EXAMINING THE FEASIBILITY OF VERMICOMPOSTING BIOSOLIDS PRIOR TO LAND APPLICATION TO REMOVE TRICLOSAN AND METHYL TRICLOSAN

by

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ABSTRACT

Examining the feasibility of vermicomposting municipal biosolids prior to land application to remove triclosan and methyl triclosan

Whitney P. Weibel

More than 4 million dry tons of nutrient-rich biosolids are applied to land every year in the United States. Current wastewater treatment plants (WWTPs) are ineffective in removing all pharmaceuticals and personal care products (PPCPs) from biosolids prior to land application. Triclosan (TCS) is a widely used broad-spectrum bacteriostatic. Both TCS and its degraded form, methyl triclosan (Me-TCS), are hydrophobic and accumulate in biosolids. As potential endocrine disruptors, removing TCS and Me-TCS from biosolids is crucial. The feasibility of using earthworms (*Eisenia fetida*) to vermicompost biosolids sourced from the City of Tacoma, Pierce County and the City of Lynden was examined. Method development included determining the ratio of biosolids to paper mulch that would allow for earthworm survival. Potassium, phosphorus, total organic carbon, total Kjehldahl nitrogen (TKN), and pH were also evaluated in the biosolids based on earthworm survival and reproduction. Total organic carbon appeared to be positively associated with earthworm survival and TNK inversely so, which is believed to be due to the presence of ammonia that is toxic to earthworms. Due to instrument uncertainty and the lack of replication, the effect of earthworms on TCS concentrations was inconclusive for all three biosolids sources. If earthworms were left in the substrate for more time, perhaps there would have been a discernable difference in measured TCS and Me-TCS concentrations. However, the presence of earthworms increased the concentrations of Me-TCS, compared to a control, in the substrate composed of the City of Tacoma's biosolids. The difference observed in the City of Tacoma's biosolids is believed to be due to the initial concentration of TCS that then degraded into Me-TCS. Based on past research (Domínguez, Aira, and Gómez-Brandón, 2010), it is believed microbes excreted in *E. fetida's* feces contributed to the increase in Me-TCS formation the City of Tacoma's biosolids.

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List of Acronyms

BAF	Bioaccumulation factor
C	Celsius
EQ	Exceptional quality
g	Gram
GC/MS/MS	Gas chromatograph double mass spectrometer
K	Potassium
kg	Kilogram
L	Liter
µg	Microgram
M	Mean
Me-TCS	Methyl triclosan
NH_3	Ammonia
NH_4^+	Ammonium
P	Phosphorus
PPCP	Pharmaceuticals and personal care products
SD	Standard deviation
SE	Standard error
TCC	Triclocarban
TCS	Triclosan
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
U. S. EPA	United States Environmental Protection Agency
U. S. FDA	United State Food and Drug Administration
WWTP	Wastewater treatment plant

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Do mischief.

1. Introduction

In the United States, wastewater treatment plants (WWTPs) generate over eight million dry tons of biosolids annually, which are the end result of the wastewater treatment process (U. S. Environmental Protection Agency, 1999). Biosolids are generated from both residential and industrial wastewater treatment and can contain heavy metals, pathogens, plus pharmaceuticals and personal care products (PPCPs) such as hormones, steroids, antibiotics soaps, shampoos, and household cleaners (Xia, Bhandari, Das, & Pillar, 2005). Rather than incinerating or dumping biosolids in landfills, a number of cities around the world reuse the biosolids to amend soils because they have plant-available nitrogen and phosphorus (Jacobs & McCreary, 2001). At least 55 percent of biosolids produced in the U. S. are reused in a beneficial manner: applied to land for restoration purposes, and in forestry and agricultural practices (North East Biosolids & Residuals Association, 2007). Despite the contamination, the processed biosolids are a nutrient-rich soil additive that homeowners, farmers, landscapers and forest managers can use (Outwater, 1994).

While most pathogens and heavy metals are removed from the final product, per government standards, anthropogenic chemical compounds have been found to persist in the final product destined for land application (Kinney et al., 2006). For example, one study found that one third to one half of the anthropogenic contaminants were not removed and were consistently present in biosolids, suggesting current wastewater treatment processes are not effective at removing PPCPs from biosolids (Kinney et al., 2006). The ecosystems where biosolids are applied can become contaminated with the chemicals, which in turn can be taken up by flora and fauna and leach into water sources

(Prosser & Sibley, 2015). Solutions are needed to remove these contaminants from the biosolids before they are scattered throughout the environment.

This study focuses on using earthworms as a potential solution for removing PPCPs from biosolids destined for land application. Earthworms have been used to process biosolids, to enrich and aerate the soil and make nutrients, like phosphorus and nitrogen, readily available for plant use (Rajpal, Bhargava, Chopra, & Kumar, 2014; Sinha, Bharambe, & Chaudhari, 2008; Sinha, Herat, Bharambe, & Brahamhatt, 2009; Singh & Suthar, 2012; Suthar & Singh, 2008). Earthworms have also been found to take up, or bioaccumulate, chemical contaminants and heavy metals found in biosolids-amended soils (Higgins, Paesani, Chalew, Halden, & Hundal, 2011; Kinney et al., 2006, 2008, 2010, 2012; Macherius et al., 2014, Pannu, O'Connor, & Toor, 2012). However, their ability to bioaccumulate PPCPs from biosolids derived from WWTPs for purposes of removal remains largely unexplored.

Biosolids are a great alternative to chemical fertilizers to enrich soils, but if the biosolids are polluted with anthropogenic chemical contaminants, application of biosolids may be doing the environment a disservice. Furthermore, if the anthropogenic chemical contaminants are antimicrobials they may render beneficial bacteria and other microorganisms ineffective (World Health Organization, 2016). Triclosan (TCS) and its degraded form, methyl triclosan (Me-TCS), are anthropogenic chemical contaminants, which are also antimicrobials, used in common household and industry products. Triclosan has endocrine disrupting properties seen in rats (Jung, An, Choi, & Jeung, 2012) as well as human tissue (Ahn et al., 2008; Gee, Charles, Taylor, & Darbre, 2008). Additionally, researchers have observed positive associations between TCS exposure and

earlier onset of puberty (Wolff et al., 2010) and increased body mass index (Lankester, Patel, Cullen, Ley & Parsonnet, 2013).

Triclosan and Me-TCS are also hydrophobic and therefore are more readily found in biosolids rather than the processed wastewater (Chenxi, Spongberg, & Witter, 2008; Lozano, Rice, Ramirez & Torrents, 2013; Ying & Kookana, 2007). If earthworms are capable of bioaccumulating antimicrobial agents, like TCS and Me-TCS, perhaps they can be used to remove them and other contaminants that are not being removed by current WWTP processes in order to produce a cleaner product for land application and soil amendment purposes. The possibility of incorporating earthworms into large scale processing of biosolids, specifically for removal of TCS, and Me-TCS, has to the extent of my knowledge yet to be evaluated and has significant potential for research opportunities. Evaluating earthworms' ability to bioaccumulate and subsequently remove TCS and Me-TCS from biosolids prior to land application is an initial step to determining the potential of PPCP removal from biosolids.

To understand how earthworms can be incorporated in the biosolids process we must first understand TCS, Me-TCS, and other PPCPs, their source, impact on humans, and route into the environment. The historical use of land-applied biosolids and impact on the environment is discussed as well as the fate and prevalence of TCS and Me-TCS in the environment. Next, to demonstrate why a solution is needed, the shortcomings of current wastewater treatment processes are explored; specifically, the challenge of removing anthropogenic chemical contaminants, which can enter the environment through the land application of biosolids. A discussion of bioremediation, a mechanism used to remove chemicals and other anthropogenic contaminants from soils, will follow.

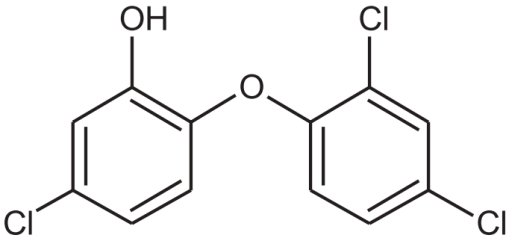
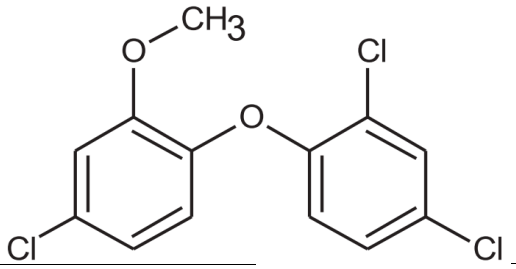
Because there is an appreciable use for the biosolids waste and with future limits on the disposal of biosolids in expensive and spatially limited landfills, a solution is needed to reduce the amount of pollutants in the biosolids that we add to the environment. Finally, this thesis is discussed and how it fills gaps in the literature and expands the possibility of incorporating earthworms into processing of biosolids.

2.1. Triclosan and methyl triclosan

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether, see Table 2.1) is a synthetic broad-spectrum bactericide (Orhan, Kut, & Gunseoglu, 2007; Reiss, Lewis, & Griffin, 2009). Methyl triclosan is the biodegraded product of TCS and has been found to be more persistent in soils with a half-life four times that of TCS (104 days versus 443 days; Lozano, Rice, Ramirez & Torrents, 2012). While the exact process is not entirely known, Me-TCS is most likely formed by microbial methylation within WWTPs (Boehmer, Ruedel, Wenzel & Schroeter-Kermani, 2004) during aerobic digestion and in anoxic conditions (Chen et al., 2011). The "cleaned" wastewater that leaves the treatment plant is called the effluent and concentrations of Me-TCS, relative to TCS, were found to be greater than three times that of the wastewater that comes into the treatment plant, which is called the influent (Lindström et al., 2002). The half-life of TCS depends on the medium in which it is found: air-borne TCS has a half-life of one day, 60 days in water, 120 days in soil, and 540 days in sediment; the researchers offered no explanation for these differences in the TCS half-lives they calculated from models they created but is perhaps due to oxygen availability (Halden & Paull, 2005). However, other research indicates that TCS has a half-life of over one year in water (Ciba-Geugy Limited, 1990, as cited in Ohron, 2014). In sewage sludge TCS has been observed to have a half-life of

315 to 770 days depending on its depth within a settling bed (Chen, Pauly, Rehfus & Bester, 2009). This variation in half-life is most likely due to environmental factors such as pH, temperature, and oxygen levels. The long half-life of TCS in sewage sludge suggests the need for alternative methods for its removal.

Table 2.1. Physiochemical properties of triclosan and methyl triclosan

Triclosan 	Chemical formula: $C_{12}H_7Cl_3O_2$ CAS number: 3380-34-5 Molecular mass: 289.54 g mol^{-1} log K_{ow}: 4.2-4.8 Melting point: 55°C (131°F) Boiling point: 120°C (248°F) Water solubility: 4.621 mg L^{-1}
Methyl triclosan 	Chemical formula: $C_{13}H_9Cl_3O_2$ CAS number: 4640-01-1 Molecular mass: 303.56 g mol^{-1} log K_{ow}: 5 Melting point: 43-45°C (109-113°F) Boiling point: 358.7°C (678°F) Water solubility: 0.4 mg L^{-1}

Sources: Chen et al., 2011; National Center for Biotechnology Information (n.d.); Toronto Research Chemicals (n.d.).
Note: Chemical Abstract Services is abbreviated as CAS. The partition coefficient is log K_{ow}, which is the ratio of a chemical's concentration in octanol relative to the chemical's concentration in water; it is an indicator of a compound's lipophilicity. Compounds can be considered highly lipophilic with measurements up to 6 and 6.5 (Organization for Economic Cooperation and Development, 2002).

The European Commission's Scientific Committee on Consumer Safety (SCCS) (2010) stated that there is little known regarding the biochemistry of the biodegradation of TCS. The SCCS states that the Minnesota Biocatalysis and Biodegradation Database, which was inaccessible during the time of this writing, has nothing documented for TCS yet the database claims to contain information regarding xenobiotic, or synthetic, chemical compounds (University of Minnesota, 2016). Therefore, there is a gap in knowledge of biodegradation of TCS and intermediary products.

2.2. Sources of triclosan and other pharmaceutical and personal care products

Triclosan was patented in 1964 and introduced as a surgical scrub in United States in 1972 (Halden, 2014; Jones, Jampani, Newman, & Lee, 2000) and was quickly incorporated into many home and personal care products, including toothpastes, cosmetics, soaps and even plastics (Reiss et al., 2009). By 1978 it was detected in aquatic environments and sediments (Hites & Lopez-Avila, 1979; Jungclaus, Avila, & Hites, 1978) and by 1981 Me-TCS was detected in fish (Miyazaki, Yamagishi, & Matsumoto, 1984).

During the writing of this thesis, the United States Food and Drug Administration (U. S. FDA) enacted a rule banning the use of 19 antiseptic chemicals, including TCS, in over-the-counter products intended for consumer use as a wash, which is to go into effect September 6, 2017 (Safety and effectiveness of consumer antiseptics, 2016). It must be stressed that this ban is only for over-the-counter consumer antiseptic washes; the ban does not include products used in food service, hospitals, in products that are not rinsed off after use, or its use in materials such as plastics and other items. Therefore, TCS and Me-TCS will continue be used and enter WWTPs.

The U. S. Environmental Protection Agency (U. S. EPA) lists 88 synonyms for triclosan. Some of the most common in consumer goods include Microban®, Biofresh®, Irgasan® DP 300, Lexol 300, and Cloxifenolum (EPA, 2016; Glaser, 2004; entire list can be found in Appendix A). According to Microban®'s website (2016), working in multiple industries, products are infused with TCS during the manufacturing process. This process of infusing materials with anti-microbial TCS creates a material that is inhabitable for microbes. As one would expect it can be found in medical products such

as scrubs, masks, and polymer-based storage containers. However, it is also found in everyday household materials such as air filters, kitchen and bathroom fixtures, carpets, countertops, insulation, door hardware, paints, tile, and grout; not to mention clothing, cutting boards, and children's toys. Microban® also markets products for commercial use from food prep and storage, baby changing stations and highchairs, elevator buttons and paper towel dispensers.

In general, TCS can be found in hand and dish soaps, face wash, toothpaste and toothbrushes, cosmetics, deodorant, household cleaners, as well as various other household personal care products and wares (Glaser, 2004). About 96 percent of products that contain TCS are ultimately washed down the drain to WWTPs (Reiss, Mackay, Habig, & Griffin, 2002).

Antibacterial hand soaps, for consumer use, can contain TCS concentrations ranging from 0.1% to 0.45% (weight/volume). This amount results in the total concentration of TCS in your average bottle of hand soap to contain 0.221 g/mL to 0.994 g/mL, respectively.

2.3. Triclosan's impact on humans

The direct impact of TCS on human health is not totally understood but it has been determined that TCS-resistant microbes can become more abundant merely through exposure to TCS (McNamara, LaPara, & Novak, 2014). The possibility of antibiotic resistance prompted Minnesota's Governor, Mark Dayton, to sign a bill that will go into effect January 2017 banning all products containing TCS (Landau & Young, 2014). Also, in an effort to "'protect our Great Lakes and water supplies'" New York State Senator, Tim Kennedy, announced legislation that would prohibit the sale of household cleaning

products containing TCS, and derivatives such as triclocarban, in the state of New York (Pignataro, 2015). In September 2006 Germany disallowed the use of TCS in plastics in contact with food (European Commission, 2010). The United States Food and Drug Administration (FDA) (2013) states that there is no evidence to support the idea that using antibacterial soap is any better than using regular soap when washing hands. It has even been argued that using antibacterial soap may lead to a false sense of security leading individuals to not wash their hands as well because they believe antibacterial soap does a better job than regular soap (Alliance for the Prudent Use of Antibiotics, 2011). On September 2, 2016, the FDA banned the use of TCS and triclocarban (TCC), along with 17 other chemicals, from soaps but the U. S. EPA will still allow it in other household textiles, such as cutting boards, toys, and hairbrushes (Safety and Effectiveness of Consumer Antiseptics, 2016). Methyl triclosan is not included in the list of banned chemicals because it is merely a byproduct of TCS, not a chemical that is produced for a specific use, other than laboratory testing. These recent events regarding the banning of TCS indicates a growing concern over the wide spread use in many consumer products.

Researchers have evaluated the presence of TCS in humans, specifically testing breast milk of nursing mothers, blood, and urine. Of 36 nursing mothers, those who used toothpaste, deodorant or soap that contained TCS had higher concentrations of the contaminant in their breast milk (0.022 to 0.95 μg/g) compared to mothers who did not use PPCPs not containing TCS (<0.018 to 0.35 μg/g) (Allmyr, Adolfsson-Erici, McLachlan, & Sandborgh-Englund, 2006). It should be noted that TCS concentrations were also measured in the blood plasma of each mother and significantly more TCS was

found there than in the milk, indicating the infants were not exposed to TCS as much as their mothers were. Additionally, every participant in the study had TCS present in their body indicating that PPCPs are only one route of TCS exposure for the mothers since some participants indicated not using products containing TCS.

The National Health and Nutrition Examination Survey, conducted annually by the Centers for Disease Control and Prevention in the U. S., was the source of 2,517 urine samples evaluated for TCS concentrations (Calafat, Ye, Wong, Reidy, & Needham, 2008). Triclosan was present in 74.6 percent of the samples with values ranging from 2.4 to 3,790 µg/L. The researchers found concentrations correlated to age and socio-economic status. Specifically, concentrations of TCS were highest among individuals in their thirties and those in higher-income households.

Triclosan is considered an endocrine disruptor, indicating it interferes with the hormonal system in mammals (Chen et al., 2008). Exposure to TCS is also considered a potential contributor to the development of breast cancer, and may otherwise be harmful to the immune systems of mammals (Bertelsen et al., 2013; Clayton, Erin, Todd, Dowd & Aiello, 2010). Studies evaluating the effect TCS has on humans are limited but there are studies that utilize rats to test TCS endocrine disrupting properties. Jung (2012) studied the effects TCS has on rats' hormone receptors, finding TCS blocked specific estrogen receptors. Jung's results support the potential human health risks of TCS. In vitro research found that TCS blocks estrogen and androgen receptors in tissue (Ahn et al., 2008; Gee et al., 2008). Evaluating 2,058 male and 1,979 female human participants (average age 49, 35-65), researchers found a positive association with TCS exposure, measured from a urine sample, and an increased body mass index (Lankester et al.,

2013). In 2010, Wolff et al. followed 1,151 girls (ages six to eight) through puberty to evaluate the relationship of breast and pubic hair development, in the U. S. Researchers found a slightly inverse association with pubic hair development and TCS exposure (measured from a urine sample). The results suggest a potential connection between TCS exposure and a child's development through puberty. Research on the impact TCS has on humans is, by no means, conclusive or complete but there is evidence to suggest there are negative consequences to TCS exposure whether the one being exposed is a rat or human.

2.4. Pathways by which triclosan and PPCPs enter the environment

The ecological risk of TCS and other PPCPs exposure through leaching from land-applied biosolids or in the effluent water from WWTPs has been widely reviewed (Coogan & Point, 2008; Fuchsman et al., 2010; Kreuzinger, Clara, Strenn, & Vogel, 2004; Reiss et al., 2002, 2009; Ying & Kookana, 2007; Xia et al., 2010). Researchers use either modeling or laboratory simulations to evaluate risks of TCS. Reiss et al. (2002) consider TCS concentrations in lotic, or flowing water aquatic systems, to be of little to no concern for fish and vertebrates but that some algae, especially those close to WWTP effluent discharge locations, have some risk. In 2009, Reiss et al. evaluated the TCS concentrations and associated potential risk to earthworms; again concluding no significant risk was indicated in their research. This is to be expected because TCS, even amounts up to 300 mg/kg in soil, was not lethal to earthworms but did damage the DNA of *E. fetida* (Lin, Zhou, Xie, & Liu, 2010).

Ying and Kookana (2007) conducted a preliminary risk assessment of a "worst-case scenario" of TCS concentrations in WWTP effluent (mean (*M*)=142 µg/L, maximum=434 µg/L, minimum=23 µg/L) measured in Australia and the biosolids

(*M*=5.58 mg/kg, maximum=16.69 mg/kg, minimum=0.09 mg/kg). Through their analysis of the limited available literature on the toxicity of TCS, they calculated a "risk quotient" where values greater than one indicates risk and values less than one do not. Ying and Kookana calculated the risk quotient for applying biosolids to land and in effluence from WWTPs as 1.36 and 1.5, respectively. This indicates there is a risk of TCS effecting soils when biosolids are applied to land and effecting aquatic organisms from WWTP effluence.

Coogan and Point (2008) set up an experiment where aquatic snails and algae were exposed to WWTP effluence in Texas to determine how much each would bioaccumulate TCS and Me-TCS, among other PPCPs. After two weeks of exposure snail tissue samples were tested and compared to tissue samples collected prior to exposure. Algal tissue was compared to surrounding water, rather than a pre and post-exposure comparison. The researchers found that prior to exposure, snails had TCS and Me-TCS concentrations of 5.9 µg/kg and 0.8 µh/kg, respectively, and 58.7 µg/kg (standard error (*SE*)=3.39) and 49.8 µg/kg (*SE*=2.49) after two weeks of exposure to WWTP effluence. The water surrounding the alga was measured at 0.112 µg/kg of TCS and 0.041 µg/kg of Me-TCS and after two weeks of exposure TCS and Me-TCS algal concentrations measured 162 µg/kg (*SE*=17.6) and 50.4 µg/kg (*SE*=5.21). This research indicates there is definitely bioaccumulation of TCS and its degraded form Me-TCS by the snails and algae tested in this study.

Fair et al. (2009) evaluated the plasma of wild Atlantic bottlenose dolphins (*Tursiops truncatus*) blood samples for the presence of TCS. Of 26 individual dolphins sampled, 27% had detectable levels of TCS (≥0.033 µg/g) in blood samples. While the

levels detected in sampled dolphins does pose a lethal threat, the effects of chronic exposure is unknown. The researchers express concern with the increasing human population living in coastal communities leading to an increase in WWTP effluence into waters inhabited by dolphins.

Through the land application of biosolids, PPCPs can leach into groundwater (Kreuzinger et al., 2004; Xia et al., 2010). Triclosan and other PPCPs can potentially accumulate in the food chain and travel to higher trophic levels through the uptake of plants and animals living in soils amended with biosolids (Kelsey, Colino, & White, 2005; Kinney et al., 2010; Harris et al., 2000; Wild, Harrad, & Jones, 1994). Xia et al. (2010) observed leaching of TCS in biosolids-amended soils in their evaluation of field-applied biosolids. By measuring TCS concentrations at various depths in a field that was amended with biosolids, they determined that there is a potential for TCS leaching but believe transformation of TCS was likely the cause for decreased concentration measurements in soil samples.

Agyin-Birikorang, Miller, and O'Connor (2010) evaluated the difference between soil, biosolids, and biosolids-amended soils and retention of TCS. Due to its hydrophobic nature, TCS is more likely to be in biosolids rather than WWTPs' effluent. Within the biosolids tested, the TCS appeared to be retained whereas in the unamended sandy soils, greater mobility, or leaching, of TCS was observed. Overall, they found that substrates with high organic carbon resulted in lower mobility of the compound, compared to substrates with higher organic carbon.

2.5. Historical use of land-applied biosolids in the United States

Regulations established back in the 1970's focused mostly on pollutants common during that time (heavy metals) and pathogen reduction. Pharmaceuticals and personal care products were not addressed because they were not of concern, but as more PPCPs are becoming more readily available and developed at accelerated rates, they are getting more attention (Hildebrandt, 2007).

The processing of biosolids for consumer use results in a mostly odorless soil with minimal pathogens; the amount of pathogens allowed in biosolids designated for land-application use must meet specific U. S. Environmental Protection Agency (U. S. EPA) standards to qualify as Class A or B Biosolids (U. S. EPA, 2016). Compared to anaerobic digestion, the final product of aerobically digested sludge is considered to have a higher fertilizer value, in terms of available nitrogen, phosphorus, potassium, pH, and carbon-nitrogen ratio (Outwater, 1994).

2.6. Environmental impacts of triclosan and methyl triclosan

Triclosan has been found to be toxic in aquatic and terrestrial ecosystems. In a survey of 139 U. S. streams TCS was among the seven most common anthropogenic compounds detected. Effluent, or the processed wastewater, from WWTPs, and land application of biosolids are the primary routes for TCS to enter the environment (Ying & Kookana, 2007). In fish, TCS has been observed to cause lengthening of fins and changes in sex ratios (la Farré, Pérez, Kantiani, & Barceló, 2008) and in frogs, disruption in thyroid hormone and associated gene expression has been observed, affecting the metamorphosis process from tadpole to frog (Veldhoen et al., 2006). While very few

studies have evaluated the effects of Me-TCS, one study did fine it to be toxic to blood cells in abalone (Gaume et al., 2012).

Triclosan and Me-TCS have been found to be toxic to aquatic organisms by fragmenting or causing irreversible damage to DNA (Binelli, Cogni, Parolini, Riva, & Provini, 2009; DeLorenzo et al., 2008; la Farré et al., 2008). Both TCS and Me-TCS have been found to bioaccumulate in fish, algae, earthworms, and snails (Balmer et al., 2004; Boehmer, Ruedel, Wenzel, & Schroeter-Kermani, 2004; Coogan, Edziyie, La Point, & Venables, 2007; Higgins et al., 2009/2011; Kinney et al., 2008; Snyder, O'Connor, % McAvoy, 2011). Fish and aquatic invertebrates ingest triclosan by feeding on organisms that live in contaminated soils. The concentration of Me-TCS was measured in fish in various lakes in Switzerland WWTP effluence. The concentrations of Me-TCS measured up to 35 μg/g (wet weight) compared to no detectable levels of Me-TCS in fish from a remote lake receiving no WWTP effluence (Balmer et al., 2004). Although it has not been studied directly, it is assumed that Me-TCS could have effects similar as TCS in the environment and, consequently, organisms (Reiss et al., 2009).

Triclosan has also been shown to move through the food web affecting different species of birds and mammals. Triclosan and Me-TCS are lipophilic, meaning they readily pass through cells' walls and accumulate in fats and lipids (Chedgzoy, Winckle & Heard, 2002). The accumulation of TCS and Me-TCS allows for the contaminants to move up through the food chain through consumption of plants or animals, like earthworms or soil microorganisms, by birds and fish (Reiss et al., 2009).

2.7. Factors affecting triclosan and methyl triclosan stability

There are many processes, such as biodegradation, methylation, chlorination, photolysis, and combustion that can transform TCS into other compounds. Biological methylation of TCS results in Me-TCS (Bester, 2005; Boehmer et al., 2004). Photolysis, or photodegradation, of TCS in aqueous solutions results in 2,8-dichlorodibenzodioxin and other dioxin derivatives (Aranami & Readman, 2007; Latch, Packer, Arnold, & McNeill, 2003; Mezcua et al., 2004; Lores, Llompart, Sanchez-Prado, Garcia-Jares, & Cela, 2005; Sanchez-Prado et al., 2006) which the U. S. EPA (2015) states are persistent organic pollutants, are highly toxic, and carcinogenic, and can accumulate in the food chain. Combustion of TCS leads to another dioxin, the formation of di- and trichlorodibenzo-p-dioxin (Kanetoshi, Ogawa, Katsura, Okui, & Kaneshima, 1988). Chlorophenols, specifically 2,4,6-trichlorophenol and 2,4-dichlorophenol (Kanetoshi Ogawa, Katsura, & Kaneshima, 1987; Rule, Ebbett, & Vikesland, 2005; Canosa et al., 2005; Greyshock & Vikesland, 2006), are transformations of TCS that are on the U. S. EPA's "Priority Pollutant List" (Effluent limitations guidelines and standards, 2013). Finally, when TCS reacts with chlorine, such as right before treated water is discharged from a WWTP, chloroform is formed (Fiss, Rule, & Vikesland, 2007; Greyshock & Vikesland, 2006; Rule et al., 2005). Chloroform was used as an anesthetic for nearly 100 years, from the 1847 to the beginning of its decline in 1932 (Wawersik, 1996). In humans, chloroform was found to cause jaundice of the liver, depression of the central nervous system decreasing respiratory rates, as well as effects on the heart and kidneys (U. S. EPA, 2016). Chloroform is not believed to be a major concern in harming the environment or human exposure to chloroform in the environment unless there is a spill

or some other occurrence of extremely high quantities being exposed to nature (Scottish Environment Protection Agency, n.d.). However, the U. S. EPA has classified chloroform as a probable carcinogen to humans (U. S. EPA, 2000).

The product of transformed and degraded TCS can result in toxic and non-toxic compounds (National Industrial Chemicals Notifications and Assessment Scheme [NICNAS], 2009). Under dark, anaerobic conditions, TCS is quite stable (McAvoy, Schatowitz, Jacob, Hauk, & Eckhoff, 2002). However, in aerobic conditions TCS degrades more readily into Me-TCS and, both being hydrophobic, tend to concentrate in the solids and are removed from the water during the wastewater treatment process. When TCS enters a WWTP most stays within the dewatered biosolids even after extensive plant processes and treatments (Lozano et al, 2013; Chenxi et al., 2008; Ying & Kookana, 2007). On average, 96 percent of TCS that entered WWTPs was "eliminated" from the wastewater effluent but 30 percent was found in the sludge (Bester, 2003). The researcher did not test for any other compounds that could have transformed from TCS in the study, which may explain the 66 percent of unaccounted for TCS. Methyl triclosan occurs at much lower concentrations than TCS. However, Me-TCS is more hydrophobic, persistent in the environment because is more resistant to biodegradation and photolysis, and more lipophilic making it more readily bioaccumulative than its parent form, (Dann & Hontela, 2011; Mackay, & Barnthouse, 2010; NICNAS, 2009). Another factor that can affect the transformation of TCS is whether oxygen is present or not.

To evaluate the effect oxygen has on the degradation of TCS and the formation of Me-TCS, Chen et al. (2011) measured the change of TCS and Me-TCS concentrations in sludge in three environments, aerobic, anaerobic and anoxic before and after an 80-hour

period. Adding a constant flow of oxygen through the substrate created the aerobic condition, a constant flushing of nitrogen gas and potassium nitrate (KNO_3) maintained the anaerobic and anoxic conditions, respectively. They found, in the aerobic environment, TCS concentrations decreased by 49 percent (30 µg/L to 15 µg/L) whereas Me-TCS concentrations increased by 16 percent (4.5 µg/L to 5 µg/L). The anoxic environment decreased TCS concentrations by 16 percent (32 µg/L to 29 µg/L) and a 17 percent increase in Me-TCS concentration (4.1 µg/L to 4.8 µg/L). Anaerobic environment decreased TCS concentrations 11 percent (32 µg/L to 28 µg/L) but no change in Me-TCS concentrations was detected. Overall, they determined that only one percent of TCS degraded into Me-TCS in aerobic conditions, less in anoxic conditions, and no TCS was observed to degrade into Me-TCS in anaerobic conditions.

In their evaluation of the effects of natural conditions on the degradation of TCS and formation of Me-TCS from biosolids applied to fields, Butler, Whelan, Sakrabani and Van Egmond (2012) observed a seasonal affect. They measured the greatest decrease in TCS concentrations between July and October when soil moisture was low and temperatures were warm. In loamy sand soil a 59 percent decrease in TCS was measured, whereas in sandy clay loam and clay soils a 72 and 74 percent decrease in TCS concentration was measured, respectively. They believe the temperature was a factor in its impact on microbial activity affecting TCS biodegradation and moisture in soil was believed to create a more anaerobic condition, in which TCS did not degraded into Me-TCS. They also measured the formation of Me-TCS over the course of a year observing 66% percent of TCS degrade into Me-TCS in sandy clay loam, 64 percent in loamy sand, and 39 percent in clay soils.

Biosolids are typically stored in piles or tanks prior to land application, such as on a farm or in a garden. Chenxi et al. (2008) evaluated the persistence of seven pharmaceuticals and one antibacterial compound, TCS, in stored biosolids over a 77-day period. They tested the biological degradation of the compounds in the biosolids in aerobic and anaerobic conditions, with and without light, at varied lengths of time. Continuously pumping air through the biosolids in a bucket created the aerobic condition and putting a lid on the bucket to restrict airflow created the anaerobic condition. Triclosan, along with three other pharmaceuticals, showed no change in concentration in any of the conditions tested. Considering previous research suggested a reduction of TCS, Chenxi et al. attribute their results of no change on the "strong affiliation of TCS to the organic-rich particles in biosolids and the resulting strong sorption might prevent TCS from photo and biodegrading" (p. 516). Their reasoning for attributing the organic-rich particles for TCS not degrading is the organic particles inhibit electron transfer from TCS, thus restricting degradation (Reineke, 2001 – as cited in Chenxi et al. 2008). Given that triclosan concentrations were not affected by aerobic or anaerobic storage conditions or length of time stored in Chenxi et al.'s 2008 study, alternative mechanisms are needed to remove this compound from biosolids, perhaps through the bioaccumulation of the material in earthworms.

2.8. Current wastewater treatment processes

Anthropogenic contaminants are present in biosolids because wastewater treatment processes are not 100 percent effective at removing said chemical compounds. The primary process WWTPs utilize is called digestion. Similar to how the human stomach breaks down the food we eat, wastewater digestion incorporates the natural

ability of microorganisms and bacteria to break down organic matter and pathogens within the incoming wastewater, also called the influent. There two primary methods of digestion is aerobic and anaerobic digestion. Anthropogenic chemical contaminants enter WWTPs and are not all removed by current wastewater processes (Deegan et al., 2011).

Aerobic digestion requires the pumping of oxygen into processing tanks holding the influent. Heat is naturally produced from the microorganisms breaking down the plethora of organic matter within the influent converting it into carbon dioxide. Aerobic digestion is typically utilized in smaller WWTPs, with capacities of less than five million gallons per day. It is a faster process where the liquid influent is processed in the tanks for 12-24 hours (Outwater, 1994; Thompson, D., personal communication, February 6, 2015). Aerobic is more costly than anaerobic digestion because of energy needed to pump oxygen into the processing tanks but produces a mostly odorless and more biologically stable product that also has higher fertilizer value than anaerobically digested wastewater (Outwater, 1994).

Anaerobic digestion takes place in the absence of oxygen. This is to encourage bacteria to convert fats, carbohydrates, proteins into organic acids and alcohols, which are then converted into carbon dioxide and methane. In some cases the anaerobic wastewater treatment process can result in net energy production because the methane created is then used by the WWTP (Marchaim, 1992).

Following either anaerobic or aerobic digestion, sewage sludge is piped into pools where the heavy solids sink to the bottom where they are removed from the pool from a drain located at the bottom of the pool. The heavier solids at the bottom of the tank, called biosolids, are dewatered, or pressed to remove excess water, before being used for

land-application purposes, incinerated, or taken to the landfill.

McAvoy et al. (2002) evaluated the concentration of TCS in the influent and effluent wastewater from five WWTPs in Ohio and the digested sludge from three plants. When it comes to aerobic versus anaerobic sludge digestion, McAvoy et al. found a greater decrease in TCS concentrations in the two WWTPs that aerobically digested sludge (14.7 and 12.2 µg/g of TCS in undigested sludge down to 4.2 and 1.5 µg/g in digested sludge, respectively) compared to another plant, which utilized anaerobic digestion, which saw no change in TCS concentrations. Interestingly, the WWTP that anaerobically digests their sludge had the lowest concentration of TCS in the influent wastewater yet showed the concentrations of TCS in the effluent to more than double, from 7.5 µg/g to 15.6 µg/g (dry weight). The authors attribute a 50 percent reduction in the overall amount of solids during the digestion process to this increase in TCS concentration. Triclosan is synthetic and cannot be created except in a lab, by humans (Orhan et al., 2007; Reiss et al., 2009). What is fascinating is Me-TCS was determined present in the influent and effluent wastewater in all five of the WWTPs evaluated, but accurate concentrations could not be determined because they were generally below detectible levels. The exception was for the WWTP that anaerobically digests their sludge, where Me-TCS was not detected after processing the wastewater. While the authors did not discuss this finding, considering the hydrophobic nature of Me-TCS, assuming it concentrated in the solids during the wastewater treatment process is not unreasonable. Either way, the Me-TCS concentrations at the end of the digestion process of the sludge did not greatly differ between the anaerobic (0.13 µg/g, dry weight) and two aerobic (0.17 µg/g and 0.13 µg/g, dry weight) WWTPs.

There are more than 16,583 publicly owned wastewater treatment plants in the U. S. (LeBlanc, Matthews, & Richard, 2009). In 2004, 51 percent of the WWTPs in the U. S. used anaerobic digestion of wastewater and sewage sludge (EPA, 2006). Therefore, based on McAvoy et al.'s (2002) findings, more than half of the WWTPs in the U. S. are not removing as much TCS as WWTPs utilizing aerobic digestion for wastewater treatment.

Wastewater treatment plants' effluence is the primary route for PPCPs to enter the environment. This indicates current wastewater processes are not removing the contaminants (Gao, Ding, Li, & Xagoraraki, 2012; Miao, Bishay, Chen, & Metcalfe, 2004; Vienoa, Tuhkanen, & Kronberg, 2007). Furthermore, the majority of PPCPs are hydrophobic and end up in the biosolids during the wastewater treatment process (Kinney et at., 2006; Strachan, Nelson, & Sommers, 1983). Therefore, it is expected that TCS and Me-TCS will behave similarly and become concentrated in the biosolids.

