AN EXAMINATION OF THE TOXICOLOGICAL EFFECTS OF THE LEACHATE FROM BIORENTENTION SYSTEM SUBSTRATES ON ZEBRAFISH, *DANIO RERIO*

by

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ABSTRACT

An Examination of the Toxicological Effects of the Leachate from Bioretention System Substrates on Zebrafish, *Danio rerio*

Maria G. Redig

Stormwater pollution is a major concern in urbanized areas with an increased amount of impervious surfaces. These surfaces accumulate contaminants from vehicles and other sources. During rain events, these chemicals are washed into drains, ditches, ponds, wetlands and streams nearby. Bioretention structures are the most commonly used type of stormwater conveyance feature. They are composed of mixtures of sand, bark, and compost. Compost used in bioretention systems binds with pollutants and removes them from the stormwater. Recent studies have also shown that in certain situations composts have the potential to leach heavy metals, adding to the stormwater pollution issue. In this study, gravel and soil were tested to determine if they were adding to the contaminant load when used in bioretention systems. The toxic response of the leachate was measured using Zebrafish as a freshwater model organism. By testing leachate of clean water flushed through bioretention systems, it was found that extremely high levels of heavy metals were leached from the substrates; however, as more water was flushed through, these metal concentrations drastically decreased, indicating that conditioning the bioretention systems is an appropriate means to reducing heavy metal load on aquatic systems. When contaminated stormwater runoff was flushed through the bioretention soils and the gravel, the metal levels decreased. Zinc was reduced by 75%, copper by 44%, nickel by 11%, lead by 56% and cadmium by 33%. Metal concentrations alone are not an adequate indication of ecological health. Dissolved organic carbon, pH and other cations present in the water all affect the toxicity of metals. The leachate from the clean water passing through the bioretention structures was found to be not toxic to Zebrafish embryos even though the metal concentrations were elevated. Stormwater runoff displayed a toxic effect to the Zebrafish by causing reduced eye size and an enlarged periventral area, indicating blood pooling near the heart. Treating the stormwater through a bioretention system decreased this toxic response. This shows that even though the substrates used in this experiment leached elevated levels of heavy metals, it was not enough to cause toxicity. Ultimately, the soils were successful in the removal of metals and subsequently the toxicity of the stormwater, however this was after conditioning of the composts, which may prove to be a necessary component to the use of composts in bioretention systems.

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1. INTRODUCTION

Stormwater is responsible for a majority of water pollution in urban waterways, even more so than pollution from sewage treatment plants and industrial wastewaters (Joerger, 2008). Pollutants that land on roadways eventually get washed away by rain, becoming stormwater, and have the potential to end up in rivers, lakes, and marine environments. As of 2008, there were 43 marine species in the Puget Sound which were at risk, endangered or even threatened with extinction (Joerger, 2008). These species includes orcas, groundfish, abalone, salmon and marine birds. One of the leading reasons of this threat to viable populations of species is due to stormwater contamination (Joerger, 2008). The severity of the ecosystem effects of the large toxic mixture of pollution are still being studied, but it is safe to say that the effects are detrimental and stormwater needs to be studied further. Heavy metals and polycyclic aromatic hydrocarbons (PAHs) are of particular concern to the Puget Sound region due to the effects they have on the fish populations (Department of Ecology, 2011).

Stormwater conveyance features, such as bioretention structures, are meant to be a means of contaminant removal before the stormwater enters larger bodies of water, such as the ocean, however, recent data has shown that compost and soils used in bioretention structures, may leach metals (Cambier et al., 2014)(Kaschl, Römheld, & Chen, 2002)(Page, Harbottle, Cleall, & Hutchings, 2014). This has not been studied extensively, but if bioretention systems do leach metals, they would have the potential to add to the metal contamination in the stormwater. Though, this relationship is not clear, as soil and compost has also been shown to bind with and remove metals by adsorption

(Good, O’Sullivan, Wicke, & Cochrane, 2012)(Geronimo, Maniquiz-Redillas, Tobio, & Kim, 2014)(McIntyre et al., 2014).

Soils also bind with polycyclic aromatic hydrocarbons (PAHs) (McIntyre et al., 2014). This property is one reason which makes bioretention systems successful in treating stormwater. McIntyre et al. (2014) found that stormwater treated through bioretention structures resulted in a 95% reduction in total PAH concentration. The concern is that bioretention systems may not only be successfully removing PAHs and other organic contaminants from the stormwater, but they also may be adding heavy metals.

In order to better analyze the impacts of bioretention systems, the scholarly literature was reviewed, focusing on stormwater contaminants, toxicity to marine organisms and the use of bioretention systems. The literature showed a general lack of research in the performance of soils and the ability of bioretention systems to act as either stormwater pollution treatment or pollutant generators by leaching metals. This thesis research addressed the concern of metal leaching in bioretention systems by analyzing the leachate when clean water was flushed through them. Metal concentrations were measured initially, as well as over time, as more water was flushed through. This tested a potential to condition the soils and reduce leachate metal concentrations. The analysis was compared to the metal removal efficiency of the bioretention systems when contaminated stormwater runoff was flushed through.

The next part of this research assessed the toxicity of the leachate from the bioretention systems. Zebrafish were used as a model freshwater aquatic organism.

Toxicity endpoints such as hatch rate, growth and mortality were analyzed to test if the metals in the leachate had a toxic effect. To assess the toxicity potential for other contaminants leaching through the stormwater, such as PAHs, heart development was also measured, including pericardial area, periventral area and heartbeat. While metals can also cause heart defects, those toxic endpoints would have better signified a response to organic pollutants in the stormwater itself (Incardona, Collier, & Scholz, 2004).

By comparing the metal concentrations in the leachate to the toxicity to aquatic organisms, this thesis was designed to determine if bioretention systems are generating contamination and if that pollution is toxic enough to potentially cause an ecological impact. Furthermore, by assessing which toxic endpoints were observed (i.e. growth versus heart defects) if could be determined if the toxicity to the zebrafish was likely caused by metals leaching from the soils, or pollutants from the stormwater itself making it through the bioretention structures.

2. LITERATURE REVIEW

Stormwater pollution in a rising concern in urban waterways. It is incredibly difficult to regulate, being that it is a broad category, encompassing many different pollutants from a variety of sources. There are numerous studies on the toxic impact of the individual pollutants, but not many on the mixtures found in an actual urban environment. State and local governments are attempting to combat stormwater pollution with green stormwater infrastructure (GSI) techniques. Some of these techniques are new and lacking in research on the effectiveness of them.

2.1 STORMWATER CONCERN

There are two categories of stormwater pollution: point sources and non-point sources. The Clean Water Act defines point source pollution as "any discernible, confined and discrete conveyance, including but not limited to any pipe, ditch, channel, tunnel, conduit, well, discrete fissure, container, rolling stock, concentrated animal feeding operation, or vessel or other floating craft, from which pollutants are or may be discharged. This term does not include agricultural storm water discharges and return flows from irrigated agriculture" (EPA, 2015). Point source pollution is regulated within the individual states by National Pollutant Discharge Elimination System (NPDES) permits (EPA, 2015). Non-point source pollution is much more difficult to control and regulate. Unlike pollution from a point source, non-point source pollution can come from many sources. Non-point source pollution encompasses all the contaminants that end up on the ground, waterways or in the atmosphere that cannot be tracked to a single source

(EPA, 2015). It is, basically, all the stormwater pollution that is not point source pollution. With rainfall flowing over the ground, the contaminants get carried away and deposited into larger water bodies. These pollution sources can include fertilizers, pesticides, oils, fuels, sediments off of construction sites or agricultural areas, nutrients and bacteria or even pollution from atmospheric deposition (EPA, 2015). The initial discharge of these contaminants may be far below the levels to be concerned with, but they accumulate and create toxic mixtures that are difficult to control or treat. It is non-point source pollution that is the main threat to waterways and aquatic organisms (Department of Ecology, 2011).

Another issue with stormwater is the amount of water that urban areas have to control. With the rapid increase in development, more of the land is urbanized and covered with impervious surfaces. Major storm events can cause too much water for city stormwater drains to handle. A large amount of rain can overflow the stormwater conveyance features, allowing for large, fast flushes of the contaminants to enter larger water bodies in a short time period, rather than to recharge the groundwater. When overflowed, the stormwater goes untreated, dumping a mixture of toxic contamination into the waterways. This results in a very high concentration of contaminants in a short period of time. A stormwater conveyance feature is anything that moves stormwater in a system. Many are designed to allow for slow infiltration into the groundwater. Stormwater conveyance features that allow for infiltration are a means of treatment of the stormwater as well.

2.2 STORMWATER CONTAMINANTS

Stormwater contaminants include any contaminant that collects on the land and is flushed into a stormwater conveyance features. Commonly this includes metals from vehicles and rooftops, polycyclic aromatic hydrocarbons (PAHs) from fuel drips, refueling vehicles and fossil fuel combustion, and pesticides and other chemicals from farming applications (Norton, Serdar, Colton, Jack, & Lester, 2011). Heavy metals and PAHs are the largest concern in stormwater pollution due to the high concentrations and the fact that they are difficult to control (Norton et al., 2011).

2.2.1 HEAVY METALS

Metals can enter the aquatic environment in a variety of ways. Metals get washed into the stormwater drainage systems off of fields, roads, and parking lots. Heavy metals that are the largest threat to aquatic species include copper, zinc, cadmium, mercury, lead and arsenic (Norton, Serdar, Colton, Jack, & Lester, 2011). According to Dengler and Brasino (2007), runoff, from cars in particular, is a huge source of metals pollution. Copper, cobalt, cadmium, barium, aluminum, lead, nickel and zinc are all used in brake pads and tires on vehicles (Scholz et al., 2011). Furthermore, copper is used in building materials (roofs), and is common in agriculture and individual home pesticides (Norton et al., 2011). The amount of copper from pesticide applications and the release of copper from roofing materials are not as easy to quantify. The source of these metals vary in each particular area. In this thesis, Puget Sound will be a focus, due to the amount of research that has been conducted in this watershed. Table 1, below, shows the amount of metals and their main sources in the Puget Sound, Washington and demonstrates the issues with stormwater by showing that surface run-off delivered by stormwater is a major source of these toxic metals (Norton, Serdar, Colton, Jack, & Lester, 2011).

Table 1. Department of Ecology total metal release into the Puget Sound Basin catchment (metric tons/year) by the major sources and the total load that ends up in the Puget Sound water by the major pathways (Norton et al., 2011).

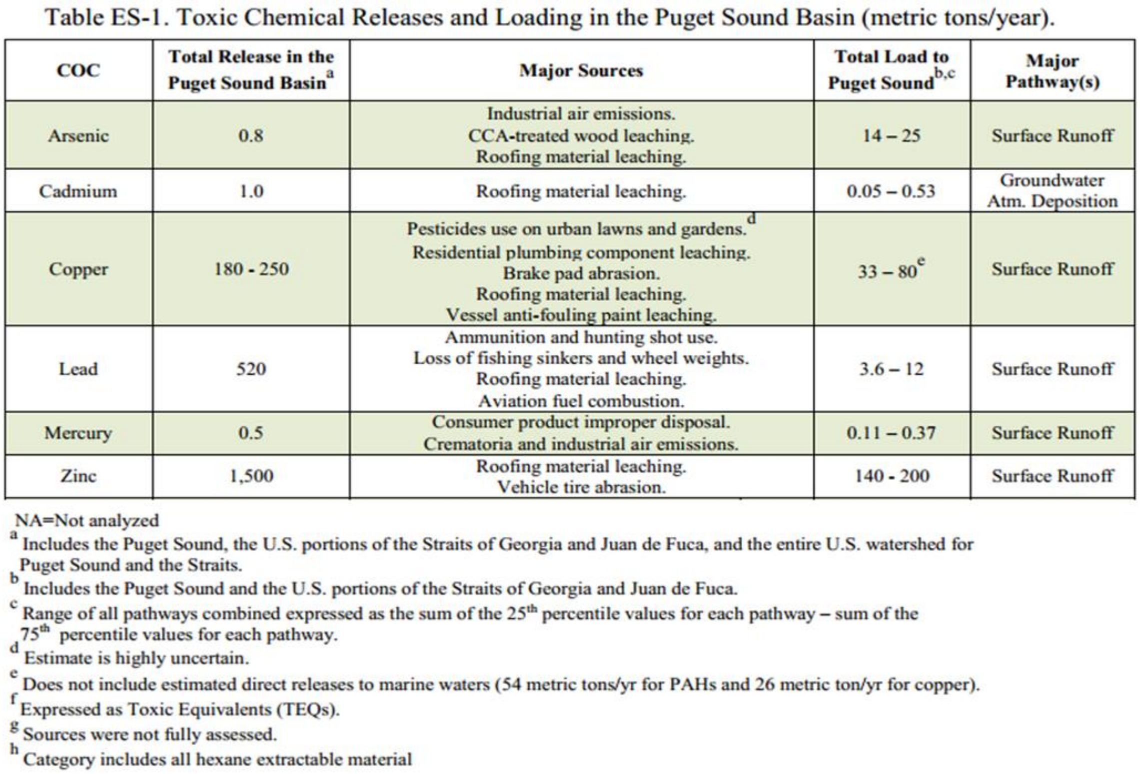


Table ES-1. Toxic Chemical Releases and Loading in the Puget Sound Basin (metric tons/year).

COC	Total Release in the Puget Sound Basin[a]	Major Sources	Total Load to Puget Sound[b,c]	Major Pathway(s)
Arsenic	0.8	Industrial air emissions. CCA-treated wood leaching. Roofing material leaching.	14 – 25	Surface Runoff
Cadmium	1.0	Roofing material leaching.	0.05 – 0.53	Groundwater Atm. Deposition
Copper	180 - 250	Pesticides use on urban lawns and gardens.[d] Residential plumbing component leaching. Brake pad abrasion. Roofing material leaching. Vessel anti-fouling paint leaching.	33 – 80[e]	Surface Runoff
Lead	520	Ammunition and hunting shot use. Loss of fishing sinkers and wheel weights. Roofing material leaching. Aviation fuel combustion.	3.6 – 12	Surface Runoff
Mercury	0.5	Consumer product improper disposal. Crematoria and industrial air emissions.	0.11 – 0.37	Surface Runoff
Zinc	1,500	Roofing material leaching. Vehicle tire abrasion.	140 - 200	Surface Runoff

NA=Not analyzed

[a] Includes the Puget Sound, the U.S. portions of the Straits of Georgia and Juan de Fuca, and the entire U.S. watershed for Puget Sound and the Straits.

[b] Includes the Puget Sound and the U.S. portions of the Straits of Georgia and Juan de Fuca.

[c] Range of all pathways combined expressed as the sum of the 25th percentile values for each pathway – sum of the 75th percentile values for each pathway.

[d] Estimate is highly uncertain.

[e] Does not include estimated direct releases to marine waters (54 metric tons/yr for PAHs and 26 metric ton/yr for copper).

[f] Expressed as Toxic Equivalents (TEQs).

[g] Sources were not fully assessed.

[h] Category includes all hexane extractable material

Much of the metals that are deposited into a catchment area end up in lakes and streams. Table 1 shows some differences in the total release to Puget Sound Basin and the total load to the Puget Sound. With the exception of arsenic, all the metals have a higher release than a load to the Sound. This indicates that some metals are left to accumulate in streams, lakes, or near shore areas. Stormwater pollution in freshwater systems is a major issue. Copper and zinc are particularly high in this region. One common main sources of copper and zinc is off of vehicles and ends up in the Puget Sound from surface runoff (Norton et al., 2011).

2.2.2 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are another concern with stormwater pollution. They are simply a combination of two or more fused aromatic rings and have hundreds of different forms. PAHs are found in different mixtures due to a variety of sources. Some are naturally occurring as there is a low level of background concentration but the largest source is from human activity, mostly from the use of fossil fuels (Rand, 1995) (Newman & Unger, 2003) (Istenič, Arias, Matamoros, Vollertsen, & Brix, 2011). The PAHs can be from petroleum spills and leaks, creosote oil, burning of organic material such as brush fires, wastewater from refineries, as well as municipal and industrial effluents (Rand, 1995). PAHs are in fossil fuels in varying concentrations. Fossil fuels can collect on the roadways from cars, oil, fuel spills, pesticides or industrial practices. Stormwater run-off flushes the petroleum products off the roadways and into the stormwater systems (Norton et al., 2011). The amount released into Puget Sound watershed from each source is depicted in Figure 1 below. The majority of PAHs are from the incomplete combustion of organic material in woodstoves and fireplaces. Since the PAHs are not very volatile they sorb to particulates. This results in atmospheric deposition on the land and roadways. The next highest sources are from the use of creosote followed by gasoline.

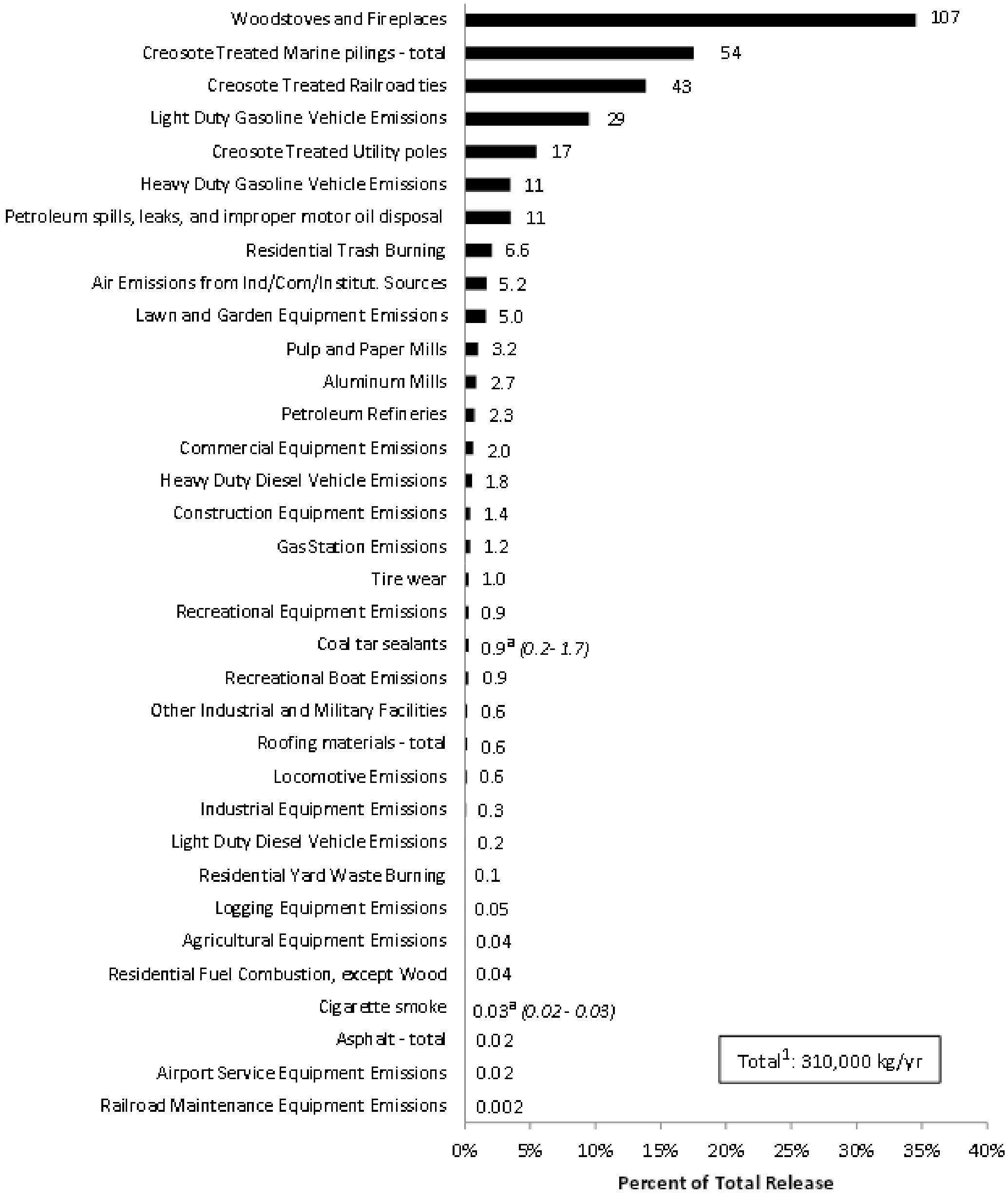


Figure 1. Estimated PAH total release into the Puget Sound watershed. Values shown are in thousands of kg/year (Norton et al., 2011).

