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SCIENCE FOR A SUSTAINABLE DEVELOPMENT

MOSQUITO VECTORS OF DISEASE: SPATIAL BIODIVERSITY, DRIVERS OF CHANGE AND RISK

"MODIRISK"

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SCIENCE FOR A SUSTAINABLE DEVELOPMENT
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Biodiversity

FINAL REPORT PHASE 1

MOSQUITO VECTORS OF DISEASE: SPATIAL BIODIVERSITY, DRIVERS OF CHANGE AND RISK

"MODIRISK"

SD/BD/04A

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Mars 2009

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Wim Van Bortel, Patrick Grootaert, Thierry Hance, Guy Hendrickx, William Takken ***Mosquito Vectors of Disease : Spatial Biodiversity, Drivers of Change and Risk "MODIRISK"*** Final Report Phase 1. Brussels : Belgian Science Policy 2009 – 42 p. (Research Programme Science for a Sustainable Development)

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1 Context and objectives

Ongoing eco-climatic changes create suitable conditions for the (re)emergence of vector-borne diseases in Europe. Of these, mosquito-borne diseases are prime candidates (e.g. recent West Nile Fever events, records of introduction/spread of exotic *Aedes albopictus* in Europe, outbreaks of Chikungunya in Italy and Dengue in Europe overseas territories). 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. Thus the objectives of the project are:

1. To inventorize endemic and invading mosquito species in Belgium considering environmental and taxonomic elements of biodiversity;
    a. To conduct grid-based cross-sectional surveys in Belgium representative of various habitats;
    b. To conduct complementary sampling in selected key habitats in The Netherlands;
    c. To establish and maintain a reference archive of biological samples.
2. To assess the population dynamics of endemic and invasive mosquito species and their interrelationship and in particular to:
    a. Assess the population dynamics of endemic mosquito populations known as potential arbovirus vectors at the landscape scale in selected field sites in Belgium during one sampling season;
    b. To assess the status of selected invasive and spreading endemic species to determine whether they are 'temporary records of non established species', 'established species in confined foci' or 'established spreading species'.
3. To model mosquito biodiversity distribution at a one km resolution in the Benelux, and in particular to:
    a. Develop/validate distribution models for all mosquito species using state of the art multivariate analysis techniques and, low and medium resolution remotely sensed and ground measured eco-climatic time series as predictor variables;
    b. Test/validate best performance adapted spatial sampling approaches, including different trapping systems, based on spatial model outputs and propose a standardized methodology for similar future research activities.
4. To disseminate project outputs to scientific community, end users and the general public

MODIRISK will provide data on the distribution of endemic and invasive mosquitoes in Belgium, and their population status. It will supply predictive spatial models on the presence/absence of mosquitoes and will contribute to understand the impact of eco-climatic changes on their distribution. 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. The project will also provide an adapted spatial sampling strategy approach for the monitoring of endemic and invasive arbovirus vectors and on different trapping systems that can by used in such a monitoring systems. During the project an expertise in mosquito research will be built up in different institutions in Belgium.

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.

MODIRISK fills up an essential knowledge gap in Europe, and the expansion of model outputs through linking up with a project in The Netherlands will result in more robust results and prepare for later expansion of activities in Europe.

2 Methodology

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. CO_2-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 CO_2-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; Anderson, Ithaca, personal communication). Furthermore it is the only commercial available trap type that allows a certain autonomy which was necessary for the trapping scheme. It is also the main trap used in a Dutch ongoing study which teams up with MODIRISK and thus will enable to integrate these data in the MODIRISK modelling approach.

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.

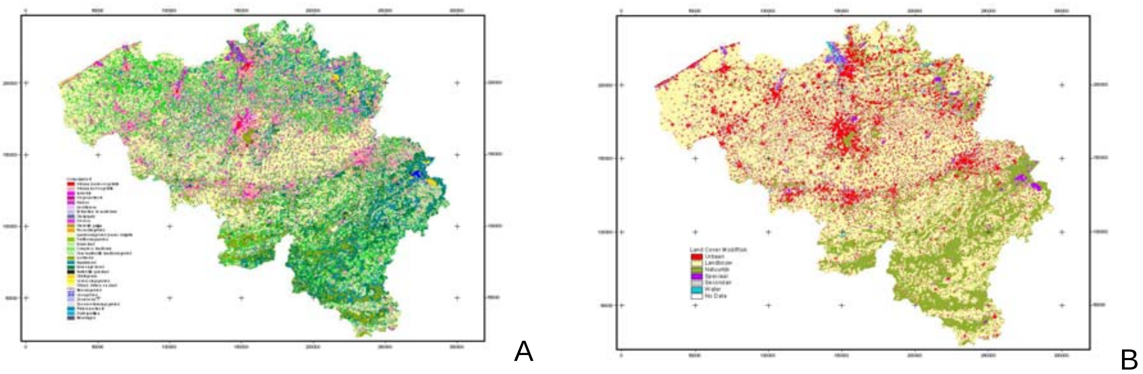

Figure 1. (a) Corine Land Cover Classification and (b): Aggregated Land Cover Classes

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 1) and each point received a random set of X and Y coordinates.

Table 1. 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.

2.2 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 is showing 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 2), the field teams can identify each sampling point through the interactive map. The interactive map allows full capabilities of zooming (Figure 3) and panning. The sampling points are colour coded in the same manner as on the public web site. They can visualize the sampling per season, per year or for the total study period. For each sampling point, the field teams can query which team is responsible for trapping, and during which season the point has to be sampled. The high resolution satellite imagery background (Figure 3) enables a rapid assessment of accessibility and contributes to efficient planning of the field visits.

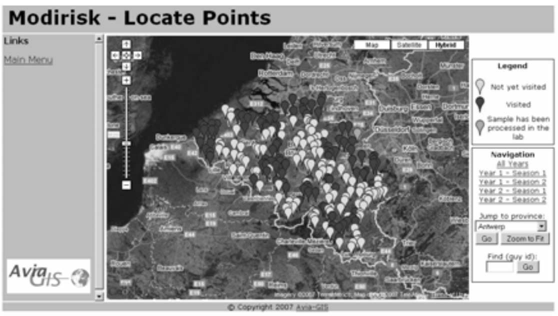

Figure 2. *Point locator on private website*

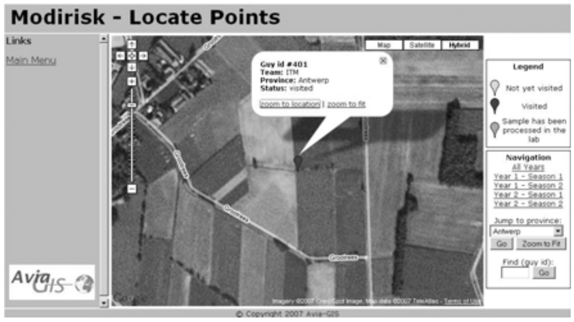

Figure 3. *Zooming capabilities*

In the administration section, the field teams can complete the sampling forms. The data from the first form is uploaded from the field teams' PDA. The other forms have to be completed manually. Once the data is uploaded or entered, the data base is automatically updated. The status of each sampling point on the web-maps automatically reflects any change in the database (e.g. from not visited to visited when data is uploaded from the PDA to the database server or from visited to processed when field form 2/3 have been completed).

Software development for PDA
Thirty PDAs (Pocket PCs) were compared using the following criteria: cost, software, memory, processor, screen, GPS included, external storage memory cards, wireless and/or Bluetooth availability. From this list, the Fujitsu Siemens LOOX N560 was selected because of its high quality and the respective performance of its screen, processor and memory. The PDA is running Windows Mobile 5 and has a built-in GPS.

The field form was implemented on the PDA. All functionalities were individually tested: communication between PDA and database server through RDA, data storage in the SQL Server Mobile database, editing data in the database, user interface for field form, integration and communication with the TomTom Navigator through the .NET classes for navigating to the sample points (either using coordinates or using addresses) and taking GPS coordinates for a sample point. 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 is available to all MODIRISK partners.

