Research Paper

Higher effectiveness of photoinactivation of bacterial spores, UV resistant vegetative bacteria and mold spores with 222 nm compared to 254 nm wavelength

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Eleven selected species of vegetative bacteria, bacteria spores and mold spores were irradiated with different doses of UV radiation of a 222 nm krypton-chloride excimer lamp and a 254 nm mercury lamp under laboratory conditions. Then the inactivation curves were determined. The necessary UV fluences for a respective reduction were higher for the excimer lamp for the tested vegetative bacteria of *Bacillus cereus*, *Arthrobacter nicotinovorans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and slightly higher for the spores of *Streptomyces griseus* and *Clostridium pasteurianum*. However, less than 250 J/m^2 UV fluence with 222 nm was sufficient for a 4-log reduction, depending on the species. On the other hand, the UV fluences for the 254 nm mercury lamp were much higher for the bacterial spores of *Bacillus cereus*, *Thermoactinomyces griseus* and the bacteria of *Deinococcus radiodurans* and slightly higher for the mold spores of *Aspergillus niger* and *Penicillium expansum*.
The results show that especially the germs with a higher UV resistance and those with more effective repair mechanisms can be inactivated more efficiently by the 222 nm excimer lamp. This may be due to the fact that low UV fluence mainly affects radiation sensitive microorganisms by DNA damage whereas at higher UV fluence (various) mechanisms of protein damage can presumably be held responsible for inactivation.

Keywords: Excimer lamp / mercury lamp / disinfection / microorganism / drinking water

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1 Introduction

Biological effectiveness of UV radiation varies markedly, depending on the wavelength used for the irradiation [1]. This is caused by varying degrees of absorption of different biomolecules such as DNA, membranes or proteins. According to the kind of chemical bond between the elements of the molecule, these are able to absorb the energy of UV light of different wavelengths in a selective way [2]. In most cases, this energy absorption results in damage of the molecule. If this damage occurs to a large extent and cannot be repaired, it harms and in case of sufficiently high UV fluence even kills the organism.

A wavelength of 254 nm is traditionally used for inactivation of microorganisms. This wavelength corresponds approximately to the absorption maximum of DNA at about 260 nm and can easily be obtained with mercury lamps. At irradiation with UV light at this wavelength there is a great extent of damage in the DNA such as strand breaks, photo products and linking-ups, also with proteins. It is a disadvantage of this wavelength that the cells have different mechanisms to repair DNA damage [3]. The light dependent photoreactivation is especially effective. In a bacteria suspension with *E. coli* cells of which 99.99% have been inactivated by irradiation with 254 nm UV light, only 90% killed cells can be found after a 2-hour-exposition to artificial sunlight. This leads to

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problems especially for large-scale use of UV inactivation of microorganisms when processing drinking water and sewage, i.e. when inactivated cells can get out and are exposed to sunlight.

This effect can be stopped partially by using wavelengths which are absorbed by other molecules than DNA. Proteins have a strong absorption maximum at 220 nm. UV radiation of approximately this wavelength can be generated with a 222 nm KrCl-excimer lamp. Examinations comparing the photoreactivation of *E. coli* after irradiation with 222 nm and 254 nm have shown that the irradiation is considerably less effective at 222 nm than at 254 nm [4]. Furthermore, additional tests showed that *Bacillus subtilis* spores can be inactivated much better with UV radiation having a wavelength of 222 nm than with 254 nm [5, 6] whereas the vegetative forms of *B. subtilis, Enterococcus faecalis, Candida albicans* and *E. coli* can be inactivated better with 254 nm.

In this examination, the effectiveness of photoinactivation with 222 nm wavelength compared to 254 nm wavelength of further microorganisms is tested. For this, the inactivation curves for both wavelengths of the microorganisms were taken. Out of the most important groups different aerobic spore-producing bacteria and mold spores, the anaerobic and also spore-producing bacterium *Clostridium pasteurianum,* some important microorganisms relevant for drinking water and sewage and the highly radiation-resistant *Deinococcus radiodurans*, which has extremely effective repair mechanisms, were chosen.