Kinney et al. (2006) investigated the presence of PPCPs and other contaminants in biosolids destined for land application. Nine different WWTPs' biosolids were collected from seven states within the U. S. Of those nine WWTPs, 87 compounds were screened for and every sample of biosolids contained at least 30 of these compounds, some as many as 55 contaminants. Twenty-five contaminants were found in *every* sample. Triclosan was one of the 87 contaminants tested for and was present in all of the WWTP biosolids samples. The TCS concentration across the nine WWTPs ranged from 1.2 µg/g to 32.9 µg/g, by dry weight (mean and median concentration 10.5 µg/g and 10.2 µg/g, respectively). The most common contaminants were fairly consistent across all the samples analyzed. This is surprising because the WWTPs, from which the biosolids

samples were obtained, did not have similar methods in the production of biosolids and have varied population demographics from which the biosolids originated. This indicates that regardless of the biosolids source's population demographics and the treatment processes utilized by WWTPs PPCPs persist and remain in the biosolids that are then applied to land. Through the land application of biosolids the environment is being polluted with anthropogenic contaminants, which can result in flora and fauna taking up the contaminants and potentially moving through the food chain (Prosser & Sibley, 2015).

2.9. Other wastewater treatment options

Currently, there are a number of options for treating wastewater but many are cost prohibitive or are not effective in the removal of PPCPs (see Table 2.2, below). Especially in arid climates, reclaimed water (WWTP effluent) is pumped into constructed wetlands called treatment wetlands. The purpose of treatment wetlands is to further improve water quality after the primary wastewater treatment processes (U. S. EPA, 1993). An evaluation of contaminant concentrations of the inlet and outlet of a treatment wetland, which receives two million gallons per day with average water retention time of three to four days, showed a decrease in some contaminants but there was a six percent increase in TCS during summer months. During winter months, however, researchers measured TCS concentrations to decrease by 29 percent at the inlet and outlet. The potential reason for the increase in concentration was not discussed in the published report but researchers did collect fish living in the water. These fish were not measured for TCS concentrations but perhaps the fish and other micro biota has something to do with the differences observed (Barber, Keefe, Antweiler, Taylor, & Wass, 2006).

Table 2.2. Wastewater treatment cost and effectiveness of PPCP removal summary for Washington State

Treatment	Percentage of facilities using treatment	Relative Cost	Relative Effectiveness of PPCP removal
Primary	100%	--	--
Secondary	100%	--	--
Filtration	20%	--	--
Activated Sludge	--	--	--
Microfiltration	0%	Very expensive	Poor
Ultrafiltration	0%	Very expensive	Poor
Nanofiltration	0%	Very expensive	Excellent
Granular Activated Carbon	0%	--	Excellent
Powdered Activated Carbon	0%	--	Excellent
Reverse Osmosis	--	Very expensive	Excellent
Riverbank Filtration	--	--	Poor
Membrane Bioreactor	15%	Very expensive	--
Electrodialysis Reversal	0%		--
Ozonation	Few	Expensive	Excellent
Flocculation	--	--	Poor

Note: Jones, 2008, as cited in Lubliner, Redding, & Ragsdale, 2010

2.10. Bioremediation

Remediation is the process of removing pollutants or contaminants, such as heavy metals (Barker & Bryson, 2002), petroleum (Atlas, 1995), waste from drilling (Rojas-Avelizapa, Roldan-Carrillo, Zegarra-Martinez, Munoz-Colunga, & Fernandez-Linares, 2007), and other hazardous materials (Sayara, Borràs, Caminal, Sarrà, & Sánchez, 2011) from soils, sediments, ground water, or surface water. Bioremediation is the utilization of natural processes for remediation treatments. There are various types of bioremediation. To name a few: anaerobic and aerobic remediation utilize microbes in the absence and presence (respectively) of oxygen to degrade the pollutants or contaminants (Russell et al., 2011); phytoremediation utilizes plants to uptake contaminants in soils before being harvested (Salt et al., 1995); mycoremediation involves mushrooms to sequester contaminants in the fruit bodies which are then harvested and disposed of (Bhatt,

Cajthaml, & Šašek, 2002); vermiremediation incorporates earthworms to bioaccumulate contaminants and heavy metals (Chachina, Voronkova, & Baklanova, 2015; Dabke, 2013). In addition, vermicomposted organic matter has been found to show an increase in total nitrogen, available phosphorus and a desirable decrease in the carbon to nitrogen ratio (Suthar & Singh, 2008).

Utilizing earthworms for the purposes of removing anthropogenic contaminants from biosolids is the chosen form of bioremediation for a number of reasons. Sinha et al. (2009) put it perfectly, "vermicomposting is a self-promoted, self-regulated, self-improving, self-driven, self-powered and self-enhanced, low or no energy requiring zero-waste technology, easy to construct, operate and maintain" (p 880). Meaning that earthworms self-regulate their population, where if there is enough food, they multiply and if food is scarce, they do not (Edwards & Bohlen, 1996). Additionally, they improve the substrate in which they find themselves in that they neutralize soil pH, aerate the soil through tunneling and improve water retention (Ismail, 1998, as cited in Gajalakshmi, Ramasamy & Abbasi, 2001)

2.11. Vermiremediation

Earthworms have been studied in soils that have been amended with biosolids and have shown to bioaccumulate triclosan and other anthropogenic contaminants (Higgins et al., 2011; Kinney et al., 2010; Lin et al., 2010; Pannu et al., 2012; Snyder et al., 2011).

To evaluate anthropogenic contaminants in soils treated with biosolids researchers have looked at bioaccumulation of these contaminants in various species of earthworms (Higgins et al., 2011; Kinney et al., 2006, 2008, 2010; Macherius et al., 2014). Kinney et al. (2008) assessed the use of earthworms as a diagnostic tool for evaluating the presence

of 77 anthropogenic contaminants in soils amended with biosolids, manure, or land not amended. Soil and earthworm samples were collected from the four commercial agronomic production sites; two of three sites received an application of biosolids or manure 31 days prior to sample collection; the third site had no known history of biosolids or manure amendments. Prior to application, the manure and biosolids were tested for contaminants so researchers knew what contaminants to expect in the soil or earthworm samples to be collected in the future. It does not appear the soil was sampled and measured for contaminants prior to the application of the swine manure or biosolids. Triclosan was found in the biosolids (10.5 µg/g, dry weight), but not in the manure collected for testing prior to land application. After 31 days, researchers measured TCS concentrations in the soil and worms to be 160 µg/g and 1,740 µg/g, dry weight, respectively in the biosolids-amended treatment. One hundred and fifty six days following the application of biosolids, the researchers collected samples again and found TCS concentrations to have decreased in the soil, to 96 µg/g, dry weight, and an increase in TCS concentrations in the worms, 2,610 µg/g, dry weight. Contaminants, such as some fragrances and detergent metabolites, bioaccumulated within earthworms at quantifiable levels, yet the soils did not have any discernible measure. The biosolids had detectable levels of some contaminants, so researchers knew the contaminants might be present in the soil after the biosolids application. The findings from this study indicate earthworms magnify anthropogenic contaminants present in soils and may be used as indicators for contaminants that may be below detectible concentrations. Even though contaminants may be below detectable levels, they can still accumulate in flora and fauna and work their way up trophic levels of the food chain.

Kinney et al. (2008) also calculated the bioaccumulation factor (BAF), which is the ratio of the mean concentration of contaminants found inside the earthworm to the mean concentration of contaminants to the corresponding soil that had detectable levels. Researchers use the BAF to determine the ratio by which earthworms are able to bioaccumulate a contaminant, relative to the concentration of the contamination in the soil (Organization for Economic Co-operation and Development, 2002). Kinney et al. found that of all the contaminants detected in the worm and soil samples, TCS had the highest BAF of 27; biogenic sterol cholesterol had the second-highest BAF of 21.4 (which has no known direct threat to environmental ecology but can biotransform into testosterone (Fernandes, Cruz, Angelova, Pinheiro, & Cabral, 2003)). The results from this study indicate the higher concentrations of TCS detected in the worms could only be due to the application and presence of biosolids.

Snyder, O'Connor, and McAvoy (2011) evaluated bioaccumulation of triclocarban (TCC) by earthworms (*E. fetida*). Related to TCS, TCC also has antibacterial properties and is also a possible endocrine-disruptor (Chen et al., 2008; Diamanti-Kandarakis et al., 2009) and is found in personal care products that end up in biosolids applied to land. Snyder et al. (2011) mixed biosolids that contained measureable amounts of TCC with three types of soil: fine sand, silty clay loam, and artificial soil, and added earthworms. Based on real-life application rates of biosolids in agricultural settings, the TCC concentration in the amended test soils was estimated to be 6.9 μg/g, calculated from the measured biosolids concentration and load rate. After 31 days the worms were removed from the fine sand, silty clam loam and artificial soils and concentrations of TCC in the earthworm tissue was recorded as 127±14 μg/g, 142±8.4 μg/g, and 36.5±0.89

µg/g, respectively. The difference in the concentrations of bioaccumulated TCC was attributed to the difference the amount of organic matter, the earthworms' main food source, within each soil. In the artificial soil, the organic matter was 2.5 and 10 times greater than the silty clay loam and fine sand, respectively, which provided a higher volume of uncontaminated food for the worms to consume. Snyder et al. also calculated the BAF for the fine sand, silty clam loam and artificial soils, 18±3.5, 20±2.1, and 5.2±0.22, respectively. The BAF values correspond with the concentration of TCC bioaccumulated by the worms from the soil.

Looking exclusively at TCS, Pannu et al. (2012) evaluated earthworm bioaccumulation from fine sand and silty loam clay soil in a laboratory. The soil samples in this study were spiked with TCS of varying concentrations (0.05, 0.07, 0.1, 0.15, 0.55 and 1 µg/g) and earthworms remained in the soil for a period of 28 days. Bioaccumulation was assessed through the calculation of BAF in which average values for the fine sand and silty loam clay, regardless of the spiked TCS amount, was 6.5±0.84 µg/g and 12±3.08 µg/g, respectively. These values follow the trend seen in Snyder et al.'s (2011) findings described above. Using the values from Snyder et al.'s research, Pannu et al. determined the BAF values for TCC in the fine sand and silty loam clay soils were greater but not significantly different than the BAF values calculated for TCS.

Higgins et al. (2011) evaluated bioaccumulation of TCS and TCC by *E. fetida*. The researchers conducted their study evaluating the rate of bioaccumulation of the two contaminants after earthworms were in contaminated and control soils for 1 day, 5, 7, 9, 14, and 21 days. They found rapid and consistent TCC accumulation by *E. fetida* but inconclusive evidence for TCS, from biosolids-amended soils. Substrates were created

with “high” and “low” concentrations of TCS and TCC, which were established by adjusting the amount of biosolids applied to each test soil to reach the desired concentrations. The researchers concluded the lack of relationship between TCS exposure and accumulation by earthworms was most likely due to TCS transforming into degraded compounds, such as Me-TCS, once the worms had consumed the parent contaminant.

There have only been a few studies evaluating Me-TCS bioaccumulation in earthworms. One of the studies investigated bioaccumulation of TCC, TCS, and Me-TCS in the soil and earthworm four years after biosolids were applied to a plot (Macherius et al, 2014). All three compounds were detected in the soil. Triclosan and TCC concentrations in the soil (0.0015 µg/g and 0.013 µg/g, respectively) decreased 100 to 1,000 times compared to their concentrations in the biosolids (10.9 µg/g and 4.94 µg/g, respectively) applied four years prior. Methyl triclosan had a concentration six times that of TCS (0.009 µg/g and 0.0015 µg/g, respectively) in the soil four years following the application of biosolids. Similar results were observed in earthworm bioaccumulation of Me-TCS which was at least double that of TCS or triclocarban in endogenic earthworms (Figure 2.1). This study supports the importance of studying a chemical compound as well as its transformative or degraded form. Prior to this study, there was no published research demonstrating bioaccumulation of Me-TCS in earthworms.

2.12. Earthworm anatomy and reproduction

Earthworms are made up of mostly water and fat (Washington State University Whatcom Extension, 2016) making them ideal for lipid-bonding contaminants, like TCS and Me-TCS. *Eisenia fetida* is more commonly known as red wiggler, redworm, tiger worm, or the red Californian earthworm. They are in the kingdom Anamalia, phylum

Figure 2.1. Concentrations of triclocarban, triclosan, and methyl triclosan in anecic or endogenic earthworms from fields amended with biosolids.

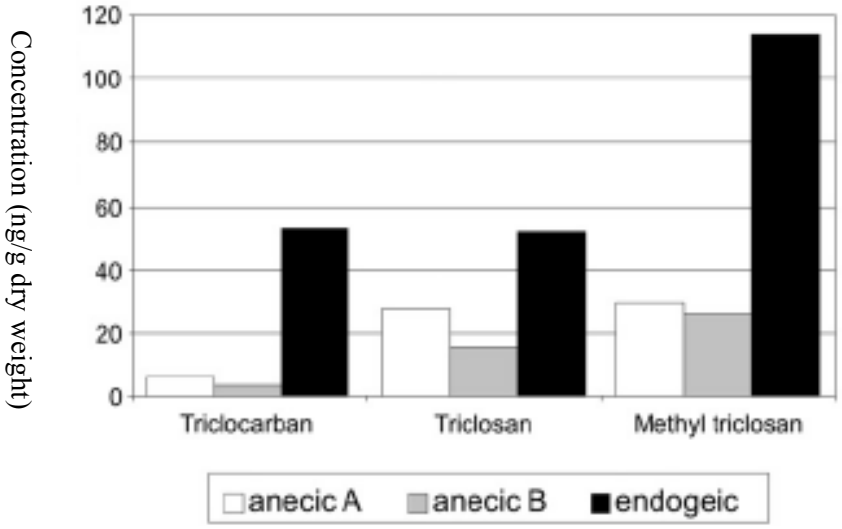


Figure 2.1. Concentrations of triclocarban, triclosan, and methyl triclosan in samples of anecic or endogenic earthworms from fields that were amended with biosolids four years prior. Endogenic earthworms live in the upper layers of soil in the area surrounding plants' roots. Anecic earthworms create vertical burrows that can be up to six feet deep (Macherius et al. 2014).

Annelida, class Clitellata, order Heplotaxida, family Lumbricidae, genus *Eisenia*, and species *fetida*. They live primarily in leaf litter, mulch and manure. Like all earthworms, *E. fetida* are hermaphroditic, but two earthworms are required for reproduction. After copulation, each worm creates a cocoon from which 2-5 baby worms will hatch after approximately 32-72 days. After hatching, *E. fetida* reach sexual maturity within 53-76 days (Edwards, 1988, as cited in Edwards & Bohlen, 1996).

2.13. Current study

There is a need for some way to effectively remove TCS and Me-TCS from biosolids prior to land application because it is clear from the presented information that current wastewater treatment processes are not effective; TCS and Me-TCS persist in the biosolids produced. In a review of literature to evaluate the economic potential of vermicomposting municipal solid waste researchers concluded that vermicomposting is a

great alternative to filling dumps and that by using vermicomposted municipal waste can benefit the quality of soils to which the vermicast is applied (Singh, R., Singh, P., Araujo, Ibrahim, & Sulaiman, 2011).

Taking into consideration the potential economic benefits of vermicomposting municipal waste and the fact that earthworms can bioaccumulate contaminants that persist in municipal biosolids following wastewater treatment processes, a solution may be at hand. Combining these two theories to create a product, vermicomposted municipal biosolids, that can improve soils, utilize our waste in a beneficial way, and reduce polluting our environment seems to be a benefit all around.

Earthworms have shown to improve the nutrients available for plants, improve the microbial community, and aerate soils (Edwards & Bohlen, 1996). In addition, they have been observed bioaccumulating anthropogenic contaminants from soils amended with biosolids suggesting the land applied biosolids did not have all the anthropogenic contaminants removed during processing (Higgins et al., 2011; Kinney et al., 2006, 2008, 2010; Macherius et al., 2014). To my knowledge, there are no studies evaluating the bioaccumulation of TCS and Me-TCS by earthworms for the purpose of removing or filtering such contaminants out of biosolids prior to land application. Based on the fact that WWTPs are unable to effectively remove TCS and Me-TCS, all known anthropogenic contaminants, this research is clearly needed. If earthworms can remove anthropogenic contaminants from biosolids, then incorporating vermiculture into the wastewater treatment process may result in a more environmentally friendly product for land application; one that may be economically feasible and possibly profitable.

The City of Tacoma, for example, sells biosolids as a product called TAGRO, short for Tacoma Grown, to anyone who wants to use the material (City of Tacoma, 2013a). At times, they have even had to close their gates because they had run out of product to sell (Cohen, 2015). TAGRO is sold by the truckload from the WWTP and in bags at local Ace Hardware stores in addition to a number of other locally run garden stores (City of Tacoma, 2013b). Customers can even fill a few buckets worth for free to try it out or if they only need a small amount (personal observation). TAGRO is made with biosolids classified as Class A EQ (Exceptional Quality), the U. S. EPA's highest rating for biosolids (City of Tacoma, 2013c). Class A EQ biosolids meet and exceed Class A standards in pathogen and heavy metals reduction (U. S. EPA, 1999). So, while the product is safe for humans to use the contaminants that are not removed in the wastewater treatment process are being applied to land in the form of individuals' lawns and gardens and commercially on fields and forests.

Whether earthworms can be used to remove chemical contaminants from biosolids prior to land application will be determined by comparing the concentrations of TCS and Me-TCS in biosolids after vermicomposting to a control of substrate to which earthworms are absent. The application of earthworms to biosolids for the distinct purpose of removing anthropogenic contaminants has yet to be evaluated.

3. Biosolids

Biosolids were collected from three WWTPs in Western Washington (The City of Tacoma, Lynden, and Pierce County), each utilizing a different process of biosolids digestion (see Table 3.1). The sites were selected because they were willing to participate in this study.

The City of Tacoma's Central Wastewater Treatment Plant in Tacoma, WA, utilizes dual-digestion in processing sludge. Influent is first digested aerobically for 8 to 12 hours before it is anaerobically digested for 30 days prior to settling out solids to be dewatered. The process produces Class A Exceptional Quality biosolids, which are combined with sawdust and sand to create TAGRO that is then sold to residents and local businesses. The Plant serves a population of approximately 258,000 individuals (Morris Pumps, 2008).

Pierce County's Chambers Creek Regional Wastewater Treatment Plant, in University Place, WA, utilizes anaerobic digestion to process the treatment plant's influent. Sludge is processed for 30-35 days before it is sent to settling tanks to separate out the solids to be dewatered to create Class B biosolids. Biosolids were collected for this study prior to the Plant's final step of heating the biosolids to create their final Class A biosolids fertilizer product because of access and ease of collecting enough material. Additionally, following the final heating step the final product consists of small, desiccated pellets and would have to be re-hydrated for the purposes of this study. The Plant serves a population of nearly 288,000 (Tobin, A., personal communication, October 21, 2016).

The City of Lynden, located in Whatcom County, WA, utilizes aerobic digestion to process the Plant's influent. Influent is processed for 25-30 days before solids are separated out and dewatered to create Class B biosolids. The Plant's serves a population of approximately 13,000 individuals (Goree, T., personal communication, October 25, 2016).

Table 3.1. Characteristics of the wastewater treatment plants from which biosolids were obtained (Washington State).

Wastewater treatment plant	Wastewater treated gal/per day	Population served	Primary treatment process
City of Tacoma	38 million	258,000 [b]	Dual[a]
Pierce County	17.4 million[c]	288,000[c]	Anaerobic
City of Lynden	1.2 million[d]	13,000[d]	Aerobic

Note. [a]Dual indicates that the biosolids are processed aerobically and anaerobically prior to biosolids collection. [c]Information obtained from Morris Pumps, 2008. [c]Information obtained from Tobin, A., personal communication, 2016. [d]Information obtained from Goree, T., personal communication, October 25, 2016. All other information obtained from Northwest Biosolids Management Association's website (http://www.nwbiosolids.org/membership_agencies.htm)

4. Earthworms

Eisenia fetida, or red wigglers, were purchased from three different suppliers in Western Washington, depending on availability. Worms were purchased from Yelm Earthworms and Castings located in Yelm, 3 in 1 Earthworms located in Poulsbo, and Northwest Redworms located in Camas.

Upon receipt, worms were separated from the substrate in which they were transported, by way of tabling. Tabling is a process where the worms and substrate are placed on a table and a light is shined down upon the surface of the pile to encourage the worms to travel to the bottom of the pile. Substrate is removed as the worms continue to travel towards the table, away from the light. At the end of the tabling process, there are only worms left at which point they were weighed and added to appropriate treatments.

5. Pilot study

An initial pilot study was conducted to ensure worms could survive in the substrate mixture of biosolids, from the City of Tacoma, and paper mulch using a ratio of four parts biosolids to three parts paper mulch by wet weight (Ndegwa, Thompson, & Das, 2000). Unfortunately, values were mistaken and 20 percent moisture was used in the

calculations of the moisture content of the biosolids, rather than the actual 80 percent, and considered the paper mulch dry or zero percent moisture rather than the seven percent that it is. This mistake was not realized until later in the study; the assumed or correct percent moisture used in each of the following sections in this study is identified in each part. After the mistake of percent moisture was realized, the ratio of biosolids to paper was recalculated and the actual ratio for this pilot study was closer to 1 to 4.

The substrate for the pilot study was prepared by adding biosolids and paper mulch, at a ratio of four to three (wet weight), to the worm bin and mixed together by hand. Distilled water was added to achieve the moisture content required for the earthworms to survive, approximately 80 percent moisture (Ndegwa, Thompson, & Das, 2000), which was determined by look and feel. Earthworms were separated from the substrate in which they were transported and placed in a pile on the surface of the substrate the day after it was prepared.

The worm bin was comprised of one 68-liter polyethylene plastic containers (42 cm high, 61 cm wide, 41 cm deep) with a surface area of 0.24 m^2. To create the ideal stocking density for vermicomposting biosolids of 1.6 kg of worms/m^2 (Ndegwa, Thompson, & Das, 2000), 384 g of *E. fetida* were needed. In one pound (453 g) there are approximately 1,000 sexually mature red wigglers; therefore 845 adult earthworms were added to the worm bin (Yelm Earthworms and Castings, personal communication, 2015).

Paper mulch was added to the biosolids to provide bedding and a carbon supplement for the worms. Premium Paper 100, a 100% hand sorted recycled newsprint without added dye, was purchased from Applegate Mulch. The bin was filled in a single-batch with enough substrate to equal 0.75 kg of the biosolids and paper mulch (wet

weight) mixture per kg of worms per day, for the anticipated duration of the experiment (Ndegwa & Thompson, 2000). The depth of substrate did not exceed 0.3 m, suggesting that material heating from microbial decomposition would not occur (Lindgern, Pettersson, Kaspersson, Jonsson, & Lingvall, 1985). Distilled water was sprayed on the surface of the substrate to maintain the moisture content earthworms require throughout the experiment.

When checking the worms the morning after they were added to the substrate, most of the worms were found crawling up the sides attempting to escape, or had succeeded in escaping from the worm bin. Thinking the substrate appeared heavy on paper mulch, two handfuls of 100 percent biosolids were applied to half of the bin's surface. Worms will not stay in a bin if there is not enough food, water, or oxygen (Fong & Hewitt, 2016). Additionally, more water was added to the substrate, as it appeared on the dry side. Earthworms are photophobic (Chengelis, 1990) so a light was kept on the lid of the worm bin, which had half-inch holes drilled for air and a little light, to encourage the worms to burrow into the substrate.

By the second day, most all of the worms had burrowed down into the substrate that had not had additional biosolids applied to the surface. After noticing there were no worms on the half of the bin to which the additional dually digested biosolids were applied, the two handfuls of biosolids were removed because there was no other reason for all the earthworms to be in the substrate on the opposite end of the bin. This observation led to the idea there was something about the biosolids that was repulsive or at least not appealing to the earthworms. Otherwise, it is believed the earthworms did not try to escape after the second day because the initial moisture content was not sufficient

for a habitable environment.

In the literature, only a few studies indicate use of anaerobically digested biosolids (Benitez, Sainz, & Nogales, 2005; Gaylor, Harvey, & Hale, 2013; Prosser, Lissemore, Topp, & Sibley, 2014) whereas the majority of studies did not specify whether the biosolids were aerobically or anaerobically digested. Further research was conducted after (Experiment III: Bulking material and concentrations, described in section 8 below), which suggested that anaerobically digested biosolids, similar to the City of Tacoma and like that Pierce County's biosolids, are toxic to earthworms (Hartenstein et al., 1981).

The pilot study began October 26, 2015 and concluded November 30, 2015. During the 35-day study, there were no further mass escapes and the earthworms thrived and were even reproducing, as evident by the presence of cocoons, indicating favorable environmental conditions.

6. Experiment I: 89 to 11

Upon successful completion of the pilot project, biosolids collected from the City of Tacoma's central WWTP were to be vermicomposted for a 45-day period. The concentration of TCS and Me-TCS were to be measured every five days for the duration of the experiment to track the change in concentration between two bins with worms and one control that contained no worms. Preliminary analysis of the City of Tacoma's biosolids indicated sufficient concentrations of TCS to where additional contamination of TCS to biosolids was not needed (M. Bozlee, personal communication, 2016).

To determine the total amount of paper mulch and biosolids necessary to feed the worms for the duration of this study, values were used from Ndegwa, Thompson, and

Das (2000). They fed the worms a mixture of biosolids and paper mulch that consisted of 89 percent biosolids and 11 percent paper mulch, by dry weight, over the entire duration of their study. These values were followed for this study, but again, the percent moisture for the biosolids was calculated as 20 percent, rather than the actual 80 percent. This miscalculation resulted in less biosolids overall than had the actual percent moisture been used. This miscalculation was noticed and corrected in Experiments II and III.

Experiment I of this study began January 15, 2016 and concluded 15 days later on January 29, 2016.

6.1. Parameters measured

If the experiment was successful, soil nutrients were to be measured (method in parentheses) at the beginning and end of the experiment and consisted of total organic carbon (EPA 9060A), phosphorus (EPA 365.4), potassium (EPA 6010C), total Kjeldahl nitrogen (SM 4500-Norg B), pH (EPA 9045D), and percent solids (SM 2540 G). Nutrients were to be measured because the final product is used for amending soil. Earthworms change the nutrients available to plants (van Groenigen et al., 2014). By measuring nutrient concentrations before and after the experiment, it could be determined whether introducing earthworms into the biosolids had an effect on said nutrients.

6.2. Substrate preparation

The substrate was prepared in the same manner as in section 5 with the following additional measures: The biosolids were allowed to off-gas for five days prior to mixing with the paper mulch and room's ambient temperature was monitored and averaged 16.8° C (standard deviation (*SD*) = 1.11).

6.3. Sample collection

On scheduled collection days, five soil samples were taken from each of the three bins (two treatments and one control). Samples were combined to make one compound sample, from which 50 grams were transferred into Whirl-Pak bags to create one compound sample for each of the two treatment bins and the one control. Samples were collected every five days for 15 days with the first sample being only substrate. The U.S. Environmental Protection Agency (1989) recommends samples of equal amounts to be taken from multiple locations within each treatment container and thoroughly mixed together to create a compound sample.

A randomly generated number table was used to pre-determine the location from which each sample was taken, on a four by six grid. Samples were taken from the center of each grid location and were stored at four degrees Celsius, or cooler, until they were transported to the City of Tacoma's Environmental Services' laboratory for analysis (U.S. EPA, 1989).

Each day, dead worms were removed and counted. The dead worms were only counted and not weighed due to desiccation and decomposition. Individuals were counted regardless of maturity; therefore a smaller, younger worm was counted the same as a larger, more mature worm.

6.4. Results

Within a week of adding the worms to the substrate, each bin lost 343 and 477 worms, or 41 and 56 percent, respectively. The percentage of dead worms was determined by converting the weight of the worms added, 384 g, to the approximate number of individuals using the ratio of 1,000 individuals per pound of earthworms

(Knipple, D., personal communication, April 1, 2016).

Nine days into the experiment, additional earthworms were purchased and added to the bins to replace the amount that had died in case the earthworms that were initially put in the bins on day one were not healthy. After the additional earthworms were added, worms continued to die, another 143 and 311 worms, or an additional 17 and 37 percent, respectively, per bin. The experiment was terminated after 15 days.

Substrate temperatures were recorded on a daily basis to ensure temperature was optimal for worm survival. The control bin's mean substrate temperature was 21.7° C (*SD* = 1.09) and the worm bins' averaged 22.0° C (*SD* = 1.09) and 23.7° C (*SD* = 1.58). The ambient temperature averaged 16.7° C (*SD* = 6.84) outside of the worm bins.

6.5. Discussion

This portion of the experiment strongly indicated there was something in their environment that was killing the worms. Kaplan, Hartenstein, Neuhauser, and Malecki (1980) determined the optimal substrate temperature for worm growth is between 20° and 29° Celsius and if pH is below five or above nine, earthworms die within a week. The substrate temperatures in this study were within the optimal limits for earthworms and the biosolids had a pH of 7.6, again, well within the range of earthworm survivability.

During the pilot study, additional biosolids were applied directly to half of the substrate surface the morning after the earthworms were added to the substrate fearing the substrate did not contain sufficient amount of feed. Upon further investigation the following day, there were few, if any, earthworms in the substrate directly below the additional biosolids. This observation suggests the earthworms did not want to be around such high concentrations of biosolids.

With this in mind, perhaps the biosolids to paper mulch ratio (89:11), calculated from Ndegwa, Thompson, and Das (2000) and subsequently used in this experiment, was too biosolids heavy. On the other hand, mixing four parts biosolids to three parts paper mulch does not seem practical if applied to a larger, possibly commercial, scale biosolids-vermicomposting process, due to required amount of paper mulch that would need to be purchased. The ratio of 89:11, biosolids to paper mulch, perhaps results in too much biosolids for healthy worm survival. It was not until after Experiment III (described in section 8 below) that it was discovered that anaerobically digested biosolids, similar to the City of Tacoma and like that of Pierce County's biosolids, are toxic to earthworms (Hartenstein et al., 1981). Without this knowledge, at the time, Experiment II of this study evaluated earthworm survival in substrate composed of the City of Tacoma's dually digested biosolids and paper mulch mixed at a ratio of 2:1 with the hypothesis that the previous experiment was too heavy in the amount of biosolids added.

7. Experiment II: 2 to 1

Considering the substrate created in Experiment I appeared to be biosolids-heavy (even with the calculations including the mistaken 20 percent moisture) and the substrate created for the pilot appeared paper mulch-heavy for practical purposes, the ratio of biosolids to paper mulch was adjusted to two parts biosolids to one part paper mulch, by dry weight. These calculations were completed using the correct percent moisture for the biosolids of 80 percent and seven percent moisture for the paper mulch.

Experiment II of this study began February 13, 2016 and concluded 20 days later on March 3, 2016.

7.1. Parameters measured

Parameters measured in this part of the experiment were the same as measured in section 6.

7.2. Substrate preparation

The same worm bin setup was used in this portion of the study, as described in section 5. The substrate was prepared the in the same manner as in section 6. The room's ambient temperature was monitored and averaged 16.8° C (*SD* = 1.11).

7.3. Sample collection

Samples were collected in the same manner as in section 6. Each day, individual worms that were dead were removed and counted. This was done, as described in section 6. Substrate temperatures were recorded on a daily basis.

7.4. Results

Earthworms began to die quickly. By Day five, 224 and 225 worms were counted as on the surface of the substrate, between the substrate and the side of the worm bins, or had escaped and died from desiccation. Worms found dead between the substrate and the sides of the worm bin were decomposed, making it difficult to accurately count the number of individuals that died. As such, it was estimated to the best of the researcher's ability.

Considering 384 g of worms (or 845 individuals) were added to each worm bin, the number of dead worms by Day 5 accounted for 26.5 and 26.6 percent, respectively, of the total earthworms added. During sampling, live worms were seen deeper in the substrate but dead and decomposed worms were also observed between the substrate and the side of the bins but were not removed because to remove them would disturb the

substrate more than desired.

After Day 5, no more earthworms were found dead on the surface of the substrate. No additional earthworms were purchased or added to the bins for this experiment. The experiment was terminated after 20 days due to the total number of worms counted as dead and the lack of live worms observed in the substrate during sampling.

Following termination of this portion of the study, the substrate from the worm bins was sifted through by hand to count the number of worms that were still alive and if any cocoons could be observed. Only seven worms were found alive in one bin and 13 in the other; no cocoons were found in either bin. The 20 surviving earthworms were placed into freshly mixed substrate that had a ratio of two to one, biosolids to paper mulch. The following day, 12 worms were found dead in or on the substrate. Eight earthworms were not accounted for in or around the container.

Substrate temperatures were recorded on a daily basis. The control bin's average substrate temperature was 22.55 (*SD* = 1.92) degrees Celsius and the worm bins' averaged 23.04 (*SD* = 2.29) and 21.7 (*SD* = 2.08) degrees Celsius.

7.5. Discussion

Even with a biosolids to paper mulch ratio of two to one, earthworms continued to quickly perish. The number of *E. fetida* counted as dead (224 and 225) and the number found alive (7 and 13) does not equal the calculated total of individuals placed in each bin (845). The totals actually account for only 27.3% and 28.2%, respectively. The discrepancy in total calculated earthworms added and the total number counted as dead and the final survivors is attributed to the fact that earthworms begin to decompose quickly, after death. Earthworms are made up of 75 to 90 percent water and when they

die, they decompose very quickly (Washington State University Whatcom Extension, 2016).

The earthworms that survived this 20-day experiment were large and robust. Perhaps age or size of the earthworms used contributes to their ability to survive in such environments. Artuso, Kennedy, Connery, Grant, and Schmidt (2010) evaluated the impact of soils amended with various concentrations biosolids on earthworm survivability. They observed significantly fewer juvenile earthworms in the substrates with the highest amount of biosolids but was not related to the presence of heavy metals, the parameter measured in this study. Therefore, the researchers believed there is another variable at play that was not measured in their study.

Kinney et al. (2012) also observed an increase in earthworm mortality and a decrease in juveniles and cocoons in soils amended with the highest amounts of biosolids. The researchers compared biosolids that had been aged for different time-periods because previous studies have shown that ageing biosolids can decrease toxicity and bioavailability, which is the ability for organisms to take-up the contaminant (Alexander, R. & Alexander, M., 1999; White, Kelsey, Hatzinger, & Alexander, 1997).

The 20 surviving earthworms were collected from the two worm bins and placed in another container of freshly mixed substrate of the exact same proportion of biosolids to paper mulch, two to one. The substrate was made from biosolids collected for this portion of the experiment so there was no difference in materials used, except they had aged in a dark container for the duration of this part of the experiment. The following day, 12 of the 20 earthworms were found dead on the surface of, or in the substrate. Eight worms were unaccounted for and were presumed to have escaped but were not found in

the area surrounding the container.

In Experiment II it was made clear there was something in the environment or substrate that was causing the earthworms to perish. Still being unaware of the toxicity of anaerobically digested biosolids, it was learned that the City of Tacoma had recently acquired a new dewatering system; they moved from a belt press to a screw press and were still in the process of determining the correct amount of polymer to add to the material. The addition of polymers is standard practice for most WWTPs that dewater biosolids (Ross, R., personal communication, 2016) but was unknown to the author, at the time, as to whether the polymer added to the biosolids during the dewatering process, was potentially causing the earthworms to perish. During the dewatering process of biosolids, polymer is added as a flocculant to improve the separation of water and solids (Oleszkiewicz & Mavinic, 2002)

The addition of polymers is standard practice including a large-scale biosolids-vermicomposting operation in Granville, Pennsylvania (Weaver, P., personal communication, 2016). Considering this facility was able to maintain healthy earthworms, even with the addition of polymers during the dewatering of biosolids, polymers were ruled out as a potential reason for the earthworms' death. The Material Safety Data Sheet for the polymer used by the City of Tacoma was obtained and no previous research turned up that indicates it is toxic in any way to earthworms (Ross, R., personal communication, February 18, 2016). Moreover, Kaplan et al. (1980) determined that, even at high concentration, inorganic additives used in the dewatering process to better congeal the material was harmless to earthworms.

However, in the large-scale biosolids-vermicomposting facility in Granville,

Pennsylvania the bedding and carbon supplement supplied to the earthworms was wood chips, rather than paper mulch. Paper mulch was used in this experiment because previous studies evaluating earthworms' ability to process biosolids also did as such (Ndegwa & Thompson, 2002; Ndegwa, Thompson, & Das, 2002).

The City of Tacoma has a bountiful supply of wood shavings that would be desirable for use of bedding if they were to start a large-scale vermicomposting operation (Thompson, D., personal communication, 2016). Perhaps the paper mulch inhibited the flow of oxygen through the substrate, because it can compress when saturated, possibly creating an anaerobic environment that is not suitable for worm survival (Weaver, P., personal communication, 2016).

