the analysis.

Results

Chemical Analysis

The quantification of dazomet and MITC in the eluates (using hydra, FETAX and carbon-filtered water medium) of soil at different pH (5.5, 6.5 and 7.5), and in both controls (CTR and CTR-E), were performed through HPLC-UV. There was no soil contamination, i.e., no presence of dazomet or MITC in the controls (both Ctr and Ctr-E). The MITC metabolite was below the HPLC-UV detection limits (LOD: 1.32 µg/mL) for all samples that were analysed. Regarding the quantification of dazomet, concentrations varied among the Ba-E eluates according to soil pH (Table 2). Increasing values of soil pH were associated with a significant decrease in the concentration of dazomet for the three test medium (Dunnett's $p<0.05$, Table 2). When comparing the concentrations of dazomet among test medium within each soil pH, significant differences were observed between hydra medium (highest measured concentration of dazomet) and both FETAX and carbon-filtered water in Ba-E 5.5. In Ba-E 6.5 and Ba-E 7.5, differences were observed between carbon-filtered water (lowest concentration of dazomet) and both hydra medium and FETAX (Dunnett's $p<0.05$).

Table 2- Measured concentration (average ± standard deviation) of dazomet in eluates of soil contaminated with Basamid® at the recommended dose (145 mg of dazomet/Kg, corresponding to 100% treatment) at the three soil pH 5.5, 6.5 and 7.5 (Ba-E-5.5, Ba-E-6.5 and Ba-E-7.5, respectively). Letters represent differences between treatments (Ba-E), within the same test medium, and symbols represent differences between medium eluates (Hydra medium, FETAX, Filtered water), within the same soil pH.

	Ba-E 5.5	Ba-E 6.5	Ba-E 7.5
	Dazomet concentration (µg/mL)		
100 % Ba-Hydra medium	$0.409 \pm 0.018^{a\#}$	$0.190 \pm 0.026^{b\#}$	$0.128 \pm 0.002^{c\#}$
100 % Ba-FETAX	$0.315 \pm 0.013^{a\$}$	$0.185 \pm 0.007^{b\#}$	$0.129 \pm 0.013^{c\#}$
100 % Ba-Carbon-filtered water	$0.344 \pm 0.043^{a\$}$	$0.129 \pm 0.008^{b\$}$	$0.088 \pm 0.005^{c\$}$

Ecotoxicity assays

The validity criteria for mortality and malformations registered in the controls of all studied species *H. viridissima*, *X. laevis* and *D. rerio* were met, namely mortality and malformations were always below 10%. The physical-chemical parameters (pH and conductivity) of each respective soil

pH eluates treatments are presented in Tables 1S-4S, of the Sup. Data. Variations between the initial and final pH values for the assay with *H. viridissima* were always below 0.96 units, and for conductivity below 545 µS/cm, for *X. laevis* variations between the initial and final pH values were below 0.7 units and conductivity below 284 µS/cm; for *D. rerio* variations between the initial and final pH values were below 1 unit and conductivity values below 61 µS/cm.

Lethal ecotoxicity of eluates: Hydra viridissima, Xenopus laevis and Danio rerio

The LD_{20} and LD_{50} of Basamid® eluates and respective 95 % confidence limits were computed for the tested species (at 48 and 96 h) and are summarized in Table 5S of Sup. Data and Table 3, respectively. Despite the standard time assessed recommended in the guidelines for a 96 h assay, the 48 h was also here monitored due to the highly volatility rate of dazomet and MITC, and thus, enabling to evaluate, across time, the effects in the tested organisms. Soil pH revealed to have an influence in the lethal toxicity caused by the eluates of soils contaminated with Basamid®, only for *H. viridissima*. With pH increase (from 5.5 to 6.5 and 7.5) and increasing time of exposure, two patterns of responses were observed. For *H. viridissima* lethal toxicity decreased with increasing soil pH, with $LD_{50,96h}$ for Ba-E 5.5 of 9.2 % and for Ba-E 7.5 of 21.2 % (Table 3, Behrens-Fisher 'test $p<0.05$). Soil pH did not significantly influence Basamid® toxicity regarding *X. laevis* tadpoles and *D. rerio* as shown by the similar values of the $LD_{50,96h}$: 5.7 % for Ba-E 5.5, 4.9 % for Ba-E 6.5, and 6.9 % for Ba-E 7.5 and $LD_{50,96h}$: 4.2 % for Ba-E 5.5, 2.1 % for Ba-E 6.5, and 2.1 % for Ba-E 7.5, respectively (Behrens-Fisher 'test $p<0.05$). Among the three tested species, *H. viridissima* presented the lower sensitivity to Basamid® eluates, regardless of soil pH (Table 3). A similar pattern of toxicity was observed when analysing the LC_{20}s or LC_{50}s computed after a 48 h exposure period. Furthermore, it was observed that, in general, the toxicity (measured by the LDs) increased in less than 2-fold over time (from 48 h to 96 h), suggesting a fast lethal effect of Basamid® in the tested species.

Table 3- Lethal dilution (%) of Ba-E eluates (obtained from soils with different pH: 5.5, 6.5 and 7.5) causing 20 and 50 % of mortality (LD_{20} and LD_{50}), and the respective 95% confidence interval, in *Hydra viridissima*, *Xenopus laevis* and *Danio rerio* after 96 h of exposure. n.c.- not possible to compute.

		Hydra viridissima	*Xenopus laevis*	*Danio rerio*
Ba-E	(%)	96h	96h	96h
5.5	LD_{20}	**6.2**[a] (4.4-8.1)	**4.5**[a] (2.4-6.7)	**3.9**[a] (n.c.)
	LD_{50}	**9.2**[a] (7.4-11.0)	**5.7**[a] (3.9-7.5)	**4.3**[a] (0-23.6)
6.5	LD_{20}	**10.4**[b] (10.2-10.4)	**3.2**[a] (1.9-4.5)	**1.8**[a] (0-5.4)
	LD_{50}	**10.6**[a] (10.5-10.6)	**4.9**[a] (3.7-6.3)	**2.1**[a] (0-14.1)
7.5	LD_{20}	**20.8**[c] (20.6-20.9)	**5.9**[a] (0-17.0)	**1.9**[a] (n.c.)
	LD_{50}	**21.2**[b] (21.0-21.9)	**6.9**[a] (2.2-11.5)	**2.1**[a] (0-25.8)

Sublethal ecotoxicity of eluates

The ED_{20} and ED_{50} for Ba-E eluates (at the three soil pH) were computed for the following endpoints: malformations for *H. viridissima*; malformations, weight, and length for *X. laevis*; and malformations for *D. rerio*. Data is summarized at Table 4.

Based on the ED_{50} for malformations, two patterns arose: an overall tendency to increased toxicity of Ba-E towards lower pH (Ba-E 5.5 and Ba-E 6.5) for *H. viridissima* and *X. laevis* (despite not statistically different between pH; Behrens-Fisher's test $p>0.05$, Table 4); while the opposite occurs in *D. rerio*, i.e., higher toxicity towards alkalinity of soil in Basamid® eluates (Ba-E 7.5; also not statistically different; Behrens-Fisher's test $p>0.05$, Table 4). Moreover, no malformations were detected at Ba-E dilutions superior to 3.75 % (for Ba-E 5.5), 15 % (for Ba-E 6.5) and 7.5 % (for all treatments) in *D. rerio*, *H. viridissima*, *and X. laevis* (all malformations conducted to death after 96 h exposure), respectively (Fig. 7S, 2S of Sup. Data). For *X. laevis,* EDs were possible to estimate for two additional sublethal endpoints: total length and body weight, for which only in the case of the former, soil pH 6.5 significantly influenced Basamid® toxicity to the tadpoles (Table 4). From the three tested species, *D. rerio* presented the highest sublethal sensitivity to the eluates while *H. viridissima* the highest sublethal tolerance (Table 4). Regarding the threshold dilutions (ED_{20}), the same tendencies were observed, with overall overlap of confidence limits (Table 4), with the exception for *X. laevis* tadpoles' weight that were thinner at Ba-E pH of 5.5 comparatively to the other two tested pHs (Table 4). No significant differences were observed for the stage of development when comparing Ba-E exposed tadpoles with those exposed to control conditions (final NF range stage of 48-49 for all surviving tadpoles at the end of the assays).

Finally, the computation of LD_x and ED_x allowed computing a teratogenic index (TI) for all species (Table 4). From the three tested species, *D. rerio* presented the highest potential to develop malformations at latter life-stages (TI of 2.12 in Ba-E 7.5), followed by *X. laevis* in Ba-E 5.5 exposure (TI of 1.8). The TI in *D. rerio* increased with alkalinity of the soil while for *X. laevis* the opposite occurred. *Hydra viridissima* was not susceptible to potential teratogenic effects (TI values < 1.5) (Table 4).

Table 4- Malformations, total length, and weight (only for *X. laevis*) in (%) of Ba-E-eluates (obtained from soils with different pH: 5.5, 6.5 and 7.5) causing 20 and 50 % of mortality (LD_{20} and LD_{50}), and the respective 95% confidence interval, in *Hydra viridissima*, *Xenopus laevis* and *Danio rerio* after 96 h of exposure. n.c.- not possible to compute. (a, b) represent homologous groups within the ED_{20} and ED_{50} of each respective soil pH within the same species, TI-Teratogenic Index.

