Plants and fungal diversity analysed by DNA Metabarcoding on canadian pollen sample of honeybees (Apis Mellifera L.) along an urban-rural gradient and across seasons

Auteur : Decolle, Alicia
Promoteur(s) : Francis, Frédéric; 8348
Faculté : Gembloux Agro-Bio Tech (GxABT)
Diplôme : Master en bioingénieur : gestion des forêts et des espaces naturels, à finalité spécialisée
Année académique : 2020-2021
URI/URL : http://hdl.handle.net/2268.2/13196

LIÈGE université
Gembloux
Agro-Bio Tech

“PLANTS AND FUNGAL DIVERSITY ANALYSED BY DNA METABARCODING ON CANADIAN POLLEN SAMPLE OF HONEYBEES (APIS MELLIFERA L.) ALONG AN URBAN-RURAL GRADIENT AND ACROSS SEASONS”

DECOLLE ALICIA

TRAVAIL DE FIN D’ETUDES PRESENTE EN VUE DE L’OBTENTION DU DIPLOME DE MASTER BIOINGENIEUR EN GESTION DES FORETS ET DES ESPACES NATURELS

ANNEE ACADEMIQUE 2020-2021

(CO)-PROMOTEUR(S): FREDERIC FRANCIS – AYAKO NAGASE

“PLANTS AND FUNGAL DIVERSITY ANALYSED BY DNA METABARCODING ON CANADIAN POLLEN SAMPLE OF HONEYBEES (APIS MELLIFERA L.) ALONG AN URBAN-RURAL GRADIENT AND ACROSS SEASONS”

DECOLLE ALICIA

TRAVAIL DE FIN D’ETUDES PRESENTE EN VUE DE L’OBTENTION DU DIPLOME DE MASTER BIOINGENIEUR EN GESTION DES FORETS ET DES ESPACES NATURELS

ANNEE ACADEMIQUE 2020-2021

(CO)-PROMOTEUR(S): FREDERIC FRANCIS – AYAKO NAGASE

In collaboration with Chiba University (Japan)

CHIBA
UNIVERSITY

With the financial support from Erasmus+ scholarship

Acknowledgments

First and foremost, I would like to thank my research supervisors, Mr. Françis for letting me use the offices in the entomological unit at the University of Liège (Gembloux Agro-Bio Tech) and Nagase-sensei let me realise this thesis and to support me and my work through emails and online meeting. Unfortunately, I couldn't meet you in real because of the sanitary situation that didn't allow me to travel to Japan.

I am very grateful to Gregoire Noel who followed all my work and who helps me to deals with a new software and with all the problems I encountered these past few months.

I also would like to thanks Scott McIvor for supervising the sampling in Toronto. It was nice to meet you to discuss about this thesis.

Thank you to Sugita-san for all the help she gives me for the follow-up of administrative markdowns even if the trip could not be made.

And finally thanks to my family, especially my mother for her moral support until the last minute and to my friends for the precious time they gave me when I needed it.

Abstract

The expansion of urbanised areas plays a major role in the sharp loss of biodiversity and threatens the balance of ecosystems and the production of their services to human society. Over the years, cities have become the place where vital issues are at stake for the maintenance of nature in a hostile environment. It is therefore essential to rethink the management of these environments in order to make them more attractive both for city dwellers and for the fauna and flora. These new green spaces will provide pollinators with floral resources and a place to find refuge in cities.

This thesis is part of a continuum of studies carried out in recent years, the primary aim of which is to highlight the influence that the urban-rural gradient and seasonality (variation in months) may have on the richness and composition of plant species. A complementary study aimed at revealing the role that pollinators, and more specifically honeybees Apis mellifera L., 1758, play in the vectoring of microorganisms such as fungi during foraging activities.

To this end, 44 pollen samples were collected from 13 sites in and around the city of Toronto between May and September 2020. These will be subjected to DNA analysis by metabarcoding, using the ITS marker gene, in order to perform taxonomic classification and biodiversity analyses. This classification was carried out using the naïve-bayes workbook and various reference databases and will make it possible to determine the most suitable database for the following analyses.

The taxonomic results allowed the identification of plants and fungi and their characteristics (vegetation structure - herbaceous or woody and status - exotic or native) which were linked to the 2 spatial and temporal factors.

Diversity α, or species richness was measured in 3 ways, the number of species per sample, the Shannon index and the Simpson index. It was then evaluated by applying a generalized linear model (GLM). Diversity β, or species composition was measured by the Jaccard index to calculate the Jaccard distance matrix for constrained ordination (NMDS) followed by multivariate analysis of variance (PERMANOVA) and a post-hoc test to pairwise compare compositional dissimilarities.

Species richness and composition are strongly influenced by season and month, whereas the urban-rural gradient has little influence on plants. However, fungi are slightly more dependent on this gradient. The plant and fungus species identified in the samples have characteristics that can have both harmful and beneficial impacts on their environment.

This master thesis aims to provide decision support for future (re)development of green spaces or greening of streets and buildings in urban and peri-urban environments. It will also shed light on the role of honeybees in the spread of micro-organisms, such as fungi, and its consequences on flowers, pollinator colonies and humans.

Résumé

L'expansion des milieux urbanisés jouant un rôle prépondérant dans la forte perte de biodiversité menace l'équilibre des écosystèmes et la production de leurs services envers la société humaine. Les villes sont devenues au fil des années le lieu d'enjeux vitaux pour le maintien de la Nature en milieu hostile. Il est donc primordial de repenser la gestion de ces environnements afin des les rendre plus attractive tant pour les citadins que pour la faune et la flore. Ces nouveaux espaces verts offriront aux pollinisateurs les ressources florales et un lieu où trouver refuge en villes.

Ce mémoire s'inscrit dans le continuum des études réalisées ces dernières années et dont le but premier est de mettre en évidence l'influence que pourrait avoir le gradient urbain-rural et la saisonnalité (variation des mois) sur la richesse et la composition en espèces de plantes. Une étude complémentaire visant à révéler le rôle que les pollinisateurs, et plus particulièrement les abeilles domestique *Apis mellifera* L., 1758, joue dans la vectorisation de microorganismes tel que des mycètes lors des activités de butinage.

Pour ce faire, 44 échantillons de pollen ont été collecté dans 13 sites situés dans la ville de Toronto et ses environs entre mai et septembre 2020. Ceux-ci seront soumis à une analyse de l'ADN par métabarcodage, en utilisant le gène marqueur ITS, dans le but de réaliser une classification taxonomique et de réaliser les analyses de biodiversité. Cette classification a été menée en utilisant le classeur naïves-bayes ainsi que différentes bases de données de références et permettront de déterminer la base de données la plus adaptée pour réaliser les analyses suivantes.

Les résultats taxonomiques ont permis l'identification de plantes et de mycètes et leurs caractéristiques (structure de la végétation – herbacée ou ligneuse et le statut – exotique ou natif) qui ont été mis en lien avec les 2 facteurs spatiaux et temporels.

La diversité α, ou richesse en espèces a été mesurée de 3 façons, le nombre d'espèce par échantillon, l'indice de Shannon et l'indice de Simpson. Elle a ensuite été évaluée par l'application d'un modèle linéaire généralisé (GLM). La diversité β, ou composition en espèces a été mesurée par l'indice de Jaccard permettant de calculer la matrice de distance de Jaccard afin de procéder à une ordination contrainte (NMDS) suivie d'une analyse multivariée de la variance (PERMANOVA) et d'un test post-hoc pour comparer par paire les dissimilarités de composition.

La richesse et la composition en espèce sont fortement influencée par les saisons et les mois alors que le gradient urbain-rural n'influe que très peu sur les plantes. Cependant les mycètes sont légèrement plus dépendants de ce gradient. Les espèces de plantes et de mycètes identifiés dans les échantillons possèdes des caractéristiques pouvant engendrer des impacts tant dommageables que bénéfiques sur leur environnement.

Ce mémoire de master vise à fournir une aide à la décision pour de futurs projets de (ré)aménagement d'espaces verts ou de verdissement de rues et de bâtiments dans des environnements urbains et périurbains. Il apportera également un éclairage sur le rôle de l'abeille domestique dans la propagation de micro-organismes, tels que les champignons, et ses conséquences sur les fleurs, les colonies de pollinisateurs et les humains.

Contents

List of Figures

List of Tables

1. Introduction

1.1 Pollinators: Importance and decline

Demographic growth and climate change place food security at the top of worldwide challenges. Indeed, food security is essentially linked to pollinators' existence and their close interactions with plants (Marshman, Blay-Palmer, and Landman 2019). Agricultural production of fruits, vegetables and wild flowering plants depends mainly on pollination services provided by animal vectors, which account for 85-90% of the sexual reproduction of flowering plants (van der Sluijs and Vaage 2016; Ollerton, Winfree, and Tarrant 2011). Pollination is responsible for approximately one third of the world food production volume, and 40% of the nutrient supply (van der Sluijs and Vaage 2016; Marshman, Blay-Palmer, and Landman 2019; Eilers et al. 2011). This reliance depends on the climate zone, varying from 78% for tempered areas to 94% for tropical ones (Ollerton, Winfree, and Tarrant 2011). In addition to that, they are indispensable to transfer essential micronutrients, vitamins and/or minerals between flowers for human diet. Their extinction is of great concern.

Pollination services are provided by numerous and diversified animals. The most symbolic ones are insects such as bees, bumblebees, butterflies and hoverflies. Moreover, birds (colibri), mammals (bats) and other animals with coats could be another biotic vector of pollination. Somes scientists hypothesise that in cities the possible negative impact that bee colonies, if present in excess, could have on wild pollinator populations as a result of strong competition for the same food resource (Ropars et al. 2019) It is therefore important to moderate the introduction of domesticated species for the benefit of wild species.

Scientists agree that the world is currently facing its sixth mass extinction. Numerous studies highlight the alarming rate of biodiversity loss over the past centuries (Nazarevich 2015; Oke et al. 2021; Samways 2017; Wagler 2017) specially due to anthropogenic pressures (Cardinale et al. 2012). Land-use change (Otto et al. 2016), habitat loss, fragmentation and/or degradation, pollution, landscape homogenisation, electromagnetic waves (Favre 2011; Kumar et al. 2020), agricultural intensification, pathogens and parasites (*Varroa mites*, *Nosema* sp.) (Sandhu et al. 2016; Tantillo et al. 2015), introduction of non-natives bees, fungicides and pesticides (Goulson et al. 2015) have been highlighted as potential threats to pollinator survival. In general, it is a combination of some of these threats that leads to irreversible and damaging consequences to pollinator populations. In the short-term, land use for urbanisation shows a negative impact on pollinators such as honeybees, but in the long-term, colonies set up in urban areas are stronger and more resistant to certain parasitic fungi such as Nosema sp. (Samuelson, Gill, and Leadbeater 2020). The nutritional quality and the richness of pollen is also higher compared to rural areas. Pollinator's loss also creates disturbances affecting ecosystem resilience and sustainability. In China, productors have to hire people to recreate pollination services due to the lack of pollinators, which is less effective in terms of economy and sustainability (van der Sluijs and Vaage 2016). One study estimates the annual economic value of pollination at €153 billion (Gallai et al. 2009). Humans are therefore highly dependent on their environment.

1.2 Apis mellifera L.

1.2.1 Description of the species

A. mellifera is an insect belonging to the Hymenoptera order and the family Apidae family that was endemic to three regions: Europe, Africa and the Near East. The species have been introduced to other continents through the years by human activities (Eickwort 1990). Today, the endemic European

honeybee is found on every continent except Antarctica. The evolution of honeybees, as well as other pollinator insects, is correlated with angiosperm species, as one cannot live without the other. This mutualistic relationship has been implicated in the biodiversity of both kingdoms, reproduction and the evolution of plant traits (Sauquet et al. 2017; Bronstein, Alarcón, and Geber 2006).

Among all the pollinator species existing in the world, the honeybee is one of the most effective in pollination services. it has therefore been domesticated and managed mainly for agricultural and environmental purposes (Pirk, Crewe, and Moritz 2017). For example to play the role of a bio-indicator, a biomarker to monitor the quality of the environment ((heavy-)metal concentration (Zaric et al. 2018; Sadeghi et al. 2012), presence of metallothioneins and pesticides (Badiou-Bénéteau et al. 2013)) , a pollinating agent or a biological control agent (delivery of fungicide, for example against grey mold *Botrytis cinerea* Pers. on strawberries) (Hokkanen, Menzler-Hokkanen, and Lahdenpera 2015).

1.2.2 Honeybees' colonies

A. mellifera belongs to the eusocial bees. Several generations of honeybees live in society and share the work for the well-being of the colony (Nimmo 2015). Honeybees are organised in different castes to allocate tasks necessary for their survival. Depending on their caste, individuals have developed morphological, physiological and behavioural adaptations to carry out their work (Wright, Nicolson, and Shafir 2018). The first division separates females and males. Female individuals are split in reproductively viable (queens) and non-reproductively viable (workers) subdivisions. The division of males, also called drones, corresponds to less than 2% of the hive population whose only role is to fertilise the queen. Once they are no longer useful, they are driven out of the hive (Schönleben et al. 2007). The future queen is selected by workers among the larvae. Once selected, the larva receives a special diet of large quantities of royal jelly (Schönleben et al. 2007). The queen, responsible for reproduction, lays around 1000 to 2000 eggs daily (Münch and Amdam 2010). Depending on the needs of the colony, the queen will either produce drones (unfertilised eggs) or workers (fertilised eggs) (Nimmo 2015; Schönleben et al. 2007). When the reproduction rate of the actual queen drops and jeopardises the survival of the colony, workers select a new larva. Workers perform a variety of tasks such as maintaining the hive, rearing larvae, providing water to regulate the temperature of the hive, foraging and producing honey, locating food sources or new places to set up a new colony, etc (Nimmo 2015).

Honeybees' societies, which could be associated with the image of a superorganism, have a complex organisation based on several means to communicate. Chemical and olfactory communications take place through the secretion of different types of pheromones (Moritz and Fuchs 1998; Münch and Amdam 2010; Nimmo 2015). Two kinds of pheromones can be secreted, primers and releasers. Primers are intended to modify the behaviour of bees in the long term (e.g. to inhibit reproduction of workers). The latter have a short-term effect to induce an immediate change in behaviour (Trhlin and Rajchard 2011; Paoli and Galizia 2021). In the case of bees, each caste produces different pheromones to regulate the activity of the colony and promote social cohesion. Olfactory communication is not only used to interpret the information transmitted by the colony's pheromones but also to detect and analyse the odours in their environment. These may be associated with danger or food sources (Paoli and Galizia 2021; Suwannapong, Michael, and Eric 2012). They also use dance languages to share their foraging strategies according to floral composition in their environment and to find potential nesting

sites. Through this communication, workers transmit a kind of map including distances and directions to the desired sites (S. Zhang, Si, and Pahl 2012; Suwannapong, Michael, and Eric 2012; Menzel, De Marco, and Greggers 2006).

Honeybees' lifespan varies according to the needs of the colony, the caste and the environment of work of the individuals in it, ranging from a few weeks to a few years. This ageing plasticity is a characteristic of eusocial insects and is linked to floral resources (Münch and Amdam 2010). The workers' tasks are assigned according to age, this organisation is called the polytheism of castes. Young workers start with tasks inside the beehive such as rearing, cleaning and storing pollen. After 2 to 3 weeks, they can be assigned to foraging activities outside the beehive (Winston 1987).

In temperate climates, A. mellifera populations differ within the year. Indeed, worker bees are divided into two seasonal groups: winter bees and summer bees that have differences in behaviour, physiology, longevity and immunology (Kunc et al. 2019; Steinmann et al. 2015). The lifespan of the winter populations can go up to eight months compared to the summer populations which live up to one month and a half. Therefore, colony collapse disorder which occurs mainly in winter can strongly impact colony survival (Kunc et al. 2019).

Nutritional requirements of the colony differ during the year and according to seasonal population, caste and age of the individuals. Winter bees survive thanks to the food collected and stored as honey or beebread by summer bees. Some cases of cannibalism have been observed in colonies when protein was scarce in winter (Crailsheim 1990). The development, productivity and survival of bees and colonies depend on the presence of various nutrients in their food such as carbohydrates, proteins, lipids, vitamins and minerals and on water (Haydak 1970; Brodschneider and Crailsheim 2010). The nutritious quality of the food relies on the diversity of pollen and nectar foraged and stored (Di Pasquale et al. 2013). However, this quality is reduced by the drying of the food during storage (Haydak 1970).

1.2.3 Foraging activities

One of the most important tasks of honeybees is to provide food for the colony throughout the year. For this, the workers are willing to sacrifice their fitness for the survival of the colony (Moritz and Fuchs 1998). As honeybees are generalist foragers, they will visit many plant species to diversify the pollen and the nectar collected. They also harvest water and plant resins called propolis (Louveaux 1958; Seeley 1985). If food is abundant, it is not impossible that they select the species they will visit (Nicholls and Hempel de Ibarra 2017; Wright, Nicolson, and Shafir 2018). But if the quality and/or quantity of pollen is insufficient, the foragers will compensate for this deficit by increasing the amount of pollen collected (Pernal and Currie 2001). For this purpose, bees visit more plants, more species, which increases the probability of transmission of parasites and pathogens, such as the parasitic fungus species *Nosema apis* Zander, to foragers and colonies (Figueroa et al. 2019). As honeybees visit four time more flowers than to solitary bees and hoverflies, they can be an important threat to their species and wild native bees (Albrecht et al. 2012).

Foraging is not only beneficial to honeybees but also to flowering plants as they are in a mutualistic relationship. Indeed, plant species provide pollen and nectar to bees as a reward to ensure the cross-breeding between flowers (Marshman, Blay-Palmer, and Landman 2019; Nicholls and Hempel de

Ibarra 2017). Several plant species have developed adaptations or behaviours to attract particular pollinators by releasing chemical and olfactory signals or by adopting colours or morphological shape (the Orchidaceae family is a great example) (Piñeiro Fernández et al. 2019).

Pollen and nectar are the main components of their diet. Pollen is the primary source of protein to support the proper development of individuals and the only supply of nitrogenous elements, it is also rich in lipids and sugar (Deveza et al. 2015). Nectar, constituted of carbohydrates, is essential to provide energy for activities of the colony (Louveaux 1958). As the need for nectar is greater than the need for pollen, more workers are specialised in collecting nectar only (Pernal and Currie 2001). This ratio of pollen-collecting workers, nectar-collecting workers and workers able to collect both is adapted to the needs of the colony and the time of year (Pernal and Currie 2001). At the same time, all the flowers do not produce nectar and pollen (Von Frisch 1967). Pollen collections are carried by foragers in what is called “pollen basket” located on their hind legs (Hodges 1952). Nectar collections are collected thanks to their tongue.

