Biosorption of Pharmaceuticals and Noble Metals Nanoparticles by Algal Turf Communities

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BIOSORPTION OF PHARMACEUTICALS AND NOBLE METALS NANOPARTICLES BY ALGAL TURF COMMUNITIES

by

Haider Abid Faraj

A dissertation submitted to the Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy Chemistry Western Michigan University April 2017

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Pharmaceuticals and nanoparticles are increasingly released into the aquatic ecosystem, while removing these contaminants from waters can be challenging. This study explores the use of periphytic algal turf communities to remove these contaminants. Initially algal turfs from different locations and aquatic systems were analyzed for pharmaceuticals commonly observed in wastewater outflows. Lab-grown turfs were exposed to a mixture of six representative pharmaceuticals of different functionality (acid, base, neutral and steroidal). Analysis of water column and the algal biomass showed pharmaceutical removal rates varied, specific to each pharmaceutical. Caffeine and estradiol had the highest removal rates while carbamazepine was not removed. The relative biomass density of the algae exposed to pharmaceuticals was decreased, suggesting the mixture of pharmaceuticals used may have had a negative effect on the stability of the algal turfs.

Algae exposed to pharmaceuticals were analyzed by CHN and an ashing protocol to determine carbohydrate, lipid, protein, inorganic carbon (CO$_3^{2-}$) and oxygen content. The data showed that the pharmaceuticals did not significantly affect the chemical compositions of the biomass during the exposure. Two factor ANOVA was used and there was no statistically significant ($\alpha = 0.05$). Experiments also showed that pharmaceuticals decreased the biomass density after exposure. Chlorophyll fluorescence intensity and absorbance of algae exposed to nanoparticles did not show statistically significant ($t$ test $> \alpha = 0.05$).

Lab-grown algal turfs were also exposed to silver and gold nanoparticles and to the corresponding to silver and gold ions. Atomic absorption measurement was used to quantify noble metal biosorption by the algal turfs.
Biosorption was fast, essentially reaching maximum biosorption within 24 hours. The rate constants were almost same for low and high contaminant concentrations. Experiments showed that AgNPs and Ag (I) ions had a small effect on the biomass density. However AuNPs and Au (III) ions did cause decreased biomass density after exposure. Chlorophyll fluorescence intensity and absorbance of algae exposed to nanoparticles did not show statistically significant. The effect of nanoparticles on the compositions of algal biomass was not statistically significant (t test > α = 0.05).

Confocal laser microscopy was used to locate the nanoparticles in the algal cells and bloodworms. To do so, algal turf biomass was exposed to fluorescent paracetamol (PA)-modified AuNPs from the media. Confocal laser micrograph images showed that the PA-AuNPs, at least, are most likely inside the algal cells, though they are most concentrated in the cell walls.

To test whether nanoparticle contaminants sorbed by algal turfs can move to a higher trophic level. Bloodworms (Chironomidae sp. larvae) were fed fluorescent PA-AuNPs exposed algae for seven days, while another group of bloodworms were exposed to PA-AuNPs solution for the same interval. Confocal laser micrograph images showed that nanoparticles are present in, though at lower concentration, than for the bloodworm exposed directly to the PA-AuNPs. Nanoparticles can transfer from one trophic level to another.
Read in the name of your Lord who created
Created man, out of a (mere) clot of congealed blood
Read, and your Lord is the most Generous
Who taught by the pen
Taught man that which he knew not.
- Holy Quran 96:1-5

Ask, and it shall be given you; seek, and ye shall find; knock, and it shall be opened unto you
- Matthew 7:7

Teach me knowledge and good judgment, for I trust your commands
- Psalm 119:66
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Motivation

One of the most important environmental issues is to have clean water for use daily for drinking, cleaning and agriculture. Water contaminants, such as pharmaceuticals and nanoparticles, are global problems and are among the most costly to be removed from wastewater streams.\(^1\) Pharmaceutical and nanoparticle contamination can have a deleterious effect on water quality, human health, and the economy.\(^2\) Contaminants reach the water from diverse sources including point and non-point sources. Point sources are any discernible, discrete source from which pollution is discharged e.g. any pipe, ditch, well or concentrated animal feeding operation.\(^3\) Non-point sources introduce pollution to the water from many diffuse sources, such as: over-fertilized agricultural lands, urban areas with extra usage of chemicals, and runoff from such areas. While point sources can usually be identified for remediation, non-point sources pose particularly serious ecological concerns for water quality, since they can be hard to identify and even harder to control.\(^4\)

The effects of pharmaceutical and nanoparticle pollution have the potential to be extensive because typical wastewater treatment processes do not remove these materials, and high percentages of the mobilized pharmaceuticals and nanoparticles are transported from surface waters to streams and rivers, and end up in coastal regions.\(^5\) The increased pharmaceutical and nanoparticle loads include possible links to multiple health problems in humans and aquatic animals. The health issues of having high concentrations of pharmaceuticals and nanoparticles in water include reproductive problems in fish and poison fish and other organisms.\(^6,7\)

1.2 How Pharmaceuticals Enter the Aquatic Environment

Generally, pharmaceuticals are absorbed by humans or animals after intake and then attacked by degradation processes. However, significant portions of the original pharmaceuticals often are excreted in an unmetabolized form or as active metabolites via urine or feces that are emitted into raw sewage, which may or may not be treated.\(^8,9\) Some pollutants escape degradation in wastewater treatment plants (WWTPs) and enter the environment.\(^8,10\) Flushing of unused or expired medication and drug-containing waste from manufacturing facilities can also contribute to environmental contamination.\(^9\) Patient excretion following therapy is widely considered to be the
primary pathway into the environment, while flushing unused medicines down the toilet appears to be of minor importance.\textsuperscript{11} North American waterways were found to contain traces of active pharmaceutical ingredients (APIs) from a wide spectrum of therapeutic classes such as contraceptives, painkillers, antibiotics, anticancer drugs and blood-pressure medications.\textsuperscript{12} Antibiotics and hormones were found with the greatest frequency.\textsuperscript{11} Trace amounts of drugs have been found in the tap water of some communities at concentrations ranging between 20 parts per billion (ppb) to less than one part per trillion (ppt).\textsuperscript{12,13} While these concentrations are small, drugs often have a physiological effect in small quantities. The United States Geological Survey study has established a baseline for pharmaceuticals as environmental contaminants in the United States. The fates of human and veterinary drugs after urinary and fecal excretions are quite different. The excreted human pharmaceuticals pass through sewage treatment plants prior to entering rivers or streams. Veterinary drugs are more likely to directly contaminate soil and groundwater without any sewage treatment. Manure is an important resource for topsoil dressing. After rainfall, surface waters can be polluted with human or veterinary drugs by runoff from fields treated with digested sludge or livestock slurries. The groundwater can also be contaminated.\textsuperscript{14}

1.3 Pharmaceuticals in the Environment

Over the last decade, many researchers studied the effect of pharmaceuticals once they had become present in the waters and how they were released into the environment.\textsuperscript{15} The presence of contaminants in water has become a serious concern in recent times. Water pollution has been a significant problem for the past 60 years in the United States.\textsuperscript{16} There have been efforts to address water pollution since the Federal Water Pollution Control Amendments of 1972 and those efforts evolved further with the Clean Water Act of 1977 and the Water Quality Act of 1987.\textsuperscript{17} The problem is that the contaminants have been found in waters and they do not respond to the treatments.

1.4 Effects of Pharmaceuticals on Aquatic Organisms

The effects of pharmaceuticals on aquatic life have been the key point of most studies.\textsuperscript{18} This shows how risky some substances may be to the health of humans and animals. Some believe that medicines are present in low concentrations in aquatic environments, and thus their effect is insignificant.\textsuperscript{19} In the future, the concentrations of pharmaceuticals in aquatic systems could become higher and more risky to both humans and animals.\textsuperscript{20}
There is an increase in released pharmaceuticals into aquatic systems because of the increase in human population and the use of prescription and non-prescription drugs.\textsuperscript{21} The fact that there is a higher percentage of prescription drugs being abused than cocaine or heroin, demonstrates an increasing problem. This situation adds to the problem of pharmaceuticals getting into the aquatic environment and the water supply because of the quantity that is consumed and then transferred into the environment through wastewater outflows.\textsuperscript{22}

Many researchers conducted their tests and experiments through the use of fish as the recipient of the pharmaceuticals. Fish are affected to a greater degree by a smaller amount of pharmaceuticals than humans.\textsuperscript{23,24} Since fish live in water, they are directly affected and are the first organisms to be impacted by the pharmaceutical pollutants that result from the release of pharmaceuticals into water sources.\textsuperscript{25} A study performed in England tested the bodies of water surrounding five Waste Water Treatment Plants to learn about the effects that the discharge from these plants had on the environment and the levels at which they were present in the water.\textsuperscript{26}

For example, in Weinberg and Klaper (2014), they studied the effects of pharmaceuticals on fish,\textsuperscript{27} the experiment was supposed to be conducted for a week, but had to be stopped because the fish died after 24 hours. The stress of the antibiotics present made the fish dispense a milky mucus and it was determined that the continuation of the experiment would kill them.\textsuperscript{28} Other experiments conducted using single concentrations of pharmaceuticals found effects such as reduced egg fertilization, reduced male characteristics, histological changes in the liver and gills and increased deformities.\textsuperscript{29} Another experiment was conducted with zebra fish that were introduced into ecological relevant levels of pharmaceuticals.\textsuperscript{30} The fishes’ genetic structure, as well as ability to function, was affected.\textsuperscript{31,32} Other researchers found that bacteria in water might be building up a resistance to pharmaceuticals present because of the low concentrations of pharmaceuticals in the water.\textsuperscript{33,34}

1.5 How Nanoparticles Enter the Aquatic Environment

The contamination of aquatic systems by silver and gold nanoparticles and metal ions because of mining and other anthropogenic activities has produced increasing concerns globally.\textsuperscript{35} Nanoparticles enter the environment through different exposure ways, including solid and liquid waste from domestic sources and industrial activity, accidental spillages, and atmospheric emissions. Additional types of source that need to be assessed are those resulting from the use of products that contain nanoparticles (NPs) such as sunscreens and paints. These paths may allow
NPs to disperse throughout the environment. A few published studies have focused on the fate of nanoparticles in aquatic systems and they are harmful to both humans and other organisms.

Different methods have been used to remove heavy metals from waters. The high costs of some of these methods bring an interest in lower-cost technologies that make use of plants (phytoremediation) and algae (phycoremediation). These new approaches can remove metal-contaminated water by exploiting the potential of algae to accumulate metal ions in their biomass. The use of algae is particularly attractive due to their large surface area to volume ratios. These organisms also possess high-affinity metal binding functionalities on their cell walls, via hydroxyl, sulphhydryl, carboxyl and amino groups that enable algae to bind up to 10% of their biomass as metals. The adsorptive removal of metal ions from contaminated water is also of considerable interest due to the ease of the adsorption process and the availability of a wide range of adsorbents.

1.6 Effect of Nanoparticles/Metals on Aquatic Organisms

The production of nanomaterials is increasing dramatically with an expected resultant increase in the dispersion of nanoparticles (NPs) into the environment. While many of these NPs aggregate and form larger assemblages, some particles are resistant to aggregation and retain high surface area and high mobility, particularly in aquatic environments. Due to the high surface area and binding constants of NPs, nanoparticles tend to absorb small colloids that may aggregate and even separate out from the water column by differences in settling velocity. This results in a transfer of other pollutions such as metals and polymers to the sediments where the NPs and those materials can also be taken up by organisms. In general, nanoparticles are not considered as a single unit, since they tend to form larger groupings of multiple units, through various mechanisms of association. The possible environmental impact, therefore, needs to be assessed for each type of nanomaterial in its own right. In the case of water systems, the most probable behavior of NPs is to form clusters. These colloids might have higher toxicity than single nanoparticles because of reactivity of nanoparticles with other contaminants in the ecosystem.

1.6.1 Silver Nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials because of their strong broad-spectrum antimicrobial activity with applications in textiles, personal care products, food storage containers, laundry additives, home appliances, paints, and even food
supplements. On the basis of these uses, it is likely that AgNPs will be released to the aquatic environment, be a source of AgNP aggregates and dissolved silver ions (Ag(I)), and possibly exert toxic effects on aquatic organisms. Although evidence has shown that both AgNPs and Ag(I) are toxic to bacteria and some aquatic organisms and marine invertebrates, it is unclear whether toxicity is specifically related to NP properties or is due to the effects of dissolved Ag(I). For example, AgNPs appear to cause bacterial cell death by pitting cell membranes, increasing permeability and disturbing respiration. In contrast, Ag(I) interacts with the thiol groups of proteins, resulting in inactivation of vital enzymes, disruption of bacterial membrane integrity and an increase in permeability and likely affecting DNA replication. When compared on the basis of total mass added, the toxicity of AgNPs to organisms has generally been found to be lower than that of Ag (I).

Recent studies showed that reactive oxygen species can mediate the transformation between AgNPs and Ag (I) via an electron-charging and discharging process. For instance, superoxides can reduce Ag (I) to AgNPs, whereas H$_2$O$_2$ can oxidize AgNPs to Ag (I). Furthermore, some data showed that AgNPs and Ag (I) can enhance reactive oxygen species generation which affects bacterial activity.

1.6.2 Gold nanoparticles (AuNPs)

Gold nanoparticles are used in variety of applications including industrial catalysis, chemical sensing, drug delivery, electrical, and optical technology. AuNPs could be released into the ecosystem in several ways and could create effects of different kinds on environments and organisms.

Because of the extensive use of nanoparticles in numerous products, these pollutants will find their way into aquatic environments, with the potential for consequences to human health and animals. Despite an increasing number of studies, their fate and behavior remains largely unknown. Studies have suggested that AuNPs can be bioconcentrated and bioaccumulated. These studies, however, do not indicate the effects of AuNPs characteristics on transfer through different trophic levels.

1.7 Water Contaminant Remediation Methods

The removal of pharmaceuticals and nanoparticles from contaminated waters is required in order to avoid the challenging conditions associated in wastewater treatment plants. Removal of contaminants from water did not start until the 1700’s, when the first water filters for local
application were introduced. A hundred years later, the first water treatment plant was built in Scotland in 1804 where sand filters were used. The process was slow, and sand filters were large and required constant cleaning and maintenance.

In the 1850’s, it was found that diseases, such as cholera, traveled and spread easily through water because of the absence of developed technologies in water treatment. The 1900s brought increased technology and new methods for treating water. Today, wastewater treatment plants in developed countries are efficient but they are not designed to remove pharmaceuticals or nanoparticles. They have been used for many years to help make wastewater clean enough for release into the environment. However, this does not seem to be fixing the pharmaceutical problem. Scientists have found rising levels of medicines in what was believed to be clean drinking water. These contaminants have been found in public water, wells, and rivers.

The main options for contaminant removal from water involve either physicochemical or biological methods. Some common physicochemical methods involve precipitation using chemical addition (chlorination), coagulation-flocculation, ion exchange, electrodialysis and reverse osmosis. Biological methods include the construction of wetlands and the strategic use of bacteria and algae. Unfortunately, pharmaceuticals and nanoparticles are often poorly treated by conventional methods. Only about 10% of pharmaceuticals are removed at the treatment plants while 20% ends up in the sludge and 70% ends up in the aquatic environment.

1.7.1 Physicochemical Remediation

1.7.1.1 Chlorination

Chlorination is one of the most common methods of water treatment around the world. Chlorine is added to the water to combat bacteria and viruses; a large portion of water treatment plants utilize this system for treating water. When used as treatment, there needs to be a certain level of free chlorine, which is the excess chlorine that did not react with anything, to combat bacteria along the way to its final destination. The accepted level is a concentration of 0.3-0.5 mg/L. This method generally does not remove all pharmaceuticals and nanoparticles from the wastewater.

1.7.1.2 Flocculation-Coagulation

The method of flocculation is used for bringing out materials that are dissolved in the water so that they can be collected on a filter. Flocculation was originally designed by Wilfred F. Langelier for the use of heavy metals and solids.
Coagulation is somewhat similar to flocculation in that the goal is to make larger particles that will either settle down or be able to be filtered. The difference is that coagulation combines the particles using positive charges from iron or aluminum salts. This step is usually what is done prior to flocculation and both methods are used in combination to be an effective step in the water purification process. Ternes and a few colleagues conducted an experiment with Flocculation using iron (III) chloride and the results showed no real effect on the pharmaceuticals present. Many other similar experiments have confirmed these data, so flocculation and coagulation can be taken out of the list for methods to treat water for pharmaceuticals.

1.7.1.3 Ozone

Ozone is a selective oxidant and primarily attacks electron rich structures in molecules, such as double bonds, and is therefore efficient in breaking down pharmaceuticals. With ozone technology, ozone can be used to effectively remove pharmaceutical residue at a low cost. Many customers are positively surprised by the low cost of water treatment, on volume basis. Studies also suggest that ozonation should be succeeded by sand filters to ensure an even more effective cleaning of the water. Using 0.5 mg/L it “was shown to reduce the concentrations of diclofenac and carbamazepine by more than 90 %”. The results for other pharmaceuticals showed less efficiency in reacting with ozonation. With an increase in ozone to 1.5 mg/L, bezafibrate only decreased total concentration by 50 %. With an increase of ozone to 3 mg/L, which is an extremely high level of ozone, there was only a 30 % decrease in clofibrac acid levels. Ozonation was very effective in oxidizing carbamazepine and diclofenac.

1.7.1.4 Adsorption

Adsorption is another way to remove contaminants from water. The process involves a solid surface that reacts with liquids to form a film. Adsorption can be either physisorption or chemisorption. Physisorption is an interaction involving van der Waals forces that are weaker than the bonds that form through chemisorption. Granular Activated Carbon (GAC) is commonly used as an adsorbant. Due to its high surface area (500 m$^2$/g) it is very effective and can even be used when a person swallows a toxic chemical. In water treatment, the water moves through a thick bed of activated carbon for the water to be cleaned. Different particles and pharmaceuticals require a certain throughput, or rate of entry, for it to interact efficiently with the activated carbon; each pharmaceutical is different. Ternes concluded in one of his experiments that the
pharmaceuticals that were strongly adsorbed with GAC can be removed efficiently from water under real conditions.\textsuperscript{71}

1.7.2 Disadvantages of Physicochemical Remediation

The problem with all methods above is that other chemicals are required to remove the contaminants from water such as chlorine, ozone or iron compounds, and the products need to be disposed in specific areas or dumpsters which might also end in the groundwater. The chlorine residual, even at low concentrations, is toxic to aquatic life and may require dechlorination. All forms of chlorine are highly corrosive and toxic.\textsuperscript{79} Thus, storage, shipping, and handling pose safety risks.\textsuperscript{80} Chlorine oxidizes organic matter in wastewater, sometimes creating compounds that could be harmful to humans and the environment.\textsuperscript{81} The chloride content of the wastewater is increased. Certain types of microorganisms have shown resistance to low doses of chlorine.\textsuperscript{82} The long-term effects of discharging chlorinated compounds into the environment are unknown.\textsuperscript{82}

In the ozone method, there is no guarantee that ozonation will react with all the pharmaceutical compounds.\textsuperscript{83} After taking into consideration the many types of particles in the wastewater that are in high quantities, and the fact that pharmaceuticals are in low concentrations, it can be inferred that in real scenarios less pharmaceuticals will be able to react with ozonation and then be filtered than was shown in experiments. As with ozonation, adsorption is only so effective against pharmaceuticals in real conditions due to the competition that the different contaminants have with sorbents.

1.7.3 Biological Remediation

Biological remediation is generally a process of using microorganisms and plants as a safe and less expensive method to remove hazardous chemicals from water or soil.\textsuperscript{84} Plants have an inherent ability to detoxify some xenobiotics in soil by direct uptake of the contaminants, followed by subsequent transformation using enzymes similar to detoxification enzymes in mammals, transport and product accumulation.\textsuperscript{85}

Literature shows that one of the best methods for treatment is biological methods, as a result these methods then have a reduced environmental impact.\textsuperscript{86} The biological process also produces fewer biosolids.\textsuperscript{87}

1.8 Algae

Algae can range from single-celled species to large seaweeds over 50 meters long and they are found in nearly every ecosystem on earth. Algae is a functional description, and do not fall
within a single group linked by a common ancestor; they do not exclusively have a specific group of defining features. They are divided into 9 major groups or phyla and each phylum of algae share a set of characteristics. The major groups of algae and their species almost all have three things in common. These similarities form the most common definition of algae. First, most are photosynthetic oxygenic producers of organic compounds. Secondly, most are aquatic. Lastly, most are smaller and less complex than land plants. The exceptions are heterotrophic and mixotrophic algae, algae that occur in non-aquatic habitats, and so-called macroalgae. 

Algae have the ability to fix carbon dioxide gas. Algae absorb CO$_2$ present in the atmosphere during photosynthesis, capturing it as biomass through increased growth. The algae assimilate the CO$_2$, nitrogen, and phosphorus into lipids, proteins and carbohydrates. The production of these co-products could give the biomass value in other markets such as the agriculture and biofuel industries.