PAHs are lipophilic, meaning that they are found more in oil than in water. In a stormwater conveyance feature, this means that they will sorb to soils, sediments or oil, rather than staying in the water. Once in Puget Sound, they will be found bound to sediment. This also means that once ingested in an organism they will have an affinity for the fats and can accumulate up the food chain (Norton et al., 2011).

There are high molecular weight PAHs and low molecular weight PAHs. Low molecular weight PAHs tend to be in elevated concentrations in fossil fuels, whereas high molecular weight PAHs are from the incomplete combustion of those fossil fuels or other organics, like wood (Norton et al., 2011). (Newman & Unger, 2003). Combustion in an engine is not an efficient process; it leads to a high concentration of incompletely combusted fuel (Newman & Unger, 2003). High molecular weight PAHs are more of a concern. They are larger and are more lipophilic as well as less volatile (Istenič, Arias, Matamoros, Vollertsen, & Brix, 2011). This makes them less water soluble and form a stronger affinity for soils and sediments. The Clean Water Act listed the following 16 PAHs as being priority pollutants:

Low Molecular Weight PAHs (LPAHs)
- Acenaphthene
- Acenaphthylene
- Anthracene
- Fluorene
- Naphthalene
- Phenanthrene

High Molecular Weight PAHs (HPAHs)
- Benzo(a)anthracene*
- Benzo(a)pyrene*
- Benzo(b)fluoranthene*
- Benzo(k)fluoranthene*
- Benzo(g,h,i)perylene
- Chrysene*
- Dibenzo(a,h)anthracene*
- Fluoranthene
- Indeno(1,2,3c,d)pyrene*
- Pyrene

* Probable human carcinogens (cPAHs) by EPA (Norton et al., 2011)

Levels of PAHs are difficult to measure because there are so many types. In the Puget Sound area, based on limited data, it is estimated that the freshwater concentrations range from 0.1 – 1.0 µg/L, with the marine waters being slightly higher (Norton et al., 2011). The sediment, however, is where the PAHs accumulate. It is estimated that the freshwater and marine sediments in the Puget Sound area are 100- 1,000 µg/kg with an approximation of about ten-fold higher in urban bays (Norton et al., 2011). These levels would be comparable to other urban environments.

PAHs can degrade naturally and can even be broken down and metabolized by organisms so the environmental concentrations varies widely and depend on many factors such as sunlight, medium accumulated in (soil, water pavement, etc), or even presence and abundance of microbes (Rand, 1995). There is a natural process of photodegradation over time, but this strong affinity for soils makes them difficult to breakdown naturally, as they would on the surface of water. This makes the concentration in sediment much higher than in surface soils (Norton et al., 2011). They also biodegrade over time when not attached to the soil, but because of the huge variety of PAH compounds and the different half-lives, this process is highly variable and experimental data ranges anywhere from a 2 day half-life to a 1.9 year half-life ("Technical Factsheet on: POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)," n.d.). Microorganisms can assist in the degrading of the PAH compounds and actually use the PAHs as a carbon and energy source forming non-toxic products, such as cell biomass, carbon dioxide and water (Atlas & Cerniglia, 1995) (Lundstedt et al., 2007). This process is known as bioremediation.

2.3 EFFECTS TO AQUATIC ORGANISMS

Metals and PAHs have both been found to be toxic to aquatic organisms. This is a concern in the freshwater streams and lakes where the stormwater initially gets discharged. Levels of toxicity very between metal type, PAH type and mixture concentrations. In this thesis, metal concentrations will be analyzed against the toxic response measured, but PAH toxicity endpoints will also be considered.

2.3.1 METAL TOXICITY

Most of the metals that are of concern can be found naturally occurring within the aquatic environment. They become harmful due to the increased concentration from human activity (Newman, & Unger, 2003). There are a number of ways that metals are toxic to fish, including adversely affecting the immune system and liver, the olfactory system and the ability to regulate ion uptake, transport and kidney function (Rand, 1995). Metals are usually found in ionic form or as simple compounds readily available for uptake and accumulation (Rand, 1995). Copper and zinc are necessary for cellular function (Rand, 1995). These essential elements are only beneficial for ionoregulation at low levels. At higher levels they become toxic; this effect is known as hormesis (Newman & Unger, 2003). The threshold value for the essential metals is different among species and continues to be studied for fish.

The metallothionein protein, in the fish liver, binds to the metal ions for detoxification purposes (Newman & Unger, 2003). This process reduces the over-abundance of essential and non-essential metals. Metallothionein can lead to a metal resistance by the liver by producing more of the protein and making the threshold for toxicity higher. But the production of more metallothionein could also mean less production of other detoxifying proteins, causing the fish to be susceptible to injury by other contaminants (Newman & Unger, 2003).

Besides metallothionein, which is a detoxifying protein, there are also environmental stress proteins that are produced in response to heavy metal exposure. There are a wide array of proteins that are rapidly produced as a defense against an environmental stress, such as that caused by toxic metals (Rand, 1995). They weaken the fish and decrease the function of the immune system making disease, cancer, and gene mutations more common (Rand, 1995). This is known as immunosuppression and is a reduction in the fish defense mechanism making them more susceptible to pathogens (Rand, 1995).

One way in which immunosuppression may occur is through exposing fish to metal ions that disrupt and alter ionoregulation in fish (Scholz et al., 2011). The metal ions bind to the gill surface leading to damage of the gills themselves as well as the blocking of essential sodium and potassium ion uptake (Landis & Yu, 2004). This effect is observed with many metals because of the common positive charge of a metal ion in water. The sodium/potassium ATPase channel would normally be used for sodium and potassium uptake and regulation, but because of the common positive charge of the metal ions, the transport channel can be blocked by the accumulation of metals. This simple metal accumulation on the gill surface can directly impact the health of the fish and cause the development of tumors, as well as impair other biological functions which require the ions that are being blocked (Rand, 1995).

Salmon in particular are sensitive to accumulations on the gill surface due to the fact that they transition from saltwater to freshwater fish in order to spawn in freshwater streams. This adjusts how the gill surface functions in terms of osmoregularity and the regulation of sodium and potassium uptake (Scholz et al., 2011). Metal accumulated on the gill surface in a saltwater fish may be more of a functional problem once it transitions into a freshwater fish. This also affects the general ability to acclimate to the freshwater system (Scholz et al., 2011). The gill surface is not the only site that metal acts on. Heavy metals also alter the olfactory system of the fish, which affects the sense of smell.

The effects of copper on the olfactory system of juvenile Coho salmon have been studied in lab experiments. In a study done by McIntyre et al. (2012), Coho salmon were exposed to copper and then allowed to be in the same tank as predator fish. They demonstrated that the “alarm response was absent in prey fish…exposed Coho were unresponsive to their chemosensory environment, unprepared to evade nearby predators, and significantly less likely to survive an attack sequence" (McIntyre, Baldwin, Beauchamp & Scholz, 2012). The olfactory system is very important in salmon because they use the sensory neuron output to get information about the surrounding environment, in this case the predators nearby. In juvenile salmon, the normal response to prey in the environment is to become motionless. In freshwater at low copper concentrations of 2 to 20 µg/L, copper ions can block the sensory receptor neurons and inhibit the response to prey in the environment. In concentrations above 20 µg/L, the copper can cause cell death of the olfactory receptor neurons (McIntyre et al., 2012).

The copper disrupts and distorts the neuron output and the fish becomes confused and cannot detect predators nearby (McIntyre et al., 2012). The prey is more disadvantaged than the predator because trout (the predator) are visual hunters so the olfactory disruptor does not affect the ability to hunt. The olfactory system is not only used for avoidance of predators, it is essential for the recognition of family and for the synchronization for spawning salmon to find mates (McIntyre et al., 2012). The fish also use olfactory chemical cues for their migration pattern as forms of memory to return to the stream they are from to spawn, as well as determine their habitat quality (Baldwin et al., 2003). The inhibitions of the olfactory system has the potential to be detrimental to salmon species. Because of the common charges of metal ions and the similarities in the fish olfactory system, a similar effect could occur with other fish dependent on sense of smell, as well as with other metals. The effect on the olfactory system has also been studied in Chinook Salmon, Rainbow Trout, Brown Trout, Fathead Minnow, Colorado Pikeminnow and Tilapia (Sandahl et al., 2007).

Juvenile salmon in rivers out of the Puget Sound catchment were showing neurotoxic effects at levels as low as 2 µg/L copper (McIntyre et al., 2012), and exhibiting a lack of predator avoidance behavior at levels as low as 0.7 µg/L (Norton et al., 2011). Spawning salmon and juvenile salmon have been shown to avoid point sources of copper contamination, but in a situation such as stormwater pollution, where there is not a point source, the fish are unable to avoid the contamination (Sandahl et al., 2007). Norton et al. with the Department of Ecology reported near shore (freshwater) and off shore (marine) concentrations of copper in the Puget Sound are at an average of 5 µg/l and 2 µg/l respectively (2001). Freshwater streams in California were found to have dissolved copper concentrations ranging from 3.4 – 64.5 µg/l. These levels are representative of freshwater in an urban watershed area (Sandahl et al., 2007). This shows how metal concentrations in urban watersheds are high enough to cause a toxic effect.

2.3.2 PAH TOXICITY

The other main contaminant of concern is polycyclic aromatic hydrocarbons (PAHs). High levels of PAHs are a concern for human and ecological health (Sun, Liu, Jin, & Gao, 2013). PAHs range in potency and toxicity, and are most harmful by causing DNA damage, some being carcinogens, mutagens, or even teratogens (Rand, 1995). This means there is an increase in cancer to exposed aquatic organisms, they cause mutations to occur within the cells of an organism at all stages of life, and they can cause deformities to the forming embryos through the mother’s exposure (Rand, 1995).

Most PAHs are toxic by the process of biotransformation. They are readily absorbed across lipid membranes and accumulate in the fats, gills, skin and digestive tract of aquatic organisms (Rand, 1995). The body's reaction to this lipophilic nature is an attempt to metabolize the compounds by detoxification. Certain detox genes are produced in response to PAH ingestion and can be measured in different areas of an organism to indicate PAH exposure. Enzymes, such as cytochrome P450 monooxygenase, are produced in response to the elevated levels of PAHs. PAHs in the body induce the gene cytochrome cyp1A for production of this enzyme (Rand, 1995). In summary, the enzyme is produced and catalyzes a series of reactions which break down the PAHs and make the components water-soluble. This can make a chemical more or less toxic. In the case of PAHs, it is a process which detoxifies the contaminant from the body, but the byproducts can be harmful. Benzo (a) pyrene, for example, is not toxic on its own. The hydrolysis of benzo (a) pyrene creates a byproduct known as benzo (a) pyrene diol epoxide. This byproduct is extremely carcinogenic and can form covalent bonds with DNA, resulting in DNA point mutations, ultimately causing cancer (Rand, 1995). In fish, the process of biotransformation is most commonly observed in the liver, creating liver tumors.

Other PAHs in the body promote the production of free radicals and interfere with the normal function of coping with oxidative stress (Newman & Unger, 2003). This process can occur near DNA and cause DNA damage leading to cancer and other genotoxic effects such as mutations (Newman & Unger, 2003). DNA damage and mutations can be an indication of PAH exposure. If the rate at which a contaminant can be detoxified from the organism is lower than the rate of accumulation, than PAHs will accumulate in the lipids of the organisms. This creates the potential of bioaccumulation and biomagnification as the PAHs move up the food chain (Rand, 1995).

PAHs are also considered immunotoxic (toxic to the immune system) as well as cardiotoxic (disrupts normal heart function). They have been shown to result in inflammatory gill damage (Rand, 1995). When the immune system is required and the fish has inflamed gills, the antibody production is drastically decreased. This makes the fish vulnerable to other contaminants and diseases. Other studies on fish embryos show a decrease in size and development rate, specifically in the head and eye sizes of the fish (Incardona et al., 2004). PAHs are cardiotoxic by causing deformities in the heart of fish embryos. Incardona et al. (2004) measured an increase in the amount of fish embryos with arrhythmia (uneven heart beats), pericardial edema (fluid accumulation in the heart area), circulatory stasis (lack of blood flow) and unlooped hearts (lack of distinct heart chambers). This shows that the development, as well as the function, of the heart is effected by PAH exposed in the embryonic stages (Incardona et al., 2004).

2.4 STORMWATER MANAGEMENT

Nationwide, understanding of stormwater pollution is of growing importance (Ormond, Mundy, Mary Weber, & Friedman, 2010). There is a push for innovative solutions to the non-point pollution problem. Updated development planning is implemented to control the flow of the water from over-whelming the city stormwater infrastructure. Another goal is to decrease anthropogenic impacts by containing the contaminants coming off the highways, parking lots, roofing and other developed areas.

The development approaches are known as Best Management Practices (BMP), Low Impact Development (LID), or Green Stormwater Infrastructure (GSI). They aim for development of cost effective and sustainable stormwater solutions (Ormond et al., 2010).

2.4.1 LOW IMPACT DEVELOPMENT

Low impact development (LID) is an approach to the stormwater control problem which attempts to use structures and nature functionally for stormwater control and pollution reduction. The idea is to improve landscapes which would already be used or to increase the perviousness of surfaces (EPA, 2015a). LID ultimately attempts to transform stormwater from being viewed as a waste source to more of a resource. The goals of LID are, according to the EPA (2015a), to maintain groundwater quality and recharge, to reduce stormwater pollutant loads, to protect streams and channels, to prevent overbank flooding, and to safely control extreme floods.

One of the major issues with increased urban areas and impervious surfaces is that all the water that naturally falls on the ground concentrates on the roadways and ends up in storm drains flowing back to the rivers and ocean. As a result of this the groundwater is left with little recharge. Another issue is that when larger storm events happen, the stormwater pipes can get overloaded and cause a flooding and overflow of the sewage system. This is extremely detrimental because in many cases it can lead to untreated water being diverted directly into the water bodies, creating a larger toxic control problem. By using LID principles home owners, as well as municipalities, can harness stormwater to recharge the groundwater as well as reduce the contaminant load to larger waterways. “Applied on a broad scale, LID can maintain or restore a watershed's hydrologic and ecological functions” (US EPA, 2015). A few of the LID approaches are bioretention ponds, rain gardens, vegetated rooftops, rain barrels, grassed channels and permeable pavements (EPA, 2015).

2.4.2 BIORETENTION SYSTEMS

Bioretention systems are landscape designs that are used to control stormwater runoff. Ideally, they are shallow depressions with shrubs, trees and grasses planted in them or areas with no plants, covered with gravel or bark (Dietz & Clausen, 2005). They are placed adjacent to roads and parking lots and are used to divert stormwater and road runoff. They can also be used on residential areas to capture rainfall from individual homes and rooftop runoff. According to Hinman (2009), with the WSU extension campus, “Bioretention is one of the most common applied and adaptable integrated management practices in the low impact development approach”.

The two main types of bioretention systems are rain gardens and bioretention ponds. The premise behind a rain garden is to allow for slow ground filtration of stormwater so that the water from the rooftops and streets will flow in and permeate the garden to be re-entered into the groundwater supply. This helps to recharge the groundwater, securing and controlling the freshwater supply. They also act as a pollution control technique for the treatment of stormwater. The water can filter through the soil column allowing the contaminants in the stormwater to be retained by adsorption or taken up by plants in the gardens (Dietz & Clausen, 2006). In this way, the rain garden can act as a natural way to provide pollutant treatment (Dietz & Clausen, 2006).

Bioretention ponds are very similar to rain gardens with one exception. Rain gardens are not meant to have any standing water, while bioretention ponds are. A rain garden is a sort or bioretention area in that is designed to retain the contaminants and allow for water to pass through, whereas, a bioretention pond is designed for larger quantities of overflow water from a road system. These ponds are a standing pool of stormwater (EPA, 2015).

The ponds are designed to capture the stormwater and allow for the slow settling of the contaminants to the bottom of the pond. The ponds usually have an overflow designed to divert untreated water into a stream or channel or into another stormwater feature. One issue with bioretention ponds is that they tend to have an extremely high concentration of stormwater contaminants, specifically copper and zinc (Wium-Andersen, Nielsen, Hvitved-Jakobsen, & Vollertsen, 2011). This does indicate that the pollutants are being stopped from entering larger streams and water bodies, but it also poses a concern because these ponds create mini contaminated ecosystems for many different species. Retention ponds are usually constructed in highly urbanized areas where natural habitat is difficult to access. These ponds create a place for birds, insects and amphibians to survive and breed.

Both rain gardens and bioretention ponds help lessen the load on other stormwater conveyance features to avoid the burden of heavy rain and storm events. Collectively they will be referred to as bioretention systems for the remainder of this thesis. While bioretention systems are very valuable in the absorption of stormwater contaminants and lessening the impact of flow in storm events, there is concern of the effectiveness in treatment of contaminants in stormwater runoff.

2.5 METALS IN BIORETENTION SUBSTRATES

Some studies show that bioretention systems aid in the binding capacity and removal of metals while other studies show that the composts in these systems can leach metals (McIntyre et al., 2014)(Geronimo et al., 2014)(Page et al., 2014)(Good et al., 2012). Composts are known to have high concentrations of metals. Much of this metal is naturally occurring, but land use can also add to metals in the composts (Cambier et al., 2014). Using compost from agricultural lands in particular can have elevated levels of metals due to the use of different fertilizers and pesticides (Cambier et al., 2014). Metals in the soils can do three things: accumulate in the soil and create a larger environmental issue, leach from the soil and contaminate the groundwater, or be taken up by plants and potentially enter the food web (Kaschl et al., 2002). The purpose of a bioretention system is to allow for the accumulation of the metals in the soils and ultimate retention of the metals. If the metals are leaching out at a greater rate than they are being retained, then they are adding to stormwater pollution.

In a study of different stages of compost production, it was found that compost can leach out metals regardless of what stage of decomposition they are in (Page et al., 2014). Metal concentrations in dry compost were initially measured. Deionized water was allowed to pass through the compost and the resulting heavy metal extractability was measured. The metals that leached out were highest for nickel and zinc, with 13.4 % and 29% of the metals leaching out respectively. This study did, however, find that the water soluble forms of the metals were in very low concentrations. Most of the metal leached out of the compost were in complexes and not in free ionic form (Page et al., 2014). In this study pH was also measured. The leachate water became more basic from flushing through the compost, from 5.64 to 6.5. This indicates a retention of H+ in the compost (Page et al., 2014).

Another study found elevated levels of copper, zinc, cadmium and lead in the compost leachate at 37, 259, 0.21 and 4.5 mg/l respectively (Zhao, Lian, & Duo, 2011). In a separate study of compost over time, the compost was allowed to age for 2-10 years. All samples were similar in the leachate of metals. This leachate was found to contain elevated levels of metals, however the only metal leaching out of the compost was zinc (Cambier et al., 2014). Municipal solid waste composts are measured with higher levels of heavy metals than background soil concentrations and there is concern of contaminating groundwater (Zhao et al., 2011). Multiple experiments show pH as the leading factor in metal leaching. Metal leaching is effected by pH by processes outlined in the cation exchange capacity and the nature of soils to prefer certain cation over others. In a more acidic environment, hydrogen ions could displace other metal ions, resulting in metal leaching (Cambier et al., 2014). One study found that copper, zinc, nickel and chromium were all leached out of compost, but at levels below drinking water standards. There was a correlation with both pH and dissolved organic matter and the amount of copper leached (Kaschl et al., 2002). All of the studies mentioned show leaching of zinc and other metals out the composts. The concentrations vary widely. All of these studies were done with clean water being flushed through compost. There is still a question of what happens when the compost is merely a component of the soil as well as what happens when contaminated stormwater is flushed through the soils.

