Inhibition of Bacterial and Phytoplanktonic Metabolic Activity in the Lower River Rhine by Ditallowdimethylammonium Chloride

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The effects of a quaternary ammonium compound, ditallowdimethylammonium chloride (DTDMAC), on natural populations of bacteria and phytoplankton from the lower River Rhine were examined to estimate their sensitivity to the discharges of cationic surfactants in the river basin. In short-term experiments, significant decreases in the growth rate of bacterioplankton and in the photosynthetic rate of phytoplankton were observed at a nominal concentration of 0.03 to 0.1 mg of DTDMAC liter⁻¹. Nitrification was measured with an ion-selective electrode and by the rate of acid production in ammonium-spiked river water and was found to be only sensitive to the addition of concentrations higher than 1 mg of DTDMAC liter⁻¹. This does not support an earlier suggestion that ammonium-oxidizing bacteria are specifically sensitive to quaternary ammonium compounds. The effect of DTDMAC on thymidine incorporation was shown to depend strongly on the concentration of suspended material, which varied with the sampling date. This effect was also quantified in experimental manipulations with Rhine water. Calculations on the partitioning of DTDMAC between water and suspended matter confirmed the role of suspended solids and showed that an increase of the dissolved DTDMAC concentration in Rhine water by circa 0.01 mg liter⁻¹ leads to a slight inhibition of the growth of heterotrophic bacteria. It is concluded that a total concentration of circa 0.01 mg of DTDMAC liter⁻¹ measured in the River Rhine is likely to have biological consequences.

MATERIALS AND METHODS

Water samples containing their natural communities of bacteria and phytoplankton were collected from the lower River Rhine at station Lobith, close to the German-Dutch border (863 km from Lake Constance). Samples of 20 to 40 liters were taken with a plastic bucket from a platform located circa 20 m from the river bank and were transported to the laboratory in polypropylene jerry jugs within 3 h. The experiments were done four times, 7 March, 12 April, 30 May, and 7 June 1990.

The substance DTDMAC was provided as a technical product by Procter and Gamble European Technical Center S.A. According to the manufacturer’s specification, it contained 77% DTDMAC, 1.7% monolallowtrimethylammonium chloride, and 13.3% isopropanol in water. In this form, the substance is used in fabric softeners. The substance was heated in a water bath at 75°C to facilitate dilution in demineralized water; suspensions of 0.1 and 1 g liter⁻¹ were prepared. The concentrations used were 0.03 to 10 mg liter⁻¹ of this suspension in six logarithmic steps. Since DTDMAC tends to adsorb onto solids, e.g., glassware, successful precautions were taken to diminish adsorption onto the walls of the test vessels (25a). Glass vessels (60 ml and 1 liter) were initially filled with a suspension of 0.1 g of DTDMAC liter⁻¹ for 24 h to coat the walls. Subsequently, the glassware was rinsed three times with 99% chloroform and 99.8% methanol, respectively. To obtain an equilibrium between the wall and the test concentrations, the flasks were rinsed two times with river water amended with the test concentrations. Furthermore, the same bottles were always used for the same DTDMAC concentrations. The concentrations used in the experiments were based on concentrations of the technical product added and were not corrected for impurities. Effects of isopropanol were neglected, but no-effect concentrations (NOEC) of the isomeric n-propanol were 2 or 3 orders of magnitude higher than the highest solvent concen-

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The dry weight of suspended material in river water was determined with preweighed (1.5 h, 75°C) cellulose acetate filters (Sartorius SM 12305; pore size, 1.2 \(\mu\)m). Dry weight was measured after 1 h of drying at 75°C. Chlorophyll \(a\) concentrations were determined in extracts (80% ethanol, 75°C) of glass fiber-filtered material larger than 0.3 \(\mu\)m (Schleicher & Schuell no. 6). Extinction was measured at 665 and 750 nm before and after the addition of HCl to a final concentration of 4 mM. The concentration of chlorophyll \(a\) was calculated by following the Netherlands Standards method (6).

