Reviewing Pesticide Impacts on Frogs to Suggest Management Applications in the Gambles Mill Eco-Corridor

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ABSTRACT

For years, frog populations have been declining due to a variety of anthropogenic sources, including pesticide use. Pesticides work by inhibiting bodily functions in their target pest species, though they frequently have unintentional impacts on other life forms in an ecosystem. Some researchers have evaluated these effects, but their studies mainly focus on finding an LC50 - a concentration that will kill 50% of their test species sample. However, these LC50 levels are often higher than what would be found in nature, and pesticides have been shown to still impact species at lower concentrations. Thus, this study attempted to summarize literature that included these lower concentrations to identify the level at which three target pesticides began to disrupt frog function. The target chemicals were atrazine, carbaryl, and glyphosate, all of which are commonly used pesticides or pesticide bases. This study was done specifically in the context of management for the Gambles Mill Eco-Corridor (referred to as the Eco-Corridor) in Richmond, Virginia, the site of a recent creek restoration project with a focus on storm water management and ecosystem health. To make results more applicable to the study area, four frog species with known or potential ranges in the Eco-Corridor were selected: The Gray Treefrog (*Hyla versicolor*), the Spring Peeper (*Pseudacris crucifer*), the American Bullfrog (*Lithobates catesbeiana*), and the Eastern Spadefoot (*Scaphiopus holbrookii*). The goal of this study wass to determine if the selected frog species and pesticides could be connected to provide motivation for the use of frogs as indicator species and contribute insight into water testing requirements for the Eco-Corridor. The results suggested that atrazine is impactful at 0.0025ppm, glyphosate is impactful at 0.018ppm and carbaryl is impactful at 0.07ppm for one or more of the target frog species. These lowest concentrations of concern can be used to set detection thresholds when conducting water quality testing in the Eco-Corridor. This study also discussed potential sources of pesticides for the Eco-Corridor and suggested water quality testing locations. However, the results showed a lack of consistency in literature availability for the target species. Thus, *P. crucifer* was recommended as an indicator species, largely due to capture availability.

INTRODUCTION

Of the class Amphibia, the most well-known global representative is undeniably the anuran, the zoological order consisting of frogs and toads. In fact, frogs and toads make up around 90% of all amphibian species. Like most amphibians, frogs are ectothermic, meaning their body temperatures rely on their ambient environment for homeostasis. They can also be fully or partially cutaneous, which means they rely on ambient moisture to respire. This puts frog species worldwide in danger, as the changing global climate threatens to increase temperatures and alter precipitation patterns (Cummins, 2003). In addition to climate change, frogs also face shrinking diversity from human-driven habitat loss, animal-driven ecosystem engineering, frog poaching for food-related trade, fungal diseases like Chytridiomycosis, and interspecies competition (Gratwicke et al., 2010; Ringler et al., 2015).

To adapt to their environments, frogs use a variety of behavior patterns that can be generally distinguished into three categories: semi-fossorial, semi-arboreal, and semi-aquatic. While most amphibian species have the ability to burrow, fossorial amphibians are noted to spend most of their lives beneath the ground, only surfacing occasionally to breed and forage (Székely et al., 2018). Since frogs dig backwards, semi-fossorial frogs' burrows are not permanent and usually collapse after exit (Johnson & Hembree, 2015). In amphibians, fossorial behavior represents greater adaptation to dry climates, as some semi-fossorial frogs can stay burrowed for up to nine months and are activated by even light rainfall (Ruibal et al., 1969). Arboreal frogs are known for their climbing acumen, spending most of their adult lives in trees. Many semi-arboreal frog species are members of the Family *Hylidae*. These frogs are believed to use mucus present on their toepads to induce wet adhesion, allowing them to stick to most surfaces (Langowski et al., 2018). Semi-aquatic frogs are the most abundant type of frog and divide their time between the water and moist land. Many semi-aquatic frogs belong to the Genus *Lithobates*. These frogs' habits are less defined and the amount of time they spend in the water depends on the individual species.

For humans, the global decline of frogs presents a major dilemma since many frog species provide people with invaluable ecosystem and health services. Frogs have long been used in medicine, with the South African clawed frog being touted as the world's first pregnancy test, while other frog species like the Chinese brown frog have historically been used in traditional remedies for their antimicrobial qualities (Bieniarz, 1950; Jin et al., 2009). Consuming a diet of small invertebrates such as flies, mosquitos, and earthworms, frogs act as wildly successful biological pest-managers in all life stages (Chowdhary et al., 2018). And as pest species become progressively more tolerant to modern agricultural control methods, frogs will only become increasingly essential for pest control (Zhelev et al., 2018). As indicator species, frogs provide value to complex or recently disturbed habitats by acting as biotic representatives for whole groups within an ecosystem, allowing environmental managers to focus their monitoring onto a single, more attainable species (Lindenmayer & Likens, 2011). Due to their sensitivity to environmental changes and reliance on consistent conditions for survival, frogs are particularly valuable indicator species for wetland habitats which can often be difficult to manage and monitor (Naito et al., 2012).

Despite these valuable ecosystem services, most of the negative changes that frogs face have been caused by human action and development. In fact, anthropogenic land-use change drives population-declines for an estimated 88% of amphibians, whose habitat has been threatened by both chemical contamination and ecosystem destruction (Taylor & Paszkowski, 2017). In general, environmental pollution is believed to be most impactful on frogs during the tadpole and embryonic life stages. Yet in most ecosystems, it is rare for one contaminant to be potent enough to cause death at any stage, meaning the individuals in polluted environments must live out their lives with reduced fitness (James & Semlitsch, 2011). Conversely, some built environments can actually benefit frogs by providing refuge from anthropogenic destruction. For example, some storm water management systems have potential to provide habitat for frogs and other aquatic species during drought (Halliday et al., 2015).

As the global community has become more in tune with environmental issues, improving storm water management has developed into a major consideration for cities and localities alike. As part of a project first conceptualized in 2011, the University of Richmond in Richmond, Virginia worked with the environmental solutions firm "RES" to improve storm water management near their campus by restoring an adjacent stream named "Little Westham Creek" (*University Facilities - University of Richmond*, n.d.). This project, now completed, saw the reestablishment of the creek's bed, the introduction of improved flood zones, the expulsion of invasive plant species, and the addition of a newly paved walking path through the surrounding

3,094-foot-long Gambles Mill Eco-Corridor (Joireman, n.d.). With goals to improve ecosystem health and biodiversity around the creek, and provide new spaces for environmental learning, the Gambles Mill Eco-Corridor restoration project represents an opportune moment to evaluate the current knowledge surrounding potential risk factors for local water systems (Joireman, n.d.). One of those risk factors is the use of pesticides in the areas around the Gambles Mill Eco-Corridor.

