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Validation of Microbial Recovery of Pharmaceutically Important Gram-negative Bacteria from Peroxygen/Silver based Disinfectants and Evaluation of their Degree of Corrosiveness

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Abstract: Evaluation of a neutralization method and the degree of corrosiveness of disinfectants is a crucial initial step that precedes biocidal agents efficacy study. One of the major factors affecting the choice of the disinfectants is their aggressiveness towards materials of construction in the pharmaceutical facility. Two dilutions of each of four commercially used peroxygen/Ag^+-based disinfectants were examined against four Gram-negative microorganisms using Fluid Thioglycolate Medium (FTM) broth for both chemical and physical neutralization. The acceptance criteria of a test group was ≥ 70 % of the control group for each replicate group namely neutralizer efficacy and neutralizer toxicity. Statistical analysis using One-way analysis of variance (ANOVA) was carried out to confirm success/failure of a suspect result. By this analysis; five suspect treatments passed the neutralizer efficacy test. Modified FTM-thiosulfate (FTMT); FTM neutralizer, was tested under the same conditions against the same index of microorganisms and compared to a previously used and tested FTM neutralizer. Neutralization of the modified FTM neutralizing broth was more effective than the previously reported FTM with peroxygens/Ag^+ disinfectants especially 1:100 dilution. Using higher dilutions has minimized the reaction time in the present study. In addition, a gravimetric corrosion test was performed to determine the degree of corrosiveness of disinfectants. Bixco showed greatest corrosion rate when compared with other commercially used peroxygens which were not significantly different from the control. Corrosion test is a useful tool to determine the preference among a range of disinfectants especially if they belong to the same class.

Key words: Peroxygen/Ag^+, Neutralizing broth, Gram-negative bacteria**,** Fluid Thioglycolate Medium, Corrosion test.

Introduction

Diminishing of biocidal activity of chemical compounds is a crucial initial prerequisite for many assessment studies that are focused on the evaluation of the potencies of the antimicrobial components and recovery of bioburden from controlled environment. Neutralization procedure assessment should be carried out for each organism/disinfectant combination, to demonstrate the ability of the culture medium to support growth of any viable microorganism. In general, neutralization and microbial recovery studies are designed per compendial guidelines methods for validation of microbial recovery as detailed in the United States Pharmacopeia (USP) Chapter <1227> "Validation of Microbial Recovery from Pharmaceutical Articles" as described by Clontz [1].

The residual amount of the biocidal agent can be neutralized by chemical neutralizers; several classes of biocides have well-established chemical neutralizers [2] and examples of these are noted in (Table 1**)** as illustrated by Sutton, *et.al*.[3]. How-

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Table 1. Previously reported efficacy against biocides and toxicity on microbial cells for neutralizers [3]

Neutralizer*	Antimicrobial compound to be neutralized	Toxicity on microorganisms
Bisulphate	Glutaraldehyde, Mercurials	Non-spore forming bacteria
Dilution	Phenolics, Alcohol, Glutaraldehyde	None
Glycine	Glutaraldehyde	Growing Cells
Lecithin	Parabens, Bis-Biguanides, Quaternary Ammonium Compounds (QACs)	Bacteria
Magnesium or Calcium ions	EDTA	None
Polysorbate	QACs, Iodine, Parabens	None
Thioglycolate	Mercurials	Spores and Staphylococci
Thiosulphate	Glutaraldehyde, Halogens, Mercurials	Staphylococci

*All neutralization methods are based on chemical compounds except dilution technique

ever, a main challenge of neutralization of biocides using chemical compounds is the potential toxicity demonstrated by many classes of neutralizers. Thus, the evaluation of a chemical neutralizer scheme should consider the examination of the potential toxicity of the neutralizer as well as its efficacy. A common example of the toxicity of some neutralizer components is that of thioglycolate against Staphylococci; in addition to spores [4-7]. Another example is thiosulfate toxicity against Staphylococci [5,8-10].

Complete abolishing of disinfectants activity is crucial for the accuracy of a biocidal efficiency determination, as microbicidal effect is usually assessed as still-viable along definite time interval and suppression of microbial growth by the remaining non-neutralized amounts of biocide may lead to over estimated measures of microbicidal activity [11]. A work done by Russell describes three principle criteria to judge effectiveness of the neutralizer. First of which; the neutralizer must effectively stop the effect of the biocidal solution. Secondly, the neutralizer must not itself be unduly toxic to the challenge organisms. Finally, both the neutralizer and the active agent must not react to form a toxic byproduct. On the same line, three methods have been published describing methods of neutralizer evaluation [12].

Unfortunately, there is no ideal disinfectant, antiseptic or preservative. All chemical agents have their limitations either in terms of their antimicrobial activity, resistance to organic matter, stability, incompatibility, irritancy, toxicity or corrosivity. To overcome such limitations of an individual agent, formulations consisting of combinations of agents are available. For example, some combinations are synergistic, e.g. hydrogen peroxide (intermediate antibacterial activity level) and peroxygen compounds. The germicidal properties of hydrogen peroxide (H_2O_2) have been known for more than a century, but the use of low concentrations of unstable solutions did little for its reputation. However, stabilized solutions are now available and due to its unusual properties and antimicrobial activity, hydrogen peroxide has a valuable role for specific applications. Concentrations of 3-6 % are effective for general disinfection purposes. Peracetic acid (CH_3CO_3H) is the peroxide of acetic acid and is a more potent biocide than hydrogen peroxide, with excellent rapid biocidal activity against bacteria. Similarly, silver have long been known to have antibacterial properties and preparations of these metal were among the earliest used antiseptics, silver will inhibit enzymes in the membrane, and for that matter in the cytoplasm, which contain thiol, -SH, groups [13,14].

To obtain accurate data a from neutralization study, considering the inoculums count of each organism incorporated in the study is a must. The most common ranges to be accepted for countable numbers of colonies on a plate are 30-300 [15,16]. However, experimental studies have dem-

onstrated very poor accuracy in plate counts below 25 CFU per plate at which Error is 1/5 of the mean. Theoretically, it can be argued that because the CFU follow the Poisson distribution, the error of the estimate is the square root of the average [17]. This leads to results as those illustrated in (Table 2), Equations (1) and (2) were derived from (Table 2) for count range from 1 to 30 CFU/plate. Equations (3) and (4) are derived from (1) and (2), respectively by applying the inverse of the Logarithm:

$$\text{Log E.P.} = 2 - (\text{Log N.C./2}) \quad \text{(Eq.1)}$$

$$2 \text{ Log S.E.} = \text{Log N.C} \quad \text{(Eq. 2)}$$

$$\text{E.P.} = 10^{(2-((\text{Log N.C.})/2))} \quad \text{(Eq. 3)}$$

$$\text{S.E.} = 10^{((\text{Log N.C.})/2)} \quad \text{(Eq. 4)}$$

Where, E.P. = is the error as percent of mean. S.E. = is the standard error. N.C. = is the number of CFU/plate.

Equation (3) demonstrates that increasing the number of CFU/plate reduces error percent of the mean. However, at the same time -from Equation (4) -standard error increases but in less magnitude (1 to 5.48) than that of the error percent of the mean (100 % to 18.3 %) when the number of CFU increases from 1 to 30 per plate.

Regarding the process risk assessment; a variety of tools has been used in pharmaceutical industry to determine the critical points in the process that are at high risk of being contaminated with microorganisms [18]. On the same line, *Hazard Analysis and Critical Control Points (HACCP)*, which was developed in the 1970s by the U.S. Department of Agriculture to address food safety, is a systematic, proactive, and preventative tool to identify, assess, and prevent or reduce the potential risks that can occur at specific steps in a process. Through the risk analysis process, critical control points are identified and monitored [19].

