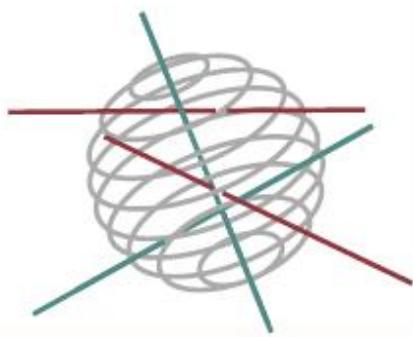




SCIENCE FOR A SUSTAINABLE DEVELOPMENT



Mosquito vectors of disease: spatial biodiversity,
drivers of change, and risk

"MODIRISK"

V. VERSTEIRT, E. DE CLERCQ, W. DEKONINCK, D. DAMIENS,
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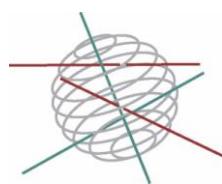
ATMOSPHERE AND TERRESTRIAL AND MARINE ECOSYSTEMS



TRANSVERSAL ACTIONS



SCIENCE FOR A SUSTAINABLE DEVELOPMENT (SSD)



Biodiversity

FINAL REPORT

MODIRISK

Mosquito vectors of disease: spatial biodiversity, drivers of change, and risk

"MODIRISK"

SD/BD/04

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ACRONYMS

AVHRR	Advanced Very High Resolution Radiometer
COI	Cytochrome oxidase I
DNA	Deoxyribonucleic acid
GPS	Global Positioning System
IIS	Microsoft Internet Information Services
ITMA	Institute of Tropical Medicine (Antwerp)
ITS2	Internal Transcribed Spacer 2
LST	Land Surface Temperature
MaT	Maximum Apparent Temperature
MGRS	Military Grid Reference System
MODIS	Moderate Resolution Imaging Spectroradiometer
MS SQL	Microsoft Structured Query Language
mtDNA	Mitochondrial DNA
NOAA	National Oceanographic and Atmospheric Administration
PBS	Potential Breeding Site
PCR	Polymerase Chain Reaction
PDA	Personal Digital Assistant
PHP	Hypertext Preprocessor
RBINS	Royal Belgian Institute of Natural Sciences (Brussels)
RDA	Remote Data Access
rDNA	Ribosomal DNA
ROK	Republic of Korea
SBS	Small Business Server
UCL	Université Catholique de Louvain (Louvain)
UTM	Universal Transverse Mercator
WUR	Wageningen University and Research Centre

SUMMARY

Knowledge of the taxonomic and functional biodiversity of both endemic and invading vector mosquito species as well as the factors driving change, is missing in Belgium. Acquiring this knowledge is an essential step towards understanding current risk and preparing for future treads. Therefore the objectives of the project MODIRISK (Mosquito vectors of disease: spatial biodiversity, drivers of change, and risk) were (1) to inventory endemic and invading mosquito species in Belgium considering environmental and taxonomic elements of biodiversity, (2) to assess the population dynamics of endemic and invasive mosquito species and their interrelationship (3) to model mosquito biodiversity distribution at a one km resolution in the Benelux, and (4) to disseminate project outputs to the scientific community, end users and the general public. During the first phase (years 2007-2008), the project focused on the inventory activities; setting-up laboratory experiments for studying life history traits of *Culex pipiens* in relation to temperature and the first selection of models based on the field results. Whilst during the second phase of the project (years 2009-2010) the focus was on the spatial model building and validation, on the longitudinal study and dynamics of selected indigenous and exotic species that were found during the inventory of the first phase and on more population genetic driven research.

Sampling design and biodiversity outcomes of the mosquito inventory.

The cross-sectional field survey was conducted in 2007 and 2008 by use of a network of CO₂-baited Mosquito Magnet Liberty Plus traps throughout Belgium in three key habitats. These habitats (urban, agriculture and nature) were selected based on the Corine database. Twenty seven traps operated simultaneously (nine per team, three teams leaded by three partner institutes). Each trap operated seven days on one study site after which it was placed on the next study site. During the inventory 936 randomly selected sites were selected of which 97% were sampled. Additionally sites in import risk areas were sampled to evaluate the presence of exotic mosquito species in Belgium. A MODIRISK website, a palm-to-web tool and a database were developed which serve now as an example to implement a cluster of spatial mosquito sampling and modelling projects in several European countries as part of the IAP program of ESA (European Space Agency). After two years of intensive inventory and based on as well morphological as molecular identification 23 Culicidae species belonging to 5 genera were found. The number of species caught is close to the expected number of species (about 27 species) possibly present in Belgium. Two exotic species were found, *Aedes japonicus japonicus* in the province of Namur and *Aedes (Finlaya) koreicus* in the province of Limburg. Biodiversity differed amongst the sampled habitats. Diversity indices indicate the highest richness in natural habitats, although also urban areas score well (which could have important implications on transmission risks). There were lower in rural areas.

At the Royal Belgian Institute of Natural Sciences (RBINS) about 1400 mosquito-specimens from the Belgian collection of the Entomology Department were screened and if needed, added to the collection. All these and previous Belgian records were added to a newly established database CULIBEL. This database will be integrated into the Belgian Biodiversity Platform and will be kept updated by RBINS. Both RBINS and MODIRISK collections were used to compare recent and old data distributions (UTM 10x10km squares). A trend criterion was made of well surveyed grid cells and a decline of diversity near larger cities could be

observed. An increase of distribution area was observed for several potential mosquito vectors having the capacity to use artificial containers as breeding sites. For 23 species there is a relative change in distribution area in 56 (10x10km) grid cells.

A molecular archive was constructed of all collected species based on the DNA barcoding region at the ITMA. Moreover, a larval molecular identification assay was developed to rapidly detect and identify possible invasive species.

Modeling indigenous mosquitoes

A spatial data archive of low resolution remote sensing data was developed. Eco-climatic zones were identified using an unsupervised k-means clustering. In the first phase, the objective was to determine whether the data extracted from the MODIS data-series was useful for the prediction of mosquito distribution. The distribution models were tested on two species namely *Anopheles claviger* and *Aedes cinereus/geminus*, for which a training sample was selected. The explanatory variables, composed of 28 data layers were standardised to facilitate model output interpretation. The stepwise regression procedure was successful in ruling out a considerable number of explanatory variables without decreasing the predictive value of the models. Using this model a distinct distribution over the study area was obtained for both species showing that eco-climatic variables are paramount to explain this variation. In the second phase models were generated for those species present in more than 20 sampled sites. Fourteen species fit these conditions and were further analyzed using random regression "forest" statistics. Finally, the model was used to make a map of environmental suitability (ranging between 0 and 100 %) for the entire area of the BeNeLux. Field validation in Belgium of the preliminary models was done according to the same protocol as for the inventory during July-October 2009 in 73 sites (97% of planned sites) and May-August 2010 in 74 sites (99% of planned sites). During the same period (in 2009 and 2010) a similar validation took place in the Netherlands in respectively 53 and 55 sites. The north-east of the country showed a high environmental suitability for a majority of the considered species, such as *Aedes cinereus*, *Anopheles maculipennis*, *Ae. vexans*, *Culiseta annulata*, *Coquillettidia richiardii*, *Ae. cantans*, and *Ae. punctor*. In the southern part of the country a wedge-shaped band of high environmental suitability was noticed.

Ecology, biology and population genetics of selected indigenous and exotic vectors

During the second phase of the project, longitudinal studies were conducted on selected indigenous and exotic species found during the first phase of the project. RBINS followed a severe nuisance problem of *Anopheles plumbeus* in Torhout after complaints to the MODIRISK project of some of the inhabitants of that area. It was found to be present at very high numbers, being aggressive to humans and breeding in old abandoned piggeries. This problem of high nuisance has also been reported in other provinces (Antwerp and Liege). A similar development of *An. plumbeus* was observed in the Netherlands (WUR). The population of *Aedes japonicus* at Natoye was surveyed by UCL. The results of as well morphological as molecular studies showed that the species is numerously abundant at the second hand tyre company but can also colonise natural breeding sites, like tree holes, nearby. Furthermore, this species competes with *Culex pipiens*, delaying its development until *Ae. japonicus* starts to decline. Lab experiences performed by UCL using safe insectory

facilities of EID (Montpellier) confirmed the high competition of *Ae. japonicus* larvae in the presence of *Cx pipiens* larvae for food. Molecular studies showed that in 2009 three separate gene pools were present, evincing that probably three importation events occurred. However in 2010, mixing of these genetic groups occurred. This may increase genetic diversity and so increase the species invasive potential. Another exotic species, *Aedes koreicus*, found near Maasmechelen was followed by ITMA. This species has previously never been reported outside its natural region of Asia but seemed to be well established in Maasmechelen but still not spreading. *Ae. koreicus* is not readily attracted to the currently used traps and was not found to be aggressive to humans; however the species is, in the former USSR, suspect to be a vector of Japanese Encephalitis. Furthermore the species colonises both natural and artificial habitats which can encompass a risk of spreading in Belgium.

A laboratory colony of *Culex pipiens* was set-up at Université Catholique de Louvain (UCL) to study the impact of temperature on life history traits of the most wide spread mosquito species from Belgium. Males emerged 1,2 to 5,4 days before females and time between pupation and emergence increased with low temperatures. Although not excessive, larvae reared at low temperatures (T15, T11) gave bigger adults than the larvae reared at high temperatures (T28, T20, T35 and T40). Furthermore the results show that temperature influences the time required to obtain copulation in *Cx pipiens*. The parameter temperature has a great influence on development and mating activity of *Cx pipiens* and the results of these laboratory tests will be included in transmission models.

Members of the *Anopheles* genus as well as the population structure of *Culex pipiens* were genetically analyzed due to their potential as indigenous vectors. Different molecular methods were applied, tested and compared. For *Anopheles* species rDNA marker seem to work best, which coincide with literature data; although some inconsistencies occurred. Using microsatellite markers two genetic groups were observed in the *Culex pipiens* individuals tested, indicating the possible presence of two described forms: *Cx pipiens pipiens* and *Cx pipiens molestus*. Its presence as well in same habitat as time frame have implications on vector monitoring and transmission risk particularly when hybrids are present acting as potential bridging vectors between bird and human populations.

Overall, the results of the modeling indicate that a stratified random sample is a good methodology for selection sampling locations for mosquito monitoring. However for future monitoring this strategy is labor intensive and costly thus it was necessary to analyze how the sampling size could be reduced without reducing the accuracy of the model outputs. Based on a simulation of four indicator species, one trap per 300km² resolution (in stead of one trap every 30km² used in present study) would be appropriate for further monitoring. Based on these four indicator species, hotspots can be identified for intensifying trapping. These findings will make future monitoring more efficient and less expensive.

Key words: inventory, Belgium, The Netherlands, indigenous and exotic species, modeling, sampling strategy, biodiversity, vector population studies, molecular identification, DNA barcoding, *Aedes japonicus*, *Aedes koreicus*, *Anopheles plumbeus*, *Anopheles maculipennis* s.l., *Culex pipiens*

1 INTRODUCTION

Increased globalization, landscape management and changing socio economic behavior create suitable conditions for the (re)emergence of vector-borne diseases in Europe. It was estimated that during the decade 1990-2000 nearly a third (29%) of the recorded events related to emerging infectious diseases were due to vector-borne diseases (Hendrickx & Lancelot 2010). Furthermore, Reiter (2010) stated that especially the importation and establishment of vector-borne pathogens that have a relatively low profile in their current habitat is a serious danger to Europe and throughout the world. Of these, mosquito-borne diseases are prime candidates. Recent outbreaks of West Nile virus in Romania and Greece (Papa et al. 2010, Sirbu et al. 2011) and autochthonous transmission of Dengue and Chikungunya in southern France (La Ruche et al. 2010), indicate the existing danger of local transmissions. A key factor in this increased susceptibility of Europe to vector borne diseases is the rapidly expanding transportation of humans and goods. Due to this intensification of worldwide trade and tourism which increases indeed the likelihood of human-mediated introduction of organisms, posing a risk to biodiversity, economy and human health (Tatem et al. 2006, Lines 2007). The probability of a country or region for bioinvasions appears generally to be correlated to the extent of international trade it conducts (economic variable) and to its national wealth and human population density (demographic variable) (Pysek et al. 2010). In the last decade, dramatic increase in traffic has been observed between eastern Asia and Europe and North America (Tatem 2009). Due to the mix of economic and demographic influences combined with the further increasing speed and pervasion of modern transport networks, global trade, transport and tourism are becoming more and more pivotal in the spread of vectors and the infectious diseases they transmit (Wilson 1995., Wilson et al. 2009, Pysek et al. 2010). There are three possible scenarios that may influence the risk of transmission and outbreaks of arbovirusses: the import of an exotic species that can transmit an arbovirus, the import of an arbovirus that is transmitted by an exotic established mosquito, the import of an arbovirus that is transmitted by indigenous species. Of all mosquitoes worldwide, Aedines seem to have the highest invasive potential as the eggs of most species tolerate considerable periods of desiccation thus surviving long transports (Reiter & Sprenger 1987). Furthermore, many *Aedes* species prefer small and often man-made containers as oviposition sites, including used tyres and 'lucky bamboo' containers (Eritja et al. 2005), which are frequently moved across international borders. In southern Europe, past and recent accidental importations of mosquito vector species such as *Aedes (Stegomyia) aegypti* (L.) and *Aedes (Stegomyia) albopictus* (Skuse) have created suitable conditions for local transmission of arbovirusses (Christophers 1960, Eritja et al. 2005, Angelini et al. 2007, Fontenille et al. 2007, Angelini et al. 2008). *Aedes* species imported into northern Europe include *Ae. albopictus* (Schaffner et al. 2004), *Aedes (Finlaya) japonicus japonicus* (Theobald) (Schaffner et al. 2009, Versteirt et al. 2009), and *Aedes (Ochlerotatus) atropalpus* (Coquillett) (Scholte et al. 2009). Besides these *Aedes* species, the invasive potential of *Culex* and *Anopheles* species should not be underestimated, although it seems that these introductions were more frequently in past eras. *Culex pipiens* was introduced into the USA more than hundred years ago (Kesavaraju et al. 2011) and *Culex*

quinquefasciatus was introduced into Australia and New Zealand around 1848 (Lounibos 2002), both established successfully. Many *Anopheles* species were imported into North and South America due to increased trade in early 1900. For example, the African malaria vector *Anopheles gambiae* s.l arrived from West Africa in 1930 and was rapidly established and spreading into northeastern Brazil (Lounibos 2002). Larvae or adults of this anopheline are believed to have traveled by air or fastpassenger ship from Dakar, Senegal, to Natal, Brazil (Lounibos 2002), where the first malaria epidemic attributable to the species occurred in March–May, 1930. Although malaria was endemic in northeastern Brazil, the native anopheline vectors were less efficient transmitters compared with the highly anthropophilic and endophilic *An. arabiensis*. However Anopheline mosquitoes, among which are the vectors of malaria, are less successful in colonization new regions.

Not only importation of a vector species can cause problems, also the introduction of pathogens poses a risk for human and animal health. Global travel has grown continuously during the last 2 centuries, with especially a massive increase in the last 50 years (www.world-tourism.org). Worldwide tourism allows humans to interact with microbes and spread pathogens to new locations and populations (Mavroidi 2008). This massive increase in international travel has reduced or even eliminated natural geographic barriers and increased the spread of infectious diseases worldwide. In addition, the world population continues to grow rapidly causing more and more people to live closer together, which again increases the risk of spreading diseases. Apart from humans travelling, the transport of infected animals can cause the same problems. During the Bluetongue outbreaks in Europe, the transport of sheep and cattle became very restricted by the European Commission to avoid outbreaks in new locations. The outbreak of WNV in the USA in 1999 started in New York City and it is assumed that the pathogen was imported along with an exotic bird in a zoo nearby (Lanciotti et al. 1999). Globalization is therefore potentially a far greater challenge to public health in Europe than any future changes in climate would be.

Besides abiotic risk factors and the hazards posed by invading mosquitoes, biotic factors linked to indigenous and exotic species can also play a role in possible transmission and outbreaks of mosquito borne diseases. For instance, spatial distribution of adult mosquitoes is associated with various environmental and climatic factors. Alterations of these factors can produce significant differences in the distribution of adult mosquito populations which may have important implications for current risk on (re)emergence and epidemiology of vector-borne diseases and the implementation of control strategies. Climate can influence the host, pathogen and vector populations in several ways and thereby increase or decrease the incidence or prevalence of arboviral diseases. For example, it can cause a shift in the geographical distribution and the density of host and vector populations, potentially bringing the two in closer contact and increasing the prevalence of an infection (Mills et al. 2010). Recurring year to year fluctuating in day and night temperature and precipitation will have an impact on oviposition strategies, larval development and overall larval and adult survival, thus effecting directly population density of many vector species in Belgium. Temperatures can also have profound effects on the development of pathogens and pathogen loads in arthropod vectors (Sutherst 2004, Gage et al. 2008). However, knowledge of the taxonomic and functional biodiversity of both indigenous and invading vector mosquito species as well as the factors driving change is missing in Belgium. Except for a paper published in 2004,

recent information on the presence and distribution of indigenous and invasive mosquito (Diptera: Culicidae) species in Belgium is lacking. Before that, the sole published information relates to three studies. In the beginning of the previous century the first species list on Belgian mosquitoes was published after extensive collection campaigns of insects all over Belgium (Goetghebuer 1910). This list was extended and a new publication on Culicidae present in Belgium appeared in 1925 (Goetghebuer). In the 1940s the presence of malaria mosquitoes and associated malaria transmission risk was studied at several occasions in Belgium (Rodhain and van Hoof 1942, Rodhain and van Hoof 1943, Rodhain and Van Mechelen 1944) and during the early 1950's mosquito nuisance was investigated around the city of Antwerp (Wanson 1952). Albeit a large collection is present at the Royal Belgian Institute of Natural Sciences (RBINS) many of this collected material was never identified. In 1991 a checklist of Belgian Culicidae was created comprising 24 species present in the RBINS collection (Gosseries and Goddeeris 1991) collected mainly between 1910 and 1960. Moreover, records are sometimes clustered in space and time, as, for example, between 1940 and 1950 mosquitoes were mainly collected around Ghent and Brussels (Dekoninck et al. 2011). Based on all this literature information, the identification key for European mosquito species (Schaffner et al. 2001) record 26 species possibly present in Belgium, although a total of 30 different species were mentioned in all previously published lists, some were discarded due to the low probability of occurrence in Belgium.

Acquiring detailed knowledge on distribution and population dynamics of Belgian mosquitoes is an essential step to anticipate, prevent or prepare for the establishment and spread of vector-borne diseases. National presence-absence maps are the first step for understanding current risk and preparing for future threats and one of the main objectives of this study was to compile predictive modeling maps which give an overview of the current distribution of the Belgian mosquito fauna. Next to distribution data, information on bionomics and population dynamics of mosquito species is crucial in the evaluation of the possible risk and in the preparedness to propose management politics of mosquito species (Parmesan and Yohe 2003, Zell 2004).

The inventory done in the framework of the MODIRISK project is based on a random (statistical) approach that is designed for model building. This is unique in Europe (and even in world) since most models are based on historical records. Based on the experience gained during MODIRISK a cost-effective sampling strategy will be designed for use in follow-up and similar studies. Modeling will mainly assist in defining the minimal field sample needed to produce acceptable distribution maps, and how these samples are best distributed in space. Furthermore it contributes to the development of state of the art scientific tools integrating collection-based information technology at various resolutions with geographic mapping efforts and remote sensing driven continuous distribution models. This enables to better describe the spatial distribution of mosquito biodiversity, and to understand how these are organized in communities and habitats.

In this report we describe the objectives and the applied methodology of the MODIRISK (Mosquito vectors of disease: spatial biodiversity, drivers of change, and risk) project and we discuss main results and achievements to formulate in the end recommendations for preparedness and responsiveness. The project directly contributes to discovering biodiversity

and monitoring/predicting its changes, and actively prepares to address issues such as the assessment of impacts of biodiversity change with particular reference to new invasive mosquito species and the risk to introduce new pathogens. An improved understanding of the biodiversity of mosquito vectors is an essential step towards an improved understanding of the ecology of the diseases they transmit. Looking at Europe, vector borne diseases are becoming more and more a topical issue that we cannot simply ignore.

MODIRISK fits in the science plan of the global initiative Diversitas, which was one of the main drivers of the 'Research programme Science for a Sustainable Development (SSD)'. MODIRISK directly contributes to discovering biodiversity and monitoring/predicting its changes, and actively prepares to address issues such as the assessment of impacts of biodiversity change with particular reference to new invasive species and the risk to introduce new pathogens (impact on health). These are two of the three key topics, respectively addressed in the three 'core projects' of Diversitas.

The filling of an essential knowledge gap in Europe, and the expansion of model outputs through linking up with a project in The Netherlands, enables the project to produce more robust results and to prepare better for later expansion of activities in Europe. MODIRISK plays its role as interplay between newly gained insights and the end-users. MODIRISK participated to the risk assessment group of the Scientific Institute of Public Health concerning the presence of the exotic vector species *Aedes. j. japonicus* and *Ae. koreicus* and participated to meetings of the European Centres for Disease Prevention and control Control (ECDC). Links were made with the Belgium Forum on Invasive Species by participating to the discussions on the 'Guidelines for environmental impact assessment and list classification of non-native organisms in Belgium' and by acting as member of the scientific committee of the 'Science Meeting Aliens" conference on biological invasion (11th of May 2009). MODIRISK was consulted by the AGORA project on 'set-up of monitoring of potential effects of climate change on human health and on the health of animals. In January 2009 MODIRISK organised a workshop on vector control in Belgium bringing together persons potentially involved in the decision making process on vector control, other stakeholders and interested persons. A strong plea was made to set-up entomological surveillance to follow the situation and to evaluate the spread of the exotic species in Belgium. Moreover a flow chart of competence and responsibilities of the different authorities potentially involved in vector control should be established. Members of the MODIRISK project attended to several meetings and workshops on national and international level. Moreover MODIRISK was asked to take part in a number of national (VIRORISK, CULIMON, BC42W) as international (VBORNET, Vecmap) projects. MODIRISK has become the contact for numerous questions and problems relating biting insects especially mosquitoes and biting midges.

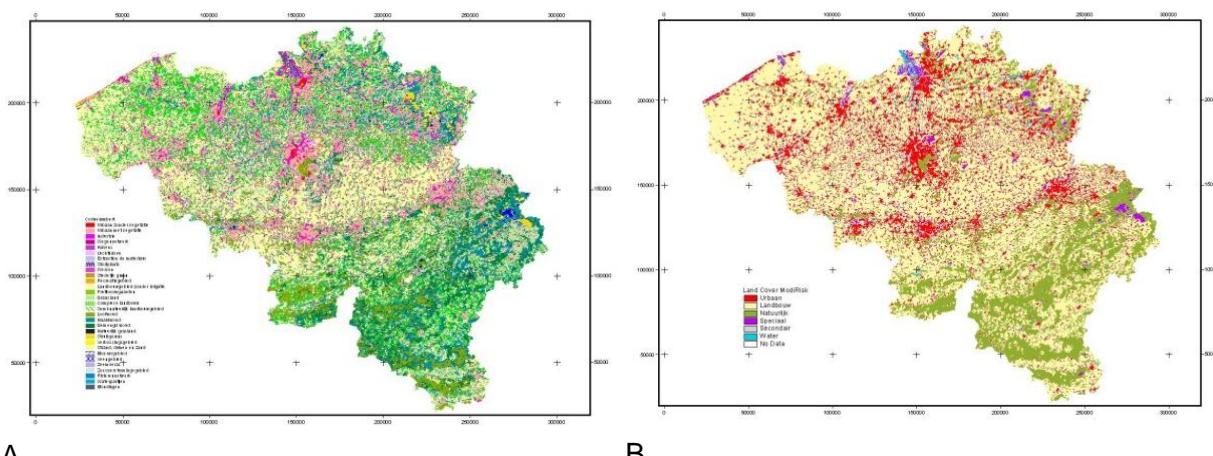
The report is subdivided into different important themes as biodiversity of indigenous and exotic mosquitoes in Belgium and The Netherlands; ecology and population dynamics of mosquitoes in Belgium; biology and genetic population structure of mosquitoes in Belgium and application for surveillance systems in Belgium and Europe

2 BIODIVERSITY OF MOSQUITOES IN BELGIUM AND THE NETHERLANDS

2.1 Sample design of the inventory of Belgian Culicidae

Cross-sectional field surveys were conducted during the first phase of the project to inventorize Culicidae. CO₂-baited traps were used throughout Belgium in a grid-based sampling approach where different habitats in each grid were sampled. One trapping device, Mosquito Magnet Liberty Plus which is a high performance CO₂-baited trap was used. In a recent study it outperformed both in number of specimens and number of genera collected compared to seven other trap systems (Dennett et al. 2004). Furthermore it is the only commercial available trap type that allows a certain autonomy which was necessary for our trapping scheme. This trap runs on propane gas which is converted into CO₂, which is a good attractant for most mosquitoes. This CO₂ leaves the trap through a central tube with a constant flow (500 ml/min). Attracted insects are drawn into the apparatus and trapped in a small net. Mosquitoes are killed by placing the nets in the freezer at -20°C. This trap was also employed in the Dutch studies.

Using the Corine Land Cover (2000) classification (NGI, 2004), potential mosquito habitats were delineated. The Corine Land Cover Classes were regrouped in 6 classes. These classes are shown in Figure 1.



A

B

Figure 1. (a) Corine Land Cover Classification and (b): Aggregated Land Cover Classes

This data layer was overlayed with the Military Grid Reference System (MGRS) which is used internationally for species mapping such as mammals (Amori et al. 2002) and birds (Hagemeir and Blair 1997). The MGRS is an extension of the UTM system. Across Belgium, 312 10x10km MGRS cells are identified. Per cell an average of three points is to be sampled, thus the total number of sample points amounts to 936. Per aggregated class the number of points assigned was proportional to its total surface (Table) and each point received a random set of X and Y coordinates.

Table I. Number of samples per stratum

Class	Pixels	Percentage	Samples
Urban	769723	17.7 %	173
Agriculture	2514014	57.7 %	564
Natural	888272	20.4 %	199
Specific	56479	1.3 %	13
Secondary	103411	2.4 %	23

Given the random location each point was assigned to a full address, i.e. street, house number, and postal code using the geocoding functionality from ArcView3.2 and based on the geocoding street network data layer (TeleAtlas MultiStreetNet). Each point was initially linked to the nearest street segment (i.e. a segment of a street between cross roads) using a spatial join. The house number was generated randomly within the range of house numbers of that street. If there were no houses in the street segment, and the point belonged to the urban category, the nearest street segment with houses was used. If the point belonged to the nature or agriculture category, it only received a street name and no house number. The survey was conducted over a period of two years with the first set of 250 points sampled in the first half of the season (referred to as 'spring') of 2007, the second set in the second half of the season (referred to as 'summer') in 2007 and the third and fourth set similarly spread in 2008 (Figure 2). No sampling was conducted during winter. In 2009 and 2010 additional samples were taken to validate model outputs (see 3.1).

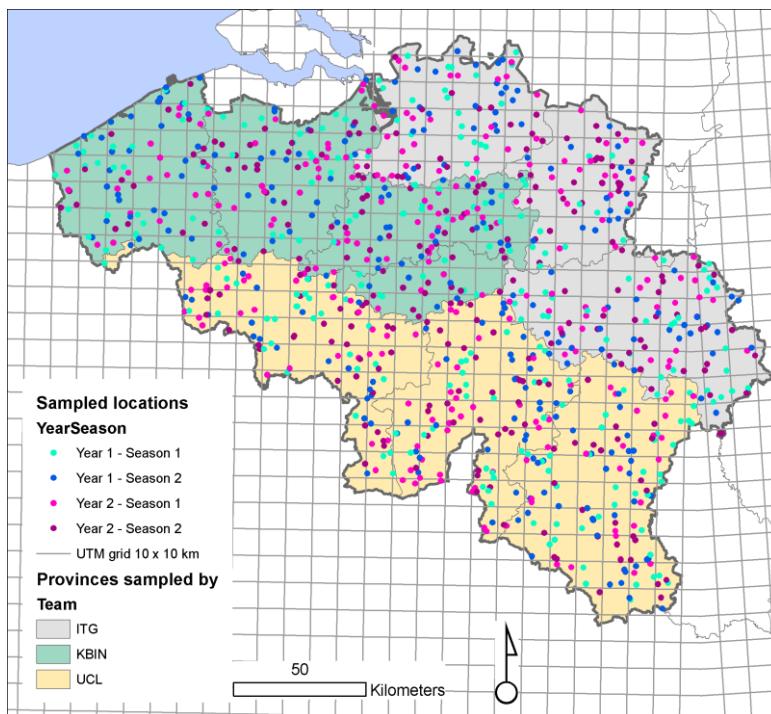


Figure 2: Sampling scheme for the extensive survey of Belgium

2.1.1 Tools developed to facilitate the field work

Websites

A general website was designed providing information on the project and the project outputs (www.modirisk.be). This website provides a link to a site composed of a public part as well as a private part. User access security was implemented to limit the access to the private part. The public part showed the general progress of the field work (<http://modirisk.avia-gis.com/>). Each sample point is colour coded according to its status (not sampled, visited, processed). The private website has two basic functionalities: determining the location of the sampling point and filling out the different forms. Using the sampling point locator (Figure 3), the field teams could identify each sampling point through the interactive map. This map allowed full capabilities of zooming (Figure 4) and panning. The sampling points were colour coded in the same manner as on the public web site. For each sampling point, the field teams could query which team was responsible for trapping, and during which season the point had to be sampled. The high resolution satellite imagery background (Figure 4) enabled a rapid assessment of accessibility and contributed to efficient planning of the field visits.

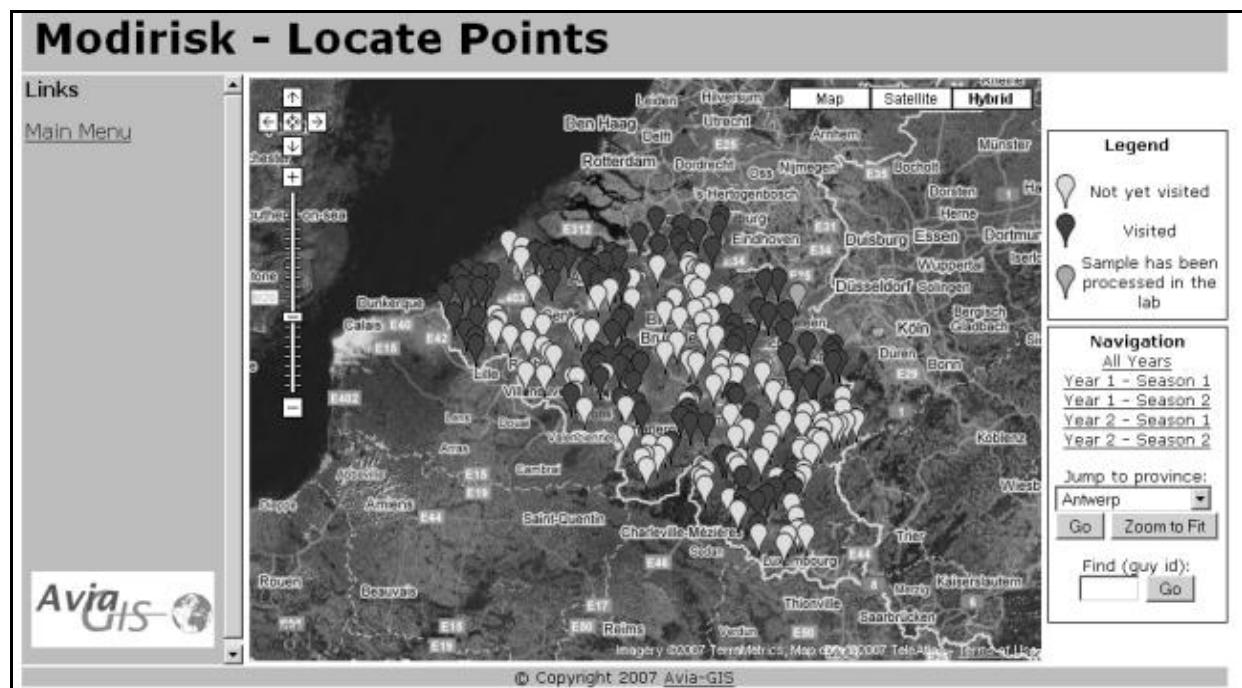


Figure 3. Point locator on private website

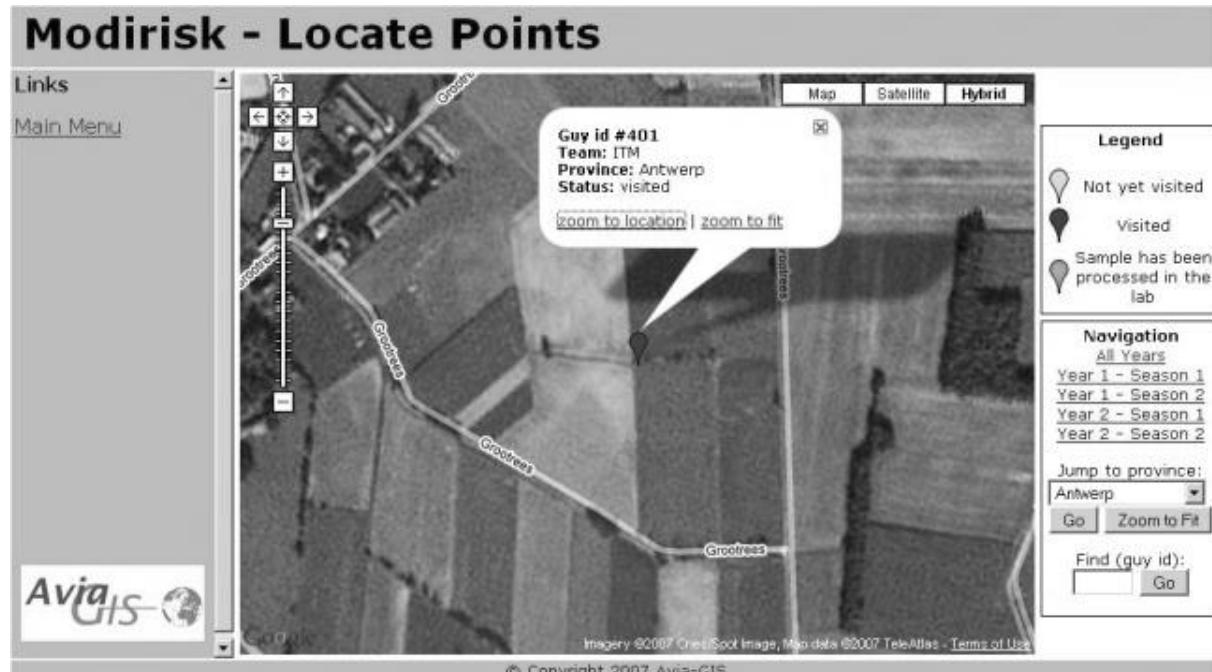


Figure 4. Zooming capabilities

In the administration section, the field teams could complete the sampling forms. The data from the first form was uploaded from the field teams' PDA. The other forms had to be completed manually. Once the data was uploaded or entered, the data base was automatically updated. The status of each sampling point on the web-maps automatically reflected any change in the database. For validation purposes both the website and the database were extended. On the website the listing page was updated in order to avoid mixing of the original points and the validation points.

Software development for PDA

The Fujitsu Siemens LOOX N560 was selected (after comparison with 29 others) because of its high quality and the respective performance of its screen, processor and memory. The PDA was running Windows Mobile 5 and had a built-in GPS. The field form was implemented on the PDA. All functionalities were individually tested. During the test phase, the software was further refined and the user interface was optimized. A manual was written for the software, and uploaded to the main private MODIRISK website. Permanent on-line help and troubleshooting was available to all MODIRISK partners. The software downloaded the coordinates from the central database server to the MS SQL Server Mobile database on the PDA. The user could query and edit the data, and all changes were uploaded after the field visit directly in the central MS SQL Server database.

Database setup

The database server uses Windows Server 2003 SBS R2 as operating system, and is running IIS with PHP for site development, MS SQL Server for database development and SQL Server Mobile Tools to allow remote access from a PDA. Three types of MODIRISK forms were prepared by the MODIRISK coordinator and adapted during a group session at

ITM: (1) Field form, (2) Morphological identification form, (3) Mosquito storage form. Based on these, relevant tables (Figure 5) were developed by Avia-GIS, implemented in the database, and transferred to the web server.

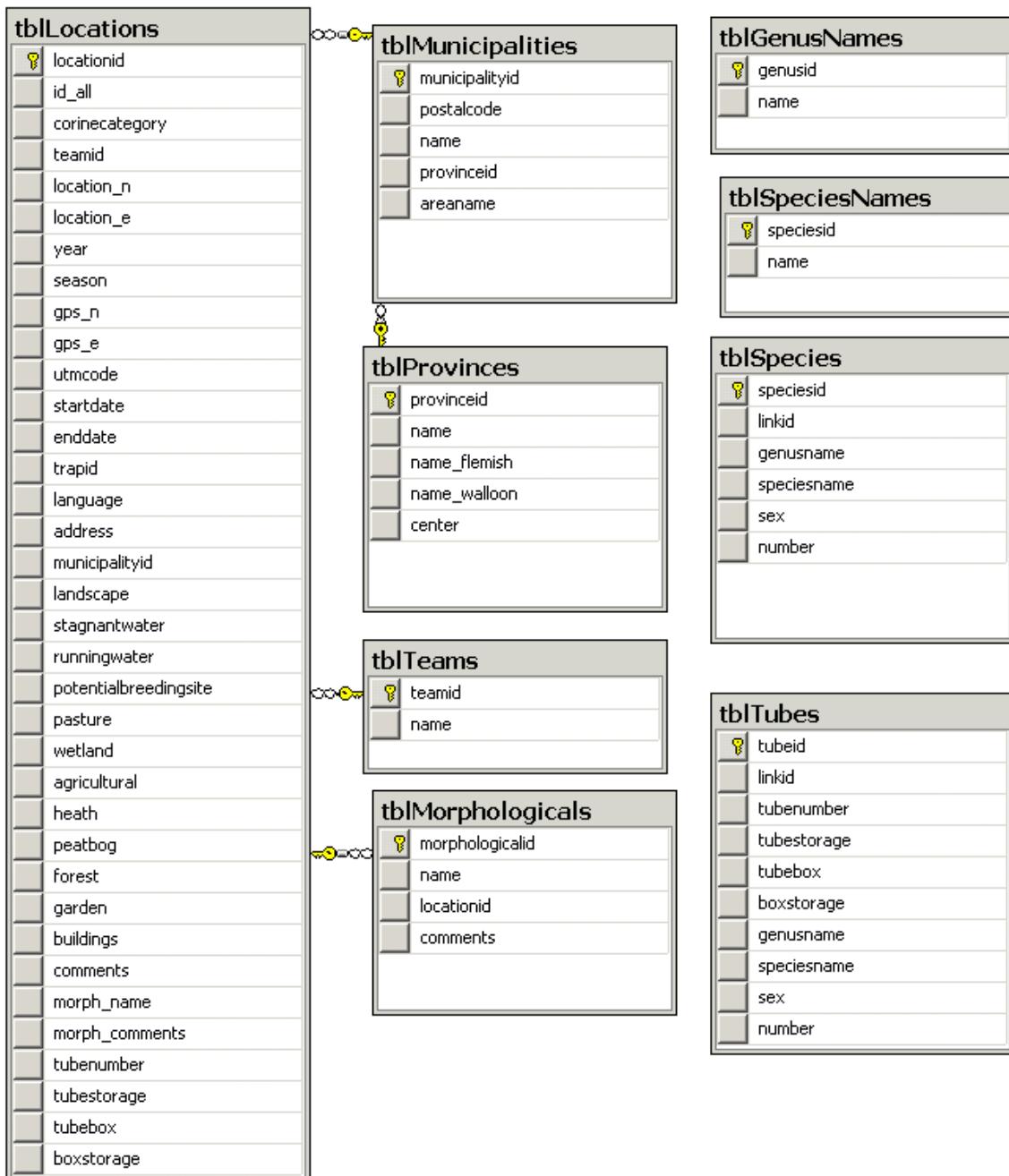


Figure 5. Tables present in the MS SQL Server Database

The database was modified for validation accordingly: the points selected were entered in sample point database and in order to distinguish them from the original data points the ID numbering was in a different ID range.

During the validation phase, forms for identification and validation were edited and added to the private website of MODIRISK. The identification forms allowed the users to fill out data with respect to the morphological identification of species collected in the trap (Form 4), the

morphological identification of the tube content of the collected adults (Form 5) and larvae (Form 6). The database was adapted to allow the storage of the data coming from these 3 new forms.

2.2 Spatial distribution and biodiversity of indigenous and exotic mosquitoes in Belgium

2.2.1 Mosquito sampling and morphological identification

Mosquitoes were sampled from May till October 2007 and 2008 (inventory) and from August till October 2009 and May till August 2010 (validation) according to the sampling design explained above. Three teams were involved in the field collections namely, ITM, RBINS and UCL. In order to standardize the field work a written standard protocol was elaborated by the ITM in cooperation with the other field teams.

Before the implementation of the field work a short training in the use of the Mosquito Magnet Liberty plus trap was organised from 3-4 April 2007 at Wageningen University. During the field work, twenty seven traps operated simultaneously (9 by team). Each trap operated seven days on one study site after which it was placed on the next study site. During the first phase of the project (inventory), field work was done on Monday, Tuesday, and Wednesday: each day three traps were emptied and replaced. The remaining days were used for the organisation of the field work and the morphological identification of the collected mosquitoes. During the second phase of the project (validation), each field team was free to organise the field work as suited.

Morphological identification was done mainly using the electronic identification key of Schaffner et al. (2001) and the paper key of Becker et al. (2003). Data were stored into the web based data base as described above.

Project staff (2 persons ITM, 2 persons RBINS, 1 person UCL, 1 person WUR) was trained during two sessions in state of the art taxonomic identification using morphological techniques based on reference collection from an expert in European morphological mosquito identification and field collected mosquitoes. Moreover, in July 2007 and January 2009 a quality control was done by this international expert. All teams performed well and improved their identifications skills during the training (from 88% accuracy before the second training to almost 100% at the end of the second training). Further verification of performed identification (validation) was done internally.

2.2.2 Molecular identification of the *Anopheles maculipennis* complex

Members of the *Anopheles maculipennis* complex were identified to species level using a specific designed PCR-RFLP method (Nicolecu et al. 2004) of the Internal Transcribed Spacer 2 (ITS2) region. DNA extraction was performed using the protocol described by Collins et al. (1987) after which the ITS2 region was amplified using primers described by Collins and Paskewitz (1996). Consequently the positive amplification products were digested using CfoI restriction enzyme (Roche Molecular Biochemicals Ltd, Sussex, England). Restriction fragments were visualised on a 3% agarose gel. A selected number (5

of each species) of positive ITS2 PCR amplifications were send for sequencing (Genoscreen, Lille, France).

2.2.3 Data analysis

Input data was analysed at the main Corine habitat. All traps used in sites belonging to one of the five defined main habitat groups were treated as a single input point to test habitat effect. Alpha diversity was calculated using three different indices. Simpson's index of diversity, $1 - D = 1 - \sum [n_i * (n_i - 1) / N * (N - 1)]$, where n_i is number of the i th species and N is the number of individuals in the studied habitat, as a measurement for the probability that two randomly selected individuals in an area belong to different species. The closer 1-D is to one, the more diverse the habitat is. Shannon-Wiener index ($H' = - \sum p_i * \ln p_i$, where p_i is the proportion of the i th species in the studied habitat) was used as a measure of community heterogeneity (Krebs 1989); evenness ($E' = S / \ln(H')$, where S is the total number of species) calculates how individuals are distributed among species habitat. Rarefaction based estimates were calculated using EcoSim (Gotelli & Entsminger 2001) to estimate and compare the relative abundance and the density of mosquito species among habitats. The use of rarefaction allows comparison of the number of species in samples of different sizes by limiting the sample to the smallest size in the set of populations and calculating the species richness. Individual based rarefaction curves were created in GraphPad.

2.2.4 Spatial distribution and mosquito diversity (2007 and 2008)¹

Based on the above described sampling strategy 936 sites were randomly identified in three key habitats (urban, agriculture and natural); 97% of these were effectively sampled. Additionally 45 import risk areas were included in the inventory of which 27 areas were located in natural habitat (IRA-nature,in total 37 traps) and 18 companies (IRA-industry, total 24 traps). The import risk areas for exotic mosquitoes included zoos, safari parks, second hand tire import/storage companies, lucky bamboo importers, harbours and airports. Risk areas for import of pathogens included protected areas involving presence of large numbers of migratory birds. Table II summarizes the actual number of sites sampled in each category.

Table II Overview of the number of study sites.

Habitats	Selected	YEAR 1 (2007)		YEAR 2 (2008)	
		To be done	Done	To be done	Done
Urban	173	82	81	91	90
Agriculture	564	283	280	281	277
Natural	199	101	90	98	94
Import risk areas	42	21	26	21	37

¹ Note: data gathered during field work validation in 2009 & 2010 has been used for model validation (see xxx) and is included in this part of the report

Species diversity, abundance and evenness

A total of 26533 individuals, morphologically belonging to 22 species and 5 genera, were collected and morphologically identified (Figure 6). The most species rich genus in Belgium was *Aedes* whereas *Coquillettidia* was only represented by 1 species. The five most abundant species were *Culex pipiens* (61.62%), *Coquillettidia richiardii* (15.43%), *Aedes cinereus/geminus* (5.04%), *Anopheles claviger* (3.52%) and *Aedes vexans* (2.93%). The high abundance of *Cq richiardii* is however due to one study site, a nature reserve in the harbour of Antwerp, where more than 3700 specimens of this species were collected. The species was caught only in 38 study sites. Interestingly is the general occurrence of *Anopheles* species, mainly *An. claviger* and *An. plumbeus*. Two exotic mosquito species were collected. *Aedes japonicus japonicus*² was found in a second hand tyre company (Versteirt et al. 2009) whereas *Aedes koreicus* was found in a randomly selected site near an old sand mine in nature restoration but close to a recycle company and industrial zone (Versteirt et al) (see also 3.6.).

Eighty individuals of *An. maculipennis* s.l. were collected of which 67 were molecularly identified using the protocol of Nicolescu et al (2004). Forty three individuals belonged to *An.maculipennis* s.s. (64.18%), 24 to *An. messae*. This PCR-RFLP does not make the differentiation between *An. messae* and the recently described and closely related *An. daciae* (Nicolescu et al. 2004). Although positive ITS2 amplifications of *An. messae* were sequenced no clear separation between the latter species could be observed. Both species were especially present in the northern part of Belgium, in 6 sites they occurred sympatric, in 24 other positive sites either one of them occurred (7 sites positive for *An. messae*, 17 for *An.s maculipennis* s.s.).

Combining morphological and molecular identification, a total of 23 species were collected during the inventory. Seen individuals of *Ae. geminus* and *Ae. cinereus* can only be morphological separated with certainty based on the shape of male genitalia and seen little is known on the medical importance of these sibling species, individuals were not further distinguished.

