INTRODUCTION

Preventing microbial contamination in the cell culture laboratory is essential to keep cell cultures contamination-free: a prerequisite to obtain reliable results and avoid costly and time-consuming damage control measures. The water required for heating baths, cooling systems, and particularly, trays of humidified CO_2 incubators is highly susceptible to contaminations, due to the inbuilt ideal environmental conditions for microbial growth (e.g. temperature, humidity).
WaterShield™ is an effective disinfecting and cleaning agent, intended as a water-additive for open water containers, active against a wide range of microorganisms. Therefore, adding WaterShield™ to water reservoirs, especially to humidity pans of cell culture CO_2 incubators, can greatly impact the reliability, duration, and costs of cell culture research.
Its formulation (as a 200X concentrated solution) includes surfactants, quaternary ammonium compounds and complexing agents, but no aldehydes, phenols, alcohols, or azides, and more generally, no active ingredient that can evaporate. WaterShield™ also contains a pigment conferring the solution a dark blue color. The light blue color of the diluted solution indicates the active status of the additive or the lack of possible contaminations. Due to the long-term incubation of cell cultures in humidified CO_2 incubators, one critical aspect of using water additives is the safety for cell samples. From a merely chemical perspective, the lack of potentially evaporating compounds in WaterShield™ should guarantee its safe use, when following the specific recommendations.
In this study, we assessed whether WaterShield™ retained its protective action, especially against representative fungi and bacteria, after 4 weeks use in water pans for CO_2 incubators humidification, in comparison with unsupplemented sterile water. In parallel, we monitored the color change of the diluted WaterShield™, in the attempt to verify the existence of a correlation between activity and indicator color intensity.
In addition, we investigated whether WaterShield™-supplemented water in evaporation-promoting conditions induced cytotoxic effects on mammalian cells.

PROCEDURES

1. Antifungal action of WaterShield™ over 4 weeks

Autoclaved water, supplemented or not with WaterShield™ at the recommended concentration (1:200; 5 ml in 1 liter H_2O), was added to the open water containers of 2 incubators (37 °C), at timepoint 0 (T0) for up to 4 weeks (T4). During the following 4 weeks, the incubators were handled as usual. At the time of addition as well as after 4 weeks, samples were collected from both water containers to verify whether antifungal activity of WaterShield™ decayed over time. Additionally, an aliquot of each sample was spiked with *Candida albicans* at the concentration of 100 CFU/ml, supplemented with 10 % Sabouraud medium to promote *C. albicans* growth during the following 6 days (37 °C). At that point, diluted samples (1: 100, 1: 1000, and 1: 10000) were grown on Sabouraud Agar plates for further 2 days. Colonies formation was documented by acquiring pictures every 24 h after plating.

2. Antibacterial action of WaterShield™ over 4 weeks

As described above, autoclaved water with or without WaterShield™ (1:200) was incubated in open water containers at 37 °C for up to 4 weeks. The use of the cell culture incubator during this time interval was nor limited or modified. Samples were collected from the freshly prepared WaterShield™ water solution (T0) or after 4 weeks incubation in humidified CO_2 incubator. An aliquot of each sample was spiked with *Bacillus subtilis* at the concentration of 100 CFU/ml, supplemented with 20 % LB medium to favor bacterial growth, and preincubated at 37 °C for 6 days. Dilutions of the samples (1: 1000; 1: 10000; 1: 100000) were plated onto LB agar plates and kept at 37 °C for further 2 days. Pictures of the cultured plates were acquired every 24 h after plating.

3. Monitoring of WaterShield™ (diluted) color change

The effect of time on WaterShield™ blue staining in open water containers was investigated by adding WaterShield™ to autoclaved water as recommended (1:200; 5 ml in 1 liter H_2O) and mixing thoroughly in sterile conditions. The solution was transferred to open water reservoirs of a cell culture incubator and stored for up to 4 weeks. Samples were collected at T0 (freshly prepared) and after 4 weeks. Absorbance at 664 nm was measured in duplicate immediately after collection by using a Beckman Coulter spectrophotometer UV/Vis Reader. The same batch of water without Wa-

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terShield™ was used as blank. Absorbance values were plotted against time (in weeks). Color intensity was documented also by acquiring pictures of the photometer cuvettes.

