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

Juliana A. Galhardi[1,2]*, Peiying Wang[3]*, Vinicius Bueno[4], Subhasis Ghoshal[4], Valérie Gravel[5], Kevin J. Wilkinson[2], Stéphane Bayen[3¥]

[1] Institute of Science and Technology, Laboratory of Environmental Nanotechnology, São Paulo State University (UNESP), 18087-180 Sorocaba, SP, Brazil

[2] Dept. of Chemistry, Université de Montréal, 1375 Ave. Thérèse-Lavoie-Roux, Montréal, QC, Canada

[3] Department of Food Science and Agricultural Chemistry, McGill University, Ste-Anne-de-Bellevue, QC, Canada

[4] Department of Civil Engineering, McGill University, H3A 0C3 Montreal, QC, Canada

[5] Department of Plant Science, McGill University, Ste-Anne-de-Bellevue, QC, Canada

*co-first authors

Accepted for publication: *Environ. Sci.: Nano***,** 2022**,** 9, 3833-3843; DOI: 10.1039/D2EN00329E

[¥] Corresponding author current address and email:

Department of Food Science and Agricultural Chemistry

McGill University, 21111 Lakeshore, Ste-Anne-de-Bellevue

Quebec, Canada, H9X 3V9

Email: stephane.bayen@mcgill.ca

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. None of the pesticide formulations appeared to systematically affect soil enzyme activity. 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.

1. Introduction

Synthetic nanoparticles (NPs, ≤100 nm particle size) are increasingly incorporated into products and applications in agriculture (1, 2). 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 (3, 4). 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 (5, 6). 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 (7).

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 (8, 9). 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; (10)) addition to agricultural soils is likely to alter the biogeochemical cycling of trace elements and organic substances in soils (3). Some early studies indicated that Ag NPs could perturb soil nutrient cycling (11), while $Cu(OH)_2$ nanopesticides have been shown to affect microbial diversity (6). Indeed, nanopesticides were postulated to have a higher bioavailability when compared to their conventional forms (6).

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 (12). As both AZOX and BFT may have some impacts on soils (e.g. bacterial communities, (13); (14)) or may be toxic to aquatic organisms (5), 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 (2). 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 (1). In another study, AZOX-loaded mesoporous silica NPs exhibited better fungicidal activity than AZOX alone (15). Although the beneficial effects of silica for plants are well established (16), $nSiO_2$ have been shown to exhibit acute toxic effects *in vivo* (17) and to affect plant biomass and nutrient content (18).

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 (19). 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 (20) and since pesticides and fungicides are commonly applied to the production of this fruit (21).

2. Material and methods

2.1 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. (22). Particles sizes were previously characterized by Diaz et al. (23) (Allosperse® - 7 nm), Kah et al. (3) (Allosperse®-BFT – 333 to 424 nm), Zhang et al. (2) (Allosperse®-AZOX - <100 nm), and Bueno and Ghoshal (24) ($nSiO_2$ - 258nm). $nSiO_2$ were loaded with the active ingredients to produce nanoencapsulated $nSiO_2$–AZOX (19.1 mg AZOX in 1 g $nSiO_2$) and $nSiO_2$–BFT (20.3 mg BFT in 1g $nSiO_2$). 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 (D_4-azoxystrobin and D_5-bifenthin) 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).

2.2 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 S1). 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 (25, 26). 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)** $nSiO_2$ only; **(iii)** 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 (details are provided in the Supplementary Materials, Section C), 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 D_4-AZOX and 60 μg L^{-1} of D_5-BFT. 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 S2 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.

2.3 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. (27) and summarized in the Supplementary Materials. 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}) (27). The instrumental detection limits for AZOX and BFT were 0.3 pg and 2.2 pg, respectively.

2.4 Degradation products of pesticides in samples

LC/MS data were screened for potential metabolites and degradation products of AZOX or BFT for the different matrices 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 S2 & S3), based on formulae reported in the literature (28-31). 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).

2.5 Soil enzyme activities

Extracellular enzymes in soils can be sensitive indicators of changes of soil quality and fertility (32). Soil enzyme activities: β-D-glucosidase (MUB-C), phosphomonoesterase (MUB-P), arylsulfatase (MUB-S) and leucine-aminopeptidase (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. (11), as summarized in the Supplementary Materials.

2.6 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 (33) (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) (34). 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 (35) 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 (36) trained with the Greengenes database (37).

