Impact of standard test protocols on sporicidal efficacy

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Summary

Background: There is an increase in commercially available sporicidal formulations. Any comparison of sporicidal data from the literature is hampered by the number of different standard tests available and the use of diverse test conditions including bacterial strains and endospore preparation.

Aims: To evaluate the effect of sporicidal standard tests on the apparent activity of eight biocides against *Clostridium difficile* and *Bacillus subtilis*.

Methods: The activity of eight biocidal formulations including two oxidising agents, two aldehydes, three didecyldimethylammonium chloride and amine formulations, and sodium hypochlorite were evaluated using four standard sporicidal tests, BS EN 14347, BS EN13704, ASTM E2197-11 and AOAC MB-15-03 against *B. subtilis* (ACTC 19659) and *C. difficile* (NCTC 11209) spores.

Findings: *C. difficile* spores were more susceptible than *B. subtilis* ones to the sporicides, regardless of the method used. There were differences in sporicidal activity between methods at five min but not at 60 min exposure. DDAC and amine based products were not sporicidal when neutralised appropriately. Neutralisation validation was confirmed for these biocides using the reporting format described in the BS EN standard tests, although looking at raw data neutralisation failed.

Conclusions: The different methods, whether based on suspension or carrier tests, produced similar sporicidal inactivation data. This study suggests that detailed neutralisation validation data should be reported to ensure that neutralisation of the active is effective. Failure to do so may lead to erroneous sporicidal claims.

Introduction

Bacterial endospores are far less susceptible to biocidal products than their vegetative counterparts.[1-3] Sporicide is the term used to define biocidal products that can destroy spores, although the term sporistatic has also been used.[1,2,4] The mechanisms leading to a sporistatic or sporicidal effect have recently been reviewed.[5] The structure of the endospores explains their resistance to biocidal products, notably the presence of spore coats, small acid soluble proteins (SASPs), a highly compressed spore membrane and low water content.[3] To measure the efficacy of sporicides against specific bacterial endospores a number of standard sporicidal tests are available.[4] In Europe, there are not yet specific test protocols to measure the efficacy of sporicides against *Clostridium difficile*, although recently Fraise and colleagues[6] proposed a UK-suspension test against this pathogen. The use of different standard protocols against different spore formers and different bacterial strains make the comparison of sporicidal activity of biocidal products difficult.[7,8] Test parameters such as concentration of biocide, contact time, spore strain, concentration of spores, spore preparation and purification, and organic load often differs between studies. The neutralisation of the biocide/biocidal products is also important to determine their sporicidal effect,[4] but is not always effective potentially leading to inappropriate product claims.[5,9] Empirically only a small number of biocides principally oxidising and alkylating agents have been shown to be sporicidal.[1,2,3,7]

This study aims to compare the activity of a number of biocides/biocidal products against *Bacillus subtilis* (the standard strain in EN tests) and *C. difficile* using a number of standard test protocols commonly used in Europe and the USA.

Materials and Methods

Bacterial strains

Two spore producing bacteria were used in all testing procedures: *Clostridium difficile* (NCTC 11209) and *Bacillus subtilis* (ACTC 19659). Both bacteria are relevant to standard disinfectant testing procedures. Vegetative bacterial cells for both strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at -80ºC (+-1ºC). Liquid spore stock cultures of *C. difficile* were cultured using the Clospore method.[10] This liquid medium was chosen as it enables the production of large concentrations of purified *C. difficile* spores.[6,10] *Bacillus subtilis* liquid spore cultures were prepared in accordance with the ASTM method E2197-11.[11] Spore suspensions were washed, re-suspended in phosphate buffered saline (Fisher Scientific, Loughborough, UK) and stored at 4ºC for one month before use. Regular enumeration and sterility checks were performed to ensure spore stock purity. Total spore count was measured using a haemocytometer. The percentage of germinating spores was estimated by comparing total count and viable spore count (after germination) for each bacteria. The percentage of germinating spores was 88.06% for *C. difficile* and 83.49% for *B subitilis*. A viable count was performed prior to each test. The average counts of viable spore stock for *B. subtilis* and *C. difficile* were 7.02 ± 0.59 and 7.39 ± 019 Log_{10} respectively.