2 Materials and methods

2.1 UV source

A collimated beam device (WEDECO AG Water Technology) with interchangeable lamp units was used for irradiation as described previously [4]. The UV fluences were measured with a Bentham Spectrometer DM 150 double monochromator with a 200…450 nm standard sensing head and verified for the excimer lamp every month with *Bacillus subtilis* spores strain ATCC 6051 as biological dosimeter after the previously investigated inactivation curve [6]. For the mercury lamp the irradiance was verified every week with a Spectroline DRC 100H digital radiometer with a DIX-254A UV-C sensor (Spectronics Corporation).

2.2 Organisms and their cultivation

Aspergillus niger ATCC 32625 spores and *Penicillium expansum* ATCC 36200 spores (American Type Culture Collection, Manassas, VA): Spores were taken from a slant culture with a sterile cotton bud and applied evenly on YGC-Agar (yeast extract (Oxoid) 5.0 g, glucose (Merck) 20.0 g, chloramphenicol (Selective Supplement OXOID) 0.1 g and agar (Oxoid) 12.0 g per litre aqua demin). The plates were incubated at 30°C for 5 days. To harvest the spores, 3.0 g sterile quartz sand was dispersed on the plate. Then the plate was shaken for one minute by hand. The mixture of sand and spores was removed by being washed with sterile aqua demin with 0.001% Tween 80 and collected in a 300 mL Erlenmeyer flask. To remove the spores from the sand, the suspension was sonicated for 2 min in an ultrasonic bath (Sonorex RK 102 company Bandelin Electronics, Berlin). The supernatant was removed and centrifuged at 1000 rcf for 10 min at 20°C. Then, the pellet was resuspended in 100 mL sterile aqua demin. This stock solution was stored in the fridge at 4°C.

Bacillus cereus ATCC 11778 spores: 1…2 colonies were plated evenly on 5 Nutrient Agar plates (meat extract (Merck) 3.0 g, tryptone (Oxoid) 5.0 g, agar (Oxoid) 15.0 g, with 10.0 mg $MnSO_4 \cdot H_2O$ per litre aqua demin). After 72 h at 30 °C the harvest of the spores was done by resuspending the colonies on each plate in 10 mL sterile aqua demin with a cotton bud. The solution was sonicated for 2 min in an ultrasonic bath (Sonorex RK 102 company Bandelin Electronics, Berlin) and centrifuged (Biofuge 28 RS, company Heraeus) with 3000 rcf for 15 min at 20°C. Afterwards, the pellet was resuspended in 100 mL sterile aqua demin. This was repeated two times. Then, the solution was sounded again for 2 min. Vegetative cells were eliminated by heating the solution for 10 min at 80°C. This stock solution was also stored in the fridge at 4°C.

Bacillus cereus ATCC 11778 vegetative bacteria, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853: 1…2 colonies were plated to Standard-I-Nutrient medium (Merck) and incubated at 30°C for 24 h. Once again, 1…2 colonies were inoculated in 100 mL Standard-I-Nutrient broth (Oxoid) for 14…20 h. Bacteria were harvested by centrifugation (Biofuge 28 RS, company Heraeus) with 1000 rcf for 10 min at 20°C, the pellet was resuspended in 100 mL 0.65% NaCl and filtrated through an 8.0 µm filter (cellulose-nitrate filter, Sartorius).

Arthrobacter nicotinovorans ATCC 49919 and *Deinococcus radiodurans* ATCC 13939: The same procedure as described for *B. cereus* vegetative bacteria, *S. aureus* and *P. aeruginosa* was done only with Corynebacterium Agar (tryptone (Oxoid) 10.0 g, yeast extract (Oxoid) 5.0 g, glucose (Merck) 5.0 g, NaCl (Merck) 5.0 g, agar (Oxoid) 15.0 g per litre aqua demin).

Clostridium pasteurianum ATCC 6013 spores: 1…2 colonies were plated to 5 GYE-agar plates (glucose (Merck) 20.0 g, yeast extract (Oxoid) 10.0 g, $CaCO_3$ (Merck) 10.0 g and agar (Oxoid) 17.0 g per litre aqua demin) under anaerobic conditions in a nitrogen atmosphere. The plates

were put in an air sealed 2.0 L preserving glass with an Anaerogen bag (Oxoid atmosphere generation system) for 2.5 L and incubated for 72 h at 37°C. Spores were harvested and stored under aerogenic conditions as it was described for *B. cereus* spores.

