J. Hyg., Camb. (1977), **78**, 175
Printed in Great Britain

The germicidal effect of the open air in different parts of The Netherlands

By G. de Mik and Ida de Groot

Medical Biological Laboratory TNO, 139 Lange Kleiweg, Rijswijk 2100, The Netherlands

(*Received 4 August* 1976)

SUMMARY

Using the microthread technique the survival of *Escherichia coli* MRE 162 in open air was measured in different parts of The Netherlands.

The presence of bactericidal compounds (open air factor = OAF) could be demonstrated on several days and quantitated in relative units of OAF concentration.

In the absence of ozone the OAF concentration was always low. In the presence of ozone the OAF concentration was dependent on wind direction. At the selected microthread exposure sites air from areas with high traffic intensity contributed more to OAF production than air from industrial areas. OAF production is probably related to the nature of hydrocarbons in the air.

INTRODUCTION

In 1968 May & Druett described a technique (the 'microthread' technique) which made it possible to subject micro-organisms to any gaseous environment of interest for extended periods of time, during which their loss of viability might be assayed. In this procedure airborne micro-organisms are held on ultrafine spider threads, to provide a 'captive' aerosol.

Using this technique it was demonstrated that the survival rate of *Escherichia coli* 162 exposed to open air is very low on certain days, in contrast with the survival in 'clean' air at the same relative humidity and temperature (Druett & Packman, 1968).

Not only *Escherichia coli*, but also *Serratia marcescens*, *Franciscella tularensis*, *Brucella suis*, *Staphylococcus epidermidis*, and a group C *Streptococcus* appeared sensitive to open air (May, Druett & Packman, 1969). It was concluded from these observations that there is a factor existing in the open air, which is toxic to *E. coli* and other organisms, and which was called the 'open air factor' (OAF).

The nature of the OAF proved difficult to identify. There was no correlation between the amount of OAF present as measured by the *E. coli* decay rate and concentrations of atmospheric ozone, sulphur dioxide, oxides of nitrogen, formaldehyde or air ions (Druett & May, 1968). A surprising experimental finding was the rapidity with which OAF disappeared in any form of enclosure, the rate of decomposition being dependent on the chemical nature of the surface of the

container. These data suggested that OAF is a metastable compound and is only stable in free air. These properties of OAF agreed with those of the class B phytotoxicants, components of photochemical smog. In laboratory experiments the bactericidal properties of air containing a mixture of olefin vapour and ozone could indeed be demonstrated (Dark & Nash, 1970). Since the concentrations of known bactericidal products in the atmosphere do not cause rapid viable decay, it is strongly suspected that OAF is a gaseous product formed by a reaction between ozone and olefin.

Olefins are present in the atmosphere in various concentrations dependent on the geographic and environmental conditions. In The Netherlands the most important sources are traffic and (petro)chemical industries.

Tests carried out in England clearly showed the association between urban areas and the toxicity of air for the test micro-organism. The high decay downwind of an oil refinery supported the suggestion that olefins may be the principal contributors to the formation of OAF in England (Harper, 1973).

Ozone is a natural constituent of clean air at high altitudes. It is brought down by turbulence and mixes with air in the lower atmosphere giving concentrations up to 100 $\mu g/m^3$ (5 parts/10^8). It may also be generated by photochemical smog giving concentrations up to 1000 $\mu g/m^3$ as reported for Los Angeles.

Ozone determinations in outdoor air indicate that smog-forming conditions occur in the western part of The Netherlands (Guicherit, Jeltes & Lindqvist, 1972). In 1969 and 1970 there were 25 days on which ozone values of more than 200 $\mu g/m^3$ were recorded. Further indications for smog formation were obtained from measurements of nitrogen oxides, hydrocarbons, and aldehydes.

In view of these findings it could be expected that OAF activity could also be detected in The Netherlands, especially in the western part with its large industrial complexes such as oil refineries and chemical plants. In order to see if there is any OAF-activity detectable in this country and if this activity correlates with any of the atmospheric pollutants which are normally recorded we have measured, using the microthread technique, the survival of *Escherichia coli* in air in various parts of The Netherlands.

MATERIALS AND METHODS

Growth and storage of test organisms.

The test strain *Escherichia coli* (MRE no. 162) was obtained from the Microbiological Research Establishment, Porton, G.B. The strain is kept in plastic capillary tubes in liquid nitrogen. Cultures are prepared by inoculating 100 ml cooked meat medium with the contents of one capillary. After 5 h shaking at 37 °C, 1 ml of the broth is inoculated into 200 ml tryptone medium. The culture is incubated at 37 °C under shaking for 18 h and then contains approximately 4×10^9 cells per ml. When kept at 4 °C this culture can be used during a period of about 1 month without significant changes in survival characteristics.

