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Journal

Water Environment Research, 85(12)

ISSN

1061-4303

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Publication Date

2013-12-01

DOI

10.2175/106143012x13560205144335

Peer reviewed

Published in final edited form as:
Water Environ Res. 2013 December ; 85(12): 2237–2242.

Effects of Triclosan and biosolids on microbial community composition in an agricultural soil

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Abstract

Triclosan (TCS) is a widely used antimicrobial agent found at high concentrations in biosolids produced during municipal wastewater treatment. The effect of adding TCS, in the presence or absence of biosolids, on the composition of an agricultural soil microbial community was measured using phospholipid fatty acid analysis (PLFA). Most changes observed in microbial community composition were attributable to addition of biosolids or passage of time, with smaller changes due to TCS exposure, regardless of the biosolids presence. TCS slightly reduced the relative abundance of Gram positive and negative bacteria and fungi, both with or without biosolids. Bacteria were more sensitive than eukaryotes, consistent with the mode of action of TCS, which selectively targets fatty acid synthesis and disrupts cell membranes of bacteria. TCS slightly increased biomarkers of microbial stress, but stress biomarkers were lower in all biosolid treated soils, presumably due to increased availability of nutrients mitigating potential TCS toxicity.

Keywords

Soil microbial community; Biosolid; Triclosan; Phospholipid fatty acid (PLFA); Antimicrobial; Personal care products

Introduction

Triclosan (TCS, MW=289.5, [2,4,4'-trichloro-2'-hydroxydiphenylether]) is a non-agricultural pesticide widely used as an antibacterial agent in common medical, household and personal care products (e.g., medicated skin cream, mouthwash, shampoo, soap, detergent, toothpaste, cosmetics, hand sanitizers) in the range of 0.1%–0.3% (w/w). The use of TCS has increased worldwide over the last 30 years (Sabaliunas *et al*., 2003). The broad household use of products containing TCS results in the discharge of TCS to municipal wastewater treatment plants, and it has been detected in effluents (0.10–2.70 $\mu g \cdot L^{-1}$) and sewage sludge (0.028–15.6 $\mu g \cdot g^{-1}$ dry weight (dw)) in Europe and the United States (Singer *et al*., 2002; Waltman *et al*., 2006; Ying and Kookana, 2007). TCS has been thought to act non-specifically by attacking and destroying bacterial membranes. The mode of action of

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TCS on bacteria is through inhibition of fatty acid synthesis by targeting enzymes specific to bacteria (Escalada *et al*., 2005; McMurry *et al*., 1998). Since fatty acid biosynthesis is a fundamental process for cell growth and function; the ability to inhibit this makes TCS a particularly effective antimicrobial compound.

Biosolids are the nutrient-rich byproduct of wastewater treatment operations and large quantities are generated. For example, approximately 750,000 dry tons is produced annually in California and 54% of these biosolids are applied on agricultural lands, 16% are composted and the remaining 30% goes to landfills (http://www.ciwmb.ca.gov/Organics/Biosolids). Concerns about potential health and environmental effects of land application of biosolids include possible off-site transport of pathogens, heavy metals, and trace organic constituents such as TCS (Lozano *et al*., 2010; Ying *et al*., 2007). A less explored set of potential impacts is how TCS and other biosolid-borne contaminants affect ecosystem processes and associated soil microbial communities. Potential impacts on soil microorganisms are important to assess since these organisms mediate much of the nitrogen, carbon and phosphorous dynamics in soil, biodegrade contaminants, create soil structure, decompose organic compounds, and play a major role in soil organic matter formation (Alef, 1995; Forster, 1995).

We hypothesized that biosolids containing TCS would have detrimental effects on soil microbial communities by decreasing biomass and altering community composition in agricultural soil. Our objectives were to evaluate the effects of increasing amounts of TCS on soil microbial community composition in the presence and absence of biosolids. We used phospholipid fatty acid (PLFA) analysis to characterize the response of microbial communities; the method provides information about microbial community composition, biomass, and diversity (Zelles, 1999). Experiments in which TCS was added to soil without biosolids allowed the relative effects of biosolid and TCS addition on microbial community composition and function to be compared and also provided a “secondary control” because TCS-free municipal biosolids are essentially unavailable in the United States (USEPA, 2009).