The taxonomic biodiversity differed among the three main habitats. A large number of species has been found in the import risk areas whereas it only represented a small portion (6-7%) of the sample sites. Most species (21) were caught in the IRA-nature habitat (4% of the study sites) followed by rural (56% of the study sites), and nature habitat (20% of the study sites) where respectively 20 and 19 species were collected. In sites classified as urban, representing 17% of the study sites, 16 species were collected; lowest number of species were collected in IRA_industry sites (Table III). Total abundance was highest in rural sites, largely due to a high number of *Cx pipiens* individuals (>10 000); whilst the mean value was actually the lowest (20,97 individuals per trap). Furthermore, individual based rarefaction

² Reinert, (2000) divided the genus *Aedes* Meigen into genera *Aedes* and *Ochlerotatus* (Lynch Arribalizaga) on the basis of "consistent primary characters" and supplemental features. *Ochlerotatus* was elevated to generic rank and was further divided into two sections based on features of the fourth-instar larvae and pupae. However the controversy surrounding this separation has left non-taxonomists in doubt. The authors would follow in this article the taxonomy of Reinert (2000). *Aedes japonicus japonicus* is one of morphologically similar subspecies originating from Japan, Korea, Taiwan, eastern China and Russia, but seen the other species do not occur here we will hereafter use *Aedes japonicus* to refer to this species.

curves suggest that, for equal sample size, species richness in rural habitats is only slightly lower than in habitats with highest species richness (Figure 7). Species diversity (H' + Simpson) and evenness (E') were both higher in nature and IRA_nature sites than in other, which can also be observed by the rarefaction curve. Individual based rarefaction curves suggest that for equal sample sizes, species richness might be slightly higher in nature and IRA_nature than in urban and rural areas; lowest species richness could be expected in IRA-industry although the rarefaction curve of this latter habitat is still far from reaching a plateau. Half of the (morphological) identified species were shared amongst habitat.

Looking at the species occurring in all main Corine habitats, eight species seem very common in Belgium: *Ae. cantans*, *Ae. cinereus/geminus*, *An. claviger*, *An. plumbeus*, *Cq richiardii*, *Cs annulata*, *Cx pipens* and *Cx torrentium*. Although these species are found over a large range of spatial divisions, some species were more collected in one habitat. *Anopheles claviger* was especially found in sites classified as rural (44%) or IRA_nature (39%); *An. plumbeus* was mostly present in rural (51%) habitats. *Coquillettidia richiardii* was found in high abundances in IRA-nature habitats (98%). Other species, which are not exclusively linked to one of the spatial levels, show a distinct and sometimes somewhat unexpected preferences. *Aedes vexans*, for example, was especially found in sites classified as urban habitat (78,5%).

Table III. Taxonomic diversity by main Corine habitat

	Main Corine habitat				
	Urban	Rural	Nature	IRA_nature	IRA_industry
Number of sites sampled	153	568	183	37	27
Number of species collected	16	20	19	21	12
Total of specimens collected	3992	12100	2553	6857	1031
Mean number of specimens per trap	26,09	21,30	13,95	185,32	38,19
Species diversity (Simpson)	0,476	0,299	0,765	0,605	0,099
Species diversity (Shannon H')	1,107	0,830	1,951	1,354	0,274
Species Eveness (Shannon E')	0,399	0,277	0,663	0,438	0,110

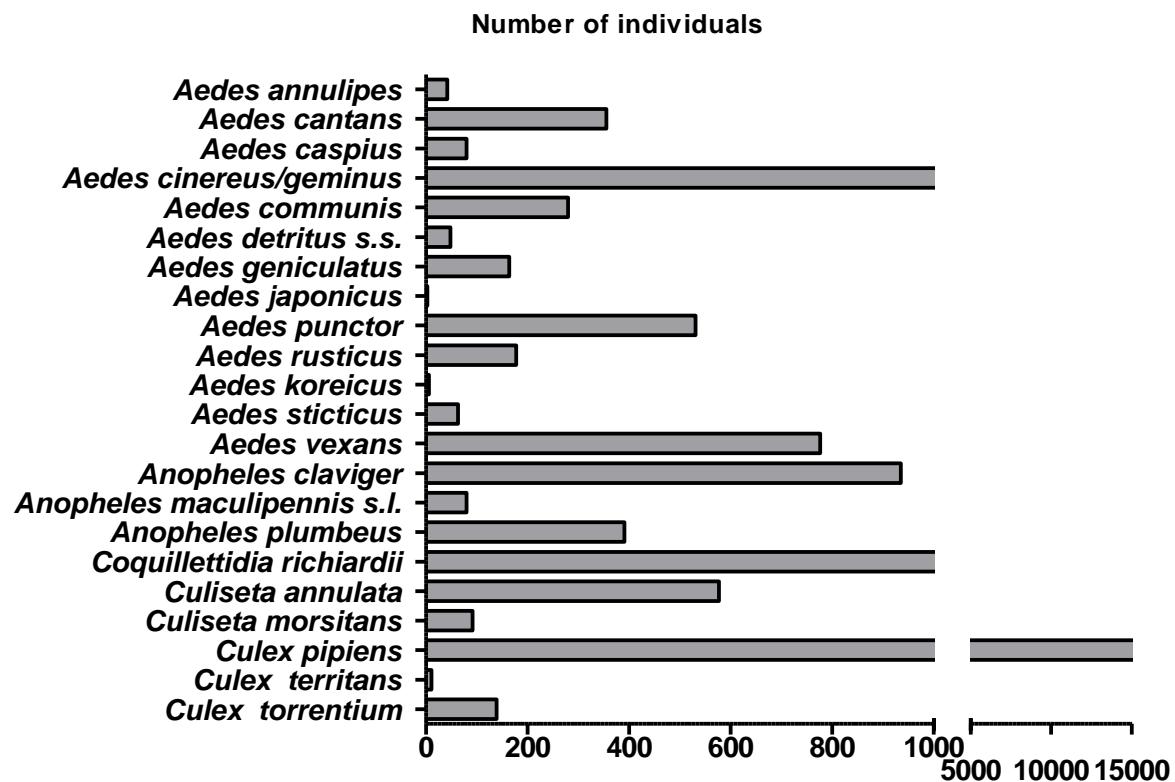


Figure 6 Species collected during the two years inventory study (2007-2008).

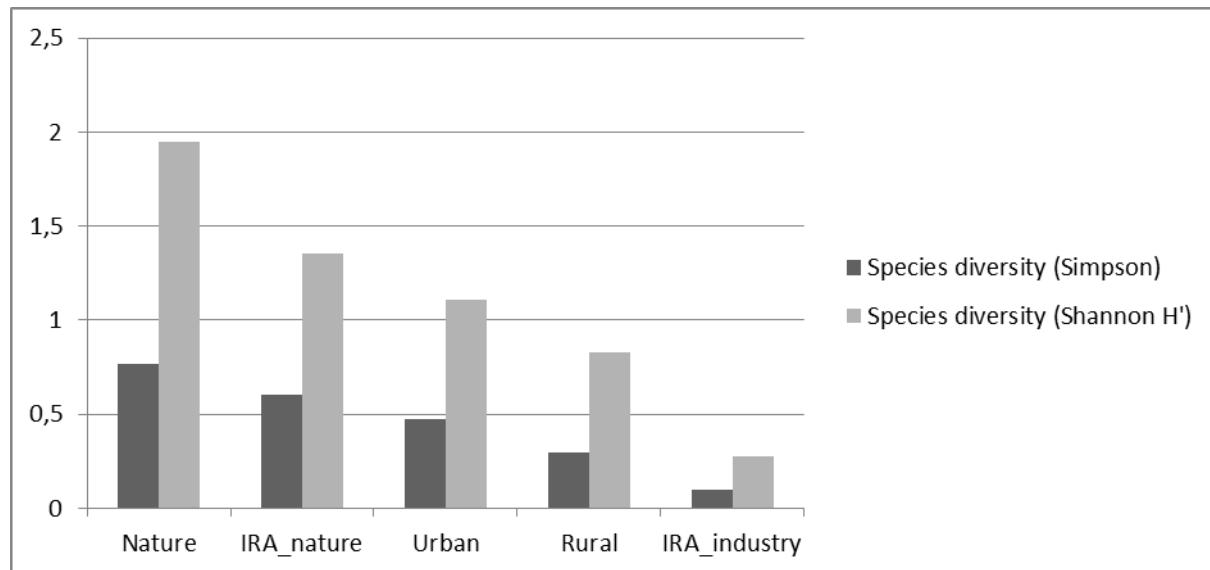


Figure 7: Species diversity in main Corine habitat based on Simpson (black) and Shannon (Grey) diversity indices

2.3 The importance of museum collections to basic invertebrate inventories

2.3.1 Voucher specimens

In the beginning of past century many mosquitoes were collected all over Belgium by dipterologists as M. Goetghebuer and M. Bequaert who both built up the most representative and rich collections of Belgian Diptera, preserved at RBINS (Grootaert et al., 1991). In the Belgian Culicidae collection of RBINS four subcollections are present: a general collection, two subcollections (Goetghebeur and Becquart), and a subcollection of unidentified specimens i.e. the supplements. The subcollection Bequaert was mainly collected between 1912-1958 and counts 135 voucher specimens. The subcollection Goetghebuer was collected between 1909-1946 (mainly between the period 1910-1930) and counts 269 specimens. In the general collection 241 specimens are present all of them collected between 1878-1967 (mainly between 1880-1925). The supplements are the largest subcollection with 737 specimens collected between 1892-2005 (mainly during 1920-1960).

2.3.2 Identification

All voucher specimens from the available collections were re-identified at the species level using Schaffner et al., 2001.

2.3.3 Re-evaluation of RBINS mosquito collection

The most recent checklist of the Belgian Culicidae counted 24 species, which was the number of identified species found in RBINS collection and additional species mentioned in the card-indexes of RBINS (Gosseries and Goddeeris 1991). The latter authors suggested at that time that the real number of species to be expected to occur in Belgium being approximately 50. However since 1991 only a few mosquito species were added to the Belgian fauna; *Culex hortensis* (Versteirt et al. 2009) and *Culiseta ochroptera* (Schaffner pers. com.). All 1381 specimens (24 species) in RBINS collections were reidentified and digitised. Most of the specimens (77%) were collected between 1910 and 1960 (Figure 8). Most specimens were collected between 1940 and 1950. The intensity of research and mosquito-sampling fluctuated during this period, as revealed by the number of voucher specimens per decade (Figure 8). The oldest specimens (collected in 1878) are deposit in the general collection. In this collection 16 species were discovered, in the subcollection Bequaert, the subcollection Goetghebuer and in the supplements respectively 18 species, 21 species and 20 species were counted.

Culex pipiens and *Culiseta annulata* were the most abundant recorded species present in the collection. Also many voucher specimens of *Aedes punctor* were present.

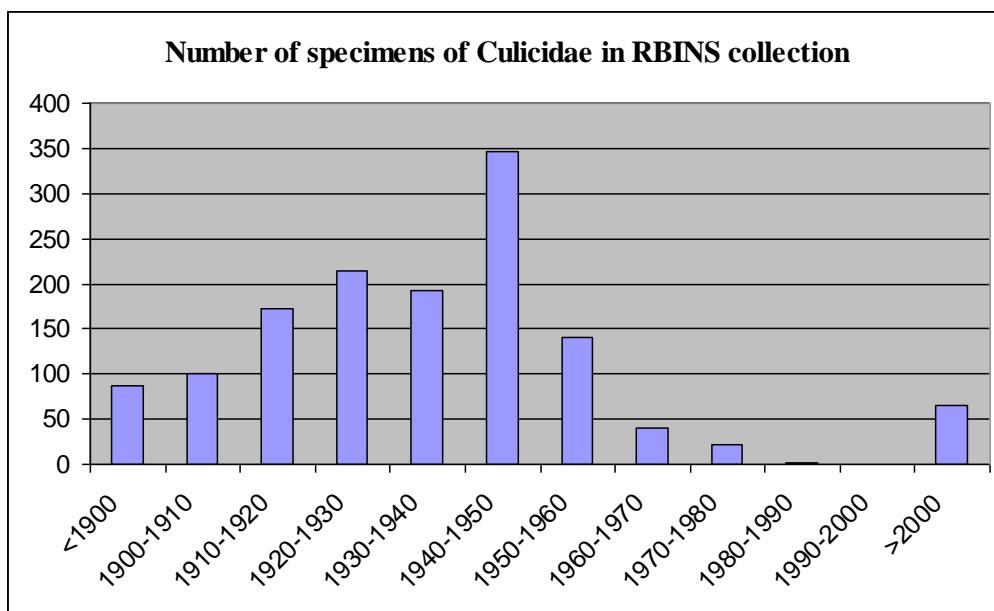


Figure 8: Number of identified voucher specimens for each considered period

Records of rare and interesting species

During the re-evaluation some so far seldomly recorded mosquito species were discovered in the supplements. A female of *Cx hortensis*, only recently added to the Belgian fauna (Versteirt et al. 2009), was collected by Bequaert in Aywaille, Nonceveux on 6/vii/1947. Other remarkable records are retrieved from two sites were Goetghebuer collected *Cs fumipennis*: on 1/vii/1920 and 11/vi/1914 in Hockai near Stavelot (Prov. Liège), probably in bogs and wet heathland vegetations of the Hautes Fagnes. Later it was also collected in Lovendem, Vinderhoute (Prov. Eastern-Flanders) on 9/5/1920. Two specimens of *Cs subochrea* were found in the collection of BEQUAERT: one male collected at Destelbergen, Heusden (Prov. Eastern-Flanders), 2.viii.1944 and one female collected in Blankenberge, (Prov. Western-Flanders), 6.xi.1955. No evidence was found that introductions of exotic mosquitoes were noticed or invasive specimens were collected prior to 2000.

Establishment of a MODIRISK-subcollection in RBINS mosquito collection

At RBINS a new Culicidae subcollection with specimens collected during the MODIRISK project was generated. Of all species collected during the project specimens of at least 5-10 sites are stored (if the species was collected in more than 5-10 sites). Moreover all partners involved in the morphological identification started their own reference collection with help from the expert. Therefore 1-5 correctly identified specimens per species were pinned and stored in insect boxes.

2.3.4 Calculating trends in mosquito distribution in Belgium

Both datasets were used to test whether or not trends in changing mosquito diversity were observed for several regions in Belgium. Results of this large scaled survey provided us with an estimated present observed mosquito diversity (hereafter called PresDiv). Estimated former observed mosquito diversity was obtained from the revision of RBINS mosquito-

collection (hereafter called ForDiv). All present and former records (i.e. a species collected at a given site (locality on the label) on a given date) were attributed to 10x10km grid cells of the UTM (Universal Transverse Mercator) projection, hereafter called grid cells. To compare the present diversity with that obtained from voucher specimens we restricted our analyses to grid cells where at least two species were found (ForDiv and PresDiv \geq 2; in most cases at least *Culex pipiens* and *Culiseta annulata* present).

Methodology

For each of these grid cells a trend criterion (degree of decline or increase) was calculated (Dufrêne and Desender 2007, Desender et al. 2008). The degree of change in diversity for each grid cell (TREND) was calculated by $TREND = [PresDiv - (ForDiv * COR)] / [(ForDiv * COR) + PresDiv]$, where PresDiv and ForDiv are the number of mosquitoes observed per grid cell and COR the correction factor to correct for difference in sampling intensity and methods during both inventory systems. This correction factor was calculated by dividing the sum of PresDiv by the sum of ForDiv for all well studied grid cells. Here the correction factor was + 1.215. By calculating this TREND we generate a value between -1 (all diversity lost) and +1 (all diversity new) for each well surveyed grid cell.

To obtain relative changes in distribution area for 23 mosquito species, a linear regression was performed of the logit-transformed proportions from the recent inventory as a function of the logit-transformed proportions retrieved from the revision of RBINS collection. Proportions in both surveys were calculated as $P=(x+1)/(n+1)$ where x is the number of recorded grid cells for a given species and n is the total number of grid cells surveyed. The logit-transformed proportions were calculated as $\text{logit}(P)=\ln[P/(1-P)]$ (Telfer et al. 2002).

The index of relative change in distribution area for each species was calculated by its standardized residual from the fitted regression line (Van Landuyt et al. 2008). Species that obtained positive values of this index had relatively increased their distribution area. Species that obtained negative values of this index had relatively decreased their distribution area. Values equal to zero indicate no relative change in distribution area for this mosquito species between the two survey period

Results

Mainly near big cities mosquito diversity has declined because of the loss of undisturbed natural habitats and their inhabiting mosquito species. On the other hand in the north of the country and the Kempen region some grid cells show an increase in mosquito diversity (Figure 9).

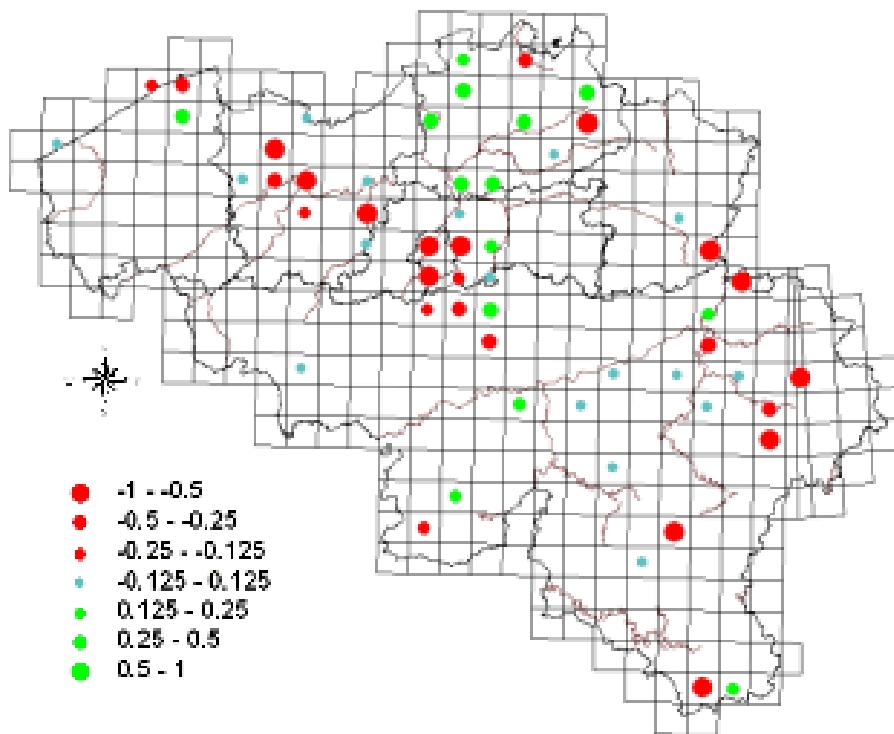


Figure 9: Trends in mosquito diversity for well studied grid cells based on mosquito diversity obtained from voucher specimens and from the recent inventory.

An estimation of the relative changes in distribution area of most of the Belgian mosquito species revealed that eight species have a positive index of relative change in distribution area and might be considered as species that enlarged their relative distribution area recently. Amongst them are two *An.* species and the *An. maculipennis* s.l. complex. Also *Ae. vexans* and *Ae. cinereus/geminus* have a positive relative index of change in distribution area. The highest indexes of change in distribution area were retrieved for *Cx torrentium*, *Cx pipiens* and *An. plumbeus*. that recently also use man-made habitats as larval breeding site as used tires and waste waters, have enlarged their distribution area.

2.4 Set up a molecular reference archive of Belgian mosquitoes based on the DNA barcoding region

2.4.1 General molecular approach

A general molecular identification framework based on the DNA-barcoding approach is developed for the Culicidae from Belgium. This framework will allow verifying the species for which the morphological identification is problematic or when sibling species are involved. Furthermore DNA barcoding will be an essential tool for the fast and reliable identification of possible invading species. The development of a comprehensive DNA barcodes dataset of the Belgian species based on fresh field material will contribute to disentangle possible taxonomic problems and improve further species identification. Based on the currently available information markers other than COI, have been explored to improve the differentiating power of the DNA barcoding like ND4 (for *Anopheles* and *Aedes* species) and

D3 (for *Anopheles* species). Two major regions of interest (ITS2-rDNA and COI-mtDNA) have been PCR-amplified and sequenced. The PCR on ITS2 is done according to the method described in Van Bortel et al. (2000) whilst for the COI PCR the universal DNA-barcoding primers are used (Folmer et al. 1994). Protocols for the ND4 regions were done based on Cameron et al (2010) for *Aedes* species and based on Fonseca et al. (2001) for *Anopheles* species; whilst PCR on D3 was done according to the method of Sharpe et al 1999. The protocol for the microsatellite PCR was adapted from Keyghobadi et al. (2004) whilst that for the CQ11 locus from Bahnck and Fonseca (2006).

2.4.2 DNA barcoding of Belgian mosquito species from MODIRISK

A total of 964 individuals (20 species) were molecularly identified, amplifying the COI mtDNA barcoding region and the ITS2 rDNA region. Sequence results were good for the COI region. The ITS2 showed different copies in the same individual with a returning deletion/insertion at a fixed site. Especially *Anopheles* species showed this multi-copy phenomenon. The COI sequences were assembled and aligned using ClustalW (BioEdit) and neighbour joining tree was constructed using Mega4. Preliminary analysis (neighbour joining, Mega4) of the COI sequence results confirms the utility of the COI region for species identification. Most branches of the tree are supported by bootstraps values higher than 70% (for most species even 90% and more). On genus level most branches are not supported, hence for more phylogenetic studies other markers were tested as well from the mitochondrial as from the ribosomal DNA (like for *Anopheles* species). It seems that depending on the studied genus, other markers and methods should be employed. A well supported and integrated identification system for field material will be created usable in a broader European context (Biosurveillance and vector control programmes). Furthermore, as Sequences of all species will be submitted to GenBank so they are freely available for researchers and students worldwide these results will be available for worldwide mosquito taxonomy studies. This work has been done in the cooperation with the JEMU (RBINS and RMCA) project in the framework of their flagship project Barcodes for TwoWings (BC42W).

2.4.3 A larval assay to rapidly and correctly identify mosquito species

Correct identification of the vector is one of the important factors in the study of arboviral diseases (Cook et al., 2005). In addition, the precise identification of the target species has direct medical and practical implications, particularly in developing vector control strategies. Identifying larvae is not easy and often problems are encountered in separating larvae of endemic and exotic container mosquito species (Beebe et al. 2007). However some already described tests work only on a specific targeted genus. Moreover, preliminary test results of Belgian mosquito larvae (based on the rDNA ITS2 region, and the mtDNA ND4 and COI region) showed that DNA extracted from field larvae was highly contaminated by organic matter competing in the PCR reactions.

After several tests assessing the best way in which larvae (lab and field material) could be killed, preserved and DNA extracted to increase the percentage of positive results after PCR amplification, an easy and efficient protocol is set up that will be generally applicable.

2.5 Longitudinal data of Dutch mosquitoes for validation of habitat characteristics, mosquito species composition and abundance

2.5.1 Methodology

Longitudinal studies were conducted in 55 selected sites across The Netherlands in the late summer of 2009 and the spring of 2010. The aim was to assess whether mosquito presence and abundance in the selected sites were correlated to those found in Belgium. It was assumed, though, that as the typical meadow landscapes as found in much of the North West of The Netherlands are not present in Belgium, differences in mosquito species composition and/or abundance might occur.

Mosquito sampling and identification:

Mosquitoes were sampled with CO₂ traps used throughout the Modirisk project. Each site was sampled for one week continuously, once between August and October 2009, and once again between April and June 2010. In this way, spring and fall populations of mosquitoes were expected to be found. Samples taken to Wageningen for taxonomic identification using the key provided by Schaffner (Schaffner 1993). Members of the *An. maculipennis* complex were further identified to species using a PCR of the ITS2 region (Marinucci et al. 1999).

Study sites

A total of 55 sites distributed across The Netherlands were studied (Figure 10). Study sites included agricultural and urban sites, forest, dunes and marshes, as well as one harbour (Rotterdam) and one airport site (Schiphol, Amsterdam) (Table IV). More than 56% of the sites were classified as agricultural, and included mostly meadows, as well as 2 greenhouses. At greenhouses, traps were placed outdoors, in the vicinity of the main entrance used for loading cargo.



Figure 10. Locations of LP CO₂ traps in The Netherlands in 2009 and 2010

Table IV: Detailed overview landscapes sampled in The Netherlands (2009- 2010)					
	Type	Specification	No. locations	Perc.	
1	Agricultural	Pastures	Cattle	12	21.8
			Sheep	5	9.1
			Horses	5	9.1
		Stable	Chickens	2	3.6
			Pigs	1	1.8
		Crop production		4	7.3
2	Greenhouses			2	3.6
3	Urban			8	14.5
4	Forest			5	9.1
5	Dunes			2	3.6
6	Marsh	Peatbog (high)		2	3.6
		Peatbog (low)		2	3.6
		Freshwater		2	3.6
		Brackish		1	1.8
7	Harbour			1	1.8
8	Airport			1	1.8
	Total			55	100

2.5.2 Results

Twenty two species of Culicidae were found (Table V). *Cx pipiens*, *An. plumbeus* and *Ae. cantans* were the most abundant species. There were marked differences in phenology among the species collected. Species that only were present in the spring include *Cx torrentium*, *Ae. punctor* while *Ae. cantans* was much more abundant in the spring than in the late summer. No species were only present in the late summer, although *Cx pipiens*, *Cs annulata*, *Cq richardii* and *An. maculipennis* were much more abundant in late summer than in the spring. Table VI summarizes the distribution of the most common species found per landscape type. Both *Cx pipiens* and *Cx torrentium* were common in industrial areas like the airport. *Cs annulata* was equally common in agricultural, urban areas and marshes. *Cs morsitans* preferred marshes but was also found in forests and in agricultural areas. *Coquillettidia richardii* preferred marshes, but was also common in urban areas. *Anopheles maculipennis* clearly preferred agricultural areas. *Anopheles claviger* was most common in dunes and to a slightly lesser extent in marshes, although only two sites "dunes" were sampled, while the number of marshes studied was higher. *Anopheles plumbeus* was by far most common in agricultural areas. This species was abundant on only a few farms (in high numbers) while on most other farms it was absent (see 3.3). Most *Aedes*-species preferred forests and were to lesser extend found in marshes or agricultural areas. Only *Ae. vexans* and *Ae.cinereus* appeared to prefer marshes and agricultural areas.

Species	July, Sept. 2009	August, 2010	April, May, June	Total
<i>Culex pipiens</i>	1069	145		1214
<i>Culex torrentium</i>	0	33		33
<i>Culex territans</i>	3	5		8
<i>Culex modestus</i>	1	0		1
<i>Culiseta annulata</i>	92	12		104
<i>Culiseta suborchea</i>	0	1		1
<i>Culiseta morsitans</i>	2	12		14
<i>Culiseta fumipennis</i>	2	1		3
<i>Coquillettidia richardii</i>	104	9		113
<i>Anopheles maculipennis</i>	142	18		160
<i>Anopheles claviger</i>	20	39		59
<i>Anopheles plumbeus</i>	483	661		1144
<i>Aedes vexans</i>	0	6		6
<i>Aedes cantans</i>	72	1035		1107
<i>Aedes riparius</i>	20	57		77
<i>Aedes annulipes</i>	5	58		63
<i>Aedes communis</i>	0	8		8
<i>Aedes cinereus/geminus</i>	70	81		151
<i>Aedes punctor</i>	0	427		427
<i>Aedes leucomelus</i>	0	9		9
<i>Aedes rusticus</i>	0	20		20
<i>Aedes geniculatus</i>	0	1		1

Table VI: Distribution (%) of most common mosquitoes caught per landscape type per week (2009 & 2010).							
Species	Agricultural	Forest	Urban	Marsh	Dunes	Harbour	Airport
<i>Culex pipiens</i>	15.5	2.6	9.2	2.8	2.7	24.0	43.2
<i>Culex torrentium</i>	2.2	0.0	0.0	0.0	5.1	92.7	0.0
<i>Culiseta annulata</i>	26.5	5.3	38.0	30.2	0.0	0.0	0.0
<i>Culiseta morsitans</i>	31.4	15.0	0.0	53.6	0.0	0.0	0.0
<i>Coquillettidia richardii</i>	8.8	3.3	37.2	50.7	0.0	0.0	0.0
<i>Anopheles maculipennis</i>	64.7	0.0	12.3	16.0	7.0	0.0	0.0
<i>Anopheles claviger</i>	1.5	3.1	9.6	28.4	57.4	0.0	0.0
<i>Anopheles plumbeus</i>	98.0	0.5	1.6	0.0	0.0	0.0	0.0
<i>Aedes cantans</i>	15.8	75.0	0.2	9.0	0.0	0.0	0.0
<i>Aedes annulipes</i>	43.3	42.1	3.3	11.3	0.0	0.0	0.0
<i>Aedes riparius</i>	4.2	90.2	1.0	4.6	0.0	0.0	0.0
<i>Aedes punctor</i>	2.6	85.9	0.9	10.7	0.0	0.0	0.0
<i>Aedes rusticus</i>	2.8	97.2	0.0	0.0	0.0	0.0	0.0
<i>Aedes vexans</i>	31.1	0.0	0.0	68.9	0.0	0.0	0.0
<i>Aedes cinereus/geminus</i>	0.3	6.5	7.0	86.2	0.0	0.0	0.0

Table VII shows the species composition of the *Anopheles maculipennis* complex in The Netherlands: *An. maculipennis* s.s., *An. messeae* and *An. atroparvus*. The by far most common species of this complex was *An. messeae* (95.1 %) and to a lesser extent the species *An. maculipennis* s.s. (4.3 %) and *An. atroparvus* (0.6 %). *Anopheles messeae* was found all over the country but clearly preferred the pastures on lowland peatbogs in the west of The Netherlands. *Anopheles atroparvus* was found only once, in the natural reserve "The Putten" near Camperduin in Noord Holland. This open brackish marshland consists of areas with shallow open water and broad reed beds. *Anopheles maculipennis* s.s. was found especially in agricultural areas in the East of the country. Species of the *An. maculipennis* complex were found more in the late summer of 2009 than in the spring of 2010. From previous studies in The Netherlands it was found that populations of *An. maculipennis* s.l. develop slowly in the spring, to peak in late July/August.

Table VII: Speciescomposition of the <i>Anopheles maculipennis</i> complex (2009 & 2010).				
	2009		2010	
	Number	Percentage	Number	Percentage
<i>Anopheles messeae</i>	139	95.9	16	88.9
<i>Anopheles atroparvus</i>	1	0.7	0	0.0
<i>Anopheles maculipennis</i> s.s.	5	3.4	2	11.1
Total	145	100	18	100.0

A seasonal distribution of mosquitoes is shown in Table VIII, which includes the mean number of mosquitoes caught per location per month. *Culex pipiens* appears to be most common in late summer and the early autumn, while *Cs annulata*, *An.maculipennis* and *Cq richardii* were most common in July. *Anopheles claviger* was most common in spring, while *Anopheles plumbeus* was most common in June and July. Most *Aedes* species reached their summit in numbers in spring. Mosquitoes that appeared earliest in spring are *Culex pipiens*, *Cx territans*, *Cx torrentium*, *Cs annulata*, *An.maculipennis* and *An. claviger*. Of the *Aedes* species, *Aedes punctor* was usually the first, followed by *Ae. cantans*, *Ae. riparius*, *Ae. annulipes* and *Ae. rusticus*. *Aedes cinereus* and *Ae. vexans* appeared just a bit later. Mosquitoes that were still active in September were *Cx pipiens*, *Cs annulata*, *Cs morsitans*, *Cs fumipennis*, *An. claviger*, *An. maculipennis*, *Cq richardi* and *Ae. cinereus*. Only *Cx pipiens* and *Cs annulata* remained active till late in the autumn (Oct, Nov). *Culex pipiens* was even still caught at the beginning of December 2009 in some old forests in the Betuwe! It seems that a number of the mosquito species that appear early in the spring were still active late in the autumn

Species	2009			2010		
	July	August	September	April	May	June
<i>Culex pipiens</i>	12.1	23.4	35.2	0.6	0.8	4.6
<i>Culex torrentium</i>	0	0	0	0.1	0.1	1.5
<i>Culex territans</i>	0.2	0	0	0.1	0.0	0.2
<i>Culex modestus</i>	0.1	0	0	0.0	0.0	0.0
<i>Culiseta annulata</i>	2.4	1.3	2	0.4	0.0	0.3
<i>Culiseta suborchea</i>	0	0	0	0.1	0.0	0.0
<i>Culiseta morsitans</i>	0	0	0.3	0.0	0.5	0.1
<i>Culiseta fumipennis</i>	0	0	0.2	0.0	0.0	0.1
<i>Coquillettidia richardii</i>	3.3	0.5	4	0.0	0.0	0.6
<i>Anopheles maculipennis</i>	5.8	1	0.5	0.3	0.2	0.5
<i>Anopheles claviger</i>	0.2	0.6	0.5	0.9	0.9	0.3
<i>Anopheles plumbeus</i>	23	1	0	0.0	0.0	14.5
<i>Aedes vexans</i>	0	0	0	0.0	0.2	0.1
<i>Aedes cantans</i>	3.4	0.2	0	0.4	27.3	30.3
<i>Aedes riparius</i>	1	0	0	0.1	0.4	3.1
<i>Aedes annulipes</i>	0.3	0	0	0.0	0.0	3.9
<i>Aedes communis</i>	0	0	0	0.0	0.1	0.4
<i>Aedes cinereus</i>	0	2.8	0.2	0.0	0.3	4.9
<i>Aedes punctor</i>	0	0	0	2.5	3.0	21.2
<i>Aedes leucomelus</i>	0	0	0	0.0	0.3	0.2
<i>Aedes rusticus</i>	0	0	0	0.0	0.1	1.1
<i>Aedes geniculatus</i>	0	0	0	0.0	0.0	0.1

By trapping mosquitoes in a comparable way during one week in the late spring (mid April to mid June) as well as late summer (July till the beginning of September) on 55 sites evenly spread over the country, a good impression of the distribution of the different mosquito species in The Netherlands was obtained. Most locations have been chosen to verify the model that predicts the presence or absence of mosquitoes on the basis of the habitat. If, by chance, many farms would have been chosen with suitable breeding places for *An. plumbeus*, their numbers found would be manyfold of what would have been found if farms would have been chosen where breeding places for this species are absent. The three most commonly found mosquitoes in this survey were *Cx pipiens*, *An. plumbeus* and *Ae. cantans*. Mosquitoes that are typical for brackish water habitats were almost absent from the catches. Only one specimen of *An. atroparvus* was found. Although this species was historically present in high abundance (Swellengrebel and de Buck 1938), this species is rarely found in recent years (Takken et al. 2002), presumably because of a change in habitat and significant change in water quality, from being brackish to fresh. The sibling species *An. messeae*, by contrast, is widely present in all ditches in agricultural areas. Other species, which are typical for this habitat, like *Ae. caspius*, *Ae. dorsalis* and *Ae. detritus*, were not found at all. It is unlikely that these species are absent in The Netherlands, although they were previously

found (Takken et al 2007). Probably the lack of places with a suitable habitat for these species that were chosen for this research is the reason for their absence. By comparing the mosquito species and abundance recorded in the present study with those obtained in the Belgian study, using GIS referenced habitats, the data from The Netherlands can be scaled up and related to the landscape types as mentioned above.

Distribution of *Culex pipiens* in The Netherlands

We focus here on the distribution of *Cx pipiens*, as this was the species that dominated the collections and was found throughout the country (Figure 11 and 12). Distribution of other species are available from the Laboratory of Entomology at Wageningen University. Figure 11 shows the distribution of *Cx pipiens* in late summer of 2009 and Figure 12 shows the distribution of this species during late spring of 2010. The red dots present the places where traps were present, but where no *Cx pipiens* were found, the light blue dots present the number caught in a logarithmic scale. In late summer of 2009 *Cx pipiens* was much more common than in the late spring of 2010, with spatial differences in abundance. In the spring this species was in the southern part a bit more common than in the rest of the country (Figure 12), which is in line with previous studies (Huijben et al. 2007). As with *An. maculipennis* spp., populations of *Cx pipiens* are low in the spring and develop slowly until they reach a population threshold level at the end of June, when population development rises rapidly.

Several other species show a dishomogeneous distribution in the country. For example, *An. plumbeus*, *Cq richardii*, *Ae. cantans* and *Ae. punctor* show a distinct segregated distribution pattern, which can be linked to habitat type. Other species were more evenly distributed across habitats/landscapes, or were too few in numbers to draw any conclusion about habitat preference. These include *Cx modestus*, *Cs fumipennis*, *Ae. vexans*, *Ae. communis*, *Ae. leucomelus* and *Ae. geniculatus*, of which <10 specimens were found. The near absence of *Ae. vexans* is remarkable, given the ubiquitous distribution of this species elsewhere in Europe, where it often reaches a high nuisance level (Becker 1997). However, this absence has been observed before, and is possibly due to unsuitable vegetation and/or lack of inundations at the time when the eggs need to hatch (Takken et al., 2007).

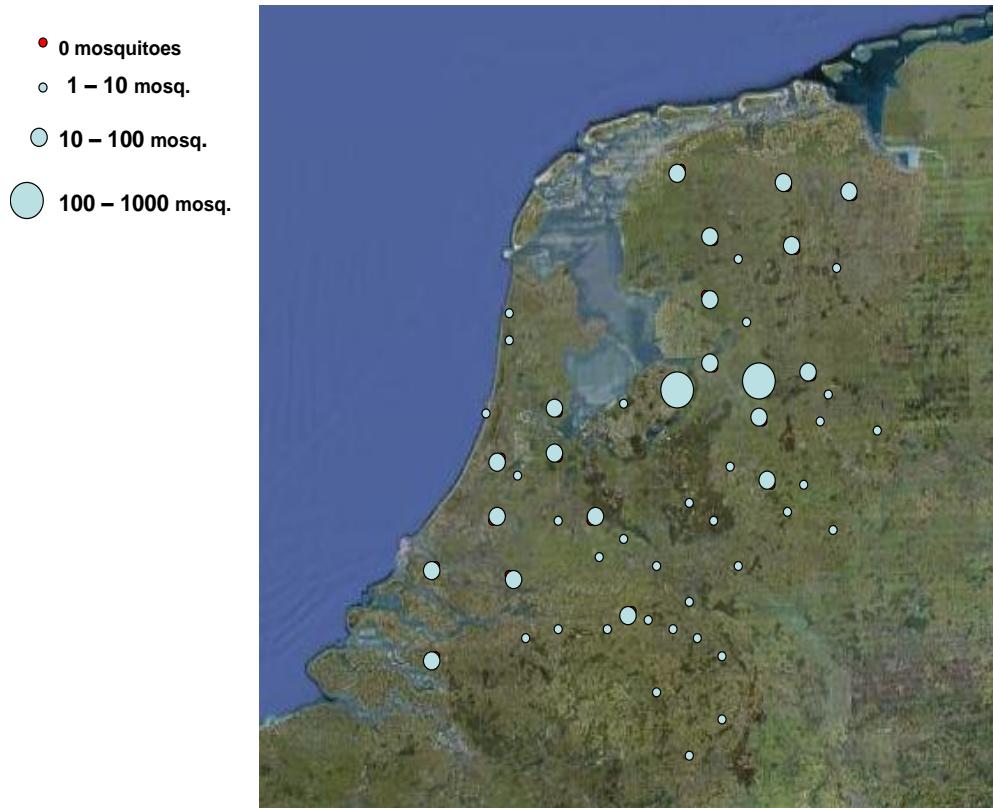


Figure 11: Logarithmic number and distribution of caught *Culex pipiens* during late summer of 2009

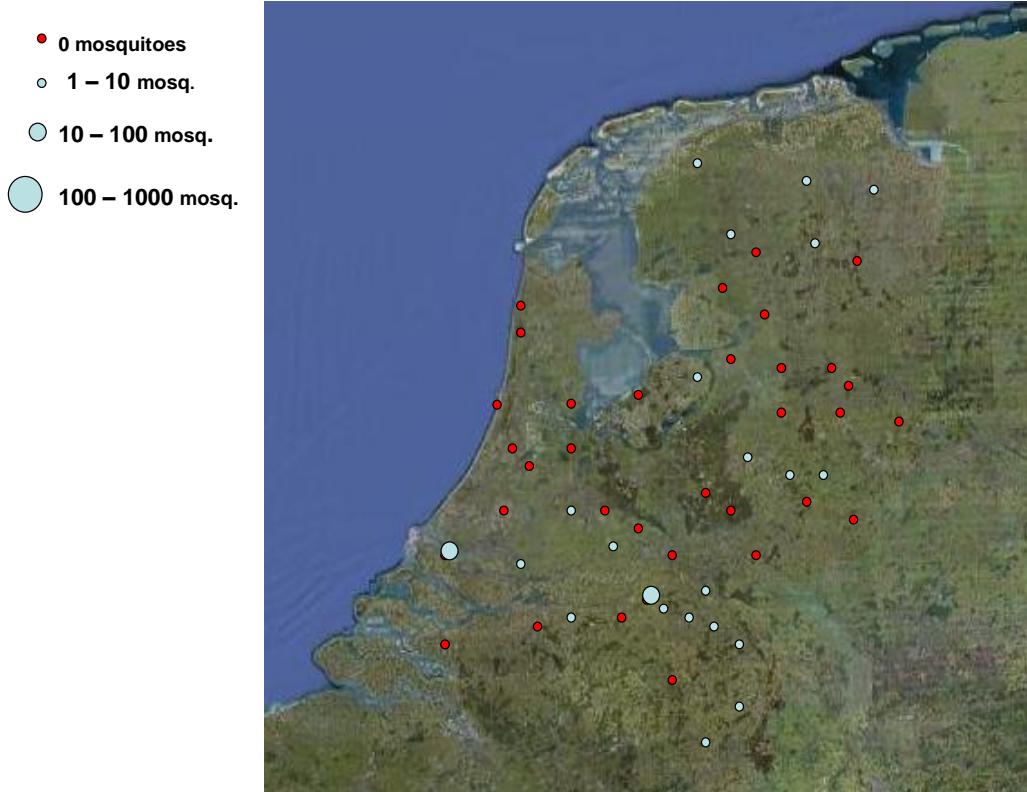


Figure 12: Logarithmic number and distribution of caught *Culex pipiens* during early summer of 2010

3 ECOLOGY AND POPULATION DYNAMICS OF MOSQUITOES IN BELGIUM

3.1 Spatial modelling of indigenous mosquito species in Belgium; detection of hotspots and implications for field sampling design and risk analysis in Europe

3.1.1 Methodology

3.1.1.1 Development of a spatial data archive

Low resolution remote sensing

NOAA AVHRR data has been archived from the following online archive: <http://www.class.noaa.gov/saa/products/>. Each individual image has been visually inspected for noise. If the noise level was too high, then the image was not included in the Avia-GIS archive, this to avoid contamination of the archive and corruption of the processing chain and the processed images. The NOAA data starting from 2000 were then processed using the Avia-GIS software. To remove cloud contamination 10 day composites using the Maximum Apparent Temperature (MaT) algorithm (Cihlar 1994) were created. These images also had automatic geometric correction using the ground control points included with the satellite image. The images were then subjected to further noise removal.

Images from the MODIS sensor were used to derive similar variables. MODIS data were ordered through the following data gateway: <http://elpdl03.cr.usgs.gov/pub/mswelcome/>. Images from the MODIS sensor were used to derive additional variables. Both Land Surface Temperature (LST-day and -night) and Vegetation Indices (VI: EVI and NDVI) for the years 2004, 2005, 2006, 2007 and 2008 were used to detect yearly trends. After Fourier transformation, the first three harmonics were included as predictor variables (Scharlemann et all. 2008). The number of days with a mean temperature above (i) 0 °C, (ii) 5 °C and (iii) 12,5 C were derived from the MODIS images for the year 2007 and 2008.

NOAA data has been used the most extensively in the past, mainly due to its extensive historical archive. Since the beginning of 2000, MODIS imagery is an alternative to the NOAA archive. Its main features are higher spectral resolution, and higher spatial resolution for several bands. Moreover, ready-made derived products are also available to the general public. The temperature profiles from NOAA and MODIS were compared to a limited ground truth meteorological data set (August–September 2006). The meteorological data set includes hourly observations of temperature. To allow for comparison, the time of recording the land surface temperature (LST) from MODIS was extracted from the science data set using the MODIS Reprojection Tool. The accuracy of the satellite derived temperature was assessed through the calculation of the root mean square error and the bias. A good fit was found between the profiles extracted from both NOAA and MODIS data.

In a paper by Scharlemann et al. (2008), a cubic spline interpolation technique was tested for seasonality extraction to be used as input for species distribution modelling. This novel algorithm of spline interpolation followed by regular resampling of the composited satellite data was developed to produce a 5-day interval MODIS time series that could then be

subjected to standard temporal Fourier processing methods. This algorithm was found to capture the input amplitude and phase information correctly. This algorithm was applied to all MODIS image time series to remove missing data pixels. This includes the following environmental parameters: NDVI, EVI and Land Surface Temperature (LST) from 2000 up to 2010.

Other spatial data sources

All relevant data layers needed to map mosquito habitats and plan standardized spatial sampling of mosquitoes were collected. The following data layers have been archived: land use and land cover classification, administrative boundaries, geocoding data layers (MultiNet Street data). In January 2008 a session with the field teams was organised to create a shortlist of data layers that can be useful for further analysis.

Information on land cover was derived from the CORINE dataset (JRC-IES 2005). The percentage of the three Level-1 land cover classes, *i.e.* urban, agriculture and natural, within a one-kilometre-pixel was computed. Within the forest class, cover percentage was also computed for three forest types: broadleaved, coniferous, and mixed forest. Human population pressure on the landscape was assessed by population density expressed by the number of inhabitants/km² (GWPv3.0 by SEDAC 2000). Other environmental data layers were the available water capacity of topsoil (JRC-IES 2009), distance to waterways (GfK 2009) and the GTOPO30 elevation (USGS 1996), mean of total yearly precipitation and mean of monthly mean of precipitation were obtained from the WORLDCLIM dataset (Hijmans et al. 2005).

All data layers were clipped to the extent of the study area and resampled to a 1- and 5-km resolution. GIS manipulations were performed using ArcGIS9.3 (ESRI 2009).

Eco-climatic seasonality analysis

Fourier analysis is a family of mathematical techniques, all based on decomposing signals into sinusoids. Through the use of a Fourier transform, any real world signal can be split into basic sine/cosine waves, each at a different frequency. The more sinusoids included, the better the approximation of the real-world signal. Each of the harmonic frequencies is defined by a magnitude (amplitude) and a phase. The phase indicates how to shift the harmonic before adding it to the sum.

Fourier analysis is ideally suited for summarizing seasonal variables (Rogers et al. 1996) because seasonal activity is a driven factor for vegetative status, vector abundance etc. The seasonal dynamics directly influence vector population dynamics. The importance of Fourier derived products has been shown by Rogers et al. (1996) in prediction tse tse fly distribution, and by Baylis et al. (2002) and Purse et al. (2004) for predicting *Culicoides imicola*. All satellite data has been processed using the Fourier analysis. For each of the environmental parameters, the first three harmonics were retained for further analysis.

Eco-climatic zones were identified using an unsupervised k-means clustering. This clustering was performed on the following variables:

- Altitude
- First three Fourier transforms on EVI, NDVI, LST(day) and LST(night)
- Precipitation : minimum, mean, and maximum values

- Land cover:
- Proportion of artificial surfaces,
- Proportion of agriculture areas,
- Proportion of natural vegetation

3.1.1.2 Development of spatial distribution models

Entomological data.

