Development of a LC-MS-based approach to study the uptake, impact and thermal degradation of nanoencapsulated pesticides (azoxystrobin and bifenthrin) in strawberry plants and fruits.

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Abstract

Sustainable agricultural practices are required to meet the demand of a rapidly increasing global population. In this context, nanoencapsulated pesticides (NEPs) have emerged as a strategy to protect plants and agricultural products from fungi, insects, and others with the promise of a higher efficacy of the active ingredients, minimal environmental impacts and reduced undesirable consequences as compared to conventional pesticides. It is therefore anticipated that the development and the wider usage of NEPs will result in a greater exposure for humans and the environment. In order to properly evaluate their potential risks, a better understanding of the fate of NEPs under realistic conditions is needed. However, there are currently few comprehensive studies which have analyzed the fate, uptake and impact of NEPs in agricultural products under carefully controlled conditions. In fact, the analytical methods for NEPs in currently available pesticide experiments are the same as those applied conventional pesticides. The efficiency of those analytical methods for pesticides encapsuled into nanocarriers has not been validated. The main objective of my research was to develop an analytical strategy to investigate NEPs in strawberry plants, and to compare the fate from field to fork and potential effects on the plant (phenology parameters and phenolic compounds) between conventional and NEPs.

In Chapter 3, analytical methods based on high performance liquid chromatography hyphenated to quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS) were firstly optimized and validated to investigate NEP residues in strawberry tissues and soil. Good performance of the methods including recovery, matrix effect, precision and detection limits were achieved for two NEPs (Allosperse® and $nSiO_2$) loaded azoxystrobin (AZOX) and bifenthrin (BFT) with different physicochemical properties. In Chapter 4, AZOX and BFT loaded into polymeric (Allosperse®) and $nSiO_2$ were applied in strawberry plants in a controlled field system

over two growing seasons, and their effects on plants were evaluated. AZOX was detected and quantified in leachate, soil, leaves, roots and strawberries. Nanocarriers appeared to reduce slightly the AZOX bioaccumulation in fruits. Encapsulation with Allosperse® modified the soil mobility of AZOX.BFT was not detected in strawberries in any of the formulations, confirming that nanocarriers did not modify the non-systemic behavior of this insecticide. Generally, NEPs had no effects on the strawberry plant growth and soil microbial populations. In Chapter 5, a deeper investigation of fruit composition revealed that AZOX and BFT NEPs had small but significant impacts on the total phenolic content (TPC) and profiles in strawberries, compared with conventional AZOX and BFT formulations. Overall, even though NEPs had no apparent effects on the plant phenological parameters, they generated some subtle but significant changes at the molecular level in the plant tissues. In Chapter 6, the thermal degradation kinetics and pathways of AZOX in three formulations (conventional, Allosperse® and $nSiO_2$) were investigated in water, spiked strawberry and incurred strawberry models to understand the fate of the pesticide during thermal food processing. The thermal degradation of AZOX followed the first-order kinetics in the water system (100°C) for all the formulations. Nanocarriers slightly reduced the thermal degradation rate of AZOX in strawberries. A non-targeted workflow was applied to screen extracts for the presence of thermal products (TDPs) of AZOX. Nanoencapsulation did not generate new TDPs for AZOX. Six, four and two TDPs were detected in water, spiked and incurred strawberry models, respectively, and were matched with compounds reported in the literature. This study investigated the thermal degradation pathways of conventional-AZOX and AZOX NEPs for the first time.

Overall, this research demonstrated that encapsulation of a pesticide into a nanocarrier can result in small but measurable changes in the fate and behavior of the active ingredient. The most

notable change impacted the behavior of the target pesticide during their extraction and quantification with method developed for conventional pesticides. Limited effects on plants and soil microorganisms were observed in a realistic experiment conducted under controlled conditions. This research provides new tools for the assessment of NEPs, and contributes to a better assessment of the risk associated with this new technology.

Résumé

Des pratiques agricoles durables sont nécessaires pour répondre à la demande d'une population mondiale en croissance rapide. Dans ce contexte, les pesticides nanoencapsulés (NEPs) sont apparus comme une nouvelle stratégie pour protéger les plantes et les produits agricoles contre les champignons, les insectes et autres avec la promesse d'une plus grande efficacité des ingrédients actifs, des impacts environnementaux minimaux et des conséquences indésirables réduites par rapport aux pesticides conventionnels. Le développement et l'usage plus répandu des NEPs vont probablement conduire a une exposition plus grande a ces substances parmi les populations et dans l'environnement. Une meilleure compréhension du devenir des NEPs en conditions réelles est donc nécessaire pour évaluer correctement les risques potentiels. A date pourtant, peu d'études ont été menée sur le devenir, l'accumulation et les impacts des NEPs dans les systèmes agricoles de manière contrôlée. En fait, les méthodes d'analyse des NEP dans les expériences précédentes sur les pesticides sont les mêmes que celles des pesticides conventionnels. L'efficacité de ces méthodes analytiques pour les pesticides encapsulés dans des nanotransporteurs n'a pas été validée. L'objectif principal de ma recherche a été de développer une stratégie analytique pour étudier les NEPs dans les fraisiers, et de comparer leur devenir du champ à la fourchette et leurs effets potentiels sur la plante (paramètres phénologiques et composés phénoliques) par rapport aux pesticides conventionnels.

Dans le chapitre 3, des méthodes analytiques basées sur la chromatographie liquide à haute performance couplée à l'analyse par spectrométrie de masse à temps de vol quadrupole (HPLC-QTOF-MS) ont d'abord été optimisées et validées pour étudier les résidus de NEP dans les tissus des fraisiers et dans les sols. De bonnes performances des méthodes comprenant la récupération, l'effet de matrice, la précision et les limites de détection ont été obtenues pour deux NEPs

(Allosperse® et nSiO2) chargées d'azoxystrobine (AZOX) et de bifenthrine (BFT) avec des propriétés physicochimiques différentes. Dans le chapitre 4, AZOX et BFT chargés dans un polymère (Allosperse®) et $nSiO_2$ ont été appliqués sur des fraisiers en conditions contrôlées pendant deux saisons de croissance, et leurs effets sur les plantes ont été évalués. L'AZOX a été détecté et quantifié dans l'eau de lessivage, le sol, les feuilles, les racines et les fraises. Les formulations Allosperse® et $nSiO_2$ ont réduit la bioaccumulation d'AZOX dans les fruits. L'encapsulation avec Allosperse® a modifié la mobilité au sol d'AZOX. L'insecticide non systémique – BFT dans toutes les formulations n'a pas été détecté dans les fraises. Globalement, les NEP n'ont pas d'effet sur les paramètres phénologiques de la plante. Dans le chapitre 5, l'analyse plus en détails de la composition des fruits a révélé que les NEPs d'AZOX et de BFT ont un impact faible mais significatif sur la teneur en phénols (TPC) et le profil des fraises, par rapport aux AZOX et BFT en formulations conventionnelles. Globalement, même si les effets des NEP sur les paramètres phénologiques des plantes ne sont pas apparents, ils peuvent avoir des impacts subtiles mais significatifs sur les plantes au niveau moléculaire.

Dans le chapitre 6, la cinétique et les voies de dégradation thermique d'AZOX dans trois formulations (conventionnelle, Allosperse® et $nSiO_2$) ont été étudiées dans des modèles d'eau, de fraise enrichie et de fraise contaminée, afin de comprendre le devenir des résidus pendant les procédés de cuisson ou de transformation des aliments. La dégradation thermique d'AZOX a suivi une cinétique de premier ordre dans le modèle eau (100°C) pour toutes les différentes formulations. Les nanotransporteurs ont légèrement réduit le taux de dégradation thermique de l'AZOX dans les fraises. Un flux de travail en analyse non-ciblée a été appliqué pour filtrer les extraits pour la présence de produits thermiques (TDP) d'AZOX. Les nanotransporteurs n'ont pas généré de nouveaux TDPs. Six, quatre et deux TDP ont été détectés dans l'eau, les modèles de fraises dopés

et contaminés, respectivement, et correspondent à des structures rapportées dans la littérature. Cette étude a étudié pour la première fois les voies de dégradation thermique des NEP conventionnels-AZOX et AZOX.

Dans l'ensemble, cette recherche a démontré que la présence de nanotransporteurs pouvait entraîner une modification légère mais mesurable du devenir et du comportement des NEPs. En particulier, l'encapsulation du pesticide a impacte le comportement du compose actif lors de l'extraction et la quantification du pesticide dans les matrices. Peu d'effets ont été enregistrés sur les plantes et les micro-organismes du sol en conditions réelles d'exposition. Cette recherche a défini de nouveaux outils analytiques pour la gestion des NEPs et contribue à une meilleure évaluation des risques associes avec cette nouvelle technologie.

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Contribution of Authors

This thesis is presented in a manuscript format and consists of seven chapters. Chapter 1 presented a general introduction of nanopesticides application in agricultural systems and the research objectives of the thesis. Chapter 2 contains an up-to-date review of the literature leading to the identification of knowledge gaps related to the fate, impacts and thermal degradation of conventional and nanoencapsulated pesticides in plants and the food chain. Chapters 3 to 6 are four manuscripts arranged sequentially through connecting text. Chapter 3 has been published in the journal *Talanta*. The manuscripts of Chapters 4 to 6 were in preparation. Finally, Chapter 7 was an overall conclusion of the whole thesis and the recommendations for future research.

The present author was responsible for the concepts, design of field work and experiments, laboratory work, data acquisition, data analysis and manuscript preparation in all chapters, except chapter 4. The thesis supervisor, Dr. Stéphane Bayen, and co-supervisor, Dr. Valérie Gravel, oversaw the progression of the experimental work and were involved, along with all co-authors, in the editing of the manuscripts before submission. Dr. Vinicius Bueno and Dr. Subhasis Ghoshal produced the $nSiO_2$-based nanoencapsulation pesticides used in the experiments. In Chapter 4, Dr. Juliana Galhardi was also the joint first author. She completed the soil extraction and the soil enzyme activity analyses and also was involved in the manuscript writing. Dr. Vinicius Bueno completed the soil microbial community assays. Dr. Kevin James Wilkinson, Dr. Subhasis Ghoshal, Dr. Stéphane Bayen and Dr. Valérie Gravel contributed to the experimental design and manuscript preparation. In Chapter 5, undergraduate students Stefania Reino and Alexandra Roginski analyzed the total phenolic content of strawberries collected in the second growth year.

Publications

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Conference Presentations

Wang P., Galhardi J., Gravel V., Liu L., Bueno V., Ghoshal S. Wilkinson K., Bayen S. Comparison of the fate of conventional and nanoencapsulated pesticides in an agricultural field of strawberries. American Chemical Society (ACS) Fall 2021. 22-26 August 2021. (Oral)

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samples. Society of Environmental Toxicology and Chemistry (SETAC) North America 41st Annual Meeting, virtual, 15-19 November 2020. (Poster)

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Abbreviations

AZOX	Azoxystrobin
AzFA	Azoxystrobin free acid
BFT	Bifenthrin
AAFC	Agriculture and Agri-Food Canada
ACN	Acetonitrile
AI	Active ingredient
ANOVA	A nested analysis of variance
APCI	Atmospheric Pressure Chemical Ionization
CMCS	Carboxymethyl chitosan
D_4-AZOX	Azoxystrobin-d4
D5-BFT	Bifenthrin-d5
ECD	Electro capture detector
EIC	Extracted ion chromatograms
ESI	Electrospray ionization
FC	Folin-Ciocalteu
FPD	Flame photometric detector
GAE	Gallic acid equivalents
GC	Gas chromatography
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IDLs	Instrument detection limits

LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
m/z	Mass charge ratio
MDLs	Method detection limits
MgSO4	Magnesium sulfate
MQLs	Method quantification limits
MRLs	Maximum residue limits
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSN	Mesoporous silica nanoparticles
ND	No detected
NEPs	Nanoencapsulated pesticides
NH4Ac	Ammonium acetate
NPD	Nitrogen phosphorous detector
NPs	Nanoparticles
NSERC	Natural Sciences and Engineering Research Council of Canada
OM	Organic material
PAA	Polyacrylic acid
PCA	Principal compound analysis
PHSNs	Hollow silica nanoparticles
PMRA	Pest Management Regulatory Agency

PSA	Primary secondary amine
PTFE	Polytetrafluoroethylene
QqQ	Triple quadrupole
QToF	Quadrupole time-of-flight
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RSD%	Precision, relative standard deviation
RT	Retention time
r^2	The coefficient of determination
S/N	Signal-to-noise ratio
$nSiO_2$	Nano silica dioxide
SPE	Solid phase extraction
TIC	Total ion chromatogram
TDP	Thermal degradation product
TOF	Time-of-flight
TPC	Total phenolic content
USDA	U.S. Department of Agriculture
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

Chapter 1. General Introduction

Strawberries combine a range of excellent features for consumers including an attractive appearance, a good taste, a high nutritional value, and can be used as ingredient in the preparation of a wide range of value-added products. Their remarkable nutritional quality, combining vitamin C, folate and phenolic constituents (Giampieri et al., 2012), makes them one of the fruits with the highest oxygen radical absorbance capacity (Wolfe & Liu, 2007). As a result, strawberries have become popular globally and their global consumption has increased steadily in the years to reach 9.2 million tons in 2017 (IndexBox, 2019). In Canada, strawberries are grown in all provinces. According to Agriculture and Agri-Food Canada (2005), most of the strawberry production is concentrated in Quebec (36%) and Ontario (32%).

Pests and diseases can impact both the quantity and quality of strawberry fruits and can affect considerably strawberry productivity (Abrol & Anil, 2009; Pandey, Shankar, & Sharma, 2012). For strawberry cultures, insect pests such as whiteflies, bugs, aphids, mites, and several fungi-related diseases such as powdery mildew, anthracnose, leather rot, and leaf scorch can induce yield losses. The primary pest and fungi control strategy in strawberry cultures is pesticide use (Garrido et al., 2011). Strawberry plants are quite sensitive to insects, and application of synthetic pyrethroids (e.g., bifenthrin (BFT), fenvalerate) is often recommended as a management strategy for insect pests. Foliar sprays or drench applications of Azoxystrobin (AZOX), a fungicide approved for use on more than 80 different crops across 72 countries (Herrero et al., 2015), can significantly reduce fungi-related diseases in strawberry plants.

Although pesticides prevent some yield losses in the strawberry supply chain, pesticide residues also represent a risk for the environment and human health. For example, the indiscriminate use of pesticides has led to insect resistance and environmental pollution (Yang et

al., 2007). Pesticide usage in the agriculture sector accounted for around 90% of worldwide usage (6 billion pounds) (US EPA, 2017). Regrettably, relatively large proportions of pesticides applied through conventional methods do not reach their target and may end up contaminating the environment (Pimentel & Burgess, 2012). Currently, the use of bifenthrin, which exhibits high acute lethal toxicity to aquatic species, is increasing in agricultural activities (Weston, 2005).

Nanotechnologies have recently gained much attention in this field since they could help the development of more sustainable agricultural practices while maintaining high crop yields. Nanopesticides, in particular, are designed currently to achieve a more efficient usage of the active ingredients (AIs) through the smart and targeted delivery of the substance, therefore reducing application rates of AIs. After many years of research, nanopesticides have started to make their way to the market (Walker et al., 2017). Several commercial nanopesticides have already been validated for specific agriculture crops, such as corn, cotton, dry beans, potatoes, soybeans (Vive Crop Protection, 2021). Because of their excellent performance in managing the pests, AZOX and BFT have appeared as candidate AIs for inclusion into nanocarriers to produce nanopesticides. To date, nanopesticides including AZOX or BFT as AI, marketed as AZteroid®FC or Bifender®FC, have been registered for some crops (not including strawberry) in the US but not in Canada (Vive Crop Protection, 2021).

Although nanopesticides are suggested to lower risks for the environment and human health compared to their conventional pesticide formulation counterparts, there are still few available data to confirm this feature. For example, there are still few systematic comparisons of the conventional pesticides versus nanopesticides on the treated plants under field conditions (Kah et al., 2018), and notably none for strawberry plants and fruits. Pesticides may undergo metabolism or degradation after application in the field via multiple pathways depending on the chemical

structure of the pesticides, the organism, environmental conditions, and metabolic factors (Van et al., 2003). To the best of my knowledge, there are no published studies on possible differences in the degradation/metabolism of nanopesticides in cultivated plants. In addition, conventionally formulated pesticides are known to interfere with the synthesis of the plant's primary and secondary products, such as phenolic profiles (Lydon & Duke, 1989; Herms et al., 2002; Sundravadana et al., 2007; Debona et al., 2018). No research has been done to investigate the impact of nanopesticides on plant metabolites. Data on the fate of the residues in soil, plants, edible parts such as fruits are critically needed to fully assess the risks and benefits of nanopesticides.

In the end, strawberries are often consumed directly as fresh fruits but are also processed into several value-added products (Sharma et al.,2013; Nile & Park, 2014). Post-harvest processing steps, such as thermal treatments, are also often applied to minimize losses and increase profit. Pesticide residues may degrade during thermal processing steps. The *Codex Alimentarius* recommends investigating the pesticide residues and identifying the breakdown or reaction products generated by processing (2008). The levels of pesticides such as dichlofluanid, procymidone and iprodione were shown to decrease during the production of strawberry juice and wine (50% to 100%) but remain relatively high in the strawberry jam (Hendawi, Romeh, & Mekky, 2013; Will & Kruger, 1999). To date, there is no study on the fate of nanopesticides during food processing.

The analysis of pesticide residues in environmental and food matrixes is essential to understand all the various aspects abovementioned related to the fate and the risks of pesticides. The traditional analytical approach to monitor pesticide residues in soil, water, plant and food samples relies on the extraction and the detection/quantification with instruments such as gas/liquid chromatography (GC/LC), mass spectrometry (MS) or tandem mass spectrometry

(MS/MS) (Christensen, Granby, & Rabolle, 2003; Looser et al., 2006; Fernandes et al., 2011; Souza et al., 2014). Recently, a novel approach, named Quick Easy Cheap Effective Rugged and Safe (QuEChERS), has become extremely popular for the routine analysis of pesticides. This approach involves an extraction step with acetonitrile, followed by clean-up steps based on dispersive solid-phase extraction with MgSO4, primary secondary amine (PSA) and/or C18 sorbents. The recoveries of all the conventional pesticides under study are in the range of 46-128% (European Commission, 2015). The limit of detection (LOD) for all compounds met maximum residue limits (MRLs). To date, no analytical method has been validated to analyze nanopesticides in the literature.

1.1 Research hypothesis

The present study was conducted with the hypothesis that:

Hypothesis 1: The analytical behaviour of active pesticide ingredients differs for NEPs and conventional pesticide formulations, and, as a result, existing analytical methods validated for conventional pesticides are not applicable for nanoencapsulated pesticides.

Hypothesis 2: The fate of nanoencapsulated pesticides in a soil/strawberry plant system and effects on plant growth are different compared to conventional pesticide formulations, as a result of a change in the physicochemical properties of the pesticide.

Hypothesis 3: Nanoencapsulation of the pesticides can modify the phenolic content of strawberry fruits compared to the conventional pesticides due to a higher efficiency of nanopesticides and lower self-defence mechanisms in the plants.

Hypothesis 4: Nanoencapsulation of the pesticides can modify the thermal degradation rate and products of AZOX in strawberry fruits.

1.2 Research objectives

The overall goal of my research is to investigate the accumulation and the impact of AZOX and BFT, applied either as conventional and nanopesticide formulations, in strawberry plants, and to understand the fate of their residues during the post-harvest processing of strawberry fruits. More specifically, the research objectives are:

AIM 1: To develop and validate a method for the simultaneous analysis of the residues of two pesticides (azoxystrobin and bifenthrin) in soil and strawberry fruits, when applied in their conventional formulation or encapsulated in different classes of nanocarriers (Allosperse® and nano-SiO_2).

AIM 2: To describe the fate of azoxystrobin and bifenthrin nanopesticides in a controlled soil-strawberry plant system, and to determine the possible bioaccumulation of nanopesticides in plants.

AIM 3: To determine the impact of AZOX and BFT NEP treatments on the metabolites and notably the phenolic profile of strawberry fruits.

AIM 4: To assess the degradation of the conventional and AZOX nanopesticides during the thermal processing of strawberry fruits and the potential degradation products. Targeted and nontargeted analytical methods will be developed to study AZOX thermal degradation products.

Chapter 2. Literature Review

A relatively large proportion of pesticides applied as conventional formulations is not reaching their target (Pimentel & Burgess, 2012). In this context, nanoencapsulated pesticides (NEPs) have been developed as a strategy to protect crops from pests, promising higher active ingredient efficacy than conventional pesticides and decreased environmental impacts. Recently, nanoencapsulated pesticides have been introduced in the market. To understand the challenges associated with use of nanopesticides for fruits such as strawberries, it important to review the literature on strawberry plants (morphology and metabolites composition), on the fate of conventional and nanopesticides, and on analytical methods for pesticide residues and phenolic compounds. The goal of this section was to identify gaps in the current scientific knowledge related to the fate, impacts and thermal degradation of NEPs in strawberry plants and fruits under real conditions.

2.1 Strawberry

2.1.1 Strawberry plants

The cultivated strawberry is a perennial plant member of the Rosaceae (rose) family (Agriculture and Agri-Food Canada, 2005). The most cultivated strawberry, *Fragaria* × *ananassa*, is a hybrid of *Fragaria chiloensis* (L.) Duch. and *Fragaria virginiana* (Duch.), two wild species from America (Thomas, Murray & Murphy, 2016). The varieties of strawberries can be divided into three groups based on the harvest period: June-bearing, ever-bearing, and day-neutral (Agriculture and Agri-Food Canada, 2005).

June-bearings are the most popular and common type, especially in northern climates. They produce the largest fruits over a period of two to three weeks in June. Most June-bearing

strawberries are *Fragaria* × *ananassa* species. In Quebec, the most popular *Fragaria* × *ananassa* species are Annapolis, Chambly, Harmonie, Honeoye, Kent, La Cle des Champs, Mira, Saint Laurent, Saint-Pierre, and Yamaska. Furthermore, June-bearing can be classified into Early-, Mid- and Late seasons (Agriculture and Agri-Food Canada, 2005).

Ever-bearings generally produce two or three harvests per year. One is in the spring, and another is in the late summer or early fall. Most ever-bearing strawberry types are also *Fragaria* × *ananassa*, but some are the species of *Fragaria vesca*. Ever-bearing is not recommended in Quebec by the Agriculture and Agri-Food Canada (2005).

Day neutral plants produce a good yield in their first year (Agriculture and Agri-Food Canada, 2005). Day neutral plants show insensitivity to photoperiod, flowering and fruiting at the same rate throughout a growing season of dynamic changes in day-length (Durner et al. 1984). The recommended day neutral varieties in Quebec are Albion, Charlotte, Mara des bois, Monterey, and Seascape. Seascape belongs to *Fragaria* × *ananassa* variety. The University of California developed this species in 1991. The peak production of Seascape is in August and early September. Day neutral is highly successful for northeastern locations in North America.

The strawberry plant is a typical hardy, perennial, rosette plant. Every strawberry plant has a compressed stem (crown) from which roots, trifoliate leaves, stolons (runners), and inflorescences emerge (Figure 2.1). The strawberry plant is composed of the root and shoot system. The root system is located mainly (80%-90%) in the upper 15 cm of soil (Ellis et al., 2006). However, 20 cm of soil is the lowest limit for strawberry growth. The roots' growth follows a fixed pattern of three new roots emerging from each side of the base of every new leaf. Generally, there are 20 to 35 primary active roots in the mature period (Ellis et al., 2006).

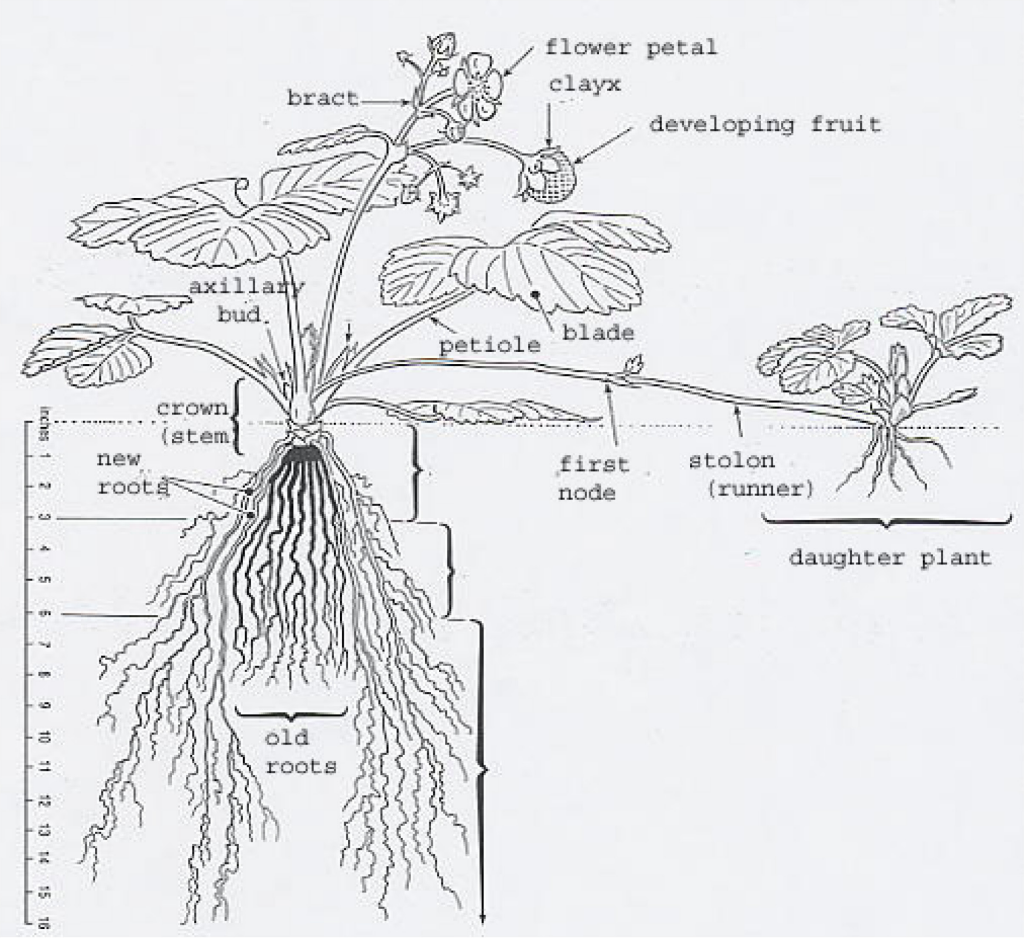


Figure 2. 1 Schematic morphology of a strawberry plant (Strand, 2008)

The shoot system of the strawberry plants includes the petiolate leaves, the inflorescence, and the crown (Poling, 2012). Some axillary buds can develop into branches with long internodes called runners, which produce new leaves and roots at the nodes. The runners are used for next year's propagation. The flowers and fruits are produced on a stalk that emerges from an axillary bud (Figure 2.1).

2.1.1 Strawberry composition

The interest of consumers in nutraceutical-rich foods, especially fruits and vegetables, is increasing due to the increased knowledge about the association between nutrition and health (Paredes-Lopez et al., 2010). Thereby the consumption of natural products, for example, strawberries have become popular globally and their global consumption has increased steadily in the years to reach 9.2 million tons in 2017 (IndexBox, 2019). Strawberries are widely consumed fresh or as processed food products (jams, juices) and are quite popular for they contain high content of essential nutrients (e.g., essential fatty acids, vitamins and minerals) and beneficial phytochemicals such as phenolic compounds, which have biological activities (e.g., antioxidant) in human health (Proteggente et al., 2002; Halvorsen et al., 2006). Strawberry plants' vegetative parts and roots can also be used in infusions and decoctions for different medicinal purposes (Dias et al., 2015a).

The general nutrient composition of strawberry plant tissues is presented in Table 2.1. A detailed nutrient composition of the vegetative parts and roots of strawberry plants has been reported by Dias et al. (2015a). Compared to vegetative parts and roots, strawberry fruits generally contain a relatively higher concentration of lipids and proteins, and smaller amounts of carbohydrates. Vegetative parts contain a higher ash content than roots and strawberry fruits. According to the profile of strawberry nutrients, the strawberry is a good source of dietary fiber, minerals, and essential fatty acids for human nutrition (Proteggente et al., 2002; Scalzo et al., 2005; US Department of Agriculture, 2010).

Table 2. 1 Nutrient composition of vegetative parts, roots and fruits of strawberry plants (adapted from Proteggente et al., 2002; Scalzo et al., 2005; US Department of Agriculture, 2010; Dias et al., 2015a)

	Vegetative parts	Roots	Fruits
Total lipid (g per 100g dry weight basis)	2.9	1.6	3.3
Proteins (g per 100g dry weight basis)	6.4	3.9	7.4
Ash (g per 100g dry weight basis)	7.5	5.9	0.4
Carbohydrates (g per 100g dry weight basis)	83.2	88.6	84.9
Water (%)	64	65	91

Relatively high antioxidant and anti-inflammation properties have been reported in strawberry fruits and may be partly attributed to phenolic compounds (Costantino et al., 1992; Prior et al., 1998). In a comparative study by Wolfe and Liu (2007), strawberries had the highest oxygen radical absorbance capacity among fruits such as plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear, and honeydew melon, presumably due to their rich phytochemical composition.

Phenolic compounds, a wide set of variable secondary metabolites of plants, share a common structural backbone comprised of one or many aromatic benzene rings along with a minimum of one hydroxyl functional group, which conduct the antioxidant capacity (Cheynier, 2012). In general, plant tissues may contain up to several grams per kilogram of phenolics (Daniel et al., 1999). The synthesis of phenolic compounds can be modulated by external stimuli such as pathogen infections, temperature, UV and chemical stresses (Li, Tsao & Deng, 2012; Chowdhary et al., 2021). In fact, phenolics serve as a self-defence mechanism by scavenging free radicals due to an o-benzoquinone counterpart, metal chelation and endogenous antioxidant system

upregulation against oxidation damage, microbial pathogens and herbivorous insects (Chowdhary et al., 2021).

Interest in phenolic compounds has increased due to their various health-promoting properties to prevent chronic diseases. Their health benefits are mainly attributed to their antioxidant and anti-inflammatory properties that could prevent and/or treat cardiovascular diseases, neurodegenerative disorders, cancers, diabetes, among others (Perez-Jimenez et al., 2010; Singh, Holvoet, & Mercenier, 2011). For example, plant-derived phenols have shown *in vitro* potential for decreasing the risk of developing type 2 diabetes, cardiovascular diseases, neurological disorders, inflammatory diseases, and cancers (Pinto, Lajolo & Genovese, 2008; Cassidy et al., 2013). The antioxidant potential of phenolic compounds also protects biological macromolecules, namely proteins and nucleic acids, from oxidative stress or from an imbalanced production of free radicals in the body (Ellis et al., 2011).

Flavonoids (anthocyanins, flavonols, and flavanols) are the primary class of phenolic compounds in strawberries, but other families of phenolics such as hydrolyzable tannins and phenolic acids are also present in fruits (See Table 2.2) (Hakkinen & Torronen, 2000). Condensed tannins are minor constituents in strawberry fruits (Kahkonen, Hopia, & Heinonen, 2001; Maatta, Kamal, & Torronen, 2004).

Anthocyanins are the major polyphenolic compounds in strawberries (Clifford, 2000), and are responsible for the red color of strawberries. The total content of anthocyanins generally ranges from 150 to 600 mg/kg in fresh strawberries (Lopes et al., 2002). Although glucose seems to be the most common sugar in strawberry anthocyanins, other sugars (e.g., rutinose, arabinose, and rhamnose) have also been conjugated with phenolic compounds (Silva et al., 2007). Therefore, pelargonidin-3-glucoside is the dominant anthocyanin in strawberry (Aaby, Skrede, & Wrolstad,

2005; Aaby, Ekeberg, & Skrede, 2007). Anthocyanin concentrations in different strawberry cultivars at different locations were not affected by environmental conditions but significant differences were observed among cultivars (Carbone et al., 2009; Aaby et al., 2011). The study of dietary anthocyanins' functional effects revealed that anthocyanins might prevent and manage type 2 diabetes by protecting pancreatic beta cells, decreasing starch digestion due to the suppression of enzyme activity, and inhibiting advanced glycation end-product formation (Xiao & Hogger, 2015).

Ellagitannins (ETs) are detected only in berries from the Rosaceae family (e.g., strawberry and raspberry) at reported levels ranging from 21.7 to 83.2 mg per 100g fresh weight (Koponen et al., 2007). ETs content of strawberries is from 25 to 59 mg per 100g fresh weight (Mattila & Kumpulainen, 2002). ETs are the combinations of ellagic acid and hexahydroxydiphenic acid with glucose (hexahydroxydiphenoyl-glucose or HHDP), with a wide range of structures including monomers (e.g., ellagic acid glycosides), oligomers (e.g., sanguiin H-6, the most typical ET in the strawberry) and complex polymers (Aaby, Ekeberg, & Skrede, 2007; Giampieri et al., 2012; Skupien & Oszmianski, 2004). The hydrolysis of ETs releases ellagic acid. Ellagitannins and ellagic acid improve human health by affecting intestinal immune function and activating the short-chain fatty acids excretion (Kawabata, Yoshioka, & Terao, 2019).

Strawberries also contain small amounts of other phenolics. The identified flavonols in strawberries are the derivatives of quercetin and kaempferol (Aaby, Ekeberg, & Skrede, 2007). Flavanols in strawberries are in monomeric (catechins) and polymeric forms (procyanidins) (Aaby, Ekeberg, & Skrede, 2007). Besides antioxidant properties, flavonoids could also modulate the protein functions through interactions between their hydroxyl groups and amino and carbonyl groups in proteins to exhibit anti-inflammatory function. Therefore, flavonoids are involved in

gene expression and cell signaling, leading to protective effects throughout the human body (EFSA, 2015).

Strawberries also contain a variety of phenolic acids, including derivatives of hydroxycinnamic acids (e.g., *p*-coumaric acid and caffeic acid) and hydroxybenzoic acids (e.g., dihydroxybenzoic acids, gallic acid and vanilla acid) (Aaby, Ekeberg, & Skrede, 2007; Maatta, Kamal, & Torronen, 2004). The predominant phenolic acids in strawberries are hydroxybenzoic acids and *p*-coumaric acid. Phenolic acids are known for diverse biological applications. The critical advantage is antioxidants due to avert the damage of cells resulting from free-radical oxidation reactions. Phenolic acids in diet also promote the anti-inflammation capacity (Kumar & Goel, 2019).

Table 2. 2 Phenolic composition reported in of strawberries (adapted from Aaby, Ekeberg, & Skrede, 2007; Dias et al., 2015a; Dias et al., 2015b; Silva et al., 2007; Simirgiotis & Schmeda, 2010).

Class	Group	Fruits
Flavonoids	Flavonols	Quercetin-(glucoside or rutinoside)
		Kaempherol-3-(glucoside or malonylglucoside)
	Flavanols	Proanthocyanidin B1
		Proanthocyanidin B2
		Proanthocyanidin trimer
		(+)-catechin
	Anthocyanins	Cyanidin-3-(glucoside or rutinoside or malonylglucoside)
		Pelargonidin-3-(glucoside or rutinoside or malonylglucoside or arabinoside or diglucoside etc.)
Phenolic acids		p-Coumaric acid, gallic acid, hydroxybenzoic acid, caffeic acid, vanillic acid
Hydrolyzable tannins	Ellagitannins	Ellagitannin
		Sanguiin H-6
		Ellagic acid

2.2 Pesticides

Powdery mildew (*Podosphaera aphanis*), a type of fungal disease, has been observed to be responsible for up to 30% yield losses in Seascape strawberry cultures (Carisse et al., 2013). Other fungal diseases for strawberry plants include fruit rot caused by anthracnose fruit rot (*Colletotrichum acutatum*), gray mold (*Botrytis cinereal*), crown rot (*Phytophthora cactorum*), and some foliar diseases such as leaf scorch (*Diplocarpon earlianum*), Ramularia leaf spot (*Mycosphaerella brunnea*) and angular leaf spot (*Xanthomonas fragariae*) (Sharma et at., 2019). Arthropod pests cause some decreases in strawberry yields (Solomon et al. 2001), including two-spotted spider mites, western flower thrips, aphids, whiteflies, and spotted wing fruit flies. Strawberry softening and decaying rates are very high due to various pathogens including *Rhizopus stolonifera* Vuill and *Mucor* sp. (Angioni et al., 2004). In order to minimize the loss caused by those various pathogens, pesticides have been introduced in strawberry production.

Pesticides are chemical compounds used to prevent, destroy, repel and kill pests, including insects (i.e., insecticides), rodents (i.e., rodenticides), fungi (i.e., fungicides), unwanted plants such as weed (herbicides), and microorganisms (i.e., bactericides) (WHO, 2018). There are 1383 active substances used worldwide and 1 billion pounds in the U.S. in 2009 (Alavanja, 2009). Pesticides can also be organized by their chemical classes, i.e., as groups of compounds sharing some structural similarities. For example, strobilurins are a group of fungicides including azoxystrobin (AZOX), pyraclostrobin, fluoxastrobin, kresoxim-methyl, trifloxystrobin, picoxystrobin, mandestrobin, and metominostrobin, which share a basic chemical structure named β - methoxyacrylate moiety. Pyrethroids as a group of insecticides such as allethrin, bifenthrin, cyfluthrin, cypermethrin, and cyphenothrin have an alcohol group of the ester of chrysanthemic acid derivatives.

The losses of food yield caused by fungi, insects, and weeds are significant. In response, farmers use a considerable amount of pesticides to minimize these losses, and around 5.6 billion pounds of pesticides have been used wordwide per year (Atwood & Paisley-Jones, 2017). The release of pesticide residues from agriculture may become pollution issue with adverse effects on humans, animals, and the environment. Therefore, the use of pesticides has been managed and monitored by governments and organizations. The Pest Management Regulatory Agency (PMRA) are responsible for the assessment and registration of pesticides used in Canada. Before the pesticide registration, applicants have to submit some required information, including product chemistry and performances, toxicity study to determine hazard to humans, animals, and organisms, exposure studies, environmental fate studies, and residue studies (Government of Canada, 2019). Pesticide metabolite residues commonly exist in food, and the *Codex Alimentarius* (2008) has suggested that pesticide metabolites should also be analyzed in food, The analysis of all pesticide metabolites is, however, not commonly included in the routine surveillance program.

2.2.1 Azoxystrobin

The systemic, broad-spectrum azoxystrobin (AZOX) was first introduced in 1992 by J. R. Godwin. AZOX inhibits the electron transport system by binding the Qo site of cytochrome b and cytochrome c1 to inhibit mitochondrial respiration (Von, Gribble, & Trumpower, 1986). AZOX has been registered mainly for wheat, fruit including strawberries and grapes, and vegetables in about 70 countries on 80 crops (Ghosh & Singh, 2009b). Forty-three AZOX pesticide products are currently fully registered in Canada (Health Canada, 2022). Because of its highly efficient and broad-spectrum character, AZOX occupies a large portion of the market share (Lu et al, 2019). The physical properties of AZOX are stable (Table 2.3) and AZOX is indispensable for modern

agriculture. Due to widespread application, AZOX residue has been detected in many waters and crops with a concentration up to 6 mg/kg (Chen et al., 2021). AZOX eventually transferred and accumulated in the non-target organisms, causing adverse effects on aquatic organisms, as well as human health risk (Rodrigues, Lopes, & Pardal, 2013).

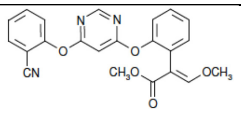
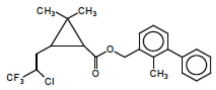
AZOX, under recommended dosage, can control foliar and soil-borne fungal diseases, including anthracnose (*Colletotrichum fragariae*), leather rot (*Phytophthora cactorum*), powdery mildew (*Podosphaera aphanis*) and suppression of botrytis (*Botrytis cinerea*) on the foliage of strawberry plants, and soil-borne diseases such as seeding root rot and basal stem rot related to *Rhizoctonia solani* (Sharma et al., 2019). AZOX provides excellent activity against the leather rot of strawberries, a disease responsible for up to 30% loss of fruits (Rebollar, Madden, & Ellis, 2007). The current Maximum Residue Level (MRL) of AZOX in strawberries is 10 mg/kg (Health Canada, 2016).

2.2.2 Bifenthrin

Bifenthrin (BFT) is listed as a moderately hazardous compound by the WHO and is one of the most important insecticides presently (Zhao et al., 2021). BFT is a non-systemic, broad-spectrum synthetic pyrethroid insecticide, commonly used to control a range of pests sucking and biting leaves in a wide range of crops. BFT is an organofluorine compound with a cyclopropanecarboxylate ester. It works on contact and ingestion to paralyze the insect, causing death by interfering with nerve cells' ability to transfer signals like most pyrethroid pesticides (Yang & Li, 2015). BFT was first produced and developed in 1984 by FMC Corporation in America and first evaluated by JMPR in 1992 (FAO, 2017). Because of high toxicity to aquatic organisms, BFT is classified as "restricted use pesticides", which should be sold or used by

Certified Pesticide Applicators. However, the usage of BFT is still extensive and has become increasingly popular since 2000 (Feo, Eljarrat, & Barcelo, 2010; Luo & Zhang, 2011). The consumer sales of BFT in California in 2005 was 4759 kg AI (Krieger, 2010). BFT has been registered mainly for cereals, cotton, corn, alfalfa, hay, grass seed, some fruits, including strawberries, ornamentals, and vegetables. In the strawberry cultures, BFT targets aphids, armyworms, flea beetles, plant bugs, spittlebugs, stink bugs, strawberry clippers, strawberry sap beetle, strawberry root weevil and spider mites. The MRL of BFT in strawberries is 1 mg/kg (Codex Alimentarius, 1995).

Table 2. 3 Structure, formula and physicochemical properties of azoxystrobin and bifenthrin (adapted from PubChem, 2019)

	Azoxystrobin	Bifenthrin
Molecular Formula	$C_{22}H_{17}N_3O_5$	$C_{23}H_{22}ClF_3O^2$
Structure		
Molecular weight g/mol	403.392	422.872
Melting Point ℃	116	69
Density g/cm3	1.096	1.024
Solubility (in water) mg/L	6	<0.001
Vapor pressure @25℃ (mm Hg)	$7.3*10^{-14}$	$1.34*10^{-8}$
Log K_{ow} @ 20 ℃	3.7	6.6
Stability	stable in the presence of light	Stable in natural daylight and water
ADI mg/kg/day	0.2	0.015
LD50 Rat Oral g/kg	5	0.375

2.2.3 Environmental fate of pesticides

The use of pesticides in agriculture may lead to the residues in the environment. The fate of pesticides includes the transport of parent compounds in the soil, air and water. Pesticides may bind to soil matter, or freely dissolve in water and bind to sediments, or volatilize into the atmosphere. The pesticides bio-uptake and metabolism by organism is also an important part of the pesticides fate. Some pesticides are systemic which can transfer into the plants and lead to bioaccumulation

After field application (soil drenching or foliar spraying), AZOX and BFT have low volatilization rates in soil because of their low vapor pressure (see Table 2.3). Reported half-lives of AZOX in agricultural soils range from 58 to 87 days (Edwards et al., 2016), while for BFT, reported half-life in soil is 125.3 ± 13.3 days (Kah et al., 2016). Once AZOX reached the soil, it interacts with some organic and mineral constituents, or undergoes physical and biological transformations (Bending, Lincoln, & Edmondson, 2006). Joseph reported AZOX in sterile soil had a shorter half-life of 14 days than in nonsterile soils (1999). BFT has a low solubility in water and a correspondingly strong tendency to bind to soil or sediment. BFT is relatively photostable with a 106 days photodegradation half-life (Fecko, 1999). Additionally, the dissipation half-lives of BFT are in the range from 122 to 345 days in various soil conditions and BFT residues in the soils are affected by many factors, such as pH, micro-organisms, moisture status, and the soil types (Ghosh & Singh, 2009a; Kah et al., 2016; Xu et al., 2018).

2.2.4 Effects of pesticides on phenolic compounds

Pesticides have not only effects on pests but also impact crops, possibly resulting in undesired effects on the biological functions of plants. For example, some pesticides have also

been reported to influence the flavor of fruits and vegetables (Gunther & Gunther, 2013). Furthermore, the application of high level of fungicides such as AZOX on strawberries have been suggested to inhibit the stimulation of self-defence mechanisms, such as increasing the synthesis of phenolic compounds by decreasing pest pressures (Abountiolas et al., 2018). Therefore, it is essential to know about the biological effects of these pesticides on their protected plants. No data have been reported on the effect of BFT on plant phenolics.

Pesticides could modulate the levels of phenolic compounds in the plant in several ways. For example, external stimuli such as microbial infections with low or high doses have been proposed to enhance or suppress the accumulation of certain phenolic compounds in plants (Belles et al., 2006). As fungicides can reduce the microbiological infections, the synthesis of some phenolic compounds associated with plant defense strategies could be also stimulated, leading to the accumulation of phenolic compounds in plants. Systemic pesticides, taken up by the plants through leaves or roots, may directly modulate their physiology. These exogenous chemicals may then interfere with the synthesis of the plant's primary and secondary products (Lydon & Duke, 1989). Previous studies investigated the difference in total phenolic content (TPC) in strawberry plants from organic vs. conventional cultivation. TPC values in organic or lower-fungicide (including AZOX) strawberries were higher than in conventionally cultivated strawberries (Hakkinen & Torronen, 2000; Olsson et al., 2006; Reganold et al., 2010; Fernandes et al., 2012; Abountiolas et al., 2018), since no pesticides or low level of pesticides in strawberry plants could encourage the synthesis of phenolic compounds serving as self-defence mechanisms to decrease pest stresses. The same results were observed when AZOX was applied to tobacco (Herms et al., 2002) and rice plants (Sundravadana et al., 2007; Debona et al., 2018; Abed et al., 2018).

As herbicides target plants themselves, the effects of herbicide substances on the crops to protect have attracted a lot of attention. In particular, the mechanisms of herbicides impacting the phenolics' synthesis were to reduce the carbon fixation or block the shikimate pathway to interfere with the formation of aromatic amino acids (Daniel et al., 1999). The pathways of fungicides and insecticides that impact the synthesis of plant individual phenolic compounds are still unclear. Further studies are needed to assess the mechanisms of fungicides and insecticides working on phenolic synthesis.