Materials and Methods

Chemicals, Soil and Biosolid

Triclosan was purchased from Fluka (Buchs, Swizerland). Yolo silt loam (mixed, nonacid, thermic Typic Xerothent) was collected from the Student Experimental Farm at the University of California, Davis (34.533_S, 138.733_E) at a depth of 0 to 15 cm. The soil was passed through a 2 mm sieve and stored at 4 °C until use. Biosolids originated from a municipal wastewater treatment plant in Southern California that employed a conventional activated sludge treatment system followed by aerobic sludge digestion. Biosolids from this system were selected for study because they had the lowest concentration of TCS among those collected from 10 different wastewater treatment plants in California (Ogunyoku and Young, in review). The soil and biosolid physiochemical properties are reported in Table 1 and were determined using standard techniques (Nelson and Sommers, 1982).

Microcosm experiments

The soils were moistened to 40% water-holding capacity, which is equivalent to 18% water content in our experiments, and pre-incubated for 7 days at 25°C to allow time for normal microbial activity to recover to a constant level after disturbance. The pre-incubated 50 g (dw) of soil was weighed into 200 ml glass bottles to make three replicates per treatment. For the biosolid amended soil (SB) sample, 20 mg/g of biosolid (dw, w/w) was added. Each treatment sample was then spiked with TCS to achieve final concentrations of 10 or 50 mg/

kg using TCS stock solutions prepared in acetone, as recommended by Waller and KooKana (2009). This spiking level was chosen as a conservative upper bound on anticipated soil concentrations in the field. The lower spiking level (10 mg/kg) is below the mean concentration observed in US biosolids (16 mg/kg) and the higher level (50 mg/kg) is below the 95th percentile (62 mg/kg) for US biosolids (USEPA, 2009); adding biosolids to soils at typical application rates would produce soil concentrations ~50–200 times lower. Control samples were also prepared with acetone only. After that, the solvent was allowed to evaporate inside the fume hood before the samples were thoroughly mixed. The microcosms were incubated in the dark at 25°C for 0, 7 and 30 days. Every week, each vial was opened to help keep conditions aerobic and the water content of each set of samples was measured and water was added as needed to maintain target moisture levels.

At each sampling time, the remaining TCS was measured by drying 3–5 g samples at 70°C for 24 hours and homogenizing with a mortar and pestle. Replicate 1 g subsamples of each dried sample were placed in centrifuge tubes, spiked with deuterated trichlorocarban (TCC, 3, 4, 4'-trichlorocarbanilide, TCC-d7) in methanol, air dried under a fume hood to remove the methanol, and then mixed well. Extraction was performed by adding 15 mL of 1:1 acetone and methanol to the centrifuge tube. Samples were extracted on a shaker table for 24 hours at 295 rpm and 55 °C and then centrifuged for 30 min at 4,100 g. The supernatant was diluted as needed to ensure that the concentration remained within the linear portion of the calibration curve. The extracts were analyzed for TCS using LC-MS/MS. Additional details regarding the extraction and analysis procedures can be found in Ogunyoku & Young (in Prep). Recoveries of deuterated TCC ranged from 63–115% during extraction and analysis.

PLFA analysis

PLFA analysis was performed as previously reported (Bossio *et al*., 1998; Cordova-Kreylos *et al*., 2006). Dry samples (8 g) were freeze-dried and total lipids were extracted with a one-phase chloroform/methanol/phosphate buffer solvent mix. Phospholipids were separated with a solid phase extraction column (0.58 Si; Supelco Inc., Bellafonte, PA) and the individual phospholipid fatty acids were analyzed using a Hewlett-Packard 6890 Gas Chromatograph fitted with a 25 m Ultra 2 column (5% phenylmethylpolysiloxane; J & W Scientific, Folsom, CA). Fatty acids were identified using the Sherlock software (Microbial ID, Inc, Newark, DE).