The software downloads the coordinates from the central database server to the MS SQL Server Mobile database on the PDA. The user can query and edit the data, and all changes are 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 4) were developed by Avia-GIS, implemented in the database, and transferred to the web server.

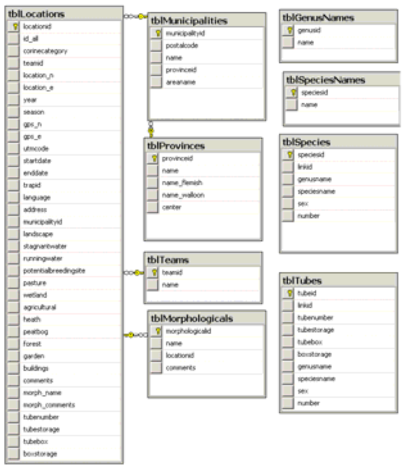

Figure 4. *Tables present in the MS SQL Server Database*

2.3 Mosquito sampling and morphological identification

Mosquitoes were sampled from May till October 2007 and 2008 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 coordinator and adapted during a group session at ITM.

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 traps per team). Each trap operated seven days on one study site after which it was placed on the next study site. 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. Morphological identification was done 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 in state of the art taxonomic identification using morphological techniques. Training was provided by an expert in European morphological mosquito identification based on reference collection from the expert and from field collected mosquitoes. To assure quality of morphological identification the expert was re-invited to perform a quality check of the mosquitoes identified in the course of the project.

All partners involved in the morphological identification started their own reference collection. Therefore 1-5 correctly identified specimens per species were pinned and stored in insect boxes. The remaining specimens were stored in small tubes for subsequent molecular identification.

2.4 Molecular identification of mosquito species

A general molecular identification framework based on the DNA-barcoding approach is being 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.
Two regions of interest (ITS2-rDNA and COI-mtDNA) will be 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 (Folmar et al 1994).

The molecular identification will focus on species for which the morphological identification is problematic or when sibling species are involved. The groups that will be looked at are (1) *Anopheles claviger* vs *An. plumbeus*, (2) *Culex pipiens complex* and (3) *Anopheles maculipennis* complex. Other groups such as *Aedes cinereus/geminus* will be included as well.

2.5 Laboratory tests on mosquito behaviour related to temperature

Establishment of mosquito colonies

A colony of *Culex pipiens* was started up at UCL and a first assay was made to colonise *Ochlerotatus japonicus japonicus*. Therefore, larvae of *Cx. pipiens* were collected on the UCL campus (Univ. Catholique de Louvain, Louvain La Neuve, Belgium) early October 2007 in a pond. The colony is maintained at 28°C, 50% relative humidity with a photoperiod of 15:9 (L:D). Larvae are reared in small plastic containers with dechlorinated tap water and fed on a diet of Tetramin ground fish food. Adults are kept in screened cages and provided constant access to a 10% sucrose solution. A rat blood meal is 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. Preliminary tests were needed. Actually, mosquitoes mate in swarms, males flying with females in open space. To normalize the experiments, forced mating was tested (one virgin male, one virgin female into a 1l-jar). A total of 16 pairs were made. All spermatecae 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. Experiments on the length of the gonotrophic cycle at T28 have already started (May 2008).

Larval and adult life history traits at varying temperatures
The effects of thermal conditions on larval development were tested under four temperatures (T11, T15, T20 and T28 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.

Mating activity at varying temperatures
To estimate pre-copulation time (i.e. the time between the couple creation and the first mating) at different temperature, 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 at the study temperatures (T11, T15, T20 and T28 degrees). Ten couples were dissected each day from day 2 to day where all females were inseminated. Females were killed with ethanol and then spermateca dissected under binocular microscope. Dissections were done in a PBS solution following the procedure of Damiens and Boivin (2005). The mating percentage was evaluated as the number of females with filled spermateca.

2.6 Development of a spatial data archive

Low resolution remote sensing
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 to date: 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.

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, 1997) 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.

MODIS data were ordered through the following data gateway:

http://elpdl03.cr.usgs.gov/pub/imswelcome/. The 8 days composites for land surface temperature (LST) at 1km were ordered, just as the vegetation indices (NDVI and EVI) at 1km spatial resolution.

The 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.

A cubic spline interpolation was applied to de-noise MODIS data series.
In a recent 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 2007. This database will be completed with data from the years 2008 and 2009, at the moment that these datasets become available to the public.

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 a.o. 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* (2001) 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 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

2.7 Development of a spatial distribution models

For all sample points (ca. 1000) the presence/absence of a given mosquito species has been recorded. On this database the spatial distribution models were developed. 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 *An. claviger* and *Ae. cinereus*.

For both species, a training sample was selected, divided over both the presence and absence category. The explanatory variables, composed of 28 data layers (i.e. first three amplitudes and phases of the Fourier transforms, and mean values for day-time land surface temperature (LST), night-time LST, NDVI and EVI), were standardised prior to the statistical modelling to facilitate model output interpretation. Per training site, the corresponding values were extracted from each raster data layer.

The most common approach for species distribution modelling is to apply a logistical regression function that describes the probability of a given species occurring at a given location (Brown et al., 2004). Stepwise logistic regression was employed to construct the regression model. Model selection was based upon the Akaike's Information Criterion (AIC, Equation 1) This criterion makes a trade-off between the complexity of a model and its goodness of fit (Kutner et al., 2005). Using this procedure, the model with the lowest AIC value is retained.

Equation 1. $AIC = n \ln SSE + 2p$

Where:

n = number of observations

SSE = residual sum of squares

p = number of variables in the model

In a second phase, the species distribution is predicted for the entire study area.

3 Results

3.1 Inventory

3.1.1 Overview of the collections

Based on the above described sampling strategy 936 sites were randomly identified in three key habitats (urban, agriculture and natural) and 42 sites had to be selected manually in specific habitats. In total 97% of the randomly selected sites was effectively sampled and 63 import risk areas were included in the inventory (Table 2). 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 2. 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

A total of 26508 individuals, belonging to 23 species and 6 genera, were collected and morphologically identified (Figure 5). The number of species will certainly increase because some species complexes, such as *Anopheles maculipennis* complex, still need to be identified by molecular means. The most species rich genus in Belgium is *Ochlerotatus* whereas *Coquillettidia* is only represented by 1 species. The most abundant species was *Culex pipiens* which is found in a large variety of breeding sites. *Coquillettidia richiardii* was the second most prevalent mosquito. This 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 in only 38 study sites. Interestingly is the general occurrence of *Anopheles* species, mainly *Anopheles claviger and Anopheles plumbeus.* The latter is becoming a nuisance species in The Netherlands and Belgium and has been implicated in malaria transmission in Germany. Therefore in-depth study of *An. plumbeus* is needed. Other potential vector species are present: *Aedes vexans* and *Cx pipiens* are known vectors of West Nile Virus.