2.3 Nanopesticides

Relatively large proportions of pesticides applied through conventional methods do not reach their target (Pimentel & Burgess, 2012). Such indiscriminate use and misuse of pesticides have caused damage to the environment. In the end, the initial intended benefits of the pesticides are weakening due to the non-target impact, ineffectiveness of formulation, and emerging resistance in the environment among pests. As an emerging technique, nanotechnology has the potential to revolutionize pesticide applications and develop a new generation of pesticides. The nanoformulation can change the physiochemical properties of pesticides, e.g., surface area, solubility, and mobility. Some industries have already started developing nanopesticides and applied them to different agriculture crops (Kah, Weniger, & Hofinann, 2016; Xu et al., 2018). There are three major types of novel nanopesticides, including nanoencapsulation, nanoemulsification, and nanoparticles (Ranjan, Dasgupta, & Lichtfouse, 2016). The present study focused on nanoencapsulation pesticides.

A first breakthrough of nanoencapsulation pesticide is the nanocarriers (polycaprolactone and polylactic acid nanospheres) loaded with insecticides ethiprole (Boehm et al., 2003). In this case, the nanoformulation enhanced penetration of the AI through the leaves. Furthermore,

controlled release nanoencapsulated fungicide (tebuconazole and chlorothalonil) has been observed with 100-250 nm nanocarriers of polyvinyl pyridine and polyvinyl pyridine co-styrene (Liu et al., 2001). Later, some inorganic nanoparticles such as $nSiO_2$ were produced as carriers for various pesticides (Wang et al., 2004).

2.3.1 Nanocarrier categories

Nanocarriers can be either organic or inorganic. The organic nanocarriers, also called "soft" nanocarriers, include chitosan, starch, cellulose, and polyester materials (Chhipa, 2017). Chitosan nanocarriers have been reported to deliver several pesticides in plants (Ding et al., 2011; Feng & Zhang, 2011). Polyhydroxyvalerate-based nanocarriers has been used to control the release rate of specific herbicides (Grillo et al., 2011; Lobo et al., 2011). Furthermore, amino group functionalized mesoporous silica nanoparticles (MSN) and carboxymethyl chitosan (CMCS) were combined to make a new type of nanoencapsulation of AZOX (AZOX-MSN-CMCS), which enhanced pesticides loading and improved fungicidal activity against tomato late blight disease under the same doses of active ingredient applied (Xu et al., 2018). Polyacrylic acid NEPs (Allosperse®) are currently marketed by Vive Crop Protection. According to Vive Crop Protection (2021), Allosperse® generates a better mixing of the products and improves foliar penetration, rainfastness, coverage performance and sustainability. Therefore, encapsulation in organic nanocarriers is expected to increase pesticides' efficiency.

Inorganic nanoparticles are called "hard" nanoparticles. In recent years, developments have been made to produce inorganic carrier materials for the controlled release of pesticides. Inorganic nanoencapsulation materials may offer a nontoxic, biocompatible, stable alternative, and controlled-release application (Radin et al., 2005). SiO_2 is an earth-abundant and bioinert material,

and SiO_2 nanoparticles ($nSiO_2$) have already been used as nanocarriers for the smart delivery of medicines (Chen et al., 2004; Li et al., 2004; Wang et al., 2015). In agriculture, $nSiO_2$ was shown to have positive effects on the plant uptake of pesticides (Wong et al., 2017). It could also help diseased plants increase their stress resistance capability (Kanto et al., 2004). Furthermore, inorganic compounds such as SiO_2 are micronutrients for plants, which have low toxicity potential and thus are a promising encapsulation medium of nanocarriers for agrochemicals (Bueno & Ghoshal, 2020).

2.3.2 Environmental fate and mobility of nanopesticides

The nanocarriers are expected to have significant impacts on the characteristics of active ingredients. NEPs have shown controlled release, slow degradation of AI, and target impact. For example, in a study on the impacts of different nanocarriers on the properties of BFT in soil, the sorption of BFT to soil was shown to be increased by the nanocarriers (Kah, Weniger, & Hofinann, 2016). In this case, nanocarriers are able to change the fate of pesticides AI before or/and after application. Because of the lack of efficient analytical methods for environmental samples with complex matrixes, to date, most published studies are focused on the aqueous environmental samples for nanoparticle analysis (Simonet & Valcarcel, 2009; Weinberg, Galyean, & Leopold, 2011). Thus, the environmental fate of nanopesticides still represents a knowledge gap.

2.3.3 Distinct effects of nanopesticides on plants

The effect of nanoparticles on plants has been reviewed recently (Ali et al., 2021). Some nanoparticles have been found to enhance seed germination, plant growth, and fruiting while some other can negatively affect plant growth. Field trials with application of Allosperse® products

(AZteroid® (azoxystrobin) and Bifender® (bifenthrin)) have been reported for corn, soybean, potato and sugar beet. Producer feedback and field observations suggested that nanopesticides improved plant growth (e.g., root mass) (R&D NEWS, 2018). Compared with conventional formulations, the toxicity of pesticides at high doses was reduced when encapsulated in $nSiO_2$, leading to an enhancement of the plant biomass (Bueno et al., 2021). As discussed in section 2.2.4, pesticides can modulate plant growth and metabolites. Few studies have evaluated the effect of NEPs on primary and secondary metabolites (incl. phenolic compounds). In one study, the botanical pesticide, Osthole, loaded in polymer-based nanocarriers did not impact strawberry fruit quality, including fruit weight, sugar content, and vitamin C content (Yan et al., 2021).

NPs and nanocarriers may modify pesticide uptake in plants. For example, in one study of uptake and translocation of SiO_2-Coated ZnO nanoparticles in plants (Gao et al., 2021), the SiO_2 shell enhances the uptake of ZnO nanoparticles in *Solanum lycopersicum*. In another study, pyraoxystrobin within MSNs enhanced the upward transportation rate of the fungicide 3.5-fold in cucumber plants (Xu et al., 2021). Moreover, 2.7-fold higher AZOX in conventional formulation uptake per unit dry biomass after ten days of exposure than in porous hollow SiO_2 nanocarriers. However, the uptake was 3-fold higher for the nanoformulation after 20 days of exposure (Bueno et al., 2021). In the same study, the biomass of plants heavily increased (3.85-fold) by the NEP compared to the conventional pesticide. Nanoparticles can be taken up by plants through direct penetration and transport through the stomatal opening (Raliya et al., 2016). Notably, nanocarrier smaller than the pores of the root epidermal cell walls (5-20 nm) could also be taken up by plants (Pacheco & Buzea, 2018). For example, Allosperse®, with a constant hydrodynamic diameter of about 7 nm, could be absorbed by the plant roots (Diaz, Peyrot & Wilkinson, 2015). Nanoparticles with a size over 250 nm were also shown to be absorbed by tomato leaves and internalized through

the plants. Moreover, 200-300 nm $nSiO_2$ were detected in different cucumber plant parts (Zhao et al., 2018). As nanocarriers can also enter plant tissues, they could modify plants' uptake and bioaccumulation of pesticides AI.

Because agriculture is the beginning of the food chain and in direct contact with the environment, the risk assessment of nanopesticides is necessary before the nanopesticides application leads to irreversible damage. There are however some major knowledge gaps in performing a sound evaluation of the risks and benefits of nanopesticides. Most notably, few comprehensive and systemic studies evaluated the efficacy and environmental impact of nano-agrochemicals under actual field conditions (Kah et al., 2018).

2.4 Stability and degradation of pesticides in the environment and following food processing

2.4.1 Pesticide degradation and transformation in the environment

Pesticides can be degraded, metabolized, or transformed in plants and the environment through biotransformation and chemical reactions. Pesticide biotransformation may occur via multiple processes including oxidation, reduction, hydrolysis, and conjugation. Metabolic pathways vary among pesticides, organisms (including fungi, plant species and animals) and environmental conditions (Van et al., 2003). The degradation of pesticides often leads to detoxification of priority substances. However, for some pesticides, the metabolites can be comparable or even more toxic than their parent compounds (Dekant, Melching-Kollmuss & Kalberlah, 2010). Key metabolites have to be included in the risk assessment of pesticide residues if they are found in a significant amount and are toxic for humans, as illustrated by the case study of 3-hydroxy-carbofuran, the metabolite of carbofuran (Lan et al., 2020).

The major metabolite of AZOX in aerobic and anaerobic conditions soil is azoxystrobin free acid (R234886; AzFA) (European Food Safety Authority, 2010). In a water column test, AZOX has a half-life of 13 days, and AZOX tend to degrade into AzFA (European Food Safety Authority, 2010). In a leaching experiment, the result indicated that AZOX is immobile but the mobility of R234886, R401553 and R402173 are high (Ghosh & Singh, 2009b).

The degradation of AZOX has been studied in some plants, including wheat, grapes, peanuts, and rice (Hassellov et al., 2008; Tiede et al., 2008). AZOX metabolism was shown to be relatively complex in these crops, with at least 23 metabolites detected (Figure 2.2) (FAO, 2009). The relative contents of the parent AZOX and its metabolites were different among the different plant tissues. For example, wheat grains harvested 13 days after the application of AZOX contained much lower levels (0.075 mg/kg) than in wheat forage and straw (2 – 9 mg/kg). Although

the metabolite profiles were complex and corresponded to a large fraction of the AZOX residues, individual metabolite concentrations were low (<9%). The top high residue of AZOX metabolites in wheat, grapes, peanuts' hull and hay, and rice were similar. However, the highest residues in peanuts nut were unique, oleic acid (30.9%) and linoleic acid (11.2%). The potential reason may be the high-fat content of the peanuts nut. Except for the peanut, the primary metabolites in samples were R234886, R71395, R400753, R40553, R71395, and R402987, which were also detected in soil metabolism. In recent studies, researchers determined the metabolism of AZOX in lettuce harvested 2 and 4 weeks after the application of AZOX (Gautam, Etzerodt, & Fomsgaard, 2017; Gautam & Fomsgaard, 2017). Two new metabolites (M4 and M6) were detected (Figure 2.3). In another study of Gautam, Elhiti, and Fomsgaard (2018), maize root was used as a model system to investigate the biotransformation of AZOX in plants. Different suites of metabolites were identified compared to the lettuce study.

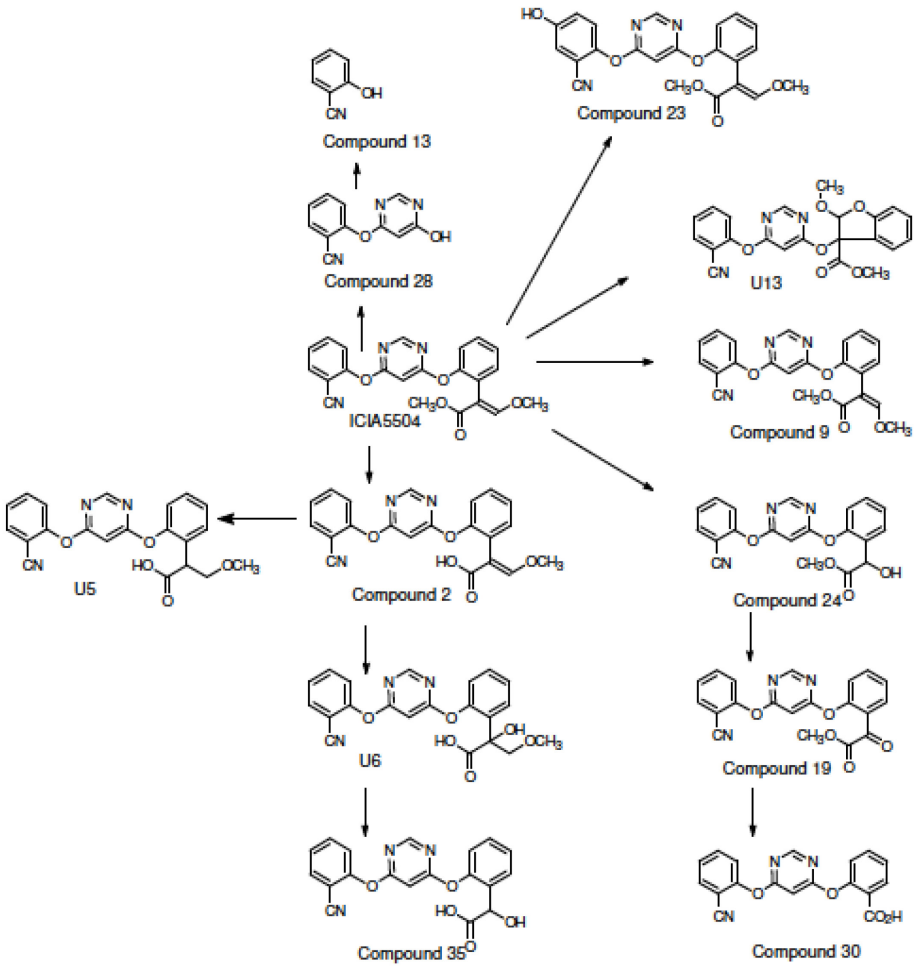


Figure 2. 2 Proposed metabolic pathway of azoxystrobin in plants (FAO, 2009)

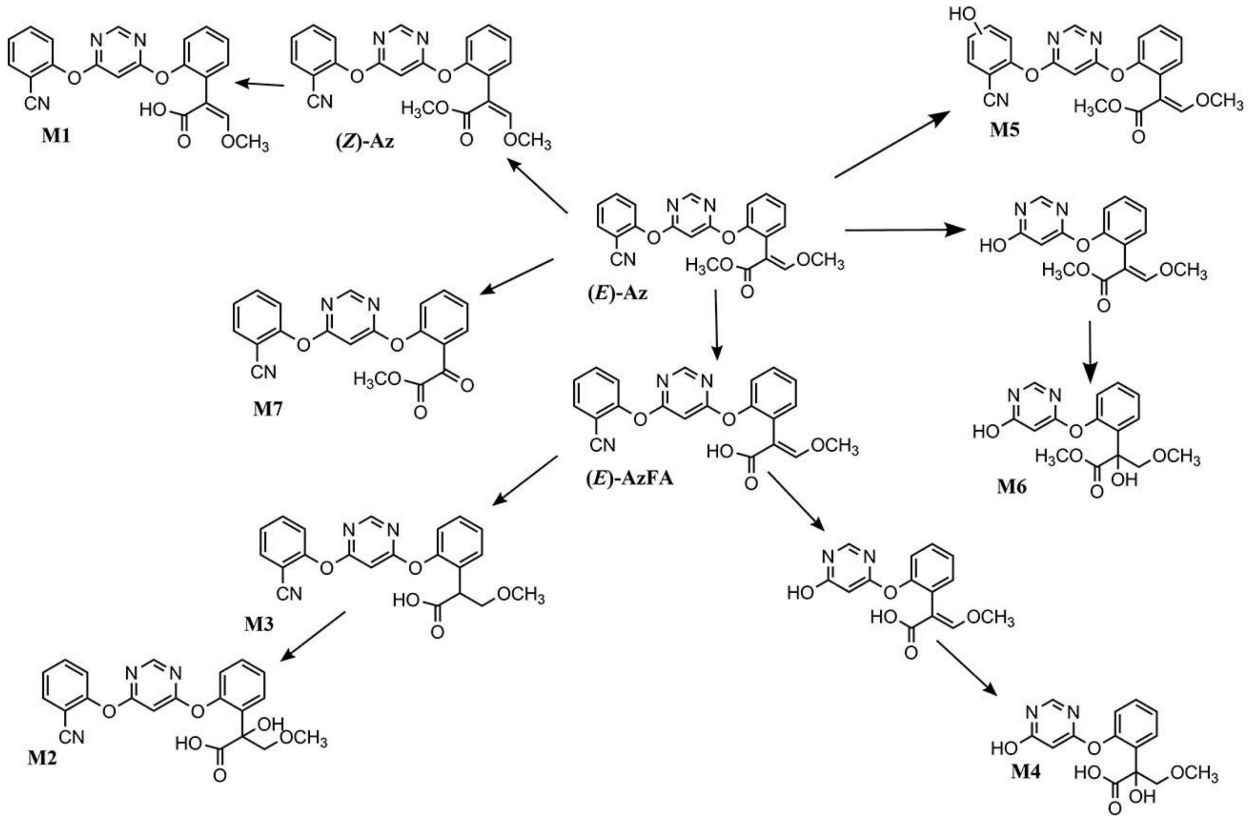


Figure 2. 3 Proposed biotransformation pathway of azoxystrobin in lettuce (Gautam, Elhiti, & Fomsgaard, 2018)

In aquatic environments, BFT is usually adsorbed onto sediment and suspended particles because of its very low water solubility (Mukherjee, Singh, & Govil, 2010). BFT is relatively stable under aerobic and anaerobic conditions in sediments, with a half-life ranged from 8 to 17 months at 20 ℃ (Gan, Lee, Liu, Haver, & Kabashima, 2005). In this study, less than 10% of the initial concentration of BFT were detected to have degraded in sediments, and the major metabolite of was 4'-hydroxy BFT (3-5% of the initial BFT content). This result is consistent with a previous study by Fecko (1999) showing that the major biotic pathway of bifenthrin degradation in the environment is hydrolysis into 4'-hydroxy BFT. Minor pathways of BFT degradation include ester cleavage, hydroxylation and oxidation into TFP acid (*Cis, trans*-3-(2-chloro-3,3,3-trifluoro-1-

propenyl)-2,2-dimethyl-cyclopropanecarboxylic acid), BP alcohol, and BP aldehyde (Fecko, 1999).

Studies on the metabolism of BFT in plants have been reported for apples, potatoes, and maize (Figure 2.4) (FAO, 2010). Spray application of phenyl ring- (PH-) and cyclopropyl ring- (CP-) ^{14}C labeled BFT was used in the field cultures of apples, revealed that most of the ^{14}C BFT residues remained in peel rather than in the pulp. BFT was relatively stable, and the parent compounds consisted of more than 80% of the initial concentration after 28 days in leaves and fruit parts. The major metabolite of BFT was biphenyl acid (2.6% of PH-^{14}C). Similar studies were reported for potatoes and maize, though, in the potato study, more metabolites were reported including TFP acid and 4'-hydroxy BFT as the dominant species. In the maize study, other metabolites such as BP-alcohol and BP-aldehyde were identified in maize leaves. The metabolism pathways in plants are similar with those in the environment.

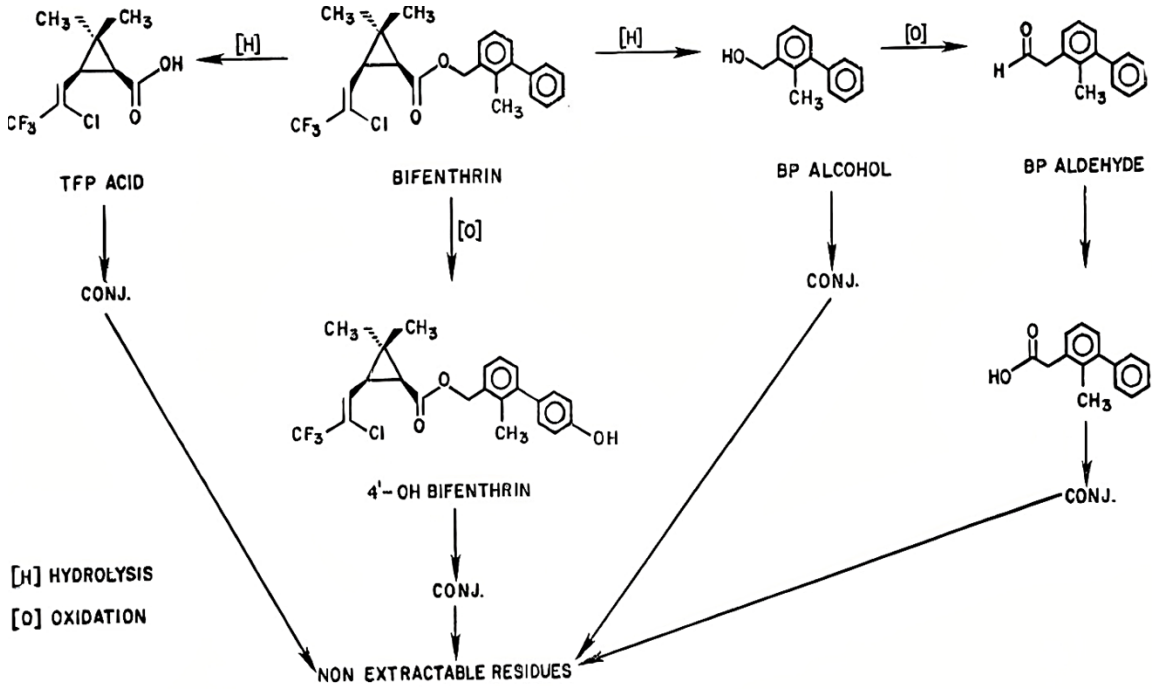


Figure 2. 4 Proposed metabolic pathway of bifenthrin in/on plants (FAO, 2010).

2.4.2 Pesticide degradation and transformation during food processing

Strawberries are sold as raw agriculture products but also be produced into high-value processed commodities such as strawberry jam. Pesticide residue levels are often observed to reduce substantially during food processing such as washing and cooking (Kaushik, Satya, & Naik, 2009). Some pesticides levels in foods are increased by thermal treatment due to moisture loss (Amvrazi, 2011). In one study about the effect of food processing on removing pesticide residues, the Log K_{ow} value of pesticide was the key factor affecting the final residue after food processing (Huan et al., 2015). For example, blanching reduced residues with low K_{ow} while stir-frying and frying were more effective to residues with high K_{ow}. The effect of food processing on AZOX (Log Kow = 3.7) and BFT (Log Kow = 6.6) residue levels has been reviewed below.

Washing is the common step in both household and commercial food preparation. Washing can effectively remove pesticide residues, especially polar pesticide, loosely bound to the fruit surface (Aguilera et al., 2012). Studies on grape (Lentza, Avramides, & Kokkinaki, 2006) and zucchini (Aguilera et al., 2012) indicated that from 75% to 80% of AZOX was removed during washing. For strawberries, 45% AZOX could be removed by tap water (Angioni et al., 2004). In the study of spinach and perilla leaf, AZOX was completely removed by washing (Yang et al., 2012). The effect of washing on removing BFT on rice (Shakoori et al., 2018) and cowpea (Huan et al., 2015) was not obvious. Therefore, washing is efficient at removing surface AZOX, but not for BFT. The reason may be related to the difference of water solubility between AZOX and BFT. Furthermore, washing would not remove pesticides inside the fruits or vegetables (Teixeira et al., 2004).

Thermal processing for food encompasses various techniques, including boiling, blanching, and frying. The stability of AZOX during thermal processing was investigated in several food matrixes. For example, 11-92% decrease was observed for the AZOX level in peanuts after boiling for 30 min (Hou et al., 2017). However, Aguilera et al. (2012. found that heating zucchini for 30 min did not reduce the concentration of AZOX since the water lost 35% during cooking. The thermal removal effectivity of BFT also has been analysis by Huan et al. (2015). In this study, frying was an effective way to eliminate BFT on cowpea. Additionally, the concentration of BFT in frying oil increased over time. In another study, sterilization (121°C; 15 min) had no obvious effect on removing BFT residue in wheat (Dordevic et al., 2013). The low degradation of BFT may be caused by its lower vapor pressure and very low water solubility.

Overall, the cooking process could decrease AZOX and BFT levels in some food matrixes due to diffusion, volatilization and thermal degradation. Instead, the concentration of AZOX increases as a result of condensation. However, to the best of my knowledge, there are no published studies on the thermal degradation kinetics, pathways and products of neither conventional nor nanoencapsulated AZOX and BFT in food. Nanocarriers such as $nSiO_2$ have excellent thermal stability under high temperatures (Feng et al., 2012). Thus, nanocarriers may protect the active ingredients during thermal processing and modify the thermal stability of pesticides. In order to fully assess the risks associated with nanopesticides in diet, it is necessary to investigate the thermal degradation of conventional and nanopesticides during food processing.

2.5 Analytical method for pesticides

Pesticide residue analysis is essential for pesticide control and the protection of human health. There is a great interest in investigating pesticides in foods, including (common, less common and new) pesticides and their metabolites. LC or GC coupled with MS or MS/MS instruments is currently the state-of-the-art tool for pesticide analysis. Pesticides in the complex matrix can be identified and quantified by mass charge ratio (*m/*z) and retention time (RT) of precursors and/or fragments using the combination of LC or GC and MS or MS/MS. Pesticide residue determination can be targeted and non-targeted, depending on the ultimate purpose of the analysis.

2.5.1 Target analysis

Target analysis requires pesticide standards before the analysis of real samples. In the process of method development, a list of target compounds is prepared and characterized. The obtained information (e.g., RT, *m/z* and spectrum) is used to analyze food samples. Target analysis can be used to monitor MRLs of pesticides in food. The tandem mass spectrometer based on triple quadrupole (QqQ) system has high specificity and low LODs, which is a common instrument for pesticide target analysis. Target analysis typically cover a limited number (about 200) of know compounds (Garcia-Reyes et al., 2007). However, more than 1000 pesticides AI have been applied to crops (Garcia-Reyes et al., 2007).

2.5.2 Non-target analysis

The most significant difference between targeted and non-targeted analysis is that non-target analysis dose not rely on the use of pure analytical standards of the analyte. There are two

types of non-targeted analysis: suspects screening and non-target screening (Krauss, Singer & Hollender, 2010). Non-target compounds include unauthorized, unregistered, banned, and new pesticides, and their metabolites, which can be persistent and hazardous as their parent compounds. These compounds' reference standards are currently not available. In method development, suspects screening needs to make a list of suspect ions. Non-target screening can detect unexpected compounds by data filtering and peak detection. Information (e.g., RT, *m/z*, or spectrum) on compounds is not required before testing food samples. HRMS instruments such as Time-of-flight (TOF) and Orbitrap, frequently used for non-target analysis to obtain the full spectrum without additional injections.

The investigation of transformation products is essential for evaluating stability and toxicity of pesticides. Although pesticide residues in food have been a concern for decades, the analysis of pesticide metabolites is not usually included in routine monitoring programs. *Codex Alimentarius* therefore recommends the analysis of pesticide decomposition and their reaction products generated during processing (2008).

Recently, some studies have proposed the determination strategy of pesticide transformation products in food. Due to the lack of commercial standards, literature, and databases, identifying unknown compounds is a challenging task. It has been reported that LC or GC combined with QToF MS can successfully identify unknown compounds in foods without prior use of standards (Garcia-Reyes, Molina-Diaz, & Hassellov, 2007; Lacorte & Fernandez, 2006; Cervera et al., 2012, Saito-Shida et al., 2021). Because of the full-scan measurement, the peak of interest can be deduced. Fragments generated in the instrument can provide additional information (e.g., mass and elemental compositions) for confirmation (Garcia-Reyes et al., 2007).

2.5.3 Extraction methods of pesticides

Analysis of pesticide residues usually requires extraction of pesticide residues from complex food matrixes, as the amount of pesticide residues in actual food tends to be low (trace) and food substrates contain many potentially interfering endogenous compounds. Because pesticides contain a variety of compounds, covering a range of physical and chemical properties, the selection of appropriate solvents and extraction methods is a necessary condition for pesticide analysis. Recently, QuEChERS, as a popular extraction method, combines liquid-liquid extraction (LLE) with dispersive solid-phase extraction (SPE) as a clean-up step. Extraction methods for non-target analysis should have a wide range and cover as many compounds as possible. To this aim, the QuEChERS method has been widely used in the analysis of pesticides in food. Some modifications have improved the scope of the method, such as acetate buffering or citrate buffering, for compounds with various properties (Lehotay et al., 2010). The QuEChERS method has been used to detect hundreds of pesticides in various foods with satisfactory results (Garcia et al., 2007; Pico et al., 2018). This method can extract a variety of pesticides, including AZOX and BFT, from fruits (e.g., strawberries) and vegetables (Lehotay, 2007). There are also other extraction methods for AZOX and BFT in fruits (e.g., strawberries) and vegetables. Tables 2.4 and 2.5 detail some of the commonly used analytical methods reported in the literature for the extraction and determination of AZOX and BFT in food, plant parts, and soil.

2.5.4 Analytical method of nanopesticides

Due to the lack of research methods, most of the published studies have focused on the analysis of nanoparticle in aqueous environmental samples with relatively simple matrixes (Simonet & Valcarcel, 2009; Weinberg, Galyean, & Leopold, 2011). The analytical determination

of nanoparticles in food matrixes requires appropriate sample preparations capable of isolating and extracting nanoparticles from complex matrixes that may contain compositions similar to those of nanocarriers. It seems impossible to investigate the uptake and biodistribution of "soft" nanoparticles (e.g., Allosperse®) in plants (Singh et al., 2014). Moreover, the most common characterization techniques for "hard" nanoparticles, such as light scattering and electron microscopy, are not appropriate for weakly scattering, low electron density polymer nanocarriers (Diaz, Peyrot & Wilkinson, 2015). Furthermore, the low concentration and small particle size of nanopesticides increase the difficulty (Gallego-Urrea, Tuoriniemi & Hassellov, 2011). Thus, there is a lack of analytical methods for polymer nanoparticles. To the best of our knowledge, NEPs has been analyzed using the same method as conventional pesticides in previous nanopesticides experiments. The efficiency of these analytical methods for pesticides encapsulated in nanocarriers has not been validated.

Table 2. 4 Key methods reported in the literature for the analysis of azoxystrobin (AZOX) in food, plant tissues and soil.

Matrix	Extraction	Determination	Reference
Soil	Solid phase extraction	GC-MS/MS	(Kumar et al., 2018)
Soil, Leaves, Potato	QuEChERS	LC-QqQ-MS/MS	(Yu et al., 2018)
Soil, leaf, roots, stem	Solvent extraction	LC-MS/MS	(Wang et al., 2017)
Strawberry	Solvent extraction	GC- NPD	(Angioni et al., 2004)
Strawberry	The solid-liquid method of extraction with low-temperature partition	GC- ECD	(Heleno et al., 2014)
Strawberry	QuEChERS and matrix solid phase dispersion	GC-ECD/NPD LC-MS/MS	(Jankowska, Lozowicka, & Kaczynski, 2019)
Leaves	QuEChERS	LC-QqQ-MS/MS	(Ornek & Durmusoglu, 2018)
Roots, leaves, stem, soil	Solvent extraction	GC	(Hou et al., 2016)
Brassica species	Solvent extraction	UPLC-QToF-MS	(Bauer et al., 2018)

LC-liquid chromatography; GC-gas chromatography; MS-mass spectrometry; MS/MS-tandem mass spectrometry; QqQ-triple quadrupole; NPD-nitrogen phosphorous detector; ECD-electron capture detector; QToF-quadrupole time-of-flight

Table 2. 5 Key methods reported in the literature for the analysis of bifenthrin in food, plant tissues and soil.

Matrix	Extraction Method	Instrument	Reference
Soil	Solvent extraction system equipped with 34 mL stainless steel extraction cells	GC-QqQ-MS/MS	(Martinez et al., 2010)
Soil	Solvent extraction, then purified by florisil SPE	GC- FPD GC-ECD	(Han et al., 2017)
Soil	Solvent extraction with rotatry vacuum evaporator, then florisil SPE	GC-FPD GC-ECD	(Liu et al., 2016)
Soil	Solvent extraction with sodium chloride (NaCl)	GC-ECD	(Shi et al., 2016)
Soil	SPE	GC-ECD	(Chauhan, Monga, & Kumari, 2012)
Soil	Solvent extraction with Liquid-Liquid extraction, then SPE clean-up step	GCL-ECD	(Mukherjee, Singh, & Govil, 2010)
Soil	Solvent extraction with SPME procedure	GC-MS	(Markovic et al., 2010)
Strawberry	PDMS/DVB fiber	GC-MS	(Beltran et al., 2003)

Matrix	Extraction Method	Instrument	Reference
Strawberry	QuEChERS	GC-MS/MS	(Fernandes et al., 2012)
Tea	Solvent extraction with SPE clean-up	GC-MS	(Dayarathna et al., 2013)
Leaves	Subcritical butane extraction	GC-MS	(Zhang et al., 2017)
Leaves	QuEChERS and SPE columns or Carbon X Plus column	GC-QqQ-MS/MS	(Hayward, Wong, & Park, 2015)
Tea	Solvent extraction with SPE clean-up	GC-MS	(Chen et al., 2012)
Roots	Solvent extraction with LLP (separatory funnel) then SPE clean-up	GC-ECD GC-MS.	(Hwang, Lee, & Kim, 2014)
Tomato	Solvent extraction	GC-TOF-MS	(Hlihor et al., 2019)
Egg	QuEChERS	HPLC	(Kim & Hur, 2018)
Wheat grains	ACN extraction, then SPE-Strata-X-column as clean-up	LC-TOF-MS LC- DAD	(Savi et al., 2016)
Banana	QuEChERS	HPLC-MS/MS	(Raphealla et al., 2013)

Matrix	Extraction Method	Instrument	Reference
Dried hops	QuEChERS	UHPLC-hybrid quadrupole–Orbitrap-MS	(Dusek, Jandovska, & Olsovska, 2018)
Celery, kale, avocado, lime, brown rice flour	QuEChERS	LC-MS/MS	(Kowalski, Lupo, & Cochran, 2013)
Strawberries	QuEChERS	LC-QqQ-MS/MS	(Stachniuk et al., 2017)

LC-liquid chromatography; GC-gas chromatography; MS-mass spectrometry; MS/MS-tandem mass spectrometry; QqQ-triple quadrupole; ECD-electron capture detector; QToF-quadrupole time-of-flight; DAD-diode array detector; FPD-flame photometric detector.

2.6 Analysis of phenolic compounds and other metabolites in plant matrixes.

The investigation of plant metabolites (primary or secondary metabolites) in crops can be used to understand the effects of pesticides on plants (Abountiolas et al., 2018; Ganugi et al., 2020). Abountiolas et al. (2018) extracted strawberry metabolites with acetone and then separated phenolics from sugars, acids and other water-soluble compounds using C_{18} Sep-Pack cartridge. The extracted metabolites were identified and quantified using LC with several detectors (e.g., photodiode array detector for phenolics, refractive index detector for sugars, and diode array detector for ascorbic acid). In another study of tomato metabolites, Ganugi et al. (2020) used an LC-QToF-MS for non-target metabolite analysis and found that herbicides could alter the secondary metabolism of tomato plants.

Phenolic compounds have received increasing attention for their potential health benefits. TPC of strawberries is usually determined by Folin-Ciocalteu method (FC). The principle of this method is to oxidize phenolics with phosphotungstic-phosphomolybdic reagent to obtain a chromophore (blue). The FC reagent method is one of the most used methods for the determination of phenolic content. However, the reagent measures can react with any reducing substance (e.g., ascorbic acid). Therefore, the results obtained by FC method are overestimated (Huang, Ou, & Prior, 2005). Because phenolics are the most abundant antioxidants in most plants, FC can be used to estimate the total antioxidant capacity in fruits and vegetables (Prior, Wu, & Schaich, 2005).

The extraction efficiency is the main impact factor for analyzing the individual phenolic. Some methods to extract phenolic compounds in food had been investigated in previous studies (Table 2.6). Extraction methods can be divided into chemical extractions and enzymatic extractions. Different organic solvents (e.g., acetone, methanol, and ethanol) with or without the addition of acids (e.g., HCl) were used to extract phenolics from well homogeneous fresh or freeze-

dried fruits. Extraction times for shaking (or sonicating) range from a few minutes to several hours (Aaby et al., 2007; Moze et al., 2011). The extraction temperature was in the range of 25 - 100°C. According to the review, the selection of extraction methods and extraction parameters of phenolic compounds should be determined according to research objectives, food matrixes, available instruments, time, and other factors.

Mass spectrometry is a powerful technique for analyzing individual phenolics because of its high sensitivity and can be combined with different chromatographic techniques (Table 2.6). For example, phenolic compounds in aqueous extracts of strawberry were qualitatively and quantitatively determined by LC/MS with direct injection (Kafkas et al., 2018). Ionization techniques in MS include ion spray and ion desorption. Ion-spray techniques include electrospray ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). ESI is more commonly used to ionize polar and nonvolatile molecules such as tannins and anthocyanins. APCI is used for less polar and nonionic compounds such as flavonols, flavones, flavanones and chalcones. Ion-desorption techniques include fast atomic bombardment and matrix-assisted laser desorption ionization.

Table 2. 6 Previous research about the analysis of phenolic compounds in plants.

Compounds	Matrix	Sample treatment	Determination Technique	Reference
Phenolic compounds TPC	Strawberry	Liquid nitrogen homogenized, then 70% acetone extracted then mixed with Chloroform (1:1 v/v); Or C18 Sep-Pak cartridges	TP: FC assay Phenolic compounds: HPLC with DAD, coulometric array detector and MS	(Aaby, Skrede, & Wrolstad, 2005)
TPC	Rice plant	Chemical extraction: 80% methanol	FC assay	(Abed et al., 2018)
TPC	Wine, fruit, juices, plant tissues	Methanol	FC assay	(Singleton, Orthofer, & Lamuela-Raventós, 1999)
TPC	Vegetables and fruits	Methanol	Enzyme determination	(Saura, Serrano, & Goni, 2007)
TPC	Strawberries, Arabidopsis leaf	Methanol with formic acid	LC-photodiode array-QToF-MS/MS	(De et al., 2007)

Compounds	Matrix	Sample treatment	Determination Technique	Reference
Phenolic compounds	Almond	Deionized water at 100□C water bath for 30 min; or 10% ethanol for 42 hours; or 80% methanol for 24 hours; or petroleum ether for 24 hours; or microwave assisted extraction in deionized water; or boiling deionized water for 5 min.	LC-QToF-MS/MS GC-QToF-MS/MS	(Kaneria et al., 2018)
Phenolic compounds	Citrus fruit	53% of ethanol in water	LC-QToF-MS/MS	(Ledesma et al., 2017)
Phenolic compounds	Cherry juice	Filtration	LC-QToF-MS/MS HPLC	(Toydemir et al., 2012)
Phenolic compounds	Strawberry and blackcurrant	50% methanol with 1.2m hydrochloric acid (HCl)	HPLC	(Hakkinen et al., 1998)
Phenolic compounds	Strawberry	Liquid nitrogen milled, then acetone extraction	HPLC with DAD, MS, coulometric array, or UV-vis	(Aaby et al., 2007)

Compounds	Matrix	Sample treatment	Determination Technique	Reference
Phenolic compounds	Strawberry (freeze-dried and fresh)	Methanol with 0.1% HCl or 70% acetone	HPLC with ESI-MS and DAD	(Seeram et al., 2006)
TPC	Fruits (including strawberry) and vegetables	Chemical extraction: methanol Enzymatic extraction: pancreatin	FC assay	(Alvarez et al., 2016)
Phenolic compounds	Berries (including Strawberry)	50% (v/v) methanol with HCl and an antioxidant (*tert*-butylhydroquinone and ascorbic acid)	HPLC with DAD and UV-vis detection	(Hakkinen & Torronen, 2000)
TPC	Strawberry	Acetone, water with acetic acid (70:29.5:0.5 v/v)	FC assay	(Asami et al., 2003)
Phenolic compounds	Bilberry and blueberry	Methanol	LC-MS/MS	(Moze et al., 2011)

2.7 Conclusion

In reviewing the nano- and conventional pesticides, NEPs appears to have unique characteristics and a potentially different environmental behavior from their conventional formulations. Few studies have actually investigated the fate, effects or thermal degradation of the NEPs. In order to properly assess the potential risks associated with NEPs, it is necessary to have a validated analytical method specifically for NEPs. To the best of our knowledge, the analytical methods for NEPs in previous nanopesticides experiments were the same as conventional pesticides. The efficiency of those analytical methods for pesticides encapsulated in nanocarriers has not been validated.

This review found that conventional pesticides could modulate the plant phenolic compounds. However, few comprehensive studies have analyzed the fate, uptake, and impact of any conventional or NEPs from a well-controlled agricultural field. Moreover, there is limited understanding of conventional and nano- pesticides' thermal degradation products in foods.

The distinct environmental fate and effects of NEPs on plant growth and metabolites compared to conventional pesticides are unknown. Therefore, the main objective of my research was to develop an analytical strategy to investigate NEPs in strawberry plants and to compare the fate and potential effects (phenology parameters and phenolic compounds) of conventional pesticides and NEPs on plants.

Connecting Text

Chapter 2 provided an up-to-date overview of the scientific literature on the analysis, fate and effects of conventional pesticides of AZOX and BFT and the current knowledge gaps for nanopesticides. Based on the review, most studies on nanopesticides are focused on developing and characterizing their properties and functions. However, there is a lack of information about the analytical behavior of NEPs for the accurate assessment of their fate in the field and food chain. Chapter 3 provides a comprehensive study of the impact of extraction time, solvent volume and material composition used in extraction to determine NEPs of AZOX and BFT in plants (fruits, leaves, and roots) and soils with different compositions. Analytical methods based on the state-of-the-art approach QuEChERS were developed and validated for NEPs in plants. Chapter 3 was published in *Talanta*: Wang, P., Galhardi, J. A., Liu, L., Bueno, V., Ghoshal, S., Gravel, V., Wilkinson, K. J. & Bayen, S. (2022). Development of an LC-MS-based method to study the fate of nanoencapsulated pesticides in soils and strawberry plant. *Talanta*, 123093.

Chapter 3. Development of an LC-MS-based method to study the fate of nanoencapsulated pesticides in soils and strawberry plant

3.1 Abstract

The increased production and use of nanopesticides will increase the likelihood of their exposure to humans and the environment. In order to properly evaluate their risk, it will be necessary to rigorously quantify their concentrations in major environmental compartments including water, soil and food. Due to major differences in the characteristics of their formulation, it is unclear whether analytical techniques that have been developed for conventional pesticides will allow quantification of the nano-forms. Therefore, it is necessary to develop and validate analytical techniques for the quantification of nanopesticides in foods and the environment. The goal of this study was to validate a method for analyzing the active ingredients of two pesticides with different physicochemical properties: azoxystrobin (AZOX, a fungicide, log K_{ow} 3.7) and bifenthrin (BFT, an insecticide, log K_{ow} 6.6) that were applied to agricultural soils, either as a conventional formulation or encapsulated in nanoparticles (either Allosperse® or porous hollow $nSiO_2$). Pesticide-free strawberry plants (*Fragaria* × *ananassa*) and three different agricultural soils were spiked with the active ingredients (azoxystrobin and bifenthrin), in either conventional or nano formulations. A modified QuEChERS approach was used to extract the pesticides from the strawberry plants (roots, leaves and fruits) and a solvent extraction (1:2 acetonitrile) was employed for the soils. Samples were analyzed by liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry in order to determine method detection limits, recoveries, precision and matrix effects for both the "conventional" and nanoencapsulated pesticides. Results for the modified method indicated good recoveries and precision for the analysis of the nanoencapsulated pesticides from strawberries and agricultural soils, with recoveries ranging from 85-127% (AZOX) and 68-138% (BFT). The results indicated that the presence of the nanoencapsulants had significant effects on the efficiency of extraction and the quantification of the active ingredients.

The modified analytical methods were successfully used to measure strawberry and soil samples from a field experiment, providing the means to explore the fate of nanoencapsulated pesticides in food and environmental matrices.

Keywords: Nanoencapsulated pesticides; Azoxystrobin; Bifenthrin; Soil; Strawberry, Liquid chromatography–mass spectrometry.

3.2 Introduction

Sustainable agricultural practices, potentially implicating nanotechnology, are required to meet the demand of a rapidly increasing global population (Rodrigues et al., 2017; Hofmann et al., 2020). Nanopesticides, particles with at least one dimension in the 1-100 nm size range (Iavicoli, Leso, Beezhold & Shvedova, 2017), have been developed with the promise of a higher efficacy of the active ingredients, minimal environmental impacts and reduced undesirable consequences as compared to conventional pesticides (Rodrigues et al., 2013; Camara et al., 2019). Although nanopesticides have great potential to increase crop productivity, their potential risks have also raised concerns (Adisa et al., 2019), especially with respect to their toxicity or changes to the fate (aging, mobility, etc.) of the active ingredients in the environment (Hofmann et al., 2020; Singh et al., 2020). Since some nanopesticides have been shown to be systemic for plants (Melissa et al., 2013; Zhao et al., 2017; Zhao et al., 2018; Dong et al., 2020; Mathur & Roy, 2020), there is a need to investigate if nanoencapsulation could modify the fate of active ingredients. In previous pesticide residual experiments, nanoencapsulated pesticides were analyzed by traditional methods for conventional pesticides (Liang et al., 2017; Zhao et al., 2017; Zhao et al., 2018). The efficiency of those analytical methods for pesticides capsuled in nanocarriers has not been validated.

Among the most promising nanopesticides are those where the active ingredient is encapsulated within nanomaterials comprised of lipid and polymer carriers (e.g., polyacrylates), inorganic nanoparticles such as SiO_2 or carbon nanotubes (Chhipa, 2017; Kumar et al., 2019). Due to interactions of the pesticides with the nanocarriers, modifications to the solubility of the active ingredients and analytical difficulties associated with their extraction, the analytical approach required for the quantification of nanopesticides is likely to differ from the ones that have been developed for conventional pesticides (Mohd Firdaus et al., 2018; Adisa et al., 2019). There is

presently little information in the literature on the extraction and quantification of nanopesticides in plants and soils.

Azoxystrobin (AZOX, log K_{ow} 3.7) and bifenthrin (BFT, log K_{ow} 6.6) are among the active ingredients currently being incorporated into nanocarriers for commercialization for crop protection (Vive Crop Protection, 2021). AZOX is a major strobilurin fungicide, with annual global sales reaching 1.2 billion in 2014 (Cao et al., 2016). AZOX inhibits mitochondrial respiration via a blockage of the electron transfer between cytochromes *b* and *c*1, leading to an oxidative stress in the target fungus (Zhang et al., 2020). BFT is a pyrethroid insecticide, which is neurotoxic to insects by interfering with the nerve cells' ability to transfer signals (Yang & Li, 2015). Both AZOX and BFT have been applied to strawberry crops in order to increase their yield (Abrol & Anil, 2009; Pandey, Shankar & Sharma, 2012).

The extraction of pesticides from plants and soils can be challenging due to their affinity with organic matter (Harrison, Bull & Michaelides, 2013). Among the various extraction methods reported for conventional pesticide analysis in food, QuEChERS (quick, easy, cheap, effective, rugged, and safe) has emerged as a popular method (Lehotay, 2007). Nonetheless, methodologies for the simultaneous extraction and analysis of the nano-based pesticides still need development (Singh et al., 2014). Since extraction shaking time and solvent volumes are known to affect the recovery of the pesticides from fruit matrices (Jia et al., 2010), these parameters need to be optimized. Furthermore, BFT is relatively hydrophobic (log K_{ow} = 6.6), so its affinity with plastic materials may be relatively high (Guo et al., 2020), implying that the type of materials used for sample preparation may impact the recoveries of the target analytes.