PLFA biomass was expressed in nanomoles (nmol) of PLFA per gram (dw) of soil or SB. The biomass values were averaged from triplicate samples for each TCS concentration (0, 10 and 50 mg/kg) and incubation time (0, 7 and 30 days) of soil and SB. The following biomarkers and ratios were used: Total PLFA (sum of all PLFA detected, nmol/g dw soil), number of PLFA detected, Fungi/Bacteria (18:2 ω6,9c/i15:0 + a 15:0 + 15:0 + i16:0 + 16:1 ω5c + i17:0 + a17:0 + 17:0cy + 17:0 + 19:0 cy), Gram positive (sum of all branched PLFA), Gram negative (sum of all monounsaturated PLFA), actinomycetes (10Me PLFAs), Fungi (18:2 ω6,9c), and eukaryotes (sum of 20: 20:4 ω6,9,12,15c, 20:2 ω6,9c). The following two ratios of PLFA were also used as indicators of nutritional stress in bacterial communities: 17cy/precursor (17:0cy/16:1 ω7c) and Saturated/Monounsaturated fatty acid. The suffixes `c' and `t' stand for *cis* and *trans*, the prefixes `i', `ai', and `Me' indicate to iso, anteiso, and mid-chain methyl branching, and the prefix `cy' refers to cyclopropyl rings.

Statistical analysis

Analysis of variance (ANOVA) was performed using SAS (version 9.2, SAS Ins., Cary, NC). The statistical significance of differences was assessed using the Levene's test of means at the 5% confidence level ($p < 0.05$). Microbial community fingerprints and correlation with environmental variables (e.g., TCS, biosolid addition, and incubation time)

were analyzed by canonical correspondence analysis (CCA) (Lepš and Šmilauer, 2003) carried out with the CANOCO software (Microcomputer Power, Ithaca, NY). Only lipids that were detected in >90% of the samples were used for multivariate analysis.

Results and Discussion

As expected, the biosolids contained far larger amounts of nitrogen (N) and carbon (C) than the Yolo soil (Table 1). Even though the biosolids constituted less than 2% (w/w) of the amended soil, they contributed nearly 50% of the total nitrogen and 40% of the total carbon in the amended soil system. The biosolids contained an abundance of nutrients accumulated as by-products of sewage treatment in forms likely to be more labile than equivalent nutrients present in the soil. As will be discussed further, the greater availability of C and N in the SB than soil treatments had a strong influence on some of the results, especially at the early time points. In the following section, therefore, it is useful to remember that all SB treatments contain more available C and N than all soil treatments.

Biodegradation of TCS

The initial concentration of TCS in unspiked SB samples was very low (0.18 mg/kg), fell below the quantitation limit for TCS after 7 days, and was not detectable after 30 days of incubation. Significant TCS biodegradation was observed in spiked soil and SB samples during incubation and the data were well described using a first order model as indicated by linear plots (R^2>0.92) of $\ln(C/C_0)$ against time (Fig. 1). Degradation trends were consistent at the two spiking levels (10 or 50 mg/kg) for each sample type but biosolid addition significantly reduced degradation rates at both spiking levels compared with un-amended samples. The percentage of TCS removed was approximately two times greater in soil than in SB samples. Approximately 80% of the TCS was removed over 30 days in soil treated with either 10 mg/kg or 50 mg/kg of TCS, but no more than 30% was transformed in the corresponding SB microcosms. The reduced biodegradation in the SB microcosms may have resulted from the ~40% higher carbon content in the SB microcosms, which would be expected to increase the soil-water distribution coefficient (K_d) by a comparable amount. Reduced TCS concentration in soil pore water would be expected to slow biotransformation, potentially in a nonlinear fashion. Another possible contributor to the slower degradation of TCS in SB is the greater availability of alternative, likely more easily degradable, carbon sources in SB than soil microcosms, reducing the use of TCS as a substrate. Selective biodegradation of one carbon source, and inhibition of the degradation of other chemicals also present, has been observed for mixtures of chemicals in aquifers (Deeb *et al*., 2001).

To assess which of these mechanisms was controlling, measured Freundlich isotherm parameters for TCS adsorption on biosolid amended Yolo soil (K_F =2.02, n = 0.66; Ogunyoku and Young, in Prep) were used to calculate equilibrium pore water concentrations in the soil and SB microcosms over the course of the experiment. Using estimated pore water concentrations of moistened soil and SB samples, instead of total soil concentrations to perform half-life calculations, resulted in modest increases in the rate constants (increased by ~50%) and decreases in half-lives (reduced by 30%) of soil samples and did not narrow the significant gap between half lives in soil and SB (data not shown). This suggests that the primary reason for the slower degradation of TCS in biosolid amended soils is the increase in more labile forms of carbon because organic material is highly porous and has a lower particle density.

