Science of the Total Environment 583 (2017) 292–299

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Comparative evaluation of four biosolids formulations on the effects of triclosan on plant-arbuscular mycorrhizal fungal interactions in three crop species

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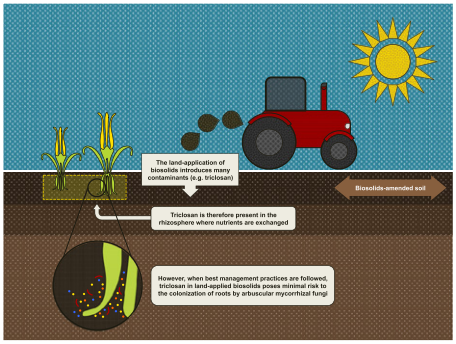
HIGHLIGHTS

- Triclosan in municipal biosolids poses minimal risk to arbuscular mycorrhizal fungi.
- No triclosan effects in three plant species grown in four formulations of biosolids.
- Amending soil with biosolids and inoculating with fungi can enhance crop growth.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 November 2016
Received in revised form 11 January 2017
Accepted 11 January 2017
Available online 16 January 2017

Editor: Jay Gan

Keywords:
Agriculture
Arbuscular mycorrhizal fungi
Biosolids
Triclosan

ABSTRACT

Triclosan (TCS) is an antimicrobial ingredient found in personal care products that include soaps, shampoos, and other sanitation goods. TCS is moderately hydrophobic and has been shown to be resistant to wastewater treatment and thus accumulates in biosolids. Biosolids are commonly applied to agricultural land but little is known about the risk that TCS in biosolids poses to soil fungal communities following land application. The purpose of this study was to characterize the effects of TCS on the symbiotic colonization of roots in three field crops (soybean, corn, and spring wheat) by arbuscular mycorrhizal fungi (AMF) in soils amended with four different types of biosolids (liquid, dewatered, composted, alkaline and hydrolyzed). Crops were grown to maturity in pot-exposure systems under controlled temperature settings. Biosolids treatments were spiked with concentrations of TCS typically found in amended fields. Analysis of AMF colonization by hyphae, and the production of arbuscules and vesicles indicated no significant TCS concentration-dependent effects in the three plant species for any of the biosolids formulations. The data indicate that TCS present in municipal biosolids applied to agricultural lands likely poses minimal risks to AMF or its establishment of a symbiotic relationship in the three species tested.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are the most widespread plant symbiont in the world (Simon et al., 1993). They serve to mitigate

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http://dx.doi.org/10.1016/j.scitotenv.2017.01.067
0048-9697/

nutrient deficiency, strengthen soil fertility, and assist hosts to develop resistance towards soil-borne plant pathogens (Jansa et al., 2006; Ortas, 2012; Pellegrino et al., 2011; Smith and Read, 2008). The use of AMF symbionts as a biofertilizer in crop systems has been encouraged for many years; inoculating with AMF continues to receive recognition as a biological tool in sustainable agriculture as it has been shown to increase productivity (Hart and Forsythe, 2012; Hart and Trevors, 2005; Hillis et al., 2008; Jansa et al., 2006; Pellegrino et al., 2011; Ortas, 2012; Prosser et al., 2015). One study revealed a 37% increase in yield due to AMF inoculation and subsequent colonization with roots of crops and pasture herbs (McGonigle, 1988). A later study on horticultural and leguminous plants revealed that AMF inoculation increased the uptake of nutrients and plant growth in fumigated and non-fumigated soils, except for field crops grown in phosphorus-rich conditions where nutrients were readily available and the support of AMF to transfer nutrients was less needed (Ortas, 2012). The application of these findings in nutrient-rich conditions such as biosolids-amended soil should be investigated in order to determine whether the synergistic role between AMF and terrestrial plants is viable.

Amending soil with biosolids enhances nutrient recycling, soil tilth, and crop productivity (Hazard et al., 2014; Prosser et al., 2015). However, biosolids contain a variety of emerging substances of concern (ESOCs), including pharmaceuticals and personal care products (PPCPs) (CCME, 2010; Miller et al., 2016; Prosser et al., 2015). While recent studies indicate that the long-term effects of PPCPs on plant health are likely small (Aryal and Reinhold, 2011; Boxall et al., 2012; Holling et al., 2012; Pannu et al., 2012; Prosser et al., 2014b; Sabourin et al., 2012; Wu et al., 2010), potential effects on plant-AMF relationships are poorly documented (Hazard et al., 2014; Prosser et al., 2015). Consequently, there remains a concern whether PPCPs in biosolids that are commonly detected in agricultural systems, such as triclosan (TCS), could adversely affect plant-AMF relationships and pose a risk to the health and development of crop species (Prosser et al., 2015).

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether CAS 3380-34-5) is an antimicrobial agent commonly used in soaps, shampoos, deodorants, and household detergents (Table 1) (Giuliano and Rybak, 2015). After use, TCS is rinsed down drains and received in wastewater treatment plants (WWTPs). Sewage waste and water received in WWTPs undergo a series of treatment processes so that the solid portion can be further stabilized into biosolids (CCME, 2012). Despite this wastewater treatment process, TCS is only partially degraded and can accumulate in biosolids (CCME, 2010).

