Ammonium, nitrate, and proton flux in fern gametophytes

By

EM CREASY

B.Sc. University of Victoria, 2024

A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF SCIENCE (HONS.)

in the Department of

BIOLOGY,

UNIVERSITY OF VICTORIA

Supervisors

Dr. Barbara Hawkins
(Department of Biology, University of Victoria)

Dr. Patrick von Aderkas
(Department of Biology, University of Victoria)

External examiner

Andy MacKinnon
(Retired)

Table of Contents

List of Tables

List of Figures

Acknowledgements

Heartfelt thanks to Samantha Robbins, Daisuke Yamakawa, and Dr. Barbara Hawkins for their MIFE expertise and troubleshooting help, and to Dr. Patrick von Aderkas, Alistair Knox, Kailey Strachan, Simar, Liberty, Olivia, and Jadyn for their incredible fern parenting skills and endless support.

Abstract

Total net NH_4^+, NO_3^-, and H^+ fluxes were investigated in three species of fern gametophytes: *Polystichum munitum*, *Christella dentata*, and *Cyrtonium falcatum*. Flux measurements were performed with a microelectrode ion flux measurement (MIFE) system. Overall, net NH_4^+ influx was seen in all gametophytes. Net NO_3^- efflux and net H^+ influx was seen in most gametophytes. The gametophyte generation of *C. dentata* and *C. falcatum* displayed greater NO_3^- efflux than the sporophyte generation. On average, male *P. munitum* gametophytes displayed greater per-unit-area NH_4^+ uptake than female/hermaphrodite gametophytes, though the latter showed greater NH_4^+ uptake at the whole-organism level. This finding suggests that fern gametophytes may conform to the expectations of nutrient-based sex determination. This study was the first to perform precise ion flux measurements at discrete positions on fern gametophytes and sporophytes. This research provides greater insight into the nutrient ecology of fern gametophytes, an under-studied field.

Chapter 1. Introduction

1.1 Fern lifecycle and reproduction

Life cycle

The life cycle of all vascular plants alternates between two generations or phases: the diploid sporophytic phase and the haploid gametophytic phase (Banks, 1999). In land plants other than bryophytes, the sporophytic phase includes the larger, asexual, spore-producing plant, i.e. the sporophyte. The gametophytic stage is the ephemeral, sexual, gamete-producing plant, i.e. the gametophyte. Unlike any other extant land plants, ferns exhibit independent generations in their life cycle; thus, the haploid gametophyte and diploid sporophyte are completely free-living. The phenomenon of independent alternation of generations marks an evolutionary transition from the gametophyte-dependent sporophyte seen in bryophytes to the sporophyte-dependent gametophyte in seed-bearing plants (Kinosan & Wolf, 2022). As the most primitive vascular plants, ferns share characteristics of both bryophytes and seed-bearing plants. Their unique evolutionary position enables fern gametophytes to be useful experimental systems for understanding plant development (Banks, 1999). Because their gametophytic and sporophytic phases are independent, the specific mechanisms underlying their separate growth and development can be studied. Much remains unknown of the functions unique to the gametophyte, including sex determination. The ease of studying fern gametophytes in a non-destructive manner is a major reason why ferns are excellent study organisms. Additionally, their shared

characteristics with both more primitive and more advanced plants suggests that these mechanisms may be applicable to other plant clades.

The fern life cycle begins with the single-celled haploid spore, produced by meiosis within the sporophyte plant. Dormant spores, which can remain viable for decades, eventually break dormancy and germinate with adequate moisture, temperature, and light (Miller, 1968). These processes are comparable to those in angiosperm seeds, suggesting that they may be regulated by similar mechanisms and require the synthesis of like proteins and enzymes (Banks, 1999). The rupturing of the fern spore during germination results in a first asymmetric division of the nucleus, comparable to that seen in the angiosperm microspore, followed by further divisions that result in a rhizoid initial and a protonemal initial (Banks,1999). The rhizoid initial is a single cell that functions in nutrient absorption and anchorage. The protonema initial ultimately forms a photosynthetic prothallus that is a single cell-layer thick at maturity (Banks, 1999).

Gametangia, the reproductive structures, develop from the lateral meristem initiated by the prothallus. The entire developmental process does not take long: in the model fern *Ceratopteris richardii*, the hermaphroditic gametophyte exhibits the characteristic heart-shape with many rhizoids and gametangia after only two weeks (Banks, 1999). During fertilization, water facilitates the movement of sperm from the antheridia to the archegonium, the multicellular flask-shaped structure that houses the egg. Successful fertilization results in the development of a diploid zygote and the termination of meristematic division of the gametophyte. Finally, the development of the sporophyte results in the death of the gametophyte (Banks, 1999).

Asexual modes of reproduction

Apogamy is an asexual method of reproduction found in approximately 10% of fern species (Walker, 1985). In apogamy, the sporophyte develops from the gametophyte without fertilization. Apogamous sporophytes show differences in development compared to fertilized sporophytes: they mature faster than their fertilized counterparts, and generally develop a leaf prior to roots (the opposite is observed in fertilized ferns). Due to their rapid growth and no need for water to facilitate fertilization, apogamous ferns have a competitive advantage in dry regions with short growing seasons (Sharpe & Mehltreter, 2010).

Both fern gametophytes and sporophytes can persist indefinitely through vegetative growth. Vegetative propagules, known as gemmae, can disperse and produce a new gametophyte thallus. Gemmae consist of small strings and plates of cells that are produced by the parent thallus. At maturity, they abscise from the parent and can be dispersed by wind, animals, water, and/or gravity (Farrar et al., 2008). Gemmae-producing gametophytes have strap-shaped and ribbon-like morphologies. The cordiform fern gametophytes used in this study do not reproduce vegetatively (Farrar et al., 2008). Cordiform gametophytes are characterized by their butterfly or heart-like shape and an annual life cycle. At maturity, these gametophytes have a single apical meristem within an apical notch. Most Polypodiales are cordiform (Farrar et al., 2008).

1.2 Sex differentiation in ferns

Many fern species produce sexually dimorphic gametophytes: small male gametophytes with antheridia, or larger hermaphroditic and female gametophytes with archegonia (Banks, 1999). Additionally, most fern species are homosporous, meaning they produce one type of spore. In homosporous ferns, the sex of the gametophyte is determined post-spore germination by external factors. Mainly studied in angiosperms, this phenomenon is called environmental sex determination (ESD) and is affected by factors such as local mate competition, resource availability, and sex-specific mortality (Charnov & Bull, 1977). Two models exist for the dominant form of ESD in angiosperms - sexual lability - where sex change occurs bidirectionally. Though sex change occurs unidirectionally in fern gametophytes, the principles of these models are still pertinent. In the ‘patchy environment model’, female to male/hermaphrodite sex change occurs due to stress-inducing conditions, including shading, drought, and cold. In the ‘size advantage model’, sex change occurs based on size, where larger individuals become female and smaller ones become male, due to the higher resource investment needed to produce seeds compared to pollen (Charnov & Bull, 1977). These theories can be applied to fern gametophytes to understand how sex is differentiated. For example, unfavourable conditions such as crowding and nutrient unavailability may result in male gametophytes due to fewer resources needed to produce sperm compared to eggs.

Light is a major factor regulating gametophytic growth and development (Banks, 1999; Farrar et al., 2008). Cordiform gametophytes, like all those included in this study, are adapted to short-lived habitats produced by small-scale natural disturbance on the forest floor; these conditions promote access to light and reduced competition (Farrar et

al., 2008). A study by DeSoto et al. (2008) found that low nutrient availability and crowding resulted in *Woodwardia radicans* gametophytes developing as small males, a result compatible with ESD. However, the extent to which environmental factors such as light intensity, temperature, and available soil nutrients influence gametophytic sex expression remains poorly understood for the majority of fern species (Sharpe & Mehltreter, 2010).

Of all factors involved in ESD, antheridiogens have received the most attention (DeSoto et al., 2008). Antheridiogens are gibberellin-like pheromones that are synthesized and secreted by hermaphroditic and female gametophytes, inducing male development in surrounding immature gametophytes (Banks, 1999). The effect of the antheridiogen extends to 20 cm around the mature female gametophyte, and can affect both its own species and others with chemically similar antheridiogens (Schneller, 2008). Antheridiogens suppress the development of the lateral meristem in males, resulting in a multicellular single-layered gametophyte (Banks, 1999). Fertilization of the female gametophyte halts antheridiogen production, resulting in nearby male gametophytes developing archegonia and becoming hermaphroditic (Schneller, 2008). However, this effect may be age-dependent; for example, in *Ceratopteris richardii*, the gametophyte loses its ability to respond to antheridiogens after approximately five days (Banks, 1999). While the presence of antheridiogens has not yet been investigated in many fern species, including *Polystichum munitum*, other ESD impacts can occur in conjunction with or in absence of this pheromonal effect.

1.3 Gametophyte resource acquisition

Fern gametophytes are capable of living for extended periods of time (i.e. years) if they are not fertilized and disturbance is limited. They can grow in areas where fern sporophytes cannot, and tolerate higher stress than sporophytes (Farrar et al., 2008). In contrast to sporophytes, gametophytes have neither xylem, nor effective waxy cuticles, nor stomata: instead, they rely on the ability to tolerate and recover from fluctuations in water availability (Farrar et al., 2008). Nutrient uptake occurs over the entire gametophytic tissue surface, and unlike in sporophytes, rhizoids are not directly involved in nutrient uptake (Richardson & Walker, 2010). Thus, in terms of physiology the fern gametophyte is more comparable to a bryophyte, whereas the vascular tissue-containing sporophyte is more comparable to seed plants (Farrar et al., 2008).

While the effect of nutrient availability on fern sex determination is poorly studied, the literature demonstrates a trend of unfavourable growth conditions, such as poor substrate or high density, resulting in a higher ratio of small, male gametophytes to large female ones (Miller, 1968; DeSoto et al., 2008). However, if male gametophytes are moved to favourable growth conditions, they will differentiate both types of sex organs (Miller, 1968). The inability of fern gametophytes to acquire necessary amounts of essential mineral nutrients has been shown to limit prothallial growth and produce unisexual male plants or even completely sterile plants (Miller, 1968). Thus, it can be said that the accessibility of resources (both their availability and the gametophyte's ability to acquire them) has an impact on the development of fern reproductive structures. Ultimately, the literature suggests that there exists a direct link between growth conditions, including mineral nutrient access, and sex, and that female and

hermaphroditic gametophytes require more favourable conditions than males in order to mature. This suggests that archegonia/eggs are more expensive to produce and maintain than antheridia/sperm.

1.4 Plant nutrition

Mineral nutrients are essential to the successful growth, development, and metabolism of all plants. Plants require 14 essential nutrients, without which they cannot complete the vegetative or reproductive stage of their life cycle (Roy et al., 2006). Nitrogen is the essential mineral nutrient required in the greatest quantities. Composing 1-4% of plant tissues, nitrogen is required for the formation of chlorophyll and RuBisCo, as well as being a major component of both amino acids and nucleotides (Roy et al., 2006; Lewis, 1986; Turetsky, 2003). Additionally, nitrogen is involved in compounds critical to energy transfer and metabolism, i.e. adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD), respectively (Lewis, 1986). In temperate regions, nitrogen is considered the dominant limiting nutrient for plants. This phenomenon is due to the inability of plants to metabolize dinitrogen gas (N_2). The notable exceptions are those plants in symbiotic associations with nitrogen-fixing bacteria (von Wirén et al., 1997). Therefore, plants that do not form symbioses with bacteria that engage in N_2 fixation rely on the processes of mineralization, immobilization, remineralization, and sometimes nitrification to absorb bio-available nitrogen (Lewis, 1986).

Since nutrient limitation is one of the most significant factors affecting plant growth in natural ecosystems, it is expected that these conditions have exerted strong

selective pressure for organisms to evolve mechanisms to optimize growth under limiting conditions, including maximizing uptake and metabolism rates for that particular nutrient (Harder & Dijkhuizen, 1983). Ammonium (NH_4^+) and nitrate (NO_3^-) are the primary forms by which plants take up nitrogen due to their abundance and accessibility (von Wirén et al., 1997). Once assimilated, NO_3^- and nitrite (NO_2^-) are reduced (Turetsky, 2003). When both NO_3^- and NH_4^+ are available in equal amounts, plants generally preferentially take up NH_4^+. This selective uptake may be due to the lower energy requirement for cation assimilation (Reid & Hayes, 2003). Additionally, low pH has been shown to inhibit NO_3^- assimilation (Rudolph et al., 1993; Turetsky, 2003). The preference of NO_3^- versus NH_4^+ may also be determined by the plant's overall cation/anion balance. In addition to its inorganic form, nitrogen may be taken up as organic N (amino acids, peptides, etc) (von Wirén et al., 1997). These may be more prevalent in N-limiting environments such as forests and tundra, where mineralization rates of organic matter are low (von Wirén et al., 1997; Reid & Hayes, 2003). As with other nutrients, the uptake of NO_3^- and NH_4^+ is strongly downregulated if internal nitrogen levels are high in the plant. This regulation is likely effectuated by the downstream metabolites of NO_3^- and NH_4^+ rather than high concentrations of the ions themselves (Reid & Hayes, 2003).

In the nitrogen-limited environment of the Pacific Northwest (PNW), vegetation has evolved in response to moisture, temperature, and nutrient regimes. Since most decomposition and nutrient release from organic litter occurs during the wet, cool, dormant winter season, plants have evolved to accumulate nutrients during these conditions. Another unique aspect of the nutrient regimes of the PNW is that nitrogen

often becomes available through large episodic losses from infrequent but large-scale wildfires. Thus, plants must be adapted to survive low nutrient conditions and to conservatively use the nutrients they acquire (Waring & Franklin, 1979). Better understanding of the processes underlying nitrogen acquisition and flux in abundant understory species, such as ferns, is needed to understand their role in broader ecosystem nutrient cycling (Richardson & Walker, 2010).

1.5 Current knowledge of fern nutrient ecology

Ferns are capable of growing on soils that range greatly in levels of nutrient availability. They can colonize and survive in nutrient-poor, dry, and cold environments, from sand dunes, to tundra, to early-successional soils (Richardson & Walker, 2010). Through decomposition, ferns may contribute to the formation of soil organic matter and litter, bolstering ecosystem health. The resorption of nutrients affects plant fitness on nutrient-poor soils and can affect both soil quality and ecosystem nutrient cycling. A conclusion in the limited literature is that terrestrial ferns are able to maximize their diversity on nutrient-rich soils, but they make the greatest proportional contribution to biomass on nutrient-poor soils (Richardson & Walker, 2010).