Several new corrosive antimicrobial-based biocidal agents such as chlorine and peroxygens that are commercially available contain anticorrosive substances. Corrosion Inhibitors (ex. 3-amino-5-mercapto-1,2,4-triazole (AMTA)) are widely used in the protection of metals such as copper against corrosion in different corrosive media [20]. Similarly, carbon steels are commercially available metals, which are used in the fabrication of reaction vessels, storage tanks, etc. Corrosion behavior of steels is widely investigated in inorganic acids, salts, non-oxidation organic acids, alkaline solutions, and marine media, and many inhibitors were also suggested to retard the corrosion of steel in the above-mentioned solutions. However, corrosion behavior and corrosion inhibition of carbon steel in oxidation media, especially in strong oxidizing disinfectant solutions were rather scant. Strong oxidizing disinfectant solutions have wide range of biocidal activity; they can be used to kill bacteria, algae, yeasts, moulds, fungi and viruses [21]. Peroxygen disinfectants are very active, and

Table 2. Error as a percentage of mean for plate counts covering range from 1 to 30 CFU/plate and standard error [17]

CFU per Plate	Standard Error	Error as % of Mean	CFU per Plate	Standard Error	Error as % of Mean	CFU per Plate	Standard Error	Error as % of Mean
30	5.48	18.3	20	4.47	22.4	10	3.16	31.6
29	5.39	18.6	19	4.36	22.9	9	3.00	33.3
28	5.29	18.9	18	4.24	23.6	8	2.83	35.4
27	5.20	19.2	17	4.12	24.3	7	2.65	37.8
26	5.10	19.6	16	4.00	25.0	6	2.45	40.8
25	5.00	20.0	15	3.87	25.8	5	2.24	44.7
24	4.90	20.4	14	3.74	26.7	4	2.00	50.0
23	4.80	20.9	13	3.61	27.7	3	1.73	57.7
22	4.69	21.3	12	3.46	28.9	2	1.41	70.7
21	4.58	21.8	11	3.32	30.2	1	1.00	100.0

are not affected by organic matter, but some products may be corrosive to alloys, aluminum and plain steel. Peracid solutions are a mixture of peracetic acid, hydrogen peroxide and acetic acid; the two latter compounds possess a synergistic effect with peracetic acid. They are extremely effective biocides and have no toxic residuals [22], but their acidic pH makes them corrosive to some materials when used at high concentrations. Mechanistically, peracids are active against all types of microorganisms, including spores, and retain activity in the presence of organic matter [23].

The current study was designed to optimize the use of combined chemical neutralization and dilution for the recovery of Gram-negative microorganisms using commercial peroxygen-based biocidal agents. Herein, two studies were carried out, firstly: disinfection validation program and the degree of its corrosiveness. The second study involved the microbial recovery in the presence of residual disinfectant in environmental monitoring (EM) media.

Materials and methods

Preparation of microbial suspension

Standard strains were purchased from the American Type of Culture Collection (ATCC, Manassas, Virginia) and handled as per a standard procedure. As for the bacterial environmental isolates, they were isolated and identified using the automated microbial biochemical identification system VITEK® 2 (bioMérieux, Inc., 100 Rodol-phe Street, Durham, NC 27712). All the culture media were purchased from OXOID (Basing-stoke, Hampshire) and chemicals from Sigma-Alrich (St. Louis, MO 63103). (Table 3) shows the list of microorganisms used in current study, their sources, family and some of their biochemical reactions.

Standardized stable suspensions of test strains were prepared and used as stated by their suppliers. Seed-lot culture maintenance techniques (seed-lot systems) were used so that the viable microorganisms used for inoculation are not more than 5 passages removed from the original master seed-lot. All organisms were stored at -80°C in a validated -86°C Ultra low temperature freezer (-86 Degree ULT Freezers, Qingdÿ Shandong, China) in validated cryogenic environment and reactivated only prior to the study conduction using standard method illustrated by the supplier. All media were sterilized by autoclaving in a steam sterilizer (FEDEGARI FOB3, Fedegari Autoclavi SpA, SS 235 km 8, 27010 Albuzzano (PV), Italy). All pH measurements and weighing procedures were done using Mettler-Toledo S20 Seven Easy™ pH Meter and XPE Analytical Balance, respectively (Mettler-Toledo, LLC 1900 Polaris Parkway Columbus, OH 43240).

Suspensions were quantified by making serial dilutions and performing duplicate plate counts using conditions and media suitable for each microorganism to choose suspensions of concentration $3x10^2$-10^3 CFU/50-100 µl as working suspensions. Microbial test suspensions should be used as soon as the results of serial dilutions could be enumerated using a digital colony counter (Digital Colony

Table 3. List of Gram-negative rod microorganisms challenged in neutralizer validation study with the source, family and some of the biochemical reactions of each

Challenged microorganisms	Source	Family	Catalase/Oxidase/ Indole
Escherichia coli	ATCC8739	*Enterobacteriaceae*	+/-/+
Pseudomonas aeruginosa	ATCC9027	*Pseudomonadaceae*	+/+/-
Salmonella enterica subsp. Enterica serovar Typhimurium	ATCC14028	*Enterobacteriaceae*	+/-/-
Sphingomonas picamobilis	EM isolate[a]	Sphingomonadaceae	+/+/-

a = Slow-growing biofilm former organism isolated from early processing stages in water treatment station and identified by VITEK 2 System version 5.02 (bioMerieux, France)

Counter Model: 361, Laxman Mahtre Rd. Navagaon, Dahisar West, Mumbai, India).

Neutralization validation study of peroxygen-based biocidal agents

The purpose of this study was to ensure that the assumed contact time is optimum. In other words, the time is valid for both the neutralizing agent to efficiently stop the action of the tested sanitizer, and at the same time the neutralizing agent should not have any inhibitory or toxic effect on any of the microorganisms. It was suggested that two comparisons among three populations would be performed. The first study was concerned with the *Neutralizer Efficacy* (NE) which can be determined by evaluating the number of survivors in the neutralizing broth in the presence and the absence of the biocide. Furthermore, the ability of the neutralizing broth alone to allow the survival was a second important consideration in this analysis. The second comparison involved the *Neutralizer Toxicity* (NT). This aspect of neutralization was determined by comparing survivors in the neutralizing medium without the biocide with the viability (growth) control [3,24].

The test solutions were freshly prepared and diluted in the same conditions that simulated actual usage environment of biocidal agents using the highest concentration which is 5 % (v/v) as recommended by the manufacturer. These commercial disinfectants were denoted Bixco (hydrogen peroxide/Ag^{+}), BafD (hydrogen peroxide/Ag^{+}), Pur (hydrogen peroxide/peraceticacid/Ag^{+}) and Mil (hydrogen peroxide/Ag^{+}). Using a neutralizing broth as diluent 1:10 and 1:100 (v/v) dilutions of test solution- i.e. Disinfectant final concentrations/10 ml of neutralizers were 5 % and 0.5 % (v/v), respectively were made at working concentration, then 1 ml is transferred of each dilution to each of duplicate petridishes this is test group. Neutralizer exposed group is prepared in parallel in the same manner as test group but using sterile saline or buffer instead of test solution. While viability control group is prepared using peptone water without test solutions or neutralizing broth. Organisms were prepared so that the required inoculums did not exceed 0.5-1.0 % (v/v) of the total volume in the tubes. Neutralizers used in the study were of normal strength for Fluid Thioglycolate Medium Broth (FTM) and double strength for Fluid Thioglycolate Medium Thiosulfate (FTMT).

Inoculums of each of the used microorganisms were added to each of the above described tubes so that the final count per plate of positive control was range 30 to 100 CFU per plate. Then about 20 ml of molten suitable medium at 45°C was added; allowed to solidifying, then incubating at suitable temperature for 30-35°C for 3 days in BD 115 incubator (BINDER GmbH, ImMittleren Ösch 5 D-78532 Tuttlingen). After that; duplicate plate counts were prepared for the 3 groups. The negative control study for each media with the same volume of diluents or neutralizers added was carried out to ensure sterility of all used materials. All the tests and control groups were performed in triplicates for each microorganism, disinfectant and dilution. Residual peroxide was determined using a semi-quantitative colorimetric method by peroxide test strips (Peroxid-Test: MQuant™, Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany) at intervals in plain neutralizer with biocidal agents at two dilutions used in the test.

Gravimetric corrosion test

Gravimetric corrosion measurement technique was mainly based on the percentage weight-loss regime of the test specimens. In applying this method, the weights of test specimens (hollow cylindrical rings) were obtained before and after a specified time interval of 96 hours (final weight, $mt_{fin.}$). The initial weights (initial weight, $mt_{int.}$) of the test specimens were recorded before immersion in the test solution while the change in weights was taken after the test pieces were rinsed in water and dried using a filter paper [25]. The gravimetric measurements were reproduced twice at room temperature range of 20 to 25°C. Control coupons were exposed to the same conditions of washing and time but not to the test solutions. The initial and final weights of control coupons were denoted by $mc_{int.}$ and $mc_{fin.}$, respectively [26]. The test was accelerated by using easily corroded steel material of density approximately 7.6 g/cm^3 and

challenged by the maximum working concentration of disinfectant solutions of 5 % (v/v) and purified water USP as control vehicle.