2.7 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.

3. Results and Discussion

3.1 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 S3 and S4). For all of the formulations (conventional AI, Allosperse®-AZOX and $nSiO_2$-AZOX), m_{AZOX} were the highest from day 52 to 68, before decreasing to near background levels at day 68, whereas C_{AZOX} were highest on days 25 and 52. The decreasing C_{AZOX} from day 52 were consistent with the profile for the cumulative precipitation (Figure S5), 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., ≤ 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 (5) 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 (3), 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.

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

Concentrations of the pesticides extracted from the soils are shown in Figure 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 $C/C_{0\ BFT}$, Fig. 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 (38), and for BFT, which is 125.3 ± 13.3 days (3),

the decreasing C_{AZOX} can be attributed to chemical or enzyme degradation, assimilation by soil organisms, uptake by crops and leaching from the soil (13, 15). The observed decrease of C_{BFT} with time is likely related to chemical or enzymatic degradation and assimilation by soil organisms (1, 3).

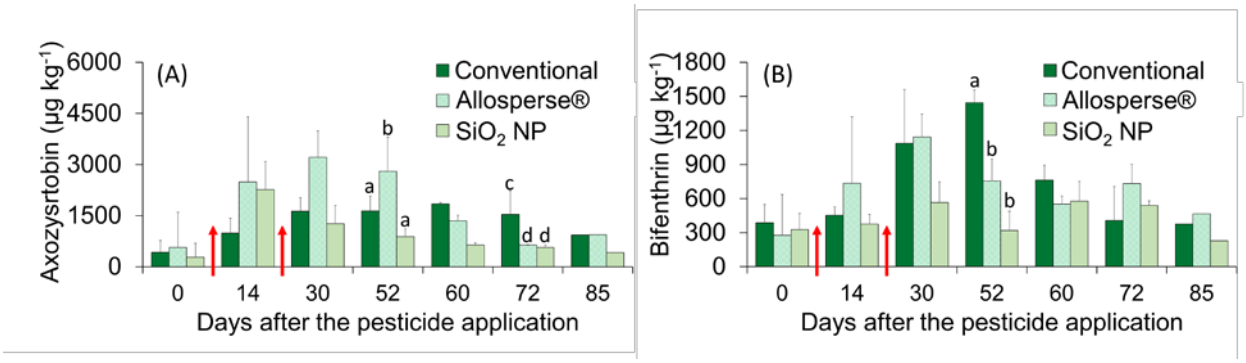

Figure 1 Concentrations of AZOX (A) and BFT (B) (conventional and nano forms) in the soils in the second experimental year as a function of time following the application of the pesticide formulations. Red arrows indicate when the addition of the treatments to the soils occurred (days 0 and 14). For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

The nanoformulations appeared to impact the mobility of the AIs in the soil. 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 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 S6). 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 could be more mobile and less associated with the soil. For the BFT, these results appear to contrast with Kah et al. (3), 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.

3.3 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 2 for several exposure times. Although AZOX levels were significantly lower on day 21 in the $nSiO_2$-AZOX exposures (Fig. 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 (Fig. 2B). Nonetheless, this contrasted with year 1 data, which showed a higher BF for the Allosphere® in comparison to the other treatments (Figure S8B).