Streptomyces griseus ATCC 10137 spores: 1…2 colonies were plated evenly on 5 GYM plates (glucose (Merck) 4.0 g, yeast extract (Oxoid) 4.0 g, malt extract (Merck) 10.0 g, $CaCO_3$ (Merck) 2.0 g, agar (Oxoid) 12.0 g per litre aqua demin). After 5 days at 28°C the harvest of the spores was done by resuspending the colonies on each plate in 10 mL sterile aqua demin with a cotton bud. The solution was sonicated for 2 min in an ultrasonic bath (Sonorex RK 102 company Bandelin Electronics, Berlin) and centrifuged (Biofuge 28 RS, company Heraeus) with 5000 rcf for 15 min at 20°C. Then, the pellet was resuspended in 100 mL sterile aqua demin. This was repeated two times. After that, the solution was sounded again for 2 min and filtrated through an 8.0 mm filter (cellulose-nitrate-filter, Sartorius). The stock solution was stored in the fridge at 4°C.

Thermoactinomyces vulgaris ATCC 43649 spores. The same procedure as described for *S. griseus* was done only with Czapek Peptone Agar for 7 days (sucrose (Merck) 30.0 g, $NaNO_3$ (Merck) 3.0 g, K_2HPO_4 (Merck) 1.0 g, $MgSO_4 \cdot 7H_2O$ (Merck) 0.5 g, KCL (Merck) 0.5 g, $FeSO_4 \cdot 7H_2O$ (Merck) 0.01 g, yeast extract (Oxoid) 2.0 g, tryptone (Oxoid) 5.0 g, agar (Oxoid) 15.0 g per litre aqua demin), centrifugation with 2600 rcf and filtration through a 12.0 μm filter.

2.3 Standardization of bacteria titer

For irradiation the titer of the test suspension was standardized at $1 \cdot 10^5$ vegetative bacteria/mL with a photometric method as described previously [4]. The number of cfu of the spore stock solutions was only one time determined because the spores will keep in the fridge at 4°C for several months, as well as the spores of the anaerobe *C. pasteurianum*. Hence, simply by diluting the stock suspension a concentration for the irradiation of $1 \cdot 10^5$ spores/mL is reached (for *C. pasteurianum* $1 \cdot 10^6$ spores/mL). The spores were irradiated in sterile aqua demin and the vegetative bacteria in 0.65% NaCl. The transmission of each solution for the irradiation was measured with a photometer (Hitachi U-1100 Spectrometer).

2.4 Irradiation and evaluation of the results

The irradiation was done as described previously [4]. Each time 25 mL of test suspension were irradiated for different periods of times in 85 mm standard polystyrene Petri dishes (arithmetical thickness of 4.4 mm) without intermixing and shade effects. The measured transmissions (Hitachi U-1100 Spectrometer, quartz glass cuvette against 0.65% NaCl) of all bacteria solutions used for irradiation were between 96.5…98.0% for both wavelengths. In order to determine the exact inactivation kinetic, five duplicate samples of each of the microorganisms were irradiated. After irradiation, *A. niger* and *P. expansum* spores were plated in pour-plate method on YGC-Agar and incubated for 5 days at 30°C. *B. cereus* spores and vegetative bacteria, *S. aureus* and *P. aeruginosa* were plated in pour-plate method on PC-Agar (tryptone (Oxoid) 5.0 g, yeast extract (Oxoid) 2.5 g, glucose (Oxoid) 1.0 g and agar (Oxoid) 10.0 g per litre aqua demin) and incubated for 24 h at 30°C. *A. nicotinovorans* and *D. radiodurans* were plated in pour-plate method on Corynebacterium Agar and incubated at 30°C for 24 h for *A. nicotinovorans* and 72 h for *D. radiodurans*. *C. pasteurianum* spores were plated in pour-plate method on GYE-Agar and incubated in air sealed 2.0 L preserving glasses each with an Anaerogen bag for 2.5 L and incubated for 72 h at 37°C. *S. griseus* spores were plated on GYM-Agar and incubated for 72 h at 28°C. *T. vulgaris* spores were plated in pour-plate method on Czapek Peptone Agar and incubated for 20 h at 50°C. The evaluation of the results was done as described previously according to the regulations of the DVGW and the ÖNORM, which lay down the requirements and testing of plants for the disinfection of water using ultraviolet radiation [7, 8]. The dose reduction factor was determined and plotted logarithmically as function of the irradiance.

