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Antimicrobial activity of electrolyzed oxidizing water using standard in-vitro test procedures for the evaluation of chemical disinfectants

INAUGURAL-DISSERTATION

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Meinen Eltern

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1 Zusammenfassung

Elektrolysiertes, oxidierendes Wasser (EOW) ist ein Konzept zur Inaktivierung von mikrobiologischen Verunreinigungen und weist eine starke antimikrobielle Wirksamkeit gegen ein breites Spektrum von Mikroorganismen auf. In der vorliegenden Studie wurde EOW durch Elektrolyse von 0.1% Natriumchloridlösung in einer Elektrolysezelle hergestellt, in der eine Membran Anode und Kathode trennte, wodurch zwei separate Kammern entstanden. Die Bestimmung der antimikrobiellen Wirksamkeit von EOW wurde gemäss Standardmethoden der Deutschen Veterinärmedizinischen Gesellschaft (DVG) für die Testung von chemischen Desinfektionsmitteln durchgeführt. Die Prüfung der sporiziden Wirkung erfolgte nach DIN/EN 13704. *Enterococcus faecium*, *Mycobacterium avium* subspecies *avium*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* und Endosporen von *Bacillus subtilis* subspecies *spizizenii* wurden anodischem EOW (pH, 3.0 ± 0.1; Redoxpotential (ORP), +1100 ± 50 mV; freies Chlor, 400 ± 0.021 mg/L Cl2) und kombiniertem EOW (7:3 Anode : Kathode, vol/vol; pH, 8.3 ± 0.1; ORP, 930-950 mV; freies Chlor, 271 ± 0.01 mg/L Cl2) ausgesetzt. In Wasser standardisierter Härte (WSH) wurden alle Bakterienstämme durch 30 minütigen Kontakt mit maximal 10.0% anodischem EOW (≈40.0 mg/L Cl2) oder 50.0% kombiniertem EOW (≈135.5 mg/L Cl2) vollständig inaktiviert. Die Empfindlichkeit der Teststämme gegenüber anodischem EOW war folgendermassen: *P. mirabilis* > *S. aureus* > *M. avium* ssp. *avium* > *E. faecium* > *P. aeruginosa*. *P. mirabilis* und *S. aureus* konnten nach 5 minütiger Einwirkzeit von 7.5% anodischem EOW (≈30.0 mg/L Cl2) nicht mehr nachgewiesen werden. *C. albicans* konnte durch 5 minütige Behandlung mit 5.0% anodischem EOW (≈20.0 mg/L Cl2) vollständig abgetötet werden. Sporen von *B. subtilis* konnten durch 60 minütige Einwirkzeit von 80% anodischem EOW (≈320 mg/L Cl_2), nicht jedoch durch 80% kombiniertes EOW (≈217 mg/L Cl_2) inaktiviert werden. Sowohl anodisches, wie auch kombiniertes EOW zeigten in Standardnährmedien oder bei Zugabe von 20.0% Rinderserum zum WSH keine antimikrobielle Wirkung mehr. Es sind deshalb noch weitergehende Tests notwendig, um die Anwendbarkeit von EOW als Desinfektionsmittel unter Feldbedingungen in Tierhaltungsbetrieben zu evaluieren.