The literature demonstrates mixed results in terms of the nutrient concentrations of ferns compared to seed plants. Some large-scale studies have found no difference in N concentration between ferns and seed plants, while others have concluded that ferns have lower P and N concentrations (Richardson & Walker, 2010). These discrepancies are likely the result of the broad scale of the studies, which do not control for differences in environment. Interestingly, ferns demonstrate low leaf calcium concentrations,

resulting in much higher N:Ca ratios than other vascular plants. This could be due to the fact that most fern species, except Polypodiaceae, may have evolved in cation-poor environments, leading to the evolution of cell wall structures with low cation content (Richardson & Walker, 2010; Amatangelo & Vitousek, 2008).

1.6 Nitrogen uptake in roots

Uptake of nitrogen in its various forms occurs primarily across the plasma membrane of root epidermal and cortex cells, notably in root hairs. However, uptake systems for mineral N forms are also present at the plasma membrane of leaf cells (von Wirén et al., 1997). Nutrient uptake is commonly associated with the action of H^+-ATPases in the plant plasma membrane, which establish electrical and proton gradients that drive the uptake of ions, including cations and anions (Reid & Hayes, 2003). There are three main types of transport mechanisms in the plasma membrane of plants: channels, carriers, and cotransporters. Nutrient cation uptake occurs via channels, and is passive, while nutrient anion uptake occurs via cotransport with H^+, and is active (Reid & Hayes, 2003).

In fern sporophytes, nutrient uptake occurs via root hairs that grow along the rhizome, the thickened, mostly subterranean shoot involved in anchorage (Richardson and Walker, 2010). There is no evidence that the rhizome is directly involved in nutrient uptake, though this phenomenon has been seen in a species of sedge (Brooker et al., 1999). Mycorrhizal symbioses, particularly endomycorrhizae, may play a role in nutrient acquisition for ferns in dry environments (Richardson and Walker, 2010).

1.7 Nitrogen uptake in gametophytes

Little research has focused on nitrogen uptake in gametophytes. The form in which nitrogen is supplied may influence the growth of the gametophyte. While some species can grow with ammonium as the sole nitrogen source, development is often abnormal with excessive filamentous growth and no two-dimensional development (Miller, 1968). Prior to this work, no studies have explored nitrogen or other mineral ion fluxes in fern gametophytes. However, a need has been expressed to better understand the dynamics underlying fern gametophyte nutrition, considering that this phase is essential to the development of the sporophyte (Farrar et al., 2008).

Recent work by Hawkins et al. (2017) used a microelectrode ion flux measurement (MIFE) system to measure inorganic N uptake rates and N form preferences in 21 common mosses from varying locations in British Columbia, Canada. The premise of the study was that unlike other land plants, mosses never evolved roots or vascular tissue, and thus water and nutrient uptake occur over the entire plant surface (Hawkins et al., 2017). Specifically, the majority of uptake occurs across the surface of the gametophore, rather than through the rhizoids (Turetsky, 2003). Therefore, the authors measured nitrogen-form uptake on the leafy tissue of each moss species using a MIFE system (Hawkins et al., 2017). The authors had success with the methods employed and significant differences among moss species corroborated by the literature were found. However, the authors noted that compared to MIFE experimentation on plant roots and mycorrhizae, overall NH_4^+ and NO_3^- uptake patterns were less consistent in moss gametophores (Hawkins et al., 2017).

1.8 Effect of pH on nutrient uptake and fern ecology

Soil pH affects nitrogen ion availability, uptake, and assimilation by plants (Hawkins & Robbins, 2010). Specifically, it influences the electrical potential difference between the plasma membrane and the surrounding environment (Hawkins & Robbins, 2010; Reid & Hayes, 2003). A negative surface potential is created by excess negative charges on phospholipids and the ionization of acidic side groups on membrane proteins. The surface potential is sensitive to pH: it becomes more negative with increasing pH due to the dissociation of these acidic amino acids. This phenomenon can affect the K_m of transport systems (Reid & Hayes, 2003).

Inorganic nutrient ions can move with, or in opposition to, H^+ flux across membranes, depending on whether they are positively or negatively charged (Hawkins & Robbins, 2010). The pH of the environment can thus directly affect nutrient uptake as it can influence the amount and activity of H^+-ATPase proteins, which are responsible for establishing the electrical and proton gradients that drive ion uptake (Reid & Hayes, 2003; Hawkins & Robbins, 2010). Plants excrete oppositely charged ions to compensate for those taken up in order to preserve electroneutrality in their tissues. It has been proven that H^+ is the compensating ion in the case of excess cation uptake and that there is a relationship between H^+ flux and NH_4^+/NO_3^- flux (Darrah, 1993).

Low pH has been demonstrated to inhibit NO_3^- assimilation (Rudolph et al., 1993; Turetsky, 2003). Hawkins and Robbins (2010) found that in *Glycine max*, *Pseudotsuga menziesii*, and *Pinus contorta*, mean net NO_3^- uptake was significantly greater at pH 7 than pH 4. In both *P. menziesii* and *P. contorta*, there was no significant effect of pH of

NH_4^+ uptake, but there was significant effect of pH on NH_4^+ flux at varying N concentrations (Hawkins & Robbins, 2010).

In fern gametophytes, a pH of 5-6 has been shown to be optimal for spore germination and gametophyte growth. However, the optimal pH for the gametophyte's physiological processes will be influenced by the habitat to which the fern is adapted (Miller, 1968).

1.9 Microelectrode ion flux measurement system (MIFE) overview

The MIFE™ system, developed in 1996 by Dr. Ian Newman at the University of Tasmania, is an effective approach to monitor and quantify ion fluxes in living plant and animal tissue (Newman, 2001). Originally called 'microelectrode ion flux estimation' (MIFE), the technology is now known as the 'microelectrode ion flux measurement system' but the acronym remains the same. The concept of using electrochemical potentials measured at the surface of plant tissues to calculate ion flux of the tissue was first proposed by Bill Lucas at the 1984 NATO Advanced Studies Institute in Italy (Lucas & Kochian, 1986). Newman experimented with this theory on corn seedling roots, examining the stoichiometry of H^+ and potassium (K^+) fluxes, prior to developing the MIFE system (Newman et al., 1987).

MIFE is based on the theory that molecules in solution move according to the force of diffusion; i.e. from regions of high concentration to low concentration. Additionally, ions are under the influence of electric forces; thus, the movement is from high electrochemical potential to low (Newman, 2001). Net ion movement by diffusion is calculated from the measured electrochemical gradient and the mobility and

concentration of the ion in solution. It is important that the solution is not disturbed by excessively rapid movement. Sufficient time is needed for steady, diffusion-limited conditions to be established when changing the testing solution (Newman, 2001). The MIFE system has three major underlying assumptions: 1) that there is no bulk solution flow, so that ionic movement is based entirely on diffusion under the influence of electric and chemical forces in the solution; 2) that the measurement is close to the tissue surface; and 3) that the ionic movement is normal to the surface (Newman, 2001).

Microelectrodes specific to each ion being measured are prepared prior to use of the MIFE. The microelectrodes contain two components: a filling solution and a liquid ion exchanger (LIX). Both these components are specific to the nutrient ion being studied. LIX are available commercially for a wide range of nutrient ions. The filling solution is prepared in-lab using a sufficient concentration of the ion of interest in order to stabilize its electrochemical potential (Newman, 2001). Microelectrodes are calibrated prior to measurement in a series of concentrations covering the expected range of ions being examined.

The system functions by automatically moving the chamber containing the plant, which is attached to a computer-controlled micromanipulator. The chamber moves up and down in a 10-second square-wave cycle: it alternates between two positions that are 40 µm apart (Figure 1). Ion concentration is automatically calculated from the electrochemical potential at each position. Ion flux can then be calculated from the difference in electrochemical potential at these positions (Hawkins et al., 2017). A higher electrochemical potential at the more distant measurement position would thus

signify a net influx into the tissue sample for a positive ion, and a net efflux for a negative ion (Newman, 2001).

Advantages of MIFE

The MIFE system has many advantages over other technologies in quantifying nutrient ion fluxes in plants. The non-invasive nature of MIFE may provide more accurate measurements of nutrient ion fluxes, as entering the tissue is known to affect the *in situ* electrophysiological processes (Shabala et al., 2013). Before the advent of non-invasive ion flux measurements, electric currents were directly measured using the vibrating probe, developed by Jaffe & Nuccitelli in 1974. However, this methodology was problematic as it lacked specificity, which prevented researchers from differentiating which specific ions were being transported, i.e. which ions contributed to the electric current (Newman, 2001). The ability to not only study specific ions, but to study several concurrently (i.e. up to four ions), allows for the study of the stoichiometry of membrane transporters (Shabala et al., 2013). Other advantages to the MIFE method include its high temporal (i.e. circa 5 seconds) and spatial (i.e. several microns) resolution, which has allowed for a much more precise functional characterization of ion transport systems in different regions of plant tissues (Newman, 2001; Shabala et al., 2013). Additionally, the MIFE is able to run limitlessly (i.e. for hours or days) (Shabala et al., 2013).

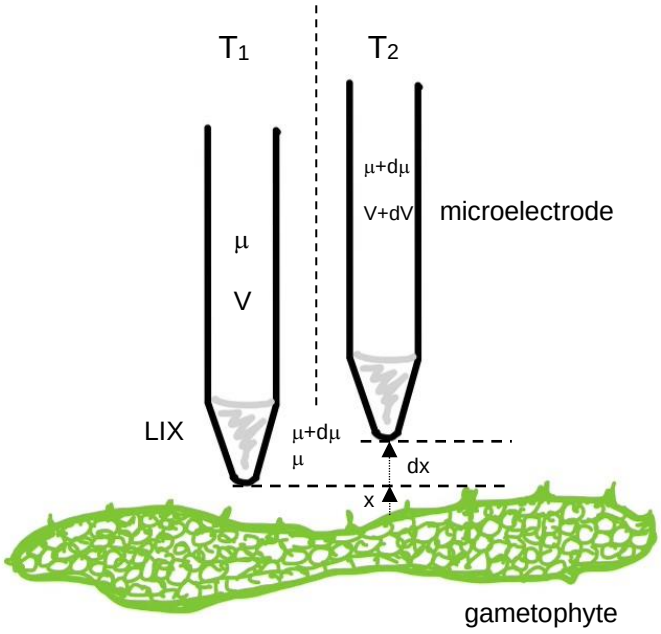

Figure 1. Illustration of a close-up of the MIFE system. A microelectrode with a liquid ion exchanger (LIX) tip is moved through a known distance (dx) and an electrometer measures the concurrent change in electrode voltage (dV). Microelectrodes are separated temporally, not spatially (i.e. positions T_1 and T_2 occur 10 seconds apart over the same section of tissue). Figure adapted from Newman (2001).

1.10 MIFE applications

The majority of experiments using MIFE technology have focused on examining nutrient ion net fluxes in roots. As all vascular plants rely on roots for both structural support and assimilation of water, minerals, and nutrients, there is great scientific and economic importance to understanding the specific ion fluxes in different root regions and the underlying transport mechanisms. Due to their particular significance and commercial availability of LIX, studies using MIFE have focused on measuring H^+, K^+, NH_4^+, NO_3^-, sodium (Na^+), calcium (Ca^{2+}), and chloride (Cl^-). The only major mineral ion without commercially available LIX is phosphate (Newman, 2001).

A notable use of the MIFE system has been in studying salinity tolerance in crop plants. For example, MIFE has allowed researchers to determine the key role of cytosolic K^+ homeostasis in salinity tolerance of barley and wheat genotypes. Thus, MIFE can be used to screen plant accessions for tolerance, enabling plant breeders to successfully develop salinity-tolerant crops (Shabala et al., 2012). Though the majority of experiments have focused on ion fluxes in roots, particularly in crop plants, ion fluxes have also been studied using MIFE in leaf mesophyll and other tissues, as well as protoplasts and bacterial biofilms (Newman, 2001), and recently, mosses (Hawkins et al., 2017).

1.11 Selection of fern species

The primitive and conserved features of Polypodiophyta make ferns choice organisms for studying many fundamental aspects of plant development (Banks, 1999). As ferns are the second most diverse lineage of vascular land plants, they are critical for

ecosystem health and function (George & Bazzaz, 1999; Krieg & Chambers, 2022). Notably, they are an important understory plant characterizing many ecosystems, including in the Pacific Northwest. Like all land plants, Polypodiophyta has two alternating and morphologically variant growth forms: the diploid sporophyte and the haploid gametophyte. However, unlike other vascular plants, these two growth forms are free-living in Polypodiophya organisms, meaning that the majority of their lifespan is nutritionally and physically independent of one another (Farrar et al., 2008). Thus, Polypodiophyta are the ideal organisms in which to compare nutrient uptake in the sporophyte versus gametophyte growth form. As the membrane transporters involved in nutrient uptake are generally evolutionarily conserved across organisms (Reid & Hayes, 2003), this research can be applied to other plant types that possess non-independent alternation of generations to better understand the role of each form in the acquisition of nutrients.

I chose to use *Polystichum munitum*, commonly known as western swordfern, for the majority of my experiments. *P. munitum* is one of the most abundant fern species in the Pacific Northwest, making its spores easily collectible (Pojar & MacKinnon, 2016). Additionally, abundant populations of *P. munitum* have been reared and maintained in the von Aderkas lab since 2022 with great success. To assess the effect of generation (i.e. gametophyte versus sporophyte) on nutrient ion flux, I chose to use apogamous species to eliminate the potential effect of genetic variation from recombination in sexually reproducing species. I used two different apogamous fern species: *Cyrtonium falcatum*, the Japanese holly fern, and *Christella dentata*, the soft fern. *C. falcatum* is a terrestrial fern native to temperate eastern Asia (Gibson et al., 1984). *C. dentata* is a

terrestrial fern endemic to the tropical and subtropical regions of Africa, Asia, and Asia Pacific. It has been reported to be invasive in multiple countries (Xu et al., 2022). These two apogamous ferns are morphologically different and grow in different ecosystem types, which is interesting to consider in terms of ion flux variance.

1.12 Objectives and hypotheses

This study seeks to use a microelectrode ion flux measurement system to characterize the ion fluxes of NH_4^+, NO_3^-, and H^+ in fern gametophytes. Specifically, my study has three objectives: 1) to examine the effect of pH on net flux of NO_3^-, NH_4^+, and H^+ in female *Polystichum munitum* gametophytes; 2) to examine the effect of sex (female/hermaphrodite versus male) on net flux of NO_3^-, NH_4^+, and H^+ in *P. munitum* gametophytes; and 3) to examine net flux of NO_3^-, NH_4^+, and H^+ in the sporophyte versus gametophyte generations of two apogamous fern species, *Christella dentata* and *Cyrtonium falcatum*.

