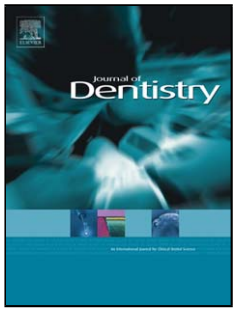

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Lack of cytotoxicity by Trustwater Ecasol™ used to maintain good quality dental unit waterline output water in keratinocyte monolayer and reconstituted human oral epithelial tissue models

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Short Title: Biosafety of Ecasol demonstrated *in vitro*

KEYWORDS: Trustwater Ecasol™; superoxidised water; hypochlorous acid; biosafety; cytotoxicity testing; RHE; keratinocytes; dental unit waterlines; residual disinfectant

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Summary

We previously showed that residual treatment of dental chair unit (DCU) supply water using the electrochemically-activated solution Trustwater Ecasol™ (2.5 ppm) provided an effective long-term solution to the problem of dental unit waterline (DUWL) biofilm resulting in DUWL output water quality consistently superior to potable water.

Objectives: To investigate the cytoxicity of Ecasol using cultured keratinocyte monolayers and reconstituted human oral epithelial (RHE) tissue and to extend the study of Ecasol's effectiveness in maintaining the microbiological quality of DUWL output water.

Methods: TR146 human keratinocyte monolayers and RHE tissues were exposed to Ecasol (2.5-100 ppm) for 1 h periods after removal of growth medium and washing with phosphate buffered saline (PBS). Experiments were repeated using Ecasol that had been exposed for 30 min to 1-2 µg/mL bovine serum albumin (BSA), equivalent to protein concentrations in saliva. To quantitatively determine cytotoxic effects on monolayers following Ecasol exposure, the Alamar Blue proliferation assay (assesses cell viability) and the Trypan Blue exclusion assay (assesses plasma membrane integrity), were used. Cytotoxicity effects on RHE tissues were assessed by the Alamar Blue assay and by histopathology.

Results: Ecasol at >5.0 ppm resulted in significant ($P<0.001$) cytotoxicity to keratinocyte monolayers following a 1 h exposure. These effects, however, were completely negated by BSA pretreatment of Ecasol. No cytotoxicity was observed in the more complex RHE tissue at any of the Ecasol concentrations tested. In a 60-week study of 10 DCUs, tested weekly, the average density of aerobic heterotrophic bacteria in Ecasol-treated (2.5 ppm) DCU supply water was < 1 cfu/mL and in DUWL output water was 6.5 cfu/mL.

Conclusions: Ecasol present as a residual disinfectant in DUWL output water is very unlikely to have adverse effects on human oral tissues at levels effective in maintaining DUWL output water quality at better than potable standard water quality.

1. Introduction

Dental chair units (DCUs) contain an intricate network of interconnected narrow-bore flexible plastic waterlines (DUWLs) that provide water to irrigate tooth surfaces during dental procedures using three-in-one air/water syringes. This water is also used to provide cooling to dental instruments during use, including turbine and conventional handpieces and ultrasonic scalers, as heat generated during instrument use can be injurious to teeth. [1-7] DUWLs also provide water to other DCU outlets such as the DCU spittoon or cuspidor bowl and the patient cupfiller used for oral rinsing during and after dental procedures. [3-4] Water supplied to DCUs may be provided directly from a potable quality mains supply or from bottle reservoirs in the DCU which are replenished with water as necessary. [3-4] Water storage tanks filled by mains water often provide the water supplied to DCUs in dental hospitals and clinics equipped with large numbers of DCUs. [3,4,8]

Over the last four decades many studies have shown that DUWL output water is frequently heavily contaminated with microorganisms, predominantly aerobic Gram-negative heterotrophic environmental bacterial species. [3,4,9-24] Microbial contamination of DUWL output water results from the growth and development of microbial biofilms on the internal surfaces of DUWLs. [3,4] These biofilms are formed mainly by microorganisms arriving in low numbers in DCU supply water, such as mains water, which adhere to the internal surface of DUWLs and secrete a protective matrix of complex polysaccharides. [3,4,25,26] Water flow within DUWLs is laminar and accordingly the flow at the lumen surfaces is minimal relative to that at the centre of the lumen, permitting biofilm to form readily. [3,4] Water stagnation within DUWLs when DCUs are not being used, such as at night and over weekends, facilitates the growth of biofilm. Subsequently, planktonic forms of microorganisms and pieces of biofilm are released to seed biofilm formation elsewhere in the waterline network. [3,4] Planktonic cells and by-products including bacterial endotoxin present in DUWL water are aerosolised by DCU-supplied instruments such as ultrasonic scalers and turbine dental handpieces, thus exposing patients and staff to these microorganisms , biofilm fragments and to bacterial endotoxins. [27-30]

Currently, there is no mandatory European Union (EU) quality standard for DUWL output water. However, the quality of DUWL output water should be consistent with, or at least approximate to, potable water quality standards because DCUs are classified as medical devices according to the European Medical Devices Directive. [3,4,31,32] The present potable water standard for aerobic heterotrophic bacteria in the EU and the USA do not specify an upper limit, although water sold in bottles or containers in the EU should not exceed 100 cfu/mL. [33,34] The Centers for Disease Control and Prevention (CDC) guidelines for infection control in dental health-care

settings recommend a maximum level of aerobic heterotrophic bacteria in DUWL output water of ≤500 cfu/mL. [35] In addition, the American Dental Association (ADA) in 1995 proposed a target limit of ≤200 cfu/mL for the year 2000, but this has proven quite difficult to achieve in practice. [3,4,36] The most effective approach to sustaining DUWL output water of good microbiological quality has been regular or continuous treatment of DUWLs using a disinfectant, biocide or cleaning agent that removes biofilm or inhibits its growth. [1-4,19-23,37-42] A broad range of DUWL treatment products have been developed and marketed in recent years, many of which have been reported to be effective at controlling DUWL biofilm. [3,4,22,42] Furthermore, some manufacturers have developed DCU models with integrated semi-automated or automated DUWL cleaning systems that facilitate the regular cleaning of DUWLs with effective disinfectants that eliminate biofilm. [1-3] However studies have shown that consistent provision of good quality DUWL output water from DCUs equipped with these cleaning systems was dependent on rigorous implementation of the disinfection protocol by staff undertaking DUWL disinfection. [2] In 2009, we reported on the development at the Dublin Dental Hospital of a large-scale system capable of automatically and consistently maintaining the microbiological and chemical quality of DCU supply and output water at better than potable quality simultaneously, in multiple DCUs (>100) over a two year period. [8] The principle of the system was based on sequential filtration of potable-quality mains water using a series of specific filters to provide DCU supply water of consistent physical and chemical composition. This water was then stored in a large holding tank that supplied the hospitals' 103 DCUs via a recirculating ring main. Prior to circulation, filtered water was treated with 2.5 ppm of the electrochemically activated mixed oxidant solution Ecasol™ at neutral pH, to control microbial growth and eliminate biofilm formation in DUWLs and in the associated water distribution network and storage tank. Over the two year study period, DCU supply water and output water aerobic heterotrophic bacterial counts averaged <1 and 18.1 cfu/mL, respectively, which correlated with the absence of biofilm in DUWLs. [8] This approach provided a robust solution to the problem of DUWL biofilm, together with significant economic benefits in reduced equipment maintenance, consumable materials and labour.