2 Summary

Electrolyzed oxidizing water (EOW) is a relatively new concept to inactivate microbiological contaminants and has been shown to impart strong antimicrobial properties against a broad spectrum of pathogens. In the present study, EOW was produced by electrolysis of a 0.1% sodium chloride solution in an electrolysis chamber, where the anode and the cathode are separated by a diaphragm to form two compartments. Standards of the German Association of Veterinary Medicine (DVG) for the evaluation of chemical disinfectants were used to assess the antimicrobial efficacy of EOW. *Enterococcus faecium*, Mycobacterium *avium* subspecies *avium*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and endospores of *Bacillus subtilis* subspecies *spizizenii* were exposed to anode EOW (pH, 3.0 ± 0.1; oxidation-reduction potential (ORP), +1100 ± 50 mV; free chlorine, 400 ± 0.021 mg/L Cl_2) and combined EOW (7:3 anode : cathode, vol/vol; pH, 8.3 ± 0.1; ORP, 930-950 mV; free chlorine, 271 ± 0.01 mg/L Cl_2). In water of standardized hardness (WSH), all bacterial strains were completely inactivated by a 30 min exposure to maximum 10.0% anode EOW (≈40.0 mg/L Cl_2) or 50.0% combined EOW (≈135.5 mg/L Cl_2). The sensitivity ranking order for anode EOW to the bacterial test strains was *P. mirabilis* > *S. aureus* > *M. avium* ssp. *avium* > *E. faecium* > *P. aeruginosa*. *P. mirabilis* and *S. aureus* decreased to undetectable levels after 5 min of exposure to 7.5% anode EOW (≈30.0 mg/L Cl_2). *C. albicans* was completely inactivated by a 5 min exposure to 5.0% anode EOW (≈20.0 mg/L Cl2). *B. subtilis* spores were inactivated by 60 min of exposure to 80.0% anode EOW (≈320 mg/L Cl_2) but not by 80% combined EOW (≈217 mg/L Cl_2). Both, anode and combined EOW exhibited no antimicrobial activities in standardized nutrient broth or after addition of 20.0% bovine serum to the WSH. Further research is necessary to evaluate EOW as a disinfectant under operating conditions in animal production facilities.

3 Introduction

In intensive livestock housing, appropriate cleaning and disinfection procedures are essential to prevent disease and economic loss due to infections. Disinfectants for use in on-farm biosecurity measures should (i) have a high fast-acting antimicrobial potency even in heavily contaminated areas, (ii) be effective at ordinary temperatures when diluted with water, and readily mix with water, (iii) not remain strongly toxic after their application nor excessively irritating, (iv) not be corrosive, and (v) be free of strong and objectionable odours.

Electrolyzed oxidizing water (EOW) has been attracting attention as a disinfectant due to its strong microbicidal activities on a broad variety of bacterial pathogens including mycobacteria as well as bacterial endospores (Tanaka et al., 1996; Venkitanarayanan et al., 1999; Selkon et al., 1999; Loshon et al., 2001). Moreover, EOW appeared to have an inactivative potential against bacterial endotoxin, exerts fungicidal activity, and is described to inactivate bacterial or fungal toxins probably by break down of such hazardous substances into non-harmful components (Suzuki et al., 2002 a,b).

EOW is generated by electrolysis of a dilute aqueous solution of sodium chloride (NaCl) in an electrolysis chamber where the anode (+ electrode) and cathode (- electrode) are separated by a cationic membrane to form two compartments (Kumon, 1997; Fig. 1). The anode acidic EOW has a low pH of 2.3 to 3.0, a high oxidation-reduction potential (ORP) greater than 1000 mV, and contains relative concentrations of chlorine (Cl_2), hypochlorous acid (HOCl) and hypochlorite (OCl^-; Sharma and Demirci, 2003). The alkaline solution from the cathode has a high pH and low ORP (Kumon, 1997). The physical properties and chemical composition of EOW varies dependent on concentration of NaCl, amperage level, time of electrolysis or water flow rate (Kiura et al., 2002; Hsu S. Y., 2003; Nakajima et al., 2004).

A variety of applications of EOW in agriculture, medicine, and food sanitation have been described so far (Sharma and Demirci, 2003). Due to the reported broad spectrum of microbicidal activities, EOW is proving to be of considerable interest as a disinfectant in animal husbandry and veterinary medicine. However, before any approval can be given to apply EOW as an antimicrobial treatment of e.g. food animal husbandry facilities, candidate substances must be thoroughly evaluated

(Yilmaz and Kaleta, 2003). In the present study, the procedures specified by the German Association of Veterinary Medicine (Deutsche Veterinärmedizinische Gesellschaft, DVG, http://www.dvg.net) and by the German Institute of Standardisation (Deutsches Institut für Normung, DIN, http://www2.din.de) for the evaluation of chemical disinfectants were used to assess the microbicidal efficacy of EOW.