### 1.8.1 Contaminant Removal by Algae

Algae are excellent for nutrient removal processes, as they exhibit several-fold higher nitrogen and phosphorus concentrations than other plants, about 10 % and 1 % dry weight, respectively. Algae have been shown to reduce residual concentrations of nitrogen and phosphorus to vanishingly low levels. Algae have also been shown to have an excellent capability to remove heavy metals from water. Research performed by Doshi and others showed that Cladophora sp. can take up 347, 168, 819, and 504 mg/g (dry mass) of Cr$^{+3}$, Cr$_2$O$_7^{-2}$, Cu$^{+2}$, and Ni$^{+2}$, respectively. Cadmium bioaccumulation has been demonstrated using three different species of algae: *Chlorella pyrenoidosa*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. In addition algae have been successfully used to monitor different heavy metals such as Co, Cr, Cu, Fe, Mn, Ni, and Pb levels in sea water. These contaminants are toxic to algae but algae are used in other applications after harvest.

The implementation of algae for nutrient removal is advantageous over other methods because it rests on the principles of natural ecosystems and therefore is not environmentally dangerous. Also wastewater treatment plants have been using algae for years because algae have also been shown to have an excellent power to remove heavy metals from water such as cadmium, zinc, nickel, lead, copper, mercury, and chromium.

W.J. Oswald and C.G. Golueke used algae to remove contaminants from water in 1963. They used large-scale raceway ponds to cultivate microalgae on wastewater and then converted
the algal biomass to methane gas. One of the major and practical limitations in developing algal contaminant removal systems is the harvesting or separation of algal biomass from the treated water discharge, an efficient removal of algal biomass is essential for recycling of the wastewater.\textsuperscript{97}

Algae have demonstrated metal removal by either passive sorption to dead biomass or removal by living cells.\textsuperscript{40} To date, most studies have focused on metal removal using dead biomass, although viable algae have shown promise in metal removal from domestic wastewater or synthetic media.

1.9 Benefits of Algae Biomass

Literature shows that algae is a great source for a lot of chemicals and raw materials.\textsuperscript{98} The natural products and nutrient content found in algae give the biomass many possible uses and provide for the possibility that the biomass can be recycled. Algae are used by producers of vitamins, minerals, proteins, and fatty acids and could be used for antioxidants.\textsuperscript{99} Antioxidants are key compounds to fight against various diseases such as cancer, chronic inflammation, atherosclerosis, cardiovascular disorder and antibiotics substances capable of inhibiting bacteria, viruses, and fungi.\textsuperscript{100,101} One of the most powerful soluble antioxidants, polyphenols and phycobiliproteins, was found in algae.\textsuperscript{102} For example, the Japanese widely use \textit{Spirulina} as feed additives in the fish industry and it improved the growth rates and coloration and higher survival rates of cherry salmon, sea bream, mackerel, and koi carp.\textsuperscript{103} In the context of this work, algae used to remove contaminants from water column so it would be preferred to be recycled instead of using the algal biomass as a source of vitamins, minerals, proteins, and fatty acids for human or animal uses.

1.10 Recycling of Algal Biomass

Once algae have been used for the removal of contaminants, the algal biomass needs to be disposed of or re-used in a proper way. The disposal of the algae represents an opportunity to recycle the biomass for other purposes. Algae have a greater overall biomass yield and can produce more energy per hectare compared to terrestrial crops.\textsuperscript{104} Algae, like most biomass, are composed of proteins, lipids, carbohydrates and other components. These characteristics give the harvested algal biomass value as a feedstock for biofuel production. The carbohydrate portion of the biomass has potential to be extracted and fermented into bio-alcohol fuels and the lipid portion has capacity to be converted into bio-diesel.\textsuperscript{105} The growth rate of algae is high and the overall biomass
productivity from the ATS™ system ranges from 40-70 dry g/m²/day in the summer\textsuperscript{106}, depending on latitude, to 5-20 dry g/m²/day during the winter months.\textsuperscript{107}

In 2014, researchers succeeded in converting algae oil into high-quality chemical raw materials. This provides the foundation for the use of algae as a basic chemical component for a broad spectrum of materials and products, beyond the use of algae as a substitute for crude oil.\textsuperscript{108} Another researcher synthesized thermoplastics from algae and polymer when the thermoplastics contain 70\% of the aquatic algae.\textsuperscript{109}

1.11 Evaluation of the Algae

1.11.1 Relative Biomass Density Measurement

The measurement of algal biomass is common in studies and may be especially important in studies that address nutrient enrichment or toxicity.\textsuperscript{110} High nutrient concentrations can affect recreational water users when the nutrients produce dense growths of algae and aquatic vegetation, which are undesirable.\textsuperscript{111} Use of waters for a public water supply can be affected if algal blooms result in an unpleasant taste and odor in the treated water. Fisheries are positively affected by increased primary algal production resulting from increased nutrient loads.\textsuperscript{112} However, when eutrophication begins to reduce dissolved oxygen concentrations substantially, fisheries can be adversely affected.\textsuperscript{112}

1.11.2 Importance of Ash Analysis

Ash is the portion of the biomass that is non-combustible, and contains mineral and other inorganic material.\textsuperscript{113} It is important to assess the chemical and physical properties of the biomass for purposes such as alternative fuel production. Generally, lower ash content is more favorable for biofuel applications because ash content is closely related with the total caloric value.\textsuperscript{114} Under normal conditions, when the ash content is increased, the caloric value of biomass fuels is reduced. Additionally, ash is a pressing concern for biomass combustion; it can cause slagging, fouling, and bed agglomeration in the combustion device, which can reduce performance and damage equipment.\textsuperscript{115} The biomass components that form the ash are wide-ranging, and hinge on biomass type, type of soil and harvesting. Since ash is the solid byproduct left after combustion it builds up and must be removed and disposed of correctly. Disposal can become even more complicated and expensive when toxic material such as pharmaceutical and nanoparticles are present in the ash. These issues serve to reduce the economic efficiency of using biomass as a fuel source.\textsuperscript{116}
Organic and inorganic contents can be approximately calculated by weight loss measurements in sequential heating of the sample.\textsuperscript{113,114} For example, to calculate the organic content, the sample is oxidized to carbon dioxide and ash at temperatures ranging from 200 to 500ºC. Carbon dioxide evolution from carbonate forms happens at temperatures between approximately 500 and 850 ºC.\textsuperscript{114} Weight losses connected with H\textsubscript{2}O and CO\textsubscript{2} evolutions are simply calculated by recording sample masses before and after controlled heating. The difference in weight between the 500 and 850 ºC ashes may be assumed to result from the loss of carbon dioxide during carbonate mineral breakdown.

\subsection*{1.11.3 Importance of CHN Elemental Analysis}

CHN analysis is one of the important techniques used to characterize biomass and new synthetic organic compounds.\textsuperscript{117} The weight percent of carbon, hydrogen, and nitrogen content in biomass are commonly referred to as the ultimate analysis. The term comes from the object of the analysis, which is the determination of the eventual components, the elements, of the substance.\textsuperscript{118} The information gained from this analysis can be used to estimate the value of the biomass for use as a biofuel. Many mathematical models have been developed to correlate final analysis information with higher heating values.\textsuperscript{119, 120} The atomic ratios of C, H and N are also used to classify the heating value of biofuels.

CHN analysis data can also be used to estimate the carbohydrate, lipid, and protein composition of algae biomass. A widely accepted method for determining the protein content in algae biomass is to use the nitrogen content calculated from the analysis and a conversion factor, which will yield the protein content.\textsuperscript{121,122} Also carbohydrate and lipid contents are directly related to the carbon content and can also be calculated by the CHN equation developed by Gnaiger.\textsuperscript{123} This information is important for determining which applications are optimal for the algal biomass and for which are not. Elevated lipids or carbohydrates are good indications that the biomass may have biofuel potential.

\subsection*{1.11.4 Chlorophyll a}

Chlorophyll is the green pigment in plants and algal cells and it is essential for energy fixation in the process of photosynthesis.\textsuperscript{124} Chlorophyll is used as an estimator of algal biomass in lakes and streams.\textsuperscript{125} It is used to measure algal biomass present per area of stream bottom that is relatively unaffected by non-algal substances. Chlorophyll provides an evaluation for measuring
algal weight and volume, and acts as a link between nutrient concentration and other biological phenomena in aquatic systems.

The amount of chlorophyll in algal cells varies based on the health and growth of the cells. Nutrients and other chemicals (contaminants) in water, together with light and temperature, affect the biomass production of algae in streams and lakes. In this study nutrients, light and temperature are constant parameters during the experiment. Pharmaceuticals and nanoparticles were added to the water column (media) as contaminants and chlorophyll was monitored using fluorescence and UV-Vis spectroscopies.

![Chemical structure of chlorophyll a.](image)

Figure 1-1. Chemical structure of chlorophyll a.

### 1.13.5 Detection Methods for Chlorophylls

A variety of methods have been developed to detect chemicals such as organic or inorganic molecules. Detection methods such as atomic absorption spectroscopy, atomic emission spectroscopy, inductively coupled plasma spectroscopy, X-ray fluorescence spectrometry, microprobes or electrochemistry have been used. These conventional detection methods have several drawbacks limiting their use for rapid and sensitive detection under field conditions. They require well-trained laboratory professionals, expensive equipment, sample pretreatment, mobile phase(s) and long analysis times. Because of that these methods are complex and expensive, which restricts their use in a laboratory.

For the past few years, there has been a growing interest in using fluorescence and UV-Visible spectroscopy for the detection of physiologically, environmentally and industrially important analytes. Their rapid nature of detection, high sensitivity, easy operational use, versatility and cost effectiveness make them suitable for rapid, sensitive and on-time monitoring of analytes.
1.13.6 Fluorescence Spectroscopy

Fluorescence is a property where molecules absorb energy at a particular (shorter) wavelength and emit at another (longer) wavelength.\textsuperscript{135} When a molecule absorbs energy at a particular wavelength it becomes excited from the ground state to one of the vibrational energy levels of one of the excited states. Then the excited electron falls back to the lowest vibrational level of the first excited state through internal conversion and/or vibrational relaxation. This electron then falls back to the ground state through several processes. If electrons in the first excited state fall back directly to the ground state emitting light, the process is known as fluorescence.\textsuperscript{136}

![Figure 1-2: The origin of chlorophyll fluorescence: basic aspects (adopted from Amarendra N., Chlorophyll Fluorescence in Plant Biology).\textsuperscript{136}]

1.13.7 UV-Visible Spectroscopy

UV-Vis spectroscopy refers to absorption spectroscopy in the ultraviolet (200-400 nm) - visible (400-800 nm) spectral region.\textsuperscript{137} Molecules having $\sigma$-electrons, $\pi$-electrons or non-bonding electrons that absorb radiation in the ultraviolet or visible region to excite these electrons to higher anti-bonding molecular orbitals. This causes organic molecules to undergo electronic transitions.\textsuperscript{138,139} There are also two other types of transitions. 1) d-d transitions: this type of transitions can be observed when transition metal ions containing partially filled d orbitals absorb energy and promote their electrons to an excited state.\textsuperscript{140} 2) Charge transfer transitions: these strong absorptions can be found when metal ion chelates with an organic ligand to form a charge transfer complex.\textsuperscript{141}
1.14 Research Objectives and Organization of Dissertation

The goal of this research is to study the removal of toxic contaminants that were released into the environment, such as surface water or ground water, particularly from wastewater treatment plant outflows. The target contaminants in this work were six pharmaceuticals and two types of nanoparticles. The target pharmaceuticals were selected based on previous literature studies, algae sample pre-experiments and the presence of these medicines in the environment. The nanoparticles and their metal ions were selected because of the huge concern of most current chemical researchers and the numerous applications for their use.44, 45, 46

The second chapter discusses the different methods used to extract pharmaceuticals from algae to test our hypothesis about biosorption of pharmaceuticals into algae by using different solvents and GC-Ms. Algae from three different locations in the United States were tested; 1) Goldsworth Valley Pond, Kalamazoo, MI. 2) Muskegon county wastewater reclamation plant, Muskegon, MI, and 3) Great Wicomico River, Virginia. A number of solvents was evaluated to isolate the pharmaceuticals from the algae. GC-Ms technique was used to analyze the extracts for any pharmaceuticals. The second chapter also focuses on extraction of pharmaceuticals from water using solid phase extraction (SPE). An ATS-like system was built as described in the literature to grow algae on screens. Algal Nutrients were added and culture medium will be monitored through pH and temperature. Silver and gold nanoparticles were prepared and characterized using UV-Vis and fluorescence spectroscopies. This chapter also included preparing and characterizing paracetamol-AuNP composite that can fluoresce at 402 nm. Bloodworms (Chironomid midge
larvae) were exposed to fluorescent nanoparticles as a solution and as embedded in algae to see if the nanoparticles transfer from contaminant environments to one of the organisms in the environment, bloodworms.

In the third chapter, periphytic algal communities were grown on a mesh support then expose to and analyze for different classes of pharmaceuticals. This information was needed to determine possible uses for the biomass, beyond its use to remove pharmaceuticals from the wastewater model. Pharmaceutical removal by algae was quantified using HPLC techniques. Experiments were performed on different exposure times of pharmaceuticals. Relative biomass density was calculated depending on the weights of dried algal biomass per area. Compositions of algal biomass were evaluated using by measuring organic and inorganic contents at 500 °C and 850 °C and CHN technique (hydrogen and nitrogen contents) and determining the chlorophyll fluorescence intensity and ultraviolet.

In the fourth chapter discusses preparation, characterization and separation of silver and gold nanoparticles and silver and gold ions. Nitric acid digestion method was used to dissolve the metal nanoparticles and an atomic absorption method was used to evaluate the nanoparticle concentrations in the biomass. High concentrations of a mixture of gold/silver nanoparticles were used to examine the ability of algae to remove them with high efficiency. Algae biomass was evaluated by measuring organic and inorganic contents at 500 °C and 850 °C and CHN technique (hydrogen and nitrogen contents) and determining the chlorophyll fluorescence intensity and ultraviolet. Confocal laser microscopy was used to locate the fluorescent nanoparticles in the algae biomass.

In the fifth chapter, the primary results and knowledge gained from this study are summarized. The limitations and implications of this research are assessed, and ideas for future work are suggested.
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CHAPTER 2

EXPERIMENTAL METHODS

2.1 Objective

The goals of this chapter were: 1) To find the best method to extract pharmaceuticals from algal biomass and water samples. Several solvents were used to isolate the pharmaceuticals from the algae. 2) To find the best method to analyze the pharmaceuticals from algae and water. 3) To find the common pharmaceuticals present in algae grown in different locations/environments. 4) Target nanoparticles were prepared and characterized using TEM, UV-Vis and fluorescence spectroscopies. 5) Paracetamol-AuNP (PA-AuNP) composite was prepared and characterized. This composite was used to locate the uptake in the algae, intracellular or extracellular. 6) Bloodworms were fed the algae already exposed to the PA-AuNP composite to see if the PA-AuNP transfers from the algae to a higher trophic level. Bloodworms were exposed directly to a PA-AuNP solution. Confocal laser fluorescence microscope was used to help locate the nanoparticles in the algae and bloodworm tissues.

2.2 Materials

The following materials were used: methanol, ethanol, water, ethyl acetate, acetonitrile and hexane. All solvents were purchased from Sigma-Aldrich with high purity 99.9% except for water, which was deionized by reverse-osmosis. Potassium chloride, magnesium sulfate, sodium nitrate, potassium phosphate monobasic, silicic acid, ferric sulfate, boric acid, molybdenum oxide and manganese dichloride, aspirin, carbamazepine, caffeine, 17β-estradiol, ibuprofen, bezafibrate, acetonitrile, acetic acid HPLC grade, ammonium hydroxide, acetone ACS grade and EDTA standard were purchased from Sigma-Aldrich, Milwaukee, WI.

Sodium borohydrate, gold chloride trihydrate, silver acetate, sodium citrate tribasic, paracetamol, HNO₃ (70%) and KBr were purchased from Sigma-Aldrich, Milwaukee, WI. 100 Chironomid midge larvae were obtained from Sachs Systems Aquaculture Inc., St Augustine, FL. The preservation solution was freshly made up from water: ethanol: formalin (6:3:1). Two mL autosampler vials with 9 mm PTFE septa caps were purchased from VWR International.
2.3 Locations of Algae Used

2.3.1 Goldsworth Valley Ponds, Kalamazoo, MI

Goldsworth Pond is a fresh water pond located in an urban area.\(^1\) The water here is eutrophic and receives excess nutrient inputs from the surrounding urban area. Additionally, it is known that fertilizer is used on the grassy areas around the pond and these nutrients certainly run off into the pond.\(^1\) The algae that naturally grow at this location are largely composed of filamentous green algae and diatoms. Biomass was collected from an ATS-like system from April 2010 through October 2011. Samples were not collected from this location during winter months. Algae samples were harvested intermittently throughout each growing season.

Water from this location was used to seed the algal turf communities grown and experimentally treated throughout this work.

2.3.2 Muskegon Wastewater Treatment Plant, Muskegon, MI

The Muskegon Wastewater Treatment Plant location is used to treat municipal and industrial wastes from the surrounding areas.\(^1\) The water is very high in nutrients and other contaminants. Algal biomass was grown at this location using the ATS™ system and samples were harvested from July to August 2011. The biomass was harvested about once a week. The algal biomass from this location was largely green filamentous algae and cyanobacteria.

2.3.3 Great Wicomico River, Virginia

The Great Wicomico River is located on the eastern coast and feeds into the Atlantic Ocean. The water level is affected by tidal changes, causing the water to change from fresh to brackish conditions in a cyclical pattern over time.\(^1\) The algae that grow on the ATSTM substrate in this river are mainly green algae and filamentous diatoms. Samples were harvested from this location from July 2010 to May 2011. Samples were harvested weekly during the summer and spring months and intermittently during the fall and winter.\(^1\)
Table 2-1. Summary of algal biomass location data.

<table>
<thead>
<tr>
<th>Location</th>
<th>Code</th>
<th>Harvest Duration</th>
<th>Water Source</th>
<th>Algae Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldsworth Valley Pond, Kalamazoo, MI</td>
<td>AGH</td>
<td>4/2010-10/2011</td>
<td>Fresh water, Eutrophic, Urban Run off</td>
<td>Greens and Diatoms</td>
</tr>
<tr>
<td>Muskegon County Wastewater Treatment Plant, Muskegon, MI</td>
<td>AMS</td>
<td>7/2011-8/2011</td>
<td>High Nutrients, Wastewater</td>
<td>Greens</td>
</tr>
<tr>
<td>Great Wicomico River, Virginia</td>
<td>AES</td>
<td>7/2010-5/2011</td>
<td>Fresh water/Salty Mix/Tidal</td>
<td>Green algae and Filamentous Diatoms</td>
</tr>
</tbody>
</table>

Figure 2-1. Map of the three locations of algae used.

2.4 Instruments

GC/MS analysis was performed using a HP 6890 Model equipped with a 5973 N MSD mass selective detector and an HP 7683 Series auto sampler. Shimadzu CBM20A/Lite HPLC System equipped with a SPD-20A spectrometer was used. Separation was completed using an
Agilent Zorbex SB-C18 (2.1x150 mm, 5µm) column. UV-Vis Scanning Spectrophotometer (UV-2101PC, SHIMADZU), was run with UV-2101PC software. Fluorescence Spectrophotometer (F-2500, HITACHI) was run with FL solution v.2 software. A PRECISION, Oven model 45EG, SN: 600101175 was used to dry the algae. Adventurer OHAUS, SN: G1541202091547, was used to weigh the samples. Ultrasonic water bath (2210 Branson) was used to extract chlorophyll a from the algae biomass. pH and temperature probes were used to monitor pH, and temperature of water column.

A Transmission Electron Microscope (JEOL, JEM-1230) was used to measure the size of the prepared gold and silver nanoparticles and find the size distribution of both. Digital Micrograph software was used to take pictures. MARS5 Digester, SN: DS-9015 was programmed. 100% power, at 80° C RAMP 15 min, PSI 300. Atomic Absorption Spectroscopy, iCE3300 FL AA system, SN: AA01134503 was used to determine the concentrations of NPs in Algae. SOLAAR security software V. 2.01 was used to measure the absorbance. Confocal laser microscopy, Nikon Eclipse Ti, Nikon C2 was used to measure the fluorescence of the paracetamol-gold nanoparticle composite.

2.5 Extraction from Solid Samples

Before doing any extraction, algal samples from the three locations were washed under a stream of deionized water for 5 mins to remove extracellular metals, left to drain into a waste container, and then dried for 2 hours at 105 °C. After that, algal samples were ground to destroy the initial matrix structure and to increase the surface area that can be easily penetrated by the solvent. Algae were milled to a powder using a mortar and pestle before any extraction methods were performed.

2.5.1 Ultrasonic Extraction

It has been reported that ultrasonic extraction showed better recoveries for most target pharmaceuticals than accelerated solvent extraction or pressurized fluid extraction, an extraction method that raises the solvent temperature above its boiling point but keeps it in the liquid phase by increasing the pressure.\(^2\)\(^3\) One of the studies also showed that sonication extraction using methanol or acetonitrile resulted in a higher extraction efficiency compared to other solvents such as acetone, ethyl acetate, and methyl-tert-butyl ether.\(^2\) An alternative to this is to use pulsed electric fields\(^4\) to destroy the cell walls so the solvent can penetrate into the cells and dissolve the target
compounds and facilitate extraction. However, this is mainly used for a small scale\(^5\), since with such fields it is difficult to adjust penetration depth and energy efficiency with larger scales.

Samples of 50 mg of algae were mixed with 10 mL of only one extracting solvent (acetonitrile, methanol, acetone, and ethyl acetate) in a time placed in an ultrasonic cleaning bath (with water as a coupling liquid), sonicated for 60 min at 45 °C, and filtered through a 0.2 µm microporous membrane (PTFE), syringe filter. The filtrate was collected and the solid was extracted again with the same volume of fresh solvent. The extract was analyzed using GC-MS.

