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Latitudinal effect on mosquitoes' microbiota

Bachelor thesis

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Annotation

The aim of this thesis was to find environmental influences on the microbiota of *Culex pipiens* mosquito, while investigating the 16S ribosomal RNA composition of the samples, collected over different latitudes in Europe.

Declaration

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abstract

Culex pipiens mosquitoes are vectors of different widespread pathogens, and therefore have a huge influence in public health. Especially the composition of the microbiota can play an important role in mosquito infection and disease transmission to other living organisms. Therefore, the different influencing factors and their effects on the mosquito microbiota are of special interest.

In this study, the focus will lie on the different biogeographical patterns that may influence the microbiota composition of *Culex pipiens* mosquitoes. The main focus will be on latitude, but also the longitude, the country, the localities within them and the habitat of the collecting site will be considered. The main hypothesis of this work is that if the latitude (directly associated with temperature) has an influence on the microbiota of the mosquito, we can foresee the impact of the climate change on the composition of the microbiota and, thus, on vector-borne diseases spread.

First of all, the mosquitoes were collected in eight European countries, morphologically identified, catalogued, and preserved in AllProtect buffer. After this process the *Allprep96 DNA/RNA Kit* was used to extract the DNA and the RNA of the different mosquito samples individually. This was followed by PCR and sequencing. The outcoming results were compared qualitatively using microbiome taxonomic profiles, and quantitatively using alpha diversity indices and statistical tests.

All the samples were molecularly confirmed to be *Culex pipiens pipiens*. In the statistical analyses only latitude, longitude and localities showed significant differences ($p < 0.05$), when checking for the alpha diversity of the microbiome including *Wolbachia*, but not when excluding this endosymbiont. Moreover, the comparison of localities within countries showed different outcomes for each country, significant differences in Italy, Slovakia and The Netherlands were obtained. No local influences within the countries Spain and Sweden were seen. Local influences could only be observed in the *Wolbachia* proportion, and not in the remaining composition of the microbiota. This geographical difference in the *Wolbachia* percentage is not, however, directly associated with country or latitude. Also, the habitat has no influence on the composition of the microbiota. This research's outcome should contribute to the understanding of the environmental factors driving the microbiota composition in mosquitoes, and ultimately help in the fight against mosquito-borne diseases. Future studies should further delve into the influence of particular environmental factors on the mosquitoes' microbiota.

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1 Introduction

1.1 Mosquitoes as vectors of disease

The group Diptera includes well-known insects such as flies and mosquitoes (Rozo-Lopez and Mengual, 2015). There are more than 3555 mosquito species (Diptera: Culicidae) currently existing worldwide (Jayakrishnan et al., 2018). We can differentiate between invasive and native populations of mosquitoes (Zittra, 2013). The native populations of mosquitoes are those that naturally originate from a certain region, like *Anopheles maculipennis* in Europe, whose distribution is shown in Figure 1.

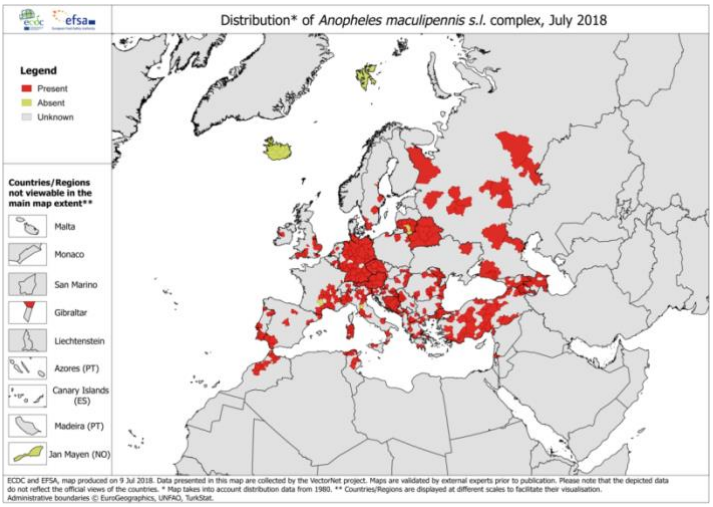

Figure 1 Native distribution of Anopheles maculipennis s.l. complex in Europe: July 2018 (*ECDC, 2018)*

In contrast, invasive mosquitoes are those that are foreign species in the regions, which influences the habitat in a harmful way (Juliano and Lounibos, 2005). This negative impact distinguishes the invasive species from non-native species, which are also foreign in the region, but not endangering the environment. These invasive species cause billions of damages per year (Tobin, 2018). For this reason, great effort is put into surveillance programs to detect the spread of invasive mosquito populations, as illustrated for European and Mediterranean countries in Figure 2. The darker blue areas depict populations where vector surveillance is on-going, whereas the lighter blue areas represent regions where no vector surveillance activities are being carried out, and the grey areas show regions where data is not available.

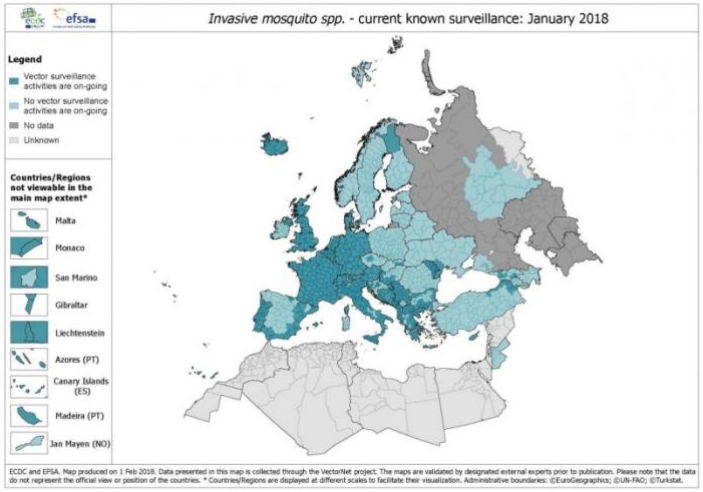

Figure 2 Invasive mosquito species in Europe – known surveillance: January 2018 (ECDC, 2018)

Mosquitoes can be a big threat, especially for humans, livestock and other animals, when transferring pathogens from their own microbiota (Marcantonio et al., 2015; Schaffner et al., 2013). In fact, these disease-causing agents are increasing in high rates. There are a lot of diseases that can be transferred from mosquitoes to human beings, for example the West Nile virus, malaria, yellow fever and dengue fever (Fang, 2010).

Pathogens vectored by mosquitoes are responsible for several millions of deaths per year and the caused diseases' prevalence is much higher according to the WHO executive summary (https://www.who.int/whr/1996/media_centre/executive_summary1/en/index9.html (accessed 28.11.2018)). The pathogens that can be transmitted by mosquitoes are divided into three big groups: viruses, bacteria and protozoa (Federici, 2009). In the case of viruses and protozoans, the mosquitoes get infected by biting an infected animal (Andreadis, 1985; Olson et al., 1996). The other group of pathogens transmitted by mosquitoes, bacteria, only need contact for transmission to occur. That is the reason why the most common infections in insects are due to bacteria (Wagner, 2004). After infection, viruses replicate in the midgut and can infect humans afterwards during the ingestion of the following bloodmeal (Olson et al., 1996). For bacteria and protozoans there is no replication necessary, and transmission to humans is just mechanical (Gubler, 2009).

The best-known mosquitoes belong to the genera *Aedes, Culex, Anopheles, Culiseta, Coquillettidia* and *Ochlerotatus.* Particularly, the most dangerous disease carriers are from the genera *Culex, Anopheles* and *Aedes*, which are described in more detail below (Akorli et al., 2016; Baldacchino et al., 2015; Harbach, 2012).

The *Anopheles* genus is spread over the whole world and includes around 420 species. These species mainly transmit the malaria parasite (Holt et al., 2002). Malaria is a well-known

disease vectored by mosquitoes. It is caused by the parasite *Plasmodium* and is transmitted by the female *Anopheles* mosquitoes (Boissière et al., 2012; Holt et al., 2002).

The *Aedes* genus includes more than 950 species, especially native to the tropical regions. As a result of environmental changes due to human activities, they are also spreading to new areas, where they are considered as invasive species. This species vectors several viral diseases, for example, the Zika fever and the dengue fever (Christophers, 1960). Dengue fever causes 50 million infected people per year (Guha-Sapir and Schimmer, 2005). It is in fact one of the most important mosquito-borne virus diseases, with 2.5 billion people in the world at risk of getting infected (Xi et al., 2008). This illness is mostly found in the tropical areas of the world (Gubler and Clark, 1995), and the infected mosquito transports the virus after infection for its whole life (Xi et al., 2008). The dengue fever virus is closely related to the West Nile virus and the yellow fever virus.

The *Culex* genus includes 769 species, which occur in the tropics, subtropics, temperate and Holarctic areas of the world (Almirón et al., 1995). A lot of these species feed on humans, other mammals or birds. The *Culex* mosquitoes transmit for example the West Nile fever or the Rift Valley fever (Fawzy and Helmy, 2019; Hayes et al., 2005). From 1999 to 2010 there were more than 2.5 million people infected by West Nile virus (WNV) (Colpitts et al., 2012). WNV is widespread in Africa, Europe, the Middle East, North America and West Asia (Campbell et al., 2002). The virus is transmitted by mosquitoes that feed upon infected birds or other infected hosts. Humans, and especially horses, are the most well-known "dead-end" hosts for the virus, so they suffer the disease, but do not spread the infection towards other mammals (Campbell et al., 2002). As one of the most widespread and epidemiologically relevant species, this mosquito will be the object of this research, and thus it will be discussed in more detail below.

1.2 The microbiota and its interaction with the vectored pathogens

Every living organism hosts a wide range of microorganisms, its microbiota. The microbiota is indispensable for the survival of the host, as it provides many important functions, like metabolizing food (e.g. polysaccharides and polyphenols; (Rowland et al., 2018)), producing vitamins, regulating the immune system, or breaking down toxins, which protects the host from diseases or inflammation (Engel and Moran, 2013).

A disturbance in the right composition of the microbiome (i.e. dysbiosis) can have the effect of a disease for the host (Phillips, 2009). In the field of vector-borne disease control, one of the most relevant microbiota functions is the effect on vector competence.

Insects carry microbes in their body, some of them with pathogenic effects (e.g. life shortening; (Romoli and Gendrin, 2018)), and some of them living in symbiosis (Engel and Moran, 2013). For example, the tsetse fly, transmitter of the African sleeping sickness (Kennedy, 2008), hosts three main bacteria: *Wolbachia, Wigglesorthia* and *Sodalis.* Ongoing studies involving *Sodalis* could lead to a big step forward in the research of the influence of bacteria on diseases (Wang et al., 2013). Ticks are also important transmitters of different diseases, and their microbiomes differ between species and are well described (Gall et al., 2016). It has been previously shown that the microbiome of larvae, kept in sterile conditions, increases blood feeding and decreases infection by the Lyme disease bacterium *Borellia.* So potentially the feeding way of the ticks could lead to new developments in the research of vector-borne pathogens and the resulting diseases mediated by the microbiota (Narasimhan et al., 2014). There are also studies about microorganisms that could be used to reduce the lifespan of the mosquitoes or to block the pathogens or parasites' proliferation through natural competition mechanisms or by expression of anti-pathogen molecules genetically introduced by paratransgenesis (Muturi et al., 2018). Particularly in mosquitoes, the microbiota can produce several metabolites and toxins that kill the pathogens, or limit host colonization by reducing the available nutrients, interfering with the high nutrient requirement of the parasites (Ramirez et al., 2014). In recent studies, a bacterium that improves the defense against malaria and dengue pathogens, which could also prevent infections of these diseases, was found (Ramirez et al., 2014). Some other studies lay their focus on the role of antibiotics in the interaction with the microbiome (Gendrin et al., 2015), or the role of enzymes and the immune system (Gendrin et al., 2017, 2013). For example, antibiotics, transferred from the human blood to the microbiota, can influence the capacity of the transmission of malaria (Gendrin et al., 2015).

To get more opportunities to reduce mosquito-borne diseases, it is important to understand the interaction of the microbiota and the pathogen. In the past, the main tools to fight against these diseases relied on the containment of biting rates and mosquito populations. For this purpose, several methods, such as pesticide application, adulticide and larvicide treatments and source reduction, were used to eliminate mosquito populations and to prevent their reproduction

(Alphey et al., 2010; Baldacchino et al., 2015; Medlock et al., 2012). The source reduction is focuses on reducing breeding sites and is often used in combination with pesticides, which often leads to better control of the mosquito problem (Medlock et al., 2012). However, these methods may lose effectivity over time, for example with the development of resistance in the mosquito populations (Hegde et al., 2015). As a consequence, many recent studies focus on the development of new control strategies and most of these new research lines have their foundation in the closer examination of the microbiota and its functionality (Hegde et al., 2015).

1.3 Environmental influences on the mosquitoes' microbiota

An essential piece of knowledge, necessary to better understand the microbiome assemblage in mosquitoes and how to use it to fight against the vectored diseases, are the environmental factors that affect the microbiota composition. The environment plays a key role in several characteristics and behaviours of all animals, including insects (Collier et al., 1982). As an essential part of most living beings, the microbiota is not indifferent to environmental changes either (Spor et al., 2011). In fact, the environmental influence on the microbiome has been described for many organisms, including fish (Sullam et al., 2012), birds (i.e. chicken (Kers et al., 2018), and mammals (i.e. humans (Phillips, 2009)). Insects' microbiota also has been found to vary according to different environmental factors, like the way of feeding in field crickets (Ng et al., 2018) or the larval breeding site and season of breeding in *Anopheles* mosquitoes (Akorli et al., 2016). Other environmental conditions have an impact on the mosquito microbiota as well, such as water conditions (Saab et al., 2020), diet (Muturi et al., 2019), temperature (Novakova et al., 2017), species (Muturi et al., 2016), seasonality (Akorli et al., 2016), locality (Akorli et al., 2016), climate (Mandrioli and Emilia, 2012; Marcantonio et al., 2015). The influence of the seasonality and locality has been particularly studied in *Anopheles gambiae* and *coluzzii.* The diversity of their microbiomes in the dry season is higher than the diversity in the rainy season. Furthermore, the diversity is higher in urban localities for *Anopheles coluzzii* (Akorli et al., 2016). Wang et al. (2018) showed that the bacterial component in the aquatic habitat of the larvae of *Aedes albopictus* has a huge impact on the larval development. When testing the development in an antibiotic treated aquatic environment, the development is nearly not existing, while the different food supplements have little impact as long as they are available. The differences in bacteria of the microbiota

between laboratory and field collected mosquitoes have also been estimated, showing that Proteobacteria are the most common bacteria found in both, but other dominant phyla (namely Bacteroidetes and Actinobacteria) have inverted their relative abundance positions.