		Hydra viridissima	*Xenopus laevis*			*Danio rerio*
		Malformation	Malformation	Total length	Weight	Malformation
Ba-E	**(%)**	96h	96h	96h	96h	96h
5.5	ED_{20}	**5.9**[a] (4.5-7.2)	**2.1**[a] (1.3-2.7)	**6.6**[a] (4.0-9.2)	**0.9**[a] (0.2-1.7)	**1.7**[a] (1.1-2.2)
	ED_{50}	**7.7**[a] (6.9-8.4)	**3.3**[a] (2.5-4.9)	**14.0**[a] (10.2- 17.8)	**3.5**[a] (1.9-5.1)	**2.4**[a] (1.8-3.2)
	TI	**1.2**	**1.8**			**1.8**
6.5	ED_{20}	**5.6**[a] (4.9-6.4)	**3.5**[a] (3.3-3.7)	**7.5**[a] (6.8-8.2)	**3.5**[b] (0.9-6.1)	**0.7**[a] (0.4-0.9)
	ED_{50}	**7.9**[a] (7.3-8.4)	**3.8**[a] (3.5-3.9)	**8.9**[b] (7.1-10.8)	**6.8** [a] (2.5-11.1)	**1.2**[a] (0.9-1.6)
	TI	**1.4**	**1.3**			**1.8**
7.5	ED_{20}	**16.4**[a] (0-OL)	**4.1**[a] (1.8- OL)	**10.6**[a] (6.6-14.6)	**4.1**[b] (1.0-7.1)	**0.6**[a] (0.37 -0.8)
	ED_{50}	**17.4**[a] (0-OL)	**17.5**[a] (5.4- n.c.)	**15.4**[a] (11.9-18.9)	**7.5**[a] (3.1-11.9)	**1.0**[a] (0.7-1.4)
	TI	**1.2**	**0.4**			**2.1**

Discussion

Dazomet, the precursor of methyl isothiocyanate (MITC), present in Basamid® commercial formulation, has been in the market for more than ten years. The revision of its regulatory status was postponed by the European Commission to the year of 2023 (2020b, 2020a). Over the past years, attention has been drawn on the potential adverse effects that MITC may pose to the biota. Despite the particular information regarding microorganisms' activity and pathogens (Eo and Park, 2014; Mao et al., 2017; Nicola et al., 2017; Ślusarski and Pietr, 2009), a paucity of information exists in what concerns effects over non-target biota. Moreover, such gap knowledge is deepened when bringing into account realistic exposure scenarios mimicking the entrance of leachates and runoffs into the aquatic systems. Therefore, this research work focused its attention on the effects of eluates from soils contaminated with a commercial formulation of dazomet on three different aquatic ecological receptors, and how soil characteristics (namely soil pH) may influence such toxicity.

Results from the quantification of dazomet in Basamid® eluates through HPLC-UV analysis showed a significantly lower dazomet concentration at higher alkalinity soils. As expected, and in agreement with the literature (e.g., Fang et al., 2018; Ren et al., 2022; Gabriel et al*. submitted*), increased soil alkalinity resulted in higher dazomet fumigation rate, thus leading to the detection of lower fumigant concentrations at pH 7.5 comparatively to pH 5.5 and 6.5. Supporting our data, Ren

et al. (2022) reported a reduction on the half-life of dazomet by 13.9 to 47.4 % at pH 9 comparatively to pH 5 (depending on the size of the granules of dazomet), indicating that dazomet hydrolysis occurs faster under alkaline conditions. These results come to strength the conclusion that soil pH plays a major role in the fate and toxicity of Basamid®. Adding to the influence of pH, though at a lower extension, test media (FETAX, carbon-filtered water or Hydra medium) may also had posed some role on dazomet final concentrations, with an overall tendency for higher concentrations of the fungicide in *H. viridissima* medium. This result may probably be explained by the different chemical composition of the three media, namely by the higher proportions of cations (e.g., Ca^{2+} or H^{+}) in hydra medium and FETAX. Cation's presence in solution has already been reported to influence dazomet degradation. For example, Consolazio (2017) showed that concentration of iron of 0.4 and 0.8 mM caused an increase of 71% and 190% in degradation rates of dazomet comparatively to iron-free water. Likewise, it may be also hypothesized that buffering agents (as EDTA that is present in the hydra medium) may have played a parallel role namely interacting with the fungicide or forming complex intermediates with the ions present in the natural soil used (e.g., Ferreira et al., 2015). Regarding the major metabolite that results from the degradation of dazomet, MITC, no conclusions could be drawn from the influence of soil pH or test medium on its degradation, since it was below the detection limit for all the analysed samples. The high solubility of MITC in water and its low adsorption to the soil would anticipate its appearance in the eluates; however, under conditions of unsaturated soil (as those used to contaminate the soil with Basamid®), its volatilization rate and degradation are very high, and most probably, that is the reason why it could not be detected in the eluates (EFSA 2010).

Regarding the lethal ecotoxicity of the eluates at three different soil pHs, overall, two main patterns of toxicity could be observed: similar toxicity across pHs (*X. laevis* and *D. rerio*), and decreased toxicity with increasing pH (*H. viridissima*). Gabriel et al. (*submitted)* work on the same line of research, also reported different patterns of response in two producer's species (the microalgae *Raphidocelis subcapitata* and the macrophyte *Lemna minor*) after exposure to Ba-E dilutions under different soil pH levels. The authors observed that with increased pH, Ba-E toxicity decreased to microalgae, whereas an increase in soil pH increased the toxicity of Ba-E to the macrophyte. Since the chemical analyses have indicated a reduction of dazomet concentration with increased pH, it is supposed that the differential sensitivity is mainly attributed to species physiological and morphological differences, as previously hypothesized by Gabriel et al. (*submitted*) for the two producers, and/or with the different standard conditions of exposure, namely temperature levels. Despite here all selected species belong to the same trophic level, the whole different ontogenetic developments may lead to different responses to dazomet exposure. Hydras are considered as a less complex but highly sensitive to chemical contamination (Lee et al., 2020; Quinn et al., 2012; Venâncio et al., 2021). Despite, it was the least sensitivity organism among the three species, fact that may be explained with two reasons pointed out by Quinn et al. (2012): i) the ability of the hydras to compact the body and regenerate, allowing to maintain the integrity of the cells; and ii) the lower sensitivity of hydras towards organic contamination due to important

enzymatic activity of phase II biotransformation of molecules in the detoxification of these compounds. Regarding the amphibian and the fish species, similar patterns of toxicity to chemical exposure were observed, which can be attributed to the reason that as vertebrates they share more analogies during their embryonic development (Ismail et al., 2019; Modra et al., 2011; Shinya et al., 2014). Although, it must be highlighted that exposure to Ba-E in *D. rerio* took place during the embryo stage, whilst in *X. laevis* occurred at later stage of development (stage NF 45). In addition, as previously mentioned, one must account for species specific assay standard-requirements such as temperature (20 °C, 23 °C, and 26 °C for the cnidarians, the amphibia, and the fish, respectively) that may also contribute for the differential patterns of toxicity that were observed. It is known that increased temperature promotes faster dazomet hydrolyzation. For instance, Ren and colleagues (2022) aiming at testing dazomet hydrolysis under different temperature regimes, verified that the half-time of the compound was decreased up to 6 times more when temperature was risen from 5 to 35 °C. Therefore, it can be hypothesised that during the assays at 23 and 26 °C a higher transformation of dazomet into MITC and its subsequent volatilization occurred comparatively to that at 20 °C, which could have contributed to a higher exposure to this secondary metabolite that has been identified to be the most responsible for the toxicity induced by Basamid® (EFSA 2010).

It is noteworthy also that sublethal effects, i.e., malformations, were detected at lower dilutions corresponding to 20% of contamination doses equivalent to 145 mg/kg soil (100% Basamid® eluate). For example, hydra malformations were mainly related to the morphology of the tentacles, which can severely impair the hydra's ability to capture food due to their sessile behaviour (Venâncio et al., 2021; Wilby et al., 1990), whilst the most severe malformations observed for both vertebrate species were mostly oedemas, clots, and spine deformities, that not only interfere with the normal respiratory function but also interfere with organisms' foraging ability for food or to escape from predators and seek refuge (Mori et al., 2018). To underline that alike other PPPs from the dithiocarbamates group, dazomet has a lipophilic nature, meaning that it may easily cross biological barriers. Thus, even if not inducing mortality, major sublethal effects may be triggered at very low doses specially in vertebrate species since it may present a neurotoxic and a pituitary-thyroid axis disruptive behaviour, potentially leading to changes in sexual maturity, reproduction, or even the development of tumours (Rath et al., 2011), reason which reinforces the need to revise PPP products, such as dazomet. Regardless the data here presented, in addition to that previously delivered by Gabriel et al. (*submitted*), it is highlighted the need to generate toxicity data for organisms of the same trophic group to increase the robustness of potential environmental protection values that may be generated, as well as, whenever possible, include data for amphibians separately from fish as toxicity may vary substantially.