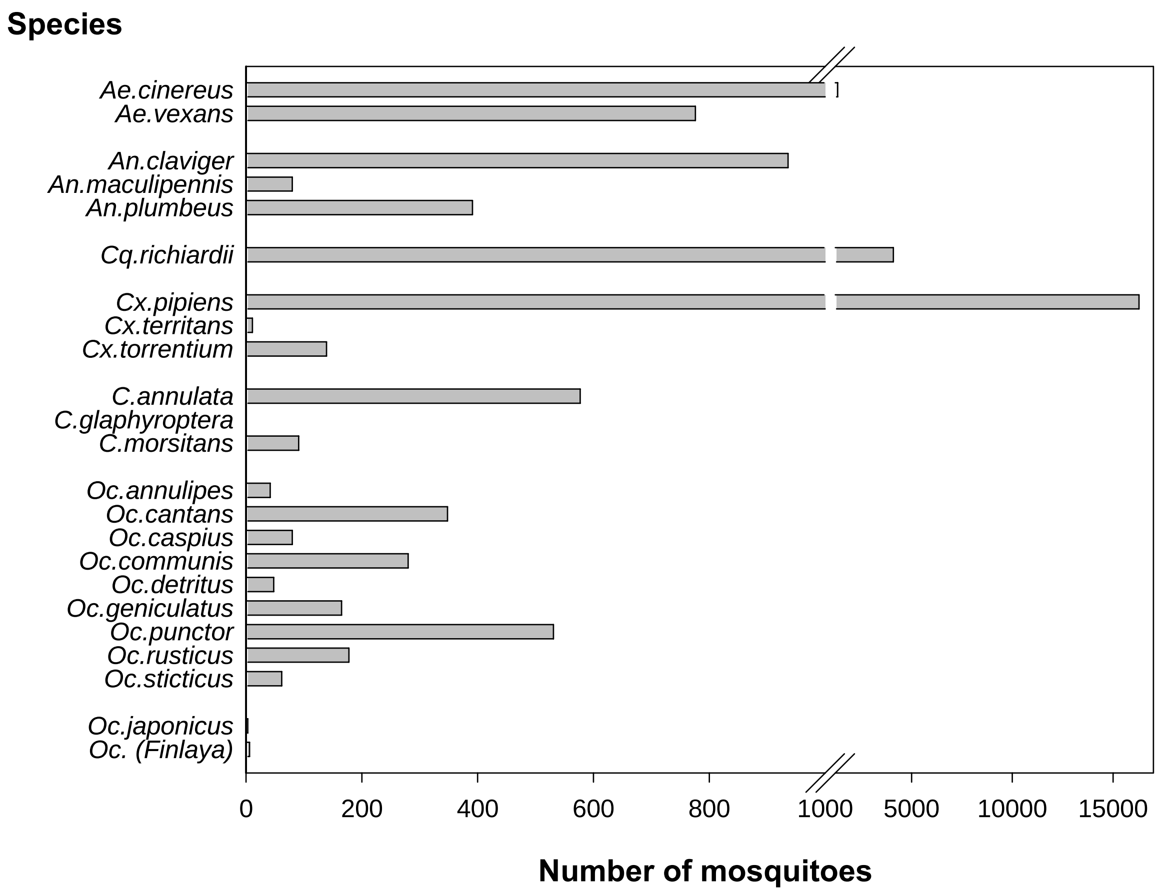

Figure 5. Species collected during the two years inventory study (2007-2008). Ae. = *Aedes*, An. = *Anopheles*, Cq. = *Coquillettidia*, Cx. = *Culex*, C. = *Culiseta*, Oc. = *Ochlerotatus*

The taxonomic biodiversity among the three main habitats differed. A large number of species has been found in the import risk areas whereas it only represented a small portion (6%) of the sample sites. But the three different key habitats (nature, agriculture and urban) are represented in the import risk areas. In each the agriculture habitat (56% of the study sites) and the nature habitat (20% of the study sites) 20 species were collected. In habitats classified as urban, representing 17% of the study sites, 16 species were collected (Table 3). The distribution of the different genera and species clearly displays spatial patterns and are suitable for the development of spatial models (Figure 6).

Table 3. Overview of the collections per habitat.

Habitat	Number of traps	Numbers of species	Mean number of mosquitoes per trap		
			Mean number	CI95%	Range
Urban	171	16	23.1	(17.0 – 29.2)	[0 – 624]
Agriculture	557	20	21.4	(18.3 – 24.5)	[0 – 993]
Nature	195	20	12.9	(9.7 – 16.1)	[0 – 252]
Import Risk area	63	21	123.3	(70.3 – 176.3)	[0 – 3780]

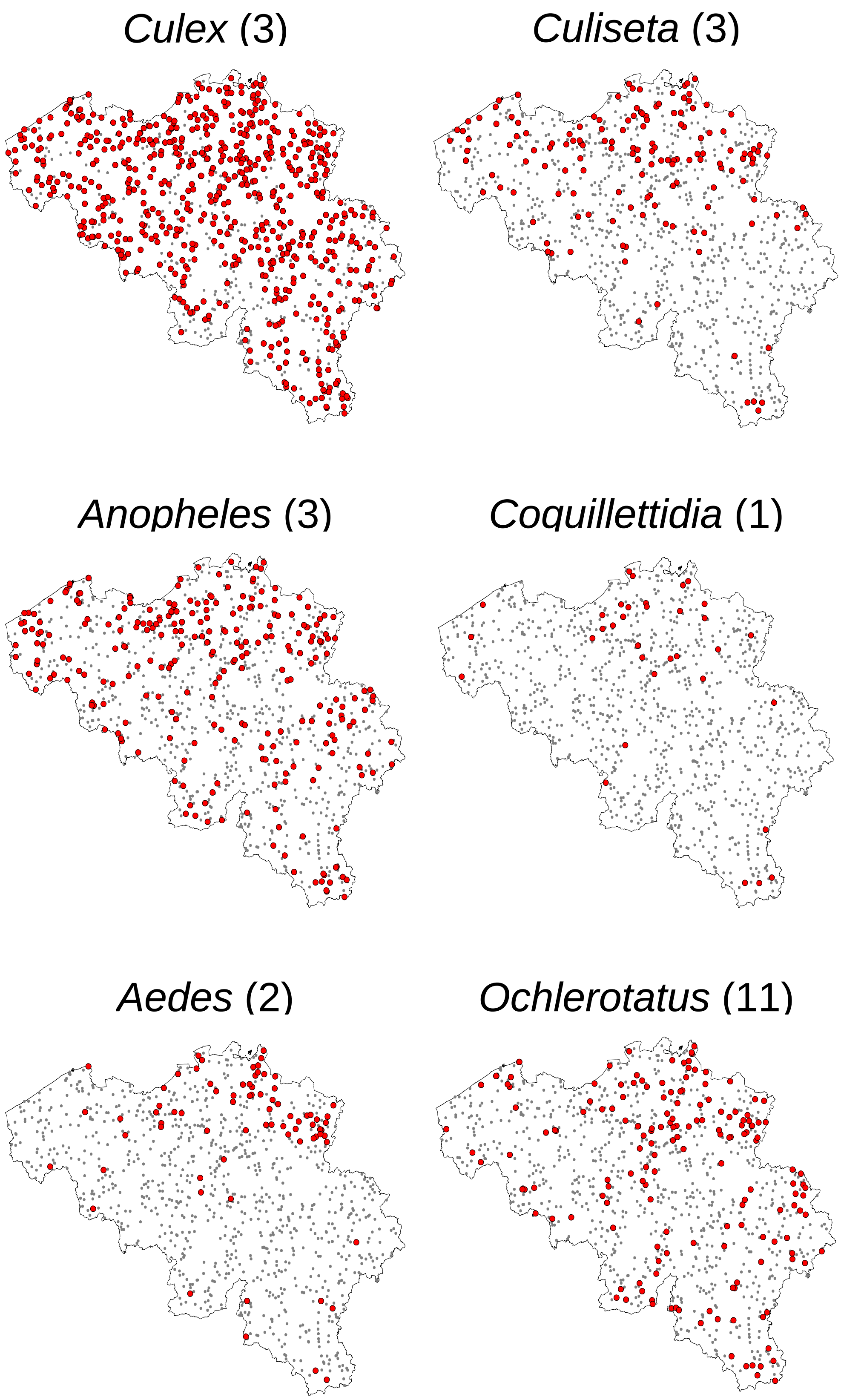

Figure 6. Spatial distribution of the different genera. Between brackets the number of species found. Red dots: the presence of the genus, Gray dots: study site where genus was not found.

3.1.2 Invasive species

The potential vector species *Oc.* (*Finlaya*) *j. japonicus* (Theobald) (Diptera: Culicidae) has been found in southern Belgium (Natoye, Namur) in 2002. Its presence at two tyre companies in the area as both adult and larval stages was confirmed in 2007 and 2008 using both morphological and molecular identification tools (ITS2 and COI region). To date, it seems that *Oc. j. japonicus* is well-established at least on one site, even if recurrent infestations cannot be completely ruled out yet. The species was probably introduced through the international trade in second-hand tyres, a known pathway of exotic species introduction. This is the first record for Belgium of an exotic mosquito species that established successfully.