The goal of this paper is to develop and validate a method for the extraction and quantification of AZOX and BFT from agricultural soils and strawberry plants (roots, leaves and

fruits), for compounds that are either present in their conventional form or encapsulated with two important types of nanoparticles: polyacrylic acid nanoparticles (Allosperse®) and porous hollow nano-sized SiO_2. Precision, matrix effects and recoveries of the methods were determined. The methods were then applied to field samples for further validation.

3.3 Methods

3.3.1 Chemicals and reagents

Analytical standards of the pure compounds, azoxystrobin (AZOX) (≥98%, CAS#131860-33-8) and bifenthrin (BFT) (≥98.0%, CAS#82657-04-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated internal standards (D_4-azoxystrobin and D_5-bifenthin) were purchased from Toronto Research Chemicals (North York, ON, Canada). HPLC grade solvents (water, acetonitrile (ACN), and methanol), anhydrous magnesium sulphate, sodium acetate, LC/MS grade formic acid and ammonium acetate (NH_4Ac) were obtained from Fisher Chemicals (Pittsburgh, PA, USA). Primary and secondary amine (PSA) salts were purchased from Agilent (Santa Clara, CA, USA). Allosperse® is a polymeric nanoparticle, comprised of polyacrylic acid, that is used as a nanocarrier for the pesticides (AZOX, BFT). Allosperse®-AZOX and Allosperse®-BFT were prepared and supplied by Vive Crop Protection Inc. (Toronto, Canada). Porous hollow silica nanoparticles ($nSiO_2$) were synthesized as reported in an earlier study (Bueno & Ghoshal, 2020). The feasibility of loading dissolved solutes into the $nSiO_2$ were also evaluated in that study. For the experiments conducted in this study, the $nSiO_2$ was loaded with the analytical standards to produce $nSiO_2$–AZOX and $nSiO_2$–BFT as described above for the Allosperse®. Stock solutions of the nanopesticides used for method validation were prepared in methanol.

3.3.2 Field samples

A controlled field experiment was carried at the Macdonald Campus of McGill University, Sainte-Anne-de-Bellevue, QC, Canada. Strawberry plants (*Fragaria* x *ananassa* Duch. "Seascape"), were cultivated under field conditions (n = 5) and exposed to seven different treatments: (i) control ("pesticide-free" soil); (ii) BFT; (iii) AZOX; (iv) Allosperse® containing BFT; (v) Allosperse® containing AZOX; (vi) $nSiO_2$ containing BFT; (vii) $nSiO_2$ containing AZOX (0.22 $mg.kg^{-1}$ of the active ingredient). Treatments with AZOX all contained 7.6 mg active ingredient / pot; treatments with BFT all contained 7.98 mg active ingredient / pot based on the US EPA guidelines (2015a; 2015b). Strawberry plants without fruit (Pépinière Lareault, Canada) were planted in the first week of June and the treatments was applied twice: 15 and 30 days after planting, following the instructions for commercial pesticides. In summary, 200 mL of the different formulations were diluted to 1 L using irrigation water, which was then used to drench on the soils of each pot (n = 5), avoiding the direct contact of the solutions with the plants. Strawberry plants and the corresponding soil samples were collected 30 days after the first exposition prior to treatment using the methodology described in section 3.3.3 and 3.3.4.

3.3.3 Extraction of the pesticides from the strawberry plants

Initial tests to adapt the extraction method for nanoencapsulated pesticides in plant tissues and the subsequent method validation tests were conducted on strawberry tissues from plants grown in pesticide-free soils (See section 3.3.2). Fruits were homogenized in a stainless-steel blender. Leaves and roots were freeze dried and homogenized. All field samples were stored in at -20 °C until analysis.

Pesticide extraction for the strawberry plants was adapted from a method based on the original QuEChERS approach (AOAC, 2007). The method was scaled to a smaller sample size (2 g) in order to accommodate field samples that may be available in limited amounts on some harvest days. In the present study, pesticide recovery was assessed for strawberry samples (spiked at 10 μg kg^{-1} for AZOX or BFT) for several shaking times (1, 5, 15 and 30 min) and solvent volumes (2 and 4 mL). Two types of centrifuge tubes (glass and plastic) were tested for the extraction of conventional and Allosperse®-BFT, spiked at 10 μg kg^{-1} and 1000 μg kg^{-1} (concentration corresponds to the active ingredient). The mass-labeled standards D_4-AZOX and D_5-BFT were spiked in the strawberry plant samples at 40 and 60 μg kg^{-1}, respectively. For the extraction, 2 g of homogenized fruit or 0.2 g of homogenized dried leaves and roots (n = 3) were weighed in a 15 mL plastic centrifuge tube to which 4 mL of 1% acetic acid in acetonitrile, 0.8 g of magnesium sulphate and 0.2 g of sodium acetate were added. Solutions were vortexed for 15 minutes then centrifuged at 2240 × g (5 min, 20 °C). One mL of the supernatant was transferred to centrifuge tubes containing 50 mg PSA and 150 mg of $MgSO_4$. Solutions were then vortexed for 1 min, centrifuged (2240 × g, 5 min, 20 °C), and filtered through a 0.22 μm polytetrafluoroethylene (PTFE, Chrom4; Thuringen, Germany) filter into HPLC vials.

3.3.4 Extraction of the pesticides from the soil samples

Method validation was performed on three different types of soils collected in Quebec, Canada (Table 3.1), including a clay soil (relatively rich in organic matter – OM; 6.1%), a loamy sand soil (intermediate OM content; 4.7%), and a loam soil (lower OM content; 3.6%) (Table 3.1). Soil 1 corresponded to the soil used for the strawberry crop described in section 3.3.2. Soils were dried at room temperature until constant weight, sieved through a 2 mm nylon mesh, then ground

to a fine powder. Prior to the extraction, soils (n = 3) were spiked with 10 µg kg^{-1} or 1000 µg kg^{-1} of the different treatments (AZOX, BFT, Allosperse®, Allosperse®-AZOX, Allosperse®-BFT, $nSiO_2$, $nSiO_2$-AZOX and $nSiO_2$–BFT) and with deuterated standards (40 ug kg^{-1} of D_4-AZOX and 60 ug kg^{-1} of D_5-BFT). Samples were then vortexed for 1 min and left to equilibrate for at least one hour prior to extraction. The extraction method was adapted from Kah et al. (2016) and consisted in shaking 1 g of dried and sieved (2 mm) soil in 2 mL of ACN for 1 hour at 20 rpm on a vertical shaker at room temperature; followed by centrifugation (1882 × g; 5 min, 20 °C) and filtration of the supernatant through 0.22 µm filters into HPLC glass vials.

Table 3. 1 Characteristics of the three agricultural soils used for method validation.

	% sand	**% silt**	**% clay**	**Soil texture class**	**pH**	**% OM[1]**
Soil 1	30	31	38	clay	7.2	6.1
Soil 2	81	14	5	loamy sand	6.9	4.7
Soil 3	53	32	15	loam	7.2	3.6

1. OM is Organic material.

3.3.5 Instrumental analysis

Extracts were analyzed with an Agilent 1290 Infinity II liquid chromatograph (LC) coupled to a 6545 QTOF mass spectrometer (Agilent Technologies, Santa Clara, USA) operating in positive electrospray ionization mode. The LC separation was conducted on a Poroshell 120 phenyl hexyl column (Agilent Technologies; 2.7 µm × 3.0 mm × 100 mm) fitted with a Poroshell 120 EC-C18 (2.7 µm × 3.0 mm × 5 mm) guard column. Elution was performed in gradient mode (0.4 mL min^{-1}) using A = water and B = Acetonitrile: Methanol (1:1), both containing 0.1% formic acid and 5 mM NH_4Ac (0 min: 70% A; 0-3 min: B increased from 30 to 100%; 3-6 min: 100% B;

6-8 min: B decreased from 100% to 30%). The injection volume was 10 µL and the column temperature was maintained at 30°C. Nitrogen was used as the drying gas (110°C, 12 L min^{-1}). Samples were run in the *All Ions MS/MS* mode. The fragmentor voltage was 110 V and MS data was acquired in the 50-750 *m/z* range. The following *m/z* were extracted from total ion chromatogram (TIC) (±10 ppm) for quantification: 404.1247 for AZOX and 440.1604 for BFT. The qualifier ions for AZOX and BFT were 372.0971 *m/z* and 181.1009 *m/z*, respectively.

3.3.6 Linearity, IDLs, MDLs and MQLs

Calibration curve linearity was evaluated from the coefficient of determination (r^2) using injections of the standards prepared in acetonitrile at 1, 5, 10, 25, 50, and 100 ng mL^{-1}. Instrument detection limits (IDLs) were calculated as the amount of analyte injected that resulted in a signal-to-noise ratio (S/N) of 3, as determined from the lowest standard of the calibration curve in pure solvent (Indrayanto, 2018). Method detection limits (MDLs) were assessed as 3σ of the response obtained for procedural blanks. Method quantification limits (MQLs) were determined from 10σ of the procedural blanks.

3.3.7 Recoveries, matrix effects and precision

Recoveries, matrix effects and precision were assessed for conventional AZOX and BFT (AZOX and BFT spiked together), Allosperse®-AZOX, Allosperse®-BFT, $nSiO_2$–AZOX and $nSiO_2$–BFT, for all plant and soil samples. As of 2021, maximum residue limits (MRLs) for AZOX and BFT in strawberry fruits in Canada are 10 and 3 mg kg^{-1}, respectively (Government of Canada, 2016, 2018). For soils, spiking concentrations were set according to residue levels commonly reported in agricultural soils: AZOX in the range of 30 - 250 µg kg^{-1} (Silva et al., 2019); and BFT

in the range of 2.28 to 112.9 μg kg^{-1} (Leyva-Morales et al., 2015). Recovery was determined by spiking the homogenized samples prior to extraction with both pesticides and their mass-labeled surrogates. For each treatment (AZOX, BFT, Allosperse®, Allosperse®-AZOX, Allosperse®-BFT, $nSiO_2$, $nSiO_2$-AZOX and $nSiO_2$–BFT), samples (n = 3) were spiked at two levels: strawberries and soils (10 μg kg^{-1} and 1000 μg kg^{-1}); leaves and roots (20 μg kg^{-1} and 1000 μg kg^{-1}). Recoveries of the pesticides were considered acceptable when in the 70-120% range (Rutkowska, Lozowicka & Kaczynski, 2018).

Matrix effects were studied by comparing the slope of a matrix-matched calibration curve with the slope of the calibration curve in pure solvent. Four different concentrations (10, 25, 50, and 100 μg kg^{-1}, n = 3) were added to each matrix in order to assess matrix effects according to:

$$\text{Matrix effect (\%)} = (1-B/A) \times 100 \quad (1)$$

where A is the average peak area obtained for a given concentration of standard in the pure solvent and B is the average peak area obtained for the sample extracts (Chambers et al., 2007). Intraday and interday precision were determined from the analysis of samples (n = 5) spiked at a level of 100 μg kg^{-1} spike for each pesticide.

3.3.8 Statistical analysis

Analysis of variance (one-way ANOVA, Microsoft Excel) was used to identify differences among results obtained for different pesticide formulations and different types of samples, by applying a confidence range of 95% ($\alpha = 0.05$, n = 3). When differences were identified, Tukey's test was then used to determine which pairs of means were statistically different ($p<0.05$). In the figures, error bars represent standard deviations (n = 3).

3.4 Results and Discussion

3.4.1 Instrument validation

Instrument validation was performed for the LC-MS analysis (Table 3.2). Good instrumental linearity was achieved ($r^2 > 0.999$) in the range of 10-1000 pg injected for AZOX and 50-1000 pg for BFT. Low IDLs for AZOX and BFT were obtained (0.3 pg and 2.2 pg). Mass measurement errors were generally below 2.5 ppm for both pesticides among the various formulations (Table S3.3 and S3.4). As can be seen in Figs. S2 and S3, *m/z* and retention times were similar for the target compounds when they were prepared in extracts or when they were present as pure active ingredients or encapsulated into the different nanocarriers. The relative intensities of the qualifier and quantifier ions for both AZOX and BFT in acetonitrile and samples (Table S3.5) were acceptable according to the SANCO/12495/2011 guideline (European Commission, 2012).

Table 3. 2 Instrument validation for the LC-MS analysis of AZOX and BFT

Target analytes	RT (min)	Formulation	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	IDLs[a] (pg)	r^2 [b]
AZOX	3.72	$C_{22}H_{17}N_3O_5$	404.1247	372.0971	0.3	0.9997
BFT	4.97	$C_{23}H_{22}ClF_3O_2$	440.1604	181.1009	2.2	0.9981

[a]. IDLs are the instrument detection limits. [b]. r^2 is the coefficient of variation of calibration curve

3.4.2 Development and validation of the methods

In the initial tests, the performances of the solvent extraction methods for AZOX and BFT in soil samples were acceptable. For strawberries, initial tests conducted with the original approach (AOAC, 2007) gave acceptable recoveries for the three forms of AZOX pesticides. On the other hand, BFT (conventional and Allosperse®) was not detectable in samples spiked at 10 μg kg^{-1}

(Figure S3.1). In order to increase the recovery of the BFT (conventional and Allosperse®) to acceptable levels, several conditions were tested, including the use of different tube materials (glass, plastic), variable extraction solvent volumes, and shaking times. The developed extraction method was then applied to all strawberry plant matrices (strawberry, leaves and roots).

3.4.2.1 Development of an extraction method for the strawberries

Initially, when using plastic centrifuge tubes, only 23 ± 32% of the conventional BFT was recovered from the spiked strawberry samples (10 μg kg^{-1}) and no signal was detected in the Allosperse®-BFT treatment. By increasing the extraction solvent volumes from 2 to 4 mL (Figure S3.1), recoveries for Allosperse®-BFT increased to 61 ± 4%. For both BFT formulations, recoveries were improved further when switching to glass centrifuge tubes: 78 ± 17% for the conventional BFT and 60 ± 4% for the Allosperse®-BFT (Figure S3.1). Note that when using the longer extraction times (15 min), acceptable recoveries for plastic centrifuge tubes were also obtained (80 ± 12% for conventional BFT and 98 ± 4% for Allosperse®-BFT). Considering the efficiency, cost and labor-consumption, the final conditions for the extraction combined the plastic tube, 4 mL of solvent and 15 min of shaking time. Given our initial observation that 15 minutes of shaking improved the extraction efficiency, a subsequent optimization below examined the role of shaking time (1, 5, 15 and 30 minutes). This point is important given that the two nanocarriers provide slow release of the loaded pesticides (Walker et al., 2017).

Shaking time had no perceptible influence on the extraction of AZOX, for any of the formulations and recoveries were already acceptable when using 1 min shaking (Figure 3.1). Furthermore, there were no significant differences observed when comparing the extraction of the conventional AZOX with respect to the two nanocarriers (Allosperse® and porous hollow nano-silica).

On the other hand, for BFT, recoveries were improved ($p < 0.05$) for Allosperse® and the conventional formulations of the longer shaking times (5, 15 or 30 min). For BFT, the nanoencapsulated pesticides generally had better recoveries than the conventional ones (Figure 3.1). This may be linked to a faster release rate of the pesticides from those nanoparticles compared with conventional pesticides, which is controlled by many factors, including shell thickness, pore size, inner polarity and the solubility of the active ingredient (Botterhuis, Sun, Magusin, Van Santen & Sommerdijk, 2006; Yao, Shi, Jin, Li & Zhang, 2010). Pesticide encapsulation has also been shown to modify the hydrophobic partitioning of pesticides (Slattery et al., 2019). Although the basic AOAC QuEChERS method was efficient and accurate with respect to the extraction of the AZOX and the silica nanopesticides, the increased extraction times clearly improved the efficiency of the Allosperse® encapsulated and conventional pesticide formulations.

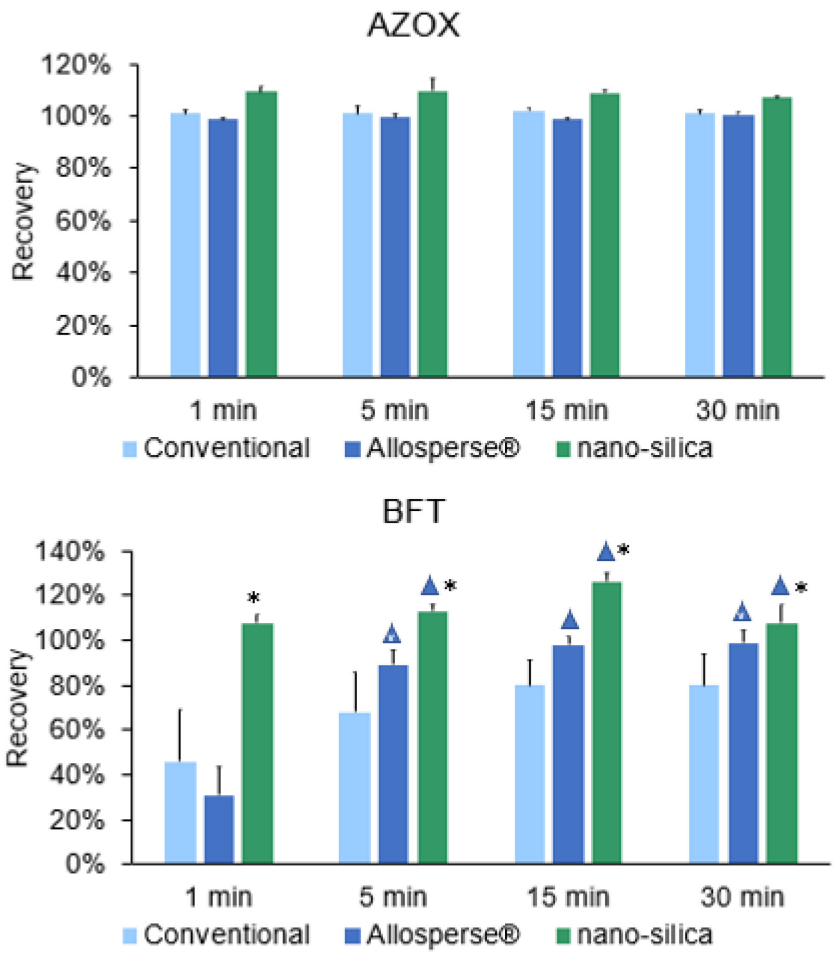


Figure 3. 1 The recovery of AZOX and BFT pesticide (conventional and nanoencapsulated, 10 µg kg^{-1}) from strawberries using QuEChERS with different extraction time (extraction solvent volume: 4 mL; plastic centrifuge tubes; n = 3). Δ indicates a significantly higher recovery with respect to the 1 min extraction time for a given formulation; * indicates a significantly different recovery when compared to results for the conventional pesticides for an identical extraction time.

3.4.2.2 Validation of the developed extraction method for strawberry plant matrices

Recoveries, matrix effects, precisions and MDLs were assessed using the above method for both the conventional pesticide formulation and the nanopesticides (Table 3.3). MDLs ranged

from 0.02 to 0.65 µg kg^{-1} among the various plant matrices for azoxystrobin, and from 0.03 to 0.36 µg kg^{-1} for bifenthrin. These MDLs were comparable or lower than those reported in the literature (Chauhan, Monga & Kumari, 2012; Vera et al., 2013; Bhattacharyya & Roy, 2014). For the lowest spiking level, recoveries ranged from 80 ± 12% to 125 ± 2% for the conventional formulations; from 87 ± 10% to 126 ± 6% for the Allosperse® and from 103 ± 13% to 126 ± 4% for the $nSiO_2$-based nanopesticides. Recoveries were also satisfactory when plant samples were spiked with the higher concentration of conventional pesticides (between 88 ± 6% and 111 ± 12%); Allosperse® (83 ± 4% to 138 ± 14%) and $nSiO_2$ (68 ± 3% to 118 ± 6%). There was an important improvement in the recovery of BFT from no detection to 80% (Figure S3.1), when using the modified method. For strawberries spiked with 10 µg kg^{-1} (lower spiked level), recoveries (87 ± 10%-126 ± 6%) were higher than those at the higher spiked level. These lower pesticide concentrations correspond to levels that were found in the strawberries taken from the experimental field (Figure S3.4), and 2–98 ug kg^{-1} of AZOX residue levels and 2-85 ug kg^{-1} of BFT residue levels reported by the U.S. Dept. of Agriculture (USDA, 2019). Furthermore, the precision (RSD%) was in the range of 1.99-16.71% (Table 3.3, Table S3.2) for both AZOX and BFT in the plant samples. This confirms the good performance of the modified method.

Note that the above recovery values were obtained after correction for matrix effects. In LC-ESI-MS, matrix effects are commonly caused by coeluting compounds, including endogenous metabolites, impurities or degradation products found in the extract (Chambers et al., 2007). These substances can promote or compete with the target analyte for the available charges in the ion source, which may either cause an increase (enhancement) or decrease (suppression) in the detector response as compared to the analyte in pure solvent. When the average matrix effect

exceeds ± 20%, the matrix is considered to have a significant effect on quantitative determinations (European Commission, 2017).

For AZOX, matrix effects were not significant (<±20%). For BFT, matrix effects were below 20% except for two observed matrix effect values linked to the $nSiO_2$ formulation: 27 ± 1% in the strawberries (fruit) and 43 ± 6% in the strawberry roots (Table 3.3). When comparing the conventional-BFT and the nanopesticides, several significant ($p < 0.05$) matrix effects were observed for both the Allosperse®-BFT and $nSiO_2$-BFT (Table S3.1). These results again demonstrate that impact of nanoencapsulation on the extraction of BFT from the strawberry samples.

Mass-labeled surrogates can be added prior to extraction to correct for matrix effects (Niessen, Manini & Andreoli, 2006). In the present study, the use of D_4-AZOX and D_5-BFT indeed reduced the effect of the matrix on the quantification. The combination of longer extraction times and higher solvent volumes and the use of labeled pesticides allowed us to attain the higher recoveries discussed above for the nanopesticides in the strawberries. Similar recoveries of 90.6-116.2% have been reported for the extraction of a nanoformulation of pyridalyl from tomatoes using a different QuEChERS protocol (Saini, Gopal, Kumar, Gogoi, & Srivastava, 2015).

Table 3. 3 Recoveries (%) and matrix effect (%) of the pesticides in the conventional and nano formulations for samples of strawberry, leaves, roots and soils (n = 3).

Azoxystrobin						
Matrix	Treatment	Recovery % (n = 3)		Matrix Effect % (n = 3)	Precision % (n = 5)	MDLs[1] µg kg^{-1} (n = 5)
		Spiked @0.01mg kg^{-1} (wet wt.)	Spiked @1 mg kg^{-1} (wet wt.)			
Soil 1	Conventional	105 ± 3	109 ± 19	-2.3 ± 7	1.27	0.65
	Allosperse®	85 ± 2	110 ± 1	-8.5 ± 5.3		
	$nSiO_2$	108 ± 3	127 ± 1	-4.9 ± 5.6		
Soil 2	Conventional	120 ± 2	122 ± 1	-29 ± 6		
	Allosperse®	87 ± 4	104 ± 1	-11 ± 3		
	$nSiO_2$	117 ± 2	125 ± 1	-12 ± 1		
Soil 3	Conventional	126 ± 1	125 ± 1	-14 ± 1		
	Allosperse®	126 ± 4	102 ± 2	36.5 ± 5		
	$nSiO_2$	107 ± 3	122 ± 5	-12 ± 9		
Strawberry	Conventional	102 ± 1	97 ± 6	-19 ± 2	3.90	0.14
	Allosperse®	99 ± 1	88 ± 3	-15 ± 1		
	$nSiO_2$	109 ± 1	111 ± 5	-9 ± 3		
		Spiked @0.02 mg kg^{-1} (dry wt.)	Spiked @1 mg kg^{-1} (dry wt.)			
Leaves	Conventional	93 ± 3	91 ± 1	-3 ± 6	4.33	0.02
	Allosperse®	114 ± 26	115 ± 5	-13 ± 3		
	$nSiO_2$	108 ± 13	116 ± 5	-10 ± 2		
Roots	Conventional	125 ± 2	94 ± 1	-11 ± 8	1.99	0.07
	Allosperse®	115 ± 16	114 ± 8	-15 ± 8		
	$nSiO_2$	113 ± 15	118 ± 6	-13 ± 2		

Bifenthrin						
Matrix	Treatment	Recovery % (n = 3)		Matrix Effect % (n = 3)	Precision % (n = 5)	MDLs [a] µg kg^{-1} (n = 5)
		Spiked @0.01 mg kg^{-1} (wet wt.)	Spiked @1 mg kg^{-1} (wet wt.)			
Soil 1	Conventional	92 ± 9	104 ± 4	66 ± 3		
	Allosperse®	84 ± 3	92 ± 3	57 ± 9		
	$nSiO_2$	91 ± 6	106 ± 5	33 ± 10		
Soil 2	Conventional	91 ± 1	93 ± 2	-80 ± 8		
	Allosperse®	78 ± 3	81 ± 2	-60 ± 8	2.36	0.36
	$nSiO_2$	83 ± 5	95 ± 2	-72 ± 9		
Soil 3	Conventional	86 ± 3	98 ± 2	-51 ± 4		
	Allosperse®	71 ± 1	78 ± 5	-73 ± 15		
	$nSiO_2$	86 ± 2	101 ± 1	-71 ± 11		
Strawberry	Conventional	80 ± 12	88 ± 6	0.1 ± 2	16.71	0.03
	Allosperse®	98 ± 4	87 ± 4	15 ± 5		
	$nSiO_2$	126 ± 4	68 ± 3	27 ± 1		
		Spiked @0.02 mg kg^{-1} (dry wt.)	Spiked @1 mg kg^{-1} (dry wt.)			
Leaves	Conventional	107 ± 8	111 ± 12	7 ± 8	8.72	0.08
	Allosperse®	126 ± 6	138 ± 14	20 ± 7		
	$nSiO_2$	103 ± 13	98 ± 8	18 ± 5		
Roots	Conventional	115 ± 3	99 ± 1	20 ± 19	2.97	0.25
	Allosperse®	87 ± 10	114 ± 15	4 ± 21		
	$nSiO_2$	107 ± 9	79 ± 4	43 ± 6		

[a]. MDLs are method detection limits

3.4.2.3 Validation of the developed extraction method for soil

Recoveries were also assessed for three types of agricultural soils. For the lowest spiking level, recoveries ranged from 86-126% for the conventional formulations; from 71-126% for the Allosperse® and from 83-117% for the $nSiO_2$-based nanopesticides. Recoveries were also satisfactory when the soils were spiked with the higher concentration of conventional pesticides (98-122%); Allosperse® (78-110%) and $nSiO_2$ (95-127%). These recoveries were thus comparable to those reported using a QuEChERS approach for the multi-pesticide extraction of several conventional formulations in soils (range of 70 to 120%, MQL for AZOX = 0.01 mg kg^{-1}; Silva et al., 2019). Furthermore, results were similar to those obtained by an accelerated solvent extraction (dichloromethane:acetone, 50:50, v/v) and analysis by GC coupled to selective detectors (reported recoveries for conventional AZOX ranged from 78-130%, MQL = 6.432 µg kg^{-1}; BFT ranged from 71-126%, MQL = 4.779 µg kg^{-1}) (Leyva-Morales et al., 2015). Overall, the extraction procedure proposed here was appropriate for the fast quantification of the different formulations of the two different pesticides in the soils, with a MQL lower than previously reported.

Matrix effects (Table 3.3) were significant (>±20%) for all three of the BFT formulations, for all of the tested soils and for conventional AZOX (Soil 2) and Allosperse®-AZOX (Soil 3). Matrix effects were generally less important for the AZOX formulations as compared to the BFT formulations, although some different tendencies were observed based upon the type of soil examined (Table S3.1, $p < 0.05$). For Soil 1, which was the most OM rich soil, an enhancement of the signal was observed for BFT, whereas for Soil 2 and Soil 3, the signal was suppressed. For example, it was possible to observe a slightly higher recovery for BFT-SiO_2 extracted from Soil 1 when compared to the other soils (Table 3.3; Table S3.1, $p < 0.05$). Matrix effects appeared to be related to the soil, the pesticide type and to the nature of the formulation. Clearly, the addition of

the internal standards D_4-AZOX and D_5-BFT was necessary to compensate for matrix effects and to improve the precision and robustness of the analytical method (Tan and Awaiye 2013; Stachniuk and Fornal 2016; Hu et al., 2016). Overall, the recoveries (Table 3.3) and precision (Table S3.2) were consistent with an accurate, simultaneous extraction of these two pesticides in their formulations, from different soils.

3.4.3 Application to real samples

Chromatograms obtained for strawberries, leaves, roots and soils that were exposed to the different pesticide formulations showed clear, symmetrical peaks at 3.7 min for AZOX and 5.0 min for BFT (Figure 3.2). The chromatograms for AZOX and BFT standards in ACN solvent were also shown in Figure 3.2 (Panels 4, 8, 12, 16, 20, 24, 28, and 32), and were used to quantify the target compounds in sample extracts. AZOX could be detected in all treated plant matrices (range: 1-400 μg kg^{-1}) and in soil samples (range 500- 3000 μg kg^{-1}). BFT was detected in leaves, roots and soils, but not in the fruit (strawberries).

Before widespread the application of those nano herbicides, a reliable and comprehensive risk assessment will be necessary to ensure environmental safety and protect the human health (Kah et al., 2016). Because the standard guidelines for pesticide characterization in environmental and food samples have been established for conventional formulations, the adjusted analytical techniques presented here will be required in order to quantify the nanoformulations and therefore allow a reliable and comprehensive risk assessment, prior to the registration, commercialization and widespread the application of those nano pesticides (Kah et al., 2016; Li et al., 2019).

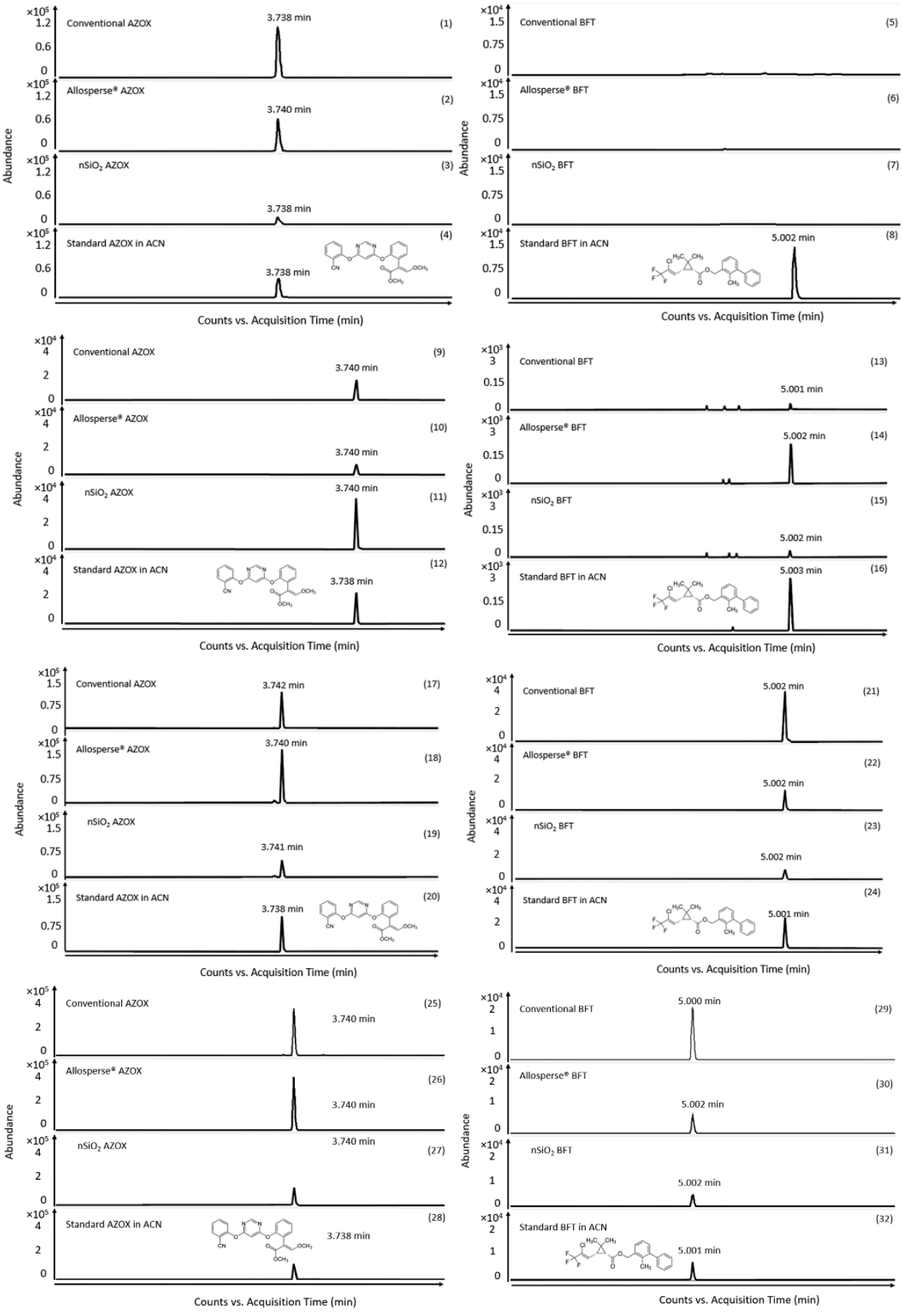

Conventional AZOX
3.738 min
(1)
Allosperse® AZOX
3.740 min
(2)
nSiO2 AZOX
3.738 min
(3)
Standard AZOX in ACN
3.738 min
(4)
Abundance
Counts vs. Acquisition Time (min)
Conventional BFT
(5)
Allosperse® BFT
(6)
nSiO2 BFT
(7)
Standard BFT in ACN
5.002 min
(8)
Abundance
Counts vs. Acquisition Time (min)
Conventional AZOX
3.740 min
(9)
Allosperse® AZOX
3.740 min
(10)
nSiO2 AZOX
3.740 min
(11)
Standard AZOX in ACN
3.738 min
(12)
Abundance
Counts vs. Acquisition Time (min)
Conventional BFT
5.001 min
(13)
Allosperse® BFT
5.002 min
(14)
nSiO2 BFT
5.002 min
(15)
Standard BFT in ACN
5.003 min
(16)
Abundance
Counts vs. Acquisition Time (min)
Conventional AZOX
3.742 min
(17)
Allosperse® AZOX
3.740 min
(18)
nSiO2 AZOX
3.741 min
(19)
Standard AZOX in ACN
3.738 min
(20)
Abundance
Counts vs. Acquisition Time (min)
Conventional BFT
5.002 min
(21)
Allosperse® BFT
5.002 min
(22)
nSiO2 BFT
5.002 min
(23)
Standard BFT in ACN
5.001 min
(24)
Abundance
Counts vs. Acquisition Time (min)
Conventional AZOX
3.740 min
(25)
Allosperse® AZOX
3.740 min
(26)
nSiO2 AZOX
3.740 min
(27)
Standard AZOX in ACN
3.738 min
(28)
Abundance
Counts vs. Acquisition Time (min)
Conventional BFT
5.000 min
(29)
Allosperse® BFT
5.002 min
(30)
nSiO2 BFT
5.002 min
(31)
Standard BFT in ACN
5.001 min
(32)
Abundance
Counts vs. Acquisition Time (min)

Figure 3. 2 Extracted ion chromatograms at m/z 404.1250 for conventional, Allosperse®, $nSiO_2$ and ACN standard - AZOX (Treated strawberry extract: Panel 1-4; Leaf extract: Panel 9-12; Root extract: Panel 17-20; Soil extract: Panel 25-28). Extracted ion chromatograms at m/z 440.1604 for conventional, Allosperse®, $nSiO_2$ and ACN standard - AZOX (Treated strawberry extract: Panel 5-8; Leaf extract: Panel 13-16; Root extract: Panel 21-24; Soil extract: Panel 29-32).

3.5 Conclusion

This paper described rapid and accurate analytical techniques for analyzing nano-based pesticides in strawberry plants and agricultural soils with different characteristics. For the strawberries, a QuEChERS technique was modified, followed by LC-QToF-MS. Extraction time and solvent volume were successfully optimized. For the extraction of the fungicide AZOX in strawberries, plastic extraction tubes were shown to have minimal impact on the recovery of the conventional and nano formulations of the pesticide. When extracting BFT from the fruits, the use of doubling extraction solvent and longer extraction time were shown to give improved recoveries. For 3 different agricultural soils, acceptable recovery and precision could be obtained when using the modified extraction. Given the significant matrix effects that were observed, the use of stable isotopes (D_4-AZOX and D_5-BFT) as internal standards was necessary to properly quantify these emerging products. Because BFT and AZOX are major pesticides from different classes, the modified procedures may be useful for rapid and efficient extractions of other nanopesticides from similar samples, increasing the possibilities for research on nano enabled pesticides and facilitating a more complete understanding of the effects of the nanopesticides on these systems.

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3.8 Supplementary materials

Table S3. 1 Results of ANOVA and Tukey's Post Hoc Test for comparison of the matrix effect between the different pesticide formulations and the classes of samples. The mean difference is significant at a level of 0.05.

		Categories	*p* value Azoxystrobin	*p* value Bifenthrin
Pesticide formulation	Conventional	Soil 1 x Soil 2	0.006*	0.004*
		Soil 1 x Soil 3	0.216	0.004*
		Soil 1 x Strawberry	0.095	0.004*
		Soil 3 x Soil 2	0.084	0.004*
		Soil 2 x Strawberry	0.370	0.004*
		Soil 3 x Strawberry	0.824	0.004*
	Allosperse®	Soil 1 x Soil 2	0.930	0.004*
		Soil 1 x Soil 3	0.004*	0.004*
		Soil 1 x Strawberry	0.604	0.033*
		Soil 3 x Soil 2	0.004*	0.592
		Soil 2 x Strawberry	0.877	0.005*
		Soil 3 x Strawberry	0.004*	0.004*
	$nSiO_2$	Soil 1 x Soil 2	0.647	0.004*
		Soil 1 x Soil 3	0.687	0.004*
		Soil 1 x Strawberry	0.904	0.918
		Soil 3 x Soil 2	1.000	1.000
		Soil 2 x Strawberry	0.977	0.004*
		Soil 3 x Strawberry	0.986	0.004*
Classes of samples	Soil 1	Conventional x Allosperse®	0.587	0.565
		Conventional x $nSiO_2$	0.907	0.018*
		Allosperse® x $nSiO_2$	0.823	0.063
	Soil 2	Conventional x Allosperse®	0.012*	0.149
		Conventional x $nSiO_2$	0.017*	0.703
		Allosperse® x $nSiO_2$	0.957	0.407
	Soil 3	Conventional x Allosperse®	0.004*	0.171
		Conventional x $nSiO_2$	0.948	0.218
		Allosperse® x $nSiO_2$	0.004*	0.980
	Strawberry	Conventional x Allosperse®	0.289	0.041*
		Conventional x $nSiO_2$	0.053	0.030*
		Allosperse® x $nSiO_2$	0.217	0.071

Significant differences between the compared groups ($p < 0.05$) are indicated by an asterisk *.

Table S3. 2 Precision (%) of the pesticide extractions (mixture of AZOX and BIF, 100 μg kg^{-1}) for strawberry fruits and soil.

		Precision (%)	
		AZOX	**BFT**
Strawberry	Intraday (n=5)	3.9	16.7
	Interday (n=4)	3.9	15.3
Soil 1	Intraday (n=5)	1.27	2.36
	Interday (n=4)	3.23	2.85

Table S3. 3 Mean (n=3) mass measurement errors (ppm) for the two pesticide compounds (AZOX and BFT) in pure solvent (ACN) and among the various formulations in strawberry extracts.

Pesticides	***m/z***	**Mass measurement error (ppm) of standard**	**Mass measurement error (ppm) in strawberry extract**	**Significant (p<0.05)**
Allosperse®-AZOX	404.1247	1.4 ± 0.3	1.8 ± 0.2	Yes
$nSiO_2$-AZOX	404.1247	1.4 ± 0.3	1.5 ± 0.1	No
AZOX	404.1247	1.4 ± 0.3	-2.5 ± 0.7	Yes
BFT	440.1604	0.3 ± 0.3	-3.7 ± 0.5	Yes
Allosperse®-BFT	440.1604	0.3 ± 0.3	-4.3 ± 0.4	Yes
$nSiO_2$-BFT	440.1604	0.3 ± 0.3	0.6 ± 0.2	Yes
Allosperse®-AZOX Fragment	372.0971	-2.3 ± 0.2	-1.6 ± 0.2	Yes
$nSiO_2$-AZOX Fragment	372.0971	-2.3 ± 0.2	0.5 ± 0.7	Yes
AZOX Fragment	372.0971	-2.3 ± 0.2	-1.7 ± 0.2	Yes
BFT Fragment	181.1009	0.4 ± 1.7	-1.1 ± 0.8	No
Allosperse®-BFT Fragment	181.1009	0.4 ± 1.7	-0.9 ± 1.2	No
$nSiO_2$-BFT Fragment	181.1009	0.4 ± 1.7	0.4 ± 0.5	No

Table S3. 4 Mean (n=3) mass measurement errors (ppm) for the two pesticide compounds (AZOX and BFT) in pure solvent (ACN) and among the various formulations in soil extracts.

Pesticides	***m/z***	**Mass measurement error (ppm) of standard**	**Mass measurement error (ppm) in soil extract**	**Significant (p<0.05)**
Allosperse®-AZOX	404.1247	1.3 ± 1.2	2.5 ± 0.2	No
$nSiO_2$-AZOX	404.1247	1.3 ± 1.2	1.0 ± 1.9	No
AZOX	404.1247	1.3 ± 1.2	1.5 ± 0.4	No
BFT	440.1604	0.5 ± 0.2	0.6 ± 0.8	No
Allosperse®-BFT	440.1604	0.5 ± 0.2	0.3 ± 0.2	No
$nSiO_2$-BFT	440.1604	0.5 ± 0.2	-0.2± 0.1	Yes
Allosperse®-AZOX Fragment	372.0971	-2.3 ± 0.2	-1.8 ± 0.1	Yes
$nSiO_2$-AZOX Fragment	372.0971	-2.3 ± 0.2	-2.0 ± 0.4	Yes
AZOX Fragment	372.0971	-2.3 ± 0.2	-1.9 ± 0.1	No
BFT Fragment	181.1009	0.4 ± 1.7	1.5 ± 1.5	No
Allosperse®-BFT Fragment	181.1009	0.4 ± 1.7	1.9 ± 1.0	No
$nSiO_2$-BFT Fragment	181.1009	0.4 ± 1.7	1.4 ± 0.5	No

Table S3. 5 The relative intensities of qualifier (372.0971 *m/z* for AZOX and 181.1009 *m/z* for BFT) to quantifier ions (404.1247 *m/z* for AZOX and 440.1604 *m/z* for BFT) in pure acetonitrile and in soil, strawberry, root and leave extracts (n=3).

Pesticide	**Matrix**	**Formulation**	**Relative intensity (% of base peak)**	**Ratio[a]**	**Significant among formulations (p<0.05)**
AZOX	Solvent	Acetonitrile	6.42 ± 0.2%	-	
	Soil	Conventional	6.36 ± 0.54%	-0.81%	No
		Allosperse®	6.15 ± 0.08%	-4.08%	
		$nSiO_2$	6.38 ± 0.33%	-0.63%	
	Strawberry	Conventional	3.26 ± 0.08%	-49.19%	Yes
		Allosperse®	3.28 ± 0.03%	-48.84%	
		$nSiO_2$	6.51 ± 0.19%	1.42%	
	Leaves	Conventional	9 ± 0.61%	40.25%	No
		Allosperse®	8.76 ± 0.49%	36.56%	
		$nSiO_2$	8.9 ± 0.39%	38.66%	
	Roots	Conventional	9 ± 0.61%	6.05%	No
		Allosperse®	8.76 ± 0.49%	3.15%	
		$nSiO_2$	8.9 ± 0.39%	8.72%	
BFT	Solvent	Acetonitrile	16.02 ± 1.32%	-	
	Soil	Conventional	17.2 ± 0.72%	7.40%	No
		Allosperse®	17.88 ± 0.57%	11.64%	
		$nSiO_2$	18.48 ± 1.53%	15.38%	
	Strawberry	Conventional	16.09 ± 0.39%	0.46%	No
		Allosperse®	16.64 ± 0.66%	3.90%	
		$nSiO_2$	16.05 ± 0.87%	0.18%	
	Leaves	Conventional	19.76 ± 1.48%	23.39%	No
		Allosperse®	20.53 ± 0.85%	28.17%	
		$nSiO_2$	19.63 ± 3.81%	22.57%	
	Roots	Conventional	12.69 ± 2.3%	-20.77%	No
		Allosperse®	12.13 ± 0.3%	-24.25%	
		$nSiO_2$	14.07 ± 1.44%	-12.17%	

[a] If the relative intensity (% of base peak) <10%, the default recommended maximum permitted tolerances should be ±50%; If the relative intensity (% of base peak) in 10% to 20% range, the default recommended maximum permitted tolerances should be ±30%, according to Document No. SANCO/12495/2011.

