Increased resistance of environmental anaerobic spores to inactivation by UV

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Abstract Water Company Europoort started a pilot plant (MP) UV study to determine the UV-fluence to meet the Dutch drinking water standards. The results of large volume sampling of this pilot plant demonstrated that environmental spores of sulphite-reducing clostridia (SSRC) were highly resistant against UV. With the pilot plant at a flow of 180 m³/h a challenge test was conducted to compare the susceptibility of environmental SSRC and lab-cultured spores of C. perfringens, MS2 bacteriophages and Bacillus subtilis. The latter was dosed as a biodosimeter with a calibrated UV_{253.7nm} sensitivity to confirm the germicidal fluence. This test demonstrated that environmental SSRC were a factor of 1.6 more resistant against UV than the lab-cultured spores of C. perfringens. Furthermore, the results of these environmental SSRC indicated that they are more resistant to UV than other relevant micro-organisms for drinking water safety. Environmental SSRC calibrated with biodosimetry and determined with large volumes sampling is a potential parameter for on-site verification of the efficacy of UV-systems. Further research is necessary to determine to what extent the UV susceptibility of SSRC is consistent in the water and to find the cause of the increased resistance of environmental micro-organisms.

Keywords Environmental clostridia spores; increased resistance; on-line fluence verification; UV-disinfection

Introduction UV irradiation is an attractive alternative disinfection process in drinking water treatment because of its efficacy against pathogenic bacteria, viruses and protozoa. Water Company Europoort (WBE) investigates the application of UV disinfection as an alternative for chlorination as primary disinfection and started a pilot plant study with a medium pressure UV system (180 m³/h). The objective was to determine the required UV-fluence (irradiation intensity per surface area and contact time) to meet the standards of the Dutch Drinking Water Decree (VROM, 2001). Spores of sulphite-reducing clostridia (SSRC), which include spores of Clostridium perfringens, are a drinking water standard in The Netherlands since 1984. These resistant bacterial spores were thought to require relatively high UV- fluences for inactivation. This was studied by assessing the inactivation of these spores in the pilot plant by large volume sampling. To achieve compliance with the standard for this parameter in more than 99% of the samples, Van der Kooij et al. (1994) calculated that the SSRC concentration in drinking water should be below 0.035 CFU/l. Results presented in this paper show that SSRC are more resistant against UV than other relevant micro-organisms, including pathogens. Hence, these spores turned out to be the determining parameter for the required fluence.

Methods Water treatment and the UV installation
The feed water of the UV installation (Berson, In-line 1000) was river Meuse water pre-treated by impoundment reservoirs, micro-strainers, coagulation and floc removal and
granular activated carbon filtration. The pilot UV installation consisted of two serial reactors operated at water flows between 40 and 180 m$^3$/h.

During the pilot plant challenge-test (Figure 1) only one reactor was used with a flow of 180 m$^3$/hour. In the UV-reactor 4 quartz tubes were placed in tilted square (tangential flow) position and fitted with medium pressure lamps (B2020). The process was operated at three (calculated) UV-fluences (770, 960 and 1150 J/m$^2$). Flow, UV transmission were measured and lamp output was measured with an UV-sensor located at a distance of 1 cm from one of the quartz tubes. The different fluences were obtained by changing the power input of the lamps to keep the flow distribution through the reactor constant.

The age of the lamps in reactor 1 and 2 varied between 34 and 304 irradiation days. The UV output of each lamp was tested before the experiments. In reactor 2, the reactor operated during the challenge test, four lamps were placed with the smallest differences in output.

**Inactivation capacity under normal operation**

Inactivation capacity of this pilot plant UV-disinfection was assessed with environmental spores of sulphite-reducing clostridia (SSRC) present in the feed water. The concentration of these spores in the influent and effluent of the installation was determined with large volume sampling using a special on-site membrane filtration device (Hijnen et al., 2000).

