ABSTRACT

Title of Document: AN INVESTIGATION OF THE RELATIONSHIP BETWEEN THE LEVEL OF ANTIBIOTIC RESISTANCE OF *ESCHERICHIA COLI* IN NON-TIDAL WETLANDS AND COMMON WETLAND HEALTH FACTORS

Neil Agarwal, Sean Ahearn, Erik Dudziak, Sehba Khan, Daniel Marcin, Matthew Shofnos, Emily Skoda, Padmasini Venkatachari, Robert Vocke III

Directed By: Dr. David Tilley, Ph.D.
Environmental Science & Technology

This report investigated the prevalence of antibiotic resistance among *Escherichia coli* in the water of 13 non-tidal mitigation wetlands in Maryland, and its relation to land use and wetland health. At each site, land use, surface and sub-surface water samples, soil samples, and vegetation cover were collected. From the water samples, individual colonies of *E. coli* were isolated and tested, using the disc diffusion method, for resistance to the antibiotics ampicillin, ciprofloxacin, erythromycin, sulfisoxazole, and tetracycline. According to soils, vegetation and water quality improvement criteria the wetlands function like healthy wetlands. The wetlands' *E. coli* exhibit resistance to all of the antibiotics tested, except for ciprofloxacin. There were statistically significant relationships found between land use and antibiotic resistance, vegetation, soil and water chemistry. Surprisingly, *E. coli* in wetlands with smaller stocks of carbon and nitrogen in their soil exhibited more resistance to tetracycline, possibly indicating that soil quality plays an important role in fostering or fighting antibiotic resistance. The work demonstrates that antibiotic resistance is present in Maryland's wetlands, but that its spread could be subdued by healthy wetlands.

AN INVESTIGATION OF THE RELATIONSHIP BETWEEN THE LEVEL OF
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By

Team CRABS
(Chesapeake Revitalization and Alternative Bay Solutions)

Neil Agarwal
Sean Ahearn
Erik Dudziak
Sehba Khan
Daniel Marcin
Matthew Shofnos
Emily Skoda
Padmasini Venkatachari
Robert Vocke III

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Advisory Committee:
Dr. David Tilley, Ph.D, Mentor
Dr. Andrew Baldwin, Ph.D, Discussant
Ms. Denise Clearwater, Discussant
Dr. Sam Joseph, Ph.D, Discussant
Mr. Todd Nichols, Discussant
Mr. Peter Sharpe, Discussant

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1. Introduction

1.1 The CRABS Team

The Gemstone program at the University of Maryland, College Park is a unique program that brings together talented undergraduate students of varying disciplines to work on multi-year interdisciplinary team research projects. Team CRABS was formed in 2005, and composed of nine undergraduate students in the Gemstone program studying the health of wetlands and waterways throughout the state of Maryland. This team performed its research under the auspices of Dr. David Tilley of the University of Maryland's Department of Environmental Science and Technology.

Team CRABS was borne of its members' common concern for environmental issues and the health of the Chesapeake Bay watershed. In selecting a research topic, CRABS decided to focus on the Chesapeake Bay's wetlands; in light of growing evidence that wetlands act as ecological "filters" that reduce the flow of pollutants through a watershed, it has become increasingly clear that the health of wetlands is closely tied to that of the watershed as a whole. Being that these research interests aligned with the priorities of the Maryland Department of the Environment (MDE), the team's research was predominantly funded by a grant from the agency's Wetlands and Waterways Program.

The Maryland Department of the Environment is the state of Maryland's chief environmental agency. Its mission is to protect and restore the quality of Maryland's air, water, and land resources, among other endeavors. Founded in 1987, the MDE works to enforce and regulate health standards, growth issues, and environmental

emergencies throughout the state. Accordingly, the MDE is required to comply with the Clean Water Act of 1972. This act requires each state to "assess the quality of their waters every two years and publish a list of those waters not meeting the water quality standards set for them" (U. S. Environmental Protection Agency, 1972).

1.2 The Status of Maryland's Non-Tidal Wetlands

Wetlands are unique transitional ecosystems – neither strictly aquatic nor terrestrial – which are saturated by water often enough during their growing seasons to develop characteristic hydric soils and support specially-adapted hydrophytic vegetation. In particular, a fundamental characteristic of wetland soils is that they are anaerobic; as a result of being inundated by water they are less able to receive oxygen from the atmosphere. Wetlands are particularly worthy of attention because beyond their own inherent complexity they have a wide variety of benefits to both humans and the broader environment.

One of the most evident benefits of wetlands is their role in protecting biological diversity. They are home to innumerable species of plants, animals, and microbes, including a number of economically important species and over one-third of all threatened and endangered species in the United States (U.S. Environmental Protection Agency, 1995). Wetlands are also important for their ability to reduce shoreline erosion and to lessen the impact of flooding and droughts. Because of their transitional hydrology, wetlands can take up storm water during periods of high rainfall while slowly releasing it during periods of low rainfall. Such hydrological stabilization is important for both humans and the ecosystem as a whole.

Another function of wetlands, and perhaps the one most pertinent to the present study, is their ability to improve water quality. Wetlands have a number of characteristics that allow them to naturally take up sediments and harmful chemicals: they reduce water velocity and allow for deposition of suspended sediments, their shallow water allows for a high rate of chemical exchange between ground and water, and their high productivity in combination with their diversity of biogeochemical processes facilitates the uptake and/or degradation of numerous chemicals (Mitsch & Gosselink, 2000). In the scheme of an entire watershed, wetlands are often situated between developed areas and low-lying aquatic systems like rivers and lakes. As such, water draining through a watershed can improve in quality as it passes through one or more wetlands before reaching a more traditional waterway. Considerable scientific attention has been focused on this idea, and an entire subfield is now devoted to the use of wetlands to treat polluted water (Kadlec & Knight, 1996).

Unfortunately, the benefits of wetlands have not always been so well-recognized. In the past, human destruction of wetlands has been a considerable problem, with the U.S. having lost more than 50% of its original wetland area in less than two centuries (Balcombe et al., 2005). Even as the issue of water pollution began drawing concern towards the second half of the twentieth century, wetland protection often fell by the wayside. Most notably, when first enacted in 1972, the United States' Clean Water Act became a significant step in protecting the water quality of the nation's navigable waterways, but failed to include any mention of wetlands.

In 1975, the Supreme Court cases *United States v. Holland* and *Natural Resources Defense Council v. Calloway* reinterpreted the Clean Water Act's definition of water bodies to include wetlands (Mitch & Gosselink, 2000). As a result, under Section 404 of the act, anyone wanting to dredge or fill a wetland now had to obtain a permit from the U.S. Army Corps of Engineers. This permit process requires that wetland losses are avoided or minimized when possible, and that any unavoidable losses of wetland function are compensated for through the creation or restoration of so-called mitigation wetlands (National Research Council, 2001).

The use of mitigation wetlands in the U.S. has become a major route by which wetland functions are preserved. In 1989, President Bush officially adopted the goal of "no net loss" as his administration's policy towards wetland conservation (National Research Council, 2001). In the wake of this policy, mitigation wetlands created under Section 404 were responsible for an estimated net gain of 500 square km of wetland area between 1993 and 1999 (Mitsch & Gosselink, 2000).

Despite this recent progress in the realm wetland preservation, a separate issue is that existing wetlands are still under stress from water pollution resulting from human activity. The ability of wetlands to reduce water pollution has been discussed, but it should be noted that wetlands can also be impacted by the same pollutants they filter out. For example, a study by Barber at al. (2006a) on the fate of pollutants in a treatment wetland revealed that many of the toxins filtered out by that wetland ended up accumulating in its fish and compromising their health.

Nitrogen and phosphorous are two of the most known and studied environmental water pollutants. As plant nutrients, they are extremely important for

their use as agricultural fertilizers. However, when released into the environment in high quantities, nitrogen and phosphorous can cause imbalances to aquatic ecosystems in a phenomenon referred to as eutrophication. Though eutrophication is generally associated with deeper-water systems, it can also be detrimental to wetlands. Indeed, it has been noted many times that nutrient overabundance in a wetland can result in the dominance of certain plant species at the expense of others, reducing overall wetland biodiversity (Bedford, Walbridge, & Aldous, 1999; Tilman & Lehman, 2001). Isolatable point sources of nutrients and other pollutants (e.g. factory outflows, landfills, etc.) are generally regulated under applicable laws, but non-point source pollution originates as runoff from various ill-defined sources (e.g. lawns, farms, roadways, etc.) and is thus particularly difficult to account for and regulate.

Because wetland ecosystems protect downstream waterways from water pollution and are simultaneously impacted by pollution, monitoring wetland water quality and other aspects of wetland health and function is of utmost importance. Mitigation wetlands require special attention; while the intended purpose of mitigation wetlands is to replace lost wetland function, research has shown that many either do not fulfill their permit requirements or are unable to fully match natural reference sites in terms of value and function (National Research Council, 2001). Furthermore, the impacts of human development are particularly pertinent to the study of mitigation wetlands, due to the fact that placement of mitigation sites is often based on cost and convenience as opposed to ecological optimality. In other words, mitigation wetlands are often "artificially" placed in developed areas where they may

be more heavily affected by human activity. One recent study found wetland plant diversity to be affected by surrounding land use (Houlahan, Keddy, Makkay, & Findlay, 2006). In light of this discussion, it is likely that there exist many more such relationships between human activity and wetland function.

1.3 The Problem: Pharmaceutical Runoff

Pharmaceuticals and personal care products are all but ubiquitous in the modern world, and as their use among humans and farm animals continues, so does the potential for their entry into the surrounding environment. In normal use, up to 90% of pharmaceuticals pass through their host system non-metabolized, while even metabolized portions may continue to pose an environmental risk (Jones, Voulvoulis, & Lester, 2003). Additionally, unused pharmaceuticals are often subject to improper disposal. Dietrich, Webb, and Petry (2002) note that the same properties that give pharmaceuticals the ability to function in the body also make them potentially persistent and harmful to aquatic ecosystems: pharmaceuticals must inherently interact with living systems, they are often designed for stability within such systems, and their proper bodily transport often requires that they be water soluble.

It was not until the 1990s that the harmful potential of pharmaceuticals began to gain more widespread scientific attention (Dietrich et al., 2002). However, the development of analytical methods capable of detecting pharmaceuticals in the environment was, and is, often a limiting factor in the study of this topic (Daughton & Ternes, 1999; Bruchet et al., 2005). Early studies were relatively sparse and generally focused on narrow aspects of the issue; many authors noted the need for a

clearer picture of the general extent of pharmaceutical contamination. In 2002, a landmark study from the United States Geological Survey (Kolpin et al., 2002) presented the first nationwide picture of the occurrence of pharmaceuticals in waterways – it found organic wastewater contaminants in 80% of streams studied. Compounds detected in the study originated in agricultural, industrial, and residential sources, and included antibiotics, antidepressants, disinfectants, fragrances, the metabolites of detergents, prescription and nonprescription painkillers, and hormonally active steroids. The study noted that though concentrations were generally low, many compounds do not have guidelines as to what represents a safe level.

There are in fact numerous factors complicating the issue of what constitutes a "safe level." Many manmade compounds are not easily biodegradable and will accumulate in the environment regardless of their rate of release. But even for pharmaceuticals that degrade with relative ease, guidelines on acute ecological toxicity only cover a portion of the picture. Constant and continual release of degradable compounds can present a chronic exposure risk that is difficult to account for, since organisms may spend their entire lives exposed to a compound in minute concentrations. Ecological harm may accumulate so slowly that it is difficult or impossible to discern its original source (Daughton & Ternes, 1999; Jones et al., 2000; Halling-Sørensen et al., 1998). Furthermore, simultaneous occurrence of multiple contaminants is hindering efforts to establish causal relationships to individual compounds and creating the potential for complex interactions between different contaminants (Kolpin et al., 2002; Jones et al., 2003; Boxall, Kolpin,

Halling-Sørensen, & Tolls, 2003). Finally, analytical techniques used in studies of

pharmaceutical prevalence have generally required a preexisting knowledge of

analyte compounds, and as such have been of limited use in elucidating the chemical

changes the pharmaceuticals may undergo before and after their release into the

environment. Undetected metabolites may remain active, or may even be

transformed back into their parent compounds soon after their release into the

environment (Glassmeyer & Shoemaker, 2005; Ternes, 2001; Halling-Sørensen et al.,

1998). Recent advancements in analytical chemistry show hope in overcoming this

issue (Kosjek, Heath, Petrović, & Barceló, 2007).

Driven by the need to account for these complexities, research detailing the

environmental fate and effects of pharmaceuticals has intensified in recent years. For

example, Carballa et al. (2005) studied the fate of thirteen pharmaceutical and

personal care compounds in a sewage treatment plant in northwest Spain. Their study

revealed a significant presence of eight compounds, and found that upon passing

through the treatment plant, concentrations of most compounds were reduced

substantially but not fully. Barber et al. (2006) measured for various organic and

inorganic contaminants in the Boulder Creek watershed of Colorado, including

twelve pharmaceutical compounds. In general, they found that contaminant

concentrations increased in areas of high population density and spiked immediately

downstream of wastewater treatment plants, and noted a possible correlation to

endocrine disruptions in native fish. Cleuvers (2003) performed lab tests of the

toxicity of ten pharmaceutical compounds on three different aquatic organisms.

Measured levels of acute toxicity for individual compounds were much greater than

their likely level of occurrence in the environment. However, the study found the toxicities of certain mixtures to be greater than the sum of their parts, affirming the potential for additive interactions. Liu and Williams (2007) studied the sunlight-induced degradation of several β-blocker pharmaceuticals, and were able to explicitly identify multiple degradation products for the drug propranolol. They noted the potential usefulness of this pathway as a propranolol attenuation mechanism, but did not discuss the potential effects of newly formed products. Meanwhile, a study by Bedner and MacCrehan (2006) revealed that acetaminophen, the active ingredient in many painkillers, can be transformed into decidedly more toxic compounds during chlorination in wastewater treatment plants. Runnalls, Hala, and Sumpter (2007) studied the effects of clofibric acid, a persistent metabolite of the cardiovascular drug clofibrate. They found that concentrations of clofibric acid almost as low as those reported in the environment are capable of causing reproductive impairments to the fathead minnow over a three week period. While it has been noted that human exposure to pharmaceutical compounds may occur when ground and surface water are used to produce drinking water (Pauwels & Verstraete, 2006), the EPA asserts that no studies thus far have revealed any human effects from the release of pharmaceuticals (U.S. Environmental Protection Agency, 2007).

Among the varying studies of pharmaceuticals in the environment, antibiotics have been identified as an area of particular concern. Since the year 1942, when Anne Miller became the first person to be successfully treated using penicillin (Oransky, 2002), literally hundreds of new antibiotic compounds have been discovered or developed (Neu, 1992). Antibiotics are an extremely important class of

pharmaceuticals – their use encompasses both human and veterinary medicine, and extends to non-therapeutic uses such as growth promotion in farm animals. The widespread use of antibiotics is reflected by the fact that it is very common for antibiotics to be detected in studies of the pharmaceuticals in the environment. However, an oft-cited drawback to the widespread use of antibiotics is that it creates a selective pressure that favors the development and spread of bacteria that are resistant to treatment by antibiotics. In under a century, the spread of antibiotic resistant bacteria has become a major issue; countless studies have documented cases of bacteria becoming resistant to commonly used antibiotics, to the point where many of the most widely used antibiotics have lost much of their original effectiveness. The overall trend in the spread of antibiotic resistance creates a vicious cycle that threatens the use of existing antibiotics while constantly necessitating the increased development and use of new ones.

In itself, the issue of antibiotic resistance has been the subject of much research. Antibiotic resistance has been a well-known concern to both the scientific and lay communities for many years, and since antibiotic resistance poses a recognized threat to human medicine in addition to the environment, its study is naturally of high priority. However, research conducted to date has generally leaned towards studying human isolates from human locales – it is only recently that scientists have begun to study how antibiotic resistance may operate in the open environment. Yet environmental studies of antibiotic resistance are significant for at least two reasons: it is important not to overlook the spread of antibiotic resistance as a form of pollution that can adversely natural ecosystems, and it is important to

realize that environmental locales may be sites for the exchange of antibiotic resistance genes, which can subsequently have adverse effects on human medicine.

Indeed, recent research by Halda-Alija (2004) and Biyela et al. (2004) indicates that wetlands and waterways may retain, and possibly facilitate the spread of, antibiotic resistant pathogens. Halda-Alija (2004) demonstrated the presence of several species of antibiotic resistant pathogenic bacteria in wetlands in Mississippi, and concluded that wetlands may retain genes for antibiotic resistance but not necessarily aid in their dissemination. Biyela et al. (2004) found antibiotic resistant bacteria and their corresponding genetic elements in water samples taken from the Mhlathuze River in South Africa, and revealed a strong correlation between the resistance profiles of bacteria in the environment and bacteria in stool samples of nearby human diarrhea patients. The authors wrote that "the prevalence of antibiotic resistance was directly related to the frequency of antibiotic usage," and went on to conclude that the Mhlathuze River "not only played a role as a reservoir but also was a medium of spread and evolution of antibiotic resistance."

In wetlands, antibiotics and antibiotic resistance may also have subtle effects on the microbial communities that play important roles in many aspects of a wetland's biogeochemistry. Examples of non-target microbes that may potentially be affected by antibiotics include photosynthetic cyanobacteria, nitrifying and denitrifying bacteria, and decomposers residing in the wetland detritus layer (Jones et al., 2003; Maul, Schuler, Belden, Whiles, & Lydy, 2006). Changes that begin at the level of bacteria may cascade through the food chain (Daughton & Ternes, 1999),

creating disturbances to the wetland ecosystem that fall well outside the bounds of simple considerations of acute or chronic toxicity.

The topic of antibiotic resistance in the environment is poorly understood and subject to myriad complexities. Given the likelihood of continued increases in the use of antibiotics and impingement of human activity on the environment, it should be abundantly clear that further study of this issue is merited.

1.4 Objectives

In order to add to the current body of wetland research and to help fill the significant research gaps surrounding the unknown effects of pharmaceutical runoff, the primary objective of this project was to assess the overall impact of human activity, broadly defined in this paper as "human factors," on specific indicators of wetland health. The fundamental research question guiding this project was:

How do human factors affect the health and function of Maryland's wetlands?

Though this research question initially appeared direct in nature, it quickly proved to be quite complicated and multi-faceted in its scope. For this study, "human factors" were defined by two parameters: the land use surrounding each wetland site, and the prevalence of antibiotic resistance found at each site. This definition helped further split the fundamental research question into the pursuit of two more specific objectives:

1. To use common indicators of wetland health and function to determine if Maryland's wetlands are functioning properly, and to understand how they are impacted by surrounding land use.

2. To assess the prevalence of antibiotic resistance in Maryland's wetlands, and to determine its relationship to other indicators of wetland health and function.

1.5 Outline of Study

Mitigation wetland sites were selected for sampling with assistance from the Maryland Department of the Environment.

Objective 1 was accomplished through the use of standard, well-studied environmental sampling protocols that involved collecting vegetation, soil, and water samples from each wetland site. The soil and water samples were analyzed for specific characteristics and chemical composition, and standardized techniques were used to quantify and qualify the vegetation found at each site. This collected data was then compared to accepted measures of wetland health and to site land use designations determined through the use of a geographic information system (GIS). Significant correlations were then evaluated and used to provide an overall assessment of the functioning of each of the Maryland wetland sites tested.

Objective 2, required a more novel research strategy due to the scarcity of published bacterial antibiotic resistance data for wetlands. To complete this objective, water samples collected at each site were tested for the presence of *Escherichia coli*. If *E. coli* was present, a disc diffusion method was used to

determine the bacterial population's degree of resistance to several different antibiotics. A standardized procedure was created for these lab analyses in order to maintain consistency and validity across the scope of the study. Comparing the resistance results to Objective 1's determination of wetland health at each site allowed for correlations to be drawn between the level of antibiotic resistance and the functioning of the wetland.

1.6 General Study Hypotheses

- Wetlands heavily impacted by human development will exhibit higher levels of nutrients in soil and water and lower plant diversity than wetland in natural settings.

- Wetlands heavily impacted by human development will exhibit higher levels of antibiotic resistance than more natural wetlands due to the higher volumes of pharmaceutical pollution entering them.

2. Literature Review

2.1 Wetlands

2.1.1 Mitigation Wetlands

Mitigation wetlands are artificially constructed to combat the consistent loss of wetland acreage. To compensate for this consistent, unavoidable loss, the U.S. Army Corps of Engineers and the MDE issue permits to restore, enhance, or create wetlands (Brinson & Rheinhardt, 1996). In order to do this, non-wetlands are converted to jurisdictional wetland status. Typically, this requires excavating a depression that connects with the ground water table, or by hydraulically connecting the site to a source of ground water (Brinson & Rheinhardt, 1996).

As can be seen in the most recent report from the United States Fish and Wildlife Service (USFWS), we are still losing significant acreage of the nation's wetlands (United States Fish and Wildlife Service, 1997). When the amendments were added to the Clean Water Act in 1977, a federal "no net loss" policy regarding wetlands was endorsed. The primary tool to combat wetland loss was the large-scale construction of mitigation wetlands.

Working in conjunction with the MDE, thirteen mitigation wetland sites were chosen to complete this research due to the opportune level of access provided by the government and the mutually beneficial baseline data collection used to complete our research as well as contribute to the MDE's body of data for Maryland's waterways.

2.2 Wetland Assessment Protocols

2.2.1 Vegetation

Wetland vegetation is a valuable indicator of a wetland's function, and it is a primary factor considered when classifying wetlands. Plant community structure is viewed as "one of the best indicators of the factors that shape wetlands within their landscape" (Bedford, 1996). Several studies have indicated that vegetation composition influences various factors, including, but not limited to, groundwater chemistry, erosion, and diversity of wildlife (Balcombe, 2005). In addition, vegetation study assists in delineating the boundaries of the regions under study (Cowardin et al., 1979). For example, under some classification systems, areas dominated by certain types of vegetation are not considered as part of what is defined as a "wetland" (Cowardin et al., 1979). In addition, plant composition provides a greater insight into the wetland under study, for it has been shown to influence various other characteristics of the wetland. Finally, identification of vegetation does not require extensive knowledge, and the skills necessary to classify species can be acquired relatively easily.

2.2.1.1 Classification Systems

As noted by the EPA, several wetland classification systems have emerged in an attempt to "reduce variability … and enable more sensitivity in detecting differences between least impacted and impaired wetlands" (Fennessy, 2004). These systems are based on numerous factors, including hydrology, landscape features, and plant composition. The majority of techniques are more quantitatively based, while

more experienced researchers may rely on more qualitative methods. The most prominent systems include the Anderson Classification System, the Cowardin Classification System, and the General Wetland Vegetation Classification System (GWVCS) (Dieck & Robinson, 2004). Each system was developed with specific goals in mind: the Anderson System consists of a two-tier hierarchy to be used with remote sensing systems; the Cowardin System is more detailed and classifies regions by focusing on ecologically similar habitats; and the GWVCS builds on the Cowardin system (Dieck & Robinson, 2004). Another prominent technique, and the one employed in this study, is the North Carolina Vegetation Survey (NCVS). Finally, a quantitative method discussed is the Transect Method.

2.2.1.2 History

The Anderson Classification System was first published in the early 1970s as a revision to an existing classification system presented in the U.S. Geological Survey Circular 671. The Anderson system was designed specifically so that it would be agreeable to data from satellite, aircraft, and other remote sensing sources (Anderson, et al. 1976). The goal was to provide a uniform method (at a basic level) to categorize land use and cover for federal and state purposes. The Anderson System has a multilevel structure to accommodate different sensors that provide data at various resolutions depending on altitude and scale. Level I classification is designed for use with the LANDSAT program, which is a joint coalition between NASA and the U.S. Geological Survey to gather satellite photography of the Earth. Level II is for use with high-altitude data (40,000 feet or above), level III is for use with mid-

altitude data (10,000 – 40,000 feet), and level IV is for use with low-altitude data (below 10,000 feet), but these generalizations are not limiting "It is intended that these latter levels of categorization will be developed by the user groups themselves, so that their specific needs may be satisfied by the categories they introduce into the structure" (Anderson, et al., 1976). For this reason, the system was left open-ended and detailed specifications were not given, thereby giving respective users flexibility in defining their own categories at higher levels of classification. Data at levels I and II is probably more useful for individuals seeking information on a nationwide or statewide basis, while levels III and IV are more useful to those seeking information on a more local basis.

Based on their survey, Anderson et al. proposed nine level I categories: urban or built-up land, agricultural land, rangeland, forest land, water, wetland, barren land, tundra, and perennial snow or ice. These level I divisions were further divided for a total of 37 level II categories. For example, urban or built-up land is divided as follows:

Table 1: Example of urban or built-up land level II categories

Level I	Level II
1 Urban or Built-up Land	1.1 Residential
	1.2 Commercial and Services
	1.3 Industrial
	1.4 Transportation, Communications, and Utilities
	1.5 Industrial and Commercial Complexes
	1.6 Mixed Urban or Built-up Land
	1.7 Other Urban or Built-up Land

Within this framework, users may create additional distinctions. Residential, for instance, may be further classified in level III as single-family units, residential hotels, mobile home parks, etc. (Anderson, et al., 1976).

Unlike the Anderson System, the remaining classification systems discussed do not such a broad, top-down approach. At the highest level, the Cowardin method identifies five main categories that incorporate the various elements such as landscape and vegetation to characterize a wetland. These categories are: Marine, Estuarine, Riverine, Lacustrine, and Palustrine. In order to identify the plant communities themselves and better define a wetland, scientists have subdivided these categories as follows:

Table 2: Subsytems of wetlands derived from the Cowardin method

System	Subsystem
Marine	Subtidal
	Intertidal
Estuarine	Subtidal
	Intertidal
Riverine	Tidal
	Lower Perennial
	Upper Perennial
	Intermittent
Lacustrine	Littoral
	Limnetic
Palustrine	None

The subsystems are further divided based on substrate, soils, water chemistry, or vegetation. An example is the Emergent Wetland, which can be found under the latter four systems. Emergent wetlands are characterized by the presence of grasses, sedges, and other herbaceous species. The plants are often perennial, and Emergent

Wetlands can be found throughout the United States. They are also known as marshes, meadows, and sloughs. Further information can be found in *Classification of Wetlands and Deepwater Habitats of the U.S.* (Cowardin et al., 1979).

The GWVCS builds on the Cowardin System and is the one endorsed by the U.S. Geological Survey in their General Classification Handbook for Floodplain Vegetation in Large River Systems (Dieck & Robinson, 2004) The GWVCS further extends the Cowardin method by identifying 31 major classes within six hydrologic domains. These domains range from those in which water is constantly present to those where it is rarely present. The process begins with obtaining aerial photographs, either color-infrared (CIR) or true-color, though the former is preferred. This is often done in late summer, for this is when aquatic vegetation is at the height of its growth. Areas that appear debatable in the photographs are then visited and detailed observations are recorded. The aerial photographs are subsequently analyzed using a stereoscope; the vegetation is categorized based on the 31 classes, and other factors such as density are determined. In a final step, the results of this interpretive work are digitally processed using geographic mapping software in order to reference them with real world coordinates (Dieck & Robinson, 2004).

2.2.1.3 Vegetation Sampling Methods

Vegetation sampling methods have been developed to serve a variety of purposes, and selecting the correct method for a particular study is difficult. Two major methods, the line intercept method and the cover/nested cover quadrants method, will be discussed here because of their merits in regard to this study.

The line-intercept, or transect method is a quantitative means of assessing the vegetation of a wetland. It involves setting up transects across a site and recording the occurrence of plant species along the transect line at predetermined intervals. Transect lines can range in length from millimeters to kilometers, depending on their intended application. This method is particularly useful and applicable in sites where the vegetation is sparsely distributed, or in areas populated by tall trees. It is a tedious method in areas where plants are small, interwoven, tussocky, or densely populated. The transect method can be easily adapted for use in small or large areas, which makes it attractive to many state and federal environmental agencies. In Maryland specifically, its use is required by the Maryland Department of the Environment when wetlands exceed 5 acres (Bonham 1989).

The cover quadrants method is a well-established technique used when counting individual stems is impractical. It involves visually estimating leaf area cover with respect to a prescribed plot. Multiple graduated quadrants can be "nested" to form layers of quadrants that give an idea of the prevalence of species, as well as total leaf area cover (Bonham 1989). An example of this survey method is the North Carolina Vegetation Survey. Established in 1987, the main goal of the NCVS is to categorize the natural plant life of North Carolina and nearby states (Peet, et al., 1997). This will better enable researchers to interpret the interactions between plant life and the general environment and to monitor those relationships on a long-term basis. The underlying idea behind the NCVS is to create a flexible method of vegetation classification to satisfy numerous purposes. It employs a module, quadrant, based approach to determining the plot layout. All measurements and

observations are taken within plots consisting of multiple 10 x 10 m modules. The number of modules utilized is left to the discretion of the user, thereby providing flexibility. In each module, both cover and stem data are collected (Peet, et al., 1997). According to Peet et al., "Percentage cover represents a crude estimate of the vertical projection of leaf area and other aboveground parts (not leaf area index) and is thus an index of a species' potential contribution to community production." The presence of a species, on the other hand, is measured by the stem count. In order to be considered within a module, the species must have at least one stem originating from the soil within the module. Further measurement specifications are detailed in Section 3.2.2.1: Plot Setup.

The NCVS method was chosen as the vegetation sampling method for this study. The flexibility and reliability of the method were primary motivators in this decision, as well as the established familiarity of the field samplers with the method.

2.2.2 Water Quality

Every body of water has an individual pattern of physical and chemical characteristics determined largely by the climatic, geomorphologic and geochemical conditions prevailing in the drainage basin and the underlying aquifer (Chapman, 2006). Certain variables can provide a strong picture of water quality at a particular site and can act as key indicators to the overall health of a wetland. Total quantities of dissolved solids indicate the condition of water bodies of a similar nature. Mineral content, which is determined from the amount of total dissolved solids, is also an essential feature of water quality that results from the balance between dissolution

and precipitation. Minerals and nutrients are necessary for wetlands to thrive, but an excess of nutrients can prove harmful. Excessive nutrient inputs from sewage effluent, agriculture, or internal loading caused by fish foraging and excretion can destroy wetland vegetation (Wersal, 2006). Thus, the specific level of nutrients can indicate much about the health of a wetland. Lastly, oxygen content influences the solubility of metals and the presence of oxygen is necessary for many forms of biological life. Hence, dissolved oxygen is also a vital factor to consider when addressing the health of any water body.

Hydrological processes are important factors in making determinations about vegetation communities, wildlife habitat, nutrient cycling, and other wetland functions (Mitsch & Gosselink, 2000). However, obtaining accurate water quality data is typically both expensive and time-consuming; therefore, water quality data for wetlands is scarce and only known for a few scattered wetlands in any one area (Kusler, 1998). If it is not feasible to assess wetlands through extensive sampling, then a quicker assessment method is needed. One of the MDE's long term goals is to use this research study's data to develop an improved rapid assessment method. This rapid assessment method would only incorporate data that is relatively easy and quick to obtain in order to assess the health of the wetland.

Water bodies can be classified by their water quality characteristics. It is often easy to identify a certain body of water as a wetland, but in some environments (especially where wetlands and upland areas converge) it can be difficult to distinguish the boundaries of a wetland. Some bodies of water may exhibit unusual water quality data and would be difficult to classify, but in general, water quality data

is an accurate indicator of water body type. It is also possible to further classify a wetland as tidal, non-tidal, mitigation, etc. Researchers at the University of Michigan have devised a hydro geomorphic (HGM) wetland assessment method that can assess the functional condition of a specific wetland on the basis of a range of physical conditions. HGM groups wetlands into seven different wetland classes and provides an ecologically-sound means for classifying, assessing, and comparing wetland hydrodynamics and related functions (Merkey, 2006).

Once a body of water is classified as a wetland, it can be compared to other existing wetlands. Water quality data, such as mineral content, can be analyzed and compared to data from wetlands that are already known to be healthy or unhealthy. Certain existing standards, including beneficial use (e.g., drinking, swimming), numeric (e.g., allowable concentrations of pollutants) and narrative components (e.g., unacceptable surface conditions), have been developed by governmental and environmental agencies. These can be used to judge water quality data and to draw comparisons between bodies and among restoration and purification plans.

In selecting this project's water quality variables, we placed a high importance on the levels of nitrogen compounds present. Plants and micro-organisms are constantly converting inorganic nitrogen to organic forms and thus the cycling of nitrogen is necessary for all living organisms. Inorganic nitrogen occurs in a range of oxidation states and based on the different levels of nitrogen compounds one can determine in which stage of the nitrogen cycle the environment is operating (Chapman, 1996).

The nitrogen compound variables that we decided to monitor were: Ammonia (NH3), Nitrite (NO2-), Nitrate (NO3-) + Nitrite, and Total Nitrogen (N). Since we did not have the capability to test for these compounds ourselves, we decided to send the water samples collected to Appalachian Lab for chemical analysis, where they could also be quickly tested for Total Phosphorous. Ammonia occurs naturally in wetlands and results from the breakdown of nitrogenous matter in the water. It is also sometimes discharged into the wetlands through industrial waste. High concentrations of ammonia are toxic to aquatic life given the pH level. Unpolluted waters contain small amounts of ammonia and ammonia compounds, usually <0.1 mg l-1 as nitrogen. Total ammonia concentrations measured in surface waters are typically less than 0.2 mg l-1 N but may reach 2-3 mg l-1 N (Chapman, 1996). Higher concentrations are suggestive of some type of organic pollution, thus ammonia was selected as a variable to indicate organic pollution.