The teratogenic indexes for Basamid® were possible to determine for each species within each soil pH, considering that indexes > 1.5 mean a greater potential for the development of severe malformations at later life stages. In the cnidarian *H. viridissima* and *X. laevis* (except pH 5.5) there was no indication of a potential teratogenic effect since all values were below the threshold value of

1.5. In case of *H. viridissima* such may be related to its ability to regenerate body parts as previously mentioned (e.g., Quinn et al., 2012; Venâncio et al., 2021); although for *X. laevis* was mostly due to the proximity between the LD_{50} and the ED_{50} indicative of "all-or-nothing" effect and to the fact that organisms were exposed at the developmental stages of tadpole, all the embryogenesis had already occurred (Williams et al., 2015). Zebrafish had a higher potential to develop malformations on further advanced life-stages comparatively to the other two species, most probably because it was exposed to Basamid® during all embryogenesis, maximizing the probability of occurring malformations. Two studies conducted by Haendel and colleagues (2004) and Tilton and colleagues (2006) shown that dithiocarbamates group that degrades in MITC caused higher teratogenicity on zebrafish related to collagen formation and somitogenesis, leading to notochord distortions at early stages in the zebrafish development. Furthermore, Ducharme and colleagues (2013) led a meta-analysis study of the teratogenicity of 133 chemicals to zebrafish, including fumigant MITC, where data obtained corroborated the present results. Although no concentration level is reported, since the work in question is a general classification of this extensive list of chemicals into three levels of teratogenicity (high, medium or low teratogenicity), the MITC was within the group of chemicals with the lowest teratogenicity (Ducharme et al., 2013). A TI for MITC of 4.68 for zebrafish was estimated, which is in line with our findings. Despite the lower TI obtained from the present work (2.12 at Ba-E 7.5 in *D. rerio*) comparatively to those reported by Ducharme and colleagues (2013) in zebrafish, the index is of the same order of magnitude. Moreover, it can be addressed as low teratogen when considering also the closed to the limited 1.5 index assessed of *H. viridissima* and *X. laevis* at the same conditions. Despite low, one must acknowledge its presence, and therefore organisms' behaviour and fitness of these organisms, in late life stages and in the environment, might be threatened and compromise the ability to feed, escape to predators and/or survival. Additionally, these organisms are commonly used as proxies for human studies and data extrapolation for human exposure (Gonçalves et al., 2020). Since the application in field is proceeded by man, the direct exposure is inevitable. Following the severe effects shown here, following the contamination of soils at the recommended dose, it is imperative the revision of Basamid® envision both environmental and human health.

Conclusion

These findings suggest the importance of PPP evaluation at its commercial formulation in different complexity organisms at relevant and realistic exposure scenarios. This research work showed that Basamid® applied to the soil in recommended doses induced severe effects to aquatic organisms even through indirect exposure as eluates of the soil. Soil pH was a major factor regarding the fumigant toxicity to the cnidarian, with higher soil pH levels inducing lower lethal toxicity, whereas, for *X. laevis* and *D. rerio* soil pH did not seem to induce a significant effect. Nevertheless, different endpoints of sublethal effects of different organisms should be considered in what concerns PPP risk assessment in order to gather a more accurate environmental and ecotoxicological framework. The risk assessment of Basamid® along with general PPP should be

aligned with the realistic events in the environment to further consider more effective and protective measures to organisms of different complexity and trophic level.

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Chapter VI- Ecotoxicity of eluates obtained from Basamid® contaminated soils is pH dependent: a study with *Hydra viridissima, Xenopus laevis* and *Danio rerio*.

Supplementary data

Tables

Table 1S- Summary of laboratory-culture conditions of *Hydra viridissima*, *Xenopus laevis* and *Danio rerio*. [a]Commercially available in pet stores, [b]Additional Vitamin Supplementation (XE40 Mucedola, Italy), [b]Zebrafish Management Ltd

	Hydra viridissima	*Xenopus laevis*	*Danio rerio*
Culture Medium	*Hydra* medium	FETAX	Carbon Filtered Water
Food	Brine shrimp nauplii[a]	*Tenebrio molitor*[b]	ZM-400 fish food[c]
Feeding (times/ week)	1-2	3	Daily
Temperature (ºC)	20 ± 2	23 ± 2	27 ± 2
Photoperiod (L:D h)	16:8	14:10	14:10
Conductivity (µS/cm)	± 500	± 550	± 500

Table 2S- Initial (i) and final (f) pH and conductivity (C, µS/cm) values measured in the test solutions used to perform the *Hydra viridissima* malformation test (96 h): Ctr-control consisting of hydra medium, Ctr-E-eluates obtained from non-contaminated soils and 0.94 to 60%-dilutions of the eluates obtained from Basamid® contaminated soils at three soil pH of 5.5, 6.5 and 7.5.

	pH 5.5				pH 6.5				pH 7.5			
Dilutions (%)	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf	pHi	Ci	pHf	Cf
Ctr	7.07	494	7.35	622	7.07	550	7.18	605	7.07	599	8.03	652
Ctr-E	7.82	869	7.10	324	7.95	549	7.19	584	7.73	871	8.10	458
0.94	7.08	342	7.39	573	7.51	548	7.36	543	8.06	600	8.18	650
1.88	7.12	276	7.39	572	7.54	548	7.39	540	8.13	597	8.24	649
3.75	7.14	281	7.41	569	7.58	549	7.40	545	8.12	597	8.23	649
7.5	7.11	257	7.42	555	7.55	553	7.35	545	8.07	596	8.19	644
15	7.15	241	7.41	553	7.52	555	7.33	551	7.97	598	8.12	646
30	7.12	251	7.41	522	7.41	563	7.29	560	7.82	615	7.97	797
60	7.12	266	7.39	529	7.26	576	7.10	574	7.63	743	8.03	803

Table 3S- Initial (i) and final (f) pH and conductivity (C, µS/cm) values measured in the test solutions used to perform the *Xenopus laevis* malformation test (96 h): Ctr-control consisting of FETAX medium, Ctr-E-eluates obtained from non-contaminated soils and 0.94 to 60%-dilutions of the eluates obtained from Basamid®-contaminated soils at three soil pH of 5.5, 6.5 and 7.5.

	pH 5.5				pH 6.5				pH 7.5			
Dilutions (%)	pHi	pHf	Ci	Cf	pHi	pHf	Ci	Cf	pHi	pHf	Ci	Cf
Ctr	7.80	7.90	551	656	8.11	8.01	644	651	7.68	7.90	552	601
Ctr-E	7.80	8.07	754	745	8.57	8.37	861	868	7.49	8.18	684	712
0.47	8.02	8.10	549	606	8.35	8.10	645	652	7.75	8.15	554	590
0.94	8.03	8.08	550	599	8.26	8.10	638	645	7.85	8.14	552	589
1.88	8.03	8.05	547	593	8.20	8.10	637	644	7.88	8.30	548	589
3.75	8.02	8.04	546	593	8.15	8.07	652	659	7.92	8.14	550	583
7.5	8.02	8.04	548	592	8.12	8.07	659	666	7.91	8.08	562	596
15	7.99	8.04	560	603	8.19	8.09	661	668	7.90	8.07	581	612
30	7.82	8.04	603	646	8.20	8.06	665	672	7.82	8.05	622	650

Table 4S- Initial (i) and final (f) pH and conductivity (C, µS/cm) values measured in the test solutions used to perform the *Danio rerio* malformation test (96 h): Ctr-control consisting of Carbon Filtered Water, Ctr-E-eluates obtained from non-contaminated soils and 0.94 to 60%-dilutions of the eluates obtained from Basamid®-contaminated soils at three soil pH of 5.5, 6.5 and 7.5.

	pH 5.5				pH 6.5				pH 7.5			
Dilutions (%)	pHi	pHf	Ci	Cf	pHi	pHf	Ci	Cf	pHi	pHf	Ci	Cf
Ctr	7.86	7.93	516	606	7.86	7.93	516	606	7.86	7.93	516	606
Ctr-E	6.49	7.52	997	1058	7.27	8.52	1162	1223	7.19	8.58	1206	1265
0.47	7.59	8.01	829	894	7.71	8.18	827	883	7.67	8.23	830	884
0.94	7.77	8.07	824	875	7.74	8.08	819	865	7.72	8.11	818	863
1.88	7.78	8.04	823	875	7.77	8.08	820	868	7.72	8.09	819	865
3.75	7.49	8.05	823	875	7.77	8.06	826	871	7.72	8.08	825	873
7.5	7.73	8.04	824	870	7.74	8.07	836	882	7.7	8.08	836	910
15	7.59	7.99	843	894	7.68	8.09	855	907	7.67	8.08	857	952
30	7.37	7.93	869	919	7.59	8.11	899	950	7.62	8.15	898	862

Chapter VI- Ecotoxicity of eluates obtained from Basamid® contaminated soils is pH dependent: a study with *Hydra viridissima, Xenopus laevis* and *Danio rerio*.