2.6 EFFECTIVENESS OF BIORETENTION

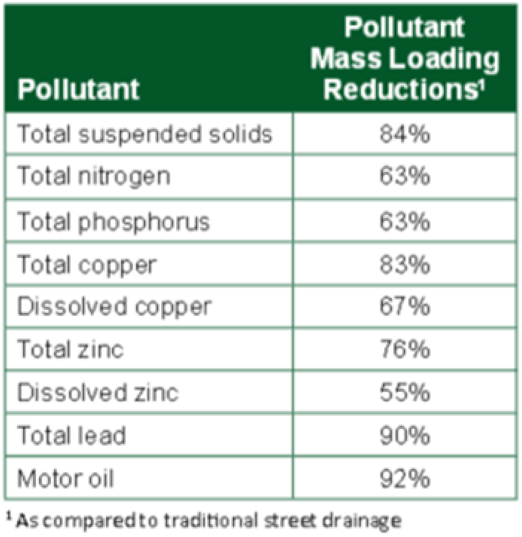
Bioretention systems have been shown to be effective at controlling the flow of water off of impervious surfaces as well as treating the stormwater. This ultimately reduces the potential to overflow stormwater conveyance features, which could cause large flushes of highly concentrated stormwater into larger bodies of water. Contradictory to studies finding the leaching of metals, many studies find that composts, used in bioretention systems, are effective at retaining metals by absorption.

2.6.1 PREVIOUS STUDIES

The Northwest Cascade Project from 2004 through 2006 was aimed at using a combination of LID approaches in an average neighborhood and found that the pollutant load from stormwater was significantly decreased with LID technology (Table 2) (EPA, 2012) . Total copper was reduced by 83%, total zinc by 76%, total lead by 90% and motor oil, which contains a majority of the PAHs was reduced by 92% (EPA, 2012).

Another similar study found that when synthetic stormwater (water spiked to replicate stormwater contaminants) was leached through compost, the compost retained 93% of the copper, 88% of the zinc and 97% of the lead. Based on this data the relative sorption affinity of the composts were lead > copper > zinc (Seelsaen, McLaughlan, Moore, & Stuetz, 2007). At the beginning of the study, the pH of the compost was adjusted with hydrochloric acid, HCl, to be around 5. This was done to load the soils with hydrogen ions and test the efficiency of the soils. This compost was also found to leach very high concentrations of dissolved organic carbon (DOC) (Seelsaen et al., 2007).

Table 2. Pollutant mass loading reductions from a combination of LID approaches done by The Northwest Cascade Project from 2004 through 2006 in Seattle, Washington (EPA, 2012).



Pollutant	Pollutant Mass Loading Reductions[1]
Total suspended solids	84%
Total nitrogen	63%
Total phosphorus	63%
Total copper	83%
Dissolved copper	67%
Total zinc	76%
Dissolved zinc	55%
Total lead	90%
Motor oil	92%

[1] As compared to traditional street drainage

McIntyre et al. (2014) performed a study on zebrafish embryos, using them as biological indicators of the effectiveness of green stormwater infrastructure treatment, in this case, bioretention systems. The fish were tested with untreated stormwater runoff as well as runoff that had been treated by soil filtration. As expected, the untreated stormwater was highly contaminated with PAHs and resulted in an array of heart conditions, as well as reduced growth, reduced eye size and swim bladder inflation (McIntyre et al., 2014). The bioretention treatment of the stormwater, by flushing through a bioretention system, was successful in reducing nearly all developmental toxicity (McIntyre et al., 2014). The concentrations of dissolved metals were reduced by 99% for zinc, 72% for copper, 31% for nickel, 91% for lead, and 95% for cadmium. The PAHs were reduced 95% by the treatment (McIntyre et al., 2014).

2.6.2 CURRENT RESEARCH

There is research currently being conducted by Jenifer McIntyre and her colleagues at the Washington State University (WSU) Green Stormwater Infrastructure (GSI) Facility in Puyallup, Washington studying bioretention treatment. They have set up columns with bioretention soil medium and gravel to study the effectiveness of bioretention treatment. Some column have plants planted on top of the bioretention systems and some have a mulch layer instead. They allowed collected stormwater runoff to flush through the soil columns and captured the leachate to do toxicity tests with zebrafish on gene expression. The preliminary results of their study show significant upregulation of detox (cyp1a) and cardiac injury genes in the fish exposed to highway runoff (Figure 2) (McIntyre et al., unpublished). These are genes are produced in excess when an organisms is exposed to environmental stressors. Each gene indicated a different kind of environmental stress. The cardiac injury genes are an indication of pollutant exposure causing cardiotoxicity. Detox genes, such as cyp1a, provide metabolic protection against contaminants such as high molecular weight PAHs (McIntyre et. al, unpublished). Metallothionein (mt2) is the gene upregulated for detox due to exposure to metals. An upregulation would be observed with an increase in contaminants in the system.

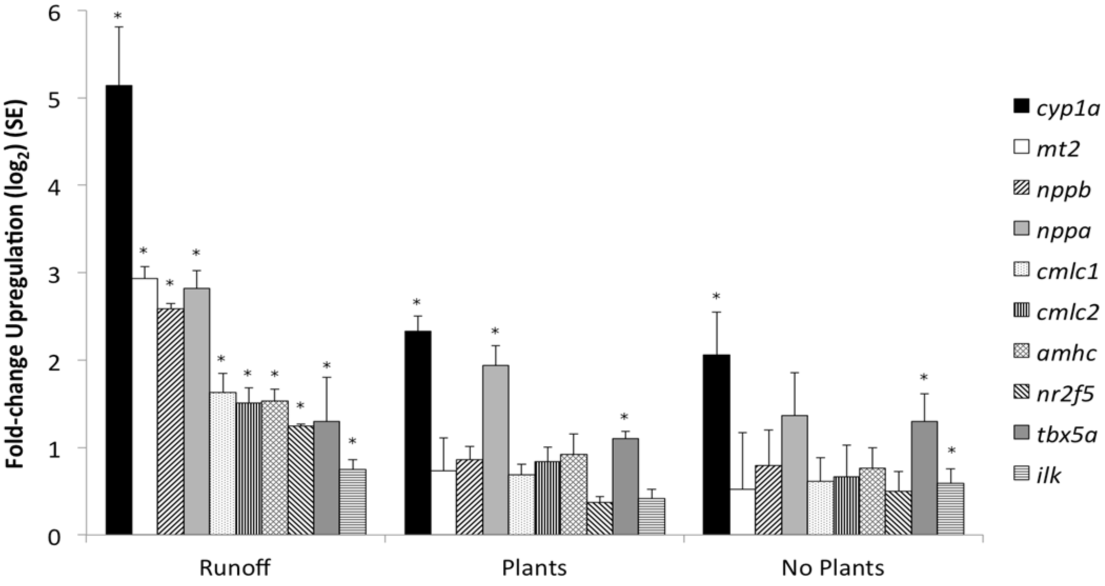


Figure 2. Zebrafish upregulation of detox and cardiac injury genes from exposure to untreated stormwater runoff and stormwater runoff treated through bioretention columns with and without plants (McIntyre et. al, unpublished).

Filtering the runoff through the bioretention cells significantly reduced expression of those genes, but some still showed significant upregulation compared to controls. The cyp1a gene was still significantly elevated after treatment of the stormwater runoff. Metallothionein (mt2) was significantly reduced in the treatment with and without plants. This indicated that it is more probable that PAHs rather than metals were responsible for the toxic effects observed (Figure 2). The results from this test indicate that there is still a toxic response from the bioretention system leachate of the treated stormwater. The leachate from columns themselves were never tested with clean water being flushed through. There is not conclusive evidence that the toxic effects of the leachate where from the stormwater that had passed through or if it was from contaminants leaching out of the soils.

These examples all used toxic stormwater runoff to measure retention capability in the soils. The bioretention systems in all of those studies were shown to remove a majority of the metals and PAHs, but this was only when the stormwater was contaminated to begin with. Soils and composts have already been shown to have metal in them. For a thorough analysis on bioretention systems it is important to consider what can leach out of the material on its own.

Bioretention systems may be primarily used to treat stormwater, but in some applications they are used to control the flow of water. Residential areas use rain gardens primarily to allow for water filtration to recharge the ground water. This prevents some of the flow onto impervious surfaces. In this situation, the water that flushes through the retention systems is not significantly contaminated compared to stormwater runoff in an urban setting. There is research that suggests in certain situations, metals could be leaching out of the compost. However this has not been extensively studied in the application of bioretention systems. It is possible that there are contaminants from the stormwater which pass through the bioretention systems, but it is also possible that the substrates themselves in the bioretention systems are leaching enough metal to cause a toxic effect

3. THESIS RESEARCH

There is still question of the toxicological impact of bioretention systems on aquatic organisms. This thesis research was designed to answer the following research questions:

- Could the substrates in bioretention systems be a source of metal pollution? If so, is it from the soil mixture or the gravel?
- As more water is passed through a soil column, does the amount of metals in the leachate decrease?
- What is the biological response of the contaminant mixture generated from bioretention systems to Zebrafish?
- Does the pollutant removal efficiency of bioretention systems outweigh the pollution generating behavior observed?

This research will help to better understand the contaminant removal potential of different substrates. It will also add to the understanding of the pollution generating potential of bioretention systems. Overall, this data could be used to determine if bioretention systems are an adequate treatment for stormwater or if they could be adding to stormwater pollution by leaching metals into aquatic systems.

3.1 METHODS

To address the research questions outlined above, bioretention system columns were set up in a greenhouse at the Green Stormwater Infrastructure Facility (GSI) at the Puyallup Extension Campus of Washington State University (WSU) in Puyallup, Washington. Clean water was leached through the columns at varying times from August 2014 through January 2015. There were first eight separate flush tests, each a week apart, then there was a conditioning period for the columns, where a large amount of water was flushed through in a 3 week period. After the conditioning with the clean water, stormwater runoff was collected and treated through though the columns. The stormwater runoff collection and leach was done in February 2015. The leachate of the individual tests were measured for heavy metals. Zebrafish toxicity tests were conducted at the Northwest Fisheries Science Center of the National Oceanic and Atmospheric Administration (NOAA) facility in Seattle, Washington. A comparative analysis was done between the leachate of the clean water and the leachate of the collected stormwater runoff.

3.1.1 BIORETENTION COLUMN SET-UP

The soil columns were designed to replicate a bioretention structure in compliance with recommendations for flow control and pollutant removal (Palmer, Poor, Hinman, & Stark, 2013) (Hinman, 2009). A typical design for a bioretention system, as recommended by the Department of Ecology and the Seattle Municipal Stormwater Code, includes a mineral aggregate drainage layer at the base, followed by a bioretention soil media (BSM), with either mulch, plants, or a ponding zone on the top (Hinman, 2012). To allow for a better understanding of the BSM and gravel layers, the top layer was not included in this study. Plants were eliminated given that they do not significantly improve removal of contaminants (Palmer et al., 2013). This research compared the leachates from two substrates used in bioretention systems, the bioretention soil medium (BSM) and the gravel drainage layer.

Three separate columns were set up. The first column was filled with all bioretention soil medium (BSM). The next column was filled with the gravel representative of a drainage layer in a rain garden. The final column was both BSM and gravel. The bioretention soil medium (BSM) was a composition of 60% mineral aggregate, 15% compost, 15% finely shredded cedar bark and 10% drinking water treatment residuals (WTR) (Palmer et al., 2013). This ratio is accepted by the Department of Ecology from bioretention system stormwater treatment (Hinman, 2009). The WTRs used were amorphous aluminum hydroxides from the Anacortes Water Treatment Plant in Anacortes, WA. They are produced when aluminum sulfate is added to water for treatment. The WTR are a byproduct of flocculation and are obtained in the precipitate that forms. They were dried after use in the drinking water treatment and sieved to remove large clumps. When added to the BSM mixture they were a fine grained material (Palmer et al., 2013). Since they are composed of aluminum and hydrogen, and this study is not measuring aluminum, there is no concern of them being a source of the metals measured. The compost used was from an all organic compost company, Cedar Grove, in Seattle, WA. The compost was Type 1 feedstock, which was derived from materials such as yard, garden, wood, agricultural residuals and pre-vegetative food wastes (Palmer et al., 2013)(Hinman, 2009). The compost used in this mixture was in compliance with Washington Administrative Code (WAC) 173-350 which requires the organic matter content to be between 45 and 60 percent, have a pH between 5.5 and 8, a carbon:nitrogen ratio between 20:1 and 25:1, and less than one percent of manufactured inert materials (Hinman, 2009). There is also metal regulations for this compost. The metals had to meet the requirements in Table 3, below, in order to be in compliance with WAC 173-350.

Table 3. The limits in metal concentration (mg/kg dry weight) for compost to be in compliance with WAC 173-350 (Hinman, 2009).

Metal	Limit
Arsenic	≤ 20 ppm
Cadmium	≤ 10 ppm
Copper	≤ 750 ppm
Lead	≤ 150 ppm
Nickel	≤ 210 ppm
Zinc	≤ 1400 ppm

For the gravel substrate a 3/4" Seattle Type 26 sandy gravel was used. The gradation size distribution for this gravel layer as well as the BSM mineral aggregate are within the Department of Ecology guidelines as shown in Table 4 (Hinman, 2009). This was the same BSM and gravel used in the research by Palmer et al. (2013) and McIntyre et al. (2014).

Table 4. BSM mineral aggregate and gravel layer aggregate gradation size distribution (Palmer et al., 2013).

	Sieve Size	Percent Passing
BSM Mineral Aggregate	3/8 inch	100
	U.S. No. 4	95-100
	U.S. No. 10	75-90
	U.S. No. 40	25-40
	U.S. No. 100	4-10
	U.S. No. 200	2-5
Gravel Layer Aggregate	3/4 inch	100
	1/4 inch	30-60
	U.S. No. 8	20-50
	U.S. No. 50	3-12
	U.S. No. 200	0-1

According to the *Bioretention Soil Mix Review and Recommendation for Western Washington*, a BSM in the ratio used for this experiment would have a highorganic matter content and a high cation exchange capacity (Hinman, 2009). Theoretically the mixture of materials used for this BSM should have a cation exchage capacity of ≥ 5 meq/100 grams of dry soil.

The columns were constructed in polyvinyl chloride (PVC) pipes measuring 10.2 cm in diameter. They were open on the top and had a plastic screen on the bottom to avoid the loss of substrate material. The columns were placed on a wooden frame with plastic funnels under them, draining into 4 L amber glass carboys to catch the leachate. The PVC columns and funnels were rinsed with DI water before filling with the substrates. Each column was filled to the 50 cm mark with the testing material. This amount was adequate because the majority of the PAHs are sequestered in the top few centimeters of the bioretention systems and the majority of metals in the top 20 cm (Hunt, Davis, & Traver, 2012).

For the column with both BSM and gravel, the first 16 cm were gravel and the remaining 34 cm was BSM for a total of 50 cm. This ratio was proportionally representative of previous experiments (Palmer et al., 2013). The construction design is depicted below in Figure 3. After each 10 cm of substrate was added, the material was gently compressed by shaking and pressing down on it. This was done to avoid the creation of any voids or inconsistency in the amount of material. More material was added to ensure an equal 50 cm of total substrate for each column after compression.

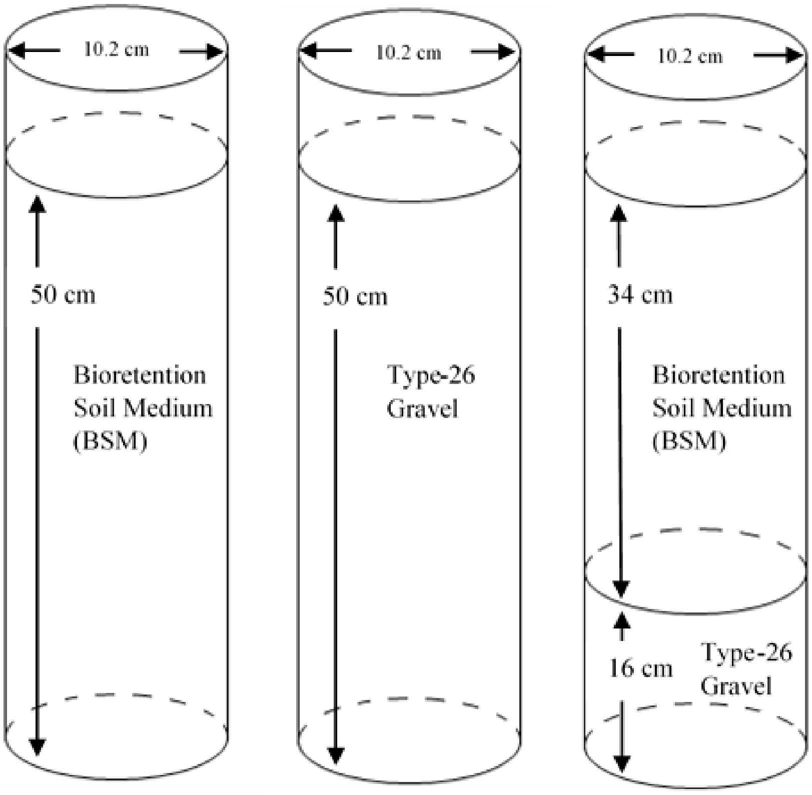


Figure 3. Construction design of the three bioretention substrate columns.

3.1.2 BIORENTENTION TREATMENT AND LEACHATE COLLECTION

A 1 g/L Instant Ocean® Sea Salt solution was made using deionized water which had also been filtered through a Brita and a Millipore filter. This solution is the same as the embryonic medium solution used for culturing the laboratory zebrafish and replicates previous zebrafish toxicity tests (McIntyre et al., 2014). It is a freshwater solution made with the essential minerals needed for zebrafish survival. When doing a toxicity test, it is important to be sure that the response measured is from the manipulated variable, in this case, the contaminants in the leachate. Using an embryonic medium solution decreases the chance that the response is due to the control water being different than solution the zebrafish is accustomed to. The same solution was used for each treatment column, which was pumped into the columns at a flow rate of 25 mL/min to replicate Palmer et al. (2013) and McIntyre et al. (2014). Each tube was attached to an irrigation head made with plastic disks and hypodermic needles to allow for drips similar to an average rainfall (0.05 mm/s). This is the rate at which the water would infiltrate into the substrates and was standardized for the size of the column used. The irrigation heads were fastened above the individual columns.

For each water collection period, three liters were allowed to drip into the columns. The amount of water retained in soil due to saturation was about one liter. After approximately two hours, two liters of leachate was collected in each corresponding glass carboy. In between each individual leach test, the carboys were rinsed three times with DI water to avoid cross-contamination. The glass carboys were shaken to homogenize prior to distribution into the separate analysis containers and storage jars. The caps were composed of Polytetrafluoroethylene (PTFE)-lined, polypropylene.

For the dissolved metal, calcium, magnesium, hardness, and alkalinity (no headspace) tests, plastic bottles were used. These samples were unpreserved. For the dissolved organic carbon, and pH tests, amber glass bottles were used. This was also unpreserved. An amber glass bottle was also used for the total organic carbon tests. This sample was preserved with sulfuric acid (H_2SO_4). All samples were placed on ice and transported for analysis within 24 hours. For future toxicity testing, four to six amber glass jars with at least 150 ml of leachate were frozen within 24 hours. The column flush was completed a total of eight times. There was at minimum one week between each flush and collecting period where the columns were allowed to completely dry out to mimic natural rainfall events. These eight flushes were all measured as outlined in the chemical analysis section and samples were frozen for each flush.

Next, the columns were flushed with more water to test if the retention medium could be conditioned prior to use to decrease the concentration of contaminants in the leachate. This portion of the experiment was not used for the toxicity tests. It was only done to test for the conditioning potential. In order to replicate the practices in the experiment by Palmer et al. in 2013, water from a garden hose was used rather than the embryonic medium as before. This was the source of water for that test. Each column received a flush of 34 L over a two week period using the same water distribution methods as before. After the 34 L flush of tap water, three additional liters of the tap water was dripped through and collected for chemical analysis.

3.1.3 HIGHWAY STORMWATER RUNOFF

Stormwater runoff was collected from Highway 520 in Seattle, Washington in February 2015 on the second day of a rain event. This portion of the highway is rated at 60,000 annual average daily traffic (AADT), meaning that an average of 60,000 vehicles use the bridge each day and is considered a high volume urban highway (McIntyre, unpublished). There is an elevated portion of the highway with a downspout routed into the National Oceanic and Atmospheric Administrative (NOAA) Northwest Fisheries Science Center (NWFSC) parking lot. The stormwater was collected into a large stainless steel cistern. The stormwater is not acidic enough to be concerned with the stainless steel leaching metals. This portion of the highway has guardrails and no plants or soil. This is important because the only chemicals present in any stormwater collected would be from the roadway. There is no obvious potential from contamination from pesticides, lawn care, roof materials or other stormwater pollution besides what comes off vehicles or from asphalt. The main contaminants of concern in these samples are heavy metals and PAHs.