### 2.5.2 Soxhlet Extraction

Soxhlet extraction, originally designed for the extraction of a lipid from a solid material, is a very efficient form of extraction\(^6\). It consists of a distillation flask placed in a heater, thimble holder and condenser. The solvent is heated to reflux and penetrates the solid material. The disadvantage of this method is the solute is always at the boiling temperature of the solvent, which may cause damage of thermally labile compounds.

1 g of dried powdered algae was packed in filter paper then placed in a thimble holder that was filled with 150 mL of solvent. When liquid reaches the overflow level, liquid moved through the siphon and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. Temperature and time depended on the solvent used. Methanol and methyl acetate were individually used as solvents in this method.

### 2.5.3 Steeping Technique

Steeping is a technique widely used in the wine industry and was adopted to remove the pharmaceuticals from the medicinal plants and algae. Basically steeping involved soaking raw materials in a container with a solvent at room temperature for 3 days or more with frequent agitation\(^7\). The principal of this method is to soften and break down the plant’s cell wall to release the soluble phytochemicals. In this method, the choice of solvents will determine the type of compound extracted from the samples. Organic solvents such as methanol and ethanol are recommended to extract pharmaceuticals from plants, since enzymes in plants will be denatured, preserving the solute undamaged\(^8\).

50 mg of algae was ground using a mortar and pestle to turn the dried algae into a powder. Then the dried powdered algae was mixed with 15 mL of methanol and left for three days at room temperature. The procedure was done for all algae samples, AGH, AMS, and AES. Extracts were
filtered using a PTFE 0.2 µm syringe filter. Samples were transferred into GC-MS vials for analysis.

2.6 Extraction from Liquid Samples

2.6.1 Solid-phase Extraction (SPE)

Solid-phase extraction was the first, and probably the most, successful extraction procedure that involves the utilization of sorbent materials in the sample preparation field. It is widely used in the environmental\textsuperscript{9}, biological\textsuperscript{10}, and food\textsuperscript{11} fields. Solid-phase extraction was introduced in 1990 by Arthur and Pawliszyn\textsuperscript{12} and became commercially available in 1993. The principle of SPE depends on the partitioning of analytes between two phases; aqueous sample and solid phase. Generally, during extraction the aqueous sample is preloaded in an immobilized sorbent phase such as fused silica\textsuperscript{12} or stainless steel\textsuperscript{13}. Then, a solvent elutes analytes. The efficiency of extraction depends on the distribution coefficient and the thickness of the coating phase.\textsuperscript{14}

2.7 GC-MS Analysis

Separation was completed using a 30 m x 0.32 mm x 0.25 µm Stabilwax (PEG) column from Resteck (Bellefonte, PA, USA). High purity helium was used as the carrier gas. The GC inlet temperature was kept at 240 °C with inlet purge of 10.4 mL min\textsuperscript{-1} starting at 0.5 minutes. The column flow was 15.5 mL min\textsuperscript{-1} of helium. The pressure was set to 8.47 psi with a split ratio of 5:1. The initial column temperature was 125 °C held for 3 minutes and increased 15 °C min\textsuperscript{-1} until reaching 175 °C and held for 5 mins, then the temperature was increased 10 °C min\textsuperscript{-1} until reaching the final temperature of 225 °C and held for 10 minutes for total run time of 26 minutes. The transfer line from the GC to the MSD detector was held at 240 °C. The mass selective detector was run in scan mode from 50-300 amu with a solvent delay of 2.5 minutes.

2.8 Algal Growth System or Algal Turf Scrubber -ATS™

Algal Turf Scrubber -ATS™ is a commercial technology, invented by Walter Adey and developed by HydroMentia, Inc, that is used to grow attached, filamentous algae species to capture the energy of sunlight and produce algal biomass, as a turf, from CO\textsubscript{2} and nutrients.\textsuperscript{15} The set up for the system is pictured in Schematic 2-1. The design involves using a fine mesh plastic screen support for the growing of the algae. The screen is then fit into a shallow trough through which the water is pumped. On the opposite end of the trough, water is allowed to return to the main source through gravity drainpipes. The screens require periodic harvesting to ensure new growth. This is accomplished by first elevating the screens from the water in the trough to allow them to
drain. The algal turfs are harvested by scraping biomass from the upper screen surface. The basal filaments that remain intertwined in the mesh of the screen send up new growth to replace what has been removed. Optimal harvest intervals range from 7 to 20 days.

![Schematic 2-1. Illustration representation of the basic ATS-like system.](image)

2.8.1 Periphytic Algal Growth

The ATS system is pictured in Figure 2-2 that was built in the lab. A fine mesh plastic screen was used to support the algae. The screen was then fitted into a shallow trough through which the water was pumped. Algae grew on the screens, which can then be removed to harvest the algae. The screens were made from 0.08636 m wide by 0.0047625 m thick hollow aluminum screen frames, nylon corners, and black PVC-coated fiberglass screen material. The screen area was 0.14732 m by 0.15113 m, which equates to 0.0222 m² while the mesh size was 0.0011684 m. The area does not include the frames. On the other side of the trough, water returned to the container by gravitational means.

Each floway consists of four 5 ft long sections and the flashes were bolted with a sheet of aluminum and sealed with epoxy resins. The sections were fabricated using 0.063 inch thick 6061-T6 aluminum; the cross-section is seen in Figure 2-3. The troughs were supported by stands made of treated lumber.
2.8.2 Algal Nutrients and Culture Medium

Algae were left 21 days to grow on the troughs. Nutrients were added to distilled water weekly and freshly prepared from the following chemicals: 2.5 mmol L$^{-1}$ K$^+$, 2 mmol L$^{-1}$ Mg$^{2+}$, 2 mmol L$^{-1}$ Ca$^{2+}$, 2 mmol L$^{-1}$ Mg$^{2+}$, 2 mmol L$^{-1}$ SO$_4^{2-}$, 6 mmol L$^{-1}$ NO$_3^{-}$, 0.5 mmol L$^{-1}$ H$_2$PO$_4^{-}$, 10 µmol L$^{-1}$ Fe$^{3+}$, 10 µmol L$^{-1}$ H$_3$BO$_3$, 1 µmol L$^{-1}$ Mn$^{2+}$, 0.5 µmol L$^{-1}$ Cu$^{2+}$, 0.1 µmol L$^{-1}$ Fe$^{3+}$.17

The algae community was grown in Western Michigan University lab in Kalamazoo, Michigan for three weeks in four troughs and 20 L nutrient solution was recirculated using pumps. In the fourth week of growth, the experiment took place by adding the target contaminants and samples were harvested for analysis of the accumulation of contaminants. Two fluorescence tube (48")-F40 T12 Bulbs were used over (12") each turf to grow algae. This type of tube is used for plants and aquariums and purchased from L.G Sourcing, Inc, N. Wilkesboro, NC.
2.9 Typical experimental Protocol

a) Grow algal turf on “seeded” screen for 21 days
b) Introduce contaminant (or mixture), adjusting pH if necessary
   • A mixture of six pharmaceuticals at two different concentrations, 3.5 and 5 ppm
   • A mixture of ibuprofen and bezafibrate at concentration 3.5 ppm.
   • Ibuprofen at concentration 3.5 ppm.
   • Bezafibrate at concentration 3.5 ppm.
   • Silver nanoparticles at concentration 2.475 ppm.
   • Gold nanoparticles at concentration 4.475 ppm.
   • A mixture of 2.475 ppm of AgNPs and 4.475 ppm of AuNPs.
   • A mixture of 4.95 ppm of AgNPs and 9 ppm of AuNPs.
c) Take triplicate turf samples from each of three treatment and one untreated, control trough at intervals before and after treatment.
d) Analyze algal biomass
   • Concentration of the contaminant in the algal biomass
   • Organic and inorganic carbon contents.
   • CHN content.
e) Analyze circulating water
   • Concentration of the contaminant in the water column.

2.10 Pharmaceutical Experimental Section

2.10.1 Parameters Controlled

2.10.1.1 Flow Rates of Pumps

Stirring of the media is necessary to achieve contact between the sorbent and water phase which contains necessary nutrients for algae growth. The experiments were performed at a flow rate of 580±15 mL/min. This measurement was done using a 100 mL volume cylinder and water was pumped into the cylinder for 10 sec. Measurements were repeated three times to take the average as shown in Figure 2-4. The error bars were measured depending on triplicate runs.
2.10.1.2 Water Temperature

Water temperature was measured in the container where water and dissolved nutrients were recycled. The temperature sensor was read by a PASCO PS-2169 Water Quality interface. PASCO Capstone software was used to log a measurement from the sensor using a personal computer. The measurement was in the morning between 8 and 10 AM. The change in temperature was $20 \pm 3 ^\circ C$.
2.10.1.3 Acidity of the Media

To run the experiment under the same conditions as the control, pH was measured during the experimental period. A pH probe sensor was used and read by a PASCO Water Quality interface. PASCO Capstone software was used to log a measurement from the sensor using a personal computer.

2.10.2 Solubility of Pharmaceuticals/Adjust the pH

Not all pharmaceuticals used in this study were soluble in water; aspirin and caffeine were easily soluble but not the others. 40 mL of 0.03 M of NaOH (5.05 g of NaOH in 250 mL) were prepared and added to the mixture of pharmaceuticals in 1L to form sodium salts. The mixture was mechanically stirred for 1 hour until all the precipitated pharmaceuticals had disappeared. The pH for all the containers including the control was around 9. The pH was monitored using a pH PASCO probe and the sensor was read by a PS-2169 Water Quality interface. PASCO Capstone software was also used to log a measurement from the sensor every three minutes using a personal computer. Figure 2-6 presents the pH values of growth media during the experiment. The pH values were monitored between 9 ± 0.5 as seen in the Figure 2-6 (the change pH was around 1). pH was adjusted to all growth media by using sodium hydroxide (0.03 M) and hydrochloric acid (0.053 M).

![Figure 2-6. pH values of the growth media in a typical experiment.](image-url)
2.10.3 Calibration of a Micro-Pipette

Given the low concentrations of the pharmaceuticals, one needs to be more accurate in volume measurements. To do that, a micropipette was calibrated using Milli-Q water at 25 °C each time the micropipette was used. Figure 2-7 refers to accuracy of the pipette. The micropipette was set up at different volumes to draw Milli-Q water. Water was weighed three times to take the average and find the error bars.

![Figure 2-7](image)

Figure 2-7. Calibration of the micropipette used in the study. Some error bars (Standard Deviation) are smaller than the point symbols.

2.10.4 Preparation of Standards

A known amount of the pharmaceuticals was weighed and then diluted in 20 mL of HPLC methanol. This solution is the stock solution and specific volumes were withdrawn and diluted into 2 mL to create five standard solutions with concentrations of 1, 3, 5, 10, and 20 mg/L. Solutions of known initial concentrations were created from the stock solution for each experimental treatment. This was done by pipetting the stock solution into vials with 0.1% HCl-HPLC methanol.

To determine the unknown concentration of the treated samples, standard concentration curves were established for each pharmaceutical using the HPLC and UV detector at wavelengths of 220, 235, 270, 265, 218, and 285 nm to detect ibuprofen, aspirin, caffeine, carbamazepine, bezafibrate, β-Estradiol.

Quantitation was performed by first creating calibration curves using a set of five standards to check linearity of signal. The peak area of each analysis was integrated by using the available
system software (Chemoleon). The quantity of compound in each sample was calculated from the calibration curve.

2.10.5 Extracting pharmaceuticals from Algae

Pharmaceuticals in algae samples were extracted using the ultrasonication method with 0.1% HCl-HPLC grade methanol and filtered through 0.2 µm PFTE filters. Typically, 30 mg of dried algal samples were sonicated for 60 min, at 40 °C and then filtered.

2.10.6 Extracting Pharmaceuticals from Water

Pharmaceuticals were extracted using solid phase extraction (SPE) cartridges based on EPA method 1694. SPE Oasis HLB cartridges are made of hydrophobic-lipophilic copolymers. They can retain acidic, basic, and neutral compounds that were used to clean up the samples. Because sodium hydroxide was used to form the pharmaceutical salts, that are soluble in water, 1 mL of 0.1% HCl was added to 3 mL of water to neutralize the pharmaceuticals, pH measurement was around 7. In this context, acidic and neutral analytes are those pharmaceuticals that are eluted off the SPE Oasis HBL cartridge with methanol, whereas basic analytes are those that are eluted by methanol in 5% ammonium hydroxide.

Before loading the sample extracts, the SPE cartridges were preconditioned with 5 mL of water then 3 mL of methanol by gravity. Sample extracts were passed through the SPE cartridges, using a vacuum to control the flow rate at 3-5 mL/min. Acidic and neutral analytes in each sample were first eluted with methanol (2 × 2 mL) into a vial using a vacuum. Basic analytes retained on the cartridge material were then eluted by methanol solution (2 × 2 mL) containing ammonium hydroxide (5 %) into another vial.

2.10.7 HPLC Analysis

Concentrations of extracted pharmaceuticals were determined by HPLC. All target pharmaceuticals were separated by HPLC (2695 module). A gradient separation was achieved using two mobile phases. Aspirin and ibuprofen analyses were carried out using a 60:40 water: acetonitrile phase with a flow rate of 1.00 mL/min for 5 mins. Caffeine and carbamazepine were analyzed using a 50:50 methanol: water mobile phase with a flow rate of 0.800 mL/min for 3.5 and 5 mins. B-estradiol was analyzed using a 70:30 methanol: water mobile phase with a flow rate of 1.000 mL/min for 4 mins. Bezafibrate was carried out using a 70:30 water: acetonitrile (pH= 4.2) mobile phase with a flow rate of 0.750 mL/min for 3 min.
2.11 Nanoparticle Experimental Section

2.11.1 Synthesis and Stability of Silver and Gold Nanoparticles

In reported synthesis methods of NPs, a reducing and a stabilizing agent are added to avoid the aggregation of the particles. The type of stabilizer affects the selection of further biofunctionalization strategies, and for certain strategies a good colloidal stability at a broad range of pH and ionic strength is required. Synthesis of silver and gold nanoparticles is rather easy even though the size of the nanoparticles is sensitive to the temperature and reaction time. Sizes of the synthesized silver and gold nanoparticles are characterized using the absorption literature values because of the lack of a scanning electron microscope to characterize the size precisely.

Two main parameters need to be considered for an analysis of nanoparticles. One is the stability of the solution, and the second is the size and shape of the NPs. Stability of the nanoparticles can be achieved by proper addition of a stabilizing agent like sodium citrate. Control of the size and shape of nanoparticles is done with the seed growth method, which offers a narrow size distribution with spherical particles and prevents nucleation.

2.11.2 Preparation of AgNPs and AuNPs Nanoparticles

Silver and gold nanoparticles were prepared per the literature. Monodisperse citrate-stabilized silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) were synthesized by the reduction of silver acetate (Ag-OAc) or gold tetrachloroaurate trihydrate (HAuCl₄) with sodium borohydride (NaBH₄). In an Erlenmeyer flask, a mixture of 18.5 ml of deionized water, 0.5 ml of 0.01M sodium citrate, and 0.5 ml of 0.01 M Ag-OA or HAuCl₄ were stirred for 5 min at 10°C. During AgNPs and AuNPs syntheses protocols, 0.5 ml of 0.01M NaBH₄ was added slowly to the reaction mixture and immediately after addition of NaBH₄ stirring was stopped. The solution color was changed to gold-yellow, indicating the formation of AgNPs. The final concentration of Ag nanoparticle was 2.5x 10⁻⁴ M (26.75 ppm) and for AuNPs was 2.5x 10⁻⁴ M (44.75 ppm). AgNPs and AuNPs were also stored in the dark at 14°C. The chemical reactions involved in the synthesis of silver and gold nanoparticles are given below.

\[
\text{Ag-OAc} + \text{NaBH₄} \rightarrow \text{Ag} + 1/2\text{H₂} + 1/2\text{B₂H₆} + \text{NaOAc} \quad \text{Equation 2-1}
\]

\[
\text{HAuCl₄} + 4\text{NaBH₄} \rightarrow \text{Au} + 5/2\text{H₂} + 2\text{B₂H₆} + 4\text{NaCl} \quad \text{Equation 2-2}
\]
2.11.3 Synthesis of Paracetamol Dimer (PD)–Au NPs Composite

Paracetamol dimer (PD)–AuNPs composite was prepared according to the literature. Briefly, 1.5 mL of 0.01 M of paracetamol (0.3804g in 250 mL of MiliQ water) and 1500 µL of 0.3 M NaOH (3.0456g in 250 mL of MiliQ water) were mixed together in a conical flask containing 100 mL of de-ionized water while heating at 90 °C with constant stirring. After 10 min, 1.5 mL of 0.01M of HAuCl₄ solution was added drop by drop. The appearance of red color immediately after the addition of gold salt indicated the formation of AuNPs. For the complete reduction of gold, heating was continued for another 30 min.

2.11.4 Digestion of AgNPs and AuNPs Samples

For total metal analysis exposed and unexposed algae samples were digested in HNO₃ using the MARS5 Digester. Approximately 30 mg of dried algae samples were weighed and put in beakers then 10 mL of concentrated nitric acid 70% was added. The MARS5 (microwave digester) was programmed for 100% power for 10 minutes at 80 ºC and then held for 5 mins, all grounded algae disappeared. After that, algae samples were left for 20 minutes to cool down and then filtered. The samples were diluted in Milli-Q water into a 25 mL volumetric flask and analyzed by Atomic Absorption spectroscopy (AAS). The wavelength used to collect the data were 242.8 nm for gold and 328.1 nm for silver.

2.11.5 Standards and Calibration Curves

All standard solutions of AgNPs, AuNPs, Ag (I) and Au (III) were prepared from stock solutions that contained 24.75 mg/L of Ag and 44.75 mg/L of Au. Five standard solutions were made to the calibration curves. All stock solutions and standards were freshly prepared before analyzing.

Standard solutions were treated as the algae biomass samples, where specific volumes of each nanoparticle or ion and 10 mL of concentrated nitric acid 70 % were added in a beaker then digested for 10 mins in a microwave digester at 80 ºC and held for 5 mins. Samples were then left 20 mins to cool down to room temperature. Samples were transferred to 25 mL volumetric flasks and were diluted to the mark with Milli-Q water. Absorbance was measured for all standards using Atomic Absorption Spectroscopy (AAS).
2.11.6 Water Chemistry Analysis

To determine the concentration of nanoparticles or metal ions that were removed, 10 mL of water samples were filtered by 0.45 µm PTFE to remove any particles or algae and then 10 mL of concentrated nitric acid (70 %) was added. The mixture was put in the microwave digester for 10 mins at 80°C with 100 % power. After 10 mins the samples were left for 20 min to cool down and the volume was filled to 25 mL mark with Milli-Q water. The supernatant solution was used for nanoparticle/metal ion analysis by AAS.

2.11.7 Bloodworm Experiment

Bloodworms were divided to three groups as shown in Table 2-2. Group A was the control experiment. Group B was the bloodworms fed algae containing fluorescent nanoparticles and group C was the bloodworms exposed to untreated algae.

Table 2-2. Bloodworm experimental design.

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 bloodworms • sacrificed</td>
<td>25 bloodworms • Fed PA-NP-treated algae (5 days) • Moved to clean beaker • Fed untreated algae (2 days)</td>
<td>25 bloodworms • Fed untreated algae (7 days)</td>
</tr>
</tbody>
</table>

To analyze the metal content, the bloodworms were digested using 10 mL of concentrated nitric acid then placed into the MARS5 Digester that was programmed to 100 % power, at 80° C RAMP for 15 min, PSI 300, then held 10 mins to digest the nanoparticles in bloodworm tissues. The vials were moved out of the digester and MilliQ-water was added to the 20 mL mark.

Atomic absorption spectroscopy was used to determine the concentrations of NPs in the bloodworms. SOLAAR security software V. 2.01 was used to measure the absorbance. Each sample was measured three times and the average was recorded.

2.12 Evaluation of the Algae

2.12.1 Relative Biomass Density Measurement

To find the effect of pharmaceuticals on the growth rate of algae, screens were lifted from the turfs and specific areas of algae were harvested at 0, 1, 3, 5, 60, 1440, 2880, and 5760 min by
using a 1 inch diameter PVC tube, see Figure 2-8. Triplicate samples were harvested from each trough. The PVC tube was used to select the area and then the specific algal biomass was removed by a spatula. The algae biomass was washed under a stream of deionized water for 5 mins (water temperature was 20±3 °C) and then dried by oven at 105 °C for two hours, then algae were hand ground and weighed. Then biomass density was calculated in triplicate.

\[
\text{Biomass density} = \frac{\text{mass of dried algae}}{\text{area}} \quad \text{.......... Equation 2-3}
\]

\[
\text{Relative biomass density} = \frac{\text{biomass density at any time}}{\text{biomass density at time 0}} \quad \text{......Equation 2-4}
\]

Figure 2-8. Algal biomass samples.

