

Nationwide inventory of mosquito biodiversity (Diptera: Culicidae) in Belgium, Europe

V. Versteirt¹, S. Boyer², D. Damiens², E.M. De Clercq⁴,
W. Dekoninck³, E. Ducheyne⁴, P. Grootaert³, C. Garros²,
T. Hance², G. Hendrickx⁴, M. Coosemans^{1,5*}
and W. Van Bortel¹

¹Department of Biomedical Science, Vector Biology Group, Medical Entomology Unit, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium: ²Biodiversity research Centre, Earth and Life Institute, Université catholique de Louvain, Place Croix Sud 4/5, B-1348 Louvain-La-Neuve, Belgium: ³Department of Entomology, Royal Belgian Institute of Natural Sciences, Vautierstraat 29, B-1000 Brussels, Belgium: ⁴Avia-GIS, Risschotlei 33, B-2980 Zoersel, Belgium: ⁵Department of Biomedical Sciences, Faculty of Pharmaceutical, Veterinary and Biomedical Sciences, University of Antwerp, Universiteitsplein 1, B-261 0 Antwerpen (Wilrijk), Belgium

Abstract

To advance our restricted knowledge on mosquito biodiversity and distribution in Belgium, a national inventory started in 2007 (MODIRISK) based on a random selection of 936 collection points in three main environmental types: urban, rural and natural areas. Additionally, 64 sites were selected because of the risk of importing a vector or pathogen in these sites. Each site was sampled once between May and October 2007 and once in 2008 using Mosquito Magnet Liberty Plus traps. Diversity in pre-defined habitat types was calculated using three indices. The association between species and environmental types was assessed using a correspondence analysis. Twenty-three mosquito species belonging to traditionally recognized genera were found, including 21 indigenous and two exotic species. Highest species diversity (Simpson 0.765) and species richness (20 species) was observed in natural areas, although urban sites scored also well (Simpson 0.476, 16 species). Four clusters could be distinguished based on the correspondence analysis. The first one is related to human modified landscapes (such as urban, rural and industrial sites). A second is composed of species not associated with a specific habitat type, including the now widely distributed *Anopheles plumbeus*. A third group includes species commonly found in restored natural or bird migration areas, and a fourth cluster is composed of forest species. Outcomes of this study demonstrate the effectiveness of the designed sampling scheme and support the choice of the trap type. Obtained results of this first country-wide inventory of the Culicidae in Belgium may serve as a basis for risk assessment of emerging mosquito-borne diseases.

*Author for correspondence
Fax: +32 3 2476359
E-mail: mcoosemans@itg.be

Keywords: inventory, sampling strategy, biodiversity, invasive species, Culicidae

(Accepted 3 July 2012; First published online 13 September 2012)

Introduction

In general, the term biodiversity comprises the diversity of species and their complex interplay with the abiotic, non-living, features of their environment. Biodiversity relates to the range of genes, species and ecosystems and can be divided into a structural, functional and spatial component changing over time and space (Gaston, 1996). Gaining knowledge on these different components of mosquito biodiversity is essential to understand the current risk of new invading species and disease transmission in a region (Manguin & Boëte, 2011). The *structural* biodiversity component encompasses species richness based on morphological or molecular characteristics and their relative abundance. The *functional* aspect comprises the ecology of different species and their habitat preferences, their influence on ecosystem functioning, and the species interactions (e.g. competition, the influence of invasive species). *Spatial* biodiversity, in turn, covers the occurrence of species in a given region or habitat, which is essential to predict species variance with changing environmental and climatic conditions. Despite the importance of these components in risk assessments, little attention has been paid to the distribution and biodiversity of mosquitoes in northern Europe, except for some scattered studies (Schäfer, 2004). Studies focused mainly on indigenous vector species like *Anopheles* spp. in southern Europe (Patsoula *et al.*, 2007; Ponçon *et al.*, 2007; Di Luca *et al.*, 2009; Vicente *et al.*, 2011) and invasive (potential vector) species such as *Aedes albopictus* (= *Stegomyia albopicta* in the phylogenetic classification of Reinert *et al.*, 2009) and *Ae. j. japonicus* (also *Ochlerotatus j. japonicus* or *Hulecoeteomyia j. japonicus*) (see Medlock *et al.*, 2012) but disregarded information about the spatial distribution of other indigenous (adult) mosquito species. Overall, recent knowledge on the different aspects of biodiversity of both indigenous and invading vector mosquito species is currently missing in many northern European countries, including Belgium. Besides a paper published in 2004 reporting the occurrence of *Aedes albopictus* in Belgium (Schaffner *et al.*, 2004), only a few publications are available on mosquitoes in Belgium. Some scattered records of mosquito species in Belgium were mentioned by Goetghebuer (1925) in the beginning of the 20th century. In the 1940s, the presence of malarial mosquitoes and associated malaria transmission risk was studied at several locations in Belgium (Rodhain & van Hoof, 1942, 1943; Rodhain & Van Mechelen, 1944); and, during the early 1950s, mosquito nuisance was investigated around the city of Antwerp (Wanson, 1952). Although a large collection is present at the Royal Belgian Institute of Natural Sciences (RBINS), the majority of this material was never identified. In 1991, a checklist of Belgian Culicidae was created comprising 24 species present in the RBINS collection and additional species mentioned in card-indexes (Gosseries & Goddeeris, 1991), based on historical data mainly collected between 1909 and 1958. Moreover, records are sometimes clustered in space and time, as, for example, between 1940 and 1950 mosquitoes were mainly collected around Ghent and Brussels (Dekoninck *et al.*, 2011a). No exotic mosquito species were recorded in this checklist. Acquiring information on the biodiversity

of Culicidae is an essential step towards understanding the current risk of diseases transmitted by mosquitoes and preparing for future threats. In that framework, a national inventory started in 2007 (MODIRISK, www.modirisk.be). It was based on a specific and unique sampling strategy designed to allow a rapid overview of the Belgium Culicidae.

