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DISSERTAÇÃO DE MESTRADO

TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Assessmet of toxicity and behavior of Cu formulations in vineyard soils

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Cristiana Patrícia Gonçalves Paiva, Assessment of toxicity and behavior of Cu formulations in vineyard soils

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SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

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Assessment of toxicity and behavior of Cu formulations in vineyard soils

Dissertation for the degree of Master in
Environmental Contamination and Toxicology, presented to
Institute of Biomedical Sciences Abel Salazar
of the University of Porto

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I would like to acknowledge the financial support from:

- Project AgriFood XXI - NORTE-01-0145-FEDER-000041, financed by European Regional Development Fund (FEDER) through North Portugal Regional Operational Programme (Norte 2020).
- GreenUPorto though the summer school scholarship “Verão com Ciência – Hands on Science for Sustainable AgriFood production: From the Soil to the Fork”, promoted by FCT, in collaboration with Direção Geral do Ensino.

Agradecimentos

Gostaria de começar por agradecer à minha orientadora Dr. Anabela Cachada, não só por todo o apoio e constante acompanhamento, mas acima de tudo pelo exemplo. Mostrou-me o melhor que podemos ser no mundo da ciência, exigentes, cuidados, atentos ao pormenor, críticos e resilientes na adversidade. A Anabela sempre conseguiu ser tudo isto, sem nunca descurar a sensibilidade, a preocupação e a simpatia para comigo. Devo-lhe todo o trabalho que aqui apresento, e ainda parte da minha visão e atitude enquanto alguém que quer trabalhar no mundo da investigação científica.

Agradecer também à professora Ruth Pereira, que no meio de todos os seus inúmeros projetos, conseguiu tempo para me acolher, ouvir e incluir em projetos, dando-me a oportunidade de fazer o que me faz feliz. Agradeço todas as suas ideias e entusiamo de alguém que, apesar de extremamente experiente, continua a estimar todo o trabalho que realizamos.

Agradecer ainda aos que disponibilizaram o seu tempo, conhecimento, meios e experiência na realização de partes fundamentais deste trabalho, particularmente à Dr. Carla Patinha e ao professor Carlos Pereia. Um agradecimento também muito especial à Dr. Tatiana Andreani, por também ter disposto do seu tempo e conhecimento em trabalhar comigo de perto para a realização de uma componente essencial deste trabalho, e ainda pela sua amizade.

A todos os que constituem o LabRisk, por fazerem de todo este processo uma experiência inesquecível: à Inês, Saúl, Sirine, Márcia, Miguel e Andrés, e especialmente à Beatriz Fernandes, pela ajuda e companheirismo. A todos eles devo as melhores memórias destes últimos meses.

A título pessoal, não posso deixar de agradecer à minha família, por sempre ter estado ao meu lado, mesmo nos momentos em que me tornava a pior companhia quando me deixava levar pela frustração e pelo cansaço. Mas sei que também será com eles que irei partilhar toda a alegria por ver este trabalho concluído. E por fim, deixo o meu maior obrigada ao Gonçalo, pelo carinho e apoio, pelo orgulho e pela paciência, mas o mais importante, pelo exemplo. Por ser a verdadeira definição de persistência, integridade e dedicação. Agradeço-lhe por todos os dias em que não me senti capaz de concluir este trabalho por mim, pois nesses momentos sei que sempre o fiz por ele.

Abstract

Agricultural production will need to take a huge leap in order to fulfill future world needs, and all at the cost of soil degradation, loss of biodiversity and aquatic pollution if the current agricultural practices remain the same. In this sense, organic agriculture has been promoted as being less harmful towards the environment than conventional farming, since there is the perception that applied approaches are more sustainable. However, in organic farming, the use of plant protection products or fertilizers isn't prohibited, and are used when required, as long as they're natural or naturally derived products. Specifically, in the context of organic viticulture, products like fungicides are essential, and, since synthetic organic fungicides are prohibited in European organic agriculture, this sector is highly dependent of Cu-based inorganic fungicides.

Cu-based fungicides have been used for more than a century, with a pronounced application in vineyards, as protective products against fungal diseases, like downy mildew (*Plasmopara viticola)*. The extensive and intensive use of these products has led to environmental values of Cu found in soils that can be critical towards non-target and beneficial organisms, with vineyards soils in the European Union showing the highest mean concentration of Cu among any other crops. Due to this, and to recent limitations imposed to Cu-fungicides use, new formulations are making their way into the market, appealing to higher efficiency and environmental benefits. Some new commercial products have already been approved in organic agriculture, which are presented as formulations with reduced particle size, that in turn are told to result in higher coating areas, higher resistance to wash-off by precipitation and higher bioavailability of Cu ions.

Taking all this into account, the present work aimed to understand if traditional formulations of Cu can be used in a safe and sustainable way in organic agriculture and farming in general, allowing to evaluate if there is a true necessity for replacement of previous Cu-based products by new formulations. To enlighten these questions, a field study was carried out in vineyards from the Douro region (Portugal), in order to assess the extent environmental impacts of the use of Cu-based fungicides in an established organic vineyard. Also, when so little is known about the performance, environmental costs and the fate of new Cu-formulations in the environment, a comprehensive assessment of the composition and effectiveness of new technological advanced formulations of Cu was performed.

The integration of data obtained from experimental work accomplished in this work allowed to understand that high contents of Cu in vineyard soils won't always compromise organisms' viability. Furthermore, if existent, impacts of Cu towards organisms can, in some cases, be surpassed and biological communities can transcend these effects, showing the

ability of the ecosystem to recover. Results also allowed for the realization that new and poorly studied formulations are being introduced and used in agriculture, at the cost of unknown environmental risks. Also, there's a probability that predicted efficiency of new Cu formulations might not be revealed in practice, meaning that higher doses of these products can likely be needed to equate traditional formulations performance.

Keywords: viticulture, organic management, copper, fungicides, soil health

Resumo

A produção agrícola atual terá de ser aumentada de forma a dar resposta às futuras necessidades nutricionais mundiais, a custo da degradação do solo, perda de biodiversidade e da poluição aquática, se as atuais agrícolas se mantiverem. Por este motivo, a agricultura biológica tem sido promovida como sendo menos prejudicial para o a ambiente do que a agricultura convencional, uma vez que existe a perceção de que as suas abordagens são mais sustentáveis. No entanto, na agricultura biológica, a utilização de produtos fitossanitários não está interdita, e são utilizados quando necessário, desde que sejam ou derivem de produtos naturais. Mais especificamente, no contexto da viticultura biológica, a utilização de produtos como fungicidas é fundamental, e, uma vez que a utilização de fungicidas orgânicos sintéticos é proibida na agricultura biológica Europeia, este setor encontra-se altamente dependente de fungicidas inorgânicos à base de cobre.

Os fungicidas à base Cu têm vindo a ser usados há já mais de um século, com uma aplicação muito pronunciada nas vinhas, enquanto agentes protetores de doenças fúngicas, como o míldio (*Plasmopara viticola*). O uso extensivo e intensivo destes produtos tem levado a concentrações ambientais de Cu em solos que podem atingir níveis críticos para organismos benéficos e não alvo, sendo que os solos vinícolas na União Europeia são os que demonstram a maior concentração média de Cu de entre todas as outras utilizações do solo. Neste sentido, e devido às recentes limitações impostas à utilização de fungicidas à base de Cu, novas formulações podem ser agora encontradas no mercado, apelando a maior eficiência e mais benefícios ambientais. Alguns destes produtos já se encontram homologados para a agricultura biológica, sendo que se tratam de formulações com um tamanho reduzido de partículas, que resultam em maiores áreas de cobertura, com maior resistência à lixiviação pela ação da chuva, e à maior biodisponibilidade de iões Cu^{2+}.

Tendo tudo isto em consideração, o presente trabalho pretendeu compreender se as formulações tradicionais de Cu podem ser utilizadas de forma segura e sustentável de forma geral em agricultura e na agricultura biológica, permitindo avaliar se de facto existe uma necessidade evidente da substituição dos produtos de Cu até então disponíveis pelas novas formulações. De forma a esclarecer estas questões, um estudo de campo foi conduzido nas vinhas da região do Douro (Portugal), a fim de avaliar os impactes atuais da utilização de fungicidas à base de Cu numa quinta em modo de produção biológica já estabelecida. Além disso, quando ainda tão pouco é conhecido sobre o desempenho, custos ambientais e o destino destas novas formulações de Cu, foi realizado um estudo da composição e eficácia destas novas formulações de tecnologia avançada.

A integração dos resultados obtidos neste trabalho experimental permitiu compreender que, elevados conteúdos de Cu presentes em solos vinícolas, nem sempre se irão refletir na diminuição da viabilidade dos organismos. Da mesma forma, quando existentes, os impactes do Cu na biodiversidade podem ser ultrapassados e as comunidades biológicas poderão conseguir transcender estes efeitos, demonstrando a capacidade de recuperação do sistema, em certas condições. Ademais, os restantes resultados também permitiram a perceção de que novas formulações e produtos estão a ser inseridos e utilizados na agricultura com informações insuficientes, com o possível custo de riscos ambientais desconhecidos. Aliás, poderá mesmo existir a probabilidade de que a eficiência esperada destas novas formulações não se revele na prática, o que poderá significar que doses superiores destes produtos terão que ser utilizadas para igualar os efeitos das formulações tradicionais.

Palavras-Chave: viticultura, agricultura biológica, cobre, fungicidas, qualidade do solo

Index

Figure index

Table index

Abbreviations

$\%I_r$ – Percentage Inhibition of Growth Rate

AAS - Atomic Absorption Spectrometry

BM – Bordeaux Mixture

CH – Champion

DGR_{ab} – Daily Growth Rate

Dh - Hydrodynamic Diameter

DLS - Dynamic Light Scattering

EC - Electric Conductivity

EU - European Union

ICP-MS - Inductively Coupled Plasma-Mass Spectrometry

KD - Kados

KO – Kocide OPTI

ME – Major Elements

MEA – Malt Extract agar

NPs – Nanoparticles

NTAF-Cu - New Technological Advanced Formulations of Cu

OM – Organic matter

PI – Polydispersity Index

PPPs – Plant Protection Products

PTEs - Potentially Toxic Elements

RD – Recommended Dose

ROS - Reactive Oxygen Species

SEM - Scanning Electron Microscopy

SOM – Soil organic matter

TE – Trace Elements

XRF - Wavelength-Dispersive X-Ray Fluorescence

Z-Ave - Mean Particle Size

Chapter I. General introduction

1.1. General Introduction

The soil not only provides tangible and evident services for humans, as a source of biomass (which include agriculture and forestry) and raw materials, but also supports several other ecosystem services through its functions, from nutrient cycling and water quality regulation to life support and accommodation, and even climate regulation (FAO, 2015). As a whole, soil provides most of the food for both humans and animals, but it's also a source of pathogens and new novel compounds (Pepper, 2013). Nonetheless, more than ever, humans have the capacity to significantly transform soils, and consequently the ecosystems that it supports, by changing its composition, structure, vegetation cover, topography and, ultimately, by changing the climate (Pepper, 2013). For instance, with coming climate change, extreme events like droughts and inundations are expected in the future European climate, leading to soil salinization, its compaction, erosion and ultimately land degradation. As a consequence, crops will become more vulnerable and the need for already scarce resources will rise (Falloon & Betts, 2010). Also, with accelerated soil erosion by water, wind and agricultural tillage, onsite effects are expected, as soil productivity may be reduced due to nutrient losses and lower water holding capacities. On the other hand, offsite effects are foreseen, since particles detached from soil can carry nutrients and contaminants to new locations and compartments (FAO, 2015).

Agriculture highly depends on the soil compartment, and currently feeds over seven billion people, while it's also one of the main causes of environmental degradation (Clark & Tilman, 2017). In the next decades, the world population growth will be accompanied by the need for more food, which will result in a 6% higher occupation of soil by agricultural areas (Muller *et al.*, 2017), being predicted that agricultural yields must be raised by 60 to 100% by 2050 to suppress world needs, at the cost of soil degradation, loss of biodiversity and aquatic pollution if the current agricultural practices remain the same (Meemken & Qaim, 2018). As a result of intensive agriculture, animals and organisms that inhabit soils are less diverse, with fewer and taxonomically closer species, which ultimately leads to less complex food webs with less trophic levels (Tsiafouli *et al.*, 2015). Biodiversity will be impacted differently by intensive farming, being that increasing impacts will be verified from the more mobile to the more sessile organisms (German *et al.*, 2017), meaning that less mobile species are naturally more vulnerable or not capable of a more extensive use of resources,

As the demand for food rises, it is essential to keep the productivity of agricultural systems, or otherwise more natural habitats will need to be converted for agricultural purposes, with the consequent loss of areas reasonably free of human influence (German *et al.*, 2017). This way, the high productivity of a farming system is a relevant environmental factor (Seufert & Ramankutty, 2017) that cannot be neglected when rethinking the current

strategies used in agricultural production. The efficiency and revenue of agricultural systems tends to be positively correlated to ecosystem services indicators, like soil quality and the presence of species that contribute for ecosystem services, like pollinators and worms, as these support the productivity of crops (German *et al.*, 2017).

On the other hand, large scale and intensive agriculture with substantial yields is highly dependent on the use of fertilizers and pesticides, that in turn can represent negative environmental impacts when excessively used, by adversely affecting benefic species, which include parasites and predators that control pests, pollinating insects and microorganisms that promote soil fertility (Walker, 2014). In this sense, organic agriculture is promoted as being less harmful towards the environment than conventional farming, because the use of natural fertilizers and the application of natural pest controls are taken as more sustainable approaches (Clark & Tilman, 2017). Indeed, the benefits of organic agriculture have been shown in terms of water quality (less pesticide contamination) and for biodiversity and overall soil health, especially for organism groups like plants and pollinators (Seufert & Ramankutty, 2017) and with greater soil microbial abundance and activity *(*Lori *et al.*, 2017) and with improvements in soil organic matter (SOM) (Tuomisto *et al.*, 2012). Also, it has been suggested that the inclusion of agricultural fields under organic production in a scenery of intensive farming might contribute to more diverse landscapes, resulting in more efficient pest control by the diversification of cultures and habitats for species of “natural enemies” (Muneret *et al.*, 2018).

However, in organic farming the use of plant protection products (PPPs), fertilizers and soil conditioners isn’t prohibited, and are used when required, as long as they’re natural or naturally-derived products (Council Regulation (EC) No 834/2007), with even exceptional inputs of chemically synthesized organic products. Besides, while the use of smaller doses of these naturally-derived compounds is promoted, natural origin doesn’t automatically reflects less toxicity towards the environment, being important the extensive understanding of the application of compounds like Cu or S in organic farming (Muneret *et al.*, 2018). Equally important is the use of fertilizers, and whilst the use of their synthetic forms isn’t allowed in organic agriculture, other natural sources are used, like manure or compost. However, inputs in the form of manure or compost don’t contribute with newly fixed N or P, from the global cycle of N and P point of view, meaning that organic farming depends on the reuse of N and P already introduced to the cycle (Seufert & Ramankutty, 2017). Thus, the loss of N and P through leaching from the soil to water systems it’s not exclusive to mineral/synthetic fertilization, and in organic systems leaching of N per unit of output is, on average, higher than in conventional systems, due to low efficiency rates of use of the N provided by these sources: there’s generally a lag between the need for N by crops and the bioavailability of N given by these natural fertilizers (Seufert & Ramankutty, 2017). The

irrational and overuse of fertilizers, regardless of its provenance, can lead to severe cases of eutrophication of water masses, since this process is highly promoted by inputs of N and P (Vitousek *et al.*, 1997). This being said, the exclusive use of organic amendments can't, on its one, improve nutrient use efficiency, and management practices need to be based on prediction and optimization of nutrient needs, supporting productivity whilst reducing nutrient losses to the atmosphere or aquatic systems (FAO, 2015).

The advocated advantages of organic agriculture might actually not be as environmentally relevant as expected, or its principles may not be used as a one-size-fits-all approach when it comes to defining guidelines for a more sustainable future of agriculture. Considering the productivity of a farming system a highly important environmental factor, since it is imperative to assess the advantages per unit of output, the benefits of organic farming might not be relevant when considering the yields (Seufert & Ramankutty, 2017). In fact, organic crops require agricultural areas 25-100% bigger, with eutrophication potentials 35% higher, per unit of output, than conventional ones (Clark & Tilman, 2017), with yields 19-25% lower for most of the crops planted (specially for cultures like cereals) and exceptions of high productivity only for certain crops (like legumes or hay) (Seufert & Ramankutty, 2017). For unit of output, it becomes clear that environmental benefits promoted by organic farming are less pronounced or even nonexistent: organic agriculture has lower yields than conventional farming, meaning that a bigger area of soil for organic conversion would be needed to produce the same amount of food from conventional systems (Muller *et al.*, 2017). In fact, if the response to the need for more food is made by a conversion to 100% organic agriculture, the world might require to convert 16-33% more soil for agriculture production, to suit the same nutritional needs (Muller *et al.*, 2017). When comparing production systems as a whole (like conventional vs. organic), instead of comparing individual choices, it becomes clear that certain measures, when applied, entail consequences for other factors: for instance, conventional regimes with high yields have a lower worm presence and activity when compared to organic ones; nevertheless, individual approaches like the use of manure or the reduction of soil tillage can improve both endpoints at the same time, without calling into question the production mode integrally (German *et al.*, 2017).

Organic farming represented 1% of all global cultivated area in 2018, and the search for organic products is on the rise, reaching also the grape and wine sector (Meemken & Qaim, 2018) . Actually, vineyards are particularly sensitive to changes in the quality of their soils, which in turn is susceptible to degradation, largely because of its typical layout (normally located in high slope areas, an important factor for the quality of grapes), that makes them especially prone to erosion and leaching of important components for soil fertility (Navel & Martins, 2014). In the European Union (EU), viticulture represents a sector

of high economic relevance, especially in the Mediterranean region, with around 45% of the world's total area under vines (Eurostat, 2017).

In viticulture, fungicides are essential, and, since synthetic organic fungicides are prohibited in European organic agriculture, this sector is highly dependent of Cu-based inorganic fungicides, which are allowed (Komárek *et al.*, 2010). Cu-based fungicides have been used since 1850, with a pronounced application in vineyards, as protective products against fungal diseases, like downy mildew (*Plasmopara viticola*) (Ruyters *et al.*, 2013). The very well-known Bordeaux Mixture [$CuSO_4$ + $Ca(OH)_2$] is one example, which has been used for more than a century, and more recently other Cu-based alternatives emerged, like, for example, Copper(II) Hydroxide [$Cu(OH)_2$] or Copper Oxychloride [$3Cu(OH)_2 \bullet CuCl_2$] (Kelepertzis *et al.*, 2018). The extensive and intensive use of these products resulted, due to leaching of treated vines and deposition of senescent leaves, in accumulation of Cu in vineyard soils, besides the Cu that may be already present in soils derived from the geological parent material (Komárek *et al.*, 2010). This phenomenon is aggravated in wetter areas, as in temperate or tropical climates, due to the higher number of treatments of vineyards with Cu, as well as their cumulative application with the growing age of vineyards. This, combined with Cu low mobility in soils, results in a tendency for its accumulation in vineyard soils (Patinha *et al*., 2018).

The toxicity of contaminants towards soil living organisms may not be directly related with their total content, with greater significance being given to their bioavailability (Navel & Martins, 2014). In addition, the potentially adverse effects of trace elements on the environment will depend on their association with mineral fractions, that will highly influence the mobility trough the soil profile up to other environmental compartments *(*Kelepertzis *et al.*, 2018). Thus, not only concentrations of Cu in soils must be taken in consideration when assessing its toxicity and impacts towards the environment, but also its (bio)availability, that will depend on soil features, particularly its pH: acidic soils will rise Cu bioavailability, as well as its capacity to migrate through the soil profile, reaching water masses more easily (Komárek *et al.*, 2010). The accumulation of Cu in soils derived from the intensive use of Cu-based fungicides can be revealed as a harmful factor, not for vine health – since these have deep roots and Cu tends to be accumulated in the topsoil – but for other organisms (Ruyters *et al.*, 2013). Environmental values of Cu commonly found in soils under inputs of Cu-based fungicides are shown to be toxic towards non-target organisms, like worms, *Vibrio fischeri*, *P. subcapitata* and *Daphnia magna* and microbial communities (Komárek *et al.*, 2010). The mean concentration of Cu in soils of the EU is 16.85 mg kg^{-1}, with vineyards soils being the land use with the highest mean concentration of Cu among other crops – 49.26 mg kg^{-1} (Ballabio *et al.*, 2018). Concentrations above 33 mg kg^{-1} are considered critical to worm communities (Komárek *et al.*, 2010), and a soil screening value ranging

between 26.3 and 31.8 mg kg^{-1} has been proposed to guarantee the protection of terrestrial elements and ecosystems functioning (Caetano *et al*., 2016). However, the state of how Cu is present in soils is determinant on how it exerts its toxicity – for instance, the adsorption of Cu on SOM it's probably the most important form of complexation of Cu in soils, and this complex represents less toxicity when compared to free Cu^{2+}. That being said, agricultural practices applied to vineyard systems will be a major factor influencing the behavior and toxicity of Cu, in the way that different practices will promote differences in soil properties, also influencing the complexation or solubilization of Cu (Navel & Martins, 2014). For instance, the addiction of manure or compost to soils will increase soil aggregation and SOM, which can lead to a protective effect for living communities since complexation processes of Cu with SOM are promoted (Navel & Martins, 2014).

In times when maximization of the yields of crops and the reduction of food waste are extremely relevant, the loss of agricultural products due to plant diseases still represent losses of about 25% (Malandrakis *et al.*, 2019). With now an alternative to conventional Cu-based fungicides being considered a necessity, new formulations, with new encapsulation technologies and with reduction of particle size (reaching in some cases the nano size), have been pointed as a more sustainable and reasonable approach. Besides, with the growing evidence of resistance events in plant pathogens, like fungi, it's extremely important the development of new fungicide products (Kim *et al.*, 2017). However, the processes of absorption, bioaccumulation and biotransformation of these new products, and especially the ones based on nanoparticles (NPs), by soil and water biotic communities aren't still clarified (Walker *et al*., 2017), despite their pointed advantages like less toxic PPPs and lower amounts of extracted minerals from the environment (Kim *et al.*, 2017).

Metallic NPs efficiency has been proven against plant pathogens, pests and parasites (Khot *et al*., 2012), being interesting the application of nanotechnology to the development of Cu-based PPPs, taking advantage of its enhanced properties at the micro and nanoscale (Malandrakis *et al.*, 2019). Due to this, newer formulations of Cu-based fungicides are making their way into the market, supported by the announced advantages when comparing to traditional formulations, both in terms of efficiency and environmental benefits. Some new commercial products have already been approved in organic agriculture, which are presented as formulations with reduced particle size, that in turn are told to result in higher coating areas, higher resistance to wash-off by precipitation and higher bioavailability of Cu ions. These properties, however, seem to be predicted by physical and chemical properties of traditional Cu forms (like Cu hydroxide) when presented as smaller particles, and not by actual evidences of their performance under physiological and environmental conditions. Besides, the properties claimed by the formulations may also represent lower toxic doses of Cu for plants and other non-target organisms when

compared to traditional forms of Cu (Ameh & Sayes, 2019). Especially, being Cu a micronutrient for plants, a higher risk for food safety is a concern, since plants tend to excessively accumulate Cu in their tissues when compared to other elements (Ballabio *et al.*, 2018). This may have consequences also to plant health as Cu shows phytotoxicity at higher doses when its foliar uptake takes place (Xiong *et al.*, 2017).

Beyond the already known consequences of Cu when introduced improperly into the environment, is its use inappropriate in all circumstances and conditions? Or can Cu-based fungicides be used in a safe and sustainable way in organic agriculture and farming in general, when in appropriate doses and respective mitigation approaches? These are especially important questions when so little is known about the true environmental costs and the fate micro and NPs in the environment. With all of this in mind, the present work aims to answer the following questions: (Q1) Can different Cu-based formulations be part of a sustainable viticulture? (Q2) Is there a real necessity for replacement of previous Cu-based products by new formulations? (Q3) Do fungicide labels and safety-sheets provide realistic information about their content? (Q4) Do new technological advanced formulations of Cu (NTAF-Cu) truly offer high efficiency with less total Cu? To enlighten all of these questions, several tasks were outlined. A field study was carried out in vineyards from the Douro region (Portugal), in order to assess the extent and environmental impacts of the use of Cu-based fungicides in an established organic vineyard (Q1 and Q2). This task was accomplished by the analysis of physical, chemical, and biochemical properties of soil samples combined with a battery of ecotoxicological assays aimed in assessing the bioavailability and the direct effects of soils receiving Cu inputs. Further understanding of commercially available fungicides was achieved by assessing composition and particle size of bulk and dissolved formulations (Q3). Besides, the toxicity and efficiency of different Cu-based fungicides to fungi species was also assessed through the execution of mycelia growth inhibition assays (Q4). Results and conclusions from this work will be presented through the following chapters, as well as further insight into the methodology used to obtain them.

Chapter II. How toxic and persistent are Cu-based fungicide formulations? A field study in the Douro vineyards

1.2. Introduction

Soil quality has been defined as "*the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health*" (Doran & Parkin, 1994), a definition that comprises the key principle that soil must function effectively at the present and in the future. In this sense, sustainable soil management must include practices that, at the long term, maintain or enhance soil biodiversity and ecosystem services without significantly impairing soil functions that enable those services (FAO, 2015). Soil functions are settled in a complex balance between faster processes, that happen at the small scale, and progressively slower and larger scale events, which ultimately make soils a non-stochastic system (Lavelle *et al.*, 2006). Thus, any disturbance to this order of events will most likely impact and modify ecosystem services provided by soils. As already discussed, humans can negatively impact the soil ecosystem in several ways, through land-use purposes, including agriculture, urbanization or deforestation, contamination, climate change, between others. However, when looking at ecosystem services provided by vineyards, these are particularly threatened: the perennial nature of vineyards implies that agricultural management procedures are intensively performed, year after year, to obtain high quality grapes with reasonable yields, through chemical or mechanical weeding, tillage, pruning and pesticide application (Salome *et al.*, 2016). Biotic communities of perennial crops are differentially affected by agricultural practices than those from annual crops, and although perennial cultures might actually provide habitat services and resources that enhance biodiversity, these are also the ones that receive higher inputs of pesticides and damaging treatments (Muneret *et al.*, 2019). Such recurrent practices can lead to soil erosion and compaction, pollution, loss of OM and ultimately of biodiversity, this being especially true for vineyards in Mediterranean regions, where viticulture is strongly implemented, and oftentimes in soils inappropriate for other crops (Salome *et al.*, 2014).

Downy mildew, as previously referred, is one of the most devastating infections that reach vineyards. This fungal disease was introduced in Europe in the late 19th century, which resulted in susceptible crops of *Vitis vinifera*, that still represent the great majority of the viticultural area in Europe and which require the greatest number of treatments from all grape-vine diseases (Boso *et al*., 2019). As soon as temperatures rise above 11ºC, primary infections of *P. viticola* take place after the occurrence of rains, that disperse the zoospores from the soil to younger leaves and grapes. After a period of incubation, *P. viticola* is established in the vine and can disperse and cause outbreaks throughout vineyards. For this reason, prophylactic treatments are usually advised and applied before the ending of the incubation period, avoiding the dissemination of infection *(*Weitbrecht *et al.*, 2021). In

organic viticulture these preventive treatments are exclusively restricted to Cu-based fungicides, which can compromise biodiversity in an already simplified landscape such as vineyards, that provide a limited habitat function to a richer diversity of organisms (Paiola *et al.*, 2020). After understanding the vulnerability of vineyard systems and its soil quality, it becomes evident the necessity to ensure the proper assessment of their soil health and the impact of agricultural management practices towards biodiversity and soil function.