The effects of DTDMAC on natural phytoplankton assemblages were determined after 4 h of preexposure to DTDMAC on a rolling device (100 rpm; Greiner Technik, Solingen, Germany) under continuous illumination (45 W m\(^{-2}\) at 2°C). The photosynthetic rate was assessed by adding 0.1 \(\mu\)Ci of \(\text{H}^3\text{CO}_3^-\) (50 to 60 mCi/mmol; Amersham Corp.), using the procedure described in detail by Tubbing et al. (30a). Two glass vessels (60 ml) with 50 ml of river water were incubated in the light, while one vessel was incubated in the dark. Directly after the addition of labeled bicarbonate and after 60 min of incubation, subsamples of 10 and 40 ml, respectively, were filtered on cellulose nitrate filters (Sartorius; pore size, 0.45 \(\mu\)m; 25 mm). The filters were transferred to scintillation vials containing 1 ml of methanol acidified with acetic acid (5%, vol/vol). After slow evaporation of the liquid on a heating plate, the filters were dissolved in propyl acetate (Merck); subsequently, 7 ml of scintillation liquid (Instagel; Packard) was added. The total radioactivity added to the glass vessels was determined by transferring 0.3 ml from each vessel into a scintillation vial containing 1 ml of Carbosorb (Merck); subsequently, 20 ml of scintillation liquid was added. The radioactivity was counted with a liquid scintillation counter (Packard model 1500). Photosynthetic rates were expressed as micrograms of carbon per liter per hour, as described by Vollenweider (36), using the inorganic carbon content of river water determined titrimetrically.

The effects of DTDMAC on bacterial thymidine incorporation were determined after 2 h of preexposure, unless stated otherwise, with DTDMAC on a rolling device (100 rpm; 20°C in darkness). The incorporation of \([\text{methyl-}^3\text{H}]\)thymidine (40 to 60 mCi/mmol; Amersham Corp.) into trichloroacetic acid-insoluble material was measured by the method of Fuhrman and Azam (7) and described in detail by Tubbing and Admiraal (30). In short, samples of 50 ml were incubated with 5 nM of labeled thymidine. After 0, 10, 20, and 30 min, 10-ml subsamples were poured into an equal volume of an ice-cold 10% solution of trichloroacetic acid supplemented with unlabeled thymidine. After 30 min of extraction on ice, the cold trichloroacetic acid-insoluble material was collected on cellulose nitrate filters. Each filter was washed with an ice-cold 5% solution of trichloroacetic acid and subsequently put in a scintillation vial containing propyl acetate (Merck). After the filters had dissolved, 10 ml of scintillation liquid was added and the radioactivity was measured by liquid scintillation counting (Packard model 1550). Counting efficiency was determined with a standard of tritiated water (Amersham Corp.). The rates of thymidine incorporation were estimated by calculating the slopes of regression lines representing incorporated thymidine versus time and were expressed as nanomolar units per hour. The incorporation of thymidine was linear with the incubation time, and the correlation coefficient amounted to 0.95 even after adding toxicants.

**Experimental manipulations.** To measure the effect of suspended matter, Rhine water sampled on 7 June 1990 was diluted with 0.22-\(\mu\)m-filtered Rhine water (Millipore) before different concentrations of DTDMAC (0 to 10 mg liter\(^{-1}\)) were added. The final concentrations of suspended matter were 39.7, 15.9, and 4 mg liter\(^{-1}\), measured as dry weight; for photosynthesis, only the two highest concentrations of suspended matter were chosen. Directly after dilution of Rhine water, the different concentrations of DTDMAC were added and the effect of thymidine incorporation and photosynthesis was measured by the methods described above. The samples without the addition of DTDMAC served as controls.

Chronic effects of DTDMAC on thymidine incorporation were studied by incubating water samples (30 May 1990) with a concentration range of DTDMAC for 48 h in darkness at 20°C. After 2, 24, and 48 h, the thymidine incorporation rate was measured. The effect of DTDMAC is shown as percentages of the control at the different times.