I hypothesize that *P. munitum* gametophytes will favour NH_4^+ uptake over NO_3^- uptake, particularly at lower pH, as is demonstrated in bryophyte gametophores (Reid & Hayes, 2003; Rudolph et al., 1993). Additionally, I expect to see overall greater nitrogen influx at pH 5, as the environment to which these specimens are adapted is slightly acidic. Furthermore, I hypothesize that the larger, female and hermaphroditic gametophytes will exhibit greater rates of both NO_3^- and NH_4^+ uptake than smaller, male gametophytes, due to the greater nutritional/energetic requirements of archegonia and greater overall growth requirements. I again expect uptake of NH_4+ to be greater than that of NO_3^- (Reid & Hayes, 2003). I hypothesize that there will be greater NH_4+ and

NO_3^- influx in the gametophyte generation than the sporophyte generation due to the lack of effective cuticle and more active development during the gametophyte phase.

Chapter 2. Methods

2.1 Gametophyte production

Gametophytes were reared as per Knox (2023). Reproductive *Polystichum munitum* fronds were collected in Mystic Vale (University of Victoria, Victoria, BC) by Patrick von Aderkas and Kailey Strachan during the summer of 2023 and dried for 24 hours to facilitate spore release. Spores were stored at 4°C until sown. *Christella dentata* and *Cyrtomium falcatum* spores were purchased from the American Spore Exchange. All spores were sown directly onto sterile soil. Sterile soil was prepared by filling 2-inch-deep rectangular plastic seeding pots with MySoil Organic Starter Mix. Soil was hydrated with distilled water. Pots containing hydrated soil were placed into Ziploc bags, which were then sealed and heated until the internal soil temperature reached 70°C. The sealed bags ensured a sterile environment and helped maintain consistent humidity during gametophyte development. Bags were placed in a growth chamber set to 22°C with a 12-hour day/night cycle.

2.2 Gametophyte selection and preparation

Intact and representative female and hermaphrodite *P. munitum* gametophytes, *C. dentata* gametophytes, and *C. falcatum* gametophytes were selected and removed from their populations using forceps immediately before treatment. Male *P. munitum* gametophytes were removed using forceps and repotted days prior to treatment. Soil particles or debris were removed prior to treatment for all gametophytes. Prior to ion flux

measurement, gametophytes were tied to a Perspex strip and conditioned in a test tube in 60 mL of 500 µm NH_4NO_3 + 200 µm $CaSO_4 \cdot 2H_2O$ solution for 30-60 minutes. Aeration was maintained using a bubbler.

2.3 Preparation of microelectrodes

Microelectrodes were prepared as per Hawkins et al. (2017). Electrode blanks were pulled from 1.5 mm borosilicate glass capillaries, dried in an oven at 220°C for 5 hours, and silanized with tributylchlorosilane (catalog no. 90796, Fluka, Sigma-Aldrich Canada Ltd., Oakville, ON). Cooled microelectrode blanks were backfilled with 200 mM NH_4Cl for NH_4^+, 500 mM KNO_3 + 100 mM KCl for NO_3^-, and 15 mM NaCl + 40 mM KH_2PO_4 for H^+. Each of these solutions was adjusted as needed to pH 5 using H_2SO_4 and NaOH. Electrode tips were then filled with commercially available ion-selective H^+ or NH_4^+ LIX (Fluka catalog no. 95297 and 09882, respectively), or a NO_3^--selective LIX containing 0.5% methyltridodecylammoniumnitrate, 0.084% methyltriphenylphosphonium bromide and 99.4% n-phenyloctylether (Plassard et al. 2002). Once prepared, microelectrodes were mounted on an electrode holder (MMT-5, Narishige, Tokyo, Japan) providing three-dimensional positioning. Microelectrodes were calibrated with a set of known standards.

2.4 Ion flux measurements

Measurements of ion net flux were performed as per Hawkins et al. (2017). Prior to measurement, gametophytes were removed from the test tube and placed in a 2.75 cm W x 22.5 cm L x 2.0 cm D tray filled with 40 mL of the 500 µm NH_4NO_3 + 200 µm

$CaSO_4 \cdot 2H_2O$ solution. The tray was carefully placed under the microelectrodes. The microelectrodes were then positioned 3-4 µm apart by hand using a Leitz Wetzlar Germany compound microscope. The chamber was attached to a computer-controlled micromanipulator (PatchMan NP2, Eppendorf AG, Hamburg, Germany) and was manipulated using the Eppendorf PatchMan NP2 joystick so that the microelectrodes were positioned in a line 5-40 µm above the gametophyte surface. During flux measurements, the microelectrode ion flux measurement (MIFE) computer moved the chamber containing the gametophyte up and down, relative to the microelectrodes, between two positions 40 µm apart, in a 10 second square-wave cycle. The concentration of each ion was calculated from its electrochemical potential at each position. The flux of each ion was later calculated from the measurements of the difference in the electrochemical potential between these positions (Shabala et al., 1997).

2.5 Gametophyte weight and surface area calculations

Gametophytes were oven dried at 220℃ after flux measurements. Dried gametophytes were individually weighed using an ATI CAHN model C-44 scale. In the case of *C. dentata* and *C. falcatum*, where the intact sporophyte/gametophyte was measured, both generations were weighed together. To calculate average surface area of male and female/hermaphrodite *P. munitum* gametophytes, 10 high resolution photographs of male gametophytes and female/hermaphrodite gametophytes were taken with a scale bar for reference. Each photographed gametophyte was weighed using an ATI CAHN model C-44 scale. Surface area of each photographed

gametophyte was calculated in ImageJ using the Freehand Tool. A linear regression of surface area and weight was performed in R to extrapolate this relationship and determine the surface area for each of the gametophytes measured using MIFE. Each flux measurement was multiplied by surface area to calculate uptake rates for the entire gametophyte.

2.6 Statistical analyses

Net fluxes of NH_4^+, NO_3^-, and H^+ in *P. munitum* male and female/hermaphrodite gametophytes were analyzed with Welch Two Sample t-tests in R (v.4.2.3), assuming unequal variance. Linear regressions were performed for each ion for each group to assess whether there was a significant relationship between the ion net flux and the concentration of the measurement solution. A significant relationship was found between NH_4^+ flux and concentration in female/hermaphrodite *P. munitum* gametophytes ($p = 0.00006$); thus only flux measurements at concentrations ranging from 400-700 mmol were retained as they did not display a significant relationship ($p = 0.8$). After unusable measurements were removed, the sample sizes for NH_4^+ flux comparisons were n_{male} (n_M) = 8 and $n_{female/hermaphrodite}$ ($n_{F/H}$) = 7; the sample sizes for NO_3^- flux comparisons were $n_M = 16$ and $n_{F/H} = 15$; and the sample sizes for H^+ flux comparisons were $n_M = 16$ and $n_{F/H} = 15$.

Net fluxes of NH_4^+, NO_3^-, and H^+ at pH 5 versus pH 7 in *P. munitum* female/hermaphrodite gametophytes were analyzed with Welch Two Sample t-tests in R (v4.2.3). The sample size of each group was 10.

Net fluxes of NH_4^+, NO_3^-, and H^+ in the sporophyte versus gametophyte generation in *C. dentata* and *C. falcatum* gametophytes were analyzed with a General Linear Model ANOVA to determine differences in net flux among species, generations, and their interaction. All tests were performed with SAS PROC GLM using Type III Sums of Squares for unbalanced experimental designs. The sample sizes for NH_4^+, NO_3^-, and H^+ flux comparisons, after the removal of unusable measurements, are displayed in Table 1.

Table 1. Sample sizes of each generation/species used for NH_4^+, NO_3^-, and H^+ flux comparisons.

Ion	Generation	Species	Sample size (n)
NH_4^+	Sporophyte	*C. dentata*	4
NH_4^+	Gametophyte	*C. dentata*	3
NH_4^+	Sporophyte	*C. falcatum*	3
NH_4^+	Gametophyte	*C. falcatum*	2
NO_3^-	Sporophyte	*C. dentata*	4
NO_3^-	Gametophyte	*C. dentata*	3
NO_3^-	Sporophyte	*C. falcatum*	3
NO_3^-	Gametophyte	*C. falcatum*	3
H^+	Sporophyte	*C. dentata*	4
H^+	Gametophyte	*C. dentata*	3
H^+	Sporophyte	*C. falcatum*	3
H^+	Gametophyte	*C. falcatum*	3

Chapter 3. Results

3.1 Effect of pH on ion flux

There was no significant difference in NH_4^+ net flux in female/hermaphrodite *P. munitum* gametophytes at pH 5 versus pH 7 ($p = 0.5742$) (Figure 2); however, there was greater variability at pH 7.

There was greater NO_3^- net flux in female/hermaphrodite *P. munitum* gametophytes at pH 7 versus pH 5, with NO_3^- efflux observed at pH 5 ($p = 0.0026$) (Figure 3).

There was a significantly greater H^+ net influx in female/hermaphrodite *P. munitum* gametophytes at pH 5 versus pH 7, with near-zero flux at pH 7 ($p = 0.016$) (Figure 4).

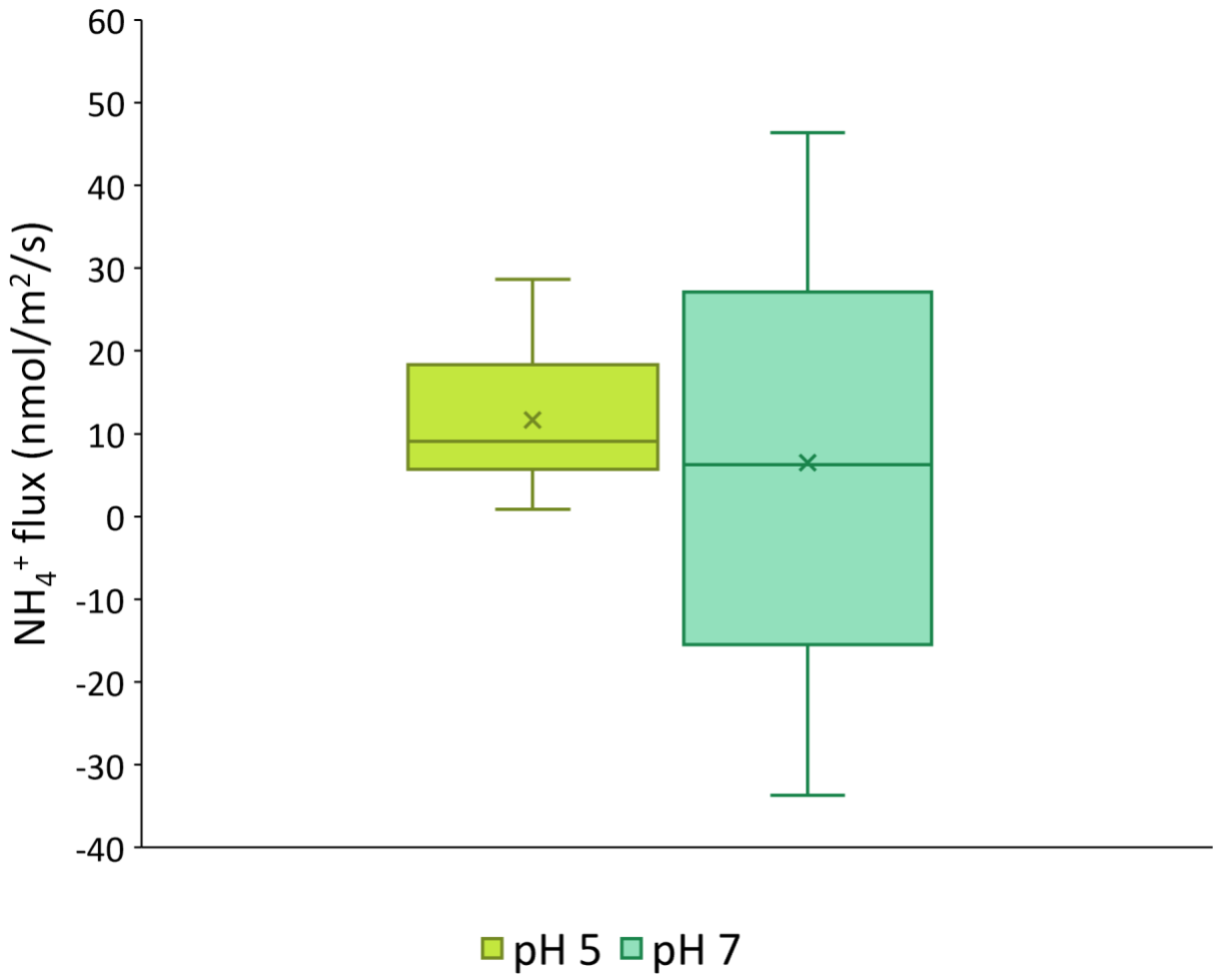

Figure 2. Box and whisker plot of the effect of pH on NH_4^+ flux in female/hermaphrodite *P. munitum* gametophytes (n = 10). For all box and whisker plots, minimum and maximum values are indicated by the ends of the whiskers; the mean is indicated by the "X"; the median is indicated by the centre line; and outliers are indicated by points.

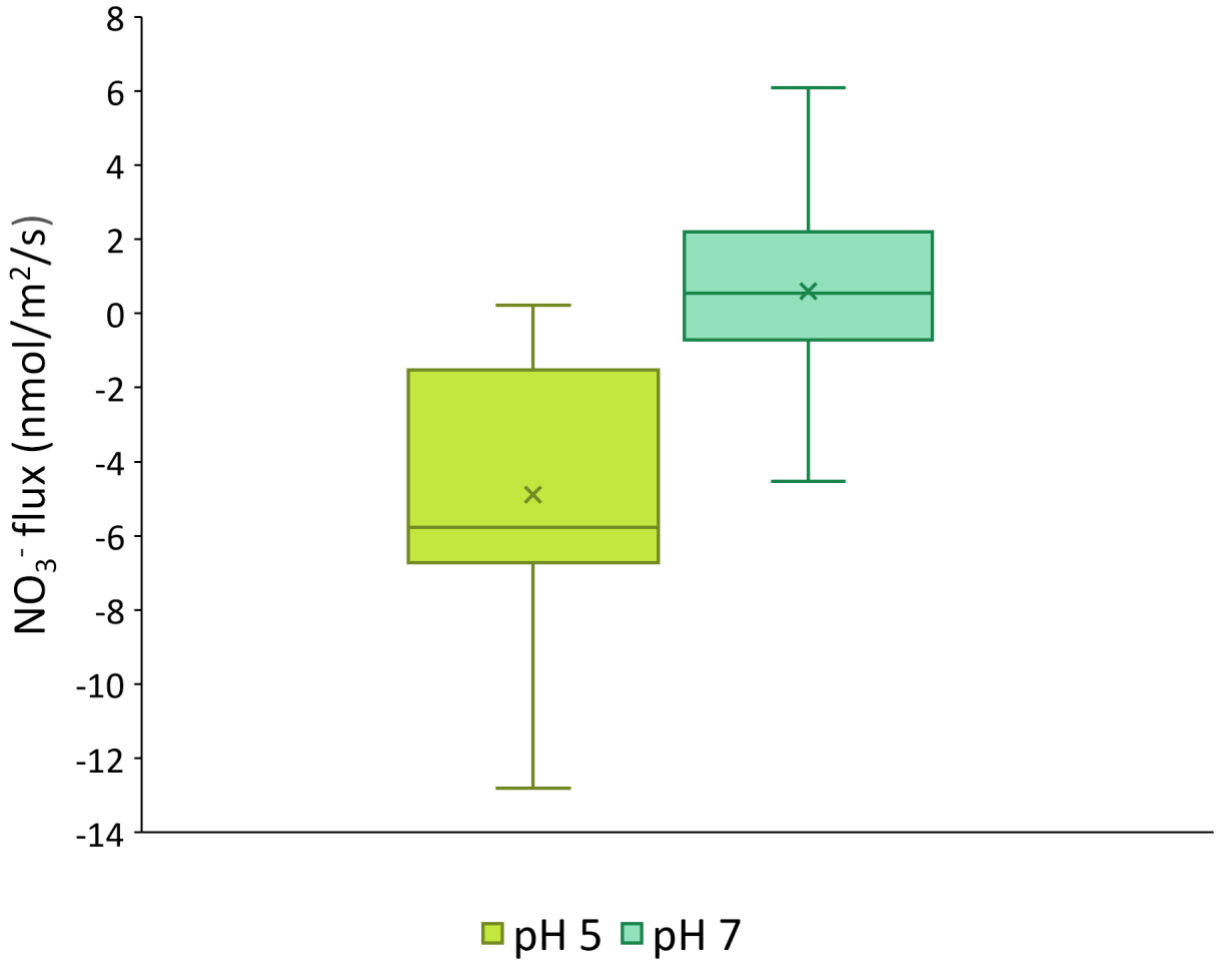

Figure 3. Box and whisker plot of the effect of pH on NO_3^- flux in female/hermaphrodite *P. munitum* gametophytes (n = 10). Negative flux values indicate efflux.