Since soil functions aren't directly measurable, finding tools that allow for the quantification of soil health is a challenge, so there's a need to rely on tools that integrate information about soil quality deriving from single parameters (Marzaioli *et al.*, 2010). Thus, soil quality assessment uses soil attributes that reflect changes in response to environmental conditions and management practices, measuring physical, chemical and biological properties over time (Oliver *et al*., 2013). Overall physical and chemical parameters often used as soil quality indicators are: SOM, pH, nutrient composition, water storage and soil texture (Bünemann *et al*., 2018). These, in turn, will highly influence and support the comprehension of the result of bioindicators (Salome *et al*., 2014), which allow to evaluate parameters that make soil a living system: biological processes can be more sensitive to disturbances in soil, functioning as an integrative tool to predict environmental risk (Nogueira Cardoso & Lopes Alves, 2012). Furthermore, soil, as an ecosystem, has unique constraints that differ from any other compartment: natural compaction of soil is usually a limiting factor for the movement of organisms, aeration and water storage. Such limiting properties can only be antagonized by intense biological or physical processes, the majority being performed by soil engineers (Lavelle *et al.*, 2006). In this sense, the assessment of soils ability to provision habitat for key communities of soil invertebrates can function as a clear evidence that soil health promoting events are occurring and that soil habitat function is assured for other biological diverse communities (van Leeuwen *et al*., 2019). For this reason, soil organisms are used for more than three decades in ecotoxicological assays, more specifically, important functional groups like oligochaetes, collembolans or enchytraeids, since they are present in the vast majority of ecosystems in permanent contact with soil, performing relevant environmental roles, whilst reproducing quickly and being maintained easily as lab cultures (Nogueira Cardoso & Lopes Alves, 2012).

The ecotoxicological risk assessment has two main dimensions: predictive, in which possible toxic effects of compounds are predicted and toxicity limits for their presence in the environment are established; and a diagnostic approach, with the aim to estimate the real environmental risk of contaminated areas, proposing strategies for mitigation and risk reduction (van Gestel, 2012). For both, in order to obtain a relevant evaluation, it is recommended the performance of feasible and standardized ecotoxicological assays with

a battery of species (van Gestel, 2012). Also, it is extremely important to account for different routes of exposure and for (bio)availability of contaminants in soils, especially for metals like Cu, in which the soil matrix and its abiotic factors highly influence its toxicity and mobility through the soil profile (Maisto *et al*., 2011). Taking this into account, the use of elutriates for the exposure of aquatic organisms in ecotoxicological assays can be a suitable complementation to tests of direct exposure of terrestrial organisms, as it allows for the simultaneous assessment of toxicity of contaminants and their mobility in the environment (Antunes *et al*., 2010). Likewise, it is of extreme importance not only to proceed to the quantification of total soil contaminants, but also to further understand contaminants (bio)availability through selective chemical extractions of these from the soil matrix (Kelepertzis *et al*., 2018) and inference the degree of contamination of soils by determining the concentration of these in tissues of organisms that compose the soil biota (Hendrickx *et al*., 2004).

Besides the ecological relevance of the meso and macrofauna for soil quality and ecosystem services, soil microfauna and microbiological communities are a fundamental part of soil function. Altogether, they're responsible for biological and biochemical processes that participate in the C, N, P and S cycles, since they mediate the mineralization and humification of organic substrates (Nogueira Cardoso & Lopes Alves, 2012). Also, vineyards soils microbiological populations can be compromised by the use of Cu-based fungicides, with fungal and bacterial communities being more active between than within rows of vines, the opposite of Cu distribution in these systems (Mackie *et al*., 2013), a possible evidence of negative consequences of Cu towards these organisms.

The combination of the analysis of bioindicators through ecotoxicological assays that comprise different functional and taxonomic groups with different exposure routes, together with the assessment of physical and chemical parameters, can offer a detailed insight for soil quality evaluation (Salome *et al*., 2014). In this context, the present chapter intended to evaluate the immediate effects of Cu-based fungicides and their persistence in the environment, following a diagnostic approach, by using soil samples from a 15 years old vineyard under organic management in the Douro Demarcated Region. The degree of contamination and the potential risk towards biodiversity was assessed, for both terrestrial and aquatic organisms, by direct exposure to vineyard soils and to their leachates, respectively. Sampling took place in two periods, during the application of fungicides (July 2018) and six months after (January 2019), to understand the impacts of Cu and their persistence, as well as the ability of the ecosystem to recover from these. The integration of the results from physical and chemical analysis of soil samples with the results from ecotoxicological assays to both microbial communities, soil invertebrates and aquatic organisms allowed to understand if Cu-based fungicides can be used in a sustainable way,

or if their use bring environmental consequences that cannot be ignored, calling for their substitution for more environmentally friendly options.

2. Materials and Methods

2.1. Study area

The study was conducted in the Douro Demarcated Region, in a farm belonging to Real Companhia Velha, "Quinta do Síbio", located in Vale do Roncão, Alijó (Figure 1). The predominant exposure is to the South quadrant and it is located at altitudes between 120 and 300 meters, with a slope of 40%[1].The vineyards are placed on narrow terraces, supported by schist walls. The climate is characterized by high summer temperatures and a marked water deficit in the summer which results in a high level of aridity. The soils of this area can be classified as lithosols (Carta dos Solos, 1: 1.000.000). They are rocky soils without defined horizons and are located just above the mother rock (greywacke-schist complex). Therefore, many of these soils were formed by the human intervention that needed to break the rocks to implant the vineyards. In this sense, these wine-growing soils are also often called by anthrosols.

The grape varieties in this farm are Touriga Nacional, Touriga Franca, Sousão, Tinto Cão, Tinta Amarela and Tinta Francisca. The farm is under organic production management, meaning that phytosanitary treatments are restricted to copper and sulfur-based fungicides as well as to the use of pheromones for sexual confusion of pest communities. Fertilization of soil is made by the incorporation of organic matter in the form of pellets made from livestock manure every three years.

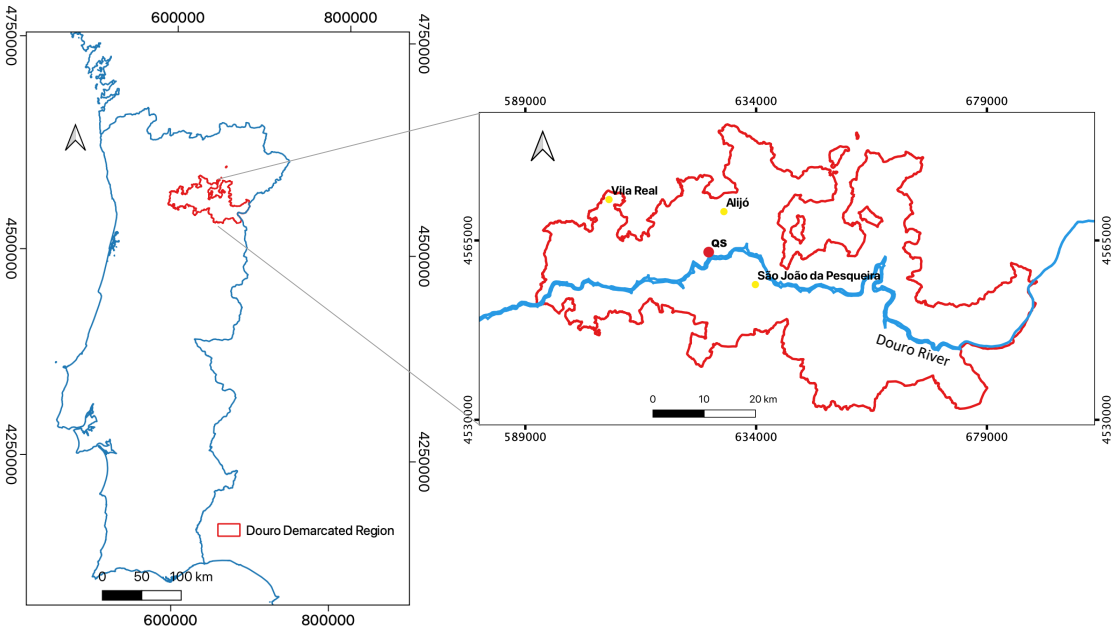

Figure 1. Localization of the Douro Demarcated Region and of "Quinta do Síbio", located in Vale do Roncão, Alijó, represented in the map by "QS".

[1] https://www.realcompanhiavelha.pt/pages/quintas/8

2.2. Sampling design, samples collection and pre-treatment

In the plot selected for this study, the vines, "Touriga Nacional" variety, had 15 years old, in order to obtain soil samples with low levels of Cu contamination due to successive accumulation through cumulative treatments. The sampling design was based on a previous work conducted by (Costa, 2018) (Figure 2a), thus seven sampling points from the previous study were selected in order to obtain a spatial distribution within the plot (Figure 2b).

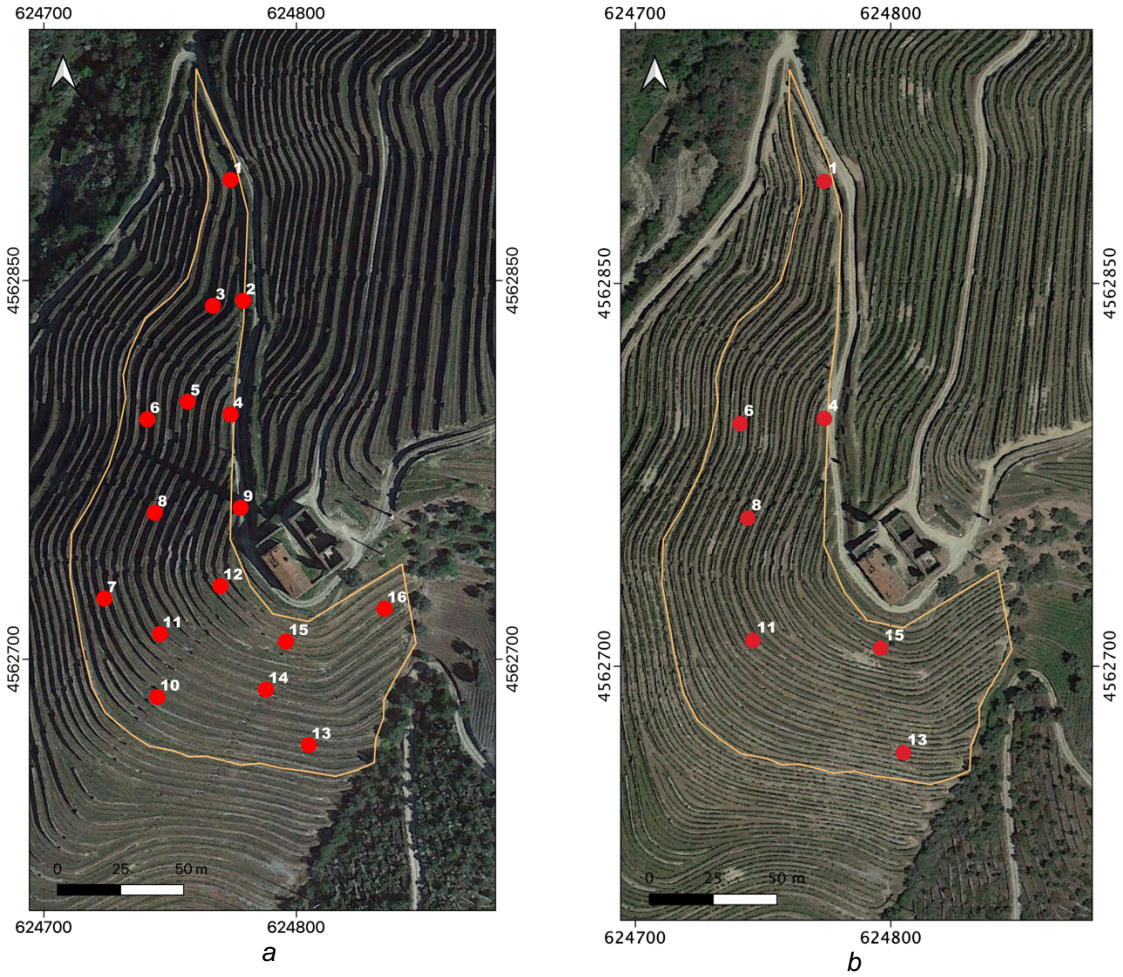

Figure 2. Schematic representation of (a) sampling design from the previous study of Costa (2018) and (b) sampling points selected for the present work.

The sampling was done in two periods: 18th July 2018, during the period of Cu and S application (Table 1); and 12th February 2019, six months after the last phytosanitary treatment. In each sampling point, a composite sample was collected for physical-chemical characterization, soil microbial parameters and ecotoxicological assays. Each composite

soil sample consisted in three sub-samples of superficial soil (0-10 cm): one collected near the vine stem (in the line) and the other two in each side of the line, about 0.5 m away.

For the chemical analysis (pH, organic matter, electric conductivity, and inorganic elements), 1 kg of soil was collected and stored in plastic bags until arrival. Once in the lab, the soil was placed in trays, one per sample, and oven dried at 40°C. After this, the samples were manually sieved, and the fraction lower than 2mm was stored at room temperature until analysis. For inorganic elements analysis, samples were further grounded in an agate mill and then stored in plastic containers. For soil enzyme's activity, samples were stored in plastic bags and refrigerated until arrival to the lab. Once in the lab, they were immediately frozen at -20°C. Before the analysis, the samples were slowly thaw at 4°C, manually sieved and the fraction lower than 2 mm was used for analysis. For the ecotoxicological assays, around 6 kg of soil was collected into plastic bags and once in the lab, the soil was dried at room temperature and manually sieved at <4 mm. Samples were kept at room temperature until testing.

Table 1. PPPs application in 2018 in "Quinta do Síbio", provided by Real Companhia Velha.

Period	Problem	Type of intervention	Treatment	Dose/ha
14-18 May	Powdery mildew	Preventive fungicide	S	20 Kg
21-24 May	Grape moth	Pheromone Diffusers	-	500 dif
04-08 June	Powdery mildew	Preventive fungicide	S	20 Kg
18-20 June	Powdery mildew	Preventive fungicide	S	20 Kg
26-30 June	Mildew	Preventive fungicide	Cu	3 Kg
09-11 July	Powdery mildew	Preventive fungicide	S	20 Kg
	Mildew		Cu	3 Kg
16-23 July	Mildew	Preventive fungicide	Cu	3 Kg
30 July - 01 August	Protection against sunburn	Application in more exposed plots	Kaolin clay	25 Kg

2.3. Determination of physical and chemical parameters

The pH of soil samples was determined in both soil:water and soil:KCl (1 M) suspension (1:5 m/v), as described in ISO 10390:1994 (ISO, 2002). To do so, 10 g of soil from each

sample were mechanically stirred with 50 mL of deionized water (for pHw) or KCl solution (for pHKCl) during 5 minutes. The mixtures remained resting for about 24 hours and the supernatant's pH was measured in the suspension using a previously calibrated pH meter (Edge®, Hanna Instruments). Electric conductivity (EC) was measured in the supernatant at rest of the soil:water suspension used for pH, using a conductivity meter (Edge®, Hanna Instruments).

The soil organic matter (OM) was measured by loss-on-ignition at 450°C for 8h. This method determines the total soil organic matter content based on the weight loss of a soil sample, previously dried at 105°C ($Soil_{105°C}$), after ignition at 450°C, for 8 hours ($Soil_{450°C}$). After this period, the crucibles containing the ignited soil samples were left in a desiccator, and then were weighted to the nearest 10 mg. The percentage of organic matter in the soil samples was calculated using equation 1.

$$Total\ OM\% = \frac{Soil_{105℃} - Soil_{450℃}}{Soil_{105℃}} * 100$$

(Eq 1)

2.4. Determination of potentially toxic elements concentration in soil samples

The pseudo-total concentration of major and trace elements was determined by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) after digestion in an heating block (DigiPREP MS, SCP Science), with a mixture of HNO3:HCl (3:1), following the method 3051A from USEPA (EPA, 2007). The extracts were analyzed for 17 chemical elements: Mg, Al, P, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Ba, and Pb. To evaluate the accuracy and the precision of the analytical method, procedure blanks, and certified reference materials were included in each analytical batch. The replicate analysis of the soil gave an uncertainty of <10% for these inorganic elements. The results of blank analysis were always below the detection limit and recoveries of reference materials (Till 1 and ERM-CC141 LOAM SOIL) were within the certified value.

The available content of Cu was assessed using calcium chloride (0.01 M), according to Houba *et al.* (2000). Briefly, a 1:10 (w/v) suspension was prepared and, after 2h of agitation, samples were centrifuged (3000 rpm for 10 min). The supernatant was acidified and Cu concentration was determined by ICP-MS.

2.5. Determination of soil microbial parameters

Soil enzymes were used as an indirect method for assessment of microbial activity of soils and as potential indicators of soil quality. For the determination of soil's enzymatic activity, 1g of thawed soil was weighted into six centrifuge tubes, with 3 being used as controls and 3 as analytical replicates. The enzymatic activity was measured using the methodologies described by *Schinner, et al*. (1996) and adapted to a microplate reader as previously described by Antunes *et al*. (2011). Soil moisture content was determined by considering the difference of weight after drying at 105 °C for 24 hours.

2.5.1. Acid phosphatase activity

For the determination of acid phosphatases activity, a buffered solution of p-nitrophenyl phosphate (pH 6.5 with MUB) was added to the samples and then incubated for 2h at 35°C. The p-nitrophenol (pNP) released by phosphomonoesterase activity was extracted with sodium hydroxide, which produces a yellow coloration, measurable spectrophotometrically at 405nm. The control samples were treated in a similar way, but the substrate solution was only added after the incubation period. The concentration of p-nitrophenol (pNP) produced was determined using a standard calibration curve (absorbance vs 7 standard solutions with concentrations ranging from 0 to 30 µg pNP mL^{-1}). The enzymatic activity was calculated using equation 4.

$$\text{Acid phosphatase activity} = \frac{\frac{(L - C) \times V \times D}{W} \times \frac{100}{\%dm}}{2} \ (\mu g\ pNP.\ g^{-1} dm.\ h^{-1}) \quad \text{(Eq. 4)}$$

Where:

L – Mean concentration of the sample (µg pNP mL^{-1});

C – Mean concentration of the control value (µg pNP mL^{-1});

V – Incubation volume (5mL);

D – Dilution factor of the supernatant (2);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.5.2. Arylsulfatase Activity

For the determination of arylsulfatase activity in soil samples, a 0.02M potassium-p-nitrophenylsulfate solution (prepared with an acetate buffer 0.5M, pH 8.5) was added to the

falcon tubes which were incubated for 1 hour at 37°C. The nitrophenol (pNP) released by the arylsulfatase activity was then extracted and colored with sodium hydroxide 0.5M and measured spectrophotometrically at 420nm. The samples used as controls were treated following the same overall procedure, but the substrate solution was added after the incubation. The concentration of p-nitrophenol (pNP) produced was determined using a standard calibration curve (absorbance vs 7 standard solutions with concentrations ranging from 0 to 20 µg pNP mL^{-1}). The enzymatic activity was calculated using Equation 2.

$$\text{Arylsulfatase activity} = \frac{(\text{L} - \text{C}) \times \text{V}}{\text{W}} \times \frac{100}{\%\text{dm}} \left(\mu\text{g pNP.gsoil}^{-1}.\text{h}^{-1}\right)$$

(Eq. 2)

Where:

L – Mean concentration of the samples (µg pNP mL^{-1});

C – Mean concentration of the control (µg pNP mL^{-1});

V – Incubation volume (10mL);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.5.3. Cellulase activity

For the determination of the cellulase activity samples were incubated for 24h at 50ºC, with only acetate buffer (2M) in the control samples, and with both acetate buffer and CM-cellulose as the substrate in samples. The reduced sugars released during incubation from the degradation of the substrate cause the reduction of potassium hexacyanoferrate (III) in an alkaline solution. Reduced hexacyanoferrate (III) reacts with ferric ammonium sulfate in an acid solution to form a complex of ferric hexacyanoferrate (II) (known as Prussian blue), which can be determined colorimetrically by reading in a spectrophotometer at 690nm. The activity of cellulase is expressed as µg of glucose (GE), calculated using a standard calibration curve (absorbance vs 6 standard solutions with concentrations ranging from 0 to 7.5 µg GE mL^{-1}). The activity was calculated using equation 7.

$$\frac{(\text{L} - \text{C}) * \text{V} * \text{D}}{\text{W}} * \frac{100}{\%\text{dm}} / \text{T} \left(\mu\text{g GE g}^{-1}\text{dm . h}^{-1}\right)$$

(Eq. 7)

Where:

L – Mean concentration of the samples (µg GE mL^{-1});

C – Mean concentration of controls (µg GE mL^{-1});

V – Incubation volume (3mL);

D – Dilution factor of the supernatant (40);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.5.4. Dehydrogenase activity

For the determination of the activity of dehydrogenases, the samples were suspended in a 1% triphenyltetrazolic chloride solution (prepared in TRIS buffer, 0.1 M) and incubated for 24 hours, at 40°C. The triphenylformate (TPF) produced was extracted with acetone and measured spectrophotometrically at 546 nm. The control samples were treated in a similar way but instead of the substrate solution, TRIS buffer was added before incubation. The concentration of TPF produced was determined using a standard calibration curve (absorbance vs 10 standard solutions with concentrations ranging from 0 to 100 µg TPF mL^{-1}). The enzyme activity was calculated using equation 3.

$$\text{Dehydrogenases activity} = \frac{\frac{(\mathrm{L}-\mathrm{C})\times \mathrm{V}}{\mathrm{W}} \times \frac{100}{\%\mathrm{dm}}}{24} \ (\mu\mathrm{g\ TPF\ .\ g^{-1}dm.\ h^{-1}})$$

(Eq. 3)

Where:

L – Mean concentration of the samples (µg TPF mL^{-1});

C – Mean concentration of the controls (µg TPF mL^{-1});

V – Incubation volume (6mL);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.5.5. Nitrogen mineralization

For the determination of the nitrogen mineralization, the samples were incubated with deionized water for 7 days at 40°C. During this period, the nitrogen organic forms originate an inorganic form of nitrogen (preponderantly ammonium ion, $NH4^+$), which is determined by a modification of the Berthelot reaction, after extraction with potassium chloride. This reaction is based in the reaction between sodium salicylate and ammonia (NH_3) in the presence of sodium dichloroisocyanurate, forming a green complex in alkaline conditions. The sodium nitroprusside is used as a catalyzer to increase the method's sensibility. The

released inorganic nitrogen is measured spectrophotometrically at 690nm. The control samples were treated in a similar way, but they were incubated at -20°C. The concentration of nitrogen (N) produced was determined using a standard calibration curve (absorbance vs 6 standard solutions with concentrations ranging from 0 to 1.6 µg $NH4^+$ mL^{-1}). The activity was calculated using equation 5.

$$\text{Nitrogen mineralization} = \frac{\frac{(\mathrm{L}-\mathrm{C})\times \mathrm{V}\times \mathrm{D}}{\mathrm{W}}\times\frac{100}{\%\mathrm{dm}}}{7}\ (\mu\mathrm{g\ N.\ g^{-1}dm.\ d^{-1}})$$

(Eq. 5)

Where:

L – Mean concentration of the samples (µg N mL-1);

C – Mean concentration of the controls (µg N mL-1);

V – Incubation volume (6mL);

D – Dilution factor of the supernatant (10);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.5.6. Urease activity

For the determination of the urease activity of soils, samples were incubated for 2h at 37ºC with a borate buffer (0.1M) in the control samples, and with both borate buffer and a buffered urea solution as a substrate for samples. The released ammonium during incubation from the degradation of the substrate were extracted with a potassium chloride solution, and determined by the reaction of sodium salicylate with NH_3 in the presence of sodium dichloroisocyanurate, which forms a green-colored complex under alkaline pH conditions – determination based on the modified Berthelot reaction. Sodium nitroprusside is used as a catalyst and increases the sensitivity of the method. The formed green complex can be determined colorimetrically by reading in a spectrophotometer at 690nm. The activity of urease is expressed as µg of N, calculated using a standard calibration curve (absorbance vs 6 standard solutions with concentrations ranging from 0 to 30 µg NH_4^+ mL^{-1}). The activity was calculated using equation 6.

$$\frac{(\mathrm{L}-\mathrm{C})*\mathrm{V}}{\mathrm{W}}*\frac{100}{\%\mathrm{dm}}/\mathrm{T}\ (\mu\mathrm{g\ N\ .\ g^{-1}dm\ .\ 2h^{-1}})$$

(Eq. 6)

Where:

L – Mean concentration of the samples (µg N mL^{-1});

C – Mean concentration of controls (µg N mL^{-1});

V – Incubation volume (10.5 mL);

W – Initial soil weight (1g);

%dm – Percentage of dry matter (100-%humidity).

2.6. Ecotoxicological assays

2.6.1. Indirect exposure of aquatic organisms to vineyard soils

2.6.1.1. Microtox

In order to assess toxicity of soil samples towards aquatic bacteria, a bioluminescence assay with *Aliivibrio fischeri* (Microtox® test) was performed using a Microtox 500 Analyzer, following the protocol provided by the manufacturer (AZUR Environmental, 1998). The *Basic Solid-Phase Test* was chosen in the software MicrotoxOmni Azur, since it is an acute toxicity test, commonly used for solid matrices (soils and sediments). For this assay, a soil suspension was prepared with 17.5 mL of solid-phase diluent and 3.5 g of soil, stirred for 10 minutes. After this, 2mL of the soil suspension was placed in a glass cuvette from which a series of dilutions were made. The initial bioluminescence was determined before exposing the bacteria to the soil suspension dilutions. Afterwards, the soil suspension dilutions were added to the bacteria and the bioluminescence was read after 5, 15 and 30 minutes of exposure. With this assay, the EC_{20} and EC_{50} (effective concentrations for 20 and 50% bioluminescence inhibition) are estimated with a 95% confidence interval. However, when it wasn't possible to estimate ECx values, results were expressed as the highest effect (HE) after 30 minutes of exposure.

2.6.1.2. Growth inhibition of freshwater alga exposed to soil elutriates

The freshwater alga *Raphidocelis subcapitata* was used in growth inhibition assays, performed according to an adaptation of the standard OECD protocol 201 (OECD, 2011), being exposed to elutriates obtained from soil samples. The culture of *R. subcapitata* was obtained by inoculation in MBL medium enriched with vitamins, after exposure to continuous light at 24 ± 1°C for 72 hours. The microalgae were then counted using a Neubauer chamber and the concentration was adjusted to 1×10^4 cells mL^{-1} by dilution. The soil elutriates were made by preparing suspensions of 1:4 (w/v) of the samples with Woods Hole MBL medium. The suspensions were mechanically agitated for 24 hours at room temperature, being

centrifuged after at 3900 rpm for 5 minutes. Each elutriate was then tested individually at a concentration of 100%. This assay was an adaptation to the original protocol since it was carried in a 24-well sterile plates (Figure 3), using four replicates per sample, plus the control. The first row (A1 to A6) of the plate was filled with 2mL of water to maintain appropriate humidity, and wells 1B, 1C and 1D were used as controls with 900 µL of MBL inoculated with 100 µL of the *R. subcapitata* dilution. The remaining wells were filled with 900 µL of soil elutriate plus 100 µL of the inoculum. The plates were incubated at continuous light at 24 ± 1°C for 72 hours, with agitation. After this period, the quantification of *R. subcapitata* was performed using a Neubauer chamber, for both controls and samples. The percent inhibition of growth for each treatment was calculated using Equation 8.

$$\% I_R = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

(Eq. 8)

Where:

% Ir – percent inhibition in average specific growth rate;

μ_c – mean value for average specific growth rate (µ) in the control group;

μ_t – average specific growth rate for the treatment replicate.