The electrochemical activation (ECA) technology involves the generation of electrochemically activated solutions by passing a dilute NaCl solution through an electric field in a Flow-through Electrolytic Module (FEM), segregating the ions formed and producing two oppositely charged solutions with altered physical and chemical properties. [43] Electrochemical activation changes the state of the salt solution from a stable to a metastable state. The positively charged solution (anolyte) usually has a redox value of +600 mV, and consists of a mixture of unstable mixed oxidants (mainly hypochlorous acid) in a physically excited state that is capable

of penetrating biofilms and is highly microbicidal. The negatively charged antioxidant solution (catholyte) has detergent-like properties, typically a pH of 11, a redox value of -600 mV and consists predominantly of sodium hydroxide in an excited state. These active ion species are short lived with a half-life of usually less than 48 hours. [43]

ECA technology was pioneered in Russia in the 1970s where ECA solutions have been used extensively for over 30 years, for drinking water disinfection, swimming pool disinfection, as the general disinfectant in hospitals, as wound irrigants, as nebulised inhalant sprays and many other infection control applications with no apparent harmful effects. [44-48] Since the 1970s, several generations of FEM have been developed, with the FEM-3 being one of the more recent. [45,46] Production of ECA solutions with consistent quality and properties was technically difficult prior to the advent of FEM-3 technology. FEM-3-based ECA technology outside Russia is owned by and has been further refined by the Trustwater Group (Clonmel, Ireland). [8] Anolyte (Ecasol™) produced by Trustwater ECA generators has a neutral pH, very much in contrast to anolyte produced by earlier ECA generators from other manufacturers, which was often acidic and highly corrosive. [43,46,49] There are few quantitative scientific publications outside of Russia that investigated potential health effects of ECA solutions, although they have been marketed widely in the US and Japan for both human and animal use. [3,4,8,43,45,46,49] The US Food and Drug Administration consider Ecasol suitable for food processing applications. [8]

The first objective of the present study was to investigate the cytotoxic effects of Trustwater Ecasol™ (hereafter referred to as Ecasol) used as a residual treatment to control DUWL biofilm by using cultured keratinocyte monolayers and a reconstituted human oral epithelial tissue model system *in vitro*. The second objective was to further investigate the efficacy of residual Ecasol at maintaining good microbiological DUWL output water.

2. Materials and methods

2.1 Collection and processing of DCU supply and output water samples

Each week, for 60 consecutive weeks, after flushing for 1 min, samples (20 ml) of DCU output water were collected directly from the operator's air/water syringe waterline from 10 Planmeca Prostyle Compact DCUs located in three separate clinics in the Dublin Dental Hospital as described previously. [8] Samples were also taken from the processed mains water supply to DCUs. The water had been filtered and treated with 2.5 ppm Ecasol as described previously. [8] Residual free available chlorine (FAC) in water samples was neutralised using a 1:1 dilution of 0.5% (w/v) sodium thiosulphate. Water samples were cultured in duplicate on R2A agar plates (Lab M Ltd., Bury, Lancashire, United Kingdom) to determine total aerobic heterotrophic bacterial density as described previously. [1,2,8,20] After 10 days incubation at 20-22˚C, plates were examined and colonies counted using a Flash and Go™ automatic colony counter (IUL Instruments Ltd., Barcelona, Spain). [8] R2A agar is the medium of choice for monitoring heterotrophic bacterial counts in water as it permits the recovery of significantly more organisms than conventional, more nutritious culture media, at 20°C compared to 35°C. Higher counts of bacteria are recovered on this media following prolonged incubation (i.e. 10 days) ensuring that the maximum number of bacteria are detected.[50] The inclusion of sodium pyruvate in R2A medium also leads to enhanced recovery of chlorine stressed bacteria from water. [50.51]

2.1. Chemicals, reagents and cell culture media

Unless otherwise indicated, all chemicals and reagents used were of analytical grade or molecular biology grade and were purchased from Sigma-Aldrich Ireland Ltd. (Arklow, Wicklow, Republic of Ireland).

2.2. Ecasol™

The disinfectant solution Ecasol™ was produced by electrochemical activation (ECA) using a Trustwater model 120 ECA generator (Trustwater, Clonmel, County Tipperary, Ireland) equipped with four FEM-3 type flow-through electrolytic modules (FEMs). [8] The generator was supplied with pre-treated potable-quality mains water supplied to the Dublin Dental Hospital together with a saturated NaCl solution to give a final concentration of 0.2% (w/v). Water from the mains potable supply to the Dublin Dental Hospital was subjected to sequential filtration through a number of specific water filter types in order to provide DCU supply water of consistent physical and chemical composition, all as described previously. [8] The Trustwater model 120 ECA generator produces Ecasol at neutral pH having an oxidation-reduction potential

of +900 mV ±100 mV and consisting of approximately 200 ppm metastable oxidants (predominantly hypochlorous acid ~158 ppm, hypochlorite ion ~ 42 ppm, ozone < 1 ppm, chlorine dioxide < 2.5 ppm, chloric acid <1.5 ppm and chlorous acid <3 ppm). [8] The activated oxidants (Ecasol's activated state lasts for a period of up to 48 h), which are formed initially are in dynamic equilibrium and gradually revert to the initial ingredients (i.e. water supplied and 0.2% (w/v) NaCl) after time. [8] The formed substances are additionally in an electrochemically-energised state, which relaxes gradually over a period of 24-48 h. [8] Ecasol initially contains energised microbubbles formed at the FEM's electrode interface. [45] These also dissipate by cavitation over a period of 12-24 h. Freshly generated Ecasol was stored on ice and was used within 10-15 min. Ecasol was diluted in sterile phosphate-buffered saline (PBS) to the required concentration (i.e. 2.5, 5.0, 10.0 and 100 ppm).

2.3. Measurement of residual free available chlorine in Ecasol

Free available chlorine (FAC) of Ecasol was measured using a Hach Pocket Colorimeter II (Hach Company, Iowa, USA) analysis system, which uses *N*,*N*-diethyl-*p*-phenylenediamine to react with free chlorine and form a red solution, whose colour intensity is proportional to the FAC concentration. The equipment was used according to the manufacturer's instructions.

2.4. TR146 cell culture

The TR146 cell line was originally derived from a biopsy of a squamous cell carcinoma of the human buccal mucosa and was kindly provided by Imperial Cancer Research Technology (London, UK). [52,53] The culture medium for the cell line consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% (v/v) foetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin sulphate (100 µg/mL). Cells were maintained in a humidified atmosphere at 37°C in 5% ~~(v/v)~~ CO_2. Cells were detached from culturing flasks by treatment with trypsin–EDTA (0.25% (w/v)) and subcultivated for further studies with proliferating cells. For studies with proliferating cells, the cells were seeded in flat-bottomed Cellstar® 96-well culture plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 2 x 10^4 cells/well and cultured for 48 h until 70-90% confluent.

2.5. Reconstituted oral epithelium (RHE) tissue

The reconstituted human epithelium (RHE) model used in the study is a three-dimensional tissue culture model consisting of TR146 cells grown on polycarbonate filters. [54,55] RHE tissues were purchased from SkinEthic Laboratories (Nice, France). When cultivated *in vitro* on a polycarbonate filter at the air liquid interface in a chemically defined medium, the transformed

human keratinocytes of the cell line TR146 form an epithelial tissue, devoid of stratum corneum, but histologically resembling the mucosa of the oral cavity. This tissue model does not fully differentiate, but does form a non-keratinising oral epithelium that has been extensively used for biocompatibility studies. On arrival at the laboratory, the RHE tissues (0.50 cm^2) were removed from the shipping agar and cleaned of residual agar. Individual tissue samples were immediately placed into single wells of Cellstar® 24-well plates (Greiner Bio-One) and incubated with 0.5 ml of maintenance medium (SkinEthic) for 48 h in a humidified atmosphere at 37°C and 5% ~~(v/v)~~ CO_2.

