2011 NCIMS Conference

PMO Scientific Data

Contents

Sanitizer and Detergent SDS Sheets

Efficacy Data and Reports

Toxicology Reports

Materials Compatibility Report

USDA & FDA Letter of No Objection

Dairy Usage Examples

Sanitizer and Detergent SDS Sheets

MATERIAL SAFETY DATA SHEET

Date: 26th Oct 2009
Revision No.: 5.0
Aversol Detergent

1. Chemical Product and Company Identification

PRODUCT NAME: Aversol
GENERAL USE: Detergent
CHEMICAL FAMILY: Base

MANUFACTURER

Trustwater,
Unit 1, Gurtnafleur Business Park,
Clonmel,
Co. Tipperary,
Ireland.

COMMENTS: To the best of our knowledge, this Material Safety Data Sheet conforms to the requirements of US OSHA 29 CFR 1910.1200, 91/155/EEC and Canadian Hazardous Products Act.

2. Composition Information on Ingredients

Ingredient	CAS-No	EINECS-NO	Wt/Vol %
Water	7732-18-5	231-791-2	92% - 99.76%
Sodium chloride	7647-14-5	231-598-3	0.2% – 4%
Sodium Hydroxide	1310-73-2	215-185-5	0.04% – 4%

COMMENTS: Product composition ranges shown are typical values for health, safety and environmental use and are not intended as specifications.

3. Hazards Identification

EMERGENCY OVERVIEW
Appearance: clear liquid. **Danger!** Causes eye and skin burns. May cause severe respiratory tract irritation with possible burns. May cause severe digestive tract irritation with possible burns. Corrosive to aluminum. Eye contact may result in permanent eye damage.
Target Organs: Eyes, skin, mucous membranes.

Potential Health Effects
Eye: Causes eye burns. May cause lacrimation (tearing), blurred vision, and photophobia. May cause chemical conjunctivitis and corneal damage.
Skin: Causes skin burns. May cause deep, penetrating ulcers of the skin. May cause skin rash (in milder cases), and cold and clammy skin with cyanosis or pale color.
Ingestion: May cause severe and permanent damage to the digestive tract. Causes gastrointestinal tract burns. May cause perforation of the digestive tract. Causes severe pain, nausea, vomiting, diarrhea, and shock. May cause systemic effects.
Inhalation: Irritation may lead to chemical pneumonitis and pulmonary edema. Causes severe irritation of upper respiratory tract with coughing, burns, breathing difficulty, and possible coma. Causes chemical burns to the respiratory tract. Aspiration may lead to pulmonary edema. May cause systemic effects.
Chronic: Prolonged or repeated skin contact may cause dermatitis. Effects may be delayed.

4. First Aid Measures

Eyes: In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical aid immediately
Skin: In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid immediately. Wash clothing before reuse.
Ingestion: If swallowed, do NOT induce vomiting. Get medical aid immediately. If victim is fully conscious, give a cupful of water. Never give anything by mouth to an unconscious person.
Inhalation: If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical aid.
Notes to Physician: Treat symptomatically and supportively.

5. Fire Fighting Measures

General Information: Substance is non-combustible. As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Use water spray to keep fire-exposed containers cool. Contact with metals may evolve flammable hydrogen gas.

Extinguishing Media: Use water spray to cool fire-exposed containers. Substance is non-combustible; use agent most appropriate to extinguish surrounding fire.

Flash Point:	Not applicable.
Autoignition Temperature:	Not applicable.
Explosion Limits, Lower:	Not available.
Upper:	Not available.

6. Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.

Spills/Leaks: Absorb spill with inert material (e.g. vermiculite, sand or earth), then place in suitable container. Avoid runoff into storm sewers and ditches which lead to waterways. Clean up spills immediately, observing precautions in the Protective Equipment section. Provide ventilation.

7. Handling and Storage

Handling: Wash thoroughly after handling. Do not get in eyes, on skin, or on clothing. Keep container tightly closed. Discard contaminated shoes. Use only with adequate ventilation. Do not breathe spray or mist.

Storage: Keep container closed when not in use. Store in a cool, dry, well-ventilated area away from incompatible substances. Keep away from strong acids. Keep away from metals. Keep away from flammable liquids. Keep away from organic halogens.

8. Exposure Controls, Personal Protection

Engineering Controls: Use process enclosure, local exhaust ventilation, or other engineering controls to control airborne levels below recommended exposure limits. Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower.

Exposure Limits

Chemical Name	ACGIH	NIOSH	OSHA - Final PELs
Water	none listed	none listed	none listed
Sodium Chloride	none listed	none listed	none listed
Sodium hydroxide	2 mg/m3 Ceiling	10 mg/m3 IDLH	2 mg/m3 TWA

Personal Protective Equipment
Eyes: Wear chemical goggles.
Skin: Wear appropriate protective gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant a respirator's use.

9. Physical and Chemical Properties

Physical State: Liquid
Appearance: clear
Odor: none reported
pH: Alkaline
Vapor Pressure: 14 mm Hg
Vapor Density: >1.0
Evaporation Rate: Not available.
Viscosity: >1 (ether=1)
Boiling Point: 100 °C – 105 °C
Freezing/Melting Point: 0 °C - -10 °C
Decomposition Temperature: Not available.
Solubility: Soluble.
Specific Gravity/Density: 1.00 – 1.04

10. Stability and Reactivity

Chemical Stability: Stable at room temperature in closed containers under normal storage and handling conditions.
Conditions to Avoid: Acids.
Incompatibilities with Other Materials: Metals, acids, aluminum, tin, zinc.
Hazardous Decomposition Products: Toxic fumes of sodium oxide.
Hazardous Polymerization: Will not occur.

11. Toxicity Information

Eye Effects (Rabbit)
Draize test, rabbit, eye: 400 μg Mild;
Draize test, rabbit, eye: 1% Severe;
Draize test, rabbit, eye: 50 μg/24H Severe;
Draize test, rabbit, eye: 1 mg/24H Severe;
Skin Effects (Rabbit): Draize test, rabbit: 500 mg/24H Severe;<BR.
Carcinogenicity: Not listed by ACGIH, IARC, NIOSH, NTP, or OSHA.
Teratogenicity: No information found.
Reproductive Effects: No information found.
Neurotoxicity: No information found.
Mutagenicity: No information found.

12. Ecological Information

No Information available

13. Disposal Considerations

Dilute with water and flush to sewer if local ordinances allow, otherwise, whatever cannot be saved for recovery or recycling should be managed in an appropriate and approved waste disposal facility. Processing, use or contamination of this product may change the waste management options. State and local disposal regulations may differ from federal disposal regulations. Dispose of container and unused contents in accordance with federal, state and local requirements.

14. Transport Information

Domestic (Land, D.O.T.)

Proper Shipping Name: SODIUM HYDROXIDE SOLUTION
Hazard Class: 8
UN/NA: UN1824
Packing Group: II

International (Water, I.M.O.)

Proper Shipping Name: SODIUM HYDROXIDE, SOLUTION
Hazard Class: 8
UN/NA: UN1824
Packing Group: II

15. Regulatory Information

US FEDERAL

TSCA
CAS# 7732-18-5 is listed on the TSCA inventory.
CAS# 1310-73-2 is listed on the TSCA inventory.
CAS# 7647-14-5 is listed on the TSCA inventory.
Health & Safety Reporting List
None of the chemicals are on the Health & Safety Reporting List.
Chemical Test Rules
None of the chemicals in this product are under a Chemical Test Rule.
Section 12b
None of the chemicals are listed under TSCA Section 12b.
TSCA Significant New Use Rule
None of the chemicals in this material have a SNUR under TSCA.

SARA

CERCLA Hazardous Substances and corresponding RQs
CAS# 1310-73-2: 1000 lb final RQ; 454 kg final RQ
SARA Section 302 Extremely Hazardous Substances
None of the chemicals in this product have a TPQ.
SARA Codes
CAS # 1310-73-2: acute, reactive.
CAS # 7647-14-5: acute.
Section 313
No chemicals are reportable under Section 313.

Clean Air Act:
This material does not contain any hazardous air pollutants. This material does not contain any Class 1 Ozone depletors. This material does not contain any Class 2 Ozone depletors.
Clean Water Act:

CAS# 1310-73-2 is listed as a Hazardous Substance under the CWA. None of the chemicals in this product are listed as Priority Pollutants under the CWA. None of the chemicals in this product are listed as Toxic Pollutants under the CWA.

OSHA:

None of the chemicals in this product are considered highly hazardous by OSHA.

STATE

CAS# 7732-18-5 and CAS # 7647-14-5 are not present on state lists from CA, PA, MN, MA, FL, or NJ.

CAS# 1310-73-2 can be found on the following state right to know lists: California, New Jersey, Pennsylvania, Minnesota, Massachusetts. California No Significant Risk Level: None of the chemicals in this product are listed.

European/International Regulations

European Labeling in Accordance with EC Directives

Hazard Symbols: C

Risk Phrases:

R 34 Causes burns.

Safety Phrases:

S 26 In case of contact with eyes rinse immediately with plenty of water and seek medical advice.

S 37/39 Wear suitable gloves and eye/face protection.

S 45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Exposure Limits

CAS# 1310-73-2: OEL-AUSTRALIA:TWA 2 mg/m3 OEL-BELGIUM:STEL 2 mg/m3 OEL-DENMARK:TWA 2 mg/m3 OEL-FINLAND:TWA 2 mg/m3 OEL-FRANCE:TWA 2 mg /m3 OEL-GERMANY:TWA 2 mg/m3 OEL-JAPAN:STEL 2 mg/m3 OEL-THE NETHERLANDS:TWA 2 mg/m3 OEL-THE PHILIPPINES:TWA 2 mg/m3 OEL-SWEDEN:TWA 2 mg/m3 OEL-SWITZERLAND:TWA 2 mg/m3;STEL 4 mg/m3 OEL-THAILAND:TWA 2 mg/m3 OEL-TURKEY:TWA 2 mg/m3 OEL-UNITED KINGDOM:TWA 2 mg/m3;STEL 2 mg/m3 OEL IN BULGARIA, COLOMBIA, JORDAN, KOREA check ACGIH TLV OEL IN NEW ZEALAND, SINGAPORE, VIETNAM check ACGI TLV

This Safety Data Sheet does not constitute a workplace risk assessment. The data given here is based on current knowledge and experience. This Safety Data Sheet describes the product terms of safety requirements and does not signify any warranty with regard to the product's properties.

16. Other Information

Manufacturer Disclaimer: Information given herein is offered in good faith as accurate, but without guarantee. Conditions of use and suitability of the product for particular uses are beyond our control; all risks of use of the product are therefore assumed by the user. Nothing is intended as a recommendation for uses which infringe valid patents or as extending license under valid patents. Appropriate warnings and safe handling procedures should be provided to handlers and users.

SAFETY DATA SHEET

Date: 3rd June 2009
Revision No.: 4
Ecasol Biocide

1. Chemical Product and Company Identification

PRODUCT NAME: Ecasol
GENERAL USE: Biocide
CHEMICAL FAMILY: Weak Acid

MANUFACTURER

Trustwater,
Unit 1, Gurtnafleur Business Park,
Clonmel,
Co. Tipperary,
Ireland.

COMMENTS: To the best of our knowledge, this Material Safety Data Sheet conforms to the requirements of US OSHA 29 CFR 1910.1200, 91/155/EEC and Canadian Hazardous Products Act.

2. Composition Information on Ingredients

Ingredient	**CAS-No**	**EINECS-NO**	**Wt/Vol %**
Water			99.7%
Sodium chloride	7647-14-5	231-598-3	0.2%
After activation			**1000 ppm (0.1 %) oxidants**
Hypochlorous acid	7790-92-3	232-232-5	<791 ppm (0.791%)
Hypochlorite ion	7681-52-9	231-668-3	<209 ppm (0.209%)
Ozone	10028-15-6	233-069-2	<1.4 ppm (0.00014%)
Chlorine dioxide	10049-04-4	233-162-8	<2.5 ppm (0.00025%)
Chloric acid	7790-93-4	232-233-0	<1.5 ppm (0.00015%)
Chlorous acid			<3.0 ppm (0.0003%)

COMMENTS: Product composition ranges shown are typical values for health, safety and environmental use and are not intended as specifications.

3. Hazards Identification

Emergency Overview: Hazardous gas evolved on contact with acid
Physical Appearance: Colorless liquid with chlorine odor
Immediate Concerns: No hazard expected under normal conditions of use

Potential Health Effects:
Eye: May cause irritation.
Skin: May cause irritation.
Ingestion: May cause irritation of the digestive tract.
Inhalation: May cause respiratory tract irritation
Chronic: Prolonged or repeated skin contact may cause dermatitis. Effects may be delayed.

4. First Aid Measures

Eyes: Immediately flush eyes with plenty of water for several minutes.
Skin: Remove affected clothing and wash skin with plenty of water.
Ingestion: Do not induce vomiting. Give plenty of water to drink. Seek medical attention if and when effects occur.
Inhalation: Remove from further exposure. Seek medical attention if any effects occur.
Notes to Physician: Treat symptomatically and supportively.

5. Fire Fighting Measures

General Information: Substance is non-combustible. As in any fire, wear a self-contained breathing apparatus in pressure-demand, MSHA/NIOSH (approved or equivalent), and full protective gear. Use water spray to keep fire-exposed containers cool. Contact with metals may evolve flammable hydrogen gas.

Extinguishing Media: Use water spray to cool fire-exposed containers. Substance is non-combustible; use agent most appropriate to extinguish surrounding fire.

Flash Point:	Not applicable.
Autoignition Temperature:	Not applicable.
Explosion Limits, Lower:	Not available.
Upper:	Not available.

6. Accidental Release Measures

General Information: Use proper personal protective equipment as indicated in Section 8.

Spills/Leaks: Absorb spill with inert material (e.g. vermiculite, sand or earth), then place in suitable container. Avoid runoff into storm sewers and ditches which lead to waterways. Clean up spills immediately, observing precautions in the Protective Equipment section. Provide ventilation.

7. Handling and Storage

Handling: No special precautions necessary or use with adequate ventilation. Avoid contact with eyes, skin, and clothing. Avoid ingestion and inhalation.

Storage: Store in a suitable container below 25 ºC.

8. Exposure Controls, Personal Protection

Engineering Controls: Facilities storing or utilizing this material should be equipped with an eyewash facility and a safety shower. Use adequate ventilation to keep airborne concentrations low.

OSHA Vacated PELs: No OSHA Vacated PELs are listed for the chemical components of this solution

Personal Protective Equipment
Eyes: Wear chemical goggles.
Skin: Wear appropriate protective gloves to prevent skin exposure.
Clothing: Wear appropriate protective clothing to prevent skin exposure.
Respirators: A respiratory protection program that meets OSHA's 29 CFR 1910.134 and ANSI Z88.2 requirements or European Standard EN 149 must be followed whenever workplace conditions warrant a respirator's use.

9. Physical and Chemical Properties

Physical State:	Liquid
Appearance :	clear
Odour:	chlorine odour
pH:	7 ± 0.3
Vapor Pressure:	Not available
Vapor density:	Not available.
Evaporation Rate:	Not available.
Viscosity:	Not available.
Boiling Point:	> 100 °C
Freezing/Melting Point:	< 0 °C
Decomposition Temperature:	Not available.
Solubility:	Fully soluble in water
Specific Gravity/Density:	> 1 g cm^{-3}

10. Stability and Reactivity

Chemical Stability: Stable under normal ambient conditions of temperature and pressure.
Conditions to avoid: High temperatures, incompatible materials
Incompatibilities with other materials: Strong oxidizing agents, strong acids.
Hazardous Decomposition Products: Chlorine, Hydrochloric Acid
Hazardous Polymerisation: Will not occur.

11. Toxicity Information

Dermal LD50:	Not available
Oral LD50 (rat):	>5000 mg/kg
Inhalation LC50:	Not Available
Eye effects (rabbit):	Negative.
Skin Effects (rabbit):	Negative.
Sensitization (rabbit):	Negative
Mutagenicity (Ames test):	Negative
Reproductive Effects:	Not Available
Teratogenic Effects:	Not Available

12. Ecological Information

Environmental Fate:	No Information found
Environmental Toxicity:	Toxic to aquatic life

13. Disposal Considerations

Dilute with water and flush to sewer if local ordinances allow, otherwise, whatever cannot be saved for recovery or recycling should be managed in an appropriate and approved waste disposal facility. Processing, use or contamination of this product may change the waste management options. State and local disposal regulations may differ from federal disposal regulations. Dispose of container and unused contents in accordance with federal, state and local requirements.

14. Transport Information

Not classified as hazardous for transportation per ASTM G31.42 testing.

15. Regulatory Information

United States: Not available.
Canada: Not available.
European Community EEC Label and Symbol Classification: R31

This Safety Data Sheet does not constitute a workplace risk assessment. The data given here is based on current knowledge and experience. This Safety Data Sheet describes the product terms of safety requirements and does not signify any warranty with regard to the product's properties.

16. Other Information

Manufacturer Disclaimer: Information given herein is offered in good faith as accurate, but without guarantee. Conditions of use and suitability of the product for particular uses are beyond our control; all risks of use of the product are therefore assumed by the user. Nothing is intended as a recommendation for uses which infringe valid patents or as extending license under valid patents. Appropriate warnings and safe handling procedures should be provided to handlers and users.

Efficacy Data and Reports

Moyne Institute
University of Dublin
Department of Microbiology
Trinity College
Dublin 2
Ireland

Dr R.J. Russell

Immunology & Applied Microbiology Section

Phone: 01-6081194
Fax: 01-6799294
E-Mail: rrussell@tcd.ie

OPINION

ANOLYTE PRODUCED BY HYDROFEM REACTORS USING CONTROLLED PROCESS AT NEUTRAL PH

After research and testing since late 2003 using a Hydrofem reactor, I can attest to the following:

- All Hydrofem work in my laboratory only required municipal potable water supply as input and standard grade sodium chloride as the only input additive to yield effective anolyte
- Anolyte produced under preset reactor conditions was always of uniform quality
- All research work carried out used only anolyte produced at neutral pH
- The anolyte produced contained nothing other than the activated and ionised constituents of water and salt
- The activation state subsided after several days, returning anolyte to being slightly saline water
- Anolyte killed a wide range of vegetative bacterial cells (particularly aquatic gram negative species) with less than 2 minutes exposure. Bacillus spores were completely eliminated after 10 minutes exposure.
- Anolyte removed stubborn biofilm from pipework in dental and medical appliances after overnight exposure and maintained this state while present
- No residues remained in pipework after discontinuing use and biofilms re-established almost immediately
- No corrosive or aggressive effects have been observed with metal or plastic pipework over a three month period

- There have been no Health & Safety issues with regard to skin contact, allergies or inhalation effects. Anolyte used as a mouthwash showed no detrimental effect on the mucosae yet reduced oral microbial load by over 1000-fold
- I have reviewed over 60 publications and scientific reports on electrochemically activated water covering toxicity testing, health and safety issues, electro-chemistry and applied trials. This together with my own research observations lead me to conclude that anolyte produced at neutral pH is not hazardous to health in normal use and may be treated as dilute salt water containing 200ppm available free chlorine and having a REDOX activity of over +1000mV. Its physical chemistry is not totally understood yet. It is not the same as sodium hypochlorite diluted to 200 ppm free chlorine in effectiveness (being much better), acidity (being neutral), odour (being much less) or corrosiveness (being much less). A number of different manufacturers have produced equipment for generating anolytes but these have varied in quality, stability of output and particularly pH and aggressiveness.
- Anolyte, produced at neutral pH, has borne out the claims of efficacy for microbial disinfection, control and elimination made for it by Hydrofem Ltd when used according to their protocols. I have satisfactorily been able to replicate their data.

SIGNED: .....

R.J. Russell B.A. (Mod), Ph.D.
(Senior Lecturer in Microbiology)

16th March 2004

microchem
Lancaster Laboratories Europe

Mr Edmond O'Reilly
Hydrofem Ltd
Gurtnafluer Business Park,
Clonmel,
Co Tipperary

Sample Type : Hydrofem Anolyte Solutions **Date Received:** 28/08/02
Date Analysed: 28/08/02

TEST	METHOD
*** Microbial Analysis**	Clients own DEV 023 (1)

LAB NO	BATCH NO
0227089	Hydrofem Anolyte Solutions

For results see pages 2 - 5

Comments: PO#: None given

Signed _____ Date 10/09/2002
Anita Geoghegan Ph D
Director

1 OBJECTIVE:

Development of a procedure to determine the bactericidal properties of Hydrofem anolyte solution using a contact time of 2 minutes.

2 REFERENCE:

Microbiological Analysis of Hydrofem Anolyte solutions – DEV 023 (1)

3 PROCEDURE:

3.1 Materials

Tryptone Soy agar (TSA)
Sabaourand Dextrose agar (SDA)
Neutralizer – Sterile sodium thiosulphate (0.1N)
Sterile Phosphate buffer
Sterile peptone saline with 0.05% polysorbate 80

3.2 Organism Preparation

3.2.1 The following organisms were cultured on either TSA or SDA as indicated:

Organism	Strain	Culture agar	Culture conditions
Escherichia.coli	NCIMB 8545	TSA	30-35°C x 1 day
Salmonella Typhimurium	ATCC 13311		
Listeria monocytogenes	NCTC 11994		
Staphylococcus aureus	NCIMB 9518		
Streptococcus faecalis	NCIMB 775		
Pseudomonas aeruginosa	NCIMB 9027		
Aspergillus niger	ATCC 16404	SDA	20-25°C x 5 days
Candida albicans	ATCC 10231		

Signed _____ Date 10/09/2002
Anita Geoghegan Ph D
Director

3.2.2 After appropriate incubation times, the organisms were harvested using 2mls of sterile peptone saline with 0.05% polysorbate 80 into sterile test tubes.

3.2.3 Using sterile peptone saline with 0.05% polysorbate 80, serial dilutions were prepared for each of the organisms to obtain approximately 10^9 cfu/ml.

3.2.4 The inoculum was confirmed for each organism using the plate count technique with the above culture agar. All plates were incubated at 30-35°C for 2-3 days.

3.2.5 The organism preparations were stored at 2-8°C until required but for no longer than one week.

3.3 Preparation of the sample

3.3.1 The sample was received in the diluted state from Hydrofem Ltd. This was the working suspension and no further dilution/preparation was required prior to analysis.

3.3.2 The sample was analysed on the day of receipt.

3.3.3 The pH and free chlorine levels were analysed upon receipt of the sample by the chemistry department at Microchem laboratories using Standard Operating Procedures.

3.4 Analysis procedure

3.4.1 The sample was dispensed in 9 x 99.9ml aliquots in sterile 250ml containers. Eight of these aliquots represented the test solutions. The remaining aliquot represented a negative sample control.

3.4.2 9 x 99.9ml aliquots of sterile phosphate buffer were dispensed into sterile 250ml containers. Eight of these aliquots represented positive controls and the remaining aliquot represented a negative control.

3.4.3 Each solution was tempered at 20 +/- 2°C for 15 minutes approximately prior to analysis.

Signed _____ Date 10/09/2002 03/06/2010

Anita Geoghegan Ph D
Director

3.4.4 0.1ml of the working suspension of one of the above cultures was added to one aliquot of sample and one aliquot of phosphate buffer. Each container was incubated at 20+/-2°C immediately after inoculation and a stopwatch was set for two minutes.

3.4.5 After two minutes, a 1ml aliquot was removed from each container and added to 9ml aliquots of neutraliser solution tempered to 20+/-2°C prior to use.