When biosolids are applied to agricultural fields, TCS can resist degradation and movement within the rhizosphere where they are typically applied (Briggs et al., 1982; McAvoy et al., 2002; Reiss et al., 2009). The relative immobility of TCS in soil is supported by Briggs et al. (1982) who found that hydrophobic chemicals (log $K_{OW} > 4$) will adsorb to lipid-rich root structures and resist translocation from roots to shoots in plants. This corroborates a number of studies that found little uptake of TCS in plants grown in biosolids-amended soils (Al-Rajab et al., 2015; Edwards et al., 2009; Pannu et al., 2012; Prosser et al., 2014a).

Although most studies indicate negligible uptake of TCS from soil in above-or-belowground tissues, in terrestrial plants TCS in biosolids may interact with AMF activity in the rhizosphere of field crops where vital nutrients are exchanged (Prosser et al., 2015). Recent laboratory studies have suggested possible mycotoxic effects of TCS on AMF colonization in the rhizosphere of wetland plants (Twanabasu et al., 2013b). Whether the exposure pathway for wetland plants can be extrapolated to terrestrial plants is uncertain. To date, only three laboratory studies have evaluated the potential risks of TCS to field crops via plant-AMF interactions (Prosser et al., 2015; Twanabasu et al., 2013a; Twanabasu et al., 2013b).

Current literature indicates that the application of biosolids may not significantly affect plant-AMF interactions (Barbarick et al., 2004; Hazard et al., 2014; Madejón et al., 2010; Prosser et al., 2015; Sullivan et al., 2006). However, this conclusion is based on assessments using single biosolids formulations, typically dewatered biosolids, and it is uncertain if these conclusions hold for different formulations of biosolids. In this study, we assessed for the potential effects of TCS in biosolids on AMF in field crops grown in soil amended with four formulations of biosolids: liquid, dewatered, composted, and alkaline hydrolyzed. The aim of this study was to determine if four formulations of biosolids, with and without TCS at various spiked concentrations, affects the colonization (penetration into the root, and establishment of arbuscules and vesicles) of AMF in root tissues of three common crop species (soybean, corn, and spring wheat) in laboratory pot studies. Biosolids were applied to emulate current best management practices (BMPs) in the province of Ontario, Canada.

2. Materials and methods

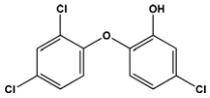
2.1. Soil and biosolids

Fallow soil was obtained from an agriculture field in Guelph, Ontario, Canada (Latitude: 43.577997, Longitude: −80.224128). The field was not treated with biosolids or pesticides for over ten years. Anaerobically digested liquid municipal biosolids (LMB), anaerobically digested dewatered municipal biosolids (DMB), compost municipal biosolids (CMB), and alkaline-hydrolyzed municipal biosolids (AMB), were obtained from various WWTPs across Canada. Samples of soil and biosolids were analyzed for physical and chemical properties and are reported in Table S1. A full analysis of the soil and biosolids properties is reported in Shahmohamadloo et al. (2016).

2.2. Experimental design

The experimental design of this study is reported in Shahmohamadloo et al. (2016) and is modified from Prosser et al. (2015). Corn (*Zea mays* var. *saccharata*), soybean (*Glycine* max), and spring wheat (*Triticum aestivum*) plants were selected for this study. Corn (Variety HZ982GT, Syngenta) seeds were planted in 4-L plastic pots (24.8 cm × 19.2 cm) (Stuewe and Sons, Tangent, OR, USA); soybean (Variety S20-Z9, Syngenta) and spring wheat (Variety 5604, Syngenta) plants were planted in 3-L plastic pots (20.3 cm × 14.3 cm) (ITML, Brantford, ON). Prior to seeding, the rate of amendment for each type of biosolids was calculated using NMAN3, a software designed by the

Table 1
Physicochemical properties of triclosan.

Compound	Application	Structure	Solubility (25 °C)	Acid/base	pKa	Log K_{OW}	$t_{1/2}$ in soil (days)
Triclosan	Antimicrobial		4.6 mg/L[a]	Weak acid	7.9[b]	4.8[c]	12.7[d]–83[e]

[a] Halden and Paull, 2005.
[b] Loftsson et al., 2005.
[c] Zhao et al., 2013.
[d] Xu et al., 2009.
[e] Cha and Cupples, 2010.

Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) (OMAF, 2012a, 2012b; Prosser et al., 2014a). The rates of amendment for each type of biosolids were based on crop type, soil properties (pH, nutrient content), biosolids properties (pH, nutrient content, concentration of metals, and pathogenic microorganisms), and field properties (soil series, soil texture, tillable area, minimum depth to bedrock, and area for material) (OMAF, 2012b). The rates of amendment, corresponding to LMB, DMB, CMB, and AMB were, respectively: 9, 15, 15, and 15 t dry weight (d.w.)/ha. LMB corresponded to 511.6, 324.1, and 324.1 g wet weight (w.w.) of biosolids per pot for corn, soybean, and spring wheat plants, respectively. DMB corresponded to 252.6, 160.0, and 160.0 g w.w. of biosolids per pot for corn, soybean, and spring wheat plants, respectively. CMB corresponded to 148.8, 94.3, and 94.3 g w.w. of biosolids per pot for corn, soybean, and spring wheat plants, respectively. AMB corresponded to 82.2, 52.1, and 52.1 g w.w. of biosolids per pot for corn, soybean, and spring wheat plants, respectively.