Rosso et al. (2018) compared the gut microbiota of *Aedes albopictus* from different locations: Italy, France and Vietnam. They showed differences at phylum level, where Proteobacteria had higher percentages in the Italian samples than in the French and Vietnamese samples. The relative abundance of Bacteroidetes, Actinobacteria and Firmicutes in the Italian samples were, therefore, lower. Also, the percentage of unidentified organisms was much higher in French and Vietnamese samples than in the Italian ones. These and other recent studies concerning the influence of the seasons and the vicinity to urban areas (Akorli et al., 2016), but also other factors like housing, climate, litter or available diet (Kers et al., 2018), have confirmed the environmental influence in the microbiome. However, in spite of the available knowledge, many aspects of one of the most widespread mosquito species' microbiome, the vector of WNV *Culex pipiens* (*Cx. pipiens*), remains largely unknown. One of such aspects is the impact of different environmental and biogeographical factors in *Cx. pipiens* biology, including its microbiome. The increasing concern about global warming and its consequences makes it even more urgent to assess how biogeographical patterns, and their concomitant environmental variables, affect the microbiome of *Cx. pipiens*.

2 Aims

In this research work the main focus will lie on the microbiota of the *Cx. pipiens* mosquito. The goal is to check the biogeographical patterns in the mosquitoes' microbiota. For this purpose, samples were collected in different European countries along a gradient of latitudes. Microbiome composition will be assessed by sequencing of the 16S rRNA gene.

The specific aims of this thesis are:

1) To describe the latitudinal patterns of the microbiome in *Cx. pipiens* populations of Europe.
2) To assess some of the environmental factors that could potentially cause the geographical patterns found.
3) To discuss the potential effect of climate change in the microbiome-mosquito dynamics, and its relevance for mosquito-borne diseases epidemiology.

3 Materials and methods

3.1 Study organism: *Culex pipiens* and its microbiome

One of the most widespread and epidemiologically relevant species of mosquitoes is *Cx. pipiens*. This mosquito species is also known as the northern house mosquito (Robich et al., 2007). Its distribution includes North America, Europe and Asia (Harbach, 2012). In contrast to other mosquitoes, *Cx. pipiens* survives also the winter months with snow (Brugman et al., 2018). Before the winter season, they eat a lot of nectar to get fat reserves in their body for the cold time (Zittra, 2013). This species lays their eggs in stagnant water, often in gardens (Zittra, 2013). For this reason, the storage of water in the urban regions can lead to a problem of increasing populations of mosquitoes in the cities (Brugman et al., 2018).
Cx. pipiens belongs to the family Culicidae and the subfamily Culicinae (Brugman et al., 2018). *Cx. pipiens* has several sister groups, including *Culex torrentium* (Werblow et al., 2014), *Culex quinquefasciatus* (Cornel et al., 2003), *Culex australicus* (Harbach, 2012) and *Culex globocoxitus* (Harbach, 2012). The *Cx. pipiens* species contains also two forms. The first one is the *pipiens* form, and the second one is known as *molestus* form (Martínez-de la Puente et al., 2016). *Cx. pipiens pipiens* and *Cx. pipiens molestus* forms differ in general in their ecological and behavioural characteristics (Brugman et al., 2018). *Cx. pipiens molestus* lives preferably in underground areas, but in Europe they were also seen living above ground (Amraoui et al., 2012). *Cx. pipiens pipiens* lives preferably above the ground all over the world (Martínez-de la Puente et al., 2016), but has also been seen to live underground in Europe (Amraoui et al., 2012). From the morphological point of view, these two subspecies are identical, and their brown-greyish appearance is similar. The only differences are found in the genomic regions flanking the CQ11 microsatellite locus of their genomes, which is used as a marker to differentiate them (Kent et al., 2007; Shaikevich et al., 2016).

Cx. pipiens is the main vector of the WNV in Europe (Turell et al., 2001). As a vector of disease, it is thus important to have a closer look on *Cx. pipiens* microbiome, because as mentioned before, the interaction between pathogens and microbiomes play a role in vector competence. It is known that a regular microbiome is essential for *Cx. pipiens* normal development to an adult stage (Jayakrishnan et al., 2018). Recent studies about the microbiota

of *Cx. pipiens* show that this species has 195 bacterial Operational Taxonomy Units (OTUs) belonging to 9 phyla and 54 families (Muturi et al., 2016).
The dominant phylum (99%) is Proteobacteria, mostly Alphaproteobacteria (94.37%), and the second most abundant phylum is Firmicutes (0.53%) (Muturi et al., 2016). Within Alphaproteobacteria *Wolbachia* makes 76-98% of the whole microbiota of *Cx. pipiens*. In general, *Wolbachia* causes reproductive alterations in the host: male killing, parthenogenesis, feminization and cytoplasmic incompatibility (IC) (Muturi et al., 2016). These facts together can shorten the life span of the mosquito, and in the case of *Cx. pipiens* it causes IC between uninfected females and infected males. Furthermore, it confers a fitness advantage to the infected females, which can accelerate the process of invading the host population (Muturi et al., 2016).

3.2 Sampling and storage of the mosquito samples

Cx. pipiens mosquitoes were collected between June and August 2018 (from 08.06.2018 to 29.08.2018) in eight European countries: Austria, Czech Republic, Italy, Portugal, Slovakia, Spain, Sweden and The Netherlands. The samples were collected between 36.98875N and 60.4158N latitude and between -8.793116E and 21.2574722E longitude. The samples from each country included different populations and different habitats (e.g. urban, semi-urban, industrial, marsh and garden/rural). They were sampled using standard traps, BG sentinel and CDC traps. With these methods, only host-seeking mosquito females are collected.
The samples were morphologically identified by our collaborators from each of these countries, preserved in AllProtect tissue reagent (Qiagen), and sent to our laboratory for further processing. Storage was carried out at -20°C until RNA/DNA extraction was performed.

3.3 DNA and RNA Extraction

Prior to extraction, legs and wings were removed from each individual with forceps in sterile conditions. After this step, the samples were individually washed first in sterile phosphate buffer solution (PBS), then in absolute ethanol and finally in sterile PBS again. The first PBS wash was used to remove the preservation buffer, the absolute ethanol was used to remove possible contaminants, and the last PBS washing was used to remove the ethanol that could interfere with the extraction. The advantages of PBS are that it keeps the pH value and cleans the sample without disruption.

Following the washing steps, each sample was placed in a tube with 350 µl of RLT buffer, where homogenisation was performed to disrupt the tissues and eventually the cell walls. The homogenized samples were subject to DNA and RNA extraction following the Allprep96 DNA/RNA Kit (Qiagen) indications. First, the samples were pipetted into the DNA extraction plate, placed on top of the S-block and then centrifuged at 6000 rpm for four minutes, until all the liquid went through. In this step, the RNA is present in the S-block, while the DNA remains in the plate columns.

The DNA was further washed with two buffers, AW1 and AW2, using centrifugation for four minutes at 6000 rpm each time. Then 50 µl of PCR-clean water were added twice, incubated for five minutes, and centrifuged at 6000 rpm for four minutes. The eluted DNA was stored in the freezer at -20 °C.

The RNA was placed into the corresponding RNA plate and washed with 350 µl of 70% ethanol, pipetting up and down three times, and centrifuged four minutes at 6000 rpm. Washing proceeded by adding sequentially 800 µl RW1 buffer, 800 µl of RPE buffer, and 800 µl RPE buffer. Between each buffer addition, centrifugation for four minutes at 6000 rpm was performed. To ensure complete removal of the ethanol from the previous buffers, the last centrifugation was carried out at 6000 rpm for 10 minutes. Then, two elution steps with 45 µl of RNase-free water each, were performed, incubating for a minute before centrifugation for four minutes at 6000 rpm. The RNA samples were stored in an ultra-freezer at -80 °C.

3.4 Polymerase chain reaction (PCR)

Two different PCRs were carried out on the mosquito samples: one to identify the species (Smith and Fonseca, 2004) and another one to identify the subspecies (Bahnck and Fonseca, 2006) within the morphologically undistinguishable *Cx. pipiens* complex. PCR fundaments, including amplification steps, are shown in Figure 3. The different reaction mixtures, primers and amplification programs used, are summarized in Table 1.

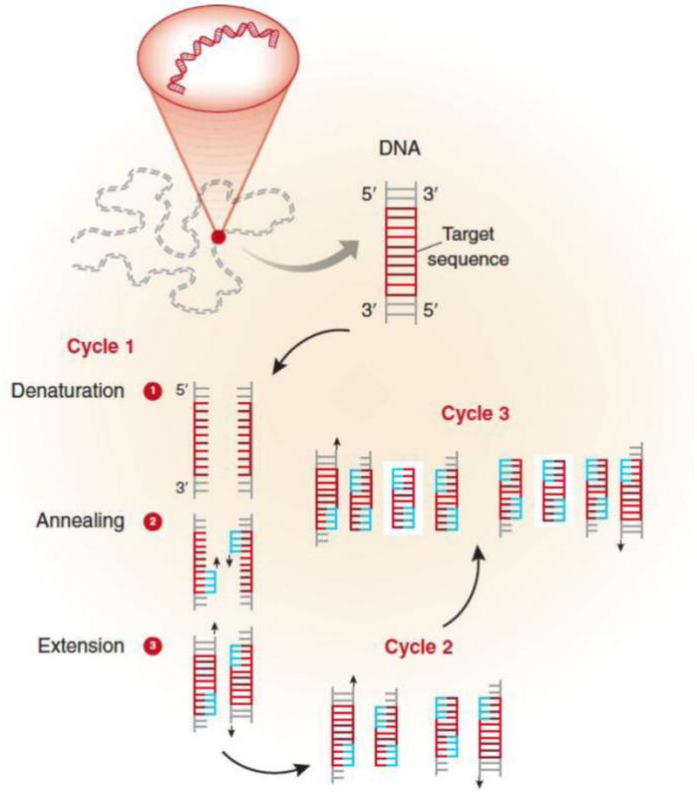

Figure 3 PCR fundaments (Garibyan and Avashia, 2013)

The Master Mix used for PCR contained 1x PCR buffer (including Taq polymerase and dNTPs) and the appropriate primers for each analysis. For every PCR a negative control was included using 1 µl water instead of the DNA template.

Table 1 PCR mixtures and primers

Identification of species	Identification of subspecies
10 µl PCR buffer + Taq polymerase 5 µl water 2 µl B1246s 1 µl ACEpip 1 µl ACEtorr 1 µl DNA	10 µl PCR buffer + Taq polymerase 2.5 µl water 1.5 µl CQ11F 1.5 µl pipCQ11R 1 µl molCQ11R 1 µl DNA
ACEpip 5´-GGAAACAACGACGTATGTACT-3´ ACEtorr 5´-TGCCTGTGCTACCAGTGATGTT-3´ B1246s 5´-TGGAGCCTCCTCTTCACGGC-3´	CQ11F2 5´-GATCCTAGCAAGCGAGAAC-3´ pipCQ11R 5´-CATGTTGAGCTTCGGTGAA-3´ molCQ11R 5´- CCCTCCAGTAAGGTATCAAC-3´

For the identification of the species, the amplification program consisted of one cycle at 94°C for five minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for one minute, and one cycle at 72°C for five minutes (Smith and Fonseca, 2004).

The next PCR was the one for the identification of the subspecies, *Cx. pipiens pipiens* or *Cx. pipiens molestus*. The thermocycling conditions were 94°C for 5 minutes and then 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 40 seconds, concluding with a final 5 minutes extension at 72°C (Bahnck and Fonseca, 2006).

3.5 Gel electrophoresis

To evaluate the PCR results, agarose gel electrophoresis was performed for each marker and sample. 2% gels were prepared in either 96-well format or 32-well format. 8 µl of each sample were mixed with 2 µl of loading buffer. For comparison of the samples, 5 µl of the 100 bp ladder was loaded on the edges of each row in the gels. The gels were run at 210V for around 50 minutes. Afterwards, the gels were observed under UV light and the results interpreted according to the respective publications. For the identification of *Cx. pipiens* species, a band between 634-636 base pairs (bp) was expected. The subspecies were subsequently distinguished between *Cx. pipiens pipiens* that showed a band around 200 bp and *Cx. pipiens molestus* that showed a band at 250 bp. Hybrid forms could also be detected when bands of both sizes were observed simultaneously.

3.6 Library preparation and microbiome sequencing

The libraries for microbiome analyses were performed according to the EMP protocol (http://www.earthmicrobiome.org/protocols-and-standards/16s/ (accessed on 07.05.2020)) using the forward primer 515F (Parada et al., 2016) and the reverse primer 926R (Parada et al., 2016; Quince et al., 2011), modified to include a double-barcoding strategy. The samples were labelled with barcodes to be able to distinguish them, since they were mixed in a single sequencing run. The amplified region was the V4–V5 of the 16S rRNA gene. PCR was performed in 96-well plates under following thermocycling conditions: 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, and a final extension step of 72 °C for 10 minutes. The PCR mix was prepared as stated in Table 2.

After the PCR was performed, the presence of the PCR product was tested using agarose gels with expected band size of approximately 500 bp. PCR products were cleaned up using AMPure XP magnetic beads (Beckman Coulter). Equal amounts of each amplicon were mixed in tubes according to the concentrations measured in a Synergy H1 microplate reader (Biotek).

Table 2 Library preparation: PCR mixtures and primers

PCR library preparation
13 µL PCR-grade water 10 µL Master Mix 0.5 µL forward primer (10 µM) 0.5 µL reversed primer (10 µM) 1 µL sample DNA
Forward primer: 1. 5′ Illuminaadapter 2. Golaybarcode 3. Forward primer pad 4. Forward primer linker 5. Forward primer (515F) AATGATACGGCGACCACCGAGATCTACACGCTXXXXXXXXXXXXTATGGTAATTGT GTGYCAGCMGCCGCGGTAA
Reversed primer: 1. Reverse complement of 3′ Illuminaadapter 2. Barcode 3. Reverse primer pad 4. Reverse primer linker 5. Reverse primer (926R) CAAGCAGAAGACGGCATACGAGAT XXXXXAGTCAGCCAGCCCCGYCAATTYMTTTRAGTTT

The library was sent for sequencing in an Illumina MiSeq run (two reads of 300 bp each with v2 chemistry) using custom primers for Read 1 (3' AATGATACGGCGACCACCGAGATCTACACGCT 5'), Read 2 (3' AGTCAGCCAGCCCCGYCAATTYMTTTRAGTTT 5') and Index read (3' AAACTYAAAKRAATTGRCGGGGCTGGCTGACT 5').