At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of CFU recovered from the challenge product is not less than 70 % of that recovered from the inoculums control (USP<1227>, 2013) and test for outlier data within each set of groups was conducted using statistical package software to confirm the results homogeneity and validity [27]. If there were suspect results within the groups, One-way ANOVA was performed on Log 10 transformed results to confirm significance followed by Dunnett's Multiple Comparison Test which was used to confirm success or failure of the test and Tukey's Multiple Comparison Test to compare between individual groups at α <0.001. The gravimetric corrosion test calculations were carried on according to the method described by Vasilescu et al. [26]. The weight loss (Δm, mg), the corrosion rate (K_{Corr}, mg.cm ^{H2}. h ^{H1}) were calculated as follow:

Δm_c :$mc_{int.}$ - $mc_{fin.}$$\Delta m_t$ = $mt_{int.}$ - $mt_{fin.}$$\Delta m$ = Δm_t - Δm_c

KCorr = Δm/A.t

Where,

Δm_c : weight loss of the control material, mg.

Δm_t : weight loss of the test material, mg.

Δm: the actual weight loss of the test material due to exposure to specific corroding substance to which control material is not exposed mg.

$mc_{int.}$:the initial weight of control coupon material prior the initiation of the test, mg.

$mc_{fin.}$:the final weight of control coupon material after the completion of the test, mg.

$mt_{int.}$:the initial weight of test coupon material prior the initiation of the test, mg.

$mt_{fin.}$:the final weight of test coupon material after the completion of the test, mg.

K_{Corr} : the corrosion rate, $mg.cm^{-2}.h^{-1}$.

t : the exposure time, hours.

A: the surface area of the coupon and is the exposure time in hours.

The penetration index (P, mm. $year^{-1}$) in metallic mass was also determined using the following expression:

$P = (K_{Corr}/\rho) \times 8.76$

Where,

P : the penetration index, $mm.year^{-1}$.

ρ : the density of metal, g/cm^3

8.76 : conversion factor, taken into account that one year has 8760 hours.

According to the penetration index stability of the metal coupons against the liquid tested materials used in this study and the degree of corrosion could be determinedas detailed by some researchers [26]. Finally, all statistical analysis was performed using GraphPad Prism version 5.00.288 for the corrosion test and version 6.01 for the microbial recovery test. Any interpretation or complex calculation was performed using Microsoft Excel 2007.

Results

NT study revealed that both FTM and FTMT did not possess any adverse or toxic effects on tested Gram-negative microorganisms and passed the acceptance criteria. The recovery ratio of the four microorganisms was e"1 except for 0.97 for *Salmonella enterica subsp. entericaserovar Typhimurium* with FTMT. All NT results did not require statistical analysis by Dunnett's multiple comparisons test and they were not significantly different from each other using Tukey's multiple comparisons test at α<0.001. The preliminary NT test results were presented in (Table 4) as geometric means and subjected to comparison with each other and against USP<1227>acceptance criterion in (Fig. 1).

Table (4) shows the geometric means of NE ratio for the four microorganisms with four disinfectants at two dilution levels. Statistical analysis was conducted on the transformed microbial counts of the four microorganisms' groups including five suspect results then they were subjected to Dunnett's Multiple Comparison Test at α<0.001 which revealed non-significance of the difference between means. These suspect groups were from *Pseudomonas aeruginosa* and *Sphingomonas paucamobilis* in FTM with Pur and Bixco, respectively and from *Salmonella enterica* with Mil and Pur and *Sphingomonas paucamobilis* with Bixco in FTMT. These results are demonstrated based on the USP <1227>

Table 4. NT and NE ratios derived utilizing the geometric mean of the recovery in the different populations of NT and NE for the tested Gram-negative bacilli against Commercial peroxygen/silver biocidal agents at two dilution levels: 1:10 and 1:100 (v/v)

G. mean[a]			*E.coli*		*S.enterica*		*P.aeruginosa*		*S.paucimobilis*	
			FTM	FTMT	FTM	FTMT	FTM	FTMT	FTM	FTMT
NT			1.07	1.14	1.07	0.97	1.20	1.10	1.11	1.08
	Bixco	1:10	0.75	1.13	0.81	0.90	0.43	0.83	0.67[b]	0.58[b]
		1:100	0.87	1.15	0.81	1.47	0.78	1.07	0.89	0.98
	BafD	1:10	0.09	0.58	0.72	0.76	NA	0.26	NA	NA
NE		1:100	0.56	0.90	0.80	1.10	0.49	0.86	0.96	1.00
	Mil	1:10	0.22	0.59	0.62	0.67[b]	NA	0.27	NA	NA
		1:100	0.55	0.88	0.81	0.83	0.36	0.82	1.01	1.05
	Pur	1:10	0.22	0.43	0.23	0.73[b]	NA	0.26	NA	NA
		1:100	0.54	0.75	0.54	1.02	0.58[b]	0.84	0.86	1.02

a = Geometric means

NA = Not applicable as the replicate contains one zero or more

b = Group set having result(s) below 70 % but passed statistical test for NE

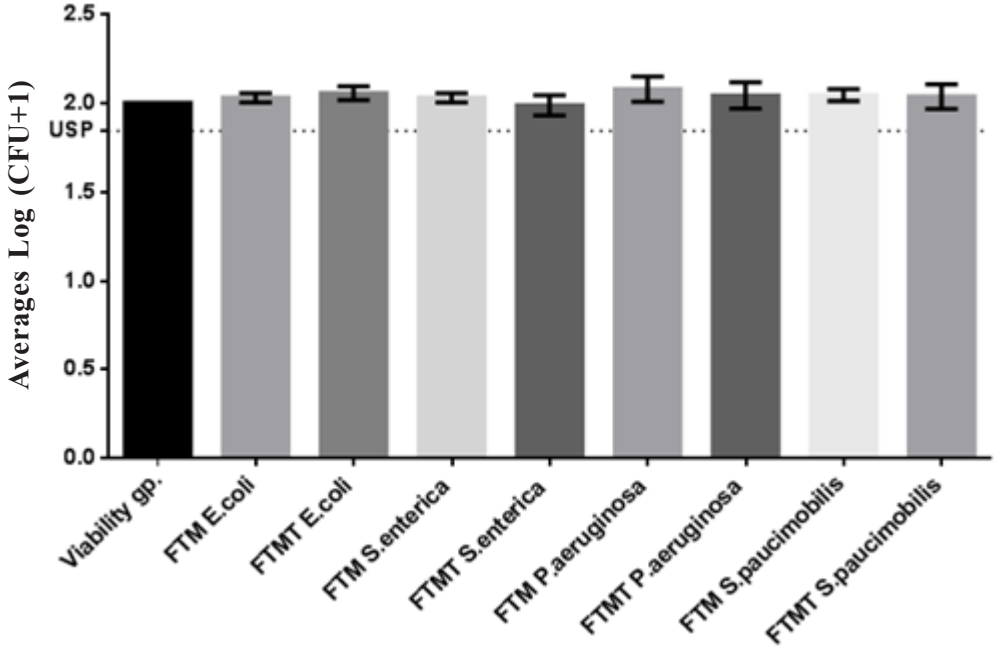

Fig. 1. Viability control bar of FTM and FTMT for *Escherichia coli* ATCC 8739, *Salmonella enterica subsp. enterica serovar Typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027 and *Sphingomonas picamobilis* and NT of both chemical neutralizers on specified Gram-negative rods. All results are expressed as averages of Log10 transformed (CFU+1) ± S.D. Dashed line represents acceptance criteria for both neutralizers according to United States Pharmacopeia Harmonized USP<1227>Validation of Microbial Recovery from Pharmacopoeial Articles at 70 %. (Results were analyzed using GraphPad Prism for Windows version 6.01)

acceptance criterion in (Figs. 2, 3, 4 and 5).

Statistical comparison between groups in NE study of the four microorganisms with the four biocidal agents revealed superiority of FTMT over FTM in terms of microbial recovery at both dilutions with Bixco especially showing greatest recovery over the other three disinfectants. These results were illustrated in (Figs. 2, 3, 4 and 5). By comparing FTMT and FTM neutralization profile of all groups, it was found that FTM total rate of success and failure was 14 and 18 per 32 while that of FTMT was 23 and 9 per 32 test groups, respectively. The ease of neutralization of commercial peroxygen disinfectants was in the following decreasing order: Bixco (15/16)>BafD (8/16)>Mil=Pur (7/16). By increasing dilution ratio from 1:10 to 1:100 more than half of the failed results passed the test and the failed microbial count plates decreased to about half for both FTM and FTMT together. However, for FTM alone there is 2.5 increase in the rate of success when dilution ratio was increased from 1:10 to 1:100 while for FTMT, the success of total microbial recovery increased from less than 50 % to 100 % thus, the impact of ten-times dilution of peroxygen-based disinfectants on neutralization success of microbial recovery was significant and increased the microbial recovery by more than a factor of two. In terms of total microbial recovery from the four disinfectants after neutralization *Salmonella enteric* demonstrated highest recovery rate from the chemical neutralization study (81 %) followed by *Sphingomonas paucimobilus* (63 %) then *Pseudomonas aeruginosa* and *Escherichia coli* (44 %).