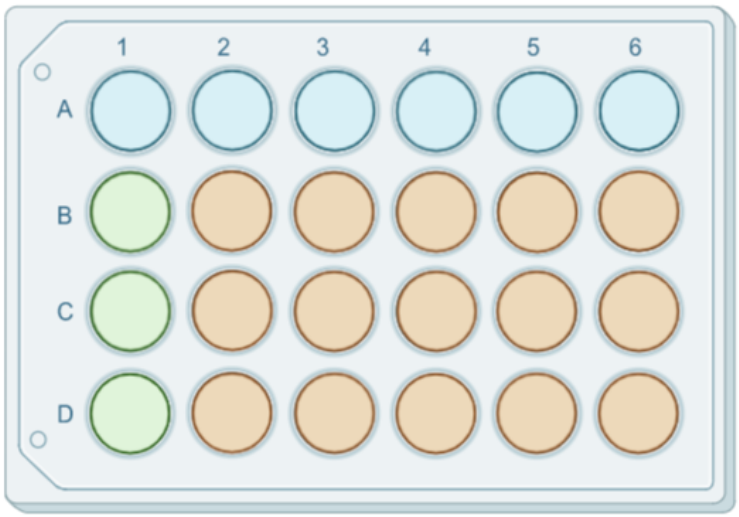

Figure 3. Schematic representation of loaded microplate used in R. subcapitata growth inhibition assays. The color blue represents wells filled with water, green the control wells, and brown the wells loaded with samples.

2.6.1.3. Growth inhibition of freshwater aquatic plant exposed to soil elutriates

Lemna minor was used as a freshwater aquatic plant to perform growth inhibition assays, which were carried following an adaptation to OECD 221 (OECD, 2006). The aquatic plants

for testing were maintained as cultures in Steinberg culture medium, in an acclimated chamber with a photoperiod of 16hL:8hD at 24 ± 2 °C. To perform this assay, soil elutriates were prepared with 12.5 g of the soil sample and 50 mL of Steinberg, left stirring for 24 hours. After this period, the suspension was centrifuged at 3900 rpm for 5 minutes and the elutriate obtained from the supernatant. A 6-well plate was used in the assays for each sample, with three wells being used as controls (with only 12 mL of Steinberg) and the other three wells were filled with 12 mL of the soil elutriate. A set of plants with a total of 9 fronds were selected and added to the six wells, with an extra three replicates of sets of plants being dried at 60 °C for the initial dry weight. After seven days of exposure, the number of fronds were counted and then dried at 60 °C until achieving a stable weight. The growth inhibition rate was calculated according to the average specific growth rate (Equation 9) and yield (Equation 10).

Equation 1. Calculation of the percent inhibition of growth rate.

$$\%\, I_r = \frac{\mu_c - \mu_t}{\mu_c} \times 100 \qquad \text{(Eq. 9)}$$

Where,

$\%\, I_r$ – percent inhibition in average specific growth rate;

μ_c – mean value for μ in the control;

μ_t – mean value for μ in the treatment group.

$$\%\, I_y = \frac{b_c - b_t}{b_c} \times 100 \qquad \text{(Eq. 10)}$$

Where,

$\%\, I_y$ – percent reduction in yield;

b_c – final biomass minus starting biomass for the control group;

b_t – final biomass minus starting biomass in the treatment group.

2.6.2. Direct exposure of terrestrial organisms to vineyard soils

2.6.2.1. Artificial soil and water holding capacity

An artificial soil was prepared in order to be used in ecotoxicological assays with terrestrial organisms as a control soil, according to OECD guidelines Test No. 222 (OECD, 2016a). It was made by combining 5% of sphagnum peat as a source of OM, 20% kaolin clay, 74.7% dried sand and 0.3% calcium carbonate to obtain a pH of 6.0 ± 0.5. The maximum water

holding capacity (WHC) of both artificial soil and vineyard soil samples was determined according to the standard protocol ISO 17512-1 (2008), as described by Rodríguez-Seijo *et al*. (2017).

2.6.2.2. Avoidance assays with *Eisenia fetida*

Avoidance assays with the earthworm *Eisenia fetida* were performed using organisms from lab cultures of standard age and size. The earthworms were maintained in plastic boxes (10–50 L) containing a substrate composed of 50:50 sphagnum peat and sterilized horse manure (dry and defaunated through two freeze–thawing cycles: 48h at -20°C followed by 48h at 65°C), with $CaCO_3$ to adjust the substrate pH (6.0 ± 0.5), which was kept moist with deionized water. The earthworms were fed every 2 weeks with oatmeal previously hydrated with deionized water. For the avoidance test with *E. fetida*, the ISO guideline No. 17512-1:2008 (ISO, 2008) was followed. Individuals with a weight for each between 0.30 and 0.60g were selected from cultures and left to acclimate in artificial soil for two days prior the assay. In order to obtain a dual section chamber, rectangular plastic containers were used and divided in two compartments by a removable cardboard split. The artificial soil was used as a control, being placed in both of the compartments, to assess normal worm behavior and guarantee normal distribution of worms throughout the container when at normal conditions. For vineyard soils, samples were placed in pairs, meaning that in one side of the chamber there was a sample from July 2018 and its respective sample in the other side from February 2019. Both soils had their humidity adjusted to 50% of the WHC. Five replicates were prepared and the split was removed, following the addition of 10 organisms per replicate. The assays were kept at 20 ± 2°C and a 16h L:8h D photoperiod. After the 48h test period, the split was reintroduced in the marked position and the individuals were counted in each compartment containing the control and the test soil. If any earthworm was not found it was assumed as dead. Earthworms that were between soils were considered as being in the soil to which the organism's head was directed to.

2.6.2.3. Reproduction assays with Collembolans

The reproductive output of *Folsomia candida* was assessed in reproduction tests in sampled soils, according to OECD guidelines Test No. 232 (OECD, 2016b). *F. candida* cultures were kept in containers with culturing substrate constituted by 1:10:10 of activated charcoal, distilled water and plaster of Paris, respectively. They were cultured at 20 ± 2 ºC, at a light-dark cycle of 16hL-8hD, and transferred to newly prepared plaster of Paris/charcoal substrate weekly, being fed with dried baker's yeast and kept moist with distilled water. To perform the assays, synchronized animals with 9-12 days were used. The same artificial

soil as described before was used as the control soil. The test was carried in plastic containers, with 30g of dry weight soil humidified to 50% of WHC with deionized water, in which 10 individuals were added for each replicate, with five replicates for each sample and for the control (Figure 4). The assay was carried for a total of 28 days at 20 ± 2 ºC, at a light-dark cycle of 16hL-8hD, with twice a week maintenance by feeding with dried yeast and ensured conservation of humidity. At the end of the test, mortality and reproduction were assessed by extracting collembolans from the soil and proceeding to its counting using ImageJ. The assay is considered valid if the mean adult mortality doesn't exceed 20% and the mean number of juveniles per vessel is at least 100.

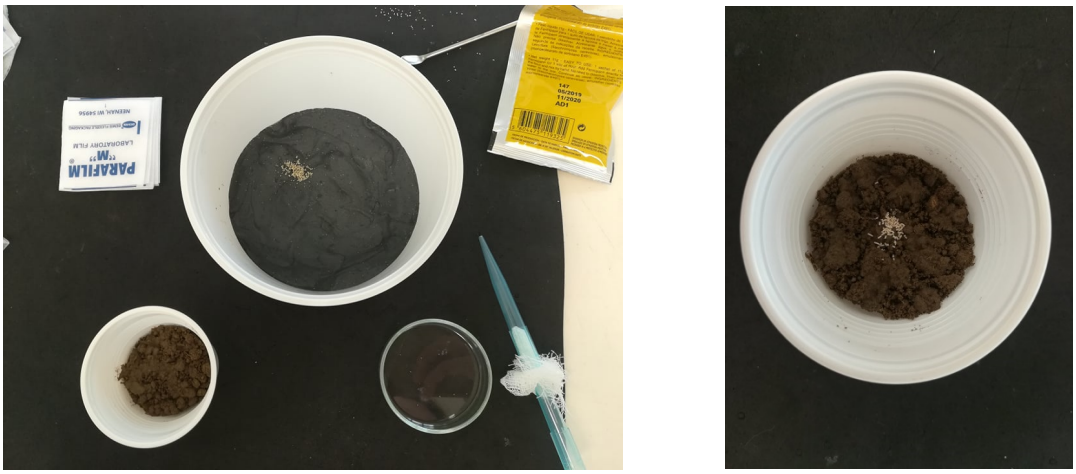

Figure 4. Photos of the preparation of F. candida reproduction test (left) and a prepared replicate using a sample soil (right).

2.6.2.4. Reproduction assays with *E. Fetida*

Earthworm reproduction tests were carried with *E. fetida* exposed to vineyard soil samples, according to OECD guidelines Test No. 222 (OECD, 2016a). Organisms were kept as lab cultures as described before for avoidance tests, and the same artificial soil was used for controls. Adult organisms with a visible clitellum were selected from synchronized cultures, with 0.30-0.60 g, and acclimatized for two days in artificial soil. Experimental design consisted of plastic containers to which were added 500g of either artificial soil (for the control) or soil samples, humidified at 50%of WHC with deionized water, with five and three replicates, respectively. A set of 10 worms were added per container, and the assay took place at 20 ± 2 ºC, at a light-dark cycle of 16hL-8hD, being weekly fed with sterilized, dry and sieved horse manure and replenished as necessary with deionized water. At the 28th day, adults were removed and weighed, and the remaining soil with cocoons were left another 4 weeks being only fed once, with moisture maintenance. Assays were considered valid when adult mortality was less than 10% and each replicate produced ≥ 30 juveniles at the end of the 28 and 56 days, respectively.

2.6.2.4.1. Bioaccumulation of Cu of Eisenia fetida after exposure to vineyard soils

After 28 days, adult individuals of the reproduction assay were collected to evaluate the bioaccumulation of Cu in the body tissue. Earthworms were removed, rinsed with deionized water and allowed to egest their gastrointestinal tract for 24 h in plastic containers with a moist filter paper (OECD, 2010). After that, individuals were weighted and immediately frozen (−20 °C). Before analysis, samples were dried in an oven at 60 °C during 48h and the weighted again. The earthworms were digested with an acid mixture of 6mL of HNO_3 and 2mL of H_2O_23 (Ultra-pure reagents) in a heating block (DigiPREP MS, SCP Science). The digests were diluted to 50 mL with Milli-Q water, and the Cu content (dry weight basis) were determined by ICP-MS. Each extraction batch included the analysis of blanks (always below detection limit) and reference materials (ERM-CE278k Mussel Tissue, BCR-710 Oyster Tissue, and SRM 2976 Mussel Tissue) which was within the certified value. Besides, the bioconcentration factor (BCF) was calculated as the ratio of the Cu concentration in the earthworm (C_w) to that in the soil (C_s).

2.7. Data analysis

All endpoints were evaluated using at least three replicates for each sampling point. Physical and chemical parameters of the two sampling periods were compared using a t-test. For some specific parameters, when normality of results wasn't verified, a non-parametric test was used (Mann-Whitney). A one-way ANOVA was used to compare potentially toxic elements in the two sampling periods and in the background, when normality of results wasn't verified, a non-parametric test was used (Kruskal-Wallis). Results of microbial parameters and ecotoxicological assays were compared using a t-test. For avoidance assays, a two-tailed t-test was used to test the hypothesis of no significant avoidance in the dual controls, and an one-tailed Fischer Exact Test was performed to test the null hypothesis of no significant avoidance of the test soils. All statistical procedures were performed in Prism 8 (GraphPad Software Inc, USA).

3. Results and discussion

3.1. Physical chemical characterization of vineyard soils

Table 2 shows the results of general parameters determined in vineyard soils for both sampling periods. EC values appear to be higher in July, decreasing in February. These results may be explained by the application of fungicides in July, that presumably increased salt contents of soils. As raining events happened during fall up until February, leaching of these salts may have happened, justifying lower results obtained in this sampling period. However, due to the high variability of results observed, differences were not statistically significant ($p>0.05$). The pH remained similar in both sampling periods, although it may have been expected a decrease in July, due to S applications before the sampling of soils (Table 1), since S is rapidly transformed into sulfates, which increases soil acidity (Hinckley *et al*., 2011). However, due to soil's buffer capacity, and to the fact that this decrease in soil pH seems to be rapidly replenished to pre-application levels after twelve days, as reported in the same study, a similar pH for both sampling periods can be considered ordinary.

OM contents of samples decreased from July to February, however, differences weren't statistically significant ($p>0.05$), which can be due to high variability of results, especially for February samples. Still, and considering the decrease of OM, there seems that a loss of OM is happening through the years: when comparing these results with the ones obtained in the previous study (3.37 ± 0.78) (Costa, 2018), which was conducted in January 2017, this trend seems more evident. Nevertheless, values are considered low for most of the results, and, according to Costa (2018), they are related and in accordance with the nature of background soils.

Table 2. Determined general parameters of vineyard soils for both sampling periods.

Parameter	July 2018	February 2019
EC (µS cm^{-1})	90.4 ± 44.6	48.9 ± 19.1
pH_w	6.06 ± 0.13	5.95 ± 0.30
pH_{KCl}	5.54 ± 0.21	5.43 ± 0.46
OM (%)	3.1 ± 0.3	2.5 ± 0.9

Regarding potentially toxic elements (PTEs) analysis of soils, background levels from the previous study were considered (Costa, 2018). These results were obtained from five forest soils collected next to the study vineyards, and plotted in Figure 5 to allow interpretation of results obtained from analysis of sample soils. The first graph contains pseudo-total contents of major elements (ME), which are nutrients, and the second one some trace elements (TE), determined by ICP-MS. Detailed and complete results are

presented in Appendix Table A1 and Table A2. By the analysis of results, it is possible to see that P content of sample soils are significantly higher than background values, which can possibly be due to amendments of soils with animal manure pellets, since they are used as a source of OM and nutrients, including P. Concerning TE, some may be of anthropogenic origin, or derived from soil parent materials, being that some of these TE may be considered PTEs. Cu values in soil samples are much higher than background levels, for both sampling periods, and, in fact, the mean concentration of Cu of both periods (103.2 mg kg^{-1}) is more than double the mean concentration of vineyard soils in the EU (49.26 mg kg^{-1}) (Ballabio *et al.*, 2018). Since differences between samples and background values are so clear, there's a high possibility that Cu content of vineyard soils is due to anthropogenic enrichment, probably resultant from cumulative Cu-fungicides application in this context. Also, geography of these particular vineyards may be influencing Cu inputs, since these are located in a slope and organized in terraces, they may be receiving leachates and sediments rich in Cu from vineyards above. Although no significant differences were found for As between samples and background, its concentrations should still be highlighted, since they can be classified as high levels when compared to soil-As guidelines generally established for plant production (20-50 mg kg^{-1}) (Ravenscroft *et al.*, 2009). Even so, these concentrations are probably characteristic of the nature of these soils.

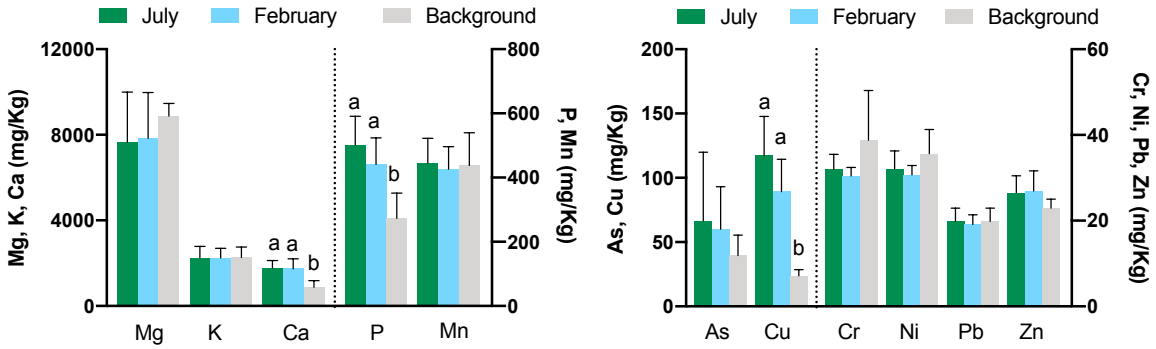

Figure 5. Major elements (left) and trace elements (right) for background and soil samples, determined by ICP-MS. Letters a and b represent statistically different mean values.

The available content of Cu of soils, determined by ICP-MS after extraction of soils with $CaCl_2$, is presented in Figure 6. Although concentrations in July samples are significantly higher ($p<0.05$) than February samples, the overall content of available Cu for both sampling periods is very low, being that the available fraction (Figure 6b) represents less than 1% of the pseudo-total concentrations of Cu. Also, differences between the two sampling periods reflect the total contents of Cu for each period, meaning that in July Cu isn't probably more available, but instead this value is higher due to higher concentrations of total Cu in July.

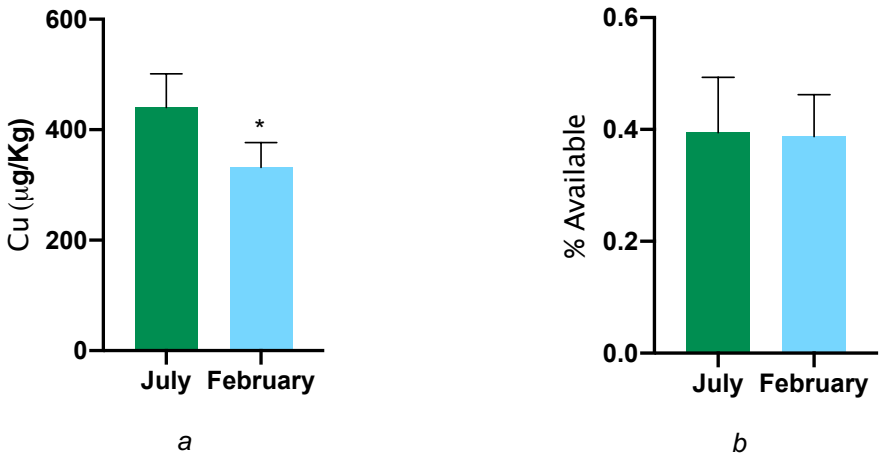

*Figure 6. Available Cu content of soil samples determined by soil extractions with CaCl2 and analyzed by ICP-MS, for both sampling periods. Graph (a) represents determined Cu contents as µg Kg^{-1} and graph (b) the available % of Cu from the pseudo-total concentrations. * represents statistically significant differences.*

Figure 7 represents the results obtained from the analysis of bioaccumulation of Cu by *E. fetida* after analysis by ICP-MS of digested tissues. Results show statistically significant differences between bioaccumulation of organisms exposed to July or February samples ($p<0.05$), meaning that earthworms accumulated more Cu in their tissues when exposed to soils collected when fungicides treatments took place. These results reflect both the total and the available Cu content of soils, since *E. fetida* accumulated more Cu in soils where its total and available content was superior. Also, Cu values present in the tissues of earthworms exposed to July samples deserve to be highlighted, since environmental concerns may be at cause. Determined concentrations (35.5 ± 8.1 mg Kg^{-1}) may be compromising *E. fetida* reproduction ability, since a threshold value of 40 mg Kg^{-1} has been suggested concerning cocoon production of earthworms (Ma, 2005), with EC_{50} values varying from 15.5 to 62.5 mg Kg^{-1} for *E. fetida* in particular (Duan *et al.*, 2016).

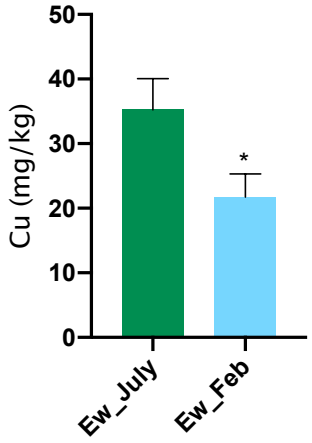

*Figure 7. Bioaccumulation of Cu by E. fetida determined by ICP-MS. * represents statistically significant differences.*

3.2. Soil microbial parameters

Results obtained for all the six soil enzymes assessed are represented in Figure 8. Significant differences were obtained between sampling periods, but not correlating with Cu contents of soils: enzymes activities, specifically cellulase, dehydrogenase and urease activities, were higher for samples collected in July than those from February. Apparently, superior contents of Cu in July samples weren't responsible for a decrease in microbiological activity of the soil microfauna. Instead, either soil or environmental conditions of July promoted important nutrient cycle processes. Indeed, microbial and enzyme activities reflect the combined effects of environmental factors, like temperature and humidity, meaning that a temporal variability in soil enzyme activities can be expected (Paz-ferreiro *et al*., 2011). This is especially true for urease, which is highly regulated by climate (Lebrun *et al*., 2012). However, for dehydrogenase, higher activities are usually found in winter, due to higher levels of soil humidity (Paz-ferreiro *et al.*, 2011). Nevertheless, Mediterranean ecosystems have high summer temperatures with low rainfall, meaning that, in July, conditions wouldn't be prosperous for microbial communities. However, established communities in such specific conditions might actually thrive under sub-optimal conditions (Yuste *et al*., 2014).

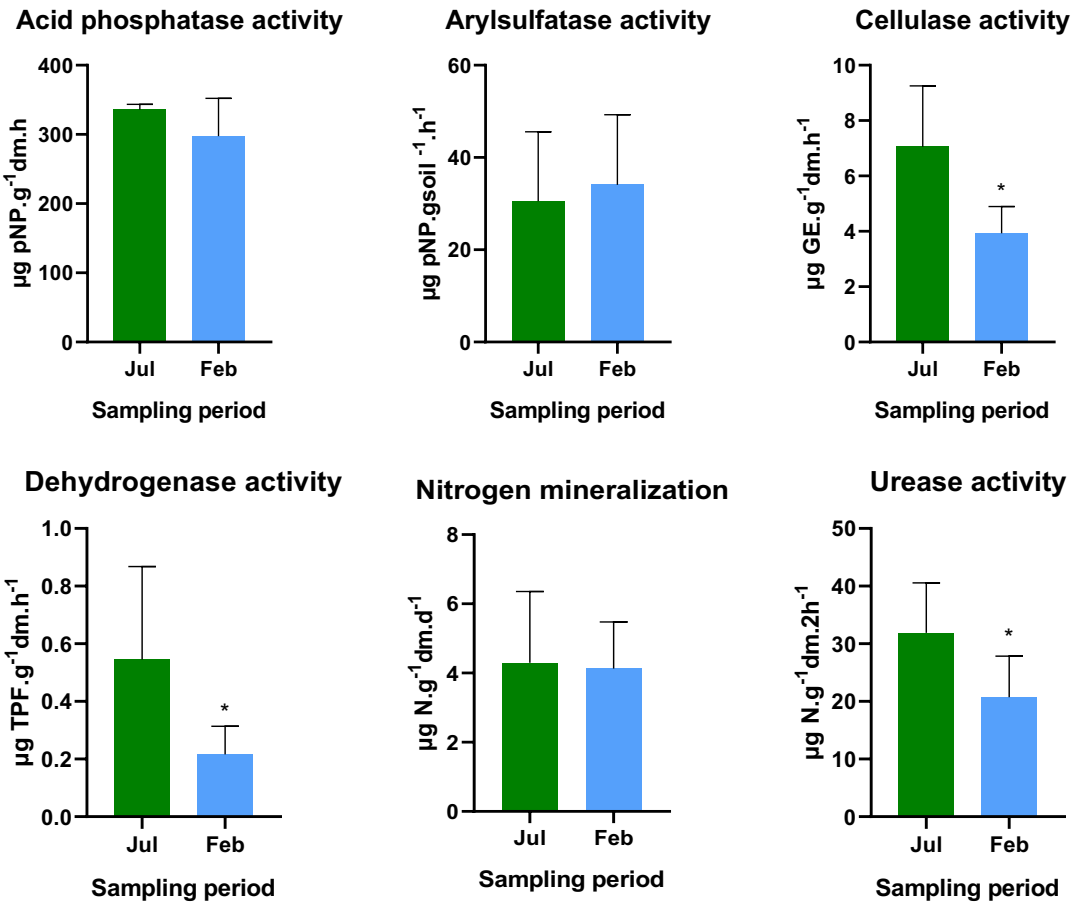

*Figure 8. Results of all the microbial parameters assessed for both sampling periods. * represents statistically significant differences.*

Briefly, the results obtained regarding soil enzymes, might elucidate the influence of environmental conditions towards microbial activity of soils, which in this case seem to explain the differences between sampling periods, rather than soil enrichment with Cu in July.

3.3. Ecotoxicological assays

3.3.1. Indirect exposure of aquatic organisms to vineyard soils

Figure 8 represents results obtained from the Microtox® assay, after exposure of *Aliivibrio fischeri* to soil solutions. The results didn't allow for the determination of EC_{20} or EC_{50}, so they are plotted as the percentage of highest inhibitory effect at 30 minutes of exposure. Significant differences between both sampling periods were found, with highest inhibition of bioluminescence being shown for July samples.

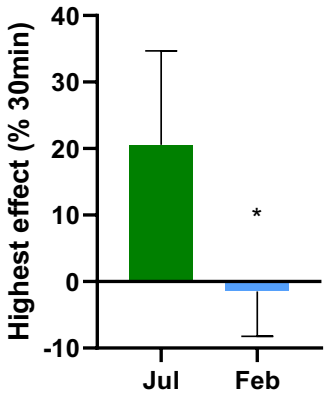

*Figure 9. Microtox® results presented as highest effect at 30 min (%). * represents statistically significant differences.*

Growth inhibition assays of the freshwater alga *Raphidocelis subcapitata* didn't show toxicity of soil elutriates towards the growth of *R. subcapitata*, for neither of the sampling periods. Results won't be integrally shown, due to high variability of results (Mean values for July: -0.5 ± 9.5; mean values for February: -0.3 ± 7.6). Such high variability can be explained since some elutriate samples even promoted the growth of algae, which can be expected, since elutriates won't only have soil contaminants available in the aqueous phase, but will also be enriched with OM and nutrients, which can stimulate organisms viability (Antunes *et al.*, 2010).

Regarding growth inhibition assays *of Lemna minor* exposed to soil elutriates, results are presented in Figure 9. Differences between the growth performance of *L. minor* weren't found for neither of the two sampling periods, and for neither of the endpoints assessed (number of fronds and dry weight).

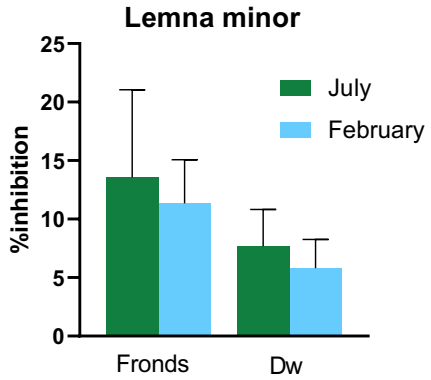

Figure 10. Results of growth inhibition assays Lemna minor exposed to soil elutriates presented as %inhibition of number of fronds and dry weight.