2.6. Measurement of cell damage following Ecasol exposure

Cytotoxicity of Ecasol on TR146 cells grown as monolayers was measured using two assays including the Alamar Blue cell proliferation assay and the Trypan Blue exclusion assay, both as described previously. [56-59] Alamar Blue is a water-soluble non-toxic dye (also known as Resazurin) that has been used previously for quantifying the *in vitro* viability of a variety of cell types. Once added to cell cultures, the oxidized form of Alamar Blue is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes. This redox reaction is accompanied by a shift in colour of the culture medium from indigo blue to fluorescent pink, which can easily be measured by colourimetric or fluorometric reading.

For studies with Alamar Blue, cells were seeded in flat-bottomed Cellstar 96-well culture plates at a density of 2 x 10^4 cells/well and cultured for 48 h until 70-90% confluent. The culture medium was then removed and each well was washed twice with 200 µl of sterile PBS. Cells were then treated with 200 µl of respective concentrations of Ecasol for 1 h. After exposure, the Ecasol was removed and the cells were washed twice with DMEM cell culture medium followed by incubation with 200 µl of 10% (v/v) Alamar Blue (Tox-8 kit, Sigma-Aldrich) in DMEM. Wells were mixed by tapping gently on the side of the plate and incubated at 37°C in 5% ~~(v/v)~~ CO_2 for 24 h. Absorbance was measured using a Tecan Genios (Tecan, Mannedorf, Switzerland) plate-reader at 540 nm. Readings were expressed as a percentage of those obtained with non-Ecasol-treated control cells, which were exposed to PBS only. Each experiment was performed in triplicate. Alamar Blue was also used to assess the effect of various concentrations of Ecasol on RHE tissue samples. Prior to exposure to Ecasol, culture medium was removed from the tissue samples, followed by washing with PBS. Then 200 µl of the respective Ecasol concentration was applied onto the surface of individual RHE samples in duplicate. Following incubation for 1 h at 37°C in 5% ~~(v/v)~~ CO_2, Ecasol was removed and tissues were washed with

RHE maintenance medium and then 200 µl of 10% (v/v) Alamar Blue was added followed by incubation for 4 h at 37°C in 5% ~~(v/v)~~ CO_2. Absorbance was measured as above at 540 nm. For both experiments with TR146 monolayers and RHE tissue samples, addition of 200 µl 1% (v/v) Triton X 100 was used as a positive control for cell damage.

For the Trypan Blue assay with monolayers, cells were seeded into 6 well cell culture plates (Greiner Bio-one) at a density of 1.8 x 10^5 cells/well and cultured for 48 h until 70-90% confluent. Growth medium was removed and the cells washed twice with sterile PBS. One ml aliquots of each Ecasol concentration tested were placed onto the cells and incubated for 1 h at 37°C in 5% ~~(v/v)~~ CO_2. Following incubation, the percentage of viable cells in each well was determined using Trypan Blue dye exclusion. Cells were stained with 500 µl 0.2% (w/v) Trypan Blue in PBS and then examined by light microscopy using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan). Photographic images were recorded at random from three separate areas of each cell culture well. The percentage viability of cells in each culture tested was calculated based on examination of an average of 1,500 cells in each case.

Measurement of levels of glutathione (GSH) and lactate dehydrogenase (LDH) activity released from monolayers and RHE tissues were also used as alternative methods to assess cell damage following exposure to Ecasol as both activities are altered during oxidative stress and cell injury. Monolayers and RHE tissue samples were exposed to Ecasol as described above. After 1 h of exposure, the monolayers and RHE tissue samples were washed gently with PBS and Dulbecco's Modified Eagle's Medium Modified (DME) supplemented with 0.584 gm/L L-glutamine was added. LDH leakage was measured after 1 h and 24 h of incubation in DME with at 37°C. LDH activity was measured using a Cyto-tox 96 kit (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions using a Tecan Genios plate reader and recorded as a percentage of LDH activity released from cells lysed with 0.1% (v/v) Triton X for 20 min at 37°C. GSH activity was measured using a GSH-Glo Glutathione assay kit (Promega) according to the manufacturer's instructions.

2.7. Protection of cells from damage by Ecasol by exogenous protein

The protective affect of bovine serum albumin (BSA) on the cytotoxicity of Ecasol was also investigated. Briefly, a range of concentrations of BSA (1-10 mg/mL) was mixed with each test concentration of Ecasol evaluated on TR146 monolayers. Ecasol/protein mixtures were incubated at 37˚C in a water bath for 30 min prior to addition to monolayers as described above.

2.8. Light microscopy of RHE tissue samples

RHE tissue samples for histological investigation were initially fixed in 4% (v/v) formalin. Paraffin sections 5 µm thick were cut, dehydrated and stained with hematoxylin-eosin using standard procedures. Prepared samples were examined by light microscopy using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan).

2.9. Statistical Analysis

To evaluate the differences in the relative cytotoxicity of various Ecasol concentrations, variance analyses were conducted using one-way ANOVA (GraphPad Prism version 3.0 statistics programme; GraphPad Software Inc., San Diego, CA) with significance level of 95%. Data are presented as mean ± SEM. Experiments were performed in triplicate. $P < 0.05$ was considered statistically significant.

3. Results

The main purpose of the present study was to investigate the biocompatibility of low concentrations of Ecasol using both TR146 cell monolayers and reconstituted oral epithelial (RHE) tissue.

3.1. Neutralisation of Ecasol by cell culture media

Preliminary experiments revealed that addition of Ecasol (2.5-100 ppm free available chlorine (FAC)) to the TR146 cell growth medium (DMEM supplemented by 10% (v/v) foetal bovine serum) resulted in the complete neutralisation of Ecasol's oxidative activity as determined by measurement of FAC levels. Similar results were observed for the maintenance medium used to support RHE tissue samples. In both cases, levels of FAC were reduced to undetectable levels following one minute contact with either growth or maintenance media. For cytotoxicity testing therefore, all growth or maintenance media was first removed from TR146 monolayers or RHE tissue samples, and was followed by three separate washes with PBS prior to exposure to Ecasol. Addition of Ecasol to PBS at a range of concentrations (2.5-100 ppm FAC) showed no reduction in FAC at any Ecasol concentration tested (2.5-100 ppm FAC) and such solutions were used for cytotoxicity testing.

3.2. Effect of Ecasol on proliferation and membrane integrity of oral keratinocyte monolayers

Potential cytotoxicity of Ecasol on TR146 keratinocyte monolayers was assessed by monitoring cell proliferation and cell membrane integrity using the Alamar Blue and Trypan Blue cell proliferation and membrane integrity assays. Fig. 1 shows the effect of Ecasol at various concentrations on TR146 keratinocyte proliferation following a 1 h contact time relative to the PBS-only control. Similar findings were obtained using both assays in that increasing concentrations of Ecasol resulted in a decrease in viability of the cells. Using both assays, the use of Ecasol at 2.5 ppm had no significantly adverse effects on the cells with regard to both proliferation (89.6 ± 12%) and cell membrane integrity (99.35 ± 1%) ($P > 0.05$). Ecasol at 5 ppm had a significant effect on cell proliferation (72 ± 9.0%) ($P < 0.01$) (Fig. 1a) but had no significant effect ($P > 0.05$) on membrane integrity (90 ± 4%) of the TR146 monolayers (Fig. 1b). However, Ecasol concentrations of 10 ppm and 100 ppm significantly reduced both cell viability and cell membrane integrity ($P < 0.001$) (Fig. 1). Ecasol at 100 ppm had the greatest adverse affect reducing both cell proliferation and membrane permeability to an average of 2%

of the controls (Fig. 1), whereas 10 ppm reduced proliferation to 23 ± 3% and membrane integrity to 57.99 ± 29%, respectively, of the controls (Fig. 1).