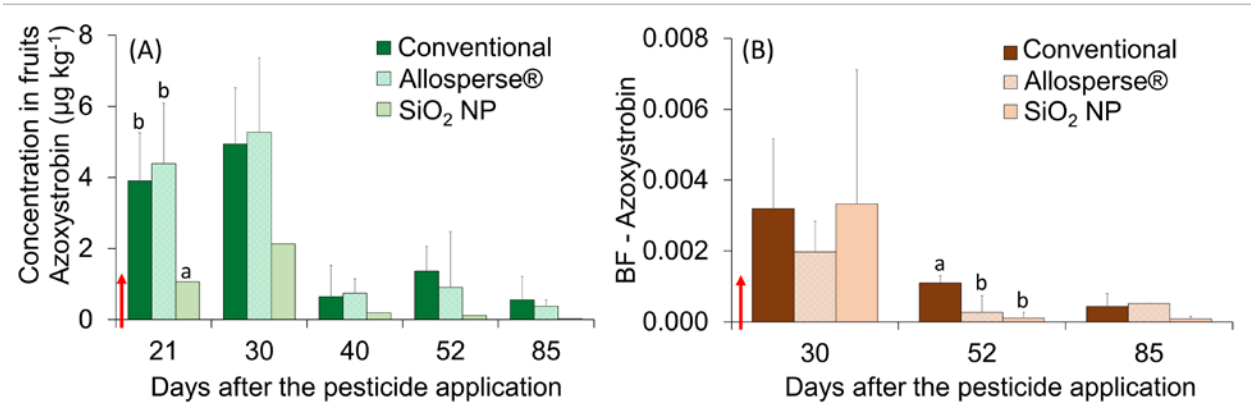

Figure 2 Concentration of azoxystrobin (conventional and nanoencapsulated) in the fruits (A) and the calculated bioaccumulation factors (B; BF = concentration in the fruits divided by the concentration in the soils) for AZOX following different exposition times (days) beginning at the first dosage application. 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 (Fisher's least significant 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 2A), resulting in larger BFs at the beginning of the growing season (Figure 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 is the major metabolite of AZOX. It was detected in the roots on the last sampling day, which is consistent with AZOX being metabolized by the plants (Figure S9). No significant differences in the concentrations of the free acid were observed when comparing 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 higher for the conventional AZOX ($p<0.05$) than for the nanopesticides (Figure 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 (39).

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 S10, Figure S11). 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 (40). 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 (41). 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 S1), 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 S12a,b), number of flowers and leaves (Figure S12c), or biomass (plant without fruits) (Figure S12d). This contrasts somewhat to results of Bueno et al. (39), 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.

3.4 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 (6). 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 3) were observed, generally in the first day after pesticide application.