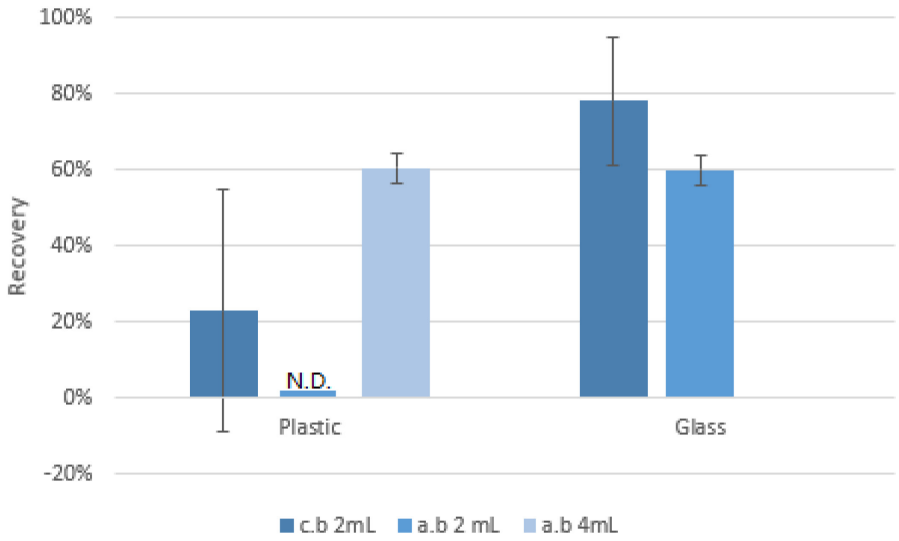

Figure S3. 1 Recovery of Allosperse®-BFT (a.b) and Conventional BFT (c.b) in strawberry samples when extracted with plastic or glass centrifuge vials, and 2 mL or 4 mL extraction solvent (Shaking time: 1 min; spiked standard:10 ug kg^{-1})

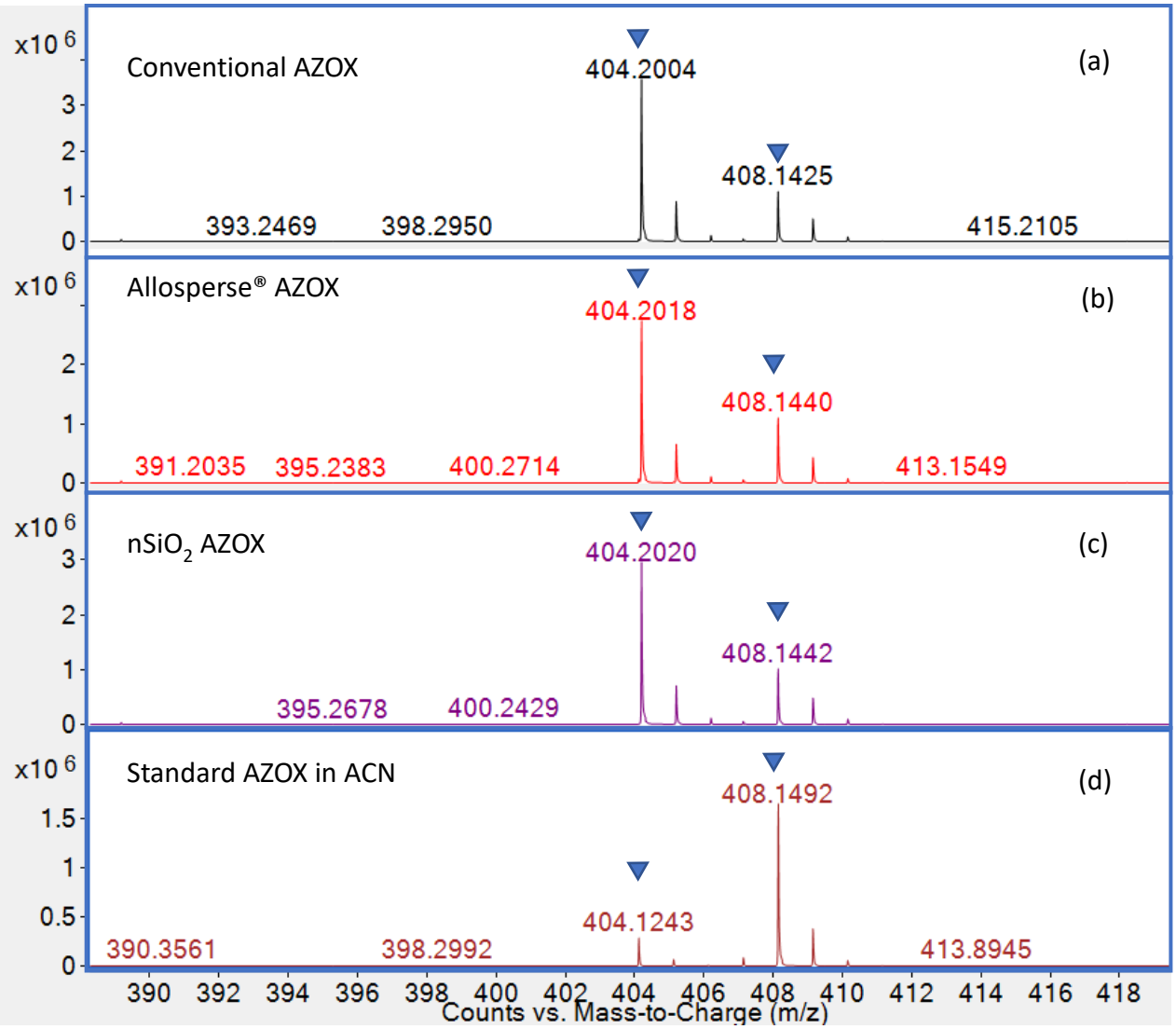

Figure S3. 2 All Ions MS/MS spectra for molecular AZOX (around 404.2004 m/z at RT=3.738 min) in a conventional AZOX treated strawberry extract (Panel a); All Ions MS/MS spectra for molecular AZOX (around 404.2018 m/z at RT=3.740 min) in a Allosperse®-AZOX treated strawberry extract (Panel b); All Ions MS/MS spectra for molecular AZOX (around 404.2020 m/z at RT=3.738 min) in a $nSiO_2$-AZOX treated strawberry extract (Panel c); All Ions MS/MS spectra for molecular AZOX (around 404.1243 m/z at RT=3.733 min) in pure standard in ACN solution (Panel d).

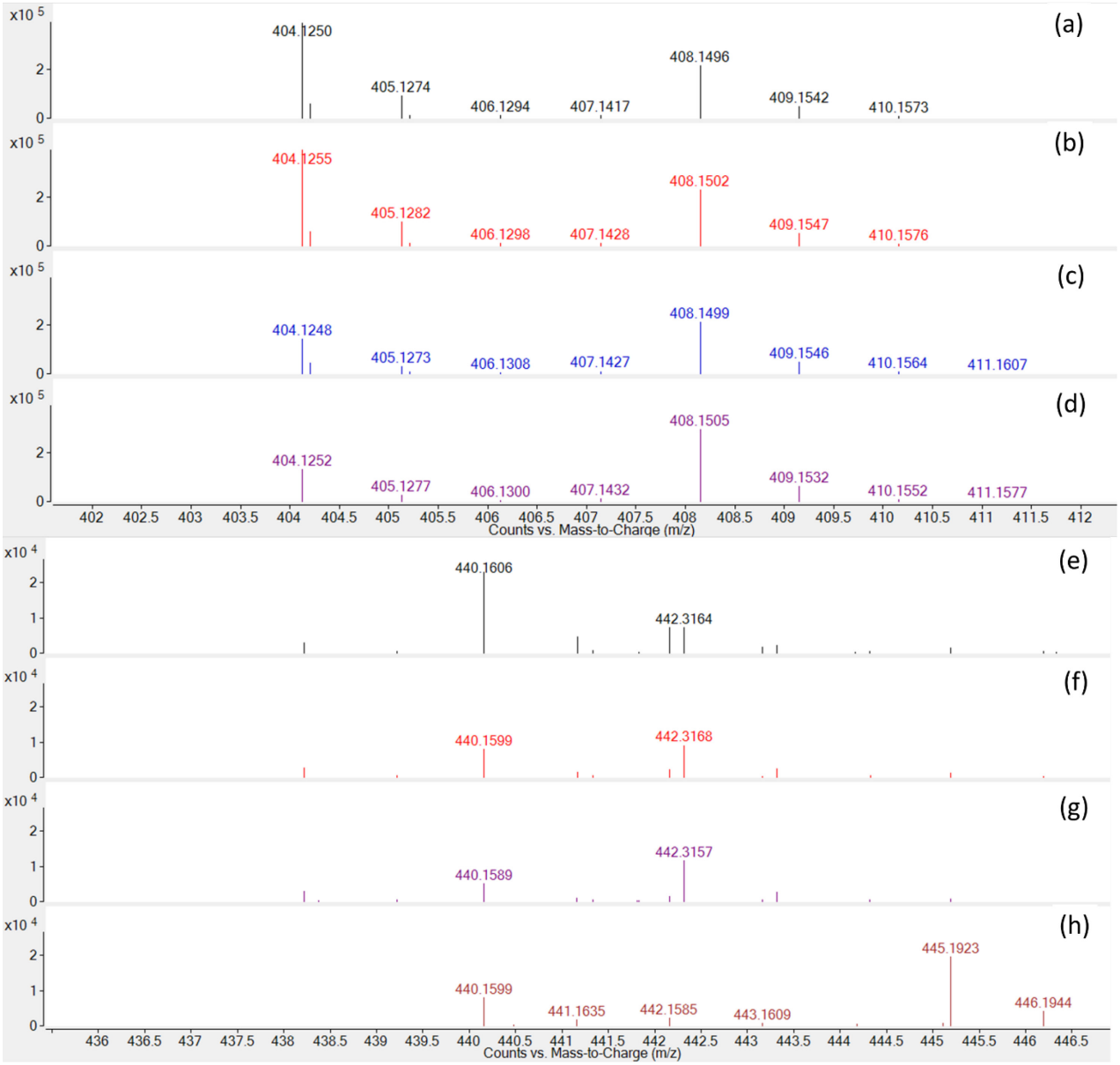

Figure S3. 3 All Ions MS/MS spectra for molecular AZOX (around 404.1255 m/z at RT=3.707) in a conventional AZOX soil extract (Panel a); All Ions MS/MS spectra for molecular AZOX (around 404.1225 m/z at RT=3.705) in a Allosperse®-AZOX treated soil extract (Panel b); All Ions MS/MS spectra for molecular AZOX (around 404.1248 m/z at RT=3.706) in a $nSiO_2$-AZOX treated soil extract (Panel c); All Ions MS/MS spectra for molecular AZOX (around 404.1252 m/z at RT=3.701) in pure standard in ACN solution (Panel d). All Ions MS/MS spectra for molecular BFT (around 440.1606 m/z at RT=4.979) in a conventional BFT soil extract (Panel e); All Ions

MS/MS spectra for molecular BFT (around 440.1599 m/z at RT=4.982) in a Allosperse®-BFT treated soil extract (Panel f); All Ions MS/MS spectra for molecular BFT (around 440.1589 m/z at RT=4.981) in a $nSiO_2$-BFT treated soil extract (Panel g); All Ions MS/MS spectra for molecular BFT (around 440.1599 m/z at RT=4.984) in pure standard in ACN solution (Panel h).

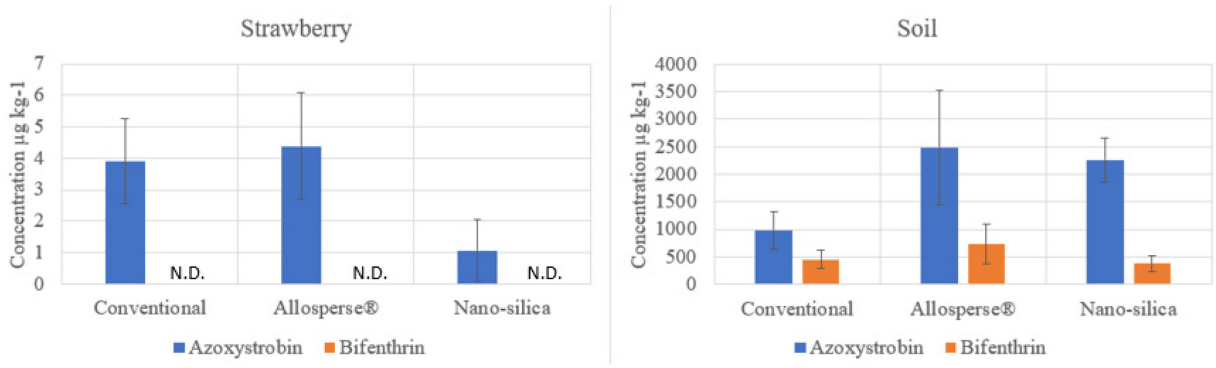

Figure S3. 4 Concentration of the pesticides (conventional and nano) in the field plots and strawberry samples after 30 days of exposure to the nanopesticides. N.D. = not detected.

Connecting Text

The development and validation of specific analytical methods for NEPs in the different matrixes (plant tissues and soils) were presented in Chapter 3. Based on the results obtained, validated analytical methods based on QuEChERS for plants and solvent extraction for soils were shown to have good recoveries, precision and low detection limits for both AZOX and BFT in both nanoencapsulation and conventional formulations. In Chapter 4, the validated analytical methods were applied to incurred field samples collected from a well-controlled strawberry culture system treated with NEPs of AZOX and BFT. This experiment investigated the distinct fate and effects of the NEPs on plant growth compared with their conventional formulations equivalent via analyzing the irrigation leachates, plants and soils. Chapter 4 will be submitted as "Field evaluation of the potential effects of polymer and silica-based nanopesticides on strawberries and agricultural soils" (Juliana A. Galhardi*, Peiying Wang*, Vinicius Bueno, Subhasis Ghoshal, Gravel, V., Kevin J. Wilkinson, Stéphane Bayen)

Chapter 4. Field evaluation of the potential effects of polymer and silica-based nanopesticides on strawberries and agricultural soils

4.1 Abstract

Polymeric and SiO_2 nanoparticles can be used as nanocarriers to improve the efficacy of pesticide delivery in agriculture. However, the environmental fate and potential risks of this type of nanopesticides in agroecosystems remain poorly understood. In this study, two separate active ingredients, azoxystrobin (AZOX) and bifenthrin (BFT), loaded into two different types of nanocarriers (Allosperse® polymeric nanoparticles and SiO_2 nanoparticles), were applied to strawberry plants under realistic field conditions over two growing seasons. The pesticide concentration profiles in soil and plant tissues, plant growth and soil microorganisms were compared among treatments. Although the encapsulation appeared to reduce sorption of the active ingredients (AI) to the soils, few of the sensitive indicators of ecosystem health showed any differences when compared to controls. Bioaccumulation of the AI by the strawberry plants and fruit was similar for classical and nano-applications of the AI. No significant differences were observed among the conventional, nanopesticide or control treatments in terms of fruit mass, number of flowers and leaves, or biomass. Finally, the soil microbial composition (Shannon indices, PCoA plots) and function (soil enzyme activity) only showed some transient, initial effects to the pesticides, but did not distinguish among formulations.

Keywords: nanopesticides; uptake; soil enzyme activity; soil bacterial community; SiO_2 nanoparticles, polymeric nanoparticles.

4.2 Introduction

Synthetic nanoparticles (NPs, ≤100 nm particle size) are increasingly incorporated into products and applications in agriculture (Mohd Firdaus et al., 2018; Zhang et al., 2020). For example, polymeric nanocarriers and metal oxide NPs are being used in fertilizers, growth regulators and pesticides, to control their release or to facilitate target-specific delivery (Kah et al., 2016; Wang et al., 2016). Nanopesticides are being designed with the ambition to deliver the active ingredients (AIs) more efficiently, reduce impacts to non-target organisms and provide longer pest protection (Petosa et al., 2017; Zhang et al., 2019). This technology has the potential to reduce the ecological risks associated with pesticides with respect to more conventional formulations, while more efficiently contributing to crop protection (Hofmann et al., 2020).

Despite the prospects of nanotechnology in agriculture, the environmental fate and the ecological risks of nanomaterials have not been fully documented, in particular, for nanomaterials that may be in contact with crops and foods (Dan et al., 2015; Prasad et al., 2017). Due to the high specific surface area of the NPs and thus their high capacity for adsorption or partitioning, their direct or indirect (e.g., biosolids; Asadishad et al., 2018) addition to agricultural soils is likely to alter the biogeochemical cycling of trace elements and organic substances in soils (Kah et al., 2016). Some early studies indicated that Ag NPs could perturb soil nutrient cycling (Peyrot et al., 2014), while $Cu(OH)_2$ nanopesticides have been shown to affect microbial diversity (Zhang et al., 2019). Indeed, nanopesticides were postulated to have a higher bioavailability when compared to their conventional forms (Zhang et al., 2019).

Azoxystrobin (AZOX, log K_{ow} 3.7), a major strobilurin fungicide, and bifenthrin (BFT, log K_{ow} 6.6), a pyrethroid insecticide, are commonly used in agriculture, including strawberry production. These active ingredients are being incorporated into commercially available

polyacrylic acid (PAA) based nanocarriers (e.g., Allosperse®, from Vive Crop) for crop protection. In addition, silica nanoparticles ($nSiO_2$) have emerged as a new product to control the release of drugs or pesticides based upon stimuli-response (Liang et al., 2020). As both AZOX and BFT may have some impacts on soils (e.g. bacterial communities, Wang et al. (2020); Mukherjee et al. (2020)) or may be toxic to aquatic organisms (Petosa et al., 2017), nanoencapsulation could be seen as a strategy to mitigate the potential ecological risks associated with their use in agriculture. In a controlled experiment, the toxicity of an encapsulated form of AZOX (i.e. Allosperse®) was significantly lower for zebrafish than was its conventional formulation (Zhang et al., 2020). In contrast, earthworms exposed to BFT-Allosperse® accumulated ~50% more of the AI than those exposed to the conventional formulation. However, while most of the conventional BFT was found in external earthworm tissues, BFT applied as a nanopesticide was mainly detected in the gut and therefore not internalized (Mohd Firdaus et al., 2018). In another study, AZOX-loaded mesoporous silica NPs exhibited better fungicidal activity than AZOX alone (Xu et al., 2018). Although the beneficial effects of silica for plants are well established (Rastogi et al., 2019), $nSiO_2$ have been shown to exhibit acute toxic effects *in vivo* (Murugadoss et al., 2017) and to affect plant biomass and nutrient content (Le et al., 2014).

Given the differences observed between the conventional formulations and the nanopesticides with respect to their bioavailability and mobility in soils, it is essential to determine NP fate under realistic conditions if one is to properly evaluate their environmental risk (Walker et al., 2017). However, there are only few comprehensive studies that have analyzed the fate, uptake, and impact of nanopesticides in field experiments, under reasonable usage scenarios. Therefore, the overall objective of this study was to evaluate the environmental effects of several nanopesticides that were based on commercially available polymer and silica-based nanocarriers.

Field mesocosm (pot strawberry culture under real weather/irrigation conditions) assessments were performed over 2 growing seasons by comparing the treatments with nanopesticides to both control (no treatment) and conventional formulation treatments. The specific objectives were to: **a)** compare the uptake of the AIs (AZOX, BFT) by strawberry plants and fruits; **b)** assess the effects of the different pesticide formulations on the biological properties of the soil, including enzyme activity (glucopyranoside, phosphomonoesterase, arylsulfatase, and β-D-glucosidase) and the microbial community structure. Soil microbiota were evaluated as a non-target organism and surrogate for the health of the soils through the measurements of function (soil enzyme activities) and microbial community structure. Strawberry plants were used as the test crop since they have the ability to accumulate pesticides into the fruits following assimilation from the roots, which represents a vegetal source for human exposure (Dias et al., 2015) and since pesticides and fungicides are commonly applied to the production of this fruit (Warner et al., 2021).

4.3 Material and methods

4.3.1 Polymeric and SiO_2 based nanopesticides

The pesticides AZOX (96.5% AI), BFT (98.5% AI), AZOX-Allosperse® (18.4% AI) and BFT-Allosperse® (19.3% AI), as well as a mixture of the dispersant agents contained in all of the nanoformulations were obtained from Vive Crop Protection Inc (Mississauga, Canada). Hollow $nSiO_2$ used in the first experimental year were those acquired from Materium Innovations (Granby, Canada), while those used in the second year were synthetized according to Bueno et al. (2022). Particles sizes were previously characterized by Diaz et al. (2015) (Allosperse® - 7 nm), Kah et al. (2016) (Allosperse®-BFT – 333 to 424 nm), Zhang et al. (2020) (Allosperse®-AZOX - <100 nm), and Bueno and Ghoshal (2020) ($nSiO_2$ - 258nm). $nSiO_2$ were loaded with the active

ingredients to produce nanoencapsulated $nSiO_2$–AZOX and $nSiO_2$–BFT. Stock solutions of the nanoformulations were prepared in Milli-Q water (R>18 MΩ cm; TOC < 2 μg C L^{-1}). Stock solutions of AZOX and BFT contained the same proportions of dispersive agents to AI as the nanoformulations provided by Vive Crop Protection Inc. Analytical standards of the pure compounds, AZOX (≥98%, CAS#131860-33-8), and BFT (≥98.0%, CAS#82657-04-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated internal standards (azoxystrobin-d_4 and bifenthin-d_5) and azoxystrobin free acid (R234886, AzFA) were purchased from Toronto Research Chemicals (North York, ON, Canada). HPLC grade solvents (water, acetonitrile (ACN), and methanol), anhydrous magnesium sulphate, sodium acetate, LC/MS grade formic acid and ammonium acetate were obtained from Fisher (Pittsburgh, PS, USA).

4.3.2 Field experiments

The field experiment was carried out at the Macdonald Campus of McGill University (Ste-Anne-de-Bellevue, QC, Canada), over two growing seasons, under realistic field conditions. During the first experimental year, the experimental design was optimized (i.e. methods for collection and preparation of the soil, preparation of the pots, placement of the pots in the field, construction of the irrigation and fertilization systems, etc.). At the end of the growing season of the first experimental year, some final samples were collected, plants were removed from the pots, pots with soil were covered with a black polyethylene sheet (to protect against weathering) and then left outdoors over the winter. Therefore, the soil used in the second growing season was that which contained residual pesticide concentrations (as would a real-world field site). New strawberry (bare root) plants were planted in the second year and soil and strawberry samples were collected at a higher frequency than year one in order to provide a higher resolution on the concentration profiles.

The agricultural soil used for this experiment was characterized as clayey soil with the following characteristics: pH 7.2, 6.1 % organic matter, 183 mg kg^{-1} of P, 3999 mg kg^{-1} of Ca, 325 mg kg^{-1} of Mg, 349 mg kg^{-1} of K, 717 mg kg^{-1} of Al, 6.4 mg of N kg^{-1} as NO_3^{-1} and 2.5 mg of N kg^{-1} as NH_4^+ (soil characterization methods are provided in the Supplementary Material). Forty-five, 20 L-polyethylene pots, each containing 18 kg of soil, were arranged randomly on a black plastic polyethylene tarp (5 rows × 9 columns). The tarp was used as a secondary containment to prevent any transfer of pesticide residues to the soil. Pots were positioned on a wood structure at a height of 30 cm above ground in order to collect any excess water leaching from the soil under each pot. Four strawberry bare root plants (*Fragaria* × *ananassa* "Seascape", Pépinière Lareault, QC, Canada) were planted in each pot (Figure S4.1). Irrigation with pesticide-free water was performed on a daily basis, whereas fertilization was performed weekly.

Strawberries were planted in early June and pesticide treatments were applied twice (15 and 30 days after transplantation), according to the suggested maximum application dosages for the commercial conventional pesticide formulations (USEPA, 2015a; USEPA, 2015b). Treatments with AZOX all contained 7.6 mg active ingredient / pot, whereas treatments with BFT contained 7.98 mg active ingredient / pot. A drench method was used for the application of the different treatments in order to better control the amounts of pesticides applied to each pot, particularly avoiding losses to the surroundings, such as air. In addition, the drench application allowed us to better assess uptake by the plants through the roots and the effects of the treatments on the soil microorganisms. Nine different conditions were evaluated in replicate (n = 5): **(i)** Control (no nanoparticle and pesticide added); **(ii)** 0.04 mg kg^{-1} of $nSiO_2$ only; **(iii)** 0.04 mg kg^{-1} of Allosperse® only; **(iv)** BFT; **(v)** AZOX; **(vi)** $nSiO_2$ -BFT; **(vii)** $nSiO_2$ -AZOX; **(viii)** Allosperse®-BFT; and **(ix)** Allosperse®-AZOX. Dispersants were added in **(i)** to **(vii)** in order to reproduce the

amounts present in the nanoformulations provided by Vive Crop Protection. For each formulation, a 1 L stock solution was first prepared in ultrapure water where it was left to equilibrate for 24 hours prior to field application. In the field, stock solutions were separated into five 200 mL aliquots, which were diluted to 1 L using the irrigation water and then applied using a soil drench in each of the 5 pot replicates, carefully avoiding direct contact of the solutions with the plants.

Strawberries, soil and leachate samples were collected for pesticide residue analysis by sampling only the three rows in the middle of the field to avoid edge effects. For the ***leachates***, volumes were recorded continuously for each pot. Aliquots of the leachates were collected and filtered into glass vials (0.22 μm PTFE filter, Chrom4; Thuringen, Germany) for pesticide analysis. Prior to LC-MS analysis, leachate samples were spiked with internal standards: 40 μg L^{-1} of AZOX-d_5 and 60 μg L^{-1} of BFT-d_5. For the ***soils***, three subsamples were collected from each pot, 72 and 85 days after the application of the formulations in the first experimental year, and 14, 30, 52, 60, 72, and 85 days after the application of the formulations in the second experimental year. Subsamples were homogenized in an aluminum tray, transferred to a 20 mL glass flask, and stored at -20 ºC until extraction. For the measurements of pesticide residues in the ***strawberries***, sampling was performed on days 23, 33, 53, 63 and 73 days post-application in the first experimental year, and 21, 26, 40, 52 and 85 after pesticide application in the second year. ***Leaves and roots*** were sampled uniquely at day 85, i.e., the last experimental day, for both experimental years. All the plant samples were stored in glass vials at -20 ºC prior to extraction. Figure S4.2 shows an overview of the sample collection timeline.

Phenological data was acquired for one plant from each of the three middle pots and included the plant biomass (without the fruits) in addition to the number of leaves and the number of flower stalks at the end of the exposure period. The ripe fruit yields for each pot were also

recorded during the growing season. At the end of the season, three plants from different pots were collected from each treatment. They were air dried in order to measure plant biomass.

4.3.3 Pesticide analysis in soils and plants

AZOX and BFT in the strawberry plant tissues and soils were analyzed using a LC-QTOF-MS-based method, recently developed by Wang et al. (2022) and summarized in the Supplementary Material. Method detection limits (MDL) and method quantification limits (MQL) were: AZOX in strawberry (MDL = 0.14 μg kg^{-1}, MQL = 0.46 μg kg^{-1}), BFT in strawberry (MDL = 0.03 μg kg^{-1}, MQL = 0.10 μg kg^{-1}), AZOX in soil (MDL = 0.65 μg kg^{-1}, MQL = 2.15 μg kg^{-1}), BFT in soil (MDL = 0.36 μg kg^{-1}, MQL = 1.2 μg kg^{-1}) (Wang et al., 2022). The instrumental detection limits for AZOX and BFT were 0.3 pg and 2.2 pg, respectively.

4.3.4 Degradation products of pesticides in samples

LC/MS data were screened for potential metabolites and degradation products of AZOX or BFT for the different matrixes and treatments. First, LC/MS data were aligned using the Agilent Masshunter Profinder (Agilent Technologies, USA), using tolerances of 0.15 min for the retention times (RT) and 10 ppm for the mass differences. A library of AZOX and BFT metabolites was prepared using the Agilent Masshunter PCDL software (Agilent Technologies, Table S4.2 & S4.3), based on formulae reported in the literature (Fecko, 1999; FAO, 2009; 2010; Gautam, Etzerodt & Fomsgaard, 2017). The library was used to screen the LC/MS data for possible metabolites of AZOX and BFT. The MS/MS spectra of those metabolites were manually compared with spectra from the literature to increase confidence in the identification. The identity of the AZOX free acid (AzFA), a major degradation product of AZOX, was confirmed using the pure reference standard

(matching RT=3.491 min and ion at 372.0971 *m/z)*. The signals for selected compounds of interest were compared across the pesticide and control treatments using the Agilent Masshunter Qualitative Analysis software (Agilent Technologies).

4.3.5 Soil enzyme activities

Extracellular enzymes in soils can be sensitive indicators of changes of soil quality and fertility (Galhardi et al., 2020). Soil enzyme activities: glucopyranoside (MUB-C), phosphomonoesterase (MUB-P), arylsulfatase (MUB-S) and β-D-glucosidase (AMC-N) were measured immediately after sampling the soils in 15 mL Falcon tubes at 1, 7, 14, 30, 60, and 85 days after the application of the treatments in the second experimental year. Enzyme activities were determined according to Peyrot et al. (2014), as summarized in the Supplementary Materials.

4.3.6 Microbial community assays

Genomic DNA (gDNA) was extracted from soil that was randomly collected from the pots on days 0 (first day of first experimental year), 356 (first day of the second experimental year) and 455 (last day of the second experimental year). In summary, 250 mg of dry soil (N = 3) was processed using a DNeasy PowerSoil Pro kit (Qiagen) in order to obtain gDNA suspensions ready for downstream applications. Quality control on the extracted gDNA was performed by quantifying the DNA content using the PicoGreen method (Susan et al., 1996) (Invitrogen Quant-iT PicoGreen dsDNA Assay Kit, Thermo Fisher). The V4 region of the 16S rRNA gene in archaea and bacteria was amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplified sequences were analyzed on a Ilumina MiSeq instrument using the PE250 protocol.

The sequence reads were processed using the QIIME2 pipeline (version 2019.4) (Bolyen et al., 2019). The processing included: pairing forward and reverse sequence reads, demultiplexing sequences by linking the barcode information with the corresponding samples, denoising the amplicon sequence data with the DADA2 pipeline (Callahan et al., 2016) and truncating at positions 20 on the left, and 220 on the right, when quality started to drop significantly. Taxonomic ranks were assigned to the 16S rRNA processed sequences using Naïve Bayes Taxonomic Classifier (Wang et al., 2007) trained with the Greengenes database (McDonald et al., 2012).

4.3.7 Statistical analysis

One way analysis of variance (ANOVA) followed by a Tukey's test were used to identify significant differences as a function of time, using $p<0.05$ to denote statistical significance. A two-way ANOVA ($p<0.05$) was used to identify differences among the different AIs (AZOX and BFT) and the different formulations (conventional and nanoformulations based on Allosperse® and $nSiO_2$). All data are presented as means ± standard deviations for values obtained from at least three independently performed experiments. Shannon's index and β-diversity metrics used for Principal Coordinate Analysis (PCoA) were performed using the q2-diversity pipeline.

4.4 Results and Discussion

4.4.1 Leaching from the soils depends on the pesticide formulation

4.4.2 Leaching from the soils depended mainly on the pesticide and less on the formulation

For AZOX, concentrations and the cumulative mass (m_{AZOX}) were measured over time in the leachate solutions (Figures S4.3 and S4.4). For all of the formulations (conventional AI, Allosperse®-AZOX and $nSiO_2$-AZOX), m_{AZOX} were highest from day 25 to 52, before decreasing

to near background levels at day 68. The decreasing concentrations of pesticide from day 52 were consistent with the profile for the cumulative precipitation (Figure S4.5), where high precipitation rates were observed up to day 52 prior to dropping down between days 52 and 72. The final m_{AZOX} in the leachates represented 0.10%, 0.20% and 0.09% of the initial amounts added to each pot for the conventional, Allosperse® and $nSiO_2$ treatments, respectively. Although all losses to leaching were small (i.e., $\leq$ 0.2%), the results suggest that the Allosperse® encapsulated AZOX were more water soluble and thus more mobile than the other formulations (for the Allosperse®, AZOX in the leachate was significantly higher than for the conventional and $nSiO_2$ treatments on days 39 and 52).

In contrast, BFT concentrations in the leachates were always below the MDLs, which is consistent with previous results (Petosa et al., 2017) that showed very limited mobility of a conventional formulation due to the high affinity of the BFT for the soil ($\log K_{ow} = 6.6$). Although nanoencapsulation of the BFT (poly(methacrylic acid) based nanocarriers) could have improved the mobility of the AIs (Kah et al., 2016), that was not observed here where no BFT could be detected in the leachate. Nonetheless, it should be noted that leachate concentrations are largely influenced by the sampling interval and the rainfall volumes, implying that tendencies in the concentration data have to be carefully interpreted.

4.4.3 Formulation and nanocarrier type affected the mobility of pesticides in soils

Concentrations of the pesticides extracted from the soils are shown in Figure 4.1. Pesticides were not detected in any of the control samples. In the second experimental year, C_{AZOX} and C_{BFT} measurements at day 0 correspond to the quantities remaining from the first experimental year, which were not significantly different from concentrations from the end of the first

experimental year (p<0.05). The slight increase of C_{AZOX} and C_{BFT} on day 30 is mainly related to the addition of the second dose of pesticide to the soils, which occurred just after sampling on day 14. Subsequently, C_{AZOX} and C_{BFT} (also $C/C_{0\ AZOX}$ and $C/C_{0\ BFT}$, Figure S6) decreased after days 60 or 52, respectively, for the conventional formulations. Given the reported half-lives in agricultural soils of AZOX which ranges from 58 to 87 days (Edwards et al., 2016), and for BFT, which is 125.3 ± 13.3 days (Kah et al., 2016), the decreasing C_{AZOX} can be attributed to chemical or enzyme degradation, assimilation by soil organisms, uptake by crops and leaching from the soil (Xu et al., 2018; Wang et al., 2020). The observed decrease of C_{BFT} with time is likely related to chemical or enzymatic degradation and assimilation by soil organisms (Kah et al., 2016; Mohd Firdaus et al., 2018).

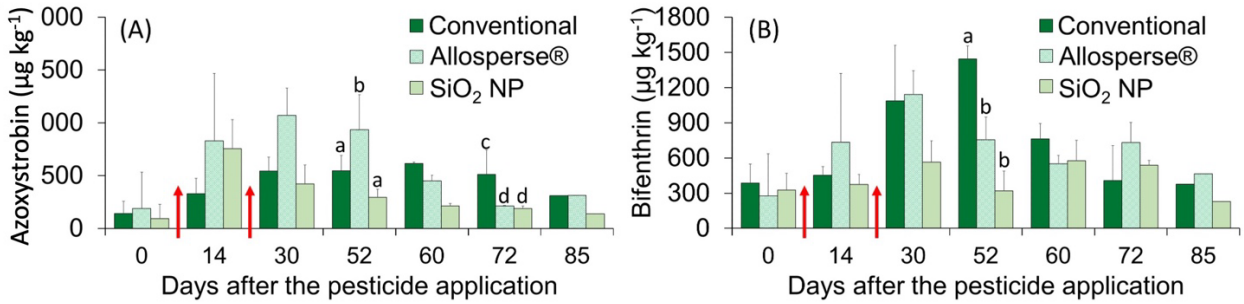

Figure 4. 1 Concentrations of AZOX (A) and BFT (B) (conventional and nano forms) in the soils in the second experimental year as a function of time following the application of the pesticide formulations. Red arrows indicate when the addition of the treatments to the soils occurred (days 0 and 14). For a specific timepoint, significant differences (ANOVA) between different formulations are represented by different letters, according to Tukey's test. Data are means ± standard deviation (SD), n = 3.

Generally speaking, the nanoformulations appeared to be more mobile than the conventional formulations. For example, a 45% decrease in conventional AZOX was observed between days 30 to 85 as compared to a 62% decrease for Allosperse®-AZOX and a 59% decrease for $nSiO_2$-AZOX, reflecting perhaps an increased sorption of the conventional pesticide to the soil when not encapsulated by the nanocarriers (Figure 4.1). The more pronounced decrease of AZOX in the nanoformulations might imply an increased availability for the plants and soil microorganisms. For both pesticides, the nanoformulations appeared to increase soil mobility as the peak in soil associated compound occurred earlier (AZOX: day 30 for the Allosperse® and day 14 for the SiO_2 as compared to day 60 for the conventional formulation; BFT: day 30 for the Allosperse® and SiO_2 NP as compared to day 52 for the conventional formulation). When compared to its maximum measured concentration in the soil, BFT concentrations on day 85 represented a 70% reduction for the conventional formulation as compared to a 71% reduction for the Allosperse and a 69% reduction for the SiO_2. Indeed, on day 52, a significantly higher concentration of BFT was measured in the soil with respect to either of the nano-formulations (Figure S4.6). Similarly, for AZOX, concentrations of the conventional formulation were the highest at the end of the field experiment, consistent with an increased leaching of the nano-formulations. All of these indicators suggest that the nanoformulations were more mobile and less associated with the soil. For the BFT, these results appear to contrast with Kah et al. (2016), who showed increased sorption to soil, therefore lower mobility, when it was encapsulated in a polymeric nanoparticle. In addition to mobility, reduced pesticide concentrations in soil may be due to other processes such as plant uptake or degradation. Therefore, the effects of nanocarriers on pesticide residues in soil should be analyzed in detail.

4.4.4 Nano-formulations had a limited impact on bioaccumulation or plant growth

BFT levels were below the MDL for all plant tissue samples. This was expected as BFT is a non-systemic pesticide, and nanoencapsulation did not modify this behavior. AZOX concentrations in the strawberry plant tissues (fruits, leaves and roots) and their bioaccumulation factors (BFs) are given in Figure 4.2 for several exposure times. Although AZOX levels were significantly lower on day 21 in the $nSiO_2$-AZOX exposures (Figure 2A), no differences were observed when concentrations were normalized to the measured concentrations in the soil (i.e. bioaccumulation factors, BF). In fact, when comparing BF, significant differences were only observed at day 52, where the conventional formulation of AZOX appeared to be more strongly accumulated (Figure 2B). Nonetheless, this contrasted with year 1 data, which showed a higher BF for the Allosphere® in comparison to the other treatments (Figure S4.8B).

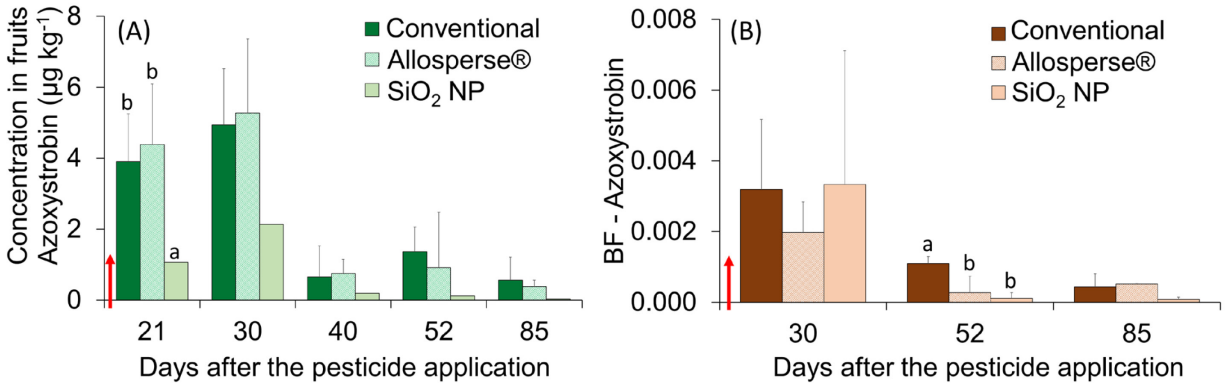

Figure 4. 2 Concentration of azoxystrobin (conventional and nanoencapsulated) in the fruits (A) and the calculated bioaccumulation factors (B; BF = concentration in the fruits divided by the concentration in the soils) for AZOX following different exposition times (days) beginning at the

first dosage application. Red arrows indicate when the addition of the treatments to the soils occurred. Pesticides were applied at days 0 and 14. Significant differences (ANOVA) between different formulations at the same sampling date are represented by different letters (Tukey's test, $p<0.05$). Data are the means ± SD, n = 3. BF were only calculated for the three sampling days when both soil and fruits were collected concurrently.

In contrast to the soil concentrations (all formulations) that peaked on days 52-60, C_{AZOX} in the strawberries were at their maxima earlier in the exposure period (day 21 or 30) (Figure 4.2A), resulting in larger BFs at the beginning of the growing season (Figure 4.2B). From day 40, lower C_{AZOX} were observed in the fruits, possibly reflecting some metabolism/degradation of the AZOX by the plants, in addition to a decrease of C_{AZOX} in the soil. Nonetheless, there were no AZOX metabolites detected in the fruits, leaves or soils. For example, the free acid of AZOX is its major metabolite. It was detected in the roots on the last sampling day, which is consistent with AZOX being metabolized by the plants (Figure S4.9). No significant differences in the concentrations of the free acid were observed between the conventional and nanoformulations (AZOX: 35.2 ± 8.2; Allosperse®-AZOX: 64.8 ± 36.6; nSiO_2-AZOX: 46.6 ± 34).

Although the C_{AZOX} in strawberry fruits were similar in conventional and Allosperse® formulations up to day 40, at day 52, the BF was slightly higher for the conventional AZOX ($p<0.05$) than for the nanopesticides (Figure 4.2), indicating that AZOX might be more bioaccessible when in the conventional formulation. Nanocarriers are thought to reduce the bioaccessibility and therefore the plant uptake of AZOX, due to the slow-release rate of the AI from the nanoparticles (Bueno et al., 2021). Because the C_{AZOX} is much lower than MRLs and the

difference among conventional and nano pesticides is small, the relevant toxicity of nanopesticides to humans is negligible.

AZOX residues were analyzed in the plant tissues at the end of the growing season (day 85). The highest levels were recorded in the roots (up to 74.51 μg kg^{-1}), followed by the leaves (up to 3.00 μg kg^{-1}) and the fruits (up to 1.29 μg kg^{-1}) for both experimental years (Figure S4.10, Figure S4.11). AZOX can be taken up in the roots mainly by passive transport and is more likely to accumulate in organelles with a higher lipid content (Ju et al., 2019). These results are in line with those obtained for rice exposed to fenoxil encapsulated into mesoporous $nSiO_2$, which also showed absorption by the roots and translocation to above ground tissues (Zhu et al., 2018). BFs increased in the order fruits < leaves < roots. There were no significant differences observed among the different pesticide formulations for any of the BFs for leaves or roots. Based on the higher transfer factors (TFs) for the AZOX (Table S4.1), transfer from the roots to the leaves was facilitated as compared to that from the leaves to the fruits, for both experimental years. Similar to the BFs, no significant differences (p>0.05) were observed among TFs for different formulations of pesticides.

No significant differences were observed among the conventional, nanopesticide or control treatments in terms of fruit mass (Figure S4.12a,b), number of flowers and leaves (Figure S4.12c), or biomass (plant without fruits) (Figure S4.12d). This contrasts somewhat to results of Bueno et al. (2021), who reported that exposure to relatively high levels (20 μg/leaf) of AZOX and $nSiO_2$-AZOX negatively impacted the growth of tomatoes under controlled hydroponic conditions. Under the present realistic field conditions using recommended exposure levels, none of the pesticide treatments had a inhibitory impact on the growth of the strawberries.

Overall, although the nanocarriers showed some small effects on the mobility of the AZOX in the soils, effects on the TFs and BFs of the plants were negligible and no effects of the different formulations could be seen on growth.

4.4.5 Nano-formulations had limited impact on the soil enzymes

Nanopesticides have previously been shown to affect soil enzymes. For example, $Cu(OH)_2$ nanopesticides have been shown to affect soil bacterial abundance, diversity, and community structure as compared to a conventional commercial formulations (Zhang et al., 2019). In this work, none of the pesticide formulations appeared to systematically affect soil enzyme activity. Only a few differences with respect to the control treatments (dotted horizontal lines, Figure 4.3) were observed, generally in the first day after pesticide application.

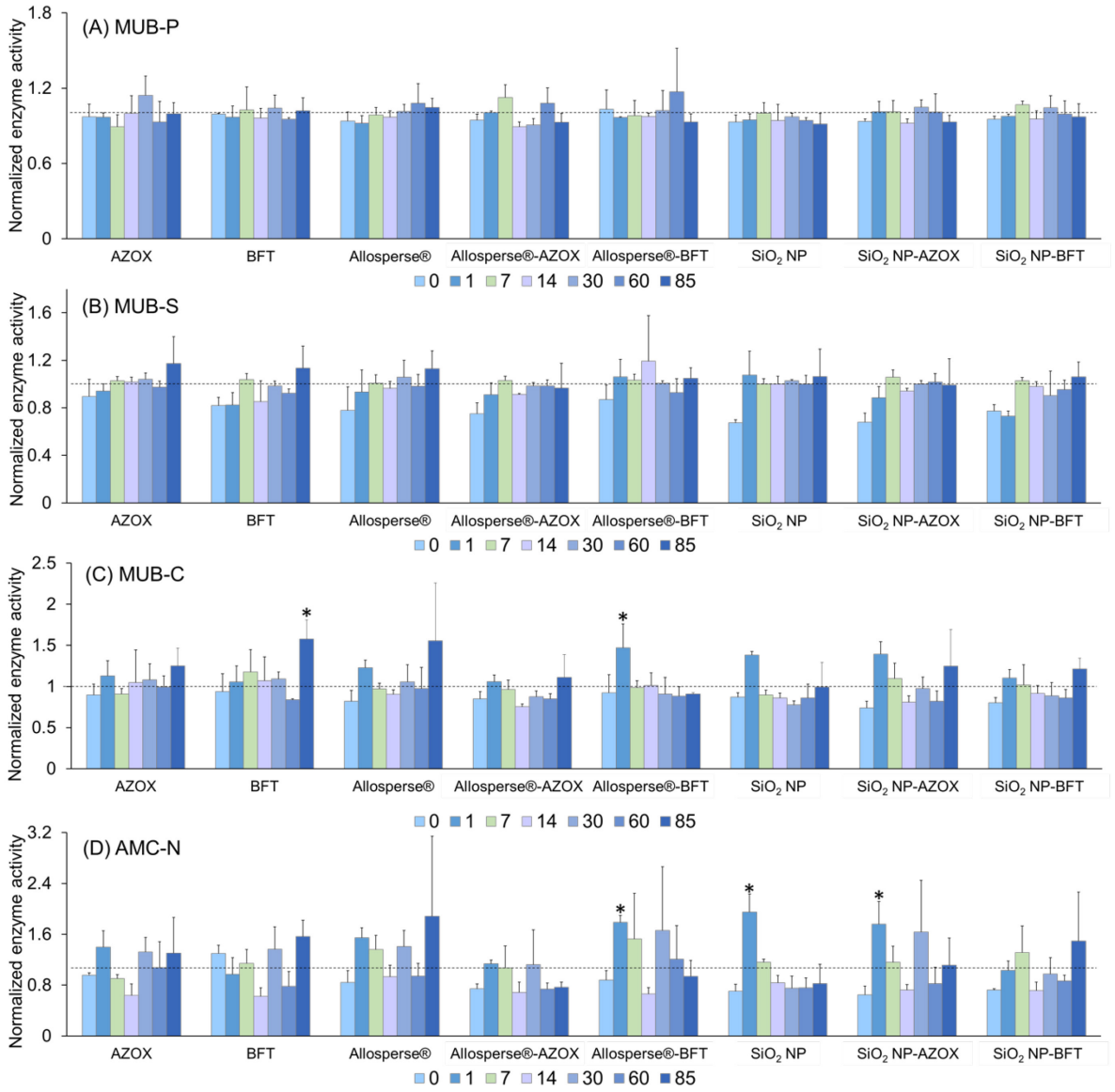

Figure 4. 3 Soil enzyme activities for β-D-glucosidase (MUB-C), phosphomonoesterase (MUB-P), arylsulfatase (MUB-S) and leucine-aminopeptidase (AMC-N) in soils treated with the different pesticide formulations in the second experimental year. Sampling occurred at different times (days) following the first application (t = 0), which occurred 15 days after the transplantation of the strawberries. Enzyme activities can be compared to values obtained for the nanoparticle-free and pesticide-free samples (i.e., dashed line). Error bars indicate one standard deviation of the

mean obtained from 3 biological replicates and 2 technical replicates (n = 6). Activities that were significantly different from controls (p<0.05) are indicated by an asterisk *.