4. WaterShield™ effect on mammalian cells viability

The mammalian kidney cell line Vero was cultured in presence of DMEM supplemented with 5 % FCS, at 37 °C, 5 % CO2 until confluency (70 - 90 %). 3 days before the experiment, cells were passaged by trypsinization and transferred to 6-well cell culture plates at the density of 105 cells/well (in triplicates). Next, they were incubated (37 °C, 5 % CO_2) in confined incubator chambers and exposed to water containers filled with autoclaved water supplemented or not with WaterShield™ at the recommended concentration (1:200; 7.5 ml in 15 ml) or 10-fold higher (1:20; 75 ml in 15 ml H2O). After 3 days exposure to the above-mentioned conditions in confined environments, cell viability was assessed according to the Mossmann method (Mosmann T. J Immunol Methods. 1983; 65(1-2):55-63). Briefly, MTT (3-[4,5-Dimethyl-2]-2,5-diphenyl-2H-tetrazoliumbromid) 0.5 mg/ml was added to wells and incubated for 3 hours at 37 °C to allow the formation of formazan crystals. Shortly before measurement, the crystals were dissolved by replacing the MTT-medium mixture with DMSO 100 %. Measurement of absorbance at 562 nm was performed with Eppendorf Biophotometer UV/Vis Reader after dilution of the samples in PBS (1:10). Viability of cells placed in humidified CO_2 incubator without any additive (control cells) was set to 100 % for normalization and comparisons. The experiment was repeated 2 to 3 times.

RESULTS

1. Antifungal action of WaterShield™ over 4 weeks

Results of the fungi culture experiments showed that freshly added WaterShield™ completely blocked *C. albicans* growth in Sabouraud-medium-supplemented water, as indicated by the absence of colonies on the Sabouraud agar plates (Fig. 1C). In contrast, considerable growth could be observed when WaterShield™ was absent (Fig. 1A and B). These results could be fully reproduced after 4 weeks-use of WaterShield™ in humidified CO_2 incubator at 37 °C (Fig. 1D), indicating no significant loss of antifungal activity of the additive in 4 weeks.

2. Antibacterial action of WaterShield™ over 4 weeks

B. subtilis-contaminated, LB-supplemented water displayed substantial colony formation after 6 days liquid and 2 days solid culture (Fig. 1E). Pre-incubating this sample for 4 weeks resulted in comparable bacterial growth (Fig. 1F). Conversely, freshly added as well as pre-incubated WaterShield™ (for 4 weeks) completely blocked *B. subtilis* growth (Fig. 1G and H). Therefore, these results did not show any loss of antibacterial activity of the additive after 4 weeks incubation in humidified CO_2 incubator at 37 °C.

3. Monitoring of WaterShield™ (diluted) color change

Once diluted in H_2O and mixed, the WaterShield™ solution (1:200) appeared light blue (Fig. 2A). Upon 4 weeks incubation in the water tray of humidified CO_2 incubator, WaterShield™-supplemented water faded, turning to a slightly fainter light blue tone (Fig. 2B). Even though the absorbance measurement at 664 nm of samples from Fig. 2A and B confirmed the observed color decay (Fig. 2C), it should be noted that a 4 weeks incubation did not result in a dramatic color change of the WaterShield™ solution (e.g. colorless).

4. WaterShield™ effect on mammalian cells viability

3 days exposure to WaterShield™-supplemented water added to open containers in evaporation-promoting conditions did not produce any cytotoxic effects on the mammalian cell line Vero, compared to controls (non supplemented water), as shown by the results of the viability test MTT (Fig. 3).