It is hard to say whether the earthworms did better or worst in this substrate, compared to the substrate used in Experiment I. It was assumed that they would do better because the ratio of biosolids to paper mulch was lighter on the biosolids, but the rate at which they perished would not support that hypothesis. Therefore, in Experiment III that follows, four smaller containers were filled with different substrates to evaluate whether one substrate was more harmful or suitable for earthworm survival. Wood shavings, supplied by the City of Tacoma, were used to create a substrate of proportions similar to the substrate used in the large-scale biosolids-vermicomposting facility (Weaver, P., personal communication, 2016). To determine if there was a component of the substrate that was causing the worms to perish, total organic carbon, total nitrogen, phosphorus, potassium, and pH were measured when the worms were put into each container.

8. Experiment III: Bulking material and concentration

This portion of the study was completed in an attempt to determine which parameters within the substrate resulted in earthworm survival. The following variables were examined by The City of Tacoma's Environmental Services' Laboratory: pH, potassium (K), phosphorus (P), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), which is the sum of organic nitrogen, ammonia (NH_3) and ammonium (NH_4^+).

Experiment III began February 28, 2016 and concluded, 30 days later, on March 28, 2016.

8.1. Parameters measured

The parameters measured in section 6 and 7 were also measured in this section, with the exclusion of substrate temperature because little fluctuation had been observed in Experiments II and I. The detailed laboratory methods for testing TCS and Me-TCS, which follow EPA Method 8270D for semi-volatile organics PPCP, and can be found in Appendix B. Additionally, the weight of worms and number of cocoons produced was measured at the end of the 30-day experiment.

8.2. Substrate preparation

Dually digested biosolids were spread onto plastic sheeting to a depth of a few centimeters and allowed to off-gas for 10 days after being collected directly from dewatering from the City of Tacoma's Central Wastewater Treatment Plant. In Experiments I and II, biosolids were allowed to off-gas for five days, however the biosolids for Experiment III were allowed the extra time in an effort to allow more ammonia to off-gas as earthworms are sensitive to ammonia (Edwards & Bohlen, 1996).

After off gas time period, the biosolids still had a strong ammonia odor (similar to Experiments I and II).

Four mixtures were created for this part of the experiment to test worm survival in biosolids substrates with the addition of the paper mulch or wood shavings, mixed with different concentrations of biosolids. Further, the substrates and the biosolids themselves were examined to assess if they were causing the earthworms to perish.

The substrate created for the Pilot (section 5) was replicated to see if it was an initial fluke the worms survived and flourished in the material. The ratio of biosolids to paper mulch by dry weight, using the correct percent moisture, for the initial pilot was 1:4. The second substrate consisted of two parts biosolids and one part wood shavings by volume. This substrate was created based on the process by which a successful large-scale vermicomposting of biosolids, in Granville, PA, operated (Weaver, P., personal communication, 2016). The third substrate was the true ratio of 89:11, biosolids to paper mulch by dry weight, based on correct and accurate percent moisture. The last substrate was 100 percent biosolids to rule out any potential effect the addition of paper mulch or wood shavings may have interacted with the biosolids creating an uninhabitable environment for the earthworms. Distilled water was added to achieve approximately 80 percent moisture.

In addition, the possibility that the earthworms were shocked or overly stressed when placed on the biosolids substrate, led to a new approach of stacking two containers with the earthworms in a familiar substrate in the lower container and, with holes drilled in the bottom of the upper container, so that earthworms could move up into the novel substrate as they pleased (see Figure 8.1; Monroy, Aira, & Dominguez, 2009). Collecting

vermicompost from the source where the earthworms were purchased ensured the worms would be in material with which they were familiar. This material contained the organic matter in which the worms were raised, as well as their feces. The worm supplier utilizes coconut coir as bedding for their worm-growing operation and some was obtained for the experiment. One part coconut coir was mixed with two parts vermicompost. The coconut coir was added to provide bedding and increase the volume of substrate in the compost. Coconut coir's nutrient value is relatively low (Richards, 2006) therefore its addition would reduce the amount of feed available to the worms, encouraging them to utilize the biosolids substrate while still providing habitable environment.

Figure 8.1. Diagram of stacked containers used in Experiment III: Bulking material and concentration.

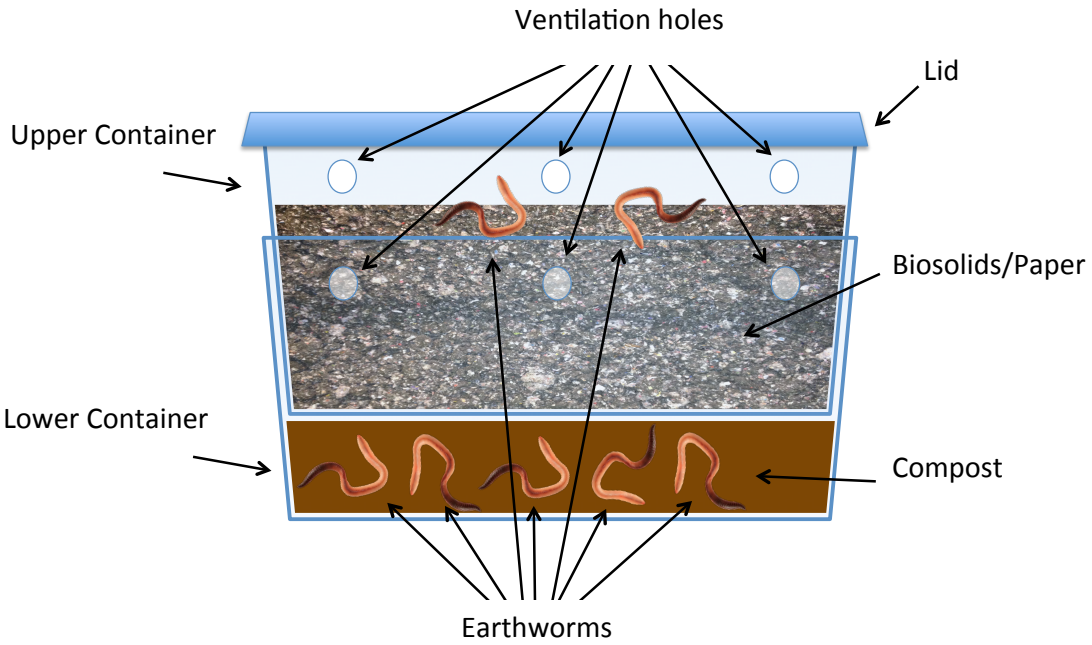


Figure 8.1. Schematic of the preparation of stacked containers of substrate, compost, and earthworms. Upper container is in contact with the substrate in the lower container. Two thirds of the earthworms (by weight) were placed on the surface of the compost, prior to stacking the upper container with the test substrate. One third of the earthworms (by weight) were placed on top of the test substrate in the upper container).

Container size was reduced from Experiment I and II to 1.24-liter polypropylene plastic containers (12 cm high, 18 cm wide, 18 cm deep) for a surface area of 0.03 m^2 because smaller amounts of material were needed, and fewer earthworms were utilized. Each container's base was wrapped in foil (Kwon & Xia, 2012**)** because the containers were clear and earthworms are photophobic (Phillips, Checkai, Chester, Wentsel, & Major, 1994). Ventilation holes were drilled into each of the four containers' lids and to the sides of each container. The containers of earthworms and biosolids substrates were not disturbed for the duration of this portion, Experiment III.

8.3. Sample collection

All substrate samples were collected to analyze pH, potassium (K), phosphorus (P), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), which is the sum of organic nitrogen, ammonia (NH_3) and ammonium (NH_4^+) prior to the addition of the earthworms and at the end of the 30-day experiment. Additionally, a sample of the repeat of the pilot study's substrate was collected prior to the addition of worms and after the 30-day experiment ended to be tested for TCS and Me-TCS concentrations at the City of Tacoma's Environmental Services Laboratory. The laboratory methods for each test in listed in section 6.1. Only the repeated pilot substrate was tested for TCS and Me-TCS concentrations because it was the only substrate to have substantial earthworm survival at the end of the 30 days.

8.4. Results

Of the 31 grams of worms added to each substrate, the pilot repeat saw an increase in total weight of worms to 44 grams, suggesting the worms were actually growing. The biosolids and wood shavings substrate resulted in a 20-gram decrease of

live worm weight, for a total of 11 grams at the end of the experiment. The 89 to 11, biosolids to paper mulch, substrate and the biosolids-only substrate, had only five grams and 0.7 grams, of live worms, respectively, at the end of the experiment (see Figure 8.2). Cocoons were observed only in the repeated pilot substrate and the biosolids and wood shavings test substrate where 51 cocoons were counted in the repeated pilot substrate and only one cocoon found in the biosolids and wood shavings substrate.

Figure 8.2. Total weight (g) of earthworms (*E. fetida*) alive in each substrate after 30-day experiment.

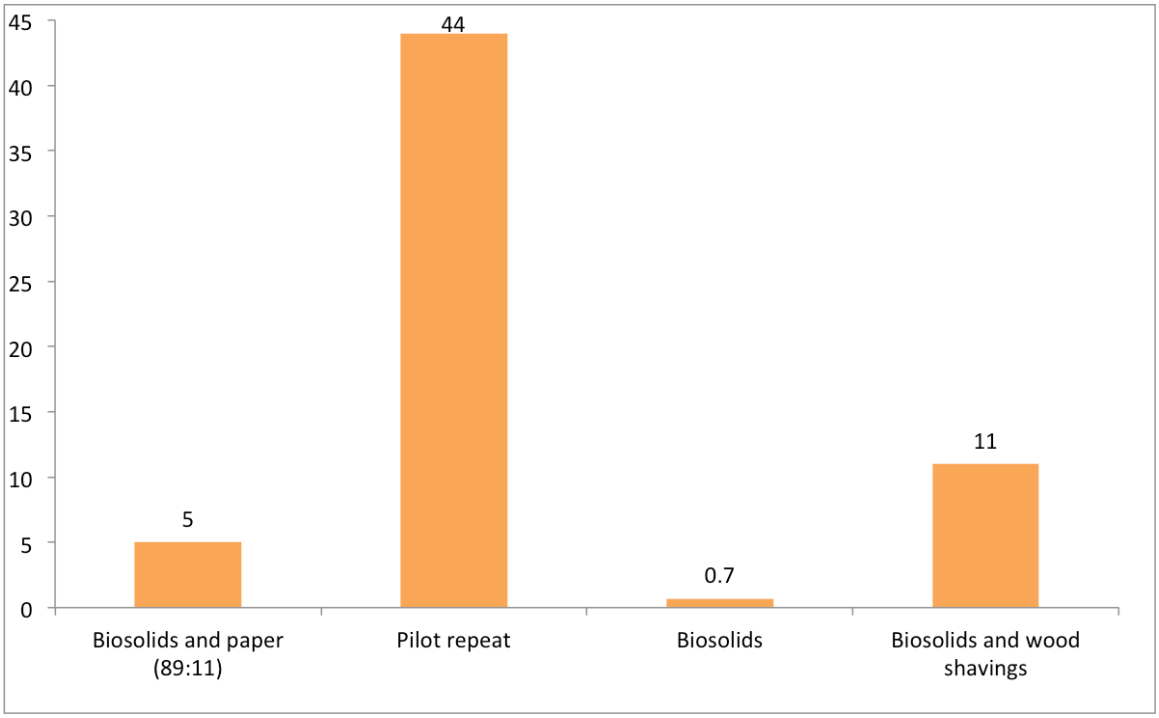


Figure 8.2. Weight (grams) of live worms in each substrate. The initial weight of worms added to each container was 31 g, therefore values greater than 31 g indicate growth, whereas values less than 31 g indicate death and decomposition of worms.

The biosolids-only substrate had the highest amount of TKN (48.7 g/kg) followed by the biosolids and paper mulch (89:11), biosolids and wood shavings, and the repeat of the pilot substrate (42.9 g/kg, 30.5 g/kg, and 19.5 g/kg, respectively; see Figure 8.4). The biosolids-only substrate had the greatest amount of phosphorus (27.7 g/kg) followed by

biosolids and paper mulch (89:11), biosolids and wood shavings, and the repeated pilot substrate (25.8 g/kg, 20.7 g/kg, and 13.4 g/kg, respectively). The highest amount of potassium was measured in the biosolids-only substrate (2,310 mg/kg), followed by biosolids and wood shavings, biosolids and paper mulch (89:11), and then the repeated pilot substrate (2,190 mg/kg, 2,130 mg/kg, and 1,070 mg/kg, respectively).

Figure 8.3. Amount of total Kjeldahl nitrogen, phosphorus, and potassium measured in the four test substrates prior to the addition of earhtworms (*E. fetida*).

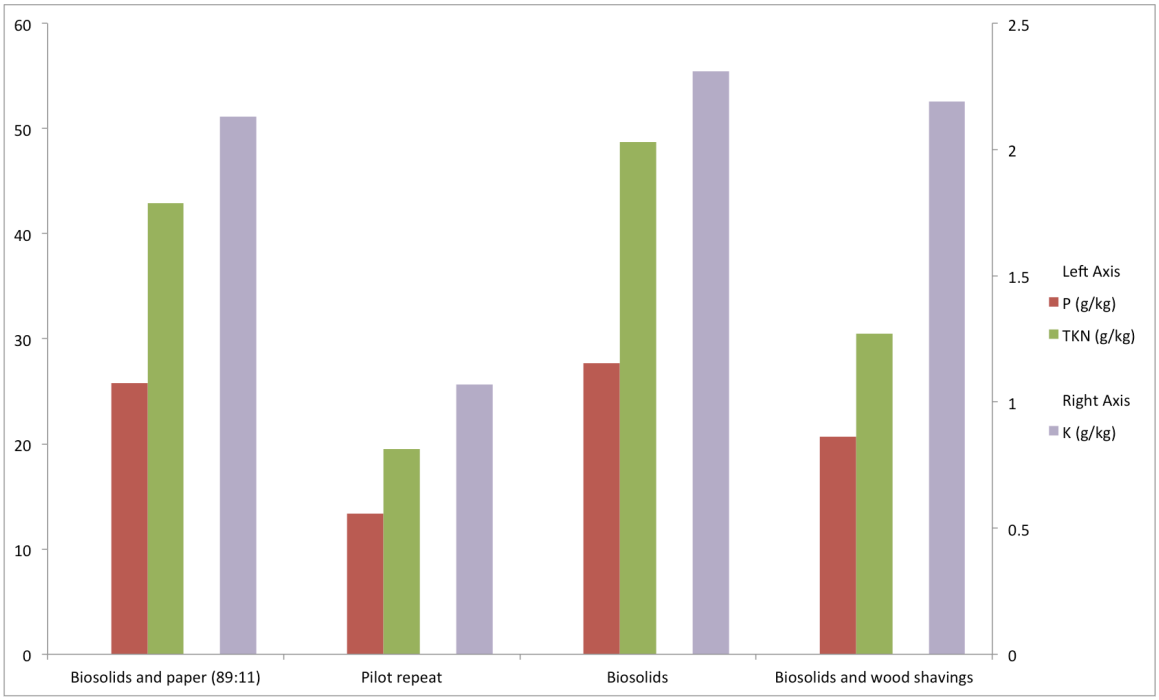


Figure 8.3. Amount of total Kjeldahl nitrogen (TKN), phosphorus (P), and potassium (K) in biosolids and paper mulch substrate (89:11, by dry weight), repeat of the pilot substrate (1:4, by dry weight, biosolids to paper mulch), biosolids-only substrate, and biosolids mixed with wood shavings (2:1, by volume).

Total organic carbon was highest in the pilot repeat (389 g/kg), followed closely by biosolids and wood shavings, biosolids and paper mulch (89:11), and the biosolids-only substrate (321 g/kg, 320 g/kg, and 309 g/kg, respectively; see Figure 8.4).

The pH of the four substrates ranged from 7.2 (biosolids and wood shavings) to 7.7 (biosolids and paper mulch, 89:11), which is well within earthworms' pH tolerance of five to nine (Kaplan et al., 1980).

Figure 8.4. Total organic carbon (g/kg) measured in the four test substrates prior to the addition of earhtworms (*E. fetida*).

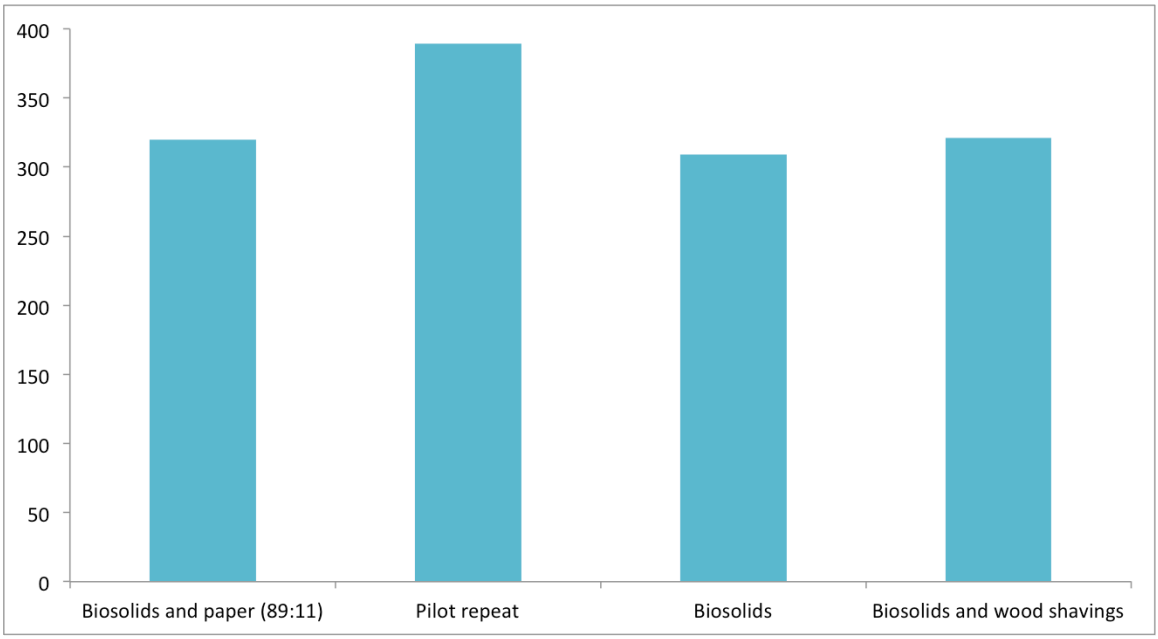


Figure 8.4. Amount of total organic carbon (TOC) in biosolids and paper mulch substrate (89:11, by dry weight), repeat of the pilot substrate, biosolids-only substrate, and biosolids mixed with wood shavings (2:1, by volume).

8.4.1 Triclosan and methyl triclosan concentrations

The TCS concentrations in the repeated pilot substrate decreased from 3,200 µg/kg to 880 µg/kg (75%) from before and after earthworm exposure. Methyl triclosan concentrations increased from an undetectable level (minimum detection limit = 5 µg/kg) in the repeated pilot substrate before earthworms were added to 29 µg/kg (480%) after 30 days of exposure to earthworms (see Figure 8.5).

Figure 8.5. Concentration of triclosan and methyl triclosan before and after vermicomposting repeated pilot substrate.

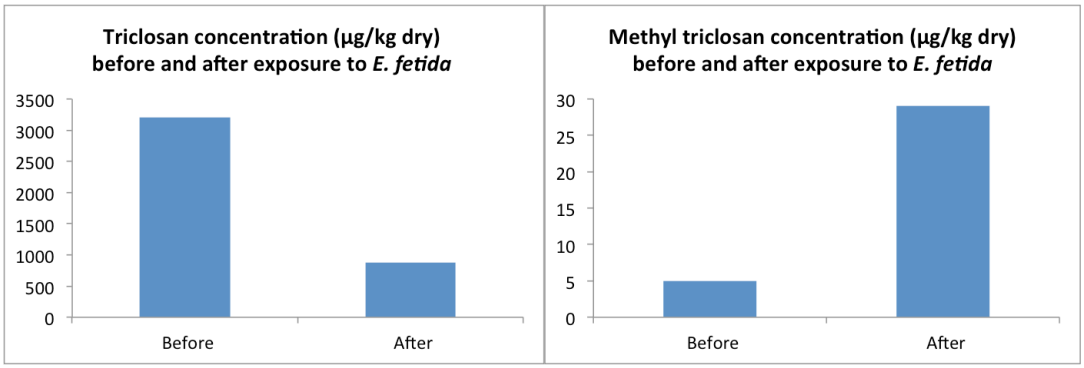


Figure 8.5. Triclosan and methyl triclosan concentration (µg/kg dry) in repeated pilot substrate (1:4 biosolids to paper mulch, by dry weight) prior to earthworms (*E. fetida*) and after 30 days of vermicomposting.

8.5. Discussion

The largest amount of worm survival was seen in the repeated pilot substrate, which actually showed an overall increase in live earthworm weight. This substrate contained the lowest concentration of biosolids suggesting it may be potentially the cause for the earthworms' inability to survive. Interestingly, the biosolids-only substrate had the least amount of surviving earthworms, only one individual (0.7 grams), and had the highest amount of TKN while the repeated pilot had the lowest TNK but most surviving earthworms. Wei and Liu (2005) found that high ammonia nitrogen concentration inhibited growth and were initially toxic to earthworms. Unfortunately, the test available in the current experiment, TKN, is the sum of organic nitrogen, ammonia, and ammonium so it is impossible to determine which nitrogen compound concentration is high and therefore the cause of the toxic environment. Edwards (1988, as cited in Edwards & Bohlen, 1996) found that earthworms will leave a substrate once it becomes anaerobic because they are very sensitive to ammonia and will not survive in substrates containing high ammonia levels. The aerobic digestion of sludge creates ammonia but it

is typically released into the atmosphere (Maramba, 1978), like in an open tank similar to the City of Lynden's WWTP. Anaerobic digestion retains the ammonia that is produced (Maramba, 1978), sometimes to levels that can actually become toxic and inhibit the microbes from digesting and stabilizing the raw sewage sludge (Hansen, Angelidaki, & Ahring, 1998).

The production of cocoons appeared to follow the trend of surviving earthworms, which makes sense since there must be mature individuals in order to reproduce. However, viability of cocoons was not evaluated. Reinecke, A., Reinecke, S., and Maboeta, (2001) evaluated the effect metal toxicity on *E. fetida* reproduction and cocoon viability. While they did not observe a difference in cocoon production of the worms in contaminated substrate, compared to a control, they did observe a decrease in cocoon viability in the soil contaminated with sublethal amounts of toxins.

Total organic carbon was measured highest in the repeated pilot substrate (389 g/kg) with the biosolids and wood shavings and 89:11 substrates second and third closest (321 and 320 g/kg, respectively) and biosolids last with 309 g/kg of TOC. Interestingly, the number of individual earthworms at the end of the 30-day experiment followed the same pattern where the repeated pilot substrate had 44 individuals at the end, followed by the biosolids and wood shavings and 89:11 substrates with 11, five, and one individual (respectively). Additionally, the repeated pilot substrate had 51 cocoons where as the only other substrate to have any cocoons was the biosolids and wood shavings where only one cocoon was counted. Earthworms need carbohydrates, or carbon, and protein to survive (Avis, 2011). Stachell (1967, as cited in Edwards & Bohlen, 1996) observed a positive correlation between palatability and soluble carbohydrates in *E. fetida.* Perhaps

the earthworms in this experiment found the carbon-based paper mulch palatable in that they are able to thrive in the repeated pilot substrate better than in the biosolids and wood shavings substrate or the 89:11 substrate, that did not have nearly as much paper mulch to biosolids as the repeated pilot substrate.

Knowing the TCS and Me-TCS concentrations before and after earthworm exposure, the amount of expected amount of TCS degrading into Me-TCS can be mathematically extrapolated. As mentioned earlier in section 2.7, Chen et al. (2011) determined one percent of TCS transformed into Me-TCS in aerobic laboratory conditions while Butler et al. (2012) measured up to 66 percent of TCS transforming into Me-TCS in sandy loam clay soil during warm, dry months in a field setting. If the minimum, one percent, of 3,200 µg/kg of TCS transformed into Me-TCS were applied the total would be 32 µg/kg of Me-TCS (Chen et al., 2011). Whereas, if the maximum, 66 percent (Butler et al., 2012), of TCS transformed into Me-TCS, 2,112 µg/kg of Me-TCS would be the expected amount observed.

Based on the expected percent of Me-TCS formation (one to 66) from TCS degradation, the current study more closely aligns with Chen et al.'s (2011) findings of one percent of TCS accounts for the formation of the measured Me-TCS. In the present study, the formation of Me-TCS accounts for only one percent of the 75 percent decrease in TCS concentration. The formation of Me-TCS cannot explain the total decrease in TCS concentration, which would indicate there are other factors involved in the further reduction of TCS observed. Something other than the formation of Me-TCS caused the other 2,320 µg/kg of TCS to not be present in the substrate after exposure to earthworms.

To see Me-TCS concentrations below the level of detection is expected because it is only formed through the process of TCS degradation. Therefore, with time, TCS would degrade and Me-TCS concentration would be expected to increase, as seen in Figure 8.5. The biosolids collected from the City of Tacoma were collected directly after dewatering, the last stage of the wastewater treatment process. The plant does not age their biosolids; they are used immediately and were collected as such.

The European Commission (2010) states TCS is degraded by photolysis (exposure to light), chlorination, ozone treatment, and aerobic bacterial hydrolysis or the breakdown of chemicals by bacteria in water. At The City of Tacoma's WWTP where the biosolids for this experiment were collected, chlorination does not occur until just prior to release of effluent water back into the environment, after separation of the biosolids; so no chlorine was introduced to cause degradation. The substrate was collected directly following the dewatering process at the WWTP and was mostly kept in the dark throughout the experiment, additionally, ozone treatment is not incorporated at The City of Tacoma's WWTP. Aerobic bacterial hydrolysis is the only factor the European Commission lists as a primary degrader of TCS that cannot be ruled out in the current experiment. However, the presence of earthworms may have an impact on the observed decrease TCS concentration but further research will be needed to fully support this.

The consideration that earthworms may be responsible for the decrease in measured TCS concentration is consistent with findings from previous research that earthworms can bioaccumulate TCS (Higgins et al., 2011; Kinney et al., 2006, 2008, 2010; Macherius et al., 2014). Unfortunately, only the repeated pilot substrate had earthworms survive in the material for evaluation of TCS and Me-TCS concentration

In search of the impact of potassium and phosphorus on earthworm survival, no studies were found that would indicate there is any amount of either chemical that inhibits earthworm survival or which causes death. However, phosphorus was positively associated with earthworm survival.

It turns out that anaerobic sludge can be toxic to earthworms (Hartenstein, 1981). Masciandaro, Ceccanti, and Garcia (2000) found that when anaerobically digested biosolids were spread onto fields the amount of earthworms that left the area was positively correlated with an increasing amount of biosolids. This is supported in the current research in that very few worms survived in the biosolids-only substrate. Additionally, when anaerobically digested biosolids were applied to the surface of half of the substrate in the initial pilot study (section 5) the majority of earthworms appeared to move to the area of substrate without added biosolids.

As the amount of material added to the biosolids was increased, from 89:11 (by dry weight) biosolids to paper, 2:1 (by volume) biosolids and wood, and the repeated pilot substrate (1:4, biosolids to paper, by dry weight) the amount of worms that survived also increased. This suggests that while the anaerobically digested biosolids are toxic to earthworms, an environment can be created in which the earthworms can survive by the addition of other material or bedding.

Once Hartenstein's publication was discovered, and subsequently confirmed here in Experiment III, biosolids processed differently were sourced. In Experiment IV, that follows, biosolids from the City of Lynden, that processes their incoming wastewater aerobically, Pierce County that processes their incoming wastewater anaerobically, and from the City of Tacoma that utilizes a dual digestion process of aerobic followed by

anaerobic digestion were obtained and earthworm survival and reproduction was. The purpose for including Pierce County's anaerobically digested sludge was to determine if the earthworms had not survived in The City of Tacoma's biosolids due to it being anaerobically digested as the second step of the dual digestion process or perhaps another unknown factor.

9. Experiment IV: Three biosolids sources

Through further discussion, Weaver, P. (2016) stated he had the most success with vermicomposting aerobically digested sludge. Upon researching peer-reviewed literature, it appears others have found anaerobically digested sludge to be toxic to earthworms (Hartenstein, 1981; Masciandaro et al., 2000). There are two possibilities for why anaerobically digested biosolids are toxic to earthworms, one being an oxygen deficiency in the substrate because of limited compaction, and therefore minimal aeration, and the other is the anaerobic process, utilized at WWTPs, results in toxic compounds (Masciandaro et al., 2000). The biosolids used in Experiments I, II, and III were all dually digested, initially aerobically followed by anaerobically digestion. Therefore, aerobically digested, Class B biosolids were obtained from the City of Lynden, Washington and anaerobically digested, Class B biosolids were obtained from Pierce County's Chambers Creek Wastewater Treatment Plant to compare worm survival, reproduction, and ultimately contaminant concentrations to that of the City of Tacoma's Central Wastewater Treatment Plant's Class A Exceptional Quality (EQ) biosolids.

The primary difference between Class A and B biosolids is the amount of pathogens allowed in the final product. In Class A biosolids, pathogen levels must be

nearly eliminated from the material whereas Class B biosolids can have pathogens to a certain level. Class B biosolids tend to have more plant available nitrogen and are therefore preferred by farmers but are more regulated and have more restrictions on usage (Oregon Association of Clean Water Agencies, 2009). Class A EQ biosolids meet and exceed Class A standards in pathogen and heavy metals reduction (U. S. EPA, 1999). According to a 2004 survey, 23 percent of biosolids were processed to the Class A level and 34 percent were processed to a Class B level (North East Biosolids and Residuals Association, 2007).

9.0.1. Substrate preference

While anaerobically digested biosolids have been shown to be toxic to earthworms (current study; Hartenstein, 1981), they were able to survive and thrive in the Pilot study's substrate (section 5). Therefore, at that concentration of biosolids and carbon supplement, the biosolids were habitable but may not be preferred by earthworms. An additional test was performed to determine whether aerobically or anaerobically digested biosolids are more preferable to earthworms, compared to compost.

Experiment IV had a staggered start time because of biosolids availability. Beginning April 4, 2016 and concluding on May 15, 2016 each treatment lasted 35 days. The number of days was increased from Experiment III, which was 30 days, to ensure the earthworms had enough time to process the material to test before and after concentrations of TCS and Me-TCS.

9.1. Parameters measured

Parameters measured in Experiment III were the same as measured in section 8 of this study, in addition to substrate temperatures. All samples, before, the control, and after earthworm exposure, were measured for concentrations of TCS and Me-TCS.

9.1.1. Substrate preference

The parameters measured in testing the earthworms' substrate preference was number and weight of earthworms added initially, and again after 35 days. Cocoons were also counted but the viability of cocoons was not tested or measured.

9.2. Substrate preparation

Biosolids were spread onto plastic sheeting to a depth of a few centimeters and allowed to off-gas for 12 days after being collected from each WWTP and prior to being mixed with paper mulch and distilled water. In Experiment III, the biosolids were allowed to off-gas for 10 days. An additional two days was added here, in Experiment IV, because the time was available and may have allowed for even more of the ammonia smell to off-gas.

The substrate mixture was a ratio of four parts biosolids to three parts paper mulch, by dry weight. The ratio of 4:3, biosolids to paper mulch, was chosen because a ratio of 2:1, in Experiment II, resulted in major earthworm mortality and there was success in the pilot study of which the substrate was prepared at a ratio of 4:3, biosolids to paper mulch, but by wet weight, 1:4 by dry weight. In an effort to determine whether the earthworms are capable of bioaccumulating TCS and Me-TCS from biosolids, effectively removing the contaminants, the worms needed to survive and therefore a substrate suitable for survival, rather than practical application, was chosen.

Three different substrates were created from biosolids from the three WWTPs, for a total of nine unique substrates. No replicates were created due to limitations in funding. These substrates were created to test whether the presence of earthworms had an impact on TCS and Me-TCS concentrations in a biosolids and paper mulch substrate and the substrate preference of *E. fetida* between biosolids and compost.

Each container's base was wrapped in foil because earthworms prefer dark conditions (Kwon & Xia, 2012**)**. All containers were checked regularly for mold growing on the surface of the substrate and sides of the container; any observed mold was removed and the amount of material removed with the mold was weighed and recorded. Each container had a lid in which ventilation holes had been drilled. Thermometers were placed through a ventilation hole in the lid of each container into the substrates and remained there for the duration of the experiment to obtain daily temperatures.

The same containers used in Experiment III were also used here. Additionally, the stacked container approach (see Figure 9.1; Monroy et al., 2009) was used in the set up evaluating the presence of earthworms' influence on concentrations of TCS and Me-TCS in the substrates; otherwise, all other treatments, including the control, were not stacked containers.

For each biosolids and paper mulch treatment, a large batch was prepared for each biosolids source and divided between two containers; one in which earthworms were added and the other was the control, which was allowed to age the duration of the 35-day experiment. The preparation of the three containers evaluating earthworms' preference is described below in section 9.2.1.

Compost was collected from Northwest Redworms, in Camas, WA at the time earthworms were acquired. The compost consists of horse manure, grass clippings, and sawdust pellets, which is turned and aged for more than one year. Northwest Redworms grow their worms in this compost. For this experiment, four parts compost and one part peat moss (wet weight) was mixed to create a substrate that would be familiar to the earthworms, limiting the shock and stress of being placed in a novel substrate, while not providing enough that they would be able to survive by simply consuming the familiar compost. In this section of the experiment the ratio of compost to peat moss, a coconut coir alternative, was increased from Experiment III (2:1) to allow enough bedding for earthworms to utilize in the substrate preference sub-experiment.

Figure 9.1. Diagram of stacked containers used in Experiment IV: Three biosolids sources.

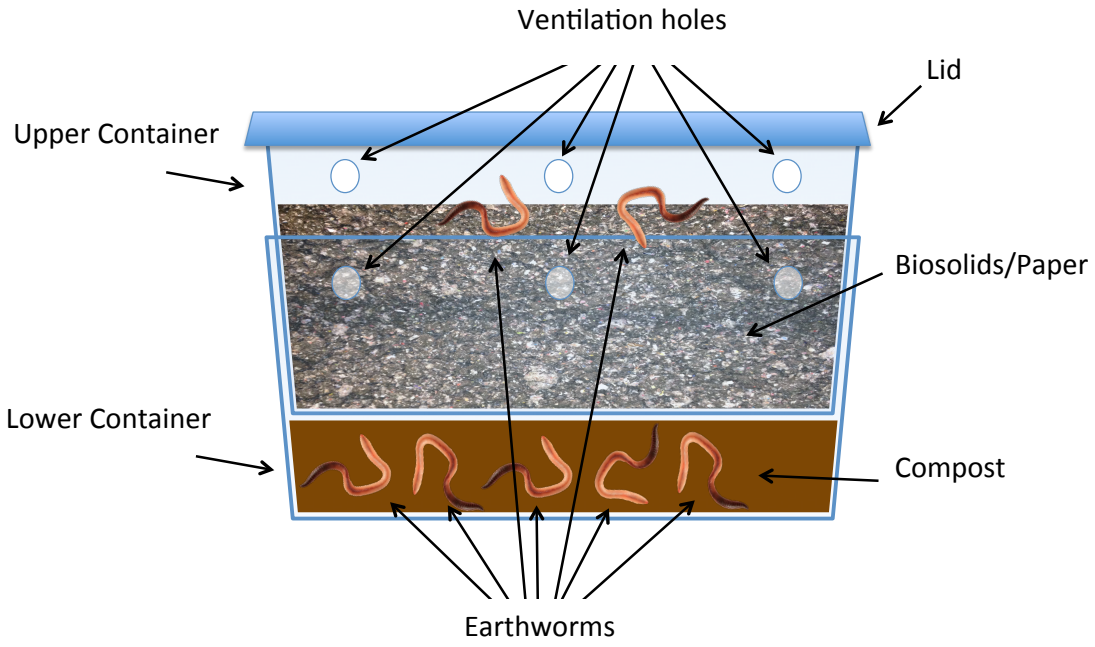


Figure 9.1. Schematic of the preparation of stacked containers of substrate, compost, and earthworms. Upper container, with holes drilled into bottom to allow earthworms to travel between substrates, is in contact with the substrate in the lower container. Two thirds of the earthworms (by weight) were placed on the surface of the compost, prior to stacking the upper container with the test substrate. One third of the earthworms (by weight) were placed on top of the test substrate in the upper container.

9.2.1. Substrate preference

A sub-experiment was created to test earthworm preference between biosolids from each of the three sources and a compost mixture. Layered on top of moistened paper mulch, compost and peat moss mixture and biosolids each evenly covered half of the paper mulch surface (see Figure 9.2). Distilled water was added to the biosolids and paper mulch to achieve approximately 80 percent moisture (by weight). There was no control substrate this substrate-preference sub-experiment.