Nitrate (NO3-) is the main form of combined nitrogen found in wetlands, and can be reduced to nitrite (NO2-) by the denitrification process. Nitrate has many natural sources, including plant and animal debris, and is an essential nutrient for wetland plants. The level of nitrate plus nitrite in surface water gives an indication of both the nutrient status and the level of organic pollution. Because of this, a combined quantification of nitrate and nitrite are included in almost all water quality surveys, especially background monitoring programs such as ours. When influenced by human activities, surface waters can have nitrate concentrations up to 5 mg l-1 NO3--N, but often less than 1 mg l-1 NO3-N (Chapman, 1996). Concentrations in excess of 5 mg l-1 NO3--N usually indicate pollution by human or animal waste or

fertilizer run-off. In cases of extreme pollution, concentrations may reach 200 mg l-1 NO3--N (Chapman, 1996). The World Health Organization (WHO) recommended that the maximum limit for NO3- in drinking water should be 50 mg l-1 (or 11.3 mg l-1 as NO3-N) (Chapman, ed., 1996), and classified waters with higher concentrations as representing a significant health risk. Nitrite concentrations in freshwaters are usually very low by comparison, 0.001 mg l-1 NO2--N, and rarely higher than 1 mg l-1 NO2--N (Chapman, 1996). Total nitrogen levels, while subject to seasonal fluctuations, are used as a general indicator of pollution.

Phosphorous is also a necessary nutrient for living organisms. It is usually the limiting nutrient for algal growth. Artificial increases in phosphorous levels due to unnatural activity are the leading cause of eutrophication (Chapman, 1996). The weathering of phosphorus-bearing rocks and the decomposition of organic matter are the principal sources of phosphorous. Because phosphorous plays such an instrumental role in the biological cycle, it is also included in almost all basic water quality surveys and monitoring programs. As stated earlier, the presence of high phosphorous concentrations are largely responsible for eutrophic conditions and, like nitrogen, can also indicate the presence of pollution. One must maintain an accurate knowledge of phosphorous levels in order to sufficiently manage a wetland site. Although there can be considerable seasonal fluctuations, in most natural surface waters phosphorus ranges from 0.005 to0.020 mg l-1 PO43--P. Concentrations as low as 0.001 mg l-1 PO43--P may be found in some pristine waters and as high as 200 mg l-1 PO43--P in some enclosed saline waters. Average groundwater levels are about 0.02 mg l-1 PO43--P (Chapman, 1996).

Dissolved oxygen (DO) readings of surface water are also useful in evaluating the health of a wetland. The organisms that are responsible for self-purification processes are dependent upon oxygen.   Dissolved oxygen readings depend on both temperature and the level of biological activity present. DO percentages can and do change, both over a seasonal basis and on a daily basis. In still waters, pockets of both high and low concentrations of DO can be found in close proximity to one another.   Since oxygen is involved in a majority of chemical and biological processes, DO is also a very common variable used in water quality assessments. In fresh-waters dissolved oxygen (DO) at sea level ranges from 15 mg l-1 at 0° C to 8 mg l-1 at 25° C. Concentrations in unpolluted waters are usually close to, but less than, 10 mg l-1 (Chapman, 1996). Concentrations below 5 mg l-1 may adversely affect the functioning and survival of biological communities and below 2 mg l-1 may lead to the death of most fish.

Finally, the temperature of the water must be taken into consideration when evaluating all other variables. Water temperature, especially surface water temperature, can fluctuate greatly because it is influenced by so many factors, including season, time of day, and cloud cover. Water temperature affects many chemical, physical, and biological processes and thus effects the concentrations of many other variables (Chapman, 1996). As temperature increases, the speed of chemical reactions also increases and the solubility of gas in the water decreases. Water temperature influences plant performance, especially photosynthetic rates (Pilon & Santamaria 2002), however, extremely high water temperatures can reduced photosynthetic rates and have a negative effect on the ecosystem (Spencer, 1986).

Also, higher temperatures can lead to increased respiration rates and thus increased oxygen consumption. So, when evaluating water quality data, unusually low DO readings must always be evaluated with respect to temperature in order to accurately analyze its context. Groundwater, such as those samples collected from lysimeters, maintain a relatively constant temperature somewhere close to the mean annual air temperature.

2.2.3 Soil

The Federal Manual for Identifying and Delineating Jurisdictional Wetlands was published in 1987 with the help from four agencies: the USFWS, the EPA, and the United States Army Corps of Engineers, and the Soil Conservation Service. According to this manual, in order for an ecosystem to be classified as a wetland it must meet three criteria: it must possess hydrophytic vegetation, wetland hydrology, and hydric soils, thus establishing the importance of soils in the classification of wetlands (Lilly, 1993). In most ecosystems, soil plays an important role in overall function; however, a special condition exists in wetlands because the soil is frequently saturated with water. It is not uncommon to find a layer of standing water at most wetland sites throughout the growing season (USDA, 1998). As the ground becomes saturated with water, water molecules begin to fill pore space once occupied by air, thus depriving the soil of oxygen. As water fills the gaps found between the soil peds, the rate of oxygen diffusion through the soil is greatly reduced. The reduced rate of oxygen circulation in the soil results in anaerobic conditions. It is these anaerobic conditions that are responsible for the presence of wetland vegetation.

When there is a lack of oxygen, other chemicals must be used in the chemical transformation process, including: nitrogen, manganese, sulfur, and iron. The transformation reactions of these chemicals enable wetlands to act as sinks and filtration mechanisms for nitrogen and sulfur pollution.

An important equation in determining the existence of a hydric soil is the Nernst equation. Nernst equation: $E_H = E_0 + 2.3[RT/nF]\log[\{ox\}/\{red\}]$, is the equation used to calculate the redox potential of a soil (Mausbach et al, 1994). Redox potential, also referred to as oxidation-reduction potential, is a measurement used to quantify the electrochemical reduction of wetland soils. With an abundance of oxygen in the soil, the redox potential should be between +400 and +700 mV; however in an anaerobic soil, such as those found in wetlands, the redox potential fluctuates between –400 and +400 mV. As oxygen becomes unavailable, organic substrates look to donate electrons to substances other than oxygen, thus lowering the redox potential value. The first terminal acceptors of electrons in anaerobic soils are nitrates (NO_3^-) (Mausbach et al, 1994).

Nitrates are often found in wetland soils and are reduced to nitrites (NO_2^-) and ultimately to N_2O and N_2. Nitrogen is often identified as one of the most limiting nutrients of wetland soils. Fertilizers used by humans add a great deal of unnatural nitrates to the nitrogen cycle. If anaerobic soils were not able to process out this excess nitrate, then pollution would present an even bigger challenge. Through the process of denitrification, excess nitrates in the soil lithosphere are converted into gaseous forms of nitrogen, specifically N_2 and N_2O. Nitrogen often enters the soil in the form of nitrates or ammonium (NH_4^+), from either crop residues or fertilizers.

When there is a deficiency of oxygen, symbiotic microorganisms in the soil convert the NO_3^- into nitrogen oxide and nitrogen gases, which are able to escape into the atmosphere (Mausbach et al, 1994). Plants are also responsible for the uptake of a large amount of nitrogen from the soil in the form of nitrates. Nitrates are a pollution concern because they easily leach from the soil and pollute our waterways (Reppert et al, 1979).

Iron and manganese are also important nutrients for they are also reduced in chemical transformations when oxygen is absent. Typically, iron is found in its oxidized state as $Fe(OH)_3$. Oxidized iron is easily identifiable because the soil often has a reddish or brown hue. Soils in wetlands are often identified with a grayish color because the iron in these soils is in its reduced form ($Fe(OH)_2$). A depleted or reduced matrix in a soil profile occurs when iron is either removed or reduced by chemical transformations. The reduced forms of both iron and manganese are mobile; therefore, they have a tendency to accumulate in pore spaces. Reduced iron and manganese are stripped from the soil peds, leaving behind the dull gray color of the mineral matrix (Soil Survey Staff, 1999).

Often during the growing season the water table fluctuates. This fluctuation of the water table can be identified by the presence of substances known as mottles. Root channels and macropores often contain sources of oxygen in anaerobic soils. Root channels, often referred to as oxidized rhizospheres, have available oxygen because oxygen escapes from the roots of plants into the surrounding root channel. Once this oxygen contacts the soil, it is able to transform iron and manganese back

into their oxidized states. This condition can be identified by the presence of reddish soil surrounding the root channels of plants (Richardson, et al, 2000).

A final terminal electron receptor in the electron chain is sulfate ($SO_4{}^{2-}$). Sulfate reduction in wetland soils is often caused by the presence of microorganisms. Bacteria are able to convert sulfates into hydrogen sulfide, by using sulfur as a terminal electron receptor in anaerobic respiration. The release of this hydrogen sulfide gas is responsible for the rotten egg odor which is prevalent at wetland sites (Mausbach et al, 1994).

Another important characteristic of wetland soils is the presence of a large layer of organic matter near the surface of the soil profile. This organic matter, often found in the form of peat or muck, plays an important role as an energy source for the many chemical transformations which occur in wetland soils. Dead plants replenish the soil with nutrients as they are broken down by the many microorganisms present in soil (Richardson, et al, 2000).

2.3 Antibiotics

2.3.1 Methods of Analysis

Testing for the presence of antibiotics in the environment encompasses two major possibilities: direct analytical testing of drug concentrations and indirect microbiological testing of resistance patterns in samples of collected bacteria.

Direct testing techniques usually involve the use of gas or liquid chromatography in combination with mass spectrometry. Ahrer, Scherwenk, and Buchberger (2001) note that until 2001, analysis of chemicals in surface waters was

mostly conducted using gas chromatography in combination with mass spectrometry. However, since then, additional research has been performed exploring other technologies, such as the use of capillary electrophoresis with mass spectrometry. In particular, liquid chromatography with tandem mass spectrometry is becoming more prevalent because the method is highly specific, enabling better detection of compounds. The technique also allows for the separation and delineation between ions of different compounds with the same molecular mass (Fatta et al., 2007).

Such direct testing methods tend to require sophisticated equipment and an involved process of sample preparation. Through a process known as derivatization, water must be pretreated in order to yield optimal results (Ahrer, Scherwenk, & Buchberger, 2001). This procedure may require the use of highly toxic or carcinogenic compounds. The traditional process flow includes filtration, solid phase extraction, and/or derivatization prior to the actual sample analysis via liquid or gas chromatography and mass spectrometry (Fatta et al., 2007).

In comparison, microbiological testing of bacterial resistance to antibiotics tends to be significantly less involved and less expensive. Based on the generally accepted assumption that levels of antibiotic resistance increase with increasing prevalence of antibiotics themselves, testing for antibiotic resistance amongst wetland bacteria is thus a more feasible, albeit indirect, method of testing for the impact of antibiotics in a wetland. To our knowledge, all resistance testing techniques measure minimum inhibitory concentration (MIC), the lowest concentration of a drug that will prevent the growth of bacteria. In the United States and much of the world, the authority on determining standard for bacterial resistance testing is the Clinical and

Laboratory Standards Institute (CLSI). Several methods exist to test for antimicrobial resistance, the most common of which are the disc diffusion and broth dilution tests.

The disc diffusion technique is the solid equivalent of the broth dilution technique (discussed below) and is often preferred because it enables one to simultaneously test for multiple drugs (Lorian, 2005). Perhaps the most well-established method is the Kirby-Bauer disc diffusion assay, developed in 1966. This method involves either plating a "lawn" of bacteria onto a selective medium, such as Mueller Hinton agar, or inoculating the medium with the culture itself, and then placing small paper discs with known concentrations of antimicrobials onto the plate. After a given period of incubation, usually 18–24 hours, there will likely be an area around the disc in which the presence of antibiotic has prevented bacterial growth. The diameter of this so-called "zone of inhibition" is measured, and bacterial sensitivity or resistance to the antibiotic is determined by comparing this diameter to an established standard. Smaller diameters indicate higher concentrations of antibiotic required to inhibit bacterial growth, and thus indicate higher levels of bacterial resistance (Bauer et al., 1966). A similar method is the gradient method. Commercially known as Etest, this technique uses plastic strips preloaded with antibiotics at various concentrations. Numerous strips are placed on a plate in a spoke-like fashion, and an ellipse-shaped clearing around the strip results after incubation. The MIC is determined by the intersection of the ellipse with the test strip (AB BIODISK).

Often, disc diffusion techniques yield poor categorizations for large macromolecules such as vancomycin. Because these drugs have large molecular

weights, they take longer to diffuse into agar growth media, resulting in smaller differences in the sizes of the zones of inhibition. This makes it difficult to differentiate between resistant and susceptible bacteria. To overcome this problem, prediffusion methods have been developed. For example, Neo-Sensitabs are dry crystalline antimicrobial tablets that enhance susceptibility profiles (Katz, Luperchio, & Thorne, in press).

The broth dilution, or microdilution, test is another means of testing for antibacterial resistance. Various dilutions of a given drug are prepared (usually a minimum of ten) and loaded into microwells, inoculated with bacteria, and incubated. Chemical indicators are usually added to aid in the visualization of bacterial growth. After incubation, the turbidity and MIC are determined (Hyman et al., 2002). Susceptibility is evaluated by comparing these measurements with established standards. Additionally, commercial advances have made plates preloaded with antibiotics available.

Finally, efforts have been made to automate the testing process. One such method is the Cobas-Bact technique developed by Roche Diagnostics. This technique evaluates antimicrobial susceptibility in less than five hours. After several rounds of incubation and centrifugation, turbidity of the inoculum is measured, and the system computer determines resistance based on growth of the bacteria. However, a study conducted by Murray, Niles, and Heeren (1987) found discrepancies when this method was compared to traditional disc diffusion and broth dilution techniques.

In this study, we elected to use the Kirby-Bauer disc diffusion method. Previous studies (Drew et al., 1972; Dornbusch et al., 1975; Gaudreau & Gilbert,

1997) have repeatedly shown that when performed correctly this method remains a simple and inexpensive, yet reliable, means of testing for antibiotic resistance. The issue of poor susceptibility profiles for vancomycin, one of the antibiotics we selected, was not a problem for us, since we were using the drug as a negative control. In addition, we possessed the facilities and equipment required to use this technique. Conversely, we did not have access to the specialized equipment necessary for direct testing or broth dilution. *Escherichia coli* was selected as a model organism due to its prevalence in the environment and the ease of isolating and testing for this bacterium.

2.3.2 Selection of Antibiotics

Six antibiotics were selected for evaluation in this study: erythromycin, ciprofloxacin, ampicillin, sulfisoxazole, tetracycline, and vancomycin. Based on our review of previous literature, these antibiotics are among those that are most commonly tested. In addition, each antibiotic is representative of a different class of drugs. Vancomycin was selected as a negative control.

2.3.3 A Brief Background of Antibiotics Chosen for This Study

Erythromycin

Erythromycin is a macrolide drug that is often used to treat acne, strep throat, syphilis, and other infections caused by bacteria. With a range of activity that is slightly broader than that of penicillin, erythromycin is a common alternative for individuals who are allergic to penicillin. The drug possesses bactericidal properties,

meaning that it has the ability to kill bacteria versus simply inhibiting bacterial growth, but the exact mechanism of action is still not fully understood. The prevailing theory is that erythromycin binds to a subunit of bacterial ribosome and inhibits protein synthesis.

Ciprofloxacin

Ciprofloxacin is a broad spectrum antibiotic that is regularly used to treat various infections. It belongs to a group of bactericidal compounds known as fluoroquinolones. Furthermore, ciprofloxacin affects both Gram-positive and Gram-negative bacteria by targeting the enzymes topoisomerase IV and DNA gyrase, respectively. Both of these enzymes are essential for bacterial DNA replication. By inhibiting DNA replication and transcription, ciprofloxacin leads to chromosomal breaks and eventual death of the cell. Neither of the two enzymes is present in eukaryotic cells, which is why ciprofloxacin is safe for human use.

Ampicillin

Ampicillin belongs to the aminopenicillin family, within the broader class of drugs known as beta-lactam antibiotics. Beta-lactam drugs are bactericidal and were initially thought to only affect Gram-positive bacteria. However, recent developments indicate that they are effective against various strains of Gram-negative bacteria as well. Ampicillin acts by interrupting synthesis of the peptidoglycan layer of the cell wall. By competitively inhibiting the enzyme transpeptidase – a penicillin-

binding protein (PBP) – ampicillin disrupts the final step of peptidoglycan synthesis and eventually leads to cell lysis.

Two main mechanisms of bacterial resistance toward beta-lactam drugs exist. This class of drugs is characterized by what is known as a beta-lactam ring, which plays a critical role in drug interactions with PBPs. If this ring is not intact, it could lead to bacterial resistance of the drug. Bacteria that are able to produce enzymes such as beta-lactamase and penicillinase are able to hydrolyze this ring and disrupt its structure and overall effectiveness. Resistance also emerges if the PBPs are altered in some way, making it difficult for the drug to bind. This is seen in infections such as methicillin-resistant *Staphylococcus aureus* (MRSA).

Sulfisoxazole

Sulfisoxazole is one of several sulfonamide-based drugs, or sulfa drugs. Though some of these drugs do not possess antibacterial properties, sulfisoxazole does have bacteriostatic activity, meaning that it can inhibit growth, against both Gram-positive and Gram-negative bacteria. The compound acts as a competitive inhibitor of an enzyme critical to folate synthesis. Folate is essential for DNA and RNA synthesis, and thus sulfisoxazole effectively hinders cell division. The drug is only effective versus bacterial cells, because mammals do not produce folate; instead, it is a dietary requirement.

Tetracycline

Tetracyclines are broad spectrum antibiotics that are often used to treat acne and ulcers, in addition to having several dental applications. This class of drugs works by binding to the bacterial ribosome, thereby inhibiting protein production and subsequent growth. Mechanisms of resistance towards tetracyclines include inactivation via enzymes or through the production of proteins that effectively pump the drug out of the cell. Another method is that of ribosomal protection, in which a resistance gene encodes a protein, which performs one of many functions in order to defend the ribosome, including blocking the binding of or dislodging already bound tetracycline.

Vancomycin

Vancomycin is primarily effective against Gram-positive bacteria, for it acts by inhibiting cell wall synthesis; the outer membrane of Gram-negative bacteria impedes the molecule's entry into the cell, and therefore vancomycin is unable to impact the cell wall. Over the years, the drug gained popularity due to the fact that staphylococci had difficulty gaining resistance towards it, as opposed to their rapid development of resistance towards penicillin. However, vancomycin is for the most part used only as a drug of last resort due to its strength and nature of side effects. In our study, vancomycin was chosen as a negative control due to its activity and potency.

2.3.4 Prevalence of Antibiotic Resistance

Antibiotic resistance of bacteria is a growing problem, particularly in the hospital setting. There has also been concern regarding vulnerability of patients in related settings, such as nursing homes (Weiner et al., 1999). According to the World Health Organization, up to 60 percent of nosocomial, or hospital-acquired, infections in the United States are caused by drug-resistant bacteria. In addition, these microbes could potentially be resistant to as many as 10 different antibiotics (World Health Organization, 1996). Part of the problem stems from the fact that while it could take up to decades to develop a drug, these same drugs may not be effective for as long a period of time due to the quick nature of resistance transfer amongst bacteria. Additionally, Clark, Patterson, and Lynch (2003) note that heightened use of broad spectrum antibiotics such as the beta-lactamases has contributed to multidrug resistance. On the other end of the spectrum is the fact that sanitation and other asceptic standards must be maintained; this could be an issue in third-world nations.

While it is generally accepted that there is an increasing prevalence of antibiotic-resistant bacteria in hospitals, it is difficult to quantify this increase because of the lack of consistency of trends in all hospitals (Fridkin et al, 2002). A majority of the studies to date have focused on intensive care units (ICUs); due to the quick pace required in an ICU, there may not be adequate time to follow proper sanitation techniques, and there is likely an increased risk of spreading resistance (Fridkin & Gaynes, 1999). Reports from the Center for Disease Control (CDC) reaffirm this notion and assert that patients receiving treatment in ICUs are at an increased risk for nosocomial infections including pneumonia, urinary tract infection, and other

bloodstream infections (Fridkin & Gaynes, 1999). Additionally, Vincent et al. (1995)

found that over 20 percent of patients admitted to ICUs in Western European

hospitals developed ICU-acquired infections.

Ampicillin, tetracycline, and ciprofloxacin were among the major antibiotics

that we encountered in our survey of prior studies of resistance in hospitals. Estrada-

García et al. (2005) analyzed isolates of *E. coli* from children in Mexico that had been

hospitalized for diarrhea. Among these diarrheogenic isolates, it was found that 73

percent were resistant to ampicillin. In another study, Oteo et al. (2005) surveyed 32

Spanish hospitals and found that among the 7,098 invasive *E. coli* isolates, 59.9

percent were resistant to ampicillin. Conversely, resistance of *E. coli* or other gram-

negative species to ciprofloxacin was relatively low in most of the studies reviewed,

ranging from 19.3 percent (Oteo et al., 2005) to approximately 40 percent (Aksaray et

al., 2000). However, it has been documented that resistance towards the drug is

steadily increasing. A survey by Neuhauser et al. (2003) noted a decrease in the

susceptibility of gram-negative bacteria towards ciprofloxacin from 86 percent in

1994 to 76 percent in 2000 – that is, an increase in resistance from 14 percent to 24

percent.

Moving from the hospital to the community setting, Bartoloni et al. (2006)

examined the occurrence of antibiotic-resistant *E. coli* in the feces of children living

in urban neighborhoods of Peru and Bolivia. Once again, highest resistance was seen

towards ampicillin (95 percent) and tetracycline (93 percent). In addition, a random

sample of these isolates was selected and tested for multiple drug resistance patterns

(i.e. combinations of drugs). Ninety percent of these isolates exhibited multidrug

resistance, and two of the three most common combinations included ampicillin and tetracycline. Estrada- García et al. (2005) reported slightly lower multiple resistance results – approximately 58 percent of samples were resistant to three or more drugs. Nevertheless, these statistics highlight the importance of studying antibiotic resistance in conditions other than those found in hospitals.

Some research has been conducted reviewing bacterial resistance patterns in the natural environment. For example, Zuccato et al. (2000) note that drugs such as erythromycin usually have long half-lives and are not biodegradable, and as a result their presence in the environment often persists, sometimes for over a year. In fact, erythromycin has been detected in surface waters more frequently than other antibiotics; however, these levels have been noted to be below those which would foster resistance bacteria (Summers, 2002).

Widespread resistance towards ciprofloxacin has been seen, despite its label as a "drug of last resort." Initially, practitioners were cautious about using the drug due to its broad spectrum activity and potent nature, but this is no longer the case. Additionally, analogues of the drug have been employed much more in agriculture and farming. Until September 2005, enrofloxacin (also known as Baytril), another drug in the fluoroquinolone family, was commonly used in poultry to prevent illness and to boost growth. Studies have found that in the ten year period following the drug's introduction in 1995, enrofloxacin resulted in increased bacterial resistance to ciprofloxacin by approximately 21 percent (Truant, 2005). In another study, antimicrobial resistance patterns of *Neisseria gonorrhoeae* were studied over a four year period in Korea. The investigators found that ciprofloxacin resistant isolates

increased dramatically after the recommendation to use the drug for therapeutic purposes. Specifically, the percent of resistant isolates increased from just 1 percent in 1999 to 48.8 percent in 2002 (Yoo et al., 2004).

The extensive use of ampicillin has lead to widespread resistance, particularly in the hospital setting as noted above. However, resistance is prevalent in the environment as well. A study conducted in 2002 that surveyed 15 U.S. rivers found that ampicillin was ineffective against up to 50 percent of the bacteria sampled (Ash et al., 2002). In addition, no strong pattern could be found regarding the resistant isolates, as they were found in rural as well as urban areas (Raloff, 1999).

Current research indicates that resistance to sulfisoxazole is not extremely prevalent. In a study conducted by Sayah et al. (2005) investigating the antimicrobial resistance patterns of *E. coli* in various environments, only a 13.3 percent resistance among isolates was noted. Similarly, McKeon et al. examined over 250 bacterial isolates (*E. coli* included) from rural water sources in West Virginia, and detected less than 10.0 percent resistance to sulfisoxazole (McKeon et al., 1995).

Research shows that there is indeed a concern over the prevalence of tetracycline-resistant bacteria in the environment. Tetracycline is a common drug used in farming and swine cultivation, and in general, links have been suggested between such use and drug-resistant infections in humans (Chee-Sanford et al., 2001). In the general environment, Sayah et al. (2005) found a 27.3 percent resistance to tetracycline among isolates, while McKeon et al. (1995) noted 32.3 percent resistance. In addition, Chee-Sanford et al. (2001) conducted a study to assess the prevalence of tetracycline-resistant genes in lagoons and groundwater surrounding

two swine facilities. Published in 2001, their results confirmed that all classes of genes conferring ribosomal protection were present in bacterial isolates. These results were significant, since groundwater is a major constituent of drinking water, suggesting that despite intense processing, resistant bacteria have the potential to make their way into our everyday water sources.

Though vancomycin is an incredibly strong drug, resistance towards it has been an emerging problem, particularly in the hospital setting since it is used as a "last line of defense." Vancomycin resistant enterococci (VRE) were first confirmed in the mid-1980's, and since then, multi-drug resistant VRE have also been seen (Rice, 2001). In addition, VRE have been isolated from both hospital and residential wastewater environments (Harwood et al, 2001).

Our high level survey seems to indicate that bacterial resistance seen towards antibiotics in the natural setting is comparable in that seen in the hospital environment.

3. Methods

3.1 Site Selection

Team CRABS studied thirteen wetland sites around Maryland, selected in cooperation with MDE. All wetlands were non-tidal, mitigation sites. These sites were selected to be representative of the each type of mitigation wetland present around the state, including emergent, scrub-shrub, and forested wetlands, but they were also selected on the basis of convenience and feasibility of obtaining landowner permission to visit the sites. Figure 1 is a map of Maryland showing the location of the thirteen sites, and Table 3 gives brief descriptions of these sites.

Figure 1: Map of the 13 Maryland wetland sites used in this study
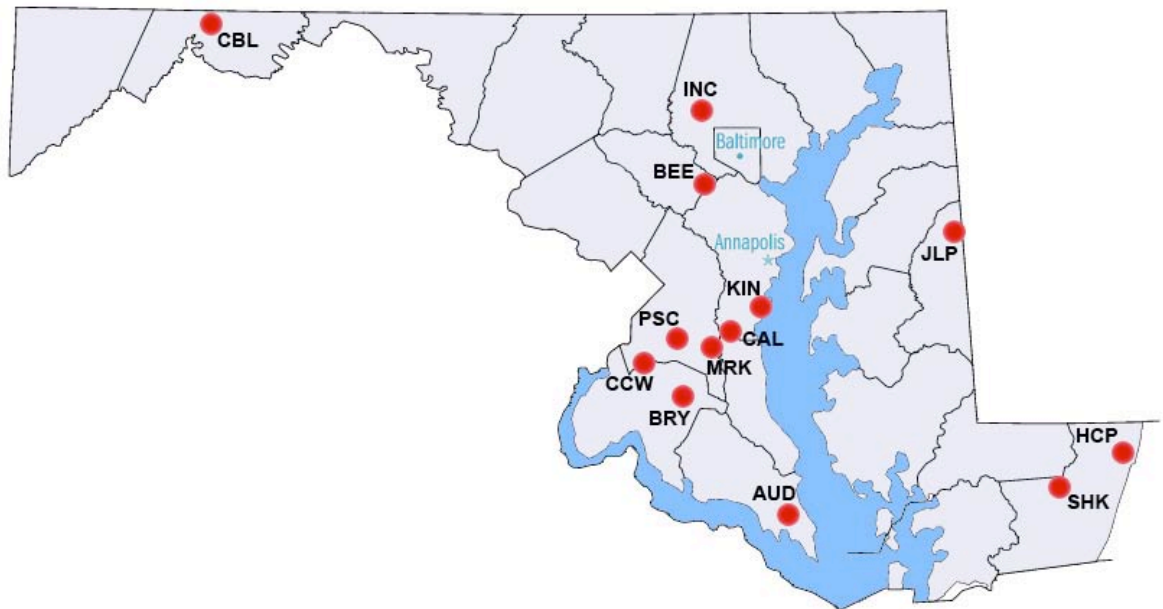
Table 3: Brief descriptions of the 13 wetland sited used in this study

Code	Name	Brief description
AUD	Aud	Small densely vegetated and wet site near the St. Mary's River, just downhill from a horse farm
BEE	Beehive	Small dug out site in Howard County in a residential neighborhood. Train tracks run on the edge.
BRY	Bryantown	Large site located just off of Route 5 in Charles County in a mainly agricultural and residential area
CAL	Calvert	Very small site located on the edge on Calvert County just off of Route 4 at the Route 260 overpass. Very wet.
CBL	Cumberland	Small site nestled in the Appalachian Mountain foothills, just north of Interstate 70
CCW	Waldorf	Charles County site a few miles west of Waldorf, MD. Small yet diverse, shows elements of forests, lakes, and plains
HCP	Herring Creek Park	Site in West Ocean City dominated by phragmites, very wet, only site located in Coastal Bays Watershed
INC	Irvine Nature Center	Former farmland in central Baltimore County, allowed to naturally transform into a wetland
JLP	Jackson Lane Preserve	Large site near Maryland/Delaware border operated by The Nature Conservancy. Fairly flat with several ponds.
KIN	Kinder	Usually dry site within 5 miles of the Chesapeake Bay. Heavily covered by low growing sedges and rushes.
MRK	Merkle WMA	Site just off of the Patuxent River. Constructed wetland which is part of a much larger wildlife management area.
PSC	Piscataway Stream Valley Park	Located near Route 301, east of Route 5. Large ponds in a formerly agricultural area.
SHK	Shockley	Densely forested site in Snow Hill in Worcester County. Areas classified as wetlands usually contain long, narrow ponds.

3.2 Methods of Data Collection

3.2.1 Land Use

The very first step in our research was to analyze the land surrounding each of our

thirteen sites. Following the example of previous studies, it was important to begin

with a land use study as it would help form the framework for the rest of our research.

The standard tool used in studying land use is Geographical Information Systems,

GIS. According to Environmental Systems Research Institute (ERSI), "A geographic

information system (GIS) integrates hardware, software, and data for capturing, managing, analyzing, and displaying all forms of geographically referenced information." (ERSI). Therefore, before we conduct our study we had to determine what GIS software to use, what type of data to examine, and where we would draw this data from.

In performing a GIS study, a researcher could chose from a wide variety of a data types including: watershed data, soil series data, average rainfall, transportation systems, elevation, sewage transport, roadways, population density, land use classification, and more. For purposes of our study we wanted to focus specifically on the land use surrounding our sites.

The software we chose to use was a program entitled GISHydro2000. GISHydro2000 was developed by the University of Maryland's Department of Civil and Environmental Engineering, in collaboration with the Maryland State Highway Administration in 1997. The software and its databases are updated by Dr. Glen Moglen, a professor from the University of Maryland's Civil and Environmental Engineering department. The program combines a GIS platform (ArcView3.x) with a database including land use, soils, drainage areas, watershed, channel delineation, peak discharge estimates data. Through using this program, we were able to extract relevant land use data from Maryland Office of Planning, 2002 into the ArcView3.x software and perform a data analysis using program tools.

After identifying surrounding land use classifications as the object of analysis, we first had to establish buffer zones around each site. We decided to examine the surrounding land use for both a 1000m and 2000m circular buffer around each site.
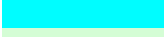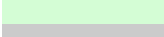
To create the buffers we first had to identify a center point of reference for each

wetland site. These site reference points were identified with the help of Google

Earth and GPS coordinates taken on site. The reference points were chosen for their

close proximity to the center of each wetland site. The coordinates of the center

points were recorded so they could later be imported into GISHyrdo2000. After

uploading the 13 reference points (corresponding to our thirteen sites) to

GISHyrdo2000, we drew a 1000m circular buffer around each point with ArcView

tools. Later 2000m circular buffers were also drawn, as shown in Figure 2. These

buffers were all saved on a shape file on the program database so they could be

reopened for later analysis.

Figure 2: Screenshot from GISHydro2000, showing land use classifications by color, as well as 1000m and 2000m circular buffers around wetland sites
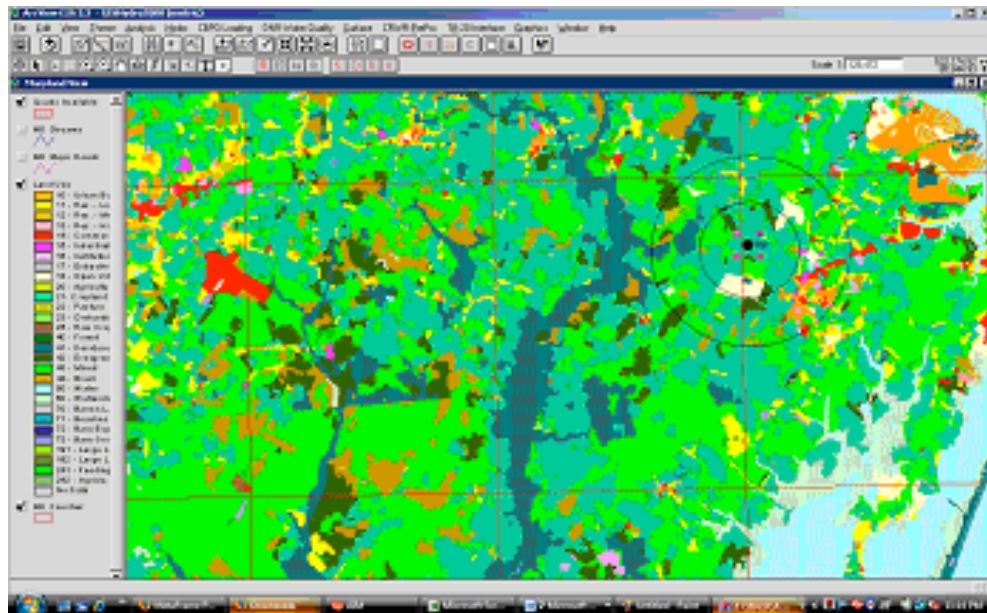
The next step was to import land use data to our ArcView program file through use of the GISHyrdo2000 database. We chose to use land use data from the Maryland Office of Planning database (2002 version). Using program tools, we separately calculated the land area of each buffer which pertained to every land use classification. For example, for both the 1000m and 2000m buffers surrounding Irvine site, we obtained square area values for low residential property, industry, pasture, brush, cropland, etc. The 29 different land use classifications can be seen in Table 4. After calculating land use areas for all thirteen sites for both buffers, we exported this data into a two excel spreadsheets for further statistical analysis.