3.4.6 The mixture was vortexed.

3.4.7 For the sample solutions, duplicate aliquots of neutralised solution were plated onto each of two petri dishes and poured with molten TSA (for bacteria) or SDA (for yeasts and moulds).

3.4.8 For the positive control, serial dilutions of the neutralised suspension using phosphate buffer were prepared to provide 10^{-2}, 10^{-3} and 10^{-4}. Each dilution was plated in duplicate onto the required agar.

3.4.9 The above procedure was repeated for each organism.

3.4.10 The procedure was repeated for the negative controls omitting the organisms.

3.4.11 All TSA plates were incubated at 30-35°C for 3 days. All SDA plates were incubated at 20-25°C for 5 days.

3.4.12 All counts were recorded and the log reduction due to the sample for each organism was calculated. (Refer to Table 1).

4 SPECIFICATIONS:

The purpose of this study was to demonstrate reduction in viability due to the sample at the stated concentration using a contact time of 2 minutes, therefore specifications were not applicable.

5 EXCLUSION AND LIMITATIONS OF THIS REPORT:

This document describes the test used to determine the bactericidal activity of the anolyte solution against the stated organisms at a contact time of 2 minutes and does not demonstrate the suitability of the procedure for analysis of the sample.

Signed _____ Date ___10/09/2002____

Anita Geoghegan Ph D
Director

TABLE 1

RECOVERY OF ORGANISM FROM SAMPLE

LAB NO: 0227089

ORGANISM	STRAIN	POSITIVE CONTROL Cfu/100ml	SAMPLE Cfu/100mls	LOG REDUCTION
Escherichia.coli	NCIMB 8545	1.1×10^7	<1	1.1×10^7
Salmonella Typhimurium	ATCC 13311	1.6×10^7	<1	1.6×10^7
Listeria monocytogenes	NCTC 11994	1.9×10^7	<1	1.9×10^7
Staphylococcus aureus	NCIMB 9518	2.4×10^7	<1	2.4×10^7
Streptococcus faecalis	NCIMB 775	7.7×10^6	<1	7.7×10^6
Pseudomonas aeruginosa	NCIMB 9027	4.8×10^6	<1	4.8×10^6
Aspergillus niger	ATCC 16404	7.0×10^4	<1	7.0×10^4
Candida albicans	ATCC 10231	6.0×10^4	<1	6.0×10^4

CONCLUSION

For each organism used in the above study, no growth was observed after a contact time of 2 minutes with the sample under the conditions specified.

DEVIATION FROM PROTOCOL

Due to the nature of growth, it was not possible to achieve an inoculum level of 10^9 Cfu/ml, therefore the organisms were used as harvested and the positive control counts recorded.

Signed _____ Date ___10/09/2002____

Anita Geoghegan Ph D
Director

University Hospital **Bonn**

PROF. DR. MED. M. EXNER

Director of the Institute for Hygiene and Public Health of the University Bonn

Institute – Sigmund-Freud-Str. 25 – D-53105 Bonn

Sigmund-Freud-Str. 25
53105 Bonn
Germany

Telephone: 0049 228 287 14022 (Direct Dial)
0049 228 287 15520 (Directorate)
Fax: 0049 228 287 19522

Company GESUNDLEBEN GmbH & Co. KG
Falkenhorst 13
D-48155 Muenster

22.02.2008

AKS Accredited testing laboratory
Registration number: xxx
National Accreditation Centre Hannover

ZLG Accredited by
Central locations of the countries for health protection with regards to medication and medicinal products
ZLG-P-470.06.02

Expert report on efficacy of Trustwater Ecaflo Systems for a short-period sanitization of biofilm-contaminated water-bearing systems

Client: GESUNDLEBEN GmbH & Co. KG

Written order from 25.07.2007

Examination duration: 02.08.2007 – 22.11.2007

DMT – Number: DMT-2007-246

Aim of the examination

The efficacy of a biocidic active ingredient (according to EG regulation 2032/2003, 1451/2007 EG and according to paragraph 11 drinking water regulation) produced by a Trustwater-Ecaflo™ System and based on active chlorine with the trade name Ecasol should be documented for a short-time treatment of biofilm in drinking water pipes.

1. Introduction

1.1 Biofilms

Biofilms are extremely successful biotic communities that enable microbial life embedded in a matrix made out of extracellular polymer substances (EPS). Nearly all microorganisms live in such synergic communities. The synergic way

of life and the different habitats in a biofilm enable to the co-habitation of mixed populations of differing species (Flemming and Wingender 2001, Flemming and Wingender 2002). When the biofilm has reached a certain size it switches to a balanced state of new growth and ablation of biofilm particles. Individual microorganisms and whole biofilm fragments can be discharged by local hydrolysis of the EPS and physical forces (e.g. currents), respectively, which can result in the release of microorganisms into the flowing water (Costerton 1995, Costerton *et al.* 1999).

The possible contamination by facultative pathogenic microorganisms, especially by *Pseudomonas aeruginosa* (Reuter *et al.* 2002, Anaissie *et al.* 2002), *Legionella pneumophila*, Acinetobacter, atypical mycobacteria or *Serratia ssp.* presents a risk of infection for water supply systems (Rahal and Urban 2000, Trautmann *et al.* 2001, Langsrud *et al.* 2003, Hall-Stoodley and Stoodley 2005, Exner *et al.* 2005).

The difficulty of biofilms in drinking water systems and connected systems is gaining increased importance (Exner *et al.* 2005, Donlan and Costerton 2002, Reuter *et al.*2002). They can lead to the contamination of drinking water in the distribution network even when the drinking water left the water supply companies in immaculate quality. Entry routes for biofilm organisms present themselves during the extraction and processing of drinking water, after the processing through pipe leaks in the pipe network, during repairs, maintenance and cleaning processes but also through retrograde contamination of drinking water extraction points and not least through employed personnel (Nagy and Olsen 1985, Le Chavallier *et al.* 1987, Block 1992).

Particularly installation in buildings of medical institutions may be a source of nosocomial infections; the underestimated importance of this became apparent in a recent publication by Exner *et al.* 2007. Some critical areas of the installation in buildings are, for example, long supply pipes, areas of stagnation, accumulations of sediment, dead-end pipes as well as warm-water containers. Since the new

legislation on the water quality for human consumption (drinking water regulation 2001) came into force on 1 January 2003, hospitals – as operators of installations in buildings – have, for example, been assigned independent responsibility for the water quality within their building's installation system (Exner and Kistemann 2004). The same is true for all other operators of buildings' installations, for example in the public domain for swimming pools, or for surgical day clinics and dental surgeries as well.

1.2 The Trustwater-Ecaflo™ Procedure

An activated aqueous disinfection solution with oxidative properties is produced via electrochemical route by the Trustwater – Ecaflo™ procedure which, according to the company, is patented worldwide. The liquid offered under the brand name Ecasol™ is a disinfection means which is produced directly at the usage site. With a pH of 7 the product lays in the pH-neutral range and it is virtually odourless. The produced solutions can show substance concentrations of ≥ 1000 mg free chlorine at a pH of 7. According to the company the neutral pH value allows safe user handling. Additionally the danger of corrosion of surfaces like, for example, fluid-bearing metal pipe lines, and material compatibility of PVC and PU hoses in dental units at a neutral pH range is minimised. According to the company the usage of Ecasol™ is unobjectionable to the law in line with the EG regulations 98/8/EG and 2032/2003 and 1451/2007 EG as well as paragraph 11 of the German drinking water regulation and its efficacy has been documented extensively in international reports. The company GESUNDLEBEN GmbH & Co. KG has already successfully used the Trustwater-Ecaflo™ procedure in the Krankenhaus (Hospital) Bad Doberan in Germany (www.hygiene.ag).

2. Materials and Methods

2.1 Quantitative suspension trial

The quantitative suspension trial is used to ascertain bactericidal and fungicide efficacy of a disinfection means and was performed in a modified manner according to standard methods of the DGHM (Deutsche Gesellschaft fuer Hygiene and Mikrobiologie – German Association for Hygiene and microbiology)

for the testing of chemical disinfection methods, 2001. *P. aeruginosa* with a germ count of 10^6 per ml was used as test organism. Neutralisation of the disinfection means was performed (according to pre-established regulation) with 0.3% sodium thiosulphate and 0.1% catalase. The tests were performed for different reaction times and at different concentrations.
All tests were performed without and with defined organic loads (0.03% bovine serum albumin BSA), respectively.

2.2 Silicone hose model

The company Deutsch & Neumann provided silicone hoses for the tests in which biofilms had been generated through continuous drinking water flow (according to Otte, 2006). At the beginning of the examinations the silicone hose was covered in a three-year-old biofilm with up to 8.4 x 10^7 colony-building units (KBE) per cm^2. The used silicone hose with biofilm measures 25 metres in length, has an inner diameter of 0.4 cm and a thickness of 1 mm.

2.2.1 <u>Circuit system in the silicone hose model for stimulation of immediate measures</u>

The efficacy of Ecasol™ was examined in a closed circuit system. A pre-defined concentration of the biocide (100 and 200 mg/l (ppm)) was pumped into 2 litres of fresh water through a biofilm-contaminated silicone hose over a defined time period (180 and 360 minutes). After the treatment the biomass was scraped out of the silicone hose, homogenised and incubated in R2A Agar at 20°C for seven days. Germ count was ascertained by heterotrophic plate count (HPC). Afterwards the bacterial load was converted into KBE per cm^2. The circulation of the fluid occurred continuously over the entire test period. The flow rate was 400ml per minute during all trials. At the beginning and end of each test the potency, pH value and redox potential were recorded.
The circuit trials were performed at differing temperatures (22°C and 37°C).

3. Results

3.1 Quantitative suspension trial without and with organic loads

In order to document the general efficacy of Ecasol suspension trials were initially performed without and with organic loads (0.03% BSA) at room temperature.
The *P. aeruginosa* concentration measure 10^6 KBE/ml.
The results without organic loads show that an Ecasol concentration of 22 ppm is sufficient to inactivate the bacteria beyond the detection limit after one minute.
With existing organic loads a reduction by 2.28 $\log_{10}$ can be reached. The reduction can be increased to 2.60 $\log_{10}$ when left up until 180 minutes.
An increase of the Ecasol concentration to 100 ppm for the same length of time leads to a slightly better result – 2.38 $\log_{10}$ steps after one minute and 2.68 $\log_{10}$ steps after 180 minutes (Fig. 1.).

3.2 Ecasol in closed circuit system

In order to examine the efficacy of the biocide substance Ecasol with regards to fighting of biofilm, higher concentrations of Ecasol (100 and 200 ppm) were used in the closed circuit system (see 2.2.1). Here the efficacy was examined at differing temperatures (22°C and 37°C) and time exposures (180 minutes and 360 minutes). The biofilms were three years old and were generated in the silicone hose model of the Institute for Hygiene and Public Health Bonn. Silicone tubes with a length of 30 cm were used. The start-up germ count was 6×10^7 KBE/cm^2, the flow speed was 400 ml/min.
A reduction of 3.15 $\log_{10}$ steps was noticed after treatment with an Ecasol concentration of 100 ppm at 22°C after 180 minutes. After a treatment time of 360 minutes the reduction of 3.78 $\log_{10}$ steps was a bit higher.
A temperature increase to 37°C lead to a reduction of 4.59 $\log_{10}$ steps after 180 minutes. After the treatment time of 360 minutes no KBE could be proven.
After treatment time of 180 minutes at an Ecasol concentration of 200 ppm at 22°C no KBE could be proven. The procedure at 37°C showed the same result (Fig. 2).
When the temperature was increased during continuous flow and increased concentration the efficacy of the biocide under investigation became stronger.

3. Discussion

The liquid biocide Ecasol produced by the Trustwater-Ecaflo procedure has an antibacterial effect. The examinations showed that bacteria that are present in a homogeneous solution are already completely inactivated within one minute (Fig. 1). As soon as an organic load is present (0.03% BSA in the examinations) Ecasol is fed (???) by this / is drained by this (???). However, the bacteria could be reduced by 2.68 $\log_{10}$ steps in the quantitative suspension trials (Fig. 1).

The efficacy of Ecasol to remove drinking water biofilm was examined in the closed circuit system and under continuous addition of fresh water. The biofilms were generated in the silicone hose model of the Institute for Hygiene and Public Health Bonn. In the circuit system it could be shown that after treatment with an Ecasol concentration of 200 ppm and after 180 minutes the germ count in the biofilm lay below the HPC detection limit; this was irrespective of the temperature (22°C and 37°C). At an Ecasol concentration of 100 ppm the results were temperature dependent. At 22°C no germs could be detected after 360 minutes, at 37°C this was already the case after 180 minutes. During continuous flow it was noticed that Ecaflo is temperature dependent.

A germ-reducing efficacy of the Trustater Ecaflo procedure without an organic load can be confirmed based on the examinations performed. Sufficient efficacy of the Trustater Ecaflo procedure at short-term treatment of drinking water biofilm can be certified based on the examinations performed.

Bonn, 22.02.2008

Prof. Dr. med M. Exner Dr. rer. Nat. J. Gebel

Hydrofem Ltd

Phenol Co-efficient Challenge

REPORT

Produced by

Southern Scientific Services Ltd

Dr Marie Kerr

Phenol Challenge

Introduction

Phenol has long been used as the standard to which disinfectant efficacy is compared. The test is used to assess and score disinfectants on a scale that is measurable and allows comparison of disinfectants.

Electrochemically Activated Solution

In brief Electrochemically Activated Solutions are generated by an electrochemical reaction where tap water is mineralized by introducing NaCl on line before entering an electrochemical cell, cathode chamber followed by an anode chamber where a voltage of direct current is applied. The reaction is simple non-complex making it possible to generate the desired quantities of Electrochemically Activated Solution onsite. However, little information exist outside of Russia on EAS and it is only in recent years that scientific publications have started to appear in Europe and America.

Objective

[a] Determine the Phenol score for Electrochemically Activated Solutions [EAS]
[b] Compare EAS to other commercially available disinfectants which will be EAS competitors in the market place.

Aim

The aim of the study is to incorporate all available data in a single report in order to progress the Electrochemically Activated Solution towards registration with the Department of Marine and the Department of Agriculture.

Phenol Co-efficient

1.0 Preparation of disinfectants for challenge test

The following list of disinfectants were assessed for their Phenol Co-efficient value against a range of NCTC strains of bacteria i.e.*Escherichia coli* and *Pseudomonas aeruginosa*.

[1] **Phenolic Compounds** - Phenol [*working concentration 50,000 ppm or 5%]*
[2] **Halogens**- Electrochemically Activated Solution [*working concentration 250 ppm or 0.025%*], Sodium Hypochlorite [*working concentration 10,000 – 20,000 ppm or 1- 2%*] and Aquatabs [Sodium dichloroisocyanurate, working concentration 5 ppm or 0.0005%]]
[3] **Virkon** [*working concentration 10,000 ppm or 1%*]
[4] **Quaternary Ammonium Compound** - Triquart P3 [working concentration 0. 3% or 3000 ppm]

2.0 Preparation of culture

Using a sterile loop remove a sufficient amount of culture of the test organism from a non-selective agar plate i.e. Plate Count Agar.
2.1 **Day 1**- Inoculate 10 ml of Brain Heart Infusion Broth incubate for 18 to 24 hrs at 37 °C with a loopful of NCTC culture.
2.2 **Day 2 -** From the 18 – 24 h culture remove a 1 ml aliquot and place in 10 ml of fresh Brain Heart Infusion Broth incubate for 18 to 24 hrs at 37 °C.
2.3 **Day 3 -** From the 18 – 24 h culture remove a 1 ml aliquot and place in 10 ml of fresh Brain Heart Infusion Broth incubate for 18 to 24 hrs at 37 °C.
The 3 day sub-culture ensures that all bacterial cells are healthy and active, giving optimal potential for growth when challenged against disinfectants.
2.4 **Day 3** – Carry out a dilution series in 9 ml of MRD to enumerate the number of colony forming units of NCTC culture.

3.0 Phenol stock

Prepared a 5% w/v of Phenol in sterile tap water, heating to ensure it is fully dissolved.

4.0 Disinfectants for challenge test

Prepare all disinfectants according to the manufacturers instructions and at the concentrations recommended for disinfection usage. Prepare further dilutions from the stocks of working disinfectants for determining the most effective concentration of test disinfectant i.e. tubes of Tryptic Soya Broth giving growth at 5 minutes but not at 10 minutes [will vary depending on the organism used].

5.0 Tryptic Soya Broth

Add 30.0g of Tryptic Soya Broth to 1 litre of demineralised water, and dissolve by heating. Where necessary adjust the pH of the dissolved broth. Dispense the Tryptic Soya Broth into 10 ml volumes and autoclave at 121 °C for 15 minutes. Store at room temp for up to 1 month.

6.0 Disfectants challenge against Phenol

6.1 **Phenol** [P]– 5% Stock - From the above stock solution of Phenol prepare and dilute the Phenol in distilled water to achieve a concentration sufficient to give a reading of positive growth after 5 minutes in Tryptic Soya Broth, but negative growth after 10 and 15 minutes.

6.2 **Virkon** [VK]– 1% Working conc. Prepare according to the manufacturers instructions at its working concentration. Prepare also a series of dilutions to achieve a concentration sufficient to give a reading of positive growth after 5 minutes in Tryptic Soya Broth, but negative growth after 10 and 15 minutes.

6.3 **Quaternary Ammonium Compound** [TQ] 0.3% Working conc. Prepare according to the manufacturers instructions at its working concentration. Prepare also a series of dilutions to achieve a concentration sufficient to give a reading of positive growth after 5 minutes in Tryptic Soya Broth, but negative growth after 10 and 15 minutes.

6.4 **Electrochemically Activated solution** [EAS] 0.025% Working conc. Prepare at its working concentration. Prepare also a series of dilutions to achieve a concentration sufficient to give a reading of positive growth after 5 minutes in Tryptic Soya Broth, but negative growth after 10 and 15 minutes

6.5 **Sodium dichloroisocyanurate** [SDI] 0.0005% Working conc. Prepare at its working concentration. Prepare also a series of dilutions to achieve a concentration sufficient to give a reading of positive growth after 5 minutes in Tryptic Soya Broth, but negative growth after 10 and 15 minutes

Note: All disinfectants were prepared and used within 5 days

7.0 Chemical Parameters of Electrochemically Activated Solution

7.1 When testing the EAS prior to performing the disinfectant challenge test measure the Free available chlorine concentration

7.2 When testing the EAS prior to performing the disinfectant challenge test measure the pH value.

8.0 Method

8.1 Dispense 5 ml of Phenol and each of the disinfectants - working concentration and their subsequent dilutions into a number of test tubes marking the disinfectant type and the concentration prepared on the individual test tubes.

8.2 To each of the disinfectants being challenged add 500 µl of the individually grown cultures i.e. E. coli NCTC 9001 and Pseudomonas aeruginosa NCTC 10662 etc noting the time of inoculation. Mix the individual tubes using a vortex in order to obtain a homogenous suspension, allowing the disinfectant to come into contact with the bacteria.

8.3 Proceeding 5, 10 and 15 minutes transfer 10 µl loopfuls from each disinfectant being challenged and add to 10 ml of Tryptic Soya Broth**.**

8.4 Prepare a positive control by inoculating 10 µl of the culture being challenged into 10 ml of Tryptic Soya Broth [*this tube will show positive growth, indicted by a cloudy suspension after 48 hours at 37 °C*]. A blank of 10 ml of Tryptic Soya Broth should also be incubated at 37°C for 48 hours [*this tube will not show positive growth i.e. the tube should be clear of any cloudiness]*

8.5 Incubate all challenge tubes including controls at 37°C for 48 hours

9.0 Interpretation of results

9.1 After the appropriate incubation period 37°C for 48 hours remove the Tryptic Soya Broth tubes and interpret as follows

Visibility in Tryptic Soya Broth	**Result**	**Visibility in Tryptic Soya Broth**	**Result**
Cloudiness visible	+	No cloudiness visible	-

9.2 Record as positive any tubes showing cloudiness. Report as negative any tubes showing no growth as indicated by a clear Tryptic Soya Broth Solution.

9.3 The positive control i.e. should show positive growth**,** while the negative control should be clear, indicating no growth. If either of these tubes do not conform with these results the test should be considered void.

10.0 Determining the Phenol Co-efficient

The Phenol Co-efficient is determined by dividing the highest dilution of the test disinfectant being tested that destroyed the micro-organisms in 10 minutes but not in 5 minutes, by the highest dilution of Phenol that destroyed the micro-organisms in 10 minutes but not in 5 minutes.

11.0 Calculation of Phenol Co-efficient

From the results obtained calculate the Phenol Co-efficient

Example
Assume a 1/20 dilution of Phenol kills E. coli within 10 minutes and a 1/300 dilution of test disinfectant kills E. coli within 10 minutes. The calculation of the Phenol Co-efficient is as follows;

Phenol Co-efficient = Test disinfectant/Phenol
PC = 300/20 = 15
PC 15
Lysol is 15 times more effective than Phenol in killing E. coli

12.0 Hydrofem Ltd Equipment

The Hydrofem equipment was used to manufacture the required concentration of Electrochemically Activated Solution with the required Free Available Chlorine [FAC] and pH. Table 1 below indicates the setting required to achieve various concentrations of FAC.