Previous research shows that TCS biodegrades within weeks to months in aerobic soils (Christensen, 1994; Xu *et al*., 2009; Ying *et al*., 2007), although Chenxi *et al*., (2008) found no TCS degradation in biosolids stored under aerobic or anaerobic conditions, Kinney *et al*., (2008) observed a 40% decrease in TCS concentrations over a 4-month period following an

agricultural biosolids application. Because the slopes of the lines in Fig. 1 are not significantly different ($p<0.05$) as a function of spiking level (10 or 50 mg/kg), the slopes were averaged for each treatment type, yielding apparent first order rate constants of 0.093±4% d^{-1} for soil samples and 0.024±41% d^{-1} for SB samples where the percent error represents the relative percent difference between the 10 mg/kg and 50 mg/kg degradation curves. These apparent rate constants translate to half-life estimates of 7.5 d in soils and 29 d in biosolid amended soil. The estimated half-life of TCS in soil is within the range of previously reported half-lives of from 2.5 to 58 d in soil (Wu *et al*., 2009; Ying *et al*., 2007). The half-life determined here in biosolid amended soils is lower than the one available literature value of 107.4 d (Lozano *et al*., 2010).

PLFA Results

The microbial biomass decreased in the TCS spiked samples (10 or 50 mg/kg) after 7 or 30 days of incubation in comparison with the unspiked controls, for both soil and SB, and the decline was statistically significant ($p < 0.05$) at 50 mg/kg (Fig 2, top). Although exposure to TCS caused declines in biomass in both soil and SB microcosms, the total microbial biomass was two times higher in SB than soil (Fig. 2) probably due to the increased availability of nutrients and/or possibly due to addition of biosolid associated microorganisms in the latter (Svenningsen *et al*., 2011).

The total number of PLFAs ranged from 42–47 in soil and 48–59 in SB (Fig.2, bottom). No significant change in numbers of PLFAs was evident with increasing dosage of TCS for any incubation time ($p < 0.05$) suggesting that TCS addition did not adversely affect microbial diversity.

Microbes respond to various stresses by modifying cell membranes, for example by transforming the *cis* double bond of 16:1ω7c to cy17:0, which is more stable and not easily metabolized by the bacteria, reducing the impact of environmental stressors (Chaudhary *et al*., 2005; Zelles *et al*., 1992). Consequently, the ratio of cy17 to its precursor has been employed as an indicator of microbial stress that has been associated with slow growth of microorganisms (Bossio and Scow, 1998; Kieft *et al*., 1994). Increases in this stress biomarker were observed in both soil and SB samples as TCS concentrations increased (Fig. 3), suggesting that TCS has a negative effect on the growth of soil microorganisms. The overall level of cy17 to its precursor is lower in SB than soil samples, suggesting that nutrients contributed by the biosolids reduce stress on the microbial community. Our study agreed with a previous study showed that carbon added to soil led to a reduction in the cy17 fatty acid (Drenovsky *et al*., 2004)

TCS additions, however, increased the stress marker compared with that detected in the corresponding samples with no added TCS. A broader implication of this result is that presence of biosolids may mitigate the toxic effects of chemicals in soil, or chemicals added in combination with biosolids, on soil microbial communities.

Groupings of microbial communities, based on CCA analysis of their composition as estimated by PLFA, were distinguished primarily by whether they were in soil or SB treatments (first axis, 84.8% of variation in data) and secondarily by time since spiking (day 0, 7 or 30; second axis, 8.7%) (Fig. 4). To isolate the effects of biosolids and TCS amendments on microbial community composition, the data was analyzed using pCCA considering TCS and biosolid amendment as environmental variables, and incubation time as a covariable (Fig. 5). This confirmed the results of the CCA indicating that the strongest determinant of microbial community composition (92.1% of the variation described by the first axis) was addition of biosolids to soil. TCS concentration, on the second axis, described

only 3.6% of the variation, showing TCS effects were overshadowed by the effects of biosolid amendment.