For their first exit from the hive, foragers study their immediate environment to spot spatial landmarks, new food sources, analyse floral composition (including the nutritional quality of their pollen and nectar) to optimize their works and their movements for future collections. Once back at the hive, foragers recruit new workers and share their navigational memory and foraging strategies with them, through the waggle dance (S. Zhang, Si, and Pahl 2012). The forager can inform the recruits of the presence of a flowering site by detailing the distance to the hive and the direction to the sun (Seeley 1985; Von Frisch 1967). The agitation caused by the waggle dance results in the dispersion of pollen odours which are also used to determine whether the forager is moving towards or away from the target site (Von Frisch 1967).

Foraging activities are highly dependent on the weather and the time. Temperature and relative humidity are the main factors which have an impact on the realisation of this task (Vasudeva and Lokesha 1993; Abou-Shaara et al. 2017). The availability of food depends on the flowering period of the plant species, so foraging activities are quite limited in time. As these activities cost a lot of energy, the workers do not have the possibility to travel too far from the hive. Generally, foragers collect as close to their hive as possible (less than one kilometre), but as the flowering season draws to a close, they must venture further afield to meet the colony's needs (Beekman and Ratnieks 2000)

1.2.4 Honeybees, a vector of micro-organisms

Like plants and in addition to abiotic vectors, several micro-organisms and pathogens, such as fungi, oomycetes, bacteria, yeasts and viruses, have also adapted to make their dispersal by pollinators possible. Flowering plants offer a unique microbiome, providing microbial communities a suitable host for their development and proliferation, which is visited by pollinators serving as vectors to colonise new hosts (Binoy 2018; Trivedi et al. 2020). Some plant pathogens can influence plant-pollinator interactions by altering the attractive traits of flowering plants or by replacing pollen by sporidia in the anther sacs (Schäfer et al. 2010). During foraging activities, bees can carry fungal microorganisms from flower to flower or from flower to hive via the nectar and/or pollen they collect, or via their bodies. Floral nectar is known to be composed by a high abundance and diversity of bacterial and yeast communities able to withstand high sugar levels in nectar (Aizenberg-Gershtein, Izhaki, and Halpern 2013; Mcart et al. 2014). Pollen is considered as a unique microhabitat for micro-organisms

communities, which are essential for the long-term preservation of the stored pollen and for meeting the nutritional needs of bees (Dharampal et al. 2019).

Once these microbial individuals have been transferred to new plant hosts, they will enter the plant either by taking advantage of an existing opening (fungi, bacteria) or by penetrating the host directly (fungi), or via vectors such as insects (viruses) (Card, Pearson, and Clover 2007). While most of the micro-organisms are carried on the pollen, viruses can be found inside the pollen. This happens when the virus has infected a plant and modified its reproduction (Card, Pearson, and Clover 2007). However, there is little information on the transmission of viruses to subsequent generations in plant species (Binoy 2018). Although these microbial communities can have a significant negative impact on agricultural yields and crops quality, causing, along with insect damage, the loss of about 20% of the world's crops (Bebber and Gurr 2015; Fisher, Gow, and Gurr 2016), the relationship between these individuals and the host plant is not always detrimental, but it can also be neutral or beneficial for plants (Scortichini and Katsy 2014; Trivedi et al. 2020). Plants-microbiota association confers beneficial traits to their host, such as promoting plant growth and health, helps them to cope with the abiotic stresses they encounter (Rodriguez et al. 2019; Trivedi et al. 2020; Vannette 2020).

Interactions between microbial communities and bees can be of several types and can result in both benefits and harms: an example of mutualistic relationships is that plant microbiota provide immunity, keeping the bees healthy by constituting a source of nutrients in floral nectar and pollen in exchange for the dispersal achieved by the bees. The presence of microbes in pollen is essential for the proper development, health and survival of larvae and honeybees (Dharampal et al. 2019). Other microbes can threaten the fitness, the longevity of the colony, the bee brood and the honeybee welfare (Tantillo et al. 2015; Mcart et al. 2014). It is the case of the parasitic fungus *N. apis*, some single-strand RNA viruses of the *Dicistroviridae* and *Iflaviridae* families (Tantillo et al. 2015). In combination with abiotic factors, these pathogens can be the one of the causes of epidemic phenomena such as CCD, colony collapse disorder, which leads to the loss of bee colonies through hive desertion. The presence of the ectoparasitic mite Varroa destructor, vector of viruses such as ABPV (Acute Bee Paralysis Virus) and DWV (Deformed Wing Virus), strongly increases these loss phenomena, becoming the first cause of CCD (Posada-Florez et al. 2020; Nazzi et al. 2014; Kang et al. 2015). A study highlighted the rapidity with which this species parasitizes foragers when the mite is found on flowers (about 49% of the mites studied infested the bee on first contact). However, it is difficult to quantify this mode of transmission and to define whether it is possible that it occurs in the opposite direction, from the bee to the flower (Peck, Smith, and Seeley 2016).

Honeybees have developed defences by collecting propolis that have antiseptic properties against certain microbial communities. For example, ferulic acid acts as an antimicrobial agent against various viruses, bacteria and fungi (Seeley 1985).

1.3 Urban beekeeping

The exponential growth of the world's population is accompanied by rapidly expanding urbanisation and an increase in the area devoted to agriculture and industries. This land-use change leads to the fragmentation of natural environments and the alteration of their connectivity, disrupting the balance of ecosystems. It also has a strong influence on the life habits of pollinators (Sari 2020; Z. Liu, He, and Wu 2016; Wilson et al. 2016; Otto et al. 2016). The availability of food and habitat for new hives becomes scarce, forcing pollinators to travel longer distances to cover the needs of the colony.

Moreover, rural areas are gradually becoming less hospitable to them than cities as a result of homogenisation of the landscape (in terms of floral resource) and the use of pesticides (Ollerton et al. 2014).

In this context, urban beekeeping emerged and became more present worldwide. Initially imported to produce bio products such as honey and wax for centuries (Kohsaka, Park, and Uchiyama 2017), honeybees are nowadays used mainly to compensate for the lack of wild pollinators to provide pollination services for agricultural projects. *A. mellifera* is strongly associated with biodiversity protection projects, this image has made it very popular with city dwellers to the point that it is considered a MIMS., a massively introduced Managed Species (Geslin et al. 2017). Honeybees are also considered as a threat to local wild pollinators by competing for the same food resources and ecological niche (McCune 2018) and by being a possible vector of diseases (Douglas B. Sponsler and Bratman 2020; Fürst et al. 2014).

In the past, cities were considered to be an unfavourable environment for all pollinators due to the low availability of floral resources and nesting sites (Bates et al. 2011). However, recent studies highlight that pollinator species richness is higher in rural than in urban areas, except for the *Apis* genus of the *Hymenoptera* family that acclimatise well in the urban environment (Theodorou et al. 2020; Marquardt et al. 2020). The low pesticide use and localized abundance of plant diversity through the importation of ornamental plants provides a more hospitable environment than the monocultures found in rural areas, especially if these environments are connected by ecotones (Theodorou et al. 2020; Marquardt et al. 2020; Lowenstein, Matteson, and Minor 2019). However, the temperature of cities is not suitable for all pollinators, and its concrete environment and manicured parks limit nesting opportunities. All these factors favour generalist eusocial species living in hives whose behavioural plasticity allows them to adapt more easily to anthropized and disturbed environments (McCune 2018).

Green spaces and vegetated infrastructures in the cities therefore have an important role to play in welcoming pollinators to the city, both for beekeeping and wildlife. It is essential to rethink the management of these environments to make them more attractive for both society and biodiversity.

1.4 Plants and Fungi metagenomic from pollen sample

1.4.1 The advent of bioinformatics

In order to determine the current state of ecosystems and the quality of services provided to humans such as pollination, scientists can rely on biodiversity index such as the presence/absence and the abundance of indicator species essential to the proper functioning of these environments (Pavan-Kumar, Gireesh-Babu, and Lakra 2015). One of the techniques used to identify these species is to carry out floristic and faunal surveys based on visual identification in the field. For micro-flora and micro-fauna organisms or elements such as pollen, fungi spores, bacteria and microbes, identification is more complex. For this, palynology, the study of pollen grains and spores, is employed. Once collected and prepared, the samples are analysed under the microscope, then, pollen grains and spores are classified using dichotomous keys and compared to reference collections (Galimberti et al. 2014). However, these methods are time-consuming, need to be carried out at times of the year when species can be accurately identified (for surveys in the field), do not allow species identification of large numbers of samples or surveys with high taxonomic accuracy, and require specialists in palynological, taxonomic, botanical and zoological sciences (even in this case, the identification is still subjective), etc. (Bell et al.

2017; Gous et al. 2019). To overcome these problems, scientists from all fields of study have investigated several areas.

One of the most promising areas is the study of molecular biology and ecology of the species using bioinformatics tools, investigated since the 1980s. From this point on, DNA can be analysed for taxonomic purposes by recognising short gene sequences, called barcodes, thanks to the amplification of genetic markers, called primers. These barcodes are unique to each species (Kress et al. 2015; Kress and Erickson 2008). This approach, called DNA barcoding, does not allow the identification of more than one species at a time (Pavan-Kumar, Gireesh-Babu, and Lakra 2015), the barcode reading technology used being Sanger sequencing (L. Liu et al. 2012). It has therefore been coupled with bioinformatic tools, such as Next-Generation Sequencing (NGS) technologies, allowing the simultaneous sequencing of reads and, consequently, the identification of all taxa present in a sample, reducing analysis time and giving results with high taxonomic resolution (L. Liu et al. 2012; Pavan-Kumar, Gireesh-Babu, and Lakra 2015; Gous et al. 2019). Originally used to identify microbial communities, this approach, called DNA metabarcoding, has been extended to other kingdoms such as plant or fungal kingdoms. However, this method also has drawbacks, it cannot work if the reference database is not complete enough, it can only give qualitative information of the ecosystem composition of fauna and flora.

1.4.2 Pollen and spore DNA metabarcoding

DNA metabarcoding analysis consists of four steps: DNA extraction, amplification, sequencing and analysis (Keller et al. 2015). Firstly, DNA is extracted from the sample collected and prepare for the next step. Then, searchers select the primer (a standardized barcode marker of the genome) to isolate and amplify it through the PCR method. The amplicons obtained are then read to determine their nucleotide order using Next-generation sequencing technologies and classified according to their base sequences (Bell et al. 2017). These sequences are compared with an adapted and exhaustive reference database (depending on the primers, the species studied), enabling them to be assigned a taxonomy and subsequently to carry out biodiversity analysis or to highlight the presence of certain species such as pathogens, parasites, indicators or invasive species (Hebert and Gregory 2005). This information obtained through the new applications of DNA metabarcoding analysis can provide support for the management of natural areas, ecosystems and biodiversity in general (Bell et al. 2016; Kress et al. 2015).

Advantages and drawbacks of plants and fungi primers

Environmental DNA (eDNA) samples, such as pollen sample, are generally multi-species from several kingdoms (e.g. plant and fungal kingdom, etc). It is therefore necessary to test which classification techniques and which primers are the most suitable to perform taxonomic assignment and biodiversity analysis according to the aims of the study (Gous et al. 2019).

For plant kingdom, several genetic marker regions can be used as primer. The first type of primers is based on DNA region of plastid genome such as the *rbcL*, *matK*, *trnL* and *trnH-psbA* markers, the second type is based on a region of the nuclear ribosomal DNA called the internal transcribed spacers (*ITS*) (Gous et al. 2019; Hollingsworth, Graham, and Little 2011; Kress et al. 2015; Bell et al. 2016). The rbcL marker is easily amplified by PCR but is not one of the best for taxonomic resolution, unlike the matK marker (better resolution but more complicated amplification). To improve analysis for taxonomic purposes, these two markers are often combined and have several barcoding applications

(Hollingsworth, Graham, and Little 2011). For certain land plant species, the trnH-psbA marker offers a better discrimination rate and taxonomic resolution and is easy to amplify than the rbcL-matK combination but has a weakness during sequencing, leading to problems of unidirectional reads (Hollingsworth et al. 2009). The trnL marker shows a lower resolution than the other markers because of the low intraspecific variation of the trnL intron, not allowing a biodiversity analysis to be carried out afterwards. To compensate, this marker has some advantages: the high conservation of the primers used for amplification make this procedure very robust and the exhaustive trnL database that already exists for the species identification (Taberlet et al. 2007).

Compared to the first type of primers, which can be used to identify species which have chloroplasts (plants and algae), the ITS region is known as one of the major DNA barcode markers for plants and the universal one for fungi (Schoch et al. 2012). However, it shows less efficiency for animal identification (X. C. Wang et al. 2015). The ITS region can be divided into two markers, the ITS1 and ITS2 subregions. Both allow for high taxonomic resolution (down to the species level) but ITS1 is more efficient for species discrimination, for PCR amplification (due to ITS-u1 and ITS-u2 allowing for higher matching success) and for sequencing (due to their lower CG content in the base sequence) than the ITS2 region for eukaryotic species (Cheng et al. 2016; X. C. Wang et al. 2015; Kress et al. 2005).

The identification of multiple species by a single primer can reduce the risk of prediction error in taxonomic assignment (Gous et al. 2019; Schoch et al. 2012). ITS region has the advantage to have a high resolution of inter- and intraspecific variability, even if the DNA sample is degraded (Cheng et al. 2016). For fungal kingdom, other barcode markers can be used such as the nuclear ribosomal DNA region called the large subunit 25-28S RNA (LSU) (Oh et al. 2014), or the small subunit 18S RNA (SSU) (Demirel 2016). Among these primers, SSU shows the lowest taxonomic resolution, the two others give similar taxonomic results (Brown, Rigdon-Huss, and Jumpponen 2014; Demirel 2016).

Taxonomy analysis – OTU vs ASV

Taxonomy assignment can be performed thanks to microbiome bioinformatics platforms such as Qiime2 (Quantitative Insights Into Microbial Ecology vers. 2). DNA metabarcoding analysis allows two different methods of taxonomy analysis : the construction of operational taxonomic units (OTUs) thanks to UCLUST and UPARSE pipelines and the classification under the amplicon sequence variants (ASVs) name thanks to DADA2, Deblur or UNOISE pipelines (Callahan, McMurdie, and Holmes 2017; Caruso et al. 2019). OTU clustering approach gathers read sequences that have less than 3% of dissimilarities. This consensus sequence created limits the influence of sequencing errors (Blaxter et al. 2005). However, it also generates a bias, by grouping certain sequences, although highly similar, under the same OTU. Depending on the OTU clustering technique used (de novo, open or closed reference), OTUs can be compared or not to other samples or studies (Callahan, McMurdie, and Holmes 2017).

To overcome these drawbacks, researchers have developed other denoising pipelines, whose output files are the ASV table (Callahan et al. 2016; Caruso et al. 2019). The ASV approach does not aim to find a consensus but identifies the exact sequences and counts the number of times they appear in the sample which avoids the creation of a bias due to the clustering as for OTUs. ASV can therefore be compared to other samples of amplicons or other studies and do not depend on reference dataset. A de novo process is used to differentiate sequencing errors from the biological variability of ASV, as errors are less likely to occur in large numbers in a sample. ASV also shows a higher taxonomic resolution and accuracy than OTU clustering (Callahan, McMurdie, and Holmes 2017; Callahan et al.

2019). Finally, each OTU/ASV is read and assigned to a species and a pairwise identity score to state the probability of identification according to the reference dataset.

Taxonomy analyses – taxonomic classifier

Once these OTUs/ASVs have been obtained, taxonomic assignment is carried out by means of algorithms. Depending on the desired resolution and the type of studies conducted, one algorithm can be preferred to another as they offer various taxonomic classification performances (Bokulich et al. 2018). Among the most used alignment-based classifiers for marker-gene amplicon sequences, there are the VSEARCH and BLAST consensus classifiers and the Naive Bayes classifier.

VSEARCH-based consensus classifier is based on heuristic reasoning by performing an alignment and by counting how many nucleotides the OTU/ASV has in common with the reference sequences and assigns the consensus taxonomy corresponding to these reference sequences. Once the entire database has been read, the best match is selected (Rognes et al. 2016).

BLAST-based consensus works in a similar way to VSEARCH but instead of taking only the best match it will associate to each OTU/ASV a top of N potential species with the first N percentage of identity exceeding a given percentage of identity threshold (the number N of hypothetical species and the percentage are defined by the controller). This method will therefore not scan the entire reference database (Camacho et al. 2009).

Compared to the other two algorithms, the Naive Bayes classifier does not rely on consensus. This classifier will apply Bayes' theorem by making the "naive" assumption of conditional independence between features that can identify a nucleotide sequence (Q. Wang et al. 2007). Although considered simplistic, this classification offers good taxonomic results that can compete with other classifiers. However, it requires training of the classifier with the reference database to improve confidence in the taxonomic classification obtained (Newton and Roeselers 2012).

1.5 Objectives

Over the years, the world population continues to grow. It is predicted that it will exceed 8 billion by 2023. To house and feed this growing population, cities are expanding, natural areas are being fragmented and converted to crops, upsetting the balance of ecosystems and threatening global biodiversity. By 2030, cities will be home to around 5 billion people (Ash et al. 2008). Consequently, the management of anthropized environments, whose landscape matrix is highly fragmented, and the maintenance of biodiversity in them are becoming a major issue in the creation/expansion of cities.

Urban green spaces in actual cities constitute a small percentage of the land use and only few streets and buildings are vegetated. These are the only source of food for pollinators, which are so important for the proper functioning of our society. Ecosystem services created by these environments are essential not only for fauna and flora by providing food, ecological niche but also for the quality of life of the inhabitants, the quality of the environment (air, temperature, CO^2 capture), the economy, etc (Loures, Santos, and Panagopoulos 2007; J. Zhang et al. 2019). Urban parks and vegetated places are also the site of a high concentration of microorganisms, such as fungi, because of the presence of plants which provide them an unique microbiome (Kasprzyk et al. 2021). They can be vectored by pollinators, other animals or the air. The transmission of these fungi can have a beneficial, neutral or detrimental impact on flowering plants, crops, bee colonies, other pollinators, as they compete for the same limited food resource, and humans because of their allergenic properties (Kasprzyk et al. 2021).