Table 1 Shows machine setting used to achieve various Free Avaialble Chlorine levels

Flow	Current	pH level	mV	Free Available Chlorine [FAC]
1.0	6-7 amp	6.95 – 7.10	20-25	200
1.2	6-7 amp	6.95 – 7.10	20-25	160
1.6	6-7 amp	6.95 – 7.10	20-25	140
2.0	6-7 amp	6.95 – 7.10	20-25	100

Table 2 Result of enumeration of test organism *Escherichia coli* NCTC 9001

Test organism	Dilutions	Result	Average	Log cfu per ml
Escherichia coli NCTC 9001	10^{-5} 10^{-6} 10^{-7}	tntc,tntc,tntc 77,86,67 8,10,7	76,666,666	7.8

Table 3 Result of enumeration of test organism *Pseudomonas aeruginosa* NCTC 10662

Test organism	Dilutions	Result	Average	Log cfu per ml
Pseudomonas aeruginosa NCTC 10662	10^{-5} 10^{-6} 10^{-7}	tntc,tntc,tntc 70,69,54 7,8,13	64,333,333	7.8

13.0 Phenol results for *Escherichia coli*

Escherichia coli NCTC 9001

Disinfectant Phenol 5% 50,000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
Average	**3**	**13000 13000 13000**	**11700 -14300**

Disinfectant Electrochemical Activated Solution [EAS] 0.025% 250 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3	**125 150 125**	**120 -140**
Average		**130**	
Phenol Co-efficient score/range		**96**	**89-104**

Disinfectant Triquart 0.3% 3000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3	**900 900 800**	**780 -954**
Average		**867**	
Phenol Co-efficient score/range		**15**	**13-16**

Disinfectant Hypochlorite 1.5% 15000ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3	**300 400 300**	**300-366**
Average		**333**	
Phenol Co-efficient value		**38**	**34-42**

Virkon 1.0% 10,000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	1	**5000**	**4500-5500**
Average		**5000**	
Phenol Co-efficient score/range		**3**	**2-3**

Sodium dichloroisocyanurate 0.0005% *5ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3		
		250 400 250	**270-330**
Average		**300**	
Phenol Co-efficient score/range		**42**	**38-46**

N = number of tests performed
A factor of 10 – 15% accuracy is applied by industry [Verbal Communication with Industry]
*Note Although the working concentration was stated on the label as 5ppm this concentration was not found to give any bacterial kill, so the concentration had to be increased

14.0 Phenol Co-efficient calculations – *Escherichia coli*

Calculations

Phenol co-efficient values

Test organism *Escherichia coli*

Electro-chemically Activated Solution
Calculation Test/phenol 385/4 = 96

Triquart
Calculation Test/phenol 65/4 = 15

Hypochlorite
Calculation Test/phenol 150/4 = 38

Virkon
Calculation Test/phenol 10/4 = 3

Sodium dichloroisocyanurate
Calculation Test/phenol 167/4 = 42

15.0 Phenol results for - *Pseudomas aeruginosa*

Pseudomonas aeruginosa NCTC 10662

Disinfectant Phenol 5% 50,000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3	**11,000 11,000 12,000**	**10350- 12650**
Average		**11500**	

Disinfectant Electrochemical Activated Solution [EAS] 0.025% 250 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	3	**190 190 220**	**180-220**
Average		**200**	
Phenol Co-efficient score/range		**63**	**57-69**

Disinfectant Triquart 0.03% 3000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	2	**700 700**	**630-770**
Average		**700**	
Phenol Co-efficient score/range		**18**	**16-18**

Disinfectant Hypochlorite 1.5% 15000 ppm	**N**	**Results** Positive growth after 5 min but not after 10 in TSB	**Accuracy range ±10%**
	2	**800 800**	**680-920**
Average		**800**	
Phenol Co-efficient score/range		**16**	**14-18**

N = number of tests performed
A factor of 10 – 15% accuracy is applied by industry [Verbal Communication with Industry]

16.0 Phenol Co-efficient calculations – *Pseudomonas aeruginosa*

Calculations

Phenol co-efficient values

Test organism *Pseudomonas aeruginosa*
Electro-chemically Activated Solution
Calculation Test/phenol 250/4 = 63

Triquart
Calculation Test/phenol 71/4 = 18

Hypochlorite
Calculation Test/phenol 63/4 = 16

17.0 Summary of Phenol results

Table 4 Summary of Phenol Co-efficient scores for a range of disinfectants

Test disfectants	Conc limit ppm	Test organism	Phenol score	Phenol range
Electrochemically Activated Solution	130	*Escherichia coli*	96	**89-104**
Triquart P3	867		15	**13-16**
Hypochlorite	333		38	**34-42**
Virkon	5000		3	**2-3**
Sodium dichloroisocyanurate	300		42	**38-46**
Electrochemically Activated Solution	200	*Pseudomonas aeruginosa*	63	**57-69**
Triquart	700		18	**16-18**
Hypochlorite	800		16	**14-18**

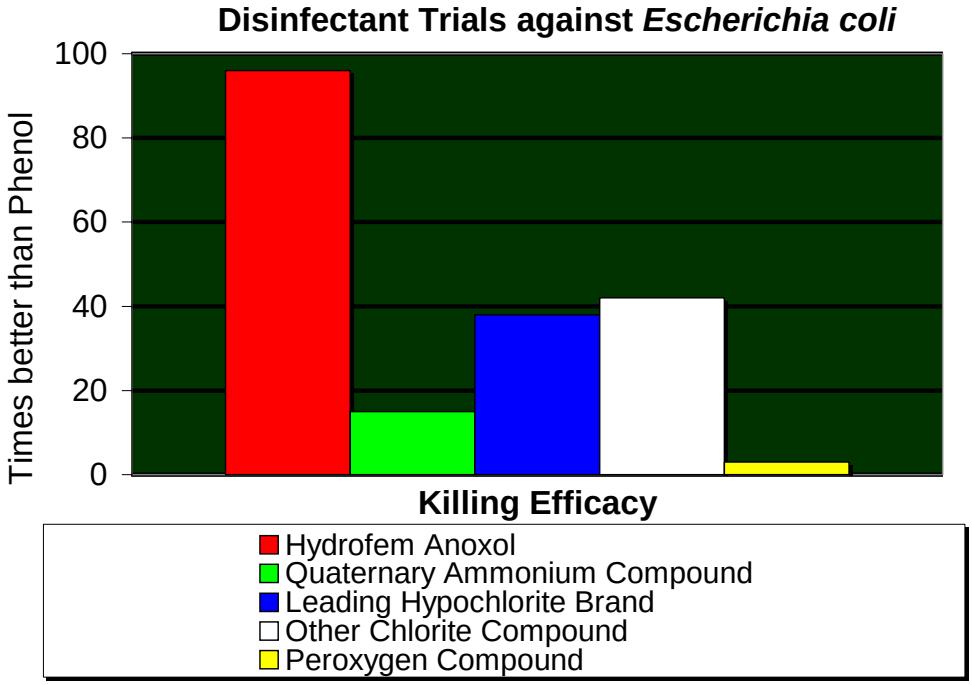


Figure 1. The Phenol Scores for a range of disinfectants challenged with *Escherichia coli*

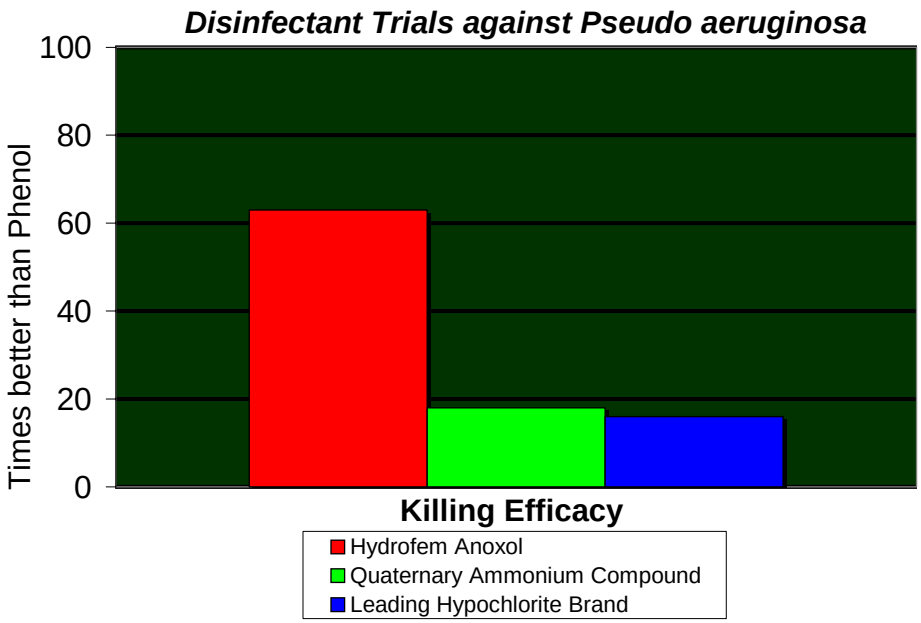


Figure 2. The Phenol Scores for a range of disinfectants challenged with *Pseudomonas aeruginosa*

18.0 Direct inoculation study

Recovery of organism from sample

Table 5 Details a range of bacteria inoculated into Electrochemically Activated Solution and plated after a contact time of 2 minutes

Organism	**Strain**	**Positive control cfu/100 ml**	**EAS cfu/100 ml**	**Log reduction**
Escherichia coli	NCIMB 8545	1.1×10^7	<1	1.1×10^7
Salmonella typhimurium	ATCC 13311	1.6×10^7	<1	1.6×10^7
Listeria monocytogenes	NCTC 11994	1.9×10^7	<1	1.9×10^7
Staphlococcus aureus	NCIMB 9518	2.4×10^7	<1	2.4×10^7
Streptococcus faecalis	NCIMB 775	7.7×10^6	<1	7.7×10^6
Pseudomonas aeruginosa	NCIMB 9027	4.8×10^6	<1	4.8×10^6
Aspergillus niger	ATCC 16404	7.0×10^4	<1	7.0×10^4
Candida albicans	ATCC 10231	6.0×10^4	<1	6.0×10^4

Carried out by MicroChem Dungarvan

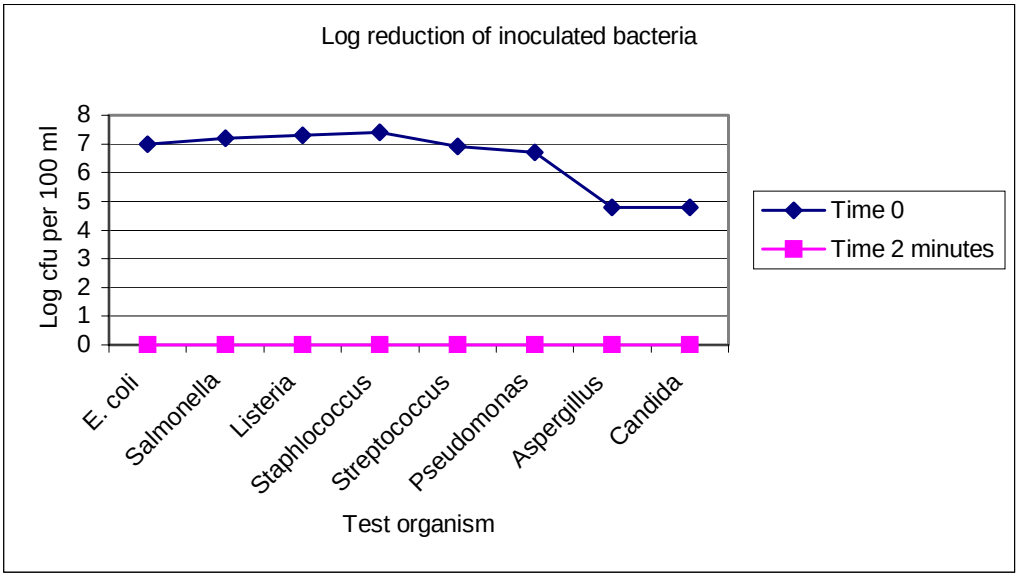


Figure 3 Log reduction for a range of bacteria inoculated into in Electrochemically Activated Solution and left for a contact time of 2 minutes, before enumeration on a non-selective agar

19.0 Results of direct inoculation study

No growth was observed after 2 minutes contact time, giving a complete kill for all test organisms

20.0 Conclusion

The results of the study carried out indicates that Electrochemically Activated solutions are more effective that the standard range of disinfectants on the market.

The efficiency is dependent on the test organism used. When *Escherichia coli* was used as the challenge test organism results ranged from 83 for EAS to 3 for Virkon. When *Pseudomonas aeruginosa* was used as the challenge test organism results ranged from 63 for EAS to 16 for Hypochlorite.

Direct inoculation studies performed on a wide range of test organisms lead to complete elimination after a 2 minute contact time.

FINAL STUDY REPORT

STUDY TITLE

Virucidal efficacy of two different concentrations of ECA Anolyte against feline calicivirus

PRODUCT IDENTITY

ECA Anolyte

SPONSOR

Trustwater c/o

Johnson Diversified Products

PERFORMING LABORATORY AND AUTHOR

Sagar M. Goyal, DVM, PhD
Professor of Virology,
Department of Veterinary Population Medicine,
University of Minnesota, 1333 Gortner Ave,
Saint Paul, MN 55108, USA.

STUDY COMPLETION DATE

February 27, 2010

REPORT SUBMITTED ON

March 1, 2010

DISCLAIMER

This study was not done under GLP conditions

STUDY REPORT

GENERAL STUDY INFORMATION

Study title: Virucidal efficacy of two different concentrations of ECA Anolyte against feline calicivirus

Sponsor: Johnson Diversified Products
1408 Northland Drive, Suite 407
Mendota Heights, MN 55120-1013

Test Facility: Virology Laboratory
1333 Gortner Avenue
University of Minnesota
Saint Paul, MN 55108

TEST SUBSTANCE IDENTITY

Test substance name: ECA Anolyte. According to the sponsor, the ECA Anolyte was prepared on February 19, 2010 at 8:00 am.

Test Substance Characterization: Characterization of ECA Anolyte as to content, stability, and ppm equivalent of chlorine etc. is the responsibility of the sponsor. The sponsor has indicated that the freshly generated solution delivered to the lab titrated at roughly700ppm FAC. On receipt at the Performing Laboratory, the pH of undiluted solution was 6.2.

Concentrations of ECA Anolyte and time points to be tested for virucidal efficacy: Two experiments were done. In first experiment 9:30 hrs old solution was used. In the second experiment, the solution was 56:30 hrs old. The concentrations of ECA Anolyte tested at three different contact times (1, 2, and 5 minutes) are listed in Table 1. The dilutions for each experiment were prepared immediately before use in sterile distilled water.

Table 1. Concentrations of ECA Anolyte and time points

Time	Concentrations of ECA Anolyte	
5 min	500ppm	150ppm
2 min	500ppm	150ppm
1 min	500ppm	150ppm

Dilutions of Test Substance: The ECA Anolyte was diluted at the Performing Laboratory. The dilutions were made in sterile distilled water as per Table 2. After the dilutions were made, their actual pH was measured and recorded. The dilutions for each experiment were prepared immediately before use. The solutions were 9:30 and 56:30 hrs old at the time of experiment 1 and 2, respectively. Between experiments, the solution was stored at room temperature in dark.

Table 2. Dilutions of ECA Anolyte and their characteristics

Dilution No.	ppm needed for the test	Amount of initial solution (1000 ppm)	Amount of diluent	Actual ppm measured	pH	
					Expt. 1	Expt. 2
A	500	70.0 mL	30.0 mL	500	6.32	5.90
B	150	21.4 mL	78.6 mL	150	6.32	5.93

Study Dates and times:

Experiment 1:
Date and time of ECA preparation: February 19, 2010 at 8:00 am
Date and time ECA received in the Lab: February 19, 2010 at 10:30 am
Date and Time dilutions were made: February 19, 2010 at 5:30 pm
Date and Time solutions were used in testing: February 19, 2010 at 6:00 pm
Study Initiation Date and time: February 19, 2010 at 5:30 pm
Study completion date: February 25, 2010
Final report date: March 1, 2010

Experiment 2:
Date and time of ECA preparation: February 19, 2010 at 8:00 am
Date and time ECA received in the Lab: February 19, 2010 at 10:30 am
Date and Time dilutions were made: February 21, 2010 at 4:30 pm
Date and Time solutions were used in testing: February 21, 2010 at 5:00 pm
Study Initiation Date and time: February 21, 2010 at 4:30 pm
Study completion date: February 27, 2010
Final report date: March 1, 2010

OBJECTIVE

To evaluate the virucidal efficacy of two different concentrations ECA Anolyte against feline calicivirus in a surface test. The feline calicivirus belongs to the family Caliciviridae and is normally used as a surrogate for human Norovirus because the latter virus cannot be grown and titrated adequately in vitro.

SUMMARY OF RESULTS

Test substance: ECA Anolyte.

Dilutions tested: 500 ppm and 150 ppm

Challenge virus: Feline calicivirus strain 255.

Host cells: Crandell-Reese feline kidney (CRFK) cells.

Exposure temperature: Ambient room temperature (approx. 23ºC).

Exposure time: 1, 2, and 5 minutes.

Growth medium: Minimum essential medium (MEM) with Earle's salt (Cell grow, Media Tech, VA, USA) containing antibiotics (150 IU/mL penicillin, 150 µg/mL streptomycin, 50 µg/mL neomycin and 1 µg/mL fungizone), 8% fetal bovine serum, 0.05% trypsin, and Edamin S as additive.

Maintenance Media: Same medium as above except with 4% donor horse serum instead of fetal bovine serum.

Efficacy results: ECA Anolyte met the test criteria specified in the protocol. Under these test conditions, the results indicate 99.999% inactivation of feline calicivirus within one minute.

TEST PROTOCOL

1. **Virus**

   The 255 strain of feline calicivirus used in the present study was obtained from the National Veterinary Sciences Laboratory, Ames, IA. The stock virus was prepared by collecting the supernatant culture fluid from 75-100% infected CRFK cells. The cells were disrupted by freeze-thaw cycle (repeated thrice) and cell debris was removed by centrifugation at 2,000g for 10 minutes at 4^0C. The supernatant was removed, aliquoted (1 ml each in cryovials), and stored at -80^0C until the day of use. On the day of experiment, one aliquot of virus was removed and thawed just before the experiment. The stock virus, when tested, showed cytopathic effects (CPE) consistent with FCV on CRFK cells. The cytopathic effects observed were: rounding of cells followed by the disintegration of monolayer.

2. **Cell culture**

   The CRFK cells are used routinely in our laboratory. This cell line has historically been used as the cell line for propagation, detection, and titration of feline calicivirus. For titration purposes, the cells were seeded into 96-well microtiter plates and incubated at 37^0C in a humidified atmosphere of 5% CO_2. The plates were examined daily to determine confluency of the cells and at the time of experiment the confluency of the cells was >80%.

TEST METHOD

1. **Preparation of Test Substance**

   Test substance (ECA Anolyte) was prepared by the sponsor of the study and delivered to the Performing laboratory at 10:30 am on February 19, 2010. On receipt, the test substance was in solution phase as determined by visual observation. Two experiments were done. The first experiment was started at 5:30pm on February 19, 2010 and the second experiment on February 21, 2010 at 4:30pm. See page 2 for dilutions tested and how they were prepared.

2. **Preparation of Virus Films**

   Films of virus were prepared by spreading 0.1 ml of virus inoculum uniformly over the bottom of all wells of a 6-well plate and allowed to dry for 30 min at room temperature (about 23^0C) in a biosafety cabinet. The wells were labeled A through F.

3. **Treatment of Virus Films with Test Substance**

   ECA Anolyte at 500ppm was placed in wells A, B, and C @ 2mL/well. Well D, E, and F served as negative controls; they each received 2 mL of phosphate buffered saline (PBS) instead of ECA. *(Note: This is a deviation from the earlier protocol in which a volume of 5 mL was used. We have reduced the volume to 2 mL so as not to unnecessarily reduce virus concentration).* The plate was placed on an orbital shaker and oscillated at 120 rpm. After contact times of 1, 2, and 5 minutes, the ECA and PBS solutions were pipeted and aspirated from their respective wells. All solutions (100 µL aliquots) were immediately diluted by mixing with 900 µL of the maintenance medium so that the activity of ECA and PBS was stopped at the indicated times. Similar test was done with 150ppm of ECA Anolyte. The whole experiment was repeated with the two dilutions (prepared fresh at the time of use) on February 21, 2010.

4. **Infectivity Assays**

   Serial 10-fold dilutions of all samples were prepared immediately in the maintenance medium. All dilutions were inoculated in CRFK cells (4 wells/dilution) grown in 96-well

tissue culture plates. Cell controls were inoculated with culture media only. The plates were incubated at 37^0C in a humidified atmosphere of 5% CO_2. The cells were observed microscopically for 5 days post infection for the appearance of virus specific CPE (cytopathic effects).

PROTOCOL CHANGES

Protocol Amendments: No protocol amendments were required for this study.

Protocol Deviations: No protocol deviations occurred during this study.

DATA ANALYSIS

Viral titers are expressed as-$\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$), respectively, calculated by the method as described by Spearman and Karber.

$$\text{log of 1}^{st}\text{ dilution inoculated} - \left(\left(\left\{\frac{\text{sum of \% mortality at each dilution}}{100}\right\} - 0.5\right) \text{X (log of dilution)}\right)$$

STUDY ACCEPTANCE CRITERIA

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control films; 2) that the cell controls be negative for infectivity. Note: An efficacious product must demonstrate at least 4 $\log_{10}$ virus inactivation.

SUTY RETENTION

Record Retention

All of the original raw data developed exclusively for this study shall be archived at the Virology Laboratory, Room 350-352, 1333 Gortner Ave, Saint Paul, MN 55108. These original data include, but are not limited to the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original study protocol.

6. Copy of final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention
The test substance will be returned following study completion per Sponsor-approved protocol. It is the responsibility of the Sponsor to retain a sample of the test material.

REFERENCES

1. Annual Book of ASTM Standards 2000, Section 11 Water and Environmental Technology Volume 11.05 Biological Effects and Environmental Fate: Biotechnology; Pesticides, E1053-97.
2. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, NJ. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.
3. Environmental Protection Agency Federal Register: August 25. 2000 (Volume 65, Number 166).
4. Inactivation of feline Calidvirus, a Norwalk virus surrogate. Journal of Hospital Infection (1999) 41:51-57.
5. Taku, A., Gulati, B.R., Allwood, P. B., Palazzi, K., Hedberg, C.W., and Goyal, S.M. 2002. Concentration and detection of caliciviruses from food-contact surfaces. J. Food Protect. 65:999-1004.
6. Gulati, B.R., Allwood, P.B., Hedberg, C.W., and Goyal, S.M. 2001. Efficacy of commonly used disinfectants for the inactivation of calicivirus on strawberry, lettuce, and a food-contact surface. J. Food Protect. 64:1430-1434.

STUDY RESULTS

Results of tests with ECA Anolyte ready-to-use solution, exposed to feline calicivirus in the presence of 4% donor horse serum as organic soil load at room temperature (approx. 23^0C) for 1, 2, and 5 minutes are shown in Tables 3 to 10.

Experiment 1: The input virus titer (not dried) was $10^{7.25}$ $TCID_{50}$/0.1 mL. The titer of the dried virus controls ranged from $10^{6.50}$ to $10^{7.25}$ $TCID_{50}$/0.1 mL at various time points (see Tables 3 and 5). The minimum concentration of ECA Anolyte required to produce 5 $\log_{10}$ reduction in virus titer within 1 minute was 150 ppm.

Experiment 2: The input virus titer (not dried) was $10^{6.75}$ $TCID_{50}$/0.1 mL. The titer of the dried virus controls ranged from $10^{6.50}$ to $10^{7.00}$ $TCID_{50}$/0.1 mL at various time points (see Tables 7 and 9). The minimum concentration of ECA Anolyte required to produce 5 $\log_{10}$ reduction in virus titer within 1 minute was 150 ppm.

STUDY CONCLUSION

Under the conditions of this investigation, the ECA Anolyte at 500 ppm and 150 ppm was able to inactivate 5 $\log_{10}$ of FCV within 1 minute at room temperature. In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or the integrity of the data.