Pesticide use represents just one of the major anthropogenic challenges currently threatening frog populations. Pesticides are chemical compounds that target different portions of a pest species' bodily functions to inhibit their growth and reduce their presence in an ecosystem, usually for human benefit. Pesticides can be broken down into 4 different categories: herbicides which target plants, fungicides which target fungi, rodenticides which target rodents, and insecticides which target insects. In Virginia, around 14,000 pesticide products are registered for use each year, many of which are popular name brands such as Roundup® or Sevin® (Virginia Department of Agriculture and Consumer Services (VDACS), n.d.). While pesticides are usually very effective at eradicating their targets, they almost always have additional unintended effects on other key ecosystem players. Due to their permeable skin, amphibians are often the most susceptible to these externalities, which can lead to severely reduced fitness or death in many frog species (Quaranta et al., 2009). By running off during rain events, pesticides applied onto the lawns of homes, businesses, and especially farms can enter nearby surface water systems until they are eventually ingested by aquatic species or neutralized by micro-organisms. One of the main concerns with pesticide presence in water stems from the use of surface water as brooding sites for frogs, where the pesticides can freely disrupt larval development. In the embryonic and tadpole life stages, frogs are particularly vulnerable to their ambient conditions as chemicals can easily penetrate the larvae's protective barrier and alter the frogs' formative processes.

In scientific research, not all pesticides are tested equally. Due to the huge number of pesticides available in the United States, checking for pesticides in natural environments can be difficult. Currently, pesticide testing is routinely done by governmental agencies like the US Geological Survey, though their lowest thresholds for detection are often set too high to detect concentrations that could impact aquatic life (Food and Agriculture Organization of the United Nations (FAO), n.d.). However, pesticides can be naturally broken down in both surface water and soil through microbial processes and metabolic action (Food and Agriculture Organization of the United Nations (FAO), n.d.). Currently, water quality test kits for a majority of widely used pesticides are commercially available, though these tests are designed to indicate high parts per million (ppm) limits in drinking water (SimpleLab, n.d.). For lower ppm testing, enzyme-linked immunosorbent assay (ELISA) test kits can be purchased, though these kits are often expensive and require prior chemical expertise (Creative Diagnostics, n.d.).

In current studies, the most commonly tested pesticides appear to be atrazine-based pesticides, glyphosate-based pesticides, and carbaryl-based insecticides. Atrazine is most commonly used in corn fields but is also found on many lawns and golf courses across the southern USA. It is the most commonly found pesticide in drinking water as well (Gilliom et al., 2006). The glyphosate-based herbicide "Roundup®" is currently the most commonly used pesticide in the world due to its effectiveness at inhibiting aromatic amino acid production in plants (Rissoli et al., 2016). The agricultural giant Monsanto has also developed genetically modified "Roundup Ready®" seeds that are designed to be immune to the potent amino acid blocking capabilities of the pesticide, allowing farms to spray Roundup® indiscriminately. Carbaryl also holds its own as one of the world's most frequently used insecticides, explaining its frequency in scientific literature (Relyea & Mills, 2001). In their methods, many pesticide studies will choose to test just one or two target chemicals and will typically focus their observations on mortality rates. These studies usually aim to determine and report the levels at which their test pesticides would kill 50% of the test species after a single large dosage is suspended in air or water, notated in a format called the "LC50" (ScienceDirect, n.d.). These LC50s are used for safety purposes to determine the toxicity of a chemical for human and animal safety ("Pesticide Toxicity and Hazard," 2017). Yet, LC50 levels are usually very high, ranging somewhere between 1.5-5ppm for most highly toxic pesticides, and these levels are very unlikely to be found in uncontrolled settings. One study found that even after direct, accidental spraying of the chemical atrazine over a stream, the parts per million concentrations would not exceed 1 (Wan et. al, 2006). However, in addition to the LC50, some studies also report the milder effects of lower toxin concentrations, where disturbances can be observed in test species' function, but fatality is not common. This lower concentration level is not typically regarded as useful, but these lower concentrations can reveal previously unconsidered problems for ecosystem health and help forewarn about potential dangers in the future.

Therefore, this study reviews current literature on atrazine, carbaryl, and glyphosate-based pesticides to determine their lowest stated problematic concentrations in surface water. Then, the study uses first-hand experience and the Virginia Herpetological Society's (*Virginia Herpetological Society*, n.d.) guide on native frogs to select 4 frog species that could act as indicators for these pesticides in the Gambles Mill Eco-Corridor (referred to as "Eco-Corridor"). These chosen frog species represent the spectrum of different frog behavior patterns: The Gray Treefrog (*Hyla versicolor* - arboreal), the Spring Peeper (*Pseudacris crucifer* – arboreal/terrestrial), the American Bullfrog (*Lithobates catesbeiana* – aquatic), and the Eastern Spadefoot (*Scaphiopus holbrookii* – fossorial). This paper then describes potential habitat for these frog species and suggests which species could be the best potential indicator species to use in the Eco-Corridor. This paper also synthesizes the current literature on lowest impactful pesticide concentrations for these frog species to suggest water testing thresholds, sources of pesticides for the Eco-Corridor watershed, and best water testing locations within the Eco-Corridor. The main goal of this study is to answer whether the selected frog species and pesticides can be connected to provide motivation for using frogs as indicator species and contribute insight into water testing requirements for the Eco-Corridor.

STUDY AREA AND METHODOLOGY

The study area for this project was the Gambles Mill Eco-Corridor at the University of Richmond in Richmond, Virginia, a varied ecosystem with recent heavy disturbances from a large stream restoration project. The Gambles Mill Eco-Corridor contains a wide variety of ecological niches, including forested land, wetland, developed land, and meadows. These habitats include both long-established and newly constructed territories. As well, the stream restoration project introduced a storm water raingarden, increased standing water in wetlands, and established numerous viable amphibian breeding locations in the floodplains (B. Siegfried II, personal communication). The corridor is enclosed by the Country Club of Virginia to the east, a moderately trafficked road to the south (River Road), and the University of Richmond campus to the north and west (Figure 1).

Using available research tools such as the University of Richmond OneSearch and Web of Science, peer reviewed journal articles pertaining to different pesticide interactions for each above-mentioned target frog species were ascertained. Selected articles were then read through and scrutinized to determine whether their findings were likely applicable to the study area. As the goal was to collect all pertinent literature, there were no limitations to maximum or minimum number of articles used.

As well, to best assess the selected pesticides a thorough baseline understanding was established through use of the University of Richmond OneSearch and government databases on the target pesticides. For each pesticide, all available studies on the interaction between the toxins and target frog species were considered and read. After assessing all available literature, summaries of each pesticide's interactions with the selected frog species were created. The lowest disruptive concentrations were organized into a table using Excel (Table 1). All results were converted to parts per million for consistency.

RESULTS

Atrazine

Atrazine is the second most used herbicide in the United States, famed for its abilities to disrupt key proteins in plant photosynthesis (Almberg et al., 2018). Atrazine is controversial because it is a known endocrine disruptor that is shown to delay puberty and lengthen menstrual cycles in rats, leading it to be banned in the European Union (Zorrilla et al., 2010). The maximum allowed atrazine concentration in US drinking water is 0.003ppm, though atrazine concentrations have been found at levels over 0.5ppm in some waterways around the world (GovInfo, n.d.; Rimayi et al., 2018). In water, atrazine has a half-life of 96.5 days when exposed to sunlight and 252.6 days without sunlight (averages) (Farruggia, 2016).