The honey bee, widely used in urban beekeeping, is a generalist pollinator that represents a significant economic value (Allsopp, de Lange, and Veldtman 2008). However, this species is heavily imported into the world for beekeeping leading to a strong competition with native pollinators (Ropars et al. 2019). Foraging activities of honeybees are usually performed not far from their hive. However, as the green spaces in/around the city centre are fragmented and food resources are inconsistent (in quality and/or quantity) throughout the seasons, foraging behaviour is modified accordingly to meet the needs of the colony (Harrison and Winfree 2015). The health and survival of bee colonies depend on the quantity and quality of the pollen harvested, but also on its diversity. Indeed, pollen diversity can improve the immunity of honeybees (Di Pasquale et al. 2013; D. B. Sponsler and Johnson 2015). The floral composition is therefore decisive for the well-being of the colony and must be considered in urban greening projects.

To understand urban ecology and to improve the integration of nature in the cities of the future, this research will highlight plants and fungi species richness and composition along temporal and spatial gradients thanks to the analysis of pollen samples foraged by honeybees. The objectives are:

- The identification of plant and fungi species and their impact on urban and peri-urban flora and fauna and on humans. This will be carried out by DNA metabarcoding analysis, and various taxonomies will be compared to define the most suitable for this study.
- The analysis of alpha diversity, i.e. the species richness of plants and fungi along the urban-peri-urban gradient and according to the seasons.
- The analysis of beta diversity, i.e. the species composition of plants and fungi and their variation along the urban-peri-urban gradient and as a function of the seasons.

This master thesis aims to provide support for decision making for future green space (re)development projects or street and building greening projects in urban to peri-urban environments. It will also shed some light on the role of the domesticated bee in the spread of microorganisms, such as fungi, and its consequences on flowers, pollinator colonies and humans.

2. Materials and methods

2.1 Study area

This study was conducted on 13 sites in the city of Toronto and its surroundings, included in the Greater Toronto Area (GTA), in Southern Ontario, Canada. This region, considered as the economic centre of Canada, is made up of urban and rural areas, protected natural areas gathered under the name "Green Belt" and agricultural areas. The 13 sites used in this study are mainly located in urban areas, except for C-Farm, Meadow Sweet and Caledon which are in more rural areas (Figure 1).

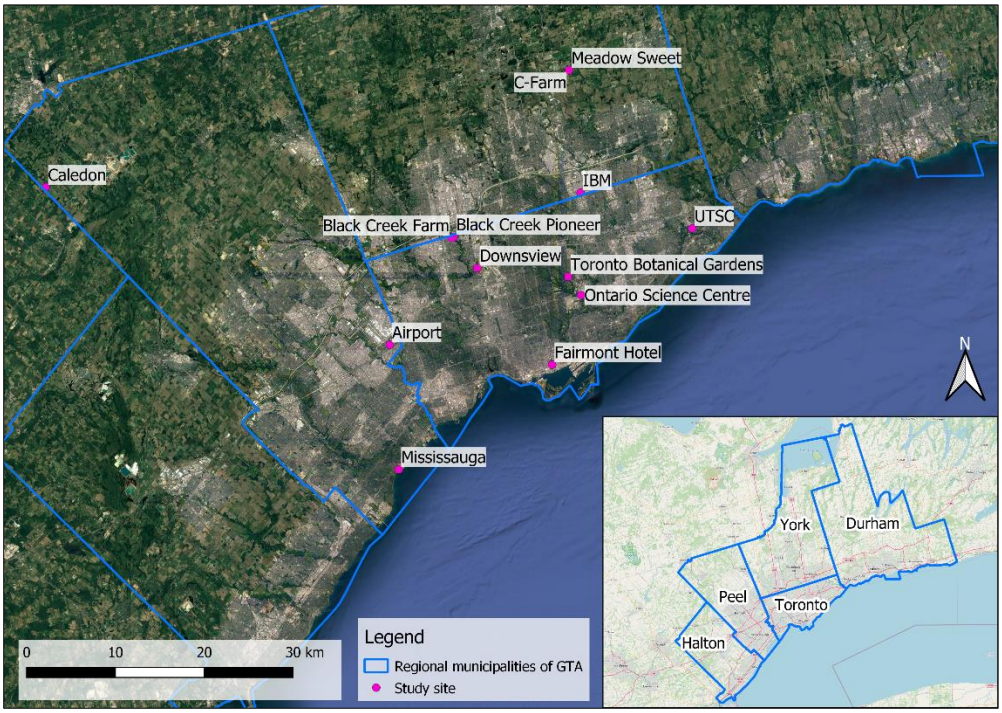

Figure 1: Distribution of the 13 pollen sample collection sites in and around Toronto, Canada (Made with QGIS using OpenStreetMap & Google Earth - Landsat / Copernicus / NOAA satellites)

The abiotic characteristics of the sites may play a role in the foraging activities of the bees. The elevation varies between 74 to 430 m above the sea level and the maximum distance between the 2 most distant sites is 73 km. The difference in altitude also affects temperature and precipitation. The temperature in 2020 is also lower than the monthly averages due to the prolonged winter during the spring. Despite these observations, these factors were considered as constant and the activity of each hive sampled as identical.

2.2 Pollen collection

Each site houses several beehives managed by local beekeepers. Among these hives, one hive per site was selected to carry out the sampling. The selection of hives by beekeepers was based on the colony activity at the end of winter. This is an important criterion to ensure that the pollen traps will not be detrimental to their survival and the development of future generations of honeybees (Owayss et al. 2012). For the sampling, a plastic trap was permanently installed at the entrance of the hive (Figure 2). It has a removable piece, the trellis, which is installed for approximately half a day. The trellis is designed to partially scrape the pollen basket from the hind legs of honeybees entering the hive, causing the pollen grains to fall into the collector.

With the participation of the beekeepers, a total of 44 pollen samples were collected. This collection was carried out monthly between the 21st and 23rd day of the month and between May and September 2020. This variation in days can be explained by the collectors' schedule and weather conditions. More information about these samples can be retrieved in the annexes of this thesis (Annexe A).

The pollen collected, is stored in jars and frozen at -20°C. These jars are then sent to the analysis laboratory (in Japan) to be prepared in solution to perform DNA metabarcoding analysis.

Figure 2 : Pollen trap installed on a beehive © Scott MacIvor

2.3 DNA metabarcoding analysis: DNA extraction, amplification and sequencing

DNA metabarcoding analysis is a revolutionary approach to rapid and accurate species identification and biodiversity analysis from environmental DNA samples. To do this, the sample will be prepared for analysis, the DNA will be extracted, and a gene will be amplified and then sequenced to allow taxonomic classification.

3.3.1 DNA extraction

To carry out the DNA analysis, sub-samples were taken from the total mass of each sample collected and frozen. The average mass of these subsamples is 0.705 g. Some samples do not have enough mass, so the subsamples are adapted accordingly.

DNA was extracted from the pollen samples by adding a Lysis Solution F (from the Nippon Gene kit) and then grinding the mixture at 1500 rpm for 2 minutes using a 'Shake Master Neo' (bms, Shinjuku, Tokyo, Japan), a cell destructive equipment. Once ground, the mixture was left to stand at 65°C for 10 minutes. The sample is then centrifuged at 12,000 x g for 1 minute to remove the supernatant. A Nippon purification solution and chloroform were added to the DNA solution, which was shaken before being returned to the centrifuge at the same speed for 15 minutes.
The purified solution of any supernatant is now ready for further analysis.

3.3.2 DNA amplification & sequencing

Once the DNA has been extracted and cleaned, it will be used to prepare amplicon libraries following the PCR (Polymerase Chain Reaction) protocol (McFrederick and Rehan 2019). To obtain the best performance for DNA analysis and species identification, four primers pairs were tested to amplify different genomic target regions (18S-MiseqF/5.8S-MiseqR, ITS-S2F/ITS4R, ITS1-u1 / ITS1-u2, ITS2-u3 / ITS2-u4). Among these, the primers pair 18S ITS1-u1/5.8S ITS1-u2, amplifying the ITS1 region, was

selected (Cheng et al. 2016). This 2 primers pair allows forward and reverse reads of the ITS1 region and therefore improves the coverage of the region and reduces the risk of error during PCR step.

The amplicon sequences were then sequenced to determine their nucleotide order using Next-generation sequencing technologies and saved in FASTQ files. Two files were created per sample, saving complementary reverse and forward reads. Both amplification and sequencing step were performed using the paired-end sequencing and MiSeq Illumina platform (Illumina, San Diego, CA, USA).

Among the 44 samples of the study, 2 were removed during this process as their quality were not sufficient to allows further analysis.

2.4 Bioinformatics analyses

The data were analysed with QIIME2 (Bolyen et al. 2019), a microbiome bioinformatics platform (using Python code). The analysis followed these steps to transform raw sequences (libraries) into features such as ASV which were subsequently used for taxonomic classification: Firstly, the raw sequence data were imported, aligned and demultiplexed using Casava 1.8. demultiplexed format for the FASTQ files during the importation of the data. Secondly, a plugin called DADA2 (Callahan et al. 2016) was applied on the demultiplexed sequences in order to improve the quality of the data by filtering the data thanks to its error model implemented of quality data, trimming, denoising and merging the paired-end reads. Of the reads, the primers linked to the ITS1 region of each sequence are trimmed (the forward primer is 21 nucleotides long and the reverse primer is 18 nucleotides long). The reads were also truncated at 250 (based on the information linked to the demultiplexed sequences), the length remaining of the sequences allows the merging of paired-end reads. The results obtain after processing by the DADA2 plugin is a list of amplicon sequence variants (ASVs), a feature table indicating which ASV is present in each sample and their statistics. These ASVs were considered as the representative sequences and were compared to the naïve-bayes classifier to assign the taxonomy to the sequences of the samples.

The feature table from DADA2 was filtered based on the taxonomic results to remove mitochondria and chloroplast sequences. The feature table was filtered to remove ASVs with a frequency of less than 10 within each sample and across all samples. These filters allow for better resolution and limit false positives. The results are then converted into a file with a ‘biom format’ and exported.

2.4.1 Reference databases

The taxonomic classification was performed using 4 different reference databases called Toronto, Ontario, UNITE and NCBI databases, ranked respectively from smallest to largest in terms of number of plants species included in the database (for the number of fungi species, the UNITE database is the richest, other databases having the same source to customized them). Toronto, Ontario and NCBI databases were customised databases. Toronto database was based on a list of 1937 vascular plants present in the city of Toronto (Cadotte 2021). This exhaustive list of species is considered as the closest, taxonomically speaking, to the species naturally present in this region and is based on available taxonomic and biogeographic information. Ontario database was based on a list of 3524 vascular plants present in Canada, called VASCAN, developed by the Biodiversity Centre of the University of Montreal (Desmet and Brouilet 2013). The resources (reference sequences and taxonomies) used to create them can be retrieved on the National Centre for Biotechnology Information (NCBI) Genbank data (www.ncbi.nlm.nih.gov/genbank). Fungal resources are from a NCBI bioproject resulting from an international collaboration of specialists with the aim of creating a set of ITS dataset for fungi

(http://www.ncbi.nlm.nih.gov/bioproject/PRJNA177353) (Schoch et al. 2014). Plants and fungi reference sequences and taxonomies were downloaded on QIIME2 thanks to the 'RESCRIPt' python package and were merged to create the classifier (Robeson et al. 2020). UNITE database, specialised in the identification of fungi by using the ITS marker-gene, was downloaded in the UNITE website (https://unite.ut.ee/repository.php) ((Abarenkov et al. 2020).

Here is all the characteristics for the reference databases used for taxonomic classification:

Table 1: Characteristics of the different reference databases used for taxonomic classification. The number between () is the maximum number of species present in the taxonomy (including all unidentified species).

Databases	Toronto database	Ontario database (NCBI)	UNITE database	NCBI database (all plants and fungi)
number of species in the database	Plants: 1.723 sp Fungi: 12.441 sp	Plants: 2.785 sp Fungi: 12.441 sp	Plants: 24.238 sp (34.279 sp) Fungi: 19.032 sp (58.437 sp) other kingdoms: 4.005 sp (9.650 sp)	Plants: 126.215 sp Fungi: 12.441 sp
Percent of Toronto species present in another database	/	Plants: 89,7 % Fungi: 100 %	Plants: 28,5 % Fungi: 47 %	Plants: 100 % Fungi: 100 %
Data source	Plants : (Cadotte 2021), NCBI Genbank Fungi : NCBI Bioproject	Plants : VASCAN, NCBI Genbank Fungi : NCBI Bioproject	Plants & Fungi : all Eukaryote UNITE database	Plants : NCBI Genbank Fungi : NCBI Bioproject

Remark: In the taxonomy of the UNITE database, all species under the label "unidentified" have been removed. This remove may underestimate the actual number of species in the taxonomy.

The comparisons between the different taxonomic results and the sample species composition were carried out thanks to Microsoft Excel software (version 16.0.14228.20216 (64-bit)).

2.4.2 Classification method

Taxonomic classification was performed using the Naïve-Bayes classification. This classification is based on the probabilistic Bayes theorem coupled with a naïve hypothesis (hypothesis with a strong independence) that suppose a characteristic for a species is independent of the existence of other characteristics allowing the identification of this species.

$$P(A|B) = \frac{P(B|A)\,P(A)}{P(B)}$$

This formula is the Bayes theorem and gives the probability of A (e.g., a species) given B (e.g., a sequence). $P(A|B)$ is the probability of a species matching a sequence in the sample. As the naïve hypothesis considers all characteristics as independent, the formula can be adapted as follows:

$$P(A = k|B_1, \dots, B_n) = \frac{P(B_1|A = k) * \dots * P(B_n|A = k) * P(A = k)}{P(B_1) * \dots * P(B_n)}$$

The naïve-bayes classifier can be mathematically written as follows:

$$A = argmax_A\, P(A) \prod_{i=1}^{n} P(B_i|A)$$

This classifier will analyse the probabilities of all species that could match the sequence of the sample and select the one with the maximum probability.

To assign taxonomy, a naïve bayes classifier was trained with the Toronto reference sequences and taxonomy and tested to obtain taxonomic assignment on the representative sequences of the sample. This action is carried out thanks to the 'q2-feature-classifier' plugin and the parameter to keep only those taxonomic ranks that were identified with 97% confidence. These results will be analysed and interpreted in terms of species richness and composition.

2.5 Urban-Rural gradient creation

The urban-rural gradient is characterised by the percentage of urbanised areas. Urbanised areas include buildings, concrete car parks, roads, railways and airport runways. They can therefore be defined as all the impermeable surfaces where vegetation cannot develop. Wasteland areas and quarries are not included in these areas because they can host ruderal and pioneer species in a hostile environment.

The transport network data used to create the urbanised area shapefiles were downloaded from the open-source data available on the Government of Canada website (https://ouvert.canada.ca/fr). These data were published in 2017. The other urbanised areas were created by combining visuals from Google Earth data and Open Street Map data.

The digitalisation process of urbanised areas was done using QGIS software (version 3.10.14-A Coruña). The percentage of urbanised areas was calculated within a 2,2 km radius of each sampled hive. This distance corresponds to the mean distance that honeybees travel between the hive and the forage site and was also chosen so that the digitisation of the urbanised areas would not be too time consuming (Seeley 1995). The delimitation of 2,2 km radius circle was adapted when the hive is located within 2.2 km of Lake Ontario (Figure 3).

$$\%_{urbanised\ area} = \frac{digitalised\ surfaces\ (ha)}{surface\ of\ the\ 2{,}2\ km\ circle\ (ha)}\ x\ 100$$

This percentage is ranged between 0 and 76 %. 0 corresponds to the rural area with a smaller area where soils are impermeable and 76% corresponds to the most urbanised area (the city centre).

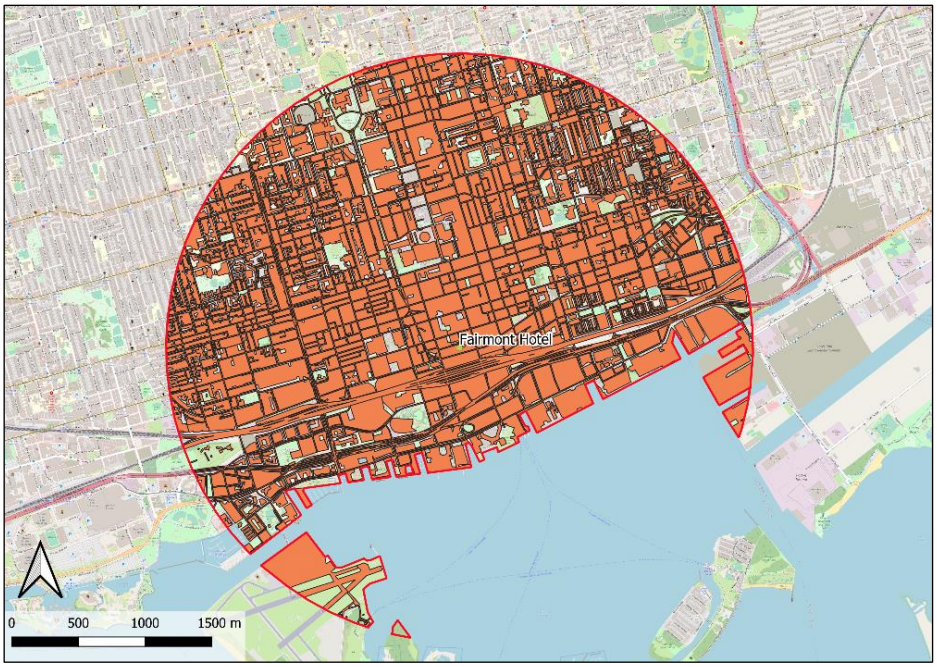

Figure 3: Digitalisation of the urbanised areas in the Fairmont Hotel site (Made with QGIS using OpenStreetMap)

An underestimation of the surfaces occupied by roads in urban areas must be considered, as the buffer applied to the whole transport network is identical along the urban-rural gradient and therefore does not allow the width of certain urban roads to be covered.

Vegetated projects, installed on some buildings (e.g. green roofs), have been included in the urbanised areas although their contribution to the plant diversity of the city is not negligible. Their digitisation would have been too complex in some cases.

2.6 Biodiversity Analysis

Alpha and beta diversities were performed on the Toronto taxonomic results combined with the ASV table containing the number of reads per ASV and were analysed across sites, along an urban-rural gradient and the months (seasons) using Rstudio software (R version 1.4.1103). The results were analysed with a qualitative approach thanks to the presence-absence data of the species per sample as the number of reads cannot be interpreted as abundance of a species in a sample. The plant and fungus data were processed separately to obtain the alpha and beta diversity for each kingdom.

