

Nanoparticles for 3-methyl-5-phenyl-pyrazoline-1-(S-benzyldithiocarbazate) delivery: Synthesis, Characterization and Eco-cytotoxicity studies

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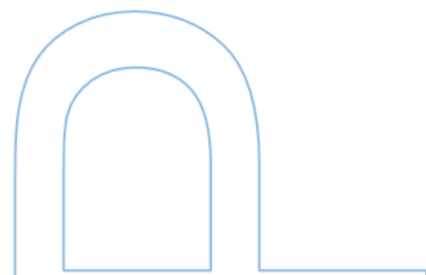
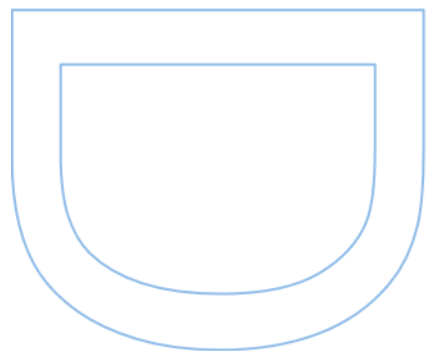
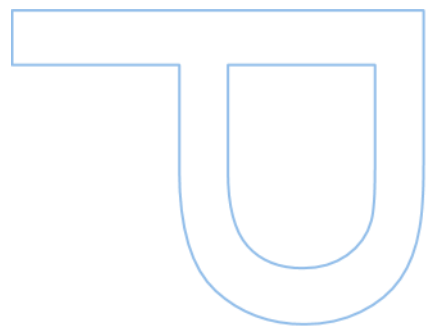
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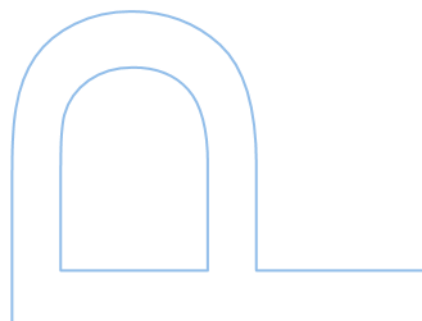
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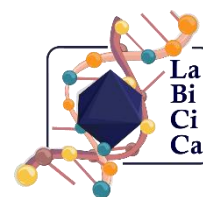
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Resumo

Atualmente, existem diferentes tipos de pesticidas para o controlo de pragas e doenças nas lavouras. Entretanto, pouco se conhece sobre os fungicidas para o controlo dos fungos *Moniliophthora perniciosa*, *Phytophthora theobromicola* e *Phytophthora palmivora* que são motivo de grande preocupação, devido a perdas significativas nas plantações cacaueiras brasileiras. Nessa perspectiva, destaca-se o uso de fungicidas químicos, como os compostos orgânicos ditiocarbazatos, com notáveis aplicações biológicas. No entanto, a exploração total da atividade biológica de 3-metil-5-fenil-pirazolina-1-(S-benzilditiocarbazato) (DTC) é limitada devido à sua fácil degradação e baixa solubilidade em soluções aquosas. O carregamento de DTC em Nanopartículas de Sílica Mesoporosa (MSiNP) e Nanopartícula Lipídica (LN) pode ser uma excelente estratégia para melhorar a solubilidade do DTC em meio aquoso. Neste sentido, o principal objetivo deste Projeto de Doutorado foi a produção, caracterização físico-química das nanopartículas MSiNP e LN carregadas com DTC, bem como a avaliação da eficácia e da segurança humana e ambiental destes compostos a serem utilizados como fungicida em práticas agrícolas. Como resultado, as nanopartículas (NPs) apresentaram uma distribuição de tamanho homogênea e boa estabilidade física, com alta capacidade de carregamento e eficiência de encapsulamento do DTC. A avaliação da atividade antifúngica *in vitro* de DTC e NPs resultou em uma inibição acima de 30% para todos os fungos testados, sendo mais eficientes que o fungicida Tricovab aplicado para *M. perniciosa*. No entanto, particularmente para MSiNP e MSiNP-DTC não houve inibição do crescimento fúngico, o que pode estar relacionado às características intrínsecas dessas NPs. As avaliações de segurança em células humanas HaCaT (queratinócitos normais) e células A431 (carcinoma de células escamosas), em organismos aquáticos (*Raphidocelis subcapitata*, *Lemna minor* e a bactéria marinha *Aliivibrio fischeri*) e na comunidade microbiana do solo indicaram que o DTC e as NPs apresentaram baixa ou nenhuma toxicidade nas concentrações testadas para essas células e organismos. Portanto, no presente estudo, comparando os efeitos das MSiNP-DTC e das LN-DTC, concluímos que as LN demonstram ser um excelente carreador para o DTC e pode ser uma alternativa promissora para uso como nanofungicidas no controlo dos fungos que diretamente atacam as plantações de cacau já que, além de inibirem significativamente o crescimento de fungos, também apresentaram melhores resultados que o fungicida comercializado.

Palavras-chave: Base de Schiff, Nanofungicidas, Eco-citotoxicidade, Avaliações de Segurança

Abstract

Currently, there are different types of pesticides for the control of pests and diseases in crops. However, little is known about fungicides for the control of fungi *Moniliophthora perniciosa*, *Phytophthora theobromicola* and *Phytophthora palmivora* that are of great concern, due to significant losses in Brazilian cocoa plantations. From this perspective, the use of chemical fungicides stands out, such as the organic compounds dithiocarbazates, with notable biological applications. Nevertheless, full exploitation of the biological activity of 3-methyl-5-phenyl-pyrazoline-1-(S-benzylthiocarbamate) (DTC) is limited due to its easy degradation and poor solubility in aqueous solutions. The loading of DTC into Mesoporous Silica Nanoparticles (MSiNP) and Lipid Nanoparticle (LN) can be an excellent strategy to improve the solubility of DTC in the aqueous medium. In this sense, the main objective of this Doctoral Project was the design, physicochemical characterization of nanoparticles MSiNP and LN loaded with DTC, as well as the evaluation of efficacy and human and environmental safety of these compounds to be used as a fungicide in agricultural practices. As a result, the nanoparticles (NPs) showed a homogeneous size distribution and good physical stability, with high loading capacity and DTC encapsulation efficiency. The evaluation of the *in vitro* antifungal activity of DTC and NPs showed inhibition above 30% for all fungi tested, being more efficient than the fungicide Tricovab applied for *M. perniciosa*. However, particularly for MSiNP and MSiNP-DTC there was no inhibition of fungal growth, which may be related to the intrinsic characteristics of these NPs. The safety assessments with human HaCaT cells (normal keratinocytes) and A431 cells (squamous cell carcinoma), with aquatic organisms, (*Raphidocelis subcapitata*, *Lemna minor* and the marine bacteria *Aliivibrio fischeri*) and with the soil microbial community indicated that DTC and NPs showed no or low toxicity at the tested concentrations to these cells and organisms. Therefore, in the present study, comparing the effects of MSiNP-DTC and LN-DTC, the results allowed to conclude that LN seem to be an excellent carrier for DTC and can be a successful alternative for use as nanofungicides to control the fungi that directly attack cocoa crops, since not only inhibited significantly the fungal growth, but also presented better results than the marketed fungicide.

Keywords: Schiff Base, Nanofungicides, Eco-cytotoxicity, Safety Assessments

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List of abbreviations

% DL: Drug Load expressed as %

% WHC_{max}: Water-Holding Capacity expressed as %

%EE: Loading Efficiency expressed as %

A431 cells: Human Skin Cancer Cells

A549: Human lung epithelium cancer

AB: Alamar Blue

AI: Active Ingredient

BET: Brunauer–Emmett–Teller

BOD: Biochemical Oxygen Demand

DLS: Dynamic Light Scattering

dm: % dry matter

DMSO: Dimethylsulfoxide

DSC: Differential Scanning Calorimetry

DTA: Differential Thermal Analysis

DTC: 3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate)

EC₂₀: 20% maximal effective concentration

EC₅₀: 50% maximal effective concentration

FBS: foetal bovine serum

FTIR: Fourier-Transform Infrared Spectroscopy

h.e.: highest effect

HaCaT cells: Human Normal Kertotinocytes

HCT116: Human colorectal carcinoma

ISO: International Organization for Standardization

K562: Human chronic myeloid leukemia

LN: Lipid Nanoparticles

LN-DTC: DTC- loaded Lipid Nanoparticles

LOEC: Low Observed Effect Concentrations

MSiNP: Mesoporous Silica Nanoparticles

MSiNP-DTC: DTC- loaded Mesoporous Silica Nanoparticles

NC: Could Not be Calculated

NLC: Nanostructured Lipid Carriers

NMs: Nanomaterials

NOEC: No Observed Effect Concentrations

NPs: Nanoparticles

NT: Not Toxic

O/W: Oil/Water emulsion

OECD: Organization for Economic Co-operation and Development

OM: Organic Matter Content (%)

PDI: Polydispersity Index

PhCH₂Cl: Benzyl Chloride

pNP: p-nitrophenol

ROS: Reactive Oxygen Species

S.D.: Standard Deviation

SAA: Soil Arylsulfatase Activity

SAPA: Soil Acid Phosphatase Activity

SDA: Soil Dehydrogenase Activity

SiNPs: Silica Nanoparticles

SL: Solid Lipid

SLN: Solid Lipid Nanoparticles

SMA: Soil Mineralization Activity

sp: spore

TEM: Transmission Electron Microscope

TG: Thermogravimetry

TPF: Triphenylformazan

TTC: Triphenyltetrazolium chloride

Z-Ave: Average particle size

ZP: Zeta Potential

1 Introduction

1.1 The use of Fungicides in Agricultural Practices

On a planet with more than 8 billion people, around of 200 thousand people die annually worldwide due to the direct impact of pesticides [1]. Chemical substances used to prevent, destroy, repel, or mitigate crop pests such as insects, rodents, weeds, and microorganisms are defined as pesticides [2]. With the development of agricultural production, plant pathogens, biological pests and genetically modified or disease-resistant crop varieties began to emerge and gain more attention [3]. Currently, a total of 883 active substances are associated with pesticidal action and use in agriculture, which 255 are fungicides, 272 are insecticides and 356 are herbicides [4].

Several chemical compounds have been used as pesticides, such as polycyclic aromatic hydrocarbons (PAHs), carbamates, organochlorines. Among different pesticides, pyrethroids, organophosphates and chlorinated compounds (DDT, hexachlorocyclohexane, aldrin and dieldrin) are the most widely used pesticides in developing countries due to their low cost, easy availability in local markets and versatility against various pests [5].

Within the different classes of pesticides, fungicides include physical, chemical, or biological agents intended to combat fungal pathogens. These microorganisms are widely used in agricultural systems to control diseases and preserve the yield and quality of crops [6]. Fungicides can act by different modes when applied to foliage. Some compounds, such as chloroneb, mancozeb, and thiram act through contact, protecting the plant surface as a shield. However, these active ingredients (AI) are short-lived and in general can suffer physical degradation from environmental effects (pollution, rain, ultraviolet light, etc.). Cyazofamid, iprodione, pyraclostrobin are fungicides that act both on the surface and can also penetrate the plant leaf, while azoxystrobin, boscalid, propiconazole are penetrants compounds that act by moving through the xylem and phloem [7].

In tropical countries, such as Brazil, where high temperatures and humidity facilitate the rapid multiplication of pests and fungi, the intensive use of pesticides helps to protect crops against pests (insects, mites, nematodes and rodents), phytopathogens (fungi, viruses and bacteria) and weeds, reducing crop loss by 30%. Regarding fungicides, only a small part of what is applied remains in the plant. In general, despite the heavy use of pesticides, only 1% of the total is used to control insect pests on target plants, the remainder enters or

reaches non-target plants and environmental media. About 55% is lost during fungicide application, which is found in the soil, water or even air, reducing its efficiency [8]. As a result, pesticide contamination significantly pollutes the environment and causes adverse impacts on human health [9].

For example, edolfuran, widely used in Brazil as insecticide, more than 99% is misused and remains in the environment as a pollutant [5]. In the same country, the cost passed on by the government in the environmental, social and health areas due to acute intoxication to the intensive use of pesticides, mainly insecticides and herbicides for corn, is currently around 64% and could reach 85% in 2029 [10]. In addition, the use of fungicides in cocoa plantations in Brazil, does not reach more than 30% efficiency, can lead to the loss of more than 75% of production and, consequently, increase of the costs for the producers, as well as soil and fruit contamination [11].

Thus, in view of the growing use of pesticide compounds, much must be taken into account, mainly due to the fact that pesticides directly affect human health in the short term (acute effects), as well as generating chronic adverse effects that may occur months or years after their application. Acute effects include burning eyes, skin rashes, blisters, partial or permanent blindness, nausea, dizziness, diarrhea, while cancers, birth defects, reproductive damage, neurological and endocrine system disruptions are examples of chronic effects [2].

In addition to their potential negative effects on human health, the use of pesticides can also lead to various deleterious effects on the environment (contamination of water, soil and air, toxic effects on non-target organisms). In particular, adverse effects on non-target organisms by: water contamination affecting the aquatic species through its accumulation; soil contamination affecting its pH, stability, solubility that directly affects the soil microbiota diversity; damage to non-target plants and rotating crops by herbicide/fungicides residues remaining in the field or by high application rate/misapplication or unfavorable environmental conditions during and after pesticide application [12].

Therefore, strong efforts are urgently needed to minimize the risks of pesticides on human and ecosystem's health and to protect resources. In this context, new chemical compounds proposed for use as fungicides must be characterized by high toxicity to pathogenic agents and, simultaneously, low toxicity to non-target organisms and human health. They must also be durable, susceptible to biodegradation in such a way that they do not accumulate in the environment [13].

1.2 Schiff Base Derived from Dithiocarbazate

Dithiocarbazates, compounds which belong to the Schiff base class, have been highlighted as possible pesticides, more specifically fungicides to be used as better alternatives to existing ones. Schiff's base reaction, the classic reaction discovered by Hugo Schiff in 1864 [14], is a versatile tool in organic synthesis, which originates organic compounds, that have at least one $R_2C=N$ - group and are obtained by the condensation of a primary amine with a carbonyl group [15]. They play an important role in the development of coordination compounds, as they form stable complexes with a large number of transition metals [16]. Since the Schiff bases of biologically active heterocycles are also pharmacologically active, they attracted high interest in the medicinal field [17], and also expanded to the applications in electroplating, metallurgy, photography [18]. Further, these compounds have been used as pesticides in the agriculture practices [19].

Dithiocarbazate ligands are Schiff bases with great chelating capacity due to their diversified binding sites [20]. The coordination chemistry of dithiocarbazates has aroused considerable interest due to their ability to form various metal complexes with different coordination numbers and geometries [21].

Dithiocarbazates $[NH_2NHCS_2]$ have sulfur and nitrogen atoms (substituted derivatives) in their constitution that are the main responsible for their donor properties which can be modified or adapted by means of suitable substituents in the side chain [22]. Usually, dithiocarbazate can be easily formed by condensation of thiazide with certain aldehydes and ketones. The thione to thiol tautomeric transformation schematically represented in Figure 1 of dithiocarbazate plays a critical role in building distinct coordination geometries around metal centers that results a required significant change in color [16]. The number of synthesized ligands continues to increase due to the intriguing observation that different ligands show different biological properties, even though they may only slightly differ in their molecular structure [23].

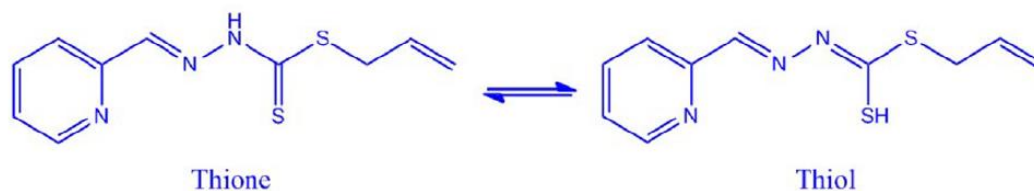


Figure 1. Representative tautomeric transformation of thione to thiol of Dithiocarbazate (Takjoo *et al.*, 2018)

Most of the published works related to dithiocarbazates aim at obtaining chelators (metal-coordinated dithiocarbazates). Metal ions, as a catalytic center, play a very important role in the electron transfer process with Schiff bases as they are easy to lose electrons and form empty electron orbits, which are typical “Lewis acids”, resulting in an improvement of properties of these compounds [24]. For example, the chelation of metals with dithiocarbazate increases the ability of the complex to cross the cell membrane, increasing its bacterial activity. This occurs due to the delocalization of π -electrons in the chelate ring, favoring its permeation through the lipid layer of the cell membrane [25].

It has also been shown that transition metal Co(II), Ni(II), Cu(II) and Zn(II) complexes of Schiff bases derived from 1,2,4-triazole compounds exhibit greater antifungal activity than their precursor Schiff base [26]. A study conducted by Nanjundan et al., 2017 demonstrated that derivatives of dithiocarbazates (S-allyldithiocarbazate) complexed with metallic ions Ni(II), Cu(II) and Zn(II) showed significant activity against the human cervical cancer cell line (HeLa) even at micromolar concentrations, but significantly less toxic to normal Vero cell line [27].

However, all the biological and pharmacological properties such as anticancer, antifungal, anti-inflammatory, antibacterial activities, mainly for neglected diseases such as malaria, trypanosomiasis and tuberculosis come from the Schiff base binding compounds [28,29], being only potentiated when linked to metal ions, although the synthesis of these complexes are considered costly and involve several steps.

Regarding the application of Schiff's bases as a fungicide, as far as we know, there are only works published for dithiocarbamates which are a group of compounds containing disulfide substituents with various toxicities and are widely used in agriculture. Depending on the carbon chain structure of the molecule, dithiocarbamates can be divided into three major types: (1) dimethyldithiocarbamates (DMDCs): Thiram and Ferbam; (2) ethylene-bis-dithiocarbamates (EBDCs): Mancozeb, Maneb, Zineb and Mitram; and (3) propylene-bis-dithiocarbamates (PBDCs): Propineb [30].

Dithiocarbamates have been applied as fungicides and are known worldwide due to their low cost, relatively lower acute toxicity, and lower persistence in the environment. However, recent studies show that these compounds are toxic to humans and the environment mainly due to their bioaccumulation. For example, the compound Zineb was phased out in several countries after it was found to have a strong inhibitory effect on thyroid peroxidase (TPO) that can lead to reductions in circulating levels of thyroxine (T4) [31].

In vitro and *in vivo* studies confirmed that exposure to Maneb and Mancozeb causes the generation of reactive oxygen species (ROS) and alterations in antioxidant defense systems that lead to increased markers of oxidative stress and mitochondrial inhibition and consequently induces apoptosis in fibroblasts and mouse peripheral blood cells and human lymphocytes [32]. Studies have revealed that frequent exposure to Thiram is tremendously hazardous to human beings, producing impairments in the liver, nerves system and reproduction system [33].

Specifically for aquatic organisms, acute Thiram exposure induced oxidative damage in *Daphnia magna* and demonstrated toxicity in *Cyprinus carpio*, causing glycogen depletion in the liver, as well as muscle and elevated blood glucose levels. Studies have linked embryonic thiram exposure to reproductive toxicity, thyroid dysfunction, spinal curvature, and abnormal craniofacial development in zebrafish [34]. It is also considered toxic to many animals, including chickens, goats, mice and fish [35]. Exposure to Propineb triggers abnormalities in different organ systems in zebrafish and suggests the physiological complexity of the response to Propineb [36]. Mancozeb toxicity in aquatic organisms has been reported to be moderate to high for *Daphnia magna* and zebrafish (*Danio rerio*). Chronic exposure to Mancozeb also causes changes in gonadal structure and function in female rats [37,38].

In general, compounds based on dithiocarbamates were found to be moderately toxic bactericides, and their toxicity can be significantly increased when heavy metal ions are included and cause poisoning by food exposure or skin contact. In addition, they have been classified as a Group 3 carcinogen by the International Agency for Research on Cancer (IARC) [31,39]. As reported, major efforts are urgently needed to minimize the risks of using these compounds as fungicides on human and ecosystem health. In this context, new chemical compounds proposed for use as fungicides must be characterized by high toxicity to pathogenic agents and, simultaneously, low toxicity to non-target organisms and human health. They must also be durable, susceptible to biodegradation so that they do not accumulate in the environment [13].

1.2.1 DTC: (3-methyl-5-phenyl-pyrazol-1-(S-benzylthiocarbamate))

Given the large amount of dithiocarbamate compounds derived from the Schiff base, 3-methyl-5-phenyl-pyrazoline-1-(S-benzylthiocarbamate) named as DTC has attracted great attention specially for its ease of manufacture obtaining almost 100% of the product after its purification. Considering the biological properties of DTC this compound presents as a promising agent to treat a variety of diseases.

Carneiro *et al.*, 2014 showed that the dithiocarbamate derivative (5-hydroxy-3-methyl-5-phenyl-pyrazoline-1-(S-benzylthiocarbamate) (H₂bdtc)), presented advantages over the currently used drug, bezonidazole (BZN) against *Trypanosoma cruzi*. In addition, dithiocarbamate decreased inflammation and damage to the liver and heart, leading to a possible development of new drug to be used against Chagas disease [40].

It is possible to obtain DTC by acid-catalyzed conversion, adding two drops of concentrated HCl in the second step of the synthesis described schematically in Figure 2. At the end, compounds of different colors are produced, white for H₂bdtc and yellow for DTC [41].

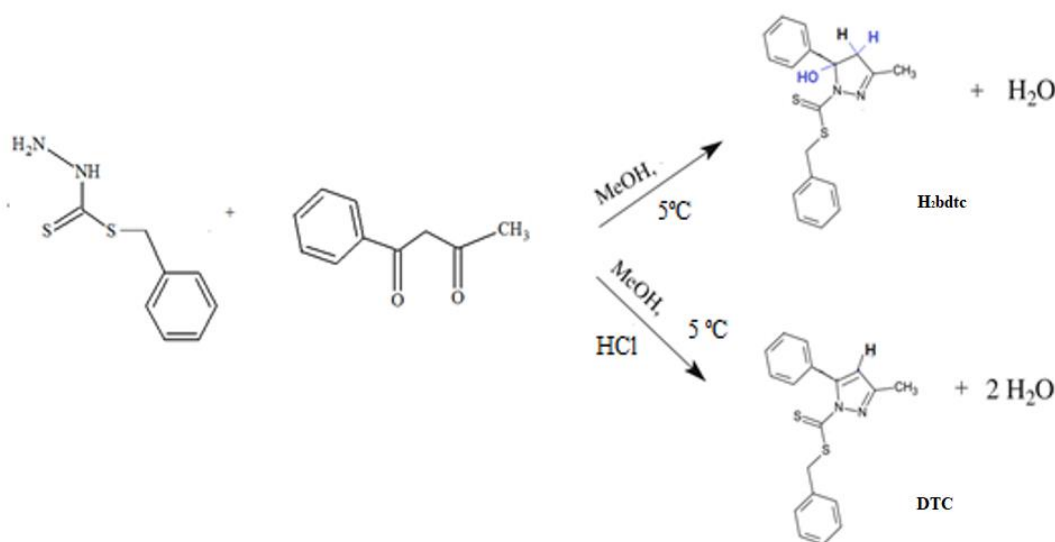


Figure 2. Scheme of the simplified synthetic route of two dithiocarbamate derivatives: 5-hydroxy-3-methyl-5-phenyl-pyrazole-1-(S-benzylthiocarbamate) (H₂bdtc) and 3-methyl-5-phenyl-pyrazol-1-(S-benzylthiocarbamate) (DTC). Adapted from (De Sousa *et al.*, 2011)

The compound referred as DTC, its synthesis and structural analysis were previously reported by De Sousa *et al.*, 2011 [42]. Recently, characterized by various techniques and density functional theory (DFT), calculations were performed to evaluate its electronic characteristics. In order to enhance its properties, DTC has been chelated with ruthenium (II) [28], and due to its low solubility in aqueous media, DTC has been encapsulated in biodegradable polymeric nanoparticles of poly(lactic acid) (PLA) aiming potential drug delivery application [29]. Molecular interactions between the surfactant Pluronic F127 (copolymer that has a tendency to self-assemble into micelles in aqueous medium) and DTC in aqueous solutions were also investigated by spectroscopy methods, in an attempt to improve DTC solubility and evaluate its possible applications. As a result, DTC-based micellar system prevents the DTC decomposition, increases solubility, and show potential application for disease treatments, since its immunomodulatory action on the macrophages may be tools on further studies for the development of novel microbicide or anti-inflammatory drugs [43].

Considering the published studies related to the biological properties of DTC and its possible applications, the present work focuses on the use of DTC as a fungicidal agent in agricultural practices, since dithiocarbazates have excellent antimicrobial characteristics and it is not necessary to include heavy metals in their structure, which could lead to increased toxicity to humans and environment. Although DTC exhibits notable potential biological properties, its low solubility and poor stability in aqueous media limit its application and bioavailability, making it necessary to conceive innovative designs by encapsulating DTC in nanocarriers, for example, in order to protect DTC against premature degradation to enhance its solubility in aqueous media and provide controlled release of DTC, thus contributing to greater efficiency and reduction of application doses in the agriculture practices.

1.3 Nanofungicides in Agricultural Activity

The use of Nanomaterials (NMs) has emerged as a promising way to control pests and pathogens in agricultural practices. The use of pesticide encapsulated in NMs can lead to a delivery of AI to the desired crop providing target delivery of the required dose (reducing toxicity to non-target species) promoting improvement, nutrition, and growth of plants, disease control and post-harvest quality [44]. Numerous nanoformulations with different types of materials (polymers, lipids, silica, metals) have been investigated for the encapsulation of fungicide AI. A study conducted by Taha *et al.*, 2023 showed that selenium

nanoparticles (Se-NPs) alone or in combination with SiO₂-NPs was efficient in the control of leaf spot caused by *Alternaria alternata* (*Phaseolus vulgaris* L.) on bean plants when compared to commercial formulations (Difenoconazole). The *in vitro* study showed that 100 ppm of Se-NPs had an effectiveness rate of 85.1% on the mycelial growth of *A. alternata*, followed by combined applications (Se + SiO₂ in half doses) with an effectiveness rate of 77.8%. Field studies showed that nano-Se and nano-Se associated with SiO₂-NPs significantly decreased the severity of the *A. alternata* disease, increasing leaf weight by 38.3%, number of leaves per plant by 25.7%, chlorophyll A by 24%, chlorophyll B by 17.5% and total seed yield dry by 30%. In addition, Se-NPs significantly increased the enzymatic capacity of the enzymes catalase, peroxidase and Polyphenol oxidase and the antioxidant activity in the leaves [45].

Other studies have focused on zinc, copper, gold and silver nanoparticles, as well as their oxides. ZnO-NPs allowed the development of cheaper, more reliable and ecofriendly nanofungicides, to determine their antifungal capacity on *Mycena citricolor* and *Colletotrichum* sp., ZnO-NPs showed an efficient antifungal capacity, with ~97% growth inhibition for *M. citricolor* and ~93% for *Colletotrichum* sp. With particle size <100 nm, ZnO-NPs acted in a way that they were easily absorbed in the fungi by being absorbed through the cell membrane causing cell dysfunction. Due to their physicochemical properties such as high surface area, they can directly or indirectly produce ROS, which affect cell wall proteins without causing adverse effects such as plant death [46].

Polymers and liposomes have been widely used as organic carriers to encapsulate pesticides. Poly(ethylene glycol) has been used as NMs aiding in the delivery of AI such as imidazole, clotrimazole, econazole nitrate and miconazole nitrate for the effective and efficient management of plant diseases, reducing the adverse effects on the environment. Nanoformulations with particle sizes below 100 nm and containing clotrimazole at concentration of 0.5% showed excellent *in vitro* antifungal activity, with a median effective dose (ED₅₀) of 1.18 µg mL⁻¹ against *Rhizoctonia solani*, while the nanoformulations containing econazole nitrate showed an ED₅₀ of 5.25 µg mL⁻¹ against *Sclerotium rolfsii* and the nanoformulations with miconazole nitrate showed an ED₅₀ of 1.49 and 1.82 µg mL⁻¹ against *Macrophomina phaseolina* and *Fusarium oxysporum*, respectively [47].

Nanofungicides have been considered as safe and efficient in pest control and have drawn the attention of the scientific community and the agro-industrial sector due to their ability to increase efficiency against the target organism and reduce side effects, mainly on non-target

organisms, overcoming some limitations presented by conventional pesticides. However, due to advantages such as their large specific surface areas, nanofungicides have become an important topic in the field of research on environmental hazards which may become a new class of pollutants that threaten human health and the balance of ecosystems [48].

A review by Chen *et al.*, 2019 reports that the various types of carbon NMs, such as fullerene, carbon nanotubes, and graphene, induce effects on microbial communities (manifested in community structure, biomass, and abundance of microorganisms). As a result, it was found that such effect is dependent on the type of microorganism, concentration of carbon NMs and external environment and act by directly destroying the electrostatic balance or cell membrane and organelles of microbial cells, as well as to produce oxidative stress, which induces changes in lipids and proteins in microbial cells, causing abnormal metabolism of microorganisms and further cell damage [31].

Some studies demonstrated that Ag-NPs from 10 to 80 nm affect early life stage development, which includes spinal cord deformities, cardiac arrhythmia, and survival in zebrafish [50]. These NPs also accumulate in the gills and liver tissue, affecting the fish's ability to cope with low oxygen levels and inducing oxidative stress [51]. In human cells, Ag-NPs cause endoplasmic reticulum stress signalling and induce apoptosis [52].

Au-NPs can be considered toxic to aquatic biota and can induce cellular toxicity through different mechanisms, such as oxidative stress, cell membrane disruption, inflammation and DNA damage [53]. Fe-NPs at concentration of 10 mg mL⁻¹ showed to be toxic to the zebrafish embryo, causing mortality, delayed hatching and malformation. In general, metallic NPs can cause effects on embryo development, oxidative stress and bioaccumulation, but these effects depend on the exposure time, concentrations and application dose for a given type of exposure to the environment [54].

In this way, due to the vast types and models of NMs currently used for AI loading for possible application as a nanofungicide, it becomes necessary to choose a way that has advantages in relation to the amount of AI loading and mainly that it does not have (or reduces) the risks associated with humans and the environment.

1.3.1 Mesoporous Silica Nanoparticles

The structures of Mesoporous Silica Nanoparticles (MSiNP) have become familiar for material scientists for 40 years and the first independent synthesis was achieved in 1990, conducted by researchers in Japan. Later, in 1992, it was synthesized by Mobil Corporation Laboratories, where it was named as “molecular 41 sieves” (M41S) [55]. The most frequently reported families are MCM-n (Mobil Corporation Matter), SBA-n (Santa Barbara), MSU-n (Michigan State University), KIT-n (Korean Institute of Technology), FSM-n (folded sheet materials), FDU (Fudan University) and AMS-n (anionic mesoporous silica) [56].

There are a variety of methods, which are represented in Figure 3, that can be used to produce MSiNP. However, in general, the synthetic route relays in three different processes: (i) the sol–gel process for producing silica; (ii) the use of surfactants as structure directing agents for producing mesoporous materials; (iii) and Stöber method modification under dilute conditions to yield spherical nanoparticles [57].

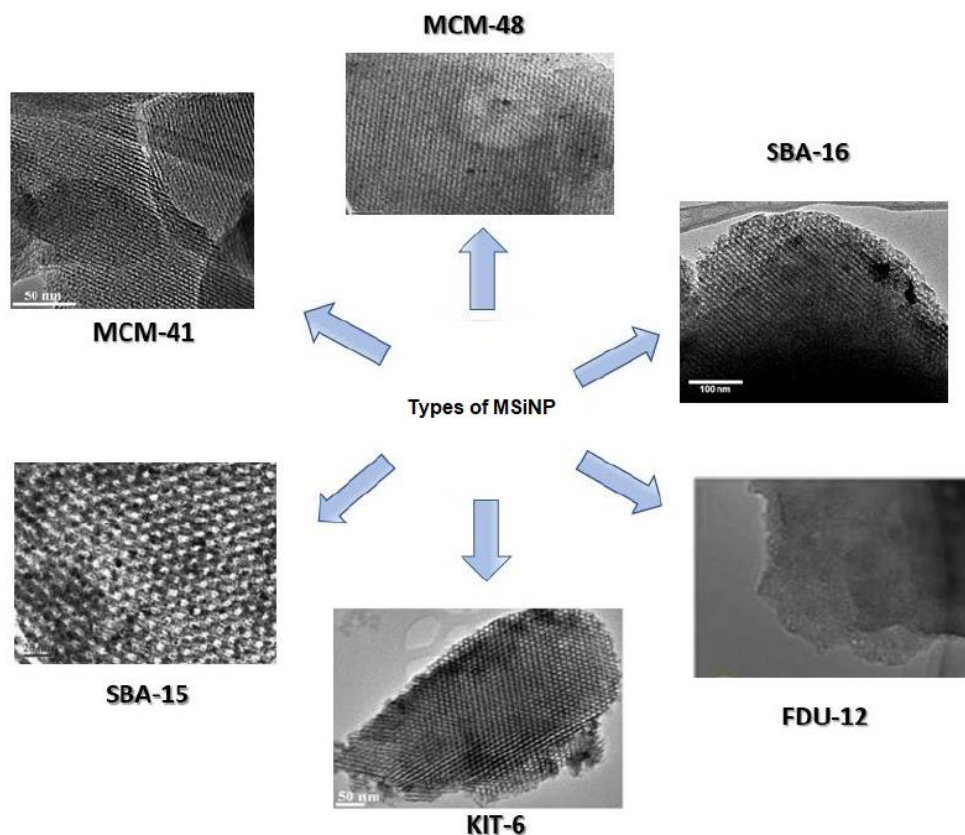


Figure 3. Some types of MSiNP, MCM-n (Mobil Corporation Matter), SBA-n (Santa Barbara), FDU-n (Fudan University) and KIT-n (Korean Institute of Technology). Adapted from (Sreeharsha *et al.*, 2022).

First reported by Stöber, Fink and Bohn, in 1968, the synthesis of colloidal spherical silica particles is based on an ammonia-catalyzed sol–gel reaction where hydrolysis of tetraethylorthosilicate (TEOS) occurs in the presence of water and low molecular weight alcohols like ethanol, that is followed by subsequent condensation [58]. The Stöber method, is one of the most used processes for silica syntheses due to its low cost and easy operation [59] leading to highly tailorable particle size and surface properties [60].

Mesoporous silica materials obtained by the Stöber method are attractive as NMs, offering a large specific surface area, a high pore volume and an ordered/disordered pore network. Loading drugs into the silica pores suppresses AI crystallization due to confinement effects, leading to an amorphous formulation with greater physical stability, in particular for pore sizes smaller than the critical AI nucleus size. Due to their amorphous nature, mesoporous formulations generally exhibit dissolution profiles with rapid AI release and generation of a supersaturated solution, leading to better bioavailability compared to a crystalline compound [61–63].

In the field of agriculture, materials based on silica have been utilized to improve plant resistance against different biotic and abiotic stresses and, along these lines, MSiNP have attracted considerable interest to produce different formulations for fungal control [64]. In addition, beneficial effects of MSiNP on the growth of various crops, such as reducing the infection and damage degree of plant disease, as well as improving plant stress tolerance, have been reported.

Recent reviews have already concentrated on the use of MSiNP as NMs for pesticides to achieve intelligent transportation and targeted release, such as adsorbents to remove pesticides from aqueous media or enrich trace pesticides before quantitative determination, and as well as a support of catalysts or sensors to degrade or detect pesticide residues [65]. Kaziem *et al.*, 2021 proposed a system with MSiNP containing the fungicide chlorothalonil (CHT). In this work, β -glucanase enzyme-responsive nanovehicle was used for controlling chlorothalonil (CHT) release in the plant vascular system (CHT@MSNs- β -glucans) by attaching β -glucans extracted from the yeast cell wall on MSiNP pore rims for the management of rice blast (*Magnaporthe grisea*). As a result, CHT@MSNs- β -glucans showed less biotoxicity for *Daphnia magna*, and less toxic effects for the soil microbial community than the commercial product (CHT-WP). It is considered a promising approach to achieve maximum employment as pesticides and minimum environmental and human risks [66].

Another work published by Pan *et al.*, 2022 reports the use of MSiNP as a carrier complexed by ferric ion and polymethacrylic acid (PMAA/Fe³⁺) to deliver the AI prochloraz (Pro) for the smart treatment of wilt disease. Pro is known as a broad-spectrum, high-efficiency imidazole fungicide. However, the use of Pro to prevent foliar disease is limited due to poor conductivity and light instability. When added to NMs such as MSiNP, it was able to promote excellent antifungal activity against *Rhizoctonia solani* compared to AI isolated at the same concentration, providing a new measure of defense to crops against wilt disease [67].

In this way, in view of the numerous advantages mentioned above for the application of MSiNP, these NPs become promising transport systems for carrying DTC, which is a hydrophobic compound, increasing its solubility in aqueous media. Due to the large surface area of MSiNP, DTC can be adhered to MSiNP pores leading to a high loading efficiency.

1.3.2 Lipid Nanoparticles

Lipid nanoparticles (LN) were also chosen to encapsulate DTC. LN can be considered less toxic and more biocompatible compared with inorganic or polymeric NPs [68]. The first generation of LN was reported in the early 1990s and is known as SLN (Solid Lipid Nanoparticles), originally developed by replacing the oil in an O/W (oil/water) emulsion with a solid lipid at room temperature, in which the lipid is dispersed in the aqueous phase and both phases are stabilized by surfactants to prevent aggregation/agglomeration of NPs [69]. However, SLN presents some potential problems mainly due to their stability during storage, since they crystallize during the preparation method promoting drug expulsion when stored. Other disadvantages of SLN are the low drug payload and high-water content [70]. On the other hand, representing a second generation of LN, NLC (Nanostructured Lipid Carriers) were developed to overcome the problems associated to SLN. In this case, NLC are constituted by a mixture of solid and liquid lipid, which results in an increase of the space to accommodate AI, thus ensuring higher drug load values than SLN as shown in Figure 4, preventing drug loss during storage [71,72].

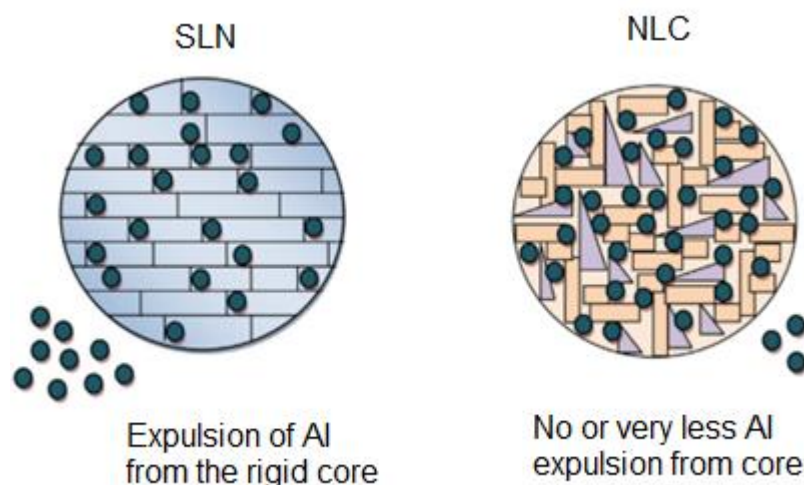


Figure 4. Illustration of stability of SLN and NLC during storage. Adapted from (Salvi & Pawar, 2019).

LN can be produced by techniques considered “green”, without the use of organic solvents, like high shear and ultrasonic homogenization, high pressure homogenization, hot and cold homogenization, solvent emulsification/evaporation and microemulsion [73], using mostly biodegradable and biocompatible lipids [74]. In addition, these systems have good physicochemical stability [75], promote the controlled release of AI and their production offers competitive costs and ease of manufacture [76].

Since LN are essentially composed of lipid matrix, these NPs have the ability to interact with various types of bacterial and fungal cells and can also facilitate the antimicrobial activity of AI, improving the diffusion properties across biological membranes due to the nanosized and lipophilic nature [77–79] .

Although LN have a remarkably wide application range and have been used to control the release of several AI in different routes of administration (nasal, oral, dermal, ocular and cerebral) [80–83], for use as pesticides, LN has been extensively, investigated with main use being for insecticide delivery. Recently, Maroofpour *et al.*, 2021, showed that the encapsulation of the insecticide pirimicarb in NLC was effective against the cabbage aphid, *Brevicoryne brassicae* (Linnaeus) (Hemiptera: *Aphididae*) and its natural enemy, the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: *Chrysopidae*) [84]. Nanoencapsulation of pirimicarb increased its toxicity to cabbage aphids by 12.6 times compared to its commercial formulation. In addition, nanoformulation reduced the

environmental impacts caused by conventional insecticides. However, many studies are based on the encapsulation of essential oils used as insecticides to combat pathogens in agriculture [78,85,86].

There are few works that refer the use of LN for fungicide loading. An example is the work developed by Campos *et al.*, 2015, in which the authors use LN as NMs to prevent photodegradation, volatilization, leaching and mainly to reduce the impacts on the environment and human health caused by the fungicides Carbendazim (MBC) (methyl-2-benzimidazole carbamate) and tebuconazole (TBZ) ((RS)-1-(4-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol) offering new options for the treatment and prevention of fungal diseases in plants [87]. LN have also been used as NMs to deliver Palmarosa essential oil (PEO), an alternative to synthetic fungicides to control the contamination by food spoilage fungi such as *Aspergillus nomius*. This system showed 100% of inhibition and prevented the deterioration of Brazil nuts by *A. nomius* [88].

Therefore, in addition to the properties mentioned above, the choice of LN for the encapsulation of compounds with low aqueous solubility is also based on the biocompatibility of these NPs, since the type of material used in the synthesis of NPs can cause toxic effects on the environment and in human health [89]. Due to their composition by physiological lipids, the use of LN for pesticide AI encapsulation can be a promising strategy to ensure the safe and effective application of DTC.

1.4 Evaluation of *in vitro* Antifungal Activity

The prevention and control of fungal diseases using chemical approaches constitute an important part of modern agricultural practices. However, the application of antimicrobial agents in the agriculture has raised relevant concerns in the scientific community and regulatory agencies, such as: resistance and microbial adaptation to commercially available compounds; decline in valuable natural resources, including loss of biodiversity, soil depletion, and damage inflicted on people and the environment [90].

In this context, fungal diseases are responsible for increasing losses in cocoa production, in addition to reducing planted areas in Central and South America [91]. Cocoa (*Theobroma cacao*) is considered a traditional and essential product of Brazilian agriculture, and Brazil is one of the largest producers of cocoa worldwide [92].

Among the infections originated by fungi that affect cacao cultivation, it has been identified several destructive diseases, such as the brown rot and cancer caused by *Phytophthora palmivora* and *Phytophthora theobromicola*, followed by striated vascular wilt induced by *Oncobasidium theobromae*, *Ceratocystis wilt* caused by *Ceratocystis wilt* monilia and witches' broom caused by *Moniliophthora roreri* and *Moniliophthora perniciosa*, respectively. Other active pathogens are fungi of the genus *Aspergillus* and *Penicillium*, bacteria of the genus *Bacillus* and yeast of the genus *Geotrichum*, which are active in the production of mycotoxins that tend to persist during the processing phase of the raw material [93–95].

Witches' broom (*M. perniciosa* and *M. roreri*) and black pod rot (*P. palmivora* and *P. theobromicola*) are two of the world's most dangerous cocoa diseases [90] and in the 1970s these infections became responsible for losses of more than 90% in cocoa production in Brazil, having a significant impact on socioeconomic development [96]. Witches' broom disease is known to induce overgrowth and hypertrophy in the branches, fruits and flower cushions, lead to plant death after successive attacks of the pathogen, favored by abiotic factors. Figure 5 displays a cocoa plant and fruit infected [97].



Figure 5. Infection of the cocoa plant and fruit by *M. perniciosa* (Witches' broom). Photo from the cocoa plantation belonging to M CCS - Mars Center for Cocoa Science.

Currently, a strategy used to reduce the growth of the fungi is the application of a commercial biofungicide known as Tricovab® and developed by CEPLAC (Comissão Executiva do Plano da Lavoura Cacaueira) applied since 1999. The AI of this formulation is the fungi *Trichoderma stromaticum*, which interacts with *M. pernicioso* via competition mechanisms by nutrients, antibiosis and mycoparasitism. Despite efficiency reports reaching 30%, the product does not offer complete confidence to farmers, since it can have interaction between plants, pathogens, microenvironment and biocontrol agents, in addition to experimental conditions, which can influence the success of biocontrol and generate conflicting results regarding its efficiency [94,98]. There are several studies that tested different agents to inhibit *M. pernicioso*, such as compounds derived from triazoles, strobilurins and cupric fungicides [99–101], essential oils and plant extracts [102–104] and other microorganisms [105,106]. However, all these agents described above have showed low efficiency to control *M. pernicioso*.

Despite witches' broom being the disease that causes the most losses in cocoa production, black pod rot disease caused by *P. palmivora* and *P. theobromicola* has also drawn attention with losses between 20% and 40% of the cocoa crop [107].