Another exotic mosquito species was found in an at random chosen site, an old sand mine in nature restoration but close to a recycle company and industrial zone, indicating that the sampling set-up was effective, also for the detection of exotic/invasive species. The species was identified up to subgenus level as *Ochlerotatus* subgenus Finlaya. It strongly resemblances *Oc. j. japonicus* or *Oc. koreicus* (see Table 4 for an overview of the characteristics of each species), but the identification up to species level needs further investigation. This will be done in collaboration with the Smithsonian (Washington DC) and Rutgers University (New Brunswick). The site was sampled more thoroughly after detection of the mosquito species and was checked for natural and artificial breeding sites as well as for adults. Larvae were encountered in artificial (such as discarded tyres and rusted pots) as well as in natural (water filled tracks/ holes in dirt road) sites. Moreover other adults were found in an area 500m away from the first sampling point (on the opposite site of a road with heavy traffic) indicated its possible establishment.

Table 4. Overview of the morphological characteristics of the unknown *Ochlerotatus* species and differences with the closely related *Oc. japonicus* and *Oc. koreicus species*.

species characteristics	***Ochlerotatus sp.***	***Ochlerotatus j. japonicus***	***Ochlerotatus koreicus***
palp	entirely black	idem	idem
proboscis	entirely black	idem	idem
head	black with broad median white stripe + small triangular white lateral bands	black with small median white stripe + broad comma shaped white lateral bands	Broad patch of white scales close to eye
abdomen	White median + basal lateral band Variation: only median or only lateral bands present	Always white median + basal lateral bands	Very thin median band + always clear white basal lateral bands
Hind tarsus 4	pale basal band	black, sometimes some scales	pale basal band
Hind tarsus 5	pale basal band	entirely black	entirely black; sometimes few pale scales
Hind femur	large pale ventral apical patch + pale basal band	idem	idem
subspiracular area	10-20 white scales	no white scales	20-30 broad white scales

scutum	White coloured stripes, 1 median + posterior (curved) dorsocentral stripe	Yellowish brown coloured stripes, 1 median+ anterior dorsocentral stripe + posterior dorsocentral stripe	Yellowish brown coloured stripes, 1 media+ narrow anterior dorsocentral stripe + posterior dorsocentral stripe
scales on posterior pronotum (ppn)	Mixed broad & narrow white and some black scales	Some creamy narrow curved scales dorsally	Broad white scales, sometimes some black scales (usually a few narrow curved scales also present)?

3.1.3 Morphological and molecular identification

Morphological quality control

The necessary expertise in morphological identification of Culicidae was built up during the different training sessions. In July 2007 and January 2009 a quality control was done by an international expert. Thirty study sites per team were selected for the quality control. Of each site a sample of mosquitoes belonging to different species was re-analysed. 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). Where needed some systematic errors were corrected.

DNA Barcoding

A total of 964 individuals (17 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 (Figure 7). Most branches of the tree are supported by bootstraps values higher than 70% (for most species even 90% and more). At genus level most branches are not supported, hence for more phylogenetic studies other markers will be tested as well from the mitochondrial as from the ribosomal DNA.

Visualisation of the COI alignments reveals discrepancies with *An. claviger* and *An. plumbeus*, indicating a possible taxonomy problem of the species. One clade of the subfamily Anophilinae comprises only *An. plumbeus* specimens, whilst 2 other *An. plumbeus* mosquitoes group together with *An. claviger* specimens, indicating a high affinity between species. Other morphologically based misidentifications are revealed and difficulties solved when visualising the data; *Aecingem2* (morphologically identified as *Ae. cinereus*) will probably be an *Aedes vexans* and *Ochdetcomm* (morphologically identified as *Ochlerotatus communis*) is clearly an *Ochlerotatus detritus*. With the inventory being completed the DNA barcoding can be further established for all species found during the project. The selection of all 6 populations/species has been done, samples are at the ITM and the first extractions are done. Based on the amplification results, minimum 10 samples/species will be sent for sequencing. The developed DNA-barcoding system will be used to identify the members of the different species complexes.

Study on Culex pipiens complex

Several *Cx pipiens* populations were analysed (COI and ITS2) with PCR (ITS2) and PCR-RFLP (COI) to identify the members of the *Cx pipiens* complex. The *Cx pipiens* complex can be divided into *Cx torrentium* and *Cx pipiens pipiens.* The later is devided into *Cx p. pipiens* and *Cx p. pipiens* biotype molestus. The taxonomic status of the later two is however not well established. 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.

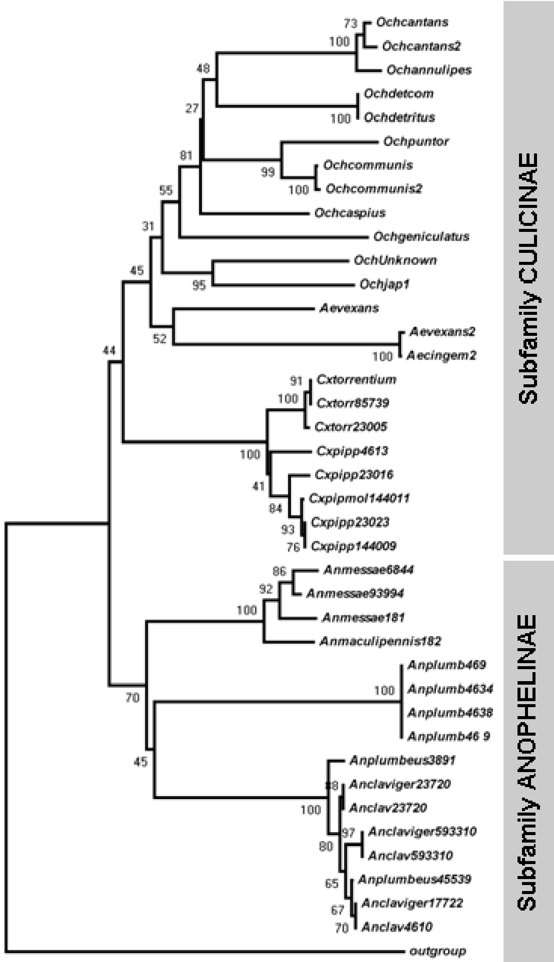

Figure 7. Preliminary neighbour joining tree based sequence alignments of COI region (outgroup = wasp)

3.1.4 Reference archive & link with biodiversity platform

At the RBINS all un-identified already mounted mosquito-specimens from the Belgian collection of the Entomology Department were identified and added to the identified collection (Collection of Goetghebuer). About 4000 specimen were screened during winter 2007-2008 and summer 2008 at the RBINS. At the end all these records and many other individual records and records from other projects at the RBINS will be added to a newly established database CULIBEL (all Belgian Culicidae records). Next, this database will be integrated into the Belgian Biodiversity Platform and will be kept updated by RBINS.

3.2 *Population dynamics*

3.2.1 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 *Cx 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 mid May 2008. The adults were morphologically identified and only *Cx pipiens* individuals were released into the three reproductive cages.

3.2.2 Larval and adult life history traits at varying temperatures

The effects of thermal conditions on larval development were tested under four temperatures (T11, T15, T20 and T28 °C). Pupation time and emergence time were calculated for four replicates for each temperature. Each replicate was constituted of one egg raft. Both the pupation time and the emergence time increased with low temperatures. 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 (Figure 8).