Biosolids treatments BS1–BS6 were spiked with TCS at varying concentrations, and left for 24 h to allow for equilibration and solvent evaporation. The solvent carrier for TCS was methanol. Treatment BS1 (biosolids only) received no TCS, with the aim of determining and testing the inherent concentration of TCS in each type of biosolids. Treatments BS2 to BS6 were spiked with TCS to produce nominal concentrations of 25,000, 75,000, 150,000, 300,000, and 600,000 ng/g d.w., respectively. Treatments BS2 to BS4 represent TCS concentrations usually detected in municipal biosolids generated in Canada and the United States (Clarke and Smith, 2011). Although treatments BS5 and BS6 are unlikely exposure scenarios, they were added in order to assess whether TCS could elicit an adverse response to plant-AMF relationships. Soil control (no biosolids and no solvent), solvent control (no biosolids plus solvent), no-AMF soil control (no biosolids), and no-AMF biosolids control treatments were also included in the experimental design. In order to mimic the tilling of biosolids into agricultural fields in Ontario, Canada, biosolids were thoroughly mixed in with soil to a depth of 15 cm in each pot for 3 min using a gloved hand (Prosser et al., 2015). After spiking and soil amendment, all treatment groups were left for 48 h to allow for the evaporation of methanol and equilibration of TCS before AMF inoculation and seeding.

To ensure close emulation of an agricultural field (both in terms of fungal species abundance and composition and seeding practices), the soil control, solvent control, and treatments BS1 to BS6 were inoculated with an AMF powder (Micronized Endomycorrhizal Inoculant, BioOrganics™, New Hope, PA, US) that contained a minimum of 10 spores/cm^3 of *Glomus aggregatum*, *Glomus etunicatum*, *Glomus intraradices*, and *Glomus mosseae*, and 2 spores/cm^3 of *Glomus clarum*, *Glomus monosporus*, *Gigaspora margarita*, and *Paraglomus brasilianum*. The inoculant was mixed approximately 8 cm deep in each pot for 3 min using a gloved hand. Following the recommended rate of application, soybean and spring wheat pots received 18.9 g of AMF inoculant, and corn pots received 25.2 g of AMF inoculant. The no-AMF controls did not receive AMF inoculant.

Corn, soybean, and spring wheat experiments received ten, five, and five replicate pots per treatment, respectively. We applied a greater number of replicates for corn compared to soybean and wheat because only a single corn plant could be grown per pot (compared to several plants for soybean and wheat) and we wanted to ensure that sufficient biomass was available to assess plant-AMF relationships. Three additional replicate pots for each treatment in all experiments were reserved for TCS analysis in the soil. All treatments in corn, soybean, and spring wheat experiments received three, seven, and eight seeds, respectively, and were sown 50 mm, 50 mm, and 30 mm deep, respectively. After seeding, corn, soybean, and spring wheat experiments were watered with 500 mL, 250 mL, and 250 mL deionized (DI) water per pot, respectively, followed by daily watering at the same amounts.

Corn experiments were conducted in a greenhouse (19 to 31 °C, 32 to 93% relative humidity), while soybean and spring wheat experiments were conducted in a growth chamber (23 ± 1 °C day, 20 ± 1 °C night, 16:8 h day: night, 60 ± 10% relative humidity, and 299 ± 87 μmol photons/m^2·s). All experiments followed a completely randomized design, and pots were randomly repositioned on the bench, once a week, using a random number table.

Corn, soybean, and spring wheat plants were grown for 85, 80, and 70 days, respectively. After plant emergence, corn, soybean, and spring wheat were randomly thinned, using a random number generator, to one, five, and five plants per pot, respectively. Percent emergence, wet root mass, wet and dry shoot mass, and shoot height of plants were measured for each treatment group. These data are presented in Shahmohamadloo et al. (2016) and are not considered further here.

2.3. Quantifying AMF colonization

At the end of each experiment, roots from five plants were randomly selected from pots in each treatment. All roots were thoroughly washed with DI water to remove soil and stored in 70% ethanol at 4 °C until staining (Brundrett et al., 1985; Prosser et al., 2015). To prepare for staining, roots were removed from 70% ethanol, thoroughly rinsed with DI water, placed in 10% KOH and autoclaved at 120 °C for 15 min. After autoclaving, roots were removed from 10% KOH, thoroughly rinsed with DI water, and placed in pure white vinegar for ≥1 h at 23 °C. Roots were then removed from vinegar, thoroughly rinsed with DI water, and placed in 0.03% chlorazol black E solution (e.g., 0.03 g in 100 mL of 1:1:1 ratio of glycerol, 80% lactic acid, water) for 20 h at 23 °C. Roots were removed from chlorazol black E solution, thoroughly washed and stored in 1:1 DI water and glycerol solution at 4 °C for a minimum of 3 days before being mounted.