Glucosidase is an important hydrolyze for the decomposition of organic matter in soils by producing smaller molecules that are used by soil microorganisms as an energy supply (Li et al., 2017), whereas leucine aminopeptidase is a hydrolyze involved in the acquisition of nitrogen by microorganisms by cleaving N-terminal residues from proteins and peptides (Matsui et al., 2006). AMC-N activities were significantly higher from controls one day after the application of $nSiO_2$, $nSiO_2$-AZOX, and Allosperse®-BFT, while MUB-C was significantly higher from controls one day after the application of Allosperse®-BFT (p<0.05). The present results are in line with a previous investigation that reported no effect or a stimulatory effect of pesticides on glucosidase activity, possibly due to the supplementary source of energy to the soil bacteria (Li et al., 2017). If the polymeric nanoparticles and the AI are considered as extra sources of carbon and organic matter to the soils, such amendments could improve microbial synthesis of extracellular enzymes and liberate further nutrients, which in turn would positively affect the soil microbiota and enhance the activities of the soil enzymes (Nottingham et al., 2012). At these dose levels, AMC-N and MUB-C were sensitive short-term indicators of the impacts of the nanopesticides (especially for Allosperse®-BFT and SiO_2-AZOX), however, enzyme activities appeared to return to control levels after 24 h.

Arylsulfatase, an essential hydrolase that controls the availability of sulfur in agricultural soils (Chen et al., 2019), and acid phosphatase, which plays an important role for the cycling of phosphorous (a limiting nutrient for crops), have also been proposed as sensitive environmental indicators for the effects of pesticides and nanomaterials in soils (Riah et al., 2014; Kwak et al.,

2017). For example, under controlled laboratory conditions, AZOX had an inhibitory effect on MUB-P and indicated risks to living organisms (Baćmaga et al., 2015). However, for the low level field exposures used here, neither enzyme was significantly affected by the treatments.

The overall lack of systemic, extensive effects of the AIs (conventional or encapsulated) to the soil enzymes suggests that the nanopesticides do not have a significant higher risk to the soil microbiota as compared to the conventional AIs. Such results are consistent with previous work showing no apparent difference in dehydrogenase activity for conventional BFT and BFT encapsulated into a polymeric NP (Kah et al., 2016). The activity of the enzymes appeared to be more responsive to the exposure time and environmental conditions than the different treatments. It is nonetheless important to note that this study focused on the four main soil hydrolases, whereas impacts to other soil enzymes may differ. Further research is needed to ensure that novel nano-based pesticides safeguard soil microbiota (Galhardi et al., 2020). Experiments could involve testing the effects of the nano-based pesticides in different types of soil or for variable fertilization rates and crop management practices. Finally, more differences would be expected for higher application rates, i.e. higher than the rates recommended by the manufacturers.

4.4.6 Nano-formulations had limited impact on the soil microbial community

Similar to the results for the enzyme activities, no systematic, significant effects were observed for the microbial community composition following the treatments (Figs. 4-5). The Shannon Index was between 8 and 9 for all samples (Figure S4.13), which indicates that the microbial community was very rich (which is usually the case for agricultural soil communities). It would thus appear that all formulations, including the polymer and nano-silica based nanopesticides, had limited effects on the soil biodiversity, which is similar to results that were

obtained when measuring the effects of copper-based nanopesticides for a different agricultural soil (Carley et al., 2020). Nonetheless, some small subtle changes occurred when comparing data obtained following the first and second experimental years (Figures 4.4 and S4.14). The most noticeable change in the first experimental year was the increase of *Acidobacteria* and the decrease of *Crenarachaeota* (the only large *Archea* group) and *Actinobacteria* at day 85 (with respect to day 0), especially for the control, AZOX, Allosperse®-BFT and $nSiO_2$ (Figure 14). Similar results were observed in year 2 (Figure 4) with a large but transient increase in relative abundance of *Acidobacteria* (25% for Allosperse®-AZOX and 10% for Allosperse®-BFT) and a large but transient decrease in *Actinobacteria* (25% for Allosperse®-AZOX and 10% for Allosperse®-BFT). In both cases, perturbations to the soil microbial community appeared to be attenuated with time, returning to near control levels when measured 85 days after pesticide addition. Changes in the microbial community composition appeared to be more related to length of exposure time rather than the actual pesticide treatments.

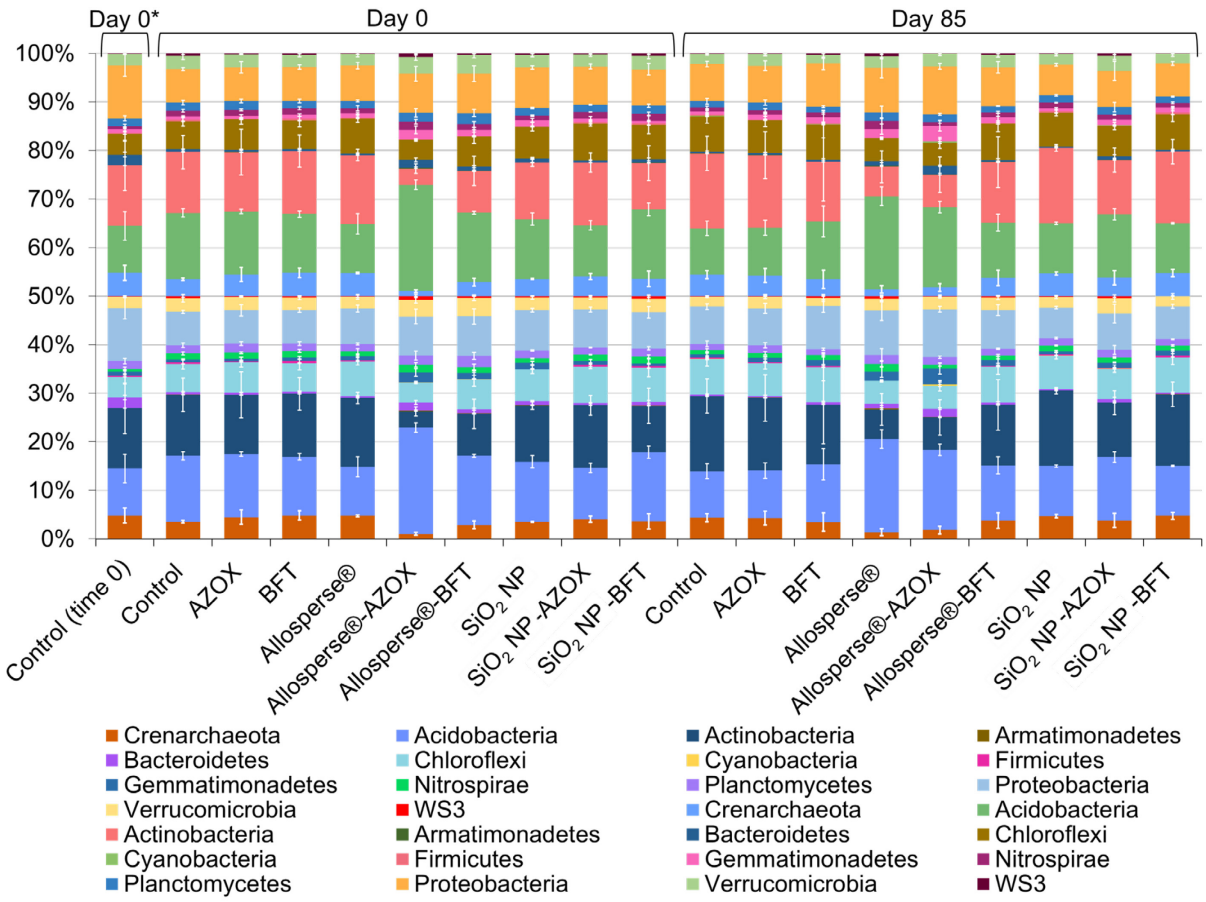

Figure 4. 4 Relative abundance plot of the soil microbial community composition from the second experimental year. *Day zero of the first experimental year refers to the soils before the pesticide application. Days 0 (before pesticide application) and 85 (after pesticide application) of the second experimental year.

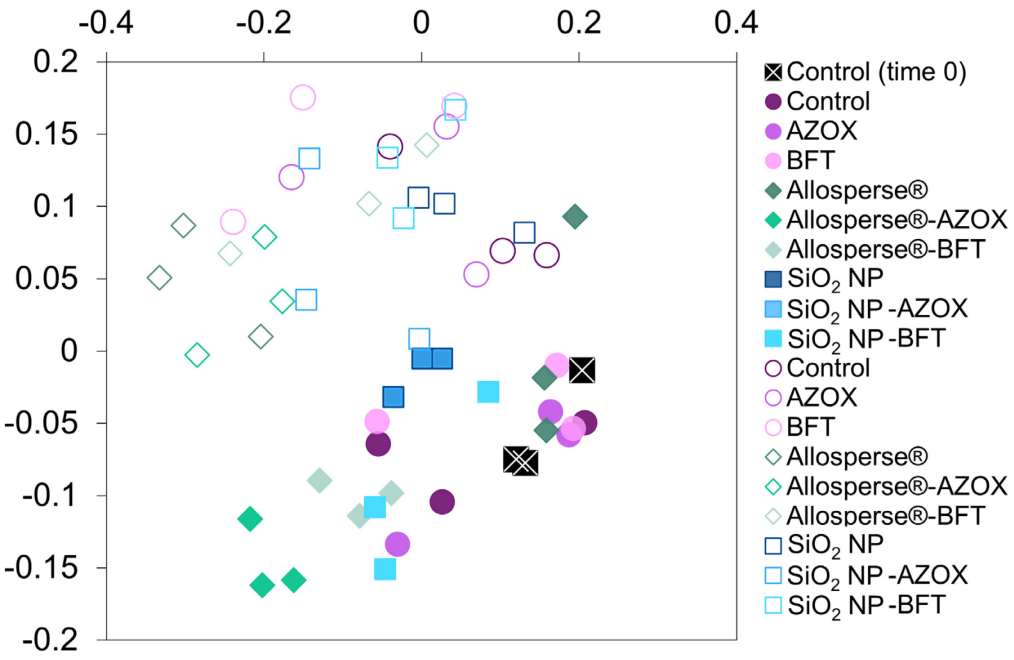

Figure 4. 5 PCoA plot of the soil microbial community composition from the days 0 (filled symbols) and 85 (hollow symbols) of the second experimental year. Control (time zero) refers to results from the first experimental year before the pesticide application.

Recall that second year, day 0, samples refer to the initial microbial community composition, one year after the initial treatments. With a few exceptions, only subtle changes were observed among treatments or between days for the phylum-level soil microbial community composition (Figure 4). For example, following the treatment with Allosperse®-AZOX, *Chrenarcheaota* decreased by 8% on day 0 and by 6% on day 85 with respect to the control. Similarly, following the treatment with Allosperse®-BFT, *Chrenarcheaota* decreased by 4% on day 0 and by 3% on day 85 of the second experimental year. Indeed, the soil's ability to resist and recover to its healthy state in response to destabilizing influences, in this case the addition of nanopesticides, is well established (i.e. soil resilience; Seybold et al., 1999). Similarly, for

terrestrial mesocosms, Carley et al. (2020) observed no significant long-term effects on soil biodiversity from the repeated exposure to $Cu(OH)_2$ nanopesticides. Although some initial shifts in soil microbial community composition were more evident in the treatments with Allosperse®, they did not seem to have a longer term influence in the plant growth or soil health. Indeed, observed changes could have been related to a secondary change in the soil ecosystem, such as pH, since previous studies have shown that soil pH controlled the abundance and diversity of these phyla (Lehtovirta et al., 2009; Sait et al., 2006).

A principal coordinate analysis (PCoA) obtained from the β-diversity analysis of the bacterial and archaea communities (Figure 4.5) showed no clear trend with respect to whether a given treatment had unique impact on microbial diversity. There were small differences among the nanocarrier systems (e.g., Allosperse®-based formulations clustered to the left of the figure whereas $nSiO_2$ and the conventional formulations clustered to the right). Nonetheless, the most significant differences were due to time with most of the data points below 0 in the *y-axis* representing the day 0 (control) and day 1 treatments with most of the points above 0 on the y-axis corresponding to day 85 data. These results indicate that changes in the microbial community composition appeared to be more related to exposure time than to the pesticide treatments. Similar conclusions were reached for the PCoA analysis of year 1 data (Figure S4.15).

Figure S4.16 shows the Shannon diversity indices during the first exposure year. The Shannon diversity index remained constant throughout the study for all of the treatments (Figure S4.13, S4.16), which is in line with other studies using nanopesticides. Zhang et al. (2020), for instance, reported Shannon indices between 9-10, and which did not vary significantly for different treatments, including Cu-based nanopesticides in agricultural soils.

4.5 Conclusions

Overall, it is clear that even when employing the maximum concentrations of pesticides suggested by the manufacturer and fairly sensitive indicators of ecosystem stress, none of the treatments seemed to have significant impacts on the strawberries or on the microbial communities in the soil. Concentrations of the AI in the strawberries were below the maximum permissible dose for human ingestion (Government of Canada, 2016) for all sampling times. Differences between the treatments with conventional pesticides and the nanopesticides were generally not noteworthy. The largest observed changes were related to time, with some indicators of a small initial stress, immediately after application, followed by a return towards control values after a short period (~days). The activities of MUB-C and AMC-N, as well as the microbial community composition appeared to be the most sensitive indicators of ecosystem health for these pesticides. Some small differences on pesticide retention were noted. For example, the Allosperse® formulation of AZOX appeared to be less retained by the soil than the classical formulation, even in the presence of an equivalent concentration of dispersants. Although minimal or no effects of the nanopesticides were observed with respect to pesticide accumulation, strawberry plant growth or soil microorganism composition or function, our findings demonstrate nonetheless that encapsulation into the nanocarriers might lead to some subtle differences in the behavior in environmental systems. Further research will be needed to assess release kinetics of the AIs from the nanocarriers under field conditions and the role of additional formulation components on the function and bioavailability of these emerging products.

4.6 Acknowledgments

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4.8 Supplementary materials

A) Methods to characterize the soil properties

(i) pH: A 1:1 (w/v) solution of soil to water was shaken for 30 min, then left to rest for 1 hour. The measurements were done on an Accumet AR15 research pH meter (Thermal Fisher).

(ii) pH buffering: the SMP single-buffer procedure (Shoemaker et al. 1961) is applied to estimate the lime requirement.

(iii) % Organic matter by Loss on Ignition (LOI): A (previously heated to 105℃ for 24 hours) sample is burned at 360 ℃ for 4 hours. The difference in weight between the two steps is attributed to loss of organic matter (expressed as a percent).

(iv) Available P, K, Ca, Mg, Na, Al, Cu, Zn, Mn and Fe: A multi-element extraction was performed using the Mehlich III solution (a mixture of acetic acid, ammonium nitrate, ammonium fluoride, nitric acid and EDTA). A colorimetric technique was used for the determination of P (Lachat flow injection analysis). P was measured at 880 nm following complexation with ammonium molydate in a reducing solution of ascorbic acid. The determination of metals was performed by Atomic Absorption Spectrophotometry using standards prepared in adequate matrixes and dilutions. Quantification was performed on a Varian 220FS.

(v) Extractable Ammonium and Nitrates in soils: An extraction is performed using a 1:10 soil-to-2M KCl solution, which was shaken for 1 hour. The filtrate is analyzed by colorimetry for the determination of N as NH_4 and N as NO_3 on a multi-channel Lachat autoanalyser. Ammonium is determined following heating of the solution with salicylate and hypochlorite in an alkaline phosphate buffer. The green color is measured colorimetrically at 660 nm using flow injection analysis. Nitrates were measured following reduction to nitrites in a copperized cadmium column. The magenta color is measured colorimetrically at 520 nm on a Lachat flow injection instrument.

(vi) Particle Size Distribution (hydrometer method): The hydrometer is used to measure the density of the material in suspension. Readings were performed at specific intervals according to settling times of grain sizes (considering temperature).

B) Pesticide analysis in soils and plants

AZOX and BFT were analyzed in the ***strawberry plant tissues and soil*** based on a LC-QTOF-MS method developed by Wang et al. (2022), which was an approach adapted from the QuEChERS technique (Lehotay, 2007), and validated for both the conventional and nanoformulations of the pesticides. In short, 2 g of homogenized fruit (n = 3) was weighed in 15-mL plastic centrifuge tubes in which 4 mL of 1% acetic acid in acetonitrile, 0.8 g of magnesium sulphate (Fisher Chemicals) and 0.2 g of sodium acetate (Fisher Chemicals) were added. Internal standards (40 µg kg^{-1} of AZOX-d_4 and 60 µg kg^{-1} of BFT-d_5) were spiked into each sample. Solutions were vortexed for 15 minutes then centrifuged at 2240 x *g* (5 min, 20°C). One mL of the extract was transferred to centrifuge tubes containing 50 mg of a Primary Secondary Amine (PSA, Agilent) and 150 mg of $MgSO_4$. Solutions were then vortexed for 1 min, centrifuged (2240 x g, 20°C) for 5 min and filtered through a 0.22 µm PTFE filter (Polytetrafluoroethylene, Chrom4; Thuringen, Germany) into HPLC vials (Agilent) for analysis.

Soils were dried at room temperature until constant weight, sieved through a 2-mm nylon mesh, then ground to a fine powder. Prior to the extraction, soils (n = 3) were spiked with internal standards (40 µg kg^{-1} of AZOX-d_4 and 60 µg kg^{-1} of BFT-d_5). Samples were then vortexed for 1 min and left at least one hour prior to extraction. The extraction method was adapted from Kah et al. (2016) and consisted of shaking (rotary shaker, 20 rpm) 1 g of dried and sieved (2 mm) soil in 2 mL of ACN for 1 hour at room temperature. Samples were then centrifuged (1882 × *g*; 5 min,

20°C) and the supernatant was filtered through 0.22 μm filters into glass HPLC vials. Leachate solutions from the pots were sampled in the field using a glass syringe, stored in glass flasks, and filtered through 0.22 μm filters into glass HPLC vials.

C) LC-QTOF-MS instrumental analysis

Leachate, soil and plant extracts were analyzed on an Agilent 1290 Infinity II liquid chromatograph (LC) coupled to a 6545 QTOF mass spectrometer (Agilent Technologies, Santa Clara, USA) operating in positive electrospray ionization mode. The LC separation was conducted on a Poroshell 120 phenyl hexyl column (Agilent Technologies; 2.7 μm × 3.0 mm × 100 mm) fitted with a Poroshell 120 EC-C18 (2.7 μm × 3.0 mm × 5 mm) guard column. Elution was performed in gradient mode (0.4 mL min^{-1}) using A = water and B = Acetonitrile: Methanol (1:1), both containing 0.1% formic acid and 5 mM NH_4Ac (0 min: 70% A; 0-3 min: B increased from 30 to 100%; 3-6 min: 100% B; 6-8 min: B decreased from 100% to 30%). The injection volume was 10 μL and the column temperature was maintained at 30°C. Nitrogen was used as the drying gas (110°C, 12 L min^{-1}). Samples were run in the *All Ions MS/MS* mode. The fragmentor voltage was 110 V and MS data was acquired in the 50-750 *m/z* range. The following *m/z* were extracted from total ion chromatogram (TIC) (±10 ppm) for quantification: 404.1247 for AZOX and 440.1604 for BFT. The qualifier ions for AZOX and BFT are 372.0971 *m/z* and 181.1009 *m/z*, respectively.

D) Soil enzyme activity analysis

Glucosidases are widely responsible for the supply of energy in soil microorganisms through the decomposition of organic matter. **Phosphatases**, originating from soil microorganisms,

hydrolyzes phosphorus into its bioavailable forms, which is important to maintain crop yields (Li et al., 2015). The mineralization of sulfur, an essential element for plant growth, from organic sulfates is mediated by the hydrolase **arylsulfatase** (Chen et al., 2019). **Leucine aminopeptidase** are metallopeptidases that cleave N-terminal residues from proteins and peptides (Matsui et al., 2006). Therefore, these four enzymes provide a sensitive indicator of soil microbial changes which could be induced by nanomaterials or pesticides in agricultural soils (Galhardi et al., 2020).

The activity of soil phosphomonoesterase, arylsulfatase, β-D-glucosidase, and leucine-aminopeptidase were determined according to Peyrot et al. (2014) using the fluorescent substrates 4-methylumbelliferone-phosphate (MUB-P), 4-methylumbelliferone-sulfate (MUB-S), 4-methylumbelliferone-glucopyranoside (MUB-C), and L-leucine-7-amino-4-methyl coumarin (AMC-N), respectively (Glycosynth, England). The fluorophores 4-methylumbelliferone (MUB) and 7-amino-4-methyl coumarin (AMC) were purchased from Sigma-Aldrich. Stock solutions of MUB (5 mM) and AMC (15 mM) were prepared in dimethylsulfoxide. Working solutions of the MUB and AMC (10.0, 8.0, 6.0, 4.0, 2.0, 1.0, 0.5, 0.1 μM) and the substrates (50 μM MUB-P, 100 μM MUB-C, 500 μM MUB-S, 50 μM AMC-N) were prepared in the buffer solution, using a similar pH as the soils (phosphate buffer, pH 7.2).

Enzymes were extracted from the soils by adding 0.5 g of soil (n = 3) to 25 mL of the buffer solution and then rotating the solutions for 30 min on a tube rotator (Fisher Scientific Tube Rotator) at 20 rpm. Mixtures were subsequently centrifuged for 5 min at 1882 × g and the supernatants were filtered over 0.22 μm filters into glass HPLC vials. For each sample, there were 6 analytical replicates, and after the addition of 150 μL of enzyme substrates and 50 μL of the soil extract solution to multiwell plates, samples were incubated under constant stirring (24 h, 30 °C). Fluorescence intensities were measured using excitation wavelengths of 330 nm (MUB) or 360

nm (AMC), with a fluorescent emission of 460 nm (Infinite M200, Tecan). The results were calculated by subtracting the average signal of both the blanks (soils) and the background wells from each sample. Enzyme activities were expressed as nmol MUB or AMC g^{-1} h^{-1} and normalization was performed against the control samples (no treatment added) to obtain a relative percentage of enzyme activity.

E) Figures

Figure S4. 1 Field set-up and the development of the strawberry plants over the duration of the experiment.

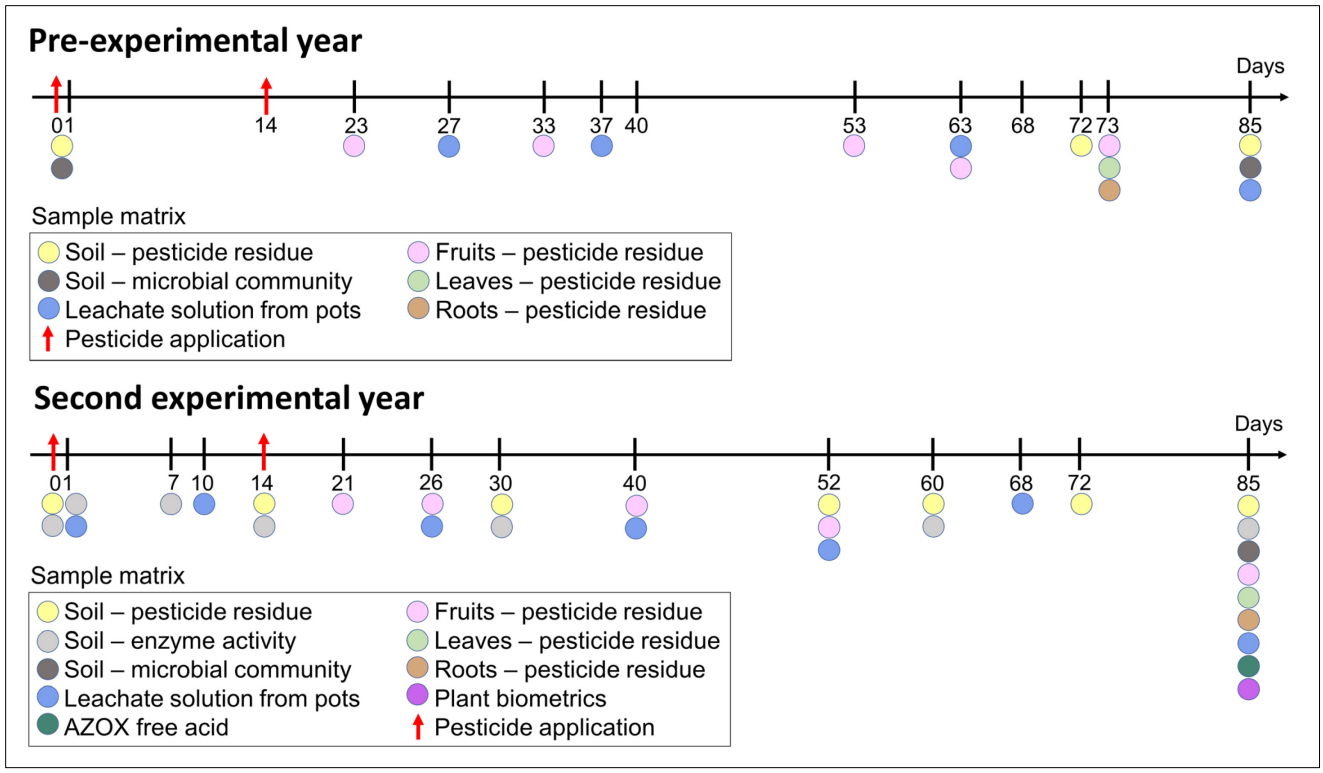


Figure S4. 2 Overview of the sample collection timeline.

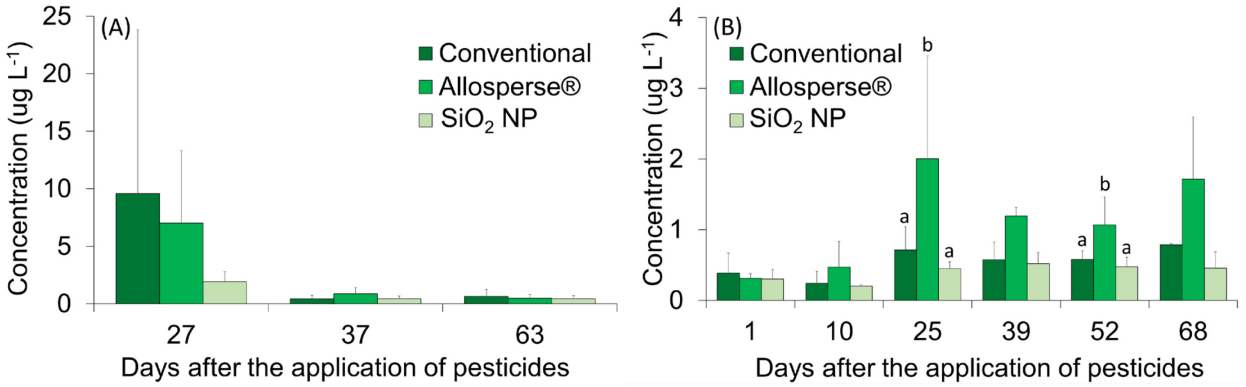


Figure S4. 3 Azoxystrobin levels in the leachate solution sampled from each pot (n = 3) on different days counting from the application of the pesticides in the field. Data from the first experimental year are presented in (A), whereas datain (B).

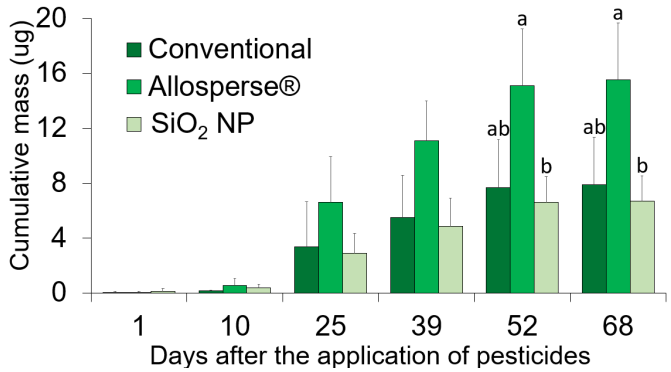


Figure S4. 4 Cumulative azoxystrobin mass in the leachate solution sampled from each pot (n = 3) in different days counting from the pesticide application on field in the second experimental year.

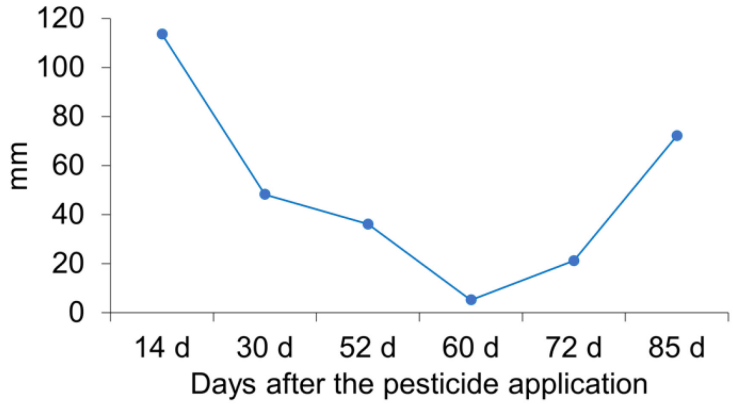


Figure S4. 5 Cumulative precipitation records between the sampling days for the second growing season.

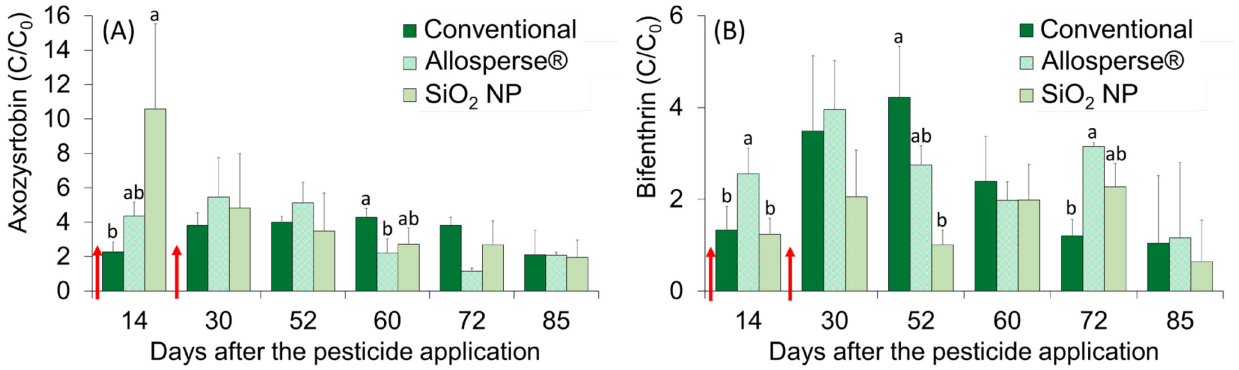


Figure S4. 6 Normalized concentrations (concentration at a given time, C, divided by the concentration at day zero, C_0) of AZOX (A) and BFT (B) (conventional and nano formulations) in the soils in the second experimental year as a function of time following the application of the formulations. Red arrows indicate when the addition of the treatments to the soils occurred. Significant differences (ANOVA) between different formulations are represented by different letters, according to Tukey's test. Data are means ± standard deviations (SD), n = 3.

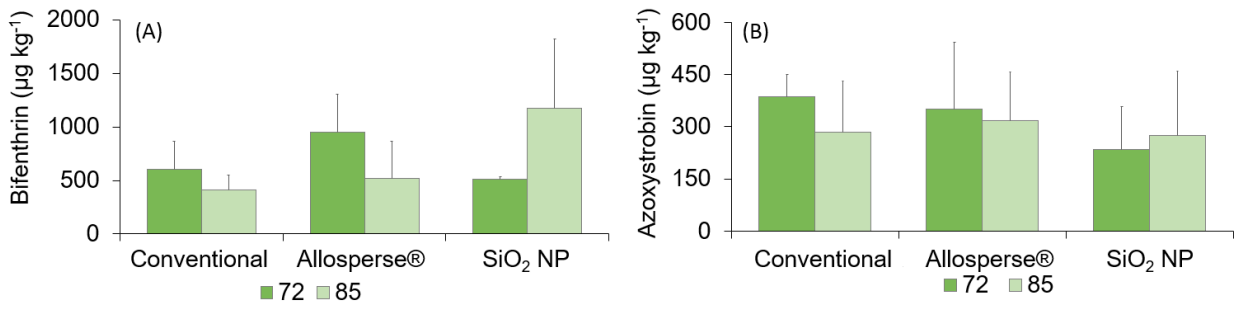


Figure S4. 7 Concentrations of AZOX (A) and BFT (B) (conventional and nano) in the soils in the pre-experimental year as a function of time following the application of the pesticide formulations. Samples on day 14 were sampled just before the second application of the treatments to the soils. Statistically significant differences between the different formulations are represented by different letters, according to Tukey's test. Data are means ± standard deviations (SD), n = 3.

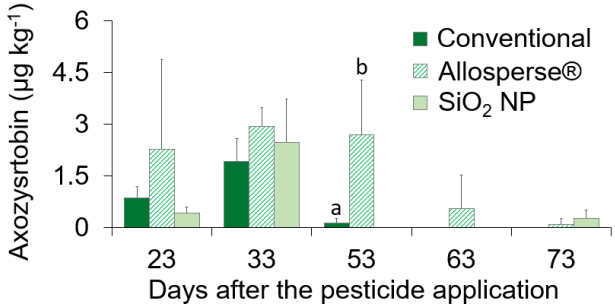


Figure S4. 8 Concentration of Azoxystrobin (conventional and nano formulations) in the fruit samples from the first experimental year at different exposure times (days) counting from the first dosage application. Statistically significant differences between different formulations at the same sampling dates are represented by different letters, according to Tukey's test. Data are means ± SD, n = 3.

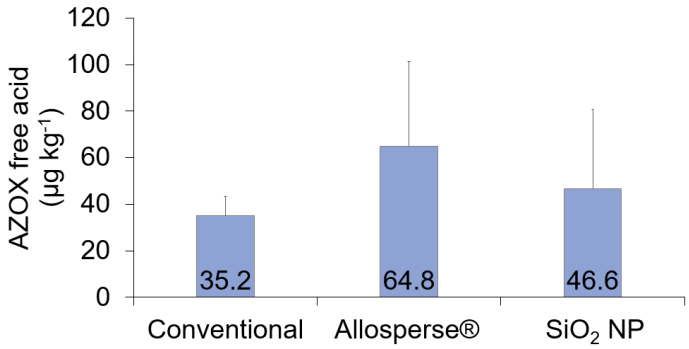


Figure S4. 9 Concentrations of Azoxystrobin free acid in the strawberry roots sampled on the last day of the second experimental year. (Data are means ± SD, n = 3)

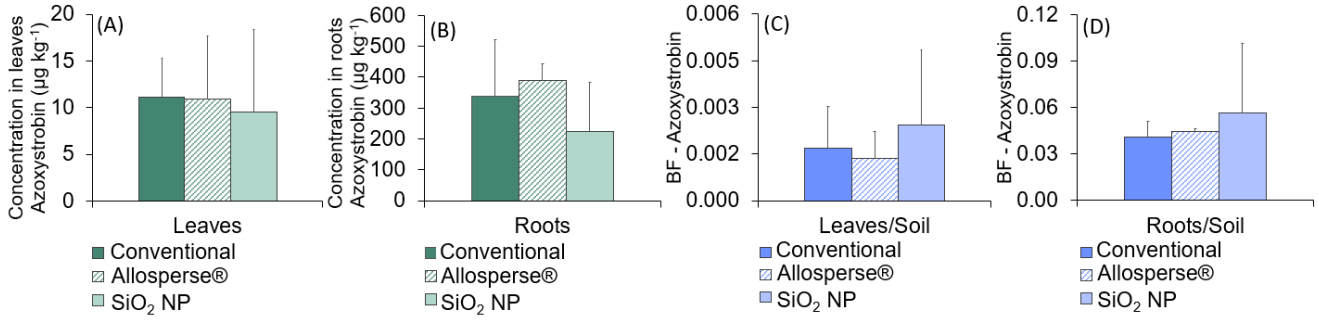


Figure S4. 10 Concentrations of Azoxystrobin (conventional and nano formulations) in the leaves (A) and root samples (B) and bioaccumulation factors (BF, concentration in the plant divided by the concentration in the soils) from soil to leaves (C) and soil to roots (D) on day 85, i.e., the last sampling day of the second experimental year. No significant differences were found between the different formulations at $p < 0.05$. Data are means ± SD, n = 3.

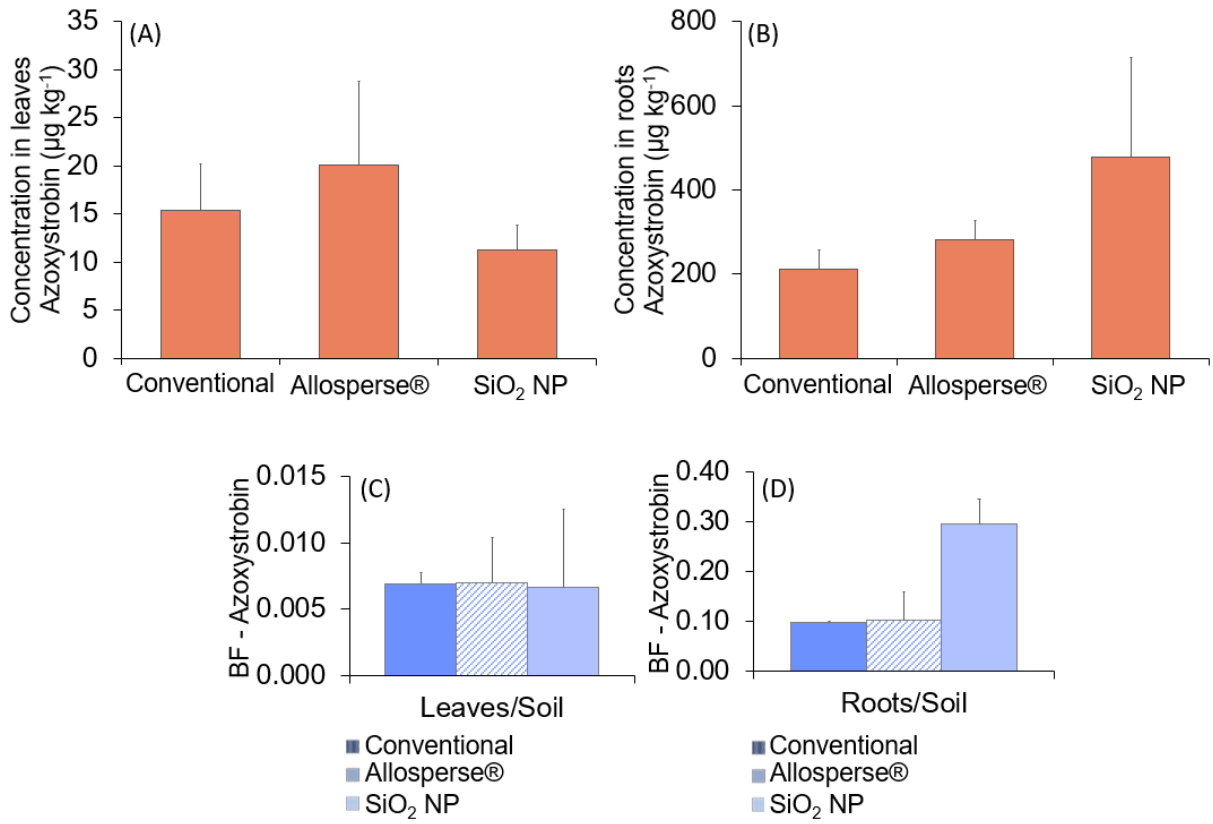


Figure S4. 11 Concentration of azoxystrobin (conventional and nano formulations) in the leaves (A) and roots (B) and bioaccumulation factors (ratios of the concentrations in the plant tissue vs. soil) for leaves (C) and roots (D) from the last sampling day of the pre-experimental year. No significant differences were found between the different formulations at $p < 0.05$. Data are mean ± SD, n = 3.

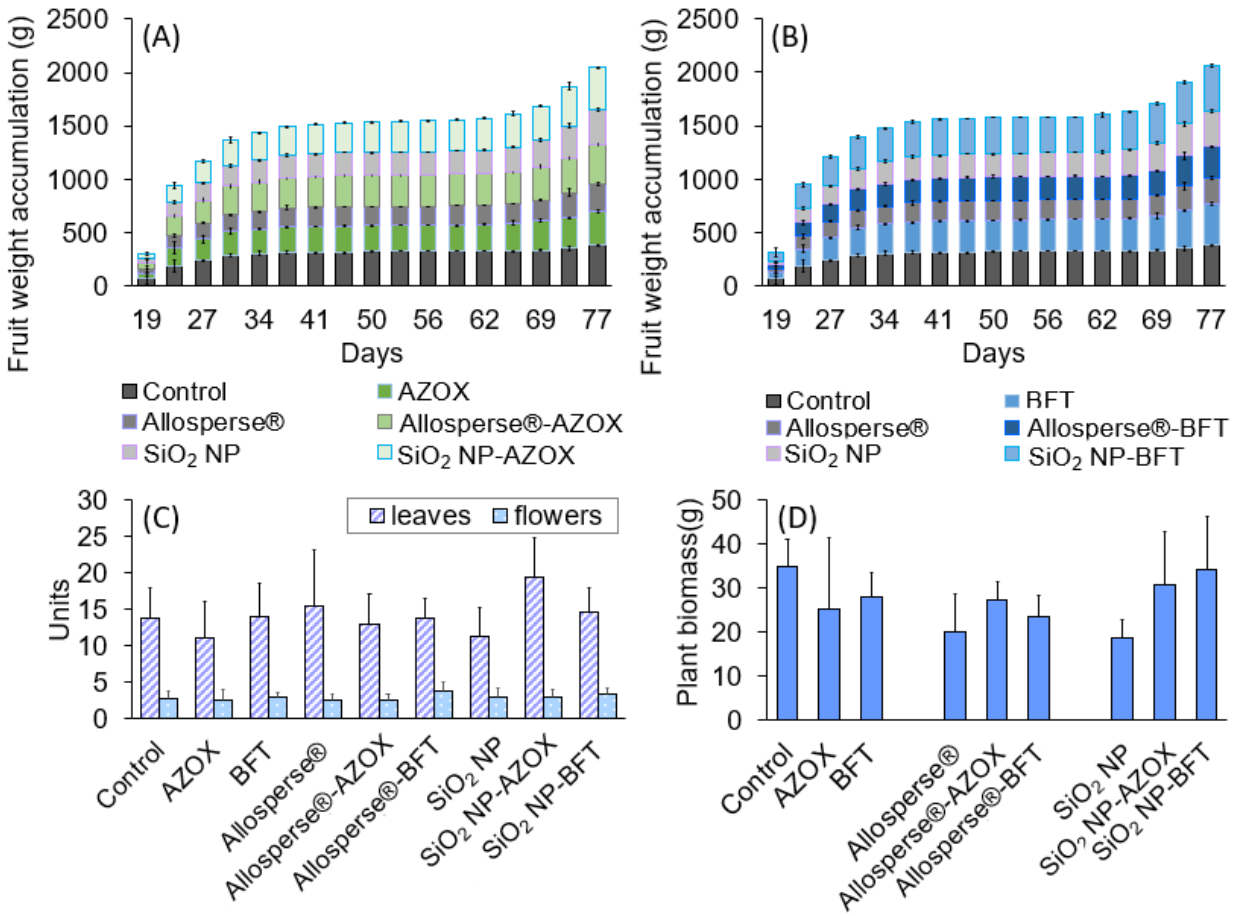


Figure S4. 12 A) and B) Accumulation of the strawberry fruit (g/per pot) mass over time (days) counting from the first dosage application (A=AZOX; B=BFT) of the second experimental year. C) Number of flowers (units/per plant) and leaves for the strawberry plants at the end of the experiment. D) Biomass (g dry weight) of the strawberry plants analyzed at the end of the experiment. Control samples refer to the nanoparticle-free and pesticide-free samples. AZOX = Azoxystrobin; BFT = Bifenthrin.

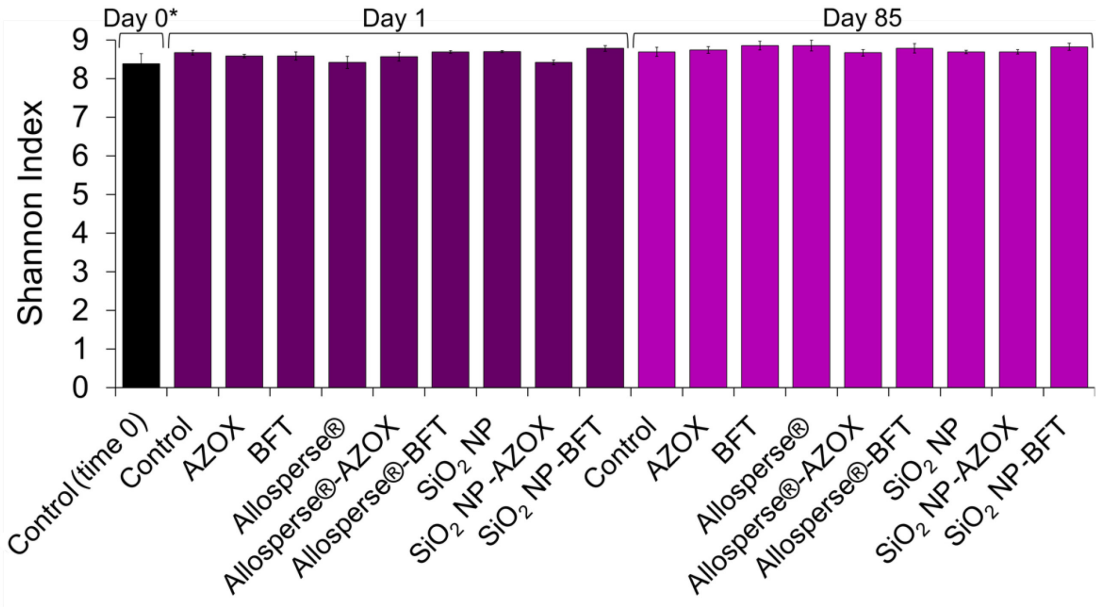


Figure S4. 13 Shannon index for all of soil treatments from the second experimental year. *Day zero of the first experimental year. Days 0 and 85 of the second experimental year.