In the modeling approach, the trap was the sampling unit. Table IX gives an overview of the field data, not in number of individuals, but in number of traps in which individuals of a given species were found. In order to construct a robust and statistically sound model, a minimum of 20 positive (containing the species) and 20 negative (not containing the species) traps were needed. For some mosquito species, not enough positive traps were available. No models were constructed for these 'rare' species, e.g. *Culex territans*, *Culiseta morsitans*. Other individuals were damaged to a degree that made identification impossible, which lead to a species name 'sp'. No models were constructed for these species either. The list of species excluded from analysis can be found in the last column of Table IX

Table IX: Summary of field data based on the trap as sample unit. Positive trap: a trap in which the species is present			
Species name	Positive traps	Individuals	Model
<i>Aedes cinereus/geminus</i>	74	1,328	<i>Aedes cinereus/geminus</i>
<i>Aedes sp</i>	3	8	<i>Excluded</i>
<i>Aedes vexans</i>	29	776	<i>Aedes vexans</i>
<i>Anopheles claviger</i>	185	935	<i>Anopheles claviger</i>
<i>Anopheles maculipennis s.l.</i>	45	80	<i>Anopheles maculipennis s.l.</i>
<i>Anopheles plumbeus</i>	114	391	<i>Anopheles plumbeus</i>
<i>Anopheles sp</i>	4	5	<i>Excluded</i>
<i>Coquillettidia richiardii</i>	38	4,095	<i>Coquillettidia richiardii</i>
<i>Culex pipiens</i>	698	16,338	<i>Culex pipiens</i>
<i>Culex sp</i>	1	5	<i>Excluded</i>
<i>Culex territans</i>	7	11	<i>Excluded</i>
<i>Culex torrentium</i>	75	139	<i>Culex torrentium</i>
<i>Culiseta annulata</i>	162	577	<i>Culiseta annulata</i>
<i>Culiseta morsitans</i>	17	92	<i>Excluded</i>
<i>Culiseta sp</i>	2	2	<i>Excluded</i>
<i>Aedes annulipes</i>	8	42	<i>Aedes annulipes/cantans</i>
<i>Aedes cantans</i>	63	348	<i>Aedes annulipes/cantans</i>
<i>Aedes caspius</i>	12	80	<i>Excluded</i>
<i>Aedes communis</i>	33	280	<i>Aedes communis</i>
<i>Aedes detritus s.s.</i>	4	48	<i>Excluded</i>
<i>Aedes geniculatus</i>	48	164	<i>Aedes geniculatus</i>
<i>Aedes japonicus</i>	1	3	<i>Excluded</i>
<i>Aedes punctor</i>	65	531	<i>Aedes punctor</i>
<i>Aedes rusticus</i>	33	178	<i>Aedes rusticus</i>
<i>Aedes sp</i>	12	37	<i>Excluded</i>
<i>Aedes sticticus</i>	13	63	<i>Excluded</i>

In Table X, summary information is given on the species that were most widespread and most abundant in the traps.

Table X. Presence data for the most abundant and easy-to-catch species in Belgium. The column 'total' indicates the number of positive traps, also expressed as a percentage (%) of the total number of traps. The column 'N' gives the total number of individuals caught. The column 'N/+Tr' lists the average number of individuals per positive trap.

Species	Total	%	N	N/+Tr
<i>Aedes vexans</i>	29	2.97%	776	26.76
<i>Coquillettidia richiardii</i>	38	3.89%	4,095	107.76
<i>Aedes cinereus/geminus</i>	74	7.57%	1,328	17.95
<i>Culex pipiens</i>	698	71.37%	16,338	23.41

In Figure 133 the number of species found in each trap is visualized. The majority of the traps contained only one or two species. Rarely more than five mosquito species were recorded in one trap. This gives an idea how difficult it is to catch all mosquito species present at a given location. The maximum number of mosquito species found in one and the same trap was 12.

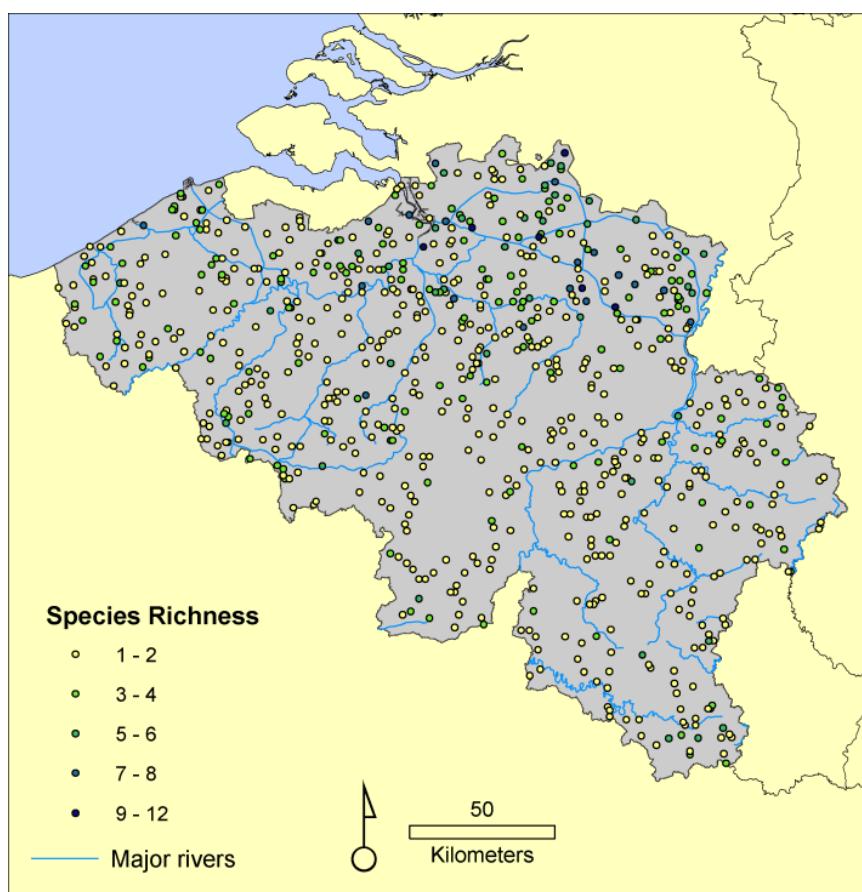


Figure 13. Species richness as observed in the traps.

Explanatory variables

In order to predict the presence/absence of a given mosquito species, all data layers defined under 2.1 were used as explanatory variables.

Modelling approach.

In order to obtain a balanced data set for the construction of the models, a subsample of the available sample was taken. For each species, the number of traps reporting respectively a presence or an absence of the species was counted. If the number of presences for a given species was lower than the number of absences, all traps reporting a presence were retained in the calibration data set. A sample of the same size was randomly drawn from all traps reporting an absence of the species. If the number of presences was superior to the number of absences, the procedure was inverted; all absences were retained in the data set, and the presences were randomly drawn.

This calibration data set was then used to construct a model for each mosquito species. Models were generated using Random Forests (RF) (Breiman 2001). Random Forests is a robust ensemble learning technique, which can be applied to model probability maps, expressing the probability of occurrence, through a random classification forest or abundance maps through a random regression forest. The technique consistently outperforms traditional modelling techniques such as logistic regression (Cutler et al. 2007, Peters et al. 2007). Random classification forests have been used to assess if temperature and precipitation affected the minimum infection rate of *Culex* species for West Nile Virus in Illinois (Ruiz et al. 2010) and to model the current spatial distribution of *Aedes albopictus* in Europe using a wide set of predictor variables (ECDC 2009).

Random regression forests allow both internal and external validation through a bootstrapping procedure. For each classification or regression tree, the full data set is bootstrapped, i.e. a number of data points are sampled from the complete data set with replacement. From the bootstrapped sample approximately one third of data are excluded. This set of the excluded data is referred to as the out-of-bag (OOB) data set for the tree; each tree will have a different out-of-bag data set. Since the OOB data sets are not used to build the tree, they constitute an independent validation data set for the tree.

To measure the classification error of the random classification forest, the OOB data for each tree are classified and the classification error is computed. The error values for all trees in the forest are averaged to give the overall classification error. In case of random regression forests, the error is expressed as the mean squared error between the predicted values for the OOB data and the observed data.

The performance of the model was assessed using four accuracy measures: percentage of correctly classified instances (PCC), sensitivity (sens), specificity (spec) and Area Under the Receiver Operating Curve (AUC). The AUC can be roughly interpreted as the probability that a model will correctly distinguish a true presence and a true absence (Fielding & Bell 2007). Predictor variable importance was assessed through the measurement of the decline in performance if the model is run without the variable. The performance decline was expressed as the mean decrease in GINI index. This index is unit-less, and can only be interpreted as a relative indication of variable importance. It does not allow to compare the

importance of a given variable between two models, but rather indicates the relative importance of two variables within one model.

Finally, the model was used to make a map of environmental suitability (ranging between 0 and 100 %) for the entire area of Belgium, the Netherlands and Luxembourg. Environmental suitability was expressed as a value between 0 (low suitability) and 100 (high suitability). When the probability for species presence was higher than 50, this location was considered to be a suitable habitat for the mosquito species under consideration.

All statistical analyses and modelling were performed in R2.10.1 statistical language environment (R Development Core Team 2006), using of the R-packages rgdal (version 0.6-25) and randomForest (version 4.5-34).

Biodiversity Hotspot mapping.

Co-occurrence approach

The main output of the species modeling was an environmental suitability for the presence of a given species on a given location in the Benelux. A species co-occurrence map was derived by counting the number of species for which the environmental suitability was higher than 50 %. In the remainder of this document, this map is referred to as "diversity map".

Species richness approach

An alternative map of hotspots of mosquito presence was obtained by modeling the species richness based on the number of species caught in each trap (Figure 13). Random forest was again used as modeling technique. Instead of predicting two classes (absence or presence of a given mosquito species), the model predicts the number of species caught in each location. An ensemble of regression trees was constructed to this aim.

3.1.1.3 Unique field validation of the spatial models

Selection of validation points

Several validation points were sampled, ca. 50 situated in the Netherlands, and 150 additional sites were visited in Belgium. In the Netherlands, trap sites were selected using a stratified sampling approach discriminating between urban, rural, natural and import-risk habitats. The sampling strategy was specifically designed to prepare for spatial distribution modelling at a one kilometre resolution. In Belgium, the sites were selected based on the obtained model outputs. The line of thought is specified in the remainder of this paragraph. When comparing the model output with the field data, two kinds of errors occurred. False negatives are locations where the model predicted no suitable habitat for a given species, but the field data contained individuals caught at this location. False positives indicated the inverse error: model outputs indicate that the environment was suitable for the species, but no individuals were caught in the field. False negatives were assumed to be an error of the model, while false positives could be caused by chance. It was considered possible that not all species present at a location were caught in the traps. For every given species, the number of false positives were summed, and each trap was given a weight Lambda using the following equation:

$$\Lambda = \sum_i^m \mathbf{1} / T t_i$$

Where

m = nr of species modelled (7 in this case)

$T t_i$ = nr of false positives for species i

The traps with the highest value for Lambda computed on summer species were selected for field validation during 2009. The traps with the highest value for Lambda computed on spring species were selected for field validation during 2010 (Figure 14).

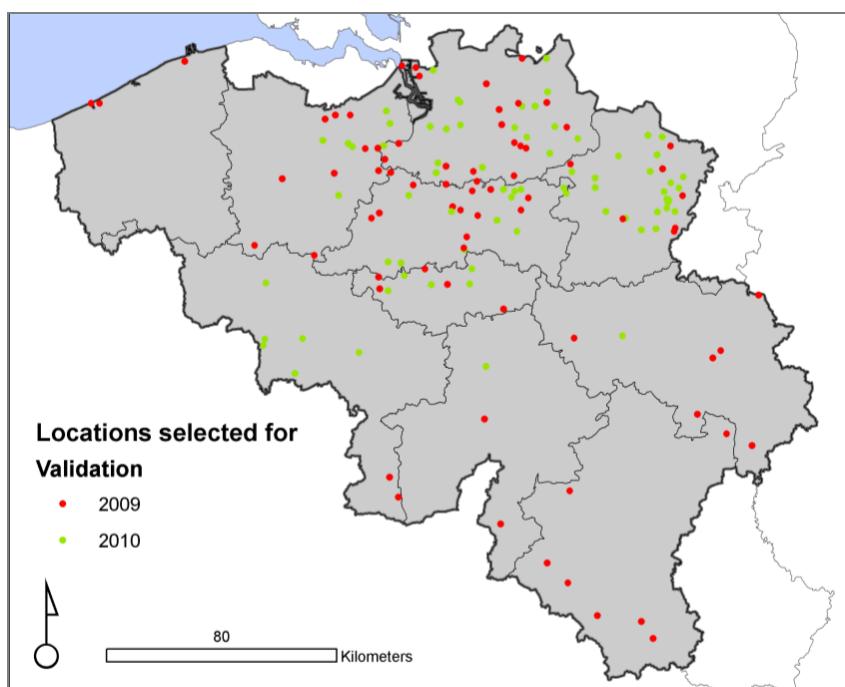


Figure 14. Distribution of the selected validation points in Belgium. Points sampled in 2009 are depicted in red; points sampled in 2010 are depicted in green.

3.1.2 Modelling outcomes

3.1.2.1 Eco-climatic zones

The scree plot and silhouette plot from the unsupervised k-means clustering are found in Figure 15. No further decrease in Wilk's Lambda was observed when increasing the number of clusters to more than 6, and thus six clusters were distinguished in the dataset. The clear gaps between the clusters in the silhouette plot indicate that the separation between the clusters was distinct.

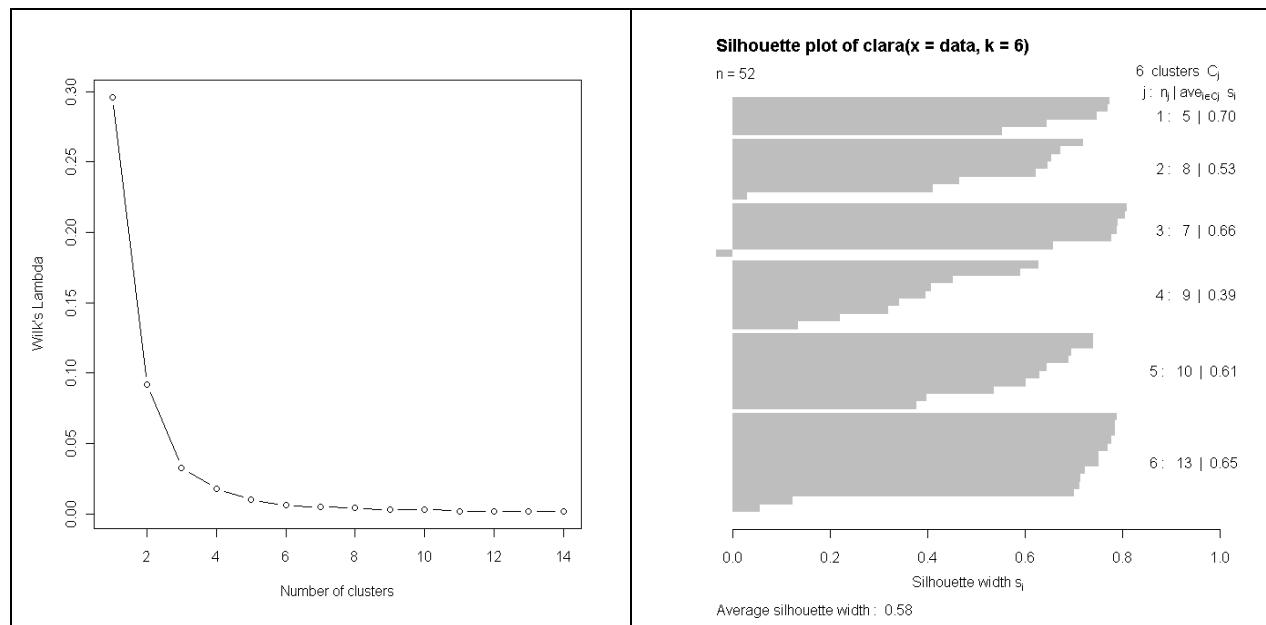


Figure 15. In the scree plot (left), no decrease in Wilk's Lambda is seen when the number of clusters, and thus 6 clusters are identified. The silhouette plot (right) indicates that there is a clear distinction between the clusters.

The geographical distribution of the eco-climatic zones is shown in Figure 16. The characteristics of the identified eco-climatic zones can be found in Table XI.

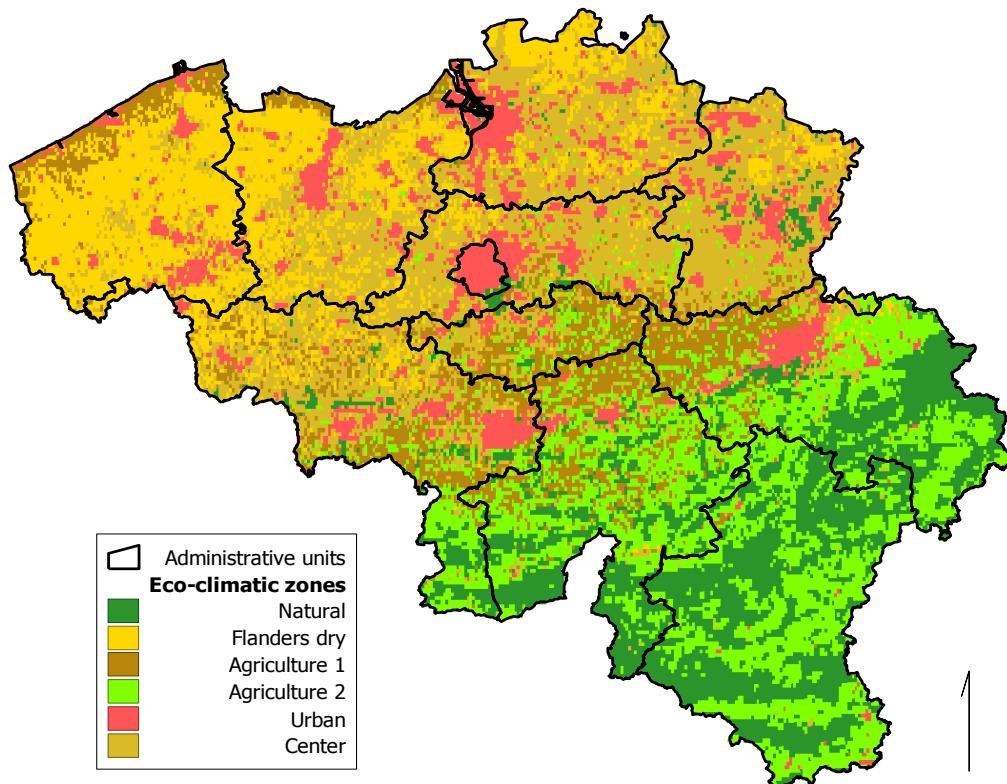


Figure 16: the geographical distribution of the distinguished eco-climatic zones

Table XI: Characteristics of the identified eco-climatic zones, the suffix _a0 refers to the annual mean, _a1 respectively _p1 to the amplitude and the phase of the first Fourier harmonic, _a2 respectively _p2 to the amplitude and the phase of the second Fourier harmonic, _a3 respectively _p3 to the amplitude and the phase of the third Fourier harmonic. The table contains the cluster means for each of these variables.

	Natural	Flanders dry	Agriculture 1	Agriculture 2	Urban	Center
Altitude	362.62	21.82	118.32	291.66	52.23	49.68
EVI_a0	0.38	0.44	0.36	0.46	0.27	0.40
EVI_a1	0.15	0.11	0.16	0.15	0.09	0.13
EVI_a2	0.04	0.03	0.05	0.04	0.02	0.03
EVI_a3	0.03	0.02	0.03	0.03	0.02	0.02
EVI_p1	175.56	180.22	185.71	180.92	178.48	175.03
EVI_p2	272.85	180.30	210.01	70.41	142.36	169.18
EVI_p3	127.11	236.47	112.73	162.87	139.93	143.13
% NATURAL	0.77	0.03	0.06	0.16	0.06	0.11
% AGRICULTURE	0.18	0.84	0.82	0.70	0.20	0.62
% ARTIFICIAL	0.04	0.13	0.12	0.14	0.71	0.26
T_DAY_a0	285.17	287.67	287.00	286.17	288.84	287.51
T_DAY_a1	11.04	11.39	11.38	11.56	12.44	11.46
T_DAY_a2	1.55	1.09	1.25	1.44	0.99	1.06
T_DAY_a3	0.50	0.27	0.47	0.56	0.40	0.33
T_DAY_p1	179.32	181.48	178.37	179.07	181.75	180.85
T_DAY_p2	84.95	65.84	92.86	88.90	70.05	75.81
T_DAY_p3	288.98	185.88	271.39	302.68	257.93	265.36
T_NIGHT_a0	277.80	278.91	278.58	277.55	279.89	279.04
T_NIGHT_a1	8.72	7.59	7.96	8.47	8.56	8.17
T_NIGHT_a2	0.40	0.37	0.35	0.40	0.31	0.28
T_NIGHT_a3	0.42	0.29	0.38	0.44	0.37	0.37
T_NIGHT_p1	168.18	164.93	166.67	167.74	167.84	167.29
T_NIGHT_p2	101.35	269.86	278.68	229.38	269.39	278.02
T_NIGHT_p3	285.81	172.51	266.01	294.73	277.41	228.42
NDVI_a0	0.74	0.68	0.60	0.74	0.52	0.67
NDVI_a1	0.11	0.06	0.12	0.06	0.08	0.09
NDVI_a2	0.04	0.05	0.08	0.04	0.03	0.04
NDVI_a3	0.04	0.04	0.05	0.03	0.03	0.03
NDVI_p1	149.60	167.32	198.53	177.93	156.80	159.95
NDVI_p2	138.24	166.29	212.92	92.42	152.74	153.04
NDVI_p3	155.03	197.18	150.34	160.53	130.34	101.64
PREC_MAX	105.40	75.26	79.55	95.74	76.42	75.74
PREC_MEAN	84.95	61.71	68.78	80.02	66.05	65.96
PREC_MIN	69.52	46.21	54.81	65.60	51.46	51.29

3.1.2.2 Model selection

Environmental suitability

The results for the predicted environmental suitability are in Figure . The north-east of the country showed a high environmental suitability for a majority of the considered species, such as *Ae. cinereus*, *An. maculipennis*, *Ae. vexans*, *Cs. annulata*, *Cq. richiardii*, *Ae. cantans*, and *Ae. punctor*. In the southern part of the country a wedge-shaped band of high environmental suitability was noticed.

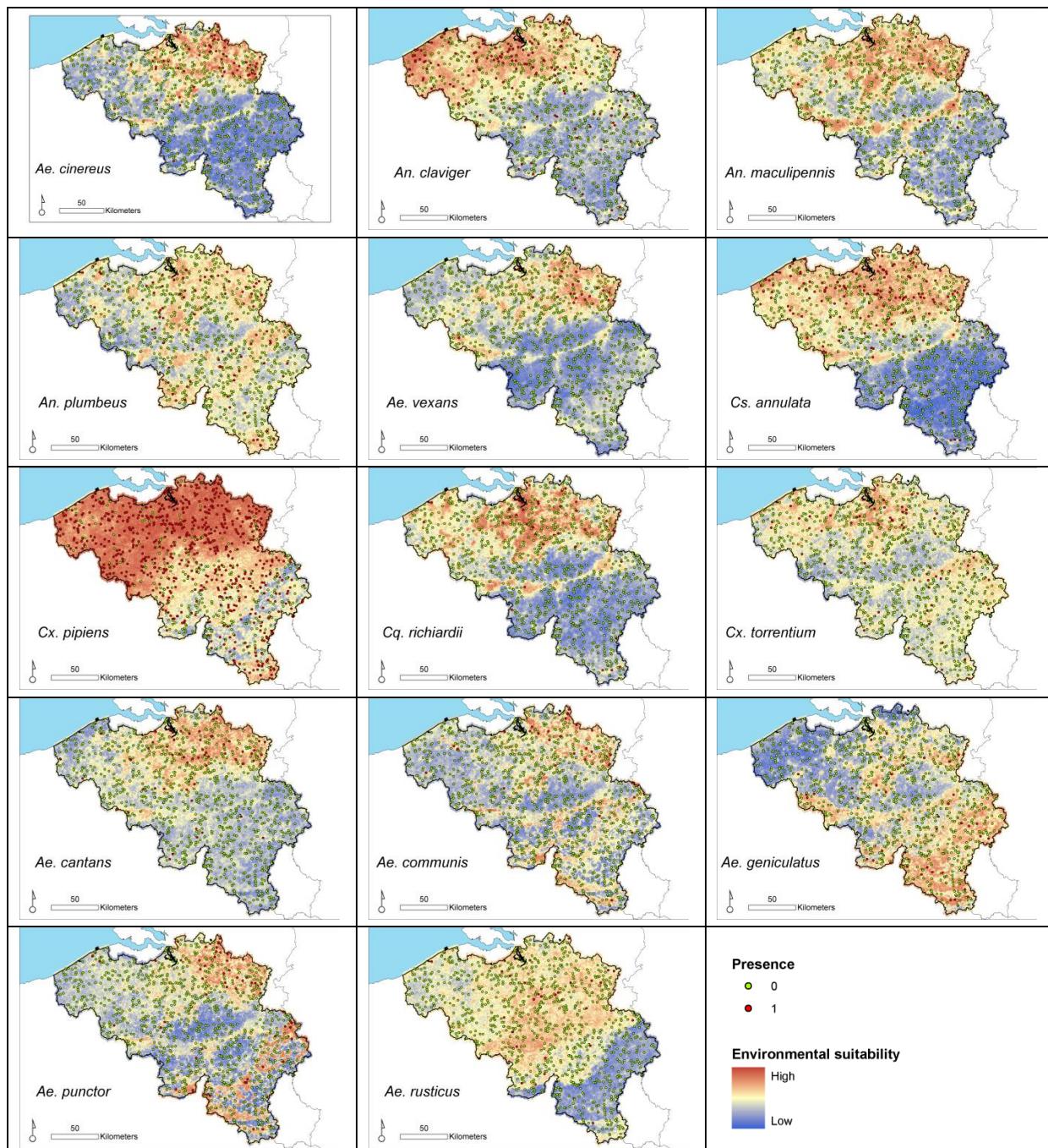


Figure 17. Predictions of environmental suitability for the given mosquito species, using the full dataset. The dots indicate whether the species under consideration was caught on the sampled locations.

Table XII. Variable importance for each variable and each model, as expressed by the mean decrease in GINI. The ten variables contributing most to each model are indicated in bold.

	Mean Decrease Gini														
Variable	Ae. cinereus	Ae. vexans	An. claviger	An. plumbeus	Cx. pipiens	Cx. torrentium	Cs. annulata	An. maculipennis	Cq. richiardii	Ae. cantans	Ae. communis	Ae. geniculatus	Ae. punctor	Ae. rusticus	
agriculture_p	1.47	0.24	1.94	1.62	5.69	1.66	2.12	0.74	0.72	2.57	1.00	1.34	2.03	0.56	
awc	1.17	0.39	1.31	0.97	3.15	0.86	1.77	0.22	0.24	0.69	0.50	0.41	0.42	0.47	
corine	0.20	0.07	0.64	0.41	1.40	0.72	0.61	0.16	0.12	0.34	0.10	0.14	0.31	0.31	
d_protected	3.62	0.81	3.21	1.92	6.45	1.49	3.03	0.64	1.92	2.52	1.03	0.52	1.19	0.37	
d_urban	2.03	0.63	4.32	3.46	8.89	1.21	4.36	1.33	1.18	0.92	0.91	0.93	1.20	0.62	
d_water	1.03	1.50	2.35	1.81	6.93	1.11	2.14	1.60	0.36	0.89	0.34	0.51	1.11	0.86	
dem	2.34	0.90	7.99	1.86	16.68	2.57	7.50	0.80	1.35	1.46	0.32	0.94	1.18	0.74	
broadleaved	0.40	0.06	1.82	0.79	2.51	0.41	0.60	0.11	0.23	0.38	0.08	0.86	0.35	0.12	
coniferous	0.51	0.12	0.69	0.39	1.49	0.34	0.44	0.24	0.15	0.18	0.18	0.23	0.65	0.05	
forest_mixed	0.36	0.02	1.58	0.75	3.61	0.53	0.49	0.15	0.11	0.21	0.99	0.51	0.91	0.26	
natural_p	0.94	0.13	1.37	0.71	6.33	0.56	1.15	0.28	0.13	0.74	1.19	1.33	2.38	0.75	
prec_yearly	2.70	0.82	6.33	2.37	14.74	1.30	6.31	0.99	0.91	1.68	0.46	0.52	1.35	0.59	
prec_yearly_max	1.75	0.57	2.73	1.24	11.76	1.13	4.71	0.49	0.49	1.28	0.25	1.06	0.78	0.52	
prec_yearly_min	2.50	0.60	5.99	1.13	13.99	1.05	5.06	0.86	1.17	1.33	0.40	0.56	0.88	0.46	
evi_max	0.97	0.38	2.97	2.02	6.21	1.66	3.01	0.79	0.61	1.47	0.33	1.01	1.03	0.65	
evi_min	0.90	0.36	2.76	1.80	7.60	1.67	2.35	0.73	0.43	1.28	0.41	1.53	1.10	0.55	
eviamplitude0	1.24	0.60	2.82	2.39	7.30	1.92	2.45	0.66	0.45	1.12	0.37	1.19	1.16	0.70	
eviamplitude1	0.85	0.41	3.09	2.33	6.53	2.28	2.26	0.51	0.62	0.95	0.58	0.63	1.06	0.31	
eviamplitude2	0.83	0.24	3.04	2.22	7.69	1.18	2.17	1.16	0.39	1.12	0.39	0.87	0.90	0.46	
eviamplitude3	0.78	0.41	3.48	1.86	7.59	1.18	2.06	0.70	0.50	0.65	0.42	0.59	1.01	0.66	
eviphase1	0.85	0.43	2.95	2.21	7.80	1.22	2.64	0.84	0.53	1.08	0.57	0.89	0.71	0.90	
eviphase2	0.88	0.40	3.12	2.18	6.48	1.14	2.54	0.71	0.39	1.04	0.83	0.90	2.17	0.73	
eviphase3	0.99	0.38	4.07	3.07	6.41	1.08	2.49	0.93	0.76	1.09	0.80	0.66	0.57	0.64	
freezingdays	0.53	1.13	0.96	0.79	3.04	0.29	0.91	0.13	0.16	0.23	0.29	0.38	0.63	0.31	
freezingnights7	0.86	0.28	2.70	1.59	6.44	1.13	4.16	1.14	0.46	0.86	0.47	0.51	0.52	0.55	
lstday_max	1.01	0.51	3.55	2.88	9.79	1.02	2.81	0.63	1.04	0.92	0.42	0.84	1.55	0.67	
lstday_min	1.13	0.45	3.93	2.08	9.02	1.18	2.75	0.68	0.59	0.97	0.66	0.69	0.77	0.56	

Istday_a0	1.50	0.43	4.93	2.23	13.06	1.09	4.93	0.76	1.25	0.99	1.06	0.94	1.51	1.12
Istday_a1	2.60	0.43	3.77	2.16	6.78	1.21	2.19	1.05	0.55	1.37	1.42	0.70	0.79	0.59
Istday_a2	0.99	0.42	2.82	2.03	10.84	1.22	2.37	0.57	0.67	2.54	0.30	0.51	1.03	0.87
Istday_a3	0.77	0.31	3.14	3.27	6.70	1.95	2.41	0.50	1.51	1.13	0.62	0.61	0.83	0.63
Istday_p1	2.18	1.17	3.93	2.95	7.30	1.43	2.73	2.54	0.59	2.21	0.57	0.73	1.33	0.71
Istday_p2	1.50	0.53	3.03	2.07	8.89	1.13	2.86	0.74	1.30	1.49	0.50	0.68	1.29	0.95
Istday_p3	1.22	0.41	3.84	3.23	7.64	1.79	2.62	0.86	0.65	0.98	0.43	0.54	0.80	0.48
Istnight_max	5.28	1.70	3.58	3.50	6.66	2.06	4.94	1.91	2.23	2.92	1.59	1.27	4.32	0.64
Istnight_min	2.37	0.38	5.60	1.84	8.89	1.59	5.09	0.90	1.67	0.91	0.34	0.87	1.04	0.54
Istnight_a0	3.86	0.80	4.68	2.47	9.12	2.69	5.15	1.45	1.56	1.77	1.15	0.99	1.76	0.92
Istnight_a1	1.28	0.82	3.84	1.86	6.97	1.49	2.59	0.79	0.55	0.93	0.41	2.51	0.97	0.55
Istnight_a2	0.91	0.33	3.59	2.33	8.45	1.21	3.25	0.86	0.35	1.68	0.54	1.24	0.88	0.53
Istnight_a3	0.92	0.25	3.13	2.15	7.38	1.91	3.38	0.87	0.51	1.20	0.44	1.18	1.01	0.54
Istnight_p1	1.34	1.71	3.86	2.04	7.18	1.38	2.98	0.63	0.50	1.37	0.44	1.36	0.70	0.72
Istnight_p2	1.00	0.43	3.65	1.93	7.26	1.62	3.65	1.02	0.35	1.28	0.49	1.42	1.18	0.79
Istnight_p3	0.69	0.44	4.63	1.86	7.53	1.64	2.51	1.19	0.49	1.00	0.49	0.98	1.34	0.70
ndvi_max	1.62	0.30	3.02	2.96	7.11	1.09	2.60	0.54	0.34	1.25	0.82	0.81	1.54	0.60
ndvi_min	0.87	0.44	3.40	2.06	6.54	1.17	2.43	0.88	0.29	1.40	0.81	0.56	2.57	0.43
ndvi_a0	1.07	0.26	2.74	2.55	7.94	1.44	2.78	0.62	0.51	1.43	0.95	0.67	2.47	1.02
ndvi_a1	0.69	0.22	3.38	2.74	6.50	1.28	2.10	0.62	0.46	1.13	0.47	1.12	1.09	0.73
ndvi_a2	1.01	1.06	3.03	2.44	8.15	1.27	2.97	0.99	0.41	1.07	0.50	1.07	1.62	0.38
ndvi_a3	0.78	0.45	3.42	2.16	7.38	2.37	2.93	0.82	0.36	1.13	0.44	0.93	0.68	0.44
ndvi_p1	1.07	0.40	3.16	2.06	8.46	1.31	2.74	0.79	0.75	1.14	0.55	0.68	1.06	1.00
ndvi_p2	0.72	0.34	2.95	2.62	6.65	1.30	2.75	0.64	0.51	0.94	0.47	0.64	0.95	0.50
ndvi_p3	0.84	0.37	3.83	2.44	6.66	1.01	2.48	0.62	0.46	0.84	0.52	0.99	0.86	0.42
population	1.31	0.48	4.37	2.98	8.37	1.44	2.89	1.08	1.06	1.10	0.68	1.18	1.25	0.53
urban_p	0.67	0.17	1.95	1.31	4.34	2.04	1.66	0.55	0.42	0.79	0.26	0.73	0.74	0.37

The importance of each variable for the models of the 14 mosquito species was assessed by the mean decrease in GINI coefficient (Table XII). The ten variables with the highest mean decrease in GINI were indicated in bold.

1) *Aedes cinereus*

The presence of *Ae. cinereus* was explained by the distance of the location to protected areas and urban zones. Also elevation was an important predictive variable. Regarding precipitation, yearly precipitation and the precipitation of the driest month were important. Vegetation indices did not yield a high importance value. Temperature was also related to the presence of *Ae. cinereus*, day temperatures were important, more precisely the first harmonic, defined by a1 and p1, indicating the temperature difference between summer and winter temperatures, and the moment that spring starts. Night temperatures were also explaining presence, but seasonal differences were not. Minimum, maximum and mean annual night temperature (lstdnight_a0) featured in the list of the ten most important variables.

2) *Aedes vexans*

Aedes vexans' presence was explained by the distance to protected areas and water. Urban areas were less important. Elevation explained part of the environmental suitability. Precipitation played a less prominent role than for *Ae. cinereus*, only yearly precipitation featured among the ten most important variables. Five out of ten most important variables were related to temperature: freezing days and the timing of spring (lstday_p1) on the one hand, and maximum night temperature and the amplitude and timing of the summer-winter variation on the other hand. Finally, one vegetation index featured among the most important variables: ndvi_a2, indicating fluctuations in vegetation greenness within seasons.

3) *Anopheles claviger*

This species had a relation with urban areas and population density. Also precipitation, both yearly and minimal rainfall, influenced the environmental suitability. Regarding temperature, yearly averages (a0 for both day and night temperature) and minimum night temperature were among the ten selected variables.

4) *Anopheles plumbeus*

A link to urbanised areas (d_urban and population) was again noticed for *An. plumbeus*. Day temperatures, its variation and minimum night temperatures were also related to environmental suitability. In contrast to the species described above, vegetation indices comprised three out of ten most important variables. Maximum greenness, as well as inter- and intra-seasonal variation was related to the presence of this species.

5) *Culex pipiens*

For this species, not one variable related to land cover or vegetation greenness was retained in the top-10 list. In contrast, all variables connected to precipitation were selected. Day temperature was also related to environmental suitability, especially day temperature, both minimum values, as yearly means, and the size of the variation throughout the year. For night temperature, minimum and yearly means were retained.

6) *Culex torrentium*

Environmental suitability for *Cx. torrentium* was related to the proportion of urban area in the surroundings. Vegetation, and the size of its' variation provided three variables for the top-ten list. The remainder of this list was populated by both day and night temperature and its variability.

7) *Culiseta annulata*

Presence of *Cs annulata* was related to precipitation and temperature. All three variables related to precipitation were selected, and regarding temperature, night temperature (minimum, maximum and mean values) were most determining. Finally, the elevation and the distance to urban areas were important factors.

8) *Anopheles maculipennis*

For this species, distance to water and urban, as well as the population density were important. Precipitation did not feature among the variables of the top-ten list. Temperature-related variables made out the bulk of the important variables, including freezing nights, the difference between summer and winter temperatures, the onset of spring, and mean night temperatures throughout the year.

9) *Coquillettidia richiardii*

For *Cq richiardii*, six out of ten most important variables were related to temperature, both day and night variables were selected. Other factors determining environmental suitability were distance to protected and urban areas, elevation and the precipitation of the driest month.

10) *Aedes cantans*

The presence of *Ae. cantans* was explained by the presence of agriculture, and the distance to protected areas. Yearly precipitation and maximum vegetation greenness also featured in the top-ten list. For night temperature, both the annual mean and minimum were retained. Interseasonal variability in both day and night temperature were also important in predicting environmental suitability.

11) *Aedes communis*

For *Ae. communis*, land cover was important, more specifically the proportion of agriculture, mixed forest and natural land cover and the distance to protected and urban areas. Precipitation and vegetation greenness was less important than temperature, the mean day and night temperature, as well as the maximum night temperature and the temperature difference between summer and winter featured amongst the most contributing variables.

12) *Aedes geniculatus*

The environmental suitability of *Ae. geniculatus* was determined by the proportion of agriculture and natural land cover, and population density. Extra information was provided by minimum and mean vegetation greenness and night temperature, more specifically maximum night temperature and the variation in night temperature over the year

13) *Aedes punctor*

Aedes punctor was similar to *Ae. geniculatus* in the sense that it was related to the proportion of agriculture and natural land cover. In the top-ten list featured generally maximum values : lstday_max, ltnight_max, ndvi_max, as well as mean values: ltnight_a0 and ndvi_a0. Interseasonal variation in vegetation greenness delivered two of the top-ten variables: evi_p2 and ndvi_a2.

14) *Aedes rusticus*

This species was determined by the distance to water and the proportion of natural land cover. Precipitation did not feature amongst the top-ten variables. The onset of spring was indicated by two variables (evi_p1 and ndvi_p1). Environmental suitability was related to three mean values: lstday_a0, ltnight_a0 and ndvi_a0.

Validation of the Environmental Suitability maps

The accuracy of the models for the 14 species was variable (Table XIII). For several species, the result was good, and a PCC of more than 70 % and a AUC higher than 0.8 was obtained. The following species were predicted to a satisfactory level: *Ae. cinereus*, *Ae. vexans*, *Cx. pipiens*, *Ae. communis*, *Ae. punctor*. Fair accuracy was obtained for *An. claviger*, *Cs. annulata*, *Cq. richiardii*, and *Ae. cantans*. On the contrary, the models for *An. plumbeus*, *Cx. torrentium*, *An. maculipennis*, *Ae. geniculatus* and *Ae. rusticus* were considered poor. High sensitivity values (100 % for all species) indicated that the models make an overestimation. This can be explained by the presence of false absences, or the failure of a trap to capture the species even if it is present in the environment in which the trap was positioned.

Table XIII. Accuracy measures for the predictions constructed for each of the 14 modeled species

Species	PCC	AUC	Sens	Spec
<i>Ae. cinereus</i>	82%	0.84	100%	81%
<i>Ae. vexans</i>	79%	0.80	100%	78%
<i>An. claviger</i>	79%	0.68	100%	74%
<i>An. plumbeus</i>	65%	0.62	100%	61%
<i>Cx. pipiens</i>	99%	0.72	100%	97%
<i>Cx. torrentium</i>	69%	0.59	100%	66%
<i>Cs. annulata</i>	72%	0.72	100%	67%
<i>An. maculipennis</i>	62%	0.66	100%	60%
<i>Cq. richiardii</i>	68%	0.78	100%	67%
<i>Ae. cantans</i>	77%	0.72	100%	75%
<i>Ae. communis</i>	72%	0.84	100%	72%
<i>Ae. geniculatus</i>	64%	0.7	100%	62%
<i>Ae. punctor</i>	75%	0.82	100%	74%
<i>Ae. rusticus</i>	54%	0.56	100%	53%

Extrapolation of Environmental Suitability to The Netherlands

Extrapolation of a model outside the geographical zone of the training data, is always risky. The accuracy of the extrapolation of the 14 built models is listed in Table XIV and XV. In the confusion matrices (Table XV), most of the 55 elements were on the diagonal axis, and thus correctly classified. The elements that were wrongly classified were mostly model overestimations, locations where the model predicts a species presence but none were caught. This proportion was particularly high for *An. plumbeus*, *Cx torrentium*, *Cs annulata*, *An. maculipennis*, and *Ae. rusticus*. For *Cx pipiens*, only presences were predicted, and only 3 out of 55 traps did not capture this species. The accuracy measures are found in Table XIII. The prediction of *An. claviger* had a very poor accuracy (PCC 32 %), caused by a low specificity (0.10). The model for *Ae. communis* also had a poor accuracy, since the model predicted that only one out of 55 locations was suitable for this species. Moreover, no specimens of *Ae. communis* was caught at this location. The same applies for *Ae. geniculatus*.

Table XIV. Confusion matrices for model extrapolations for several species in the Netherlands in 2009-2010

Species	pred\obs	1	0
<i>Aedes cinereus</i>	1	3	10
	0	5	37
<i>Aedes vexans</i>	1	1	9
	0	1	44
<i>Anopheles claviger</i>	1	13	36
	0	2	4
<i>Anopheles plumbeus</i>	1	5	23
	0	5	22
<i>Culex pipiens</i>	1	52	3
	0	0	0
<i>Culex torrentium</i>	1	4	27
	0	2	22
<i>Culiseta annulata</i>	1	21	18
	0	8	8
<i>Anopheles maculipennis</i>	1	10	20
	0	14	11
<i>Coquillettidia richiardii</i>	1	7	15
	0	9	24
<i>Aedes cantans</i>	1	2	14
	0	7	32
<i>Aedes communis</i>	1	0	12
	0	1	42
<i>Aedes geniculatus</i>	1	0	10
	0	1	44
<i>Aedes punctor</i>	1	2	15
	0	12	26
<i>Aedes rusticus</i>	1	2	24
	0	1	28

Table XV: Accuracy measures for model extrapolations for several species in the Netherlands in 2009-2010

Species	PCC	Sens	Spec
<i>Aedes cinereus</i>	80%	0.38	0.79
<i>Aedes vexans</i>	83%	0.50	0.83
<i>Anopheles claviger</i>	32%	0.87	0.10
<i>Anopheles plumbeus</i>	54%	0.50	0.49
<i>Culex pipiens</i>	95%	1.00	0.00
<i>Culex torrentium</i>	49%	0.67	0.45
<i>Culiseta annulata</i>	62%	0.72	0.31
<i>Anopheles maculipennis</i>	51%	0.42	0.35
<i>Coquillettidia richiardii</i>	67%	0.44	0.62
<i>Aedes cantans</i>	71%	0.22	0.70
<i>Aedes. communis</i>	78%	0.00	0.78
<i>Aedes geniculatus</i>	81%	0.00	0.81
<i>Aedes punctor</i>	65%	0.14	0.63
<i>Aedes rusticus</i>	56%	0.67	0.54

Mosquito hotspot mapping

Using the predictions of environmental suitability for the given species, a species diversity map was created (Figure 18). This provided an indication of hotspots of mosquito diversity in Belgium. The north-eastern part of the country features a high diversity of mosquito species. Also the area in the southern tip, near the larger city of Arlon has a high species diversity. This also holds true for an east-west band running through the southern part of the country. When overlaying this diversity map with the locations where less common species were found, it was striking that all rare species also occurred in the high diversity zone.

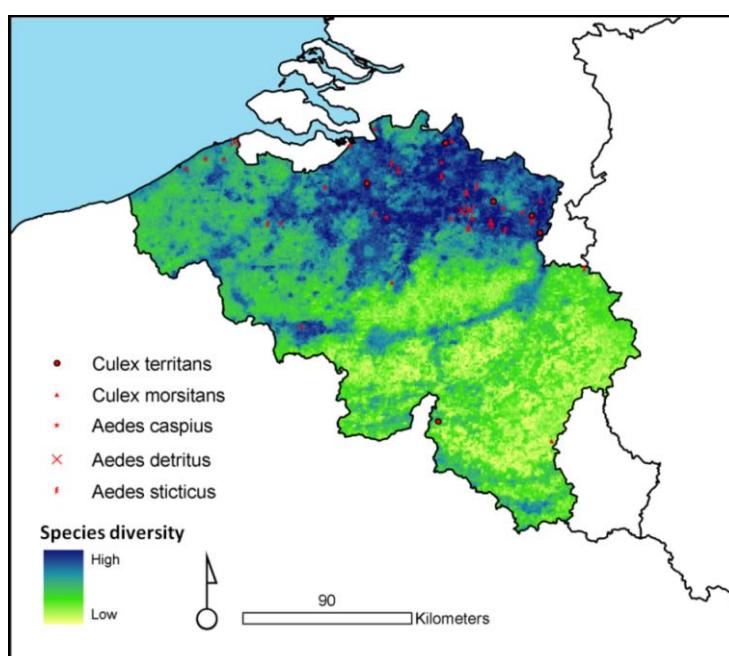


Figure 18. Species diversity map for 14 species ; different symbols (in red) were used to indicate the locations where rare species were caught.