Table 5S- Lethal dilution (%) of Ba-E eluates (obtained from soils with different pH: 5.5, 6.5 and 7.5) causing 20 and 50 % of mortality (LD_{20} and LD_{50}), and the respective 95% confidence interval, in *Hydra viridissima*, *Xenopus laevis* and *Danio rerio* after 48 h of exposure. n.c.- not possible to compute.

		Hydra viridissima	*Xenopus laevis*	*Danio rerio*
Ba-E	(%)	**48h**	**48h**	**48h**
5.5	*LD20*	**7.9**[a] (6.0-9.7)	**6.4**[a] (4.3-8.4)	**7.5** [a] (6.8-8.2)
	LD50	**10.6**[a] (8.8-12.4)	**9.9**[a] (8.0-11.9)	**8.2** [a] (8.5-10.2)
6.5	*LD20*	**10.4**[b] **(n.c)**	**6.3** [a] (n.c)	**3.0** [a] (n.c)
	LD50	**10.6**[a] (10.5-10.6)	**9.7** [a] (n.c)	**3.8** [a] (n.c)
7.5	*LD20*	**20.8**[c] (20.6-20.9)	**8.2** [a] (4.9-11.5)	**3.5** [a] (3.3-3.7)
	LD50	**21.2**[b] (21.0-21.3)	**11.2** [a] (8.2-14.2)	**3.7** [a] (3.5-3.9)

Figures

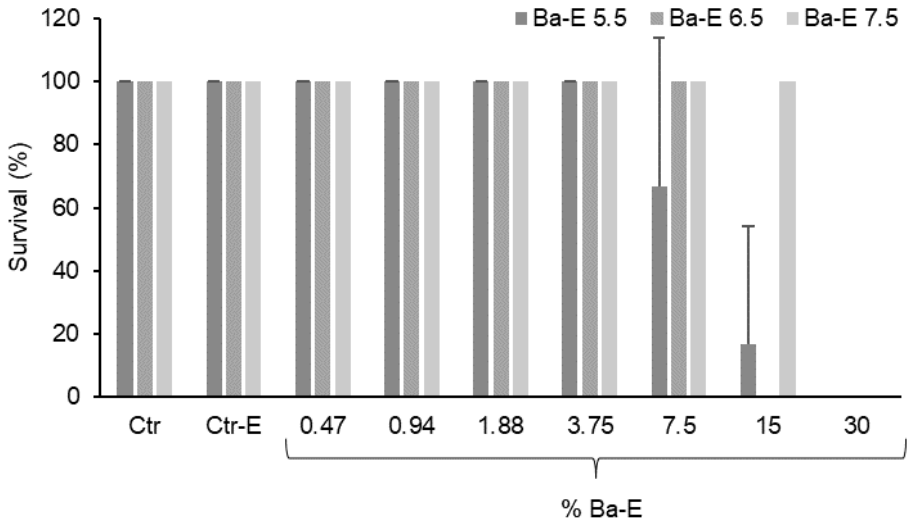

Figure 1S- Survival rate (%) of *Hydra viridissima* exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of hydra culture medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

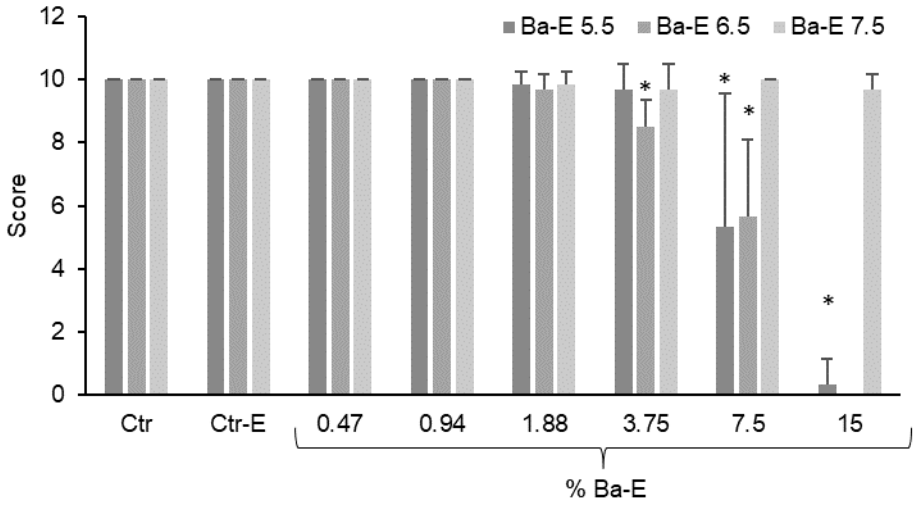

Figure 2S- Score of *Hydra viridissima* malformation exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of hydra culture medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

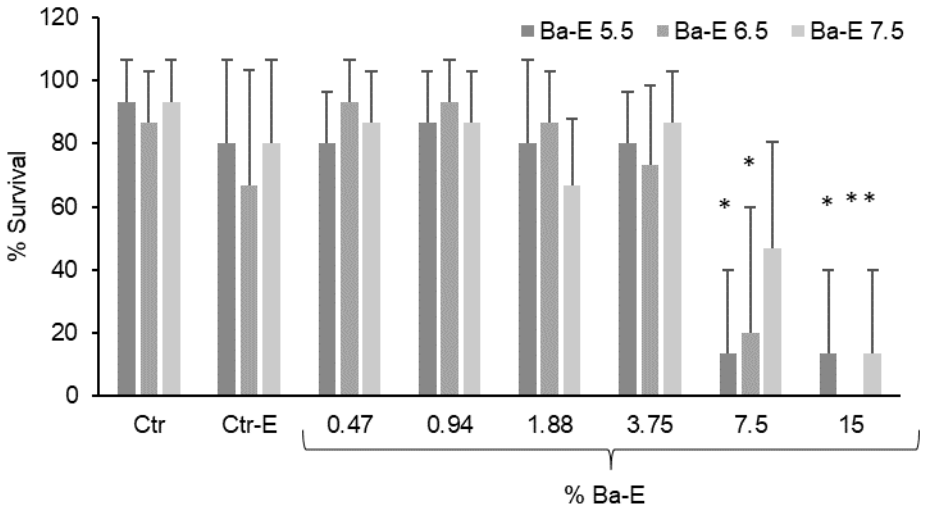

Figure 3S- Survival rate (%) of *Xenopus laevis* exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of FETAX medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

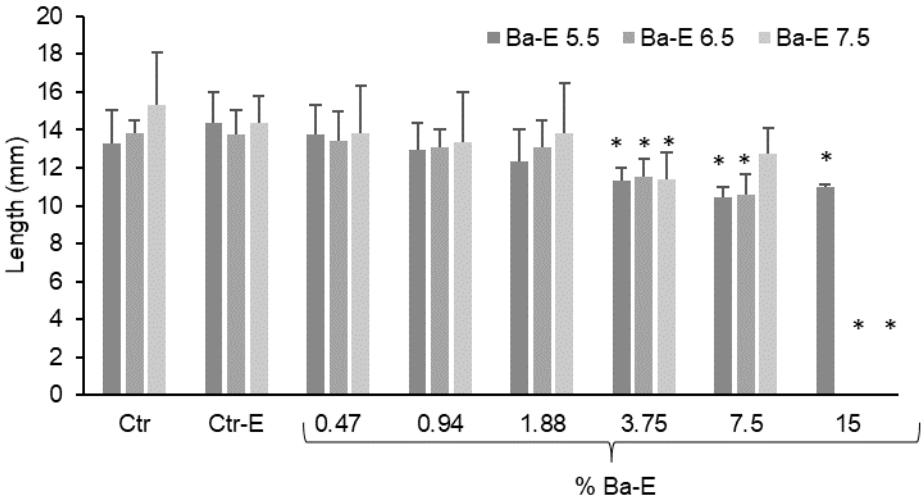

Figure 4S- Total body length of *Xenopus laevis* exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of FETAX medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

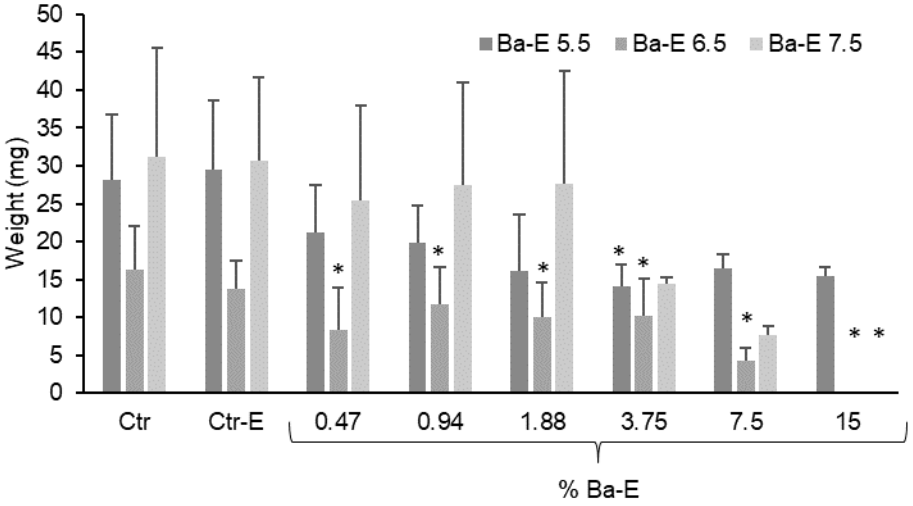

Figure 5S- Body weight of *Xenopus laevis* exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of FETAX medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