Biosolid amendments caused an approximately two-fold increase in PLFA biomarkers for Gram-positive bacteria, actinomycetes and eukaryotes in SB compared to soil samples (Table 2). Even larger increases were observed in biomarkers for fungi and Gram-negative bacteria, which were up to three times higher in SB than soil. Again, these changes were likely due to increased nutrient availability in the biosolid amended samples and/or the biomass added along with the biosolids, consistent with previous studies that found that the fatty acid 18:2 ω6, 9c and monounsaturates were increased by addition of these materials (Bossio and Scow, 1998; Zelles *et al*., 1992).

The effect of TCS on microbial community composition was greater in soil than SB. Spiking with 10 or 50 mg/kg TCS decreased the abundance of Gram positive and Gram negative bacteria as well as fungi, with reductions ranging from 14 to 27% by day 30. Additionally, actinomycetes, which are Gram positive bacteria, were reduced in the 50 mg/kg TCS samples after 30 days of incubation (Table 2). Eukaryotes were negatively affected after 7 and 30 days of incubation at both concentrations of TCS in soil but not SB samples. Biomass results for all microbial groups were consistent in suggesting that the presence of biosolids mitigated the potential toxicity of TCS. It is important to note that the spiking levels used here (10 or 50 mg/kg on a soil basis) are similar to levels found in the upper half of U.S. biosolids, but would be unlikely to be achieved in biosolid amended soils even after continued long term application. Therefore, the effects observed at the 10 or 50 mg/kg spiking levels should be viewed as a conservative upper bound on potential effects expected in the field. In addition, since all of the results in this study are based on an observation period of 30 d, the extent to which the observed effects persist is not known. Future studies should, in particular, investigate longer term changes in community structure in response to addition of biosolids both with and without specific contaminants.

Conclusion

Biosolid addition to soil had a pronounced effect on microbial community composition. TCS effects at the community level were small and were overshadowed by the impacts of biosolid addition and the passage of time. All available measures of community composition using PLFA data suggest that the community changes imparted by biosolids addition are positive, causing increases in microbial biomass, microbial diversity, and reducing stress biomarker production. TCS addition slightly reduced the relative abundance of Gram positive and Gram negative bacteria and fungi and led to an increase in a bacterial stress biomarker in soil, both with or without biosolids. Bacteria appeared to be more sensitive than eukaryotes to TCS addition in the absence of biosolids, consistent with the reported mode of action of TCS, which targets fatty acid synthesis and disrupts cell membranes in bacteria.

Acknowledgments

Research reported in this publication was supported by Superfund Research Grant of University of California at Davis and by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number P42ES004699. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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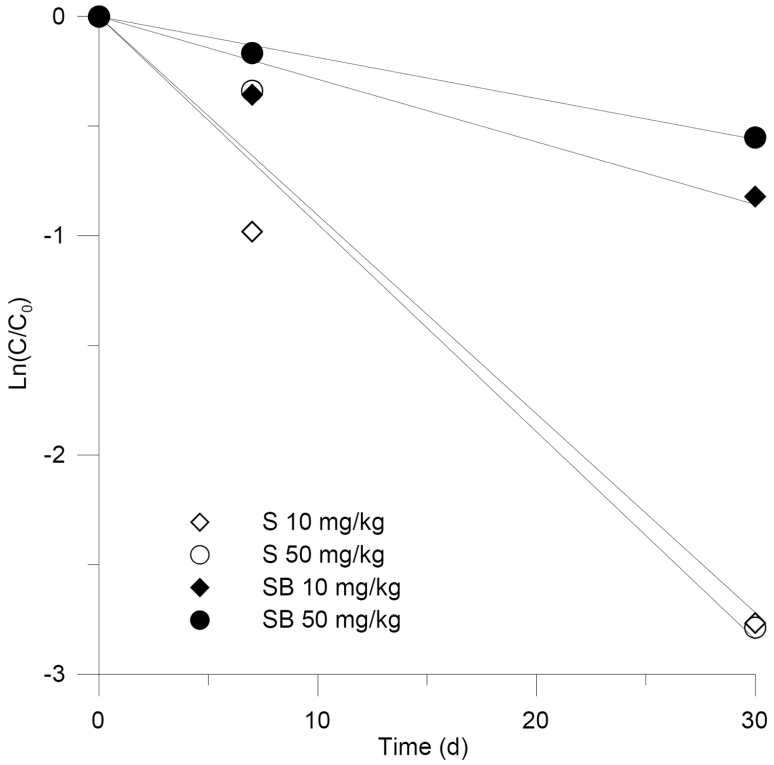

Fig. 1.
Degradation of TCS on soil (S) or biosolid amended soil (SB) spiked at 10 or 50 mg/kg.