3 Results

The UV fluence/reduction response curves for the tested mold and bacteria spores are presented in Figure 1, the curves for the vegetative bacteria in Figure 2. The necessary UV fluences with both wavelengths for reductions of 1…4-log steps and 90…99.99% respectively for all tested microorganisms are presented in Table 1. The inactivation curves for both wavelengths are very close for the two kinds of mold spores, the curves of *P. expansum* even cross each other twice (cf. Fig. 1). Furthermore, the curves do not necessarily show the typical sigmoid gradient of the other inactivation curves but are rather irregular. The values of the necessary UV fluences for the corresponding inactivations differ only slightly for both wavelengths. However, the inactivation at 222 nm seems to be more effective. For a 3-log reduction of *Aspergillus niger* spores 3250 J/m^2 at 222 nm are necessary, at 254 nm 3700 J/m^2 (cf. Table 1). For *P. expansum* the UV fluence is 420 J/m^2 at 222 nm and 490 J/m^2 at 254 nm. For *A. niger* and 222 nm even 4300 J/m^2 and for 254 nm 5600 J/m^2 are necessary for a reduction of 4-log steps. Therefore, these

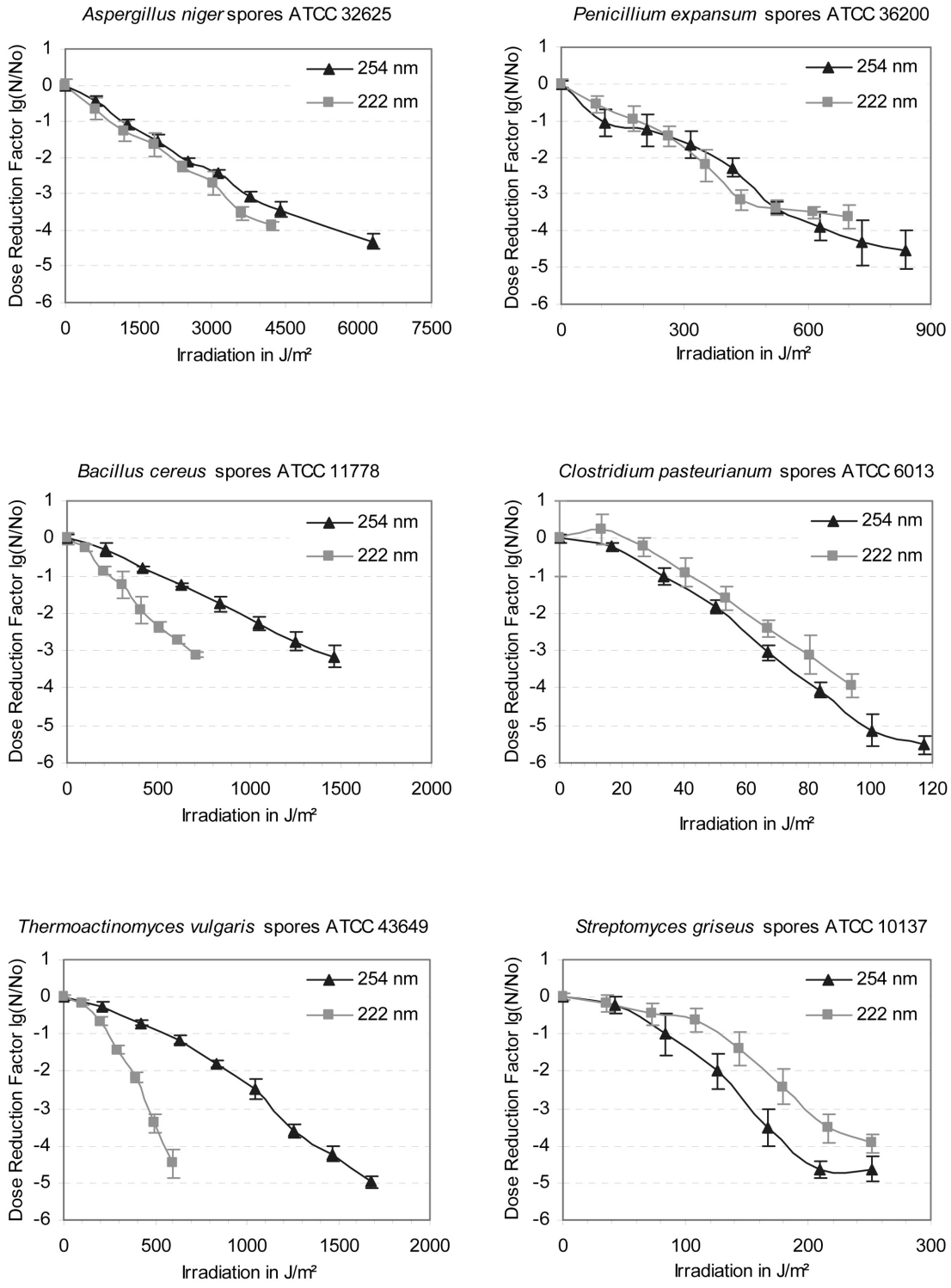

Figure 1. UV fluence/reduction response curves for different mold and bacteria spores. All symbols indicate the results of five independent series of experiments. Error bars denote the standard deviations.

spores show the highest resistance against UV radiation among all tested germs. *P. expansum* fungus spores can be inactivated correspondingly with only a seventh of the UV fluence. There is considerably less UV fluence necessary for a corresponding reduction of the spores of *B. cereus* and *T. vulgaris* at 222 nm than at 254 nm. The values