Stained roots were randomly selected from each treatment and mounted horizontally on microscope slides using light white corn syrup as the medium. A single 24 × 50 mm cover slip was used to cover the roots on each slide. Ten root segments were placed on each slide; three slides were made for each treatment. Slides were placed in the dark at 23 °C for ≥3 days. The method developed by McGonigle et al. (1990) was used for quantifying the colonization of roots by AMF. Intersections of roots were analyzed for hyphae, arbuscules, and vesicles using a 200× magnification compound microscope. Total percent colonization of roots by hyphae, and total percent production by presence of arbuscules and vesicles, were scored for a total of 100 intersections observed in each replicate.

2.4. Sample preparation and TCS chemical analysis

Replicates from treatment BS1 were randomly sampled at the end of each experiment in triplicate. Samples from each experiment were submitted to the Ministry of the Environment and Climate Change (MOECC), Ontario, Canada, so that the concentrations of a suite of contaminants that were measured in each formulation of biosolids could be determined. These values are reported in the supplementary information of Shahmohamadloo et al. (2016). Techniques used to prepare soil and plant samples for chemical analysis, and the analysis of chemical residues, metals, nutrients, and cations are described in Shahmohamadloo et al. (2016).

The nominal and measured concentrations of TCS in biosolids-amended soil at the beginning and end of the experiments were determined for spring wheat and corn, and are presented in full detail by Shahmohamadloo et al. (2016). In all cases, the values presented are based on measured concentrations. The percent difference

between nominal and measured concentrations for the TCS in treatments BS2 to BS6 for corn tests were: 40.2–83.2% for LMB; 31.0–96.4% for DMB; 51.0–97.2% for CMB; 79.4–94.3% for AMB. The percent difference between nominal and measured concentrations for TCS in treatments BS2 to BS6 for spring wheat tests were: 36.4–82.9% for LMB; 45.5–65.4% for DMB; 44.8–78.1% for CMB; 35.8–95.6% for AMB.

2.5. Data analysis

Data was tested for normality using the Shapiro-Wilk test, and equal variance using the Levene's test. When normality was met, a one-way ANOVA ($\alpha = 0.05$) was performed to evaluate if there was a significant difference in percent colonization by presence of hyphae, and total percent production by presence of arbuscules, and vesicles among treatments. A Kruskal-Wallis one-way ANOVA on ranks ($\alpha = 0.05$) was performed when normality was not met. If a significant difference between treatments was identified by the ANOVA, a post hoc Tukey's test ($\alpha = 0.05$) was performed to compare all treatment means. Linear regression ($\alpha = 0.05$) was performed to model the relationship between the colonization by AMF and concentration of TCS. Statistical analysis was performed using Sigma Stat (Version 3.5, Systat Software, San Jose, CA, US).

3. Results

3.1. Zea mays var. saccharata

TCS had no significant effect ($p > 0.05$) on total percent colonization of roots by hyphae, and total percent production of arbuscules or vesicles in corn, as indicated by the absence of a concentration-response relationship across treatments (Fig. 1; Supplemental Data, Figs. S1 and S2).

Biosolids-amendment did not adversely impact colonization by AMF. Accounting for all endpoints, percent colonization was greatest between roots and hyphae, ranging from 30 to 50%, followed by percent