Bacillus globigii (syn. *B. subtilis* var. *niger*), which is used as 'tracer organism' (see below), is grown in peptone water. After 3 days incubation at 37 °C under shaking the suspension is kept at room temperature for 7–9 days, during which

period vegetative cells will autolyse. The spores are washed 5 times and resuspended in distilled water. The spore suspension, the titre of which is approximately 5×10^9 spores per ml, can be preserved for several months at 4 °C. Before use the suspension is heated at 65 °C for 10 min in order to kill any remaining vegetative cells.

Spray-suspension

This is prepared by diluting and mixing samples of both stock suspensions in such a way as to give a final concentration of 1×10^9 cells per ml of each organism in a medium consisting of equal parts of spent *E. coli* culture fluid (tryptone) and distilled water.

The microthread technique

The procedures described by May & Druett (1968) were closely followed. For loading the microthreads with micro-organisms a modified Henderson apparatus was constructed and operated as described by Druett (1969). The main part of this system is a stainless-steel tube called 'the sow' which contains 20 holders with metal frames supporting the spider threads. For sampling the frames are removed from the sow.

Frame loading and sampling

At the exposure site the modified Henderson apparatus is installed in the open air to ensure that the equipment is at nearly ambient temperature. The relative humidity (r.h.) in the apparatus is adjusted to give the same value as the r.h. expected in the field during the exposure period. In this way conditions during loading do not differ greatly from those likely to be met during subesquent exposure in the field.

Aerosols are produced by a 3-jet Collison nebulizer (May, 1973) containing 20 ml of the spray suspension, which produces 8 l of aerosol per min. Before and after aerosolization the suspension is titrated in duplicate. The frames are loaded by a 30 s exposure to the aerosol; the aerosol is passed through the sow with a linear velocity of 0·185 m/s. The load of *E. coli* and *B. globigii* spores per frame is about 20000–30000 of each. After the 30 s period of loading clean preconditioned air is drawn through the sow for 1 min in order to remove all free organisms; the sow is then closed at both ends.

Samples are obtained by shaking each frame separately in a sampling cell containing 7 ml peptone water or phosphate buffer manucol sucrose solution (PMBS). The individual frames are assessed at frequent intervals during the exposure period. Samples are kept in an ice bath until plating in 7-fold on tryptone agar in suitable dilutions so as to yield 100–200 colonies per plate. Colony counts of *E. coli* and *B. globigii* are made on the same plate.

Exposure of the organisms to the open air

For exposure to outdoor air the 5–10 loaded frames are transferred from the sow to a 'roundabout' (Druett & May, 1969) with a circular plastic wall. The position

Fig. 1. Location of the microthread exposure sites in The Netherlands.

of the microthreads is 1 m above ground level. At least two control frames are kept in the closed sow at the same temperature and r.h. during the experiment.

Three exposure sites are located in the western part of The Netherlands, Delft, Vlaardingen and Hellevoetsluis, and the fourth, Soesterberg, is located in the central area of the country (Fig. 1). These sites were selected for two reasons. First, to be able to utilize the air pollution data, which are continuously recorded at certain stations, the exposures of *E. coli* were carried out near such sampling stations, except for Soesterberg. Secondly, for studying OAF activity, the western part of the country where the highest degrees of air pollution occur, is the most interesting one. Delft is situated along the busy highway between Rotterdam and The Hague. Vlaardingen is situated at the Nieuwe Waterweg with its harbours and large industrial complexes such as oil refineries and chemical plants. Hellevoetsluis is situated at the coast, the degree of pollution there being strongly dependent on wind direction. Soesterberg was chosen to study the activity of open air in an area without large industries.

Evaluation of the survival and presentation of the results

The ratio between the numbers of *E. coli* and *B. globigii* spores in the spray suspension is taken as a 100 % value of viability (average of titres measured before and after nebulization).

On several occasions a linear relation can be found between the values of the logarithm of viability (S) and exposure time (t). Only in these cases an inactivation rate k (May *et al.* 1969) can be calculated according to the formula:

$$S_t = S_o\, e^{-kt} \quad \text{or} \quad k = \frac{\ln S_o - \ln S_t}{t},$$

The survival percentage, the inactivation rate, the linear regression and the test on linearity (Hald, 1952) were calculated with a digital computer PDP-8/I.