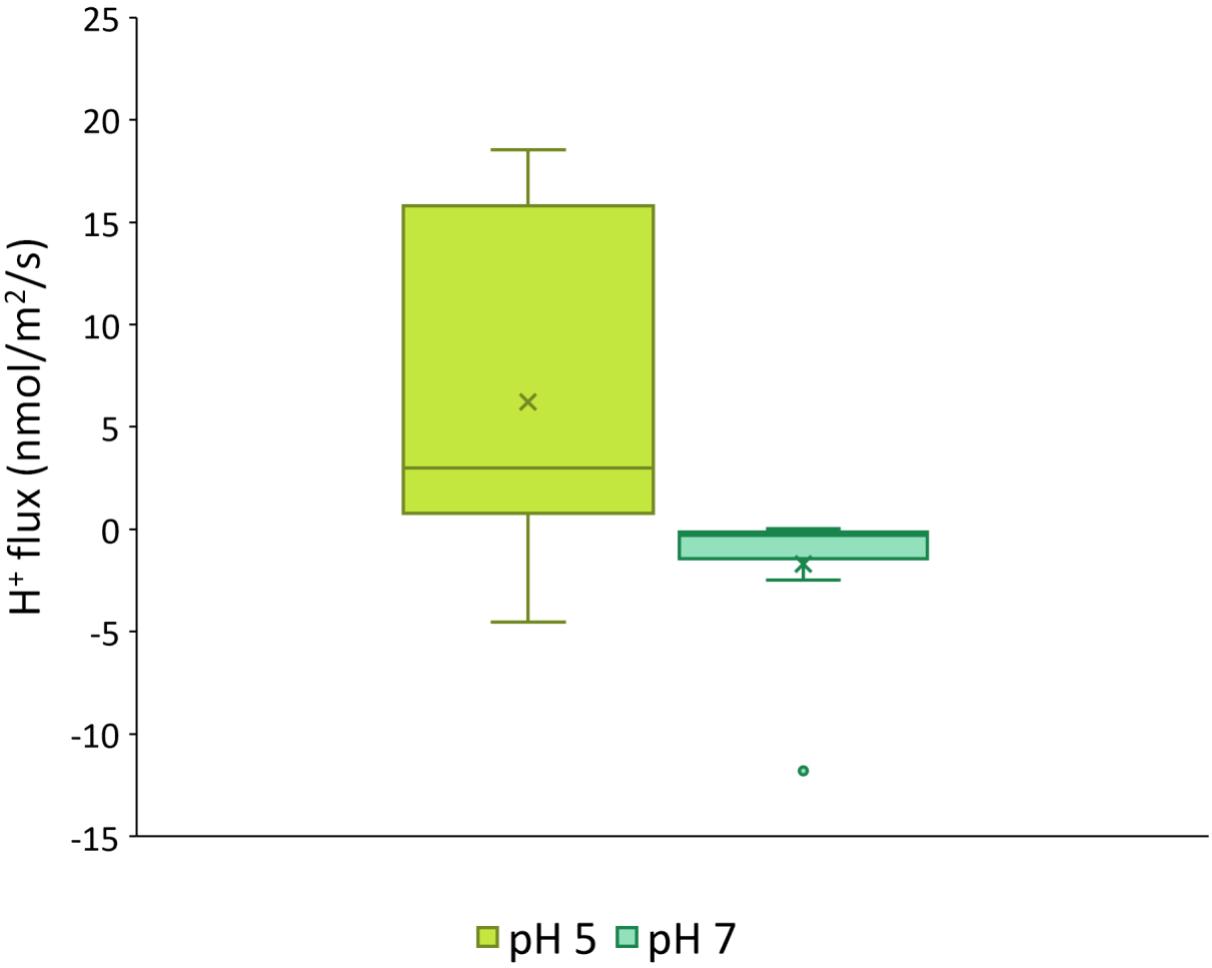

Figure 4. Box and whisker plot of the effect of pH on H^+ flux in female/hermaphrodite *P. munitum* gametophytes (n = 10).

3.2 Effect of sex on ion flux

For all three ions, flux range was much greater for male gametophytes than female/hermaphrodite gametophytes.

On average, NH_4^+ uptake was greater in male *P. munitum* gametophytes than female/hermaphrodite *P. munitum* gametophytes, but the difference was not significant ($p = 0.092$) (Figure 5).

On average, NO_3^- net efflux was greater in female/hermaphrodite *P. munitum* gametophytes than male *P. munitum* gametophytes, but the difference was not significant ($p = 0.083$) (Figure 6).

There was significantly greater H^+ net uptake in male than in female/hermaphrodite *P. munitum* gametophytes ($p = 0.00006$) (Figure 7).

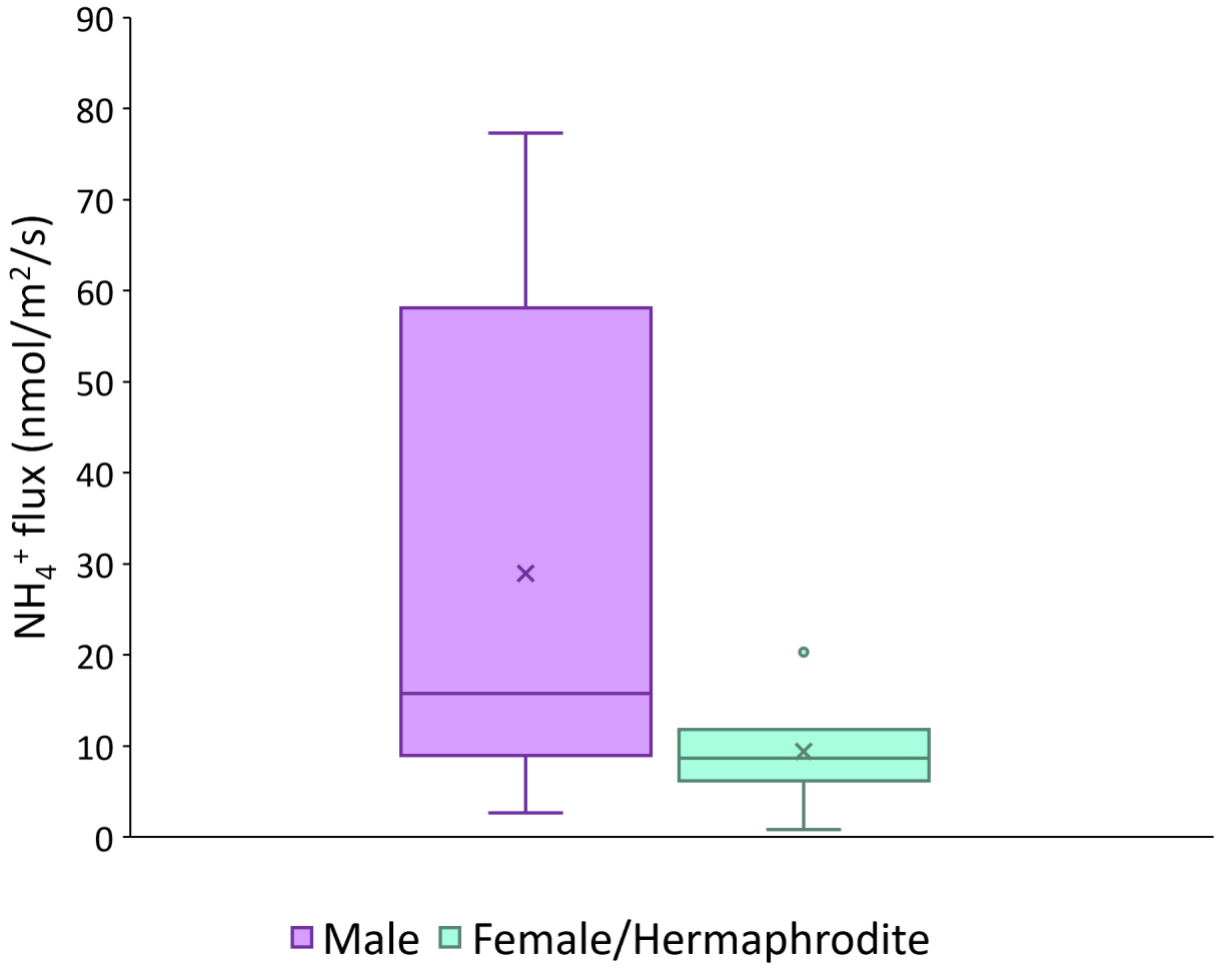

Figure 5. Box and whisker plot of NH_4^+ flux in male versus female/hermaphrodite *P. munitum* gametophytes ($n_M = 8$, $n_{F/H} = 7$).

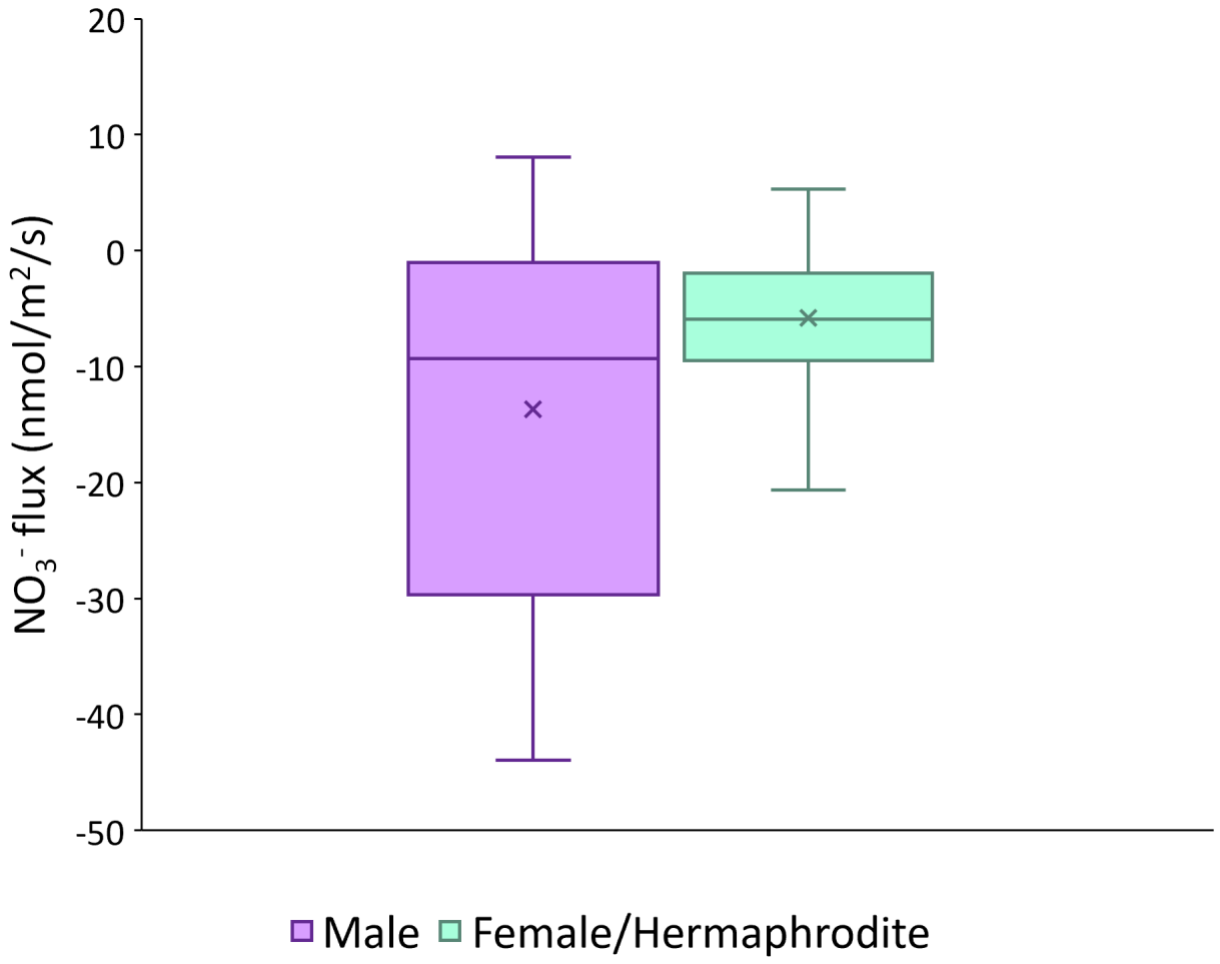

Figure 6. Box and whisker plot of NO_3^- flux in male versus female/hermaphrodite *P. munitum* gametophytes (n_M = 16, $n_{F/H}$ = 15).

