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Use of nuclear receptor luciferase-based bioassays to detect endocrine active chemicals in a biosolids-biochar amended soil

Carolyn G. Anderson^a, Geetika Joshi^a, Daniel A. Bair^a, Charlotte Oriol^b, Guochun He^c, Sanjai J. Parikh^a, Michael S. Denison^c, Kate M. Scow^a

Abstract

Biosolids are a potentially valuable source of carbon and nutrients for agricultural soils; however, potential unintended impacts on human health and the environment must be considered. Virtually all biosolids contain trace amounts endocrine-disrupting chemicals derived from human use of pharmaceuticals and personal care products (PPCPs). One potential way to reduce the bioavailability of PPCPs is to co-apply biosolids with biochar to soil, because biochar's chemical (e.g., aromaticity) and physical properties (e.g., surface area) give it a high affinity to bind many organic chemicals in the environment. We developed a soil-specific extraction method and utilized a luciferase-based bioassay (CALUX) to detect endocrine active chemicals in a biosolids-biochar co-amendment soil greenhouse study. Both biochar (walnut shell, 900 °C) and biosolids had positive impacts on carrot and lettuce biomass accumulation over our study period. However, the walnut shell biochar stimulated aryl hydrocarbon receptor activity, suggesting the presence of potential endocrine active chemicals in the biochar. Since the biochar rate tested (100 t ha⁻¹) is above the average agronomic rate (10-20 t ha⁻¹), endocrine effects would not be expected in most environmental applications. The effect of high temperature biochars on endocrine system pathways must be explored further, using both quantitative analytical tools to identify potential endocrine active chemicals and highly sensitive bioanalytical assays such as CALUX to measure the resulting biological activity of such compounds.

Keywords

Biosolids, biochar, land application, endocrine disruption, CALUX bioassay

1. Introduction

Biosolids are the repository for a substantial portion of nutrients consumed in the form of food. Returning biosolids to food production systems can help “close the loop” in nutrient cycling (DeLuca, 2009). A challenge is that biosolids contain not only plant nutrients, but also pharmaceuticals (Table A.1) (U.S. Environmental Protection Agency, 2009).

Many organic contaminants commonly found in biosolids, such as pharmaceuticals and personal care products, sorb strongly to organic matter (Aristilde and Sposito, 2010; Carmosini and Lee, 2009; Rogers, 1996) and clays (Carmosini and Lee, 2009; Carrasquillo et al., 2008) due in part to their low solubility and the interactive effects of their multiple functional groups (MacKay and Seremet, 2008). Many pharmaceuticals exhibit a high sorption affinity to biosolids (Wu et al., 2009). For example, the antibiotic ciprofloxacin is not significantly removed after methanogenic wastewater treatment due to its high sorption properties (Golet et al., 2003). The antibacterials triclosan (TCS) and triclocarban (TCC) have low water solubility, high octanol-water partitioning coefficients (K_{ow}), and high organic carbon-water partitioning coefficients (K_{oc}) (Table A.2), and thus have a moderate affinity for solids. These compounds accumulate in biosolids and are found in nearly all wastewater treatment plant (WWTP) effluents (U.S. Environmental Protection Agency, 2009). Additionally, biodegradation studies have demonstrated that some pharmaceuticals or their residues can persist in the soil environment for over six months after application (Boxall et al., 2006). Together, these properties suggest that pharmaceuticals applied to land in biosolids could accumulate and persist in the environment (Näslund et al., 2008)).

Ciprofloxacin, TCS, and TCC are classified as “contaminants of emerging concern” by the US EPA, a designation for substances present at trace but potentially ecotoxicologically critical

concentrations (Lozano et al., 2010). While many pharmaceutical residues in environmental samples are relatively low in concentration, especially compared to the prescribed human dosage, non-specific bioactivity of these drugs might reach environmentally-relevant levels, causing adverse effects on soil biota and biologically-driven ecosystem services (Dolliver et al., 2007; Jones-Lepp et al., 2010; Kumar et al., 2005; Lillenberg et al., 2010). It is of particular concern that several emerging contaminants, including TCS (Foran et al., 2000) and TCC (Chen et al., 2008) have capacity for endocrine-disrupting activity, or disruption of normal hormonal functions in biota. Since land application is a common means for disposal of biosolids produced at US wastewater treatment facilities (National Research Council, 2002), cost-effective technologies are needed to prevent associated pharmaceuticals from contaminating the environment or affecting human health.