A three liter sample of the collected stormwater was flushed through each of the bioretention columns and two liters of leachate was collected in the same procedure as described above. This was done a few hours after collection to avoid any breakdown of PAHs in the samples as recommended by previous research (McIntyre et al., 2014). The leachate from the treatment was collected and frozen or sent for analysis within a few hours.

3.1.4 CHEMICAL ANALYSIS

All samples were transported within 24 hours to Analytical Resource Incorporated (ARI) Laboratory in Tukwila, Washington. ARI conducted all chemical analysis using US Environmental Protection Agency (EPA) methods or EPA approved in-house methods. Three water samples were measured for the conventional water chemistry parameters: 1) The water from the 8th flush with the Instant Ocean® Sea Salt solution, 2) the final conditioning flush with tap water and 3) the stormwater runoff through the bioretention treatments. These tests consisted of measuring calcium (In-house method 6010C), magnesium (In-house method 6010C), alkalinity (In-house method SM 2320), dissolved organic carbon (DOC) (EPA method 9060), total organic carbon (TOC) (EPA method 9060), and pH (EPA method 150.1). All leachate samples, the untreated the Instant Ocean® Sea Salt solution (1 g/L), untreated tap water, and the untreated stormwater runoff, were measured for total arsenic, chromium, copper, lead, nickel, and zinc (In-house method 200.8) using an inductively coupled plasma mass spectrometer (ICP-MS). PAH measurements were not taken, but samples from the stormwater runoff leach test were preserved with methylene chloride and frozen for possible testing in the future. The first four leachate tests were also analyzed for cadmium and silver. The levels were below detection so this analysis was not continued for the remainder of the uncontaminated water treatment, but analysis was continued for the stormwater runoff test.

3.1.5 ZEBRAFISH TOXICITY TESTING

The biological portion of this research was conducted to determine if there was a toxic response to aquatic organisms from the leachate. Two separate toxicity tests were done. The first toxicity test was with the leachate from the Instant Ocean® Sea Salt solution treated through the BSM plus gravel column. Leach number two, four and eight were all tested against a control. The second toxicity test was with the stormwater runoff. The untreated stormwater runoff, the leach through the BSM, the leach through the BSM/gravel and the leach through the gravel column were all tested against a lab control. The lab control was the same composition as the embryonic medium used in the uncontaminated water tests. The methods used are in replication of a previous experiment (McIntyre et al., 2014). Zebrafish were used because as embryos they are transparent, so the developmental changes and heart function can be visually measured. They were also used because the formation of their heart and other organs happens within hours. A toxic response can be measured with a relatively short exposure. Wild type (AB) zebrafish were tested. The zebrafish were cultured and spawned at the National Oceanic and Atmospheric Administration Northwest Fisheries Science Center (NOAA NWFSC) in Seattle, Washington.

The day before egg collection, two separate containers each containing three males and three females were set up. The morning before collection the water was changed to ensure that the old eggs were disposed of. The Zebrafish were then allowed to spawn and two to three hours later the eggs were collected, rinsed and put into a petri dish in the incubator. The water samples were taken from the freezer and placed in a warm water bath for thawing.

Once the eggs were between the eight cell and high stage cell stages, (1.25-3.5 hours post fertilization) based on the guidelines in *Stages of Embryonic Development of the Zebrafish*, they were picked through to get the healthiest looking ones for the experiment (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The eggs closest to the ideal pictures were chosen (Figure 4, Images C-H). Eggs were chosen that had even cell division and were symmetrical. Each petri dish was given 15 eggs by collection with a glass pipette. There were three replicates for each treatment and the control. The control used was an embryo rearing medium made with 1 g/L Instant Ocean® Sea Salt. The residual water was removed and the eggs were dosed with 10 milliliters of leachate water (or control water) per petri dish. The dishes were covered and the positions were randomized before placement in the incubator at 28.5 °C.

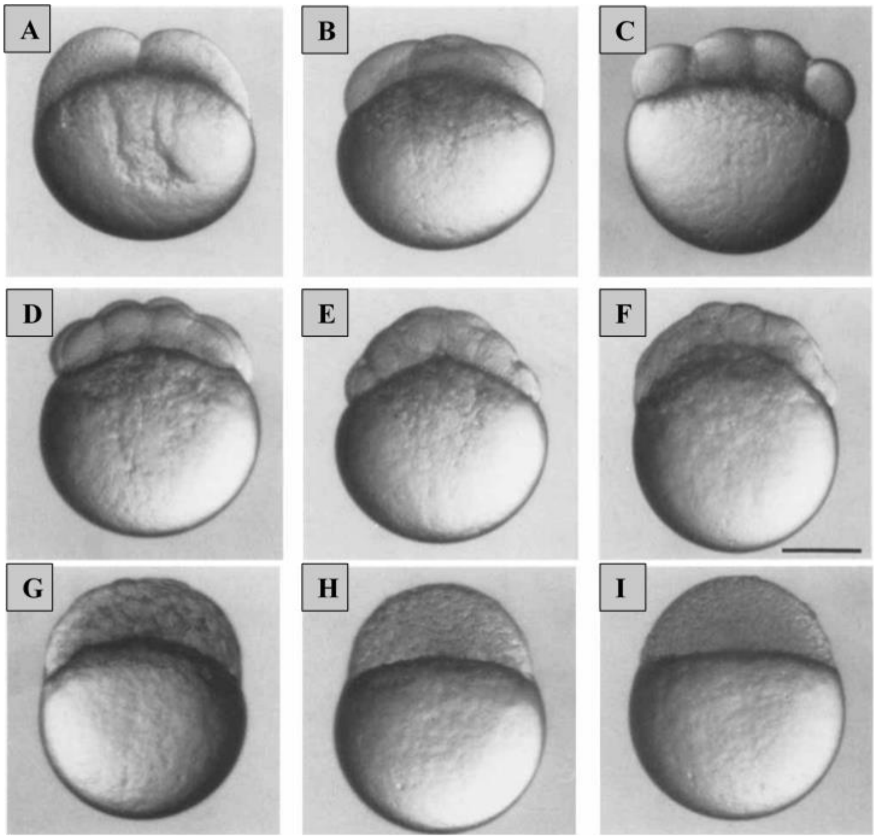


Figure 4. Stages of Zebrafish embryo development. A: Two-cell stage (0.75 hr). B: Four-cell stage (1 hr). C: Eight-cell stage (1.25 hr). D: Sixteen-cell stage (1.5 hr). E: Thirty-two cell stage (1.75 hr). F: Sixty-four cell stage (2 hr). G: 256-cell stage (2.5 hr). H: High stage (3.5 hr). I: Transition between high and oblong stages (3.5 hr). (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995)

After 24 hours, the water was replaced with the same initial water (i.e. treatment or control water) and any dead eggs were removed. Survival count was measured by counting the number of dead embryos per replicate. The dish was placed back in the incubator until the following day. At the 48 hour post exposure point, the petri dishes were analyzed in random order. Based on the previous work by McIntyre et al. (2014), 48 hours was sufficient for the responses measured; the heart is developed enough at this point to determine toxicity. Zebrafish pigmentation begins to form after 48 hours making it difficult to visualize heart defects and see the heart development. The hatch rate was measured as the amount of embryos per replicate that had hatched from the chorion. At 48 hours this was an insignificant measurement and was not included in the analysis.

All embryos that were still unhatched were manually dechorionated by using tweezers to rip the chorion without injuring the embryo. Once all the embryos were dechorionated, the dirty water was pipetted out and replaced with clean embryonic medium water. A few drops of tricaine methanesulfonate (250 µg/L MS-222) was added to the dishes to anesthetize the embryos to avoid twitching and movement during imaging.

A daub of 3% methylcellulose was put on the bottom of a plastic petri dish and spread to be as flat as possible with minimal bubbles. The embryos were then removed with a dropper and put on top of the methylcellulose. Excess water was removed from the surface. One at a time, the embryos were shifted away from each other and oriented in the same direction. They were shifted on their sides facing the left with the eyes stacked, creating uniformity in the imaging. Each embryo was analyzed under a Nikon SMZ800 stereomicroscope (Nikon Instruments Inc., Melville, NY) and imaged with a Fire-I 400 digital camera (Unibrain Inc., San Ramon, CA). A five second video was also taken of the periventral area by focusing the microscope on the heart. This was repeated for each replicate and each treatment group as well as the control.

3.2 ANALYSIS

3.2.1 IMAGE AND VIDEO ANALYSIS

The still images were measured using ImageJ 1.48v software (National Institutes of Health). These measurements included the total length of the embryo, which is the sum of the length from the tail through the notochord to the ear plus the length straight through the middle of eye to the end of the embryo (Figure 5A). Consistent use of this method normalized measurements for kinks and head angle between the different embryos. The eye area was measured by tracing the circumference the eye (Figure 5B). The periventral and pericardial area was also measured. The periventral area consists of the area with the heart as well as any area of fluid accumulation under the yolk sac (Figure 5D). The pericardial area is the area where the heart and surrounding fluid are (Figure 5C). This excludes the area under the yolk sac where the blood initially flows before entering the heart chamber. Since all the measurements were done in the ImageJ program at the same microscope zoom setting (2X for length and 6.3X for heart measurements), the images will all be compared in pixels rather than a metric unit. The videos were used to analyze heart function and determine the borders of the periventral and pericardial area. Periventral blood pooling was identified when there was blood pooled in the periventral area that did not get pumped into the pericardial area where the heart was. The heart rate was counted in each 5 second clip and multiplied to get beats per minute.

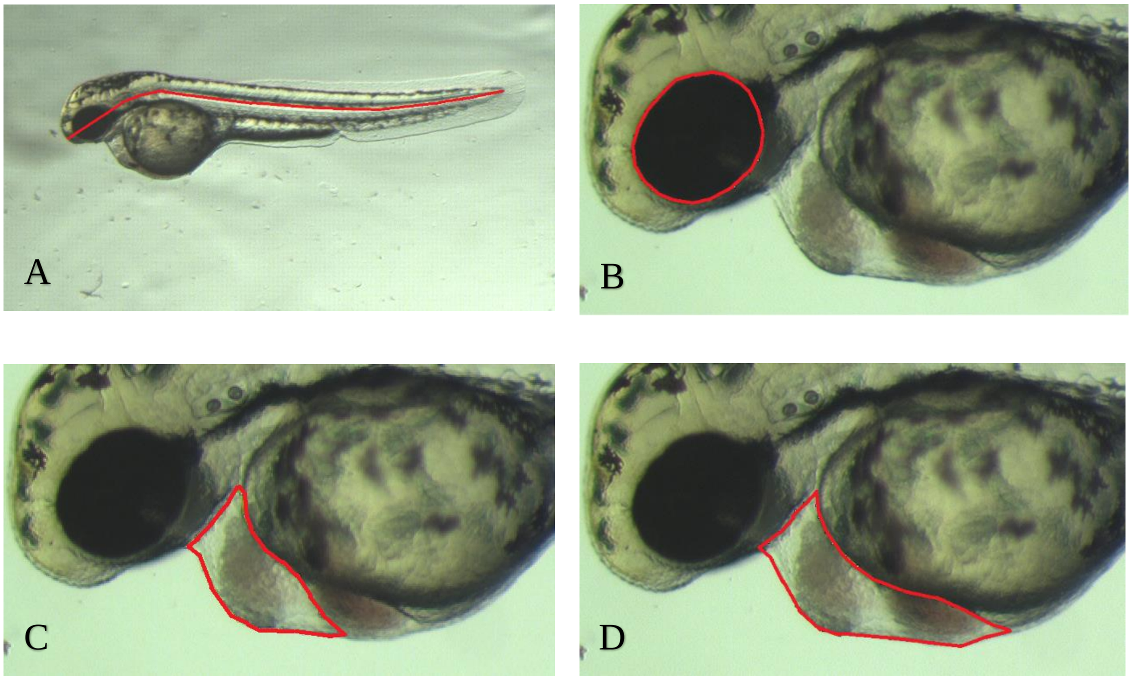


Figure 5. Zebrafish image measurements. A) Length, B) Eye Area, C) Pericardial area, and D) Periventral area.

3.2.2 STATISTICAL ANALYSIS

For the toxicological results, all statistics were done using JMP Pro 11.0.0 (SAS Institute Inc.). The statistical means of each replicate and measurement (i.e. length, eye area, pericardial area) were analyzed to compare the treatments to the control. The measurements were first tested for normality and equal variance using Shapiro-Wilk and Levene's tests respectively. None of the treatments met the assumptions of both the Shapiro-Wilk and Levene's test using a p-value of 0.05. Since the ANOVA test is considered to be robust in the analysis of environmental tests it was still used to remain consistent with previous Zebrafish toxicity tests (McIntyre et al., 2014). After the ANOVA, a Dunnett's post-hoc analysis was done to test the statistical difference compared to the control group. For the second toxicological study with the stormwater, a Dunnett's post-hoc analysis was also done with the stormwater as the control to test the differences between the stormwater and the treatments through the bioretention substrates.

3.3 RESULTS

The results for this thesis research are broken up into conventional chemistry results, metal concentrations in the leachate when uncontaminated water was treated through bioretention systems, metal concentration in stormwater before and after treatment and toxicological results. These results will be analyzed further in the discussion section.

3.3.1 CONVENTIONAL CHEMISTRY RESULTS

Alkalinity, pH, magnesium, calcium, total organic carbon (TOC) and dissolved organic carbon (DOC) were all measured in the following three tests: 1) the leachate of the 8th water flush with the Instant Ocean® Sea Salt solution, 2) the leachate after the conditioning period with the tap water, and 3) the stormwater runoff leachate.

The pH in the leachate of the 8th Instant Ocean® Sea Salt solution being treated through the bioretention columns did not change very much. Before treatment the solution was 7.39. It lowered when filtered through either of the columns, with the lowest being the leachate from the BSM column at a pH of 7.07. The pH of the BSM plus gravel and the gravel only column were both 7.28. For the conditioning treatment, the initial pH before treatment was 7.35, which was very similar to the 8th leach test. With treatment through the BSM, BSM plus gravel and gravel only column, the pH decreased to 7.19, 7.14 and 7.18 respectively. The untreated stormwater runoff had a pH of 7.1. Treatment of the stormwater runoff had an opposite effect on pH as the treatment on uncontaminated water. With treatment through the bioretention columns the pH increased to 7.35 through the BSM column, 7.61 through the BSM plus gravel column and 7.81 through the gravel only column (Figure 6).

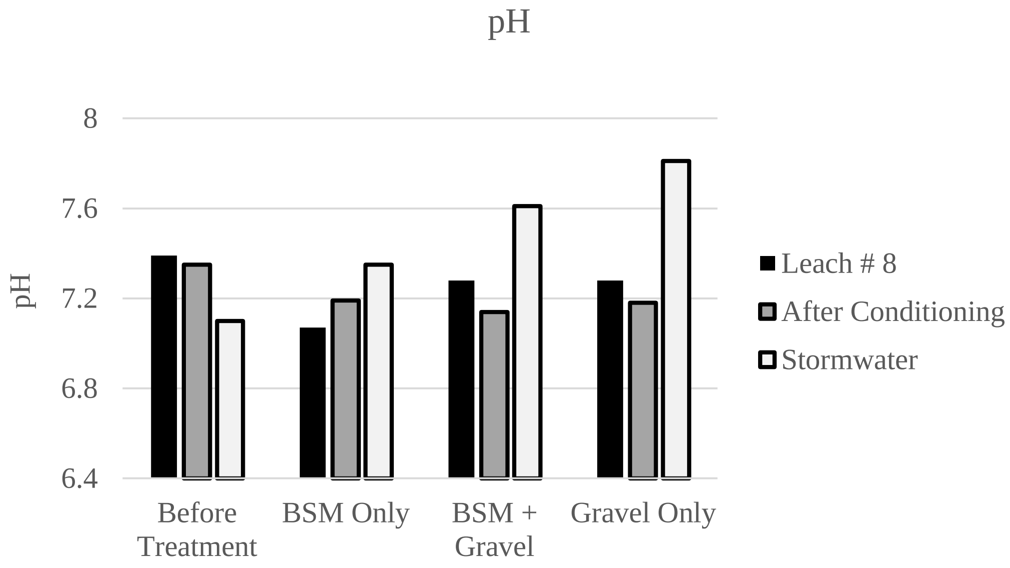


Figure 6. pH of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars are not depicted but are ± 0.01 which is the reporting limit.

The alkalinity increased with treatment through the bioretention columns for both the 8th uncontaminated water flush as well as the leachate of the uncontaminated water after the conditioning period. In both tests, it increased more in the treatment through the BSM and the BSM plus gravel columns compared to the gravel only column. The alkalinity of stormwater runoff was much higher than the uncontamianted water before treatment, at 52 mg/L $CaCO_3$, whereas the uncontamianted water for the 8th flush and the flush after conditioning was only 3.7 and 3.9 mg $CaCO_3$/L respectively. The treatment of stormwater runoff through the BSM or the BSM plus gravel decreased the alkalinity, while the treatment through the gravel increased it (Figure 7).

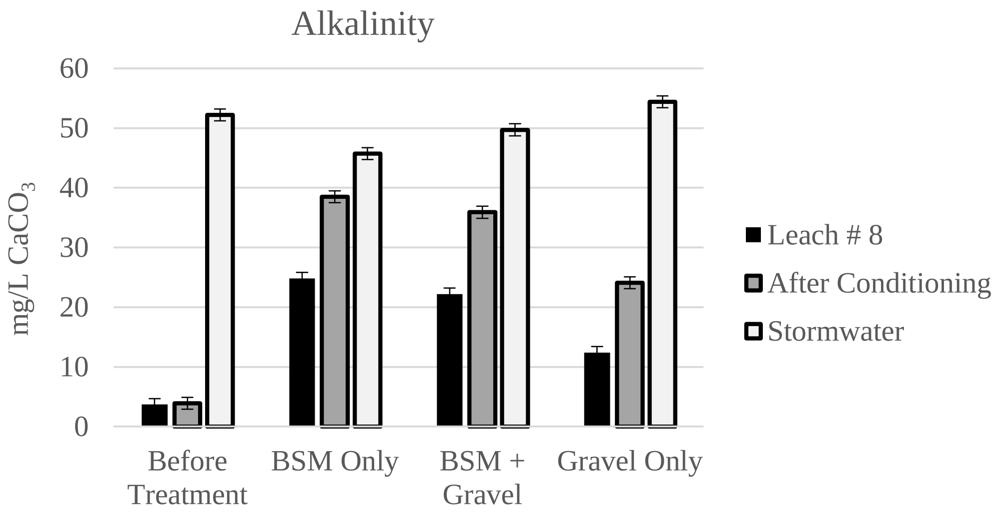


Figure 7. Alkalinity in mg/L $CaCO_3$ of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars show ± 1 mg/L $CaCO_3$ which is the reporting limit.

The initial conentration of calcium was higher in the stormwater runoff than the uncontaminated Instant Ocean® Sea Salt solution. Treatment of the 8th leachate test with uncontamianted water through the bioretention columns increased the calcium. The highest concentration was from the treatment through the gravel only column. This was the same pattern with the leachate of the test after the conditioning. The concentrations of the calcium after conditioning were much higher than the 8th leachate test. An opposite pattern was observed for the treatment of stormwater runoff. Calcium decreased with treatment of stormwater runoff with the treatment through the gravel only column being the lowest (Figure 8).