Oomycete pathogens of the genus *Phytophthora* are part of a distinct group of fungal-like eukaryotic microbes which share similar morphological properties to fungi, but also exhibit characteristics unique to plants, such as the cell wall composed by cellulose.

Another feature is that its mycelium is composed of hyaline, branched and non-septate filaments, while fungal hyphae are septate. The dissemination of *Phytophthora* occurs through the contamination of plants by deposition of the asexual sporangia of the fungi, which can germinate directly forming germ tubes and hyphae represented in Figure 6 or can release motile asexual spores called zoospores. This dispersion occurs especially by wind or water. Zoospores spread by humidity and water splashes from plant to plant can be controlled when growing in greenhouses, but it becomes almost impossible in the open air [95,108].

The management of diseases caused by *Phytophthora* species, in addition to being technically difficult, can be very expensive, since they are soil-dwelling pathogens that have a high capacity for survival. Chemical control of these diseases is only cost-effective when the price of cocoa is high and the crop is under high disease pressure, and even then, agents such as copper hydroxide may not save more than 20% of a crop infected with black pod rot [108,109].

Development of new fungicide formulations that are more effective and safer for the environment is one of the great challenges in agricultural scenario, especially in the case of pest control associated with lesions in cocoa crop. NMs can be an excellent alternative for the incorporation of fungicides, increasing the effectiveness and decreasing the toxicity of these compounds, which is a cause for concern regarding the effects and mode of action of AI in agriculture.

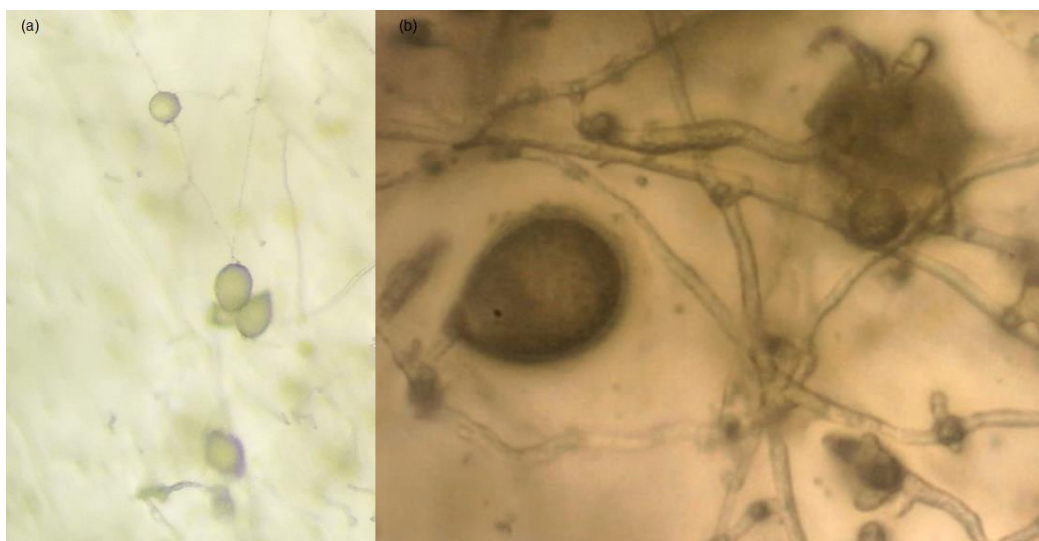


Figure 6. The characteristic morphology of *Phytophthora* (a) Papillate sporangium (mature) and (b) Terminal sporangium and chlamydospore forming new hyphal extension. Photo from *Phytophthora* germination plates from the MCCS laboratory - Mars Center for Cocoa Science.

1.5 Safety Assessment of the use of Nanofungicides

1.5.1 Safety Evaluation in Human Cell Lines

Application of pesticides in agriculture practices, as a way to improve crop yields, raises public and environmental concerns due to the critical effects which pesticides can cause to living forms and the environment. The inadequate handling and prolonged exposure to pesticides have registered several side effects, including skin and eye irritation, nausea, headache, vomiting and shortness of breath. Furthermore, it has been shown that conventional agrochemicals can confer cytotoxic and genotoxic changes upon continuous exposure to farmers and landowners [110].

For example, Triazole fungicides are a large family of organic heterocyclic compounds containing 1,2,4-triazole ring [111,112]. Compounds such as tebuconazole and hexaconazole have been classified by the US EPA as Group C-Possible Human Carcinogen because of their multiple chronic toxic effects on organisms. Tebuconazole increased cell viability of HeLa (human cervical carcinoma), A549 (human lung epithelium cancer) and HCT116 (human colorectal carcinoma) in a time- and concentration-dependent manner, whilst decreased cell viability of K562 (human chronic myeloid leukemia) cells. However, hexaconazole revealed stronger inhibitory activity on HeLa, HCT116, and K562 cells as the exposure time elongated and the concentrations increased. Both of hexaconazole and tebuconazole have a potential to develop a wider negative impact on different human organs[112].

In vitro studies with Mancozeb classified as a dithiocarbamate, indicated a strong reduction in cell viability of the HepG2 cell line (human hepatoma cell line), to below 50%, for mancozeb concentrations ranging 1–100 ppm that after 48h caused total cell death, suggesting that mancozeb is strongly cytotoxic to human hepatocytes [113]. Maneb in turn causes drastic induced bioenergetics alterations of SK-N-AS human neuroblastoma cells, including an acute reduction in ATP-bound mitochondrial respiration, inhibition of glycolysis and eradication of mitochondrial fuel flexibility, directly related to the risk factor for Parkinson's disease [114].

To minimize the pesticide side effects, NMs have been used as excellent tools for new regulated formulations. Metal nano-oxides and different biopolymers have been explored to develop nanopesticides that apparently offer a targeted solution minimizing the side effects of pesticides. However, some studies have proven that these NMs can also induce relevant toxicological effects mainly due to their photodegradation in the environment. For the human health, NMs can cause toxicological effects due to their greater ability to enter the human body through inhalation, ingestion and skin contact, compromising the immune system response and inducing pathological effects such as oxidative damage and inflammation [115,116].

Thus, understanding the biophysical-chemical interactions is very important from the point of view of safety and the development of AI and NMs-based systems for the delivery of different compounds, since the cytotoxicity of these compounds refers to the toxic effect of a poisonous agent on cells by inhibiting cell growth and may occasionally result in cell death [117].

Interactions between NMs and biological membranes including cells, tissues and organisms, is a dynamic process influenced by the properties of the nanosystem, such as, size, surface of area, shape/angle of curvature, porosity/surface crystallinity, hydrophobicity/hydrophilicity, chemical functionality, surface charge, and composition. On the other hand, the nano-biointerfaces are also influenced by the composition of the biological systems (proteins, membranes, phospholipids, organelles, endocytic vesicles, DNA and biological fluids) [118].

The class of nanopesticides containing inorganic NPs as active ingredients, including various metallic oxides, such as silicon dioxide (SiO_2), zinc oxide (ZnO), titanium dioxide (TiO_2), silver (Ag), copper (Cu), aluminum (Al) and iron (Fe), is commonly used against fungi, bacteria and other plant pathogens [119]. Cu NPs are effective against Gram-positive and Gram-negative bacteria and fungal disease pathogens like *Fusarium* sp., *Phytophthora infestance*, *Xanthomonas oryzae* and *Xanthomonas campestris* at concentration of 25% w/w; 0.054 wt% and 2.5 mg mL^{-1} [120]. However, the use of Cu-based NPs and their compounds can lead to toxic/genotoxic effects in organisms, and their accumulation in the human body is associated with the pathogenesis of liver disorders and neurodegenerative disorders [119].

Azoxystrobin loaded MSiNP modified with carboxymethyl-chitosan have been explored as a sustainable fungicide for tomato plants. The results showed an increase in the loading content, controlled release driven by pH and better fungicidal effect [121]. However, some studies have reported that excessive exposure to SiNP can result in cytotoxic effects. SiNP can induce oxidative stress at 3 and 24h post-treatment *in vitro*, suggesting that the mitochondrial pathway mediated by oxidative stress can lead to apoptosis, contributing to hepatotoxicity. Furthermore, it has been reported that Kupffer cells (cells lining hepatocellular plaques) can be activated by SiNP and contribute to hepatotoxicity by releasing bioactive mediators such as tumor necrosis factor, ROS and nitric oxide [122]; and could induce cytotoxicity to different cancer cells, including human lung cancer cells and glioblastoma [123]. In contrast, MSiNP characterized by chemical stability and inertness [124], as well as their high surface area and large pore volume [125], in general, present lower cellular cytotoxicity in comparison with other types of SiNP.

For organic NMs, some of them are not toxic to cells at certain doses. Liposomes, lipid micelles, SLN, and nanoemulsions are the major classes of lipid nanosystems that have provided us with promising platforms in order to entrapment and nanovectorisation of

lipophilic particles. These classes of NMs are well tolerated since they are formulated based on a solvent-free process and using only regulatory approved components [126].

However, even SLN/NLC formulations that are considered as non-toxic NMs, the low particle size (<300 nm) and lipophilic nature of these NMs may affect their penetration into cells and induce a stronger effect in cell viability [127]. A study conducted by Mendes *et al.*, 2015, shows that changes in the population density of the nanosystems (number mL⁻¹) resulted in cytotoxicity responses, comparing different NMs (nanospheres, polymeric nanocapsules, liposomes and NLC) with sizes between 136 and 214 nm [118]. The effect of NMs were investigated using human lymphocytes by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay and NLC induced highest cytotoxic effects, in relation the other NMs, reaching a reduction in the viability of 58% (compared to control) at the concentration of 2.1×10^{10} nanoparticles mL⁻¹.

1.5.2 Environmental Safety Evaluation on Aquatic Organisms

Despite the use of pesticides being a concern due to their high degree of toxicity to humans and the environment, the development and application of nanopesticides as a solution for the controlled release of AI, increasing the AI efficiency and minimizing its impact on the environment has also drawn attention, especially due to the limited information about the risks of nanopesticides associated with different organisms, such as bioavailability, accumulation, mode of action and toxicity.

In addition to their use in the agrochemical sector, current estimates indicate that NPs are in more than 1800 commercial products for various applications, including electronics, energy, biomedical, cosmetics, defense, automotive, among others. Therefore, at any stage in the life of NPs, they can be released into the environment, requiring efforts to understand and mitigate potential risks to the environment and human health [128,129].

The wide application of NPs in different fields increases the possibility of their release into the environment by human and animal excretion, mechanical processes (abrasion, scraping and sanding), washing, diffusion, matrix degradation (photo, thermal and hydrolytic) and incineration [130]. Once released into the environment, these NPs can exert oxidative stress, DNA damage and cell cycle interruption in human cells [131], it can interact with different enzymes involved in the metabolic pathway of algae, thus altering the normal

metabolic pathway of cells; has already been proven to interact with plant cells, thereby increasing antioxidant enzyme activity [132].

Monitoring the environmental effects is generally based on evaluating the synthesized AI and NPs added to water, soil or sediment, using standardized test following Organization for Economic Co-operation and Development (OECD) or standards developed by the International Organization for Standardization (ISO) [133]. The assessment of risks to human health, is specified in the European Union by the “Guideline on the Environmental Risk assessment of Medical Products for Humans” [134].

Different ecotoxicological tests with non-target species are widely used to assess the effect of AI and NPs on environment, for example, algae *Raphidocelis subcapitata* as a model species commonly used for evaluating the toxicity of chemical compounds [135]. These algae have been proposed as key indicators to assess water quality and the ecotoxicity of chemicals because they are known as primary producers in the food chain and play a key role in maintaining the function of ecosystems [136]. Some environmental factors are taken into account for influencing the toxicity of NPs on algae,: (i) light intensity when altered can cause damage to multiple targets in algae species, such as toxin production and oxidative stress; (ii) pH changes can affect the availability of CO₂ and essential nutrients, as well as algae metabolism; (iii) low nutrients availability can reduce the algae growth and (iv) the presence of natural organic matter can interact with NPs increasing their toxicity [137].

Another ecotoxicological test used to evaluate the safety of NPs is based on the assays with duckweed species (members of the Lemnaceae family). Duckweed growth inhibition tests are used to predict potential hazards of chemicals and contaminants to aquatic acrophytes in natural ecosystems [138]. The accumulation of NPs in the medium with *Lemna minor* which is the most used duckweed species model for laboratory toxicity tests [139], causes adverse effects on the plant, such as reduced growth, reduced levels of photosynthetic pigments, alteration of the chloroplast ultrastructure, increase in the activities of antioxidant enzymes, increase in lipid peroxidation and decrease in chlorophyll and protein contents [140]. In this way, the effects of NPs on vegetative growth can be quantified based on the evaluation of variables such as number of leaves and dry biomass and subsequent calculation of the average growth rate per day.

The Gram-negative marine bacterial species *Aliivibrio fischeri* has also been widely used in toxicity testing because its bioluminescence is sensitive to exogenous chemicals, and it is easy to observe light variation [141]. Microtox® assay, through the inhibition of *A. fischeri*

bioluminescence, is performed by correlating the emission of light proportional to the metabolic activity of the bacterial population under favorable environmental conditions, with the toxicity of the test substance [142]. Thus, when exposed to toxic NPs, there is a reduction in the production of light by *A. fischeri*, affecting the respiratory process of the bacteria, which can then be quantified after 5-30 min of exposure to the medium, measured using a photometer [143].

In general, the effects of NPs are usually associated with their physicochemical properties including: shape, size range, agglomeration/aggregation, time and extent of agglomeration, elemental composition, surface chemistry/area/charge, crystalline structure, solubility/dispersibility and material coverage [144,145]. Andreani *et al.*, 2017, concluded that the reduction in the average particle size of the nano-silver led to a lower toxicity on different human cell lines (Caco-2, HepG2, SV-80 and HaCaT), with the opposite observed for the aquatic species tested (*A. fischeri* and *R. subcapitata*), while for Cu-NPs, the consequent reduction in the average particle size, changes in toxicity for both human cell lines and aquatic species were clearly variable [146].

Due to the great use and application of SiNP, there is a considerable number of publications regarding the exposure of these NPs to environmental organisms such as fish, algae, crustaceans, bacteria, and plants. Results such as for algae and bacteria showed that they generally have a comparatively low sensitivity, probably due to their cell wall which forms a protective barrier against exposure to these NPs. However, the toxicity of SiNP is directly dependent on their physicochemical properties, and thus it becomes necessary to assess their environmental safety [147–149].

Although there are several studies with different types of SiNP, to the best of our knowledge, there are limited toxicological studies on the effects of DTC as well as LN in the environment. Thus, it is still essential to evaluate the safety of NMs on aquatic organisms, as each nanoparticle has its own characteristics. In addition, the interaction with different types of AI can also contribute for the toxicity of NPs on the environment.

1.5.3 Environmental Safety Evaluation on Soil Microbial Community

Pesticides have positive effects on crop yield and productivity, their application can lead to several hazardous impacts on soil biodiversity. Pesticides usually render the soil brittle,

reduce soil respiration by 35%, and decrease the activities of some macroorganisms in the soil, such as earthworms. Although the use of pesticides can induce beneficial effects on the soil microbiota, such as the increase of soil microbial biomass carbon after the application of glyphosate [150] and the increase of the soil bacterial population after the application of cypermethrin [151], pesticides can negatively affect the soil biochemical parameters and/or soil microbial biomass by affecting carbon microbial function nitrogen cycle [152], inhibiting the activity of certain soil enzymes [153] and by limiting their biological services in the production of some plant growth-promoting traits, such as siderophores, nitrogen and indole-3-acetic [154].

A systematic review conducted by Chowdhury *et al*, 2008 highlighted that the application of the fungicides Captan, Thiram (dithiocarbamate) and Verdasan, at the tested concentrations of 5 and 50 $\mu\text{g g}^{-1}$, caused a 40% decrease in soil biomass, altering the balance of fungi and bacteria, and thus reducing the activity of soil microorganisms due to the toxicity of these compounds [155].

Fungicides such as triazoles have also been studied in the laboratory (maintained at 23 °C, 16h:8h (light:dark) photoperiod (approx. 4000 lx) and under field conditions (collected from the top layer of soil (0–20 cm) and preserved in a refrigerator) to assess their impact on soil microbiota and soil enzymatic activities. Difenoconazole (DIF) at a concentration of 0.04 mg DIF kg^{-1} soil and incubation in the laboratory up to 84 days, caused an acute decrease in microbial activity in the soil because microorganisms spend more energy in the processes of detoxification than in microbial growth [156]. When the fungicide was applied in a dose of 0.150 mg DIF g^{-1} soil, there was a reduction of 90.16% in dehydrogenase activity after 21 days in controlled laboratory conditions; for urease 33.84% under field conditions and 29.63% under laboratory conditions; a reduction of 56.98% for phosphatase under field conditions and, an increase of protease activity by 40.24% in the laboratory at controlled conditions [157]. Thus, this study allowed to verify that high doses of this fungicide greatly disturb the structure of microbial communities in the soil and generally lead to a decrease in this population and a decrease in the activities of enzymes found in the soil, being necessary to evaluate the impacts caused by the application of these compounds in the long term in the properties and soil activity [158].

As previously mentioned, NMs appear as an alternative to reduce the impacts caused by the direct application of pesticides. However, very little information is available on how they affect the soil microbial community. It is well known that NMs can change the bioavailability

of toxins or nutrients; can interact with natural organic compounds; or with toxic organic compounds that would amplify or alleviate their toxicity. Although the toxicity mechanisms have not yet been completely elucidated, possible mechanisms may include disruption of membranes or membrane potential, protein oxidation, genotoxicity, disruption of energy transduction, formation of ROS and release of toxic constituents [159,160].

Some studies indicated that Ag-NPs at high concentrations (100 and 1000 mg kg⁻¹ of soil) reduce the enzymatic activities, especially of urease. Aged TiO₂-NPs (91 mg kg⁻¹ soil) stimulated urease activity, but decreased catalase and peroxidase activities after 9 months of incubation. ZnO-NPs (45 mg kg⁻¹ soil) reduced protease, catalase and peroxidase activities. TiO₂ and ZnO-NPs also reduce soil respiration after 15 and 60 days of incubation at concentrations of 500, 1000 and 2000 mg kg⁻¹ of soil [161]. It was also found that Fe-NPs incubated in three different soil textures significantly reduced microbial biomass by 29%, due to inhibition of ammonia oxidation potential, suggesting possible changes in the microbial community. At the highest concentration of Fe₂O₃ (40 mg kg⁻¹), these NPs acted as electron acceptor for hydrogen, reducing dehydrogenase activity [162]. Other studies indicated that PLGA-NPs inhibited ammonia oxidation by more than 50% when the PLGA concentration was greater than 0.05 mg L⁻¹ or more, while negligible effects were observed on nitrite oxidation [163].

Based on the above considerations, it is crucial to evaluate the impact of new AI and NMs as potential fungicides on soil microbiota. This assessment can be performed through changes in the microbial community determined by the study of functional parameters such as dehydrogenase enzymes, acid phosphatase, arylsulfatase and nitrogen mineralization. Dehydrogenase occurs in all living microbial cells, and it is linked with microbial respiratory processes. This intracellular enzyme is an indicator of overall microbial activity of soils [164]. Phosphatase is an enzyme that hydrolyzes compounds of organic phosphorus esters and anhydrides of phosphoric acid, plays a critical role in phosphorus cycle and is correlated to phosphorus stress and plant growth [165]. Arylsulfatases are typically widespread in soils and responsible for the hydrolysis of sulfate esters in the soil secreted by bacteria into the external environment as a response to sulfur limitation [166]. Soil nitrogen mineralization is considered a critical soil nitrogen cycling process that determines its availability in the soil and is associated to the productivity and health of ecosystems [167].

Most studies regarding the impacts of NPs on soil microbiota refer to metallic NPs, especially due to their wide use as antimicrobial agents. However, there are no data in the literature

that ensure the effects of MSiNP on the soil microbial community, while for LN, to the best of our knowledge, there was only one study conducted by our group relating the effect of LN loaded with lambda-cyhalothrin on soil microbial parameters [77].

2 Aims of the work

3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate) has received significant attention from the scientific community due to their numerous biological effects, such as the antimicrobial characteristics. However, the exploitation of such properties is limited due to DTC poor water solubility and easy degradation in aqueous solutions. The nanoencapsulation of DTC can be an excellent strategy to overcome these drawbacks. In this sense, the main objective of this Doctoral Project was the design, physicochemical characterization, and safety evaluation of Mesoporous Silica Nanoparticles (MSiNP) and Lipid Nanoparticles (LN) loaded with DTC to be used as a potential fungicide in the agriculture practices.

Based on the considerations above, the specific objectives of the present Thesis were:

- (i) Synthesis and physicochemical characterization of DTC;
- (ii) Design and physicochemical of MSiNP and LN loaded and unloaded with DTC using various analytical techniques to assess their stability over time and the interaction of nanoparticles with DTC;
- (iii) Evaluation of the fungicidal effect of DTC, MSiNP and LN loaded and unloaded with DTC on *Moniliophthora perniciosa*, *Phytophthora palmivora* and *Phytophthora theobromicola* in the Brazilian cocoa crops;
- (iii) Safety evaluation of MSiNP and LN loaded and unloaded with DTC to human health and environment, using human cell lines, aquatic and terrestrial organisms, respectively.

Part of the work developed for the DTC and MSiNP/MSiNP-DTC such as Synthesis and physicochemical characterization and Safety evaluation on aquatic organisms was published in the scientific article “Menezes, T., Bouguerra, S., Andreani, T., Pereira, R., Pereira, C. Preparation, Characterization, and Environmental Safety Assessment of Dithiocarbazate Loaded Mesoporous Silica Nanoparticles. *Nanomaterials*, 13(2), 370, 2023. doi: 10.3390/nano13020370.”

3 Materials and Methods

3.1 Reagents

All chemicals used were of analytical reagent grade and of the highest purity commercially available and used without further purification. Deionized water (Millipore®, Darmstadt, Germany) was used for all preparations of aqueous solutions. Hexadecyltrimethylammonium bromide (CTAB, ≥99%), tetraethyl orthosilicate (TE-OS, ≥99%), absolute ethyl alcohol (Fermont, ≥99.5%), N₂H₄.H₂O (≥98%), CS₂ (anhydrous, ≥99%), PhCH₂Cl (≥99%), HCl (≥37%), MeOH (anhydrous, ≥99.8%), and 4-phenyl-2,4-butanedione were supplied by Sigma-Aldrich. Precirol® ATO 5 (Glyceryl palmitostearate, Gattefosse S.A., Saint-Priest, France) was used as a solid lipid, and Capryol 90® (Propylene glycol monocaprylate, Gattefosse S.A., Saint-Priest, France) used as a liquid lipid. Soy lecithin used as amphipathic surfactant was obtained from VWR (Alfragide, Portugal). The surfactant TegoCare 450® was supplied by Evonik Industries (Essen, Germany). Dimethyl sulfoxide (DMSO) was used as a solubilizing agent to dissolve DTC (3-methyl-5-phenyl-pyrazoline-1-(S-benzylthiocarbamate)) and obtained from Sigma-Aldrich (Germany).

The solid culture media used for the growth of the mycelia and maintenance of the tested fungi were the CPD medium (Potato, Bacteriological Agar and Dextrose) 2% in distilled water, Tomato medium (Tomato extract, Calcium Carbonate, Bacteriological Agar) 5% in distilled water and Lit medium (Carrot, Tomato Extract, Calcium Carbonate, Bacteriological Agar) 3% in distilled water. All media were prepared and autoclaved at the MCCS-Mars Center for Cocoa Science, then kept in BOD (Biochemical Oxygen Demand) for further use.

Dulbecco's Modified Eagle's Medium (DMEM), Foetal Bovine Serum (FBS), penicillin/streptomycin, L-glutamine, 0.05% trypsin-EDTA and Alamar Blue (AB) were purchased from Gibco (Alfagene, Invitrogen, Portugal).

3.2 Synthesis

3.2.1 3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate) – DTC

The synthesis and structural analysis of the compound referred as DTC ($C_{18}H_{16}N_2S_2$), were previously performed and described by De Sousa *et al.*, 2011, and reproduced in the Department of Chemistry of FCUP according to the procedure reported in the literature by Menezes *et al.*, 2023 and represented schematically in Figure 7. Briefly, KOH (11.4 g) was dissolved in 90% ethanol, adding $N_2H_4 \cdot H_2O$ (10.2 mL) and cooling the mixture to 0 °C in an ice-salt bath. CS_2 (19.24 mL) was added dropwise under constant and vigorous stirring (700 rpm) for 1h. Subsequently, two layers were observed: one upper yellow and a lower brown layer. The upper lower was discarded and the brown layer was dissolved in 40% cold ethanol keeping the reaction at approximately between 5 and 7 °C. Once the temperature was reached (5/7 °C), $PhCH_2Cl$ (27.5 mL) was added dropwise to the mixture with vigorous magnetic stirring for 30 min. The white solid that was formed was filtered, washed with ultrapure water and, after drying at room temperature, it was recrystallized in methanol and dried over anhydrous $CaCl_2$. Then, a solution of 1-phenyl-1,3-butanedione (0.811 g) in methanol (10 mL) was added to a solution of S-benzylidithiocarbazate (0.991 g) in methanol (10 mL) under reflux with addition of two drops of HCl 37% to the reaction medium under employing an acid-catalyzed conversion, maintaining a reflux for 2h and temperature of 64 °C. A yellow solid was obtained, subsequently filtered, washed with methanol, and dried at room temperature over anhydrous $CaCl_2$. The product was also recrystallized in methanol to obtain pure yellow crystals, resulting in a total mass of 0.8728 g and yield of 95.81%.

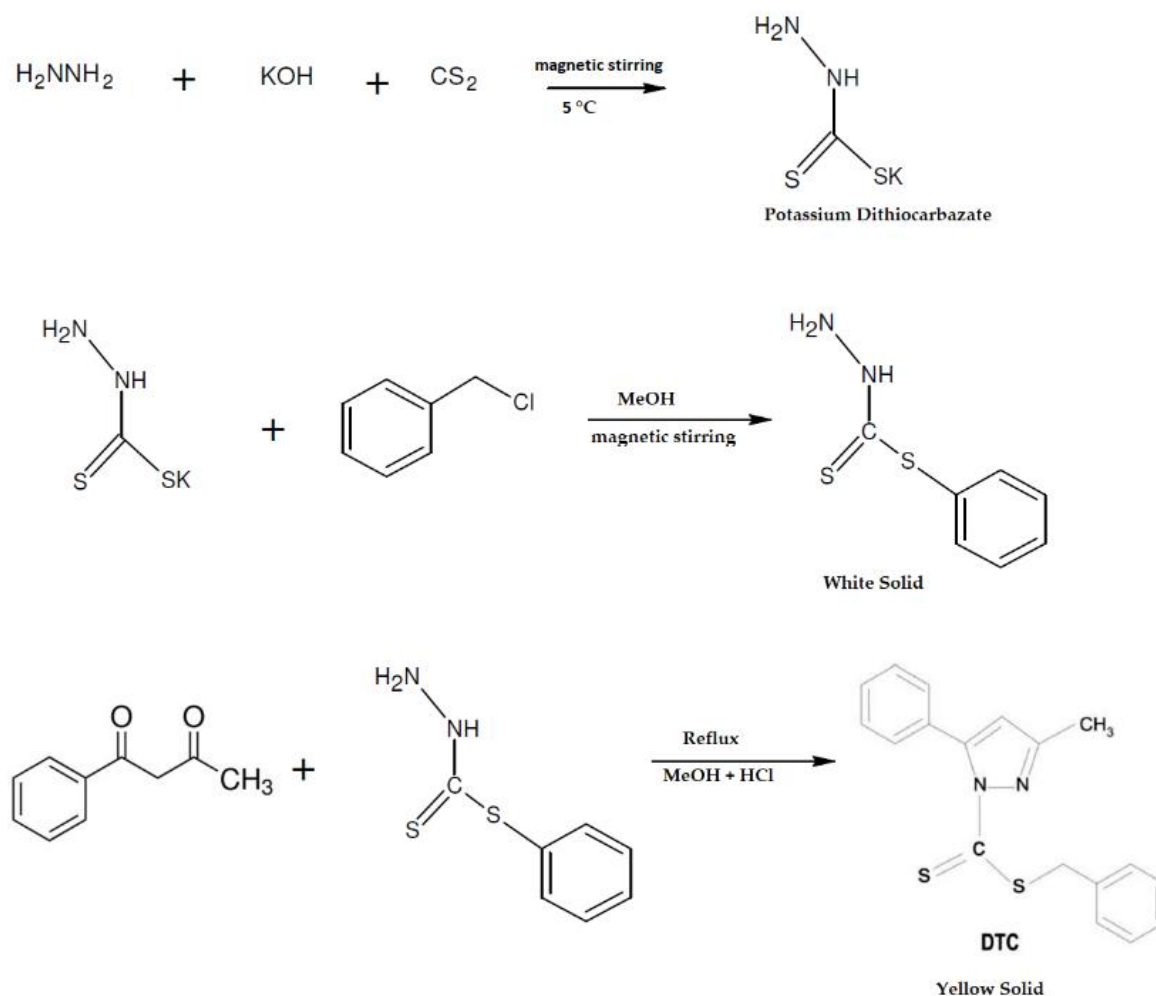


Figure 7. Schematic representation of the synthesis process of DTC (3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate)) (Menezes *et al.*, 2023).

3.2.2 Mesoporous Silica Nanoparticles (MSiNP and MSiNP-DTC)

Synthesis of MSiNP was performed by Stöber method following the published work from Paula et al., 2012 and reproduced in the Department of Chemistry of FCUP according to the procedure reported in the literature by Menezes et al., 2023, under a high concentration of precursors, resulting in NP with a highly uniform spherical shape represented schematically in Figure 8. For this procedure, 750 mg of cetyltri-methylammonium bromide (CTAB) were dissolved in 20 mL of an aqueous solution of NH_3 (0.048 mol L^{-1}) followed by homogenization under magnetic stirring (300 rpm) in a round bottom distillation flask connected to a condenser reflux setup at 5°C (to prevent evaporation of ethanol). To this solution, 3.2 mL of absolute ethanol were added as a co-solvent and the mixture was homogenized for 15 min, at 60°C . Sequentially, 2.5 mL of tetraethyl orthosilicate (TEOS) (1.2 mmol) were added to the solution and the flask kept at the same temperature for 2h with agitation (300 rpm). At the end of the reaction, MSiNP were separated from the solution by centrifugation and washed with absolute ethanol before extraction of the soft mold (CTAB). Finally, MSiNP was washed twice with absolute ethanol and dried at 60°C for 24h. The residual CTAB was removed in an inert atmosphere (N_2) at 550°C with a rate of temperature increase of 3°C min^{-1} for 5h. For MSiNP-DTC, 5 mg of DTC were solubilized in 10 mL of ethanol at 37°C followed by the addition of 100 mg of MSiNP under magnetic stirring (300 rpm) for 2h. Then, the suspension was centrifuged at 15000 rpm and MSiNP-DTC were washed with distilled water, separated by a second centrifuge, and dried at 60°C overnight. MSiNP and MSiNP-DTC in powder were stored in the dark at room temperature for further analysis.

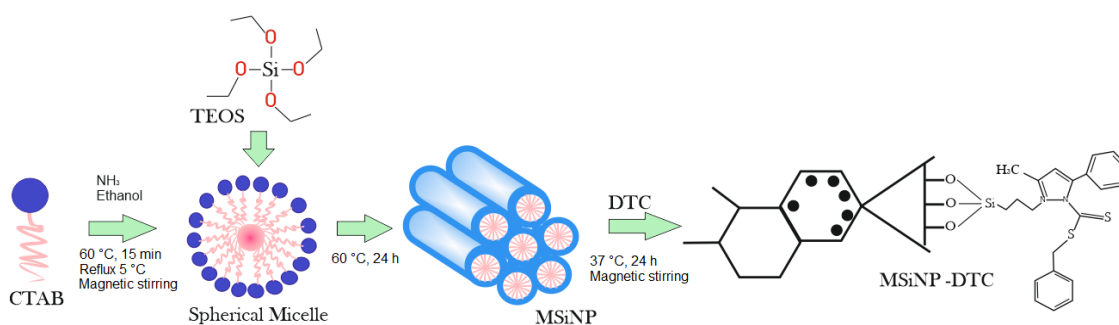


Figure 8. Schematic representation of the synthesis process of MSiNP and MSiNP-DTC (Menezes *et al.*, 2023).

3.2.3 Optimization of the synthesis of Lipid Nanoparticles (LN and LN-DTC)

LN-DTC synthesis was performed as reported by Ganilho *et al.*, 2022 with slight modifications and represented schematically in Figure 9, using an oil/water (O/W) emulsion under high shear homogenization and no organic solvents. The lipid phase composed of a mixture of DTC, Precirol ATO 5, Soy Lecithin, Capryol 90® and TegoCare 450® was heated to a temperature of 5-10 °C above solid lipid (Precirol) melting point (~65 °C). Separately, the aqueous phase was heated to the same temperature and subsequently added to the lipid phase under high shear homogenization (18G impeller, Ultra-Turrax T25), applying an intensity of 15000 rpm for 10 min at constant temperature. After that, the dispersion was placed on ice bath for 10 min to form LN-DTC. A similar procedure without the DTC incorporation were carried out to prepare the unload LN.

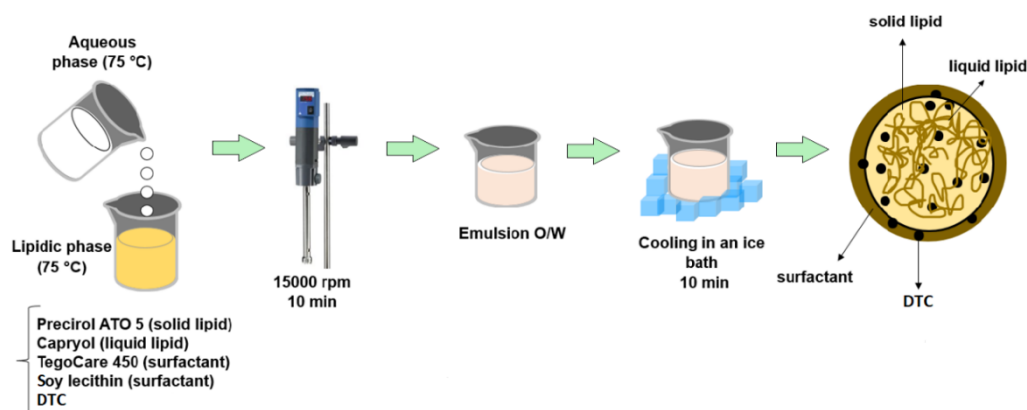


Figure 9. Schematic representation of the synthesis process of LN and LN-DTC.

Several variables can affect the properties of LN. Types of Lipids (solid and liquid) and their concentrations may influence the load capacity and stability of LN, as well as may affect the solubility and release of the AI incorporated in LN. In addition, surfactants and their concentrations also may influence the characteristics of LN, namely, the particle size, encapsulation efficiency and load capacity [170]. In this sense, in the present work, the

experimental factorial design was applied to optimize the design of LN by reducing the number of essential syntheses and estimate the effects on LN properties of diverse variables simultaneously. The concentrations of all compounds that constitute LN were selected according to previous studies in our laboratory, which demonstrated to maintain LN stability after DTC encapsulation. Thus, the influence of Precirol ATO 5 and soy lecithin concentrations was evaluated on the physicochemical properties of LN-DTC using a 2^3 factorial design with triplicate of central point to select the best formulation with minimal experimental procedures. The triplicate of central point was used to estimate the experimental error and observe the repeatability of the synthesis. The experiment required a total of 11 experiments. For each factor, the lower and upper values were represented by a (-1) and a (+1), and the central point was represented by (0), as summarized in Table 1. Statistical differences were determined using analysis of variance (ANOVA), followed by Tukey's test for comparisons between groups. The significance level was considered as 95% ($p < 0.05$). Factorial design data were analysed using STATISTICA 7.0. For all experiments, the amount of Capryol 90, TegoCare 450 and DTC was 66 mg, 33 mg, and 2 mg, respectively. The total amount of the formulation (Total amount of the formulation=10 g) was assumed by ultra-purified water.

Table 1. Preparation of LN-DTC. Factorial design, providing the lower (-1), upper (+1), and (0) central point values for each independent variable (Precirol ATO 5 and Soy lecithin).

Factor	Coded Levels		
	Low level (-1)	Medium level (0)	High level (+1)
Precirol ATO 5 (mg)	16	40	100
Soy Lecithin (mg)	16	40	100

3.3 Physicochemical Characterization of Nanoparticles

3.3.1 Average Particle Size, Polydispersity Index, and Zeta Potential Analysis

Average particle size (Z-Ave) and polydispersity index (PDI) of the prepared MSiNP/MSiNP-DTC and LN/LN-DTC were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS90 (Malvern, United Kingdom). The zeta potential (ZP) was determined by applying an electric field to the samples, and the value of the ZP was obtained by measuring the velocity of the electrophoretic mobility of the particles using the Doppler laser anemometry technique. Measurements were performed in triplicate for each sample at 25 °C using a Malvern Zetasizer Nano ZS (Malvern Instruments, Works, United Kingdom). Measurements were performed in triplicate for each sample at 25 °C and the data are represented by mean $\pm$ standard deviation (S.D.).

Prior analysis, MSiNP and MSiNP-DTC were suspended in ultrapure water (200 mg L⁻¹) by sonication for 10 min in an ultrasonic bath (Ultrasonic Cleaner, SONICA®, Soltec, Milan, Italy). Afterwards, 1 mL of suspension was used for the analysis. LN and LN-DTC formulations were diluted in ultrapure water immediately before the measurements (1:100 (v/v)) to obtain an opalescent dispersion. Measurements of Z-Ave, PDI and ZP were performed after synthesis.

Samples were stored at 25 °C, and the variations of Z-Ave, PDI, and ZP were recorded at predetermined time intervals 0, 30, 60, and 210 days after storage to evaluate nanoparticles stability over time.

3.3.2 Loading Efficiency

Loading efficiency was determined using T70 UV-VIS Spectrometer PG Instruments Ltd at a length of 303 nm. MSiNP-DTC (5 mg) were dispersed in 1 mL of ethanol and centrifuged at 13500 rpm for 30 min. Then, 200 μ L of supernatant were removed and diluted with ethanol to 500 μ L.

In the case of LN-DTC, the concentration of encapsulated DTC was indirectly measured after centrifuging LN-DTC (500 μ L) dissolved in dimethyl sulfoxide (DMSO) (1:1) for 40 min at 6000 rpm, at 25 °C in a centrifuge equipped with a membrane concentrator (Amicon Ultra

15, NMWL 3 K, Merck Millipore Ltd, Ireland). The concentration of free DTC in the diluted filtrate was determined using 100 µL and then diluted with DMSO to 200 µL. Data were expressed as mean ± S.D of three measurements.

The loading efficiency (% LE) (1) and the drug load (% DL) (2) were determined by the following Eqs. (1) and (2), respectively.

$$LE = \frac{(Amount\ of\ initial\ DTC - Amount\ of\ free\ DTC)}{Amount\ of\ initial\ DTC} \times 100 \quad (1)$$

$$DL = \frac{(Amount\ of\ initial\ DTC - Amount\ of\ free\ DTC)}{Total\ Nanoparticles} \times 100 \quad (2)$$

3.3.3 Lyophilization of LN and LN-DTC

The optimized LN/LN-DTC were lyophilized using a BenchTop Pro with Omnitronics™ (SP Scientific, Genevac LTD) without a cryoprotectant agent for 24h (-85.3 °C and 53 mT). This technique can improve the long-term physicochemical stability of NPs and avoid degradation reactions such as hydrolysis [171]. In the present study, the freeze-drying process was used to obtain the NPs in powder to perform TEM, FT-IR, Raman spectroscopy, thermal analysis and the safety tests.

3.3.4 Transmission Electron Microscope (TEM) Analysis

The morphology of NPs was checked by TEM (Hitachi model H8100, with an LaB6 filament and using an accelerating voltage of 200 kV). Images were acquired with an Olympus Keenview CCD camera (formerly Soft Imaging). MSiNP/MSiNP-DTC and LN/LN-DTC (after freeze-drying) were dispersed in ultrapure water (conductivity 0.14 µS cm⁻¹) by sonication

for 30 min in an ultrasonic bath and placed on copper grids covered with a carbon coating for TEM observations.

3.3.5 Fourier-Transform Infrared (FT-IR) spectroscopy

Spectroscopy in the infrared region was used to evaluate the interaction at the level of functional groups of DTC and NPs. FT-IR spectra were performed using a Bruker Vector 22 spectrometer in the 400 to 4000 cm^{-1} region and for LN-DTC it is represented from 2000 to 4000 cm^{-1} for better identification of the peaks. Loaded and unloaded MSiNP and LN (after freeze-drying) were mixed with suitable amount of micronized KBr powder and compressed into disks with a force of 10 kN using a manual press of the tablet. For each spectrum, an interferogram of 100 scans was collected with a resolution of 4 cm^{-1} in the medium infrared region.

3.3.6 Raman Spectroscopy

Raman spectrophotometric technique was used as a complement to FT-IR due to some DTC bands not being easily detected by FT-IR. The analysis of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC was performed using a Raman spectrometer that consists of a High-efficiency inVia Qontor Renishaw confocal spectrometer with a focal length of 250 mm and a spectral range of detection from 200 to 1064 nm, based on lenses with direct coupling to an upright microscope. 1024x256 pixel CCD detector cooled by the Peltier effect to -70 °C. High efficiency 1200-, 1800- and 2400-lines mm^{-1} diffraction gratings with high spectral resolution better than 2 cm^{-1} . Excitation lasers in the spectral range of 100 to 4000 cm^{-1} .

3.3.7 Thermal Analysis

Thermal characterization of MSiNP/MSiNP-DTC and LN/LN-DTC was performed by differential scanning calorimetry (DSC) and simultaneous thermal analysis (thermogravimetry (TG) and differential thermal analysis (DTA)) techniques. The DSC studies were performed using a DSC 7020 (Hitachi High Tech Science Corporation, Tokyo, Japan). Each sample (about 10 mg) was placed in an aluminium pan (7.5 μL), which was hermetically sealed. Scans were carried out from 25 to 600 °C at a heating rate of 5 °C min^{-1} under nitrogen atmosphere (100 mL min^{-1}). The TG and DTA analyses were performed using

simultaneous thermal analysis (STA) (STA 7200RV, Hitachi High-Tech Science Corporation, Japan). Each sample was accurately weighed in aluminium pans, and the scans were performed from 25 to 400 °C at a heating rate of 10 °C min⁻¹ under nitrogen atmosphere (200 mL min⁻¹).

3.3.8 Nitrogen Adsorption

Surface areas of the different mesoporous silica grades were determined by nitrogen adsorption at -196 °C using a TriStar II Plus 2.02 from Micrometrics. Specific surface areas (SBET) were calculated by using the Brunauer–Emmett–Teller (BET) method (Brunauer, *et al.* 1938). Pore size distribution and pore volume were estimated from the desorption branch of the N₂ ad-sorption–desorption isotherms by applying the Barrett–Joyner–Halenda (BJH) method (Barrett, *et al.* 1951). Measurements were performed in triplicate for MSiNP and MSiNP-DTC and the data were represented by mean ± S.D.

3.4 *In vitro* Antifungal Activity

3.4.1 Halo Growth Test

Three different species, such as *M. pernicios*a (Mp4145) obtained by the Fungi Biology Laboratory (LBF), from the Center of Biotechnology and Genetics (CBG) at the Universidade Estadual de Santa Cruz (UESC), *P. theobromicola* (MCCS-MB-01982) and *P. palmivora* (MCCS-MB-01919) obtained through the partnership with the multinational company MCCS - Mars Center for Cocoa Science, were exposed to DTC, MSiNP, MSiNP-DTC, LN and LN-DTC to evaluate the fungicide effects of such samples. Before testing, all species were incubated at 25 °C in CPD medium 2%, Tomato medium 5% and Lit medium 3%, kept in BOD for 7 and 4 days and then exposed to different treatments.

The halo growth tests were performed in sterile Petri dishes (diameter: 85 mm) comparing the effect of the treatments with the control group. The medium (prepared with sterile distilled water and after autoclaving) was placed on the surface of the plate (approx. 20 mL medium/plate), and after its solidification, all samples were applied and spread (500 µL) on the surface, except for the control group which was applied and spread with sterilized water. Inoculation of Petri dishes was performed with a sample of mycelium (approx. 5 mm), taken from the outer edge of actively growing colonies and placed in the center of the Petri dish.

Three replicates were prepared for the control and for each concentration, with incubation in BOD (25 °C) for 7 days for *M. pernicioso* and 4 days for *P. theobromicola* and *P. palmivora*. During the test, the diameters of the growing mycelia (2 equidistant axes were defined per plate) were measured every day. The test was considered completed when the mycelia of the control group completed the entire surface of the plate.