Biochar, a carbonized byproduct of the pyrolysis of organic biomass, is an increasingly common soil amendment and may provide agronomic benefits (Laird et al., 2010; Lehmann et al., 2003; Van Zwieten et al., 2010). Using biochar as a co-amendment with biosolids could potentially reduce bioavailability and toxicity of endocrine-disrupting chemicals (EDCs) found in biosolids. Because of its high aromaticity and surface area, biochar can effectively sorb many organic chemicals introduced into the environment (Jung et al., 2013; Yao et al., 2012). This, in turn, could decrease bioavailability and limit bioaccumulation of EDCs in plants (Khan et al., 2013; Yu et al., 2009). While use of biochar has been investigated in treating heavy metals (Park et al., 2011) and pesticides (Yu et al., 2009), the effect of biochar co-amendment with biosolids on the bioavailability and resulting activity of EDCs is unknown. The physical-chemical properties of biochar (e.g., pH, surface area, elemental composition) vary depending on parameters such as feedstock and pyrolysis temperature (Mukome et al., 2013b), and biochars

produced via gasification have been shown to have high levels of EDCs such as polycyclic aromatic hydrocarbons (PAHs) (Cole et al., 2012; Hale et al., 2012).

Pharmaceutical residues in environmental samples are often analyzed using chemical methods such as liquid chromatography tandem mass spectrometry (LC/MS/MS), which can resolve trace environmental concentrations of specific compounds of known mass with high precision. However, such analyses measure a total concentration and not the concentration of the contaminant that will actually be biologically available.

An alternative approach is application of the Chemically-Activated Luciferase Expression (CALUX) bioassay (Denison et al., 2004) that can measure the combined ability of chemicals extracted from environmental matrices, including biosolids- and biochar-amended soil, to stimulate hormone receptor signaling pathways. These CALUX bioassays are recombinant cell lines that contain a stably transfected nuclear receptor responsive firefly luciferase reporter gene and they respond to activators/inhibitors of these receptors in a chemical-, time-, concentration- and receptor-specific manner with the induction/inhibition of luciferase activity and measurement of light output (Denison et al., 2004). CALUX bioassays based on estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) and aryl hydrocarbon receptor (AhR) have been previously developed and used to determine the total biological activity (activation/inhibition) of a chemical(s) or extract on a given receptor pathway (Giudice and Young, 2011; Lorenzen et al., 2004; Topp et al., 2006; Zhang et al., 2009). Measurement of endocrine active chemicals in biosolids with CALUX bioassays have been used as a method both to characterize such activity (Lorenzen et al., 2004; Topp et al., 2006; Zhang et al., 2009) and for environmental mobilization studies (Giudice and Young, 2011). In addition to showing high sensitivity and specificity for known endocrine-disrupting hormone receptor

agonists and antagonists (van der Linden et al., 2008), the CALUX bioassay can provide an estimate of the overall activity of mixtures of compounds such as may be found in biosolids or in soil after biosolids application.

In this study, we measured the individual and combined effects of biochar and biosolids co-amendments on endocrine active chemicals in soil from greenhouse-grown lettuce and carrots. We utilized CALUX bioassays to specifically target androgen, estrogen, aryl hydrocarbon, and glucocorticoid/progesterone receptors, and hypothesized that biochar preferentially sorbs organic pharmaceuticals decreasing their bioavailability in the soil and thus potential to produce endocrine disrupting effects. Therefore, we expected to see lower bioassay activity levels in the biosolids-biochar treatments with high biochar application rates.

2. Materials and methods

2.1. Soil, biosolids, and biochar

A greenhouse experiment was conducted using a loamy sand (Columbia series, Aquic Xerofluvent) collected from USDA-NRCS Lockeford Plant Materials Center (Lockeford, CA). Soil particle size distribution was determined using the hydrometer method (82.5% sand, 6.0% clay) and water-holding capacity was determined using a common field method whereby soils were saturated and allowed to drain until drainage stopped (0.52 g g^{-1}). Biosolids were provided by the Woodland Wastewater Treatment Plant (Woodland, CA), derived from an extended aeration activated sludge process. Macronutrient (N, P, K) data for soil and biosolids were determined by the combustion method for total N (AOAC Official Method 972.43), a KCl extraction using a flow injection analyzer for $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ (Table A.3) (Knepel, 2003), the Olsen-P method for bioavailable P (Prokopy, 1995), and ammonium acetate displacement for