This paper, therefore, elaborately describes the applied sampling protocol, as it was specifically developed for this study. Furthermore, our aims were (i) to update the current knowledge on mosquito species present in Belgium, (ii) to assess structural biodiversity based on different species richness indices and (iii) assess spatial biodiversity according to the main environmental types.

Material and methods

Sampling design

Cross-sectional field surveys were conducted using the Corine (2000) Land Cover classification (NGI, 2004), delineating potential mosquito habitats. The Corine Land Cover Classes were regrouped in six classes: (i) urban biotopes, (ii) land in agricultural cycle, (iii) natural terrestrial environmental type, (iv) mosquito specific areas (ports, airports, dump sites, moors and heat land, inland marshes, salt marshes), (v) secondary sites (industrial and commercial units, roads, mines and mineral extraction sites, construction sites) and (vi) water bodies. The last two were not included in the inventory. The first three were retained for a random selection of sampling sites and were renamed as (i) urban, (ii) rural and (iii) natural. A fourth class was additionally defined as selected sites, those prone to an introduction of vectors or pathogens, subdivided into import risk areas industry (IRA-industry) and importation risk areas (IRA). The former included used tire importation/storage companies, bamboo importers, harbours and airports and are mainly areas at risk for importation of exotic mosquito species. The IRA included zoos, safari parks, protected areas involving presence of large numbers of migratory birds where importation of pathogens is possible. This data layer was then overlaid with the Military Grid Reference System (MGRS) (Hagemer & Blair, 1997), which is an extension of the UTM system. A total of 312 10×10km MGRS cells are identified across Belgium. The aim was to sample per cell an average of three representative environmental types, representing a total of 936 sampling points. The number of points assigned for each Corine land cover aggregated class was proportional to its total surface in Belgium, and each point received a random set of X and Y coordinates. 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.

Table 1. Taxonomic diversity (Simpson index, 1-D; Shannon-Wiener diversity, H'; Shannon-Wiener Evenness, E') by main Corine habitat type.

	Habitat				
	Urban	Rural	Nature	IRA_nature	IRA_industry
Number of sites sampled	171	577	184	37	26
Number of species collected	16	20	20	21	11
Total of specimens collected	3992	12100	2553	6857	1031
Mean number of specimens per trap	23.4	21.0	13.9	185.3	38.2
Simpson Index	0.476	0.299	0.765	0.605	0.099
Shannon-Wiener diversity	1.107	0.830	1.951	1.354	0.274
Shannon-Wiener evenness	0.399	0.277	0.663	0.445	0.114

Table 2. The total number of specimens collected per species and the number of study sites where the species were caught.

Species	Abbreviation	Total number of specimens collected (% of total)	Number of sites where the species was found (% of all sites)	Environmental type
<i>Ae. annulipes</i>	Aeanni	42 (0.16)	8 (0.80)	rural, natural and IRA_natural
<i>Ae. cantans</i>	Aecant	355 (1.34)	63 (6.31)	all
<i>Ae. caspius</i>	Aecasp	80 (0.30)	12 (1.20)	urban, rural and IRA_natural
<i>Ae. cinereus/geminus</i>	Aecine	1338 (5.04)	74 (7.41)	all
<i>Ae. detritus</i>	Aedetr	48 (0.18)	4 (0.40)	rural and IRA_natural
<i>Ae. geniculatus</i>	Aegeni	164 (0.62)	48 (4.81)	All
<i>Ae. j. japonicus</i> *	Aejapo	3 (0.01)	1 (0.10)	IRA_industry
<i>Ae. koreicus</i> *	Aekore	6 (0.02)	2 (0.20)	natural
<i>Ae. punctor</i>	Aepunc	531 (2.00)	65 (6.51)	All
<i>Ae. rusticus</i>	Aerust	178 (0.67)	33 (3.30)	urban, rural, natural and IRA_natural
<i>Ae. sticticus</i>	Aestic	63 (0.24)	13 (1.30)	urban, rural, natural and IRA_natural
<i>Ae. vexans</i>	Aevexa	776 (2.92)	29 (2.90)	urban, rural, natural and IRA_natural
<i>Ae. communis</i>	Aecomm	280 (1.06)	33 (3.30)	all
<i>An. maculipennis</i>	Anmacu	43 (0.16)	23 (2.3)	urban, rural, natural and IRA_natural
<i>An. messeae</i>		24 (0.09)	13 (1.3)	urban, rural, natural and IRA_natural
<i>An. plumbeus</i>	Anplum	391 (1.47)	114 (11.41)	All
<i>Anopheles claviger</i>	Anclav	935 (3.52)	185 (18.52)	All
<i>Coquillettidia richiardii</i>	Cqrich	4095 (15.43)	38 (3.80)	All
<i>Cs morsitans</i>	Csmors	92 (0.35)	17 (1.70)	rural, natural and IRA_natural
<i>Culex pipiens</i>	Cxpipi	16,349 (61.62)	698 (69.87)	All
<i>Culiseta annulata</i>	Csanna	577 (2.17)	162 (16.22)	All
<i>Cx territans</i>	Cxterr	11 (0.04)	7 (0.70)	rural, natural and IRA_natural
<i>Cx torrentium</i>	Cxtorr	139 (0.52)	75 (7.51)	All

* exotic species.