Using these, forward barcode and the DNA sequence of interest are analysed in Read 1, while Read 2 includes only the DNA sequence of interest (the reverse barcode is sequenced independently in the Index read).

3.7 Data processing and statistical analyses

The fastq files obtained from the sequencer were processed using the standard Illumina 16S amplicon sequencing pipeline of our laboratory (created by Sonia M. Rodríguez-Ruano (Rodríguez-Ruano et al., 2020)). This pipeline contains the following main steps, which will be explained in more detail afterwards: preparation of metadata and barcodes, extraction of barcodes from sequencing files and demultiplexing, sequence quality filtering, creating the OTU (Operational Taxonomic Unit) table, taxonomical assignments, and final filtering.

The files received from the sequencer include the Read 1 (forward read), the Read 2 (reverse read), and the Index read (barcode read used to identify the sequences and assign them to the samples in the metadata). In the first step, preparation of metadata and barcodes, the barcodes were matched with the samples in the metadata. Afterwards, the forward and reverse indexes were prepared using QIIME1 (Caporaso et al., 2010). The demultiplexing was performed using USEARCH (Edgar, 2010). In this step, the sequences were separated according to their barcodes and then forward and reverse reads were merged together for each sample, using a minimum overlap of 70 bp. In the sequence quality filtering step, the sequences of low quality were filtered out using USEARCH. The primers were also removed, and the length of the sequences was checked and trimmed, because different sequence lengths interfere with the alignment in the next step. While creating the OTU table, the clustering of the sequences was performed matching the sequences with 97% similarity using USEARCH. This generated an OTU table that contains the number of occurrences of every OTU per sample. Using the OTU representative sequences also obtained in this step, the taxonomy of the OTUs was identified with BLAST against the SILVA database truncated for SSU of 16S rDNA. The last step, a final filtering, was performed in QIIME1. In this step spurious sequences, as well as Archaea, Chloroplast and Mitochondrial sequences were filtered out, to retain only Bacteria in the results. Contaminants identified in the negative controls and pathogens present in the mosquito samples were also removed. In this way we ensured that all possible contamination during the experiments and potential infections in mosquitoes were not reflected in our bacterial microbiome data.

As an additional step, since we mentioned that *Wolbachia* is really prevalent and abundant in *Cx. pipiens*, we also generated a data set without *Wolbachia*. This extra file is helpful for the evaluation of our results, since this bacterium, which is maternally transmitted, takes up the largest part of the mosquito's microbiome and would not allow for the adequate assessment

of the rest of the mosquito microbiota, which is the part that could be potentially affected by the environmental factors tested.

Alpha diversity indexes were calculated using USEARCH (https://drive5.com/usearch/manual/cmd_alpha_div.html (accessed on 08.04.2020)). The different alpha diversity indexes used, were: richness (amount of OTUs per sample), Shannon index (combination of richness and evenness), dominance (focuses on the most abundant OTUs) and equitability (also known as evenness; measure for the even distribution of OTUs) (https://drive5.com/usearch/manual/alpha_metrics.html (accessed on 08.04.2020)).

The general information and figures in the Results section were created in Excel. All statistical analyses were performed in R. All figures and tests were performed with the datasets including and excluding *Wolbachia*.

The Kruskal-Wallis rank sum test, from the R "stats" package, was used for comparing all alpha-diversity indexes for the different categories: country, habitat, locality, latitude grouped by one degree and longitude grouped by one degree. In summary, eight different countries, four habitats, 20 localities, seven latitude groups clustered by one degree and ten longitude groups clustered by one degree were prepared.

The samples of the same countries were clustered, and the latitudes and longitudes of the countries are summarized in Table 3 and Table 4.

Table 3 Countries and their latitude ranges

Spain	36.98875N to 37.25222N
Portugal	37.851652N to 41.3342222N
Italy	44.947222N to 45.754027N
Austria	48.141296N to 48.294784N
Slovakia	47.7622778N to 48.74475N
Czech Republic	49.252984N
The Netherlands	52.05198N to 52.69772N
Sweden	60.1043N to 60.4158N

Table 4 Countries and their longitude ranges

Portugal	-8.793116E to -6.9634444E
Spain	-6.968481E to -6.443033E
The Netherlands	4.96832E to 6.64558E
Italy	11.018483E to 13.4694492E
Czech Republic	14.092055E
Austria	16.299404E to 16.431640E
Sweden	16.7516E to 17.2315E
Slovakia	17.0713333E to 21.2574722E

For the countries in which samples were collected in more than one locality, namely Italy, The Netherlands, Slovakia, Spain and Sweden, the localities within each country were compared to each other. There were three localities in Italy, four in The Netherlands, four in Slovakia, three in Spain and three in Sweden. In the data set without *Wolbachia*, the only change was that one of the three localities in Sweden did not reach enough sample number and had to be removed from the analyses, leaving only 19 localities to be analysed.

4 Results

4.1 Evaluation of gel electrophoresis

In the first part of the experiment, 110 mosquito samples were prepared for DNA/RNA extraction. Out of these samples, the PCRs for species (ACE marker) and subspecies (CQ11 marker) were done and the outcome of these PCRs was tested using agarose gels. Gels for all samples were prepared, but here just two examples are shown for every marker: species (ACE, Figure 4) and subspecies (CQ11, Figure 5).

The following results were obtained for the species evaluation:
Six samples (5,5%) showed no band in the gel, indicating a negative result for the species tested. For the rest of samples, 104 (94.5%), the result was a band at 600 bp, indicative of *Cx. pipiens*. In Figure 4, all visible bands indicated *Cx. pipiens* species at 600 bp and the empty spaces indicated negative tested samples.

The results for the subspecies identification were as follows:
Three samples (2.9%) were negative. Three samples (2.9%) showed multiple bands, which could be an indication of unspecific amplification. One sample (1%) showed a band at 700 bp. The *Cx. pipiens pipiens* subspecies was indicated by a band at 200 bp, which occurred in 97 samples (93.2%). In Figure 5, all of the before mentioned bands can be seen: the lower bands at 200 and the higher ones at 700 bp.

In summary, 97 samples were tested positive for species *Cx. pipiens* and subspecies *Cx. pipiens pipiens*. The samples tested negative or showing different bands than expected were removed from the data set. These included samples from Austria (N = 1), Czech Republic (N = 1), Italy (N = 4), Portugal (N = 3), Spain (N = 1), Sweden (N = 1) and The Netherlands (N = 2).

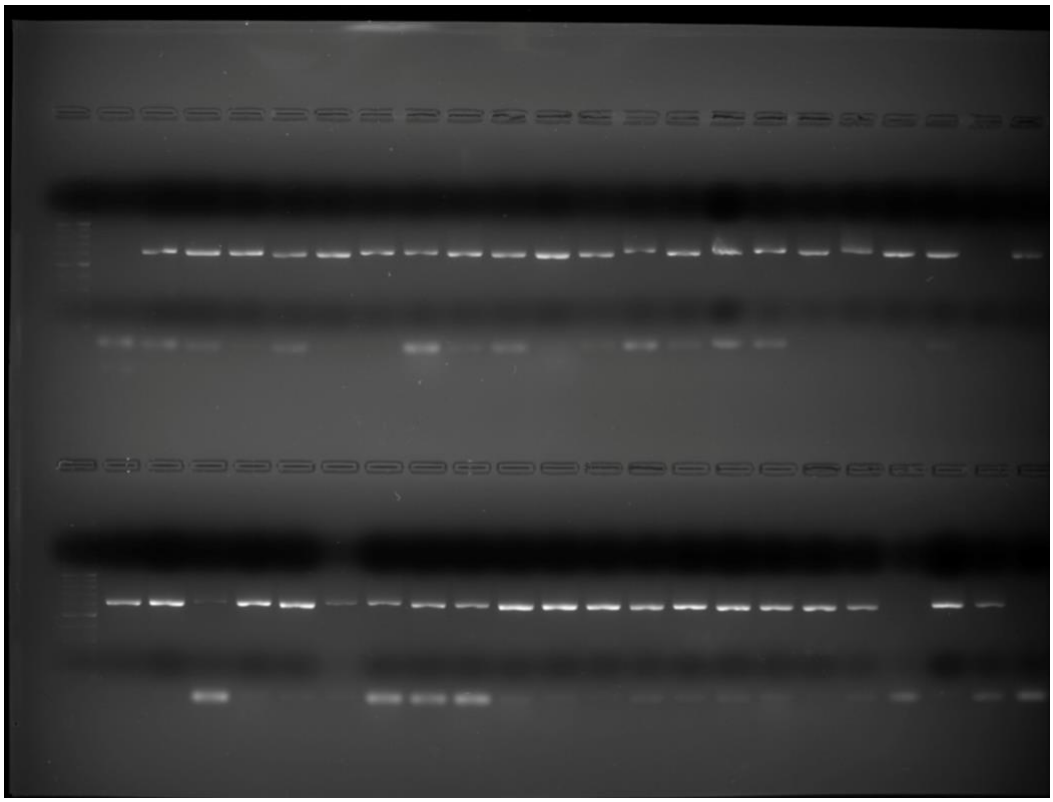

Figure 4 Agarose gel for determining species (ACE)

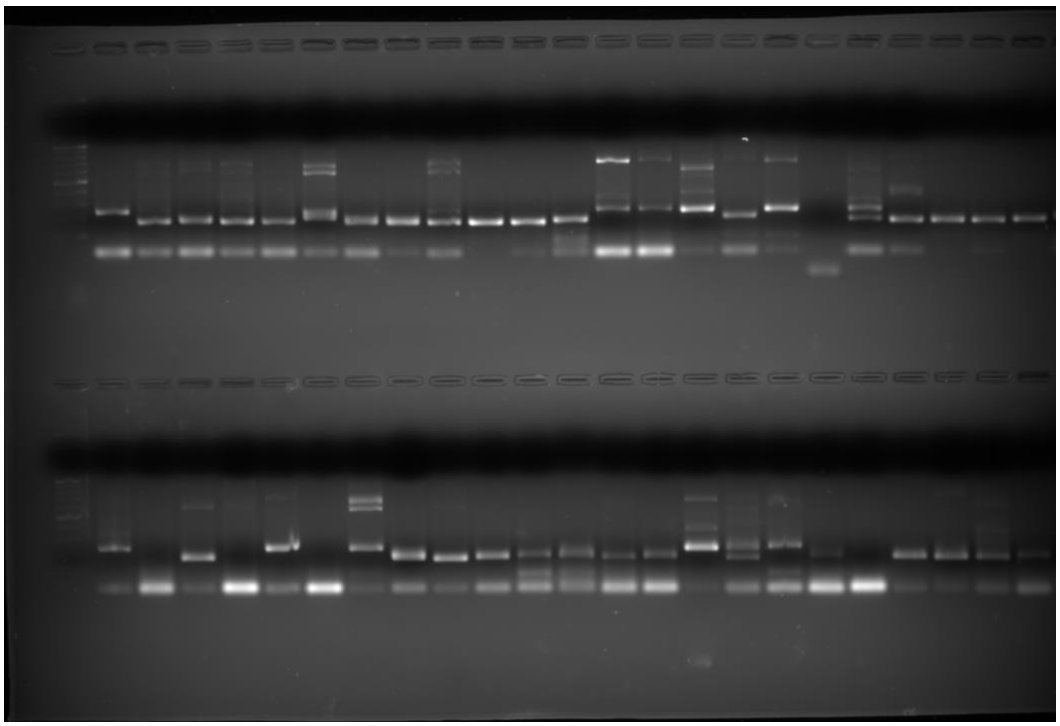

Figure 5 Agarose gel for determining subspecies (CQ11)

4.2 Evaluation of general information of DNA

110 samples were sent in for sequencing for all the countries sampled. The 13 samples that were tested negative for *Cx. pipiens pipiens* in the gels were not used for further analyses. First, the data was analysed including *Wolbachia*: Austria (N = 16), Czech Republic (N = 3), Italy (N = 16), Portugal (N = 7), Slovakia (N = 15), Spain (N = 11), Sweden (N = 12) and The Netherlands (N = 17). In the analyses without *Wolbachia* the following numbers of samples were left after the filtering: Austria (N = 7), Czech Republic (N = 2), Italy (N = 9), Portugal (N = 3), Slovakia (N = 8), Spain (N = 3), Sweden (N = 4) and The Netherlands (N = 12).

In the first case, 70 OTUs were found in total. The distribution of the OTUs can be seen in Figure 6. 41 OTUs (58.6%) belonged to the phylum Proteobacteria, 11 OTUs (15.7%) to the phylum Firmicutes. 10% belonged to the phylum Bacteroidetes, 7.2% were Actinobacteria, 5.7% were Planctomycetes and 1.4% belonged to each Cyanobacteria and Deinococcus-Thermus phyla. OTU 1 was identified as *Wolbachia*, which was the dominating bacterium with 855761 reads (88%) in total.

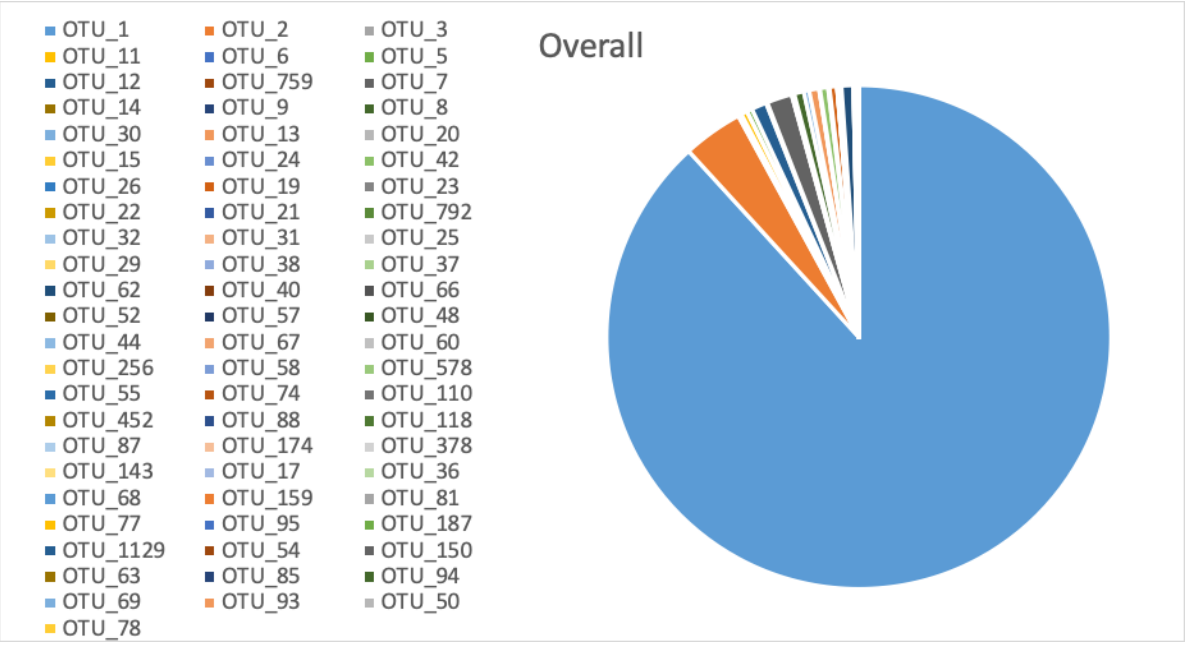

Figure 6 Overall OTU distribution with Wolbachia

The result of the analyses without *Wolbachia* included 48 samples. The distribution of the OTUs over all samples can be seen in Figure 7. Within these mosquitoes, 62 OTUs could be found. 36 (58.1%) OTUs belonged to the phylum Proteobacteria, 11 (17.7%) were Firmicutes, 11.3% were Bacteroidetes and 8.1% were Actinobacteria. 3.2% belonged to the phylum Planctomycetes and 1.6% were Deinococcus-Thermus. The dominant OTU, after *Wolbachia* was removed, was OTU 2, the Proteobacteria *Erwinia*, with 11347 counts (24%). Generally, the distribution of the rest of OTUs was quite even, around 0,002-8%.

