# Microbiota of Arctic mosquitoes

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Annotation

The aims of this thesis are:

1. Describe the microbiome of the mosquito Aedes nigripes, a unique species inhabiting Arctic areas, in comparison with other mosquito species from temperate climates.

2. Evaluate the seasonal (i.e. different weather conditions) effect on Ae. nigripes microbiome.

3. Discuss the potential of Ae. nigripes to become a vector of viruses according to the possible vector-pathogen-microbiome interactions that could occur in the event of global warming.

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 form, 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

In spite of being one of the tiniest organisms in the world, mosquitoes have a significant role in ecology and human health. Here we describe and analyze microbiota of Aedes nigripes mosquitoes collected in Svalbard archipelago during summer 2017 and 2018. Samples were collected on five different localities during July and August. Aedes nigripes as the most abundant and widely spread mosquito in the Arctic shows high abundance during the summer period, becoming a pest to vertebrates including humans. Microbiota present in mosquitoes includes bacteria, fungi and viruses that can affect their ability to transmit pathogens. We used 16S rRNA gene sequencing methods to identify bacteria present in our samples. As a result we got four major genera: Dietzia, Buttiauxella, Pseudomonas and Sphingomonas from which Buttiauxella is not commonly found in other Aedes species. In addition to that, we discussed the overall effects of climate change on mosquito habitat, ecology and, more important, its effects on mosquito abundance. With warmer climate and more breathing sites available, there will be enlarged mosquito populations able to spread viruses and diseases beyond their current areas.

Aims

1. Describe the microbiome of the mosquito Aedes nigripes, a unique species inhabiting Arctic areas, in comparison with other mosquito species from temperate climates.

2. Evaluate the seasonal (i.e. different weather conditions) effect on Ae. nigripes microbiome.

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# 1.Introduction

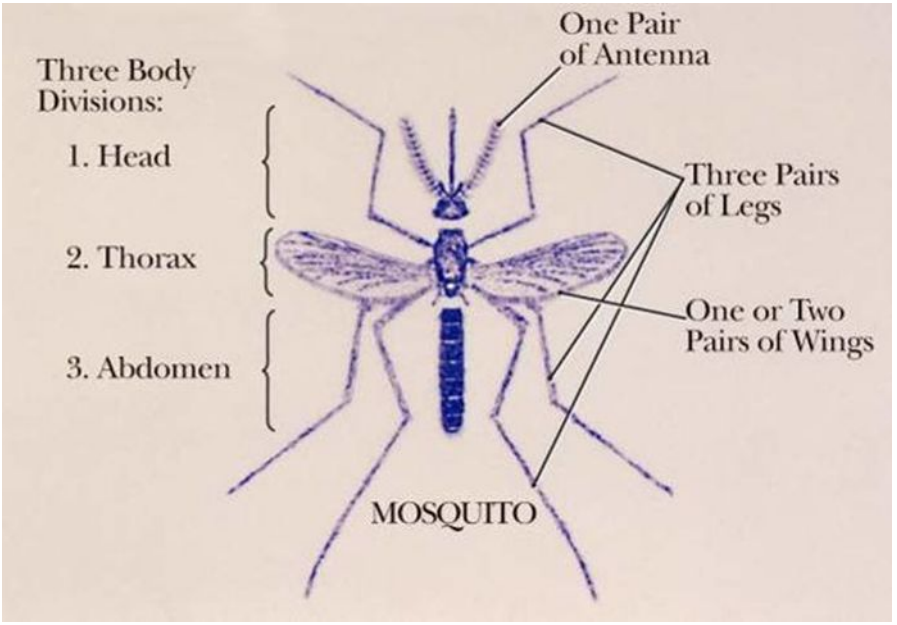
Insects have inhabited earth for about 350 million years compared with less than 2 million for humans (Chinery, 1973). During this time they have evolved into the largest group of animals, with 900 thousand species discovered and described to date. They appear all around the world and have adapted to life in almost every type of habitat in all different climate areas (Ross, 1982).

Their importance in ecosystems is great, especially in aquatic ecology (Borror, 1954). Insects are the main source of food for many other insects and small fishes (Borror, 1954). Insects also pollinate different plants including crops, which ensures fruit and seed production. Some other insects, like ladybirds, act by controlling the populations of other insects (for example pests) they use as a source of food (Borror, 1954). Another example of the role insects play in human's economy are the production of honey, beeswax and silk. On the other hand, insects also represent human enemies, because they feed in a varied source of natural food. This can affect economically relevant crops or have negative consequences for human health, i.e. transferring insect-borne diseases (Ross, 1982). Within the deadliest animals, mosquitoes as insect vectors of disease kill about 2.7 million people a year (WHO Executive summary, 2019).

## 1.1. Insect physiology

Insects are very unusual animals when considering their structure. It can be said that they are inside out because their skeleton is on the outside, or upside down because their nerve cord extends along the lower side of the body and the heart lies above the alimentary canal (Borror, 1954). Due to their skeleton being outside, insects are limited to very small sizes, which enables them to live in places that are unreachable for most other animals. Insect size range is from 0.25 to 330 mm length and 0.5 to 330 mm wingspread (Borror, 1954), with an average size of 6 mm in length (Ross, 1982). They possess no lungs, but breathe through tiny holes in the abdomen. Air entering these spiracles is being distributed throughout the whole body

through tracheae, so the heart does not have its usual function of allowing transport of oxygen to tissues (Borror, 1954). Insects are also the only invertebrates with wings and this is the main reason for their dominance since they can easily leave a place when it becomes unsuitable for living (Borror, 1954).




Taken from: Effects of weather on mosquito biology, behavior, and potential for west nile virus transmission on the southern high plains of texas (Bradford et al. 2005)

As seen from the picture, the body of a mosquito consists of 3 main parts: head, thorax and abdomen. The adult head comes out of the thorax and has the mouthparts in the front position, one pair of antennae, and simple lateral eyes, while the typical shape of the larval head can be either oval or ovate (Snodgrass, 1959). As of the second part, the thorax, it has a simple oval form and bears the wings, legs and respiratory spiracles (Snodgrass, 1959). As an important remark, not all insects have wings, and there are two suborders in this large group of animals according to that characteristic: Apterygota (″without wings″) and Pterygota (″with wings″) (Horn, 1976). The last part of the insect body is the abdomen, which appears divided into ten segments (eighth and ninth segments are combined). Respiratory apparatus is located on the eight segment, the ninth bears the gonopore, and the tenth segment contains male or female reproductive organs, depending on the sex of the insect (Horn, 1994). Insects can reproduce quite quickly and massively. For example Drosophila flies can reproduce 25 generations in a single year, which means approximately $10^{41}$ flies (Borror, 1973). In addition, contrary to most animals where an egg usually develops into a single individual, insect eggs can divide into 18, 60 or even 1000 new individuals (Borror, 1973).

In addition to their reproductive potential, there are other factors that contribute to the dominance of insects throughout the world. These are: flight capacity, adaptability, size, exoskeleton, resistance to dessication, respiration, complete metamorphosis and defense system (Thomas et al., 2000). For instance, flight capacity enables insects to seek food or shelter in different places, while adaptability allows them to tolerate various environmental conditions (Thomas et al.,

2000). Since insects are very small, this advantage allows them to exploit numerous ecological niches inaccessible to other animals, while their exoskeleton, made of chitin, provides them strength and rigidity giving them protection but still allowing them to be flexible enough in given circumstances (Thomas, 2000). Spiracles play a major role in water loss prevention as well as for the respiratory system, where their closing mechanism can admit air and prevent excessive water loss (Thomas, 2000).

There are insects that undergo very little change as they grow e.g. Blattaria ( Borror, 1973), while the majority of insects (82%) go through metamorphosis as they develop from zygote to adulthood (Horn, 1978). The majority of insects have a life cycle that develops from an egg into a worm-like larvae, which grows by periodically shedding their outer skin (together with the linings of the foregut, hindgut and breathing tubes), finally transforming into an inactive pupal stage from which the winged adult emerges (Borror, 1973). Metamorphosis is not as common in other groups of animals, which is why they successfully created such a diverse group based on terrestrial habitats (Horn, 1978).