Figure 9.2. Diagram of divided containers used in Experiment IV: Three biosolids sources

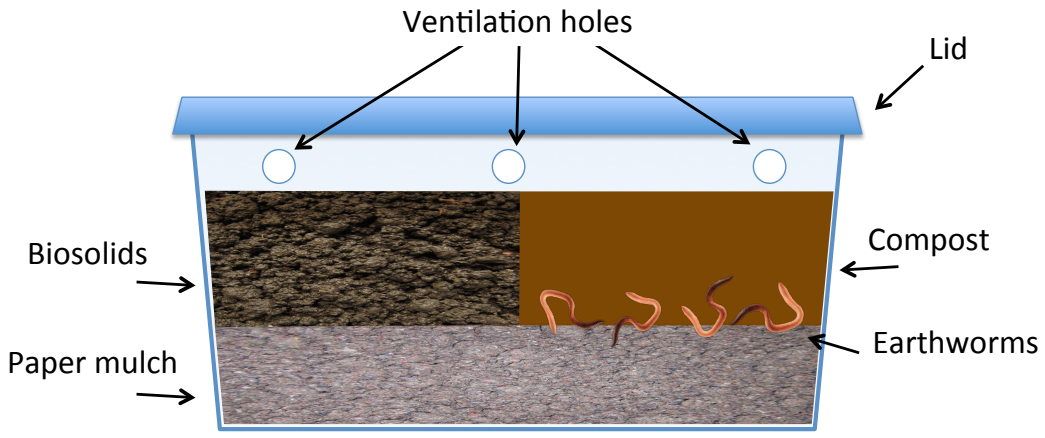


Figure 9.2. Schematic of the preparation of divided containers of biosolids, compost, and earthworms. Paper mulch was evenly spread across the bottom of the container. Half of the paper mulch was covered with biosolids while the other half was covered with compost. All the worms were sandwiched between the paper mulch and compost at the beginning of the experiment.

9.3. Sample collection

Samples were collected from all substrates for laboratory analysis prior to the addition of the worms. After the 35-day experiment, each substrate was sorted by hand to remove and count all earthworms and cocoons and each substrate was well mixed before

being packed into individual Whirl-Pak bags. Samples were frozen until they were transported to the City of Tacoma's Environmental Services' laboratory. All biosolids and paper mulch substrate samples were collected to analyze the percent solids, pH, TCS and Me-TCS concentrations, pH, potassium (K), phosphorus (P), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), which is the sum of organic nitrogen, ammonia (NH_3) and ammonium (NH_4^+) prior to the addition of the worms and at the end of the 35-day experiment. Laboratory methods used for each test are listed in section 6.1.

9.4. Results

Substrate and ambient room temperatures were recorded on a daily basis and are presented in Table 9.1.

Table 9.1. Mean (standard deviation) temperatures of substrate and ambient room temperature.

Source	Biosolids control	Divided substrate with worms	Biosolids and paper control	Biosolids and paper with worms	Ambient room temperature
Lynden	18.4 (1.9)	18.0 (1.9)	18.4 (1.7)	17.8 (1.7)	17.3 (1.8)
Tacoma	17.3 (1.7)	18.8 (1.8)	17.5 (1.6)	17.7 (1.7)	17.6 (1.7)
Pierce County	19.1 (1.8)	18.1 (1.8)	18.0 (2.2)	18.5 (1.8)	17.8 (1.8)

Note. Mean (standard deviation) temperature, in Celsius, of ambient room temperature and substrates made from the City of Lynden, Tacoma, and Pierce County's biosolids and paper mulch.

9.4.1. Earthworm survival

Of the two substrates created using The City of Tacoma's dually digested biosolids, all the worms survived the duration of the 35-day experiment. The divided and biosolids and paper mulch substrates created using the City of Lynden's aerobically digested biosolids saw a loss of one (4%) to three (12.5%) earthworms, respectively. Substrates created using Pierce County's anaerobically digested biosolids resulted in a decrease of eight earthworms (45%) Interestingly, the total number of earthworms added

to the divided substrate composed of Pierce County biosolids and compost showed an increase of one individual worm (5.9%; see Figure 9.3); this is assumed a counting error and is discussed further in section 9.5.1).

Figure 9.3. Number of earthworms added to each substrate at the beginning and after 35-day experiment.

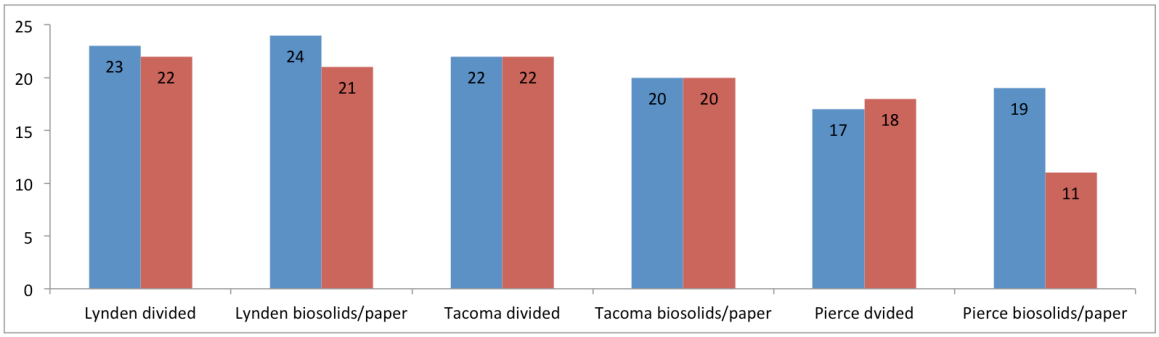


Figure 9.3. The number of earthworms added to each substrate at the beginning (blue) and the number of earthworms remaining at the end (red) of the 35-day experiment.

The total weight of earthworms added to each substrate at the beginning of this experiment was 30 grams. The number of earthworms added to each container was counted, as well, for both the biosolids and paper mulch substrates and divided containers. The total weight and number of earthworms added to each container was used to calculate the weight per earthworm at time they were added as well as at the end of the 35-day trial. Figure 9.4 illustrates the weight per earthworm in the substrates composed of four parts biosolids to three parts paper mulch, by dry weight. Initially the City of Lynden had the lowest weight per earthworm (1.25 g/earthworm), followed by the City of Tacoma and Pierce County (1.5 g/earthworm and 1.63 g/earthworm, respectively). At the end of the 35-day experiment, the earthworms in the substrate composed of Pierce County's biosolids weighed 0.94 grams per earthworm and had lost the most amount of weight per earthworm (0.69 g/earthworm lost). Earthworms in the substrate composed of the City of Tacoma's biosolids weighed 1.17 grams per earthworm, which is an overall

loss of 0.33 grams per earthworm. Earthworms in the substrate composed of the City of Lynden's biosolids weighed 1.08 grams per earthworm and had the smallest amount of loss of weight per earthworm (0.17 g/earthworm lost).

Figure 9.4. The weight (g) per earthworm in substrates composed of three parts paper mulch and four parts biosolids sourced from the City of Lynden, Tacoma, and Pierce County at the beginning and end of the 35-day experiment.

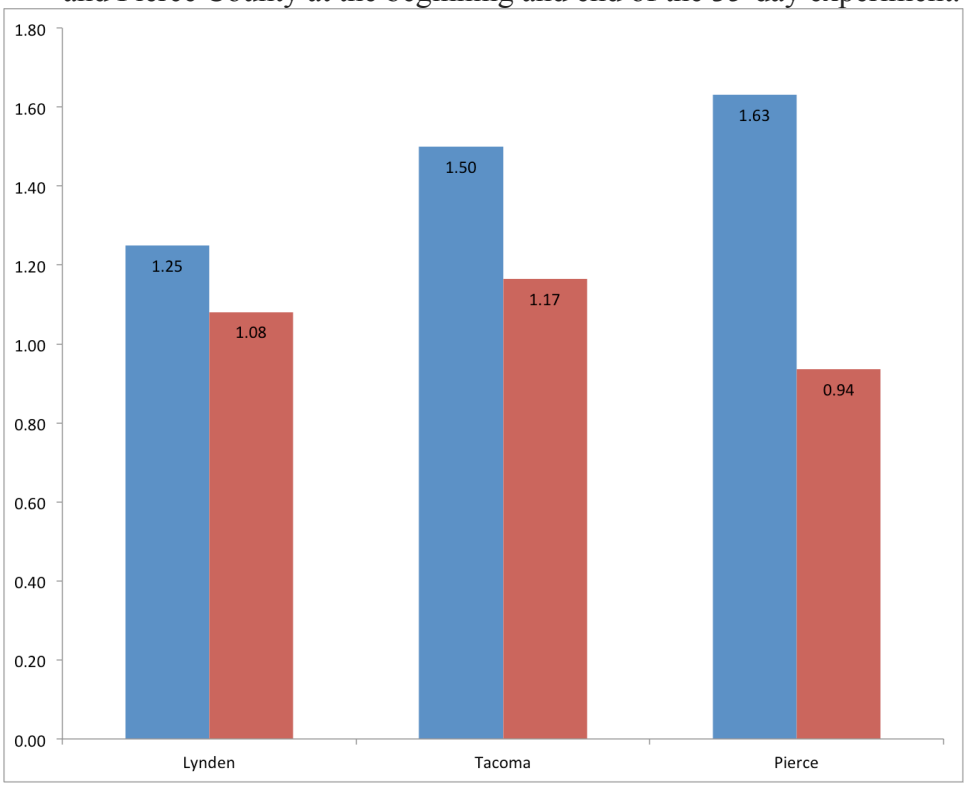


Figure 9.4. The weight (g) per earthworm in substrates composed of three parts paper mulch and four parts biosolids sourced from the City of Lynden, Tacoma, and Pierce County at the beginning (blue) and end (red) of the 35-day experiment.

Similarly, the divided substrate containers followed the same pattern as the biosolids and paper mulch substrates. When the earthworms were added to the substrate the City of Lynden had the lowest weight per earthworm (1.3 g/earthworm; see Figure 9.5), followed by the City of Tacoma and Pierce County (1.36 g/earthworm and 1.76 g/earthworm, respectively). At the end of the 35-day trial, the earthworms in the divided container with Pierce County's biosolids weighed 1.13 grams per earthworm and had lost

the most amount of weight per earthworm (0.63 g/earthworm lost). Earthworms in the divided container with the City of Tacoma's biosolids weighed 1.13 grams per earthworm, which is an overall loss of 0.23 grams per earthworm. Earthworms in the divided container with the City of Lynden's biosolids weighed 1.24 grams per earthworm and had the smallest amount of loss of weight per earthworm (0.06 g/earthworm lost).

Figure 9.5. The weight (g) per earthworm in containers with divided substrates composed of compost and biosolids sourced from the City of Lynden, Tacoma, and Pierce County at the beginning and end of the 35-day experiment.

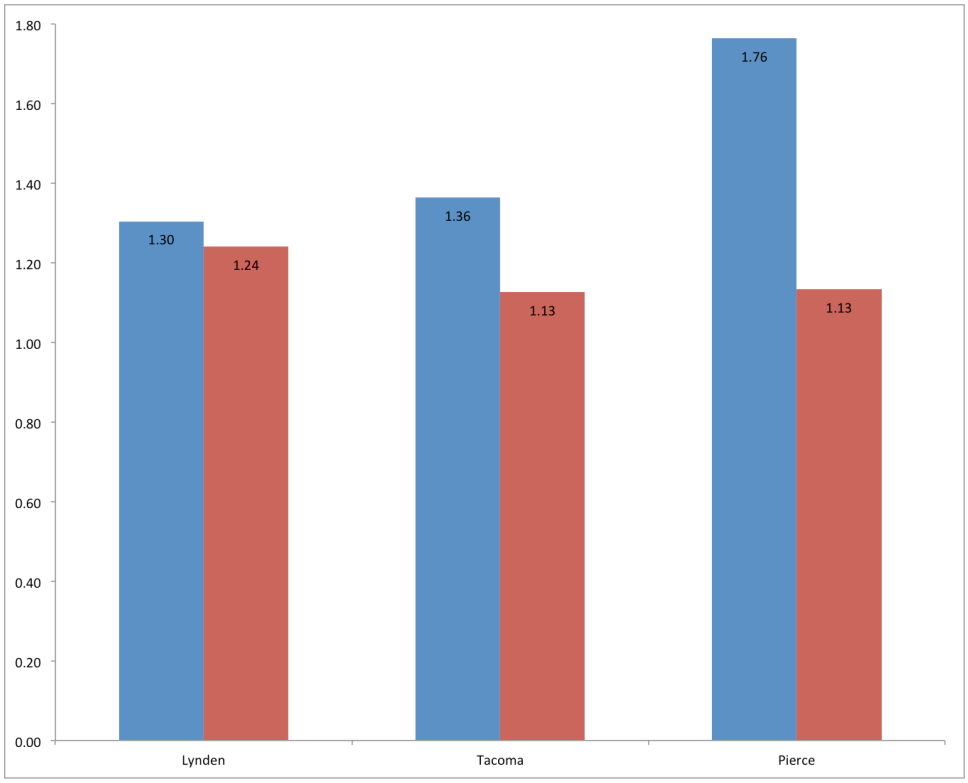


Figure 9.5. The weight (g) per earthworm in containers with divided substrates composed of compost and biosolids sourced from the City of Lynden, Tacoma, and Pierce County at the beginning (blue) and end (red) of the 35-day experiment.

9.4.2 Reproduction

Earthworm cocoons were counted at the conclusion of this 35-day experiment for the preference sub-experiment (see Figure 9.6) and the biosolids and paper mulch

substrates (see Figure 9.7). Cocoon production within the compost and biosolids substrates in the divided containers sub-experiment showed similar tends across the three biosolids sources. The majority of cocoons were found in the compost (248, 252, and 200) compared to the biosolids (53, 35, and 12) of the City of Lynden, Tacoma, and Pierce County, respectively.

In the substrates composed of biosolids mixed with paper mulch the City of Lynden had the most cocoons (303) followed by the City of Tacoma with 271 and then Pierce County had the fewest cocoons with 114 counted at the end of the 35-day experiment.

Figure 9.6. Total number of cocoons counted within divided substrates composed of biosolids from the City of Lynden, Tacoma, and Pierce County and compost.

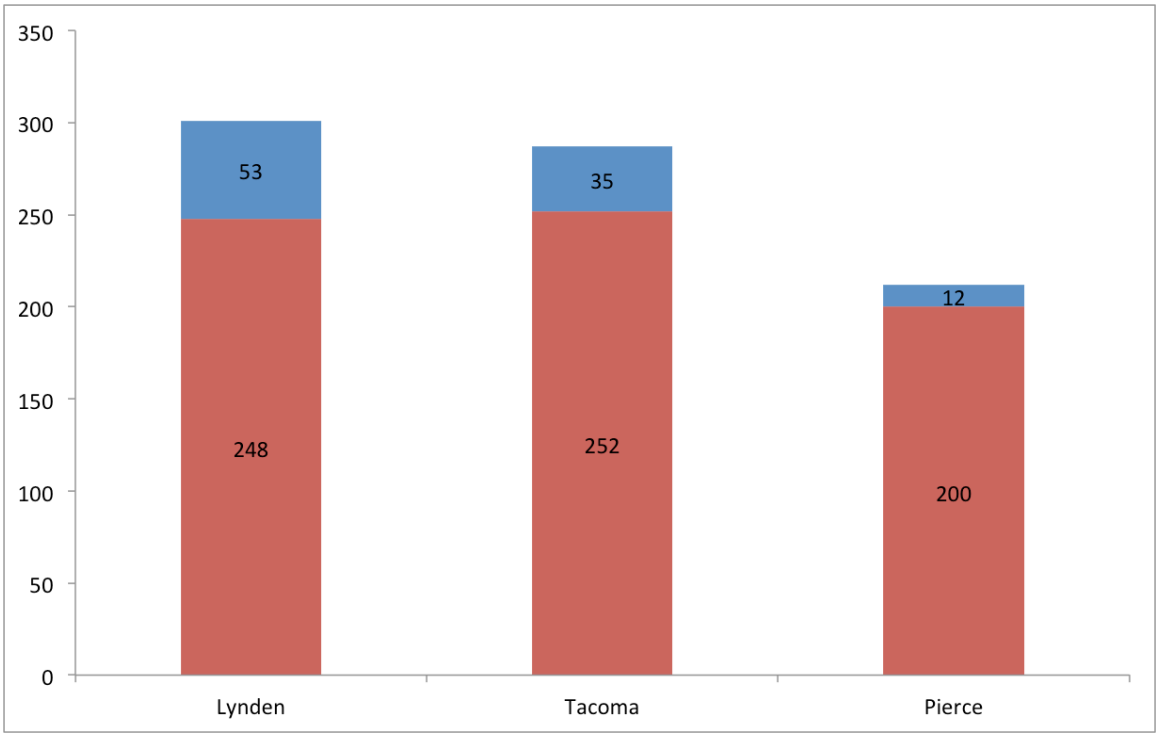


Figure 9.6. The total number of cocoons counted within divided substrates composed of biosolids from the City of Lynden, Tacoma and Pierce County (blue) and the number of cocoons counted in the side composed of compost (red).

Figure 9.7. Total number of cocoons counted within each substrate consisting of biosolids from the City of Lynden, Tacoma, and Pierce County.

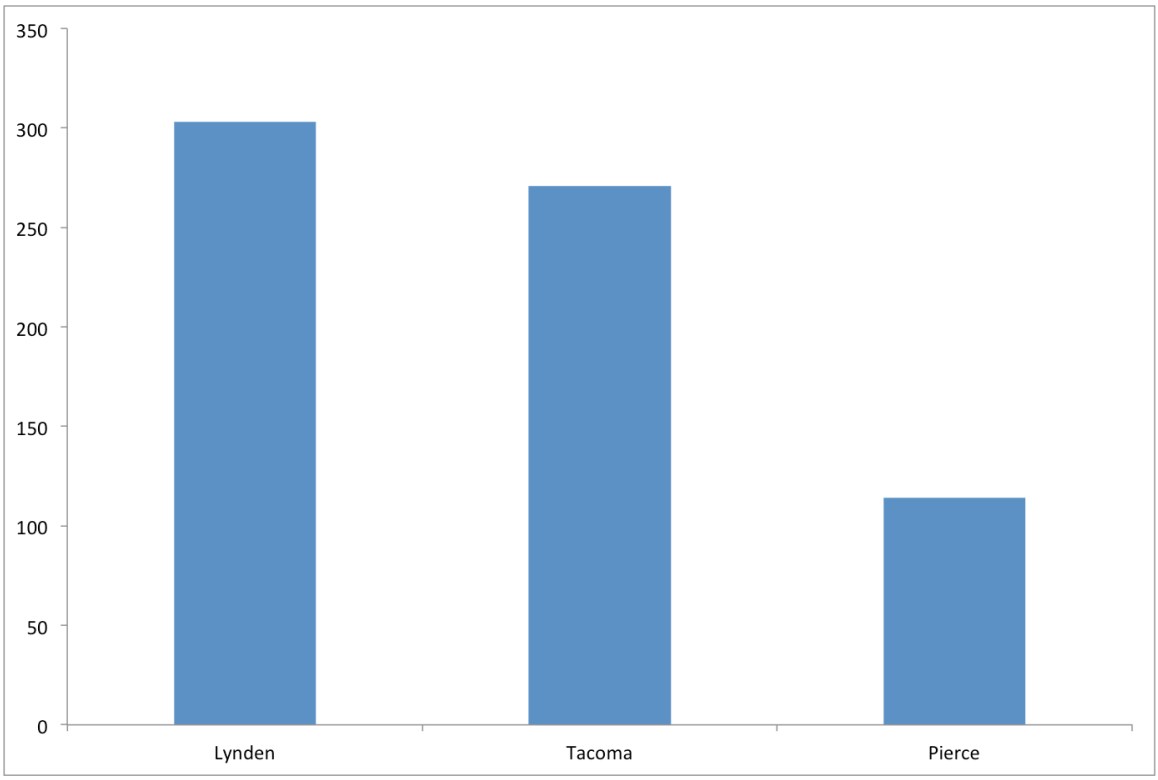


Figure 9.7. Total number of cocoons counted within each substrate consisting of four parts biosolids, from the City of Lynden, Tacoma, and Pierce County, and three parts paper mulch after 35 days of exposure to earthworms.

9.4.3. Triclosan and methyl triclosan concentration

Triclosan concentrations were measured in the biosolids and paper mulch substrates for Pierce County, the City of Tacoma, and the City of Lynden before and after earthworm exposure and the control (see Figure 9.8). The City of Tacoma's Environmental Services' laboratory, that analyzed the samples, allows for 20 percent uncertainty between soil sample duplicates, due to GC/MS/MS calibrations (Bozlee, M., personal communication, November 21, 2016). Therefore, a 20 percent uncertainty has been applied to the measured TCS and Me-TCS values.

The TCS concentration in the City of Lynden's substrate prior to the addition of earthworms was 48 µg/kg (± 9.6). The substrates, after earthworm exposure and the control, which was allowed to simply age throughout the duration of the 35-day experiment, both had TCS concentrations below the limit of detection (39 µg/kg). After bring exposed to earthworms for 35 days the TCS concentration appeared to decrease in the substrates created with Pierce County and City of Tacoma's biosolids mixed with paper mulch (17% and 16%, respectively) but the 20 percent instrument uncertainty results in overlapping of error bars, indicating inconclusive change (see Figure 9.8). For the control (where no worms were added), there was no difference in TCS concentration between the substrates made with Pierce County's biosolids before and after the experiment (3,500 ± 700 µg/kg). The biosolids and paper mulch substrate made with the City of Tacoma's biosolids measured 4,300 ± 860 µg/kg before and 5,200 ± 1,040 in the control after the 35-day experiment. This 20.9 percent increase is believed to be due to the instrument uncertainty and is discussed further in section 9.5.3.

Methyl triclosan concentrations within the substrates composed of paper mulch and biosolids from the City of Lynden, Tacoma and Pierce County varied greatly within and between substrates (see Figure 9.9). Concentration of Me-TCS in the substrate made with the City of Lynden's aerobically digested biosolids appeared to decrease from the beginning of the study (110 ± 22 µg/kg), in the control at the end of the 35-day experiment (82 ± 16.4 µg/kg), and in the substrate exposed to earthworms (75 ± 15 µg/kg). However, the 20 percent instrument uncertainty results in overlap of the error bars indicating and inconclusive difference in values. The substrate composed of

Figure 9.8. Triclosan concentrations before and after *E. fetida* exposure and control, for substrates compose of biosolids and paper mulch from the City of Tacoma and Pierce County.

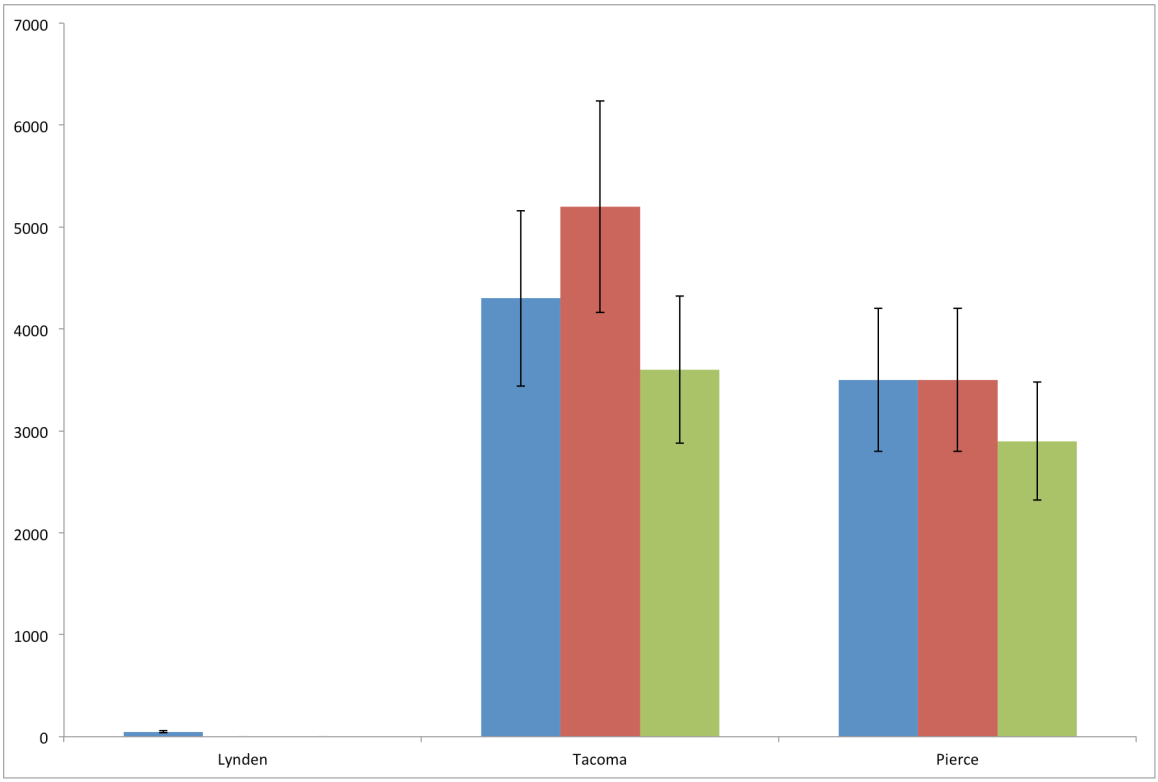


Figure 9.8. Triclosan concentrations (µg/kg) in substrates composed of paper mulch and biosolids from the City of Lynden, Tacoma, and Pierce County at the beginning of the experiment (blue), in the control after the experiment (containing no worms)(red), and after exposure to *E. fetida* (green). Error bars represent 20 % instrument uncertainty. For each WWTPs' biosolids and paper mulch substrate, a large batch was prepared and divided between two containers; one in which earthworms were added and the other was the control, which was allowed to age the duration of the 35-day experiment.

the City of Tacoma's dually digested biosolids saw the largest difference between the substrate at the beginning of the study (5 ± 1 µg/kg), the control (59 ± 11.8 µg/hg) and the substrate exposed to earthworms (160 ± 32 µg/kg). The Me-TCS concentrations in the substrate made with Pierce County's anaerobically digested biosolids appeared to increase slightly between the samples at the beginning (14 ± 2.8 µg/kg), the control substrate after (15 ± 3 µg/kg) and the substrate exposed to earthworms (16 ± 3.2 µg/kg).

There is no discernable difference when the 20 percent uncertainty is applied to these values.

Figure 9.9. Methyl triclosan concentrations (µg/kg) in substrates composed of paper mulch and biosolids from the City of Lynden, Tacoma, and Pierce County before and after *E. fetida* exposure and control.

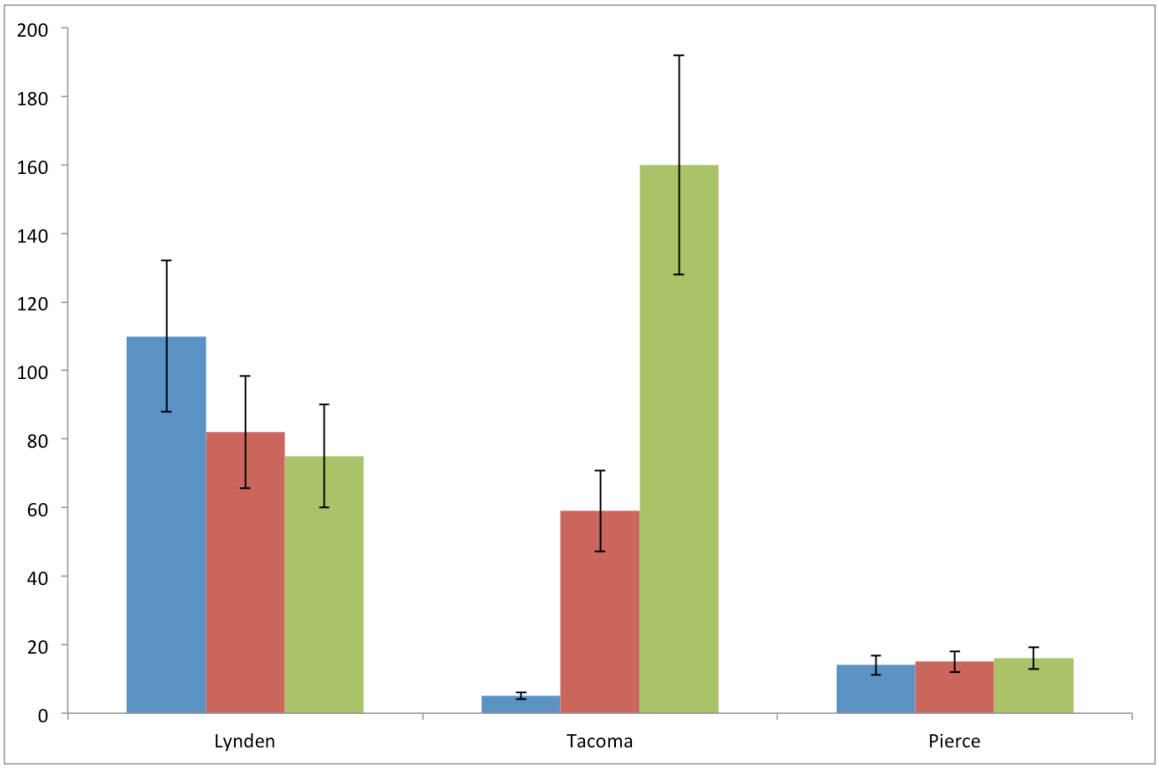


Figure 9.9. Methyl triclosan concentrations (µg/kg) in substrates composed of paper mulch and biosolids from the City of Lynden, Tacoma, and Pierce County at the beginning of the experiment (blue), in the control after the experiment (containing no worms)(red), and after exposure to *E. fetida* (green). Error bars represent 20 % instrument uncertainty. For each WWTPs' biosolids and paper mulch substrate, a large batch was prepared and divided between two containers; one in which earthworms were added and the other was the control, which was allowed to age the duration of the 35-day experiment.

9.4.4. *Eisenia fetida* substrate preference

Substrate preference was determined by counting the number of earthworms and cocoons within the biosolids or compost, and the paper mulch under each substrate, on the final day of the experiment. After the 35-days the worms spent in the substrates, the

paper mulch was well incorporated into the corresponding substrate above it. In the divided substrate composed of the City of Lynden's biosolids and compost, 64 percent (14 earthworms) of the earthworms at the end of the experiment (22 earthworms) were found in the compost while only 36 percent (8 earthworms) were found in the biosolids (see Figure 9.10). Similarly, for the City of Tacoma 36 percent (8 earthworms) of the earthworms at the end of the experiment (22 earthworms) were found in the biosolids and 64 percent (14 earthworms) were found in the compost. Unexpectedly, the majority of the earthworms 72 percent (13 earthworms) of the earthworms at the end of the experiment (18 earthworms) were found in Pierce County's biosolids while 27 percent (5 earthworms) were found in the compost.

Figure 9.10. Percent of total earthworms at the end of the 35-day experiment in divided containers found in the compost and either Pierce County, City of Tacoma, or City of Lynden's biosolids.

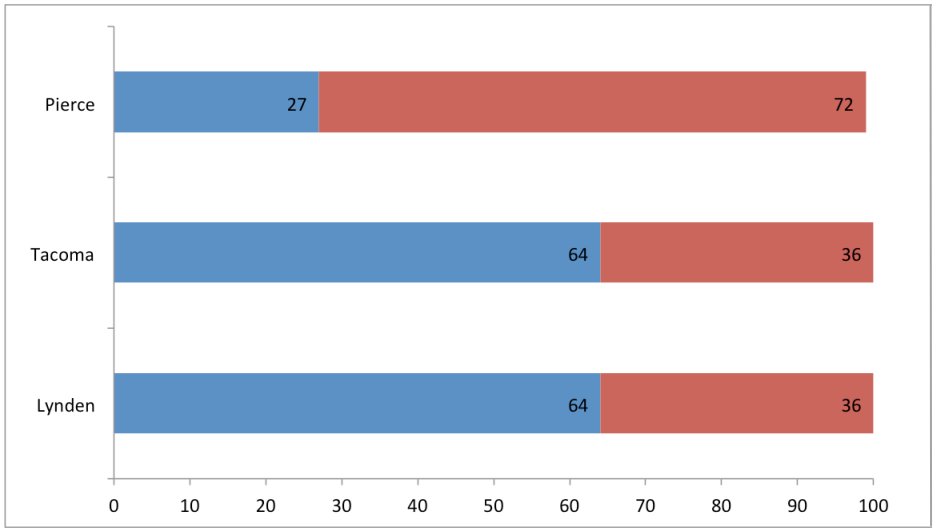


Figure 9.10. Percent of total earthworms at the end of the 35-day experiment in the divided containers found in the compost (blue) and either Pierce County, City of Tacoma, or City of Lynden's biosolids (red).

9.4.5. Nutrients

Potassium (K) and phosphorus (P) concentrations were measured for each paper mulch and biosolids substrate from the City of Lynden, Tacoma, and Pierce County

before worms were added, only, due to cost restrictions and because no literature could be found suggesting it would affect the survival or growth of the earthworms (see Table 9.2). Total organic carbon (TOC), total Kjeldahl nitrogen (TKN, see Figure 9.12), and pH were measured for each paper mulch and biosolids substrate before earthworms were added, post exposure and for the control (no worms).

Table 9.2. Nutrients of substrates composed of paper mulch and biosolids from the City of Lynden, Tacoma, and Pierce County.

Source		TOC (g/kg)	TKN (g/kg)	pH	K (g/kg)	P (g/kg)
Lynden	Before	331	34	6.5	3.3	17
	After worms	332	30.6	5.9	-	-
	Control	369	38.8	6.1	-	-
Tacoma	Before	395	21.7	7.7	1.39	13
	After worms	339	25.3	7.3	-	-
	Control	340	22.7	7.1	-	-
Pierce County	Before	393	36.4	8.2	1.36	15.5
	After worms	418	39	6.3	-	-
	Control	387	38.1	6.2	-	-

Note. Total organic carbon (TOC), total Kjeldahl nitrogen (TKN), pH, potassium (K) and phosphorus (P) of substrates composed of three parts paper mulch to four parts biosolids (by dry weight) sourced from the City of Lynden, Tacoma, and Pierce County. Substrates were sampled before and after exposure to *E. fetida* and a control substrate (no earthworms added) which was allowed to age for the 35-day period.

The TKN (g/kg) was measured for the City of Lynden, Tacoma and Pierce County's biosolids and the substrate consisting of four parts biosolids and three parts paper mulch (by dry weight). Of the three sources, Pierce County had the highest amount of TKN in both the biosolids and the biosolids and paper mulch mixture (80 and 36.4 g/kg, respectively), the City of Lynden had the second highest amount of TKN in the biosolids and biosolids and paper mulch mixture (68.3 and 34 g/kg, respectively), and the City of Tacoma had the lowest amount of TKN in the biosolids and biosolids and paper mulch mixture (46.3 and 21.7 g/kg, respectively).

Figure 9.11. Total Kjeldahl nitrogen (g/kg) in the biosolids and substrates composed of three parts paper mulch to four parts biosolids (by dry weight) from City of Lynden, Tacoma, and Pierce County before *E. fetida* were added.

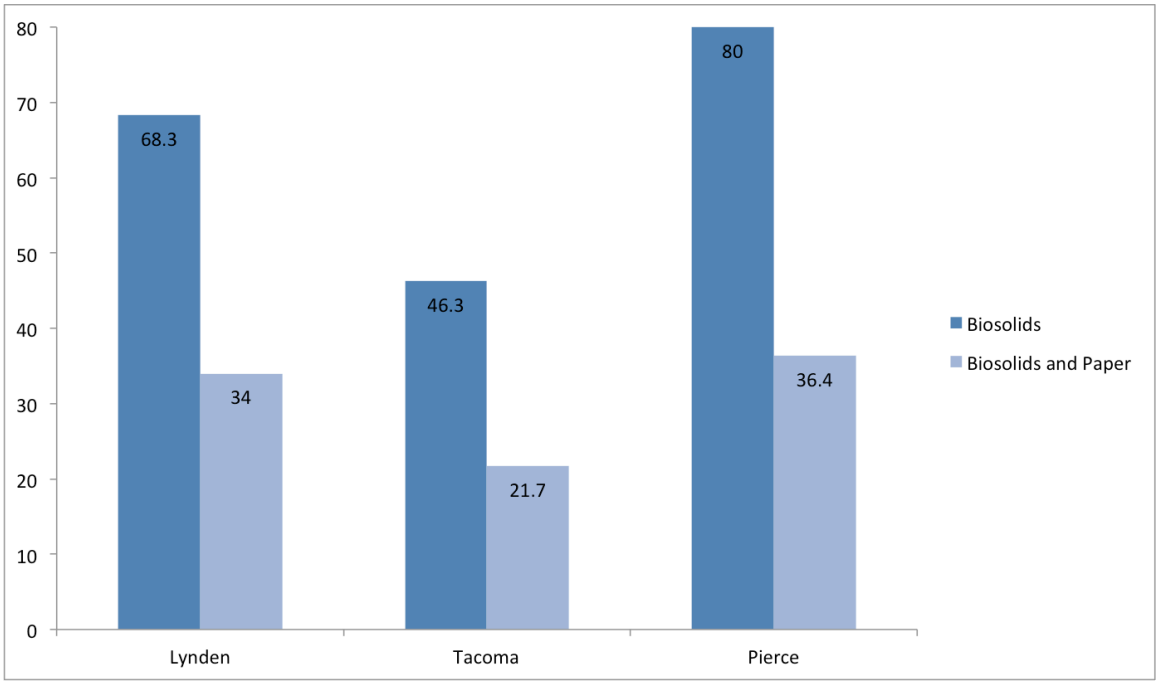


Figure 9.11. Total Kjeldahl nitrogen (g/kg) in the biosolids (dark grey) and substrates composed of three parts paper mulch to four parts biosolids (by dry weight; light grey) from City of Lynden, Tacoma, and Pierce County before earthworms were added.