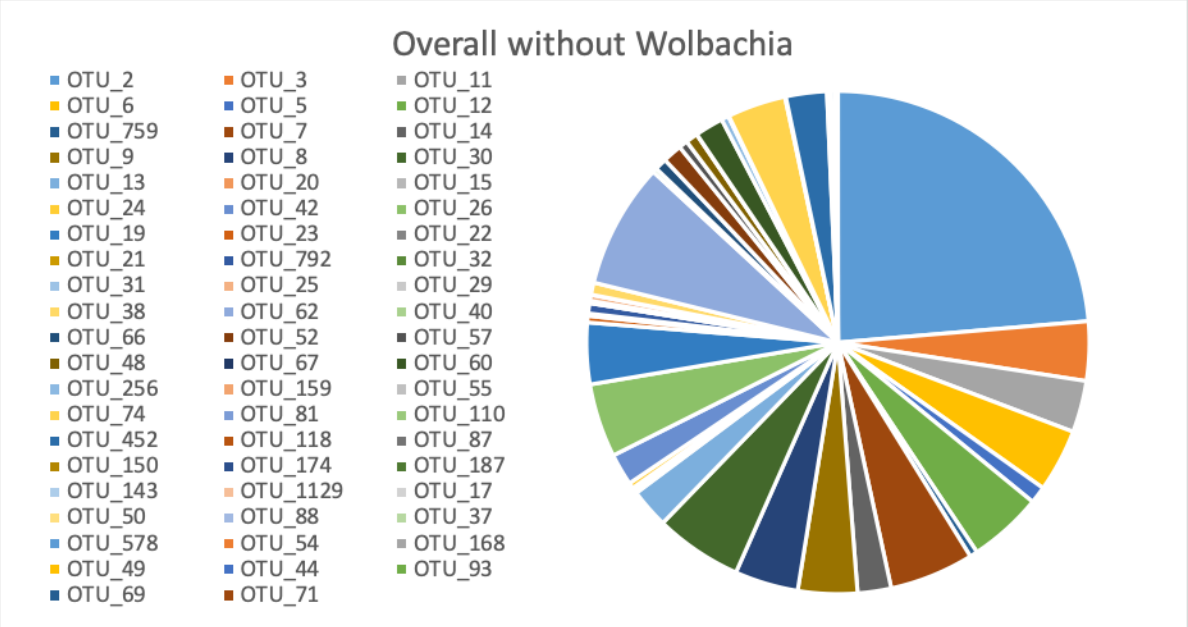

Figure 7 Overall OTU distribution without Wolbachia

When comparing Figure 6 to Figure 7, it can be seen immediately that the diversity of OTUs other than *Wolbachia*, can be only seen when removing that dominating bacterium.

The distribution of OTUs over the different countries sorted by latitude is shown in Figure 8 and Figure 9, with and without *Wolbachia* respectively.

In Figure 8, OTU 1 (*Wolbachia*) accounts for 91% (Spain), 84.5% (Portugal), 86.8% (Italy), 96.6% (Austria), 90% (Slovakia), 71.2% (Czech Republic), 81.7% (The Netherlands), and 89.9% (Sweden) of the total reads for each country. The second most abundant OTU observed for Spain, Czech Republic, The Netherlands, Sweden and Austria was OTU 2 (*Erwinia*) with 3.1%, 28.6%, 7.8%, 7.2% and 1.5% of the reads, respectively. In Portugal, OTU 13 (*Pseudomonas*) was the second most abundant OTU with 8.2% of the reads, while for Italy and Slovakia OTU 7 (*Orbus*) with 8.4% and OTU 62 (*Dietzia*) with 4.3%, respectively, were the second most abundant OTUs.

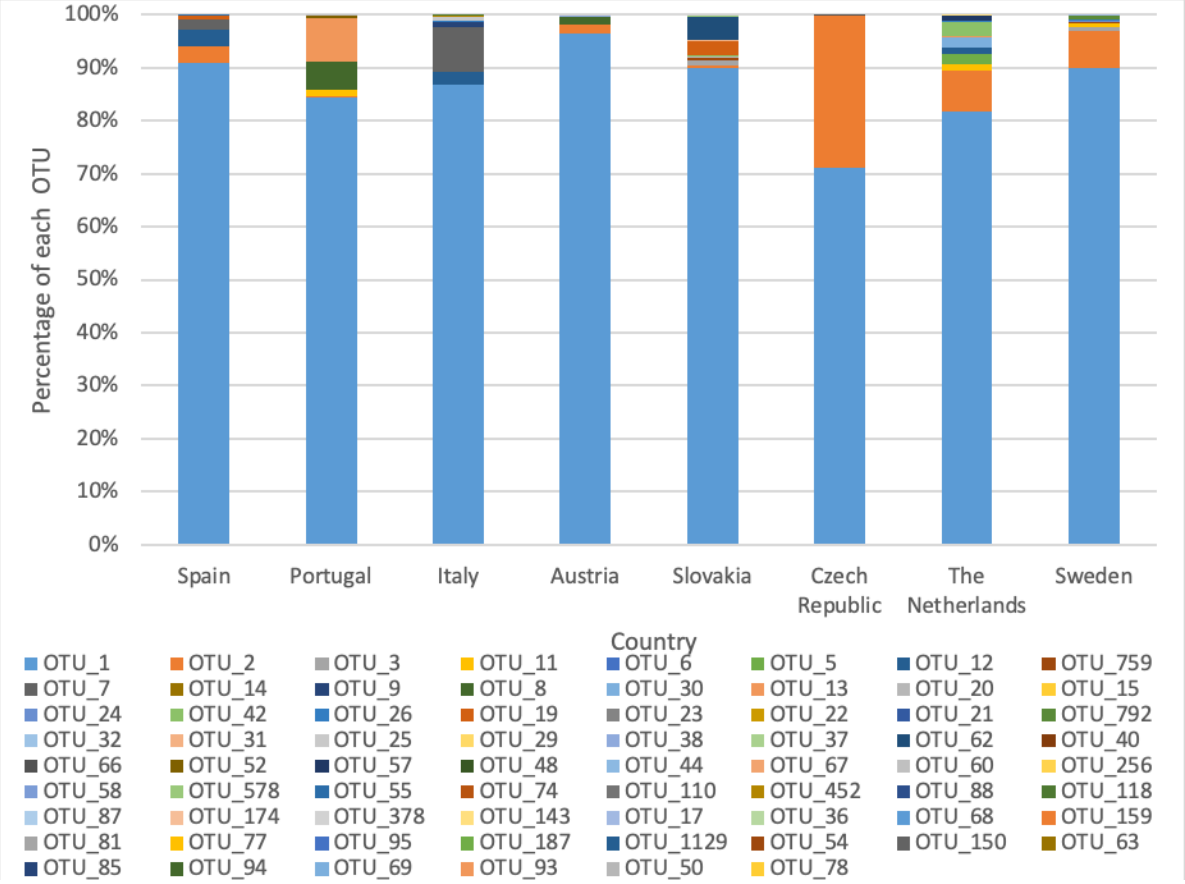

Figure 8 Comparison of OTU composition between countries sorted by latitude with Wolbachia

In Figure 9, the countries are compared and sorted according to their latitude. The dominant OTUs when removing *Wolbachia* were OTU 2, *Erwinia*, (Spain, 32.4%; Austria, 49%; Slovakia, 23.4%; Czech Republic, 52.7%; The Netherlands, 23.1%; Sweden, 26.3% of the

remaining reads), OTU 8, *Thorsellia*, (Portugal, 32.1%) and OTU 7, *Orbus*, (Italy, 24.1%). The next most abundant OTUs were OTU 19, *Asaia*, (Spain, 29.8%), OTU 13, *Pseudomonas*, (Portugal, 29.3%), OTU 9, *Rickettsia*, (Italy, 20.1%), OTU 8, *Thorsellia*, (Austria, 14.2%), OTU 74, *Rhodocyclaceae* (uncultured group) (Slovakia, 22.5%), OTU 62, *Dietzia*, (Czech Republic, 15%; Sweden, 20.9%) and OTU 30, *Pantoea*, (The Netherlands, 12.2%).

Even though in the analyses including *Wolbachia* OTU 13 was the most abundant besides OTU 1, this outcome shifted excluding *Wolbachia* to OTU 8, due to the decrease of Portugal samples remaining in the analyses. The same shift occurred also in the Slovakian samples.

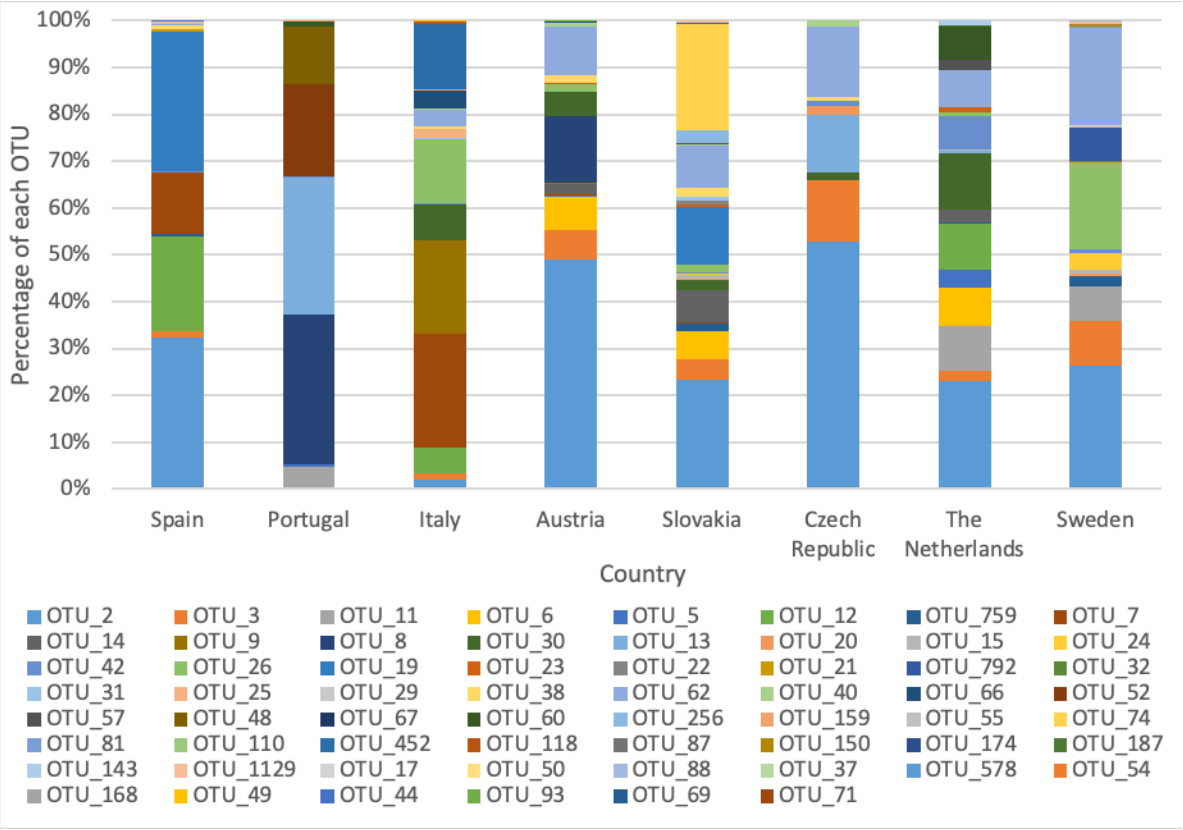

Figure 9 Comparison of OTU composition between countries sorted by latitude without Wolbachia

The second variable analysed was the habitat: garden/rural (N = 44), industrial (N = 21), urban (N = 9) and semi-urban (N = 8). Figure 10 shows the taxonomic profiles including *Wolbachia*. The values of OTU 1 (*Wolbachia*) were 91.6% (garden/rural), 80.2% (industrial), 83.6% (urban) and 99.5% (semi-urban). The second most abundant OTUs were OTU 7 (3.5%, garden/rural), OTU 2 (6.4%, industrial), OTU 62 (7.2%, urban) and OTU 74 (0.3%, semi-urban).

The different habitats were also analysed without *Wolbachia*: garden/rural (N = 20), industrial (N = 14), urban (N = 6) and semi-urban (N = 2) (Figure 11). In this case the most and second most abundant OTUs for garden/rural habitat were OTU 2 (27.2%) and OTU 7 (12.9%), for industrial habitat were OTU 2 (19.9%) and OTU 30 (10.5%), for urban habitat OTU 2 (16.9%) and OTU 19 (15.5%), and for the semi-urban habitat OTU 74 (90.1%) and OTU 26 (2%).
Also, in these analyses, due to the decrease of samples in the different categories, when excluding *Wolbachia*, shifts of OTU dominance occurred (garden/rural and urban habitat data were affected in this case).