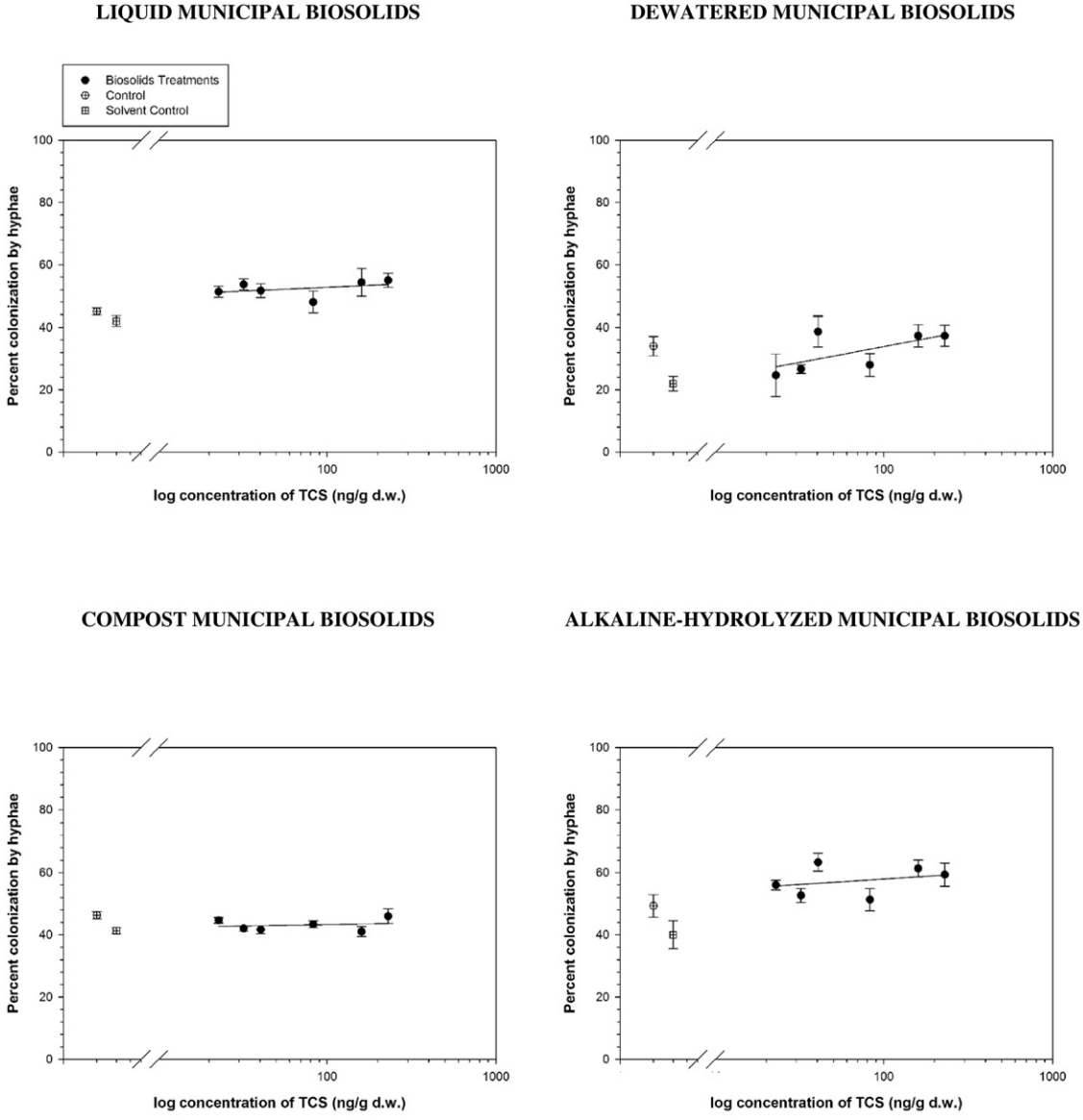


Fig. 1. Mean percent colonization by hyphae for corn plants grown in soil without biosolids and TCS (Control and Solvent Control), and biosolids-amended soils with increasing concentrations of TCS (BS1 to BS6). Bars represent standard error. The legend applies to each figure shown.

production of arbuscules and vesicles, ranging from 5 to 10% and 10–30%, respectively (Fig. 1; Supplemental Data, Figs. S1 and S2). When compared to control groups, AMB demonstrated higher percent colonization of roots by hyphae and greater production of arbuscules and vesicles; colonization by hyphae and production of arbuscules and vesicles was similar across LMB, DMB, and CMB (Fig. 1; Supplemental Data, Figs. S1 and S2).

3.2. Glycine max

No significant effect ($p > 0.05$) was observed between TCS and soybean on total percent colonization of roots by hyphae, with the exception of AMB ($p = 0.043$), and total percent production by presence of arbuscules and vesicles (Fig. 2; Supplemental Data, Figs. S3 and S4).

AMF colonization was not impacted by amendment with biosolids across all treatments. Percent colonization of roots by hyphae, and percent production of roots by arbuscules and vesicles ranged from 20 to 50%, 5–20%, and 15–20%, respectively (Fig. 2; Supplemental Data, Figs. S3 and S4). When compared to the control groups, LMB demonstrated higher percent colonization of roots by hyphae, and percent production by arbuscules and vesicles; CMB and AMB showed a decrease in percent colonization by arbuscules and vesicles. There was no production of arbuscules detected in roots exposed to AMB-amended soil.

3.3. Triticum aestivum

No concentration-response relationship ($p > 0.05$) was established between TCS and total percent colonization of roots by hyphae, and total percent production by presence of arbuscules and vesicles in spring wheat (Fig. 3; Supplemental Data, Figs. S5 and S6).

The amendment of biosolids to soil had no adverse impact on colonization by AMF. Percent colonization by hyphae in the control and biosolids groups ranged from 15 to 40%, while percent production by arbuscules and vesicles ranged from 3 to 8% and 5–10%, respectively (Fig. 3; Supplemental Data, Figs. S5 and S6). All biosolids types remained near the same level of percent colonization by hyphae and production by arbuscules and vesicles, relative to the control groups. There was no production of arbuscules detected in roots exposed to AMB-amended soil.