exchangeable K (Thomas, 1982). The application rate of biosolids used was 63.1 g biosolids kg⁻¹ soil based on plant demand for nitrogen, calculated at a rate of 200 kg N ha⁻¹ to 30 cm (Evanylo et al., 2006). Non biosolids-amended treatments received the equivalent N in mineral fertilizer. Based on the 16-16-16 fertilizer (Lilly Miller Brands, Walnut Creek, CA, USA) used, 87.4 kg ha⁻¹ phosphorus, 166 kg ha⁻¹ potassium and 62.5 kg ha⁻¹ sulfur were also applied. The biochar was made from walnut shells (*Juglans californica*) using a Biomax 50 downdraft gasifier at Dixon Ridge Farms (Winters, CA, USA) (Mukome et al., 2013a), as part of a waste-to-energy process (Pujol Pereira et al., 2016). Detailed information on the physical and chemical characteristics of this biochar have been detailed elsewhere (Mukome et al., 2013b). The pyrolysis temperature was 900 °C, resulting in an ash content of 46.6% and a specific surface area of 227.1 m² g⁻¹ and total N of 0.47% (Mukome et al., 2013b). The walnut shell biochar was chosen specifically because it has been shown to perform well compared to other chars as a sorbent for pharmaceutical (and other) contaminants (Bair et al., 2016).

2.2 Chemicals

Biosolids were spiked with ciprofloxacin, TCS, and TCC (Sigma-Aldrich, St. Louis, MO, USA), at concentrations slightly higher than the maximum concentrations found in municipal biosolids from throughout the US (Table A.1) (U.S. Environmental Protection Agency, 2009). The spiking levels were determined by a mass balance approach to estimate the amount of plant-available pharmaceuticals based on sorption coefficients of the soil, biosolids, and biochar. The pharmaceuticals were spiked in air-dried biosolids in 0.1% formic acid in methanol, at concentrations of 100 mg kg⁻¹ ciprofloxacin, 200 mg kg⁻¹ TCS, and 500 mg kg⁻¹ TCC. The methanol was allowed to evaporate in a fume hood for 24 h before application of the biosolids to soil.

2.3 Experimental design and sample collection

A leaf crop (lettuce, *Lactuca sativa* L.) and root crop (carrot, *Daucus carota*) were planted in 4-L pots of 2 mm-sieved soil in a factorial design that combined three levels of biochar application (0, 10, and 100 t ha⁻¹), and two levels of biosolids application (0 and 20 t ha⁻¹). A high biochar application (100 t ha⁻¹) was chosen to assess if this rate resulted in more benefits (e.g., increased plant growth or decreased pharmaceutical bioavailability) than a more standard application rate (10 t ha⁻¹). A high biochar application rate (100 t ha⁻¹) has been shown to maximize crop productivity (Jeffery et al., 2011), and while this rate may not be economically feasible in broadcast application, targeted (banded) application may allow for high targeted rates of biochar inputs (Blackwell et al., 2010). Each biosolids-biochar treatment consisted of five replicates per plant type. Water-holding capacity was determined as detailed above for each biosolids-biochar treatment, and was maintained across treatments at 60-70% with DI water. Plants were grown in a temperature- and light-controlled greenhouse (Hoagland Hall, UC Davis, CA) from November 30, 2012, until harvest on January 30, 2013 (62 d). Upon harvest, soil samples were taken and deep-frozen (-80 °C) until further analysis. Above- and below-ground plant biomass values were determined after freeze-drying plant samples for 48 h.