While all substrates were prepared to be 80 percent moisture (or 20 percent solids), some variation was observed when sampled at the laboratory but nothing to the extent that would indicate conditions not favorable to earthworm survival (Edwards & Bohlen, 1996).

9.5. Discussion

9.5.1. Earthworm survival

Within the substrates composed of four parts biosolids and three parts paper mulch, the one made with Pierce County's biosolids had nearly half of the earthworms added, perish (see Figure 9.3). In the substrates made with the City of Lynden's biosolids, there was very little loss of earthworm life. The substrates composed of the City of

Tacoma's dually digested biosolids saw no loss of earthworms in either the biosolids and paper mulch mixture or the divided substrate containers.

The greatest loss of earthworms was observed in the biosolids and paper mulch substrates composed of the City of Lynden's aerobically (12.5 % of total earthworms added has perished) and Pierce County's anaerobically (42.1% of total earthworms added has perished) digested biosolids. The City of Lynden's divided biosolids and compost container was the only biosolids source that lost earthworms (4%). It is believed that fewer earthworms perished in the divided containers, compared to the biosolids and paper mulch substrates, because the earthworms had a more habitable option, the compost, that was exposed to air other than the biosolids and paper mulch mixture, which had the compost sandwiched between the lower and upper containers. However, the divided substrate composed of Pierce County's biosolids had 76 percent of the surviving earthworms in the biosolids and experienced the greatest loss of earthworms compared to substrates made with the City of Tacoma and Lynden's biosolids. Considering most of the earthworms within the divided containers with the City of Tacoma and Lynden's biosolids were found in the compost it was unexpected to find the majority of earthworms in Pierce County's biosolids. Because the source of biosolids was the only difference between the containers and the divided container with Pierce County's biosolids experienced the most loss of the three sources the biosolids are believed to be reason for the earthworms inability to thrive, but finding the majority in the biosolids was unexpected and unexplainable.

The increase in the total number of earthworms in the divided substrate composed of Pierce County's biosolids is attributed to a counting error. The chance one worm left a

container, travelled between the two, and ended up in this container is possible but does not seem probable. Additionally, it would be impossible for a cocoon to have hatched and worm matured in the 35-day period of this study as it takes four months for *E. fetida* to mature from fertilization (Tripathi & Bhardwaj, 2004). One other possibility is that there was freshly hatched earthworm on one of the counted adult worms that was not seen when placed into the substrate. However, every effort was made to ensure only the adult worms were being added to test substrates. Therefore, a counting error is attributed to the observed increase.

When comparing the weight per earthworm within each substrate before and after the 35-day experiment, regardless of whether the substrate was the biosolids and paper mulch mixture or the divided container with biosolids and compost, the greatest loss of weight per earthworm was observed in the substrates composed of Pierce County's biosolids and the smallest weight loss was observed in the substrates composed of the City of Lynden's biosolids (see Figure 9.4 and 9.5). In their research evaluating survival and growth of *E. fetida* Kaplan et al. (1980) found that anaerobically digested sludge did not contain sufficient nutrients for growth in the form of weight gain, in addition to various environmental factors that affected growth and survival. The environmental factors that Kaplan et al. found as impacting growth and survival of *E. fetida*, temperature, soil moisture, and pH, were maintained as consistent as possible between containers or were within optimal parameters in this study. What Kaplan et al. noticed, regarding the use of sludge or biosolids, is that if anaerobically digested material was used, layering or mixing it with soil resulted in greater earthworm growth. Additionally, Neuhauser, Kaplan, Malecki, and Hartenstein (1980) determined that the presence of soil

increases earthworm growth due to the inorganic matter in the soil. Neuhauser et al. also observed that the particle size of the material was inversely related to earthworm weight gain; the smaller the material the greater weight gain was observed.

In the current study, the biosolids were not sieved to ensure size but when preparing the substrates there was an observable and tactile difference in the biosolids. The City of Lynden's biosolids were smooth, almost clay like, while the biosolids from Pierce County were much more coarse and did not absorb water well. Based on the findings by Neuhauser et al. (1980) it is possible the particulate size of the biosolids resulted in the difference in weight per earthworm at the end of the 35-day study.

9.5.2. Reproduction

A large amount of cocoons were produced in all the divided compost-biosolids substrates, with the majority of the cocoons found in the compost, regardless of the source of the biosolids. While the same amount of compost was provided in the substrates that were a mixture of biosolids and paper mulch and in the divided substrate containers, the only difference is the compost in the divided container was in the upper portion and may have increased the amount of available oxygen, which was not a parameter measured in this experiment. This exposure to air may have created a more ideal environment for the earthworms

Edwards and Bohlen (1996) found that temperature and moisture are correlated to cocoon production and growth. However, there was no remarkable difference in temperature or moisture in the present experiment. The Pierce County biosolids and paper mulch substrate had the fewest amount of cocoons between the three biosolids sources but that would be expected when the overall total of earthworms decreased by

nearly half over the course of the experiment. Additionally, regardless of the substrate treatment, those made with Pierce County's biosolids had the lowest number of cocoons compared to the other substrates made with the City of Tacoma or Lynden's biosolids. The only major difference in nutrients between the three biosolids sources is the TKN of the substrate before earthworms were added (see Figure 9.12). The biosolids and paper mulch substrate created with Pierce County's biosolids had TKN of 36.4 g/kg. However, this is not that much different from the substrate created with the City of Lynden's biosolids (34 g/kg). While the substrate created with the City of Tacoma's biosolids had TKN of (21.7 g/kg). Unfortunately, due to the limitations of the TKN test, it is not possible to determine which, organic nitrogen, ammonia (NH_3) and ammonium (NH_4^+), is responsible for the impact the TKN may or may not have had on earthworm reproduction.

Perhaps the particulate size of the biosolids contributed to the reproduction success by earthworms. While cocoon production does not follow the trend of observed tactile differences between the biosolids, Pierce County being the most coarse and the City of Lynden being the least coarse (by personal observation), perhaps the size differed just enough to result in sufficient versus insufficient nutrition for the earthworms. Another factor that may be at play, which was not quantitatively measured, is that the City of Tacoma's WWTP utilizes a dual-digestion process. The wastewater that enters the treatment plant is first aerobically digested before it is anaerobically digested. The effect an initial aerobic digestion has on the biosolids does not stand out in the metrics measured in this study.

9.5.3. Triclosan and methyl triclosan concentration

The concentration of TCS in the substrate made with the City of Lynden's biosolids was above quantifiable limit (30 µg/kg) only in the sample tested at the beginning of the experiment. Therefore only the substrates created with the City of Tacoma and Pierce County's biosolids are compared here regarding TCS concentration. Overall, TCS concentrations decreased by 17 and 16 percent in the substrates made with City of Tacoma and Pierce County's biosolids, respectively, from before and after exposure to earthworms. However, when the 20 percent uncertainty is applied to the TCS values measured, the error bars overlap indicating inconclusive results (see Figure 9.8).

Triclosan is a synthetic chemical compound that does not exist in the natural environment (U. S. EPA, 2010). That said, within the substrates made with the City of Tacoma's biosolids, TCS concentration increased by 20.9 percent in the control substrate compared to the sample tested in the beginning (4,300 ± 860 to 5,200 ± 1,040 µg/kg). The uncertainty in laboratory measurements of TCS and Me-TCS in soil samples is typically about 20 percent (M. Bozlee, personal communication, November 21, 2016). The error bars, for the before and control substrate made with the City of Tacoma's biosolids, overlap indicating they may be the same concentration and the difference in the values measured is due to the instrument uncertainty. Overall, there is no discernable difference within and across biosolids sources.

The substrate made with the City of Tacoma's biosolids that was exposed to earthworms had a TCS concentration of 3,600 ± 720 µg/kg, which is a 16 percent decrease from the beginning of the 35-day study. Initially, the substrate made with Pierce County's biosolids had TCS concentration of 3,500 ± 700 µg/kg before exposure and

2,900 ± 580 µg/kg after 35 days of exposure to earthworms; the difference between the measured values of TCS is 600 µg/kg but with the 20 percent instrument uncertainty, the values overlap indicating no discernable difference. No difference in TCS concentration was observed in the control substrate that did not have earthworms added and was allowed to age throughout the 35-day trial.

Approximately one to 66 percent of TCS degrades into Me-TCS through biological mythelation (Butler et al., 2012; Chen et al., 2011). The difference in TCS concentrations observed in the current study between the biosolids from the City of Tacoma and Pierce County and paper mulch substrates before and after exposure to earthworms is greater than one percent of the initial TCS concentrations but less than 66 percent. The City of Tacoma's biosolids and paper mulch substrate had TCS concentration of 4,300 ± 860 µg/kg before exposure and 3,600 ± 720 µg/kg after exposure to earthworms; the difference between the measured values of TCS is 700 µg/kg, which is greater than one percent of the starting concentration (43 µg/kg) yet far less than the possible 66 percent (2,838 µg/kg).

If the TCS degradation in this experiment is like that observed by Chen et al. (2011) and Butler et al. (2012), one and 66 percent, respectively, we would expect there to be 43 µg/kg to 2,838 µg/kg of Me-TCS formed in the substrates composed of the City of Tacoma's biosolids and 34 µg/kg to 2,310 µg/kg of Me-TCS formed in the substrates composed of Pierce County's biosolids. Again, the process by which TCS degrades into Me-TCS is not entirely known, it is likely due to microbial methylation (Boehmer et al., 2004).

Interestingly, and unexpectedly, in the substrate composed of the City of Tacoma's biosolids 59 ± 11.8 µg/kg of Me-TCS was formed in the control substrate (no earthworms added) and 160 ± 32 µg/kg of Me-TCS in the substrate with earthworms. This does not align the results that had been anticipated that Me-TCS concentrations would be less in the substrate with earthworms because past research has observed their bioaccumulation of TCS and Me-TCS (Macherius et al., 2014). When comparing the values with the 20 percent uncertainty the error bars do not overlap indicating a discernable difference between Me-TCS concentrations between the substrate made with the City of Tacoma's biosolids before, after exposure to earthworms, and the control. However, because there were no replicates to compare these numbers to, there is no way to know whether these numbers are typical, therefore repeating this experiment, with replicates, is necessary to draw founded conclusions.

As TCS concentrations decrease due to degradation, Me-TCS concentrations are expected to increase. The Me-TCS concentrations in the substrates made with Pierce County's biosolids remained stable between the samples collected in the beginning of the trial (14 ± 2.8 µg/kg) compared to that of the substrate exposed to earthworms (16 ± 3.2 µg/kg) and the control (15 ± 3 µg/kg). Methyl triclosan concentrations within the City of Lynden's biosolids and paper mulch substrate decreased more so in the substrate exposed to *E. fetida* (110 ± 22 µg/kg initially, to 75 ± 15 µg/kg) compared to the control substrate (110 ± 22 µg/kg initially to 82 ± 16.4 µg/kg; see Figure 9.7). This may support the possibility that the earthworms' bioaccumulated the TCS and Me-TCS (Macherius et al., 2014) as the TCS concentrations decreased as well, but the 20 percent instrument uncertainty, the values overlap indicating inconclusive results. However, it is unlikely the

total amount of Me-TCS observed to decrease was only due to degraded because it is known to persist longer in the environment than its parent compound, TCS (Lindström et al., 2002). It did appear to decrease some in between the beginning and the control of the biosolids and paper mulch substrate made with the City of Lynden's biosolids, but not a difference that is conclusive due to instrument uncertainty.

Interestingly, the substrates made with the City of Tacoma's biosolids measured the greatest increase in Me-TCS concentrations. At the beginning of the trial the concentration of Me-TCS measured below the detectible limit of 5 µg/kg. At the end of the 35-day experiment, the control substrate, not exposed to earthworms, measured 59 ± 11.8 µg/kg of Me-TCS and the substrate exposed to earthworms had 160 ± 32 µg/kg of Me-TCS. The difference in Me-TCS concentration observed in the substrates made with the City of Tacoma's biosolids was greater than anticipated. The substrate exposed to the earthworms was expected to have the lowest Me-TCS concentration because it was hypothesized the earthworms would bioaccumulate the chemical compound, which was clearly not the case (see Figure 9.7).

Putting aside the TCS concentration increase of 900 µg/kg observed in the control substrate after the 35-day trial compared to the initial substrate, a 700 µg/kg difference was observed in the TCS concentration from the initial substrate and the substrate exposed to earthworms for 35 days. Twenty-two percent of TCS degrading into Me-TCS is possible (Butler et al., 2012) but is nearly 100 times greater than what was measured in the substrate made with Pierce County's biosolids that was exposed to earthworms (see Figure 9.12).

Figure 9.12. The difference in triclosan concentration that can be explained by the formation of methyl triclosan in substrates composed of paper mulch and biosolids sourced from the City of Tacoma and Pierce County after 35-day exposure to *E. fetida*.

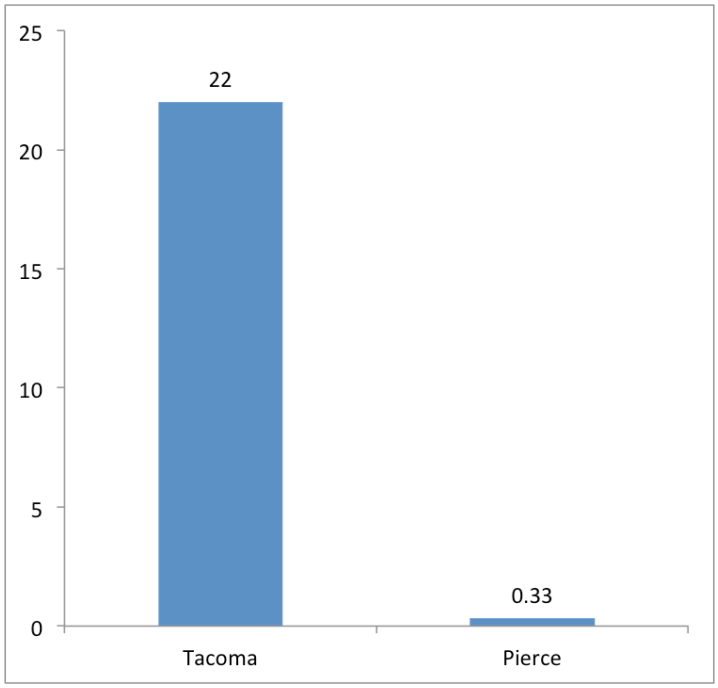


Figure 9.12. The total difference of measured triclosan concentration from the initial substrate compared to substrate exposed to earthworms for 35 days, which can be explained by the formation of Me-TCS during the same time period for substrates composed of biosolids from the City of Tacoma and Pierce County.

The City of Tacoma measured higher levels of Me-TCS in the substrates exposed to earthworms compared to the control substrates. In the biosolids and paper mulch substrate made with the City of Tacoma's biosolids, there was a sharp increase in Me-TCS concentration between the initial substrate tested, control substrate, and the substrate exposed to earthworms. While Me-TCS concentrations were expected to increase as TCS degraded the increase observed in the substrates made with the City of Tacoma's biosolids was greater than anticipated. The City of Lynden may not have followed suit because there was half as much TCS in the substrate compared to Me-TCS. However, a 32 percent decrease in Me-TCS concentration was observed in the substrate exposed to

earthworms while Me-TCS concentration decreased by 25 percent in the control substrate, not exposed to earthworms; evidence that there is something affecting the Me-TCS concentration that can not be attributed to the presence of earthworms.

The substrate composed of Pierce County's biosolids had TCS concentration of 3,500 ± 700 µg/kg before exposure and 2,900 ± 580 µg/kg after exposure to earthworms; the difference between the measured values of TCS is 600 µg/kg. The concentration of Me-TCS at the beginning of this experiment in Pierce County's biosolids measured 14 ± 2.8 µg/kg and at the end increased to 16 ± 3.2 µg/kg in the substrate that was exposed to earthworms while TCS concentrations decreased from 3,500 ± 700 to 2,900 ± 580 µg/kg in the same substrate. Triclosan concentrations decreasing by 600 µg/kg cannot not only be due to degradation into Me-TCS because Me-TCS concentration only increased by 2 µg/kg. This suggests there are other factors affecting the decrease in overall TCS concentration. While it is possible the earthworms bioaccumulated the additional TCS, especially because past research supports this (Higgins et al., 2011; Kinney et al., 2006, 2008, 2010; Macherius et al., 2014), it cannot be ruled out that there were other factors influencing the decrease in TCS. Therefore, either the earthworm's bioaccumulated the majority of the TCS that did not degrade into Me-TCS or it degraded into another compound that was not measured in the current study. If the TCS was bioaccumulated by the earthworms, one would expect to see increased concentrations of TCS in the earthworm tissues and if the TCS degraded into another compound, we would expect to see the concentrations of that degraded compound increase when comparing substrates before and after earthworm exposure.

A possible mechanism that was not measured in this study is the effect the earthworms have the microbial community in the substrate. One factor that was not measured that may have impacted the TCS and Me-TCS concentrations is the impact earthworms have on the microbial community (European Commission, 2010). Domínguez, Aira, and Gómez-Brandón (2010) evaluated microbial activity in the presence and absence of earthworms. They found that when earthworms were present, microbes in the soil were more effective at utilizing available energy compared to a control, which was conducted without added earthworms. Over a four-week period, microbial respiration increased nearly 90 percent simply through the process of earthworms consuming and defecating soil (Scheu, 1987). Additionally, Gómez-Brandón, Aira, Lores, and Domínguez (2011) looked at the microbes in manure and microbes excreted within earthworm casts. Finding that while earthworms decreased the overall biomass, or amount, of microbes in the substrate, yet the activity of the microbe community did not change, even with fewer microbes present after earthworm digestion.

Turning back to the current study, perhaps the more active microbes excreted by the earthworms in their casts were breaking down the TCS into Me-TCS, which is then bioaccumulated by the earthworms. This may explain the increase in Me-TCS concentrations seen in substrates that were exposed to earthworms created with the City of Tacoma's biosolids. While the exact process by which TCS degrades into Me-TCS is not entirely known, Me-TCS is most likely formed by microbial methylation (Boehmer et al., 2004). Perhaps the microbes excreted by earthworms assisted in the microbial methylation that is believed to be the process by which Me-TCS is formed.

It would be interesting to see what would have happened had the earthworms been kept in the substrates for a longer period of time. If given more time, they may bioaccumulate more TCS and Me-TCS through consumption of the contaminated substrate and through direct contact with the compounds in the substrate in which they are living. The amount of TCS present in the substrates composed of the City of Tacoma and Pierce County's biosolids may have seen a reduction had the earthworms been allowed more time to process the material. Additionally, if the microbes are indeed increasing the rate at which TCS degrades into Me-TCS, increasing the time the earthworms are in and consuming the substrate would further increase the microbes' activity as well. Because Me-TCS is more lipophilic than TCS it would be more readily bioaccumulated by the earthworms in the substrate.

9.5.4 *Eisenia fetida* substrate preference

The divided container approach was taken to determine whether earthworms prefer aerobically digested sludge or anaerobically digested sludge over compost. While more worms did survive, it did not appear as though there was a difference in the preference of dually versus aerobically digested biosolids because substrates made with the City of Tacoma and Lynden's biosolids had healthy worm survival while anaerobically digested Pierce County did not.

Dually digested City of Tacoma and aerobically digested City of Lynden had the majority of the earthworms in their biosolids. Whereas, both the divided container and the biosolids and paper mulch mixture created with anaerobically digested Pierce County's biosolids had the majority of the earthworms in the compost, indicating a preference for the compost over the biosolids. The reason for this difference is not

understood and cannot be explained with the data from this thesis. Based on knowledge gained and past research (Hartenstein, 1981; Masciandaro et al., 2000; Weaver, P., personal communication, 2016) it was expected that earthworms would mist likely be in the aerobically digested biosolids and less likely to be in the anaerobically digested biosolids, but that was not the case in this experiment.

9.5.5. Nutrients

Tacoma experienced no loss of earthworms and had lowest TKN (21.7 g/kg) in divided and biosolids and paper mulch substrate followed by substrates composed of the City of Lynden and Pierce County's biosolids (34 g/kg and 36.4 g/kg, respectively) and experienced loss of earthworms in a similar fashion (City of Lynden=12.5% and Pierce County = 42.1%; see Figure 9.14). As for cocoon production, Pierce County had the greatest amount of TKN and lowest number of cocoons (200) but the pattern does not hold when it comes to the City of Tacoma and Lynden, in that Lynden had a higher TKN value than Tacoma but more cocoons (Lynden = 252 and Tacoma = 248; see Figure 9.13).

The correlation of the TOC in the substrate to the survival of earthworms observed in Experiment III was also seen here, in Experiment IV. As the TOC increased, so did the total number of individual earthworms alive at the end of the 35-day experiment.

Figure 9.13. Initial total Kjeldahl nitrogen (TNK) and percent of total worms added that died in substrate made of three parts paper mulch and four parts biosolids from the City of Tacoma, Lynden, and Pierce County.

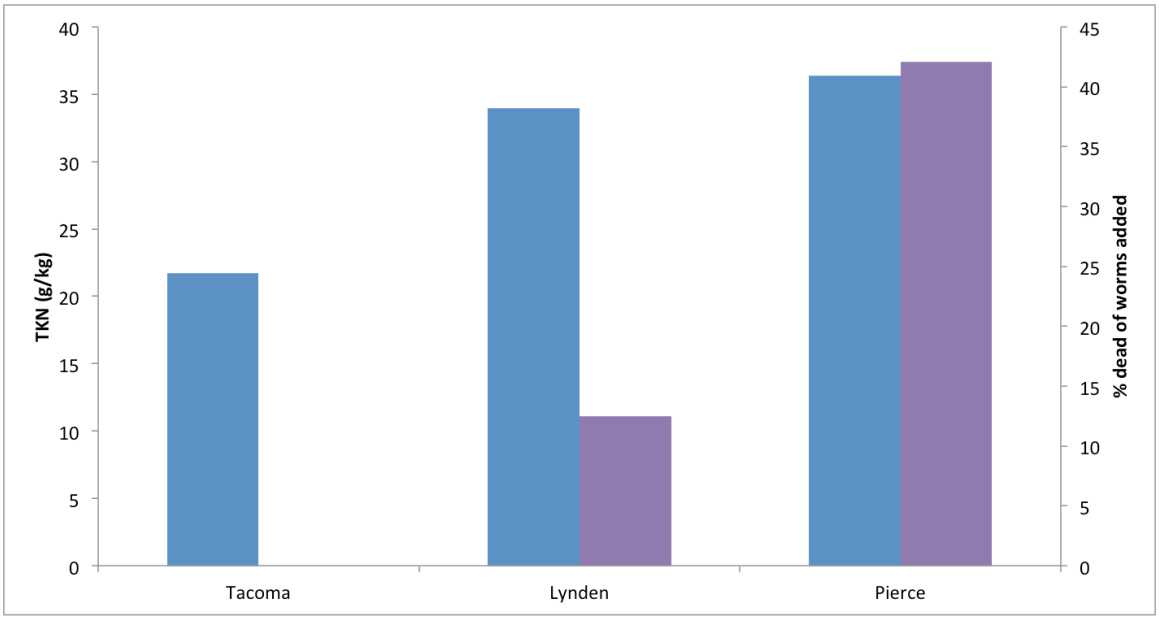


Figure 9.13. Total Kjeldahl nitrogen (g/kg, blue) within substrate prior to the addition of earthworms and percent of total earthworms added that died (purple) in substrate made of three parts paper mulch and four parts biosolids sourced from the City of Tacoma, Lynden, and Pierce County.

10. Conclusion

The question at the heart of this thesis is whether the presence of earthworms (*E. fetida*) effects TCS and Me-TCS concentrations in biosolids destined for land application. The answer is maybe yes and no. The "maybe" is because there were no replicates in these experiments due to financial limitations and due to the unanticipated methods development (Experiments I, II, and III). However, the results obtained from laboratory analysis do suggest Me-TCS concentrations increased in the presence of earthworms in the substrates composed of the City of It is unclear whether the repeated Pilot substrate (in Experiment III) had an elevated Me-TCS concentration after earthworm exposure because there was no control substrate that did not have earthworms with which to compare.

As for the TCS and Me-TCS concentrations in the biosolids, it is possible the presence of earthworms may have reduced the overall concentrations, more so than the microbial degradation of TCS into Me-TCS alone. It is unclear whether TCS degraded into compounds other than Me-TCS, as they were not tested in this study, or if the earthworms bioaccumulated the contaminants, as they were not tested for TCS and Me-TCS. It is indicative that there may be a relationship between the presence of *E. fetida* in biosolids and an increase in Me-TCS concentrations, in the City of Tacoma's biosolids.

This study established first steps in developing methods for the use of biosolids from Pierce County, the City of Tacoma, and Lynden in the application of biosolids-vermicomposting. Future research can evaluate the TCS and Me-TCS concentrations in the substrates as well as the earthworms. Due to the lack of replicates, it is impossible to know whether the observations and results in this study are representative of trends one could anticipate seeing if repeated. A simple study including replicates would allow for stronger data and conclusions. However, there are some interesting findings that can shed light down hallways of knowledge towards possible avenues of successful PPCP removal. All PPCPs will not be banned as the U. S. and Germany have done with TCS because some are necessary. Even though TCS was banned, we do not know the effects of long-term exposure on wildlife and the environment to know how things will react; much less how the degraded forms of TCS will affect us and the environment in the future.

Overall, the initial steps of this study support the findings of Hartenstein et al. (1981) that anaerobically digested biosolids create a toxic environment for earthworms. Even though the biosolids obtained from the City of Tacoma are dually digested, initially

aerobic followed by anaerobic digestion, the earthworms were not able to survive in biosolids-heavy substrates. However, with the addition of sufficient carbon-based bedding, the earthworms were able to survive and thrive. By creating substrates composed of aerobically digested biosolids from the City of Lynden and anaerobically digested biosolids from Pierce County for comparison the results are not so straightforward. More earthworms survived in substrate made with aerobically digested biosolids compared to substrate made with anaerobically digested biosolids but the dually processed biosolids from the City of Tacoma resulted in no loss of earthworms.

Something to note is that the initial steps in determining the appropriate biosolids to paper mulch ratio was based on the City of Tacoma's biosolids. The C to N ratios of the substrates composed of Pierce County and the City of Lynden's biosolids mixed with the same ratio of paper mulch were 11 and 10, respectively. Compared to the City of Tacoma, it is easy to see they have more nitrogen as the City of Tacoma's C to N was 18 Therefore, perhaps with further testing appropriate C to N ratios can be accomplished using biosolids from Pierce County and the City of Lynden. Additionally, simply allowing the earthworms more time to process the biosolids and be in contact with the substrate may result in clearer results.

If earthworms are indeed capable of bioaccumulating, and effectively removing, TCS and Me-TCS from biosolids, they may be utilized in the removal of the contaminants, and perhaps other PPCPs, prior to land application. Deegan et al. (2011) reviewed a variety of wastewater treatment options and their ability and efficiency in removing PPCPs. They evaluated published literature testing wastewater treatments that have been added to traditional secondary sewage treatment: aerobic digestion, anaerobic

digestion and oxidation ditches, which are ditches with mechanized agitators to create an aerobic environment. These additional treatments include membrane filtration, reverse osmosis and activated carbon and can be costly and time consuming to incorporate into an already existing WWTP. Unfortunately, they found that there is no solution for removing all PPCPs that enter a WWTP.

Washington State's Department of Ecology tested the influent, effluent and biosolids of five WWTPs in the Pacific Northwest for 172 organic compounds, including 72 PPCPs, 27 hormones/steroids, and 73 semi-volatile organics (Lubliner et al., 2010). Of the all samples collected and tested, every sample had detectible levels of PPCPs. Only 12 of the 172 compounds (7%) were not detected following secondary wastewater treatment technologies, mentioned in the previous paragraph) and were not preset in the biosolids. Triclosan was of detectable levels after the secondary treatment, and in the biosolids, but not in wastewater after a tertiary treatment (defined by Lubliner et al. as a chemical addition, filtration, or nutrient removal). Interestingly, approximately 20 percent (mostly polycyclic aromatic hydrocarbons or PAHs) of the 172 compounds were detected only in the biosolids further supporting the need to develop a method for removing these contaminating compounds prior to the land-application of biosolids. Biosolids are rich in nutrients and great for amending soils but their application on land with the anthropogenic contaminants only pollutes our environment and puts organisms at risk. This research illuminates one potential avenue for a potential solution, the utilization of earthworms.

References

Agyin‐Birikorang, S., Miller, M., & O'Connor, G. A. (2010). Retention‐release characteristics of triclocarban and triclosan in biosolids, soils, and biosolids‐amended soils. *Environmental Toxicology and Chemistry*, *29*(9), 1925-1933.

Ahn, K. C., Zhao, B., Chen, J., Cherednichenko, G., Sanmarti, E., Denison, M. S., ... & Gee, S. J. (2008). In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. *Environ Health Perspect*, *116*(9), 1203-1210.

Aiello, A. E., Larson, E. L., & Levy, S. B. (2007). Consumer antibacterial soaps: effective or just risky? *Clinical Infectious Diseases*, *45*(Supplement 2), S137-S147.

Alexander, R. R., & Alexander, M. (1999). Genotoxicity of two polycyclic aromatic hydrocarbons declines as they age in soil. *Environmental Toxicology and Chemistry*, *18*(6), 1140-1143.

Alliance for the Prudent Use of Antibiotics. (2011). *Triclosan: White paper prepared by The Alliance for the Prudent Use of Antibiotics (APUA)*. Retrieved November 12, 2015 from http://www.tufts.edu/med/apua/consumers/personal_home_21_4240495089.pdf

Allmyr, M., Adolfsson-Erici, M., McLachlan, M. S., & Sandborgh-Englund, G. (2006). Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. *Science of the Total Environment*, *372*(1), 87-93.

American Biogas Council Frequent Questions. (n.d.). Retrieved from http://www.americanbiogascouncil.org/biogas_questions.asp

Anderson, J. M., & Ingram, J. S. I. (1993). Soil organic matter and organic carbon. *Tropical Soil Biology and Fertility*, 62-66.

Aranami, K., & Readman, J. W. (2007). Photolytic degradation of triclosan in freshwater and seawater. *Chemosphere*, *66*(6), 1052-1056.

Artuso, N., Kennedy, T. F., Connery, J., Grant, J., & Schmidt, O. (2010). Effects of biosolids at varying rates on earthworms (*Eisenia fetida*) and springtails (*Folsomia candida*). *Applied and Environmental Soil Science*,*2011*.

Atlas, R. M. (1995). Bioremediation of petroleum pollutants. *International Biodeterioration & Biodegradation*, *35*(1), 317-327.

Avis, R. (2011, April 2). Everything you need to know about composting with worms. *Permaculture Research Institute*. Retrieved from http://permaculturenews.org/2011/04/02/everything-you-need-to-know-about-composting-with-worms/

Balmer, M. E., Poiger, T., Droz, C., Romanin, K., Bergqvist, P. A., Müller, M. D., & Buser, H. R. (2004). Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. *Environmental Science & Technology*, *38*(2), 390-395.

Barber, L. B., Keefe, S. H., Antweiler, R. C., Taylor, H. E., & Wass, R. D. (2006). Accumulation of contaminants in fish from wastewater treatment wetlands. *Environmental Science & Technology*, *40*(2), 603-611.

Barker, A. V., & Bryson, G. M. (2002). Bioremediation of heavy metals and organic toxicants by composting. *The Scientific World Journal*, *2*, 407-420.

Benitez, E., Sainz, H., & Nogales, R. (2005). Hydrolytic enzyme activities of extracted humic substances during the vermicomposting of a lignocellulosic olive waste. *Bioresource Technology*, *96*(7), 785-790.

Bertelsen, R. J., Longnecker, M. P., Løvik, M., Calafat, A. M., Carlsen, K. H., London, S. J., & Lødrup Carlsen, K. C. (2013). Triclosan exposure and allergic sensitization in Norwegian children. *Allergy*, *68*(1), 84-91.

Bester, K. (2005). Fate of triclosan and triclosan-methyl in sewage treatment plants and surface waters. *Archives of Environmental Contamination and Toxicology*, *49*(1), 9-17.

Bhatt, M., Cajthaml, T., & Šašek, V. (2002). Mycoremediation of PAH-contaminated soil. *Folia Microbiologica*, *47*(3), 255-258.

Binelli, A., Cogni, D., Parolini, M., Riva, C., & Provini, A. (2009). In vivo experiments for the evaluation of genotoxic and cytotoxic effects of triclosan in Zebra mussel hemocytes. *Aquatic Toxicology*, *91*(3), 238-244.

Boehmer, W., Ruedel, H., Wenzel, A., & Schroeter-Kermani, C. (2004). Retrospective monitoring of triclosan and methyl-triclosan in fish: results from the German environmental specimen bank. *Organohalogen Compounds*, *66*, 1516-1521.

Butler, E., Whelan, M. J., Sakrabani, R., & Van Egmond, R. (2012). Fate of triclosan in field soils receiving sewage sludge. *Environmental Pollution*, *167*, 101-109.

Calafat, A. M., Ye, X., Wong, L. Y., Reidy, J. A., & Needham, L. L. (2008). Urinary concentrations of triclosan in the US population: 2003-2004. *Environmental Health Perspectives*, *116*(3), 303.

Canosa, P., Morales, S., Rodriguez, I., Rubi, E., Cela, R., & Gomez, M. (2005). Aquatic degradation of triclosan and formation of toxic chlorophenols in presence of low concentrations of free chlorine. *Analytical and Bioanalytical Chemistry*, *383*(7-8), 1119-1126.

Chachina, S. B., Voronkova, N. A., & Baklanova, O. N. (2015). Biological remediation of the engine lubricant oil-contaminated soil with three kinds of earthworms, *Eisenia Fetida*, *Eisenia Andrei*, *Dendrobena Veneta*, and a mixture of microorganisms. *Procedia Engineering*, *113*, 113-123.

Chedgzoy, P., Winckle, G., & Heard, C. M. (2002). Triclosan: release from transdermal adhesive formulations and in vitro permeation across human epidermal membranes. *International Journal of Pharmaceutics*, *235*(1), 229-236.

Chen, J., Ahn, K. C., Gee, N. A., Ahmed, M. I., Duleba, A. J., Zhao, L., ... & Lasley, B. L. (2008). Triclocarban enhances testosterone action: a new type of endocrine disruptor?. *Endocrinology*, *149*(3), 1173-1179.

Chen, X., Pauly, U., Rehfus, S., & Bester, K. (2009). Personal care compounds in a reed bed sludge treatment system. *Chemosphere*, *76*(8), 1094-1101.

Chengelis, C. P. (1990). Examples of alternative use in toxicology for common species. *International Journal of Toxicology*, *9*(3), 319-342.

Chen, X., Nielsen, J. L., Furgal, K., Liu, Y., Lolas, I. B., & Bester, K. (2011). Biodegradation of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions. *Chemosphere*, *84*(4), 452-456.

Chenxi, W., Spongberg, A. L., & Witter, J. D. (2008). Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. *Chemosphere, 73*(4), 511-518.

City of Tacoma, Environmental Services. (2013a). *TAGRO*. Retrieved from https://www.cityoftacoma.org/cms/one.aspx?objectId=16884

City of Tacoma, Environmental Services. (2013b). *TAGRO: Purchase locations*. Retrieved from https://www.cityoftacoma.org/government/city_departments/environmentalservices/tagro/tagro_home_users/purchase_locations

City of Tacoma, Environmental Services. (2013c). *TAGRO: Safety*. Retrieved from https://www.cityoftacoma.org/government/city_departments/environmentalservices/tagro/tagro_safety

Clayton, R., Erin, M., Todd, M., Dowd, J. B., & Aiello, A. E. (2010). The impact of Bisphenol A and Triclosan on immune parameters in the U. S. population, NHANES 2003 a 2006. *Environmental Health Perspectives*, *119*(3), 390-396.

Cohen, L. (2015, June 29). 'Crappy' solution to soil shortage: U.S. Open human waste. *KOMO News*. Retrieved from http://komonews.com/news/local/crappy-solution-to-soil-shortage-us-open-human-waste

Coogan, M. A., Edziyie, R. E., La Point, T. W., & Venables, B. J. (2007). Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant receiving stream. *Chemosphere, 67*(10), 1911-1918.

Coogan, M. A., & Point, T. W. L. (2008). Snail bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas, USA, stream affected by

wastewater treatment plant runoff. *Environmental Toxicology & Chemistry*, *27*(8), 1788-1793.

Dabke, S. V. (2013). Vermi-remediation of heavy metal-contaminated soil. *Journal of Health and Pollution*, *3*(4), 4-10.

Dann, A. B., & Hontela, A. (2011). Triclosan: environmental exposure, toxicity and mechanisms of action. *Journal of Applied Toxicology*, *31*(4), 285-311.