4 Materials and methods

4.1 Electrolyzed oxidizing water

EOW was generated with the Stericold® electrolysis device (Biostel® Schweiz AG, Buttikon, Switzerland). The principle of the apparatus is given in Fig. 1. The generator was connected to a water faucet. A 0.1% solution of NaCl in tap water automatically provided from a continuous supply of a saturated aqueous NaCl solution was pumped into the electrolytic cell at a rate of 1.3 L/min. Under these conditions, the 0.1% NaCl solution was electrolyzed at room temperature for 46 s. The current passing through the electrolysis cell and the voltage between the electrodes was set at 12 A and 10 V, respectively. Electrolyzed solutions from the electrodes were automatically provided at separate outlets. The generator was allowed to run for 10 min before collecting solutions for analyses so that the amperage level was stabilized to the set value of 12 A. The electrolyzed solutions were collected in sterile containers and were used within 30 min for microbiological studies. Samples for the determination of physical and chemical properties of electrolyzed solutions were collected simultaneously and analysed immediately. The pH was determined by using a digital pH meter (Metrohm AG, Herisau, Switzerland) according to established standards (Anonymous, 1999a). The ORP was measured with an ORP meter (SenTix ORP Redox, WTW Wissenschaftlich Technische Werkstätte, Weilheim, Germany). Total and free chlorine were determined by a photometric detection of the products from the oxidation of ABTS (2.2-Acino-bis-(3-ethylenbenzothiazolin)-6-sulphonate) with chlorine species. EOW from the anode was characterized by the following parameters: pH, 3.0 ± 0.1; ORP, +1100 ± 50 mV; free chlorine concentration, 400 ± 0.021 mg/L Cl_2). Analogous values for electrolyzed water from the cathode were a pH of 11-13 and an ORP of -850 ± 50 mV. Anode

EOW and combined EOW (7:3 anode:cathode, vol/vol; pH, 8.3 ± 0.1; ORP, 930 - 950 mV; free chlorine, 271 ± 0.01 mg/L Cl_2) were subjected to analyses of antimicrobial activities.

4.2 Microorganisms

The following test strains were used in accordance with the proposed guidelines of the DVG for the testing of disinfectants in livestock husbandry (Anonymous, 2000b): *Enterococcus* (*E.*) *faecium* Kulmbach strain 2 (DSM 2918), *Mycobacterium* (*M.*) *avium* subspecies (ssp.) *avium* (ATCC 15769), *Proteus* (*P.*) *mirabilis* (ATCC 14153), *Pseudomonas* (*P.*) *aeruginosa* (ATCC 15442), and *Staphylococcus* (*S.*) *aureus* (ATCC 6538). *Candida* (*C.*) *albicans* (ATCC 10231) was used as the yeast representative. Test strains were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany). The growth condition for the microorganisms followed recommendations of the supplier (http://www.dsmz.de/dsmzhome.htm). Prior to testing, each strain was subcultured at least twice onto suitable media to ensure purity and viability. Purified spores of *Bacillus* (*B.*) *subtilis* ssp. *spizizenii* (ATCC 6633) for the evaluation of the sporicidal activity of chemical disinfectants according to DIN/EN 13704 (Anonymous, 2002c) were purchased from Institute Pasteur (Paris, France; http://www.pasteur.fr/externe).