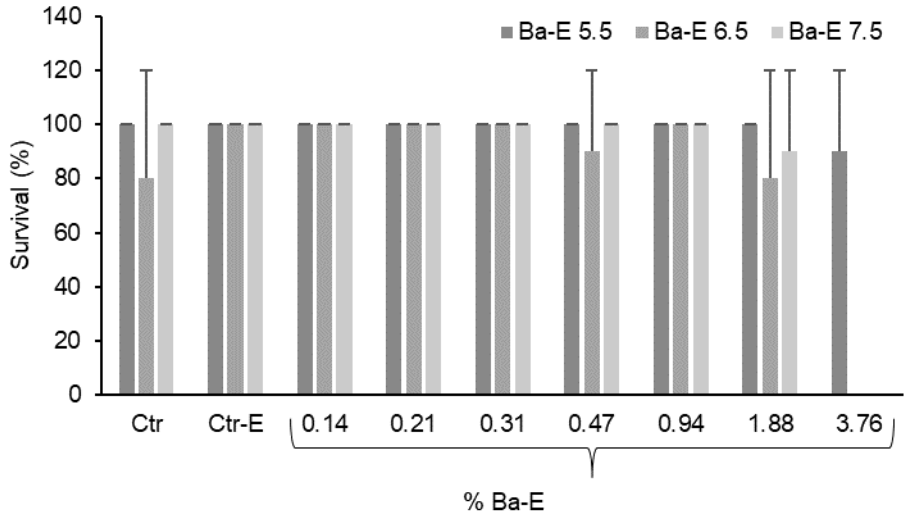

Figure 6S- Survival rate (%) of *Danio rerio* exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of Carbon Filtered Water medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

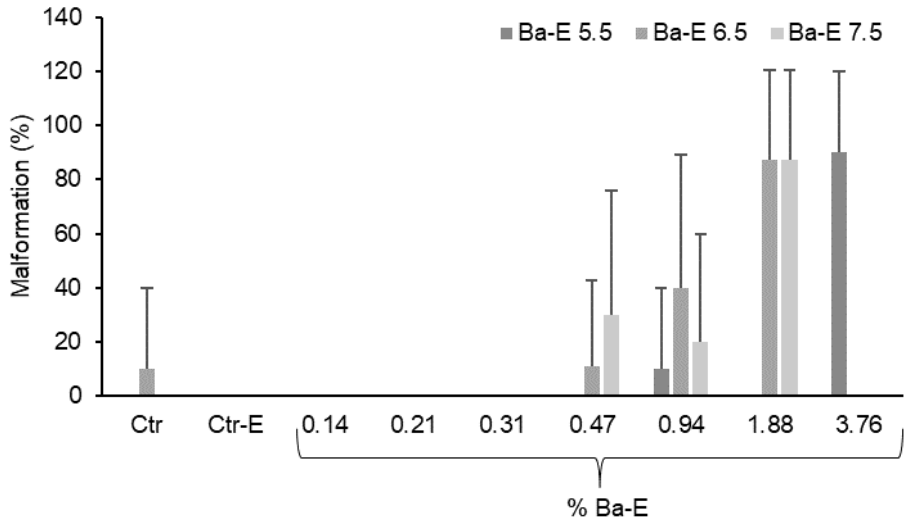

Figure 7S- Percentage (%) of *Danio rerio* organisms with malformation, exposed, for 96 h, to dilutions of the eluates obtained from Basamid®-contaminated soils at different soil pH (5.5, 6.5 and 7.5, from darker grey to lighter, respectively). Ctr-control consisting of Carbon Filtered Water medium, Ctr-E-control consisting of eluates obtained from non-contaminated soils at the three pHs. Error bars represent standard deviation.

Chapter VII

General Discussion and Conclusions

General approach and achieved objectives

Concerning the worldwide demand for food along with the increasing rate of Human population, agriculture will remain to be the primary source of food provisions (Tilman et al. 2002). Thus, it is expected the continuous use of agrochemicals as fertilizers and pesticides (such as Basamid® fumigant) in agriculture activities (Datta et al 2016). Consequently, the pollution of the atmosphere, soil and water, due to agrochemical application and respective leachates/runoffs, is foreseen to persist and potentiate their accumulation in the environmental matrices and biota, over time. Even though the use of these chemicals is controlled by restrictive regulations implemented by regulatory entities and authorities (EFSA, EC, EU members), the process between new evaluations of agrochemicals is too long, corresponding mostly to a period of 10 years. Also, in some cases, the assessment of some active ingredients may be postponed, enlarging further that period, which may contribute to the prolonged use of substances that may pose high risks to the environment. This is the case of the active ingredient dazomet, which respective evaluation revision has been postponed for more than a decade until 2023 (EC 2020). This long period until the new evaluation, without the generation of new and relevant knowledge to improve and reduce uncertainties on the ecological risk of this fumigant, may contribute to serious adverse effects in the ecosystems receiving this PPP. Actually, dazomet and its degradation products (namely MITC) have been reported to present risk to biota and the environment. Therefore, the extended period for the new evaluation, potentiates the risks that this PPP may cause in the different environmental compartments, some of which may result in irreversible effects. Thus, it is of most importance to generate further knowledge, under more relevant exposure scenarios, on the effects that Basamid® and its active ingredient (dazomet) may cause on both terrestrial and aquatic ecosystems aiming to provide stronger evidence and reduce the uncertainties for the new risk assessment and respective evaluation.

The current situation and effects of PPP in the environment, such as Basamid® (a commercial formulation of dazomet) across time needs to be addressed. The knowledge gap regarding the effects that this PPP may pose to soil and aquatic non-target organisms must be fulfilled. Primarily due to the postponed re-evaluation from the authorities and continuous application until new revision (EC2020). Secondarily, due to the lack of data on dazomet effects to organisms from different environmental ecosystems: only one study was reported until date on earthworms (Mao et al. 2017) and soil microorganisms (Eo et al. 2014; Nicola et al. 2017; Fang et al. 2018, 2020), no studies were reported for aquatic organisms and no long-term effects of dazomet were presented until now. Furthermore, the influence of environmental parameters on its ecotoxicity is also of much relevance, since some works have already reported the influence of soil parameters, such us humidity, organic matter and urea content (Zhang and Wang 2007; Fang et al. 2017). While Zhang and Wang (2007) observed higher rates of atmospheric emissions at higher irrigation time, Fang and colleagues (2017) observed that higher temperature and higher soil pH increased dazomet hydrolysis reaction, therefore, increasing its gas release rate.

With the intention of coping with some of these major gaps, this work intended to explore the effects of soil pH in Basamid® ecotoxicity through the following specific goals: i) to determine the

ecotoxicity of Basamid® in non-target soil organisms across different values of soil pH, ii) investigate the capacity of soil non-target organisms to recolonize contaminated soil, across different values of soil pH; iii) to assess the influence of soil pH on the ecotoxicity of Basamid® eluates in different trophic level of non-target organisms from freshwater systems and, iv) to establish recommendations for a safer use of Basamid® as it regards to soil pH management within agricultural activities.

Therefore, with a sounder and more accurate knowledge on the effects of soil pH on the ecotoxicity of Basamid® to non-target organisms, both terrestrial and freshwater, it is aimed to contribute to establish strategies for environmental management and mitigation, and to promote a sustainable and greener practice within agriculture and the use of PPP, specifically Basamid®. In this context, the research developed within the presented work contributed with the new knowledge described in the following topics.

Influence of soil pH on the ecotoxicity of Basamid® in non-target soil organisms and on their capacity for recolonization.

Soil organisms are directly affected by Basamid® application, since this fumigant is commonly applied to the soil prior sowing/planting, to eradicate edaphic pathogens/target species (e.g., nematode, fungi). However, this direct mode of application will promote the exposure of non-target organisms as well.

In this work, four non-target soil species were studied regarding the influence of soil pH in the ecotoxicity of Basamid® on each species' reproduction (Chapter II). Also, the avoidance behaviour of earthworms to soil spiked with Basamid® was evaluated along with the respective ability of recolonizing a contaminated soil, across time through aged-contaminated soils (Chapter III). Results showed that the exposure to Basamid® was critically lethal to all tested soil organisms and significantly affected reproduction at concentrations (LOEC of 0.2 mg a.i./Kg soil) considerably lower than the recommended dose (145.7 mg a.i./Kg soil). From the four tested species: *F. candida, E. crypticus, H. aculeifer* and *E. andrei*, the collembolan and enchytraeid presented the highest sensitivity to the fumigant, in opposition of the acari and earthworm, which showed the lowest sensitivity to Basamid® regardless of the soil pH.