2.6.1 Alpha diversity analysis

The α diversity can be defined as the richness of species within an area, ecosystem or habitat on a local scale, i.e. the number of distinct species in each site studied (Whittaker 1972).

Alpha diversity was measured in three different ways using the absence/presence matrix of species according to the characteristics of the samples: the first is the species richness (e.g. the number of species observed per sample), the second is based on the Shannon's diversity index and the last on the Simpson's diversity index. These measures were done using the 'Phyloseq' package (version 1.36.0).

Shannon's diversity index (H') varies from 0 (no diversity) to ln(S) (a maximum of diversity).

$$H' = -\sum_{i=1}^{S} p_i \ln (p_i)$$

Simpson's diversity index (D) varies from 0 (no diversity) to 1-1/S (a maximum of diversity).

$$D = 1 - \sum_{i=1}^{S} p_i^2$$

Where:

p_i : the proportion of the total sample represented by the species i.
i: a species of the study area.
S: the total number of distinct species.

Compared to species richness, the Shannon and Simpson indices decrease the weight of the presence of rare species in the samples.

A Generalised Linear Regression Model (GLM) with a gaussian error distribution was applied to the number of species to evaluate the effect of the months and the urban-rural gradient (environmental factors) on this richness. The null hypothesis is that there is no interaction between the dependent variable and one or both predictor variables. This statistical analysis was performed using stat package. The linear regression was added in the alpha diversity graphs with a confidence interval of 0,95.

Residual analysis was carried out to check compliance with the conditions for the application of GLM analysis. A Shapiro test was used to evaluate the homogeneity of variance.

The graphical representations were generated using the ggplot2 package (Wickham 2010).

2.6.2 Beta diversity analysis

The β diversity can be define as the differentiation of species composition between several areas, ecosystems or habitats (Whittaker 1972). To measure this dissimilarity between the different sites, the Jaccard similarity index (S) from the R package 'vegan' was used.

This Jaccard similarity index can be written as follows for the analyse between 2 samples:

$$\beta_{jac} = \frac{a}{(a + b + c)}$$

Where:

a: number of species present in the 2 samples.
b & c: number of species absent from one of the 2 samples.

The Jaccard distance matrix, obtained thanks to this index, was considered as the response variable to apply a permutational multivariate analysis of variance (PERMANOVA) using the 'Adonis' function with 999 permutations from the 'Vegan' package (Oksanen et al. 2012). The factors used were the urban-rural gradient and the months. If this analysis shows significant differences, a pairwise post-hoc test with Bonferroni correction using the pairwise.adonis function from the 'pairwiseAdonis' R package (Martinez Arbizu 2020). The null hypothesis of this test is that there is no difference between 2 factor combinations.

To visualise the variation in species composition as a function of spatial and temporal factors, non-metric multidimensional scaling (NMDS) was performed using 'ggplot2' package (Wickham 2010). This NMDS is an iterative and non-parametric ordination based on a Jaccard distance matrix.

3. Results

3.1. Taxonomy analysis

Taxonomic classification was performed with 4 different databases: 3 customised databases (Toronto, Ontario, NCBI) and 1 downloaded database (UNITE). The comparison was made before filtering the results according to the number of reads of the ASVs (only the percentage of identity at 97% was applied). The table of comparison of the different results can be retrieved in the appendices of this thesis (annexe B). A sample of these results are presented as follows:

Table 2: Sample of the comparison table for the Toronto taxonomic results

Toronto taxonomic results							
ASV	**kingdom**	**phylum**	**class**	**order**	**family**	**genus**	**species**
ASV 1	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	Starmerella	Starmerella bombicola
ASV 2	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	Starmerella	Starmerella bombicola
ASV 3	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	Starmerella	Starmerella bombicola
ASV 4	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae sedis	Starmerella	Starmerella bombicola
ASV 5	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycopsidaceae	Saccharomycopsis	Saccharomycopsis capsularis

The taxonomic results contain 1.111 ASVs and were ranked in descending order of the number of readings of an ASV in all samples.

To highlight the precision of the taxonomic resolution, the results were analysed by database and compared (Figures 4 & 5). This comparison helped to decide on the most appropriate reference database to carry out the biodiversity measurements on samples from an area geographically limited to the Greater Toronto Area.

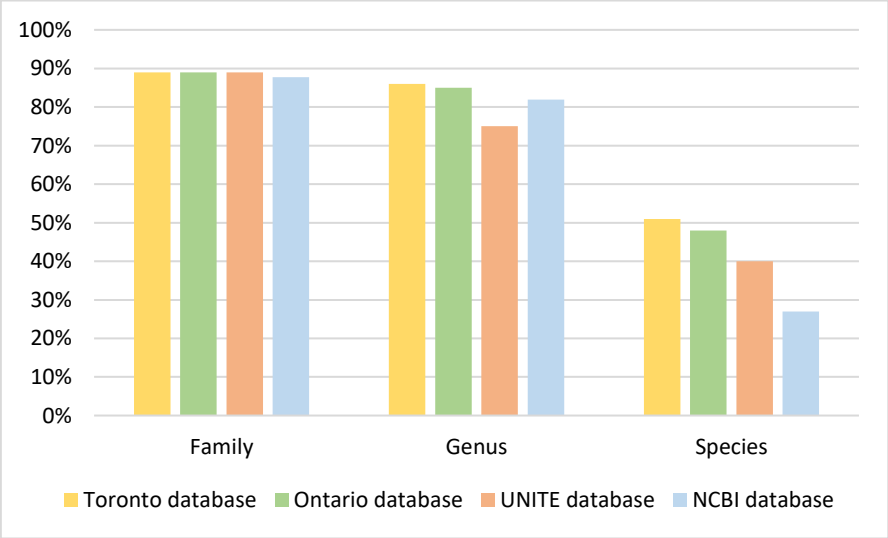

Figure 4 : Percentage of taxa identified a taxonomic rank with 97 % certainty for the 4 different databases

The comparison shows that, overall, the number of taxa identified for each taxonomic rank decreases with increasing taxonomic resolution. The study of the results according to each rank shows that, for the family taxonomic rank, the number of taxa identified remains stable regardless of the database used. The results obtained for the genus level remain substantially stable except for the UNITE database which goes down slightly. For the species rank, the number of taxa identified up to the species level shows a strong decrease for all databases with a more significant reduction with the rise of the number of species constituting the reference databases.

The number of plants and fungi species in the reference database therefore has an influence on taxonomic resolution. A database containing species present in the geographical and/or climatic region is therefore more suitable for carrying out biodiversity analyses at the species level than a general database covering a wider range of species present on earth. Moreover, this choice also reduces the risk of mismatches between the sequences representative of the sample and species whose probability of occurrence on the sites studied is (almost) zero.

The analysis of the number of families and species according to the database used also demonstrates this reduction in taxonomic resolution (Figure 5).

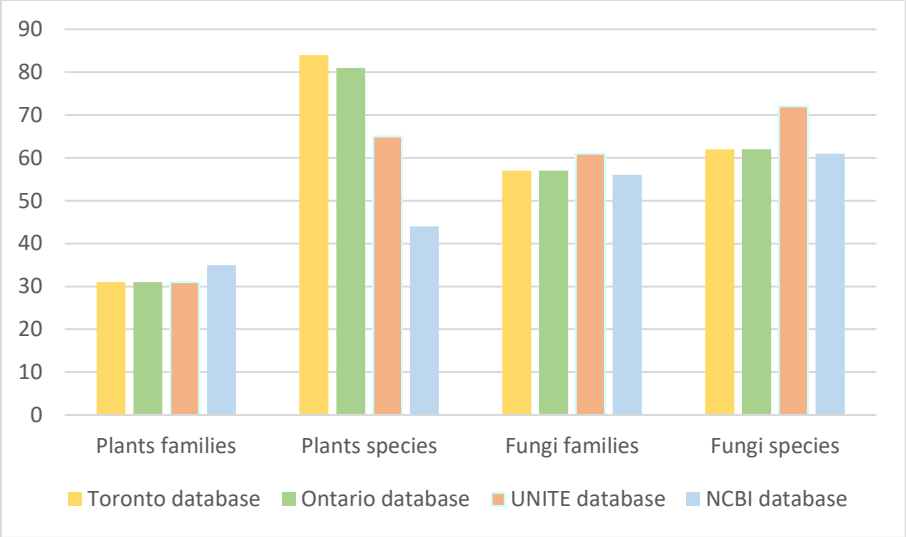

Figure 5 : Number of distinct families and species identified in the 4 taxonomic results

While for the taxonomic rank of families the results are essentially stable for both plants and fungi, for the rank of species, the number of distinct plant species decreases with the increase of the number of plants species in the reference database. The number of fungi species/families is identical for the custom databases as they are based on the same source (NCBI Bioproject). The UNITE database shows a higher number of fungus families and species compared to the other results. This shows there is a slight weakness in the identification of fungi due to a lack of information in the other databases.

The correspondence between Toronto and the 3 other databases results was analysed to highlight whether the ASVs were indeed identified as the same species between them (Table 3). Among these species for the Toronto results, 173 species are fungi and 391 species are plants.

Table 3: Percent of correspondence of plants and fungi species between the Toronto results and the 3 other results

	Ontario results	**UNITE results**	**NCBI results**

Correspondence of plant species	87%	28,4%	17 %
Correspondence of fungi species	*	42%	*

*As the source for the fungi data is the same for all 3 custom databases, only the match for UNITE fungi is calculated.

The difference between the Toronto and Ontario reference databases for the plants species does not fully explain the percentage of matches, as most species that do not match between the two results are present in both databases. As said before, fungi species have a 100% match.

The UNITE results show that unlike the results for Ontario, the match here is mainly related to a lack of adapted data for plants in the UNITE reference database.

Of the few taxa identified to species for the NCBI results, very few match with the plant taxa of Toronto although all species are present in both databases.

3.2. Composition of the sample

The floral and fungal composition of the samples was analysed from the taxonomic results of the Toronto database and the ASV table containing the number of ASVs read per sample. Data were filtered according to the number of ASV reads, removing ASVs with less than 10 reads per and for all samples. A total of 79 plants species and 46 fungi species was recorded in the investigated study sites. The list of fungi and plant species can be retrieved can be found in the annexes of the report (annexe C).

3.3.1 Plant species

Among the 79 plant species, the 3 species that were observed in the most samples are: *Trifolium repens* L. (30 samples), *Sonchus arvensis* L. (15 samples) and *Trifolium pratense* L. (13 samples). The 3 most represented plant families, in terms of number of distinct species, are: Asteraceae (31 sp), Fabaceae (9 sp) and Brassicaceae (7 sp).

Among these identified species, there are:

- 1 pest species from the genus Rhamnus (*R. cathartica* L.).
- Some highly allergenic species such as *Ambrosia trifida* L.
- Several cultivated species found in few samples, such as the *Cucumis melo* L., *Cucumis sativus* L. and *Lactuca serriola* L., *Allium tuberosum* Rottler ex Spreng. 1825, *Cichorium intybus* L., 1753
- Several invasive species such as Alliaria petiolata

The structure of the vegetation is composed of 5 plant strata and sub-strata: herbaceous, graminoids, vines, shrubs and trees. These elements have been grouped into 2 categories: Herbaceous species (Herbaceous, Graminoid, vine) and Woody species (Shrub, trees). The species were also divided into two classes: native species and exotic species. Their distribution in all samples (Figure 6) as well as their distribution according to the 2 factors (Figures 7 & 8) was carried out.

Figure 6: Percentage of Herbaceous - Woody & Native - Exotic species in samples

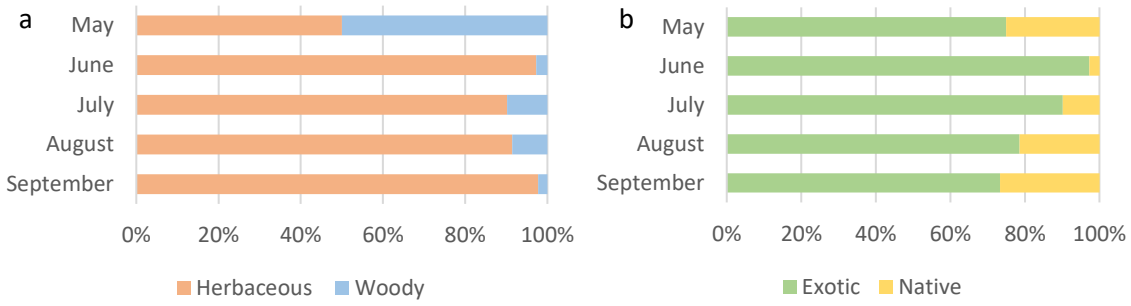

Figure 7: Percentage of Herbaceous - Woody species (a) and Exotic - Native species (b) according to the months

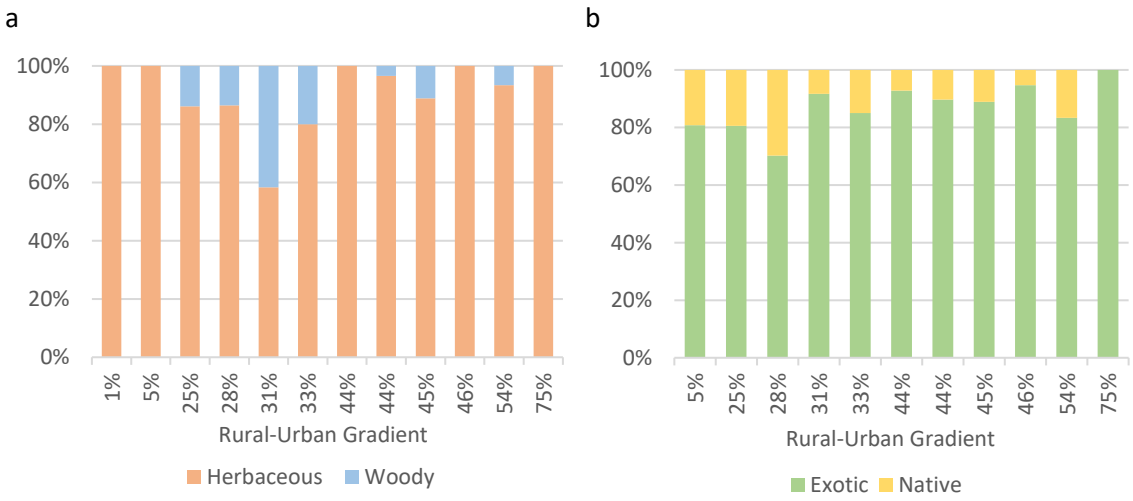

Figure 8: Percentage of Herbaceous - Woody species (a) and Exotic - Native species (b) according to the Rural-Urban Gradient

The vegetation in and around Toronto is dominated by herbaceous species (91%). The proportion of trees and shrubs tends to decrease with the month but decreases sharply in June and increases slightly in July. The proportion of trees and shrubs shows an increasing presence with the urbanisation of the area, reaching a maximum in the urban neighbourhoods and then it declines as we move closer to the city centre. No trees were identified in rural areas and in the hyper-centre of Toronto.

Regarding 'Exotic' and 'Native' status, exotic species dominate the landscape (85%). Except for May, the collection of pollen of species native to the region by the bees seems to follow an increasing trend

with the months. The distribution of species according to status along the rural-urban gradient is rather heterogeneous and does not show a clear trend. Nevertheless, it is possible to observe a greater proportion of native species in rural areas than in urban areas, with a total absence of native species in downtown Toronto.

3.3.2 Fungi species

Among the 46 fungi species, the 3 species that were observed in the most samples are: *Starmerella bombicola* C.A. Rosa & Lachance (31 samples), *Mucor circinelloides* Tiegh. (28 samples), Mucor falcatus Schipper (24 samples). The 3 most represented fungi families, in terms of number of distinct species, are: Saccharomycetaceae (6 sp), Saccharomycetales – Incertae sedis (6 sp) and Mucoraceae (5 sp).

The 10 most present fungi species were analysed in terms of influences/effects on plants, pollinators and Human (Table 4).

Table 4: Influences, effects and uses of the top 10 fungi species on their environment (plants, pollinators and Human).

Fungal species	Influence (beneficial +, neutral 0, detrimental -, ND: no data)			Examples of uses by Human	# of reads	# of sample Observed in	References
	Plant	Pollinator	Human				
S. bombicola	0	(+)	0	Producer of biosurfactant, vinification	508646	31	(De Graeve et al. 2018)
M. circinelloides	0	(+)	-	Biodiesel production	211501	28	(Wagner et al. 2019), (Carvalho et al. 2017)
Z. rouxii	0	ND	ND	Food and vine spoilage agent, fermentation, and sugar concentrates	131620	18	(Pitt and Hocking 2009)
Saccharomycopsis capsularis	ND	ND	-, 0		109624	3	(Ejdas 2014)
M. falcatus	ND	ND	-		63203	24	(Wagner et al. 2019)
Starmerella lactis-condensi	0	ND	ND	Vinification (fermentation, spoilage effects)	56692	10	(Čadež et al. 2020)
Mucor lusitanicus	ND	(+)	-	Ethanol producer, source of lipids and carotenes	38742	17	(Wagner et al. 2019), (Navarro-Mendoza et al. 2019)
Starmerella jinningensis	ND	ND	ND		36830	22	
Moniliella pollinis	ND	ND	ND	Polysaccharide and polyol	8954	18	(Sarkar, Hennebert,

				(Erythritol) producer			and Mayaudon 1986), (Thoa et al. 2015)
Lachancea quebecensis	ND	ND	ND		7275	2	

() Indirect beneficial, detrimental influences.

The influence of fungi on their environment is poorly documented, with only the best-known species having a well-documented impact on the subject.

In all samples, 4 species (8.7%) of fungi are detrimental to plant survival. These are the following species: *P. coronati-agrostidis* M. Liu & Hambl., 2013 and *P. coronati-brevispora* M. Liu & Hambl., 2013, *Alternaria rosae* E.G.Simmons & C.F.Hill and *Stemphylium loti* J.H. Graham, 1953. The other species, for which data are available, do not show an adverse effect on plants. Fungi use flowers as substrates for their growth.

The impact of fungi on human health, when documented, is mainly negative. Of the top 10 species, 40% affect human health.

Indirect positive effects can be observed between fungi and pollinators, mainly because the latter contribute to the nutritional value of their food (via pollen and nectar)

3.3. α diversity

The raw data consists of 2.457.263 number of reads and 1.111 ASVs (features) distributed in 42 samples. After applying various filters, the data is as follows: 1.664.808 number of reads, 1.111 ASVs with 563 identified up to the species level. The number of distinct plant and fungus species is 79 and 46 respectively.

