INTRODUCTION

During the first half of the 20th century malaria was common in the coastal provinces of The Netherlands. For example, in Amsterdam more than 2400 cases of malaria were registered in 1946 (1). The most prevalent parasite was Plasmodium vivax, but at least during the first decade of the century P. malariae was also present. The disease was transmitted by the mosquito Anopheles atroparvus Van Thiel. A. atroparvus is a member of the A. maculipennis complex, which in The Netherlands further consists of A. messeae Falleroni and A. maculipennis s.s. Meigen (2, 3). Other anopheline species that have been recorded from The Netherlands are A. claviger and A. plumbeus. Of these, only A. plumbeus has been incriminated as a malaria vector in western Europe. A. claviger is a vector in the Mediterranean (4). In The Netherlands the disease was limited mostly to the coastal provinces because of the optimal breeding conditions for A. atroparvus in brackish waters (5). Further inland, in less brackish or freshwater systems, A. atroparvus could not develop into the high-density populations required for malaria transmission.

Apart from the uniqueness of the single vector, there was one more typical character associated with malaria transmission in The Netherlands: people became infected with the parasites by the bite of mosquitoes in the autumn (September–October) but developed the disease only 8–10 months later (May–July), unlike in the tropics where the incubation time of the disease is only 2 weeks (6). The onset of the disease in the early summer coincided with the emergence of the vector mosquitoes from hibernation. As is common with this type of P. vivax, many people were asymptomatic carriers and served as an infectious reservoir for the mosquitoes in September–October.

Immediately following World War II, anti-malaria activities were undertaken by indoor spraying with insecticides (DDT) and active surveillance and treatment of Plasmodium carriers with quinine. The last case of indigenous malaria in The Netherlands was reported in 1961 (1, 7).

During the second half of 20th century, major engineering works in The Netherlands caused the transition of many brack-
ish waters to freshwater systems. Also, the introduction of synthetic detergents caused heavy pollution of mosquito larval habitats. It is believed that both developments combined caused the near extinction of *A. atroparvus* from The Netherlands, and with it the continued risk of malaria transmission (8). However, during the last decades of the 20th century, anti-pollution measures and a strong interest in environmental conservation resulted in a strong improvement of the water quality. In addition, plans have been developed to restore some of the formerly brackish waters to their original state by allowing seawater to re-enter wetland areas (9, 10). Thus, the ecological conditions under which *A. atroparvus* once thrived, are expected to return. The question is, will this be followed by a recovery of *A. atroparvus* populations and, consequently, a return of malaria risk in The Netherlands?

The lower delta of the Rhine and Meuse rivers was malarious up to World War II (5) and malaria vectors were common throughout the delta (2). The present study was undertaken to investigate the current distribution of the malaria mosquito and its population dynamics in the delta of the Rhine and Meuse rivers in the southwestern part of The Netherlands, with particular emphasis on the ecology of the aquatic mosquito population. The results will be discussed considering the likelihood that occasional outbreaks of malaria might occur in The Netherlands as a result of adequate anopheline mosquito densities.

**DESCRIPTION OF THE STUDY AREA**

The study was conducted in the lower delta of the rivers Rhine and Meuse, south of Rotterdam. To the west, the study area is bordered by the North Sea and to the north and east by the rivers Noord, Beneden Merwede, Oude Maas and Nieuwe Waterweg. The rivers Nieuwe Merwede and Hollandsch Diep, Lake Volkerak and Lake Grevelingen form the southern limit (Fig. 1). The study area comprises 1500 km² and is intersected by many small and large streams, lakes and waterways. As much of the land is below sea level, ditches, and canals have been created to drain the area permanently by using pumping stations. The artificial drainage of the area causes an enhanced seepage of seawater into surface waters. The Nieuwe Waterweg, Oude Maas, Hollandsch Diep and Haringvliet are the most important waterways in the area, carrying the bulk of the water from the Rhine and the Meuse to the North Sea. In the study area, there were originally 3 open-sea arms: the Nieuwe Waterweg, the Haringvliet and the Grevelingen/Volkerak. To protect the south-west of The Netherlands from catastrophic coastal flooding, as occurred in 1953, the “Delta Plan” was made to provide for the closure of all tidal inlets in the study area, with the exception of the Nieuwe Waterweg. In 1970, the Haringvliet was closed off from the sea by means of a dam with outlet sluices. As a result, the Haringvliet turned into a freshwater river system with a strongly reduced tidal range (about 30 cm). In 1972, the Grevelingen was closed off from the sea and the Volkerak was embanked from the Grevelingen and Oosterschelde in 1965 and 1987, respectively. This resulted in a brackish/salty Lake Grevelingen and a freshwater Lake Volkerak. The coastal flatlands behind the dikes along the Haringvliet and Hollandsch Diep, which traditionally contained brackish to salty groundwater, gradually turned to freshwater areas. Indeed, the study area now contains several points where freshwater is being collected for household water supply.