Nitrification was measured by the decrease of the ammonium concentration in river water in darkness with an ion-selective electrode (Ammonia Probe Kit, model 8002-8; Electronic Instruments Limited, Chersey, Surrey, England), accompanied by pH determinations to determine the acid production involved in oxidation of ammonium. In recent years, the highest concentrations of ammonium in Rhine water amounted to 2 mg of NH\(_4\)\(^+\) liter\(^{-1}\). To estimate the decrease of the ammonium concentration, we added 10 mg of ammonium chloride liter\(^{-1}\) to the water. The ammonium electrode was calibrated with known concentrations of ammonium (0 to 10 mg liter\(^{-1}\); its response was insensitive to exposure to the DTDMAC solutions. This procedure was chosen to allow for a distinct oxidation of the ammonium to be measured. In an earlier stage of the study, ammonium oxidation was measured exclusively by measuring NH\(_4\)\(^+\), NO\(_2^-\), and NO\(_3^-\) chemically (1). However, DTDMAC seemed to interfere with the automatic analysis of these compounds. A specific inhibitor of nitrification, allyl thioura (Merck; 5 mg liter\(^{-1}\)), was added to some of the flasks with different concentrations of DTDMAC to ensure that the observed decrease of the ammonium concentration in the other flasks was due only to ammonium-oxidizing bacteria.

The effect of DTDMAC on photosynthesis and thymidine incorporation was described by a logistic response model (11). The concentrations of DTDMAC which cause 10 and 50% reduction of the activities (EC\(_{10}\) and EC\(_{50}\), respectively) were reported. To fit the logistic curves for the activities to the experimental data, we used a nonlinear least-squares method described by van Beilen et al. (31). Two independent dose-effect curves were fitted separately to give two EC\(_{50}\) values. The standard deviation was derived from these values by using the \(n - 1\) method. An analysis of variance with the software package Genstat 5 was used by the method of Van Gestel and Ma (32) to test the different dose-response curves observed in the field and in experimental manipulations of river water. Two logit models were fitted to the percentage effect data; the first model had the same intercept and slope for all months (see Fig. 2) or concentrations of suspended matter (see Fig. 4), and the second model allowed for different slopes and intercepts. Under the null hypothesis that all treatments have the same slope and intercept, the difference in adequacy between the two models, as measured by the reduction in residual deviance when going from the simple to the more complex model, follows a \(\chi^2\) distri-
bution. The number of degrees of freedom equals $2 \times (\text{the number of treatments or months } - 1)$ (25). A measure of the adequacy of a model such as is $[1 - (\text{residual deviance/total deviance})] \times 100\%$. EC$_{50}$ values of DTDMAC-inhibited nitrification and no-observed-effect concentrations were obtained by graphical interpolation of the dose-response curves.

**RESULTS**

The observation period from March to June 1990, shown in Table 1, was marked by seasonal plankton successional stages, a period with high discharge and high concentrations of suspended matter (7 March), and a period of low discharge and low concentrations of suspended matter (12 April). The chlorophyll $a$ content ranged from circa 5 µg liter$^{-1}$ in March to 140 µg liter$^{-1}$ in May; the rates of bacterial thymidine incorporation varied between 0.091 and 0.209 nM h$^{-1}$. The nitrification rate in ammonium-spiked Rhine water was measured on three occasions and was the highest in June.

Figure 1 shows that photosynthesis of three of the phytoplankton samples was not notably affected by nominal concentrations up to 1 mg of DTDMAC liter$^{-1}$; higher concentrations of DTDMAC significantly ($P < 0.05$) reduced the photosynthesis. Only for the samples obtained on 7 March was photosynthesis gradually inhibited by increasing test concentrations. This high sensitivity occurred during a period of very low algal abundance, indicated by the chlorophyll $a$ concentration (Table 1).

Bacterial thymidine incorporation (Fig. 2) decreased when 0.03 to 0.3 mg of DTDMAC liter$^{-1}$ was added; the inhibition was more pronounced at higher concentrations. Data from 12 April showed that the incorporation rate of thymidine was particularly affected, indicated by a 45% inhibition by the lowest test concentration. This increased inhibition coincided with minimal amounts of suspended matter (Table 1). The EC$_{50}$ values of DTDMAC on the other dates varied between 0.6 and 1.6 mg of DTDMAC liter$^{-1}$.

The effect of DTDMAC on nitrification observed on 7 June is shown in Fig. 3 as a representative observation. The inhibition of the oxidation of ammonia by DTDMAC, measured polarographically, correlated with reduced acid production. The addition of allyl thiourea, a well-known inhibitor of nitrification (3), inhibited ammonium oxidation completely. Again, no obvious changes in the ammonium concentration and pH were observed. Experiments similar to the one presented in Fig. 3 were done with water from 7 March and 30 May and showed essentially the same results, although the oxidation rates of ammonium were lower.