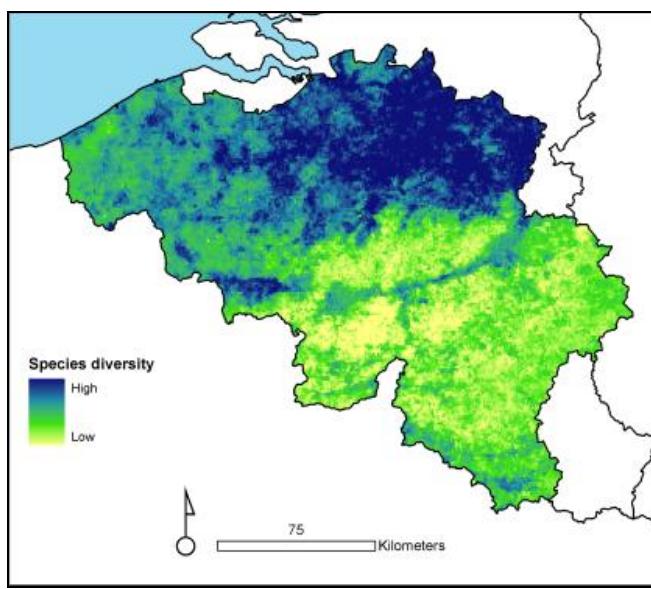
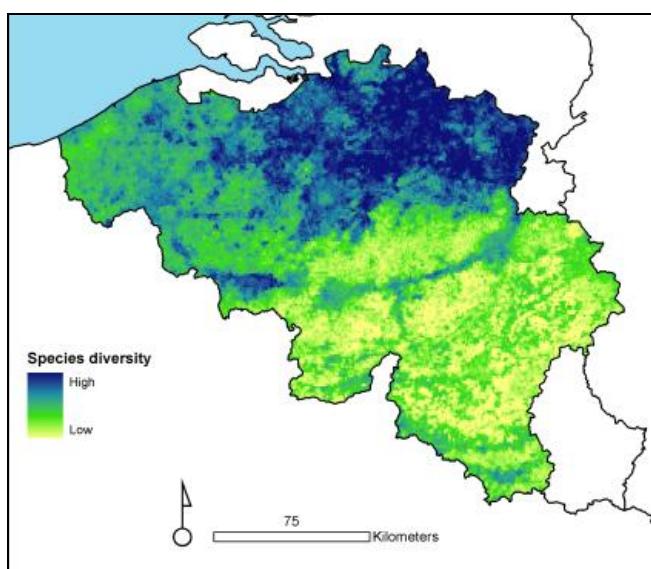
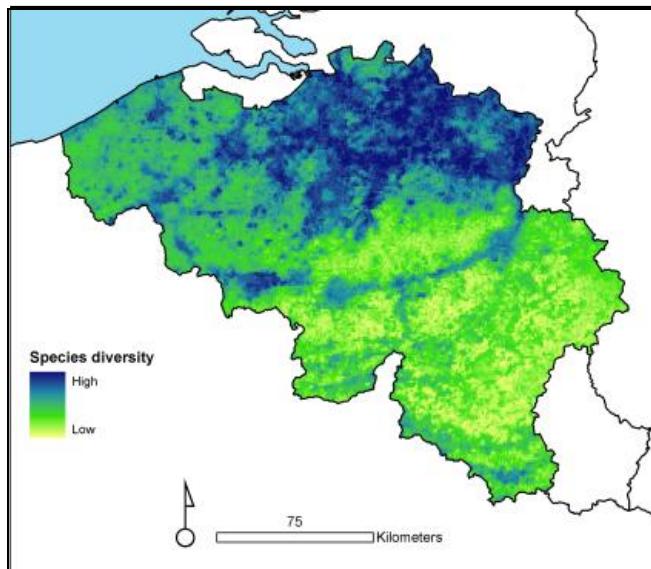


Figure 19 Comparison of the species diversity map, based on respectively 14, 8 or 4 species

In Figure 19, three versions of the species diversity map were compared. When building the species diversity map based on the eight best models, a similar trend could be observed than on the species diversity map based on all constructed models. Taking this logic further, we constructed the species diversity map based on the four most common species (listed in previous Tables). Again, the same pattern was observed on this map. In Figure 19, the species diversity based on 14 species was compared with the species diversity based on respectively eight and four species was made, and the correlation between these was computed. A R^2 of 0.94 was observed when the habitat area for eight species was used; R^2 was 0.85 when the environmental suitability for four species was used (Figure 20). This result leads to the conclusion that if the goal is to obtain a rapid assessment of the species diversity of mosquito in a region, it suffices to obtain a good model for the four most common mosquito species. In Belgium, these four species were *Ae. cinereus*, *Ae. vexans*, *Cq richiardii* and *Cx pipiens*.

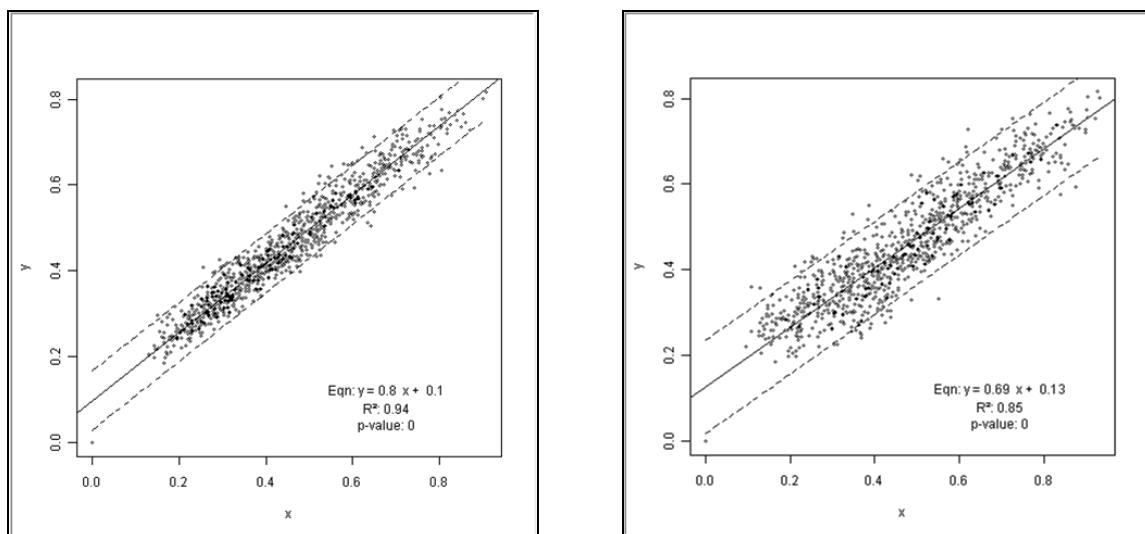


Figure 20. Scatterplots of co-occurrence values based on 14 species (y-axis) and respectively 8 (left) and 4 (right) species (x-axis).

A second approach in constructing a map of hotspots of mosquito diversity comprised modelling the species richness based on the species richness, as observed in the traps. Before feeding the observed species richness into the model, this variable was tested for spatial autocorrelation using a semi-variogram (Figure 21). The semi-variogram did not show a clear trend, so it could be concluded that no spatial correlation was present. The traps were several kilometres apart, which reduces the chance of finding spatial autocorrelation between the observed species richness of two neighbouring traps.

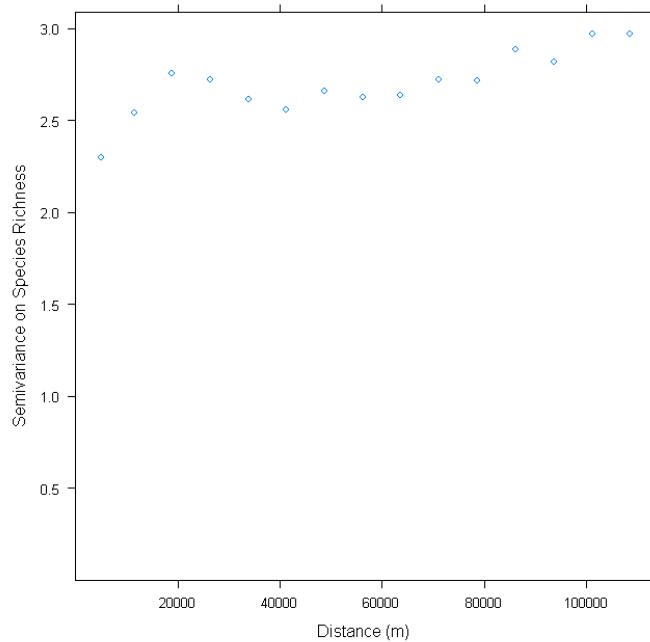


Figure 21. Semi-variogram for the observed species richness in the traps.

The predicted species richness was low in most locations (Figure 22), since most data points (*i.e.* observed data) that were fed into the model equally had low species richness. The highest predicted species richness was 8 (versus an observed maximum of 12 species in one trap).

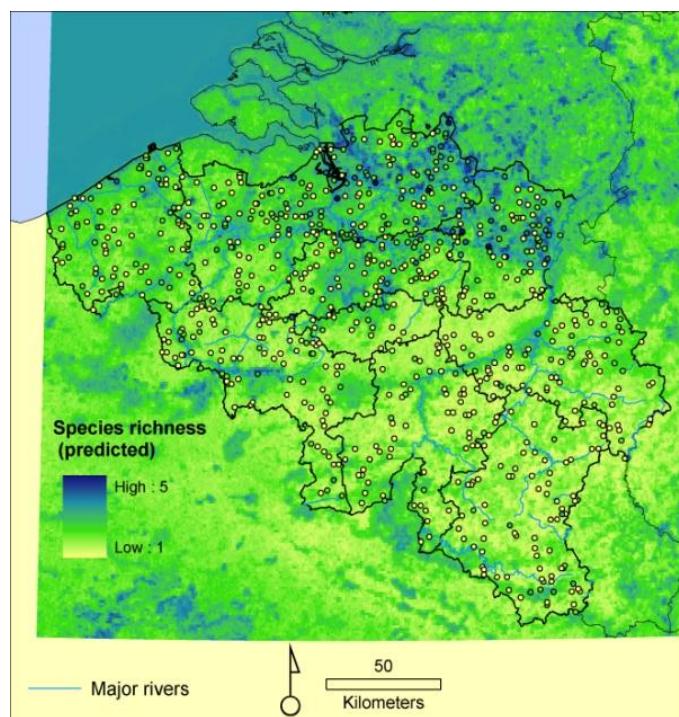


Figure 22. Map of predicted species richness.

3.2 *Anopheles plumbeus*: shift of habitat and risk for autochthonous malaria in Belgium

3.2.1 Methodology

Adult monitoring and larval sampling of *An. plumbeus* was done at one of the nuisance sites, a particular locality in Torhout (51°04'13.84"N and 3°07'33.59"E), situated 350 m of the Groenhovebos forest complex during spring and summer 2009 (Figure 22). Two MMLP traps (MMLP01 and MMLP02) were installed at the site which operated 48 hours, fortnightly starting at 12:00 20th of April 2009 until 10th of October 2009. The traps were emptied 13 times.

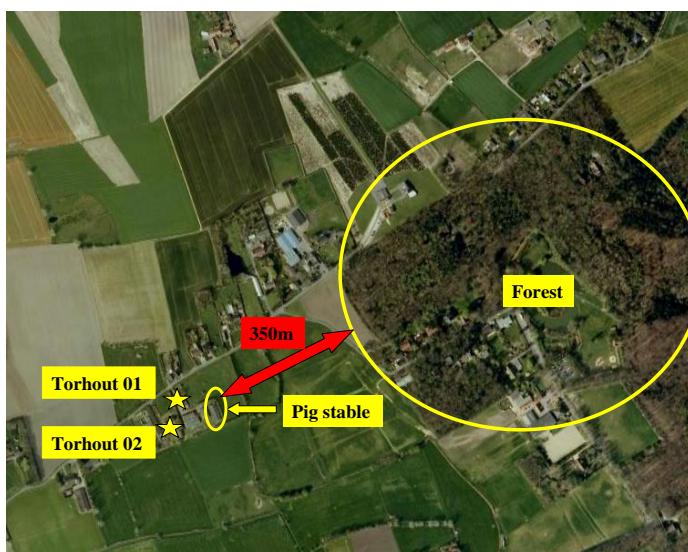


Figure 22: The pest sites in Torhout, situated 350 m of the Groenhovebos forest complex.

Larval study

In order to unravel potential larval breeding places of *An. plumbeus* in urban habitats we surveyed from May 2009 onwards one of the sites where outbreaks of *An. plumbeus* were reported. We therefore screened all potential natural and human-created habitats, hereafter called potential breeding sites (PBS in total 26 see Figure 23.), that could act as breeding sites at the pest site.



Figure 23: Overview of all sampled PBS (1 to 26) at the pest site in Torhout

In each PBS a net (12 cm width and 17 cm large) and 500 ml-capacity dipper were used three times per survey. We explored nine potential natural mosquito breeding sites (PBS01-PBS10) at two different periods (beginning of July and end of September) in 2009 and eight human-created potential breeding sites in September 2009 (PBS11-PBS18). Moreover eight artificial breeding containers (small flower pots with $\varnothing = 18$ cm, height = 20 cm) were installed at the study site (four PBS at each edge of the garden in one line, 20 meters separated from each other) in June 2009 (PBS19-PBS26 in red on Figure 23) and inspected for the presence of larvae simultaneously with the other PBS. They were filled with an infusion-baited mixture (Scott et al. 2001).

Phenology

In order to understand phenology, the dynamics of its populations and adult competition of this species in both natural sites and urban sites we monitored adult mosquitoes at two localities. We sampled mosquitoes at two different localities in Western Flanders in the same eco-climatic region, during one complete active season from May until October:

- an urban-rural landscape was sampled: 2 sites at Torhout near Groenhove forest complex (TH01 and TH02) (Figure 24)
- a natural landscape was sampled: 2 sites at Ruijselede, Vorte Bossen (RL01 and RL02) (Figure 25).

Each locality was sampled with six traps (3 types, 2 of each type). Following traps were used: (1) Mosquito Magnet ® Liberty Plus (called MMLP01 and MMLP02); (2) BG Sentinel trap (called B01 and B02); (3) the CDC J. Hocke Gravid traps (called G01 and G02) which collect the egg laying female mosquitoes.

Traps were placed in such a manner that they did not interfere or that the interference was reduced to the minimum. Near each MMLP a data logger for temperature and humidity recording was placed (<http://www.mulder-hardenberg.com>). The sites were sampled fortnightly for two consecutive days from the 20th of April 2009 until the 5th of October 2009, in total 13 adult sampling moments took place.

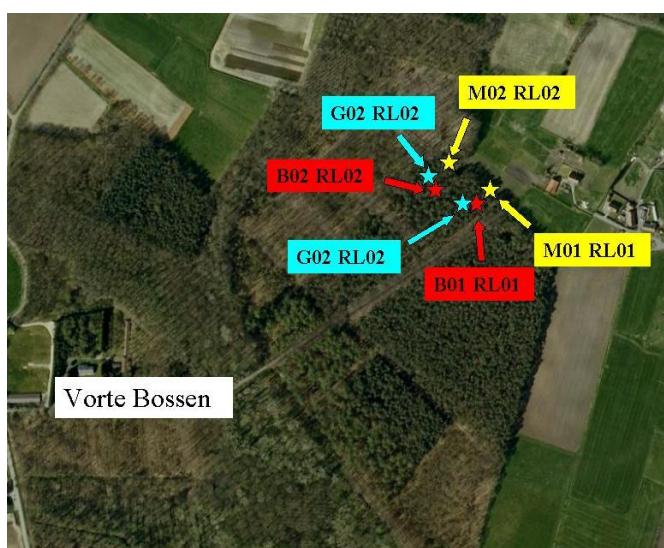


Figure 24. Detailed overview of the position of the mosquito traps at the Vorte Bossen in Ruijselede (RL), with M=MMLP, B=BG Sentinel trap and G=Gravid trap.

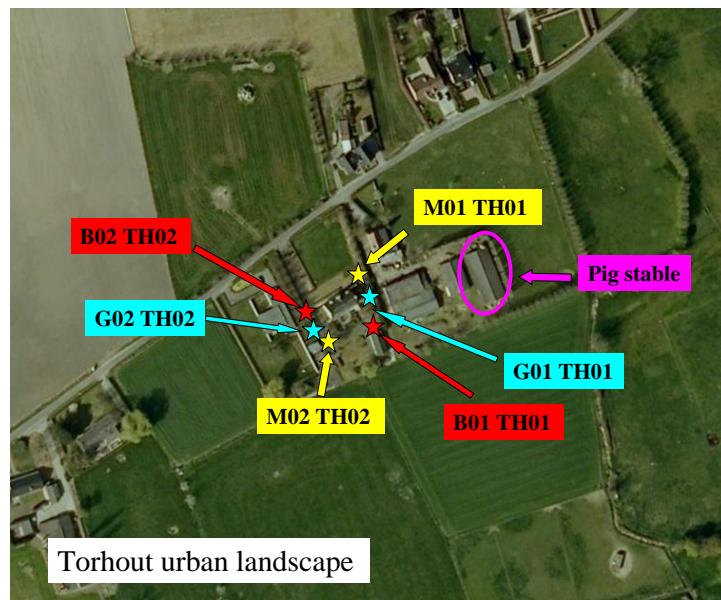


Figure 25. Detailed overview of the position of the mosquito traps at the pest site in Torhout (TH), with M=MMLP, B=BG Sentinel trap and G=Gravid trap

3.2.2 Results

Larval survey

Larvae of three different mosquito species were found at the different PBS sampled in the present study (Table XVI). Besides *Culex pipiens*, found in 73 % of the surveyed breeding sites, *Cs annulata* larvae (7 PBS) also appeared to be present in both natural and human-created PBS. *Anopheles plumbeus* larvae were found in only four surveyed sites, albeit in often large numbers. The most important breeding site was found in an abandoned yet uncleaned pig stable, where larvae breed in subterranean manure collection tanks in the water on top of the manure. Here, more than 30 larvae were found in a single net sweep. Nevertheless, smaller larval numbers were found in PBS15 (a pond in the garden) and PBS18 (wet sewerage along the road), indicating that females also used other human created PBS to lay eggs. Although present at the study site in Torhout, second hand tires (PBS11, 12 and 13) were not colonized by *An. plumbeus*. Nevertheless studies by Schaffner et al. (2004) and Versteirt et al. (2009) demonstrated that they are a potential human induced breeding site for this mosquito elsewhere in Belgium.

Table XVI: Surveyed potential mosquito breeding sites at the *Anopheles plumbeus* pest site and the average number of larvae collected during one survey with AN PLU=*Anopheles plumbeus*, CX PIP=*Culex pipiens* and CS ANN=*Culiseta annulata*.

Code of the site	Description of the site	n larvae/survey		
		AN PLU	CX PIP	CS ANN
PBS01	pond in sheep meadow	/	17	/
PBS02	small brooklet along small path in fields	/	8	/
PBS03	small brooklet at the side of the road	/	12	6
PBS04	small brooklet at the side of the road	/	1	10
PBS05	very small brooklet near agricultural (maize) field	/	1	4
PBS06	very small brooklet near agricultural (maize) field	/	/	/
PBS07	very small brooklet near agricultural (maize) field	/	/	1
PBS08	very small brooklet near garden	/	/	/
PBS09	very small brooklet near garden and compost heap	/	/	/
PBS10	lower and upper case cavity in Willow trees along the road	/	/	/
PBS11	small second used tyre	/	6	/
PBS12	large second used tyre	/	7	/
PBS13	very large second hand tyre	/	3	19
PBS14	pond in garden with fish	/	/	/
PBS15	pond in garden with fish	2	/	/
PBS16	subterranean manure collection tank in pig stable	105	4	/
PBS17	manure pit of pig stable	52	19	/
PBS18	sewerage along the road	1	3	/
PBS19	artificial breeding site 1 shady, closed vegetation	/	28	/
PBS20	artificial breeding site 2 sunny, open vegetation	/	9	/
PBS21	artificial breeding site 3 shady, closed vegetation	/	18	/
PBS22	artificial breeding site 4 sunny, open vegetation	/	17	5
PBS23	artificial breeding site 5 shady, under hedge	/	18	/
PBS24	artificial breeding site 6 shady, closed vegetation	/	12	/
PBS25	artificial breeding site 7 sunny, open vegetation	/	24	/
PBS26	artificial breeding site 8 sunny, open vegetation	/	13	2

Adult survey

At the nuisance site adults were found foraging in neighboring gardens where they preferred places shaded with trees and hedges. Adults were collected from beginning of May until the beginning of September for MMLP02 and the end of September for MMLP01 (Table XVII). Large numbers of adult *An. plumbeus* were collected in the beginning of June for MMLP02 (5034 during two days in beginning of June and 7121 half June) and half July and half August for MMLP01 (2038 and 1785 reps.).

Table XVII: Number of adult *An. plumbeus* collected during 48 hours fortnightly from 20th of April until 7th of October 2009 with MMLP

Start date	End date	MMPL01	MMPL02
20 April 2009	22 April 2009	0	0
4 May 2009	6 May 2009	1	0
18 May 2009	20 May 2009	754	1224
1 June 2009	3 June 2009	182	5034
15 June 2009	17 June 2009	728	7121
29 June 2009	1 July 2009	1471	2054
13 July 2009	15 July 2009	2038	442
27 July 2009	29 July 2009	513	924
10 Aug. 2009	12 Aug. 2009	1785	1132
24 Aug. 2009	26 Aug. 2009	180	340
7 Sept. 2009	9 Sept. 2009	631	663
21 Sept. 2009	23 Sept. 2009	101	0
5 Oct. 2009	7 Oct. 2009	0	0

Phenology

In total 33682 adult mosquitoes were collected at the four sites with the twelve traps. Twelve species were collected at the Vorte Bossen in Ruiselede and five species in the pest locality, the garden in Torhout. The MMLP-traps collected 83% of all collected mosquitoes, the BG-Sentinel traps 13% and the Gravid-traps 4%. Both males and females were collected with the traps. However 94% of the mosquitoes collected with MMLP and 81% collected with the BG-Sentinel were females, whereas the gravid trap collected as much females as males of *An. plumbeus* (resp. 49.7 % and 50.3%). 97.66 % of all collected *An. plumbeus* were caught in Torhout 01 and Torhout 02. In this locality hardly any specimens of other mosquito species were collected. At all the sampled sites the activity-peak of *An. plumbeus* was between the end of June and the end of July. In the forest at Ruiselede almost all other species were collected in the spring before June.

At the nuisance site

During the first three months of the sampling we found a positive correlation between the average temperature and the number of adult *An. plumbeus* collected (Figure 26). After this building up of the population we found no longer a positive correlation between the number of adult mosquitoes and the average temperature at the site. These results suggest a characteristically phenology of a successful (nuisance) species in a habitat with the right temperature, food availability and without limiting factors and lack of competition, at least for this part of the season. After the population reached his peak density, probably the abiotic conditions for quick development of the larvae in the manure pit of the abandoned pig stables become less favorable. This might result in a decrease of the population. Maybe the manure pits become too dry or the composition of the stagnant water changes. More research is needed to confirm this. From the beginning of August the numbers of adult *An. plumbeus*

collected at both sites decreases and at the beginning of October *An. plumbeus* were no longer collected. Probably at that time the temperature was already too cold for this species.

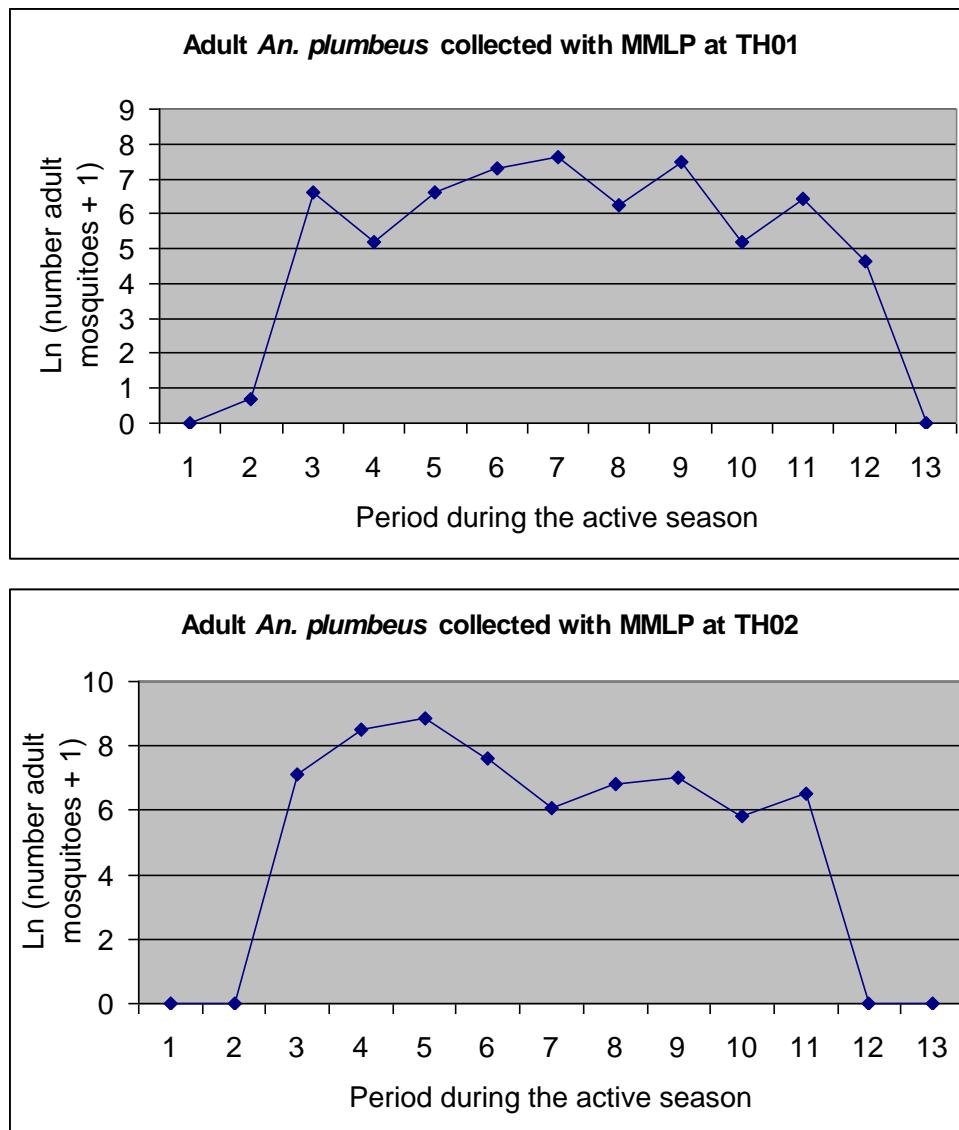


Figure 26. Phenology of adult *An. plumbeus* collected with MMLP at TH01 and TH02.

At the forest site

In the forest site the number of adult *An. plumbeus* was much lower than in the urban pest site (Figure 27). Moreover we collected more other species in the forest than in the urban site. Most of the *Aedes* species were collected early in the active season. Later on from half July the only two species collected in the forest were *An. plumbeus* and *Culex pipiens*.

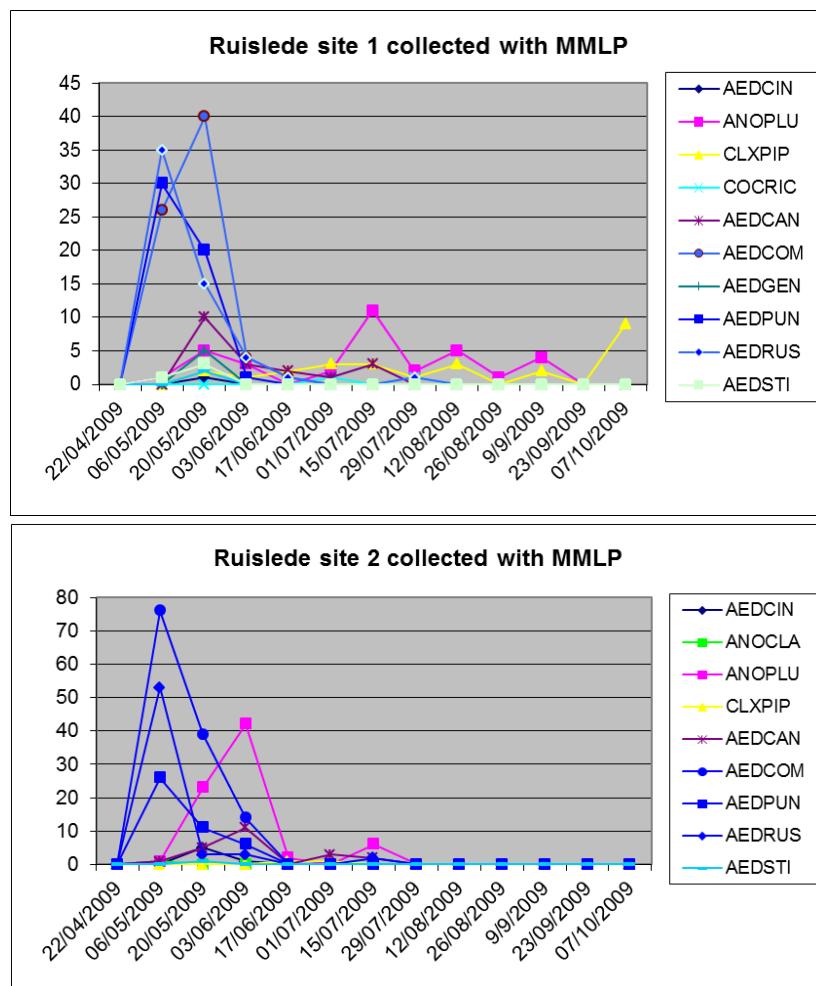


Figure 27. Number of collected adult mosquitoes for all species found in the forest sites, with AEDCIN=Ades cinereus, ANOCLA=Anopheles claviger, ANOPLU=Anopheles plumbeus, CLXPIP=Culex pipiens, AECAN=Aedes cantans, AECOM=Aedes communis, AEPUN=Aedes punctor, AERUS=Aedes rusticus and AESTI=Aedes sticticus

A different phenology of *An. plumbeus* and its sympatric mosquito species was observed in the forest compared to the urban pest site. In the latter the adult population was built up very quickly from half May forward and reaches a peak density of adult *An. plumbeus* mosquitoes half June, half July. In the forest there is no such building up of the population. We only see some small peaks of activity and adult density spread over the active season.

Probably these differences can be explained by the origin and features of the larval breeding places. In the urban site there is a large breeding site: the manure pit. In the forest the larval breeding sites are small tree-holes. Moreover these tree-holes are more dispersed over the forest site and this could result in a more as well temporal as spatial scattered emergence of the adult mosquitoes. In the manure pit in the urban site, characteristics of the larval breeding site are more uniform all over the active season, resulting in a more continuously emergence of adult mosquitoes.

Expanded distribution of *An. plumbeus* in Belgium

According to historic descriptions on its distribution and habitat preference, *An. plumbeus* was not recorded in Belgium before 1925 (Goetghebuer 1925). A re-evaluation of RBINS

mosquito collections (see 2.3) revealed that the first record of *An. plumbeus* goes back to 12-VII-1938 when Goetghebuer collected this species in Rouvroy near Torgny. Between its first discovery in Torgny and 1975 this species was only occasionally collected in Belgium and the majority of these records stem from highly forested regions (Figure 28).

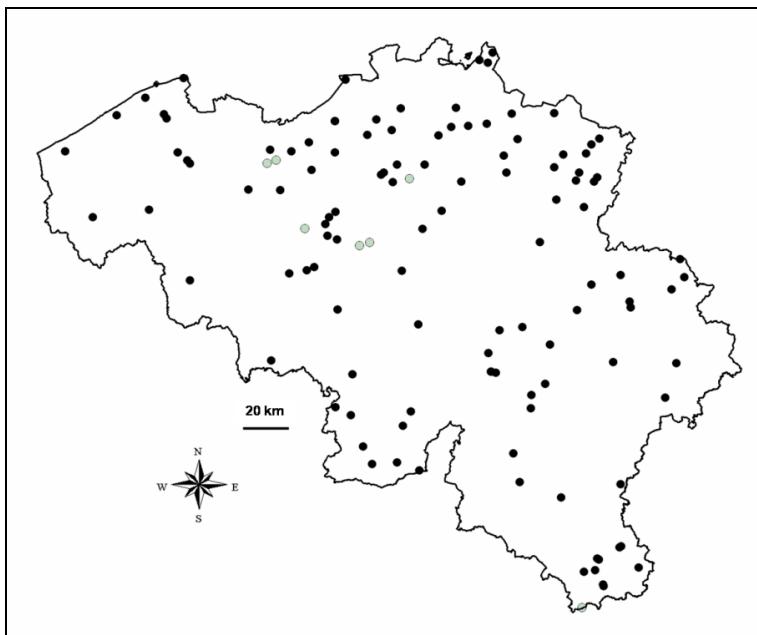


Figure 28: Former records and present distribution of *Anopheles plumbeus* in Belgium, black dots records 2007-2008, grey dots records <1975

To demonstrate the expanded present distribution rate of *An. plumbeus*, distribution data of mosquitoes obtained during the MODIRISK-project were retrieved. *An. plumbeus* was found at 114 localities spread over Belgium. We recorded 30 observations in urban habitats (17.3 % of all urban sampled sites), 47 in agriculture habitat (8.3 % of all agriculture sampled sites) and 37 in natural habitats (18.6 % of all natural sampled sites).

3.3 *Anopheles plumbeus* nuisance in the Netherlands

3.3.1 Methodology

In June 2006 Wageningen University was alerted by inhabitants of Lent village (near Nijmegen) about a plague of nuisance mosquitoes, that were aggressively biting outdoors throughout the evening. A preliminary survey with CO₂-baited traps revealed the unexpected high densities of *An. plumbeus*. Prior, nuisance caused by this mosquito species had not been reported in The Netherlands.

The natural habitat of *An. plumbeus* are old deciduous forests where they use rainwater-filled treeholes as oviposition sites (Becker et al 2003). Since a few years *An. plumbeus* has become settled at farms with fluid manure storage basins or with abandoned rainwater-filled car tyres. In It often concerned former (pig)farms where these basins are no longer filled with manure but with rainwater. *Culex pipiens* and *Cs annulata* also use these basins as oviposition sites. In some places this resulted in a huge nuisance in the summer caused by

the hugh number of *An. plumbeus* that emerged. To get an overview of the population dynamics of *An. plumbeus* in his old- as well as in its new habitat, their numbers were monitored in 2009 and in 2010 on three (former) farms and in three old deciduous forests.

Description of study sites:

The mosquito collections were done in three farms and at three natural forest areas. Farm1 was in Veur Lent, near Nijmegen (Gelderland). The other two farms were in Geffen and Mill (North Brabant). Forest sites were in Oosterhout, Loenen aan de Waal and Hemmen (Gelderland). Two other farms (Geffen and Mill) were only surveilled in 2010.

Veur Lent: The farm is on the edge of the village Veur Lent in a small scaled agricultural landscape. The village is situated on both sides of the dyke that prevents water of the river Waal to flood the inland. On the other side of this river is the town Nijmegen. The centre of the nuisance caused by *An. plumbeus* was at a former pigfarm close to the there present rainwaterfilled manure basin. Close to that there was in 2008 also a pile of old tyres present in which in some of them larvae of *An. plumbeus* were found. However, in 2009 these tyres were covered with plasticand no mosquito larvae were found here anymore.

Geffen: This former pig farm is situated on the edge of the village Geffen. There are two basins for fluid manure in which there is now only ground- or rainwater present. The water is still very black caused by a high amount of organic material. The surroundings consists mainly of a smallscaled agricultural landscape (pastures alternating with maize fields).

Mill: The farm is situated on the edge of the village Mill. On this former cattle farm two big fluid manure basins are present of which one is still being used. It concernes manure of a cattle farm from nearby. The other basin is filled with rather clear rainwater. In the surrounding there are a lot more farms with storage of fluid manure underground but here it concerns mainly pig farms.

Oosterhout Forest: This forest is situated 3.5 km northwest of Veur Lent and can be characterized as the *Violo odoratae ulmetum*. This is a typical riverine forest in which oaks (*Quercus robur*), ashes (*Fraxinus excelsior*) and beeches (*Fagus sylvatica*) dominate among the highest and oldest trees while among the lower trees maples (*Acer pseudoplatanus*) are the most common and in the bushlayer hasel (*Corylus avellana*) and *Prunus padus*. On the ground ivy (*Hedera helix*) is the most common plant. There are many standing and lying dead trees present. The soil consists of a thick layer of litter on a sandy soil. These so called "overslaggronden" originate by a major break-through of the dyke in the past. The mean groundwaterlevel is about 2.5 m under the ground level..

Loenen aan de Waal Forest situated about 8 km northwest of Lent. It concerns an old deciduous forest of the type *Violo odoratae ulmetum*. Oaks and ashes dominate the treelayer while in the bushlayer *Sambucus nigra*, *Prunus padus*,*Crataegus monogyna* and *Corylus avellana* prevail. Among the weeds *Circaeae lutetiana*, *Stachys sylvatica* and *Festuca*

gigantea belong to the most common ones. The soil beneath is consists of sand or "zavel". The groundwaterlevel is about 2 m beneath groundlevel.

Hemmen Forest: This forest is situated about 7 km northwest of forest 2 and can be characterized as a Fraxino ulmetum typicum. The highest trees consists mainly of oaks and ashes while in the bushlayer *Corylus avelana*, *Prunus padus*, and *Symporicarpos albus* prevail. Among the weeds *Aegopodium podagraria* is one of the most common ones. The soil consists of clay and a litterlayer does not exist. The groundwaterlevel is about 1.5 m beneath the ground.

Mosquito sampling and identification:

The MMLP trap was used to catch the mosquitoes. Automated data loggers (Tinytag, UK) were placed in each of the six study sites. Maximum, minimum temperatures and relative humidity (air moisture content) were recorded at 5 min. intervals. Mosquitoes were identified using the key by Schaffner (1993). *Anopheles maculipennis* s.l. were further identified to sibling species by PCR, according to the method described in 2.5.

3.3.2 Results and discussion

Farms

In Table XVIII the total catch of mosquitoes per location is presented for the period July to December 2009. The most common species on all four locations were *Cx pipiens* and *An. plumbeus*. The high number of *An. plumbeus* in farm 1 (Veur Lent) is probably the consequence of the presence of one or a few large oviposition places of this species as the rainwater-filled basin under the former pig stable and old tyres although the latter were cover with plastic in 2009. Larvae of *An. plumbeus* are capable to stay under water for a long time once they have noticed a disturbance on the watersurface (E.J.Scholte, personal communication).

In Table XVIII the total number of mosquitoes is presented caught on the three farms and in the three forests in 2010. Again, *Cx pipiens* and *An. plumbeus* appeared to be the two most common mosquito species. When the catches of the three farms are compared then the number of caught *Cx pipiens* appeared to be less present on farm 1 (Veur Lent) than on the other two farms. *Anopheles plumbeus* appeared to be most present on farm 3 (Mill) compared to the two other farms. Oviposition places of *An. plumbeus* could not be found in 2010. This in spite of the fact that the basins with fluid manure or rainwater that were present on all three farms had been sampled for mosquito larvae many times (weekly, with several dips per basin). Only larvae of *Cx pipiens* and *Cs annulata* were found in underground basins filled with rainwater. These two species had also been found in rainwaterfilled tyres .The species diversity among mosquitoes was on farm 3 less then on the other two farms; *Aedes* species were absent.

Forests.

Although the three forests are quite comparable concerning their vegetation, major differences were found in the species composition of the mosquitoes present. The high

number of *Cx pipiens* in 2009 in forest nr 2 (Loenen an de Waal) has probably been caused by a roost (Table XVII) since the species feeds mainly on birds. Indeed, after several visits in the evening and in the early morning there appeared to be a sleeping place of carrion crows, which group consisted of several dozens of birds. Table XVIII as well as Table XVIII shows that in forest 1 (Oosterhout) the lowest number of individuals and species were caught. This is probably because this forest is the driest of the three forests with the lowest mean groundwaterlevel in summer as well as in winter. In contradiction, forest nr 3 (Hemmen) had the highest number of mosquitoes and species diversity caught. The soil here consists of clay and the groundwaterlevel usually is high. Even after a drought mosquitoes can always find somewhere ovipositionplaces. Illustrative for this is the fact that in this forest *Cx territans*, a species that parasitises amphibians, has been repeatedly found in 2009 and 2010.

The number of *An. plumbeus* caught in the three forests were much lower then those found at the three farms (in 2010 only 6.4% was collected in the forests). Of the three forests, most *An. plumbeus* were found in forest nr 3 (Hemmen). Both observations can ben explained by the availability and size of oviposition sites for this speices.

Table XVIII - Total number of mosquitoes caught in 2009 on four sites in the Betuwe (farm 1, forests 1, 2 and 3)

	Veur Lent	Oosterhout	Loenen a/d Waal	Hemmen
<i>Culex pipiens</i>	259	62	1252	307
<i>Culex torrentium</i>	0	6	4	4
<i>Culex territans</i>	0	0	3	4
<i>Culex modestus</i>	1	0	4	0
<i>Culiseta annulata</i>	1	2	3	8
<i>Culiseta morsitans</i>	0	0	0	7
<i>Coquillettidia richardii</i>	1	0	0	0
<i>Anopheles maculipennis</i>	1	1	2	2
<i>Anopheles plumbeus</i>	829	50	13	188
<i>Anopheles claviger</i>	27	0	0	1
<i>Aedes cantans</i>	1	1	0	0
<i>Aedes riparius</i>	0	0	1	0
<i>Aedes rusticus</i>	5	0	0	0
<i>Aedes communis</i>	1	0	1	1
<i>Aedes cinereus</i>	1	0	0	0

The high number of caught *Culiseta* species in forest 3 (Hemmen) is also remarkably, also as far as the number of species is concerned. Especially *Cs morsitans* was common in forest 3 in 2010. Quit remarkable was the presence in this forest of the rather rare species *Cs fumipennis* and *Cs suborchea*. In forest 2 (Loenen a/d Waal) as well as in forest 3 (Hemmen) several *Aedes* species (*Ae. cantans* and *Ae.rusticus*) were common and can be a nuisance when they have their peak activity. Other *Aedes* species were found only occasionally like *Ae. cinereus/geminus* and *Ae. vexans* which were mainly found on farm 1 (Veur lent).

	Farm 1	Farm 2	Farm 3	Forest 1	Forest 2	Forest 3
	Veur Lent	Geffen	Mill	Ooster- hout	Loenen	Hemmen
<i>Culex pipiens</i>	169	1489	1703	34	250	257
<i>Culex torrentium</i>	30	164	222	9	38	34
<i>Culex territans</i>		2				2
<i>Culiseta annulata</i>	3	26	1	1	10	14
<i>Culiseta suborchea</i>		2			1	2
<i>Culiseta morsitans</i>		1			3	38
<i>Culiseta fumipennis</i>					1	1
<i>Coquillettidia richardii</i>		2			1	
<i>Anopheles maculipennis</i>	4	16	5	2	4	4
<i>Anopheles plumbeus</i>	669	775	1516	32	21	149
<i>Anopheles claviger</i>		16		1	1	2
<i>Aedes vexans</i>	2					
<i>Aedes cantans</i>	7	1			4	31
<i>Aedes annulipes</i>						1
<i>Aedes punctor</i>					3	1
<i>Aedes rusticus</i>	2	1			6	5
<i>Aedes communis</i>					1	1
<i>Aedes cinereus</i>	2					3

Phenology of *Anopheles plumbeus* at the study sites

In Figure 29 the number of *An.plumbeus* on the four places in the Betuwe (farm 1 and the forests 1,2 and 3) is shown during 2009. On farm 1 (Veur Lent) the species shows two large peaks, of the first one the peak is reached beginning of June and the second at the end of August. The flow of the number of *An.plumbeus* found in the three forests is not synchronic. When, for instance, in forest 1 a peak has been reached in the other forest their number has gone down and a minimum was reached. This difference among the three forests probably has been caused by differences in the date on which eggs were laid and the growth rate of the larvae. These differences in oviposition time is translated in the date that the first mosquitoes appear. The presence of two big peaks of *An. plumbeus* on farm 1 (Veur lent) and many small peaks in the three forests probably is related to the presence of one or a few big oviposition sites at this farm and (many) more oviposition places (waterfilled treeholes) in the three forests. The number of *An. plumbeus* found on farm 1 (Veur Lent) are a multitude of the number found of this species in the three forests. This is in agreement with the already observed pattern that *An. plumbeus* occurs in its new habitat (farms with abandoned basins for fluid manure) in huge numbers while in their natural habitat (old deciduous forests) their numbers are generally moderate. In their new (man-made) habitat this species can cause a large nuisance.

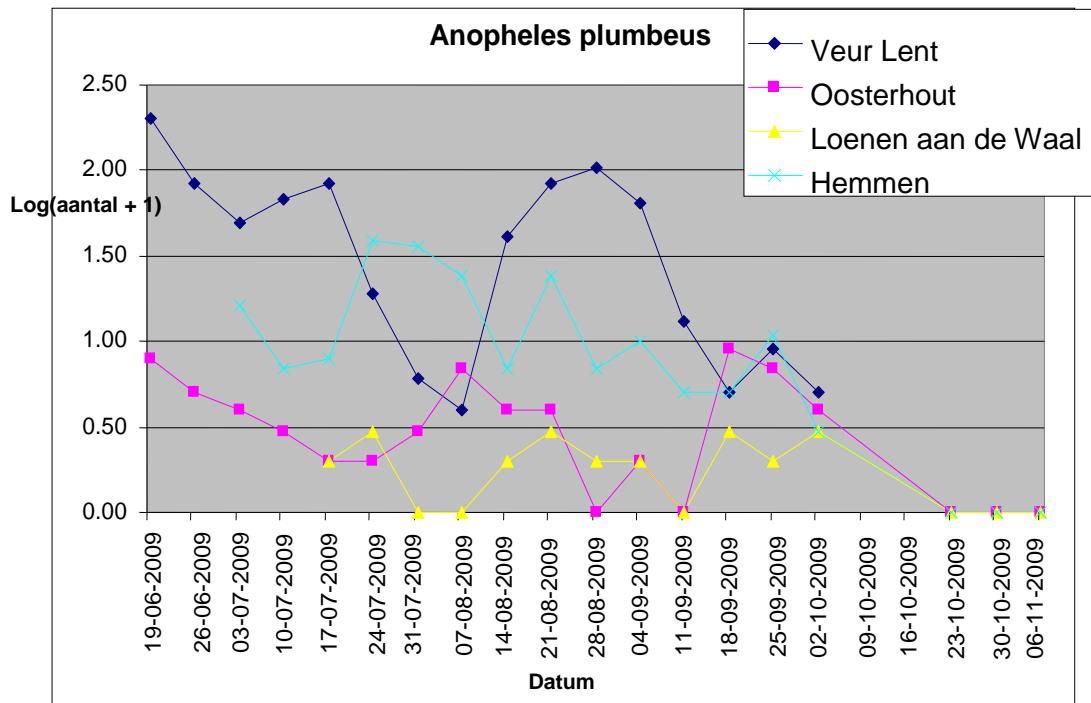


Figure 29. Number of *Anopheles plumbeus* on farm 1 and forests 1,2 and 3 from mid June till the end of October 2009.