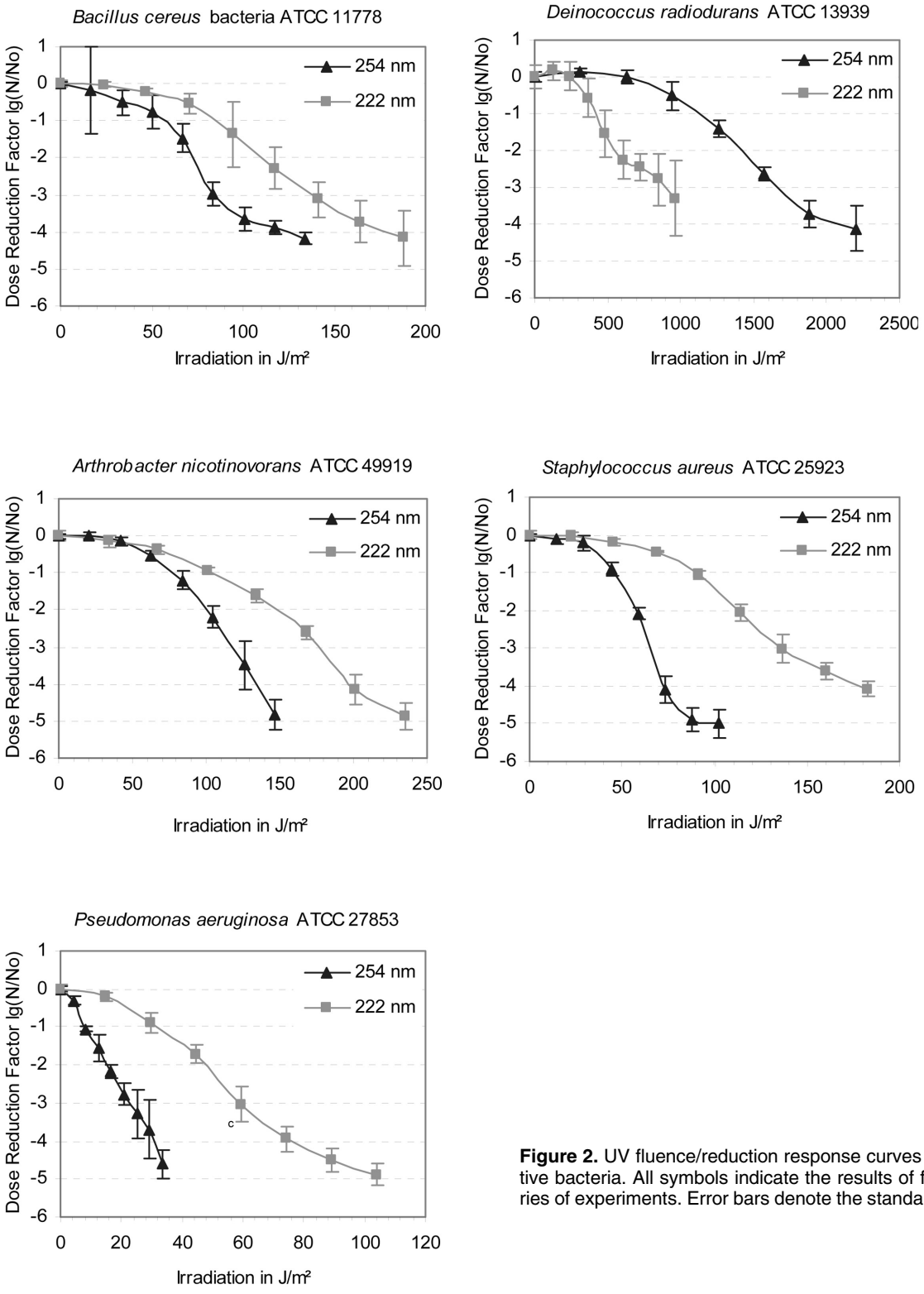

Figure 2. UV fluence/reduction response curves for different vegetative bacteria. All symbols indicate the results of five independent series of experiments. Error bars denote the standard deviations.

of the inactivation of *B. cereus* spores by 3-log steps are for 222 nm 690 J/m² and for 254 nm 1400 J/m². For *T. vulgaris* the values are 550 J/m² and 1400 J/m² for a 4-log reduction. Consequently, these bacteria are almost 10 times more resistant against UV radiation of these wavelengths compared with the spores of *C. pasteurianum* and *S. gri-*

Table 1. Necessary UV fluences for a 1-…4-log reduction of different microorganisms with 222 nm and 254 nm UV radiation.

		UV fluence in J/m²			
Tested microorganisms	lg(N/N_0)	−1	−2	−3	−4
Aspergillus niger	222 nm	900	2200	3250	4300
spores ATCC 32625	254 nm	1150	2450	3700	5600
Penicillium expansum	222 nm	180	330	420	–
spores ATCC 36200	254 nm	110	380	490	650
Bacillus cereus	222 nm	250	430	690	–
spores ATCC 11778	254 nm	520	930	1400	–
Clostridium pasteurianum	222 nm	43	61	79	96
spores ATCC 6013	254 nm	34	53	67	84
Thermoactinomyces vulgaris	222 nm	250	380	460	550
spores ATCC 43649	254 nm	550	900	1150	1400
Streptomyces griseus	222 nm	127	168	200	255
spores ATCC 10137	254 nm	85	126	154	182
Bacillus cereus	222 nm	85	111	137	179
veg. bacteria ATCC 11778	254 nm	58	73	85	123
Deinococcus radiodurans	222 nm	440	570	910	–
ATCC 13939	254 nm	1130	1420	1700	2050
Arthrobacter nicotinovorans	222 nm	104	149	177	198
ATCC 49919	254 nm	80	102	120	135
Staphylococcus aureus	222 nm	93	115	138	178
ATCC 25923	254 nm	44	60	73	95
Pseudomonas aeruginosa	222 nm	31	48	59	75
ATCC 27853	254 nm	8	16	23	31