Overall, looking at results obtained from ecotoxicological assays of aquatic organisms, soil elutriates obtained from July samples only seem to have been toxic at inhibiting the bioluminescence activity of the aquatic bacteria *A. fischeri*. Even though results obtained from Microtox® for July samples were significant, there's still a visible high

variability of values between sampling points. Also, considering that we weren't able to determine toxicity towards any other of the organisms tested, it seems likely that soil contaminants, and particularly Cu, weren't available and interchangeable into the aqueous phase. This might indicate that, despite high levels of Cu in samples, this might exist in more stable forms and with low mobility through the soil profile, and also evidencing soil buffer capacity.

3.3.2. Direct exposure of terrestrial organisms to vineyard soils

Regarding avoidance assays performed with *E. fetida,* the normal behavior of worms was validated ($p>0.05$), meaning that they didn't show any preference for either sides of the dual chamber at control conditions. When exposed to soils collected from both sampling periods, earthworms didn't prefer soils from February rather than those from July ($p>0.05$), as represented in Figure10. This can presumably mean that Cu application in vineyards didn't compromise soil habitat function to *E. fetida*.

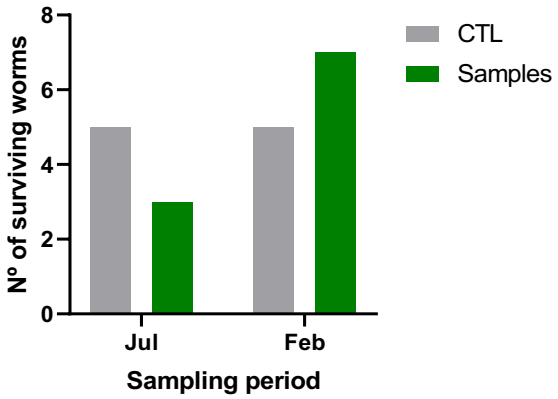

Figure 11. Results of avoidance assays with E. fetida exposed to soil samples from both sampling periods. Results presented as nº of survival worms for each sample at the end of the assay.

Concerning reproduction assays with *E. fetida*, results won't be shown since tests didn't validate (number of juveniles at the end of 54 days < 30 for the controls).

Chapter III. The toxicity of Cu-based commercial formulations to different fungi species

1. Introduction

Cu is used in agriculture as a fungicide and algicide, and Cu ions (Cu^{2+}) are their active compound, exerting its antimycotic behavior by direct contact in an non-specific way (Martins *et al.*, 2012; Vallières & Avery, 2017). Although its multi-site mode of action isn't completely understood, it has been described that Cu exerts its toxicity by compromising fungal protein synthesis and inducing the formation of reactive oxygen species (ROS) (Vallières & Avery, 2017), which will inducepermeabilization and lipidic peroxidation of cellular membranes, and even the denaturation of nucleic acids (Oussou-Azo *et al.*, 2020). Also, given the multi-site action of Cu ions, and its interaction with well-preserved mechanisms, benefits of the use of Cu-based fungicides are raised when discussing resistance events, since the risk is lower than for other specific fungicides (Malandrakis *et al.*, 2020).

Cu-based fungicides are of extreme importance in organic agriculture, since they fulfill the premise of being a non-synthetic compound and the only effective product to cope with certain fungal diseases (Cabús *et al.*, 2017). These are used in crops in a preventive way, meaning that treatments are applied before the occurrence of rains that favor the spore production of fungi and their dispersal, with Cu being less effective as a curative treatment for fungal diseases (Cabús *et al.*, 2017). Once applied, Cu-based fungicides leave a protective film on leaves, which will act as a Cu deposit, that when in contact with water will release free Cu ions, exerting its expected toxicity (Lamichhane *et al.*, 2018).

Cu is used in fungicides formulations in various forms , including the Bordeaux mixture [$CuSO_4$ + $Ca(OH)_2$], Cu oxychloride [$3Cu(OH)_2 \cdot CuCl_2$], basic copper sulphate [$CuSO_4 \cdot 3Cu(OH)_2$], cuprous oxide (Cu_2O), copper hydroxide [$Cu(OH)_2$], among others (Komárek *et al.*, 2009). Therefore, it's also important to address that not all forms of Cu and their respective commercial formulations will result in equal outcomes when managing fungal infections: for example, for certain fungi, Cu oxychloride and Cu hydroxide seem to exert more fungistatic effects, while formulations like the Bordeaux Mixture appear to work better as fungicides (Martins *et al.*, 2012). Also, different fungi will react differently to Cu fungicides, for instance, oomycetes have cellulosic cell walls, while ascomycetes have chitin as their main cell wall component, making the latter less sensitive to Cu ions (Banik & Pérez-de-luque, 2017).

Adding to the fact that the choice of Cu-based fungicides has to me made considering a myriad of factors, like weather forecasts and type of infection, finding strategies to cope with fungal diseases is particularly difficult because, like fungi, their hosts (plants) are also eukaryotes, making it a challenge to find specific treatments for these pathogens (Vallières & Avery, 2017). Also, although Cu's broad spectrum action as a

fungicide is an advantage from a resistance point of view, this same aspect can be harmful towards benefic species of fungi, that function as natural biologic control agents for pests (Martins *et al.*, 2012). Moreover, whilst not expected, development of resistance of fungi against Cu use is becoming a major issue, with the fast development of resistance in a large number of plant pathogens, like the oomycete *Plasmopara viticola* (Malandrakis *et al.*, 2020), the causal agent of grapevine downy mildew, a disease treated with Cu in organic farming.

In the recent years, with Cu accumulation in soils and its potential negative outcomes for the environment, concern has turn to the broad and extensive use of Cu-based fungicides. Successive restrictions to its use have been applied in the EU since 2002 (Commission Regulation (EC) No 473/2002), with the recent legislation aimed in limiting Cu use in agriculture to 28 kg/ha over a period of 7 years (i.e. on average 4 kg/ha/year) (Commission Implementing Regulation (EU) 2018/1981). With these restrictions, but with the same necessity for treatments of crops from fungal diseases, it is expected that farmers increase the use of Cu fertilizers, with the purpose of fighting crop losses without including these values into account for Cu-based fungicides totals (Lamichhane *et al.*, 2018).

With the rising pursuit of new alternatives for Cu-based fungicides, NPs have been pointed as a solution to reduce Cu inputs in the environment, due to their expected high efficiency with lower application doses (Malandrakis *et al.*, 2020). The physical and chemical properties of a given metal are different when looking at the nanoscale, when comparing to its bulk form, being that their antibiological properties seem more pronounced when a metal is presented as NPs (Oussou-Azo *et al.*, 2020). They might offer a slower release of their active compound (Malandrakis et al., 2019), and their high surface area when compared to their volume seems to be the major factor influencing NPs efficiency, since it provides a larger interaction area with biological membranes (Oussou-Azo *et al.*, 2020).

When taking into consideration NPs as fungicides, it is extremely important to consider that toxicity and efficiency of NPs might not be proportionally similar with their respective commercial bulk form: for instance, when considering a traditional formulation of Cu hydroxide, with higher efficiency for a certain infection when compared to Cu sulfate, it doesn't mean that the same rule applies to both of their nanoformulations (Malandrakis *et al.*, 2019). Also, NPs are used as part of new commercial fungicides formulations in conjugation with coadjuvants, since it allows to improve NPs behavior (Banik & Pérez-de-luque, 2017) by inhibiting NPs typical agglomeration when in physiological conditions (Oussou-Azo *et al.*, 2020), which results in better performing results, but can also influence its toxicity and environmental impacts.

Whilst being highly promising, NPs still lack further understanding of its mechanisms of toxicity towards target and non-target organisms, as well as deeper knowledge on how to take full advantage of its properties. However, NTAF-Cu are being introduced in the market for antifungal treatments, especially new formulated fungicides with reduced particle size. As an example, in Portugal, several NTAF-Cu have been added to the list of approved products for the control of vineyards infections in organic farming. While the manufacturers and labels promote them as improved formulations, thanks to their smaller particle size and higher efficiency, they are easily included as options because they maintain the same active compound previously approved for traditional formulations. Nevertheless, the same active compounds in new improved formulations can provide additional benefits when compared to their traditional counterparts, and these new products rely on their technology to use less elemental copper than other products without compromising protection rates, since they promise more bio-available copper. Higher efficiency of these alternatives is also promoted thanks to their claims of superior performance in residual effects, rainfastness and foliar coverage areas. Brands foment NTAF-Cu as a way to reduce rates of Cu applied with no effect on beneficial species of non-target organisms.

The aims of this chapter were to assess and understand how newly introduced formulations provide accurate information about their content in labels and safety-sheets, and evaluate their effect in the growth of fungi, under in vitro conditions, when compared to more traditional formulations. To achieve these aims, the composition of different Cu-based commercially available fungicides as well as their particle size was assessed. Moreover, assays of mycelia growth inhibition were carried for two different species (one not responsible for vine infections, and the other a known vine pathogen). Three different formulations of $Cu(OH)_2$, a traditional one and two newer formulations were used: Champion WP (CH) (Nufarm), Kados (KD) (Certis) and Kocide Opti (KO) (Certis), respectively. The composition analysis was achieved by Wavelength-Dispersive X-Ray Fluorescence (XRF), the particle size of bulk formulations accomplished by Scanning Electron Microscopy (SEM) and the particle size of dissolved formulations in water was assessed by Dynamic Light Scattering (DLS). Fungi species were exposed to these same formulations, as well as to the Bordeaux Mixture (BM), which was used as the more traditional formulation of $CuSO_4$, and Cu sulphate (Merck), with the goal of reproducing the results of a positive control for comparison.

2. Materials and methods

2.1. Tested fungicides

Three commercial formulations, with their active ingredient being in the form of copper hydroxide [$Cu(OH)_2$] were tested: a traditional wettable powder - Champion® WP (CH), from Nufarm; and two newer water-dispersible granules formulations - Kados® (KD) from Genyen and Kocide Opti® (KO) from Certis. Another traditional wettable powder formulation was used, but in which Cu is in the form of copper sulphate ($CuSO_4$): Bordeaux Mixture (BM). Copper sulphate, not being used on its own as a fungicide in the treatment of vineyards, was used in the form of pentahydrate copper (II) sulphate ($CuSO_4 \cdot 5H_2O$) (Merck) as a positive control. All the selected commercial fungicides are approved for organic agriculture in Portugal.

2.2. Characterization of Cu-based formulations

The characterization of the composition and size of particles was made for $Cu(OH)_2$ formulations, with the purpose of fully understand how new formulations differentiate from traditional ones, and how their physical and chemical properties vary between each other. The goal was also to clarify and verify the information provided in labels and safety sheets that go along with these products. A characterization of formulations in their powder form was performed by scanning electron microscopy (SEM - FlexSEM 1000, Hitachi), which allowed for visualization of particles and the semi-quantification of formulations. Further, the diameter of particles was measured using ImageJ. The chemical composition of the commercial formulations was also determined by wavelength-dispersive X-ray fluorescence (XRF) using an Axios PW4400/40 X-ray (Marvel Panalytical) fluorescence wavelength dispersive spectrometer, which allows to perform a quantification without any sample pre-treatment. In order to confirm the XRF results, a further quantification of some elements was performed by Atomic Absorption Spectrometry (AAS, Avanta Σ GBC) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent 7700), after *aqua regia* digestion in a heating block (DigiPREP MS, SCP Science), using three replicates of each formulation. Dynamic light scattering (DLS - Avid Nano W130i) was used to determine the hydrodynamic diameter (Dh) of formulations and the polydispersity of particles. All formulations were diluted with ultrapure water to obtain a stock solution of 500 mg/L, from which three aliquots were taken and diluted to obtain three solutions with a concentration of 30 mg/L, which were analyzed.

2.3. Test species

One Basidiomycota and one Ascomycota species were used, *Lentinus sajor caju* and *Botrytis cinerea*, respectively. Whilst the first isn't responsible for infections in vines or grapes, the latter is responsible for a disease called "grey mold", or as commonly called "botrytis bunch rot" in viticulture. Both species were cultured at 25ºC. for 8 days, in Malt Extract Agar (MEA) (Thermo Scientific™ Oxoid™ Malt Extract Agar, dehydrated) before assays were conducted.

2.4. Exposure of *Lentinus sajor caju* to Cu-solutions

In a first experiment, *Lentinus sajor caju* was exposed to Cu-solutions in Petri dishes (diameter: 9mm) containing MEA (prepared with ultrapure water). Four commercial formulations (CH, KD, KO, BM) were tested at different concentrations, considering the RD of each one. Thus, for CH, KD and KO 5 concentrations were tested (1.5, 3, 5, 7.5 and 10 g/L), whereas for the BM, two concentrations were used (20 and 60 g/L), since the purpose of using BM was mainly to set up the concentrations for further experiments. The positive control, $CuSO_4$, was also tested at two different concentrations (4 and 12g/L). For each formulation a solution at the highest concentration was prepared using sterilized ultrapure water, and the remaining solutions were prepared by dilution. Solutions were kept under agitation and 500 µL were collected and spread evenly in the agar surface. A negative control was prepared by spreading only sterilized ultrapure water on the agar surface. Following this step, Petri dishes were inoculated with a circular 7mm mycelia plug removed from the edge of actively growing colonies, which were placed in the center of the agar. For each treatment, including the control, five replicates were prepared, and ensuing incubation was carried at 28ºC in the dark. During the assay, the diameters of the growing mycelia of each replicate were measured daily, using a ruler. Each measurement was taken three times. The assay was considered concluded when the mycelia of the control had covered the surface of the agar (with a mean diameter of 8.5 mm). The daily growth rate (DGR_{ab}) (mm day^{-1}) was calculated following Equation 11, based on the work of Venâncio *et al.* (2017).

$$DGR_{ab} = \frac{Db - Da}{t_b - t_a}\ mm\ day^{-1} \quad \text{(Eq. 11)}$$

Where:

D_b – mean diameter at the end of the assay (mm);

D_a – diameter at the beginning (7mm);
t_b-t_a – exposure time interval (in days).

The percentage inhibition of growth rate (%I_r) was then calculated following Equation 12.

$$\% I_r = \frac{DGR_c - DGR_t}{DGR_c} * 100$$

(Eq. 12)

Where:
DGRc – mean value for DGR in the control (mm day^{-1});
DGRt – mean value for DGR in the treatment (mm day^{-1}).

2.5. Exposure of fungi to amended MEA

In a second experiment, both species of fungi were exposed to MEA amended with three different formulations (CH, KO an BM) and with $CuSO_4$. The choice of formulations and concentrations to be tested was based on the results of the first experiment. First, it was conducted an experiment with *Lentinus sajor caju,* in which fungicides were weighted to obtain mediums with the following concentrations: 3 and 10 g/L for CH and KO; 20 and 60 g/L for BM; 4 and 12 g/L for $CuSO_4$. In a second step, an assay with *Botrytis cinerea* was carried out with a higher number of concentrations: 0.75, 1.5, 3 and 5 g/L for CH; 1.5, 3, 5 and 10 g/L for KO; 10, 20 and 40g/L for BM; 0.5, 1, 2 and 4g/L for $CuSO_4$.

All fungicides were incorporated in the medium post-autoclaving, with the MEA under constant agitation whilst still liquid, at a temperature of 40ºC. The mixtures were kept at agitation at 40ºC for 5 minutes to ensure homogenous incorporation of formulations, and then poured into the 9mm Petri dishes. For the control plates, the same procedure was replicated but without the addition of any fungicide. The inoculation of Petri dishes was performed with a circular 7mm mycelia plug removed from the edge of actively growing colonies. Five replicates were prepared for each treatment, and incubation took place in the dark, at 28ºC for *L. sajor caju* and 25ºC for *B. cinerea*. The remaining steps of the experiment occurred as previously described for the first experiment.

2.6. Determination of Cu content in solutions and amended MEA

The concentration of Cu in each solution and MEA media used in the fungal assays was determined by AAS (Avanta Σ GBC). For Cu-solutions, samples were acidified with 1% of nitric acid and diluted appropriately so that the final concentrations were within the limits of the calibration curve. For the analysis of effective Cu concentrations in MEA used in the

fungal assay, 1 ml of the medium was digested with *aqua regia* using a heating block (DigiPREP MS, SCP Science). The digests were diluted to 50 mL with ultrapure water and the Cu content was determined by AAS.

2.7. Data analysis

All endpoints were evaluated considering at least three replicates per treatment. Results of the characterization of Cu-based formulations are presented as mean ± standard deviation (SD). The effects of solutions of the three Cu-based formulations CH, KD and KO towards *L. sajor caju* were evaluated using a two-way ANOVA, defining as fixed factors the type of formulation and the tested concentrations. In cases of significant differences for any of the factors, a one-way ANOVA was performed. Effects of solutions of BM and $CuSO_4$ were evaluated using a one-way ANOVA. Whenever $p \leq 0.05$, the post-hoc Tukey's test was used to compare the mean of each group. For the effects of the exposure of *B. cinerea* to amended agar, an one-way ANOVA was used and, whenever $p \leq 0.05$, the post-hoc Dunnet's test was used to compare the mean of each group with the control. All statistical procedures were performed in Prism 8 (Graphpad Software Inc, USA).

3. Results and discussion

3.1. Characterization of Cu-based formulations

Overall properties, like Cu content, composition, toxicity to aquatic organisms and recommended doses (RD) for vines, are presented in Table 3. All information presented was given in information provided by brands, in the form of labels and technical or safety data sheets (SDS). The main features that differentiate the three formulations are: their Cu content (reduced in newer formulations when compared to CH); their formulation presentation (CH is a wettable powder whereas KD and KO are water-dispersible granules) and co-formulants present; toxicity (overall lower for KD and KO), and technological improvements in these recent formulations.

Table 3. Properties of commercial Cu hydroxide-based fungicides, as found on labels, technical and safety sheets, provided by respective brands.

Name	Champion WP	Kados	Kocide Opti
Brand	Nufarm	Certis	Certis
Cu content	50% (w/w)	35% (w/w)	30% (w/w)
Composition	$Cu(OH)_2$ (76.7%)	$Cu(OH)_2$ (50-70%) $Na_4P_2O_7$ (5-10%) NaOH (<2.5%) $C_{14}H_{26}O_2$ (<0.5%)	$Cu(OH)_2$ (25-50%) NaOH (<2.5%)
Formulation	Wet powder	Water dispersible granules	Water dispersible microgranules
Toxicity			
LC_{50} (96h) *O. mykiss*	0.0165 mg/L*	4.79 mg/L	0.24 mg/L
EC_{50} (48h) *D. magna*	0.038 mg/L*	1.61 mg/L	0.118 mg/L
EC_{50} (72h) *S. capricornutum*	0.0229 mg/L	-------	0.0516 mg/L
NOEC (21 days) *D. magna*	-------	0.0025 mg/L	0.012 mg/L
RD (for vines)	300 g/hL	200-300 g/hL	350 g/hL
Features	------	BioActive™ technology	BioActive™ technology

* tested substance: $Cu(OH)_2$

NTAF-Cu have lower Cu contents, but the RDs are similar to those from the traditional formulation (CH), which means that, according to the manufacturers, similar application doses from these can result in the same protection against fungal diseases than

their traditional counterparts, with less Cu. Regarding the composition of formulations, SDSs have undergone updates through the years, with different presentations being brought when describing their components. The most recent versions where used when constructing Table 3 (CH: V1.0 from 24/09/2019 – Appendix Figure A1; KD: V1.3.0 from 05/08/2020 – Appendix Figure A2; KO: SDS V1.2.2 from 01/04/2020 – Appendix Figure A3). However, looking to older versions of the same products enables for a greater insight on these formulations' composition. According to the SDS V1.0 from 12/03/2018 (Appendix Figure A4), CH formulation has sodium lauryl sulfate [$CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_nOSO_3Na$] as a co-formulant in a percentage ranging from 0 to 5%, besides $Cu(OH)_2$, the only substance described in the SDS V1.0 from 24/09/2019. Also, regarding KO, from an older SDS (V1.1 from 29/01/2018, Appendix Figure A5) it's possible to see that polyacrylic acid [$(C_3H_4O_2)_n$] is also used as a co-formulant (5-10%) in addition to NaOH.

Regarding toxicity, the lower values observed for the traditional formulation show a higher toxicity towards aquatic organisms, which is in line with the concentration of Cu present, however it should be noted that with exception of *S. capricornutum*, the tested substance was $Cu(OH)_2$ and not the formulation. On the other hand, the difference in toxicity for the two NTAF-Cu should be related with other factors rather than the Cu content, since this is lower in the most toxic formulation. One can possibly presume that such a difference should be due to different co-formulants or the different particle size.

Looking at different versions of SDSs that were produced over time, something relevant can be observed: whilst maintaining the same product, different SDSs have come out with different toxicity values towards aquatic organisms. For instance, for KO, the LC_{50} values for *O. mykiss* and EC_{50} for *D. magna* were 4.79 mg/L and 1.61 mg/L, respectively, according to the SDS V1.1 from 29/01/2018 (Appendix Figure A5). However, the new SDS V1.2.2 from 01/04/2020 presents much lower values (as seen on Table 3). So, even though the product was kept the same, the recent SDS refers to a more toxic description of the formulation. Indeed, the more recent toxicity data is in line with the data provided by the previous supplier of the Kocide® brand, Dupont, as can be found in SDS V4.0 from 30/11/2015 (Appendix Figure A6). This means that after Kocide brand was bought by Certis, which is now the company responsible by KO commercialization in Portugal, although the same product was kept, SDS changed their toxicity values, presenting the same values as other Cu hydroxide-based fungicides provided by this company. Even that the toxicity values for aquatic organisms have been now updated, it is important to note that during at least 2 years the information given in the SDS was not correct.

Both KD and KO are NTAF-Cu and are characterized by their reduced particle size (1.8-2.5 µm), but they differ on their co-formulants, as seen on Table 3. However, they both have a special patented-formulant (BioActive™ technology) that binds the smaller particles

to form Cu complexes. This allows to fulfill the goal of obtaining formulations with two sources of Cu ions: reduced particle size of $Cu(OH)_2$ for immediate usage and complexed Cu for residual activity. The co-formulant, besides being used to obtain formulations with extended residual activity, also allows for better foliar coverage and persistence to wash-off by rain, and higher retention on the surface of plants as it forms an adhesive film[2]. KO is the available formulation in Europe equivalent to Kocide® 3000 (SDS V1 from 20/02/2017, Appendix Figure A7), which is commonly used and found in the existent scientific literature as an example of a nanoformulation (Adeleye *et al*., 2014; Simonin *et al*., 2018; Tan *et al*., 2018; Zhao *et al*., 2017). According to some of these studies that characterized this formulation, KO is formed by spherical composites of around 50 µm made up of $Cu(OH)_2$ particles with irregular size (from nano- to microscale) which are embedded in a primarily carbon-based matrix that breaks down when in aqueous media (Adeleye *et al.*, 2014; Conway *et al.*, 2015).

Characterization of formulations with SEM allowed for a semi-quantification of Cu and other major elements present in the three formulations (Figure 11). Despite being a very powerful non-destructive technique, the percentages obtained by SEM aren't precise, since it doesn't use any reference samples, and the total mass of the formulation is considered to be the sum the of masses of major elements (ME) detected. The XRF analysis gives a more precise quantification of the chemical composition and it's a more sensitive method which allowed to quantify ME (Table 4) and trace elements (TE) (Table 5). However, for ME results are also presented as a percentage of the total mass of ME detected, after a correction of the loss-on-ignition content. For these reasons, the two methods gave different results of percentage of Cu in each formulation. Using SEM, the Cu content was 70, 43 and 19% for CH, KD and KO, respectively (Figure 11); whereas using XRF it was 60, 43 and 33% for CH, KD and KO, respectively (Table 4). These results show different contents of Cu than those described in formulations composition provided in labels and SDSs, probably because of the quantification procedure used in both methods. However, it still shows the same decreasing ratio of Cu between formulations (CH>KD>KO). In order to obtain a precise quantification of Cu the content in the three formulations, they were analyzed by AAS after an acid digestion (Table 6). When looking to these results it's possible to conclude that the content of Cu in the formulations matches the information provided by the suppliers.

Both methods, SEM and XRF were very helpful to identify major constituents of the formulations and thus confirm that the co-formulants present in the three commercial formulations are different. Regarding SEM results, although the quantification of C and Al

[2] https://www.certiseurope.com/news-media/news/articles/news/developments-in-copper-fungicides/

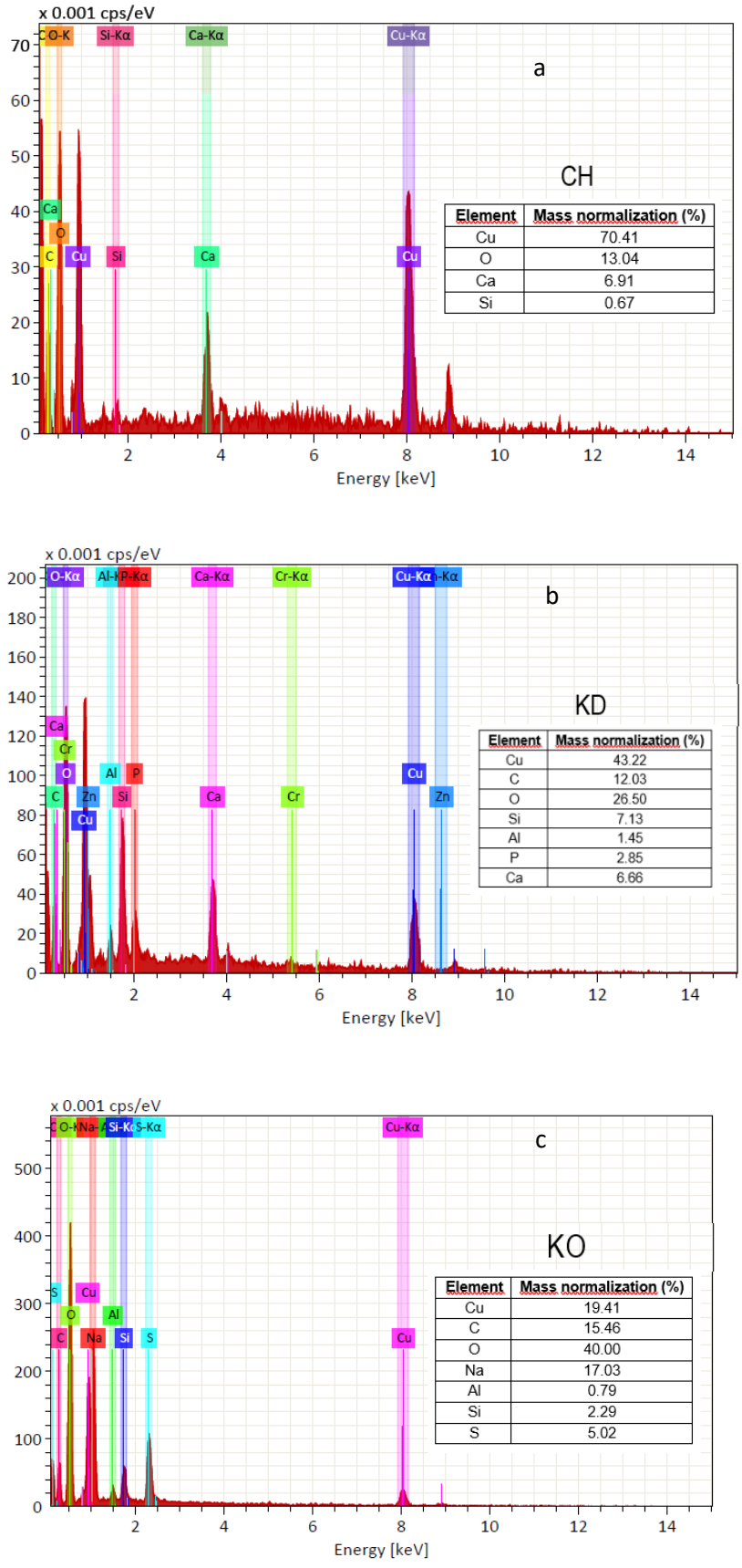

Figure 12. Semi-quantification of elements by SEM for the three Cu hydroxide formulations: a) Champion; b) Kados; c) Kocide.

may be influenced by the bracket (that is made of Al) and the adhesive tape (made of carbon) that supports the formulation powder, which interferes with readings of these elements, it's still interesting to interpret the C content of formulations. NTAF-Cu showed higher levels of C than the traditional formulation, with higher levels for KO than KD, which might support the previous idea that C is used as part of the co-formulant that revests Cu-particles (Adeleye *et al.*, 2014).