`The results illustrated in (Table 5) indicated that peroxide elimination reaction time in FTMT varied from nearly instantaneous with Bixco at both dilutions to delayed for 1.83 to 2 minute with BafD at 1:100 (v/v). Mil and Pur 1:100 (v/v) were in between both disinfectants i.e. 1.3 to 1.5 minute. BafD, Mil and Pur at 1:10 (v/v) showed continuous presence of peroxides during test time in the neutralizer indicating inadequacy of neutralization.

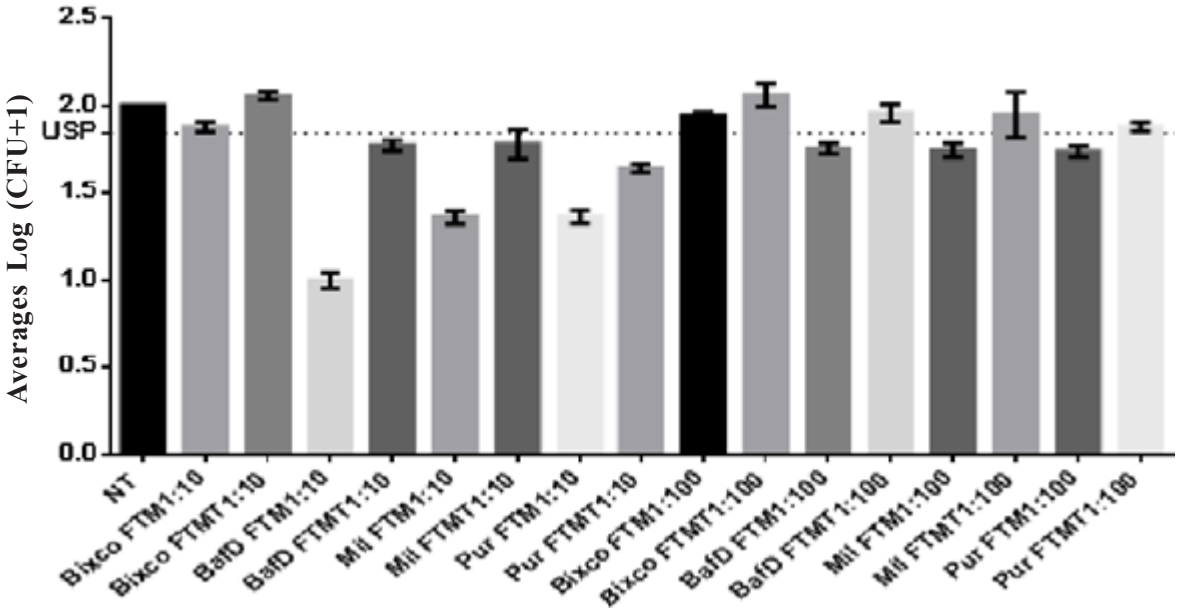

Fig. 2. NT control bar of FTM and FTMT for *Escherichia coli* ATCC 8739 and NE of both neutralizers with Bixco, BafD, Mil and Pur at two dilutions ratios. All results are expressed as averages of Log10 transformed (CFU+1) ± S.D. Dashed line represents acceptance criteria for both neutralizers according to United States Pharmacopeia Harmonized USP<1227>Validation of Microbial Recovery from Pharmacopoeial Articles at 70%. (Results were analyzed using GraphPad Prism for Windows version 6.01)

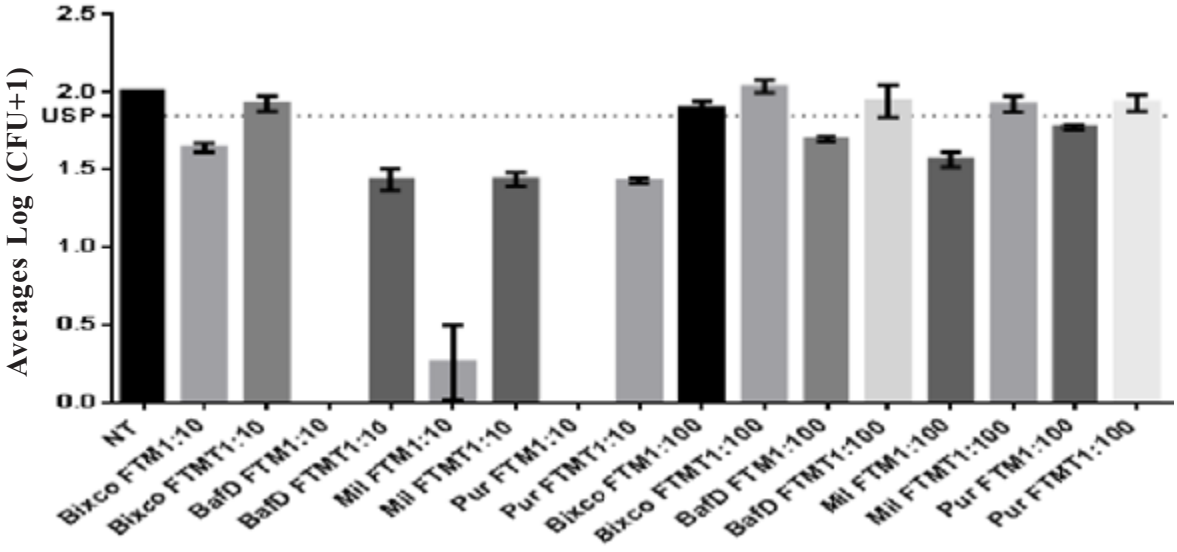

Fig. 3. NT control bar of FTM and FTMT for *Pseudomonas aeruginosa* ATCC 9027 and NE of both neutralizers with Bixco, BafD, Mil and Pur at two dilutions ratios. All results are expressed as averages of Log10 transformed (CFU+1) ± S.D. Dashed line represents acceptance criteria for both neutralizers according to United States Pharmacopeia Harmonized USP <1227> Validation of Microbial Recovery from Pharmacopoeial Articles at 70 %. (Results were analyzed using GraphPad Prism for Windows version 6.01)

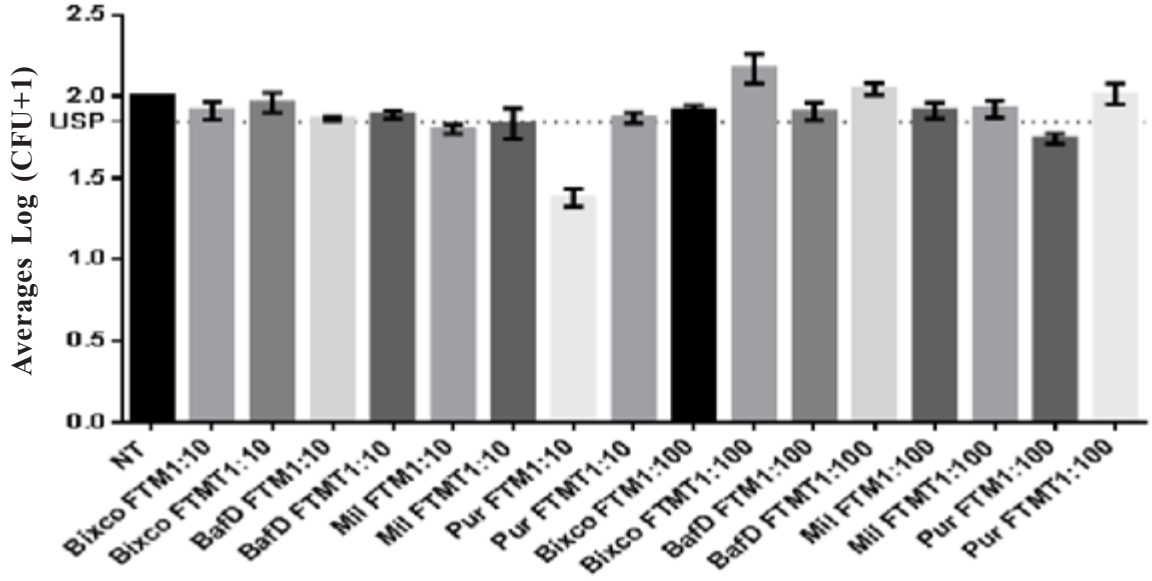

Fig. 4. NT control bar of FTM and FTMT for *Salmonella enterica subsp. enterica serovar Typhimurium* ATCC 14028 and NE of both neutralizers with Bixco, BafD, Mil and Pur at two dilutions ratios. All results are expressed as averages of Log10 transformed (CFU+1) ± S.D. Dashed line represents acceptance criteria for both neutralizers according to United States Pharmacopeia Harmonized USP <1227> Validation of Microbial Recovery from Pharmacopoeial Articles at 70 %. (Results were analyzed using GraphPad Prism for Windows version 6.01)