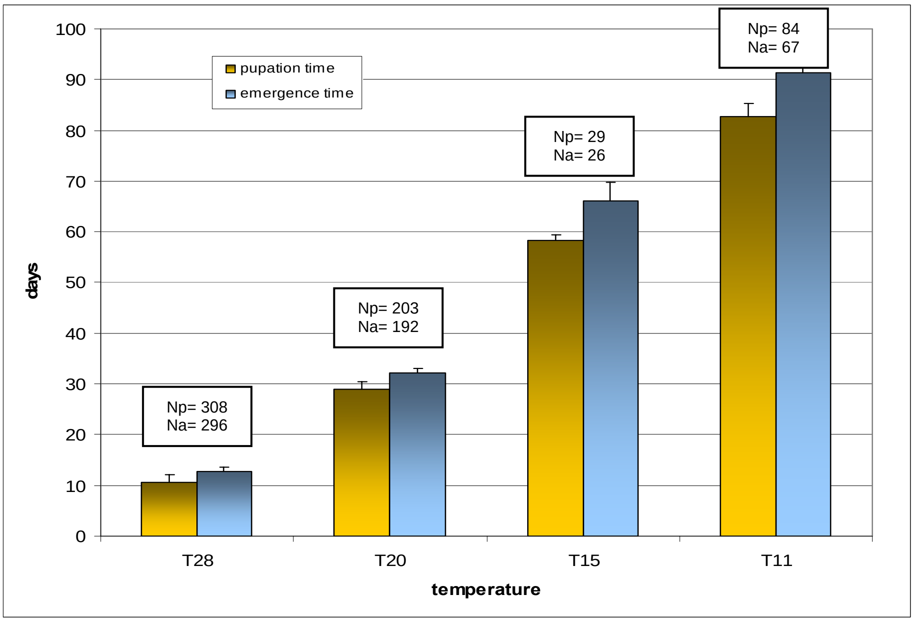

Figure 8. Pupation and emergence time at four temperatures. Np = number of pupae, Na = number of adults.

Males emerged 1.2 (T28) to 5.38 days (T15) before the females. Time between pupation and emergence increased with low temperatures, from 2.0 (T28) to 8.6 days (T11). Body size of adults was accessed under the four temperatures (mentioned above) by measuring the wing length. 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 (T28, T20) (Figure 9).

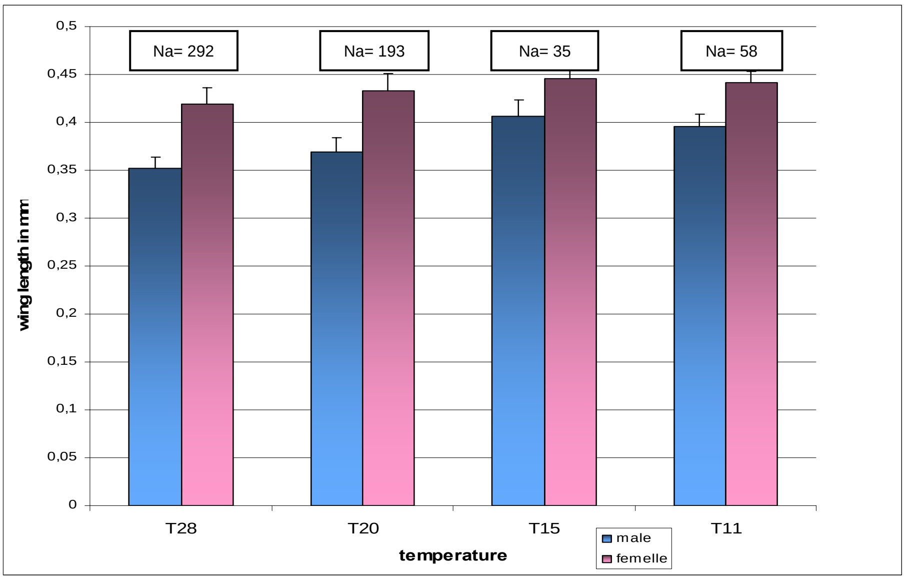

Figure 9. Wing length at four temperatures. Na = number of adults

The mating activity was observed for the first time at different temperatures. It is well know that males need time for the hemi-rotation of testes before being capable of mating. This time was estimated at 2 days. The results show that in *Cx pipiens*, temperature influence the time required to obtain copulation. Indeed, the pre-copulation time increases with the decrease of temperature (Figure 10). All females were inseminated after 3 days at 28°C and 5 days at 20°C. At 15°C, 80% of the females were inseminated after 7 days. Finally, after 7 days, no copulation has taken place at 10°C. At this point, it is uncertain if the time between the pair creation and the first mating is due to the time needed for the hemi-rotation and/or a low activity linked to coldness.

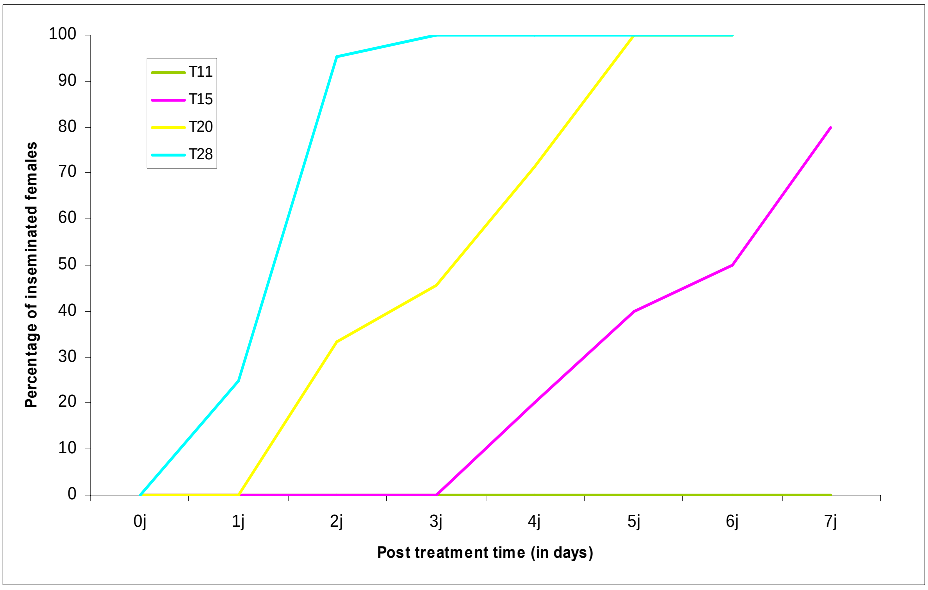

Figure 10. Pre-copulation time at four temperatures.

In conclusion, the parameter temperature has a great influence on development and mating activity of *Cx pipiens* and has to be included in transmission models and in formulas such as the vectorial capacity. Two additional temperatures (T35 and T40) have to be added to developed accurate models.

3.3 Model building

3.3.1 Eco-climatic zones

Eco-climatic zones were identified using an unsupervised k-means clustering. The scree plot and silhouette plot are found in Figure 11. No further decrease in Wilk's Lambda can be observed when increasing the number of clusters to more than 6, and thus six clusters are distinguished in the dataset. The silhouette plot indicates that the separation between the clusters is distinct.

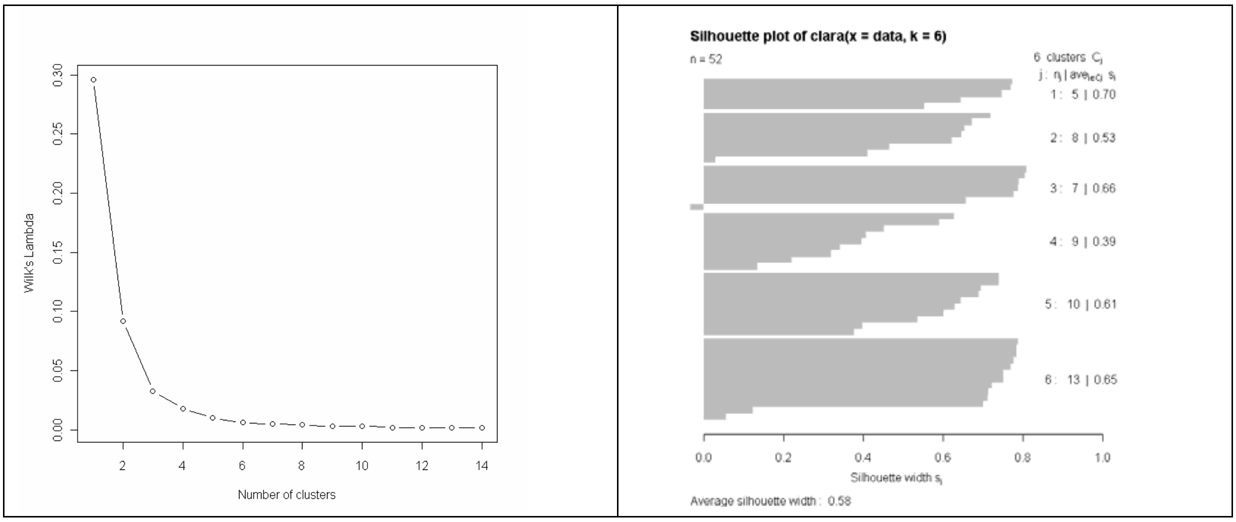

Figure 11. In the scree plot (left), no decrease in Wilk's Lambda is seen when the number of clusters, and thus 6 clusters are identified. The silhouette plot (right) indicates that there is a clear distinction between the clusters

The geographical distribution of the eco-climatic zones is shown in Figure 12. The characteristics of the identified eco-climatic zones can be found in Table 5.