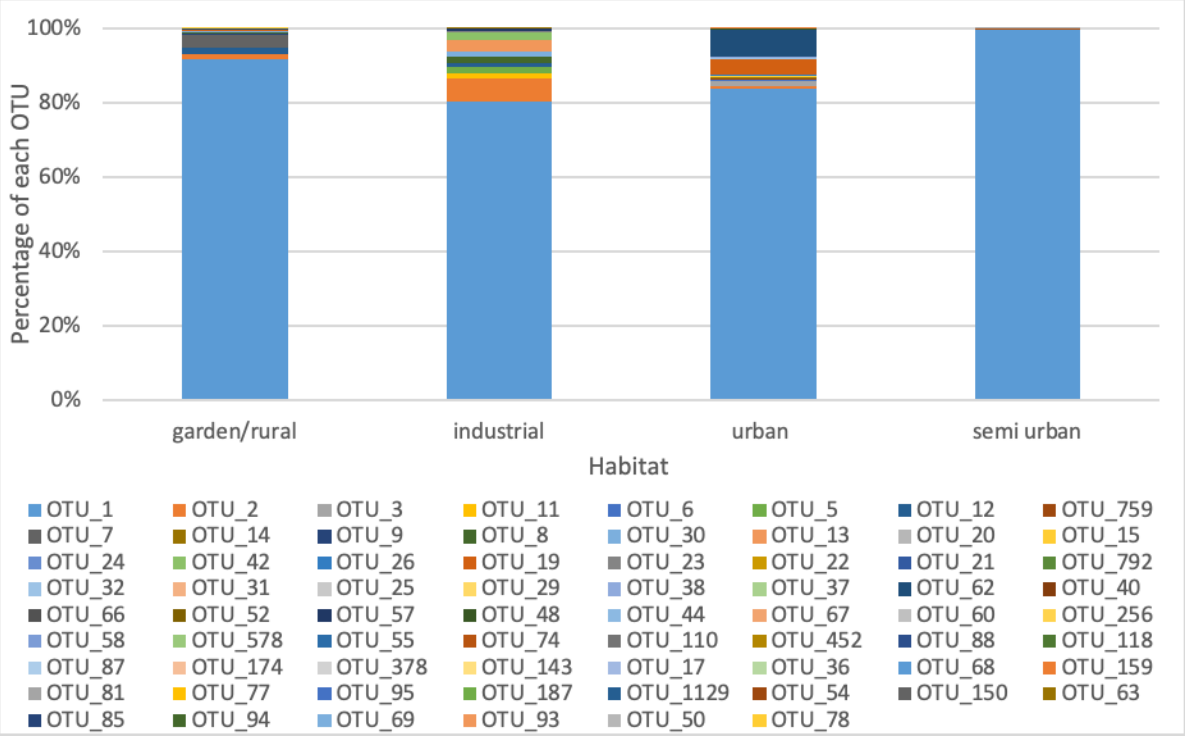

Figure 10 Comparison of OTUs composition between different habitats with Wolbachia

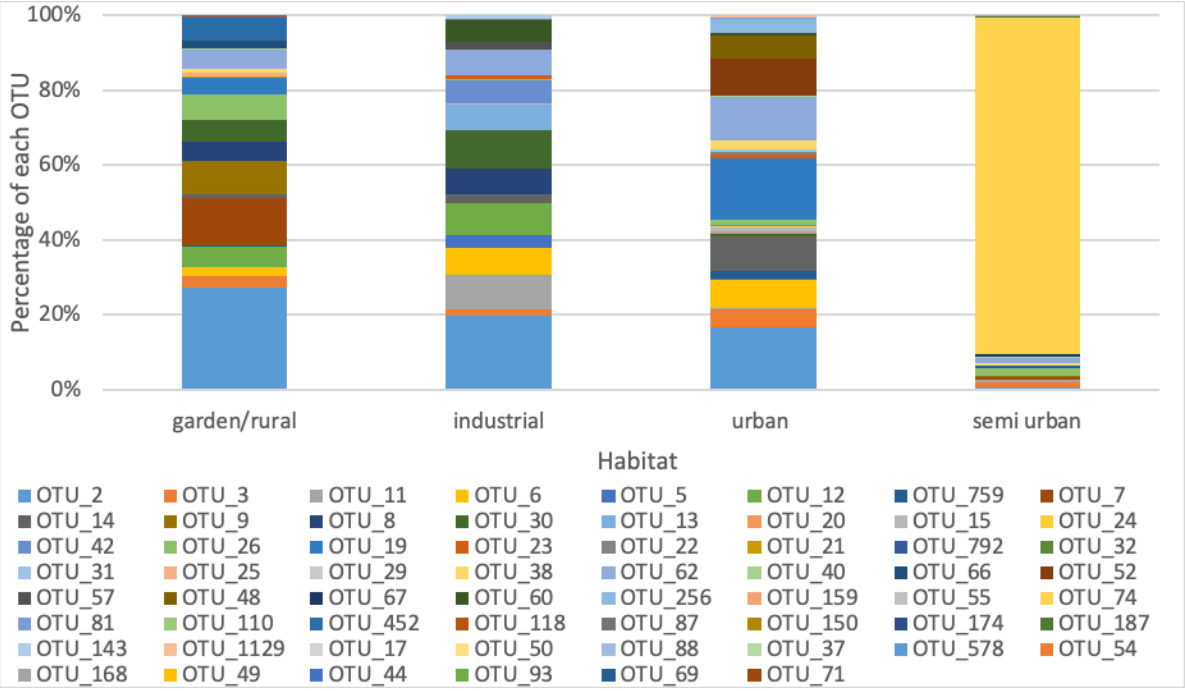

Figure 11 Comparison of OTUs composition between different habitats without Wolbachia

Lastly, all individual mosquito samples, ordered by increasing latitude, can be seen in Figure 12 (including *Wolbachia*) and 13 (excluding *Wolbachia*). Especially in the table excluding *Wolbachia*, the high inter-individual variation of the samples is clearly visible. Therefore, the finding of patterns in this highly variable data was extremely difficult, and shifts in the dominant taxa occurred, as previously stated, depending on the individuals included in each analysis (i.e. data sets with or without *Wolbachia*).

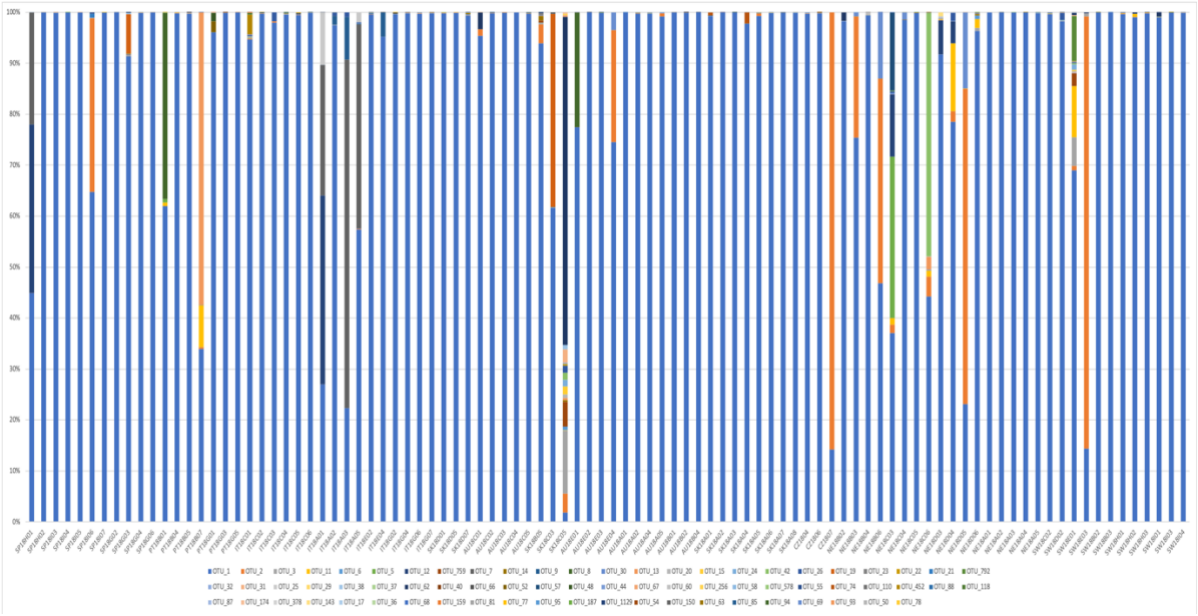

Figure 12 Microbiome of all mosquito individuals sorted by increasing latitude with Wolbachia

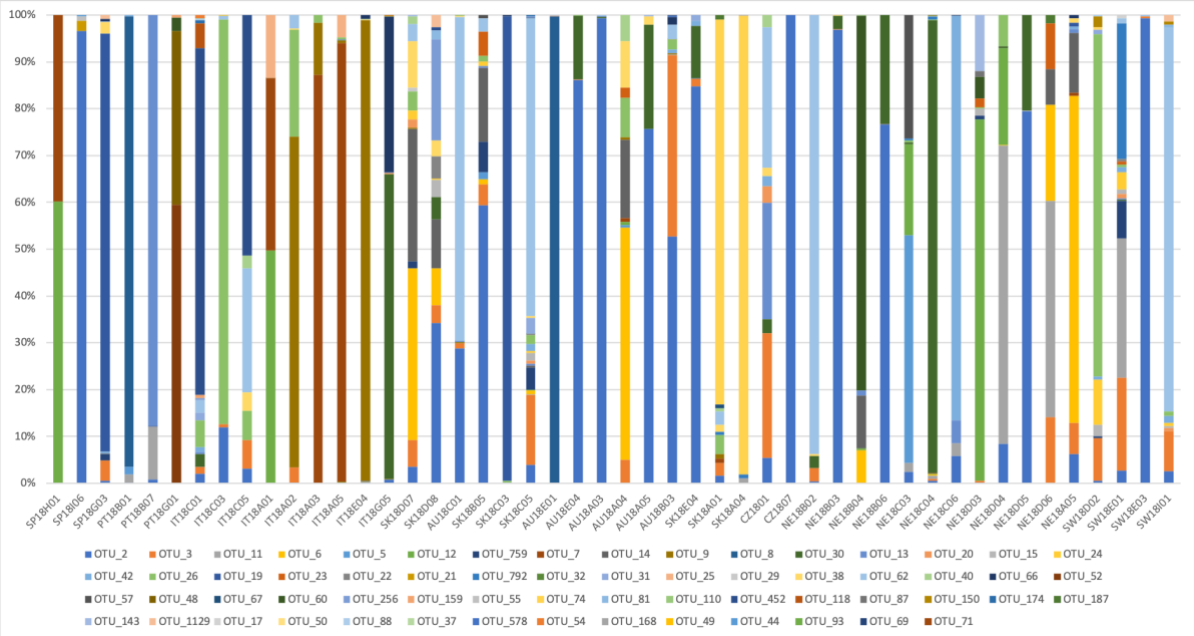

Figure 13 Microbiome of all mosquito individuals sorted by increasing latitude without Wolbachia

4.3 Alpha diversity

The effect of the different factors influencing the microbiota were tested using alpha diversity indexes (richness, dominance, Shannon index and equitability) for the *Cx. pipiens pipiens* samples. The following categories were analysed: country, habitat, locality, latitude grouped by one degree, longitude grouped by one degree and localities within the countries Italy, Slovakia, Spain, Sweden and The Netherlands.

Firstly, the analyses including *Wolbachia* are presented.
The eight different countries were compared to check for significant differences. The following results were obtained: richness (X^2 = 9.069, p = 0.248), dominance (X^2 = 8.922, p = 0.258), Shannon index (X^2 = 8.875, p = 0.262) and equitability (X^2 = 8.550, p = 0.287). All of these p-values exceed the threshold of 0.05 and therefore the compared groups are not significantly different.

The next category compared was the four different groups of habitats. The results are as follows: richness (X^2= 2.916, p = 0.405), dominance (X^2 = 4.800, p = 0.187), Shannon index (X^2 = 5.045, p = 0.169) and equitability (X^2 = 4.758, p = 0.190). Again, the groups showed no significant diversity difference with a p-value in Kruskal-Wallis rank sum test higher than 0.05.

Tests for locality showed that richness (X^2 = 32.767, p = 0.048), dominance (X^2 = 45.869, p = 0.001), Shannon index (X^2 = 46.395, p = 0.001) and equitability (X^2 = 44.936, p = 0.002) were significantly different for all localities, with a p-value under 0.05.

The latitudes clustered in groups by one degree were compared to each other as well: richness (X^2 = 5.916, p = 0.433), dominance (X^2 = 6.231, p = 0.398), Shannon index (X^2 = 6.347, p = 0.385) and equitability (X^2 = 6.184, p = 0.403). The outcome of the tests was not significant for any of the four alpha diversity indexes.

Also, the longitudes were clustered in groups by one degree and the following results were obtained: richness (X^2 = 12.014, p = 0.213), dominance (X^2 = 17.54, p = 0.041), Shannon index (X^2 = 17.971, p = 0.036) and equitability (X^2 = 17.041, p = 0.048).
In these tests the threshold of 0.05 was exceeded only in the index richness, but significant differences could be obtained in the other three indexes.

Lastly, the comparison of the localities inside the countries Italy, Slovakia, Spain, Sweden and The Netherlands showed the following results:
Italy: richness (X^2 = 0.019, p = 0.991), dominance (X^2 = 8.842, p = 0.012), Shannon index (X^2 = 9.191, p = 0.010) and equitability (X^2 = 8.842, p = 0.012);
Slovakia: richness (X^2 = 5.432, p = 0.143), dominance (X^2 = 8.492, p = 0.037), Shannon index (X^2 = 8.767, p = 0.033) and equitability (X^2 = 8.173, p = 0.043);
Spain: richness (X^2 = 0.463, p = 0.794), dominance (X^2 = 0.375, p = 0.829), Shannon index (X^2 = 0.626, p = 0.731) and equitability (X^2 = 0.333, p = 0.847);
Sweden: richness (X^2 = 4.406, p = 0.221), dominance (X^2 = 7.410, p = 0.060), Shannon index (X^2 = 7.410, p = 0.060) and equitability (X^2 = 7.667, p = 0.053);
The Netherlands: richness (X^2 = 13.297, p = 0.004), dominance (X^2 = 9.382, p = 0.025), Shannon index (X^2 = 9.469, p = 0.024) and equitability (X^2 = 9.390, p = 0.025);
Therefore, in The Netherlands all alpha diversity indexes were significantly different, while in Italy and Slovakia all indexes except richness differed significantly. In Spain and Sweden, no significant differences could be observed.

For the analysed samples excluding *Wolbachia*, no significant differences were found for the country, locality, localities inside country, and longitude analyses. In the categories habitat and latitude clustered by one degree, only the index equitability showed a significant

difference in the results: Habitat equitability (X^2 = 16.048, p = 0.003) and latitude clustered by one-degree equitability (X^2 = 14.932, p = 0.037). All other alpha indexes tested showed a p-value exceeding 0.05.