Experiment 1 (500ppm)

TABLE 3. Input virus control and dried virus control results

Dilution	Input Virus Control	Dried virus control at indicated time points:		
		1 min	2 min	5 min
Cell Control	0000	0000	0000	0000
10^{-1}	++++	++++	++++	++++
10^{-2}	++++	++++	++++	++++
10^{-3}	++++	++++	++++	++++
10^{-4}	++++	++++	++++	++++
10^{-5}	++++	++++	++++	++++
10^{-6}	++++	++++	++++	++++
10^{-7}	0+++	00++	00+0	0+00
10^{-8}	ND	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{7.25}$	$10^{7.0}$	$10^{6.75}$	$10^{6.75}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

ND = Not done

TABLE 4. Test substance (ECA Anolyte) assay results (500 ppm)

Dilution	FCV-induced CPE at indicated time points:		
	1 min	2 min	5 min
Cell Control	0000	0000	0000
10^{-1}	++++	++++	++++
10^{-2}	0000	0000	0000
10^{-3}	0000	0000	0000
10^{-4}	0000	0000	0000
10^{-5}	0000	0000	0000
10^{-6}	0000	0000	0000
10^{-7}	0000	0000	0000
10^{-8}	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

Experiment 1 (150ppm)

TABLE 5. Input virus control and dried virus control results

Dilution	Input Virus Control	Dried virus control at indicated time points:		
		1 min	2 min	5 min
Cell Control	0000	0000	0000	0000
10^{-1}	++++	++++	++++	++++
10^{-2}	++++	++++	++++	++++
10^{-3}	++++	++++	++++	++++
10^{-4}	++++	++++	++++	++++
10^{-5}	++++	++++	++++	++++
10^{-6}	++++	++++	++++	++++
10^{-7}	0+++	0+++	0000	00+0
10^{-8}	ND	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{7.25}$	$10^{7.25}$	$10^{6.5}$	$10^{6.75}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

ND = Not done

TABLE 6. Test substance (ECA Anolyte) assay results (150 ppm)

Dilution	FCV-induced CPE at indicated time points:		
	1 min	2 min	5 min
Cell Control	0000	0000	0000
10^{-1}	++++	++++	++++
10^{-2}	0000	0000	0000
10^{-3}	0000	0000	0000
10^{-4}	0000	0000	0000
10^{-5}	0000	0000	0000
10^{-6}	0000	0000	0000
10^{-7}	0000	0000	0000
10^{-8}	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

Experiment 2 (500ppm)

TABLE 7. Input virus control and dried virus control results

Dilution	Input Virus Control	Dried virus control at indicated time points:		
		1 min	2 min	5 min
Cell Control	0000	0000	0000	0000
10^{-1}	++++	++++	++++	++++
10^{-2}	++++	++++	++++	++++
10^{-3}	++++	++++	++++	++++
10^{-4}	++++	++++	++++	++++
10^{-5}	++++	++++	++++	++++
10^{-6}	++++	++++	++++	++++
10^{-7}	00+0	000+	0000	+000
10^{-8}	ND	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{6.75}$	$10^{6.75}$	$10^{6.5}$	$10^{6.75}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

ND = Not done

TABLE 8. Test substance (ECA Anolyte) assay results (500 ppm)

Dilution	FCV-induced CPE at indicated time points:		
	1 min	2 min	5 min
Cell Control	0000	0000	0000
10^{-1}	++++	++++	++++
10^{-2}	0000	0000	0000
10^{-3}	0000	0000	0000
10^{-4}	0000	0000	0000
10^{-5}	0000	0000	0000
10^{-6}	0000	0000	0000
10^{-7}	0000	0000	0000
10^{-8}	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

Experiment 2 (150ppm)

TABLE 9. Input virus control and dried virus control results

Dilution	Input Virus Control	Dried virus control at indicated time points:		
		1 min	2 min	5 min
Cell Control	0000	0000	0000	0000
10^{-1}	++++	++++	++++	++++
10^{-2}	++++	++++	++++	++++
10^{-3}	++++	++++	++++	++++
10^{-4}	++++	++++	++++	++++
10^{-5}	++++	++++	++++	++++
10^{-6}	++++	++++	++++	++++
10^{-7}	00+0	+000	00++	00+0
10^{-8}	ND	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{6.75}$	$10^{6.75}$	$10^{7.0}$	$10^{6.75}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

ND = Not done

TABLE 10. Test substance (ECA Anolyte) assay results (150 ppm)

Dilution	FCV-induced CPE at indicated time points:		
	1 min	2 min	5 min
Cell Control	0000	0000	0000
10^{-1}	++++	++++	++++
10^{-2}	0000	0000	0000
10^{-3}	0000	0000	0000
10^{-4}	0000	0000	0000
10^{-5}	0000	0000	0000
10^{-6}	0000	0000	0000
10^{-7}	0000	0000	0000
10^{-8}	0000	0000	0000
$TCID_{50}$/0.1 mL	$10^{1.5}$	$10^{1.5}$	$10^{1.5}$

+ = Positive CPE indicating the presence of virus

0 = Negative CPE indicating the absence of virus

Toxicology Reports

REPORTS

on testing the immunotoxic properties of the electrochemically activated solution ANK ANOLYTE (NEUTRAL)

Neutral ANK anolyte (hereinafter – “anolyte”) was produced with the help of STEL device (NPO Ekran) using tap water and saturated table salt solution. Anolyte was diluted with tap water to 0.01% active chlorine concentration. The solution’s concentration was controlled with the Desicont HA-01 test strips.

Mongrel mice and F1(CBAxC57BL/6) hybrid mice weighing 18-20 g and routinely fed were delivered for the study from the Stolbovaya nursery (the Russian Academy of Medical Sciences). The animals were watered with diluted anolyte for 7 days in conditions of free access to drinking bowls. Every two days the solution in the bowls was substituted with a freshly prepared one. Control mice were given ordinary tap water. On day 8, test and control animals were streamed into experimental groups to study weight and cellular composition of the central and peripheral organs of the immune system, phagocytic activity of peritoneal macrophages, chemoluminescent reaction of peritoneal mononuclear cells and the level of humoral and cellular immune response to antigen stimulation. Significance of differences was assessed by T-Student test criteria.

The effect of anolyte on weight and cellular composition of the immune system organs

Materials and methods

F1 (CBA x C57BL/6) hybrid mice were sacrificed by cervical dislocation. In anatomically isolated lymphoid organs (thymus, spleen, popliteal lymphatic nodes [hereinafter - LN]), besides the absolute number of nucleus-containing cells (NCC), their weight, and the relative quantity of cellular elements per 1 mg of tissue were determined. In bone marrow and abdominal cavity only absolute quantity of NCC was determined, in one femoral bone and abdominal cavity, respectively. In mesenteric LN, which, unlike popliteal LN, appear as clusters, only relative number of NCC was determined. The results were presented in absolute figures (organ weight in mg, total number of nucleus-containing cells in the organ in $1x10^6$) and relative values (the number of nucleus-containing cells

in $1x10^6$ per 1 mg of tissue). Each experimental group included 5 - 6 mice. The experiments were repeated 3 times [1,2].

Report 1. Thymus

Material and methods

The chest cavity of slaughtered F1 hybrid mice (CBA x C57BL/6) was opened and thymus was withdrawn and weighed. In a glass blender, cell suspension was prepared on medium 199, filtered through double nylon, and in 3%-acetic acid, in Gorjaev's count chamber, the number of nucleus-containing cells (NCC) was counted.

Experiment 1

Animal groups		Weight, mg	Number of NCC	
			Per organ, $1x10^6$	Per mg of organ weight
Control	1 2 3 4 5	42 40 48 50 26 41.2 ±4.2 (100 ±10.2)	100 116 120 112 56 101 ±11.7 (100 ±1.2)	2.3 2.9 2.5 2.2 2.15 2.43 ± 0.1 (100 ±5.3)
Test	1 2 3 4 5	42 40 48 50 26 41 .2 ±4.2 (100 ±10.2)	72 152 166 106 120 123.2 ±16.8 (122 ±16.6)	1.9 2.3 2.2 2.9 2.3 2.3 ±0.2 (115±10)

Note: here and hereafter: in brackets - % to the control

Experiment 2

Animal groups		Weight, mg	Number of NCC	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5	46 38 43 46 41 42.8 ±1.5 (100 ±10.2)	87.8 60 99.8 93.1 69.2 82 ± 7.5 (100 ±9.1)	1.9 2.1 2.3 2.0 1.7 2.0±0.1 (100 ±5)
Test	1 2 3 4 5	35 40 44 47 34 40 ±2.5 (93.5 ±5.8)	65.2 91.8 95.8 136 78.5 93.5±11.9 (114±14.5)	1.9 2.3 2.2 2.9 2.3 2.3 ±0.2 (115±10)

Experiment 3

Animal groups	No.	Weight, mg	Number of NCC	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5	20 33 33 27 24 27.4 ±2.5 (100 ±9.1)	45.4 84.7 70 55.4 26.7 56.4 ±10 (100 ±17.7)	2.3 2.6 2.1 2.1 1.1 2.0 ±0.3 (100 ±15)
Test	1 2 3 4 5	21 28 28 27 17 24.2 ± 2.2 (88.3 ± 8)	52 50 53 53 56 52.8 ±1.0 (93.6 ±1.8)	2.5 1.8 1.9 2.0 3.3 2.3 ±0.3 (115±15)

Conclusion: anolyte has no effect on thymus weight and cell composition

Report 2. Bone marrow

Material and methods

Femoral bones from slaughtered F1 hybrid mice (CBA x C57BL/6) were withdrawn and bone marrow was extracted (washed out) with the help of medium 199. Cell suspension was prepared in a glass blender, passed through a double nylon filter and centrifuged at 300 g for 10 minutes. The residue was diluted with the medium in 3% acetic acid, and the number of NCC was determined in Gorjaev's count chamber.

Experiment 1

Animal groups	No.	NCC number in two femoral bones,1x10^6	NCC number as calculated for one femoral bone, 1x10^6
Control	1 2 3 4 5	32 32 30 22 20 27.2 ±2.6 (100 ±9.6)	16 16 15 11 10 13.6 ±1.3 (100 ±9.6)
Test	1 2 3 4 5	24 36 24 24 30 27.6 ±2.4 (101. 5 ±8.8)	12 18 12 12 15 13.8 ±1.2 (101. 5 ±8.8)

Experiment 2

Animal groups	No.	NCC number in two femoral bones, 10^6	NCC number as calculated for one femoral bone, 10^6
Control	1 2 3 4 5	34 28 35 24 26 29.4 ± 2.2 (100 ±7.5)	17 15 17.5 12 13 14.7 ±1.1 (100 ±7. 5)
Test	1 2 3 4 5	27 38 32 29 24 30 ± 2.4 (102 ±8.2)	13.5 19 16 14.5 12 15± 1.2 (102 ±8.2)

Experiment 3

Animal groups	No.	NCC number in two femoral bones, 10^6	NCC number as calculated for one femoral bone, 10^6
Control	1 2 3 4 5	24 37 30 31 22 28.8 ±2.7 (100 ±9.4)	12 18.5 15 15.5 11 14.4 ±1.1 (100 ±7.5)
Test	1 2 3 4 5	29 28 34 29 32 30.4 ±1.1 (105.6 ±3.8)	14.5 14 17 14.5 16 15.2 ±0.6 (105.6 ±3.8)

Conclusion: anolyte has no effect on bone marrow cell composition

Report 3. Spleen

Material and methods

The abdominal cavity of slaughtered F1 hybrid mice (CBA x C57BL/6) was opened, spleen was extracted and weighed, then, in a glass blender with medium 199, cell suspension was prepared, filtered, and in 3% acetic acid, in Gorjaev's count chamber, the number of NCC was determined.

Experiment 1

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1	65	120	1.85
	2	74	100	1.35
	3	75	130	1.73
	4	70	130	1.86
	5	74	110	1.49
		71.6±1.9 (100 ±2.7)	118±5.8 (100 ±4.9)	1.66 ±0.1 (100 ±6)
Test	1	80	100	.25
	2	70	160	2.29
	3	58	130	2.24
	4	100	160	1.6
	5	87	150	1.72
		79 ± 7.2 (110.3±10)	140 ±11. 4 (118.6±9.7)	1.82 ±0.2 (109.6 ±12)

Experiment 2

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5	65 74 75 70 74 71.6±1.9 (100 ±2.7)	120 100 130 130 110 118 ±5.8 (100 ±4.9)	1.85 1.35 1.73 1.86 1.49 1.66 ±0.1 (100 ±6)
Test	1 2 3 4 5	80 70 58 100 87 79 ± 7.2 (110.3 ±10)	100 160 130 160 150 140 ±11. 4 (118.6±9.7)	1.25 2.29 2.24 1.6 1.72 1.82 ±0.2 (109.6 ±12)

Experiment 3

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5 6	114 69 80 79 93 107 90.3 ± 7.2 (100 ±8)	131 78 86 142 153 140 121 .6± 12.9 (100 ± 10.6)	1.1 1.1 1.1 1.8 1.6 1.3 1.3±0.1 (100 ±6)
Test	1 2 3 4 5 6	97 87 66 89 120 110 94.8± 7.7 (105 ±8.5)	127 98 96 151 173 120 127.5 ±12.3 (104.9±10.1)	1.3 1.1 1.5 1.7 1.4 1.1 1.4±0.1 (107.6 ±7.7)

Conclusion: anolyte has no effect on spleen weight and cell composition.

Report 4. Popliteal lymphatic node

Material and methods

The skin of slaughtered F1 hybrid mice (CBA x C57BL/6) was cut in popliteal space area. Popliteal lymphatic node was extracted and, in a glass blender with medium 199, cell suspension was prepared and filtered; then in 3% acetic acid, in Gorjaev's count chamber, the number of NCC was determined.

Experiment 1

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1	2	2.1	1.05
	2	2	2.8	1.4
	3	2	5.5	2.75
	4	2	2.07	1.04
	5	1.5	4.8	3.2
		1.9±0.1 (100 ±5.3)	3.45± 0.7 (100 ±20.3)	1.82 ±0.48 (100 ±26.4)
Test	1	2	7.2	3.6
	2	2	5.2	2.6
	3	2	3.8	1.9
	4	1.5	7.4	4.9
	5	1.5	7.5	5.0
		1.8±0.12 (94. 7 ±6.3)	6.2 ± 0.74 * (179.7±21.4)	3.6 ±0.6* (197.8 ±33)

Note: here and hereafter: * - $p < 0.05$

Experiment 2

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5	2 2 2.5 1.5 1.5 1.9 ±0.2 (100 ±10.5)	2 2.5 2 2 2 2.1 ±0.1 (11 0.5 ±5.3)	1.1 1.3 1.5 3 2.5 1.9 ±0.4 (100 ±21)
Test	1 2 3 4 5	2 2.5 2 2 2 2.1 ±0.1 (110.5 ±5.3)	6 6.4 7.3 7.1 5.2 6.4 ±0.4* (193.9±12.1)	3 2.6 3.7 3.6 2.6 3.1 ±0.2* (163.2 ±10.5)

Experiment 3

Animal groups	No.	Weight, mg	NCC number	
			Per organ, 10^6	Per mg of organ weight
Control	1 2 3 4 5	2.8 1.5 2 1.5 1 1.8 ±0.3 (100 ±6.7)	1.4 1.3 1.5 1.2 1.2 1.3±0.06 (100 ±4.6	1.5 0.9 0.8 0.8 1.2 0.8 ±0.1 (100 ±12.5)
Test	1 2 3 4 5	0.5 1 1.8 1.8 1.3 1.3 ±0.2 (72.2 ±11.1)	1.8 2.3 2 2.1 2.3 2.1 ±0.1 * (161.5±7.7)	1.8 2.3 2 2.1 2.3 2.1 ±0.1 * (161. 5± 7.7)

Conclusion: anolyte introduction has no effect on the mass of popliteal lymphatic nodes, but increases the number of cells and relative content of cells per mg of organ weight.

Report 5. Mesenteric lymphatic node

Material and methods

The abdominal cavity of sacrificed F1 hybrid mice (CBA x C57BL/6) was opened, one of mesenteric lymphatic nodes was withdrawn and, in a glass blender, with medium199, cell suspension was prepared, filtered, and in 3% acetic acid in Gogjaev's count chamber, the number of NCC per mg of organ weight was determined.

Experiment 1

Animal groups	No.	NCC number per mg of organ weight
Control	1 2 3 4 5 6	13.5 4 5.1 3.7 4.3 4.1 4.1 ±0.2 (100 ±4.9)
Test	1 2 3 4 5 6	3.1 3.7 4.4 4.3 4.6 7.6 4.6 ±0.6 (112.2 ±14.6)

Experiment 2

Animal groups	No.	NCC number per mg of organ weight
Control	1 2 3 4 5	3.6 4.1 3.6 3.9 4.2 3.9 ±0.1 (100 ±2.6)
Test	1 2 3 4 5	3.8 4.2 3.7 4.8 3.6 4 ±0.2 (102.6 ±0.2)

Experiment 3

Animal groups	No.	NCC number per mg of organ weight
Control	1 2 3 4 5	4 4.1 3.9 4.2 3.8 4 ±0.1 (100 ±2.5)
Test	1 2 3 4 5	4.3 4 3.7 3.9 3.5 4 ±0.2 (102.6 ±0.2)

Conclusion: introduction of anolyte has no effect on cell composition of mesenteric lymphatic nodes.

Report 6. Peritoneal cavity

Material and methods

F1 hybrid mice (CBA x C57BL/6) were sacrificed, and 5-ml portions of medium 199 containing 5 units of heparin per 1 ml were introduced. After massaging the abdominal wall, cell suspension was extracted with a syringe. Peritoneal exudate taken from 10 mice of each group (control, test) was collected, centrifuged in test-tubes treated with silicon at 200 g for 10 minutes, and in 3% acetic acid, in Gorjaev's count chamber, the number of NCC was determined [3].

No. of experiment	Animal groups	NCC number, 10^6
1	Control	114.5
	Test	99.8
2	Control	78.8
	Test	89.4
3	Control	80.8
	Test	90.0

Conclusion: introduction of anolyte has no effect on the number of nucleus-containing cells in peritoneal cavity.

Report 7. The effect of anolyte on primary T-dependent humoral immune response

Material and methods

Humoral immunity was assessed by the level of primary T-cell dependent immune response to sheep erythrocytes. For this purpose, 24 hours after watering with anolyte solution (test) or tap water (control), F1 hybrid mice (CBA x C57BL/6) were immunized intraperitoneally with erythrocytes in suboptimal ($5x10^7$ cells/mouse) or optimal ($1x10^8$ cells/mouse) dose. After 4 days, the level of primary humoral immune response was determined using the method of local hemolysis in the liquid phase [3]. For this purpose, a mixture of splenocytes, sheep erythrocytes and guinea pig complement was placed into a glass chamber, incubated for 60 minutes in a thermostat at 37°C, and the number of hemolytic patches (hemolysis areas) corresponding to the number of antibody-forming

cells (AFC) was counted. The quantity of AFC was calculated for the spleen and the number of nucleus-containing cells in the spleen (NCC). To assess the titer of antibodies-hemagglutinins, the collected blood was incubated for an hour in a thermostat at 37° C and for 24 hours at 4°C, then blood serum was separated, centrifuged at 400 g for 15 minutes, and heated to 56°C to inactivate the complement. Serum treated in this manner was titrated by double dilution in a buffered with phosphates saline solution of pH 7.2, in plastic microplanchettes, and 0.8% sheep erythrocyte solution was added as test-antigen. Antibody titer was assessed by final dilution with erythrocyte agglutination, and expressed in -log2. To isolate antibodies of IgM and IgG classes in total hemagglutinins, prior to titration, part of the serum was incubated for an hour at room temperature with an equal volume of 0.1M 2-mercaptoethanol (2-me), which inactivates antibodies of IgM class (2-me sensitive) and does not affect class IgG antibodies (2-me resistant) [4]. Each experimental group of animals contained 5-8 individuals. The experiments were repeated 2-3 times.

Inducing antibody formation with suboptimal antigen dose (ЭБ 5 x10^7)

Experiment 1

Animal groups	No.	Spleen		AFC number		Titer of hemagglutinins, -log2	
		Weight, mg	NCC, 10^6	Per spleen x10^3	Per1x10^6 NCC	Ig M	Ig G
Control	1	123	146.3	34	232.4	6	0
	2	129	132.5	35	264.2	7	0
	3	129	133	32	240.6	7	0
	4	123	119.7	44	367.6	7	0
	5	122	172.9	38	219.8	7	0
	6	121	139.7	22	157.5	6	0
	7	121	136.3	50	366.8	8	0
	8	95	172.9	52	300.8	7	0
		120.4 ±3.8	144.2 ±6.8	38.4 ± 3.5	268.7 ± 25.8	6.9 ±0.24	0
		(100 ±3.2)	(100 ±4.7)	(100±9.1)	(100 ±9.6)	(100±3.5)	(0)
Test	1	156	159.6	30	188	7	0
	2	134	133	20	195.5	7	0
	3	131	146.3	28	191.4	7	0
	4	98	159.6	16	100.3	6	0
	5	147	146.3	34	232.4	7	0
	6	117	153	32	209.2	7	0
	7	132	239.4	24	100.3	6	0
	8	176	186.2	28	150.4	7	0
	9	113	173	28	161.8	7	0
	10	140	173	50	289	7	0
		133.8 ±5	166.9 ±9.4	28.8 ±1.1	189.8 ±10.4	6.8 ±0.1	
		(111±4.2)	(116±6.5)	(75 ± 2.9)	(70.6 ± 3.9)	(98.6±1.4)	(0)

Experiment 2

Группы животных	No.	Spleen		AFC number		Titer of hemagglutinins, -log2	
		Weight mg	NCC, 10^6	Per spleen $1x10^3$	Per $1x10^6$ NCC	IgM	LgG
Control	1	134	190	53	278.9	7	0
	2	125	120	37	308.3	8	0
	3	138	145	48	331	7	0
	4	121	116	30	367.6	8	0
	5	144	173	45	258.6	7	0
	6	139	180	46	157.5	6	0
	7	140	164	39	255.6	7	0
		120±3.8 (100*3.2)	144.2±6.8 (100±4.7)	42.6±2.9 (100±6.8)	279.6±25.4 (100±9.6)	7.1±0.3 (100±3.5)	(0)
Test	1	145	170	41	241	6	0
	2	127	149	32	214.8	6	0
	3	142	158	39	246.8	8	0
	4	138	124	32	258	6	0
	5	115	135	45	333.3	8	0
	6	150	192	54	281.3	7	0
	7	122	171	43	251.5	8	0
		134.1 ±4.9 (111±4.1)	157 ±8.8 (109±6.1)	40.9±2.9 (96±6.8)	261 ±14.2 (93.3±5.1)	7 ±0.4 (98.6±5.6)	0 (0)

Report 8. Inducing antibody formation with optimal antigen dose (ЭБ,1x10^8)

Experiment 1

Animal groups	No.	Spleen		AFC number		Titer of hemagglutinins, -log2	
		Weight, mg	NCC, 10^6	Per spleen 1x10^3	Per1 0^6 NCC	IgM	IgG
Control	1	113	187.6	152	810.2	12	0
	2	96	154	76	493.5	11	0
	3	156	160.8	92	572.1	12	0
	4	117	207.7	164	789.6	12	0
	5	123	201	124	617	12	0
		122.2 ± 10	182.2 ±10.7	122±16.9	656±62	12	
		(100 ±8.2)	(100 ±5.9)	(100±13.9)	(100±9.4)	11.8±0.2 (100±1.7)	(0)
Test	1	139	187.6	136	724.9	12	0
	2	117	274.7	132	480.5	12	0
	3	135	180.8	84	464.6	12	0
	4	124	160.3	72	449.2	11	0
	5	118	194.3	80	411.7	11	0
		126.6 ±4.5	199.7 ±19.6	101±13.7	506±55.9	1 1	(0)
		(103.6±3.7)	(109.6±10.8)	(82.9±11.3	(77.1 ±8.5)	(98.3±2)	

Experiment 2

Animal groups	No.	Spleen		AFC number		Titer of hemagglutinins, -log2	
		Weight, mg	NCC, 10^6	Per spleen $x10^3$	Per 10^6 NCC	IgM	LgG
Control	1	89	80	92.8	1160	9	0
	2	118	266.7	104	390	10	0
	3	99	146.7	103.3	774.7	10	0
	4	119	166.7	105.6	633.5	12	0
	5	105	133.3	108.8	816.2	11	0
	6	117	113.3	115.2	1016.8	12	0
	7	105	213.2	88	412.6	8	0
	8	85	93.3	72	771.7	10	0
		104.6 ±4.6	151.7±22.2	98.7 ±4.9	746.9±94.7	10.3 ±0.5	
		(100± 4.4)	(100±14.6)	(100 ±5)	(100±12.7)	(100 ±4.9)	(0)
Test	1	109	180	116.8	648.9	10	0
	2	107	86.7	92.8	1070.4	12	0
	3	121	120	123.2	1026.7	12	0
	4	112	120	97.6	813.3	9	0
	5	102	106.7	84.8	794.8	8	0
	6	126	120	86.8	720	8	0
	7	112	73.3	64	873.1	10	0
		11 3.4 ±3.2	115±12.8	95.1 ±7.6	849±58.2	9.96±0.6	(0)
		(108.8±3.1)	(75.9 ± 8)	(96.4±7.7)	(114±7.8)	(96.1 ±5.8)	

Experiment 3

Animal groups	No.	Spleen		AFC number		Titer of hemagglutinins, -log2	
		Weight, mg	NCC, 106	Per spleen x10^3	Per 10^6 NCC	IgM	IgG
Control	1 2 3 4 5 6	133 142 131 138 139 128 135.2 ±2.2 (100 ±1.6)	233 273 332 281 287 220 271 ± 16.5 (100 ± 6.1)	112 69 118 134 217 171 136.8± 21 (100 ±15.3	480.7 252.7 354.4 473.5 756.1 777.3 515.8 ± 35.3 (100±6.8)	8 7 8 9 10 10 8.7 ±0.3 (100 ±5.7)	0 0 0 0 0 0 (0)
Test	1 2 3 4 5 6	12 6 131 134 132 127 128.8 ±1.7 (95.3 ±1.3)	186.7 266.7 273.3 233.3 223.2 248.4 238.6 ±13 (87.9 ± 4.8)	92.8 128 80.4 131.6 91.2 132.1 109.4± 9.7 (80 ± 7.1)	498. 1 292.7 566.8 407.7 513.4 462.8 ± 40.4 (89.8± 7.8)	9 8 7 10 9 10 8.8 ±0.5 (101.1 ± 5.7)	0 0 0 0 0 0 (0)

Conclusion: Anolyte introduction has no effect on the number of AFC in the spleen and the titer of hemagglutinins in blood serum. Only antibodies of class IgM are found among hemagglutinins. No class IgG antibodies have been found.