**The challenge test**

To determine the inactivation of lab-cultured SSRC a challenge test with spores of *Clostridium perfringens* (D10) was performed. Simultaneously, the germicidal fluence of the installation was determined with spores of *Bacillus subtilis* ATCC 6633 with a calibrated UV$_{253.7\text{nm}}$ sensitivity (obtained from R. Sommer, University of Vienna). Together with these spores the F-specific RNA bacteriophage MS2 was dosed as a surrogate for virus inactivation by UV.

The installation was adapted for the test. To ensure proper mixing of the dosed microorganisms, dosing occurred in a by-pass that fed into the main feed water and a static mixer was installed before the UV-reactor (Figure 1). To ensure a proper assessment of the concentration of micro-organisms leaving the UV-installation a static mixer was also installed after the UV reactor and before the effluent sampling point. In a 200 litre stainless steel vessel with a mechanical stirrer 100 litre GAC filtrate was inoculated with the micro-organisms and dosed with a flow of 30 l/h to the bypass (3 m$^3$/h) of the inlet of the UV installation. After 5, 10 and 15 minutes of challenge in- and effluent samples were collected in 1 litre sterile bottles. Two different persons sampled in- and effluent to prevent cross-contamination. For the spores, samples were analysed in duplicate.

**Microbiological methods**

Environmental SSRC in the influent and effluent of the UV installation were determined (Hijnen et al., 1997) using Perfringens Agar Base medium (PAB, Oxoid CM5878). Sample volumes of 100 ml were pasteurised in a water bath at 70±1°C for 30 minutes prior to
filtration. With sample volumes of 1 litre or more membrane filters were pasteurised after sample filtration in the liquefied medium placed in an oven at 70±1°C for 30 minutes. Preparation of the spore suspension of *C. perfringens* (D10) was performed as previously described (Hijnen et al., 2002) and the enumeration was identical to the SSRC method.

Freeze-dried spores of *Bacillus subtilis* ATCC 6633 (10^{11}/gram; Sporodos-Biodosimeter batch 2000/1) were derived from the University of Vienna. 30–50 mg of this material was suspended in 10 ml sterile water. These spores were enumerated as aerobic colony count on Plate Count agar (Difco 247940) incubated at 37±1°C for 24 hours.

Spore suspensions of *C. perfringens* and *B. subtilis* were pre-treated prior to the inoculation. 10 ml of these suspensions was homogenised by sonification during 30 seconds (Branson Digital Sonifier) and filtered through an 8 mm sterilised membrane filter to eliminate aggregates.

Pre-culturing, storage and enumeration of MS2 phages was performed as described in ISO 10905. The MS2 suspension was filtered over a 0.22 mm sterilised membrane filter before the inoculation to reduce the presence of aggregates.

### The calculated UV-fluence, the germicidal UV-fluence and the inactivation capacity

The UV fluence (mJ/cm²) of the installation was calculated with the Berson model BUV3D. With the UV transmission (T₁₀) of the water (90%) the radiance distribution in the reactor was calculated with the point source summation method. The average UV fluence was derived from these radiance data, the average residence time (min) and the water flow. The UV fluence was regulated with the power output of the lamps (3 steps) and the water flow to compensate for lamp age, lamp fouling and water quality variation. Wipers automatically clean the lamps once every hour.

The germicidal fluence or Reduction Equivalent Fluence (REF) was determined from the inactivation data of *B. subtilis*, the biodosimeter. For the Sporodos-Biodosimeter batch 2000/1 the following equation was used

\[
\text{REF} = 145.149 \log \frac{C_{\text{out}}}{C_{\text{in}}} + 100.623
\]

where \(C_{\text{out}}\) and \(C_{\text{in}}\) are the in- and outgoing spore concentrations (n/l), respectively, and \(\text{REF}\) the calculated UV fluence (J/m²) at 253.7 nm.

The inactivation of the micro-organisms or the decimal elimination capacity (DEC; log) of the UV installation is calculated from the average micro-organism concentration (n/l) in the influent and effluent,

\[
\text{DEC} = \log C_{\text{in}}^\text{ps} - \log C_{\text{out}}^\text{ps}
\]

The actual inactivation was calculated from the simultaneously determined in- and outgoing concentration (n/l). Statistical analysis was performed with Microsoft Excel.