Because DMEM, the culture medium used for TR146 monolayer growth, was shown to rapidly neutralise FAC in Ecasol (section 3.1.), we hypothesised that exposure of Ecasol to organic material would result in neutralisation of FAC activity and thus potential for cytotoxicity. An additional series of experiments were performed in which Ecasol at the same range of FAC concentrations used above, was pre-exposed for 30 min to varying concentrations (1-2 mg/mL) of bovine serum albumin (BSA) prior to being added to TR146 monolayers (Fig. 2). BSA was used as an analogue for protein found in human saliva and in the oral cavity. Addition of BSA had a dramatic affect on the level of FAC in Ecasol. Addition of 1 mg/mL BSA to Ecasol at 100 ppm FAC reduced the FAC level to 0.4 ppm (±0.05). Similarly addition of 1 mg/mL BSA to Ecasol at 10 ppm FAC reduced the FAC level to 0.01 ppm (±0.03). Furthermore, addition of unstimulated whole saliva (1:1 (v/v)) to Ecasol at 100 and 10 ppm FAC for 30 min reduced the FAC level to undetectable levels in both cases (data not shown). Pretreatment of Ecasol at 10 and 100 ppm with BSA completely abolished the cytotoxic effects observed with non-BSA treated Ecasol monolayers at either concentration as determined by both the Alamar Blue and Trypan Blue assays (Fig. 2). Addition of BSA only to TR146 monolayers had no detectable cytotoxic effect using both assays (data not shown).

Attempts to assess Ecasol-induced damage to keratinocyte monolayers independently by measuring release of LDH and GSH activity were unsuccessful as reproducible results were not obtainable. Preliminary experiments indicated that Ecasol interferes with LDH and GSH assays and so it was concluded that these assays were not reliable indicators for investigating Ecasol biocompatibility.

3.3. Effect of Ecasol on the viability of RHE tissue

The cytotoxic effects of Ecasol were also investigated using RHE tissue samples using the Alamar Blue viability assay. RHE samples were pre-washed three times with PBS to remove any residual maintenance medium prior to the addition of Ecasol. It was found that concentrations of Ecasol up to 100 ppm had no significant affect ($P > 0.05$) on viability following 1 h exposure (Fig. 3). Histopathological analysis of Ecasol-treated RHE samples showed no detectable damage (Fig. 4). In contrast, extensive damage was evident with Triton-X 100-treated RHE samples with the presence of large vacuoles clearly evident in the tissues. (Fig. 4).

3.4. Efficacy of Ecasol disinfection of DUWLs and FAC of DUWL output water

A previous study from our laboratory demonstrated that treatment of filtered mains water with 2.5 ppm Ecasol maintained the aerobic heterotrophic bacterial cell density of both DUWL supply (average < 1cfu/mL) and output water (average 18.1 cfu/mL) at significantly better than potable water for a two year study period. [8] This investigation was further extended in the present study. For a period of 60 consecutive weeks, processed mains water (i.e. filtered and Ecasol-treated water) from the 8000-L tank supplying the Dublin Dental Hospital's 103 DCUs and output water from DUWLs from 10 test DCUs were tested weekly for density of aerobic heterotrophic bacteria as well as residual FAC levels. The residual FAC level varied from week to week (Fig. 5), with a mean average of 1.6 ppm. The average bacterial density in DUWL output water from the operator's air/water syringe DUWL from the 10 sentinel DCUs included in the 60-week study period was 6.3 cfu/mL (Table 1). The average bacterial density from processed supply water during the 60-week study period was ≤1 cfu/mL (Table 1).

4. Discussion

The purpose of the present study was to investigate potential toxic effects elicited by a range of concentrations of the ECA solution Ecasol on oral keratinocyte monolayers as well as RHE tissues *in vitro*. Previously we have shown that the use of 2.5 ppm Ecasol as a residual disinfectant in DCU supply water provides an effective and robust long-term solution to DUWL biofilm management and consistently maintains the microbiological quality of DUWL output water at better than potable quality standards. [8] There are few quantitative scientific publications outside of Russia that have investigated potential health effects of ECA solutions, although they have been used extensively for over 30 years with no reported harmful effects. [44-48]

In the present study, oral keratinocyte monolayers of TR146 cells were selected as a preliminary model for assessing the biocompatibility of Ecasol because their survival rate is high and they have been shown to be suitable for cytotoxicity testing if reproducibility is a prerequisite. [60-62] Furthermore, keratinocytes are the first cells to be exposed to potential irritants *in vivo*. Several reporter assays for quantitatively determining potential adverse effects of Ecasol on TR146 monolayers were investigated. Efforts to assess Ecasol-induced damage to TR146 monolayers by measuring release of cellular LDH and GSH activity following Ecasol exposure proved unreliable. Both GSH and LDH are stable intracellular enzymes, present in all cell types, and are rapidly released into the cell culture medium upon damage of the plasma membrane. Subsequent experiments revealed that the addition of 1 mg/mL of BSA to Ecasol completely inactivated FAC in Ecasol. These findings suggested that Ecasol probably interacts with LDH and GSH, making accurate measurement of enzyme activity impossible and thus the use of such enzyme activities as a measure of TR146 cell damage due to Ecasol, unreliable. Previous studies demonstrated that exposure of cells to hypochlorous acid (HOCl), the main oxidant in Ecasol, lead to a decrease in intracellular level of GSH and LDH. [63] Furthermore, Whiteman and co-workers found that HOCl interfered with LDH release assays when working with human chondrosarcoma cells. [64] Because of these findings it was concluded that alternative assays post-Ecasol treatment would have to be used. The Alamar Blue assay, a quantitative colorimetric assay that relies on the reduction of Alamar Blue by mitochondrial enzymes involved in respiration, was found to be a reliable assay. The dye is taken up by proliferating cells where reduction is accompanied by a change in colour of the dye from blue to fluorescent pink, a change that can be monitored quantitatively by absorbance. Monolayers of keratinocytes were exposed to various concentrations of Ecasol for 1 h, the Ecasol removed and the cells washed with PBS followed by staining with Alamar Blue. Using this assay Ecasol was found to significantly affect TR146 cell proliferation at 10 ppm and 100 ppm and to a lesser extent at 5

ppm (Fig. 1a). The greatest effect was observed at 100 ppm where cell proliferation was reduced to 2% of PBS-treated control cells (Fig. 1a). This effect was negated when TR146 cells were exposed to Ecasol that had been pre-exposed to BSA where no significant effect on cell viability was observed at any of the Ecasol concentrations tested (Fig. 2a). The effect of Ecasol on TR146 keratinocyte monolayers was further investigated using the Trypan Blue assay, which relies on the exclusion of the dye from cells with an intact plasma membrane. Cells with plasma membrane damage show uptake by the dye, an effect that can be monitored by light microscopy. Monolayers of keratinocytes were exposed to various concentrations of Ecasol for 1 h, the Ecasol removed and the cells washed with sterile PBS followed by staining with Trypan Blue. Following staining, cells were observed directly by light microscopy of the monolayers *in situ* and the number of stained cells expressed as a percentage of the total number of cells counted. Using this approach, concentrations of Ecasol of 5 ppm and 10 ppm resulted in significant TR146 keratinocyte membrane permeability with the greatest affects observed at 100 ppm (Fig. 1b). No significant damage was observed using concentrations <10 ppm (Fig. 1b). As with the previous experiments using Alamar Blue, cell damage due to Ecasol was completely negated by prior treatment of Ecasol with BSA (Fig. 2b).