The EC$_{10}$ and EC$_{50}$ values of DTDMAC-inhibited microbial activity are summarized in Table 2. Thymidine incorporation of heterotrophic bacteria seems to be more sensitive than ammonium oxidation of nitrifiers and photosynthesis of phytoplankton. Only on 7 March, when the phytoplankton biomass was very low, was the photosynthetic rate more sensitive to DTDMAC than bacterial growth.

Effects of suspended matter on thymidine incorporation, measured separately on 7 June, indicated that at lower

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**TABLE 1.** Dry weight, chlorophyll $a$, and rates of photosynthesis, thymidine incorporation, and nitrification in microbial communities from the River Rhine in 1990

<table>
<thead>
<tr>
<th>Date (1990)</th>
<th>Dry wt (mg liter$^{-1}$)</th>
<th>Chlorophyll $a$ (µg liter$^{-1}$)</th>
<th>Photosynthesis (µg of C liter$^{-1}$$^{\text{a}}$)</th>
<th>Thymidine incorporation (nM h$^{-1}$)</th>
<th>Nitrification (mg of NH$_4^+$ liter$^{-1}$ day$^{-1}$)$^{\text{b}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 March</td>
<td>56.2</td>
<td>4.8</td>
<td>3.7</td>
<td>0.209</td>
<td>1.7</td>
</tr>
<tr>
<td>12 April</td>
<td>25.0</td>
<td>56.6</td>
<td>158.8</td>
<td>0.140</td>
<td>NM$^{\text{b}}$</td>
</tr>
<tr>
<td>30 May</td>
<td>34.8</td>
<td>139.1</td>
<td>536.0</td>
<td>0.096</td>
<td>&gt;1.6</td>
</tr>
<tr>
<td>7 June</td>
<td>39.7</td>
<td>109.1</td>
<td>409.6</td>
<td>0.091</td>
<td>6.5</td>
</tr>
</tbody>
</table>

$^{\text{a}}$ Photosynthesis and thymidine incorporation, mean of two replicates. Nitrification, highest rate observed.

$^{\text{b}}$ NM, not measured.
amounts of suspended matter the inhibition by DTDMAC (0 to 10 mg of DTDMAC liter\(^{-1}\)) was considerably enhanced (Fig. 4A). Even the lowest test concentration (0.03 mg liter\(^{-1}\)) had an inhibitory effect when the lowest amount of suspended material (4 mg liter\(^{-1}\)) was encountered. The incorporation rate of thymidine diminished proportionally to the dilution with filtered water, so that the manipulation itself did not affect the metabolic activity. Among phytoplankton (Fig. 4B), no large differences in the photosynthetic rate were observed at different concentrations of suspended matter. The EC\(_{10}\) and EC\(_{50}\) values of DTDMAC-inhibited photosynthesis were lower at the lower concentrations of suspended matter. However, no significant differences between the two dose-response curves were observed.

To determine whether growth and adaptation of microbial communities to DTDMAC occurred, we exposed heterotrophic bacteria to a concentration range of DTDMAC for periods ranging up to 48 h. Within 24 h of exposure in the laboratory, the thymidine incorporation rate in the control increased by a factor of more than three. It seems that the initially strong inhibition of thymidine incorporation at high DTDMAC concentrations (0.3 to 10 mg liter\(^{-1}\)) is relieved after 24 h of exposure. The EC\(_{10}\) value changed from 0.12 mg of DTDMAC liter\(^{-1}\) after 2 h to 0.26 mg of DTDMAC liter\(^{-1}\) after 24 h of exposure. The EC\(_{50}\) value changed from 0.66 to 2.9 mg of DTDMAC liter\(^{-1}\), respectively. Between 24 and 48 h, the EC\(_{10}\) and EC\(_{50}\) values remained constant. The percent

**TABLE 2. Effects of the concentration of DTDMAC on photosynthetic rate, thymidine incorporation, and oxidation of ammonium (nitrification)**