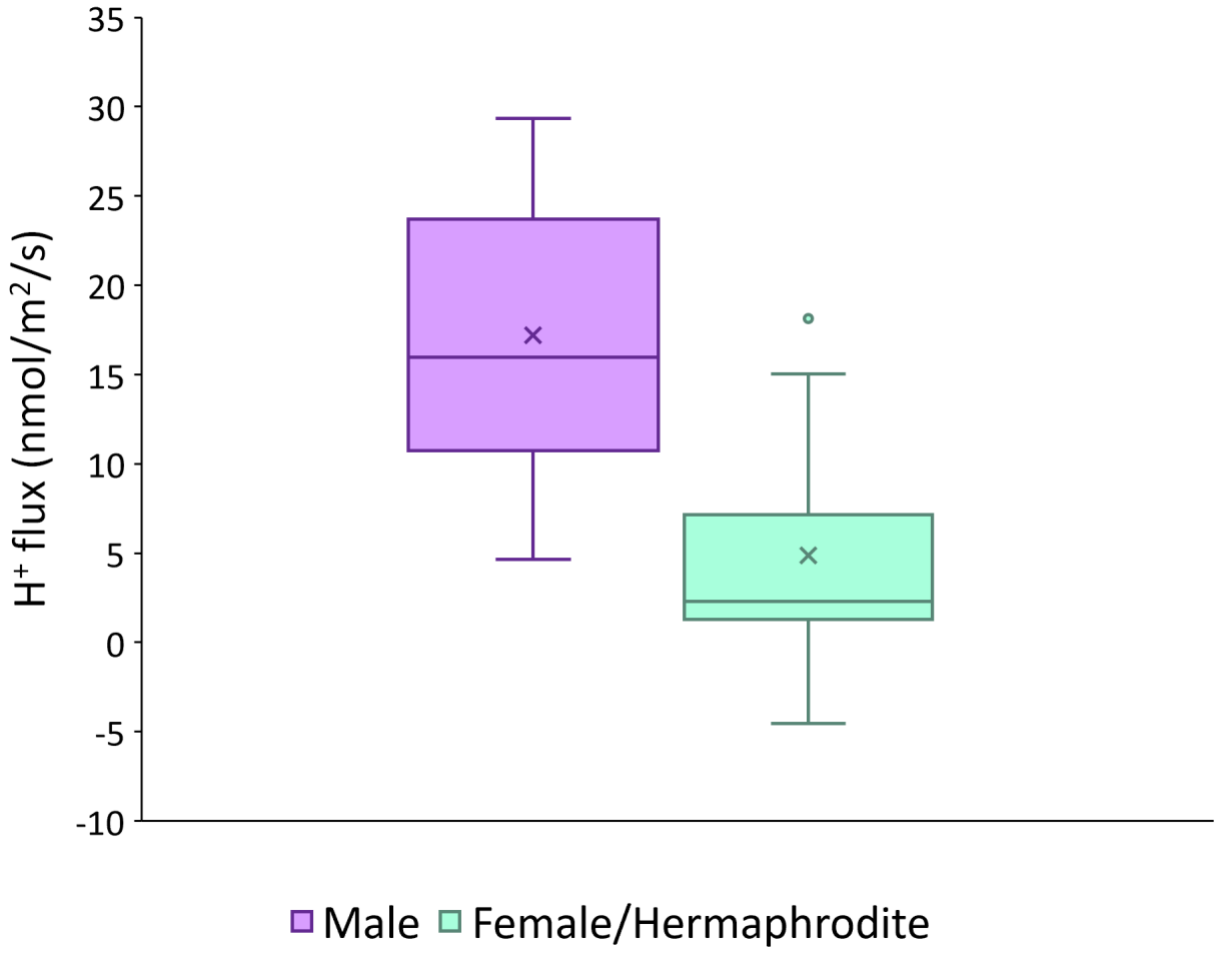

Figure 7. Box and whisker plot of H^+ flux in male versus female/hermaphrodite *P. munitum* gametophytes ($n_M = 16$, $n_{F/H} = 15$).

3.3 Effect of generation on ion flux

There was no significant difference in NH_4^+ flux between *C. dentata* (*M* = -1.84; *SE* = 4.51) and *C. falcatum* (*M* = 8.04; *SE* = 2.89) (p = 0.19), between sporophytes (*M* = 3.09; *SE* = 4.25) and gametophytes (*M* = 1.47; *SE* = 3.62) (p = 0.64), or the interaction between species and generation (p = 0.13).

There was significantly greater NO_3^- uptake in the sporophyte generation, compared to the gametophyte (p = 0.053) (Figure 8), but no significant difference between *C. dentata* (*M* = -2.76; *SE* = 7.03) and *C. falcatum* (*M* = 0.68; *SE* = 8.11) (p = 0.27) or between the interaction of species and generation (p = 0.97).

There was significantly greater H^+ uptake in *C. dentata* compared to *C. falcatum* (p = 0.028) (Figure 9), but no difference between sporophytes (*M* = 2.13; *SE* = 0.21) and gametophytes (*M* = 4.43; *SE* = 0.22) (p = 0.26) or between the interaction of species and generation (p = 0.29). There was no H^+ flux in either the sporophyte or gametophyte generation in *C. falcatum* (Figure 9).

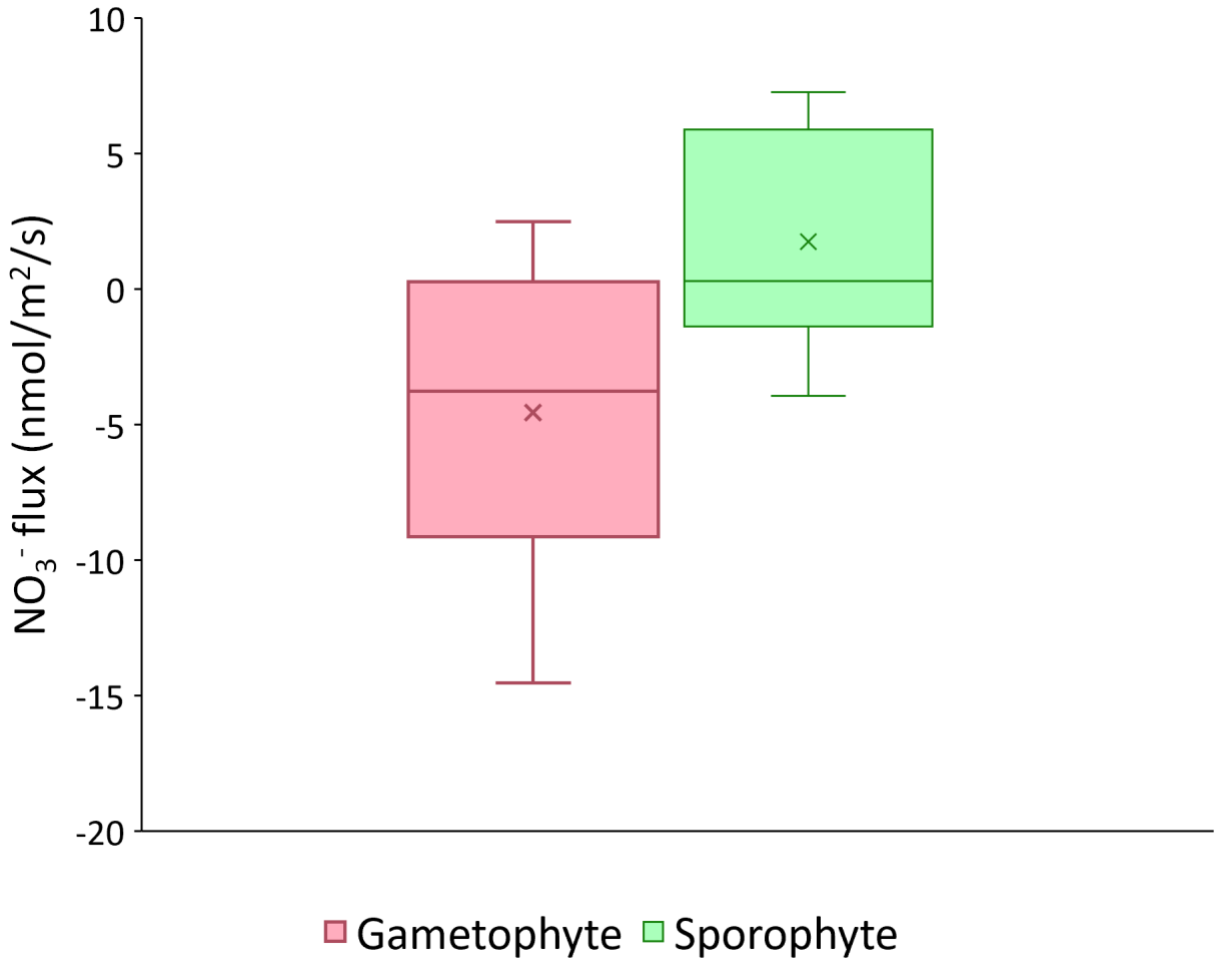

Figure 8. Box and whisker plot of NO_3^- flux in gametophyte versus sporophyte tissues of *C. dentata* and *C. falcatum* ferns ($n_{gametophyte}$ = 6; $n_{sporophyte}$ = 7).

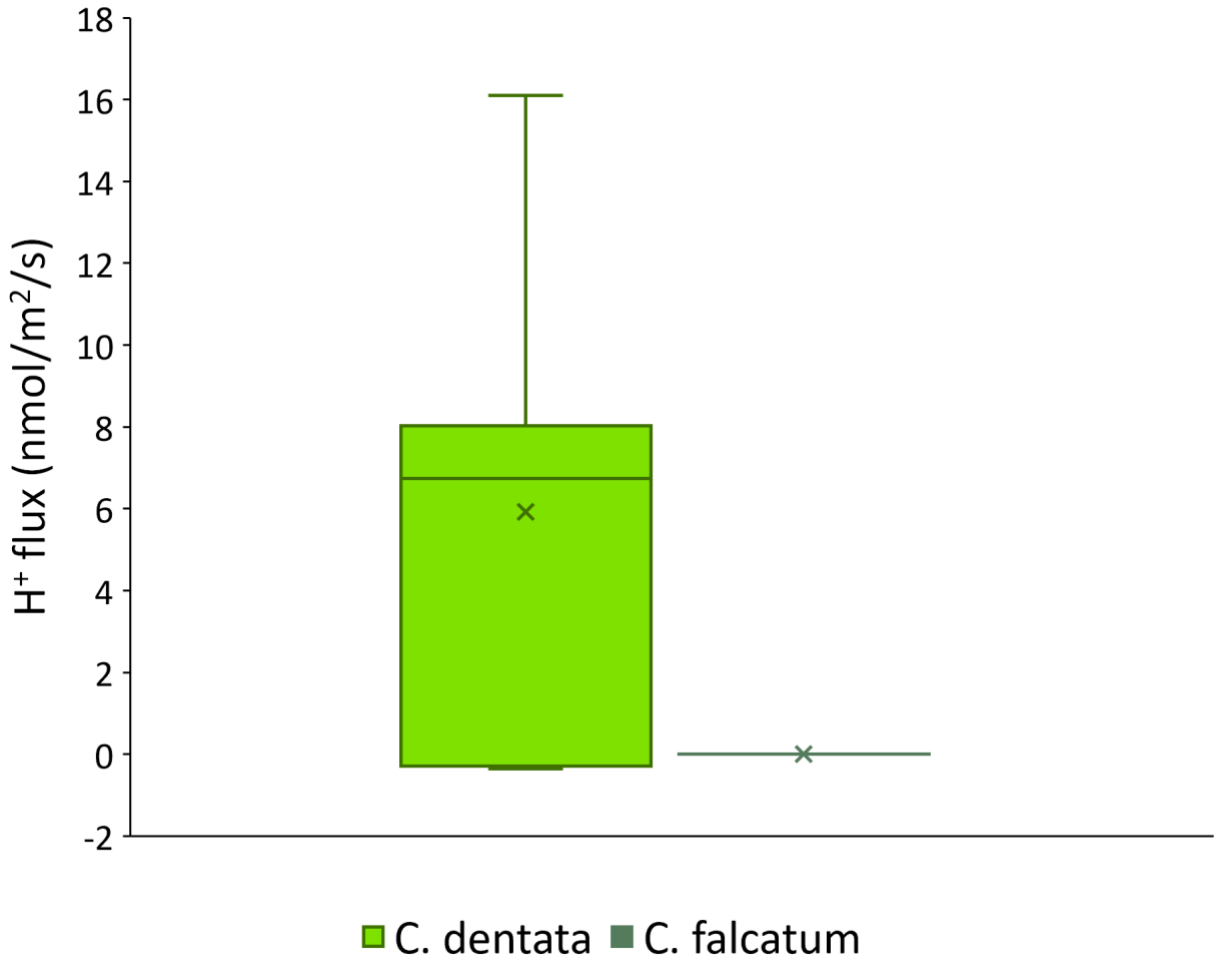

Figure 9. Box and whisker plot of H^+ flux in both sporophyte and gametophyte generations in *C. dentata* versus *C. falcatum* ferns ($n_{C.dentata} = 7$; $n_{Cfalcatum} = 6$).

3.4 Calculation of total ion flux

The average weight of the male gametophytes sampled was 0.013 mg (*SE* = 0.0027 mg) and the average weight of the female/hermaphrodite gametophytes was 0.65 mg (*SE* = 0.11). When these values were multiplied by the respective weight:surface area ratios, the average estimated surface area of the male gametophytes was 0.49 mm^2 and the average estimated surface area of the female/hermaphrodite gametophytes was 45.54 mm^2.

When the net ion flux results per unit area were multiplied by total surface area, female/hermaphrodite *P. munitum* gametophytes exhibited significantly higher NH_4^+ total uptake than male *P. munitum* gametophytes ($p = 0.039$) (Figure 10). Female/hermaphrodite *P. munitum* gametophytes exhibited significantly higher total NO_3^- efflux than male *P. munitum* gametophytes (Figure 11). Female/hermaphrodite gametophytes also exhibited significantly higher total H^+ influx than male gametophytes ($p = 0.0058$) (Figure 12).