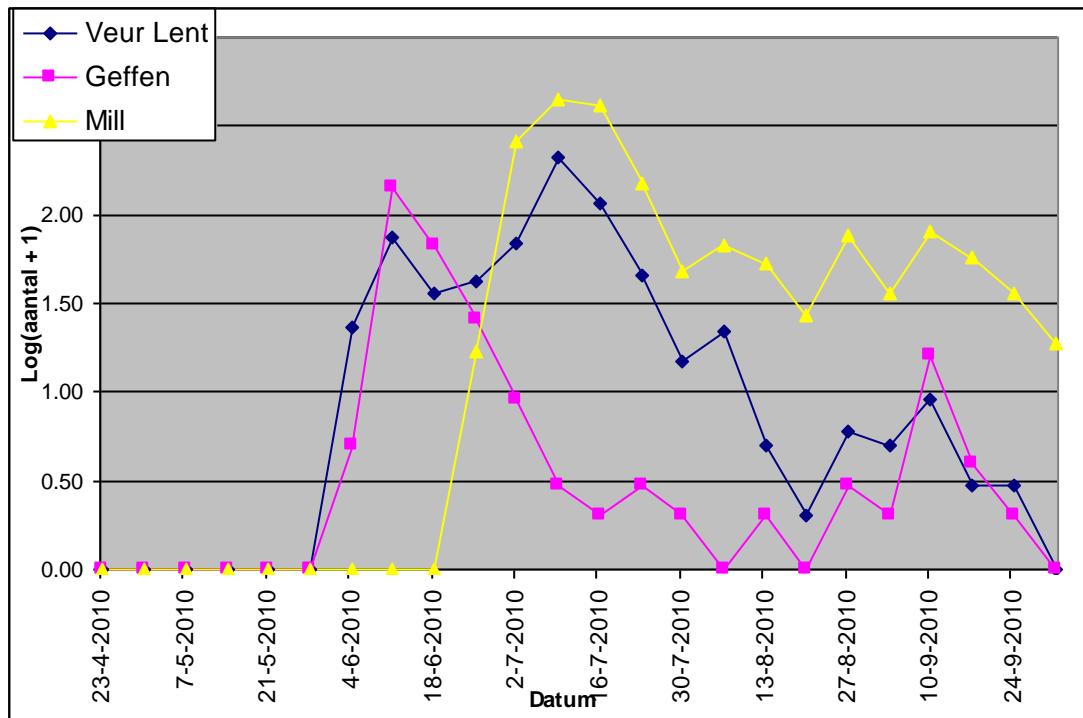


Figure 30. Number of *Anopheles plumbeus* from the end of April till October 2010 on the three farms.

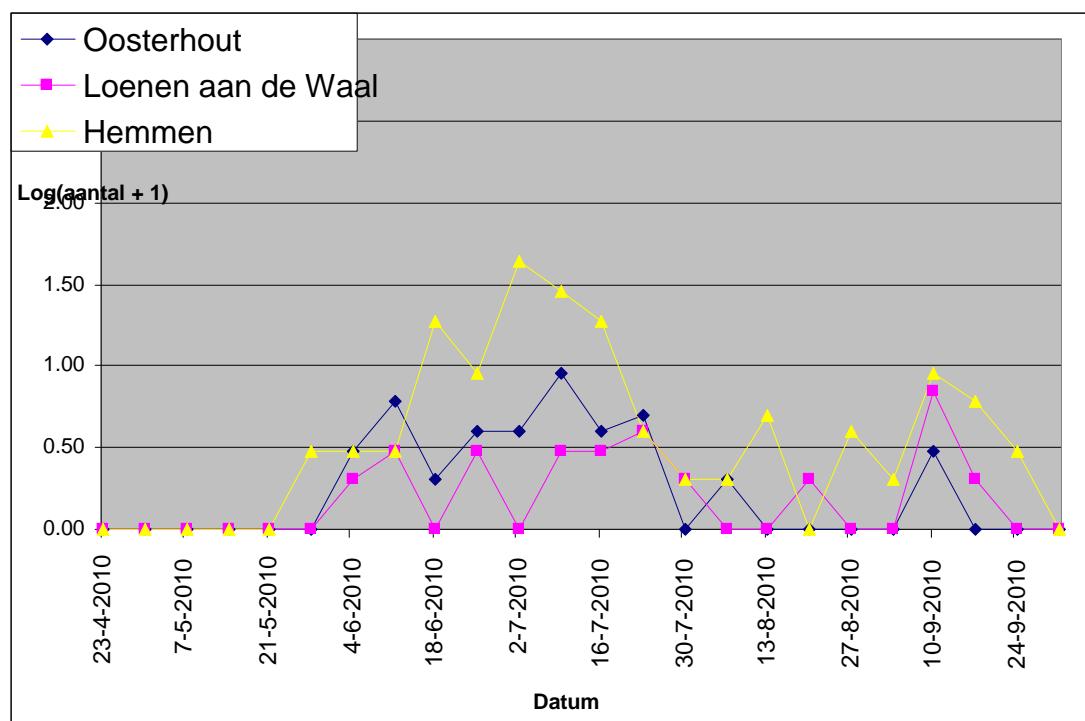


Figure 31. Number of *Anopheles plumbeus* in the three forests from the end of April till October 2010.

In figure 30 the number of *An. plumbeus* is shown on the three farms in 2010. It is clear that the pattern of their numbers on the three farms is only partly comparable. When in Veur lent and in Geffen a population peak can be observed at mid July, their numbers are still almost zero in Mill. It is clear that next to weather conditions also local factors play an important role. On all three farms a maximum is reached at mid August. The season of *An. plumbeus* is relatively short. On the farms as well as in the forests the first mosquitoes of this species have been observed at the end of May while the last ones were caught at the end of September or the start of October (Figure 30 and 31). An exception is the farm 3 (Mill) where not earlier than mid June the species appeared and was still present at the beginning of October. When comparing forests to farms, the same patterns was observed as in 2009.

Phenology and co-occurrence of *Culex pipiens* at pest sites

In Figure 32 the number of *Cx pipiens* is shown from mid June till December 2009 on the 5 studied sites in the Betuwe. There is a general tendency that on all locations a maximum is reached around 10th of October. Only in the forests 2 (Loenen) and 3 (Hemmen) there was er a short early peak around 10th of July in forest 2 and around 7th of August in forest 3. *Cx pipiens* was still found till beginning of December. This late presence is probably caused by the extremely warm November. This extraordinary mild weather lasted till about 10th of December.

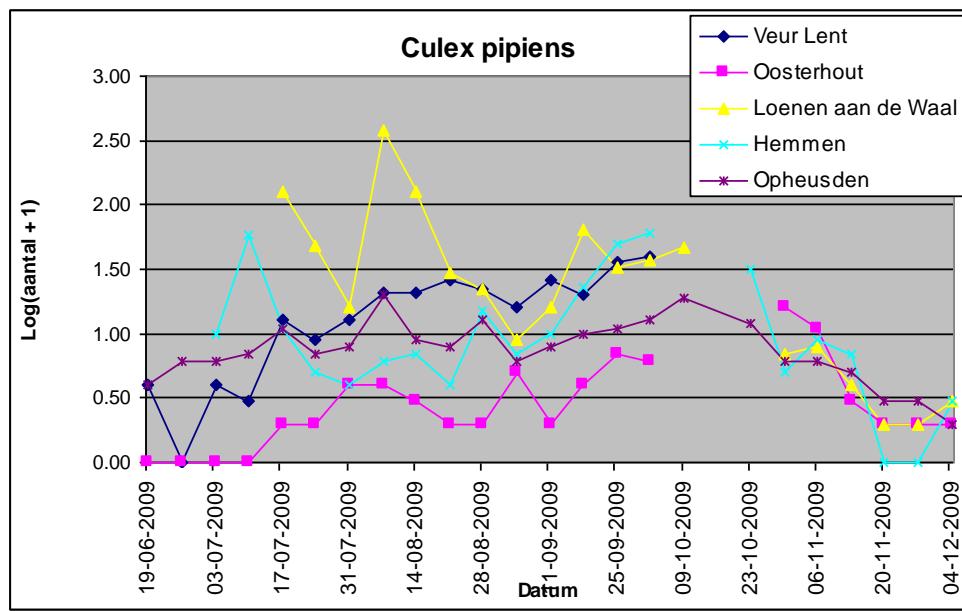


Figure 32. Number of *Culex pipiens* on the four locations in the Betuwe (farm 1 and forests 1,2 and 3) from mid June till the end of October 2009.

Culex pipiens showed a peak around mid July at all three locations after which a new maximum has been reached by the end of September (Figure 33). In 2009 *Cx pipiens* reached a maximum in numbers at late September-early October. In contradiction to *An. plumbeus* the season of *Cx pipiens* was long. The first mosquitoes appeared at the farms already in April and the last ones can be observed at the beginning of November (E. Viennet, unpublished data) or even early December (Figure 32). The start of the season in the forests is a bit later than at the farms (early May, Figures 33 and 34). *Culex pipiens* was, like *An. plumbeus*, a lot more common at the farms than in the forests. This is probably because at the farms the density of animals is a lot higher than in the forests.

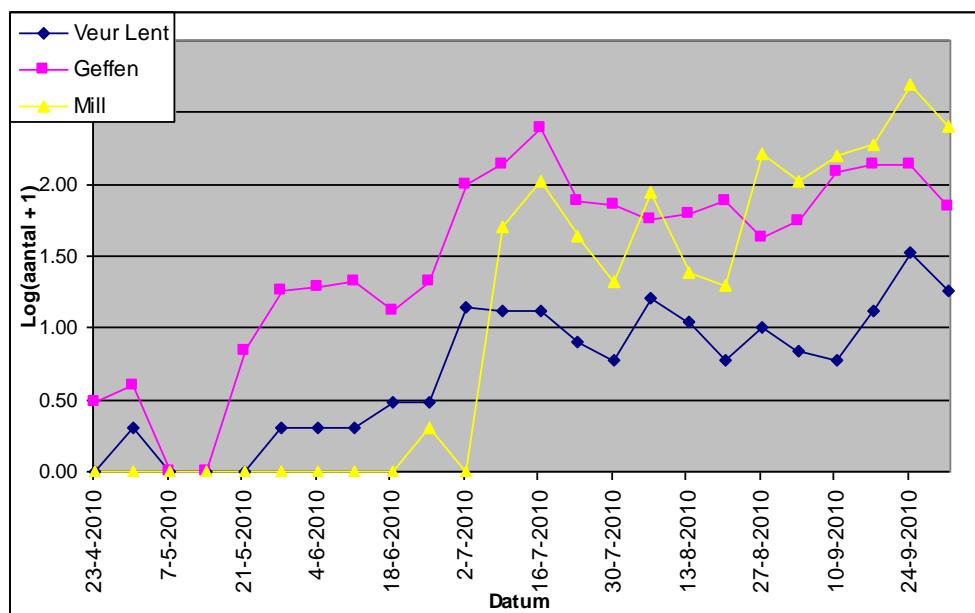
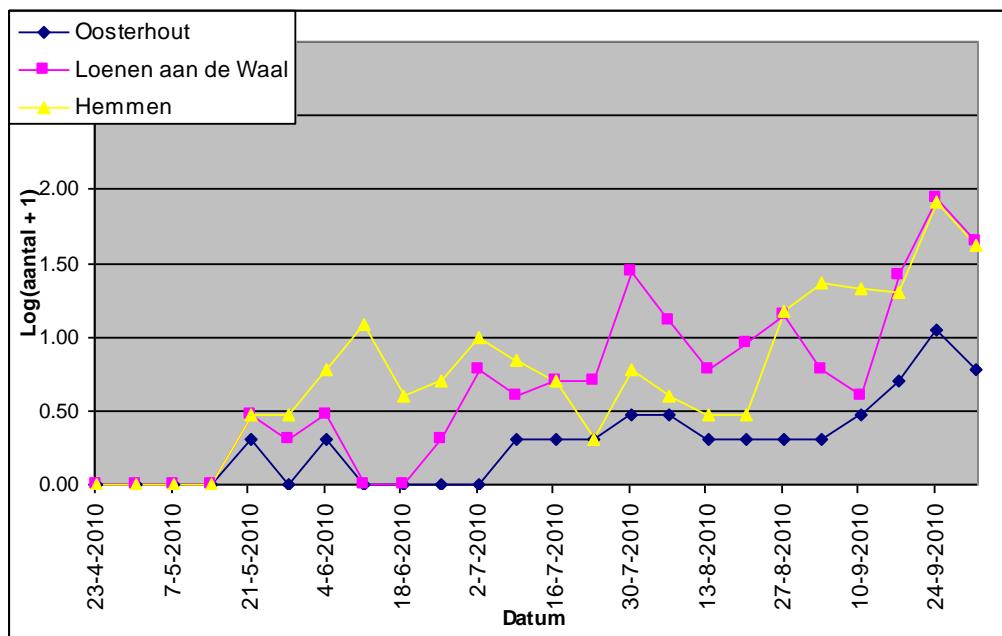


Figure 33. Number of *Culex pipiens* on the three farms from the end of April till October 2010.

Figure 34. Number of *Culex pipiens* in the three forests from the end of April till October 2010.

Relation with the microclimate

The microclimate was more moderate and moist in the forests than around the farms. The minimum-airmoisture content never reached a level below 60 % in forest 1 (Oosterhout, Figure 35) and rarely in forest 3 (Hemmen) while this happened regularly at farm 1 (Veur Lent). The mean airmoisture content was in the forests constantly higher than at the farms (Figure 36). In the forests it rarely happened that the maximum-airmoisture content per day was lower than 100 %. Occasionally in forest 3 (Hemmen) it was continuously 100 % during several days in a row (data not shown). Till beginning of December 2009 the minimum temperature in the three forests never dropped below zero, at the farms this happened several times during that autumn (not shown in the figures).

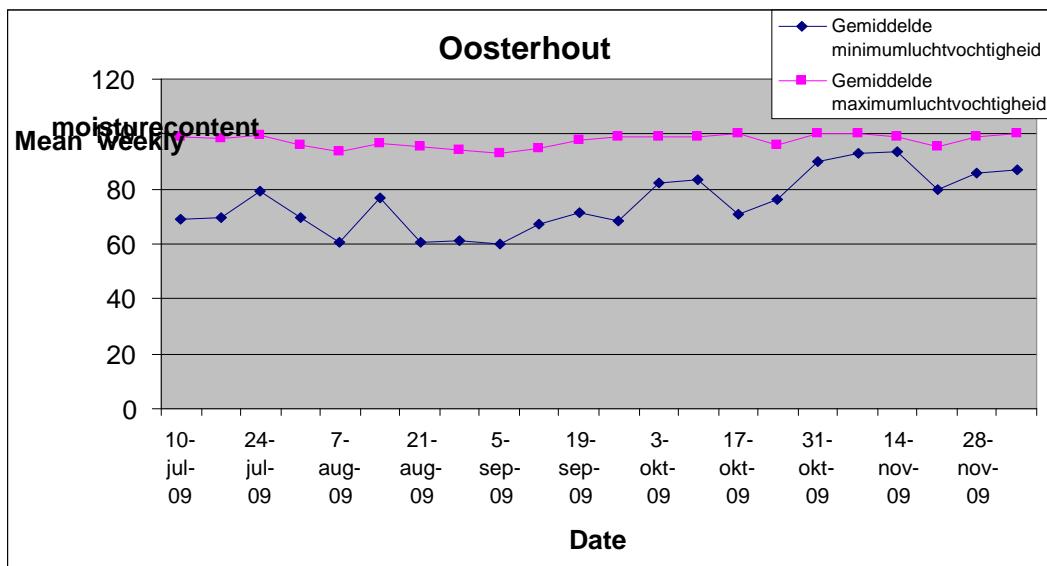


Figure 35. Mean minimum- and maximum air moisture content per week in forest 1 (July-Dec 2009).

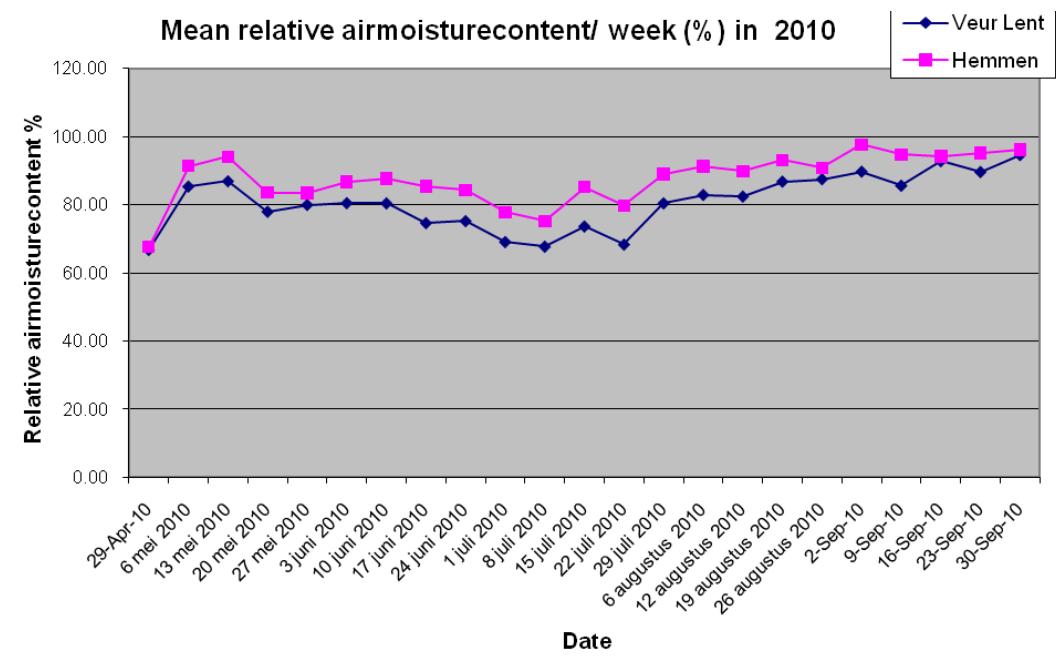


Figure 36. Mean relative air moisture content per week (%) at Veur Lent and in Hemmen in 2010.

Figures 37 and 38 show the mean minimum- and maximumtemperature per week from April till October 2010 in Veur Lent and in Hemmen forest. In the summer of 2010 the temperature at the farm passed the 30°C in mid July. In contrast the microclimate in the three forests is a lot more moderate then at the three farms. Besides that the mean temperature appeared to be slightly higher at the farms than in the forests (data not shown).

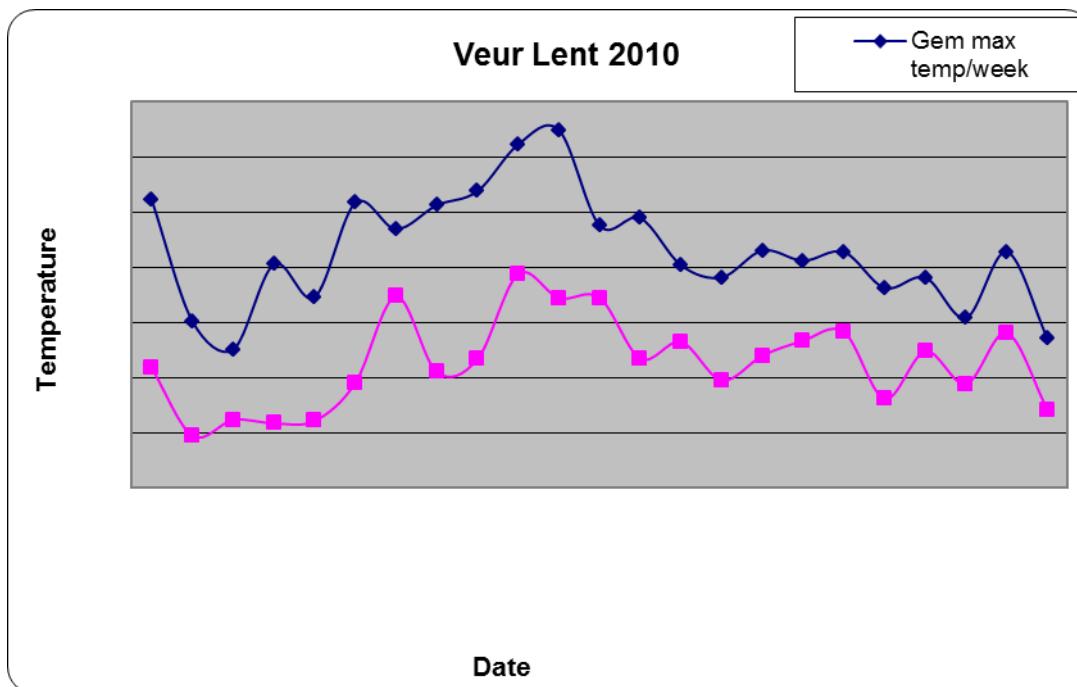


Figure 37. Mean minimum- and maximumtemperature per week at Veur Lent in 2010.

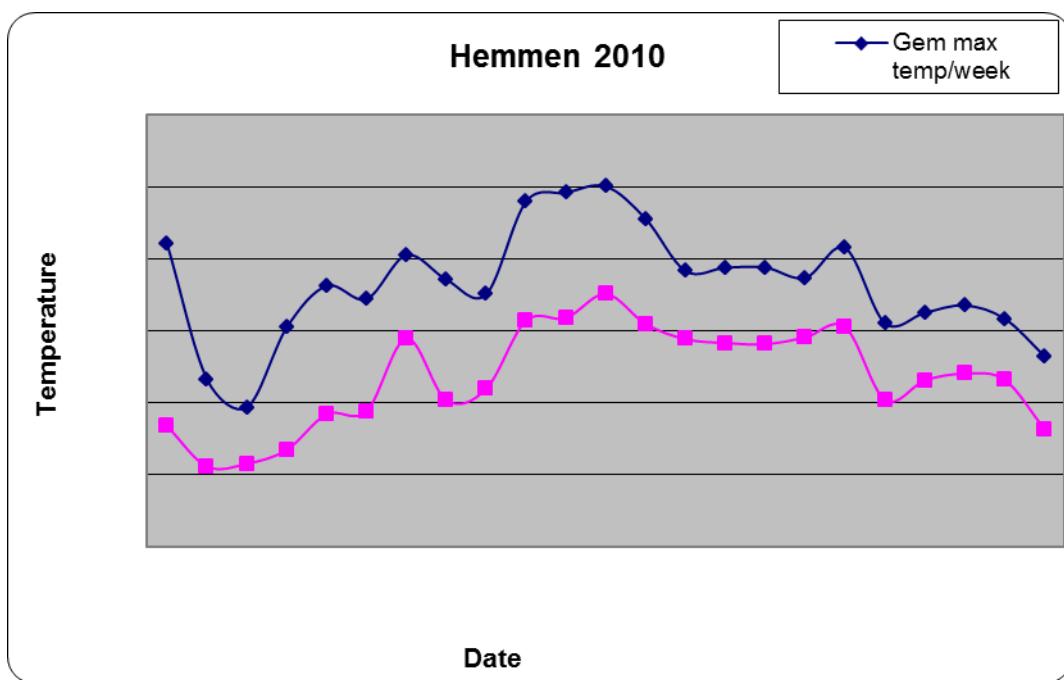


Figure 38. Mean minimum- and maximum temperature per week in Hemmen in 2010.

Figure 39 shows that the number of *An. plumbeus* is positively correlated with the mean temperature per week and the mean maximum- and minimum temperature per week (not shown). This correlation is highly significant ($P<0.001$). Figure 40 indicates that also the number of *Cx pipiens* found per week is positively correlated with the mean temperature per week. This correlation is weak but still significant ($P=0.008$).

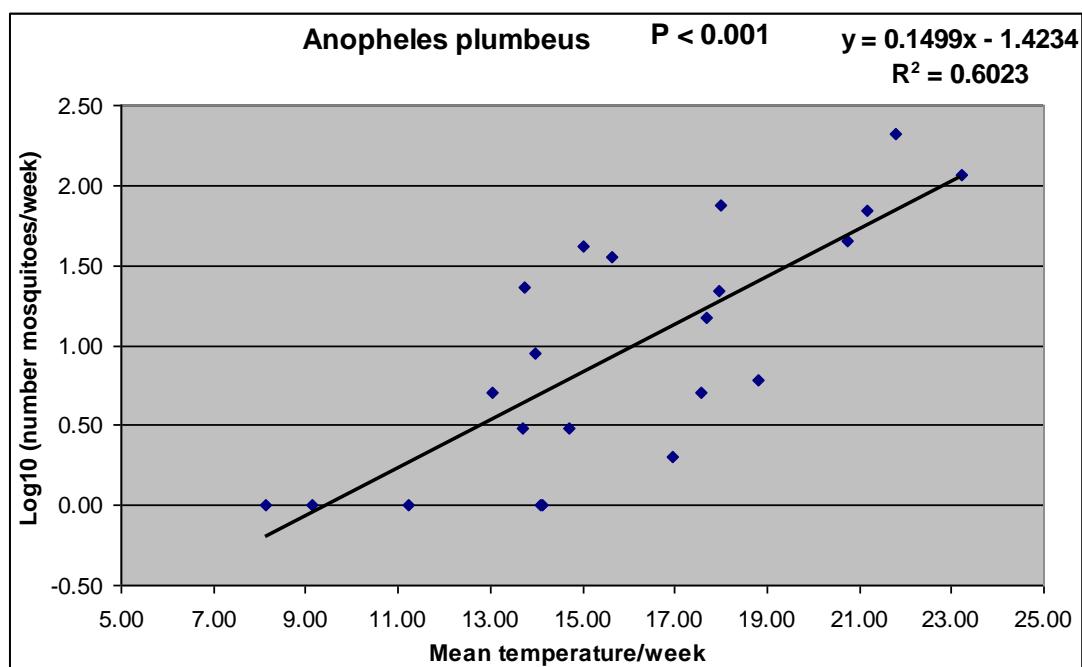
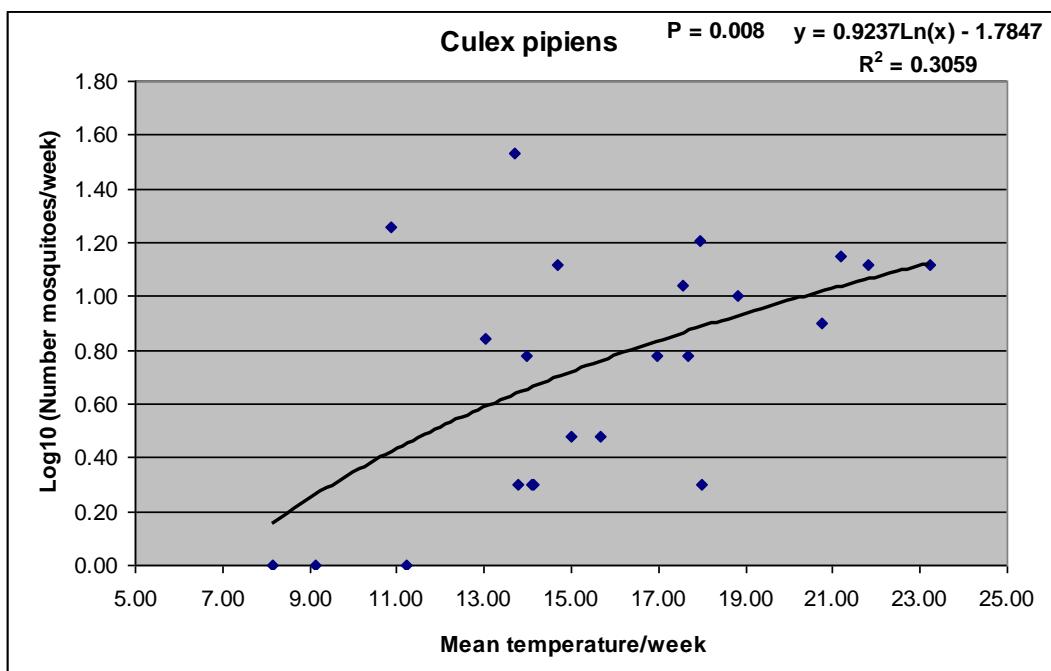


Figure 39. Relation between the mean week temperature and the \log_{10} of the total number of *Anopheles plumbeus* caught per week.



Figuur 40. Relation between mean weekly temperature and the total number of *Cx pipiens* per week.

The correlation between the mean saturation deficit per week and the number of *An. plumbeus* caught per week is positive (Figure 41); so dryer conditions are favourable. This might explain why the species occurs in higher abundance during late summer, when the saturation deficit can be higher than in early summer. The correlation found is highly significant ($P < 0.001$). For *Cx pipiens* the correlation is very weak ($P = 0.626$) and the trendline goes almost horizontal. So within the found range of mean saturation deficits *Cx pipiens* seems not to have a preference for higher or lower saturation deficits (Figure 42).

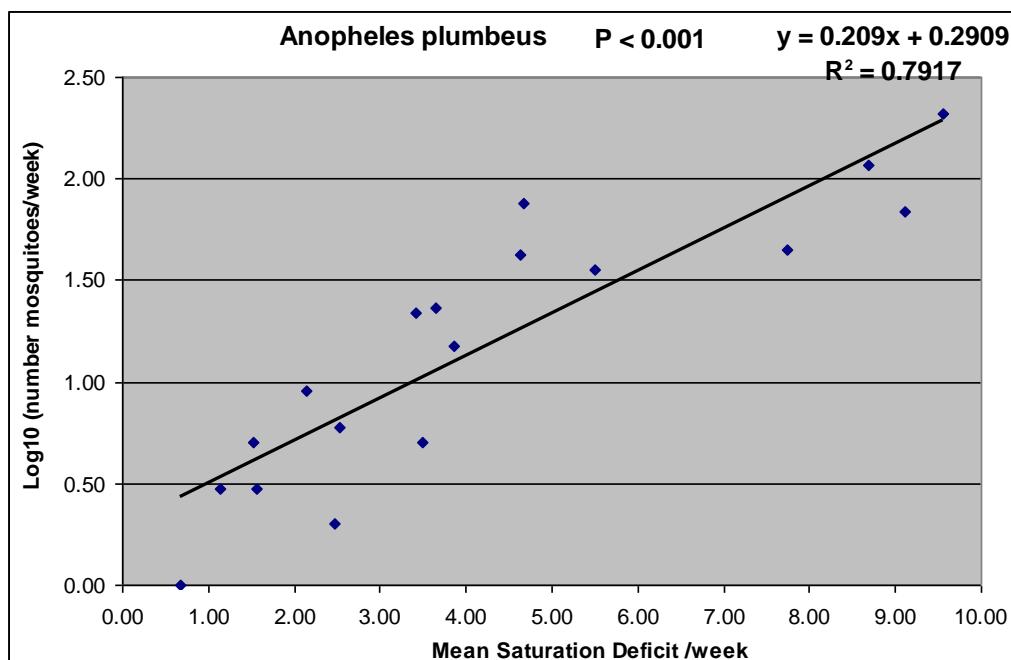


Figure 41. Correlation between mean saturation deficit and the log10 of total number of *An. plumbeus* per week.

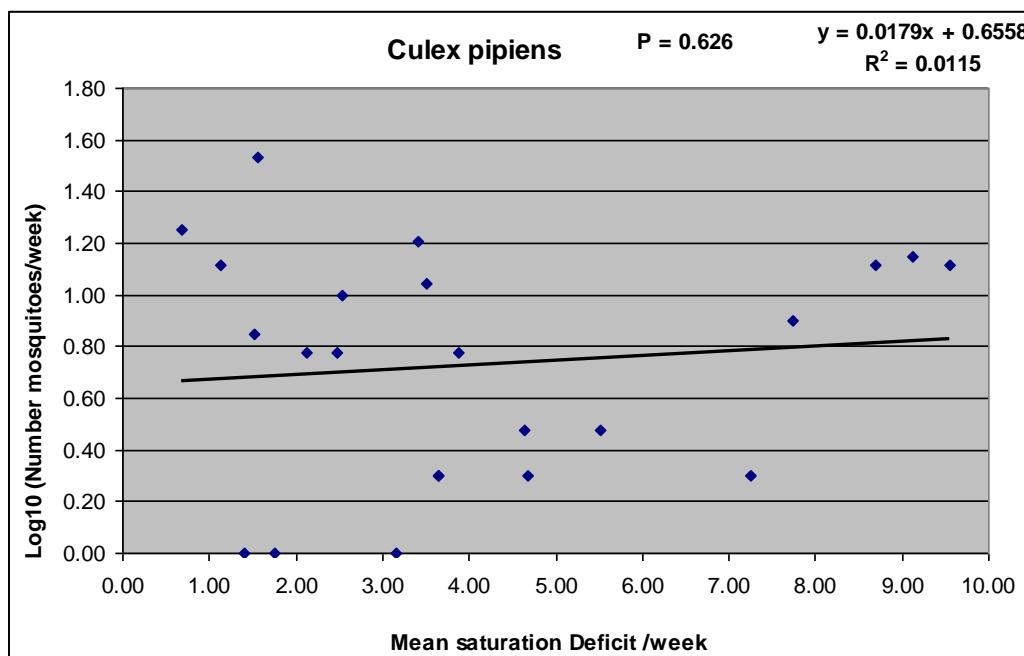


Figure 42. Correlation between mean saturation deficit and total number of *Cx pipiens* per week.

Anopheles plumbeus appeared to be more common in its urban habitat than in its natural habitat. The urban habitat of this species consists of farms with stables that often are unused. Under these stables fluid manure storage basins are present, in use or abandoned and now filled with rainwater. On other farms there can be a storage of old used tyres which are partly filled with rainwater. Both are favourite breeding sites of *An. plumbeus*. The natural habitat of *An. plumbeus* consists of old deciduous forests with a lot of lying and standing dead wood. In which it uses rainwater-filled treeholes as breeding sites. There is a difference in the phenology of *An. plumbeus* found between its old natural habitat (forests) and its new habitat (farms). At farms there are a few big peaks while in the forests there are (many) more small peaks. It is likely that this is related with one or a few big breedingsites at the farms and many small breedingsites in the forests. The duration of the season of *Anopheles plumbeus* is relatively short. At the end of May the first individuals appear and at late September-early October the last ones disappear. *Anopheles plumbeus* seems to be a warmth-loving mosquito. When the mean week temperature is higher, their numbers found increased as well. They also favour dryer conditions; within the measured moisture content range there is a tendency for higher numbers of *An. plumbeus* with decreasing moisture content or increasing saturation deficit.

From these studies it is obvious that *An. plumbeus* can, locally, be an important nuisance mosquito. It is unclear whether this is a novel ecological phenomenon, in which the mosquito has adapted to a new environment which is highly favourable (stable, permanent breeding sites in the vicinity of blood hosts). The species readily bites humans, although we do not know its degree of anthropophily: given its natural habitat in woodland, it is more likely to be a generalist or ornithophilic species. Currently, *An. plumbeus* is the most likely potential vector of tropical *Plasmodium falciparum* in Europe (Shute 1956, Kruger et al. 2001, Eling et al. 2003) and for this reason the ecology and population dynamics of this species should be closely watched in the Netherlands.

Culex pipiens is common in urban as well as in rural areas. Like *An. plumbeus*, *Cx pipiens* appeared to be more common on the farms than in the forests. Among the forests there were large variations in density. The high number of trapped *Cx pipiens* recorded in forest nr 2 (Loenen a/d Waal) probably was caused by a sleeping place of carrion crows as *Cx pipiens* is principally ornithophilic. The species density seemed to reach a peak in 2009 around 10th of October. Only in two of the three forests (2 and 3) there was a peak much earlier in summer. In 2010 *Culex pipiens* reached its maximum at the end of September, in the forests as well as around the farms. The season of *Cx pipiens* can be long (from April till December). found, *Cx pipiens* does not clearly prefer higher or lower temperatures or higher or lower air moisture contents. There was a weak positive tendency for higher air moisture contents and mean week temperatures of 15 till 20°C. We conclude that, as with the study of the distribution of Culicidae around the Netherlands, *Cx pipiens* is the dominant species among our catches, and therefore remains a species to be closely watched, as it is a potential vector of West Nile virus and other vector-borne pathogens.

3.4 Repeated introduction of the exotic *Aedes japonicus* as suggested by microsatellite markers

3.4.1 Methodology

Study Sites

Two Belgian second-hand tire companies located in the village of Natoye (Namur) were surveyed. Sites were named Natoye1 (50.3389587° N, 5.044879° E) and Natoye2 (50.33588° N, 5.0714698° E). The companies import mainly tires for trucks and heavy vehicles originating from various countries (only from Europe). Tires are stacked outside and many are exposed to rainfall and contain water and organic material like decomposing leaves. Landcover consists largely of deciduous forests, gardens, cultivated fields around Natoye1 and gardens, cultivated field and meadows around Natoye 2.

Adult survey

The sites were sampled two consecutive days (12:00 am-12:00 am) during 13 visits every two weeks from 20th of April 2009 until 5th of October 2009 with three types of traps (see 3.2).

Larval survey

Summer 2009

Sampling for immature mosquitoes was conducted on the two Natoye sites during four visits in May, August, September and October 2009. During each visit an extensive inventory of potential breeding sites (all water-holding sites) and mosquito immature stages was done at the company and in a perimeter of 500 m of the tire stock. At each visit, about 20 potential breeding sites were found and observed from the company. At the company main observed sites were tyres and artificial container such as abandoned buckets. *Aedes japonicus* larvae were collected from potential breeding sources by using a pipette and white plastic bowl, and transported alive to the laboratory in vials labeled with breeding sites specific identification

details. Once in the lab, the larvae were first transferred with a dropper to a small cup or bowl with fresh clean water as a washing procedure. The procedure was repeated till total elimination of debris or sediment. Larvae were killed by a thermal shock with hot water (60°C). Larvae were then collected with a fine pipette and put in a vial filled with a solution of ethanol at 80%. Treated larvae were then identified using a stereoscopic microscope and an identification key. Pupae were reared to adult in a secured lab for identification.

Spring 2010

Additional larval surveys have been made during the spring 2010 in the site of Natoye1 (50.3389587° N, 5.044879° E) to verify the recurrent presence of *Ae. japonicus*. Five visits have been done between April and June. During each visit, known breeding sites have been sampled: six tyres, one artificial container, abandoned bucket and a natural site (tree hole). *Aedes japonicus* larvae and pupae were collected from the breeding sources by using a pipette and white plastic bowl, and transported alive to the laboratory in vials labeled with breeding sites specific identification details. In the lab, the larvae were then transferred in small plastic containers with dechlorinated tap water as used in *Cx pipiens* colony and reared to adult in a secured cage at room temperature for identification.

Morphological Identification.

Mosquito larvae and adults were identified using identification keys of Tanaka et al. (1979), Schaffner et al. (2001), Schaffner (2003) and Becker et al. (2003).

Blood meals identification.

54 females of *Ae. japonicus*, captured in gravid traps in Natoye during the 2009 campaign, were immediately frozen at -80°C after identification. Abdomen were then dissected with sterile pincers and DNA was extracted with salt/chloroform extraction protocol (Maniatis et al., 1982). The concentration and purity were determined from the A₂₆₀/A₂₈₀ ratio using a spectrophotometer. The concentration of all samples was then homogenized at 50ng/µL. Presence of host blood was determined by PCR, and amplified products were visualized on 2% agarose gel (see Table XX for primer pairs). DNA of each sample was analyzed for the presence of mammal blood using a primer pair, specific to a region of the cytochrome b mitochondrial gene (Cyt b), designed by Ngo and Kramer (2003). Samples positive to the presence of mammalian blood were then subjected to a multiplex PCR to discriminate mammal species among dog, human, cow and sheep/goat (Kent and Norris., 2005). Presence of avian blood was tested with a primer pair that specifically amplify the NADH dehydrogenase subunit 2 (ND2) mitochondrial gene (Sorenson et al., 1999). Undetermined mammals and avian products were sequenced using 377 DNA analyzer (Applied Biosystems). Multiplex PCR reactions were performed using Qiagen Multiplex PCR kit.

Table XX: Primer sequences for the polymerase chain reaction blood meal identification assay			
PCR	primer name	size	Source
Mammal	MAMMAL-For	772	Ngo and Kramer 2003
	MAMMAL-Rev		
Avian	L5216-For	1041	Sorenson et al., 1999
	H6313-Rev		
Multiplex mammals	Human741F	334	Kent and Norris., 2005
	Goat894F	132	
	Cow121F	561	
	Dog368F	680	
	UNREV1025	-	

Genetic structure of Natoye population

Studies on invasive species have shown that often alien species established in a new environment remains at low density and does not become invasive for sometimes years. After a certain time called lag period, the population suddenly starts to grow exponentially and become invasive. A hypothesis is that the lag period is necessary for invasive species to prepare it adaption to his new environment. Lag period is mostly described and documented for invasive plant species for which lag period can vary from a very short period (herbacae species) to several years (50 years for some invasive plants in Hawaii) (Daehler 2009). Genetic analysis of invasive population can provide relevant information on the genetic structure and on the invasive potential of an introduced population.

To assess possible genetic variation in the Belgian population of *Ae. japonicus*, we used the same set of microsatellite loci as markers used to study the American population of *Ae. japonicus* (Fonseca et al. 2001). Microsatellites because of their neutrality and their high mutation rate are ideal markers for population genetic studies. Comparison with *Ae. japonicus* from Japan has also been performed. Analysed mosquitoes were obtained from adults, trapped through CDC Gravid trap (model 1712, John W. Hock Co., Gainesville, FL), and from larvae collected within tires, in a recycling second-hand tires company in Natoye (Namur, Belgium). In total, during the longitudinal survey of *Ae. japonicus* (Damiens et al, submitted), 123 specimens were collected between July 28 and November 20, 2009. In addition, 311 additional *Ae. japonicus* collected between April 28 and June 30, 2010 were added in the analysis to follow the evolution of genetic diversity of the population among years. DNA was extracted with salt/chloroform extraction protocol (Maniatis et al. 1982). The concentration and purity were determined from the A_{260}/A_{280} ratio using a spectrophotometer. The concentration of all samples was then homogenized at 50ng/ μ L. Samples were analysed with a set of seven microsatellite loci specific to *Ae. japonicus* (Widdel et al. 2005). Multiplex PCR reactions were performed in an ABI thermocycler, using Qiagen multiplex kit. PCR conditions were an activation step of 15 minutes at 95°C, followed by 35 cycles of 30 seconds at 94°C, 90 seconds at 57°C, 90 seconds at 72°C. Final step was 10 minutes at 72°C. amplified fragments were determined using sequencer and analysed using GeneMapper Software version 3.7 (Applied Biosystems). Genetic structure of Belgian *Ae. japonicus* was investigated using Bayesian multilocus analysis executed in the program STRUCTURE (Pritchard et al., 2000). An estimate of the most likely amount of genetic

groups composing the Belgian population was performed using the method of Evano (Evano et al. 2005). Using the program Structure 2.0 (Pritchard et al. 2000), we determined the amount of homogeneous groups (clusters) composing the population with a Bayesian multilocus analysis. We used the uncorrelated allele model with admixture (Structure infer the alpha). For k=1 to 10, we run 20 simulations using 10.000 burning steps and 100.000 MCMC steps (Markov Chain Monte Carlo). The true amount of clusters (K) composing the population was determined using the formula described by Evanno et al (2005) to calculate ΔK .

$$\Delta K = \frac{m | L(K+1) - 2L(K) + L(K-1) |}{s[L(K)]}$$

With $L(K)$ representing the mean likelihood given by the program as $\text{LnP}(D)$; "m" as the mean value and 's' as the standard deviation. The individuals were then assigned in a genetic group and clustering model was plotted

3.4.2 Results

Adult survey

During the sampling season, 753 adult mosquitoes were collected from both sites belonging to five species for Natoye1 and four species for Natoye2 (Table XXI). The largest number of mosquitoes was collected in the CDC Gravid traps (473 mosquitoes) while 214 and 66 mosquitoes were found respectively in the MMLP traps and in the BG sentinel traps. In both sites, the most abundant species was *Cx pipiens* s.l. (63.2%). *Ae. geniculatus* (16.1%), *An. claviger* (6.9%) and *Cx torrentium* (1.9%) were also found. *Ae. japonicus* individuals were found only on Natoye1 (11.9% of the number of sampled individuals). *Ae. japonicus* was firstly recorded at Natoye1 mid June (Figure 43). The population increased then from June to August and decreased till October. *Aedes japonicus* appeared to be a late species while *Ae. geniculatus* appeared earlier in the season but disappeared rapidly at the end of July.

Table XXI Distribution of mosquito species by sites (2009)

		<i>Cx pipiens</i>	<i>Cx torrentium</i>	<i>An. claviger</i>	<i>Ae. geniculatus</i>	<i>Ae. japonicus</i>
Natoye 1	Number	91	6	21	35	90
	percentage	37,5	2,5	8,6	14,4	37,0
Natoye 2	Number	385	8	31	86	0
	Percentage	75,5	1,6	6,1	16,9	0

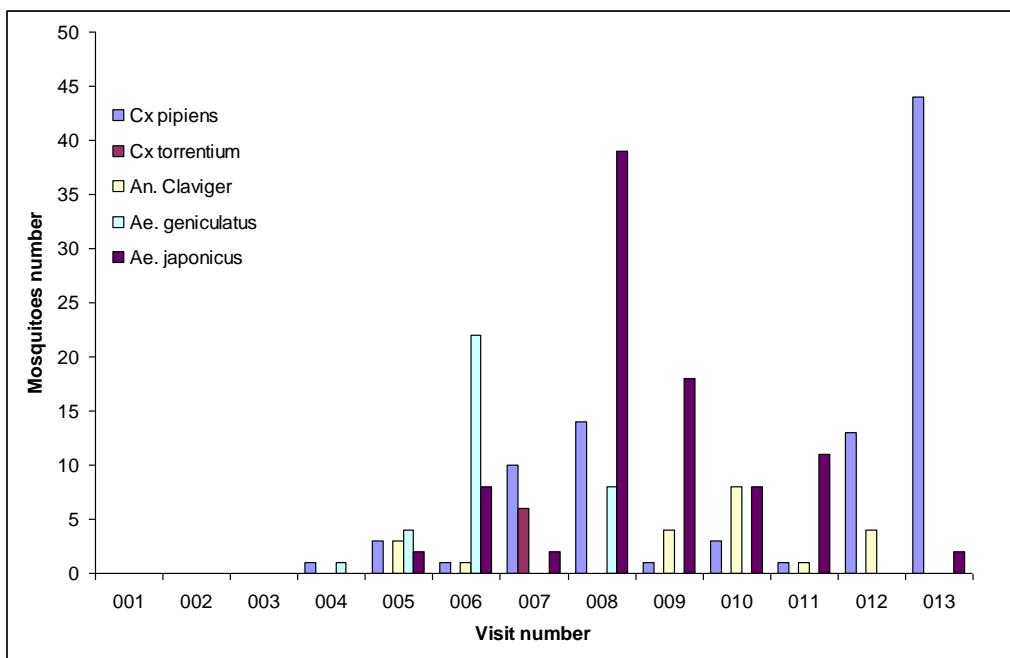


Figure 43: Total number of mosquitoes of the 5 different species found in the three trap types during the 13 visits (every two weeks from the 20th of April 2009 to the 5th of October 2009) at Natoye 1.

In Natoye2, *Ae. japonicus* was not recorded (Figure 44). *Cx pipiens* population increased from June to August and decreased in September. The population of *Cx pipiens* appears to be more important than in Natoye1 suggesting that the presence of *Ae.japonicus* could be detrimental for *Cx pipiens*. Several breeding sites present the presence of *Cx pipiens* larvae and *Ae. japonicus*, meaning that competition probably occurred in the field. As explained in the competition experiment section (see 3.5), presence of *Ae japonicus* leads to an increase of *Cx pipiens* larval mortality.