All of the means and standard deviations of the alpha diversity indexes of the categories that were tested are presented and summarized in Table 5 and Table 6, including and excluding *Wolbachia*, respectively.

Table 5 Mean and standard deviations of alpha diversity indexes with Wolbachia

incl. Wolbachia			**Dominace**		**Equitability**		**Richness**		**Shannon_index**	
Category	**Subcategory**	**N**	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
countries	Austria	16	0,0551	0,1260	0,0628	0,1355	5,7500	2,7928	0,1464	0,2954
	CZ	3	0,0846	0,1381	0,2036	0,3346	6,3333	4,5092	0,2171	0,3230
	Italy	16	0,1274	0,2253	0,1206	0,1828	8,1875	3,3708	0,3522	0,5631
	Portugal	7	0,1599	0,2448	0,1413	0,1987	6,2857	1,7995	0,3920	0,5580
	Slovakia	15	0,0871	0,1794	0,0790	0,1396	8,0000	4,8990	0,2836	0,5733
	Spain	11	0,1157	0,2245	0,1045	0,2013	5,4545	2,9787	0,2820	0,5241
	Sweden	12	0,0714	0,1539	0,0742	0,1377	7,5833	4,3996	0,2354	0,4933
	The Netherlands	17	0,2053	0,2617	0,1866	0,2256	6,7059	2,6638	0,5554	0,6794
habitat	garden/rural	44	0,0965	0,1900	0,0945	0,1678	6,6136	3,2149	0,2568	0,4616
	urban	9	0,1396	0,2208	0,1208	0,1208	9,2222	5,7179	0,4442	0,7098
	semi-urban	8	0,0098	0,0146	0,0180	0,0256	5,7500	2,6592	0,0430	0,0530
	industrial	21	0,2157	0,2615	0,1925	0,2228	6,7143	2,4319	0,5657	0,6623
locality	Bratislava	3	0,3860	0,2370	0,3110	0,1778	14,3333	7,0946	1,1943	0,8486
	Bredforsen mitt	6	0,0084	0,0084	0,0155	0,0134	6,8333	2,5626	0,0440	0,0388
	Calatilla	4	0,0405	0,0790	0,0355	0,0643	6,5000	4,5092	0,1278	0,2415
	Celestino Mutis	5	0,0938	0,2069	0,0823	0,1748	4,8000	2,1679	0,2111	0,4522
	Delta del Po	6	0,0279	0,0384	0,0374	0,0384	8,8333	4,9160	0,1169	0,1425
	Emmeloord	5	0,0014	0,0018	0,0041	0,0034	3,8000	1,4832	0,0086	0,0100
	Foce dell Isonzo	4	0,0046	0,0015	0,0096	0,0031	8,0000	1,6330	0,0278	0,0063
	Hardenberg	4	0,2823	0,2117	0,2548	0,1603	9,2500	1,5000	0,8065	0,4848
	Isola della Scala	6	0,3087	0,2956	0,2778	0,2258	7,6667	2,7325	0,8038	0,7318
	Komarno	3	0,0061	0,0057	0,0117	0,0075	7,6667	3,5119	0,0357	0,0303
	Kosice	8	0,0098	0,0146	0,0180	0,0256	5,7500	2,6592	0,0430	0,0530
	Lelystad	4	0,3323	0,3704	0,2769	0,2995	8,7500	1,7078	0,9216	1,0193
	Lindangsbacken	4	0,2010	0,2304	0,1977	0,1966	10,5000	6,2450	0,6371	0,7526
	Montfoort	4	0,2562	0,2846	0,2562	0,2775	5,7500	0,5000	0,6218	0,6485
	Palacio Donana	2	0,3209	0,4527	0,2979	0,4173	5,0000	1,4142	0,7678	1,0778
	Podrecany	1	-	-	-	-	-	-	-	-
	Salja	2	0,0009	0,0004	0,0031	0,0013	4,0000	0,0000	0,0063	0,0026
latitude	47,7622778 - 48,74475N	31	0,0706	0,1524	0,0707	0,1354	6,8387	4,0505	0,2128	0,4493
	49.252984N	3	0,0389	0,0905	0,0927	0,2193	7,2857	2,9841	0,1089	0,2122
	44.947222 - 45.754027N	16	0,1274	0,2253	0,1206	0,1828	8,1875	3,3708	0,3522	0,5631
	52.05198 - 52.69772N	17	0,2053	0,2617	0,1866	0,2256	6,7059	2,6638	0,5554	0,6794
	36.98875 - 37.851652N	15	0,1542	0,2431	0,1346	0,2101	5,8000	2,6511	0,3692	0,5631
	41.3342222N	3	0,0266	0,0431	0,0396	0,0610	5,6667	2,5166	0,1026	0,1581
	60.1043 - 60.4158N	12	0,0714	0,1539	0,0742	0,1377	7,5833	4,3996	0,2354	0,4933
longitude	16.299404 - 17.2222E	31	0,0934	0,1729	0,0912	0,1537	7,2903	4,5401	0,2823	0,5242
	13.4694492 - 14.092055E	7	0,0389	0,0905	0,0927	0,2193	7,2857	2,9841	0,1089	0,2122
	4.96832 - 5.74737E	13	0,1816	0,2784	0,1656	0,2438	5,9231	2,4651	0,4782	0,7274
	-6.443033- -6.924128E	14	0,0966	0,2013	0,0906	0,1803	5,5000	2,7942	0,2436	0,4701
	18.0556667 - 19.6051944E	4	0,0176	0,0235	0,0271	0,0315	7,7500	2,8723	0,0818	0,0954
	21.2574722E	8	0,0098	0,0146	0,0180	0,0256	5,7500	2,6592	0,0430	0,0530
	6.64558E	4	0,2823	0,2117	0,2548	0,1603	9,2500	1,5000	0,8065	0,4848
	11.018483E	6	0,3087	0,2956	0,2778	0,2258	7,6667	2,7325	0,8038	0,7318
	12.279425E	6	0,0279	0,0384	0,0374	0,0384	8,8333	4,9160	0,1169	0,1425
	-8.793116E	4	0,2599	0,2958	0,2175	0,2417	6,7500	1,2583	0,6091	0,6779
AllSamples	All	97	0,1156	0,2030	0,1127	0,1808	6,9175	3,5316	0,3195	0,5321

Table 6 Mean and standard deviations of alpha diversity indexes without Wolbachia

excl. Wolbachia			Dominace		Equitability		Richness		Shannon_index	
Categorie	**Subcategory**	**N**	mean	s.d.	mean	s.d.	mean	s.d.	mean	s.d.
countries	Austria	7	0,3895	0,2462	0,3728	0,2145	7,0000	3,4641	1,0753	0,7845
	CZ	2	0,3850	0,5445	-	-	5,0000	5,6569	1,2200	1,7253
	Italy	9	0,3586	0,2147	0,3853	0,2245	7,6667	4,4721	1,0224	0,6077
	Portugal	3	0,2650	0,2216	0,3320	0,2320	4,3333	0,5774	0,6767	0,4450
	Slovakia	8	0,4252	0,3095	0,4105	0,2648	10,8750	5,8904	1,4947	1,0826
	Spain	3	0,2484	0,2106	0,4417	0,4603	5,3333	4,1633	0,6607	0,3678
	Sweden	4	0,3892	0,3176	0,3611	0,2542	10,2500	5,3774	1,3197	1,0467
	The Netherlands	12	0,3465	0,2146	0,4105	0,2117	6,4167	2,7455	1,0428	0,6046
habitat	garden/rural	20	0,3462	0,2119	0,3865	0,2441	7,0000	3,8586	0,9711	0,6103
	urban	6	0,5468	0,2842	0,5200	0,2559	11,5000	6,5651	1,8161	1,0396
	semi-urban	2	0,1797	0,1984	0,2150	0,1867	8,0000	5,6569	0,7030	0,7594
	industrial	14	0,3277	0,2141	0,3815	0,2109	6,1429	2,6270	0,9559	0,6012
locality	Bratislava	3	0,3990	0,3308	0,3607	0,2814	12,3333	8,5049	1,3954	1,1341
	Bredforsen mitt	1	-	-	-	-	-	-	-	-
	Calatilla	1	-	-	-	-	-	-	-	-
	Celestino Mutis	1	-	-	-	-	-	-	-	-
	Delta del Po	3	0,4463	0,2085	0,4660	0,2163	10,0000	7,0000	1,4450	0,6909
	Emmeloord	1	-	-	-	-	-	-	-	-
	Foce dell Isonzo	1	-	-	-	-	-	-	-	-
	Hardenberg	4	0,4908	0,1709	0,5590	0,1858	6,2500	2,8723	1,3970	0,5564
	Isola della Scala	5	0,2847	0,2346	0,3499	0,2644	5,8000	2,3875	0,7614	0,5295
	Komarno	2	0,7885	0,0304	0,7285	0,0474	14,0000	0,0000	2,7750	0,1768
	Kosice	2	0,1797	0,1984	0,2150	0,1867	8,0000	5,6569	0,7030	0,7594
	Lelystad	3	0,3222	0,3023	0,3435	0,2573	8,0000	3,0000	0,9673	0,7682
	Lindangsbacken	3	0,4126	0,3847	0,3717	0,3103	10,0000	6,5574	1,3796	1,2735
	Montfoort	4	0,2206	0,1523	0,2970	0,1825	4,5000	1,2910	0,6133	0,3543
	Palacio Donana	1	-	-	-	-	-	-	-	-
	Podrecany	1	-	-	-	-	-	-	-	-
latitude	47,7622778 - 48,74475N	15	0,4099	0,2742	0,3943	0,2363	9,2143	5,2209	1,3149	0,9561
	49.252984N	2	0,3850	0,5445	-	-	5,0000	5,6569	1,2200	1,7253
	44.947222 - 45.754027N	9	0,3586	0,2147	0,3853	0,2245	7,6667	4,4721	1,0224	0,6077
	52.05198 - 52.69772N	12	0,3583	0,2146	0,4105	0,2117	6,4167	2,7455	1,0428	0,6046
	36.98875 - 37.851652N	5	0,2067	0,1674	0,3480	0,3550	5,0000	3,0000	0,5704	0,3071
	41.3342222N	1	-	-	-	-	-	-	-	-
	60.1043 - 60.4158N	4	0,3892	0,3176	0,3611	0,2542	10,2500	5,3774	1,3197	1,0467
longitude	16.299404 - 17.2222E	14	0,3916	0,2621	0,3664	0,2204	9,2308	5,4338	1,2244	0,8753
	13.4694492 - 14.092055E	3	0,4117	0,3878	-	-	6,6667	4,9329	1,1667	1,2235
	4.96832 - 5.74737E	8	0,2921	0,2117	0,3363	0,1917	6,5000	2,8785	0,8656	0,5780
	-6.443033 - -6.924128E	4	0,3131	0,2151	0,4765	0,3823	5,0000	3,4641	0,7855	0,3905
	18.0556667 - 19.6051944E	3	0,6150	0,3013	0,5907	0,2411	11,3333	4,6188	2,1217	1,1385
	21.2574722E	2	0,1797	0,1984	0,2150	0,1867	8,0000	5,6569	0,7030	0,7594
	6.64558E	4	0,4908	0,1709	0,5590	0,1858	6,2500	2,8723	1,3970	0,5564
	11.018483E	5	0,2847	0,2346	0,3499	0,2644	5,8000	2,3875	0,7614	0,5295
	12.279425E	3	0,4463	0,2085	0,4660	0,2163	10,0000	7,0000	1,4450	0,6909
	-8.793116E	2	0,1441	0,1018	0,2075	0,1209	4,5000	0,7071	0,4350	0,2135
AllSamples	All	48	0,3645	0,2442	-	-	7,5532	4,4075	1,1033	0,7783

5 Discussion

The microbiota of a mosquito contains many different microorganisms, which form a different community for every individual (Strand, 2018). The different microorganisms have various influences on the hosting animals (Moeller et al., 2019), especially in mosquitoes (Saab et al., 2020). Some of these microorganisms can have an impact on mosquitoes' vector competence and, therefore, studying the mosquitoes' microbiota should help to gain knowledge about the fight against vector-borne diseases (Novakova et al., 2017; Thongsripong et al., 2018). The *Cx. pipiens* mosquito is of special interest, due to the transmission of the WNV. This virus does not spread in Northern Europe, but is present in Southern, Central and Eastern Europe (Sambri et al., 2013). With this background, it is important to investigate possible

environmental influences on the *Cx. pipiens* mosquitoes' microbiota that could may be a reason for this distribution of WNV, and other epidemiological latitudinal patterns. In addition, sampling from different latitudes (and thus different climate) could also help to understand the effect of climate change in the mosquito microbiome.

The goal of this thesis was to find an effect of different biogeographical variables, mainly latitude, but also longitude, habitat, country and location of sampling, on the composition of the microbiota of *Cx. pipiens* mosquitoes.

First of all, all our mosquito samples were screened and identified by molecular methods up to subspecies within the *Cx. pipiens* species. The general distribution of OTUs we found confirms previous studies' results (Muturi et al., 2016; Novakova et al., 2017), with *Wolbachia* (our OTU 1) as the most common OTU in *Cx. pipiens* mosquitoes. This bacterium is known to stimulate the immune response in other insects (Wong et al., 2011), and has the same effect in mosquitoes (Dennison et al., 2014). Actually, it is known that this bacterium can affect the vector competence of mosquitoes, such as limiting the infection and replication rate of dengue virus in *Aedes* mosquitoes (Frentiu et al., 2014) and reducing the life span of *Aedes* mosquitoes (McMeniman et al., 2009). For this reason, the results of different *Wolbachia* distributions in various countries or localities of this study could have a positive impact on disease research (Niang et al., 2018).

The microbiota was also analysed without *Wolbachia* in order to identify other main bacteria that are not vertically transmitted and could be of interest to check for environmental influences. Using this approach, we found mainly bacteria from the phyla Proteobacteria and Firmicutes. The high abundances of these two phyla are also confirmed by other studies (Muturi et al., 2016; Zotzmann et al., 2017). The classes Alphaproteobacteria and Gammaproteobacteria were the most abundant ones within the phylum Proteobacteria, while for the second most occurring phylum, Firmicutes, the classes of Bacilli and Clostridia were dominant. These results are in agreement with previous reports of the *Cx. pipiens* mosquito microbiota (Muturi et al., 2016). The second most abundant bacterium found in our samples, *Erwinia,* is also a typical bacterium found in the *Cx. pipiens* mosquito (Thongsripong et al., 2018).

The insight into the local differences and their influences on the microbiota was obtained using different alpha diversity indexes: richness, dominance, Shannon index and equitability.