Table 4: The 29 different land use classifications, sorted by color

Land Use Classification	Color Code	LDI Values
Urban Build-up		
Residential - Low Density		20.51
Residential - Medium Density		26.5
Residential - High Density		29.5
Commercial		30.57
Industrial		32.2
Institutional		30.57
Extractive		35.51
Open Urban Land		5.65
Agriculture		
Cropland		4.99
Pasture		2.09
Orchards		6.45
Row Crops		9.11
Forest		
Deciduous		
Evergreen		
Mixed		
Brush		
Water		
Wetlands		
Barren Land		
Beaches		
Bare Exposed Rock		

Bare Ground	
Large Lot Agriculture	
Large Lot Forest	
Feeding Operations	20.21
Agriculture Buildings	20.21
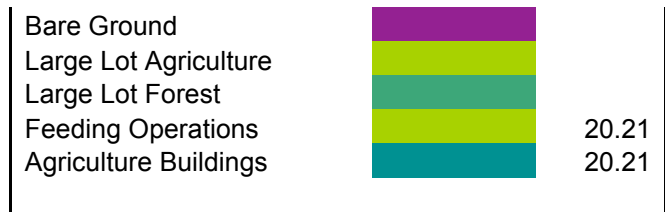
In Microsoft excel, all land use classification categories were assigned to one of the following groups: agricultural, urban, natural. The breakdown of classification was as follows:

Table 5: 29 land use classifications condensed into three classifications to better suit CRABS' study

Condensed Classification	Original Classifications
Agricultural	Agriculture, Cropland, Pasture, Orchards, Row Crops, Large Lot Agriculture, Feeding Operations, Agriculture Buildings
Urban	Urban Build-up, Residential (Low, Medium, High), Commercial, Industrial, Institutional, Extractive
Natural	Forest, Deciduous, Evergreen, Mixed, Brush, Water, Wetlands, Barren Land, Beaches, Bare Exposed Rock, Bare Ground

Following these group delineations, land use areas were aggregated in order to establish overall land use data for Agricultural, Urban, and Natural. These values would later be used in calculating Landscape Development Intensity (LDI) values for all thirteen sites.

Landscape Development Intensity (LDI) Index is a measure commonly used in analyzing the impact surrounding land use has on a site of study. For our research, LDI was the most important factor in determining the impact land use had on the location of our 13 sites. LDI is a weighted average calculation using both an index

coefficient and a percentage. Every land use is assigned a value (the coefficient). From our study using GISHydro2000 we were able to determine the percentage of each land use found in the buffers surrounding all thirteen sites. LDI values were calculated for both the 1000m buffer and 2000m buffer.

Example of LDI Calculation:

LDI = (% Residential Low Density) (LDI Value) + (% Cropland) (LDI Value) + (% Evergreen) (LDI Value) + (% Mixed) (LDI Value) + (% Brush) (LDI Value) + (% Feeding Ops) (LDI value)

LDI = (0.355) (20.51) + (0.134)(4.99) + (0.098) (0) + (0.245) (0) + (0.189) (20.21)

In addition to calculating LDI values, the thirteen sites were further grouped along three broad land use classification categories: Agricultural, Urban, and Natural. Sites were grouped according by computing Agricultural:Urban ratios.

Table 6: CRABS' 13 wetland sites organized by land use classification

Site Classification	Wetland Site
Agricultural	Irvine, Kinder, Jackson Lane, Bryantown
Natural	Beehive, Herring Creek, Calvert, Cumberland
Urban	Aud, Piscataway, Waldorf, Merkle, Shockley

These land use groups would be used later in portraying results from other areas of our study.

3.2.2 Field Work

3.2.2.1 Plot Setup

At each wetland site, three representative plots were chosen within the

wetland boundary. Field researchers picked the starting point with a variety of

methods. Sometimes, location was picked to ensure that plots would cover distinct

areas of the wetlands. Other times, plots were chosen so as to be sufficiently far apart

from one another, when size was an issue. Sometimes, plot location was chosen with

random methods. The method for plot setup was modified from the North Carolina

Vegetation Survey (NCVS) (Peet et al., 1998). In the NCVS method, schematically

represented in Figure 3 below, plastic poles are inserted vertically into the ground and

used to mark off a 20 meter by 50 meter rectangular plot boundary. This plot was

divided into ten 10 meter by 10 meter subplots, or 'modules,' numbered as shown in

Figure 3. Intensive vegetation sampling is performed in module numbers 2, 3, 8, and

9.

Figure 3: Diagram of a normal NCVS plot

Circles represent plastic poles used to mark off the plot.
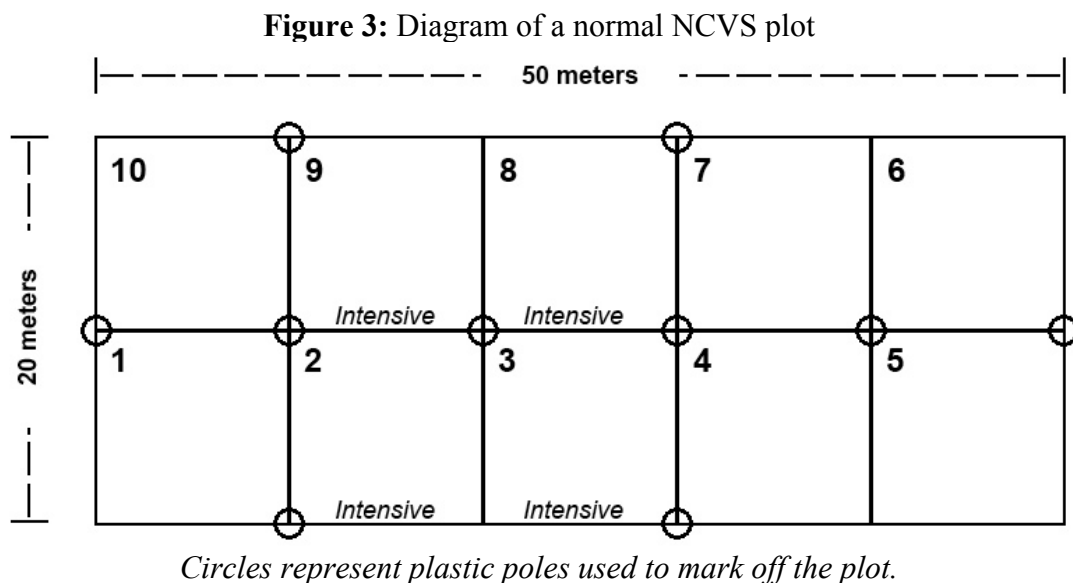
In our modified plot setup method, a 20 meter by 30 meter rectangle was marked off with poles, and divided into six 10 meter by 10 meter subplots. To maintain sequential module numbering and for ease of remembrance, the modules normally numbered 8, 9, and 10 in the NCVS method were respectively renumbered 4, 5, and 6 (see Figure 4). Intensive vegetation sampling was conducted in modules 2 and 5 of the modified plot.

Figure 4: Schematic diagram showing truncation and renumbering of the standard NCVS plot
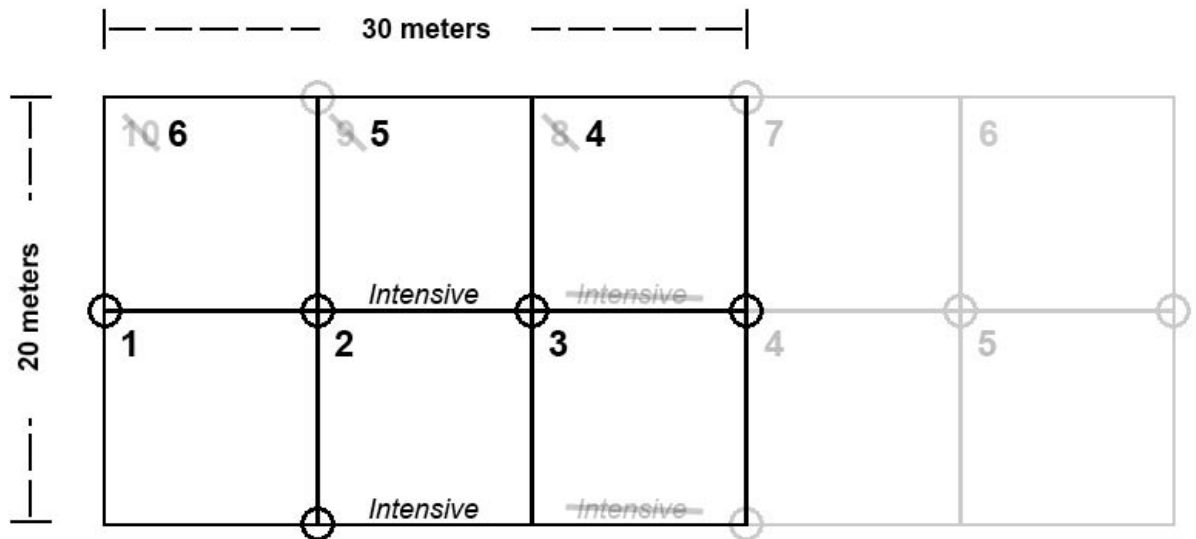
After the plot was set up, GPS coordinates were taken at the start pole, and an azimuth was used to obtain the directional bearing of the plot's center axis line. The center axis line is the line connecting the start and end poles (see Figure 5).

We used a modified survey method to allow us to cover more ground with similar results. Using the NCVS 50 by 20 plot size, we would have had four intensive modules, rather than two. However, the team decided that our time could

be more efficiently spent by surveying three 30 by 20 plots, which would have

encompassed 6 intensive modules, rather than surveying two 50 by 20 plots, covering

8 intensive modules. We felt that gaining information for more plots spread out

across each wetland outweighed the cost of losing intensive modules adjacent to

already-sampled intensive modules.

Figure 5: Schematic diagram of a completed Team CRABS plot
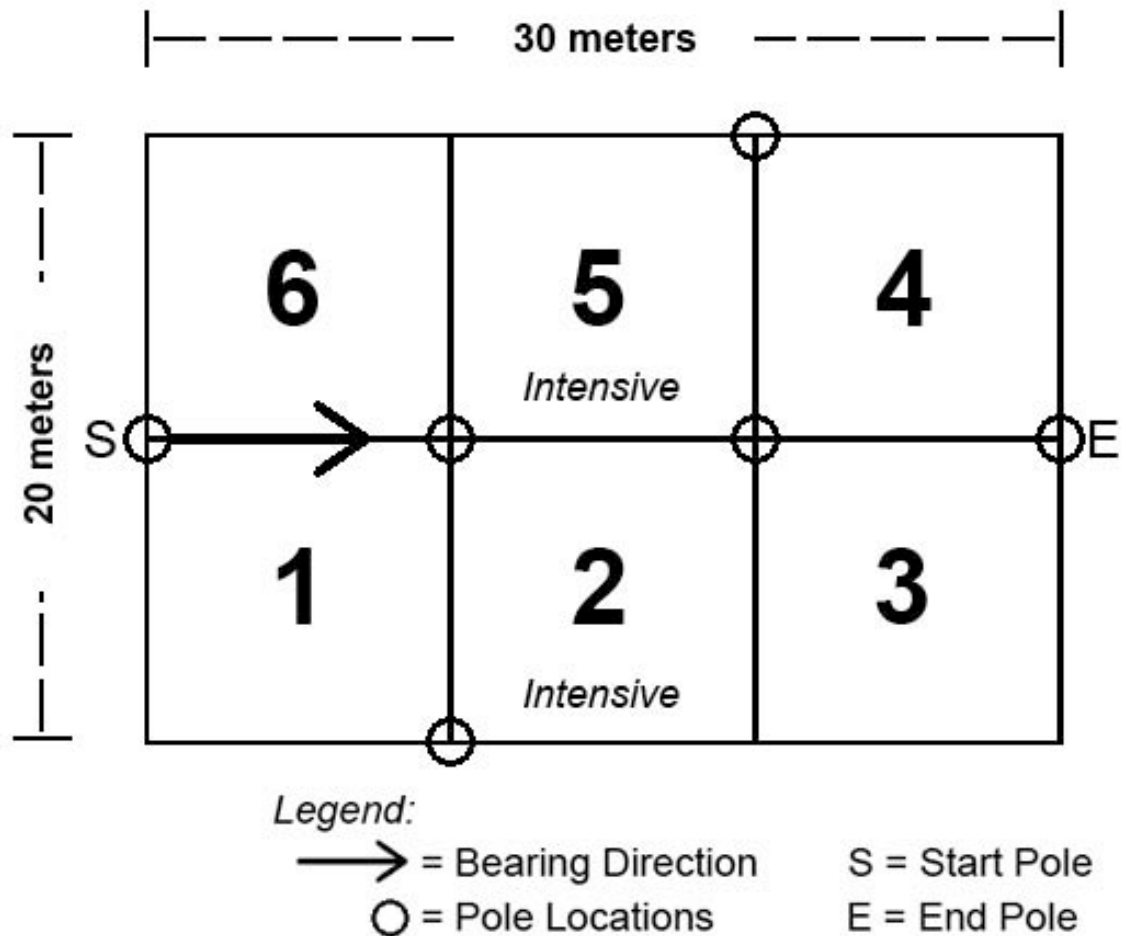
Start pole and direction of center axis are noted.

3.2.2.2 Vegetation Sampling

Vegetation sampling was also adapted from the NCVS method (Peet, et al., 1998) and focused primarily on measuring both presence of plant species and the leaf area cover of those species. The overall procedure involved setting up a series of nested squares, called "depths," inside the two intensive modules (i.e. modules 2 and 5). Working from the smallest to the largest of these nested squares, new plants were identified and established as present. Once presence was established for all plants, each species was classified into 1 of 10 different classes of leaf area cover.

Specifically, the nested depths were set up as shown in and Table 7. Because the smallest depth (depth 5) is 10,000 times smaller than the module as a whole (depth 1), this method has the advantage of balancing specificity and detail with overall efficiency.

Figure 6: Method of setting up nested depths within intensive module
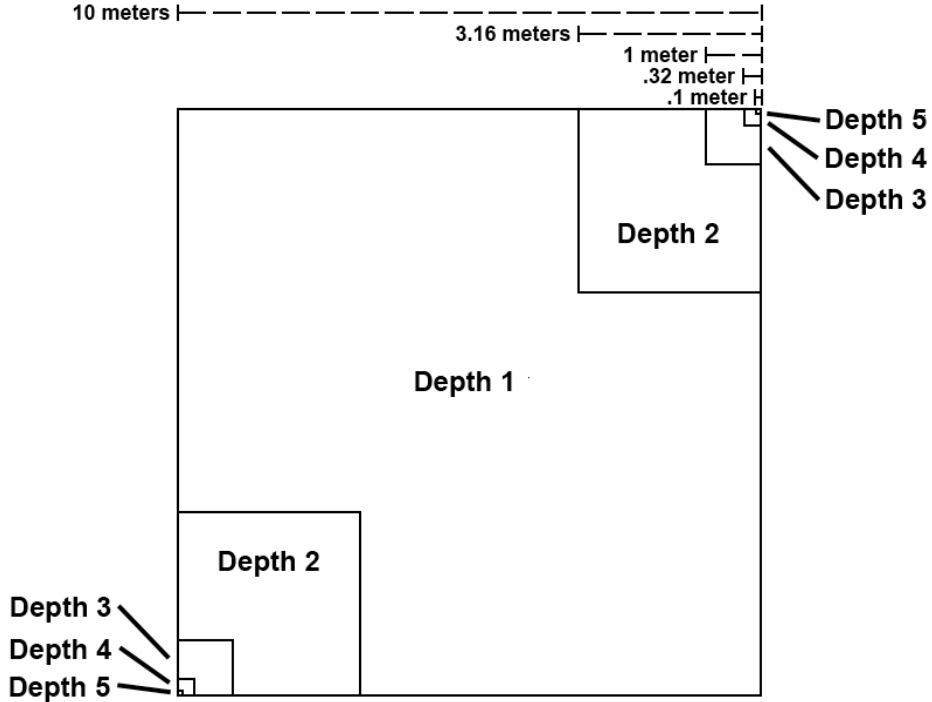
The largest square in this figure represents the entire module, and is labeled "depth 1." Each successive depth from 2 through 5 is one-tenth the area of the previous depth. Note that since the depths travel in the direction of two corners, there is only one depth 1, but two each of depths 2 through 5. The bottom left corner is referred to as corner 2, and the top right corner is referred to as corner 4, for reasons described in (Peet, et al., 1998). Compare this figure to figure 3d, and note that in both module 2 and in module 5, the locations of corners 2 and 4 coincide with the placement of plastic poles; this was intentional.

Table 7: Dimensions of each intensive depth

Depth	Dimensions (m x m)	Area (m^2)
5	0.1 x 0.1	0.01
4	0.32 x 0.32	0.1
3	1 x 1	1
2	3.16 x 3.16	10
1	10 x 10	100
0*	---	---

Asterisk denotes the "overhang" depth, and applies to plants that broke the vertical plane of the module but did not actually have a stem inside the module.

Beginning at depth 5 of corner 2 of intensive module 2 and working outwards, all plants were identified and marked as being present in that depth. After depth 5 of corner 2, depths were visited in the following order: depths 4, 3, and 2 of corner 2, followed by depths 5 through 2 of corner 4. Finally, the researchers moved to depth 1 (the entire module), and marked down any plants not previously found. If a plant was found overhanging the vertical plane of any part of the module, but did not have a stem actually inside the module, it was noted as being in "depth 0."

Note that for each corner, plants were only marked down as being in the first depth in which they were found, since presence in that depth automatically implied presence in all depths containing it. For example, if a plant was found in depth 5 of corner 2, and again in depth 3 of corner 2, it did not need to be marked down twice. However, if a plant was found in depth 5 of corner 2 and again in depth 3 of corner 4, it did need to be marked down twice, since those two depths do not overlap.

After this presence sampling was completed, each species was subjectively categorized into a leaf area cover class, based on a visual estimate of total percentage of the module covered by that species' leaves. Table 8 shows the 10 different leaf area cover classes.

Table 8: Leaf area cover classes

Cover Class	Estimated Leaf Area Cover
1	Trace
2	0-1%
3	1-2%
4	2-5%
5	5-10%
6	10-25%
7	25-50%
8	50-75%
9	75-95%
*	95-100%

After completing the presence class and cover class procedure for the first intensive module, the same procedure was repeated in the second intensive module (i.e. module 5). At this point, presence and cover of plants in these two intensive modules were catalogued separately, but had not been considered together. Thus, to consider the plot as a whole, a residual walkthrough was performed, in which all 4 remaining modules of the entire plot were visited, and any plants not previously found were recorded. Finally, estimates were made of leaf area cover of every species found in the plot, based on their leaf area cover of the plot as a whole.

Since in general each site had three plots, this whole vegetation procedure was repeated two additional times at each wetland site. All vegetation data was recorded on standard NCVS data sheets. Plants were identified using *Newcomb's Wildflower Guide* (Newcomb, 1989), *Peterson's Tree and Shrub Guide* (Peterson, 1973), and *Grasses* (Brown, 1992), combined with the expertise of the investigators. Plants that

could not be identified on site were bagged, labeled, and taken back to a lab for identification with the help of University of Maryland botanists.

3.2.2.3 Soil Sampling

Inside each plot, two cylindrical soil cores were taken at the same time and location. The first soil core was analyzed on site for basic physical characteristics, and the second soil core was sent to UMCES Appalachian Laboratory for chemical analysis of nutrient content, including total nitrogen, total carbon, carbon/nitrogen ratio, and total phosphorous.

At each plot, the first (on-site) soil profile was extracted to a depth of 50 ± 5 cm using a steel soil auger. The soil was carefully transferred to a half-pipe (see Figure 3e), where it was laid out and divided into broad color horizons. The top and bottom depths of each horizon were noted. Individual horizons were qualitatively characterized based on organic content, hydric characteristics such as gleying and mottling, and soil color. Soil color was evaluated using a Munsell soil color chart. In addition to the handwritten field notes and drawings of the profile, photographs of each soil profile were taken.

The second soil sample was obtained from within 0.5 meters of the location where the first soil sample was taken, using a steel soil auger. This soil core was taken down to a depth where the soil remained uniform for at least 15 cm; this depth was noted. The entire sample was then bagged, labeled, and stored at 4 °C for transport back to a lab where it could be temporarily stored. Stored soil samples were

kept refrigerated, and sent to UMCES Appalachian Laboratory within fourteen days of original collection.

3.2.2.4 Water Sampling

Water samples were collected from within each plot for two different types of laboratory analysis: (1) chemical nutrient analysis and (2) determination of the antibiotic resistance profile of *E. coli*. A large set of surface and subsurface water samples was collected for chemical nutrient analysis, and a smaller set consisting solely of surface water samples was collected for antibiotic resistance characterization.

For the samples collected for nutrient analysis, a consistent number of samples was not obtained; the researchers simply attempted to collect both a surface and a subsurface water sample as often as possible from every plot. However, due to hydrologic variations between sites this was not always possible; some sites simply lacked water. Thus the number of samples varied between sites.

Surface water for use in nutrient analysis was collected in non-sterile polypropylene bottles, labeled, and chilled to 4° C to be transported back to a base laboratory for temporary storage. Subsurface water samples for use in nutrient analysis were collected by use of a SoilMoisture Corp. suction lysimeter, planted with its base at a depth of between 45 cm and 55 cm. After 24 hours, the subsurface water that had been pulled into each lysimeter was collected in a non-sterile polypropylene bottle, labeled, and chilled to 4° C for transport back to the base lab for temporary storage. All water samples were stored for up to one week in a freezer, before being

sent to a lab for chemical nutrient analysis (see Section 3.2.3.2: Chemical Analysis of Water Samples).

For the water samples collected for antibiotic resistance testing, a single surface sample was collected from within each plot, subject to availability of surface water at the time of collection. In general, between one and three distinct surface water samples were collected from each of the thirteen wetland sites.

Surface samples for *E. coli* antibiotic resistance analysis were collected using sterile NASCO WhirlPak bags. Samples were then immediately chilled to 4° C to arrest bacterial growth, and within 24 hours they were cultured for *E. coli* and tested for antibiotic resistance in the base lab.

At one site (Kinder), surface water was not available in any plot, so instead a sterilized suction lysimeter was used to collect a subsurface sample. The lysimeter was sterilized by soaking it in 6% hydrogen peroxide for 30 minutes, rinsing the lysimeter tubing with 30% hydrogen peroxide, and then rinsing all parts of the lysimeter with sterile distilled water. The lysimeter was sealed off and allowed to sit for at least 24 hours, so that any residual hydrogen peroxide could degrade. The subsurface water collected in this lysimeter was transferred to a sterile WhirlPak bag, and thereafter treated in the same manner as other samples. Note also that no water samples were ever obtained from Bryantown, surface or otherwise.

Date, time, and location were recorded any time a water sample was taken for any purpose during the course of research. When possible, pH and dissolved oxygen (DO) readings were also taken on-site at the same time. However, due to equipment failures during experimentation, pH and DO data are incomplete.

3.2.3 Lab Work

3.2.3.1 Chemical Analysis of Soils

Sediment samples were analyzed for nutrient content at UMCES Appalachian Laboratory in Frostburg, MD. Each sample was analyzed for total phosphorous via acid digestion, total available phosphorous via Mehlich III extraction, total nitrogen, and total carbon.

The acid digestion method for extracting phosphorous from sediments is intended to detect soil phosphorous in its totality. It involves subjecting sediment samples to a harsh acidic reagent, removing all forms of phosphorous and converting them to orthophosphate (Fishman, 1993). Mehlich III extraction involves agitating sediment samples in the presence of a gentler reagent, causing only a portion of total phosphorous to be converted to orthophosphate. The amount of phosphate extracted by the Mehlich III technique is intended to mimic the amount of soil phosphorous available to wetland biota (Tran & Simard, 1993).

For both methods of extraction, orthophosphate concentration was then measured using an automated colorimetric technique. Orthophosphate samples are treated with several acidic reagents, resulting in the formation of a blue complex which absorbs light at 880 nm. A spectrophotometer is then used in combination with an automated Flow Injection Analysis system to measure light absorbance and thus determine concentration of orthophosphate (Clesceri, Greenberg, & Eaton, 1998, Method 4500-P G).

Soil total carbon and soil total nitrogen were both measured using the Dumas combustion technique. In this method, sediment samples are dried and pulverized,

and then placed in a furnace where they are combusted at extremely high temperatures in the presence of pure oxygen (O_2). Combustion removes all carbon and nitrogen from the solid phase and converts it into gaseous combustion products. Resulting gases are carried away in inert helium gas and then separated using gas chromatography. Finally, they are measured using a thermal conductivity detector (Bremner, 1996).

3.2.3.2 Chemical Analysis of Water Samples

Water samples were analyzed at UMCES Appalachian Laboratory for total phosphorous and four types of nitrogen: total nitrogen, nitrogen in the form of nitrite and nitrate ($NO_2^- + NO_3^-$), nitrogen in the form of nitrite (NO_2^-), and nitrogen in the form of ammonia (NH_3).

Total phosphorous was extracted from water samples using a manual digestion method similar to the acid digestion technique used in soil analysis. Addition of an acid reagent converts phosphorous compounds to orthophosphate. Further addition of reagents causes the formation of a blue complex, which is measured automatically using a spectrophotometer and a Flow Injection Analysis system (Clesceri et al., 1998, Method 4500-P H).

Total nitrogen was extracted from water samples using a different manual digestion technique. In this technique, the water sample is exposed to a persulfate solution at high temperature, which causes the conversion of all nitrogen compounds into nitrate. The sample is subsequently treated with cadmium to reduce nitrate to nitrite, and further treated with sulfanilamide and N-(1-naphthyl)ethylenediamine

dihydrochloride to yield a magenta dye absorbing at 540 nm. The concentration of dye is then determined spetrophotometrically via Flow Injection Analysis (Clesceri et al., 1998, Method 4500-N C).

To determine the concentration of nitrite + nitrate, the same procedure is performed without the initial persulfate digestion step. As a result, only nitrate and nitrite are incorporated into the magenta dye during cadmium reduction and dye treatment. Dye concentration is still measured spectrophotometrically (Clesceri et al., 1998, Method $4500\text{-}NO_3^-$ I).

Finally, to determine the concentration of nitrite alone, the cadmium reduction step is also omitted from the procedure. As a result, only the nitrite that was initially present in the water sample reacts with the sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride to form the resulting magenta dye (Clesceri et al., 1998, Method $4500\text{-}NO_3^-$ I).

Ammonia nitrogen was determined using a separate colorimetric technique. In the technique for ammonia, the water sample is mixed with several reagents, and the dissolved ammonia reacts with these reagents to form indophenol blue. Indophenol blue concentration is then measured spectrophotometrically with Flow Injection Analysis (U.S. Environmental Protection Agency, 1999, Method 350.1).

3.2.3.3 Antibiotic Resistance Testing of Water Samples

The methods for antibiotic resistance testing were adapted from a study entitled *Microbial Source Tracking of Escherichia coli in a Constructed Wetland* (Orosz-Coghlan, et al., 2006). Most supplies were obtained from Fisher Scientific,

unless noted. Three 10x serial dilutions were performed for each sample of wetland water, using a 0.9 percent saline solution to normalize volume, creating solutions of 100%, 10%, 1%, and 0.1% of the original concentration. *E. coli* was then isolated from these samples using m-ColiBlue24$^{©}$ broth culture media and following the procedures as instructed. Each of the four dilutions was filtered through a separate filter with 0.45nm pores small enough to capture bacteria. In general, 50 milliliters were passed through the filter for each dilution; variations in volume (due to availability at sites) were noted and later factored into calculated colony counts.

The filters now contained the bacteria trapped from the diluted solution. Each filter was placed in a sterile Petri dish on top of an absorbent pad containing a uniform volume of m-ColiBlue24$^{©}$ broth culture media, and the entire dish was incubated at 37° C for 18 to 24 hours. The basis for using this media is bacterial growth selectivity: m-ColiBlue24$^{©}$ only supports coliform bacteria. *E. coli* grow as blue colonies on the plate, while non-*E. Coli* coliforms grow as red colonies. Once the incubation period was complete, both total coliform and *E. coli* coliform counts were taken and standardized as colony forming units per milliliter (CFU/mL).

Figure 7: Example of a Millipore m-ColiBlue plate
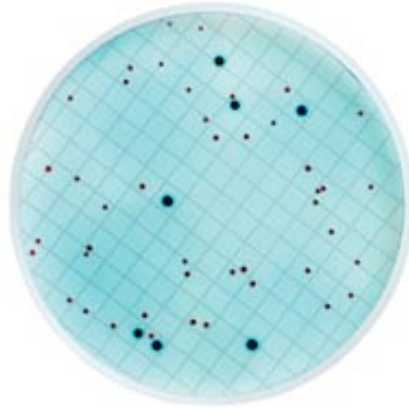
Red (total coliform) and blue (E. coli) colonies are visible. Image from
www,millipore.com.

A minimum of three individual blue *E. coli* colonies were randomly selected

from among the four plates prepared for each site. Often, it was difficult to select an

isolated *E. coli* colony that made no contact with another colony of any type; in other

instances, no *E. coli* colonies were detected. The selected isolates were then streaked

onto separate non-selective Tripticase Soy agar (TSA) plates in order to proliferate

the bacteria. The TSA plates were incubated at 37° C for 24 hours. After 24 hours, a

single colony on each plate was selected, and the BD BBL[©] Enterotube II test was

used to confirm the identity of the sample. This procedure consists of 15 simultaneous

biochemical tests that allow the identification of Gram-negative species. Enterotubes

were incubated at 37° C for 24 hours. At the same time that the enterotube test was

performed, a sterile toothpick was used to obtain a portion of the same colony and

inoculate 5 milliliters of separate sterilized TS broth solution. The broth solutions

were cultured at 37° C for 24 hours inside a shaking incubator.

E. coli antibiotic resistance was determined using the standard Kirby-Bauer

method for testing antibiotic resistance (Wikler, 2006). In accordance with this

standard protocol, after 24 hours, each broth culture was uniformly plated onto two plates of Mueller Hinton agar. Ideally, turbidity of each culture should have been measured in order to comply with McFarland standards of bacterial suspensions. However, we did not have access to the equipment required to carry out this step; instead, after a visual check of turbidity for consistency among samples, we uniformly plated 2 milliliters of each broth culture. Since all cultures were growing in similar conditions for the same period of time, we believe that there would not have been a significant difference in the concentrations of the solutions. Due to the plating technique, after incubation, an effective "lawn" of bacteria results, and individual colonies cannot be distinguished. BBL Antibiotic Sensitivity discs with standard dosages were obtained: erythromycin, 15μg; ciprofloxacin, 5 μg; ampicillin, 10 μg; sulfisoxazole, 25 μg; tetracycline, 30 μg; and vancomycin, 30 μg. The discs were then spaced at least 5 centimeters apart on the plates, three on each of the two plates. These two plates were then incubated for exactly 24 hours at 37° C.

After 24 hours, zone of clearance of the bacterial lawn around each of the antibiotic discs was measured and recorded. The total diameter of each circular clearance, including the disc itself, was recorded. However, if there was no visible clearance around the disc, a value of 0 mm was recorded. Evaluation of resistance for a particular antibiotic was based on standard zone diameters taken from the *Clinical and Laboratory Standards Institute* (Wikler et al., 2006).

3.3 Methods of Data Analysis

Upon completion of data collection, all data was imported into Microsoft Excel and the STATA Data and Analysis Package (Statacorp., ver. 9). Two types of statistical tests were then performed to determine significant correlations between all the different measurements taken in this study: least-squares regression and t-tests.

Least-squares regression is a statistical means for determining whether there exists a significant relationship between two variables. Microsoft Excel was used to run a least-squares regression on every possible two-variable combination, resulting in the creation of a large correlation matrix containing a list of all statistically significant relationships and their associated two-tailed p-values. A p-value is essentially a measure of statistical confidence, with lower p-values indicating greater confidence.

T-tests were particularly appropriate for testing the relationship of antibiotic resistance to other variables. A t-test is used to determine whether there is a statistically significant difference between the characteristics of two groups; in this study the two groups used for each antibiotic were wetland sites with resistant isolates and wetland sites with only susceptible isolates. A t-test was run between resistance/susceptibility to each antibiotic and each of the other wetland variables, with the main result being a two-tailed p-value. If this p-value was less than 0.05, its respective correlation was reported as being statistically significant. If this p-value was between 0.05 and 0.10, its correlation was reported as being almost statistically significant.