2.4 Sample preparation and CALUX analysis

To directly assess the bioavailability of these compounds from soil, all samples were extracted with 18.2 MΩ-cm water (Barnstead Nanopure) instead of organic solvents (Andersson et al., 2009; Hale et al., 2012; Kanematsu et al., 2009). All references to the water extraction are to 18.2 MΩ-cm water. All extraction glassware was washed, rinsed in water, acid-washed in 1N H₂SO₄ and autoclaved for 30 min. Two grams of 2 mm-sieved air-dried soil in glass centrifuge tubes with Teflon-lined caps was extracted with 30 mL water in an ultrasonication bath for 30

min in the dark, followed by centrifugation at 3500 rpm for 20 min. The supernatant was filtered through Whatman GF/F glass fiber filters using Buchner funnels into glass culture tubes and the filtrate acidified to pH 2 with HCl. The sample was then concentrated using solid phase Oasis HLB 6-cc cartridges (Waters Corporation, Milford, MA) (Giudice and Young, 2011), and evaporated to dryness in glass vials at room temperature under a gentle stream of nitrogen. The dried residues were resuspended in 50 μ L of DMSO and analyzed using CALUX bioassays to determine the presence and relative concentration of activators of ER, AR, GR, PR and AhR signaling pathways (ER: 17 β Estradiol, AR: dihydroxytestosterone, GR: dexamethasone, PR: progesterone, and AhR: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)). CALUX bioassays were carried out as previously described (Giudice and Young, 2011) and the results expressed relative to the activity obtained with a maximal inducing concentration of the positive control chemicals for each receptor-selective CALUX bioassay, normalized per gram dry soil to allow for cross-treatment comparison.

2.5 LC/MS/MS

HPLC-ESI/MS/MS was used to determine recovery efficiency of the extraction method (SI, Tables A.4-6).

2.6 Statistical analysis

All statistical analyses were performed using the R computing package (R Core Team, 2016) version 3.2.4. For analysis of the CALUX bioassay, data from the lettuce and carrot trials were pooled. Two-way analysis of variance (ANOVA) was performed for CALUX bioassay and plant biomass data with biosolids and biochar levels as factors. We found no significant effect of the biosolids \times biochar interaction and therefore proceeded with analysis with biosolids and biochar as main effects. Tukey's honest significant differences (HSD) were used as post hoc tests to

assess treatment differences. Differences were determined to be statistically significant if $p < 0.05$. The relationship between CALUX bioassay activity levels and plant growth were assessed using regression analysis.

2.7 Data and analysis code availability

All data and analysis code will be available at <https://github.com/carolynanderson/calux> [Note that data and analysis code will be uploaded upon publication].

3. Results and discussion

3.1 CALUX bioassay detects endocrine active chemicals in biosolids-biochar co-amended soil

Soil samples from both the lettuce and carrot trials show low levels of AhR activity, in all combinations of biosolids-biochar treatments (Figure 1, Table 1). The addition of biosolids significantly increased the AhR activity in soils compared to no biosolids treatments (mean values from 4.7% to 6.9%), as did the addition of biochar compared to no biochar treatments (mean values from 4.3% to 7.4%), but this was only significant at the highest biochar application rate (100 t ha^{-1}). These results suggest that biosolids and biochar contain AhR active chemicals; this is supported by previous studies reporting the presence of the AhR agonist TCS in biosolids in trace quantities (Ahn et al., 2008). Interestingly, extracts from soil samples revealed no activators of the ER, AR, GR or PR CALUX bioassays (data not shown); the presence of antagonists of these receptors was not determined.