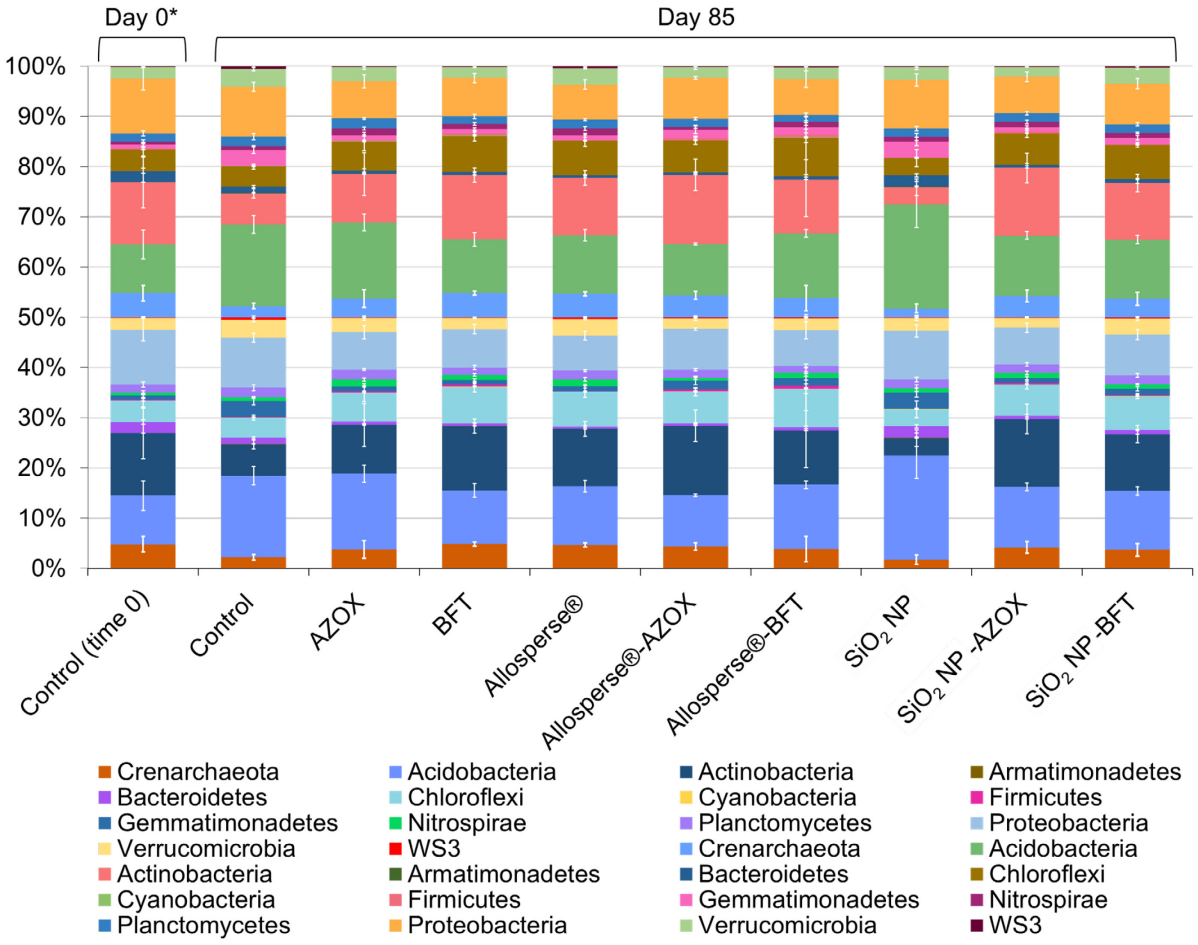


Figure S4. 14 Relative abundance plot of the soil microbial community composition analyzed in the first experimental year. *Day zero of the first experimental year refers to the soils before the pesticide application.

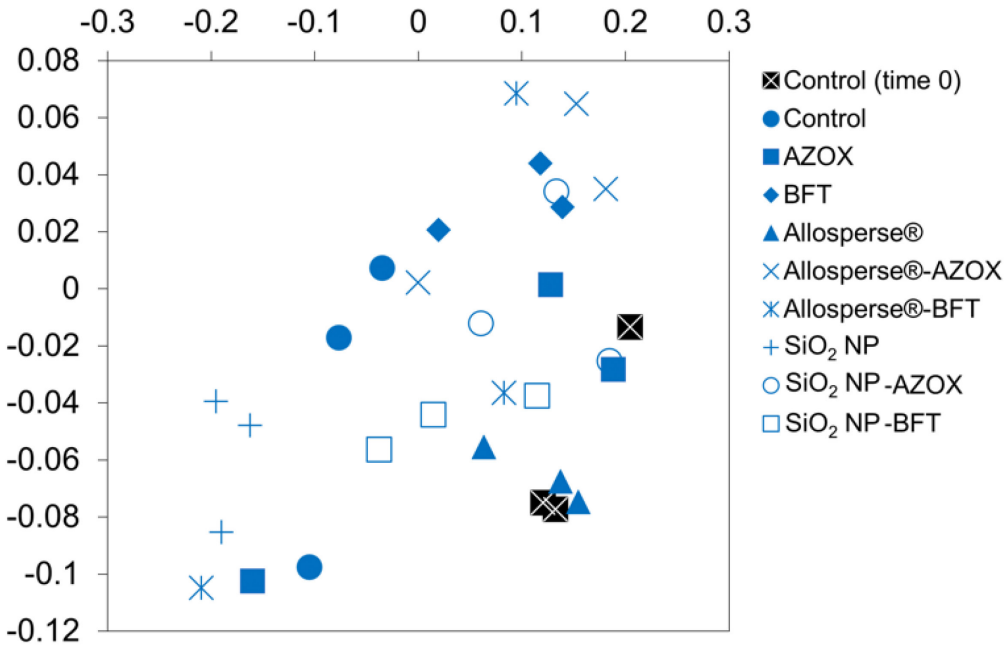


Figure S4. 15 PCoA plot of the soil microbial community composition analyzed in the first experimental year.

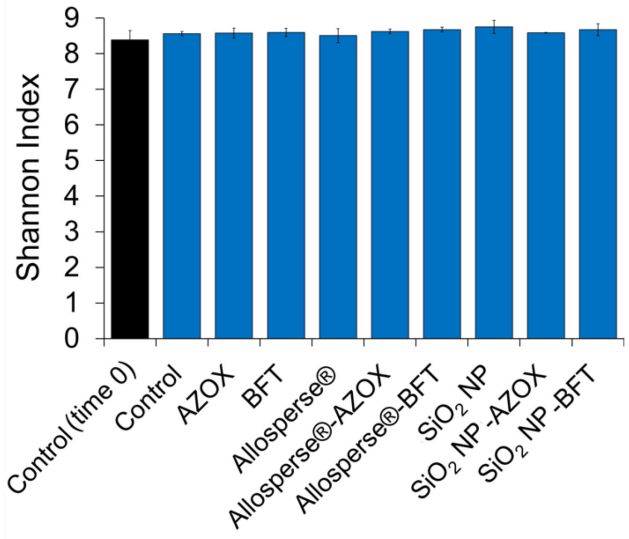


Figure S4. 16 Shannon index for all of the soil treatments analyzed in the first experimental year.

F) Tables

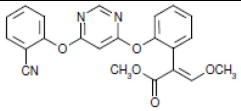
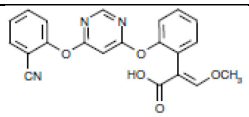
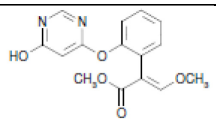
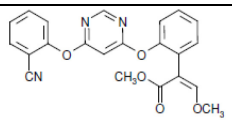
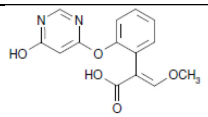
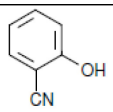
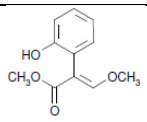
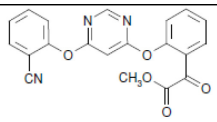
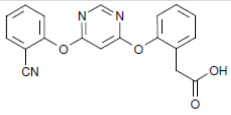
Table S4. 1 - Transfer factors for the different pesticide formulations calculated on day 85 of the second experimental year.

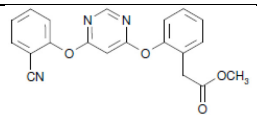
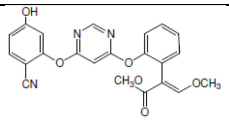
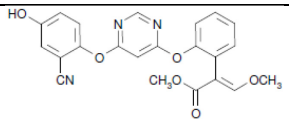
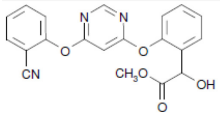
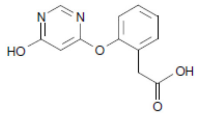
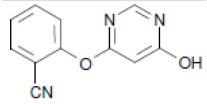
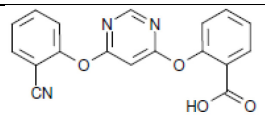
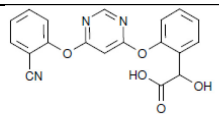
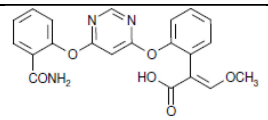
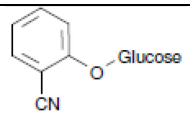
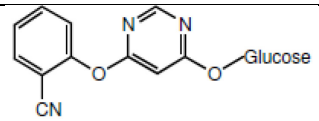
	Formulation	**First experimental year**	**Second experimental year**
$TF_{fruits/leaves}$ AZOX	Conventional	n.a.	$2.5 \times 10^{-2} \pm 2.2 \times 10^{-2}$
	Allosperse®	$1.1 \times 10^{-4} \pm 1.1 \times 10^{-4}$	$2.1 \times 10^{-2} \pm 1.3 \times 10^{-2}$
	$nSiO_2$	$3.6 \times 10^{-4} \pm 3.9 \times 10^{-4}$	$2.8 \times 10^{-3} \pm 3 \times 10^{-3}$
$TF_{fruits/roots}$ AZOX	Conventional	n.a.	$6.5 \times 10^{-4} \pm 5.9 \times 10^{-4}$
	Allosperse®	$4.6 \times 10^{-6} \pm 4.6 \times 10^{-6}$	$4.9 \times 10^{-4} \pm 1.9 \times 10^{-4}$
	$nSiO_2$	$1.2 \times 10^{-5} \pm 1.5 \times 10^{-5}$	$9.6 \times 10^{-5} \pm 1.1 \times 10^{-4}$
$TF_{leaves/roots}$ AZOX	Conventional	$7.5 \times 10^{-2} \pm 2.7 \times 10^{-2}$	$3.8 \times 10^{-2} \pm 2.1 \times 10^{-2}$
	Allosperse®	$7.7 \times 10^{-2} \pm 4.6 \times 10^{-2}$	$2.8 \times 10^{-2} \pm 1.6 \times 10^{-2}$
	$nSiO_2$	$2.6 \times 10^{-2} \pm 9.1 \times 10^{-3}$	$3.9 \times 10^{-2} \pm 8.5 \times 10^{-3}$

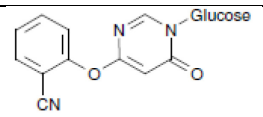
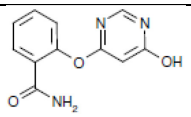
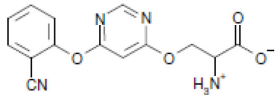
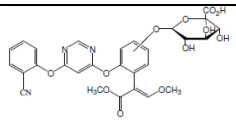
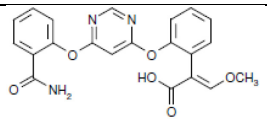
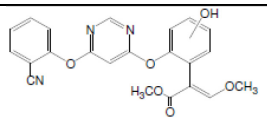
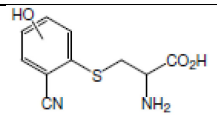
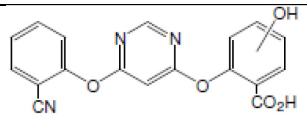
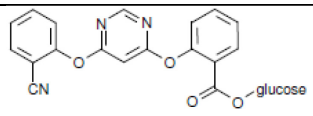
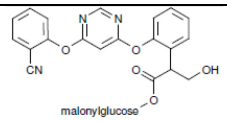
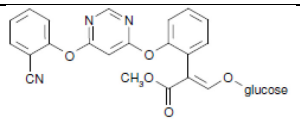
No significant differences were found between the different formulations at $p<0.05$. Data are mean ± SD, n = 3.

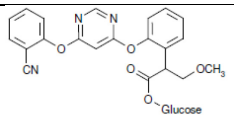
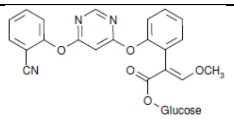
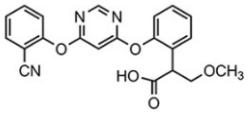
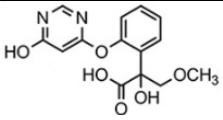
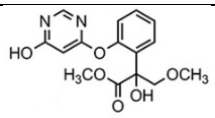
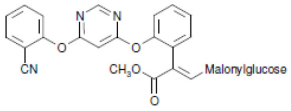
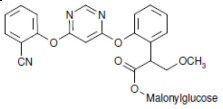
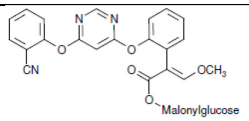
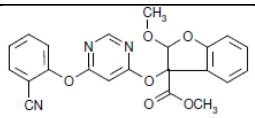
n.a. = not available since levels were below the MDLs in most samples.

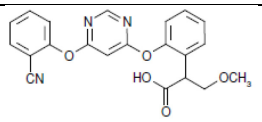
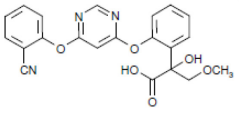
Table S4. 2 - Azoxystrobin metabolites and degradation products (including identification number or letter, manufacturer code number, formula, *m/z* and structure) in the environment reported in the literature.

Compound[a]	**Manufacturer code**[b]	**Formular**	**Molecular Weight**	**Structure**	**Reference**
Compound 01 (azoxystrobin)	ICIA5504	$C_{22}H_{17}N_3O_5$	403.1168		(FAO, 2009)
Compound 02 (azoxystrobin free acid)	R234886	$C_{21}H_{15}N_3O_5$	389.1012		(FAO, 2009)
Compound 03	R219227	$C_{15}H_{14}N_2O_5$	302.0903		(FAO, 2009)
Compound 09	R230310	$C_{22}H_{17}N_3O_5$	403.1168		(FAO, 2009)
Compound 10	R232493	$C_{14}H_{12}N_2O_6$	304.0695		(FAO, 2009)
Compound 13	R71395	C_7H_5NO	119.0371		(FAO, 2009)
Compound 18	R176586	$C_{11}H_{12}O_4$	208.0736		(FAO, 2009)
Compound 19	R230309	$C_{20}H_{13}N_3O_5$	375.0855		(FAO, 2009)
Compound 20	R400050	$C_{19}H_{13}N_3O_4$	347.0906		(FAO, 2009)

Compound 21	R400051	$C_{20}H_{15}N_3O_4$	361.1063		(FAO, 2009)
Compound 22	R400297	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)
Compound 23	R400299	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)
Compound 24	R400753	$C_{20}H_{15}N_3O_5$	377.1012		(FAO, 2009)
Compound 26	R401487	$C_{12}H_{10}N_2O_4$	246.0641		(FAO, 2009)
Compound 28	R401553	$C_{11}H_7N_3O_2$	213.0538		(FAO, 2009)
Compound 30	R402173	$C_{18}H_{11}N_3O_4$	333.0750		(FAO, 2009)
Compound 35/U3	R402987	$C_{19}H_{13}N_3O_5$	363.0855		(FAO, 2009)
Compound 36	R403314	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)
Compound 40	R405270	$C_{13}H_{15}NO_7$	297.0849		(FAO, 2009)
Compound 41	-	$C_{17}H_{17}N_3O_8$	391.1016		(FAO, 2009)

Compound 42	R405287	$C_{17}H_{15}N_3O_8$	389.0859		(FAO, 2009)
Compound C	-	$C_{11}H_9N_3O_3$	231.0644		(FAO, 2009)
Compound G2	-	$C_{14}H_{12}N_4O_4$	300.0859		(FAO, 2009)
Compound K1	-	$C_{29}H_{29}N_3O_{12}$	611.1751		(FAO, 2009)
Compound K2	-	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)
Compound L1	-	$C_{23}H_{21}N_3O_6$	435.1430		(FAO, 2009)
Compound L4	-	$C_{11}H_{14}N_2O_3S$	254.0725		(FAO, 2009)
Compound L9	-	$C_{19}H_{15}N_3O_5$	365.1012		(FAO, 2009)
Compound M1	-	$C_{24}H_{21}N_3O_9$	495.1278		(FAO, 2009)
Compound M2	-	$C_{29}H_{27}N_3O_{13}$	625.1544		(FAO, 2009)
Compound M3	-	$C_{27}H_{25}N_3O_{10}$	551.1540		(FAO, 2009)

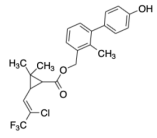
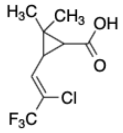
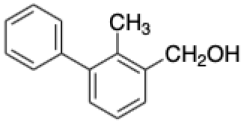
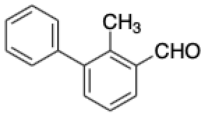
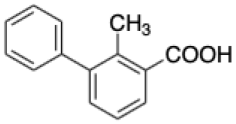
Compound N1	-	$C_{27}H_{27}N_3O_{10}$	553.1696		(FAO, 2009)
Compound N2	-	$C_{27}H_{25}N_3O_{10}$	551.1540		(FAO, 2009)
Compound New M3	-	$C_{21}H_{17}N_3O_5$	391.1168		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound New M4	-	$C_{14}H_{14}N_2O_6$	306.0852		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound New M6	-	$C_{15}H_{16}N_2O_6$	320.1008		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound O1	-	$C_{30}H_{27}N_3O_{13}$	637.1544		(FAO, 2009)
Compound O2	-	$C_{30}H_{29}N_3O_{13}$	639.1700		(FAO, 2009)
Compound O3	-	$C_{30}H_{29}N_3O_{13}$	639.1700		(FAO, 2009)
Compound U13	-	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)

Compound U5	-	$C_{21}H_{17}N_3O_5$	391.1168		(FAO, 2009)
Compound U6	-	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)

[a] The compound: number and letters were commonly used in the literature, except the "new M3, M4, and M6", which is found in the study of Gautam, Etzerodt & Fomsgaard (2017).

[b] Manufacturer codes of azoxystrobin metabolites were usually used as compounds ID in the literature.

Table S4. 3 - Bifenthrin metabolites and degradation products (including compounds name, formula, *m/z* and structure) in the environment reported in the literature.

Compound	Formular	Structure	Molecular Weight	Reference
4'OH-BFT	$C_{23}H_{22}ClF_3O_3$		438.12096	(Fecko, 1999; FAO, 2010)
TFP acid	$C_9H_{10}ClF_3O_2$		242.03214	(Fecko, 1999; FAO, 2010)
Biphenyl alcohol (BP alcohol)	$C_{14}H_{14}O$		198.10447	(Fecko, 1999; FAO, 2010)
BP aldehyde	$C_{14}H_{12}O$		196.08882	(Fecko, 1999; FAO, 2010)
Biphenyl acid (BP acid)	$C_{14}H_{12}O_2$		212.08374	(Fecko, 1999; FAO, 2010)

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Connection Text

Chapter 4 reported the environmental fate and potential effects of NEPs of AZOX and BFT on agroecosystems under realistic field conditions. Based on the results obtained, various pesticide treatments did not significantly impact the plant phenology parameters (strawberry yield, biomass of plant without fruits, number of flavors, leaves and runners). From the literature review, pesticides are known to modulate plant metabolites at the molecular level, inducing the phenolic compounds in the various plant tissues. Chapter 5 investigated the impacts of the nanoformulations on the total phenolic content (TPC), the levels of individual phenolic compounds and the phenolic profiles of the incurred strawberry fruits collected from the field. Chapter 5 will be submitted to *Agriculture and Food Science* as "Effect of nanopesticides (azoxystrobin and bifenthrin) on the phenolic content and profiles in strawberry fruits (*Fragaria* × *ananassa*)" (Peiying Wang, Juliana A. Galhardi, Lan Liu, Vinicius Bueno, Subhasis Ghoshal, Valérie Gravel, Kevin J. Wilkinson, Stéphane Bayen)

Chapter 5. Effect of nanopesticides (azoxystrobin and bifenthrin) on the phenolic content and metabolic profiles in strawberries (*Fragaria* × *ananassa*)

5.1 Abstract

Conventional pesticides can cause side effects on plant metabolism. To date, the effect of nanoencapsulated pesticides (NEPs) on the phenolic content of plants has not been reported. In this study, a comparative evaluation of the phenolic contents and metabolic profiles was performed in strawberry fruits from plants treated under controlled field conditions with different pesticide formulations (conventional, or pesticides nanoencapsulated in polymeric (Allosperse®) or porous hollow SiO_2) and different active ingredients (azoxystrobin and bifenthrin). There were small but significant differences observed in the phenolic contents, metabolite profiles and the individual phenolic profiles among the formulations for both azoxystrobin and bifenthrin. The impact of NEPs on the levels of individual phenolic compounds were not consistent when compared with the conventional pesticides. Even though the effects of NEPs on the strawberry plant phenological parameters are not obvious, NEPs may have a significant impact on the plant metabolism, observable at the molecular level.

Keywords: nanoencapsulated pesticides, phenolic compounds, strawberry, metabolites, LC-QTOF-MS

5.2 Introduction

Despite the development of pesticide resistance in some pests, the current context of a shortage of arable land coupled with human population growth stimulate the use of ever larger amounts of pesticides and the production of novel formulations to improve crop yield[1]. Recent developments in nanotechnology have led to a new era in agrochemicals designed to reduce some of the undesirable consequences of conventional pesticide use[2]. Nanopesticides, particles with at least one dimension in the 1-100 nm size range[3], have been developed with the promise of a higher efficacy of the active ingredients (AIs) of the pesticides[2].

Understanding the fate and impacts of pesticides in plants is key to the development of new formulations. Although, the modes of actions of pesticides are well described in their target pest species[4], relatively less information is generally available regarding their impact on plant metabolism[5], which in turn may affect plant growth or the quality of fruits[1]. The analysis of metabolite profiles can provide additional insights on how a plant responds to biotic or abiotic stresses[6]. Although concerns associated with possible side effects of nanopesticides on plant growth and metabolism have been raised, studies on the effects of nanopesticides on plant metabolism are limited.

Phenolic compounds, a wide set of secondary metabolites of plants, share a common structural backbone comprised of one or many aromatic benzene rings along with a minimum of one hydroxyl functional group, which are responsible for the antioxidant capacity[7]. Phenolics account for several milligrams per gram of the plant tissues including monoaromatic phenolic compounds (e.g., gallic acid and caffeic acid), and polyphenols such as stilbenes, flavonoids, and polymers derived from these various groups[7]. The synthesis of phenolic compounds is stimulated by external stimuli such as pathogen infections, temperature, UV and chemical stresses[8,9].

Phenolics serve as a self-defence mechanism against damage due to oxidation, microbial pathogens and herbivorous insects[9]. Some conventional pesticides including herbicides, fungicides and, to a lesser extent, insecticides have been shown to impact/modulate the synthesis of phenolic compounds in plants through several mechanisms, such us inducing the production of phenolics to ward off microbial attack[10]. Azoxystrobin (AZOX), as a systemic fungicide, has beneficial physiological effects on crop yield and promotes the synthesis of secondary metabolites in cucumber[11]. Sundravadana et al.[12] showed that treatment with AZOX resulted in an increased total phenolic content (TPC) in rice leaves from blast pathogen treated plants. Increased TPC was also observed for powdery mildew infected chili leaves after treatment with AZOX[13].

Dietary phenolic compounds, encountered in vegetables, fruits, cereals for example, are of interest for their role in color and taste, but also their diverse benefits to humans in terms of antioxidant activity, anticancer and anti-diabetes properties.[14] For example, plant-derived phenols have shown *in vitro* potential for decreasing the risk of developing type 2 diabetes, cardiovascular disease, neurological disorders, inflammatory diseases and cancer[15]. The antioxidant potential of phenolic compounds also protects biological macromolecules, namely proteins and nucleic acids, from oxidative stress or an imbalanced production of free radicals in the body[16]. Strawberries are a good source of high content of diverse dietary phenolic compounds[17]. Pesticides are regularly used in strawberry production to control disease and increase yield. The effect of bifenthrin (BFT) on plant phenolics has not been reported in the literature, but treatments of strawberry plants with high levels of fungicides such as AZOX could inhibit the stimulation of self-defence mechanisms, including the synthesis of phenolic compounds, by decreasing pest pressures[18]. To date, the impact of pesticide nanoencapsulation on plant phenolics and fruit quality has not been reported.

As a part of a broader study on the fate and effects of nanoencapsulated pesticides in a well-controlled strawberry field[19], the present study compared the impact of nanoencapsulation (with two different formulations, Allosperse® and $nSiO_2$ and two AI: AZOX and BFT) on TPC levels and phenolic profiles in strawberries. Experiments were performed over 2 growing seasons in two continuous years, for various exposure periods, under realistic field conditions. During the first experimental year, the experimental design was set up and adjusted (methods for collection and preparation of samples, preparation of the pots, displacement of the pots in the field, construction of the irrigation and fertilization systems, and others). TPCs were recorded for both years, and individual phenolic profiles were obtained for the samples collected in the second year.

5.3 Material and methods

5.3.1 *Chemicals and reagents*

Folin-Ciocalteu reagent, sodium carbonate and analytical standards of azoxystrobin (≥98%), bifenthrin (≥98%), p-coumaric acid (CAS#501-98-4), catechin (≥98%), ellagic acid (≥97%), quercetin (≥95%), pelargonidin-3-glucoside chloride(≥98%), procyanidin B1 (CAS#20315-25-7), procyanidin B2 (CAS#29106-49-8), gallic acid (≥98%), 4-hydroxybenzoic acid (≥99%), 3,4-dihydroxybenzaldehyde (≥97%), 2,5-dihydroxybenzoic acid (≥98%), caffeic acid (≥98%), kaempferol (≥97%), kaempferol 3-glucoside (CAS#480-10-4), cyanidin N-3-o-glucoside chloride (≥97%) and internal standards of 2-hydroxybenzoic acid-D6 ((≥98%) and catechin-2,3,4-^{13}C3 (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade solvents (water and methanol) were obtained from Fisher Chemicals (Pittsburgh, PA, USA) and LC/MS grade formic acid from Agilent Technologies (Santa Clara, CA, USA). Allosperse® is a polymeric nanoparticle, synthesized from polyacrylic acid that is used as a nanocarrier for the pesticides (AZOX, BFT). Allosperse®-AZOX and Allosperse®-BFT were prepared and supplied by Vive Crop Protection Inc. (Mississauga, Canada). Porous hollow silica nanoparticles ($nSiO_2$) were synthesized as reported in an earlier study[20]. For the experiments conducted in this study, the $nSiO_2$ was loaded with the analytical standards to produce $nSiO_2$–AZOX and $nSiO_2$–BFT. Stock solutions of the phenolic compounds were prepared in methanol. In a preliminary test, the stability of the phenolic standards including p-coumaric acid, catechin, ellagic acid, quercetin, procyanidin B1 and B2, 1000 μg/L was assessed at 25°C for four hours and <7% degradation was recorded. Therefore, the phenolics were considered sufficiently stable during extraction steps (around 1.5 hours).

5.3.2 *Controlled field exposure experiment*

A controlled field experiment was carried out at the Horticultural Center of the Macdonald Campus of McGill University, Sainte-Anne-de-Bellevue, QC, Canada[19]. Strawberry plants (*Fragaria* × *ananassa* Duch. cv. "Seascape"), were cultivated under field conditions and exposed to nine different treatments (Table 5.1). Treatments with AZOX all contained 7.6 mg active ingredient / pot whereas all treatments with BFT contained 8.0 mg active ingredient / pot. Each pot (81 cm × 22 cm × 17 cm) contained four plants. The pots were randomly positioned in the field, keeping a distance of 1.5 meter between pots. Five pots were prepared for each treatment, but only the three replicates from the middle rows of the field were used for characterization in this study in order to avoid '*edge effects*'[21]. The "pesticide-free" soil used to grow strawberries was obtained from the McGill Macdonald farm. Strawberry bare root plants (Pépinière Lareault, Canada) were transplanted in the first week of June and pesticide treatments were applied twice: 15 and 30 days after planting, following the instructions for commercial pesticides. The fruits from each pot were harvested twice per week and accumulated to make one single weekly sample. Counting from the first day of treatment application, the sampling dates for the first year were recorded as day 25, 30, 40, 55, 65, and 78. For the second year, sampling occurred on days 25, 30, 44, 55, and 81.

Table 5. 1 Pesticide treatments applied on strawberry plants

Group	Treatment ID	Composition		
		Active ingredient	Nanocarrier	Dispersant
Conventional	control	-	-	yes
	conventional - AZOX	AZOX	-	yes
	conventional - BFT	BFT	-	yes
Allosperse®	Allosperse®	-	Allosperse®	yes
	Allosperse® - AZOX	AZOX	Allosperse®	yes
	Allosperse® - BFT	BFT	Allosperse®	yes
$nSiO_2$	$nSiO_2$	-	$nSiO_2$	yes
	$nSiO_2$ - AZOX	AZOX	$nSiO_2$	yes
	$nSiO_2$ - BFT	BFT	$nSiO_2$	yes

[a]Dispersant: surfactants, antifreeze, biocide and others.

5.3.3 *Extraction of the phenolic compounds*

For each batch of strawberries, fresh fruits were homogenized in a stainless-steel blender. All processed homogenates were stored at -80°C until analysis. The extraction method was adapted from the method described by Singleton et al. with modifications[22]. In short, approximately 1 gram of homogenized strawberry sample was combined with 10 mL of methanol. During the extraction, samples were stirred at 200 rpm, at room temperature, for 1 hour. Then, the extract was centrifuged at 1000×g for 20 minutes. The resulting supernatant was stored in 20-mL polypropylene tubes at -20°C, prior to the analysis of phenolics.

5.3.4 *Determination of total phenolic content (TPC)*

TPC was determined in strawberry extracts using the Folin-Ciocalteu method with gallic acid as a reference[22]. In short, 0.1 mL of the strawberry extract was mixed with 0.5 mL of Folin-Ciocalteu diluted reagent (1:10 v/v in water). The resulting solution was then allowed to stand for 5 min at 25℃, and 1.7 mL of sodium carbonate solution (20% w/v; 2 grams of sodium carbonate + 10 mL of Milli-Q water) was added along with 10 mL of MilliQ water. The final mixture was allowed to sit for 20 minutes in the dark. One mL of the mixture was used to measure absorbance at a wavelength of 735 nm on a Genesys 30 Vis spectrophotometer (ThermoFisher). The results were expressed as mg gallic acid equivalents (GAE) per g of fresh sample. Standards (0-400 mg/L gallic acid in methanol, n=3) were prepared and tested to build the calibration curve. QC samples (300 mg/L gallic acid in methanol; n=6) were put through the same extraction processing as the strawberry samples, in order to assess recoveries and intraday precision (%RSD).

Determination of individual phenolic compounds

Individual phenolics were analyzed following a method based on Kajdzanoska et al.[23] Strawberry extracts filtered through a 0.22 μm polytetrafluoroethylene filter (PTFE, Chrom4; Thuringen, Germany) were analyzed using Agilent 1290 Infinity II liquid chromatography (LC) coupled to a 6545 QTOF mass spectrometer (Agilent Technologies, Santa Clara, USA), operating in both positive (ESI+) and negative electrospray ionization (ESI-) modes. The LC separation was conducted on a Poroshell 120 phenyl hexyl column (Agilent Technologies; 2.7 μm × 3.0 mm × 100 mm) fitted with a Poroshell 120 EC-C18 (2.7 μm × 3.0 mm × 5 mm) guard column. Elution was performed in gradient mode (0.4 mL min^{-1}) using A:water and B:methanol, both containing 0.1% formic acid (0 min: 70% A; 0-3 min: B increased from 30 to 100%; 3-6 min: 100% B; 6-8

min: B decreased from 100% to 30%). The injection volume was 6 μL, and the column temperature was maintained at 30°C. Nitrogen was used as the drying gas (110°C, 12 L min^{-1}). The fragmentor voltage was 150 V and MS data was acquired in the 50-750 *m/z* range in the full scan mode. Individual anthocyanins in the strawberries were identified and quantified in ESI+. Two anthocyanin glycosides belonging to cyanidin and pelargonidin anthocyanins were analyzed in the fruits. Other phenolic compounds were determined in ESI-. Identification was performed through comparison with the pure analytical standards (retention time, *m/z*).

Method validation included assessment of the linearity of the calibration response (r^2), method detection limits (MDLs; 3σ of the response obtained for procedural blanks or the concentration of the lowest calibration point in a signal-to-noise ratio (S/N) of 3) and intraday precision (RSD%, n=6 replicates from one sample pool).

5.3.5 *Determination of phenolic and metabolic profiles*

Principal component analysis (PCA) is recognized as an effective method for data grouping[24]. PCA was applied to investigate the influence of the nanoencapsulated pesticides on metabolites and phenolic profiles in ESI negative mode, which includes all phenolics, except anthocyanins. In each analysis, samples were harvested from the same sampling day. First, LC/MS data were aligned using the Agilent Masshunter Profinder, setting the tolerance for retention times (RT) and mass differences to 0.15 min and 10 ppm, respectively. Because the methanol extracts contained not only phenolic compounds but also other metabolites such as amino acids, nucleosides, fatty acids, amines, carbohydrates, vitamins and hormones[25], the obtained dataset was used to investigate the overall metabolite profiles in the strawberries. A statistical comparison of the metabolite profiles among the samples was completed using MassHunter Profiler Professional.

Samples were grouped according to pesticide active ingredients ("No pesticides", "AZOX" or "BFT") and pesticide formulations ("conventional", "Allosperse®" and "$nSiO_2$").

5.3.6 *Statistical analysis*

Analysis of variance (ANOVA, SPSS) was used to identify differences between treatments for the different pesticide formulations, by applying a confidence range of 95% (α=0.05, n=3). The results reported for strawberries were based on triplicate extractions (3 replicates from 3 different pots). Significant differences ($p \leq 0.05$) between means were evaluated by using Tukey's multiple-comparisons test. The sampling days (Year1 is from Day1 to Day6; Year 2 is from Day1 to Day5), pesticide active ingredients (*No pesticides*, *AZOX* and *BFT*), pesticide formulations (*conventional*, *Allosperse®*, and *$nSiO_2$*) were fixed factors. A general linear model was used to determine the effects of all experimental factors.

5.4 Results and Discussion

5.4.1 *Total phenolic content (TPC)*

The total phenolic content of strawberry samples (expressed as mg GAE/g fresh weight tissue) was determined by the Folin-Ciocalteu method (Figure 5.1). Instrument response was linear over the 0-400 mg/L gallic acid range (r^2>0.99, n=3). Based on spiked samples (n=6), the intraday precision (RSD%) was 2%, and the recoveries averaged 116%. The total phenolic content in the strawberries in this controlled experiment ranged from 1.554 to 2.984 mg gallic acid equivalent (GAE)/g across the treatments over the two years.

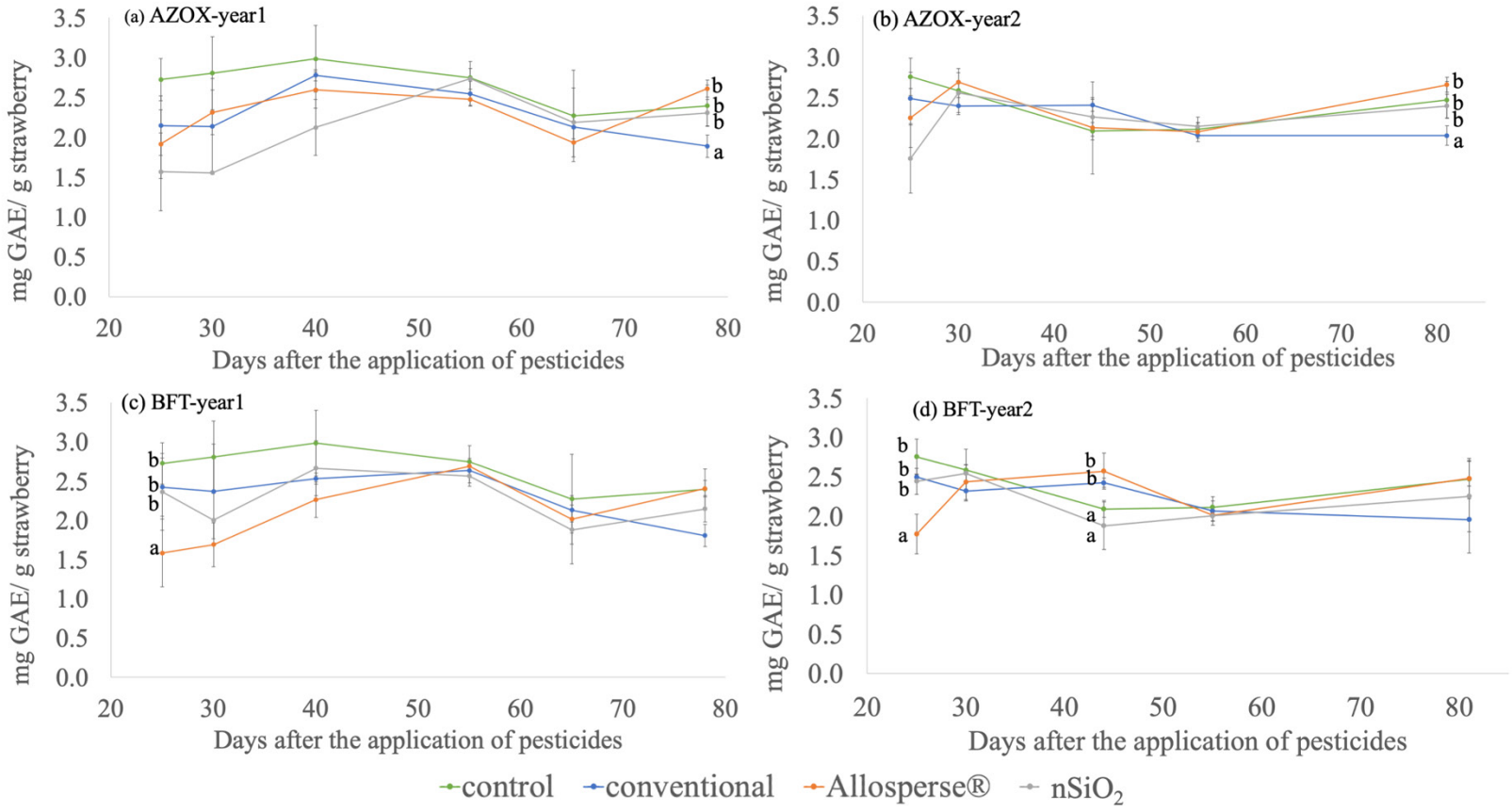

Figure 5. 1 Total phenolic content (mg GAE/g fresh weight tissue) of the strawberries harvested from plants treated with different pesticides: (a) AZOX-1st year (control, conventional, Allosperse® and $nSiO_2$); (b) AZOX-2nd year (control, conventional, Allosperse® and $nSiO_2$); (c) BFT-1st year (control, conventional, Allosperse® and $nSiO_2$); (d) BFT-2nd year (control, conventional, Allosperse® and

$nSiO_2$). GAE-gallic acid equivalent. Values represent mg of gallic acid equivalent per gram of fruit tissue mass. Data are shown as means and standard deviations (n=3). Statistically significant differences between different formulations at the same sampling date are represented by different letters ($p<0.05$)

In Figure 5.1, during the entire growing season, TPCs for AZOX treatments were only significantly different ($p<0.05$) for the last sampling dates for both years. The Allosperse®-AZOX and $nSiO_2$-AZOX treatments gave significantly higher TPCs (1.34 and 1.20-fold, respectively), compared to the treatment with conventional-AZOX. However, statistically significant differences ($p<0.05$) among the BFT treatments were found at the beginning of the growing season for both years. TPCs in the strawberries grown with the conventional-BFT and $nSiO_2$-BFT treatments were 40% and 28% greater, respectively, than the Allosperse®-BFT. The differences between the AZOX and BFT treatments indicated that the nanocarriers could give AZOX a longer-term effect as compared to the BFT. Therefore, the same nanocarrier could generate different effects on TPC for different AIs.

Initially, TPC levels in the control strawberries were higher as compared to treatments of AZOX and BFT. In line with the literature where TPC values in organic or treated with low level fungicide treatments (including AZOX) were higher than in conventionally cultivated strawberries[18,26,27], these results show that the synthesis of phenolic compounds could be stimulated in untreated strawberry plants, or in plants treated with low dose of pesticides, as a self-defence mechanism to decrease stresses to the plants.

In the three-way ANOVA analysis (Table S5.2, Supplemental Information), the fixed factor and interactions related to the pesticide formulations - "formulation", "day × formulation" and "day × formulation × AI" indicated significant effects on the TPC in both the first year and second year. For the simple effect – "formulation", the TPC values in the $nSiO_2$ formulation were lower (2.22±0.05 mg GAE/g strawberry) than for the conventional formulation (2.43±0.05 mg GAE/g strawberry) in the 1st year. TPC levels for the Allosperse® formulation (2.24±0.05 mg GAE/g strawberry) were lower but not statistically, compared to the conventional formulation.

Plants synthesize and accumulate phenolic compounds as plant growth regulators or in response to pathogen and/or herbivore attacks[9]. The lower TPC may be caused by the relatively higher efficiency of the nanopesticides in controlling fungus and insects. Thus, the nanocarriers ($nSiO_2$ and Allosperse®) showed some inhibitory effects on the TPC in strawberries.

The strawberry plants were grown under field conditions where environmental factors such as temperature, solar radiation and climate can have considerably large effects on the phenolic profiles in plants[8]. The fixed factor - "day" showed a significant effect on TPC in both years, which clearly reflects the impact of the changing environmental factors overtime in this study. The two-way interaction "day × formulation" had a significant effect on TPC in both years, so a combination of formulations and environmental factors could modulate TPC. Moreover, the three-way interaction "day × formulation × AI" showed significant differences on TPC in year 2. Thus, the impact of pesticide formulations on TPC depends on the effects of AIs and days. The significant effects of nanopesticides on TPC varied from pesticide to pesticide and from time to time.

The Folin-Ciocalteu method can detect variations in the sums of oxidizable compounds including not only phenolic compounds, but also ascorbic acid and lipophilic antioxidants[28]. Ascorbic acid (around 0.621 mg/g) in fresh organic strawberries was 9.7% higher than in conventional strawberries[26]. The effects of pesticide formulations on ascorbic acid may have had a non-negligible influence on TPC values. In order to obtain more information about the effect of nanoencapsulated pesticides on phenolic compounds, individual phenolic compounds were investigated in the strawberries collected in year 2.

5.4.2 Individual phenolic compounds

The RT, linear range, r^2, MDLs and intraday precision (RSD%) are presented in Table 5.2 for individual phenolics. Mass measurement errors in LC-QTOF-MS were below 5 ppm, which is comparable with the literature[29]. Based on the literature and preliminary tests, levels of individual phenolic compounds in the strawberries were expected to vary over a wide range, from 1 µg/kg to 30 mg/kg. Calibration ranges were adjusted accordingly and the linearity of the method was validated for individual phenolic compounds (r^2 obtained using linear regression analysis were >0.985). The MDLs for most compounds were low (<5 µg/kg), except for pelargonidin-3-glucoside, ellagic acid, procyanidins and 2,5-dihydroxybenzoic acid. As the concentration of ellagic acid and procyanidins in strawberries are expected to be in mg/kg level (Table S5.1), this method was considered to be sufficiently sensitive for all compounds. The intraday precision of this method was assessed from the relative standard deviation (RSD%, n=6) on the analysis of a strawberry batch and was satisfactory, with values consistently below 10%.

Table 5. 2 Identification of phenolic compounds by LC-ESI-QToF/MS

Compounds	Group	Molecular formula	Molecular weight	Ion adduct	*m/z*[a]	Mass measurement error (ppm) in solvent	RT[b] (min)	Range (µg/L)	r^2[c]	MDL[d] (µg/kg)	Intraday precision RSD[e]% (n=6)	Reference for *m/z*
Pelargonidin-3-glucoside	Anthocyanin	$C_{21}H_{21}O_{10}$	468.0824	[M]+	433.1135	-0.73	2.275	100-30,000	0.999	11.21	6%	30
Cyanidin N-3-o-glucoside	Anthocyanin	$C_{21}H_{21}O_{11}$	484.0773	[M]+	449.10845	-0.89	3.015	5-500	0.996	2.77	7%	31
Ellagic acid	Ellagic acid	$C_{14}H_6O_8$	302.0063	[M-H]-	300.999	3.23	3.046	100-15000	0.999	61.81	10%	32
Catechin	Flavan-3-ols	$C_{15}H_{14}O_6$	290.0791	[M-H]-	289.0713	0.14	1.961	10-25000	0.999	0.55	2%	31
Procyanidin B2	Flavan-3-ols	$C_{30}H_{26}O_{12}$	578.1425	[M-H]-	577.1346	-1.00	1.878	5-1000	0.994	15.18	4%	31
Procyanidin B1	Flavan-3-ols	$C_{30}H_{26}O_{12}$	578.1425	[M-H]-	577.1346	-1.44	1.461	1000-25000	0.985	242.25	3%	31
Quercetin	Flavonol	$C_{15}H_{10}O_7$	302.0427	[M-H]-	301.0349	-0.66	3.385	10-250	0.995	1.25	4%	33
Kaempferol	Flavonol	$C_{15}H_{10}O_6$	286.0478	[M-H]-	285.0405	-0.94	3.675	5-250	0.992	4.39	7%	34
Kaempferol 3-glucoside	Flavonol	$C_{21}H_{20}O_{11}$	448.1006	[M-H]-	447.0928	-0.67	3.034	1-100	0.997	3.02	3%	32
p-coumaric acid	Phenolic acid	$C_9H_8O_3$	164.0474	[M-H]-	163.0395	0.68	2.772	1-1000	0.995	0.97	9%	33
Gallic acid	phenolic acid	$C_7H_6O_5$	170.0216	[M-H]-	169.0137	1.51	1.571	5-1000	0.997	3.98	3%	33
4-hydroxybenzoic acid	Phenolic acid	$C_7H_6O_3$	138.0317	[M-H]-	137.0239	1.15	2.442	1-250	0.992	3.10	9%	33
2,5-Dihydroxybenzoic acid	Phenolic acid	$C_7H_6O_4$	154.0266	[M-H]-	153.0188	-0.64	2.454	5-1000	0.995	20.37	7%	35
Caffeic acid	Phenolic acid	$C_9H_8O_4$	180.0423	[M-H]-	179.0345	1.29	2.452	5-250	0.997	2.44	8%	34
3,4-dihydroxybenzaldehyde	Phenolic aldehyde	$C_7H_6O_3$	138.0317	[M-H]-	137.0239	1.05	2.266	1-750	0.991	0.56	2%	36

[a]Mass charge ratio; [b]Retention time; [c]Linearity of the calibration response; [d]Method detection limit; [e]Relative standard deviation

Anthocyanins

Pelargonidin-3-glucoside was a dominant anthocyanin in the strawberries (Table S5.1 and S3), consistent with the literature[37]. Plargonidin-3-glucoside levels showed significant differences between the conventional and nanoformulations (Table 5.3, $p<0.05$). Plargonidin-3-glucoside levels decreased from conventional>$nSiO_2$>Allosperse®, though this effect was mild as it represented, on average, a 5% increase compared to Allosperse® and $nSiO_2$ treatments. Genetic, developmental, and environmental factors all regulate anthocyanin synthesis and degradation. Anthocyanins play a vital role in plant survival as they repel herbivores[38]. Insect feeding and fungal infection could lead to an increased accumulation of anthocyanins in plants[39,40]. In a previous study, anthocyanin-enriched tomatoes exhibited lower susceptibility to gray mold than low anthocyanin containing fruits[41]. A decrease of plargonidin-3-glucoside levels in nanopesticide treated strawberry plants may be related to the high efficiency of nanoencapsulated pesticides to control and eliminate the fungal and insect stresses. Weak pathogen stresses will lead to a reduced self-defence mechanism of the plants against fungi and insects when compared with the conventional pesticides.