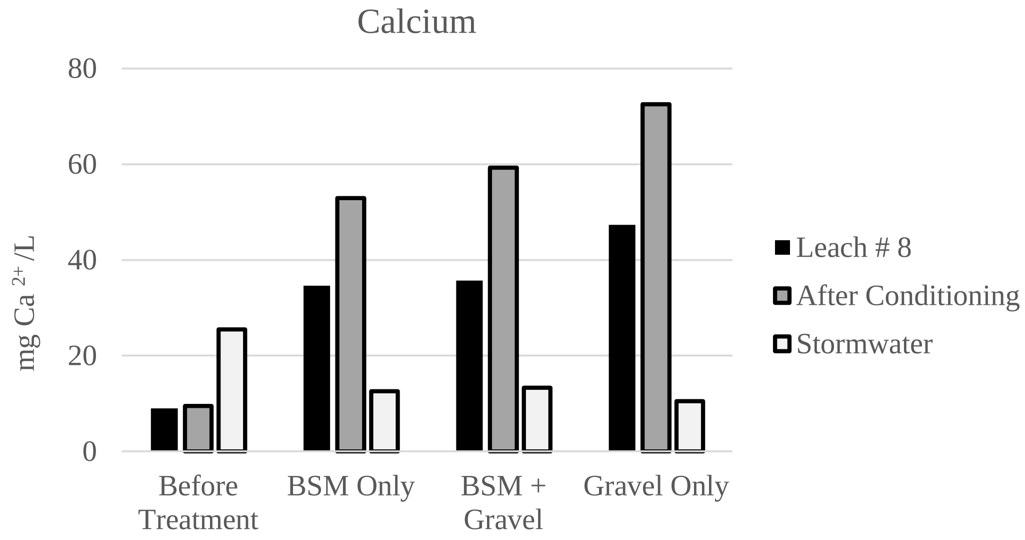


Figure 8. Calcium concentration in mg Ca^{2+}/L of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars are not depicted but are ± 0.05 mg Ca^{2+}/L which is the reporting limit.

The magnesium in the Instant Ocean® Sea Salt solution before treatment for the 8th leachate test as well as the test after the conditioning period was much higher than the untreated stormwater runoff. The 8th leachate test magnesium concentration decreased with treatment through the bioretention columns while the concentrations after the conditioning period as well as the treatment of stormwater runoff stayed relatively stable (Figure 9).

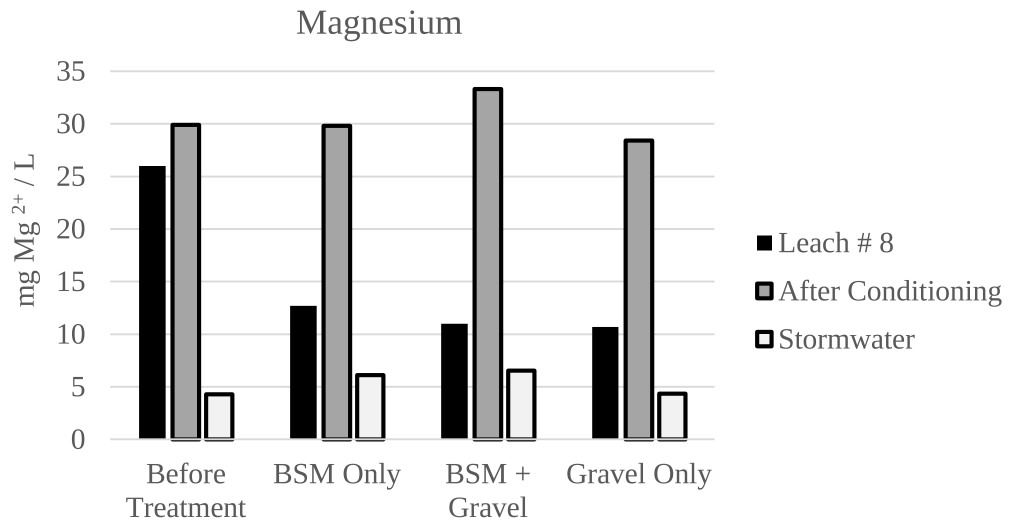


Figure 9. Magnesium concentration in mg Mg^{2+}/L of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars are not depicted but are ± 0.05 mg Mg^{2+}/L which is the reporting limit.

Initially there was a very low concentration of dissolved organic carbon (DOC) in the Instant Ocean® Sea Salt solution in the 8th leach test as well as the test after the conditioning period. With treatment during the 8th leach test through the BSM and BSM plus gravel columns this concentrations increased greatly. After the conditioning period the concentration did not change as drastically with treatment through the columns. The stormwater runoff initially had a much higher concentration of DOC comparred to the other uncontaminated water flushes. Following the same pattern as the 8th leach test, the concentrations increased with treamtent through the BSM and the BSM plus gravel columns. None of the tests showed a change in DOC with treatment through the gravel column (Figure 10).

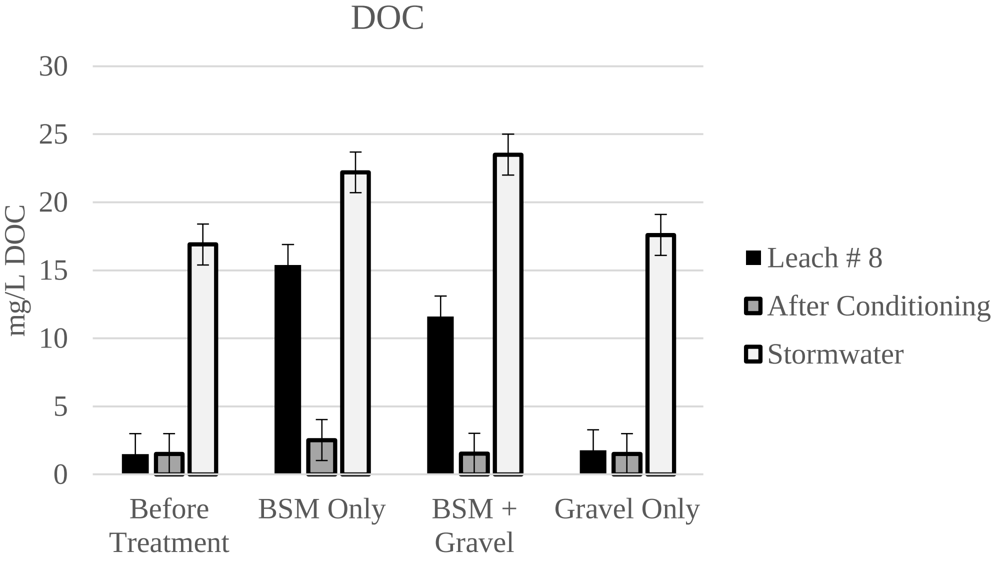


Figure 10. Dissolved organic carbon concentration in mg DOC /L of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars are ± 1.5 mg DOC/L which is the reporting limit.

In the treatment of the Instant Ocean® Sea Salt solution in the 8th leachate test there was an increase in total organic carbon (TOC) in the BSM and BSM plus gravel treatments but not the gravel alone. This was the same pattern as observed in the DOC measurements. After the conditioning period there was very low concentrations of TOC. The untreated stormwater runoff had very high concentrations of TOC. The concentrations degreased with treatment through the BSM and the BSM plus gravel columns (Figure 11).

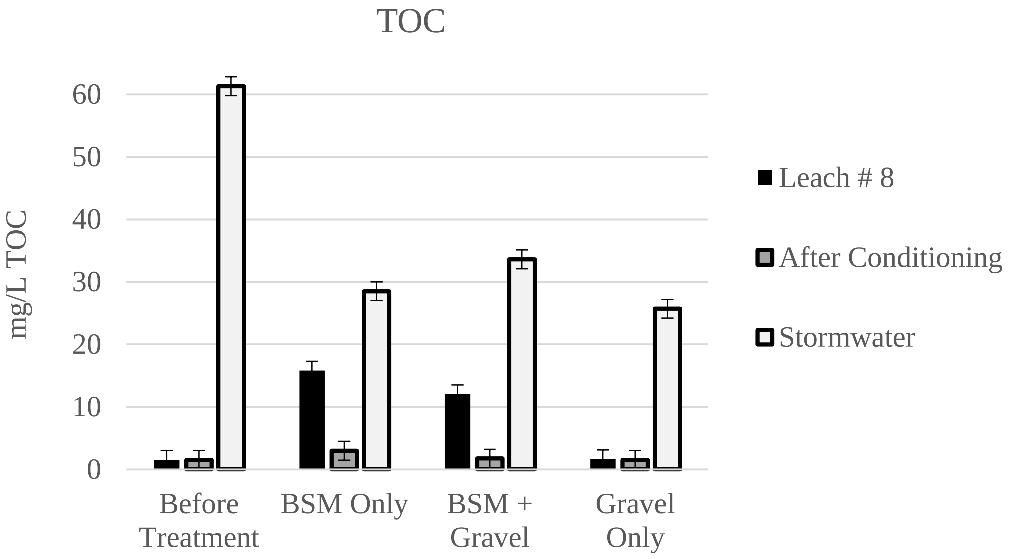


Figure 11. Total organic carbon concentration in mg TOC /L of the 8th leach test with uncontamianted water, leachate after conditioning and treatment of stormwater runoff initially and after being treated through the columns for the conventional water chemistry parameters. Error bars are ± 1.5 mg TOC/L which is the reporting limit.

3.3.2 METAL FROM UNCONTAMINATED WATER TREATMENT

In the treatment of the Instant Ocean® Sea Salt solution through the bioretention soil medium (BSM) only column there was a general trend of an increase in metal concentration in the first few flushes. The zinc and copper concentrations were the highest, peaking around 35 µg/L for zinc and 31.7 µg/L for copper. The first eight flushes were completed when 24 liters had passed through the column. The conditioning period was completed when 61 liters had been flushed through the columns. After conditioning all of the metal concentrations were under 10 µg/L which was lower than the values of the first flush through the treatment column. The values for the metal analysis as well as the detection limits are in Appendix A. A graph of the metal concentrations are shown in Figure 12. Cadmium and silver were excluded because the levels were below detection.

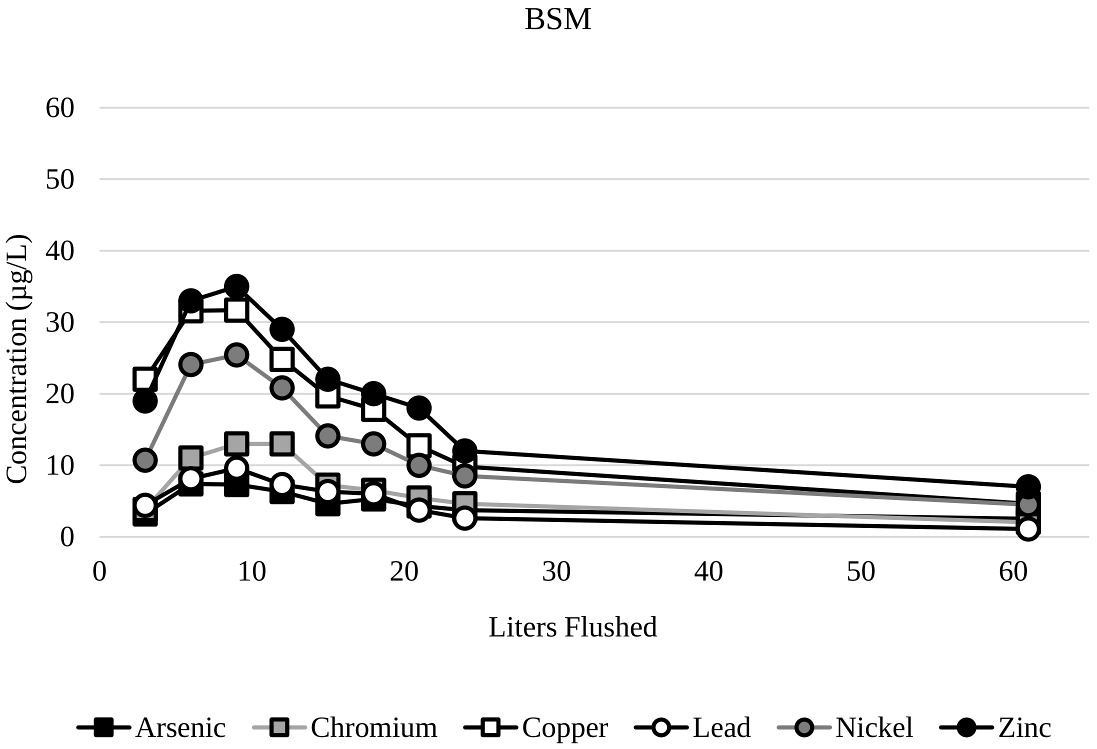


Figure 12. Metal concentrations in µg/L in the leachate of the bioretention soil medium (BSM) during the first eight flushes and the measurements after the conditioning period shown as a function of how many liters were flushed through the column.

The increase in metal concentration in the bioretention soil medium plus the gravel layer follows the same pattern as the bioretention soil medium only column. There was a general increase in the first few flush periods followed by a decline in metal concentrations. Again the zinc and copper concentrations were the highest in the leachate. Zinc peaked at 28 µg/L. The copper was much higher in the BSM plus gravel column compared to the BSM only column, peaking at 60.1 µg/L. After the conditioning period at 61 liters, all metal concentrations were under 10 µg/L just as in the BSM only column (Figure 13).

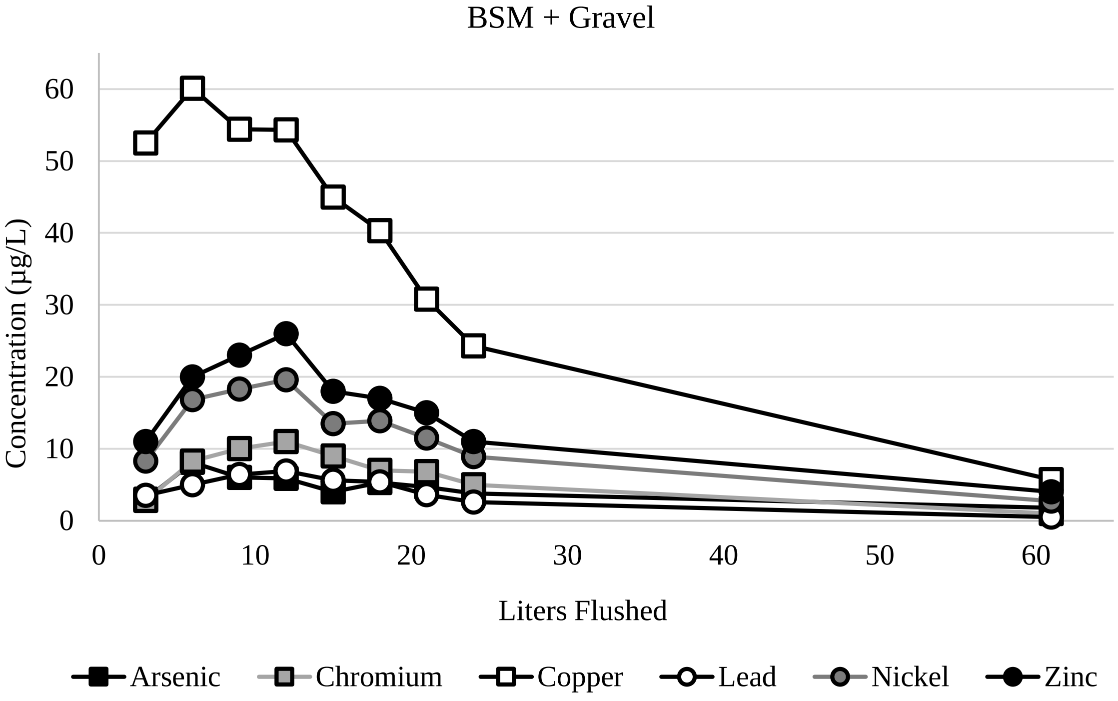


Figure 13. Metal concentrations in µg/L in the leachate of the bioretention soil medium (BSM) plus the gravel layer during the first eight flushes and the measurements after the conditioning period shown as a function of how many liters were flushed through the column.

The gravel layer treatment did not result in an overall increase in metal concentrations of the leachate, however it is important to note that the concentrations of arsenic, copper and chromium all increased as more water was flushed through and then decreased in the end of the conditioning. The arsenic concentration at the end of the conditioning period was higher than the first flush for the gravel layer and lower than the first flush for the BSM and BSM plus gravel treatments. For the chromium, copper, lead, nickel and zinc, the metal concnetrations at the end of conditioning were lower than the leachate of the first flush for all treatments. The gravel alone layer produced little above detection for lead and nickel and was below detection for zinc. The metal leaching from the gravel column was never over 10 µg/L (Figure 14).

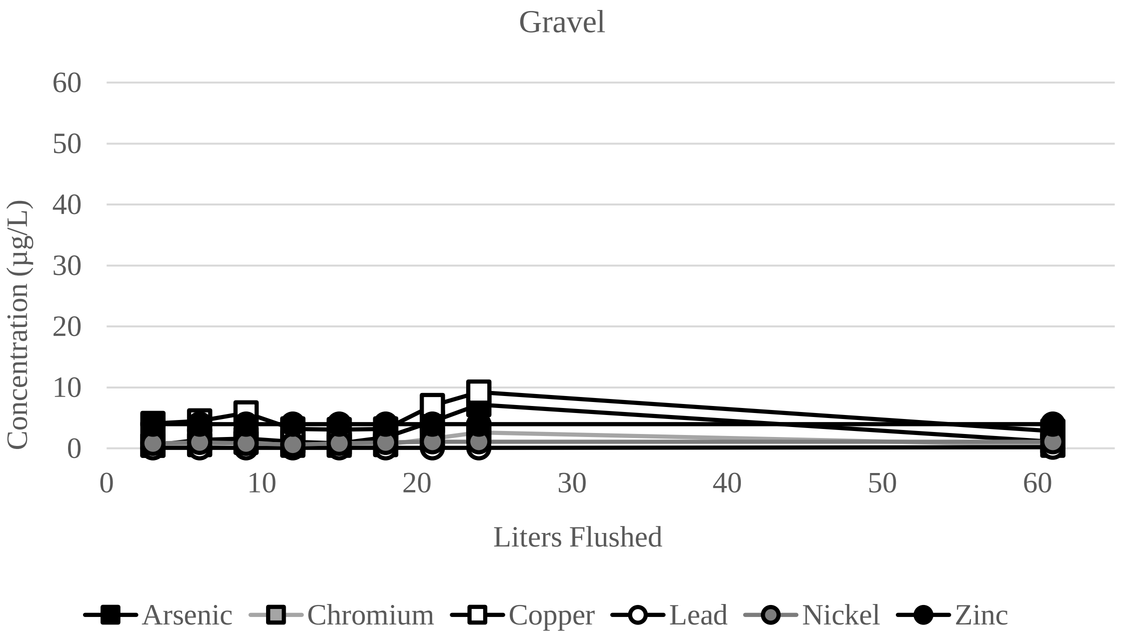


Figure 14. Metal concentrations in µg/L in the leachate of the gravel only during the first eight flushes and the measurements after the conditioning period shown as a function of how many liters were flushed through the column.

3.3.3 METALS FROM TREATING STORMWATER RUNOFF

The concentrations of metals in the untreated stormwater runoff were the highest for zinc and copper with 420 µg/L and 107 µg/L respectively. The levels for each metal in the untreated stormwater runoff are shown in Figure 14. When stormwater runoff was passed though the different treatment columns most had a reduction in concentration. Arsenic was the only metal that increased as it was treated and this was only observed in the BSM plus gravel treatment column. The arsenic, chromium and nickel concentrations were lowest in the gravel leachate and highest in the BSM plus gravel leachate. The copper, lead and zinc were all lowest in the BSM leachate indicating the retention of the highest concentration in the soil mixture (Figure 15).