The tested samples were DMSO at 3% (v/v) (22 mg DMSO L⁻¹), DTC at 100 mg L⁻¹, LN at 1600 mg SL (Solid Lipid) L⁻¹, and LN-DTC at 200 mg DTC L⁻¹. The selected concentrations were based on previous studies (preliminary tests), which indicated that such concentrations were more effective to control fungal growth. Although, the effect of MSiNP and MSiNP-DTC was also evaluated in the preliminary tests, these NPs exerted no inhibitory effects on the growth of three fungal species (data not shown), and thus, they were not selected to be tested for the subsequent assays.

3.4.2 Evaluation of the Fungicide effect on Cocoa Fruit

Resistance evaluation of cocoa fruits (FB206) was carried out with young fruits against the species *P. theobromicola* (MCCS-MB-01960) and *P. palmivora* (MCCS-MB-01994), which were obtained through the partnership with the multinational company MCCS - Mars Center for Cocoa Science. Sporangia were collected from hyphae clippings in Tomato 5% and lit 3% medium, respectively, inoculated in 10 mL of liquid medium for five days and incubated in BOD at 27 °C under continuous light. After the incubation period, stress procedures were performed to remove the liquid medium for 24 hours. Then, the second stress was carried out with the addition of 10 mL of cold water to the plates and maintained for 20 minutes, followed by counting the spore number in a Neubauer chamber and dilution of the medium to a final spore (sp) concentration of 7.5×10^{-4} (sp mL⁻¹). After that, 20 µL of the medium containing the spores were added in the cocoa fruit, followed by the addition of 20 µL of the solution of the respective compounds and controls using a piece of properly autoclaved filter paper. All fruits were properly identified and kept under controlled conditions inside crates and at an ambient temperature of 27 °C, represented in Figure 10. The assay was maintained for 7 days and the fruits were exposed to different treatments: control groups treated with distilled water and treated with aqueous solution with DMSO at 3% (v/v) (22 mg L⁻¹) without spores to check possible contaminations in the fruit; control groups treated with distilled water and treated with aqueous solution of DMSO at 22 mg L⁻¹ with spores);

DTC at 100 mg L⁻¹ dispersed in the aqueous solution of DMSO at 3% (v/v) ; aqueous dispersions of LN (1600 mg SL L⁻¹) and LN-DTC (200 mg DTC L⁻¹) with spores. These concentrations were selected based on the *in vitro* assay (Halo growth test), which induced highest fungi growth inhibition. MSiNP and MSiNP-DTC were not evaluated in this trial, since there was no inhibition on *M. pernicioso*, *P. theobromicola* and *P. palmivora* in the *in vitro* assays. The diameters of the growing lesions in the fruits were measured every day (2 equidistant axes defined per lesion) and the results were expressed as Diameter, Height and Interaction (DHI) of the lesion caused by the fungi.

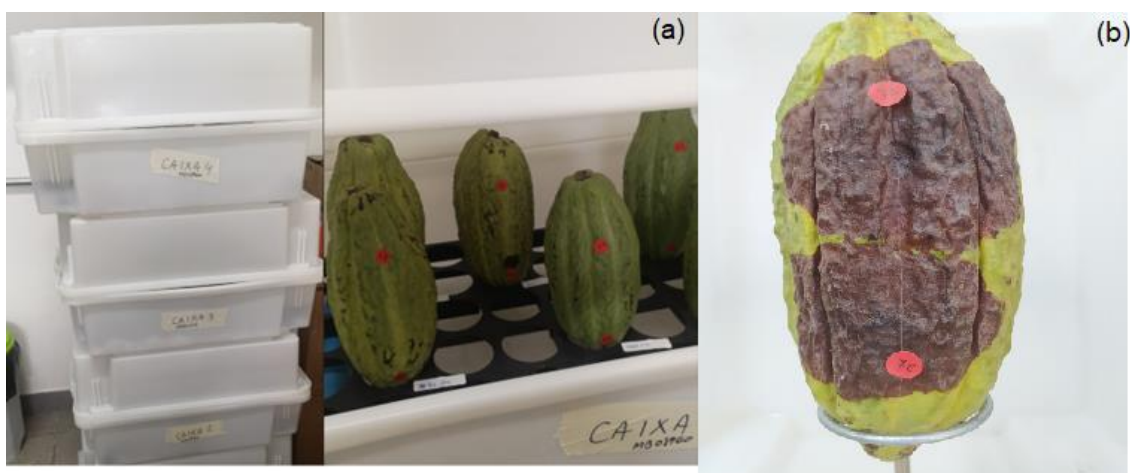


Figure 10. Design for resistance evaluation test of cocoa fruits under controlled conditions inside crates at 27 °C (a) and growth of lesions on cocoa fruit (b).

3.5 Effect Evaluation on Human Cell Lines

3.5.1 Cell Cultures and Maintenance

The experiments were conducted at the Cell Biology and Bioenergetic Laboratory of the Center for Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Vila Real, Portugal. The effect of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC on human normal keratinocytes (HaCaT cells) and human skin cancer cells (A431cells) was evaluated using Alamar Blue (AB) assay. HaCaT and A431 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) foetal bovine serum (FBS), antibiotics (100 U mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin) and 1 mM L-glutamine in an atmosphere of 5% CO₂ / 95% air, at 37 °C with controlled humidity.

3.5.2 *In vitro* test with Human Cell Lines

The safety of NPs on selected human cells was evaluated by comparing the proliferation rate and viability of non-exposed HaCaT and A431 cells (control) with exposed HaCaT and A431 cells, to appropriate concentrations during defined periods of time (see later), using the AB reduction method.

For the cytotoxicity assay cells were detached from the culture flasks with trypsin, counted and seeded in 96-well microplates at a density of 5×10^4 cells mL^{-1} ($100 \mu\text{L well}^{-1}$). DTC was previously suspended in an aqueous solution of DMSO at 0.5% (v/v) before the assay. DTC in DMSO 0.5% (v/v), MSiNP and LN loaded with DTC were diluted in FBS-free culture media to various concentrations: 0, 1, 2.5, 5, 7.5 and 10 mg DTC L^{-1} . Unloaded MSiNP and LN were also diluted in FBS-free culture media and tested at concentrations of 0, 26.7, 66.7, 133.4, 200 and 266.7 mg MSiNP L^{-1} , while for unloaded LN, the concentrations tested were 0, 10.7, 26.7, 53.4, 80, 106.7 mg SL (solid lipid) L^{-1} .

Then, 24h after seeding, the culture medium was removed and replaced by media containing NPs (at defined concentrations). For each concentration of NPs, microplates were prepared for 24h and 48h exposure. After the exposure time (24 or 48h), the medium containing the NPs (and the control) was removed and replaced by FBS-free medium supplemented with 10% (v/v) of AB. The absorbance was read occurred about 4h after AB addition, at 570 and 620 nm using a microplate reader (Multiskan Ascent). The percentage of AB reduction was calculated according to the equation 3:

$$\%AB \text{ reduction} = \frac{(\epsilon_{ox \lambda_2})(A\lambda_1) - (\epsilon_{ox \lambda_1})(A\lambda_2)}{(\epsilon_{red \lambda_1})(A\lambda_2) - (\epsilon_{red \lambda_2})(A\lambda_1)} \times 100 \quad (3)$$

where, $\epsilon_{ox \lambda_1}$ is the molar extinction coefficient of oxidized AB at 570 nm, $\epsilon_{ox \lambda_2}$ is the molar extinction coefficient of oxidized AB at 620 nm, $\epsilon_{red \lambda_1}$ is the molar extinction coefficient of reduced AB at 570 nm, $\epsilon_{red \lambda_2}$ is the molar extinction coefficient of reduced AB at 620 nm, $A\lambda_1$ and $A\lambda_2$ are the absorbance of test wells at 570 and 620 nm, respectively, and $A\lambda_1$ and $A\lambda_2$ are the absorbance of the negative control wells (media plus AB but no cells) at 570 and 620 nm, respectively.

3.6 Effect Evaluation on Aquatic Organisms

3.6.1 Preparation of Solutions and Dispersions

For the growth inhibition tests with *R. subcapitata* and *L. minor*, a stock aqueous solution of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC (after freeze-drying) was prepared at a concentration of 200 mg L⁻¹ followed by homogenization using an ultrasound bath for 10 min. The maximum concentration tested of encapsulated DTC in MSiNP and LN was 7.2 mg L⁻¹ and 2.9 mg L⁻¹, respectively. DTC stock solution (200 mg L⁻¹) was prepared by dissolving DTC in aqueous solution of DMSO at 0.5% (v/v). Subsequently, the stocks solutions of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC were diluted in the Woods Hole MBL (medium for algae assays) and Steinberg culture medium (medium for lemna assays) in different concentrations of DTC and NPs: 0.0, 8.8, 13.2, 19.8, 29.6, 44.4, 66.7, 100 and 150 mg L⁻¹, while the concentrations of DTC in MSiNP-DTC was 0.0, 0.42, 0.63, 0.95, 1.42, 2.1, 3.2, 4.8 and 7.2 mg L⁻¹ and 0.0, 0.17, 0.26, 0.38, 0.57, 0.86, 1.3, 1.9 and 2.9 mg L⁻¹ in LN-DTC. For the bioluminescence assays with *A. fischeri*, the same procedures for preparing the stock solutions of DTC and NPs were performed as described above for *R. subcapitata* and *L. minor* tests.

3.6.2 Microtox test: *A. fischeri*

The toxicity test was performed with *A. fischeri* bacteria by Microtox® model 500 Toxicity Analyzer (Modern Water, New Castle, DE) following the 81.9% basic test protocol (Azur, 1998) which tests a range of serial dilutions from 81.9 to 0.32 (dilution factor of 2), using the marine bacteria test medium NaCl 2%, measured after 5, 15 and 30 min of exposure. EC₅₀ and EC₂₀ values for bioluminescence inhibition were computed for each contact time and samples using the Azotox® MicrotoxOmni Software version V1.18.

3.6.3 *R. subcapitata* growth inhibition test

R. subcapitata growth inhibition assay was performed according to the standard OECD protocol 201 [135]. The microalgae were obtained from axenic batch cultures maintained in Woods Hole MBL medium at continuous light exposure (cool white fluorescent illumination of 100 µE.m⁻².s⁻¹) and a temperature of 21 ± 2 °C. Algae were exposed in 24-well sterile plates in three replicates (wells) per each concentration plus three replicates for the control.

In each well, 900 µL of the dispersions were added at different concentrations followed by the addition of 100 µL of algal inoculum, to an initial cell concentration of 10^4 cells mL⁻¹. Microplates were maintained in continuous agitation for a period of 72h under the same conditions described for cultures maintenance. cells were kept on an orbital shaker. The cells of each replicate were counted using a Neubauer chamber by using an optical microscope. The average specific growth rate of algae exposed to DTC, MSiNP/MSiNP-DTC and LN/LN-DTC for a specific period was calculated as the logarithmic increase in biomass from the equation for each single well of controls and treatments:

$$\mu_{i-j} = \frac{\ln(X_j) - \ln(X_i)}{t_j - t_i} (\text{day}^{-1})$$

where:

- μ_{i-j} : average specific growth rate from time i to j
- X_i : biomass at time i
- X_j : biomass at time j
- t: time period from i to j

3.6.4 Growth test with *L. Minor*

L. minor growth inhibition test was performed according to the OECD guideline 221 [139]. Cultures are maintained in axenic condition, with a light intensity 6.000-10.000 Lux, obtained with a cool white fluorescent illumination, temperature (21 ± 2 °C). The test was performed at a 16^L:8^D h photoperiod and 20 ± 1 °C, for 7 days. A total of 3 colonies with three visible fronds each were initially exposed per replicate (three replicates per concentration plus the control). The exposures were carried out in sterilized 6-well microplates, filled with 12 mL of each treatment/control. After 7 days of exposure in the same conditions described for the culture maintenance, all the fronds were collected from each microplate well, counted, and dried at 60 °C to a stable weight. The effect of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC on *L. minor* growth was evaluated according to the growth rate of *L. minor* calculated based on the number of fronds and on the dry weight of the fronds after 7 days of exposure. The

average specific growth rate was calculated as the logarithmic increase in the growth variables number of fronds and dry weight using the above mentioned formula for each replicate of control and treatments.

3.7 Effect Evaluation on Soil Microbial Community

3.7.1 Test soil

In the present study, natural soil was collected from the 0–20 cm topsoil layer in the region of Vairão (Vila do Conde, Portugal), in the open fields of the GreenUPorto Research Centre, with no historical application of pesticides at least in the last three decades and sieved through a 2 mm mesh size. This type of soil was selected due to its similarity to the soils used for the cocoa cultivation (sandy loam and neutral pH). The soil physicochemical characterization presented in Table 2 was performed in LabRisk using five replicates for all the following parameters: pH (H₂O and KCl (1 mol L⁻¹)), conductivity, organic matter content (%OM), humidity, and water-holding capacity (% WHC_{max}). The pH and conductivity were determined in a 1:5 (v/v) soil suspension. After 60 ± 10 min of agitation, the suspension was allowed to stand for 60 min before measuring pH with a pre-calibrated multiparameter (Edge, HANNA Instruments, Póvoa de Varzim, Portugal) [172]. Conductivity was recorded with the pre-calibrated meter on the H₂O suspension [173]. For OM content, soil samples were dried at 105 °C until constant weight and then obtained by loss on ignition of dried soil samples at 450 °C, for 8h, and expressed in percentage [174]. The humidity of the soil was calculated by gravimetric method in an oven at 105 °C until constant mass [174]. For WHC_{max} (%), soil samples were introduced in polypropylene containers whose bottom was replaced with filter paper and immersed in water for 3h. Subsequently, soil samples were drained with successive exchanges of absorbent paper for 2h and dried at 105 °C.

Table 2. Physicochemical characterization (mean values ± standard deviation (S.D.)) of the natural soil.

Natural Soil	pH (H ₂ O)	pH (KCl)	Conductivity (mS cm ⁻¹)	%OM	%WHC _{max}
	6.46 ± 0.0020	5.39 ± 0.32	0.77 ± 0.045	5.21 ± 0.15	43 ± 0.15

% OM, percentage of organic matter; % WHC_{max}, water-holding capacity in percentage of dry mass.

3.7.2 Preparations of Dispersions and Soil Homogenization

To evaluate the effect of DMSO, DTC and LN/LN-DTC on soil microbial community, the test was performed at concentrations of 0, 0.025, 0.5, 1, 2 and 5 mg DMSO Kg⁻¹ soil for DMSO; 0, 1.7, 3.3, 6.7, 13.3 and 33.3 mg DTC Kg⁻¹ soil for DTC and LN-DTC, while for unloaded LN, the concentrations tested were 0, 26.7, 66.7, 133.4, 200 and 266.7 mg SL Kg⁻¹ soil. For the test, a stock suspension of DTC was prepared in an aqueous solution of DMSO 3% (v/v).

The DTC stock solution and LN and LN-DTC formulations were diluted at different concentrations as described above, in the amount of distilled water required to adjust the soil WHC_{max} to 80%. Then, the soil was spiked with the selected concentrations for all the samples (3 replicates per concentration). All replicates were incubated for two periods (48h and 15 days) at 24 ± 2°C. The selection of the incubation times and the temperature were based on the lack of knowledge regarding the DTC degradation time in soil (since DTC is a new compound) and the annual average temperature in cocoa cultivation regions, respectively. During the incubation period, the soil moisture was continuously monitored and readjusted with deionized water if necessary. After the incubation period, 3 sub-replicates of 1g from each replicate was added to Falcon tubes. In total, 9 sub-replicates were made for each concentration. In this study, changes in the activity of three enzyme were measured (dehydrogenase, acid phosphatase, and arylsulfatase). Additionally, nitrogen mineralization was also evaluated.

3.7.3 Dehydrogenase Activity

Triphenyltetrazolium chloride (TTC) solution (10 g L⁻¹) in 0.1 mol L⁻¹ Tris buffer at pH 7.6 was used to incubate the soil samples at 40 °C for 24h, while the blank test tubes were incubated only with 0.1 mol L⁻¹ Tris buffer at pH 7.6. Reduced TTC produces triphenylformazan (TPF) which is extracted with acetone, forming a pink colored complex measured at 546 nm. Dehydrogenase enzymes' activity was determined from a standard curve obtained for TPF in acetone and expressed in µg TPF g⁻¹ dm (% dry matter) h⁻¹ [175].

3.7.4 Acid Phosphatase Enzyme

Soil was incubated at 37°C for 1h with p-nitrophenylphosphate solution (115 mmol L⁻¹) in pH 6.5 standard buffer and the blank tubes were incubated only with pH 6.5 standard buffer.

Sodium hydroxide (0.5 mol L^{-1}) was used to extract the p-nitrophenol (pNP) released through phosphomonoesterase activity, obtaining a yellow complex measured at 400 nm. Acid phosphatase enzyme activity was determined from a standard curve and expressed in $\mu\text{g pNP g}^{-1} \text{ dm h}^{-1}$ [176].

3.7.5 Arylsulfatase Enzyme

P-nitrophenyl sulfate (0.02 mol L^{-1}) in 0.5 mol L^{-1} acetate buffer at pH 5.8 was used to incubate the soil samples at 37°C for 1h, while the blank tubes were incubated only with sodium acetate trihydrate buffer (0.5 mol L^{-1}) at a pH of 5.8. Arylsulfatase activity releases nitrophenol (pNP) which is extracted with 0.5 mol L^{-1} NaOH forming a yellow color measured at 420 nm. The activity of the arylsulfatase enzyme was determined from a standard curve and expressed in $\mu\text{g pNP g}^{-1} \text{ dm h}^{-1}$ [177].

3.7.6 Nitrogen Mineralization

The blank and soil samples were incubated at 40°C for 7 days with deionized water. In this process, inorganic compounds of nitrogen are metabolized generating organic forms, such as NH_4^+ , and are determined according to the modified Berthelot reaction after extraction with $\text{KCl } 2 \text{ mol L}^{-1}$. A green-colored complex is obtained after the reaction of ammonia with sodium salicylate and sodium nitroprusside in the presence of sodium dichloroisocyanurate. This complex was then measured spectrophotometrically at 690 nm and nitrogen mineralization was determined from a standard curve using defined concentrations of NH_4^+ , in aqueous solution, and expressed as $\mu\text{g NH}_4^+ \text{ g}^{-1} \text{ dm d}^{-1}$ [178].

3.7.7 Statistical Analysis

All data were expressed as mean values $\pm$ S.D and all graphs were performed using Prisma GraphPad 8 software (La Jolla, California, USA).

For the analysis of average particle size (Z-Ave), PDI and ZP of the NPs, the significant differences between 0 days and other periods for the same LN and between LN with and without encapsulated DTC for the same period were calculated by repeated measures analysis of variance (ANOVA). Significant statistical differences were considered for a significance level of $\alpha=0.05$.

For the experimental factorial design, the Statistica 13 software (TIBCO Software Inc, Palo Alto, Tulsa, California, USA) was used, through ANOVA test to determine the significance and interactions between the factors. Significant statistical differences were considered for a significance level of $\alpha=0.05$. Graphic representations were performed using the Statistica 13 software.

The analysis of the effect on fungi was performed by ANOVA followed by Tukey's-multiple comparisons test to check for differences between samples. Aquatic organisms, microbial community, and human cells was performed by ANOVA followed by a Dunnett's-multiple comparison test to check for differences from the control group. A p -value <0.05 was considered statistically significant. No observed effect concentrations (NOEC) and low observed effect concentrations (LOEC) were determined based on the previously described analysis. The EC_{50} and EC_{20} values were determined by applying non-linear least squares regression analysis using STATISTICA® 7.0 software (StatSoft, Inc., Tulsa, OK, USA). A level of significance $\alpha=0.05$ was used.

4 Results and discussion

4.1 Evaluation of the Average Size, Homogeneity and Surface Charge of Nanoparticles

Regardless of the application of NPs, determining some physical-chemical parameters is of paramount importance. For example, particle size, shape, surface chemistry are essential properties of NPs, which play an important role in the quality control of NPs production [179]. Therefore, the measurement and control of these parameters are crucial for optimizing the synthesis of NPs. Through DLS measurements, it is possible to evaluate the behavior of NPs in the liquid phase an aqueous medium and other solvents by monitoring their mean hydrodynamic particle size (Z-Ave), their size distribution (indicated by polydispersity index (PDI)) and their stability (indicated by zeta potential (ZP)) [180]. In general, NPs with particle size between 1 and 100 nm have high surface to volume ratios and can be used for drug delivery, imaging agents, and can penetrate areas inaccessible to larger molecules. However, it has also been found that the particles with smallest sizes (NPs < 100 nm) can cause undesirable effects, such as crossing the blood-brain barrier and causing immune reactions, as well as damage to the cell membrane [181], and, to date, a great deal of research on the effect of size of NPs has been concentrated in the field of toxicity [182].

Depending on the particle size distribution, the numerical PDI values ranging from 0.0 (for a perfectly uniform sample with respect to particle size) to 1.0 (for a highly polydisperse sample with populations of various particle sizes) can be measured for the dispersions of NPs. In AI delivery applications, PDI values ≤ 0.3 are considered and can indicate a homogenous population of NPs [183].

The ionization of chemical functional groups on the surface of NPs, or the absorption of suspended ions (for example, opposite charges), increases or decreases the surface charge and can be determined through the ZP. Extremely positive or negative ZP values cause large repulsive forces, whereas repulsion between particles with similar electric charge prevents aggregation, and accordingly ensures easy redispersion. In the case of a combined electrostatic and steric stabilization, minimum ZP values of ± 30 mV are desirable and indicate that the dispersions can maintain their stability for long periods of time [184].

MSiNP were synthesized using a positively charged CTAB model and NH_3 as catalytic agent in aqueous medium, through hydrolysis and condensation of tetraethoxysilane. MSiNP were

loaded with DTC and after their resuspension in aqueous medium, MSiNP and MSiNP-DTC were evaluated according to Z-Ave, PDI and ZP as represented in Table 3.

The results show a Z-Ave ranging of 168.0 ± 3.9 nm for unloaded MSiNP of 176 ± 1 nm for MSiNP-DTC, which corroborated that the encapsulation of the DTC in MSiNP did not significantly change the particle size ($p > 0.05$). Concerning the PDI, the obtained values for unloaded and loaded MSiNP suggest monodispersing formulations with mean values of 0.29 ± 0.02 for MSiNP which, compared to MSiNP-DTC, suffered a significant increase ($p < 0.05$) to 0.38 ± 0.04 . The ZP values for MSiNP changed from positive $+17.0 \pm 0.2$ mV to negative values -12.0 ± 0.4 mV after calcination in a nitrogen atmosphere, due to neutralization of the ionizable fraction (residual silanol groups (Si-OH)). After surface modification with DTC, there were no significant changes ($p > 0.05$) in ZP values of MSiNP. However, obtaining higher ZP values (in modulus) for MSiNP-DTC (-22 ± 0.3 mV) compared to MSiNP may indicate an improvement in the stability of MSiNP-DTC in the aqueous medium.

Table 3. Average hydrodynamic size $\pm$ standard deviation (Z-Ave $\pm$ S.D.), polydispersity index (PDI $\pm$ S.D.), and average zeta potential (ZP $\pm$ S.D.) recorded for MSiNP, MSiNP calcined, and MSiNP-DTC dispersed at 200 mg L⁻¹ in water.

SAMPLES	Z-Ave $\pm$ S.D. (nm)	PDI $\pm$ S.D.	ZP $\pm$ S.D. (mV)
MSiNP	168 ± 3	0.30 ± 0.02	$+17.0 \pm 0.2$
MSiNP calcined	168 ± 4	0.29 ± 0.02	-12.0 ± 0.4
MSiNP-DTC	176 ± 1	0.38 ± 0.04	-22.0 ± 0.3

4.2 Optimization of Lipid Nanoparticles (LN)

Different factors can affect the size and stability of LN such as type and concentration of lipid constituents, solid lipid/oil ratio, surfactant type and concentration, sonication parameters and cooling rate of pre-emulsion [185]. With the aim of obtaining homogeneous LN dispersions with reduced average particle size and high stability in aqueous media, a factorial design was performed to verify the appropriate concentration of solid lipid and surfactant for DTC encapsulation in LN.

The mean particle size is highly affected by the type and concentration of the surfactant (s) used for LN synthesis. The increase of surfactant concentration can result in a decrease of nanoparticle sizes [186]. In addition, the concentration and ratio of oil and solid lipid are also responsible for the variations of particle size [187]. Using factorial design 2^3 , the influence of the concentration of solid lipid (Precirol ATO 5) as well as the concentration of Soy lecithin on Z-Ave, PDI and ZP of LN-DTC was investigated. A total of 11 experiments were performed and the results of the experimental design are shown in Table 4 (factorial points).

From the data obtained, the Z-Ave of LN-DTC ranged from 150.0 ± 0.4 nm (F1) to 236 ± 3 nm (F11) and the PDI ranged between 0.23 ± 0.02 (F3) and 0.27 ± 0.02 (F7). For ZP values, the results were between -53.0 ± 0.3 (F1) and -38.0 ± 0.9 mV (F7) (Table 4). To identify the significant effects and interactions, ANOVA statistical tests were performed for each parameter. Figure 9 shows the Pareto graphs and illustrates the surface response graphs (Z-Ave, PDI and ZP values) of the experimental design. The results reported in Table 6 indicate that the concentrations of Precirol and Lecithin had no significant effect on the PDI ($p > 0.05$). However, there was a significant effect in relation to Z-Ave and ZP ($p > 0.05$) (Table 5 and 7). In Figure 11A, it is possible to see that increasing the concentration of Precirol, the average particle size also increases, that is, Precirol significantly influences the Z-Ave (Figure 11A). From the data obtained or ZP analysis, it was observed that the greater amount of Precirol or Soy lecithin led to a lower surface charge of nanoparticles (Figure 11A and C).

After factorial design, LN-DTC with the best values of Z-Ave, PDI and ZP were chosen for the subsequent assays. To summarize, the optimized LN formulation was composed of 0.17% (m/m) Precirol and Soy lecithin, 0.70% (m/m) Capryol 90, 0.35% (m/m) Tego Care 450 and 0.021 % (m/m) DTC.

Table 4. Influence of Precirol ATO 5 and Soy lecithin on Z-Ave, PDI and ZP of LN-DTC.

Formulations	LEVELS OF FACTORS		MEASURED RESPONSES		
	Precirol	Soy Lecithin	Z-Ave $\pm$ S.D. (nm)	PDI $\pm$ S.D.	ZP $\pm$ S.D. (mV)
1	-1	-1	150.0 $\pm$ 0.4	0.24 $\pm$ 0.01	-53.0 $\pm$ 0.3
2	-1	0	165 $\pm$ 1	0.25 $\pm$ 0.01	-47 $\pm$ 1
3	-1	+1	161 $\pm$ 2	0.23 $\pm$ 0.02	-45 $\pm$ 1
4	0	-1	181 $\pm$ 4	0.21 $\pm$ 0.02	-45 $\pm$ 1
5	0	+1	184 $\pm$ 1	0.26 $\pm$ 0.01	-44 $\pm$ 1
6	+1	+1	178 $\pm$ 3	0.26 $\pm$ 0.01	-42 $\pm$ 1
7	+1	0	224 $\pm$ 7	0.27 $\pm$ 0.02	-38 $\pm$ 1
8	+1	-1	236 $\pm$ 3	0.24 $\pm$ 0.02	-41 $\pm$ 1
CENTRE POINTS					
9	0	0	180.0 $\pm$ 0.5	0.25 $\pm$ 0.01	-46 $\pm$ 1
10	0	0	186 $\pm$ 4	0.25 $\pm$ 0.03	-45.0 $\pm$ 0.5
11	0	0	180 $\pm$ 4	0.25 $\pm$ 0.02	-44 $\pm$ 1

Table 5. Evaluation of Precirol and Soy Lecithin and their interactions. Analysis of Z-Ave by analysis of variance (ANOVA) statistical test.

Factors and their Interactions	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p -value
(1) Precirol	2621.440	1	2621.440	329.9187	0.000364
(2) Lecithin	566.440	1	566.440	71.2887	0.003487
1by2	1211.040	1	1211.040	152.4142	0.001145
Error	23.837	3	7.946		
Total	4422.757	6			

Table 6. Evaluation of Precirol and Soy Lecithin and their interactions. Analysis of PDI by analysis of variance (ANOVA) statistical test.

Factors and their Interactions	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p -value
(1) Precirol	0.000225	1	0.000225	7.000000	0.077274
(2) Lecithin	0.000025	1	0.000025	0.777778	0.442763
1by2	0.000225	1	0.000225	7.000000	0.077274
Error	0.000096	3	0.000032		
Total	0.000571	6			

Table 7. Evaluation of Precirol and Soy Lecithin and their interactions. Analysis of ZP (mV) by analysis of variance (ANOVA) statistical test.

Factors and their Interactions	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p -value
(1) Precirol	54.02250	1	54.02250	46.74864	0.006402
(2) Lecithin	12.60250	1	12.60250	10.90564	0.045652
1by2	23.52250	1	23.52250	20.35531	0.020348
Error	3.46679	3	1.15560		
Total	93.61429	6			

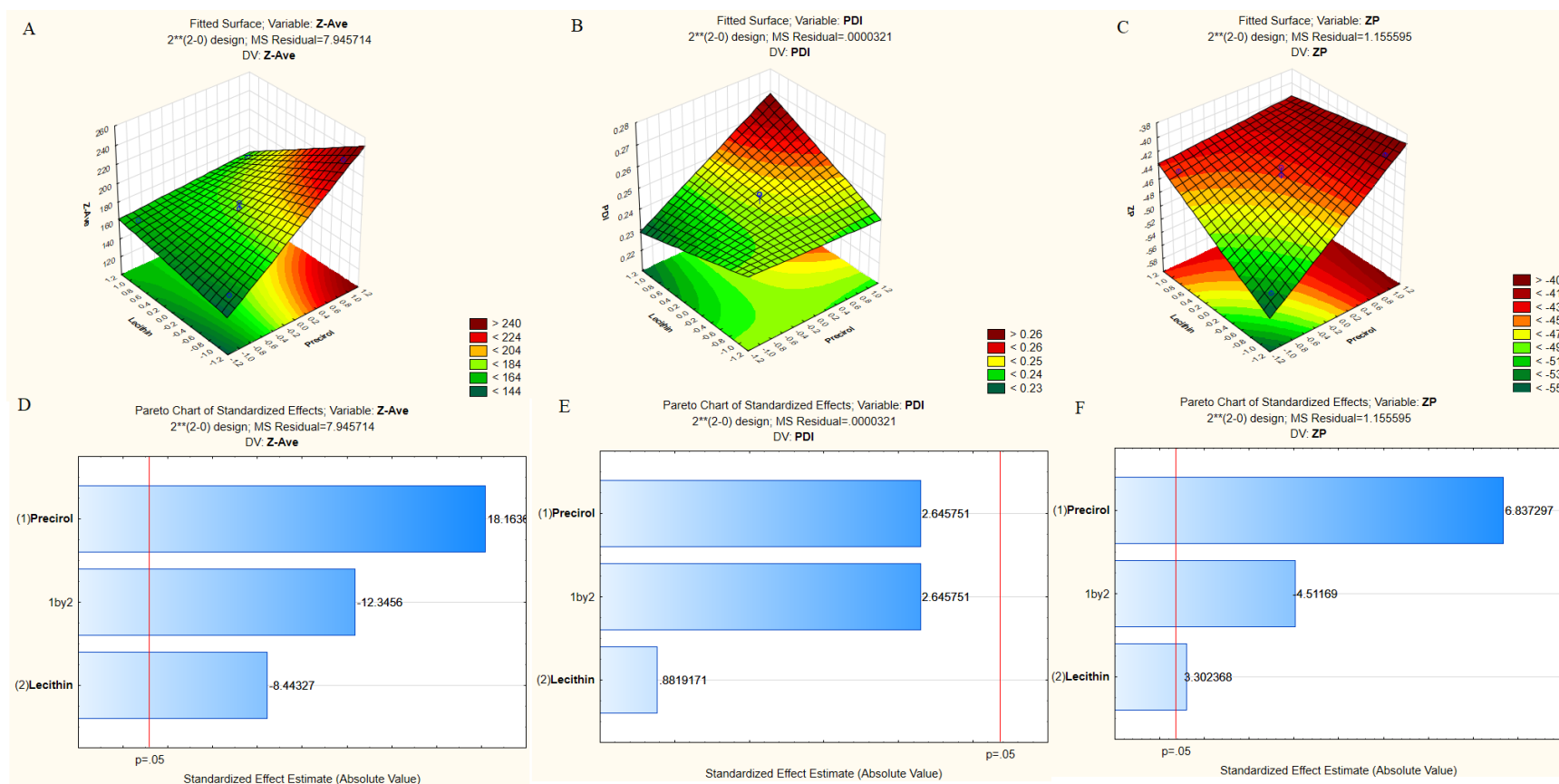


Figure 11. Graphical representation of the interaction between the independent variables on LN-DTC properties (factorial design experiment set in Table 5). Surface response plots of the effect of Precirol and Lecithin on Z-Ave (A), PDI (B) and ZP (C). Pareto chart of Precirol ATO 5 and Soy lecithin on Z-Ave (D), PDI (E) and ZP (F). Vertical lines in Pareto chart indicate the critical effect at 95% confidence level.

4.3 Evaluation of the Stability of Lipid Nanoparticles

After loading the DTC into LN, the stability of LN-DTC and unloaded LN was analysed over time, in relation to Z-Ave, PDI and ZP, after 30, 60, 210 days of storage at 25 °C, as shown in Figure 12.

The evaluation of the stability of the synthesized LN is related to the fact that these formulations tend to sediment, flocculate and gelify over time, being necessary to verify if these matrices tend to resist recrystallization. In this way, stable dispersions preserve the Z-Ave, PDI and ZP over time and are essential to ensure not only a homogeneous distribution of the particles, but also a greater loading efficiency and controlled release of the encapsulated/loading AI [188].

According to Figure 12, Z-Ave values ranged from 134 ± 1 to 143.0 ± 0.3 nm for LN and 144 ± 4 to 188 ± 1 nm for LN-DTC and significant differences were observed for both NPs in the period of 210 days of storage ($p < 0.05$). The PDI values varied between 0.200 ± 0.003 and 0.26 ± 0.01 for LN and between 0.170 ± 0.007 and 0.25 ± 0.01 for LN-DTC. Therefore, the PDI measurements indicated that the loaded and unloaded LN can be considered as homogenous dispersions, since the PDI values were below 0.3. Comparing with day 0 and with LN, a significant reduction of PDI is observed for LN-DTC after 210 days ($p < 0.05$), and thus, we can consider that despite having an increase in particle size, encapsulation of DTC in LN showed high stability over time.

The ZP values varied between -39.0 ± 0.2 to -54.0 ± 0.8 mV for LN and -42 ± 1 to -53.0 ± 0.3 mV for LN-DTC, noting that the LN, and after DTC incorporation, ZP values for LN-DTC were lower (in modulus) compared with unloaded LN, over time. Despite this reduction, the ZP values indicated that LN-DTC maintain their colloidal stability, which prevents the occurrence of particle aggregation [189].

These results could be confirmed with the visual analysis of the formulations, which presented good appearance and fluidity, without the presence of flocculated or sedimented particles immediately after production (0 days) and after 210 days of storage

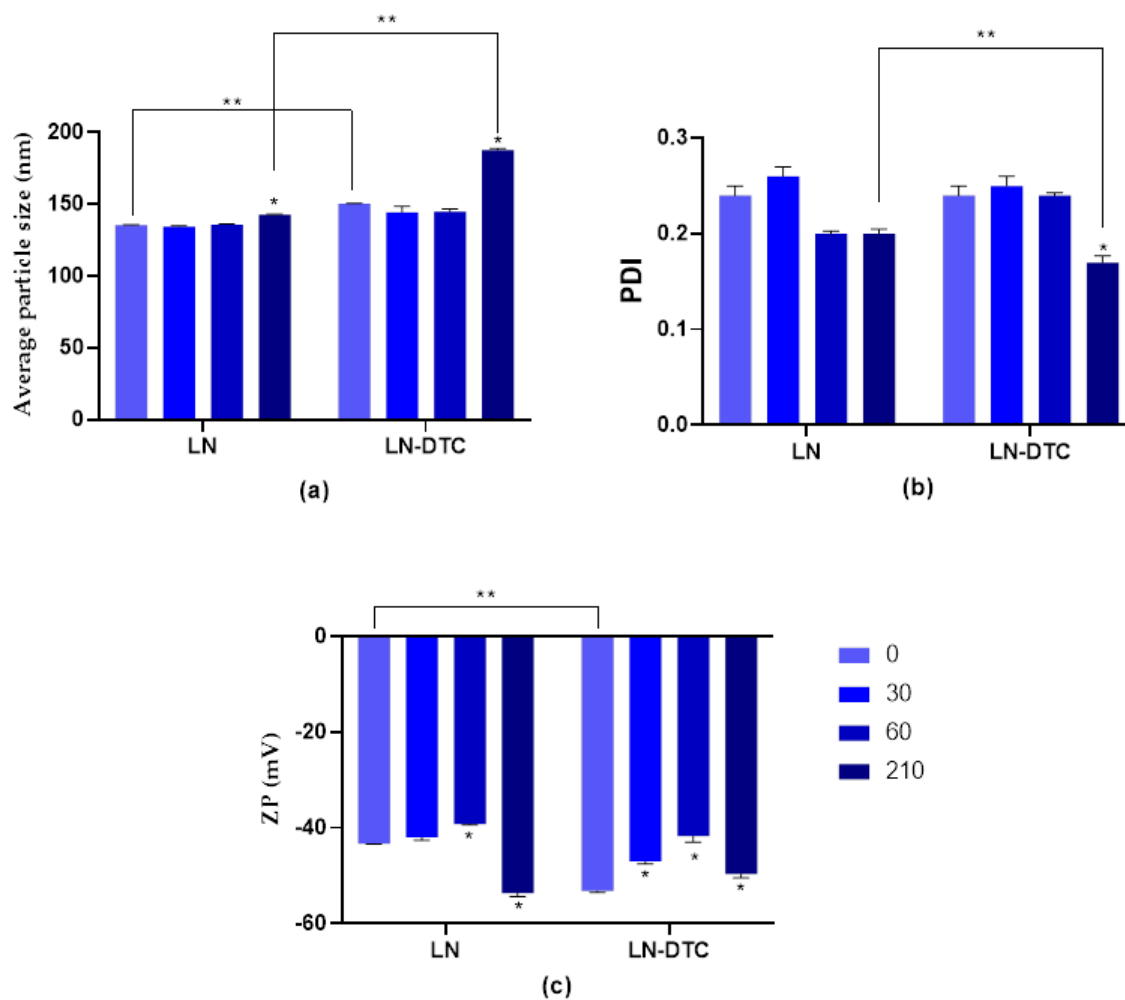


Figure 12. Average particle size (Z-Ave) (nm) (a), PDI (b), and ZP (mV) (c) of synthesized LN and LN-DTC (0 days) and after 30, 60, and 210 days of storage at 25 °C. Results are expressed as mean $\pm$ S.D. (n=3). Significant differences between 0 days and other periods for the same LN are represented by (*), $p < 0.05$ (ANOVA repeated measures). Significant differences between unloaded LN and LN-DTC for the same period are represented by (**), $p < 0.05$ (ANOVA repeated measures).

4.4 Physical-chemical Characterization of DTC and Nanoparticles

4.4.1 Loading Efficiency

It is known that inorganic carriers such as MSiNP tend to have high loading capacity, but low encapsulation efficiency, unlike organic carriers such as micelles, liposomes and polymeric nanoparticles [190]. However, in the present study, MSiNP-DTC maintained high drug loading capacity ($4.790 \pm 0.004\%$) and encapsulation efficiency ($95.77 \pm 0.08\%$). Loading capacity and the encapsulation efficiency were higher than in some similar systems which results from the strong electrostatic and hydrogen bonding interactions between the AI and the carriers.

For LN-DTC, an encapsulation efficiency and a loading capacity of $99.39 \pm 0.01\%$ and $4.39 \pm 0.01\%$, respectively, were determined, showing that the DTC has high affinity to the lipid matrix. Owing to the nature of LN with a high lipid content (ideal for solubilizing lipophilic compounds), high trapping efficiency of DTC was observed, which is a compound with low solubility in aqueous media (solubility in water = 0.08 mg L^{-1} at room temperature) due to the homogeneous solubilization of the drug within the lipid and formation of a rigid solid nucleus after cooling that slows the exit of the trapped drug [191].

4.4.2 Lyophilization of LN and LN-DTC

Freeze-drying has been routinely applied to increase the long-term stability of NPs upon storage. In the present study, only LN were freeze dried for further physicochemical analysis. To avoid any external influences such as temperature and light, which are of paramount importance for long-term stability during storage and transport, LN and LN-DTC were lyophilized in the absence of cryoprotectants followed by sample reconstitution in ultra-pure water. To reconstitute the NPs in water, aqueous dispersions of NPs were homogenized in an ultrasonic bath for 10 min followed by their physicochemical characterization related to Z-Ave, PDI and ZP analysis. The obtained results from freeze drying of NPs indicated $124.0 \pm 0.2 \text{ nm}$; 0.26 ± 0.01 and $-49.0 \pm 0.3 \text{ mV}$, respectively, for unloaded LN and a $134 \pm 3 \text{ nm}$; 0.37 ± 0.01 and $-50 \pm 1 \text{ mV}$, respectively, for LN-DTC.

4.4.3 Transmission Electron Microscope (TEM) analysis

TEM analysis was performed to evaluate the size distributions, structure, and morphology of NPs. TEM image in Figure 13 revealed the formation of spherical MSiNP with particle size around 48 ± 5 nm for MSiNP and 47 ± 6 nm for MSiNP-DTC. Moreover, the image clearly shows the mesoporous structure of NPs and shows that surface modification with DTC did not have any impact on size, shape, and morphology of the MSiNP. The reduction in mean diameter observed in TEM compared to Z-Ave in DLS is in accordance, since the dry structure analysed by TEM has to be smaller than the same structure with its solvation layer analysed by DLS [192]. TEM analysis performed for LN and LN-DTC are shown in Figure 14. It is known that the drying process and imperfect crystallization of lipids can result in sample deformation. However, the images revealed that there was no change in the lyophilization process, which maintained the spherical morphology of the particles with particle size around 30 ± 3 nm for LN and 38 ± 4 nm for LN-DTC. No crystallization of DTC was observed on the surface of LN-DTC, and its loading on LN did not alter the NPs' morphology/size. Thus, we can suggest that the lipid matrix used for LN synthesis solidified upon cooling, but remained in an amorphous state, improving the accommodation of DTC in the lipid matrix [193–196].

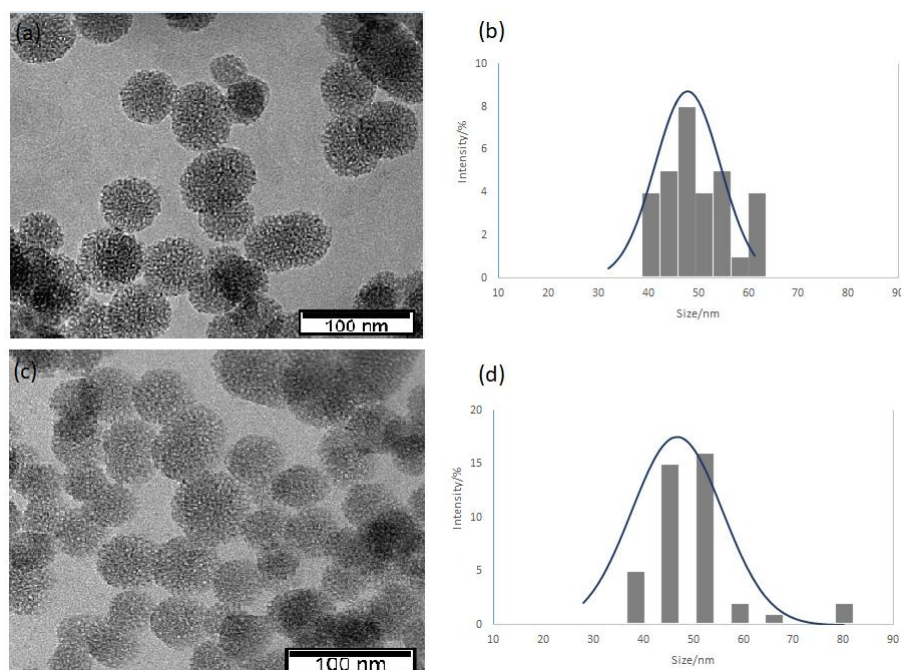


Figure 13. TEM images of (a) MSiNP and (c) MSiNP-DTC and their respective size distribution histograms (b,d). Images obtained by the IST-microlab laboratory, Lisbon.