Sampling trap

The CO₂ baited trap Mosquito Magnet Liberty Plus (Woodstream Corp., Lititz, PA, USA) was used throughout Belgium for mosquito sampling. This trap is a CO₂-baited trap which outperformed both in number of specimens and number of genera collected compared to seven other trap systems (Dennett *et al.*, 2004). Furthermore it is the only available trap type that allows the autonomy needed for the trapping scheme. This trap works on a counter flow technique, in which one fan exhausts CO₂, heat and moisture from

the bottom outlet, while the other fan draws air from the bottom inlet and forces it out at the top, causing a suction that pulls mosquitoes into a net.

Sampling frequency

Mosquitoes were sampled from May till October in 2007 and 2008 according to the sampling design explained above. Each year, the entire country was sampled twice to counteract possible seasonal influences in each region. The first sampling

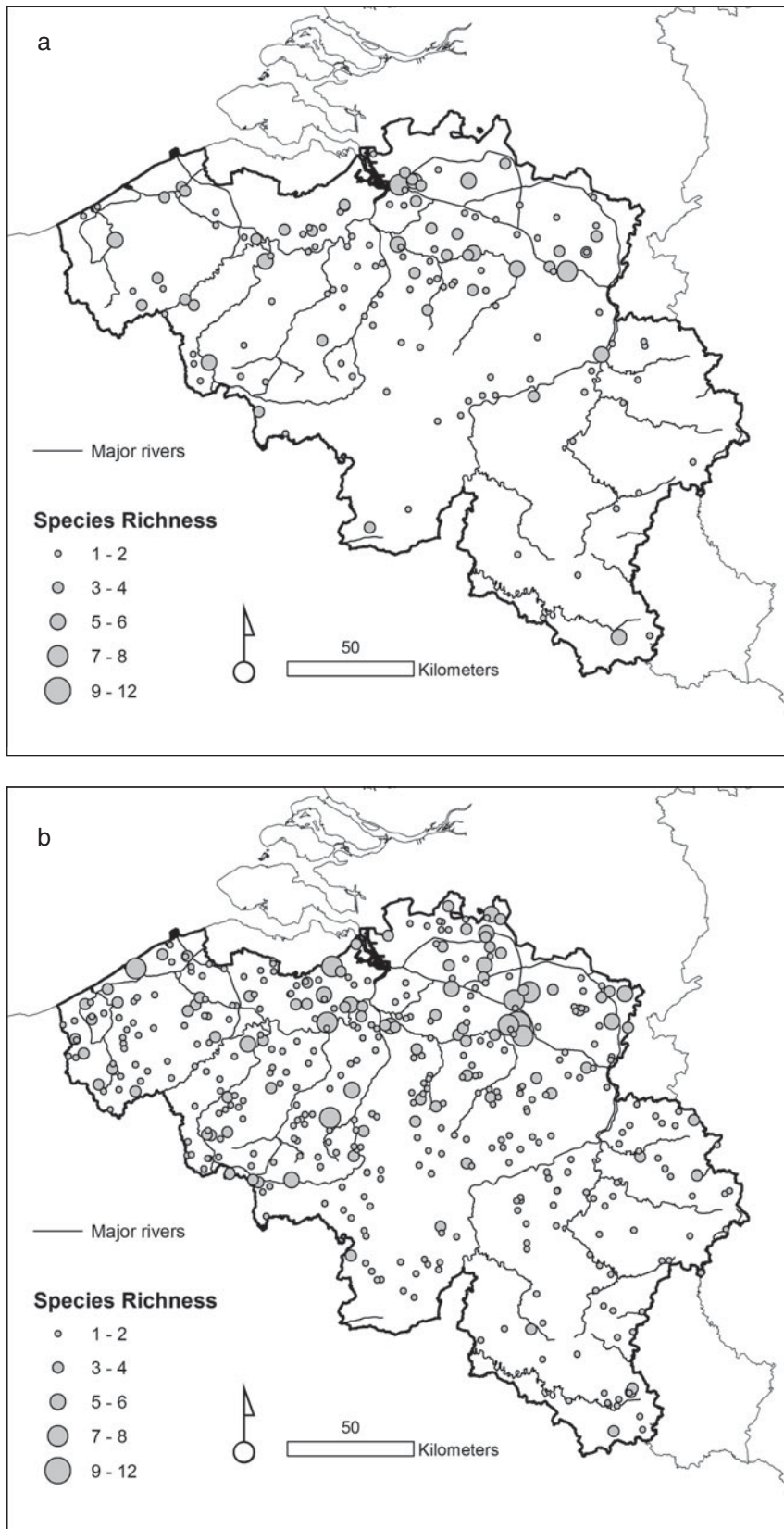


Fig. 1. For Figure legend see opposite page.