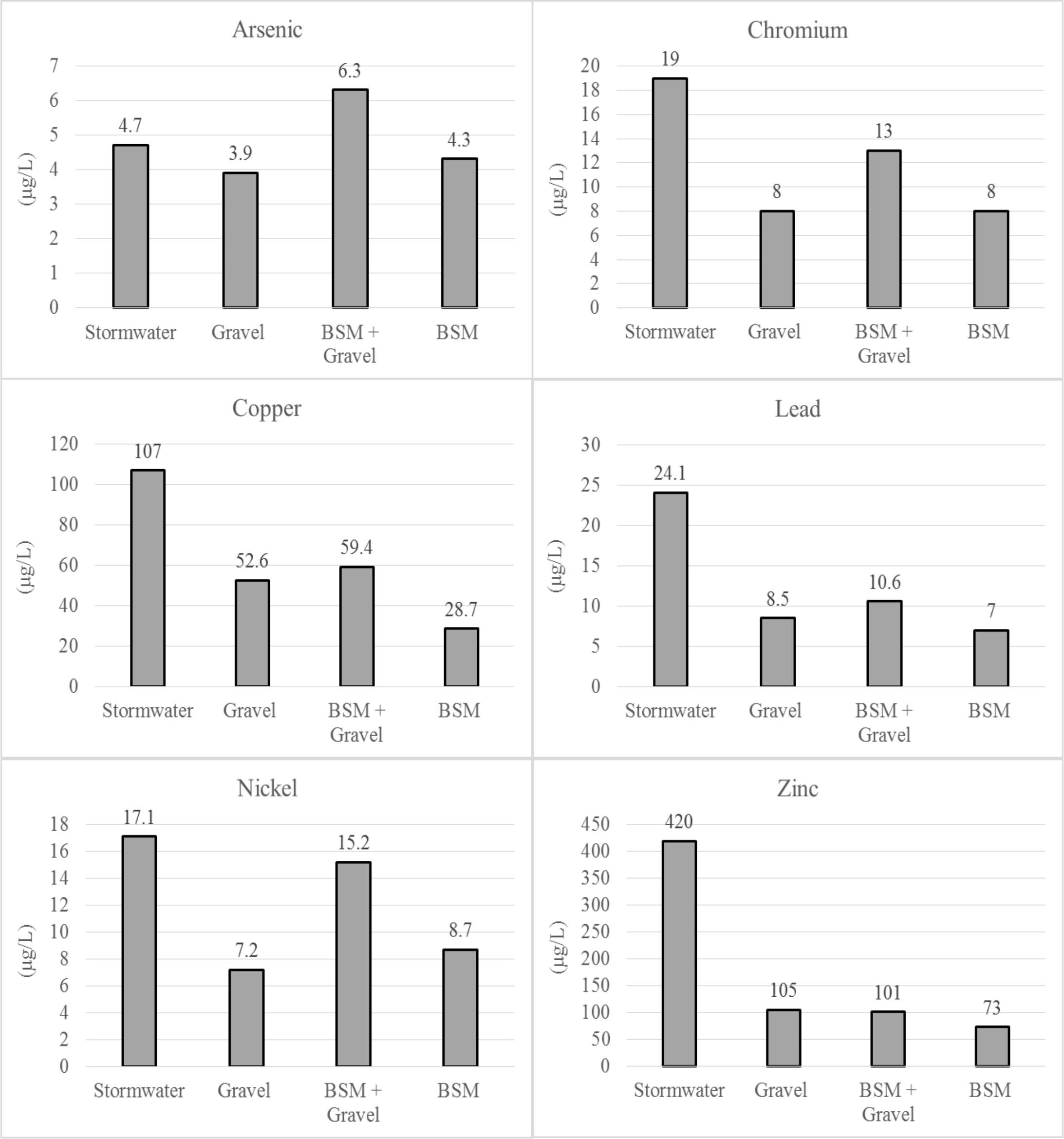


Figure 15. Metal concentrations in µg/L of the leachate of stormwater runoff treated through the different experimental columns. Stormwater indicates untreated stormwater runoff. Values are in µg/L. Error is ± the reporting limit. For arsenic this is 0.2 µg/L, for chromium it is 0.5 µg/L, for copper it is 0.5 µg/L, for lead it is 0.1 µg/Lm for nickel it is 0.5 µg/L and for zinc it is 4 µg/L.

3.3.4 TOXICOLOGICAL RESULTS- UNCONTAMINATED WATER

The second, fourth and eighth leachates of uncontaminated water through the BSM plus gravel layer were used for toxicity analysis. The zebrafish tested did not show a significant difference from the control in any of the parameters measured with a p value of 0.05 (Table 5). The measurements for length, eye area, periventral area, pericardial area and heart rate are shown in Figures 16-20.

Table 5. Results from one-way ANOVA for leachate of uncontaminated water passing through different treatment columns for measurements of length, eye area, periventral area (PVA), pericardial area (PCA) and heart rate. F ratios for comparison between averages of replicates and different columns. No values were statistically significant ($p < 0.05$).

	Length	**Eye Area**	**PVA**	**PCA**	**Heart Rate**
F ratio	0.92	2.6949	0.707	0.6617	1.0503
P Value	0.4738	0.1166	0.5743	0.5984	0.4219

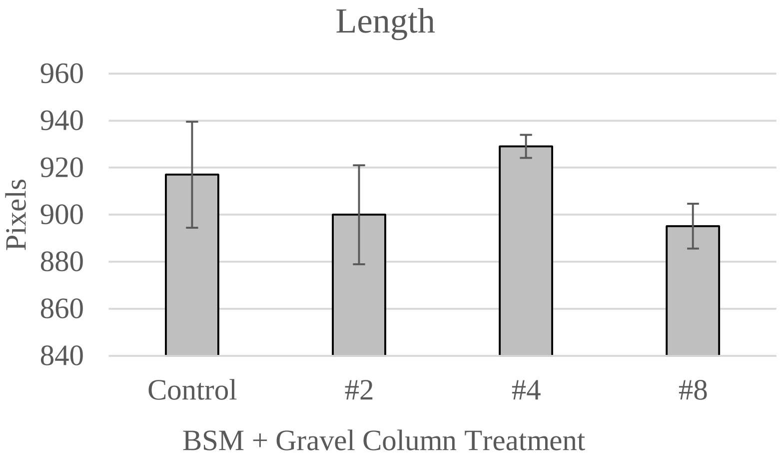


Figure 16. Zebrafish lengh in pixels measured at 48 hours. Zebrafish embryos exposed to Instant Ocean® Sea Salt solution (control) treated through bioretention soil medium plus gravel for the 2nd, 4th and 8th leach test. Results shown with ± 1 Standard Error.

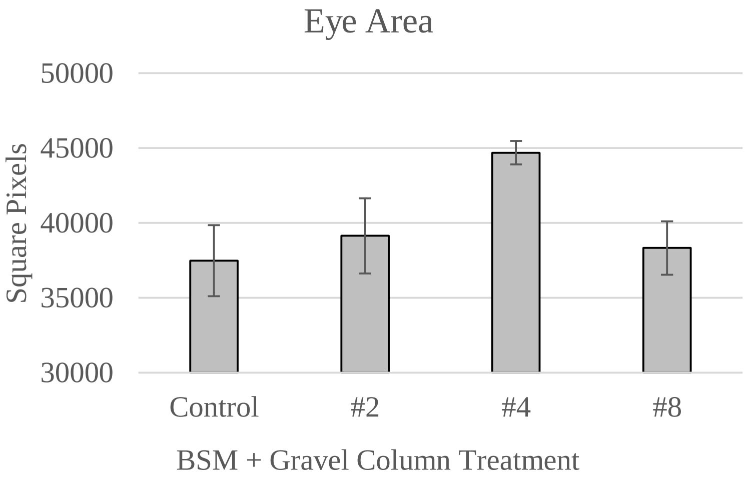


Figure 17. Zebrafish eye area in square pixels measured at 48 hours. Zebrafish embryos exposed to Instant Ocean® Sea Salt solution (control) treated through bioretention soil medium plus gravel for the 2nd, 4th and 8th leach test. Results shown with ± 1 Standard Error.

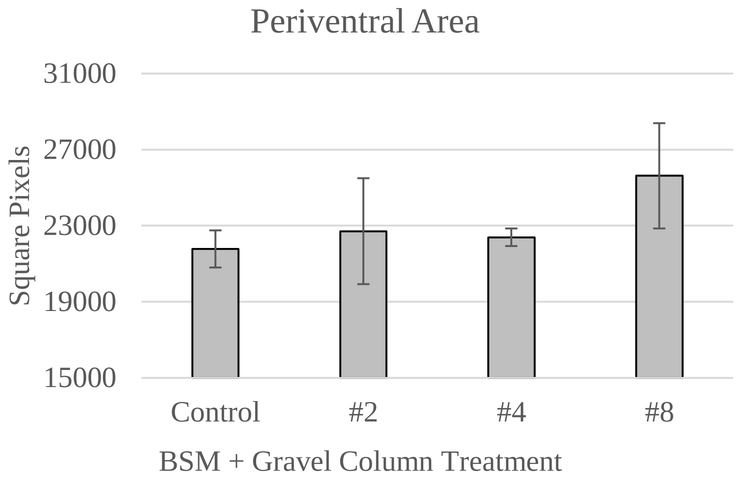


Figure 18. Zebrafish periventral area in square pixels measured at 48 hours. Zebrafish embryos exposed to Instant Ocean® Sea Salt solution (control) treated through bioretention soil medium plus gravel for the 2nd, 4th and 8th leach test. Results shown with ± 1 Standard Error.

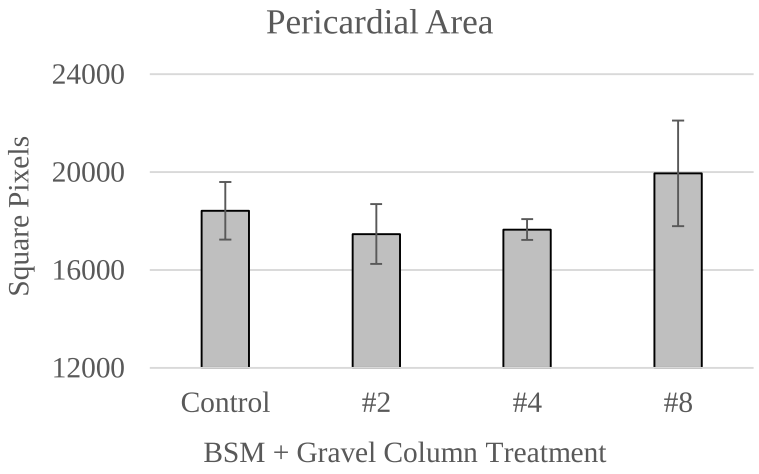


Figure 19. Zebrafish pericardial area in square pixels measured at 48 hours. Zebrafish embryos exposed to Instant Ocean® Sea Salt solution (control) treated through bioretention soil medium plus gravel for the 2nd, 4th and 8th leach test. Results shown with ± 1 Standard Error.

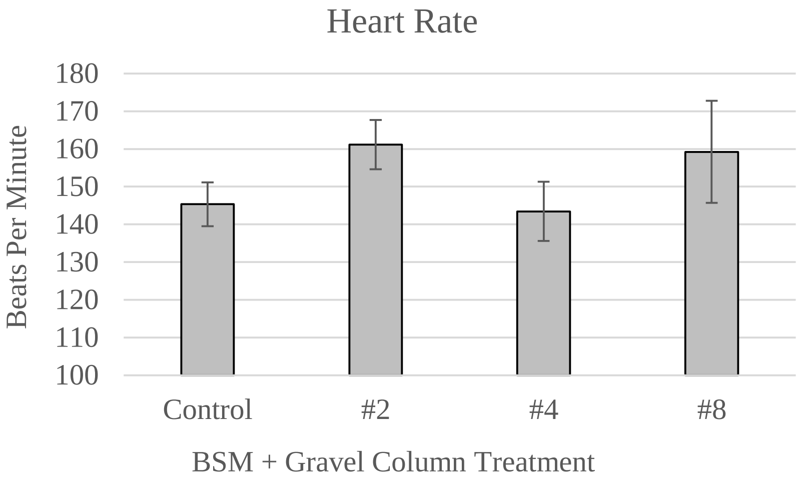


Figure 20. Zebrafish heart rate in beats per minute measured at 48 hours. Zebrafish embryos exposed to Instant Ocean® Sea Salt solution (control) treated through bioretention soil medium plus gravel for the 2nd, 4th and 8th leach test. Results shown with ± 1 Standard Error.

3.3.5 TOXICOLOGICAL RESULTS- STORMWATER RUNOFF

For the stormwater runoff that was collected, the leachate from each treatment columns was used for toxicity testing, as well as the untreated stormwater itself. The results of the one way ANOVA are in Table 6. There was no significant difference in the length, eye area or heart rate of the zebrafish when exposed to stormwater or treated stormwater. (Figure 21, 22 and 25). For the eye size, while the overall test did not show significance, the results of the Dunnett's post hoc analysis did show that the untreated stormwater runoff had significantly smaller eyes than the control water (P=0.0197) (Figure 22). The untreated stormwater runoff and the treatment with only the gravel layer had a significantly larger periventral area compared to the control. This was based on the Dunnett's post hoc analysis comparing to the control with a p value of 0.0199 for the untreated stormwater runoff and a p value of 0.0237 for the gravel layer compared to the control (Figure 23). There was no significant difference in the pericardial area of the different treatments when compared to the control (Figure 24).

Table 6. Results from one-way ANOVA for leachate of stormwater runoff passing through different treatment columns for measurements of length, eye area, periventral area (PVA), pericardial area (PCA) and heart rate. F ratios for comparison between averages of replicates and different columns. Values in bold indicate statistical significance ($p < 0.05$).

	Length	**Eye Area**	**PVA**	**PCA**	**Heart Rate**
F ratio	0.5282	3.3382	4.6721	4.8256	1.4797
P Value	0.718	0.0556	**0.0219**	**0.0199**	0.2967

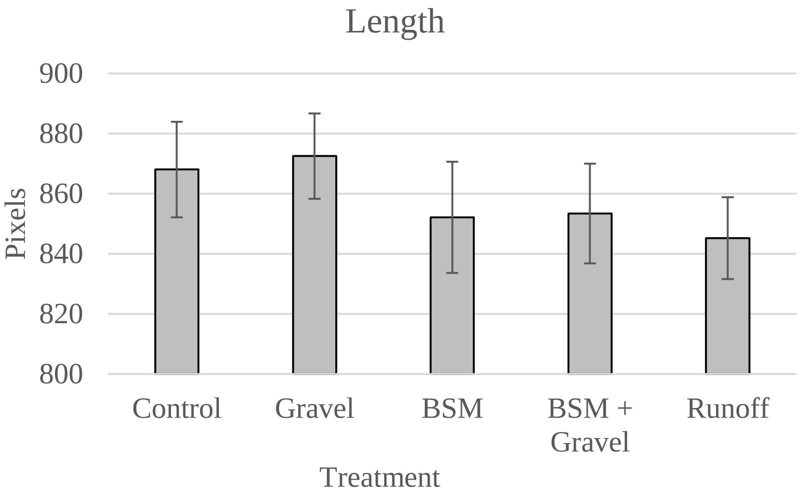


Figure 21. Zebrafish length in pixels measured at 48 hours. Zebrafish embryos exposed to untreated stormwater runoff (runoff) treated through the different bioretention substrate columns. Instant Ocean® Sea Salt solution was used for the control. Results shown with ± 1 Standard Error.

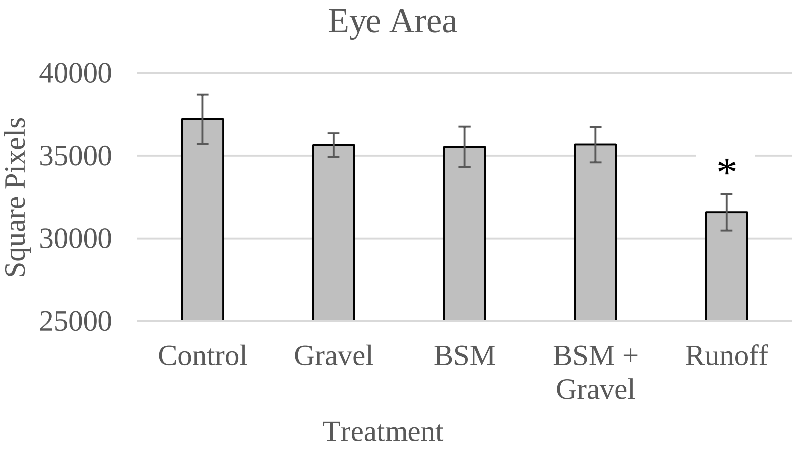


Figure 22. Zebrafish eye area in square pixels measured at 48 hours. Zebrafish embryos exposed to untreated stormwater runoff (runoff) treated through the different bioretention substrate columns. Instant Ocean® Sea Salt solution was used for the control. Results shown with ± 1 Standard Error. The * indicates significance ($p<0.05$) from the results of the Dunnett's post hoc analysis comparing each mean to the control.

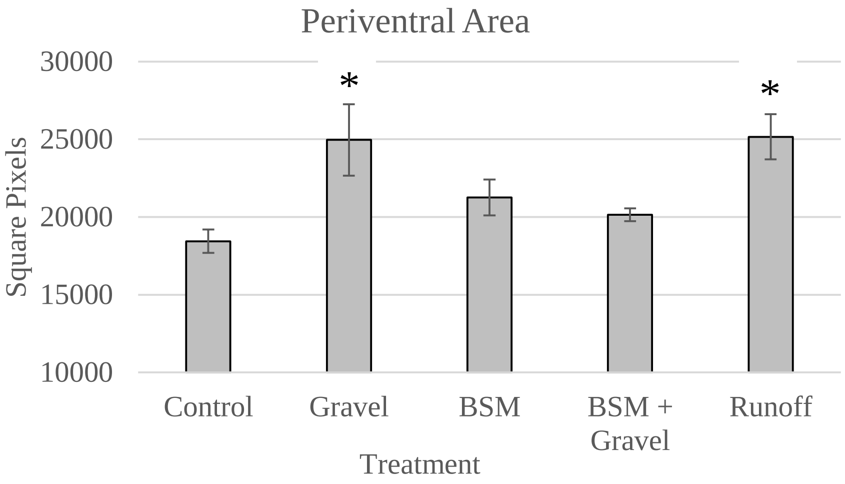


Figure 23. Zebrafish periventral area in square pixels measured at 48 hours. Zebrafish embryos exposed to untreated stormwater runoff (runoff) treated through the different bioretention substrate columns. Instant Ocean® Sea Salt solution was used for the control. Results shown with ± 1 Standard Error. The * indicates significance ($p<0.05$) from the results of the Dunnett's post hoc analysis comparing each mean to the control.

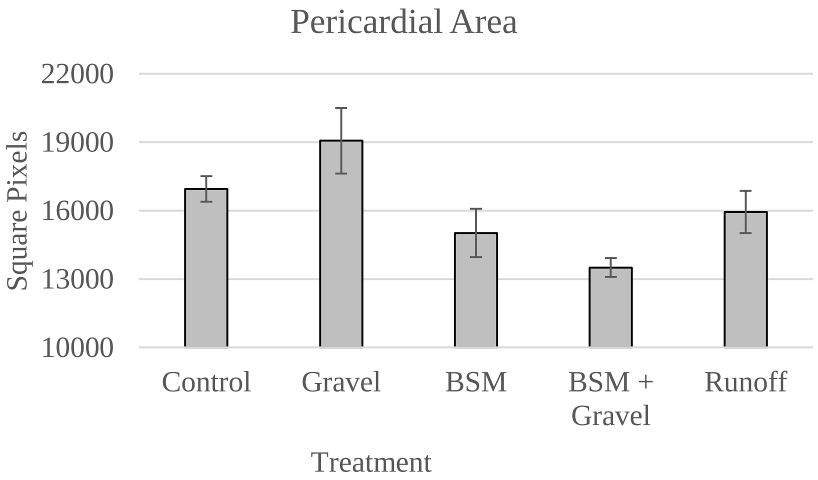


Figure 24. Zebrafish pericardial area in square pixels measured at 48 hours. Zebrafish embryos exposed to untreated stormwater runoff (runoff) treated through the different bioretention substrate columns. Instant Ocean® Sea Salt solution was used for the control. Results shown with ± 1 Standard Error.

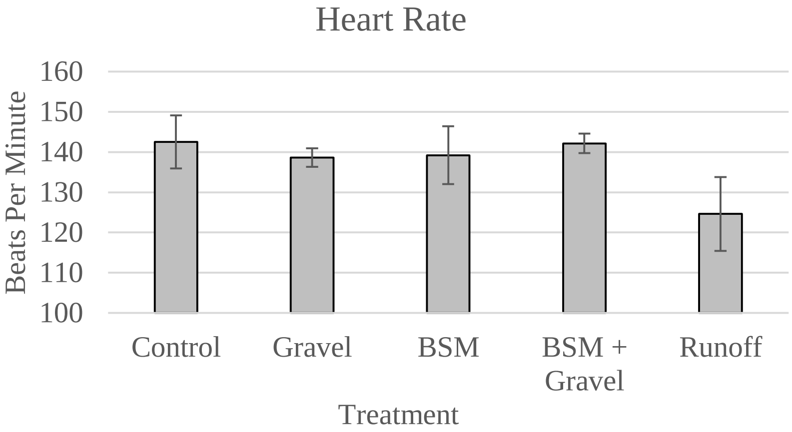


Figure 25. Zebrafish heart rate in beats per minute measured at 48 hours. Zebrafish embryos exposed to untreated stormwater runoff (runoff) treated through the different bioretention substrate columns. Instant Ocean® Sea Salt solution was used for the control. Results shown with ± 1 Standard Error.