4.3 Standardizing inocula

The preparation of the inocula followed standard procedures provided by the DVG guidelines (Anonymous, 2000b). Gram-negative and Gram-positive test strains were grown on Tryptic Soy Agar (TSA, P05012A, Oxoid, Wesel, Germany). Growth from an isolated colony of each strain was transferred into 10.0 mL of Tryptic soy broth (TSB, CM129, Oxoid). After overnight incubation at 37°C, broth cultures were checked for purity by microscopic examination of Gram-stained smears. A 10.0 µL aliquot from a pure broth culture was transferred into 10.0 mL TSB and incubated at 37°C for 18 h. Passage in TSB was performed twice. The second broth culture passage contained approximately 1.0×10^8 - 1.0×10^9 CFU/mL. To ensure that the

final inoculum concentration routinely obtained approximates this default, CFU/mL of inocula prepared from different strains were measured periodically by serial dilution and spread plate counting on TSA according to DIN 10161 (Anonymous, 1984d; Foster et al., 1986).

M. avium ssp. *avium* was grown on commercially available agar slants of Loewenstein-Jensen Medium (LJM, TV102C, Oxoid; Malone et al., 2003). When sufficient growth was encountered at the three-week reading of pure cultures, colonies were harvested in sterile saline (0.9 M NaCl in Aqua dest.) and were vigorously vortexed. The mycobacterial suspension was adjusted to McFarland turbidity standard 2 representing approximately 1.0 x 10^8 - 1.0 x 10^9 CFU/mL.

Inocula from purified *B. subtilis* ssp. *spizizenii* endospores were prepared as follows: The number of viable spores per millilitre of the original suspension was determined by serial dilution and pour plating of the spore suspension into duplicate Glucose Yeast (GY) agar plates formulated according DIN/EN 13704. After incubation for 72 h at 30°C, the average CFU/mL for the duplicate plates was determined and used to adjust the concentration of viable spores in the original suspension to the required standard of 1.5 - 5.0 x 10^6 CFU/mL.

C. albicans was subcultured at least twice onto TSA supplemented with 2.0% dextrose. Colonies from pure cultures were harvested in sterile saline and were vigorously vortexed. The yeast suspension was clarified from massive clumps of yeast cells and agar residues by filtration through columns consisting of 10-ml syringes filled with sterile glass wool (Jürgens, Hannover, Germany) and was adjusted to the turbidity of a 2 McFarland standard (≈10^8 - 10^9 cells/ml).

4.4 Bacteriostatic and fungistatic efficacy

The bacteriostatic efficacy of anode and combined EOW was determined to the bacteria *E. faecium*, *S. aureus*, *P. mirabilis*, and *P. aeruginosa*. Test concentrations of both EOWs were full strength, and 50.0, 25.0, 10.0, and 1.0%, respectively in sterile water of standardized hardness (WSH: Aqua bidest. containing 2.42 mM $CaCl_2$ and 0.61 mM $MgSO_4$). A volume of 5.0 ml of each EOW dilution was transferred to separate, sterile screw-cap tubes containing 5.0 ml of double concentrated TSB. To each tube, 0.1 ml of a 1:10-diluted inoculum (equivalent to

~1.0 x 10^7 – 1.0 x 10^8 CFU/mL) was then added. Tubes were vortexed for 10 s to ensure homogenization, and were incubated for 72 h at 37°C. Growth controls were incorporated for each test and consisted of a 1:1 mixture of sterile WSH with double concentrated TSB and of a bacterial inoculum as described above. Testing of growth inhibition of bacteria was carried out by using aqueous phenol (1.0, 0.50, 0.25, 0.10, and 0.01% in WSH) instead of EOW dilutions. The fungistatic efficacy of both EOWs was determined accordingly using *C. albicans* as the test strain, TSB supplemented with 4.0% dextrose as the growth medium, and an incubation of 96 h at 37°C. 3.0% formalin and its dilutions in WSH as mentioned above were used as inhibition controls of *C. albicans*. All tests were performed at least in duplicate. Growth of bacteria or fungi resulted in a visible clouding of the culture medium. The lowest concentration of EOW that inhibited visible growth was recorded as the minimum bacteriostatic or fungistatic concentration.