The northern and eastern parts of the study area form part of the urban conglomerate of mainport Rotterdam and are densely populated as well as heavily industrialized. Rotterdam harbor is the world’s largest commercial port, with ships entering through the Nieuwe Waterweg. By contrast, the center and southern parts of the study area are remarkably rural and dominated by agricultural activities. The seashores are used for port activities (west of Rotterdam), tourism and/or nature reserves.

Three areas were selected for detailed studies of larval populations. These were Preekhil (5°29'50"E, 42°40'10"N), Brielle (7°02'82"E, 43°44'54"N) and Poortugaal (8°72'00"E, 43°29'50"N) (Fig. 1). The study site in Preekhil is a relatively undisturbed grassland polder adjacent to a nature reserve, far...
collected anopheline larvae were reared in small collection and sorted. In the laboratory, field-collec-
tion day. All larvae and pupae present were col-
lected and stored singly in 70% alcohol. According to the precipitin method (15). The head and thorax of the mosquitoes were kept for identification by PCR (see below).

**MATERIAL AND METHODS**

**Measurement of Chlorinity**

The electrolytic conductivity of larval habitats was measured with a conductivity reader (model WTW - LF196, Retsch, Ochten, The Netherlands). By calibration with solutions of known Cl contents, electrolytic values were transformed to Cl values. For the purpose of this study, we define water as 'fresh' when the Cl content is less than 300 mg L⁻¹. Brackish water has Cl contents ranging from 300–17 000 mg L⁻¹. Water containing > 17 000 mg Cl L⁻¹ is considered salty. The Cl contents of seawater are approximately 19 000 mg L⁻¹ (11).

**Measurement of Temperature, Rainfall and Relative Humidity**

The meteorological conditions during the study period were ob-
tained from the Royal Netherlands Meteorological Institute’s (KNMI) field station in Wilhelminadorp, approximately 40 km south of the study area. Cloud cover for the areas close to Rot-
tterdam was derived from the meteorological station at Rotter-
dam Airport. Water temperatures from all sample sites were measured continuously during the months of August and September to estimate the population density and develop-
ment. Using geographical information about the total water sur-
face area at each study site, the minimum and maximum number of larvae per m² and per km² of surface water were estimated.

**Mosquito Sampling**

Larval stages of mosquitoes were collected by dipping with a white soup ladle; one dip was calibrated to collect 135 cm² of surface water. The ladle was gently immersed until the edge was just under the water surface. After filling, it was quickly lifted out and inspected for the presence of mosquito larvae and/or pupae. Depen-
ding on the number of larvae collected, 50 or 100 dips were taken per site on each collection day. All larvae and pupae present were collected and sorted. In the laboratory, field-col-
lected anopheline larvae were reared in small pans until 4th larval instar or pupation and emerg-
ence of the adult mosquito (14). Larvae were fed Tetramin® fishfood. Adult mosquitoes were collected with aspirators from animal shelters. After morphological identification, blood meals were collected on filter paper and dried over silica gel. Bloodmeal identification was done according to the precipitin method (15). The species identification was done according to the precipitin method (15). The head and thorax of the mosquitoes were kept for identification by PCR (see below).

**Species Identification**

Mosquitoes were sorted to species or genus (A. maculipennis s.l.) based on larval or adult mor-
phological characteristics. Most larvae were reared to 4th instar and stored singly in 70% alcohol until molecular identification using a PCR technique (16). Some field collected adults of A. maculipennis s.l. were also identified by PCR. Ribosomal DNA was isolated from alcohol-stored larvae or dried adults by incubating an entire mosquito in 100 µl Bender buffer (0.1M NaCl, 0.1M Tris-
HCl pH 7.5, 0.05M EDTA pH 7.5–8, 0.5% SDS) at 65°C for 30 min, after which 50 µl of pre-chilled KAc was added, mixed well and placed on ice for a further 30 min. Following centrifugation for 5 min at 14 000 rpm, 200 µl of ethanol were added and DNA isolated by precipitation at −20°C for 30–60 min. The supernatant was then removed by pipette and the DNA dried under vacuum for 30 min at room temperature, before re-suspend-
ing in 50 µl water and storing at 4°C. Each DNA isolate was diluted by x10 for PCR. The PCR identification employed a Perkin Elmer 480 thermal cycler (Cetus Corp., USA) using the oligonucleotide primers and protocols described by Proft et al. (16). DNA isolates from A. atroparvus (Leiden strain, kept in the Wageningen Entomology laboratory), A. messeae and A. maculipennis s.s. (courtesy of J. Proft, University of Bonn, Ger-
many) were used as controls in each PCR procedure and electro-
phoresis.