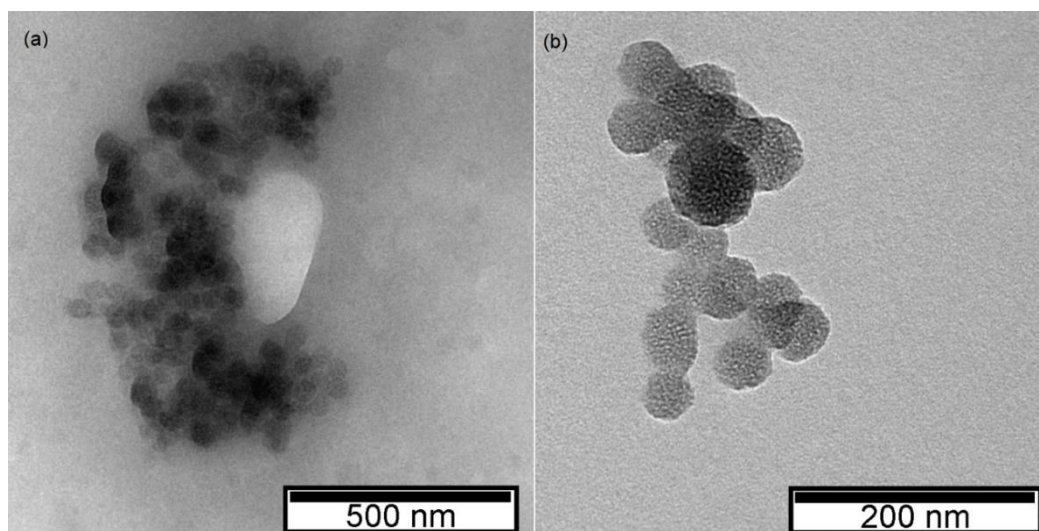


Figure 14. TEM images of (a) LN and (b) LN-DTC after lyophilization. Images obtained by the IST-microlab laboratory, Lisbon.

4.4.4 Fourier Transform Infrared (FT-IR)

FT-IR spectra of DTC, MSiNP and LN unloaded and loaded with DTC are shown in Figure 15, and 16 (A and B), respectively. DTC presents important bands that must be monitored to verify if there was adequate formation of the compound and if interaction with the matrix in question occurred. As it was necessary to repeat the synthesis of the compound, we verified the formation of the main bands, thione group referring to the vibration mode $\nu(\text{C}=\text{S})$ and generally appears quite intensity in the region of 1050 cm^{-1} , was observed with a shift to 1085 cm^{-1} . DTC also has two intense bands in 1574 and 1031 cm^{-1} , which can be attributed to the $\nu(\text{C}=\text{N})$ and $\nu(\text{N}-\text{N})$ modes, respectively. And finally, the last two bands at 846 and 696 cm^{-1} refer to vibration band $\nu(\text{SCS})$ and $\nu(\text{CSC})$. Special attention must be paid to these bands, since a possible shift in their frequencies can be an indication of DTC loading in matrix, which, a shift to lower frequencies is associated with a reduced order of bonding, while the shift to higher frequencies corresponds to the increase in connection order [28,197–199].

Figure 16 (A) shows the FT-IR spectra from MSiNP (a), MSiNP calcined (b) and MSiNP-DTC (c). The asymmetric vibration band of the O-H group is observed at 3450 cm^{-1} . Absorption band around 1096 cm^{-1} is due to the vibrations of asymmetric stretching of Si-O-Si, which indicates the presence of silica in the MSiNP, while the peaks at 807 and 468 cm^{-1} attributed to the groups Si-O-Si are symmetrical stretching and flexing vibrations,

respectively. After calcination in a nitrogen atmosphere, the ionizable fraction (residual silanol groups (Si-OH)) of the nanoparticles was neutralized at 2931, 2844 and 1508 cm^{-1} .

However, only the band at 963 cm^{-1} corresponding to the stretching vibration of the Si-OH surface groups remains after calcination. In the spectrum of MSiNP-DTC, it was not observed the appearance of new bands. The main bands of DTC that were reported above suffered displacement increasing the intensity of the bands related to silica. The peak at 1627 cm^{-1} , we can attribute to the mode the vibration band $\nu(\text{C}=\text{N})$ which in the DTC appears at 1574 cm^{-1} undergoing shift to high frequencies corresponding to the increase in the connection order. The same happened for the vibration band $\nu(\text{C}=\text{S})$ appearing in the DTC at 1085 cm^{-1} , but when loaded onto silica it increases its binding order to 1096 cm^{-1} . Differently, the vibration bands $\nu(\text{N}-\text{N})$; $\nu(\text{SCS})$ and $\nu(\text{CSC})$ change to lower frequencies, so the graph shows peaks with low intensities appearing at 963, 796 and 475 cm^{-1} , respectively when compared to pure DTC [200–203].

The FTIR spectra of LN and LN-DTC are shown in Figure 16 (B). In both spectra (a and b), similar peaks are observed at 2916 cm^{-1} referring to the characteristic C-H stretching of glyceryl palmitostearate present in Precirol ATO 5 and at 2850 cm^{-1} from Capryol 90 referring to C-H aliphatic. The high intensity peak at 1730 cm^{-1} and 1469 cm^{-1} correspond to the carbonyl group $\nu(\text{C}=\text{O})$ and the $\nu(\text{C}-\text{H})$ bending, respectively, and are derived from the compounds Precirol, Soy Lecithin and Capriol 90. And finally, the peak at 719 cm^{-1} corresponds to the $\nu(\text{C}=\text{C})$ band of Soy Lecithin [204–206]. In the LN-DTC spectrum Figure 16 (B-(b)), we observe only three new slightly weak peaks when compared to the LN spectrum. These peaks have been shifted compared to pure DTC and appear at 1308, 1143 and 852 cm^{-1} for $\nu(\text{C}-\text{N})$, $\nu(\text{N}-\text{N})$ and $\nu(\text{C}-\text{S})$ in the LN-DTC matrix. At 1469 cm^{-1} we also observed a certain increase in the intensity of the peak that is attributed to the binding mode $\nu(\text{C}=\text{N})$, also occurring at 722 cm^{-1} referring to $\nu(\text{SCS})$ in the DTC compound.

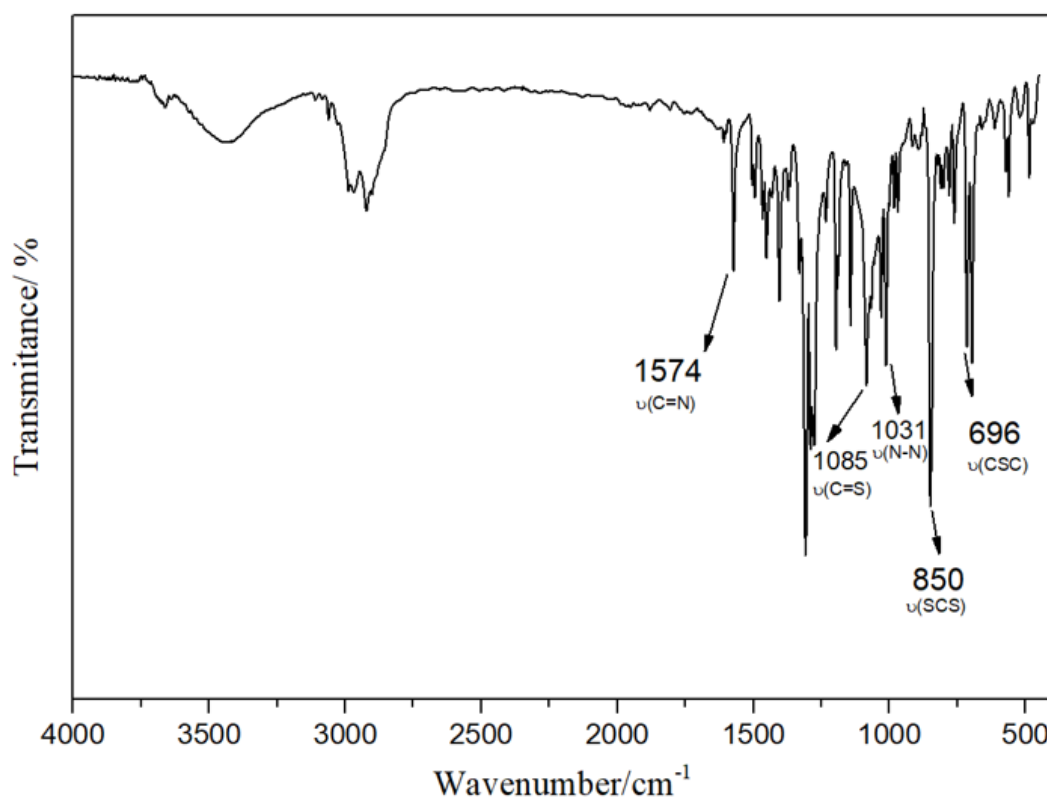


Figure 15. FT-IR of the compound DTC (3-methyl-5-phenyl-pyrazoline-1-(S-benzylthiocarbazate) in KBr.

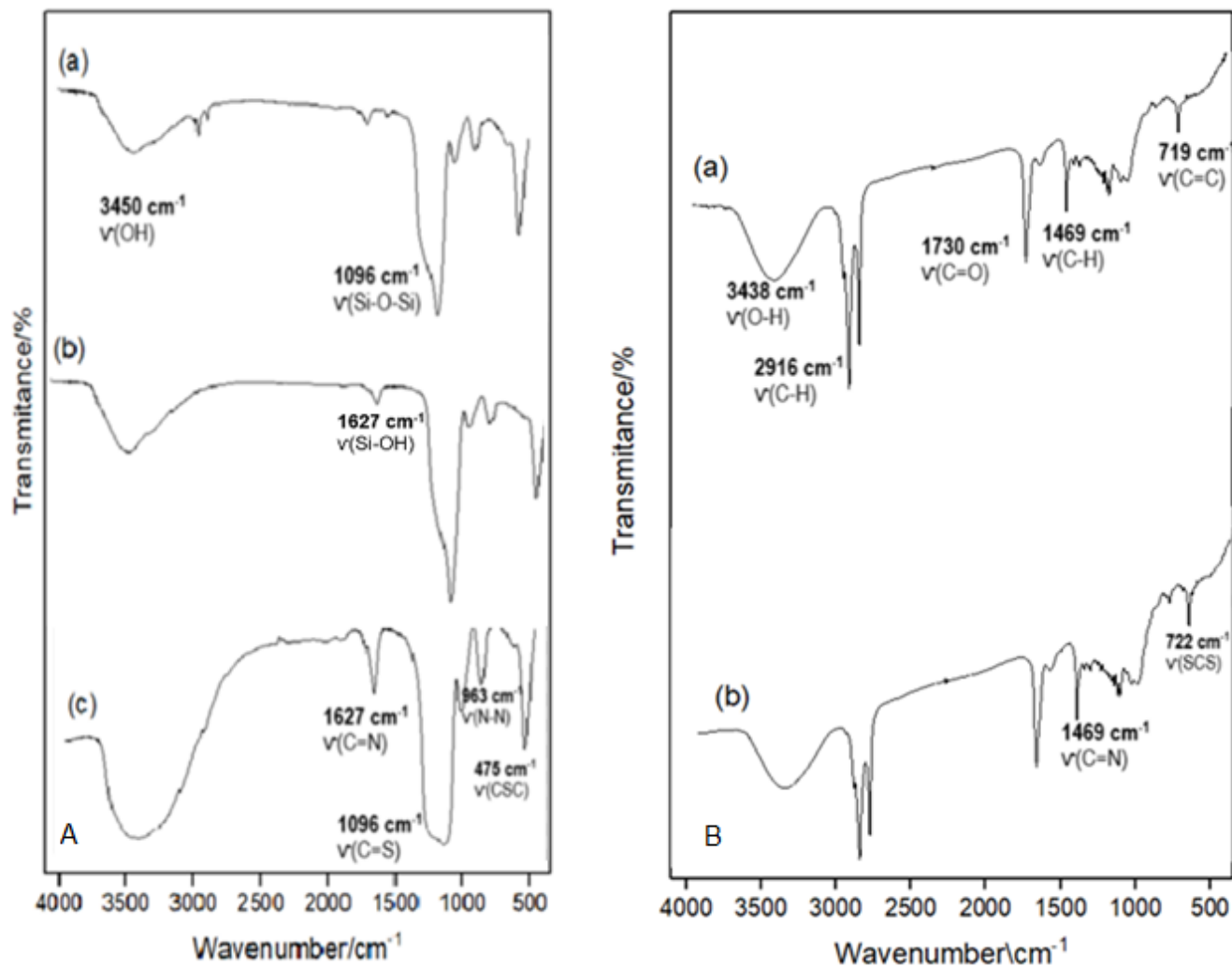


Figure 16. FT-IR of MSiNP (a), MSiNP calcined (b) and MSiNP-DTC (c) in KBr (A) and LN (a) and LN-DTC (b) in KBr (B).

4.4.5 Raman Spectroscopy

A popular technique in the area of biological, pharmaceutical also in medicine sciences is Raman Spectroscopy, because it is a non-destructive technique and does not require sample preparation [207]. The difference between the Infrared (IR) and the Raman Spectroscopy is basically related to the fact that FTIR relies on the change in the molecular dipole moment, while the change in molecular polarizability induces the Raman bands. Strong IR bands are associated with polar groups in fingerprints, while non-polar groups produce strong Raman spectra. Therefore, Raman spectroscopy can detect some undetectable information in the infrared spectrometer, such as C-C single bond, S-S single bond, N=N double bond, and C=C double bond [208].

Complementary to the IR, Raman Spectroscopy was performed in order to evaluate the possible interactions between the components with DTC. Initially, the main peaks of the DTC compound were defined. The characteristic functional $\nu(\text{C-S})$ stretching mode is observed in 711 cm^{-1} . Stretching mode of aromatic ring groups appears at 998 cm^{-1} , the peaks in 1029 , 1595 and 2907 cm^{-1} refer to the stretching modes $\nu(\text{C=S})$, $\nu(\text{C=N})$ and $\nu(\text{C-H})$, respectively and clearly highlighted in Figure 17 [22,209,210].

Characteristic vibrational modes of the embedded DTC are not clearly displayed in the Raman spectra of the MSiNP and MSiNP-DTC as they are obscured by the host's hydroxyl vibrations. We generally assign the bands at 810 and 970 cm^{-1} to siloxane bridges (Si-O-Si) and surface silanol (Si-OH) groups of MSiNP and in (Figure 18 (A-(a))) we see that there is only one weak band at 1372 cm^{-1} due to spectral overlap. However, in the MSiNP-DTC (Figure 18 (A-(b))) spectrum we observe a significant increase in the intensity of this band and no additional DTC band is detected. This could be due to the high interaction between the DTC and the MSiNP structure or the low amount of DTC intervention in the MSiNP channels [211–213].

Figure 18 (B) shows the Raman spectra of the NLC formulations compared to their respective formulation containing DTC. The peaks observed in LN (Figure 18 (B-(a))) are basically from the compounds Precirol and Capryol 90. Peaks at 1296 cm^{-1} are attributed to the twisting of CH_2 and between 800 and 900 cm^{-1} to the oscillation of CH_3 . The peak at 1442 cm^{-1} is related to CH_2 bonds, while the peaks at 1132 and 1064 cm^{-1} refer to C-C asymmetric and symmetric stretching, respectively. Weak peak in 1736 cm^{-1} is related to the carbonyl bond $\nu(\text{C=O})$ and the peaks at 2885 and 2849 cm^{-1} are assigned asymmetric and symmetric CH, respectively [214–216]. When we look at the LN-DTC (Figure 18 (B-(b)))

spectrum, we observe the reduction in intensity and the formation of new peaks coming from the DTC, which does not appear intensely in the IR spectrum. Analyzing the spectrum, it is possible to observe bands at 471 and 715 cm^{-1} referring to the $\nu(\text{C-S})$ bond. The peak related to the aromatic ring of the compound DTC appears at 1004 cm^{-1} and at 1200 cm^{-1} the $\nu(\text{C=S})$ bond, while the peak at 1613 cm^{-1} refers to $\nu(\text{C=N})$. Compared with the spectrum of pure DTC, we can observe the main peaks in LN-DTC. However, these peaks suffered displacement as indicated in Figure 18.

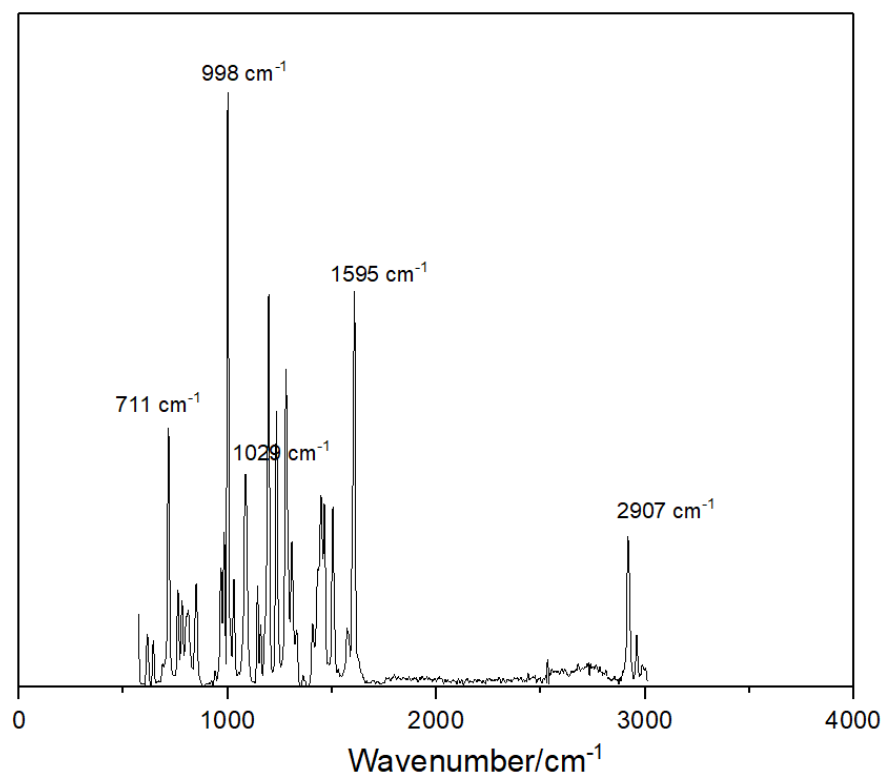


Figure 17. Raman spectra of DTC (3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate)).

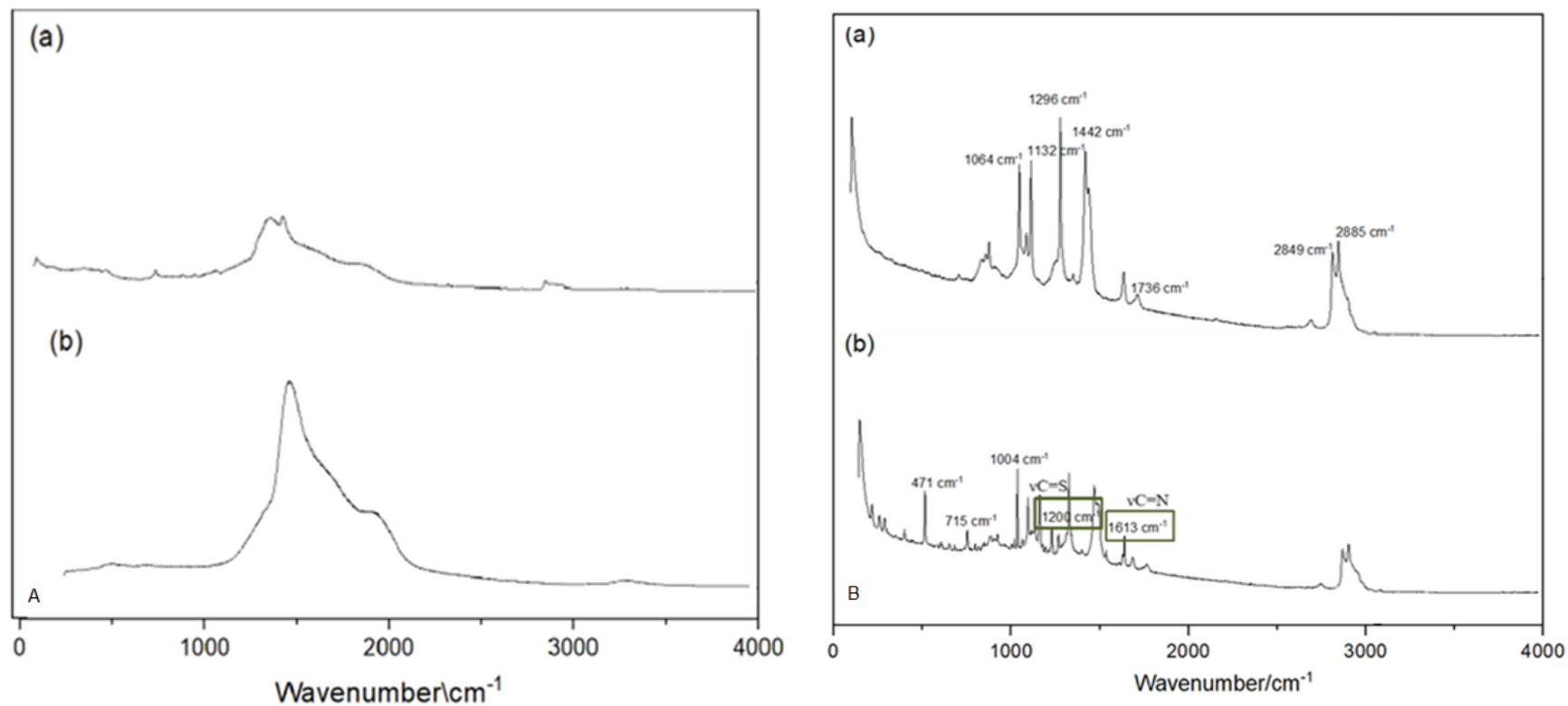


Figure 18. Experimental Raman spectra of (a) MSiNP and (b) MSiNP-DTC (A) and (a) LN and (b) LN-DTC (B).

4.4.6 Thermal Analysis

Thermal studies of all compounds were carried out within a temperature range of 25–600 °C under the dynamic atmosphere of nitrogen to investigate their thermal stability. According to studies conducted by Costa *et al.*, 2019, DTC (Figure 19) has a melting point of 107 °C and a decomposition or evaporation temperature of the sample with a mass loss of around 95% temperature of 300 °C. As shown in Figure 20 (a), the thermogram obtained for MSiNP did not show any transition in the temperature range of 25-600 °C. However, a melting endothermic peak at around 104 °C was detected in the thermogram of MSiNP-DTC, confirming that DTC was incorporated into MSiNP (Figure 20 (b)).

TG analysis showed that the mass loss was 17.12% and 12.52% for MSiNP and MSiNP-DTC, respectively. As shown in thermal curves, DTA thermograms for MSiNP and MSiNP-DTC showed an intensive endothermic reaction in the range of 20–100 °C. This peak is correlated to the loss of physically adsorbed water from the surface and chemically adsorbed water bonded to Si-OH through the hydrogen bond.

Regarding the results obtained for LN, the TG analysis showed that the mass loss for unloaded LN was 84.11% (Figure 21 (a)), while for LN-DTC, it was 84.29% (Figure 21 (b)). This result supports the idea that DTC can be thermodynamically stable under the production conditions applied for the preparation of LN. As for the DSC analysis, there is a peak at 50 °C attributed to the lipid Precirol ATO 5, which generally has a melting point at 66.2 °C, suggesting that LN synthesis leads to the formation of a less ordered structure of the lipid matrix requiring less energy to reach the fusion state [205]. The value obtained for the melting point of the DTC was not detected after encapsulation in LN, indicating that the DTC is completely dispersed in the lipid matrix, and it is possible that the DTC induces changes in the lipid structure from crystalline to amorphous in order to increase its accommodation [217–219].

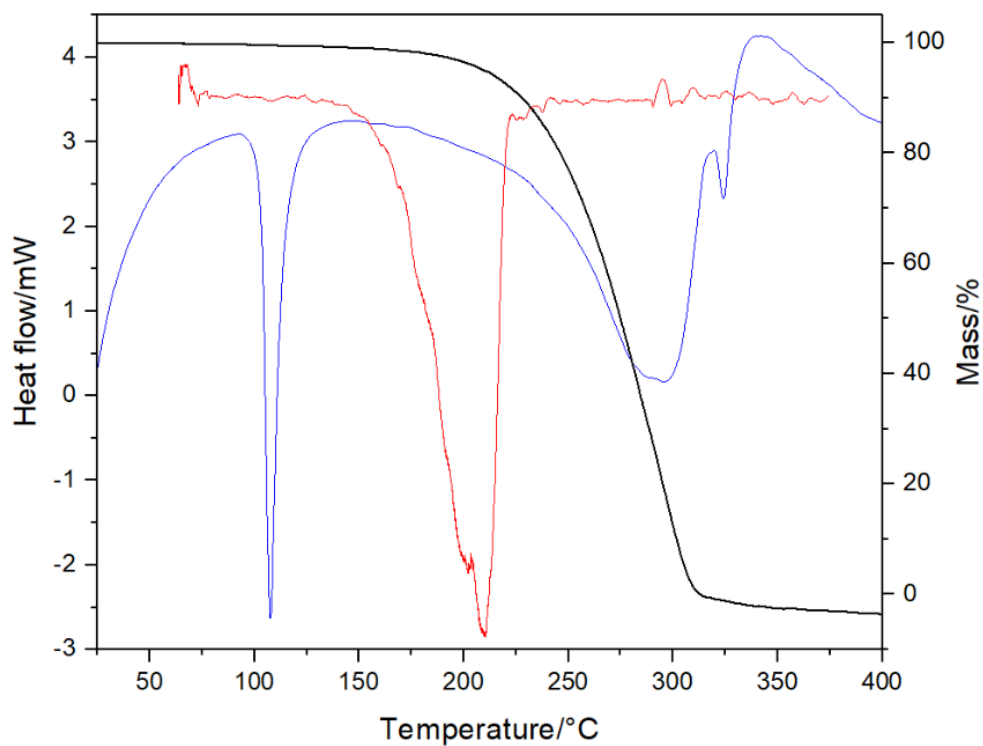


Figure 19. Thermal analysis of DTC (3-methyl-5-phenyl-pyrazoline-1-(S-benzylidithiocarbazate)): thermogravimetry (TG) (%) (black line), differential thermal analysis (DTA) (red line), and differential scanning calorimetry (DSC) (blue line). Adapted from: Costa *et al.*, 2019.

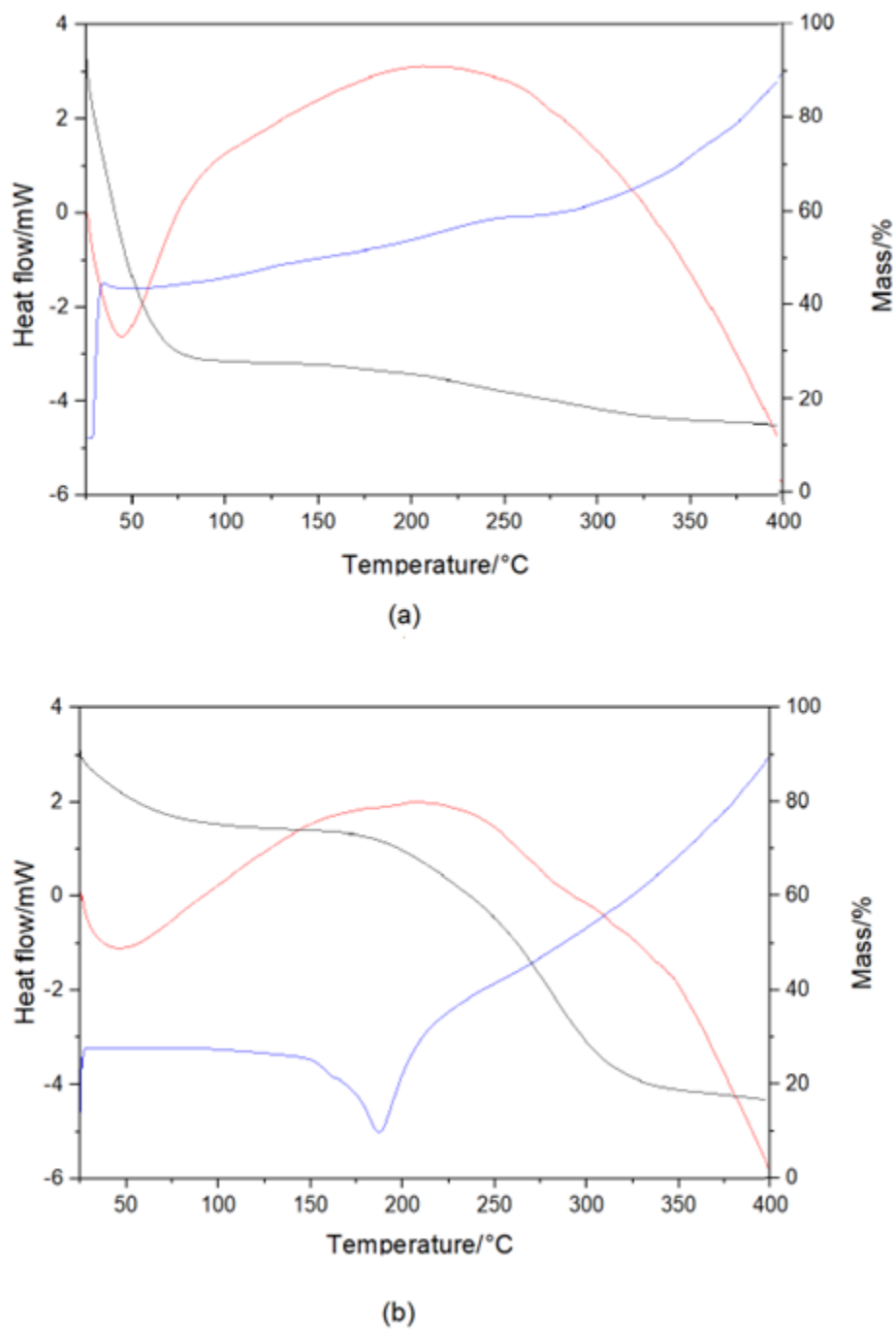
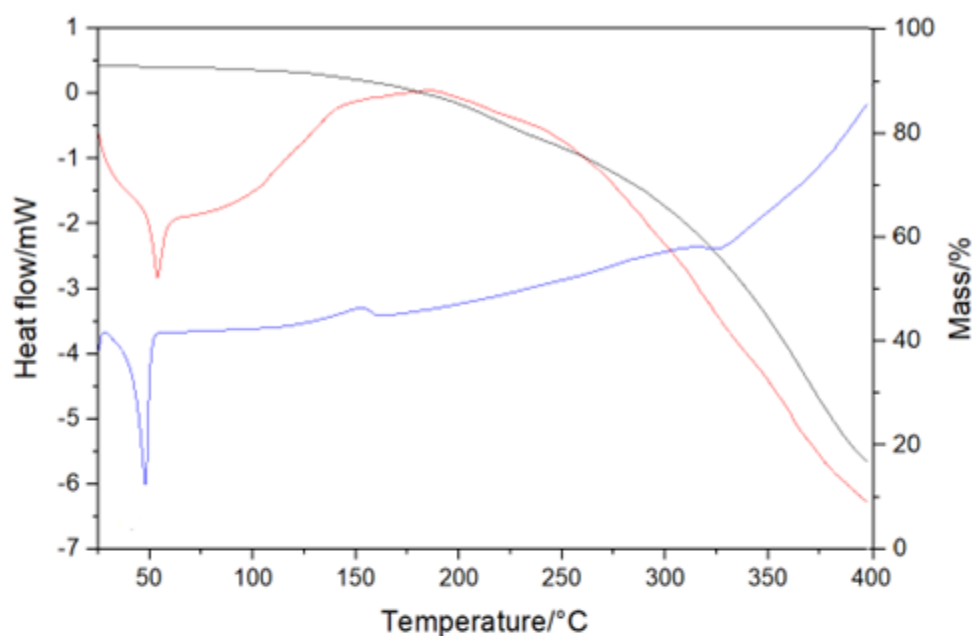
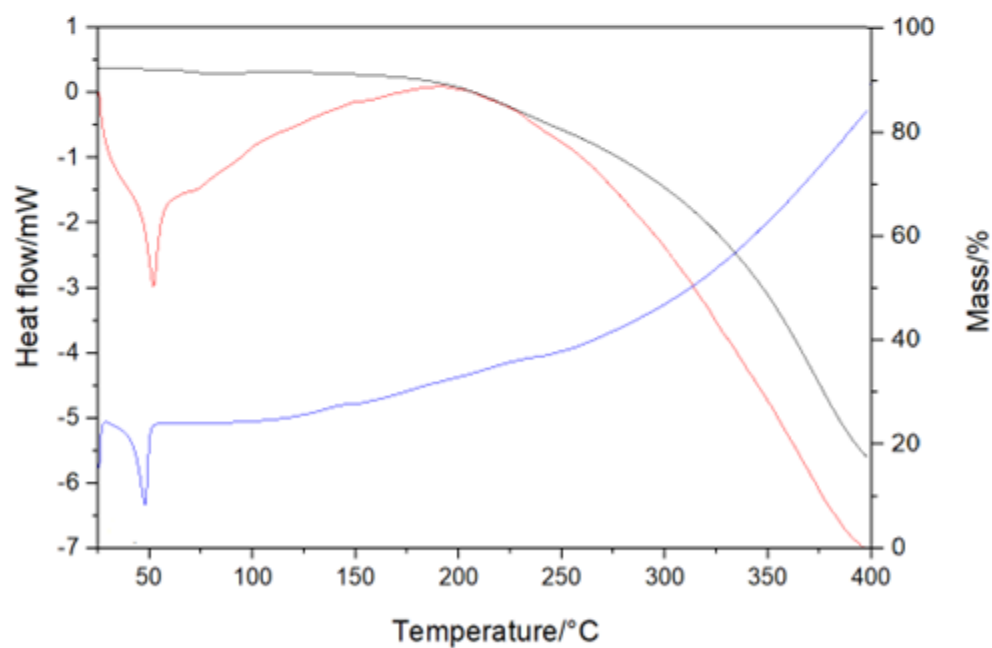


Figure 20. Thermal analysis of MSiNP (a) and MSiNP-DTC (b): thermogravimetry (TG) (%) (black line), differential thermal analysis (DTA) (red line), and differential scanning calorimetry (DSC) (blue line).



(a)

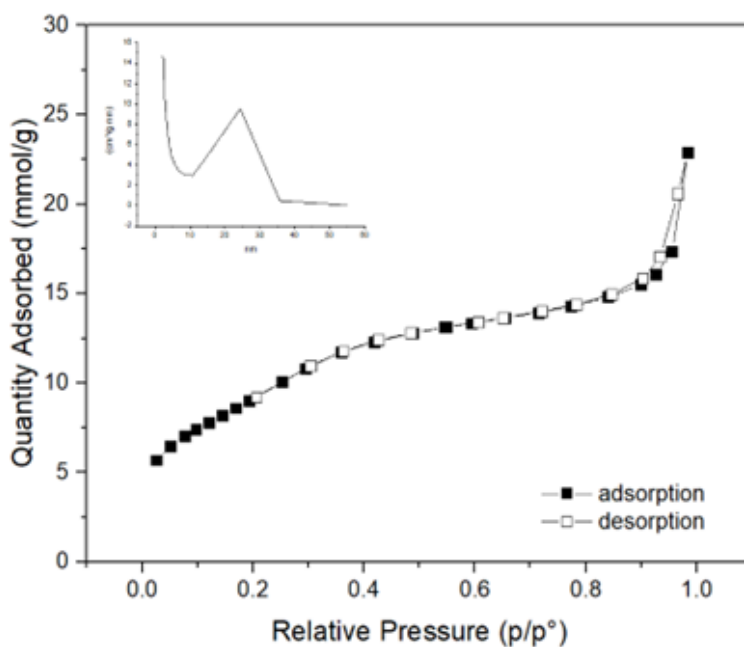


(b)

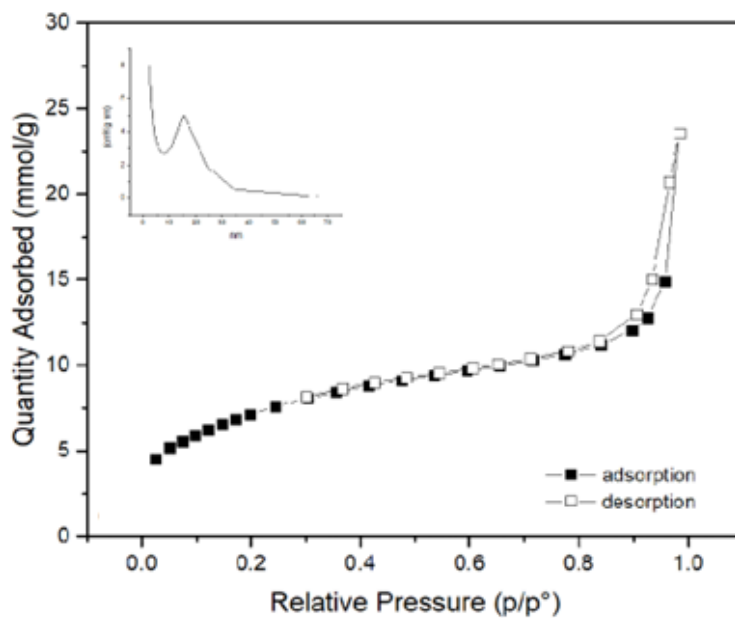
Figure 21. Thermal analysis of LN (a) and LN-DTC (b): thermogravimetry (TG) (%), differential thermal analysis (DTA) (red line), and differential scanning calorimetry (DSC) (blue line).

4.4.7 Nitrogen Adsorption

BET adsorption isotherms are routinely used to determine the specific surface areas of porous materials. The specific surface area is undoubtedly the most significant morphological characteristic of solid substances in applications related to porous structures, such as industrial adsorbents, catalysts, pigments, cement, polymers, among others [220,221]. The BET surface area and pore-size distribution were calculated by N₂ adsorption–desorption isotherms of the MSiNP and MSiNP-DTC, as reported in Figure 22 (a and b), which correspond to type IV with an H1 hysteresis loop according to the IUPAC, typical for materials containing mesoporous structures with cylindrical pores [222,223]. MSiNP exhibited a high BET surface area of $1021 \pm 15 \text{ m}^2 \text{ g}^{-1}$, a pore volume of $1.61 \pm 0.04 \text{ cm}^3 \text{ g}^{-1}$, and a mean pore size of $24.3 \pm 0.2 \text{ nm}$ all characteristics favorable for loading. For the MSiNP-DTC, the measured surface area and pore volume were estimated to be $618 \pm 15 \text{ m}^2 \text{ g}^{-1}$ and $1.01 \pm 0.01 \text{ cm}^3 \text{ g}^{-1}$, respectively, while the mean pore size calculated from the N₂ adsorption–desorption isotherm was $15.2 \pm 0.2 \text{ nm}$. Although such a change in the textural properties occurred after the DTC incorporation, the MSiNP-DTC still maintained the typical mesoporous structure during the immobilization process. The reductions in the surface area, volume, and mean pore size suggest that the DTC was present in the MSiNP matrix in various ways, such as adsorbed/adhered to the surface and inside the pore.



(a)



(b)

Figure 22. Nitrogen isotherms from MSiNP (a) and MSiNP-DTC (b) and plots of the normalized differential volume as a function of pore diameter as inset.

4.5 Evaluation of *in vitro* Antifungal Activity of DTC and Nanoparticles

The pathogenic disease caused by *M. perniciosa* which affects cocoa crops in Brazil is still a constant concern due to the difficulty in controlling the growth of the fungi leading to great economic losses for the producers. Currently, disease management is based on the application of biofungicide Tricovab®, containing *T. stromaticum* conidia as an active ingredient. Its mode of action is related to microparasitism, and therefore, treated plants are not protected against new infections [11,98]. Thus, considering the need for a new fungicide that acts effectively in the control of *M. perniciosa*, *in vitro* tests were performed with DTC and NPs.

As described in Materials and Methods, the effect evaluation of the samples on fungi growth was based on preliminary tests to select the effective concentrations to be tested in the contaminated cocoa fruits. In this context, from preliminary tests, a high growth inhibition of *M. perniciosa* (Mp4145), *P. theobromicola* (MCCS-MB-01982) and *P. palmivora* (MCCS-MB-01919) was observed after the fungi being exposed to DTC at 100 mg L⁻¹, LN-DTC at 200 mg DTC L⁻¹ and LN at 1600 mg SL⁻¹ (data not shown).

MSiNP and MSiNP-DTC exerted no toxic effects on *M. perniciosa*, *P. theobromicola* and *P. palmivora* in all concentrations tested (data not shown). Although several works have reported the effect of SiNP on different fungal pathogens, such as on *P. infestans*, *Phomopsis asparagi* (Sacc.) and *Aspergillus niger* [224,225] most of these studies do not evaluate the effect of unloaded SiNP, and thus, their effect as antimicrobial agents can come from their encapsulated AI or from the synergic interaction between NPs and AI. In addition, when the effect of SiNP is evaluated, the studies often do not relate the fungal inhibition with the physicochemical parameters of SiNP, such as particle size, surface area, morphology, and surface charge [225,226]. Possible explanations for the absence of the MSiNP effect on fungal inhibition can be attributed to NPs long carbon chain which can serve as a nutritional resource for fungi or can interact with other compounds present in the medium. Another factor is related to the negative surface charge of the synthesized MSiNP, since it was verified that NPs with positive charges can provide significant toxicity when compared to the anionic and/or neutral NPs [227]. Regarding the effect of MSiNP-DTC, the amount of DTC loaded may not have been sufficient to act efficiently for inhibiting the fungal growth, at least for *M. perniciosa*.

In the case of LN, although their composition can also serve as nutritional source for fungi, the toxicity depends on the nature and concentration of the lipids and of the surfactants [228]. In addition, the encapsulation of DTC in LN can also contribute to this reduction in fungal growth since DTC is totally dispersed in the lipid matrix, which does not occur in the case of MSiNP where DTC is adsorbed on the MSiNP surface.

Comparing the survival percentages of *M. perniciosa* when exposed to DMSO, DTC and LN and LN-DTC, in Figure 23 (a), we can see that DMSO did not induce a significant fungal inhibition compared with the control group. However, the results showed that DTC exerted a significant ($p < 0.05$) inhibition of 30.9% on the fungal growth *M. perniciosa* compared with control. It is known that dithiocarbazates have a wide spectrum of antimicrobial properties and that these compounds, such as DTC even belonging to the same class, can act in different ways in the biological target. Although there are no studies regarding the effect of DTC on *M. perniciosa*, the activity on different pathogens (bacteria and fungi) is well described for a wide range of dithiocarbazates, and even their main degradation product CS_2 tested against *M. perniciosa* presented inhibition of 61.7% with only one application of 50 μ L [229]. Thus, in the present work, the low percentage of inhibition attributed to DTC can be due to the method of application on the plates and/or conditioning of the plates.

LN and LN-DTC induced a significant growth inhibition of *M. perniciosa* of 33.9% and 35.2%, respectively, compared with the control to (Figure 23 (a), being relatively more efficient than the currently used biofungicide Tricovab®, whose inhibition does not exceed 30%, and part of this percentage of Tricovab® inhibition can be lost during the application, which is dependent on the season and the way it is applied in the cocoa plantation [230]. As these LN are composed of lipids, they have the ability to interact with various types of bacterial and fungal cells and can also facilitate the antimicrobial activity of DTC by improving the diffusion properties through biological membranes due to its nanosized and lipophilic nature [88].

Compared to *M. perniciosa*, the species *P. theobromicola* and *P. palmivora* colonize all parts of the cacao tree, causing black rot, stem canker and leaf blight in nurseries [231]. These infections in the fruit and leaves of cocoa act in a similar way influenced by the tropical climate of the region, by insects and harvesting tools that enhance to spread the sporangium infecting new plants for many years in a vicious and devastating cycle [232].

Figure 23 (b) shown that for *P. theobromicola* (MCCS-MB-01982) there were significant differences between DTC and LN, as well as between DTC and LN-DTC, indicating that for

P. theobromicola the use of DTC as a fungicide is more efficient, since DMSO does not inhibit fungal growth. The DTC inhibition percentage reached 42.3%, followed by LN-DTC with 36% and LN with only 29%. For *P. palmivora* (MCCS-MB-01919) (Figure 23 (c)), the results were 4.6% inhibition for DMSO, which was not significant compared to the control ($p>0.05$), followed by 42.5% for DTC, 43.3% LN and 47.1% LN-DTC, indicating that these results are more relevant in relation to those obtained for *M. pernicioso*. Considering that there are no data in the literature regarding the inhibition of *P. theobromicola* and *P. palmivora* caused by LN, and that there is no fungicide which can be applied to interrupt or reduce the impacts caused by such fungi, the effect evaluation of the compounds as possible fungicides is of extreme relevance for solving these problems.