But probably the most important feature of insects is they are cold-blooded. Their body temperature easily adapts to the temperature of the environment; when environment temperature drops, their body temperature follows and vice versa (Borror, 1973). Their physiological processes can be slowed down which helps them to survive hard environmental conditions. Some can also survive long freezing periods without consumption of food (Borror, 1973). This allows some mosquito species, for example, to overwinter as adults, or to colonize a priori inhospitable places like the Arctic. Exactly this kind of mosquito is Aedes nigripes or, as we can call it, “ice mosquito”. They are characterized by the ability to withstand very low temperatures (below zero Celsius degrees) during the winter, while the summers when they reproduce are also cold in comparison with other mosquito-borne areas.

## 1.2. Mosquitoes as relevant insects in disease transmission

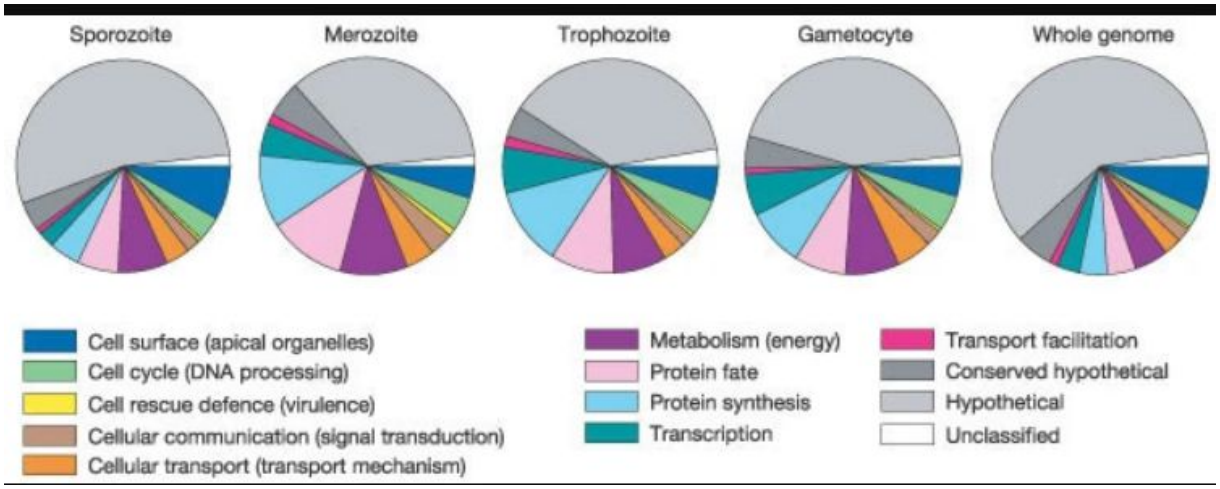
Mosquitoes (Diptera: Culicidae) comprise 3500 different species within the insect group, being one of the most diverse groups of insects. There are fossil records of mosquitoes as old as 226 million years ago (Borror, 1973). Mosquitoes differ from their closest insect families because they possess wing veins and margin. Not all mosquitoes bite, in fact only females possess mandibles that allow them to feed on blood. After the blood meal, females are able to lay a batch of eggs on or near water; then most aquatic larvae consume algae and organic remains, while only few species prey other mosquito larvae (e.g. the most known representatives belonging to genus Toxorhynchites, which can also be named elephant mosquito or mosquito eater) (Horn, 1978).

Apart from their ecological aspects, mosquitoes have been for decades number one world's health threat, particularly representatives from the genera Anopheles, Culex, Aedes (Kjanijou et al., 2012). From the 3500 different mosquito species, only about 200 species bite humans, and not all of them are equally attracted to them (Mukabana et al., 2002). Some research has shown that human age, size and other factors can also affect the way mosquitoes are being attracted (Lindsay et al., 2002). For example certain physiological changes such as the ontogenic development of skin glands and variations that affect the skin microbiota, and more generally, skin odor determine mosquito attraction (Braks et al., 1999). What also attracts mosquitoes is the $CO_2$ gas humans release by breathing, as well as lactic and carboxylic acid (Keswani and Bellare, 2006). Lactic acid is produced in muscle cells when the body breaks down carbohydrates to use for energy, usually when oxygen levels are low (Barell, 2019). Carboxylic acids are formed by oxidation of aldehydes and are used for formation of fat in the body (Badea and Radu, 2018).

When feeding on blood, female mosquitoes become one of the dominant disease transmitters, spreading various infections such as yellow fever (spread by Aedes species mostly in equatorial Africa and South America; McGuinness and Wu, 2019), malaria (spread by Anopheles

mosquitoes mainly in America, Africa and Asian-Pacific areas) and filariasis (spread by Aedes and Culex species in Africa, the Pacific and Asia) (Horn, 1978). Aedes species (mainly Aedes aegypti and Aedes albopictus) are also able to transmit medically important arboviruses to animals as well as humans, including West Nile (WNV) (Roehrig et al., 2002), dengue (DENV) (Martina, 2009), Zika (ZIKV) (Petersen et al., 2016) and chikungunya (CHIKV) viruses (Grandadam et al., 2011).

But definitely one of the most devastating mosquito-borne diseases is malaria, world's leading disease with 290 million cases and around 1 million deaths per year (Smith et al., 2014). Malaria is caused by one of these five species of the protist genus Plasmodium: P. vivax, P. ovale, P. malarie, P. falciparum, and P. knowlesi (Sadanand, 2010). The female mosquito bites infected hosts acquiring gametocytes of the parasite that arrive to the mosquito gut and develop into gametes. The fecundation of gametes produces diploid zygotes that mature into oocytes in the midgut epithelium. After an incubation time of two weeks in the midgut, the sporozoites are released first into the hemolymph and then into the salivary glands, where they can be eventually transmitted to a new human host through mosquito bites (Sadanand, 2010).The life cycle of Plasmodium parasite through mosquito is a complex and sensitive process with malaria transmission as a result (Vlachou et al., 2006). The parasite requires specialized protein expression to live in the host environment of invertebrates and vertebrates as well as for intracellular and extracellular survival, invading a variety of cells and evading host immune responses (Florens et al., 2002). Life cycle contains five different stages with variable expression of unique proteins (ranging between 20 and 49% depending on the stage), while only 6% of proteins are common to all stages (Florens et al., 2002). The full Plasmodium life cycle (with its five different stages) can be seen in the figure below according to its proteomic profile.




Taken from: A proteomic view of the Plasmodium falciparum life cycle (Florens et al., 2002)

After malaria, lymphatic filariasis is the second most common mosquito borne disease, being present in more than 80 tropical and subtropical areas with 120 million of people infected and one billion exposed to risk of infection (Wynd et al., 2007). In general, lymphatic filariasis can be complicated to detect since it causes a lot of other clinical and subclinical diseases like chronic lymphoedema, elephantiasis and hydrocele, while parasite associated immunosuppression would be shown only when tested for it (Wynd et al., 2007). First successfully treated cases were reported in 1947, when disease was treated with 1-diethyl-carbamyl-4-methylpiperazine hydrochloride (Hetrazan). Three years later, Tokyo university in Suganuma revealed that synthesized 1-dimethyl carbamoyl-4-methyl-piperazine citrate (Supatonin) also showed positive results (Otsuji, 2011). Hetrazan is still widely used as the best treatment for lymphatic filariasis (Ottesen, 2006), while Supatonin is still undergoing research for improvements (Otsuji, 2011).

### 1.3. Aedes nigripes and climate change

By virtue of its tiny structure, mosquitoes are able to live in different ecological niches that vary from tundra, forests and the tropics to deserts of different ranges in temperature, where they can breed in various water bodies, including water containers filled by rainfall, drainage and seepage. In particular, the mosquito object of this research is Aedes nigripes (Culler et al., 2015), the most

abundant species in the Arctic circle, which includes Northern European countries, and the Northern parts of Canada, United States (Alaska) and Russia (Culler et al., 2015).


Taken from: Controls on Arctic mosquito (Aedes nigripes) populations in western Greenland (DeSiervo et al.,2018).

Taking into consideration the fact that Aedes nigripes occupies areas with cold and long winters in the tundra, mosquito host offer is modest and mainly based on larger mammals e.g. moose, reindeers or brown bears (Schäfer and Lundström, 2001).