4. Results & Discussion

4.1 Land Use

Computerized analysis of the land use at and around a wetland site is an important initial step in understanding the wetland's most basic characteristics. Landscape Development Intensity (LDI) is a common index used to provide an approximate gauge of how much a wetland site is impacted by surrounding human development. LDI combines individual land use types into one value by use of a weighted summation formula. In this study, LDI was calculated twice for each wetland site: once within a 1000 meter circular buffer extending out from the center of the site, and once within a 2000 meter circular buffer. LDI values for the thirteen sites are shown in Figure 8.

Figure 8: LDI values from 1000m and 2000m circular buffers surrounding each of the 13 study sites
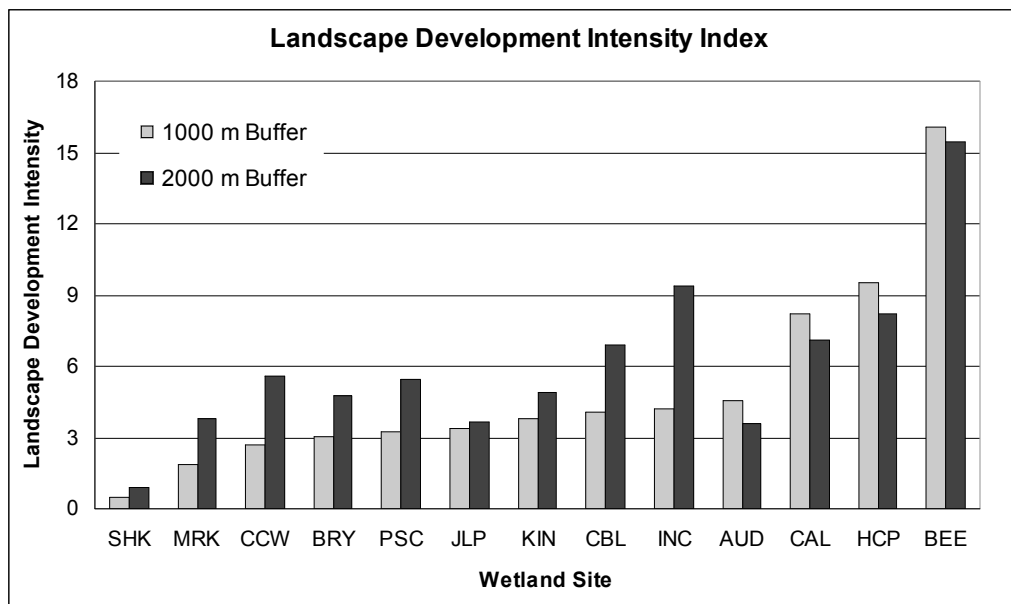
LDI is convenient in that it provides a unified measure of the impact of land development on a wetland, but at the same time it does not provide details as to the type of land use that causes this impact. Thus, land use was further broken down into the three broad classifications of 'agricultural', 'urban', and 'natural'. Figure 9 to Figure 11 show the percentage of land around the study sites falling into each of these three categories. As with LDI, tabulation was performed within two circular buffers: one of radius 1000m and the other of radius 2000m.

Figure 9: Percentage of land used for agricultural purposes in the areas surrounding each of the 13 study sites
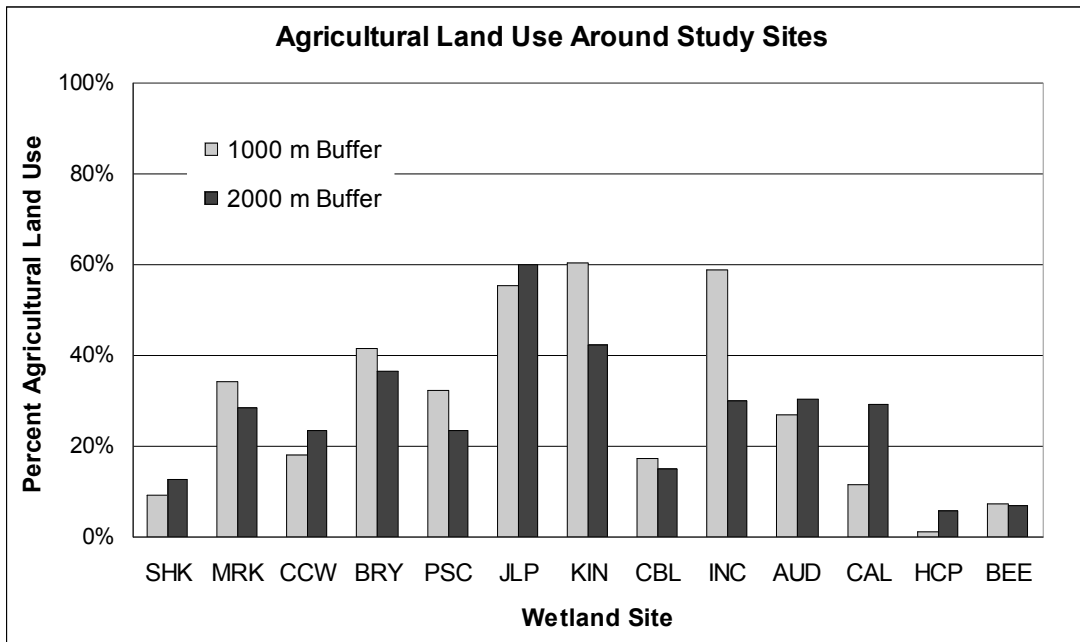
Land use tabulation was performed within two circular buffers extending around the center of each site - the first buffer had a radius of 1000 meters and the second buffer had a radius of 2000 meters.

Figure 10: Percentage of urban land in the area surrounding each of the 13 study sites
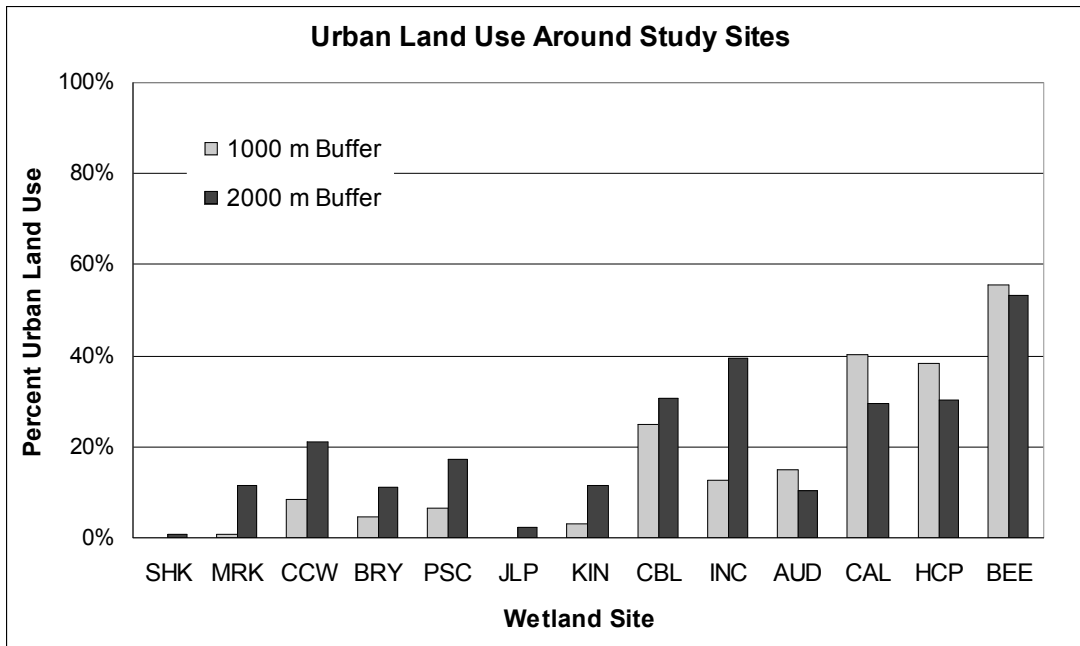
Land use tabulation was performed within two circular buffers extending from the center of each site - the first buffer had a radius of 1000 meters and the second buffer had a radius of 2000 meters.

Figure 11: Percentage of 'natural' land use in the area surrounding each of the 13 study sites
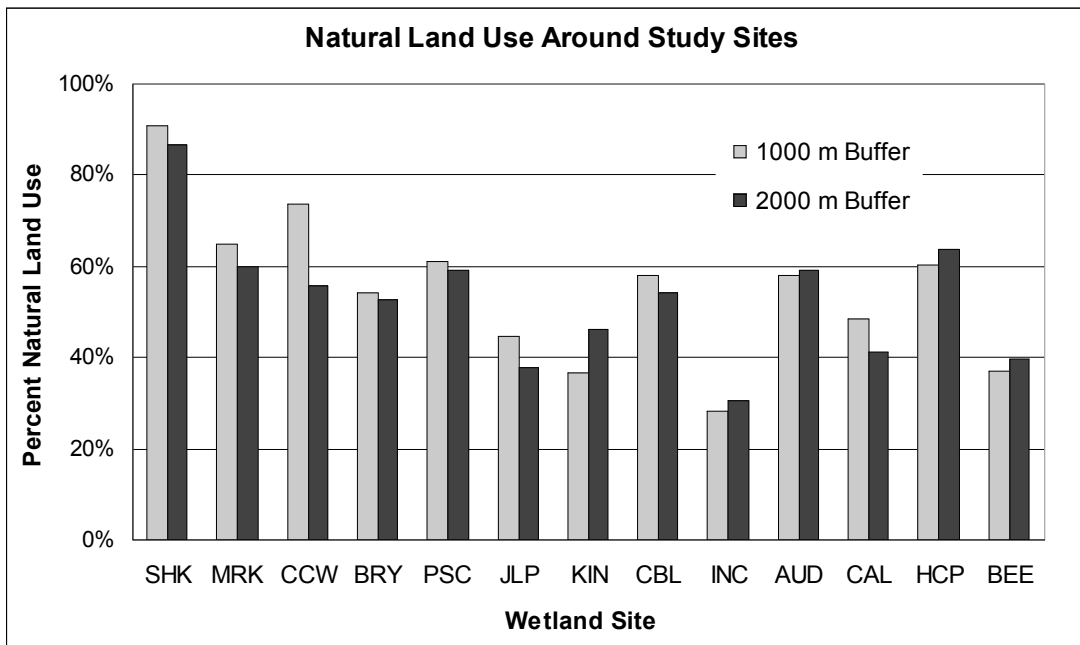
Land use tabulation was performed within two circular buffers extending from the center of each site - the first buffer had a radius of 1000 meters and the second buffer had a radius of 2000 meters.

Moving from left to right in Figure 8 to Figure 11, wetland sites are arranged in order of increasing LDI within their 1000m buffers. The most visible trend is that urban land use also tends to increase from left to right, while natural land use tends to decrease in this direction. Thus LDI is directly correlated to percentage urban land use and inversely correlated to natural land use; this trend is easily understood, given that LDI is a measurement of human impact. Urban land use constitutes the highest level of human impact on an ecosystem, while natural land use constitutes the lowest level of human impact.

Indeed Shockley site, which had the lowest LDI values for both the 1000 and 2000m buffers (0.457 and 0.888 respectively), also had the highest percentage of natural land use. More specifically, mixed and brush land use made up over 90% of the total buffer. As expected, Shockley had very low levels of agricultural and urban land use. In fact, no urban land use was identified until the buffer was expanded to 2000m. It can be concluded that Shockley is the wetland site least affected by human development.

On the opposite end of the spectrum, Beehive was the site with the highest LDI values (LDI = 16.085 and 15.459). As noted earlier, high LDI values are associated with either high percentage of urban or agricultural land use. Beehive site, because of its location in Elkridge, MD, had the greatest percentage of urban land use. Three of the most abundantly found land uses were residential, industrial, and institutional. After visiting Beehive site, it is clear the site is located in a highly populate residential neighborhood.

Herring Creek had the second highest LDI for the 1000m buffer (9.532), which was nearly twice that of average site LDI. Unexpectedly, of the three main land use classifications, natural land was the most prevalent. Both water and wetlands were found in the top five most commonly occurring land use classifications. Herring Creek's high LDI value can be explained by it's a relatively high level of urban land use (38.484%). Agricultural land use was almost nonexistent.

With respect to the 1000m buffer, Calvert had the third highest LDI value (8.1834). A large portion of CAL's development intensity can be contributed to urban land use, and more specifically, low density residential. The presence of residential lands can be attributed to the sites' close proximity to MD route four. In addition, a large percentage (49%) of land use was characterized as natural habitat, most notably deciduous forest.

Aud's LDI index (4.524) was just slightly below the site average. An overwhelming majority of the overlaying 1000m buffer (58%) was classified as natural habitat. Natural habitat classifications found around AUD include mixed, wetlands, and water. Significant levels of both agriculture and urban land uses were found as well (27.02% and 14.88% respectively). The agriculture lands are linked to a strong presence of cropland and the urban lands are associated with low density residential housing surrounding the site.

Irvine had the fifth highest LDI index of the 13 sites. A large percentage of the surrounding land use was classified as Agriculture, and more specifically cropland. Irvine was established on a former farm, and large fields still occupy a

large portion of the site; therefore, a large percentage of agricultural land use is to be expected. When the buffer was expanded, Irvine's close proximity to Baltimore city suburbs is reflected in the increase in urban land use.

Cumberland had a slightly lower LDI than the average (4.044), due to the large amount of natural landscape. Around the Cumberland site, the five most prevalent land use classifications were Mixed, Pasture, Deciduous, Residential, and Open Urban. Cumberland was located in the foothills of the Appalachian Mountains; therefore, there was very little human development. This lack of human influence is reflected in the low LDI value.

Kinder had a lower LDI than the average (3.7792). In the 1000m buffer around the site, there was very little urban development (2.94%) because the site was located in a rural area of Anne Arundel County. There was an abundance of agriculture land (60.42%) because Kinder was located in between two large farms. As the buffer surrounding Kinder was expanded to 2000m, the impact of these two farms on LDI was reduced. At this site there was also a strong presence of natural habitat (36.64%). The majority of natural land can be classified as deciduous forest.

Jackson Lane has a LDI number well below the average (3.3854). No urban land was found in the 1000m buffer around the site because Jackson Lane is located in a remote area of Caroline County. Over 90% of the land use found in the site's 1000m buffer consists of either Cropland or Deciduous Forest. This lack of urban development and high percentage of natural environment (44.80%) explain Jackson Lane's low LDI value.

Averages for LDI, Agriculture, Urban, and Natural were calculated as follows for the thirteen sites:

Average LDI (1000m Buffer) = 5.00

Average LDI (2000m Buffer) = 6.12

Average % Agriculture (1000m Buffer) = 28.7%

Average % Agriculture (2000m Buffer) = 26.5%

Average % Urban (1000m Buffer) = 16.2%

Average % Urban (2000m Buffer) = 20.7%

Average % Natural (1000m Buffer) = 55.2%

Average % Natural (2000m Buffer) = 52.8%

As seen from the above averaged data is can be determined that as the buffer was expanded the LDI value increased (by 1.12). In also examining the averaged data, percent urban land use also increased; however, both percent agriculture and percent urban decreased.

The LDI values for both the 1000m buffer and 2000m buffer were compared to one another in order to identify significant changes. A difference in LDI (LDI2000-LDI1000) was calculated to determine significant increases or decreases. In examining these LDI differences, it was determined that nine of the 13 sites saw their LDI values increase as the buffer zone was expanded. In addition, three sites showed a significant increase in value of at least 2.5. These sites included Irvine, Cumberland, and Waldorf. A large increase in LDI demonstrates that the impact on the buffer area is amplified as the buffer is expanded. After determining significant increases in LDI, it was important to investigate and determine the relative causes.

Irvine observed the largest increase in LDI of the 13 sites. In examining the other data, it is clear that as the buffer was increased, high density urban land was found. A larger portion of the buffer consisted of more highly developed lands; thus, resulting in a large LDI value. This increase in LDI and % Urban land use could indicate a possible area of concern for the site. Waldorf site also had a notably higher LDI value for its 2000m buffer. In examining other data concerning Waldorf, it was clear that this increase is due to the decrease of natural land and corresponding increase in urban development. Natural land uses, such as Mixed Forest or Brush, have very little impact on sites; therefore they are valued as very low on the LDI Index. Conversely, urban land uses, such as Residential or Industrial lands, have a much higher impact on the surrounding environment. As was the case with Waldorf, more urban land corresponded directly to a large increase in LDI. Finally, Cumberland saw an increase of LDI of 2.857. This increase in LDI again corresponds directly with an increase in percent urban land use (5.799%) and a decrease in percent natural land use (3.538%). Although the increase in percent urban land use is not very large, the LDI increase can be explained by the high level of development found in the 2000m buffer. Urban land uses with an extremely high LDI coefficient are the cause for Cumberland's LDI increasing.

4.2 Vegetation Sampling

The study of wetland plants is essential to fully understand a wetland's function. Wetlands are a sink for nutrients and wetland plants greatly influence water chemistry and the cycling of those nutrients. They are primarily responsible for the

water filtering and cleansing that has been widely observed (Gersberg et al, 1986).

They are also the base of the food chain and even provide a habitat for other

taxonomic groups (Cronk and Mitsch 1994). Team CRABS sampled vegetation at 36

separate plots across Maryland and identified 256 separate species.

4.2.1 Species Leaf Area Cover and Land Use

Figure 12 shows the dominant plant community in the agricultural sites and

traces the leaf cover through the natural and urban sites. The y-axis shows the per-

plot average midpoint leaf area cover over all of the agricultural, natural, or urban

sites. That is, it shows what percent of the average plot would be covered by a given

species. The x-axis shows the dominant plant community by species. Figure 13 and

Figure 14 are identical to Figure 12, except they track the dominant plant community

in the natural and urban sites, respectively.

Figure 12: Top ten species by cover at agricultural sites, along with their cover at natural and urban sites
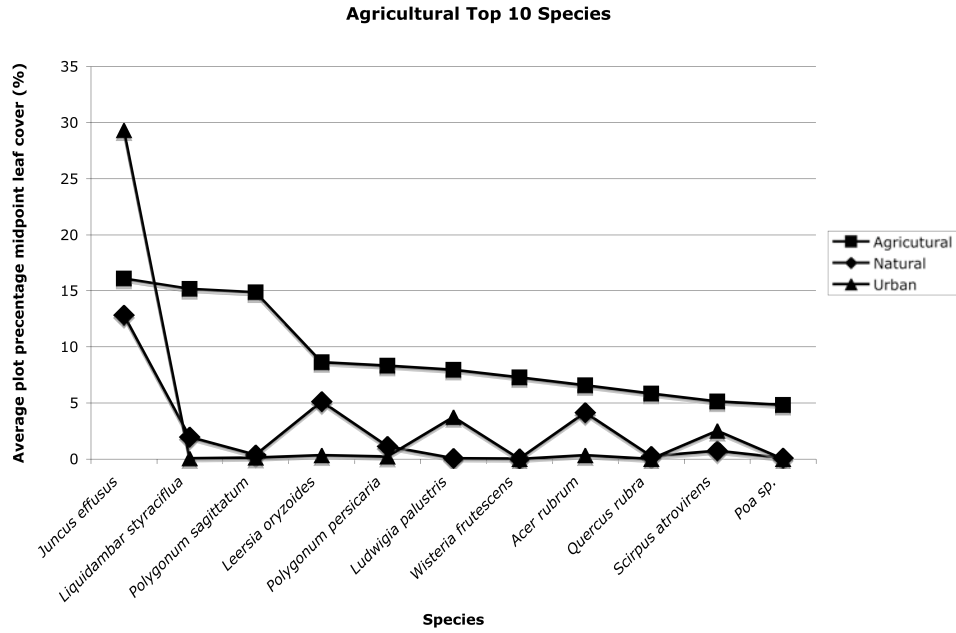
Agricultural sites included Irvine Nature Center, Jackson Lane Preserve, Kinder Site, and Bryantown.

Figure 13: Top ten species by cover at natural sites, along with their cover at agricultural and urban sites
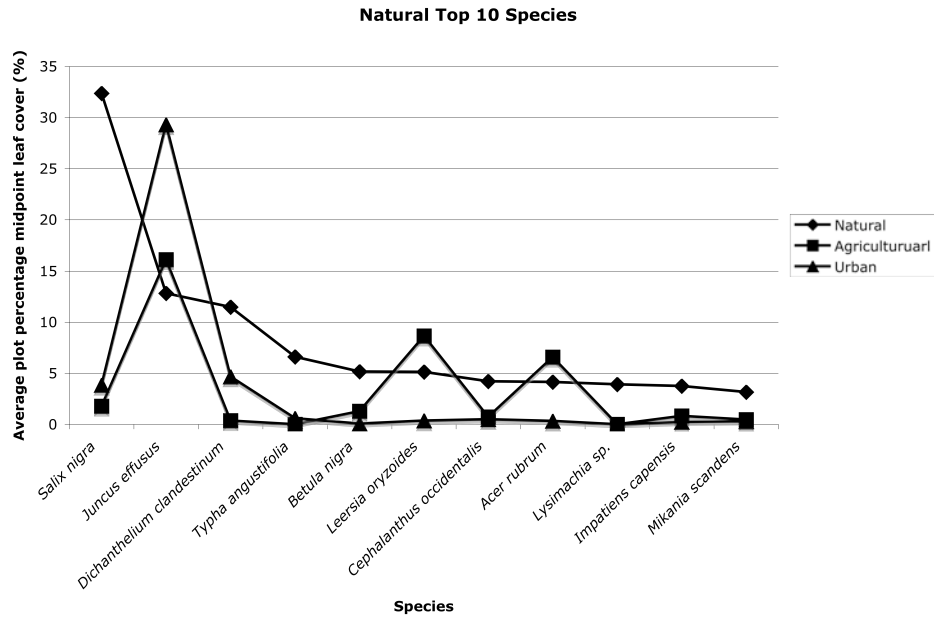
Natural sites include Shockley, MD 228 Site, Merkle Wildlife Refuge, Piscataway Stream Valley Park, and Aud.

Figure 14: Top ten species by cover at urban sites, along with their cover at agricultural and natural sites
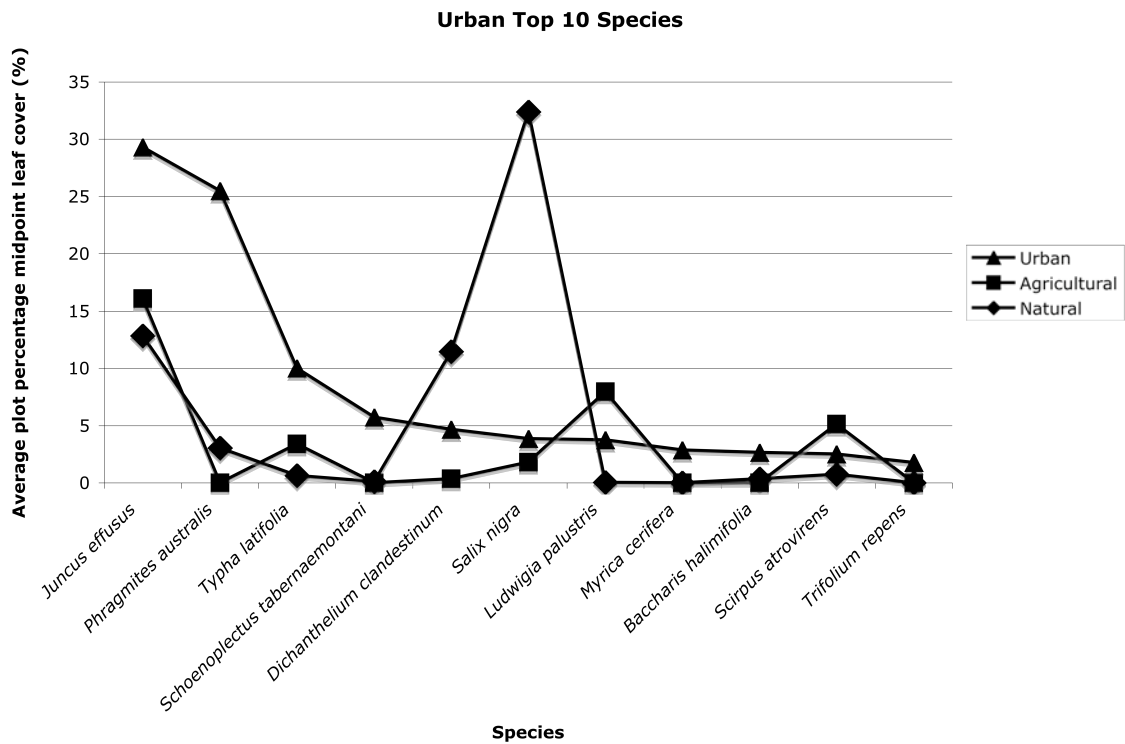
Urban sites include the US 220 Site, Beehive Site, MD 4/ MD 260 Site, and Herring Creek Park.

There are some clear relationships shown between dominant land use type and the dominant plant community. In Figure 12, the agricultural and urban dominant plant distributions are remarkably different. Likewise, in Figure 13, the natural and urban plant community dominant cover distribution show clear differences. Figure 14 does not show a clear enough trend between any of the three land use types to form any conclusions.

The natural and agricultural sites generally have more woody species than the urban sites, which are dominated by herbaceous plants. There may be some factor

such as frequent or intense flooding, or more nutrient loading, that prevent the woody species from taking a hold in the urban wetland environment.

Qualitatively, these results suggest that land use has a great impact on the dominant vegetation seen in mitigation wetlands. For example, if vegetation sampling turned up a dominant species distribution similar to the agricultural sites in Figure 12, especially with a high occurrence of *Salix nigra*, it would be reasonable to conclude that the surrounding land is primarily designated as agricultural. Being more cautious, another conclusion could be that the surrounding land is probably not urbanized. This information could also be used to guess at dominant plant community based on GIS land use analysis. Similarly, Figure 13 indicates that an average species distribution similar to that of the natural sites makes it likely that the surrounding land is not urban.

4.2.2 Wetland Vegetation Prevalence Index

Table 9 states the five different indicator designations for wetland status, based on the vegetation present. Table 10 lists the top 15 species by percent cover found in all plots that Team CRABS sampled, along with their wetland indicator status.

Table 9: Wetland indicator status categories for plant species

Wetland Indicator Status	Probability of Occurrence in Wetlands (%)	Probability of Occurrence in Non-Wetlands (%)	Weight
Obligate wetland (OBL)	>99	<1	1
Facultative wetland (FACW)	67-99	1-33	2
Facultative (FAC)	24-66	34-66	3
Facultative upland (FACU)	1-33	67-99	4
Upland (UPL)	<1	>99	5

Table from Cronk and Fennessy, 2001.

Table 10: Top 15 species by leaf area cover over all sites sampled by CRABS, along with their indicator status

Species	Indicator Status
Juncus effusus	FACW
Salix nigra	FACW
Phragmites australis	FACW
Dichanthelium clandestinum	FACU
Liquidambar styraciflua	FAC
Leersia oryzoides	OBL
Polygonum sagittatum	OBL
Typha latifolia	OBL
Acer rubrum	FAC
Ludwigia palustris	OBL
Polygonum persicaria	FACW
Typha angustifolia	OBL
Juncus canadensis	OBL
Scirpus atrovirens	OBL
Betula nigra	FACW

This data is of interest when considering the overall state of wetlands.

The indicators were developed to supplement wetland delineation methods employed by the Army Corps of Engineers (Cronk and Fennessy, 2001). When evaluating each site, the top ten species by cover were found and assigned a weight according to their indicator status. A weighted average of the indicator value, also called the prevalence index (Wentworth et al. 1988), was then found at each site according to the following formula:

$$Weighted\ Average_{j} = \frac{\sum_{i=1}^{p} C_{ij}W_{i}}{\sum_{i=1}^{p} C_{ij}}$$

W_i is the indicator weight of species i, C_{ij} is the midpoint cover of species i in plot j, and p is the total number of species in plot j. The results are shown in Figure 15, with each site's value being the average of the weighted average of each plot in the site. The y-axis ranges from 1 (site totally comprised of OBL species) to 5 (site totally comprised of UPL species). Any site that scores <3.0 is considered a wetland site.

Figure 15: Wetland vegetation prevalence index for all sites
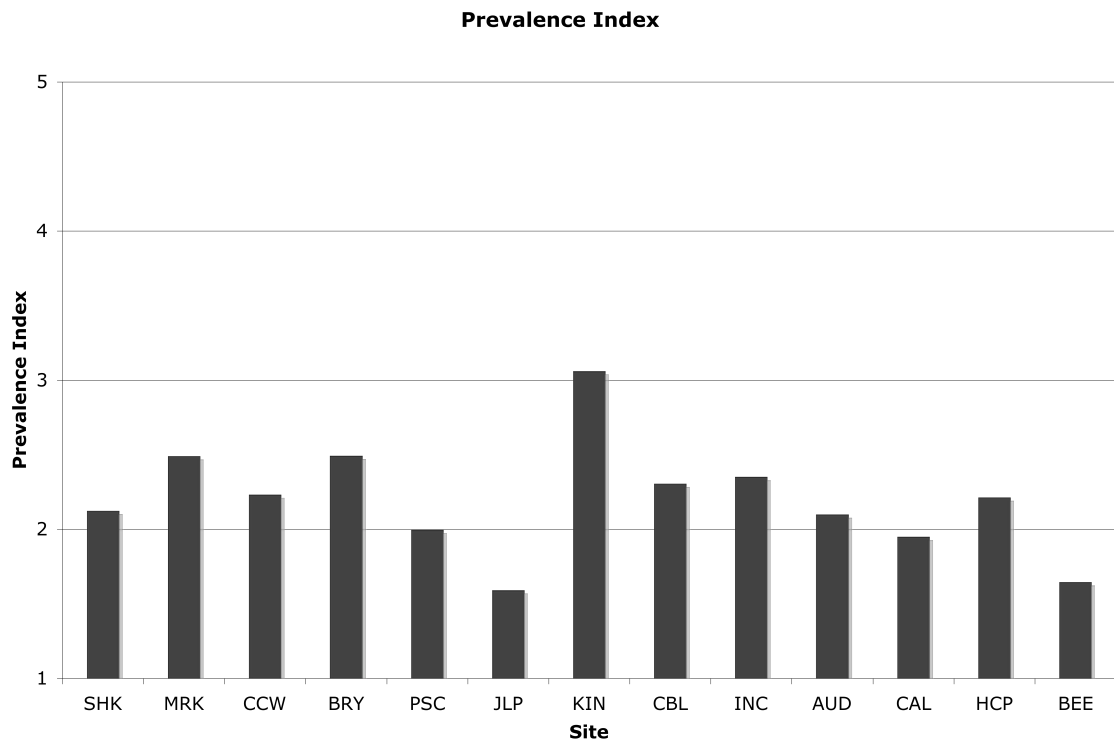
Any value below 3.0 is accepted as an indication of the presence of a wetland.

Only one of the sites, Kinder, had a prevalence index over 3.0. This is an encouraging result as it indicates that the mitigation wetlands in Maryland are performing well. The sites in Figure 15 are arranged according to their ratio of natural to urban land use, with most natural on the left. Although trends related to land use and plant life were seen in other analysis, there appears to be no trend here. This makes sense because in most cases, the presence of water in a wetland is dictated first by topography, and then by land use and other factors. Since the presence of wetland plants is primarily dictated by hydrology, prevalence index should not be related to land use. However, in a case where a wetland has a large area of impervious surfaces, or some other mechanism that would increase runoff during rain storms in its watershed, the prevalence index may shift toward FACW species that favor more constant inundation.

4.2.3 Shannon Diversity Index

The Shannon diversity index (SDI) is a measure of the biodiversity in a plot. It is maximized by an even distribution of leaf area cover between species and it increases with increasing species. It can be calculated according to the following formula (Shannon and Weaver, 1949):

$$H = -\sum_{i=1}^{S} p_i \log p_i$$

H is the diversity index, s is the number of species in a plot, and p_i is the ratio of the leaf area cover of the species to the combined leaf area cover of all species in the plot. This study used a log base of 2. Figure 16 shows the diversity index of each site.

Shannon Diversity Index
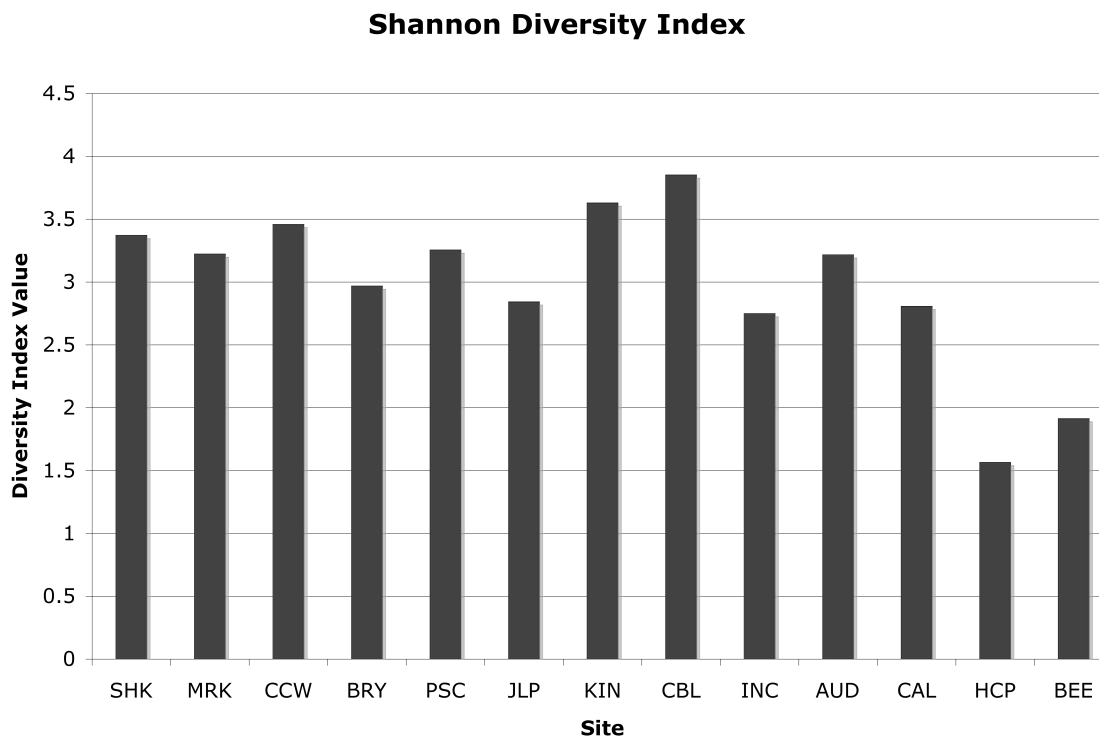
The higher the index value, the more evenly distributed species there are in the sampled area.