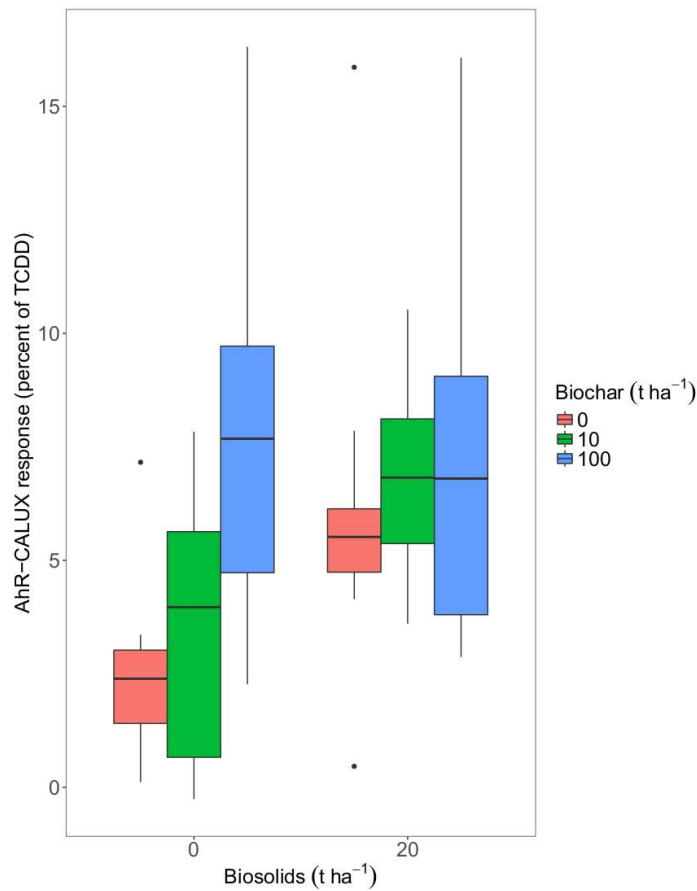


Figure 1. Aryl hydrocarbon receptor (AhR) activity in pooled soil extracts from biosolids-biochar treatments, as measured by the AhR-CALUX bioassay. AhR activity as expressed as relative light units normalized to the activity observed with a maximally inducing concentration of TCDD. Box plots show medians with the middle bars, surrounded on the top and bottom by the first and third quartiles, respectively. The highest and lowest data points within 1.5 times the interquartile range are shown by the whiskers; outliers are represented as points. [single column fitting image]

Table 1: Two-way ANOVA of AhR activity levels, with biosolids and biochar application rates as factors. Differences were determined to be statistically significant if $p < 0.05$.

Degrees of freedom, d.f.	Sum of squares, SS	Mean squares, MS	F-statistic	p-value
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Biosolids	1	75.31	75.31	6.13	0.0163
Biochar	2	123.18	61.59	5.02	0.0099
Residuals	56	687.68	12.28		

More specifically, in non-biosolids amended treatments, AhR activity increased with increasing biochar application rate (Figure 1), suggesting that this 900 °C walnut shell biochar itself may be a source for AhR active compounds. Based on this result, an extensive non-target analysis for biochar-associated compounds which may responsible for increased AhR activity is warranted. However, some of the compounds in biochar that could increase AhR activity include PAHs and dioxins (Quilliam et al., 2013; Stevens et al., 2009), both byproducts of biochar production, depending on the feedstock and production process and temperature (Hajaligol et al., 2001; Hale et al., 2012). Specifically, PAH levels exceeding environmental standards have been detected in biochars produced via gasification (i.e., in the presence of oxygen) (Cole et al., 2012; Hale et al., 2012), the method used to form the walnut shell biochar used in this study. This is in contrast to biochars formed via pyrolysis (i.e., in oxygen-limited conditions), which show decreasing PAH concentrations with increasing pyrolysis temperatures (Hale et al., 2012; Keiluweit et al., 2012; Zielińska and Oleszczuk, 2016). It is important to note that while the highest biochar application rate tested in our study (100 t ha⁻¹) was over 10 times the most common agronomic rate used in land application, benefits (as measured by crop productivity) have been demonstrated with biochar applications at this rate (Glaser et al., 2002; Jeffery et al., 2011). However, high rates of biochar application have also been shown to cause a change in microbial community composition and a physiological response to stress in Gram-negative

bacteria, in addition to a significant reduction in soil nitrate (Ippolito et al., 2014). Additionally, our use of an environmentally-relevant water-based extraction method most likely contributed to low concentrations of PAHs and dioxins extracted from these samples compared to that which could be obtained with a nonpolar solvent extraction method (e.g. methanol, toluene) (Andersson et al., 2009; Hale et al., 2012; Kanematsu et al., 2009), suggesting that endocrine active compounds may not be of significant concern under environmental conditions.

The relationship between individual biochar feedstock properties and the concentration and composition of PAHs and other AhR ligands is still poorly understood, as is the environmental fate of PAHs in a soil application of biochar (Bucheli et al., 2015; Dutta et al., 2016). Given that the AhR can bind a wide range of structurally diverse chemicals (DeGroot et al., 2011; Denison et al., 2011), biochar and biosolids could contain a variety of active chemicals, the identity of which remains to be determined. This warrants more research to assess AhR activity-inducing compounds in different biochars, and determine whether the presence of these compounds has negative implications for environmental or human health. The CALUX method is a novel approach that can directly and sensitively detect such activity, and can be used in tandem with traditional analytical approaches which by themselves may not be sufficient (Mayer et al., 2016).