Formulations, biocides and neutralisation

Eight formulated biocides were tested for their sporicidal activity at five and 60 min with four different standard test procedures (Table 1). All disinfectants were provided by Anios (Lille, France) except for sodium hypochlorite which was purchased from Fisher Scientific (Loughborough, UK). The disinfectants were as follows: glutaraldehyde (GTA; tested at 2% v/v; pH 6.0); *ortho*-phthalaldehyde (OPA; tested at 0.55 and 0.65% v/v; pH 7.0); didecyldimethylammonium chloride (DDAC; labelled 191501; tested at 1%; pH 6.0); bis (aminopropyl) laurylamine (labelled 191502; tested at 1% w/v; pH 11.5); a combination of DDAC (1% w/v) and bis (aminopropyl) laurylamine (1% w/v) (labelled 191503; pH 11.0); two oxidisers: ANIOXY-TWIN (tested at 1200 ppm) and ANIOSEPT ACTIV (tested at 2% v/v); sodium hypochlorite (NaOCl; tested at 5000 ppm; pH 7.8). ANIOSEPT ACTIV was made 2 h before use. All tests were performed at 20ºC in clean conditions (Table 1).

The aldehydes, oxidisers, and sodium hypochlorite were neutralised after five and 60 min contact time with a solution composed of 5 g/L sodium thiosulphate, 30 g/L tween 80, 30 g/L saponin, 1 g/L L-histidine and 3 g/L azolectin (Fisher Scientific, Loughborough, UK). This universal neutraliser was initially used to quench the activity of DDAC and amines when the BS EN14347[12] protocol was used. Filtration neutralisation according to the BS EN 13704[13] was subsequently used for all test protocols.

Neutralisation toxicity and efficacy to quench each biocide were confirmed with the aldehyde, oxidising agents and sodium hypochlorite. The failure of chemical neutralisation to quench the activity of DDAC and amines was further investigated whereby both chemical neutralisation and neutralisation by filtration were compared following exposure to biocides at three concentrations (0.5, 1 and 2% v/v).

Modification to standardised testing procedures

Four sporicidal test protocols were used in this study; the BS EN 14347[12],the BS EN 13704,[13] the ASTM E2197–11,[11] and the AOAC MB-15-03.[14] Due to the nature of this study standardised test methods were modified somewhat to ensure test consistency. We were interested in studying the effect of the test procedures themselves on sporicidal activity and not the effect of the spore preparation and viable count enumeration protocols. With this in mind, all testing procedures followed enumeration with the pour plating method in accordance with BS EN 14347.[12] Apart from the preparation of spore stock and enumeration of viable count following exposure, the test procedures described in the standard were strictly followed.

Reproducibility

Unless otherwise mentioned, tests were carried out in triplicate on three separate occasions. The data analysed were normally distributed (Shapiro-Wilk test; $P>0.05$) and with this in mind t-test ANOVA, MANOVA and post-hoc Tukey tests were conducted to analyse the results using SPSS® software where appropriate.

Results

This study produced data that enables an understanding of the effect of the test protocols on the sporicidal activity of biocides against two distinct endospores. For the purpose of this study a biocide formulation was deemed to be sporicidal if it achieved >4 $\log_{10}$ reduction in spore number. Our results showed that GTA was not sporicidal even after 60 min exposure (Fig. 1 & 2). The other aldehyde, OPA, also failed to achieve a 4 $\log_{10}$ reduction in *B. subtilis* spores even after 60 min contact (Fig. 2b), but was sporicidal against *C. difficile* spores after 60 min exposure (Fig. 1b). The DDAC and amine formulations tested were not sporicidal when neutralisation by filtration was used (Fig. 1 & 2). The effect of chemical neutralisation *vs.* neutralisation by filtration is further developed later. The two oxidising formulations ANIOSEPT ACTIV and ANIOXY-TWIN were sporicidal after 60 min exposure (Fig. 1b & 2b). The sporicidal activity attained with the oxidising formulations depended upon the test performed (Post-hoc Tukey; $P<0.005$; 95% CI). Overall the oxidising formulations performed significantly better (MANOVA, $P=0.0000$; 95% CI) than the other biocides tested regardless of the spore strain and contact time despite that on occasions they failed to achieve a four $\log_{10}$ reduction in spore number; ANIOXY-TWIN only achieved a 3.65 ± 0.00 $\log_{10}$ reduction with *B. subtilis* spores using the AOAC MB-15-03 after five min contact (Fig. 2b), and ANIOSEPT ACTIV produced only a 2.12 ± 0.11 against *C. difficile* spores with the ASTM E2197 at 5 min (Fig. 1a) and 2.15 ± 0.00 $\log_{10}$ reduction in *B. subtilis* spores when tested with the AOAC MB-15-03 at five min (Fig. 2a).