Table 4. Quantification of major elements (ME) in Cu hydroxide formulations by XRF. TE stands for trace element; ND stands for elements not detected. Values presented in bold represent those higher than 1%.

Element (%)	CH	KD	KO
Cu	**60.3**	**43.4**	**32.8**
Al	0.145	0.807	**1.33**
Ba	0.090	0.055	0.047
Ca	**5.59**	**7.77**	0.208
Ce	TE	0.010	0.012
Cl	0.017	0.031	0.226
Fe	0.018	0.396	0.757
Hf	0.483	0.292	0.234
K	0.022	0.099	0.087
Mg	0.037	0.193	0.261
Mn	0.021	0.007	0.016
Na	ND	**3.32**	**10.8**
P	0.060	**1.43**	0.067
Pb	0.013	TE	TE
S	0.836	0.099	**4.38**
Si	**1.10**	**2.67**	**3.98**
Sn	TE	0.018	0.015
Ta	0.768	0.455	0.373
Ti	ND	0.029	0.040
Zn	ND	0.187	ND

Other major elements present in higher quantities, like Na, P, S, can, in most cases, be justified by other elements used in formulations as co-formulants, as described before. For example, KD has sources of Na and P in its formulation, as well as for S in CH. Even so, other elements like Si and Ca don't come listed in labels of formulations and quantification by XRF shows significant values of these elements in almost all three formulations. In fact, brands don't provide the total composition of their formulations, so these elements may be added but not clarified in the ingredients list. Furthermore, KO is the formulation with more major elements in high quantities that don't come clarified in its provided ingredients list, like Na, Al, Si and S, which might suggest the use of some of these elements as co-formulants to obtain a NTAF-Cu.

Several trace elements were detected in Cu-formulations and quantified by XRF, as can be observed in Table 5.

Table 5. Quantification of trace elements (TE) of Cu hydroxide formulations by XRF; ME stands for major element; ND stands for values below the detection limit.

Element (ppm)	CH	KD	KO
Ag	ND	5.3	ND
Br	ND	7.7	6.3
Cd	ND	9.9	5.4
Ce	38.2	ME	ME
Cr	25.2	57.1	74.4
Ge	10.7	5.8	5.2
La	43.1	49.2	69.6
Mo	5.1	3.8	3.4
Nb	ND	2.0	2.2
Nd	8.9	48.3	37.2
Pb	ME	47.8	36.3
Rb	5.8	6.1	5.8
Sc	89.9	60.8	33.9
Sn	16.4	ME	ME
Sr	65.8	28.2	15.3
V	ND	5.9	7.6
Y	1.1	3.8	5.6
Zr	ND	20.3	30.3

The presence of some of these elements in formulations can be related with the co-formulants used or they can be of natural origin, since they can be present in the ore used as raw material for Cu extraction. However, the hypothesis that cross-contamination during analysis may have occurred should not be ignored, as well as the interferences and chemical noise in the analysis of XRF. This is especially true for several elements that belong to the rare-earth elements were detected, like Sc, La and Nd. In order to confirm which are the elements that result from contamination or interferences during analysis, rather than be present in the composition of the formulations, a quantification was performed by AAS and ICP-MS (Table 6).

*Table 6. Quantification of Cu and other elements in the three formulations, by AAS (marked with an *) or ICP-MS.*

Elements	CH	KD	KO
Cu* (%)	50.9 ± 3.5	35.5 ± 0.9	30.8 ± 3.6
Ag (mg/Kg)	5.67	5.22	7.36
Ce (mg/Kg)	0.330	1.38	3.07
Cr (mg/Kg)	ND	ND	3.05
La (mg/Kg)	0.095	0.619	1.51
Mo (mg/Kg)	2.31	2.99	5.47
Nd (mg/Kg)	ND	0.501	1.56
Ni (mg/Kg)	ND	73.4	38.0
Pb *(mg/Kg)	38.3 ± 1.2	13.6 ± 6.5	10.7 ± 3.2
Rb (mg/Kg)	5.62	6.11	6.55
Sb (mg/kg)	3.61	3.85	5.34
Sn (mg/Kg)	11.9	33.2	18.5
Sr (mg/Kg)	75.4	30.4	17.1
Zn* (%)	ND	21.7 ± 1.6	0.0359 ± 0.0002

Results of TE are different between techniques (XRF or AAS and ICP-MS), but even though values aren't the same, the trend of ratios is maintained. AAS or ICP revealed the presence of Zn, Pb, Cr, and Ni. However, only Pb seems to follow increasing contents of Cu, which likely means that Pb is happening in formulations due to natural contamination of the ore used to extract Cu. These other TE might be related with co-formulants. Sn and Sr also seem to be at high concentrations for CH, this might reveal a higher presence of

these metals in the raw material used for the extraction of Cu utilized to produce CH formulation.

The use of SEM also allowed to assess differences in the aspect and surface of the formulations through the analysis of micrographs (Figure 12), as well as to measure particles diameter (Table 7).

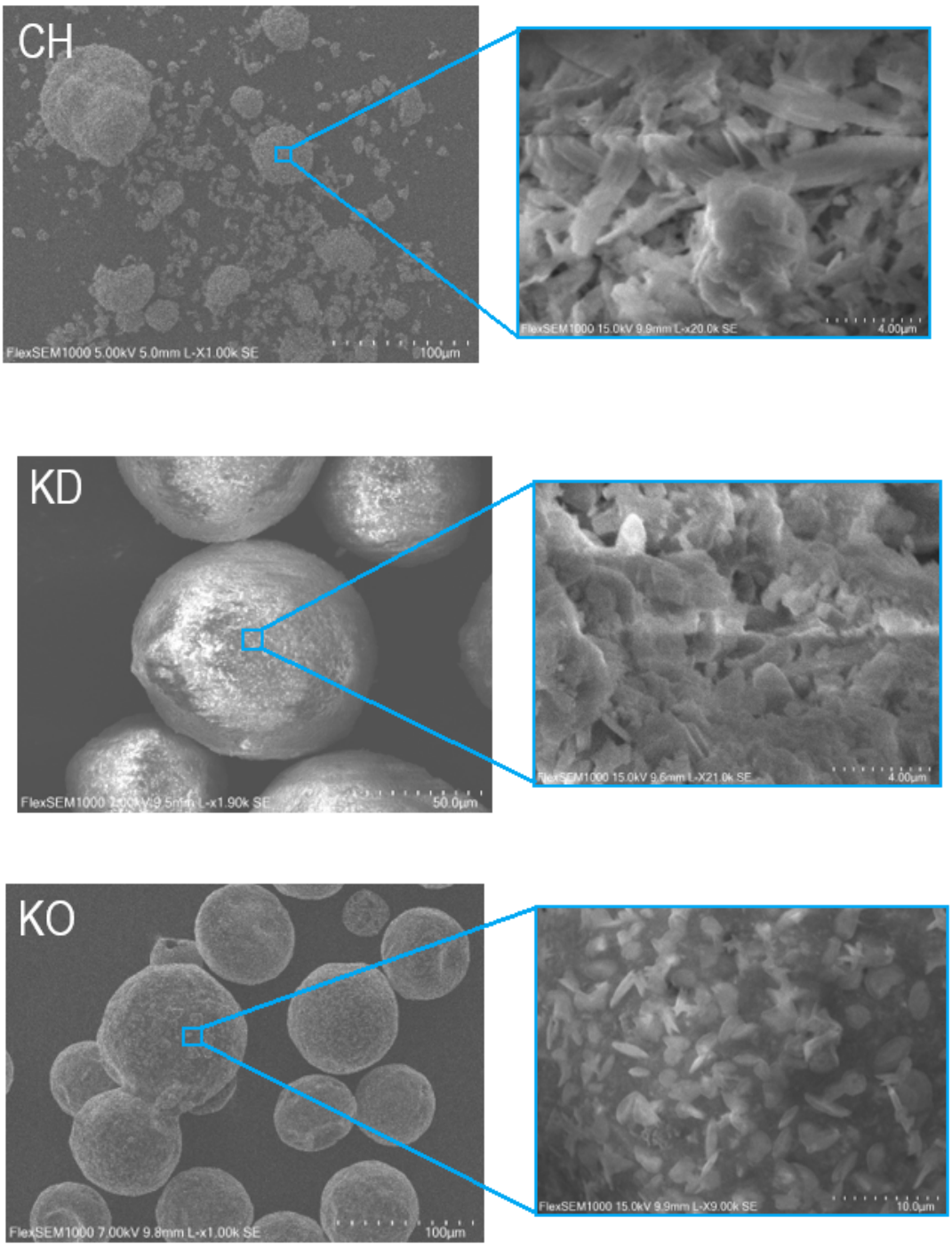

Figure 13. SEM micrographs of CH, KD and KO, with insert showing close-up of surface.

Through the analysis of images obtained from SEM (Figure 12), especially the ones showing a close-up of the surface of formulations particles, it seems possible that NTAF-Cu have particles with a higher surface area, as one can see by the intricate aspect of the surface, when compared to the coarser look of CH particles surface. From the results of measurements of particles through the analysis of micrographs obtained from SEM it is possible to observe that, without dissolution of formulations, CH particles present the lowest mean diameter size (KD>KO>CH), as can be observed in Table 7. Indeed, this formulation comes as a powder whereas the NTAF-Cu formulations have the form of granules. Also, to be noted, for CH the analysis by SEM produced a low number of micrographs that allowed to measure particles using ImageJ, which might explain low values of standard deviations despite the highly heterogeneous look of particles, as can be observed in Figure 12.

DLS analysis results were presented as the mean Dh and polydispersity of the main peak of assessed formulations (Table 7). This decision was made instead of representing the mean particle size (Z-ave) and polydispersity index (PI), because the great majority of readings provided Z-ave values with high standard deviations, and PIs constantly greater than 0.7, which indicates that the samples have a very broad size distribution and are probably not suitable for DLS analysis. However, results were still presented because some interesting conclusions may be taken from these, even if with limitations. Foremost, results suggest that, when dissolved in water, CH forms larger particles than KD and KO. CH formulation didn't form any NPs when dissolved, and its particle mean size is clearly far larger than the purpose of DLS analysis, with high polydispersity, revealing a heterogeneous arrangement of particles of CH when in solution. For both KD and KO their main mass peaks revealed that most particles, as in terms of mass, were at the high end of the nanoscale (10^2nm), whilst their high polydispersity reveals a very broad size distribution of particles. This could mean that, when dissolved, formulations of KD and KO form some larger particles and a majority of nano-sized particles, meaning that larger particles are influencing the quality of the reading provided by DLS, resulting in high polydispersity values and high variability of sizes. Still, whilst produced values of Dh cannot be considered due to the limitations of the results, we can possible assume that these NTAF-Cu, despite being produced at the microscale, when dissolved in water form particles around 10^2nm and some larger particles. This can perhaps validate the Bio-Active™ technology of KD and KO.

This characterization of Cu-formulations allowed for a better insight on how NTAF-Cu perform when compared to traditional formulations, as well as clarifying labels and information provided by brands that commercialize these products. Summarizing the main aspects from all the information discussed above (Table 7), some conclusions may be drawn. Foremost, particle size of formulations as described in labels and technical information might be related to particle size when formulations are dissolved in water, since

SEM results show far larger diameters for particles than those mentioned by brands (1.8-2.5 µm). Diameters of particles from dry powders of these new formulations are actually bigger than those from CH. However, when in solution, KD and KO seem to produce smaller Cu particles than CH. Without considering actual measurements from DLS results, due to the limitations of this technique with broad size distribution solutions, we can still possibly assume that CH forms particles still at the microscale when dissolved, whilst KD and KO possibly produce two distinct Cu hydroxide particles: the majority at the nanoscale, and most likely the ones responsible for immediate toxicity, and some other bigger particles, which can possible be the advertised complexes used for residual effects. Furthermore, quantification techniques of elements produced different values for the determination of the Cu content of formulations. When looking at XRF results, at first it could seem that these formulations actually contain superior contents of Cu than those described in the ingredient's composition. However, considering AAS analysis, which is the only one used that doesn't perform any mass normalization when expressing results, Cu contents match those indicated by producers, meaning that labels information is most likely accurate regarding this information. Also, all the analysis performed (SEM, XRF, AAS and ICP-MS) allowed to determine the presence of several elements, probably used as co-formulants since high concentration values were found, that don't come described in the ingredient lists of these products, which might have to be included when analyzing environmental concerns of the application of such formulations.

Table 7. Summarized physicochemical properties of Cu hydroxide formulations.

Property	**CH**	**KD**	**KO**
Particle diameter (µm)[a]	46.46 ± 18.50	109.38 ± 35.93	70.36 ± 16.83
Hydrodynamic diameter (nm)[b]	1684.04 ± 2190.53	131.10 ± 55.71	145.28 ± 163.23
Polydispersity (%)[b]	238.85 ± 24.15	83.27 ± 20.35	116.26 ± 48.07
Copper content (%)[c]	50.9 ± 3.5	35.5 ± 0.9	30.8 ± 3.6
Other ME present[a,d]	O, Ca, Si	C, O, Si, Al, P, Ca, Na	C, O, Na, Al, Si, S
Other TE present [c]	Pb, Sn, Sr	Ni, Pb, Sn, Sr Zn	Cr, Ni, Pb, Sn, Sr, Zn

[a] Dry powder measured with SEM; [b] Measured via DLS in ultra-purified water; [c] AAS/ICP-MS analysis; [d] Analysis done by XRF.

3.2. Exposure of *Lentinus sajor caju* to Cu-solutions

The mycelium of *L. sajor caju* required 11 to 12 days to cover all the surface of MEA in the control Petri dishes. The technique used in this first experiment, however, presented some limitations. Although the experimental design was based on similar experiments, that tested the toxicity of NPs to this same species of fungus (Galindo *et al.*, 2013), some problems were encountered when using solutions as the form of exposure of the mycelium to Cu-formulations. Firstly, between the 4th and 5th day of the experiment, some new growth spots were starting to get noticed in the agar of several petri dishes (as seen on Figure 13). This was probably due to water/solution that still remained at the top of the agar, which dispersed fragments of the mycelia plug to other parts of the petri dish. This can have potentially influenced the results, especially the measurements taken from day 4th and onwards, as these new spots grew and couldn't be distinguished from the radial growth of the mycelia plug in the center. Another problem that was clearly visible when assembling the experiment was the heterogeneous distribution of Cu-solutions on the agar. When added to water, formulations don't get fully dissolved, with particles still remaining visible and even depositing as time passes. This resulted in a spotty distribution on the top of the agar, even with solutions being carefully spread. The same was not enlightened in the work of Galindo *et al.* (2013), as NPs were used as already homogenized dispersions for further dilutions. This might suggest that solutions made from commercially available formulations, that don't dissolve completely in water and don't form homogenous solutions, are probably not the best vehicle to expose fungi mycelia to Cu-formulations, because the growing mycelia encounters different degrees of contamination throughout the agar.

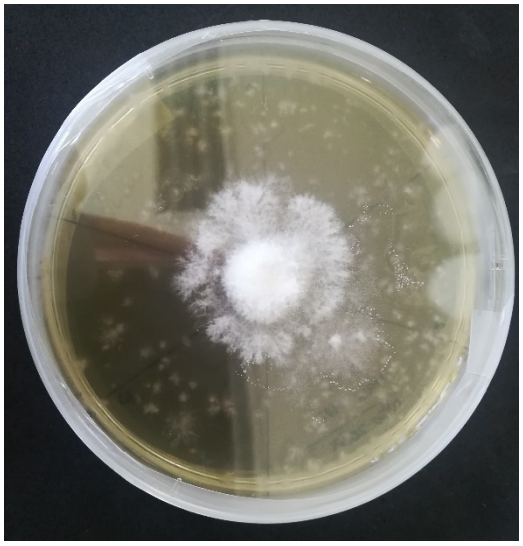

Figure 14. Petri dish of L. sajor caju exposed to KO at 0.89g Cu/L at the 4th day of experiment.

Figure 14 shows the mean diameter of *L. sajor caju* mycelium over the time, after exposure to different formulations (of both $CuOH_2$ and $CuSO_4$), expressed in its Cu content.

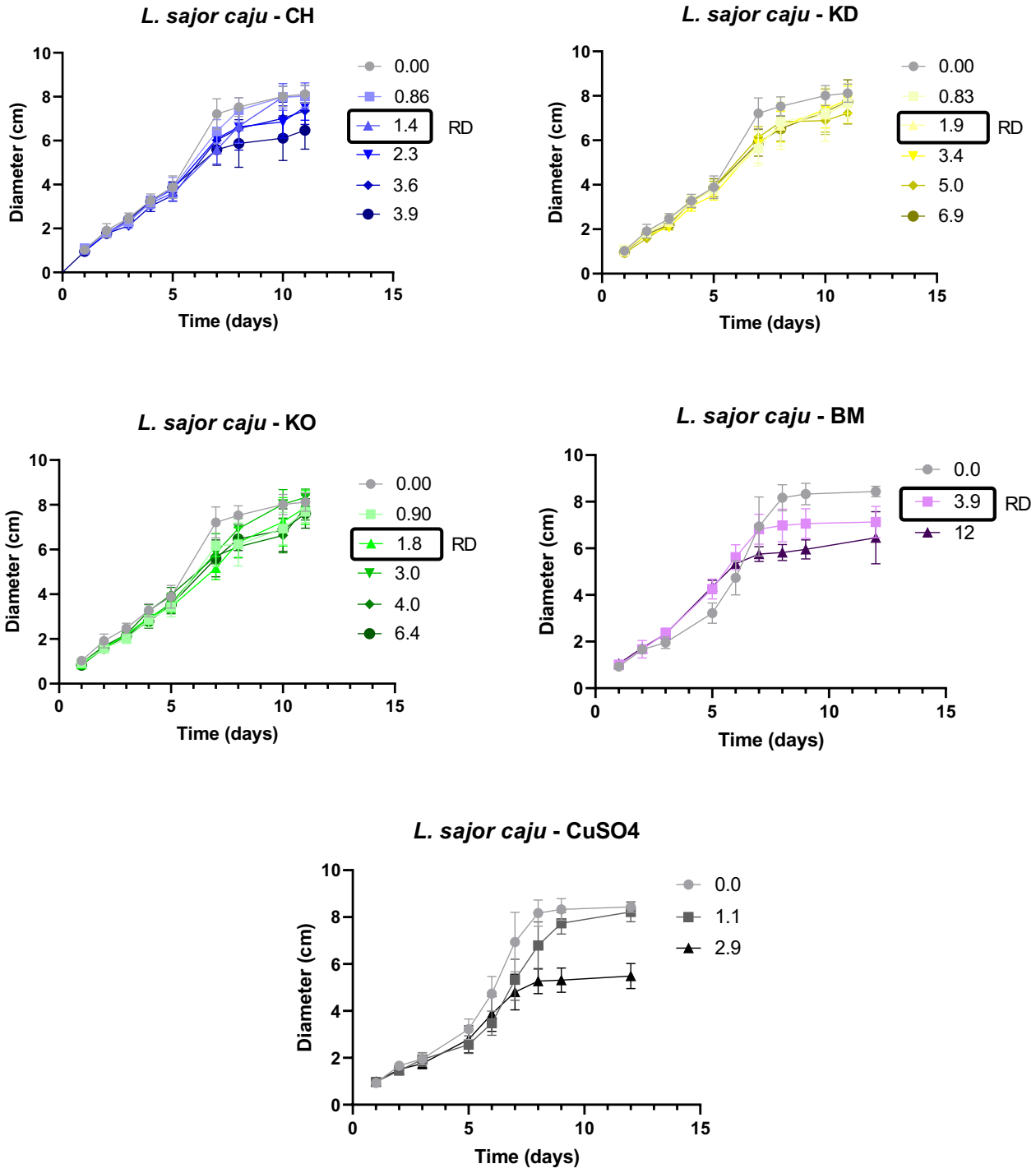

Figure 15. Mean diameter (cm) and standard deviation (error bars) of L. sajor caju mycelium after exposure to Cu-solutions of formulations and $CuSO_4$ (gCu/L).

The correspondence between Cu content in the formulation and the concentration of formulation can be found in Table 8.

Table 8. Percentage of inhibition of growth of L. sajor caju mycelium after exposure to solutions of Cu formulations and $CuSO_4$.

Formulation	[formulation] g/L	Expected [Cu] %	[Cu]* g/L	[Cu]* %	% Inhibition end assay
CH	1.5	50	0.86	58	1.6 ± 6.5
	3.0		1.4	47	1.2 ± 6.1
	5.0		2.3	46	8.1 ± 5.8
	7.5		3.6	35	10 ± 8
	10		3.9	39	22 ± 12
KD	1.5	35	0.83	55	5.7 ± 7.4
	3.0		1.9	64	6.1 ± 12
	5.0		3.4	69	4.0 ± 7.3
	7.5		5.0	67	12 ± 4
	10		6.9	69	5.3 ± 12
KO	1.5	30	0.90	60	5.9 ± 5.7
	3.0		1.8	59	3.3 ± 7.7
	5.0		3.0	60	-2.7 ± 4.7
	7.5		4.0	53	3.4 ± 5.8
	10		6.4	64	6.9 ± 9.2
BM	20	20	3.9	20	17 ± 8
	60		12	21	26 ± 15
$CuSO_4$	4.0	26	1.1	29	2.8 ± 3.3
	12		2.9	24	38 ± 4

*Concentration of Cu determined by AAS.

From the analysis of data in this table, it becomes clear that solutions made from formulations didn't had Cu contents corresponding to what was expected. Stock solutions were prepared by properly weighing formulations and dissolving them in water to obtain desired concentrations, as manufacturer instructions for regular use of these products. However, it became clear that for all formulations (with exception of $CuSO_4$) the result were highly heterogeneous solutions, with suspended particles as described before. Tested

solutions were aliquots from these stock solutions, and, even though stock solutions were kept at constant agitation when pipetting, there was a concern of obtaining highly variable aliquots. Thus, it was decided to analyze results regarding Cu concentrations determined by AAS, since these were the real values of Cu that *L. sajor caju* was exposed to, confirming the suspects about the heterogeneity of the solutions. Even so, conclusions may be drawn by the effectiveness of the different forms of Cu provided by different formulations.

From Figure 14 we can possibly see that differences between treated samples and the control seemed to appear only after the 8th day, and they seem to be more evident for traditional formulations (CH and BM) and for $CuSO_4$. However, due to already discussed limitations of this experimental design, measurements might have been influenced from the off-radial growth of mycelium, so we decided to strict our analysis to data obtained only from measurements of the last day of the assay. Results of $\%I_r$ are presented in Table 8.

Further conclusions require statistical analysis of results, so a two-way ANOVA was performed comparing the three formulations of $Cu(OH)_2$ and their concentrations, followed by an one-way ANOVA with a Tukey's multiple comparisons test. The two-way analysis revealed that there is a significant interaction between formulations and concentrations (p<0.05), and that concentrations had a significant effect in the diameter of mycelium of *L. sajor caju* (p<0.05) (Appendix Table A3). However, this only indicates that at least one group of measurements had a mean value significantly different from at least one of the mean measurements of the remaining groups. Thus, looking at the results produced by the *post-hoc* analysis it is possible to see that only for CH it was observed a significant difference (p<0.05) between the higher concentration and the four lowest concentrations in the last day of the assay (Appendix Table A4). This means that none of the tested concentrations of Cu of NTAF-Cu were able to significantly inhibit the growth of *L. sajor caju* mycelium, even those above the RD. The same can be observed by looking at $\%I_r$ (Table 8) and plotted in Figure 15. Likewise, the dose-response effect of CH seems to be coherent with increasing concentrations of CH, which isn't verified for the NTAF-Cu.

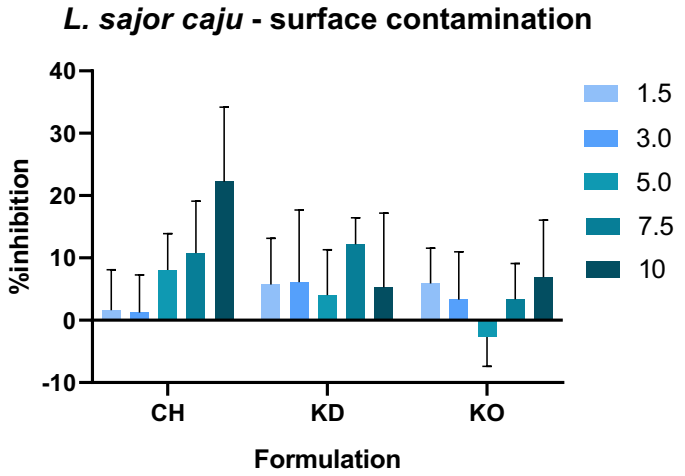

Figure 16. Percentage of growth inhibition of L. sajor caju mycelium after exposure to solutions of $Cu(OH)_2$ formulations (g/L).

For data obtained from the exposure of *L. sajor caju* to BM and $CuSO_4$ an one-way ANOVA was performed for each formulation, followed by a Tukey's multiple comparisons test. By the analysis of the output from the one-way test (Appendix Table A5) it is possible to conclude that there's at least two treatments that resulted in significantly different diameters of mycelium ($p<0.05$). The *post-hoc* test (Appendix Table A6) shows that both treatments of BM and the highest treatment of $CuSO_4$ (12g/L) had significantly reduced mycelium growth when compared to the control ($p<0.05$). Altogether, we can possibly conclude that traditional treatments (CH and BM) and $CuSO_4$ were the only capable of significantly reducing the growth of *L. sajor caju* mycelium, at least for the conditions tested, showing higher efficiency than NTAF-Cu.

However, due to all the limitations of the experiment design of this experiment, it was decided to change our experimental design in the second experiment.

3.3. Exposure of fungi to amended MEA

In a second experiment both *L. sajor caju* and *B. cinerea* were exposed to MEA amended with Cu-formulations and $CuSO_4$. This approach was selected based on the work of Martins *et al.* (2012), where amended agar medium was used for similar purposes. It was expected that, by contaminating the agar and not its surface with solutions, the problem of dispersion of mycelia fragments could be resolved, as no free liquid would be at the top of the agar. Also, it could possibly allow for a better dispersal of Cu-formulations through the agar, taking advantage of MEA viscosity and the lack of necessity to make stock solutions. Thus, a preliminary experiment to set up the best working methodology was performed, where *L.*

sajor caju mycelium was exposed only to two formulations of $Cu(OH)_2$ (CH and KO), leaving KD out, to maintain only one traditional formulation and the most recent NTAF-Cu, and to BM and $CuSO_4$. Only two concentrations were tested (a low and a higher one), to understand the range of concentrations that should be tested next.