All of the tests using the data set without *Wolbachia* resulted in non-significant differences. Therefore, environmental influences could not be seen in the gut microbiota fraction of our data.

On the other hand, all tests including *Wolbachia* showed significant differences for localities, but not for countries (including different localities) or habitats. These results confirm that the microbiota diversity varies all over the world at a local level (Akorli et al., 2016; Muturi et al., 2018; Novakova et al., 2017; Rosso et al., 2018; Zouache et al., 2011). The effect of latitude and longitude was analysed by grouping areas by one-degree latitude and longitude, where only the longitude groups showed significant differences.

The already mentioned non-significant differences ($p > 0.05$) among the different countries (Austria, Czech Republic, Italy, Portugal, Slovakia, Spain, Sweden and The Netherlands) using different alpha diversity indexes, are contrary to previous studies. Rosso et al. (2018) found microbiota differences according to the country, at the genus level between Italy and France, Italy and Vietnam and between France and Vietnam. When comparing the alpha diversity, Italy showed significant differences to both Vietnam and France, but Vietnam and France were not significantly different. Also, the differences of the mosquito species, *Aedes* in the case of Rosso et al. (2018) and *Culex* in our case, can have potential impacts on possible environmental influences due to the microbiota host species-specificity (Novakova et al., 2017). Two of the countries, Vietnam and France, were from different continents with very different climate conditions: tropical climate in Vietnam (Kuwata et al., 2013) and oceanic climate in France (Bessat and Buigues, 2001). The climate zones of our study are not so different, as they adjoin each other, and therefore showed more similarities (Lauer and Frankenberg, 1986). Neither in our case, with similar climate zones, nor in the case of distinct climate zones, differences could be shown. Furthermore, in the study of Minard et al. (2015), *Aedes albopictus* samples, also from France and Vietnam, were compared to each other. In this study, individual samples were grouped, and no significant differences were found on the country level, which is in contrast to Rosso et al. (2018). In our case it can be seen that all analysed samples showed a high variability and therefore, finding patterns was hard and no significant differences among countries could be found, like in the case of Minard et al. (2015).

The different localities within each country were also compared. Only Italy, Slovakia, Spain, Sweden and The Netherlands had different collecting localities to test. Only the localities inside Italy, Slovakia and The Netherlands showed significant differences in the microbiome alpha diversity. The localities inside Italy, Slovakia, Spain, Sweden and The Netherlands showed a latitudinal difference between 0.3 and 0.8 degrees. The longitudinal differences of

the localities in Sweden and Spain were of 0.5 degrees, whereas the localities inside Italy, Slovakia and The Netherlands showed longitudinal distances between 1.7 and 4.1 degrees. These differences match the significant differences found also when comparing the longitude groups. Further research will be necessary to unravel the factors behind these local-longitudinal patterns.

The last factor analysed, the habitat, did not show significant differences neither including nor excluding *Wolbachia*, even if we considered relatively different settings (garden/rural, industrial, urban and semi-urban). In the study of Möhlmann (2019), who compared also three European countries and their habitats, there was also no significant influence of the habitat on the *Cx. pipiens* mosquitoes' microbiome. Compared to studies of other mosquito species, e.g. *Aedes*, the habitat can have an influence on the microbiome through factors like the aquatic environment of the breeding site (Coon et al., 2016). In the study of Coon et al. (2016), larvae of different sampling sites and different mosquito species were collected, pooled and analysed. Mainly bacteria of sampling site water were found in their samples. However, our sampling includes adult mosquitoes, and even if they may keep some bacteria from the larval environment, the effect of this factor may be much lower (Minard et al., 2013).

Future directions should include dealing with a wider distribution of countries, latitude and longitude ranges, for example localities distributed not only over the Northern hemisphere, but all over the world. In addition, studies with higher sample numbers and including more target variables (such as temperature) would increase the results' statistical power. Our approach, searching for influences on the microbiome including and excluding *Wolbachia* for the *Cx. pipiens* mosquito, may be useful in further studies, as we have shown each dataset provides different information.

6 Conclusion

The main aim of this thesis was to find environmental factors, which have an influence on the composition of the microbiota of *Cx. pipiens pipiens* mosquitoes in Europe. This species is the main vector of WNV, and therefore of huge interest for the fight against mosquito-borne diseases. The multiple biogeographical factors tested showed no clear results. The already known differences of the microbiome at the locality level have been confirmed and local influences on the *Wolbachia* relative abundance were observed. The fact that the rest of the microbiome without *Wolbachia* does not seem to be significantly influenced by external factors, indicates that the differences seen with *Wolbachia* may be determined by mosquito population (genetic background), and not by actual environmental effects. However, all of these results are influenced by the high inter-individual variability of the microbiome found in the samples of this study. Further research can lay its focus on the expansion of latitudinal and longitudinal ranges over the whole world, extending both the variables recorded for each site and the species of mosquitoes collected.

7 References

Akorli, J., Gendrin, M., Pels, N.A.P., Yeboah-Manu, D., Christophides, G.K., Wilson, M.D., 2016. Seasonality and Locality Affect the Diversity of *Anopheles gambiae* and *Anopheles coluzzii* Midgut Microbiota from Ghana. PLoS ONE 11. https://doi.org/10.1371/journal.pone.0157529

Almirón, W.R., Humeres, S.G., Gardenal, C.N., 1995. Distribution and hybridization between *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in Argentina. Mem. Inst. Oswaldo Cruz 90, 469–473. https://doi.org/10.1590/S0074-02761995000400007

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W., Dobson, S.L., 2010. Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis. Vector Borne Zoonotic Dis. 10, 295–311. https://doi.org/10.1089/vbz.2009.0014

Amraoui, F., Tijane, M., Sarih, M., Failloux, A.-B., 2012. Molecular evidence of *Culex pipiens* form *molestus* and hybrids *pipiens/molestus* in Morocco, North Africa. Parasit. Vectors 5, 83. https://doi.org/10.1186/1756-3305-5-83

Andreadis, T.G., 1985. Experimental transmission of a microsporidian pathogen from mosquitoes to an alternate copepod host. Proc. Natl. Acad. Sci. 82, 5574–5577. https://doi.org/10.1073/pnas.82.16.5574

Bahnck, C.M., Fonseca, D.M., 2006. Rapid assay to identify the two genetic forms of *Culex (Culex) pipiens* L. (Diptera: Culicidae) and hybrid populations. Am. J. Trop. Med. Hyg. 75, 251–255.

Baldacchino, F., Caputo, B., Chandre, F., Drago, A., Torre, A. della, Montarsi, F., Rizzoli, A., 2015. Control methods against invasive *Aedes* mosquitoes in Europe: a review. Pest Manag. Sci. 71, 1471–1485. https://doi.org/10.1002/ps.4044

Bessat, F., Buigues, D., 2001. Two centuries of variation in coral growth in a massive Porites colony from Moorea (French Polynesia): a response of ocean-atmosphere variability from south central Pacific. Palaeogeogr. Palaeoclimatol. Palaeoecol. 175, 381–392. https://doi.org/10.1016/S0031-0182(01)00381-9

Boissière, A., Tchioffo, M.T., Bachar, D., Abate, L., Marie, A., Nsango, S.E., Shahbazkia, H.R., Awono-Ambene, P.H., Levashina, E.A., Christen, R., Morlais, I., 2012. Midgut Microbiota of the Malaria Mosquito Vector *Anopheles gambiae* and Interactions with Plasmodium falciparum Infection. PLOS Pathog. 8, e1002742. https://doi.org/10.1371/journal.ppat.1002742

Brugman, V.A., Hernández-Triana, L.M., Medlock, J.M., Fooks, A.R., Carpenter, S., Johnson, N., 2018. The Role of *Culex pipiens* L. (Diptera: Culicidae) in Virus Transmission in Europe. Int. J. Environ. Res. Public. Health 15. https://doi.org/10.3390/ijerph15020389

Campbell, G.L., Marfin, A.A., Lanciotti, R.S., Gubler, D.J., 2002. West Nile virus. Lancet Infect. Dis. 2, 519–529. https://doi.org/10.1016/S1473-3099(02)00368-7

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Christophers, S.R., 1960. *Aedes aegypti*: the yellow fever mosquito. CUP Archive.

Collier, R.J., Beede, D.K., Thatcher, W.W., Israel, L.A., Wilcox, C.J., 1982. Influences of Environment and Its Modification on Dairy Animal Health and Production1. J. Dairy Sci. 65, 2213–2227. https://doi.org/10.3168/jds.S0022-0302(82)82484-3

Colpitts, T.M., Conway, M.J., Montgomery, R.R., Fikrig, E., 2012. West Nile Virus: Biology, Transmission, and Human Infection. Clin. Microbiol. Rev. 25, 635–648. https://doi.org/10.1128/CMR.00045-12

Coon, K.L., Brown, M.R., Strand, M.R., 2016. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol. Ecol. 25, 5806–5826. https://doi.org/10.1111/mec.13877

Cornel, A.J., Mcabee, R.D., Rasgon, J., Stanich, M.A., Scott, T.W., Coetzee, M., 2003. Differences in Extent of Genetic Introgression Between Sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa. J. Med. Entomol. 40, 36–51. https://doi.org/10.1603/0022-2585-40.1.36

Dennison, N.J., Jupatanakul, N., Dimopoulos, G., 2014. The mosquito microbiota influences vector competence for human pathogens. Curr. Opin. Insect Sci. 3, 6–13. https://doi.org/10.1016/j.cois.2014.07.004

ECDC; 2018. European Centre for Disease Prevention and Control and European Food Safety Authority. Mosquito maps [internet]. Stockholm, Available from: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease- data/mosquito-maps (accessed 2020/18/05)

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Engel, P., Moran, N.A., 2013. The gut microbiota of insects – diversity in structure and function. FEMS Microbiol. Rev. 37, 699–735. https://doi.org/10.1111/1574-6976.12025

Fang, J., 2010. Ecology: A world without mosquitoes. Nature 466, 432–434. https://doi.org/10.1038/466432a

Fawzy, M., Helmy, Y.A., 2019. The One Health Approach is Necessary for the Control of Rift Valley Fever Infections in Egypt: A Comprehensive Review. Viruses 11. https://doi.org/10.3390/v11020139

Federici, B.A., 2009. Chapter 193 - Pathogens of Insects, in: Resh, V.H., Cardé, R.T. (Eds.), Encyclopedia of Insects (Second Edition). Academic Press, San Diego, pp. 757–765. https://doi.org/10.1016/B978-0-12-374144-8.00202-2

Frentiu, F.D., Zakir, T., Walker, T., Popovici, J., Pyke, A.T., van den Hurk, A., McGraw, E.A., O'Neill, S.L., 2014. Limited Dengue Virus Replication in Field-Collected *Aedes aegypti* Mosquitoes Infected with *Wolbachia*. PLoS Negl. Trop. Dis. 8. https://doi.org/10.1371/journal.pntd.0002688

Gall, C.A., Reif, K.E., Scoles, G.A., Mason, K.L., Mousel, M., Noh, S.M., Brayton, K.A., 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. ISME J. 10, 1846–1855. https://doi.org/10.1038/ismej.2015.266

Garibyan, L., Avashia, N., 2013. Research Techniques Made Simple: Polymerase Chain Reaction (PCR). J. Invest. Dermatol. 133, e6. https://doi.org/10.1038/jid.2013.1

Gendrin, M., Rodgers, F.H., Yerbanga, R.S., Ouédraogo, J.B., Basáñez, M.-G., Cohuet, A., Christophides, G.K., 2015. Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. Nat. Commun. 6. https://doi.org/10.1038/ncomms6921

Gendrin, M., Turlure, F., Rodgers, F.H., Cohuet, A., Morlais, I., Christophides, G.K., 2017. The Peptidoglycan Recognition Proteins PGRPLA and PGRPLB Regulate *Anopheles* Immunity to Bacteria and Affect Infection by Plasmodium. J. Innate Immun. 9, 333–342. https://doi.org/10.1159/000452797

Gendrin, M., Zaidman-Rémy, A., Broderick, N.A., Paredes, J., Poidevin, M., Roussel, A., Lemaitre, B., 2013. Functional Analysis of PGRP-LA in Drosophila Immunity. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0069742

Gubler, D.J., 2009. Vector-borne diseases. Rev. Sci. Tech. 28, 583–588. https://doi.org/10.20506/rst.28.2.1904

Gubler, D.J., Clark, G.G., 1995. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. 1, 55–57.

Guha-Sapir, D., Schimmer, B., 2005. Dengue fever: new paradigms for a changing epidemiology. Emerg. Themes Epidemiol. 2, 1. https://doi.org/10.1186/1742-7622-2-1

Harbach, R.E., 2012. *Culex pipiens*: Species Versus Species Complex – Taxonomic History and Perspective. J. Am. Mosq. Control Assoc. 28, 10–23. https://doi.org/10.2987/8756-971X-28.4.10

Hayes, E.B., Komar, N., Nasci, R.S., Montgomery, S.P., O'Leary, D.R., Campbell, G.L., 2005. Epidemiology and Transmission Dynamics of West Nile Virus Disease. Emerg. Infect. Dis. 11, 1167–1173. https://doi.org/10.3201/eid1108.050289a

Hegde, S., Rasgon, J.L., Hughes, G.L., 2015. The microbiome modulates arbovirus transmission in mosquitoes. Curr. Opin. Virol., SI: 15 Virus–vector interactions•Viral immunology 15, 97–102. https://doi.org/10.1016/j.coviro.2015.08.011

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.M.C., Wides, R., Salzberg, S.L., Loftus, B., Yandell, M., Majoros, W.H., Rusch, D.B., Lai, Z., Kraft, C.L., Abril, J.F., Anthouard, V., Arensburger, P., Atkinson, P.W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chaturverdi, K., Christophides, G.K., Chrystal, M.A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C.A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M.E., Hladun, S.L., Hogan, J.R., Hong, Y.S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.-J., Lobo, N.F., Lopez, J.R., Malek, J.A., McIntosh, T.C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S.D., O'Brochta, D.A., Pfannkoch, C., Qi, R., Regier, M.A., Remington, K., Shao, H., Sharakhova, M.V., Sitter, C.D., Shetty, J., Smith, T.J., Strong, R., Sun, J., Thomasova, D., Ton, L.Q., Topalis, P., Tu, Z., Unger, M.F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K.J., Wortman, J.R., Wu, M., Yao, A., Zdobnov, E.M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S.C., Zhimulev, I., Coluzzi, M., della Torre, A., Roth, C.W., Louis, C., Kalush, F., Mural, R.J., Myers, E.W., Adams, M.D., Smith, H.O., Broder, S., Gardner, M.J., Fraser, C.M., Birney, E., Bork, P., Brey, P.T., Venter, J.C., Weissenbach, J., Kafatos, F.C., Collins, F.H., Hoffman, S.L., 2002. The genome sequence of the malaria mosquito *Anopheles gambiae*. Science 298, 129–149. https://doi.org/10.1126/science.1076181

Jayakrishnan, L., Sudhikumar, A.V., Aneesh, E.M., 2018. Role of gut inhabitants on vectorial capacity of mosquitoes. J. Vector Borne Dis. 55, 69. https://doi.org/10.4103/0972-9062.242567

Juliano, S.A., Lounibos, L.P., 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol. Lett. 8, 558–574. https://doi.org/10.1111/j.1461-0248.2005.00755.x

Kennedy, P.G.E., 2008. The continuing problem of human African trypanosomiasis (sleeping sickness). Ann. Neurol. 64, 116–126. https://doi.org/10.1002/ana.21429

Kent, R.J., Harrington, L.C., Norris, D.E., 2007. Genetic Differences Between *Culex pipiens* f. *molestus* and *Culex pipiens pipiens* (Diptera: Culicidae) in New York. J. Med. Entomol. 44, 50–59.