While the developmental cycle of Aedes nigripes takes up to 8 days, the total life cycle varies from days to months (Lundström et al., 2013). Aedes nigripes life cycle begins with egg hatching and development in shallow temporary ponds during May and June, followed by adult emergence as well as mating and seeking of blood meal (Culler, 2015). The cycle ends with the laying of matured eggs in the drying margins of ponds which will become the larval habitat during the following spring (Corbet and Danks, 1973).

An important characteristic of this species is the autogeny (i.e. the capacity of reproducing without a blood meal). This was first recorded in an experiment performed by Philip S. Corbett in 1984 where different groups of Aedes nigripes mosquitoes were exposed to a blood meal, a sugar and nectar diet, or demineralized water. The first group fully developed ovaries and oocytes, while the second group also developed ovaries and oocytes but not fully as the first one. This research suggested that autogeny is facultative in female Aedes nigripes and would be directly associated with decreased fecundity. An additional important fact is the occurrence of intersexes in Aedes nigripes. Arthropods are usually sexually dimorphic, differentiating between male and female. However, very rarely, individuals containing both male and female morphological characters have been found. This occurrence is known as intersex (Brust, 1968). Intersexes are genetically uniform, meaning they are complete male, female or intermediate in every tissue, but some tissues have tendency to show sexual phenotypes contrary to the genetic sex (Periera et al., 2010). In an experiment performed by Reinhart A. Bust in 1965 where larvae of arctic and subarctic mosquitoes were reared at higher temperatures than those they experience in the open field, it was demonstrated that the intersexes were caused by elevated rearing temperatures. The effect of high temperatures on intersexes is passed to offspring but weakens as the number of generations increases (Ning et al., 2019). The effect of the increase of intersexes in mosquito populations as temperatures rise due to global climate warming remains to be explored. As larvae and pupae, mosquitoes are an easy prey for other members of the food chain (Culler and Ayres, 2015), in spite of which the larvae of Aedes nigripes increase rapidly in thawed lakes of the vast tundra. Recent research shows that, due to global warming and the early thawing of lakes in the Arctic, mosquitoes emerge much earlier as well as in higher numbers than they were in the middle of the last century (Fang, 2015). With increasing temperatures, the larvae development period has decreased, which has a positive effect on mosquito survival as they develop more rapidly and are less exposed to predators. For example, if the temperature rises by 2 °C in Kangerlussuaq, Greenland, the chance of Aedes nigripes survival would increase by 53%, resulting in a higher number of mosquitoes (Culler, 2015). In this scenario, their life cycle will also overlap with the caribou calving season, which allows female mosquitoes to feed on bigger and less motile herds, which will have a negative impact on caribou populations (Fang, 2015). Another negative consequence of the increase in temperatures caused by global warming is that the natural mosquito habitat itself expands, which can cause a greater transmission of infectious diseases by disease agents and mosquitoes in emerging new areas (Culler, 2015).

In fact, transmission of infectious and vector borne diseases, such as malaria, are under great influence of climate change and global warming, which leads to their higher incidence and wider geographic range over time (Bai et al., 2013). Rainfall, humidity and increased temperature show significant impact on mosquito rate of multiplication which in return accelerate salivary infection, therefore increasing the likelihood for successful transmission of pathogens to another host (Reiter, 2001). However, the progress of climate change is undetermined and arbovirus ecology is complicated, so it is possible that some areas will show increased arbovirus activity and human infection, but the possibility of increase in transmission will depend on locality, vector, host and

human factors (Russell, 1999). Referring to the above-mentioned work by Culler et al. and the fact that mosquito survival increases by 53% with just 2 °C temperature increase, we can assume that ongoing climate changes and global warming will positively affect mosquito abundance and reproduction in the Arctic. This fact has been supported by the result of the study of the population dynamics of the mosquito vectors of the Rift Valley fever in Senegal (Biteye et al., 2020), which recorded a positive increase in the number of collected mosquitoes in different weather conditions and biotopes. In this scenario, how to prevent further spread of mosquito-borne diseases and the mass appearance of mosquitoes in new areas is a main field of research. In particular, the Aedes nigripes biology in this context, including the most recent notions of the microbiome as a key player in host processes, has been little explored.

## 1.4 Mosquito microbiome

Microbiota has a major role for overall health of the host, including nutrient metabolism, maintenance of structural integrity of the gut mucosal barrier and protection against pathogens. It has also been found to be crucial for overall homeostasis of their host (Mishra and Mishra, 2018), and associated with multiple human diseases ranging from inflammatory bowel disease to metabolic disorders like diabetes, or up to allergic diseases (Walker, 2015). Microbiomes are, thus, in close interaction with the host, thereby the host is no longer labelled as an isolated entity; it is considered to be a chimera or holobiont (Guégan et al., 2018).

Recent research on the mosquito microbiome has revealed that it includes bacteria, fungi (mycobiota), protists and viruses, namely mosquito-specific viruses (MSV) and transmitted pathogens (Guégan et al., 2018). The first endogenous mosquito-specific viruses identified in Aedes and Culex sp. mosquitoes were the cell combining agent virus (CFAV), Kamiti River virus (KRV) and Culex flavivirus (CxFV) (Guégan et al., 2018).

The composition and diversity of bacteria, the most studied component of the microbiota in mosquitoes, are affected by sugar and/or blood-meal intake, which increases inter-individual dissimilarities. Blood-meal causes, for example, progress in oxidative conditions in the gut through the restriction of microbial communities' constitution and structure (Guégan et al., 2018). Particularly, the mosquito gut represents an ecosystem where the complex, intimately associated microbiome influences multiple host traits (e.g. immunity), making the research on microbial community structure and its dynamics in mosquito a requirement to understand the symbiotic relationship between mosquito and its gut microbial residents, including pathogens (Wang et al., 2011). Interaction relationships between the host immune system and the symbiotic bacteria, which include mechanisms to control the mammalian host immune system, for example, regulates their cooperative relationship (Pang et al., 2016). Even though microbial interactions are a strong force in shaping insect microbiome communities, and determining vector competence of the

insect host, symbiont interactions are still unclear due to the nature of co-occurrence and co-exclusion interactions within the microbiome (Hegde et al., 2018).

After investigating the midgut bacterial flora of Aedes triseriatus using quantitative aerobic bacterial culture, most frequently found bacteria were Serratia marcescens, Klebsiella ozaenae, Pseudomonas aeruginosa, and Enterobacter agglomerans (Demaio et al., 1996). Using 16S rRNA gene sequence analysis for investigating the microbiota of Aedes albopictus and Aedes aegypti, it was found that phylum Proteobacteria was dominant, with Firmicutes, Bacteroidetes and Actinobacteria following. The dominant bacteria in both species was Enterobacter cloacae. Bacillus aryabhattai was dominant in Ae. albopictus and Stenotrophomonas maltophilia in Ae. aegypti (Yadav et al., 2015). Similarly, three major phyla, namely Proteobacteria, Firmicutes and Actinobacteria, were found in Aedes albopictus mosquito samples analysed by Moro et al. (2013). However, no studies of the microbiota of another member of the Aedes genus, Aedes nigripes, have been made to date in spite of their very particular habitat compared to other species of the genus.

## 2. Material and methods

### 2.1. Sampling site and mosquito collection

Svalbard is a Norwegian archipelago in the Arctic Ocean about midway between continental Norway and the North Pole. The islands of the group range from 74° to 81° North latitude, and from 10° to 35° East longitude. About 60% of Svalbard's land area is glaciated and characterized as high latitude with permafrost occurring both in mountains and lowlands, although this is decreasing due to climate change (Schuler et al., 2020). The glaciation is affected by local climate; precipitation and, thus, glaciation show an increase in altitude and to the west, which is the main source of moist air. Northern air masses carry a lot of snow, which is the main cause for the vast glaciation of Nordaustlandet and Kvitøya, with their wide ice caps (Dowdeswell et al., 2010). Aedes nigripes is the only mosquito species widely and abundantly distributed in this area (Oleg Ditrich, personal communication).