In current studies, atrazine was found to have an array of disruptive impacts on *H. versicolor* tadpoles at 1.25ppm, particularly in relation to energy usage for detoxification, tissue repair, and homeostasis maintenance (Snyder et al., 2017). Similarly, *L. catesbeiana* tadpoles were shown to face lipid degradation, higher lipid levels, and increased cholesterol levels when exposed to atrazine at a very low 0.0025ppm level, though no mortality was observed (Dornelles & Oliveira, 2016). Low concentrations (0.003ppm – US EPA standard for safe drinking water) of atrazine significantly reduced the survival of late-stage *P. crucifer* individuals, though high concentrations increased *P. crucifer* survival in early-stage individuals (Storrs & Kiesecker, 2004). For *L. catesbeiana*, one author suggested that atrazine would not be toxic even up to 16 ppm (Wan et al., 2006). Thus, disruptive and lethal concentrations of atrazine seem to vary between species, but the lowest ppm demonstrating species health concerns was 0.0025ppm (Table 1).

Glyphosate - Roundup®

Glyphosate-based herbicides are one of the most commonly used pesticides in the world with popular name brands including Roundup® and Rodeo®. These herbicides work by constraining the production of amino acids, reducing plants' abilities to synthesize proteins (Hanlon & Parris, 2014). The highest recorded concentration of glyphosate in a waterbody as of 1980 was 5.2ppm (Edwards et al., 1980). Roundup® has a half-life of between 7-70 days (Giesy et al., 2000). In the United States, around 4 billion kilograms of glyphosate-based herbicides are applied to homes, gardens, forest, wetlands and 820 million hectares of cropland each year (National Center for Food & Agricultural Policy (NCFAP), n.d.).

Roundup® is suggested to have little to no impact on the survival of *P. crucifer* tadpoles at 3.8ppm and may actually be beneficial for them by killing predatory insects (Relyea, 2005). One study noted that pure glyphosate impaired oxygen uptake and distribution for *L. catesbeiana* tadpoles at 1ppm, though they distinguished pure glyphosate from the Roundup Original® and Roundup Transorb R® products (Rissoli et al., 2016). This study proposed that while glyphosate itself was toxic, the "inert" components mentioned on Roundup® packaging did in fact have impacts on tadpole oxygen uptake (Rissoli et al., 2016). This was suggested to have stemmed from the inert compounds differentially impacting the tadpoles' protective epithelial wall, which they partially use to breathe (Rissoli et al., 2016). At a low 0.018ppm concentration, glyphosate was demonstrated to increase lipids, increase cholesterol, and decrease proteins in *L. catesbeiana*, making it more difficult for the tadpoles to maintain homeostasis (Dornelles & Oliveira, 2016). Thus, for glyphosate-based pesticides, the lowest level of concern is 0.018ppm (Table 1).

Carbaryl - Sevin®

Carbaryl, more commonly known as the insecticide brand Sevin®, is also one of the most frequently used pesticides worldwide. In the United States alone, around 10-15 million pounds of carbaryl are sprayed annually, which includes application for approximately 28 million homes and 31 million gardens (Relyea & Mills, 2001). Carbaryl impacts target species by inhibiting nervous system function at low concentrations and paralyzing individuals at high concentrations (Bridges 2009). Carbaryl's half-life ranges from 2.4 hours to 90 days in water depending on temperature, sunlight, and water microbiome (Hanlon & Parris 2014). Carbaryl has been observed at concentrations as high as 4.8ppm in waterbodies (Hanlon & Parris 2014).

Carbaryl-based pesticides like Sevin® were found to be particularly devastating to *H. versicolor* tadpoles, with 1ppm of carbaryl causing 60% mortality after only 6 days (Relyea & Mills, 2001). These effects were worsened by 2-4 times in the presence of predatory stressors, as even at 0.07ppm predatory presence yielded over 40% mortality after 12 days (Relyea & Mills, 2001). However, without predatory stressors, carbaryl was not found to reduce *H. versicolor* tadpole survivorship even at 0.54ppm. At 0.5ppm, carbaryl was found to significantly slow the hatching time in *L. catesbeiana* embryos (Puglis & Boone, 2007). Since the study area is not a controlled environment, predatory presence is unpredictable, so the lowest disruptive concentration may be higher than 0.07ppm, though 0.07ppm should remain as the lowest level of concern (Table 1).

Literature Gaps

There is a severe lack of literature regarding the Eastern Spadefoot, both in general and in regard to chemical interactions. No articles could be located discussing their interactions with any target pesticides. Despite the Eastern Spadefoot's wide habitat range and local abundance, they are likely not as well studied because of their preference to remain beneath the surface and explosive breeding patterns (only breed after severe rain events) which make them more difficult to collect in all life stages. Since Eastern Spadefoots spend so much of their time surrounded by soil, further research on the effects of toxic soil elements such as copper, lead, and pesticides on toad survival would be interesting.

The literature review results are summarized in Table 1 and Table 2.

APPLICATIONS

These results reveal major differences in literature availability for the target frog species. Previous research describing low concentration was not able to be consistently located for any of the frogs, so literature availability cannot be heavily weighted in selecting an indicator species (Table 1). However, this does not mean that generalized lowest concentrations of concern (depicted in Table 2) are invalid for use in determining useful pesticide detection thresholds. As such, the application of these results will vary between section.

To apply the results of this literature review to management in the Gambles Mill Eco-corridor, several items need to be established. First, current frog habitat needs to be established. This should include frog population estimations, locations of likely frog habitat, and understanding of frog behavior. Next, sources for pesticide run-off need to be identified. Then finally, best water testing locations should be described. The following sections will aim to do so in hopes of developing useful strategies for ecosystem management.

Estimating Frog Populations

To employ these results in the Gambles Mill Eco-Corridor and make use of frogs as indicator species, one may want to first establish frog population estimates. Determining frog occupation in the Eco-Corridor will allow managers to view whether frog populations are increasing or decreasing, revealing the ecosystem's on-going health.

For researchers to determine frog populations within a habitat, a variety of methods can be used. The most generally accepted method for determining frog populations is mark-recapture with statistical analysis, with frog toe clippings noted as the most common method for marking without decreased survivability (Ackleh et al., 2012; Ginnan et al., 2014). There are multiple methods for estimating populations using mark-recapture, though for this population, Schnabel mark-recapture methods should be followed. This would allow for the most accurate results, which is necessary to observe population changes over time. Lincoln-Peterson methods may also be used, though they tend to overestimate population size by only using two days of capture data (Olmos Alcoy, 2013). While frog populations are not guaranteed to be closed for these mark-recapture studies, it's doubtful that a significant amount of migration would occur during a survey period due to the short time-frame, abundance of water sources, and presence of available frog habitat in the Eco-Corridor. In addition to abundance, frog body length, body mass, and presence of deformities should also be noted (Simon et al., 2010). These factors can help indicate increasing or decreasing presence of pollutants such as pesticides. For marking the frogs, toe-clipping is a proven safe method, as is use of fluorescent elastomers.