4.5 Bactericidal and fungicidal efficacy

Test strains to determine the bactericidal and fungicidal efficacy were *E. faecium*, *M. avium* ssp. *avium*, *P. mirabilis*, *P. aeruginosa*, *S. aureus* , and *C. albicans*. The following concentrations of test solutions in WSH were used: anode EOW, 80.0, 25.0, 10.0, 7.5, 5.0, 1.0%; combined EOW, 80.0, 50.0, 25.0, 20.0, 10.0, 5.0, 1.0%. A volume of 10.0 mL of each EOW dilution was given to separate sterile tubes. To each tube 0.1 mL of standardized bacterial and yeast inoculum was added. After vortexing, samples were incubated at 22°C (ambient temperature) for 5, 15, 30 and 60 min. WSH was used as the growth control. Inactivation controls consisted of 1.0% phenol for inhibition of Gram-negative and Gram-positive bacteria and 3.0% formalin for *C. albicans* and *M. avium* ssp. *avium*. At each time point, a 0.1 mL aliquot from each sample and each control was transferred into a sterile tube containing 10.0 mL of appropriate culture medium (bacteria, TSB; *C. albicans*, TSB + 2.0% dextrose). Samples were incubated at 37°C for 72 h (bacteria) or 96 h (*C. albicans*). In the testing of *M. avium* ssp. *avium*, 0.1 mL aliquots were inoculated onto LJM. Agar slants were incubated at 37°C for four weeks. Growth of surviving bacteria or yeasts was indicated either by visible clouding of the broth culture medium or growth of characteristic *M. avium* ssp. *avium* colonies on LJM. The identity of

growing microorganisms was confirmed by random checks using standard laboratory procedures (Muskens et al., 2003; Paolicchi et al., 2003; Quinn et al., 1994). To evaluate the interference of organic matter (protein) with the microbicidal activity of EOWs, inactivation tests were performed with the final concentrations of EOWs as given above and a final protein load of 20.0% bovine serum (Gibco™, Invitrogen AG, Basel, Switzerland). All tests were performed at least in duplicate.

4.6 Sporicidal efficacy

Sporicidal activity was tested against endospores of *B. subtilis* ssp. *spizizenii*. Final concentrations of anode and combined EOW were 80.0, 70.0, and 60.0%. All tests were carried out in a water bath at a temperature of 20°C ±1°C. A 1.0 mL volume of the standardized endospore suspension equivalent to 1.5 – 5.0 x 10^6 CFU/mL was given to separate sterile tubes. To each tube 1.0 mL of a sterile 0.03% bovine serum albumin solution in WSH was added. After vortexing and 2 min incubation at ambient temperature, 8.0 mL of each EOW concentration was added. The tubes were vortexed and incubated for 60 min in a water bath at 20°C ±1°C. To prevent any post-incubation effect of probably active EOW residues, aliquots of 1.0 mL from each sample were mixed immediately after termination of incubation with 8.0 mL sodium thiosulphate and 1.0 mL of sterile distilled water, and samples were incubated for another 5 min. 1.0 mL of each sample was then transferred in duplicate into a sterile Petri dish. 15.0 mL of melted GYA with a temperature of 45°C was added and thoroughly mixed with the sample. Solidified agar plates were incubated at 30°C for 72 h. Colonies were enumerated to calculate the rate of surviving endospores. Controls performed in tandem with each test series confirmed that the test conditions including thiosulphate treatment to neutralize EOW did not induce any inactivation of endospores. All tests were performed at least in duplicate.

5 Results

5.1 Bacteriostatic and fungistatic efficacy

Bacteriostatic and fungistatic effects were evaluated against *E. faecium*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, and *C. albicans*, respectively by exposure of

standardized inocula of microorganisms to a range of anode and combined EOW concentrations in a defined nutrient broth. 72 h or 96 h of exposure to a final concentration of maximum 50% anode or combined EOW (equivalent to [≈] 200 and 135 mg/L Cl_2, respectively) did not achieve any bacteriostatic or fungistatic efficacy.