Sodium hypochlorite (5000 ppm) was used as a positive control owing to the amount of information in the literature on the sporicidal activity of this biocide. Sodium hypochlorite was sporicidal against *C. difficile* after five min exposure regardless of the test protocol used (Fig. 1a) but sporicidal against *B. subtilis* only after 60 min contact (Fig. 1b & 2 b). There was a significant difference (One-Way ANOVA, $P=0.0000$; 95% CI) in susceptibility to sodium

hypochlorite between *C. difficile* and *B. subtilis* spores, the former being more susceptible, regardless of the protocols and contact time used.

Overall *C. difficile* spores were more susceptible than *B. subtilis* spores to the biocide tested regardless of the test used (MANOVA and post-hoc Tukey, P=0.0000; 95% CI). When the type of test is considered, there was no significant difference (Post-hoc Tukey; P>0.05 ; 95% CI) in the biocide efficacy against spores between suspension (BS EN 14347 and BS EN13704) and carrier (AOAC MB-15-03 and ASTM E2197). Overall BS EN14347 produced better sporicidal activity (Post-hoc Tukey, P<0.05 ; 95% CI).

To investigate the appropriateness of the neutralisation method, the efficacy of chemical neutralisation *vs.* filtration neutralisation was investigated further using the BS EN 13704 protocol, which measures the efficacy of the neutralisation method to quench the activity of the biocide using three concentrations of a given formulation (Table 2). According to our results and normal reporting of the data following the layout of the standard, both chemical and filtration neutralisation validation passed (Table 2) when the DDAC and amine formulations were investigated. However, when one looks further at the reporting details, it was clear that chemical neutralisation failed to inactivate the DDAC and amine formulations with no viable count being observed at the lowest dilutions (Table 3).

Discussion

This study made a number of interesting observations. The first one is that *C. difficile* spores were more susceptible than *B. subtilis* ones. This is the first time that a study investigated the activity of different biocidal formulations against the spores of two different species conjointly using the same test protocols. The comparison of sporicidal activity between products and spore strains has been difficult to date because different methods, contact time, spore concentration and spore preparation protocols have been used.[7,8] The preparation of *C. difficile* spore inoculum including spore purification, age of spore stock and

types of recovery media used have been shown to affect spore viability and/or sporicidal activity.[10] Here, we used the Clospore method[10] for all *C. difficile* spore preparation and purification, not only ensuring spore stock consistency and viability but also generating a spore concentration high enough to demonstrate >4 $\log_{10}$ reduction in spore number regardless of the test protocol used. In Europe, there is no standard test yet available for evaluating the sporicidal activity of a product against *C. difficile* with parameters relevant to the healthcare industry.[4,6] The recent publication of a suspension test specific to *C. difficile* is timely.[6] Surface tests are preferred to evaluate the activity of sporicides on surfaces, notably because suspension tests are often considered to be less stringent than carrier tests.[7] Here, we observed that there were no significant differences in test performance between surface (ASTM E2197 and AOAC MB-15-03) and suspension tests (BS EN14347 and BS EN13704), however, the suspension test BS EN 14347 performing generally better than the other tests performed. The potential impact of "super-dormant" spores[15] was not considered in this despite that the percentage of germinating spores was <90%. None of the standard sporicidal tests specified that "super-dormant" spores need to be measured. Comparing the effect of sporicides based on spore viability might underestimate the susceptibility of the overall spore suspension used.