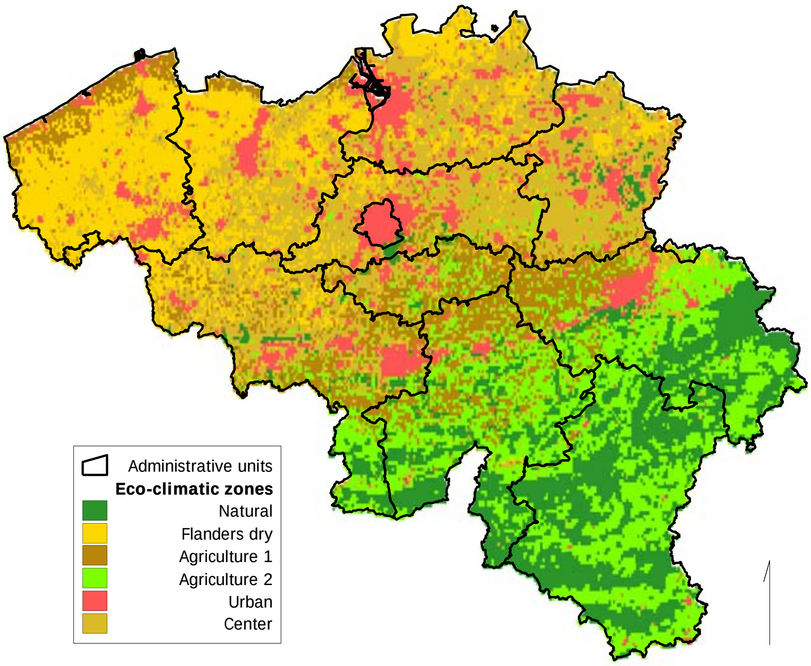

Figure 12. Eco-climatic zones in Belgium.

Table 5. 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_P1	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

3.3.2 Model selection

The stepwise regression procedure was successful in ruling out a considerable number of explanatory variables. This did however not decrease the predictive value of the models. For *An. claviger* 63.64 % of the absences are correctly classified, and 81.48% of the presences are correct. For *Ae. cinereus* 93.75 % of the observed absences is correctly classified, as compared to 86.67 % of the observed presences.

The explanatory variables that were retained in the respective model for the selected species can be found in Table 6 and Table 7. *An. claviger* individuals have a larger probability to occur in areas with relatively lower vegetation abundance and higher bi-annual vegetation variation*. Ae. cinereus* prefers higher amounts of vegetation, and smaller variations in vegetation amount throughout the year.

It was thus proven that different mosquito species show a distinct distribution over the study area, and that eco-climatic variables are paramount to explain a part of this variation.

Table 6. Variables retained in the model for spatial distribution of *Anopheles claviger*

Variable	Parameter estimate (β)
EVI_amplitude0	- 0.289756
EVI_amplitude2	+ 0.624217
LSTday_amplitude1	- 0.817186
LSTday_phase1	+ 0.811779
LSTnight_phase1	- 0.322869

Table 7. Variables retained in the model for the spatial distribution of *Aedes cinereus*

Variable	Parameter estimate (β)
EVI_amplitude0	+ 1.792545
EVI_amplitude2	- 1.416803
EVI_amplitude3	- 2.180805
LSTday_amplitude1	- 2.779280
LSTday_amplitude2	+ 4.713714
LSTnight_amplitude0	+ 6.786369
LSTnight_amplitude1	- 0.100548
LSTnight_phase3	- 1.312313

In a second phase, these models are used to provide a country-wide prediction. The resulting distribution maps are visualised in Figure 13 for *An. claviger* and Figure 14 for *Ae. cinereus*. A first observation is that *An. claviger* features a more general distribution over the country than *Ae. cinereus*. In the southern part of the country, the preferred habitats of both species tend to occur in the same geographic areas, situated mostly the south east. In the northern part of the country, the predicted probability for *An. claviger* is high in the west, whereas the probability of occurrence for *Ae. cinereus* is much lower in the west. The latter species features high probabilities of occurrence in the north east.