The samples used in this study were collected at three sites in the Svalbard Archipelago during summer 2017 and 2018 using an entomological aspirator. Samples were collected in 2017 in Nostoc (July 10 and 22), Brucebyen (July 10th and 27th) and Elba (July 12th). Samples in 2018 were collected in Tempelt (August 13th) and Petunia (August 4th) localities. In total 36 samples were analyzed, distributed as shown in Table 1.

| | | |
|---|---|---|
| Nostoc | July 10th 2017<br>July 22nd 2017 | 4 samples<br>7 samples |

| | | |
|---|---|---|
| Brucebyen | July 10th 2017<br>July 27th 2017 | 4 samples<br>5 samples |
| Elba | July 12th 2017 | 4 samples |
| Templet | August 13th 2018 | 6 samples |
| Petunia | August 4th 2018 | 6 samples |

Table 1. Distribution of mosquito samples collected in different locations and dates in Svalbard.

All samples were stored in 96% ethanol and then frozen at -20 °C for storage.

2.2. DNA extraction

Prior to extraction, in sterile conditions, we separated the extremities from the rest of the body to use only the thorax and head of the specimens (i.e. where the gastrointestinal tract containing the microbiota is). Then each sample was washed in 96% ethanol to remove any external contamination, followed by sterile PBS (phosphate buffered saline) to remove ethanol traces that would interfere with the extraction. Samples were individually homogenized in RLT Plus buffer using sterile pestles for extraction with the Allprep DNA/RNA kit (Qiagen) in a 96-well plate following the manufacturer instructions and eluted in 200 μL of MilliQ ultrapure water. Eluted DNA was stored at -20 °C for further analyses. RNA was eluted in ultrapure, RNase free water and was stored at -80 °C. Only DNA samples corresponding to the 36 specimens collected were used in the current study.

2.3. PCR amplification and screening

PCR stands for polymerase chain reaction and represents a commonly used biological method to produce multiple copies of a specific DNA region. Very efficient, rapid and able to amplify various DNA sequences. Once the DNA has been successfully amplified, the product can be sequenced, analyzed by gel electrophoresis or cloned into plasmids. For a particular PCR process, target DNA is needed as well as specific primers to target it. Besides, a thermostable DNA polymerase (Taq polymerase) and nucleotides are required. The overall process consists of 3 steps: denaturation, annealing and extension/elongation. The first one, denaturation, occurs at 90-95 °C, where double-stranded DNA separates into two single strands, thanks to the fact that hydrogen bonds between bases are weak and breakable at high temperatures. The melting temperature is where 50% of the dsDNA is denatured and is determined by G+C content and ions concentration. Second step allows primers to anneal or bind the target sequence by lowering temperature to approximately 50-60 °C. For the final step, temperature rises again to around 75 °C, where DNA polymerase extends the sequence from primers. Primers, as necessary part of this process, are single strands of nucleic acids usually very short (20-30 bases) specific to the target DNA sequence portion, commonly used in pairs known as forward and reverse primers. After primers anneal to the complementary DNA sequence, Taq DNA polymerase (a recombinant

thermo stable DNA polymerase from the organism Thermus aquaticus) is used to replicate the DNA strands by synthesis (always in 5' to 3' direction). In this step a new double stranded DNA molecule, identical to the original one, is produced.

For 16S bacterial gene amplification, we followed the EMP 16S protocol, which is designed to detect prokaryotes (bacteria and certain archaea). We used the primer pair 515F–806R to amplify the V4 hypervariable region of the 16S SSU rRNA gene. These primers and primer constructs were designed by Caporaso et al. (2011,2012), while the modifications of primer degeneracy were included by the labs of Fuhrmann (Parada et al., 2016) and Apprill (Apprill et al., 2015). Forward-barcoded constructs were redesigned by Walters et al. (2016). Primer sequences without linker, barcode or adapter are:

Forward: GTGYCAGCMGCCGCGGTAA

Reverse: GGACTACNVGGGTWTCTAAT

For PCR mixture we used the following: PCR grade water (13.0 µl), PCR master-mix (Q5 High-Fidelity 2X Master Mix , 10.0 µl), forward primer (10 µM, 0,5 µl), reverse primer (10 µM, 0,5 µl), and 1 µl of template DNA which brings up total volume of 25 µl. The PCR process goes through 3 main stages, with two steps at the beginning and the end, and a cycle repeated 35 times in the middle: first, initial denaturation occurs at 94 °C for 3 minutes. The cycle starts with 60 seconds at 94 °C (denaturation), followed by 50 °C for 60 seconds (annealing of primers) before rising again to 72 °C for 90 seconds. The last step (elongation) occurs at 72 °C for 10 minutes.

In addition, a specific DNA amplification to screen for recent blood-meal was also performed with a combination of reverse and forward primers that amplified a fragment of the mosquito COI gene: Mod_RepCOI_F + VertCOI_ 7194_R. PCR reactions were carried out with an initial denaturation temperature of 94º C for 3 min followed by 40 cycles of denaturation at 94º C for 40 s, hybridization at 48.5° C for 30 s and extension at 72º C for a min, followed by a final extension at 72º C for 7 min (Reeves et al., 2018).

All PCR reactions included a negative control.

In order to examine if the PCR was successful, agarose gel electrophoresis for size separation of PCR products was performed. The sizes were compared to a DNA ladder (100 bp), a molecular weight marker composed of known size DNA fragments run on the gel alongside PCR products. Before amplicons of each sample were run on agarose gel, they were stained with GelRed® Nucleic Acid Gel Stain so they could be visible under UV light after gel electrophoresis. Band size for the V4 region of the 16S rRNA gene amplified with 515F-806R primers is around 350 bp, while the expected band size for the blood meal presence is around 300 bp.

## 2.4. Microbiome sequencing

For microbiome sequencing, amplicons of the 16S rRNA V4 region were cleaned up using AMPure XP magnetic beads (Beckman Coulter), and their concentrations measured in a Synergy H1 microplate reader (Biotek). Equal amount of each samples' amplicons were combined into a single sterile tube and the library was sent for sequencing in an Illumina MiSeq nano run (v2 chemistry, 2x250 bp output) using the primers described in the EMP protocol webpage (https://www.earthmicrobiome.org/protocols-and-standards/16s/). Our samples were sequenced along with positive and negative controls. 5-10% PhiX was added to the sequencing to make the sample composition more complex before running libraries, as a requirement of paired-end 16S sequencing on the Illumina platform.

## 2.5. Data analysis and statistics

After getting files from the sequencer, multiple computational steps were performed for 16S microbiome data analysis. First, we prepared the metadata in the right format (avoiding, for example, non-alphanumeric characters and spaces) in order to avoid any errors and compatibility issues in further analyses. After obtaining the metadata, the barcode file was prepared using a text/spreadsheet editor. Afterwards, we did the demultiplexing according to the barcodes. Quality of the reads was then checked in FastQC and sequences trimmed using the USEARCH command: fastx_truncate. Demultiplexed and trimmed outputs were merged into a single fastq file. One last step before creating the OTU table was to filter the sequences, removing the primers with the usearch command fastx_truncate. Then, an OTU table was created. We made a representative set of sequences for OTU picking using the USEARCH command fastx_uniques. Afterwards, generating an OTU table was performed in 2 steps, using the USEARCH commands fastq_filter to prepare the original dataset for alignment with the representative sequences, and then usearch_global to get the OTU table, aligning and clustering the sequences at 97% similarity.

For the taxonomic assignments, we used the SILVA database truncated for SSU of 16S rDNA (Bacteria, Archaea and Eukarya) and we formatted the database for BLAST. Taxonomic assignments were obtained for the representative sequences using standalone BLAST and then added to the OTU table. As a final step, we filtered the OTU table in QIIME 1 according to taxonomy. Low abundant OTUs were removed, and sequencing effort was normalized by rarefaction for all the samples. We generated the OTU table in two formats: text format was used for text/spreadsheet editors, and biom format was used for QIIME 1. These final output files were used for further analysis in QIIME 1, R and other tools.