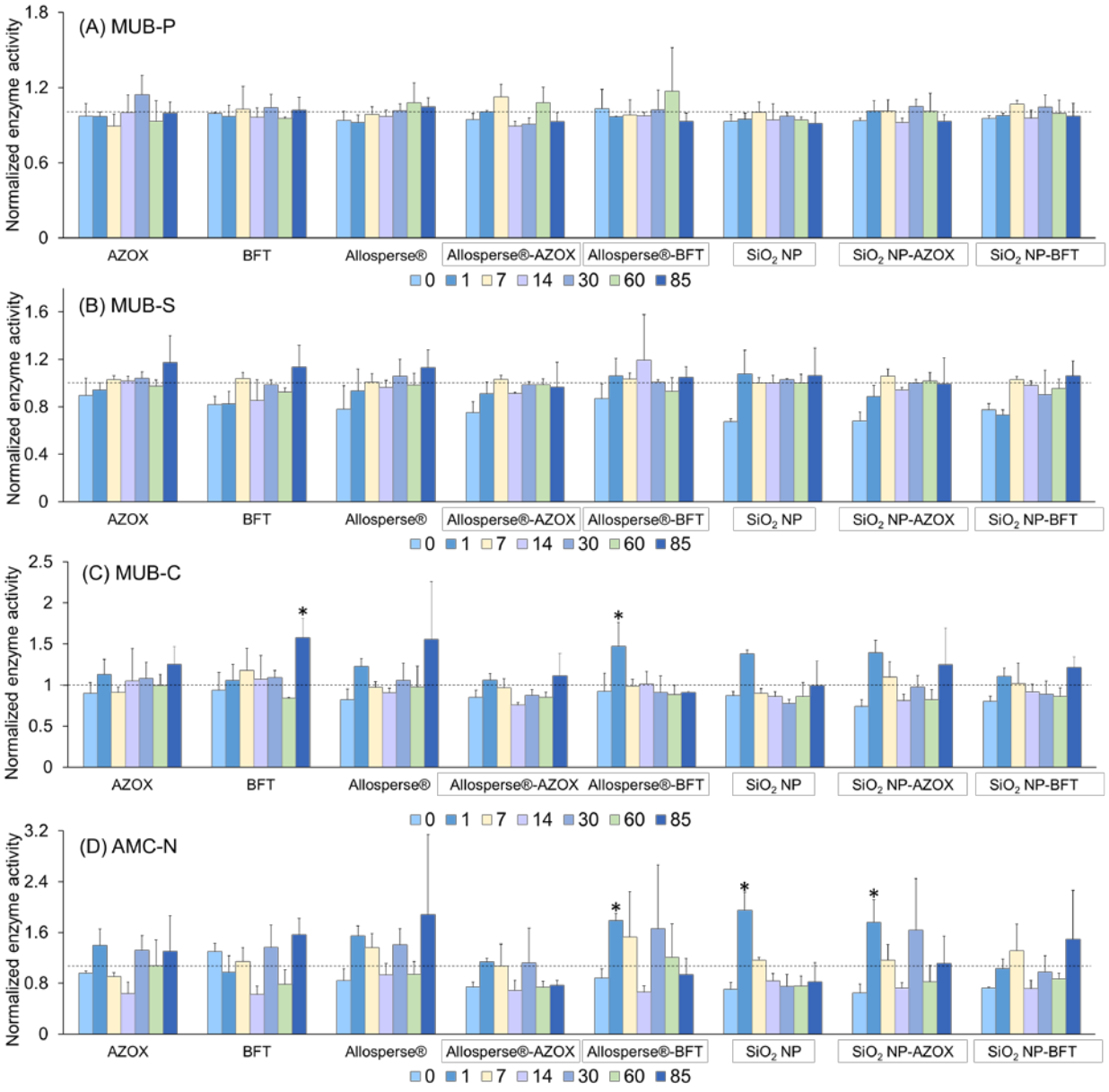

Figure 3 Soil enzyme activities for β-D-glucosidase (MUB-C), phosphomonoesterase (MUB-P), arylsulfatase (MUB-S) and leucine-aminopeptidase (AMC-N) in soils treated with the different pesticide formulations in the second experimental year. Sampling occurred at different times (days) following the first 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 (42), whereas leucine aminopeptidase is a hydrolyze involved in the acquisition of nitrogen by microorganisms by cleaving N-terminal residues from proteins and peptides (43). 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 (42). 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 (44). 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 (45), 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 (46, 47). For example, under controlled laboratory conditions, AZOX had an inhibitory effect on MUB-P and indicated risks to living organisms (48).

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 (3). 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 (32). 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.

3.5 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 S13), 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 (49). Nonetheless, some small subtle changes occurred when comparing data obtained following the first and second experimental years (Figures 4 and S14). The most noticeable change in the first experimental year was the increase of *Acidobacteria* and the decrease of *Crenarchaeota* (the only large *Archea* group) and *Actinobacteria* at day 85 (with respect to day 0), especially for the control, AZOX, Allosperse®-BFT and $nSiO_2$ (Fig. S14). Similar results were observed in year 2 (Fig. 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.