Deegan, A. M., Shaik, B., Nolan, K., Urell, K., Oelgemöller, M., Tobin, J., & Morrissey, A. (2011). Treatment options for wastewater effluents from pharmaceutical companies. *International Journal of Environmental Science & Technology*, *8*(3), 649-666.

DeLorenzo, M. E., Keller, J. M., Arthur, C. D., Finnegan, M. C., Harper, H. E., Winder, V. L., & Zdankiewicz, D. L. (2008). Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl‐triclosan in estuarine systems. *Environmental Toxicology*, *23*(2), 224-232.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., ... & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293-342.

Domínguez, J., Aira, M., & Gómez-Brandón, M. (2010). Vermicomposting: earthworms enhance the work of microbes. In *Microbes at work* (pp. 93-114). Springer Berlin Heidelberg.

Edwards, C. A., & Bohlen, P. J. (1996). *Biology and Ecology of Earthworms* (Vol. 3). Springer Science & Business Media.

Effluent limitations guidelines and standards for the steam electric power generating point source category; Proposed rule, 78 Fed. Reg. 110 (June 7, 2013) (to be codified at 40 C.F.R. pt. 423)

European Commission, Scientific Committee on Consumer Safety. (2010). *Opinion on triclosan (antimicrobial resistance)* (SCCP/1251/09). Retrieved from http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_023.pdf

Fair, P. A., Lee, H. B., Adams, J., Darling, C., Pacepavicius, G., Alaee, M., ... & Muir, D. (2009). Occurrence of triclosan in plasma of wild Atlantic bottlenose dolphins (*Tursiops truncatus*) and in their environment. *Environmental Pollution*, *157*(8), 2248-2254.

Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M., & Cabral, J. M. S. (2003). Microbial conversion of steroid compounds: recent developments. *Enzyme and Microbial Technology*, *32*(6), 688-705.

Fiss, E. M., Rule, K. L., & Vikesland, P. J. (2007). Formation of chloroform and other chlorinated byproducts by chlorination of triclosan-containing antibacterial products. *Environmental Science & Technology*, *41*(7), 2387-2394.

Fong, J. & Hewitt, P. (2016). *Troubleshooting worm bins*. Cornell Composting. Retrieved on September 28, 2016 from http://compost.css.cornell.edu/worms/troubleshoot.html

Fuchsman, P., Lyndall, J., Bock, M., Lauren, D., Barber, T., Leigh, K., ... & Capdevielle, M. (2010). Terrestrial ecological risk evaluation for triclosan in land‐applied biosolids. *Integrated Environmental Assessment and Management*,*6*(3), 405-418.

Gajalakshmi, S., Ramasamy, E. V., & Abbasi, S. A. (2001). Potential of two epigeic and two anecic earthworm species in vermicomposting of water hyacinth. *Bioresource Technology*, *76*(3), 177-181.

Gao, P., Ding, Y., Li, H., & Xagoraraki, I. (2012). Occurrence of pharmaceuticals in a municipal wastewater treatment plant: Mass balance and removal processes. *Chemosphere, 88*, 17-24.

Gaume, B., Bourgougnon, N., Auzoux-Bordenave, S., Roig, B., Le Bot, B., & Bedoux, G. (2012). In vitro effects of triclosan and methyl-triclosan on the marine gastropod Haliotis tuberculata. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *156*(2), 87-94.

Gaylor, M. O., Harvey, E., & Hale, R. C. (2013). Polybrominated diphenyl ether (PBDE) accumulation by earthworms (*Eisenia fetida*) exposed to biosolids-, polyurethane foam microparticle-, and penta-BDE-amended soils. *Environmental Science & Technology*, *47*(23), 13831-13839.

Gee, R. H., Charles, A., Taylor, N., & Darbre, P. D. (2008). Oestrogenic and androgenic activity of triclosan in breast cancer cells. *Journal of Applied Toxicology*, *28*(1), 78-91.

Glaser, A. (2004). The ubiquitous triclosan. *A common antibacterial agent exposed. Pesticides and You*, *24*, 12-17.

Gómez-Brandón, M., Aira, M., Lores, M., & Domínguez, J. (2011). Epigeic earthworms exert a bottleneck effect on microbial communities through gut associated processes. *PloS one*, *6*(9), e24786.

Greyshock, A. E., & Vikesland, P. J. (2006). Triclosan reactivity in chloraminated waters. *Environmental Science & Technology*, *40*(8), 2615-2622.

Halden, R. U. (2014). On the need and speed of regulating triclosan and triclocarban in the United States. *Environmental Science & Technology*, *48*(7), 3603-3611.

Halden, R. U., & Paull, D. H. (2005). Co-occurrence of triclocarban and triclosan in US water resources. *Environmental Science & Technology*, *39*(6), 1420-1426.

Hansen, K. H., Angelidaki, I., & Ahring, B. K. (1998). Anaerobic digestion of swine manure: Inhibition by ammonia. *Water Research*, *32*(1), 5-12.

Harris, M. L., Wilson, L. K., Elliott, J. E., Bishop, C. A., Tomlin, A. D., & Henning, K. V. (2000). Transfer of DDT and metabolites from fruit orchard soils to American robins (*Turdus migratorius*) twenty years after agricultural use of DDT in Canada. *Archives of Environmental Contamination and Toxicology*, *39*(2), 205-220.

Hartenstein, R. (1981). Use of *Eisenia foetida* in organic recycling based on laboratory experiments. In *Workshop on the role of earthworms in the stabilization of organic residues. M. Appelhof-Compiler* (Vol. 1, pp. 155-165).

Higgins, C. P., Paesani, Z. J., Chalew, T. E. A., & Halden, R. U. (2009). Bioaccumulation of triclocarban in *Lumbriculus variegatus*. *Environmental Toxicology & Chemistry*, *28*(12), 2580-2586.

Higgins, C. P., Paesani, Z. J., Chalew, T. E. A., Halden, R. U., & Hundal, L. S. (2011). Persistence of triclocarban and triclosan in soils after land application of biosolids and bioaccumulation in *Eisenia foetida*. *Environmental Toxicology & Chemistry*, *30*(3), 556-563.

Hildebrandt, P. (2007). Pharmaceuticals and personal care products: An issue of growing concern for OWT. *Onsite Water Treatment, 3*(1). Retrieved from http://www.foresterpress.com/ow_0701_ppcp.html

Hites, R. A., & Lopez-Avila, V. (1979). Identification of organic compounds in an industrial waste water. *Analytical Chemistry*, *51*(14), 1452A-1456A.

Jacobs, L. W., & McCreary, D. S. (2001). *Applying Biosolids to Land in Michigan*. Michigan State University Extension.

Jones, R. D., Jampani, H. B., Newman, J. L., & Lee, A. S. (2000). Triclosan: a review of effectiveness and safety in health care settings. *American Journal of Infection Control, 28*(2), 184-196.

Jung, E. M., An, B. S., Choi, K. C., & Jeung, E. B. (2012). Potential estrogenic activity of triclosan in the uterus of immature rats and rat pituitary GH3 cells. *Toxicology letters*, *208*(2), 142-148.

Jungclaus, G., Avila, V., & Hites, R. (1978). Organic compounds in an industrial wastewater: A case study of their environmental impact. *Environmental Science & Technology*, *12*(1), 88-96.

Kanetoshi, A., Ogawa, H., Katsura, E., & Kaneshima, H. (1987). Chlorination of Irgasan DP300 and formation of dioxins from its chlorinated derivatives. *Journal of Chromatography A*, *389*, 139-153.

Kanetoshi, A., Ogawa, H., Katsura, E., Okui, T., & Kaneshima, H. (1988). Disposition and excretion of Irgasan® DP300 and its chlorinated derivatives in mice. *Archives of Environmental Contamination and Toxicology*, *17*(5), 637-644.

Kaplan, D. L., Hartenstein, R., Neuhauser, E. F., & Malecki, M. R. (1980). Physicochemical requirements in the environment of the earthworm *Eisenia foetida*. *Soil Biology and Biochemistry*, *12*(4), 347-352.

Kelsey, J. W., Colino, A., & White, J. C. (2005). Effect of species differences, pollutant concentration, and residence time in soil on the bioaccumulation of 2, 2‐BIS (p‐chlorophenyl)‐1, 1‐dichloroethylene by three earthworm species. *Environmental Toxicology & Chemistry*, *24*(3), 703-708.

Kinney, C. A., Campbell, B. R., Thompson, R., Furlong, E. T., Kolpin, D. W., Burkhardt, M. R., ... & Hay, A. G. (2012). Earthworm bioassays and seedling emergence for monitoring toxicity, aging and bioaccumulation of anthropogenic waste indicator compounds in biosolids–amended soil. *Science of the Total Environment*, *433*, 507-515.

Kinney, C. A., Furlong, E. T., Kolpin, D. W., Burkhardt, M. R., Zaugg, S. D., Werner, S. L., ... & Benotti, M. J. (2008). Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environmental Science & Technology*, *42*(6), 1863-1870.

Kinney, C. A., Furlong, E. T., Kolpin, D. W., Zaugg, S. D., Burkhardt, M. R., Bossio, J. P., & Werner, S. L. (2010). Earthworms: Diagnostic Indicators of Wastewater Derived Anthropogenic Organic Contaminants in Terrestrial Environments. In *ACS symposium series* (Vol. 1048, pp. 297-317). Oxford University Press.

Kinney, C. A., Furlong, E. T., Zaugg, S. D., Burkhardt, M. R., Werner, S. L., Cahill, J. D., & Jorgensen, G. R. (2006). Survey of organic wastewater contaminants in

biosolids destined for land application. *Environmental Science & Technology*, *40*(23), 7207-7215.

Kreuzinger, N., Clara, M., Strenn, B., & Vogel, B. (2004). Investigation on the behaviour of selected pharmaceuticals in the groundwater after infiltration of treated wastewater. *Water Science & Technology*, *50*(2), 221-228.

Kwon, J. W., & Xia, K. (2012). Fate of triclosan and triclocarban in soil columns with and without biosolids surface application. *Environmental Toxicology & Chemistry*, *31*(2), 262-269.

la Farré, M., Pérez, S., Kantiani, L., & Barceló, D. (2008). Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *TrAC Trends in Analytical Chemistry*, *27*(11), 991-1007.

Landau, E. & Young, S. (2014). Minnesota issues ban on antibacterial ingredient. *CNN*. Retrieved November 12, 2015 from http://www.cnn.com/2014/05/21/health/triclosan-ban-antibacterial/

Lankester, J., Patel, C., Cullen, M. R., Ley, C., & Parsonnet, J. (2013). Urinary triclosan is associated with elevated body mass index in NHANES. *PloS one*, *8*(11), e80057.

Latch, D. E., Packer, J. L., Arnold, W. A., & McNeill, K. (2003). Photochemical conversion of triclosan to 2, 8-dichlorodibenzo-p-dioxin in aqueous solution. *Journal of Photochemistry and Photobiology A: Chemistry*, *158*(1), 63-66.

LeBlanc, R. J., Matthews, P., & Richard, R. P. (Eds.). (2009). *Global atlas of excreta, wastewater sludge, and biosolids management: Moving forward the sustainable and welcome uses of a global resource*.

Lin, D., Zhou, Q., Xie, X., & Liu, Y. (2010). Potential biochemical and genetic toxicity of triclosan as an emerging pollutant on earthworms (*Eisenia fetida*). *Chemosphere*, *81*(10), 1328-1333.

Lindgren, S., Pettersson, K., Kaspersson, A., Jonsson, A., & Lingvall, P. (1985). Microbial dynamics during aerobic deterioration of silages. *Journal of the Science of Food and Agriculture*, *36*(9), 765-774.

Lindström, A., Buerge, I. J., Poiger, T., Bergqvist, P. A., Müller, M. D., & Buser, H. R. (2002). Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environmental Science & Technology*, *36*(11), 2322-2329.

Lozano, N., Rice, C. P., Ramirez, M., & Torrents, A. (2012). Fate of triclosan and methyltriclosan in soil from biosolids application. *Environmental Pollution*, *160*, 103-108.

Lozano, N., Rice, C. P., Ramirez, M., & Torrents, A. (2013). Fate of triclocarban, triclosan and methyltriclosan during wastewater and biosolids treatment processes. *Water Research, 47*, 4519-4527.

Lubliner, B., Redding, M. B., & Ragsdale, D. (2010). *Pharmaceuticals and personal care products in municipal wastewater and their removal by nutrient treatment technologies*. Washington State Department of Ecology.

Macherius, A., Lapen, D. R., Reemtsma, T., Römbke, J., Topp, E., & Coors, A. (2014). Triclocarban, triclosan and its transformation product methyl triclosan in native earthworm species four years after a commercial-scale biosolids application. *Science of the Total Environment*, *472*, 235-238.

Mackay, D., & Barnthouse, L. (2010). Integrated risk assessment of household chemicals and consumer products: addressing concerns about triclosan. *Integrated Environmental Assessment and Management*, *6*(3), 390-392.

Maramba, F. D. (1978). *Biogas and waste recycling: The Philippine experience*. Metro Manila, Philippines: Regal Printing Company.

Marchaim, U. (1992). *Biogas processes for sustainable development* (No. 95-96). Food & Agriculture Organization of the United Nations.

Masciandaro, G., Ceccanti, B., & Garcia, C. (2000). "In situ" vermicomposting of biological sludges and impacts on soil quality. *Soil Biology and Biochemistry*, *32*(7), 1015-1024.

McAvoy, D. C., Schatowitz, B., Jacob, M., Hauk, A. & Eckhoff, W. S. (2002). Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology & Chemistry, 21*(7), 1323-1329.

McNamara, P. J., LaPara, T. M., & Novak, P. J. (2014). The impacts of triclosan on anaerobic community structures, function, and antimicrobial resistance. *Environmental Science & Technology*, *48*(13), 7393-7400.

Miao, X. S., Bishay, F., Chen, M., & Metcalfe, C. D. (2004). Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environmental Science & Technology*, *38*(13), 3533-3541.

Microban. (n.d.). Retrieved December 5, 2016 from https://www.microban.com/

Miyazaki, T., Yamagishi, T., & Matsumoto, M. (1984). Residues of 4-chloro-1-(2, 4-dichlorophenoxy)-2-methoxybenzene (triclosan methyl) in aquatic biota. *Bulletin of Environmental Contamination and Toxicology*, *32*(1), 227-232.

Morris Pumps. (2008). *Project Profile: Central waste water treatment pant Tacoma, WA*. Retrieved from http://www.yccpump.com/assets/tacoma_central_WWTP_profile.pdf

Monroy, F., Aira, M., & Domínguez, J. (2009). Reduction of total coliform numbers during vermicomposting is caused by short-term direct effects of earthworms on microorganisms and depends on the dose of application of pig slurry. *Science of the Total Environment*, *407*(20), 5411-5416.

National Center for Biotechnology Information. *PubChem Compound Database*. (n.d.). Retrieved from https://pubchem.ncbi.nlm.nih.gov/

Ndegwa, P.M., & Thompson, S.A., (2000). Effects of C-to-N ratio on vermicomposting of biosolids. *Bioresource Technology, 75*, 7-12.

Ndegwa, P.M., Thompson, S.A., & Das, K.C. (2000). Effects of stocking density and feeding rate on vermicomposting of biosolids. *Bioresource Technology*, *71*, 5-12.

Neuhauser, E. F., Kaplan, D. L., Malecki, M. R., & Hartenstein, R. (1980). Materials supporting weight gain by the earthworm *Eisenia foetida* in waste conversion systems. *Agricultural Wastes*, *2*(1), 43-60.

NICNAS, T. (2009). Priority existing chemical assessment report no. 30. *National Industrial Chemicals Notification and Assessment Scheme, Department of Health and Ageing, Australian Government, Sydney, Australia*.

Northwest Biosolids Management Association. *Membership agencies*. (n.d.). Retrieved from http://www.nwbiosolids.org/membership_agencies.htm

North East Biosolids and Residuals Association. (2007). A national biosolids regulation, quality, end use & disposal survey: Final report. *NEBRA, Tamworth, NH*.

Oleszkiewicz, J. A., & Mavinic, D. S. (2002). Wastewater biosolids: an overview of processing, treatment, and management. *Journal of Environmental Engineering and Science*, *1*(2), 75-88.

Oregon Association of Clean Water Agencies. (2009). *Biosolids Fact Sheet*. Retrieved from http://www.nwbiosolids.org/pubs/OR_BiosolidsFactSheet%209_09p.pdf

Organization for Economic Cooperation and Development. (2002). *Guidelines for the Testing of Chemicals / OECD Series on Testing and Assessment Guidance Document on the Use of the Harmonised System for the Classification of Chemicals which are Hazardous for the Aquatic Environment*. OECD Publishing.

Orhon, A. K. (2014). *Triclosan in biological wastewater treatment: Fate, kinetics and population dynamics aspects* (Masters thesis, Middle East Technical University, Ankara, Turkey). Retrieved from http://etd.lib.metu.edu.tr/upload/12617360/index.pdf

Orhan, M., Kut, D., & Gunesoglu, C. (2007). Use of triclosan as antibacterial agent in textiles. *Indian Journal of Fibre and Textile Research*, *32*(1), 114-118.

Outwater, A. B. (1994). *Reuse of sludge and minor wastewater residuals*. Boca Raton, FL: CRC Press, Inc.

Pannu, M. W., O'Connor, G. A., & Toor, G. S. (2012). Toxicity and bioaccumulation of biosolids‐borne triclosan in terrestrial organisms. *Environmental Toxicology & Chemistry*, *31*(3), 646-653.

Phillips, C. T., Checkai, R. T., Chester, N. A., Wentsel, R. S., & Major, M. A. (1994). *Toxicity Testing of Soil Samples from Joliet Army Ammunition Plant, Illinois* (No. ERDEC-TR-137). Edgewood Research Development and Engineering Center Aberdeen Proving Ground, MD.

Pignataro, T. J. (2015, October 1). The chemicals in some everyday soaps threaten Great Lakes waster quality. *The Buffalo Times*. Retrieved from http://buffalonews.com/

Prosser, R. S., Lissemore, L., Topp, E., & Sibley, P. K. (2014). Bioaccumulation of triclosan and triclocarban in plants grown in soils amended with municipal dewatered biosolids. *Environmental Toxicology & Chemistry*, *33*(5), 975-984.

Prosser, R. S., & Sibley, P. K. (2015). Human health risk assessment of pharmaceuticals and personal care products in plant tissue due to biosolids and manure amendments, and wastewater irrigation. *Environment International*, *75*, 223-233.

Rajpal, A., Bhargava, R., Chopra, A. K., & Kumar T. (2014). Vermistabilization and nutrient enhancement of anaerobic digestate through earthworm species *Perionyx excavatus* and *Perionyx sansibaricus*. *Journal of Material Cycles and Waste Management, 16*, 219-226.

Reinecke, A. J., Reinecke, S. A., & Maboeta, M. S. (2001). Cocoon production and viability as endpoints in toxicity testing of heavy metals with three earthworm species. *Pedobiologia*, *45*(1), 61-68.

Reiss, R., Lewis, G., & Griffin, J. (2009). An ecological risk assessment for triclosan in the terrestrial environment. *Environmental Toxicology & Chemistry*, *28*(7), 1546-1556.

Reiss, R., Mackay, N., Habig, C., & Griffin, J. (2002). An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environmental Toxicology & Chemistry*, *21*(11), 2483-2492.

Richards, D. (2006, June 2). *Coir is sustainable alternative to peat moss in the garden*. Retrieved from Oregon State University, Extension Service website: http://extension.oregonstate.edu/gardening/coir-sustainable-alternative-peat-moss-garden

Rojas-Avelizapa, N. G., Roldan-Carrillo, T., Zegarra-Martinez, H., Munoz-Colunga, A. M., & Fernandez-Linares, L. C. (2007). A field trial for an ex-situ bioremediation of a drilling mud-polluted site. *Chemosphere*, *66*(9), 1595-1600.

Rule, K. L., Ebbett, V. R., & Vikesland, P. J. (2005). Formation of chloroform and chlorinated organics by free-chlorine-mediated oxidation of triclosan. *Environmental Science & Technology*, *39*(9), 3176-3185.

Russell, J. R., Huang, J., Anand, P., Kucera, K., Sandoval, A. G., Dantzler, K. W., ... & Strobel, S. A. (2011). Biodegradation of polyester polyurethane by endophytic fungi. *Applied and Environmental Microbiology*, *77*(17), 6076-6084.

Safety and effectiveness of consumer antiseptics; Topical antimicrobial drug products for over-the-counter human use, 81 Fed. Reg. 172 (September 6, 2016) (to be codified at 21 C.F.R. pt. 310)

Salt, D. E., Blaylock, M., Kumar, N. P., Dushenkov, V., Ensley, B. D., Chet, I., & Raskin, I. (1995). Phytoremediation: A novel strategy for the removal of toxic metals from the environment using plants. *Nature Biotechnology*, *13*(5), 468-474.

Sánchez-Prado, L., Llompart, M., Lores, M., Fernández-Álvarez, M., García-Jares, C., & Cela, R. (2006). Further research on the photo-SPME of triclosan. *Analytical and Bioanalytical Chemistry*, *384*(7-8), 1548-1557.

Sayara, T., Borràs, E., Caminal, G., Sarrà, M., & Sánchez, A. (2011). Bioremediation of PAHs-contaminated soil through composting: influence of bioaugmentation and biostimulation on contaminant biodegradation. *International Biodeterioration & Biodegradation*, *65*(6), 859-865.

Scheu, S. (1987). Microbial activity and nutrient dynamics in earthworm casts (*Lumbricidae*). *Biology and Fertility of Soils*, *5*(3), 230-234.

Scottish Environment Protection Agency. (n.d.). *Scottish pollutant release inventory: Chloroform*. Retrieved from http://apps.sepa.org.uk/spripa/Pages/SubstanceInformation.aspx?pid=30

Simard, R. R. (1993). Ammonium acetate-extractable elements. *Soil sampling and methods of analysis*, 39-42.

Singh, D., & Suthar, S. (2012). Vermicomposting of herbal pharmaceutical industry waste: Earthworm growth, plant-available nutrient and microbial quality of end materials. *Bioresource Technology, 112*, 179-185.

Singh, R. P., Singh, P., Araujo, A. S., Ibrahim, M. H., & Sulaiman, O. (2011). Management of urban solid waste: Vermicomposting a sustainable option. *Resources, Conservation and Recycling*, *55*(7), 719-729.

Sinha, R. K., Bharambe, G., & Chaudhari, U. (2008). Sewage treatment by vermifiltration with synchronous treatment of sludge by earthworms: a low-cost sustainable technology over conventional systems with potential for decentralization. *The Environmentalist*, *28*(4), 409-420.

Sinha, R. K., Herat, S., Bharambe, G., & Brahambhatt, A. (2009). Vermistabilization of sewage sludge (biosolids) by earthworms: converting a potential biohazard destined for landfill disposal into a pathogen free, nutritive & safe biofertilizer for farms. *Waste Management & Research*.

Snyder, E. H., O'Connor, G. A., & McAvoy, D. C. (2011). Toxicity and bioaccumulation of biosolids-borne triclocarban (TCC) in terrestrial organisms. *Chemosphere*, *82*(3), 460-467.

Strachan, S. D., Nelson, D. W., & Sommers, L. E. (1983). Sewage sludge components extractable with nonaqueous solvents. *Journal of Environmental Quality*, *12*(1), 69-74.

Suthar, S., & Singh, S. (2008). Vermicomposting of domestic waste by using two epigeic earthworms (Perionyx excavatus and Perionyx sansibaricus). *International Journal of Environmental Science & Technology*, *5*(1), 99-106.

Toronto Research Chemicals. (n.d.). Retrieved from http://www.trc-canada.com/

Tripathi, G., & Bhardwaj, P. (2004). Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg). *Bioresource Technology*, *92*(3), 275-283.

U. S. Environmental Protection Agency. (2000). *Chloroform: Hazard summary*. (CAS 67-66-3). Retrieved from https://www.epa.gov/sites/production/files/2016-09/documents/chloroform.pdf

U. S. Environmental Protection Agency. (2016, July). *Biosolids: Frequently asked questions about biosolids*. Retrieved from https://www.epa.gov/biosolids/frequent-questions-about-biosolids

U. S. Environmental Protection Agency. (2015, December). *Learn about dioxin*. Retrieved from http://www.epa.gov/dioxin/learn-about-dioxin

U. S. Environmental Protection Agency. (2004). Method 9045D: Soil and waste pH. *Test methods for evaluating solid waste, physical/chemical methods*. Retrieved from https://www.epa.gov/sites/production/files/2015-12/documents/9045d.pdf

U. S. Environmental Protection Agency, Office of Pesticide Programs. (2010, March). *Triclosan facts*. Retrieved from https://archive.epa.gov/pesticides/reregistration/web/html/triclosan_fs.html

U. S. Environmental Protection Agency, Office of Pesticide Programs. (2016, November). *Triclosan*. Retrieved from https://ofmpub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:3:::NO:1,3,31,7,12,25:P3_XCHEMICAL_ID:4124

U. S. Environmental Protection Agency, Office of Solid Waste and Emergency Response. (1999, September). *Biosolids generation, use, and disposal in the United States*. (EPA-530-R-99-009). Retrieved February 8, 2015, from http://www.epa.gov/wastes/conserve/composting/pubs/biosolid.pdf

U. S. Environmental Protection Agency, Office of Water. (1989, August). *POTW sludge sampling and analysis guidance document.* (EPA-833-B-89-100). Retrieved from http://www.epa.gov/npdes/pubs/owm012.pdf

U. S. Environmental Protection Agency, Office of Wastewater Management. (1999, September). *A plain English guide to the EPA Part 503 Biosolids Rule*. (EPA-832-R-93-003). Retrieved from http://water.epa.gov/scitech/wastetech/biosolids/503pe_index.cfm

U. S. Environmental Protection Agency, Office of Wastewater Management. (1993, September). *Constructed wetlands for wastewater treatment and wildlife habitat*. (EPA831-R-93-005). Retrieved from http://water.epa.gov/type/wetlands/upload/constructed-wetlands.pdf

U. S. Food and Drug Administration, Consumer Health Information. (2013). *FDA taking a closer look at 'antibacterial' soap*. Retrieved from http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm378393.htm

University of Minnesota. (2016). *University of Minnesota biocatalysis and biodegradation database.* Retrieved November 18, 2016 from https://www.msi.umn.edu/content/university-minnesota-biocatalysis-and-biodegradation-database

van Groenigen, J. W., Lubbers, I. M., Vos, H. M., Brown, G. G., De Deyn, G. B., & van Groenigen, K. J. (2014). Earthworms increase plant production: A meta-analysis. *Scientific Reports*, *4*, 6365.

Veldhoen, N., Skirrow, R. C., Osachoff, H., Wigmore, H., Clapson, D. J., Gunderson, M. P., ... & Helbing, C. C. (2006). The bactericidal agent triclosan modulates thyroid

hormone-associated gene expression and disrupts postembryonic anuran development. *Aquatic Toxicology*, *80*(3), 217-227.

Vieno, N., Tuhkanen, T., & Kronberg, L. (2007). Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Research*, *41*(5), 1001-1012.

Washington State University Whatcom Extension. (2016). Composting with redworms. Retrieved from http://whatcom.wsu.edu/ag/compost/Redwormsedit.htm

Wawersik, J. (1996). [History of chloroform anesthesia]. *Anaesthesiologie und Reanimation*, *22*(6), 144-152.

Wei, Y., & Liu, J. (2005). The discharged excess sludge treated by Oligochaeta. *Water Science and Technology*, *52*(10-11), 265-272.

White, J. C., Kelsey, J. W., Hatzinger, P. B., & Alexander, M. (1997). Factors affecting sequestration and bioavailability of phenanthrene in soils. *Environmental Toxicology & Chemistry*, *16*(10), 2040-2045.

Wild, S. R., Harrad, S. J., & Jones, K. C. (1994). The influence of sewage sludge applications to agricultural land on human exposure to polychlorinated dibenzo-p-dioxins (PCDDs) and-furans (PCDFs). *Environmental Pollution*,*83*(3), 357-369.

Wolff, M. S., Teitelbaum, S. L., Pinney, S. M., Windham, G., Liao, L., Biro, F., ... & Calafat, A. M. (2010). Investigation of relationships between urinary biomarkers of phytoestrogens, phthalates, and phenols and pubertal stages in girls. *Environmental health perspectives*, 1039-1046.

World Health Organization. (2016). *Antimicrobial Resistance* [Fact sheet]. Retrieved from http://www.who.int/mediacentre/factsheets/fs194/en/

Xia, K., Bhandari, A., Das, K., & Pillar, G. (2005). Occurrence and fate of pharmaceuticals and personal care products (PPCPs) in biosolids. *Journal of Environmental Quality*, *34*(1), 91-104.

Xia, K., Hundal, L. S., Kumar, K., Armbrust, K., Cox, A. E., & Granato, T. C. (2010). Triclocarban, triclosan, polybrominated diphenyl ethers, and 4‐nonylphenol in biosolids and in soil receiving 33‐year biosolids application. *Environmental Toxicology and Chemistry*, *29*(3), 597-605.

Ying, G. G. & Kookana, R. S. (2007). Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environmental International, 33*, 199-205.

Appendix A

United States Environmental Protection Agency's list of 88 synonyms for triclosan:

1. TCL
2. 72779
3. T1872
4. 524190
5. C12059
6. D06226
7. DP-300
8. IN1424
9. S00100
10. Trisan
11. CH 3565
12. CH-3565
13. CID5564
14. D014260
15. DB08604
16. Irgasan
17. AC-10667
18. AC1L1KMN
19. Aquasept
20. I01-2897
21. LS-67854
22. Manusept
23. Sapoderm
24. 222-182-2
25. 3380-34-5
26. CHEMBL849
27. CPD0-1227
28. HSDB 7194
29. Lexol 300
30. TL8002539
31. 88032-08-0
32. C12H7Cl3O2
33. CCRIS 9253
34. Cliniclean
35. Cloxifenol
36. HMS2093L17
37. 112099-35-1
38. 164325-69-3
39. 261921-78-2
40. BRN 2057142
41. Triclosanum
42. CHEBI:164200
43. CPD000471847
44. Cloxifenolum
45. MLS001066347
46. MLS001074876
47. MLS001335937
48. MLS001335938
49. SAM002554907
50. SMR000471847
51. Irgasan DP300
52. Microshield T
53. Oxy Skin Wash
54. Irgasan DP 300
55. NCGC00159417-02
56. NCGC00159417-03
57. NCGC00159417-04
58. UNII-4NM5039Y5X
59. Stri-Dex Face Wash
60. Triclosan; Irgasan
61. MolPort-003-666-702
62. Triclosan (USP/INN)
63. SSL Brand of Triclosan
64. Stri-Dex Cleansing Bar
65. Triclosan Reckitt Brand
66. SterZac Bath Concentrate
67. Clearasil Daily Face Wash
68. Ster Zac Bath Concentrate
69. Ster-Zac Bath Concentrate
70. Dermtek Brand of Triclosan
71. Reckitt Brand of Triclosan
72. Triclosan Pharmachem Brand
73. Stri-Dex cleansing bar (TN)
74. Pharmachem Brand of Triclosan
75. Trans Canaderm Brand of Triclosan
76. GlaxoSmithKline Brand of Triclosan
77. Procter & Gamble Brand of Triclosan
78. Johnson & Johnson Brand of Triclosan
79. 5-CHLORO-2-(2,4-DICHLOROPHENOXY)PHENOL
80. 5-Chloro-2-(2,4-dichloro-phenoxy)-phenol
81. 2,4,4'-Trichloro-2'-hydroxydiphenyl ether
82. 2-Hydroxy-2',4,4'-trichlorodiphenyl Ether
83. Phenol, 5-chloro-2-(2,4-dichlorophenoxy)-
84. 2,4,4'-Trichloro-2'-hydroxy diphenyl ether
85. Ether, 2'-hydroxy-2,4,4'-trichlorodiphenyl
86. Phenyl ether, 2'-hydroxy-2,4,4'-trichloro-
87. Irgasan DP-300
88. 5-Chloro-2-(2,4-dichlorophenoxy)phenol

Appendix B

Standard Operating Procedure

Pharmaceutical and Personal Care Products by EPA Method 8270D

City of Tacoma
Environmental Services Laboratory

Stuart Magoon Assistant Division Manager	Date
Greg Perez Senior Environmental Lab Analyst – Organics Lead	Date
Lori Zboralski Senior Environmental Lab Analyst – QA Officer, LIMS Administrator	Date

Disclaimer:

Please note that the City of Tacoma's Environmental Services Laboratory Standard Operating Procedures (SOPs) are adapted from published methods. They are intended for internal use only and are specific to the equipment, personnel, and samples analyzed at the Environmental Services Laboratory. This SOP is not intended for use by other laboratories nor does it supplant official published methods. Distribution of this SOP does not constitute an endorsement of a particular procedure or method.

Any reference to specific equipment, manufacturer, or supplies is for descriptive purposes only and does not constitute an endorsement of a particular product or service by the author or by the City of Tacoma.

Although the lab follows the SOP in most instances, there may be instances in which the lab uses an alternative methodology or procedure with quality assurance and management approval.

The method is for "research only". It has not been vetted through our normal validation process.

Currently, the SOP document review process is not complete for this first version and thus unsigned.

SOP Revision History

Revision Date	Rev Number	Summary of Changes	Sections	Reviser(s)
1/8/2016	1.0	New SOP	all	Mark Bozlee

1.0 Scope and Application

1.1 This document is the Standard Operating Procedure (SOP) for the analysis of Pharmaceutical and Personal Care Products by method SW846 8270D. Refer to the Project and Sample Analysis Request Form in Element for project specific compounds and reporting limits. The following compounds, including typical MRLs, can be determined by this method:

S8270_BNA		
Analyte	**MRL**	**Units**
Triclosan	20	ng/g
Methyl Triclosan	10	ng/g

1.2 The analysis portion of this method is to be used by, or under the direct supervision of, analysts experienced in the use of Agilent gas chromatography/mass spectrometry (GC/MS/MS) systems, MassHunter software and in the interpretation of mass spectral data.

2.0 Summary of Procedure

2.1 The samples consisting of biosolids and paper mulch are milled using a Cryomill without liquid nitrogen (SOP 1022 Cryomill Sample Processing) followed by a vortex and sonication extraction.

2.2 The semivolatile compounds are introduced into the GC/MS/MS by injecting the sample extract into a GC equipped with a narrow-bore fused-silica capillary column. The GC column is temperature-programmed to separate the analytes, which are then detected with a tandem MS connected to the gas chromatograph. Analytes eluted from the capillary column are introduced into the mass spectrometer via a direct connection.

2.3 A characteristic (precursor) m/z is further broken down into a characteristic daughter (product) m/z for each compound and quantitated. An additional daughter ion (qualifier ion) is also measured for even further identification. An individual compound is identified by comparing the GC retention time, a precursor ion, a product qualifier ion, and ratio of qualifier to quantifier ion to an authentic standard. See Table 14.1 The concentration is determined by using the response of the product quantitative ion and a multipoint calibration of the target analytes with isotope dilution technique. Isotope dilution provides automatic correction of the target analyte concentrations.

3.0 Interferences

3.1 Solvents, reagents, glassware, and other sample processing hardware may yield artifacts and/or interferences to sample analysis. All of these materials must be demonstrated to be free from interferences under the conditions of the analysis by analyzing method blanks. Also refer to SW-846 Method 8000 for a discussion of interferences.

3.2 Raw GC/MS/MS data from all blanks, samples, and spikes must be evaluated for interferences. Determine if the source of interference is in the preparation of the samples and take corrective action to eliminate the problem. Contamination by carryover can occur whenever high-

concentration and low-concentration samples are sequentially analyzed. To reduce carryover, the sample syringe must be rinsed with solvent between sample injections. Whenever an unusually concentrated sample is encountered, it should be followed by the analysis of solvent to check for cross contamination.

4.0 Safety

4.3 Refer to Chemical Hygiene and Laboratory Health and Safety Plan for standard lab safety practices. See Section 13.0.

4.4 The toxicity or carcinogenicity of each reagent used in this method have not been precisely defined; however, treat each chemical compound as a potential health hazard. Reduce exposure to these chemicals to the lowest possible level by whatever means available. Prepare primary standards of these toxic compounds in a hood.

5.0 Equipment and Supplies

5.1 Vials – 4, 8 and 12 mL, amber glass, with polytetrafluoroethylene (PTFE)-lined screw cap.

5.2 Gas tight syringes, various volumes

5.3 Hamilton Digital Dilutor - \\fspwes01\general\qa\sop\5019 Maintenance and Operation of Hamilton Digital Diluter_v4.pdf

5.4 Metal Spatula

5.5 Vortex mixer

5.6 Sonic bath

5.7 Single use aluminum weighing pan

5.8 100 ml volumetric flask, ground glass joint with stopper

5.9 Balance accurate to 0.0001g (Mettler MS 3045 S/N B021037549). See SOP ..\Current\1015_Analytical Balance Calibration and Maintenance_v2.pdf

5.10 Syringe filters (0.45 micron) and self-filtering autosampler vials, (0.2 micron)

5.11 GC/MS/MS System

5.11.1 Agilent 7890 GC complete with all required accessories including syringes, columns, and gases. The GC includes a front multi-mode inlet (MMI) capable of large volume injection and a rear split/splitless inlet.