Population estimates are also not always required. If the goal is to compare a target ecosystem with other habitats in the area, presence or lack thereof is sufficient. For estimating species richness, sound loggers provide a valuable resource by recording frog calls to identify which species are present in an ecosystem, but this method is not as widely esteemed for estimating populations (Čeirāns et al., 2020). Richness estimates would allow managers to compare their study area with others in terms of species richness.

It's important to note that while frogs may spend most of their time in one area, they can travel distances sometimes up to nearly 2000 meters to reach breeding ponds (Kovar et al., 2009; Marsh et al., 2000). Many male frogs will exhibit breeding site fidelity, emphasizing the use case for confined population estimates (Blaustein et al., 1994). As well, the presence of conspecifics at a site is important for the continued recruitment of new individuals, so understanding current population dynamics is vital for predicting how populations may change over time (Buxton et al., 2015).

Current Frog Habitat

To apply scientific literature to a specific habitat, as many similarities need to be established as possible. Here, choosing four diverse frog species with known or assumed ranges in the University of Richmond Eco-Corridor allowed for a well-rounded basis of information to amplify site specific applications. Information from the Virginia Herpetological Society (Virginia Herpetological Society, n.d.) and scientific literature were used to provide insight into each of these species seen below, with photo references provided at the end. Figure 4 depicts areas of the Eco-Corridor referenced here:

1. Gray Treefrog - *Hyla versicolor* (Photo Reference 1):

Gray treefrogs are small, nocturnal, and arboreal. The current distribution of *H. versicolor* is highly disputed, as the identifying factors between Gray Treefrogs and Cope's Gray Treefrogs (*Hyla chrysoscelis*) are very similar, though there are believed to be *H. versicolor* groups found throughout the midwestern, southern, and eastern portions of the United States. Gray Treefrog tadpoles are believed to have been spotted in the University of Richmond Eco-Corridor as recent as the Fall of 2020, making this species highly relevant to the focus area. Gray Treefrog eggs are laid in water.

Since *H. versicolor* is a small nocturnal arboreal frog, doing traditional mark-recapture studies can be difficult. One recommendation is to conduct mark-recapture when temperatures begin to cool, since *H. versicolor* begins to move closer to the ground in preparation for overwintering (Johnson et al., 2008). This would allow for easier capturing, as *H. versicolor* specimen would occur more frequently at reachable heights. Sampling can also be conducted during the day (when they are inactive) to increase ease of capture. Artificial pipe refugia could be created as well to allow for consistent capture locations within the study area (Johnson et al., 2008). Another option would be to conduct surveying at night and have researchers divide the study area into quadrants, using gray treefrog calls to pinpoint frog locations for capture (Pham et al., 2007).

Gray Treefrogs surveying in the Eco-Corridor would be most successful within the forested area between the creek and walking path. However, the Gray Treefrog tadpoles were spotted by Dr. Kristine Graysen, a Biology professor at the University of Richmond, in the vernal pools and raingarden along the pathway. While tadpoles are great for knowing frog species presence, they provide little in terms of use for population estimating, as methods for tadpole mark-recapture are not as developed and many tadpoles may perish during pre-metamorphic life stages (Lima et al., 2018). Thus, adult Gray Treefrogs are better suited for mark-recapture population estimating.

2. <u>Spring Peeper - *Pseudacris crucifer* (Photo Reference 2):</u>

Spring Peepers are nocturnal, terrestrial frogs with the capabilities to climb trees, though they are more frequently found on the forest floor. The optimal habitat for Spring Peepers has been described as "moist, upland woods with shallow ponds," but they are also found in many wetlands at the source of streams. Spring Peepers are distributed throughout a majority of the eastern continental U.S.A. Spring Peepers are believed to have been heard in the University of Richmond Eco-Corridor over the summer of 2020, making them an appropriate species to consider. Spring Peeper eggs are laid in water.

Spring Peepers were also noted to have been present in the Gambles Mill Eco-Corridor, as Jennifer Sevin, visiting Biology lecturer at the University of Richmond recognized them by call in the Spring of 2020. While Spring Peepers are nocturnal like the Gray Treefrog, they do not spend most of their time in treetops and prefer to be under leaf litter on the forest floor. Thus, hand-collecting individuals for mark-recapture should not be difficult if collection is done at night using audio cues. Spring Peepers are also likely to be found in the forested area between the creek and the path. They may also be found in the wetlands further down the creek, surrounding the forested areas.

3. <u>American Bullfrog - *Lithobates catesbeiana* (Photo Reference 3):</u>

American Bullfrogs are a notably large species that spend most of their lives in warmer aquatic habitats where they can be found in pond shallows or along stream shorelines. While the native range of the American Bullfrog spans across most of the eastern USA and into the Great Plains region, they were introduced throughout the world for culinary purposes where they now run rampant as an invasive species. Despite being competitively dominant, American Bullfrog populations have been declining due to pollution, habitat loss, and pesticide introduction. American Bullfrog eggs are laid in water.

American Bullfrogs are highly mobile and can sometimes be difficult to capture. Since *L. catesbeiana* is an invasive species worldwide and in the Western USA, much research has gone into Bullfrog capture and removal methods. Some of these techniques can also work for population estimating. Multiple capture traps, which are essentially large cages with one-way plastic doors, were shown to be highly effective at capturing male bullfrogs. (Snow & Witmer, 2011). Capture for American Bullfrogs is recommended to take place in early fall or summer (Howell et al., 2020). For those interested in using multiple capture traps, they would likely have to be purchased or retrofitted from traps used to catch Cane Toads (*Rhinella marina*). Traps should be set out in the evening and checked in the morning to avoid desiccation of capture individuals. American Bullfrogs could also be hand-captured at night, though this method requires skill and can be difficult if the frogs cannot be spotted by sight or sound. Bullfrog tadpole populations can also be estimated using double fyke net capture methods (Louette et al., 2013). It is unknown if American Bullfrogs are currently in the Gambles Mill Eco-Corridor, but their likely habitat would be in the wetlands and floodplains, particularly along the creek edges and in vernal pools.

4. Eastern Spadefoot - *Scaphiopus holbrookii* (Photo Reference 4):

Eastern Spadefoots are a fossorial frog species with a wide range across most of the eastern coast of the USA. Eastern Spadefoots spend most of their life underground and breed explosively following major rain events, which can make them difficult to survey. These frogs will often return to the same burrowing areas for years on end. Of the frog species considered, this is the only species that can also be classified as a toad. Eastern Spadefoot eggs are laid in water.