2.12.2 Ash Analysis

To determine the quantity of ash in each algal sample the ASTM E1755 standard test method for ash in biomass was employed.\(^{24}\) Previously dried algae samples were each placed in a 20-mL glass scintillation vial and heated at 105 °C for 2 hours, capped, and allowed to cool in a desiccator prior to being analyzed. Meanwhile, each algae sample was pulverized and ground into fine particles using a mortar and pestle. 50 ±5 mg of each sample was accurately weighed to the nearest tenth of a microgram into an evaporating dish. Most samples were analyzed once except the samples that were harvested at 5760 minutes analyzed in triplicate and the content of ash was averaged. The dried algae samples were then put in the furnace at 500°C for 5 minutes and cooled down to the room temperature ~20 °C. The samples were weighed and the mass was recorded then inorganic and organic carbon contents were calculated using the following equation:

\[
\text{Inorganic carbon content} = (\text{ash at 500 °C} - \text{ash at 850 °C}) \times \frac{12}{44} \quad \text{...... Equation 2-5}
\]
Organic carbon content = (Total carbon (from CHN data) – inorganic carbon)

……. Equation 2-6

This method assumes that all the organic carbon has been volatilized at 500 °C. Any remaining carbon in the biomass is bound in the inorganic form of CaCO$_3$, which is not volatile under 850 °C.

2.12.3 Carbon, Hydrogen, and Nitrogen Analysis

Carbon, hydrogen and nitrogen contents were analyzed using a Leco Corporation Truespec® CHN analyzer from LECO Corporation, St. Joseph, MI. Samples were first dried at 105 °C for two hours and stored in a desiccator to prevent them from collecting moisture. The instrument was calibrated before each analysis using 100 mg ± 3 samples of pure EDTA standard purchased from LECO and blanks. All samples and standards were weighed using a Sartorius balance to the nearest tenth of a microgram into tin foil caps. All samples were analyzed in triplicate. Each sample was analyzed in duplicate or triplicate and the content of carbon, hydrogen and nitrogen was averaged. During the combustion process the CHN instrument has a temperature of 950 °C, which means it is capable of volatilizing organic and inorganic carbon. This allows for the determination of the organic carbon fraction by simply subtracting out the inorganic carbon portion in the ash analysis.

![Figure 2-9. Calibration Curve using pure EDTA standard. (■) C%, (■) H% and (■) N%.](image)

Samples previously ashed at 500 °C, to a constant weight, were used to analyze the percentage of inorganic carbon in the biomass. This method assumes that all the organic carbon
had been volatilized at this temperature. Any remaining carbon in the biomass is presumed to be bound in the inorganic form of CaCO3, which is not volatile under 825°C. During the combustion process the CHN instrument attained a temperature of 950 °C, which means it volatilized both organic and inorganic carbon fractions. The organic fraction is determined by simply subtracting out the inorganic carbon quantity in the ash. All samples were run in triplicate except where noted. CHN values were reported as a percentage of the total dry biomass on a g/g basis. The values were reported this way because carbon may constitutes both the organic and inorganic fractions of the biomass. Oxygen content was determined according to the equation below.

\[
\text{Oxygen content} = 100 - (C \text{ wt }\% + H \text{ wt }\% + N \text{ wt }\% + \text{ash wt }\%) \ldots \text{ Equation 2-7}
\]

### 2.12.4 Extraction of Chlorophyll

Freshly grown algae in the lab was washed by deionized water for 5 mins then dried and ground. The washing process was done using a piece of cloth that did not allow to algal cells to escape through the pores. This specific area of algal biomass, approximately 7.29 cm², was harvested then dried for two hours in the furnace at 105 °C. After two hours, samples were taken out of the furnace and left to cool down to room temperature and then ground using a mortar and pestle. Samples were moved to test tubes and 10 mL of acetone were added to each test tube. Incubation was performed at 45 °C for 60 min in an ultrasonic bath to activate the enzyme chlorophyllase which is present naturally in algae. After incubation, the algae were filtered using 0.2 µm PEFE filters and extracts were put in vials. All extracts were used to measure the UV-Vis and fluorescence spectra.
2.12.4.1 Spectrophotometry Measurements

The fluorescence and UV-Vis measurements were carried out in a 1-cm path length quartz cuvette. The illumination of the chlorophyll a solution (3 mL) in acetone was carried out using collimated light from a xenon lamp. A red cut-off filter which allowed wavelengths greater than 640 nm was used. All UV-Vis operations with chlorophyll a solutions were carried out with wavelengths between 400-750 nm and 640-750 nm for fluorescence. Excitation wavelength of the chlorophyll a used in fluorescence measurements was 640 nm. The solutions were measured on the same day as preparation. All experiments were carried out in the presence of air. The fluorescence emission spectra were recorded with a Fluorescence Spectrophotometer (F-2500, HITACHI), and FL solution v.2 software. Absorption spectra were recorded with UV-Vis scanning spectrophotometer (UV-2101PC, SHIMADZU), and UV-2101PC software.

2.12.5 Statistics

CHN, fluorescence and UV-Vis absorbance experiments were conducted for three troughs and one trough control with three replicates of each. Triplicate turf samples were measured from each of three treatments and one control trough at intervals before and after treatment. Absolute values represented only one run done for this experiment. The standard deviation and means were calculated using the \( n \) values of each experiment standard. Arithmetic mean and sample standard deviation \((n = 3)\) are given in tables and figures in the Results and Discussion Section. The
significance of the main effects was determined using analysis of variance (ANOVA). Student's t-test was used to determine whether there were significant differences between the levels. For all statistical analysis, an \( \alpha \) of 0.05 was used and a probability of \( p \leq 0.05 \) was considered significant. Microsoft excel 2013 was used for all statistical analysis.

2.13 References


Seyyed Mohammad Hossein Abtahi, Synthesis and characterization of metallic nanoparticles with photoactivated surface chemistries, thesis, 2013


CHAPTER 3
BIOSORPTION OF PHARMACEUTICALS FROM WATER COLUMN BY
ALGAL PERIPHYTIC TURFS

3.1 Objectives

The main objectives of this chapter were 1) To study the biosorption of the pharmaceuticals by algal biomass from water column. 2) To study the effect of the exposure time of a mixture of pharmaceuticals on algae. 3) To compare between the concentration of the pharmaceuticals in the biomass and water at different times of experiment. And 4) to assess the effect of pharmaceutical mixtures on algal biomass. There has been minimal previous work done on the examination of the sorption of contaminants by algae, but researchers have been testing the water not the algae and noticed that pharmaceutical concentrations decreased with time. Therefore, algal turfs were exposed to a mixture of pharmaceuticals with a concentration of 3.5 mg/L and 5mg/L for each pharmaceutical and then a mixture of 3.5 mg/L of ibuprofen and bezafibrate. The pharmaceuticals selected depended on previous extraction methods from algae and their ability to transfer to the algal biomass. The compounds explored were aspirin, caffeine, ibuprofen, carbamazepine, β-estradiol and bezafibrate. Ibuprofen and β-estradiol were selected because they are known pollutants that persist in natural aquatic environments and were found in algal extractions in previous work. Aspirin and caffeine were selected because they are soluble in water and they are used in large quantities. Additionally, these compounds are considered because they are all substituted aromatic ring(s). Relative biomass density was measured based on specific dried algal biomass area and a signal of the effect of the pharmaceuticals on the algae communities.

The algal biomass after the exposure to a mixture of pharmaceuticals at different concentrations were evaluated by several techniques depending on carbon, nitrogen, hydrogen elements and chlorophyll a. 1) Mineral and other inorganic materials were measured using two techniques; a) Ash by oven at 500°C and 850°C and b) a CHN tool was used as well to determine carbon, nitrogen and hydrogen elements. 2) Separating chlorophyll a from the algal biomass by acetone and ultrasonic technique at 45 °C for an hour. 3) Fluorescence intensity and ultraviolet spectroscopies were used to compare the concentration of chlorophyll a in the exposed and unexposed algae.
### 3.2 Pharmaceuticals Used in the Study

Table 3-1. Overview of pharmaceuticals considered for use in this study

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CAS Number</th>
<th>$K_a$</th>
<th>$pK_{ow}$</th>
<th>% saturation @ 20L</th>
<th>Chemical structure</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>50-78-2</td>
<td>0.5</td>
<td>1.19</td>
<td>0.17%</td>
<td><img src="image" alt="Aspirin structure" /></td>
<td>an analgesic to relieve minor aches and pains</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>41859-67-0</td>
<td>0.83</td>
<td>4.25</td>
<td>14%</td>
<td><img src="image" alt="Bezafibrate structure" /></td>
<td>Lipid re the treatment of hyperlipidaemia-gulator</td>
</tr>
<tr>
<td>Caffeine</td>
<td>58-08-2</td>
<td>0.4</td>
<td>0.16</td>
<td>36%</td>
<td><img src="image" alt="Caffeine structure" /></td>
<td>Mental alertness, painkillers</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>15687-27-1</td>
<td>0.9</td>
<td>3.5</td>
<td>0.04%</td>
<td><img src="image" alt="Ibuprofen structure" /></td>
<td>headache, dental pain, menstrual cramps, muscle aches, fever, minor aches and pain</td>
</tr>
<tr>
<td>Estradiol</td>
<td>50-28-2</td>
<td>0.7</td>
<td>3.94</td>
<td>211%</td>
<td><img src="image" alt="Estradiol structure" /></td>
<td>Hormone replacement, Blocking estrogens</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>298-46-4, 85756-57-6</td>
<td>3.9</td>
<td>2.45</td>
<td>43%</td>
<td><img src="image" alt="Carbamazepine structure" /></td>
<td>Antiepileptic, bipolar disorder, and trigeminal neuralgia</td>
</tr>
</tbody>
</table>
3.2.1 Aspirin

Aspirin is a salicylate compound. In many countries, it has been detected with low concentrations in many environmental samples, for example sewage-treatment plant effluents, surface water, seawater, and ground water. Non-steroidal anti-inflammatory drugs including aspirin and paracetamol are among the most commonly used pharmaceuticals.

Recently, common non-steroidal anti-inflammatory agents have shown hepatotoxicity, including aspirin in therapeutic doses. It has been said that about 50% of patients taking aspirin regularly develop mild, dose-related liver damage and cancer. It is noted that aspirin, the longest established non-prescription analgesic, seems to have the greatest potential for hepatotoxicity currently. In 2009, 2,437,163 people died for numerous reasons, one of these reasons is aspirin. This shows the overall issue of negative side-effects in contrast to its benefits.

3.2.2 Carbamazepine

Carbamazepine is an anti-epileptic drug that lowers the level at which a spasm is triggered and therefore lowers the hyperesthesia of neural cells in the brain. It is used to treat bipolar depression and used to control seizures. Carbamazepine can affect blood levels of certain other drugs, making them less effective or causing increased side effects. It may cause serious blood problems or a life-threatening skin rash. The drug is degraded in the liver to active metabolites which are as active as the parent drug. Only 2 to 3% of the administered dose is excreted in an unchanged forms.

Carbamazepine has been proposed as an anthropogenic marker in water bodies. Studies in Europe and North America have shown that carbamazepine is one of the most frequently detected pharmaceuticals in wastewater treatment plant effluents and in river water at concentrations of up to 2.3 µg/L. In German wastewaters, carbamazepine was detected and presented in treated wastewater, surface water, and drinking water at concentrations of up to 6.3, 1.1, and 0.03 µg/L, respectively.

3.2.3 Caffeine

Caffeine is an alkaloid that occurs in more than 60 plant species (e.g., in the seeds of the coffee, cacao, and cola tree and in the leaves of the tea tree). It is an ingredient of a variety of beverages and of numerous food products. Caffeine enhances the effect of certain analgesics in cough, cold, and headache medicines. It is used as a cardiac, cerebral, and respiratory stimulant and as a diuretic. Considering its presence in beverages and foods, caffeine is probably the most
widely consumed drug in the world.\textsuperscript{15} It is produced commercially as a byproduct from the decaffeination of coffee.

Caffeine is a potential chemical marker for domestic wastewater contamination.\textsuperscript{15,16} Caffeine is released into the aquatic environment, and consequently found in wastewater, surface water, and groundwater.\textsuperscript{17,18} Meanwhile, results of toxicology studies have revealed that some pharmaceuticals are suspected to have direct toxicity to certain aquatic organisms.\textsuperscript{19,20}

Few studies have shown the relation between the concentration of caffeine and the anthropogenic burden by domestic wastewater.\textsuperscript{21,22} Regional mass balances have yet to be established, and the fate of caffeine in natural waters has barely been investigated. Researchers discovered that caffeine levels strongly correlate with levels of fecal coliform bacteria. Caffeine is a particularly good marker for human fecal contamination because agricultural and industrial sources of fecal coliforms generally do not release caffeine into the environment.\textsuperscript{23}

3.2.4 \(\beta\)-Estradiol

\(\beta\)-Estradiol is one of the chemical estrogens that are of an increasing public concern because it is an endocrine disrupting compound which has increased in water supplies over time causing higher levels of exposure to wild life resulting in adverse health conditions.\textsuperscript{24} They are produced naturally by humans and animals, used for personal care, and are excreted in urine and feces as inactive polar conjugates.\textsuperscript{25} The conjugates can be converted back to active forms by bacterial enzymes in the raw wastewater and during the wastewater treatment processes. Estrogens survived from the treatment processes are subsequently released into the environment through effluent.\textsuperscript{25} Thus, treated wastewater is considered one of the most likely estrogenic sources released into the environment. Numerous laboratory and field studies have been focused on the fate of estrogens in wastewater treatment plants in the past decades.\textsuperscript{25} Many studies have suggested that estrogens in treated wastewater are responsible for male fish feminization and sexual disruption in many aquatic organisms.\textsuperscript{26,27,28} A study verified that exposure to 17\(\alpha\)-estradiol affected the reproduction of marine male fish.\textsuperscript{27}

3.2.5 Ibuprofen

Ibuprofen possesses analgesic, antipyretic and anti-inflammatory properties. In addition, it relieves symptoms of arthritis, rheumatic disorders, and fever when taken orally. Ibuprofen has been found in various waters.\textsuperscript{29} Throughout the past decade’s quantitative studies have been conducted on many types of pharmaceuticals released into the environment including ibuprofen.
One study showed that 77-85 % of ingested ibuprofen is excreted in urine. Another study, in 2002, was performed on 139 sources of water across the United States to test for hundreds of different pharmaceuticals. It was found that 10 % of these sources had traces of ibuprofen. In 2002, a study found 0.90– 2.11 µg/L of ibuprofen in raw and reclaimed waters and nearly 1.35 µg/L in drinking water. Five years later, the same streams across America were tested and the concentration was found to have increased almost to 1.0µg/L above levels in 2002.

3.2.6 Bezafibrate

Bezafibrate is a known activator of peroxisome proliferator-activated receptor, a region of tissue that responds specifically to a particular neurotransmitter, hormone, antigen, or other substance. Bezafibrate improves markers of combined hyperlipidemia, effectively reducing low density lipids and triglycerides and improving high density lipid levels. A study of dyslipidemia patients with diabetes or hyperglycemia showed that bezafibrate significantly reduces hemoglobin concentration as a function of baseline HbA1c levels, regardless of concurrent use of antidiabetic drugs. The main toxicity of bezafibrate is hepatic (abnormal liver enzymes), myopathy, and rarely, rhabdomyolysis. Bezafibrate is excreted mainly via urine, 50 % of the administered dose in unchanged form and 20 % in the form of glucuronides.

Table 3-2 shows the concentrations of some pharmaceuticals found in waters from different areas. In this research, higher concentrations of the pharmaceuticals were used so they can be detected in the HPLC instrument after being extracted from algal samples.
3.3 Results and Discussion

3.3.1 Selection of Extraction Method and Solvent

A series of preliminary experiments was performed to investigate the best extraction method and effects of extraction conditions to recover any pharmaceuticals from algal biomass samples. We started the extraction process with algae harvested from the Muskegon wastewater treatment plant location. There was a good reason to believe that algae at that location was contaminated by pharmaceuticals. Some methods, Soxhlet and steeping technique, did not show any pharmaceuticals in the extracts. Phytol is an organic compound that contains a double bond and hydroxyl group in its structure. It can be used to synthesize vitamin E and K1, which might come from the algae themselves. Phytol was seen in the algal extracts when using methanol and hexane. Glycerin is a polyol compound and it is found in all known lipids. Pinane is used in the synthesis of linalool and it was seen in the extract when cyclohexane was used as solvent.

Solvent types are the most influential parameter in almost all of the methods. Several solvents with different polarities; methanol, ethanol, acetone, ethyl acetate, hexane, cyclohexane, chloroform, toluene, and water, were tested. The solubility of pharmaceuticals in a solvent is
largely a function of the polarity of that solvent. Semi-polar solvents such as methanol and acetone may induce a certain degree of polarity in non-polar molecules.\textsuperscript{39}

Temperature is an important factor in the extraction methods, but it should be very carefully and should not exceed the temperatures that breakdown targets into unmeasurable compounds. Phytol has been seen in all three algae extracts and it might already be found in the chemical structure of the algae biomass so phytol will not be studied any further. Glycerin has been seen in the AMS and AES extracts but pyrrol and estrone derivatives have not, see Table 3-3.

Table 3-3. Organic compounds extracted by Soxhlet method using mixture of alcohols.

<table>
<thead>
<tr>
<th>Pharma</th>
<th>60% MeOH:40EtOH /Soxhlet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGH</td>
</tr>
<tr>
<td>Phytol</td>
<td>+</td>
</tr>
<tr>
<td>Glycerin</td>
<td>-</td>
</tr>
<tr>
<td>Pyrrol- derivative</td>
<td>-</td>
</tr>
<tr>
<td>Estron- derivative</td>
<td>-</td>
</tr>
</tbody>
</table>

+S/N > 10 (by GC/MS), - S/N < 10

A steeping method may be an alternative method that is more easy, applicable, convenient and less costly method.\textsuperscript{40} Two solvents were used; semi-polar (methanol) and non-polar (hexane). Pharmaceuticals were not observed in the algal extracts of this method, although, all this extraction method resulted in crude extracts containing a mixture of long chain paraffins and other aromatic compounds which are not useful for this study as outlined in the objectives above.\textsuperscript{41} As seen in Table 3-4, pharmaceutical derivatives pyrrol, estrone, phytol, and glycerin were observed.

Table 3-4. Organic compounds observed from steeping method.

<table>
<thead>
<tr>
<th>Pharma</th>
<th>Methanol/ Maceration</th>
<th>Hexane / Maceration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGH</td>
<td>AMS</td>
</tr>
<tr>
<td>Phytol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pyrrol- derivative</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Estron- derivative</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+S/N > 10 (by GC/MS), - S/N < 10

The ultrasonic extraction method was evaluated and this method showed a couple of pharmaceuticals in the algae extracts, but solvent types also influenced the ultrasonic method.\textsuperscript{42}
Because the high temperature may cause degradation of compounds, 40 °C was used in the ultrasonic method for one hour. Using methanol as the extraction solvent led to better detections in GC-MS for pharmaceuticals. Methanol, therefore, was the only solvent used in this project to obtain pharmaceutical extracts, see Table 3-5. Some organic compounds such as estradiol and methenamine were found in both pond water and AGH. That means that these compounds were absorbed by the algae, transferred from the water into algal biomass. Other compounds such as phytol and cholestra- derivative were observed just in algal extract that means these compounds were extracted from the algae itself. All compounds detected in AMS were found in the wastewater. Limits of detection (LODs) and limits of quantification (LOQs) were not determined in this study.

Table 3-5. Pharmaceuticals found in algae biomass and waters (ultrasonic method-methanol)

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>Pond Water AGH location</th>
<th>Algae AGH</th>
<th>Algae AES</th>
<th>Algae AMS</th>
<th>Wastewater AMS location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Estradiol</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cholesta- derivative</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phytol</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyrrol-derivative</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methenamine</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ S/N > 10 (by GC/MS), - S/N < 10

3.3.2 Pharmaceutical Detection

The removal of pharmaceuticals from algae biomass samples harvested from three different locations in the United States was assessed. Media used to feed the algae were different according to the locations where the algae were harvested. Because the water column contains a number of the contaminants, pharmaceuticals are expected to be present in the water and transfer into algae biomass. In our laboratory experiments not all extracts contained pharmaceuticals. That suggests pharmaceuticals detected are dependent on the locations of algae harvest. The pharmaceuticals in some water columns might be low to be detected when the GC-MS was used or there were no
pharmaceuticals. The contamination of the raw water is influenced mainly by its percentage of treated wastewater.

Some pharmaceuticals and hormones, namely β-Estradiol and ibuprofen, were found to consistently accumulate in algae where algae were exposed to any level of pharmaceuticals present in water, see Table 3-5. Several studies showed that ibuprofen, aspirin, caffeine and β-Estradiol were frequently detected in surface water and wastewater in the USA and around the world, such as Italy and Spain.\textsuperscript{43,44,45} Finding these pharmaceuticals in the algae extractions can lead us to believe that they can typically be transferred into algae cells or attached to the cell walls. Literature shows that carbamazepine was detected in water\textsuperscript{45} in several areas but it is not detected in the algae extracts in our experiments. This could be due to the fact that the water where the algae were harvested does not contain carbamazepine or carbamazepine concentration were lower than detectable levels of GC-MS.

![Figure 3-1. Gas Chromatography of extraction from AMS.](image)
Figure 3-2. Mass spectra of ibuprofen from algae extraction (top) comparing to ibuprofen spectra from the GCMs library (bottom).

Figure 3-3. Mass spectra of estrone from AMS (top) comparing to estrone from the GCMs library (bottom).
Figure 3-4. Gas Chromatography of the extraction of AGH.