**RESULTS**

The general survey for the presence of anopheline larvae in the study area was conducted in July 1999. Population studies of A. maculipennis in Preekhyl, Brielle and Poortugaal were done in August and September 1999.
Meteorological Conditions during the Study Period
In Figure 2 the minimum and maximum temperatures of the ambient air and of the water surface in Brielle are shown from 16 March to 14 September 1999. This period corresponds with the expected activity of adult *A. maculipennis* s.l. in The Netherlands (2). On average, at the surface, the temperature of the water was 2.5°C higher than that of the ambient air. Rainfall during the study period (March–September) was 451.6 mm, with peaks in March (95.4 mm), June (71.3 mm) and August (106.5 mm). In 1999, the months of July and September were relatively dry compared to historical averages. Mean temperatures in July and August were above the historical values, with mean temperatures of 19.1 and 18.3°C, respectively.

Current State of Freshwater and Brackish Waters
Analysis of 125 sites throughout the study area showed that the chlorinity of the waters varied from 100 to 14 838 mg Cl L⁻¹. Sites in direct and open connection with the North Sea and exposed to large tidal changes were salty. The water level at these sites varied with 100–175 cm between tides, depending on the distance from the open sea. This applies to sites along the Nieuwe Waterweg, Nieuwe Maas, and Oude Maas. Formerly salty and/or brackish areas, now closed off from the sea and exposed to continuous flushing with Rhine or Meuse waters, were fresh or slightly brackish. In several polders, ditches contained brackish water while the surrounding surface waters are fresh. This is due to seepage from salt deposits below the surface.

Distribution of Anopheline Larval Habitats and Estimated Densities of *Anopheles maculipennis* s.l.
Fourty-seven of the 125 sites sampled for anopheline larvae contained *A. maculipennis* s.l. (Fig. 3). In 4 of the 47 sites *A. claviger* was also present, jointly with *A. maculipennis* s.l. Larval densities varied from 0.01–4.40 per dip, equivalent to 7.4–325.93 lar...
vae per m$^2$ of water surface (Table 1). The estimated densities of larval populations in Preekhil, Brielle, and Poortugaal varied between 161 099–8 599 190; 311 136–9 126 040; and 121 525–1 619 783 per km$^2$, respectively. Larval densities were considerably higher in Brielle than in the other 2 areas but the mean proportion of 4$^\text{th}$ larval stages varied considerably between the 3 sites, from 30% in Preekhil, 5% in Brielle, and 10% in Poortugaal. With an estimated survival rate of 80% from fourth larval stage to adult, the daily production of adult mosquitoes per km$^2$ varied between 9722 in Poortugaal and 2 063 805 in Preekhil (Table 1), depending on inclusion of 30% of the water surface area, or of the entire water surface area. There was no correlation between larval density and chlorinity of the water (Fig. 4).

**Species Composition and Blood Hosts**

Morphological identification revealed the presence of *A. maculipennis* s.l. and *A. claviger*. 150 larvae of *A. maculipennis* s.l. from 21 sites were identified to species using the PCR technique. Four specimens (2.7%) were *A. atroparvus*, the others *A. messeae*. *A. atroparvus* was found in 3 different sites (Albrandswaard, Beninger Slikken, and Preekhil) (Fig. 1). *A. maculipennis* s.l. that were collected as adult mosquitoes in animal shelters, were identified as *A. messeae*. All females appeared to have recently blood fed, and analyses of 30 blood meals showed that 64% originated from cattle, 28% from horse and 8% from sheep.

**Population Dynamics of Anopheles maculipennis s.l.**

The distribution of larval stages in the 3 sentinel sites from late July until mid-September 1999 is shown in Figure 5. These data are based on the sum of 100 dips per site per sampling day. In some cases they reflect the mean of 2 successive days. In Preekhil the number of larvae reached a peak in mid-August. In contrast, in Brielle the highest larval density was observed on 16$\text{th}$ July (440 larvae per 100 dips, data not shown), and from 29$\text{th}$ July larval densities remained stable until early September, after which date the numbers decreased mildly. After an initially high number of larvae in early August in Poortugaal, the density remained low during the rest of August, to rise again in early September. On 20 September, no larvae were found anymore in the three study sites.