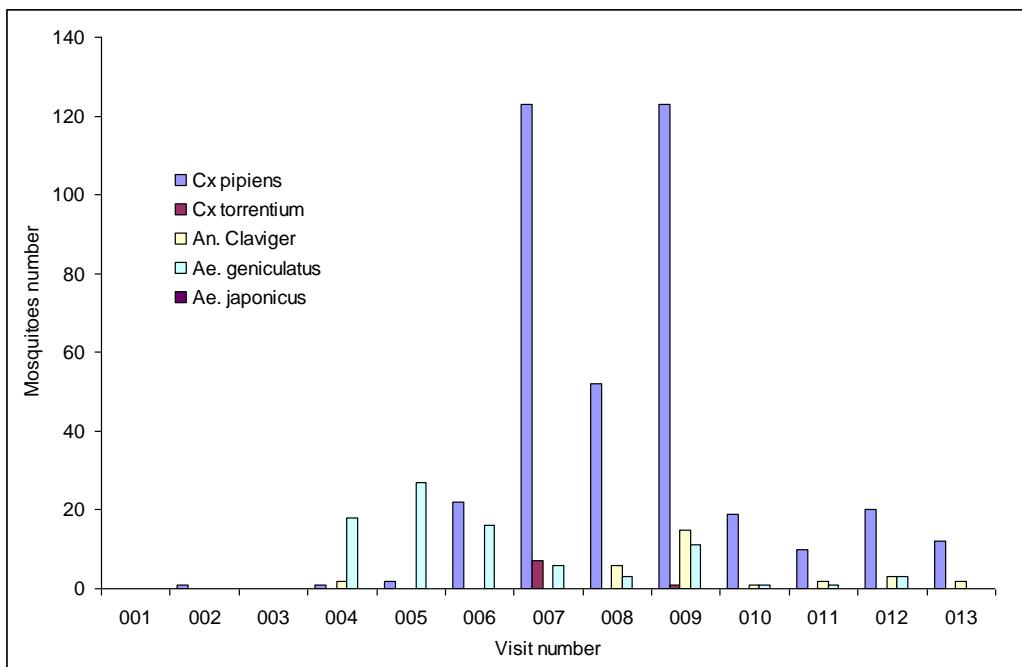


Figure 44: Total number of mosquitoes of the 4 different species found in the three trap types during the 13 visits (every two weeks from the 20th of April 2009 to the 5th of October 2009) at Natoye2.

Larval survey

Summer 2009

A total of 71 and 65 positive breeding sites were identified respectively in Natoye1 and Natoye2. In Natoye1, 786 larvae belonging to 10 species were collected and 922 larvae belonging to 7 species were collected in Natoye2. From the 786 collected larvae, the first observation of *Ae. japonicus* larvae was in May. A total of 323 *Ae. japonicus* larvae were collected only around Natoye1, the majority of which were recovered from tyres (138 larvae; 42.7%) and other artificial sites as buckets in the company (143 larvae, 44.3%). Although less used, natural sites as one tree hole (25 meters from company) also harbored notable numbers of larvae (41 larvae, 12.7%). Finally one larvae was found in a puddle (about 100 meters from company). *Cx pipiens* larvae were also found in 25 sites of the 32 sites where *Ae. japonicus* larvae were present. *Cx torrentium* were also recorded in 3 sites of those 32. All larval stages were mixed in these two cases.

Spring 2010

For the five visits, a total of 226 *Ae. japonicus* larvae were collected only around Natoye 1 (Table XXII). The majority of which were recovered from tyres (188 larvae; 83.2%), 29 larvae were found from a bucket (12.8%) and 9 larvae in a tree hole (4.0%).

Table XXII: Number of *Aedes japonicus* larvae found during the five visits during the Spring 2010 at Natoye 1

	09/04	28/04	12/05	19/05	02/06
Tyres	0	56	67	51	14
Bucket	0	3	26	0	0
Tree hole	0	7	2	0	0
Total	0	66	95	51	14

Blood meals identification.

From the 54 female mosquitoes analyzed, 23 were positive for the presence of a blood meal. Majority of them were single meals (23 meals, 92%), and only two mosquitoes (8%) were fed on two species. Analysis of the blood meal sources identified mammals as the only source of blood. Human was the most frequent host from the mammal blood meal source (65.2%), followed by cow.(34.8%). Four samples were positive for the amplification of the avian fragment but the sequencing did not give any results. Those samples were then discarded.

Genetic structure of Natoye population

Bayesian multilocus analysis done with *Structure* has revealed that the Belgian population of *Ae. japonicus* was composed by three different genetic groups (Figure 45). The three groups were determined as the most probable genetic structure composing the population.

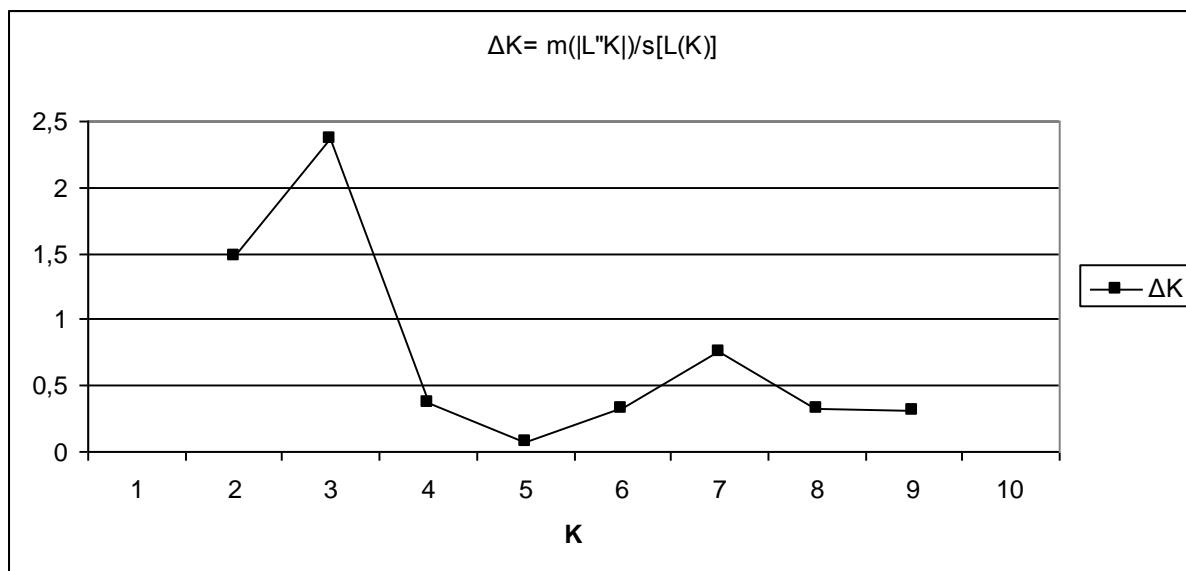


Figure 45: Values of ΔK associated with putative amount of clusters composing the Belgian population of *Ae. japonicus*. The highest value of ΔK depicted the real K .

We plotted the cluster representation of the three genetic groups in 2009 with *Structure* (Figure 46). It can be observed that mosquitoes within the three genetic groups have a strong microsatellite signature association. We can hypothesis that those three separate groups most likely derived from separate introductions in the site of Natoye. Those multiple introductions first raised the problem of contaminated tyres that are transported to Natoye. Here we show that the introduction of *Ae. japonicus* was not an single incident but that contaminated tyres are regularly bring on the site. An efficient eradication program of *Ae. japonicus* should then integrate this possibility. A systematic decontamination of arriving tyres or an out of water storage of the tyres should be envisaged to avoid any reinfestation of the site post treatment.

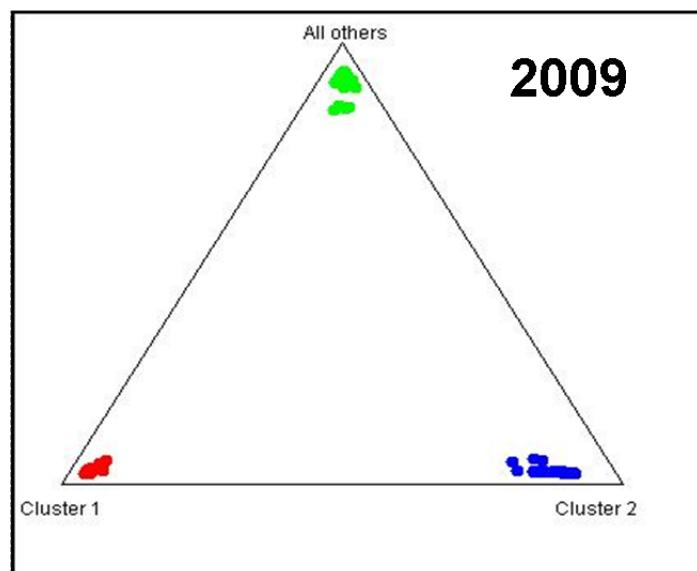


Figure 46 Plotted representation of the three genetic groups determined by *Structure*. Each point represents an individual mosquito genetic combination and the colour represent the assigned genetic group.

The analysis of the mosquitoes collected in 2010 was then added to the model (Figure 47) we can observe a mixing of the three genetic groups. Those results seem to be consistent with the ones obtained by studying genetic structure of *Ae. japonicus* in US. In her analysis of the American invasive population of *Ae. japonicus* (Fonseca et al. 2010), the author also reveal that multiple introductions have probably form the actual population. The two genetic groups composing the American population were also genetically recombining among groups between the years of follow-up. The resulting genetic mixing we observed in Belgium could possibly increase the genetic diversity of the population and thus enhance the invasive capacity of the species. Indeed, the occurrence of multiple introductions raised the problem of the gene flow occurring on the site. We can hypothesis that after having obtain sufficient genetic diversity, in order to fully adapt to his environment, the *Ae. japonicus* species would enter into wide population extension and disseminate uncontrollably. Thus eradication measures should be started quickly before next step occur.

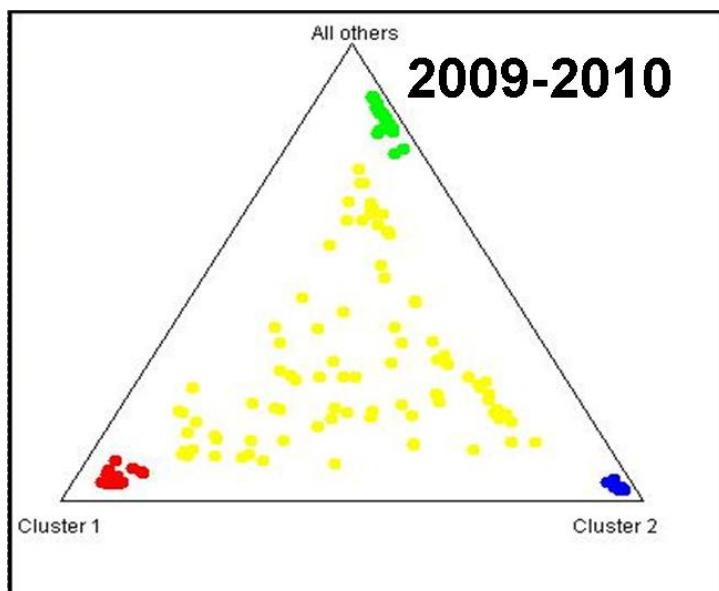


Figure 47: Cluster representation of the three genetic groups determined in 2009. The yellow plots represent the individuals collected in 2010 and their genetic distance to the ones analysed in 2009.

3.5 Competitive interactions between larvae of *Aedes japonicus* and *Culex pipiens* under laboratory conditions

3.5.1 Methodology

***Aedes japonicus* colony establishment**

In the past two years, only two successful laboratory establishments of *Ae japonicus* have been reported (Williges et al. 2008; Hoshino et al. 2010). This species seems rather difficult to maintain in laboratory due to its reproductive behaviour and the difficulty to obtain mated females. Moreover, it requires secure installations to avoid possible environmental contamination by this exotic invasive species. This kind of confined insectaries does not exist in Belgium. In consequence, a colony had to be established at the EID laboratory in

Montpellier (Entente Interdépartementale pour la Démoustication, Dr. Lagneau). the different steps to rear the species and the problems encountered are described. Eggs were collected in Natoye (Namur). These were then brought to the experimental laboratory of the EID in Montpellier (France). Rearing was done in their confined (P3) insectarium specially designed to keep and study invasive mosquito species, under 70% of relative humidity, 27°C, and a photoperiod of 16:8 (L:D). *Aedes japonicus* eggs were placed into white plastic trays (30x18x5cm) and submerged into 1.5L of dechlorinated water. The egg hatching was stimulated by adding 5mg of Vitamin C per liter to lower the concentration of oxygen in the rearing water.

Competition experiment between *Culex pipiens* and *Aedes japonicus*

By comparing two collection sites in Natoye, Damiens et al. (submitted) have stated that *Ae. japonicus* was only present in one location, Natoye1, in sympatry with *Cx pipiens* mosquitoes, whereas, in the other location, Natoye2, only native species where present. The presence of larvae of *Ae. japonicus* and *Cx pipiens* within the same larval breeding sites have revealed a possible larval competition between both species. Moreover, the distribution of *Cx pipiens* species in Natoye1 showed a reduced distribution compared to the one in Natoye2. This reduction also corresponds to the peak of emergence of *Ae. japonicus*, suggesting a negative effect of *Ae. japonicus* on the *Cx pipiens* population. To evaluate this hypothesis, we tested the effect of larval coexistence on the survival of larvae under different environmental conditions (food availability and temperature).

Larvae of *Ae. japonicus* were obtained by hatching eggs coming from the laboratory colony established in Japan. *Culex pipiens* larvae were obtained from the laboratory colony established in Louvain-la Neuve. Larvae used for this experiment have been collected two to three hours after hatching. Larvae were introduced by group of 10 in white plastic vials in the density of 1 larvae per 5 mL. The vials were then placed in a climate-controlled closet under a photoperiod LD 16:8h and 70% relative humidity. Three replicates were done for each conditions. The vials with single species were composed by 10 larvae of one specie (*Cx pipiens* or *Ae. japonicus*), the mixed species condition was done by introducing 5 larvae of *Cx pipiens* and 5 larvae of *Ae. japonicus* in the same vial. For all conditions (single, mixed-species, optimal, starvation, 25 and 15 degrees) 3 replicates were constituted.

Winter survival of *Aedes japonicus*

One Belgian company of second-hand tires reconditioning (corresponding to Natoye1 (50.3389587° N, 5.044879° E)) located in the village of Natoye (Namur) where *Ae. japonicus* have been found was surveyed during the Autumn and Winter of 2009-2010. A larval survey has been done during eight visits between November and March. During each visit, known breeding sites have been sampled: six tyres, one artificial container: abandoned bucket and a natural site as a tree hole. *Aedes japonicus* larvae and pupae were collected from the breeding sources by using a pipette and white plastic bowl, and transported alive to the laboratory in vials labeled with breeding sites specific identification details. On the lab, the larvae were then transferred in small plastic containers with dechlorinated tap water as used in *Cx pipiens* colony and reared to adult in a secured cage at room temperature for identification. Adults were killed at emergence.

3.5.2 Results

Set up of the *Aedes japonicus* colony

Despite all efforts undertaken to establish a colony of *Ae. japonicus*, no hatching was observed from the first batch of eggs sent to Montpellier, beginning of October 2010. Another assay was then performed end of October 2010 with newly collected eggs. Again no hatching was observed despite several stimulations with addition of Vitamin C. A possible explanation of this failure is the sensitivity of *Ae. japonicus* to desiccation (Williges et al. 2008), but the eggs were stored in tubes constantly humidified through wetted cotton and the observation of the eggs under binocular did not reveal any signs of desiccation. Another possibility is egg diapause. A part of the eggs was then stored at cold temperature (4°C) for a month before being submitted to a hatching stimulation.

At the same time, larvae were also collected in Natoye, identified and placed in the confined insectarium of EID. Larvae were kept in plastic trays (30x18x5cm), and submerged into 1.5L of dechlorinated water with constant water emulsion. They were fed with finely ground dried puppy food. Pupae were individually collected and placed into a 40x40x40cm cage for emergence. Adults were fed with a solution of 10% honey and females were fed twice a week on an anesthesia guinea pig. Despite their readily blood-feeding behaviour, the females did not lay eggs. The cause could be the absence of free-mating in captivity. To improve free-mating we followed recommendations of Dr Tsuda (NIH, Japan), who succeeded in establishing a local colony in Japan (Hoshino et al. 2010). We first increased the cage size to 120x40x40cm in order to create a vertical colon to enhance mosquito flight. Then we obscured the top of the cage with a chinked aluminium foil to mimic the sun passing through tree leaves in forest. Despite our efforts and the addition of new larvae collected in November 2010 in Natoye, the mosquitoes never succeeded in reproducing.

At last, eggs of *Ae. japonicus*, from the colony established in Japan (Hoshino et al. 2010), received in the EID laboratory were used to create a colony that was reared following the previous conditions. After having successfully obtained adults, we encountered the same reproductive difficulties with the Japanese originated mosquitoes. To supplement natural copulations, forced-mating pairs of mosquitoes were performed following the technique of Gerber et al. (1994). Nevertheless, we never succeeded in obtaining female egg laying. The colony went then to extinction despite our efforts to maintain it. Our main difficulty was the absence of free-mating in captivity, and the absence of egg laying of the females despite their blood feeding behaviour.

Competition experiment between *Culex pipiens* and *Aedes japonicus*

Either starved or fed, under 25°C and 15°C, mortality of *Culex pipiens* larvae was always higher when mixed with *Ae. japonicus* than alone (Figure 48, 49 and 50). We observed the reverse effect for *Ae. japonicus* for which a higher mortality of larvae was recorded when alone. *Ae. japonicus* larvae seem highly competitive and affect negatively the survival of *Cx pipiens*. The presence of intraspecific competition for *Ae. japonicus* during the experiment can explain the higher mortality of *Ae. japonicus* when alone. Whereas in mixed conditions, the intraspecific competition will be partially replaced by an interspecific competition with *Cx pipiens*, which results in the increased mortality of *Cx pipiens*. However, no aggressive behaviour of *Ae. japonicus* toward *Cx pipiens* was observed and a possible explanation to

the reduction of mortality could be due to a better or faster foraging behaviour of *Ae. japonicus* larvae. These interesting and new results may explain the phenology of both species when together in Natoye1. In case of expansion of *Ae. japonicus* in Belgium, a strong competitive exclusion shall probably appear leading to a change in the dominant species in our country.

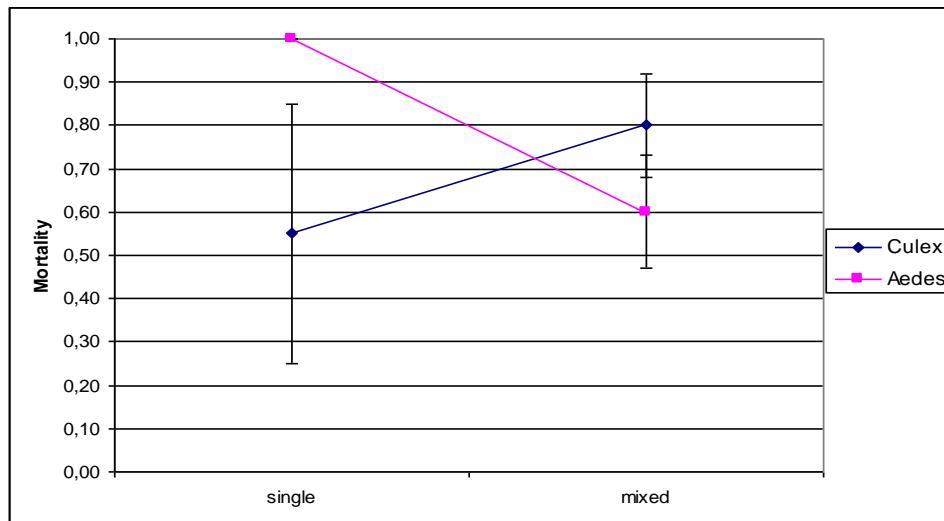


Figure 48: Mortality of larvae of *C. pipiens* and *Ae. Japonicus* in starvation conditions (at 25°C), in single and mixed conditions. Errors bars represent standard error of mean based on replicates.

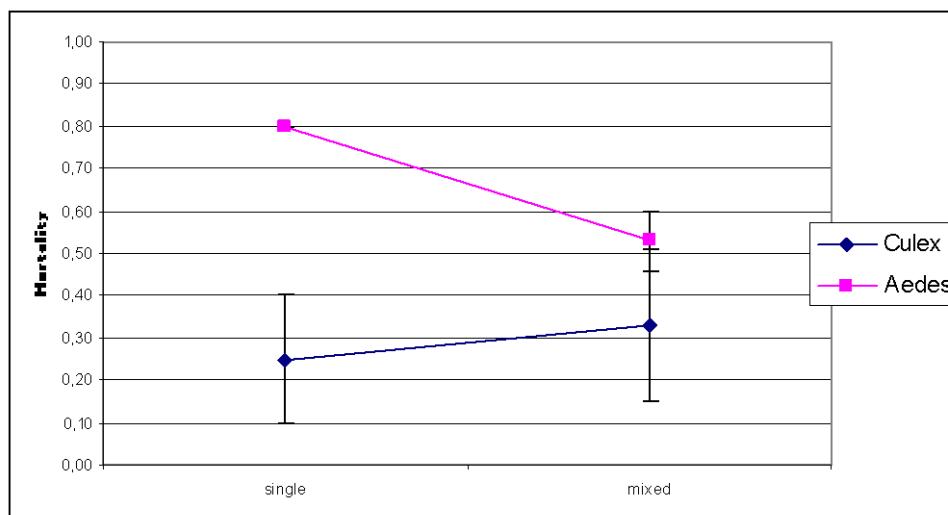


Figure 49: Mortality of larvae of *C. pipiens* and *Ae. japonicus* in "optimal conditions" (25°C). Errors bars represent standard error of mean based on replicates. The error bars represent the standart error.

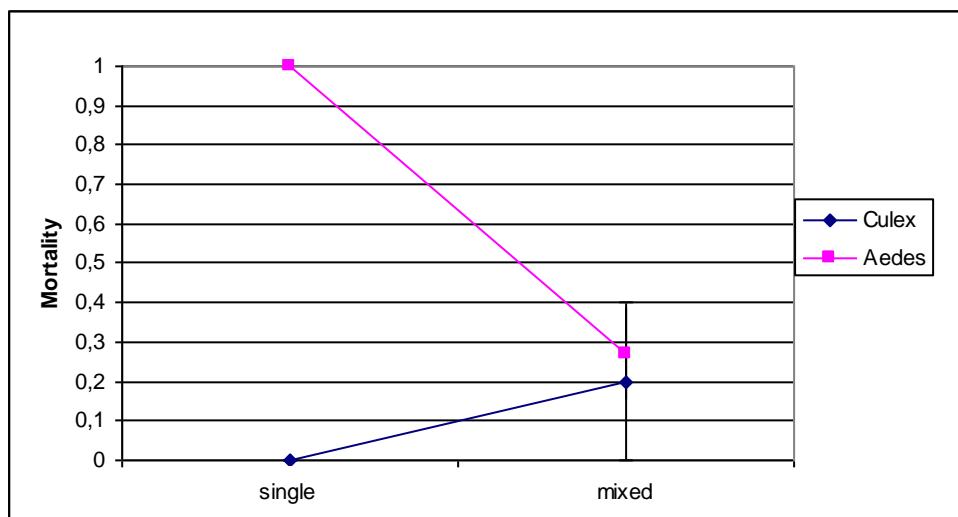


Figure 50: Mortality of larvae of *C. pipiens* and *Ae. japonicus* at 15 degrees in single and mixed conditions. Errors bars represent standard error of mean based on replicates.

Winter survival of *Aedes japonicus*

Table XXIII shows the number of pupae that was found during Autumn and Winter. No larvae have been found in breeding sites during this period and one adult female was found in a tyre on 20th November 2010.

Table XXIII: number of <i>Aedes japonicus</i> pupae found in Natoye 1 during the Autumn winter 2009-2010								
Date	20/11	12/01	22/01	27/01	12/02	18/02	24/02	03/03
	30	0	5	6	0	50	50	0

From December 2009 to beginning of February 2010 most of the water present in breeding site was frozen. Several piece of ice were collected in the tyres and taken to the lab to allow melting. No larvae or pupae have been found in ice. Some pupae collected in January have been allowed to develop and emerge. It takes about one month to emerge suggesting that a diapause or a dormance allow *Aedes japonicus* to survive during the winter. The pupae found in February have been found in a new breeding site consisting on a pound appeared after the thaw.

3.6 Arrival and acclimatisation of the exotic mosquito species *Aedes koreicus* in Belgium, Europe

A new exotic mosquito species was found for the first time in Belgium and also in Europe in 2008 at a single site. Despite differences found between morphological characteristics described in the literature and field specimens, the species was identified as *Ae. koreicus* based on detailed morphological and molecular analyses.

3.6.1 Methodology

Study Sites

The area surveyed was an old sand quarry near the national park Hoge Kempen and the industrial park of Maasmechelen, which harbours several recycling companies. One site was the initial reference site, a small mixed forest fragment with birch, oak and pine next to the industrial zone (MA1). The other subsite (MA2) was situated in the opposite of the road, in a narrow strip of mixed forest adjacent to a large nature reserve (heath) (Figure 51). Land cover consists largely of mixed small forest, moorland, sand quarry and a large industrial zone.



Figure 51. The two sampled subsites at Maasmechelen (MA1 and MA2)

Adult survey

Each site or area was sampled thoroughly, with preferably each site/area consisting of different subsites. Each of this subsite was then again sampled with a minimum of 6 traps (3 types, 2 of each type). The traps chosen are: the Mosquito Magnet Liberty Plus (MMLP), the BG Sentinel and Gravid trap (CDC, J. Hocke model). MMLP were used because of the fact that these traps were used during the inventory whilst BG Sentinels are often used to attract *Aedes* species and Gravid traps for *Culex* species.

The sites were sampled fortnightly during 14 visits every two weeks from the 20th of April 2009 until the 12th of October 2009.

Larval survey

An extensive inventory of potential breeding sites was conducted on both subsites during three visits once in the beginning of the season (May), once during summer (Mid-June) and

once at the end of the season (Mid-September) at both subsites. Emphasis was on as well finding possible natural as industrial breeding sites. At each visit, about 20-30 potential breeding sites were screened. At the industrial zone artificial containers such as abandoned water recipients and tyres were sampled. All found larvae were collected from potential breeding sources by using a pipette, small sieves and white plastic bowl, and transported alive to the laboratory in vials labeled with breeding sites specific identification details. Once in the lab, the larvae were killed by a thermal shock with hot water (60°C). Larvae were then collected with a fine pipette and put in a vial filled with a solution of ethanol at 80%. Treated larvae were then identified using a stereoscopic microscope and an identification key of Schaffner et al. (2001). Some larvae and all pupae were reared to adult in a secured lab for identification.

3.6.2 Results

In total, 1953 adult mosquitoes were collected from both studied subsites belonging to sixteen species. In MA1 all sixteen species were found (1415 individuals) where in MA2 fifteen species were caught (538 individuals). The Mosquito Magnet Liberty Plus (MMLP) caught the highest number of individuals and species (1704 adults, 16 species) where as with the BG Sentinel 10 species were caught but in low numbers (only 82 individuals) and in the CDC Gravid traps 6 species (166 individuals were collected). Only 69 adult mosquitoes of *Aedes koreicus* were sampled in both subsites. Gravid traps yielded the highest number of this target species (46 individuals) whilst the MMLP caught 23 individuals and none were found in the BG Sentinel. The species appeared first in MA1 (mid May) and was found about a month later in MA2 (end June, see Figure 52)

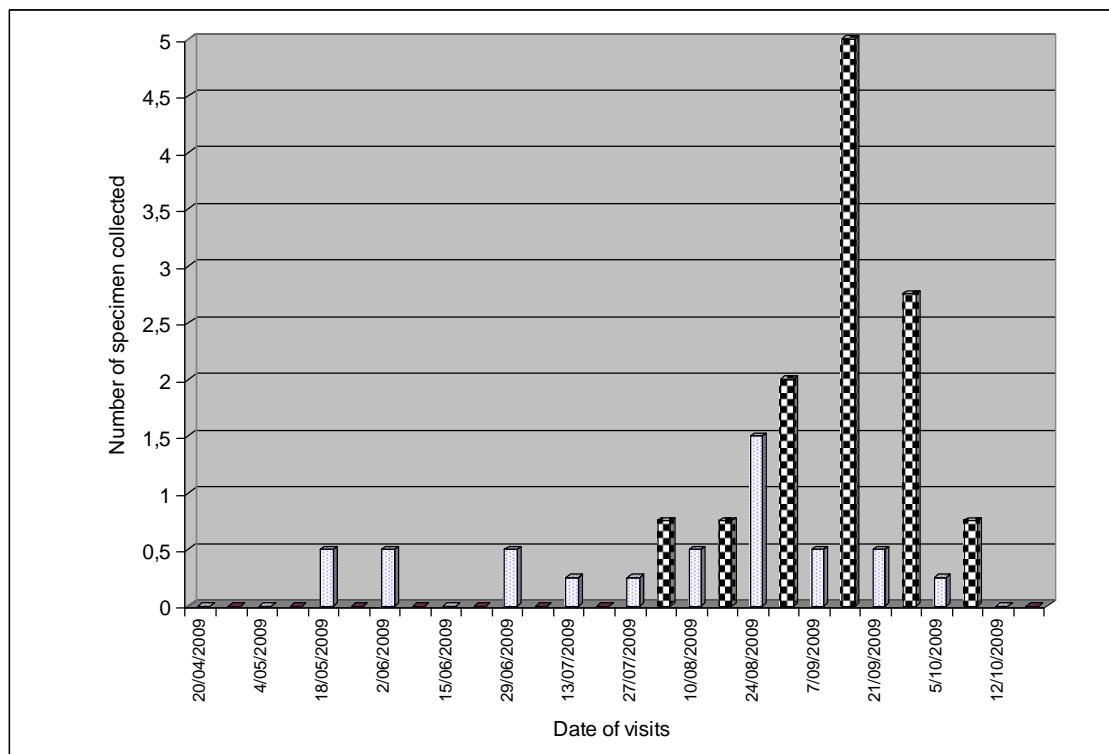


Figure 52. The percentage of *Aedes koreicus* present at MA1 (dotted) and MA2 (block) per visit.

Immature survey

On each of the three visits, the same breeding sites (ca 30/visit) were screened on the presence of larvae. In total 20 positive sites were identified in MA1, no positive sites were found in MA2. No natural breeding sites were identified despite profound survey of the surrounding forests and moors. Breeding sites positive for *Ae. koreicus* were also positive for *Cx pipiens* s.l. and *Cs annulata* (Figure 53). In total 67 larvae of the target species were collected. Due to the very dry summer period, no collections were made in July-August. Each month all 17 placed gravid traps were monitored; however only 2 where found positive for *Aedes koreicus* eggs (molecular identification). Not surprisingly these positive sites were 2 traps placed near the industrial zone of Maasmechelen.

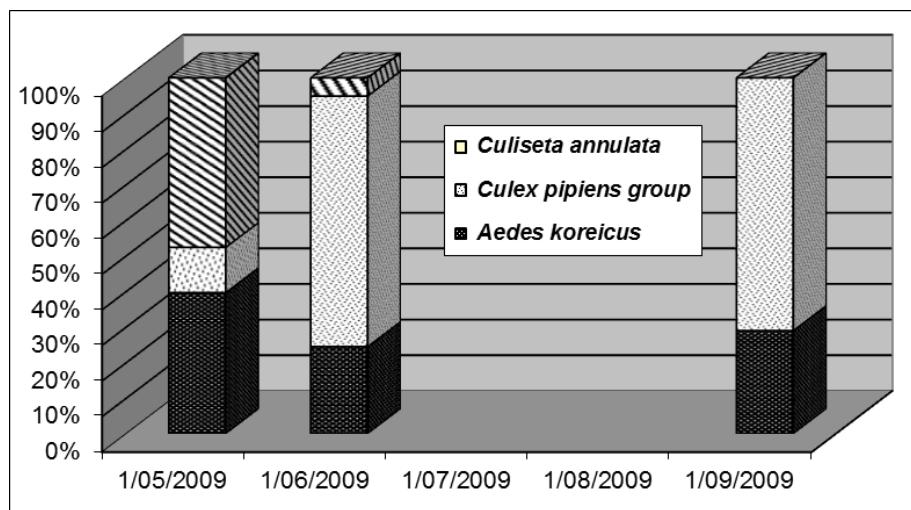


Figure 53. Percentage of *Aedes koreicus* and associated species per visit

The *Ae. koreicus* population found in a natural area in the eastern part of Belgium is the first evidence that the species is capable of successfully surviving long distance transport and to settle outside its region of origin. The species can be regarded as acclimatised at its new environment since adults and immatures were found on different occasions. Neither the route of importation nor the precise origin of the species could be determined. However, the most likely importation pathway will be through the second hand tyre trade, although other pathways cannot be ruled out. In northern Europe, almost half of the first introductions of mosquitoes reported are most probably due to transport by international (often intercontinental) trade, mainly of used tyres (Scholte and Schaffner 2007) or, as described for one case (*Ae. albopictus*), of Lucky Bamboo (Scholte et al. 2008), whilst importation through ground traffic (road or ferry) originating from an infested area in Europe could be responsible for the current spread of *Ae. albopictus* in Europe (Scholte and Schaffner 2007). Worldwide tyre trade is generally considered as an important route for invasive mosquitoes. For example in the USA, of all species collected from discarded tyres, seven of them were introduced through the international tyre trade (Joy and Sullivan 2005, Kaufman et al. 2005). ROK has only a short history of automobile industry (Jang et al. 1998) but this industry is booming in the last decade, with some companies exporting 30,000 ~ 50,000 used tyres per month to middle-east Asian and Caribbean countries (info sheet Eastern Point 2010).

4 BIOLOGY AND GENETIC POPULATION STRUCTURE OF MOSQUITOES

4.1 Influence of temperature on oviposition and larval development of *Culex pipiens* in laboratory conditions

4.1.1 Methodology

***Culex pipiens* colony establishment**

A colony of *Culex pipiens* was started at UCL. Therefore, larvae of *Cx. pipiens* were collected on the UCL campus (Louvain La Neuve, Belgium) in early October 2007 in a pond. The mosquitoes were molecularly identified as *Cx. pipiens pipiens* at ITM. The colony was maintained at 28°C, 50% relative humidity with a photoperiod of 15:9 (L:D). Larvae were reared in small plastic containers with dechlorinated tap water and fed on a diet of Tetramin ground fish food. Adults were kept in screened cages and provided constant access to a 10% sucrose solution. A rat blood meal was offered every two days using the cotton-stick feeding technique (Moutailler et al. 2007).

Oviposition at varying temperatures

The gonotrophic cycle (time between blood meal and oviposition) is important to access the vectorial capacity of mosquito populations. Temperature plays a role in the length of this cycle. The effects of thermal conditions on the length of the gonotrophic cycle started early May 2008. To normalize the experiments, forced mating was tested (one virgin male, one virgin female into a 1l-jar) although normally this species mates in swarms in open spaces. A total of 16 pairs were made. All spermathecae of females were dissected after 4 days to observe sperm. A total 14 females were positive for sperm after four days thus leading to a 87,5% of successful mate. Some pairs were given a blood meal and the females laid eggs after roughly three days. This means that *Cx. pipiens* can mate in close space (small jar) without swarming.

Gonotrophic cycles

The gonotrophic cycle (time between blood meal and oviposition) is important to access the vectorial capacity of mosquito populations. Temperature plays a role in the length of this cycle. To study the effects of thermal conditions on the length of the gonotrophic cycle, Five mated females were placed together in a small cage with water and sugar meal. Females were blood fed once and the water was checked every day to determine the presence of egg rafts. Time between blood feeding and egg raft apparition was noted.

Larval and adult life history traits at varying temperatures

Larval: The effects of thermal conditions on larval development (pupation and emergence time) were tested under eight temperatures (T11, T15, T20, T28, T30, T32, T35 and T40 degrees). One egg raft was placed in a plastic tray containing dechlorinated tap water. Each day, the number of pupae was counted. Each new pupa was isolated in a small plastic cup. The time at pupation and the time at emergence were both recorded. After emergence,

adults were sexed and then killed by freezing. Five egg rafts (replicates) were tested for each temperature. Measurement of wing length (from the base of costa vein to distal extreme of R3 vein, excluding the fringe setae) was used as indicator of imaginal body size. All emergent adults were measured. Measurements were done using a stereoscopic microscope with an ocular micrometer.

Adult: The effects of thermal conditions on adult survival (pupation and emergence time) were tested under six temperatures (T11, T15, T20, T28, T35 and T40 degrees). Pupae were isolated before emergence to determine precise day of emergence. Five adults of same sex were placed together in a small cage with water and sugar meal. Fifteen male and 15 females were thus observe every day to determine their longevity under the six temperatures.

Mating activity at varying temperatures

Surprisingly, little data are available on the reproductive strategy of mosquitoes including common species like *C. pipiens*. However, these data are needed to understand the pattern of population growth of these species and also the relationships between blood meals and population growth (gonotrophic cycle). As temperature influences strongly these traits, we compared several conditions to determine temperature threshold. To estimate pre-copulation time (i.e. the time between the pair formation and the first mating) at different temperatures, one virgin male and female were places in a 1-liter glass jar containing a cup of water. A sucrose solution was given *ad libidum* on the top mesh. The jars were then placed under 11, 15, 20 and 28 C° temperatures. Ten pairs were dissected every day from day 2 to the day where all females were inseminated. Females were killed in ethanol and dissected under binocular microscope to obtain their spermatheca. Dissections were done in a Phosphate buffered saline solution following the procedure of Damiens and Boivin (2005). The mating percentage was evaluated as the number of females with filled spermatheca.

Flight performance at low temperatures. (task 2.1.2)

Because of a 21% budget reduction, we were obliged to limit or drop some of the activities according to the initial proposal. Reductions in the activities were made in such a way that the main outcomes of the project can be achieved. We focused on the validation of the assessment of the population dynamics of endemic and invasive mosquito species and their interrelationship. As a practical consequence of the budget reduction for the 2 years renewal, it was not possible to hire the personal for more than 20 months. Choice had to be made between priority experiments. So we decide to concentrate our means on longitudinal surveys, model validation samplings, mating strategy and competition analyses. These choices were announced in the activity report intended for the intermediate evaluation.

4.1.2 Results

Set-up of the *Culex pipiens* colony

Different protocols have been tested to maximize the productivity of the colony of *Cx. pipiens* (temperature, relative humidity, larval food, blood meal). Two membrane feeder systems

(parafilm and skin membrane) and a cotton-stick feeding system (Moutailler et al. 2007) were tested to feed the females. The latter system showed a higher percentage of engorged females and thus we decided to adopt this feeding system. In May 2008, the *Culex pipiens* colony was well established at UCL's lab under slightly changed conditions: 28°C, 45% HR, 14:10 (L:D). The cotton-stick feeding method was slightly improved. Three cages are maintained for adult reproduction. To avoid inbreeding, larvae from the field (UCL campus, Louvain La Neuve, Belgium) were collected regularly. The adults were morphologically identified and only *Cx. pipiens* individuals were released into the three reproductive cages.

Mating activity experiments

The time between the beginning of the mating activity decrease with the temperature. Indeed, pairs begin to mate after 1, 2 and 5 days respectively at 28, 20 and 15°C. At 10 °C, no mating takes place between male and female. All pairs were mated after 3, 4 and 7 days respectively at 28, 20 and 15°C (Figure 54).

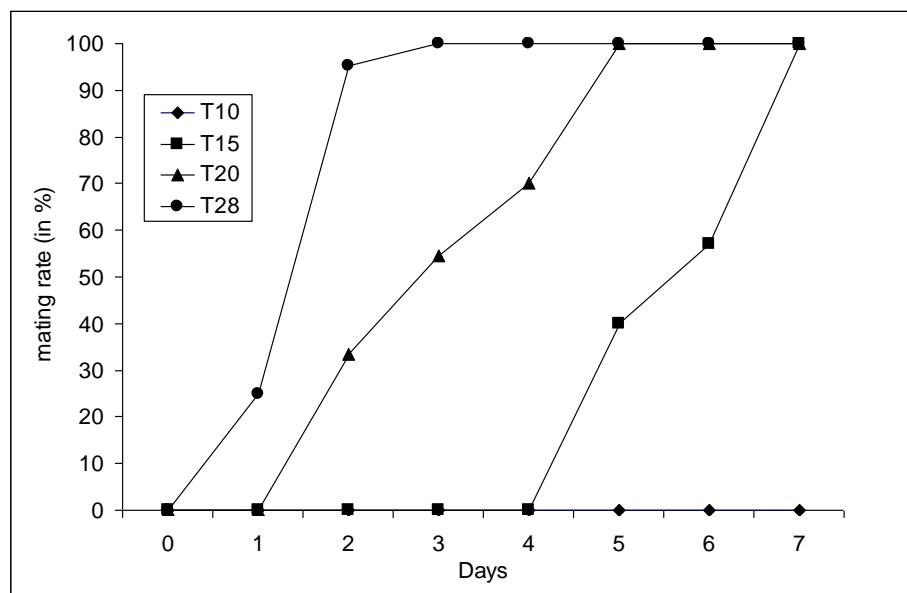


Figure 54. Kinetic of mating rate according to the temperature.

Gonotrophic cycles

The effects of thermal conditions on time between blood meal and oviposition (length of the gonotrophic cycle) have been estimated for three temperatures. The experiment is ongoing and more data ($n=1$ for T15, $n=2$ for T20 and $n=5$ for T28) are needed to confirm the observed trend showing that the length of gonotrophic cycle decreases with increasing temperatures (Figure 55).

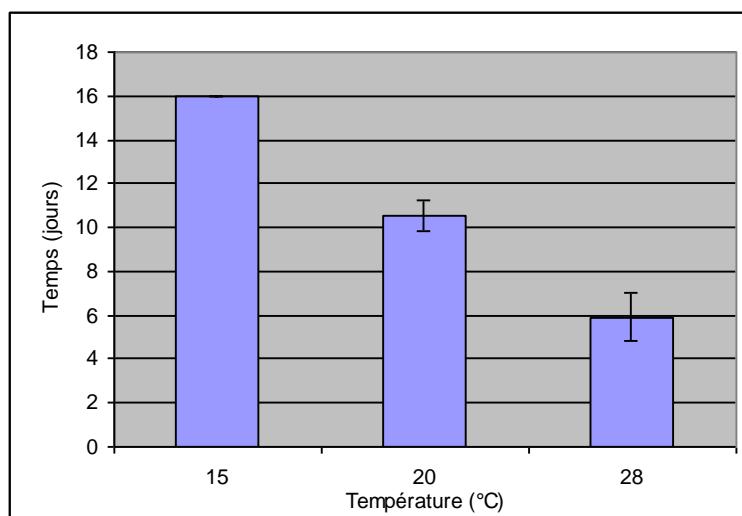


Figure 55. Mean time (\pm SD) between blood meal and oviposition in days for T15, 20 and 28

Development time of larvae at varying temperatures

Both the pupation time and the emergence time increased with low temperatures (Figure 56). It ranges from 10,6 (T28) to 82,7 days (T11) for the pupation time; and from 12,6 (T28) to 91,4 days (T11) for the emergence time. Time between pupation and emergence increased with low temperatures, from 2,0 (T28) to 8,6 days (T11). The pupation time and the emergence time at T30 is similar to T28. Protandry was observed regardless the temperature with the males emerging 1,2 (T28) to 5,4 days (T15) before the females. At 32, 35 and 40°C, no emergence occurred. At 32, mortality occurred mainly during larval stage. At 35, although all the eggs have hatched, no larvae reached the pupal stage (Figure 57). At 40, no eggs have hatched. Females were always bigger than males regardless of the temperature. Although not excessive, larvae reared at low temperatures (T15, T11) gave bigger adults than the larvae reared at high temperatures (T30, T28, T20).

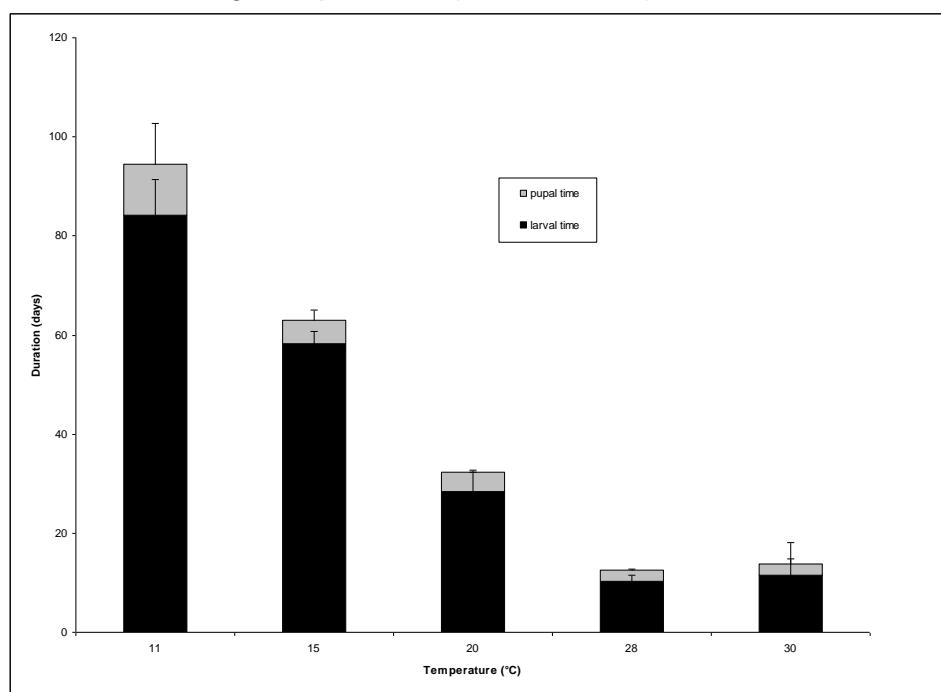
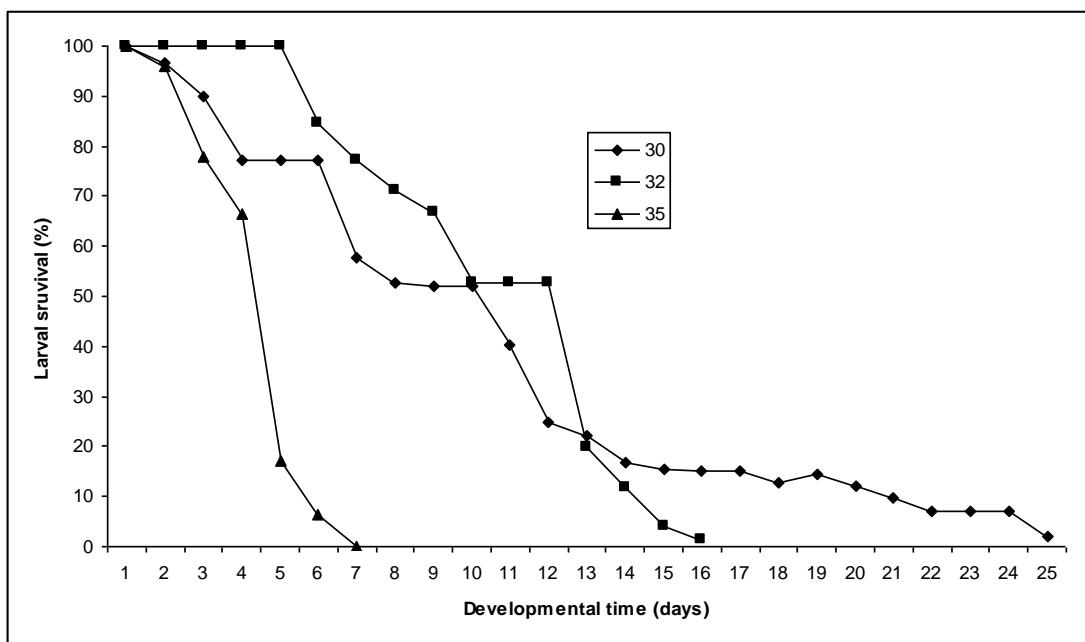


Figure 56: Larval and pupation time (Mean \pm SE) in days according to temperature.