Alpha diversity indexes were calculated from the OTU table. Alpha diversity represents diversity in a single sample. Metrics like Shannon, Chao1, dominance, equitability and richness were measured. Richness represents a number of species (or OTUs) in the sample while Shannon's index accounts for both abundance and evenness of the species present. Dominance index

describes the probability that two randomly selected reads will belong to different OTU's, while equitability measures species evenness. Chao1 metric is an estimator of the number of species that try to extrapolate richness to include some rare species in a community that may have not been observed due to methodological reasons. Alpha diversity metrics use different units and cannot be compared with each other. The alpha diversity file is created from an OTU table by using the USEARCH command alpha_div. The input OTU table must be in QIIME classic format. After successfully creating the alpha diversity file, we performed Kruskal-Wallis tests in RStudio (kruskal.test function) for the different alpha-diversity metrics measured. Kruskal-Wallis tests were performed in order to see if there were any statistically significant differences between two or more groups of an independent variable on a continuous or ordinal dependent variable. Values obtained from tests are chi-squared and p-values that tell if there is significant difference among the analysed data. We also generated box plots in RStudio (boxplot function) as visual representation of our alpha-diversity data.

## 3. Results

### 3.1 Gel electrophoresis

The 16S library construction was checked on gel electrophoresis. Figure 1 is an example of the gels performed and shows 2 different bands: The higher band (approximately 700 bp) corresponds to host 18S rRNA unspecific amplification, while the smaller band (approximately 400 bp) is the 16S rRNA from the bacteria that was sequenced for the microbiome analysis. The well at the beginning of each lane is the 100 bp ladder.

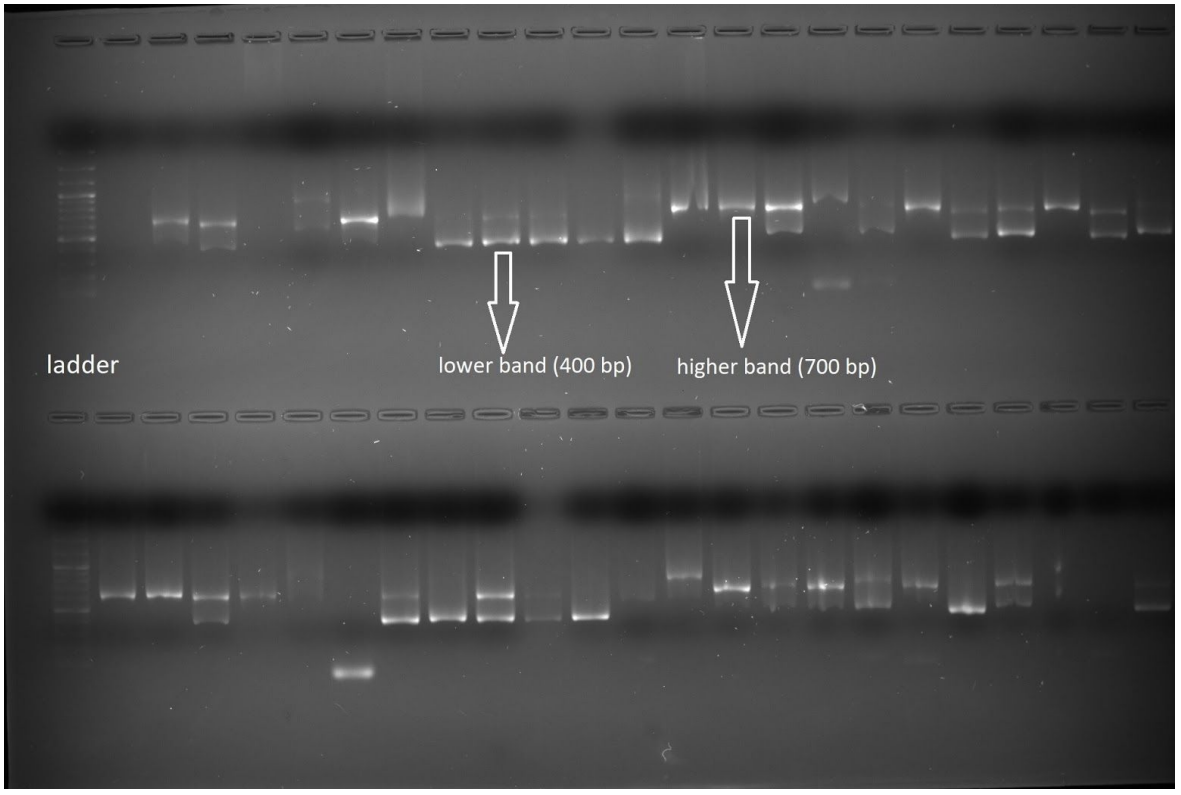



Figure 1. Gel electrophoresis of the PCR amplification for the 16S library.

In Figure 2 we show an example of gel electrophoresis we runned for blood meal screening, where the positive band is of around 300 bp. We tried to use the results of this PCR, but the protocol described in Reeves et al. (2018) publication was optimized for a different mosquito species, and we found few positive bands and many unspecific ones for Aedes nigripes, which made the results difficult to interpret.