<table>
<thead>
<tr>
<th>Date (1990)</th>
<th>Photosynthesis (mg liter(^{-1}))</th>
<th>Thymidine incorporation rate (mg liter(^{-1}))</th>
<th>Nitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{10})</td>
<td>EC(_{50})</td>
<td>EC(_{10})</td>
</tr>
<tr>
<td>7 March</td>
<td>0.04 ± 0.001</td>
<td>0.51 ± 0.001</td>
<td>0.27 ± 0.018</td>
</tr>
<tr>
<td>12 April</td>
<td>1.49 ± 0.34</td>
<td>3.64 ± 0.32</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>30 May</td>
<td>1.48 ± 0.52</td>
<td>3.62 ± 0.78</td>
<td>0.11 ± 0.029</td>
</tr>
<tr>
<td>7 June</td>
<td>1.54 ± 0.38</td>
<td>3.98 ± 0.54</td>
<td>0.05 ± 0.007</td>
</tr>
</tbody>
</table>

\(^a\) The EC\(_{10}\) and EC\(_{50}\) values ± standard deviation for photosynthesis and thymidine incorporation were calculated according to a logistic dose-response curve (11, 31). The no-observed-effect concentration (NOEC) and the EC\(_{50}\) values of DTDMAC on nitrification were obtained by graphical interpolation.

\(^b\) NM, not measured.
inhibition of thymidine incorporation after 2 h of exposure to different concentrations of DTDMAC was consistent with results obtained from the data of 30 May (Fig. 2).

**DISCUSSION**

Suspended matter reduced the availability of DTDMAC to heterotrophic bacteria, most likely by providing surfaces for adsorption of DTDMAC. Kappeler (17) and Lewis and Wee (23) also found that the effect of circa 10 mg of DTDMAC liter$^{-1}$ on bluegills (*Lepomis macrochirus*) after 96 h of exposure was negligible when the suspended matter concentrations exceeded 50 mg liter$^{-1}$. The role of suspended matter was also indicated in several more controlled laboratory studies in which the toxic effects of DTDMAC and other QACs were very pronounced (17, 19, 20, 22). In the present study, the variable impact of suspended matter was studied by diution of natural water samples with particle-free water. The influence of suspended matter was described quantitatively by transforming the added concentrations of DTDMAC in river water to the concentration of DTDMAC in the water phase by using the sediment-water partition coefficient of DTDMAC ($K_{pw} = 8.5 \times 10^4$ kg liter$^{-1}$ [33; based on the report of Kappeler (17)]). These calculated concentrations of dissolved DTDMAC in the water phase appeared to be a more clear-cut causative factor than the total DTDMAC concentration (Fig. 5A and B). The EC$_{50}$ values at 4, 15.9, and 39.7 mg of suspended matter liter$^{-1}$ (calculated by the method of van Beelen et al. [31]; see Materials and Methods) amounted to 0.079 ($\pm$ 0.038), 0.211 ($\pm$ 0.021), and 0.557 ($\pm$ 0.062) mg of total DTDMAC liter$^{-1}$, respectively, and to 0.067 ($\pm$ 0.018), 0.073 ($\pm$ 0.013), and 0.177 ($\pm$ 0.008) mg of dissolved DTDMAC liter$^{-1}$, respectively. A more uniform response to dissolved rather than to total DTDMAC was also shown by the method of Van Gestel and Ma (32) (see Materials and Methods). The adequacy of the total DTDMAC concentration amounted to 66% ($X^2 = 320$; df = 6), whereas dissolved DTDMAC led to an adequacy of 82% ($X^2 = 135$; df = 6). Following the same statistical method, the seasonal observations of thymidine inhibition (Fig. 2) were more adequately correlated to dissolved DTDMAC (adequacy of 70%, $X^2 = 269$; df = 6) than to total DTDMAC (adequacy of 75%, $X^2 = 197$; df = 6). Although the relationship between bacterial growth and the concentrations of dissolved DTDMAC still showed significant ($P < 0.01$) differences at different concentrations of suspended matter, the variability observed in response to the total concentration of DTDMAC was reduced. These results appeared to substantiate the earlier conclusions that suspended matter reduced the toxicity of DTDMAC. The mitigating effect of suspended matter for the toxic effect of DTDMAC observed here also supports the observations by Gerike (8) and Ginn (10) indicating strong adsorption onto solids. However, it is remarkable that minor additions of circa 0.01 mg of dissolved DTDMAC liter$^{-1}$ added to Rhine water have biological effects, despite the presence of anionic detergents which mitigate the effects of QACs by complex formation or by increasing biodegradation (13, 15, 18, 24, 38).