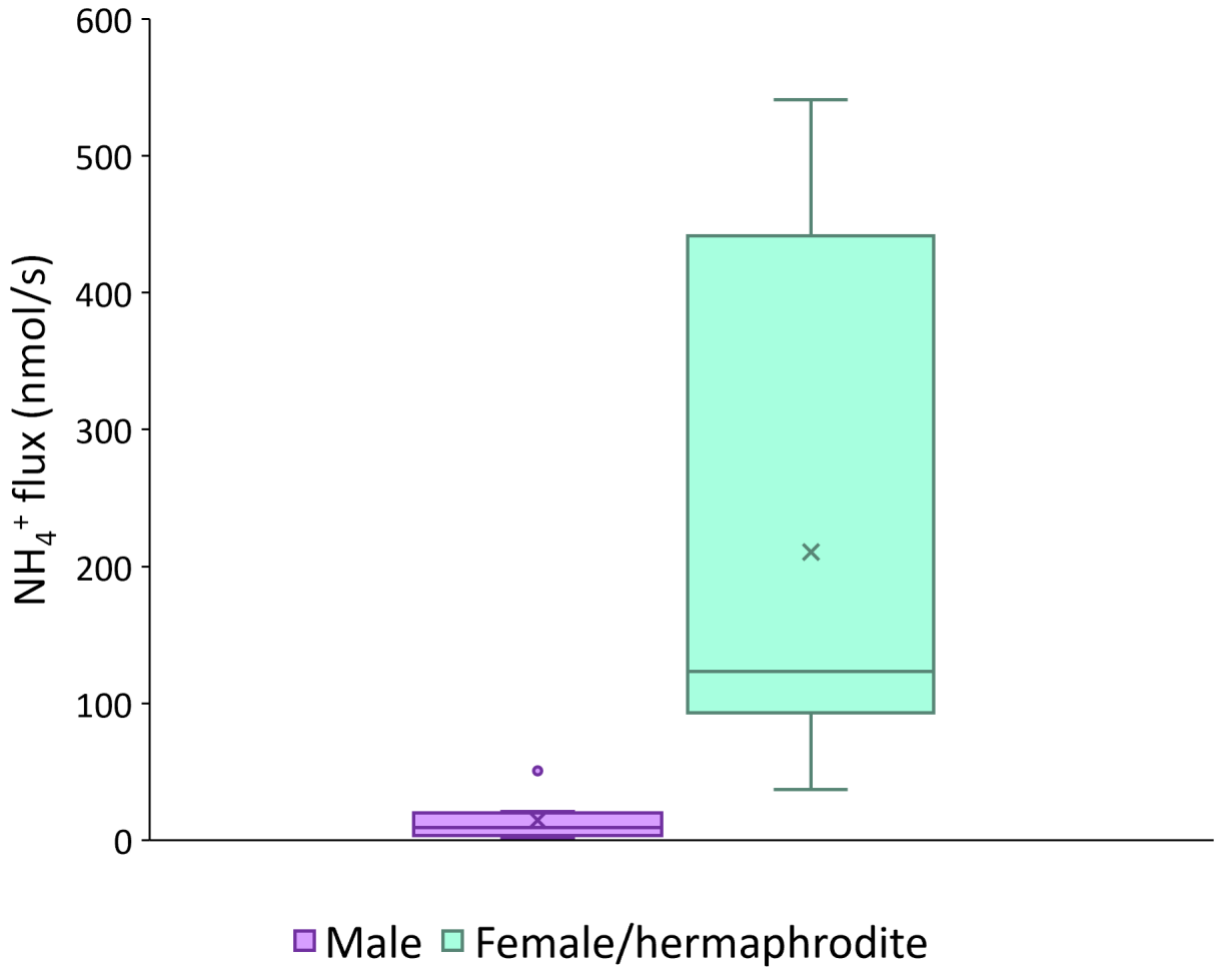

Figure 10. Box and whisker plot of total NH_4^+ flux in male versus female/hermaphrodite *P. munitum* gametophytes calculated for the entire tissue surface area ($n_M = 8$, $n_{F/H} = 7$).

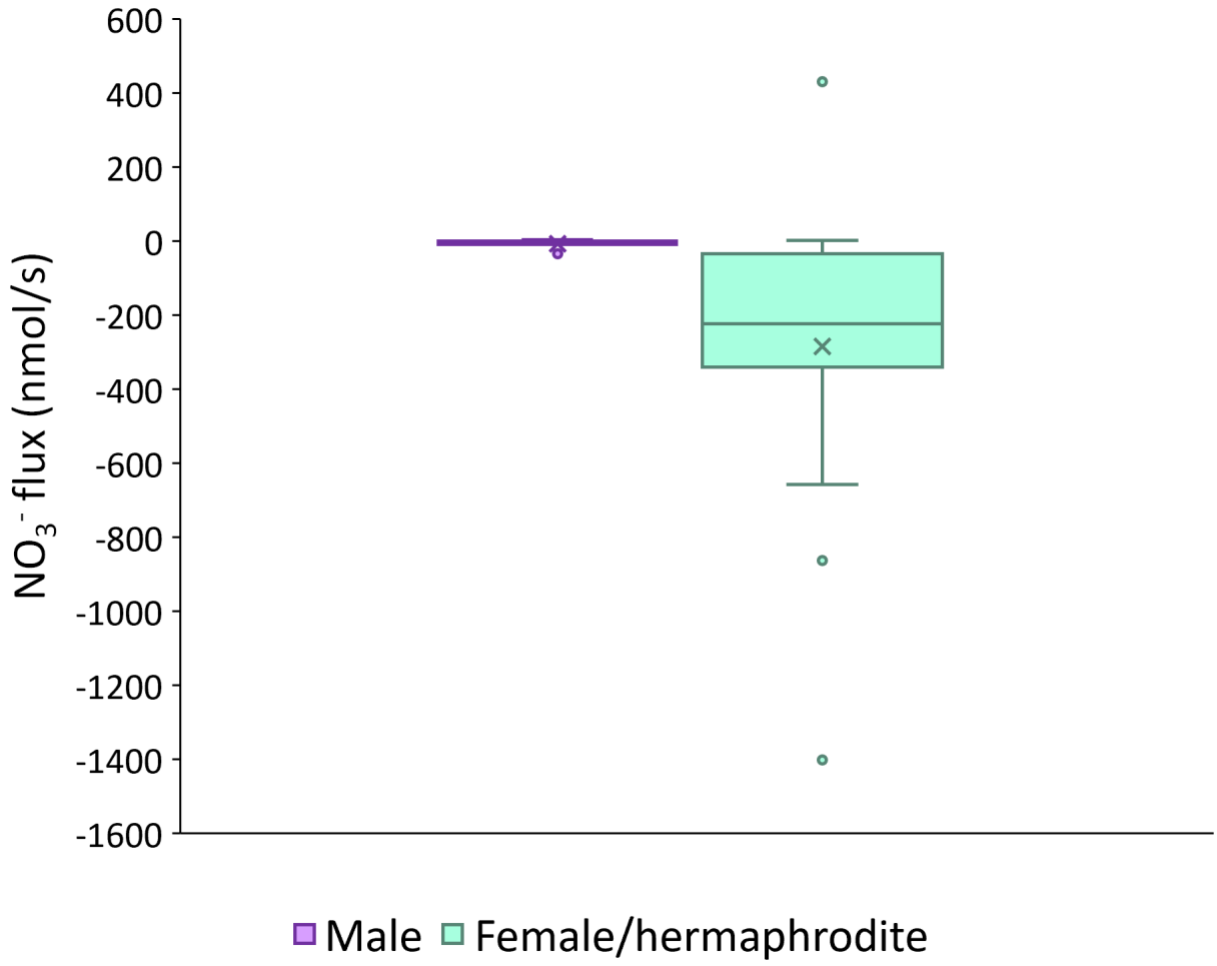

Figure 11. Box and whisker plot of total NO_3^- flux in male versus female/hermaphrodite *P. munitum* gametophytes calculated for the entire tissue surface area (n_M = 16, $n_{F/H}$ = 15).

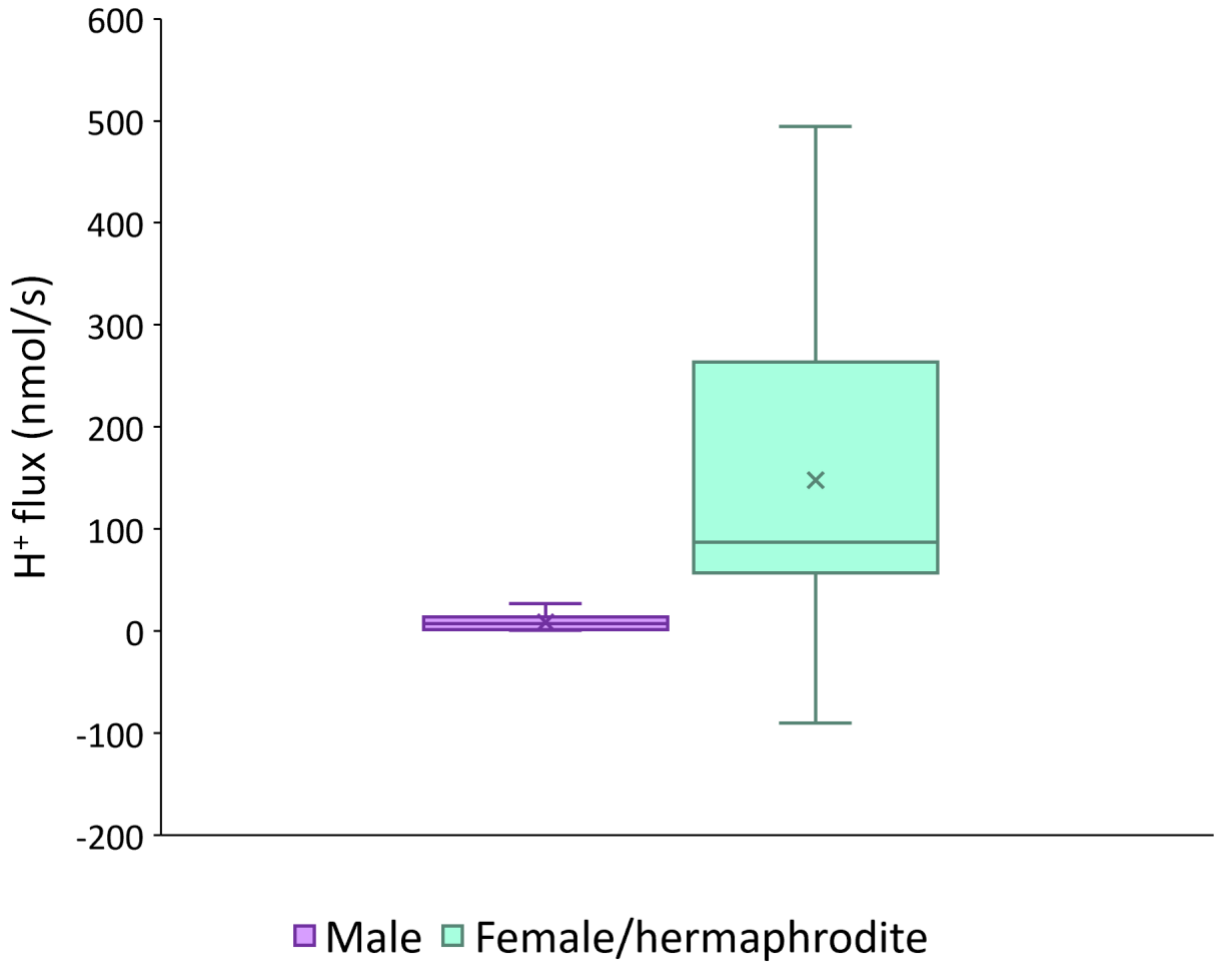

Figure 12. Box and whisker plot of total H^+ flux in male versus female/hermaphrodite *P. munitum* gametophytes calculated for the entire tissue surface area (n_M = 16, $n_{F/H}$ = 15).

Chapter 4. Discussion

To our knowledge, this study is the first to measure nutrient ion flux in fern gametophytes using a MIFE system. This methodology was based on the theory that nutrient acquisition occurs over the entire gametophyte tissue surface (Richardson & Walker, 2010), and thus nutrient uptake could be directly and instantaneously quantified at the gametophyte surface. The methodology proved successful in quantifying these fluxes.

4.1 Assessing overall trends in NH_4^+, NO_3^-, and H^+ flux

I hypothesized that *P. munitum* gametophytes would preferentially take up NH_4^+ over NO_3^-, as is demonstrated in the literature for bryophyte gametophores (Hawkins et al., 2017; Reid & Hayes, 2003; Rudolph et al., 1993). In all of the experiments conducted at pH 5, the majority of NO_3^- flux was efflux, whereas NH_4^+ was always taken up by gametophytes. In moss gametophores, whether net NH_4^+ flux was positive or negative was influenced by species (Hawkins et al., 2017). Relatively high NH_4^+ flux could be due to a high cation exchange capacity in fern gametophytes, as is the case with mosses (Hawkins et al., 2017; Turetsky, 2003). Additionally, NH_4^+ is generally the preferred form of inorganic nitrogen of plants, likely due to its lower energy requirement for assimilation (Reid & Hayes, 2003). With the consistent efflux of NO_3^-, it follows that NH_4^+ uptake would be high in order to support the growth and development of the gametophytes.

A 2009 study by Jampeetong and Brix assessed NH_4^+ and NO_3^- uptake dynamics in *Salvinia natans*, commonly known as floating fern. *S. natans* is a unique tropical/subtropical fern that can rapidly form mats on water surfaces through vegetative reproduction. Though evolutionarily different than *P. munitum*'s gametophytes, the two are comparable in terms of nutrient uptake as it occurs across the entire surface area, rather than through roots. *S. natans* does not have true roots, instead possessing a rootlike submerged leaf that is likely to function as true roots do, absorbing nutrients from the water (Jampeetong & Brix, 2009). The authors examined the growth of *S. natans* supplied with NH_4^+ alone and with NO_3^- versus NO_3^- alone, determining that the two former treatments resulted in significantly higher growth than the latter. Additionally, *S. natans* could take up both NH_4^+ and NO_3^-, but the uptake rate of NH_4^+ was 6-14 times higher than the uptake rate of NO_3^-. Though the method of measuring N uptake rates were different, the results for both my organism of study and *S. natans* are comparable.

The result of total net NO_3^- efflux seen in the majority of the experiments was contrary to my hypothesis. A potential explanation for this result could be that the acidic environment in which the experiments were conducted caused leaching of this anionic compound. However, the rates of NO_3^- efflux were surprising high, especially considering Hawkins et al. (2017) found net NO_3^- influx in virtually all moss species sampled. This suggests that there may be other mechanisms underlying fern nutrient acquisition that are responsible for this result. While NH_4^+ can be directly assimilated, cellular NO_3^- must first be reduced to NH_4^+ before assimilation into organic compounds (Reid & Hayes, 2003). This process requires two enzymes, nitrate reductase and nitrite

reductase (Tischner, 2000). Therefore, another possible explanation for the significant NO_3^- efflux is that fern gametophytes lack the enzymes necessary for NO_3^- assimilation.

While I did not specifically determine a hypothesis for H^+ flux, seeing that the ion was primarily used as an indicator for the NH_4^+ and NO_3^- measurements, it is interesting that H^+ influx was almost always seen. In other studies that utilize the MIFE system to quantify ion flux, H^+ efflux is the norm. In the 2017 study conducted by Hawkins et al., on which my methods are based, high H^+ efflux was seen in most moss species. The authors found that location and species had a significant effect on net H^+ flux, with mosses obtained from locations with high rainfall exhibiting greater proton efflux than those from the drier location of Victoria, BC (Hawkins et al., 2017). Hawkins et al. (2017) posited that mosses from moist areas could have a greater number of H^+ transporters in order to facilitate ion uptake in these conditions.