The sites are again arranged by land use "naturalness" from left to right, with left being the sites with the largest percentage of surrounding natural land use. Some of the most diverse sites (Kinder, Calvert) did not rate low on the prevalence index (i.e. did not have a lot of wetland plants). The combination of upland and wetland ecosystem probably increased the number of species found at these sites, and thus increased the diversity index.

Figure 17 is a scatter plot of the diversity index. The x-axis is dimensionless, as it simply represents the ratio of natural to urban land use, with most natural on the left. There is a noticeable trend from high diversity in natural environments to low diversity in urban environments. This could be because wetlands in urban

environments have a greater nutrient and pollution inflow, and thus the less pollution

tolerant species may not be able grow.

Figure 17: Shannon diversity index with the x-axis showing the sites sorted from more natural to less natural by the ratio of natural to urban land use
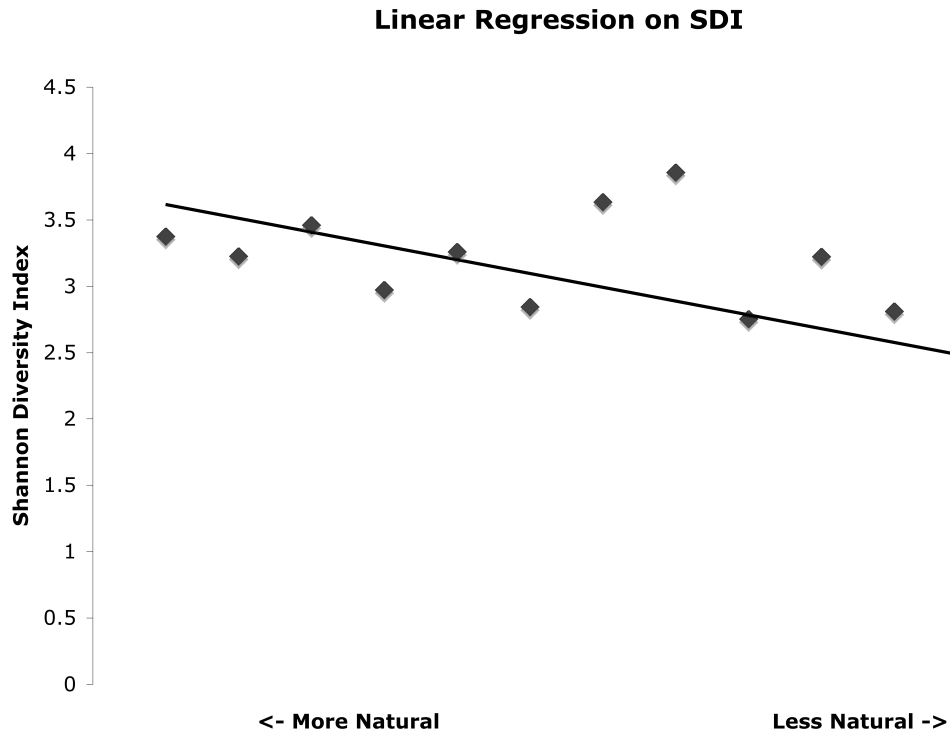
The linear regression R^2 value is shown in the upper right corner. This trend is statistically significant at the 5% level.

4.3 Water Sampling

Surface and sub-surface water quality measurements were obtained for total

nitrogen, organic nitrogen, nitrite, nitrate, ammonia, and total phosphorous.

A study released by the Florida Department of Environmental Protection in

2005 sampled local wetlands in order to find biological indicators for developing the

Florida Wetland Condition Index (Brown & Reiss, 2005). The wetlands were

grouped into three categories: Agricultural, Urban, and Reference. Because this study

grouped their wetlands into land-use categories similar to the way we did, we decided

that it was a valuable tool for comparing out water quality data to other wetlands. We

too classified our wetlands as agricultural and urban, but our third category was

natural. For the sake of comparison, we will treat the Florida study's reference

wetlands the same as our natural wetlands.

It also helped that the Florida study sampled for many of the same nutrients

as we did. While the wetlands in Florida are in a vastly different ecosystem than our

wetlands, they can still be used as a tool for comparison. Even if you look at our

wetlands alone, they too come from different ecosystems. We did not expect to find

exactly similar water quality data, but we did hope that there would be some

similarities. We looked at other studies to compare our numbers to (such as studies

conducted in North Carolina and Ohio), but the Florida study was the most similar to

ours. Also, it was beneficial that the Florida study was conducted in 2005 and that it

was relatively new data.

4.3.1 Total Nitrogen

In 2006, the site with the lowest level of total nitrogen present in surface water

samples was Calvert with a reading of 1.219 mg-N/L (Figure 18). The highest level

was found at Bryantown with a reading of 16.834 mg-N/L. The second highest level

was at Aud with a reading of 14.224 mg-N/L.

In 2007, the site with the lowest level of total nitrogen in surface water

samples was once again Calvert and the highest level was now found at Cumberland.

In the sub-surface samples, the average had fallen from 3.252 mg-N/L of total nitrogen in 2006 to 0.2005 mg-N/L in 2007. As with nitrite+nitrate, the site with the lowest level in 2006 (Piscataway with 1.101 mg-N/L) was higher than the highest level the following year (MRK with 1.0418 mg-N/L). Total nitrogen levels were much higher in 2006 for reasons that are not known to us.

The highest reading in 2006 was Bryantown with 7.438 mg-N/L. Bryantown had unusually high levels of total nitrogen at the surface water level as well as sub-surface.

Again, our correlation index showed that there was a possible correlation between the level of urban land use present and one of the constituent levels, this time 2006 total nitrogen levels. Figure 18 shows the total nitrogen levels for the 2006 sub-surface and surface water samples.

Figure 18: Total nitrogen levels across all sites in 2006
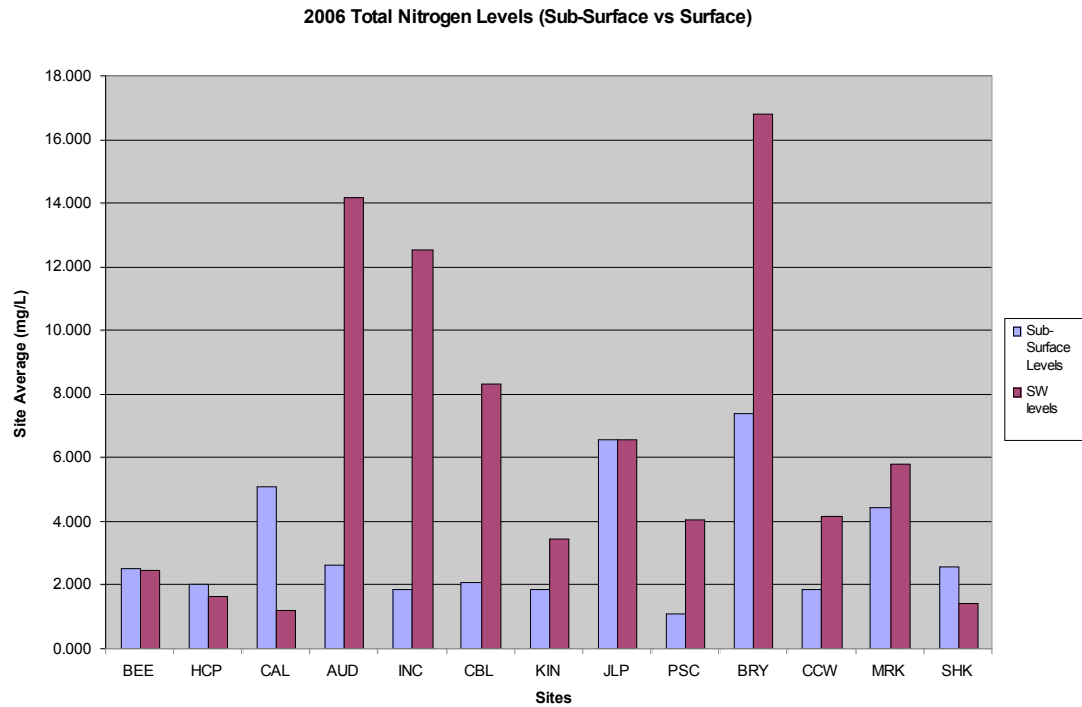
As stated earlier the sites are shown in order with the LDI rankings for each site, with the highest on the left. If the correlation held true, it would make sense that the sites with the higher LDI rankings (those on the left) would have higher total nitrogen levels. Looking at the figure, this is not obvious to the naked eye. In order to further examine this correlation, more samples are needed.

4.3.2 Organic Nitrogen

In 2006 surface water samples the site with the highest organic nitrogen average was Irvine with a reading of 21.542 mg-N/L. The site with the lowest average was Piscataway with 1.19 mg-N/L.

The 2006 surface water average for all sites was 5.396 mg-N/L.

In 2006 sub-surface water samples the site with the highest organic nitrogen average was Bryantown with 3.324 mg-N/L. The site with the lowest average was Herring Creek with a reading of 0.809 mg-N/L.

The 2006 sub-surface water average for all sites was 1.573 mg-N/L.

One reason that surface water sample averages could be so much higher is that the layer of organic matter mixes in with the surface water, while the lysimeters take water samples from a depth in the ground which is almost always below the organic matter.

In 2007 there were no organic nitrogen readings for either surface or sub-surface water samples.

Figure 19 shows the organic nitrogen levels for the 2006 sub-surface and surface water samples.

Figure 19: Organic nitrogen levels across all sites in 2006

2006 Organic Nitrogen Levels (Sub-Surface vs Surface)
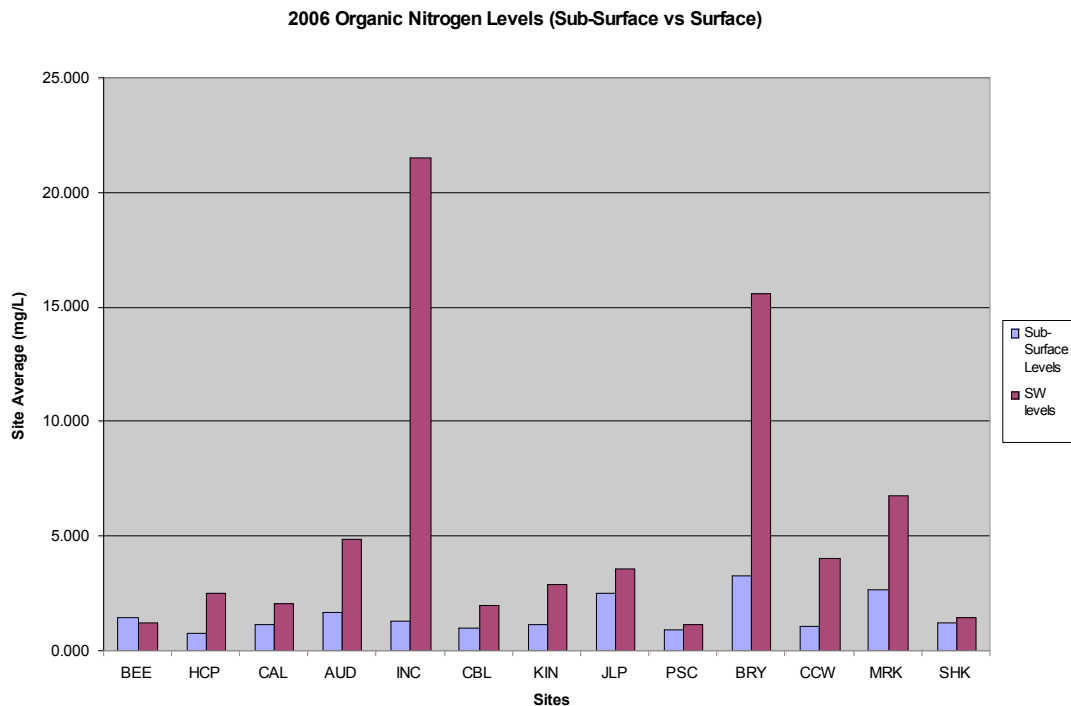
4.3.3 Nitrite

In 2006 the site with the least amount of nitrite in surface water samples was Herring Creek with a reading of 0.004 mg-N/L. The site with the highest amount of nitrate was Bryantown with 0.21 mg-/L with the next highest being Merkle with 0.030 mg-N/L.

In 2007 Merkle had a higher reading than the year before and Bryantown dropped drastically so that now Merkle was the highest with a reading of 0.0539 mg-N/L and Bryantown was second with 0.0154 mg-N/L. Instead of Herring Creek being the lowest reading in 2007 three sites (Calvert, Beehive, and Irvine) measured below detection limits. Herring Creek did have the lowest reading among those sites that were able to be detected, with a reading of 0.0010 mg-N/L.

The average nitrite reading for surface water samples was 0.026 mg-N/L in 2006, but that fell to 0.0131 mg-N/L in 2007. A large part of this can be accounted for by an unusually high 2006 reading for Bryantown.

There was an unusually high reading for nitrite levels in the sub-surface water samples as well. In 2006, Waldorf registered 26.005 mg-N/L of nitrite in the sub-surface samples. This was by far the highest reading for both surface and sub-surface samples. The next highest site was Jackson Lane with 0.378 mg-N/L. The lowest two readings were Beehive and Piscataway with 0.015 and 0.020 mg-N/L, respectively.

In 2007 Waldorf dropped to a lower level with a reading of 0.2320 mg-N/L, and the highest reading for a site was now Bryantown with 0.5826 mg-N/L. Surprisingly, Beehive was now the second highest with a reading of 0.3513 mg-N/L, whereas it had had the lowest levels the year before. The lowest readings for 2007 were Shockley and Piscataway with 0.0326 and 0.0493 mg-N/L, respectively.

The average level of nitrite in the 2006 sub-surface samples for all 13 sites was 2.096 mg-N/L, distorted by the unusually high Waldorf reading. In 2007, the samples had much more parity, and the average was 0.1948 mg-N/L. Overall, the 2006 numbers were very much higher than 2007. Figure 20 shows the difference between 2006 sub-surface nitrite and 2007 sub-surface nitrite. Make sure to remember that the Waldorf reading was over 26 mg-N/L in 2006, thus it does not fit on the figure.

Figure 20: Sub-surface nitrite levels across all sites in 2006 and in 2007

2007 Nitrite Levels vs. 2006 Nitrite Levels (Sub-Surface)
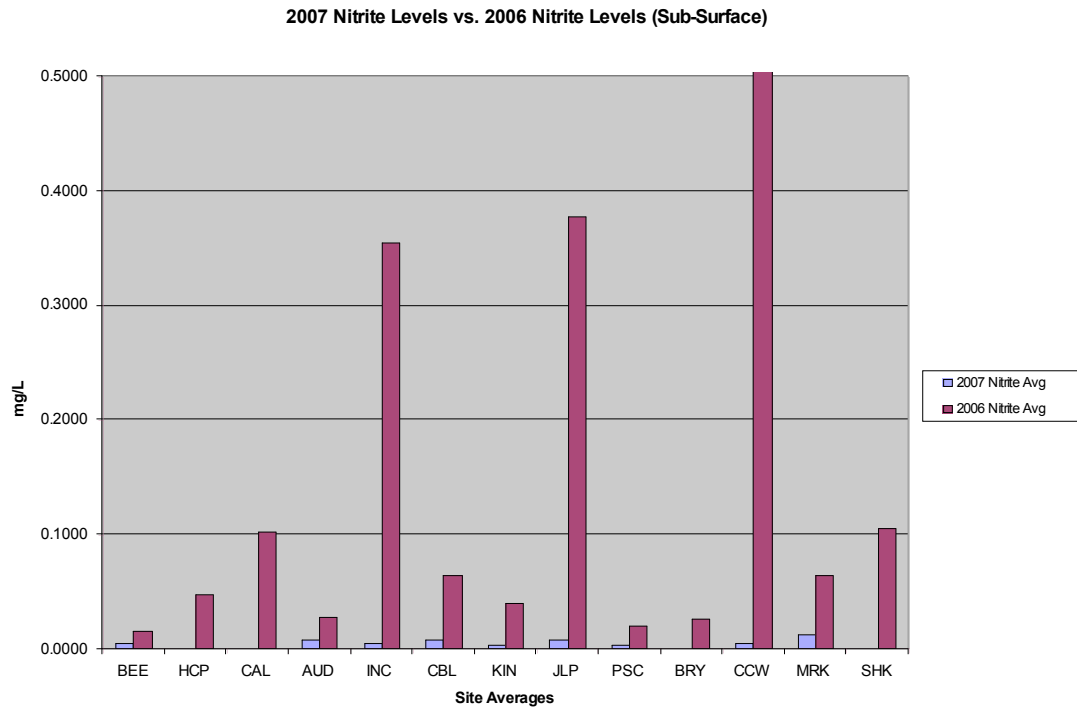
4.3.4 Nitrate

In 2006 surface water samples, the site with the highest nitrate average was Merkle with a reading of 0.862 mg-N/L. The site with the lowest average was Kinder with 0.001 mg-N/L without considering the seven sites (Shockely, Jackson Lane, Piscataway, Irvine, Beehive, Bryantown, and Aud) that were below the detection limit.

In 2006 sub-surface water samples, the site with the highest nitrate average was Bryantown with a reading of 3.871 mg-N/L. The site with the lowest nitrate average was Piscataway with 0.061 mg-N/L unless you consider Waldorf, which had an average nitrate reading of -38.54 mg-N/L. This distorted the entire 2006 sub-surface water nitrate average.

The 2006 site average for sub-surface ammonia was -2.07 mg-N/L due to CCW's negative reading.

There were no 2007 readings for nitrate for either surface or sub-surface samples.

Our correlation index showed that there was a possible correlation between 2006 sub-surface levels of nitrate and the level of urban land use. Figure 21 shows the 2006 nitrate levels for surface samples at each site. Because of Waldorf's negative reading, it is just 0.00 mg-N/L for the figure.

Figure 21: Surface water nitrate levels for all sites in 2006

2006 Surface Water Nitrate Levels
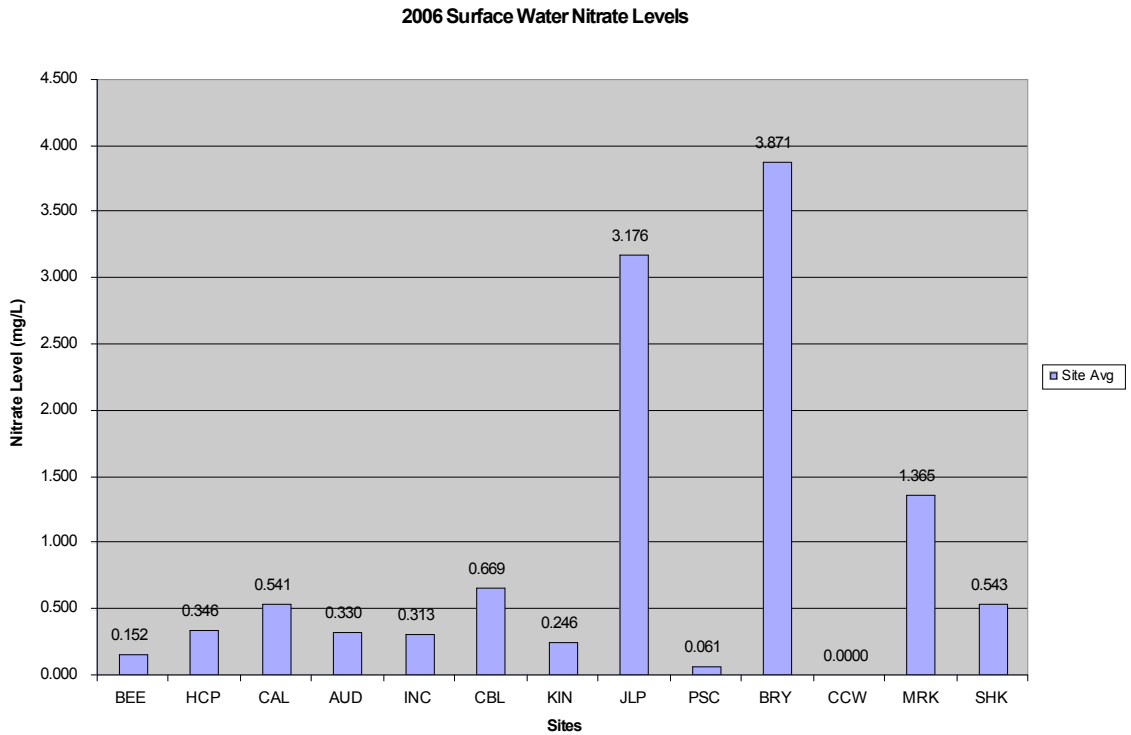
The sites are shown in order with the LDI rankings for each site, with the highest on the left. Because sites with higher LDI rankings typically are indicative of

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a higher level of urban land use, it would make sense that the sites with the higher

LDI rankings (those on the left) should have had higher nitrate levels. Instead, the

site averages on the right were much higher (note that CCW had a negative reading,

thus the 0.00 site average). This suggests that more samples are needed to further

examine the possible correlation between land use and sub-surface nitrate levels.

4.3.5 Ammonia

In 2006, the site with the lowest ammonia reading in surface water samples

was Calvert, with 0.027 mg-N/L. The second lowest reading came from Herring

Creek 0.031 mg-N/L. The highest reading was Aud with 1.725 mg-N/L.

In 2007 the site with the lowest ammonia reading for surface water was

Herring Creek, with 0.0223 mg-N/L. The second lowest reading came from Calvert

with 0.0233 mg-N/L (Calvert and Herring Creek flip-flopped from 2006 to 2007).

The highest reading was once again Aud, with 2.0669 mg-N/L. Both years the

second highest ammonia reading for surface water came from Bryantown.

Seeing that Aud is downhill from a horse farm, it is not surprising that it

would have the highest level of ammonia in surface water for both years. Unusually

high ammonia levels can be linked with fertilizer usage.

Regarding the sub-surface samples, the lowest reading in 2006 came from

Bryantown with 0.217 mg-N/L. The second lowest reading came from Piscataway

with 0.303 mg-N/L. The highest reading was from Shockley with 1.153 mg-N/L.

In 2007, numbers changed drastically. Instead of having the highest reading,

Shockley now had the lowest reading among the thirteen sites with 0.0326 mg-N/L.

Shockley was the site that was the most natural of all sites. There was no urban

percentage of land-use and it was classified as 91% natural. The fact that such a

drastic drop in ammonia level occurred over the course of a year in a wetland that was

almost completely isolated is something that could be pursued further. The second

lowest reading was Piscataway with 0.0493 mg-N/L. The site with the highest

reading was now Bryantown with 0.5826 mg-N/L. Bryantown had been the site with

the lowest ammonia reading among sub-surface samples the year before. The sites

with the highest and lowest readings had completely flip-flopped from 2006 to 2007.

Both years the site with the second highest reading was Beehive.

     Figure 22 shows a visual comparison between sub-surface and surface

ammonia levels for 2007, and Figure 23 shows a comparison between 2006 and 2007

sub-surface ammonia readings.

Figure 22: Sub-surface and surface ammonia levels in 2007
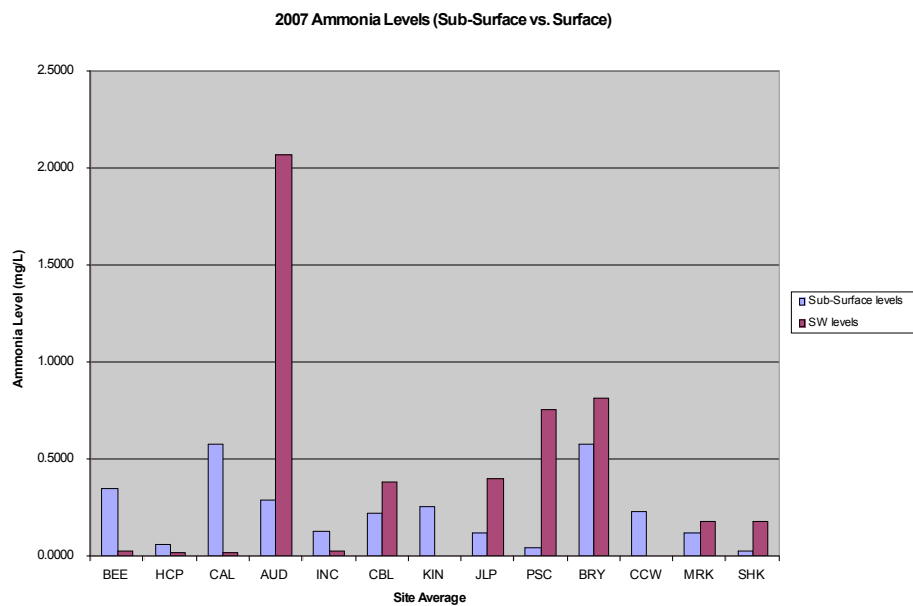
Figure 23: Sub-surface ammonia levels in 2006 and in 2007
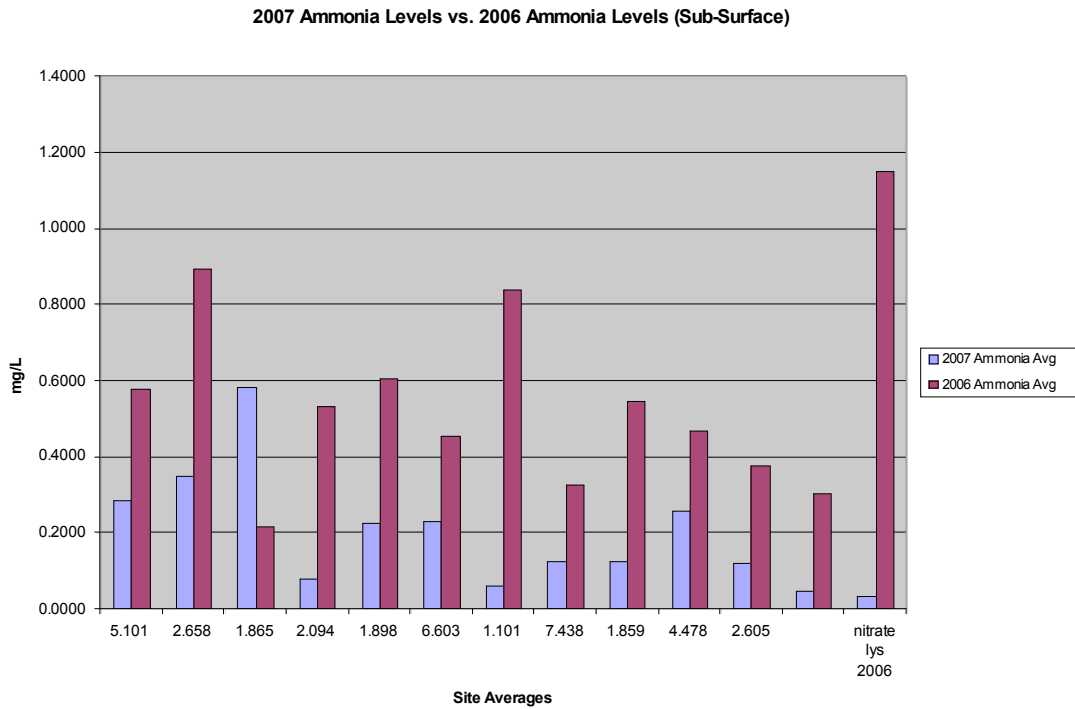
The sites are listed in order of LDI (Land Development Index) rankings. This rates a wetland based on the surrounding land use present. In general, relatively higher LDI rankings correspond with a higher percentage of urban land use. The wetlands with the highest LDI ranking are on the left. Notice that the sites with the highest LDI rankings have higher sub-surface ammonia levels than surface ammonia levels. Overall, sub-surface and surface ammonia do seem to follow each other. Note that Kinder and Waldorf did not have surface water ammonia readings for 2007.

In the Florida study that we are using as comparison, there were 30 reference wetlands tested for ammonia levels and the average came out to 0.15 mg/L (Brown & Reiss, 2005). In wetlands that we classified as predominately natural (similar to the Florida studies classification of "reference"), surface water samples had an ammonia

level average of 0.484 mg-N/L in 2006 and 0.797 mg-N/L in 2007. The fact that these numbers are higher than the Florida numbers can be attributed to the fact that most of our natural wetlands were not 100% natural.

Because we only had 13 sites, we wanted to have an even allotment of natural, urban, and agricultural wetlands. We had 5 natural wetlands, 4 urban, and 4 agricultural. Because of this, there were some minor discrepancies. For example, CAL was 49% natural, 40% urban and 11% agricultural. It had to be labeled urban for statistical purposes because there were 5 sites that were more than 49% natural. A more complicated method of allotting each wetland a certain weight based on each percentage land-use could be developed in the future.

There were 19 agricultural wetlands in the Florida study that had an ammonia level average of 0.33 mg-N/L (Brown & Reiss, 2005). This less than our 2006 ammonia average of 0.601 mg-N/L, but much closer to the 2007 ammonia average of 0.4158 mg-N/L for surface water in wetlands that we also classified as predominately agricultural.

Finally, there were 26 urban wetlands in the Florida study that had an ammonia level average of 0.19 mg-N/L (Brown & Reiss, 2005). Our urban wetlands had a 2006 ammonia average of 0.212 mg-N/L and a 2007 ammonia average of only 0.115 mg-N/L in surface water samples. The large gap is because we only had one wetland (Beehive) that was predominately urban, and its water quality numbers differed across the board from 2006 to 2007.

Figure 24 compares the surface water ammonia averages from the Florida study to our 2007 numbers.

Figure 24: Ammonia level averages from the Florida study and from CRABS'
Maryland study

Florida vs. Maryland ammonia levels
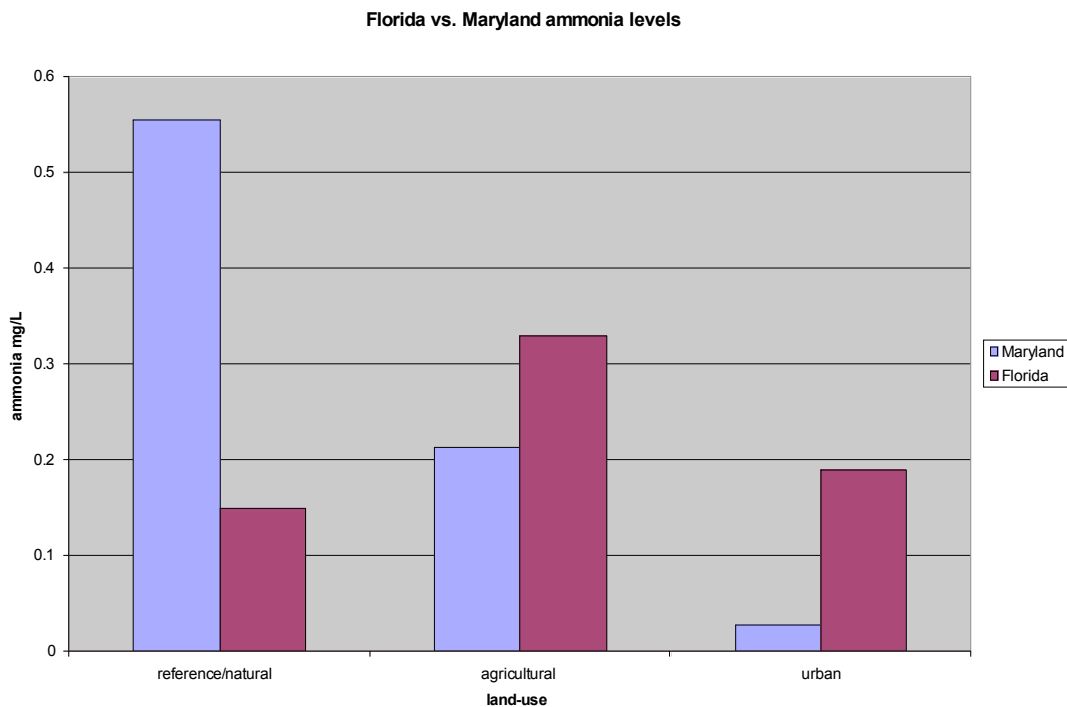
4.3.6 Phosphorous

In 2006, the site with the lowest phosphorous reading in surface water
samples was Shockley with 0.065 mg-N/L. The second lowest reading came from
Herring Creek with 0.094 mg-N/L. The highest reading was Aud with 12.487 mg-
N/L.