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

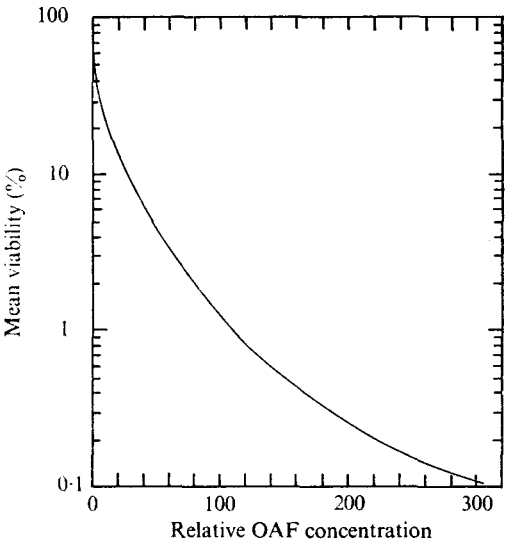


Fig. 2. Theoretical relation between 'Mean Viability' and relative OAF concentration.

However, 57 % of the measurements did not meet the requirements of the test. According to Harper (1973), in these cases the result of a series of exposures can be conveniently expressed by the 'mean viability'. This is the arithmetic mean of the percentage survival after 30, 60 and 120 min exposure.

Calculation of the OAF concentration

Cox, Hood & Baxter (1973), using a mathematical model for the toxic working mechanism of OAF (in analogy with that of oxygen), developed an equation by which, at a given OAF concentration, the percentage survival of *E. coli* in the open air could be expressed as a function of the exposure time *t*. Using this equation, in which a value of 10 was arbitrarily assigned to the OAF concentration, a typical OAF-induced decay curve for *E. coli* was analysed, and in this way the values for the various constants included in the formula could be defined. From the formula thus obtained a family of decay curves for other values of OAF concentration was calculated, and by comparing these with a number of experimental decay curves a relative value of OAF concentration could be assigned to the latter. Cox *et al.* (1973) were able to show that this method applied in practice, thus allowing a direct comparison of the relative OAF concentrations.

Using the formula and the constants given by Cox *et al.* we calculated the 'mean viability' values of theoretical *E. coli* decay curves at various relative OAF concentrations. The results are shown in Fig. 2.

From our experimental decay curves the 'mean viability' values were also calculated, and by comparing these figures with those in Fig. 2 relative OAF concentrations could be assigned to the experimental curves, thus translating a curve into a single parameter.

Table 1. *Survival percentages, inactivation rate, and 'mean viability' of* E. coli 162 *on microthreads in the closed sow at various RH values at* 22 °C

r.h. (%)	Survivals (%) Exposure time (min) 5	30	60	90	120	240	Inactivation rate (min^{-1})	Mean viability (%)
50	100	100	90	90	90	90	0·0001	93·3
60	98	90	82	77	75	73	0·0010	82·3
68	98	87	84	80	77	66	0·0014	82·7
75	100	90	60	74	64	40	0·0037	71·3
79	96	91	85	80	77	—	0·0022	84·3
96	100	98	95	94	91	—	0·0009	94·7

Data of air pollutant concentrations

Concentrations of ozone (O_3), sulphur dioxide (SO_2), nitric oxide (NO), nitrogen dioxide (NO_2), acetylene (C_2H_2), ethylene (C_2H_4), propene (C_3H_6) and propane (C_3H_8) are continually recorded by the Atmospheric Pollution Division of the TNO Research Institute for Environmental Hygiene, to study smog formation.

Media

Cooked-meat medium was prepared by dissolving Oxoid cooked meat medium (no. CM 82) in distilled water (1 tablet per 10 ml, pH 7·6).

Tryptone medium contains 20 g tryptone (Oxoid), 3 g NaCl, 5 ml 1 M-K_2HPO_4, 1 ml 1 M ferric citrate, 4 ml 1 M-$MgSO_4$ and 1 ml 1 M-$CaCl_2$ per l. The pH is adjusted to 7·2 with H_2SO_4.

Peptone water contains 1% peptone (George D. Gurr, London) in tap water, pH 6·2–6·4.

Tryptone agar contains 1% tryptone (Difco), 0·5% NaCl and 1·5% Agar (Difco). After sterilization glucose is added to a concentration of 1%.

Phosphate buffer manucol sucrose solution (PBMS) contains 4·5 g KH_2PO_4, 0·5 g $(NH_4)_2SO_4$, 0·5 g NH_4Cl, 2·5 g sodium alginate and 342 g sucrose per l, pH 7·6.

RESULTS

Effect of collecting fluid

For collecting the exposed organisms peptone water was used in all experiments. In order to compare the results with those published by the British investigators (May & Druett, 1968; Druett & Packman, 1968), in a number of experiments the survival percentages in the closed sow as well as in the open air were measured by sampling both in PBMS and in peptone water. The survival percentages measured with these collecting fluids were highly correlated ($r = 0·966$ with 95% confidence limits of 0·920 and 0·986). Moreover, the paired t test showed no significant difference, $t = 0·77$, $f = 22$, $P_2 = 0·45$.