To obtain a more accurate assessment of potential adverse affects of Ecasol on human oral mucosa reconstituted human oral epithelium (RHE) tissue generated from TR146 cells was used as an alternative model to TR146 monolayers for investigating the biocompatibility of Ecasol. RHE tissues have been used extensively previously for biocompatibility studies and as a model system for investigating microbial pathogenicity. [65-70] RHE tissues are structurally more complex than keratinocyte monolayers and more closely resemble the physiological environment of the oral cavity. A range of concentrations of Ecasol were applied to the surface of RHE tissue samples for 1 h periods, and following removal of Ecasol and washing with PBS, viability was assessed using the Alamar Blue assay. Alamar Blue was used because it is more sensitive for detecting cell damage than Trypan Blue but is non-toxic, which allowed for post-experimental histological processing of tissue samples. None of the concentrations of Ecasol tested (2.5-100 ppm) resulted in a significant reduction in RHE cell proliferation (Fig. 3), whereas a significant reduction was observed following exposure of RHE to 1% (v/v) Triton-X (reduced to 11 ± 0.4% of PBS-treated control tissues) (Fig. 3). Histological examination of Ecasol-treated RHE tissues showed no observable damage when compared to the PBS-treated controls (Fig. 4). In contrast, RHE treated with Triton-X showed visible damage and vacuolisation of cells (Fig. 4). These findings with the more complex RHE epithelial cell model demonstrated that Ecasol caused no significant damage to the tissues at any of the concentrations tested (2.5-100 ppm).

Ecasol is a metastable solution consisting of activated mixed oxidants (200 ppm) that are formed in dynamic equilibrium and gradually revert to the initial ingredients (i.e. supplied water and 0.2% NaCl) over time. Hypocholorous acid (HOCl) is the principal oxidant (~ 158 ppm) of freshly generated Ecasol. [8] HOCl is known to be a potent microbicidal agent produced by neutrophils in the human body. [71] In the present study preliminary findings revealed that FAC in Ecasol was reduced to undetectable levels when added to cell culture medium or RHE maintenance medium. For this reason all culture media was removed and residual culture media washed off with sterile PBS prior to Ecasol exposure tests. Ecasol was diluted in PBS as this was found to be well tolerated by cells during treatment and did not affect the level of FAC in Ecasol. This was to achieve the most accurate biocompatibility information relating to Ecasol without interference from organic material not relating to the monolayer or RHE tissues being tested. Due to the influence of organic material on FAC in Ecasol, a series of experiments were performed to explore the influence of protein on the level of toxicity elicited from the monolayers following exposure to Ecasol. Bovine serum albumin (BSA) at 1-2 mg/mL was added to Ecasol for a period of 30 min prior to exposure to monolayers and RHE tissues. The level of protein in normal human saliva is estimated to be 1-2 mg/mL. [72] Addition of 1 mg/mL BSA to Ecasol concentrations that caused the most damage to monolayers (i.e. 10 and 100 ppm) resulted in almost instantaneous depletion of FAC to undetectable levels and subsequent complete negation of toxic effects on monolayers (Fig. 2). Human saliva when added to Ecasol (100 ppm) was also found to cause instantaneous depletion of FAC to undetectable levels. A similar finding was found by Kotula *et al*. who found that chlorine reduction was dependant on chlorine concentration and the amount/source of organic material. [73] Another study by Kouoh *et al*. found that BSA inhibited the amounts of superoxide anions, hydrogen peroxide and HOCl produced by human neutrophils. [74] The authors proposed that the mechanism through which BSA acts may result from a simple chemical interaction with reactive oxygen intermediates produced rather than an intracellular mechanism. Furthermore, a recent study by Rajabalian *et al*. evaluated the cytoxicity of Persica mouthwash on human and mouse cell lines found that reduced cytotoxic effects were observed in the presence of foetal calf serum. [75]

A previous study from our laboratory showed that residual treatment of DCU supply water with low levels of Ecasol (2.5 ppm) was very effective at controlling biofilm in DUWLs and maintaining DUWL output water quality (average 18.1 cfu/mL aerobic heterotrophic bacteria) at better than potable quality continuously for a two year period. [8] In the present study we extended this long-term monitoring of the effectiveness of Ecasol (2.5 ppm) as a residual waterline disinfectant for more than an additional year and confirmed the results of the original study. The average bacterial density in DUWL output water from 10 sentinal DCUs tested

weekly for the 60-week period was 6.2 cfu/mL (Table 1). No evidence for DCU component corrosion or other adverse affects were observed during the 60 week study period. The FAC level of DUWL supply water to and output water from the 10 DCUs was also monitored weekly during the 60 week period. The average input FAC was 2.5 ppm and the average output FAC 1.6 ppm (Fig. 5). The FAC of Ecasol in DUWLs or in the associated water distribution network can be reduced by reaction with organic material, including microorganisms. In general, the more closely the output FAC compares to the input FAC, the 'cleaner' the system, i.e. the less organic material is present to reduce the FAC.

5. Conclusions

The findings of this study and previous studies from our laboratory showed that residual treatment of DCU supply water with low concentrations (2.5 ppm) of the pH-neutral ECA solution Ecasol provides a robust and effective long-term means of controlling DUWL biofilm and provides DUWL output water the quality of which is superior to potable water. In addition, the findings of the present study demonstrate unequivocally that the level of Ecasol used to treat DCU supply water (i.e. 2.5 ppm) had no adverse affect on the cell viability of oral keratinocyte monolayers. Ecasol concentrations >2.5 ppm did adversely affect cell viability of oral keratinocyte monolayers but this effect was negated by the presence of exogenous protein concentrations (i.e. 1-2 µg/mL) equivalent to those found in human saliva due to inactivation of FAC in Ecasol. Furthermore Ecasol concentrations up to 100 ppm, 40-times higher than the level used to treat DCU supply water (i.e. 2.5 ppm FAC) and 62.5-times higher than the average Ecasol FAC concentration present in DUWL output water (i.e. 1.65 ppm), had no cytotoxic effect on the more complex RHE tissue model, which is more reflective of epithelial tissues present in the oral cavity. All of these findings show that Ecasol present as a residual disinfectant in DUWL output water is very unlikely to have any adverse effects on human oral tissues during patient treatment.

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Table 1. Average density of aerobic heterotrophic bacteria and average FAC in 60 consecutive weekly water samples from DUWL output water from ten DCUs supplied with Ecasol treated water

DCU	Average bacterial density in DUWL output water[b] (cfu/mL)	Average FAC concentration in DUWL output water[b] (ppm)
1	7.9	1.7
2	1.4	1.5
3	3.4	1.5
4	2.1	1.7
5	7.1	1.5
6	3.1	1.4
7	17.8	1.6
8	15.8	1.7
9	1.9	1.5
10	2.3	1.7
Overall	6.3	1.6
Treated[a] supply water	!1.0	2.5 ± 1.1

[a] Filtered and Ecasol-treated (2.5 ppm) mains water supplied to DCUs. [8]

[b]Water samples were taken from the operator's three-in-one air/water syringe from each DCU.