A positive association was observed regarding the influence of soil pH in the ecotoxicity of Basamid® for all studied species, except for the earthworms, for which no significant influence of soil pH was observed. These results could be explained by the expected acceleration in the breakdown reaction of Basamid® at higher soil pH values, which could potentiate the toxic effects of dazomet, MITC and other secondary metabolites resulting from the degradation of Basamid® (Nicholls 1988; FAO 2001; EFSA 2010; Fang et al. 2018). On the other hand, the absence of any influence of soil pH on the toxicity to Basamid® to earthworms could be related to a high activity of earthworms in the soil (Jänsch et al. 2005) and larger body size (compared to the other soil organisms). The large body size of earthworms concurrently to their high activity, contributes to increase the mixture of the soil by forming channels and, thus, allowing it to "breath" (Jänsch et al. 2005). Hence, this higher activity caused by earthworms mobility can lead to a higher diffusion of

Basamid® and in consequence, acceleration of the fumigant degradation (Prider and Williams 2014; Huang et al. 2019a). This could have contributed to supress the effect of pH, by the increased diffusion of the compound resulting from the earthworms' activity in the soil. On the other hand, organic matter (horse manure) which is added to earthworms as food, can also be a contributing factor for the disperse effect of soil pH on Basamid® toxicity to the earthworms. As reported by Fang and colleagues (2018), a higher organic matter, namely chicken manure, would increase dazomet half-life in soil, which means, higher time frame to dissipate from the soil. Hence, the higher organic content in earthworms' tests could be resulting as a buffering agent or a trade-off mechanism to the fumigant reaction balancing with the effect of soil pH. Observed by the same authors, the alkalinity of the soil increases dazomet hydrolysis, therefore, the conjugation of pH and organic matter can be annulling the effect of one parameter alone, leading to similar effects of dazomet in all tested soil pH.

Lastly, the cocoon structure could also be an additional protective barrier to the exterior since until juveniles hatching, most of the Basamid® has probably been degraded from the soil (the maximum DT_{50} of dazomet is around 7 d, depending on soil properties, EFSA 2010).

Nevertheless, since the survival and reproduction of these organisms were severely affected at concentrations of Basamid® lower than the recommended dose and at all soil pHs, effects on the populations and community diversity of this group of organisms are also expected to occur. Given that earthworms play an important role in the soil structure and quality, it is of most importance to investigate their ability to recolonize soils previously contaminated with Basamid®. Thus, it was studied the capacity of earthworms to recolonize soils (with the three pH values) contaminated with a similar range of Basamid® concentrations (only sublethal concentrations) over time (Chapter III). For this, the methodology used to run avoidance assays with *E. andrei* was applied. Results from this test revealed the possibility of soil recolonization by the earthworms, based on a "no avoidance" response across time. Therefore, after multiple independent avoidances tests across time, the earthworms were starting to not avoid the contaminated side which leads to the principal of ecosystem recovery (Brock et al., 2006; Clements and Rohr, 2009). The no avoidance behaviour, i.e., the moment that conditions would be favourable for a recolonization to occur, started sooner at higher alkalinity of the soil (from day 14 of the experience, at the recommended dose), while at the lowest tested soil pH (5.5) a longer time was needed (56 days) to observe a no avoidance response. These observations corroborate once more the pH influence on dazomet fumigant reaction, in which faster degradation of the fumigant from the soil occurs at higher soil pH values. These findings bring major insights concerning the recovery of communities in previous impacted environments and the capacity to recolonize, even at some level of pollution (Brock et al., 2006; Clements and Rohr, 2009; Renaud et al., 2022). The avoidance test showed potential to provide valuable information regarding the time needed for re-colonization/recovery of impacted areas and an important complement for the standard assays of lower tiers of the environmental risk assessment.

Influence of soil pH on the ecotoxicity Basamid® eluates to non-target freshwater organisms.

As already discussed, the effects of Basamid® in edaphic communities were variable with environmental factors such as soil pH and species specific sensitivity, nevertheless, it is consistent the sever toxicity in soil non-target organisms at concentrations much lower than the recommended dose (EFSA 2010; BVBA/SPRL 2014). Regarding the effects of this fumigant in freshwater organisms, few data is available, although, evidence has been reported to the effective leachate and run-off contamination of this compound in freshwater systems and groundwater (USEPA 2008; EFSA 2010). Therefore, studies were conducted to investigate the effects of Basamid® soil eluates, at the same three soil pH values as described previously, to three groups of aquatic species of different trophic levels: primary producers, primary consumers, and secondary consumers (Chapter IV, V and VI). Overall, the results were consistent in what concerns the sever effects of Basamid® to the aquatic system organisms, regardless of the soil pH. The exposure occurred through soil eluates of Basamid® in which the recommended dose (RD) was assessed as the 100% eluate from a soil contaminated with the RD. For the majority of all tested aquatic species, soil pH presented to be a significant factor regarding the toxicity of Basamid® on each organism, independently of each specific sensitivity. Also, soil pH influenced significantly, the concentration of Basamid® in soil, with lower concentrations at higher soil pH. Thus, soil pH effect along with different species-specific culture medium can influence Basamid®'s chemical reaction in soil such as adsorption, occlusion in the soil and the velocity of the fumigation reaction. Regarding the influence of soil pH in Basamid® toxicity it can be reported that from in the primary producers tested, *R. subcapitata* was more sensitive to the eluates of the lower soil pHs, whilst *L. minor* was more sensitive to the higher soil pH of the eluates. From the primary consumers both species were more sensitive towards the lower soil pH tested regarding lethal exposure, although, sublethal effects based on reproduction tests revealed that *D. magna* was more sensitive to the lower soil pHs eluates while *B. calyciflorus* to the highest soil pHs exposure. Lastly, for the secondary consumers, *H. viridissima* was more sensitive to lower pH soils while both vertebrates *X. laevis* and *D. rerio* were not affected by the influence of soil pH on Basamid® eluates toxicity.

As previously mentioned, dazomet undergoes to a faster gas release reaction with increased humidity (EFSA 2010; Fang et al. 2018). This chemical reaction process would explain the lower expected effects on aquatic organisms due to the consequent faster dissipation of the fumigant when in contact with humidity. However, this result did not occur, and the sensitivity of the aquatic organisms were critically higher even at such lower levels of the fumigant. On a real scenario of drainage or run-off, the aquatic organisms could not respond with such severer effects since the reaching concentration from soil to water could be partially retained, however, the sensitivity of the aquatic organisms was clear even at dilutions greater than 0.47% of Basamid®.

Altogether, the effect of soil pH in Basamid®'s reaction and the leachates and runoffs occurrences pose major risks to the aquatic systems affecting severely organisms from all different levels of the trophic web.

The effect of soil pH on Basamid® - a simulation to integrate a risk assessment to aquatic and terrestrial organisms

From the data generated in this study, it was possible to infer that: Basamid® fumigant constitutes a highly toxic PPP to non-target organisms from terrestrial and freshwater ecosystems; and soil pH represents a significant factor influencing Basamid®' ecotoxicity, also for both terrestrial and aquatic ecosystems. At the recommended dose and lower, all of the non-target organisms tested were severely affected by Basamid® exposure. Higher values of soil pH led to a higher Basamid® toxicity for terrestrial organisms but a clear association between soil pH and the toxicity of Basamid® eluates was not found in aquatic organisms. Regarding the higher toxicity in more alkaline soil, the main reason could be related to the faster degradation of the parent compound, generating secondary metabolites that are more toxic. On the contrary, the aquatic species had a variable response regarding soil pH x Basamid® what can be related to the process of the eluates obtention (12 h of agitation in soil with $CaCO_3$ addition) and pH specificity. Furthermore, each species-specific culture medium could have some additional influence regarding the availability of Basamid® and its chemical fate.

In this study lethal and sublethal effects were assessed and consequently different endpoints were analysed. Considering the sublethal effects it was evaluated multiple endpoints as reproduction, avoidance behaviour, growth, embryogenic development, malformations, body size and weight, which all revealed to be suitable for the toxicity assessment, even regarding the lower DT_{50} of the fumigant. Therefore, due to the great volume of data available from the study, an exercise of risk calculation for terrestrial and aquatic species was here simulated for the fumigant. Only standard organisms were considered for both approaches due to organisms' high sensitivity, representativeness of different groups and respective standard guidelines.