All larval stages and pupae were present at most times and there was no clear trend related to cohorts of eggs deposited simultaneously.

**Vegetation Type and Presence of Anopheles maculipennis s.l.**

There was a wide variation of floristic and geomorphological characteristics within the sites where larvae of *A. maculipennis* s.l. were found. The most commonly found plant species in sites with a prevalence of *A. maculipennis* s.l. were *Phragmites australis* and *Enteromorpha* sp. followed by *Ceratophyllum demersum* and *Elodea nutalii*. *A. maculipennis* s.l. was also found in ditches overgrown with dense communities of only *Phragmitis australis*.

**DISCUSSION**

The results show that *A. maculipennis* s.l. is widely distributed in South Holland. All aquatic sites, from ditches and shallow pools in polders to creeks, canals and small lakes on former tidal areas along the Haringvliet may contain larvae of *A. maculipennis* s.l. We did not observe a greater propensity for larvae in rural areas than in areas adjacent to residential sites, such as the town of Brielle. Also, larvae were found in canals and ponds within towns. There was a high propensity for larvae to be present on top of floating vegetation such as *Enteromorpha* and *Ceratophyllum* spp. This is in agreement with previous findings in this area by Van der Torren (2). Surprisingly, we also found many larvae in ditches over-
grown with common reed. To our knowledge, the latter ecosystem has not previously been described as a habitat of *A. maculipennis* s.l. Mud flats that are exposed to tidal fluctuations and which are bordering a fast-flowing river like the Oude Maas (e.g. the nature reserve near Rhoon), cannot be considered larval habitats of this species even though these areas are often densely overgrown with reeds. Even small pools on elevated areas on these mud flats did not contain mosquito larvae. We suggest that these tidal areas are too turbulent for mosquito eggs or larvae, as these are likely to be washed into the sea during infrequent flooding.

As only 2.7% of our larval collections consisted of *A. atroparvus*, we consider most specimens found to be *A. messeae* and the discussion will mostly concern this member of the *A. maculipennis* complex. We found only one published reference on the density and distribution of *A. messeae* in south Holland (2) and, based on this publication and our own observations, we suggest that the current distribution of this species is not very different from that in 1935. Larval densities were also not much different from the 1935 data, and it is possible that *A. messeae* has not undergone a marked ecological change since Van der Torren’s study (2). We found the species in many varied habitats, from dominantly reeds (*Phragmites australis*) to floating weeds and algae, to relatively open ditches inside a forest, to clear water in a small lake in the dunes. These latter findings are different from Van der Torren (2), who mentioned that the species is limited to wide ditches with a dense mass of floating vegetation. The near absence of *A. atroparvus* from the study area was surprising, as 60 years ago this species was the most abundant one found on the island of Goeree-Overflakkee. At that time, *A. messeae* was rare on this island. Similarly, *A. atroparvus* was also the most common anopheline of Voorme-Putten island. Indeed, some inhabitants of a retirement home in Rockanje village contracted malaria (*P. vivax*) in 1950 (17), which is further evidence of the historical presence of *A. atroparvus* in this area. The present distribution data of *A. maculipennis* s.l. in the study area suggests that *A. atroparvus* has either lost most of its former habitat to *A. messeae*, or that the former species has become nearly extinct from the area, whereas the latter is still present in its former densities. Our larval collections and subsequent estimates of the population density in Preekhil, Brielle, and Poortugaal, suggest that *A. messeae* does not reach nuisance levels. The species rarely bites man during the summer (5, 18), goes into complete diapause during the fall and winter, and seems to be able to survive on wildlife and domestic grazers. These changes become evident by comparing the 1935 distribution map with that of 1999 (Fig. 6) from which it is apparent that the geographic distribution of *A. atroparvus* has diminished markedly compared to its former distribution. The reasons for the near extinction of *A. atroparvus* may have been the change of the water salinity, pollution of the water by detergents (8) or lack of suitable feeding and resting sites during the winter or possibly all these factors combined. Today, few farmhouses are attached to livestock stables, and the stables are of a much different construction than before, having few dark, sheltered sites where mosquitoes can hibernate.