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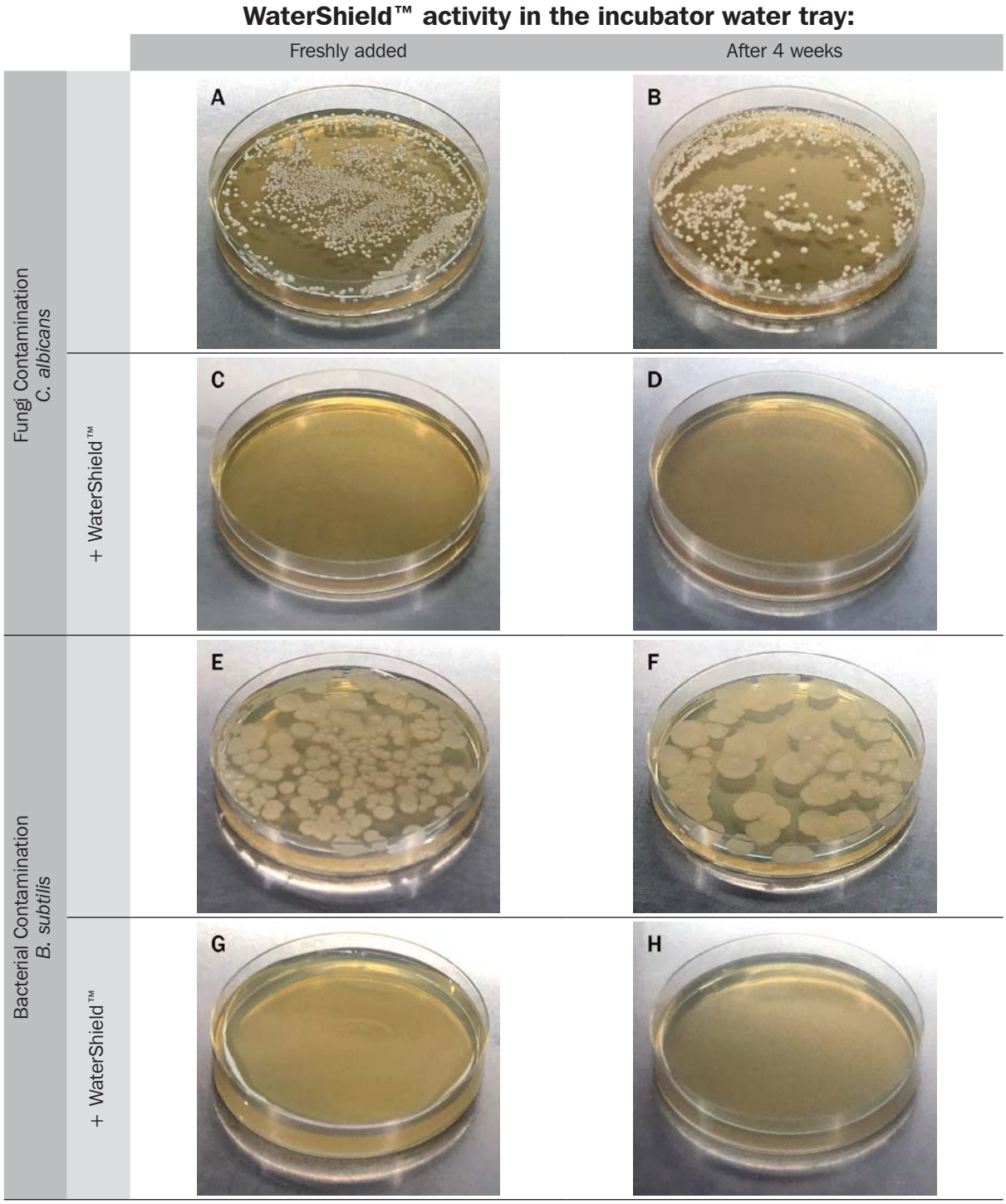

Figure 1. Antifungal and antibacterial action of WaterShield™ over time. Water samples collected from water containers at T0 (freshly added) or after 4 weeks storage at 37 °C, supplemented (C, D, G, H) or not (A, B, E, F) with WaterShield™ (1:200) and contaminated with fungi (*Candida albicans*, A-D) or bacteria (*Bacillus subtilis*, E-H). All samples were spiked with 100 CFU/ml of the respective species, grown for 6 days as liquid culture at 37 °C and after dilution (1:100 for *C. albicans*; 1:1000 *for B. subtilis*) grown on the corresponding agar plates for the following 2 days.