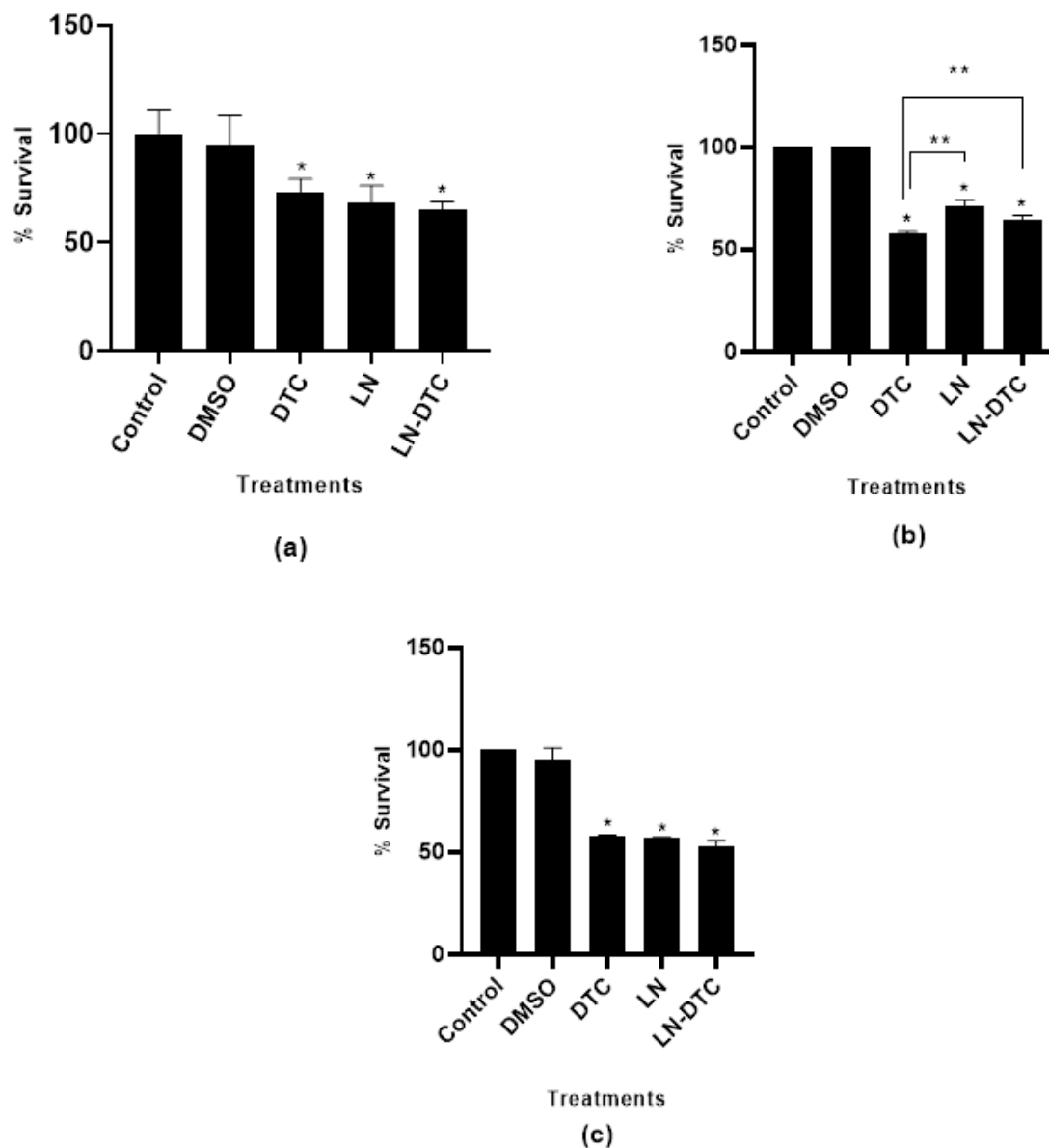


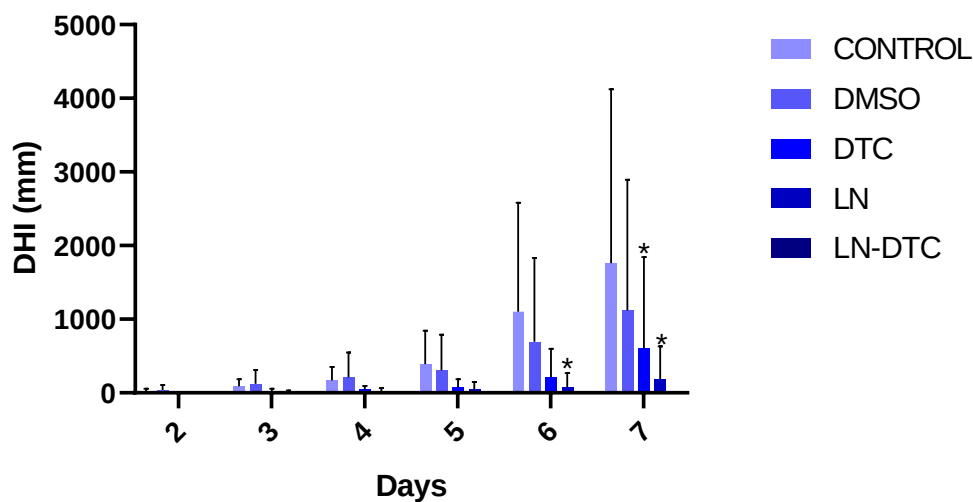
Figure 23. Mean Percentage (%) of survival for (a) *M. pernicioso* (UESC-Mp4145), (b) *P. theobromicola* (MCCS-MB-01982) and (c) *P. palmivora* (MCCS-MB-01919) after exposure to DMSO at 3% (v/v) (22 mg DMSO L⁻¹), DTC (100 mg L⁻¹) dispersed in the aqueous solution of DMSO at 3% (v/v), LN (1600 mg SL L⁻¹) and LN-DTC (200 mg DTC L⁻¹). *Corresponds to significant differences between the respective control and the treatments. Significant differences between DTC, unloaded LN and LN-DTC for the same period are represented by (**), (two-way ANOVA followed by Tukey's multiple comparisons test: $p < 0.05$). The control group is considered as 100%.

The inhibition growth of *P. theobromicola* (MCCS-MB-01960) and *P. palmivora* (MCCS-MB-01994) after exposure to samples was also evaluated on cocoa fruit. This test was only performed with DTC, LN and LN-DTC, since the results from *in vitro studies* with plates indicated no inhibitory effects of MSiNP/MSiNP-DTC on selected fungi species. Following the procedure defined by MARs Cocoa, the test with cocoa fruits was evaluated based on the measurement of the damage caused by the fungi, which must be minimized by adding the samples to the solution containing the fungi through the sporangium. Subsequently, the lesion on the fruits was measured every day during the 7 days of incubation. The results were expressed through a calculation based on the Diameter, Height, and Interaction of the lesion (DHI), in which the greater the resistance of the fungi to the applied fungicide, the greater the lesion caused on the fruit peel, which may affect the pulp and grains of the fruit cocoa, causing great losses in crops. Figure 24 ((a) and (b)) show the DHI obtained for *P. theobromicola* and *P. palmivora*, respectively, and as there was no growth of the lesions on the first day of incubation, the lesions were measured from the second day.

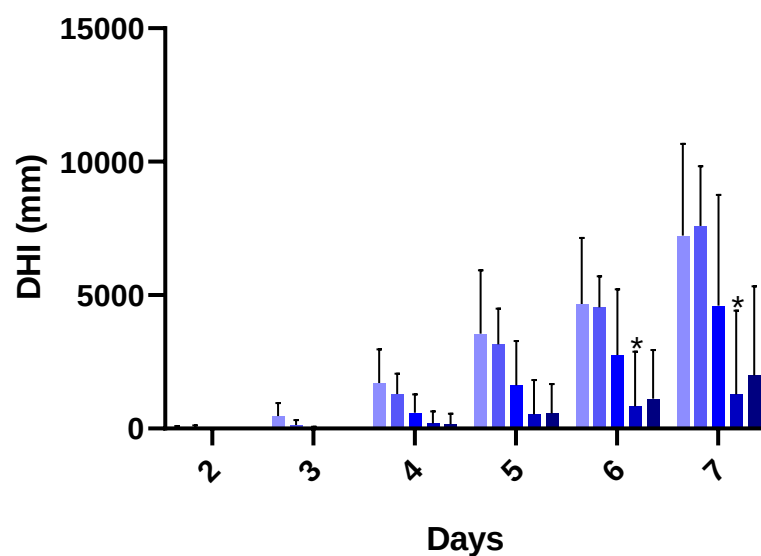
In correlation with the *in vitro* assays, for *P. theobromicola*, the application of DMSO in the fruit did not induce fungal inhibition compared with the control group (Figure 29 (a)), i.e., there is growth of lesions in all cocoa fruits exposed to DMSO. In this sense, the results indicate that DMSO does not interfere with the effects of DTC on fungal inhibition. The application of DTC and LN allowed the development of the lesions, and the fungal inhibition was significant only after 6 and 7 days of exposure ($p < 0.05$). On the other hand, the LN-DTC were the most efficient when applied to the fruit and it was verified that none of the fruits developed lesions, preventing the growth of the fungi until the seventh day of incubation in a controlled environment.

For the assay in contaminated fruits with *P. palmivora*, it was observed that the application of DMSO and LN-DTC did not significantly inhibit the development of lesions caused by the fungi compared with the control (Figure 24 (b)). Contrarily, LN significantly inhibited the fungal growth after 6 and 7 days of exposure, compared with the control group (Figure 29 (b)).

In this way, with the results of the test on the cocoa fruit, we observed that, in general, the application of LN-DTC as fungicides to inhibit the growth of the fungi *P. theobromicola* and *P. palmivora* were efficient since they significantly reduced the damage caused by these fungi compared to the control.



(a)



(b)

Figure 24. Evaluation of resistance cocoa pods (FB206) (measured as DHI of the lesions) against *P. theobromicola* (MCCS-MB-01960) (a) and *P. palmivora* (MCCS-MB-01994) (b) exposed to different treatments: control groups with water and DMSO at 3% (v/v) (22 mg DMSO L⁻¹), DTC (100 mg L⁻¹) dispersed in the aqueous solution of DMSO at 3% (v/v), LN (1600 mg SL L⁻¹) and LN-DTC (200 mg DTC L⁻¹). *Corresponds to significant differences between the respective control and the treatments. Significant differences between DTC, unloaded

LN and LN-DTC for the same period are represented by (**), (two-way ANOVA followed by Tukey's multiple comparisons test: $p < 0.05$).

4.6 Safety Evaluation of DTC and Nanoparticles in Human Cell Lines

As described previously in the present study, the conventional application of pesticides is not sustainable and can lead to hazardous effects to environment and human health. A particular group that can be exposed to excessive levels of pesticides and other toxic compounds existing in the formulations are those that need to manipulate the pesticide previously and during application. The routes of exposure to pesticides for the general populations (not only in agriculture practices but also in domestic applications) can be through ingestion of contaminated food with pesticide residues, inhalation or/and direct skin contact with pesticides during preparation and spraying [233]. As skin is the largest organ in the human body and is primary exposure route to pesticides the safety evaluation of synthesized NPs using two different human cells lines derived from skin (HaCaT and A431) was performed to mimic the possible human exposure pathway to pesticides.

HaCaT (normal keratinocytes), is a well-characterized and immortalized cell line able to proliferate and differentiate, and it has been shown to be a good model in toxicology. HaCaT cells show good predictive results and constitute an appropriate model for studying several pesticide effects, and A431 cells (epidermoid carcinoma) have also been widely used to assess pesticide toxicity [234], due to their sensitive to low concentrations ($\leq 1 \text{ ng mL}^{-1}$). In addition, A431 cells have been used as a model to evaluate the early events after the interaction of the epidermal growth factor (EGF) with its specific cell surface receptor (EGFR) [235].

Figure 25 and Figure 26 show the cell viability data for HaCaT and A431 incubated with DTC (a), MSiNP/MSiNP-DTC (b and c) and LN/LN-DTC (d and e) after 24h and 48h. In general, all samples tested were not toxic to both cell lines at tested concentrations (cell viability > 70%) [236].

According to results, DTC significantly induced HaCaT cell viability after 48h of exposure at concentrations $\geq 5 \text{ mg DTC L}^{-1}$ ($p < 0.05$) compared with the control group (Figure 25 (a)). The same pattern was observed for LN and LN-DTC, which both formulations stimulated significantly compared with the control, the HaCaT cell viability in a dose-dependent manner after 24 and 48h of exposure (Figure 25 (d and e)). However, the viability of HaCaT cells was not significantly affected by MSiNP and MSiNP-DTC in the range of concentrations tested (Figure 25 (b and c)).

Although all samples did not induce toxicity on A431 cells, after exposure to DTC, the cell viability was decreased significantly compared with the control, especially for the highest concentrations at both exposure times (Figure 26 (a)).

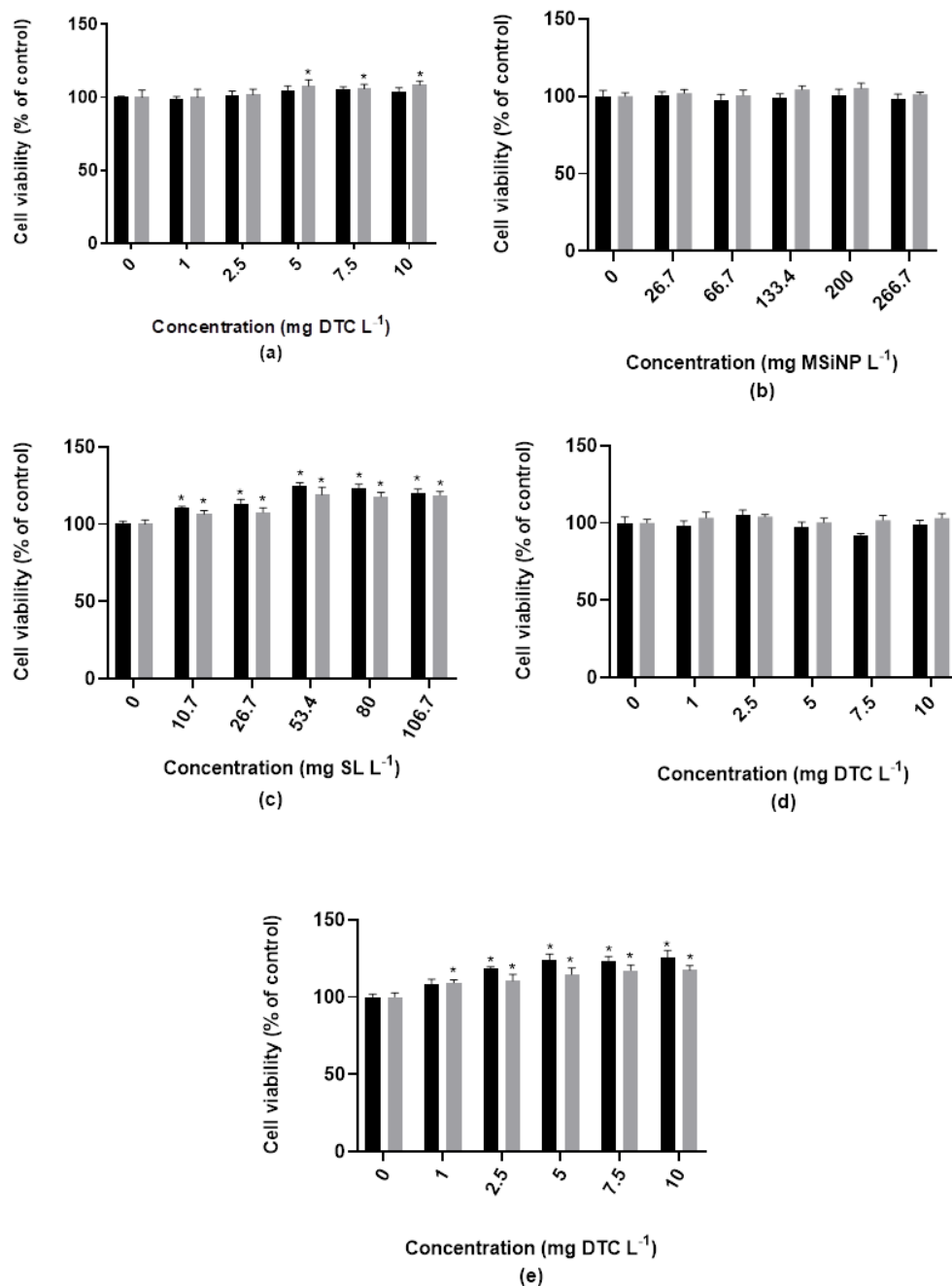


Figure 25. Viability of HaCaT cells after 24h (black bar) and 48h (gray bar) of exposure to DTC suspended in 0.5% (v/v) DMSO (a), MSiNP (b), MSiNP-DTC (c) LN (d) and LN-DTC (e). In the case of LN, the concentrations indicated are of solid lipid (SL) used in the synthesis of LN. The same concentrations of SL were tested for LN-DTC. In the case of MSiNP, the same concentrations of MSiNP were tested for MSiNP-DTC. Cell viability is expressed as % of control. Asterisks indicate significant differences in cell viability compared with the control group (non-exposed group) (ANOVA followed by Dunnett's test, $p < 0.05$).

Unloaded MSiNP and loaded MSiNP with DTC were not toxic to A431 cells at all concentrations tested after 24 and 48h of exposure (Figure 26 (b and c)) and it appears that MSiNP could protect DTC from degradation or even reduce its toxicity due to its incorporation into MSiNP structure.

Regarding LN and LN-DTC effects on A431 cell viability, the results were similar to those obtained for HaCaT cells, which LN and LN-DTC significantly stimulated A431 cell viability compared with the control group after both exposure times.

LN have been widely explored as systems for dermal delivery, due to their film-forming characteristics, skin hydration, controlled occlusion, increased skin bioavailability and physical stability [237]. Although, it is expected that the LN are not toxic to skin cells, their effect is dependent on the particle composition, as well as their concentration in the medium in addition to the loaded active ingredient.

As mentioned, the toxicity of LN on human cells is related to the particle size as well as its composition, for example the type of surfactant. The type of surfactant may lead to cell membrane compromise resulting in cell apoptosis/necrosis, however this concrete biochemical process is yet to be elucidated [238]. Despite the significant effects from the LN with and without DTC, it is observed that both for A431 and mainly for HaCaT these effects are of increased cellular activity compared to the control. The composition of the cell membrane of the skin is basically composed of phospholipids, which is part of the composition of the surfactant soy lecithin. The surfactant used, although non-toxic themselves, could even improve their tolerability when bound to LN. Binding of surfactant to LN surface decreases its availability to interact with cells and thus decreases its toxicity [236]. as the LN are dispersed in an aqueous medium, they probably remain in their entirety in the culture medium and can serve as a nutrient for cell proliferation and thus increase the cellular activity of HaCaT and A431. Since DTC is solubilized in the LN matrix, we can also infer that there is no interaction between the DTC and the cell membrane and, consequently, the nanoencapsulation of DTC in LN also increases cell activity, thus reducing the impacts that the DTC can cause when in contact with skin cells.

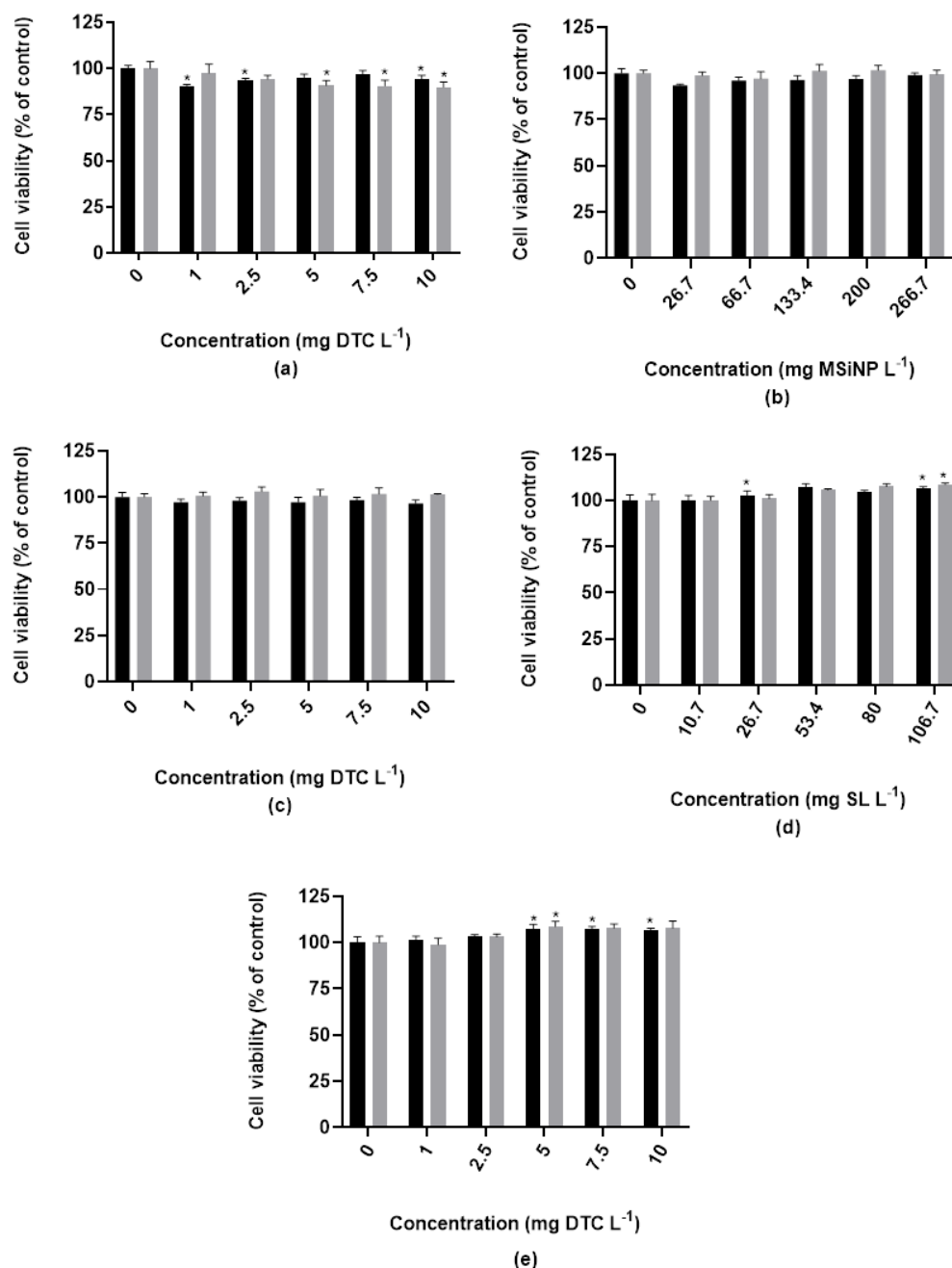


Figure 26. Viability of A431 cells after 24h (black bar) and 48h (gray bar) of exposure to DTC suspended in 0.5% (v/v) DMSO (a), MSiNP (b), MSiNP-DTC (c), LN (d) and LN-DTC (e). In the case of LN, the concentrations indicated are of solid lipid (SL) used in the synthesis of LN. The same concentrations of SL were tested for LN-DTC. In the case of MSiNP, the same concentrations of MSiNP were tested for MSiNP-DTC. Cell viability is expressed as % of control. Asterisks indicate significant differences in cell viability compared with the control group (non-exposed group) (ANOVA followed by Dunnett's test, p<0.05).

4.7 Environmental Safety Evaluation of DTC and Nanoparticles on Aquatic Organisms

4.7.1 Average Particle Size, Polydispersity Index, and Zeta Potential Analysis in Different Culture Media

As DTC is not soluble in aqueous media, an aqueous solution of DMSO at 0.5% (v/v) was used as a solubilizing agent for the DTC. DMSO in an aqueous solution was previously tested on *A. fischeri* at 5% (v/v) [239] and on *R. subcapitata* at 1% (v/v), showing no toxicity for both species [146]. Therefore, based on this consideration, in the present work, the effect of DMSO at 0.5% (v/v) was only tested on *L. minor*. In general, there is a relationship between particle size and its toxicity, as it is associated that smaller particles can easily penetrate cell membranes and thus induce higher cytotoxicity [240]. The particle aggregation/agglomeration state can also influence the toxicity of NPs and depends on the particle concentration, pH, ionic strength, ionic composition and concentration, and other characteristics of the aqueous medium in which the NPs were dispersed [241]. According to Table 8, the most notable increase in size was recorded for MSiNP-DTC in Steinberg medium (pH= 7.5 ± 0.1) and can be attributed to the possible higher ionic strength of this medium, which reduced the ZP (in modulus) of NPs. Another fact may be related to the modification of the silica surface with DTC, which led to the formation of large agglomerates in alkaline solution in Steinberg medium, which were almost twice as large as in neutral and acidic solutions. This may be related to the dissociation of hydrogen bonds on the surface of MSiNP and delocalization of electron pairs π (pi) of the C-N and C-S bonds of the DTC, leading to a strong interaction between the MSiNP and the DTC and resulting in an increase in strength ionic [242,243].

The results obtained for LN resuspended in ultrapure water indicated a reduction in Z-Ave and PDI and an increase in ZP, resulting in the non-agglomeration of the particles, which allows inferring that after lyophilization, the LN remain stable maintaining their initial characteristics. On the contrary, when resuspended in culture media, it clearly showed the formation of particle agglomerates with some reduction in ZP when in Steinberg medium.

Table 8. Average hydrodynamic size $\pm$ standard deviation (Z-Ave $\pm$ S.D.), polydispersity index (PDI $\pm$ S.D.), and average zeta potential (ZP $\pm$ S.D.) recorded for MSiNP/MSiNP-DTC and LN/LN-DTC dispersed at 200 mg L⁻¹ in different assays' culture media.

SAMPLES	MEDIA	Z-AVE $\pm$ S.D. (nm)	PDI $\pm$ S.D.	ZP $\pm$ S.D. (mV)
MSiNP	MBL	186 $\pm$ 17	0.39 $\pm$ 0.03	-30.0 $\pm$ 0.3
	Steinberg	189 $\pm$ 6	0.45 $\pm$ 0.03	-10.0 $\pm$ 0.4
MSiNP-DTC	MBL	200 $\pm$ 9	0.41 $\pm$ 0.04	-29.0 $\pm$ 0.4
	Steinberg	282 $\pm$ 31	0.47 $\pm$ 0.05	-11.0 $\pm$ 0.4
LN	MBL	166 $\pm$ 4	0.61 $\pm$ 0.06	-40.0 $\pm$ 0.7
	Steinberg	111.0 $\pm$ 0.2	0.27 $\pm$ 0.01	-25.0 $\pm$ 0.6
LN-DTC	MBL	156 $\pm$ 4	0.52 $\pm$ 0.02	-43.0 $\pm$ 0.1
	Steinberg	160.0 $\pm$ 0.9	0.31 $\pm$ 0.02	-25.0 $\pm$ 0.9

4.7.2 Microtox test: *A. fischeri*

Table 9 shows the % of highest effect and EC_x values for *A. fischeri* after 5, 15 and 30 min of exposure. The concentration of the dispersions tested for DTC, MSiNP/MSiNP-DTC and LN/LN-DTC was 150 mg L⁻¹, which was then tested for different dilutions separated by a factor of two, according to the protocol of the basic test [244].

As DTC has low solubility in aqueous media (solubility in water=0.08 mg L⁻¹ at room temperature), this compound was dispersed in an aqueous solution of DMSO at 0.5% (v/v) to proceed with the serial dilutions from 81.9 to 0.32 (dilution factor of 2). As previously mentioned, DMSO at 5% (v/v) was not toxic for *A. fischeri* [239], and thus, DMSO at 5% (v/v) was not tested in the present work. As shown in Table 9, DTC up to 30 min of exposure was not toxic (highest effect: h.e. 2.6%) to *A. fischeri*, which we can infer that there was no degradation of DTC, that is, there was no release of one of its by-products, sulfur dioxide (CS₂) considered highly toxic to marine life [245].

The toxicity of MSiNP-DTC increased when compared to the non-loaded MSiNP. However, the effect recorded was slightly above the 20% of bioluminescence inhibition after 30 min of exposure. As there was no change between MSiNP and MSiNP-DTC regarding size and charge, the fact that MSiNP-DTC is more toxic is due to the synergistic interaction between DTC and the particle, which may be responsible for a higher interaction of the NPs with the biological membranes, at least for the bacteria *A. fischeri*.

No information in the literature was found regarding the toxicity of LN, and compared to the organic compound DTC, the lipid matrix is much more toxic, being LN more toxic after 30 min of exposure ($EC_{50}=92.6 \text{ mg L}^{-1}$) than LN-DTC ($EC_{50}=104.9 \text{ mg L}^{-1}$). This can be explained by the fact that long alkyl chains are incorporated into the phospholipid bilayer of bacterial membranes, leading to nonspecific disturbances of structural functioning, thus increasing their toxicity [143]. In the case of LN-DTC, the LN matrix may favor the protection of the DTC, preventing its degradation in the culture medium, leading to low toxicity for *A. fischeri*.

Table 9. *A. fischeri* toxicity results obtained from bioluminescence test (in mg L⁻¹) after exposure to DTC, MSiNP/MSiNP-DTC and LN/LN-DTC at 5, 15 and 30 min of exposure (95% confidence interval, inside parenthesis). The maximum tested concentration of DTC and NPs was 150 mg L⁻¹. Data are Highest Effect (%) expressed in bioluminescence inhibition percentage and EC₅₀/EC₂₀ values.

SAMPLES	EC ₅₀ /(mg L ⁻¹)			EC ₂₀ /(mg L ⁻¹)		
	5 min	15 min	30 min	5 min	15 min	30 min
DTC	NC (h.e. 6.2%)	NC (h.e. 6.5%)	NC (h.e. 2.6%)	NC (h.e. 6.2%)	NC (h.e. 6.5%)	NC (h.e. 2.6%)
MSiNP	NC (h.e.17.4%)	NT	NC (h.e. 8.5%)	NC (h.e. 17.4%)	NC (h.e. 13.4%)	NC (h.e. 8.5%)
MSiNP-DTC	NC (h.e. 11.5%)	NC (h.e. 8.7%)	NC (h.e. 13.9%)	15.3 (0.03 - >1000)	NC (h.e. 8.7%)	114 (1.3 - >1000)
LN	83.1 (35.2 - 196.3)	92.7 (34.7 - 247.4)	92.6 (33.0 - 259.6)	20.5 (10.9 - 38.6)	21.8 (11 - 43.2)	26.7 (12.2 - 58.7)
LN-DTC	99.8 (58.6 - 170.1)	113 (41 - 311.8)	104.9 (56.7 - 194.3)	28.3 (17.1 - 46.8)	17.7 (8.9 - 34.9)	30.5 (17.6 - 52.6)

Note. EC₅₀/EC₂₀: effect concentrations causing 50 or 20% of bioluminescence inhibition, respectively; NC: could not be calculated; NT: not toxic; h.e.: highest effect.

The concentrations of DTC in the NPs were 0.0, 0.42, 0.63, 0.95, 1.42, 2.1, 3.2, 4.8 and 7.2 mg L⁻¹ for MSiNP-DTC and 0.0, 0.17, 0.26, 0.38, 0.57, 0.86, 1.3, 1.9 and 2.9 mg L⁻¹ for LN-DTC.

4.7.3 *R. subcapitata* growth inhibition test

Algae are at the base of the aquatic food chain and are important because any adverse effect of NMs in their environment can have repercussions on the entire aquatic ecosystem. Therefore, in recent decades there has been substantial research into the impacts of NMs on algae and their environment as it is essential to determine whether there are influences of NMs on the inhibition of algae growth subsequent toxicity [246]. Toxicity in algae is also influenced by the inherent properties of NPs, such as their size, shape, concentration and their coating layers, and these properties can lead to defensive behaviors of algae through stimulation of the antioxidant system to remove ROS induced by NPs [247].

Some authors proposed a classification based on EC_{50} values to classify the effect of substances on microalgae and called it the Passino and Smith Classification (PSC). Therefore, NPs are classified as very toxic ($EC_{50} < 1 \text{ mg L}^{-1}$); toxic ($1\text{--}10 \text{ mg L}^{-1}$), harmful ($10\text{--}100 \text{ mg L}^{-1}$), or not harmful ($>100 \text{ mg L}^{-1}$) [248]. The test results with the algae *R. subcapitata* are shown in Figure 27 with the respective EC_{50} and EC_{20} in Table 10.

As depicted in Figure 27 (a), for DTC ($EC_{20}=120 \text{ mg L}^{-1}$, showing in Table 10), significant growth inhibition was recorded for almost all concentrations tested up to a maximum of 29.6% inhibition in growth rate of algae in the highest concentration (150 mg L^{-1}) when compared to the control. As it is an organic compound with an intense yellow color, the high concentration of DTC in the suspension probably indirectly caused the unavailability of light crucial for algae growth. According to PSC classification, MSiNP proved to be non-toxic to *R. subcapitata* (Figure 27 (b)), with only 3.2% inhibition of algae growth rate at the highest concentration tested, while MSiNP-DTC was slightly toxic (13.5%), but compared to pure DTC, MSiNP manages to protect DTC from degradation in the culture medium, reducing its toxicity, so this toxicity may come from the size of MSiNP-DTC ($200 \pm 9 \text{ nm}$) which despite not being small enough to cross the membrane, can agglomerate/aggregate in the aqueous medium reducing the nutrition of the algae.

Regarding to LN data, the growth curves showed that for unloaded LN ($EC_{20}=144 \text{ mg L}^{-1}$, Table 9) a significant difference was observed in the growth rate of algae compared with the control group at the concentration of 150 mg L^{-1} , where growth inhibition was 31.8% and for LN-DTC ($EC_{20}=92 \text{ mg L}^{-1}$, Table 10) was 30%. The effect on algae in the highest concentration tested for LN may be related to the high particle agglomeration of LN in the MBL culture medium ($PDI > 0.3$), leading to NPs deposition on the algal cell membrane, and

preventing the algal growth by inhibiting necessary nutrients, especially after exposure to unloaded LN [249].

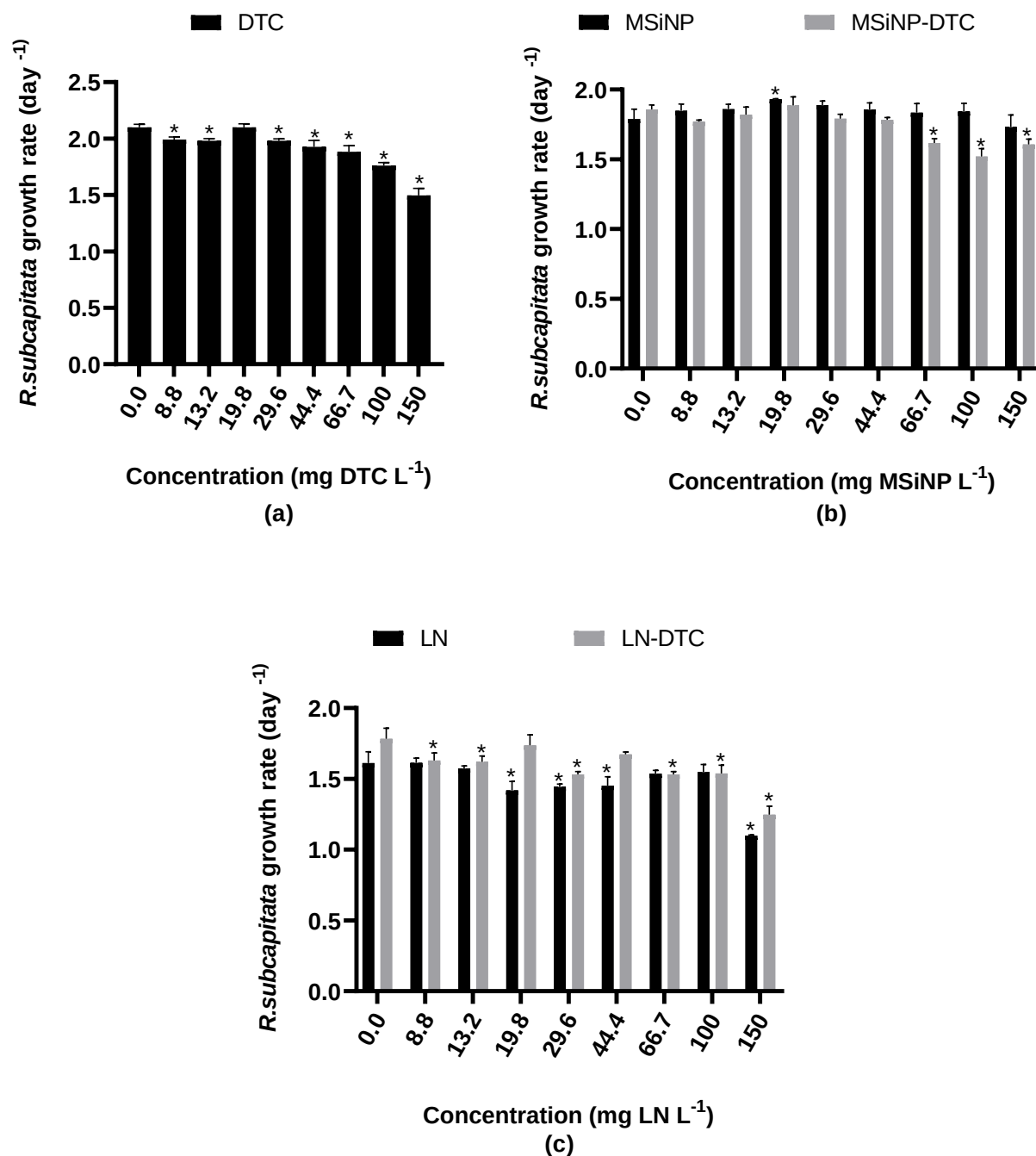


Figure 27. Average *R. subcapitata* growth rate after 72h of exposure to DTC (a), MSiNP/MSiNP-DTC (b) and LN/LN-DTC (c). Error bars represent the standard deviation, and *corresponds to significant differences between the respective control and the treatments (Dunnett's test: $p < 0.05$). The concentrations of DTC in the NPs were 0.0, 0.42, 0.63, 0.95, 1.42, 2.1, 3.2, 4.8 and 7.2 mg L⁻¹ for MSiNP-DTC and 0.0, 0.17, 0.26, 0.38, 0.57, 0.86, 1.3, 1.9 and 2.9 mg L⁻¹ for LN-DTC.

4.7.4 *L. minor* growth inhibition test

L. minor is of great ecological importance and economic value, as they are producers and food source for fish, invertebrates, and waterfowl [250]. The effects of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC on *L. minor* growth assessed by the number of fronds and dry weight after 7 days of exposure are shown in Figure 28 and Table 10. In Figure 28 (a) and (b) we see that at 150 mg L⁻¹ for both DTC and MSiNP, there were significant effects on *L. minor*. The effect for the DTC is in the dry weight with 32.1% of inhibition, while for the number of fronds the effect was 17.5%, considered as non-toxic. As DTC was dissolved in DMSO 0.5% (v/v), the influence of DMSO on toxicity to *L. minor* was also tested. DMSO has a significant effect on *L. minor* growth that contributes to the inhibitory effect caused by DTC. In addition, some morphological differences in the fronds compared with the control group were noted after exposure to DMSO. Regarding MSiNP the inhibition effect at 150 mg L⁻¹ was greater on the dry weight (24.4%) for the MSiNP than on the number of fronds (18.6%), while for the MSiNP-DTC, the opposite was observed, with 22% and 35.2%, respectively. Although this change in toxicity is not remarkable, agglomeration/aggregation of MSiNP in Steinberg medium may influence this effect mainly after loading with DTC. As can be seen in Table 8, there was a considerable reduction in ZP values of MSiNP in Steinberg medium when compared with MSiNP dispersed in water, followed by an increase in Z-Ave values especially for MSiNP-DTC. Aggregation may have caused the superficial attachment of the particles to plant cell walls with strong adhesion, preventing *L. minor* from getting enough food and nutrition.

Evaluating the impacts of LN and LN-DTC (Figure 28 (d) and (e)), the obtained data showed significant effects on *L. minor* from 150 mg L⁻¹, being greater on the frond number (32.6%) for LN than on dry weight (20%), while for LN-DTC, the % of inhibition was 22% and 18%, for frond number and for dry weight, respectively. LN are well known in the pharmaceutical field for having several advantages in drug delivery due to their encapsulation stability. However, few studies report the application of LN for pesticide encapsulation [251]. Since no changes in the particle size of LN were observed after their dispersion in Steinberg medium as shown in Table 8, a possible explanation for the inhibition of *L. minor* growth caused by LN may be related to the matrix composition itself. The physical state of the NMs lipid matrix has a significant effect on its penetration into plant roots and on its further transport to the stem [252].

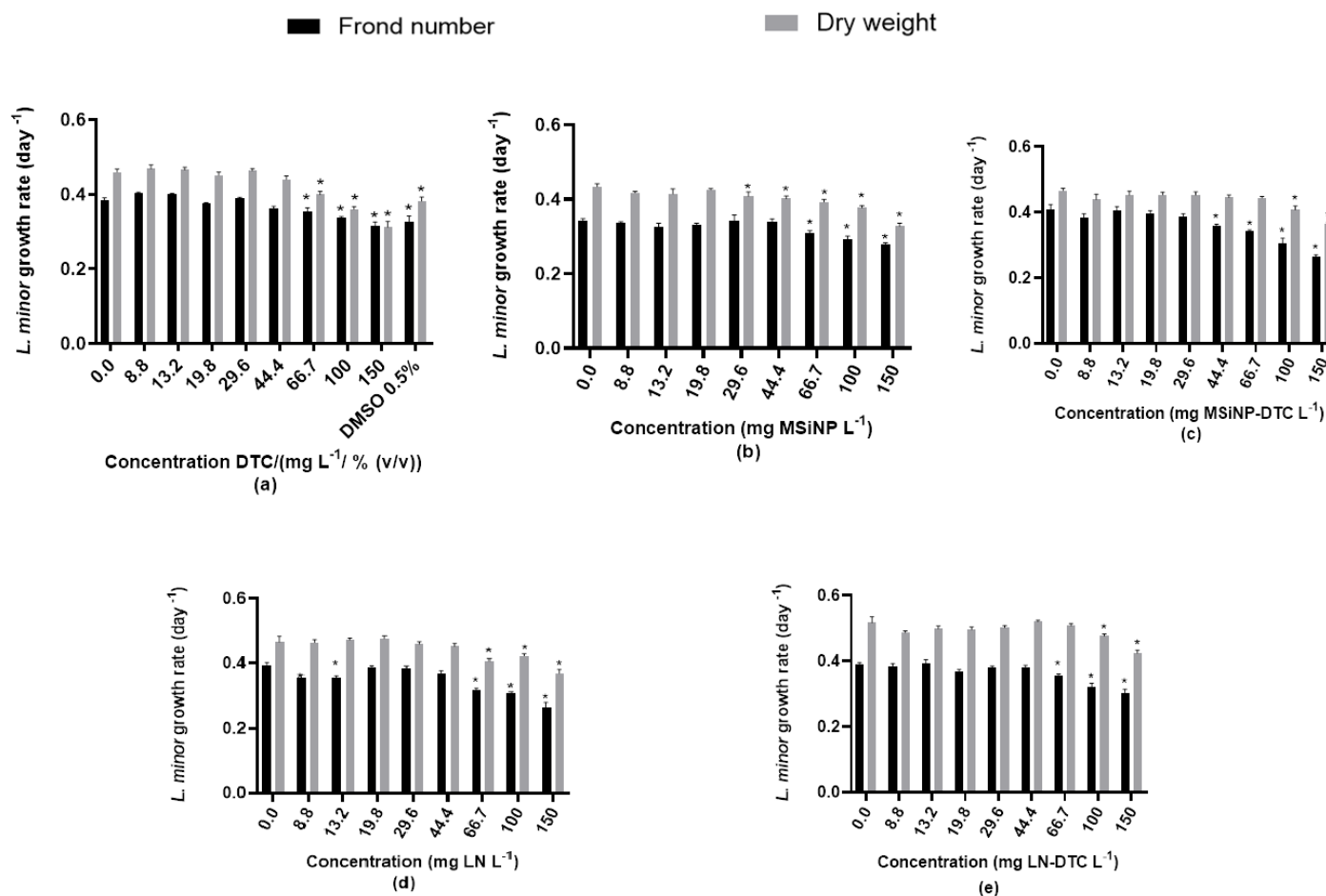


Figure 28. Average growth rate (day⁻¹) calculated based on frond's number (black bars) and on frond's dry weight (grey bars) of *L. minor* exposed to DTC and DMSO 0.5% (v/v) (a), MSiNP (b), MSiNP-DTC (c), LN (d) and LN-DTC (e). Error bars represent the standard deviation, and * corresponds to significant differences from the control (Dunnett's test: $p < 0.05$). The concentrations of DTC in the NPs were 0.0, 0.42, 0.63, 0.95, 1.42, 2.1, 3.2, 4.8 and 7.2 mg L⁻¹ for MSiNP-DTC and 0.0, 0.17, 0.26, 0.38, 0.57, 0.86, 1.3, 1.9 and 2.9 mg L⁻¹ for LN-DTC.