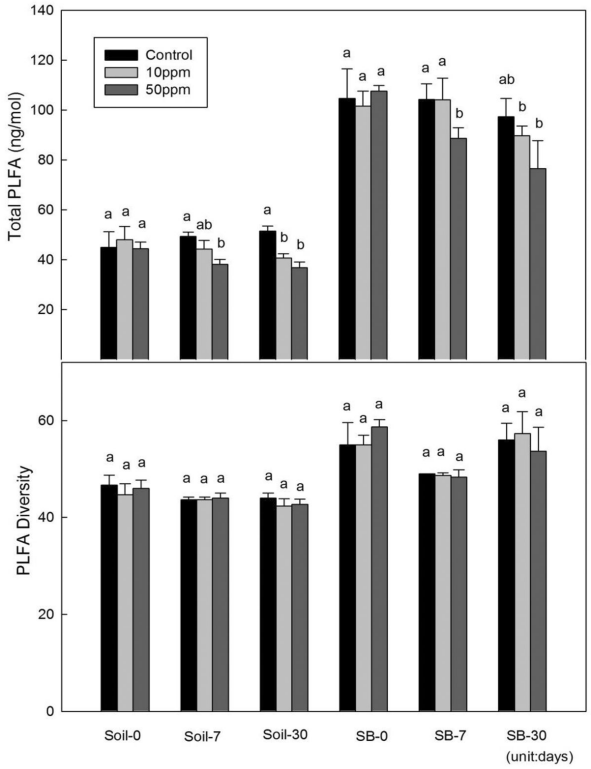

Fig. 2.

Total biomass and PLFA numbers in agricultural soil (S) and biosolid-amended agricultural soil (SB) with 0, 10 and 50 mg/kg spiked TCS after 0, 7 and 30 days of incubation. Error bars indicate standard deviations. Different letters above the bars indicate significant differences, as determined by ANOVA.

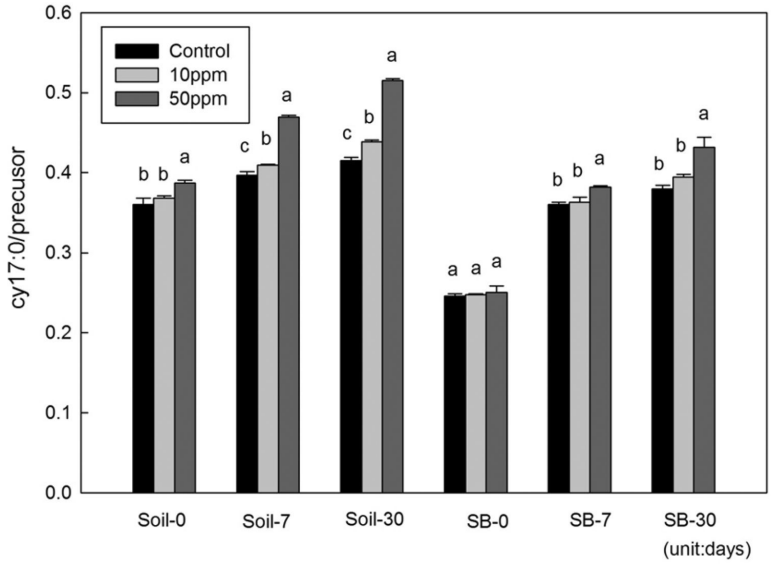

Fig. 3.

Stress biomarker of cy17/precursor in agricultural soil (S) and biosolid-amended agricultural soil (SB) with 0, 10 and 50 mg/kg of added TCS after 0, 7 and 30 days of incubation. Error bars indicate standard deviations. Different letters above the bars indicate significant differences, as determined by ANOVA.