Figure 3-5. Mass spectra of estradiol extracted from AGH (top) comparing to estradiol from GC/Ms library (bottom).
3.3.3 Identification of Algae

Algae grown in the lab and used in this study were identified using a microscope. An algae community was formed from two predominantly species *Spirogyra sp.* about 60 % of the community and *Chlorella sp.* about 30 % as shown in Figure 3-6. These two species were found in more than one locations, different troughs. *Spirogyra sp.* is a genus of filamentous green algae of the order Zygnematales, named for the helical or spiral. There are more than 400 species of Spirogyra in the world. Spirogyra measures approximately 10 to 100 µm in width and may grow to several centimeters in length. *Chlorella sp.* is a genus of single-cell green algae belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 µm in diameter. *Spirogyra sp.* and *Chlorella sp.* have the green pigments (chlorophyll a and b) in its chloroplast. Through photosynthesis, it reproduces quickly, just needing carbon dioxide, water, sunlight, and a minor amount of nutrients.

![Spirogyra sp.](image1)

![Chlorella sp.](image2)

Figure 3-6. Predominant species in the algae community grown and used in the lab.

3.3.4 Aspirin and Caffeine

Figure 3-7 and Figure 3-8 show the concentration of aspirin and caffeine, respectively, in the algal biomass. It is seen the concentration of the aspirin (pK\text{ow} = 1.19) and the caffeine (pK\text{ow} = 0.16) increased with exposure time and the initial concentration of the contaminants. At higher concentrations, algae take in more pharmaceuticals into the biomass body. Control experiment did not show any aspirin and caffeine in algal biomass. For neutral chemicals, hydrophobicity (usually expressed as pK\text{ow}) has been assumed to be the most important property involved in the uptake of
chemicals into plants from the medium\(^{48}\) as the degree of uptake appears to be proportional to the octanol-water partition coefficient.\(^{49}\) Briggs and his coworkers suggested that uptake of neutral chemicals can be represented by a Gaussian distribution.\(^{49}\) Maximum translocation of chemicals can be seen at a pK\(_{ow}\) \(\sim 1.78\) in comparison to particularly hydrophobic (high pK\(_{ow}\)) and hydrophilic (low pK\(_{ow}\)) chemicals which are taken up to a lesser extent. The uptake into algal biomass can more likely be attributable to pK\(_{ow}\) of the compound. In general, organic chemicals with pK\(_{ow}\) less than 4 are expected to have low translocation capacity. The concentrations of aspirin and caffeine in algal biomass were statistically significant (t test = 0.034 for aspirin and 0.0839 for caffeine).

Figure 3-7. Concentration of aspirin in algal biomass. (■) control, (□) L.C (3.5 ppm), (■) and H.C (5 ppm). Error bars represent sample standard deviations of averaged data.
3.3.5 Carbamazepine

Figure 3-9 shows that carbamazepine is not removable by algal turf communities where it stays in the water column at the same concentration during the four days allotted for the experiment. It prefers to reside in the aqueous phase rather than attach to the adsorbent as demonstrated by the low dissociation constant (K_d) values discussed in literature.\textsuperscript{50}

Literature shows that the differences in lipid contents in plants may also be an important factor and can affect the sorption of hydrophobic chemicals.\textsuperscript{51,52} The reported lipid content illustrates that *Spirogyra sp.* (11-21% biomass) and *Chlorella sp.* (14-22 % biomass)\textsuperscript{53} contain few amounts of lipids which may explain why there was no observed uptake of carbamazepine. However, similarly to results of Wu and his coworkers, differences in plant uptake behavior could not be only attributable to differences in lipid content. Based on the results presented, the factors that affect the uptake of pharmaceuticals into algae include physicochemical properties of the pharmaceuticals and species type. A previous study also highlighted that the uptake can vary between a mixture and a single compound exposure.\textsuperscript{54} Therefore to conclude, the uptake of chemicals into algae is a complex process controlled by a combination of species and chemical factors.
3.3.6 β-Estradiol

Figure 3-10 shows the uptake of β-estradiol by algal turf communities. The concentration of β-estradiol increased with the time of exposure and the initial concentration of β-estradiol in the water column affected the uptake by algal turf communities. The continuous-flow tests showed that about 84% of the β-estradiol was removed from the water column, based on the initial concentration and the final concentration of estradiol in the water column. Concerning the removal mechanisms, it has been generally accepted that different processes like biosorption, biodegradation, and photolytic degradation might play an important role in the removal of estrogens from wastewater.\textsuperscript{55} Although there might be some differences due to algal uptake, one can still guess that these processes probably played the same role on the removal of β-estradiol from the water column by algae.
3.3.7 Ibuprofen and bezafibrate

Figure 3-11 and Figure 3-12 show the uptake of ibuprofen and bezafibrate, respectively, by algal turf communities. Biosorption tests using algae showed that detectable amounts of ibuprofen could be seen after one hour harvesting. The concentration of ibuprofen and bezafibrate increased with the exposure time and the initial concentration of ibuprofen and bezafibrate in the water column. This indicates that the algae are effective for ibuprofen removal at the tested concentrations of 3.5 and 5 mg/L. Biosorption experiments carried out on control samples that did not spike with ibuprofen, showed no biosorption of the ibuprofen over the experiment test period. Similar results can be seen in Figure 3-12 for bezafibrate. It can be said that the concentrations of bezafibrate in algal biomass stay constant without any changes after the first 24 hours of the experiment.
Figure 3-11. Concentration of ibuprofen in algal biomass. (■) control, (▲) L.C (3.5 ppm), (●) and H.C (5 ppm). Error bars represent sample standard deviations of averaged data.

Figure 3-12. Concentration of bezafibrate in algal biomass. (■) control and (▲) L.C (3.5 ppm), (●) and H.C (5 ppm). Error bars represent sample standard deviations of averaged data.

Figure 3-13 shows the concentrations of both ibuprofen and bezafibrate were higher when they were individually spiked into the media. But when the mixture of them was made, the concentration of the ibuprofen and bezafibrate dropped. That might be because of the limited
functional groups on the algal cell walls that the pharmaceuticals could attack or might be that the pores could fill quickly from both pharmaceuticals.

![Graph showing concentrations of ibuprofen and bezafibrate in algae.](image)

Figure 3-13. Concentrations of ibuprofen and bezafibrate in algae. (■) control, (▲) ibuprofen only, (▲) ibuprofen in the mixture, (▲) bezafibrate only and (▲) bezafibrate in the mixture.

### 3.3.8 Adsorption Kinetics

In order to clarify the adsorption kinetics of pharmaceuticals onto algal turf communities, the Lagergren pseudo-first order kinetic model was applied to the experimental data. The Lagergren pseudo-first-order rate equation used by researchers to study the kinetics of pharmaceutical adsorption is as follows:

\[
\ln (A_0 - A_t) = \ln(A_t) - kt \quad \text{Equation 3-1}
\]

Where \( A_t \) (mg/L) is the amount of the pharmaceutical adsorbed at \( t \) (min) and \( k \) is the rate constant of the adsorption (min\(^{-1}\)). Figure 3-14 shows the pseudo-first-order model for the kinetics of ibuprofen adsorption on algae.

The rate constant, \( k \), of this equation is listed in Table 3-6. It is clear from the results that the adsorption of pharmaceuticals onto algal turf communities follows well the pseudo-first-order kinetics.
Figure 3-14. Concentrations of ibuprofen in (■) algal biomass and (□) water. Some error bars (S.D to the average) are smaller than the symbol.

Because not all data sets continue sufficient shot-time measurement, an initial-rates analysis was not universally feasible. Thus, we performed a least-squared analysis over the entire time sequence data using the first-order equation for product formation solving for \( C_{eq} \) and \( k \) as fit parameters.

\[
C_t = C_{eq}(1 - e^{-kt}) \quad \text{Equation 3-2}
\]

An example is shown in Figure 3-15. Similar analyses were performed for other pharmaceuticals.

Table 3-6 shows the effects of varying initial concentration (3.5 and 5 mg/L) on the rate constants of pharmaceutical removals. Rate constants for pharmaceutical removal were similar in high and low concentrations.

Table 3-6. Rate constants of pharmaceutical absorption by algae at 3.5 and 5 ppm.

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
<th>Rate constants / mg.L(^{-1}).min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 3.5 mg/L</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.0811</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>0.0151</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.0191</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>0.1916</td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.0261</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Not observed</td>
</tr>
</tbody>
</table>
Figure 3-15. Ibuprofen concentration against time and its fitted curve. (■) experimental data (L.C) and its (•) fitted curve and (▲) experimental data (H.C) and its (∗) fitted curve.

Figure 3-16. Estradiol concentration against time and its fitted curve. (■) experimental data (L.C) and its (∗) fitted curve and (▲) experimental data (H.C) and its (∗) fitted curve.
3.3.9 Pharmaceuticals in Water

Figure 3-17 shows the concentrations of the pharmaceuticals used in this study in circulating water during the experiment. The importance of biosorption for pharmaceutical removal was observed over the first 60 min. Algal turf communities have different abilities to adsorb pharmaceuticals from the water column. Literature shows that pharmaceuticals in wastewater are not only taken on by activated sludge but are also degraded by microorganisms. One might also find a difference in pharmaceutical concentrations between day 1 and day 4 in the circulating water because the pH of the media slightly increases with the time due to the concentration of carbonate (CO$_3^{2-}$) or it might be due to the development of microorganisms at the later stage of the 4-day batch tests. Removal fractions were 70 % for aspirin, 78 % for ibuprofen, 100 % for caffeine, 89 % for bezafibrate and 88 % for estradiol. These fractions did not mean the pharmaceuticals were taken by algae alone. It might have lost in tubing and algal turf scrubber system. Concentrations of pharmaceuticals in water were decreasing with exposure time. However, carbamazepine concentration in the water column was constant, and no change. That is evidence that algae are not able to sorb the carbamazepine from the media. Four samples were measured for each data point.

![Figure 3-17. Concentrations of pharmaceuticals in water.](image)

Figure 3-17. Concentrations of pharmaceuticals in water. (■) carbamazepine, (■) estradiol, (■) caffeine, (■) bezafibrate, (■) aspirin and (■) ibuprofen.
3.3.10 Effect of Exposure Timing

Results show that most compounds had been adsorbed into algal biomass except carbamazepine. The concentrations of targeted pharmaceuticals in the algal biomass depend on exposure time and the initial concentration of the contaminant in the water. The results also indicate that in less than four days, biosorption of pharmaceuticals from water column may reach a steady level in the algal biomass. The concentrations of the pharmaceuticals were rapidly increasing with time of exposure.

The pharmaceutical concentrations in water column during the experiment decreased and confirmed that the algae were continually taking up contaminants from the water. It is likely that accumulation in algae occurred in proportion to the increased transpiration needs of a larger plant. It has been suggested previously that transpiration is the primary cause of contaminant translocation to tissues.58

3.3.11 Relative Biomass Density

Figure 3-18 and Figure 3-19 show the relative biomass density for algae exposed to mixtures of six pharmaceuticals at two different concentrations 3.5 mg/L and 5 mg/L. The mixture of pharmaceuticals in the circulating water had a negative effect on the algal biomass at both concentrations. However, the relative biomass density was not affected for unexposed algae. The relative biomass density was measured based on the total dried algal biomass in the specific area. Literature shows that aspirin inhibits phosphate uptake by the algae and that could be the cause of decreasing the relative biomass density. Also, algae contain ion channels and enzymes which could also be potentially targeted by pharmaceuticals and may initiate a response such as inhibition in the transport of essential elements required for growth.59 Previous research has highlighted the potential for pharmaceuticals to induce toxic effects on crop plants.60 The treated algae with pharmaceuticals showed relative biomass density values lower than the control ones and these differences were statistically significant (t-test < α =0.05).
3.3.12 Ash-CHN Analysis for Algae Exposed to Pharmaceuticals

Figure 3-20 shows the organic and inorganic carbon content for algae exposed to pharmaceuticals at 5760 min. The changes in the organic and inorganic content are mainly due to
breakdown of carbon-hydrogen bonds, resulting volatile loss and further concentrating the ash content in the biomass. It is suggested that each pharmaceutical will bind to the algal biomass through the functional groups on the surface of the cell walls. Reactivity of these pharmaceuticals are different based on their chemical structures, pH, and concentration. Some pharmaceuticals might be trapped between the algae layers and have more time to bind with algae on the turfs. pH was measured daily and was difficult to maintain. It was kept in range of 9 ± 0.5. This range might have allowed some pharmaceuticals to react. This discussion may explain why the organic and inorganic contents are not stable with the harvest time. At lower concentrations of pharmaceuticals, 3.5 mg/L, the inorganic content values were higher than that of higher concentrations. This change might be because binding the pharmaceuticals with the algal biomass.

Two factor ANOVA test was done to see if the P-value is greater than the significance level (0.05) was selected. The P-value for the exposed algae was 0.98. That means the effect of pharmaceuticals is not statistically significant.

![Graph showing organic and inorganic carbon content for algae exposed to pharmaceuticals at 5760 min.](image)

Figure 3-20. Organic and inorganic carbon content for algae exposed to pharmaceuticals at 5760 min. (■) Organic carbon and (□) inorganic carbon.

Figure 3-21 shows the organic, inorganic, hydrogen, nitrogen and ash compositions of algae exposed to a mixture of six pharmaceuticals (3.5 ppm). It is clear that the nitrogen content in all samples remains stable (7 ± 0.8 wt %) even with the exposure time. These results suggest that nitrogen is fixed, primarily as a protein, in the raw biomass. These sp2 bonds require more energy to break than sp bonds, causing nitrogen to be retained. Hydrogen as well stays constant during the experiments (5± 0.5 wt %).
The organic carbon content varies from a low of 25 ± 3 wt % to a high of 37± 2 wt %. The increase in organic contents during the exposure time are likely due to increase the pharmaceutical concentrations in the algae tissues. Inorganic contents during the exposure time varies from 10 wt % to 15 wt % at different exposure time. More than 50 % of the algal biomass is ash as shown in Figure 3-21. Oxygen content varies between 3 to 8 % of the biomass. The P- value was 0.99 and that means there is not statistically significant in the algal biomass during four days of exposure, P-value > 0.05.

![Figure 3-21.](image)

Figure 3-21. (■) organic, (□) inorganic, (▲) hydrogen, (▲) nitrogen, (■) ash and (■) oxygen compositions of algae exposed to a mixture of six pharmaceuticals (3.5 ppm).

Figure 3-22 represents the compositions of algae exposed to 5 ppm of pharmaceuticals. The data show that compositions of algae have lower values than of that algae exposed to 3.5 ppm of pharmaceuticals. It seems that the high concentrations of pharmaceuticals inhibit the uptake of the nutrients from the water column. Organic carbon and ash contents at 0 min and 1440 min are the lowest in this experiment and that might be because of the difference in the algal community, different algae have different percentage of lipids, proteins and carbohydrates. The oxygen content varies and has higher values than algae exposed to 3.5 ppm of pharmaceuticals. The P-value for this experiment was 0.98 and the effect of the mixture of pharmaceuticals is not statistically significant.
Figure 3-22. (■) organic, (□) inorganic, (■) hydrogen, (▲) nitrogen, (■) ash and (■) oxygen compositions of algae exposed to a mixture of six pharmaceuticals (5 mg/L).

Figure 3-23 shows that the organic content in the ibuprofen experiments is 29 ± 3 wt %. Inorganic content slightly increases with the exposure time and was 9 ± 4 wt % in the ibuprofen experiment. The total carbon (organic and inorganic carbon) content of the biomass stay same with harvest time. Hydrogen and nitrogen contents do not change during the exposure in both experiments. Oxygen content increases with the time of exposure but overall the P-value for this experiment is 0.99 and the effect of ibuprofen is not statistically significant, P-value > 0.05.

Figure 3-23. (■) organic, (□) inorganic, (■) hydrogen, (▲) nitrogen, (■) ash and (■) oxygen compositions of algae exposed to ibuprofen.
Figure 3-24 shows that the organic content in both experiments was 20 ± 2 wt %. Inorganic content stayed constant with exposure time and was 16 ± 2 wt %. The total carbon (organic and inorganic carbon) content of the biomass stayed without changing with harvest time. Hydrogen and nitrogen contents did not change during the exposure. The ash data shows the lowest in this experiment compared to other experiment when algae exposed to mixture of pharmaceuticals. The oxygen content does not change significantly during the experiment and it ranges between 20-26 wt %. The P- value for this experiment was 0.97 and the effect of bezafibrate is not statistically significant, P-value > 0.05.

Figure 3-24. (■) organic, (▲) inorganic, (□) hydrogen, (▲) nitrogen, (■) ash and (▲) oxygen compositions of algae exposed to bezafibrate.
Figure 3-25 shows that the organic-carbon contents decreased with harvest time and inorganic carbon contents increase when algae exposed to the mixture of ibuprofen and bezafibrate at 3.5 mg/L of each. The total carbon content did not change significantly. Hydrogen and nitrogen contents did not change during the exposure. The ash content decreased with the time of exposure where it was about 55% at time 0 min and went down to 40% at time 5760 min. The oxygen content increased during the experiment from 5 wt% to 10 wt%. The P-value for this experiment was 0.99 and the effect of this mixture is not statistically significant on algal biomass.

Figure 3-26 represents the compositions of algae exposed to pharmaceuticals at 5760 min. The pharmaceuticals had no effect on the total organic content. This might be caused by the
increasing of the concentrations of the pharmaceuticals inside of the algal cells, which they are organic chemical structures. In same time, the algal cells try to take the essential nutrients such as sodium and calcium from the media for photosynthesis process. Hydrogen and nitrogen contents did not change in all experiments. The oxygen content was estimated by difference and found the lowest oxygen content was for algae exposed to the mixture of six pharmaceuticals at 3.5 mg/L and the highest was for the algae exposed to bezafibrate. Bezafibrate might increase the dissolve oxygen in the algal cells. The P-value for this experiment was 0.92 (> $\alpha = 0.05$) and the effect is not statistically significant.

Figure 3-26. (■) organic, (□) inorganic, (■■) hydrogen, (■) nitrogen, (■■■) ash and (■■■■) oxygen compositions of algae exposed to pharmaceuticals for four days.

3.3.13 Effect of Pharmaceuticals on Chlorophyll Fluorescence

Before doing any chlorophyll experiments, chlorophyll was extracted from algae and then fluorescence was recorded as shown in Figure 3-27. Literature shows that chlorophyll is degraded at high temperature so to avoid this problem, all algal biomasses were dried at 105 ºC and chlorophyll was extracted from algae by acetone and it was found that the maximum fluorescence intensity was at 670 nm and that is in good agreement with the literature. The acetone used had no fluorescence intensity, no overlap, so all algae extractions were done in acetone.
Figure 3-27. Fluorescence of chlorophyll extracted from algae. (-) chlorophyll and (-) acetone.

Figure 3-28 and Figure 3-29 show that pharmaceuticals had a small effect on the fluorescence of chlorophyll. As expected untreated algae had the same chlorophyll intensities at different harvest times. Low or high concentrations of six or two pharmaceuticals showed no effect. Chlorophyll intensity of algae exposed to ibuprofen or bezafibrate had no change. It was expected that a little amount of the pharmaceuticals did not affect the algal biomass structure. The t-test for a mixture of six low concentration pharmaceuticals (3.5 ppm), high concentration (5 ppm) and the mixture of ibuprofen and bezafibrate (3.5 ppm each) were 0.69, 0.58 and 0.018, respectively. All t test values were greater than $\alpha = 0.05$ so and the effect of these combinations of pharmaceuticals is not statistically significant. The student’s t test value for ibuprofen and bezafibrate were 0.088 and 0.30 which also greater that $\alpha = 0.05$ so we all accept the null hypothesis meaning the pharmaceuticals did not affect the chlorophyll fluorescence intensity.
3.3.14 Effect of Pharmaceuticals on Chlorophyll Absorbance

Pharmaceuticals and hormones are in surface waters, but their effects on aquatic organisms and associated ecosystem properties are not well understood. To the best of our knowledge this
research will be the first one to study the effect of pharmaceuticals on algae by extracting chlorophyll and comparing the testing results to those of chlorophyll extracted from unexposed algae. Responses of algae community to six common pharmaceutical compounds (aspirin, caffeine, carbamazepine, ibuprofen, estradiol, and bezafibrate) were measured. Figure 3-30 and Figure 3-31 show chlorophyll absorbance during the exposure time. Chlorophyll absorbance for the control experiment should stay constant because algae are not exposed to pharmaceuticals. UV-Vis absorbance was measured based on the area of the samples not on the biomass weight. With that in mind, absorbance intensities increase with exposure time with all tested experiments; low and high mixtures of six pharmaceuticals, a mixture of ibuprofen and bezafibrate, and ibuprofen and bezafibrate alone. Chlorophyll absorbance for the control experiment stayed constant during the experiments. With time, pharmaceuticals in the experiment improved the chlorophyll absorbance intensities and that might be because pharmaceuticals kill the bacteria, marine viruses, and other aquatic organisms that live on the algae biomass. Error bars in the graph are based on three turfs and three samples at each time. Nine samples that were prepared for each data point except for the control where one turf and three samples that were prepared for each data point.

![Figure 3-30. Effect of pharmaceutical(s) on chlorophyll absorbance](image)

The t-test values for a mixture of six low concentration pharmaceuticals, high concentration and the mixture of ibuprofen and bezafibrate were 0.67, 0.077 and 0.19, respectively. All t test
values were greater than $\alpha = 0.05$ so the effect of these combinations of pharmaceuticals is not statistically significant. The t test value for ibuprofen and bezafibrate were 0.20 and 0.23 which also greater that $\alpha = 0.05$ so we all accept the null hypothesis meaning the pharmaceuticals did not affect the chlorophyll absorbance.