3.2 Biochar and biosolids effects on lettuce growth

Only biochar, not biosolids, significantly affected lettuce shoot growth. The presence of biochar significantly increased shoot growth (10 t ha⁻¹ biochar = 16.0±0.32 g; 100 t ha⁻¹ biochar = 16.07±0.48 g) compared to no biochar (15.22±0.69 g; Figure 2a). In non-biosolids amended treatments, the presence of biochar significantly increased lettuce shoot growth (16.15±0.33 g for 10 t ha⁻¹ biochar, 15.91±0.38 g for 100 t ha⁻¹ biochar) compared to the non-biochar treatment (15.09±0.34 g).

Both biochar and biosolids treatments had significant effects on lettuce root growth. Lettuce plants that received any level of biochar had significantly higher root biomass (10 t ha⁻¹ biochar = 12.96±0.17 g; 100 t ha⁻¹ biochar = 13.12±0.25 g), than those without biochar (12.60±0.16 g) (Figure 2b). Biochar has been shown to stimulate root growth by increasing water and nutrient availability, aeration, and soil pH (Lehmann et al., 2011) (although in the current study plants were watered regularly, and thus positive effects on water retention are probably negligible).

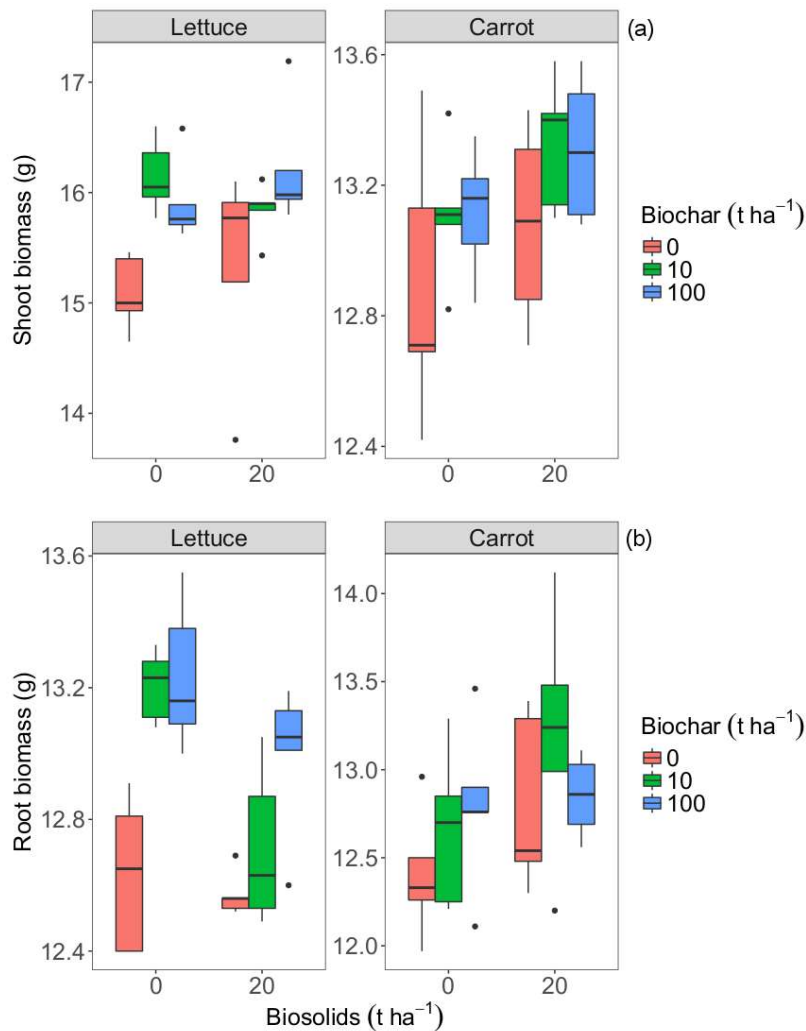


Figure 2. Growth differences in lettuce and carrot shoots (a) and roots (b) in biosolids and biochar treatments. Box plots show medians with the middle bars, surrounded on the top and bottom by the first and third quartiles,

respectively. The highest and lowest data points within 1.5 times the interquartile range are shown by the whiskers; outliers are represented as points. [1.5 column fitting image]