Table 10. Effective concentrations (EC_{50}/EC_{20}), LOEC and NOEC in $mg\ L^{-1}$ of DTC, MSiNP/MSiNP-DTC and LN/LN-DTC for the microalgae *R. subcapitata* after 72 h of exposure and for *L. minor* after 7 days of exposure (CI means the lower and upper 95% confidence interval, inside parenthesis). Maximum tested concentration of DTC and NPs was $150\ mg\ L^{-1}$.

Biota	Endpoint	Samples	$EC_{50}/mg\ L^{-1}$	$EC_{20}/mg\ L^{-1}$	LOEC/ $mg\ L^{-1}$	NOEC/ $mg\ L^{-1}$
<i>R. subcapitata</i>	Growth rate	DTC		120 (106-133)	8.8	19.8
		MSiNP		>150.0	19.8	8.8
		MSiNP-DTC	>150.0	>150.0	66.7	44.4
		LN		144 (>1000)	19.8	13.2
		LN-DTC		92 (64 - 120)	8.8	-
<i>L. minor</i>	Growth rate (Frond Number)	DTC		>150.0	66.7	44.4
		MSiNP		>150.0	66.7	44.4
		MSiNP-DTC	>150.0	81 (66 - 97)	8.8	-
		LN		57 (37 - 77)	8.8	-
		LN-DTC		71.6 (52 - 91)	66.7	44.4
<i>L. minor</i>	Growth rate (Dry Weight)	DTC		114.6 (95 -134)	66.7	44.4
		MSiNP		138 (119 -157)	29.6	19.8
		MSiNP-DTC	>150.0	148 (133- 163)	100	66.7
		LN		79 (55 -104)	66.7	44.4
		LN-DTC		127 (111 - 143)	100	66.7

Note. EC_{50}/EC_{20} -values: effect concentrations causing 50 or 20% of growth inhibition, respectively; LOEC: Low observed effect concentration; NOEC: No-observed effect concentration. The concentrations of DTC in the NPs were 0.0, 0.42, 0.63, 0.95, 1.42, 2.1, 3.2, 4.8 and $7.2\ mg\ L^{-1}$ for MSiNP-DTC and 0.0, 0.17, 0.26, 0.38, 0.57, 0.86, 1.3, 1.9 and $2.9\ mg\ L^{-1}$ for LN-DTC.

4.8 Environmental Safety Evaluation of DTC and Nanoparticles on Soil Microbial Community

Soil microorganisms play an important role in the environment through the degradation of plant and animal residues and participate in many biological processes in the soil, including nitrogen transformation, organic matter decomposition and release of nutrients and their availability, being essential for the global nutrient cycling, which is directly related to soil fertility and health. Changes in soil microorganism populations or in biotic activity can precede detectable alterations in soil physical and chemical properties, thus providing an early signal of improvement or an early warning of soil degradation [253,254].

Due to the increasing use of NMs for pesticide applications, some studies have reported that the soil microbial community can be significantly compromised after exposure to NMs, and most of these studies have been conducted to understand the impact on the soil microbial community of NMs, especially those based on metals or metal oxides [255,256] and polymeric NMs [137]. Consequently, the monitoring of microbial activity and biomass can explain the toxic effects of NMs on soil enzyme activity, and the subsequent effect of enzyme inhibition on soil quality [257].

Therefore, in the present study, the effect of the formulations was also evaluated on soil microbial parameters through the variation of enzymatic activities and nitrogen mineralization. Regarding studies of soil biochemical parameters, the present work is the first study to collect data on the effect of DTC, LN and LN-DTC, MSiNP and MSiNP-DTC were not evaluated in this trial, since there was no inhibition on *M. perniciosus*, *P. theobromicola* and *P. palmivora*.

Dehydrogenase enzyme is an indicator of soil quality and microbial activity and is also the most frequently used to determine the influence of various pollutants, such as pesticides or excessive fertilization, on the microbiological quality of soils. As they are exclusively intracellular enzymes, they are involved in cellular respiration and play an important role in the early stages of oxidation of soil organic matter [258,259].

According to the results obtained for dehydrogenase activity, after 48h of exposure, all samples significantly increased the enzyme activity at highest concentrations compared with the control group (Figure 29, Table 11). However, after 15 days of exposure, all samples increased the dehydrogenase activity compared with the control, being more noticeable for

LN-DTC and LN (Figure 29 (c and d)). The effect of a pesticide on soil microorganisms is governed not only by the intrinsic chemical and physical properties of the pesticides, but also by soil type, soil properties and prevailing environmental conditions [164]. Thus, the results indicated that the incubation time favored the activity of dehydrogenases in soil containing DMSO, DTC, LN and LN-DTC, since over time the increase in dehydrogenase activity can be directly associated to the increase in cellular respiration, and this increase is related to the decomposition of organic matter presented in the soil.

Regarding the arylsulfatase activity, after 48 hours of incubation, DTC (Figure 30 (a), Table 12) and DMSO (Figure 30 (b)) did not significantly affect the enzyme activity. However, there was an increase in the enzyme activity for LN and LN-DTC (Figure 30 (c and d)) at lower concentrations.

Arylsulfatase plays an important role in sulfur cycle by catalyzing the hydrolysis of sulfate esters and can constitute a stage limiting factor in the cycling process [260]. Thus, when there is an increase in arylsulfatase activity, there is a decrease in sulfur in the soil and this can be affected by the presence of contaminants, changes in pH and organic matter content [261,262]. As DMSO and DTC present sulfur atoms in their structure, it was assumed that there were no alterations in the sulfur cycle by these components, which can be observed for soils incubated during 48 hours, while after 15 days of incubation, there is a significant reduction of the arylsulfatase activity, compared to control which, can be associated with an increase in the amount of sulfur in the soil.

For LN, the results indicated that there was a significant increase of arylsulfatase activity at concentrations up to 13.3 mg SL kg⁻¹ soil as showing in Figure 30 (d) compared with the control, after 48h of incubation, while LN-DTC increased significantly the activity compared with the control at concentrations up to 1.7 mg DTC Kg⁻¹ soil corresponding to 53.3 mg SL kg⁻¹ soil, for the same period of incubation as showing in Figure 30 (c). However, after 15 days of incubation, LN and LN-DTC significantly increased arylsulfatase activity at the highest concentrations as can be observed in Figure 30 (a) and (b). These results can be attributed to the lower concentrations levels of sulfur in soil in comparison with soils containing DTC and DMSO presented in Figure 30 (a and b), and/or to the encapsulation of DTC into LN, which can confer a higher protection of DTC can be from degradation or can release the DTC more slowly into the soil.

Acid phosphatase is an enzyme related to the transformation of organic phosphorus into inorganic forms suitable for plants [263] and is usually produced in the soil when there is a

decrease in the concentration of phosphorus, and in alkaline soils where there is a greater availability of this element [175,264].

Regarding acid phosphatase activity, the results shown in Figure 31 and Table 11 indicate that all samples did not significantly affect the enzyme activity at both exposure time, except for LN at concentrations of 266.7 mg SL Kg⁻¹ soil after 48h of incubation (Figure 31 (d)).

The results obtained from nitrogen mineralization are shown in Figure 32 and Table 11. Mineralization of organic nitrogen (N) is a biological process [265], that is affected by many factors such as temperature, moisture, and quality of residues or their chemical composition, which is determined by the carbon to nitrogen ratio (C:N) as well as the concentration of simples and complex compounds [266]. In soils, N deposition causes acidification that might affect mineralization processes and nutrient availability, a decrease in soil respiration and changes in the structure of the microbial community [267]. After 48h of incubation, there was significant inhibition of nitrogen mineralization for DMSO and LN-DTC, especially at highest concentrations (Figure 32 (b) and (c)), while after 15 days of incubation, a significant increase of nitrogen mineralization was observed at highest concentrations for LN and LN-DTC. This stimulus may be related to the N atoms present in its structure, since LN were synthesized using soy lecithin which is rich in nitrogen and can be used as nitrogen organic source.

The evaluation of the effect of LN and LN-DTC on soil biochemical parameters showed, in general, that these nanoparticles can be considered safe for soil microbiota. In the present study, LN and LN-DTC can serve as organic source, mainly for soils after 15 days of incubation, leading to the stimulation of the activity of certain enzymes, especially the activity of dehydrogenase, as well as the enhancement of nitrogen mineralization, which are important indicators of soil quality when exposure to chemical compounds.

To the best of our knowledge, there is only one study that reports the effect of LN on soil microbiota community, indicating that despite the concentrations tested of LN to be higher than those tested in our work (660 g LS ha⁻¹ for LN) and time incubation of 15 days for the same type of soil, LN affected especially the metabolism of nitrogen, sulfur and phosphorus, and these effects seem to result from the composition and concentrations of LN constituents. As a comparison effect for dehydrogenase, there was a significant inhibition effect when increasing the concentration of LN, in which the author associates the occlusion of soil pores by the aggregation of NPs, resulting in a reduction in soil oxygenation, decreasing the microbial population. For arylsulfatase there was no significant change and in nitrogen

mineralization in agreement with our results there is a significant increase in enzyme activity. However, for acid phosphatase there is a very significant inhibition compared to our results, in which it is associated with an increase in phosphorus in the soil due to a higher concentration of soy lecithin in the synthesis of LN (Ganilho *et al.*, 2022). Despite some differences in results, it was also agreed that the effects of LN on soil do not indicate a negative impact on terrestrial biota.

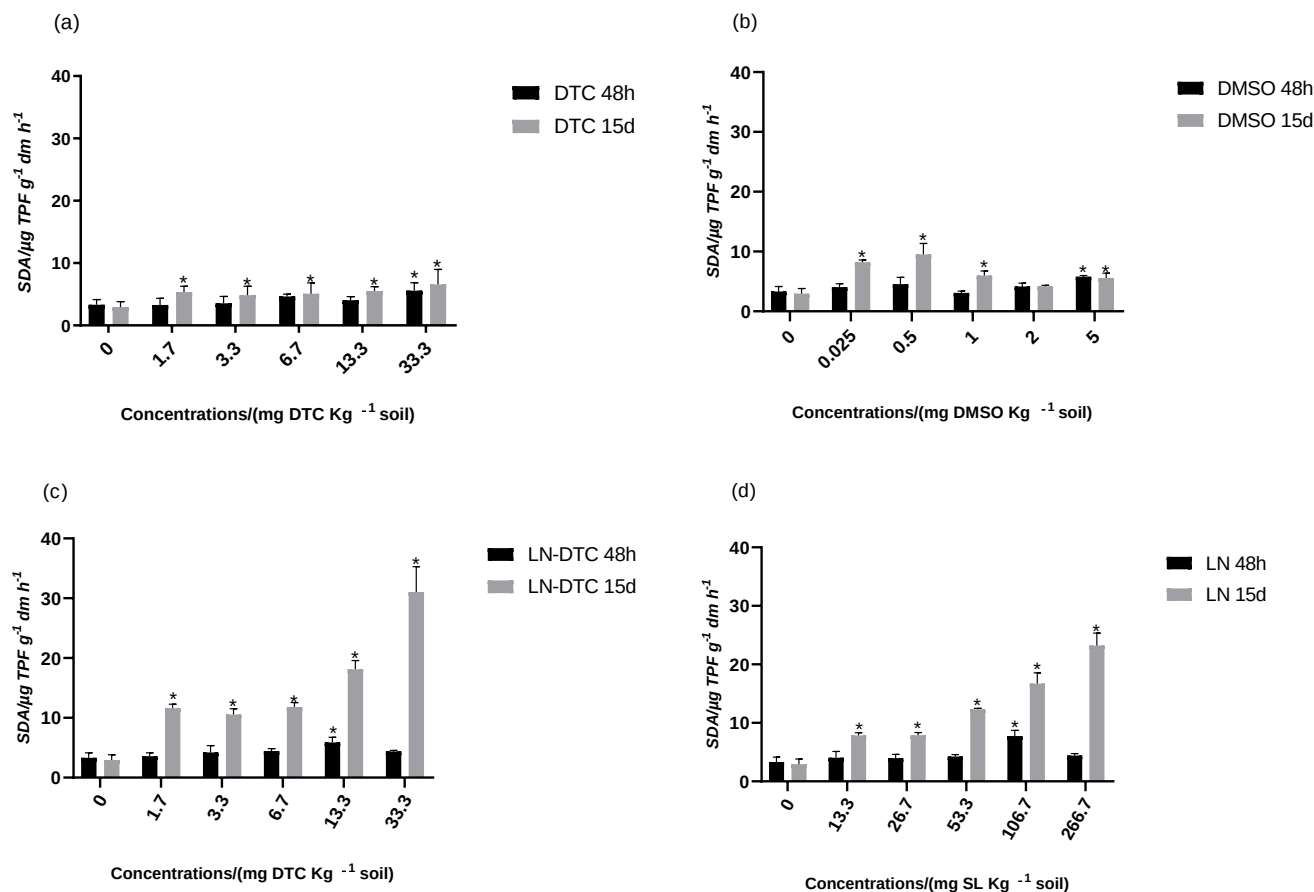


Figure 29. Soil dehydrogenase activity (SDA) in soil exposure for 48h (black bar) and 15 days (gray bar) to (a) DTC, (b) 3% DMSO in water, (c) LN-DTC and (d) LN. Concentrations tested are based on the amount of DTC and in the case of LN, the indicated concentrations are based on solid lipid (SL) used in the synthesis of LN. The same concentrations of DMSO were tested for DTC in DMSO (b). Asterisks mark significant differences from the control group (0 mg kg⁻¹ soil) ($p < 0.05$, Dunnett).

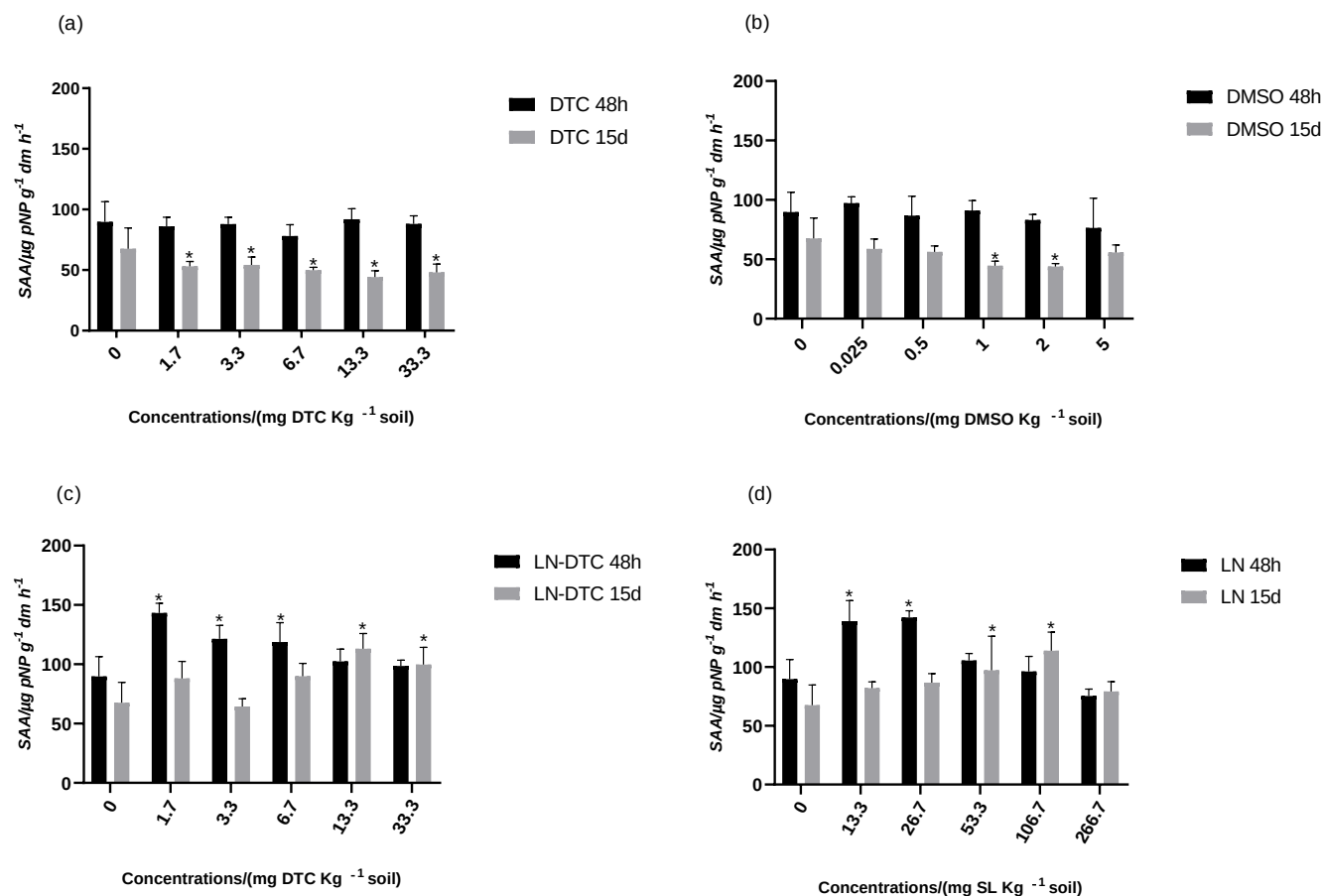


Figure 30. Soil arylsulfatase activity (SAA) in soil exposure for 48h (black bar) and 15 days (gray bar) to (a) DTC, (b) 3% DMSO in water, (c) LN-DTC and (d) LN. Concentrations tested are based on the amount of DTC and in the case of LN, the indicated concentrations are based on solid lipid (SL) used in the synthesis of LN. The same concentrations of DMSO were tested for DTC in DMSO (b). Asterisks mark significant differences from the control group (0 mg kg⁻¹ soil) ($p < 0.05$, Dunnett).

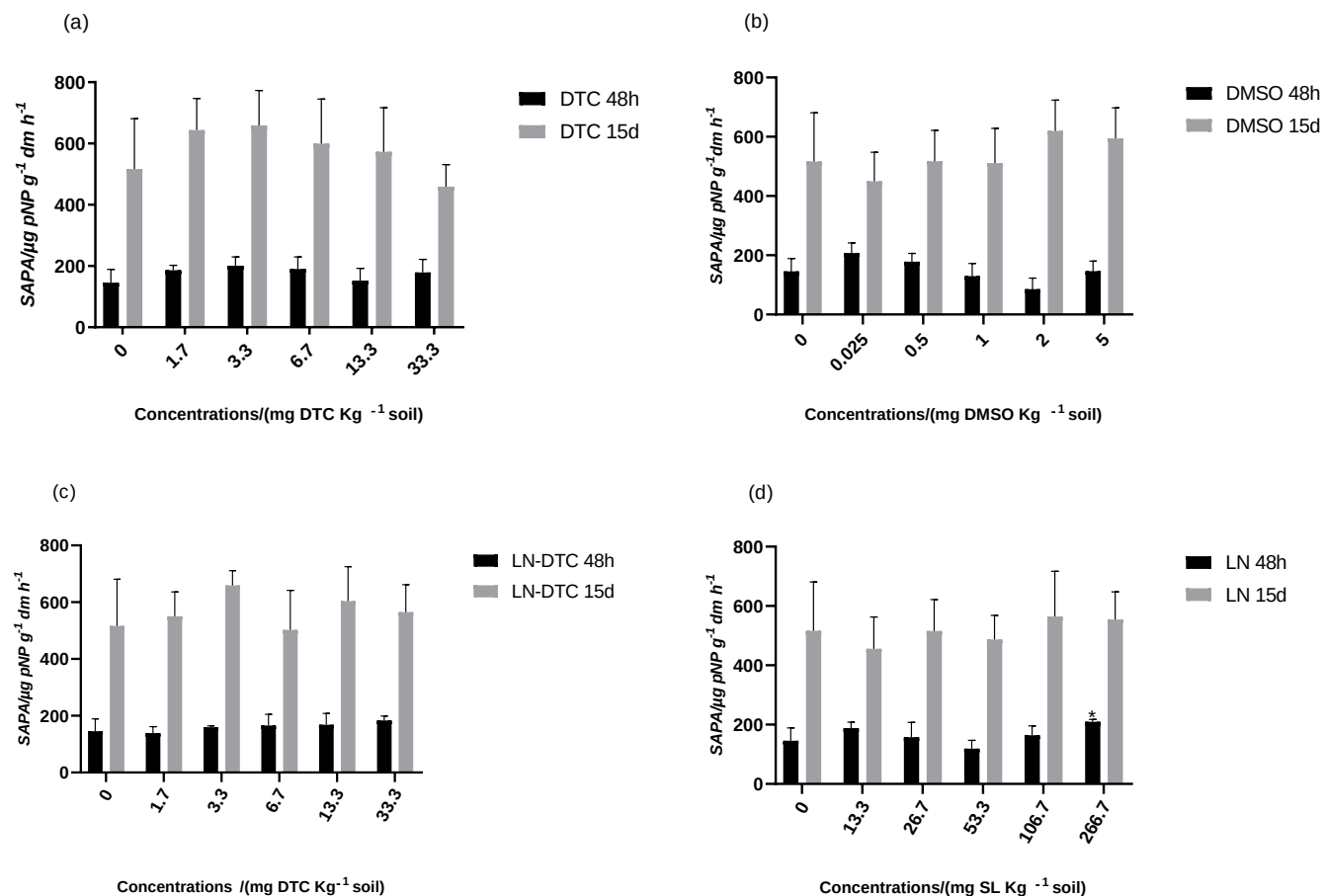


Figure 31. Soil acid phosphatase activity (SAPA) in soil exposure for 48h (black bar) and 15 days (gray bar) to (a) DTC, (b) 3% DMSO in water, (c) LN-DTC and (d) LN. Concentrations tested are based on the amount of DTC and in the case of LN, the indicated concentrations are based on solid lipid (SL) used in the synthesis of LN. The same concentrations of DMSO were tested for DTC in DMSO (b). Asterisks mark significant differences from the control group (0 mg kg⁻¹ soil) (p < 0.05, Dunnett).

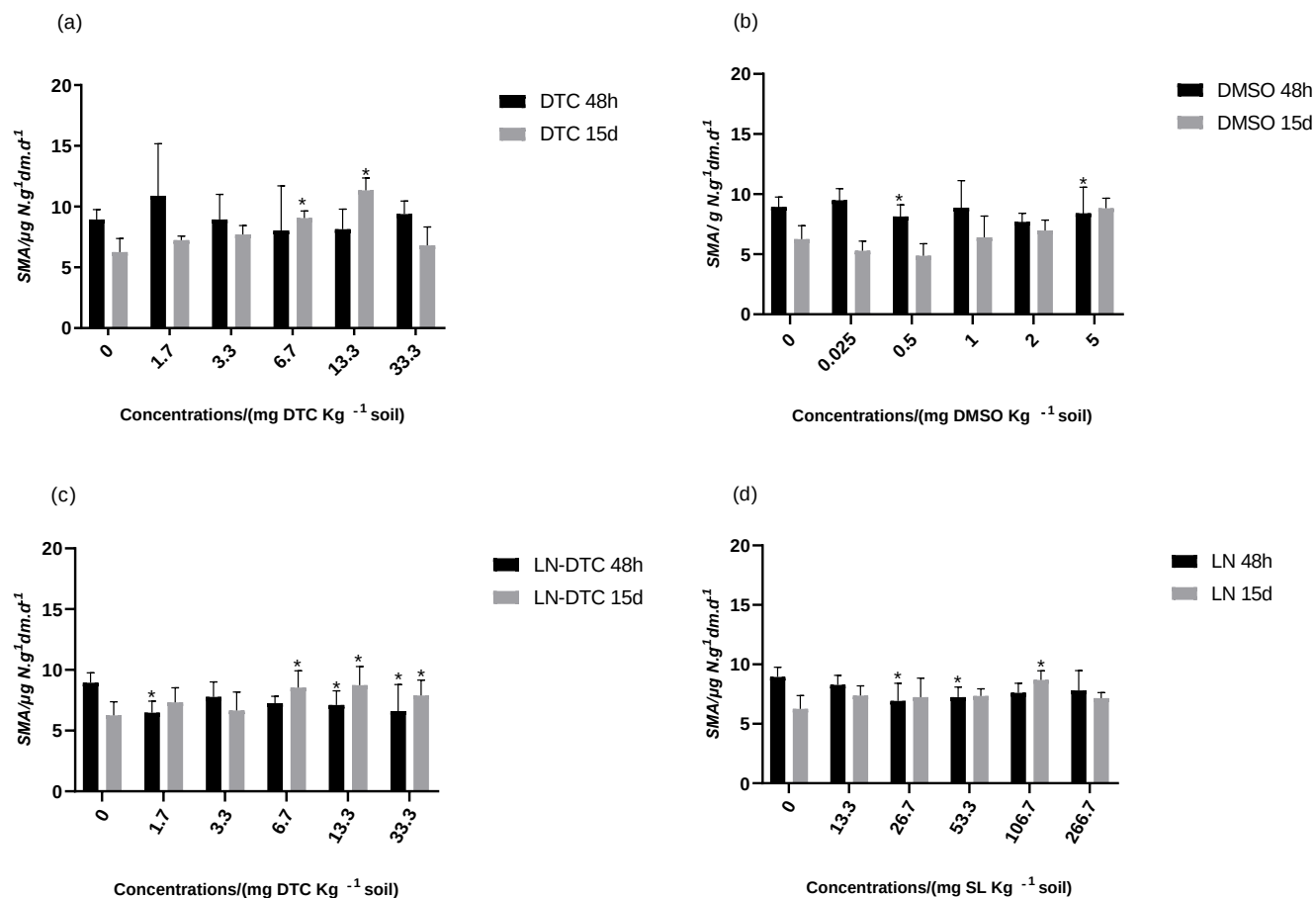


Figure 32. Soil mineralization activity (SMA) in soil exposure for 48h (black bar) and 15 days (gray bar) to (a) DTC, (b) 3% DMSO in water, (c) LN-DTC and (d) LN. Concentrations tested are based on the amount of DTC and in the case of LN, the indicated concentrations are based on solid lipid (SL) used in the synthesis of LN. The same concentrations of DMSO were tested for DTC in DMSO (b). Asterisks mark significant differences from the control group (0 mg kg^{-1} soil) ($p < 0.05$, Dunnett).

Table 11. Soil microbial parameters. Enzymatic activity of dehydrogenase, arylsulfatase, acid phosphatase and nitrogen mineralization after exposure to different concentration of aqueous solutions of DMSO, DTC in aqueous solution of DMSO, LN, and LN-DTC. The concentrations tested are based on the amount of DTC. In the case of LN, the indicated concentrations were based on solid lipid (SL) used in the synthesis of LN. DMSO concentrations were the same for DTC in DMSO. The same concentrations of SL were tested for LN-DTC. Results are represented by mean $\pm$ S.D. *values*. * Mark the significant differences in relation to the control group (0 mg Kg⁻¹).

Soil Microbial Parameters after 48 hours of exposure					
Sample	Concentrations (mg Kg ⁻¹ soil)	Dehydrogenase Activity ($\mu\text{g TPF g}^{-1}\text{dm h}^{-1}$)	Arylsulfatase Activity ($\mu\text{g pNP g}^{-1}\text{dm h}^{-1}$)	Acid Phosphatase Activity ($\mu\text{g pNP g}^{-1}\text{dm h}^{-1}$)	Nitrogen Mineralization Activity ($\mu\text{g N.g}^{-1}\text{dm.d}^{-1}$)
DMSO	0	3.3 $\pm$ 0.8	89.8 $\pm$ 16.6	145.6 $\pm$ 43.2	8.9 $\pm$ 0.8
	0.025	4.1 $\pm$ 1.2	97.8 $\pm$ 13.5	207.6 $\pm$ 56.6	9.5 $\pm$ 1.0
	0.5	4.5 $\pm$ 1.3	86.8 $\pm$ 16.4	178.5 $\pm$ 46.6	8.1 $\pm$ 1.0
	1	3.1 $\pm$ 0.4	91.0 $\pm$ 17.2	129.5 $\pm$ 46.1	8.9 $\pm$ 2.2
	2	4.1 $\pm$ 1.4	83.1 $\pm$ 15.4	85.7 $\pm$ 88.0	7.7 $\pm$ 0.7
	5	5.8* $\pm$ 1.6	76.4 $\pm$ 33.1	146.2 $\pm$ 42.6	8.4* $\pm$ 2.2
DTC	0	3.3 $\pm$ 0.8	89.8 $\pm$ 16.6	145.6 $\pm$ 43.2	8.9 $\pm$ 0.8
	1.7	3.3 $\pm$ 1.3	86.1 $\pm$ 10.8	186.3 $\pm$ 53.2	10.9 $\pm$ 4.3
	3.3	3.6 $\pm$ 1.1	88.4 $\pm$ 9.5	201.1 $\pm$ 48.2	8.9 $\pm$ 2.1
	6.7	4.7 $\pm$ 1.3	78.0 $\pm$ 13.9	190.6 $\pm$ 42.5	8.0 $\pm$ 3.7
	13.3	4 $\pm$ 1	91.9 $\pm$ 11.5	152.3 $\pm$ 39.8	8.2 $\pm$ 1.7
	33.3	5.6* $\pm$ 2.1	88.1 $\pm$ 10.4	178.8 $\pm$ 62.2	9.4 $\pm$ 1.1
LN	0	3.3 $\pm$ 0.8	89.8 $\pm$ 16.6	145.6 $\pm$ 43.2	8.9 $\pm$ 0.8
	13.3	4.1 $\pm$ 1.7	139.1* $\pm$ 29.1	188.5 $\pm$ 20.2	8.3 $\pm$ 0.8
	26.7	4.0 $\pm$ 0.8	142.2* $\pm$ 20.4	157.4 $\pm$ 50.4	6.9* $\pm$ 1.5
	53.3	4.3 $\pm$ 0.9	103.8 $\pm$ 8.5	118.7 $\pm$ 28.0	7.3* $\pm$ 0.9
	106.7	7.8* $\pm$ 2.2	96.2 $\pm$ 20.5	164.1 $\pm$ 31.6	7.6 $\pm$ 0.8
	266.7	4.4 $\pm$ 0.5	75.4 $\pm$ 22.7	209.3* $\pm$ 37.1	7.8 $\pm$ 1.7

LN-DTC	0	3.3 ± 0.8	89.8 ± 16.6	145.6 ± 43.2	8.9 ± 0.8
	1.7	3.6 ± 0.8	$142.2^* \pm 17.1$	139 ± 49	$6.5^* \pm 1.0$
	3.3	4.2 ± 1.3	$121.4^* \pm 21.6$	159.5 ± 43.0	7.8 ± 1.2
	6.7	4.5 ± 1.0	$117.0^* \pm 24.7$	165.4 ± 39.8	7.3 ± 0.6
	13.3	$5.9^* \pm 2.0$	105.9 ± 18.3	168.7 ± 39.8	$7.1^* \pm 1.8$
	33.3	4.4 ± 1.2	98.6 ± 23.3	183.9 ± 42.0	$6.6^* \pm 2.2$

Table 11. Continuation Soil microbial parameters 15 days. The concentrations tested are based on the amount of DTC. In the case of LN, the indicated concentrations were based on solid lipid (SL) used in the synthesis of LN. DMSO concentrations were the same for DTC in DMSO. The same concentrations of SL were tested for LN-DTC. Results are represented by mean $\pm$ S.D. *values*. * Mark the significant differences in relation to the control group (0 mg Kg⁻¹).

Soil Microbial Parameters after 15 days of exposure					
Sample	Concentrations (mg Kg ⁻¹ soil)	Dehydrogenase Activity (μ g TPF g ⁻¹ dm h ⁻¹)	Arylsulfatase Activity (μ g pNP g ⁻¹ dm h ⁻¹)	Acid Phosphatase Activity (μ g pNP g ⁻¹ dm h ⁻¹)	Nitrogen Mineralization Activity (μ g N.g ⁻¹ dm.d ⁻¹)
DMSO	0	2.9 $\pm$ 0.9	67.6 $\pm$ 17.2	516.5 $\pm$ 164.4	6.2 $\pm$ 1.1
	0.025	8.2* $\pm$ 1.3	58.7 $\pm$ 20.5	449.9 $\pm$ 98.0	5.3 $\pm$ 0.8
	0.5	9.5* $\pm$ 2.3	56.3 $\pm$ 10.4	517.2 $\pm$ 104.8	4.9* $\pm$ 1.0
	1	5.6 $\pm$ 1.4	44.6* $\pm$ 11.5	510.7 $\pm$ 117.5	6.4 $\pm$ 1.8
	2	4.2 $\pm$ 0.5	43.9* $\pm$ 8.4	620 $\pm$ 103	7 $\pm$ 1
	5	5.6* $\pm$ 0.9	55.8 $\pm$ 11.6	594.5 $\pm$ 102.8	8.8* $\pm$ 0.8
DTC	0	2.9 $\pm$ 0.9	67.6 $\pm$ 17.2	516.5 $\pm$ 164.4	6.3 $\pm$ 1.1
	1.7	5.3* $\pm$ 0.9	53.0* $\pm$ 10.5	643.6 $\pm$ 103.7	7.2 $\pm$ 0.5
	3.3	4.9* $\pm$ 1.4	54.3* $\pm$ 12.0	658.9 $\pm$ 114.1	7.7 $\pm$ 0.8
	6.7	5.1* $\pm$ 1.7	49.9* $\pm$ 7.5	600 $\pm$ 145	9.1* $\pm$ 1.0
	13.3	5.5* $\pm$ 0.7	44.2* $\pm$ 11.8	572.9 $\pm$ 144.4	11.3* $\pm$ 1.7
	33.3	6.6* $\pm$ 2.3	48.1* $\pm$ 12.2	458.8 $\pm$ 72.0	6.8 $\pm$ 2.3
LN	0	2.9 $\pm$ 0.9	67.6 $\pm$ 17.2	516.5 $\pm$ 164.4	6.3 $\pm$ 1.1
	13.3	7.9* $\pm$ 1.3	82.0 $\pm$ 13.4	455.4 $\pm$ 107.9	7.4 $\pm$ 0.8
	26.7	7.9* $\pm$ 1.3	86.8 $\pm$ 16.8	515.7 $\pm$ 105.9	7.2 $\pm$ 1.6
	53.3	12.3* $\pm$ 1.6	91.5* $\pm$ 18.5	486.9 $\pm$ 81.7	7.4 $\pm$ 0.6
	106.7	16.7* $\pm$ 2.0	113.9* $\pm$ 16.0	564.6 $\pm$ 152.7	8.7* $\pm$ 0.8
	266.7	23.2* $\pm$ 3.9	79.3 $\pm$ 17.5	554.3 $\pm$ 93.4	7.2 $\pm$ 0.5

LN-DTC	0	2.9 ± 0.9	67.6 ± 17.2	516.5 ± 164.4	6.3 ± 1.1
	1.7	11.6* ± 1.0	88.1 ± 24.3	550.4 ± 86.1	7.3 ± 1.2
	3.3	10.6* ± 2.4	64.4 ± 14.0	658.9 ± 52.0	6.7 ± 1.5
	6.7	11.8* ± 1.9	93.7 ± 19.3	502.5 ± 139.4	8.5* ± 1.4
	13.3	18.1* ± 2.5	105.9* ± 25.3	603.9 ± 121.2	8.7* ± 1.5
	33.3	31.1* ± 4.1	99.8* ± 30.6	565.2 ± 96.2	7.9* ± 1.3

5 Conclusions and Perspectives

Chemical pesticides have made a major contribution to combating pests and diseases. Particularly, fungicides that comprise the class of pesticides that are in ever-increasing use because fungi possess adaptability and resilience and can be found in various habitats around the globe. Among numerous species of fungi, some are pathogenic, and humans have struggled since the beginning of organized agriculture to reduce production losses.

However, although fungicides have contributed substantially to increase agricultural productivity, their excessive use has led to repercussions on human health and the environment. In this way, new chemicals and the development of safer formulations to defend sustainable and eco-friendly agriculture is of great importance, especially in the face of a growing global population, climate change and increasing fungal resistance to existing compounds. Thus, the application of nanotechnology in the agricultural sector, mainly with the use of nanofungicides to control pathogens and diseases, has aroused great interest both in the scientific community and in the industrial sector. The use of nanofungicides can decrease the amounts of chemical fungicides applied in the field and increase the efficiency of fungi control with minimal environmental and human impact. Although in the present work, DTC has been obtained with high yield (95.8%), full exploitation of its biological activities is limited due to its easy degradation and low solubility in aqueous solutions. In this sense, the main objective of this Doctoral Project was to design, characterize and evaluate the effect of DTC incorporated in MSiNP and LN on the fungi *Moniliophthora perniciosa*, *Phytophthora palmivora* and *Phytophthora theobromicola*, which are the main pathogen that attacks the cocoa crop in Brazil, as well as to evaluate their impacts on human and environmental health. MSiNP and LN are widely used as AI carriers and have proven to offer high formulation stability, increase the solubility of hydrophobic AI, protect AI against external agents and their production is simple, efficient and low cost.

The synthesized MSiNP had a Z-Ave of 168 ± 4 nm, PDI of 0.29 ± 0.02 and ZP of -12.0 ± 0.4 mV with surface area of 1021 ± 15 m² g⁻¹ and pore size of 24.3 ± 0.2 nm, while after loading with DTC, MSiNP-DTC presented a Z-Ave of 176 ± 1 nm, PDI of 0.38 ± 0.04 and ZP values of -22.0 ± 0.3 mV. However, these changes did not significantly affect the physicochemical characteristics of the MSiNP ($p > 0.05$), maintaining high drug loading capacity ($4.79 \pm 0.004\%$) and encapsulation efficiency ($95.77 \pm 0.08\%$). Contrarily, changes

in surface area ($618 \pm 15 \text{ m}^2 \text{ g}^{-1}$) and pore sizes ($15.2 \pm 0.2 \text{ nm}$) showed that MSiNP-DTC still maintained the typical mesoporous structure during the immobilization process, but these reductions suggest that the DTC was present in the MSiNP matrix in various ways, such as adsorbed/adhered to the surface and inside the pore.

LN were successfully produced without the use of organic solvents using a 3^2 factorial design to provide data on the effects of the solid lipid (Precirol) and surfactant (Soy Lecithin) on the physicochemical characteristics of LN. This information was essential to select the best nanoformulation and thus encapsulate the DTC. In this sense, the optimal values obtained in relation to the average particle size, homogeneity and surface charge were those observed for the LN composed of 0.17% (m/m) Precirol and Soy Lecithin, 0.70% (m/m) Capryol 90 and 0.35 % (m/m) Tego Care 450 with the average particle size of $135 \pm 1 \text{ nm}$, PDI 0.26 ± 0.01 and ZP $-43.0 \pm 0.1 \text{ mV}$. After encapsulation of DTC 0.021% (m/m), the mean particle size was $150 \pm 0.4 \text{ nm}$, with homogeneous size distribution and good physical stability for at least 7 months at 25°C . Furthermore, the encapsulation efficiency and load capacity values of $99.39 \pm 0.01\%$ and $4.39 \pm 0.01\%$, respectively, indicate the excellent compatibility between the DTC and the lipid matrix.

The interactions between DTC and NPs were analysed by Fourier-Transform Infrared (FTIR), Raman and thermal behavior, which confirmed the incorporation of DTC in NPs. The morphology of Unloaded and loaded MSiNP and LN with DTC were performed through Transmission Electron Microscope (TEM) showing that MSiNP presented the formation of spherical and mesoporous structure, and that LN even after the lyophilization process maintained their spherical morphology, and after the DTC incorporation, both NPs preserved their characteristics. Overall, the characterization of the materials allowed us to infer that MSiNP and LN can serve as matrices for DTC delivery in particular and possibly for wider use in drug delivery applications, improving the stability and overcoming poor solubility in water.

The evaluation of the *in vitro* antifungal activity indicated that DTC at 100 mg L^{-1} , LN-DTC at $200 \text{ mg DTC L}^{-1}$ and LN at 1600 mg SL^{-1} exerted a significant inhibition ($p < 0.05$) of 30.9%, 35.2% and 33.9% respectively, compared with the control, of the fungi *M. perniciososa*. The results from the growth inhibition studies with *P. theobromicola* demonstrated that the DTC inhibition percentage reached 42.3%, followed by LN-DTC with 36% and LN with only 29%. For *P. palmivora*, the results were 4.6% of inhibition for DMSO, which was not significant compared to the control ($p > 0.05$), followed by 42.5% for DTC, 43.3% for LN and 47.1% for

LN-DTC. With the cocoa fruits, the results showed that the DTC and NPs reduced the lesions caused by the fungi *P. theobromicola* and *P. palmivora*, and LN-DTC in contact with the fungi *P. theobromicola* completely inhibited the growth of the fungi. Considering that for *M. pernicioso*, which has only one fungicide in use with 30% efficiency depending on when and how it is applied, and that there is no control for *P. theobromicola* and *P. palmivora*, the evaluation of the effect of DTC and NPs as possible fungicides is extremely relevant. Regarding MSiNP and MSiNP-DTC, it did not exert toxic effects on *M. pernicioso*, *P. theobromicola* and *P. palmivora* in all tested concentrations. Since each fungus reacts in such a way as to create resistance to the fungicide and adapt to the environment, fungal growth in the presence of these NPs may be related to the intrinsic characteristics of MSiNP, such as the long carbon chain that can serve as a nutritional resource for fungi or it can interact with other compounds present in the medium, or even with the surface charge. In addition, in MSiNP-DTC, the amount of DTC loaded may not have been sufficient to act efficiently in inhibiting fungal growth.

The safety evaluation of compounds and nanoformulations was carried out using human skin cells, aquatic organisms and soil microbiota. The greatest toxicity to humans from the application of fungicides to the crop occurs through breathing and contact with the skin. The cytotoxicity studies with skin cells HaCaT (normal keratinocytes) and A431 cells (squamous cell carcinoma) showed that, in general, all tested samples were not toxic for both cell lines at the tested concentrations (cell viability >70%) after 24h and 48h of exposure.

The environmental safety evaluation of the new DTC formulation using different aquatic organisms, such as the microalgae *R. subcapitata*, the macrophyte *L. minor*, and the marine bacterium *A. fischeri*, showed that DTC, MSiNP, MSiNP-DTC, LN and LN-DTC at concentrations ranging from 8.8 to 150 mg L⁻¹ induced low toxicity to selected aquatic organisms. However, LN and LN-DTC showed some toxicity that may be related to the matrix composition or even to the interaction of DTC with LN.

DMSO, DTC, LN and LN-DTC showed that the activity of certain soil microbial parameters can be inhibited or stimulated. In the soil microbial test, greater alterations were observed for the soil incubated for 15 days, and for most enzymes there was an increase in enzymatic activity, especially for LN and LN-DTC. These alterations in the enzymatic activities of the soil do not necessarily correspond to harmful effects but can be attributed to the availability of nutrients.

Therefore, in the present study, these results allow us to conclude that LN demonstrated to be an excellent carrier for DTC and can be a successful alternative for use as nanofungicides to control the fungi that directly attack cocoa crops that, in addition to significantly inhibit fungal growth, also presented better results than the marketed fungicide. Also, the safety evaluation of LN-DTC indicated that this nanoformulation presented no toxicity to human health and environment. However, further studies will be performed to evaluate the effect of nanoformulations on additional non-target terrestrial species, such as on soil invertebrates (oligochaetes and collembola) and on cocoa plants infected by the fungal pathogens.