In 2007, the site with the lowest reading for phosphorous in surface water
samples was Calvert with 0.0192 mg-N/L. The second lowest reading was from
Herring Creek with 0.0228 mg-N/L. The site with the highest reading was again Aud
with 5.1056 mg-N/L.

The average reading for all 13 sites for surface water phosphorous was 1.0331 mg-N/L in 2006 and 2.369 mg-N/L in 2007, it more than doubled.

For the sub-surface samples, in 2006 the lowest phosphorous reading was from Herring Creek with 0.042 mg-N/L. The second lowest reading was from Shockley with 0.059 mg-N/L. The highest reading was from Merkle with 0.953 mg-N/L.

In 2007, the lowest reading was again from Herring Creek, but it had gone all the way down to 0.0086 mg-N/L. The second lowest reading was now Calvert with 0.0240 mg-N/L. Those two sites, along with Piscataway, Shockley, and Irvine were all below the lowest 2006 reading. The highest phosphorous reading in sub-surface water samples for 2007 was from Beehive with 0.3150 mg-N/L. This is merely one third of the highest reading from 2006. In fact, the 2007 sub-surface numbers were much lower than 2006 with an average of 0.282 mg-N/L of phosphorous in 2006 and 0.0932 mg-N/L in 2007.

Surface water phosphorous increased on average from 2006 to 2007 while sub-surface phosphorous decreased.

Figure 25 visually compares sub-surface phosphorous levels to surface phosphorous levels for 2006 and Figure 26 compares total phosphorous levels in sub-surface samples from 2006 and 2007.

Figure 25: Sub-surface and surface total phosphorous levels in 2006

Sub-Surface Total P vs. Surface Total P (2006)
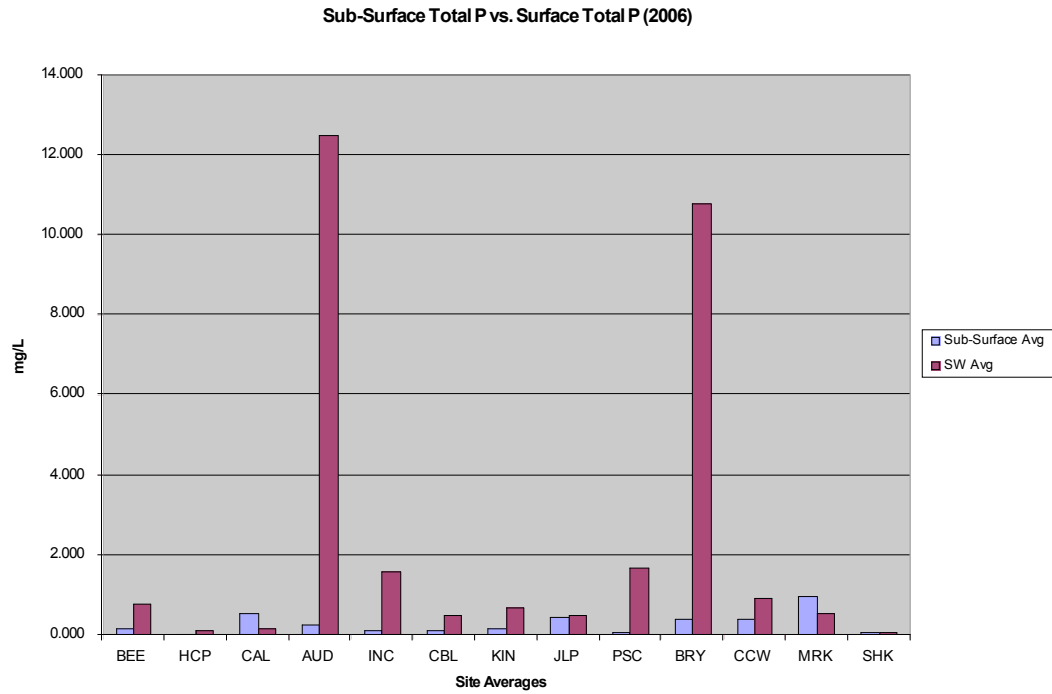
Figure 26: Sub-surface total phosphorous in 2006 and in 2007

2007 Total P levels vs. 2006 Total P levels (Sub-Surface)
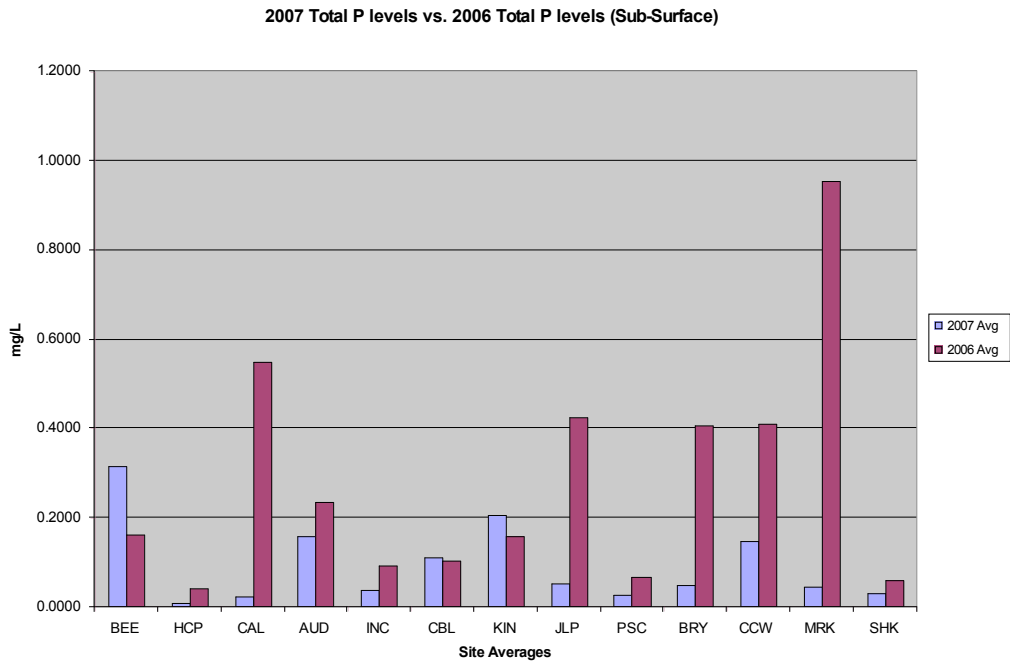
Notice that at almost all the sites, surface water levels were much higher than sub-surface levels. At some sites, the surface water levels were significantly higher.

Of 30 reference wetlands in the Florida study the average level of phosphorous was 0.08 mg-N/L (Brown & Reiss, 2005). In wetlands that we classified as natural, surface water samples had an average phosphorous level of 3.141 mg-N/L in 2006 and 2.1394 mg-N/L in 2007. Here is one example where our numbers were inexplicably higher than those of the Florida studies.

Out of 19 wetlands classified as agricultural in the Florida study, the average level of phosphorous was 0.81 mg-N/L (Brown & Reiss, 2005). In wetlands that we classified as agricultural, surface water samples had an average phosphorous level of 3.389 mg-N/L in 2006 and 0.5341 mg-N/L in 2007. These numbers are much more on par with the Florida numbers, suggesting that we classified agricultural wetlands much like they did, but our idea of natural wetlands and their idea of reference wetlands may not be as similar.

Lastly, the Florida study sampled phosphorous levels from 26 wetlands that they considered urban; the average level of phosphorous in these wetlands was 0.24 mg/L (Brown & Reiss, 2005). In wetlands that we classified as urban, surface water samples had an average phosphorous level of 0.384 mg-N/L in 2006 and only 0.301 mg-N/L in 2007. Again, this can be attributed to the fact that we had only one predominately urban wetland (Beehive) and for some reason the ammonia levels AND phosphorous levels dropped drastically from 2006 to 2007.

Figure 27 compares the surface water phosphorous levels from the Florida study to our numbers.

Figure 27: Phosphorous levels from the Florida study and from CRABS' Maryland study

Florida vs. Maryland phosphorous levels
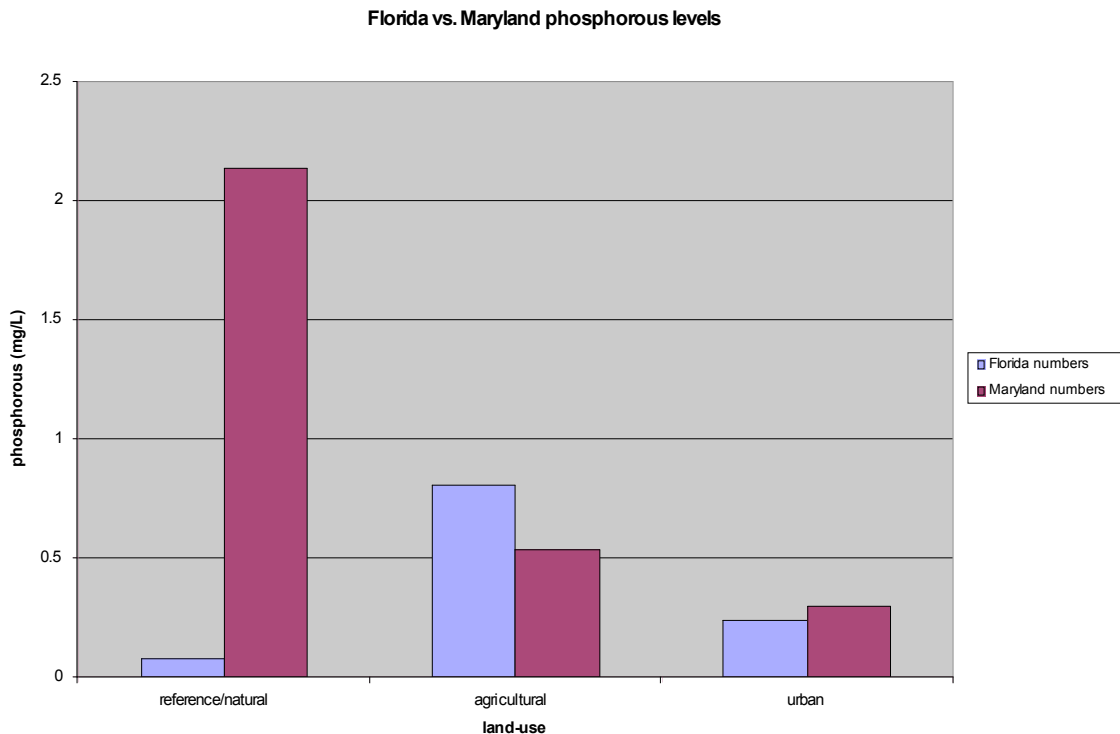
4.3.7 Ammonia versus Total Phosphorous

Out of all the constituents that we tested for, the two that showed the most correlation were ammonia and total phosphorous. In 2007 surface water samples, if the ammonia level went up, so did the total phosphorous, and vice versa. Figure 28 and Figure 29 below show the movement of the two variables. There were no samples for either Kinder or Waldorf.

Figure 28: Comparison of ammonia and total phosphorous levels across all sites, averaged
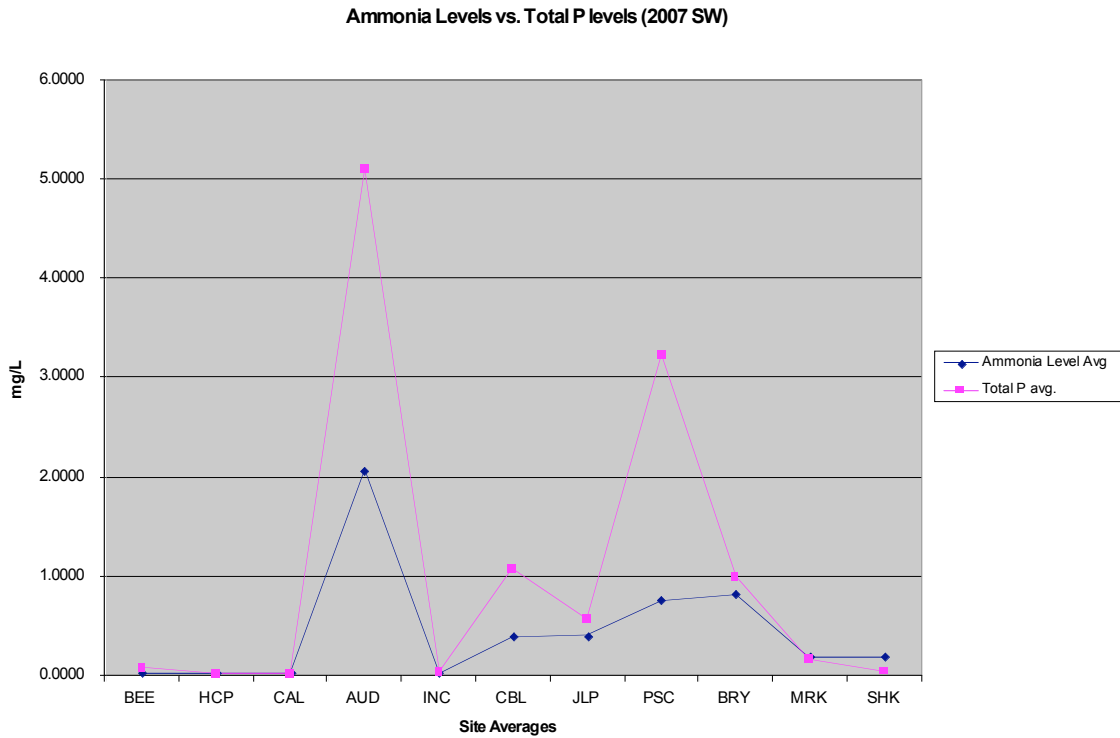
Figure 29: Regression of ammonia level versus total phosphorous level
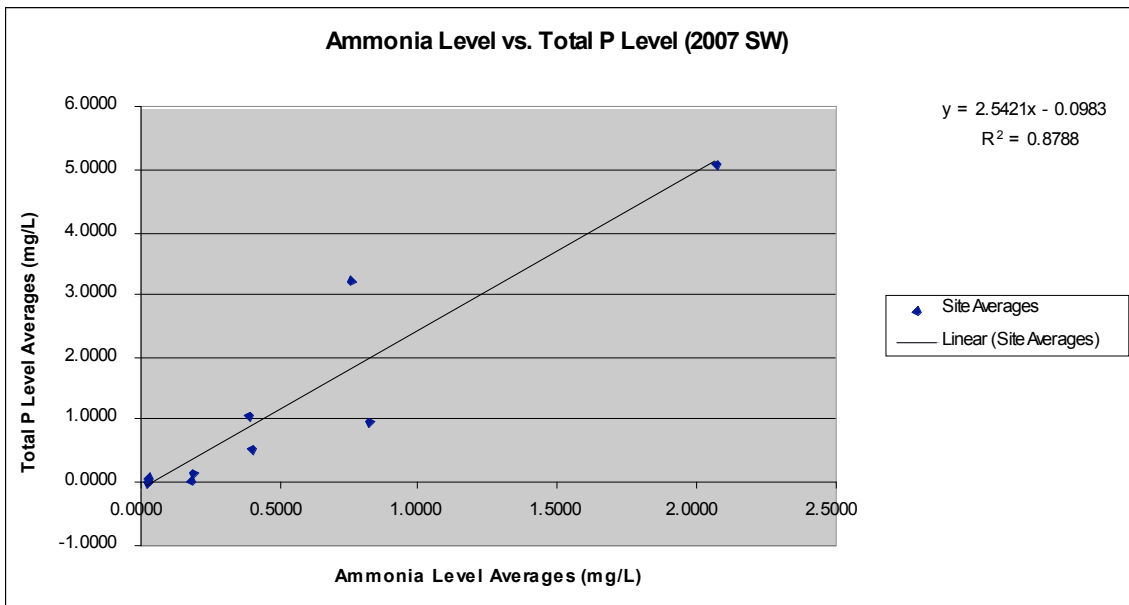
4.3.8 Limitation of Water Results

Our study shows that in various aspects of water quality, values may differ greatly from year to year. However, it is important to note that while extreme variations in nutrient levels may occur, this result may also be a figment of the study's small sample size. In order to gain confidence, these values should be taken repeatedly over time, as one-time samples can lead to a distorted conclusion.

4.4 Soil Sampling

Our soils data results were broken into two categories: data obtained through soil nutrient analysis via Applachacian Laboratory and data obtained through onsite collection. The soil samples sent to Appalachian Laboratory were analyzed for the following nutrient levels: total carbon (TC), total nitrogen (TN), total phosphorus (TP), plant available phosphorus (PAP), and carbon:nitrogen ratio (CN). Soil nutrient measurements were taken using the following unit: *milligram of nutrient / kilogram of soil basis*. Soil nutrient data was obtained for every plot at every sampled wetland site (see Table 11). An average value and standard deviation were calculated for all five nutrient levels across all sites (see Table 12). The standard deviation values for all five nutrient levels were extremely high, revealing that there was a high degree of variability between soil samples. In fact, standard deviation values exceeded the site averages for both total carbon levels and plant available phosphorus levels. This high degree of variability can be explained either by the sensitivity of the laboratory testing equipment or by large differences in nutrient composition of the soil samples.

Table 11: Soil nutrient levels from each plot at the 13 sampled wetland sites

	Total Nitrogen mg-N/kg-soil	Total Carbon mg-C/kg-soil	C:N Ratio mg-C / mg-N	Total Phosphorus mg-P/kg-soil	PA Phosphorus mg-P/kg-soil
BEE 1	420.67	6449.0	15.33	324.00	3.06
BEE 2	649.32	14324.4	22.06	445.15	6.57
BEE 3	702.39	13695.6	19.50	257.64	13.14
HCP 1	175.95	2026.5	11.52	118.57	17.95
HCP 2	431.62	7541.0	17.47	161.40	8.23
HCP 3	554.46	9442.7	17.03	314.27	9.00
CAL 1	112.74	639.2	5.67	259.34	0.20
AUD 1	732.13	10772.7	14.71	443.57	82.56
AUD 2	462.67	6541.3	14.14	398.06	58.94
AUD 3	1019.13	13627.9	13.37	449.39	88.57
INC 1	586.31	11701.7	19.96	525.10	9.58
INC 2	1562.28	33199.3	21.25	382.67	6.80
INC 3	405.03	5101.7	12.60	402.22	0.99
CBL 1	727.93	10957.2	15.05	413.12	1.41
CBL 2	820.79	11371.5	13.85	560.29	1.03
CBL 3	531.97	6721.5	12.64	501.24	1.06
KIN 1	426.76	4387.2	10.28	635.38	14.23
KIN 2	450.48	4496.4	9.98	826.65	34.11
KIN 3	492.41	5118.2	10.39	490.92	5.71
JLP 1	890.45	16981.6	19.07	330.38	30.45
JLP 2	546.45	10125.9	18.53	264.72	6.67
JLP 3	1495.17	30022.7	20.08	295.36	6.64
PSC 1	335.55	5040.8	15.02	220.02	5.38
PSC 2	157.33	1038.2	6.60	786.37	18.00
PSC 3	200.68	3001.4	14.96	293.01	36.62
BRY 1	688.47	8989.0	13.06	508.47	5.88
BRY 2	611.91	7838.0	12.81	590.03	9.46
BRY 3	343.42	4647.2	13.53	249.70	9.23
CCW 1	628.75	9652.1	15.35	532.83	23.47
CCW 2	658.51	11066.4	16.81	597.45	58.05
CCW 3	729.83	13189.8	18.07	565.71	44.59
MRK 1	466.31	10057.4	21.57	143.54	22.50
MRK 2	694.15	15553.1	22.41	244.60	19.57
MRK 3	687.67	12348.2	17.96	203.46	27.18
SHK 1	129.38	4455.6	34.44	17.65	14.78
SHK 2	3515.44	74851.8	21.29	307.58	7.29
SHK 3	665.42	29836.8	44.84	59.83	8.97

Table 12: Average value and standard deviation for soil nutrient data

Soil Nutrient Analysis Data

	Avg	St. Deviation
Total Nitrogen mg-N/kg-soil	667.836	572.546
Total Carbon mg-C/kg-soil	12075.977	12962.285
C:N Ratio mg-C / mg-N	16.843	7.004
Total Phosphorus mg-P/kg-soil	381.614	187.212
PA Phosphorus mg-P/kg-soil	19.402	21.891

For TN, the plots with the lowest levels were Calvert and Shockley 1 with values of 112.74 and 129.38 respectively. In addition, Calvert site had values significantly lower than the average values for TC, CN, and PAP. A recurring trend appeared to emerge: if a certain soil sample had atypically high or low values for one nutrient variable, than it was likely it had abnormally high or low values for another variable. Another example of this drawn conclusion is Irvine 2, which had abnormally high values for both TN (1562.28) and TC (33,199.3). In addition, even within the same site, nutrient levels between plots could be significantly different. For example, Shockley 1 had a very low TN level (129.38) and Shockley 2 had a very high TN level (3515.44).

In addition to a nutrient level analysis as described above, other important soil characteristics were examined on site. These characteristics included: mottling, gleying, organic matter thickness (OMT), and soil depth (SD). The data obtained for OMT and SD was measured using a yard stick and the values were recorded in centimeters. Mottling and gleying were analyzed using an intensity rating scheme (based on a scale of 1-10). The dotted line on the graph below (Figure 30)

demonstrates that any site with a value above three exhibits significant anaerobic soils. The averages for both mottling (m=4.36) and gleying (g=4.67) were well above this statistically significant threshold. This result indicates that as a whole, the wetland sites illustrated a significant presence of mottling and gleying.

Figure 30: Gleying and mottling intensity values from each of the 13 wetland sites
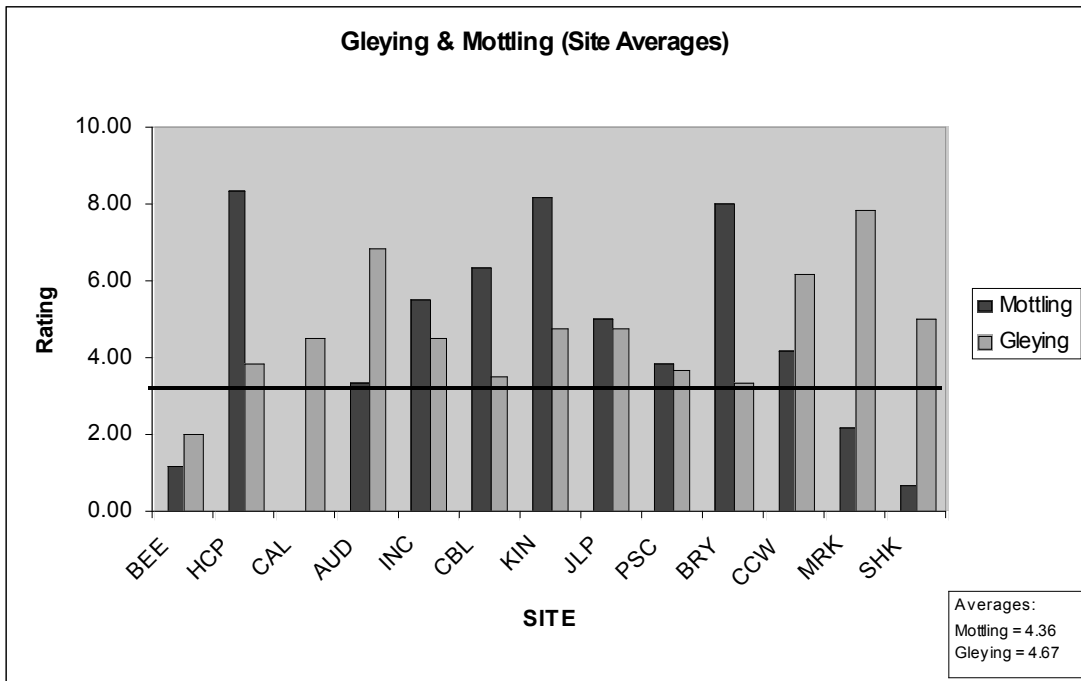
Although a very wide range of intensity values existed, nine sites demonstrated significant mottling and twelve sites demonstrated significant gleying values. Furthermore, every site we sampled revealed some presence of gleying in the soil. In addition, only one study site (Calvert) lacked any presence of mottling. The purpose of the Figure 30 is to illustrate that all study sites had characteristics of anaerobic soils. This signified that the wetland soils at the selected sites were functioning properly.

4.5 Antibiotic Resistance

4.5.1 Overall Levels of Resistance Across Sites

A total of 33 isolates of *Escherichia coli* were obtained during the sampling period in 2006, and 35 isolates were obtained in 2007, creating a final total of 68 colonies of *E. coli* isolated during the course of this study. The resistance or susceptibility of each colony to six different antibiotics was then determined via disk diffusion. Overall rates of resistance to these six antibiotics are shown in Figure 31.

Figure 31: Overall percentage of *E. coli* isolates resistant to each antibiotic tested, spanning all wetland sites as well as data from both 2006 and 2007
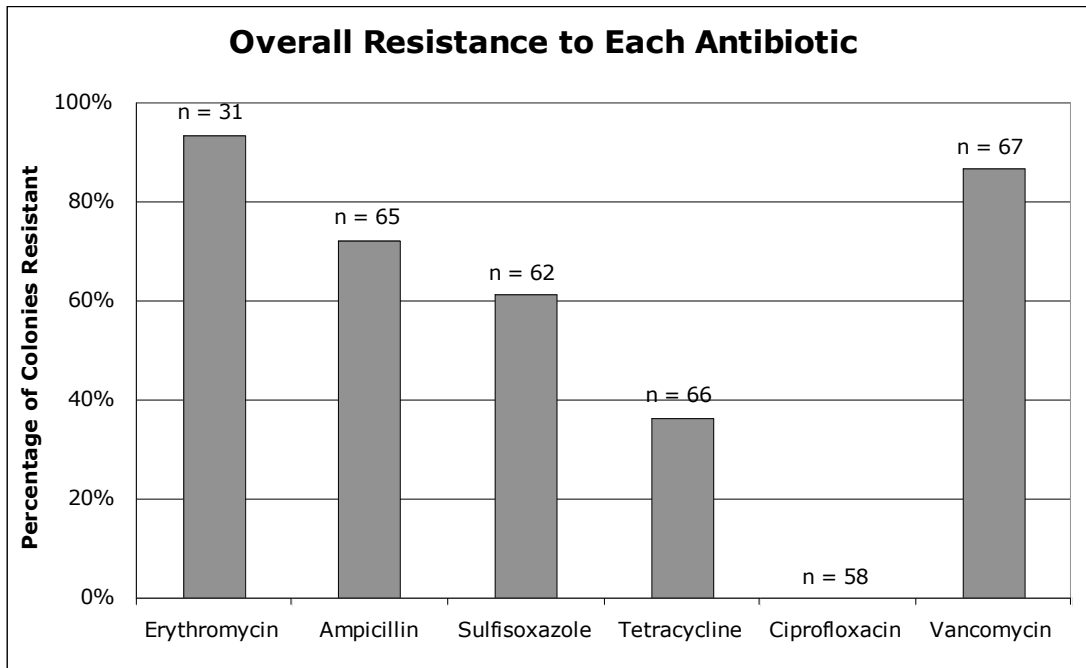
For each antibiotic, 'n' denotes the number of colonies tested against that antibiotic. Vancomycin was used as a negative control.

It is important to note that the number of colonies tested against each antibiotic, designated 'n', varies in Figure 31. This is due to experimental

inconsistencies. On occasion, the zone of inhibition around an individual antibiotic disc would be amorphous instead of circular, and thus its radius could not be measured, nor could the respective colony's resistance to that antibiotic be determined. Amorphous inhibition zones account for the minor variations in values of n. However, a more significant inconsistency is that of erythromycin; because standardized erythromycin discs were not available in 2007, only isolates from 2006 were tested against this antibiotic. As a result, the n value for erythromycin (n = 31) is about half that of the other antibiotics.

Given that the noted inconsistencies are, for the most part, minor, they do not greatly diminish the ability of our results to show overall trends in rates of *E. coli* resistance to the tested antibiotics. Erythromycin exhibited the highest percentage of resistant colonies, at 94%, while ampicillin, sulfisoxazole, and tetracycline had successively lower rates of resistance: 72%, 61%, and 36% respectively. No colonies were determined to be resistant to ciprofloxacin. An 87% resistance rate was observed towards vancomycin, which was considered to be a negative control.

Basic qualitative differences in resistance rates to the six antibiotics fall well within the realm of expectation. The fact that *E. coli* would exhibit the most resistance to erythromycin and ampicillin makes sense; both of these compounds are naturally occurring, both of them were discovered many decades ago, and both are widely used due to their broad spectrum of antibiotic activity. In contrast, ciprofloxacin is an entirely synthetic compound discovered within the past few decades, and is used less often than many other antibiotics; therefore, less resistance was expected. However, overall resistance rates for all 5 experimental antibiotics (i.e.

vancomycin excluded) appear to be higher than rates of resistance found in previous studies, as shown in Figure 32.

Figure 32: Comparison of overall *E. coli* resistance results from this study to resistance results from several similar previous studies
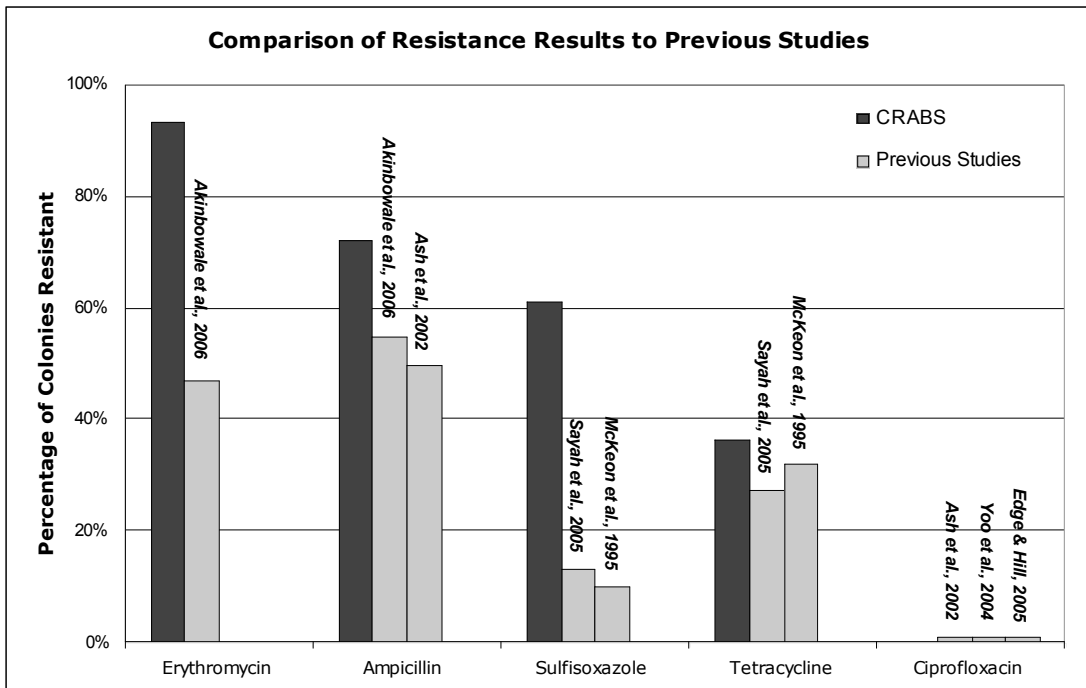
Results cannot be strictly compared due to differing methodologies and species studied, but in overall resistance rates found in this study were similar to or higher than those found in previous studies.

The rate of resistance seen towards erythromycin was significantly larger than rates of resistance found in previous research. Prior studies of other Gram-negative species, such as *Campylobacter jejuni*, have indicated no resistance to erythromycin in water samples (Levesque, 2007). A study conducted by Akinbowale et al. (2006), in which Gram-positive and Gram-negative isolates from aquacultural environments were tested for susceptibility to 19 drugs, noted only a 47.1 percent resistance to erythromycin. However, previous research by Zuccato et al. (2000) indicates that

erythromycin has a tendency to maintain its presence in the environment for long periods of time. This implies that bacterial isolates have had ample time to develop resistance to the drug, which could help explain our results.

The measured rate of resistance to ampicillin was also higher than rates seen in previous studies, though not drastically so. A recent study conducted in Australia reported 54.8 percent of 100 Gram-negative and four Gram-positive isolates to be resistant to ampicillin (Akinbowale et al., 2006), while another study conducted in the United States found 50 percent of *E. coli* isolates to be ampicillin resistant (Ash et al., 2002). As noted, the high resistance rates we found in this study are realistic given the widespread nature of penicillin use. But it should also be pointed out that β-lactam compounds do not persist in the environment, and therefore the high rate of ampicillin resistance was observed in spite of the transient nature of ampicillin in waterways. This would seem to corroborate the idea that over time, the constant release of a short-lived compound can mimic the one-time release of a long-lived compound in terms of its chronic effects.

Tetracycline results were more in line with those of previous studies. In a study conducted by Sayah et al. (2005), tetracycline resistance was found at a rate of 27.3 percent across 1,286 isolates. Our data fell within this range; we observed a 36 percent resistance rate.

The low levels of bacterial resistance to ciprofloxacin found in this study agreed with the results of other authors. A 2005 study of surface waters and fecal pollution sources near Hamilton, Ontario found resistance to ciprofloxacin in less than 1 percent of 462 isolates (Edge & Hill, 2005). Studies prior to this one also

found resistance to ciprofloxacin of less than 1 percent in *E. coli* isolates from surface water (Ash et al., 2002).