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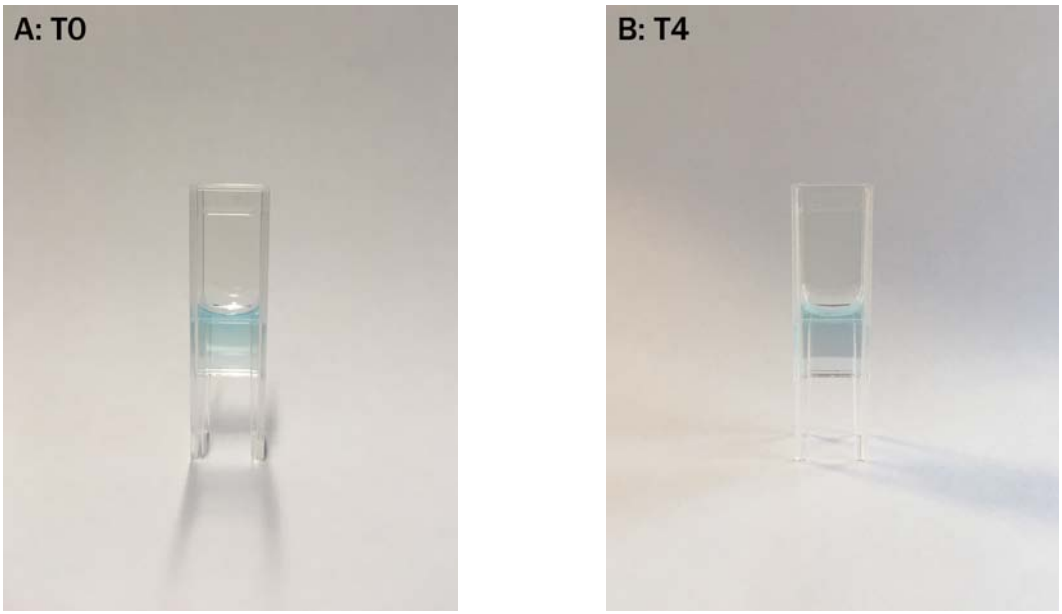

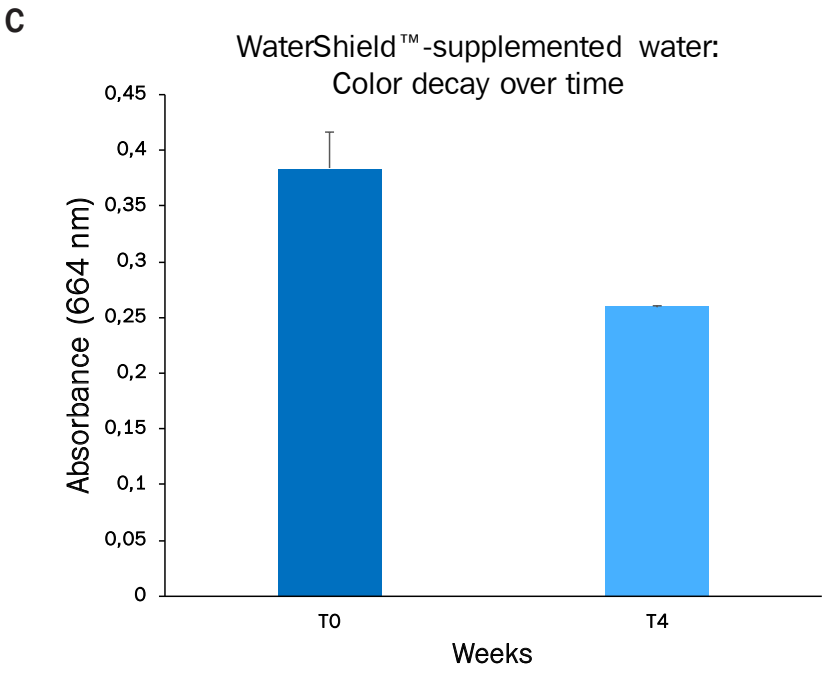

Figure 2. Blue stain of diluated WaterShield™ as an indicator of the reagent status. Due to the included blue dye, once diluted in water (T0), the WaterShield™ solution appears as in A. Over time in open containers at 37 °C, 5 % CO2, the light blue stain progressively fades, as shown in B (T4, after 4 weeks) and in the decline of absorbance at 664 nm (C).