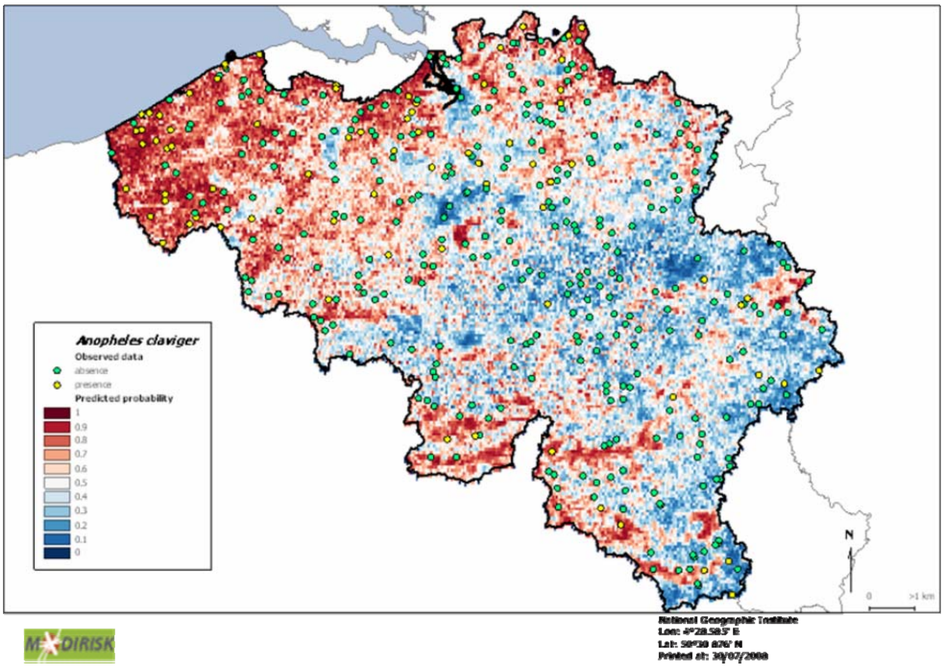

Figure 13. Presence-absence prediction for *Anopheles claviger*

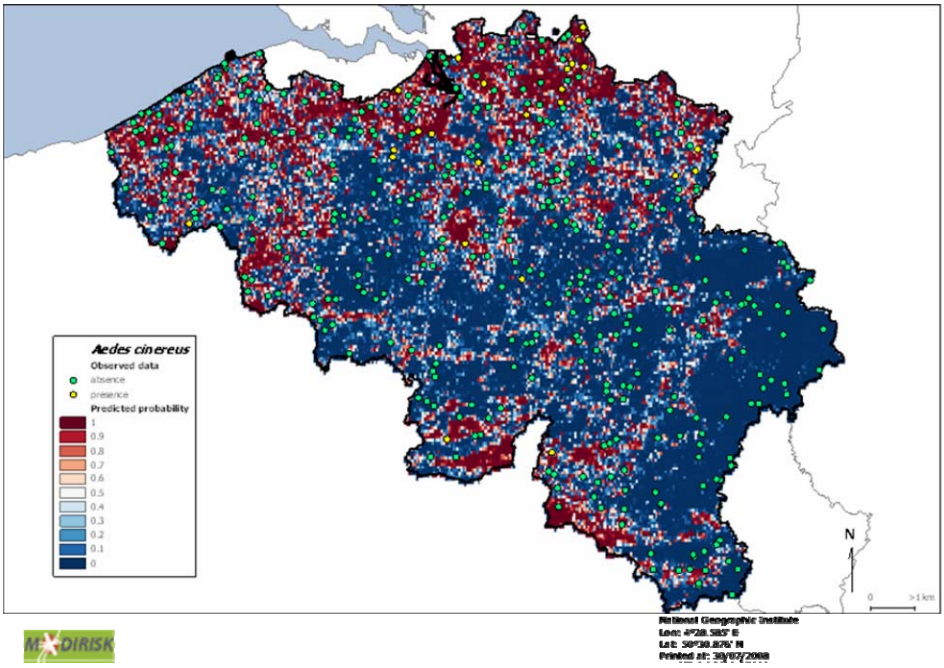

Figure 14. Presence-absence prediction for *Aedes cinereus*

Data from the longitudinal studies done in The Netherlands were transferred to the project MODIRISK. The first discussions were held in January 2008. A Master student was identified who will actually explore the possibility of integrating the longitudinal data from The Netherlands into the spatial models based on the MODIRISK data. This data analysis will be done in collaboration with the centre for Geo-Information of the WUR.

3.4 Dissemination

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. 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.

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 an 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 detials on the outcome of the workshop.

Aimed at the scientific community

The project and the spatial sampling protocol have been presented at the 4th European Mosquito Control Association Workshop in Prague, September 2007.

Oral presentations were given at the Entomology in Belgium meeting organized by IRSBN in December 2008 in Brussels.

Oral presentation at the annual ANKONA (Antwerpse koepel voor natuurstudie, febuary 2008) and LIKONA (Limburgse koepel voor Natuurstudie, January 2009) meeting were given.

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 submitted a 2 year-proposal to FSR (Fond Spécial pour la Recherche) to study the dynamics of the invasive exotic species *Oc. j. japonicus* in Namur and its epidemiological implications. The proposal passed successfully through the first step.
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.

Two partners of the MODIRISK project (ITM, RBINS) were involved in the study on the nuisance caused by biting insects near the Sea Scheldt in Gent.

4 Follow-up committee

4.1 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 sytematics of biting insects, University of Zürich.
- Marion Koopmans, Professor, unit of virology, Rijksinstituut voor Volksgezondheid en Milieu, The Netherlands.

4.2 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 resulting maps, output of the models built by MODIRISK will become available to other members of the scientific community so they can be combined with other spatial data layers (e.g. presence of birds, circulating viruses, disease outbreaks). They can also be used 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.

5 Workshop on vector control

5.1 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.

5.2 Outcomes and recommendations of the workshop

5.2.1 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 in 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.

5.2.2 The 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 *Oc. j. japonicus* found in southern Belgium.

5.2.3 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 témephos, 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 then 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.

5.2.4 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 in authorities potentially involved in vector control.

6 General discussion

Knowledge on 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 of mosquito borne diseases and preparing for future treads. Therefore the objectives of the project MODIRISK are (1) to inventorize 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 focussed on the inventory activities and the setting–up laboratory experiments for studying life history traits of *Cx pipiens* in relation to temperature. Based on the field results first model selection was done.

The cross-sectional field survey was conducted in 2007 and 2008 by use of a network of CO_2-baited traps throughout Belgium in three key habitats. These habitats (urban, agriculture and nature) were selected based on the Corine database. During the inventory 936 randomly selected sites were selected of which 97% was effectively sampled. This was only possible by the fact that three teams from three partner institutes contributed to the inventory and by the tools developed by MODIRISK to facilitate the field work. These tools including a website, a palm-to-web tool and a database and they serve 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 23 Culicidae species belonging to 6 genera were found. The number of species will certainly increase since some species complexes still need to be identified by molecular means. The number of caught species is close to the expected number of species (about 27 species) possibly present in Belgium. A molecular identification method based on the DNA barcoding approach is under development and will facilitate the identification of species complexes and species with overlapping morphological characters.

Additionally sites in import risk areas were sampled to evaluate the presence exotic mosquito species in Belgium. Two exotic species were found, *Oc. j. japonicus* in the province of Namur and *Oc. (Finlaya) sp.* in the province of Limburg. The latter could not be identified to species level and a comparative morphological study will be done at the Smithsonian (Washington DC) whilst Rutgers University (New Brunswick) will be visited to compare molecular data on this species.

Oc. (Finlaya) j. japonicus (Theobald, 1901) (sensu Reinert 2000) (= *Aedes japonicus*, Theobald) also called the Asian bush mosquito is one of four morphologically similar subspecies (*Oc. j. japonicus* Theobald, *Oc. j. amamiensis* Tanaka, *Oc. j. yaeyamensis* Tanaka and *Oc. j. shintienensis* Tai and Lien). Originating from Japan, Korea, Taiwan, eastern China and Russia (Tanaka et al. 1979), it has so far been reported in nine countries out of its native area. The first establishment outside its endemic zone was observed in the United States where it has been collected in 1998 from the eastern part of the country (Peyton et al., 1999). Since then, *Oc. j. japonicus* has spread rapidly over large parts of the USA and up till now it has been found in more than 10 states (Bevins 2007). In Europe, the species was recorded in France in 2000 from a used tyre importation platform (Schaffner et al. 2003). *Oc. j. japonicus* females are known to feed on mammals, including humans, in the field (Apperson et al. 2004) and on avian host under laboratory conditions (Sardelis et al. 2002a, 2002b, 2003) and could therefore act as a zoonotic bridge vector species. In laboratory conditions, this mosquito has been shown to be a competent vector of Eastern encephalitis virus (Sardelis et al. 2002a), La Crosse virus (Sardelis et al. 2002b), St. Louis encephalitis virus (Sardelis et al. 2003) and a highly competent vector for West Nile virus (WNV). It is even more competent as vector for WNV than *Cx pipiens.* (Sardelis & Turell 2001) which is considered as one of the main vectors (Goddard et al 2002, Sardelis et al 2001, Turell et al 2000). However, its role as a disease vector species in natural conditions in the United States, where the species has been established for almost a decade, remains unclear. Because of health risks (Rodhain 1995), a tyre trade surveillance program for *Aedes albopictus* (Skuse) has been implemented and conducted in France since 1998 focused on storage centres importing used tyres from the US, Japan or Italy (countries known to be breeding areas) (Schaffner et al. 2004). In Belgium, the Risk Assessment Group of the Scientific Institute of Public health recommended that "the precautionary principle makes it necessary to adopt measures to destroy the species". This view was confirmed during the workshop on "Vector Control in Belgium" organized by MODIRISK. Moreover 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. In the second phase of the project longitudinal studies will be done in the field sites where exotic species were observed.

A laboratory colony of *Cx pipiens* was set-up at UCL to enable the study of 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 the 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). Furthermore the results show that temperature influence 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. Two additional temperatures (T35 and T40) will be added to developed accurate models.

The inventory 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 results of the cross sectional field surveys, distribution models predicting the probability of presence of each Culicidae species will be developed and maps will be produced at a one kilometre resolution. The first selection of models has been made on *An. claviger* and *Ae. cinereus.* The models will be based on multivariate analysis techniques and use eco-climatic data as main predictor variables. Additional field surveys will be conducted in the second phase of the project in Belgium and in The Netherlands to validate and further fine tune the produced models. The model outputs will enable us to understand the factors (mainly eco-climatic, but also human driven such as land use, urbanisation) determining observed distribution patterns. When included in a GIS model, they will also enable to highlight Culicidae biodiversity hotspots 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 will be 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, will also be 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 up 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 activities and meeting such as 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.