Report 9. Studies of anolyte-induced polyclonal immune response in the spleen of mice

Material and methods

To study the possibility of anolyte-induced polyclonal activation of B-lymphocytes, antibody-forming cells (AFC) and hemagglutinins were examined in the spleen of non-immunized F1 hybrid mice (CBAxC57BL/6).

Each experimental group consisted of 5-6 animals.

The experiments were run twice.

Experiment 1

Animal groups	No.	Spleen		Number of AFC	Titer of hemagglutinins, total (IgM+IgG), -log2
		Weight, mg	NCC, 10^6	Per spleen $X10^3$	
Control	1 2 3 4 5	65 74 75 70 74 71. 6 ±1.9 (100 ±2.7)	120 100 130 130 110 220 118±5.8 (100±4.9)	0 0 0 0 0 0	0 0 0 0 0 0
Test	1 2 3 4 5	80 70 58 100 87 79 ± 7.2 (110±10)	100 160 130 160 150 140±11.4 (119±4.1)	0 0 0 0 0	0 0 0 0 0

Experiment 2

Animal groups	No.	Spleen		AFC number	Titer of hemagglutinins, total (IgM+IgG), -log2
		Weight, mg	NCC, 10^6	Per spleen $X10^3$	
Control	1	83	110	0	0
	2	81	115	0	0
	3	92	124	0	0
	4	99	128	0	0
	5	100	143	0	0
	6	75	91	0	0
		88.3 ± 4.2	130		
		(100 ±4.8)	118± 5.8		
			(100± 4.9)		
Test	1	87	133	0	0
	2	107	150	0	0
	3	79	96	0	0
	4	98	158	0	0
	5	110	127	0	0
	6	92	142	0	0
		95.5± 4.8	134±8.9		
		(108±5.4)	(114±7.5)		

Conclusion: introduction of anolyte does not lead to AFC presence in the spleen and hemagglutinins in blood serum.

Report 10. The effect of anolyte on slow-type hypersensitivity reaction

Material and methods

Cell immunity was assessed with the help of slow-type hypersensitivity reaction. For this purpose, 24 hours after watering with anolyte or tap water, F1 hybrid mice (CBA x C57BL/6) were sensitized by intraperitoneal introduction of sheep erythrocytes in the quantity of $5x10^7$ cells/mouse. 5 days later sheep erythrocytes in the quantity of 10^8 cells in 50 mcl were introduced under aponeurotic plate of one of hind paws, and 50 mcl of saline solution into the contra-lateral paw (control). 24 hours later, the mice were sacrificed and their paws were cut on the level of talocrural articulation, weighed and the reaction index was counted according to the formula: RI = (Mt - Mc) : Mc x 100, where Mt – mass of test paw, and Mc – mass of control paw [5]. Each experimental group included 9-12 animals. The experiments were repeated 3 times.

Experiment 1

Animal groups	No.	Mass of paws, mg		Index of reaction
		Right	Left	
Control	1	165	147	12.2
	2	163	146	11.6
	3	184	168	9.5
	4	168	147	14.3
	5	180	142	26.8
	6	161	142	13.4
	7	183	143	28
	8	161	142	13.4
	9	160	139	15.1
	10	163	138	18.1
				16.2 ±2
				(100 ±12.3)
Test	1	157	150	4.7
	2	163	150	8.7
	3	188	157	19.7
	4	187	159	17.6
	5	171	154	11
	6	200	160	25
	7	188	166	13.3
	8	159	141	12.8
	9	157	145	8.3
				13.5±2.1
				(83.3 ±13)

Experiment 2

Animal groups	No.	Paw mass, mg		Index of reaction
		Right	Left	
Control	1	150	128	17.2
	2	119	119	25.2
	3	150	136	10.3
	4	146	114	28.1
	5	143	120	19.2
	6	156	132	18.2
	7	171	143	19.6
	8	174	156	11.5
	9	167	145	15.2
	10	178	151	17.9
	11	165	140	17.9
	12	145	131	10.7
				17.3±1.3
				(100 ±7.5)
Test	1	157	150	4.7
	2	163	150	8.7
	3	188	157	19.7
	4	187	159	17.6
	5	171	154	11
	6	200	160	25
	7	188	166	13.3
	8	159	141	12.8
	9	157	145	8.3
				13.5±2.1
				(78 ±12.1)

Experiment 3

Animal groups	No.	Paw mass, mg		Index of reaction
		Right	Left	
Control	1	200	157	27.4
	2	172	151	13.9
	3	185	163	13.5
	4	189	147	28.6
	5	190	150	21.8
	6	203	172	18
	7	216	164	31.7
	8	191	154	24
	9	195	160	21.9
	10	198	155	27.7
	11	180	160	12.5
				21.9 ±2 (100 ±9.1)
Test	1	211	164	28.7
	2	189	164	15.2
	3	199	168	18.5
	4	187	157	19.1
	5	186	164	13.4
	6	183	153	19.6
	7	205	171	19.9
	8	160	129	24
	9	221	165	33.9
	10	192	160	20
	11	207	173	19.7
				21.1 ±1.8 (96.3 ± 8.2)

Conclusion: anolyte introduction does not affect the index of slow-type hypersensitivity reaction.

Report 11. The effect of anolyte on phagocytic activity of peritoneal macrophages

Material and methods.

The study was performed on mongrel mice. To obtain peritoneal exudate cells, on day 8 after the beginning of the experiment, all the animals were exposed to intraperitoneal introduction of 3 ml of 10% glycogen solution. 3 days later, peritoneal exudate cells were washed out of the abdominal cavity of mice sacrificed with ether, with the help of medium 199 containing 10% embryonal calf serum. Cells received from each mouse of the same group were collected in a common pool and were twice washed with medium 199 by centrifuging at 200 g during10 minutes. Cell concentration was brought to 2 million/ml and 1 ml portions of the solution were poured into silicon treated test tubes. As phagocytosis material, latex particles (Unisphere Latex, d 0,7 mkm, Serva) were used, opsonized by mouse serum for 10 minutes at 37°. After washing in medium 199, final particle concentration was $2*10^6$ in ml. Mixture consisting of 1ml macrophages and an equal volume of latex particle suspension was incubated at 37°C for 30 minutes. Smears prepared on slides were fixed in absolute alcohol and Romanowsky-Giemza stained. In each smear the number of phagocytosis-active cells and the number of latex particles ingested by them were counted and % of phagocytosis was determined – the quantity of macrophages with phagocytosis activity as well as phagocyte index – the number of ingested particles for one phagocytosis-active macrophage [2, 5]. Each experimental group included 10 animals. The experiments were repeated 3 times.

Experiment 1

Animal groups	No.	Macrophage content in 1 ml of exudate, 1x1 0^6	% of phagocytosis-active cells	Phagocytosis index
Control	1	2.7	82	4
	2	1	94	6
	3	2.5	94	8
	4	1.6	85	4
	5	2	98	4
	6	2.7	86	3
	7	3	87	2
	8	1.1	97	3
	9	2.5	95	3
	10	0.9	80	4
		2.0 ±0.3	91 ±1.8	4.1±0.5
		(100±15)	(100 ±2)	(100 ±12.2)
Test	1	4	78	4
	2	1	94	3
	3	3.8	77	4
	4	1.2	84	5
	5	1	72	5
	6	4.2	88	4
	7	1.6	92	4
	8	4	79	3
	9	1	73	4
	10	2	96	4
		2.4 ±0.5	83.3 ±2.78*	4.0 ± 0.2
		(120 ±20.8)	(91. 5 ±3.05)	(97.6 ± 0.5)

Experiment 2

Animal groups	No.	Macrophage content in 1 ml of exudate, 10^6	% of phagocytosis-active cells	Phagocytosis index
Control	1	1.3	80	4
	2	1.9	92	6
	3	1.2	94	7
	4	1.1	88	5
	5	1.5	90	7
	6	1.4	96	8
	7	2	98	7
	8	1.8	89	6
	9	1.2	87	4
	10	1.2	94	7
		1.5±0.1	90.8 ±1.6	6.1 ±0.43
		(100 ±6.7)	(100 ±1.8)	(100 ±7)
Test	1	1.1	84	3
	2	2	90	5
	3	1.4	92	8
	4	1.3	81	6
	5	2	89	6
	6	1	80 t=2.1	4
	7	0.9	92	9
	8	2.1	87	7
	9	1.8	83	5
	10	1.6	85	4
		1.52 ±0.14	86.3 ±1.4	5.7 ±0.8
		(101.3 ±9.3)	(95 ±1.5)	(93.4 ±13.1)

Experiment 3

Animal groups	No.	Macrophage content in 1 ml of exudate, 1x1 0^6	% of phagocytosis-active cells	Phagocytosis index
Control	1	1.8	84	5
	2	1.2	96	7
	3	1.5	98	8
	4	2.1	82	6
	5	1.3	90	8
	6	1.0	91	7
	7	0.9	89	4
	8	2.2	94	8
	9	2.3	97	7
	10	1.9	82	7.
		1.62 ±0.16	90.3 ±1.9	6.7 ± 0.4
		(100 ±9.9)	(100±2.1)	(100 ±6)
Test	1	2.0	84	4
	2	1.5	82	5
	3	1.2	94	9
	4	1.6	81	7
	5	1.7	80	6
	6	2.1	84	5
	7	2.0	87	4
	8	1.9	86	7
	9	1.8	83	6
	10	1.4	90	8
		1.72 ±0.1	85.1 ±1.36	6.1 ±0.53
		(106 ±6.2)	(94.2 ±1.5)	(91 ± 7.9)

Conclusion: anolyte introduction insignificantly decreases the share of cells with phagocytosis activity and has no effect on the general content of macrophages in peritoneal exudate, neither does it affect phagocytosis index.

Report 12. The effect of anolyte on chemoluminescence of peritoneal cells

Material and methods

24 hours after watering with anolyte (test) or tap water (control), F1 hybrid mice (CBA x C57BL/6) were exposed to intraperitoneal introduction of 5 ml of medium 199, and peritoneal mononuclears were washed out. The cells of the same group (control, test) were collected, transferred into plastic Petri dishes and incubated for an hour at 37°C to isolate the pool of macrophages and monocytes adhering to plastic, from non-adherent neutrophils, lymphocytes and other nucleus-containing cells. Isolated cell elements were introduced (in portions of 2.5 x 10^5 for 100 mcl of medium 199) into the cuvette of the luminometer LKB-Wallac1251 (LKB, Finland) and 350 mcl of 10^{-6} luminol solution was added with the help of an automated pipette. Chemoluminescence intensity was assessed in millivolts (mV) for 10^6 of cells after automatic addition to the cell suspension of 75 mcl suspension of zymosan (Siigma Chemical Co.) opsonized in fresh mouse serum up to the final concentration of 12.5 mg\ml. Mean value of chemoluminescence intensity was determined based on the findings of three identical measurements [2, 5].

Peritoneal mononuclears	Animal groups	Chemoluminescence, mV			
		Per 1 0^6 macrophages			Mean value
Non-adherent to plastic	Control	846	713	780	779.7
	Test	693	607	648	649.3
Adherent to plastic	Control	888	840	1035	921
	Test	555	643	588	595.3

Conclusion: Anolyte decreases the chemoluminescence intensity of cells non-adherent to plastic by 1.2 times, and that of cells adherent to plastic - by 1.5 times.

References

1. Laboratory methods of clinical studies. Reference book. Edited by V.V. Menshikov, Moscow, Meditsina Publishers, 1988, 366 pp.

2. Manual on experimental (preclinical) study of new pharmacological substances. Moscow. 2000. 398 pp.

3. Cunningham A.J., Szenberg A. Further improvements in the plaque technique for detecting single antibody-forming cells// Immunology. 1968, V.14, p. 599-601.

4. Fedoseyeva V.N., Sharetsky A.N., Aristovskaya L.V. et al. Methodical recommendations on experimental study of immunotoxic properties of environmental chemical factors. Moscow, the USSR Academy of Medical Sciences, 1989, 47 pp.

5. Khaitov R.M., Gushchin I.S., Zebrev A.I. et al. Experimental study of immunotropic activity of pharmacological preparations // Reports of the Pharmacological Committee, 1999, No.1, p. 31 -36.

Head of the Radiation Immunology Laboratory
of the MRNTs RAMN,
Doctor of Biological Science /Signed/ B.P. Surinov;

Leading Research Fellow of the Radiation Immunology Laboratory
of the MRNTs RAMN,
Candidate of Medical Science /Signed/ A.N. Sharetsky;

Signatures of B.P. Surinov and A.N. Sharetsky are certified by the stamp of the Personnel Department of the RAMN Medical Radiological Research Center and the signature of the Personnel Manager

09.09.03

journal of dentistry xxx (2010) xxx–xxx

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/jden

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

M.A. Boyle [a], M.J. O'Donnell [a], R.J. Russell [b], D.C. Coleman [a,*]

[a] Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School & Hospital, University of Dublin, Trinity College Dublin, Lincoln Place, Dublin 2, Ireland
[b] Department of Microbiology, University of Dublin, Trinity College Dublin, Dublin 2, Ireland

ARTICLE INFO

Article history:
Received 10 June 2010
Received in revised form
5 August 2010
Accepted 6 August 2010

Keywords:
Trustwater Ecasol™
Superoxidised water
Hypochlorous acid
Biosafety
Cytotoxicity testing
RHE
Keratinocytes
Dental unit waterlines
Residual disinfectant

ABSTRACT

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 mg/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.

© 2010 Elsevier Ltd. All rights reserved.

* *Corresponding author*. Tel.: +353 1 6127276; fax: +353 1 6127295.
E-mail addresses: david.coleman@dental.tcd.ie, dcoleman@dental.tcd.ie (D.C. Coleman).
0300-5712/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jdent.2010.08.005

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 $\leq$500 cfu/mL.[35] In addition, the American Dental Association (ADA) in 1995 proposed a target limit of $\leq$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 hospital's 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.[3] 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 h.[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 techni-

Please cite this article in press as: Boyle MA, et al. 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. Journal of Dentistry (2010), doi:10.1016/j.jdent.2010.08.005

cally 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 considers 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]

2.2. 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).

2.3. 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).

2.4. 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.

2.5. 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% 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, Frickenhau-

Please cite this article in press as: Boyle MA, et al. 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. Journal of Dentistry (2010), doi:10.1016/j.jdent.2010.08.005

sen, Germany) at a density of 2×10^4 cells/well and cultured for 48 h until 70–90% confluent.

2.6. 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% CO_2.

2.7. 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 oxidised 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 2×10^4 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% 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% 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% 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 1.8×10^5 cells/well and cultured for 48 h until 70–90% confluent. Growth medium was removed and the cells washed twice with sterile PBS. One millilitre aliquots of each Ecasol concentration tested were placed onto the cells and incubated for 1 h at 37 °C in 5% 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 1500 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 g/L L-glutamine was added. LDH leakage was measured after 1 h and 24 h of incubation in DME 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 100 for 20 min at 37 °C. GSH activity was measured using a GSH-Glo Glutathione assay kit (Promega) according to the manufacturer's instructions.

2.8. 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 (1–2 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.

2.9. 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).

2.10. *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 a 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 1 min 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%)

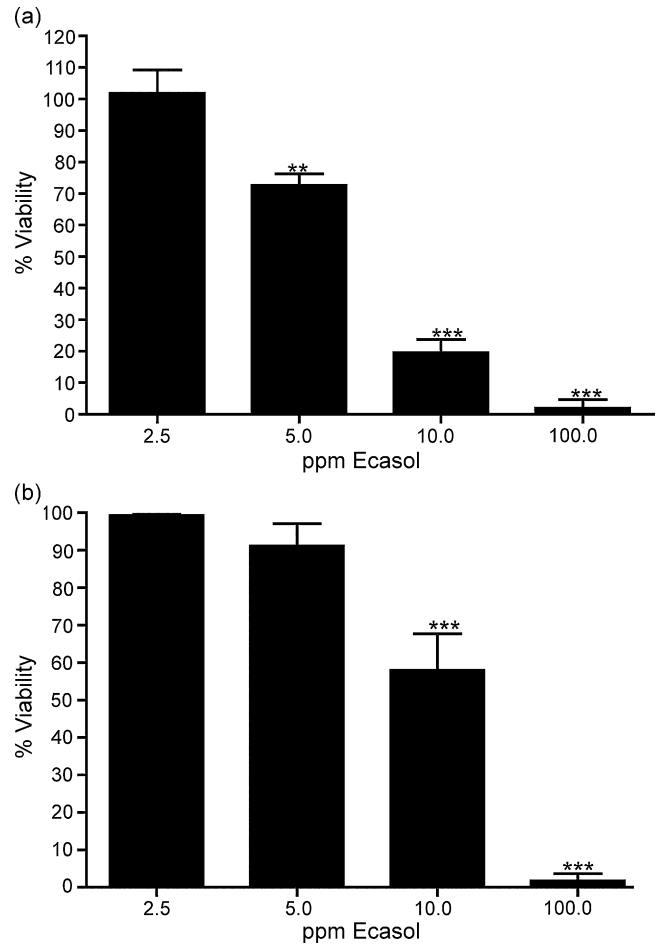


Fig. 1 – Viability (% of PBS-treated control) of TR146 monolayers following 1 h exposure to various concentrations of Ecasol (ppm FAC). Viability was assessed after 1 h using Alamar Blue as a measure of cell proliferation (a) and Trypan Blue as a measure of plasma membrane integrity (b). A significance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

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

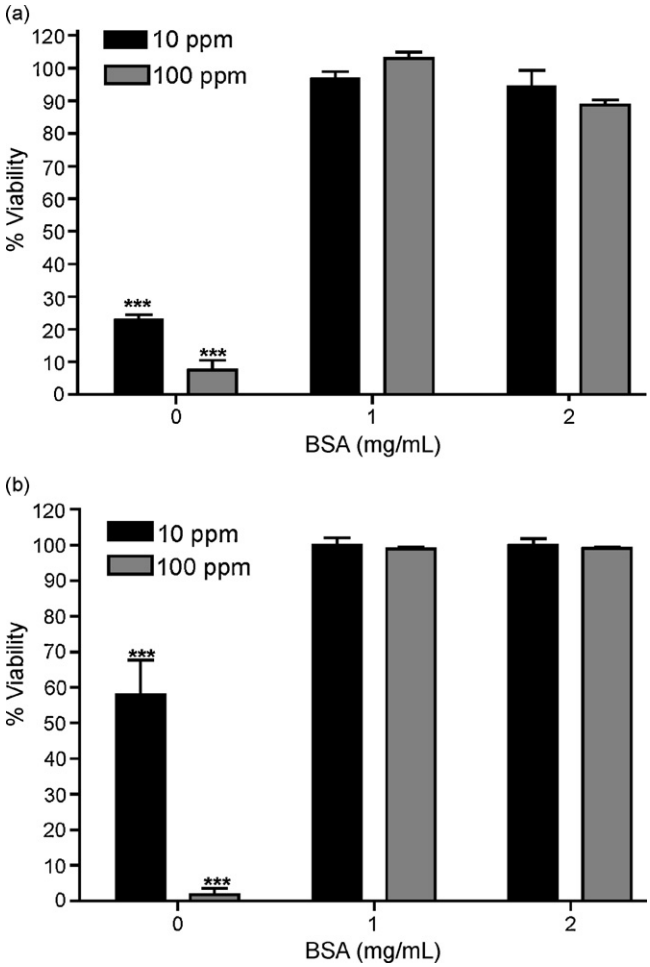


Fig. 2 – Viability (% of PBS-treated control) of TR146 monolayers following 1 h exposure to various concentrations of Ecasol (ppm FAC) to which BSA had been added (1–2 mg/mL) 30 min prior to addition to the monolayers. Viability was assessed after 1 h using Alamar Blue as a measure of cell proliferation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

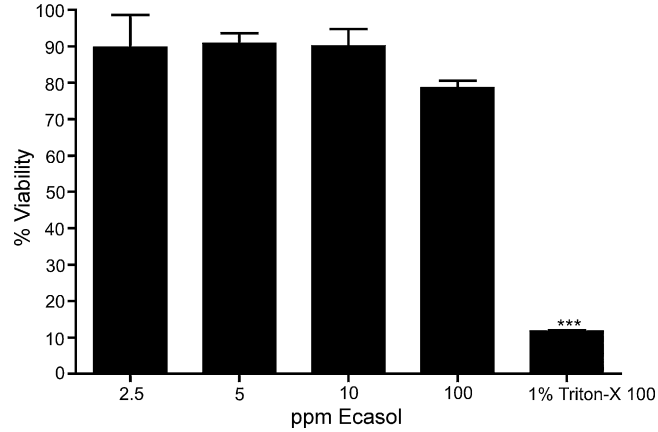


Fig. 3 – Viability (% of PBS-treated control) of RHE tissue samples following 1 h exposure to various concentrations of Ecasol (ppm FAC). Viability was assessed after 1 h using Alamar Blue as a measure of cell proliferation. Triton-X 100 was used as a positive control for RHE tissue damage. A significance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

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 < 1 cfu/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

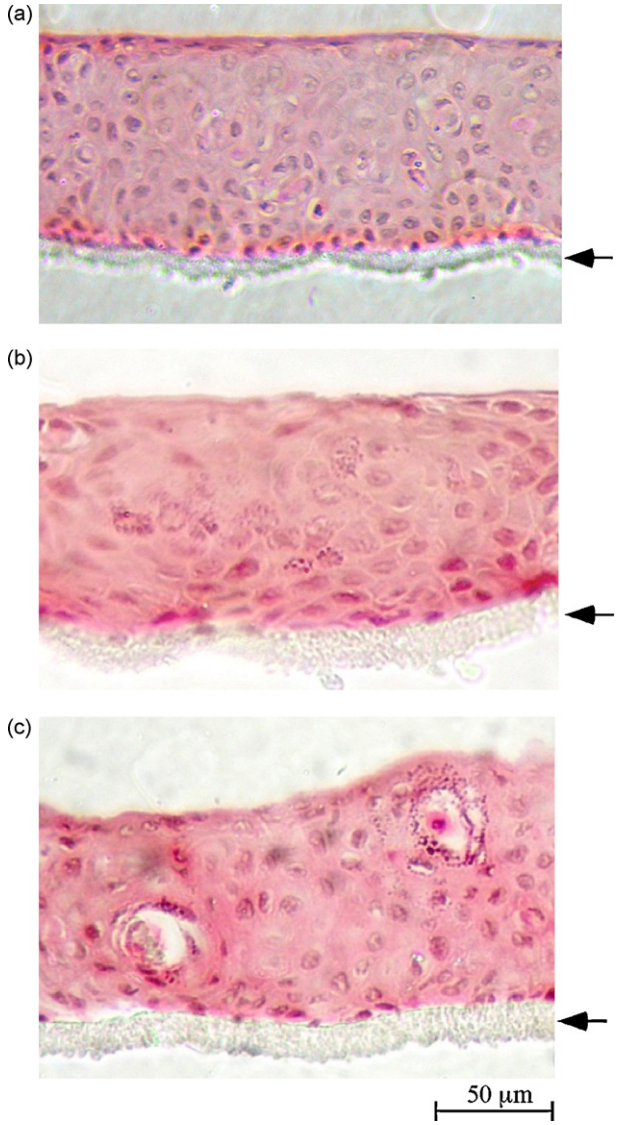


Fig. 4 – Light micrographs of RHE tissue sections stained with hematoxylin–eosin. (a) RHE tissue sample treated with PBS for 1 h; no detectable damage to the tissue is evident. (b) RHE tissue sample treated with 100 ppm FAC Ecasol for 1 h; no detectable damage to the tissue is evident. (c) RHE tissue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

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 colourimetric 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

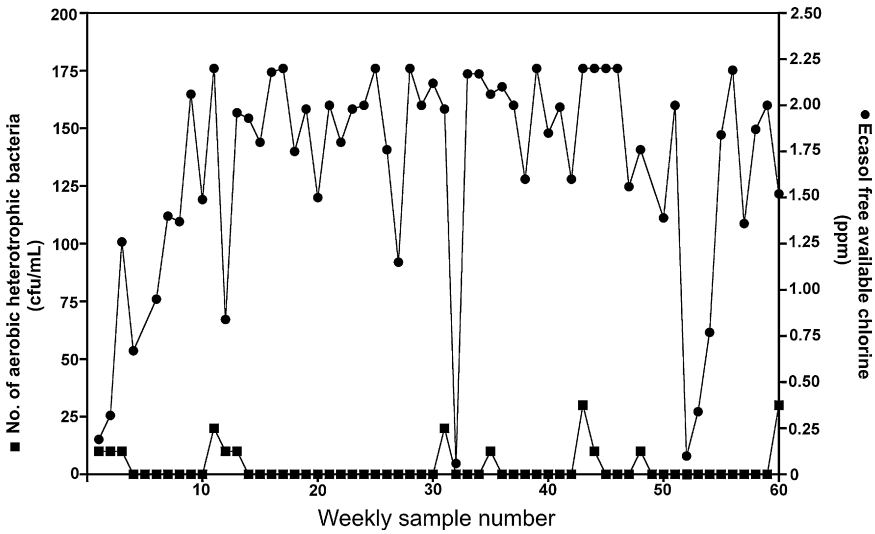


Fig. 5 – Density of aerobic heterotrophic bacteria (average 6.3 cfu/mL) and FAC (average 1.6 ppm) present in DUWL output water from the operator's three-in-one air/water waterline from DCU No. 1 during a period of 60 consecutive weeks. Similar results were obtained with nine other DCUs during the 60-week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

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).