6 References

1. Ngegba, P.M.; Cui, G.; Khalid, M.Z.; Zhong, G. Use of Botanical Pesticides in Agriculture as an Alternative to Synthetic Pesticides. *Agriculture (Switzerland)* **2022**, *12*.doi: 10.3390/agriculture12050600
2. Varghese, J. V.; Sebastian, E.M.; Iqbal, T.; Tom, A.A. Pesticide Applicators and Cancer: A Systematic Review. *Rev Environ Health* **2021**, *36*, 467–476.doi: 10.1515/reveh-2020-0121
3. Carroll, S.P.; Jørgensen, P.S.; Kinnison, M.T.; Bergstrom, C.T.; Denison, R.F.; Gluckman, P.; Smith, T.B.; Strauss, S.Y.; Tabashnik, B.E. Applying Evolutionary Biology to Address Global Challenges. *Science (1979)* **2014**, *346*.doi: 10.1126/science.1245993
4. Lykogianni, M.; Bempelou, E.; Karamaouna, F.; Aliferis, K.A. Do Pesticides Promote or Hinder Sustainability in Agriculture? The Challenge of Sustainable Use of Pesticides in Modern Agriculture. *Science of the Total Environment* **2021**, *795*.doi: 10.1016/j.scitotenv.2021.148625
5. Verma, J.P.; Jaiswal, D.K.; Sagar, R. Pesticide Relevance and Their Microbial Degradation: A-State-of-Art. *Rev Environ Sci Biotechnol* **2014**, *13*, 429–466.doi: 10.1007/s11157-014-9341-7
6. Brauer, V.S.; Rezende, C.P.; Pessoni, A.M.; De Paula, R.G.; Rangappa, K.S.; Nayaka, S.C.; Gupta, V.K.; Almeida, F. Antifungal Agents in Agriculture: Friends and Foes of Public Health. *Biomolecules* **2019**, *9*.doi: 10.3390/biom9100521
7. Tleuova, A.B.; Wielogorska, E.; Talluri, V.S.S.L.P.; Štěpánek, F.; Elliott, C.T.; Grigoriev, D.O. Recent Advances and Remaining Barriers to Producing Novel Formulations of Fungicides for Safe and Sustainable Agriculture. *Journal of Controlled Release* **2020**, *326*, 468–481.doi: 10.1016/j.jconrel.2020.07.035
8. Tsalidis, G.A. Human Health and Ecosystem Quality Benefits with Life Cycle Assessment Due to Fungicides Elimination in Agriculture. *Sustainability* **2022**, *14*.doi: 10.3390/su14020846
9. Tudi, M.; Li, H.; Li, H.; Wang, L.; Lyu, J.; Yang, L.; Tong, S.; Yu, Q.J.; Ruan, H.D.; Atabila, A.; et al. Exposure Routes and Health Risks Associated with Pesticide Application. *Toxics* **2022**, *10*.doi: 10.3390/toxics10060335
10. Sharma, A.; Kumar, V.; Shahzad, B.; Tanveer, M.; Sidhu, G.P.S.; Handa, N.; Kohli, S.K.; Yadav, P.; Bali, A.S.; Parihar, R.D.; et al. Worldwide Pesticide Usage and Its Impacts on Ecosystem. *SN Appl Sci* **2019**, *1*.doi: 10.1007/s42452-019-1485-1

11. De Souza, J.; Martins, E.D.; Luz, N.; Carlos, J.; Silva, P. *Uso Do Trichoderma Na Cultura Do Cacau Control of Post-Harvest Diseases View Project Diversidade de Fungos Da Mata Atlântica Sul Da Bahia View Project Comissão Executiva Do Plano Da Lavoura Cacaueira*, **2019**, <http://ainfo.cnptia.embrapa.br/digital/bitstream/item/207514/1/livro-trichoderma-2019.pdf>
12. Damalas, C.A.; Eleftherohorinos, I.G. Pesticide Exposure, Safety Issues, and Risk Assessment Indicators. *Int J Environ Res Public Health* **2011**, *8*, 1402–1419. doi: 10.3390/ijerph8051402
13. Jabłońska – Trypuć, A.; Wiater, J. Protective Effect of Plant Compounds in Pesticides Toxicity. *J Environ Health Sci Eng* **2022**, *20*, 1035–1045, doi:10.1007/s40201-022-00823-0.
14. Schiff, H. Mittheilungen Aus Dem Universitätslaboratorium in Pisa: Eine Neue Reihe Organischer Basen. *Annalen der Chemie und Pharmacie* **1864**, *131*, 118–119, doi:10.1002/jlac.18641310113.
15. Hussain, S.; Kiran, R.; Ahmad, M.; Saqib, M.; Munawar, K.S.; Shahid, M.; Waqas, M.; Massey, S.; Jawaria, R.; Baby, R. Synthesis, Spectroscopy and Biological Studies of Triphenyltin(IV) Derivatives with Carboxylated Schiff Bases. *Journal of the Iranian Chemical Society* **2023**, doi:10.1007/s13738-023-02746-5.
16. Boshala, A.; Flörke, U.; Yamin, B.M.; Amer, Y.O.B.; Ghaith, G.S.H.; Almughery, A.A.; Zarrouk, A.; Warad, I. Crystal Interaction, Hirshfeld Surface Analysis, and Spectral Analysis of New Dithiocarbazate Schiff Bases Derivative (LH) and Its Neutral Cis-Cu(L)₂ Complex. *J Mol Struct* **2021**, *1224*, doi:10.1016/j.molstruc.2020.129207.
17. Pradeep, S.D.; Gopalakrishnan, A.K.; Manoharan, D.K.; Soumya, R.S.; Gopalan, R.K.; Mohanan, P. V. Isatin Derived Novel Schiff Bases: An Efficient Pharmacophore for Versatile Biological Applications. *J Mol Struct* **2023**, *1271*, doi:10.1016/j.molstruc.2022.134121.
18. Kumari, N.; Singh, S.; Baral, M.; Kanungo, B.K. Schiff Bases: A Versatile Fluorescence Probe in Sensing Cations. *J Fluoresc* **2023**.
19. Zulfiqar, A.; Ahmed, D.; Fatima, R.; Yousuf, S. Green Synthesis, Urease Inhibitory Activity and Antioxidant Potential of 4-Bromo-2-(((2'-Chloro-4'-Nitrophenyl)Imino)Methyl)Phenol Schiff Base. *J Mol Struct* **2020**, *1202*, doi:10.1016/j.molstruc.2019.127263.
20. Lima, F.C.; Só, Y.A.O.; Gargano, R.; de Oliveira, D.M.; Gatto, C.C. Structural, Theoretical and Biological Activity of Mono and Binuclear Nickel(II) Complexes

- with Symmetrical and Asymmetrical 4,6-Diacetylresorcinol-Dithiocarbamate Ligands. *J Inorg Biochem* **2021**, 224, doi:10.1016/j.jinorgbio.2021.111559.
21. Takjoo, R.; Centore, R.; Hayatolgheibi, S.S. Mixed Ligand Complexes of Cadmium(II) and Copper(II) Dithiocarbamate: Synthesis, Spectral Characterization, X-Ray Crystal Structure. *Inorganica Chim Acta* **2018**, 471, 587–594, doi:10.1016/j.ica.2017.11.043.
22. Bhat, R.A.; Kumar, D. Experimental and Theoretical Study of S-Benzyl β -N-(2-Methyl-3-Phenylallylidene)Dithiocarbamate as Anti-Bacterial Agent. *Research on Chemical Intermediates* **2019**, 45, 2565–2586, doi:10.1007/s11164-019-03752-0.
23. Latif, M.A.; Tofaz, T.; Chaki, B.M.; Tariqul Islam, H.M.; Hossain, M.S.; Kudrat-E-Zahan, M. Synthesis, Characterization, and Biological Activity of the Schiff Base and Its Ni(II), Cu(II), and Zn(II) Complexes Derived from 4-(Dimethylamino)Benzaldehyde and S-Benzylthiocarbamate. *Russ J Gen Chem* **2019**, 89, 1197–1201, doi:10.1134/S107036321906015X.
24. Li, X.; Zhang, D.; Liu, Z.; Xu, Y.; Wang, D. Synthesis, Characterization of a Ternary Cu(II) Schiff Base Complex with Degradation Activity of Organophosphorus Pesticides. *Inorganica Chim Acta* **2018**, 471, 280–289, doi:10.1016/j.ica.2017.11.024.
25. Uddin, M.N.; Ahmed, S.S.; Alam, S.M.R. REVIEW: Biomedical Applications of Schiff Base Metal Complexes. *J Coord Chem* 2020, 73, 3109–3149.
26. Hanif, M.; Chohan, Z.H. Synthesis, Spectral Characterization and Biological Studies of Transition Metal(II) Complexes with Triazole Schiff Bases. *Appl Organomet Chem* **2013**, 27, 36–44, doi:10.1002/aoc.2936.
27. Nanjundan, N.; Narayanasamy, R.; Butcher, R.J.; Jasinski, J.P.; Velmurugan, K.; Nandhakumar, R.; Balakumaran, M.D.; Kalaichelvan, P.T.; Gnanasoundari, V.G. Synthesis, Crystal Structure, Biomolecular Interactions and Anticancer Properties of Ni(II), Cu(II) and Zn(II) Complexes Bearing S-Allyldithiocarbamate. *Inorganica Chim Acta* **2017**, 455, 283–297, doi:10.1016/j.ica.2016.10.035.
28. Costa, A.R.; de Menezes, T.I.; Nascimento, R.R.; dos Anjos, P.N.M.; Viana, R.B.; de Araujo Fernandes, A.G.; Santos, R.L.S.R. Ruthenium(II) Dimethylsulfoxide Complex with Pyrazole/Dithiocarbamate Ligand: Synthesis, Spectroscopy Studies, DFT Calculations and Thermal Behavior. *J Therm Anal Calorim* **2019**, 0123456789, 1683–1696, doi:10.1007/s10973-019-08185-w.
29. de Menezes, T.I.; de Oliveira Costa, R.; Sanches, R.N.F.; de Oliveira Silva, D.; Santos, R.L.S.R. Preparation and Characterization of Dithiocarbamate Schiff Base-Loaded Poly(Lactic Acid) Nanoparticles and Analytical Validation for Drug

- Quantification. *Colloid Polym Sci* **2019**, 297, 1465–1475, doi:10.1007/s00396-019-04572-9.
30. Tsen, C.M.; Yu, C.W.; Chen, S.Y.; Lin, C.L.; Chuang, C.Y. Application of Surface-Enhanced Raman Scattering in Rapid Detection of Dithiocarbamate Pesticide Residues in Foods. *Appl Surf Sci* **2021**, 558, doi:10.1016/j.apsusc.2021.149740.
31. Chen, F.; Zang, Z.; Chen, Z.; Cui, L.; Chang, Z.; Ma, A.; Yin, T.; Liang, R.; Han, Y.; Wu, Z.; et al. Nanophotosensitizer-Engineered Salmonella Bacteria with Hypoxia Targeting and Photothermal-Assisted Mutual Bioaccumulation for Solid Tumor Therapy. *Biomaterials* **2019**, 214, 119226, doi:10.1016/j.biomaterials.2019.119226.
32. Hoffman, L.; Trombetta, L.; Hardej, D. Ethylene Bisdithiocarbamate Pesticides Maneb and Mancozeb Cause Metal Overload in Human Colon Cells. *Environ Toxicol Pharmacol* **2016**, 41, 78–88, doi:10.1016/j.etap.2015.11.002.
33. Wei, X.; Liu, C.; Li, Z.; Sun, Q.; Zhang, X.; Li, Y.; Zhang, W.; Shi, J.; Zhai, X.; Zhang, D.; et al. Fabrication of a Label-Free Electrochemical Cell-Based Biosensor for Toxicity Assessment of Thiram. *Chemosphere* **2022**, 307, 135960, doi:10.1016/j.chemosphere.2022.135960.
34. Vasamsetti, B.M.K.; Chon, K.; Kim, J.; Oh, J.A.; Yoon, C.Y.; Park, H.H. Developmental Toxic Effects of Thiram on Developing Zebrafish (Danio Rerio) Embryos. *Toxics* **2022**, 10, doi:10.3390/toxics10070369.
35. Wu, Z.; Su, R. Pesticide Thiram Exposure Alters the Gut Microbial Diversity of Chickens. *Front Microbiol* **2022**, 13, doi:10.3389/fmicb.2022.966224.
36. Park, H.; You, H.H.; Song, G. Multiple Toxicity of Propineb in Developing Zebrafish Embryos: Neurotoxicity, Vascular Toxicity, and Notochord Defects in Normal Vertebrate Development. *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology* **2021**, 243, doi:10.1016/j.cbpc.2021.108993.
37. Abar Gürol, M.; Arman, S.; Yön, N.D. Effects of Mancozeb on the Testicular Histology of the Zebrafish (Danio Rerio). *Ann Limnol* **2020**, 56, doi:10.1051/limn/2020009.
38. Paganotto Leandro, L.; Siqueira de Mello, R.; da Costa- Silva, D.G.; Medina Nunes, M.E.; Rubin Lopes, A.; Kemmerich Martins, I.; Posser, T.; Franco, J.L. Behavioral Changes Occur Earlier than Redox Alterations in Developing Zebrafish Exposed to Mancozeb. *Environmental Pollution* **2021**, 268, doi:10.1016/j.envpol.2020.115783.
39. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; 2014;
40. Carneiro, Z.A.; Pedro, P.I.; Sesti-Costa, R.; Lopes, C.D.; Pereira, T.A.; Milanezi, C.M.; da Silva, M.A.P.; Lopez, R.F.V.; Silva, J.S.; Deflon, V.M. In Vitro and In Vivo

- Trypanocidal Activity of H2bdtc-Loaded Solid Lipid Nanoparticles. *PLoS Negl Trop Dis* **2014**, *8*, doi:10.1371/journal.pntd.0002847.
41. De Sousa, G.F.; Gatto, C.C.; Resck, I.S.; Deflon, V.M. Synthesis, Spectroscopic Studies and X-Ray Crystal Structures of New Pyrazoline and Pyrazole Derivatives. *J Chem Crystallogr* **2011**, *41*, 401–408, doi:10.1007/s10870-010-9896-2.
42. De Sousa, G.F.; Gatto, C.C.; Resck, I.S.; Deflon, V.M. Synthesis, Spectroscopic Studies and X-Ray Crystal Structures of New Pyrazoline and Pyrazole Derivatives. *J Chem Crystallogr* **2011**, *41*, 401–408, doi:10.1007/s10870-010-9896-2.
43. Costa, A.R.; França, R.N.; Silva-Jardim, I.; Silva, R.J.S.; Lima-Santos, J.; Salay, L.C.; Santos, R.L.S.R. Self-Assembling Micellar System Based on Pluronic and Pyrazole-Dithiocarbamate-Conjugate Stimulates Production of Nitric Oxide from Macrophages. *Colloids and Interface Science Communications* **2021**, *41*, 100378, doi:10.1016/j.colcom.2021.100378.
44. Vega-Vásquez, P.; Mosier, N.S.; Irudayaraj, J. Nanoscale Drug Delivery Systems: From Medicine to Agriculture. *Front Bioeng Biotechnol* **2020**, *8*, doi: 10.3389/fbioe.2020.00079
45. Taha, N.A.; Hamden, S.; Bayoumi, Y.A.; Elsakhawy, T.; El-Ramady, H.; Solberg, S.Ø. Nanofungicides with Selenium and Silicon Can Boost the Growth and Yield of Common Bean (*Phaseolus Vulgaris* L.) and Control *Alternaria* Leaf Spot Disease. *Microorganisms* **2023**, *11*, 728, doi:10.3390/microorganisms11030728.
46. Arciniegas-Grijalba, P.A.; Patiño-Portela, M.C.; Mosquera-Sánchez, L.P.; Guerra Sierra, B.E.; Muñoz-Florez, J.E.; Erazo-Castillo, L.A.; Rodríguez-Páez, J.E. ZnO-Based Nanofungicides: Synthesis, Characterization and Their Effect on the Coffee Fungi *Mycena Citricolor* and *Colletotrichum* Sp. *Materials Science and Engineering C* **2019**, *98*, 808–825, doi:10.1016/j.msec.2019.01.031.
47. Tippannanavar, M.; Verma, A.; Kumar, R.; Gogoi, R.; Kundu, A.; Patanjali, N. Preparation of Nanofungicides Based on Imidazole Drugs and Their Antifungal Evaluation. *J Agric Food Chem* **2020**, *68*, 4566–4578, doi:10.1021/acs.jafc.9b06387.
48. Zhu, Y.; Liu, X.; Hu, Y.; Wang, R.; Chen, M.; Wu, J.; Wang, Y.; Kang, S.; Sun, Y.; Zhu, M. Behavior, Remediation Effect and Toxicity of Nanomaterials in Water Environments. *Environ Res* **2019**, *174*, 54–60, doi: 10.1016/j.envres.2019.04.014
49. Chen, M.; Sun, Y.; Liang, J.; Zeng, G.; Li, Z.; Tang, L.; Zhu, Y.; Jiang, D.; Song, B. Understanding the Influence of Carbon Nanomaterials on Microbial Communities. *Environ Int* **2019**, *126*, 690–698, doi: 10.1016/j.envint.2019.02.005

50. Yeo, M.K.; Kang, M. Effects of Nanometer Sized Silver Materials on Biological Toxicity during Zebrafish Embryogenesis. *Bull Korean Chem Soc* **2008**, *29*, 1179–1184, doi:10.5012/bkcs.2008.29.6.1179.
51. Bilberg, K.; Malte, H.; Wang, T.; Baatrup, E. Silver Nanoparticles and Silver Nitrate Cause Respiratory Stress in Eurasian Perch (*Perca fluviatilis*). *Aquatic Toxicology* **2010**, *96*, 159–165, doi:10.1016/j.aquatox.2009.10.019.
52. Zhang, R.; Piao, M.J.; Kim, K.C.; Kim, A.D.; Choi, J.Y.; Choi, J.; Hyun, J.W. Endoplasmic Reticulum Stress Signaling Is Involved in Silver Nanoparticles-Induced Apoptosis. *International Journal of Biochemistry and Cell Biology* **2012**, *44*, 224–232, doi:10.1016/j.biocel.2011.10.019.
53. Aillon, K.L.; Xie, Y.; El-Gendy, N.; Berkland, C.J.; Forrest, M.L. Effects of Nanomaterial Physicochemical Properties on in Vivo Toxicity. *Adv Drug Deliv Rev* **2009**, *61*, 457–466, doi: 10.1016/j.addr.2009.03.010
54. Rather, M.A.; Bhat, I.A.; Sharma, N.; Sharma, R. Molecular and Cellular Toxicology of Nanomaterials with Related to Aquatic Organisms. In *Advances in Experimental Medicine and Biology*; Springer New York LLC, **2018**; Vol. 1048, pp. 263–284, doi: 10.1007/978-3-319-72041-8_16
55. Stephen, S.; Gorain, B.; Choudhury, H.; Chatterjee, B. Exploring the Role of Mesoporous Silica Nanoparticle in the Development of Novel Drug Delivery Systems. *Drug Deliv Transl Res* **2022**, *12*, 105–123, doi:10.1007/s13346-021-00935-4.
56. Oliveira, D.M.; Andrada, A.S. Synthesis of Ordered Mesoporous Silica MCM-41 with Controlled Morphology for Potential Application in Controlled Drug Delivery Systems. *Cerâmica* **2019**, *65*, 170–179, doi:10.1590/0366-69132019653742509.
57. Manzano, M.; Vallet-Regí, M. Mesoporous Silica Nanoparticles for Drug Delivery. *Adv Funct Mater* **2020**, *30*, doi:10.1002/adfm.201902634.
58. Stober, W.; Fink, A.; Ernst Bohn, D. Controlled Growth of Monodisperse Silica Spheres in the Micron Size Range. *J Colloid Interface Sci* **1968**, *26*, 62–69, doi:10.1016/0021-9797(68)90272-5.
59. Ren, G.; Su, H.; Wang, S. The Combined Method to Synthesis Silica Nanoparticle by Stöber Process. *J Solgel Sci Technol* **2020**, *96*, 108–120, doi:10.1007/s10971-020-05322-y.
60. Ghimire, P.P.; Jaroniec, M. Renaissance of Stöber Method for Synthesis of Colloidal Particles: New Developments and Opportunities. *J Colloid Interface Sci* **2021**, *584*, 838–865, doi:10.1016/j.jcis.2020.10.014.
61. Hate, S.S.; Reutzel-Edens, S.M.; Taylor, L.S. Influence of Drug-Silica Electrostatic Interactions on Drug Release from Mesoporous Silica-Based Oral Delivery

- Systems. *Mol Pharm* **2020**, *17*, 3435–3446, doi:10.1021/acs.molpharmaceut.0c00488.
62. Bakhshian Nik, A.; Zare, H.; Razavi, S.; Mohammadi, H.; Torab Ahmadi, P.; Yazdani, N.; Bayandori, M.; Rabiee, N.; Izadi Mobarakeh, J. Smart Drug Delivery: Capping Strategies for Mesoporous Silica Nanoparticles. *Microporous and Mesoporous Materials* **2020**, *299*, doi: 10.1016/j.micromeso.2020.110115
63. Kuang, Y.; Zhai, J.; Xiao, Q.; Zhao, S.; Li, C. Polysaccharide/Mesoporous Silica Nanoparticle-Based Drug Delivery Systems: A Review. *Int J Biol Macromol* **2021**, *193*, 457–473, doi: 10.1016/j.ijbiomac.2021.10.142
64. Kutawa, A.B.; Ahmad, K.; Ali, A.; Hussein, M.Z.; Wahab, M.A.A.; Adamu, A.; Ismaila, A.A.; Gunasena, M.T.; Rahman, M.Z.; Hossain, M.I. Trends in Nanotechnology and Its Potentialities to Control Plant Pathogenic Fungi: A Review. *Biology (Basel)* **2021**, *10*, doi:10.3390/biology10090881.
65. Kong, X.; Zhang, B.; Wang, J. Multiple Roles of Mesoporous Silica in Safe Pesticide Application by Nanotechnology: A Review. *J Agric Food Chem* **2021**, *69*, 6735–6754, doi:10.1021/acs.jafc.1c01091.
66. Kaziem, A.E.; Yang, L.; Lin, Y.; Kazem, A.E.; Xu, H.; Zhang, Z.X. Pathogenic Invasion-Responsive Carrier Based on Mesoporous Silica/ β -Glucan Nanoparticles for Smart Delivery of Fungicides. *ACS Sustain Chem Eng* **2021**, *9*, 9126–9138, doi:10.1021/acssuschemeng.1c02962.
67. Pan, H.; Huang, W.; Wu, L.; Hong, Q.; Hu, Z.; Wang, M.; Zhang, F. A PH Dual-Responsive Multifunctional Nanoparticle Based on Mesoporous Silica with Metal-Polymethacrylic Acid Gatekeeper for Improving Plant Protection and Nutrition. *Nanomaterials* **2022**, *12*, doi:10.3390/nano12040687.
68. Bayón-Cordero, L.; Alkorta, I.; Arana, L. Application of Solid Lipid Nanoparticles to Improve the Efficiency of Anticancer Drugs. *Nanomaterials* **2019**, *9*, doi:10.3390/nano9030474.
69. Haider, M.; Abdin, S.M.; Kamal, L.; Orive, G. Nanostructured Lipid Carriers for Delivery of Chemotherapeutics: A Review. *Pharmaceutics* **2020**, *12*.
70. Bonilla, L.; Espina, M.; Severino, P.; Cano, A.; Ettcheto, M.; Camins, A.; García, M.L.; Souto, E.B.; Sánchez-López, E. Lipid Nanoparticles for the Posterior Eye Segment. *Pharmaceutics* **2022**, *14*, doi: 10.3390/pharmaceutics14010090
71. Xu, J.; Liao, M.; Chen, Y.; Chen, L. Novel Fabrication of Marizomib-Loaded Chitosan-Coated Hydroxyapatite Nanocarriers as a Promising System for Effective Treatment of Ovarian Cancer. *Mater Res Express* **2022**, *9*, doi:10.1088/2053-1591/ac5077.

72. Cimino, C.; Maurel, O.M.; Musumeci, T.; Bonaccorso, A.; Drago, F.; Souto, E.M.B.; Pignatello, R.; Carbone, C. Essential Oils: Pharmaceutical Applications and Encapsulation Strategies into Lipid-Based Delivery Systems. *Pharmaceutics* **2021**, *13*, 1–35.doi: 10.3390/pharmaceutics13030327
73. Madkhali, O.A. Perspectives and Prospective on Solid Lipid Nanoparticles as Drug Delivery Systems. *Molecules* **2022**, *27*.doi: 10.3390/molecules27051543
74. Sharma, A.; Baldi, A. Nanostructured Lipid Carriers: A Review Production of Phyto-Pharmaceuticals by Plant Cell Technology View Project Development of Novel Drug Delivery System View Project Nanostructured Lipid Carriers: A Review. **2018**, doi:10.4172/2329-6631.1000191.
75. Tamjidi, F.; Shahedi, M.; Varshosaz, J.; Nasirpour, A. Nanostructured Lipid Carriers (NLC): A Potential Delivery System for Bioactive Food Molecules. *Innovative Food Science and Emerging Technologies* **2013**, *19*, 29–43.doi: 10.1016/j.ifset.2013.03.002
76. Severino, P.; Andreani, T.; Jäger, A.; Chaud, M. V.; Santana, M.H.A.; Silva, A.M.; Souto, E.B. Solid Lipid Nanoparticles for Hydrophilic Biotech Drugs: Optimization and Cell Viability Studies (Caco-2 & HEPG-2 Cell Lines). *Eur J Med Chem* **2014**, *81*, 28–34, doi:10.1016/j.ejmech.2014.04.084.
77. Ganilho, C.; da Silva, M.B.; Paiva, C.; de Menezes, T.I.; dos Santos, M.R.; Pereira, C.M.; Pereira, R.; Andreani, T. Environmental Safety Assessments of Lipid Nanoparticles Loaded with Lambda-Cyhalothrin. *Nanomaterials* **2022**, *12*, 2576, doi:10.3390/nano12152576.
78. Keivani Nahr, F.; Ghanbarzadeh, B.; Hamishehkar, H.; Samadi Kafil, H. Food Grade Nanostructured Lipid Carrier for Cardamom Essential Oil: Preparation, Characterization and Antimicrobial Activity. *J Funct Foods* **2018**, *40*, 1–8, doi:10.1016/j.jff.2017.09.028.
79. Yadav, J.; Jasrotia, P.; Kashyap, P.L.; Bhardwaj, A.K.; Kumar, S.; Singh, M.; Singh, G.P. Nanopesticides: Current Status and Scope for Their Application in Agriculture. *Plant Protection Science* **2022**, *58*, 1–17, doi:10.17221/102/2020-PPS.
80. Beloqui, A.; Solinís, M.Á.; Rodríguez-Gascón, A.; Almeida, A.J.; Préat, V. Nanostructured Lipid Carriers: Promising Drug Delivery Systems for Future Clinics. *Nanomedicine* **2016**, *12*, 143–161, doi:10.1016/j.nano.2015.09.004.
81. Rodrigues da Silva, G.H.; de Moura, L.D.; de Carvalho, F.V.; Geronimo, G.; Mendonça, T.C.; de Lima, F.F.; de Paula, E. Antineoplastics Encapsulated in Nanostructured Lipid Carriers. *Molecules* **2021**, *26*, doi:10.3390/molecules26226929.

82. Murthy, A.; Ravi, P.R.; Kathuria, H.; Malekar, S. Oral Bioavailability Enhancement of Raloxifene with Nanostructured Lipid Carriers. *Nanomaterials* **2020**, *10*, 1–17, doi:10.3390/nano10061085.
83. Gordillo-Galeano, A.; Mora-Huertas, C.E. Solid Lipid Nanoparticles and Nanostructured Lipid Carriers: A Review Emphasizing on Particle Structure and Drug Release. *European Journal of Pharmaceutics and Biopharmaceutics* **2018**, *133*, 285–308, doi:10.1016/j.ejpb.2018.10.017.
84. Maroofpour, N.; Mousavi, M.; Hejazi, M.J.; Iranipour, S.; Hamishehkar, H.; Desneux, N.; Biondi, A.; Haddi, K. Comparative Selectivity of Nano and Commercial Formulations of Pirimicarb on a Target Pest, *Brevicoryne Brassicae*, and Its Predator *Chrysoperla Carnea*. *Ecotoxicology* **2021**, *30*, 361–372, doi:10.1007/s10646-021-02349-x.
85. Radwan, I.T.; Baz, M.M.; Khater, H.; Selim, A.M. Nanostructured Lipid Carriers (NLC) for Biologically Active Green Tea and Fennel Natural Oils Delivery: Larvicidal and Adulticidal Activities against *Culex Pipiens*. *Molecules* **2022**, *27*, doi:10.3390/molecules27061939.
86. Tortorici, S.; Cimino, C.; Ricupero, M.; Musumeci, T.; Biondi, A.; Siscaro, G.; Carbone, C.; Zappalà, L. Nanostructured Lipid Carriers of Essential Oils as Potential Tools for the Sustainable Control of Insect Pests. *Ind Crops Prod* **2022**, *181*, doi:10.1016/j.indcrop.2022.114766.
87. Campos, E.V.R.; De Oliveira, J.L.; Da Silva, C.M.G.; Pascoli, M.; Pasquoto, T.; Lima, R.; Abhilash, P.C.; Fernandes Fraceto, L. Polymeric and Solid Lipid Nanoparticles for Sustained Release of Carbendazim and Tebuconazole in Agricultural Applications. *Sci Rep* **2015**, *5*, doi:10.1038/srep13809.
88. Uchida, D.T.; Siqueira, G.F.; dos Reis, E.M.; Hegeto, F.L.; Medina Neto, A.; Reis, A.V.; Bruschi, M.L.; Villa Nova, M.; Machinski Júnior, M. Design of Nanostructured Lipid Carriers Containing *Cymbopogon Martinii* (Palmarosa) Essential Oil against *Aspergillus Nomius*. *Molecules* **2021**, *26*, 4825, doi:10.3390/molecules26164825.
89. Andreani, T.; Nogueira, V.; Gavina, A.; Fernandes, S.; Rodrigues, J.L.; Pinto, V. V.; Ferreira, M.J.; Silva, A.M.; Pereira, C.M.; Pereira, R. Ecotoxicity to Freshwater Organisms and Cytotoxicity of Nanomaterials: Are We Generating Sufficient Data for Their Risk Assessment? *Nanomaterials* **2021**, *11*, 1–25, doi:10.3390/nano11010066.
90. Serrano, L.; Moreno, A.S.; Castillo, D.S. Del; Bonilla, J.; Romero, C.A.; Galarza, L.L.; Coronel-León, J.R. Biosurfactants Synthesized by Endophytic *Bacillus* Strains as Control of *Moniliophthora Perniciosa* and *Moniliophthora Roreri*. *Sci Agric* **2021**, *78*, 651–656, doi:10.1590/1678-992x-2020-0172.

91. Amorim, A.O.; Orlandelli, R.C.; Pamphile, J.A. Revista UNINGÁ Review ISSN 2178-2571 Ciências Agrárias e Meio Ambiente. *Rev. UNINGÁ Review* **2019**, *34*, 1–10.
92. Brazil. Ministry of Industry; External Trade and Services Cacau e Chocolate No Brasil: Desafios Na Produção e Comércio Global. **2018**, 128.
93. Copetti, M. V.; Iamanaka, B.T.; Pitt, J.I.; Taniwaki, M.H. Fungi and Mycotoxins in Cocoa: From Farm to Chocolate. *Int J Food Microbiol* **2014**, *178*, 13–20, doi:10.1016/j.ijfoodmicro.2014.02.023.
94. Oliveira, M.M.; De Assis, F.G.D.V.; Leal, P.L. Eficiência de Fungos Filamentosos No Controle Biológico Da Vassoura de Bruxa Do Cacaueiro. *SITIENTIBUS série Ciências Biológicas* **2014**, *14*, doi:10.13102/scb386.
95. Perrine-Walker, F. Phytophthora Palmivora–Cocoa Interaction. *Journal of Fungi* **2020**, *6*, 1–20, doi:10.3390/jof6030167.
96. Lisboa, D.O.; Evans, H.C.; Araújo, J.P.M.; Elias, S.G.; Barreto, R.W. Moniliophthora Perniciosa, the Mushroom Causing Witches' Broom Disease of Cacao: Insights into Its Taxonomy, Ecology and Host Range in Brazil. *Fungal Biol* **2020**, *124*, 983–1003, doi:10.1016/j.funbio.2020.09.001.
97. Gramacho, K.P.; Newman Luz, E.D.M.; Da Silva, F.S.; Lopes, U.V.; Pires, J.L.; Pereira, L. Pathogenic Variability of Moniliophthora Perniciosa in Three Agroecological Zones of the Cacao Region of Bahia, Brazil. *Crop Breeding and Applied Biotechnology* **2016**, *16*, 7–13, doi:10.1590/1984-70332016v16n1a2.
98. Nascente, A.S.; Matsumura, A.T.S.; Matsumura, A.S.; Geraldine, A.M.; Sousa, A.B.O. de; Sousa, A.C.A. de; Dias, A.R.; Santos, Á.F. dos; Tavares, A.N.G.; Pereira, A.C.P.; et al. *Trichoderma: Uso Na Agricultura*; **2019**; ISBN 978-85-7035-943-8.
99. Medeiros, F.H.V.; Pomella, A.W.V.; de Souza, J.T.; Niella, G.R.; Valle, R.; Bateman, R.P.; Fravel, D.; Vinyard, B.; Hebbar, P.K. A Novel, Integrated Method for Management of Witches' Broom Disease in Cacao in Bahia, Brazil. *Crop Protection* **2010**, *29*, 704–711, doi:10.1016/j.cropro.2010.02.006.
100. Mota, S.G.R.; Barros, T.F.; Castilho, M.S. In Vitro Screening and Chemometrics Analysis on a Series of Azole Derivatives with Fungicide Activity against Moniliophthora Perniciosa. *J Braz Chem Soc* **2010**, *21*, 510–519, doi:10.1590/S0103-50532010000300017.
101. Thomazella, D.P.T.; Teixeira, P.J.P.L.; Oliveira, H.C.; Saviani, E.E.; Rincones, J.; Toni, I.M.; Reis, O.; Garcia, O.; Meinhardt, L.W.; Salgado, I.; et al. The Hemibiotrophic Cacao Pathogen Moniliophthora Perniciosa Depends on a

- Mitochondrial Alternative Oxidase for Biotrophic Development. *New Phytologist* **2012**, *194*, 1025–1034, doi:10.1111/j.1469-8137.2012.04119.x.
102. Carvalho, L.E. de; Lima, M. da P.; Máximo, A. da C.; Pereira, E.C. da S.; Moreira, W.A. dos S.; Ferreira, A.G.; Vêras, S. de M.; Souza, M.G. De Estudo Em Raiz e Ráquis Foliar de *Spathelia Excelsa*: Fitoquímica e Atividade Frente Ao Fungo *Moniliophthora Perniciosa* Associado Ao Cupuaçuzeiro (*Theobroma Grandiflorum*). *Quim Nova* **2012**, *35*, 2237–2240, doi:10.1590/S0100-40422012001100027.
 103. Neto, A.A.P.; Santos, T.R.; Duarte, E.A.A.; de Oliveira, T.A.S.; de Andrade Silva, E.M.; Uetanabaro, A.P.T.; da Costa, A.M.; Luz, E.D.M.N.; Laranjeira, D. Yeasts Associated with Aerial Parts of *Theobroma Cacao* L. in Southern Bahia, Brazil, as Prospective Biocontrol Agents against *Moniliophthora Perniciosa*. *Trop Plant Pathol* **2021**, *46*, 109–128, doi:10.1007/s40858-020-00418-w.
 104. Costa, L.C.B.; Pinto, J.E.B.P.; Bertolucci, S.K.V.; do Bomfim Costa, J.C.; Alves, P.B.; dos Santos Niculau, E. In Vitro Antifungal Activity of *Ocimum Selloi* Essential Oil and Methylchavicol against Phytopathogenic Fungi. *Revista Ciencia Agronomica* **2015**, *46*, 428–435, doi:10.5935/1806-6690.20150023.
 105. De Souza Cabral, A.; De Carvalho, P.M.B.; Pinotti, T.; Hagler, A.N.; Leda, C.S.M.H.; Macrae, A. Killer Yeasts Inhibit the Growth of the Phytopathogen *Moniliophthora Perniciosa*, the Causal Agent of Witches' Broom Disease. *Brazilian Journal of Microbiology* **2009**, *40*, 108–110, doi:10.1590/s1517-83822009000100018.
 106. Orlandelli, R.C.; de Almeida, T.T.; Alberto, R.N.; Polonio, J.C.; Azevedo, J.L.; Pamphile, J.A. Antifungal and Proteolytic Activities of Endophytic Fungi Isolated from *Piper Hispidum* Sw. *Brazilian Journal of Microbiology* **2015**, *46*, 359–366, doi:10.1590/S1517-838246220131042.
 107. Simamora, A. V.; Hahuly, M. V.; Henuk, J.B. Endophytic Fungi as Potential Biocontrol Agents of *Phytophthora Palmivora* in the Cocoa Plant. *Biodiversitas* **2021**, *22*, 2601–2609, doi:10.13057/biodiv/d220519.
 108. Reis, A.; Feitosa, M.I. de souza; Luz, E.D.N.; Santos, Á.F. dos Espécies Fitopatogênicas de *Phytophthora*. *Revisão Anual de Patologia de Plantas* **2023**, *V*, 56–78, doi:10.31976/0104-038321v290003.
 109. Samuels, G.J.; Suarez, C.; Solis, K.; Holmes, K.A.; Thomas, S.E.; Ismaiel, A.; Evans, H.C. *Trichoderma Theobromicola* and *T. Paucisporum*: Two New Species Isolated from Cacao in South America. *Mycol Res* **2006**, *110*, 381–392, doi:10.1016/j.mycres.2006.01.009.

110. Maluin, F.N.; Hussein, M.Z.; Yusof, N.A.; Idris, A.S.; Daim, L.D.J.; Sarian, M.N.; Rajab, N.F.; Ling, S.E.; Rashid, N.; Fakurazi, S. Cytoprotection, Genoprotection, and Dermal Exposure Assessment of Chitosan-based Agronanofungicides. *Pharmaceutics* **2020**, *12*, doi:10.3390/pharmaceutics12060497.
111. Zhou, J.; Zhang, J.; Li, F.; Liu, J. Triazole Fungicide Tebuconazole Disrupts Human Placental Trophoblast Cell Functions. *J Hazard Mater* **2016**, *308*, 294–302, doi:10.1016/j.jhazmat.2016.01.055.
112. Xu, J.; Xiong, H.; Zhang, X.; Muhayimana, S.; Liu, X.; Xue, Y.; Huang, Q. Comparative Cytotoxic Effects of Five Commonly Used Triazole Alcohol Fungicides on Human Cells of Different Tissue Types. *J Environ Sci Health B* **2020**, *55*, 438–446, doi:10.1080/03601234.2019.1709377.
113. Pirozzi, A.V.A.; Stellavato, A.; La Gatta, A.; Lamberti, M.; Schiraldi, C. Mancozeb, a Fungicide Routinely Used in Agriculture, Worsens Nonalcoholic Fatty Liver Disease in the Human HepG2 Cell Model. *Toxicol Lett* **2016**, *249*, 1–4, doi:10.1016/j.toxlet.2016.03.004.
114. Anderson, C.C.; Marentette, J.O.; Rauniyar, A.K.; Prutton, K.M.; Khatri, M.; Matheson, C.; Reisz, J.A.; Reigan, P.; D'Alessandro, A.; Roede, J.R. Maneb Alters Central Carbon Metabolism and Thiol Redox Status in a Toxicant Model of Parkinson's Disease. *Free Radic Biol Med* **2021**, *162*, 65–76, doi:10.1016/j.freeradbiomed.2020.11.028.
115. Baldassarre, F.; Tatulli, G.; Vergaro, V.; Mariano, S.; Scala, V.; Nobile, C.; Pucci, N.; Dini, L.; Loreti, S.; Ciccarella, G. Sonication-Assisted Production of Fosetyl-Al Nanocrystals: Investigation of Human Toxicity and in Vitro Antibacterial Efficacy against *Xylella Fastidiosa*. *Nanomaterials* **2020**, *10*, 1–20, doi:10.3390/nano10061174.
116. Bocca, B.; Barone, F.; Petrucci, F.; Benetti, F.; Picardo, V.; Prota, V.; Amendola, G. Nanopesticides: Physico-Chemical Characterization by a Combination of Advanced Analytical Techniques. *Food and Chemical Toxicology* **2020**, *146*, doi:10.1016/j.fct.2020.111816.
117. Herdiana, Y.; Wathoni, N.; Shamsuddin, S.; Muchtaridi, M. α -Mangostin Nanoparticles Cytotoxicity and Cell Death Modalities in Breast Cancer Cell Lines. *Molecules* **2021**, *26*, doi: 10.3390/molecules26175119
118. Mendes, L.P.; Delgado, J.M.F.; Costa, A.D.A.; Vieira, M.S.; Benfica, P.L.; Lima, E.M.; Valadares, M.C. Biodegradable Nanoparticles Designed for Drug Delivery: The Number of Nanoparticles Impacts on Cytotoxicity. *Toxicology in Vitro* **2015**, *29*, 1268–1274, doi:10.1016/j.tiv.2014.12.021.

119. Demir, E.; Kansız, S.; Doğan, M.; Topel, Ö.; Akkoyunlu, G.; Kandur, M.Y.; Turna Demir, F. Hazard Assessment of the Effects of Acute and Chronic Exposure to Permethrin, Copper Hydroxide, Acephate, and Validamycin Nanopesticides on the Physiology of *Drosophila*: Novel Insights into the Cellular Internalization and Biological Effects. *Int J Mol Sci* **2022**, *23*, doi:10.3390/ijms23169121.
120. Chhipa, H. Nanofertilizers and Nanopesticides for Agriculture. *Environ Chem Lett* **2017**, *15*, 15–22. doi: 10.1007/s10311-016-0600-4
121. Chaud, M.; Souto, E.B.; Zielinska, A.; Severino, P.; Batain, F.; Oliveira-Junior, J.; Alves, T. Nanopesticides in Agriculture: Benefits and Challenge in Agricultural Productivity, Toxicological Risks to Human Health and Environment. *Toxics* **2021**, *9*. doi: 10.3390/toxics9060131
122. Lee, S.Y.; Kim, I.Y.; Heo, M.B.; Moon, J.H.; Son, J.G.; Lee, T.G. Global Proteomics to Study Silica Nanoparticle-induced Cytotoxicity and Its Mechanisms in Hepg2 Cells. *Biomolecules* **2021**, *11*, 1–21, doi:10.3390/biom11030375.
123. Wang, P.; Shen, T.; Sun, Y.; Cui, X.; Liu, C.; Yuan, Y.; Qian, J. Selective Killing of Cancer Cells by Silica Nanoparticles Due to Increased Nanoparticle Internalization and Cellular Sensitivity to Oxidative Stress. *Journal of Nanoparticle Research* **2023**, *25*, doi:10.1007/s11051-022-05643-9.
124. Kurtz-Chalot, A.; Klein, J.P.; Pourchez, J.; Boudard, D.; Bin, V.; Alcantara, G.B.; Martini, M.; Cottier, M.; Forest, V. Adsorption at Cell Surface and Cellular Uptake of Silica Nanoparticles with Different Surface Chemical Functionalizations: Impact on Cytotoxicity. *Journal of Nanoparticle Research* **2014**, *16*, doi:10.1007/s11051-014-2738-y.
125. Imperlini, E.; Massaro, F.; Buonocore, F. Antimicrobial Peptides against Bacterial Pathogens: Innovative Delivery Nanosystems for Pharmaceutical Applications. *Antibiotics* **2023**, *12*, 184, doi:10.3390/antibiotics12010184.
126. Azarnezhad, A.; Samadian, H.; Jaymand, M.; Sobhani, M.; Ahmadi, A. Toxicological Profile of Lipid-Based Nanostructures: Are They Considered as Completely Safe Nanocarriers? *Crit Rev Toxicol* **2020**, *50*, 148–176. doi: 10.1080/10408444.2020.1719974
127. Czajkowska-Kośnik, A.; Szymańska, E.; Czarnomysy, R.; Jacyna, J.; Markuszewski, M.; Basa, A.; Winnicka, K. Nanostructured Lipid Carriers Engineered as Topical Delivery of Etodolac: Optimization and Cytotoxicity Studies. *Materials* **2021**, *14*, 1–21, doi:10.3390/ma14030596.
128. Buchman, J.T.; Hudson-Smith, N. V.; Landy, K.M.; Haynes, C.L. Understanding Nanoparticle Toxicity Mechanisms to Inform Redesign Strategies to Reduce

- Environmental Impact. *Acc Chem Res* **2019**, *52*, 1632–1642, doi:10.1021/acs.accounts.9b00053.
129. Liu, P.; Liu, X.; Cheng, Y.; Zhong, S.; Shi, X.; Wang, S.; Liu, M.; Ding, J.; Zhou, W. Core-Shell Nanosystems for Self-Activated Drug-Gene Combinations against Triple-Negative Breast Cancer. *ACS Appl Mater Interfaces* **2020**, *12*, 53654–53664, doi:10.1021/acsami.0c15089.
130. Nishu, S. Das; Park, S.; Ji, Y.; Han, I.; Key, J.; Lee, T.K. The Effect of Engineered PLGA Nanoparticles on Nitrifying Bacteria in the Soil Environment. *Journal of Industrial and Engineering Chemistry* **2020**, *84*, 297–304, doi:10.1016/j.jiec.2020.01.011.
131. Koedrith, P.; Rahman, Md.M.; Jang, Y.J.; Shin, D.Y.; Seo, Y.R. Nanoparticles: Weighing the Pros and Cons from an Eco-Genotoxicological Perspective. *J Cancer Prev* **2021**, *26*, 83–97, doi:10.15430/jcp.2021.26.2.83.
132. Sarkar, R.D.; Singh, H.B.; Kalita, M.C. Enhanced Lipid Accumulation in Microalgae through Nanoparticle-Mediated Approach, for Biodiesel Production: A Mini-Review. *Heliyon* **2021**, *7*, doi:10.1016/j.heliyon.2021.e08057.
133. Hund-Rinke, K.; Diaz, C.; Jurack, A.; Klein, J.; Knopf, B.; Schlich, K.; Fernández-Cruz, M.L.; Hernández-Moreno, D.; Manier, N.; Pandard, P.; et al. Nanopharmaceuticals (Au-NPs) after Use: Experiences with a Complex Higher Tier Test Design Simulating Environmental Fate and Effect. *Ecotoxicol Environ Saf* **2021**, *227*, doi:10.1016/j.ecoenv.2021.112949.
134. Medicines Agency, E. *Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use*; 2018;
135. *Test No. 201: Alga, Growth Inhibition Test*; OECD Publishing, 2006; ISBN 9789264069923.
136. Zhang, Y.; He, D.; Chang, F.; Dang, C.; Fu, J. Combined Effects of Sulfamethoxazole and Erythromycin on a Freshwater Microalga, *Raphidocelis Subcapitata*: Toxicity and Oxidative Stress. *Antibiotics* **2021**, *10*, doi:10.3390/antibiotics10050576.
137. Xin, X.; Huang, G.; Zhang, B. Review of Aquatic Toxicity of Pharmaceuticals and Personal Care Products to Algae. *J Hazard Mater* **2021**, *410*, doi:10.1016/j.jhazmat.2020.124619
138. Oláh, V.; Hepp, A.; Gaibor Vaca, N.Y.; Tamás, M.; Mészáros, I. Retrospective Analyses of Archive Phytotoxicity Test Data Can Help in Assessing Internal Dynamics and Stability of Growth in Laboratory Duckweed Cultures. *Aquatic Toxicology* **2018**, *201*, 40–46, doi:10.1016/j.aquatox.2018.05.022.