7 Conclusions & Recommendations

During the first project year, activities focussed on the inventory of the mosquitoes. All partners were involved in the preparation and implementation of the inventory i.e. elaboration of sampling strategy, development of a palm to web data management, and implementation of the field work. Almost all sampling points could be visited and, all teams gained the necessary skills in morphological mosquito identification after the different training sessions.

The preliminary analysis of the data shows that spatial differences in mosquito presence were found and that the data will be suitable for development of spatial distribution models. The sampling strategy was designed for the model building which will provide a unique opportunity to evaluate strategies for the monitoring of mosquito species.

The sites for the longitudinal study were selected. This study will focus on both exotic species and on *Anopheles plumbeus*, which is becoming a nuisance species. The longitudinal study will address questions on spreading/establishment of the exotic species, on population dynamics and on trophic behaviour.

A colony of *Cx pipiens* was set-up at UCL. Because of security issues it was decided not to start a colony of *Oc. j. japonicus.* The laboratory is not sufficiently secured to avoid escape of this exotic mosquito species. It was decided to evaluate whether the competition experiments between *Oc. j. japonicus* and *Cx pipiens* can be set-up under field situation.

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. Two additional temperatures (T35 and T40) will be added to developed accurate models.

MODIRISK created links with different end-users. MODIRISK participated to the risk assessment group of the Scientific Institute of Public Health concerning the presence of the exotic vector species *Oc. j. japonicus* 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 (11^{th} 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 and monitoring to follow the situation and to evaluate the spread of the exotic species in Belgium. Moreover a flow chart of competences and responsibilities of the different authorities potentially involved in vector control should be established.

8 Prospects of phase 2

The second phase of the project will focus on the longitudinal study and on the model building and validation. The data of the inventory will be further analysed and link will be made with the Belgian Biodiversity platform.

Work Package 1 – To inventory endemic and invading mosquito species in Belgium considering environmental and taxonomic elements of biodiversity

Biological reference archive. Within this work package data of the inventory will be further analysed. The Biological reference archive started during the first phase of the project and will be completed during the second phase of MODIRISK. At the RBINS all un-identified already mounted mosquito-specimens from the Belgian collection of the Entomology Department started in 2008. These records and many other individual records and records from other projects at the RBINS will be added to a newly established database CULIBEL (all Belgian Culicidae records). Next, this database will be integrated into the Belgian Biodiversity Platform and will be kept updated by RBINS. From the mosquitoes collected in The Netherlands during phase 2 specimens will be stored (individually in small tubes and pinned) and will deposited at the Royal Belgian Institute of Natural Sciences and the Natural History Museum collection (Naturalis) at Leiden, The Netherlands.

Model validation. Based on the developed models sampling points that have to be re-sampled will be identified. The sampling will be performed during the months August – September 2009 and April – May 2010 after the model building (WP3). All mosquitoes will be morphologically identified and entered in a database. Molecular identification will continue. Additionally, based on the developed model sampling points will be identified in The Netherlands. The sampling will be performed during the same period as stated in Belgium by use of the CO_2-baited traps. All mosquitoes will be morphologically identified (where needed also by use of molecular methods). The data will be double entered in a database.

Molecular identifications. Molecular identification and the establishment of the DNA-barcoding for Belgium mosquitoes will continue. This DNA barcoding tool will facilitate the identification of species complexes and species with overlapping morphological characters, hence will be an important tool in the study of the taxonomic biodiversity of Culicidae.

Work package 2 – To assess the population dynamics of endemic and invasive mosquito species and their interrelationship

Laboratory tests. For identified indigenous and invasive species that are expected to share the same habitat, competition trials will be conducted under field conditions in cages in places where *Oc. j. japonicus* is present. Two questions will be asked (1) does a female still lay egg in places where larvae or eggs of another species are present? (2) are the development time and survival of larvae affected by the presence of larvae of other species?

Depending on the identified mosquito species winter survival of selected species (e.g. exotic species) will be assessed using appropriate tests e.g. aptitude for fertilized eggs to enter a diapause stage, aptitude of adults to survive in shelters with appropriate micro-climates (*Culex* group).

Additional field test. Additional field test aim at complementing knowledge acquired during the laboratory studies and will provide information on population build-up and population dynamics throughout the year. This field study will provide additional information of the presence of species in particular sites and will contribute to the validation of the models. Longitudinal data can be included in the distribution models as it will be done for existing longitudinal data from The Netherlands.

Longitudinal study in selected field sites. Longitudinal collections of mosquitoes will be done in selected field sites (WP1) by use of different traps during year 3. The longitudinal study will be oriented to sites where exotic species were found and where *An. plumbeus*, which is becoming a nuisance species in e.g. The Netherlands, is present. The combination of the longitudinal data and of published data will enable a PRA of the different study species.

Pest Risk Assessment (PRA). The combination of longitudinal and published data will enable a PRA of endemic nuisance and imported pest species i.e. exotic species such as *Oc. j. japonicus* and the endemic species *Anopheles plumbeus.* Several sites/populations will be monitored to assess the ecological (and climatic) necessities needed for establishment, maintenance and spread of each of the studied species. The spatial model of species distribution and diversity will be discussed in relationship with risk of change of land use, agricultural practice and climate. The biological data concerning temperature and competition will give an interesting basis to complete these aspects. A first list of risks to take into account will be established as a discussion basis for 'what if' scenarios.

Work Package 3 – To model mosquito species distribution/diversity at a one km scale in Benelux

Spatial distribution models. Whilst the model selection was part of phase 1, the actual modelling and validation is carried out during phase 2 of the project. The selected model approach is applied to produce distribution models and maps for each mosquito species identified during the field surveys. The procedure includes:

- The selection of a calibration and validation data set, the former being used to train the model and the latter to validate the obtained model outputs. These datasets will be used to perform a ten-fold cross-validation.
- The computing of the model equation using a standard statistical software package.
- The computing of the distribution maps using the map calculator facility in Idrisi, a raster GIS software package.

This task will be initiated during the second year of the project, but will mostly take place in the second phase (year 3-4).

The use of a split sample approach as described above enables the internal validation of model outputs. Nevertheless when mapped outputs are critically assessed distinct areas may appear where a mosquito species has been trapped, but is predicted as absent and vice versa. This may be caused mainly by (i) lack of resolution of the field sample, or (ii) need for additional predictor variable(s) to fine tune the model. Based on observed patterns for each mosquito species it then is decided to conduct additional field samples and/or to compute/include additional predictor variables.

"What-if" scenarios. Two workshops will be organized if additional funding is found to concretize the PRA and to develop possible scenarios of risks. These workshops would include, beside the member of MODIRISK consortium, the main stakeholders as well as scientists of neighboring countries dealing with the same problems.

- Workshop 1: will be organized in autumn 2009 and will deal with 'Scenario's to prevent human introduction of vector species'
- Workshop 2: will be organized in autumn 2010 and will deal with 'Scenario's to model spread of species'. Theoretical models assessing what could happen with changing

factors (such as ecological, climatic) in terms of establishment and spread of exotic and endemic species will be drawn up.

Defining a cost-effective spatial sampling strategy. Based on information gathered from the distribution model outputs and the field sampling implementation a cost-effective sampling strategy is designed for use in follow-up and similar studies. The different needs related to the sampling of all mosquito species, species groups or individual species will also be taken into consideration.
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. To achieve this, models are computed with a decreasing percentage of training data and outputs are compared to the full model output.
Field experience gained during the surveys will enable to include practical aspects such as feasibility, trap types, number of trapping sites visited per day etc.

Work package 4 - Dissemination

Public link to MODIRISK information system. Since the MODIRISK information system is now live the public link which was restricted to the spatial sampling map now gives access to the public parts of the MODIRISK information system.

Publications. Suitable international conferences will be identified and obtained results will be presented. Most peer reviewed publications (as draft or submitted) will be finalized during this phase of the project.

Meetings. One to two co-ordination meetings will be held each year. Representatives of each partner participate to each meeting. Additional experts may be asked to join the meetings and the follow-up committee meets once a year.

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