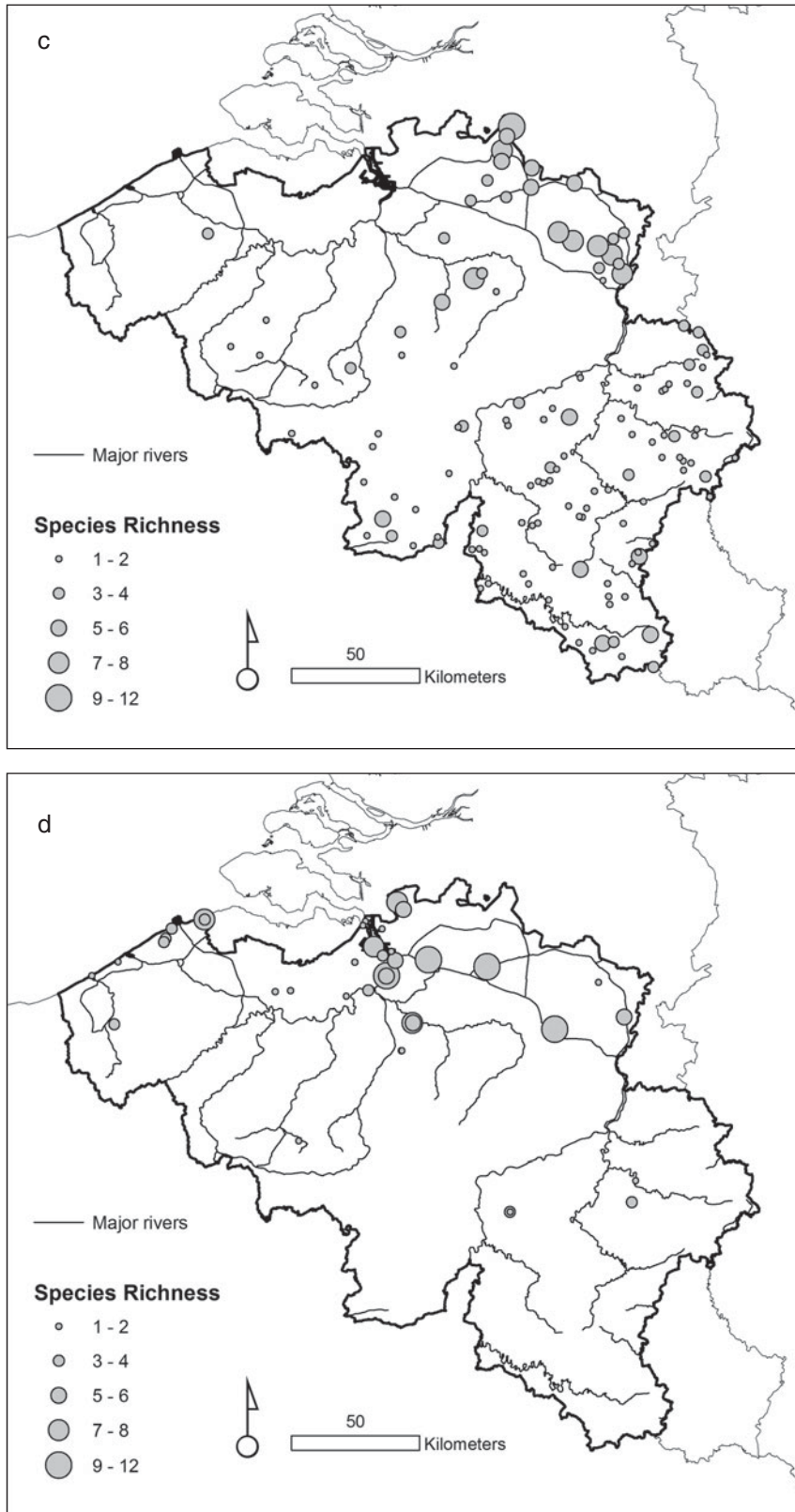


Fig. 1. (a) Observed species richness in the environmental type sampled (urban). (b) Observed species richness in the environmental type sampled (rural). (c) Observed species richness in the sampled natural environments. (d) Observed species richness in the import risk areas (natural and industrial sites combined).

period extended from May till mid-July, the second from the end of July till the beginning of October. Within each delineated MGRS cell, sampling points were randomly allocated to season and year. Although each site was only visited once, nearby sites were sampled during every period and year giving a good overview of mosquito presence in a specific area. During the inventory campaign, 27 traps operated simultaneously. Each trap operated seven days in 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 per field team (nine in total). The organization of the field work and the morphological identification of the mosquitoes were done on the other days. Each field team had a personal digital assistant (PDA) and global positioning system (GPS) to their disposal to easily locate the selected sites, record the exact GPS position of the traps and to record additional field information such as the presence or absence and artificial, natural, permanent and temporal types of habitats.

Morphological and molecular identification

Morphological identification was done using the electronic identification key of Schaffner *et al.* (2001) and the printed keys of Schaffner (1993) and Becker *et al.* (2010). Afterwards, data were stored in a specially designed web-based database. To assure quality of morphological identifications, a random sample (10%) of the identified mosquitoes was re-identified by an external expert (F. Schaffner). Voucher specimens were deposited at the Royal Belgian Institute of Natural Science. There is a growing acceptance of at least the recognition of *Ochlerotatus* as a distinct genus and recent publications (Reinert, 2000; Reinert *et al.*, 2004, 2008a,b, 2009; Shepard *et al.*, 2006) indicate justified reasons for the reclassification of the genus *Aedes* (especially elevating *Ochlerotatus* to generic rank). Recent changes to the classification of the composite genus, including the further division of the genera *Aedes* and *Ochlerotatus* have led to much confusion for non-taxonomists interested in potential vector mosquito species. For this reason, we prefer to maintain in this paper the traditional classification (pre-Reinert, 2000).