The species richness was analysed separately for the 2 kingdoms. The number of species observed the Shannon and Simpson diversity indices were compared according to the 2 factors (month, urban-rural gradient).

3.3.1 species richness

Species richness of plants was combined with a logistic regression (GLM) (Figure 9). This statistical analysis is performed using a model where the number of plants species is the dependant variable, and the 2 factors are the predictor variables. The GLM results show that the rural-urban gradient has no influence on the plant richness (t=1,01, p-value= 0.32). The null hypothesis is therefore accepted. For plants species richness, the level of significance for the months is higher than for fungi. Indeed, the results for July and August show a strong influence on species richness with highly significant differences (t= 4,05 and t= 3,83 respectively with $p < 0,001$). However, the month of September has less influence than for the fungi, while remaining significant (t= 2,30, p-value= 0,03). There is no relation for June (t= 1,27, p-value= 0,21).

Based on the graphical analysis of the residuals and on the Shapiro normality test, the 3 application conditions are fulfilled.

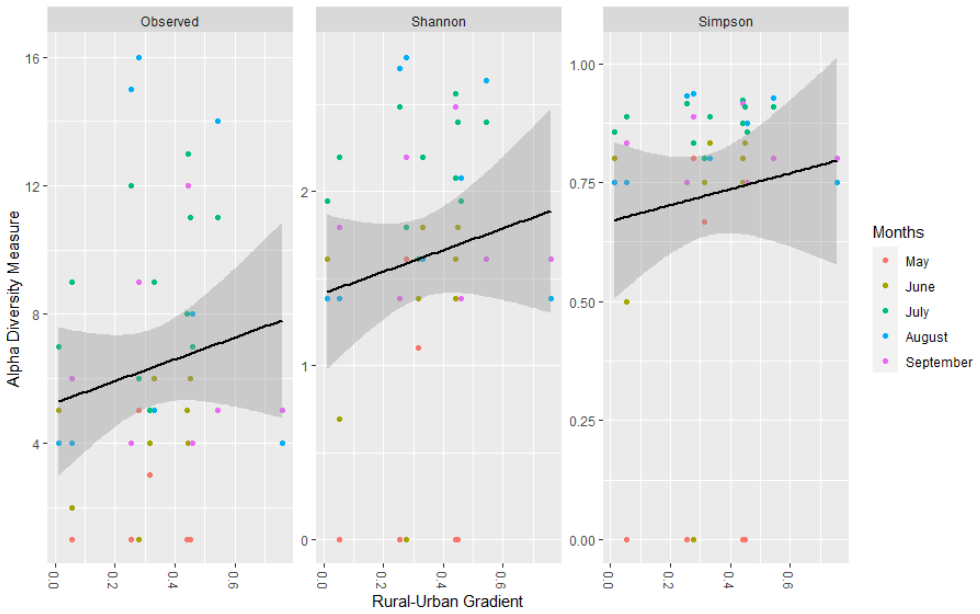

Figure 9 - Alpha diversity measures for plants species using the species richness, Shannon and Simpson indexes.

The alpha diversity graphs show, as well as fungi diversity, a positive relation between the species richness and the rural-urban gradient. However, the difference between the 3 indices is less obvious than for the fungi because the number of outliers is higher and pulls the model down. The slope of the line is also lower, which is explained by the absence of a statistical relationship between the spatial gradient and species richness. The same remark can be made here as for the fungi about the 4 outliners present in the dataset.

Species richness was then analysed only with the 'Month' factor (Figure 10). For plants, the species richness rises to reach its highest point in August and then decrease in September. The month of August is also characterised by a strong variation in species within these samples.

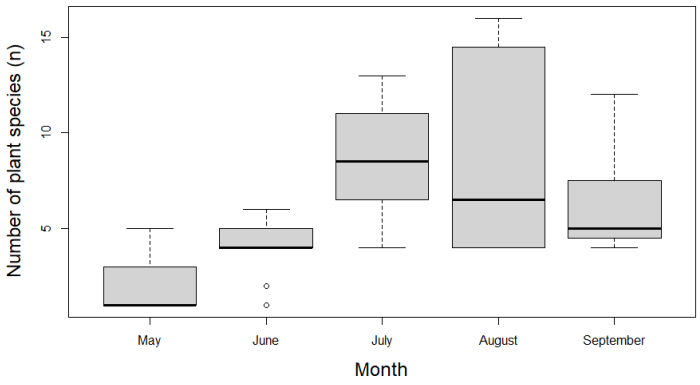

Figure 10 - Boxplot of the species richness for plant species by month.

May and June show a low species richness coupled with a low variation in species due to the presence in these two months of 'outliers' (not represented here for May) when July and August show the highest diversity of plants.

3.3.2 Fungi species richness

Species richness of fungi was combined with a logistic regression (GLM) (Figure 11). This statistical analysis is performed using a model where the number of fungi species is the dependant variable, and

the 2 factors are the predictor variables. The GLM results show that the number of species is at the limit of significances according to the rural-urban gradient, the null hypothesis can either be accepted or rejected (t= 1,85, p-value= 0,07). As for the months (compared to the results of May), there is a significant difference for August and September with the number of species (t= 2,39, p-value < 0,05 and t=2,48, p < 0,05 respectively) and there is uncertainty about the effect of July because its p-value is at the limit of significance (t=1,91, p-value= 0,06) there is no interaction for June (t=0,88, p-value=0.38).

Based on the graphical analysis of the residuals, the 3 application conditions are fulfilled.

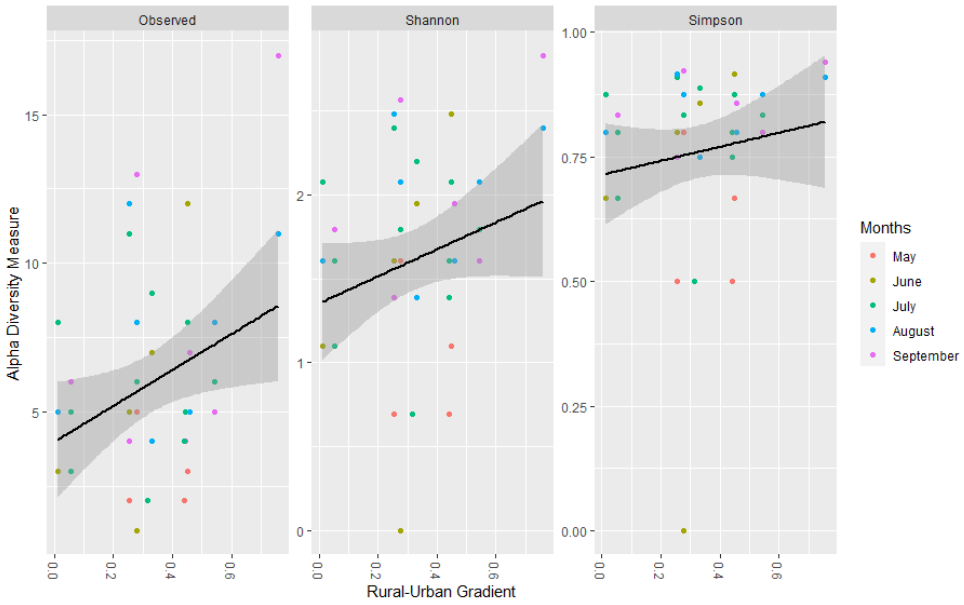

Figure 11 - Alpha diversity measures for fungi species using the species richness, Shannon and Simpson indexes.

The alpha diversity graphs show a positive relation between the species richness and the rural-urban gradient. On this graph, one of the samples (one point on the graph) has only one fungus species and could be considered an outlier. Despite this, the species will not be removed because this same point has several plant species, if this point were removed from the dataset, then the dataset would be severely depleted due to the small number of samples in this study.

Species richness was then analysed only with the 'Month' factor (Figure 12). This tends to increase from May to August and then stabilises between August and September. The species richness in September shows a higher variation between the samples composing the month. One data item in June is also considered to be an outlier, this situation has been explained previously.

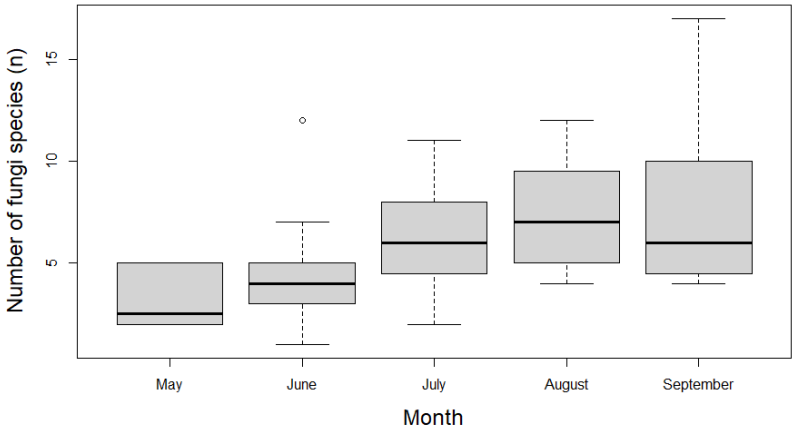

Figure 12 - Boxplot of the species richness for fungi species by month

3.4. β diversity

The species composition was analysed separately for the 2 kingdoms. Firstly, the Jaccard distance matrix was measured and analysed with a PERMANOVA approach and a pairwise to compare 2 per 2 the Permanova results. Then this matrix is used to perform a non-metric multidimensional scaling (NMDS) ordination. The results will also be observed according to the seasons. The months were divided as follows, the collection taking place around the 21st to 22nd day of the month: Spring (May), Summer (June, July and August) and Autumn (September).

3.4.1 Plant species composition

Permanova outcomes are similar to those for fungi. It shows high significant variations of species composition for the month period (F= 3,05, R^2= 0,25, P-value < 0,001) and a p-value close to significance and whose interpretation is uncertain for the rural-urban gradient (F=1,46, R^2= 0,03, P-value = 0,06). The post-hoc analysis (pairwise adonis), applied on the month factor, reveals similarities in composition between month, taken 2 per 2, sharing the same letters (letters from figure 13). Significant dissimilarities are observed for each combination (adjusted p-value = 0.01) except for the July-August and August-September combinations which share a large number of species in common in their compositions (adjusted p-value = 0.22 and adjusted p-value = 0.30 respectively). The spatial gradient has no influence on the plant species composition.

NMDS ordination shows variabilities in community composition for the month factor (Figure 13). Firstly, plant communities clustered by seasons reveal there is a difference in composition between Spring and the 2 other seasons while Fall and Summer share similarities by their overlapping ellipses. The colour gradient does not give any interpretable information, which confirms that the rural-urban gradient has no effect on the variation of species composition.

There is also a strong variation in species composition within the fall, with the points being far apart from each other. In contrast, spring shows little variation in composition due to the presence of 4 out of 6 samples being composed of only one species.

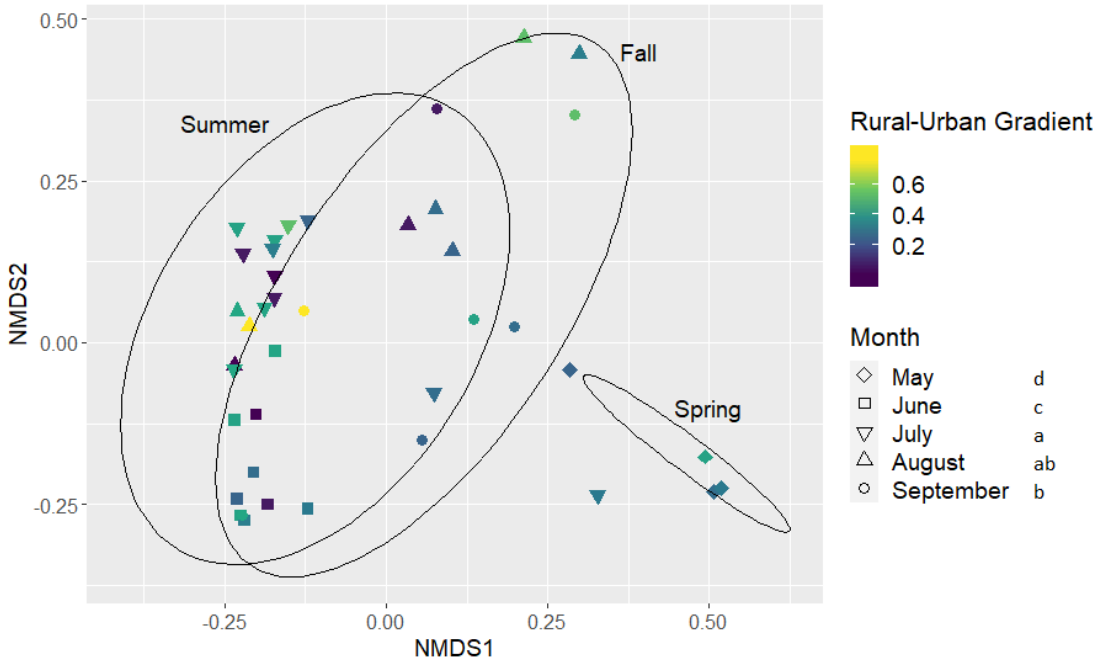

Figure 13 - Non-metric multidimensional scaling (NMDS) of plant communities. Each point corresponds to a sample and is coloured according to the rural-urban gradient with a viridis colour palette. The month are symbolized by shapes and the seasons are represented by 80% prediction confidence ellipses determined from Shepard diagram.

3.4.2 Fungi species composition

Permanova outcomes shows high significant variations of species composition for the month period (F= 2,31, R^2= 0,20, P-value < 0,001) and a p-value close to significance and whose interpretation is uncertain for the rural-urban gradient (F=1,75, R^2= 0,04, P-value = 0,06). The post-hoc analysis (pairwise adonis), applied on the month factor, reveals similarities in composition between month, taken 2 per 2, sharing the same letters (letters from figure 13). Significant dissimilarities are observed between May – July and May - August (p-value ajusted = 0,01 for both pairs). For June – August and May -September is at the limit of significance (0,05 < p-value < 0,1). The other combinations show no dissimilarities. The spatial gradient has no influence on the fungi species composition.

NMDS ordination shows variabilities in community composition for both factors (Figure 14). Firstly, fungi communities clustered by seasons reveal there is a difference in composition between Spring and Fall while summer overlaps with the other two seasons sharing some similarities in species). The colour gradient also makes it possible to highlight a particularity in these data. Indeed, the more urbanised the area becomes (i.e. the more impervious areas there are), the closer the samples (points) get to each other and therefore the more similar the composition between these points is.

There is also a strong variation in species composition within the fall, with the points being far apart from each other. The samples appear to be sorted by month from right to left on the graph, except for September which is mainly overlapping with the July and August samples.

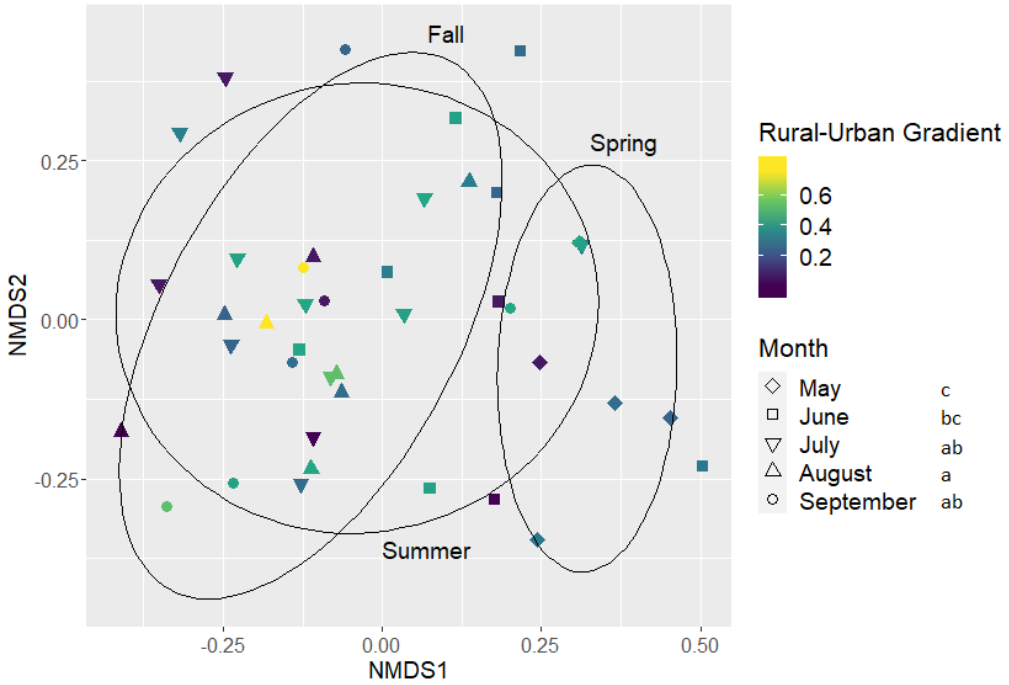

Figure 14 - Non-metric multidimensional scaling (NMDS) of fungi communities. Each point corresponds to a sample and is coloured according to the rural-urban gradient with a viridis colour palette. The month are symbolized by shapes and the seasons are represented by 80% prediction confidence ellipses determined from Shepard diagram.

4. Discussion

4.1. Taxonomy classification and comparisons

The discussion of the taxonomic results is only valid in the case of using a naive-bayes classification and the ITS region as marker-gene.

The analysis of the different taxonomic results shows differences in resolution and species identification according to the reference databases used. These were the determining factors in selecting the most suitable one in the context of this study. The reference database selected to be used for further analysis is the Toronto database. Indeed, this database contains an exhaustive list of plants naturally present in the Toronto area but doesn't cover the ornamental species that could be present in private gardens. It reveals to have the best results for the 2 factors mentioned above although the UNITE results offer a better identification for fungi species. However, there was no pre-established list of fungi for this area and no information on the geographical distribution of the fungi. This raises the question of whether the fungi data of this database is suitable for the study and provides a sufficiently complete database to not have any loss of information in the taxonomic results. As UNITE database give complementary results for some taxa, it might have been interesting to combine the data from the two databases to obtain better results, bearing in mind that these are accompanied by probable false positives.

The classification was also influenced by the number of species present in the databases, both in terms of resolution and identification. It is therefore interesting to adopt a non-generalist database for taxonomic classification at the species level using a naive-bayes classifier. However, if the study conducted only requires taxonomic analysis at the genus or family level, the effect of the size of the reference database is lessened. This size also influences the percentage of correspondence between Toronto and the 2 other customised databases (Ontario, NCBI) probably due to an overload of information, resulting in a reduction of the classifier's performance and a decrease in the quality and completeness of the results.