5.2 Bactericidal and fungicidal efficacy

Bactericidal and fungicidal activities were evaluated by exposure of standardized inocula of the microorganisms to a range of EOW concentrations in a defined aqueous system devoid of organic matter. Data are shown in Table 1. At ~22°C (ambient temperature) all microorganisms were more effective inactivated by anode EOW than by combined EOW. In general, all bacterial strains were completely inactivated by anode EOW concentrations of maximum 10.0% (≈40.0 mg/L Cl_2) after maximum 30 min of exposure. By comparison, the bactericidal activity of combined EOW was less efficient; here a minimum concentration of 50.0% (≈135.5 mg/L Cl_2) was required for a complete inactivation within 30 min of exposure. Employing inactivation values from Table 1, a sensitivity ranking order for anode EOW to the bacterial test strains is as follows: *P. mirabilis* > *S. aureus* > *M. avium* ssp. *avium* > *E. faecium* > *P. aeruginosa*. Test strains of *P. mirabilis* and *S. aureus* decreased to undetectable levels after 5 min of exposure to 7.5% anode EOW (≈30.0 mg/L Cl_2). Comparatively, the Gram-positive bacterium *E. faecium* and the Gram-negative bacterium *P. aeruginosa* exhibited a greater resistance to the bactericidal activities of anode EOW since a concentration of 25.0% (≈100.0 mg/L Cl_2) was required for a complete bactericidal effect within 5 min of exposure. *M. avium* ssp. *avium* was completely inactivated by 15 min of exposure to 10.0% anode EOW whereas a fivefold concentration of 50.0% and an extended exposure of 30 min was required to achieve complete inactivation in combined EOW. The yeast representative *C. albicans* was highly susceptible to microbicidal activities of anode EOW with complete inactivation after 5 min of exposure to a concentration of 5.0%. *C. albicans* was less susceptible to fungicidial activities of combined EOW. Here a minimum concentration of 50.0% was required for complete inactivation after 5 min of exposure (Table 1). Microbicidal activities of EOWs against vegetative cells of

bacteria and yeasts were completely abolished after addition of 20.0% bovine serum to the WSH.

5.3 Sporicidal efficacy

In order to analyse the bactericidal activities of EOWs against a highly disinfectant-resistant microorganism, standardized suspensions of endospores of *B. subtilis* ssp. *spizizenii* were exposed to defined concentrations of anode or combined EOW in an aqueous solution with a 0.003% protein load. Concentrations of maximum 80.0% combined EOW (≈217 mg/L Cl_2) did not inhibit germination of endospores. Complete inhibition of the growth of 1.5 - 5.0 x 10^6 CFU/mL *B. subtilis* spores was only observed after 60 min of exposure to 80.0% anode EOW (≈320 mg/L Cl_2).

6 Discussion

In the present study we generated EO water with a commercially available electrolysis device and examined the microbicidal efficiency of product test solutions following the guidelines outlined by the German Association of Veterinary Medicine for the testing of disinfectants in livestock husbandry.

The microbicidal properties of anode EOW are determined by its physical and chemical properties, such as low pH (~3), high ORP (+1100 mV), and large concentrations of chlorine (Cl_2, in the present case ~400 mg/L). In aqueous solutions, Cl_2 hydrolyzes rapidly into hypochlorous acid (HOCl; Fig. 1). HOCl is one of the most germicidal chlorine compounds which are by definition oxidizing agents. The oxidizing capacitiy of the HOCl is equal to the extremely active molecular Cl_2. The availability of HOCl is primarily a function of the pH, which establishes the amount of dissociation of HOCl to a hydrogen ion (H^+) and a hypochlorite ion (OCl^-; White, 1999). In general, the concentration of HOCl increases significantly as the pH decreases: At a pH above 9 and a temperature of 20°C, there is only marginal disinfecting activity since at this pH level ~96% of the free available chlorine consists of the OCl^- ion, which is a relatively poor disinfectant. This is in agreement with our finding that in comparison to anode EOW the combined EOW consisting of 70%