seus. For *C. pasteurianum* the values for a reduction of 4-log steps at 222 nm are only 96 J/m² and at 254 nm 84 J/m². For *S. griseus* the values are 255 J/m² and 182 J/m². As a result, these two germs can be inactivated more efficiently with 254 nm. For the vegetative bacteria it can easily be recognised that the wavelength of 254 nm of the mercury lamp clearly inactivates almost all germs in a more efficient way than the 222 nm of the excimer lamp. The only exception is *D. radiodurans* which is with a sufficient UV fluence of 910 J/m² at 222 nm and 1700 J/m² at 254 nm for a 3-log reduction about 10 times more resistant against UV-radiation of both wavelengths than the other tested vegetative bacteria. Furthermore, for the same reduction it needs only half of the UV fluence at 222 nm than at 254 nm. With a 4-log reduction at 179 J/m² at 222 nm and 123 J/m² at 254 nm, the vegetative bacteria of *B. cereus* are approx. 10 times more sensitive against UV-radiation of both wavelengths than the spores. For *A. nicotinovorans*, an UV fluence of 198 J/m² at 222 nm and 135 J/m² at 254 nm was sufficient for a 4-log reduction. For the same reduction, an UV fluence of 178 J/m² and 95 J/m² was necessary for *S. aureus*. Here, the wavelength of 254 nm is almost twice as effective as at 222 nm. The most UV-radiation sensitive bacteria is *P. aeruginosa* with a necessary UV fluence of 75 J/m² at 222 nm and 31 J/m² at 254 nm for a 4-log reduction. For this case, the wavelength of 254 nm is more than twice effective.

4 Discussion

Contradictory results regarding the inactivation of microorganisms can be found in literature because of the different experimental conditions and the big variability of the organisms. For example, the UV fluences for a reduction of 1- and 3-log steps for *S. aureus* ATCC 25923 are 45 J/m² and 67 J/m² [9] and in the present examination 44 J/m² and 73 J/m² showing only a slight difference. In contrast, the UV fluence for *P. aeruginosa* is for 1-log step 48 J/m² [10], but in the present examination it is only 8 J/m². Another problem is the lack of literature dealing with the inactivation of microorganisms with wavelengths less than 254 nm. Here, only few papers can be found [1, 4–6, 11].

The investigated inactivation curves of the tested germs show approximately the expected typical sigmoid gradient. The only exceptions are the alternate curve gradients of the mold spores of *A. niger* and *P. expansum* for both wavelengths. These curves are close together, i.e. the inactivation of the spores each with 222 nm and 254 nm is nearly the same. Nevertheless, the excimer lamp seems to be better for an inactivation referring to *A. niger*. The obtained values of the one curve are in the range of the standard deviations of the other curve and *vice versa*. The data for *P. expansum* are not clear, too, because of the high standard deviations. The most UV resistant germ of all tested microorganisms is *A. niger*. Together with *P. expansum* it belongs to the group of eucaryotic organisms which are higher developed in contrast to the procaryotic bacteria. But this seems to have no general influence on the UV resistance, because *P. expansum* spores are clearly more sensitive than for example the spores of *B. cereus*. Even in previous investigations, cells of the eucaryotic yeast *C. albicans* ATCC 10231 showed a reduction of 4-log steps after an UV fluence of 290 J/m² with a wavelength of 222 nm and 230 J/m² with 254 nm. This necessary UV fluence is only slightly higher than in the present investigation for the vegetative bacteria. Also responsible for the high UV resistance of *A. niger* is the black spore pigment aspergillin which is certainly able to absorb high amounts of the UV radiation to protect the cell [12]. If highly efficient repair mechanisms were responsible for the resistance, the inactivation curve would show a higher shoulder [13].

In contrast to the vegetative bacteria of the same strains, the spores of *B. cereus* and also the ones of *Bacillus subtilis* ATCC 6051, tested in previous investigations [6], can be inactivated almost twice as effective with 222 nm than with 254 nm. For the spores of *B. cereus* and *B. subtilis* other authors have investigated similar ratios as well [11] but no detailed information was given about the irradiances of the lamps used and the bacterial strains.