The importance of controlling pathogenic spore formers on surfaces demands reassurance that a sporicide/biocidal product will produce the same results regardless of the bacterial species/strain or standard tests used. Biocides that are documented as sporicides, the oxidisers,[1,2] all achieve a >4 $\log_{10}$ reduction against all spores within 60 min contact time. There were however differences in the level of sporicidal activity achieved between protocols at 60 min exposure. While some protocols demonstrated a 6 $\log_{10}$ reduction in spore number, others only showed a 4 or 5 $\log_{10}$ reduction (Fig. 1b & 2b). At five min contact time we observed differences in activity against the same spore strain between protocols particularly with the oxidising agents.

In our study GTA performed poorly in our study. This is not entirely surprising as GTA pH was 6.0. It has been well document that GTA microbicidal activity is pH dependent and a better activity is observed at an alkaline pH.[16] In addition, GTA is known to be a slow sporicide with long contact time (>1 h) necessary to achieve a significant (>5 $\log_{10}$) reduction in spore numbers.[7] Conversely, it was interesting to note that OPA (0.55% pH 7) was sporicidal against *C. difficile* after 60 min exposure (Fig. 1b) but not against *B. subtilis* (Fig. 2b). These results are in agreement with the literature; OPA 0.5% or 0.6% at room temperature was shown not to be sporicidal against *B. subtilis*,[17,18] although sporicidal activity could be restored with a higher concentration and pH (2% w/v OPA; pH8) following long exposure time (270 min).[17] OPA 0.55% (Cidex OPA®) was shown to be very effective against three strains of *C. difficile* (SJ1, PCR-ribotype 135); HU17, PCR-ribotype 133 and a hypervirulent strain, BI/NAP1/027) within 30 min contact time.[19]

The oxidising formulations showed the best activity against both spore species, although ANIOSEPT ACTIV activity at five min was at times considerably lower than that of ANIOXY-TWIN. These formulations are based on peracetic acid. However, ANIOXY-TWIN contains peracetic acid while ANIOSEPT ACTIV is based on peracetic acid generator, which might explain the differences in sporicidal activity. Overall, the results with the oxidising agents are in agreement with the literature. Oxidising agents such as peracetic acid, hydrogen peroxide and chlorine dioxide have been shown to have a significant (>5 $\log_{10}$) sporicidal activity within five-30 min against various spore genera including *B. subtilis* and *C. difficile*.[7,19] It is interesting to note that, despite the recorded sporicidal activity, the microbicidal mechanism of action between oxidisers differs,[20] notably the interaction of peracetic acid and hydrogen peroxide with spores.[21]

Sodium hypochlorite is widely used as a sporicide[7,8] and was used as a positive control in our study. It was interesting to observe that *C. difficile* spores were more susceptible to sodium hypochlorite than *B subtilis* ones, highlighting the difference in susceptibility between the two spore formers.

The DDAC and amines tested in this study did not show any sporicidal activity. This is in accordance with our knowledge of sporicides and non-sporicides.[1,2,5] Our study however showed that neutralisation validation data needs to be closely examined. We observed that the normal data reporting as described in standard tests was insufficient to demonstrate that the DDAC or amines were neutralised appropriately. To date there are still a number of amine/QAC–based products that claim to be sporicidal. Although some sporicidal claims cannot be substantiated,[9] these may be based on a correct reporting of neutralisation validation when standard test instructions are followed. Here we highlighted the discrepancy between normal reporting of neutralisation validation and a more in depth analysis of neutralisation data. This neutralisation validation issue needs to be addressed to ensure that sporicidal claims for biocidal products can be fully substantiated.

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Conflict of interest

J Criquelion and G Rauwell are employees of Laboratoire Anios. They participated to the study design, provided the biocidal products and participated to the manuscript. All data production and data analyses were carried out by Cardiff University.