Table 1 – Average density of aerobic heterotrophic bacteria and average FAC in 60 consecutive weekly water samples from DUWL output water from 10 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.

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 100 (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 100 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.

Acknowledgements

This study was supported by Enterprise Ireland grant IP/2008/0589. We thank Edmond O'Reilly, Kevin Keane and Heinrich Liesner from the Trustwater Group (Clonmel, Ireland) for engineering support relating to the application of Trustwater Ecasol™ technology. We thank Jari-Pekka Teravainen from the Planmeca Group (Helsinki, Finland) and the staff of the Planmeca Research and Development Department for providing technical and engineering support relating to Ecasol™ disinfection of DUWLs in Planmeca DCUs. We thank James Swan, Declan Clark, Damien Geoghegan and Mark Rooney for technical support with DCUs.

REFERENCES

1. O'Donnell MJ, Shore AC, Coleman DC. A novel automated waterline cleaning system that facilitates effective and consistent control of microbial biofilm contamination of dental chair unit waterlines: a one-year study. *Journal of Dentistry* 2006;**4**:648–61.
2. O'Donnell MJ, Shore AC, Russell RJ, Coleman DC. Optimisation of the long-term efficacy of dental chair waterline disinfection by the identification and rectification

of factors associated with waterline disinfection failure. *Journal of Dentistry* 2007;**35**:438–51.
3. Coleman DC, O'Donnell MJ, Shore AC, Swan J, Russell RJ. The role of manufacturers in reducing biofilms in dental chair waterlines. *Journal of Dentistry* 2007;**35**:701–11.
4. Coleman DC, O'Donnell MJ, Shore AC, Russell RJ. Biofilm problems in dental unit water systems and its practical control. *Journal of Applied Microbiology* 2009;**106**:1424–37.
5. Stanley HR. Pulpal response to dental techniques and materials. *Dental Clinics of North America* 1971;**15**:115–26.
6. Langeland K. Prevention of pulpal damage. *Dental Clinics of North America* 1972;**16**:709–32.
7. Siegel SC, von Fraunhofer JA. The effect of handpiece spray patterns on cutting efficiency. *Journal of the American Dental Association* 2002;**133**:184–8.
8. O'Donnell MJ, Boyle M, Swan J, Russell RJ, Coleman DC. A centralised, automated dental hospital water quality and biofilm management system using neutral Ecasol™maintains dental unit waterline output water at better than potable quality: a 2-year longitudinal study. *Journal of Dentistry* 2009;**37**:748–62.
9. Martin MV. The significance of the bacterial contamination of dental unit water systems. *British Dental Journal* 1987;**163**:152–4.
10. Pankhurst CL, Johnson NW, Woods RG. Microbial contamination of dental unit waterlines: the scientific argument. *International Dental Journal* 1998;**48**:359–68.
11. Mayo JA, Oertling KM, Andrieu SC. Bacterial biofilm: a source of contamination in dental air–water syringes. *Clinical Preventive Dentistry* 1990;**12**:13–20.
12. Pankhurst CL, Philpott-Howard JN. The microbiological quality of water in dental chair units. *The Journal of Hospital Infection* 1993;**23**:167–74.
13. Williams JF, Johnston AM, Johnson B, Huntington MK, Mackenzie CD. Microbial contamination of dental unit waterlines: prevalence, intensity and microbiological characteristics. *Journal of the American Dental Association* 1993;**124**:59–65.
14. Atlas RM, Williams JF, Huntington MK. Legionella contamination of dental-unit waters. *Applied and Environmental Microbiology* 1995;**61**:1208–13.
15. Barbeau J, Tanguay R, Faucher E, Trudel L, Côté L, *et al.* Multiparametric analysis of waterline contamination in dental units. *Applied and Environmental Microbiology* 1996;**62**:3954–9.
16. Meiller TF, Depaola LG, Kelley JI, Baqui AA, Turng BF, Falkler WA. Dental unit waterlines: biofilms, disinfection and recurrence. *Journal of the American Dental Association* 1999;**130**:65–72.
17. Stampi S, Zanetti F, Bergamaschi A, De Luca G. Comamonas acidovorans contamination of dental unit waters. *Letters in Applied Microbiology* 1999;**29**:52–5.
18. Mills SE. The dental unit waterline controversy: defusing the myths, defining the solutions. *Journal of the American Dental Association* 2000;**131**:1427–41.
19. Walker JT, Bradshaw DJ, Bennett AM, Fulford MR, Martin MV, Marsh PD. Microbial biofilm formation and contamination of dental-unit water systems in general dental practice. *Applied and Environmental Microbiology* 2000;**66**:3363–7.
20. Tuttlebee CM, O'Donnell MJ, Keane CT, Russell RJ, Sullivan DJ, Falkiner F, *et al.* Effective control of dental chair unit waterline biofilm and marked reduction of bacterial contamination of output water using two peroxide-based disinfectants. *The Journal of Hospital Infection* 2002;**52**:192–205.
21. Singh R, Stine OC, Smith DL, Spitznagel Jr JK, Labib ME, Williams HN. Microbial diversity of biofilms in dental unit water systems. *Applied and Environmental Microbiology* 2003;**69**:3412–20.
22. Walker JT, Bradshaw DJ, Fulford MR, Marsh PD. Microbiological evaluation of a range of disinfectant products to control mixed-species biofilm contamination in a laboratory model of a dental unit water system. *Applied and Environmental Microbiology* 2003;**69**:3327–32.
23. Walker JT, Bradshaw DJ, Finney M, Fulford MR, Frandsen E, ØStergaard E, *et al.* Microbiological evaluation of dental unit water systems in general dental practice in Europe. *European Journal of Oral Sciences* 2004;**112**:412–8.
24. Szymańska J. Bacterial contamination of water in dental unit reservoirs. *Annals of Agricultural and Environmental Medicine* 2007;**14**:137–40.
25. Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* 2000;**64**:847–67.
26. Watnick P, Kolter R. Biofilm, city of microbes. *Journal of Bacteriology* 2000;**182**:2675–9.
27. Putnins EE, Di Giovanni D, Bhullar AS. Dental unit waterline contamination and its possible implications during periodontal surgery. *Journal of Periodontology* 2001;**72**: 393–400.
28. Pankhurst CL, Coulter W, Philpott-Howard JN, Surman-Lee S, Warburton F, Challacombe S. Evaluation of the potential risk of occupational asthma in dentists exposed to contaminated dental unit waterlines. *Primary Dental Care* 2005;**12**:53–9.
29. Huntington MK, Williams JF, Mackenzie CD. Endotoxin contamination in the dental surgery. *Journal of Medical Microbiology* 2007;**56**:1230–4.
30. Pankhurst CL, Coulter WA. Do contaminated dental unit waterlines pose a risk of infection? *Journal of Dentistry* 2007;**35**:712–20.
31. Anonymous. Council Directive 93/42/EEC of 14 June 1993 concerning medical devices. *Official Journal of the European Community* 1993;L169:1–43.
32. Coleman DC, O'Donnell MJ. Guest editorial. *Journal of Dentistry* 2007;**35**:699–700.
33. Anonymous. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. *Official Journal of the European Community* 1998;L330: 32–54.
34. Anonymous. National primary drinking water standards. United States Environmental Protection Agency 2002; document number EPA 816-F-02-013.
35. Kohn WG, Collins AS, Cleveland JL, Harte JA, Eklund KJ, Malvitz DM. Guidelines for infection control in dental health-care settings – 2003. *Morbidity and Mortality Weekly Report Recommendations and Reports* 2003;**52**:1–61.
36. Anonymous. ADA statement on dental unit waterlines. *Journal of the American Dental Association* 1996;127:185–86.
37. Karpay RI, Plamondon TJ, Mills SE, Dove SB. Combining periodic and continuous sodium hypochlorite treatment to control biofilms in dental unit water systems. *Journal of the American Dental Association* 1999;**130**:957–65.
38. Smith AJ, Bagg J, Hood J. Use of chlorine dioxide to disinfect dental unit waterlines. *Journal of Hospital Infection* 2001;**49**:285–8.
39. Porteous NB, Cooley RL. Reduction of bacterial levels in dental unit waterlines. *Quintessence International* 2004;**35**:630–4.
40. Schel AJ, Marsh PD, Bradshaw DJ, Finney M, Fulford MR, Frandsen E, *et al.* Comparison of the efficacies of disinfectants to control microbial contamination in dental unit water systems in general dental practices across the European Union. *Applied and Environmental Microbiology* 2006;**72**:1380–7.
41. Szymańska J. Bacterial decontamination of DUWL biofilm using Oxygenal 6. *Annals of Agricultural and Environmental Medicine* 2006;**13**:163–7.

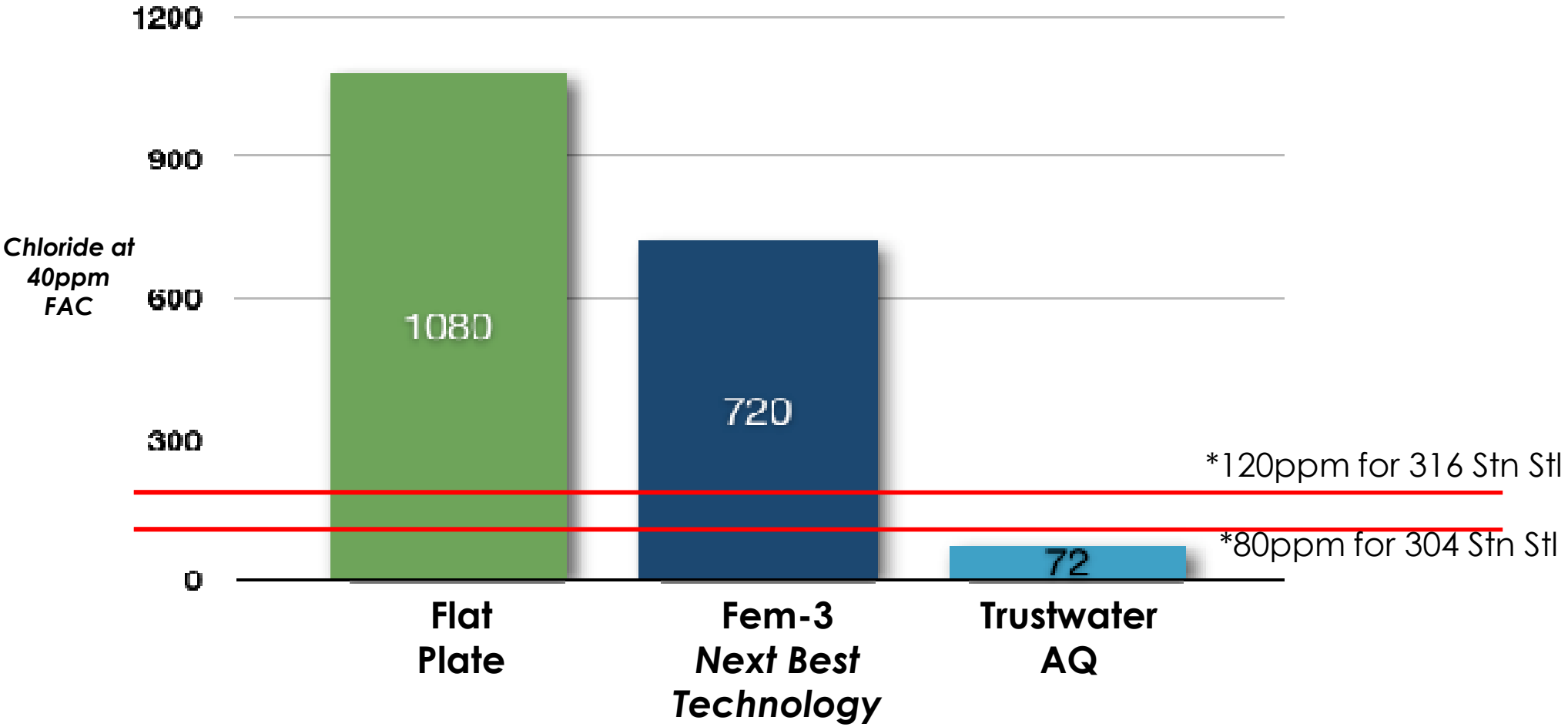
Please cite this article in press as: Boyle MA, et al. Lack of cytotoxicity by Trustwater Ecasol™ used to maintain good quality dental unit waterline output water in keratinocyte monolayer and reconstituted human oral epithelial tissue models. Journal of Dentistry (2010), doi:10.1016/j.jdent.2010.08.005

42. Walker JT, Marsh PD. Microbial biofilm formation in DUWS and their control using disinfectants. *Journal of Dentistry* 2007;**35**:721–30.
43. Bakhir V. Electrochemical activation: Vitold Bakhir Electrochemical Systems and Technologies Institute [Internet]. 129515, Moscow, Post Office box 107, Russia; c2005 [cited 2010 June 8]. Available from http://www.vbinstitute.org/terms/.
44. Bakhir VM, Zadorozhny Y, Leonov BI, Panicheva SA, Prilutsky VI. Electrochemical activation: water treatment and production of effective solutions. In: *Proceeding of the third international symposium "Electrochemical Activation in Medicine, Agriculture and Industry*. Moscow; 2001. p. 3–25.
45. Bakhir VM. Electrochemical activation; theory and practice. In: *Proceedings of the first international symposium on electrochemical activation*. Moscow; 1997. p. 38–45.
46. Bakhir V. Bibliography: Vitold Bakhir electrochemical systems and technologies institute [Internet]. 129515, Moscow, post office box 107, Russia; c2005 [cited 2010 June 8]. Available from http://www.vbinstitute.org/bibliography/.
47. Wolvos T. Advanced wound care with stable, super-oxidized water, a look at how combination therapy can optimize wound healing. *Wounds* 2006;**S1**:11–3.
48. Paola L. Treating diabetic foot ulcers with super-oxidized water, a look at advances in this pH-neutral, non-toxic treatment. *Wounds* 2006;**S1**:14–6.
49. Bakhir VM, Zadorozhny YG. Electrochemical Cell. US Patent N° 5,635,040, filed 11.03.1996, pbl. 3.06.1997. A flow-through electrochemical modular cell of FEM-3 type.
50. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. *Applied and Environmental Microbiology* 1985;**49**:1–7.
51. Calabrese JP, Bissonnette GK. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. *Applied and Environmental Microbiology* 1990;**56**:3558–64.
52. Rupniak TH, Rowlatt C, Lane EB, Steele JG, Trejdosiewicz LK, Laskiewicz B, *et al*. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *Journal of the National Cancer Institute* 1985;**75**:621–33.
53. Nielsen J, Brøndum-Nielsen EB, Christensen ME, Olin HBD, Tommerup N, Rassing MR. Filter-grown TR146 cells as an *in vitro* model of human buccal epithelial permeability. *European Journal of Oral Science* 1999;**107**:138–46.
54. Moharamzadeh K, Brook IM, Van Noort R, Scutt AM, Thornhill MH. Tissue-engineered oral mucosa: a review of the scientific literature. *Journal of Dental Research* 2007;**86**:115–24.
55. Stokes C, Moran GP, Spiering MJ, Cole GT, Coleman DC, Sullivan DJ. Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genetics and Biology* 2007;**44**:920–31.
56. Babich H, Reisbaum AG, Zuckerbraun HL. *In vitro* response of human gingival epithelial S-G cells to resveratrol. *Toxicology Letters* 2000;**114**:143–53.
57. Perrot S, Dutertre-Catella H, Martin C, Rat P, Warnet J-M. Resazurin metabolism assay is a new sensitive alternative test in isolated pig cornea. *Toxicological Science* 2003;**72**:122–9.
58. Sepet E, Bilir A, Akcin O, Aytepe Z. The effects of a caries-removing gel (Carisolv) on FM3A cell-line *in vitro*. *Journal of Dentistry* 2004;**32**:213–8.
59. Zeina B, Greenman J, Corry D, Purcell WM. Cytotoxic effects of antimicrobial photodynamic therapy on keratinocytes *in vitro*. *British Journal of Dermatology* 2002;**146**:568–73.
60. Schmalz G, Schweikl H, Hiller KA. Release of prostaglandin E2, IL-6 and IL-8 from human oral epithelial culture models after exposure to compounds of dental materials. *European Journal of Oral Science* 2000;**108**:442–8.
61. Nielsen HM, Rassing MR. Nicotine permeability across the buccal TR146 cell culture model and porcine buccal mucosa *in vitro*: effect of pH and concentration. *European Journal of Pharmaceutical Sciences* 2002;**16**:151–7.
62. Portero A, Remuñán-López C, Nielsen HM. The potential of chitosan in enhancing peptide and protein absorption across the TR146 cell culture model-an *in vitro* model of the buccal epithelium. *Pharmaceutical Research* 2002;**19**:169–74.
63. Pullar JM, Winterbourn CC, Vissers MC. Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid. *American Journal of Physiology* 1999;**277**:H1505–1512.
64. Whiteman M, Hooper DC, Scott GS, Koprowski H, Halliwell B. Inhibition of hypochlorous acid-induced cellular toxicity by nitrite. *Proceedings of the National Academy of Sciences of the United States of America* 2002;**99**:12061–6.
65. Nielsen HM, Verhoef JC, Ponec M, Rassing MR. TR146 cells grown on filters as a model of human buccal epithelium: permeability of fluorescein isothiocyanate-labelled dextrans in the presence of sodium glycocholate. *Journal of Controlled Release* 1999;**5**:223–33.
66. Eirheim HU, Bundgaard C, Nielsen HM. Evaluation of different toxicity assays applied to proliferating cells and to stratified epithelium in relation to permeability enhancement with glycocholate. *Toxicology in Vitro* 2004;**18**:649–57.
67. Boulmedarat L, Bochot A, Lesieur S, Fattal E. Evaluation of buccal methyl-beta-cyclodextrin toxicity on human oral epithelial cell culture model. *Journal of Pharmaceutical Sciences* 2005;**94**:1300–9.
68. Trombetta D, Mondello MR, Cimino F, Cristani M, Pergolizzi S, Saija. A toxic effect of nickel in an *in vitro* model of human oral epithelium. *Toxicology Letters* 2005;**159**:219–25.
69. Angelic JR, Moyes D, Macramé J, Kanzaria P, Tsichlaki E, Weindl G, *et al*. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* 2008;**154**:3266–80.
70. Spiering MJ, Moran GP, Chauvel M, Maccallum DM, Higgins J, Hokamp K, *et al*. Comparative transcript profiling of *Candida albicans* and *Candida dubliniensis* identifies SFL2, a *C. albicans* gene required for virulence in a reconstituted epithelial infection model. *Eukartotic Cell* 2010;**9**:251–65.
71. Weiss SJ, Lampert MB, Test ST. Long-lived oxidants generated by human neutrophils: characterization and bioactivity. *Science* 1983;**222**:625–8.
72. Sanjay PR, Hallikeri K, Shivashankara AR. Evaluation of salivary sialic acid, total protein, and total sugar in oral cancer: a preliminary report. *Indian Journal of Dental Research* 2008;**19**:288–91.
73. Kotula KL, Kotula AW, Rose BE, Pierson CJ, Camp M. Reduction of aqueous chlorine by organic material. *Journal of Food Protection* 1997;**60**:276–82.
74. Kouoh F, Gressier B, Luyckx M, Brunet C, Dine T, Cazin M, *et al*. Antioxidant properties of albumin: effect on oxidative metabolism of human neutrophil granulocytes. *Farmaco* 1999;**54**:695–9.
75. Rajabalian S, Mohammadi M, Mozaffari B. Cytotoxicity evaluation of Persica mouthwash on cultured human and mouse cell lines in the presence and absence of fetal calf serum. *Indian Journal of Dental Research* 2009;**20**:169–73.