The results of the larval population studies show that larval densities can increase rapidly during July and August, when the adult mosquito population is in its 3rd generation after the winter diapause. Thus, by mid-July a population of adult mosquitoes has developed and produces large numbers of eggs, which are being deposited in ditches of varying floristic composition. Inspection of half-open livestock sheds in the study area in August 1999 revealed many blood-fed *A. maculipennis* s.l., which were all identified as *A. messeae*. None of the blood meals originated from humans, but were from domestic animals, which corroborates Swellengrebel and De Buck’s (5) statement that *A. messeae* is strongly zoophilic and does not feed on humans.

The differences in larval population dynamics between the 3 sentinel sites probably reflect the heterogeneous distribution of the adult mosquitoes, which determines the number of eggs deposited in each site. The presence of first instar larvae as late as 11 September does not necessarily reflect a change in the onset of diapause by day length (2, 19), which is 15 August. Females emerging after that date no longer become reproductively active in that year. However, female mosquitoes that have initiated oogenesis before the time of critical day length may continue producing eggs until death and first instars found in September are likely the offspring of females that emerged in early August. The large variations in larval densities are probably caused by the environmental differences between the study sites and affected by such variables as vegetation, water quality (12), exposure to direct sunlight and availability of blood hosts. We included the total water surface area as potential larval sites, but this seems unlikely to be the case, and more often around 0.3 of the water surface will reflect the real situation. As a consequence,
the maximum number of emerging adult mosquitoes km$^{-2}$ day$^{-1}$ cannot exceed 619 142 (Table 1). These numbers could readily find blood on the domestic herds of animals grazing in the study areas. However, as only a small proportion of $A.\$ messeae$ will survive the winter diapause (5), mosquito populations must develop from relatively few individuals each year.

Although $A.\$ atroparvus$ and $A.\$ messeae$ were reported as equally susceptible to $Plasmodium$ vivax$^{(20)}$, the high degree of zoophilily coupled with the mostly outdoor feeding behavior during the summer, makes the likelihood for local malaria transmission by $A.\$ messeae$ remote. In The Netherlands, therefore, $A.\$ messeae$ has never been considered a malaria vector and indeed Swellengrebel and De Buck state that the presence of malaria transmission in this country was entirely caused by the blood-feeding and resting behavior of $A.\$ atroparvus$ in the fall and winter (5). Elsewhere in Europe (Sweden, Eastern Europe) $A.\$ messeae$ has been incriminated as a malaria vector (4, 21). In Germany, coastal malaria was associated with $A.\$ atroparvus$, and inland malaria with $A.\$ messeae$ (22). Because the latter reports lack data on the actual infection in the mosquito vectors, it remains difficult to explain why $A.\$ messeae$ would have been a vector in Germany, Sweden, and Eastern Europe and not in The Netherlands.

The study shows that in the 65 years since the last ecological survey of anopheine mosquitoes in the province of South Hol-}

References and Notes


12. Jacobs, A.F.G., Jetten, T.H., Lucassen, D.C., Heusinkveld, B.G. and Nieveen, J.P. 1997. Ecological and behavioral studies on $A.\$ messeae$ and $A.\$ atroparvus$ in The Netherlands, a profound ecological change has occurred, causing the near extinction of $A.\$ atroparvus$ in the study area. Since this species is the only mosquito that has been incriminated with historical malaria transmission in The Netherlands, there is no risk for the return of malaria to an endemic state, as has frequently been suggested (23, 24). Although chlorinity levels throughout the study area were reduced compared to historical (1935) levels, they were still sufficiently high to support populations of $A.\$ atroparvus$, should they have been there (2) and this was probably not the reason for the strong reduction of $A.\$ atroparvus$

We suggest that the radical change in opportunities for seeking shelter and blood meals during the fall and winter is the major cause for the recorded low densities of $A.\$ atroparvus$, preventing a population explosion as was common in the pre-World War years in many of the coastal areas of Western Europe. Therefore, it is not expected that the proposed restoration of formerly brackish areas, by re-opening former connections to the North Sea, will lead to a population increase in this malaria vector. $A.\$ messeae$ is still widely distributed in the study area, but this species is unlikely to be a malaria vector because in The Netherlands it rarely feeds on man and, unlike $A.\$ atroparvus$, does not blood feed during hibernation. In addition, the natural densities of $A.\$ messeae$ are too low to permit transmission of the malaria parasite even were mosquitoes to become infected (Takken and Jetten, unpubl. data).

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8. Mühling, W. 1966. De zoögeografische verspreiding van $A.\$ atroparvus en $A.\$ messeae in}