![Absorbance vs Harvest time](image)

Figure 3-31. Effect of pharmaceutical(s) on chlorophyll absorbance (■) control, (▲) ibuprofen and (■) bezafibrate.

### 3.4 Summary

Ibuprofen, estradiol and other pharmaceutical derivatives were detected in the algal extracts substantiating that pharmaceuticals in water are transferred into algae biomass. Other algae biomasses did not show any pharmaceuticals in the extracts that could be due to three reasons; 1) low concentration of the pharmaceuticals in the algae biomass, 2) no pharmaceuticals in the water where algae grew and 3) a different extraction method is needed. This observation may be a good approach to remove pharmaceutical contaminants in wastewater treatment plants by algae, where algae is easily and quickly grown. An algal turf scrubber system was built in the lab to grow algae for 21 days and then exposing the algae to a mixture of pharmaceuticals and then analyzing the algae biomass after specific times. Then study the effect of the time of exposure of pharmaceuticals on algae and evaluate the algae biomass. Two known pharmaceuticals, ibuprofen and estradiol, were observed in the algae extracts.
The degree of uptake varied among pharmaceuticals. The results show that algae have the ability to eliminate pharmaceuticals from the water column. Algae have different capacities for each of aspirin, caffeine, ibuprofen, bezafibrate and β-estradiol. The investigated pharmaceuticals indicate different removal rates during the treatment. Comparing the results, caffeine and estradiol evinced the best removal rates. In contrary, carbamazepine appeared to stay in the water, what confirms the results of sorption of algae in lab scale experiments. That is generally in good agreement with reported literature. The effects of exposure to these pharmaceuticals on algae and other aquatic life are largely unknown. High research costs may influence the lack of study concerning these types of products, but our data could help understand some facts about the presence of these pharmaceuticals in the water. It was noticed that algae have different abilities to take these contaminants from the water column and in the same amount of time the relative biomass density that was exposed to the mixture of pharmaceuticals was decreased. The relative biomass that was not exposed to contaminants was constant.

The t test values did not show any statistically significant for the compositions of the algal biomass exposed to pharmaceuticals. Hydrogen and nitrogen contents did not change during the time of exposure as well as types of contaminants.

Pharmaceuticals had little or no effect on the chlorophyll fluorescence intensity and absorbance. Control experiments showed the same chlorophyll fluorescence intensities at different harvest times. Chlorophyll intensity of algae exposed to ibuprofen or bezafibrate were changed slightly as shown in the plots. UV-Vis absorbance was measured based on the area of the samples not on the biomass weight. Chlorophyll absorbance for the control experiment stayed constant during the experiments. With time, pharmaceuticals improved the chlorophyll absorbance intensities and that might be because pharmaceuticals kill the bacteria, marine viruses and other aquatic organisms that live on the algal biomass. The mixtures of six pharmaceuticals did not change the chlorophyll absorbance but ibuprofen, bezafibrate and their mixture increased the chlorophyll absorbance with increased exposure time. However, the t test values showed the effect was not statistically significant.
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CHAPTER 4
BIOSORPTION OF SILVER, GOLD NANOPARTICLES AND METAL IONS FROM WATER COLUMN BY PERIPHYTIC ALGAL TURFS

4.1 Objectives

The objectives of this chapter were 1) To prepare silver and gold nanoparticles based on previous research publications and identify them using UV-Vis spectroscopy, fluorescence and TEM techniques. 2) To study the biosorption of the synthetic nanoparticles by algal biomass communities from the water column. 3) To study the length of the exposure effect on algae of the silver or gold nanoparticles. 4) To compare concentrations of the silver and gold nanoparticles and silver and gold ions in the biomass and water at predetermined times. 5) To study the effect of nanoparticles and ions on biomass density and compare the densities.

Algal turfs were exposed to nanoparticles with a concentration of 2.475 mg/L (AgNPs) and 4.475 mg/L (AuNPs) then a silver/gold mixture at a low concentration (2.475 mg/L of AgNPs; 4.475 mg/L of AuNPs) and high concentration (5 mg/L of AgNPs; 9 mg/L of AuNPs).

After the exposure, specific studies were done as listed below:

I. Test the algal biomass after exposure to silver and gold nanoparticles and their ions using techniques dependent on carbon, nitrogen and hydrogen elements, and chlorophyll a.

II. To prepare a paracetamol-gold nanoparticle composite and locate this composite in the algae biomass-at the cell wall or in the cell cytoplasm.

III. To investigate the intracellular and extracellular biosorption processes of target contaminants, which usually occur through two mechanisms;

a. Intracellular where metals are taken inside the cell body or
b. Extracellular where nanoparticles/metal ions bind to functional groups on the cell wall.

IV. To feed bloodworms algae containing gold nanoparticles to see if the nanoparticles in the algae move to the bloodworms tissue.

4.2 Fluorescence Bioimaging

Fluorescence bioimaging probably is the most widespread method in biosciences because it is sensitive and selective.\(^1\) Researchers in the last 20 years have developed a huge increase in resolution.\(^2,3\) Using synthetic fluorescent nanoparticles is the simplest method and it is basically
using strong fluorescent nanoparticles to enter cells so that they can be imaged. For example, paracetamol dimer-gold nanoparticles have been used as rapid, simple and sensitive detection of bacterial contamination for public health and the environment.\textsuperscript{4} Literature shows that the paracetamol dimer in the composite showed emission peak at 435 nm when excited at 320 nm.\textsuperscript{4} A fluorescence based method would provide an ideal background free platform in this regard. However, because of their surface free energy it was anticipated that gold nanoparticles could be attached to the cell wall of algae or pass the cell walls into the cytoplasm. The composite of paracetamol-AuNPs would serve the purpose of finding the location of the nanoparticles in the algae biomass.

4.3 Bloodworms

It was found that algae have the ability to uptake the heavy metals and pharmaceuticals from the water column.\textsuperscript{40, 5} bloodworms were used to see if the nanoparticles transfer to another trophic level. Bloodworms are the larval form of one of chironomid midges; insects rather than worms.\textsuperscript{6} They grow wild in ponds, pools, and lakes and live in the mud. Bloodworms are usually found on the bottom of shallow waters. They can grow up to 35 millimeters in length. Bloodworms are widely harvested along the Northeastern coast of the United States in fishing.\textsuperscript{7}

![Figure 4-1. Bloodworms used in the study (Chironomidae sp.).](image)

4.4 Results and Discussion

4.4.1 Characterization of Silver and Gold Nanoparticles

Different methods were used to characterize prepared nanoparticles. The most common methods used are Ultraviolet-Visible (UV-Vis) Spectroscopy and Transmission electron
microscopy (TEM). UV-Vis Spectroscopy is used to maximize the peak of the nanoparticles and can be used to estimate the size of the nanoparticles. TEM is used to take pictures of the nanoparticles and find the size distribution.

Figure 4-2 shows the maximum wavelengths of prepared silver and gold nanoparticles match with what have been mentioned in the literature. The maximum wavelength of silver nanoparticles was 410 nm and gold nanoparticles 520 nm.

Figure 4-2. Absorbance spectra of silver and gold nanoparticles synthesized. (--) AgNPs and (-) AuNPs.

Figure 4-3 and Figure 4-4 show the characterization of AgNPs and AuNPs in deionized water by TEM and size distributions. Nanoparticles size distributions were calculated by hand by drawing three squares with an area of 1 cm$^2$ on each TEM image. TEM images showed that the AgNPs and AuNPs are spherical and their sizes are between 5 to 20 nm. Analyses were performed using JEOL 2100 Field Emission Gun TEM. Samples were prepared by placing a drop of the nanoparticles on the TEM grid in a petri dish and then left for two days to dry out. This is commonly observed in previous studies using the same manufacturing procedure.
4.4.2 Characterization of the PA-AuNP Composite

Different methods have been used to characterize prepared nanoparticles. The most common methods used are UV-Vis and fluorescence spectroscopy that is used to maximize the peak of the nanoparticles and can be used to estimate the size of the nanoparticles. The composite was synthesized by reacting paracetamol (p-hydroxyacetanilide) with HAuCl₄.

The UV-Vis spectrum showed a plasmon peak of AuNPs at 525 nm. It should be mentioned here that the amount of unreacted paracetamol was less as compared to its dimer. Due to the presence of paracetamol dimer (two molecules of paracetamol link to AuNP), the composite showed a strong blue emission peak at 435 nm when excited at 320 nm, as is characteristic of the emission of the paracetamol dimer (5,50-bis(acetylamino)-2,20-dihydroxy biphenyl).
Figure 4-5. UV-Vis spectra of PA-AuNPs composite.

Figure 4-6. Fluorescence Intensity of PA-AuNPs composite.

Figure 4-7 shows the confocal laser pictures of green small dots of PA-AuNP dispersed in the nanoparticle solution. Micrograph for untreated algae did not show any green fluorescent nanoparticles. On the other hand, the PA-AuNPs were most likely inside the algal biomass cells where the nanoparticles appeared with fluorescents with bright green color. Literature studies reported that the main mechanism of metal removal is based on intracellular bioaccumulation.\textsuperscript{10} Low extracellular bioaccumulation in this study resulted from the poor proton binding capability of algal biomass surface, and it was difficult to have PA-AuNPs attach to algal cell walls because
of the small size of the nanoparticles. Intracellular bioaccumulation was clearly observed because of the high concentration of the nanoparticles inside the algal cells.

Figure 4-7. Algae community under confocal laser microscopy (top left is PA-AuNPs and top right is unexposed algae and bottom is algae exposed to PA-AuNP).

4.5 Biosorption of Silver and Gold Nanoparticles/Ions in Algae

Figure 4-8, Figure 4-9 and Figure 4-10 show the concentrations of noble nanoparticle metals in harvested algae. The high initial metal concentration in the media and the abundant availability of active sites on the surface area at the beginning of the treatment has positive effects on the rate of removal. These effects because the algae have a significant number of micro-pores and/or meso-pores responsible for an efficient diffusion to micro-pores. The lower metal concentration and the gradual occupancy of the active sites result in a weaker driving force and a less efficient sorption process, the rate of which depends on ion diffusion through the pores of the algae. The rate of metal sorption on algae, i.e. removal efficiency from the water phase, depends
on the nature of the metal. This relates to the ability for making complexes with functional groups on the algae surface, as well as to the stability in aqueous systems.

Figure 4-8. Biosorption of noble metals by algae. (■) AgNPs, (■) AuNPs, (■) AgNPs and (■) AuNPs in the mixture and (■) control.

Figure 4-9. Biosorption of noble ions by algae. (■) Ag (I) ions, (■) Au (III) ions and (■) control.
Silver nanoparticles, silver ion, gold nanoparticles and gold ions show a higher tendency for binding to oxygen functional groups of algae; therefore, it is the metal which is most efficiently removed from the water phase. In addition, differential biosorption of the ions may be ascribed to the differences in their ionic radii. The ionic radius of Au$^{3+}$ is 99 pm, while that of Ag$^{+}$ is 129 pm.\textsuperscript{11} According to the literature, the smaller the ionic radius, the greater its tendency to pass through the pores in algal cell walls into the cells. However, it can be seen the gold ion has the greater biosorption compared to that of the silver ion. That might result for two reasons 1) because the small size of the ion has more transference into the cell and the bigger size refuses to enter to the cell because of the porosity of the cell wall and, 2) the high positive charge on the gold (III) ion makes it have more ability to bind with carboxyl or hydroxyl group on the algae cell walls. After two days of the process, metal concentrations in the circulating water remain unchanged, or changes are negligible.

Many factors may influence the bioavailability of metals in algae. Regarding the physicochemical parameters, the main factors are pH, salinity, temperature, light, particulate matter, and organic matter. Besides the variations in the available metal concentrations in the
water, other factors such as water conditions, the stage of development and the variation in growth and chemical composition of the algae may influence the pattern of sorption.

4.5.1 Adsorption Kinetics

In order to clarify the adsorption kinetics of silver and gold nanoparticles into algal turf communities, a pseudo-first order kinetic model was applied to the experimental data. Figure 4-11 shows the pseudo-first-order model for the kinetics of gold nanoparticles adsorption on algae.

![Figure 4-11](image)

**Figure 4-11.** Concentrations of gold nanoparticles in (■) algal biomass and (■) water. Some error bars (S.D to the average) are smaller than the symbol.

Because not all data sets continue sufficient shot-time measurement, an initial-rates analysis was not universally feasible. Thus, we performed a least-squared analysis over the entire time sequence data using the first-order equation for product formation, solving for $C_{eq}$ and $k$ as fit parameters.

$$C_t = C_{eq}(1 - e^{-kt}) \ldots \ldots \text{Equation 4-1}$$

Examples are shown in Figure 4-12 and Figure 4-13. The rate constant, $k$, of this equation is listed in Table 4-1. It is clear from the results that the adsorption of noble metals onto algal turf communities follows well the pseudo-first-order kinetics.
Figure 4-12. AgNPs concentration against harvest time. (■) experimental data, (−) fitted curve.

Figure 4-13. Gold nanoparticle concentration against harvest time. (■) experimental data and (−) fitted curve.

Table 4-1. Rate constants of noble metal removal by algae at different initial concentrations.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Rate constants / mg.L⁻¹.min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 4.475 mg/L</td>
</tr>
<tr>
<td>AgNPs/alone</td>
<td>-</td>
</tr>
<tr>
<td>AgNPs in the mix</td>
<td>-</td>
</tr>
<tr>
<td>AuNPs/alone</td>
<td>0.00832</td>
</tr>
<tr>
<td>AuNPs in the mix</td>
<td>0.122617</td>
</tr>
<tr>
<td>Ag (I)</td>
<td>-</td>
</tr>
<tr>
<td>Au (III)</td>
<td>0.00811</td>
</tr>
</tbody>
</table>
4.5.2 Effect of Contaminant Dosage

Figure 4-8 and Figure 4-10 show the effect of nanoparticle dosage on the adsorption of AgNPs and AuNPs. Contaminant dosage is one of the important parameters in adsorption processes because it determines the capacity of an adsorbent for a given initial concentration of the adsorbate under a given set of operating conditions, water flow rate and temperature. As can be seen in these figures, the concentrations of silver and gold nanoparticles increase with increasing adsorbent dosage from 560 µg/g to 2300 µg/g for silver nanoparticles and 1350 µg/g to 4600 µg/g for gold nanoparticles at the last day of experiment. The increase in the removal efficiency may be attributed to the fact that, with an increase in the adsorbent dosage, more adsorbent surface is available for the solute to be adsorbed. These figures show that the adsorption uptake becomes almost constant after 24 hours of exposure for both nanoparticles.

4.6 Noble Nanoparticles/Ions in Water Analysis

Water analysis was done at the same time intervals as that in the nanoparticles in algae as shown in Figure 4-14 and Figure 4-15. Before spiking the nanoparticles to growth media, samples were taken for analysis to see if there was any interaction between the growth media and noble metals in this study. After adding the nanoparticles into the media, water samples were also taken. All samples were stored in 50 mL vials at 15 ºC in the refrigerator until the time of doing the digestion and analysis. It was found that the concentration of silver and gold ions decreased with the time of exposure.

The residual metal concentration in aqueous solution was determined after an exposure time of 0, 60, 1440, 2880 and 5760 min. Metal uptake, as a function of contact time, was noticed to occur in two phases. The first phase was extremely rapid or all investigated metals as nanoparticles or ions. The maximum adsorption efficiency was observed in the first 60 min of metal-algae contact, which was followed by a slow phase of metal removal spread over a longer period until equilibrium was reached after 24 hours.

Figure 4-15 indicate that the noble metal concentration in aqueous solution decreases rapidly during first 60 min and remains nearly constant after 1440 min for nanoparticles and ions, suggesting that the adsorption of both metals is fast.
Figure 4-14. Reduction of nanoparticles in water column. ( ) AgNPs, ( ) AuNPs, ( ) AgNPs in the mixture and ( ) AuNPs in the mixture and ( ) control.

Figure 4-15. Reduction of metal ions in the water column. ( ) Ag (I) ions, ( ) Au (III) ions and ( ) control.
4.6.1 Mass Balance Calculation of Silver Nanoparticles

The initial concentration of the silver nanoparticles used was 2.475 mg/L in 20 L of water. Silver mass in this solution was equal to $2.475 \text{ mg/L} \times 20 \text{ L} = 49.5 \text{ mg}$ of silver.

The total algal biomass on one the turf was about 26.5 g.

The silver concentration detected in algae biomass ranges 450 µg to 550 µg/g of dried algae. In this case the silver recovered from the total mass algae is

$$0.5 \text{ mg/g} \times 26.5 \text{ g} = 13.25 \text{ mg}$$

The percentage of silver removal = \left[\frac{13.25}{49.5}\right] \times 100 = 26.7 \%$

This removal percentage is for a four day experiment and length of the turf is 1.21 m. The concentration of silver nanoparticles in the water column reaches to approximately zero. The silver mass was not totally transferred to the algal biomass. About 73.3 % silver mass was missed in our analysis probably because the silver mass was between the layers of the algal biomass and was washed out during the preparation of the algal biomass to analyze silver nanoparticles. Nanoparticles may have adhered to surface of troughs, tubing or pumps. Algae on surfaces were not included in these calculations and incomplete collection of algal biomass during harvesting.
4.7 Relative Biomass Density

Biomass density was measured to test the effect of the silver and gold nanoparticles and silver and gold ions in the media on the algae. It seems that both silver nanoparticles and ions do not have an effect on the biomass density and relative densities stay constant as we can see in Figure 4-17 and Figure 4-18. On the other hand, both gold nanoparticles and ions have a negative effect on the biomass density, as shown in Figure 4-17 and Figure 4-19. That might be due to the bigger sizes of silver nanoparticles and ions, the ionic radius of $\text{Au}^{3+}$ is 99 pm, while that of $\text{Ag}^+$ is 129 pm, which might bind to carboxyl or other functional groups on the algal cell walls and they do not have the ability to enter to the algal cell. On the other hand, small sizes of gold nanoparticles and ions can move inside the algae cells and could change the biostructure of the cells and stop or decrease the reproduction process in the cells. The algae treated with gold nanoparticles and gold ions showed relative biomass density values lower than the control ones and these differences were statistically significant ($t$-test $< \alpha = 0.05$).

![Graph](image.png)

Figure 4-17. Relative biomass density of algae exposed to (▲) AgNPs, (■) AuNPs (■) Mixture of NPs and (▲) control.
Figure 4-18. Relative biomass density of algae exposed to (■) Ag (I) ions and (□) control.

Figure 4-19. Relative biomass density of algae exposed to (■) Au (III) ions and (□) control.
4.8 Ash-CHN Analyses for Algae Exposed to Nanoparticles

The organic carbon content was obtained and estimated directly by burning a specific quantity of dried algae at 500 °C. The inorganic carbon content was obtained by burning the leftover samples at 500 °C to 850 °C to constant weights. It was expected that the inorganic contents would increase with the time of exposure to both nanoparticles as well as silver and gold ions because these heavy metals have more resistance to the heat, and they have high melting points compared to organic compounds.

Figure 4-21 shows the organic contents vary 7 wt % to 23 wt %. The inorganic contents vary between 6 ±2 wt % to 10 ± 3 wt % for the algae biomass exposed to AgNPs at 2.475 mg/L. The algae exposed to silver and gold nanoparticles have more inorganic contents.

For comparison, silver and gold ions at the same concentrations as silver and gold nanoparticles, respectively, were examined. It was expected that the positive charges on each of the silver (I) and gold (III) ions bind strongly to the hydroxyl-carboxylic groups in cell walls. The silver and gold ion experiments had similar inorganic contents as the control, unexposed algae, and lower organic contents. The data came with the opposite of the expectations. Two factor ANOVA was done to check if there was statistically significant. It was found that the P-value was 0.608 so there is no statistically significant, P-value > 0.05.
Figure 4-21. Organic and inorganic carbon content for algae exposed to heavy nanoparticles and ions at 5760 min. (■) Organic carbon and (■) inorganic carbon.

Figure 4-22 and Figure 4-23 show that silver and gold nanoparticles, respectively, increase the contents of organic and inorganic contents. Hydrogen and nitrogen contents did not change during the exposure time. This might be because the increasing of silver nanoparticle concentration in the algae biomass during the harvest time, where the highest concentration of silver nanoparticles was 500 µg/g algae and for gold nanoparticles was 1400 µg/g algae after four days of exposure. The P-values for the algae exposed to AgNPs (2.475 ppm) and AuNPs (4.475 ppm) were 0.90 and 0.95, respectively. That means the effect of AgNPs and AuNPs was not statistically significant.
Figure 4-22. (■) organic, (○) inorganic, (□) hydrogen, (▲) nitrogen, (■) ash and (▲) oxygen compositions of algae exposed to AgNPs (2.675 ppm).

Figure 4-23. (■) organic, (○) inorganic, (□) hydrogen (▲) nitrogen, (■) ash and (▲) oxygen compositions of algae exposed to AuNPs (4.95 ppm).

Figure 4-24 shows that the effect of the low concentrations of mixture of AgNPs and AuNPs on the algae biomass, 2.475 and 4.475 ppm respectively. This mixture of nanoparticles did not change the contents of organic, inorganic, hydrogen and nitrogen for the first two days, but the data obtained showed the organic and inorganic contents of the elements decreased. Hydrogen and nitrogen contents did not change. The oxygen content varied from 25 wt % to 30 wt %. The P-
value for the algae exposed to the mixture was 0.99. That means the effect of the mixture of nanoparticles was not statistically significant.