### Results and discussion

#### Inactivation of environmental SSRC by UV

The average SSRC concentration in the feed water and the water after UV in the period between August and June was 5.0 (SD=6.0) and 0.28 (SD=0.48) per litre (n=32), respectively, indicating an average decimal elimination capacity (DEC) of 1.3 log. The actual inactivation varied between 0.20 and 2.92 log (median value of 1.50) and showed a linear relation with the calculated UV fluence of the installation (Figure 2). Assuming first-order inactivation kinetics, an inactivation constant \(k\) of 0.0132 log/(mJ/cm²) \((p<0.0001)\) was calculated.

A 2.8 log inactivation of lab-cultured spores of *Clostridium perfringens* was observed in a pilot plant scale UV installation with UV medium pressure lamps at a fluence of
100 mJ/cm² (Kruithof et al., 2002). That is twice as much inactivation as observed for environmental SSRC at the same fluence (Figure 2). This difference in efficacy could be due to differences in UV installation or differences in susceptibility of environmental and the lab-cultured anaerobic spores. To investigate this, a challenge-test with lab-cultured micro-organisms was conducted at the WBE UV-system and inactivation of environmental SSRC was determined simultaneously.

Challenge tests with lab-cultured micro-organisms

The influent concentrations of the micro-organisms dosed during this challenge-test ranged from $2.2 \times 10^4$/l spores of *C. perfringens* to $1.9 \times 10^6$ MS2 phages (Table 1). Concentrations of *C. perfringens* spores showed the highest variation.

From the actual influent and effluent concentrations in each paired samples the inactivation (log) was calculated and related to the applied UV fluence (Figure 3). For each micro-organism the magnitude of inactivation increased with the calculated fluence of the UV reactor. The environmental SSRC data were calculated from the equation in Figure 2. There was a distinct difference in susceptibility to UV between the organisms. The order of susceptibility was *B. subtilis* > MS2 phages > *C. perfringens* > environmental SSRC.

The germicidal fluence or REF of the UV reactor

With the inactivation data of *B. subtilis* (Sporodos-Biosimeter) the germicidal fluence or REF of the installation was determined and compared with the calculated fluence The REF was 55% up to 62% of the calculated fluence (Table 2).

**Table 1** The influent concentrations of the micro-organisms during the challenge test

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>B. subtilis</th>
<th>C. perfringens</th>
<th>MS2 phages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average conc. (n/l)</td>
<td>$1.6 \times 10^5$</td>
<td>$2.2 \times 10^4$</td>
<td>$1.9 \times 10^6$</td>
</tr>
<tr>
<td>SDa</td>
<td>$2.4 \times 10^4$</td>
<td>$6.5 \times 10^3$</td>
<td>$1.7 \times 10^5$</td>
</tr>
<tr>
<td>VCa (%)</td>
<td>15</td>
<td>29</td>
<td>9</td>
</tr>
</tbody>
</table>

*a SD = standard deviation; VC = variation coefficient*

**Table 2** The calculated fluence and the germicidal fluence (REF) assessed from the B. subtilis data of the challenge test

<table>
<thead>
<tr>
<th>Calc. fluence (mJ/cm²) BUV-3D</th>
<th>REF (mJ/cm²)</th>
<th>% of the calc. fluence</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>48</td>
<td>62</td>
</tr>
<tr>
<td>96</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>115</td>
<td>64</td>
<td>55</td>
</tr>
</tbody>
</table>
The method used to calculate the UV fluence of this UV installation overestimates the effective germicidal UV fluence. Consequently, the calculated fluence was adjusted to the germicidal fluence values and related to the observed inactivation of environmental SSRC, C. perfringens and MS2 phages (Figure 4).