Furthermore, for the following risk assessment exercise, only dazomet active substance was considered. From the chemical analysis, no quantification of MITC was detected in the eluate samples, however, since dazomet has a higher volatilisation rate in humid conditions, the observed toxicity could also be related to its metabolites, and further investigations should be performed to clarify their role on the observed toxicity. According to EFSA (2010) report conclusion on dazomet, the risk assessment for the aquatic organisms regards to the metabolite MITC instead of the parent substance. With humidity, dazomet dissipates faster whereas MITC tend to be present for longer time, also from EFSA (2010) conclusion, the metabolite has been classified as more toxic than the parent dazomet. On this note, and for the exercise here done of a simplistic risk assessment, the MITC will be taken into account instead of dazomet. Furthermore, and on behalf of the documentation from dazomet report conclusion (EFSA 2010) some adaptations must be attained. Since the endpoints obtained in the presented studies were reported to dazomet active substance, and the EFSA report values for the environmental risk assessment addresses the MITC metabolite, an additional assessment factor of 10 will be applied to the endpoints obtained to proceed with the environmental assessment simulation. Moreover, for the aquatic organisms, the predicted environmental concentration in surface water (PECsw), also reported in EFSA (2010) will be used (only for the purpose of this exercise, the PECsw values used for the risk calculations were not

estimated specifically for this case) to conduct the risk assessment based on the ratios of $PECsw/ED_{50}$ or (NOED). The computed ED_{50} or NOEDs obtained in the present work were used instead of the regulatory acceptable concentration (RAC) used for the aquatic risk assessment (according to the "*Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters, EFSA Journal 2013;11(7):3290*"). Furthermore, only the chronic exposure of each organism was considered to conduct the risk assessment to have the outcome of the longer exposure to dazomet, moreover, only the ED_{50} were considered instead of ED_{10} since it was the only common endpoint of all selected species, (i.e., for some species the ED_{10} was not possible to compute), allowing to conduct this risk assessment exercise. Also, only the Step 4 from FOCUS[1] calculations models were considered, and the scenarios reported in EFSA (2010) conclusion report were used. In resemblance to what occurs in regulatory environmental risk assessment, when $PECsw/ED_{50} > 1$, an unacceptable risk should be reported while $PECsw/ED_{50} < 1$ would translate an acceptable risk. Additionally, to best conduct this risk simulation, the percentage values of the endpoints were converted to concentration (µg/L) considering the concentrations obtained from the chemical analysis in the 100% Ba-Eluates.

[1] "*The FOCUS concentration estimation methodology was developed as a tiered approach with four levels of assessment. The Step 1 has been defined as a relatively simple calculation based on a maximal loading and a fixed scenario, while the Step 2 allowed multiple applications and regional variation across Europe. Step 3 of the approach consists of the scenarios developed, while Step 4 allows a detailed site-specific approach in case all Steps fail. The FOCUS surface water scenarios are a set of ten standard combinations of weather, soil and cropping data and water bodies, which collectively represent agriculture in the EU for the purposes of a Step 3 EU-level assessment of concentration estimation." (FOCUS DG SANTE, available at: https://esdac.jrc.ec.europa.eu/projects/surface-water).*

Table 1 – Simulation for aquatic organism's risk assessment in conditions of soil pH 5.5, exposed to Basamid® eluates, the Predicted Environmental Concentration in surface water (PECsw) were taken from EFSA (2010, FOCUS step 4). Green cells represent acceptable risk and orange cells represent unacceptable risk.

Simulation		***Danio rerio***	***Daphnia magna***	***Lemna minor***	***R. subcapitata***
	Endpoint[1]	ED_{50} 0.0008247	NOED 0.0000347	ED_{50} 0.0307171	ED_{50} 0.0118302
	AF*	10	10	10	10
	Endpoint*	0.00008247	0.00000347	0.00307171	0.00118302
Exposure Scenario	PEC** sw (µg/L)	**Step 4- pH 5.5**			
D3/ditch	0.033	>1	>1	>1	>1
D4/pond	0.047	>1	>1	>1	>1
D4/stream	0.701	>1	>1	>1	>1
R1/pond	0.027	>1	>1	>1	>1
R1/stream	0.431	>1	>1	>1	>1
D6/ditch	0.001	>1	>1	<1	<1
R2/stream	0.023	>1	>1	>1	>1
R3/stream	0.751	>1	>1	>1	>1
R4/stream	1.504	>1	>1	>1	>1

*Assessment factor according to the type of endpoint (based on EFSA Journal 2013;11(7):3290"), ** Step 4 PECsw of MITC after band application of dazomet at an apparent dose rate of 2/3 x 500 Kg a.s./ha=333 Kg a.s./ha prior to planting of lettuce-exposition by drainage/run-off. [1]The assessment factor of 10 was applied due to the lack of data on the endpoints for the metabolite.

Table 2 – Simulation for aquatic organism's risk assessment in conditions of soil pH 6.5, exposed to Basamid® eluates, the Predicted Environmental Concentration in surface water (PECsw) were taken from EFSA (2010, FOCUS step 4). Green cells represent acceptable risk and orange cells represent unacceptable risk.

Simulation		***Danio rerio***	***Daphnia magna***	***Lemna minor***	***R. subcapitata***
	Endpoint[1]	ED_{50} 0.000156	NOED 0.000112	ED50 0.004753	ED_{50} 0.014764
	AF*	10	10	10	10
	Endpoint*	0.0000156	0.0000112	0.0004753	0.0014764
Exposure Scenario	PEC sw (µg/L)	**Step 4- pH 6.5**			
D3/ditch	0.033	>1	>1	>1	>1
D4/pond	0.047	>1	>1	>1	>1
D4/stream	0.701	>1	>1	>1	>1
R1/pond	0.027	>1	>1	>1	>1
R1/stream	0.431	>1	>1	>1	>1
D6/ditch	0.001	>1	>1	>1	<1
R2/stream	0.023	>1	>1	>1	>1
R3/stream	0.751	>1	>1	>1	>1
R4/stream	1.504	>1	>1	>1	>1

*Assessment factor according to the type of endpoint (based on EFSA Journal 2013;11(7):3290"), ** Step 4 PECsw of MITC after band application of dazomet at an apparent dose rate of 2/3 x 500 Kg a.s./ha=333 Kg a.s./ha prior to planting of lettuce-exposition by drainage/run-off. [1]The assessment factor of 10 was applied due to the lack of data on the endpoints for the metabolite.

Table 3 – Simulation for aquatic organism's risk assessment in conditions of soil pH 7.5, exposed to Basamid® eluates, the Predicted Environmental Concentration in surface water (PECsw) were taken from EFSA (2010, FOCUS step 4). Green cells represent acceptable risk and orange cells represent unacceptable risk.

Simulation		***Danio rerio***	***Daphnia magna***	***Lemna minor***	***R. subcapitata***
	Endpoint[1]	ED_{50} 0.000868	NOED 0.0000151	ED_{50} 0.001787	ED_{50} 0.010557
	AF*	10	10	10	10
	Endpoint*	0.0000868	0.00000151	0.0001787	0.0010557
Exposure Scenario	PEC sw (µg/L)	**Step 4- pH 7.5**			
D3/ditch	0.033	>1	>1	>1	>1
D4/pond	0.047	>1	>1	>1	>1
D4/stream	0.701	>1	>1	>1	>1
R1/pond	0.027	>1	>1	>1	>1
R1/stream	0.431	>1	>1	>1	>1
D6/ditch	0.001	>1	>1	>1	<1
R2/stream	0.023	>1	>1	>1	>1
R3/stream	0.751	>1	>1	>1	>1
R4/stream	1.504	>1	>1	>1	>1

*Assessment factor according to the type of endpoint (based on EFSA Journal 2013;11(7):3290"), ** Step 4 PECsw of MITC after band application of dazomet at an apparent dose rate of 2/3 x 500 Kg a.s./ha=333 Kg a.s./ha prior to planting of lettuce-exposition by drainage/run-off. [1]The assessment factor of 10 was applied due to the lack of data on the endpoints for the metabolite.

Based on the presented simulation for risk calculation to dazomet (a.s. of Basamid® formulation), a clear unacceptable risk is derived. The concentrations causing significant effects of dazomet in the organisms are much lower than the respective PECsw (hypothetical values for the simulation exercise) except for one scenario (D6/ditch, corresponding to drainage, Table 1,2 and 3). Despite the theoretical purposes, these results were expected and probably would be close to a real situation based on the higher level of toxicity observed in these organisms. Regarding soil pH, differences were observed, the primary producer's group would be in less risk at soil pH of 5.5 whereas only the microalgae would tolerate such exposure to the fumigant for the different soil pH. Nevertheless, the disruption of the trophic web would be occurring since for *D. rerio* and *D. magna* acceptable risk was not observed and, because of that, restriction or mitigation measures on the limitation of dazomet application should be accounted as for example: non-buffer spray zone distance, reduction of nozzles or even investigate higher tier to address a more specific risk assessment.

Regarding the soil organisms, namely earthworms in the "Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC, SANCO/10329/2002 rev 2 final" is cited that "*The test is not required when both the DT90 is less than 100 days, and the number of applications is less than 3."* Therefore, just for the purpose of this simulation, the risk assessment will only contemplate both Collembola and predatory mite. Furthermore, it should be mentioned once more that the following calculations and conclusions are solely a simulation and should not be considered as a stated risk assessment for dazomet on soil organisms. The risk was assessed by

the calculation of the Toxicity/exposure ratio (TER) of *F. candida* and *H. aculeifer* to which the ratio NOEC/ PEC soil is calculated and the trigger for the risk assessment is 5 (TER > 5, acceptable risk). The selected PEC soil from EFSA 2010 were chosen for the time of the exposure test of both organisms (14 and 28 days).

Table 4 – Simulation for soil organism's risk assessment using the respective endpoints in conditions of soil pH of 5.5, 6.5 and 7.5 exposed to Basamid® concentration, dazomet PEC values were taken from EFSA 2010. Green cells represent acceptable risk and orange cells represent unacceptable risk.