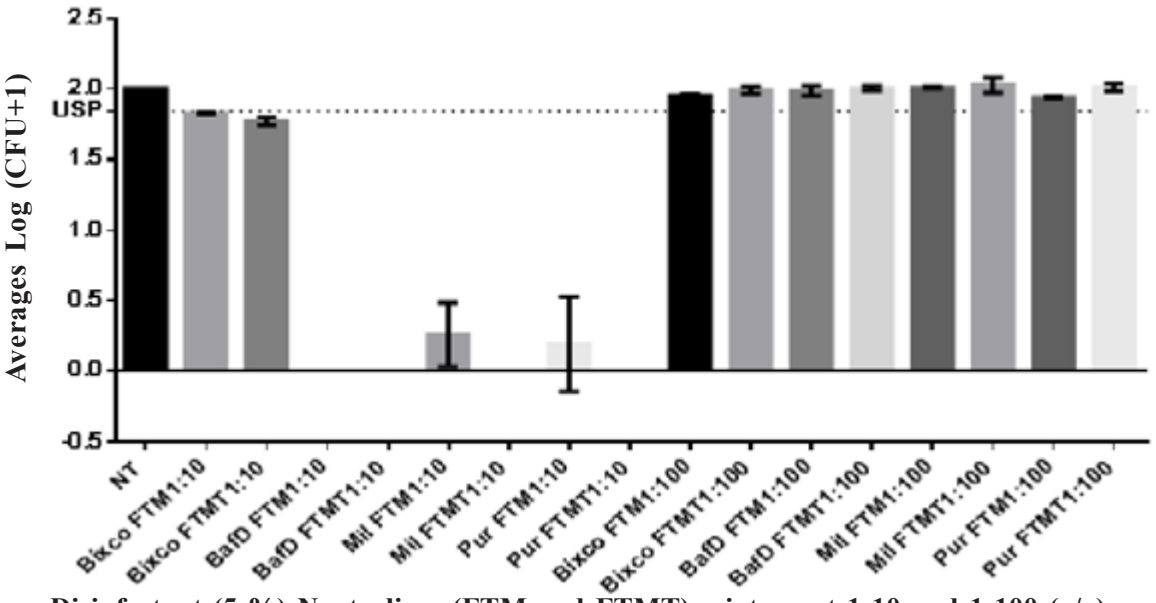

Fig. 5. NT control bar of FTM and FTMT for *Sphingomonas picamobilis* as water-born isolated from biofilm in water treatment facility and NE of both neutralizers with Bixco, BafD, Mil and Pur at two dilutions ratios. All results are expressed as averages of Log10 transformed (CFU+1) ± S.D. Dashed line represents acceptance criteria for both neutralizers according to United States Pharmacopeia Harmonized USP<1227>Validation of Microbial Recovery from Pharmacopoeial Articles at 70%. (Results were analyzed using by GraphPad Prism for Windows version 6.01)

In addition, FTM showed similar pattern but at 1:100 (v/v) dilution of BafD, Mil and Pur the time till reach undetectable level of peroxide (i.e. 0 ppm) was between 3.5 to 4 minutes (i.e. about twice the time needed for FTMT to eliminate residual peroxides level). The pH of each of FTM and FTMT, without additives, was 7.02 and 6.58 at normal laboratory temperature; respectively. However, Bixco was an exception among the tested disinfectants being rapidly and effectively neutralized with each of the two neutralizing broths. At 1:10 (v/v) dilution, the approximate average ranges of the tested residual peroxides of BafD, Mil and Pur along an interval of 15 minutes: 10-25, 5-10 and 10-25 ppm in FTM and 5-10, 5-10 and 5-10 ppm in FTMT, respectively. When test was repeated but at 1:100 (v/v) dilution till disappearance of detectable peroxides levels the results were: 10-25(3 minutes), 10-25(3 minutes) and 5-10 (2.5 minutes) ppm in FTM and 0.5 (2 minutes), 0.5 (1 minute) and 0.5-2 (1 minute) ppm in FTMT, respectively.

On the other hand, the corrosion test performed using a gravimetric method showed, according to the results demonstrated in (Fig. 6), that mass loss was significant only for Bixco only while the remaining disinfectants were not significantly different from control at $\alpha<0.001$. The relative corrosion values were arranged in a descending order as follows: Bixco (44.36), BafD (5.29), Mil (5.21) and Pur (2.64). Furthermore, (Fig. 7) showed that the corrosion rates were in the following descending order: Bixco>Purified water>Mil=BafD>Pur. However, according to the penetration index of the metal coupons exposed to each of the test solutions, the stability class/ corrosion resistance degree towards each of Bixco, purified water USP, Mil, BafD and Pur was based on the results shown in (Table 6) as follows II/4, II/3 and II/2 (for the last three), respectively. It was noted that, the result of Pur was very close to perfectly stable I/1.

Discussion

This study was carried on as a part of a major project of sanitization validation including differ-

Table 5. Semi-quantitative colorimetric Peroxide test performed on four disinfectants: Bixco, BafD, Mil and Pur at two dilutions levels: 1:10 (v/v) and 1:100 (v/v) in both FTM and FTMT

Time (minutes)	Bixco (pH 4.74)[a]				BafD (pH 4.03)[a]				Mil (pH 3.91)[a]				Pur (pH 3.25)[a]			
	FTM		FTMT		FTM		FTMT		FTM		FTMT		FTM		FTMT	
	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)	1:10 (v/v)	1:100 (v/v)
0.3-0.5	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1.25-1	-	-	-	-	+	+	+	+	+	+	+	±	+	+	+	±
1.3-1.5	-	-	-	-	+	+	+	±	+	+	+	-	+	+	+	-
1.83-2	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	-
2.5-3	-	-	-	-	+	±	+	-	+	+	+	-	+	+	+	-
3.5-4	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-
4-4.5	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-
pH[b]	6.84	6.98	6.57	6.62	6.26	6.43	6.32	6.47	6.24	6.41	6.35	6.44	6.07	6.49	6.45	6.50

(-) = Zero mg/l (ppm)
(±) = 0-0.5 mg/l (ppm)
(+) = >0.5 mg/l (ppm)
a = pH determinations were done in range20-25°C for working concentrations of the disinfectant at 5% (v/v)
b = pH determinations were done in range 20-25°C for biocidal agent-neutralizer mixture

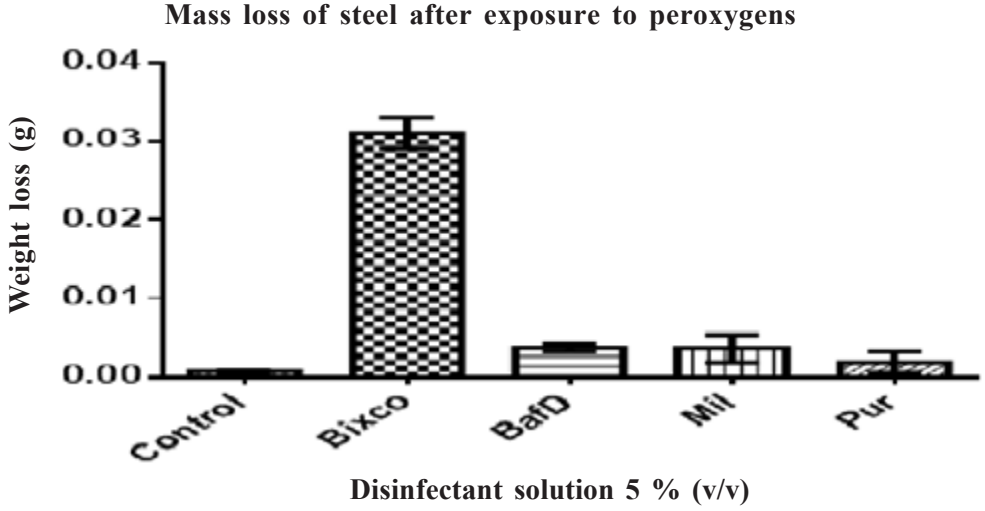

Fig. 6. Mass loss (g) of steel after 96 hours exposure at room temperature to commercial peroxygen biocidal agents and compared with control groups exposed to air only. Results are expressed as mean ± standard error of the mean (SEM). (Generated by GraphPad Prism version 5.00.288)