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

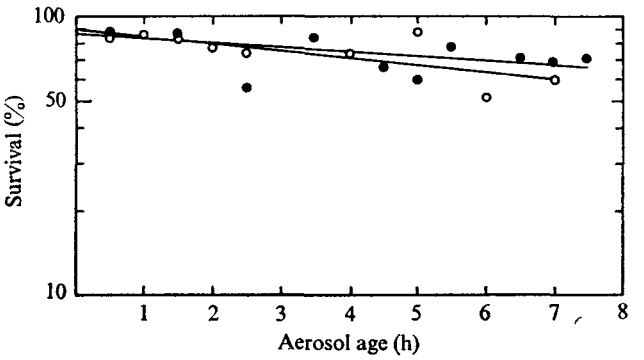


Fig. 3. Survival of *E. coli* MRE 162 on microthreads in the closed sow. ●—●, 22 °C and 96% r.h.; ○——○, 22 °C and 50% r.h.

Survival of E. coli 162 *in the closed sow*

Table 1 presents *E. coli* survivals measured in a separate series of laboratory experiments at 22 °C to various relative humidities between 50 and 100% in the closed sow.

The inactivation rates in the r.h. range between 50 and 96% do not show a striking variation; at least 60% of the *E. coli* remained viable in enclosed air for 2 h.

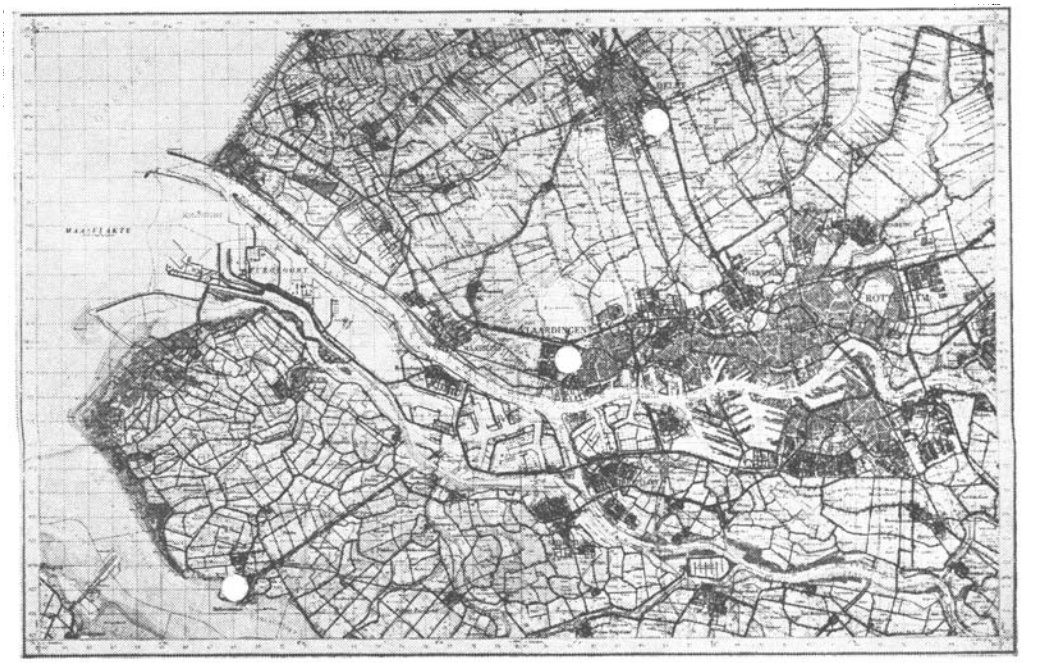
The survival in two other experiments at 22 °C in the closed sow (at 50 and 96% r.h. respectively) during a 7 h observation period was also similar (Fig. 3).