Please cite this article in press as: Boyle MA, et al. 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. Journal of Dentistry (2010), doi:10.1016/j.jdent.2010.08.005

Materials Compatibility Report

Corrosion potential @ 40ppm FAC



*Warranty limits of max acceptable Chloride in Solutions per Krones Construction Specification 1-099-00-811-0

BAM
Bundesanstalt für Materialforschung und -prüfung
D 12200 Berlin
Telefon: 0 30/81 04-0
Telefax: 0 30/81 11 29/30

Tests of the Corrosion Behaviour of Disinfectant Solutions

Aktenzeichen VI.1/14697_E

Ausfertigung 1. copy of 3 copies

Antragsteller/ Auftraggeber TRUSTWATER Ltd.
Unit 1
Gurtnafleur Business Park,
Clonmel,
Co. Tipperary,
Ireland

Objective of the report To test three electrochemically produced solutions for corrosion behavior on Stainless steel X5CrNi18-10 (Sometimes referred to as Stainless Steel 304). Trustwater markets devices for the production of disinfectant concentrates for the cleaning and disinfection of surfaces. This corrosion study examines the influence of the various active substances on the stainless austenitic steel X5CrNi18-10..

Summary The tested disinfection concentrates were adjusted by dilution with purified water so as to have uniform free chlorine content (40 ppm). The dilution of the solutions resulted in different chloride concentrations. Consequently, there are significant differences in their influence on the pitting corrosion behaviour of the tested material. The results show that at 40ppm free available chlorine the AQ solutions do not cause relevant deterioration of the corrosion of the stainless steel X5CrNi18-10 (S.S. 304).

GUTACHTEN

This report consists of pages 1 to 9.

The content of this report is related exclusively to the assessed objects.

1 Scope

Trustwater manufactures devices for the electrolytic generation of disinfectants. These disinfectants are used successfully for the cleaning and/or disinfection of surfaces (in the food industry, for example). In principle, the influence of oxidizing substances on the corrosion behaviour of stainless steel has been known for quite some time. However, an extensive test of the influence of these types of substances has not been carried out. Here, the corrosion characteristics of solutions with various chlorine/chloride-ratios are to be determined. In addition, the passive range of the tested material, here X5CrNi18-10 (1.4301), will be established and the pitting corrosion potential determined through very slow polarization. In this procedure, the electrolytes and the material determine the measurement results.

2 Examination procedure

The measurements were carried out in a measuring cell according to ASTM G150 [1].

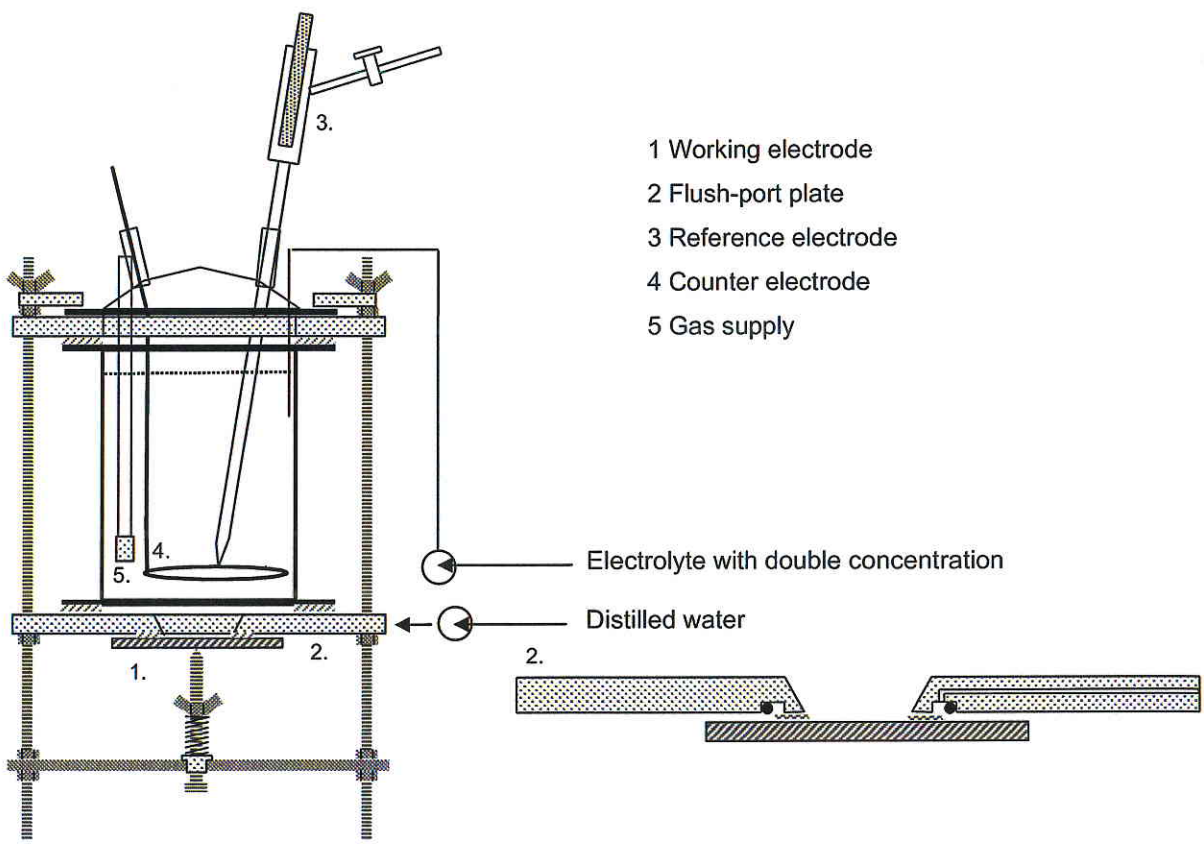


Image 1: Ilkustration of the measurement assembly

A classic electrochemical three-electrode configuration is used. The special feature is the mounting of the working electrode. Since stainless steel can tend towards crevice corrosion under certain conditions--which would prevent the determination of the pitting corrosion potential--the design was developed with an irrigated crevice. In this way, crevice corrosion can be prevented.

The measurements were carried out according to the specifications of ASTM G 61 [2]. As a deviation, the above-described measurement cell was used in order to rule out crevice corrosion problems that are to be expected for the tested material, X5CrNi18-10.

A sample of the material under test is grinded carefully (increasing up to 600 grade), in order to create a reproducible surface of the electrodes. The sample is mounted to the sample holder from below with a ring of filter paper. The crevice between the sample and the holder is flushed using a peristaltic pump and distilled water in order to prevent corrosion in the sealing crevice. Since this would dilute the electrolyte in the measurement period, an electrolyte with a double concentration of relevant substances is added at the same time and in the same amount. Where necessary, a portion of the electrolyte is removed during the measurement so as to prevent the overspill of the cell. The measurements take place at room temperature.

Solutions produced from three different disinfectant concentrates were used as electrolytes. The concentrates were supplied by the client in the following qualities: high salinity, standard salinity and low salinity produced using a plate generator and the Trustwater FEM 3 and AQ devices. The free chlorine content was determined directly before the measurement and adjusted to 40 mg/l through dilution with desalinized water. Analogue to the concentrates, the diluted solutions have three different ratios of free chlorine to chloride and thus three significantly different chloride concentrations. Sheet material from X5CrNi18-10 (1.4301) was employed for the working electrodes. The measurements were carried out with a scan rate of 0.6 V/h (10 mV/min). This slow polarization rate ensured stable conditions in the tested system during measurement. The general specifications for the execution of such electrochemical measurements are in ASTM G 61 as well as DIN EN ISO 17475 [3].

The polarization was polarized up to a limiting current of 200 uA/cm2. The potential value at 30 uA/cm2 is specified as the pitting corrosion potential.

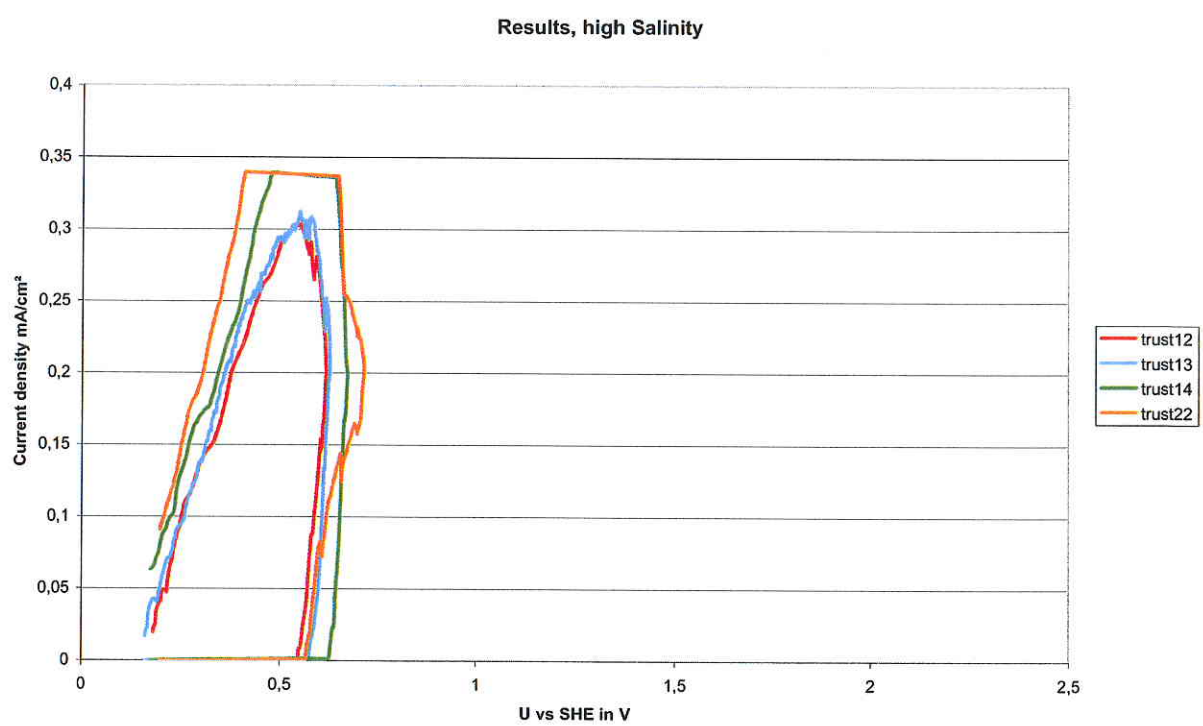
3 Results

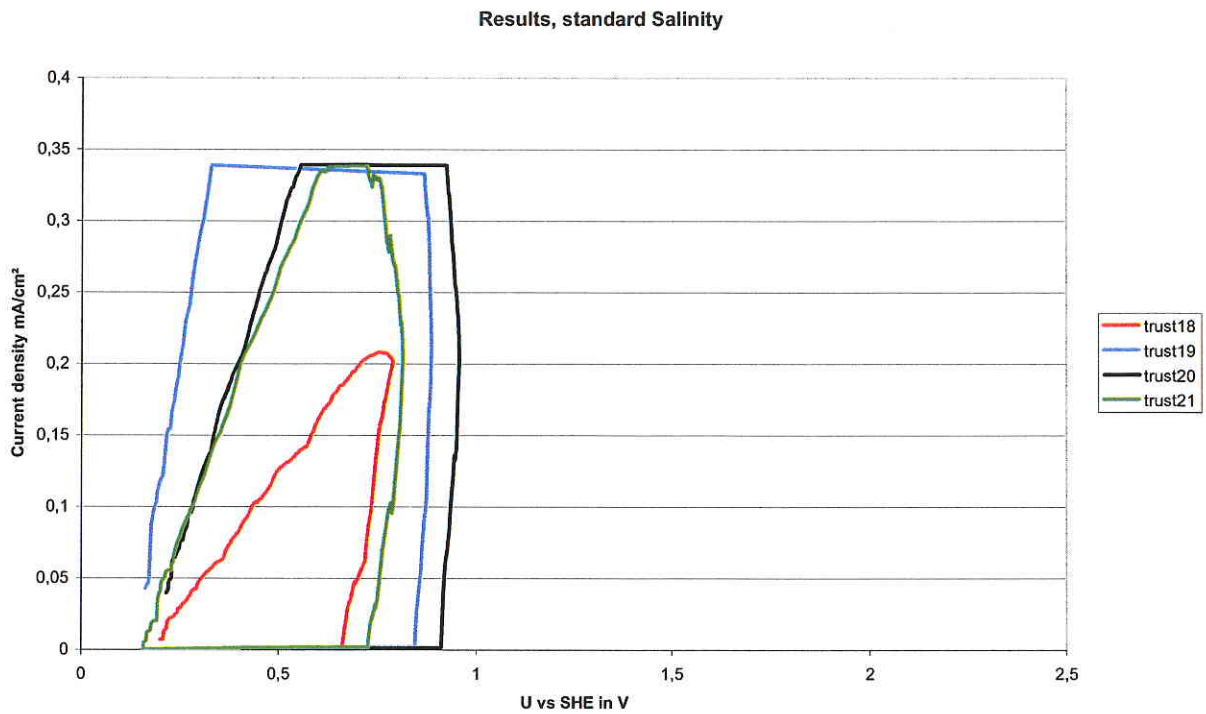
3.1 Current-Potential-Measurements

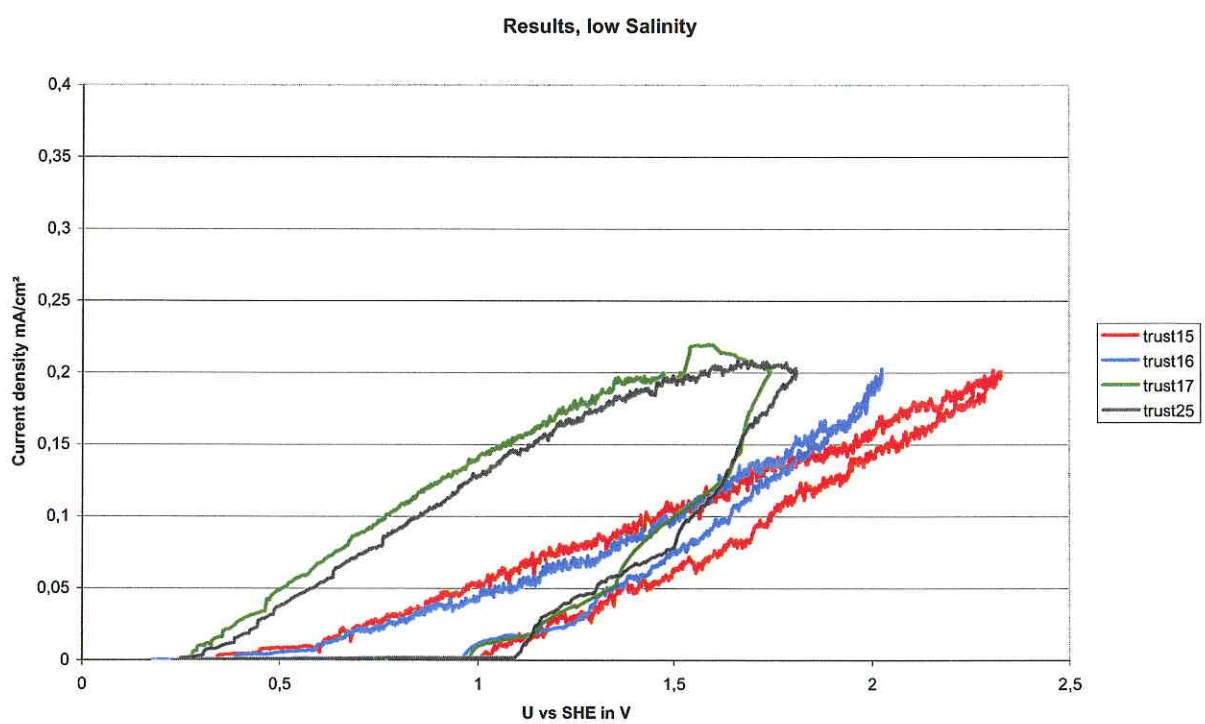
The diluted concentrates were used for the measurements. With respect to the chloride content, the following values resulted for the electrolytes:

Plate generator	High Salinity	1300 mg/l
FEM 3 generator	Standard Salinity	800 mg/l
AQ generator	Low Salinity	50 mg/l

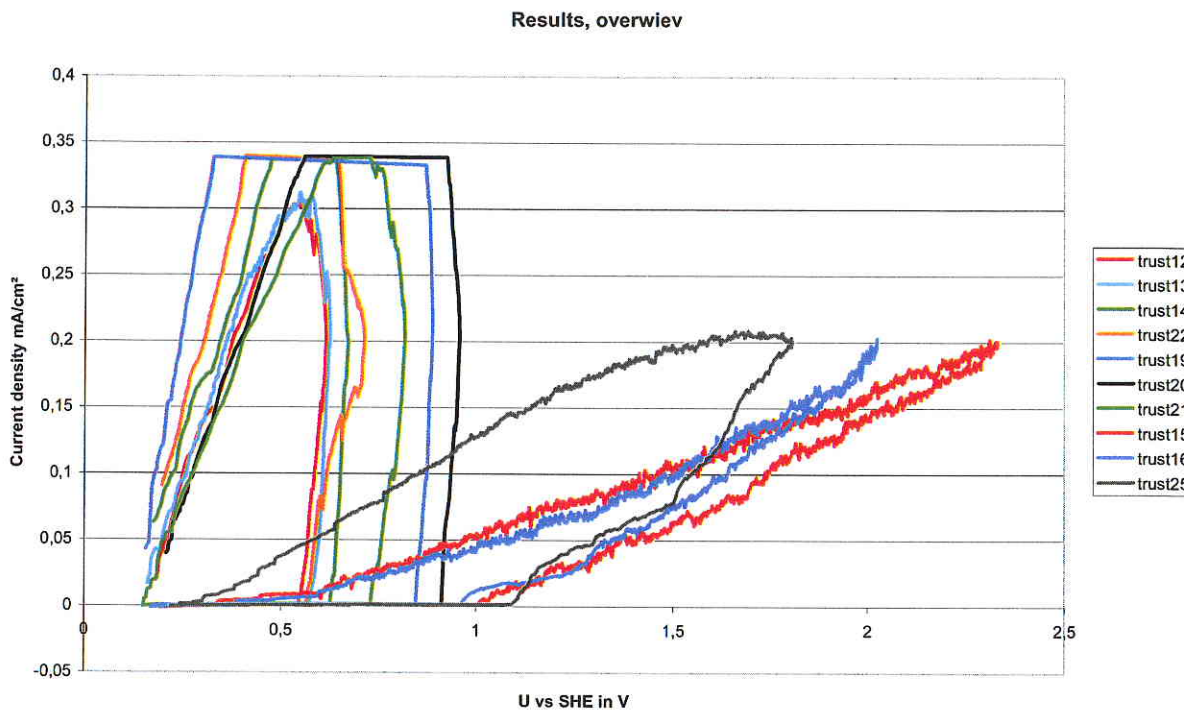
The results of the measurements of X5CrNi18-10 with the various electrolytes are shown in the following graphs. An articulate influence on the pitting corrosion potential by the variation of the chloride content of the electrolytes is observed. In addition, one notes differences in the repassivation potential behaviour in the potential return. This demonstrates the material's tendency to stop the corrosion process again after the critical potential range is exceeded--that is, to behave passively again. This can be observed in the size of the hysteresis of the curve between the upward and return flows. The return is always the upper part of the curve. The measurements demonstrate very good reproducibility.







Significant differences with respect to the various chloride concentrations can be observed.



The overview shows the different behaviour of the low salinity electrolytes even more explicit.

The pitting corrosion potentials are specified as potential values for a current of 30 µA/cm^2. The following mean values were the results of the measurents:

Table 1: Average Value of the Potential at 30 uA/cm2:

	High Salinity	Standard Salinity	Low Salinity
X5CrNi18-10	0,59 V	0,85 V	1,22 V

3.2 Determination of pit depth

In order to obtain additional assessment criteria for the corrosive action, the number and depth of the pits caused by corrosion in the test were determined pursuant to ASTM G5.

Table 2: Pits, high Salinity

Test series	Number of pits	min. pit depth	max. pit depth	Mean pit depth
Trust 12	8	113 µm	305 µm	226 µm
Trust 13	7	163 µm	397 µm	283 µm
Trust 14	8	320 µm	405 µm	358 µm
Trust 22	7	350 µm	485 µm	417 µm

Table 3: Pits, standard Salinity

Test series	Number of pits	min. pit depth	max. pit depth	Mean pit depth
Trust 19	18	385 µm	455 µm	423 µm
Trust 20	17	295 µm	460 µm	414 µm
Trust 21	7	240 µm	445 µm	367 µm

Table 4: Pits, low Salinity

Test series	Number of pits	min. pit depth	max. pit depth	Mean pit depth
Trust 15	50	245 µm	390 µm	301 µm
Trust 16	18	90 µm	530 µm	351 µm
Trust 17	6	345 µm	665 µm	499 µm
Trust 25	9	340 µm	645 µm	502 µm

No differences with respect to the corrosion behaviour can be observed in the number and the depths of the pits. Considering the test conditions, which define a polarization up to the same current density, as well as the consistently identical electrode material, this is not unexpected.

4 Discussion

The measurements carried out demonstrate that the production of the disinfectant by way of different procedures has a significant influence on the corrosion behaviour of the investigated stainless steel. In all experiments, pits were found in the measurement electrodes after the polarization experiment. This meets expectations, since the test conditions were selected in such a way that pitting corrosion would occur.

Differences between the electrolytes are clearly visible in in the run of the measurement curves--in particular, in the position of the pitting corrosion potential and the repassivation behaviour. For these criteria, there is a significant differentiation with respect to the low salinity electrolyte, which shows a by far favourable behaviour than the two electrolytes in comparison. This does not apply to the number and the depth of the pits--nor does it correspond to the test conditions, however.

The position of the pitting corrosion potential and the ability to repassivation recognizable in the measurements with the low salinity electrolytes demonstrate that no increased corrosion likelihood is to be expected for usual periods of the cleaning and disinfection application. This cannot be excluded for the substances measured in the comparison according to the standard salinity procedure, but for the low salinity procedure, it is to be accepted as likely. Decisive here is the difference in the chloride content of the examined solutions. Higher chloride contents give rise to an increased risk of corrosion.

When used for the cleaning and disinfection of surfaces composed of the tested material, no problems are to be expected in the concentration range of 40 mg/l of free chlorine. An increase of the chloride concentration could result in problematic conditions. Users of the solutions should take this into consideration.

In addition, the interaction of multiple, potentially damaging influences must be considered: In the event that the surfaces already exhibit weak points in the form of unfavourable crevices, discolorations or surface failures--i.e., issues which reduce the corrosion resistance--the stress limit of the material could be exceeded and corrosion could result. For surfaces in proper-condition, in the examined concentration range this is not to be expected according to the results of the tests.

5 Literature

1 ASTM G 150 – 99 Standard Test Method for Electrochemical Critical Pitting Temperature Testing of Stainless Steels, 2004

2 ASTM G 61 – 86 Standard Test Method for Conducting Cyclic Potentiodynamic Polarization Measurements for Localized Corrosion Susceptibility of Iron-, Nickel-, or Cobalt-Based Alloys

3 EN ISO 17475, Corrosion of metals and alloys - Electrochemical test methods - Guidelines for conducting potentiostatic and potentiodynamic polarization measurements, 2006-10

BAM Federal Institute for Materials Research and Testing
12200 Berlin,, 2010-03-19

Fachgruppe VI.1

by order

Dr. rer.nat. J.W. Erning
Oberregierungsrat

Arbeitsgruppe VI.14

by order

Dipl.-Ing. U. Klein
Technischer Regierungsamtsrat

Verteiler:
1. copy: BAM
2. to 3. copy: Client

We guarantee that this report was completed according to the best available knowledge, impartially and free from prejudice. This report can be subject to additional modifications, expansions, and, if necessary, the retraction of the report for significant reason (e.g. as a result of significant, new knowledge).