Most of the dissimilarities in distinct species identified between UNITE and Toronto are due to the difference in correspondence between the reference databases. But the correspondence is difficult to interpret due to the facts explained above but also due to the presence of synonyms for some taxa (e.g. *S. apicola* and *Candida apicola* (Hajsig) S.A. Mey. & Yarrow, 1978).

Another important point to consider when creating a customised database for taxonomic purposes is that the data available in the NCBI Genbank depends on the marker-gene used. Indeed, the use of the ITS region for plant and fungi identification is quite recent, and the datasets proposed by NCBI do not cover all the species in the Toronto and Ontario vascular plant lists.

After creating a custom reference database, a '*RESCRIPt'* tools can be used to estimate the accuracy of the classifier by testing it on the references sequences (Robeson et al. 2020). However, this only gives an idea of the reliability of the classifier and not a precise value of its accuracy. Other assessment tools in the same package assess the accuracy of classification and taxonomy, the latter being more suitable for assessing taxonomic results.

4.2. Composition of the sample

4.2.1 Plant composition:

Among the 79 plant species identified in this study, most of the species are considered as common species. According to the IUCN Red List, 30 species are classified as "Least Concern" and one species, *Aesculus hippocastanum* L., 1753, has a vulnerable status (IUCN 2021). Indeed, the latter has experienced a sharp decline in its population size worldwide, due to the emergence of diseases caused by insects and bacteria. This species was observed at Mississauga and UTSC sites. The other species have no special status

Some species pose a risk to biodiversity, human and livestock. This is the case of *Rhamnus cathartica* L., 1753, a species whose genus is subject to import and monitoring regulations imposed by the Canadian government as they are pests and therefore may cause damage to biodiversity. Another species, Alliaria petiolata (M.Bieb.) Cavara & Grande, 1913, is a species that, according to the Invasive Species Centre of Canada, is an aggressive invader of forests in Ontario.

Ambrosia trifida L. is a species that are considered to be highly allergenic during the flowering period (due to pollen) both at respiratory and epidermal level (plaques, itching) (Rasmussen et al. 2017).

Some species such as *Echium vulgare* L., 1753 and *Ageratina altissima* (L.) R.M.King & H.Rob., 1970, can cause intoxication and even death in livestock (Davis et al. 2015; Moyano et al. 2006).

The vegetation in and around Toronto represented in this study is dominated by forbs. The presence of woody species over the months tends to decrease. This is due to the flowering period of trees starting before that of herbaceous. The abrupt decrease in June can be explained by the poor weather conditions on the day of the harvest and the day before (Tmax = 17°, Tmin = 10°C, https://www.accuweather.com/fr/ca/toronto/). The proportion of woody species is also influenced by the urbanisation of the area. The maximum is reached in urban residential areas where the vegetation occupies a large place (park with lakes, rivers and gullies).

4.2.2 Fungi composition

The influence of fungi on human health is mostly negative or neutral. Indeed, species of the genus *Mucor* are the cause of diseases and infections such as mucormycosis and gastrointestinal disorders (Wagner et al. 2019). Other species such as *S. capsularis* have been found in the respiratory system of humans without causing any health problems. However, other studies affirm this species influences the activation or inhibition of the immune system and its metabolism products may be toxic or carcinogenic (Dynowska and Biedunkiewicz 2014).

Fungi are also interesting in terms of their application in several areas. For example, several species including *S. bombicola* produce sophorolipids used as biosurfactants in the pharmaceutical, cosmetic food and cleaning industries.

The main indirect positive effects existing between fungi and pollinators are: the production of fatty acid (*Starmerella* spp., *M. circinelloides*), the biosynthesis of high value lipid (*M. circinelloides*, *M. lusitanicus*) participated in the alimentation of honeybees. No negative effects were found.

Among the less present species, 2 species from the genus *Puccinia* considered as phytopathogens causing rust on several crops (*P. coronati-agrostidis* and *P. coronati-brevispora*) One discovered in the most urbanised site sampled (Fairmont Hotel site), the other in the second most rural area of the study

sites (Meadow Sweet site). This genus appears on the list of pest organisms regulated by the Canadian government. If the study of taxa is extended to the level of genera, other ASV are considered as belongs to *Puccinia* genus (but no identified up to the species). In addition to this genus, 2 other species are considered as phytopathogens: *Alternaria rosae* (which is a parasite causing alternaria blight) found in Fairmont Hotel and Downsview sites and *Stemphylium loti*, found in Fairmont Hotel site.

Apart from these species, the other fungi do not seem to have a negative or positive effect on the flower. The relationship is therefore either mutualistic if the fungus improves the attractiveness of the flowers to pollinators in exchange for a suitable substrate for the development of the fungi, or commensalistic if the situation only benefits the fungi.

As fungi spores can also be vectorized by wind, it is difficult to attribute all these species solely to the transmission by honeybees (some may have been deposited on the trap at the hive entrance). It could be the case of the fungi species which doesn't use flowers as a substrate to survive.

4.3. Analysis of α diversity

Species richness of plants is significantly influenced by the time factor (Month). There is no relationship between variation in species richness and the rural-urban gradient. This lack of interaction may be due to the presence of samples that can be considered as "outliers". Indeed, these are composed of only one species per sample (3 out of 5 samples are composed of the same species).

However, a positive trend appears for both factors. This light trend for the months can be explained by the decrease in food resources once the summer is well underway, which forces the bees to forage on a greater number of individuals and plant species. This trend declines between July/August and September following the end of the flowering season for native and relatively common species.

The months with the highest species richness, i.e. July and August, can also be explained by the presence of more samples to represent these months. The months of May and June have a low variation in species richness, which can be partly explained by a lower number of samples and the presence of outliers. Depending on the gradient, the richness is highest at sites in urban areas dominated by residential areas and where vegetation is still present.

Species richness of fungi is less significantly influenced by the time factor (Month) than plant species. However, the interpretation of the results for the rural-urban gradient is more complex because, statistically speaking, it is at the limit of significance.

For both factors, a positive trend was found. This trend for the months can be explained by the existence of a link between the presence of flowers and fungi, the more individuals/species of plants a bee visits, the greater the diversity of fungi it will encounter.

This tendency also increases with the urbanisation of the area, as the food resource is more heterogeneously distributed in urbanised areas. In addition, the closer one gets to the city centre, the sparser the vegetation becomes and the more linear the trees and shrubs, and the longer the distance the bee must travel to reach these resources. As fungus spores can remain airborne longer than pollen, the decrease in plant diversity between the last two months will not lead to a decrease in fungus diversity.

4.4. Analysis of β diversity

The species composition of the plants is also highly significantly influenced by the months. The rural-urban gradient is still in the complex situation of the significance boundary. The results of the post-hoc test show there is a strong variation in species composition between the months taken 2 by 2 except for the month of August which shares its composition between the months of July and September. This also explain the superposition of the ellipses for the summer and autumn seasons.

As with species richness, the interpretation of the results is biased by the presence of outliers as well as by the low number of samples to represent certain categories of the month factor, the season, and certain percentages of the urban-rural gradient.

The species composition of fungi shows the same statistical influence of the 2 factors than plants composition. But the gradient shows an increase in the similarity in composition with the urbanisation. Post-hoc testing shows greater similarity between months than for plants, each sharing some similarities in species composition if the months are taken 2 by 2 in calendar order.

5. Limitations

Several limitations can be highlighted:

The use of pollen collected by bees at the hive level does not provide data on the abundance of plant species. Only the incidence data (absence-presence) are representative of the samples to perform biodiversity analyses.

The taxonomic classification was performed with a custom database based on a list of plants representing the flora of a specific geographical area. Therefore, to be reproducible in other countries, it is important to have an equivalent of this list if the aim of this classification is to have a good identification at species level.

In addition, the naïve-bayes classification was done using the ITS region as a marker gene. As this marker is recent for plant and fungus identification, the datasets are not yet very complete.

To carry out this study, several assumptions were made:

- First, because the area of our study is larger than the area used to create the plant list, including sites outside of the City of Toronto, an assumption was made that this list was valid for the Greater Toronto Area.
- Another hypothesis was that the abiotic conditions of the different sites did not influence the foraging activities of the bees in any way.
- The last one stated that the hives selected for sampling had a similar activity rate and that their composition, in terms of individuals and workers, was similar.

Finally, the realisation of the rural-urban gradient is very time consuming. Indeed, all the impervious areas, except the roads, were digitised manually. Hence the limitation of the radius around the hive to the average distance a bee travels to forage.

6. Conclusion

The aim of this study was to provide decision support for the management/creation of green areas and green projects in the context of expanding urbanisation. In a second step, this study aimed to highlight the role of bees in the vectoring of fungi and their effect on the environment.

for this, pollen was collected by the species A. mellifera from 13 sites located in the city of Toronto and its surroundings between May and September 2020. DNA analysis by metabarcoding was performed on the samples using the ITS region as primers to allow reading and taxonomic classification of DNA sequences. Taxonomic results were obtained by applying a naive-bayes classification and different reference databases whose differences relate to the number of species present in the database and the data source (NCBI, UNITE). The reference database made it possible to identify 79 plants and 46 fungi.

Based on their incidence data, biodiversity analyses (alpha and beta diversity) were carried out depending on 2 factors: the months (seasonality phenomenon) and an urban-rural gradient created by digitalization. The species composition of the samples was also analysed according to the structure of the vegetation (herbaceous and woody) and the status (exotic - native) of the species.

The comparison of the taxonomic results was able to highlight the most suitable database. It consists of NCBI datasets corresponding to a predefined list of Toronto plants as well as ITS data from an NCBI bioproject for fungal species.

Among the plant species identified, the top 10 was completely constituted by common species such as *T. repens* or *S.arvensis*. Some of the 79 plant species encountered are invasive, allergenic, and toxic species. For fungi species, 4 of them are phytopathogenic species, others can impact human health or improve the nutritious quality of bee feeding. Unfortunately, as the sporidia can

The richness in plant species was found to be strongly influenced by the months and reveals an absence of interaction between it and the urban-rural gradient. richness in fungus species, when it is also significantly influenced by the months, but the influence of the spatial factor is more contrasted (at the limit of significance). The richness tends to grow with the month and declines when August's end for the plant species while for fungi, the trend stabilises between August and September.

Regarding the species composition is strongly influenced by the months and the influence of the spatial gradient is contrasted for both plants and fungi composition. The fungi composition tends to be more and more similar with the increase of impervious surfaces. Such interpretation cannot be possible for plants; thus it confirms that the spatial gradient have no influence on the species composition.

It is nevertheless necessary to remember the assumptions made on the activity of the hive, the abiotic conditions of the sites and the validation of the list of species used to create the database although the area of this research is greater than that corresponding to the listing.

To conclude, further analysis should be done to understand if honeybees will be interested in more uncommon species to be planted in parcs and gardens or if these species will prefer to forage the same common species through the seasons.

7. Bibliography

Abarenkov, Kessy, Allan Zirk, Timo Piirmann, Raivo Pöhönen, Filipp Ivanov, R. Henrik Nilsson, and Urmas Kõljalg. 2020. 'UNITE QIIME Release for Eukaryotes 2'. UNITE Community. 2020. https://doi.org/10.15156/BIO/786388.

Abou-Shaara, H. F., A. A. Owayss, Y. Y. Ibrahim, and N. K. Basuny. 2017. 'A Review of Impacts of Temperature and Relative Humidity on Various Activities of Honey Bees'. *Insectes Sociaux* 64 (4): 455–63. https://doi.org/10.1007/s00040-017-0573-8.

Aizenberg-Gershtein, Yana, Ido Izhaki, and Malka Halpern. 2013. 'Do Honeybees Shape the Bacterial Community Composition in Floral Nectar?' Edited by Hauke Smidt. *PLoS ONE* 8 (7): e67556. https://doi.org/10.1371/journal.pone.0067556.

Albrecht, Matthias, Bernhard Schmid, Yann Hautier, and Christine B. Müller. 2012. 'Diverse Pollinator Communities Enhance Plant Reproductive Success'. *Proceedings of the Royal Society B: Biological Sciences* 279 (1748): 4845–52. https://doi.org/10.1098/rspb.2012.1621.

Allsopp, Mike H., Willem J. de Lange, and Ruan Veldtman. 2008. 'Valuing Insect Pollination Services with Cost of Replacement'. Edited by Andy Hector. *PLoS ONE* 3 (9): e3128. https://doi.org/10.1371/journal.pone.0003128.

Ash, Caroline, Barbara R. Jasny, Leslie Roberts, Richard Stone, and Andrew M. Sugden. 2008. 'Reimagining Cities'. *Science*. https://doi.org/10.1126/science.319.5864.739.

Badiou-Bénéteau, A., A. Benneveau, F. Géret, H. Delatte, N. Becker, J. L. Brunet, B. Reynaud, and L. P. Belzunces. 2013. 'Honeybee Biomarkers as Promising Tools to Monitor Environmental Quality'. *Environment International* 60 (October): 31–41. https://doi.org/10.1016/j.envint.2013.07.002.

Bates, Adam J., Jon P. Sadler, Alison J. Fairbrass, Steven J. Falk, James D. Hale, and Tom J. Matthews. 2011. 'Changing Bee and Hoverfly Pollinator Assemblages along an Urban-Rural Gradient'. *PLoS ONE* 6 (8): e23459. https://doi.org/10.1371/journal.pone.0023459.

Bebber, Daniel P., and Sarah J. Gurr. 2015. 'Crop-Destroying Fungal and Oomycete Pathogens Challenge Food Security'. *Fungal Genetics and Biology* 74 (January): 62–64. https://doi.org/10.1016/j.fgb.2014.10.012.

Beekman, M., and F. L.W. Ratnieks. 2000. 'Long-Range Foraging by the Honey-Bee, Apis Mellifera L.' *Functional Ecology* 14 (4): 490–96. https://doi.org/10.1046/j.1365-2435.2000.00443.x.

Bell, Karen L., Julie Fowler, Kevin S. Burgess, Emily K. Dobbs, David Gruenewald, Brice Lawley, Connor Morozumi, and Berry J. Brosi. 2017. 'Applying Pollen DNA Metabarcoding to the Study of Plant–Pollinator Interactions'. *Applications in Plant Sciences* 5 (6): 1600124. https://doi.org/10.3732/apps.1600124.

Bell, Karen L., Natasha De Vere, Alexander Keller, Rodney T. Richardson, Annemarie Gous, Kevin S. Burgess, and Berry J. Brosi. 2016. 'Pollen DNA Barcoding: Current Applications and Future Prospects'. In *Genome*, 59:629–40. Canadian Science Publishing. https://doi.org/10.1139/gen-2015-0200.

Binoy, Ambika Manirajan. 2018. 'Bacterial and Fungal Microbiota of Flower Pollen and Potential Impact on Pollen-Related Allergies'. Gießen: Justus-Liebig-University.

Blaxter, Mark, Jenna Mann, Tom Chapman, Fran Thomas, Claire Whitton, Robin Floyd, and Eyualem Abebe. 2005. 'Defining Operational Taxonomic Units Using DNA Barcode Data'. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360 (1462): 1935–43. https://doi.org/10.1098/rstb.2005.1725.

Bokulich, Nicholas A., Benjamin D. Kaehler, Jai Ram Rideout, Matthew Dillon, Evan Bolyen, Rob Knight, Gavin A. Huttley, and J. Gregory Caporaso. 2018. 'Optimizing Taxonomic Classification of Marker-Gene Amplicon Sequences with QIIME 2's Q2-Feature-Classifier Plugin'. *Microbiome* 6 (1): 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolyen, Evan, Jai Ram Rideout, Matthew R. Dillon, Nicholas A. Bokulich, Christian C. Abnet, Gabriel A. Al-Ghalith, Harriet Alexander, et al. 2019. 'Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2'. *Nature Biotechnology* 37 (8): 852–57. https://doi.org/10.1038/s41587-019-0209-9.

Brodschneider, Robert, and Karl Crailsheim. 2010. 'Nutrition and Health in Honey Bees'. *Apidologie*. https://doi.org/10.1051/apido/2010012.

Bronstein, Judith L., Ruben Alarcón, and Monica Geber. 2006. 'The Evolution of Plant-Insect Mutualisms'. *New Phytologist* 172 (3): 412–28. https://doi.org/10.1111/j.1469-8137.2006.01864.x.

Brown, Shawn P., Anne R. Rigdon-Huss, and Ari Jumpponen. 2014. 'Analyses of ITS and LSU Gene Regions Provide Congruent Results on Fungal Community Responses'. *Fungal Ecology* 9 (1): 65–68. https://doi.org/10.1016/j.funeco.2014.02.002.

Čadež, Neža, João Drumonde-Neves, Matthias Sipiczki, Dénes Dlauchy, Teresa Lima, Célia Pais, Dorit Schuller, Ricardo Franco-Duarte, Marc-André Lachance, and Gábor Péter. 2020. 'Starmerella Vitis f.a., Sp. Nov., a Yeast Species Isolated from Flowers and Grapes'. *Antonie van Leeuwenhoek 2020 113:9* 113 (9): 1289–98. https://doi.org/10.1007/S10482-020-01438-X.

Cadotte, Marc W. 2021. 'The List of Vascular Plants for the City of Toronto'. *Ecological Solutions and Evidence* 2 (1): e12036. https://doi.org/10.1002/2688-8319.12036.

Callahan, Benjamin J., Paul J. McMurdie, and Susan P. Holmes. 2017. 'Exact Sequence Variants Should Replace Operational Taxonomic Units in Marker-Gene Data Analysis'. *ISME Journal* 11 (12): 2639–43. https://doi.org/10.1038/ismej.2017.119.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. 2016. 'DADA2: High-Resolution Sample Inference from Illumina Amplicon Data'. *Nature Methods* 13 (7): 581–83. https://doi.org/10.1038/nmeth.3869.

Callahan, Benjamin J., Joan Wong, Cheryl Heiner, Steve Oh, Casey M. Theriot, Ajay S. Gulati, Sarah K. McGill, and Michael K. Dougherty. 2019. 'High-Throughput Amplicon Sequencing of the Full-Length 16S RRNA Gene with Single-Nucleotide Resolution'. *Nucleic Acids Research* 47 (18): e103. https://doi.org/10.1093/nar/gkz569.

Camacho, Christiam, George Coulouris, Vahram Avagyan, Ning Ma, Jason Papadopoulos, Kevin Bealer, and Thomas L. Madden. 2009. 'BLAST+: Architecture and Applications'. *BMC Bioinformatics* 10 (December): 421. https://doi.org/10.1186/1471-2105-10-421.