Vancomycin was used as a negative control in this study. Because vancomycin specifically targets Gram-positive bacteria, whereas *E. coli* is Gram-negative, it was expected for *E. coli* to exhibit total resistance towards vancomycin irrespective of any kind of developed resistance. The exact reasons for the occurrence of only 87 percent resistance to vancomycin are unknown, but one possibility may be a genetic mutation.

4.5.2 Multidrug Resistance

Multidrug resistance refers to the situation in which a single bacterial colony simultaneously exhibits resistance to multiple antibiotics. The vast majority of *E. coli* isolates from this study were multidrug resistant.

Figure 33 shows multidrug resistance counts for the 23 colonies that were tested against all six antibiotics. All but one colony was resistant to more than one antibiotic, and more than half of the isolates (63 percent) were resistant to three or more antibiotics. No isolate was resistant to all six of the antibiotics tested; this would have been impossible, given that no *E. coli* isolates were found to be resistant to ciprofloxacin.

Figure 33: Multidrug resistance for *E. coli* isolates that were tested against all six antibiotics
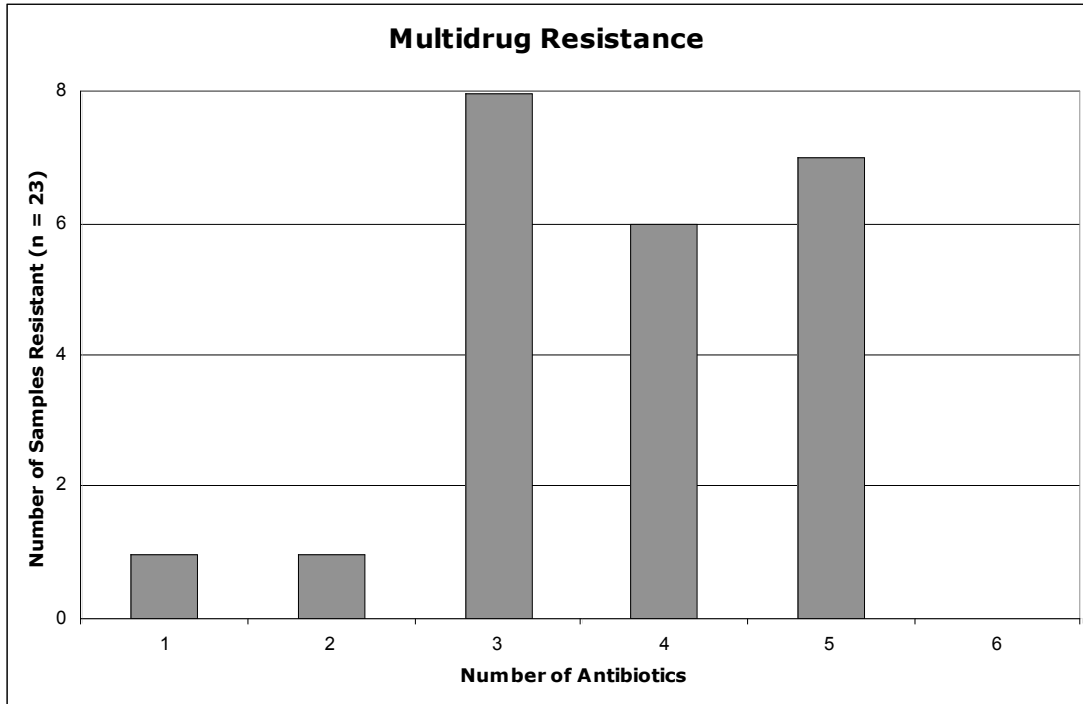
Of the 23 E. coli *isolates tested against all six antibiotics used in this study, the overwhelming majority showed simultaneous resistance to more than one antibiotic. Twenty-two colonies were resistant at least two antibiotics, 21 colonies were resistant to three or more antibiotics, and 13 colonies were resistant to four or more antibiotics.*

With respect to multi-drug resistance, our results indicate greater resistance than do those of previous studies. A survey of bacterial resistance of 250 isolates from rural water sources in West Virginia conducted by McKeon et al. (1995) noted that approximately 78 percent of all isolates exhibited resistance to multiple antibiotics. However, the study included not only *E. coli,* but also strains of *C. freundii* and *Enterobacter cloacae*. In fact, *E. coli* isolates exhibited the lowest rate of multidrug resistance of all the bacterial species studied (only 14 percent).

Prior studies have investigated the mechanisms of multidrug resistance at a molecular level. Studies of protein interactions in *E. coli* indicate that there may be a link between multi-drug resistance and the protein MsbA, which is involved in ATP production (Woebking et al., 2005). While such molecular analysis is beyond the scope of this study, it may be possible to make certain inferences about the genetic basis of antibiotic resistance. For instance, it is interesting to note that resistance to certain antibiotics was correlated to higher chances of multidrug resistance: approximately 91.30 percent of isolates resistant to erythromycin were resistant to three or more antibiotics, while only 47.93 percent of isolates resistant to sulfisoxazole were resistant to 3 or more antibiotics. This may indicate a genetic linkage of phenotypes for antibiotic resistance, in that the same chromosomes/plasmids may carry the traits for resistance to multiple antibiotics. More detailed results of this correlation can be found in Table 13 and Figure 34.

Table 13: Relationship between multidrug resistance and resistance to individual antibiotics

	Resistant to…				
If resistant to…	No resistance**	1 class	2 classes	3+ classes	Percent Resistant to 3+ classes
Erythromycin	1	0	1	21	91.30%
Tetracycline	10	0	0	13	56.52%
Ampicillin	4	1	0	18	78.26%
Sulfisoxazole	12	0	0	11	47.83%

* NOTE: Vancomycin and Ciprofloxacin were included when analyzing multidrug resistance, but each drug was not specifically evaluated since vancomycin was a control drug and no resistance was seen towards ciprofloxacin
** Implies no resistance to respective drug, therefore multidrug resistance with respect to that drug was not analyzed for given isolate

Figure 34: Relationship between multidrug resistance and resistance to individual antibiotics
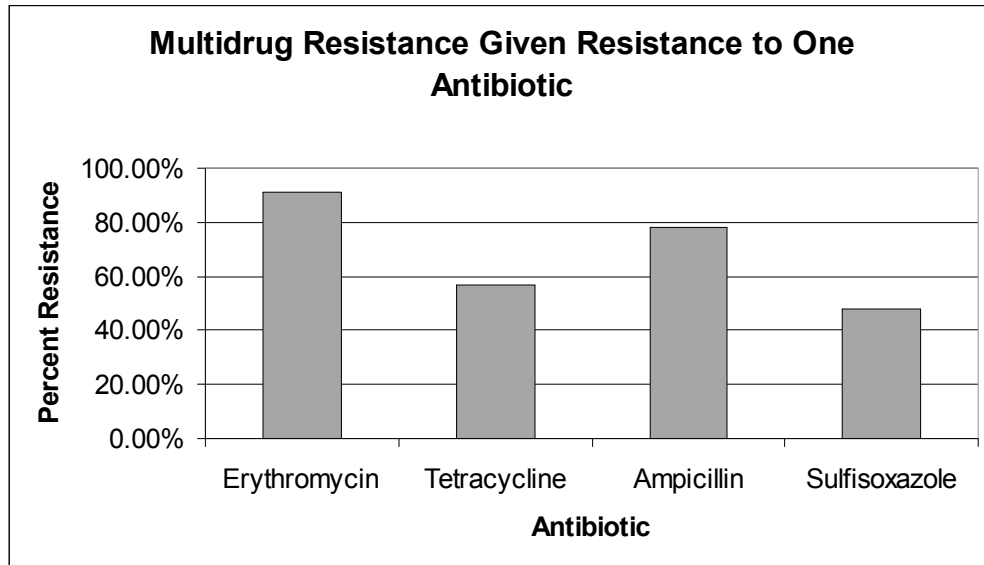
A recent antibiotic resistance study was conducted on bacterial isolates originating from catfish at three fish farms in Vietnam. Results indicated that numerous multidrug resistance profiles included ampicillin (Sarter et al., 2007). Our findings agree with such results – we found that colonies of *E. coli* resistant to ampicillin were highly likely (78.26 percent) to be multidrug resistant.

Meanwhile, Sayah et al. (2005) noted in their study that isolates resistant to multiple drugs were more likely to exhibit this multidrug resistance if a combination of agents including tetracycline was used. Our results did not indicate a strong correlation between resistance to tetracycline and multidrug resistance. Given our limited sample size, it is difficult to make a conclusive argument about this point. But the study by Sayah et al. (2005) consisted of isolates from wildlife, surface water, and farm environments, among others, and the difference in origins of isolates and environmental factors may help explain why our results are somewhat different. In

addition, Sayah et al. (2005) explain, "the multidrug resistance exhibited by *E. coli* in this study could have been the result of independent, simultaneous development of resistance to different agents or could have been the result of co-selection of resistance determinants." This assertion could very well pertain to our samples as well.

4.5.3 Antibiotic Resistance Stratified By Study Site

Antibiotic resistance was also stratified by wetland site. Percent resistance for each antibiotic at each site was calculated, and then an overall "Site Average Resistance" was calculated by taking the percent resistance for all antibiotics at a particular site and averaging them together – see Table 14 below. Irvine Nature Center had the lowest overall level of antibiotic resistance at 30.3%, while the Herring Creek Park and Shockley sites had the highest overall resistance at 75.00 percent.

Table 14: Percentages of resistant *E. coli* isolated, stratified by wetland site

Antibiotic Resistance Percentages by Wetland Site

	Ery.	Amp.	Sulf.	Tet.	Cip.	Van.	Site Average Resistance
Irvine	0	36	73	0	0	73	30.3
Kinder	0	67	83	17	0	83	41.7
Bryantown	0	75	75	50	0	100	50.0
Jackson Lane	67	67	67	17	0	83	50.0
Waldorf	100	50	0	50	0	100	50.0
Beehive	100	75	50	25	0	75	54.2
Piscataway	100	43	33	57	0	100	55.6
Aud	100	100	33	33	0	100	61.1
Cumberland	100	100	78	33	0	78	64.8
Merkle	100	100	40	56	0	100	66.0
Calvert	100	100	50	50	0	100	66.7
Herring Creek	100	100	100	100	0	50	75.0
Shockley	100	100	50	100	0	100	75.0

The rightmost column, "Site Average Resistance," is an overall average of the resistance rates to individual antibiotics at a particular site, and provides an indication of that site's overall level of antibiotic resistance. Sites are arranged in order of increasing Site Average Resistance.

Characterizing antibiotic resistance by wetland site is an interesting endeavor, but at the same it is extremely important not to place serious weight on the results in Table 14. Stratifying the resistance results of no more than 68 isolates across 13 wetland sites means the percentages listed for each site in this table are based on a miniscule sample size. There is little to no statistical significance. To account for this limitation when trying to correlate antibiotic resistance to other aspects of wetland health and function in this study, all of the resistance data should be combined into a single statistical pool and analyzed together – such an undertaking is the subject of the following section. For future studies, the number of colonies isolated and characterized must be significantly larger.

4.6 Statistical Analysis

We used statistical analysis to determine links between antibiotic resistance and other wetland characteristics. We ran a series of t tests in STATA 9.2 Data Analysis and Statistical Software. We added six dummy variables, one for each antibiotic, and assigned values of "1" for resistant and "0" for not resistant. When partitioned with respect to ciprofloxacin resistance, every sample was contained in the "not resistant" category, so running a t test was impossible. Results concerning erythromycin should also be considered with some reservation, since all but two samples showed resistance to erythromycin. We ran t tests to see if separating our samples in this way led to statistically significant differences in wetland characteristics. Results show some links between antibiotic resistance and measures of water quality, soil quality, and surrounding land use, but no links with measures of vegetation diversity

4.6.1 Relationships Amongst Wetland Health Factors

Table 15: Statistically significant relationships found amongst wetland health factors

Wetland Health Factor	Wetland Health Factor	P – value	Direction
Organic Matter Thickness	Gleying	0.024	+
Organic Matter Thickness	Surface NH_4	0.003	+
Organic Matter Thickness	Surface Total N	<0.001	+
Organic Matter Thickness	Surface Organic N	0.012	+
Organic Matter Thickness	Subsurface NO_2	0.001	+
Soil Depth	Gleying	0.006	+
Soil Depth	Soil Available P	0.044	-
Mottleing	Gleying	<0.001	+
Mottleing	Soil Total C	0.035	-
Mottleing	Soil C-N ratio	0.029	-
Mottleing	Soil Total P	0.044	+
Soil C-N ratio	Soil Total P	<0.001	-
Soil Total N	Soil Total C	<0.001	+
Soil Available P	Surface NH_4	0.034	+
Soil Available P	Surface Total P	<0.001	+
Surface NH_4	Surface NO_2	0.016	+
Surface NH_4	Surface Total N	<0.001	+
Surface NO_2	Surface Total N	0.008	+
Surface NO_2	Surface NO_3	<0.001	+
Surface NO_2	Surface Organic N	0.013	+
Surface NO_3	Subsurface $NO_2 + NO_3$	<0.001	+
Surface NO_3	Subsurface Total N	0.007	+
Surface NO_3	Subsurface NO_2	0.006	+
Subsurface $NO_2 + NO_3$	Subsurface Total N	<0.001	+
Subsurface $NO_2 + NO_3$	Subsurface Organic N	<0.001	+
Subsurface NO_3	Subsurface Organic N	<0.001	+

"Surface" refers to data from surface water samples, "subsurface" refers to data from subsurface water samples, and "soil" refers to data from soil samples.

Organic matter thickness has a positive significant relationship to surface NH_4 surface total nitrogen, surface organic nitrogen, and subsurface NO_2. An increase in the nitrogen levels in a wetland cause an increase in plant and animal abundance. This in turn can cause the organic matter thickness to increase.

Some of the very significant relationships ($p < 0.001$) are fairly obvious and intuitively understandable. Mottleing and gleying are both indicators of wetland soils, so one does not usually appear without the other in a true wet environment. Soil total

nitrogen and soil total carbon are also closely related. Wetlands tend to retain carbon

and a more productive wetland (with more total nitrogen) will be producing more

organic matter to eventually add to the soil total carbon. The other very significant

relationships (Surface NH_4 to surface total nitrogen, surface NO_2 to surface NO_3, and

subsurface $NO_2 + NO_3$ to subsurface total nitrogen) all involve nitrogen levels and are

positively correlated as would be expected from a well functioning wetland.

4.6.2 Relationships to Land Use

Table 16: Statistically significant relationships found between wetland health factors
and land use factors

Wetland Health Factor	Land Use Factor	P - value	Direction
Organic Matter Thickness	Agriculture – 1000 m buffer	0.002	+
Organic Matter Thickness	Agriculture – 2000 m buffer	0.056	+
Mottleing	Agriculture – 1000 m buffer	0.048	+
Soil C-N ratio	Nature – 1000 m buffer	0.004	+
Soil Total P	Agriculture – 1000 m buffer	0.023	+
Soil Total P	Natural – 1000 m buffer	0.041	-
Surface NH_4	Agriculture – 1000 m buffer	0.014	+
Surface Total N	Agriculture – 1000 m buffer	0.018	+
Surface Organic N	Agriculture – 1000 m buffer	0.001	+
Subsurface $NO_2 + NO_3$	Agriculture – 2000 m buffer	0.003	+
Subsurface NO_3	Agriculture – 2000 m buffer	0.005	+

*"Agriculture" and "Nature" refer respectively to the amount of agricultural and
natural land use found in a respective buffer zone.*

The significant relationships found between land use factors and wetland

health factors are detailed in Table 16. All but two of the significant relationships we

found between land use factors and wetland health factors involved agricultural land

use. The two outliers involved natural land use. Interestingly, there were no

significant relationships found between the Land Development Index (LDI) and any

wetland health factors. This indicates that the LDI is not a good predictor of soil or

water nutrient levels in a mitigation wetland when used with a 1000 or 2000 meter radius land use buffer.

Both the 1000 meter and 2000 meter agricultural land use showed a significant positive relationship to organic matter thickness. Agriculture is a large contributor to the nitrogen influx a wetland sees and nitrogen levels directly influence the productivity of a wetland. As mentioned previously, a more productive wetland would accumulate more organic matter, and thus the soil organic matter thickness would increase over time. This result is further verified by the fact that it was repeated for both size buffers and thus the chance of it being a false positive (a problem for studies with small sample sizes) is significantly reduced. A confounding variable that was not examined by this study, but influences organic matter thickness substantially is the age of the mitigation wetland in question, i.e. an older wetland should have a thicker organic layer.

The assertion that agricultural land use increases nitrogen content in a wetland is bourn out by other significant relationships found in this study. There are significant positive relationships between agricultural land use on either the 1000 meter or 2000 meter level and surface total nitrogen, surface organic nitrogen, surface ammonia, subsurface nitrate + nitrite, subsurface total nitrogen, subsurface nitrite, and subsurface organic nitrogen.

An interesting but also expected result is the relationship between soil total phosphorous and natural and agricultural land use. Agricultural land use increases soil total phosphorous, probably because of runoff from livestock yards and some

fertilizers. We would then expect to see a decrease in soil total phosphorous when a wetland is set in a more natural setting and the data bears this assumption out.

4.6.3 Relationships to Antibiotic Resistance

T-tests were performed to compare bacterial resistance data to other wetland measurements, within the categories of land use, soils, water quality, and vegetation. Figure 18 through 20 and 23 through 25 in the appendices provide lists of all variables included in statistical analysis. The variable for surface water nitrate levels was omitted from this analysis due to insufficient data. Furthermore, due to the abundance of plant species found in this study, vegetation data was not included on a plant-by-plant basis – instead, Shannon Diversity Index (described in Section 4.2.3: Shannon Diversity Index) was used to consolidate all vegetation data into a single variable representing overall plant diversity. Note also that the antibiotics vancomycin and ciprofloxacin were not included in this statistical analysis. Vancomycin was not included because it was a negative control. Ciprofloxacin was not included because there were not enough isolates in the ciprofloxacin-resistant group to mathematically allow for a t-test to be performed.

Because there were four antibiotics on which t-tests were run (tetracycline, sulfisoxazole, erythromycin, and ampicillin) and 31 variables to run them against, a total of 124 t-tests were performed for this portion of data analysis. Table 17 shows the results of all such tests.

Table 17: Results of 124 t-tests showing all statistically significant relationships between antibiotic resistance and various wetland indicators

	Bacterial Resistance to Antibiotics			
	Ampicillin	Sulfisoxazole	Erythromycin	Tetracycline
Soil Organic Matter Thickness			0.0682	
Soil Depth		0.0768		
Soil Mottling		0.0773		
Soil Gleying				
Soil Total N			0.0104	0.0579
Soil Total C			0.0237	0.0981
Soil C:N Ratio				
Soil Total P				
Soil Available P				
Surface Water Total N		0.0766		
Surface Water Ammonia		0.0705		
Surface Water Organic N			0.0042	0.0290
Surface Water Nitrite				
Surface Water Nitrite + Nitrate			0.0382	
Surface Water Total P				
Subsurface Water Total N				
Subsurface Water Ammonia		0.0436		
Subsurface Water Organic N				
Subsurface Water Nitrite				
Subsurface Water Nitrite + Nitrate			0.0302	
Subsurface Water Nitrate			0.0236	
Subsurface Water Total P		0.0522		
Plant Shannon Diversity Index				
Urban Land Use (1000 m)				
Urban Land Use (2000 m)				
Agricultural Land Use (1000 m)			0.0071	0.0754
Agricultural Land Use (2000 m)			0.0691	
Natural Land Use (1000 m)			0.0423	0.0138
Natural Land Use (2000 m)			0.0361	0.0046
Landscape Development Intensity (1000 m)				
Landscape Development Intensity (2000 m)				0.0640

Land Use, Vegetation, Water, and Soil Measurements (row group label, left vertical heading)

Shaded boxes represent significant (p < 0.05) or almost significant (0.05 < p < 0.10) correlations, with two-tailed p-values listed to four decimal places. Ciprofloxacin was excluded from this analysis due to insufficient data in its resistant group. Abbreviations: C = carbon, N = nitrogen, P = phosphorous.

Ampicillin was most notable for its lack of statistically significant relationships to other measurements. While erythromycin, sulfisoxazole, and tetracycline each had six or more significant ($p < 0.05$) or almost significant ($0.05 < p < 0.10$) relationships to other variables, ampicillin had none. The lack of statistically significant relationships for ampicillin may be due to the small sample size of isolates tested – especially since ampicillin had only three isolates in its resistant group. If the lack of relationships is due to something more than sample size considerations, a speculative conclusion might be that bacterial resistance genes to β-lactam antibiotics have now become so widespread that they are essentially present everywhere, irrespective of other environmental factors. Indeed, β-lactams have been in widespread use for decades, and ampicillin had the second highest rate of resistance out of the five non-control antibiotics in this study.

Sulfisoxazole had correlations to several soil and water quality measurements, but no correlations to land use and vegetation metrics. Resistance to sulfisoxazole had an almost significant relationship ($0.05 < p < 0.10$) with soil depth and soil mottling. As described in Section 4.6.1: Relationships Amongst Wetland Health Factors, soil depth and mottling are themselves strongly correlated with each other, so here they should be considered together. Resistant isolates tended to occur in wetlands with deeper soil layers and higher levels of mottling, both of which tend to be traits of properly functioning, anaerobic wetlands.

Sulfisoxazole resistance also had correlations to several water quality measurements, which were somewhat stronger than its correlations to soil measurements. Resistant isolates tended to be found at sites with higher levels of

surface water ammonia and surface water total nitrogen ($0.05 < p < 0.10$), lower

levels of subsurface water ammonia ($p < 0.05$), and lower levels of subsurface water

total phosphorous ($0.05 < p < 0.10$). Without further study, it would be difficult to

say whether such relationships are the result of bacterial resistance affecting wetland

biogeochemical cycling, the result of wetland biogeochemistry affecting sulfisoxazole

resistance, or whether there is some lurking cause (such as the input of runoff

containing both nutrients and pharmaceuticals) affecting both factors simultaneously.

It goes without saying that any causal scheme is certain to be complex.

Erythromycin resistance had significant correlations to several nitrite (NO_2^-)

and nitrate (NO_3^-) water quality measurements. Resistance tended to occur in

wetlands with lower subsurface water nitrate levels ($p < 0.05$), lower subsurface water

nitrite + nitrate levels ($p < 0.05$), and lower surface water nitrite + nitrate levels ($p <$

0.05). Nitrite and nitrate ions are produced during the process of nitrification, in

which ammonium nitrogen is oxidized in the upper aerobic layer of a wetland.

Therefore, the correspondence between resistance and lower levels of nitrite and

nitrate may be an indication that resistance tends to occur in more anaerobic wetlands,

in which the balance of nitrogen is towards more reduced forms like molecular

nitrogen (N_2 – produced from nitrate in the anaerobic process of denitrification) and

ammonia. It is interesting to note that this aligns with the result for sulfisoxazole

resistance, which tended to be found in more anaerobic soils.

Nitrification occurs via the actions of Gram-negative microbes such as

Nitrosomonas sp. and *Nitrobacter* sp. (Gomez, Mendez, & Lema, 1996). Since

erythromycin has actions against Gram-negative bacteria (Costanzo, Murby, & Bates,

2005), it is possible that nitrification is correlated to erythromycin resistance by some microbial-level link. One possibility is that the presence of erythromycin has some direct effect on the functions or populations of these microbes. A study by Costanzo et al. (2005) found that high doses of erythromycin were capable of reducing the rate of bacterial denitrification in aquatic sediments. That experiment, however, tested only the short-term effects of a high concentration (1000 μg/L) of antibiotic, and did not consider the long-term effects of lower concentrations, nor did it attempt to correlate these results to the occurrence of bacterial resistance. A second possibility for a microbial link between erythromycin resistance and nitrification is that nitrifying bacteria, in addition to their functions in nitrogen cycling, also have some sort of function in attenuating the spread of bacterial resistance. In this study, lower levels of erythromycin resistance corresponded to higher levels of nitrite and nitrate, and higher levels of these two molecules would intuitively correspond to properly functioning populations of nitrifying bacteria. Thus, the proper functioning of nitrifying bacteria might aid in reducing the spread of bacterial resistance.

The most striking relationships found in this data analysis were those shared by erythromycin and tetracycline; for both antibiotics, resistance was correlated to natural and agricultural surrounding land use, organic nitrogen levels in surface water, and total nitrogen and carbon levels in soil. As shown in Table 17, the fact that land use was measured within both 1000 m and 2000 m buffers means that there were a total of eight possible relationships between erythromycin/tetracycline resistance and agricultural/natural land use. Seven out of eight of these relationships turned out to be statistically significant ($p < 0.05$) or almost significant ($0.05 < p < 0.10$). Prior

to the execution of this study it was hypothesized that since humans are a major cause of the spread of antibiotic resistance, bacterial resistance would tend to occur at wetland sites that were more impacted by human development. Thus it was expected that natural land use would be associated with bacterial susceptibility, while urban and agricultural land use would be associated with bacterial resistance. Instead, the trends observed in this study were the opposite of those hypothesized; erythromycin and tetracycline resistance were found in wetlands with lower agricultural land use and higher natural land use. It may be that the relationship between land use and bacterial resistance is more complicated than initially anticipated, or it may be that land use does not play a role as significant as we previously thought.

Resistance to erythromycin and tetracycline was also associated with higher levels of soil total nitrogen and soil total carbon, as well as higher levels of surface water organic nitrogen. A visual depiction of the relationship between bacterial tetracycline resistance and soil total nitrogen & carbon is shown in Figure 35. Statistical significance values for erythromycin relationships were stronger than those for tetracycline, but tetracycline was selected for this graph because the relatively even split between tetracycline-susceptible and tetracycline-resistant isolates made for easier visualization.

Figure 35: Bacterial resistance to tetracycline plotted against soil nutrient levels
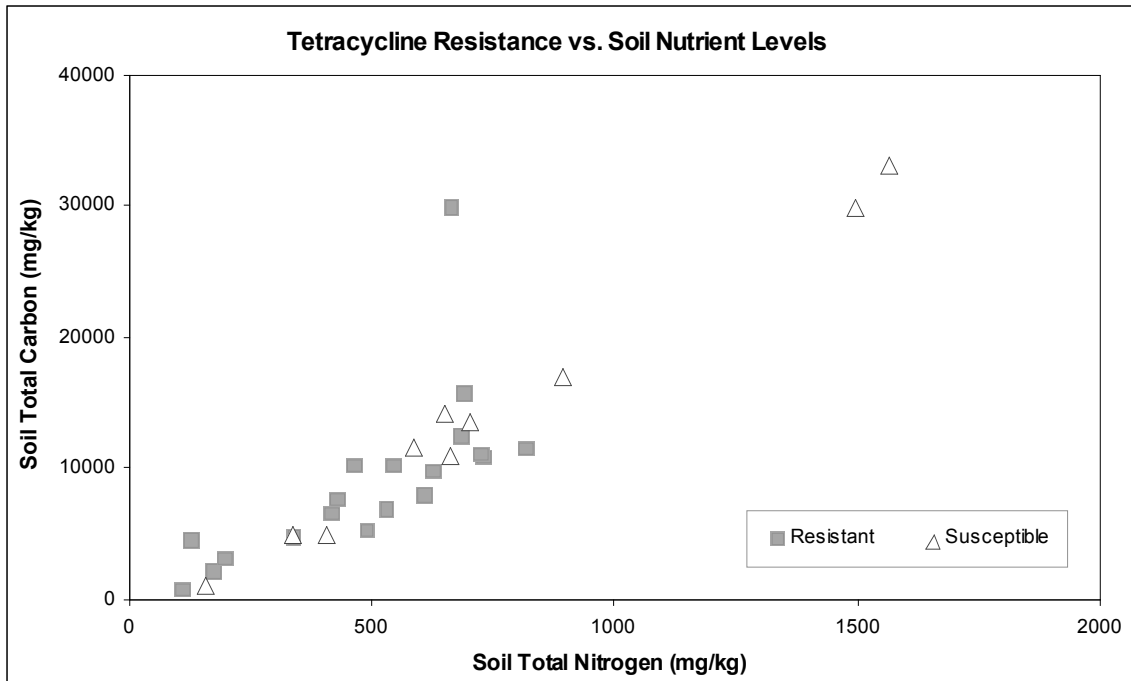
Each of the 29 points represents a different plot at which bacterial resistance data was collected. Plots in which at least one bacterial colony was resistant are marked with grey squares, while plots in which all bacterial colonies were susceptible are marked with outlined white triangles. Soil total nitrogen and carbon levels are on the axes and thus increase upwards and to the right.

In Figure 35, sites exhibiting tetracycline resistance tend to be clustered towards the bottom left, where there are lower levels of soil total nitrogen and carbon. Meanwhile, sites with susceptible isolates tend more towards the upper right. The interesting point here is that organic nitrogen, total nitrogen, and total carbon are all indicators of productive wetland systems. Therefore, sites with resistant isolates tended to be less productive, while sites with susceptible isolates tended to be more productive. The relationship held true for both tetracycline and erythromycin – more productively functioning wetland systems were associated with less bacterial resistance.

Assuming that productive wetlands have larger, healthier, and more diverse natural microbial populations, it might be possible that wetland microbes are actually responsible for mitigating the spread of antibiotic resistance. Perhaps natural bacteria are part of some biogeochemical process that results in the degradation of antibiotics, reducing the spread of antibiotic resistance. Or perhaps simple population dynamics are at work, dictating that it is more difficult for bacterial resistance genes to spread and take hold in the presumably larger bacterial populations of productive wetland systems. Any causational relationship between wetland productivity and reduced bacterial resistance could also help to explain the seemingly counterintuitive results of the agricultural/natural land use correlations to tetracycline and erythromycin: increased agricultural land use around a wetland (and the associated loss of natural land) would be associated with increased nutrients from agricultural runoff, which could cause increases in wetland productivity and an associated decrease in antibiotic resistance.

Of course, the present study was an observational study and thus it is impossible to determine actual causes behind the relationships we found. Further studies must be performed to gain a better understanding of the biogeochemical mechanisms underlying our results. Controlled microcosm studies might be particularly useful. For example, wetland microcosms could be designed in which nutrients such as nitrogen and phosphorous are varied while different antibiotics are simultaneously fed into the system at low concentrations. Resulting microbial processes could then be monitored by a variety of methods: microbial population could measured by culturing water or sediment samples and obtaining colony counts,

rates of resistance to different antibiotics could be measured as was done in the present study, time decay of antibiotics themselves could be monitored via analytical measurements, and bacterial transfer of resistance genes and associated nucleic acid plasmids could be studied via biochemical methods. The presence of a control system would help to isolate specific causal factors.

The inability to determine causal relationships should not diminish the importance of the numerous correlations found in this study. Definitive trends were noted in the results, and they are especially important due to the relative paucity of previous research in this area.

5. Conclusion

The first important conclusion reached by this study is that Maryland's mitigation wetlands on the whole appear to be functioning properly. At the 13 mitigation sites we visited, field data in the categories of water quality, soils, and vegetation tended to be indicative of productive wetland systems. For example, 12 of 13 wetland sites had a wetland vegetation prevalence index indicative of a wetland ecosystem. Every site had a least some degree of mottling and/or gleying, indicative of an anaerobic environment. Finally, nutrient levels in water sample were generally on par with those found in a similar study of wetlands in Florida. The proper functioning of the sites in this study was particularly important, because as mitigation wetlands they were designed to replace lost wetlands, and if they didn't function as wetlands then it would be impossible for them to serve their regulatory purpose.

Certain sites had anomalous characteristics. For example, the Bryantown and Aud sites had unusually high levels of surface water ammonia, surface water total phosphorous, and surface water total nitrogen. Because this trend encompassed several different measurements and multiple sampling dates, it can be reasonably assumed that it is the result of some unique characteristic of these two sites and not the result of anomalous water samples. Another example of an unusual site was the Kinder site, which was unique for its high wetland vegetation prevalence index, indicating that it was the only site in this study that could not be considered a wetland based on its vegetation.

The interesting point about Kinder is that a simple glance at it potentially foretold the same information learned through intensive vegetation sampling; upon

our first visit to the site we immediately noted that it had no standing water and was instead dominated by dry vegetation and ticks. Proper hydrology is one of the most fundamental prerequisites to a functioning wetland and without sufficient ground saturation, Kinder simply could not support the necessary hydrophytic wetland vegetation. Mitigation sites must be designed with this consideration in mind, for it would be wasteful to invest time and money attempting to create a wetland site without ensuring the proper hydrology to support it.

The second major conclusion of this study is that land use has significant relationships to field indicators of wetland health. The vast majority of statistically significant correlations found in this study were between agricultural land use and nutrient levels. Furthermore, the directions of the correlations we found agreed with our hypothesis; increasing agricultural land use, i.e. increasing human impact, correlated to increases in surface water total nitrogen, surface water organic nitrogen, and surface water ammonia, among other variables.

Land use correlations are intellectually interesting for their ability help elucidate the potential impacts of human development on wetland health, but they also serve a more practical purpose. In the future, deeper integration of land use studies with field measurements of wetland health will most likely help to make wetland monitoring strategies more efficient. Characterization of wetland sites based on surrounding land use can be made without visiting the sites themselves, so that in the future wetland managers can use existing correlations to help determine which sites need might need more in-depth attention based solely on their land use

characteristics. Such a system would allow limited resources to be used to greater

ends in the goal of environmental protection.

A third conclusion of this study is that antibiotic resistance is present in

Maryland wetlands. Antibiotic resistance was found at all thirteen sites studied, with

overall levels that were equal to or higher than levels previously reported in the

environment. Trends for individual antibiotics were similar to those reported in

previous studies, with high rates of resistance to erythromycin and ampicillin,

intermediate rates of resistance to sulfisoxazole and tetracycline, and a very low rate

of resistance to ciprofloxacin.