USDA & FDA Letter of No Objection

United States Department of Agriculture

Food Safety and Inspection Service

Washington, D.C. 20250

FEB 1 8 1998

Ms. Christie S. Chavis
Enviro-Chem Systems
A Monsanto Company
P.O. Box 14547 St.
Louis, MO 63178

RECEIVED
FEB 24 1998
CHRISTIE CHAVIS

Dear Ms. Chavis:

We have completed our review of your technology for generating aqueous chlorine solutions on-site from a brine solution using traditional electrolysis principles referred to as "Electrical Chemical Activation". We have no objections to the use of your technology in federally inspected meat, poultry, or egg products establishments.

You are correct in Stating that the Food Safety and Inspection Service (FSIS) no longer requires the prior approval of equipment used in official establishments effective September 24,1997. However, equipment must continue to meet the general standards that it be of materials and construction that will facilitate thorough cleaning and cleanliness, will not contaminate product, and will not interfere with or impede inspection procedures. Furthermore, any equipment used in federally inspected meat, poultry, or egg products establishments is still subject to the sanitation requirements and monitoring byFSIS inspectors. FSIS has authority to prevent the use of equipment which poses a threat to public health or interferes with inspection,

FSIS provided general design and construction guidelines for equipment in the Federal Register, Volume 62, No.164 (August 25,1997), pp 45033 to assist establishments in selecting equipment to be used in their operations.

Sincerely,

Will

William J. Hudnall
Assistant Deputy Administrator
Standards and Methods Review

EQUAL OPPORTUNITY IN EMPLOYMENT AND SERVICES

DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service

Food and Drug Administration
College Park, MD 20740

September 15, 2009

Trustwater USA, Inc.
Southdale Office Center
6600 France Avenue, Suite 230
Edina, Minnesota 55435

Attn: Patrick O'Shaughnessy
Chief Operating Officer

Re: Pre-Notification Consultation (PNC) 833

Dear Mr. O'Shaughnessy:

This is in response to Pre-Notification Consultation (PNC) 833, received on August 18, 2009 and submitted on behalf of Trustwater, in which our comments were requested concerning your Electrical Chemical Activation (ECA) technology. Your documents indicate that your technology applies electrolysis to a sodium chloride (brine) solution to produce chlorine sanitizing solutions containing greater than 97% chlorine. FDA does not object to this method of producing an aqueous chlorine solution for use as a sanitizer. Our letter of non-objection sent to Enviro-Chem Systems on August 8, 1997 (attached) for the form of ECA technology described in your PNC is still applicable.

Sincerely,

Julie N. Mayer, M.F.S.
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug
Administration
Washington DC 20204

August 8, 1997

RECEIVED
AUG 8 1997
CHRISTIE S CHAVIS

Ms. Christie S. Chavis
Enviro-Chem Systems
A Monsanto Company

P.O.Box 14547

St. Louis. MO 63178

Dear Ms. Chavis:

This is in response to your letters dated July 15 and 21. 1997 in which you asked that FDA confirm that your method of generating aqueous chlorine solutions for food use applications not need FDA approval. In your letters, and our meeting on July 11, you described your technology for producing chlorine as Electrical Chemical Activation (ECA) technology. Basically,'this ECA technology is an electrolysis of a sodium- chloride {brine) solution that produces chlorine solutions containing greater than 97% chlorine. You indicate that trace levels of other oxidants such as chlorine dioxide, ozone, chlorite and chlorate have been detected in the chlorine solutions at levels between 0.5 - 0.85%.

We have reviewed your submissions and we conclude that the chlorine solutions generated by ECA technology is suitable for food processing applications. Therefore, we offer no objection to the use of **ECA** technology to produce chlorine solutions for food processing applications where this technology *is* used consistent with current good manufacturing practices, Furthermore, this ECA technology does not need additional FDA approval.

We trust that the above response is satisfactory to your inquiry. If you need additional assistance, please contact us again.

Sincerely yours,

Robert L. M

Dairy Usage Examples

DISINFECTION IN A DAIRY MILKING PARLOUR USING ANOLYTE AS DISINFECTION

Prof T E Cloete and M S Thantsha, Department of Microbiology and Plant Pathology, University of Pretoria, South Africa

INTRODUCTION

Dairy products such as milk, butter, cream and cheese are all susceptible to microbial spoilage because of their chemical composition. Milk is an excellent growth medium for all of the common spoilage organisms, including molds and yeasts. Fresh, non-pasteurized milk generally contains varying numbers of microorganisms, depending on the care employed in milking, cleaning, and handling of milk utensils. Raw milk held at refrigerator temperatures for several days invariably shows the presence of several or all bacteria of the following genera: *Enterococcus, Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Microbacterium, Propionibacterium, Micrococcus,* coliforms, *Proteus, Pseudomonas, Bacillus,* and others. Those unable to grow at the usual low temperature of holding tend to be present in very low numbers. The pasteurization process eliminates all but thermoduric strains, primarily streptococci and lactobacilli, and spore formers of the genus *Bacillus* (and clostridia if present in raw milk). The spoilage of pasteurized milk is caused by the growth of heat-resistant streptococci utilizing lactose to produce lactic acid, which depresses the pH to a point (about pH 4.5) where curdling takes place. If present, lactobacilli are able to grow at pH values below that required by *Lactococcus lactis*. These organisms continue the fermentative activities and may bring the pH to 4.0 or below. If mold spores are present, these organisms begin to grow at the surface of the sour milk and raise the pH toward neutrality, thus allowing the more proteolytic bacteria such as *Pseudomonas* spp. to grow and bring about the liquefaction of the milk curd.

The use of a well balanced cleaning and sanitizing programme will aid in the production of raw milk of exceptionally high microbiological quality. Farms with well disciplined and carefully organized sanitation programmes which extend from cow preparation to bulk tank cleaning, are easily achieving Standard Plate Counts of <10 000/ml and coliforms counts of <10/ml for raw milk – standards usually not reached for fluid pasteurized milk by most processing plants in South Africa.

Chemical sanitizers are normally used and iodophors (25 ppm iodine) and chlorine (100 ppm chlorine) are the most widely used and allowed a minimum contact time of two minutes for best results.

A novel way of the electro chemical activation of water was recently introduced in South Africa. During Electro Chemical Activation (ECA) of water, a dilute saline solution is "activated" by passing through a cylindrical electrolytic cell in which the anodic and cathodic chambers are separated by a permeable membrane. Two separate streams of

activated water are produced: Anolyte with a pH range of 2-9 and an oxidation- reduction potential (ORP) of +400 mV to +1200 mV. Anolyte is an oxidizing agent due to a mixture of Free Radicals and has an antimicrobial effect. Catholyte with pH of 12 to 13 and an ORP of about –900mV, it has reducing and surfactant properties and is an antioxidant. During the process of electrochemical activation three broad classes of product are produced:

- Stable products – these are acids (in the Anolyte) and bases (in the Catholyte) which influence the pH of the solution in question, as well as other active species.
- Highly active unstable products – these include free radicals and other active ion species with a typical lifetime of less than 48 hours. Included here would be electrically and chemically active micro bubbles of electrolytic gas 0,2 – 0,5 micrometer in diameter and with concentrations up to 10^7 ml^{-1}, distributed uniformly through the solution. All these species serve to enhance the oxidation – reduction potentials (ORP) of the Anolyte.
- Quasi-stable structures – these are structures formed at or near the electrode surface as a consequence of the very high voltage drop (10^7 V cm^{-1}) in those regions. These are free structural complexes of hydrated membranes around ions, molecules, radicals and atoms. The size of these water clusters is reduced to approximately 5-6 molecules per cluster. All these features enhance the diffusion, catalytic and biocatalytic properties of the water.

The chemical composition of ECA solutions may be altered by utilizing various hydraulic arrangements linking electrolytic cell modules, together with other supplementary devices, in order to optimally address the requirements of specific areas of application. Some other variables are flow rate; hydraulic pressure; current density and voltage on the electrodes.

One of the most important problems for researchers into ECA processes' mechanism is that of the nature of the state metastable water and diluted water solutions find themselves in after unipolar electrochemical exposure. Until now this problem has not been satisfactorily solved, nevertheless it is not an obstacle to wide practical application of electrochemically activated liquids. The problem lies in the fact that it is extremely difficult to assess activation contribution of purely chemical and purely physical components of electrochemical effect on para-electrode environment.

During anode electrochemical treatment, water acidity grows. ORP increases due to the formation of stable and unstable acids (Sulfuric, hydrochloric, hypochlorous, persulfuric), as well as hydrogen peroxide, peroxo-sulfates, peroxo-carbonates, oxygen-containing chlorine compounds and different intermediate compounds arising in the process of spontaneous decomposition and interaction of the indicated substances. Also, as a result of anode electrochemical treatment surface tension somewhat decreases, electric conductivity rises, as does the content of dissolved chlorine and oxygen, concentration of hydrogen and nitrogen decreases, and water structure changes.

A range of bactericidal substances, commonly termed biocides or microbicides, are available, all of which are claimed by their agents to kill bacteria in aqueous systems quantitatively. However, different bacteria react differently to bactericides, either due to differing cell wall properties[6], or to other mechanisms of resistance, either inherent or inducible[7,8,9].

The bacterial cell membrane provides the osmotic barrier for the cell and catalyses the active transport of substances into that cell. Alternations in transmembrane potential caused by the action of electron donor or electron acceptor factors are associated with powerful electro-osmotic processes accompanied by water diffusion against ORP gradients, with resultant rupture of the membranes and outflow of the bacterial cell contents. The bacterial membrane itself has an electrical charge. The anions present in Anolyte act on this membrane. Anolyte can also disrupt other functions of the cell. Unlike "higher" organisms, single celled organisms such as bacteria obtain their energy sources form the environment immediately outside the cell. Small molecules are transported across the cell membrane via an electro-chemical gradient. Thus, any significant change in the ORP of the immediate environment has drastic consequences for the cell. Even if instantaneous death of the cell does not occur, all enzymatic functions in the membrane are affected and this will also result in loss of cell viability.

MATERIALS AND METHODS

Three milking stations (same everyday) were analysed on a daily basis after disinfection. Four different surfaces on each of the three stations were sampled each day. The four different surfaces sampled were (1) the inside of the teat cluster, (2) teat cluster top (mouth) (3) float control flow sensor inside and (4) float control flow sensor lid. A sterile swab was used to sample each surface and streaked out on nutrient agar plates. The plates were incubated at 37°C for 48h and the number of colonies formed on each plate was counted.

Sporeformers detection

Cell suspensions were prepared from the plates that gave spreaders in the dairy disinfection experiment. The colonies were suspended by adding 5 ml of sterile distilled water to the plate and suspending the colony using a sterile loop. The suspensions were transferred to test tubes and the test tubes were incubated at 80°C for 10 min. 1 ml of each of the cell suspensions was plated out on nutrient agar plate. The plates were incubated at 37°C for 48h.

Bulk tanker washing

The milk tank was washed with the usual disinfectant (control) and thereafter the different surfaces inside the tank were sampled using Rodac plates. For the experiment, the tank was washed with ECA solutions. The first wash was done with catholyte, then the tank was rinsed with tap water and the tank was finally rinsed with anolyte. The inside of the tank was sampled as for the control. The experiments were run for five days

for each disinfectant used. The plates were incubated at 37°C for 24h then numbers of colony forming units were counted.

RESULTS AND DISCUSSION

Table 1. Microbiological analysis of different surfaces in a milking parlour after disinfection.

	Control (cfu per swab)										
	1	2	3	4	5	6	7	8	9	10	11
Station 1											
FSL	Spr	>300	0	>300	14	Spr	Spr	Spr	>300	Spr	>300
FSI	Spr	119	154	115	0	>300	>300	Spr	>300	Spr	246
TCI	0	3	0	7	0	2	0	>300	105	0	0
TCT	>300	Spr	>300	>300	Spr	>300	>300	>300	>300	>300	>300
Station 2											
FSL	Spr	>300	Spr	>300	Spr	Spr	Spr	Spr	>300	Spr	Spr
FSI	279	>300	>300	>300	Spr	>300	149	Spr	98	Spr	0
TCI	0	51	0	6	61	38	1	2	0	Spr	90
TCT	>300	Spr	>300	>300	6	Spr	>300	>300	>300	Spr	>300
Station 3											
FSL	3	>300	Spr	Spr	Spr	Spr	Spr	Spr	>300	Spr	Spr
FSI	>300	>300	>300	273	9	Spr	292	>300	>300	82	>300
TCI	0	6	186	6	0	0	1	Spr	1	3	182
TCT	>300	>300	>300	>300	Spr	>300	>300	Spr	>300	Spr	>300

Spr= spreader
cfu= colony forming units
FSL= float controlled flow sensor lid
FSI= float controlled flow sensor inside
TCI= teat cluster inside
TCT=teat cluster top

The results in Table 1 and Table 2 are qualitative rather than quantitative, since it was impossible to sample exactly the same surface area, due to the nature of the sampled surface. The normally used method of disinfection (indicated as the control in the results) did have some degree of microorganism control (**Table 1**). In most cases, the teat cluster inside had the lowest level of contamination. However, most of the surfaces were not satisfactorily disinfected, indicated by the spreaders (**Table 1**), and on some surfaces where number of microorganisms exceeded the maximum number that could be counted on the plates. The relatively higher numbers of microorganisms in the float control flow sensor lid and teat cluster top, was attributed to these surfaces not being exposed to the disinfectant solutions (**Table 1 and Table 2**). The anolyte however eliminated the spreaders on these sampling sites. When anolyte was used as a disinfectant, the results were generally better. This is particularly evident when comparing the results of the FSI sampling point (**Table 1 and Table 2**)

Table 2. Microbiological analysis of different surfaces in a milking parlour after disinfection with anolyte

	Anolyte(cfu/ml)					
	1	2	3	4	5	6
Station 1						
FSL	246	94	>300	0	69	0
FSI	1	0	0	1	0	0
TCI	0	0	0	1	0	4
TCT	>300	>300	0	>300	>300	>300
Station 2						
FSL	110	5	>300	>300	185	>300
FSI	0	0	1	0	1	0
TCI	0	0	2	0	0	9
TCT	>300	>300	44	78	>300	>300
Station 3						
FSL	69	>300	Spr	>300	6	0
FSI	3	0	Spr	0	0	0
TCI	0	13	0	0	4	9
TCT	>300	164	109	>300	>300	>300

Spr= spreader
cfu= colony forming units
FSL= float control flow sensor lid
FSI= float control flow sensor inside
TCI= teat cluster inside
TCT=teat cluster top

Table 3: Growth of cells after exposure to a high temperature

Plate number	Results
1 – 18	G

G=Growth

All the spreaders were resistant to heating at 80°C (**Table 3**). This indicated that all the spreaders were a result of growth of sporeforming organisms that contaminated the milking parlour.

Table 4: Results of the tanker after cleaning with control disinfectant and ECA solutions (Catholyte followed by Anolyte)

Plate no.:	**Control**					**ECA solutions**				
	Days									
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
	cfu/cm^2									
1	Spr	288	TNTC	120	19	NG	39	NG	NG	NG
2	Spr	75	264	TNTC	2	8	9	NG	NG	NG
3	219	NG	TNTC	226	34	NG	12	33	NG	NG
4	202	126	108	258	26	44	12	Spr	NG	NG
5	TNTC	206	TNTC	Spr	6	NG	TNTC	NG	NG	NG

TNTC= Too numerous to count (> 300 cfu/ 25cm^2)

NG = No growth

Spr= Spreader

The numbers of cfu/cm^2 were higher when the tank was washed with the control disinfectant, than when it was washed with ECA solutions throughout the experimental period (Table 4). The control disinfectant was not effective for disinfecting the bulk tank, since most of the counts were higher than 300 cfu/cm^2 and spreaders (sporeformers being encountered regularly (Table 4). Effective disinfection of the milk tank was however achieved using a Catholyte wash followed by an Anolyte disinfection with most surfaces being sterilized (Table 4).

CONCLUSIONS

- The contact between the disinfectant and the surface to be disinfected is essential for removing the number of organisms.
- All spreaders were identified as spore forming organisms
- Anolyte eliminated the spore forming bacteria
- Overall, the anolyte gave better disinfection than the control disinfectant

- Where anolyte made contact with a surface, disinfection was at an acceptable level, with most surfaces being sterilized
- The combination of Catholyte followed by an Anolyte disinfection step was effective for disinfection of the bulk tank.

REFERENCES

Jay J. M. (1992). Modern Food Microbiology. 4th edition. Chapman & Hall, New York. ISBN 0-442-00733-7.

USA University Studies Milking Equipment

University Park, Pa. --- Researchers at Penn State have devised a novel way to clean and disinfect milking equipment, using little more than salt water. The new method could be a safer and cheaper alternative to conventional cleaning systems.

"Concentrated chemicals used in the conventional cleaning are stored on the farm and on contact, they can cause serious burns in the eyes and on skin," says Dr. Ali Demirci, associate professor of agricultural and biological engineering. And, he says the chemicals are also expensive.

Most farms across the United States use some form of mechanized system to milk cows. The set-up basically comprises a rubber-lined suction cup that milks the cow and transfers the milk to a central refrigerating tank, through a series of pipes.

At day's end, the whole system is cleaned in a four-step process: first the pipes are rinsed with warm water to remove the milk. Then they are flushed with a chlorinated detergent at high temperature to remove soils such as fat and protein deposits, and then with a weak acid to neutralize the detergent and remove mineral deposits.

Finally, the pipes have to be sanitized with an EPA-registered sanitizing agent before they can be used again.

Demirci and his colleagues tried to clean the milk pipes using electrolyzed oxidizing water, as other researchers had shown its effectiveness in cleaning fresh produce, eggs, etc.

Electrolyzed oxidizing water is created when electric current flowing through two electrodes – immersed in a weak salt solution and separated by a membrane – produces an alkaline and an acidic solution.

It is not as expensive as the detergents, and can be made with just a little bit of salt and water," says Demirci, whose findings are published in the December 2005 issue of Transactions of the American Society of Agricultural Engineers.

To test how the new cleaning agent measured up to conventional detergent, the researchers flushed warm milk laden with bacteria down a series of pipes set up to mimic the system on a farm, and they compared the cleaning power of both in turns.

Results showed that in between 7.5 to 10 minutes, the electrolyzed oxidizing water was as effective in removing organic matter from the pipes, as conventional treatments.

"It is not harmful to the skin, and much cheaper. The alkaline detergent and acidic rinse in conventional systems of cleaning can be replaced with this water," says Demirci.

Other researchers include Stephen P. Walker, graduate student; Robert E. Graves, professor in the Department of Agricultural and Biological Engineering; Stephen B. Spencer, professor emeritus in the Department of Dairy and Animal Science; and Robert F. Roberts, associate professor in the Department of Food Science, all at Penn State.

The U.S. Department of Agriculture funded this project.

SANI-MATIC
1915 S. Stoughton Rd. ✦ Madison, WI 53716
P: 608.222.2399 ✦ F: 608.222.5348 ✦ info@sanimatic.com
www.sanimatic.com

ECA SOLUTION – MILK CRATE CLEANING TEST

PURPOSE: To determine whether or not the solutions produced by Trustwater electrochemical generator are capable of cleaning dairy soils from milk crates.

TESTING PROCEDURE: Testing coupons were made from the flat surfaces of the milk crates. The coupons were then soiled with chocolate milk and cleaned using the following procedure.

i. Weight clean, unsoiled coupon to the .1 mg for initial weight.
ii. Soiling Coupons:
    a. Lay coupons down flat.
    b. Drop several drops of chocolate milk on coupon until majority of surface is covered with soil.
    c. Allow milk residue to air dry on surface on coupon.
    d. When dry flip all coupons over so that unsoiled surface is up.
    e. Drop several drops of chocolate milk on coupon until majority of surface is covered with soil.
    f. Allow milk residue to air dry on surface on coupon.
    g. Weight coupon to the .1 mg once both sides have completely dried.
iii. Prepare solution to be used for cleaning, diluting to the necessary strength.
iv. Record pH and conductivity of solution.
v. Heat each solution to the desired temperature and set stir bar setting to 4.
vi. Once the solution comes to temperature, add single coupon to bath and allow it to float around freely in solution. Clean for 10 minutes and remove from bath.
vii. Then hang coupon from hanging rack and allow it to air dry.
viii. Repeat steps (ii) through (vii) varying the temperature of the solution from 70°F to 150°F, for each solution.
ix. Weight cleaned coupon to the .1 mg once it has dried completely.

RESULTS: The four cleaning solutions tested were: Soft Water, Chlorinated Detergent, Ecolab's Principal, and ECA generated Aversol. Aversol was diluted until a pH of 11.5 was reached. Chlorinated Detergent and principal were used at use dilutions. Each solution was tested at room temperature (~70°F), 86°F, and 150°F. Three coupons were tested at each condition and their percent cleaned was averaged. Results are depicted in Figure 1.

SANI-MATIC
1915 S. Stoughton Rd. ✦ Madison, WI 53716
P: 608.222.2399 ✦ F: 608.222.5348 ✦ info@sanimatic.com
www.sanimatic.com

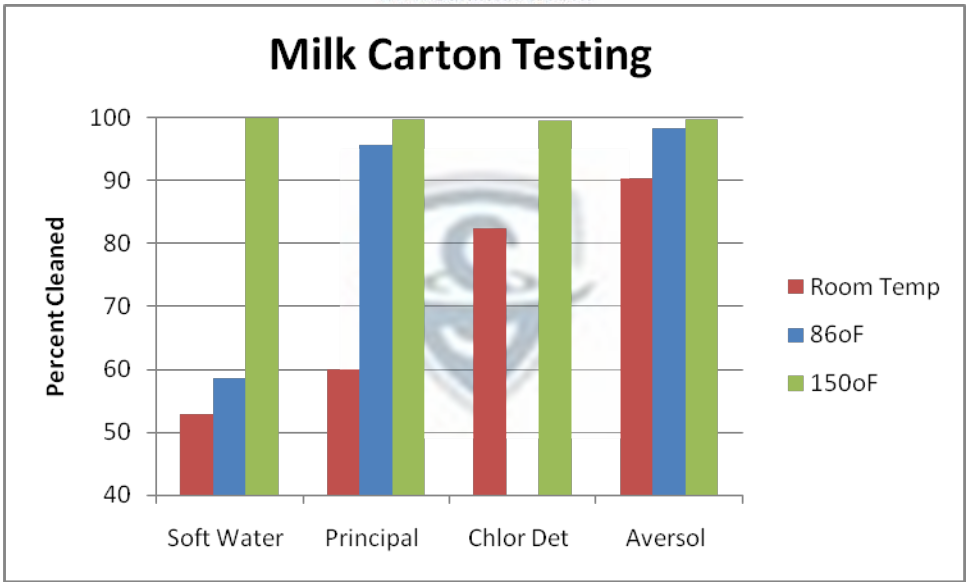


Figure 1 Bar graph of cleaning capability of each detergent at the three different temperatures.

i. Aversol out performs Principal, Chlorinated Detergent and Soft Water at room temperature and at 90°F.
ii. At current cleaning temperatures (150°F) all solutions clean equally well at 100%.
iii. At room temperature Aversol was capable of cleaning 90.3% of the soil from the surface.

Note: that the system was run at a lower agitation (speed 4). The coupons were much lighter than typical stainless steel coupons therefore the system was ran at a lower speed. If higher agitation was possibly the cleaning performance would improve.

CONCLUSION:

- Aversol at 86°F (98.4%) performs equally as well as Principal and Chlorinated detergent at 150°F.
- Aversol could be used at room temperature if higher agitation is available.
- Note that the soil conditions used in this testing may not replicate those in specific applications. Concentrations may need to be higher or lower than these, depending on the actual soil conditions.