Figure 57: Evolution of larval survival during the development of *Culex pipiens* at three temperatures.

Adult longevity at varying temperatures

The adult longevity decreased with the increasing of temperature from 83.7 ± 17.4 and 72.1 ± 17.4 days for females and males respectively at 15°C to 0.13 ± 0.35 and 0.00 ± 0.00 days for females and males respectively at 40°C (Figure 58). Females appear to live longer than males even if the difference is not significant.

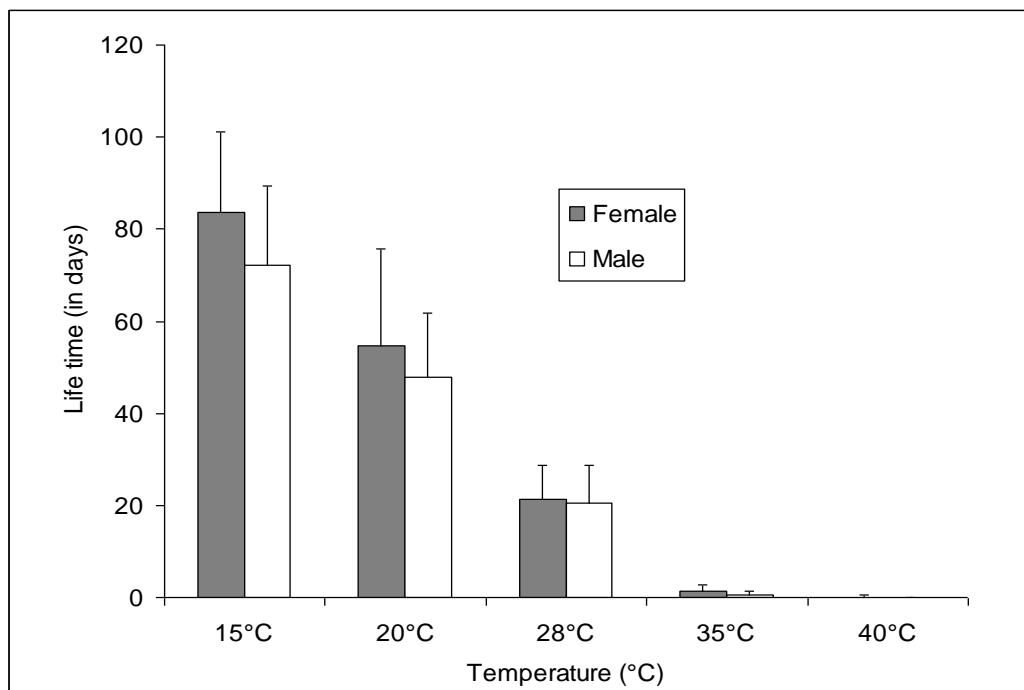


Figure 58: Male and female lifetime according to temperature.

4.2 First strong evidence of two genetic forms of *Culex pipiens* present in sympatric populations in Europe

4.2.1 Methodology

One of the most widespread mosquito species in temperate regions is *Culex pipiens* s.l. which is a good vector for different arboviruses like West Nile. It is generally assumed that *Cx pipiens* s.l. compromises two forms that differ in behaviour, habitat and vectorial capacity although this hypothesis remains disputable. However, in Northern Europe little is known on the presence of both forms although it has been described in other countries (USA, Russia, Portugal). The genetic population structure of 8 *Cx pipiens* populations (30 individuals per population) dispersed over Belgium and over 3 main habitat types (urban, agriculture, nature) were assessed using different molecular markers: microsatellites, CQ11 (flanking region) and mtDNA COI region, which were chosen based on previous studies. Analyses of the microsatellite data were run using "Structure" (for more information see 3.4.1).

4.2.2 Results

No major discrepancies between morphological and molecular identifications were encountered as 99% of all specimens identified as *Cx pipiens* or *Cx torrentium* were correctly identified. The results of the microsatellite analyses and CQ11 marker support the hypothesis of 2 different genetic entities, presumably *Culex pipiens pipiens* and *Culex pipiens molestus* (Figure 59). The number of alleles per locus (microsatellites) show a clear difference between both groups. The results of the PCR-RFLP of the COI region are different from the other two methods and show some incongruence.

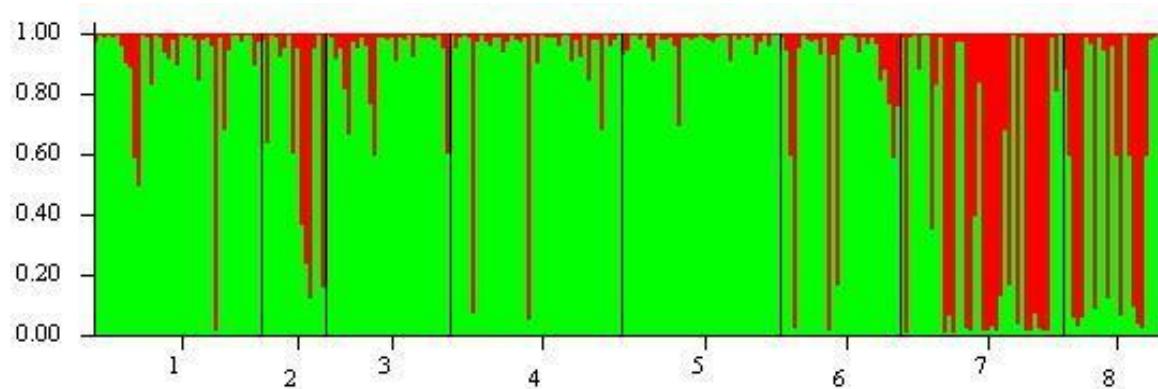


Figure 59: Individuals of each of the eight analysed populations belonged to one of the two defined genetic groups (green or red)

4.3 Genetic characterization of *Anopheles claviger* and *Anopheles plumbeus*, potential autochthonous vectors of malaria

In Belgium 5 known *Anopheles* species occur: *An. claviger*, *An. plumbeus*, *An. maculipennis* s.s., *An. messae* and *An. atroparvus*; the latter 3 all belonging to *An. maculipennis* complex which can not be separated based on morphology alone. The historic native malaria vector is *An. atroparvus*, however both *An. claviger* and *An. plumbeus* seem to be capable of transmitting respectively *Plasmodium vivax* and *Plasmodium falciparum* (Kruger et al. 2001). The identification of *An. claviger* and *An. plumbeus* is based on diminutive morphological differences and visualised basepair differences after amplification of the rDNA ITS2 region. It is however difficult to distinguish between the species based solely on morphology. Furthermore, preliminary results based on DNA sequencing studies do not show a clear separation between both species. Moreover, the hitherto used rDNA markers show intra-individual variation ("multicopy problem"), which impedes their taxonomic interpretation.

4.3.1 Methodology

Based on several molecular markers (mtDNA and rDNA) the current taxonomic description of the two taxa was studied. Different mtDNA (COI, ND4) and rDNA (ITS2, D3) were tested and protocols, especially for the rDNA ITS2 region were refined. The latter region was further exploited for *An. claviger* using cloning techniques on 4 selected individuals. Two individuals of 26 selected populations (divided over provinces and three main habitat types) were sequenced and the obtained data was analysed using BioEdit, Mega, PAUP and MDS. Additionally, of all selected individuals discriminatory characteristics, used in morphological identifications, were scored and implemented in basic analysis to compare with molecular data.

4.3.2 Results

Although some discrepancy still occurred, the distinction of *Anopheles claviger* from *An. plumbeus*, using the molecular phylogeny (based on COI and ND4), seems to match the classical morphological taxonomy. The ribosomal markers turned out to give better results than the mitochondrial markers, which is not in favor of a classification relying on the DNA barcoding. These results were also supported by the analyses based on the base composition. However seen the importance of both species as potential autochthonous malaria vectors and seen the rapid expansion of *An. plumbeus* in northern Europe, these incorrect molecular identifications need to be further unraveled. European specimens will be added to the analyses. The outcomes of this work will serve as a basis in further molecular studies on both vector species.

5 SURVEILLANCE SYSTEM ON MOSQUITOES

5.1 Accuracy of using a less intensive MODIRISK sampling strategy for surveillance

5.1.1 Defining a cost-effective spatial sampling strategy

Entomological data/Sampling Design

For this experiment, the entomological data of the cross-sectional study was used. These are described under chapter 2.1.

Predictor data

For this experiment, the full dataset of predictor variables were used. These are described under chapter 3.1.

Modelling technique

The modelling technique was Random Forests, a technique using an ensemble of classification trees. This algorithm was selected because of its robustness and its ability to make accurate model outputs, even with a limited number of observations. More detailed information about Random Forests can be found under Work Package 3.2. Model accuracy was assessed using area under the curve (AUC), percentage correctly classified (PCC), sensitivity and specificity.

Experimental Setup

During this four-year project, an impressive database was built, consisting of nearly 1,000 sampling points, randomly distributed over the area of Belgium, and evenly spread out over two years (2007,2008) and four seasons (respectively two spring and two summer seasons). This sampling scheme was described in chapter 2.1. Notwithstanding the great value of this sampling design, it would be far too labour-intensive to maintain this amount of sample points in the future for monitoring purposes. The aim was thus to analyse the effect of a decreasing amount of sample points on the mapping of mosquito diversity. In this project it has been demonstrated that a species diversity map, based on the environmental suitability of the four most common and abundant species in the traps, gives a good indication of environmental suitability of the majority of the mosquito species, both for widespread and rare species. For Belgium, the four selected species are *Aedes cinereus/geminus*, *Aedes vexans*, *Coquillettidia richiardii* and *Culex pipiens*. A cost-efficient sampling strategy would start with a first phase, during which a mosquito species diversity map is constructed with a minimum number of traps. In order to determine a minimal sampling density, a subset was made from the original database, and this subset was used to produce maps of environmental suitability for the four selected species. These maps of environmental suitability were then compared to the maps based on the entire dataset, both looking at model accuracy and general patterns/trends in the maps.

In order to test different sampling strategies, different subsets were drawn from the full dataset using a random sampling. Different sampling intensities were simulated by subsets

of different sizes, selecting respectively 90, 80, 70, 60, 50, 40, 30, 20 and 10 % of the full dataset. To test for robustness, 10 repetitions were made for each level of sampling intensity. While the original sampling approach corresponded with a sampling intensity of 1 trap per 30 km², the lowest sampling intensity corresponded with a sampling intensity of 1 trap per 300 km².

5.1.2 Results

Predictions of environmental suitability were made on several subsets of the original dataset. As the goal of these models was to construct a species diversity map with a minimum number of traps, only the environmental suitability of the four most common species were computed. The results of these models are shown in Figure 60, Figure 61, Figure 62 and Figure 63. It was striking that as the number of traps fed into the model decreased, the trends observed remained the same. Areas with high environmental suitability remained at the same place, and were clearly recognisable on the maps. Another observation that can be made based on these maps, is that the colour difference between the high and low suitable areas decreased as the number of traps was lower. This indicated that the model had more difficulty in separating suitable from non-suitable areas, which makes the colours in the map paler. The values for environmental suitability were more average than when the entire dataset was used. Nonetheless, a distinction could still be made between suitable versus non-suitable areas.

The accuracy measures for these models (Table XXIV, Table XXV and Tabel XXVI) showed, as expected, that the accuracy decreased as the number of traps fed into the model decreased. However, the loss in accuracy was acceptable to a given level. When looking at PCC (Table XXIV), two out of four models still had an accuracy higher than 0.70 when merely 40 % of the original dataset was used. Sensitivity of the models dropped only below 0.70 for one species, being *Cx. pipiens* (Table). For the other species, sensitivity was quite high; 0.89 for *Ae. cinereus*, 0.90 for *Ae. vexans* and 0.76 for *Cq. richiardii* with only 20 % of the full dataset.

Specificity values were lower to start with, so the threshold of 0.70 was reached earlier (Table XXVI). For *Cq. richiardii*, a specificity of 0.69 was observed at 80 % of the full dataset; for *Ae. vexans*, the value for specificity dropped below 0.7 at 60 %, and *Ae. cinereus* and *Cx. pipiens* featured a specificity of respectively 0.70 and 0.79 at 20 % of the full dataset.

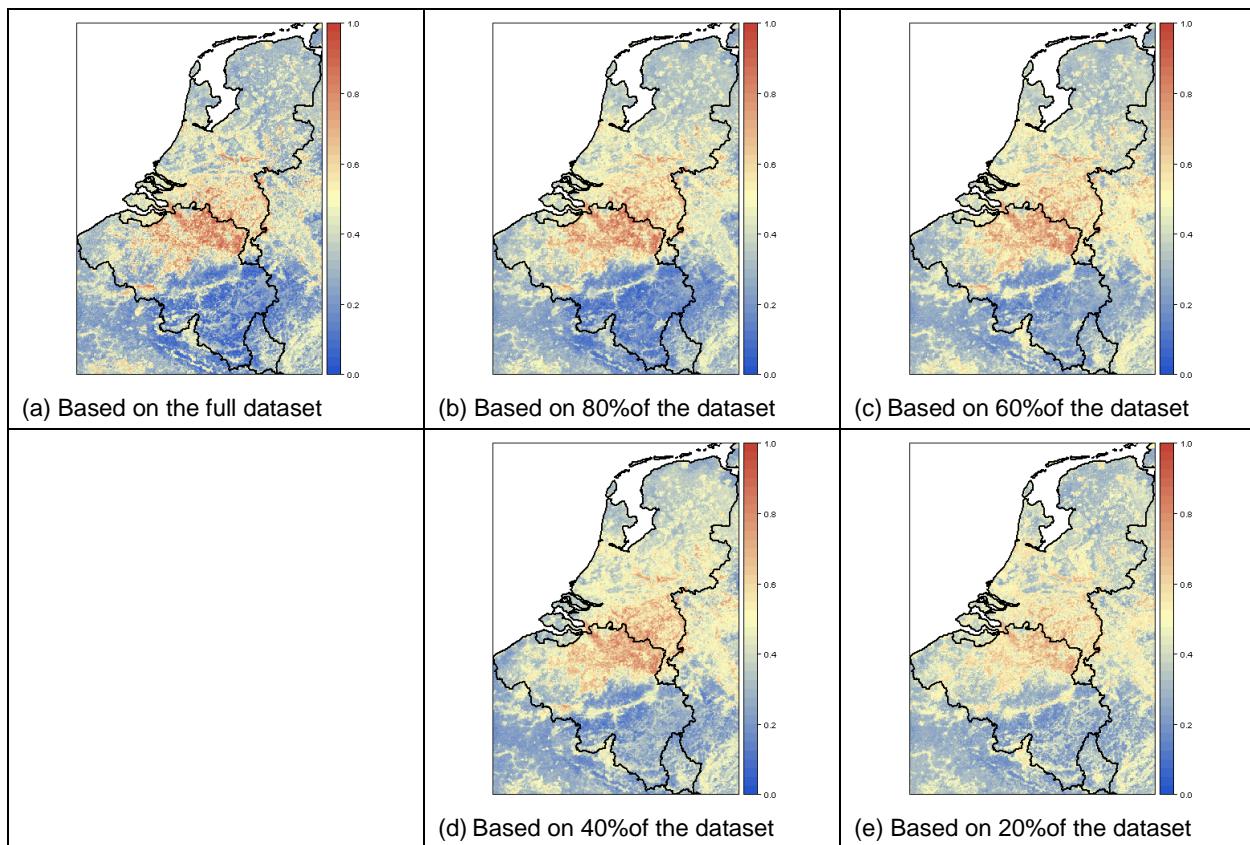


Figure 60. Predictions of environmental suitability for *Ae. cinereus*, based on a decreasing number of training points.

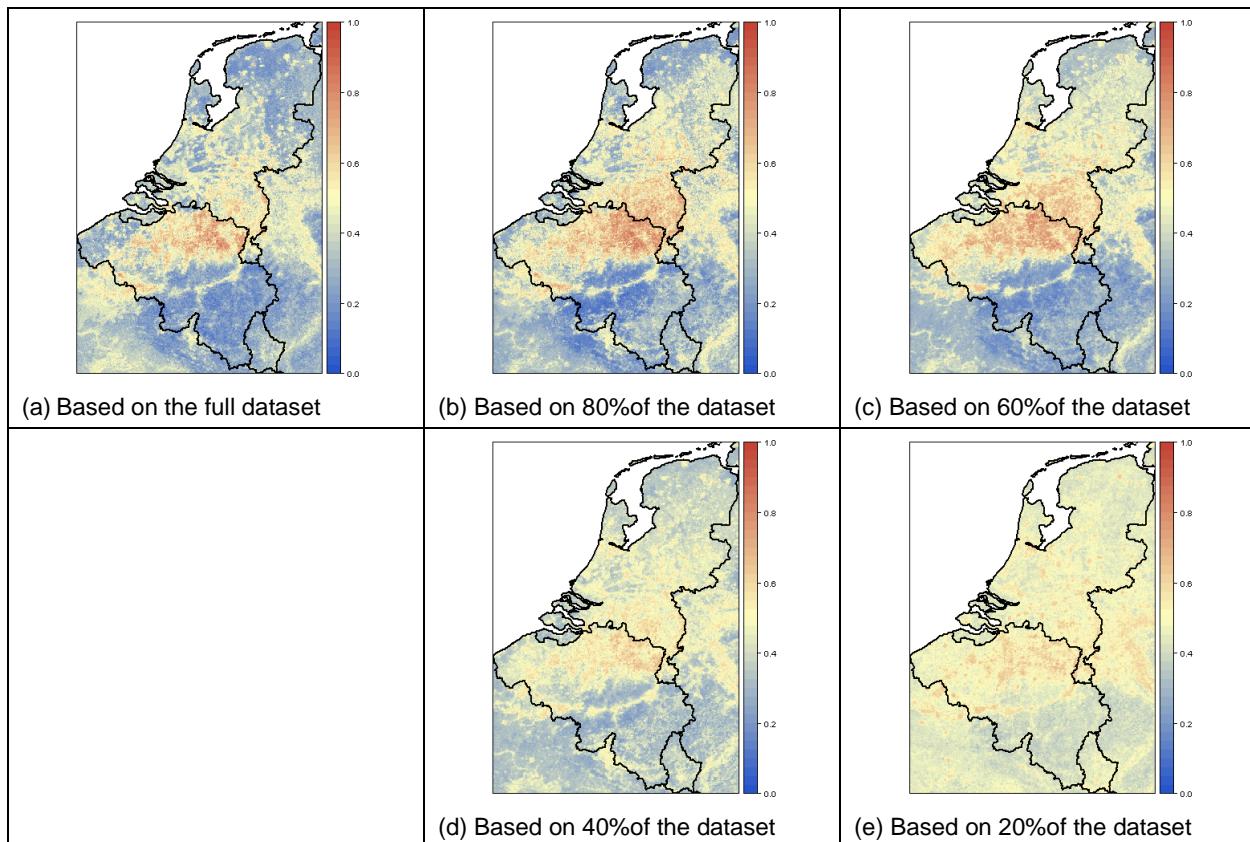


Figure 61. Predictions of environmental suitability for *Ae. vexans*, based on a decreasing number of training points.

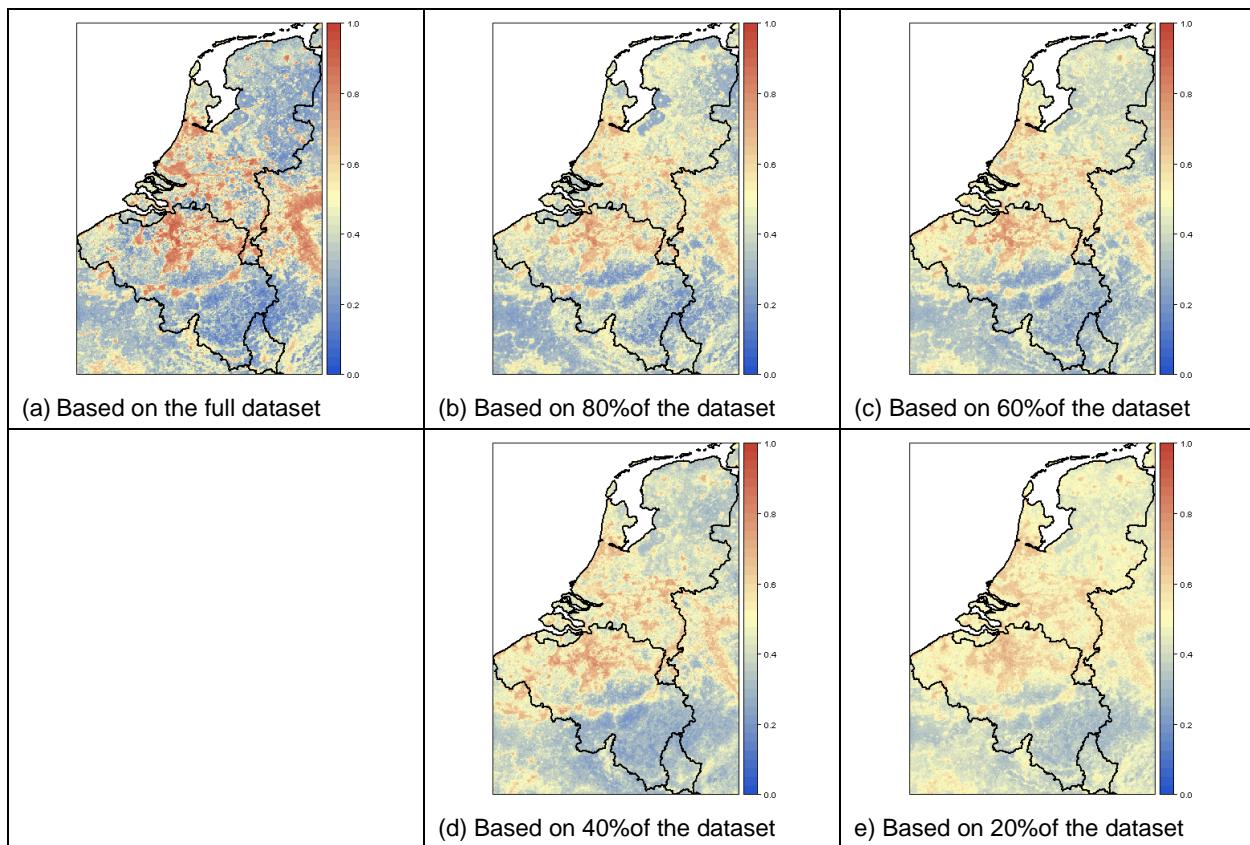


Figure 62. Predictions of environmental suitability for *Cq. richiardii*, based on a decreasing number of training points.

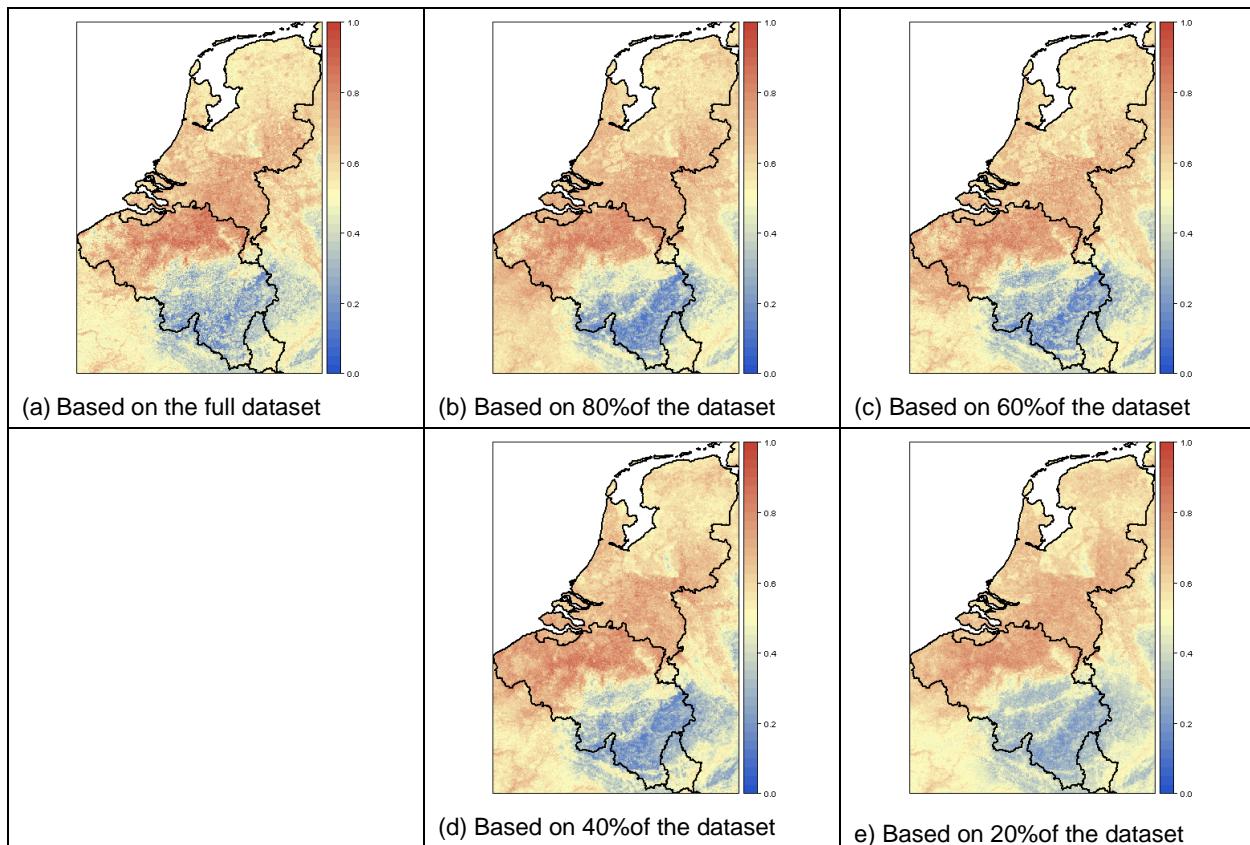


Figure 63. Predictions of environmental suitability for *Cx. pipiens*, based on a decreasing number of training points.

Table XXIV. The accuracy of the models deteriorates as fewer points were used to fit the model. Accuracy assessed by percentage correctly classified instances (PCC).					
species	100%	80%	60%	40%	20%
<i>Ae cinereus</i>	0.75	0.79	0.77	0.75	0.71
<i>Ae vexans</i>	0.75	0.71	0.65	0.60	0.54
<i>Cq richardii</i>	0.73	0.70	0.69	0.67	0.65
<i>Cx pipiens</i>	0.87	0.83	0.80	0.75	0.71

Table XXV The accuracy of the models deteriorates as fewer points were used to fit the model. Accuracy assessed by sensitivity.					
species	100%	80%	60%	40%	20%
<i>Ae cinereus</i>	0.99	0.96	0.91	0.85	0.89
<i>Ae vexans</i>	1.00	0.97	0.95	0.92	0.90
<i>Cq richardii</i>	1.00	0.94	0.88	0.82	0.76
<i>Cx pipiens</i>	0.82	0.79	0.76	0.72	0.67

Table XXVI. The accuracy of the models deteriorates as fewer points were used to fit the model. Accuracy assessed by specificity.					
species	100%	80%	60%	40%	20%
<i>Ae cinereus</i>	0.73	0.77	0.76	0.74	0.70
<i>Ae vexans</i>	0.74	0.70	0.64	0.59	0.53
<i>Cq richardii</i>	0.72	0.69	0.69	0.67	0.65
<i>Cx pipiens</i>	1.00	0.93	0.88	0.82	0.79

Reproducible sampling strategies

The obtained results indicate that a stratified random sample is a good methodology for selection sampling locations for mosquito monitoring. A first phase of an effective sampling strategy comprises a rapid assessment using 1 trap/300 km². The entomological data from this sampling is then used to model environmental suitability for the four most common species. Based on this simulation, one trap per 150km² resolution (in stead of one trap every 30km² used in present study) would be an appropriate for further monitoring. Based on these four indicator species, hotspots can be identified for intensifying trapping. These findings will make future monitoring more efficient and less expensive.

6 POLICY SUPPORT

The inventory was based on a random (statistical) approach that was designed for model building. This is unique in Europe (and even in the world) since most models are based on historical records. Based on the results of the cross sectional field surveys, distribution models predicting the probability of presence of each Culicidae species were developed and maps produced at a one kilometre resolution. These models are based on RandomForest techniques and used eco-climatic data as main predictor variables. Additional field surveys were conducted in the second phase of the project in Belgium and in The Netherlands to validate and further fine tune the produced models. These model outputs enables us to start to understand the factors (mainly eco-climatic, but also human driven such as land use, urbanisation) determining observed distribution patterns. By including these in a GIS model, Culicidae biodiversity hotspots were highlighted (areas of high co-occurrence of species) which are of prime importance when addressing the issue of emergence of diseases.

Based on the experience gained during MODIRISK a cost-effective sampling strategy was designed for use in follow-up and similar studies. Modelling will mainly assist in defining the minimal field sample needed to produce acceptable distribution maps, and how these samples are best distributed in space. The results of comparative trap trials of different trapping systems, operating on different attractants, and conducted to evaluate how trapping devices can be used for Culicidae monitoring in general and as sentinel monitoring system for invasive species in particular, was included in this integrated approach.

The project directly contributes to discovering biodiversity and monitoring/predicting its changes, and actively prepares to address issues such as the assessment of impacts of biodiversity change with particular reference to new invasive mosquito species and the risk to introduce new pathogens. An improved understanding of the biodiversity of mosquito vectors is an essential step towards an improved understanding of the ecology of the diseases they transmit. Furthermore it contributes to the development of state of the art scientific tools integrating collection-based information technology at various resolutions with geographic mapping efforts and remote sensing driven continuous distribution models. This enables to better describe the spatial distribution of mosquito biodiversity, and to understand how it is organized in communities and habitats. The filling of an essential knowledge gap in Europe, and the expansion of model outputs through linking up with a project in The Netherlands, enables the project to produce more robust results and to prepare better for later expansion of activities in Europe. MODIRISK plays its role as interplay between newly gained insights and the end-users. The partners are actively involved in different networks and meetings (with the European Centres for Disease Prevention and Control and the Belgian Forum on Invasive Species). Furthermore a workshop on "Vector Control in Belgium" was organised by MODIRISK to bring together persons potentially involved in the decision making process on vector control (such as the risk assessment group), other stakeholders and interested persons to discuss the different aspects on vector control in Belgium.

The project directly contributes to discovering biodiversity and monitoring/predicting its changes, and actively prepares to address issues such as the assessment of impacts of biodiversity change with particular reference to new invasive mosquito species and the risk to introduce new pathogens. An improved understanding of the biodiversity of mosquito vectors

is an essential step towards an improved understanding of the ecology of the diseases they transmit.

6.1 Towards predicting distribution patterns in a changing environment

6.1.1 *Aedes japonicus*: increased risk?

This longitudinal study addressed questions relevant for the development of a Pest Risk Assessment of exotic species *Aedes japonicus* and an assistance in Administrative Decision-Making. To date, it seems that *Aedes japonicus* is well-established in the site Natoye1 since significant number of larvae have been found during only three month in 2010 at the beginning of the season. However, there are currently no indications that the species, which has been present for at least seven years in Belgium, has spread from the surroundings of the second hand tyre company.

An adapted treatment could be developed from our results. The breeding sites of *Ae. japonicus* were mostly tyres and buckets localised in the company site. According to our results, a first localised treatment to control *Ae. japonicus* larvae population in the month of May could be efficient. A preventive action could also help to control population, by emptying the tyres present on the site, thought this will be complicated regarding the number of tyres and their size. Storage must also be done under a shelter or a plastic cover to avoid that rainfalls fill the tyres.

6.1.2 How to deal with outbreaks of *Anopheles plumbeus*?

When outbreaks of *An. plumbeus* are reported in sites in urban landscape, the first important thing to do is to detect the core larval breeding site in the neighborhood. In most cases - probably all in Belgium- detection should focus on manure pits and collection tanks of abandoned and uncleaned pig stables and they should be inspected intensively. When larvae are detected in such habitat, the manure pit and collection tank need a structural intervention in such way rain water can be prevented to enter the pits. However this is a very difficult task in most cases and cleaning the complete pit and collection tank is essential.

In Torhout the owner of the pig stable decided to clean his stable and manure tank in the winter of 2009. The entire collection tank was emptied and drained. Moreover drainpipes were installed in the cleaned stable. In this case the larval breeding site was removed and carried off. A second important step is a further follow-up of the pest site and screening of all other potential breeding site near stables (see 3.2). There is a strong possibility that *An. plumbeus* has colonized other small breeding sites during the peak of the active season.

After the treatment of the infested pig stable at Torhout in the winter of 2009, is seemed that the problem was solved at the pest site. However from half July 2010 again large numbers of adult *An. plumbeus* were detected in the gardens near the pig stable. *An. plumbeus* had colonized the abandoned and uncleaned pig stable next to the former core breeding site, resulting in a new and identical outbreak as in 2009. This winter this pig stable was cleaned too.

6.1.3 How to predict outbreaks of *An. plumbeus* in Flanders?

Two things are important in the prediction of other outbreaks of this mosquito in Belgium. Firstly all abandoned and uncleared pig stables near forest carry the risk to become infested in the future. In several regions in Flanders where many owners of pig stables recently received EU-funds to run down their pig-rearing activities many so far not yet colonized manure pits await colonization by *An. plumbeus*. Secondly all pig stables in the neighborhood of former and present localities of outbreaks of *An. plumbeus* should be given special attention. In Table XXVII we mention all so far known and reported outbreaks of *An. plumbeus* and the year they were reported for the first time.

Table XXVII. Sites with reported outbreaks and sites with potential outbreaks				
Community	Locality	Problems	Treated or follow-up so far	Outbreaks
Kasterlee	Isschot 14	2007	follow-up since 2010	Yes
Torhout	Groenhovestraat 25	2006	treated in 2010	Yes
Torhout	Ruitjesbosstraat	2008	follow-up since 2010	Yes
Bochelt	Oudeweg (grens NL)	2005	reported	Yes
Eben-Ernael	Rue du Couvent 10	2010	reported	Yes
Ravels	buur Jachtweg	2008	sampled during Modirisk	Yes
Diepenbeek	Kiezel	2008	sampled during Modirisk	?
Overijse	Duisburgsesteenweg	2008	sampled during Modirisk	?
Liedekerke	Laan 1	2008	sampled during Modirisk	?
Thoricourt	Rue de Silly	2007	sampled during Modirisk	?
Dilzen	Molenveld	2007	sampled during Modirisk	?

6.2 Exotic and endemic pest species

6.2.1 *Aedes japonicus*: need for a secure lab

To study invasive species such as *Aedes japonicus* in Belgium and to be able to design experiments in a proper way, developed quarantine or test for virus presence, we recommend the establishment of a secure laboratory as it was realized in Montpellier.

The confinement laboratory in Montpellier has been realized in the aim of being able to maintain non-indigenous mosquito species that can potentially become established in France under secure condition. A quarantine is also available. At the moment, two invasive mosquitoes species have been maintained in this lab: *Aedes albopictus* (Skuse) (Diptera: Culicidae) and *Aedes japonicus*. As only species with agronomical interest are listed in annexes I to V of article 77/93/CEE, possible invasive mosquitoes species are not concerned to the obligation of being maintained in a secure structure. Nevertheless the EID laboratory was built following article 95/44/CE (26 July 1995) with few adaptations for adult mosquito

maintenance: EID confinement laboratory is composed of air shower, transitional airlock, refrigerated airlock, two insectariums with airlock, four laboratory rooms and an animal housing facility. Laboratory is totally airtight and the escape of adult mosquitoes is avoided with adhesive ground system in the air shower, UV insect destructors in corridors, ventilation filters, triple sifters on evacuations pipes, inactivation of waste by autoclaving, laboratory secure suit and wearing outer garments, shoe coverings, including gloves that protect against egg contamination.

Such secure laboratory is important for any program of study of non-indigenous invasive mosquito species. It ensures the security of the environment by providing from any escape of those invasive species

6.2.2 *Aedes koreicus*

The inventory revealed the presence of a new exotic mosquito species being able of colonizing new non-native areas. *Aedes koreicus* was found for the first time in Belgium and also in Europe in 2008 at a single site. Despite differences found between morphological characteristics described in the literature and field specimens, the species was identified as *Ae. koreicus* based on detailed morphological and molecular analyses. The use of reference material from a museum collection (here Smithsonian Institution) was essential. There are a number of consistent morphological differences between Belgium and Republic of Korea mainland specimens, though there are also similarities with female adults from Jeju-do (Tanaka et al. 1979). Hence, we hypothesize that the species is imported from Jeju-do Island and not from mainland ROK. The longitudinal species seems well established in the vicinity of the industrial area. Although the species was not observed as being aggressive towards humans, its status as vector species for Japanese encephalitis (at least in the former USSR) implicates a certain human health risk.

Moreover, considering this is the third exotic mosquito vector species introduced to Belgium in the last 10 years (Schaffner et al. 2004; Versteirt et al. 2009), there could be a high invasion risk possibly associated with the country's rank in the worldwide trade in used tyres. These findings strengthen the need of surveillance in Europe and plead for monitoring of the main importation pathways of vectors. In particular, a better traceability of imported and exported goods at risk will improve the surveillance possibilities. This implies a good co-operation of import companies with the authorities which is often lacking (Piel et al. 2008). Prompt and adequate control is needed after detecting an exotic species in order to eliminate it or at least to limit its further spread. Also, mosquito control methods should be improved and adapted to target invasive species in order to increase their efficiency in elimination programs.

6.2.3 *Anopheles plumbeus*

Our observations indicate that manure tanks of abandoned and uncleaned pig and cattle stables might serve as favored breeding sites that allow unlimited population growth of *An. plumbeus* larvae during summer. This is both observed in Belgium as the Netherlands. These abandoned and uncleaned stables are neglected by the owners. Hence roofs break

down and rain water is gathered in the manure pits. Moreover all stables measure 10 by 30 meters and have (in most cases) one large collecting pit over the complete area of the stable. These pits are one meter deep and a large water volume is available for the mosquito larvae. These manure pits once colonized, allow *An. plumbeus* to reach very high larval densities in these large water volumes (10m x 30 m x 1 m volume) compared to small tree-holes and used tyres (only a few liters water).

Near these pig stables (gardens of neighbors) and within this human made ecosystem, adult *An. plumbeus* reach large densities that may pose serious nuisance to humans from May until September. This might pose particular problems in several regions in Flanders where many owners of pig stables recently received EU-funds to run down their pig-rearing activities. Hence, many so far not yet colonized manure pits await colonization by *An. plumbeus*. This habitat expansion of *An. plumbeus* could result in pest outbreaks in certain regions in Belgium and eventually could be responsible for autochthonous malaria cases. Moreover, the large population densities might facilitate colonization success of other novel human-created habitats near the abandoned pig stables. Potential sites with similar conditions are manure pits of compost mounds, sewerage with stagnant water and abandoned manure pits of stables of small cattle. Although they might not be as important and large as the manure tanks of abandoned pig stables, they might be sufficient to alter the colonization-extinction balance (Levins 1969, Hanski 1991), which could lead to a persistent presence of the species in anthropogenic influenced habitats even after removal of the core breeding site.

Besides its relevance for human health, *An. plumbeus* offers an ideal model organism to investigate the mechanisms that underlie an expansion towards novel habitats. It remains for instance unknown whether the colonization towards this novel habitat implies ecotypic differentiation, as has been observed for populations of the mosquito *Culex pipiens* in response to breeding in subterranean and human-made sites (hypogeous habitat) from natural populations all over Europe (Chevillon et al. 1998, Byrne and Nichols 1999). Also studies from the Camargue region suggests that anthropogenic changes should not be underestimated in vectorborne disease recrudescence (Ponçon et al. 2007). Understanding processes caused by habitat expansions as is the case with *An. plumbeus*, is of increasing importance when it induces the spread of species that have economic implications or a strong impact on human health.

6.2.4 Aedes albopictus

Aedes albopictus was found prior to the MODIRISK project in 2000 in a second-hand tyres company but was not recorded from the surveyed import risk stites during the project. It naturally disappeared from the site where it was found in 2002 but well established in other part of Europe (South of France, Italy, in dutch nursery importing plants as lucky bamboo...) and seem to be spreading more northwards.

6.2.5 Measurements against exotic species

All exotic species could be vectors for human diseases (as WNV, dengue, yellow fever, ...) and should therefore be quickly addressed.

Immediate measures:

For the second-hand tyres company affected:

To remove water from all tyres

To keep tyres dry (by systematic emptying)

To cover tyres at least with plastic sheets mainly for the tyres stacks bordering the wood or roof, indoor storage, shed

To avoid stagnant water on the sheets

To remove from the nearby wood, and within 1 km radius, all artificial container in the vicinity in order to avoid water accumulation and so breeding site

To maintain surveillance of larval and adult populations in a perimeter of several km at least until next summer

To spread biocide (Vectobac®) if mosquitoes population not decreased next year

To inform the population to be vigilant about stagnant water by leaflet (e.g.: water in flower pots, graveyard, old containers,...), to inform farmer about tyres utilisation, watering place

To recommend to burgomaster to distribute the folder to the population of the village

For the natural site :

To inform the population to be vigilant about stagnant water by leaflet (e.g.: water in flower pots, graveyard, old containers,...), to inform farmer about tyres utilisation, watering place

To recommend to burgomaster to distribute the folder to the population of the village

Short term measures

To maintain surveillance of mosquitoes

where they were already identified in order to estimate the impact of control measures and to adapt them if necessary

where a potential risk has been identified following final output of Modirisk project

To create a Task Force offering coordination between all initiative evaluating the risk coming from new vector as surveying possible appearance of the virus.

Mid-term measures:

To create a legal context to implement the necessary measures avoiding stagnant water in artificial container o.a. tyres.

7 DISSEMINATION

7.1 Public link to MoDiRIsk information system

7.1.1 Aimed at the general public

The field work implies a close collaboration with the general public. Therefore the project was announced through a press release and several interviews with different media were given in national and international press.

The progress of the field teams could be followed at the public site: <http://modirisk.avagis.com/>

A newsletter in three languages (Dutch, French and German) was made to inform the general public about the first results of the project (see: www.modirisk.be). This newsletter was published on the web site and hard copies were given to house owners contributing to the field work by allowing the field teams to put a trap. This newsletter and the second sampling season were announced through a press release. An additional newsletter and press statement will be released by the end of the MODIRISK project, the newsletter will be sent to all people participated in the inventory and longitudinal studies.

Several generalized presentations for a broader audience (non-scientific) were given:

Ankona (09/02/2008)

Likona (17/01/2009)

VZW Durme (09/12/2010)

The website (both at Avia-GIS and ITM) will be maintained as long as possible with links to publications and other important outcomes.

7.1.2 Aimed at the end-users

The partners of MODIRISK contributed to several meetings and critical observations were communicated with the competent authorities:

- The co-ordinator has participated to the European Centres for Disease Control (ECDC) consultation on the vector-related risk for Chikungunya virus transmission in Europe, October 2007.
- Links are made with the Belgium Forum on Invasive Species by participating to the discussions on the 'Guidelines for environmental impact assessment and list classification of non-native organisms in Belgium' and by acting as member of the scientific committee of the 'Science Meeting Aliens' conference on biological invasion (11th of May 2009).
- Several MODIRISK partners were consulted by the AGORA project on 'set-up of monitoring of potential effects of climate change on human health and on the health of animals (January 2009).

- In 2007 MODIRISK identified a recently established exotic mosquito population in a second hand tire company in Natoye (near Namur). On the initiative of the MODIRISK co-ordinator the Risk Assessment Group of the Scientific Institute of Public Health met to discuss the measures to be taken to control this potential vector species.
- On January 2009 MODIRISK organised a workshop on vector control in Belgium. See below for more details on the outcome of the workshop.
- The final meeting, on the May 10 2011 was open to the public. The program and a detailed report of the meeting can be found in the Annexes

7.2 Associated projects

- Avia-GIS initiated and successfully obtained jointly with CIRAD and 20 other partners the V-borne project as a reply to an open ECDC call. V-borne is a six month project aiming at assessing the risk of introduction, establishment and spread of vector borne diseases in Europe. The activities of MODIRISK and the need to further extend this approach to other EU countries is stressed in the final report.
- Avia-GIS initiated and is coordinator of the TigerMaps project as a reply to an open ECDC call. V-borne is a six month open call ECDC project aiming at modelling the current distribution and potential spread of *Ae. albopictus* in Europe. Two other partners are involved.
- UCL has been funded a 2 year-proposal to FSR (Fond Spécial pour la Recherche) to study the dynamics of the invasive exotic species *Aedes j. japonicus* in Namur and its epidemiological implications.
- The spatial sampling strategy and palm-to-web tool as developed by Avia-GIS serves as an example to implement a cluster of spatial mosquito sampling and modelling projects in several European countries as part of the IAP program of ESA (European Space Agency). Currently a feasibility study is being conducted upon request by ESA.
- Avia-GIS and ITM participate in the VBORNET project following an open ECDC call succeeding the V-borne project. Avia-GIS initiated and is coordinator of this project, ITM is work package leader of WP3: vector distribution and surveillance.
- ITM is partner in a cluster project of Belspo, VIRORISK (Risk Of Emergence Of Viral Diseases Driven By Eco-Climatic Changes And Socio-Economical Situations) and is in charge of the entomological data gathering and analyzing. Coordinator of the project is the Université de Liège.