The widespread prevalence of antibiotic resistance in Maryland's wetlands is

interesting because historically, antibiotic resistance has been studied only in more

traditional locales like hospitals. Meanwhile, the release of antibiotics and other

pharmaceuticals into the environment has been largely overlooked. However, this

and other studies demonstrating the presence of high rates of antibiotic resistance in

the environment reveal that the role of antibiotics in the natural world merits closer

examination. The fact that environmental antibiotic resistance rates are similar to

hospital resistance rates suggests that genes for bacterial resistance are present

everywhere, and that the effects of antibiotic resistance cannot be escaped in any

locale. A final conclusion arising from this study is that the occurrence of antibiotic

resistance has a complicated relationship to wetland health and function. In general,

more productive wetlands tended to have lower rates of antibiotic resistance. It seems

that while wetlands are sites where antibiotic resistance occurs, they may also have

the ability to "filter" out antibiotic resistance in the same way that they filter out other

pollutants. This result is a especially exciting because it suggests at a previously

unstudied function for wetlands.

A limiting factor of this study was its sample size, which made it difficult to

stratify data and to find statistically significant relationships. Beyond that, in a

system as complex as a wetland, it is difficult to determine causal relationships –

microcosm studies would be particularly helpful in the future. The present study was

an attempt, through an observational methodology, to fill in a significant gap in the

state of current scientific knowledge, and it revealed a number of open questions for

future research on the ever-changing relationship between humans and wetlands.

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Appendix A

Table 18: Soil characteristics and nutrient concentrations

Site & Plot Number	Organic Matter Layer thickness cm	Water table (cm below surface)	Sulfur smell	Presence of mottles	Presence of Gleying	Total Nitrogen mg/kg dw	Total Carbon mg/kg dw	C:N Ratio	Total Phosphorus mg/kg dw	Plant Available Phosphorus mg/kg dw
INC 1	15	Surface Water	---	6-7*	8-9*	586.31	11701.7	19.96	525.10	9.58
INC 2	15	15	---	5*	8-9*	1562.28	33199.3	21.25	382.67	6.80
INC 3	1	> 25 cm	---	5*	6-7*	405.03	5101.7	12.60	402.22	0.99
BEE 1	3	> 25 cm	---	none, orange	1-2*	420.67	6449.0	15.33	324.00	3.06
BEE 2	10	> 25 cm	---	3-4*	4-5*	649.32	14324.4	22.06	445.15	6.57
BEE 3	6	10	---	0	0	702.39	13695.6	19.50	257.64	13.14
CCW 1	1	> 25 cm	---	2-3*	0	628.75	9652.1	15.35	532.83	23.47
CCW 2	4	> 25 cm	---	7-8*	8-10*	658.51	11066.4	16.81	597.45	58.05
CCW 3	3	> 25 cm	---	2-3*	2-3*	729.83	13189.8	18.07	565.71	44.59
KIN 1	10	Surface Water	6*	8-9*	7-9*	426.76	4387.2	10.28	635.38	14.23
KIN 2	8	5	3*	7*	7*	450.48	4496.4	9.98	826.65	34.11
KIN 3	7	Surface Water	---	8-10*	8-10*	492.41	5118.2	10.39	490.92	5.71
CAL 1	2	Surface Water	8	0	0	112.74	639.2	5.67	259.34	0.20
CAL 2										
BRY 1	5	Surface Water	0	8*	6*	688.47	8989.0	13.06	508.47	5.88
BRY 2	9	Surface Water	0	7-8*	6-7*	611.91	7838.0	12.81	590.03	9.46
BRY 3	10	Surface Water	3*	8-9*	9-10*	343.42	4647.2	13.53	249.70	9.23
MRK 1	10	Surface Water	7*	4*	3*	466.31	10057.4	21.57	143.54	22.50
MRK 2	11	Surface Water	6	2.50	2.5	694.15	15553.1	22.41	244.60	19.57
MRK 3	11	Surface Water	---	0	2.5	687.67	12348.2	17.96	203.46	27.18
PSC 1(1A)	5	Surface Water	2	5*	5*	335.55	5040.8	15.02	220.02	5.38
PSC 2(1B)	9		2	4-5*	4-5*	157.33	1038.2	6.60	786.37	18.00
PSC 3	8	Surface Water	7	2	2	200.68	3001.4	14.96	293.01	36.62
SHK 1	4	10	0	0	0	129.38	4455.6	34.44	17.65	14.78
SHK 2	8	3	1	0	6--8	3515.44	74851.8	21.29	307.58	7.29
SHK 3	5	> 25 cm	1	2	9	665.42	29836.8	44.84	59.83	8.97

JLP 1	6	Surface Water		8	6--9	890.45	16981.6	19.07	330.38	30.45
JLP 2	8	Surface Water	8	7	6	546.45	10125.9	18.53	264.72	6.67
JLP 3	12	Surface Water	7	0	0	1495.17	30022.7	20.08	295.36	6.64
HCP 1	4	> 25 cm	7	9	7	175.95	2026.5	11.52	118.57	17.95
HCP 2	5	> 25 cm	6	8	58	431.62	7541.0	17.47	161.40	8.23
HCP 3	8	> 25 cm	7	8	7	554.46	9442.7	17.03	314.27	9.00
CBL 1	4	> 25 cm	0	6	0	727.93	10957.2	15.05	413.12	1.41
CBL 2	3	25	0	6	0	820.79	11371.5	13.85	560.29	1.03
CBL 3	4	> 25 cm	2	7	9	531.97	6721.5	12.64	501.24	1.06
AUD 1	7	Surface Water	6	3	9--10	732.13	10772.7	14.71	443.57	82.56
AUD 2	2	> 25 cm	0	5		462.67	6541.3	14.14	398.06	58.94
AUD 3	9	6	5	2	1--2	1019.13	13627.9	13.37	449.39	88.57

Appendix B

Table 19: Surface water quality at each plot-site, (BDL-below detection limit)

Site & Plot Number	Ammonia-N mg-N/L	Nitrite-N mg-N/L	Total Nitrogen mg-N/L	Total Phosphorus mg-P/L	Nitrate mg-N/L	Org-N mg-N/L
INC 1						
INC 2	1.078	0.0178	22.62	3.13	B.D.L.	21.54
INC 3						
BEE 1	0.015	B.D.L.	0.80	0.06	B.D.L.	0.79
BEE 2	0.282	0.0034	2.06	0.65	B.D.L.	1.77
BEE 3						
CCW 1	0.027	0.0037	1.64	0.29	B.D.L.	1.61
CCW 2	0.210	0.0120	6.76	1.54	0.0016	6.54
CCW 3						
KIN 1						
KIN 2	0.556	0.0097	3.52	0.57	B.D.L.	2.96
KIN 3	0.518	0.0150	3.46	0.81	0.0013	2.93
CAL 1	0.052	0.0080	2.25	0.35	0.0882	2.10
CAL 2	0.012	B.D.L.	0.80	0.12	0.0036	0.79
BRY 1						
BRY 2						
BRY 3	1.077	0.2104	16.83	10.81	B.D.L.	15.63
MRK 1	0.200	0.0100	12.20	0.92	B.D.L.	12.00
MRK 2	0.324	0.0145	6.68	0.93	0.0355	6.31
MRK 3	0.058	0.0652	3.94	0.10	1.6875	2.13
PSC 1(1A)	0.073	0.0043	1.17	0.15	B.D.L.	1.10
PSC 2(1B)	0.148	0.0077	1.66	0.36	B.D.L.	1.50
PSC 3	0.067	0.0019	1.04	0.42	B.D.L.	0.97
SHK 1	0.019	0.0035	1.76	0.10	B.D.L.	1.73
SHK 2	0.012	B.D.L.	0.87	0.04	B.D.L.	0.86
SHK 3	0.022	0.0042	1.86	0.10	B.D.L.	1.83
JLP 1	0.087	0.0170	3.19	0.24	B.D.L.	3.09
JLP 2	0.040	0.0088	2.76	0.35	B.D.L.	2.71
JLP 3	0.108	0.0106	5.20	0.58	B.D.L.	5.08
HCP 1	0.037	0.0033	0.96	0.05	0.0058	0.92
HCP 2	0.034	0.0061	1.02	0.07	B.D.L.	0.97
HCP 3	0.049	0.0023	5.72	0.38	0.0038	5.67
CBL 1	0.131	B.D.L.	3.30	0.36	0.0034	3.17
CBL 2	0.085	0.0022	1.09	0.11	0.0098	0.99
CBL 3	0.173	0.0038	2.13	0.11	0.0071	1.94
AUD 1	0.448	0.0057	2.72	48.02	B.D.L.	2.27
AUD 2						
AUD 3	1.217	0.0077	8.78	6.87	B.D.L.	7.56

Table 20: Sub-surface water quality at each site-plot, (BDL-below detection limit)

Site & Plot Number	Ammonia-N	Nitrite-N	Total Nitrogen	Total Phosphorus	Nitrate	Org-N
	mg-N/L	mg-N/L	mg-N/L	mg-P/L	mg-N/L	mg-N/L
INC 1	0.8052	1.5876	6.6254	0.221	0.7815	3.4511
INC 2	0.6754	0.5340	2.5244	0.1824	0.3002	1.0148
INC 3						
BEE 1	1.2102	0.0275	2.3982	0.0794	0.2295	0.9311
BEE 2	1.2097	0.0261	2.4055	0.06435	0.3168	0.8530
BEE 3	1.0866	0.0086	4.2689	0.11495	0.0959	3.0779
CCW 1	1.1854	0.0078	2.4796	0.3289	0.0008	1.2856
CCW 2	0.5188	0.0145	2.4718	1.5194	0.0992	1.8393
CCW 3						
KIN 1	0.2334	B.D.L.	0.8209	0.0189	0.3187	0.2688
KIN 2	0.832	0.145	2.658	0.144	0.5061	1.1751
KIN 3	0.918	0.007	2.000	0.150	0.1008	0.9747
CAL 1	0.4258	0.1815	3.2039	0.2244	0.4459	2.1508
CAL 2	0.7596	0.0240	7.9725	0.9396	3.8298	3.3592
BRY 1						
BRY 2	0.2170	0.0258	7.4376	0.4057	3.8705	3.3243
BRY 3						
MRK 1	0.4521	0.0869	7.6985	3.2433	1.0557	6.1038
MRK 2	0.2320	B.D.L.	2.6389	0.0302	0.0427	2.3642
MRK 3	0.8928	0.0379	7.7793	0.1222	4.0134	2.8352
PSC1(1A	0.1771	B.D.L.	0.8494	0.0935	0.0162	0.6561
PSC 2(1B)						
PSC 3	1.3270	0.0712	3.7608	0.145	0.1589	2.2037
SHK 1	3.3764	0.0364	5.4467	0.0672	0.4626	1.5713
SHK 2	0.0858	0.1733	3.1557	0.1279	1.4133	1.4833
SHK 3						
JLP 1	0.9034	0.0035	18.7948	0.9219	13.0906	4.7973
JLP 2	1.5558	0.0037	4.3501	0.74	0.1147	2.6759
JLP 3	0.4520	1.1238	13.0007	0.7274	6.7333	4.6916
HCP 1	0.3998	0.0088	3.8972	0.0655	1.4686	2.0200
HCP 2	0.4125	0.0594	2.6293	0.0712	0.5963	1.5611
HCP 3	4.0430	0.0755	5.0700	0.0894	0.1387	0.8128
CBL 1						
CBL 2	0.7249	0.1662	4.6532	0.1377	2.2312	1.5309
CBL 3	2.0149	0.0293	3.2970	0.0492	0.2544	0.9984
AUD 1	0.3886	0.0656	3.4781	0.3338	1.0229	2.0010
AUD 2	0.8058	0.0042	1.5356	0.5072	0.0893	0.6363
AUD 3	1.4069	0.0107	4.3200	0.0906	0.2696	2.6328

Appendix C

Table 21: Complete list of plant species found at each wetland site

Aud

Acer rubrum
Agrostis sp.
Alisma subcordatum
Apocynum cannabinum
Aster sp.
Aster sp. #2
Baccharis halimifolia
Baptisia tinctoria
Betula nigra
Bidens connata
Bidens sp.
Carex lurida
Carex scoparia
Carex sp.
Cephalanthus occidentalis
Cyperus strigosus
Dichanthelium clandestinum
Diospyros virginiana
Eleocharis acicularis
Erigeron annuus
Eupatorium perfoliatum
Eupatorium sp.
Fraxinus pennsylvanica
Galium tinctorium
Hypericum mutilum
Impatiens capensis
Juncus canadensis
Juncus effusus
Juncus tenuis
Leersia oryzoides
Lespedeza sp.
Lespedeza sp. #2
Liquidambar styraciflua
Liriodendron tulipifera
Ludwigia palustris
Lysimachia sp.
Mikania scandens
Mikania scandens
Panicum sp.
Parthenocissus quinquefolia

Phragmites australis
Pinus sp.
Platanus occidentalis
Polygonum persicaria
Polygonum sagittatum
Quercus rubra
Rosa palustris
Sagittaria latifolia
Salix nigra
Schoenoplectus
tabernaemontani
Toxicodendron radicans
Typha angustifolia
Typha latifolia
Vaccinium sp.
Viburnum recognitum

Beehive

Acer rubrum
Asclepias incarnata
Asclepias sp.
Asclepias sp. #2
Aster puniceus
Bidens sp.
Brasenia schreberi
Carex lurida
Carex scoparia
Carex sp.
Carex stipata
Dichanthelium clandestinum
Eleocharis quadrangulata
Eupatorium perfoliatum
Fraxinus sp.
Galium tinctorium
Impatiens capensis
Juncus canadensis
Juncus effusus
Leersia oryzoides
Ludwigia palustris
Lycopus americanus

Mikania scandens
Panicum sp.
Peltandra virginica
Pilea pumila
Platanus occidentalis
Polygonum sagittatum
Prunus sp.
Rubus sp.
Salix nigra
Schoenoplectus tabernaemontani
Sparganium erectum
Stachys sp.
Triadenum virginicum
Typha angustifolia
Typha latifolia
Typha X glauca
Ulmus sp.
Vicia cracca
Wisteria frutescens

Bryantown
Acer rubrum
Agrimonia parviflora
Agrostis gigantea
Asclepias incarnata
Asclepias sp.
Aster sp.
Betula nigra
Bidens frondosa
Boehmeria cylindrica
Carex lurida
Carex scoparia
Carex vulpinoidea
Cephalanthus occidentalis
Cuscuta gronovii
Cyperus sp.
Dichanthelium clandestinum
Diospyros virginiana
Eleocharis obtusa
Fraxinus pennsylvanica
Galium tinctorium
Hypericum mutilum
Impatiens capensis
Juncus canadensis
Juncus effusus

Juncus tenuis
Leersia oryzoides
Liquidambar styraciflua
Lycopus americanus
Mikania scandens
Nyssa sylvatica
Onoclea sensibilis
Oxalis sp.
Parthenocissus quinquefolia
Phalaris arundinacea
Poa sp.
Polygonum arifolium
Polygonum persicaria
Polygonum sagittatum
Rosa multiflora
Rosa palustris
Rubus allegheniensis
Rumex crispus
Salix nigra
Sambucus canadensis
Smilax rotundifolia
Solidago sp.
Spartina cynosuroides
Stachys sp.
Styrax grandifolius
Toxicodendron radicans
Verbena hastata
Verbena sp.
Wisteria frutescens

Calvert
Acer rubrum
Agrostis gigantea
Asclepias incarnata
Baccharis halimifolia
Betula nigra
Boehmeria cylindrica
Carex crinita
Carex lupulina
Carex lurida
Carex scoparia
Carex sp.
Cladium mariscoides
Dichanthelium clandestinum
Eleocharis obtusa
Eupatorium perfoliatum

Fraxinus pennsylvanica
Galium tinctorium
Juncus canadensis
Juncus effusus
Juncus tenuis
Leersia oryzoides
Liquidambar styraciflua
Lithospermum sp.
Lonicera japonica
Lycopus uniflorus
Mikania scandens
Oxalis stricta
Phalaris arundinacea
Polygonum arifolium
Polygonum aviculare
Polygonum sagittatum
Polygonum sp.
Populus heterophylla
Robinia pseudoacacia
Rosa palustris
Rubus allegheniensis
Salix nigra
Scirpus atrovirens
Scirpus cyperinus
Scirpus cyperinus
Solidago sp.
Toxicodendron radicans
Triadenum virginicum
Typha latifolia

Cumberland
Acer rubrum
Agrimonia sp.
Agrostis gigantea
Agrostis sp.
Allium vineale
Apocynum cannabinum
Aster sp.
Aster sp. #2
Bidens frondosa
Bidens laevis
Calystegia sepium
Carex crinita
Carex lupulina
Carex lurida
Carex scoparia

Carex squarrosa
Carex stipata
Carex vulpinoidea
Centaurea biebersteinii
Cephalanthus occidentalis
Coronilla varia
Dichanthelium clandestinum
Eleocharis acicularis
Equisetum arvense
Erigeron annuus
Eupatorium perfoliatum
Eupatorium sp.
Fraxinus pennsylvanica
Galium tinctorium
Impatiens capensis
Iris sp.
Juncus acuminatus
Juncus canadensis
Juncus effusus
Juncus sp.
Juncus tenuis
Ludwigia alternifolia
Lycopus americanus
Mimulus ringens
Osmunda cinnamomea
Panicum sp.
Plantago major
Poaceae --hairy ligule
Polygonum persicaria
Polygonum sagittatum
Polygonum sp.
Rosa palustris
Rudbeckia hirta var.
pulcherrima
Rudbeckia sp.
Rumex crispus
Schoenoplectus
tabernaemontani
Sisyrinchium angustifolium
Solanum carolinense
Solidago sp.
Solidago sp.
Solidago sp. #2
Tilia americana
Toxicodendron radicans
Trifolium repens

Typha angustifolia
Typha latifolia
Ulmus rubra
Verbena hastate

Waldorf

Acer rubrum
Achillea millefolium
Agrostis sp.
Allium vineale
Asclepias verticillata
Aster sp.
Betula nigra
Bidens laevis
Carex frankii
Carex lupulina
Carex lurida
Carex scoparia
Carex sp. #2
Carex vulpinoidea
Cephalanthus occidentalis
Cephalanthus occidentalis
Dichanthelium clandestinum
Eleocharis obtusa
Eleocharis sp.
Eupatorium sp.
Fraxinus pennsylvanica
Galium tinctorium
Iris sp.
Juncus canadensis
Juncus effusus
Juncus tenuis
Leersia oryzoides
Lespedeza virginica
Liquidambar styraciflua
Lycopus americanus
Lysimachia sp.
Mentha arvensis
Mikania scandens
Oligoneuron album
Oxalis sp.
Panicum virgatum
Panicum virgatum
Parthenocissus quinquefolia
Phalaris arundinacea
Poa sp.

Polygonum hydropiper
Polygonum persicaria
Polygonum sagittatum
Potentilla recta
Quercus bicolor
Rorippa nasturtium-aquaticum
Rosa multiflora
Rumex obtusifolius
Rumex sp.
Salix nigra
Scirpus sp.
Setaria viridis
Solidago sp.
Spartina cynosuroides
Specularia perfoliata
Toxicodendron radicans
Typha angustifolia
Typha latifolia
Veronica sp.

Herring Creek Nature Park

Acer rubrum
Agrostis gigantea
Apocynum cannabinum
Asclepias incarnata
Aster sp.
Baccharis halimifolia
Campsis radicans
Carex sp.
Cephalanthus occidentalis
Dichanthelium clandestinum
Festuca sp.
Galactia regularis
Ilex opaca
Juncus canadensis
Juncus effusus
Juncus longii
Juncus sp.
Juncus tenuis
Juniperus communis
Liquidambar styraciflua
Mikania scandens
Myrica cerifera
Phragmites australis
Pinus pungens
Pinus taeda

Poaceae --hairy ligule
Rosa palustris
Rumex crispus
Scirpus americanus
Taxodium distichum

Irvine Nature Center
Acer rubrum
Agrimonia parviflora
Agrostis gigantea
Agrostis sp.
Allium vineale
Asclepias incarnata
Betula sp.
Bidens coronata
Blephilia hirsuta
Boehmeria cylindrica
Brassicaceae sp.
Carex crinita
Carex laxiflora
Carex lurida
Carex scoparia
Carex stipata
Carex vulpinoidea
Celastrus scandens
Chenopodium album
Crataegus phaenopyrum
Eleocharis obtusa
Epigaea repens
Fragaria virginiana
Fraxinus pennsylvanica
Galium aparine
Galium tinctorium
Glyceria striata
Impatiens capensis
Juncus effusus
Juncus sp.
Juncus tenuis
Leersia oryzoides
Lindera benzoin
Lycopus uniflorus
Mentha spicata
onethera sp.
Oxalis sp.
Parthenocissus quinquefolia
Phalaris arundinacea

Polygonum arifolium
Polygonum pensylvanicum
Polygonum persicaria
Polygonum sagittatum
Polystichum acrostichoides
Quercus rubra
Ranunculus sp.
Rosa multiflora
Rubus idaeus
Rubus sp.
Rumex sp.
Scirpus atrovirens
Sisyrinchium montanum
Solidago sp.
Stachys sp. #1 (recognized)
Toxicodendron radicans
Trifolium hybridum
Trillium sp.
Typha latifolia
Ulmus americana
Viburnum recognitum
Viburnum sp.

Jackson Lane Preserve
Acer rubrum
Allium vineale
Aster sp.
Betula nigra
Bidens aristosa
Bidens connata
Bidens frondosa
Carex comosa
Carex scoparia
Carex sp.
Clethra alnifolia
Cornus amomum
Echinochloa crus-galli
Eleocharis acicularis
Eupatorium sp.
Fraxinus pennsylvanica
Hieracium sp.
Hypericum mutilum
Iris sp.
Juncus canadensis
Juncus effusus
Juncus marginatus

Juncus sp.
Juncus tenuis
Liquidambar styraciflua
Lonicera japonica
Mikania scandens
Oxalis stricta
Polygonum persicaria
Quercus alba
Quercus bicolor
Quercus lyrata
Quercus rubra
Rhexia mariana
Rhexia mariana
Rorippa islandica
Rubus allegheniensis
Rumex crispus
Salix nigra
Solidago sp.
Xanthium sp.

Limnobium spongia
Liquidambar styraciflua
Lolium pratense
Oxalis sp.
Phalaris arundinacea
Phleum pratense
Platanus occidentalis
Poa sp.
Polygonum arifolium
Polygonum hydropiper
Polygonum persicaria
Quercus bicolor
Quercus palustris
Quercus rubra
Rubus allegheniensis
Rumex crispus
Salix nigra
Scirpus atrovirens
Solanum carolinense
Viola sp.

Kinder

Agrostis gigantea
Agrostis sp.
Allium vineale
Amaranthus L. sp.
Andropogon gerardii
Asclepias incarnata
Aster sp.
Betula nigra
Carex lupulina
Carex lurida
Carex scoparia
Carex squarrosa
Carex stipata
Carex vulpinoidea
Cephalanthus occidentalis
Cornus amomum
Dactylis glomerata
Dichanthelium acuminatum
Dulichium arundinaceum
Festuca sp.
Fraxinus pennsylvanica
Galium tinctorium
Juncus effusus
Juncus tenuis
Leersia oryzoides

Merkle Wildlife Refuge

Acer rubrum
Agrostis gigantea
Asclepias incarnata
Asclepias sp.
Aster puniceus
Aster sp.
Betula nigra
Boehmeria cylindrica
Carex lurida
Carex vulpinoidea
Chamaecyparis thyoides
Crataegus iracunda
Cuscuta gronovii
Cyperus esculentus
Dichanthelium clandestinum
Distichlis spicata
Eleocharis obtusa
Erigeron annuus
Eupatorium perfoliatum
Fern sp.
Fraxinus pennsylvanica
Fraxinus sp.
Galium sp.
Galium tinctorium

Hydrangea arborescens
Hypericum mutilum
Impatiens capensis
Iris sp.
Juncus canadensis
Juncus effusus
Juncus marginatus
Juncus sp.
Juncus tenuis
Juniperus communis
Leersia oryzoides
Lespedeza violacea
Liquidambar styraciflua
Liriodendron tulipifera
Lycopus uniflorus
Mentha arvensis
Mentha spicata
Microstegium vimineum
Mikania scandens
Nyssa sylvatica
Onoclea sensibilis
Parthenocissus quinquefolia
Phalaris arundinacea
Pinus taeda
Poa sp.
Poaceae --hairy ligule
Polygonum persicaria
Polygonum sagittatum
Quercus rubra
Rhexia mariana
Rosa palustris
Rubus allegheniensis
Rumex crispus
Salix nigra
Sambucus canadensis
Scirpus cyperinus
Sisyrinchium angustifolium
Smilax rotundifolia
Solanum carolinense
Solidago sp.
Spartina cynosuroides
Toxicodendron radicans
Typha angustifolia
Typha latifolia
Vaccinium corymbosum
Verbena sp.

Veronica serpyllifolia
Veronica sp.
Viburnum recognitum
Wisteria frutescens

Piscataway Stream Valley Park
Acer rubrum
Achillea millefolium
Agrostis gigantea
Asclepias incarnata
Aster sp.
Betula nigra
Bidens connata
Carex lupulina
Carex lurida
Carex scoparia
Carex sp.
Carex vulpinoidea
Cephalanthus occidentalis
Cornus amomum
Cyperus strigosus
Dichanthelium clandestinum
Echinochloa walteri
Erigeron annuus
Eupatorium perfoliatum
Fraxinus pennsylvanica
Galium tinctorium
Impatiens capensis
Juncus canadensis
Juncus effusus
Juncus sp.
Juncus tenuis
Leersia oryzoides
Liquidambar styraciflua
Liriodendron tulipifera
Lycopus uniflorus
Mikania scandens
Osmunda cinnamomea
Parthenocissus quinquefolia
Phragmites australis
Platanus occidentalis
Polygonum hydropiperoides
Polygonum persicaria
Polygonum punctatum
Quercus alba
Quercus phellos

Rosa palustris
Rubus allegheniensis
Rumex crispus
Salix nigra
Scirpus cyperinus
Solidago sp.
Stachys sp.
Tilia americana
Toxicodendron radicans
Triadenum virginicum
Typha latifolia

Shockley
Acer rubrum
Aster sp.
Aster sp.
Cladium mariscoides
Clethra alnifolia
Cyperus sp.
Cytisus scoparius
Dichanthelium clandestinum
Dichanthelium sp.
Echinochloa muricata
Eleocharis acicularis
Eleocharis obtusa
Erigeron annuus
Ilex opaca
Iris sp.
Juncus canadensis

Juncus effusus
Juncus marginatus
Juncus sp.
Juncus sp.
Juncus tenuis
Liquidambar styraciflua
Lonicera sempervirens
Ludwigia palustris
Magnolia virginiana
Microstegium vimineum
Osmunda cinnamomea
Pinus serotina
Polygonum persicaria
Quercus alba
Rhexia mariana
Rhynchospora alba
Rosa multiflora
Rubus allegheniensis
Salix nigra
Sisyrinchium sp.
Smilax laurifolia
Smilax rotundifolia
Solidago sp.
Stachys sp.
Toxicodendron radicans
Trifolium sp.
Vaccinium sp.
Viburnum s

Appendix D

Table 22: Wetland Site Information

The following are site descriptions provided by MDE or MD SHA for the wetland assessment project.

Irvine Nature Center (INC) is located on the former site of a farm in northwest Baltimore County. Large, clear fields still occupy a major part of the property, while deciduous forests surround them. Plot 1 lies in an open field with several species of wetland vegetation, and plots two and three are in forests on opposite corners of the field containing plot 1.

Beehive (BEE) is in a fairly developed residential and industrial area in Elkridge. A railroad runs on the south side of the site. The site lies in the center of a neighborhood, with a road on one side and houses on another. The site lies approximately 10 feet lower than the surrounding area. The majority of the site holds standing water, with parts slightly above water level and others up to a foot below water. Vegetation seldom grows above 10 feet in the site.

SHA Description: Beehive Site - is a 2.4 acre site created to partially mitigate for non-tidal wetland impacts associated with the MD 100 project. The site is located on the east side of a tributary to Shallow Run, north of Loudon Avenue and east of Smith Avenue in Howard County. Shallow Run is a tributary to Deep Run. The site consists of 2.4 acres PEM creation. Construction of the site was completed in Spring 1995. The site is accessible directly from Loudon Avenue. Based on SHA-GIS data, land use within the vicinity of the mitigation site is a mix of institutional, industrial, low and medium density residential, and forested.

Waldorf (CCW) is an approximately 10 acre site with its southern border on MD 228. Most of the site is dry land. In the two southern corners, there are small lakes. Plot 1 is located just outside one of these lakes. In the middle of the site, there is a patch of forest; other than this, trees in the site grow no more than 10 feet tall. Much of the southwest corner of the site, where plot 2 is located, is covered by plants growing less than 1 inch tall. Plot 3, in the north of the site, is mostly covered with short trees, growing in dry ground.

SHA Description: MD 228 Site - is a 12.4 acre site created to partially mitigate for non-tidal wetland impacts associated with the MD 228 project. The site is located on the on the north side of MD 228, approximately one mile east of Bealle Hill Road in Charles County. The site consists of 12.4 acres of PFO/PEM creation. Construction of the site was completed in Fall 1995. The site is accessible directly from MD 228 Westbound. Based on SHA-GIS data, land use within the vicinity of the mitigation site is a mix of forested and low-density residential.

Calvert (CAL) is a small site located at the side of MD 4. Most of the site is underwater. Tree trunks were laid horizontally across the shallower of the two lakes on site. The shallower lake hosts a variety of wetland vegetation as well as our only plot on the site.

SHA Description: MD 4/MD 260 Site - is a 1.10-acre site created to mitigate for forested wetland impacts associated with the construction of the MD 4/260 interchange. This site is located within the floodplain of Lyons Creek south of the newly constructed MD 4/260 interchange and west of MD 4 in Calvert County, Maryland. Construction of the site was completed in Spring 2003. The site consists of 0.82 acre PFO creation, 0.38 acre PEM creation, and 0.16 acre bare ground. The bare ground area consists of highly acidic soils (acid sulfate soils?). SHA is currently investigating various acid-tolerant vegetation for future planting. The site is accessible directly from MD 4 Southbound. Based on SHA-GIS data, land use within the vicinity of the mitigation site is primarily forested.

Kinder (KIN) is a dry site located in a fairly rural area in Anne Arundel County. The site is located between two farms. The majority of the vegetation is low growing sedges and rushes. The surrounding area is forested on one side and farmland on the other three.

SHA Description: Kinder Site - is a 10.89-acre site created to mitigate for forested wetland impacts associated with improvements to MD 468. This site is located within a former pasture west of MD 468 and south of Sudley Road in Anne Arundel County, Maryland. Construction of the site was completed in Spring 2003. The site consists of 9.0 acres of PFO restoration/enhancement and 1.89 acres meadow/dry forest creation. This area is fenced/gated. However, the chain connecting the gate is slack enough to allow the students to "shimmy" though. Outside of the fenced area is a 18.81 acre PFO preservation area. Based on SHA-GIS data, land use within the vicinity of the mitigation site is primarily agricultural and low density residential.

Bryantown (BRY) is a densely vegetated site in Charles County. Nearly 100% of the site is covered by some sort of plant life. The site is located in a very agricultural area, with a farm on its west side. The northern part of the site is heavily forested, with tree trunks growing within a foot of each other. Standing water exists on most of the area, and a creek runs through the site.

Merkle Wildlife Management Area (MRK) hosts a 9 acre site on the banks of the Patuxent River. A majority of the site is covered with vegetation and standing water. The surrounding area is relatively undeveloped compared to other sites. Trees approximately 15 feet tall dominate each of our 3 plots.

Piscataway Stream Valley Park (PSC) is located near MD 301, a recycling center, and a police firing range. Despite all of this, the area is still fairly undeveloped. Most of the plot areas we selected were submerged. Willow is very common in sites 1 and 2, while trees are rare in plot 3.

Shockley (SHK) is a diversely vegetated site near Snow Hill on the eastern shore. Much of the area is densely forested. We chose plots in slightly depressed, submerged areas with no overhanging trees. The site overall is usually left alone to let nature run its course; some years ago, a fire burned down a significant part of the forests, but the areas are left to grow back in naturally.

Herring Creek Park (HCP) is located just a few miles from downtown Ocean City. It is surrounded by neighborhoods and is less than a mile from busy Route 50. Our plots are located off the sidewalk of the park in two submerged areas dominated by phragmites australis.

Jackson Lane Preserve (JLP) is a TNC wetland located on former farmland less than a mile from the Delaware line in Caroline County. The entire surrounding area is farmland. The area is mostly full of low growing vegetation with forest lining the border of the property. Several ponds are located throughout the wetland; we chose our three plots in these ponds.

Cumberland (CBL) is located in the foothills of the Appalachian Mountains near Cumberland, MD. The area is not densely populated at all. Some but very few people live within sight of the wetland, which is just down a hill to the side of US 220. Half of the site closer to the highway is covered in water; the other half is dry ground. Trees are very rare in the site. The site is also very close to I-70.

Aud (AUD) is located on Flat Iron Road, very close to St. Mary's River in St. Mary's County. Most notably, the wetland is downhill from a horse farm. The site consists of two slightly depressed wetlands containing usual wetland vegetation (typha latifolia, mikania scandens, etc.). Standing water is only prevalent in one of the three plots.