The Eastern Spadefoot is much more difficult to survey. Few papers exist to discuss Eastern Spadefoot capture techniques and population estimation capabilities. One study analyzed Eastern Spadefoot burrowing substrate preferences in an urban context and found that while the toads could burrow in all substrates except sod, they have the easiest time burrowing in sand, followed by mixed soil (Jansen et al., 2001). The Eastern Spadefoot was also found to prefer soil substrates topped with forest leaf litter, though they do not seem to have a preference whether the soil exists in a forest or in clear cut land (Baughman & Todd, 2007). The best time to collect Eastern Spadefoot seems to be during the morning in periods after breeding when the metamorphoses are travelling from seasonal wetland (Baughman & Todd, 2007). Since the Eastern Spadefoot is an explosive breeder, only emerging to breed after heavy rain events, it's likely that they would be found on occasions like these. During heavy rain events, researchers should listen for the crow-like call of the Eastern Spadefoot to determine if breeding is likely to occur (*Virginia Herpetological Society*, n.d.). Spadefoot eggs are also laid underwater and would be difficult to see, so researchers should wait between 3-6 weeks after a major rain event so the toads will have hatched and metamorphosed to a terrestrial form (*Virginia Herpetological Society*, n.d.). Eastern Spadefoot breeding also depends on temperature, so this may occur at nearly any time of year depending on how moderate the temperature is. Realistically, Eastern Spadefoot population estimates would probably be near impossible without modelling, so researchers may just want to wait for happenstance to observe *S. holbrookii* presence in the Eco-Corridor. Eastern Spadefoots would likely find habitat in the forested areas nearest to the creek, where soil would be soft enough to burrow into and leaf litter would be present. It is unknown whether there are current Eastern Spadefoot populations in the Gambles Mill Eco-Corridor.

Recommended Indicator Species

Spring Peepers would likely be the best indicator species for ecosystem health in the Eco-Corridor because their presence is already known, they would be easiest to survey, and they are sensitive to atrazine at low ppm concentrations. Spring Peepers have also been previously tested as indicator species, with one study suggesting that they are the best indicator for a wide range of anthropogenic stressors (Price et al., 2007). As well, Spring Peepers tend to be the first frogs to start calling in the Spring (hence their name), which could be beneficial for managers by allowing them to sample for *P. crucifer* earlier than other frog species (Lovett, 2013).

Due to the variety in amphibians' evolutionary history, not all frogs are expected to interact the same way with potential stressors (Knutson et al., 1999). This means that, while presence of Spring Peepers (or any other amphibian) is a good sign of ecosystem health, there may be underlying issues that the target species just does not react to. This justifies the selection of *P. crucifer* as an indicator species despite a lack of literature availability for its interactions with carbaryl, and the findings of its apparent lack of interaction with glyphosate at very high concentrations (Table 1; Relyea, 2005). As such, mark-recapture study efforts in the Eco-Corridor should still prioritize obtaining Spring Peeper population estimates over the other proposed indicator species

American Bullfrogs could also work as an indicator species for the Eco-Corridor, though they may be more difficult to capture, are not previously known to be in the Eco-Corridor and are not considered to be as successful indicators when compared to populations in other wetlands (Price et al., 2007). However, American Bullfrogs could be good indicators for on-going pesticide levels, as they were shown to be sensitive to two of this study's target pesticides (Table 1). There is also a large amount of available literature on the American Bullfrog, so if it were selected as an indicator species and populations were declining, researchers may be able to apply previously studied concepts to discover the reasons for the declines.

Potential Pesticide Sources

Then, for evaluating the use of pesticides in the Gambles Mill Eco-Corridor, it is important to identify the main areas where pesticides could be coming from. Since this study focused on problematic low concentrations of pesticides, it's probably that all potential sources are valid to investigate. Below are the three most likely regions where pesticides could come from. These regions all make up parts of the local watershed for the Little Westham Creek (Figure 2):

1. The County Club of Virginia – Westhampton:

Located directly adjacent to the Gambles Mill Eco-Corridor sits the Country Club of Virginia (CCV), Westhampton location. This private country club includes amenities such as tennis courts, a club house, swimming pools, and most importantly a golf course (Figure 3). The 18-hole golf course features rolling hills and a pristinely kept turf, assumedly maintained through the use of pesticides and fertilizers. While no listing of the specific chemicals used at the CCV could be found, the Virginia Department of Agriculture and Consumer Services (VDACS) maintains a public document describing the types of pesticide permits acquired by public businesses in the state of Virginia (Virginia Department of Agriculture (VDACS), n.d.). On this list, the CCV is permitted to use class 3A, 3B, and 6 pesticides (*Virginia Department of Agriculture and Consumer Services Commercial Certified Pesticide Applicators*, 2020). Class 3A covers pesticide use on ornamental plants (both indoors and outdoors), class 3B covers pesticide use on turf, and class 6 covers pesticide use to maintain public rights-of-way such as sidewalks and roads (Lee, n.d.). Unfortunately, the VDACS does not appear to distinguish which chemical-bases can be used for which class, and states that around 14,000 new pesticides products are registered in their system each year, meaning there is seemingly little to no way to determine which pesticides are being used at the CCV without firsthand knowledge or testing (Virginia Department of Agriculture (VDACS), n.d.).

As well, in 2012 the Virginia Golf Course Superintendents Association prepared a guide on best environmental management practices for golf courses in Virginia (Virginia Golf Course Superintendents Association, 2012). This manual is non-binding, indicating that golf courses are not necessarily required to follow its recommendations. However, in the manual, the use of Integrated Pest Management (IPM) is encouraged. IPM is a type of pest management that combines pre-emptive planning, biological controls, and physical barriers to reduce reliance on chemical pesticides in pest reduction, though chemicals are still often used as a last resort (*How to Prevent Water Contamination – Pesticide Environmental Stewardship*, n.d.). While this is great for the state of Virginia, there is no indication in this manual or on the CCV website that the CCV follows these guidelines, so this guide is not necessarily helpful here. However, this association could be contacted by a more official correspondent in the future, with hopes of ascertaining information about the CCV's pest control methods.

Based on available information, it still remains unclear whether pesticide runoff from the CCV would be an issue. One study estimated that only .5% of applied carbaryl runs off from golf course fairways, which translates to approximately 191 gallons/hectare per year (Haith & Duffany, 2007). This same paper also stated that it was unlikely that pesticide runoff from golf courses is a frequent occurrence, as the application of these pesticides would need to coincide with rain events to yield significant levels of runoff (Haith & Duffany, 2007). Since atrazine is noted to be commonly used on golf courses in the Southern United States, the use of atrazine would not be surprising at the CCV. If atrazine is detected in surface water near CCV, this could be problematic since studies have shown that surface water could receive a up to 75% of applied atrazine over 70 days following the first post-application rainfall event (Ng & Clegg, 1997; Wang et al., 2018). In fact, these specific results are for areas with loamy soils similar to those in the Eco-Corridor (George, 2019). As well, due to its long half-life of 168 days in water exposed to sunlight, multiple applications of atrazine throughout a single year could lead to drastic bioaccumulation (Farruggia, 2016). Thus, leaching and runoff amounts, while small, may still have an impact on the Gambles Mill Eco-Corridor. Unfortunately, the main issue still stands that there is no easy way to determine which pesticides are currently in use at the CCV.

2. <u>University of Richmond Campus:</u>

Like the Country Club of Virginia, the University of Richmond (UR) also likes to maintain a well-trimmed and weed-free lawn. Luckily, the university's methods for keeping pest species away is readily available. On the UR Facilities' website, there is the university's integrated pest management (IPM) plan that details specific approaches and pesticides used by the groundskeepers (Sandman, 2019). UR's IPM plan is reviewed annually and includes pest-specific control techniques to minimize pesticide application (Sandman, 2019).