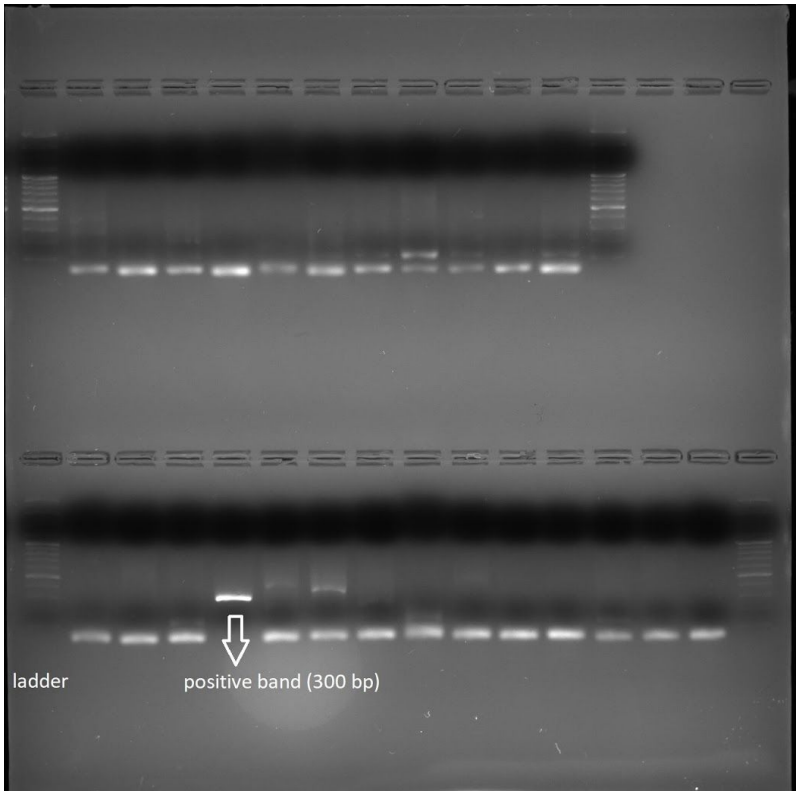



Figure 2. Gel electrophoresis for the PCR amplification for blood meal

## 3.2 Taxonomic profile of the samples

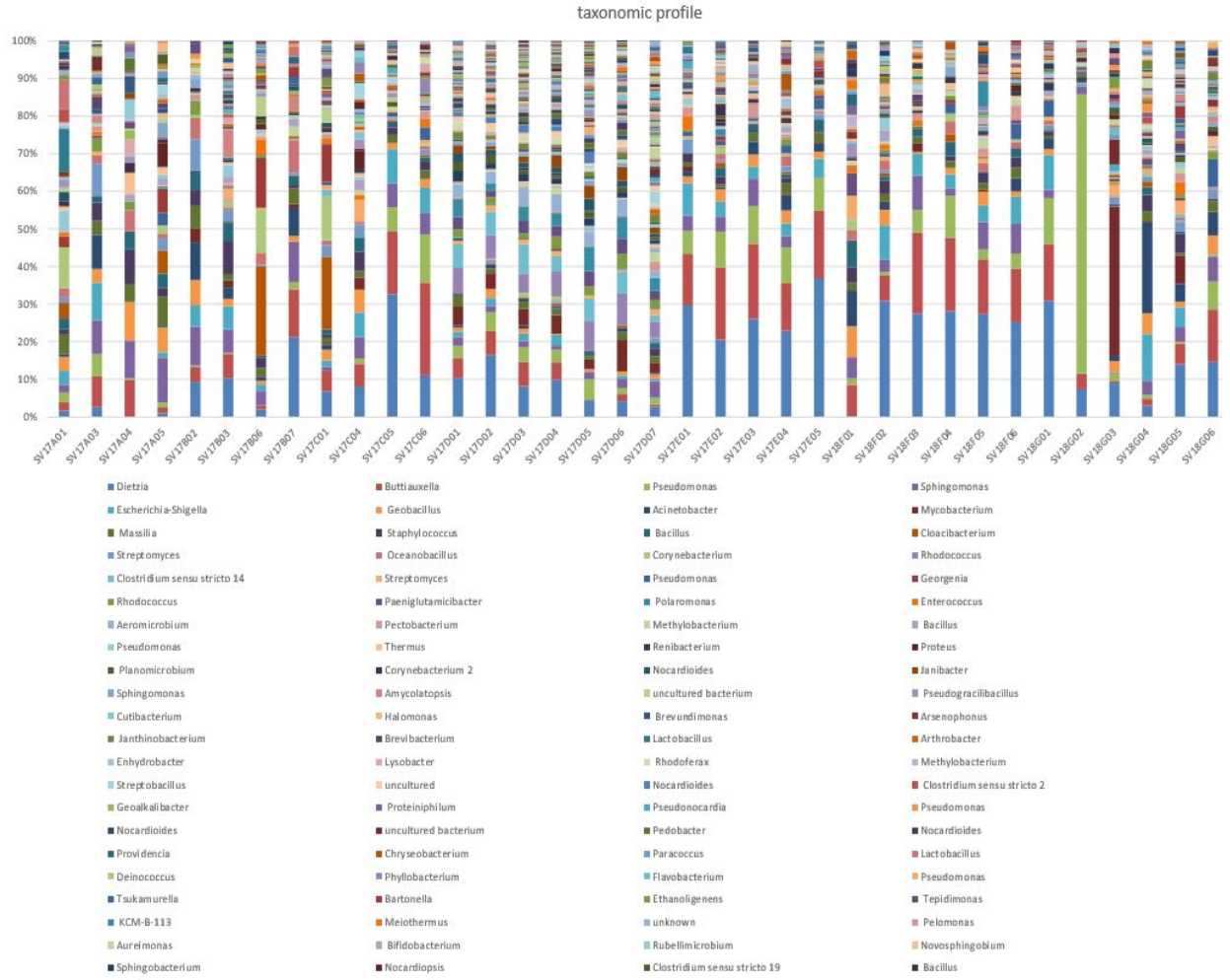



Figure 3. Taxonomic profile of the Aedes nigripes microbiome.

Figure 3 shows a visual representation (taxonomic profile) of the OTU table, with samples in the x-axis and the y-axis showing the relative abundance (in percentage) of the corresponding bacterial OTUs grouped at genus level. Bacteria species listed at the bottom are coloured and ranked from most to least abundant (starting from top left to bottom right). As seen from the list, the four major genus of bacteria present are Dietzia (14% of total reads), Buttiauxella (9% of total reads), Pseudomonas (6% of total reads) and Sphingomonas (5% of total reads).

## 3.3 Analysis of the alpha diversity of the samples

The diversity of the microbiome of the mosquito samples was described using different indexes that reflect different aspects of the bacterial community: Chao1 index (richness estimate),

equitability, dominance and Shannon index. The results by year can be seen in Figure 4. Figures 5 and 6 show, respectively, the results by date and locality of sampling.

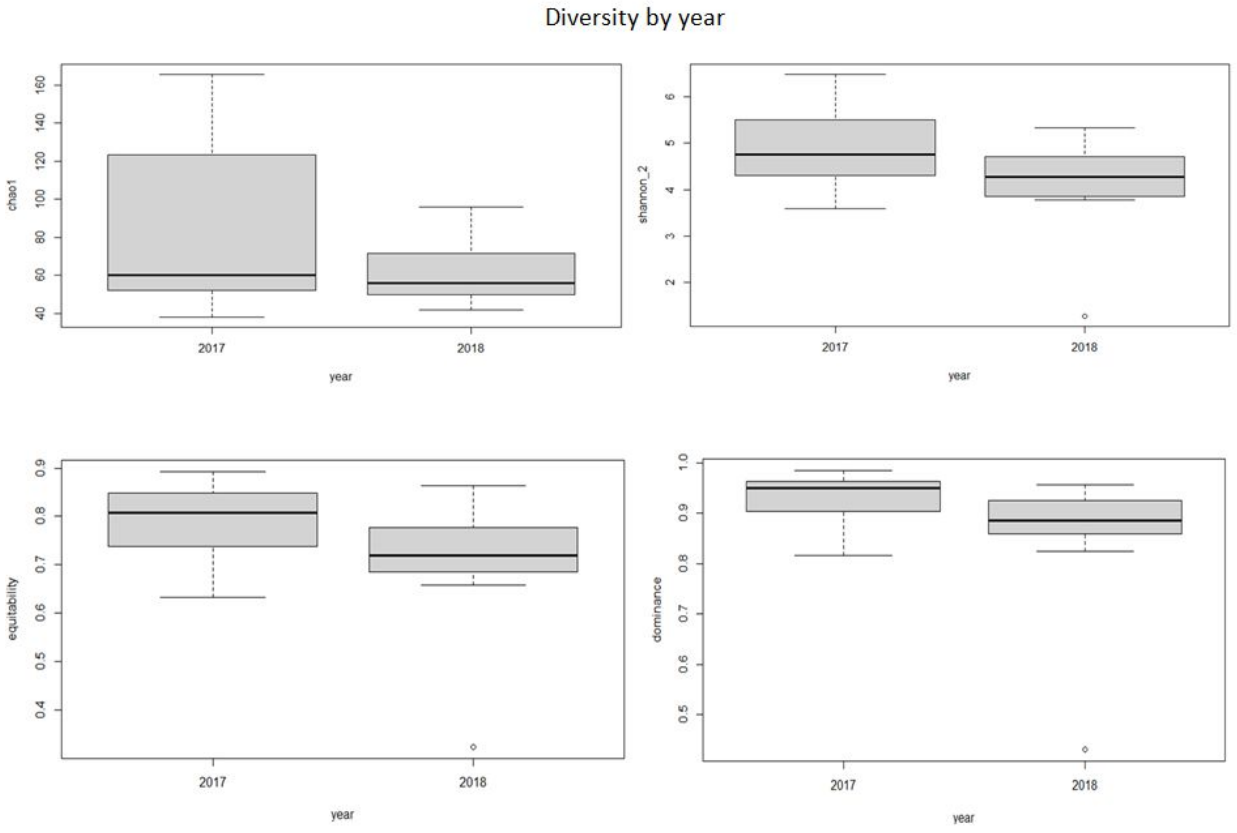



Figure 4. Alpha diversity of the mosquito microbiome in the different years they were collected (2017 and 2018).