Figure 4-24. (■) organic, (▲) inorganic, (□) hydrogen (▲) nitrogen, (■) ash and (●) oxygen compositions of algae exposed to a mixture of AgNPs & AuNPs (2.475 & 4.95 ppm).

Figure 4-25 shows that the effect of the high concentrations of mixture of AgNPs and AuNPs on the algae biomass, 4.95 and 9 ppm respectively. This mixture of nanoparticles decreased the contents of organic and inorganic after one hour of exposure. The data obtained show the hydrogen and nitrogen contents did not change with the exposure time. The P-value for the algae exposed to this mixture was 0.99. That means the effect of high concentrations of NPs was not statistically significant.
Figure 4-25. (■) organic, (□) inorganic, (■) hydrogen (■) nitrogen, (□) ash and (■) oxygen compositions of algae exposed to a mixture of AgNPs & AuNPs (4.95& 9 ppm).

Figure 4-26 and Figure 4-27 show the organic, inorganic, hydrogen and nitrogen content in algae exposed to silver and gold ions, respectively. The data show that silver and gold ions do not change the organic, hydrogen and nitrogen contents in the algae biomass but inorganic content decreases with the exposure time. The P- values for the algae exposed to Ag (I) and Au (III) ions were 0.97 and 0.99, respectively. That means the effect of silver and gold ions was not statistically significant.

Figure 4-26. (■) organic, (□) inorganic, (■) hydrogen (■) nitrogen, (□) ash and (■) oxygen compositions of algae exposed to Ag (I) ions (2.475 ppm).
Figure 4-27. (■) organic, (□) inorganic, (■) hydrogen (■) nitrogen, (■) ash and (■) oxygen compositions of algae exposed to Au (I) ions (4.475 ppm).

Figure 4-28 shows the algae exposed to silver and gold nanoparticles and ions in the last day of experiment, 5760 min. Organic content was the highest for the control sample, algae is not exposed. The lowest organic content was for algae exposed to the mixture of high concentrations of silver and gold nanoparticles. Inorganic content increased slightly for exposed algae, which was the highest for algae exposed to silver nanoparticles, 2.475 mg/L. The hydrogen and nitrogen contents were not changed with the different contaminants. These data were obtained by repeating the experiment three times for each data point and stander deviation was calculated using Microsoft excel. The P-values for the algae exposed to noble metals were 0.264. That means the effect of noble heavy metals was not statistically significant.
Figure 4-28. (■) organic, (□) inorganic, (▲) hydrogen (●) nitrogen, (■) ash and (★) oxygen compositions of algae exposed to heavy nanoparticles and ions.

It was noticed in some experiments that the data jumped higher or lower than was expected so some comparison between the data obtained from the CHN instrument and the data on the EDTA label was made. The results are shown in Table 4-2. The data of the EDTA standard obtained from the instrument are higher than that of the EDTA label so the obtained data was adjusted by multiplying them by a factor according to the equation (label data/obtained data).

Table 4-2. Comparison between EDTA data obtained from the instrument and label.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>weight [mg]</th>
<th>C</th>
<th>H</th>
<th>N</th>
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<td>41.09</td>
<td>5.51</td>
</tr>
</tbody>
</table>

4.9 Effect of Nanoparticles/Ions on Chlorophyll Fluorescence

Algae exposed to nanoparticles show different data than that of pharmaceuticals. Fluorescence intensity of algae exposed to 2.475 ppm silver and 4.475 ppm gold nanoparticles decreases as the time from harvest increases. It appears that the fluorescence intensity has no
change in the first hour of the exposure, but change is appearance in the end of the experiment at 5760 min. A mixture of both nanoparticles has a negative effect on the fluorescence intensity as shown in Figure 4-29 and Figure 4-30 where the intensity is lower than that of algae exposed to singlet nanoparticles. A mixture of high concentrations of nanoparticles (4.475 ppm of AgNPs and 9 ppm of AuNPs) has more effect on the algae than low concentrations (2.475 ppm of AgNPs and 4.475 ppm of AuNPs).

A study confirmed toxicity of low concentration $10^{-5}$ M of AgNPs for a microalgal culture. Toxicity of AgNPs for bacteria was studied and the mechanism of this effect is not clear. It is assumed that the toxic effect of AgNP may be associated with the damage of the cell membrane, oxidative stress, or interaction of Ag (I) ions with proteins and enzymes.

![Figure 4-29. Fluorescence intensity of chlorophyll exposed to silver and gold nanoparticles as a mixture at different concentrations. (■) AgNPs and (■) AuNPs.](image)

The t-test value for algae exposed to silver and gold nanoparticles were calculated and were 0.05 and 0.000128, respectively, which are equal to P-value for AgNPs and lower than P-value for AuNPs so there are statistically significant.
Figure 4-30. Chlorophyll fluorescence intensity of exposed to AgNPs and AuNPs as a mixture. (■) the mixture of 2.475 ppm of AgNPs and 4.475 ppm of AuNPs and (■) the mixture of the mixture of 5 ppm of AgNPs and 9 ppm of AuNPs.

The t-test value for algae exposed to the low mixture of 2.475 ppm of AgNPs and 4.475 ppm of AuNPs was calculated and found 0.096 greater than P-value. The t-test value for algae exposed to the low mixture of 5 ppm of AgNPs and 9 ppm of AuNPs was 0.49. The t-test values were greater than the P-value so there are not a statistically significant.

For comparison, silver and gold ions were studied to expose the algae. The same concentrations of nanoparticles were used, 2.475 ppm of Ag (I) and 4.475 ppm of Au (III). Fluorescence intensity for untreated algae was also measured. Figure 4-31 shows that fluorescence intensity of extracted chlorophyll from algae in the control experiment are stable during the exposure time. Both silver and gold ions lower the fluorescence intensity as shown in Figure 4-31. Silver and gold ions at 2.475 and 4.476 mg/L, respectively, reduced chlorophyll a fluorescence intensities to 50 % and 30 %, respectively, after being 75 % in comparison to the control after four days into the experiment. We expected that the fluorescence intensity for algae exposed to silver and gold ions at -60 and zero min would have the same values as the control, where there were no silver and gold ions in the medium. This might be because of the previous experiments where a few of the ions or nanoparticles could have been remained in tubing or pumps or might be because the growth rates of the algae on the turfs are different after a month of the experiment.
The t-test value for algae exposed to 2.475 ppm of Ag (I) was 0.56 and to 4.475 ppm of Au (III) was 0.021. The t-test values were greater than the P-value so there are not a statistically significant.

![Figure 4-31](image_url)

Figure 4-31. Fluorescence intensity of chlorophyll exposed to silver and gold ions and control. (■) Ag (I) ions, (■) Au (III) ions and (□) control.

### 4.10 Effect of Nanoparticles/Ions on Chlorophyll Absorbance

Figure 4-32 shows the response of algae community to NPs. Nanoparticles, between day 0 and 4 in a mixture of nanoparticles or alone, showed different effects on chlorophyll content.\(^{18}\) The absorbance intensity of silver nanoparticles is unstable since it decreases and increases from time to time, but overall the absorbance was increased slowly. The absorbance intensity of low-concentration mixtures of nanoparticles. Intensities at -60 and zero min should be the same, but in Figure 4-32 the intensity at zero min is higher than at -60 min. This could be because the water flow in the beginning of the turf might cause changes in the chemical structure of the algae biomass at -60 min. A literature study showed that the AgNPs concentration has a negative effect on the chlorophyll content after 16 days of an experiment, where the chlorophyll content decreases with the increasing of AgNPs concentration.\(^{18}\)

Comparison cannot be discussed among AgNPs, and both low and high concentrations of NPs because algal turfs of these experiments were done at different dates and temperatures. Algae
were left to grow for three weeks before exposure to the nanoparticles and about a month during data collection.

![Extracted chlorophyll absorbance from algae exposed to silver and gold nanoparticles at different concentrations.](image)

The algae exposed to the mixture of silver and gold nanoparticles, 2.475 ppm and 4.475 ppm respectively, showed the chlorophyll absorbance values lower than the control ones and these differences were statistically significant ((t-test = 0.0015 < P).

Figure 4-33 shows that untreated chlorophyll absorbance is stable during four days of experiment. Absorbance of chlorophyll extracted from algae exposed to silver and gold ions is decreased with exposure time. Algae exposed to silver ion had absorbance higher than that of algae exposed to gold ion in most cases. Metal accumulation in the algae bodies increased with exposure times as seen in this chapter. Silver and gold ion sorption showed a significant inhibitory effect on chlorophyll a in algae biomass. Chlorophyll contents at -60 and zero min had similar absorbance, but at 1 and 3 mins the absorbance is higher than at time zero. Chlorophyll absorbance of algae exposed to gold ion was lower than that of algae exposed to silver ion. This indicates a more destructive effect of gold metal that silver metal on the chlorophyll contents. High concentrations of gold ions might activate oxidative damage and change cell-membrane properties by lipid
peroxidation as a cupper ion\textsuperscript{19}, resulting in the inhibitory effect on the enzymes involved in chlorophyll production.

Figure 4-33. Extracted chlorophyll absorbance from algae exposed to silver and gold ions and control. (\textcolor{red}{\textbullet}) Ag (I) ions, (\textcolor{blue}{\textbullet}) Au (III) ions and (\textcolor{green}{\textbullet}) control.

\section*{4.11 Bloodworms}

The results show that the nanoparticles were not detected in atomic absorption because the nanoparticle concentration was below the detection limit but the confocal fluorescence micrograph showed a few of fluorescent nanoparticles in the bloodworms. That means that nanoparticles translocated from the algae to the bloodworms. The nanoparticles were translocated to the bloodworms that were exposed directly to PA-AuNPs solution, see Figure 4-34.

For comparison, the concentration of AuNPs in the bloodworm was 0.9545 mg/L = 0.0595 mg/g algae or 59.51 µg/g algae and the concentration of AuNPs in the algae was 1.4091 mg/L = 0.1155 mg/g algae or 115.5 µg/g algae. The concentration of AuNPs in algal biomass was almost twice (1.94) higher than that of bloodworms. It is believed that algal biomass is a better adsorbent material than bloodworms.
Figure 4-34. Bloodworms exposed to PA-AuNP in water and algae exposed to PA-AuNPs.

Confocal laser fluorescence images show that the nanoparticles appear very clear in the bloodworms exposed to PA-AuNP composite solution, as shown in Figure 4-35 but nanoparticles do not appear clearly as in the solution in the bloodworms exposed to algae containing PA-AuNP composite, as shown in Figure 4-36. This might be because the high concentration of PA-AuNP in the solution (4.728 mg/L) comparing to PA-AuNP concentration in the algae (1.182 mg/L). Confocal laser fluorescence images were enhanced using Photos software in Windows 10.

Figure 4-35. Bloodworms exposed to PA-AuNPs composite solution under confocal laser fluorescence.
4.12 Summary

In the current study, we characterized important parameters that determine the process of heavy metal sorption as nanoparticles or ions, individual or as a mixture. Biosorption of noble nanoparticles (AgNPs and AuNPs) was dependent on the following experimental conditions: contact time of NPs-algae and the initial concentration. Metal biosorption was extraordinarily fast, reaching maximum removal in the first 1440 mins (24 hrs). The initial concentration strongly affects the sorption process and it was concluded that adsorption is favored by an increase of concentration, with a concentration value of about 5 mg/L of AgNPs and 9 mg/L of AuNPs. The maximum biosorption of gold, silver nanoparticles and ions took place in the first two days of exposure. Based on the reported observations is recommended for use as an efficient and low-cost agricultural material for the removal of heavy metals from effluents. The general metal concentration order among all samples tested was AuNPs ≈ Au (III) > AgNPs ≈ Ag (I).
Relative biomass densities were evaluated for the algae exposed silver nanoparticles at 2.475 and 4.475 mg/L and gold nanoparticles at 5 and 9 mg/L and silver and gold ions at 2.475 and 4.475 mg/L, respectively. The data showed that AgNPs and Ag (I) ions have no effect on the relative biomass density but AuNPs and Au (III) ions have a larger effect. Gold nanoparticles and gold ions decreased the relative biomass density.

The organic carbon and inorganic contents were obtained and estimated directly by burning a specific amount of dried algae at 500 °C and 850 °C, respectively. The organic carbon and inorganic carbon contents varied depending on the contaminant. For comparison, silver and gold ions at the same concentrations as silver and gold nanoparticles, respectively, were examined and they had similar inorganic contents as the control and lower organic contents.

CHN data did not show any impact on heavy nanoparticles or ions in the algae biomass. This might be because the low concentration of silver and gold nanoparticles in the algae biomass, where the highest concentration of silver nanoparticles was 500 µg/g algae and for gold nanoparticles was 1400 µg/g algae, silver ion was 1600 µg/g algae and gold ion was 2000 µg/g algae after four days of exposure.

It is clear that fluorescence intensity of algae exposed to 2.475ppm silver and 4.475ppm gold nanoparticles decreased with the harvest time. Fluorescence intensity had no change in the first hour of the exposure, but it did change in the end of the experiment. A mixture of nanoparticles had a negative effect on the fluorescence. A mixture of high concentrations of nanoparticles had more of an effect on the algae than low concentrations.

Nanoparticles showed different effects on chlorophyll content. The chlorophyll content in the presence of AgNPs increased the most at 3 min of independent AgNPs. The absorbance intensities of silver nanoparticles were fluctuated from time to time but overall the absorbance is increased slowly. The absorbance intensity of a low concentration mixture of nanoparticles was decreased.

PA-AuNP composite transferred from the algae to bloodworms as the atomic absorption and confocal laser images showed. PA-AuNP in the bloodworm exposed to algae contain could not be observed in the atomic absorption because the nanoparticle concentration was below the detection limit of the instrument used but bloodworm images under confocal laser instrument showed PA-AuNP in the bloodworm tissues. Confocal laser images of bloodworms exposed to PA-AuNP solution clearly showed nanoparticles in the bloodworm tissue.
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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Introduction

To improve water quality, several methods have been used to remove contaminants, organic chemicals and inorganic chemicals. Advantages and disadvantages were viewed in chapter one. One of the methods used to eliminate contaminants is algae. Algae communities have been employed in this research to remove common chemical contaminants such as pharmaceuticals and nanoparticles in wastewater or even in surface water, and drinking water.

This research and effort focused on removal of pharmaceutical and nanoparticle from water columns. An algal turf scrubber system was used for the cultivation of lab scales of algal biomass. Pharmaceutical and nanoparticle removal from a water column is as a useful method to produce clean water that has less contaminants than EPA standards, and it is possible to remove all contaminants.

5.2 Evaluation of Pharmaceutical Extraction Methods

Ibuprofen, estradiol and other pharmaceutical derivatives were detected in some algal extracts clearly showing that pharmaceuticals in the water are transferred into algal biomass. Other algal extraction methods did not show any pharmaceuticals content. This could be due to the following reasons: 1) low concentration of the pharmaceuticals in the algae biomass, 2) no pharmaceuticals in the water where algae grew and 3) a different extraction method is needed.

The results of this study indicate that the Spirogyra sp. and Chlorella sp. community is able to remove the pharmaceuticals, nanoparticles and heavy ions from water. So, it is possible to increase the efficiency of wastewater treatment plants with flows up to 50000 L/day. These future technological innovations would be a practical chemical removal step. An algal treatment system utilizing shallow earthen ponds instead of concrete basins could be an alternative model and a focus for the future research; this could potentially lower the initial capital costs of the algal treatment system. In addition, the algal treatment system could be used as a treatment method in wastewater treatment plants that want to remove heavy metals as ions or nanoparticles, as well as pharmaceuticals in low concentrations that the normal treatment methods are not able to remove.

5.3 Algae Cultivation

Algae grown in the lab and used in this study was identified using a microscope. An algae community was formed from two predominant species Spirogyra sp. and Chlorella sp. the
percentage of each species was not determined. However, the microscope images showed *Spirogyra sp.* had higher percentage in the community about 60% and 30% of *Chlorella sp.* other species were around 10%. These percentages were estimated based on images taken by the light microscope.

5.3.1 **Recommendations**

Future work on using algal turf communities in large-scale wastewater treatment plants should include growth trials with wastewater effluent from other wastewater treatment plants to obtain a better range of growth rates and chemical removal rates. It is also necessary to determine how the algal turf communities might react to the harvesting process. In addition, the ability of algal turf communities to maintain dominance in a large-scale system should be evaluated. It might be necessary to operate a system with a mixed collection of algae, macroalgae, and microalgae because different algae species have different abilities to remove a wide range of chemicals at the same time.

Nutrients should be monitored carefully because the overdose of nutrients could kill the algae. Temperature should be maintained around 25 °C. pH of the media should stay around 8. A higher pH could result in corrosion or damage the turfs that are made of aluminum.

5.4 **Pharmaceutical Removal**

Low concentrations of pharmaceuticals were detected in wastewater treatment plants as shown in Table 3-2, however, in this study higher concentrations (more than 1000 times) were used so pharmaceuticals can be detected in algal biomass extracts by HPLC. The limit of quantification of most the pharmaceuticals in this study was 1-20 mg/L.

Six common pharmaceuticals: aspirin, carbamazepine, ibuprofen, caffeine, estradiol, and bezafibrate were removed from the water column. The results showed that algae had the ability to eliminate pharmaceuticals from the water column. The investigated pharmaceuticals indicated different removal rates during the treatment based on the chemical structure of these pharmaceuticals, size of the molecule and solubility. Comparing the results, caffeine and estradiol had the best removal rates. In contrary, to which confirms the results of sorption of algae in the lab scale experiments, carbamazepine appeared to stay in the water.

The concentration of pharmaceuticals in algal in algal biomass increased with the time of exposure and in same time increased with increasing the initial concentration in the water column. The fraction of pharmaceuticals taken up by algae differed for each pharmaceuticals. The
concentrations of pharmaceuticals in water column were decreased with the harvest time. Removal fractions were 70 % for aspirin, 78 % for ibuprofen, 100 % for caffeine, 89 % for bezafibrate, 88 % for estradiol. These fractions did not mean the pharmaceuticals were taken by algae alone. It might have lost in tubing and algal turf scrubber system.

5.4.1 Relative Biomass of Algae Exposed to Pharmaceuticals

Different uptake abilities of the algal community to contaminants from the water column were observed. The relative biomass density of exposed algae was decreased by the exposure time. The relative biomass density of untreated algae did not change during the experiment. These differences were statistically significant ($t$-test $< \alpha = 0.05$). These studies were needed for environmental and water agencies to evaluate the risk of the contaminated waters on aquatic life.

5.4.2 Ash and CHN of Algae Exposed to Pharmaceuticals

Organic and inorganic carbon contents for algae exposed to pharmaceuticals were are not stable with the harvest time. It was suggested that each pharmaceutical bonded to the algal biomass through the functional groups on the surface of the cell walls because of the chemical structures of the six pharmaceuticals used. Different reactivity of these pharmaceuticals is based on a verity of factors. Some pharmaceuticals might be trapped between the algae layers and have more time to bind with the algae. These factors might have allowed some pharmaceuticals to react. This may explain the organic and inorganic contents are not stable and declining and climbing with the harvest time. Two factor ANOVA test was done to see if the $P$-value was greater than the significance level ($0.05$) was selected. The $P$- value for the exposed algae was $0.98$. That means the effect is not statistically significant.

CHN data showed that the nitrogen content in all samples remained stable ($7 \pm 0.8$ wt %) with the exposure time. These results suggested that nitrogen is fixed, primarily as a protein, in the raw biomass. These sp2 bonds require more energy to break than sp bonds, causing nitrogen to be retained. Hydrogen as well stays constant during the experiments ($5 \pm 0.5$ wt %).

The organic carbon content varies from a low of $25 \pm 3$ wt % to a high of $37 \pm 2$ wt %. The increase in organic contents during the exposure time are likely due to increase the pharmaceutical concentrations in the algae tissues. Inorganic contents during the exposure time varies from $10$ wt % to $15$ wt % at different exposure time. The $P$- values were larger than the significance ($\alpha = 0.05$) and that means there is not statistically significant in the algal biomass during four days of exposure.
5.4.3 UV-Vis of Chlorophyll Extracted from Algae Exposed to Pharmaceuticals

Pharmaceuticals are in surface waters but their effects on aquatic organisms and associated ecosystem properties are not well understood. Chlorophyll absorbance in the beginning of the experiment should have had the same absorbance but because the water input might affect the chemical structure of the algal biomass or the water input could have moved layers of algae, UV-Vis absorbance was measured based on the area of the samples and not on the biomass weight. With that in mind, absorbance intensities increased with exposure time with all tested experiments; low and high concentration mixtures of six pharmaceuticals, a mixture of ibuprofen and bezafibrate, and ibuprofen and bezafibrate alone. Chlorophyll absorbance for the control experiment remained constant during the experiments. With time, pharmaceuticals in the experiment improved the chlorophyll absorbance and that might have been because pharmaceuticals killed the bacteria, marine viruses and other aquatic organisms that lived on the algal biomass. The effect of pharmaceuticals on the chlorophyll absorbance was not statistically significant.

5.4.4 Fluorescence of Chlorophyll Extracted from Algae Exposed to Pharmaceuticals

Before doing any chlorophyll experiment, chlorophyll was extracted from algae and then fluorescence was recorded. Chlorophyll was extracted from algae by acetone and the maximum fluorescence intensity was at 670 nm, which was in good agreement with the literature. The acetone had no fluorescence intensity, no interface, so all algae extractions were prepared in acetone.