The increased periventral area in this study was correlated with the presence of blood pooling under the yolk sac of the zebrafish. This was not a measurement that was taken in the analysis, so the reporting of this is only looking at observational trends. There was no statistics done on this and there was not a complete count. The untreated stormwater runoff had a high percentage of zebrafish which appeared to have blood pooling under the yolk sacs (Figure 26A). When treated through the gravel column, the phenotypic response was still present (Figure 26B). Treatment through the BSM column and the BSM plus gravel column did not result in as many zebrafish with blood pooling (Figure C and D).

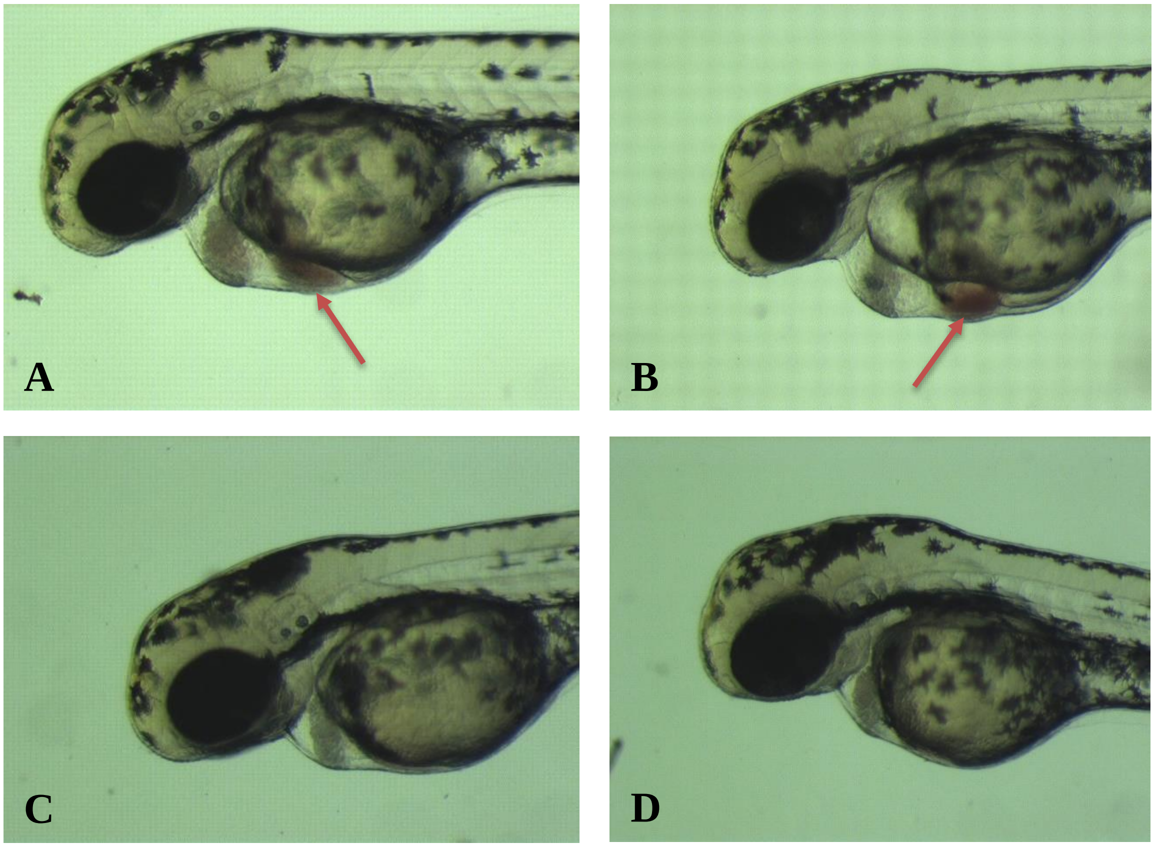


Figure 26. Representative images of zebrafish of each treatment group used in the stormwater leaching toxicity analysis. Images taken at 48 hours post fertilization. A) Untreated stormwater runoff, B) Stormwater runoff treated through the gravel column, C) Stormwater runoff treated through the BSM plus gravel column, and D) Stormwater runoff treated through BSM column. Arrows point to blood pooling under the yolk sac.

4. DISCUSSION

This thesis was designed to analyze the potential for bioretention systems to leach enough metals to cause an ecological impact. It was first found that the soils used in the bioretention systems in this study leached very high concentrations of metals. This indicated that soils can be a source of metal pollution. With conditioning of the soils, by flushing uncontaminated water through them, the concentrations were reduced. To address the ecological impact, toxicity tests were performed. The results of the toxicity tests show that the bioretention systems themselves do not cause an ecological impact, within the parameters of this experiment.

4.1 LEACHING OF METALS

The bioretention systems were flushed with eight treatments of uncontaminated water before the conditioning portion of the experiment. After the conditioning with uncontaminated water, stormwater runoff was flushed through the columns. The metal concentrations in each step of this process with will be analyzed in greater detail in order to get a better understanding of the processes which were taking place within the soils.

4.1.1 UNCONTAMINATED WATER

Both of the columns containing bioretention soil medium (BSM and BSM plus gravel) had an initial increase in metal concentration. It was predicted that the first flush would have the highest metal concentrations. In this experiment the highest metal concentrations were found between the second and fourth flush treatments. Conventional water chemistry measurements (alkalinity, pH, DOC, etc.) were not taken until the eighth water flush, so it is not definite what caused this pattern.

The cation exchange capacity (CEC) can be used to potentially explain the affinity of cations to soils. By simple definition, the numeric value of the cation exchange capacity of a soil is the concentration of negatively charged sites present in the soils and available to adsorb exchangeable cations (Sikora & Moore, 2014). The higher the CEC, the more cations the soil can adsorb. As more cations are added, the soil becomes less negative and the CEC goes down, making the soils less available to bind with the inflow of cations (Sikora & Moore, 2014). At this point of saturation, the cations begin to exchange on the binding sites based on affinity. In this thesis research, it appears that the soils were not fully saturated with cations at the beginning, resulting in a lower leaching of metals in the first few flushes. The relative affinity for the cations in average soil outlined in the cation exchange capacity is in the order as follows:

$Al^{3+} > H^{+} > Ca^{2+} > Mg^{2+} > K^{+} = NH_4^{+} > Na^{+}$ (Chapin, Matson, & Vitousek, 2011).

Since our inflow water was slightly basic and did not contain much calcium (Figure 6 and 8), there may not have been much exchange initially. In this research the inflow water had elevated concentrations of magnesium, between 25 and 30 mg/L (Figure 9). Based on the cation exchange capacity, the soils would have retained this magnesium. The cation exchange capacity (CEC) further explains the soil’s affinity for metal ions. The soils could have contained high levels of metals from the beginning, most likely from the compost, based on the compost regulations outlined in the methods section (Seelsaen et al., 2007). The Irving-Williams series compares heavy metal affinity, some of the metals are not always found in soils.

Based on the Irving-Williams series, the soils have a stronger affinity for the following heavy metals, listed in order of affinity:

$Pb^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+}$ (Seelsaen et al., 2007)

All of the heavy metals in that sequence have a higher affinity than magnesium. By the eight water flush, the bioretention soils began to have a retention of magnesium (Figure 9). This is also when the leaching of the metals was far less than at the peak. This indicates that the soils were no longer saturated with metals.

As predicted, the results of the conditioning portion of the experiment show a decline in metals as more water is passed through. The water used to flush was consistent through the first eight flushes; there was no substantial difference in mineral input or pH. The conditioning period was performed with tap water that was not tested for conventional chemistry parameters; however the flush after the conditioning period was performed with the same Instant Ocean® Sea Salt solution as the first eight flushes. Overall, the flushes appeared to rinse the columns out. The concentrations of metals after the conditioning of the BSM and the BSM plus soil column were all under 10 µg/L but appeared to be still declining (Figure 12 and 13). Other research could be used to determine if the concentrations could have become lower still by extending the conditioning period longer. As the declining slowed, it could be assumed that the soils had available negative charge to then begin retaining metals, as seen in with the application of stormwater.

The cation exchange capacity of soils and the ability for sorption of metals is highly dependent on pH (Lanno, 1998). The water used to flush the columns was slightly basic. The hydroxides added into the soil could have formed complexes with the organic matter resulting in the release of organic matter and hydrogen ions (Lanno, 1998). The hydroxides would, in turn, react with the metal already present in the soil to form hydrolyzed metal complexes in the forms of $MeOH^+$ where the Me would indicate a metal ion (Lanno, 1998). In addition to the DOC that would have been leached by just rinsing water through, this could have increased it. This in part explains why initially the columns were leaching out organic matter (Figures 10 and 11), and why the pH was decreased in the leach compared to the inflow water (Figure 6).

Based on this limited information from this research and the soil properties outlined with the cation exchange capacity it can be concluded that the soils retained magnesium and hydrogen ions in the first few flushes and, after a while, became over-saturated with cations (Lanno, 1998). This could have altered the soil chemistry and changed the CEC of the soil (Sikora & Moore, 2014). Eventually the over-saturation would lead into a leaching of cations, which would be the metals already present in the soils (Sikora & Moore, 2014).

Another potential explanation has to do with the rate of inflow of the water. After the initial flush of water the columns could have been more compressed from the water addition. Even though the columns were allowed to dry, the compression would have remained. The compact soils would have slowed the flush down, allowing for more metals to be removed from the substrates because the residence time in the water column would have been longer, however this research did not take the appropriate measurements to confirm this theory. These are just a few potential explanations. To study this in greater detail, soil samples could have been taken and analyzed for metals as well as water chemistry parameters could have been taken with each flush, rather than at the end of the experiment.

4.1.2 STORMWATER RUNOFF

Based on this principle, by the end of the conditioning period, the CEC would have been higher than the initial CEC, which could explain why metals were retained in the soils when the stormwater was flushed through (Figure 15). When stormwater runoff was flushed through these pre-conditioned columns the metal concentrations in the leachate were drastically reduced from the untreated stormwater measurements. Since soil samples were never analyzed, it is only predicted that the conditioning of soils resulted in much lower concentrations of metals than at the start of the experiment. This means that there would have been more negative sites available in the soils and a higher CEC. At that point, the capacity of the soils to retain metal ions would have been much higher. The soil columns then retained metals from the stormwater runoff. This response supports previous research and the over goal of stormwater bioretention structures.

McIntyre et al. found that the same bioretention soil medium with the gravel layer reduced zinc by 99%, copper by 72%, nickel by 31%, lead by 91% and cadmium by 95% (2014). This thesis research found that the same BSM plus gravel column resulted in a reduction in zinc by 75%, copper by 44%, nickel by 11%, lead by 56% and cadmium by 33% in the treated stormwater runoff. The study by McIntyre et al. (2014) did not precondition the soils and had a higher reduction in metal concentrations. The stormwater runoff used by McIntyre at al. (2014) was similar to this thesis research with an average pH of 6.9, compared to this research with a pH of 7.1. As far as the soil columns go, conditioning of the soils also reduces the total organic carbon in the soils (Figure 11), since McIntyre et al. (2014) did not condition the soils, if can be assumed that the soils would have had a higher organic matter content. The stormwater used in that study also contained much higher DOC ranging from 25 to 400 mg/L, whereas this thesis research used stormwater with a DOC of 17 mg/L (Figure 10). This could have bound to more metals passing through (as explained above), which would have supported the findings of a higher reduction in metal concentrations.

A study on a summary of multiple low impact development approaches as described in the literature review of this thesis, found a 76% reduction in zinc, a 83% reduction in copper and a 90% reduction in zinc (EPA, 2012). Other research on bioretention systems found a reduction in zinc of 88%, 93% for copper and 97% for zinc (Seelsaen et al., 2007). Neither of those two studies reported the pH or DOC so it is difficult to compare what could have cause the difference in the results. None of these studies used pre-conditioned soils and they all used stormwater which was more contaminated than this thesis study (McIntyre et al., 2014)(EPA, 2012)(Seelsaen et al., 2007).

4.2 TOXICOLOGICAL EFFECTS

In the treatment of the stormwater runoff, an increased periventral area in the untreated stormwater runoff as well as in the stormwater that was passed through the gravel layer was measured. This effect was not observed in the BSM or the BSM plus gravel treatments. This indicated that the BSM is the substrate responsible for reducing that toxic effect by decreasing contamination concentrations.

For the toxicity tests, the leachate from the uncontaminated water for the soil plus gravel column was tested. Zebrafish were exposed to the second, fourth and eighth leach. Out of these three samples, the highest metal concentrations were found in the second and fourth leach, with copper concentrations over 60 µg/L in the second leach and zinc concentrations at 26 µg/L in the fourth leach. There was no toxic effect from this leachate in the parameters measured. There was a toxic response in the untreated stormwater runoff and the treated stormwater through the gravel layer. In the untreated stormwater runoff, the copper was 107 µg/L and the zinc was 420 µg/L. In the stormwater treated through the gravel column the copper was 52.6 µg/L and the zinc was 105 µg/L.

The only metal that is higher in the treated stormwater compared to the treated uncontaminated water is zinc (Figure 15). The zinc concentration in the stormwater treated through the soil plus gravel column was 101 µg/L. This is very close to the zinc concentration in the stormwater passing through the gravel treatment. There are three possible explanations for what was causing the toxic effects observed. The first explanation is that the zinc was causing the toxic effect and other water chemistry parameters present (i.e., organic matter) in the soil leachate were providing a protection against the zinc toxicity, as outlined in the biotic ligand model (Rand, 1995). The second is that other contaminants, such as PAHs were leaching through the columns and causing the toxicity, this would have been removed with the BSM. The third, and most plausible possible explanation is that it was a mixture of both of these that caused a toxic response in the zebrafish.

The toxicity of metals is highly dependent on water chemistry (Bergman & Dorward-King, 1996). In freshwater fish this concept is known as the biotic ligand model (Rand, 1995). The premise behind the biotic ligand model is that the fish has a toxic site of action, known as the biotic ligand, this is usually the ion transport channels. Free metal ion in the water can bind with the biotic ligand and block essential cation exchange (Rand, 1995). Certain water paraemters effect this action, such as the concentration of dissolved organic carbon (DOC), the pH and other cations present in the water (Rand, 1995). The biotic ligand model shows that metal toxicity can be predicted with freshwater fish by measuring the metal ions, the other cations, such as calcium, magnusium and pH, and the dissolved organic carbon. High metal ions would indicate a greater chance of a toxic effects, but when there is also a high DOC or high competing cations in the solution, the toxic effect can be mitigated (Rand, 1995).

Dissolved organic carbon (DOC) in the water can bind with and form complexes with metal ions. Since metal in the ionic form is the most toxic due to the binding potential on the organisms, this makes the metals less bioavailable to an organism and therefor less toxic (Rand, 1995). This is all also affected by pH, since that is basically a presence of either hydroxide compounds or hydrogen ions (Rand, 1995). The presence of hydroxides can also bind with metals and form complexes in a basic solution. In an acidic solution there is the the presence of hydrogen ions, which are positively changed, just like the metal ions (Bergman & Dorward-King, 1996). The hydrogen binds on the biotic ligand and, in a sense neatralizes it, so that site is no longer availabel for the metals to bind to. The same action is observed with the presence of calcium and magnesium in the water. They act as competition for binding sites to an organisms.

In this research, the results of the conventional chemistry show that metals were highest in the treatments that did show a toxic effect. The pH increased with treatment of the stormwater through the bioretention columns while it decreased when the uncontamianted water was flushed though the treatment columns (Figure 6). A decreased pH would indicate more hydrogen ions, which would provide toxicity protection (Rand, 1995). The calcium and magnesium concentrations were both higher in the treated uncontaminated water than in the treated stormwater, this would also provide for protection against toxicity (Rand, 1995). For the stormwater, the calcium decreased upon treatment and the magnesium increased but only slightly (Figures 8 and 9). The DOC in the treatment of stormwater runoff was higher in the treatments containing soils leaching 22-23 mg/L dissolved carbon. This was lower than the observed DOC in the stormwater treated through the gravel layer, which had a DOC of 17.6 mg/L. This supports the toxicity observed based on the biotic ligand model and the higher DOC forming complexes with the metal ions (Rand, 1995).

Looking only at metal concentration in the leachate of the uncontamianted water passing through the columns, a toxic response would have been expected (Linbo, Baldwin, McIntyre, & Scholz, 2009). Because of the high levels of calcium, magnesium and DOC this effect could have been reduced. A toxic effect in the untreated stormwater was measured as small eye size. This was not observed in the leachate from the uncontamianted water even though the metal concentrations were very high (Figure 22). This was also not observed in any of the treatments of the stormwater runoff. This indicated that the treatement was sucessful in reducing that toxic effect. Based on the biotic ligand model, the zinc and copper ions present could have been responsible for the toxicity in the stormwater and the stormwater treated through the gravel column, but the differences were not that drastic so further research would be needed to make this conclusion. There is more than just metals in stormwater pollution. Since the only leachate which showed a toxic response was with stormwater runoff, the investigation of other pollutants is needed.

This research only measured metals. If metals were responsible for the toxicity, it would also be predicted that the other parameters such as length and hatch rate would have been effected (Linbo et al., 2009). These measurements were not significantly different than the control. The increased periventral area is an indication of blood pooling outside the heart chamber and would not be a typical response due to metal exposure (Incardona et al., 2004). This response is more indicative of exposure to an organic compound such as a PAH. Stormwater collected in the same locations from 2011 to 2012 contained total PAH concentrations ranging from 4 to 10 µg/L and was shown to be toxic to zebrafish (McIntyre et al., 2014). The samples from McIntyre et al. (2014) also had elevated metals, so the cause of the toxicity can only predicted based on comparison with the toxic response to the concentrations measured. In this thesis research, it is also plausible that the PAHs were attributing to at least some of the toxicity. In order to be positive, further research would be needed which would include measurements of how the soil treatments affect PAH measurements. The toxicity observed in the untreated stormwater and the leachate from the gravel column was most likely due to a combination of both metals as well as other contaminants present in the stormwater.

4.3 OTHER RESEARCH

A sample of the leachate of the uncontaminated water being treated through the BSM and the BSM and gravel column was used by the researchers at the WSU Puyallup GSI Facility for a qPCR (quantitative polymerase chain reaction) (McIntryre et al., unpublished). This is a test on zebrafish to analyze gene expression of cardiac injury genes and detoxification genes when exposed to contaminants. The leachate was used in the toxicity tests measuring cardiac injury genes as previously discussed. This was done specifically to focus on what was causing upregulation of cardiac injury genes in their original study. If the toxicity was due to the bioretention systems, than it would be expected that the toxicological effects could be observed in the samples of the uncontaminated water flushed through the bioretention soils. However, if the PAH's or other contaminants from the stormwater was the reason for the observed response in the previous studies, than this would not be observed with the substrate leachate alone. If the bioretention systems were not successful in retaining the contamination from the stormwater runoff, than there would be a similar response to the untreated stormwater runoff. Because stormwater contains other contaminants besides metals, which are more toxic (i.e., polycyclic aromatic hydrocarbons), the toxic response of zebrafish would be decreased with bioretention treatment of contaminated stormwater due to removing other contaminants thereby, decreasing leachate toxicity but not completely removing toxicity. However, because we are suspecting that metals could be added with treatment, we would expect some toxic response from that as well.

They used frozen samples from their previous test of runoff leached through the BSM plus gravel columns, they then compared the leachate from this research. They tested the leachate with a zebrafish qPCR assay done on groups (25-30) of zebrafish embryos at the age of 52 hpf (hours past fertilization) and measured for detox genes (cyp1a) which would be increased with exposure to PAHs and other planar aromatic hydrocarbons. They also tested for cardiac-specific (nppa, tbx5a) or cardiac-related (ilk) genes which could be an indication of other contaminants as well, not only PAHs. An upregulation would be seen with an increase in the gene due to the body producing more of it to detoxify the body.

Table 7. Quantitative polymerase chain reaction (qPCR) assay of the filtered runoff with and without plants and the control runoff of the uncontaminated water treated through the BSM and BSM with gravel columns. All values have a base of 2, so any upregulation is double the value indicated. The control water groups were leachate from this thesis research (McIntyre et al., unpublished).