The mycelia of *L. sajor caju* took a total of twelve days to occupy the surface of the agar on control plates, when the experiment was considered finished. The results of this preliminary experiment are plotted in Figure 16. Results are expressed as concentrations of Cu in the agar, determined by AAs after acid-extraction. The $\%I_r$ was also calculated and results are presented in Table 9. From the analysis of results, it is possible to observe that the highest tested concentrations were in fact too high and total inhibition of the growth of mycelium was verified for all formulations. For the DR of CH and for both concentrations of $CuSO_4$ total inhibition was also found.

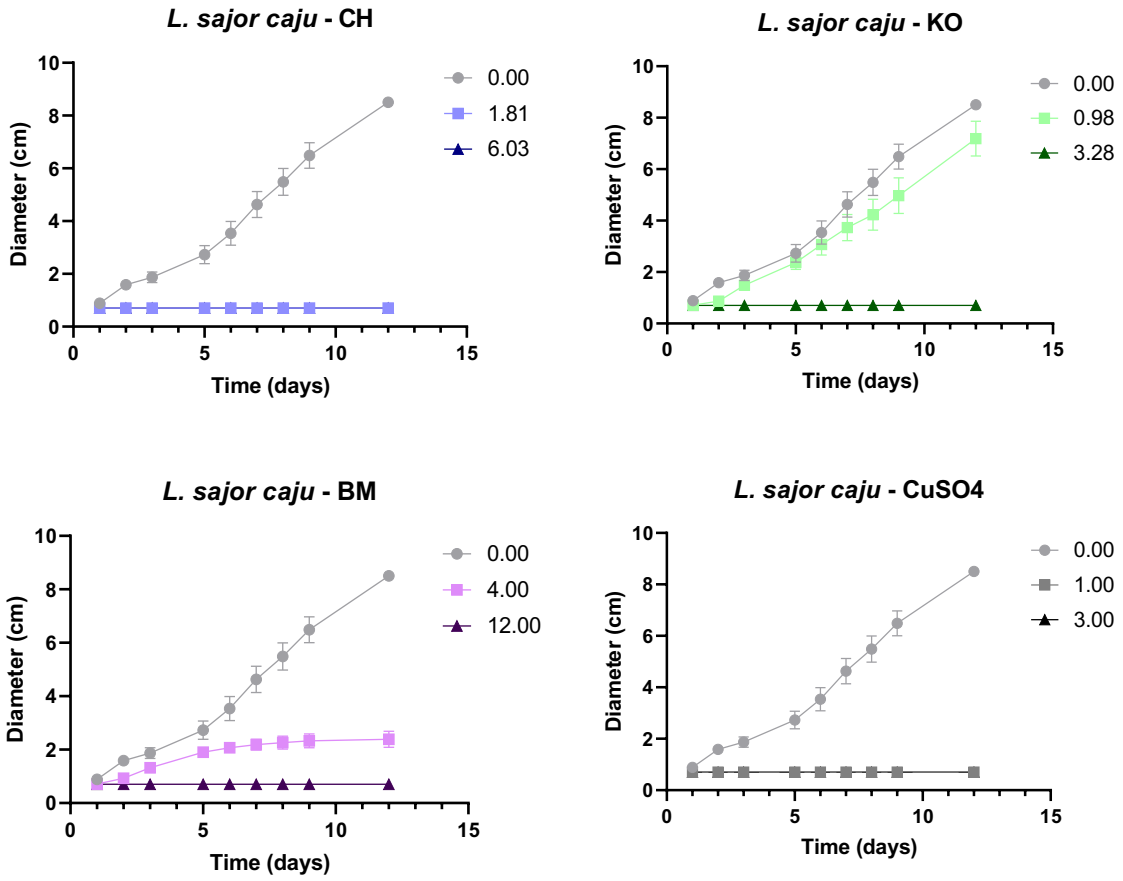

Figure 17. Mean diameter (cm) and standard deviation (error bars) of L. sajor caju mycelium after exposure to amended MEA with Cu formulations and $CuSO_4$ (gCu/L).

We then exposed *B. cinerea* to different ranges of the same Cu formulations used before, as well to $CuSO_4$. The mycelia of *B. cinerea* also took twelve days to occupy the surface of the agar on control plates, when the experiment was considered finished. Results

are plotted in Figure 17 referring to Cu contents in the agar. The $\%I_r$ were also calculated and are presented in Table 9.

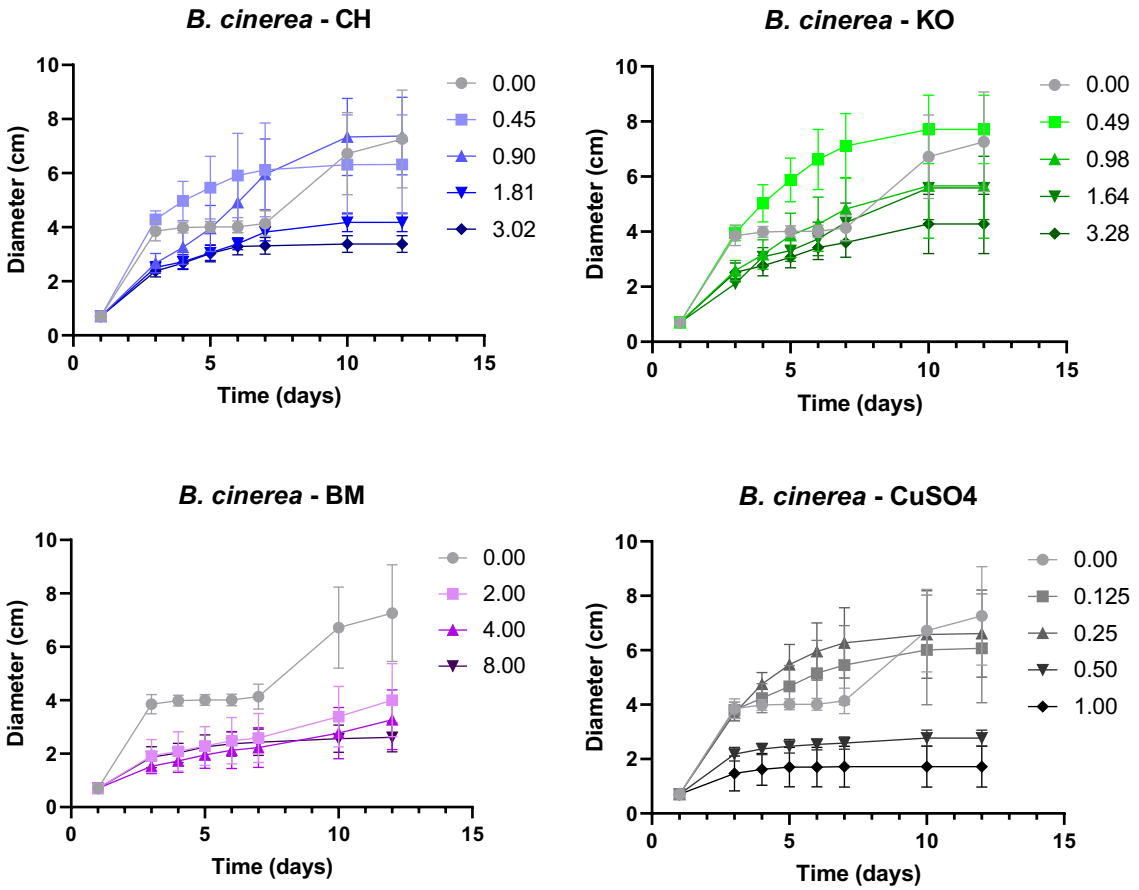

Figure 18. Mean diameter (cm) and standard deviation (error bars) of B. cinerea mycelium after exposure to amended MEA with Cu formulations and CuSO4 (gCu/L).

An one-way ANOVA was performed for measurements collected from the last day of the assay, for each Cu formulation and $CuSO_4$ that *B. cinerea* mycelia was exposed to, followed by Dunnet's multiple comparisons test (Appendix Table A7) and results are presented in Figure 18. The integrated analysis of both $\%I_r$ and the results obtained by the one-way ANOVA analysis can likely tell us that all formulations were being effective at inhibiting the growth of mycelium at doses below the RD for BM (for 10g/L) (p<0.05), or at RD and superior doses for all the others. These results presumably show that all formulations, including traditional ones (CH and BM) and the NTAF-Cu (KO) are able to inhibit fungal growth for *B. cinerea* at recommended doses of application.

Table 9. % of inhibition of growth of L. sajor caju and B. cinerea after exposure to amended MEA with Cu formulations and $CuSO_4$.

Species	Formulation	Concentration (formulation) g/L	Concentration (Cu) g/L	% Inhibition end assay
L. sajor caju	CH	3.0	1.81	100 ± 0
		10	6.03	100 ± 0
	KO	3.0	0.98	17 ± 8
		10	3.28	100 ± 0
	BM	20	4.00	78 ± 4
		60	12.00	100 ± 0
	$CuSO_4$	4.0	1.00	100 ± 0
		12	3.00	100 ± 0
B. cinerea	CH	0.75	0.45	44 ± 1
		1.5	0.90	11 ± 19
		3.0	1.81	54 ± 3
		5.0	3.02	64 ± 4
	KO	1.5	0.49	-1.3 ± 3.4
		3.0	0.98	34 ± 27
		5.0	1.64	35 ± 13
		10	3.28	52 ± 15
	BM	10.00	2.00	56 ± 19
		20.00	4.00	66 ± 16
		40.00	8.00	75 ± 6
	CuSO4	0.50	0.13	48 ± 11
		1.00	0.25	35 ± 18
		2.00	0.50	72 ± 4
		4.00	1.00	86 ± 11

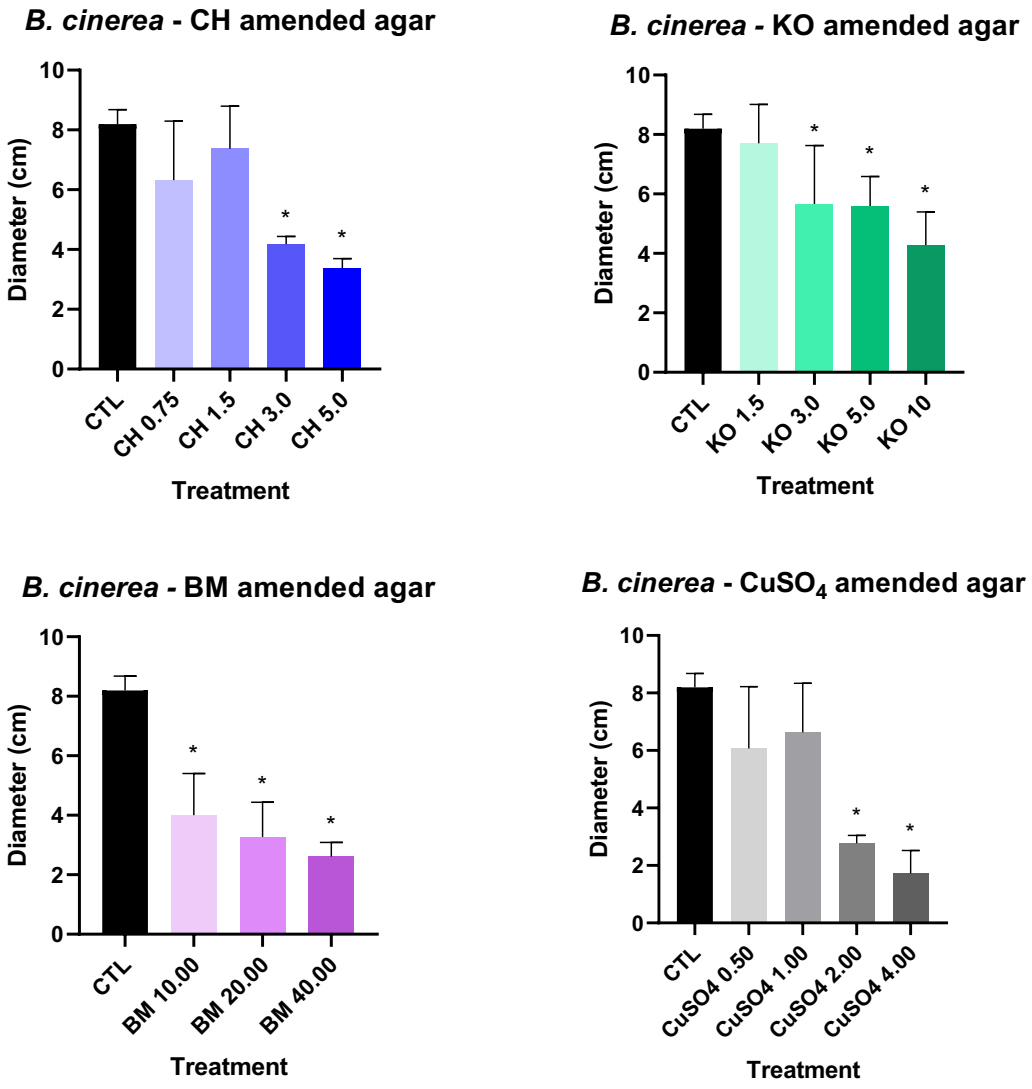

*Figure 19. Plotted results of B. cinerea mycelia exposed to Cu formulations and CuSO4. * represents statistical significant differences from the control.*

Figure 19 represents an overall summary of the information presented above, by combining the $\%I_r$ of both species of fungi when exposed to amended agar at RD of Cu formulations. From its analysis it's possible to conclude that overall, KO, the NTAF-Cu, showed the lowest efficiency in inhibiting the growing of both species, whilst traditional formulations were the most efficient. Also, *L. sajor caju* shows a particularly low sensitivity to KO, although being extremely sensitive to RD of CH and BM. Likewise, *B. cinerea* seemed to be more inhibited by the newer formulation of Cu, even at recommended doses, something that wasn't shown to *L. sajor caju*. These results, however, need to be considered with precaution, since the methodology applied probably allowed all Cu in the formulations to be available since the beginning of the assay. This means that KO, the NTAF-Cu tested, shown toxicity thanks to delivery of Cu ions that became available due to the high temperature of the agar, losing its slow release behavior. This way, the

methodology applied only allowed us to compare toxicity of absolute concentrations of Cu towards both fungi species, so high inhibition of their growth could be expected.

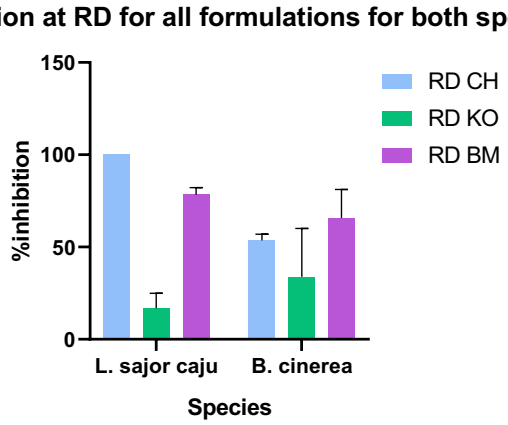

Figure 20. Graphical representation of %Ir for both fungi species exposed to amended agar with RD of CH, KO and BM.

Conclusions and Final Remarks

This work allowed for a better insight into organic viticulture and the effects of the use of Cu-based fungicides towards soil health, namely through its effects on soil micro and macrofauna, and to aquatic organisms that may be exposed to their leachates. Due to recent concerns and limitations on Cu use as a fungicide in agriculture, we found relevance in understanding the extent of its consequences on the particular scenario of the Douro vineyards. Whilst a pronounced accumulation of Cu seemed to be happening in the soils of the studied vineyard, most likely due to enrichment by the use of fungicides used to treat downy mildew, only a small fraction was readily available for organism's uptake. The same could also be concluded by the results of ecotoxicological assays to the battery of species assessed. Regarding soil organisms, the soil habitat function didn't seem to be compromised by Cu contents, since *E. fetida* didn't avoid the soil samples collected when treatments were applied. And, although earthworms have accumulated Cu in their tissues, our data couldn't allow further understanding on its effects on viability of *E. fetida* and its reproductive outcome. However, *F. candida* wasn't compromised by higher levels of Cu of soil samples from July. The integration of these data can most likely show that Cu contents of soils from "Quinta do Síbio" aren't able to compromise terrestrial organisms' fitness. Regarding indirect exposure of sampled soils, any of the tested aquatic organisms seemed to be impacted by soil constituents available at the aqueous phase, with the exception of *A. fischeri*. Altogether, when significant impacts of the exposure to soil samples from July were verified, the same weren't kept when analyzing performing results of samples from February. Such results might indicate that, if existent, impacts of Cu towards organisms are surpassed and biological communities can transcend these effects, showing the resilience of the ecosystem and the apparent lack of permanent consequences. However, these conclusions are scenario-specific, meaning that information provided by this work might not be extrapolated to other situations. For "Quinta do Síbio", and thanks to information provided by Real Companhia Velha, we were able to assess Cu inputs in these vineyards, allowing the realization that total Cu values were eligible in the current legislation. So, for these particular conditions, Cu-based fungicides seem to have been used in a sustainable way, allowing disease protection of grapevines without compromising the ecosystem functioning.

Results obtained from the first chapter of this work, however, don't and shouldn't allow for neglection of the current concerns of extensive Cu use as a fungicide, since these are sustained and relevant. Even still, the search of alternatives to Cu in agriculture has resulted in the introduction of new formulations that take advantage of technological improvements, namely through the production of reduced particle sizes and the application

of new co-formulants, that allow for better performances of lower levels of Cu. Although the necessity of alternatives is eminent, the response has been made at the cost of the introduction of poorly studied products and formulations into the market. As the results of the second chapter allowed us to realize, new formulations seem to don't provide the full description of their constituents and co-formulants, meaning that a proper evaluation of environmental concerns is being compromised. For instance, C seemed to be used in new formulations to increase the adhesiveness of Cu particles to biological membranes. Whilst this can reveal an advantage for efficiency of formulations, it can also pose risks to other non-target organisms. Likewise, the efficiency of these NTAF-Cu doesn't seem to be properly predicted and investigated, especially for physiological conditions. The work developed in this study with *L. sajor caju* and *B. cinerea* allowed not only to understand that effectiveness of NTAF-Cu may not always surpass traditional formulations, but most importantly, that traditional methods used for assess toxicity of other materials, like NPs, might not be adequate to test complex formulations like the ones tested. This way, our results allowed for the realization of two main aspects. Firstly, that new formulations, with complex and intricate formulations and mode of actions, are being introduced and used in agriculture when so little is known about their behavior. And second, that there's a probability that predicted efficiency of new Cu formulations might not be revealed in practice, meaning that higher doses of these products might actually be needed to equate traditional formulations. This could mean that, at the end, farmers would be applying higher doses, with larger inputs into the environment.

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Appendix

Table A 1. Pseudo-total contents of major elements of soil samples and background, determined by ICP-MS.

Element	July 2018	February 2019	Background value
Mg	7652±2167	7836±1978	8873±591
Al	20600±3981	22446±2873	23340±3359
Ca	1774±323	1730±436	875±307
Fe	33517±7414	29736±2412	38813±3261
K	2244±504	2247±411	2268±488
Mn	445±72	427±65	438±102
P	502±83	441±77	273±79

Table A 2. Pseudo-total contents of trace elements of soil samples and background, determined by ICP-MS.

Element	July 2018	February 2019	Background value
As	66.6±49.3	60.0±30.7	39.5±15.7
Ba	45.0±13.7	46.0±11.9	47.2±19.5
Cd	0.142±0.019	0.159±0.035	nd
Co	14.8±1.5	13.7±0.8	12.8±2.7
Cr	31.9±3.3	30.4±1.9	39.0±11.7
Cu	117±28	89.3±23.2	24.0±4.6
Ni	32.0±3.9	30.7±2.0	35.6±5.6
Pb	19.9±2.8	19.1±2.1	20.0±3.1
V	22.3±3.2	22.2±2.6	20.7±4.7
Zn	88.3±12.3	89.7±14.5	76.2±6.9

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1. Identificador do produto

CA Code (Nufarm)	: 2114
Oracle Recipe Code (Nufarm)	: OR2114
Item codes	: 100002782; 100002783
Forma do produto	: Mistura
Designação comercial	: CHAMPION WP
Type (Nufarm)	: Country Specific
Country (Nufarm)	: Portugal

1.2. Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

1.2.1. Utilizações identificadas relevantes

Categoria de uso principal	: Utilização profissional
Utilização da substância ou mistura	: Fungicida

1.2.2. Utilizações desaconselhadas

Não existem informações adicionais disponíveis

1.3. Identificação do fornecedor da ficha de dados de segurança

Fabricante
Nufarm S.A.S.
28 Boulevard Zéphirin Camélinat
92230 Gennevilliers - France
T +33140855050 - F +33147922545
FDS@nufarm.com

1.4. Número de telefone de emergência

Número de emergência : Organisme Français INRS : +33 1 45 42 59 59; Nufarm S.A.S. : +33 1 40 85 51 15

País	Organização/Empresa	Endereço	Número de emergência	Comentário
Portugal	Centro de Informação Antivenenos Instituto Nacional de Emergência Médica	Rua Almirante Barroso, 36 1000-013 Lisboa	+351 800 250 250	

SECÇÃO 2: Identificação dos perigos

2.1. Classificação da substância ou mistura

Classificação de acordo com o regulamento (CE) nº 1272/2008 [CLP]

Toxicidade aguda (via oral), categoria 4	H302
Corrosão/irritação cutânea, categoria 2:	H315
Lesões oculares graves/irritação ocular, categoria 1	H318
Perigoso para o ambiente aquático - perigo agudo, categoria 1	H400
Perigoso para o ambiente aquático - perigo crónico, categoria 1	H410

Texto completo das categorias de classificação e das advertências de perigo H: consultar a Secção 16

Efeitos adversos decorrentes das propriedades físico-químicas assim como os efeitos adversos para a saúde humana e para o ambiente

Nocivo por ingestão. Provoca lesões oculares graves. Muito tóxico para os organismos aquáticos com efeitos duradouros.

2.2. Elementos do rótulo

Rotulagem de acordo com o Regulamento (CE) nº 1272/2008 [CLP]

Pictogramas de perigo (CRE) :

GHS05 GHS07 GHS09

Palavra-sinal (CLP)	: Perigo
Advertências de perigo (CRE)	: H302 - Nocivo por ingestão. H315 - Provoca irritação cutânea.

		H318 - Provoca lesões oculares graves. H410 - Muito tóxico para os organismos aquáticos com efeitos duradouros.
Recomendações de prudência (CRE)	:	P102 - Manter fora do alcance das crianças. P270 - Não comer, beber ou fumar durante a utilização deste produto. P280 - Usar luvas de protecção/vestuário de protecção/protecção ocular/protecção facial. P301+P312 - EM CASO DE INGESTÃO: caso sinta indisposição, contacte um CENTRO DE INFORMAÇÃO ANTIVENENOS ou um médico. P305+P351+P338 - SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continuar a enxaguar. P310 - Contacte imediatamente um CENTRO DE INFORMAÇÃO ANTIVENENOS ou um médico. P391 - Recolher o produto derramado. P501 - Eliminar o conteúdo/embalagem em local adequado à recolha de resíduos perigosos.
Frases EUH	:	EUH401 - Para evitar riscos para a saúde humana e para o ambiente, respeitar as instruções de utilização.
Frases adicionais	:	SP 1 - Não poluir a água com este produto ou com a sua embalagem. SPe 3 - Para proteção dos organismos aquáticos, não aplicar em terrenos agrícolas adjacentes a águas de superfície : 5 a 50 m

2.3. Outros perigos

Esta substância/mistura não preenche os critérios PBT do anexo XIII do Regulamento REACH

Esta substância/mistura não preenche os critérios mPmB do anexo XIII do Regulamento REACH

SECÇÃO 3: Composição/informação sobre os componentes

3.1. Substâncias

Não aplicável

3.2. Misturas

Denominação	Identificador do produto	%	Classificação de acordo com o regulamento (CE) nº 1272/2008 [CLP]
di-hidróxido de cobre; hidróxido de cobre (II)	(N.º CAS) 20427-59-2 (N.º CE) 243-815-9 (Número de índice CE) 029-021-00-3	76,7	Acute Tox. 2 (Inhalation), H330 Acute Tox. 4 (Oral), H302 Eye Dam. 1, H318 Aquatic Acute 1, H400 (M=10) Aquatic Chronic 1, H410 (M=10)

Texto completo das frases H: ver secção 16

SECÇÃO 4: Medidas de primeiros socorros

4.1. Descrição das medidas de primeiros socorros

Primeiros socorros em geral	:	Em caso de indisposição, contacte um centro de informação antivenenos ou um médico.
Primeiros socorros em caso de inalação	:	Retirar a pessoa para uma zona ao ar livre e mantê-la numa posição que não dificulte a respiração. Administrar oxigénio ou praticar respiração artificial, se necessário. Em caso de indisposição, consultar um médico.
Primeiros socorros em caso de contacto com a pele	:	Em caso de contacto com a pele, retirar imediatamente toda a roupa contaminada e lavar imediata e abundantemente com água.
Primeiros socorros em caso de contacto com os olhos	:	Em caso de contacto com os olhos, enxaguar imediatamente com muita água e consultar um especialista. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continue a enxaguar. Chamar imediatamente um médico.
Primeiros socorros em caso de ingestão	:	Não induzir o vómito. Enxaguar a boca com água. Em caso de ingestão, consultar imediatamente o médico e mostrar-lhe a embalagem ou o rótulo. Fazer beber muita água.

4.2. Sintomas e efeitos mais importantes, tanto agudos como retardados

Sintomas/efeitos	:	Dores abdominais, náuseas. Vómitos.
Sintomas/efeitos em caso de contacto com os olhos	:	Lesões oculares graves.

4.3. Indicações sobre cuidados médicos urgentes e tratamentos especiais necessários

Tratamento sintomático. Proceder a uma lavagem gástrica sob vigilância médica qualificada.

SECÇÃO 5: Medidas de combate a incêndios

5.1. Meios de extinção

Meios de extinção adequados	:	Água pulverizada. Pó seco. Espuma.

10.6. Produtos de decomposição perigosos

Em condições normais de armazenamento e utilização, não devem formar-se produtos de decomposição perigosos.

SECÇÃO 11: Informação toxicológica

11.1. Informações sobre os efeitos toxicológicos

CHAMPION WP	
DL50 oral rato	500 - 2000 mg/kg (Resultados obtidos com um produto similar)
DL50 cutânea rato	> 5000 mg/kg (Resultados obtidos com um produto similar)
CL50 inalação rato (mg/l)	Não aplicável
ATE CLP (vapores)	5,06 mg/l/4h
ATE CLP (poeiras, névoa)	5,06 mg/l/4h

di-hidróxido de cobre; hidróxido de cobre (II) (20427-59-2)	
DL50 oral rato	489 - 1280 mg/kg
DL50 cutânea rato	> 2000 mg/kg
CL50 inalação rato (mg/l)	0,5 mg/l/4h fêmea

Toxicidade aguda (via oral) : Oral: Nocivo por ingestão. (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Toxicidade aguda (via cutânea) : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Toxicidade aguda (inalação) : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Corrosão/irritação cutânea : Provoca irritação cutânea.

Lesões oculares graves/irritação ocular : Provoca lesões oculares graves.