Members of the *Anopheles maculipennis* complex were identified to species level using a specifically designed PCR-RFLP method (Nicolescu *et al.*, 2004) that targets the internal transcribed spacer 2 (ITS2) region. DNA extraction was performed using the protocol described by Collins *et al.* (1987) after which the ITS2 region was amplified using primers described by Collins & Paskewitz (1996). Consequently, the positive amplification products were digested using CfoI restriction enzyme (Roche Molecular Biochemicals Ltd, Sussex, UK) (Nicolescu *et al.*, 2004). Restriction fragments were visualised on a 3% agarose gel. The PCR-RFLP does not differentiate between *Anopheles messeae* and the recently described and closely related *An. daciae* (Nicolescu *et al.*, 2004), and thus all specimens with a shared RFLP pattern were sequenced (Genoscreen, Lille, France) and compared with Genbank sequences. Additionally, a selected number (five of each species) of positive ITS2 PCR amplifications were sent for sequencing (Genoscreen, Lille, France).

Data analysis

Alpha diversity (as defined by Whittaker, 1972) in pre-defined habitat types was calculated using three different indices (Simpson and Shannon species richness indexes and species abundances through rarefaction), taking into account the number of sites sampled per habitat type. Simpson's index of diversity, $(1 - D = 1 - \sum [n_i \times (n_i - 1) / N \times (N - 1)])$, where n_i is number of the i -th species and N is the number of individuals in the studied Corine habitat), is a measurement of the probability that two randomly selected individuals in an area belong to a different species. The closer $1 - D$ is to one, the more diverse the habitat is. Shannon-Wiener index ($H' = - \sum p_i \times \ln p_i$), where p_i is the proportion of the i -th species in the studied habitat) was used as a measure of community heterogeneity (Krebs, 1989). Shannon evenness ($E' = S / \ln(H')$), where S is the total number of species calculates how individuals are distributed among species per habitat. Rarefaction based estimates were calculated using EcoSim (Gotelli & Entsminger, 2001) to estimate and compare the relative abundance and the density of mosquito species among environmental types, and thus test trapping scheme efficiency. The use of rarefaction allows comparison of the number of species in samples of different sizes as rarefaction simulates the expected number of species at a given number of individuals sampled, typically that of the smallest unit. In this paper, the sampling unit is the habitat type. It indicates how many species can be expected for a given sampling effort. Rarefaction curves generally grow rapidly at first, as the most common species are found, but level-off as only the rarest species remain to be sampled. Individual-based rarefaction curves were created in GraphPad Prism version 5.0 for Windows (GraphPad Software Inc.).

The Kruskal-Wallis one-way analysis of variance was used to test whether there is a difference in sampling results between seasons and years.

A canonical analysis (CA) was carried out to preliminary explore variation in assemblages of Culicidae between the defined habitat types. This analysis was performed using the CA package in R 2.13.0. (Greenacre & Nenadic, 2011). All species were included regardless of whether they are rare or common; absolute values were used as described by Jongman *et al.* (1995) and Legendre & Legendre (1998). Specimens belonging to the *Anopheles maculipennis* complex were included as such without taking the results of the PCR-RFLP into account, mainly due to the fact that not all individuals could be included in the molecular analysis.

Results

Based on the sampling strategy described above, 936 sites were randomly identified in three key habitats (urban, rural and natural); 97% of these were effectively sampled and mosquitoes were collected in 78% of the sites. Additionally, 45 importation risk areas were included in the inventory, of which 27 sites were located in natural areas (IRA, total of 37 traps) and 18 at industrial companies (IRA-industry, total 24 traps). Table 1 summarizes the actual number of sites sampled in each category.

Species diversity

A total of 26,533 individuals, morphologically belonging to 22 species and five traditionally recognized genera, were

collected and identified (table 2). Because individuals of *Ae. geminus* closely resemble those of *Aedes cinereus*, and identification can only be done with certainty through characters of the male genitalia, individuals were not further differentiated. The most species-rich genus in Belgium was *Aedes*, whereas *Coquillettidia* was only represented by one species. The five most abundant species were *Culex pipiens* (61.62%), *Coquillettidia richiardii* (15.43%), *Ae. cinereus* or *Ae. geminus* (5.04%), *An. claviger* (3.52%) and *Ae. vexans* (2.93%). The high abundance of *Cq. richiardii* is, however, due to one study site, a nature reserve in the harbour of Antwerp, where more than 3700 specimens of this species were collected. The species was caught in 38 study sites. Eighty individuals of *An. maculipennis* s.l. were collected, of which 67 were molecularly identified. Forty-three individuals belonged to *Anopheles maculipennis* s.s. (64.18%) and 24 to *An. messeae*. Both species were collected in higher numbers in the northern part of Belgium, in six sites they occurred sympatrically, and in 24 other sites either one of them occurred (seven sites positive for *An. messeae*, 17 for *An. maculipennis* s.s.). Positive ITS2 amplifications of *An. messeae* s.l. were sequenced but no clear separation between *An. messeae* and *An. daciae* could be observed. Combining morphological and molecular identification, a total of 23 species were collected during the inventory. Four species were collected in more than 100 study sites (table 2): *An. claviger* (18.52% of the sites), *An. plumbeus* (11.41%), *Culiseta annulata* (16.22%) and *Culex pipiens* (69.87%). Two exotic mosquito species were collected from two distinctive sites; *Ae. j. japonicus* from a used tyre trade near Namur and *Ae. koreicus* from a randomly selected site near Maasmechelen. All mosquito species were found in both years, although the abundance of these varied between years and seasons. Despite the fact that most species have a different phenology and are often characterized as 'early' or 'late' season species, the species composition was not significantly different between years and between seasons tested within each year (Kruskal-Wallis test, $df = 1$, $P > 0.05$).