Pharmaceuticals had little effect on the chlorophyll. Untreated algae had the same chlorophyll fluorescence intensities at different harvest times, and as expected these data. Both low and high concentrations of six or two pharmaceuticals showed no effect. Chlorophyll fluorescence intensity of algae exposed to ibuprofen or bezafibrate had no change. It was expected that a little amount of the pharmaceuticals did not affect the algae biomass structure. The effect of pharmaceuticals on chlorophyll fluorescence intensity was not statistically significant.

5.4.5 Recommendations

Algal turf communities were used to remove the mixture of pharmaceuticals. For future work, Algal turf communities could be used to remove the pharmaceuticals and compare the results with the results shown here. Also, Algal turfs could be used together to remove a single drug at a time instead of the mixture. Different algal communities could have several functional groups
which might be able to bind with a wide range of pharmaceuticals. pH solution could be lowered
to 7 and then study the ability of the algae community to remove pharmaceutical(s). Other drugs
and algae species could be better adsorbents than the community we used in our study. A mixture
of nanoparticles and pharmaceuticals could be used as contaminants, then be removed by the algae
community and identified by using GC-Ms and other tools.

Literature also shows that aspirin inhibits phosphate uptake by the algae and that could be
the cause of a decreasing relative biomass density. Algae as plants contain ion channels and
enzymes which could also be potentially targeted by pharmaceuticals and may initiate a response
such as inhibition in the transport of essential elements required for growth. Previous research has
highlighted the potential for pharmaceuticals to induce toxic effects on plants.

5.5 Nanoparticle Removal

Two well-known nanoparticles were prepared and characterized by UV-Vis and TEM;
silver and gold nanoparticles. They were selected based on their high quality applications, and
their presence in water. Algal turfs were also used to remove these nanoparticles from the water
column. For comparison, heavy metal ions were used; silver (I) and gold (III).

Metal biosorption was fast, reaching maximum efficiency in the first 24 hours. The initial
concentration strongly affected the biosorption process and it was concluded that adsorption was
favored by an increase of concentration. The maximum biosorption of gold, silver nanoparticles
and ions took place in the first two days of exposure, which implied rapid acclimation occurring
in response to metal deficiency during cultivation of inoculums. The rate constant of silver and
gold nanoparticles were same as silver and gold ions, which were 0.0054 and 0.0075 mg/L/min,
respectively.

5.5.1 Relative Biomass of Algae Exposed to Nanoparticles/Ions

Algal biomass density was calculated at two different concentrations of silver and gold
nanoparticles and their mixture, 2.475 and 4.475mg/L of silver nanoparticles and 5 and 9 mg/L of
gold nanoparticles. Silver and gold ions were evaluated at the same concentrations. The data
showed that AgNPs and Ag (I) had no effect on the relative biomass density, but AuNPs and Au
(III) had larger effect. It was clear gold nanoparticles decreased the relative biomass density.
5.5.2 Ash and CHN of Algae Exposed to Nanoparticles/Ions

The organic carbon and inorganic contents were obtained and estimated directly by burning a specified quantity of dried algae at 500 °C and 850 °C, respectively. The organic and inorganic carbon contents varied for algae exposed to a mixture of high silver and gold concentrations of nanoparticles than that of the control. The algae exposed to silver and gold nanoparticles had more inorganic contents. For comparison, silver and gold ions at the same concentrations as silver and gold nanoparticles, respectively, were examined and they had similar inorganic contents as the control and lower organic contents. Two factor ANOVA test was done to see if the p-value was greater than the significance level (α = 0.05) was selected. The P-value for the algae exposed to AgNPs (2.475 ppm) and AuNPs (4.475 ppm) were higher than the significance level. That means the effect of nanoparticles was not statistically significant.

Hydrogen and nitrogen contents were not affected during the exposure time. The mixture of the low concentrations of mixture of AgNPs and AuNPs on the algal biomass, 2.475 and 4.95 ppm respectively did not change the contents of organic, inorganic, hydrogen and nitrogen for the first two days, but the data obtained showed the organic and inorganic contents of the elements decreased. This mixture of the high concentrations of mixture of AgNPs and AuNPs on the algal biomass, 4.95 and 9 ppm respectively decreased the contents of organic carbon and inorganic carbon after one hour of exposure. The data of algae exposed to silver and gold ions showed that silver and gold ions did not change the organic, hydrogen and nitrogen contents in the algae biomass but inorganic content decreased with the exposure time. The effect of nanoparticles on compositions of algal biomass was not statistically significant according to P-values and t test values.

The organic content was the highest for the control sample, unexposed algae. The lowest organic content was for algae exposed to the mixture of high concentrations of silver and gold nanoparticles. The inorganic content increased slightly for exposed algae, which was the highest for algae exposed to silver nanoparticles, 2.475 mg/L. The hydrogen and nitrogen contents were not changed with the different contaminants.

5.5.3 UV-Vis of Chlorophyll Extracted from Algae Exposed to Nanoparticles

UV-Vis absorbance responded to the presence of nanoparticles showed that these nanoparticles had different effects on chlorophyll absorbance between the first and last day of the experiment. The total chlorophyll content had the most increasing effects in 3 min of independent
AgNPs. The absorbance intensity of silver nanoparticles seems to be not stable, where the intensity decreased and increased over time, but overall the absorbance is increased slowly. The absorbance intensity of low concentration mixtures of nanoparticles was decreased. Intensities at -60 and zero min should be the same, but the intensity at zero min was higher and that might be because of the water flow in the beginning of the turf that could cause changes in the chemical structure of the algae biomass at -60 min. A literature study showed that the AgNP concentration had a negative effect on the chlorophyll content after 16 days of the experiment, where the chlorophyll content decreased when of AgNP concentration was increased.

It was shown that untreated chlorophyll absorbance was stable during a four day experiment. However, chlorophyll absorbance for algae exposed to silver and gold ions was decreased with the harvest time. Silver and gold ion sorption showed a significant inhibitory effect on chlorophyll a in the algal biomass. Chlorophyll contents at -60 and zero min had approximately similar absorbance, but at 1 and 3 mins the absorbance looked higher than that at time zero. Algae exposed to silver ion had a higher absorbance than that of algae exposed to gold ion. This might indicate a more destructive effect of gold metals on the chlorophyll contents. High concentrations of gold ions might cause activated oxidative damage and change cell-membrane properties by lipid peroxidation as a cupper ion, meaning the inhibitory effect on the enzymes involved in chlorophyll production.

5.5.4 Fluorescence of Chlorophyll Extracted from Algae Exposed to Nanoparticles

Algae exposed to nanoparticles showed different absorbance patterns to that of pharmaceuticals. It was clear that fluorescence intensity of algae exposed to 2.475 ppm of silver and 4.475 ppm of gold nanoparticles decreased with the harvest time. The fluorescence intensity had no change in the first hour of the exposure, but it did change in the end of the experiment at 5760 min. A mixture of both nanoparticles had a negative effect on the fluorescence intensity where the intensity was lower than that of algae exposed to singlet nanoparticles. A mixture of high concentrations of nanoparticles had more effect on the algae than low concentrations.

Fluorescence intensity for untreated algae was also measured. Both silver and gold ions lower the fluorescence intensity. Silver and gold ions reduced chlorophyll a fluorescence intensities 50 % and 30 % respectively after being 75 % in comparison to the control after four days of the experiment. The fluorescence intensity for algae exposed to silver and gold ions at -60 and zero min would have the same values as the control, where there are no silver and gold ions.
in the medium yet. This might be because of the previous experiments, where a few amounts of the ions or nanoparticles could have been left in tubing or pumps or perhaps because the growth rates of the algae on the turfs are different after a month of the experiment.

5.5.5 *Location of Nanoparticles/ Pharmaceuticals in Algae*

Confocal laser images were used to locate nanoparticles in the algae biomass whether intracellular or extracellular bioaccumulations. To use confocal laser fluorescence, new fluorescence nanoparticles, paracetamol-AuNP composite, were prepared. Paracetamol-AuNP composite fluoresces with green light under the confocal laser instrument and is identified by UV-Vis and fluorescence.

Confocal laser pictures show that the PA-AuNPs are most likely inside the algal biomass cells, where the nanoparticles fluoresce with bright green color.\(^3\) Low extracellular bioaccumulation in our study results from the poor proton-binding capability of the algal biomass surface, and it was difficult to attach PA-AuNPs to the algal cell walls. Based on the confocal laser microscope, low extracellular bioaccumulation is likely due to the low total concentration of ionizable sites on green algae. However, other interfering factors in the water column such as cations (nutrients) might reduce extracellular bioaccumulation. Cations in media decrease the metal binding on the cell wall, and compete with the nanoparticles and silver and gold ions uptake with the cell surface, therefore, lowering extracellular bioaccumulation.

5.5.6 *Bloodworms Uptake*

Algae are the base of the food web in streams, and negatively impacts algae potentially affecting both nutrient cycles in an ecosystem and higher trophic levels that depend on algae. Silver and gold nanoparticle were not detected in bloodworm tissues after being fed exposed algal biomass because the detection limits of the atomic absorption were too high.

AuNP was detected by the atomic absorption in the bloodworms exposed to PA-AuNP solution and confocal laser fluorescence conformed the data. It was clear that bloodworms took the AuNP from the solution. On the other hand, AuNP was not detected in the bloodworm exposed to algae contain PA-AuNP. AuNPs might be below the detection limit of the atomic absorption instrument. Confocal images showed that there is a little AuNPs in the bloodworm tissue (bloodworm exposed to algae).
5.5.7 Recommendation

Relative biomass density was measured to test the effect of the silver and gold nanoparticles and silver and gold ions on the surface and internal media of the algae. It seems that both silver nanoparticles and ions did not influence the biomass and relative biomass stay constant. On the other hand, both gold nanoparticles and ions have a negative effect on the relative biomass. That might be because of the bigger sizes of silver nanoparticles and ions which might bind to carboxyl or other functional groups on the algal cell walls and they do not have the ability to enter to the algae cell. Opposite that, small sizes of gold nanoparticles and ions can move inside the algae cells and could change the biostructure of the cells and stop or decrease the reproduction process in the cells.

The complex matrix of the media that was used to grow algae biomass made it difficult to determine the actual cause of interference. The complex media of the algal biomass when measuring the concentrations of the nanoparticles in the biomass gave higher signals than what we expected for the control.

The ash procedure is not suitable for samples that have not been dried in an oven to the correct moisture content, so drying samples to a constant weight was an important process to find the ash content. If measurements are taken before a constant weight was reached, data may be influenced.

Another aspect of biochar production that needs to be evaluated is the production and identification of volatile organic compounds produced during pyrolysis. The production and identification may provide insight into the reactions that are occurring in the biomass during processing. The best technique for that is GC-Ms.

The calibration CHN instrument is important to find the inorganic, organic, lipid and protein contents. DETA standard should be used frequently. Sample weights are also an important factor when using the CHN instrument. Four stable digits should be recorded after closing the gate of the balance/scale where air does not affect the weight of samples.

Catalysts in CHN columns should be checked and they may need to be activated or replaced. Inactive catalysts can give false data which have an influence on determining the amount of inorganic, organic, lipid and protein contents. Helium, air and nitrogen tanks should be filled, or have high pressure to operate the instrument. They should be provided from the same factory where they have the same ratios of contaminants.
It is common knowledge that algae releases oxygen during the photosynthesis process. The oxygen concentration released is measureable. The amount of carbon dioxide also can be measured during the exposure of algae to pharmaceuticals or nanoparticles. This process requires building a closed system to measure the released oxygen or carbon dioxide up-take.

Phosphate is considered a nutrient in this experiment, however, it was reported that phosphate ions share similar transportation sites with some metals for bioaccumulation. Consequently, phosphate ions may compete for adsorption on extracellular biotic surfaces and may reduce metal removal efficiency. The influence of phosphate on metal removal in algae is an important topic and should be studied in the future.

We suggest an investigator to repeat the bloodworm experiment using specific time intervals as a starting point for researching detection of nanoparticles in algae. Other detection methods can be used to detect a lower level of nanoparticles. Atomic absorption spectroscopy with high resolution could also be used for this purpose.

Future research needs to determine where the nanoparticles are deposited in the bloodworms’ liver, kidney or other sites. A more definitive study is to see if the nanoparticles transfer to tissues through skin or by the consumption of exposed algae would be insightful. Other aquatic organisms might be used instead of bloodworms such as fish or algae consuming organisms.

5.6 References

Figure 6-1. Gas Chromatography of extraction from AMS/Hexane/Sand.
Figure 6-2. Gas Chromatography of extraction from AMS/Chloroform/Sand.

Figure 6-3. Gas Chromatography of extraction from AMS/Cyclohexane/Sand.
Figure 6-4. Gas Chromatography of extraction from AMS/Hexane/steeping technique.

Figure 6-5. Gas Chromatography of extraction from AMS/Toluene/Sand.
Figure 6-6. Gas Chromatography of extraction from AMS/Acetonitrile/Sand.

Figure 6-7. Gas Chromatography of extraction from AMS/Ethyl Acetate/Sand.
Figure 6-8. Gas Chromatography of extraction from AMS/Methanol/steeping technique.

Figure 6-9. Gas Chromatography of extraction from AMS/Hexane+methanol/sand.
Figure 6-10. Gas Chromatography of extraction from AMS/Ethanol/Soxhlet.
Figure 6-11. Gas Chromatography of extraction from AMS/methanol/sand.
Figure 6-12. Gas Chromatography of extraction from AMS/Ethanol/Soxhlet.
Figure 6-13. Gas Chromatography of extraction from AMS/Hexane+Chloroform/sand.

Figure 6-14. Gas Chromatography of extraction from AGH/Hexane+methanol/Sand.
Figure 6-15. Gas Chromatography of extraction from AGH/methanol/steeping technique.

Figure 6-16. Gas Chromatography of extraction from AGH/Hexane/steeping technique.
Figure 6-17. Gas Chromatography of extraction from AGH/methanol/Sand.

Figure 6-18. Gas Chromatography of extraction from AGH/ethanol/Soxhlet.
Figure 6-19. Gas Chromatography of extraction from AGH/ethanol/Soxhlet.
Figure 6-20. Gas Chromatography of extraction from AES/Hexane/steeping technique.

Figure 6-21. Gas Chromatography of extraction from AES/methanol/sand.
Figure 6-22. Gas Chromatography of extraction from AES/methanol/Ultrasonic.

Figure 6-23. Gas Chromatography of extraction from AES/40% ethanol/Soxhlet.
Figure 6-24. Gas Chromatography of extraction from AES/acetonitrile+ methanol/sand.

Figure 6-25. Gas Chromatography of extraction from AES/Acetonitrile+ methanol/sand.
Figure 6-26. Gas Chromatography of extraction from AES/methanol/sand.

Figure 6-27. Gas Chromatography of extraction from AES/acetonitrile/sand.
Figure 6-28. Gas Chromatography of extraction from AES/methanol/sand.
Figure 6-29. Gas Chromatography of extraction from AES/hexane/mechanic shaking.
Figure 6-30. Gas Chromatography of extraction from SPE/Pond water/methanol.
Figure 6-31. Gas Chromatography of extraction from SPE/Pond water/hexane.

Figure 6-32. Gas Chromatography of extraction from SPE/Pond water/ethyl acetate.
Figure 6-33. Gas Chromatography of extraction from sand/Pond water/ethyl acetate+ methanol.

Figure 6-34. Gas Chromatography of extraction from separation funnel /Pond water/chloroform.
Figure 6-35. Gas Chromatography of extraction from separation funnel /Pond water/acetonitrile.

Figure 6-36. Gas Chromatography of extraction from SPE/wastewater/methanol.
Figure 6-37. Gas Chromatography of extraction from SPE/wastewater/hexane.

Figure 6-38. pH values of algae media.
Figure 6-39. Temperature of algae media.

Figure 6-40. $O_2$ Concentration of algae media.
Figure 6-41. Conductivity of algae media.

Figure 6-42. ISE of NH$_4^+$ ion in algae media.

Figure 6-43. ISE of Nitrate Ion in algae media.
Table 6-1. Solubility of pharmaceuticals used.

<table>
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<tr>
<th></th>
<th>ASP</th>
<th>IBU</th>
<th>CAR</th>
<th>CAF</th>
<th>BEZ</th>
<th>β-EST</th>
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<tr>
<td>used in 1L / g</td>
<td>0.1537</td>
<td>0.1512</td>
<td>0.1510</td>
<td>0.1500</td>
<td>0.1515</td>
<td>0.1518</td>
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<tr>
<td>solubility / g L⁻¹</td>
<td>4.6</td>
<td>21.6</td>
<td>0.0177</td>
<td>0.021</td>
<td>0.0543</td>
<td>0.0036</td>
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<tr>
<td>% saturation @ 1L</td>
<td>3.3%</td>
<td>0.70%</td>
<td>853%</td>
<td>714%</td>
<td>279%</td>
<td>4217%</td>
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<tr>
<td>% saturation @ 20L</td>
<td>0.17%</td>
<td>0.04%</td>
<td>43%</td>
<td>36%</td>
<td>14%</td>
<td>211%</td>
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<tr>
<td>pKa</td>
<td>3.5</td>
<td>4.9</td>
<td>13.9</td>
<td>10.4</td>
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<tr>
<td>base / L</td>
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<td>0.04</td>
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<tr>
<td>Conc. / mol L⁻¹</td>
<td>0.01</td>
<td>0.001</td>
<td>0.0001</td>
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<tr>
<td>pH base</td>
<td>12</td>
<td>11</td>
<td>10</td>
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<td>Conc. @ 20L</td>
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<td>pH @ 20L</td>
<td>9.30103</td>
<td>8.30103</td>
<td>7.30102999</td>
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Figure 6-44. pH of the media after adding the mixture of pharmaceuticals in two containers.
Figure 6-45. pH of the control medium.

Figure 6-46. HPLC of aspirin standard 20 ppm.

Figure 6-47. HPLC of aspirin in water after 60 min
Figure 6-48. HPLC of aspirin in algae after 2 days.

Figure 6-49. HPLC of aspirin in control.

Figure 6-50. HPLC of Bezafibrate standard 20 ppm.
Figure 6-51. HPLC of Bezafibrate in algae in control.

Figure 6-52. HPLC of Bezafibrate in algae after 4 days.

Figure 6-53. HPLC of Bezafibrate in water at time 0 min.
Figure 6-54. HPLC of carbamazepine standard 20ppm.

Figure 6-55. HPLC of carbamazepine in algae after 4 days.

Figure 6-56. HPLC of carbamazepine in control.
Figure 6-57. HPLC of ibuprofen standard 20ppm.

Figure 6-58. HPLC of ibuprofen in control.

Figure 6-59. HPLC of ibuprofen in algae after 4 days.
Figure 6-60. HPLC of ibuprofen in water after 2 days.

Figure 6-61. HPLC of caffeine standard 20 ppm.

Figure 6-62. HPLC of caffeine in control.
Figure 6-63. HPLC of caffeine in algae after 2 days.

Figure 6-64. HPLC of caffeine in water after 60 mins.

Figure 6-65. HPLC of estradiol standard 20 ppm.
Figure 6-66. HPLC of estradiol in control.

Figure 6-67. HPLC of estradiol in algae after 4 days.

Figure 6-68. Aspirin calibration curve.
Figure 6-69. Ibuprofen calibration curve.

Figure 6-70. Caffeine calibration curve.

Figure 6-71. Carbamazepine calibration curve.
Figure 6-72. B-Estradiol calibration curve.

Figure 6-73. Bezafibrate calibration curve.

Figure 6-74. Silver nanoparticles calibration curve in water during time.
Figure 6-75. Silver nanoparticle calibration curve in nitric acid during time.

Figure 6-76. Gold nanoparticle calibration curve in water during time.

Figure 6-77. Gold nanoparticle calibration curve in nitric acid during time.
Figure 6-78. Silver nitrate calibration curve in nitric acid and water during time.

Table 6-2. t-test data for relative biomass density of HAuCl₄.

<table>
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<th>HAuCl₄</th>
<th>Control</th>
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<td>Mean</td>
<td>0.845656</td>
<td>0.985386</td>
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<tr>
<td>Variance</td>
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<td>Observations</td>
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<td>4</td>
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<tr>
<td>Hypothesized Mean Difference</td>
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<tr>
<td>t Stat</td>
<td>-2.41265</td>
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<tr>
<td>P(T&lt;=t) one-tail</td>
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<tr>
<td>t Critical one-tail</td>
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<tr>
<td>P(T&lt;=t) two-tail</td>
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<tr>
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Table 6-3. t-test data for relative biomass density of the mixture of AgNO₃

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<th>AgNO₃</th>
<th>Control</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>t Critical two-tail</td>
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Table 6-4. t-test data for relative biomass density of nanoparticles

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Figure 6-79. Uv-vis for standards

Figure 6-80. Effect of AgNPs on chlorophyll in acetone.

Figure 6-81. Effect of AgNPs on chlorophyll in acetone
Figure 6-82. Effect of AgNPs on chlorophyll in acetone.

Figure 6-83. Effect AgNPs on chlorophyll in H₂O.

Figure 6-84. Effect of AgNPs on chlorophyll.
Figure 6-85. Effect AgNPs on chlorophyll in acetone.

Figure 6-86. Effect AgNPs on chlorophyll in methanol.

Figure 6-87. Effect AgNPs on chlorophyll in H₂O.