Sensibilização respiratória ou cutânea : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Mutagenicidade em células germinativas : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Carcinogenicidade : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Toxicidade reprodutiva : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Toxicidade para órgãos-alvo específicos (STOT) - exposição única : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Toxicidade para órgãos-alvo específicos (STOT) - exposição repetida : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

Perigo de aspiração : Não classificado (Com base nos dados disponíveis, os critérios de classificação não são preenchidos)

SECÇÃO 12: Informação ecológica

12.1. Toxicidade

Ecologia - geral : Muito tóxico para os organismos aquáticos com efeitos duradouros.

Perigoso para o ambiente aquático, curto prazo (agudo) : Muito tóxico para os organismos aquáticos.

Perigoso para o ambiente aquático, longo prazo (crónico) : Muito tóxico para os organismos aquáticos com efeitos duradouros.

CHAMPION WP	
CL50 96 h peixes	0,0165 mg/l Os dados aplicam-se à substância tecnicamente ativa
CE50 48 h crustáceos	0,038 mg/l Os dados aplicam-se à substância tecnicamente ativa
CE50 72h algas	0,0229 mg/l Selenastrum capricornutum
NOEC (crónica)	0,024 mg/l Os dados aplicam-se à substância tecnicamente ativa
NOEC crónico peixes	0,0155 mg/l Os dados aplicam-se à substância tecnicamente ativa

di-hidróxido de cobre; hidróxido de cobre (II) (20427-59-2)	
CL50 96 h peixes	0,0165 mg/l Oncorhynchus mykiss (truta arco-íris)
CE50 48 h crustáceos	0,038 mg/l Daphnia magna
CE50 72h algas	0,00939 mg/l Selenastrum capricornutum
NOEC (crónica)	0,024 mg/l Daphnia pulex (Water flea)

di-hidróxido de cobre; hidróxido de cobre (II) (20427-59-2)	
NOEC crónico peixes	0,0155 mg/l Oncorhynchus mykiss (truta arco-íris);92d
LC50, Colinus virginianus (Codorniz-da-virgínia)	340 mg/kg
LC50, Toxicidade aguda, Eisenia fetida (Minhoca)	> 677,3 mg/kg
LC50, Toxicidade crónica, Eisenia fetida (Minhoca)	159 mg/kg

12.2. Persistência e degradabilidade

Não existem informações adicionais disponíveis

12.3. Potencial de bioacumulação

CHAMPION WP	
Log Pow	0,44
Potencial de bioacumulação	Baixo potencial de bioacumulação.

12.4. Mobilidade no solo

CHAMPION WP	
Mobilidade no solo	Baixa mobilidade (solo)

12.5. Resultados da avaliação PBT e mPmB

CHAMPION WP
Esta substância/mistura não preenche os critérios PBT do anexo XIII do Regulamento REACH
Esta substância/mistura não preenche os critérios mPmB do anexo XIII do Regulamento REACH

12.6. Outros efeitos adversos

Não existem informações adicionais disponíveis

SECÇÃO 13: Considerações relativas à eliminação

13.1. Métodos de tratamento de resíduos

Métodos de tratamento de resíduos : Eliminar o conteúdo/recipiente em conformidade com as instruções de triagem do agente de recolha autorizado. A embalagem vazia deverá ser lavada três vezes, fechada, inutilizada, e colocada em sacos de recolha, devendo estes serem entregues num ponto de retoma autorizado; as águas de lavagem deverão ser usadas na preparação de calda.

SECÇÃO 14: Informações relativas ao transporte

De acordo com as exigências de ADR / RID / IMDG / IATA / ADN

ADR	IMDG	IATA
14.1. Número ONU		
3077	3077	3077
14.2. Designação oficial de transporte da ONU		
MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide)	MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide)	MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide)
Descrição do documento de transporte		
UN 3077 MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide), 9, III, (-)	UN 3077 MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide), 9, III, POLUENTE MARINHO	UN 3077 MATÉRIA PERIGOSA DO PONTO DE VISTA DO AMBIENTE, SÓLIDA, N.S.A. (Copper hydroxide), 9, III
14.3. Classes de perigo para efeitos de transporte		
9	9	9
		
14.4. Grupo de embalagem		
III	III	III
14.5. Perigos para o ambiente		
Perigoso para o ambiente : Sim	Perigoso para o ambiente : Sim Poluente marinho : Sim	Perigoso para o ambiente : Sim
Não existem informações suplementares disponíveis		

Figure A 1. Parts of the SDS Champion WP, V1.0 from 24/09/2019.

Nome comercial: KADOS
No. Produto: SPU 063 C1539 PT-1
Versão actual: 1.3.0, criado em: 05.08.2020 **Versão substituída:** 1.2.3, criado em: 11.02.2020 **Região:** PT

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1 Identificador do produto

Nome comercial

KADOS

1.2 Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

Utilizações identificadas relevantes da substância ou mistura
produto fitofarmacêutico
Fungicida
Este produto destina-se ao uso professional.

utilizações contra-indicadas
Não existem informações disponíveis.

1.3 Identificação do fornecedor da ficha de dados de segurança

Endereço
Certis Europe B.V. - España
Severo Ochoa,18,2°. Bulevar Parque.
Parque Empresarial de Elche
03203 Elche - Alicante - España

Número de telefone	+34 966 651 077
No. Fax	+34 966 651 076
e-mail	certis@certiseurope.es - www.certiseurope.es

Informações relativas à ficha de dados de segurança
certis@certiseurope.es

1.4 Número de telefone de emergência

'Carechem 24 PT: +351 30880 4750

+351 800 250 250 (CIAV - Centro de Informação Antivenenos)

SECÇÃO 2: Identificação dos perigos

2.1 Classificação da substância ou mistura

classificação de acordo com o Regulamento (EC) 1272/2008 (Regulamento CLP)
Acute Tox. 4; H302
Acute Tox. 4; H332
Aquatic Chronic 1; H410
Eye Dam. 1; H318

Informações relativas à classificação
A classificação e rotulagem baseiam-se nos resultados dos testes e controlos toxicológicos efectuados sobre o produto (mistura).
A classificação e rotulagem em matéria de perigosidade de contaminação da água baseiam-se nos resultados dos testes e controlos ecotoxicológicos efectuados sobre o produto (mistura).
A classificação do produto foi conduzida mediante os métodos seguintes descritos no Artigo 9 e aplicando os critérios estabelecidos no Regulamento (CE) N.o 1272/2008:
Perigos físicos: Avaliação dos dados de acordo com o Anexo I, Parte 2
Perigos para a saúde humana e para o ambiente: Avaliação dos dados toxicológicos e ecotoxicológicos de acordo com o Anexo I, Parte 3, 4 e 5.

2.2 Elementos do rótulo

Rotulagem de acordo com o Regulamento (EC) 1272/2008 (Regulamento CLP)

Pictogramas de perigo

GHS05 GHS07 GHS09

Palavra-sinal
Perigo

Advertências de perigo

H302+H332	Nocivo por ingestão ou inalação
H318	Provoca lesões oculares graves.
H410	Muito tóxico para os organismos aquáticos com efeitos duradouros.

Advertências de perigo (UE)

EUH210	Ficha de segurança fornecida a pedido.
EUH401	Para evitar riscos para a saúde humana e para o ambiente, respeitar as instruções de utilização.

Recomendações de prudência

P102	Manter fora do alcance das crianças.
P261	Evitar respirar poeiras/fumos/gases/névoas/vapores/aerossóis.
P270	Não comer, beber ou fumar durante a utilização deste produto.
P280	Usar luvas de proteção/vestuário de proteção/proteção ocular/proteção facial.
P305+P351+P338	SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continue a enxaguar.
P310	Contacte imediatamente um CENTRO DE INFORMAÇÃO ANTIVENENOS/médico.
P301+P312	EM CASO DE INGESTÃO: caso sinta indisposição, contacte um CENTRO DE INFORMAÇÃO ANTIVENENOS/médico.
P391	Recolher o produto derramado.
P304+P340	EM CASO DE INALAÇÃO: retirar a pessoa para uma zona ao ar livre e mantê-la numa posição que não dificulte a respiração.
P501	Eliminar o conteúdo/recipiente de acordo com a legislação local/regional/ nacional/internacional.

2.3 Outros perigos

Não contaminar a água com este produto ou com sua embalagem.

SECÇÃO 3: Composição/informação sobre os componentes

3.1 Substâncias

Não aplicável. O produto não é nenhuma substância.

3.2 Misturas

Componente perigoso

N°	Denominação da substância		Recomendações adicionais	
	No. CAS / CE / índice / REACH	Classificação (EC) 1272/2008 (CLP)	Concentração	%
1	**dihidróxido-de-cobre**			
	20427-59-2 243-815-9 029-021-00-3 -	Acute Tox. 2; H330 Acute Tox. 4; H302 Aquatic Acute 1; H400 Aquatic Chronic 1; H410 Eye Dam. 1; H318	>= 50,00 - < 70,00	% (peso)
2	**Pirofosfato-de-tetrassódio**			
	7722-88-5 231-767-1 - 01-2119489794-17-0008	Eye Dam. 1; H318 Acute Tox. 4; H302	>= 5,00 - < 10,00	% (peso)
3	**hidróxido de sódio**			
	1310-73-2 215-185-5 011-002-00-6 01-2119457892-27	Skin Corr. 1A; H314 Met. Corr. 1; H290 Eye Dam. 1; H318	< 2,50	% (peso)
4	**2,4,7,9-tetrametildec-5-ino-4,7-diol**			
	126-86-3 204-809-1 - 01-2119954390-39	Aquatic Chronic 3; H412 Eye Dam. 1; H318 Skin Sens. 1B; H317	< 0,50	% (peso)

Texto completo sobre as advertências de perigo H e EUH: ver secção 16

N°	Nota	Limites de concentração específicos	Factor-M (aguda)	Factor-M (crónica)
1	-	-	M = 10	-
3	-	Skin Irrit. 2; H315: C >= 0,5% Eye Irrit. 2; H319: C >= 0,5% Skin Corr. 1B; H314: C >= 2% Skin Corr. 1A; H314: C >= 5%	-	-

SECÇÃO 4: Medidas de primeiros socorros

4.1 Descrição das medidas de primeiros socorros

Recomendações gerais

Consultar um Médico imediatamente. Despir de imediato o vestuária e os sapatos contaminados e limpá-los muito bem antes da próxima utilização. Em caso de perigo de perda de consciência, colocar e transportar em posição perfil estável. Sintomas de envenenamento podem aparecer apenas após horas; por isso é necess rio acompanhamento médico por no mínimo 48 horas.

Inalação

Remover pessoas atingidas da área de risco. Providenciar ar fresco. Em caso de respiração iregular/parada: respiração artificial necessária.

Toxicidade para órgãos-alvo específicos (STOT) – exposição única
Não existem dados disponíveis

Toxicidade para órgãos-alvo específicos (STOT) – exposição repetida	
N°	**Nome do produto**
1	**KADOS**
Avaliação/classificação	Com base nos dados disponíveis, os critérios de classificação não são preenchidos.

Perigo de aspiração
Com base nos dados disponíveis, os critérios de classificação não são preenchidos.

SECÇÃO 12: Informação ecológica

12.1 Toxicidade

Toxicidade para os peixes (aguda)			
N°	**Nome do produto**		
1	**KADOS**		
CL50		4,79	mg/l
Duração da exposição		96	h
Espécies	Oncorhynchus mykiss		
Método	OECD 203		
Origem	Produtor		

Toxicidade para os peixes (crónica)
Não existem dados disponíveis

Toxicidade para a Daphnia (aguda)			
N°	**Nome do produto**		
1	**KADOS**		
CE50		1,61	mg/l
Duração da exposição		48	h
Espécies	Daphnia		
Método	OECD 202		
Origem	Produtor		

Toxicidade para a Daphnia (crónica)			
N°	**Nome do produto**		
1	**KADOS**		
NOEC		0,0025	mg/l
Duração da exposição		21	dia(s)
Espécies	Daphnia		
Origem	Produtor		

Toxicidade para as algas (aguda)
Não existem dados disponíveis

Toxicidade para as algas (crónica)
Não existem dados disponíveis

Toxicidade em bactérias
Não existem dados disponíveis

12.2 Persistência e degradabilidade
Não existem informações disponíveis.

12.3 Potencial de bioacumulação
Não existem informações disponíveis.

12.4 Mobilidade no solo
Não existem informações disponíveis.

12.5 Resultados da avaliação PBT e mPmB
Não existem informações disponíveis.

12.6 Outros efeitos adversos
Não existem informações disponíveis.

SECÇÃO 13: Considerações relativas à eliminação

13.1 Métodos de tratamento de resíduos
Produto
O código de desperdício previsto no Catálogo Europeu de Desperdícios deve ser atribuído segundo instruções da empresa de eliminação de desperdícios local.

Figure A 2. Parts of the SDS of Kados V1.3.0 from 05/08/2020.

Nome comercial: KOCIDE OPTI
No. Produto: SPU 063 C1556/PT
Versão actual: 1.2.2, criado em: 01.04.2020 **Versão substituída:** 1.2.1, criado em: 29.01.2020 **Região:** PT

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1 Identificador do produto

Nome comercial

KOCIDE OPTI

1.2 Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

Utilizações identificadas relevantes da substância ou mistura
produto fitofarmacêutico
Fungicida

utilizações contra-indicadas
Não existem informações disponíveis.

1.3 Identificação do fornecedor da ficha de dados de segurança

Endereço
Certis Europe B.V. - España
Severo Ochoa,18,2°. Bulevar Parque.
Parque Empresarial de Elche
03203 Elche - Alicante - España

Número de telefone	+34 966 651 077
No. Fax	+34 966 651 076
e-mail	certis@certiseurope.es - www.certiseurope.es

Informações relativas à ficha de dados de segurança
certis@certiseurope.es

1.4 Número de telefone de emergência

'Carechem 24 PT: +351 30880 4750

+351 800 250 250 (CIAV - Centro de Informação Antivenenos)

SECÇÃO 2: Identificação dos perigos

2.1 Classificação da substância ou mistura

classificação de acordo com o Regulamento (EC) 1272/2008 (Regulamento CLP)
Acute Tox. 4; H302
Acute Tox. 4; H332
Aquatic Acute 1; H400
Aquatic Chronic 1; H410
Eye Irrit. 2; H319

Informações relativas à classificação
A classificação e rotulagem baseiam-se nos resultados dos testes e controlos toxicológicos efectuados sobre o produto (mistura).
A classificação e rotulagem em matéria de perigosidade de contaminação da água baseiam-se nos resultados dos testes e controlos ecotoxicológicos efectuados sobre o produto (mistura).
A classificação do produto foi conduzida mediante os métodos seguintes descritos no Artigo 9 e aplicando os critérios estabelecidos no Regulamento (CE) N.o 1272/2008:
Perigos físicos: Avaliação dos dados de acordo com o Anexo I, Parte 2
Perigos para a saúde humana e para o ambiente: Avaliação dos dados toxicológicos e ecotoxicológicos de acordo com o Anexo I, Parte 3, 4 e 5.

2.2 Elementos do rótulo

Rotulagem de acordo com o Regulamento (EC) 1272/2008 (Regulamento CLP)

Pictogramas de perigo


GHS07


GHS09

Palavra-sinal
Atenção

Advertências de perigo

H302+H332	Nocivo por ingestão ou inalação
H319	Provoca irritação ocular grave.
H410	Muito tóxico para os organismos aquáticos com efeitos duradouros.

Advertências de perigo (UE)

EUH210	Ficha de segurança fornecida a pedido.
EUH401	Para evitar riscos para a saúde humana e para o ambiente, respeitar as instruções de utilização.

Recomendações de prudência

P261	Evitar respirar poeiras/fumos/gases/névoas/vapores/aerossóis.
P270	Não comer, beber ou fumar durante a utilização deste produto.
P280	Usar luvas de proteção/vestuário de proteção/proteção ocular.
P305+P351+P338	SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continue a enxaguar.
P312	Caso sinta indisposição, contacte um CENTRO DE INFORMAÇÃO ANTIVENENOS/médico.
P337+P313	Caso a irritação ocular persista: consulte um médico.
P391	Recolher o produto derramado.
P501	Eliminar o conteúdo/recipiente em conformidade com os regulamentos locais e nacionais.

2.3 Outros perigos
Manter fora do alcance das crianças.

SECÇÃO 3: Composição/informação sobre os componentes

3.1 Substâncias
Não aplicável. O produto não é nenhuma substância.

3.2 Misturas
Componente perigoso

Nº	Denominação da substância		Recomendações adicionais	
	No. CAS / CE / índice / REACH	Classificação (EC) 1272/2008 (CLP)	Concentração	%
1	**dihidróxido-de-cobre**			
	20427-59-2 243-815-9 029-021-00-3 -	Acute Tox. 2; H330 Acute Tox. 4; H302 Aquatic Acute 1; H400 Aquatic Chronic 1; H410 Eye Dam. 1; H318	>= 25,00 - < 50,00	% (peso)
2	**hidróxido de sódio**			
	1310-73-2 215-185-5 011-002-00-6 01-2119457892-27	Skin Corr. 1A; H314 Met. Corr. 1; H290 Eye Dam. 1; H318	< 2,50	% (peso)

Texto completo sobre as advertências de perigo H e EUH: ver secção 16

Nº	Nota	Limites de concentração específicos	Factor-M (aguda)	Factor-M (crónica)
1	-	-	M = 10	-
2	-	Skin Irrit. 2; H315: C >= 0,5% Eye Irrit. 2; H319: C >= 0,5% Skin Corr. 1B; H314: C >= 2% Skin Corr. 1A; H314: C >= 5%	-	-

SECÇÃO 4: Medidas de primeiros socorros

4.1 Descrição das medidas de primeiros socorros
Recomendações gerais
Consultar um Médico imediatamente. Despir de imediato o vestuária e os sapatos contaminados e limpá-los muito bem antes da próxima utilização. Em caso de perigo de perda de consciência, colocar e transportar em posição perfil estável. Sintomas de envenenamento podem aparecer apenas após horas; por isso é necess rio acompanhamento médico por no mínimo 48 horas.

Inalação
Remover pessoas atingidas da área de risco. Providenciar ar fresco. Em caso de respiração iregular/parada: respiração artificial necessária.

Contacto com a pele
Lavar imediatamente com água e sabão.

Contacto com os olhos
Se usar lentes de contacto, retire-as. Enxaguar de imediato o olho por 10 a 15 minutos sob água corrente mantendo as pálpebras abertas e protegendo o olho não atingido. Tratamento com médico especialista (oftalmologista).

Ingestão
Enxaguar a boca e depois tomar água em abundância. Em caso de desmaio, não tratar por via oral. Não provocar vômitos.

4.2 Sintomas e efeitos mais importantes, tanto agudos como retardados
Não existem informações disponíveis.

4.3 Indicações sobre cuidados médicos urgentes e tratamentos especiais necessários
Não existem informações disponíveis.

SECÇÃO 5: Medidas de combate a incêndios

Espécies	ratazana
Método	OPPTS 870.1300
Origem	Produtor

Corrosão/irritação cutânea	
N°	**Nome do produto**
1	**KOCIDE OPTI**
Espécies	coelho
Método	OPPTS 870.2500
Origem	Produtor
Avaliação	não irritante

Lesões oculares graves/irritação ocular	
N°	**Nome do produto**
1	**KOCIDE OPTI**
Espécies	coelho
Método	OPPTS 870.2400
Origem	Produtor
Avaliação	irritante

Sensibilização respiratória ou cutânea	
N°	**Nome do produto**
1	**KOCIDE OPTI**
Via de aplicação	Pele
Espécies	porquinho-da-India
Método	Magnussen/Kligmann
Origem	Produtor
Avaliação	não sensibilizante

Mutagenicidade em células germinativas
Não existem dados disponíveis

Toxicidade na reprodutiva
Não existem dados disponíveis

Carcinogenicidade
Não existem dados disponíveis

Toxicidade para órgãos-alvo específicos (STOT) – exposição única
Não existem dados disponíveis

Toxicidade para órgãos-alvo específicos (STOT) – exposição repetida
Não existem dados disponíveis

Perigo de aspiração
Não existem dados disponíveis

SECÇÃO 12: Informação ecológica

12.1 Toxicidade

Toxicidade para os peixes (aguda)			
N°	**Nome do produto**		
1	**KOCIDE OPTI**		
CL50		0,24	mg/l
Duração da exposição		96	h
Espécies	Oncorhynchus mykiss		
Método	OECD 203		
Origem	Produtor		

Toxicidade para os peixes (crónica)
Não existem dados disponíveis

Toxicidade para a Daphnia (aguda)			
N°	**Nome do produto**		
1	**KOCIDE OPTI**		
CE50		118,0	µg/l
Duração da exposição		48	h
Espécies	Daphnia magna		
Método	OECD 202		
Origem	Produtor		

Toxicidade para a Daphnia (crónica)			
N°	**Nome do produto**		
1	**KOCIDE OPTI**		
NOEC		12	µg/l
Duração da exposição		21	dia(s)

Espécies	Daphnia magna
Método	OECD 211
Origem	Produtor

Toxicidade para as algas (aguda)			
N°	**Nome do produto**		
1	**KOCIDE OPTI**		
ErC50		51,59	µg/l
Duração da exposição		72	h
Espécies	Selenastrum capricornutum		
Método	OECD 201		
Origem	Produtor		

Toxicidade para as algas (crónica)
Não existem dados disponíveis

Toxicidade em bactérias
Não existem dados disponíveis

12.2 Persistência e degradabilidade
Não existem informações disponíveis.

12.3 Potencial de bioacumulação
Não existem informações disponíveis.

12.4 Mobilidade no solo
Não existem informações disponíveis.

12.5 Resultados da avaliação PBT e mPmB
Não existem informações disponíveis.

12.6 Outros efeitos adversos
Não existem informações disponíveis.

SECÇÃO 13: Considerações relativas à eliminação

13.1 Métodos de tratamento de resíduos

Produto
O código de desperdício previsto no Catálogo Europeu de Desperdícios deve ser atribuído segundo instruções da empresa de eliminação de desperdícios local.

Embalagens
A embalagem vazia não deverá ser lavada, sendo completamente esgotada do seu conteúdo, inutilizada e colocada em sacos de recolha, devendo estes serem entregues num centro de receção autorizado.

SECÇÃO 14: Informações relativas ao transporte

14.1 Transporte ADR/RID/ADN

Classe	9
Código de clasificação	M7
Grupo de embalagem	III
Número de perigo	90
Número ONU	UN3077
Nome técnico de expedição	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S.
Agente provocador de perigo	dihidróxido-de-cobre
Códigos de restrição em túneis	-
Etiqueta de segurança	9
Marca matéria perigosa para o ambiente	Símbolo convencional "peixe e árvore"

14.2 Transporte IMDG

Classe	9
Grupo de embalagem	III
Número ONU	UN3077
Nome e descrição	ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S.
Agente provocador de perigo	copper-dihydroxide
EmS	F-A, S-F
Etiquetas	9
Marca matéria perigosa para o ambiente	Símbolo convencional "peixe e árvore"

14.3 Transporte ICAO-TI / IATA

Classe	9
Grupo de embalagem	III
Número ONU	UN3077

Figure A 3. Parts of the SDS of Kocide Opti V1.2.2 from 01/04/2020.

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1 Identificador do produto

Nome comercial : CHAMPION WP

1.2 Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

Utilização da substância ou mistura : Fungicida

1.3 Identificação do fornecedor da ficha de dados de segurança

Companhia : Nufarm S.A.S
28 boulevard Zéphirin Camélinat
92230 Gennevilliers
Telefone : +330140855050
Telefax : +330147922545
Email endereço Pessoa responsável/editor : FDS@fr.nufarm.com

1.4 Número de telefone de emergência

Nufarm S.A.S : +33 1 40 85 51 15

Portugal : 808 250 143 – CENTRO DE INFORMAÇÃO ANTI-VENENOS
112 Número Nacional de Emergência

SECÇÃO 2: Identificação dos perigos

2.1 Classificação da substância ou mistura

Classificação (REGULAMENTO (CE) N.o 1272/2008)

Toxicidade aguda, Categoria 4	H302: Nocivo por ingestão.
Corrosão/irritação cutânea, Categoria 2	H315: Provoca irritação cutânea.
Lesões oculares graves/irritação ocular, Categoria 1	H318: Provoca lesões oculares graves.
Toxicidade aguda para o ambiente aquático, Categoria 1	H400: Muito tóxico para os organismos aquáticos.
Toxicidade crónica para o ambiente aquático, Categoria 1	H410: Muito tóxico para os organismos aquáticos com efeitos duradouros.

2.2 Elementos do rótulo

Rótulo (REGULAMENTO (CE) N.o 1272/2008)

Pictogramas de perigo :

Palavra-sinal : Perigo

Advertências de perigo : H302 Nocivo por ingestão.

		H315	Provoca irritação cutânea.
		H318	Provoca lesões oculares graves.
		H410	Muito tóxico para os organismos aquáticos com efeitos duradouros.
Recomendações de prudência	:	P102	Manter fora do alcance das crianças.
		Prevenção:	
		P270	Não comer, beber ou fumar durante a utilização deste produto.
		Resposta:	
		P301 + P312	EM CASO DE INGESTÃO: Caso sinta indisposição, contacte um CENTRO DE INFORMAÇÃO ANTIVENENOS/médico.
		P305 + P351 + P338	SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continuar a enxaguar.
		P391	Recolher o produto derramado.
		Destruição:	
		P501	Eliminar o conteúdo/embalagem em local adequado à recolha de resíduos perigosos

Etiquetagem suplementar:

EUH401 Para evitar riscos para a saúde humana e para o ambiente, respeitar as instruções de utilização.

SP1 Não poluir a água com este produto ou com a sua embalagem.

SPe3a Para proteção dos organismos aquáticos, não aplicar em terrenos agrícolas adjacentes a águas de superfície.

2.3 Outros perigos

Uma avaliação de risco químico não é necessária para esta mistura

SECÇÃO 3: Composição/informação sobre os componentes

3.2 Misturas

Natureza quimica : pó molhável

Componentes perigosos

Nome Químico	No. CAS No. CE Número de registo	Classificação (REGULAMENTO (CE) N.o 1272/2008)	Concentração [%]
Copper hydroxide	20427-59-2 243-815-9	Acute Tox. 4; H302 Acute Tox. 2; H330	88 W/W

		Eye Dam. 1; H318 Aquatic Acute 1; H400 Aquatic Chronic 1; H410	
Sodium lauryl sulfate	151-21-3 205-788-1	Flam. Sol. 2; H228 Acute Tox. 4; H302, H332 STOT SE 3; H335 Skin Irrit. 2; H315 Eye Dam. 1; H318	0W/W - 5W/W

Para o pleno texto das DECLARAÇÕES H mencionadas nesta Secção, ver a Secção 16.

SECÇÃO 4: Medidas de primeiros socorros

4.1 Descrição das medidas de primeiros socorros

Em caso de inalação : Retirar o paciente para um local arejado.

Em caso de contacto com a pele : Retirar imediatamente todo o vestuário contaminado.
Lave imediatamente todas as peças atingidas com água abundante, durante pelo menos 15 minutos

Se entrar em contacto com os olhos : Enxaguar logo com bastante água e consultar um médico.
Se a irritação dos olhos continuar, consultar um especialista.

Em caso de ingestão : Bochechar com água
NÃO provoca vómito.
Em caso de ingestão, consultar imediatamente o mé dico, e mostrar-lhe a embalagem e o rótulo.

4.2 Sintomas e efeitos mais importantes, tanto agudos como retardados

Sintomas : Não existe informação disponível.