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Table 1. Standard test protocols used in this study

Test Method	Nature of test	**Organic load:** Bovine serum albumin	**Surface material**
BS EN 14347[12]	Suspension test	N/A	N/A
BS EN 13704[13]	Suspension test	0.30%	N/A
ASTM E2197 - 11[11]	Hard surface test	0.30%	Stainless Steel
AOAC MB-15-03[14]	Hard surface test	0.30%	Porcelain

Table 2 Differences between chemical neutralisation and neutralisation by filtration for quenching the activity of amine formulations. Activity against a) *B. subtilis* spores and b) *C. difficile* spores (n=2)

a) *B. subtilis* spores

	Log_{10} Reduction								
	0191501			0191502			0191503		
	0.5%	1%	2%	0.5%	1%	2%	0.5%	1%	2%
Neutralisation	0.28	0.46	0.30	0.26	0.47	0.27	0.24	0.43	0.31
Filtration	0.25	0.44	0.27	0.73	0.48	0.86	0.27	0.46	0.50

b) *C difficile* spores

	Log_{10} Reduction								
	0191501			0191502			0191503		
	0.5%	1%	2%	0.5%	1%	2%	0.5%	1%	2%
Neutralisation	0.53	0.66	0.69	0.81	0.66	0.76	0.49	0.66	0.58
Filtration	1.00	0.18	0.82	0.81	-0.16	1.23	0.84	0.40	1.17

Table 3 Detailed colonies counted at each dilution after chemical neutralisation and filtration neutralisation for a) *B. subtilis* spores and b) C. difficile spores exposed to amine formulations

a) *B. subtilis* spores

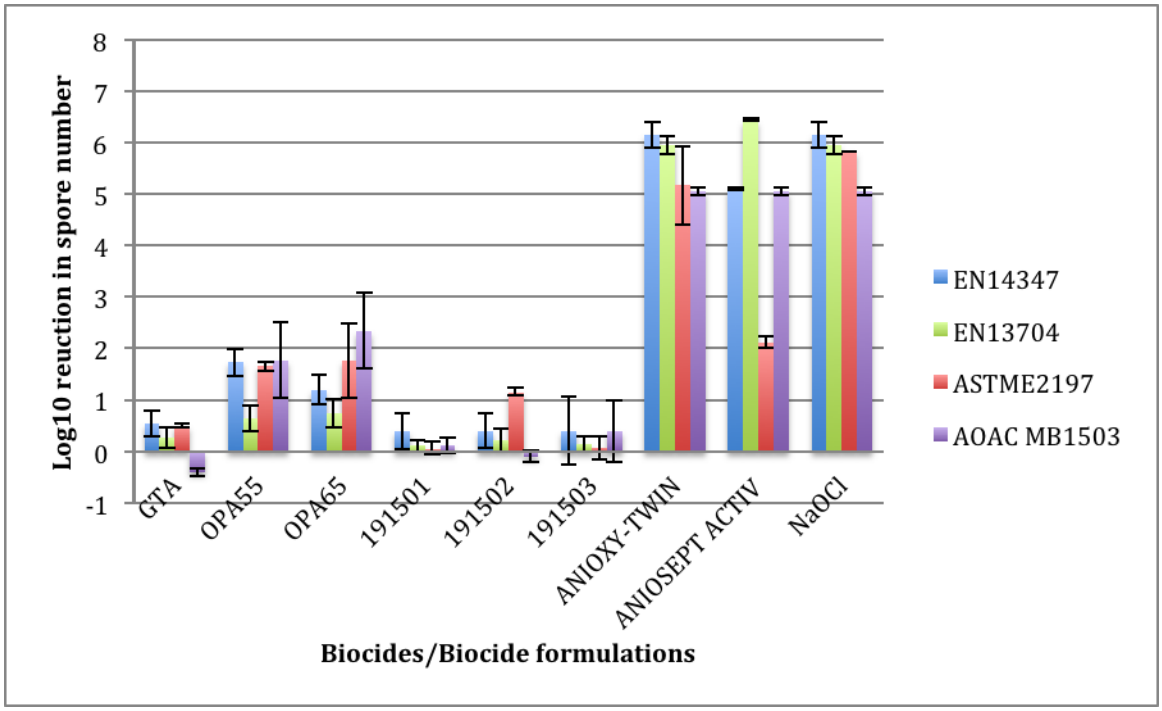
	Bacillus subtilis							
	Total colony count (CFU) following:							
	Filtration				**Neutralisation**			
Dilution	**Neat**	**-1**	**-2**	**-3**	**Neat**	**-1**	**-2**	**-3**
0191501								
0.5%	>300	>300	>300	>300	0	0	>300	294
1%	>300	>300	>300	>300	0	0	0	>300
2%	>300	>300	>300	300	0	0	0	280
0191502								
0.5%	>300	>300	>300	>300	0	0	>300	>300
1%	>300	>300	>300	>300	0	0	>300	>300
2%	>300	>300	>300	101	0	0	>300	298
0191503								
0.5%	>300	>300	>300	300	0	0	>300	>300
1%	>300	>300	>300	>300	0	0	>300	>300
2%	>300	>300	>300	199	0	0	0	276