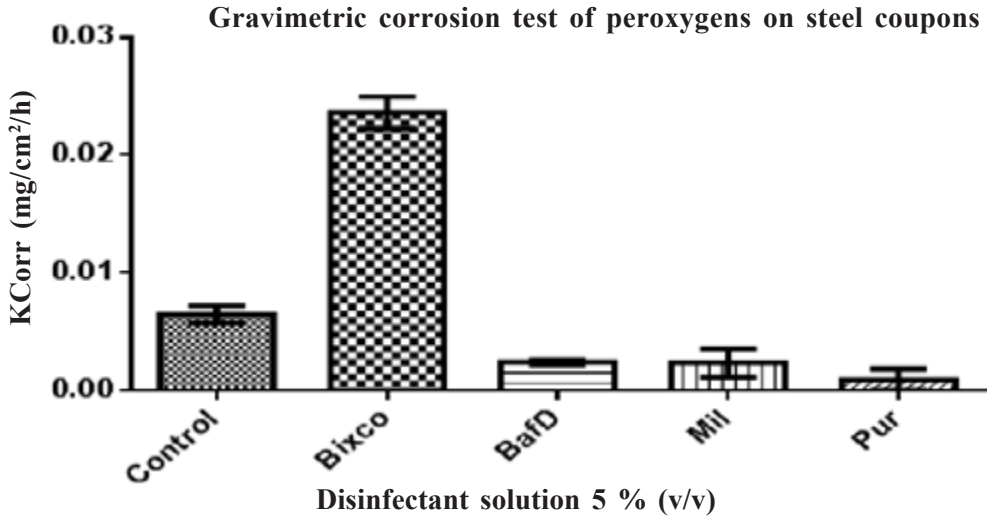

Fig. 7. Gravimetric corrosion test of commercially used peroxygen disinfectants on metal coupon material made of steel of identical geometrical shape after 96 hours exposure at room temperature. Control group consisted of material exposed to purified water only. Results are expressed as mean ± standard error of the mean (SEM). (Generated by GraphPad Prism version 5.00.288)

ent types of standard strains and environmental isolates. Gram-negative organisms are generally found in aqueous environments (e.g. water systems) and raw materials of natural origin. These types of organisms are usually pathogenic and produce toxins such as endotoxins (lipopolysaccharides in the cell wall of Gram-negative bacteria). Bacterial endotoxins cause pyrogenic (fever) reactions [1]. Three out of 4 tested microorganisms are pharmacopeial and are to be tested in pharmaceutical products as objectionable bugs that should not be present in any pharmaceutical preparation. In addition, these three organisms are members of the class of bile tolerant Gram-negative microorganisms which is also considered objectionable pharmacopeial class. EM isolate tested was added to the list as high risk organism that may contribute to a failure of water treatment station system and hence, impacting the efficiency of a classified area of sanitization, production ma-

Table 6. The classification of corrosion behavior of metallic materials according to stability class and corrosion resistance degree

P, [mm/year]	Stability class	Corrosion resistance degree	Liquids in which coupon were immersed				
			PuW[a]	Bixco	BafD	Mil	Pur
<0.001	I- Perfectly stable	1					
0.001-0.005	II- Very stable	2			0.0027	0.0026	0.0010
0.005-0.01		3	0.0076				
0.01-0.05		4		0.0272			
0.05-0.1	III- Stable	5					
0.1-0.5	IV- Relatively stable	6					
0.5-1.0		7					
1.0-5.0	V- Low stability	8					

a= Purified water USP

chine cleaning with the consequence of high possibility of compromising drug quality.

The test for neutralizer validity was performed originally between FTMT and FTM with the 3 tested standard strains to compare between the 2 tested neutralizers in their efficiency and capacity. Later, *Sphingomonas paucimobilus* was tested among other isolated microbial species to confirm the validity of FTMT for the recovery of environmental isolates. Effective neutralization of a biocidal agent is crucially important to the accuracy of information obtained from any disinfectant efficacy study [28]. The determination of NT and of NE should be a comparison between a test and a control population. NT was determined as the ratio of recovery between a viability population, and a population exposed to the neutralizer. This comparison directly examined the toxicity of the individual neutralizing media for the different microorganisms. The efficacy of a neutralizer wasdefined as the ratio of recovery between the neutralizerand the biocide, and the neutralizer exposed populations. Therefore, only the effect of the biocide in the system was measured. These ratios allowed for a threshold value (≥ 0.70) as the first test. The second test was a statistical one to confirm success or failures of the suspect results by Dunnett's Multiple Comparison Test.

As for the sodiumthioglycolate, being a thiol group containing compound, it could neutralize the activity of silver nitrate against *P. aeruginosa*. By contrast, sulfur-containing compounds such as sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation[29].It was observed that NaCl present in both neutralizing broths can aid in Ag^+ neutralization by precipitation as hazy white colloidal AgCl turbidity. Thioglycolate in the presence of hydrogen peroxide is oxidized to dithioglycolate. It is well known that sodium thiosulfate is used to neutralize hydrogen peroxide solution used in disinfection of contact lensesand it is superior over other neutralizers such as catalase in terms of stability [30]. However, the possible reaction products of thiosulfate with peroxygens are the oxidation product of thiosulfate by peroxygen such as tetrathionate and other polythionates which have no effect on microbial lethality. The selectivity of tetrathionate broth depends on the ability of the thiosulfate and tetrathionate in combination to suppress commensal coliform organisms [31]. Tetrathionate reacts with free sulfhydryl groups of enzymes and to cause their inactivation [32]. Thiosulfate can also react with sulfhydryl groups. Thus, it is suggested that tetrathionate broth interferes with the synthesis, the activity, or both, of sulfur-containing enzymes or cell wall and membrane components [33].

The lethality of tetrathionate broth is directly related to the concentrations of thiosulfate and

tetrathionate in the medium. Decreasing the concentration of either salt reduces the lethal action of the mixture. The concentrations of 0.0736 M and 0.0236 M of thiosulfate and tetrathionate, respectively (3:1 ratio), appear to be optimal [34]. The ability of a microorganism to reduce tetrathionate may play a role in decreasing the inhibition for certain species. When part of the tetrathionate is reduced, the ratio would be altered. It can be concluded that the outcome of the neutralization process may be toxic for the tested microorganisms. This part needs more investigation to be performed on the mechanism of chemical neutralization and the effect of reaction products accumulation on microbial viability. The above findings were, in part, in agreement with ours which demonstrated that *Salmonella enterica* was recovered at ≥70 % from all FTMT-peroxygen exposed groups which gave the highest recovery rate if compared with *Escherichia coli*, *Sphingomonas paucimobilus* and *Pseudomonas aeruginosa*. *Salmonella enterica* highest recovery rate from FTM is most probably attributed to its intrinsic tolerance to residual hydrogen peroxide and the support of this assumption the considerably high microbial recovery from FTM and FTMT at 1:10 (v/v) where there persistent relatively high level of the peroxides based on the results shown in (Table 6). FTM possesses much less chemical neutralization capacity (the reducing compounds components are half that of in-house made neutralizer and there is no thiosulfate) than FTMT for peroxygens because no difference in the ratio of pass/fail tests was observed between 1:10 and 1:100 groups. If this property is coupled with well-known low concentration exponents (ç) of hydrogen peroxide (peroxygen) if compared to silver nitrate (ç=0.5 and 0.9 to 1 respectively) then the intrinsic microbial resistance will play its major rule in surviving Gram-negative bacteria cells in the presence of residual amount of peroxygen disinfectants. According to USP<1072> Disinfectants and Antiseptics (2014): Biocidal activity reduction factor= (Dilution folds)^{ç}. Thus, the reduction of activity of hydrogen peroxide is about 3.2 to 10 reduction in activity only for 1:10 and 1:100, respectively while for $AgNO_3$ it is 7.9 and 10 to 63.1and 100 [35]. This means that dilution has little effect of neutralization of antimicrobial effect of peroxygen compounds much more than silver salts. However, based on the results of (Table 6), as the initial concentration of the neutralized disinfectants is getting low the time needed for neutralization becomes shorter in this study and hence increases the chance for microorganism to be recovered from the recovery medium. For FTMT neutralizer, the inhibitory effect of thiosulfate-tetrathionate combination could be altered by: first, dilution and the second factor is modifying three-to one ratio by microorganism itselfand its intrinsic resistance (ex. by production of tetrathionate reductase enzyme) and/or variation in disinfectant composition and/or concentration.