719 **Figure legends**

720

721 **Fig. 1.** Viability (% of PBS-treated control) of TR146 monolayers following 1 h exposure to
722 various concentrations of Ecasol (ppm FAC). Viability was assessed after 1 h using Alamar Blue
723 as a measure of cell proliferation (a) and Trypan Blue as a measure of plasma membrane
724 integrity (b). A significance level of $P < 0.01$ is indicated by ** and $P < 0.001$ by ***.

725 **Fig. 2.** Viability (% of PBS-treated control) of TR146 monolayers following 1 h exposure to
726 various concentrations of Ecasol (ppm FAC) to which BSA had been added (1-2 mg/mL) 30 min
727 prior to addition to the monolayers. Viability was assessed after 1 h using Alamar Blue as a
728 measure of cell proliferation (a) and Trypan Blue as a measure of plasma membrane integrity
729 (b). A significance level of $P < 0.01$ is indicated by ** and $P < 0.001$ by ***.

730 **Fig. 3.** Viability (% of PBS-treated control) of RHE tissue samples following 1 h exposure to
731 various concentrations of Ecasol (ppm FAC). Viability was assessed after 1 h using Alamar Blue
732 as a measure of cell proliferation. Triton X 100 was used as a positive control for RHE tissue
733 damage. A significance level of $P < 0.001$ is indicated by ***.

734 **Fig. 4.** Light micrographs of RHE tissue sections stained with hematoxylin-eosin. (a) RHE tissue
735 sample treated with PBS for 1 h; no detectable damage to the tissue is evident. (b) RHE tissue
736 sample treated with 100 ppm FAC Ecasol for 1 h; no detectable damage to the tissue is evident.
737 (c) RHE tissue sample treated Triton X 100 for 1 h; extensive damage to the tissue is present and
738 large vacuoles are evident. Arrows indicate the polycarbonate filter on which the RHE tissues
739 were grown.

740 **Fig. 5.** Density of aerobic heterotrophic bacteria (average 6.3 cfu/mL) and FAC (average 1.6
741 ppm) present in DUWL output water from the operator's three-in-one air/water waterline from
742 DCU No. 1 during a period of 60 consecutive weeks. Similar results were obtained with nine
743 other DCUs during the 60-week study period. The corresponding average bacterial density and
744 average FAC in the Ecasol-treated processed mains water supply was <1.0 cfu/mL and 2.5 ppm,
745 respectively, during the 60-week study period.