8 PUBLICATIONS

8.1 ITM

Peer reviewed publications

- Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M & Van Bortel W. Introduction and establishment of the exotic mosquito species *Aedes japonicus* japonicus (Diptera: Culicidae) in Belgium (2009). *Journal of Medical Entomology* (2009) 46: 1464-1467
- V. Versteirt, E. M. De Clercq, D. M. Fonseca, J. Pecor, F. Schaffner, M. Coosemans & W. Van Bortel. Arrival and acclimatisation of the exotic mosquito species *Aedes koreicus* in Belgium, Europe. submitted
- V. Versteirt, J. Pecor, D. M. Fonseca, M. Coosemans & W. Van Bortel. Description of morphological structure differences between Korean and Belgian *Aedes koreicus* supported by molecular identification. Submitted
- V. Versteirt, D. Damiens, W. Dekoninck, E.M. Declercq, M. Coosemans, W. Van Bortel. Filling the gap: an intensive survey of mosquito biodiversity (Diptera: Culicidae) in Belgium. In prep
- V. Versteirt, D. Damiens, W. Dekoninck, E.M. Declercq, M. Coosemans, W. Van Bortel. Species and vector communities of Culicidae and the potential risk for mosquito borne diseases in Belgium. In prep
- V. Versteirt, Z. Nagy, F. Breman, D. Damiens, W. Dekoninck, M. Coosemans, W. Van Bortel. DNA-barcoding of Belgian Culicidae. In prep
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Proceedings of international conferences

Oral presentation:

- Van Bortel, W., Boyer, S., Dekoninck, W., Grootaert, P., Hance, T., Hendrickx, G., Takken, W. and Versteirt, H., 2007, The MODIRISK project: a large scale survey on mosquito biodiversity as a basis for risk assessment and control. 4th EMCA Conference, Prague, Czech Republic, (xx/09/2007)
- V. Versteirt, M. Coosemans, T. Backeljau & W. Van Bortel. DNA barcoding as a molecular identification tool for Belgian mosquitoes. 2nd workshop on Entomology in Belgium. Brussels, Belgium (05/12/2008)
- V. Versteirt, M. Coosemans, F. Schaffner, C. Garros, W. Dekoninck & W. Van Bortel On the occurrence of exotic species in Belgium: the tip of the iceberg? 5th EMCA meeting Torino, Italy (10/03/2009)

- V. Versteirt, W. Dekoninck, C. Sohier, I. Deblauwe, W. Van Bortel, P. Grootaert & J.-P. Maelfait. Ceratopogonidae nuisance near the Sea Scheldt in Ghent. 5th EMCA meeting Torino, Italy (12/03/2009)
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Avia-GIS

Peer reviewed publications

- The spatial modelling of endemic mosquito species diversity hotspots in temperate Europe: implications for field sample design and spatial MBD risk analysis. A case study in the Benelux. E.M. De Clercq (1), E. Ducheyne (1), V. Versteirt (2), F. Schaffner (1), W. Dekoninck (3), T. Hance (4), W. Takken (5), M. Coosemans (2) W. Van Bortel (2), and G. Hendrickx (1)
- A model-based approach to deal with false absences in entomological data. E.M. De Clercq (1), E. Ducheyne (1), V. Versteirt (2), F. Schaffner (1), X.X.(3), X.X.(4), and G. Hendrickx (1)

RBINS

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- Dekoninck W, Hendrickx F, & Grootaert P, subm. Evidence for recent expanded distribution of arbovirus and Malaria vectors in Belgium thanks to man-made breeding sites. *Journal of Vector Ecology*.

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UCL

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- Garros C., Damiens D., Van Bortel W., Hance T. **submitted**. Effect of different thermal conditions on larval and adult life history traits of *Culex pipiens* (Diptera: Culicidae) in Belgium: consequences for WNV transmission.
- Damiens D., Ayrinhac A., Van Bortel W., Hance T. **submitted**. *Aedes japonicus* in Belgium population dynamics and blood meal.
- Ayrinhac A., Damiens D., Van Bortel W., Hance T. **In prep.** Genetic structure of *Aedes japonicus* population in Belgium

Proceedings of international conferences

Poster presentation

- International Conference EDEN 2010 “Emerging Vector-borne Diseases in a Changing European Environment”, 10-11-12th May 2010, Le Corum, Montpellier, France. Poster presentation: Longitudinal surveillance of the exotic species *Aedes j. japonicus* in a second-hand tire company in Belgium. Gauthier Martin, Thierry Hance, Wim van Bortel.

- VIIème Conférence Internationale Francophone d'Entomologie, 5 au 10 Juillet 2010, Louvain la Neuve Belgium . POSTER : Suivi longitudinal d'une espèce exotique *Aedes japonicus* dans une compagnie de rechapage de pneus en Belgique David Damiens, Gauthier Martin, Wim van Bortel, Thierry Hance.

WUR

Peer reviewed publications

- Takken W., Jacobs F. and Hendrickx G. Seasonal and geographic variations in blood feeding mosquitoes (Diptera: Culicidae) in The Netherlands. In prep
- Jacobs F., Bataille H. and Takken W. Unexpected rise of *Anopheles plumbeus* as a nuisance mosquito in The Netherlands. In prep

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ANNEX 1: COPY OF THE PUBLICATIONS (AVAILABLE ON OUR WEBSITE HTTP://WWW.BELSPPO.BE/BELSPPO/SSD/SCIENCE/PR_BIODIVERSITY_EN.STM)

ANNEX 2: MINUTES OF THE FOLLOW-UP COMMITTEE

MEMBERS OF THE FOLLOW-UP COMMITTEE

Following end-users have been identified at the start of the project.

1. **Public health:** Persons, institutions dealing with emerging infectious diseases will be interested in the predictive distribution models of potential endemic and invasive arbovirus vectors. The models will help them in assessing the risk of emerging vector-borne diseases in Belgium. Moreover the adapted spatial sampling approaches and trapping systems will be essential when monitoring of arbovirus vectors in Belgium seems to be essential from public health perspective.
2. **The veterinary and agrochemical research centre (VAR):** Many arbovirus diseases circulate among birds and other wild animals. These animals are often an important factor in the spread of the disease. Mosquito species play a role in maintaining the wild transmission cycle but are also important as bridge between the wild animals and man. The institutions dealing with the monitoring of diseases in e.g. birds will be interested in knowing where potential bridge vectors occur. By combining their disease monitoring results with mosquito distribution models they can identify possible risk areas for vector-borne transmission. The exchange of information between the VAR, Public health Institutions and the MoDiRisk will allow a better assessment of risk of emerging vector-borne diseases.
3. **Biodiversity:** This is the first large scale mosquito inventory effort in Belgium. The proposed sample strategy will give a general view of the presence/absence of endemic and exotic mosquito species and biodiversity hotspots will be identified. Concurrently the population dynamics of relevant mosquito species will be assessed. The project will provide baseline information for research groups dealing with biodiversity, impact of eco-climatic changes on mosquitoes and interrelationship between endemic and exotic species.
4. **Entomology:** the project deals with a very important family of the Diptera. Entomologists in Belgium and Europe will be interested in the results of the project and interaction with these entomologists is desirable.
5. **Dutch end user:** the Dutch government (notably the National Institute of Health and Environment, RIVM) is keen to obtain data on key-vectors in neighbouring countries in order to further assess potential health risks associated with environmental change. The project will also provide an excellent platform for bi-lateral collaboration between Belgium and the Netherlands through data sharing and exchange visits by scientists.

On the basis of this identified end-users following persons are members of the follow-up committee meeting:

- Koen De Schrijver, medical doctor, epidemiologist – infectious diseases, Vlaamse Gezondheidsinspectie.
- Sophie Quoilin, epidemiologist, Scientific Institute of Public Health
- Koen Mintiens, Head epidemiological surveillance, Veterinary and Agrichemical research centre. This person moved to an other institution. An other staff members of VAR will be asked to join the follow-up committee.
- Erny De Winne, Hoofd dienst ontsmettingen, Dienst Ontsmettingen van de Stad Brussel.
- Marc Dufrêne, Professor, project leader biodiversity in southern Belgium, Centre de Recherche de la Nature, des Forêts et du Bois.
- Erika Baus, Data Acquisition Manager - Belgian Biodiversity Platform, Royal Belgian Institute f Natural sciences. She has moved to an other institution.
- Etienne Branquart, Invasive species – Belgian Biodiversity Platform, Centre de recherche pour la nature, forêt et bois (DGRNE).
- Francis Schaffner, Professor, expert-consultant in biology and systematics of biting insects, University of Zürich.
- Marion Koopmans, Professor, unit of virology, Rijksinstituut voor Volksgezondheid en Milieu, The Netherlands.
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THE OUTCOME OF THE FOLLOW-UP COMMITTEE MEETINGS

First follow-up committee meeting

The main discussion of the first follow-up committee (November 2007) concerned the translation of the project outputs into invasive response in Belgium. It was clear from the discussion that invasive response to vector born disease outbreak is complex since different decisions/actions need to be taken where different decision levels (communes, regions, federal state) might be involved. Moreover legal aspects related to the control methods and products (safety, environmental and legal concerns, registration of products) have to be taken into account.

In order to cover the gap in transfer of the expected project outcomes to invasive response the follow-up committee recommended to work out a decision making process.

It was agreed that the co-ordinator of the MODIRISK project will prepare a framework document enumerating the different prevention/control scenarios, questions on legal aspects, decision levels, responsibilities, international context etc. This document will be sent to all members of the follow-up committee who will assist to complete and comment on the document. The final output at the end of the project would be a document giving a framework on vector control for Belgium.

Second follow-up committee meeting

The second follow-up committee meeting thoroughly discussed phases 1 and 2 of the project which resulted in the following recommendations and take home messages:

- The communication with the general public is an important factor of concern in the control of vector or exotic (invasive) species, especially to avoid panic reactions. Public health departments receive already many requests for information about dispersion of some mosquitoes (e.g. *Aedes albopictus* during the Chikungunya outbreak in Italy), prevention and expert help. MODIRISK can play a role in the distribution of correct information on mosquitoes, especially exotic species. This can be achieved through the creation of a special information page on the website where people can find information on distribution, risks imposed, what to do when encountering an exotic mosquito. The correct identification of the mosquito is therefore crucial.
- The Biodiversity Platform can also play a role in the distribution of information on the topic. Link should be further established between MODIRISK and the Biodiversity platform especially the Alien Species working group.
- The discussion on the second phase of the MODIRISK project is primarily concentrated on the model building and parameters that will be included. The models built by MODIRISK will become available to other members of the scientific community to add data (e.g. presence of birds, circulating viruses, disease outbreaks ...) and to pinpoint different kinds of "hot spots". ECDC is already involved in model building where they overlay the distribution maps of mosquitoes with other ones. Even though the risk on outbreaks is difficult to predict and therefore to model, MODIRISK aims to set the tone and should be used as a starting point for further analyses and model building.
- The testing for viruses was discussed. The question raised on what kind of sample would be sufficient to give an answer on the presence or absence of viruses in different mosquito species. Previous projects in Europe (EDEN) dealing with antibodies search has proven that it is difficult to find positive mosquitoes even though there is an ongoing outbreak. It is easier to test antibody presence in birds (EDEN). The cooperation with the bird surveillance in the framework of a West Nile survey in the RBINS is of highly interest.
- At the end questions were addressed to the members of the follow-up committee on the functioning, composition and participation of the committee and on possible ways to improve the exchange of information with the committee. Some members of follow-up

committee have resigned because of changing work activities. Some suggestions were made on possible new members of CODA, ULG and WildSurv. The possibility was raised for adding a social scientist to the follow-up committee to improve the communication to the scientific community and general public. The functioning of the committee based on annual meetings is approved. Though the organisation of special thematic workshops was encouraged.

Third follow-up committee meeting

The third follow-up committee meeting (27th January 2010) thoroughly discussed the progress of the project which resulted in the following recommendations and take home messages:

The discussion is primarily focused on the model building approach. Questions are being raised on the predictive values; their sensitivity (and the possibility to test this), the weight of the variables and the direction of influence they have.

- The ecology of some species should be taken into account; as some *Aedes* species are good flyers this could explain partly their observed distribution. Others like *Anopheles* species are less attracted by the traps used, so their distribution may be underestimated. Moreover most species are active during a specific period during the year; e.g. *Aedes rusticus* is a typical springtime species and therefore all samples after August do not have informative value for this species.
- The observed distribution pattern can be the result of the natural limits of distribution of a species; this should also be taken into account for the model refining. It remains however remarkable that the Ardennes have poor species richness, there seems not to be a clear explanation although satellite images can also differentiate between the Ardennes and other regions in Belgium. It is recommended to explore this further.
- Other factors like normal fluctuations between years of temperature and precipitation have been taken into account (all samples are divided over 4 seasons and 2 years). Each of the samples is subjected to some restraints: the year and period of the year in which the sample was taken, the trap placement and functioning of the trap at that moment, the attractiveness of the trap to a species. Specific data of each sampling point, recorded by the field teams on the moment of trap functioning, should be added to refine the models further.
- One of the options to refine models and to exclude accidental trapping of mosquito species, could be the larval sampling in different habitats and regions on different moments. However, this has also its constraints seen the labour intensity of the method. Other data (like collection material) that is geocoded could be integrated onto the biodiversity maps. In the end a map for the Benelux should be created with high and low biodiversity zones.

Longitudinal studies

- Concerning *Anopheles plumbeus* this involves the clean-up of the abandoned piggeries and subsequently sampling during the summer of 2010. In the Netherlands,

a nuisance site was followed for 3 years in a row, results will be shortly published and indicate amongst other that specimens fly up to 300m away from their breeding site. Further research in the Netherlands includes lab assays for life history traits at European temperatures. In Germany and France same nuisance has been observed for several years.

- One of the main questions on the longitudinal study of *Aedes j. japonicus* concerned the possible distribution of the species in the vicinity, but most gardens around the import site were negative. However the cemetery was not checked and this could be added in the future.

As well for the treatment of the piggeries as for the tires; biocides could be used. In February 2010 a meeting with the Walloon community will take place to discuss the treatment of the site (what to do and who will pay). The piggeries will be cleaned by the owner during Spring 2010.

Feedback to the end users?

- One of the main feedbacks to the end users will be the mosquito maps especially when it will be possible to add specific data layers to the models, adjusted to the defined question raised by the end users. The models could be used for a first risk mapping approach.
- Another feedback is the report of found exotic species as well as the nuisance of endemic ones. The question is raised how this could best be communicated to the general public. However this remains a difficult subject. At first, all information could go to the Scientific Institute of Public Health. Afterwards a general report could be communicated more broadly

Forth follow-up committee meeting

The meeting of the follow-up committee scheduled on the 16th of December 2010 had to be cancelled in a short notice due to the apologies of most members of the committee who had committed to come. An internal meeting was held instead

WORKSHOP ON VECTOR CONTROL

Background & objectives of the meeting

MODIRISK project aims at studying biodiversity of mosquitoes and monitoring and predicting its changes, and hence actively prepares to address issues on the impact of biodiversity change with particular reference to invasive species and the risk to introduce new pathogens. This interdisciplinary network is composed out of five partners namely the Institute of Tropical Medicine, the Royal Belgian Institute of Natural Sciences, the Université Catholique de Louvain, Avia-GIS and the Wageningen University.

During the inventory activities the exotic potential vector species *Oc j. japonicus* has been observed in Belgium. As a consequence, the follow up committee, assembling end-users who advise the project partners, discussed the response to invasive species in Belgium. The response to presence of invasive species and answer to the risk of vector born disease outbreak is complex since different decisions and actions need to be taken where different decision levels (communes, regions, federal state) might be involved. Moreover legal aspects related to the control methods and products (safety, environmental and legal concerns, and registration of products) have to be taken into account. In order to cover the gap in transfer of the expected MODIRISK project outcomes to invasive response the follow-up committee recommended the project to work out a decision making process. In this respect different aspects of the process should be worked out:

- The **detection** of endemic and exotic disease vectors (mosquitoes species);
- The risk **assessment** which will guide the decision on vector prevention and control;
- The **response** including the selection of control methods, insecticides and implementation aspects;
- The **monitoring** of the control measures.

Objectives of the meeting: this workshop should bring together persons potentially involved in the decision making process on vector control (such as the risk assessment group), other stakeholders and interested persons to discuss the different aspects on vector control in Belgium. Three topics related to the process outlined above will be discussed after being introduced by an expert. This workshop will guide the MODIRISK project to draft a document that can serve as a starting point to develop a framework document on vector control in Belgium as requested by the follow-up committee.

Outcomes and recommendations of the workshop

The detection of endemic and exotic disease vectors. This topic was introduced by F. Schaffner from the University of Zürich. He gave an overview of the possible biological invasions in Europe with focus on the Belgian situation. Special attention was paid on the invasion of the Asian tiger mosquito (*Aedes albopictus*) in Europe and the risks of transmission of Dengue and Chikungunya viruses.

Entomological surveillance should be set-up and maintained or extended in countries having incomplete information on the presence of exotic vector species and in regions recently colonised by exotic mosquito species. In adjacent countries and regions surveillance programmes should be set up.

Research should be promoted in two areas: (1) the development of control measures adapted to eradication from foci and prevention of the expansion of the target mosquito; (2) the evaluation of vector capacity of populations of *Ae. albopictus* for exotic viruses.

Ongoing surveillance and control is however expensive and outbreaks can be due to change in vector populations (adaptations), change in viruses (mutations) and or climate change. Therefore the development of risk maps for introduction and establishment should be enhanced.

Discussion Because of changing climatic circumstances and ongoing globalisation renewed attention is given to vector (mosquito) monitoring and control. However most projects are of a defined period (e.g. MODIRSIK for 4 years), afterwards no prolongation is foreseen. So the question raised what could be done to monitor and control the exotic vector species on long term basis. The possibilities to set-up a permanent monitoring system in Belgium need to be further explored. Such a monitoring system is needed since it is easier to control a species when it is not yet established. If an exotic vector species is not yet present importation routes should be the first target for monitoring i.e. second hand tire companies, main traffic roads, airports, harbours. In France and Switzerland the surveillance is focused on sites where mosquitoes could enter the territory (e.g. main traffic roads at borders). This surveillance is important but will not prevent introductions. It will enable to install control measures rapidly, trying to avoid establishment of the exotic species. Once the species is established, monitoring should focus on the potential spread of the mosquito species (This will be done for 1 year by MODIRISK). In the Netherlands the focus is not on the mosquito but on the disease and there is a framework for disease surveillance.

Besides a functional monitoring system, there is also a need for a flow chart of competences and responsibilities of the different authorities (national, regional governments, provinces, communes). Who will take decision, who will implement control, who will monitor the outcomes? In Italy this responsibility is scattered between the different regions and health authorities; in France the organisation of the control is done by the ministry of health but the local authorities are responsible for the actual control; in Switzerland there is a scientific team appointed to assess the problem whilst the surveillance and control are a regional matter. In Belgium, there is no political ownership but for each separate case, a health inspector is responsible.

Risk assessment which will guide the decision on vector prevention and control. In Belgium the Risk Assessment Group (RAG) of the Scientific Institute of Public Health (SIP) is responsible for the assessment of a risk. The RAG is composed out of permanent experts of the SIP, the Communities, Regions, and a representative of the Risk Management Group (RMG) and temporary experts depending on the topic. The RAG supports RMG with recommendations based on scientific evidence. The RMG decide if action should be taken or not.

On the initiative of the MODIRISK project the RAG met to discuss the measures to be taken to control the potential vector species *Aedes japonicus* found in southern Belgium.

The response including the selection of control methods, insecticides and implementation aspects. This topic was introduced by two experts. P. Ruelle from the Public Federal Service, Public Health, safety of the food chain and environment who introduced the topic on legal aspects of insecticides used for vector control and C. Jeannin from the Entente Interdépartementale Démoustication, Montpellier, France presenting the topic on vector control measures.

Legal aspects of insecticides used for vector control. In Belgium the European directive 98/8/EC is currently followed for the use of insecticides for vector control (different insecticides than those for animals and crops). This biocide legislation is under revision but this will take some time to complete (2015). The entry point will be the active ingredient and not the formulation of the product. If the European Union (EU) accepts the use of an active ingredient, it can then be used in different products by different companies. A company developing a product with that ingredient gives then the dossier to one of the member states and if that member state would approve the product, it will become valid in all member states of the EU. Now each member state decides separately which active product can be used and which not, therefore it is possible that in France or the Netherlands products are available on the market which can not be used in Belgium. Furthermore each member state may also authorise temporarily the use of product for a period not exceeding 120 days (Art 15 directive).

Discussion. The current European directive is under revision and the European Community will probably incorporate a limitation on the number of approved products in the next directive. This limitation will be based on the toxicology of the active ingredient, favouring those products with a reduced concentration of active ingredient and therefore with a lower impact on the environment but still sufficiently effective as a control measurement.

The authorisation for using Bacteria killing insects (Bti) can be asked through a fast track procedure, but still follows the same procedure of other insecticides. In the future derogation will be possible for mosquito control with insecticides for a limited period. In every case, the first step is the development of an integrated pest management; in a second step the insecticides/biocides can be used.

There is a movement on European level to develop a procedure for the treatment of emergence case (like the Chikungunya outbreak in northern Italy), but this is unfortunately not yet one of the important concerns of the European Union.

Options for mosquito and vector control. The actions France undertakes in vector monitoring and control were presented, with special attention on the surveillance and control of *Aedes albopictus* in southern France. In 3 topics (the legal context, control strategies and tools used for vector control) the situation in France is explained. On national level the European directive (98/8/EC) is followed next to two "circulaires", one on mosquito control and the use on insecticides and one about the risk on the spread of Chikungunya and Dengue in France. The aims of the control strategy are as follow:

- Preventing the vector introduction
- Slowing down his geographic spread
- Control of nuisance by mosquitoes
- Preventing or controlling outbreaks
- Reducing the disease incidence

Different strategies are used depending on the environment in which the mosquito species breed. Most control measurements taken in France imply the use of larvicides (instead of adulticides) like témetephos, Bti and Bs. Natural control of the larvae is tried with copepods. The insecticides are dispersed in different ways (such as fogging and granules) depending on the breeding site.

Both French circulaires include monitoring of the treated populations with different trapping methods and evaluation of the used insecticides. Currently, the situation of *Aedes albopictus* in southern France is one of the major concerns of mosquito control in France. The species seem to be spreading although control measurements are taking place.

Discussion. In France, it is even difficult as in other European companies to keep working products on the market (although there is a larger need for vector control than in some other countries), furthermore there is a difference between overseas areas and metropolitan France in the consent of using some products. The decrease in the choice of product is of major concern of all those working in mosquito monitoring and control.

The success of mosquito monitoring and control in southern France lies in the involvement of the different authorities: the Entente Interdépartementale Démoustication, Montpellier that performs the monitoring and control is financed by the ministry of health but the infested departments pay for the actual elimination of the pest species.

The aim of the control of *Aedes albopictus* has shifted in the past years from complete eradication of the species on a site towards the decrease in species density and vector/transmission control.

Recommendations & take home messages

- Entomological surveillance should be set-up and maintained or extended in countries having incomplete information on the presence of exotic vector species and in regions recently colonised by exotic mosquito species.
- Research should be promoted in two areas: (1) the development of control measures adapted to eradication from foci and prevention of the expansion of the target mosquito; (2) the evaluation of vector capacity of populations of *Ae. albopictus* for exotic viruses.
- The development of risk maps for introduction and establishment of exotic mosquito species should be enhanced.
- In Belgium the European directive 98/8/EC is currently followed for the use of insecticides for vector control.
- Furthermore each member state of the EU may authorise the use of product for a period not exceeding 120 days (Art 15 directive).

- The legal aspects of use of insecticides is complex and best is to contact the Public Federal Service, Public Health, safety of the food chain and environment
- Different vector control measures are possible each adapted to a specific situation (nuisance control versus outbreak control) and target species.
- Clear arrangements between the competent authorities should be made: there is a need for a flow chart of competence and responsibilities of the different authorities potentially involved in vector control.

FINAL MEETING OF THE MODIRISK PROJECT

10th of May 2011

Aim of this final meeting

- To present the final outcomes of the Modirisk project to the members of the follow-up committee and other invited guests
- To discuss and formulate recommendations from round table discussion with attendees

A welcome speech and general introduction was given by Prof. Dr. M. Coosemans, coordinator of the MODIRISK project. An overview was given of the project (main objectives and aims) including the historical situation of mosquito research in Belgium prior to the MODIRISK project and the main possible risks/ schemes of disease transmission of vectors in Belgium. Furthermore all links of MODIRISK with national and international projects were given.

Session 1: Biodiversity of mosquitoes in Belgium and The Netherlands: the pre- and post MODIRISK era; chaired by Dr. G Hendrickx (Avia-GIS)

In this session the sampling strategy and first biodiversity results were explained and discussed in detail

Model for sampling mosquitoes in Belgium and validation: E. Ducheyne

The basis for the design of the applied sampling strategy was the lack of information on national mosquito sampling campaigns worldwide. To decide the amount of samples to be taking, the rule of thumb was used; so 3 samples per grid cell of 10x10km were chosen. Furthermore several aspects had been taken into account like: major biotypes, seasonal variation and, import risks.

The methodology was as follow:

- Identify key habitats
- Calculate total surface per aggregated class
- Assign proportional number of sample points to aggregated class.
- Generate appropriate number of random sampling points within each class.

- Randomly assign sampling points within each class to two sampling seasons.
- Geocode and assign sample sites per team.

The Corine landcover and landuse (PAN European, uniform way to classify soil/landuse) was used for this methodology. It was rapidly observed that the northern part of the country is rural & urbanized whilst the southern part contains more natural areas. Most sampling points were situated in rural areas.

Once all clusters were defined, sampling points were randomly assigned over clusters, habitats and season (spatial and temporal variation). In addition import risk areas like harbors, airports and nature reserves were manually selected.

Each sampling point was geocoded and where possible linked to an address (which facilitate the preparations of the field team, ask permission + view of accessibility of point). As well these sampling points (of all teams) as the field forms were web based, could be filled in during the sampling on a palmtop and send directly to the server where it was registered and stored.

Spatial distribution and biodiversity of indigenous and exotic mosquitoes in Belgium: V.

Versteirt

First a short overview was given of the mosquito knowledge prior to the MODIRISK project and the need to make such an inventory .

After two years of intensive inventory and based on as well morphological as molecular identification 23 Culicidae (26533 specimens) species belonging to 5 genera were found of which 21 endemic and 2 exotic species. The number of caught species is close to the expected number of species (about 27 species) possibly present in Belgium. Molecular identification was done especially for the *Anopheles maculipennis* complex; only *Anopheles maculipennis* and *An. messae* were recorded, the historic malaria vector for Belgium, *An. atroparvus* was not detected. The most omnipresent species was *Culex pipiens* s.l. which was observed in almost all collection sites. Other important vector species include amongst others *Aedes vexans*, *Anopheles maculipennis* s.l., *An. plumbeus*, *An. claviger* and *Coquillettidia richiardii*.

Unexpectedly a high number of species (8) is common in all studied spatial levels, many of which have assumed vectorial capacity; amongst others *Anopheles claviger* and *Anopheles plumbeus*.

Additionally sites in import risk areas were sampled to evaluate the presence of exotic mosquito species in Belgium. Two exotic species were found, *Aedes (Finlaya) japonicus* in the province of Namur and *Aedes (Finlaya) koreicus* in the province of Limburg. Most missed indigenous species (6) are known to have very specific micro-habitat which may have been overlooked during the sampling process. They can also have been missed by sampling during the wrong season or the non-attractiveness of the trap to the species.

The taxonomic alpha diversity was estimated using three methods (Simpson, Shannon and rarefaction) at three spatial levels in which the biodiversity varied from rather poor to high. High biodiversity was observed in natural and urbanised sites and was low in rural areas; which is interesting in the framework of risk assessment. Moreover analyses showed that given the sampling effort done, most species that were expected to be found in these habitats were indeed sampled; underlining the effectiveness of the applied sampling strategy.

The importance of Museum collections to basic invertebrate inventory: a case study on the past and present mosquito diversity in Belgium.: W. Dekoninck

Within the framework of this MODIRISK-project the Belgian Culicidae collection at RBINS was revised.

There are four sub-collections in RBINS collection of Belgian Culicidae: a general collection, two sub-collections of Bequaert (from 1912-1958) and Goetghebuer (from 1909 to 1946), and a sub-collection of unidentified specimens i.e. the supplements (between 1892 and now, with the majority of specimens collected between 1920-1960). Most material in the RBINS collection was sampled between 1930-1960. None of these collections contain exotic species.

From the MODRISK project a reference archive was made (data 2007-2009). Both datasets (collections RBINS and MODIRISK) were used and recent and old data distributions were compared using UTM 10x10km squares. A trend criterion was made of well surveyed grid cells and a decline of diversity near larger cities could be observed (which is also seen for other insect groups). For 23 species there is a relative change in distribution area in 56 (10x10km) grid cells. An increase of distribution area was observed for several potential mosquito vectors having the capacity to use artificial containers as breeding sites.

MODIRISK: the Dutch extension: F. Jacobs

A synopsis was given of the study done in the Netherlands; especially on the longitudinal follow up of 55 different locations. The majority of these were situated in agricultural areas (60%); pastures, stables, cropland. As main import risk areas; the harbor of Rotterdam and airport of Amsterdam were surveyed. The same trapping tool was used during the same period of the validation period in Belgium (2009: late summer-fall; 2010: spring-early summer).

Most common species were *Culex pipiens* (widespread), *Anopheles plumbeus* (restricted distribution) and *Aedes cantans* (restricted distribution)

Spatial and temporal distribution data (same areas sampled in 2009 and 2010; only different period) were provided. A detailed overview of all results, and found species, with 9 common species were discussed in view of their main habitat.

A total of 21 species were collected of which 5 were not found in Belgium during MODIRISK. In many cases *Culex pipiens* appeared early in spring, peaked in August/September and disappeared late autumn. Only two specimens of *Anopheles atroparvus* were found whilst it was more abundant during the past. Its decline is related to deterioration of its habitat (brackish water swamps)!

Data was used for species modeling.

Questions/Remarks:

1° The question was raised that 60% of all sampling sites were in rural areas whilst analyses indicate a low biodiversity in these habitats. What are the implications for further monitoring? Especially in light of risk for transmission of diseases, should the sampling strategy be more focalized on habitats presenting a higher biodiversity. The sampling strategy for further monitoring and surveillance should be adapted to objectives. The used strategy is

appropriate to study the biodiversity at national level and to insure a good follow up of possible changes in species distribution. If the objective is to trace specific species, such as potential vectors, predictive occurrence maps will be useful in selecting the sampling sites. Specific environments can be sampled where bridge vectors (mosquitoes biting birds and other animals including humans) and/or pathogens are present, where nuisance is an important issue.

2° In light of the successful spreading of *Aedes japonicus* and other *Aedes* species in the US and Switzerland, what are the current restraints for the two exotic species in Belgium?

Although this topic is dealt later on, a number of possible explanations were suggested. As seen in other insect groups, it is a matter of time; most species coming into a new environment needs some time to adjust before spreading into this new area. This time frame can go from 5, 10 up to 50 years, but eventually the species will spread and once it has started it is very difficult to control. This also depends on the genetic richness and status of the population, recurrent import of new populations of one exotic species into an area can lead to an enriched genetic pool which in turn can be the cue to start spreading.

A clear message to the community and decision taking authorities should be formulated: elimination now before it is too late; monitoring is needed!

Session 2: Ecology and population dynamics of mosquitoes in Belgium; chaired by Dr. P. Grootaert (RBINS)

At the beginning of the second session, once again the success of this fruitful project was mentioned, especially seen the many other related projects and spin-offs.

Modeling the ecological niche of different indigenous mosquito species: E Ducheyne

Of the 21 endemic species, 14 species were included in the modeling since enough individuals were caught in enough positive sampling sites. For each of these species predictive occurrence maps were generated using different, for mosquito important, variables:

- Available water capacity of topsoil
- Digital Elevation Model
- Yearly/monthly Precipitation
- Distance to particular features
 - Waterways
 - Protected areas
- Percentage cover (% in 1 km²)
 - Broadleaved, Coniferous, Mixed forest
 - Urban, Agriculture, Natural
- Population density (inhabitants/km²)
- Temperature: day & night
- Vegetation greenness
- Fourier analysis on data of last 5 years (Mean & Interannual variation)
- Number of freezing days/nights

Random forest analyses were used to model the species distribution. Specific attention was paid to absences. Absence can mean three things: the species was not found, it is not there, the model is wrong. False prediction of absence are more important (than false prediction of occurrence) in framework of surveillance, monitoring and control of vector species. For most of the 14 species, good predictive models were obtained.

Based on the developed spatial models, 4 species could be identified as being biodiversity indicators: *Culex pipiens*, *Aedes cinereus/ geminus*, *Coquillettidia richiardii* and *Aedes vexans*. Of the six recorded rare autochthonous species, all occurred within the identified biodiversity hotspots and 70% of the positive catches where recorded in the high ranking biodiversity zone which represents only 10% of the sampled land surface. Overall 53% of the positive catches occurred on 23% of the sampled land surface, all located in the 'broader' biodiversity zone.

Anopheles plumbeus: shift of habitats and risk for autochthonous malaria in Belgium: W Dekoninck

Historic descriptions and the re-evaluation of RBINS mosquito collections revealed that the first record of *Anopheles plumbeus* goes back to 12-VII-1938 when Goetghebuer collected this species near Torgny. Between its first discovery in Torgny and 1975, this species was only occasionally collected in Belgium and the majority of these records stem from highly forested regions; where it breeds in water and debris filled tree holes. Occasionally the species can be found in manmade containers. However during the MODIRISK inventory the species was found in 114 sites (urban, rural and nature sites); which is clear evidence that the species is expanding in Belgium (like other *Anopheles* species). From 2008 onwards, cases of extreme nuisance caused by massive abundances of day-active *An. plumbeus* were reported at more than 10 suburban sites (gardens and near farms) in Flanders (Belgium). The species has expanded its habitat from forests to anthropogenic influenced habitats, which greatly enlarged its geographic distribution. Similar patterns are observed in other western-European countries where larvae were able to live in artificial containers. As the species has 2-3 generations per year and is very aggressive to humans it can cause severe nuisance problems over a long period of the year. During the longitudinal part of the project, the species was followed at a rural site in Torhout and 1 in a forest not far away. Larvae and adults were collected over the season. Larvae were mainly found in abandoned manure pits of pig stables, sewerage along the road but not in tires, nor in other artificial containers. These abandoned pig stables act as a surrogate for the natural breeding sites of the species (tree hole mimicking) and huge numbers can be obtained from this new breeding sites.

To assess how this problem originated, a transect between a small forest fragment nearby, the in-between gardens and pest site were monitored. In the pest areas only a limited number of species was present (5); in the forest a higher number (11) of species were caught and also in the human habitats between forest and pest sites many species could be found (10). It seems that the urbanized areas offer same breeding sites as forests, which can facilitate the migration of *Anopheles plumbeus* from the forest to the abandoned piggeries. So far this nuisance problem was reported from 10 sites all over Belgium.

In case of the pest site in Torhout, the piggeries were cleaned but the problem remained. The population was so big that it had already colonized new areas from which the pest problem could sustain.

Recommendations for other sites have been made: as it is a structural problem; cleaning abandoned stables will help to reduce or even eliminate the problem and will prevent colonization.

Repeated introduction of the exotic *Aedes japonicus* as suggested by microsatellite markers:

A. Ayrinhac

The origin and invasiveness (especially in US) was outlined for the vector species *Aedes japonicus*. Since its introduction in the US in 1998 it has spread rapidly in 31 North American States and parts of Canada. In Belgium it was first discovered in 2002 and studied during the MODIRISK project in 2007-2008 (inventory) and followed longitudinal in 2009-2010. The trophic behavior was tested at Natoye from female adults caught in 2009. It was observed that *Ae. japonicus* mainly fed on humans and bovines, which was surprisingly since humans are considered as secondary hosts in the US. Feeding on humans & occasionally on birds can have implementation on the risk of arboviral transmissions.

The population structure was thereafter assessed using microsatellites for 7 loci. With this technique peaks are generated for each individual and each locus and scored, these scores can be then analyzed using different statistical programs, Structure is commonly applied. In Structure each score for each loci of each individual is offered to the program after which each individual is assigned to one of the groups that was delimited by ΔK (Evanno, 2005). This is an estimate of the most likely number of genetic groups composing the Belgian population. The highest value of ΔK indicates the true K (number of genetic groups), here 3. Each mosquito has a certain probability of belonging to one of these groups. From literature is known that the US population consists of 2 distinct groups. In Belgium, in 2009 there were 3 separate groups (suggesting 3 separate introduction moments), in 2010 mixing of these genetic groups occurred. This can increase genetic diversity and so increase the species invasive potential (Kolbe et al., 2004).

Competition experiments between the exotic *Aedes japonicus* and indigenous *Culex pipiens* were done (secured lab in EID, Montpellier). Survival of single batches of *Ae japonicus* and *Cx pipiens* was compared with a mixed batches (each batch contain 10 larvae) (1:1), Either starved or fed, at 25°C or at 15°C, mortality of *Culex pipiens* larvae was always higher when mixed with *Ae. japonicus* than alone. *Aedes j. japonicus* larvae seem highly competitive and affect negatively the survival of *C. pipiens*, but seems to exhibit intraspecific competition. The species seems to be a strong competitor, capable of outcompeting indigenous species.

Questions/remarks

The discussion was mainly focusing on the invasive mosquito *Aedes japonicus* and the impact of its competitive behavior on the indigenous fauna. *Aedes japonicus* is clearly a more active competitor for food (more foraging) No cannibalism was observed.

No prediction can be made when they were introduced, nor the exact origin is known, but trade of second hand tires is for sure the origin of introduction. The samples found in Belgium belong to a different genetic cluster as compared to specimens from Japan.

The RBINS will work on population structure of *Anopheles plumbeus* using the same techniques.

Some remarks were made on the modeling, especially on some differences between the models and the biodiversity analyses. Modeling took only 14 species into account while all species were considered for the biodiversity analysis.

Session 3 Biology and genetic population structure of mosquitoes; chaired by Prof. Dr. T. Hance (UCL)

Influence of temperature on oviposition and larval development of *Culex pipiens* in laboratory conditions: T. Hance

A major factor influencing life history traits of ectotherms is temperature, effecting survival, growth, size and behavior. It can therefore have an important implementation on risk assessments: with increase temperature, there can be an increased number of generations per year, changes in the phenology (precocity and late activity), in the gonotrophic cycle including host vector contact, and in the distribution of the species. This effect of temperature on mating activity, larval development and adult body size & survival was assessed using *Culex pipiens* as a model species (widely distributed in Belgium, dominant enzootic (bird-to-bird) and bridge (bird-to-human) vector of West Nile Virus in urbanized areas in the United States). Since *Cx modestus* (considered as the main vector for West Nile virus in France) and *Cx pipiens* have the same phenology, results can extrapolated to other (more important) vector species. The colony was maintained at UCL at T28, 50% RH, 14:10 photoperiod and 7 temperatures were tested (T11, T15, T20, T28, T30, T32, T35, T40) in climatic chambers. The choice of temperature range correspond with main fluctuations possible in Belgium (meteo data from 1838-2007).

Mating: time before mating increased with low temperature; threshold 11°C (no mating). Important to know is that rotation of reproductive organs (by 25°C at least one day) in male of *Cx pipiens* need to take place before mating!

Development time = reduced with increased temperature (at 11°C 100 days – at 30°C only 20 days, but larvae/pupae mortality is very high at these high temperatures).

Body size (wing length: from the base of costa vein to distal extreme of R3 vein, excluding the fringe setae) was smaller at higher temperature.

Adult longevity: higher at lower temperature (15°C)

T11 is enough to stop mating activity but not development; at T28 development time decrease, and the number of generations increases, T35 is the upper limit.

Trade off occurs at 28°C; there is a short development time, a reduced size and reduced fecundity, and a short adult life. The optimal temperature (Brière model, using different parameters) is between 30 and 33°C. 11°C is the threshold of activity.

First evidence of two forms of *Culex pipiens* in northern Europe: implications on transmission of West Nile in humans? V. Versteirt

It is commonly accepted that *Culex pipiens* consists of two forms *Culex pipiens f pipiens* and *Culex pipiens f molestus*, morphologically indistinguishable but with a different biology. The first form is considered ornithophilic, mates in open (above ground) environment, have to take a blood meal before laying eggs and overwinters as larvae. Whilst the latter form is antropophilic, can mate in confined (below) ground species, can lay eggs for the first time without blood meal and overwinters as an adult. It is believed that hybrids between both forms can act as bridge vectors. However it remains very plastic characters and it is not possible to distinguish field caught individuals.

A blind approach was chosen to check the structure of different population in Belgium. A total of 9 populations (7 MODIRISK populations, 1 below ground sample and 1 above ground (larvae) sampled population) were used during this study. Different methods have been described in literature to make a distinction between both forms however most striking results were obtained with microsatellites afterwards analyzed with Structure (see session 2, *Aedes japonicus* for more information).

Results indicate strong evidence for the existing of two genetic forms in different populations in Belgium as well in above ground as in below ground populations, in the same habitat and period.

These findings have an implication on the transmission of several arboviruses like West Nile in which *Culex pipiens* could act as an vector. Mixed populations and hybrids in urbanized areas and nature reserves, were host, pathogen and vector can occur together during the same period pose a considerable higher risk on transmission.

Genetic characterization of *Anopheles claviger* and *An. plumbeus*, potential vectors of autochthonous malaria in Belgium: V. Versteirt

Anopheles claviger, *Anopheles plumbeus* and *Anophels maculipennis* s.l. mosquitoes were collected in Belgium in the scope of the project MODIRISK. This are close related species that are difficult to separate based on morphological characteristics alone; moreover due to sampling discriminating characteristics could be lost, complicating the correct identification.

The sequences of the mitochondrial Cytochrome Oxidase I (COI), which is the DNA barcoding region, were used to study the phylogenetic relationships between closely related species, especially of *Anopheles claviger* and *An. plumbeus*. These results were compared with the sequences of the ribosomal Internal Transcribed Spacer 2 (ITS2) and Domain-3 (D3) and the mitochondrial NADH dehydrogenase 4 (ND4). The analyses of acquired sequences consist of constructing neighbor joining trees with the bootstrap method, multidimensional scaling based on genetic distances and analyzing of the base composition of the individuals. Preliminary molecular studies showed some inconsistencies. A total of 26 populations as well sympatric as allopatric ones were used in to further study the problem. Several difficulties were encountered with all tested regions. The incongruence remained in some individuals of as well *Anopheles claviger* as *An. plumbeus* with all used marker, which is striking since these were never reported from other European studies on both species. It is assumed that *An. claviger* could consist of different genetic groups, which may be an

explanation of the observed genetic diversity. A better understanding of the species status of these anophelines is essential in identification of potential vectors of autochthonous malaria.

Questions/Remarks

The main questions were posed around the use of different molecular techniques to distinguish between genetic forms and closely related species. The genetic distances and possible parental backcrossing experiments may clarify the status of these different populations. To further study the Culex genetic structure in Belgium, larval collections should be made, also during winter in different habitats like manure pits and underground overwintering places.

Main remark on the influence of temperature on life history traits dealt with the fact that in nature temperature is constantly fluctuating whilst this is not the case in controlled lab conditions. Although this is true, it is first of all hard to mimic these fluctuations and secondly based on the range of temperatures tested a good assessment can be given of the possible field situation.

Session 4 Surveillance system on mosquitoes, vectors of diseases; chaired by Dr. Sophie Quoilin (IPH)

Dr Quoilin, as member of the Follow Up Committee, expressed her appreciation on the way the MODIRISK project was run and raised new knowledge using a multidisciplinary approach.

Risk in Belgium for mosquito borne diseases: M. Coosemans

The issue of "who will do what?" in terms of control of invasive and indigenous vector species outbreaks is addressed. Although recently the decision making authorities have shown an increased interest in this matter, it will take some time before real actions can be taken. Seen the points discussed during this meeting (several risk schemes, the presence of exotic species) and seen the rapid spreading of some invasive mosquito species in Europe, it would be advisable that preparatory measures are formulated as soon as possible.

Accuracy of using a less intensive MODIRISK sampling strategy for surveillance: E. Ducheyne

The original sampling scheme aimed at obtaining an increased knowledge on mosquito distribution and diversity. However for future monitoring this strategy is labor intensive and costly thus it was necessary to analyze how the sampling size could be reduced without reducing the accuracy of the model outputs. This reduction was done in steps of 10% from 100% (1000 points) to 10% (100 points), instead of one trap every 30km², one trap per 300km². Also the temporal sample size reduction was taken into account. The results show for different species, different thresholds; therefore the 4 found indicator species were taken into account. For these 4 species, valid results were obtained up to 1 trap per 150km² at a 1km² resolution. These findings will make future monitoring more efficient and less expensive.

Questions/Remarks

The discussion focused on the differences between Belgium and the Netherlands in sampling approach. In the Netherlands, it was chosen to sample different biotopes instead of randomly chosen points. Of course these 55 samples do not really reflect all of the Netherlands (closer to a 5x5km model output) but other studies done in Europe indicated that these 5x5km model outputs are very useful. In a study in Spain 250 traps for whole Spain were used to produce 5x5km models, and good results were obtained. Two hundred fifty traps for Spain correspond to 1 trap per 200km. For Europe 5x5km model outputs seem to be enough to produce usable risk maps.

A very interesting point was raised during this discussion, namely the longevity of these predictive model outputs. How long will this model be applicable/ up to date?? Which deadline should we set after which these models become outdated? Will the accuracy of model be lost after 5 years and on which scale?

It is generally accepted that monitoring should continue on a regular basis (every year or two) using a less intensive sampling strategy.

Recommendation should be formulated on the sample size; the speculation on temporal replicates; maybe even asking expert opinions to make a sound proposition. It all depends on the research question that needs to be answered, e.g. indigenous species versus exotic species. Health authorities will probably ask for a minimum monitoring effort, especially targeting import risk areas and invasive species. Priority should be given to exotic species that can transmit exotic pathogen and local vector (indigenous species) transmitting arboviruses. Based on the created maps, data can be extrapolated to calculate spread and possible persistence of exotic and indigenous vector species. Not only exotic species should be taken into consideration, as pathogens are also travelling and can be transmitted by indigenous vector species as it was the case for the Bluetongue outbreak. .

Round Table discussion and final remarks:

Some clear and limited recommendations should be formulated regarding biodiversity, human and animal health.

Actions are undertaken on national level in our surrounding countries and on European level should be taken into consideration in Belgium when preparing for future threats. In the Netherlands a Centre for vector monitoring and control was recently founded, responsible for surveillance & monitoring of different vector species in a large number of sites. In France invasive species are intensively surveyed especially *Aedes albopictus* (the tiger mosquito), but also indigenous species that can be vectors. There are different French institutions for control and monitoring: EID or local authority, all grouped in Adège(s). In Europe a network exists for vectors distribution and surveillance funded by the ECDC. Seen Avia-GIS is the coordinator for this network a brief summary of its aims and strategy was provided during the meeting. One of the main recommendations would be to monitor and study as well invasive as indigenous vector species, at least a minimum monitoring should be done to keep models up to date.

The issue of invasive species should also be pointed out when discussing the functionality of biodiversity: invasive species have more difficulties to establish if biodiversity is high (for

example, *Aedes japonicus* was introduced in a setting with low mosquito diversity). High biodiversity can also reduce dominance of some pest species and in the end avoid outbreak of *Culex pipiens* and *Anopheles plumbeus*. A loss of diversity can therefore have an impact on arboviral disease outbreaks and public health.

Considering the regular introduction of *Ae japonicus* in one specific site and the risk of fast expansion of this population in the future as it occurred in other countries, the meeting recommend to add this species on the Belgian list of invasive species; in future other invasive species with medical importance can be added.

A press statement will be released.

One of the main outcomes is surely the fact that this project demonstrate how important it is to have bridges between different subjects/authorities/institutes, not only on vectors but also on human and animal health; a common subject where people with different background and interest can discuss and meet. It was a fruitful initiative with many interesting and sometimes unexpected outcomes. This project was the basis from which different other initiatives were started. All participants expressed their interest and hope for a continuation of mosquito research in Belgium.