Card, S. D., M. N. Pearson, and G. R. G. Clover. 2007. 'Plant Pathogens Transmitted by Pollen'. *Australasian Plant Pathology* 36 (5): 455. https://doi.org/10.1071/AP07050.

Cardinale, Bradley J., J. Emmett Duffy, Andrew Gonzalez, David U. Hooper, Charles Perrings, Patrick Venail, Anita Narwani, et al. 2012. 'Biodiversity Loss and Its Impact on Humanity'. *Nature*. Nature Publishing Group. https://doi.org/10.1038/nature11148.

Caruso, Vincent, Xubo Song, Mark Asquith, and Lisa Karstens. 2019. 'Performance of Microbiome Sequence Inference Methods in Environments with Varying Biomass'. *MSystems* 4 (1). https://doi.org/10.1128/msystems.00163-18.

Carvalho, Ana Karine F., Leyvison Rafael V. da Conceição, João Paulo V. Silva, Victor H. Perez, and Heizir F. de Castro. 2017. 'Biodiesel Production from Mucor Circinelloides Using Ethanol and Heteropolyacid in One and Two-Step Transesterification'. *Fuel* 202 (August): 503–11. https://doi.org/10.1016/J.FUEL.2017.04.063.

Cheng, Tao, Chao Xu, Li Lei, Changhao Li, Yu Zhang, and Shiliang Zhou. 2016. 'Barcoding the Kingdom Plantae: New PCR Primers for ITS Regions of Plants with Improved Universality and Specificity'. *Molecular Ecology Resources* 16 (1): 138–49. https://doi.org/10.1111/1755-0998.12438.

Crailsheim, K. 1990. 'The Protein Balance of the Honey Bee Worker'. *Apidologie* 21 (5): 417–29. https://doi.org/10.1051/apido:19900504.

Davis, T Zane, Stephen T Lee, Mark G Collett, Bryan L Stegelmeier, Benedict T Green, Steven R Buck, and James A Pfister. 2015. 'Toxicity of White Snakeroot (Ageratina Altissima) and Chemical Extracts of White Snakeroot in Goats'. *Journal of Agricultural and Food Chemistry* 63 (7): 2092–97. https://doi.org/10.1021/JF505614Z.

Demirel, Rasime. 2016. 'Comparison of RDNA Regions (ITS, LSU, and SSU) of Some Aspergillus, Penicillium, and Talaromyces Spp.' *Turkish Journal of Botany* 40 (6): 576–83. https://doi.org/10.3906/bot-1603-12.

Desmet, Peter, and Luc Brouilet. 2013. 'Database of Vascular Plants of Canada (VASCAN): A Community Contributed Taxonomic Checklist of All Vascular Plants of Canada, Saint Pierre and Miquelon, and Greenland'. *PhytoKeys*, no. 25 (July): 55–67. https://doi.org/10.3897/PHYTOKEYS.25.3100.

Deveza, Michele Valadares, Kelly Moura Keller, Maria Cristina Affonso Lorenzon, Lucila Maria Teixeira Nunes, Érika Oliveira Sales, and Ortrud Monika Barth. 2015. 'Mycotoxicological and Palynological Profiles of Commercial Brands of Dried Bee Pollen'. *Brazilian Journal of Microbiology* 46 (4): 1171–76. https://doi.org/10.1590/S1517-838246420140316.

Dharampal, Prarthana S., Caitlin Carlson, Cameron R. Currie, and Shawn A. Steffan. 2019. 'Pollen-Borne Microbes Shape Bee Fitness'. *Proceedings of the Royal Society B: Biological Sciences* 286 (1904). https://doi.org/10.1098/rspb.2018.2894.

Eickwort, George C. 1990. 'Biogeography and Taxonomy of Honeybees'. *American Entomologist* 36 (1): 58–59. https://doi.org/10.1093/ae/36.1.58.

Eilers, Elisabeth J., Claire Kremen, Sarah Smith Greenleaf, Andrea K. Garber, and Alexandra Maria Klein. 2011. 'Contribution of Pollinator-Mediated Crops to Nutrients in the Human Food Supply'. *PLoS ONE* 6 (6): 21363. https://doi.org/10.1371/journal.pone.0021363.

Ejdas, Elżbieta. 2014. 'Fungal Infection Risk Groups among School Children'. *Acta Mycologica* 38 (1–2): 71–87. https://doi.org/10.5586/am.2003.009.

Favre, Daniel. 2011. 'Mobile Phone-Induced Honeybee Worker Piping'. *Apidologie* 42 (3): 270–79. https://doi.org/10.1007/s13592-011-0016-x.

Figueroa, Laura L., Malcolm Blinder, Cali Grincavitch, Angus Jelinek, Emilia K. Mann, Liam A. Merva, Lucy E. Metz, et al. 2019. 'Bee Pathogen Transmission Dynamics: Deposition, Persistence and Acquisition on Flowers'. *Proceedings of the Royal Society B: Biological Sciences* 286 (1903). https://doi.org/10.1098/rspb.2019.0603.

Fisher, Matthew C., Neil A.R. Gow, and Sarah J. Gurr. 2016. 'Tackling Emerging Fungal Threats to Animal Health, Food Security and Ecosystem Resilience'. *Philosophical Transactions of the Royal Society B: Biological Sciences* 371 (1709). https://doi.org/10.1098/rstb.2016.0332.

Frisch, Karl Von. 1967. *The Dance Language and Orientation of Bees*. The Belknap Press of Harvard University Press. https://doi.org/10.4159/harvard.9780674418776.

Fürst, M. A., D. P. McMahon, J. L. Osborne, R. J. Paxton, and M. J.F. Brown. 2014. 'Disease Associations between Honeybees and Bumblebees as a Threat to Wild Pollinators'. *Nature* 506 (7488): 364–66. https://doi.org/10.1038/nature12977.

Galimberti, Andrea, Fabrizio De Mattia, Ilaria Bruni, Daniela Scaccabarozzi, Anna Sandionigi, Michela Barbuto, Maurizio Casiraghi, and Massimo Labra. 2014. 'A DNA Barcoding Approach to Characterize Pollen Collected by Honeybees'. *PLoS ONE* 9 (10): e109363. https://doi.org/10.1371/journal.pone.0109363.

Gallai, Nicola, Jean Michel Salles, Josef Settele, and Bernard E. Vaissière. 2009. 'Economic Valuation of the Vulnerability of World Agriculture Confronted with Pollinator Decline'. *Ecological Economics* 68 (3): 810–21. https://doi.org/10.1016/j.ecolecon.2008.06.014.

Geslin, B., B. Gauzens, M. Baude, I. Dajoz, C. Fontaine, M. Henry, L. Ropars, O. Rollin, E. Thébault, and N. J. Vereecken. 2017. 'Massively Introduced Managed Species and Their Consequences for Plant–Pollinator Interactions'. In *Advances in Ecological Research*, 57:147–99. Academic Press Inc. https://doi.org/10.1016/bs.aecr.2016.10.007.

Goulson, D., E. Nicholls, C. Botias, and E. L. Rotheray. 2015. 'Bee Declines Driven by Combined Stress from Parasites, Pesticides, and Lack of Flowers'. *Science* 347 (6229): 1255957–1255957. https://doi.org/10.1126/science.1255957.

Gous, Annemarie, Dirk Z. H. Swanevelder, Connal D. Eardley, and Sandi Willows-Munro. 2019. 'Plant-Pollinator Interactions over Time: Pollen Metabarcoding from Bees in a Historic Collection'. *Evolutionary Applications* 12 (2): 187–97. https://doi.org/10.1111/eva.12707.

Graeve, Marilyn De, Sofie L De Maeseneire, Sophie L K W Roelants, and Wim Soetaert. 2018. 'Starmerella Bombicola, an Industrially Relevant, yet Fundamentally Underexplored Yeast'. *FEMS Yeast Research* 18 (7): 72. https://doi.org/10.1093/FEMSYR/FOY072.

Harrison, Tina, and Rachael Winfree. 2015. 'Urban Drivers of Plant-Pollinator Interactions'. *Functional Ecology* 29 (7): 879–88. https://doi.org/10.1111/1365-2435.12486.

Haydak, M.H. 1970. 'Honey Bee Nutrition'. *Annual Reviews of Entomology* 15 (6907): 143–57.

Hebert, Paul D.N., and T. Ryan Gregory. 2005. 'The Promise of DNA Barcoding for Taxonomy'. *Systematic Biology*. https://doi.org/10.1080/10635150500354886.

Hodges, Dorothy. 1952. *The Pollen Loads of the Honeybee : A Guide to Their Identification by Colour and Form*. Edited by Bee Research Association.

Hokkanen, Heikki M. T., Ingeborg Menzler-Hokkanen, and Marja-Leena Lahdenpera. 2015. 'Managing Bees for Delivering Biological Control Agents and Improved Pollination in Berry and Fruit Cultivation'. *Sustainable Agriculture Research* 4 (3): 89. https://doi.org/10.5539/sar.v4n3p89.

Hollingsworth, Peter M., Laura L. Forrest, John L. Spouge, Mehrdad Hajibabaei, Sujeevan Ratnasingham, Michelle van der Bank, Mark W. Chase, et al. 2009. 'A DNA Barcode for Land Plants'. *Proceedings of the National Academy of Sciences of the United States of America* 106 (31): 12794–97. https://doi.org/10.1073/pnas.0905845106.

Hollingsworth, Peter M., Sean W. Graham, and Damon P. Little. 2011. 'Choosing and Using a Plant DNA Barcode'. Edited by Dirk Steinke. *PLoS ONE* 6 (5): e19254. https://doi.org/10.1371/journal.pone.0019254.

IUCN. 2021. ‘The IUCN Red List of Threatened Species. Version 2021-1’. 2021. https://www.iucnredlist.org.

Kang, Yun, Krystal Blanco, Talia Davies, Ying Wang, and Gloria DeGrandi-Hoffman. 2015. ‘Disease Dynamics of Honeybees with Varroa Destructor as Parasite and Virus Vector’. *Mathematical Biosciences* 275 (May): 71–92. http://arxiv.org/abs/1505.03742.

Kasprzyk, Idalia, Agnieszka Grinn-Gofroń, Agata Ćwik, Katarzyna Kluska, Paloma Cariñanos, and Tomasz Wójcik. 2021. ‘Allergenic Fungal Spores in the Air of Urban Parks’. *Aerobiologia* 37 (1): 39–51. https://doi.org/10.1007/s10453-020-09671-7.

Keller, A., N. Danner, G. Grimmer, M. Ankenbrand, K. von der Ohe, W. von der Ohe, S. Rost, S. Härtel, and I. Steffan-Dewenter. 2015. ‘Evaluating Multiplexed Next-Generation Sequencing as a Method in Palynology for Mixed Pollen Samples’. Edited by H.-P. Mock. *Plant Biology* 17 (2): 558–66. https://doi.org/10.1111/plb.12251.

Kohsaka, Ryo, Mi Sun Park, and Yuta Uchiyama. 2017. ‘Beekeeping and Honey Production in Japan and South Korea: Past and Present’. *Journal of Ethnic Foods* 4 (2): 72–79. https://doi.org/10.1016/j.jef.2017.05.002.

Kress, W. John, and David L. Erickson. 2008. ‘DNA Barcodes: Genes, Genomics, and Bioinformatics’. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0800476105.

Kress, W. John, Carlos García-Robledo, Maria Uriarte, and David L. Erickson. 2015. ‘DNA Barcodes for Ecology, Evolution, and Conservation’. *Trends in Ecology and Evolution*. Elsevier Ltd. https://doi.org/10.1016/j.tree.2014.10.008.

Kress, W. John, Kenneth J. Wurdack, Elizabeth A. Zimmer, Lee A. Weigt, and Daniel H. Janzen. 2005. ‘Use of DNA Barcodes to Identify Flowering Plants’. *Proceedings of the National Academy of Sciences of the United States of America* 102 (23): 8369–74. https://doi.org/10.1073/pnas.0503123102.

Kumar, Sanjay, Vinay Kumar Singh, Pashupati Nath, and P. C. Joshi. 2020. ‘An Overview of Anthropogenic Electromagnetic Radiations as Risk to Pollinators and Pollination’. *Journal of Applied and Natural Science* 12 (4): 675–81. https://doi.org/10.31018/jans.v12i4.2420.

Kunc, Martin, Pavel Dobeš, Jana Hurychová, Libor Vojtek, Silvana Beani Poiani, Jiří Danihlík, Jaroslav Havlík, Dalibor Titěra, and Pavel Hyršl. 2019. ‘The Year of the Honey Bee (Apis Mellifera L.) with Respect to Its Physiology and Immunity: A Search for Biochemical Markers of Longevity’. *Insects* 10 (8): 244. https://doi.org/10.3390/insects10080244.

Liu, Lin, Yinhu Li, Siliang Li, Ni Hu, Yimin He, Ray Pong, Danni Lin, Lihua Lu, and Maggie Law. 2012. ‘Comparison of Next-Generation Sequencing Systems’. *Journal of Biomedicine and Biotechnology*. https://doi.org/10.1155/2012/251364.

Liu, Zhifeng, Chunyang He, and Jianguo Wu. 2016. ‘The Relationship between Habitat Loss and Fragmentation during Urbanization: An Empirical Evaluation from 16 World Cities’. *PLoS ONE* 11 (4): e0154613. https://doi.org/10.1371/journal.pone.0154613.

Loures, Luis, Raúl Santos, and Thomas Panagopoulos. 2007. ‘Urban Parks and Sustainable City Planning-The Case of Portimão, Portugal’. *WSEAS Transactions on Environment and Development* 3 (10): 171–80.

Louveaux, Jean. 1958. ‘Recherches Sur La Récolte Du Pollen Par Les Abeilles (Apis Mellifica L.)’. *Annales Des Abeilles* 1 (3): 113–88. https://hal.archives-ouvertes.fr/hal-00890106.

Lowenstein, David M., Kevin C. Matteson, and Emily S. Minor. 2019. 'Evaluating the Dependence of Urban Pollinators on Ornamental, Non-Native, and "Weedy" Floral Resources'. *Urban Ecosystems* 22 (2): 293–302. https://doi.org/10.1007/s11252-018-0817-z.

Marquardt, Melanie, Lydia Kienbaum, Lea Annina Kretschmer, Anja Penell, Karsten Schweikert, Ute Ruttensperger, and Peter Rosenkranz. 2020. 'Evaluation of the Importance of Ornamental Plants for Pollinators in Urban and Suburban Areas in Stuttgart, Germany'. *Urban Ecosystems*, December, 1–15. https://doi.org/10.1007/s11252-020-01085-0.

Marshman, Jennifer, Alison Blay-Palmer, and Karen Landman. 2019. 'Anthropocene Crisis: Climate Change, Pollinators, and Food Security'. *Environments* 6 (2): 22. https://doi.org/10.3390/environments6020022.

Martinez Arbizu, P. 2020. 'PairwiseAdonis: Pairwise Multilevel Comparison Using Adonis.'

Mcart, Scott H., Hauke Koch, Rebecca E. Irwin, and Lynn S. Adler. 2014. 'Arranging the Bouquet of Disease: Floral Traits and the Transmission of Plant and Animal Pathogens'. *Ecology Letters*. Blackwell Publishing Ltd. https://doi.org/10.1111/ele.12257.

McCune, Frédéric. 2018. 'Impact de l'apiculture Urbaine et Du Paysage Urbain Sur Les Abeilles Sauvages'. Université de Laval. https://corpus.ulaval.ca/jspui/handle/20.500.11794/37362.

McFrederick, Quinn S., and Sandra M. Rehan. 2019. 'Wild Bee Pollen Usage and Microbial Communities Co-Vary Across Landscapes'. *Microbial Ecology* 77 (2): 513–22. https://doi.org/10.1007/s00248-018-1232-y.

Menzel, Randolf, Rodrigo J. De Marco, and Uwe Greggers. 2006. 'Spatial Memory, Navigation and Dance Behaviour in Apis Mellifera'. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*. https://doi.org/10.1007/s00359-006-0136-3.

Moritz, Robin F.A., and Stefan Fuchs. 1998. 'Organization of Honeybee Colonies: Characteristics and Consequences of a Superorganism Concept'. *Apidologie* 29 (1–2): 7–21. https://doi.org/10.1051/apido:19980101.

Moyano, PR, A García, A Rueda, AM Molina, A Mendez, and F Infante. 2006. 'Echium Vulgare and Senecio Vulgaris Poisoning in Fighting Bulls'. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* 53 (1): 24–25. https://doi.org/10.1111/J.1439-0442.2006.00780.X.

Münch, Daniel, and Gro V. Amdam. 2010. 'The Curious Case of Aging Plasticity in Honey Bees'. *FEBS Letters* 584 (12): 2496–2503. https://doi.org/10.1016/j.febslet.2010.04.007.

Navarro-Mendoza, MI, C Pérez-Arques, S Panchal, FE Nicolás, SJ Mondo, P Ganguly, J Pangilinan, et al. 2019. 'Early Diverging Fungus Mucor Circinelloides Lacks Centromeric Histone CENP-A and Displays a Mosaic of Point and Regional Centromeres'. *Current Biology : CB* 29 (22): 3791-3802.e6. https://doi.org/10.1016/J.CUB.2019.09.024.

Nazarevich, Victor John. 2015. 'The Sixth Species Extinction Event by Humans'. *Earth Common Journal* 5 (1): 61–72. https://doi.org/10.31542/j.ecj.261.

Nazzi, F., D. Annoscia, E. Caprio, G. Di Prisco, and F. Pennacchio. 2014. 'Honeybee Immunity and Colony Losses'. *Entomologia* 2 (2): 80–86. https://doi.org/10.4081/entomologia.2014.203.

Newton, Irene L.G., and Guus Roeselers. 2012. 'The Effect of Training Set on the Classification of Honey Bee Gut Microbiota Using the Naïve Bayesian Classifier.' *BMC Microbiology* 12. https://doi.org/10.1186/1471-2180-12-221.

Nicholls, Elizabeth, and Natalie Hempel de Ibarra. 2017. 'Assessment of Pollen Rewards by Foraging

Bees'. *Functional Ecology*. Blackwell Publishing Ltd. https://doi.org/10.1111/1365-2435.12778.

Nimmo, Richie. 2015. 'The Bio-Politics of Bees: Industrial Farming and Colony Collapse Disorder'. *Humanimalia: Journal of Human/Animal Interface Studies* 6 (2): 1–20. https://www.research.manchester.ac.uk/portal/en/publications/the-biopolitics-of-bees-industrial-farming-and-colony-collapse-disorder(6fa95d30-44ad-4ab8-a327-aed3e3caa681).html.