While IPM is great alternative to non-descript pesticide application, it does still include some chemical pesticide use. In fact, the University of Richmond's IPM even includes one of this study's target pesticides. When broad-leafed or grassy weeds are located in sidewalk cracks, patios, or curbs and cannot be removed manually, spot treatment is recommended using a mixture of 2.6 ounces of glyphosate 41% (Roundup® strength), 0.55 ounces of prodiamine 65%, and one gallon of water (Sandman, 2019). When converted, this formula would end up including 19494ppm of glyphosate and 4123.74ppm of prodiamine. However, it's extremely unlikely that the entire gallon mixture would be applied, plus most of it would be broken down in the soil. Soil type, pesticide makeup, and soil arrangement are just a few of the factors that can increase or decrease pesticide runoff, and since soil compaction and makeup differs between areas of the UR campus, an exact amount of runoff would be unknown without testing (Briceño et al., 2007). Since the recommendation is for spot treatment, one can hypothetically imagine that the amount used at once would be very low, let's say 0.5% of the total gallon (though it would likely be less in actuality). This translates to 97.47ppm. If then, basing calculations off Haith & Duffany (2007) who said that 0.5% of carbaryl would runoff from golf courses, that would be of 0.5% of the 97.47ppm (that is, if carbaryl and glyphosate ran off at the same rate between golf course turf and the campus lawn). This would be approximately 0.48735ppm. So hypothetically, 0.48735ppm of the total mixture would end up as run off per spot treatment. That's a very small amount, especially when considering how diluted it would become in a body of water the size of Westhampton Lake or Little Westham Creek. However, if pesticides were applied elsewhere in large quantities or frequently, it seems clear how pesticide use could become an issue when compared to the disruptive concentrations revealed in this study.

In all likelihood, the use of IPM on the UR campus does help reduce pesticide usage to levels much lower than would otherwise be needed to maintain a campus so large (350 acres). As well, IPM does not prioritize chemical pesticide application, so the realistic amount of run off from the UR campus is likely not high. However, if pesticides are applied before storm events, some pesticide could still run off into the Westhampton Lake, which connects to the Little Westham Creek in the Eco-Corridor. As well, in the UR IPM guide, it states that facilities workers will sweep any pre-emergent pesticide pellets off paved paths when application is finished, but this does not guarantee that some pellets will not still run off from their application area (Sandman, 2019). Thus, the risk of pesticides leaching or running off and eventually reaching the Eco-Corridor should not completely ignored.

3. Surrounding Neighborhoods:

The greater Little Westham Creek watershed also expands into the neighborhoods that surround the University of Richmond as well (Figure 2). The main neighborhood in the watershed is Westham, Virginia, a subsection of Tuckahoe, Virginia. This area has an average income of around $77,000, and the homes here typically have well-kept lawns (*Tuckahoe, VA | Data USA*, n.d.; observation). Because there are so many homes to consider in this area, and so many pesticides available, it's impossible to know which pesticides would be in use if any. As well, it's likely that some of these homeowners do not tend to their own lawns, and instead hire a lawn care surface to do that for them, adding another layer of mystery. It's also more likely that these households follow less strict guidelines when apply pesticides due to lack of knowledge. Yet, pesticide use in these homes likely does still have an impact. Because the area surrounding the University of Richmond campus forms a basin leading towards Westhampton Lake, any pesticides that run off will run-off straight to the Little Westham Creek's source. As well, the Westham neighborhood has wide roads with houses relatively close together, meaning there is a significant amount of impermeable surfaces. While there is storm water drainage infrastructure in-place in this neighborhood, not all rainwater enters this system. Some will run off directly into Westhampton Lake and eventually move into Little Westham Creek, especially on roads adjacent to the lake or creek. The type of grass on a lawn also plays a role. Some grasses are more absorptive, more drought-resistant, or more hardy than other grasses. Depending on the type of grass planted on a lawn, there may be no need for pesticides on a majority of these homes' lawns, keeping pesticide use restricted to homes with installations like gardens. However, because there is no efficient way to determine the amount pesticides in use or the amount of pesticides that could run off from lawns, the surrounding neighborhoods should not be ruled out as sources for pesticides in the Eco-Corridor.

Checking for Pesticides

Lastly comes the actual identification of pesticides in the Eco-Corridor. The goal of this study is to use frogs as indicator species to denote ecosystem health, so managers should want to see if the target pesticides are present and impacting frog fitness in the Eco-Corridor. If the pesticides were present at their LC50 levels, no frog species would be present to use as indicators, but since *H. versicolor* and *P. crucifer* are known to be present in the Eco-Corridor, the LC50 isn't currently relevant to the study area. As shown above, testing is likely the only way to determine which pesticides could be in Eco-Corridor water. Other options like sending questionnaires to the surrounding neighborhoods would probably yield low responses and be difficult to implement. For this study, the CCV was attempted to be contacted, but no email address was available on their website, and phone calling was unsuccessful. To reiterate, pesticide testing has not yet been done in the Eco-Corridor, so the true presence of pesticides is currently only speculative. Test kits also do not give exact concentrations. Instead, they can be ordered to have a specific threshold for indicating presence. For example, a water body may have a concentration of 0.002 ppm, but if the threshold for the test kit is 0.005ppm, it will not indicate presence. Thus, the results of this study can be used to justify acquiring low threshold test kits to use in the Eco-Corridor. To determine where to test, a combination of probable frog habitat with potential pesticide sources can be used. Below are these locations, with visual references in Figure 4:

1. Where to Test

As mentioned before, atrazine is one of the most commonly used herbicides in agriculture and golf course settings throughout the Southeast United States (US EPA, 2015). This makes atrazine application a strong candidate for the Country Club of Virginia's preferred turf maintenance technique. The Gambles Mill Eco-Corridor is located directly downhill from the CCV, so runoff from the turf is likely to travel towards the Eco-Corridor. However, in the Eco-Corridor, there sits a stormwater raingarden which, according to Bob Siegfried II of RES, is entirely fed by the CCV. Pesticide testing should definitely be conducted within the storm water raingarden, as this area would be most indicative of the CCV's pesticide usage and it provides probable frog egg laying habitat due to its relative consistency and shallow depths (Figure 4).

Another newly altered waterway in the Eco-Corridor includes the step pools. These step pools were introduced in the Eco-Corridor as a method to reduce the energy flow of water entering Little Westham Creek (Kirkpatrick, 2019). Due to the high flow rate of the water passing through these step pools, it is unlikely that any frogs would be able to safely lay eggs in these waters (B. Siegfried, personal communication). However, one of the step pool paths flows directly down from the Country Club of Virginia (Figure 4). This means that while pesticide contamination in these pools is not of particular concern for this study, the chemicals that flow through the step pools may reach the Little Westham Creek. So, testing should be done at the highest pool in the area and at the lowest pool in the area to determine if pesticides are present in the step pools and compare the step pools' filtration capabilities.