Kers, J.G., Velkers, F.C., Fischer, E.A.J., Hermes, G.D.A., Stegeman, J.A., Smidt, H., 2018. Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.00235

Kuwata, R., Nga, P.T., Yen, N.T., Hoshino, K., Isawa, H., Higa, Y., Hoang, N.V., Trang, B.M., Loan, D.P., Phong, T.V., Sasaki, T., Tsuda, Y., Kobayashi, M., Sawabe, K., Takagi, M., 2013. Surveillance of Japanese Encephalitis Virus Infection in Mosquitoes in Vietnam from 2006 to 2008. Am. J. Trop. Med. Hyg. 88, 681–688. https://doi.org/10.4269/ajtmh.12-0407

Lauer, W., Frankenberg, P., 1986. Eine Karte der hygrothermischen Klimatypen von Europa (A Map of Hygrothermic Climatic Types of Europe). Erdkunde 40, 85–94.

Mandrioli, M., Emilia, R., 2012. Someone like it hot? Effects of global warming on insect immunity and microbiota.

Marcantonio, M., Rizzoli, A., Metz, M., Rosà, R., Marini, G., Chadwick, E., Neteler, M., 2015. Identifying the Environmental Conditions Favouring West Nile Virus Outbreaks in Europe. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0121158

Martínez-de la Puente, J., Ferraguti, M., Ruiz, S., Roiz, D., Soriguer, R.C., Figuerola, J., 2016. *Culex pipiens* forms and urbanization: effects on blood feeding sources and transmission of avian Plasmodium. Malar. J. 15. https://doi.org/10.1186/s12936-016-1643-5

McMeniman, C.J., Lane, R.V., Cass, B.N., Fong, A.W.C., Sidhu, M., Wang, Y.-F., O'Neill, S.L., 2009. Stable Introduction of a Life-Shortening *Wolbachia* Infection into the Mosquito *Aedes aegypti*. Science 323, 141–144. https://doi.org/10.1126/science.1165326

Medlock, J.M., Hansford, K.M., Schaffner, F., Versteirt, V., Hendrickx, G., Zeller, H., Bortel, W.V., 2012. A Review of the Invasive Mosquitoes in Europe: Ecology, Public Health Risks, and Control Options. Vector Borne Zoonotic Dis. 12, 435–447. https://doi.org/10.1089/vbz.2011.0814

Minard, G., Mavingui, P., Moro, C.V., 2013. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit. Vectors 6, 146. https://doi.org/10.1186/1756-3305-6-146

Minard, G., Tran, F.H., Van, V.T., Goubert, C., Bellet, C., Lambert, G., Kim, K.L.H., Thuy, T.H.T., Mavingui, P., Valiente Moro, C., 2015. French invasive *Asian tiger* mosquito populations harbor reduced bacterial microbiota and genetic diversity compared to Vietnamese autochthonous relatives. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00970

Moeller, A.H., Gomes-Neto, J.C., Mantz, S., Kittana, H., Munoz, R.R.S., Schmaltz, R.J., Ramer-Tait, A.E., Nachman, M.W., 2019. Experimental Evidence for Adaptation to Species-Specific Gut Microbiota in House Mice. mSphere 4. https://doi.org/10.1128/mSphere.00387-19

Muturi, E.J., Dunlap, C., Ramirez, J.L., Rooney, A.P., Kim, C.-H., 2019. Host blood-meal source has a strong impact on gut microbiota of *Aedes aegypti.* FEMS Microbiol. Ecol. 95. https://doi.org/10.1093/femsec/fiy213

Muturi, E.J., Kim, C.-H., Bara, J., Bach, E.M., Siddappaji, M.H., 2016. *Culex pipiens* and *Culex restuans* mosquitoes harbor distinct microbiota dominated by few bacterial taxa. Parasit. Vectors 9. https://doi.org/10.1186/s13071-016-1299-6

Muturi, E.J., Lagos-Kutz, D., Dunlap, C., Ramirez, J.L., Rooney, A.P., Hartman, G.L., Fields, C.J., Rendon, G., Kim, C.-H., 2018. Mosquito microbiota cluster by host sampling location. Parasit. Vectors 11. https://doi.org/10.1186/s13071-018-3036-9

Möhlmann, T.W.R., 2019. Mosquitoes, midges and microbiota. Wageningen University. https://edepot.wur.nl/466379 (accessed 2020/25/03)

Narasimhan, S., Rajeevan, N., Liu, L., Zhao, Y.O., Heisig, J., Pan, J., Eppler-Epstein, R., DePonte, K., Fish, D., Fikrig, E., 2014. Gut Microbiota of the Tick Vector *Ixodes scapularis* Modulate Colonization of the Lyme Disease Spirochete. Cell Host Microbe 15, 58–71. https://doi.org/10.1016/j.chom.2013.12.001

Ng, S.H., Stat, M., Bunce, M., Simmons, L.W., 2018. The influence of diet and environment on the gut microbial community of field crickets. Ecol. Evol. 8, 4704–4720. https://doi.org/10.1002/ece3.3977

Niang, E.H.A., Bassene, H., Fenollar, F., Mediannikov, O., 2018. Biological Control of Mosquito-Borne Diseases: The Potential of *Wolbachia*-Based Interventions in an IVM Framework. J. Trop. Med. 2018. https://doi.org/10.1155/2018/1470459

Novakova, E., Woodhams, D.C., Rodríguez-Ruano, S.M., Brucker, R.M., Leff, J.W., Maharaj, A., Amir, A., Knight, R., Scott, J., 2017. Mosquito Microbiome Dynamics, a Background for Prevalence and Seasonality of West Nile Virus. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00526

Olson, K.E., Higgs, S., Gaines, P.J., Powers, A.M., Davis, B.S., Kamrud, K.I., Carlson, J.O., Blair, C.D., Beaty, B.J., 1996. Genetically Engineered Resistance to Dengue-2 Virus Transmission in Mosquitoes. Science 272, 884–886. https://doi.org/10.1126/science.272.5263.884

Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environ. Microbiol. 18, 1403–1414. https://doi.org/10.1111/1462-2920.13023

Phillips, M.L., 2009. Gut Reaction: Environmental Effects on the Human Microbiota. Environ. Health Perspect. 117, A198–A205.

Quince, C., Lanzen, A., Davenport, R.J., Turnbaugh, P.J., 2011. Removing noise from pyrosequenced amplicons. BMC Bioinformatics 12, 38. https://doi.org/10.1186/1471-2105-12-38

Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G., Dimopoulos, G., 2014. Chromobacterium Csp_P Reduces Malaria and Dengue Infection in Vector Mosquitoes and Has Entomopathogenic and In Vitro Anti-pathogen Activities. PLoS Pathog. 10. https://doi.org/10.1371/journal.ppat.1004398

Robich, R.M., Rinehart, J.P., Kitchen, L.J., Denlinger, D.L., 2007. Diapause-specific gene expression in the northern house mosquito, *Culex pipiens* L., identified by suppressive subtractive hybridization. J. Insect Physiol., Physiology of Vector Arthropods 53, 235–245. https://doi.org/10.1016/j.jinsphys.2006.08.008

Rodríguez-Ruano, S.M., Juhaňáková, E., Vávra, J., Nováková, E., 2020. Methodological Insight Into Mosquito Microbiome Studies. Front. Cell. Infect. Microbiol. 10. https://doi.org/10.3389/fcimb.2020.00086

Romoli, O., Gendrin, M., 2018. The tripartite interactions between the mosquito, its microbiota and Plasmodium. Parasit. Vectors 11. https://doi.org/10.1186/s13071-018-2784-x

Rosso, F., Tagliapietra, V., Albanese, D., Pindo, M., Baldacchino, F., Arnoldi, D., Donati, C., Rizzoli, A., 2018. Reduced diversity of gut microbiota in two *Aedes mosquitoes* species in areas of recent invasion. Sci. Rep. 8, 16091. https://doi.org/10.1038/s41598-018-34640-z

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., Tuohy, K., 2018. Gut microbiota functions: metabolism of nutrients and other food components. Eur. J. Nutr. 57, 1–24. https://doi.org/10.1007/s00394-017-1445-8

Rozo-Lopez, P., Mengual, X., 2015. Mosquito species (Diptera, Culicidae) in three ecosystems from the Colombian Andes: identification through DNA barcoding and adult morphology. ZooKeys 39–64. https://doi.org/10.3897/zookeys.513.9561

Saab, S.A., Dohna, H. zu, Nilsson, L.K.J., Onorati, P., Nakhleh, J., Terenius, O., Osta, M.A., 2020. The environment and species affect gut bacteria composition in laboratory co-cultured *Anopheles gambiae* and *Aedes albopictus* mosquitoes. Sci. Rep. 10, 1–13. https://doi.org/10.1038/s41598-020-60075-6

Sambri, V., Capobianchi, M., Charrel, R., Fyodorova, M., Gaibani, P., Gould, E., Niedrig, M., Papa, A., Pierro, A., Rossini, G., Varani, S., Vocale, C., Landini, M.P., 2013. West Nile virus in Europe: emergence, epidemiology, diagnosis, treatment, and prevention. Clin. Microbiol. Infect. 19, 699–704. https://doi.org/10.1111/1469-0691.12211

Schaffner, F., Medlock, J.M., Bortel, W.V., 2013. Public health significance of invasive mosquitoes in Europe. Clin. Microbiol. Infect. 19, 685–692. https://doi.org/10.1111/1469-0691.12189

Shaikevich, E.V., Vinogradova, E.B., Bouattour, A., Gouveia de Almeida, A.P., 2016. Genetic diversity of *Culex pipiens* mosquitoes in distinct populations from Europe: contribution of *Cx. quinquefasciatus* in Mediterranean populations. Parasit. Vectors 9. https://doi.org/10.1186/s13071-016-1333-8

Smith, J.L., Fonseca, D.M., 2004. Rapid assays for identification of members of the *Culex (Culex) pipiens* complex, their hybrids, and other sibling species (Diptera: culicidae). Am. J. Trop. Med. Hyg. 70, 339–345.

Spor, A., Koren, O., Ley, R., 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat. Rev. Microbiol. 9, 279–290. https://doi.org/10.1038/nrmicro2540

Strand, M.R., 2018. Composition and functional roles of the gut microbiota in mosquitoes. Curr. Opin. Insect Sci. 28, 59–65. https://doi.org/10.1016/j.cois.2018.05.008

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'Connor, M.P., Rosen, G.L., Knight, R., Kilham, S.S., Russell, J.A., 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol. Ecol. 21, 3363–3378. https://doi.org/10.1111/j.1365-294X.2012.05552.x

Thongsripong, P., Chandler, J.A., Green, A.B., Kittayapong, P., Wilcox, B.A., Kapan, D.D., Bennett, S.N., 2018. Mosquito vector-associated microbiota: Metabarcoding bacteria and eukaryotic symbionts across habitat types in Thailand endemic for dengue and other arthropod-borne diseases. Ecol. Evol. 8, 1352–1368. https://doi.org/10.1002/ece3.3676

Tobin, P.C., 2018. Managing invasive species. F1000Research 7. https://doi.org/10.12688/f1000research.15414.1

Turell, M.J., O'Guinn, M.L., Dohm, D.J., Jones, J.W., 2001. Vector Competence of North American Mosquitoes (Diptera: Culicidae) for West Nile Virus. J. Med. Entomol. 38, 130–134. https://doi.org/10.1603/0022-2585-38.2.130

Wagner, S.J., 2004. Transfusion-transmitted bacterial infection: risks, sources and interventions. Vox Sang. 86, 157–163. https://doi.org/10.1111/j.0042-9007.2004.00410.x

Wang, J., Weiss, B.L., Aksoy, S., 2013. Tsetse fly microbiota: form and function. Front. Cell. Infect. Microbiol. 3. https://doi.org/10.3389/fcimb.2013.00069

Werblow, A., Klimpel, S., Bolius, S., Dorresteijn, A.W.C., Sauer, J., Melaun, C., 2014. Population Structure and Distribution Patterns of the Sibling Mosquito Species *Culex pipiens* and *Culex torrentium* (Diptera: Culicidae) Reveal Different Evolutionary Paths. PLOS ONE 9, e102158. https://doi.org/10.1371/journal.pone.0102158

Wong, Z.S., Hedges, L.M., Brownlie, J.C., Johnson, K.N., 2011. *Wolbachia*-Mediated Antibacterial Protection and Immune Gene Regulation in Drosophila. PLoS ONE 6. https://doi.org/10.1371/journal.pone.0025430

Xi, Z., Ramirez, J.L., Dimopoulos, G., 2008. The *Aedes aegypti* Toll Pathway Controls Dengue Virus Infection. PLOS Pathog. 4, e1000098. https://doi.org/10.1371/journal.ppat.1000098

Zittra, C., 2013. Grundlagenwissen über Stechmücken (Culicidae: Diptera) des Nationalparks Donau-Auen 10.

Zotzmann, S., Steinbrink, A., Schleich, K., Frantzmann, F., Xoumpholphakdy, C., Spaeth, M., Moro, C.V., Mavingui, P., Klimpel, S., 2017. Bacterial diversity of cosmopolitan *Culex pipiens* and invasive *Aedes japonicus* from Germany. Parasitol. Res. 116, 1899–1906. https://doi.org/10.1007/s00436-017-5466-2

Zouache, K., Raharimalala, F.N., Raquin, V., Tran-Van, V., Raveloson, L.H.R., Ravelonandro, P., Mavingui, P., 2011. Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. FEMS Microbiol. Ecol. 75, 377–389. https://doi.org/10.1111/j.1574-6941.2010.01012.x

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