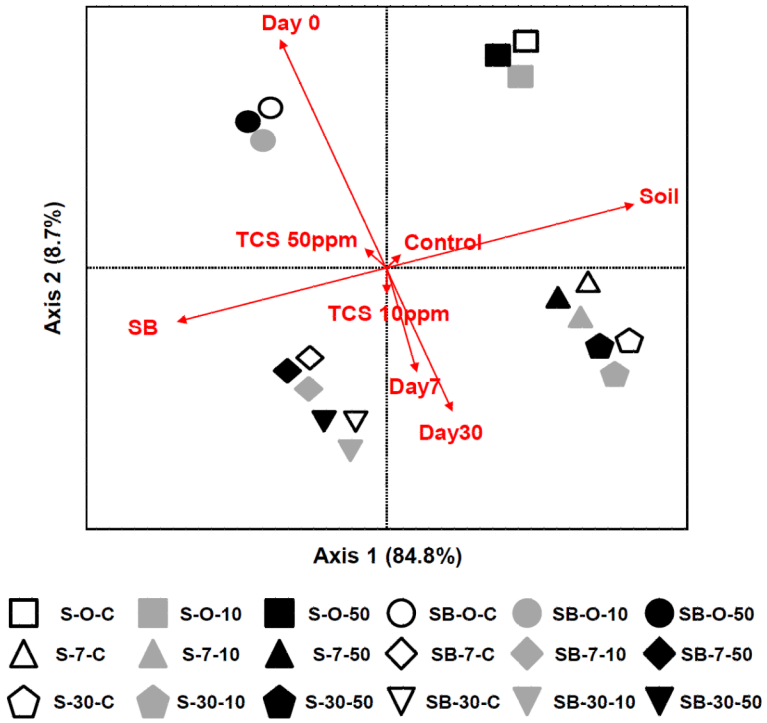

Fig. 4.

Ordination plots of CCA results for PLFA fingerprints. The TCS amounts and incubation time were environmental variables. The direction of an arrow indicates the steepest increase in the variable, and the length indicates the strength relative to other variables.

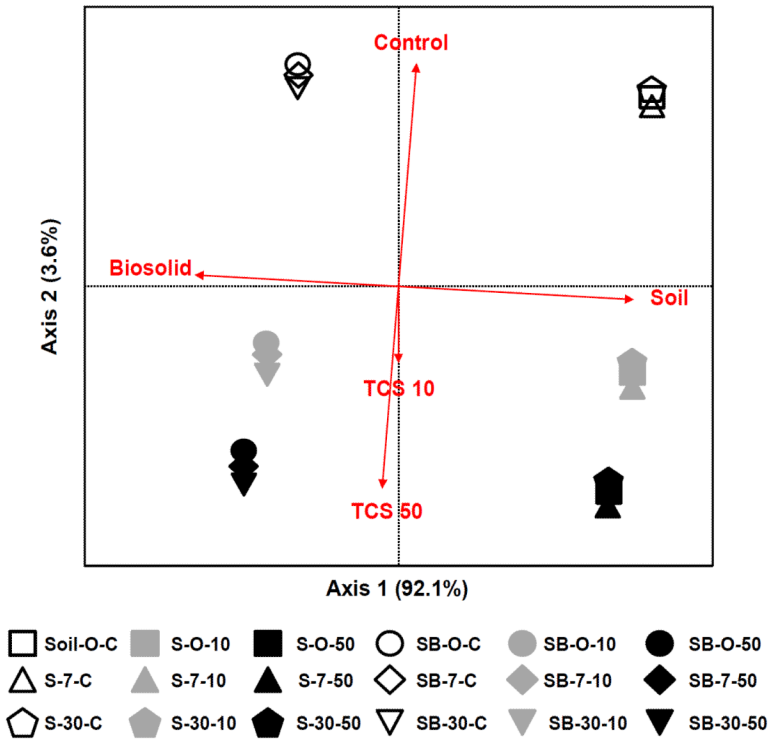

Fig. 5.

Ordination plots of pCCA results for PLFA fingerprints. TCS amounts were used as an environmental variable and incubation time was used as a covariable. The direction of an arrow indicates the steepest increase in the variable, and the length indicates the strength relative to other variables.