With the renovation of the Gambles Mill Eco-Corridor also came improved stormwater management. As the streambed was reformed, the creek's flow was slowed down which allows for increased sediment deposit into the creek bottom, which in turn filters out unwanted materials (Singh et al., 2018). The Little Westham Creek restoration also allows for greater flood mitigation in part due to floodplain connectivity and the moderate to high permeability of the surrounding loamy soil (George, 2019). Due to the reduced water flow speed associated with the restoration project, it's likely that many more pesticides will remain suspended in the creek or sink to the underlying substrate. This may both increase the amount of pesticides within the creek (by reducing the amount that flows straight through to the James River), while also beneficially increasing the overall spread of suspended pesticides, which may increase the rate at which micro-organisms can break them down (*Environ. Sci. Technol*, 2011). Thus, target pesticide testing should be conducted at the mouth of the creek (where the water flows down from Westhampton Lake), and at the end of the creek (where it is backwatered to the connecting canal) (Figure 4). This will allow for an analysis of the stream's pesticide filtration and distribution effectiveness. However, it is unlikely that any frogs would lay their eggs in these deeper running waters, so tests should also be conducted in the connecting floodplains and wetlands when vernal pools are present.

2. Difficulties with Testing

As demonstrated by this study, pesticide testing in the Gambles Mill Eco-Corridor is vital for determining ecosystem health and for establishing baselines to use for future changes. However, water quality testing is not an all-telling process. In many cases, pesticides do not work alone to reduce ecosystem well-being. Often, pesticides will interact with other elements in the water to create additional positive and negative effects. For example, one study suggested that the pesticide Roundup® (glyphosate based) may actually lengthen the lifespan of *H. versicolor* tadpoles by inhibiting the growth of *Batrachochytrium dendrobatidis* (chytrid) fungus (Hanlon & Parris, 2014). However, another study found that a mixture of atrazine, carbaryl, and glyphosate did not have this same effect on chytrid fungus exposure or viral load for *P. crucifer,* demonstrating how interactions vary between both pesticides and frogs (Jones et al., 2017). As well, some pesticides actually have a non-monotonic impact on frogs, meaning that pesticides found to be highly toxic at low concentrations may become less toxic as their concentration increases (Storrs & Kiesecker, 2004). Thus, it seems clear that presence testing for pesticides at both high and low concentrations is not perfect, but that's why indicator species like frogs can be used to tell the true story of ecosystem health.

Study Challenges

This study faced many difficulties during its working process. For one, species specific knowledge was limited in terms of literature availability. While there is a lot of scientific and governmental research on pesticides, few studies approach the topic specifically through the lens of amphibian species, and much fewer do so through the lens of the four species selected for this study. Often, multiple studies on a species would be conducted by the same person, reducing the variety in knowledge sources. As well, it is important to keep in mind that these tests occurred in lab settings, so it is difficult to apply them to real-world scenarios with confidence. Some researchers dislike controlled setting reports because they typically apply the chemicals all at once, not reflecting the slow accumulation that occurs in natural environments (*Measures of*

Toxicity, n.d.). In retrospect, the selection of target frog species was also both a hinderance and a benefit. Setting explicit parameters for literature studies helps narrow the viewpoint and increase site specific applicability, but it also greatly reduces the amount of usable research, constraining study capabilities. As displayed in Table 1, there is already a great lack of available low concentration pesticide studies, further increasing difficulty for this study.

Another major blockade for this study was the inability to conduct in-person field research. Preferably, water quality testing and frog surveying would have been carried out during the Fall 2020 semester. However, this study was limited by a lack of prior knowledge about pesticide detection methodology, lack of time, poor time management, and lack of funding to purchase specific test equipment. Also, frog species were not able to be surveyed during the study time frame due to overwintering, a process in which the frogs bury themselves underground during the colder months to help maintain homeostasis. This also meant that no acoustic presence surveys could be done as frogs only call during the warmer spring, summer, and early autumn months. In future studies, field data collection of both frog and pesticide presence should be conducted.

CONCLUSION

This study has hopefully provided an adequate framework and focus for future researchers to conduct population estimations and pesticide prevalence tests in the Gambles Mill Eco-Corridor. Overall, this study has revealed that impactful concentrations of pesticides for frogs are low, so sensitive test kits are needed. As well, feasible pesticide sources have been shown to exist for the Gambles Mill Eco-Corridor, with run off likely stemming from the Country Club of Virginia and surrounding neighborhoods. Furthermore, there do seem to be viable indicator species present in the Eco-Corridor, though there is a lack of consistent literature documenting their reactions to low concentration pesticides. Yet together, these pieces can be used to guide managers and researchers in ensuring that the Eco-Corridor remains a hub for biodiversity, and model of ecosystem health for years to come.

FIGURES AND TABLES

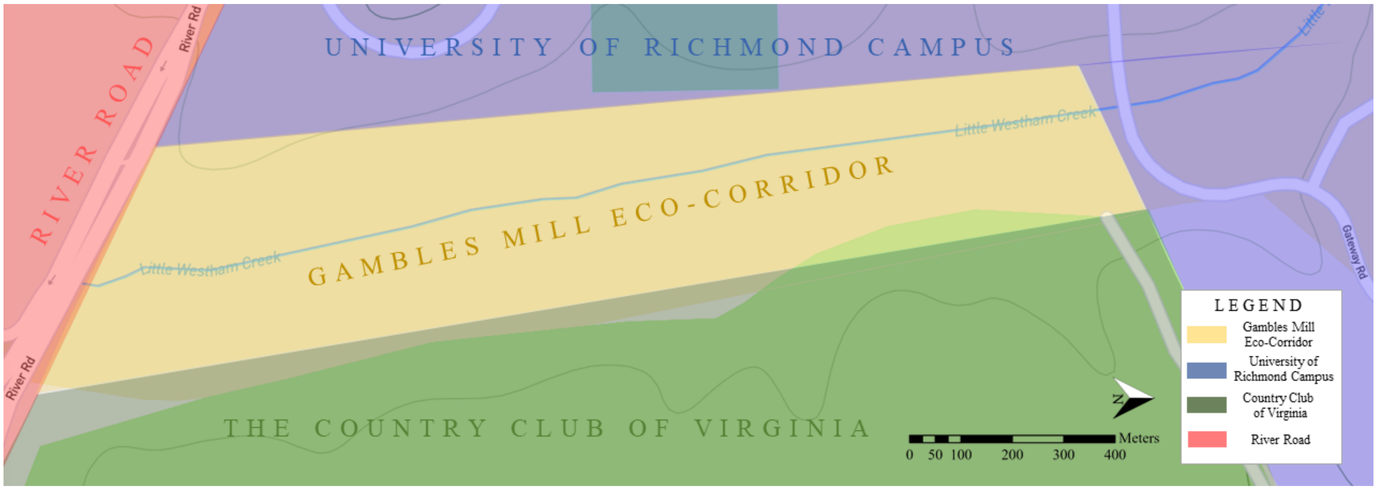

Figure 1: Map depicting the Gambles Mill Eco-Corridor and its three surrounding areas. The University of Richmond campus is to the north and west, the Country Club of Virginia is to the east, and River Road is to the south.