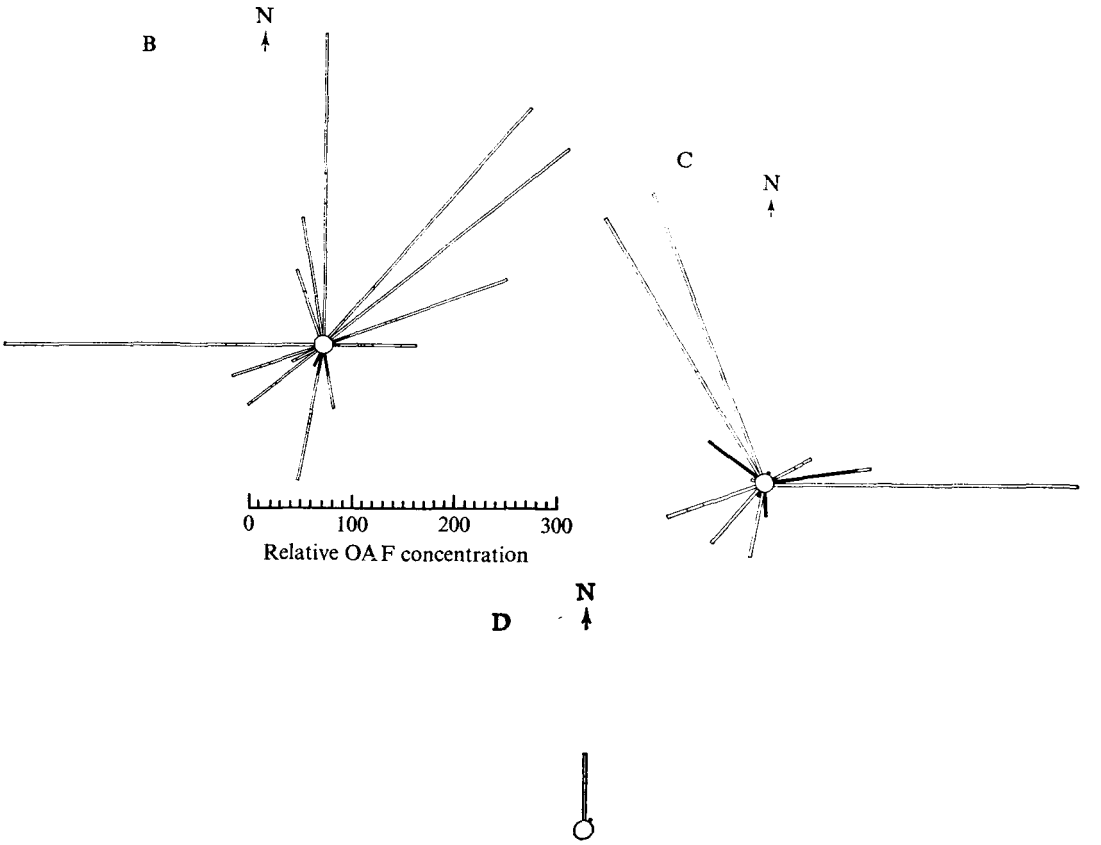
In the field experiments the relative humidity range was 45–100% at ambient temperatures of 4–25 °C. During the period of exposure of microthreads to open air, control microthreads were left in the closed sow at the same temperature and r.h. When the inactivation rate of these control frames exceeded 0·0040 min^{-1}, the results of the experiment were not used in this study.

Survival of E. coli 162 *in the open air*

The inactivation rates measured at the different exposure sites were translated into OAF concentrations with reference to Fig. 2. In Figs. 4 and 5 these concentrations have been represented by bars pointing in the direction from which the wind was blowing during the experiment. This wind direction was obtained from the nearest weather station. Open columns represent measurements at times when ozone could be detected and black columns refer to times when ozone was not detectable ($< 5\ \mu g/m^3$). At a given wind direction all viabilities measured in the absence of ozone were higher than in presence of ozone. The relative OAF concentrations were always low in the absence of ozone.

There is a marked difference in the viability values measured at Soesterberg and those at Delft and Vlaardingen. Soesterberg is located between Utrecht and Amersfoort near a busy arterial road connecting these towns. In this region, during the presence of ozone OAF concentrations are high and viabilities are very low for wind directions SW or NE, bringing urban air, but OAF concentrations are very low





Figures 4A, B, C, D. For legends see facing page.