Table 5. 3 Phenolic compound levels (mg/kg fresh strawberry) and simple effects in three-way ANOVA (three factors – day, formulations and active ingredients) test (*p* values, n=3).

	simple effect										
	day			active ingredient (AI)				formulation			
	day25	day81	*p* value	none (control)	azoxystrobin	bifenthrin	*p* value	conventional	Allosperse®	$nSiO_2$	*p* value
pelargonidin-3-glucoside	268.92 ± 3.48	232.53 ± 3.48	<0.001	256.91 ± 4.26	248.88 ± 4.26	246.38 ± 4.26	n.s.	257.93 ± 4.26^{b}	241.64 ± 4.26^{a}	252.61 ± 4.26^{ab}	0.032
cyanidin N-3-o-glucoside	0.54 ± 0.02	0.65 ± 0.02	<0.001	0.62 ± 0.02	0.6 ± 0.02	0.57 ± 0.02	n.s.	0.61 ± 0.02	0.6 ± 0.02	0.59 ± 0.02	n.s.
Total anthocyanins	269.46 ± 3.48	233.19 ± 3.48	<0.001	257.54 ± 4.27	249.48 ± 4.27	246.95 ± 4.27	n.s.	258.54 ± 4.27^{b}	242.23 ± 4.27^{a}	253.2 ± 4.27^{ab}	0.032
ellagic acid	44.03 ± 2.09	27.35 ± 2.09	<0.001	34.97 ± 2.56	37.73 ± 2.56	34.37 ± 2.56	n.s.	35.04 ± 2.56	35.62 ± 2.56	36.41 ± 2.56	n.s.
catechin	102.32 ± 2.69	85.36 ± 2.69	<0.001	97.74 ± 3.3	89.9 ± 3.3	93.88 ± 3.3	n.s.	96.79 ± 3.3	95.89 ± 3.3	88.84 ± 3.3	n.s.
procyanidin B2	3.16 ± 0.09	2.47 ± 0.09	<0.001	2.88 ± 0.11	2.72 ± 0.11	2.85 ± 0.11	n.s.	2.92 ± 0.11	2.87 ± 0.11	2.65 ± 0.11	n.s.
procyanidin B1	45.22 ± 1.04	36.74 ± 1.04	<0.001	41.97 ± 1.27	40.03 ± 1.27	40.94 ± 1.27	n.s.	41.82 ± 1.27	41.92 ± 1.27	39.19 ± 1.27	n.s.
Total flavanols	150.7 ± 3.74	124.56 ± 3.74	<0.001	142.59 ± 4.58	132.64 ± 4.58	137.67 ± 4.58	n.s.	141.53 ± 4.58	140.68 ± 4.58	130.69 ± 4.58	n.s.
quercetin	0.52 ± 0.07	1.4 ± 0.07	<0.001	0.96 ± 0.08	0.95 ± 0.08	0.97 ± 0.08	n.s.	0.77 ± 0.08^{a}	1.04 ± 0.08^{ab}	1.07 ± 0.08^{b}	0.027
kaempferol	0.65 ± 0.09	1.47 ± 0.09	<0.001	1.07 ± 0.11	1.07 ± 0.11	1.06 ± 0.11	n.s.	0.83 ± 0.11^{a}	1.27 ± 0.11^{b}	1.09 ± 0.11^{ab}	0.033
kaempferol 3-glucoside	0.35 ± 0.01	0.5 ± 0.01	<0.001	0.45 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	n.s.	0.44 ± 0.01	0.43 ± 0.01	0.41 ± 0.01	n.s.
Total flavonols	1.53 ± 0.15	3.37 ± 0.15	<0.001	2.48 ± 0.18	2.44 ± 0.18	2.43 ± 0.18	n.s.	2.04 ± 0.18^{a}	2.74 ± 0.18^{b}	2.57 ± 0.18^{ab}	0.031
p-coumaric acid	0.35 ± 0.07	1.52 ± 0.07	<0.001	0.82 ± 0.09	1.06 ± 0.09	0.93 ± 0.09	n.s.	0.84 ± 0.09	0.95 ± 0.09	1.02 ± 0.09	n.s.
gallic acid	0.03 ± 0	0.05 ± 0	<0.001	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	n.s.	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	n.s.
4-hydroxybenzoic acid	0.1 ± 0.01	0.14 ± 0.01	0.002	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	n.s.	0.11 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	n.s.
2,5-dihydroxybenzoic acid	1.94 ± 0.08	2.4 ± 0.08	<0.001	2.09 ± 0.1	2.23 ± 0.1	2.2 ± 0.1	n.s.	2.22 ± 0.1	2.22 ± 0.1	2.08 ± 0.1	n.s.
caffeic acid	0.01 ± 0	0.05 ± 0	<0.001	0.03 ± 0	0.04 ± 0	0.03 ± 0	n.s.	0.03 ± 0	0.03 ± 0	0.03 ± 0	n.s.
Total phenolic acid	2.43 ± 0.13	4.16 ± 0.13	<0.001	3.08 ± 0.16	3.51 ± 0.16	3.31 ± 0.16	n.s.	3.23 ± 0.16	3.35 ± 0.16	3.32 ± 0.16	n.s.
3,4-dihydroxybenzaldehyde	0.03 ± 0	0.03 ± 0	<0.001	0.03 ± 0	0.03 ± 0	0.03 ± 0	n.s.	0.0284 ± 0^{ab}	0.0286 ± 0^{b}	0.0274 ± 0^{a}	0.029
Total phenolic compounds	468.91 ± 6.65	393.34 ± 6.65	<0.001	441.33 ± 8.14	426.5 ± 8.14	425.54 ± 8.14	n.s.	440.96 ± 8.14	425.36 ± 8.14	427.05 ± 8.14	n.s.

Statistically significant differences for the "formulation" are represented by different letters ($p<0.05$).
n.s. Not significant, $p>0.05$

Phenolic acids, flavonols, flavanols, ellagic acid and phenolic aldehyde

The levels of ellagic acid, flavanols (catechin, procyanidin B2 and procyanidin B1), flavonols (quercetin, kaempferol and kaemperol 3-glucoside), phenolic acids (*p*-coumaric acid, gallic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and caffeic acid), and phenolic aldehyde (3,4-dihydroxybenzaldehyde) are presented in Table S5.3 (Supplemental Information). The content of these individual phenolics in strawberries were comparable with values reported in the literature for *Fragaria* × *ananassa* (Table S5.1).

For flavonols, significant differences were noted among the treatments (Table 5.3). The effect of the formulations was significant on kaempferol and quercetin. For kaempferol, levels decreased in the order Allosperse®>nSiO_2>conventional. For quercetin, levels decreased from nSiO_2>Allosperse®>conventional. In contrast with plargonidin-3-glucoside, kaempferol and quercetin contents for the conventional treatments were significantly lower than for Allosperse® (-53%) and nSiO_2 treatments (-39%), respectively. Negative correlations between the anthocyanins and flavonols have been reported for organic and conventional cultures of black and red currants[42]. In the Lou et al. study[43], grape hyacinth with low concentrations of anthocyanins had a high concentration of flavonols. The anthocyanin biosynthetic pathways are similar to those of the general flavonoids. Furthermore, competition for a common substrate - dihydroflavonols in their biosynthetic pathways could result in a negative relationship between the contents of the flavonols and anthocyanins[44]. Therefore, the present results suggest that the increased biosynthesis of quercetin and kaempferol following the nanopesticide treatments could lead to a decreased accumulation of anthocyanins.

In the literature, the most important categories of phenolic acids are hydroxycinnamic (C6-C3) and hydroxybenzoic acids (C6-C1). Gallic acid, a key hydroxybenzoic acid, showed

significant differences for the two-way interaction "day × formulation" (Table S5.4). A +125% increase in gallic acid levels were observed in the $nSiO_2$ treatment, when compared to the conventional treatment on the last sampling day (Table S5.3). Similar to anthocyanins, gallic acid contributes to the self-defence of the plants with antibiosis property on insects and fungi[45]. The high concentrations of gallic acid in $nSiO_2$ treated strawberries indicated that this self-defence mechanism was more activated than in the strawberry plants treated with conventional pesticides.

Among the other hydroxybenzoic acids and their derivatives, only the levels of 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde, PAL) in strawberries decreased from Allosperse®>conventional>$nSiO_2$ (Table 5.3). Significant differences were observed between the two nanoformulations (p<0.05). The differences between the conventional and nano- formulations were however not obvious. PAL is a degradation metabolite from complex polyphenols such as anthocyanins and phenolic acids in fruits and vegetables[46]. Overall, the Allosperse® treatments led to more biosynthesis of PAL in strawberries when compared to the conventional treatments, significantly more than $nSiO_2$ treatments. Because PAL is reported to have some biological functions such anti-inflammatory properties both *in vivo* and *in vitro*[47]. Allosperse® pesticides may have some benefits to producing high PAL levels in strawberries.

5.4.3 Phenolic profiles and total metabolite profiles

The (primary and secondary) metabolites such as ellagic acid, flavonols, anthocyanins and the ascorbic acid content and composition in strawberries are known to be influenced by pesticides[26,27]. The effect of nanoencapsulated pesticides and nanocarriers on metabolite profiles and phenolic profiles was explored using PCA. Discrimination results are presented in Figure 5.2. Figure 5.2 revealed some influence of the nanoformulations on the metabolite profiles measured

by LC-QTOF-MS, which are expected to include not only the phenolic compounds but also other metabolites such as amino acids, nucleosides, fatty acids, carbohydrates, vitamins, and hormones[25]. For the total metabolite profiles and phenolic profiles in the BFT-day25 and AZOX-d81 datasets, the three formulations (conventional, Allosperse® and $nSiO_2$) could be discriminated, confirming some actual differences among formulations. Similar with the effect of formulations on TPC values in strawberries, the effects of BFT and AZOX on both total metabolite profiles and phenolic profiles were obvious at the beginning and at the end of the growing season. The effect of nanopesticides on the strawberry plant growth was also assessed in this experiment[19], but no significant differences were observed compared to plants treated with conventional pesticides. Therefore, even though plant phenological parameters were not distinct for different pesticide formulations, some differences in the plant metabolism could be observed among the formulations.

In order to investigate the effect of nanocarriers only on the plant metabolites, strawberries treated with nanocarriers (without the pesticide AIs) were also analyzed in PCA. The size of the Allosperse® nanocarriers is smaller than the pores of the root epidermal cell walls (5-20 nm), which means it could be absorbed by the plants[48]. In addition, bioaccumulation of $nSiO_2$ nanocarriers with a diameter of ~70nm by foliar uptake has also been demonstrated in *Solanum Lycopersicon*[49]. Phenolic profiles of the control (yellow) and Allosperse® treatments were discriminated in the PCA run on the day 81 samples (Figure 5.2f). Exogenous compounds could active the detoxification mechanism of plants to against chemical stress, which was related to the synthesis and degradation of the phenolic compounds in plants[50]. One previous study reported that $Cu(OH)_2$ nanopesticides altered the metabolite profiles and reduced the antioxidant content of spinach leaves[51]. In a present study, nanocarriers of Allosperse® were small enough to be taken

up by plants and, as exogenous compounds, also seemed to have some effect on the overall phenolic profiles of the strawberries.

Several studies compared the metabolites in organic strawberries with those treated with reduced or regular amounts of pesticides[18,26,27]. They found that organic strawberries or those treated with a smaller amounts of pesticides were reported to generate better flavor qualities and higher TPC levels. In the literature, the effects of pesticides on individual phenolic compounds of strawberries were observed, but not consistently. Similarly, the present study found that the effects of pesticide formulations on the total metabolite profiles were not consistent with phenolic profiles. Moreover, the impact of the pesticide formulations on TPC and the levels of individual phenolic compounds in present study were also different. There are no studies that document how strawberries grown in the presence of nanoencapsulated pesticides perform as compared to strawberries grown under conventional treatments.

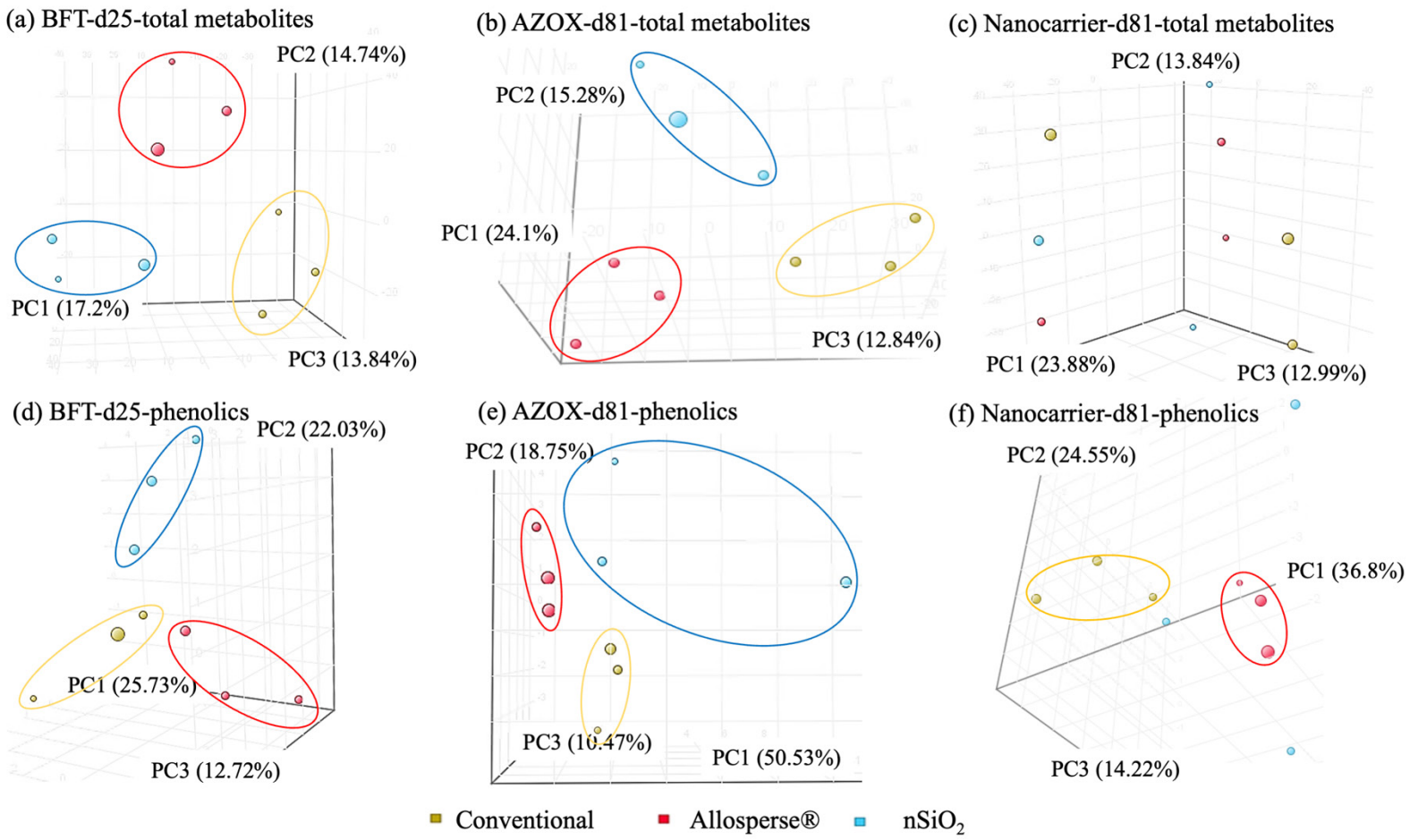

Figure 5. 2 Results of the principal component analysis (PCA) for the metabolite profiles and phenolic profiles in strawberries treated with nanocarriers, azoxystrobin (AZOX) or bifenthrin (BFT) in the different formulations (conventional, Allosperse® and $nSiO_2$) on different days (25 or 81) – (a) BFT- metabolite profiles on day 25; (b) AZOX- metabolite profiles on day 81; (c) Nanocarrier* - metabolite

profiles on day 81; (d) BFT- phenolic profiles on day 25; (e) AZOX- phenolic profiles on day 81; (f) Nanocarrier* - phenolic profiles on day 81. (*nanocarrier: no pesticidie active ingredient, only Allosperse® and $nSiO_2$)

Knowledge about the effects of nanopesticides on the total and individual phenolic compounds is essential as these compounds play an important role in the plant metabolism and health, and can impact food quality. To the best of our knowledge, this is the first report comparing the phenolic contents and profiles in strawberries treated with conventional and nanoencapsulated (Allosperse® or $nSiO_2$) AZOX and BFT pesticides in a controlled field experiment. Overall, the results support that the nanoencapsulated pesticides and their conventional formulations induced distinct effects on the strawberry metabolites including their phenolic compounds. The treatments of nanoformulations (Allosperse® and $nSiO_2$) led to decreased TPC and pelargonidin-3-glucoside levels but increased quercetin, kaempferol, and gallic acid levels in strawberries. Therefore, the impact of nanoencapsulated pesticides on individual phenolic compounds is not systematic. For different types of AI, the same nanocarriers generated different effects on the phenolic compounds at different time. Furthermore, based on the present study of phenolic profiles, nanocarriers may have some subtle effects on the plant metabolism. This study showed the distinct effects of nanopesticides on plant metabolites, but analysis of their effects is still in the early stages. Further research will thus be needed to assess the impact of diverse nanopesticides on other groups of plant metabolites.

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5.8 Supplementary materials

Table S5. 1 Comparison of the concentration of phenolic compounds quantified in field strawberries with values reported in the literature.

Name	Max mg/kg	Min mg/kg	Ave mg/kg	Lit.[a] mg/kg	Ref.
pelargonidin-3-glucoside	196.806	297.441	250.726	42.7 - 253	(Kajdzanoska, Petreska & Stefova, 2011)
cyanidin N-3-o-glucoside	0.364	0.857	0.598	11 - 27	(Kajdzanoska, Petreska & Stefova, 2011)
Ellagic acid	19.315	97.117	35.693	2 - 403	(Aaby et al., 2012)
catechin	43.154	151.955	93.839	1.7 - 86	(Kajdzanoska, Petreska & Stefova, 2011; Aksic, et al., 2019)
procyanidin B2	1.145	5.047	2.816	46 - 161	(Aaby et al., 2012)
procyanidin B1	26.370	65.181	40.980	46 - 161	(Aaby et al., 2012)
quercetin	0.338	2.370	0.960	2.45 - 3.99	(Aksic, et al., 2019)
kaempferol	0.214	2.616	1.063	0.7 - 1.74	(Aksic, et al., 2019)
kaempferol 3-glucoside	0.238	0.619	0.426	0.82 - 2.17	(Aksic, et al., 2019)
p-coumaric	0.074	3.105	0.936	0.59 - 5.27	(Aksic, et al., 2019)
gallic acid	0.007	0.178	0.038	3.6 - 8.5	(Aksic, et al., 2019)
4-hydroxybenzoic acid	0.082	0.378	0.120	0.32 - 0.96	(Kadivec, et al., 2013)
2,5-dehydroxybenzoic acid	1.488	4.144	2.173	25.5 - 30.5	(Aksic, et al., 2019)
caffeic acid	0.000	0.139	0.032	0.22 - 0.96	(Aksic, et al., 2019)
3,4-dihydroxybenzaldehyde	0.024	0.034	0.028	-	-

[a] Values were from various strawberries (*Fragaria* x *ananassa*) cultivars (Maya, Duch. Favette, Alba, and Clery).

Table S5. 2 Three-way ANOVA (three factors – day, formulations and active ingredients (AI)) for total phenolic content in strawberry fruits (*p* values, n=3).

	Year 1	Year 2
Simple effect		
day		
day1	2.201 ± 0.066^{ab}	2.304 ± 0.048^{b}
day2	2.151 ± 0.08^{ab}	2.562 ± 0.048^{c}
day3	2.466 ± 0.072^{bc}	2.206 ± 0.048^{ab}
day4	2.627 ± 0.072^{c}	2.034 ± 0.048^{a}
day5	2.068 ± 0.071^{a}	2.368 ± 0.048^{b}
day6	2.276 ± 0.066^{ab}	
p value	<0.001	<0.001
Active ingredients		
no pesticide	2.419 ± 0.049^{b}	2.355 ± 0.037
azoxystrobin	2.246 ± 0.052^{a}	2.286 ± 0.037
bifenthrin	2.229 ± 0.05^{ab}	2.243 ± 0.037
p value	0.015	n.s.
formulations		
conventional	2.428 ± 0.049^{b}	2.309 ± 0.037
Allosperse®	2.244 ± 0.051^{ab}	2.322 ± 0.037
$nSiO_2$	2.223 ± 0.051^{a}	2.253 ± 0.037
p value	0.008	n.s.
2-way interactions		
day * AI		
p value	n.s.	0.010
day * formulation		
p value	0.004	<0.001
formulation * AI		
p value	n.s.	n.s.
3-way interaction		
day * formulation * AI		
p value	n.s.	0.017

Day1-6: strawberry collected in different days (year1: 25, 30, 40, 55, 65 and 78; year2: 25, 30, 44, 55 and 81)
Statistically significant differences between different formulations at the same sampling date are represented by different letters ($p<0.05$).
n.s. Not significant, $p>0.05$
Phenolic compound content is mg/kg fresh strawberry

Table S5. 3 Phenolic compounds (mg/kg) in strawberry fruits quantified using LC-ESI-QToF/MS

Compounds	Day 25									Day 81								
	No pesticide			AZOX			BFT			No pesticide			AZOX			BFT		
	Conventional	Allospore®	$nSiO_2$	Conventional	Allospore®	$nSiO_2$	Conventional	Allospore®	$nSiO_2$	Conventional	Allospore®	$nSiO_2$	Conventional	Allospore®	$nSiO_2$	Conventional	Allospore®	$nSiO_2$
Anthocyanins																		
pelargonidin-3-glucoside	268.46 ± 17.12	265.03 ± 15.93	270.6 ± 17.51	276.84 ± 27.21	246.17 ± 18.73	270.15 ± 10.16	282.03 ± 16.89	261.59 ± 12.47	279.37 ± 15.65	251.87 ± 27.22	241.98 ± 14	243.54 ± 22.84	246.76 ± 9.67	220.18 ± 20.54	233.17 ± 30.48	221.62 ± 4.83	214.88 ± 14.83	218.82 ± 3.60
cyanidin N-3-o-glucoside	0.43 ± 0.09	0.63 ± 0.01	0.56 ± 0.07	0.65 ± 0.1	0.5 ± 0.08	0.51 ± 0.03	0.57 ± 0.10	0.45 ± 0.08	0.57 ± 0.01	0.72 ± 0.13	0.7 ± 0.11	0.69 ± 0.09	0.63 ± 0.05	0.69 ± 0.04	0.64 ± 0.10	0.63 ± 0.11	0.6 ± 0.07	0.56 ± 0.05
total anthocyanins	268.9 ± 17.03	265.66 ± 15.93	271.16 ± 17.55	277.49 ± 27.17	246.68 ± 18.80	270.67 ± 10.13	282.6 ± 16.83	262.04 ± 12.55	279.94 ± 15.65	252.59 ± 27.34	242.68 ± 13.96	244.24 ± 22.83	247.39 ± 9.72	220.87 ± 20.56	233.81 ± 30.57	222.25 ± 4.86	215.48 ± 14.76	219.38 ± 3.55
Ellagic acid																		
ellagic acid	37.18 ± 0.98	49.89 ± 9.75	44.11 ± 10.11	58.58 ± 34.58	43.2 ± 14.39	41.79 ± 3.94	39.12 ± 4.54	40.15 ± 5.14	42.28 ± 6.99	25.83 ± 1.42	26.61 ± 5.52	26.2 ± 3.13	23.62 ± 7.28	27.16 ± 3.08	32.04 ± 12.84	25.91 ± 2.88	26.73 ± 5.12	32.06 ± 10.03
Flavan-3-ols																		
catechin	95.67 ± 4.26	109.97 ± 2.29	115.95 ± 17.51	114.98 ± 33.22	96.16 ± 25.27	88.09 ± 3.59	100.14 ± 13.62	104.29 ± 9.75	95.66 ± 4.82	88.2 ± 6.88	88.8 ± 13.19	87.86 ± 9.92	86.86 ± 5.35	87.2 ± 3.00	66.1 ± 19.90	94.88 ± 6.61	88.91 ± 10.11	79.41 ± 15.17
procyanidin B2	2.94 ± 0.18	3.39 ± 0.22	3.43 ± 0.45	3.64 ± 1.23	2.95 ± 0.82	2.77 ± 0.27	3.11 ± 0.55	3.19 ± 0.28	3.07 ± 0.32	2.51 ± 0.13	2.51 ± 0.43	2.48 ± 0.23	2.53 ± 0.23	2.56 ± 0.10	1.88 ± 0.65	2.81 ± 0.19	2.65 ± 0.22	2.26 ± 0.50
procyanidin B1	43.67 ± 1.59	49.74 ± 2.82	45.27 ± 5.23	50.84 ± 13.32	43.86 ± 14.09	39.8 ± 2.2	44.39 ± 3.69	45.84 ± 2.27	43.57 ± 2.41	37.53 ± 1.51	37.56 ± 3.97	38.06 ± 1.45	35.67 ± 1.05	36.85 ± 1.70	33.15 ± 6.22	38.85 ± 2.19	37.68 ± 2.27	35.32 ± 2.42
Total flavan-3-ols	142.27 ± 5.31	163.1 ± 1.63	164.65 ± 23.11	169.46 ± 47.71	142.97 ± 40.17	130.65 ± 5.97	147.64 ± 17.70	153.31 ± 12.23	142.37 ± 7.54	128.24 ± 7.39	128.87 ± 17.24	128.4 ± 11.11	125.06 ± 6.62	126.6 ± 3.33	101.13 ± 26.59	136.54 ± 8.89	129.24 ± 12.58	117 ± 18.00
Flavonols																		
quercetin	0.54 ± 0.17	0.47 ± 0.10	0.53 ± 0.09	0.58 ± 0.22	0.46 ± 0.07	0.54 ± 0.14	0.48 ± 0.14	0.51 ± 0.09	0.6 ± 0.03	0.97 ± 0.17	1.72 ± 0.53	1.54 ± 0.43	1 ± 0.11	1.62 ± 0.35	1.5 ± 0.87	1.08 ± 0.24	1.46 ± 0.68	1.69 ± 0.27
kaempferol	0.74 ± 0.31	0.84 ± 0.22	0.46 ± 0.05	0.57 ± 0.15	0.7 ± 0.16	0.44 ± 0.06	0.46 ± 0.14	0.84 ± 0.76	0.84 ± 0.40	1.01 ± 0.17	1.72 ± 0.79	1.63 ± 0.73	1.35 ± 0.34	1.7 ± 0.47	1.64 ± 1.09	0.83 ± 0.27	1.83 ± 0.59	1.54 ± 0.42
kaempferol 3-glucoside	0.32 ± 0.10	0.37 ± 0.03	0.37 ± 0.03	0.4 ± 0.08	0.32 ± 0.07	0.36 ± 0.04	0.38 ± 0.07	0.32 ± 0.03	0.36 ± 0.04	0.56 ± 0.05	0.54 ± 0.09	0.53 ± 0.03	0.48 ± 0.03	0.54 ± 0.03	0.44 ± 0.10	0.51 ± 0.08	0.46 ± 0.03	0.41 ± 0.07
total flavonols	1.59 ± 0.36	1.68 ± 0.16	1.36 ± 0.14	1.56 ± 0.30	1.48 ± 0.18	1.33 ± 0.16	1.32 ± 0.04	1.67 ± 0.70	1.8 ± 0.34	2.54 ± 0.34	3.97 ± 1.39	3.7 ± 1.11	2.84 ± 0.33	3.87 ± 0.77	3.57 ± 2.05	2.42 ± 0.39	3.75 ± 1.10	3.64 ± 0.76
Phenolic acids																		
p-coumaric acid	0.35 ± 0.22	0.21 ± 0.17	0.35 ± 0.06	0.41 ± 0.17	0.3 ± 0.11	0.43 ± 0.18	0.39 ± 0.19	0.36 ± 0.14	0.4 ± 0.04	1.07 ± 0.19	1.56 ± 0.16	1.4 ± 0.21	1.69 ± 0.15	1.85 ± 1.10	1.68 ± 0.18	1.12 ± 0.18	1.39 ± 0.08	1.89 ± 0.89
gallic acid	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.04	0.02 ± 0.00	0.05 ± 0.03	0.1 ± 0.06	0.03 ± 0.01	0.05 ± 0.03	0.04 ± 0.01

4-hydroxybenzoic acid	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.01	0.12 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.00	0.1 ± 0.01	0.1 ± 0.02	0.11 ± 0.01	0.15 ± 0.04	0.13 ± 0.02	0.11 ± 0.02	0.13 ± 0.02	0.22 ± 0.13	0.12 ± 0.02	0.12 ± 0.01	0.16 ± 0.09
2,5-dihydroxybenzoic acid	1.84 ± 0.30	1.75 ± 0.13	1.59 ± 0.07	2.67 ± 1.28	2.08 ± 0.58	1.85 ± 0.33	1.77 ± 0.18	2.06 ± 0.46	1.88 ± 0.14	2.27 ± 0.17	2.58 ± 0.28	2.49 ± 0.37	2.24 ± 0.20	2.59 ± 0.19	1.97 ± 0.23	2.52 ± 0.25	2.29 ± 0.13	2.68 ± 0.35
caffeic acid	0.01 ± 0.00	0 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.0	0.01 ± 0.00	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.04	0.05 ± 0.03	0.04 ± 0.011	0.05 ± 0.01	0.08 ± 0.05
total phenolic acid	2.32 ± 0.48	3.26 ± 1.42	2.29 ± 0.21	2.08 ± 0.22	2.5 ± 0.71	2.56 ± 0.62	2.07 ± 0.08	2.42 ± 0.51	2.41 ± 0.16	3.52 ± 0.17	4.14 ± 0.37	3.83 ± 0.45	4.39 ± 0.48	4.69 ± 1.31	3.9 ± 0.20	4.12 ± 0.54	4.03 ± 0.24	4.86 ± 1.36
Phenolic aldehyde																		
3,4-dihydroxybenzaldehyde	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.003	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Total																		
total phenolic compounds	452.29 ± 11.23	510.37 ± 107.66	472.99 ± 12.80	482.43 ± 21.637	436.85 ± 41.33	459.75 ± 25.66	483.38 ± 20.94	446.88 ± 19.05	468.76 ± 29.49	412.76 ± 20.73	403.07 ± 20.35	390.97 ± 12.80	406.56 ± 10.19	383.23 ± 22.02	379.12 ± 21.23	406.69 ± 27.88	374.61 ± 48.11	376.96 ± 12.96

*All values are expressed as mg/kg fresh strawberry fruits ± standard deviation of three independent measurements (n=3). The phenolic compounds are classified according to the phenolic groups. The table including two batches of strawberry collected in the second year: Day number were count from the first application of treatments.

Table S5. 4 Interactions in three-way ANOVA (three factors – day, formulations and active ingredients) test for phenolic compounds in strawberry (*p* values, n=3)

	2-way interactions			3-way interaction
	day * AI	day * formulation	formulation * AI	day * formulation * AI
	p value	*p* value	*p* value	*p* value
pelargonidin-3-glucoside	0.023	n.s.	n.s.	n.s.
cyanidin N-3-o-glucoside	n.s.	n.s.	n.s.	0.019
Total anthocyanins	0.023	n.s.	n.s.	n.s.
ellagic acid	n.s.	n.s.	n.s.	n.s.
catechin	n.s.	n.s.	n.s.	n.s.
procyanidin B2	n.s.	n.s.	n.s.	n.s.
procyanidin B1	n.s.	n.s.	n.s.	n.s.
Total flavanols	n.s.	n.s.	n.s.	n.s.
quercetin	n.s.	0.018	n.s.	n.s.
kaempferol	n.s.	n.s.	n.s.	n.s.
kaempferol 3-glucoside	n.s.	n.s.	n.s.	n.s.
Total flavonols	n.s.	n.s.	n.s.	n.s.
p-coumaric acid	n.s.	n.s.	n.s.	n.s.
gallic acid	n.s.	0.013	n.s.	n.s.
4-hydroxybenzoic acid	n.s.	n.s.	n.s.	n.s.
2,5-dihydroxybenzoic acid	n.s.	n.s.	n.s.	n.s.
caffeic acid	n.s.	n.s.	n.s.	n.s.
Total phenolic acid	n.s.	n.s.	n.s.	n.s.
3,4-dihydroxybenzaldehyde	n.s.	n.s.	n.s.	0.040
Total phenolic compounds	n.s.	n.s.	n.s.	n.s.

n.s. Not significant, p>0.05

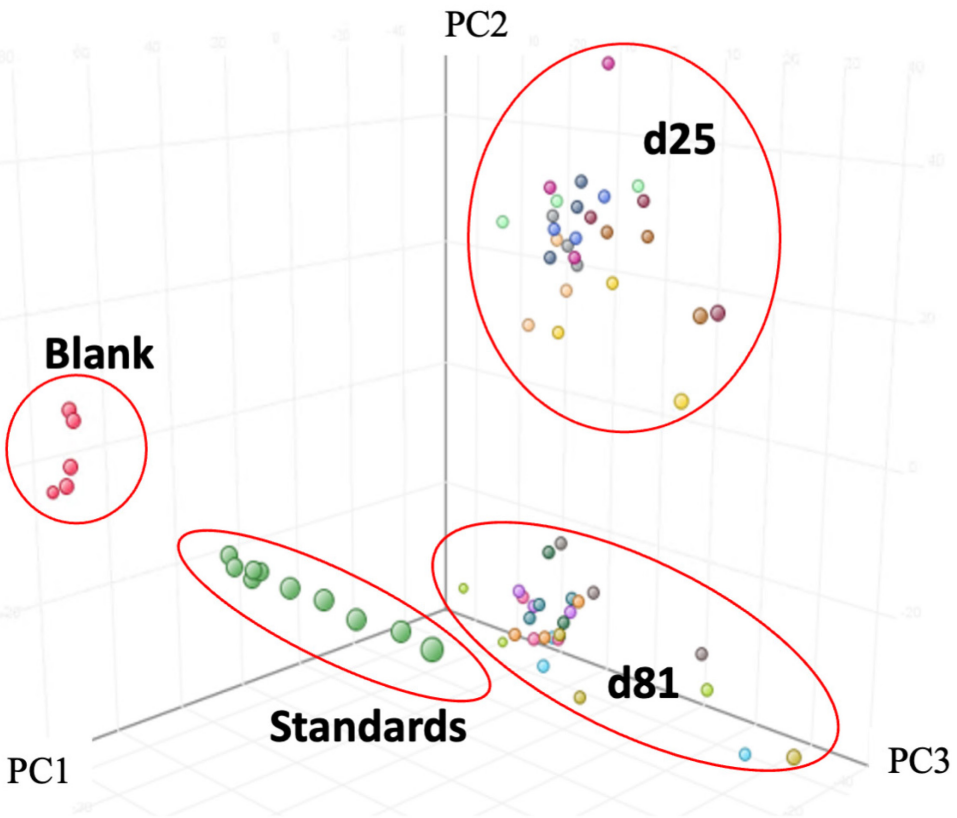

Figure S5. 1 Results of the principal component analysis (PCA) for the phenolic profiles in strawberries treated with nanocarriers, azoxystrobin (AZOX) or bifenthrin (BFT) in different formulations (conventional, Allosperse® and $nSiO_2$) in different days (25 or 81).

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Connecting Text

In Chapters 4 and 5, some effects of NEPs were verified on the environmental fate and the plant metabolism at the molecular level (e.g., total phenolic compounds and phenolic profile in strawberry fruit). In Chapter 6, in order to analyze the effect of nanocarriers on pesticide thermal degradation, the analytical method for NEPs validated in Chapter 3 was used to analyze pesticide residues from AZOX NEPs (Allosperse® and $nSiO_2$) during the thermal processing in the water, spiked strawberry and incurred strawberry models. This is the first study on the thermal degradation kinetics and products of AZOX in food. Chapter 6 will be submitted as "Non-targeted analysis of the thermal degradation of azoxystrobin (conventional and nanoencapsulated) in water, spiked strawberry and incurred strawberry models" (Peiying Wang, Juliana A. Galhardi, Lan Liu, Vinicius Bueno, Subhasis Ghoshal, Valérie Gravel, Kevin J. Wilkinson, Stéphane Bayen)

Chapter 6. Thermal degradation of conventional and nanoencapsulated azoxystrobin due to processing in water, spiked strawberry and incurred strawberry models

6.1 Abstract

Nanoecapsulated formulations of pesticides have been recently developed and some products are now marketed for specific applications in agriculture. Pesticide residues present in raw agricultural products can degrade or react during food processing steps. To date though, the fate of nanopesticides during food processing has not been well described. In this study, the thermal degradation of azoxystrobin (AZOX) in conventional and nanoencapsulated (Allosperse® and $nSiO_2$) formulations was assessed in water, spiked strawberry and incurred strawberry models. The thermal degradation followed first-order kinetics when heated at 100°C in the water model. The thermal degradation of AZOX in nanoformulations in strawberry models (18% AZOX decrease) was comparable or lower than in the conventional formulation (21%), possibly due to the nanocarriers protecting the active ingredient from hydrolytic degradation. Thermal degradation reactions for AZOX were different between the water and strawberry models. The nanoencapsulation of AZOX did not result in new TDPs in any of the matrixes. Based on the observed TDPs, AZOX thermal degradation pathways include ether cleavage, hydrolysis, demethylation and decarboxylation. Overall, nanocarriers had a slight or no impact either on the degradation rate or on the degradation product types.

Keywords: Strawberry; Thermal degradation; Azoxystrobin; Non-target analysis; Nanoencapsulated pesticide

6.2 Introduction

Small fruits such as strawberries are popular among consumers for their attractive appearance, unique taste, and high nutritive value. Strawberry production can be significantly impacted by insects and fungi, which can influence the culture yields and the quality/quantity of strawberries post-harvest[1,2]. Pesticide treatments contribute to reducing the impacts of pests and pathogens in strawberry production[3]. For example, fungicides (e.g., azoxystrobin, a systemic strobilurin) are widely used to control the decay of strawberries caused by various pathogens such as gray mold (*Botrytis cinerea)* and Rhizopus rot (*Rhizopus stolonifera*)[4]. The extensive use of pesticides is however reflected by a relatively frequent detection of pesticide residues in strawberry fruits in the market[5]. To comply with food safety regulations, pesticide residues in food commodities should not exceed limits such as maximum residue limit (MRL)– e.g., 10 mg/kg for azoxystrobin in strawberries in Canada[6]. Recently, nanoencapsulation has been introduced as a technique to increase the efficacy of pesticides and reduce the used of the active ingredients (AI) of the pesticides.

In addition to being consumed as fresh fruits, strawberries are commonly processed as an ingredient in the preparation of value-added commodities such as jams or juices. Such processing activities contribute to minimize post-harvest losses and make strawberry culture more profitable[3]. Food processing can also induce changes in the pesticide residue profiles through hydrolysis, volatilization, dissolution, metabolism, oxidation, and thermal degradation[7]. Washing steps are generally efficient at removing azoxystrobin from the surface of strawberries[4]. Thermal processing is also particularly efficient in reducing the levels of chemical residues in food[8]. The reduction of pesticide levels in food for compounds is influenced by parameters such as temperature and time, the type of food matrixes and the structure of the pesticides[9].

Strobilurin fungicides including AZOX, pyraclostrobin, fluoxastrobin, kresoxim-methyl, trifloxystrobin, picoxystrobin, mandestrobin, and metominostrobin have a similar toxiphoric group, (E)-β -methoxyacrylate moiety[10]. As one of the first synthetic strobilurin fungicides, the environmental metabolism and degradation of AZOX have been extensively studied and reported in the literature[11], however knowledge on AZOX dissipation in food is limited[12]. Depending on the cooking methods, apparent decreases and increases in the AZOX concentrations (-89% to +60%) have been reported after heating[9,13-20]. However, no studies investigate the TDPs of AZOX during thermal processing of food.

While most thermal degradation studies have reported changes in the levels of the parent pesticides, there is often little information on the newly formed degradation or transformation products. Degradation products could be comparable or even more toxic as compared to the parent compounds[21]. When toxic degradation products are found in significant amounts, they may be included in the surveillance of the parent pesticide residues, as illustrated by 3-hydroxy-carbofuran, a metabolite of carbofuran[22]. However, concerns are mounting about pesticide degradation products, as new compounds are regularly detected in food with no or little information on their toxicity. Consequently, the *Codex Alimentarius*[23] has recommended that the fate of pesticides residues during processing should be investigated in order to identify the possible breakdown or transformation products. In this context, it appears essential to identify TDPs for novel nanopesticides to produce comprehensive risk assessments. To date, the fate of nanoencapsulated pesticides during thermal processing has not been reported.

The aim of this study was to investigate the thermal degradation of AZOX in conventional and nanoencapsulated pesticide formulations, using both targeted and non-targeted analysis. LC coupled with high-resolution mass spectrometry (HRMS) has emerged as a powerful tool for

targeted and non-targeted investigations of degradation products. Targeted analysis is often applied to quantify specific degradation products, while non-targeted analysis investigates degradation product profiles and identifies unknown or unexpected compounds in the samples[24,25]. The use of spiked samples is generally recognized as inappropriate to evaluate the stability of pesticides during processing[26]. This study was therefore performed on incurred strawberries, but spiked water and strawberry models were also included for comparison. More specifically, this study aimed at identifying the thermal degradation/transformation products and compared the degradation kinetics and breakdown or reactions products of AZOX generated in these three models. Results were discussed in terms of thermal degradation pathways for AZOX. Ultimately, this study aims at determining specificities in the fate and behavior of nanoencapsulated pesticides.

6.3 Material and methods

6.3.1 Chemicals and reagents

Azoxystrobin (AZOX, CAS#131860-33-8) was purchased as a pure standard (≥98%) from Sigma-Aldrich (St. Louis, MO, USA). The deuterated analogue D_4-AZOX and azoxystrobin free acid (R234886, AzFA) were purchased from Toronto Research Chemicals (North York, ON, Canada). HPLC grade solvents (water, acetonitrile (ACN), and methanol), anhydrous magnesium sulphate, sodium acetate, LC/MS grade formic acid and ammonium acetate (NH_4Ac) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Primary Secondary Amine (PSA) salts were purchased from Agilent (Santa Clara, CA, USA). Allosperse® is a polyacrylic acid polymeric nanoparticle used as a nanocarrier for pesticides, including AZOX. Allosperse®-AZOX was prepared and supplied by Vive Crop Protection Inc. (Mississauga, Canada). The synthesis of porous hollow silica nanoparticles ($nSiO_2$) and their loading with AZOX was reported in Bueno & Ghoshal[27] and Bueno et al.[28], respectively. Stock solutions of the standards were prepared in methanol.

6.3.2 Field (incurred) strawberry samples

A controlled field experiment was carried at the Macdonald Campus of McGill University, Sainte-Anne-de-Bellevue, QC, Canada. Strawberry plants (*Fragaria* × *ananassa* Duch. "Seascape"), were cultivated under field conditions (n = 5) and exposed to different treatments: (1) control; (2) AZOX; (3) AZOX in Allosperse® nanocarriers; (4) AZOX in $nSiO_2$. Briefly, strawberry bare root plants (Pépinière Lareault, Canada) were transplanted in the first week of June 2019. Plants were treated twice (total 7.6 mg active ingredient / pot, 15 and 30 days after transplanting) using a drench application for each of the pesticide formulations. Further details on

the field experiment, plant phenology and pesticide accumulation have been described in Galhardi et al.[29] Fruits were collected and homogenized in a stainless-steel blender. All processed samples were stored at -80°C until analysis. AZOX in incurred samples were quantified in our previous study and ranged from 0.2 – 6.21 µg/kg fresh strawberry[29].

6.3.3 Spiked water and strawberry models

The degradation of AZOX in the various formulations (conventional, Allosperse®, and $nSiO_2$) was first studied in a spiked HPLC water model (100 mg/L; pH=8; n=3 for each formulation). Aliquots of each replicate (1 mL; N=5) were transferred into 2 mL amber glass vials for different processing times. Samples were placed in a water bath in a floating rack to keep the cap above the water surface. Samples were heated 100°C for 0 min (t0), 30 min (t30), 60 min (t60), 120 min (t120), and 240 min (t240). Each time point had three replicates. After heating, the vials were cooled down rapidly in cold water. Heated water samples (t240, n=6) were used for the identification of the TDPs for the spiked HPLC water (10 mg/L of the different AZOX formulations to detect as many degradation products as possible, especially those with relatively low concentrations).

Control strawberries from the field were spiked with AZOX in the three formulations at two levels (1 mg/kg and 10 µg/kg; n=3 for each formulation). The high spiking level (1 mg/kg) was used for the comparison with the spiked water (1 mg/L). The low spiking level (10 µg/kg) was comparable with concentrations measured in the harvested strawberries (incurred, around 10 µg/kg) in the field trial[29]. Aliquots (5 g) of each of the above spiked strawberry samples were transferred to 20-mL glass vials and were placed in a water bath as described above for water.

6.3.4 Extraction of the pesticides and their thermal degradation products from strawberry

AZOX extraction in strawberries was adapted from a method based on the original QuEChERS approach[30] and validated for the nanoencapsulated formulations[31]. Briefly, 2 g of homogenized strawberry sample was weighed in a 15 mL plastic centrifuge tube and spiked with D_4-AZOX (40 μg kg^{-1}). Four mL of 1% acetic acid in acetonitrile, 0.8 g of magnesium sulphate and 0.2 g of sodium acetate were added. Samples were vortexed for 15 minutes, and then centrifuged at 2240 × g (5 min, 20°C). One mL of the supernatant was transferred to centrifuge tubes containing 50 mg PSA and 150 mg of $MgSO_4$. Solutions were then vortexed for 1 min, and finally centrifuged (2240 × g, 5 min, 20°C).