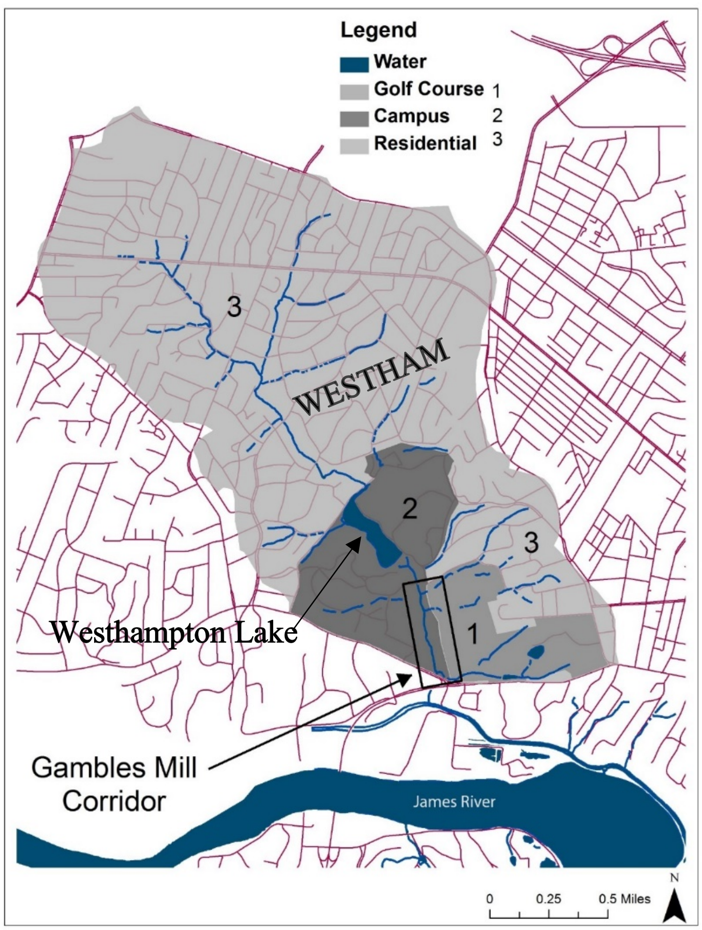

Figure 2: Map depicting the 8.4 km² Little Westham Creek Watershed. The Gambles Mill Eco-Corridor, Westhampton Lake, and Westham neighborhood are highlighted. Some annotations were added for this project. Map sourced from the University of Richmond Office of Sustainability Storymap: [*https://www.arcgis.com/apps/MapSeries/index.html?appid=2a4bc41dbe444157a1bb1ece4687b49a*](https://www.arcgis.com/apps/MapSeries/index.html?appid=2a4bc41dbe444157a1bb1ece4687b49a)

Figure 3: Map of the Country Club of Virginia (CCV)compound. This depicts the 18-hole golf course, tennis courts, pools, and other amenities. The direct border with the Gambles Mill Eco-Corridor can be seen along the western edge (left). Map sourced from the CCV website: https://www.theccv.org/documents/10184/12561/WH+Golf+Course+Illustration.pdf

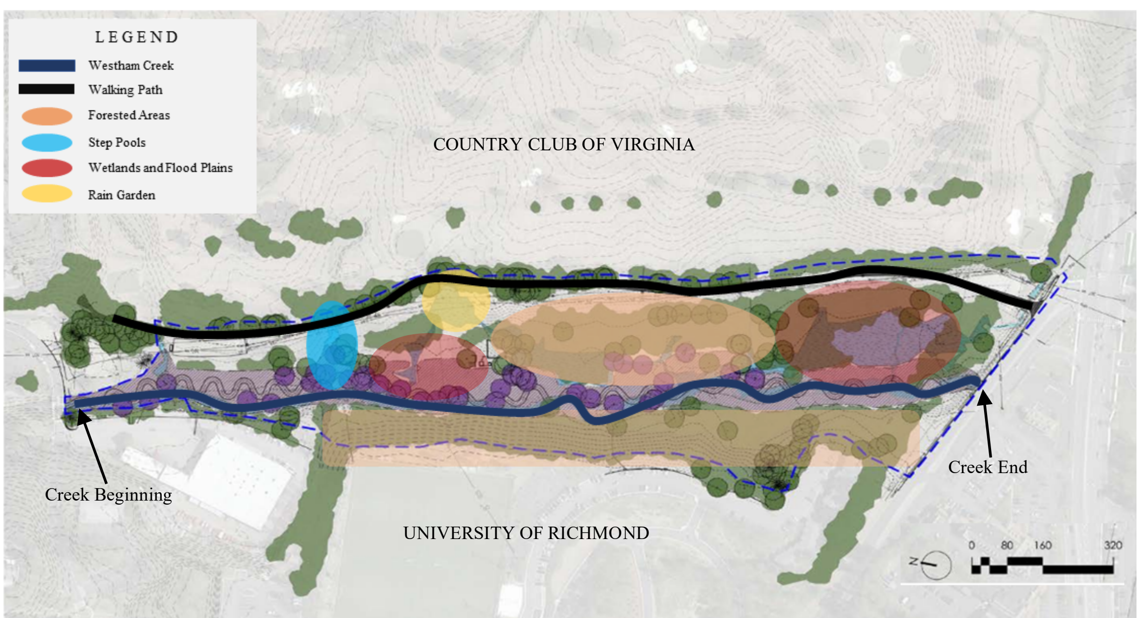

Figure 4: Map depicting the different areas of the Gambles Mill Eco-Corridor referenced throughout this paper. As no updated satellite imagery was available, all locations are approximate. Annotations were added for the purpose of this study. This map base is sourced from Water Street Studio: https://waterstreetstudio.net/work/u-of-r-gambles-mill-eco-corridor

Table 1: Results of the literature review, summarized by target frog species. N/A indicates that no studies could be located for that species and pesticide.

	Lowest Concentrations of Concern (ppm)		
	Atrazine	*Glyphosates*	*Carbaryl*
H. versicolor	1.25	N/A	0.07
P. crucifer	0.003	No Effect at 3.8	N/A
L. catesbeiana	0.0025	0.018	0.5
S. holbrookii	N/A	N/A	N/A

Table 2: Results of the literature review, summarized by chemical, lowest concentration of concern for that chemical, and the name of the study from which the results were taken.

Lowest Concentrations of Concern		
Chemical	*Concentration (ppm)*	*Study Name*
Atrazine	0.0025	Dornelles & Oliveira 2016
Glyphosates	0.018	Dornelles & Oliveira 2016
Carbaryl	0.07	Relyea & Mill 2001

PHOTO REFERENCES

Photo 1 - Gray Treefrog (Hyla versicolor)
https://fw.ky.gov/Wildlife/PublishingImages/easterngraytreefrog2.jpg

Photo 2 - Spring Peeper (Pseudacris crucifer)
https://cottagelife.com/wp-content/uploads/2019/05/shutterstock_413167021.jpg

Photo 3 - American Bullfrog (Lithobates catesbeianus)
https://upload.wikimedia.org/wikipedia/commons/a/aa/North-American-bullfrog1.jpg

Photo 4 - Eastern Spadefoot (Scaphiopus holbrookii)
https://fw.ky.gov/Wildlife/PublishingImages/Easternspadefoot2.jpg

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