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

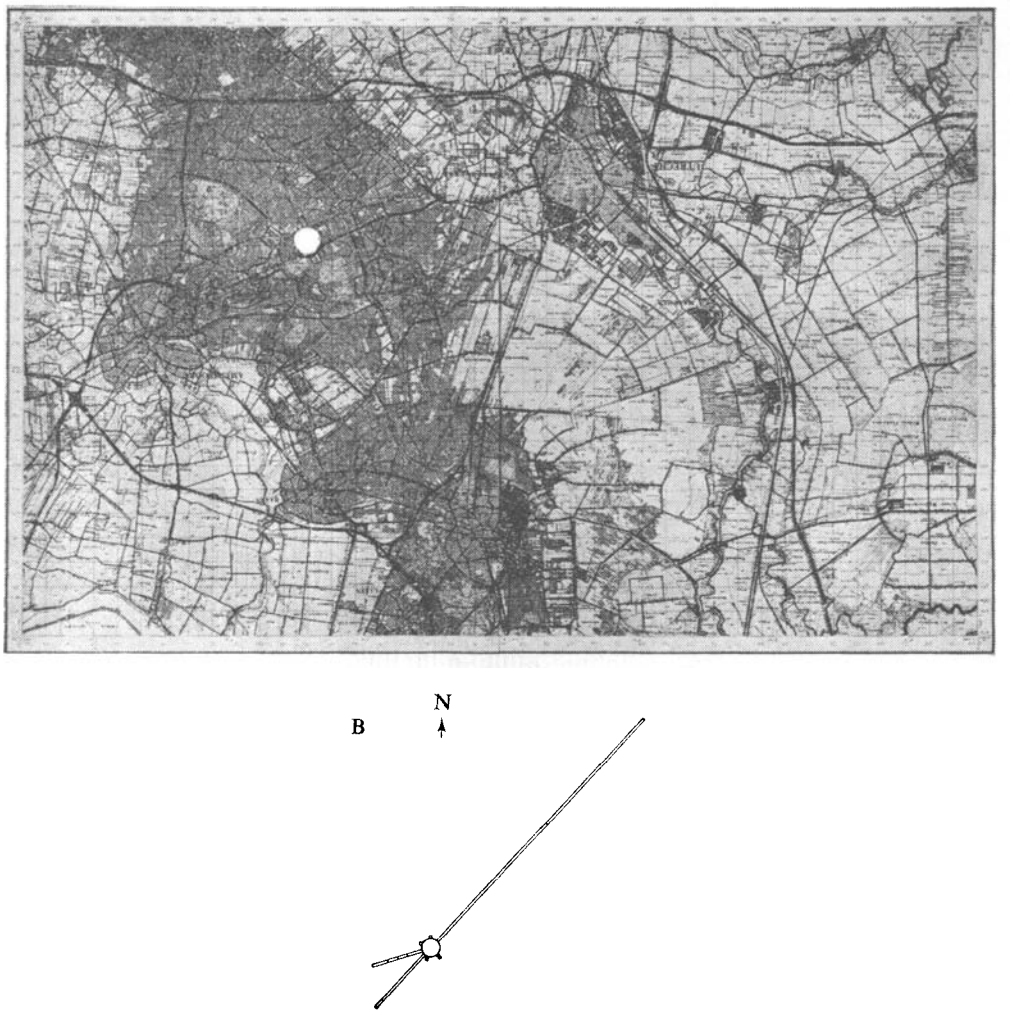


Fig. 5(A) Topographic map showing the location of the microthread exposure site at Soesterberg. (B) The dependence of the OAF concentration on wind direction at Soesterberg. For legends see Fig. 4(B).

Fig. 4(A) Topographic map showing the location of the microthread exposure sites in the western part of The Netherlands (Delft, Vlaardingen and Hellevoetsluis).

(B) The dependence of the OAF concentration on wind direction at Delft. The black columns represent OAF concentrations measured at times when the ozone concentration was below 6 $\mu g/m^3$. Columns have been drawn in the direction from which the wind was blowing. Several experiments for the same wind direction on various days (with various ozone concentrations) gave columns of different length. These are indicated by the transverse lines in the white columns.

(C) The dependence of the OAF concentration on wind direction at Vlaardingen. For legends see (B).

(D) The dependence of the OAF concentration on wind direction at Hellevoetsluis. For legends see (B).

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

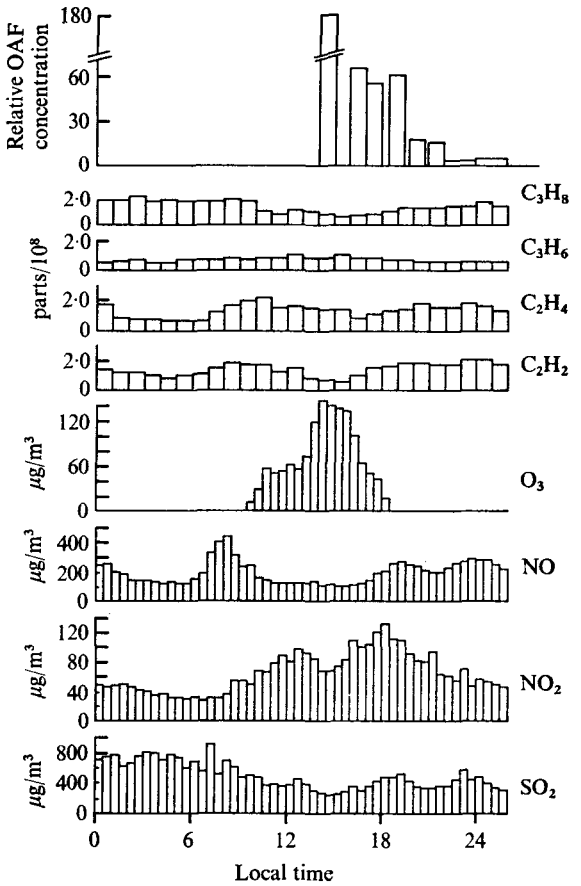


Fig. 6. Hourly and half-hourly average concentrations of various components of air pollution compared with the 'relative OAF concentration' measured at Delft from 14 h on 22 September till 2 o'clock on 23 September 1971.

for all other wind directions bringing rural air. The relative OAF concentrations (Fig. 5) are highest for NE wind.