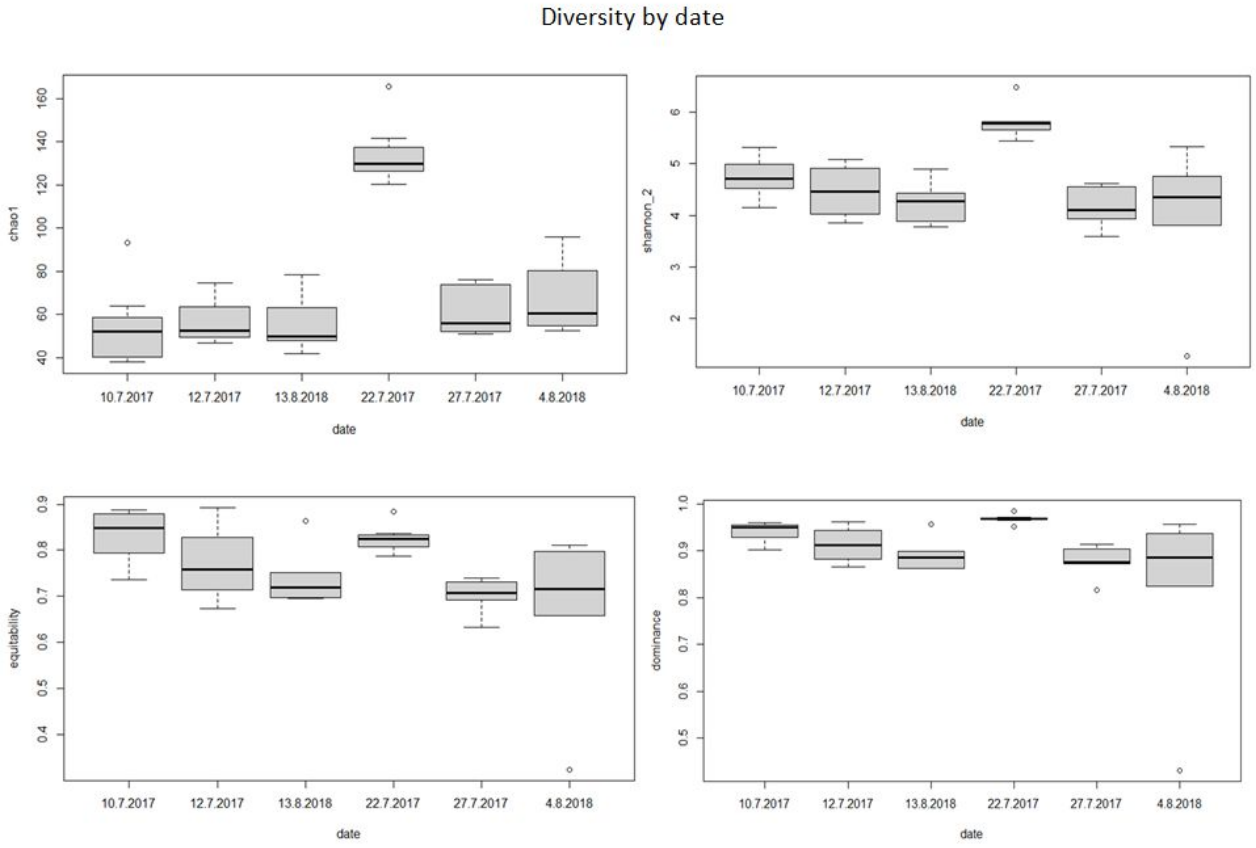



Figure 5. Alpha diversity of the mosquito microbiome in the different dates they were collected (summer).

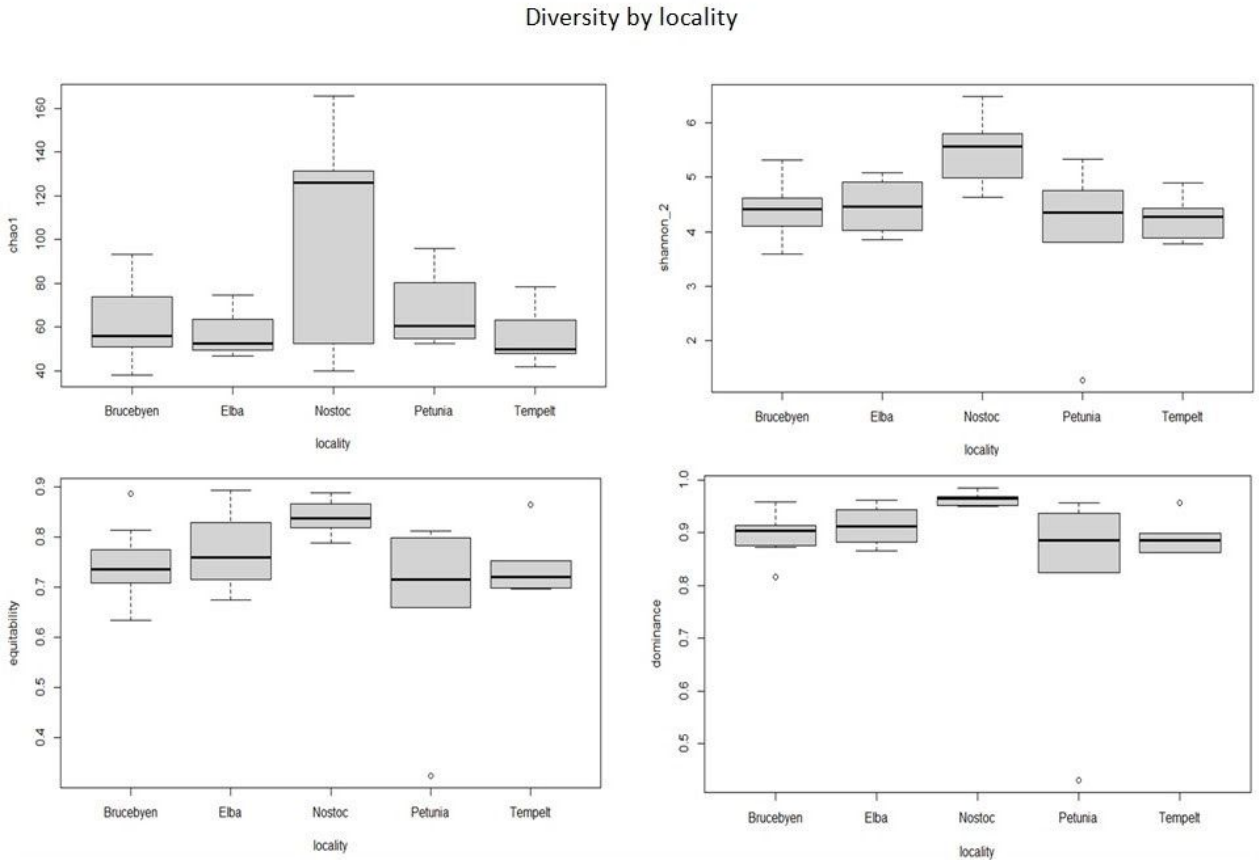



Figure 6. Alpha diversity of the mosquito microbiome in the different localities they were collected in Svalbard.

Table 2 shows the results of the Kruskal-Wallis test (p-value) for all the studied variables: year (2017 and 2018), locality (Elba, Petunia, Brucebyen, Tempelt and Nostoc) and date (6 dates during summer 2017 and 2018). Locality differences correspond to mosquito microbiome differences according to different environmental conditions and biotopes, while differences by year and date show differences in mosquito microbiome based on temperature and weather conditions.

| | Richness | Chao1 | Shannon | Dominance | Equitability |
|---|---|---|---|---|---|
| Locality | 0.098 | 0.089 | 0.002 | 0.001 | 0.009 |
| Year | 0.314 | 0.449 | 0.029 | 0.008 | 0.018 |

| | | | | | |
|---|---|---|---|---|---|
| Date | 0.001 | 0.001 | 0.001 | 0.0018 | 0.005 |

Table 2. Results of the Kruskal-Wallis tests performed for alpha-diversity in our samples. As seen from the table, three different variables were taken into account: locality, year and date, along with five alpha-diversity indexes. The significant results are p-values < 0.01 and appear highlighted in the table.