4.3 Indicações sobre cuidados médicos urgentes e tratamentos especiais necessários

Tratamento : Tratamento sintomático

SECÇÃO 5: Medidas de combate a incêndios

5.1 Meios de extinção

Meios adequados de extinção : Pulverização de água, Areia, Espuma, Dióxido de carbono (CO_2)

Meios inadequados de extinção : Jacto de água de grande volume

5.2 Perigos especiais decorrentes da substância ou mistura

Perigos específicos para : Como o produto contem componentes orgânicos

Toxicidade para órgãos-alvo específicos (STOT) - exposição única	:	Dados não disponíveis
Toxicidade para órgãos-alvo específicos (STOT) - exposição repetida	:	Dados não disponíveis

Componentes:

Toxicidade aguda por via oral	:	DL50 Ratazana: 489 - 1.280 mg/kg
Toxicidade aguda por via inalatória	:	CL50 Ratazana, fêmea: 0,5 mg/l Duração da exposição: 4 h
Toxicidade aguda por via cutânea	:	DL50 Ratazana: > 2.000 mg/kg
Corrosão/irritação cutânea	:	Espécie: Coelho Resultado: Leve irritação da pele
Lesões oculares graves/irritação ocular	:	Espécie: Coelho Resultado: Grave irritação dos olhos
Sensibilização respiratória ou cutânea	:	Espécie: Porquinho da índia Resultado: Não provoca sensibilização.

SECÇÃO 12: Informação ecológica

12.1 Toxicidade

Produto:

Toxicidade em peixes	:	CL50 (Oncorhynchus mykiss (truta arco-íris)): 0,0165 mg/l Duração da exposição: 96 h Substância teste: (copper(II)hydroxide) Método: Directrizes do Teste OECD 203
Toxicidade em dáfnias e outros invertebrados aquáticos	:	CE50 (Daphnia magna): 0,038 mg/l Duração da exposição: 48 h Substância teste: (copper(II)hydroxide) Método: OECD TG 202
Toxicidade em algas	:	CE50 (Selenastrum capricornutum (alga verde)): 0,00939 mg/l Duração da exposição: 72 h Método: Directiva 67/548/CEE, Anexo V, C.3.

Toxicidade em peixes (Toxicidade crónica)	:	NOEC: 0,0155 mg/l Duração da exposição: 92 d Espécie: Oncorhynchus mykiss (truta arco-íris) Substância teste:(copper(II)hydroxide)
Toxicidade em dáfnias e outros invertebrados aquáticos (Toxicidade crónica)	:	NOEC: 0,024 mg/l Duração da exposição: 21 d Espécie: Daphnia magna Substância teste: (copper(II)hydroxide)
Toxicidade em organismos terrestres	:	DL50: 49 µg/abelha, oral Duração da exposição: 2 d Espécie: Apis mellifera (abelhas) DL50: > 57 µg/abelha, contato Duração da exposição: 2 d Espécie: Apis mellifera (abelhas)

Componentes:

:

Toxicidade em peixes	:	CL50 (Oncorhynchus mykiss (truta arco-íris)): 0,016 mg/l Duração da exposição: 96 h
Toxicidade em dáfnias e outros invertebrados aquáticos	:	CE50 (Daphnia (Dáfnia)): 0,038 mg/l Duração da exposição: 48 h
Toxicidade em organismos terrestres	:	CL50: 340 mg/kg Espécie: Colinus virginianus (Codorniz)

12.2 Persistência e degradabilidade

Produto:

Biodegradabilidade	:	Dados não disponíveis
Eliminação Físico-Química	:	Dados não disponíveis

12.3 Potencial de bioacumulação

Produto:

Bioacumulação	:	Nenhuma bioacumulação é esperada (log P <= 4). (log Pow = coeficiente de partição P)
Coeficiente de partição n-octanol/água	:	log Pow: 0,44

12.4 Mobilidade no solo

Produto:

Mobilidade	:	Dados não disponíveis

12.5 Resultados da avaliação PBT e mPmB

Produto:

Figure A 4. Parts of the SDS of Champion WP V1.0 from 12/03/2018.

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1. Identificador do produto

Forma do produto	:	Mistura
Nome do produto	:	Kocide Opti
Código do produto	:	SPU 063 C1484
Tipo de formulação	:	Dispersíveis em agua (WG)
Ingrediente ativo	:	Hidróxido de cobre

1.2. Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

1.2.1. Utilizações identificadas relevantes

Categoria de uso principal	:	Produto fitofarmacêutico para o uso profissional. Agricultura.
Utilização da substância ou mistura	:	Fungicida

1.2.2. Usos desaconselhados

Não existe informação adicional disponível.

1.3. Identificação do fornecedor da ficha de dados de segurança

Proveedor:

Spiess-Urania Chemicals GmbH
Frankenstrasse 18 b
20097 Hamburg
Germany

Distribuidor :

Certis Europe BV Sucursal en España
Severo Ochoa, 18, 2º. Bulevar Parque.
Parque Empresarial de Elche.
03203 Elche. Alicante. España
T +34 966 651 077 - F +34 966 651 076
certis@certiseurope.es- www.certiseurope.es

1.4. Número de telefone de emergência

Número de emergência	:	Carechem24 número de emergência internacional: +44 (0) 1235 239670 Centro de Informação Antivenenos: +35 1 808 250 143

SECÇÃO 2: Identificação dos perigos

2.1. Classificação da substância ou mistura

Classificação de acordo com o regulamento (CE) nº 1272/2008 [CLP]

Acute Tox. 4 (Oral)	H302
Acute Tox. 4 (Inhalation)	H332
Aquatic Acute 1	H400
Aquatic Chronic 1	H410

Texto integral das frases H : ver a secção 16

2.2. Elementos do rótulo

Rotulagem de acordo com o Regulamento (CE) nº 1272/2008 [CLP]

Pictogramas de perigo (CLP) :

GHS07 GHS09

Palavra-sinal (CLP) : Atenção

Advertências de perigo (CLP) : H302+H332 - Nocivo por ingestão ou inalação.
H410 - Muito tóxico para os organismos aquáticos com efeitos duradouros.

Recomendações de prudência (CLP) : P261 - Evitar respirar as poeiras.
P280 - Usar luvas de protecção/vestuário de protecção/protecção ocular/protecção facial.
P270 - Não comer, beber ou fumar durante a utilização deste produto.
P305+P351+P338 - SE ENTRAR EM CONTACTO COM OS OLHOS: enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continuar a enxaguar.
P312 - Caso sinta indisposição, contacte um CENTRO DE INFORMAÇÃO ANTIVENENOS/médico.
P337+P313 - Caso a irritação ocular persista: consulte um médico.
P391 - Recolher o produto derramado.
P501 - Eliminar o conteúdo / recipiente em um local de disposição adequada de acordo com os regulamentos locais e nacionais.

Frases EUH : EUH401 - Para evitar riscos para a saúde humana e para o ambiente, respeitar as instruções de utilização.

2.3. Outros perigos

Não existe informação adicional disponível

SECÇÃO 3: Composição/informação sobre os componentes

3.1. Substância

Não aplicável.

3.2. Mistura

Nome	Identificador do produto	% (p/p)	Classificação de acordo com o regulamento (CE) nº 1272/2008 [CLP]
Copper-dihydroxide	(nº CAS) 20427-59-2 (nº CE) 243-815-9 (Número de indice) - (Nº REACH) 01-2119969283-29	25-50	Acute Tox. 4 (Oral), H302 Acute Tox. 3 (Inhalation), H331 Eye Dam. 1, H318 Aquatic Acute 1, H400 Aquatic Chronic 1, H410
Polyacrylic Acid	(nº CAS) 9003-01-4	5 - 10	Skin Irrit. 2, H315 Eye Irrit. 2, H319 STOT SE 3, H335

Texto integral das frases H : ver a secção 16.

Toxicidade para órgãos-alvo específicos (STOT) - exposição única	:	Nao classificado
Toxicidade para órgãos-alvo específicos (STOT) - exposição repetida	:	Nao classificado
Perigo de aspiração	:	Nao classificado

SECÇÃO 12: Informação ecológica

12.1. Toxicidade

Kocide Opti	
CL50 Peixe	4,79 mg/l
CE50 Daphnia	1,61 mg/l
CE50 (*Desmodesmus subspicatus*)	18,03 (72h)
NOEC Daphnia	0,0025 mg/L (21 d)

12.2. Persistência e degradabilidade

Copper-dihydroxide (20427-59-2)	
Persistência e degradabilidade	Não rapidamente biodegradável

12.3. Potencial de bioacumulação

Copper-dihydroxide (20427-59-2)	
Persistência e degradabilidade	Não rapidamente biodegradável

12.4. Mobilidade no solo

Não deixar chegar às águas subterrâneas, aos cursos de água nem à canalização.
Perigo de pol ui ção da água pot ável mesmo se f orem derramadas quanti dades muit o pequenas no subsolo.
Tóxico nas águas para os peixes e para o plâncton.
Muito tóxico para os organismos aquáticos.
Com base nos dados disponíveis sobre a eliminação/degradação e o potencial de bioacumulação, não se exclui a possibilidade de danos no ambiente a longo prazo.

12.5. Resultados da avaliação PBT e mPmB

Não existe informação adicional disponível

12.6. Outros efeitos adversos

Não existe informação adicional disponível

SECÇÃO 13: Considerações relativas à eliminação

13.1. Métodos de tratamento de resíduos

Métodos de tratamento de resíduos	:	Aplicar procedimento de lavagem tripla do recipiente vazio e colocar a água de enxaguamento no recipiente, onde a mistura é preparada. Manusear as embalagens vazias e dos resíduos, conforme estabelecido pelas autoridades competentes.
Recomendações para a eliminação dos resíduos	:	Destrua de forma segura e de acordo com os regulamentos locais e nacionais.

SECÇÃO 14: Informações relativas ao transporte

De acordo com as exigências de ADR / RID / IMDG / IATA / ADN

14.1. Número ONU

Número UN (ADR)	:	3077

Figure A 5. Parts of the SDS of Kocide OPTI V1.1 from 29/01/2018.

KOCIDE® OPTI

Versão 4.0 (substitui: Versão 3.0)
Data de revisão 30.11.2015 Ref. 130000024626

Esta Ficha de Dados de Segurança adere às normas e regulamentos de Portugal e pode não abranger os regulamentos de outros países.

SECÇÃO 1: Identificação da substância/mistura e da sociedade/empresa

1.1. Identificador do produto

Nome do produto	:	KOCIDE® OPTI
Sinónimos	:	B12015094 DPX-GFJ52 30WG

1.2. Utilizações identificadas relevantes da substância ou mistura e utilizações desaconselhadas

Utilização da substância ou mistura	:	Fungicida

1.3. Identificação do fornecedor da ficha de dados de segurança

Companhia	:	DuPont Portugal, Unipessoal Lda Campo Pequeno, nº48 - 6 Esq. - Edifício Taurus 1000-081 Lisboa Portugal
Telefone	:	+351 21 799-8030
Telefax	:	+351 21 799-8050
Email endereço	:	sds-support@che.dupont.com

1.4. Número de telefone de emergência

Número de telefone de emergência	:	+(351)-308801773 (CHEMTREC)
	:	+351 808 250 143 (CIAV Centro de Informação Anti-venenos Português)
	:	Os centros de toxicidade somente podem possuir informação exigida para produtos de acordo com a Regulação (CE) no. 1272/2008 e a legislação nacional.

SECÇÃO 2: Identificação dos perigos

2.1. Classificação da substância ou mistura

Toxicidade aguda, Categoria 4	H302: Nocivo por ingestão.
Toxicidade aguda, Categoria 4	H332: Nocivo por inalação.
Irritação ocular, Categoria 2	H319: Provoca irritação ocular grave.
Toxicidade aguda para o ambiente aquático, Categoria 1	H400: Muito tóxico para os organismos aquáticos.
Toxicidade crónica para o ambiente aquático, Categoria 1	H410: Muito tóxico para os organismos aquáticos com efeitos duradouros.

2.2. Elementos do rótulo

Esta mistura não contém nenhuma substância considerada persistente, bioacumulativa nem tóxica (PBT).
Essa mistura não contém nenhuma substância considerada muito persistente ou muito bioacumulativa (vpvB).

SECÇÃO 3: Composição/informação sobre os componentes

3.1. Substâncias

Não aplicável

3.2. Misturas

Número de registo	Classificação de acordo com a regulação (UE) 1272/2008 (CLP)	Concentração (% w/w)
Hidróxido de cobre (No. CAS20427-59-2) (No. CE243-815-9)		
	Acute Tox. 4; H302 Acute Tox. 2; H330 Eye Dam. 1; H318 Aquatic Acute 1; H400 Aquatic Chronic 1; H410	46,1 %
Polyacrylic Acid (No. CAS9003-01-4)		
	Acute Tox. 4; H332 Skin Irrit. 2; H315 Eye Irrit. 2; H319	>= 5 - < 10 %

Os produtos acima cumprem com os requisitos de registo do REACH; O(s) número de registo (s) podem não ser fornecidos porque a(s) substância(s) estão isentos(as), ainda não estão registadas no âmbito do REACH ou estão registadas no âmbito de outro processo de regulamentação (biocida, produtos fitofarmacêuticos), etc.

Para o pleno texto das DECLARAÇÕES H mencionadas nesta Secção, ver a Secção 16.

SECÇÃO 4: Primeiros socorros

4.1. Descrição das medidas de primeiros socorros

Recomendação geral : Nunca dar nada pela boca a uma pessoa inconsciente.

Inalação : Retirar o paciente para um local arejado. Após exposição prolongada, consultar um médico. Poderá ser necessária respiração artificial e/ou oxigénio.

Contacto com a pele : Despir imediatamente a roupa e os sapatos contaminados. Lavar com água morna e sabão. No caso de irritações de pele ou de recções alérgicas consultar um médico. Lavar o vestuário contaminado antes de voltar a usá-lo.

Contacto com os olhos : Se for possível, retirar as lentes de contacto, se usar. Manter o olho aberto e enxaguar lentamente e cuidadosamente com água durante 15-20 minutos. Se a irritação dos olhos continuar, consultar um especialista.

Perigo de aspiração

II A mistura não tem propriedades associadas com um potencial risco de aspiração.

SECÇÃO 12: Informação ecológica

12.1. Toxicidade

Toxicidade em peixes

Ensaio por escoamento / CL50 / 96 h / Oncorhynchus mykiss (truta arco-íris): 0,24 mg/l
Método: Directrizes do Teste OECD 203
(Dados no próprio produto) Origem da informação: Relatório interno de estudo.

Toxicidade para as plantas aquáticas

CE50r / 72 h / Pseudokirchneriella subcapitata (alga verde): 0,05159 mg/l
Método: OECD TG 201
(Dados no próprio produto) Origem da informação: Relatório interno de estudo.

Toxicidade para os invertebrados aquáticos

CE50 / 48 h / Daphnia magna: 0,118 mg/l
Método: OECD TG 202
(Dados no próprio produto) Origem da informação: Relatório interno de estudo.

Toxicidade crónica nos peixes

- Hidróxido de cobre
  NOEC / 90 d / : 0,0017 mg/l

- Polyacrylic Acid
  NOEC / 28 d / Danio rerio (peixe-zebra): > 450 mg/l
  Método: OECD TG 204
  Origem da informação: Relatório interno de estudo.

Toxicidade crónica para os invertebrados aquáticos

- Hidróxido de cobre
  NOEC / 21 d / : 0,03 mg/l

- Polyacrylic Acid
  NOEC / 21 d / Daphnia magna: 58 mg/l
  Método: ver o texto do utilizador
  Origem da informação: Relatório interno de estudo.

12.2. Persistência e degradabilidade

Biodegradabilidade

Não rapidamente biodegradável. Estimativa baseada nos dados obtidos nos ingredientes activos.

Figure A 6. Parts of the SDS of Kocide Opti from Dupont V4.0 from 30/11/2015.

SAFETY DATA SHEET

Issue Date 20-Feb-2017 **Revision Date** 20-Feb-2017 **Version** 1

1. IDENTIFICATION

Product identifier

Product Name Kocide® 3000

Other means of identification

Product Code 91411-2
Synonyms DF B12015094, DPX-GFJ52 30WG, GX-569 30WG, Kocide® Opti, Kocide® 46.1
Registration Number(s) 91411-2-70051

Recommended use of the chemical and restrictions on use

Recommended Use Fungicide
Uses advised against No information available

Details of the supplier of the safety data sheet

Manufacturer Address
Kocide LLC
9145 Guilford Road, Suite 175
Columbia, MD 21046
USA
Website: www.kocide.com

Distributed by:
Certis U.S.A. L.L.C.
9145 Guilford Road, Suite 175
Columbia, MD 21046
USA
www.certisusa.com

Emergency telephone number

Company Phone Number Certis USA +1 301-604-7340
Emergency Telephone ChemTel, Inc.: 1-800-255-3924 (outside the U.S. 1-813-248-0585)
POISON CONTROL CENTER: 800-222-1222

2. HAZARDS IDENTIFICATION

Classification

OSHA Regulatory Status
This chemical is considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200)

Acute toxicity - Oral	Category 4
Acute toxicity - Inhalation (Dusts/Mists)	Category 4
Serious eye damage/eye irritation	Category 2B
Carcinogenicity	Category 1A

Label elements

Emergency Overview

Danger

Hazard statements
Harmful if swallowed
Harmful if inhaled
Causes eye irritation
May cause cancer

Appearance Granular **Physical state** Solid **Odor** Characteristic

Precautionary Statements - Prevention
Obtain special instructions before use
Do not handle until all safety precautions have been read and understood
Use personal protective equipment as required
Wash face, hands and any exposed skin thoroughly after handling
Do not eat, drink or smoke when using this product
Avoid breathing dust/fume/gas/mist/vapors/spray
Use only outdoors or in a well-ventilated area

Precautionary Statements - Response
IF exposed or concerned: Get medical advice/attention
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
If eye irritation persists: Get medical advice/attention
IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing
IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell
Rinse mouth

Precautionary Statements - Storage
Store locked up

Precautionary Statements - Disposal
Dispose of contents/container to an approved waste disposal plant

Hazards not otherwise classified (HNOC)
Not applicable

Other Information
May be harmful in contact with skin. Very toxic to aquatic life with long lasting effects. Very toxic to aquatic life.

Unknown acute toxicity
0% of the mixture consists of ingredient(s) of unknown toxicity

3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance
Not applicable

Mixture

Chemical Name	CAS No	Weight-%
Other ingredients	Proprietary	46.9
Copper hydroxide	20427-59-2	46.1
Bentonite	1302-78-9	6 - 7
Quartz	14808-60-7	0.1 - 1

If CAS number is "proprietary", the specific chemical identity and percentage of composition has been withheld as a trade secret.

Reproductive toxicity	Based on available data, the classification criteria are not met.
STOT - single exposure	Based on available data, the classification criteria are not met.
STOT - repeated exposure	Based on available data, the classification criteria are not met.
Chronic toxicity	Avoid repeated exposure. May cause adverse liver effects.
Target Organ Effects	Eyes, kidney, liver, spleen, Respiratory system, Skin.
Other adverse effects	The following effects occurred at levels of exposure that significantly exceed those expected under labeled usage conditions.: Liver effects, Kidney effects, microcytic anemia, Information given is based on data obtained from similar substances.
Aspiration hazard	Based on available data, the classification criteria are not met.

Numerical measures of toxicity - Product Information

The following values are calculated based on chapter 3.1 of the GHS document .

ATEmix (oral)	994.00 mg/kg
ATEmix (dermal)	4,343.00 mg/kg
ATEmix (inhalation-dust/mist)	1.1 mg/l

12. ECOLOGICAL INFORMATION

Ecotoxicity

Very toxic to aquatic life with long lasting effects

96 h LC50 (Oncorhynchus mykiss (rainbow trout)) - 0.24 mg/l
48 h EC50 (Daphnia magna (Water flea)) - 0.118 mg/l

This pesticide is toxic to fish and aquatic invertebrates and may contaminate water through runoff. This product has a potential for runoff for several months or more after application. Poorly draining soils and soils with shallow water tables are more prone to produce runoff that contains this product. For terrestrial uses, do not apply directly to water, to areas where surface water is present or to intertidal areas below the mean high water mark. Do not contaminate water when disposing of equipment wash-waters or rinsate. Drift and runoff may be hazardous to aquatic organisms in waters adjacent to treated areas.

47.2 % of the mixture consists of components(s) of unknown hazards to the aquatic environment

Chemical Name	Algae/aquatic plants	Fish	Crustacea
Copper hydroxide 20427-59-2	0.00939: 72 h Pseudokirchneriella subcapitata mg/L EC50	0.135: 96 h Oncorhynchus mykiss (rainbow trout) mg/L LC50	0.0422: 48 h Daphnia magna mg/L EC50
Bentonite 1302-78-9	-	19000: 96 h Oncorhynchus mykiss mg/L LC50 static 8.0 - 19.0: 96 h Salmo gairdneri g/L LC50	-

Persistence and degradability

Not readily biodegradable.

Bioaccumulation

Not likely to bioaccumulate.

Other adverse effects

This mixture contains no substance considered to be persistent, bioaccumulating nor toxic (PBT)

13. DISPOSAL CONSIDERATIONS

Waste treatment methods

Disposal of wastes

Pesticide wastes may be acutely hazardous. Improper disposal is a violation of federal law. Disposal should be in accordance with applicable regional, national and local laws and regulations. Contact your State Pesticide or Environmental Control Agency, or the Hazardous Waste Representative at the nearest EPA Regional Office for guidance on proper disposal of waste product.

Figure A 7. Parts of the SDS V1 of Kocide 3000 from 20/02/2017.

Table A 3. One-way ANOVA results of the effects of concentrations of formulations on L. sajor caju.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	9.740	5	1.948	F (5, 24) = 6..	P=0.0008
Residual (within columns)	7.509	24	0.3129		
Total	17.25	29			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norn**	**P value sum**	
Anderson-Darling (A2*)	0.5432	0.1495	Yes	ns	
D'Agostino-Pearson omnibus (K2)	1.599	0.4494	Yes	ns	
Shapiro-Wilk (W)	0.9491	0.1599	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1353	0.1000	Yes	ns	

Table A 4. Tukey's multiple comparisons test for the effects of concentrations of formulations on L. sajor caju.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
CTR vs. CH_1.5	0.1140	-0.9798 to 1.208	No	ns	0.9995	A-B
CTR vs. CH_3	0.08800	-1.006 to 1.182	No	ns	0.9999	A-C
CTR vs. CH_5	0.5920	-0.5018 to 1.686	No	ns	0.5611	A-D
CTR vs. CH_7.5	0.7620	-0.3318 to 1.856	No	ns	0.2949	A-E
CTR vs. CH10	1.644	0.5502 to 2.738	Yes	**	0.0013	A-F
CH_1.5 vs. CH_3	-0.02600	-1.120 to 1.068	No	ns	>0.9999	B-C
CH_1.5 vs. CH_5	0.4780	-0.6158 to 1.572	No	ns	0.7543	B-D
CH_1.5 vs. CH_7.5	0.6480	-0.4458 to 1.742	No	ns	0.4656	B-E
CH_1.5 vs. CH10	1.530	0.4362 to 2.624	Yes	**	0.0028	B-F
CH_3 vs. CH_5	0.5040	-0.5898 to 1.598	No	ns	0.7123	C-D
CH_3 vs. CH_7.5	0.6740	-0.4198 to 1.768	No	ns	0.4232	C-E
CH_3 vs. CH10	1.556	0.4622 to 2.650	Yes	**	0.0024	C-F
CH_5 vs. CH_7.5	0.1700	-0.9238 to 1.264	No	ns	0.9964	D-E
CH_5 vs. CH10	1.052	-0.04182 to 2.146	No	ns	0.0643	D-F
CH_7.5 vs. CH10	0.8820	-0.2118 to 1.976	No	ns	0.1658	E-F

Table A 5. Result of one-way ANOVA performe for results of BM (a) and $CuSO_4$ (b) solutions on L. sajor caju.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	10.21	2	5.105	F (2, 12) = 8..	P=0.0056
Residual (within columns)	7.451	12	0.6209		
Total	17.66	14			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norn**	**P value sum**	
Anderson-Darling (A2*)	0.6577	0.0687	Yes	ns	
D'Agostino-Pearson omnibus (K2)	11.26	0.0036	No	**	
Shapiro-Wilk (W)	0.8742	0.0389	No	*	
Kolmogorov-Smirnov (distance)	0.2006	0.1000	Yes	ns	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	26.93	2	13.47	F (2, 12) = 20	P<0.0001
Residual (within columns)	0.7879	12	0.06566		
Total	27.72	14			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norm**	**P value sum**	
Anderson-Darling (A2*)	0.4631	0.2198	Yes	ns	
D'Agostino-Pearson omnibus (K2)	1.156	0.5609	Yes	ns	
Shapiro-Wilk (W)	0.9428	0.4187	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1835	0.1000	Yes	ns	

Table A 6. Results of Tukey's multiple comparisons test of the effects of BM and $CuSO_4$ on L. sajor caju.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
CTL vs. BM 20	1.308	-0.02155 to 2.638	No	ns	0.0539	A-B
CTL vs. BM 60	1.988	0.6585 to 3.318	Yes	**	0.0047	A-C
BM 20 vs. BM 60	0.6800	-0.6495 to 2.010	No	ns	0.3890	B-C

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	
CTL vs. CuSO4 4	0.2200	-0.2124 to 0.6524	No	ns	0.3925	A-B
CTL vs. CuSO4 12	2.946	2.514 to 3.378	Yes	****	<0.0001	A-C
CuSO4 4 vs. CuSO4 12	2.726	2.294 to 3.158	Yes	****	<0.0001	B-C

Table A 7. Results of one-way ANOVA for the effects of CH (a), KO (b), BM (c) and $CuSO_4$ (d) on B. cinerea.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	79.01	4	19.75	F (4, 19) = 14	P<0.0001
Residual (within columns)	25.17	19	1.325		
Total	104.2	23			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norm**	**P value sum**	
Anderson-Darling (A2*)	0.5722	0.1223	Yes	ns	
D'Agostino-Pearson omnibus (K2)	0.8176	0.6644	Yes	ns	
Shapiro-Wilk (W)	0.9512	0.2883	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1715	0.0660	Yes	ns	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	48.95	4	12.24	F (4, 19) = 7.	P=0.0010
Residual (within columns)	31.95	19	1.681		
Total	80.89	23			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norm**	**P value sum**	
Anderson-Darling (A2*)	0.3497	0.4440	Yes	ns	
D'Agostino-Pearson omnibus (K2)	0.7471	0.6883	Yes	ns	
Shapiro-Wilk (W)	0.9622	0.4843	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1402	0.1000	Yes	ns	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	80.55	3	26.85	F (3, 15) = 26	P<0.0001
Residual (within columns)	14.99	15	0.9997		
Total	95.55	18			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norm**	**P value sum**	
Anderson-Darling (A2*)	0.2722	0.6293	Yes	ns	
D'Agostino-Pearson omnibus (K2)	0.05213	0.9743	Yes	ns	
Shapiro-Wilk (W)	0.9678	0.7312	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1273	0.1000	Yes	ns	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	138.3	4	34.58	F (4, 19) = 19.52	P<0.0001
Residual (within columns)	33.67	19	1.772		
Total	172.0	23			
Normality of Residuals					
Test name	**Statistics**	**P value**	**Passed norm**	**P value summary**	
Anderson-Darling (A2*)	0.4916	0.1985	Yes	ns	
D'Agostino-Pearson omnibus (K2)	0.09723	0.9525	Yes	ns	
Shapiro-Wilk (W)	0.9510	0.2852	Yes	ns	
Kolmogorov-Smirnov (distance)	0.1621	0.1000	Yes	ns	