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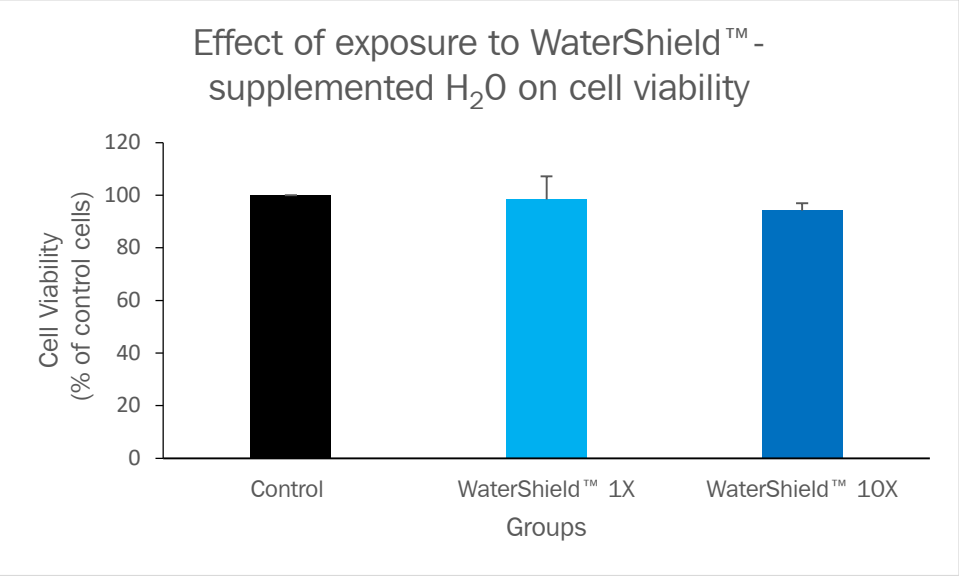

Figure 3. Lack of cytotoxicity of WaterShield™ as water additive in cell culture incubators. Viability of Vero cells after 3 days exposure to WaterShield™-containing or not water in containers and conditions favoring evaporation (e.g. humidity water pan or similar) was tested by MTT assay. Cell viability expressed as % of control cells (exposed to water-only) was not altered by the addition of WaterShield™, up to 10X the recommended concentration (1X) to incubators, suggesting that indicated use of WaterShield™ in cell culture is safe for mammalian cells. The experiment was repeated 3 times (2 in the case of WaterShield™ 10X), each with 3 replicates/condition.

CONCLUSIONS

The results of the experiments included in this study showed that adding WaterShield™ to water pans in cell culture incubators dramatically reduces the contamination risk associated to such laboratory equipment. Importantly, WaterShield™ protective action against bacteria and fungi was fully retained during 4 weeks use in a humidified CO_2 incubator tray (at 37 °C).

Furthermore, the color indicator contained in the reagent only partly decayed over time, in the incubation conditions described above. This is in line with the observation that the 4 weeks preincubated WaterShield™ solution significantly retained activity. Altogether these data suggest that the blue stain of diluted WaterShield™ reflects the activity status of the additive. A more dramatic color decay or discoloration (to colorless or yellowish) may occur in case of an increased bioburden, as in the case of a contamination, and indicates that a renewal of the water solution is necessary.

Importantly, the use of WaterShield™ in incubators for cell culture obviously raises questions about its potential cytotoxicity. According to our data and in agreement with the chemical composition of the additive (lacking any evaporating compound), the incubation of mammalian cells in presence of WaterShield™-supplemented water (incubator tray) did not affect cell viability.

In conclusion, WaterShield™ addition to humidity pans for cell culture incubators has several advantages depending on its effective disinfecting and cleaning properties and its safety for cell culture samples, experimenters, and equipment materials, compared to alternative products.

It should be noted that using WaterShield™ does not replace either a regular cleaning/disinfecting schedule for CO_2 incubators or frequent renewal or replenishment of the water tray, which remain golden rules for cell culture contamination control.

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