## 4. Discussion and conclusion

Climate change could be one of the main causes of the spread of infectious diseases and the increase in the number of infections, especially at the borders of transmission zones. These areas that were not previously areas of infection, are particularly endangered because the population does not have a built-in immune system, since they have never been exposed to the newly spread infection before (Fouque and Reeder, 2019). These assumptions were confirmed by the results of N.Johnson et al. (2018) showing a rising threat of West Nile and Usutu viruses in Eastern Mediterranean countries and a threat of exposure to other european countries, due to multiple anthropogenic changes. The problem of vector control hence requires a serious approach to find a solution. Intensive vector control strategies targeted at reducing mosquito populations in the context of global warming and rising temperatures, and prevention of disease outbreaks are today more necessary than ever (Victor et al., 2017), and some of them may rely on the microbiota to regulate vector competence of their hosts (Yordanova et al., 2018). Mosquito habitats are greatly influenced by climate changes like rainfalls or deforestation that also increase local temperature and thereby allow mosquitoes to widen their vectorial capacity (Afrane et al., 2012). Human impact on ecology also shows great influence in spreading of mosquitoes habitats and breeding sites (Reiter 2001). Warmer temperatures enhance cell metabolism and allow female mosquitoes to digest blood faster, thus increasing the need to feed more (Lee et al., 2013). In that scenario mosquito life cycle will be prolonged, and they will begin to lay eggs younger and will adapt to rapid growth rates (Kovats et al., 2001). Due to increased temperatures, winters are also not as cold, allowing some mosquito species to survive through winter (Sternberg and Thomas, 2014). Furthermore, the positive effect of constant rainfall during 2-4 weeks was shown in Oc. detrius weekly abundance (Roiz et al., 2014). It was found that more mosquitoes were collected during the period when the temperature and humidity values were the highest. Similarly positive results have been recorded by C. Zittra et al. (2017), where samples were collected from April until October in both 2015 and 2016, showing the highest number of collected samples during the 14-day mean sunshine duration, humidity and water-level maxima period in July. In addition to this fact, we can also look over to the biotopes. Observations of mosquito abundance and diversity in rural Neka township of Mazandaran province, northern Iran, showed higher Shannon index for rural areas where more breeding places like pools, streambeds, rivers and treeholes are

more present than in urban areas, allowing mosquitoes to breed and expand easier (Nikookar et al., 2015). However, it was also found that these areas under influence of rainfall deficiency may dry out which can allow more tolerant species to thrive and gain dominance. Similar case was described in the research done by S.Y. Ohba et al. (2012), where biotopes rich in water-breeding sites showed greater mosquito abundance and diversity than dried out rice paddy fields. Referring to our own results, we can observe a slight increase in numbers of samples collected in Nostoc and Brucebyen locality, rather than in Elba, Petunia and Templet, which can be correlated with the fact that Nostoc and Brucebyen are located directly on the coastline while the other localities are further in the interior of the island, perhaps providing less water surface available for mosquito breeding sites as well as a bit poorer biotope. The environmental influence can go further than the mosquito physiology and population dynamics. For example in the case of both Ae. taeniorhynchus and Cx. quinquefasciatus, the increase of temperature, precipitation and humidity enable not only mosquito development, but also vectored parasites development, increasing disease risk (Asigau and Parker, 2018). As already discussed in the introduction, the chance of Aedes nigripes survival increases by 53% if the temperature rises by only 2 °C (Culler, 2015), pointing at an increased mosquito abundance and reproduction in the coming years based on rising temperatures in the Arctic due to global warming. Considering this species, due to faster development and increased growth, they will be able to decrease their mortality rate by reducing their exposure to predators (Culler et al., 2015). So far there is no notice of arboviruses being transmitted via mosquitoes in the Arctic, or more precisely in Svalbard, probably because of current climate and biotic restrictions (Mülleorvá et al., 2018). However, with climate changes come new vertebrate host species, which could head to outbreak of arboviruses (Müllerová et al., 2018). Furthermore, the composition of mosquito microbiome (and vectored pathogens) is greatly influenced by the site of mosquito collection, which directly implies that the hosts environment plays an important role in shaping the microbiota (Muturi et al., 2018). As already mentioned, vector-borne diseases represent a major health problem and microbiota may be a key player in disease transmission. Nevertheless, the knowledge about the pathogen-vector-microbiota dynamics is not yet fully understood (Thongsripong et al., 2018). This knowledge will be essential in order to prevent pathogen transmission (Gao et al., 2019). Not only does microbiome control parasite fitness and transmission effectiveness, but also influences parasite dynamics in hosts as well (Ippolito et al., 2018). One of the most commonly found bacteria in mosquito species is Wolbachia, infecting almost all major mosquito genera like Aedes, Anopheles and Culex (Shaikevich et al., 2019). Wolbachia symbionts influence host biology in many different ways, and recent findings suggest its diversity is probably caused by strain recombination and symbiont transfers (Bogacheva et al., 2019). Beside Wolbachia, Enterobacter species have been commonly found in these mosquito genera as well (Jayakrishnan et al., 2018). Anopheles species usually show presence of Asaia proteobacterium, especially found in Anopheles gambiae, the major malaria vector in the Afro-tropical region (Damiani et al., 2010), while Culex species also show presence of B. sphaericus (Federici et al., 2003). As far as our Aedes genus is concerned, genus Asaia shows as commonly found (Scolari et al., 2019). Apart from Asaia, predominating in

the adult mosquito stage, Burkholderiaceae family dominates in the larvae and pupa stage (Alfano et al., 2019). More analysis of Aedes species microbiota discovered the presence of Elizabethkingia, Chryseobacterium and Wolbachia (Guegan et al., 2018). The presence of these bacterial genera was established in Aedes aegypti and Aedes albopictus species. Other bacteria isolated from Aedes aegypti have been identified as Bacillus sp., Bacillus subtilis and Serratia sp. (Gusmão et al., 2007). Wild Aedes aegypti and Aedes albopictus collected in Madagascar hosted bacteria belonging to Bacillus, Acitenobacter, Asaia, Delftia, Pseudomonas, Enterobacteriaceae and uncultured Gammaproteobacterium (Zouchae et al., 2011). A laboratory colony of Aedes aegypti showed similar results with most abundant genera being Bacillus, Elizabethkingia, Enterococcus, Klebsiella, Pantoea, Serratia and Sphingomonas (Terenius et al., 2012). Interestingly, it has been recently shown that field collected Aedes mosquitoes positive for S. marcescens turn out to be more permissive to dengue virus than those that do not contain this bacteria (Sun et al., 2019). Regarding our results for the microbiome of Aedes nigripes, the major bacteria genera found in our samples were Dietzia, Buttiauxella, Pseudomonas and Sphingomonas. Compared to previous research, Sphinogomonas and Pseudomonas genera occur among Culex, Anopheles and Aedes species, while Dietzia and Buttiauxella appear more often in Aedes species and rarely in the other two. Genus Dietzia has a very similar colony appearance and gram morphology to Rhodococcus and was discovered recently (R.J.Koernet et al. 2009). Based on the results of 16S ribosomal sequencing of two different strains of Rhodococcus maris and few other research, it was proposed that some Rhodococcus species should be reclassified as Dietzia (F.A.Rainey et al., 1995). Dietzia is widely present in the environment and found in soil, deep sea sediment, soda lakes, marine aquatic environments optimally with pH range from 6-10 and in presence of 10% NaCl (A.F.Yassin et al., 2006). Concering genus Buttiaxella, it is a Gram-negative, rod-shaped, non-motile bacterium (Furlan et al., 2018). The genus contains seven identified species which appear to be mainly present in mollusks like snails and slugs, fish, clams and squids, but also in soil and drinking water (Ferragut et al., 1982). Confirmed Butiauxella species are: Buttiauxella ferragutiae, Buttiauxella gaviniae, Buttiauxella brennerae, Buttiauxella izardii, Buttiauxella noackiae, Buttiauxella warmboldiae, Butiiauxella agrestis (Muller et al., 1996). It seems genus Butiauxella is rarely found in previously studied Aedes species so far, therefore, given the fact that this genus showed as second most abundant in our samples and that Aedes nigripes is generally very poorly examined, it is possible that genus Buttiauxella is characteristic of Aedes nigripes species microbiome. Other genera found in our samples include: Esherichia-Shigella, Geobacillus, Acinetobacter and Mycobacterium. These genera are also commonly found among the three most medically important mosquito vectors: Aedes, Anopheles and Culex genera (Junglen et al., 2009). Although Aedes nigripes is a fascinating and interesting species, it is seldomly described and investigated, most probably because it occupies the northern part of the globe where aquatic and terrestrial ecosystems are not as rich as in tropical regions. Harsh and long winters, fresh and short summers, ice glaciers and low temperatures make it hard to believe that something so fragile and small as a mosquito could ever feed, breed and survive in those conditions. Limited access to breeding sites and a scarce animal ecosystem seem to be

enough for Aedes nigripes to occupy the Arctic and become the most abundant and widespread mosquito species there. What will happen when, with the ongoing climate change, Aedes nigripes spreads further than the Arctic and occupy other habitats and various different regions? Would they adapt to new environments and proliferate elsewhere? Will they eventually encounter hosts or areas where pathogens are spread? Will they be able to act as novel vectors of disease in the future? All these stay as open questions to be answered by further research about this amazing species.

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