Oh, Seung-Yoon, Jonathan J Fong, Myung Soo Park, Limseok Chang, and Young Woon Lim. 2014. 'Identifying Airborne Fungi in Seoul, Korea Using Metagenomics'. *Journal of Microbiology* 52 (6): 465–72. https://doi.org/10.1007/s12275-014-3550-1.

Oke, Cathy, Sarah A. Bekessy, Niki Frantzeskaki, Judy Bush, James A. Fitzsimons, Georgia E. Garrard, Maree Grenfell, et al. 2021. 'Cities Should Respond to the Biodiversity Extinction Crisis'. *Npj Urban Sustainability* 1 (1): 1–4. https://doi.org/10.1038/s42949-020-00010-w.

Oksanen, Author Jari, F Guillaume Blanchet, Roeland Kindt, Pierre Legen-, Peter R Minchin, R B O Hara, Gavin L Simpson, Peter Solymos, and M Henry H Stevens. 2012. 'Vegan: Community Ecology Package'. *… Ecology Package …*, no. January: 263. http://mirror.bjtu.edu.cn/cran/web/packages/vegan/vegan.pdf.

Ollerton, Jeff, Hilary Erenler, Mike Edwards, and Robin Crockett. 2014. 'Extinctions of Aculeate Pollinators in Britain and the Role of Large-Scale Agricultural Changes'. *Science* 346 (6215): 1360–62. https://doi.org/10.1126/science.1257259.

Ollerton, Jeff, Rachael Winfree, and Sam Tarrant. 2011. 'How Many Flowering Plants Are Pollinated by Animals?' *Oikos* 120 (3): 321–26. https://doi.org/10.1111/j.1600-0706.2010.18644.x.

Otto, Clint R.V., Cali L. Roth, Benjamin L. Carlson, and Matthew D. Smart. 2016. 'Land-Use Change Reduces Habitat Suitability for Supporting Managed Honey Bee Colonies in the Northern Great Plains'. *Proceedings of the National Academy of Sciences of the United States of America* 113 (37): 10430–35. https://doi.org/10.1073/pnas.1603481113.

Owayss, Ayman A, Abdel-Halim M. Ismail, Karem M. Mohanny, and Rasha A. Salem. 2012. 'Effect of Pollen Trapping in Honey Bee Colonies on Brood Rearing and Honey Production in Fayoum Governorate, Egypt'. *Bulletin of the Entomological Society of Egypt* 38: 155–68.

Paoli, Marco, and Giovanni C. Galizia. 2021. 'Olfactory Coding in Honeybees'. *Cell and Tissue Research*. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00441-020-03385-5.

Pasquale, Garance Di, Marion Salignon, Yves Le Conte, Luc P. Belzunces, Axel Decourtye, André Kretzschmar, Séverine Suchail, Jean-Luc Brunet, and Cédric Alaux. 2013. 'Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter?' Edited by Jochen Zeil. *PLoS ONE* 8 (8): e72016. https://doi.org/10.1371/journal.pone.0072016.

Pavan-Kumar, Annam, P. Gireesh-Babu, and W.S. Lakra. 2015. 'DNA Metabacoding: A New Approach for Rapid Biodiversity Assessment'. *Journal of Cell Science and Molecular Biology* 2 (1): 111. http://opensciencepublications.com/fulltextarticles/JCMB-2350-0190-2-111.html.

Peck, David T., Michael L. Smith, and Thomas D. Seeley. 2016. 'Varroa Destructor Mites Can Nimbly Climb from Flowers onto Foraging Honey Bees'. Edited by James C. Nieh. *PLOS ONE* 11 (12): e0167798. https://doi.org/10.1371/journal.pone.0167798.

Pernal, Stephen F., and Robert W. Currie. 2001. 'The Influence of Pollen Quality on Foraging Behavior in Honeybees (Apis Mellifera L.)'. *Behavioral Ecology and Sociobiology* 51 (1): 53–68. https://doi.org/10.1007/s002650100412.

Piñeiro Fernández, Laura, Kelsey J.R.P. Byers, Jing Cai, Khalid E.M. Sedeek, Roman T. Kellenberger, Alessia Russo, Weihong Qi, Catharine Aquino Fournier, and Philipp M. Schlüter. 2019. 'A Phylogenomic Analysis of the Floral Transcriptomes of Sexually Deceptive and Rewarding European Orchids, Ophrys and Gymnadenia'. *Frontiers in Plant Science* 10 (November): 1553. https://doi.org/10.3389/fpls.2019.01553.

Pirk, Christian W.W., Robin M. Crewe, and Robin F.A. Moritz. 2017. 'Risks and Benefits of the Biological Interface between Managed and Wild Bee Pollinators'. *Functional Ecology* 31 (1): 47–55. https://doi.org/10.1111/1365-2435.12768.

Pitt, John I., and Ailsa D. Hocking. 2009. 'Fungi and Food Spoilage'. *Fungi and Food Spoilage*, 1–519. https://doi.org/10.1007/978-0-387-92207-2.

Posada-Florez, Francisco, Eugene V. Ryabov, Matthew C. Heerman, Yanping Chen, Jay D. Evans, Daniel E. Sonenshine, and Steven C. Cook. 2020. 'Varroa Destructor Mites Vector and Transmit Pathogenic Honey Bee Viruses Acquired from an Artificial Diet'. *PLoS ONE* 15 (11 November). https://doi.org/10.1371/journal.pone.0242688.

Rasmussen, Karen, Jakob Thyrring, Robert Muscarella, and Finn Borchsenius. 2017. 'Climate-Change-Induced Range Shifts of Three Allergenic Ragweeds (Ambrosia L.) in Europe and Their Potential Impact on Human Health'. *PeerJ* 5 (3): e3104. https://doi.org/10.7717/PEERJ.3104.

Robeson, Michael S., Devon R. O'Rourke, Benjamin D. Kaehler, Michal Ziemski, Matthew R. Dillon, Jeffrey T. Foster, and Nicholas A. Bokulich. 2020. 'RESCRIPt: Reproducible Sequence Taxonomy Reference Database Management for the Masses'. *BioRxiv*, October, 2020.10.05.326504. https://doi.org/10.1101/2020.10.05.326504.

Rodriguez, Patricia A., Michael Rothballer, Soumitra Paul Chowdhury, Thomas Nussbaumer, Caroline Gutjahr, and Pascal Falter-Braun. 2019. 'Systems Biology of Plant-Microbiome Interactions'. *Molecular Plant*. Cell Press. https://doi.org/10.1016/j.molp.2019.05.006.

Rognes, Torbjørn, Tomáš Flouri, Ben Nichols, Christopher Quince, and Frédéric Mahé. 2016. 'VSEARCH: A Versatile Open Source Tool for Metagenomics'. *PeerJ* 2016 (10): e2584. https://doi.org/10.7717/peerj.2584.

Ropars, Lise, Isabelle Dajoz, Colin Fontaine, Audrey Muratet, and Benoît Geslin. 2019. 'Wild Pollinator Activity Negatively Related to Honey Bee Colony Densities in Urban Context'. *PLoS ONE* 14 (9). https://doi.org/10.1371/journal.pone.0222316.

Sadeghi, Amin, Ali Akbar Mozafari, Reza Bahmani, and Kamran Shokri. 2012. 'Use of Honeybees as Bio-Indicators of Environmental Pollution in the Kurdistan Province of Iran'. *Journal of Apicultural Science* 56 (2): 83–88. https://doi.org/10.2478/v10289-012-0026-6.

Samuelson, Ash E., Richard J. Gill, and Ellouise Leadbeater. 2020. 'Urbanisation Is Associated with Reduced Nosema Sp. Infection, Higher Colony Strength and Higher Richness of Foraged Pollen in Honeybees'. *Apidologie* 51 (5): 746–62. https://doi.org/10.1007/s13592-020-00758-1.

Samways, David. 2017. 'Saving Living Diversity in the Face of the Unstoppable 6th Mass Extinction: A Call for Urgent International Action'. *Https://Jpopsus.Org/*, April. https://jpopsus.org/full_articles/saving-living-diversity-in-the-face-of-the-unstoppable-6th-mass-extinction-a-call-for-urgent-international-action/.

Sandhu, Harpinder, Benjamin Waterhouse, Stephane Boyer, and Steve Wratten. 2016. 'Scarcity of Ecosystem Services: An Experimental Manipulation of Declining Pollination Rates and Its Economic Consequences for Agriculture'. *PeerJ* 2016 (7): e2099. https://doi.org/10.7717/peerj.2099.

Sari, Fatih. 2020. 'Assessment of Land - Use Change Effects on Future Beekeeping Suitability Via CA-Markov Prediction Model'. *Journal of Apicultural Science* 64 (2): 263–76. https://doi.org/10.2478/jas-2020-0020.

Sarkar, J. M., G. L. Hennebert, and J. Mayaudon. 1986. 'Optimization and Characterization of an Extracellular Polysaccharide Produced ByMoniliella Pollinis'. *Biotechnology Letters 1986 8:5* 8 (5): 319–22. https://doi.org/10.1007/BF01040857.

Sauquet, Hervé, Maria Von Balthazar, Susana Magallón, James A. Doyle, Peter K. Endress, Emily J. Bailes, Erica Barroso De Morais, et al. 2017. 'The Ancestral Flower of Angiosperms and Its Early Diversification'. *Nature Communications* 8 (1): 1–10. https://doi.org/10.1038/ncomms16047.

Schäfer, Angela Maria, Martin Kemler, Robert Bauer, and Dominik Begerow. 2010. 'The Illustrated Life Cycle of Microbotryum on the Host Plant Silene Latifolia'. *Bontany* 88 (10): 875–85. https://doi.org/10.1139/B10-061.

Schoch, Conrad L., Barbara Robbertse, Vincent Robert, Duong Vu, Gianluigi Cardinali, Laszlo Irinyi, Wieland Meyer, et al. 2014. 'Finding Needles in Haystacks: Linking Scientific Names, Reference Specimens and Molecular Data for Fungi'. *Database: The Journal of Biological Databases and Curation* 2014: 1–21. https://doi.org/10.1093/DATABASE/BAU061.

Schoch, Conrad L., Keith A. Seifert, Sabine Huhndorf, Vincent Robert, John L. Spouge, C. André Levesque, Wen Chen, et al. 2012. 'Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi'. *Proceedings of the National Academy of Sciences of the United States of America* 109 (16): 6241–46. https://doi.org/10.1073/pnas.1117018109.

Schönleben, Simone, Albert Sickmann, Martin J. Mueller, and Joerg Reinders. 2007. 'Proteome Analysis of Apis Mellifera Royal Jelly'. *Analytical and Bioanalytical Chemistry* 389 (4): 1087–93. https://doi.org/10.1007/s00216-007-1498-2.

Scortichini, Marco, and Elena I. Katsy. 2014. 'Common Themes and Specific Features in the Genomes of Phytopathogenic and Plant-Beneficial Bacteria'. In *Plasticity in Plant-Growth-Promoting and Phytopathogenic Bacteria*, 9781461492:1–26. Springer New York. https://doi.org/10.1007/978-1-4614-9203-0_1.

Seeley, Thomas D. 1985. *Honeybee Ecology: A Study of Adaptation in Social Life*. Princeton, New Jersey: Princeton University Press. https://doi.org/10.2307/j.ctt7zvpg1.10.

Seeley, Thomas D. 1995. *The Wisdom of the Hive: The Social Physiology of Honey Bee Colonies*. Harvard University Press.

Sluijs, Jeroen P. van der, and Nora S. Vaage. 2016. 'Pollinators and Global Food Security: The Need for Holistic Global Stewardship'. *Food Ethics* 1 (1): 75–91. https://doi.org/10.1007/s41055-016-0003-z.

Sponsler, D. B., and R. M. Johnson. 2015. 'Honey Bee Success Predicted by Landscape Composition in Ohio, USA'. *PeerJ* 2015 (3): e838. https://doi.org/10.7717/peerj.838.

Sponsler, Douglas B., and Eve Z Bratman. 2020. 'Beekeeping In, Of, Or For The City? A Socioecological Perspective On Urban Apiculture'. *Preprint*, 1–11.

Steinmann, Nadja, Miguel Corona, Peter Neumann, and Benjamin Dainat. 2015. 'Overwintering Is Associated with Reduced Expression of Immune Genes and Higher Susceptibility to Virus Infection in Honey Bees'. Edited by Stephen J. Martin. *PLOS ONE* 10 (6): e0129956. https://doi.org/10.1371/journal.pone.0129956.

Suwannapong, Guntima, Daren Michael, and Mark Eric. 2012. 'Honeybee Communication and

Pollination'. In *New Perspectives in Plant Protection*, edited by Ali R. Bandani, 39–62. InTech. https://doi.org/10.5772/36272.

Taberlet, Pierre, Eric Coissac, François Pompanon, Ludovic Gielly, Christian Miquel, Alice Valentini, Thierry Vermat, Gérard Corthier, Christian Brochmann, and Eske Willerslev. 2007. 'Power and Limitations of the Chloroplast TrnL (UAA) Intron for Plant DNA Barcoding'. *Nucleic Acids Research* 35 (3): e14–e14. https://doi.org/10.1093/nar/gkl938.

Tantillo, Giuseppina, Marilisa Bottaro, Angela Di Pinto, Vito Martella, Pietro Di Pinto, and Valentina Terio. 2015. 'Virus Infections of Honeybees Apis Mellifera'. *Italian Journal of Food Safety*. Page Press Publications. https://doi.org/10.4081/ijfs.2015.5364.

Theodorou, Panagiotis, Rita Radzevičiūtė, Guillaume Lentendu, Belinda Kahnt, Martin Husemann, Christoph Bleidorn, Josef Settele, et al. 2020. 'Urban Areas as Hotspots for Bees and Pollination but Not a Panacea for All Insects'. *Nature Communications* 11 (1): 1–13. https://doi.org/10.1038/s41467-020-14496-6.

Thoa, Kim Vu, Nguyen Thi Minh Khanh, Dinh Duc Hien, and Le Duc Manh. 2015. 'Screening of Moniliella Yeast with High-Level Production of Erythritol'. In *Proceedings of International Scientific-Practical Conference*, 129–34. https://www.researchgate.net/publication/306250857.

Trhlin, M, and J Rajchard. 2011. 'Chemical Communication in the Honeybee (Apis Mellifera L.): A Review'. *Veterinarni Medicina*. Vol. 56.

Trivedi, Pankaj, Jan E. Leach, Susannah G. Tringe, Tongmin Sa, and Brajesh K. Singh. 2020. 'Plant–Microbiome Interactions: From Community Assembly to Plant Health'. *Nature Reviews Microbiology*. Nature Research. https://doi.org/10.1038/s41579-020-0412-1.

Vannette, Rachel L. 2020. 'The Floral Microbiome: Plant, Pollinator, and Microbial Perspectives'. *Annual Review of Ecology, Evolution, and Systematics* 51 (1): 363–86. https://doi.org/10.1146/annurev-ecolsys-011720-013401.

Vasudeva, R, and R Lokesha. 1993. 'Plant Pollinator Interactions'. *Current Science* 65 (3): 198–201.

Wagler, R. 2017. '6th Mass Extinction'. In *Encyclopedia of the Anthropocene*, 1–5:9–12. Elsevier. https://doi.org/10.1016/B978-0-12-809665-9.10477-X.

Wagner, Lysett, Sybren De Hoog, Ana Alastruey-Izquierdo, Kerstin Voigt, Oliver Kurzai, and Grit Walther. 2019. 'A Revised Species Concept for Opportunistic Mucor Species Reveals Species-Specific Antifungal Susceptibility Profiles'. *Antimicrobial Agents and Chemotherapy* 63 (8). https://doi.org/10.1128/AAC.00653-19.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. 'Naïve Bayesian Classifier for Rapid Assignment of RRNA Sequences into the New Bacterial Taxonomy'. *Applied and Environmental Microbiology* 73 (16): 5261–67. https://doi.org/10.1128/AEM.00062-07.

Wang, Xin Cun, Chang Liu, Liang Huang, Johan Bengtsson-Palme, Haimei Chen, Jian Hui Zhang, Dayong Cai, and Jian Qin Li. 2015. 'ITS1: A DNA Barcode Better than ITS2 in Eukaryotes?' *Molecular Ecology Resources* 15 (3): 573–86. https://doi.org/10.1111/1755-0998.12325.

Whittaker, R. H. 1972. 'Evolution and Measurement of Species Diversity'. *TAXON* 21 (2–3): 213–51. https://doi.org/10.2307/1218190.

Wickham, Hadley. 2010. 'Ggplot2: Elegant Graphics for Data Analysis'. *Journal of Statistical Software* 35 (July): 1–3. http://had.co.nz/ggplot2/book.

Wilson, Maxwell C., Xiao Yong Chen, Richard T. Corlett, Raphael K. Didham, Ping Ding, Robert D. Holt,

Marcel Holyoak, et al. 2016. 'Habitat Fragmentation and Biodiversity Conservation: Key Findings and Future Challenges'. *Landscape Ecology*. Springer Netherlands. https://doi.org/10.1007/s10980-015-0312-3.

Winston, Mark L. 1987. *The Biology of the Honey Bee*. Cambridge: Harvard University Press.

Wright, Geraldine A., Susan W. Nicolson, and Sharoni Shafir. 2018. 'Nutritional Physiology and Ecology of Honey Bees'. *Annual Review of Entomology*. Annual Reviews Inc. https://doi.org/10.1146/annurev-ento-020117-043423.

Zaric, Nenad M., Isidora Deljanin, Konstantin Ilijević, Ljubiša Stanisavljević, Mirjana Ristić, and Ivan Gržetić. 2018. 'Assessment of Spatial and Temporal Variations in Trace Element Concentrations Using Honeybees (Apis Mellifera) as Bioindicators'. *PeerJ* 2018 (7): e5197. https://doi.org/10.7717/peerj.5197.

Zhang, Jing, Xin Wang, Jiang Wu, and Deepika Kumari. 2019. 'Fungal Community Composition Analysis of 24 Different Urban Parks in Shanghai, China'. *Urban Ecosystems* 22 (5): 855–63. https://doi.org/10.1007/s11252-019-00867-5.

Zhang, Shaowu, Aung Si, and Mario Pahl. 2012. 'Visually Guided Decision Making in Foraging Honeybees'. *Frontiers in Neuroscience*. Frontiers Research Foundation. https://doi.org/10.3389/fnins.2012.00088.