Biochar also appears to mitigate potentially negative effects of biosolids on lettuce root growth, but only at the highest biochar application rate in this study (Figure 2). In treatments without biosolids, lettuce root biomass significantly increased with the addition of biochar (10 t ha⁻¹ biochar = 13.21±0.11; 100 t ha⁻¹ biochar = 13.24±0.22) compared to no biochar (12.63±0.23 g). In the biosolids treatments, biochar significantly increased lettuce root biomass only in the 100 t ha⁻¹ biochar treatment (13.00±0.23 g) compared to the no biochar treatment (12.57±0.07 g). Further, in the absence of biochar there were no significant differences in lettuce root growth in biosolids-amended and unamended treatments. It is possible that compounds in biosolids (e.g., salts, excess nutrients) might negatively affect lettuce root growth (Andrés et al., 2011), and these results suggest that a high biochar application rates can overcome this. Similar greenhouse studies show that biochar feedstock type is an important determinant in the plant response to biochar. Different biochar feedstocks have been shown to increase corn, grass, and barley growth (Griffin et al., 2017; Jones et al., 2012; Prendergast-Miller et al., 2014; Rajkovich et al., 2012), although contrary to the results of the current study, this effect can be negated at high biochar application rates (e.g., 91 t ha⁻¹) (Rajkovich et al., 2012). Further, the effect of biochar can differ in greenhouse vs. field studies (Jones et al., 2012; Rajkovich et al., 2012).

Together, these root and shoot growth data suggest that the walnut shell biochar has a greater impact on lettuce growth than do biosolids, at least at the given application rates. While biochar on its own has been shown to increase plant growth (Khan et al., 2013; Lehmann et al., 2003), this positive effect could be negated by toxicity from high levels of volatile organic matter in some biochars (Deenik et al., 2010; Lehmann et al., 2011). Furthermore, while biosolids have

been shown to increase crop yield (Binder et al., 2002; Christie et al., 2001; Singh and Agrawal, 2008), potential differences in lettuce biomass in the current study between biosolids and non-biosolids treatments might have been obscured by additions of N fertilizer to non-biosolids pots to remove differences in fertility among the treatments.

3.2 Biochar and biosolids effects on carrot growth

In the carrot treatments, there were no significant differences between biosolids and biochar treatments in root or shoot growth (Figure 2). In contrast to what was observed in lettuce, the addition of biosolids, regardless of biochar concentration, significantly increased carrot shoot mass. This pattern of increased plant growth with biosolids was different from that found in the lettuce plants, suggesting that there might be different growth responses between lettuce and carrot plants in the presence of compounds found in biosolids. Differences in contaminant uptake in leaf and root crops have been demonstrated and attributed to both physiological and chemical differences in the plants. For example, the mechanisms of uptake of veterinary medicines by lettuce and carrot plants have been shown to follow Gaussian and non-Gaussian relationships, respectively (Boxall et al., 2006). Differences in uptake have also been ascribed to the chemistry of the contaminant and its matrix; for example, low uptake of TCS in radish root has been attributed to low ionization of TCS in the study soil (Carter et al., 2014). Such plant growth differences warrant further research, but this is outside the scope of the current study. It may be that any negative effects of pharmaceuticals in biosolids on carrot root and shoot growth are mitigated by the high carbon and nutrient contents of biosolids. Such trends are seen in crop yields (Singh and Agrawal, 2008) and in microbially-driven soil nutrient cycling processes, which either increase or remain unaltered in the presence of biosolids (Park et al., 2013). It is also possible that pharmaceuticals or other contaminants in biosolids are sorbed strongly to the

organic matter matrix of the biosolid itself, or biochar if present, limiting plant uptake (Yao et al., 2012), although the sorption dynamics depend on the individual contaminant and biosolids or biochar matrix. Further, as previously mentioned, the uptake dynamics between lettuce versus carrot plants depend on physiological differences as well as chemical differences in the compound, which may explain why these plants responded differently to the treatments in regards to growth.