Species abundances and richness

The taxonomic diversity differed among the natural, rural, urban and importation-risk environments (fig. 1a–d). Most species (21) were caught in the IRA sites (4% of the study sites) followed by rural (56% of the study sites), and natural environmental types (20% of the study sites) where 20 species were collected (table 1). Species diversity (H' + Simpson) and evenness (E'') were both higher in natural and IRA sites than in others (table 1). Individual based rarefaction curves suggest that, for equal sample size, expected species richness in rural environmental types is slightly lower than in other environmental types with higher species richness values (fig. 2). Moreover, the curve of the rural sites is still asymptotic, predicting an increment of species with higher sampling effort. Lowest species richness was observed in IRA-industry sites, and the rarefaction curve of this latter environmental site only reached a plateau at higher calculated abundances. According to this analysis, overall species accumulation is expected to increase with a higher sampling effort in this environmental type.

The CA ordination (fig. 3) shows an incipient separation between a cluster of urban/rural/IRA-industry sites, natural and IRA sites. Axis one explains 62% (eigenvalues=0.57) and axis two 26% (eigenvalues=0.24) of the observed variance. Four clusters can be distinguished: one related to the

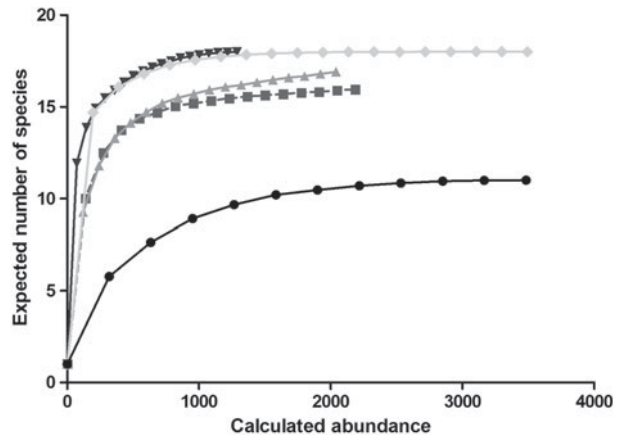


Fig. 2. Species accumulation curve using the rarefaction method where the expected species richness and its standard deviation are obtained by sampling individuals based on their corrected abundance per habitat. ■, Urban; ▲, Rural; ▼, Nature; ◆, IRA_nature; ●, IRA_industry.

urban/rural/IRA industry group with *Cx. pipiens*, *Ae. caspius*, *Ae. japonicus* and *Ae. vexans*, *Cs. annulata* and *An. maculipennis* and *An. claviger*. Secondly, a large group of species is distinguished that could not be allocated to a specific habitat type, including the now widely distributed *An. plumbeus*. A third group is discriminated, including *Cq. richiardii* and *Ae. detritus*, commonly found in IRA sites. And finally, a fourth cluster can be observed with forest species like *Ae. communis*, *Ae. geniculatus*, *Ae. punctor* and *Cs. morsitans*, as well as the exotic *Ae. koreicus*, are more readily found in sites characterized as natural.

Discussion

The structural biodiversity in Belgium was assessed through (i) morphological and molecular identification of the species, (ii) three different diversity indexes (Simpson richness, Shannon richness and evenness) and (iii) rarefaction analyses based on the pre-defined habitat types. The current inventory MODI RISK reported a total of 23 species (including two exotic species for Belgium) based on two years of intensive sampling in 971 sites scattered over Belgium. Rarefaction curves indicate that the expected number of species present was indeed sampled in almost all main environmental types. Thus, further sampling would probably not increase the observed number of species, indicating the sampling scheme worked well. Moreover, two exotic species were collected, one in a manually selected importation risk site (*Ae. j. japonicus* in Natoye, Namur) and one (*Ae. koreicus* in Maasmechelen, Limburg) from a randomly chosen site (Versteirt *et al.*, 2009, 2012), amply demonstrating the effectiveness of the sampling.

Prior to these outcomes, a checklist of Belgian Culicidae listing 24 species was published by Gosseries & Goddeeris (1991) based on historical data mainly collected between 1909 and 1958. In addition, Schaffner *et al.* (2001) listed a total of 26 species expected in Belgium based on literature data. Recent publications of Versteirt *et al.* (2009) and Dekoninck *et al.* (2011a) resulted in a total of 30 confirmed species. Eight species, recorded at least once in Belgium, were not found during the current study, namely *Ae. albopictus*, *Ae. dorsalis*,