Soil pH 5.5		*Folsomia candida*	*Hypoaspis aculeifer*
Time (d)	PEC soil mg/kg	NOEC 0.2	NOEC 1.8
0	166.6	<5	<5
14	0.0134	-	>5
28	0.0001	>5	-
Soil pH 6.5		*Folsomia candida*	*Hypoaspis aculeifer*
Time (d)	PEC soil mg/kg	NOEC 0.6	NOEC 16.3
0	166.6	<5	<5
14	0.0134	-	>5
28	0.0001	>5	-
Soil pH 7.5		*Folsomia candida*	*Hypoaspis aculeifer*
Time (d)	PEC soil mg/kg	NOEC 0.2	NOEC 1.8
0	166.6	<5	<5
14	0.0134	-	>5
28	0.0001	>5	-

Based on the resulting TERs for the soil non-target organisms, it can be clear the acute effect to the organisms, and the consequences of the acute effects could be resulting in disruption in the biodiversity of soils and in this case, mitigation or restriction measures must be applied. However, it can be also observed that, through time, the PEC in soil would decrease and based on the NOECs of *F. candida* and *H. aculeifer*, an acceptable risk would be possible over time, nevertheless, more studies would have to be investigated relating to the effects in site in the environment. For this study-case, the risk evaluation, i.e., acceptance on Basamid® exposure, was not pH dependent.

Altogether, combined with the measures applied to the aquatic organisms, the most inclusive measures should prevail in order to an acceptable risk to the environment that can cover all species passible of exposure to dazomet.

Regardless, even highlighting the fact that the used PECs were only for a simulation approach, it is possible to observe that soil pH is an influencing factor and different conclusions regarding the acceptance of the environmental risk can be derive. In this case, the management of the soil to a pH of 6.5 would be, possibly, a good measure regarding the overall effects on both aquatic and terrestrial organisms. Therefore, the soil liming practice applied in agriculture, or the management

of soil pH, could be an important tool regarding the application of Basamid® and the mediation of effects in non-target organisms and environment.

The mitigations measures should cover for all groups of organisms, based on the most sensitive species (i.e., the worst-case scenario). To be protective, the most conservative and precautionary measure that covers the most sensitive species should be applied, in order to limit the environmental risks. In this case, both groups (aquatic and terrestrial) presented no acceptable risk for dazomet exposure what translates an urgent need for the authority's revision and implementation of mitigation or restriction use of PPP with dazomet formulation.

Major conclusions and further perspectives

There are some opinions defending the limitation of PPP uses or even its complete banishment from application in the agrosystems (Datta et al., 2016; Rani et al., 2021; Sharma et al., 2020). However, to feed a still increasing Human population, the agriculture sector must be highly productive to supply food at sufficient quantities. To successfully reach such high productivities, pests' infections that are a major threat regarding crops productivity and yield must be controlled. Therefore, the complete elimination of PPP application, without adequate surrogates for controlling pests, may pose a higher concern when the productivity of the crop's depends on some level of PPP treatment or management (Meemken and Qaim, 2018; Muller et al., 2017). It is, however, important to refine better uses of PPP, and in this particular case, of dazomet, to reduce uncertainties in the risk calculation and targeting less harm to the environment. Better mechanisms should be investigated for PPP entering the environment with minor risks (e.g., nano release with higher target specificity, as already investigated by Ren et al., 2022) or the risks and effects management by the manipulation of external factors (e.g., humidity, temperature, soil pH) or even the combination of organic and conventional agriculture (e.g., use of sewage sludge with vermiculture, Meemken and Qaim, 2018). It should be considered each situation as a specific approach instead of treating crops in a large scale to a large spectrum of hypothetical problems to diminish as much as possible the pollution in the ecosystem. The management of abiotic factors can be an important practice in order to obtain a compromise within the need to deplete a crop infection, causing the less possible harm to the entire non-target ecosystem. In this work and aligned with the above mentioned, it was possible to observe a clear effect of soil pH in the toxicity of Basamid® to both terrestrial and aquatic organisms. Discarding the specificity of each species to their optimal pH, both terrestrial and aquatic ecosystems are interconnected, and the effects of soil pH can be dragged through multiple scenarios (e.g., drainage, runoff) enhancing or decreasing the effects on both groups of organisms. Here, soil pH influenced the fumigation rate of Basamid®, which led to lower concentrations of the active substance at higher soil pH and, in most of the studied species, on average, a higher toxicity at higher soil pH for terrestrial organisms and lower toxicity or equal to, for the aquatic organisms. In a real scenario, the Basamid® applied to the soils will be transported into adjacent areas by drift, drainage, and runoffs, ending in water courses. Furthermore, the DT_{50} of Basamid® can also be influenced by many factors and in specific by soil pH, what can conditionate the chemical behaviour, the duration of effects and the quantity of

Basamid® reaching the adjacent aquatic systems and, as ultimate consequence, jeopardizing the environment health.

Nevertheless, observed in this study, the toxicity of Basamid® for both groups of organisms were considerably high when compared to the recommended dose of application. Though the high ecotoxicity that Basamid® induced to terrestrial organisms, is foreseen that the recolonization of the soils where the fumigant was applied may occur over a short period of time, as suggested by the results obtained with the avoidance assays with the earthworms. Adults of *E. andrei* stop exhibiting avoidance behaviour of Basamid® contaminated soils after the application of the fumigant to the soil. The period of time needed for earthworms to stop avoiding the contaminated soils was shorter for soils with higher pH values. Thus, though a higher toxicity was observed at alkaline soils, it is expected that its recolonization will occur faster than in soils with lower pH values, due to the expected higher dissipation of the fumigant. These results suggest that the edaphic community of soils where this fumigant is applied may be recovered over time. Nevertheless, further studies on the capacity of recolonization must be done with other edaphic soils and species, and further knowledge on the long-term effects that Basamid® may pose on the reproduction of these organisms must be performed.

To sum up, it was evident the influence of soil pH on Basamid® toxicity for the majority of the tested organisms, in general, as more toxic to soil organisms at higher soil pH and less toxic to aquatic organisms at the median tested soil pH. Furthermore, a no avoidance behaviour dependent on soil pH influence and across time, allowed to investigate the potential of recolonization of earthworms exposed to contaminated soils. Altogether, the influence of soil pH based on chemical analysis, organisms' toxicity and avoidance behaviour evidenced the lower concentration of Basamid® at higher soil pH what addresses that management of the soil abiotic factors should be accounted in environmental risk assessment.

Envisioning further perspectives, there is still some major gaps to be fulfil and to bring more insights regarding Basamid® toxicity namely: investigation of the effects of Basamid® toxicity on a wider range of soil pH with more relevance to major economic interest crops; a cross-design with Basamid® and other environmental factors such as water content and temperature, factors that are known to influence Basamid® fumigant reaction and are closely linked to climate and global changes; to investigate the capacity for recolonization to previous contaminated sites to other species (including aquatic organisms) envisioning the restoring of the ecosystem; to investigate the effects in field-scenarios to cope with more realistic exposures routes and respective effects on biodiversity. Regarding specifically the aquatic system, there is still the need to investigate the effects of real scenarios of leachates and run-off, the quantification of the effective real concentrations that can enter in water bodies and respective consequences to the ecosystem as trophic level disruptions.

Regarding Basamid® fumigant risk assessment, restrictions and mitigation measures must be strengthened considering the pollution of groundwater and nearby freshwater systems. Thus, in the light of this study and since the new revision of Basamid® will be available only from 2023, some remarks and/or recommendations can be provided until new stated strategies:

I) Maximum security equipment for both operators and soil of the treated crop field. Regarding the application of Basamid®, ensure ventilation, safety buffer zones distance from water systems and the use of non-porous cover tarp to limit gas release through the atmosphere.

II) Consider the environmental factors of the soil before the application of Basamid® as: humidity, organic matter content, temperature, and soil pH. With adequate management of the soil, is possible to reduce the amount of the fumigant to exert the same result. Therefore, in the light of this study, it would be recommended to manage the soil pH regarding the surrounding environment:

- Where the possibility of Basamid® to reach water bodies is present: a manipulation of the soil pH to pH 6.5 would be advisable since it would be less toxic to soil organisms compared to the highest tested soil and less toxic, in general, to the aquatic system.
- Where the possibility of Basamid® to reach water bodies is more distant: a manipulation of soil pH to a more alkaline conditions would be a good practice based on the potential of earthworms to recolonize the soils in a shorter period comparing to the lower soil pHs.

III) In case of the impossibility of recurring to more environmentally friendly practices as organic agriculture, empathizing crops rotation, or less toxic PPP application; limit the number of Basamid® applications (for example once a year) or limit the application doses in field (for example reducing 25% of the current recommendation dose) or ally a conjugation of a lower concentration of Basamid® application (less 40% of the recommended dose for example) with the manipulation of soil pH towards a more alkaline pH, to gather the bests conditions for the maximum effect of the fumigant towards less collateral damages.

IV) Monitorization of the evolution of the crop since the moment of application until finishing the treatment. It should be made an additional effort regarding the monitorization of the quality of the ecosystem in the light of the precautionary sense respecting the ecosystem health.

V) Adapt and combine different agriculture practices. It is important to maintain the productivity and yield of the crop, and PPP should be applied when necessary and not as a commonplace practice. Furthermore, practices as rotation, use of microorganism's activity, vermiculture, should be considered in order to decrease sever effects to non-target organism and the ecosystem structure.

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