Cystine $(SCH_2CH(NH_2)CO_2H)_2$ is the amino acid formed by the oxidation of two cysteine molecules that covalently link via a disulfide bonds that cleave more rapidly at higher temperatures (ex. temperature of media preparation and sterilization) [36]. Cystine is present in FTM in half amount of that in FTMT. Cysteine amino acid has reducing properties like thioglycolate with peroxygens. However, auto oxidation is expected on standing from atmospheric oxygen in the head space of the reservoir tube thus neutralizing broth must be either prepared fresh, heated once directly before use and/or incorporation of thickening agent in the neutralizing broth (ex. agar in small amount to render media thick but not solid) which retard atmospheric oxygen diffusion. This property is found to greater extent in FTMT than FTM. Redox indicator such as resazurin is a good indicator for such situation to judge visually the presence and the degree of oxygen diffusion into the media for either reheat or discard. A further investigation is needed to measure the possible effect of auto oxidation upon storage of the neutralizer on its capacity of neutralization of peroxygen-based biocidal agents.

Considering the NT study performed for both FTM and FTMT, used herein, its results revealed that they were non-toxic and could be used in the validation program. The other important subsequent aspect is NE. The scheme followed was based on FTM as primary neutralizer that has very close composition to NIH Thioglycolate (supported by previous work). FTM Thioglycolate was found

to be non-toxic or of low toxicity against microorganisms. In-house made neutralizer FTMT is in between DEB and NIH Thioglycolate in composition. The combination of microorganism, neutralizer and disinfectant is unique and thus the success of one combination with one microorganism does not mean that same combination with other microorganisms will do accordingly [3]. However, some researchers allowed for predetermined contact time between disinfectants and neutralizers before addition of microorganisms. This sequence is different from practical situations in which disinfectant carrying microorganism is neutralized by neutralizing media either in disinfectant validation study or EM sampling. Our results showed that some disinfectants may be neutralized slower than others for the same chemical neutralizer taking some time till elimination of residual biocide to undetectable level. This time cannot be neither controlled nor calculated which may lead to exaggerated estimation of biocidal potency.

Bacteria are known to degrade H_2O_2 using two classes of enzymes: catalases and peroxidases [37]. *Salmonella typhimurium* encodes three catalases (KatE, KatN, KatG) and three peroxidases (AhpC, TsaA, Tpx) [37,38]. This fact agreed with that of the current study in which *Salmonella typhimurium* showed greatest tolerance to the residual peroxides levels in the investigated neutralizing broth. Thus, the critical factor in the microbial recovery in this test is the intrinsic bacterial cell resistance to the low level of peroxides in the neutralizing broths which was persistent at 1:10 (v/v) till more than 15 minutes but progressively decaying at 1:100 (v/v) in neutralizing broths at rate in FTMT higher than FTM. It is apparently that this tolerability to peroxygens traces was at minimum with *Escherichia coli* and *Pseudomonas aeruginosa*, maximum with *Salmonella enterica* subsp. *Entericaserovar typhimurium* with *Sphingomonas paucimobilus* in between. This is supported by (Figs. 1, 2, 3 and 4) where critical disinfectants concentrations (concentration of disinfectant in neutralizing broth that meet ≥ 70 %) could be determined roughly. Critical concentration could be determined for each disinfectant by performing serial dilutions of biocidal agent in diluting broth and determining the highest concentration that meet the predefined acceptance criterion. For example, critical concentration for Bixco was about 3.5-4 % (v/v) for *Sphingomonas paucimobilus* and >5 % (v/v) for the remaining microorganisms in FTMT.

Data transformation to Log 10 was done to approximate normal distribution following approach outlined by [39]. In Log 10 transformation, in the event where the mean count is zero, this requires the addition of "1" to each value of zero. So, that this does not distort other results, a value of "1" is added to each item of data [40].

The gravimetric corrosion measurements indicated that Mil, BafD and Pur disinfectants contain effective anticorrosive substances while Bixco does not. Normal test takes long time that may reach in some cases 90 days but using easily corroded steel material accelerated the test such that it took few days and this is important in preliminary rapid judgment for selection of the most suitable biocidal agents [41]. Long term inspection study of prolonged contact time could be done after that for the selected disinfectants on corrosion resistant materials used in the pharmaceutical facility. peracetic acid (PA) is a wide spectrum, rapid disinfectant that is more expensive than hypochlorite, but without its corrosivity and degree of inactivation by organic matter. PA is currently used as a disinfectant for flexible fiber optic endoscopes, where corrosion is highly undesirable. It is believed that PA could be used in laboratories handling mycobacteria for disinfection in areas where corrosion must be avoided [42,43]. This is in agreement with the finding in the current study where Pur gave the lowest corrosion rate if compared with the other disinfectants especially Mil and BafD (where anticorrosive substance is present in their formulae) as PA synergistic combination with hydrogen peroxide made the concentration of the later lower in the biocidal agent formula.

Finally, the results in (Table 6) are indication for that the reaction rate at ordinary room temperature played significant role for the process of chemical neutralization in addition to neutralizer capacity in this study since both FTM and FTMT at 1:100 (v/v) dilution were capable to neutralize tested disinfectants with the former reaction time

was longer (about twice the time of the later). The differences in the approximate average range of peroxide level were also significant especially at 1:100 (v/v) between both neutralizers - till complete neutralization-indicating that not only the time of exposure of microorganisms to residual disinfectants affected the viability but also the level of biocidal agents in neutralizing broths. This uncalculated time in neutralization process may give false over estimation of cleaning efficacy monitoring and/or sanitizer efficacy if microorganisms were added later to the neutralizer-disinfectant broth after certain time. During this time, innate microbial resistance played significant role for its survival till complete neutralization and gave variable observed outcomes of chemical neutralization of peroxygen-based disinfectants. Again, this could be evident from critical value. This value is between 1.5 and 2 % (v/v) for *Sphingomonas paucimobilus*, *Escherichia coli* and *Pseudomonas aeruginosa* with BafD and Mil in FTMT while for *Salmonella enterica* is ≥ 5 and 4 % (v/v) respectively. In FTMT, Pur critical concentration for organisms was in the following descending order: *Salmonella enterica* > *Sphingomonas paucimobilus*>*Pseudomonas aeruginosa* > *Escherichia coli*. For FTM, critical value of Pur for the three standard strains was less than 0.1 % (v/v). For Mil and BafD, the recovery of *Escherichia coli* and *Pseudomonas aeruginosa* was less than 0.2 % (v/v) while *Salmonella enterica* showed greater resistance with critical value of ≥ 4.5 % (v/v) approximately. Further mechanistic study is needed to provide evidence that peroxygen neutralization process takes place faster with thiosulfate than thioglycolate and cystiene in mentioned neutralizers.

It is clear from the previous findings that it is necessary in the process of chemical neutralization to achieve rapid diminishing of residual biocidal agents otherwise variable outcomes of microbial recovery will be obtained according to intrinsic microbial resistance to the disinfectant being neutralized. Thus, in the study of chemical neutralization process microorganism suspension must be added directly after mixing of disinfectant-neutralizing broth directly as the delay may mask the true neutralization process capability and hence probably leads to the skewness in the actual potency of the biocidal agent and/or misjudgment of the sensitivity of procedure of microbial recovery from residual antimicrobial substances.

Conclusion

An ideal chemical neutralizer must be non-toxic to microorganisms and neutralizes chemical biocidal agents immediately after mixing without toxic byproducts formation to microbial cells. If such purpose could not be accomplished at low dilution ration increasing dilution level may be the first aid step in achieving this goal to reduce or diminish exposure time and magnitude of toxicity of antimicrobials and/or toxic neutralization reaction products. FTMT neutralizer was effective with index commercial peroxygen-based disinfectants (Bixco, BafD, Mil and Pur) tested at 1:100 (v/v) dilution ratio. Chemical neutralizers based on redox neutralization reactions must be ensured to be in its full reduced form to obtain maximum neutralization capacity and hence efficacy for disinfectants based on oxidizers. Gravimetric corrosion test could be used as fast, simple and useful tool to judge the selection of disinfectants which are based on corrosive active biocidal compounds where they can affect life span of many materials in pharmaceutical facility.

References

1. **Clontz, L. (2009).** Microbial Limit And Bioburden Tests: Validation Approaches and Global Requirements. 2nd ed. Boca Raton: CRC Press.
2. **MacKinnon, I.H. (1974).** The use of inactivators in the evaluation of disinfectants. Journal of Hygiene. 73(02): 189-195.
3. **Sutton, S.V., Proud, D.W., Rachui, S., Brannan, D.K. (2002).** Validation of microbial recovery from disinfectants. PDA Journal of Pharmaceutical Science and Technology. 56(5): 255-266.
4. **Croshaw, B., Groves, M.J. and Lessel, B. (1964).** Some Properties of Bronopol, A New Antimicrobial Agent Active Against *Pseudomonas Aeruginosa*. Journal of Pharmacy and Pharmacology. 16 (S1), 127T-130T.