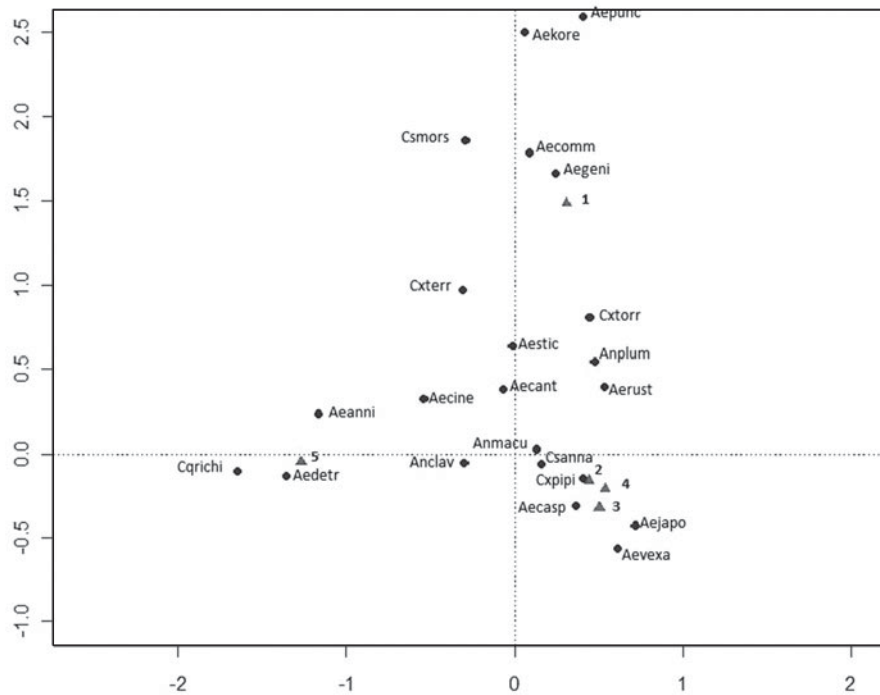


Fig. 3. Canonical analyses biplot of the mosquito fauna in Belgium with species located where they are most abundant. A total of four distinct clusters can be observed: natural sites (1) and species, IRA (5) sites and species, urban(2)/rural(3)/IRA-industry(4) sites and species and a group including species with a wider distribution range. Species abbreviations can be found in table 2.

Ae. flavescens, *An. atroparvus*, *Cx. hortensis*, *Cs. ochroptera*, *Cs. subochrea* and *Cs. fumipennis* (Gosseries & Goddeeris, 1991; Schaffner *et al.*, 2004). Missing species can be mainly linked to specific environmental types and mosquito feeding habits. *Aedes dorsalis*, *Ae. flavescens* and *An. atroparvus* are mainly associated to brackish environments, a habitat type that has become very scarce in Belgium since the destruction of brackish wetlands in the 1950s (Perillo *et al.*, 2009). Moreover, due to competition with the more ubiquitous species *An. messeae*, *An. atroparvus* has become very rare (Knottnerus, 2002). *Culex hortensis* is frequently found in the Mediterranean region, elsewhere it is rather rare and occurs only sporadically in detectable numbers (Becker *et al.*, 2010). *Culiseta ochroptera* and *Cs. subochrea* are very rare in western and central Europe and are, thus, only scarcely reported (Becker *et al.*, 2010). The absence of *Cs. fumipennis* during the inventory is difficult to explain since it is widely distributed throughout Europe. This species was, however, caught in 2010 during a revisit to some of the 971 sites (unpublished data). Pollution of its main habitat, i.e. open sunlit shallow temporary pools with rich vegetation or covered with duckweed (*Lemna* sp.) (Becker *et al.*, 2010), seems a likely explanation for this absence. *Aedes albopictus* was detected in 2000 in Vrasene (Schaffner *et al.*, 2004) in a company importing second-hand tires. The species was not found again during subsequent surveillance visits. It seems that it was naturally eliminated and up to date not reintroduced. Although trap choice could influence the number of specimens and even species caught, CO₂-baited traps are efficient in sampling most or all species present in a given area (Petric *et al.*, 1999; Hoel *et al.*, 2009; Coosemans *et al.*, 2011). Compared to Belgium, with 23 detected species during this inventory, 65 species are

present in France, 15 in Luxembourg, 27 in the Netherlands, 34 in the UK (Snow *et al.*, 1997; Schaffner, 1999; Schaffner *et al.*, 2001; Medlock & Vaux, 2009). However, it should be noted that, especially in France and the UK, a greater diversity in habitat types and eco-regions exists, offering a higher variety of suitable niches for mosquitoes with a different ecology.

Two members of the *An. maculipennis* complex were distinguished, namely *An. maculipennis* and *An. messeae*. In 2004, a new member of the *An. maculipennis* complex, viz. *An. daciae*, was described from Romania (Nicolosecu *et al.*, 2004) and was also reported from the UK (Linton *et al.*, 2005). *Anopheles daciae* was described as a different species of *An. messeae* based on egg and larval morphology and on their unique ITS2 sequence. However, none of the ITS2 sequences obtained in this study showed 100% similarity with *An. messeae* or *An. daciae* present in Genbank and BOLD. Moreover, intraspecific and intra-individual ITS2 variants in *An. messeae* was recently shown (Bezzhonova & Coryacheva, 2008), and they correspond with variants that were used to differentiate *An. messeae* and *An. daciae*. These findings indicate that the ITS2 cannot distinguish these two species.

Spatial biodiversity to assess the relationship between the pre-defined habitat types and the species occurrence was estimated by CA technique. The analysis shows a clear separation of the natural, IRA and urban/rural/IRA sites, with (mostly) typical species allocated to the different clusters. The presence of *Ae. caspius*, *Ae. vexans*, *An. maculipennis* and *An. claviger* in the urban/rural/IRA-industry group indicates, however, the fragmented landscape of Belgium, where urban and rural areas are connected to (semi-)natural sites. Currently, a worldwide trend to construct, restore and protect wetlands emerged which will create a suitable habitat in

Table 3. Overview of the found species during the MODIRISK inventory, with information on their vectorial capacity.