5.11.2 Inlet Liners - The following liners are recommended. Any liner yielding suitable chromatography may be substituted providing the same type is used for the initial calibration and sample analysis.

5.11.2.1 Single-taper 2.3-mm i.d. focus liner with inert glass wool.

5.11.3 Analytical Column – 20m x 0.18mm x 0.18µm DB-5

5.11.4 Agilent 7000 GC/MS/MS - mass selective detector capable of scanning from 35 to 1020 amu every 1 msec or less, and producing a mass spectrum which meets all the criteria of

perfluorotributylamine (PFTBA) injected through the GC inlet. See Section 8.1.

5.11.5 Agilent MassHunter data acquisition and analysis software

5.11.6 A Laboratory Information Management System (LIMS) capable of computing and storing data acquired using Mass Hunter or Chemstation software. This laboratory uses Promium® Element DataSystem® (referred to in this SOP as LIMS or Element™).

6.0 Reagents and Standards

6.1 Standard Solutions: all standards are entered into LIMS. See an example of a calibration standard entered into LIMS in Section 14.2 and a example of a standard in Section 14.3.

6.1.1 Purchase commercially prepared certified stock solutions stock solutions. Store, protected from light, at 4^{o}C or as recommended by the standard manufacturer. Check stock standard solutions frequently for signs of degradation or evaporation, especially just prior to preparing calibration standards from them. Replace stock standard solutions by the manufacturer's expiration date or sooner if comparison with quality control check samples indicates a problem.

6.1.2 Pharmaceutical Mix #2 - Available from Restek. Contains Triclosan in methanol at 200 µg/mL.

6.1.3 Methyl Triclosan – Neat analytical standard. Available from Sigma-Aldrich

6.1.3.1 Add 20 mg of Methyl Triclosan to 100 mls of acetone in a 100 ml volumetric. Mix well. Transfer solution to five 12 ml amber vials. Store at -10°C. Prepare fresh every 6 months.

6.2 Internal standard solutions

6.2.1 (13C12) Triclosan in nonane 100 ug/mL - Purchased from Cambridge Laboratories Inc. This is the working solution. The Element standard type must be 'Internal Std'. Spike each sample or calibration extraction with 5 µL of the internal standard solution, resulting in a concentration of 100 ng/ml.

6.2.2 (13C12) Methyl Triclosan in nonane 100 ug/mL - Purchased from Cambridge Laboratories Inc. This is the working solution. The Element standard type must be 'Internal Std'. Spike each sample or calibration extraction with 2 µL of the internal standard solution, resulting in a concentration of 40 ng/ml.

6.3 Surrogates - Surrogated are not used in this method. The need for surrogates is eliminated by the use of isotopic dilution. Isotopic internal standard recovery correction eliminates the need for surrogates. Surrogates may be added according to a QAPP or according to professional judgement.

6.4 Laboratory Control Sample (LCS) - Use the same source as the initial calibration standards to restrict the influence of standard accuracy on the determination of recovery through preparation and analysis.

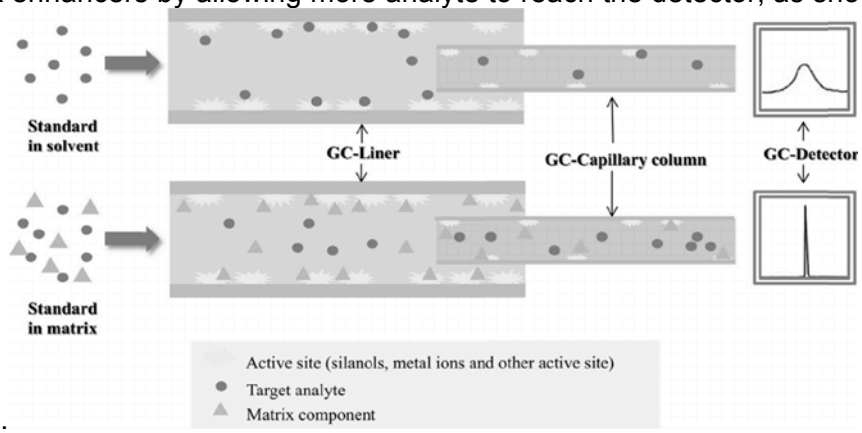
6.4.1 S8270_PPCP BS - Add 2.0 ul of the stock 200 ppm triclosan stock (Pharmaceutical Mix #1) and 2.5 ul of the 200 ppm methyl triclosan stock into a 8 ml amber vial with 0.15 g paper mulch. Add 4988.5 ul of methanol and follow the extraction procedure from 10.3.5 to 10.3.12.

6.5 Matrix Spike (MS) - Use the same source as the initial calibration standards to restrict the influence of standard accuracy on the determination of recovery through preparation and analysis

6.5.1 S8270_PPCP MS - Add 2.0 ul of the stock 200 ppm triclosan stock (Pharmaceutical Mix #1) and 2.5 ul of the 200 ppm methyl triclosan stock into a 8 ml amber vial with 0.25 g of sample. Add 4988.5 ul of methanol and follow the extraction procedure from 10.3.5 to 10.3.12.

6.6 Solvents – acetone, methylene chloride, and other appropriate solvents. All solvents are pesticide quality or equivalent

6.7 Paper mulch – Premium Paper, 100% hand sorted recycled newsprint without added dye, from Applegate Mulch. Milled (Section 10.2). Paper mulch in calibration standards and samples increase the sensitivity of methyl triclosan and triclosan. Components in the paper mulch act as matrix enhancers by allowing more analyte to reach the detector, as shown below.



6.8 Intermediate Standards (IMD Std) – Vortex to mix after all stock standard additions. Store all intermediate solutions at -10°C. Prepare fresh every 6 months or sooner if degradation is detected.

6.8.1 S8270_PPCP: Triclosan/Methyl Triclosan Mix - 2000 ppb – Add 20 ul of each 200 ppb stock to 1960 mL of methylene chloride in a 4 mL amber vial for a 2000 µg/mL solution.

6.8.2 S8270_PPCP: Triclosan/Methyl Triclosan Mix - 100 ppb – Add 50 ul of 2000 ppb Triclosan/Methyl Triclosan mix to 950 mL of methylene chloride in a 2 mL amber vial for a 2000 µg/mL solution.

6.9 Working Standards - For each calibration standard, add 0.15 g of paper mulch and the specified amount of standard to a 8 mL screw cap amber vial containing the methylene chloride ($MeCl_2$). Add the appropriate amount of IS (6.2) to each vial and vortex. Points may be added or subtracted to meet project requirements. Store at -10 °C. Prepare fresh every 3 months or

sooner if degradation is detected. Enter the Element LIMS standard type as 'Calibration'.

6.9.1 S8270_PPCP Calibration Working Standards - A typical calibration set is listed in the following table.

Std	Std Conc	µL of Triclosan Std	µL of $MeCl_2$	Conc ng/mL
CAL1	200 mg/L Stocks	25 each	4943	1000
CAL2	200 mg/L Stocks	12.5 each	4968	500
CAL3	200 mg/L Stocks	5 each	4983	200
CAL4	200 mg/L Stocks	2.5 each	4988	100
CAL5	2000 Int mix ng/ml	125	4868	50
CAL6	2000 Int mix ng/ml	62.5	4931	25
CAL7	2000 Int mix ng/ml	25	4968	10
CAL8	2000 Int mix ng/ml	12.5	4981	5
CAL9	2000 Int mix ng/ml	5	4988	2
CAL10	2000 Int mix ng/ml	2.5	4991	1
CAL11	2000 Int mix ng/ml	1.25	4992	0.5
CAL12	100 Int mix ng/mL	12.5	4981	0.25

6.9.2 S8270_PPCP continuing calibration standard – Add 0.15 g of paper mulch to a 8 ml amber vial. Add 2.5 ul of the 200 ppm stock solutions (pharmaceutical mix #2 and methyl tricolsan), 5 ul of (13C12) triclosan and 2 ul of (13C12) methyl triclosan. Add 4988 mls of methylene chloride. The target analytes are at a concentration of 100 ng/ml.

7.0 Sample Collection, Preservation and Handling

7.1 Collect samples in 8 oz Whirl-Pak container

7.2 Wrap with aluminum foil

7.3 Samples have a 14 day hold time but may be frozen at -18 °C (Freezer Room 224) per Puget Sound Protocols in order to extend the holding time from collection to extraction for up to 1 year.

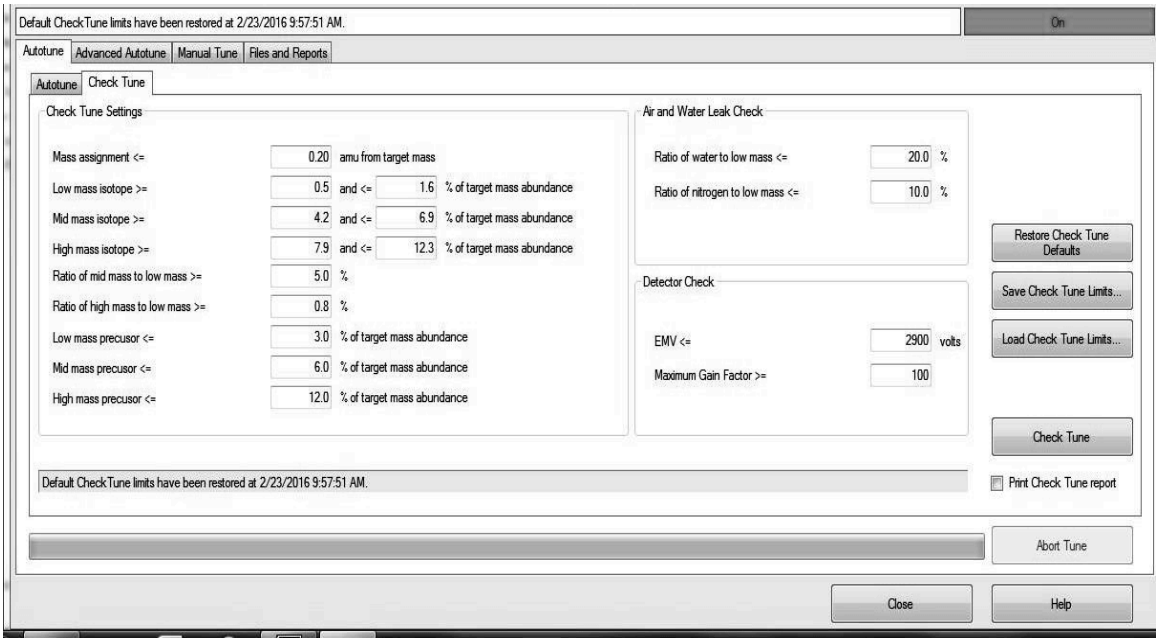
7.4 Store extracts and milled samples when not being used for analyses at -18°C. Extracts are stored in 2 ml amber vials and milled samples are stored in 40 ml amber VOA vials protected from light in screw cap vials equipped with unpierced PTFE-lined septa. Complete analysis within 40 days of extraction.

7.5 Qualify the results of any samples which exceed these limits as estimated values.

8.0 Quality Control and Method Performance

8.3 Tuning - Check tune by clicking the MS TUNE icon in the Instrument Control panel to display the Tune dialog box. Then click on the Autotune's

Check Tune tab. Click on “Check Tune”. Acceptable parameter limits are as follows: See Section 14.4 for check tune.



8.3.1 Reanalyze any samples that are injected more than 12 hours after a Autotune or Check Tune and mark the original analysis as not reportable.

8.3.2 If Check Tune does not pass, check on Autotune tab. See Section 14.5. Make sure “EI high sensitivity autotune”, “Save tune file when done” and “Default filename” are checked. Click on Autotune

8.4 Initial Demonstration of Performance (IDP) - Perform once by each analyst prior to reporting sample results. Repeat the IDP when a major change is made to the extraction, analysis method or equipment. IDP consists of the analysis of four replicates of the laboratory control sample. The IDP is acceptable if the average recovery of the four results is within the LCS limits. IDP data is stored on \\fspwes01\Transfer\7000SV1\LLOQ and IDP as a .pdf file named analyst initials_analysis name_IDP_date

8.5 Lower Limit of Quantitations (LLOQ) are determined the first time the method is performed on the instrument and repeated annually, or if there is a major change in the procedure or equipment. The LLOQ check is carried through the same preparation and analytical procedures as environmental samples and other QC samples.The verification is performed by the extraction and analysis of an LCS (or matrix spike) at 0.5 – 2 times the current LLOQ levels. Analyze in the same manner as samples. LLOQ data is stored at \\fspwes01\Transfer\7000SV1\LLOQ and IDP

8.6 Method Blank - Prepare a method blank (Batch#-BLK#) of one per day or one per 20 samples whichever is more frequent. Analyze the blank to demonstrate that the system and extraction are free from contamination. Use 0.15 g of milled paper mulch and extract as a sample (Section 10.2 and 10.3). If contaminated, evaluate if the GC system is the contamination source by analyzing an instrument blank of methylene chloride. Clean the inlet and the split vent line if GC is the source to remove higher molecular

weight target compounds that build up in the inlet as the system sits idle. Reanalyze blanks with concentrations greater than or equal to ½ the Lowest Level of Quantitation (LLOQ) after eliminating the GC as the contamination source.

8.6.1 If the blank contains a concentration greater than or equal to the LLOQ and the sample concentration is less than the LLOQ, report the LLOQ value with a "U".

8.6.2 If the sample concentration is greater than the LLOQ and within 10x the blank concentration qualify the sample concentration with a “UJ”. Complete a QC Variance form and consult project manager to determine if re-extraction is required to meet minimum project reporting limits.

8.6.3 If the sample concentration is greater than 10x the blank concentration, report the sample concentration without qualification.

8.6.4 If gross contamination exists in the blank (i.e. saturated peaks), positive sample results may require rejection and be qualified as unusable "R". Unusable data may require re-extraction. Complete a QC Variance form and consult project manager to determine if re-extraction is required.

8.7 Laboratory Control Sample - Prepare a blank spike (Batch#-BS#) the more frequent of one per 14 days or one per 20 samples as per 6.4.

8.7.1 The recovery limits are specified by the project QAPP. If not specified by the QAPP they are set by control chart of LCS recoveries or default to 70-130% if data is insufficient for control chart. Qualify the data as “J” for detects and “UJ” for non-detects if the LCS recovery is less than the lower recovery limit. Data should be qualified “R” if recoveries are below 20%. Complete a QC Variance form. If data has been “R” qualified, initiate a Corrective Action.

8.7.2 Reanalyze LCS if recovery is outside the criteria after evaluating whether GC system maintenance could improve recovery and taking any actions indicated. Consult with senior analyst if recovery is still outside the criteria to determine whether re-extraction is possible within sample holding times. Report data associated with the best recovery. Delete results in Element for non-reported LCS leaving an internal standard as an indication of the additional analysis.

8.7.3 Qualify the data with a “J” for detects and complete a QC Variance form if the LCS recovery exceeds the upper recovery limit. Do not qualify non-detects.

8.8 Matrix Spike/Matrix Spike Duplicate - Prepare one matrix spike (MS) and matrix spike duplicate (MSD) the more frequent of one set per 14 days or per 20 samples as per 6.5. Report results from the same dilution as reported for the native sample unless the MS/MSD result is over the calibration range. The recovery limits are specified by the project QAPP. If not specified by the QAPP they are set by control chart of LCS recoveries or default to 70-130% if data is insufficient for control chart.

8.8.1 Do not qualify data if the sample concentration exceeds the spike concentration by a factor of four or more.

8.8.2 No qualification of the data is necessary on MS and MSD data alone. In those instances where it can be determined that the results of the MS and MSD affect only the sample spiked, limit qualification to this sample only. However, it may be determined through the MS and MSD results that a laboratory is having a systemic problem in the analysis of one or more analytes, that affect all associated samples.

8.8.3 Reanalyze MS or MSD if recovery is outside the criteria after evaluating whether GC system maintenance could improve recovery and taking any actions indicated. Consult with senior analyst if recovery is still outside the criteria to determine whether re-extraction is possible within sample holding times. Report data associated with the best recovery. Delete results in Element for non-reported MS/MSD leaving an internal standard as an indication of the additional analysis.

8.8.4 Qualify the data with a “J” for detects and a “UJ” for non-detects if the LCS recovery is less than the lower recovery limit. Data should be qualified “R” if recoveries are below 20%. Complete a QC Variance form. If data has been “R” qualified, initiate a Corrective Action.

8.9 Internal Standards - Add 5 µL of (13C12) Triclosan and 2 ul of (C13C12) Methyl Triclosan Internal Standard Solutions prior to extraction. Compare labelled internal standard responses to the latest continuing calibration standard. The limits are retention time within 15 seconds and daughter SIM ion area within a factor of two (-50% to +100%). The retention time of each analyte and its corresponding isotope should be within +/- 6 seconds of each other for each analysis.

8.9.1 Recovery limits for isotopic dilution methods may be overly conservative (Section 11.4.4, EPA 8000D) since built in recovery correction is one of the principle advantages of isotopic calibration. Consult project manager and use professional judgement if recoveries are outside of limits.

8.9.2 In general, qualify non-detects calculated with a internal standard response <50 as estimated (J) and <20% as rejected (R). Do not qualify non-detects calculated with a high internal standard response. Consult project manager and use professional judgement.

9.0 Calibration and Standardization

9.1 Perform Initial calibration for each new instrument, and repeat when any major changes or maintenance (ion source cleaning or repair, column removal or replacement, etc.) are performed or when continuing calibration fails. Enter into LIMS the calibration curve. An example is shown in Section 14.6.

9.1.1 Prepare calibration standards at a minimum of seven concentration levels for each parameter of interest. See Section 6.9.

9.1.2 Allow standards to come to room temperature prior to analysis. Inject 1 ul of filtered extract using splitless injector. Analyze each calibration standard. Data analysis software calculates relative response factors (RR) for each compound using the equation:

Calibrate the native compounds with a labeled analog using the following equation:

$$RR = \frac{(An)\ (C1)}{(A1)\ (Cn)}$$

An = The area of the daughter m/z for the native compound
Al = The area of the daughter m/z for the labeled compound.
C1 = The concentration of the labeled compound in the calibration standard(ng/mL).
Cn = The concentration of the native compound in the calibration standard (ng/mL).

9.1.3 Use the RR for calculations. Evaluate the individual RRs compared to the calibration curve to determine if there is a consistent high or low bias indicating a problem with a particular point. Remake or reanalyze the calibration standard if a problem standard or injection is indicated. Evaluate chromatography to determine if system maintenance could improve peak shape or response enough to warrant maintenance and repeating the calibration. Evaluate linear or quadratic RR curve plots of response ratios for best fit. Linear curves must have correlation coefficients greater than 0.990 and quadratic curves must be greater than 0.995

9.1.3.1 Qualify the associated detected results as estimated (“J”) if calculated using an linear or quadratic coefficient that is out of limits. Complete a QC Variance Form.

9.1.3.2 Perform percent recovery check on each calibration point by re-fitting the response from each calibration point back into the curve. If the recalculated concentration is not within ± 20% of the standard’s true concentration or other recovery criteria outlined in a project-specific QAPP. If recoveries are failing, try different curve fits or redefine the range of quantitation. Often quadratic curve that is inversely weighted (1/x) helps accuracy at the lower concentrations.

9.1.4 Retention Time - Recheck the integration and identification of a target analyte if the retention time does not agree within +/- 15 seconds of that target analyte in the other calibration standards.

9.2 Calibration Verification

9.2.1 Calibration Check Standard (CCV) - Inject the mid-range standard at the beginning of each 12-hour period after the tuning and column performance test. Limit is the calculated result within plus/minus 20% of known value. Should this standard fail to meet those parameters, repeat the test using a fresh calibration standard.

9.2.1.1 If the CCV fails the criteria for 20% or more of the calibration analytes, or if a calculated result is not within plus/minus 20% of known value, or if an estimated value is not acceptable to the project manager prepare a new calibration curve.

9.2.1.2 IS Retention Time – Evaluate the retention times of the IS in the calibration verification standard immediately after or during data acquisition. If the retention time for any internal standard changes by more than 15 sec from that in the mid-point standard level of the most recent initial calibration sequence, then inspect the chromatographic system for malfunctions and make corrections, as required. When corrections are made, reanalyze samples analyzed while the system was malfunctioning.

9.2.1.3 IS response - If the area for any of the IS in the calibration verification standard changes by a factor of two (-50% to +100%) from that in the mid-point standard level of the most recent initial calibration sequence, inspect the mass spectrometer for malfunctions and make corrections, as appropriate. When corrections are made, reanalyze samples analyzed while the system was malfunctioning.

10.0 Procedure

10.1 Preparation

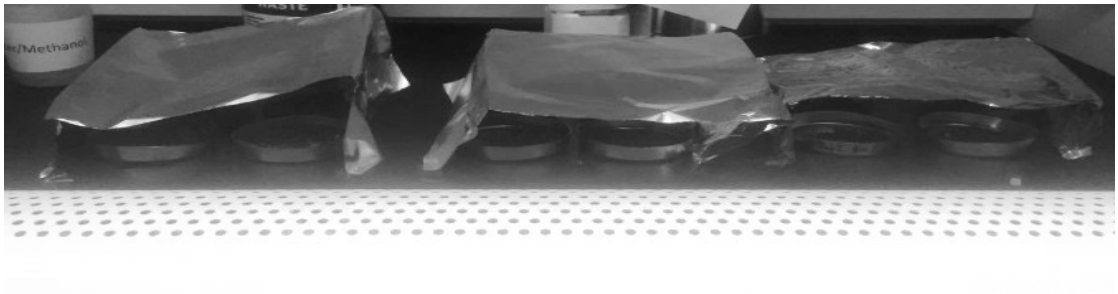
10.1.1 Obtain sample(s) from sample freezer in room 224

10.1.2 Add sample to a single use aluminum weighing pan (may need to thaw the sample a bit first)

10.1.3 Make a thin layer that covers the bottom of the pan. Two pans per sample

10.1.4 Put excess sample back into the freezer

10.1.5 Place pans under hood using aluminum foil tents over the samples to block light but allow air flow over sample. Leave hood lights off



10.1.6 Dry sample in hood at room temperature for 4 days. The biosolids/paper mix tends to clump. Separate clumps into smaller pieces after the first day and continue drying the full 4 days.

10.2 Milling

10.2.1 Using the Retsch Cryomill at room temperature (without liquid nitrogen), add air dried sample (or paper mulch for calibration standards) to a 50 ml steel grinding jar

10.2.2 Fill container roughly 2/3 full

10.2.3 Add three 10 mm steel balls

10.2.4 Follow the SOP L:\QA\SOP\Current\1022_Cryomill Sample Processing_v1.pdf, except liquid nitrogen cooling is not used

10.2.5 Several cycles of adding dried sample to steel grinding jar may be needed for each sample to have sufficient quantities

10.2.6 Combine milled samples into a 44 ml amber VOA vial using a clean spatula

10.2.7 Store milled sample in sample freezer at -20°C (Rm 224).

10.2.8 Scrupulously clean grinding jar and steel balls. Wipe off excess solids, wash with DI water and wipe clean with paper towel. Rinse apparatus and steel balls 3 times with methylene chloride and dry before next sample

10.3 Extraction

10.3.1 Add 0.25 g of milled sample or 0.15 g of milled paper mulch for calibration standards to an 8 ml amber screw cap vial

10.3.2 Record the sample weight to 0.0001 grams on the printed LIMS Bench Sheet and enter into LIMS.

10.3.3 Add appropriate amounts for calibration standards and matrix spikes directly to sample according to 6.9.1

10.3.4 Add 5 ul of (C13C12) triclosan and 2.0 ul of (C13C12) methyl triclosan to each calibration standard and sample

10.3.5 Add appropriate amounts of methylene chloride according to 6.9.1

10.3.6 Vortex 2 min

10.3.7 Sonicate 5 minutes

10.3.8 Vortex 1 min

10.3.9 Prefilter into an 4 ml amber vial using 0.45 syringe filter

10.3.10 Take 0.5 ml aliquot into self-filtering vials

10.3.11 Save extract in semi-voa freezer (Rm 231)

10.3.12 Filter using 0.2 micron self-filtering vials

10.4 Batch - In Element® LIMS select “Laboratory”and then select “Batch”. Select the Vortex micro extraction from the drop down method under “Preparation Method”. Select Semi Volatile organics from drop down menu under “Batch Department”. Select Soil from drop down menu under “Batch Matrix”. Select available methods Click on Department under “List Analyses by” and select S8270_PPCP from available analysis. Save the Batch. See example in Section 14.7.

10.5 Bench Sheet - After saving the Batch in Element® LIMS, a bench sheet is created. Save the bench sheet. See example in Section 14.8. Print the sequence using print format “seq_sxname.rpt”.

10.6 Sequence – In Element® LIMS select “Laboratory”and then select “Sequence”. Set up the initial calibration or continuing calibration sequence. Save the sequence. See example in Section 14.9. Print the sequence using print format “seq_sxname.rpt”.

10.7 Turn on the LC\MS\MS PC.

10.8 Method Setup - Load the appropriate GC acquisition method (8270_PPCP_Triclosan-MTS). Adjust the gas chromatographic operating parameters to obtain suitable chromatography.

10.8.1 Adjust the ion groupings and dwell time such that it produces at least 15 to 20 scans per chromatographic peak for quantitative analysis and 10 to 15 scans for qualitative analysis

10.8.2 Set up the Sequence in MassHunter, See Section 14.10 as an example.

10.8.3 Sequence, Run Sequence to start from the first line of Sequence, Position and Run Sequence to start sequence from a different line.

10.9 Tune Check – Perform a tune check each day to evaluate the instrument status against the manufacturer’s requirements. Autotunes and tune checks are automatically stored in the directory (Data) D:\MassHunter\GCMS/1/7000/TuneReports.

10.9.1 Check Tune – Load the 8270_PPCP acquisition method.

10.9.2 If the check tune fails, perform instrument maintenance and/or a full autotune. See Section 8.1.2 for the autotune procedure.

10.9.3 Print the autotune or checktune report. See Section 14.11 for evaluation of tune report.

10.10 Condition system with five blank injections containing milled paper mulch (Section 8.4) before calibration or continuing calibration sequence. This ensures that matrix components have sufficiently masked the active sites in the system (Section 2.4).

10.11 Calibration - Calibrate the system as described in Section 9.1. Save the analysis method as S8270_PPCP_YYYYMMDD.m

10.12 Continuing Calibration - Perform a CCV check as described in Section 9.2. Allow extracts to come to room temperature prior to analysis

10.13 Method Blank - Analyze a method blank prior to sample analyses in order to ensure that the total system (introduction device, transfer lines and GC/MS/MS system) is free of contaminants. If the method blank indicates contamination, analyze a solvent blank to demonstrate that the contamination is not a result of carryover from standards or samples.

10.14 Inject the same volume (1 ul) of the sample extract or QC extract into the GC/MS/MS system as was used for the calibration standards.

10.15 If the response for any compound exceeds the highest calibration standard, dilute the extract and reanalyze or re extract using less sample. If using diluted extract, add additional internal standard solution to the diluted extract to maintain the same concentration as in the calibration standards. Create a reshot (RE) in Element from the original sequence to import the dilution result. Label dilutions with "#X" in the Misc. Info field where # is the dilution factor and enter this # in the Sample Multiplier field. For example a 1 to 10 dilution of Outfall 230 would have Misc. Info: Outfall 230 10X and Sample Multiplier:10.

10.16 Back-up Instrument Data - Copy the analysis subdirectory to \Transfer\Instrument Backups\Instrument Name\DataYYYY.

11.0 Data analysis and Calculation

11.1 Open Agilent MassHunter quantitative analysis ('QQQ quantitative analysis' icon). Select the menu item "file" and then "new batch", navigate to the data subdirectory, double click on the data subdirectory and type the batch file name in the ".batch.bin" file box as 'S8270_PPCP_ICAL_YYYYMMDD' for an initial calibration.

11.2 Select the menu item "file" and then "add samples", select the appropriate files from the analysis subdirectory and click on "ok".

11.3 Click on the box next to the "continuing calibration" file or the initial calibration file at the CCAL level, select the menu item "method" and then "open" and "open method from existing batch". Navigate to the subdirectory of the last previous batch, click on the previous "batch.bin" file and click on "open".

11.3.1 Click on "globals setup" and compare to the example in Section 14.12 for the appropriate entries to insure no multipliers are applied to the on-column results.

11.3.2 Select the menu item "update" and then "update retention times". Select all and then "ok"

11.3.3 Select the menu item "update" and then "update qualifier ratios". Select all and then "ok"

11.3.4 Click on "exit" under "save/exit" and "yes" to apply this method to the batch.

11.4 Select the menu item "analyze" and then select "analyze batch" if the batch includes the initial calibration files. Select "quantitate batch" if the initial calibration files are not included in the batch. Analyzing a batch without the initial calibration files will overwrite the calibration, repeat Section 11.3 to restore the initial calibration files. See Section 14.13 for example batch in MassHunter quantitative analysis.

11.5 Qualitative Analysis – Evaluate the product quantitation and qualifier ions listed in Section 14.1 for individual compound information. Use the following criteria to make a qualitative identification:

11.5.1 The quantitation and qualifier ions of each parameter of interest must maximize in the same or within one scan of each other.

11.5.2 The retention time must fall within +/- 15 seconds of the authentic compound. Evaluate retention time shifts against the surrogate and internal standard for consistency.

11.5.3 The relative peak heights of the qualifier ions must fall within ±20% of the relative intensities of these ions in the authentic compound with relative intensities greater than 10%. Qualifier ion relative intensities that are less than 10% should fall within ±30% of the relative intensities of these ions in the authentic compound. Use professional judgment in interpretation where interferences are observed.

11.6 Quantitative Analysis – Quantitate identified parameters based on the integrated abundance from the product quantitation ion

11.7 Compute the concentration of each compound in the extract using the RR from the calibration data and following equation:

$$Cex\ (ng/ml) = \frac{(An)\ (C1)}{(A1)\ (RR)}$$

Cex = Concentration of the target analyte in the extract:other terms are defined in Section 9.1.2

11.8 Avoid manual integrations unless necessary when the software does not produce proper integrations

11.8.1 Investigate and evaluate any flags generated by the analysis software for outliers to the calibration or qualifier criteria.

11.8.2 Select the menu item “view” and then “compounds-at-a-glance” to view the standard and sample chromatograms side by side, if necessary, to evaluate retention time shifts. See Section 14.14.

11.8.3 Select the menu item “analyze” and then select “analyze batch” if any changes are made to the initial calibration files or the calibration type. Select “quantitate batch” if any changes are made to the continuing calibration or sample files.

11.8.4 Select the menu item “file” and then select “save batch”.

11.8.5 Generate the Element® LIMS import file. Select the menu item “report” and then “generate” and navigate to the template file “C:\MassHunter\Report Templates\Quant\en-US\Letter\ LIMS\lims export files full.xltx”. Leave “report folder” on the default option which will save the report to the batch subdirectory. Click on “ok”. The Element® LIMS file will automatically be saved in that batch’s subdirectory under “\QuantReports\batch name\lims export files full.xltx”.

11.8.6 Generate a PDF file for each calibration or sample analysis sequence. Select the menu item “report” and then “generate”, navigate to the template file “C:\MassHunter\Report Templates\Quant\en-US\Letter\ ISTD\Parts_Graphics\QuantReport_ISTD_Complete_B_05_01.xltx”. Leave “report folder” on the default option which will save to the batch subdirectory. Click in the box next to “output PDF to screen”.

Click on “ok”. The PDF will automatically be saved in that Batch’s subdirectory under \QuantReports*batch file name-1*\QuantReport_ISTD_Complete_B_05_01 .pdf.

11.9 Result Calculation

11.9.1 Sample concentration calculates in Element® LIMS using the following equation:

$$\text{Conc, (ug/kg or ng/g)} = (Cu \times V \times D)/(W \times S)$$

Where

Cu = Concentration on column (ng/ml) = IResult

V = Sample Volume (mL) = Final (mL) = 5 mls

D = Dilution factor = Diln

W = Weight of sample (g)

S = Percent solids/100 = 100% solids is default for air dried samples

11.10 Back-up Instrument Data – Copy the analysis subdirectory to \\Transfer\Instrument Backups\7000SV1\Data*YYYY.* Use a data stick for the transfer if quantitative analysis was performed on the QQQ computer.

11.11 Import into Element® LIMS

11.11.1 See Section 14.15 for a list of corresponding MassHunter and DataTool fields

11.11.2 Use \Element\DataTool\CrossTables\SemiVolatiles as the cross table. Set Units = 1 in Data Tool, units off the instrument are in µg/L.

11.11.3 The DataTool file type for import is Agilent Mass Hunter LIMS (*.xlsx). Check the box for “multiplier field” for the “take dilution factor from” window in DataTool.

11.11.4 Import the file \\Transfer\Instrument Backups\7000Sv1\ Data*YYYY**YYYYMMDD*_COT\QuantReports*batch name*\lims export files full.xlsx.

11.11.5 Merge files. Edit and replace the acquisition method name in the analysis column with “S8270_PPCP”, Instrument 1” in the “instrument” column with “7000 SV1”, ZZZ” in the “analyst” column with analyst initials if they did not import, and Element® LIMS export files full-### in the “File_Name” column with the Data File number.d as it appears in the pdf. Edit and replace the “sample name” in the “Lab_Number” column if it does not match the name as it appears in the Element® LIMS sequence.

11.12 Copy \\Transfer\Instrument Backups\7000 SV1\Data*YYYY**YYYYMMDD*_COT QuantReports*batch file name-1*\QuantReport_ISTD_Complete_B_05_01.pdf to the \\Element\Data_PDF\Sequence\ subdirectory and rename as “TYMDD##”, where TYMDD## is the Element® LIMS sequence number.

11.13 Review Results – In Element® LIMS, review the imported "IResult" column in "data review" table against the PDF.

11.13.1 Check that all dilution values are entered in the "diln" column.

11.13.2 Review any handwritten changes made to amounts or standards on the "preparation log" sheet to ensure they have been updated in Element® LIMS.

11.13.3 Investigate any red-flagged rows, correct if possible or generate an Analysis QC Variance form to document and explain the variance.

11.13.4 For initial calibration: Review the Element® LIMS calibration columns of cal type and LR COD or QR COD against the initial calibration PDF. Uncheck standard points in Element® LIMS that were not included in the calibration curve on the PDF to get the Element® LIMS columns to match for each compoud

12.0 Pollution Prevention and Waste Management

12.1 Seal the vials for disposal containing sample extracts or expired standards metal can waste container for this satellite area until disposed of by lab pack in accordance with the laboratory's waste disposal manual. Keep in use waste disposal cans in the hood in room 231. This is the designated satellite collection area for this waste stream. When waste container is full, notify the Hazardous Waste Manager for removal to Hazardous Waste Storage area.

12.2 Collect waste solvents in an appropriate waste container and dispose of in accordance with the ES Laboratory Hazardous Waste Disposal Manual.