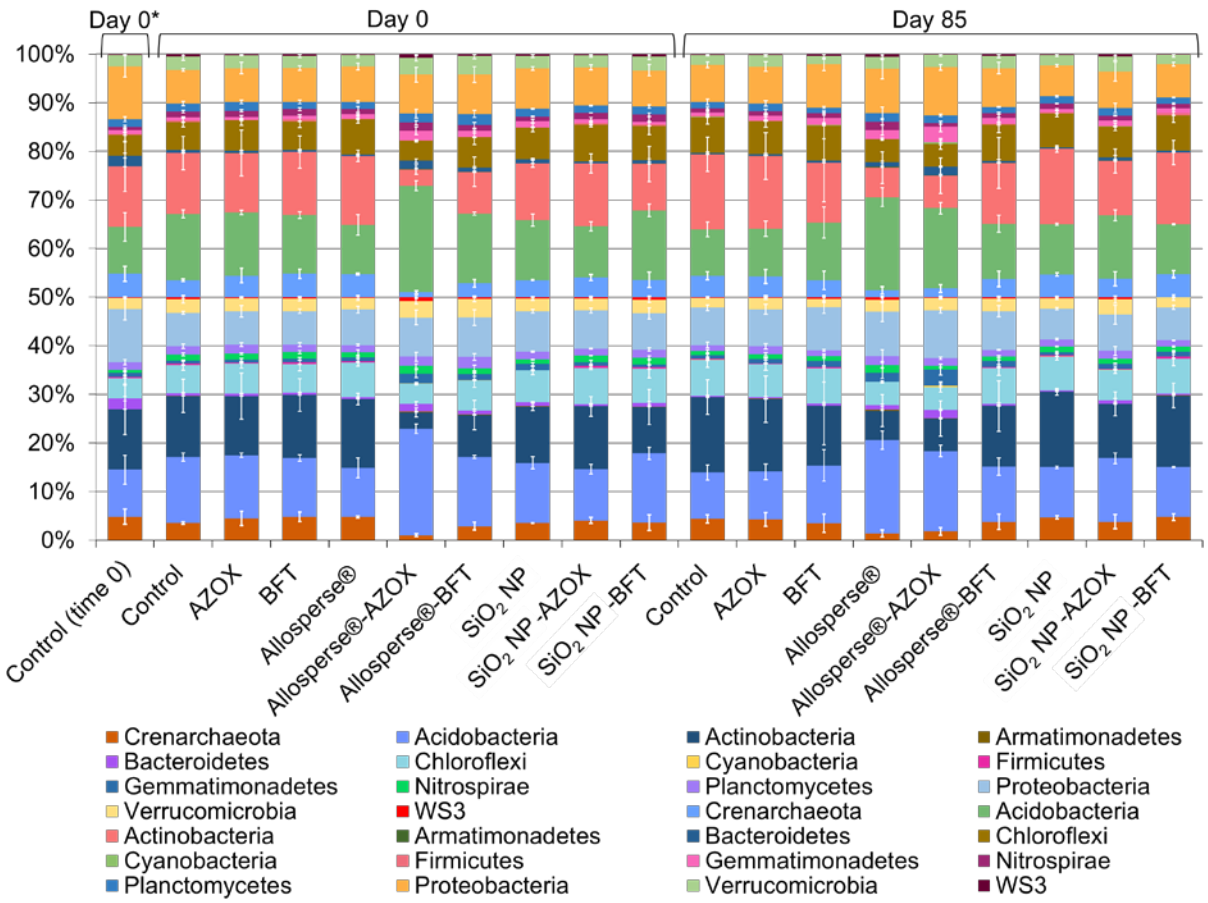

Figure 4 Relative abundance plot of the soil microbial community composition from the second experimental year. *Day zero of the first experimental year refers to the soils before the pesticide application. Days 0 (before pesticide application) and 85 (after pesticide application) of the second experimental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

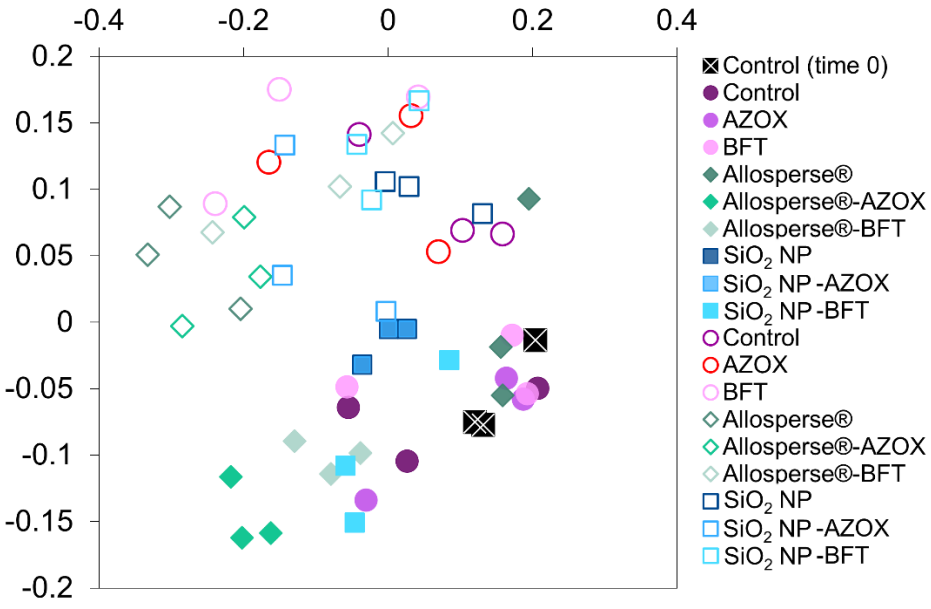

Figure 5 PCoA plot of the soil microbial community composition from the days 0 (filled symbols) and 85 (hollow symbols) of the second experimental year. Control (time zero) refers to results from the first experimental year before the pesticide application.

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 (Fig. 4). For example, following the treatment with Allosperse®-AZOX, *Crenarchaeota* decreased by 8% on day 0 and by 6% on day 85 with respect to the control. Similarly, following the treatment with Allosperse®-BFT, *Crenarchaeota* 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; (50)). Similarly, for terrestrial mesocosms, Carley et al. (49) 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 (51, 52).