A 2010 study by Hawkins and Robbins examined NH_4^+, NO_3^-, and H^+ uptake in the apical root region of conifer and soybean roots using a MIFE system. They found that *Pseudotsuga menziesii* consistently demonstrated H^+ efflux behind the root tip, while at pH 4, *Glycine max* and *Pinus contorta* demonstrated net H^+ influx in some root regions. The major takeaway from this study was that H^+ efflux at low pH may be related to the ability to maintain NH_4^+ uptake in acidic conditions. Considering that H^+ influx was the trend for the majority of the fern gametophytes studied, it may be the case that nutrient mobilization is not a top priority for the fern gametophyte. Another potential explanation could be that N transporters in the gametophyte tissue function

differently than in roots; specifically, that the proton gradient is not as critical to inorganic N uptake.

4.2 Assessing effect of pH on ion flux

The study of the effect of pH on ion flux was done as a preliminary assessment of the *P. munitum* gametophytes to determine the preferred pH for subsequent experiments. I hypothesized that the gametophytes would display greater overall NH_4^+ and NO_3^- uptake at pH 5, since the native environment to which these organisms are adapted is acidic. The results demonstrated that there was no significant difference in NH_4^+ flux between the two pH treatments; however, there was significantly greater NO_3^- efflux at pH 5, which may be explained by it being an anion that tends to be lost in acidic environments. Additionally, there is the demonstrated trend that plants preferentially uptake NH_4^+, which is seen in bryophytes (Hawkins et al., 2017).

H^+ influx occurred at pH 5, while there was near-zero flux at pH 7, which can be explained by the greater concentration of cations in a more acidic environment. Considering that the native environment of *P. munitum*, the forests of the Pacific Northwest, is acidic, I conducted the further experiments at a pH of 5 to best simulate natural conditions in vitro.

4.3 Assessing effect of sex on ion flux

I hypothesized that the larger, female/hermaphrodite gametophytes would exhibit greater rates of both NO_3^- and NH_4^+ uptake than the smaller, male gametophytes. Different results were obtained when ion flux was examined at a singular point of the

gametophyte tissue surface compared to when overall ion flux was considered across the entire tissue surface. Though not significantly different, the original ion flux data measured at a singular point demonstrated the trend that male *P. munitum* gametophytes take up more NH_4^+ than female/hermaphrodite gametophytes per-unit-area. This result was contrary to my hypothesis and suggests that male gametophytes may have high nitrogen requirements. The greater per-unit-area ion flux seen in males could be due to these gametophytes being at earlier and more active stages of development (Banks, 1999). Their much smaller size and surface area may require male gametophytes to obtain NH_4^+ at a higher rate than the significantly larger female/hermaphrodite gametophytes.

When the per-unit-area ion flux data was multiplied using a ratio of weight to surface area, the female/hermaphrodite gametophytes, which are much larger than the males, exhibited significantly higher total NH_4^+ influx, NO_3^- efflux, and H^+ influx. This suggests that at the organism level, archegonia-supporting gametophytes have greater nitrogen requirements than males.

A 2016 study by Goodnoe and Hill found that the absolute and relative content of nitrogen varies by sex in *Ceratopteris richardii* gametophytes. Though *C. richardii* possess an antheridiogen system, which has not yet been investigated in *P. munitum*, nutrient availability is still a factor underlying sex determination. That study chose to investigate ameristic versus meristic gametophytes, which translates to this study's comparisons of male gametophytes and female/hermaphrodite gametophytes, respectively. The authors found that meristic gametophytes had a higher percentage of N (6.5% $\pm$ 0.3% N) than ameristic gametophytes (5.3% $\pm$ 0.3% N). Based on these

results, the authors determined that *C. richardii* conforms to the expectations of nutrient-based sex determination as is explained in the ESD (environmental sex determination) theory proposed by Charnov and Bull (1977) (Goodnoe & Hill, 2016). This corroborates the result I elucidated - that female/hermaphrodite gametophytes demonstrate higher net NH_4^+ uptake at the organism level – and suggests that *P. munitum* also conforms to the ESD theory.

4.4 Assessing ion flux in both generations of *C. dentata* and *C. falcatum*

I hypothesized that the gametophyte generation of apogamous fern species, *C. dentata* and *C. falcatum*, would have greater NH_4^+ and NO_3^- uptake than the sporophyte generation. I also wanted to investigate whether the two different species would exhibit differences in uptake of all three ions. There was no significant difference in NH_4^+ flux between *C. dentata* and *C. falcatum*, nor between the sporophyte and gametophyte generations. However, there was a significant difference in NO_3^- flux between generations. The gametophyte generation experienced higher NO_3^- efflux than the sporophyte generation, which may be explained by the lack of a cuticle in the gametophyte generation (Farrar et al. 2008). In plant leaves, the waxy cuticle functions as a barrier to excessive transpirational water loss, in addition to providing defense against pathogens and defining organ boundaries (Yeats & Rose, 2013). The lack of stomata and effective cuticles in both fern gametophytes and bryophytes allows free exchange of solutions and gases across cell surfaces through which nutrients are taken up (Turetsky, 2003).

A surprising finding of this research was that *C. falcatum* did not exhibit any H^+ flux in either the sporophyte or gametophyte generation. This would potentially suggest a technological issue with the MIFE when doing these samples; however, I also measured the ion flux in a rhizome of this species on the same day, and it displayed H^+ influx. Thus, it appears there are no H^+ transporters in either the sporophyte or gametophyte tissue of *C. falcatum* or that H^+ transporters were not active.

4.5 Challenges

A significant challenge was in preparing and analyzing the male gametophyte samples, which had an average weight of 0.013 mg and average estimated surface area of 0.49 mm^2, compared to the female/hermaphrodite gametophytes, which had an average weight of 0.65 mg and average estimated surface area of 45.54 mm^2. It was challenging to handle the male gametophyte samples, particularly to attach them to the Perspex strip and position them under the microelectrodes. Often, the male gametophytes were impossible to focus in the field of view; this could be a source of error, as there was much less precision in positioning the microelectrodes proximal to the tissue.

Another notable limiting factor in the project was the inability to process a large number of samples due to the limitations of the methodology. The MIFE system is delicate, which led to frequent troubleshooting. Several samples had to be discarded due to electrochemical errors resulting in inaccurate measurements. Additionally, the significant relationship found between NH_4^+ flux and concentration in

female/hermaphrodite *P. munitum* gametophytes ($p = 0.00006$) resulted in a number of samples being removed from the dataset to eliminate this correlation ($p = 0.8$) and match the male gametophyte NH_4^+ concentration range for measurements of 400-700 mmol.

Chapter 5. Conclusion and perspectives

This research sought to examine NH_4^+, NO_3^-, and H^+ net fluxes in fern gametophytes under varying conditions using a microelectrode ion flux measurement system. I successfully used this methodology for fern gametophytes, which have not previously been used as an experimental material with the MIFE system. This study shows that instantaneously and non-invasively measuring nutrient ion flux in fern gametophytes is not only possible, but fairly straightforward, using MIFE. The study investigated nutrient ion flux in male versus female/hermaphrodite *P. munitum* gametophytes, finding that differences in nutrient acquisition may accompany sex differentiation. Specifically, male gametophytes have on average greater per-unit-area ion flux than female/hermaphrodite gametophytes, though the latter demonstrate greater NH_4^+ uptake at the whole-organism level. This provides evidence that fern gametophytes conform to the expectations of nutrient-based sex determination (Charnov & Bull, 1977). Additionally, the study determined that some nitrogen flux dynamics vary depending on generation. Specifically, I found that total net NO_3^- flux varies between the gametophyte and the sporophyte. This work provides insight into the nutrient ecology of fern gametophytes, which is currently a little studied field with huge potential for further research. The results suggest that certain nutrient flux dynamics in

fern gametophytes are not only different than in fern sporophytes, but also from other plants. The high total net NO_3^- efflux, as well as the consistent trend of H^+ influx seen in the study organisms suggest that fern gametophytes may possess differing ion transport mechanisms than other plants. Overall, the work contributes to the limited literature on fern gametophytes and sheds light on the nutrient requirements of this essential stage of the fern lifecycle.

Perspectives

The success of this study using a MIFE system for examining nutrient ion flux in the unconventional study organism of the fern gametophyte opens many doors to better understanding fern and lycophyte nutrient ecology. Directions for future research are extensive, and there is no shortage of questions waiting to be answered regarding the physiology of fern gametophytes.

One interesting expansion on this work could involve examining the effect of nutrient limitation on fern gametophyte nutrient ion uptake. My work served to elucidate the standard mechanisms of NH_4^+, NO_3^-, and H^+ flux under non-limited conditions, growing gametophytes on a soil substrate that had elevated nitrogen levels. However, determining these same ion flux dynamics on gametophytes that were grown in an N-limited environment could provide a more complete understanding of fern gametophyte nutrient ecology. While NO_3^- efflux was observed in the study organisms, contrary to my hypothesis, it is possible that NO_3^- influx would be seen in N-limited conditions where the preferred NH_4^+ form is not abundantly available. This further research could be important for applying results *in vivo*, where N may be limiting.

Commercially-available liquid ion exchange resins (LIX) are available for almost all of the major mineral ions, except phosphate. Thus, it is possible to apply this methodology to examine the nutrient uptake dynamics of mineral nutrients other than nitrogen. Though nitrogen is the mineral nutrient plants require in the greatest quantity, other major mineral ions are also essential to successful plant growth and development. Additionally, certain fern species may have unusual requirements for specific mineral ions. For example, *Adiantum capillus-veneris*, the southern maidenhair fern, has been shown to require elevated levels of Ca^{2+} in order to develop normally (P. von Aderkas, personal communication, November 22, 2023). In Canada, *A. capillus-veneris* is only found in two subpopulations on private lands at Fairmont Hot Springs, British Columbia. There, it resides more than 1000 km north of its main range on tufa rock faces, formed of calcium carbonate. Along the edges of hot mineral water flows, *A. capillus-veneris* can survive in this calcium-rich, humid, and warm microclimate (Environment Canada, 2013). Evidently, *A. capillus-veneris* has a great need for calcium in both *in vitro* and *in vivo* scenarios. The question of Ca^{2+} flux dynamics in *A. capillus-veneris* is just one of the possible future directions this research could take. There are innumerable fern species with unique and peculiar nutrient requirements which can be investigated using the MIFE system.

This research expands upon that of Knox (2023), which established a novel fern breeding program using *P. munitum* gametophyte, by further investigating the physiology of the bred gametophytes. Knox (2023) proposed that this breeding program could advance commercial fern production, whether for agricultural/edible or horticultural purposes. My study provides insight into the nutrient requirements of *P.*

munitum, showing the net flux dynamics of NH_4^+ and NO_3^- in this species and revealing that it may require higher concentrations of NH_4^+ than NO_3^-. Again, there is great potential for future research into broader mineral nutrient flux dynamics in fern breeding programs.

Literature cited

Amatangelo, K. L., & Vitousek, P. M. (2008). Stoichiometry of ferns in Hawaii: Implications for nutrient cycling. *Oecologia*, *157*(4), 619–627. https://doi.org/10.1007/s00442-008-1108-9

Banks, J. A. (1999). Gametophyte development in ferns. *Annual Review of Plant Physiology and Plant Molecular Biology*, *50*(1), 163–186. https://doi.org/10.1146/annurev.arplant.50.1.163

Brooker, R. W., Callaghan, T. V., & Jonasson, S. (1999). Nitrogen uptake by rhizomes of the clonal sedge *Carex bigelowii*: A previously overlooked nutritional benefit of rhizomatous growth. *New Phytologist*, *142*(1), 35–48. https://doi.org/10.1046/j.1469-8137.1999.00384.x

Charnov, E. L., & Bull, J. (1977). When is sex environmentally determined? *Nature*, *266*(5605), 828–830. https://doi.org/10.1038/266828a0

Darrah, P. R. (1993). The rhizosphere and plant nutrition: A quantitative approach. *Plant and Soil*, *155–156*(1), 1–20. https://doi.org/10.1007/BF00024980

DeSoto, L., Quintanilla, L. G., & Méndez, M. (2008). Environmental sex determination in ferns: Effects of nutrient availability and individual density in *Woodwardia radicans*. *Journal of Ecology*, *96*(6), 1319–1327. https://doi.org/10.1111/j.1365-2745.2008.01425.x

Farrar, D. R., Dassler, C., Watkins, J. E., & Skelton, C. (2008). Gametophyte ecology. In T. A. Ranker & C. H. Haufler (Eds.), *Biology and Evolution of Ferns and Lycophytes* (1st

ed., pp. 222–256). Cambridge University Press. https://doi.org/10.1017/CBO9780511541827.010

George, L. O., & Bazzaz, F. A. (1999). The fern understory as an ecological filter: Growth and survival of canopy-tree seedlings. *Ecology*, *80*(3), 846–856. https://doi.org/10.1890/0012-9658(1999)080[0846:TFUAAE]2.0.CO;2

Gibson, A. C., Calkin, H. W., & Nobel, P. S. (1984). Xylem anatomy, water flow, and hydraulic conductance in the fern *Cyrtomium falcatum*. *American Journal of Botany*, *71*(4), 564–574. https://doi.org/10.1002/j.1537-2197.1984.tb12542.x

Goodnoe, T. T., & Hill, J. P. (2016). Absolute and relative content of carbon and nitrogen differ by sex in *Ceratopteris richardii* gametophytes. *Botany*, *94*(5), 405–410. https://doi.org/10.1139/cjb-2015-0254

Harder, W., & Dijkhuizen, L. (1983). Physiological responses to nutrient limitation. *Annual Review of Microbiology*, *37*(1), 1–23. https://doi.org/10.1146/annurev.mi.37.100183.000245

Hawkins, B. J., May, E., & Robbins, S. (2017). Nitrate and ammonium uptake in 21 common species of moss from Vancouver Island, British Columbia. *Botany*, *96*(3), 201–208. https://doi.org/10.1139/cjb-2017-0154

Hawkins, B. J., & Robbins, S. (2010). pH affects ammonium, nitrate and proton fluxes in the apical region of conifer and soybean roots. *Physiologia Plantarum*, *138*(2), 238–247. https://doi.org/10.1111/j.1399-3054.2009.01317.x

Jaffe, L. F., & Nuccitelli, R. (1974). An ultrasensitive vibrating probe for measuring steady extracellular currents. *The Journal of Cell Biology*, *63*(2), 614–628. https://doi.org/10.1083/jcb.63.2.614

Jampeetong, A., & Brix, H. (2009). Nitrogen nutrition of *Salvinia natans*: Effects of inorganic nitrogen form on growth, morphology, nitrate reductase activity and uptake kinetics of ammonium and nitrate. *Aquatic Botany, 90*(1), 67–73. https://doi.org/10.1016/j.aquabot.2008.06.005

Kinosian, S. P., & Wolf, P. G. (2022). The biology of *C. richardii* as a tool to understand plant evolution. *eLife*, *11*, e75019. https://doi.org/10.7554/eLife.75019

Knox, A. (2023). *Establishing a breeding protocol for Pteridophytes* [Unpublished Honours thesis]. University of Victoria.