5. **Kayser, A. and Van der Ploeg, G. (1965).** Growth inhibition of staphylococci by sodium thiosulphate. Journal of Applied Bacteriology. 28(2): 286-293.
6. **Mossel, D.A.A. and Beerens, H. (1968).** Studies on the inhibitory properties of sodium thioglycolate on the germination of wet spores of clostridia. Journal of Hygiene. 66(02): 269-272.
7. **Hibbert, H.R. and Spencer, R. (1970).** An Investigation Of The Inhibitory Properties of Sodium Thioglycollate In Media For The Recovery Of Clostridial Spores. Journal Of Hygiene. 68(01): 131-135.
8. **Weber, G.R. and Levine, M. (1944).** Factors Affecting Germicidal Efficiency of Chlorine and Chloramine. American Journal of Public Health and the Nations Health. 34(7): 719-728.
9. **Hugo, W.B. and Newton, J.M. (1964).** The adsorption of iodine from solution by microorganisms and by serum. Journal of Pharmacy and Pharmacology. 16(1): 49-55.
10. **Cox, Claire B., Feeley, J.C. and Margaret Pittman. (1973).** Sterility Testing: Detection of Fungi and Yeasts in the Presence of Preservatives. Journal Of Biological Standardization. 1(1): 11-19.
11. **Block, S.S. (1983).** Disinfection, Sterilization and Preservation. 1st ed. Philadelphia, PA: Lea & Febiger.
12. **Russell, A.D. (1981).** Neutralization procedures in the evaluation of bactericidal activity. Disinfectants: their use and evaluation of effectiveness. Academic Press, London. p 45-59.
13. **Russell, A.D. (1990).** Bacterial spores and chemical sporicidal agents. Clinical microbiology reviews. 3(2): 99-119.
14. **Hugo, W.B. and Russell, A.D. (1998).** Pharmaceutical microbiology. 6th ed. London, UK: Blackwell Science Ltd.
15. **Maturin, L. and Peeler, J. (1998).** Chapter III: Aerobic Plate Count, Bacteriological Analysis Manual. Standard Practice for Determining Microbial Colony Counts from Water Analyzed by Plating Methods. Retrieved 10 August 2016, from http://www.fda.gov/Food/Sd.enceResearchf LaboratoryMethodsfBacteriologicaiAnalyticaiManualBAMfucrn063346.htm.
16. **Maturin, L. and Peeler, J. (2011).** Aerobic Plate Count. Bacteriological Analysis Manual. Retrieved 10 August 2016, from http://www.fda.gov/Food/Sd.enceResearchLaboratoryMethods BacteriologicalAnalyticalManual.htm.
17. **US Pharmacopoeia. (2014).** Validation of Microbial Recovery from Pharmacopoeial Articles, In: US Pharmacopoeia 37/National Formulary 32. Washington, DC: United States Pharmacopoeial Convention.
18. **World Health Organization (WHO). (2003).** Application of Hazard Analysis and Critical Control Point Methodology to Pharmaceuticals, In WHO Expert Committee on Specifications for Pharmaceutical Preparations, Thirty-Seventh report, Annex 7 (WHO Technical Report Series, No. 908), World Health Organization, Geneva.
19. **Van Schothorst, M. (1997).** A simple guide to understanding and applying the hazard analysis critical control point concept. ILSI Europe. 3rd edition. 1-30.
20. **Es-Saheb, M., Elzatahry, A.A., Sherif, E.M., Alkaraki, A.S. and Kenawy, E.R. (2012).** A novel electrospinning application for polyvinyl chloride nanofiber coating deposition as a Corrosion Inhibitor for Aluminum, Steel, and Brass in Chloride Solutions. Int. J. Electrochem. Sci. 7: 5962-5976.
21. **Qu, Q., Yuan, R., Li, L., He, Y., Luo, J., Wang, L., Xu, H., Gao, Y., Song, Y. and Ding, Z. (2013).** Corrosion Behaviour of Cold Rolled Steel in 84 Disinfectant Solutions. Int. J. Electrochem. Sci. 8: 11625-11640.
22. **Block, S.S. (2001).** Peroxygen compounds. In: Disinfection, Sterilisation and Preservation, 5th edition, Block SS (ed), Lippincott Williams & Wilkins, London.
23. **Jeffrey, D.J. (1995).** Chemicals used as disinfectants: active ingredients and enhancing additives. Revue scientifique et technique (International Office of Epizootics) 14(1): 57-74.

24. **Eissa, M.E., Ashour, M.S. and Mansy, M.S. (2012).** Neutralizer evaluation study of some microbial isolates against two strong disinfectants with and without the presence of synthetic detergent. World Applied Sciences Journal, 20(6): 823-831.
25. **Adeosun, S.O., Sekunowo, O.I., Balogun, S.A. andObiekea, V.D. (2012).** Corrosion Behaviour of Heat-Treated Aluminum-Magnesium Alloy in Chloride and EXCO Environments. International Journal of Corrosion. Article ID 927380, 9 pages.
26. **Vasilescu, M. and Vasilescu, I. (2009).** Contributions Regarding the Corrosion Behaviour of Some Al-Cu-Mg Superalloys. Revista de Chimie. 60(1): 23-28.
27. **US Pharmacopoeia. (2013).** Validation of Microbial Recovery from Pharmacopoeial Articles, In: US Pharmacopoeia 36/National Formulary 31, Washington, DC: United States Pharmacopoeial Convention.
28. **Langsrud, S. and Sundheim, G. (1998).** Factors influencing a suspension test method for antimicrobial activity of disinfectants. Journal of Applied Microbiology. 85(6): 1006-1012.
29. **Liau, S.Y., Read, D.C., Pugh, W.J., Furr, J.R. and Russell, A.D. (1997).** Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Letters in Applied Microbiology. 25(4): 279-283.
30. **Ogunbiyi, L. (1986).** The use of sodium thiosulfate for inactivating residual hydrogen peroxide on contact lenses after disinfection. Clinical and Experimental Optometry. 69(1): 16-21.
31. **Knox, R., Gell, P.G.H. and Pollock, M.R. (1943).** The selective action of tetrathionate in bacteriological media: A report to the medical research council. Journal of Hygiene. 43(03): 147-158.
32. **Parker, D.J. and Allison, W.S. (1969).** The mechanism of inactivation of glyceraldehyde 3-phosphate dehydrogenase by tetrathionate, o-iodosobenzoate, and iodine monochloride. Journal of Biological Chemistry. 244(1): 180-189.
33. **Postgate, J.R. (1963).** The examination of sulphur auxotrophs: a warning. Microbiology. 30(3): 481-484.
34. **Palumbo, S.A. and Alford, J.A. (1970).** Inhibitory action of tetrathionate enrichment broth. Applied microbiology. 20(6): 970-976.
35. **Aslaksen, M.A., Romarheim, O.H., Storebakken, T., Skrede, A. (2006).** Evaluation of content and digestibility of disulfide bonds and free thiols in unextruded and extruded diets containing fish meal and soybean protein sources. Animal Feed Science and Technology. 128(3): 320-330.
36. **US Pharmacopoeia. (2014).** Disinfectants and Antiseptics, In: US Pharmacopoeia 37/National Formulary 32. Washington, DC: United States Pharmacopoeial Convention.
36. **Imlay, J.A. (2008).** Cellular defenses against superoxide and hydrogen peroxide. Annual Review of Biochemistry. 77: 755.
37. **Sokal, R. and Rohlf, F. (1995).** Biometry. The Principles and Practice of Statistics in Biological Research. W. H. Freeman and Company, New York. p 411-422.
38. **Fang, F.C. (2011).** Antimicrobial Actions of Reactive Oxygen Species. Mbio. 2(5): e00141-11-e00141-11.
39. **Sandle, T. (2004).** An approach for the reporting of microbiological results from water systems. PDA Journal of Pharmaceutical Science and Technology. 58(4): 231-237.
40. **Standard Test Procedures For the Evaluation of Wildland Fire Chemical Products. (2000).** Test Method 5 - Corrosion Tests. [online] Available at: http://www.fs.fed.us/rm/fire/wfcs/tests/documents/stp_cont.pdf [Accessed 8 Aug. 2016].
41. **Holton, J., Nye, P. and McDonald, V. (1994).** Efficacy of selected disinfectants against Mycobacteria and Cryptosporidia. Journal of Hospital Infection. 27(2): 105-115.
42. **Griffiths, P.A., Babb, J.R. and Fraise, A.P. (1998).** Mycobacterium Terrae: A Potential Surrogate For *Mycobacterium tuberculosis* In A Standard Disinfectant Test. Journal Of Hospital Infection 38(3): 183-192.