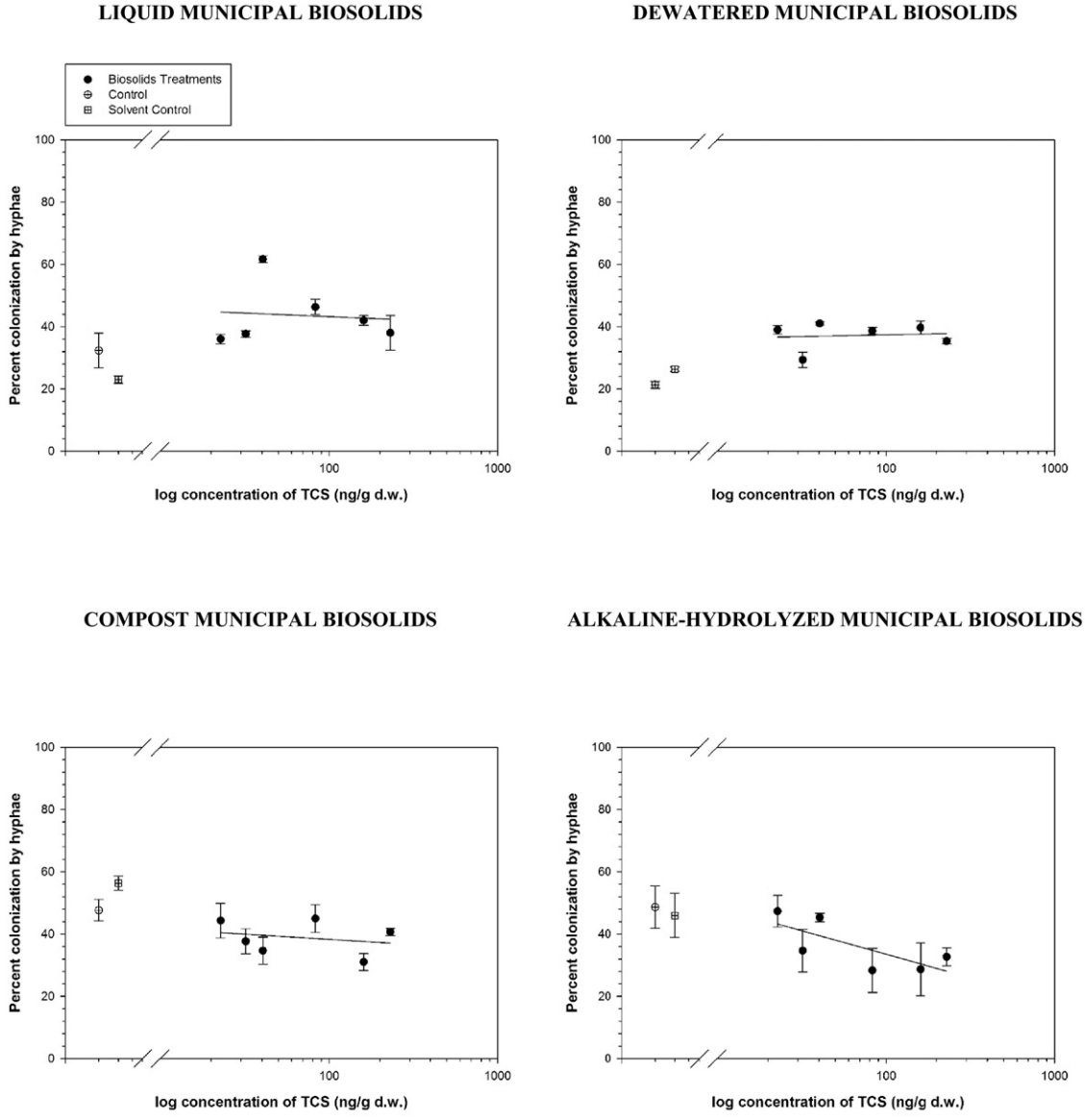


Fig. 2. Mean percent colonization by hyphae for soybean plant grown in soil without biosolids and TCS (Control and Solvent Control), and biosolids-amended soils with increasing concentrations of TCS (BS1 to BS6). Bars represent standard error. The legend applies to each figure shown.