13.0 References

Test Methods for Evaluating Solid Waste, Physical/Chemical Methods SW-846 Method 8270D

Determinative Chromatographic Separation, EPA Method 8000D

City of Tacoma Environmental Services Chemical Hygiene Health and Safety Plan 2.0", 2014

City of Tacoma Environmental Services Draft Hazardous Waste Disposal Manual_v5, 2016

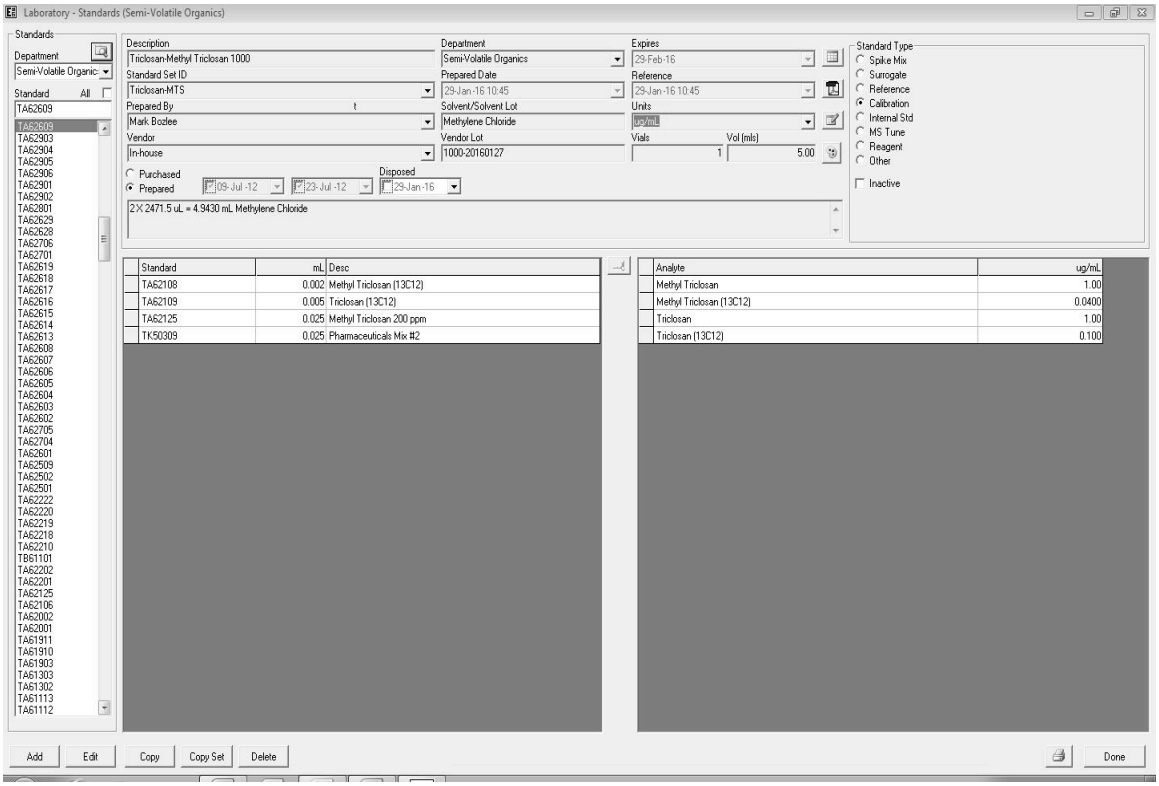
City of Tacoma Environmental Services 2015_Laboratory Quality Manual_v4, 2015

14.0 Tables, Diagrams, Flowcharts and Validation Data

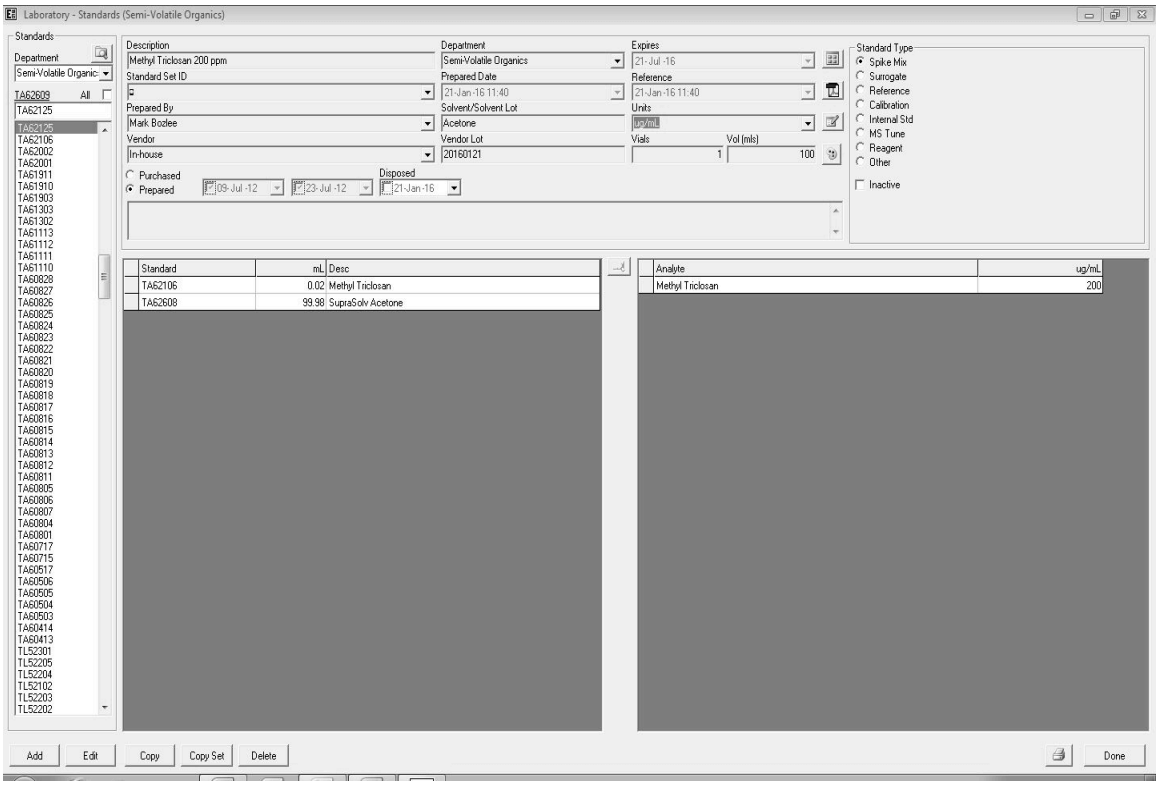
14.1 Characteristic Ions for Analytes and Internal Standard Isotope

Compound	CAS#	Precursor Ion	Daughter Ions(s)
Triclosan	3380-34-5	287.6	217.8 Quant
		217.8	155.1 Qualifier
Methyl Triclosan	4640-01-1	301.7	251.7 Quant
		251.7	188.8 Qualifier
(13C12) Triclosan		302	230 Quant
		302	119.1 Qualifier
(13C12) Methyl		313.9	263.9 Quant
		313.9	244.1 Qualifier

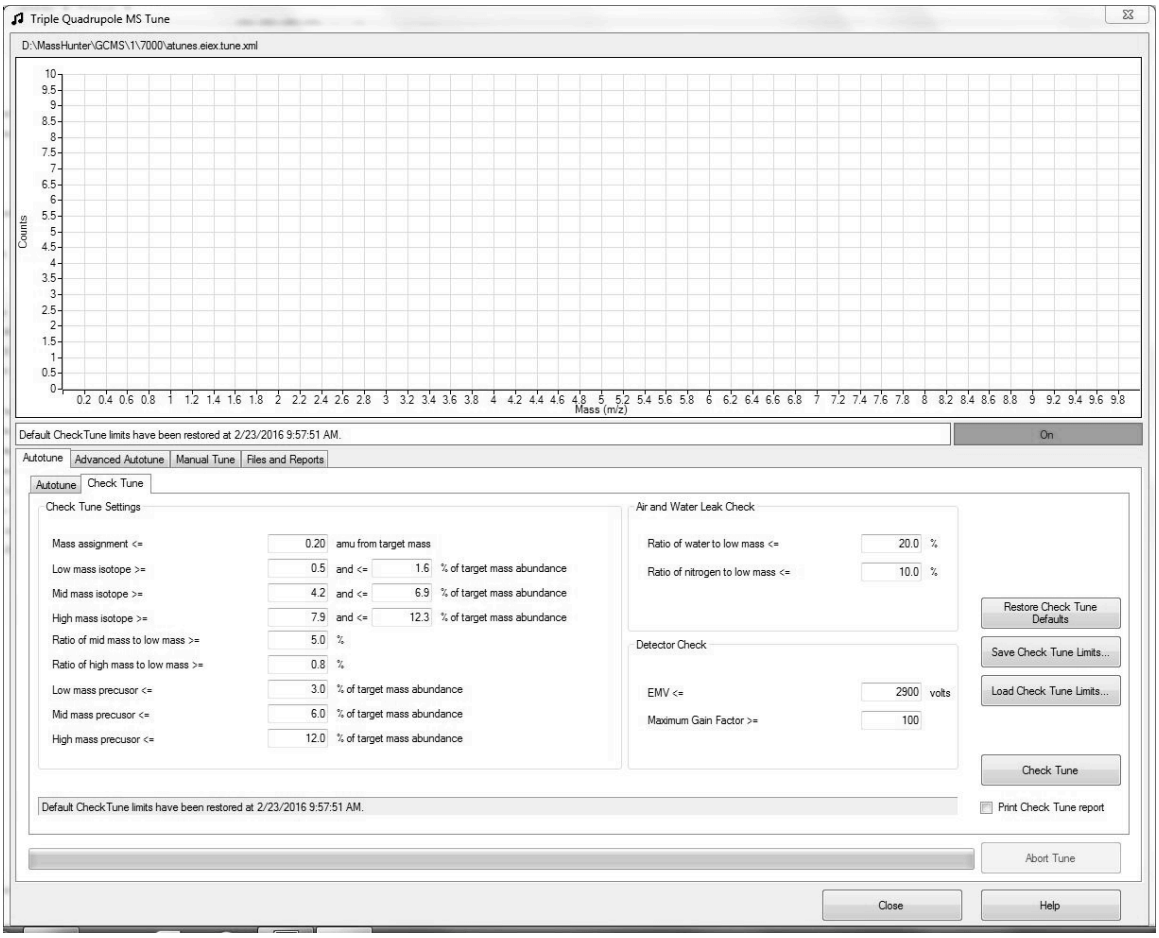
14.2 LIMS Calibration Standard



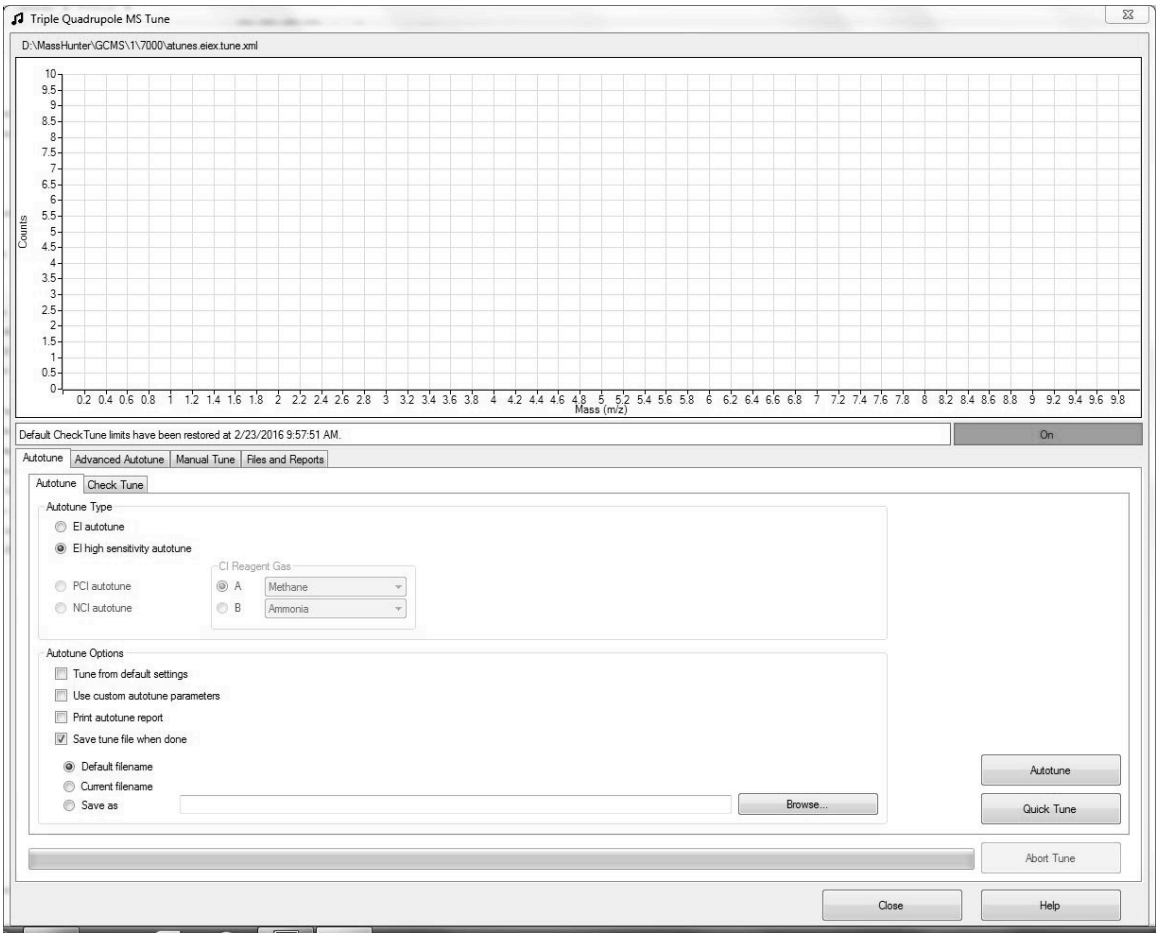
14.3 LIMS Standard



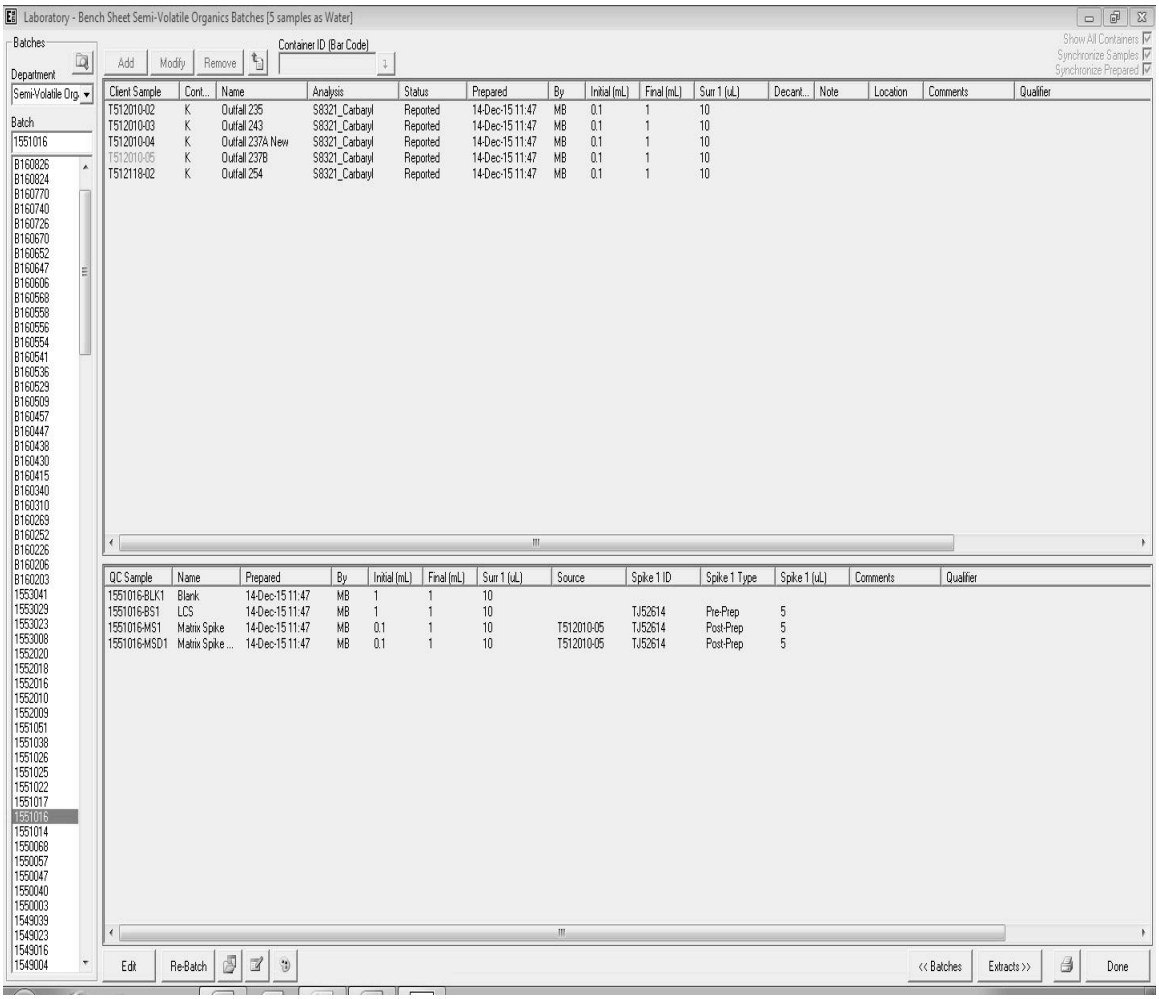
14.4 Check Tune Screen Tab



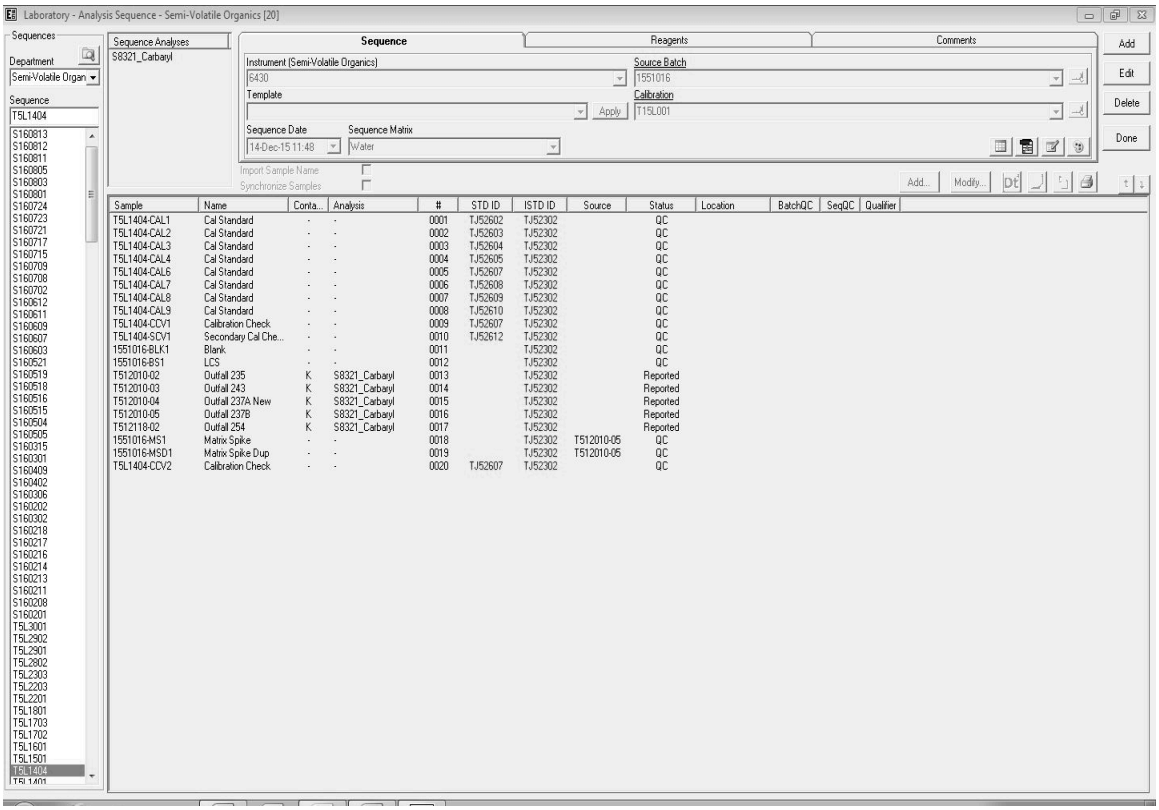
14.5 Autotune Screen



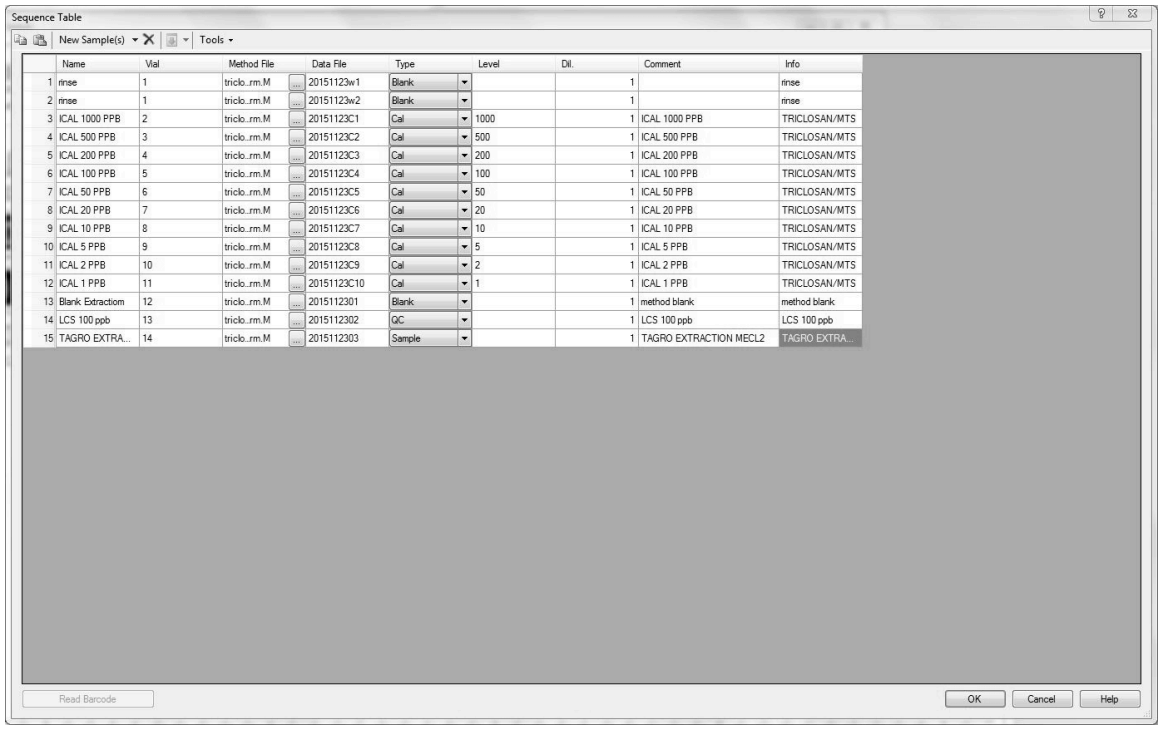
14.6 LIMS Bench Sheet



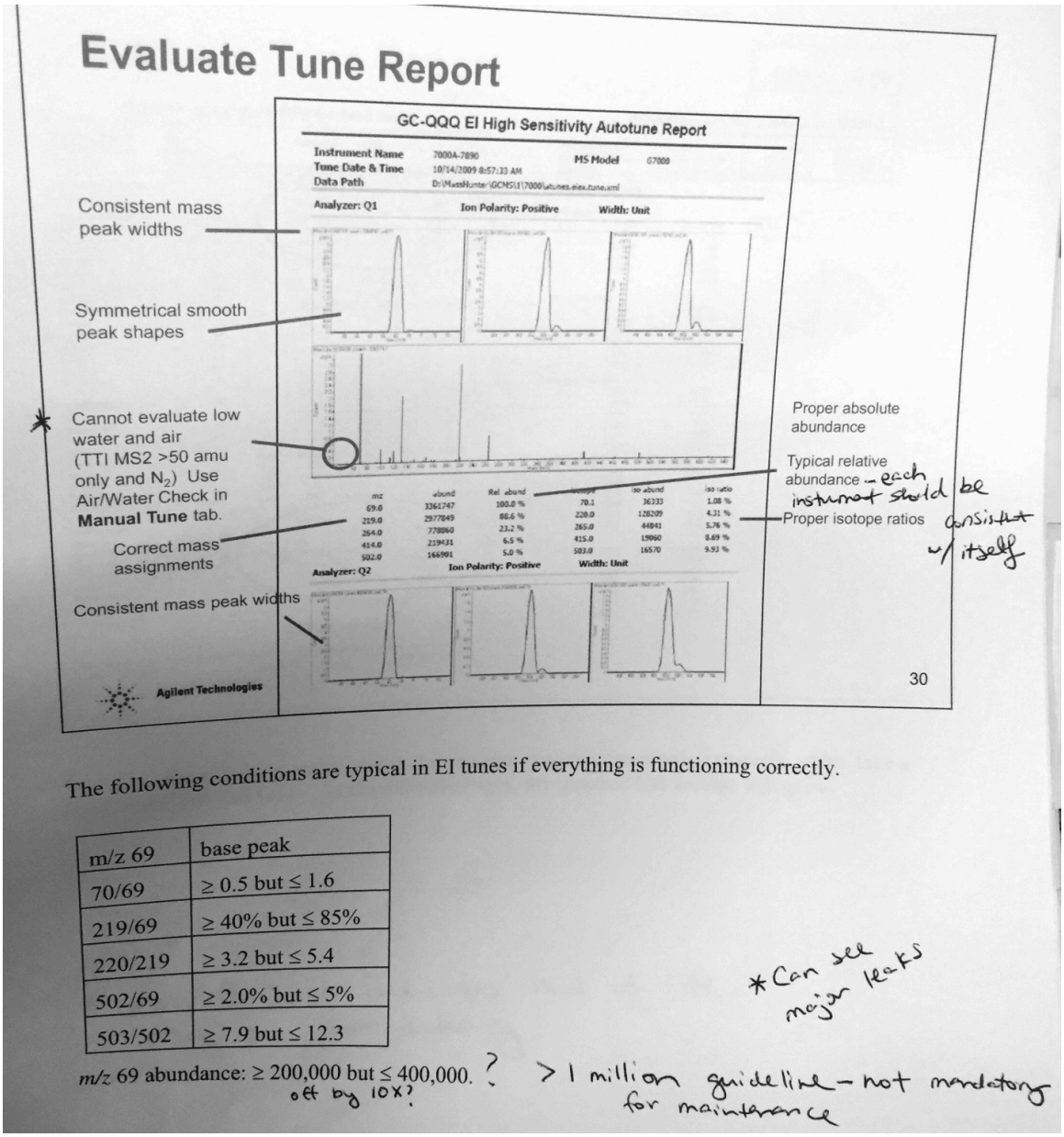
14.7 LIMS Sequence



14.8 MassHunter Sequence



14.9 Evaluate Tune Report



The following conditions are typical in EI tunes if everything is functioning correctly.

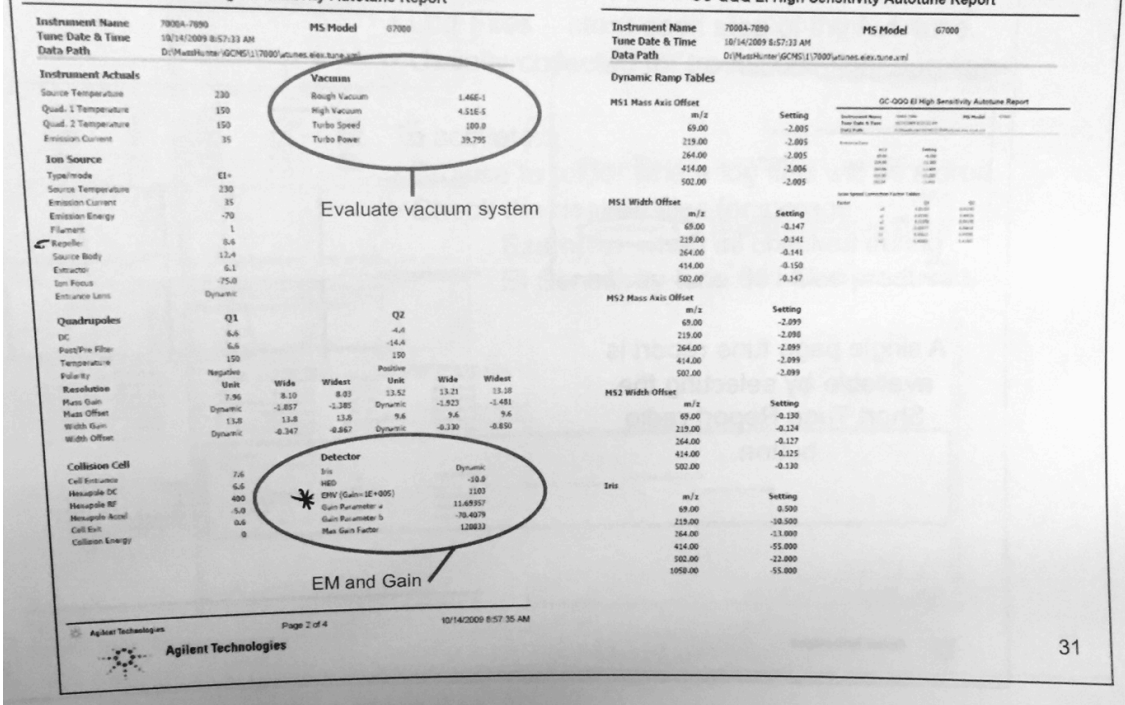
m/z 69	base peak
70/69	≥ 0.5 but ≤ 1.6
219/69	≥ 40% but ≤ 85%
220/219	≥ 3.2 but ≤ 5.4
502/69	≥ 2.0% but ≤ 5%
503/502	≥ 7.9 but ≤ 12.3

*Can see major leaks

m/z 69 abundance: ≥ 200,000 but ≤ 400,000. ? >1 million guideline – not mandatory for maintenance

off by 10X?

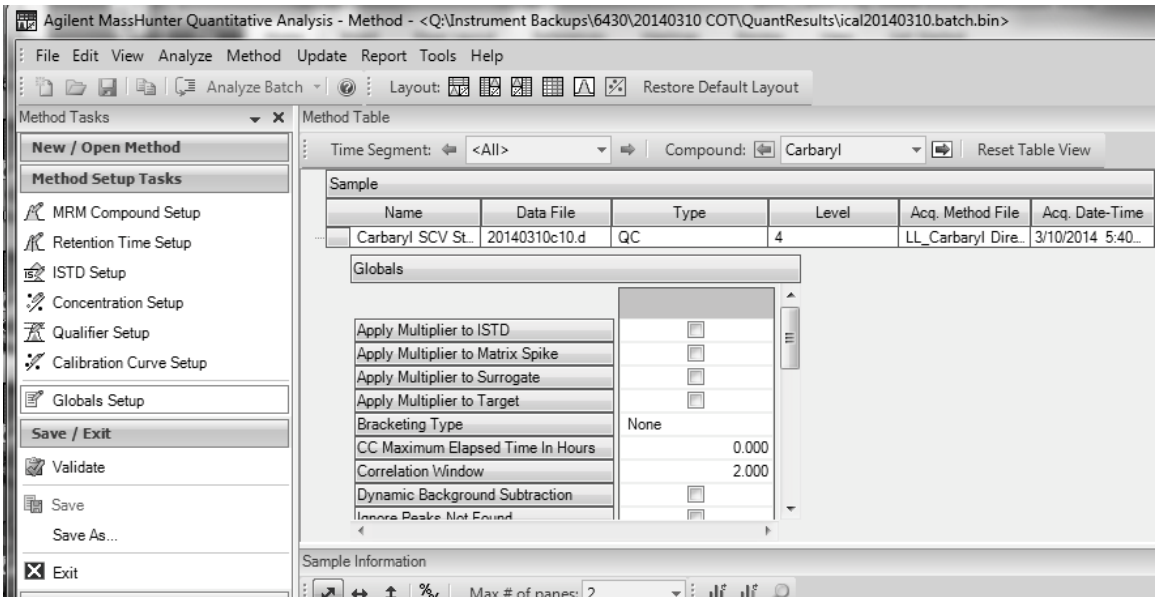
Evaluate Tune Report



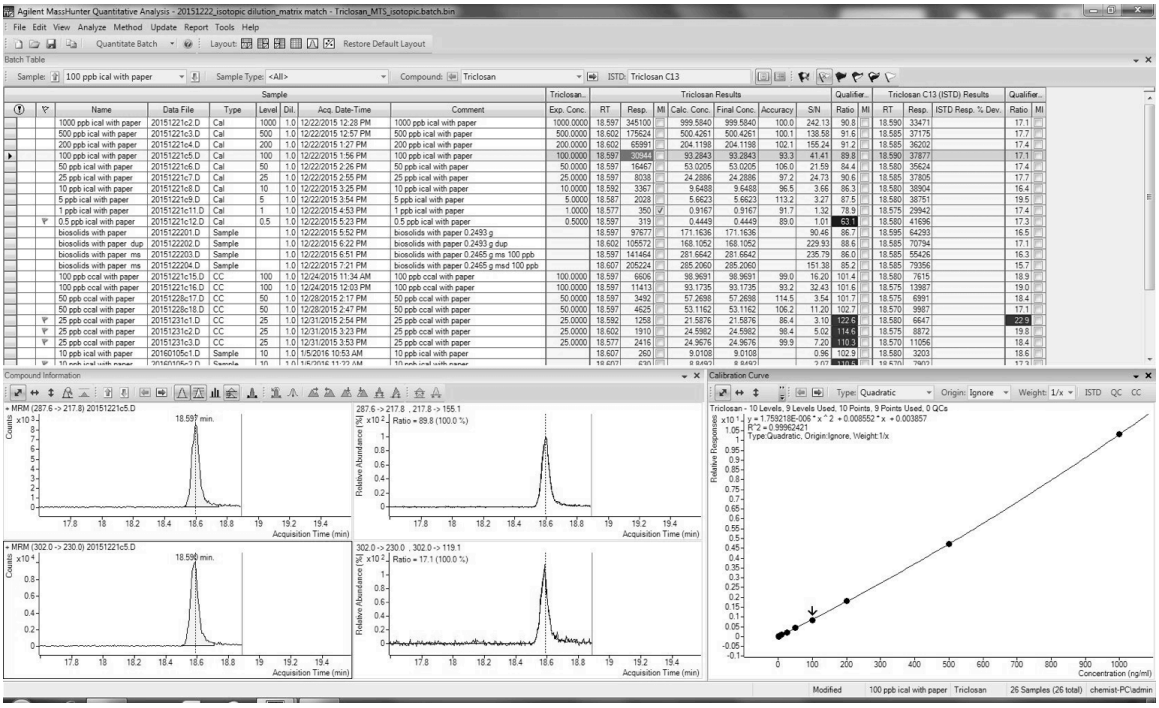
Use this opportunity to make certain the vacuum system is functioning properly. Also, take a look at the EM and Gain. As the multiplier ages, the required EM voltage will grow.

* EMV @ 2600 indicates end of life.
May see poor linearity.

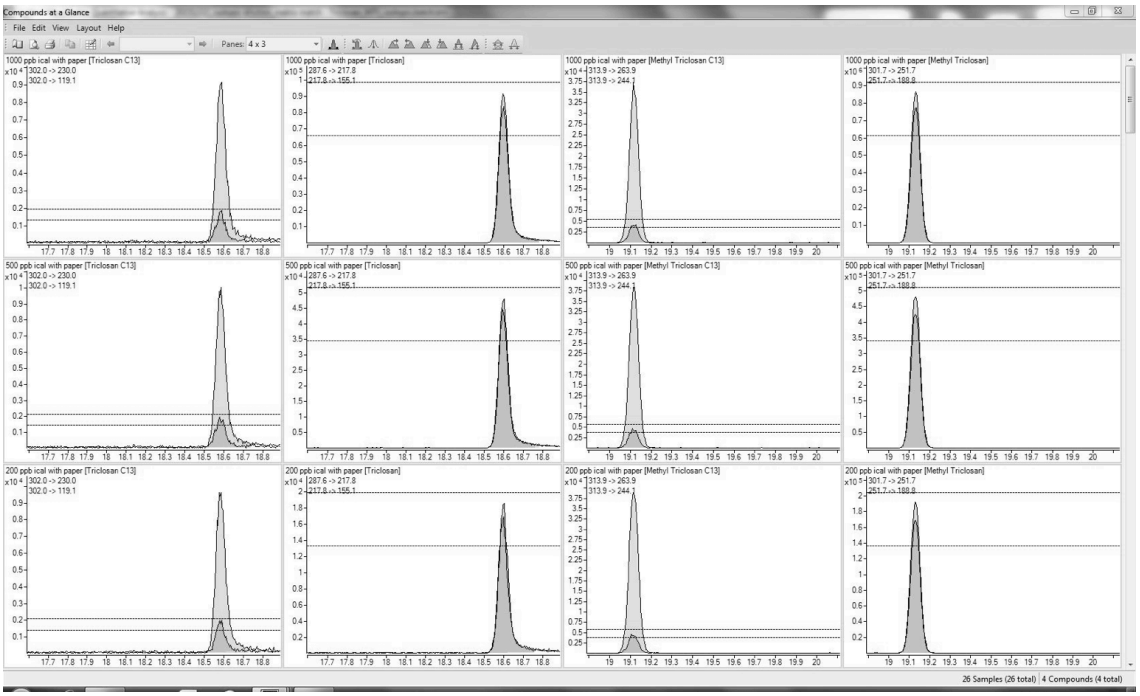
14.10 Globals Setup



14.11 MassHunter Quantitative Analysis



14.12 Compounds at a Glance



14.13 Corresponding Fields between MassHunter and DataTool

MassHunter Field	**DataTool Instrument Data**	**DataTool Merged Upload**
Data File[1]	File_Name	FileID
Sample Name[1]	Lab_Number	LabNumber
Aqu. Method File Name	Analysis	Analysis
Compound Name[4]	Analyte	Analyte
Comment[1,2]	Misc	(no upload match)
Diln.[1,2]	Dilution	Dilution
Final Conc.[7]	Result	InitialResult
Concentration Units[5]	Units	InitialUnits
AcqTime[7]	Analyzed	Analyzed
RT[7]	RTime	RT
Response[7]	Response	RESP
Instrument[6]	Instrument	Instrument
Acq. Operator[3]	Chemist	Analyst

[1]Entered in Acquisition Worklist table
[2]May be edited in QQQ Quantitative Analysis
[3]Entered under Acquisition Worklist Run Parameters
[4]Entered in QQQ Quantitative Analysis, MRM Compound Setup
[5]Entered in QQQ Quantitative Analysis, Concentration Setup
[6]Entered under Instrument Name during Agilent Configuration, the 6430 is Instrument 1
[7]Generated field based on data acquisition for the individual file