At Delft and Vlaardingen viabilities comparable with those in clean air are only found when ozone is absent, In the presence of ozone viabilities are low, and relative OAF concentrations high for nearly all wind directions.

For these towns correlation coefficients were calculated between the relative OAF concentration and the ozone concentration at the exposure site. With all wind directions, the correlation coefficient is 0·73 (N = 90). When the results are split up in such a way as to refer to the wind coming either over cities and highways (urban area) or over the industrial area near Rotterdam, then the correlation coefficients are 0·83 (N = 52) and 0·56 (N = 38) respectively, values which differ significantly.

On certain days the survival of *E. coli* 162 was measured continuously during a number of hours. The results obtained in Delft on 22 and 23 September and 1 October 1971 are given in Figs. 6 and 7 together with the hourly or half-hourly averages of the concentration of different pollutants. Fig. 6 shows that the for-

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

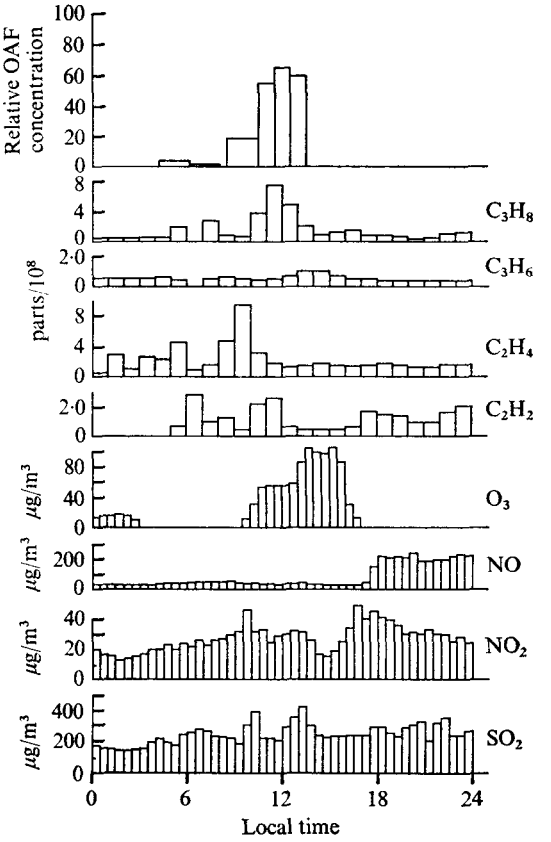


Fig. 7. Hourly and half-hourly average concentrations of various components of air pollution compared with the 'relative OAF concentration' measured at Delft from 4 o'clock till 14 h on 1 October 1971.

Table 2. *Correlation coefficients with* 95 % *confidence limits of OAF concentration and concentrations of different air pollutants measured at Delft* (N = 14)

	r	95 % limits	
OAF/O_3	0·90	0·97	0·72
OAF/NO	−0·17	0·39	−0·64
OAF/NO_2	0·24	0·68	−0·33
OAF/SO_2	−0·13	0·43	−0·62
OAF/C_3H_8	0·07	0·60	−0·50
OAF/C_3H_6	0·61	0·86	0·11
OAF/C_2H_4	−0·27	0·30	−0·70
OAF/C_2H_2	−0·34	0·23	−0·74

mation of OAF decreases in the evening after ozone has disappeared. Fig. 7 shows that the relative OAF concentration is very low early in the morning but increases about nine o'clock when smog formation starts, as a result of which ozone begins to appear.

A comparison of the OAF concentration with the concentration of the different pollutants yielded results which are given in Table 2.

https://doi.org/10.1017/S0022172400056072 Published online by Cambridge University Press

DISCUSSION

In order to compare viable decay rates as measured in the present study with those reported by the British investigators (Druett & Packman, 1968; Druett & May, 1968) the *E. coli* strain MRE 162 was used throughout our experiments and the experimental procedures described by May & Druett (1968) were closely followed. The only difference was the use of peptone water as a collecting medium in our experiments which was shown to have no influence on the results. The decay rates measured in clean air at varying relative humidities did not exceed 0·0037 min^{-1}; this is in close agreement with the results reported before (May & Druett, 1968; Druett & May, 1968; Benbough & Hood, 1971).

The decay rates of *E. coli* exposed to open air varied enormously from day to day and from site to site, ranging as widely as from 0·0018 to 0·47 min^{-1}.