746

747

Fig. 1

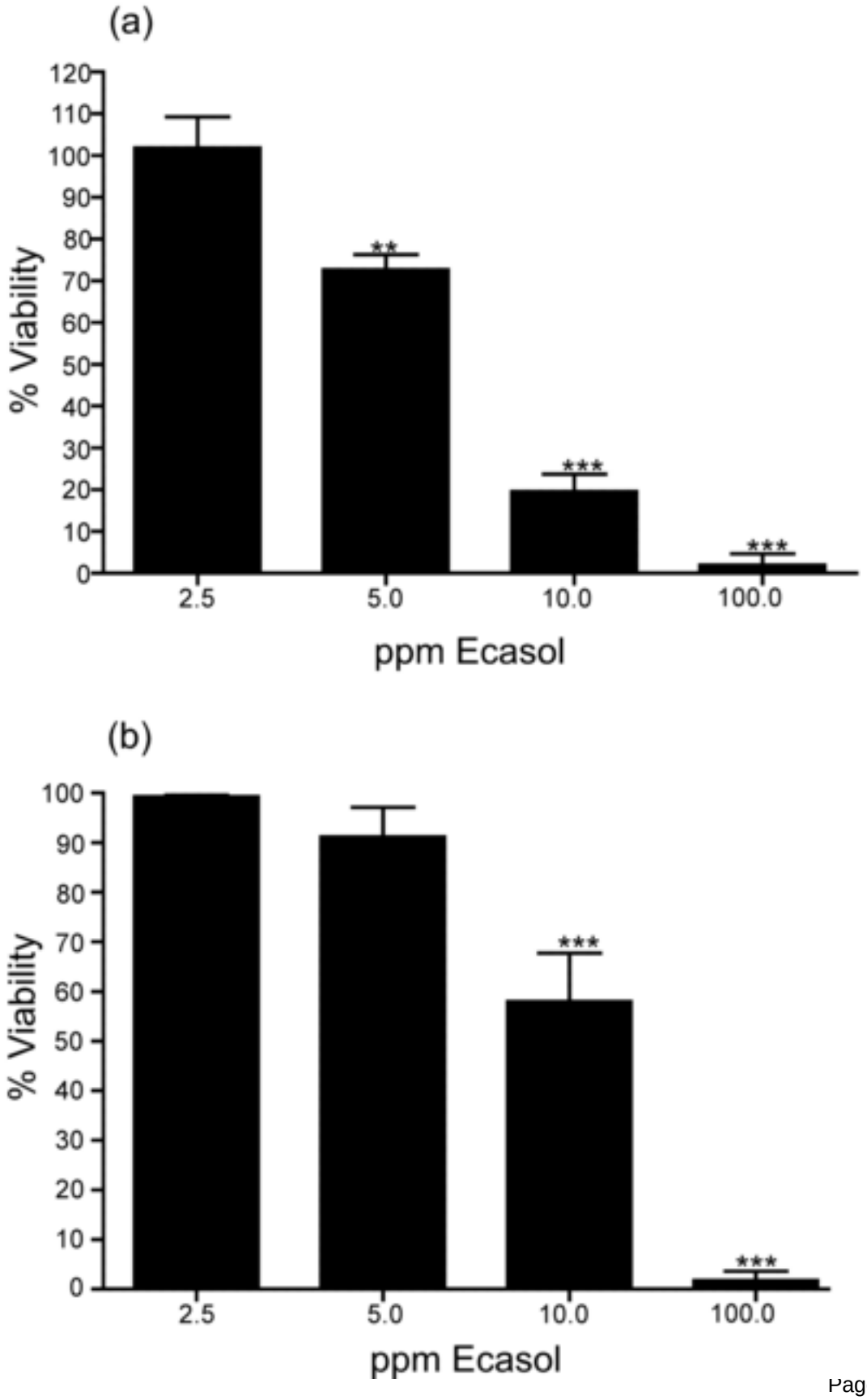


Fig. 2

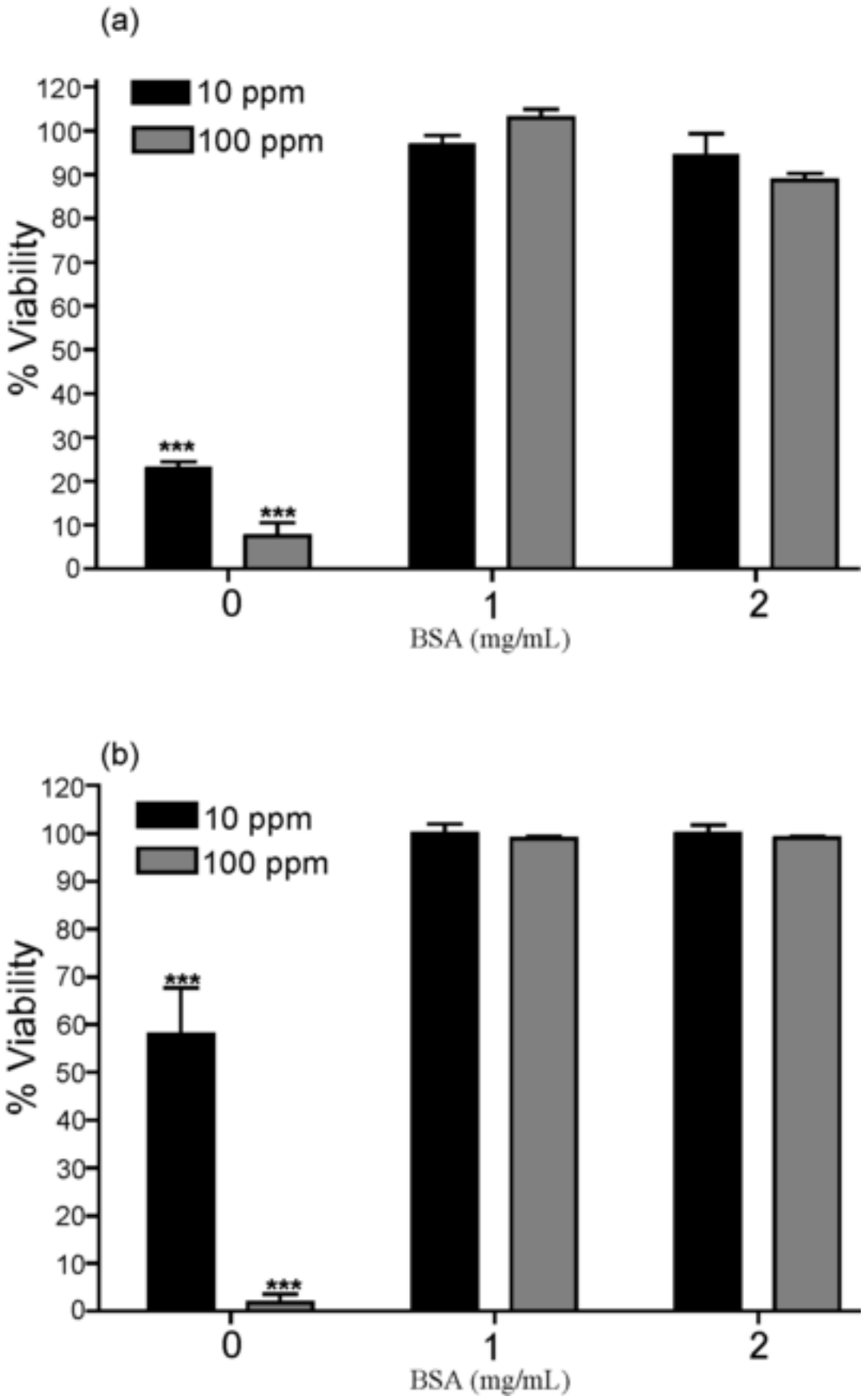
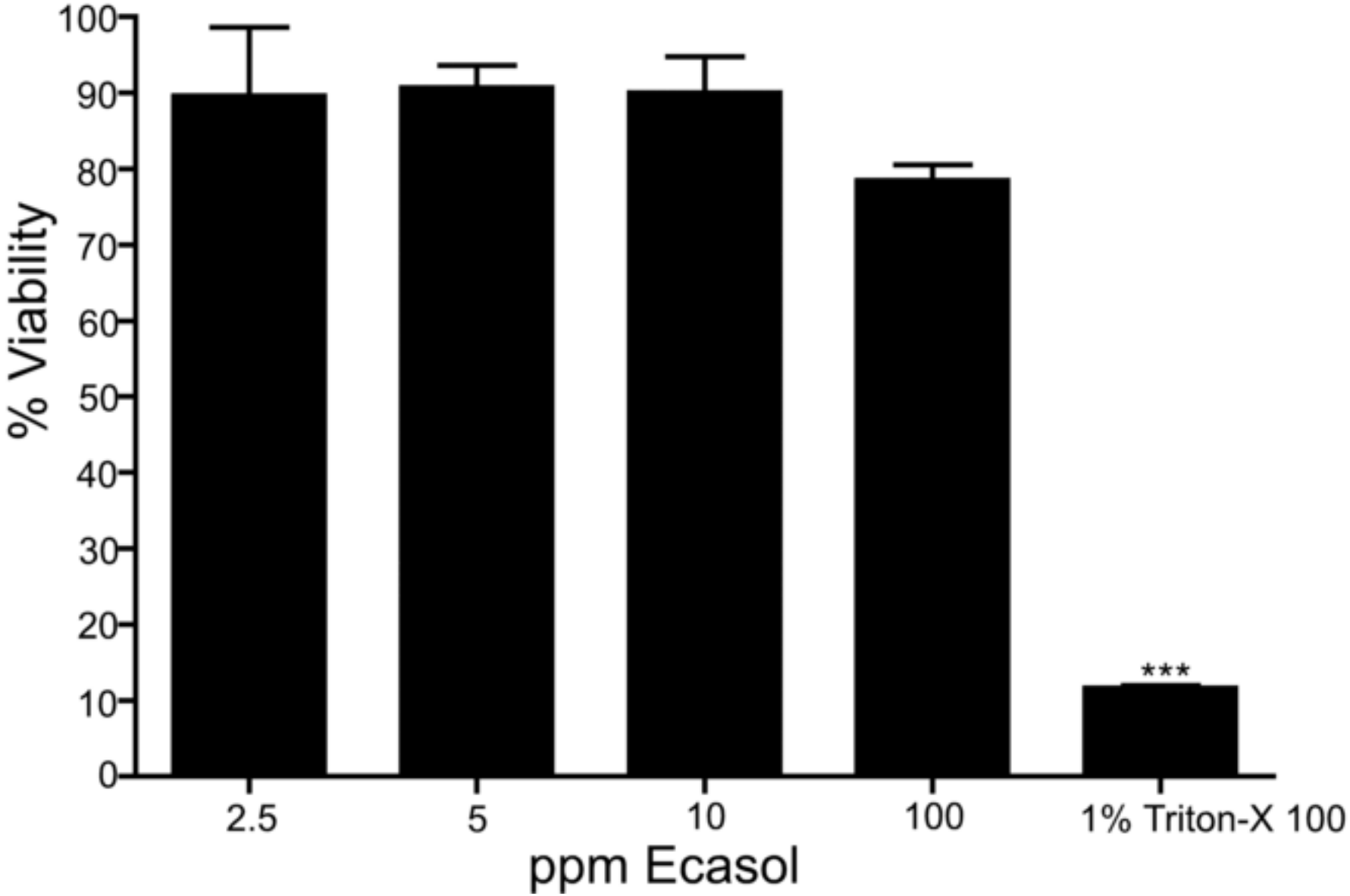