3.3 Environmental implications

While we hypothesized that the addition of biochar would decrease endocrine activity associated with biosolids treatments, high biochar application rates (100 t ha^{-1}) actually increased activity levels, albeit by a small but significant amount. This suggested that this particular biochar may be a source of AhR active compounds, which may reflect the presence of PAHs or numerous other classes of AhR agonists (DeGroot et al., 2011; Denison et al., 2011) in the biochar-amended soil. Together, these results indicate that while biochar (walnut shell, $900 \text{ }^{\circ}\text{C}$) can positively affect plant growth, it might also contribute chemicals into the soil that potentially could have endocrine-disrupting activity. The positive CALUX bioassay results presented here suggest the need for non-target analysis of the biochar to confirm and quantify which chemicals are inducing this response.

The biochar rate that induced this effect (100 t ha^{-1}) was above the average agronomic rate ($10\text{-}20 \text{ t ha}^{-1}$), so while a small increase in AhR activity was observed with the 10 t ha^{-1} sample, it is unlikely that biochar at normal environmental application levels would be of toxicological concern. Further, the AhR activity in the soil does not appear to be coupled with effects on plant growth (Figure 3, $R^2 < 0.10$ and $p > 0.10$ for all plant/tissue combinations), suggesting that low

AhR activity associated with biochar does not affect crop yield.

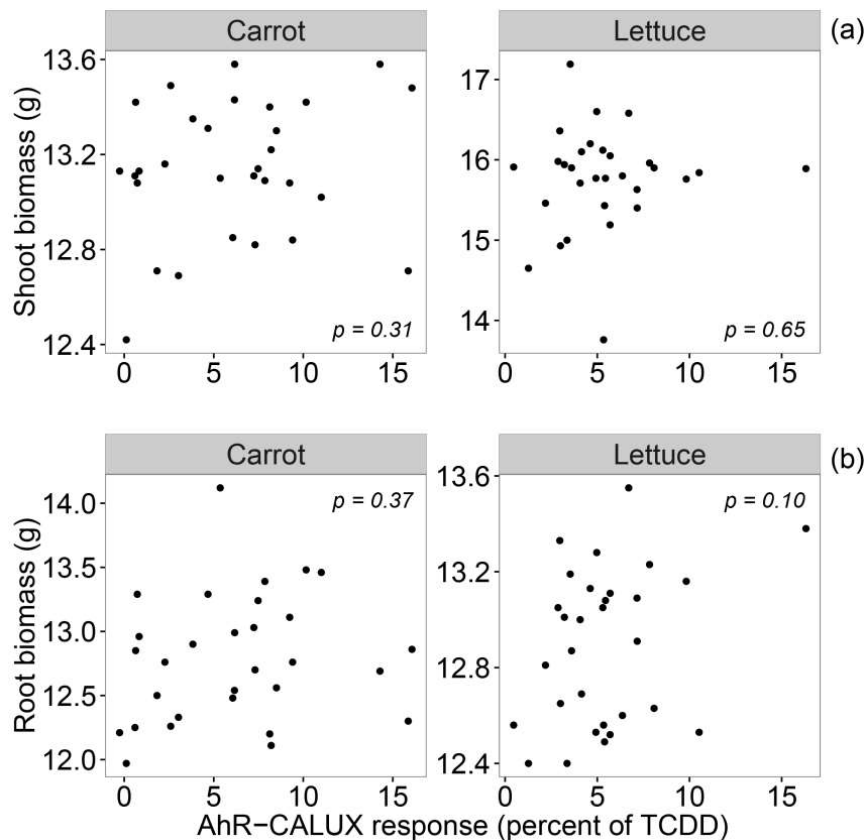


Figure 3. Regressions of plant biomass versus soil AhR activity for (a) shoots and (b) roots. P-values from linear regressions are reported in each panel. [1.5 column fitting image]

The PAHs potentially present in biochars may be taken up by crops, resulting in a potential source of exposure to humans (Zohair et al., 2006). Therefore, to ensure agronomic safety of biochar application, the presence and activity of endocrine-active compounds in biochar should be explored further, using both quantitative analytical tools to measure PAHs, dioxins, and other endocrine-active compounds. Highly sensitive bioanalytical assays such as CALUX can help to assess the biological activity of such compounds. While the biochar used in this study (walnut shell, 900 °C) is an isolated example and these results do not apply to all chars, the method

developed in this study provides a tool for rapid screening of complex environmental matrices including other biochars, and the results suggest a need to perform non-target analysis on this char. CALUX has considerable potential as a low-cost, sensitive tool to screen biological and ecological effects of complex mixtures of compounds in environmental samples.

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