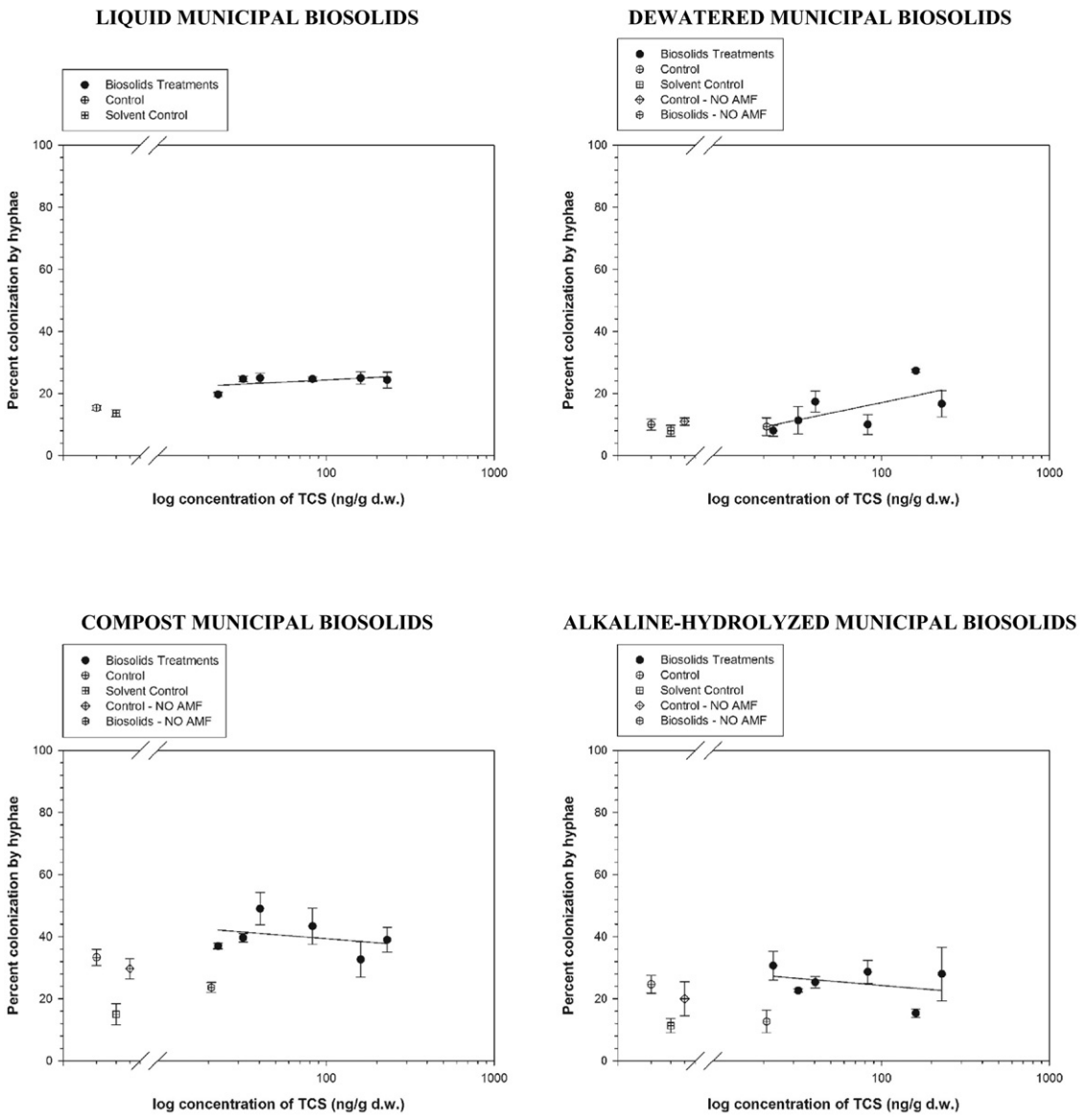


Fig. 3. Mean percent colonization by hyphae for spring wheat plant grown in soil without biosolids and TCS (Control and Solvent Control), soil without AMF (CTRL–NA), biosolids without AMF (BS–NA), and biosolids-amended soils with increasing concentrations of TCS (BS1 to BS6). Bars represent standard error.

4. Discussion

Our results found that TCS in the four formulations of biosolids presented a low risk to the colonization of AMF with roots of terrestrial plants under environmentally relevant exposure scenarios. These findings corroborate those of Prosser et al. (2015), who also found a low risk from TCS in biosolids to AMF colonization, but stand in contrast to Twanabasu et al. (2013a), who found that AMF colonization in roots of three wetland plants exposed to TCS at 0.4 μg/L in a flow-through exposure system was inhibited. As was the case for Prosser et al. (2015), our study used an agricultural soil, and results from treatments BS2 to BS4 depicted a realistic exposure scenario for TCS to terrestrial plants through pore water in biosolids-amended soil. For Twanabasu et al. (2013a), it is possible that the exposure of TCS to AMF and roots of wetland plants would have been greater due to the use of a sand substrate rather than sediment in the exposure system; the sand substrate (Twanabasu et al., 2013a) would have lacked the organic ligands typical to wetland soil, which would otherwise reduce the bioavailability of TCS. Although a significant decrease in AMF colonization by hyphae was found exclusively in AMB ($p = 0.043$) of our study, there was inconsistency among the remaining treatments and a trend could not be established between increasing concentrations of TCS and a decrease in AMF colonization by hyphae (Fig. 2).

In our study, it was noticed that the concentration of TCS decreased over the duration of all crop experiments. Growth conditions, soil properties, compound properties, and exposure medium are among the numerous factors that may have contributed to this decrease in TCS concentration, and are explored in Shahmohamadloo et al. (2016). For the purposes of this discussion, we focused on the dissipation and degradation of TCS in biosolids-amended soil. At the average soil pH in this present study, it is possible that TCS (pKa = 7.9) may have leached out of the soil (pH > 7.5) since it existed in its ionized form in all biosolids formulations (Shahmohamadloo et al., 2016). Holling et al. (2012) assessed the impact of ionization on the bioavailability or mobility of test compounds whose pKa was greater than the soil pH in the rhizosphere. Results showed that plants would not readily take up the ionized form of organic compounds compared to their neutral counterpart (Holling et al., 2012). Wu et al. (2009) showed that sorption of TCS to soil decreased with increasing pH. These findings may offer an explanation for the apparent lack of toxicity to AMF in plants in our

study, and could partially explain the significant decrease in hyphal and arbuscular colonization of three wetland plant species grown by Twanabasu et al. (2013a) in a flow-through exposure system at pH = 7.0. A look at the impact of TCS on seed emergence and plant growth in biosolids-amended soil was investigated by Shahmohamadloo et al. (2016) who concluded that leaching due to the pKa of TCS in relation to the pH of the soil, lack of sorption from spiked TCS to soil, a half-life range that is shorter than the duration of the plant experiments, and matrix spiking may have resulted in the lack of toxicity found between TCS and the plants.

Prosser et al. (2015) could not use soybean and spring wheat to determine whether TCS in biosolids impacted AMF due to low colonization levels (<2%); however, our study saw soybean and spring wheat colonization levels by hyphae between 20 and 50%, respectively. The duration of soybean and spring wheat experiments in Prosser et al. (2015) were 39 and 65 days, respectively, which was less than the duration of our experiments (80 and 70 days) and may offer an explanation for their lower colonization levels. Corn experiments in our study were grown for the same duration (85 days) as Prosser et al. (2015), and results from colonization levels by hyphae (>40% in the present study) corroborate their findings (>40%).