Fig. 3


100
90
80
70
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% Viability
***
2.5
5
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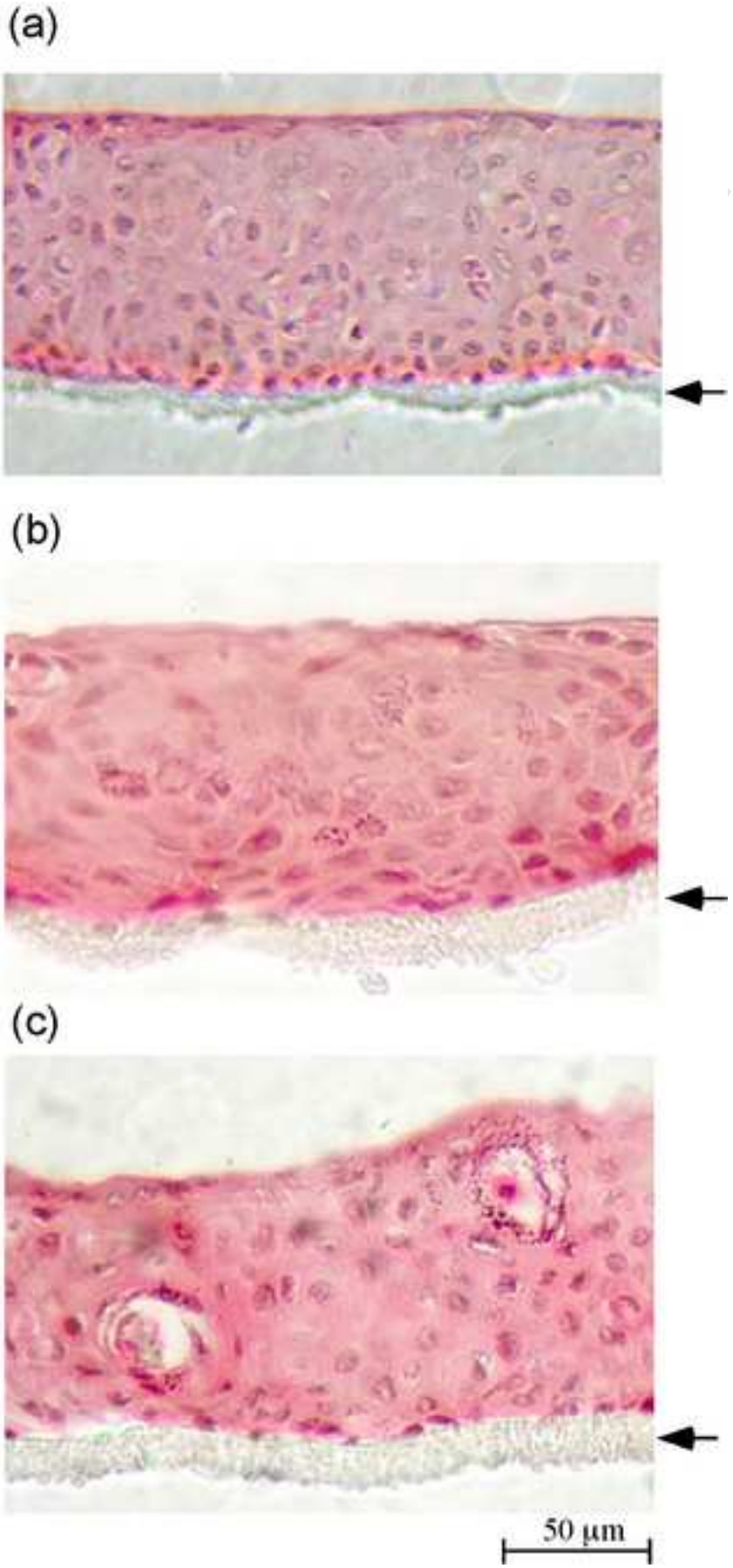
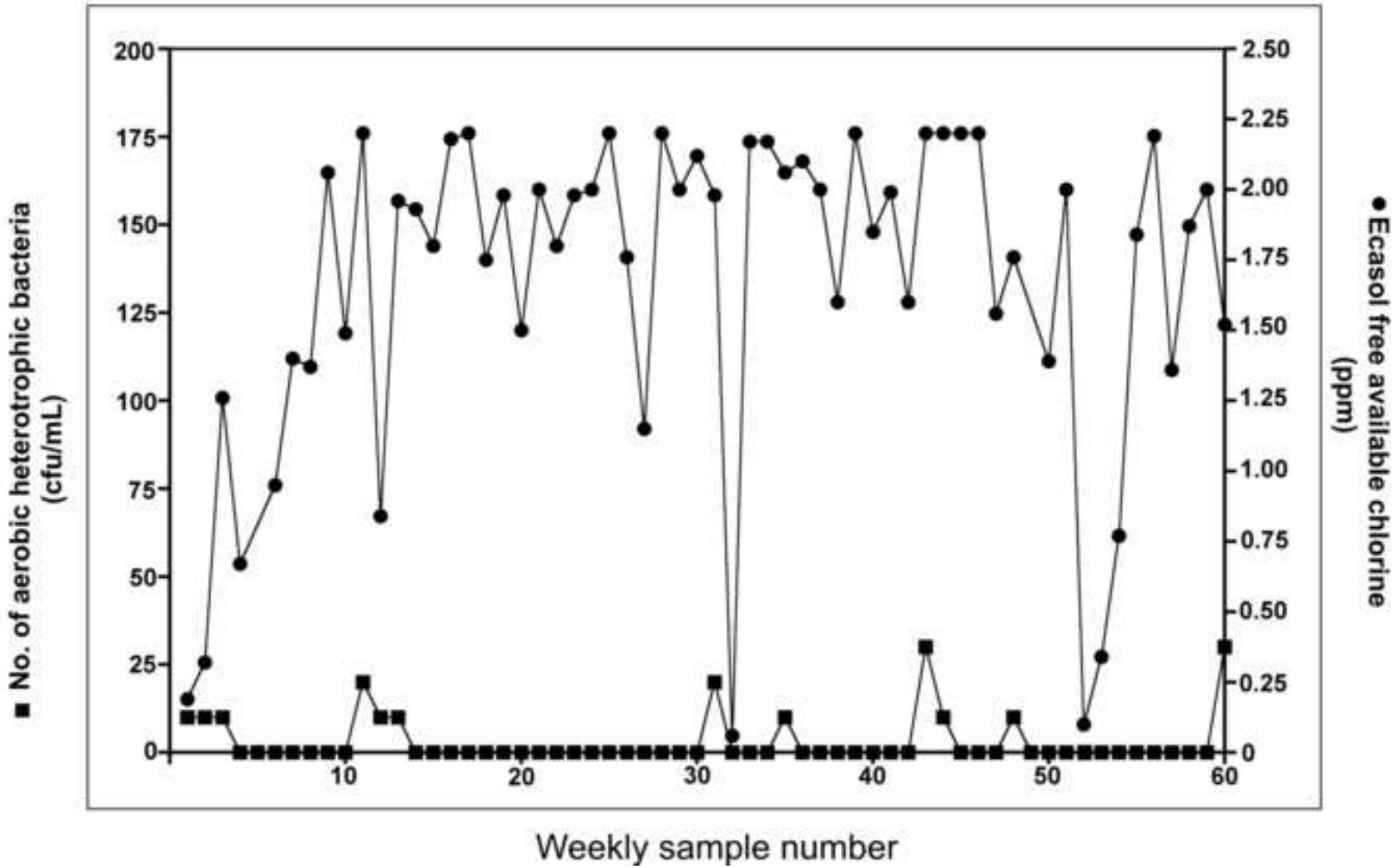
1% Triton-X 100
ppm Ecasol

Fig. 4



Figure

Fig. 4


■ No. of aerobic heterotrophic bacteria (cfu/mL)
● Ecasol free available chlorine (ppm)
Weekly sample number
200
175
150
125
100
75
50
25
0
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
10
20
30
40
50
60

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