139. *Test No. 221: Lemna Sp. Growth Inhibition Test*; OECD, 2006; ISBN 9789264016194.
140. Modlitbová, P.; Novotný, K.; Pořízka, P.; Klus, J.; Lubal, P.; Zlámalová-Gargošová, H.; Kaiser, J. Comparative Investigation of Toxicity and Bioaccumulation of Cd-Based Quantum Dots and Cd Salt in Freshwater Plant *Lemna Minor* L. *Ecotoxicol Environ Saf* **2018**, *147*, 334–341, doi:10.1016/j.ecoenv.2017.08.053.
141. Gao, Q.; Wang, J.; Ren, L.; Cheng, Y.; Lin, Z.; Li, X.G.; Sun, H. Investigations on the Influence of Energy Source on Time-Dependent Hormesis: A Case Study of Sulfadoxine to *Aliivibrio Fischeri* in Different Cultivation Systems. *Science of the Total Environment* **2021**, *775*, 145877, doi:10.1016/j.scitotenv.2021.145877.
142. Abbas, M.; Adil, M.; Ehtisham-ul-Haque, S.; Munir, B.; Yameen, M.; Ghaffar, A.; Shar, G.A.; Asif Tahir, M.; Iqbal, M. *Vibrio Fischeri* Bioluminescence Inhibition Assay for Ecotoxicity Assessment: A Review. *Science of the Total Environment* **2018**, *626*, 1295–1309, doi:10.1016/j.scitotenv.2018.01.066.
143. Ventura, S.P.M.; Marques, C.S.; Rosatella, A.A.; Afonso, C.A.M.; Gonçalves, F.; Coutinho, J.A.P. Toxicity Assessment of Various Ionic Liquid Families towards *Vibrio Fischeri* Marine Bacteria. *Ecotoxicol Environ Saf* **2012**, *76*, 162–168, doi:10.1016/j.ecoenv.2011.10.006.
144. Ameen, F.; Alsamhary, K.; Alabdullatif, J.A.; ALNadhari, S. A Review on Metal-Based Nanoparticles and Their Toxicity to Beneficial Soil Bacteria and Fungi. *Ecotoxicol Environ Saf* **2021**, *213*, doi: 10.1016/j.ecoenv.2021.112027
145. Nizam, N.U.M.; Hanafiah, M.M.; Woon, K.S. A Content Review of Life Cycle Assessment of Nanomaterials: Current Practices, Challenges, and Future Prospects. *Nanomaterials* **2021**, *11*, doi: 10.3390/nano11123324
146. Andreani, T.; Nogueira, V.; Pinto, V. V.; Ferreira, M.J.; Rasteiro, M.G.; Silva, A.M.; Pereira, R.; Pereira, C.M. Influence of the Stabilizers on the Toxicity of Metallic Nanomaterials in Aquatic Organisms and Human Cell Lines. *Science of the Total Environment* **2017**, *607–608*, 1264–1277, doi:10.1016/j.scitotenv.2017.07.098.
147. Book, F.; Backhaus, T. Aquatic Ecotoxicity of Manufactured Silica Nanoparticles: A Systematic Review and Meta-Analysis. *Science of the Total Environment* **2022**, *806*, doi: 10.1016/j.scitotenv.2021.150893
148. Book, F.; Persson, M.; Carmona, E.; Backhaus, T.; Lammel, T. Colloidal Silica Nanomaterials Reduce the Toxicity of Pesticides to Algae, Depending on Charge and Surface Area. *Environ Sci Nano* **2022**, *9*, 2402–2416, doi:10.1039/d1en01180d.
149. Book, F.; Ekvall, M.T.; Persson, M.; Lönnerud, S.; Lammel, T.; Sturve, J.; Backhaus, T. Ecotoxicity Screening of Seven Different Types of Commercial Silica

- Nanoparticles Using Cellular and Organismic Assays: Importance of Surface and Size. *NanoImpact* **2019**, *13*, 100–111, doi:10.1016/j.impact.2019.01.001.
150. Arora, S.; Sahni, D. Pesticides Effect on Soil Microbial Ecology and Enzyme Activity- An Overview. *Journal of Applied and Natural Science* **2016**, *8*, 1126–1132, doi:10.31018/jans.v8i2.929.
 151. Gundi, V.A.K.B.; Viswanath, B.; Chandra, M.S.; Kumar, V.N.; Reddy, B.R. Activities of Cellulase and Amylase in Soils as Influenced by Insecticide Interactions. *Ecotoxicol Environ Saf* **2007**, *68*, 278–285, doi:10.1016/j.ecoenv.2006.11.011.
 152. Sim, J.X.F.; Doolette, C.L.; Vasileiadis, S.; Drigo, B.; Wyrsh, E.R.; Djordjevic, S.P.; Donner, E.; Karpouzas, D.G.; Lombi, E. Pesticide Effects on Nitrogen Cycle Related Microbial Functions and Community Composition. *Science of The Total Environment* **2022**, *807*, 150734, doi:10.1016/j.scitotenv.2021.150734.
 153. Zhai, W.; Zhang, L.; Liu, H.; Zhang, C.; Liu, D.; Wang, P.; Zhou, Z. Enantioselective Degradation of Prothioconazole in Soil and the Impacts on the Enzymes and Microbial Community. *Science of The Total Environment* **2022**, *824*, 153658, doi:10.1016/j.scitotenv.2022.153658.
 154. Ayilara, M.S.; Adeleke, B.S.; Akinola, S.A.; Fayose, C.A.; Adeyemi, U.T.; Gbadegesin, L.A.; Omole, R.K.; Johnson, R.M.; Uthman, Q.O.; Babalola, O.O. Biopesticides as a Promising Alternative to Synthetic Pesticides: A Case for Microbial Pesticides, Phytopesticides, and Nanobiopesticides. *Front Microbiol* **2023**, *14*.doi: 10.3389/fmicb.2023.1040901
 155. Chowdhury, A.; Pradhan, S.; Saha, M.; Sanyal, N. *Impact of Pesticides on Soil Microbiological Parameters and Possible Bioremediation Strategies*; **2008**; Vol. 48;doi: 10.1007/s12088-008-0011-8
 156. da Rocha, A.G.; Pitombo, L.M.; Bresolin, J.D.; da Silva, W.T.L.; Espindola, E.L.G.; de Menezes Oliveira, V.B. Single and Combined Toxicity of the Pesticides Abamectin and Difenconazole on Soil Microbial Activity and Enchytraeus Crypticus Population. *SN Appl Sci* **2020**, *2*, doi:10.1007/s42452-020-3175-4.
 157. Filimon, M.N.; Voia, S.O.; Vladiu, D.L.; Isvoran, A.; Ostafe, V. Temperature Dependent Effect of Difenconazole on Enzymatic Activity from Soil. *Journal of the Serbian Chemical Society* **2015**, *80*, 1127–1137, doi:10.2298/JSC141218030F.
 158. Roman, D.L.; Voiculescu, D.I.; Filip, M.; Ostafe, V.; Isvoran, A. Effects of Triazole Fungicides on Soil Microbiota and on the Activities of Enzymes Found in Soil: A Review. *Agriculture* **2021**, *11*.doi: 10.3390/agriculture11090893

159. Dinesh, R.; Anandaraj, M.; Srinivasan, V.; Hamza, S. Engineered Nanoparticles in the Soil and Their Potential Implications to Microbial Activity. *Geoderma* **2012**, *173–174*, 19–27. doi: 10.1016/j.geoderma.2011.12.018
160. Sun, W.; Dou, F.; Li, C.; Ma, X.; Ma, L.Q. Impacts of Metallic Nanoparticles and Transformed Products on Soil Health. *Crit Rev Environ Sci Technol* **2021**, *51*, 973–1002, doi:10.1080/10643389.2020.1740546.
161. Simonin, M.; Richaume, A. Impact of Engineered Nanoparticles on the Activity, Abundance, and Diversity of Soil Microbial Communities: A Review. *Environmental Science and Pollution Research* **2015**, *22*, 13710–13723, doi:10.1007/s11356-015-4171-x.
162. Kumar, A.; Rakshit, R.; Bhowmik, A.; Mandal, N.; Das, A.; Adhikary, S. Nanoparticle-Induced Changes in Resistance and Resilience of Sensitive Microbial Indicators towards Heat Stress in Soil. *Sustainability (Switzerland)* **2019**, *11*, doi:10.3390/su11030862.
163. Nishu, S. Das; Park, S.; Ji, Y.; Han, I.; Key, J.; Lee, T.K. The Effect of Engineered PLGA Nanoparticles on Nitrifying Bacteria in the Soil Environment. *Journal of Industrial and Engineering Chemistry* **2020**, *84*, 297–304, doi:10.1016/j.jiec.2020.01.011.
164. Riah, W.; Laval, K.; Laroche-Ajzenberg, E.; Mougin, C.; Latour, X.; Trinsoutrot-Gattin, I. Effects of Pesticides on Soil Enzymes: A Review. *Environ Chem Lett* **2014**, *12*, 257–273. doi: 10.1007/s10311-014-0458-2
165. Mounissamy, V.C.; Kundu, S.; Selladurai, R.; Saha, J.K.; Biswas, A.K.; Adhikari, T.; Patra, A.K. Effect of Soil Amendments on Microbial Resilience Capacity of Acid Soil Under Copper Stress. *Bull Environ Contam Toxicol* **2017**, *99*, 625–632, doi:10.1007/s00128-017-2173-8.
166. Stressler, T.; Seidl, I.; Kuhn, A.; Fischer, L. Detection, Production, and Application of Microbial Arylsulfatases. *Appl Microbiol Biotechnol* **2016**, *100*, 9053–9067.
167. Wang, J.; Wang, F.; Dai, W.; Wang, S.; Gao, H.; Cao, L.; Sha, Z. Organic Fertilizer Made from Food Waste Improves Nitrogen Mineralization by Altering Aggregate-Associated Microbial Biomass and Enzyme Activities in Chinese Paddy Soil. *J Soils Sediments* **2023**, *23*, 1156–1168, doi:10.1007/s11368-022-03404-8.
168. Menezes, T.; Bouguerra, S.; Andreani, T.; Pereira, R.; Pereira, C. Preparation, Characterization, and Environmental Safety Assessment of Dithiocarbamate Loaded Mesoporous Silica Nanoparticles. *Nanomaterials* **2023**, *13*, 370, doi:10.3390/nano13020370.
169. Paula, A.J.; Martinez, D.S.T.; Júnior, R.T.A.; Filho, A.G.S.; Alves, O.L. Suppression of the Hemolytic Effect of Mesoporous Silica Nanoparticles after Protein Corona

- Interaction: Independence of the Surface Microchemical Environment. *J Braz Chem Soc* **2012**, 23, 1807–1814, doi:10.1590/S0103-50532012005000048.
170. Souto, E.B.; Baldim, I.; Oliveira, W.P.; Rao, R.; Yadav, N.; Gama, F.M.; Mahant, S. SLN and NLC for Topical, Dermal, and Transdermal Drug Delivery. *Expert Opin Drug Deliv* **2020**, 17, 357–377, doi:10.1080/17425247.2020.1727883.
171. Salvi, V.R.; Pawar, P. Nanostructured Lipid Carriers (NLC) System: A Novel Drug Targeting Carrier. *J Drug Deliv Sci Technol* **2019**, 51, 255–267, doi:10.1016/j.jddst.2019.02.017.
172. ISO 10390:2020 Soil, Treated Biowaste and Sludge—Determination of PH **2020**.
173. FAOUN (Food and Agriculture Organization of the United Nations) Physical and Chemical Methods of Soil and Water Analysis **1984**.
174. *Soil Analysis Handbook of Reference Methods*; CRC Press, **2018**; ISBN 9780203739433.
175. Robinson, C., Schinner, F., Ohlinger, R., Kandeler, E., & Margesin, R. Methods in Soil Biology. The Journal of Ecology, **1997**.
176. Eivazi, F.; Tabatabai, M.A. Phosphatases in Soils. *Soil Biol Biochem* **1977**, 9, 167–172, doi:10.1016/0038-0717(77)90070-0.
177. Tabatabai, M.A.; Bremner, J.M. Use of P-Nitrophenyl Phosphate for Assay of Soil Phosphatase Activity. *Soil Biol Biochem* **1969**, 1, 301–307, doi:10.1016/0038-0717(69)90012-1.
178. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M.R. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **2018**, 10, doi: 10.3390/pharmaceutics10020057
179. Shu, X.; Zhang, L.; Liao, W.; Liu, J.; Mao, L.; Yuan, F.; Gao, Y. Nanostructured Lipid Carriers (NLCs) Stabilized by Natural or Synthetic Emulsifiers for Lutein Delivery: Improved Physicochemical Stability, Antioxidant Activity, and Bioaccessibility. *Food Chem* **2023**, 403, doi:10.1016/j.foodchem.2022.134465.
180. Jia, Z.; Li, J.; Gao, L.; Yang, D.; Kanaev, A. Dynamic Light Scattering: A Powerful Tool for In Situ Nanoparticle Sizing. *Colloids and Interfaces* **2023**, 7, 15, doi:10.3390/colloids7010015.
181. Sharma, A.; Madhunapantula, S. V.; Robertson, G.P. Toxicological Considerations When Creating Nanoparticle-Based Drugs and Drug Delivery Systems. *Expert Opin Drug Metab Toxicol* **2012**, 8, 47–69, doi: 10.1517/17425255.2012.637916
182. Kim, I.; Worthen, A.J.; Johnston, K.P.; DiCarlo, D.A.; Huh, C. Size-Dependent Properties of Silica Nanoparticles for Pickering Stabilization of Emulsions and

- Foams. *Journal of Nanoparticle Research* **2016**, *18*, doi:10.1007/s11051-016-3395-0.
183. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M.R. Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems. *Pharmaceutics* **2018**, *10*, doi: 10.3390/pharmaceutics10020057
184. Lunardi, C.N.; Gomes, A.J.; Rocha, F.S.; De Tommaso, J.; Patience, G.S. Experimental Methods in Chemical Engineering: Zeta Potential. *Canadian Journal of Chemical Engineering* **2021**, *99*, 627–639. doi: 10.1002/cjce.23914
185. Sahraee, S.; Ghanbarzadeh, B.; Maryam mohammadi; Pezeshki, A.; hoseini, M. Development of Heat-Stable Gelatin-Coated Nanostructured Lipid Carriers (NLC): Colloidal and Stability Properties. *LWT* **2022**, *160*, doi:10.1016/j.lwt.2022.113265.
186. Fang, C.-L.; A. Al-Suwayeh, S.; Fang, J.-Y. Nanostructured Lipid Carriers (NLCs) for Drug Delivery and Targeting. *Recent Pat Nanotechnol* **2013**, *7*, 41–55, doi:10.2174/1872210511307010041.
187. Das, S.; Chaudhury, A. Recent Advances in Lipid Nanoparticle Formulations with Solid Matrix for Oral Drug Delivery. *AAPS PharmSciTech* **2011**, *12*, 62–76, doi:10.1208/s12249-010-9563-0.
188. Weiss, J.; Decker, E.A.; McClements, D.J.; Kristbergsson, K.; Helgason, T.; Awad, T. Solid Lipid Nanoparticles as Delivery Systems for Bioactive Food Components. *Food Biophys* **2008**, *3*, 146–154, doi:10.1007/s11483-008-9065-8.
189. Ortiz, A.C.; Yañez, O.; Salas-Huenuleo, E.; Morales, J.O. Development of a Nanostructured Lipid Carrier (NLC) by a Low-Energy Method, Comparison of Release Kinetics and Molecular Dynamics Simulation. *Pharmaceutics* **2021**, *13*, doi:10.3390/pharmaceutics13040531.
190. Hegazy, M.; Zhou, P.; Wu, G.; Wang, L.; Rahoui, N.; Taloub, N.; Huang, X.; Huang, Y. Construction of Polymer Coated Core-Shell Magnetic Mesoporous Silica Nanoparticles with Triple Responsive Drug Delivery. *Polym Chem* **2017**, *8*, 5852–5864, doi:10.1039/c7py01179b.
191. Gomaa, E.; Fathi, H.A.; Eissa, N.G.; Elsabahy, M. Methods for Preparation of Nanostructured Lipid Carriers. *Methods* **2022**, *199*, 3–8, doi:10.1016/j.ymeth.2021.05.003.
192. Awad, E.; El-Fiqi, A.; Austin, D.; Lyndon, A. Possible Effect of Lesser Galangal (*Alpinia Officinarum*) Extracts Encapsulated into Mesoporous Silica Nanoparticles on the Immune Status of Rainbow Trout (*Oncorhynchus n*). *Aquac Res* **2020**, *51*, 3674–3684, doi:10.1111/are.14717.

193. Simão, D.O.; Honorato, T.D.; Gobo, G.G.; Piva, H.L.; Goto, P.L.; Rolim, L.A.; Turrin, C.O.; Blanzat, M.; Tedesco, A.C.; Siqueira-Moura, M.P. Preparation and Cytotoxicity of Lipid Nanocarriers Containing a Hydrophobic Flavanone. *Colloids Surf A Physicochem Eng Asp* **2020**, *601*, 124982, doi:10.1016/j.colsurfa.2020.124982.
194. Vairo, C.; Basas, J.; Pastor, M.; Palau, M.; Gomis, X.; Almirante, B.; Gainza, E.; Hernandez, R.M.; Igartua, M.; Gavalda, J.; et al. In Vitro and in Vivo Antimicrobial Activity of Sodium Colistimethate and Amikacin-Loaded Nanostructured Lipid Carriers (NLC). *Nanomedicine* **2020**, *29*, 102259, doi:10.1016/j.nano.2020.102259.
195. Guilherme, V.A.; Ribeiro, L.N.M.; Alcântara, A.C.S.; Castro, S.R.; Rodrigues da Silva, G.H.; da Silva, C.G.; Breikreitz, M.C.; Clemente-Napimoga, J.; Macedo, C.G.; Abdalla, H.B.; et al. Improved Efficacy of Naproxen-Loaded NLC for Temporomandibular Joint Administration. *Sci Rep* **2019**, *9*, 1–11, doi:10.1038/s41598-019-47486-w.
196. Shimojo, A.A.M.; Fernandes, A.R. V.; Ferreira, N.R.E.; Sanchez-Lopez, E.; Santana, M.H.A.; Souto, E.B. Evaluation of the Influence of Process Parameters on the Properties of Resveratrol-Loaded NLC Using 22 Full Factorial Design. *Antioxidants* **2019**, *8*, doi:10.3390/antiox8080272.
197. Zangrando, E.; Islam, M.T.; Islam, M.A.A.A.A.; Sheikh, M.C.; Tarafder, M.T.H.; Miyatake, R.; Zahan, R.; Hossain, M.A. Synthesis, Characterization and Bio-Activity of Nickel(II) and Copper(II) Complexes of a Bidentate NS Schiff Base of S-Benzyl Dithiocarbazate. *Inorganica Chim Acta* **2015**, *427*, 278–284, doi:10.1016/j.ica.2014.12.014.
198. Crouse, K.A.; Chew, K.-B.; Tarafder, M.T.H.; Kasbollah, A.; Ali, A.M.; Yamin, B.M.; Fun, H.-K. Synthesis, Characterization and Bio-Activity of S-2-Picolylthiocarbazate (S2PDTC), Some of Its Schiff Bases and Their Ni(II) Complexes and X-Ray Structure of S-2-Picolyl-β-N-(2-Acetylpyrrole)Dithiocarbazate. *Polyhedron* **2004**, *23*, 161–168, doi:10.1016/j.poly.2003.09.025.
199. Tarafder, M.T.H.; Chew, K.B.; Crouse, K.A.; Ali, A.M.; Yamin, B.M.; Fun, H.K. Synthesis and Characterization of Cu(II), Ni(II) and Zn(II) Metal Complexes of Bidentate NS Isomeric Schiff Bases Derived from S-Methylthiocarbazate (SMDTC): Bioactivity of the Bidentate NS Isomeric Schiff Bases, Some of Their Cu(II), Ni(II) and Zn(II) . *Polyhedron* **2002**, *21*, 2683–2690, doi:10.1016/S0277-5387(02)01285-8.

200. Arriagada, F.; Günther, G.; Nos, J.; Nonell, S.; Olea-Azar, C.; Morales, J. Antioxidant Nanomaterial Based on Core–Shell Silica Nanospheres with Surface-Bound Caffeic Acid: A Promising Vehicle for Oxidation-Sensitive Drugs. *Nanomaterials* **2019**, *9*, doi:10.3390/nano9020214.
201. Nhavene, E.P.F.; da Silva, W.M.; Trivelato Junior, R.R.; Gastelois, P.L.; Venâncio, T.; Nascimento, R.; Batista, R.J.C.; Machado, C.R.; Macedo, W.A. de A.; Sousa, E.M.B. de Chitosan Grafted into Mesoporous Silica Nanoparticles as Benznidazol Carrier for Chagas Diseases Treatment. *Microporous and Mesoporous Materials* **2018**, *272*, 265–275, doi:10.1016/j.micromeso.2018.06.035.
202. Enache, D.F.; Vasile, E.; Simonescu, C.M.; Culita, D.; Vasile, E.; Oprea, O.; Pandele, A.M.; Razvan, A.; Dumitru, F.; Nechifor, G. Schiff Base-Functionalized Mesoporous Silicas (MCM-41, HMS) as Pb(II) Adsorbents. *RSC Adv* **2018**, *8*, 176–189, doi:10.1039/c7ra12310h.
203. Nikoorazm, M.; Ghorbani-Choghamarani, A.; Noori, N. Oxo-Vanadium(IV) Schiff Base Complex Supported on Modified MCM-41: A Reusable and Efficient Catalyst for the Oxidation of Sulfides and Oxidative S-S Coupling of Thiols. *Appl Organomet Chem* **2015**, *29*, 328–333, doi:10.1002/aoc.3295.
204. Siyal, F.J.; Memon, Z.; Siddiqui, R.A.; Aslam, Z.; Nisar, U.; Imad, R.; Shah, M.R. Eugenol and Liposome-Based Nanocarriers Loaded with Eugenol Protect against Anxiolytic Disorder via down Regulation of Neurokinin-1 Receptors in Mice. *Pak J Pharm Sci* **2020**, *33*, 2275–2284, doi:10.36721/PJPS.2020.33.5.SUP.2275-2284.1.
205. Kallakunta, V.R.; Tiwari, R.; Sarabu, S.; Bandari, S.; Repka, M.A. Effect of Formulation and Process Variables on Lipid Based Sustained Release Tablets via Continuous Twin Screw Granulation: A Comparative Study. *European Journal of Pharmaceutical Sciences* **2018**, *121*, 126–138, doi:10.1016/j.ejps.2018.05.007.
206. Ibrahim, T.M.; Abdallah, M.H.; El-Megrab, N.A.; El-Nahas, H.M. Upgrading of Dissolution and Anti-Hypertensive Effect of Carvedilol via Two Combined Approaches: Self-Emulsification and Liquisolid Techniques. *Drug Dev Ind Pharm* **2018**, *44*, 873–885, doi:10.1080/03639045.2017.1417421.
207. Shipp, D.W.; Sinjab, F.; Notingher, I. Raman Spectroscopy: Techniques and Applications in the Life Sciences. *Adv Opt Photonics* **2017**, *9*, 315, doi:10.1364/aop.9.000315.
208. Wang, W.; Zhang, H.; Yuan, Y.; Guo, Y.; He, S. Research Progress of Raman Spectroscopy in Drug Analysis. *AAPS PharmSciTech* **2018**, *19*, 2921–2928, doi:10.1208/s12249-018-1135-8.

209. Bingöl Alpaslan, Y.; Sert, Y.; Sohtun, W.P.; Velusamy, M.; Gökce, H. Structural, Spectral, Electronic, and Molecular Docking Investigations on N,N-Dimethyl-2-[(1E)-{[(Methylsulfanyl)Methanethiyl]Amino}imino)Methyl]Aniline. *Journal of the Chinese Chemical Society* **2021**, *68*, 971–988, doi:10.1002/jccs.202000435.
210. Bhat, R.A.; Kumar, D.; Alam, A.; Mir, B.A.; Srivastava, A.; Malla, M.A.; Mir, M.A. Synthesis, Characterization, Thermal and DFT Studies of S-Methyl- β -N-(3-(2-Nitrophenyl)Allylidene)Dithiocarbamate as Anti-Bacterial Agent. *J Mol Struct* **2018**, *1173*, 72–80, doi:10.1016/j.molstruc.2018.06.061.
211. Abdel Salam, M.S.; Betiha, M.A.; Shaban, S.A.; Elsabagh, A.M.; Abd El-Aal, R.M.; El kady, F.Y. Synthesis and Characterization of MCM-41-Supported Nano Zirconia Catalysts. *Egyptian Journal of Petroleum* **2015**, *24*, 49–57, doi:10.1016/j.ejpe.2015.02.005.
212. Borodko, Y.; Ager, J.W.; Marti, G.E.; Song, H.; Niesz, K.; Somorjai, G.A. Structure Sensitivity of Vibrational Spectra of Mesoporous Silica SBA-15 and Pt/SBA-15. *Journal of Physical Chemistry B* **2005**, *109*, 17386–17390, doi:10.1021/jp051801x.
213. Xiong, G.; Li, C.; Li, H.; Xin, Q.; Feng, Z. Direct Spectroscopic Evidence for Vanadium Species in V-MCM-41 Molecular Sieve Characterized by UV Resonance Raman Spectroscopy. *Chemical Communications* **2000**, 677–678, doi:10.1039/b000557f.
214. Araujo, V.H.S.; da Silva, P.B.; Szlachetka, I.O.; da Silva, S.W.; Fonseca-Santos, B.; Chorilli, M.; Ganassin, R.; de Oliveira, G.R.T.; da Rocha, M.C.O.; Fernandes, R.P.; et al. The Influence of NLC Composition on Curcumin Loading under a Physicochemical Perspective and in Vitro Evaluation. *Colloids Surf A Physicochem Eng Asp* **2020**, *602*, 125070, doi:10.1016/j.colsurfa.2020.125070.
215. Mitsutake, H.; Ribeiro, L.N.M.; Rodrigues da Silva, G.H.; Castro, S.R.; de Paula, E.; Poppi, R.J.; Breitzkreitz, M.C. Evaluation of Miscibility and Polymorphism of Synthetic and Natural Lipids for Nanostructured Lipid Carrier (NLC) Formulations by Raman Mapping and Multivariate Curve Resolution (MCR). *European Journal of Pharmaceutical Sciences* **2019**, *135*, 51–59, doi:10.1016/j.ejps.2019.05.002.
216. Jores, K.; Haberland, A.; Wartewig, S.; Mäder, K.; Mehnert, W. Solid Lipid Nanoparticles (SLN) and Oil-Loaded SLN Studied by Spectrofluorometry and Raman Spectroscopy. *Pharm Res* **2005**, *22*, 1887–1897, doi:10.1007/s11095-005-7148-5.
217. Savić, V.; Ilić, T.; Nikolić, I.; Marković, B.; Čalića, B.; Cekić, N.; Savić, S. Tacrolimus-Loaded Lecithin-Based Nanostructured Lipid Carrier and Nanoemulsion with Propylene Glycol Monocaprylate as a Liquid Lipid: Formulation Characterization

- and Assessment of Dermal Delivery Compared to Referent Ointment. *Int J Pharm* **2019**, 569, doi:10.1016/j.ijpharm.2019.118624.
218. Silva, A.C.; González-Mira, E.; García, M.L.; Egea, M.A.; Fonseca, J.; Silva, R.; Santos, D.; Souto, E.B.; Ferreira, D. Preparation, Characterization and Biocompatibility Studies on Risperidone-Loaded Solid Lipid Nanoparticles (SLN): High Pressure Homogenization versus Ultrasound. *Colloids Surf B Biointerfaces* **2011**, 86, 158–165, doi:10.1016/j.colsurfb.2011.03.035.
219. Doktorovová, S.; Araújo, J.; Garcia, M.L.; Rakovský, E.; Souto, E.B. Formulating Fluticasone Propionate in Novel PEG-Containing Nanostructured Lipid Carriers (PEG-NLC). *Colloids Surf B Biointerfaces* **2010**, 75, 538–542, doi:10.1016/j.colsurfb.2009.09.033.
220. Bardestani, R.; Patience, G.S.; Kaliaguine, S. Experimental Methods in Chemical Engineering: Specific Surface Area and Pore Size Distribution Measurements—BET, BJH, and DFT. *Canadian Journal of Chemical Engineering* **2019**, 97, 2781–2791, doi:10.1002/cjce.23632.
221. Tian, Y.; Wu, J. A Comprehensive Analysis of the BET Area for Nanoporous Materials. *AIChE Journal* **2018**, 64, 286–293, doi:10.1002/aic.15880.
222. Wahab, M.A.; Imae, I.; Kawakami, Y.; Kim, I.; Ha, C.S. Functionalized Periodic Mesoporous Organosilica Fibers with Longitudinal Pore Architectures under Basic Conditions. *Microporous and Mesoporous Materials* **2006**, 92, 201–211, doi:10.1016/j.micromeso.2005.12.016.
223. Talavera-Pech, W.A.; Esparza-Ruiz, A.; Quintana-Owen, P.; Vilchis-Nestor, A.R.; Carrera-Figueiras, C.; Ávila-Ortega, A. Effects of Different Amounts of APTES on Physicochemical and Structural Properties of Amino-Functionalized MCM-41-MSNs. *J Solgel Sci Technol* **2016**, 80, 697–708, doi:10.1007/s10971-016-4163-4.
224. Cao, L.; Zhang, H.; Cao, C.; Zhang, J.; Li, F.; Huang, Q. Quaternized Chitosan-Capped Mesoporous Silica Nanoparticles as Nanocarriers for Controlled Pesticide Release. *Nanomaterials* **2016**, 6, doi:10.3390/nano6070126.
225. Zhang, J.; Kothalawala, S.; Yu, C. Engineered Silica Nanomaterials in Pesticide Delivery: Challenges and Perspectives. *Environmental Pollution* **2023**, 320, doi:10.1016/j.envpol.2023.121045.doi: 10.1021/acssuschemeng.0c00649
226. Zhao, P.; Cao, L.; Ma, D.; Zhou, Z.; Huang, Q.; Pan, C. Synthesis of Pyrimethanil-Loaded Mesoporous Silica Nanoparticles and Its Distribution and Dissipation in Cucumber Plants. *Molecules* **2017**, 22, doi:10.3390/molecules22050817.
227. Kutawa, A.B.; Ahmad, K.; Ali, A.; Hussein, M.Z.; Wahab, M.A.A.; Adamu, A.; Ismaila, A.A.; Gunasena, M.T.; Rahman, M.Z.; Hossain, M.I. Trends in

- Nanotechnology and Its Potentialities to Control Plant Pathogenic Fungi: A Review. *Biology (Basel)* **2021**, *10*, doi:10.3390/biology10090881.
228. Dal Pizzol, C.; Filippin-Monteiro, F.B.; Restrepo, J.A.S.; Pittella, F.; Silva, A.H.; de Souza, P.A.; de Campos, A.M.; Creczynski-Pasa, T.B. Influence of Surfactant and Lipid Type on the Physicochemical Properties and Biocompatibility of Solid Lipid Nanoparticles. *Int J Environ Res Public Health* **2014**, *11*, 8581–8596, doi:10.3390/ijerph110808581.
 229. Chaves-López, C.; Serio, A.; Gianotti, A.; Sacchetti, G.; Ndagijimana, M.; Ciccarone, C.; Stellarini, A.; Corsetti, A.; Paparella, A. Diversity of Food-Borne *Bacillus* Volatile Compounds and Influence on Fungal Growth. *J Appl Microbiol* **2015**, *119*, 487–499, doi:10.1111/jam.12847.
 230. Medeiros, F.H.V.; Pomella, A.W.V.; de Souza, J.T.; Niella, G.R.; Valle, R.; Bateman, R.P.; Fravel, D.; Vinyard, B.; Hebbar, P.K. A Novel, Integrated Method for Management of Witches' Broom Disease in Cacao in Bahia, Brazil. *Crop Protection* **2010**, *29*, 704–711, doi:10.1016/j.cropro.2010.02.006.
 231. Decloquement, J.; Ramos-Sobrinho, R.; Elias, S.G.; Britto, D.S.; Puig, A.S.; Reis, A.; da Silva, R.A.F.; Honorato-Júnior, J.; Luz, E.D.M.N.; Pinho, D.B.; et al. *Phytophthora Theobromicola* Sp. Nov.: A New Species Causing Black Pod Disease on Cacao in Brazil. *Front Microbiol* **2021**, *12*, doi:10.3389/fmicb.2021.537399.
 232. Puig, A.S.; Quintanilla, W.; Matsumoto, T.; Keith, L.; Gutierrez, O.A.; Marelli, J.P. *Phytophthora Palmivora* Causing Disease on *Theobroma Cacao* in Hawaii. *Agriculture (Switzerland)* **2021**, *11*, doi:10.3390/agriculture11050396.
 233. Khare, P.; Singh, V.K.; Pathak, A.K.; Bala, L. Serum Deprivation Enhanced Monocrotophos Mediated Cellular Damages in Human Lung Carcinoma and Skin Keratinocyte. *Gene Rep* **2022**, *27*, 101562, doi:10.1016/j.genrep.2022.101562.
 234. Silva, A.M.; Martins-Gomes, C.; Silva, T.L.; Coutinho, T.E.; Souto, E.B.; Andreani, T. In Vitro Assessment of Pesticides Toxicity and Data Correlation with Pesticides Physicochemical Properties for Prediction of Toxicity in Gastrointestinal and Skin Contact Exposure. *Toxics* **2022**, *10*, doi:10.3390/toxics10070378.
 235. Calabrese, E. Cancer Biology and Hormesis: Human Tumor Cell Lines Commonly Display Hormetic (Biphasic) Dose Responses. *Crit Rev Toxicol* **2005**, *35*, 463–582. doi: 10.1080/10408440591034502
 236. Doktorovova, S.; Souto, E.B.; Silva, A.M. Nanotoxicology Applied to Solid Lipid Nanoparticles and Nanostructured Lipid Carriers - A Systematic Review of in Vitro Data. *European Journal of Pharmaceutics and Biopharmaceutics* **2014**, *87*, 1–18, doi:10.1016/j.ejpb.2014.02.005.

237. Rincón, M.; Silva-Abreu, M.; Espinoza, L.C.; Sosa, L.; Calpena, A.C.; Rodríguez-Lagunas, M.J.; Colom, H. Enhanced Transdermal Delivery of Pranoprofen Using a Thermo-Reversible Hydrogel Loaded with Lipid Nanocarriers for the Treatment of Local Inflammation. *Pharmaceuticals* **2022**, *15*, doi:10.3390/ph15010022.
238. Wang, J.; Xia, Q. Alpha-Lipoic Acid-Loaded Nanostructured Lipid Carrier: Sustained Release and Biocompatibility to HaCaT Cells in Vitro. *Drug Deliv* **2014**, *21*, 328–341, doi:10.3109/10717544.2013.846435.
239. Andreani, T.; Fernandes, P.M.V.; Nogueira, V.; Pinto, V. V.; Ferreira, M.J.; Rasteiro, M.G.; Pereira, R.; Pereira, C.M. The Critical Role of the Dispersant Agents in the Preparation and Ecotoxicity of Nanomaterial Suspensions. *Environmental Science and Pollution Research* **2020**, *27*, 19845–19857, doi:10.1007/s11356-020-08323-0.
240. Vale, G.; Mehennaoui, K.; Cambier, S.; Libralato, G.; Jomini, S.; Domingos, R.F. Manufactured Nanoparticles in the Aquatic Environment-Biochemical Responses on Freshwater Organisms: A Critical Overview. *Aquatic Toxicology* **2016**, *170*, 162–174, doi:10.1016/j.aquatox.2015.11.019.
241. Lekamge, S.; Miranda, A.F.; Abraham, A.; Ball, A.S.; Shukla, R.; Nugegoda, D. The Toxicity of Coated Silver Nanoparticles to the Alga *Raphidocelis Subcapitata*. *SN Appl Sci* **2020**, *2*, 1–20, doi:10.1007/s42452-020-2430-z.
242. Gorbachuk, V. V.; Yakimova, L.S.; Mostovaya, O.A.; Bizyaev, D.A.; Bukharaev, A.A.; Antipin, I.S.; Konovalov, A.I.; Zharov, I.; Stoikov, I.I. Silica Nanoparticles with Proton Donor and Proton Acceptor Groups: Synthesis and Aggregation. *Silicon* **2011**, *3*, 5–12, doi:10.1007/s12633-011-9077-8.
243. Sherif, O.E.; Issa, Y.M.; Abbas, S.M. Thermodynamic Parameters of Some Schiff Bases Derived from 5,7-Dihydroxy-6-Formyl-2-Methylbenzopyran-4-One. *J Therm Anal Calorim* **2000**, *59*, 913–926. doi: 10.1023/A:1010182527146
244. Microbics Corporation Azur Environmental Microtox® Omni Manual 1998.
245. Tilton, F.; La Du, J.K.; Vue, M.; Alzarban, N.; Tanguay, R.L. Dithiocarbamates Have a Common Toxic Effect on Zebrafish Body Axis Formation. *Toxicol Appl Pharmacol* **2006**, *216*, 55–68, doi:10.1016/j.taap.2006.04.014.
246. Mahana, A.; Guliy, O.I.; Mehta, S.K. Accumulation and Cellular Toxicity of Engineered Metallic Nanoparticle in Freshwater Microalgae: Current Status and Future Challenges. *Ecotoxicol Environ Saf* **2021**, *208*.doi: 10.1016/j.ecoenv.2020.111662
247. Nguyen, M.K.; Moon, J.Y.; Lee, Y.C. Microalgal Ecotoxicity of Nanoparticles: An Updated Review. *Ecotoxicol Environ Saf* **2020**, *201*, doi:10.1016/j.ecoenv.2020.110781.

248. Lomba, L.; Lapeña, D.; Ros, N.; Aso, E.; Cannavò, M.; Errazquin, D.; Giner, B. Ecotoxicological Study of Six Drugs in *Aliivibrio Fischeri*, *Daphnia Magna* and *Raphidocelis Subcapitata*. *Environmental Science and Pollution Research* **2020**, *27*, 9891–9900, doi:10.1007/s11356-019-07592-8.
249. Lee, S.H.; Jung, K.; Yoo, W.C.; Chung, J.; Lee, Y.W. Dispersion Stability of 14 Manufactured Nanomaterials for Ecotoxicity Tests Using *Raphidocelis Subcapitata*. *Int J Environ Res Public Health* **2022**, *19*, doi:10.3390/ijerph19127140.
250. Li, H.; Mo, F.; Li, Y.; Wang, M.; Li, Z.; Hu, H.; Deng, W.; Zhang, R. Effects of Silver(I) Toxicity on Microstructure, Biochemical Activities, and Genic Material of *Lemna Minor* L. with Special Reference to Application of Bioindicator. *Environmental Science and Pollution Research* **2020**, *27*, 22735–22748, doi:10.1007/s11356-020-08844-8.
251. Kumar, S.; Nehra, M.; Dilbaghi, N.; Marrazza, G.; Hassan, A.A.; Kim, K.H. Nano-Based Smart Pesticide Formulations: Emerging Opportunities for Agriculture. *Journal of Controlled Release* **2019**, *294*, 131–153. doi: 10.1016/j.jconrel.2018.12.012
252. Nelson, D.D.; Pan, Y.; Tikekar, R. V.; Dan, N.; Nitin, N. Compound Stability in Nanoparticles: The Effect of Solid Phase Fraction on Diffusion of Degradation Agents into Nanostructured Lipid Carriers. *Langmuir* **2017**, *33*, 14115–14122, doi:10.1021/acs.langmuir.7b03407.
253. Gonzalez-Quñones, V.; Stockdale, E.A.; Banning, N.C.; Hoyle, F.C.; Sawada, Y.; Wherrett, A.D.; Jones, D.L.; Murphy, D. V. Soil Microbial Biomass Interpretation and Consideration for Soil Monitoring. *Soil Research* **2011**, *49*, 287–304, doi:10.1071/SR10203.
254. Maddela, N.R.; Venkateswarlu, K. Soil Enzymes: Indicators of Soil Pollution. In *Insecticides–Soil Microbiota Interactions*; Springer International Publishing, **2018**; pp. 7–16. doi: 10.1007/978-3-319-66589-4_2
255. Samarajeewa, A.D.; Velicogna, J.R.; Princz, J.I.; Subasinghe, R.M.; Scroggins, R.P.; Beaudette, L.A. Effect of Silver Nano-Particles on Soil Microbial Growth, Activity and Community Diversity in a Sandy Loam Soil. *Environmental Pollution* **2017**, *220*, 504–513, doi:10.1016/j.envpol.2016.09.094.
256. Pu, S.; Yan, C.; Huang, H.; Liu, S.; Deng, D. Toxicity of Nano-CuO Particles to Maize and Microbial Community Largely Depends on Its Bioavailable Fractions. *Environmental Pollution* **2019**, *255*, doi:10.1016/j.envpol.2019.113248.
257. Lin, J.; Ma, K.; Chen, H.; Chen, Z.; Xing, B. *Influence of Different Types of Nanomaterials on Soil Enzyme Activity: 1 a Global Meta-Analysis* **2021**; 3;

258. Wolinska, A.; Stepniewski, Z. Dehydrogenase Activity in the Soil Environment. In *Dehydrogenases*; InTech, 2012.doi: 10.5772/48294
259. Januszek, K.; Błońska, E.; Długa, J.; Socha, J. Dehydrogenase Activity of Forest Soils Depends on the Assay Used. *Int Agrophys* **2015**, *29*, 47–59, doi:10.1515/intag-2015-0009.
260. Li, X.; Sarah, P. Arylsulfatase Activity of Soil Microbial Biomass along a Mediterranean-Arid Transect. *Soil Biol Biochem* **2003**, *35*, 925–934, doi:10.1016/S0038-0717(03)00143-3.
261. Makoi, J.H.J.R.; Ndakidemi, P.A. Selected Soil Enzymes: Examples of Their Potential Roles in the Ecosystem. *Afr J Biotechnol* **2008**, *7*, 181–191, doi:10.5897/AJB07.590.
262. Fageria, N.K.; Baligar, V.C. Enhancing Nitrogen Use Efficiency in Crop Plants. *Advances in Agronomy* 2005, *88*, 97–185.doi: 10.1016/S0065-2113(05)88004-6
263. Tripathi, S.; Chakraborty, A.; Chakrabarti, K.; Bandyopadhyay, B.K. Enzyme Activities and Microbial Biomass in Coastal Soils of India. *Soil Biol Biochem* **2007**, *39*, 2840–2848, doi:10.1016/j.soilbio.2007.05.027.
264. Bot, A. and B.J. The Importance of Soil Organic Matter: Key to Drought-Resistant Soil and Sustained Food Production: Food & Agriculture Org. **2005**.
265. Ali Askari; Ali Khanmirzaei; Shekoofeh Rezaei Vermicompost Enrichment Using Organic Wastes: Nitrogen Content and Mineralization. *International Journal of Recycling of Organic Waste in Agriculture* **2020**, *9*, 151–160. doi:10.30486/ijrowa.2020.1885015.1001.
266. Akbari, F.; Fallah, S.; Dahmardeh, M.; Pessarakli, M. Interaction Effects of Nitrogen and Phosphorus Fertilizer on Nitrogen Mineralization of Wheat Residues in a Calcareous Soil. *J Plant Nutr* **2020**, *43*, 1–12, doi:10.1080/01904167.2019.1659328.
267. López-Aizpún, M.; Arango-Mora, C.; Santamaría, C.; Lasheras, E.; Santamaría, J.M.; Ciganda, V.S.; Cárdenas, L.M.; Elustondo, D. Atmospheric Ammonia Concentration Modulates Soil Enzyme and Microbial Activity in an Oak Forest Affecting Soil Microbial Biomass. *Soil Biol Biochem* **2018**, *116*, 378–387, doi:10.1016/j.soilbio.2017.10.020.