		Filtered Runoff		Control Water	
Gene	Expression/Role	Plants	No Plants	BSM only	BSM + gravel
cyp1a	Whole body	2.3*	2.1*	-0.5	-0.7
nppa	Heart	1.9*	1.4	0.7*	0.5
tbx5a	Heart	1.1	1.3*	0.1	0.0
ilk	Whole body	0.4	0.6*	-0.4	-0.4

The results of this study matched what our study concluded. There was significant upregulation in filtered highway runoff in the BSM and gravel column with plants for cyp1a and nppa (Table 7) (McIntyre et al., unpublished). For the filtered highway runoff with no plants, there was significant upregulation of the cyp1a, tbx5a and ilk genes. In the leach water from this research, with the uncontaminated water going through the columns, there was only a significant upregulation of the nppa gene in the BSM only column. This could easily be explained by the fact that nppa is also sensitive to osmotic changes, so different osmolarity of the filtered runoff compared to the control water could explain that upregulation (McIntyre et al., unpublished). There was no upregulation in the other genes for the tests with the clean water running through the bioretention treatments, supporting no toxicity from the soil columns themselves. This indicates that the effects observed in the treated runoff studies were from the stormwater coming through the columns rather than the columns themselves generating enough contamination to cause toxicity. This is the same conclusion that was drawn from this thesis research and supports the findings.

4.4 ECOLOGICAL IMPLICATIONS

In the application of bioretention systems, there is no requirement to condition the soils prior to use. As shown in this research, conditioning can drastically reduce the metal concentrations leaching out. Even with no toxicity observed from the soils observed, conditioning the soils in a controlled situation should still be conducted. Bioretention systems are used as a means of removing contaminants from the stormwater. This research found that soils have the potential to leach out high levels of metal concentrations. This can add to stormwater pollution. This effect was observed when uncontaminated water was flushed through the soil medium. Metals have the ability to accumulate in an aquatic environment. Even though this study did not show a toxic effect from the metals leached out of the soil, in other aquatic environments with other water chemistry parameters they could.

Copper has been shown to be neurotoxic to freshwater fish in levels as low as 11.5 µg/L (McIntyre et al., 2014). Copper has also been shown to cause blocking of olfactory sensory neurons in salmon in freshwater at concentrations as low as 2 µg/L (Sandahl et al., 2007). The copper concentrations leaching in this thesis research were as high as 60 µg/L at one point. While they did not remain that high, after leaching the ending concentration was still around 5 µg/L. These levels are high enough to have a potential toxicological response. With the accumulation potential, this amount of metal leaching has the potential to create larger ecological concerns.

5. CONCLUSION

This research found that bioretention soil medium leaches very high concentrations of metals. This is most likely due to the compost that is present in the mixture. The highest concentrations in the leachate were zinc and copper. This is also the highest metal concentrations permitted in compost. As more water was rinsed through the soil, the metal concentration decreased, implying that conditioning the soils can reduce the metal load of the leachate. This research has shown that there was no toxic effect from the leachate coming out of soils alone. This was attributed to calcium, magnesium, pH and dissolved organic carbon providing a protection against the metal ions to the freshwater fish studied. In an aquatic environment all these factors could change. This study was not an accurate representation of changing dynamics in a freshwater system. It was purely used as a comparative analysis of toxicity to stormwater runoff, versus toxicity of bioretention system leachate.

Zinc and copper have been shown to adversely affect freshwater fish and are considered contaminants of concern in stormwater pollution. Stormwater retention structures are meant to be a means of removal of contaminants in the stormwater, but if they are also adding to metal pollution, they may need to be studied further. Bioretention systems have been shown to leach metals when uncontaminated water is applied, but retain metals when contaminated stormwater runoff is applied. If used in situations where a mixture of stormwater pollution is being treated, the overall effectiveness of bioretention systems, according to this study as well as previous research, is a reduction of pollutants.

Based on the results of this study, bioretention systems are a successful treatment for stormwater pollution. Further studies should be conducted on the use of the composts in the systems. The compost used in this study leached high concentrations of metals, it may be worth studying, to examine other compost types and test if there is a difference in metal leachate. This study also showed a drastic reduction in metal concentration in the leachate due to a conditioning of the soils by flushing them with water. Based on this, it seems that conditioning in a controlled manner, should be a practice more commonly used prior to application of composts in bioretention systems.

References

Brandenberger, J. M., Crecelius, E. A., & Louchouarn, P. (2008). Historical Inputs and Natural Recovery Rates for Heavy Metals and Organic Biomarkers in Puget Sound during the 20th Century. Environmental Science & Technology, 42(18), 6786–6790.

Balades, J.-D., Legret, M. & Madiec, H. (1995). Porous pavements: pollution management tools. Water Science and Technology. 32 (1), 49–56.

Bergman, H., & Dorward-King, E. (1996). Reassessment of Metal Criteria for Aquatic Life Protection. SETAC Press.

Chapin, F. S., Matson, P., & Vitousek, P. (2011). Principles of Terrestrial Ecosystem Ecology (2nd ed.). Springer.

Collins, K., Hunt, W., & Hathaway, J. (2008). Hydrologic Comparison of Four Types of Permeable Pavement and Standard Asphalt in Eastern North Carolina. Journal of Hydrologic Engineering, ASCE. 13 (12), 1146.

Das, K. C., & Kirkland, J. T. (2008). Quantification of water extractable contaminants from food waste and biosolids blends at different stages of composting. Compost Science & Utilization. 16(3), 200–206.

Department of Ecology. (2011, May). Toxics in surface Runoff to Puget Sound. State of Washington Department of Ecology.

Dietz, M. E., & Clausen, J. C. (2005). A Field Evaluation of Rain Garden Flow and Pollutant Treatment. Water, Air, and Soil Pollution, 167(1-4), 123–138.

EPA. (2009). Pervious Concrete Pavement. Retrieved from http://cfpub.epa.gov/npdes/stormwater/menuofbmps/index.cfm?action=browse&Rbutton=detail&bmp=137

EPA. (2012). Effectiveness of Low Impact Development: Proven LID Technologies Can Work for Your Community. Retrieved February 21, 2015, from http://water.epa.gov/polwaste/green/upload/bbfs5effectiveness.pdf

EPA. (2015). Growth and Water Resources. Watershed Academy Web. US EPA. Retrieved February 28, 2015, from http://cfpub.epa.gov/watertrain/moduleFrame.cfm?parent_object_id=133

EPA. (2015b). Low Impact Development (LID). Retrieved February 28, 2015, from http://water.epa.gov/polwaste/green/

EPA. (n.d.) Technical Factsheet on: POLYCYCLIC AROMATIC HYDROCARBONS (PAHs). *EPA*. http://www.epa.gov/ogwdw/pdfs/factsheets/soc/tech/pahs.pdf

Färm, C., & Waara, S. (2005). Treatment of stormwater using a detention pond and constructed filters. Urban Water Journal. 2(1), 51–58.

Foran, J. A., Hites, R. A., Carpenter, D. O., Hamilton, M. C., Mathews-Amos, A., & Schwager, S. J. (2004). A survey of metals in tissues of farmed Atlantic and wild Pacific salmon. Environmental Toxicology and Chemistry. 23(9), 2108–2110.

Geronimo, F. K. F., Maniquiz-Redillas, M. C., Tobio, J. A. S., & Kim, L. H. (2014). Treatment of suspended solids and heavy metals from urban stormwater runoff by a tree box filter. Water Science & Technology. 69(12), 2460.

Good, J. F., O'Sullivan, A. D., Wicke, D., & Cochrane, T. A. (2012). Contaminant removal and hydraulic conductivity of laboratory rain garden systems for stormwater treatment. Water Science & Technology. 65(12), 2154.

Grebel, J. E., Mohanty, S. K., Torkelson, A. A., Boehm, A. B., Higgins, C. P., Maxwell, R. M., … Sedlak, D. L. (2013). Engineered Infiltration Systems for Urban Stormwater Reclamation. Environmental Engineering Science. 30(8), 437–454.

Hinman, C. (2009). Bioretention Soil Mix Review and Recommendations for Western Washington. Puyallup Extension Station, Puyallup, Washington.

Hunt, W. F., Davis, A. P., & Traver, R. G. (2012). Meeting Hydrologic and Water Quality Goals through Targeted Bioretention Design. Journal of Environmental Engineering. 138(6), 698–707.

Incardona, J. P., Collier, T. K., & Scholz, N. L. (2004). Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicology and Applied Pharmacology. 196(2), 191–205.

Istenič, D., Arias, C. A., Matamoros, V., Vollertsen, J., & Brix, H. (2011). Elimination and accumulation of polycyclic aromatic hydrocarbons in urban stormwater wet detention ponds. Water Science & Technology. 64(4), 818.

Joerger, S. (2008, May). Stormwater Regulation in Puget Sound. Stormwater. 36–46.

Kamo, M., & Nagai, T. (2008). An application of the biotic ligand model to predict the toxic effects of metal mixtures. Environmental Toxicology and Chemistry. 27(7), 1479–1487.

Landis, W. G. (2004). Introduction to environmental toxicology: impacts of chemicals upon ecological systems (3rd ed.). Boca Raton: Lewis Publishers.

Lanno, R. (1998). Contaminated Soils: From Soil-Chemical Interactions to Ecosystem Management. SETAC Press.

LeFevre, G. H., Hozalski, R. M., & Novak, P. J. (2012). The role of biodegradation in limiting the accumulation of petroleum hydrocarbons in raingarden soils. Water Research. 46(20), 6753-6762.

Li, L. Y. (2006). Retention Capacity and Environmental Mobility of Pb in Soils along Highway Corridor. Water, Air, and Soil Pollution. 170(1-4), 211–227.

Linbo, T. L., Baldwin, D. H., McIntyre, J. K., & Scholz, N. L. (2009). Effects of water hardness, alkalinity, and dissolved organic carbon on the toxicity of copper to the lateral line of developing fish. Environmental Toxicology and Chemistry. 28(7), 1455–1461.

Lundstedt, S., White, P. A., Lemieux, C. L., Lynes, K. D., Lambert, I. B., Öberg, L., & Tysklind, M. (2007). Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites. *AMBIO: A Journal of the Human Environment, 36*(6). 475–485.

Malins, D. C., Anderson, K. M., Stegeman, J. J., Jaruga, P., Green, V. M., Gilman, N. K., & Dizdaroglu, M. (2006). Biomarkers Signal Contaminant Effects on the Organs of English Sole (Parophrys vetulus) from Puget Sound. Environmental Health Perspectives. 114(6), 823–829.

McIntyre, J. K., Baldwin, D. H., Beauchamp, D. A., & Scholz, N. L. (2012). Low-level copper exposures increase visibility and vulnerability of juvenile coho salmon to cutthroat trout predators. Ecological Applications. 22(5), 1460–1471.

McIntyre, J. K., Davis, J. W., Incardona, J. P., Stark, J. D., Anulacion, B. F., & Scholz, N. L. (2014). Zebrafish and clean water technology: Assessing soil bioretention as a protective treatment for toxic urban runoff. Science of The Total Environment. 500-501, 173–180.

McIntyre, J. K., Edmunds, R. C., Redig, M. G., Mudrock, E. M., Davis, J. W., Incardona, J. P., Stark, J. D., & Scholz, N. L. (unpublished). Confirmation of stormwater bioretention treatment effectiveness using molecular indicators of cardiovascular toxicity in developing fish.

Meyer, J. S., & Adams, W. J. (2010). Relationship between biotic ligand model-based water quality criteria and avoidance and olfactory responses to copper by fish. Environmental Toxicology and Chemistry.

Newman, M. C., & Unger, M. A. (2003). Fundamentals of ecotoxicology (2nd ed.). Boca Raton, FL: Lewis Publishers.

Norton, D., Serdar, D., Colton, J., Jack, R., & Lester, D. (2011, November). Control of Toxic Chemicals in Puget Sound. Washington State Department of Ecology.

Ormond, T., P. E., Mundy, B., Weber, M., A.S.L.A., & Friedman, Z. (2010). LID Meets Permaculture: Sustainable Stormwater Management in the Mountains of Western North Carolina. ASCE.

Page, K., Harbottle, M. J., Cleall, P. J., & Hutchings, T. R. (2014). Heavy metal leaching and environmental risk from the use of compost-like output as an energy crop growth substrate. Science of The Total Environment. 487, 260–271.

Peraza, M. A., Ayala-Fierro, F., Barber, D. S., Casarez, E., & Rael, L. T. (1998). Effects of Micronutrients on Metal Toxicity. Environmental Health Perspectives. 106, 203.

Petrell, R. J., & Gumulia, A. (2013). Saturated and unsaturated flow through sloped compost filter beds of different particle sizes. Water Science & Technology. 67(11), 2406.

Quan, Q., Dong, L., Li, J. K., Shen, B., & Jin, C. X. (2014). Application of Bio-retention Hydrologic Performance Tool for Urban Runoff Pollutants Removal. Nature Environment & Pollution Technology. 13(3).

Rand, G. M. (1995). Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment (2nd ed.). Washington, D.C: Taylor & Francis.

Reish, D. J., Oshida, P. S., Mearns, A. J., Ginn, T. C., & Buchanan, M. (2001). Effects of Chemicals on Microorganisms. Water Environment Research. 73(Supplement 1), 1581–1657.

Pitt, R., Field, R., Lalor, M., & Brown, M. (1995). Urban Stormwater Toxic Pollutants: Assessment, Sources, and Treatability. Water Environment Federation. 67(3), 260–275.

Quan, Q., Dong, L., Li, J. K., Shen, B., & Jin, C. X. (2014). Application of Bio-retention Hydrologic Performance Tool for Urban Runoff Pollutants Removal. Nature Environment & Pollution Technology. 13(3).

Sandahl, J. F., Baldwin, D. H., Jenkins, J. J., & Scholz, N. L. (2007). A Sensory System at the Interface between Urban Stormwater Runoff and Salmon Survival. Environmental Science & Technology. 41(8), 2998–3004.

Schell, W. R., & Nevissi, A. (1977). Heavy metals from waste disposal in central Puget Sound. Environmental Science & Technology. 11(9), 887–893.

Scholz, N. L., Myers, M. S., McCarthy, S. G., Labenia, J. S., McIntyre, J. K., Ylitalo, G. M., & Collier, T. K. (2011). Recurrent Die-Offs of Adult Coho Salmon Returning to Spawn in Puget Sound Lowland Urban Streams. PLoS ONE. 6(12), e28013.

Sébastian, C., Barraud, S., Gonzalez-Merchan, C., Perrodin, Y., & Visiedo, R. (2014). Stormwater retention basin efficiency regarding micropollutant loads and ecotoxicity. Water Science & Technology. 69(5), 974.

Seelsaen, N., McLaughlan, R., Moore, S., & Stuetz, R. M. (2007). Influence of compost characteristics on heavy metal sorption from synthetic stormwater. Water Science & Technology. 55(4), 219.

Sikora, F. J., & Moore, K. P. (2014). Soil test methods from the southeastern United States. Southern Cooperative Series Bulletin, (419).
Teng, Z. & Sansalone, J. (2004) In-situ partial exfiltration of rainfall- runoff-II: particle separation. J. Environ. Eng. ASCE 130.

Tucker, M. (2008). Compost in the Mix for Storm Water Management. BioCycle. 42–45.

Wium-Andersen, T., Nielsen, A. H., Hvitved-Jakobsen, T., & Vollertsen, J. (2011). Heavy metals, PAHs and toxicity in stormwater wet detention ponds. Water Science & Technology. 64(2), 503.

Appendix A: Metal Analysis Values

Raw data from the analytical analysis of total metal concentrations (µg/L) for the Instant Ocean® Sea Salt solution though each treatment column, flush 1 through 8 (3 L through 24 L) and the final concentration measured after conditioning of the columns (61 L). The last column is the reporting limit (RL). All values in bold are above the reporting limit.

Before Treatment

	3 L	**6 L**	**9 L**	**12 L**	**15 L**	**18 L**	**21 L**	**24 L**	**61 L**	**RL**
Arsenic	0.5	0.5	NA	NA	0.5	**0.6**	**0.8**	**0.6**	0.5	0.5
Cadmium	0.1	0.1	NA	NA	NA	NA	NA	NA	NA	0.1
Chromium	0.5	0.5	NA	NA	**0.6**	0.5	0.5	0.5	0.5	0.5
Copper	0.5	**2.1**	NA	NA	**1.7**	**1.9**	**1.2**	**0.9**	**13.4**	0.5
Lead	0.1	0.1	NA	NA	0.1	0.1	0.1	0.1	0.1	0.1
Nickel	**0.6**	0.5	NA	NA	0.5	0.5	0.5	0.5	0.5	0.5
Silver	0.2	0.2	NA	NA	NA	NA	NA	NA	NA	0.2
Zinc	4	**27**	NA	NA	**12**	**6**	**5**	**5**	**5**	4

BSM Treatment

	3 L	**6 L**	**9 L**	**12 L**	**15 L**	**18 L**	**21 L**	**24 L**	**61 L**	**RL**
Arsenic	**3.2**	**7.4**	**7.3**	**6.3**	**4.6**	**5.3**	**4.3**	**3.7**	**2.5**	0.5
Cadmium	**0.3**	**0.2**	**0.2**	**0.2**	NA	NA	NA	NA	NA	0.1
Chromium	**3.8**	**11**	**13**	**13**	**7.2**	**6.5**	**5.4**	**4.6**	**2.0**	0.5
Copper	**22**	**31.6**	**31.7**	**24.8**	**19.7**	**17.8**	**12.7**	**9.8**	**4.6**	0.5
Lead	**4.4**	**8.1**	**9.6**	**7.3**	**6.3**	**6.0**	**3.7**	**2.6**	**1.1**	0.1
Nickel	**10.7**	**24.1**	**25.4**	**20.8**	**14.1**	**13**	**10**	**8.5**	**4.5**	0.5
Silver	0.2	0.2	0.2	0.2	NA	NA	NA	NA	NA	0.2
Zinc	**19**	**33**	**35**	**29**	**22**	**20**	**18**	**12**	**7**	4

BSM + Gravel Treatment

	3 L	**6 L**	**9 L**	**12 L**	**15 L**	**18 L**	**21 L**	**24 L**	**61 L**	**RL**
Arsenic	**3**	**8.1**	**6**	**5.9**	**4**	**5.3**	**4.7**	**3.8**	**1.8**	0.5
Cadmium	0.1	0.1	0.1	0.1	NA	NA	NA	NA	NA	0.1
Chromium	**2.8**	**8.3**	**10**	**11**	**9.0**	**7.0**	**6.8**	**5.0**	**1.0**	0.5
Copper	**52.5**	**60.1**	**54.4**	**54.3**	**45**	**40.3**	**30.8**	**24.3**	**5.7**	0.5
Lead	**3.5**	**5.0**	**6.4**	**6.9**	**5.6**	**5.4**	**3.6**	**2.6**	**0.5**	0.1
Nickel	**8.3**	**16.8**	**18.3**	**19.6**	**13.5**	**13.9**	**11.5**	**8.9**	**2.7**	0.5
Silver	0.2	0.2	0.2	0.2	NA	NA	NA	NA	NA	0.2
Zinc	**11**	**20**	**23**	**26**	**18**	**17**	**15**	**11**	4	4

Gravel Treatment

	3 L	**6 L**	**9 L**	**12 L**	**15 L**	**18 L**	**21 L**	**24 L**	**61 L**	**RL**
Arsenic	0.5	**1.4**	**1.6**	**1.1**	**0.8**	**1.8**	**4.4**	**7.2**	**1.1**	0.5
Cadmium	0.1	0.1	0.1	0.1	NA	NA	NA	NA	NA	0.1
Chromium	0.5	**0.6**	**1.0**	0.5	0.5	**0.6**	**1.6**	**2.6**	0.5	0.5
Copper	**4.1**	**4.5**	**5.8**	**3.2**	**3.1**	**3.2**	**7.0**	**9.2**	**2.8**	0.5
Lead	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	**0.2**	0.1
Nickel	**0.8**	**1.0**	**0.8**	**0.6**	**0.8**	**1.0**	**1.1**	**1.1**	**1.1**	0.5
Silver	0.2	0.2	0.2	0.2	NA	NA	NA	NA	NA	0.2
Zinc	4	4	4	4	4	4	4	4	4	4

Appendix B: Substrate Concentrations by Metal Type

Metal concentrations measured in each of the different bioretention substrate columns as a function of the number of liters of Instant Ocean® Sea Salt solution that had been flushed through.