The present observations on heterotrophic bacteria in Rhine water indicate that the ambient levels of 0.006 mg of DTDMAC liter$^{-1}$ (33) at Lobith are very close to those yielding inhibitory effects, and it seems that an increased tolerance of riverine bacteria to DTDMAC, as described by Shrimp et al. (27) and Ventullo and Larson (34), is not reached. Recent studies also showed that River Rhine bacteria did not increase their tolerance to copper (30b). Preliminary experiments indicated that the apparent EC$_{50}$ value for bacterial communities exposed to DTDMAC decreased by a factor of three over 24 h. Shrimp and Schwab (26) observed adaptation to dodecyltrimethylammonium chloride in sedimentary and peryphytic compartments in an in situ environmental chamber within 5 to 10 days of exposure. However, Shrimp et al. (27) concluded that after prolonged exposure, QAC-degrading microbial communities developed a resistance to "shock-loads" of the surfactant and that communities lost their ability to rapidly degrade QACs when the surfactant input ceased.

The growth of heterotrophic bacteria, measured by incorporation of thymidine, was particularly sensitive to the addition of DTDMAC (this study) or monovalent ammonium chloride and other QACs (34). Thymidine incorporation has also been proved to be sensitive to heavy metals (16, 30), to detergents (35), and to organic compounds like polycyclic aromatic hydrocarbons and organochlorine pesticides (2, 14). There are earlier suggestions that nitrifying bacteria are more sensitive to DTDMAC than heterotrophic bacteria (4), the rationale being that the ammonium uptake in nitrifiers is particularly sensitive to QACs. This hypothesis was not supported by the present observations. On the contrary, only DTDMAC levels of milligrams per liter were inhibitory. Banyas (1) found that exposure of ammonium-spiked Rhine water led to some accumulation of
nitrite; his observations indicated that nitrite-oxidizing bacteria were probably more sensitive to DTDMAC than the ammonium-oxidizing bacteria. The longer incubation times of up to 4 days in the experiments on nitrification could also have led to some degradation of DTDMAC, resulting in a loss of DTDMAC (19, 24, 29, 37, 38). However, the results of Larson and Vashon (19) and Woltering et al. (38), who observed a turnover time of QACs in river water of 5 and 14 days, respectively, indicate that biodegradation will have minor effects in our experiments.

The photosynthetic rate of phytoplankton in Rhine water was as sensitive to DTDMAC as nitrification. This accords with results of Lewis (21) and Lewis and Hamm (22), who found that concentrations ranging from 0.6 to 6.5 mg of DTDMAC liter$^{-1}$ had a negative effect on photosynthesis of natural phytoplankton. The observation that the toxicity of DTDMAC on phytoplankton was not related to the amount of suspended matter on the four different dates and on 7 June after dilution of phytoplankton in particle-free Rhine water suggests that there are also other mechanisms which influence the toxicity of DTDMAC. One is the population size of the phytoplankton and the ratio between the concentration of DTDMAC and the cell surface; the sparse phytoplankton population was very sensitive and a dose effect rather than a concentration effect appeared to be involved. Differences in the sensitivity of phytoplankton could be caused by differences in species composition (20, 22).

Despite the many biological, physical, and chemical variables, the response of natural heterotrophic and autotrophic microbial River Rhine populations seems to be significantly affected at low nominal concentrations of DTDMAC, ranging from 0.01 to 1 mg liter$^{-1}$. Considering these results and the reported concentrations in the rivers Rhine (0.006 mg liter$^{-1}$) and Meuse (0.025 mg liter$^{-1}$) and in wastewater influents (up to 2 mg liter$^{-1}$), it seems likely that DTDMAC has an impact on microbial communities under natural conditions. This conclusion is also supported by calculations of the ecotoxicological no-adverse-effect concentration for DTDMAC of 0.016 mg liter$^{-1}$ on the basis of an extrapolation method recommended by the Dutch Health Council (12).

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