Susceptibility of SSRC and other organisms

Comparison of these results with some inactivation data in literature showed that the environmental SSRC are the most resistant against UV disinfection (Figure 4), and a factor of 1.6 more resistant than lab-cultured spores of C. perfringens.

No data were collected of the fraction of C. perfringens spores in the total count of environmental SSRC. Possibly, other clostridia species are less susceptible to UV than C. perfringens. Another possibility is that the environmental SSRC were more persistent. They are older and have been exposed to different kinds of environmental stress like UV light from solar radiation in surface water, (natural) substances with anti-microbial characteristics and low temperatures. The stress-response of environmental spores may have rendered them less susceptible to UV-irradiation. Another hypothesis is that the environmental spores are attached to or encapsulated in colloidal particles and hence protected against UV-irradiation.

In a disinfection study with wastewater and medium and low pressure UV lamps, Nieuwstad et al. (1991) also demonstrated that SSRC were the most resistant of all the

![Dose/Effect Relation](https://iwaponline.com/ws/article-pdf/4/2/55/417345/55.pdf)

**Figure 3** The dose/effect relation between the inactivation of the micro-organisms and the calculated fluence of the challenge test (range of values presented by the error bars, environmental SSRC data calculated with parameters from the equation presented in Figure 2)

**Figure 4** The required fluence for 3 log inactivation of viruses, Cryptosporidium, bacteria and bacterial spores (aerobic and anaerobic)
investigated micro-organisms (E. coli, faecal streptococci, bacteriophages and reoviruses). Moreover, in the same study environmental F-specific phages were twice as resistant to UV as lab-cultured MS2 phages. Nieuwstad et al. (1992) concluded that design of UV systems should be based on inactivation data of environmental rather than lab-cultured micro-organisms. Further research will be dedicated to elucidating the cause of increased UV-resistance of environmental micro-organisms.

SSRC as indicator for inactivation capacity under full-scale operation

Controlling the UV fluence under full-scale situations is currently performed with static sensors. However, the theoretical BUV-3D model used to calculate the fluence overestimated the REF significantly (Table 3). On-line fluence meters are subject of several studies, to obtain a tool to measure UV-efficacy on-line (Linden and Darby, 1997). Microbiological methods are less appropriate for this purpose, because they are not applicable on-line. Nevertheless, this study indicates that environmental SSRC can serve as tool to verify UV efficacy on-site on full scale treatment systems. In this study, the UV-susceptibility of these spores could be calibrated against the biodosimeter B. subtilis. This yielded the REF /inactivation-function presented in Figure 5.

This function can be used to establish the required REF to meet the drinking water standard. To reduce the observed average SSRC concentration of 5 per litre in the feed water to the concentration of 0.035/l for a 99% compliance with the standard for drinking water, 2.2 log inactivation is required. For this treatment target the UV reactor should be operated at a REF of 98 mJ/cm². Further research will be necessary to elucidate if environmental SSRC in other feed waters could also be used for this purpose.

Conclusion

Large volume sampling and challenge tests with a full-scale UV system revealed that environmental spores of sulphite-reducing clostridia are more resistant to UV than lab-cultured spores of C. perfringens. Furthermore, environmental SSRC are even more resistant against UV disinfection than the reported resistance of adenoviruses. Further research is necessary to verify if this phenomenon is present in other natural waters under different conditions and is also observed in other micro-organisms.

The biodosimeter showed that the REF of the pilot UV system was only 60% of the calculated fluence. This emphasises the need for microbiological verification of UV fluences in UV systems. The combination of biosimetry and assessment of the UV-efficacy with environmental SSRC may yield an instrument for on-site verification of the disinfection efficacy of the UV-system: the inactivation of environmental

Figure 5 The relation between the inactivation of environmental SSRC in the WBE water and the germicidal UV fluence assessed with B. subtilis with a calibrated UV<sub>253.7nm</sub> sensitivity.
SSRC can be related to the REF. A more comprehensive comparison of the relation between environmental SSRC inactivation and biodosimetry is needed to ensure its consistency.

References