6.4.5 Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis

All samples were filtered through a 0.22 μm polytetrafluoroethylene filter (Chrom4; Thuringen, Germany) and were analyzed on an Agilent 1290 Infinity II liquid chromatograph (LC) coupled to a 6545 QTOF mass spectrometer (Agilent Technologies, Santa Clara, USA), operating in both positive and negative electrospray ionization modes (2 consecutive analyses). The LC separation was conducted on a Poroshell 120 phenyl hexyl column (Agilent Technologies; 2.7 μm × 3.0 mm × 100 mm) fitted with a Poroshell 120 EC-C18 (2.7 μm × 3.0 mm × 5 mm) guard column. For both positive and negative mode, elution was performed in gradient mode (0.4 mL min^{-1}) using A=water (0.1% formic acid and 5 mM NH_4Ac) and B=ACN:methanol (1:1, v/v; 0.1% formic acid and 5 mM NH_4Ac) (0 min: 70% A; 0-3 min: B increased from 30 to 100%; 3-6 min: 100% B; 6-8 min: B decreased from 100% to 30%). The injection volume was 10 μL and the column temperature was maintained at 30°C. Nitrogen was used as the drying gas (110°C, 12 L min^{-1}). The fragmentor voltage was 110 V and MS data was acquired in the 50-750 *m/z* range in

full scan mode. Azoxystrobin TDPs were subsequently identified in the targeted MS/MS mode (optimal collision energy of 20 V). Reference ions (*m/z* at 121.0508 and 922.0098 in the positive electrospray ionization mode (ESI+); 112.9856 and 1033.9881 for the negative mode (ESI-)) were used for automatic mass recalibration of each acquired spectrum.

6.3.6 Degradation kinetics of azoxystrobin

The first-order degradation model (Eq. 1) is a common model for the degradation of chemical residues in food[8]:

$$ln[C] = ln[C_0]-k\times t \qquad \text{(Equation 1)}$$

where k is the first-order degradation rate constant (slope of the linear fit); C_0 is the initial concentration; C is the concentration after a heating time t. The first-order model needs the data have a correlation coefficient (r^2) higher than 0.90. The model was considered acceptable when p values for the data sets were <0.05 in regression statistics analysis using Microsoft Excel (Microsoft Corporation, USA).

6.3.7 Data treatment

6.3.7.1 Quantification for degradation percentage

For the quantitative analysis of AZOX, data treatment was conducted using Agilent MassHunter Quantitative Analysis (Agilent Technologies, USA). The ions at 404.1247 and 372.0971 *m/z* were selected as the quantifier and qualifier ions for AZOX, respectively, and were extracted from the full spectrum data (extraction mass window ±10 ppm). The relative response of AZOX vs. D_4-AZOX was used for quantification[31]. The thermal degradation percentages were calculated as the ratios of the AZOX concentrations after and before heating.

6.3.7.2 Identification of the thermal degradation products (TDPs)

First, chromatograms were aligned using the Agilent Masshunter Profinder (Agilent Technologies), using tolerance for retention times (RT) of 0.15 min and mass differences of 10 ppm. Extracted molecular features in heated and unheated samples were compared using the Agilent Masshunter Profiler Professional software (Agilent Technologies) to obtain a list of tentative degradation/transformation compounds. A library of AZOX metabolites and degradation products was prepared using the Agilent Masshunter PCDL software (Agilent Technologies), based on formulae reported in the literature[32,33]. This library was used to screen the LCMS data for possible TDPs of AZOX. The MS/MS spectra of those TDPs were manually compared with spectra from the literature to increase confidence in the identification. The identity of AZOX free acid, as a major degradation product of AZOX, was further confirmed based on matching signals (RT=3.491 min for ion at 372.0971 *m/z*) with the pure reference standard.

6.3.8 Statistical analysis

Analysis of variance (ANOVA) using SPSS Statistics Software 27 (IBM, USA) was used to identify differences among results obtained for different pesticide formulations, by applying a confidence range of 95% (α=0.05, n=3). The results reported for strawberries were based on triplicate extractions (3 different samples for each treatment). Significant differences ($p \leq 0.05$) between average responses were evaluated using a Tukey's multiple-comparisons test.

6.4 Results and Discussion

6.4.1 Thermal degradation kinetic of azoxystrobin in different formulations

Thermal degradation kinetics of AZOX in different formulations (conventional, Allosperse®, $nSiO_2$) were first compared to that in water heated at 100°C. AZOX concentration decreased with time for all formulations, and all degradation kinetics followed a first-order model (Figure 6.1 & Table 6.1; r^2>0.9876, p<0.05). Hydrolysis is expected to be the main degradation mechanism at pH 8[34]. The first-order degradation rate constant (k), determined from the slope (absolute value) of the linear fit ranged from 0.00244 to 0.00287 min^{-1} for the conventional formulation, from 0.00264 to 0.00292 min^{-1} for AZOX encapsulated in $nSiO_2$, and from 0.0018 to 0.00227 min^{-1} for AZOX encapsulated in Allosperse®. In the equation, the slope for the Allosperse® (0.002) was significantly lower than that of the conventional pesticide (0.0026) or $nSiO_2$ (0.0028). In other words, AZOX in the Allosperse® formulation was appeared to be more stable than the other formulations in water (100 μg/L). As the kinetics were slightly (but significantly) slower in the presence of Allosperse, the polymer nanocarrier is thought to protect the AZOX from thermal degradation.

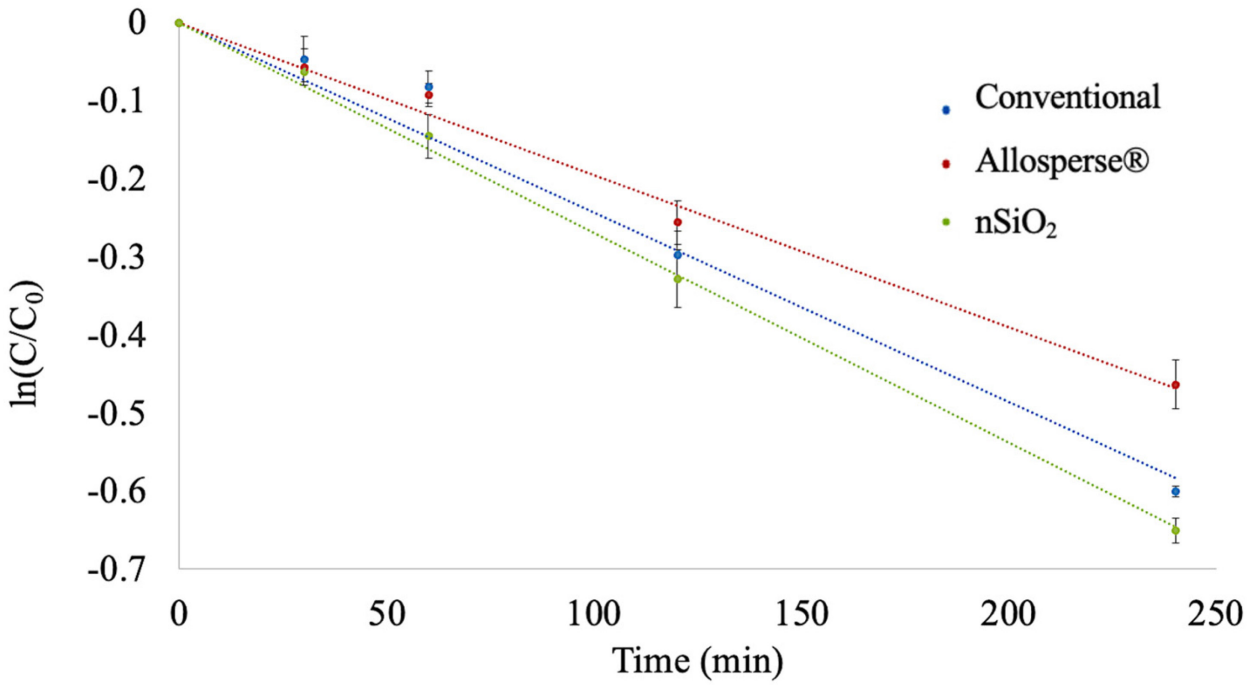

Figure 6. 1 Ln(C/C_0) as a function of time (See Eq. (1)) for three formulations of azoxystrobin (conventional, Allosperse®, and $nSiO_2$) at 100°C in water (spiked with 100 µg/L). Regression line corresponds to a linear fit. The confidence level is 95% (n=3).

Table 6. 1 Kinetics parameters of azoxystrobin thermal degradation at 100°C in water model.

	Conventional	Allosperse®	$nSiO_2$
First-order regression equation[a]	Y = -0.0026t + 0.0286	Y = -0.002t + 0.0035	Y = -0.0028t + 0.0107
r^2	0.9876	0.9926	0.9988
Rate constant (k, min^{-1})	0.00244 - 0.00287	0.0018 - 0.00227	0.00264 - 0.00292
p	3.5E-12	1.91E-11	1.12E-13

[a] Y = In C/C_0 C: concentration of azoxystrobin C_0: initial concentration of azoxystrobin; t = time

6.4.2 Degradation of azoxystrobin in different matrixes

As the thermal degradation experiments were conducted in capped glass vials, concentration decrease of AZOX was estimated to be mostly attributed to thermal degradation, and not to volatilization (AZOX is poorly volatile). The thermal degradation percentages of AZOX in different matrixes after 4 hours of heating ranged from 16 ± 2% to 45 ± 0% for the conventional formulation, from 14 ± 2% to 37 ± 2% for AZOX encapsulated in Allosperse®, and from 11 ± 2% to 48 ± 1% for AZOX encapsulated in $nSiO_2$. The thermal degradation percentages of AZOX were significantly different for the formulations in water (100 µg/L and 1000 µg/L), the spiked strawberries (10 µg/kg) and incurred strawberries (around 10 µg/kg) as shown in Figure 6.2. For the spiked and incurred strawberries, thermal degradation percentages of AZOX in the nanoformulations were comparable or lower than for the conventional formulation. Nanocarriers may reduce the thermal degradation of AZOX, as observed in the strawberry models. The capacity to prevent the degradation of the loaded pesticide AI is often highlighted as one of the key features of nanoencapsulation for pesticide applications[35]. In the present test, nanoencapsulation had no consistent impact, as a range of effects were observed depending on the type of nanocarrier, the initial pesticide concentration and the matrixes.

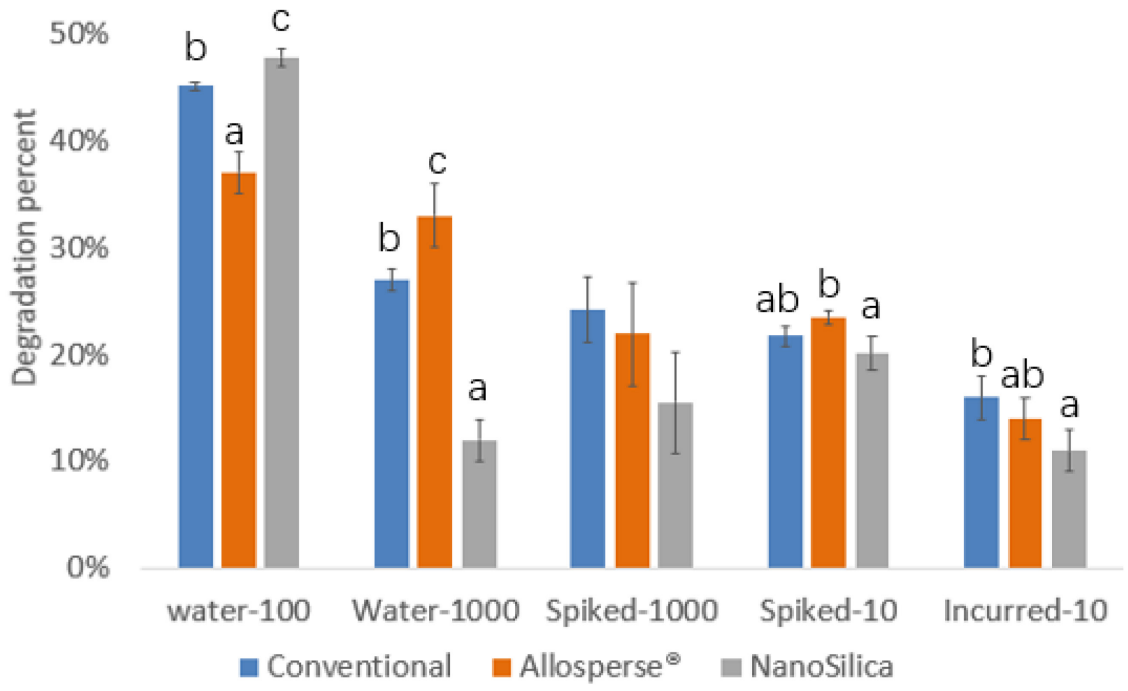

Figure 6. 2 The degradation rate of the azoxystrobin (conventional, Allosperse®, and $nSiO_2$) at 100℃ in the water (100 µg/kg and 1000 µg/kg), spiked (1000 µg/kg and 10 µg/kg) and incurred (around 10 µg/kg) strawberry models after 4 hours of heating (n=3). For each model separately, statistically significant differences between the different formulations are represented by different letters (p<0.05).

6.4.3 Identification of thermal degradation products of azoxystrobin

Compounds that may be considered as possible TDPs of AZOX in the spiked water, spiked strawberry and incurred strawberry models are listed in Table 6.2. Compounds present in both the control heated samples (matrixes without pesticide formulations) and unheated samples were eliminated from the list. In heated water and strawberries, molecular features of interest were investigated in both ESI+ and ESI- modes. Although *Codex Alimentarius* recommends investigating the breakdown or reaction products of pesticides generated by processing[23], there are

no specific guidelines for the detection of TDPs of pesticides in food. Some TDPs detected in this study could not be detected in both ESI+ and ESI- modes. Therefore, both positive and negative ESI modes should be included the method development of pesticide TDPs to detect as many TDPs as possible. Some degradation or transformation products of AZOX in the environment (water, sediments, plants and soils) have been reported in the literature[32,33]. All of these AZOX metabolites were included in the PCDL library (Table S6.1). After the targeted scan, some molecular features suspected to be TDPs could be matched with specific reported compounds based on the ion *m/z* from the library (Table 6.3).

Table 6. 2 Possible thermal degradation products of azoxystrobin identified in ESI+ or ESI- modes in spiked water, spiked strawberry and incurred strawberry models (100°C; 4 hours). ND: not detected.

Compound ID	Mass	*m/z*	RT	ESI[b] +/-	Model		
					Spiked water	Spiked strawberries	Incurred strawberries
TDP 1	208.0731	209.0806	2.906	+	ND	√	ND
TDP 2	213.0538	214.0617	2.440	+	√	√	ND
TDP 3	218.0679	219.0759	2.617	+	ND	√	ND
TDP 4	222.0527	221.0451	2.620	-	ND	√	ND
TDP 5	228.0900	229.0970	3.423	+	ND	√	ND
TDP 6	302.0903	303.0972	2.712	+	√	√	ND
TDP 7	303.1010	304.1078	3.619	+/-	√	ND	ND
TDP 8	317.0798	318.0867	3.908	+	√	ND	ND
TDP 9	321.1106	322.1172	3.717	+/-	√	ND	ND
TDP 10	325.0824	326.0892	3.622	+	√	ND	ND
TDP 11	329.0802	330.0867	3.944	+/-	√	ND	ND
TDP 12	347.0909	348.0973	3.509	+	√	ND	ND
TDP 13	351.0615	352.0683	3.937	+	√	ND	ND
TDP 14	361.0700	362.0760	2.955	+	√	ND	ND
TDP 15	361.1073	362.1141	3.783	+	√	ND	ND
TDP 16	361.1720	362.1620	3.576	+	√	ND	ND
TDP 17	369.0722	370.0790	3.498	+	√	ND	ND

Compound ID	Mass	*m/z*	RT	ESI[b] +/-	Model		
					Spiked water	Spiked strawberries	Incurred strawberries
TDP 18	375.1328	376.1391	3.922	+	√	ND	ND
TDP 19	389.1012	390.1081	3.527	+/-	√	ND	ND
TDP 20	393.0066	394.1385	3.271	+	ND	√	ND
TDP 21	405.1435	406.1503	3.502	+	√	ND	ND
TDP 22[a]	407.1118	408.1196	3.542	+	√	ND	ND
TDP 23	419.1118	420.1196	3.428	+	ND	√	ND
TDP 24	421.1273	422.1339	3.831	+/-	√	ND	ND
TDP 25	433.0650	434.0714	3.526	+	√	ND	ND
TDP 26	443.1086	444.1154	3.809	+	√	ND	ND
TDP 27	447.1543	448.1601	3.527	+	√	ND	ND
TDP 28	457.0887	456.0813	3.525	-	√	ND	ND
TDP 29	479.1795	480.1860	3.836	+	√	ND	ND
TDP 30	681.3000	682.3039	3.801	+	ND	√	ND
TDP 31	246.0641	247.0719	3.624	+	ND	ND	√
TDP 32	306.0866	307.0931	3.175	+	ND	ND	√

[a] TDP 22 was not detected in $nSiO_2$–AZOX water model. The other peaks were detected in all three formulations (conventional, Allosperse® and $nSiO_2$)

[b] ESI: Electrospray ionization

*The thermal degradation products were only detected in the samples treated with pesticides and were not present in either unheated samples nor heated control samples.

Table 6. 3 List of thermal degradation products (TDPs) tentatively identified (based on PCDL[a] library and MS/MS[b] spectra) in the water (10 µg/mL) and/or the spiked strawberries (1 µg/mg) and/or the incurred strawberries (around 10 µg/kg) after heating 4 hours at 100°C.

Compound[c] (Manufacturer code[d])	ID in this study	Model	Formula	Neutral mass	RT	Precursor ions (*m/z*) ESI+	Main fragment ions (*m/z*) ESI+	Reference
Azoxystrobin	-	water, spiked and incurred strawberry	$C_{22}H_{17}N_3O_5$	403.1169	3.738	404.12467	372.0983	36
Azoxystrobin compound 2 (R234886)	TDP 19	water	$C_{21}H_{15}N_3O_5$	389.1012	3.499	390.10902	372.0981	33
Azoxystrobin compound 3 (R219277)	TDP 6	water and spiked strawberry	$C_{15}H_{14}N_2O_5$	302.0903	2.675	303.09813	-	38
Azoxystrobin compound 18 (R176586)	TDP 1	spiked strawberry	$C_{11}H_{12}O_4$	208.0734	2.906	209.0814	-	32
Azoxystrobin compound 20 (R402173)	TDP 12	water	$C_{19}H_{13}N_3O_4$	347.0906	3.469	348.09845	-	32
Azoxystrobin compound 21	TDP 15	water	$C_{20}H_{15}N_3O_4$	361.1073	3.783	362.1141	-	37
Azoxystrobin compound 26 (R401487)	TDP 31	incurred strawberry	$C_{12}H_{10}N_2O_4$	246.0641	3.624	247.0719	-	32
Azoxystrobin compound 28 (R401553)	TDP 2	water and spiked strawberry	$C_{11}H_7N_3O_2$	213.0538	2.44	214.0617	-	37
Azoxystrobin compound 36 (R403314)	TDP 22	water	$C_{21}H_{17}N_3O_6$	407.1118	3.542	408.11959	348.0982	33,36,37

Compound[c] (Manufacturer code[d])	ID in this study	Model	Formula	Neutral mass	RT	Precursor ions (*m/z*) ESI+	Main fragment ions (*m/z*) ESI+	Reference
Azoxystrobin compound New M4	TDP 32	incurred strawberry	$C_{14}H_{14}N_2O_6$	306.0866	3.175	307.0931	-	33
Azoxystrobin compound 22	TDP 23	spiked strawberry	$C_{22}H_{17}N_3O_6$	419.1118	3.428	420.1196	-	32, 33
Azoxystrobin compound 23								32, 33
Azoxystrobin compound U13							-	32

[a] PCDL: A metabolite library made by Agilent Masshunter PCDL software
[b] MS/MS: Tandem mass spectrometry
[c] The compound: number and letters were commonly used in the literature, except the "new M4", which is found in the study of Gautam, Etzerodt & Fomsgaard (2017).
[d] Manufacturer codes of azoxystrobin metabolites were usually used as compounds ID in the literature.

6.4.3.1 Thermal degradation products in heated water

LC/MS total ion chromatograms (TICs) were obtained in full scan mode (50-750 *m/z*) for all formulations (conventional, Allosperse and $nSiO_2$). As an example, the TICs for AZOX in the $nSiO_2$ formulation (water, 10 µg/mL) before and after heating (100°C, 4 hours) are compared in Figure 6.3. As expected, the peak corresponding to AZOX decreased after 4 hours of heating. Several relatively large new peaks were observed after heating in both positive (Figure 6.3a) and negative modes (Figure 6.3b). These peaks were TDP 7, 11 and 19 (*neutral mass* 303.101, 329.0802 and 389.1012, respectively), which could be detected in both ESI- and ESI+ modes.

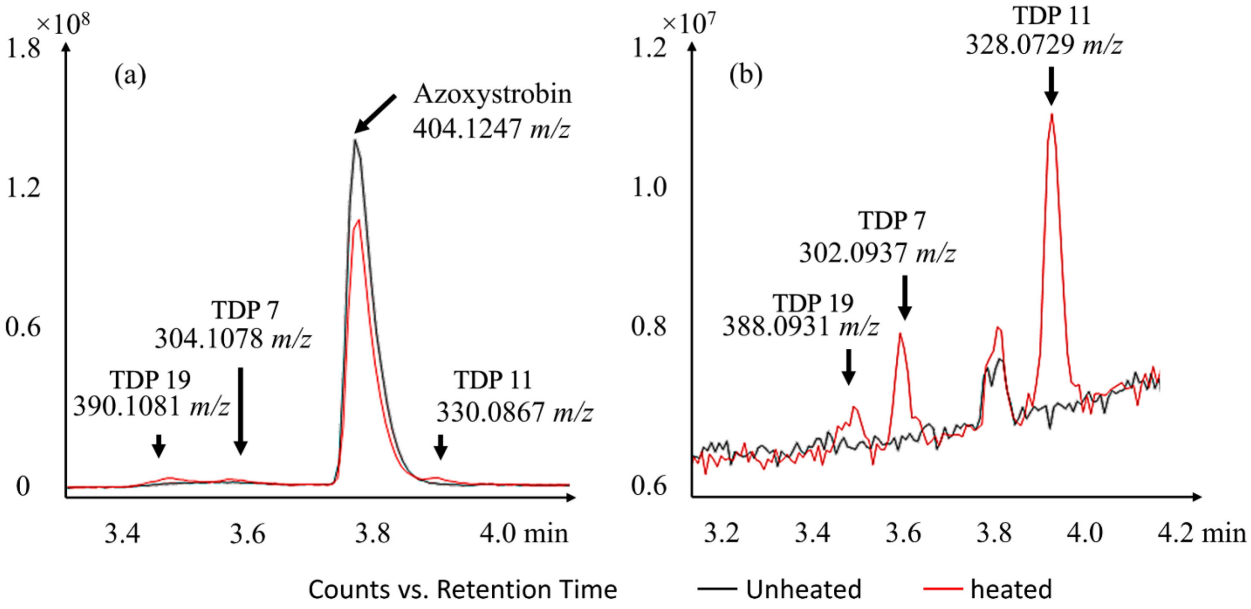

Figure 6. 3 Total Ion Chromatograms (overlap) of the azoxystrobin in $nSiO_2$ formulation in water (10 µg/mL) before and after heating for 4 hours (a: ESI+ and b: ESI-).

Beside the major degradation products of AZOX in water presented in Figure 6.3, minor degradation products, not directly visible in TICs are listed in Table 6.2. A total of 23 suspected TDPs were detected in the water. All these suspected TDPs, except TDP 22, were detected in all of the pesticide formulations (conventional, Allosperse® and $nSiO_2$) samples. The absence of TDP

22 in $nSiO_2$ samples might have been caused by the low levels of TDP 22 in the formulations, especially for the $nSiO_2$ samples, which were below the instrument detection limit. Based on the available information, the heating of nanoencapsulated AZOX did not generate new compounds compared to the conventional formulation. Moreover, six compounds (TDPs 2, 6, 12, 15, 19 and 22) in this study could be matched with substances reported the literature (Table 6.3). However, 17 other TDPs in water could not be identified due to a lack of information in the literature.

The MS/MS spectra of TDPs 19 and 22 published in the literature were matched with spectra obtained in this study (Table 6.3), with a second ion (372.0981 *m/z*) observed for AZOX TDP 19. Based on the RT (3.5 min) and MS/MS spectrum of the reference standard of AzFA, TDP 19 was confirmed to be AzFA. AzFA is a major degradation product of AZOX in the environment[39]. As it is known to be toxic to aquatic life, AzFA has been recommended for regulation in water in Denmark[40]. A fragment at 348.0982 *m/z* was recorded for TDP 22, matching with the information of R403314 reported in previous studies on the photochemical transformation of AZOX in water[33,36,37].

6.4.3.2 Thermal degradation products in the spiked strawberries

In heated spiked strawberries, nine possible TDPs (1-6, 20, 23 and 30) were detected (Table 6.2). Except for TDP 4, the other TDPs in spiked strawberry model were detected in ESI+ mode. Only two TDPs (2 and 6) were detected in both the water and spiked strawberries. For the target screening with the in-house PCDL library, four TDPs (1, 2, 6 and 23) were tentatively matched with the literature in the spiked strawberry model. Given that three degradation products of AZOX share the same formula $C_{22}H_{17}N_3O_6$, and since the literature MS/MS data were not available, the tentative identification of TDP 23 (*neutral mass* 419.1118) could not be further confirmed.

Some TDPs had a higher molar mass than the AZOX parent compound (*neutral mass* 403.388), indicating possible reactions with matrixes or other TDPs. The reactions of AZOX in water were simpler than in the food matrixes, which contain sugars, protein, etc. In environmental samples, AZOX and relevant metabolites had been found conjugated with endogenous molecules such as glucose or carboxylic or amino acids[36]. For example, TDP 2 could react with glucose to form glucosyl-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate in a plant[32]. Thus, AZOX could generate more complex TDPs in food compared to the water model.

6.4.3.3 Thermal degradation products in the heated incurred strawberries

It is important to first indicate that some degradation products of AZOX may have occured in incurred strawberries prior to thermal processing due to the metabolism or natural degradation of AZOX in the field and during storage. In the present study, once the compounds in the unheated samples were eliminated, there were no additional molecular features of interest in the heated incurred strawberries. Nonetheless, from the target screening with the PCDL library, two compounds (TDPs 31 and 32; Table 6.3) were detected in heated incurred strawberries, which were not detected in the heated control strawberry. The presence of TDP 31 and 32 may reflect some metabolism and natural degradation of AZOX in the field cultures or during storage. Although TDP 31 could be detected in both unheated and heated incurred strawberries, the peak intensity of TDP 31 in heated samples was higher than in the unheated samples, indicating the thermal degradation of AZOX to form TDP 31. All TDPs were detected across all pesticide formulations (conventional, Allosperse® and $nSiO_2$). Therefore, the nanoencapsulation of AZOX did not appear to generate new TDPs in spiked and incurred strawberry models as compared to the conventional formulation.

6.4.4 Potential degradation pathways of azoxystrobin in water

High temperatures generally accelerate the decomposition of pesticides caused by their hydrolytic degradation in water[34]. According to the tentatively identified TDPs in the previous sections, thermal degradation pathways could be proposed for AZOX (Figure 6.4). As the ether bond is unstable with heat due to a pair of lone electons on the oxygen atom, it was prone to breakage[41]. The cleavage of the ether linkages between the pyrimidinyl ring to the phenylacrylate ring and to the cyanophenyl ring of AZOX is proposed to generate TDPs 2 and 6, respectively. Oxidative o-dealkylation of AZOX could produce TDP 19, which was identified as AzFA. From the intensity of molecular ion peak in Figure 6.3, TDP 19 can be proposed as one of the major thermal products of AZOX. The cyano group (-C≡N) on the benzene ring of TDP 19 could be hydrolyzed, leading to some rearrangement reactions[42]. The cyano group may react with hydrogen ions and water molecules to form an amide group to give TDP 22. In another pathway, AZOX after demethylation, oxidation and decarboxylation would give AZOX TDP 15[10]. Then TDP 15 could also undergo demethylation to generate TDP 12.

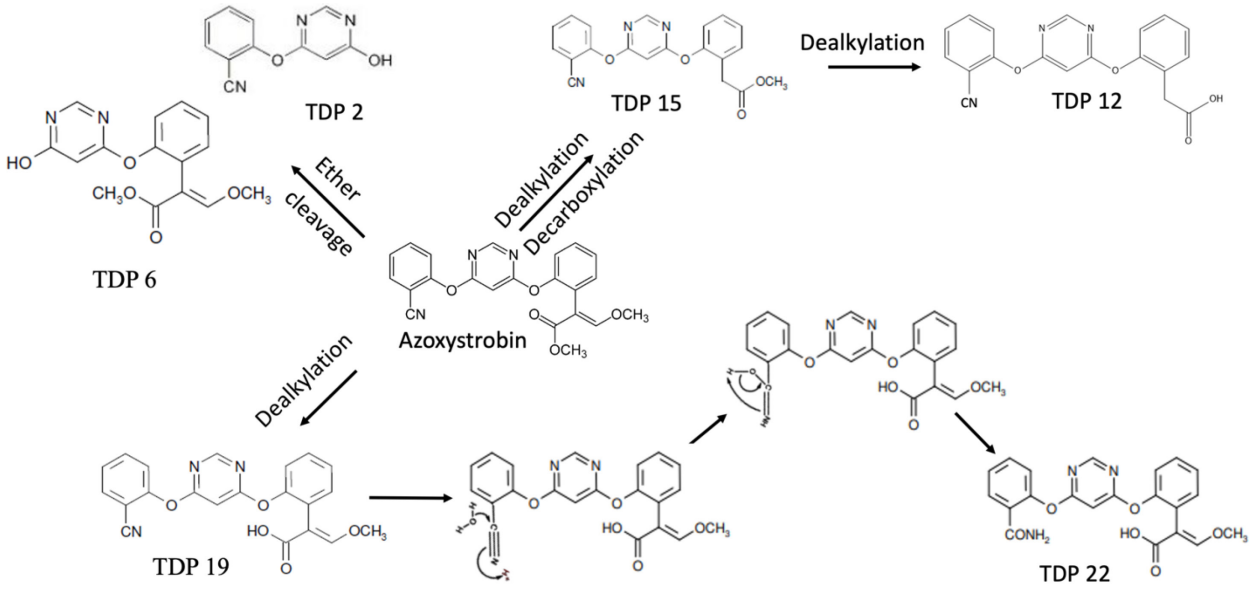

Figure 6. 4 Proposed thermal degradation pathways for azoxystrobin in water following heating for 4 hours.

This present study investigated the thermal degradation of AZOX from simple matrixes to more complex matrixes, and from laboratory control samples to 'real' samples. In conclusion, AZOX degradation for both the conventional and nanoencapsulated formulations followed first-order kinetics when heated at 100°C in the water. Different TDPs were identified in water, spiked and incurred strawberries. Nanocarriers had a slight or no impact either on the degradation rate or on the degradation product types. To the best of our knowledge, this is the first report on the TDPs of AZOX (conventional and nanoencapsulated formulations) for both water and food models. This study highlighted some knowledge gaps in our understanding of the degradation products of pesticides in the environment and during food processing. Many TDPs in water have not been reported in the literature, even some major TDPs of AZOX with relatively high intensity (e.g. TDPs 7 and 11, Figure 6.3). Toxicity studies usually focus on the parent azoxystrobin compound, and little toxicological information is available for its metabolites[43]. Therefore, further identification and toxicity studies of the unknown degradation products are necessary to fully assess the health risk which may be associated with the degradation products of AZOX.

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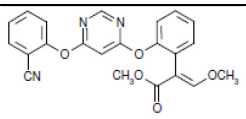
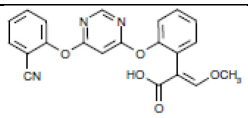
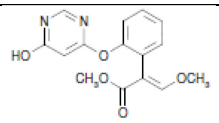
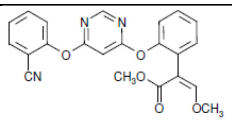
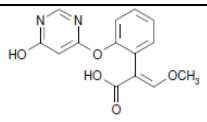
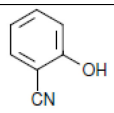
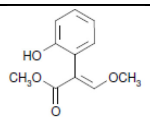
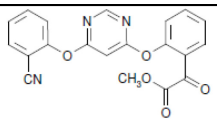
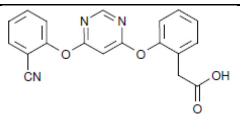
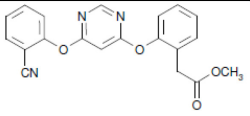
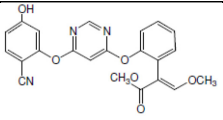
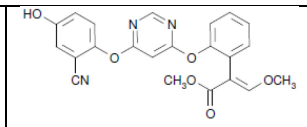
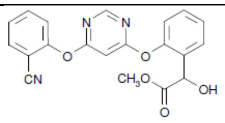
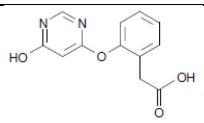
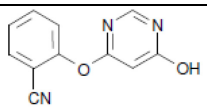
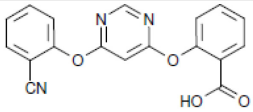
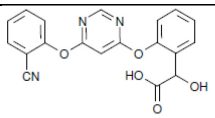
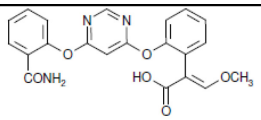
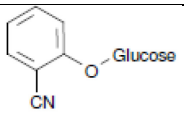
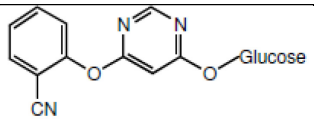
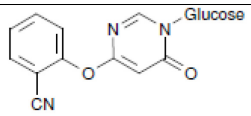
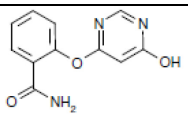
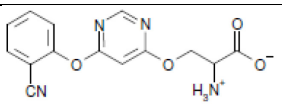
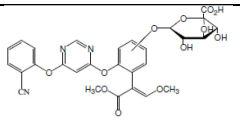
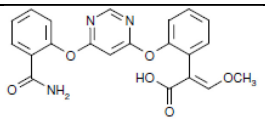
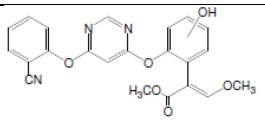
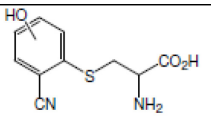
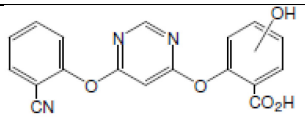
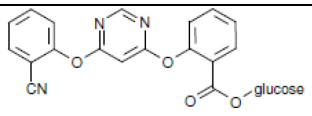
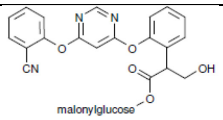
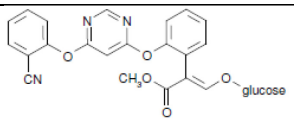
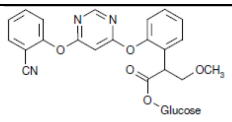
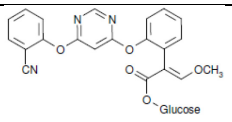
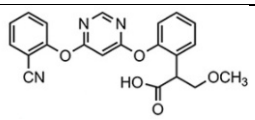
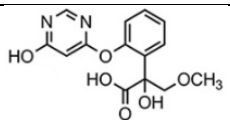
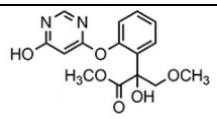
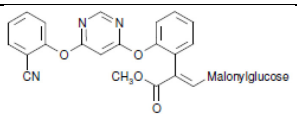
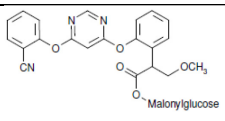
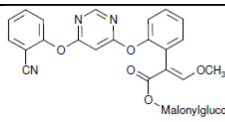
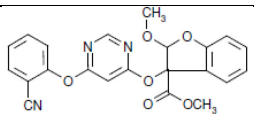
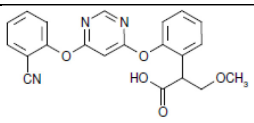
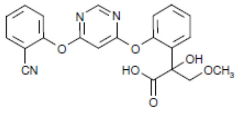
6.8 Supplementary information

Table S6. 1 Azoxystrobin metabolites and degradation products (including identification number or letter, manufacturer code number, formula, *m/z* and structure) in the environment reported in the literature.

Compound[a]	Manufacturer code[b]	Formula	Neutral mass	Structure	Reference
Compound 01 (azoxystrobin)	ICIA5504	$C_{22}H_{17}N_3O_5$	403.1168		(FAO, 2009)
Compound 02 (azoxystrobin free acid)	R234886	$C_{21}H_{15}N_3O_5$	389.1012		(FAO, 2009)
Compound 03	R219227	$C_{15}H_{14}N_2O_5$	302.0903		(FAO, 2009)
Compound 09	R230310	$C_{22}H_{17}N_3O_5$	403.1168		(FAO, 2009)
Compound 10	R232493	$C_{14}H_{12}N_2O_6$	304.0695		(FAO, 2009)
Compound 13	R71395	C_7H_5NO	119.0371		(FAO, 2009)
Compound 18	R176586	$C_{11}H_{12}O_4$	208.0736		(FAO, 2009)
Compound 19	R230309	$C_{20}H_{13}N_3O_5$	375.0855		(FAO, 2009)
Compound 20	R400050	$C_{19}H_{13}N_3O_4$	347.0906		(FAO, 2009)
Compound 21	R400051	$C_{20}H_{15}N_3O_4$	361.1063		(FAO, 2009)
Compound 22	R400297	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)

Compound 23	R400299	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)
Compound 24	R400753	$C_{20}H_{15}N_3O_5$	377.1012		(FAO, 2009)
Compound 26	R401487	$C_{12}H_{10}N_2O_4$	246.0641		(FAO, 2009)
Compound 28	R401553	$C_{11}H_7N_3O_2$	213.0538		(FAO, 2009)
Compound 30	R402173	$C_{18}H_{11}N_3O_4$	333.0750		(FAO, 2009)
Compound 35/U3	R402987	$C_{19}H_{13}N_3O_5$	363.0855		(FAO, 2009)
Compound 36	R403314	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)
Compound 40	R405270	$C_{13}H_{15}NO_7$	297.0849		(FAO, 2009)
Compound 41	-	$C_{17}H_{17}N_3O_8$	391.1016		(FAO, 2009)
Compound 42	R405287	$C_{17}H_{15}N_3O_8$	389.0859		(FAO, 2009)
Compound C	-	$C_{11}H_9N_3O_3$	231.0644		(FAO, 2009)
Compound G2	-	$C_{14}H_{12}N_4O_4$	300.0859		(FAO, 2009)
Compound K1	-	$C_{29}H_{29}N_3O_{12}$	611.1751		(FAO, 2009)
Compound K2	-	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)

Compound L1	-	$C_{23}H_{21}N_3O_6$	435.1430		(FAO, 2009)
Compound L4	-	$C_{11}H_{14}N_2O_3S$	254.0725		(FAO, 2009)
Compound L9	-	$C_{19}H_{15}N_3O_5$	365.1012		(FAO, 2009)
Compound M1	-	$C_{24}H_{21}N_3O_9$	495.1278		(FAO, 2009)
Compound M2	-	$C_{29}H_{27}N_3O_{13}$	625.1544		(FAO, 2009)
Compound M3	-	$C_{27}H_{25}N_3O_{10}$	551.1540		(FAO, 2009)
Compound N1	-	$C_{27}H_{27}N_3O_{10}$	553.1696		(FAO, 2009)
Compound N2	-	$C_{27}H_{25}N_3O_{10}$	551.1540		(FAO, 2009)
Compound New M3	-	$C_{21}H_{17}N_3O_5$	391.1168		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound New M4	-	$C_{14}H_{14}N_2O_6$	306.0852		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound New M6	-	$C_{15}H_{16}N_2O_6$	320.1008		(Gautam, Etzerodt & Fomsgaard, 2017)
Compound O1	-	$C_{30}H_{27}N_3O_{13}$	637.1544		(FAO, 2009)
Compound O2	-	$C_{30}H_{29}N_3O_{13}$	639.1700		(FAO, 2009)
Compound O3	-	$C_{30}H_{29}N_3O_{13}$	639.1700		(FAO, 2009)

Compound U13	-	$C_{22}H_{17}N_3O_6$	419.1117		(FAO, 2009)
Compound U5	-	$C_{21}H_{17}N_3O_5$	391.1168		(FAO, 2009)
Compound U6	-	$C_{21}H_{17}N_3O_6$	407.1117		(FAO, 2009)

[a]The compound: number and letters were commonly used in the literature, except the "new M3, M4, and M6", which is found in the study of Gautam, Etzerodt & Fomsgaard (2017).
[b]Manufacturer codes of azoxystrobin metabolites were usually used as compounds ID in the literature.

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Chapter 7. General Conclusions

7.1 Conclusions

This research developed an analytical strategy to investigate nanoencapsulated pesticides in strawberry plants and compared the fate from field to fork and the potential effects on the plant (phenology parameters and phenolic compounds) between conventional and NEPs. A non-targeted analytical method was used to detect the TDPs of NEPs in strawberry fruits during thermal processing.

The existing analytical method for conventional pesticides showed inappropriate performances for NEPs. Analytical methods were developed and validated to specifically extract NEPs from food and environmental samples. Increasing the extraction solvent volume and extraction time successfully improved the recoveries of the pesticide active ingredients. The validated methods were then effectively applied to quantify NEPs at trace level in different matrixes. To the best of our knowledge, this is the first paper reporting the development and validation of the analytical method for NEPs. The specific validated method increases the possibilities for future research on NEPs.

A controlled strawberry agricultural system was conducted for two years applied with the conventional and NEPs (Allosperse® and $nSiO_2$) for AZOX and BFT. The pesticide residues in leached water, soil, strawberries, leaves and roots were investigated using validated analytical methods. Allosperse®-AZOX was somehow more mobile and preferred leached out of the soil when compared to the other formulations. Based on limited evidence, nanocarriers might be reducing the bioaccessibility of AZOX. Then, a comparative evaluation of phenolic contents and profiles in strawberry fruits treated with NEPs and conventional pesticides were then performed. There were small but significant differences of total phenolic contents and profiles among pesticide

formulations (e.g., conventional, Allosperse® and $nSiO_2$) for AZOX and BFT, respectively. The impact of NEPs on each individual phenolic compound level in strawberries were not consistent when compared with the conventional pesticides. Even though the effects of NEPs, under recommended usage, on the strawberry plant phenological parameters were not observed, NEPs had some significant impact on the plant metabolism.

Finally, this research is the first to investigate the effect of thermal processing on the degradation of NEPs in food. The thermal degradation of azoxystrobin was explored in three models: water, spiked strawberry and incurred strawberry. The thermal degradation followed the first-order kinetics when heated at 100°C in the water model. The percentages of nanoformulation AZOX thermal degradation in strawberry models was comparable or lower than the conventional formulation, due to the nanocarriers protecting the pesticide active ingredient from hydrolytic degradation. Based on the observed degradation products, the thermal degradation reactions of AZOX were different in the water and strawberry models. TDPs were the same for both conventional and nanoformulations in each specific model, except for one potentially minor TDP (TDP 22) absent for the $nSiO_2$ formulation. Therefore, overall, nanoencapsulation of AZOX did not generate new TDPs.

Overall, this research demonstrated that encapsulation of a pesticide into a nanocarrier can result in small but measurable changes in the fate and behavior of the active ingredient. The most notable change impacted the behavior of the target pesticide during their extraction and quantification with method developed for conventional pesticides. Thus, the existing risk assessment of conventional pesticides is incomplete for NEPs. This research provides new tools for the assessment of NEPs, and contributes to a better assessment of the risk associated with this new technology. Limited effects on plants and soil microorganisms were observed in a realistic

experiment conducted under controlled conditions. This research also provides some knowledge of the thermal degradation of AZOX (conventional and nanoencapsulated formulation) in food.

7.2 Scientific contributions

The key novel aspects and contributions of this research were:

- This research includes the first development of analytical approaches validated for NEPs in multiple matrixes including plants (leaves, roots and fruits) and agricultural soils. This research demonstrated that, because the physicochemical properties of BFT and AZOX are modified, the development and the revalidation of method specific to NEPs is required.
- This project included a unique controlled exposure experiment conducted in a strawberry field and using dosages of pesticides aligned with actual application guidelines, which provided key data on the fate and behaviour of NEPs under realistic conditions.
- In particular, the investigation of the effects of nanopesticides on the quantity and quality of strawberry fruits allowed for a more comprehensive understanding of the effects of the NEPs in the ecosystems.
- This is the first study of the thermal degradation kinetics of AZOX, but most importantly, the first step in the understanding of the behaviour of NEPs during thermal processing. TDPs of AZOX in both conventional and nanoformulations were identified for the first time in processed food.

7.3 Recommendations for future research

Based on the results in this thesis, some recommendations for future research were presented as follow:

- Because the physicochemical properties of BFT and AZOX are quite different, the modified procedures may be useful for efficient extractions of other pesticides in various nanocarriers and different samples. These methods are required for a more complete understanding of the effects of the nanopesticides on agri-food systems.
- Although minimal or no effects of the nanopesticides were observed to the plant growth, our findings demonstrate that encapsulation into different nanocarriers might lead to subtle changes in the behavior of the AIs, which may lead to environmental and plant consequences. Further research could investigate the ecotoxicity, mobility and effects of encapsulated nanopesticides in non-target organisms, agricultural management practices, and presence of secondary contaminants such as nanocarriers on a field scale.
- NEPs generated different total phenolic content and certain individual phenolic profiles compared with conventional pesticides. Further research is significant and necessary to clarify the effect of NEPs on the phenolic synthesis mechanisms and degradation reactions in plants.
- Many TDPs of AZOX detected in the water model have not been identified. Moreover, some TDPs had a relatively higher molecular weight than the parent AZOX compound, indicating possible reactions with matrix components or other TDPs. Current knowledge on the reactions of AZOX in food matrixes is limited, and further studies about these unknown TDPs would improve risk assessments for AZOX.

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