Species	Natural infection	Lab infection	Trophic behavior
<i>Aedes annulipes</i>	Tahyna, myxomatosis		Man, mammals
<i>Ae. cantans</i>	Tahyna, WNV, flavivirus, bunyavirus, myxomatosis		Man, mammals
<i>Ae. caspius</i>	WNV, Tahyna, Francisella tularensis, Tularemia, mixomatosis	<i>Dirofilaria immitis</i> & <i>D. repens</i>	Man, mammals
<i>Ae. cinereus/geminus</i>	Sindbis, Tahyna, Tularemia		
<i>Ae. communis</i>	Batai, Inkoo, Tularemia	Tahyna	Man, mammals, birds
<i>Ae. detritus</i>	<i>Dirofilaria repens</i> , myxomatosis		Man, mammals
<i>Ae. geniculatus</i>	Tularemia	WNV, YF	Man, mammals, birds, reptiles?
<i>Ae. japonicus</i>	JE, WNV	La Crosse, CHIK, DEN, EE, StLE, WNV	Man, mammals
<i>Ae. koreicus</i>	JE?, filariosis		Man, mammals
<i>Ae. punctor</i>	Inkoo, Tularemia	Tahyna, WNV	Man, mammals
<i>Ae. rusticus</i>	?		Man, mammals, birds
<i>Ae. sticticus</i>	Inkoo, Tahyna		anthropophilic
<i>Ae. vexans</i>	WEE, EEE, CE, Tahyna, Lednice, Tularemia	<i>Dirofilaria immitis</i>	Man, mammals, birds
<i>Anopheles claviger</i>	Batai?, malaria, Tularemia, <i>Setaria labiatopapillosa</i>	myxomatosis	Man, mammals
<i>An. maculipennis</i>	Batai, malaria		Zoophilic
<i>An. messae</i>	Tahyna, Batai, malaria		Zoophilic
<i>An. plumbeus</i>	Malaria	WNV, canine filariosis	Man, mammals, birds, reptiles
<i>Coquillettidia richiardii</i>	Batai, Tahyna, WNV		Man, mammals, birds, reptiles
<i>Culiseta annulata</i>	Tahyna, avian malaria	myxomatosis	Man, mammals, birds
<i>Cs fumipennis</i>			Birds, reptiles
<i>Cs morsitans</i>	Sindbis		Birds, sometimes man
<i>Culex pipiens</i>	WNV, Sindbis, Batai, avian malaria	Tahyna, <i>Dirofilaria immitis</i>	Man, mammals, birds
<i>Cx territans</i>			Batracophilic
<i>Cx torrentium</i>	Sindbis		Birds

WEE, Western Equine Encephalitis; EEE, Eastern Equine Encephalitis; CE, California Encephalitis group; WNV, West Nile Virus; JE, Japanese Encephalitis; StLE, Saint Louis Encephalitis; YF, Yellow Fever (adapted from Becker *et al.*, 2010).

which host, vectors and pathogens can come into contact, and they might also be prone to pathogen introductions (Dale & Knight, 2008). These areas, including wetlands, marshes and tidal zones, which were categorized here as IRA and often situated near urbanized areas (like harbors), pose new hazards for the introduction and establishment of vectors and pathogen, and increase the risk of transmission and outbreaks. The urban/rural/IRA-industry cluster seems to be characterized by two species, *Ae. caspius* and *Cx. pipiens*, that can cause biting nuisance and are potential vector species of West Nile virus, Tahyna virus, Sindbis virus and *Dirofilaria immitis* amongst others (Becker *et al.*, 2010). Species having a centralized position in the CA biplot are those with a more even distribution over the different environmental types. For example, *An. plumbeus* has recently adapted to man-made artificial breeding sites and is undergoing a highly expanding distribution in Belgium (Dekoninck *et al.*, 2011b).

The inventory done in the framework of the MODI RISK project was based on a random (statistical) approach. Such a sampling scheme has some drawbacks, such as the one week-one site trapping scheme, the use of one trapping device and entailing that areas or specific habitats may have been insufficiently sampled or sampled during the wrong season (e.g. *Cs. fumipennis* habitats). However, comparing the number of found and expected species, it can be concluded that this inventory approach was successful, emphasizing its utility. One of the major advantages is certainly the fact that no prior

knowledge was required and that there was no biased choice of sampling sites. Moreover, the entire country could be sampled over different years and periods. The acquired knowledge on species presence and distribution in Belgium can be used to develop mosquito species distribution models and to assess the actual countrywide transmission risk of mosquito-borne diseases, taking into account that several ecological, biological and socio-economic factors can interfere. Based upon their ecology and biting preference, several species present in Belgium are potential vectors of pathogens, such as West Nile virus, avian malaria, Usutu, Tahyna or Sindbis virus (Becker *et al.*, 2010) (see table 3). The simultaneous temporal and spatial presence of competent vector and hosts is the first condition for arboviral transmission; however, other factors, such as their distribution and the correct combination of environmental conditions, are essential for successful and persistent transmissions (Randolph & Rogers, 2010). Indeed, the most important epidemiological parameters for risk assessments include vector diversity, spatial and temporal variation in vector abundance and pathogen prevalence in the vector population (Braks *et al.*, 2011). This knowledge is, however, lacking in most European countries (Braks *et al.*, 2011). An initiative was started to overcome this information gap by bringing entomological and public health expertise together in a European network (VBORNET). In conclusion, the present study reports a country-wide mosquito inventory with emphasis on species

richness, distribution and occurrence in the main environmental types, which is a first step toward risk assessment of mosquito-borne diseases. To keep this inventory up to date will be challenging but is highly desirable given the potential risk of mosquito-borne diseases in Europe and of changing species distributions due to climate change.

Acknowledgements

We would like to thank all people involved during the sampling (inventory and validation), private owners, companies, conservators of nature reserves and other authorities (commune, region). Many thanks to Annemie Van Ranst, Bram Wellekens, Cleo Pandelaers, Rien Dekeyzer, Marijke Wouters, Stefan Kerkhof and Charlotte Sohler without whom the field work would not been done so smoothly. We especially want to express our gratitude to Patricia Roelants and Leen Denis for assistance in (and outside) the lab. We thank FS for the technical support and the quality control of the morphological identifications. This work was funded by the Belgian Science Policy Programs (Belspo, SD/BD/04A and SD/BD/04B).

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