Table 1

Physicochemical properties of soil and biosohd.

	pH	Nitrogen (%)	Carbon (%)	C:N ratio	Initial TCS amount (mg/kg)
Biosolid	6.3	5.84	35.12	6.02	3.07
Soil	7.5	0.12	1.08	8.72	N/A[a]

[a] not applicable

Table 2

PLFA microbial groups in agricultural soil and biosohd-amended agricultural soil (SB). Biomarker averages with standard deviations are indicated. Superscript letters indicate significant differences ($p<0.05$) as determined by ANOVA.

S	0 days			7 days			30 days		
	C[a]	10ppm	50ppm	C	10ppm	50ppm	C	10ppm	50ppm
Gram +	12.39±1.65^{a}	13.63±1.47^{a}	14.44±0.93^{a}	13.44±2.53^{a}	12.28±1.11^{b}	11.21±0.60^{b}	13.33±0.49^{a}	11.05±0.76^{b}	10.39±0.36^{b}
Gram −	11.56±1.54^{a}	12.55±1.44^{a}	12.87±0.64^{a}	11.77±4.42^{a}	10.92±2.19^{a_b}	9.93±0.94^{b}	10.52±0.44^{a}	9.35±0.95^{b}	9.05±0.55^{b}
Actinomycetes	2.77±0.37^{a}	3.07±0.33^{a}	3.25±0.17^{a}	3.11±0.50^{a}	2.99±0.28^{a_b}	2.64±0.11^{b}	3.10±0.10^{a}	2.74±0.21^{b}	2.58±0.09^{c}
Fungi	1.48±0.26^{a}	1.74±0.21^{a}	1.65±0.14^{a}	1.56±0.03^{a}	1.45±0.18^{b}	1.31±0.08^{b}	1.34±0.11^{a}	1.17±0.28^{b}	1.11±0.04^{b}
Fungi/Bacteria	0.12±0.01^{a}	0.13±0.00^{a}	0.11±0.01^{a}	0.12±0.00^{a}	0.12±0.01^{a}	0.12±0.00^{a}	0.10±0.00^{a}	0.11±0.01^{a}	0.11±0.00^{a}
Eukaryotes	0.25±0.02^{a}	0.29±0.04^{a}	0.29±0.07^{a}	0.27±0.04^{a}	0.24±0.04^{a_b}	0.23±0.05^{b}	0.26±0.00^{a}	0.24±0.01^{a_b}	0.22±0.03^{b}

SB	0 days			7 days			30 days		
	C	10ppm	50ppm	C	10ppm	50ppm	C	10ppm	50ppm
Gram +	22.49±1.26^{a}	21.27±1.76^{a}	23.51±1.14^{a}	26.96±0.69^{a}	26.80±0.51^{a}	21.27±0.60^{b}	25.78±2.1^{a}	24.54±1.21^{a}	18.96±2.56^{b}
Gram −	36.83±2.07^{a}	35.71±2.88^{a}	37.40±1.18^{a}	32.83±0.28^{a}	32.48±0.55^{a}	28.27±0.46^{b}	29.04±2.36^{a}	26.46±0.94^{b}	23.86±3.99^{c}
Actinomycetes	4.55±0.23^{a}	4.38±0.36^{a}	4.79±0.25^{a}	4.74±0.12^{a_b}	5.19±0.09^{a}	4.35±0.15^{b}	5.85±0.43^{a}	6.06±0.33^{a}	4.73±0.64^{b}
Fungi	2.41±0.24^{a}	2.41±0.24^{a}	2.52±0.06^{a}	3.57±0.30^{a}	3.64±0.51^{a}	3.08±0.19^{b}	3.28±0.17^{a}	2.92±0.23^{b}	2.50±0.16^{c}
Fungi/Bacteria	0.11±0.01^{a}	0.12±0.01^{a}	0.11±0.00^{a}	0.13±0.00^{b}	0.14±0.00^{a_b}	0.15±0.01^{a}	0.13±0.00^{a}	0.13±0.01^{a}	0.14±0.01^{a}
Eukaryotes	0.41±0.18^{b}	0.60±0.21^{a}	0.44±0.17^{a}	0.50±0.01^{a}	0.54±0.03^{a}	0.49±0.03^{a}	0.31±0.01^{a}	0.30±0.02^{a}	0.33±0.05^{a}

Gram+, Gram−, Fungi and Eukaryotes PLFA expressed as % of total PLFA biomass.

[a]control sample without TCS spiking