A principal coordinate analysis (PCoA) obtained from the β-diversity analysis of the bacterial and archaea communities (Figure 5) showed no clear trend with respect to whether a given treatment had unique impact on microbial diversity. The values of the PCoA plot of the soil microbial community composition for SiO_2 NP-AZOX were interpolated at the same position as those of SiO_2 NPs for day 0 (filled symbols) of the second experimental year, indicating no differences among those sample groups. 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 S15).

Figure S16 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 S13, S16), which is in line with other studies using nanopesticides. Zhang et al. (2), 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. Conclusions

Even when employing the maximum concentrations of pesticides suggested by the manufacturer and fairly sensitive indicators of ecosystem stress, the use of nanocarriers to encapsulate and transport AIs did not appear to lead to any negative impacts on the strawberry plants or on the soil microbial communities and functions. When compared to conventional pesticide forms, nanoencapsulation did not appear to lead to measurable increases in environmental impacts. Concentrations of the AI in the strawberries were below the maximum permissible dose for human ingestion (53) 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.

5. Acknowledgments

This research was funded in part by the Natural Sciences and Engineering Research Council of Canada (grant nos. RGPIN-2016-05022, STPGP 506450-17) and the Fonds de Recherche du Québec - Nature et Technologies (FRQNT, grant no. 286120). We wish to acknowledge the financial support from the Canada Foundation for Innovation/John R. Evans Leaders Fund grant (Project #35318) research grants to SB. V.B. was supported in part by a McGill Engineering Doctoral Award. J.A.G. was supported by FRQNT (grant no. 255500). P.W. was supported by a Pylarcyk scholarship.

References

1. Mohd Firdaus MA, Agatz A, Hodson ME, Al-Khazrajy OSA, Boxall ABA. Fate, uptake, and distribution of nanoencapsulated pesticides in soil-earthworm systems and implications for environmental risk assessment. *Environ Toxicol Chem*. 2018;37(5):1420-9.
2. Zhang Y, Sheedy C, Nilsson D, Goss GG. Evaluation of interactive effects of UV light and nano encapsulation on the toxicity of azoxystrobin on zebrafish. *Nanotoxicology*. 2020;14(2):232-49.
3. Kah M, Weniger A-K, Hofmann T. Impacts of (nano) formulations on the fate of an insecticide in soil and consequences for environmental exposure assessment. *Environ Sci Technol*. 2016;50(20):10960-7.
4. Wang P, Lombi E, Zhao F-J, Kopittke PM. Nanotechnology: a new opportunity in plant sciences. *Trends Plant Sci*. 2016;21(8):699-712.
5. Petosa AR, Rajput F, Selvam O, Ohl C, Tufenkji N. Assessing the transport potential of polymeric nanocapsules developed for crop protection. *Water Res*. 2017;111:10-7.
6. Zhang X, Xu Z, Wu M, Qian X, Lin D, Zhang H, et al. Potential environmental risks of nanopesticides: Application of Cu (OH) 2 nanopesticides to soil mitigates the degradation of neonicotinoid thiacloprid. *Environ Int*. 2019;129:42-50.
7. Hofmann T, Lowry GV, Ghoshal S, Tufenkji N, Brambilla D, Dutcher JR, et al. Technology readiness and overcoming barriers to sustainably implement nanotechnology-enabled plant agriculture. *Nat Food*. 2020;1(7):416-25.
8. Dan Y, Zhang W, Xue R, Ma X, Stephan C, Shi H. Characterization of gold nanoparticle uptake by tomato plants using enzymatic extraction followed by single-particle inductively coupled plasma–mass spectrometry analysis. *Environ Sci Technol*. 2015;49(5):3007-14.
9. Prasad A, Astete CE, Bodoki AE, Windham M, Bodoki E, Sabliov CM. Zein nanoparticles uptake and translocation in hydroponically grown sugar cane plants. *J Agric Food Chem*. 2017;66(26):6544-51.
10. Asadishad B, Chahal S, Akbari A, Cianciarelli V, Azodi M, Ghoshal S, et al. Amendment of agricultural soil with metal nanoparticles: effects on soil enzyme activity and microbial community composition. *Environ Sci Technol*. 2018;52(4):1908-18.
11. Peyrot C, Wilkinson KJ, Desrosiers M, Sauvé S. Effects of silver nanoparticles on soil enzyme activities with and without added organic matter. *Environ Toxicol Chem*. 2014;33(1):115-25.
12. Liang Y, Gao Y, Wang W, Dong H, Tang R, Yang J, et al. Fabrication of smart stimuli-responsive mesoporous organosilica nano-vehicles for targeted pesticide delivery. *J Hazard Mater*. 2020;389:122075.
13. Wang X, Lu Z, Miller H, Liu J, Hou Z, Liang S, et al. Fungicide azoxystrobin induced changes on the soil microbiome. *Appl Soil Ecol*. 2020;145:103343.
14. Mukherjee I, Das SK, Kumar A, Shukla L. Sludge amendment affect the persistence, carbon mineralization and enzyme activity of atrazine and bifenthrin. *Bull Environ Contam Toxicol*. 2020;105(2):291-8.
15. Xu C, Cao L, Zhao P, Zhou Z, Cao C, Li F, et al. Emulsion-based synchronous pesticide encapsulation and surface modification of mesoporous silica nanoparticles with carboxymethyl chitosan for controlled azoxystrobin release. *Chem Eng J*. 2018;348:244-54.
16. Rastogi A, Tripathi DK, Yadav S, Chauhan DK, Živčák M, Ghorbanpour M, et al. Application of silicon nanoparticles in agriculture. *3 Biotech*. 2019;9(3):1-11.