Another investigation shows that the *B. subtilis* strain HA 101 and two derivates of it which were defective in excision repair and spore repair of spore photoproduct were investigated [5]. In addition, one of the strains carried a mutation which caused a defect in DNA polymerase I. It was revealed that the inactivation of the wild-type spores with 222 nm is better than with 254 nm, in contrast to the two defective strains. These results indicate that one reason for the better inactivation of spores with 222 nm is the damage caused to proteins like Polymerase I. Furthermore, the DNA in dormant spores of *Bacillus* and *Clostridium* species is complexed with a group of unique proteins, the α/β type SASP (small, acid-soluble proteins) [14]. These DNA protecting proteins are one reason for the high resistance of the spores, but they can be damaged more easily with 222 nm radiation than with 254 nm. Another reason is the presence of an enormous depot of pyridine-2,6-dicarboxylic acid (dipicolinic acic (DPA)) [14]. The two −COOH groups absorb the energy of UV light from about 215…225 nm in a selective way [15]. This could lead to a defect of the molecule and thus of the spores though the absorption of the pyridine is in the range of 255…265 nm. No clear evidence can be given for the spores of *C. pasteurianum* referring to the effectiveness of both wavelengths and the data collected in the present investigation. The necessary UV fluences for the excimer lamp are a little bit higher than for the mercury lamp, but the curves are very close together and the obtained values of the one curve are in the range of the standard deviations of the other curve and *vice versa*.

Remarkable is the UV sensitivity of the *Clostridium* spores although they are very similar to the *Bacillus* spores. One explanation for this could be the strictly anaerobic way of living in the soil, the natural habitat of these bacteria, which means no exposure to oxygen nor to sunlight. So special protect and repair mechanisms against UV radiation would be unnecessary. The same may be presumed for the spores of the aerobe soil bacterium *S. griseus.* They are also very sensitive to UV radiation, but they can be inactivated better with 254 nm in contrast to the spores of *T. vulgaris*. This species is also a typical soil bacterium, but shows an approximately 8 times higher UV resistance than *S. griseus* and can clearly be inactivated better with 222 nm. *T. vulgaris* is able to produce a polysaccharide layer which usually protects the cells against high temperatures. This could be a reason for the higher UV resistance.

Among the vegetative bacteria *D. radiodurans* is the most UV resistant species; even more resistant than the spores of *B. cereus*. One reason certainly are the effective repair mechanisms, clearly shown by the high shoulder of the 254 nm inactivation curve. At 222 nm this shoulder is considerably lower and even the necessary UV fluences for a respective inactivation are only about 50% of those with 254 nm. This indicates that the repair mechanisms were damaged. This thesis is also supported by the lower photoreactivation rate, done by the enzyme photolyase after irradiation with 222 nm [4].

There is another possible reason for the higher effectiveness of 222 nm regarding the inactivation especially for the UV resistant microorganisms. It could be the level of the UV fluence itself. If the UV fluence is high enough, many proteins are damaged to such an extent that there is no possibility for the cell to maintain the metabolism. This finally leads to cell death, no matter how effective the repair mechanisms are. Many procaryotic and eucaryotic cells are able to repair proteins to a certain extent [15]. They can either restore damaged polypeptides to an active stage or they can remove them [15]. However, protein synthesis is severely limited in starved bacteria [16]. Bacterial spores are in fact not starved but inactive. This also applies for the DNA repair mechanisms, which do not become active until germination and outgrowth of the spores. It can therefore be assumed that in a case where not only the DNA structure suffers damage (by irradiation with 254 nm) but also the proteins (by irradiation with 222 nm) which are responsible for various repair mechanisms the caused damage may lead to severe consequences for the cell which is indicated by the more effective inactivation of UV-resistant species.

The remaining vegetative bacteria are very UV sensitive. They all can be inactivated better with 254 nm. However, less than 200 J/m^2 UV fluence with the excimer lamp is sufficient for a 4-log reduction, depending on the species. The regulations of the DVGW [7] and the Trinkwasserverordnung [17] (Drinking Water Ordinance of Germany) prescribe for UV-devices in Germany a minimum UV fluence of 400 J/m^2 and a wavelength of 240…290 nm. With the demanded irradiation dose sufficient reductions for all tested bacteria are reached with the 222 nm wavelength of the excimer lamp. With this dose, there is a reduction of less than 4-log steps for the especially resistant microorganisms, however, the ratio still is more favourable for the excimer lamp than for the mercury lamp (cf. Table 1). Unfortunately, the wavelength of 222 nm is below the prescribed wavelength range. Therefore, this type of lamp must not be used for disinfection of drinking water at least in Germany.

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