While being exposed to the open air under the hood, the microthreads are supposed to be protected against the effects of direct sunlight and wind velocity. Since at Soesterberg very high survival rates were measured even during sunshine, the effects of daylight can be neglected. As to wind velocity, it was shown by Hood (1974) that the bacterial inactivation rate was slightly higher at a wind velocity of 2·8 m/s when OAF is measurable. This effect considerably increased at 5·7 m/s wind velocity. Since the air movements under the hood we used were low the effect of wind must have been small.

The large differences in *E. coli* survival rates were undoubtedly accounted for by the great variations in OAF concentration. The number of *B. globigii* in samples from clean air (in the sow) and in field samples was constant. There are therefore no indications that *B. globigii* spores which were used as a biological tracer are sensitive to the OAF in the atmosphere at the sites of exposure.

According to Cox, Hood & Baxter (1973) the inactivation can be expressed in terms of OAF concentration as described before. The importance of converting decay rates to OAF concentrations lies in the possibility of relating OAF to various air pollutants (ozone, sulphur dioxide, nitrogen oxides, etc.).

The data presented in this study show that OAF concentrations are relatively low in the absence of ozone (Figs. 4, 5). When ozone is present, the OAF appears to be dependent on the prevailing wind direction. This became very clear from the experiments at Soesterberg, but was also evident at Delft and Vlaardingen (Figs. 4, 5).

This dependency of OAF activity on prevailing wind directions might be explained by the assumption that rural air is 'clean', whereas urban air or air passing along the highway is 'polluted'.

Olefin sources may be roughly divided in two groups: motor traffic and (petro)-chemical industries. It was shown by Guicherit, Hoogeveen & Lindqvist (1975) that, for the city of Delft, at wind directions between 120° and 240° (roughly SE to SW), an important part of the hydrocarbons in the air originates from the industrial complexes near Rotterdam, whereas at other wind directions, the traffic was shown to be the main source.

From our observations it seems that the correlation between ozone concentration

and OAF activity is higher (R = 0·83) in situations where the most important hydrocarbon source is the motor traffic than in situations where the hydrocarbon source is industrial (R = 0·56).

The data presented in Figs. 6 and 7 and in Table 2 strongly support the hypothesis that both ozone and olefins are involved in the formation of the OAF.

The authors wish to thank K. C. Winkler and H. C. Bartlema for their stimulating discussion and L. J. Brasser and R. Guicherit of the Atmospheric Pollution Division of the TNO Research Institute for Environmental Hygiene for their permission to use the air pollution data in this study.

The authors are grateful to the Topographic Service of the Department of Defence for permission to publish the topographic maps in this paper.

REFERENCES

Benbough, J. E. & Hood, A. M. (1971). Viricidal activity of open air. *Journal of Hygiene* **69**, 619.

Cox, C. S., Hood, A. M. & Baxter, J. (1973). Method for comparing concentrations of the open-air factor. *Applied Microbiology* **26**, 640.

Dark, F. A. & Nash, T. (1970). Comparative toxicity of various ozonized olefins to bacteria suspended in air. *Journal of Hygiene* **68**, 245.

Druett, H. A. (1969). A mobile form of the Henderson apparatus. *Journal of Hygiene* **67**, 437.

Druett, H. A. & May, K. R. (1968). Unstable germicidal pollutant in rural air. *Nature, London* **220**, 395.

Druett, H. A. & May, K. R. (1969). The open air factor. *New Scientist* **41**, 579.

Druett, H. A. & Packman, L. P. (1968). Sensitive microbiological detector for air pollution. *Nature, London* **218**, 699.

Guicherit, R., Hoogeveen, A. & Lindqvist, F. (1975). Die Herkunft von C_1–C_5-Kohlenwasserstoffen im Haag und Delft. *Staub-Reinhalt. Luft* **35**, 89.

Guicherit, R., Jeltes & Lindqvist, F. (1972). Determination of the ozone concentration in outdoor air near Delft, The Netherlands. *Environmental Pollution* **3**, 91.

Hald, A. (1952). *Statistical Theory with Engineering Applications*, p. 535. New York: John Wiley & Sons.

Harper, G. J. (1973). The influence of urban and rural air on the survival of microorganisms exposed on microthreads. In *IVth International Symposium on Aerobiology* (ed. F. Ph. Hers and K. C. Winkler), p. 151. Utrecht: Oosthoek.

Hood, A. M. (1974). Open-air factors in enclosed systems. *Journal of Hygiene* **72**, 53.

May, K. R. (1973). The Collison nebulizer; description, performance and applications. *Aerosol Science* **4**, 235.

May, K. R. & Druett, H. A. (1968). A microthread technique for studying the viability of microbes in a simulated airborne state. *Journal of Microbiology* **51**, 353.

May, K. R., Druett, H. A. & Packman, L. P. (1969). Toxicity of open air to a variety of microorganisms. *Nature, London* **221**, 1146.