Krieg, C. P., & Chambers, S. M. (2022). The ecology and physiology of fern gametophytes: A methodological synthesis. *Applications in Plant Sciences*, *10*(2), e11464. https://doi.org/10.1002/aps3.11464

Lewis, O. M. A. (1986). *Plants and Nitrogen*. Cambridge University Press.

Lucas, W. J., & Kochian, L. V. (1986). Ion transport processes in corn roots: An approach utilising microelectrode techniques. In W. G. Gensler (Ed.), *Advanced Agricultural Instrumentation. Design and Use.* (pp. 402–425). Martinus Nijhoff.

Miller, J. H. (1968). Fern gametophytes as experimental material. *The Botanical Review*, *34*(4), 361–440. https://doi.org/10.1007/BF02859133

Newman, I. A. (2001). Ion transport in roots: Measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant, Cell & Environment*, *24*(1), 1–14. https://doi.org/10.1046/j.1365-3040.2001.00661.x

Newman, I. A., Kochian, L. V., Grusak, M. A., & Lucas, W. J. (1987). Fluxes of H^+ and K^+ in corn roots: Characterization and stoichiometries using ion-selective microelectrodes. *Plant Physiology*, *84*(4), 1177–1184. https://doi.org/10.1104/pp.84.4.1177

Plassard, C., Guérin-Laguette, A., Véry, A. -A., Casarin, V., & Thibaud, J. -B. (2002). Local measurements of nitrate and potassium fluxes along roots of maritime pine. Effects of ectomycorrhizal symbiosis. *Plant, Cell & Environment*, *25*(1), 75–84. https://doi.org/10.1046/j.0016-8025.2001.00810.x

Pojar, J., & MacKinnon, A. (2016). *Plants of coastal British Columbia: Including Washington, Oregon and Alaska*. Lone Pine Publishing.

Reid, R., & Hayes, J. (2003). Mechanisms and Control of Nutrient Uptake in Plants. In *International Review of Cytology* (Vol. 229, pp. 73–114). Elsevier. https://doi.org/10.1016/S0074-7696(03)29003-3

Richardson, S. J., & Walker, L. R. (2010). Nutrient ecology of ferns. In K. Mehltreter, L. R. Walker, & J. M. Sharpe (Eds.), *Fern Ecology* (1st ed., pp. 111–139). Cambridge University Press. https://doi.org/10.1017/CBO9780511844898.005

Roy, R. N., Finck, A., Blair, G.J., & Tandon, H.L.S. (2006). *Plant nutrition for food security: A guide for integrated nutrient management*. Food and Agriculture Organization of the United Nations.

Rudolph, H. J., Hohlfeld, J., Jacubowski, S., Von der Lage, P., Matlock, H., & Schmidt, H. (1993). Nitrogen metabolism of *Sphagnum*. *Advances in Bryology*, *5*, 79–105.

Schneller, J. J. (2008). Antheridiogens. In T. A. Ranker & C. H. Haufler (Eds.), *Biology and Evolution of Ferns and Lycophytes* (1st ed., pp. 134–158). Cambridge University Press. https://doi.org/10.1017/CBO9780511541827.006

Shabala, S., Cuin, T. A., Shabala, L., & Newman, I. (2012). Quantifying kinetics of net ion fluxes from plant tissues by non-invasive microelectrode measuring MIFE technique. In

S. Shabala & T. A. Cuin (Eds.), *Plant Salt Tolerance* (Vol. 913, pp. 119–134). Humana Press. https://doi.org/10.1007/978-1-61779-986-0_7

Shabala, S. N., Newman, I. A., & Morris, J. (1997). Oscillations in H^+ and Ca^{2+} ion fluxes around the elongation region of corn roots and effects of external pH. *Plant Physiology*, *113*(1), 111–118. https://doi.org/10.1104/pp.113.1.111

Shabala, S., Shabala, L., Bose, J., Cuin, T., & Newman, I. (2013). Ion flux measurements using the MIFE technique. In F. J. M. Maathuis (Ed.), *Plant Mineral Nutrients* (Vol. 953, pp. 171–183). Humana Press. https://doi.org/10.1007/978-1-62703-152-3_11

Sharpe, J. M., & Mehltreter, K. (2010). Ecological insights from fern population dynamics. In K. Mehltreter, L. R. Walker, & J. M. Sharpe (Eds.), *Fern Ecology* (1st ed., pp. 61–110). Cambridge University Press. https://doi.org/10.1017/CBO9780511844898.004

Southern maidenhair fern (Adiantum capillus-veneris): Recovery strategy 2013. (2013). Environment Canada.

Tischner, R. (2000). Nitrate uptake and reduction in higher and lower plants. *Plant, Cell & Environment*, *23*(10), 1005–1024. https://doi.org/10.1046/j.1365-3040.2000.00595.x

Turetsky, M. R. (2003). The role of bryophytes in carbon and nitrogen cycling. *The Bryologist*, *106*(3), 395–409. https://doi.org/10.1639/05

Von Wirén, N., Gazzarrini, S., & Frommer, W. B. (1997). Regulation of mineral nitrogen uptake in plants. *Plant and Soil*, *196*(2), 191–199. https://doi.org/10.1023/A:1004241722172

Walker, T. G. (1985). Some aspects of agamospory in ferns—The Braithwaite system. *Proceedings of the Royal Society of Edinburgh. Section B. Biological Sciences*, *86*, 59–66. https://doi.org/10.1017/S026972700000796X

Waring, R. H., & Franklin, J. F. (1979). Evergreen coniferous forests of the Pacific Northwest: Massive long-lived conifers dominating these forests are adapted to a winter-wet, summer-dry environment. *Science*, *204*(4400), 1380–1386. https://doi.org/10.1126/science.204.4400.1380

Xu, G., Zhang, C., Lee, S. Y., Chen, Z., & Zeng, X. (2023). The complete chloroplast genome and phylogenetic analysis of *Christella dentata* (Forssk.) Brownsey & Jermy (Thelypteridaceae). *Mitochondrial DNA Part B*, *8*(1), 181–185. https://doi.org/10.1080/23802359.2023.2168114

Yeats, T. H., & Rose, J. K. C. (2013). The formation and function of plant cuticles. *Plant Physiology*, *163*(1), 5–20. https://doi.org/10.1104/pp.113.222737

Appendix A. Measurements used for effect of pH trials.

Sample ID	Sex	pH	Average NH_4^+ flux ($nmol/m^2/s$)	NH_4^+ SD	Average NO_3^- flux ($nmol/m^2/s$)	NO_3^- SD	Average H+ flux ($nmol/m^2/s$)	H^+ SD
PM1	F	7	25.7610207	44.96186	1.5992	12.99149	0.034803	0.08852
PM2	F	7	-9.6344032	19.18583	0.740852	14.44602	-0.175907	0.077426
PM3	F	7	46.3700742	14.50234	0.182865	6.887716	-11.78524	2.761588
PM4	F	7	25.8063581	9.955001	0.6452	10.18329	-0.738148	0.110005
PM5	F	7	-0.26664194	19.52864	-4.534923	18.32713	-0.260587	0.065075
PM6	F	7	4.1564871	25.91998	-3.267458	15.43277	-0.05039	0.056534
PM7	F	7	-33.7003129	24.86916	3.969026	13.32859	-1.106658	0.054619
PM8	F	7	8.40656129	14.72659	0.144668	12.62679	-0.301594	0.095528
PM9	F	7	31.0938774	20.41259	0.428687	12.58124	-0.220919	0.063489
PM10	F	7	-33.1248129	22.59777	6.086365	14.549	-2.4749	0.148393
PM11	F	5	28.6228	13.68281	-1.967771	4.734758	4.249974	0.965905
PM12	F	5	17.7373548	11.5761	-0.209535	6.369979	-1.010484	0.626277
PM13	F	5	0.83128065	21.1186	-6.546806	20.47908	1.353445	1.868306
PM14	F	5	9.46286774	6.863248	-2.826735	16.50004	15.03985	1.771729
PM15	F	5	8.49766452	7.624092	-7.254152	9.05286	18.12141	1.226607
PM16	F	5	8.62655484	5.65399	0.213581	11.36051	7.154803	1.088244
PM17	F	5	20.2937839	4.508224	-12.80355	7.302133	18.54873	2.926949
PM18	F	5	4.16751613	4.051294	-5.922226	7.064315	1.766926	0.999834
PM19	F	5	11.7920871	9.999587	-5.955861	7.54302	1.517784	0.760577
PM20	F	5	6.18926774	8.168331	-5.609484	9.603861	-4.527865	1.47533

Appendix B. Measurements used for effect of sex trials.

Sample ID	Sex	pH	Average NH_4^+ flux ($nmol/m^2/s$)	NH_4^+ SD	Average NO_3^- flux ($nmol/m^2/s$)	NO_3^- SD	Average H+ flux ($nmol/m^2/s$)	H^+ SD
PM11	F	5	25.7610207	28.6228	13.68281	-1.967771	4.734758	4.249974
PM12	F	5	-9.6344032	17.73735	11.5761	-0.209535	6.369979	-1.010484
PM13	F	5	46.3700742	0.831281	21.1186	-6.546806	20.47908	1.353445
PM14	F	5	25.8063581	9.462868	6.863248	-2.826735	16.50004	15.03985
PM15	F	5	-0.26664194	8.497665	7.624092	-7.254152	9.05286	18.12141
PM16	F	5	4.1564871	8.626555	5.65399	0.213581	11.36051	7.154803
PM17	F	5	-33.7003129	20.29378	4.508224	-12.80355	7.302133	18.54873
PM18	F	5	8.40656129	4.167516	4.051294	-5.922226	7.064315	1.766926
PM19	F	5	31.0938774	11.79209	9.999587	-5.955861	7.54302	1.517784
PM20	F	5	-33.1248129	6.189268	8.168331	-5.609484	9.603861	-4.527865
PM21	M	5	28.6228	11.00305	17.15722	-2.269435	26.13454	16.75657
PM22	M	5	17.7373548	77.32399	7.544685	-24.8496	23.38163	18.24774
PM23	M	5	0.83128065	.	.	-31.52257	14.2391	15.21271
PM24	M	5	9.46286774	.	.	-31.27045	10.95533	14.29989
PM25	M	5	8.49766452	.	.	3.705177	14.7652	9.997342
PM26	M	5	8.62655484	65.65839	9.866981	-43.93085	12.87142	24.51936
PM27	M	5	20.2937839	2.614274	9.533898	-17.75021	19.88942	21.16833
PM28	M	5	4.16751613	14.89707	7.909645	-14.68872	13.57097	27.72554
PM29	M	5	11.7920871	16.53898	10.17553	-16.61437	20.7879	28.51473
PM30	M	5	6.18926774	35.32886	14.05894	8.073406	15.98697	29.33371
PM31	M	5	8.282013	7.991947	-0.604742	9.766467	7.522545	1.190651
PM32	M	5	.	.	-2.462265	13.20798	21.2595	11.04259
PM33	F	5	3.553235	15.95195	-3.073348	18.90731	4.289229	0.922386
PM34	M	5	9.711306	5.871598	-3.931719	17.88001	9.394361	0.284404
PM35	F	5	-0.346768	12.02703	-9.839332	14.35014	1.299377	0.90298
PM36	M	5	13.09407	5.454314	-2.718974	12.82138	13.48069	0.385134
PM37	F	5	0.630755	4.412566	-9.499403	13.85618	3.0399	0.539563
PM38	M	5	-1.579477	4.538494	0.156119	18.60815	4.660958	0.311248
PM39	F	5	0.941313	7.792396	-20.66039	18.95449	2.302955	0.715154
PM40	M	5	-6.101942	4.895888	-38.31779	13.66718	13.01317	0.330263
PM41	F	5	-3.743548	8.239734	5.322087	15.45504	0.088735	1.20909

Appendix C. Measurements used for effect of generation trials.

Sample ID	Generation	Species	Average NH_4^+ flux ($nmol/m^2/s$)	NH_4^+ SD	Average NO_3^- flux ($nmol/m^2/s$)	NO_3^- SD	Average H+ flux ($nmol/m^2/s$)	H^+ SD
IB141513	Gametophyte	*C. dentata*	-8.956839	15.43814	-14.53018	21.27893	16.09586	1.169844
IB141531	Sporophyte	*C. dentata*	2.9433	14.63983	0.30009	17.31648	-0.348771	0.92356
IB141545	Sporophyte	*C. dentata*	12.60819	9.908438	-3.942248	27.68381	8.031284	1.296741
IB141609	Sporophyte	*C. dentata*	-7.204768	9.212204	5.131268	19.37448	7.534316	0.931789
IB141625	Gametophyte	*C. dentata*	0.334087	10.59005	2.489968	13.52093	6.740458	1.288115
IB141648	Sporophyte	*C. dentata*	6.631819	13.31258	-1.387942	13.61736	-0.293274	0.823144
IB141701	Gametophyte	*C. dentata*	-19.16093	10.34228	-7.358565	17.33823	3.736968	0.711345
IB141718	Sporophyte	*C. falcatum*	11.56612	9.164602	-1.048881	20.37296	0	0
IB141734	Gametophyte	*C. falcatum*	3.87139	8.388725	-0.885655	22.9814	0	0
IB141748	Rhizome	*C. falcatum*	29.89086	13.74354	4.139839	27.1345	34.46727	4.522569
IB141804	Sporophyte	*C. falcatum*	-7.975597	6.264783	5.877797	21.46668	0	0
IB141816	Gametophyte	*C. falcatum*	1.798248	3.837305	-0.471248	16.17375	0	0
IB141834	Gametophyte	*C. falcatum*	30.93869	4.633749	-6.656726	22.26973	0	0
IB141845	Sporophyte	*C. falcatum*	.	.	7.25751	15.96225	0	0

Appendix D. Measurements used for calculations of total ion flux.

Sample ID	Tissue dry weight (mg)	Approximate surface area (mm^2)
PM11	0.46	32.00284
PM12	0.378	26.29799
PM13	0.638	44.38655
PM14	0.172	11.96628
PM15	0.209	14.54042
PM16	0.155	10.78357
PM17	0.383	26.64584
PM18	0.655	45.56926
PM19	0.538	37.42941
PM20	0.286	19.89742
PM21	0.041	1.587534
PM22	0.007	0.271042
PM23	0.026	1.006729
PM24	0.023	0.890568
PM25	0.014	0.542085
PM26	0.02	0.774407
PM27	0.013	0.503364
PM28	0.013	0.503364
PM29	0.009	0.348483
PM30	0.008	0.309763
PM31	0.008	0.309763
PM32	0.001	0.03872
PM33	1.54	107.1399
PM34	0.013	0.503364
PM35	0.962	66.92768
PM36	0.001	0.03872
PM37	1.306	90.86024
PM38	0.004	0.154881
PM39	0.975	67.83211
PM40	0.001	0.03872
PM41	1.162	80.84196