b) *C. difficile* spores

	Clostridium difficile							
	Total colony count (CFU) following:							
	Filtration				**Neutralisation**			
Dilution	**Neat**	**-1**	**-2**	**-3**	**Neat**	**-1**	**-2**	**-3**
0191501								
0.5%	>300	>300	>300	62	>300	>300	>300	187
1%	>300	>300	>300	90	0	0	>300	>300
2%	>300	>300	>300	97	0	0	>300	133
0191502								
0.5%	>300	>300	>300	51	>300	>300	>300	97
1%	>300	>300	>300	196	0	>300	>300	>300
2%	>300	>300	>300	38	0	0	>300	110
0191503								
0.5%	>300	>300	>300	50	>300	>300	>300	206
1%	>300	>300	>300	55	0	0	>300	>300
2%	>300	>300	>300	43	0	0	>301	170

Figure 1. Activity of biocide formulation against *C. difficile* spores after a) 5 min and b) 60 min exposure using the different test protocols: BS EN14247, BS EN13704, ASTM E2197 and AOAC MB-15-03 (n=3).

a) 5 min exposure



b) 60 min exposure

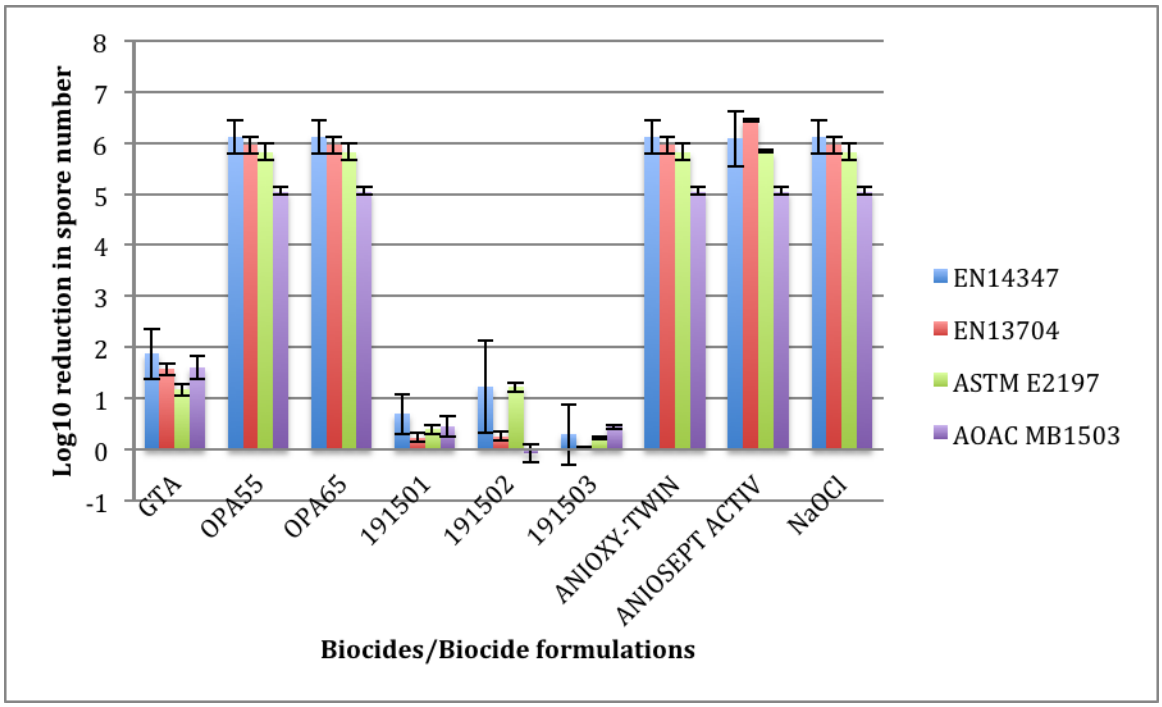
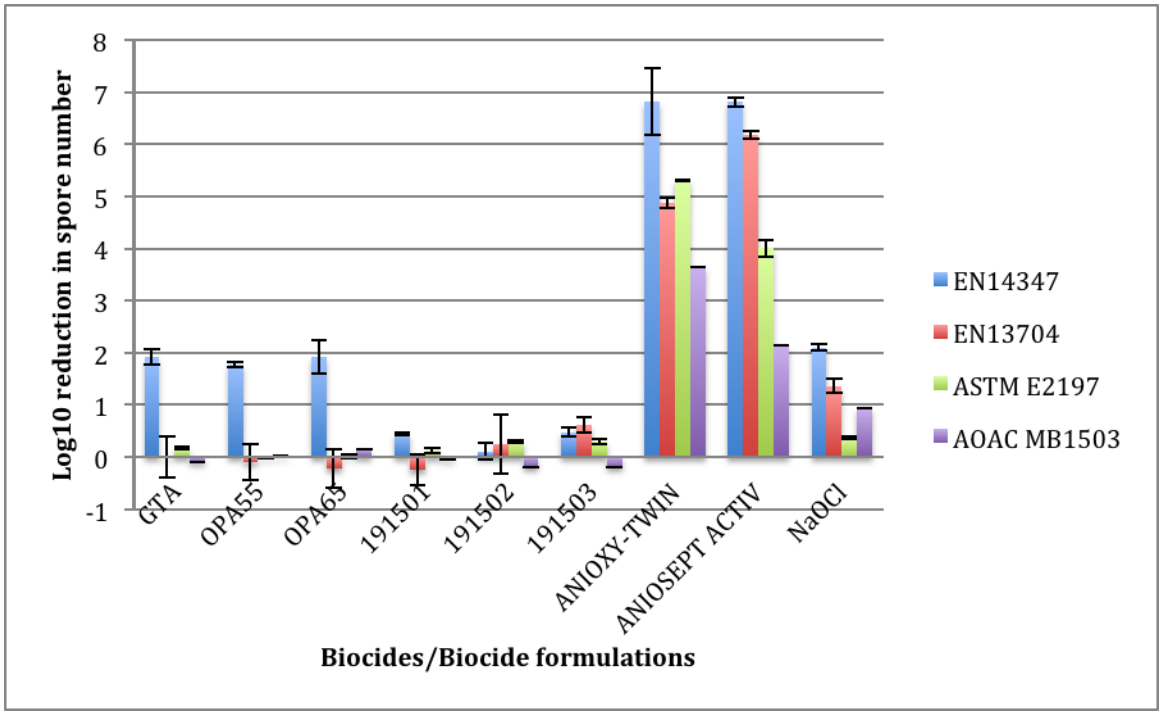


Figure 2. Activity of biocide formulation against *B. subtilis* spores after a) 5 min and b) 60 min exposure using the different test protocols: BS EN14247, BS EN13704, ASTM E2197 and AOAC MB-15-03 (n=3).

a) 5 min exposure



b) 60 min exposure