17. Murugadoss S, Lison D, Godderis L, Van Den Brule S, Mast J, Brassinne F, et al. Toxicology of silica nanoparticles: an update. *Arch Toxicol*. 2017;91(9):2967-3010.
18. Le VN, Rui Y, Gui X, Li X, Liu S, Han Y. Uptake, transport, distribution and bio-effects of SiO2 nanoparticles in Bt-transgenic cotton. *J Nanobiotechnol*. 2014;12(1):1-15.
19. Walker GW, Kookana RS, Smith NE, Kah M, Doolette CL, Reeves PT, et al. Ecological risk assessment of nano-enabled pesticides: a perspective on problem formulation. *J Agric Food Chem*. 2017;66(26):6480-6.
20. Dias MI, Barros L, Morales P, Sánchez-Mata MC, Oliveira MBP, Ferreira IC. Nutritional parameters of infusions and decoctions obtained from Fragaria vesca L. roots and vegetative parts. *LWT*. 2015;62(1):32-8.
21. Warner R, Wu B-S, MacPherson S, Lefsrud M. A review of strawberry photobiology and fruit flavonoids in controlled environments. *Front Plant Sci*. 2021;12:611893.
22. Bueno V, Bosi A, Tosco T, Ghoshal S. Mobility of solid and porous hollow SiO2 nanoparticles in saturated porous media: Impacts of surface and particle structure. *J Colloid Interface Sci*. 2022;606:480-90.
23. Diaz L, Peyrot C, Wilkinson KJ. Characterization of polymeric nanomaterials using analytical ultracentrifugation. *Environ Sci Technol*. 2015;49(12):7302-9.
24. Bueno V, Ghoshal S. Self-assembled surfactant-templated synthesis of porous hollow silica nanoparticles: mechanism of formation and feasibility of post-synthesis nanoencapsulation. *Langmuir*. 2020;36(48):14633-43.
25. USEPA. Notice of Pesticide - Azoxystrobin. 2015 [Available from: https://www3.epa.gov/pesticides/chem_search/ppls/071532-00034-20151001.pdf.
26. USEPA. Notice of Pesticide - Bifenthrin. 2015 [Available from: https://www3.epa.gov/pesticides/chem_search/ppls/083529-00048-20151030.pdf.
27. Wang P, Galhardi JA, Liu L, Bueno V, Ghoshal S, Gravel V, et al. Development of an LC-MS-based method to study the fate of nanoencapsulated pesticides in soils and strawberry plant. *Talanta*. 2022;239:123093.
28. Mastovska K. Azoxystrobin (a new evaluation). Pesticide Residues in Food. *Pesticides Residues in Food 2008*. 2008;194:1-202.
29. FAO. Bifenthrin (178). 2010 [Available from: https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation10/Bifenthrin.pdf.
30. Fecko A. Environmental fate of bifenthrin. *Environ Monitoring and Pest Mgt Branch, Dept of Pesticide Regulat*. 1999;830.
31. Gautam M, Etzerodt T, Fomsgaard IS. Quantification of azoxystrobin and identification of two novel metabolites in lettuce via liquid chromatography–quadrupole-linear ion trap (QTRAP) mass spectrometry. *Int J Environ Anal Chem*. 2017;97(5):419-30.
32. Galhardi JA, Fraceto LF, Wilkinson KJ, Ghoshal S. Soil enzyme activities as an integral part of the environmental risk assessment of nanopesticides. *J Agric Food Chem*. 2020;68(32):8514-6.
33. Susan JA, José C, Janet Rettig E. PicoGreen Quantitation of DNA: Effective Evaluation of Samples Pre-or Psost-PCR. *Nucleic Acids Res*. 1996;24(13):2623-5.
34. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-7.

35. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581-3.
36. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl Environ Microbiol*. 2007;73(16):5261-7.
37. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J*. 2012;6(3):610-8.
38. Edwards PG, Murphy TM, Lydy MJ. Fate and transport of agriculturally applied fungicidal compounds, azoxystrobin and propiconazole. *Chemosphere*. 2016;146:450-7.
39. Bueno V, Wang P, Harrisson O, Bayen S, Ghoshal S. Impacts of a porous hollow silica nanoparticle-encapsulated pesticide applied to soils on plant growth and soil microbial community. *Environ Sci: Nano*. 2022;9:1476-88.
40. Ju C, Zhang H, Yao S, Dong S, Cao D, Wang F, et al. Uptake, translocation, and subcellular distribution of azoxystrobin in wheat plant (Triticum aestivum L.). *J Agric Food Chem*. 2019;67(24):6691-9.
41. Zhu F, Liu X, Cao L, Cao C, Li F, Chen C, et al. Uptake and Distribution of Fenoxanil-Loaded Mesoporous Silica Nanoparticles in Rice Plants. *Int J Mol Sci*. 2018;19(10).
42. Li B, Chen Y, Liang W-z, Mu L, Bridges WC, Jacobson AR, et al. Influence of cerium oxide nanoparticles on the soil enzyme activities in a soil-grass microcosm system. *Geoderma*. 2017;299:54-62.
43. Matsui M, Fowler JH, Walling LL. Leucine aminopeptidases: diversity in structure and function. *Biol Chem*. 2006;387:1535-44.
44. Nottingham AT, Turner BL, Chamberlain PM, Stott AW, Tanner EVJ. Priming and microbial nutrient limitation in lowland tropical forest soils of contrasting fertility. *Biogeochemistry*. 2012;111(1-3):219-37.
45. Chen H, Liu J, Li D, Xiao K, Wang K. Controls on soil arylsulfatase activity at a regional scale. *Eur J Soil Biol*. 2019;90:9-14.
46. Riah W, Laval K, Laroche-Ajzenberg E, Mougin C, Latour X, Trinsoutrot-Gattin I. Effects of pesticides on soil enzymes: a review. *Environ Chem Lett*. 2014;12(2):257-73.
47. Kwak JI, Yoon S-J, An Y-J. Long-term effects of ZnO nanoparticles on exoenzyme activities in planted soils. *Environ Eng Res*. 2017;22(2):224-9.
48. Baćmaga M, Kucharski J, Wyszkowska J. Microbial and enzymatic activity of soil contaminated with azoxystrobin. *Environ Monit Assess*. 2015;187(10):1-15.
49. Carley LN, Panchagavi R, Song X, Davenport S, Bergemann CM, McCumber AW, et al. Long-term effects of copper nanopesticides on soil and sediment community diversity in two outdoor mesocosm experiments. *Environ Sci Technol*. 2020;54(14):8878-89.
50. Seybold CA, Herrick JE, Brejda JJ. Soil resilience: a fundamental component of soil quality. *Soil Sci*. 1999;164(4):224-34.
51. Lehtovirta LE, Prosser JI, Nicol GW. Soil pH regulates the abundance and diversity of Group 1.1 c Crenarchaeota. *FEMS Microbiol Ecol*. 2009;70(3):367-76.
52. Sait M, Davis KER, Janssen PH. Effect of pH on isolation and distribution of members of subdivision 1 of the phylum Acidobacteria occurring in soil. *Appl Environ Microbiol*. 2006;72(3):1852-7.
53. Health Canada. Proposed Maximum Residue Limit PMRL2016-10, Azoxystrobin. 2016 [Available from: https://www.canada.ca/en/health-canada/services/consumer-product-

safety/pesticides-pest-management/public/consultations/proposed-maximum-residue-limit/2016/azoxystrobin-2/document.html.