Recent studies have shown that AMF inoculation of soil can be an important biological tool in farming systems where the mycorrhizal potential of soil is lacking or depleted (Hart and Forsythe, 2012; Madejón et al., 2010; Pellegrino et al., 2011). The combined approach of amending soil with biosolids and inoculating with AMF can provide crops with enhanced access to macronutrients needed for growth (Madejón et al., 2010). Madejón et al. (2010) evaluated this approach using a native Australian grass species on sulphidic gold mine tailings and reported significant increase in plant biomass and enhanced uptake of macronutrients (nitrogen, phosphorus, potassium, calcium, and magnesium) in soils amended with biosolids and inoculated with AMF. Arguably, Madejón et al. (2010) used a polluted soil, which may not be a fair comparison with the unpolluted soil used in our study. However, plants from the present study also grew larger in the AMF-inoculated biosolids treatments compared to the controls indicating a relationship between amending soil with biosolids and inoculating with AMF. Although several studies have found that mycorrhizal colonization decreases in phosphorus-rich conditions like biosolids-amended soil (Hetrick et al., 1994), results from this study corroborate those of Madejón et al. (2010) who found AMF count was not hindered by elevated phosphorus concentrations. Mycorrhizal colonization was observed in all experiments of this study and confirms the effectiveness of AMF inoculation in the rhizosphere of corn, soybean, and spring wheat grown in soil amended with four formulations of biosolids. Therefore, when assessing the benefits of AMF and biosolids in plants and comparing results between studies, biosolids formulation, application rate, soil fungi/bacteria ratio, agricultural management, and soil composition are among the list of factors that should be considered (Hazard et al., 2014; Tian et al., 2015).

Although not statistically significant, we found that amendment with DMB resulted in the smallest colonization levels in all three plants. This finding merits further investigation into the constituents of DMB that may have caused a noticeable decrease in AMF colonization. One possibility is the concentration of metals. The DMB used in the present study had 23.8% dry matter and comparable concentrations of lead (55 μg/g dry) and zinc (820 μg/g dry); however, copper (1200 μg/g dry) in DMB relative to LMB, CMB, and AMB in the present study was 2.6, 5.7, and 2.5 times greater, respectively (Shahmohamadloo et al., 2016). Common agricultural practices, like amending soil with biosolids, gives an entryway for elevated concentrations of metals into soil, which can inhibit AMF symbiosis (Hagerberg et al., 2011). For example, a recent study that used a pasteurized biosolids with 26% dry matter found a significantly greater concentration of metals – copper (448 μg/g dry), lead (78 μg/g dry), and zinc (517 μg/g dry) – in the top layer of soil, compared to the control treatment (Hazard et al., 2014). Hagerberg et al. (2011) found that elevated concentrations of copper severely inhibited the colonization potential of the arbuscular mycorrhizal fungus *Glomus intraradices* in soils. While Hagerberg et al. (2011) did not utilize biosolids, they showed that *Glomus intraradices*, a common AMF component found in commercial inoculants, was a sensitive indicator for elevated copper concentrations in soil (Hagerberg et al., 2011). The risk, therefore, lies in the potential for copper to inhibit the colonization of commercial inoculants that contain *Glomus intraradices* (Hagerberg et al., 2011). The commercial inoculant applied in the present study contained *Glomus intraradices* and may therefore offer an explanation for the smaller AMF colonization observed in DMB. Other AMF species, like *Glomus mosseae*, have shown high tolerance in a wide spectrum of heavily polluted soils and are not hindered by elevated metals concentrations (Zarei et al., 2010). Although the total concentration of metals (Cu: 1200 μg/g dry; Pb: 55 μg/g dry; Zn: 820 μg/g dry) in our study are below the maximum allowable concentrations in biosolids (Cu: 1700 μg/g dry; Pb: 1100 μg/g dry; Zn: 4200 μg/g dry) manufactured in the province of Ontario, the toxicological impact of copper from long-term use of biosolids on AMF communities remains largely unknown (Hazard et al., 2014; OMAF, 2012a). As proposed by Hazard et al. (2014), whether AMF communities could develop resistance towards elevated concentrations of metals in soils, or conversely be depleted altogether, remains the subject of future studies.

5. Conclusions

Following current BMPs in the province of Ontario, Canada, TCS in biosolids presented a negligible risk to the colonization of AMF with the roots of three plants grown in four formulations of biosolids under environmentally relevant exposure conditions and duration scenarios. Results from this study contribute to a growing body of literature that shows the land application of municipal biosolids is a sustainable method of delivering nutrients and minerals needed for plant growth in agricultural systems. Regulators can use the information generated from this study to better understand potential risks and therefore improve current practices in sustainable biosolids management programs.

Acknowledgements

This study was funded by a University of Guelph – Canadian Water Network (CWN-MW2013-1) partnership grant. We would like to thank L. McCarthy from Ryerson University and Shelly Bonte-Gelok from Ontario Ministry of Environment and Climate Change for advice, and D. Bowes, and V. Capmourteres for assistance with experimental set up and take down at the University of Guelph.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.01.067.

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