anode and 30% cathode EOW (pH 8.3) lacked strengths of disinfecting efficacy. By way of explanation: At a pH of 8.3 and a temperature of 20°C, the percentage of undissociated HOCl is 16.1% (White, 1999). However, at a pH below 5, the percentage of undissociated HOCl in a chlorine solution is approx. 99.7%. Thus, the large amount of HOCl on account of the low pH of ~3 is considered the chief factor of the disinfecting efficacy of EO water (Vorobjeva et al., 2004; White, 1999). Due to the water-like structure, the low molecular weight, and the electrical neutrality, HOCl molecules can easily diffuse through the bacterial cell wall into the cytoplasm (Kiura et al., 2002; Len et al., 2000; White, 1999). The bactericidal mechanism is probably the irreversible oxidation of cytoplasmic enzymes in particular in the inner membrane and peripheral cytoplasm (Matsunaga et al., 1984). Whether hydrogen peroxide (H_2O_2), ozone (O_3) and chlorine dioxide (ClO_2) contribute to the microbicidal activity of EOW remains controversial, since these substances were not detected in freshly prepared EOW (Gordon et al., 1999; Vorobjeva et al., 2004).

As was confirmed in our study, anode EOW renders a strong bactericidal activitiy against Gram-positive and Gram-negative bacteria as well as against mycobacteria. However, marked differences in the sensitivity for anode EOW between the bacterial test strains were found. The Gram-negative bacterium *P. mirabilis* and the Gram-positive bacterium *S. aureus* were more susceptible to the bactericidal acitivity of anode EOW water than the Gram-negative *P. aeruginosa* and the Gram-positive *E. faecium*. The former agents were completely inactivated by 5 min exposure to 7.5% anode EOW (≈30 mg/L Cl_2) whereas the latter required a 5 min exposure to 25% (≈100mg/L Cl_2, i.e. 3.3-fold concentration) for complete inactivation. *M. avium* ssp. *avium,* a representative of the medically important *Mycobacterium* group which includes amongst others the veterinary siginificant agent of paratuberculosis (*M. avium* ssp. *paratuberculosis*) in ruminants, exhibited intermediate susceptibility with complete inactivation by 15 min exposure to 10% EOW (≈40 mg/L Cl_2). The highest germicidal activity of anode EOW was measured against the yeast representative *C. albicans*. Here a complete inactivation was obtained by a 5 min exposure to 5% EOW (≈20.0 mg/L Cl_2) and these results agree with a recent report on the relative susceptibility of *C. albicans* for antifungal activities of EO solutions (Balch et al., 2000). Overall, in a defined aqueous system containing 10% anode EOW (≈40.0 mg/L Cl_2), complete killing of vegetative forms of all test organisms was achieved within 15 to 30 min of exposition. In comparison, EOWs

exhibited only a slight sporicidal activity in that a 60 min exposure to 80% anode EOW (≈320 mg/L Cl_2) was required for complete inactivation of *B. subtilis* spores. This result appears to contradict findings of other studies which describe a complete inactivation of 10^6 to 10^9 *Bacillus* spores after 5 min exposition to EOW (Kiura etal., 2002; Vorobjeva et al., 2004). These authors, however, performed their spore disinfection experiments in aqueous solutions devoid of any protein. According to the DIN guidelines, the sporicidal activity of a disinfectant has to be examined in a 0.003% BSA solution. It is well established that nitrogenous compounds like proteins are chlorine-consuming, that means organic nitrogen (N) will react instantaneously with the free available chlorine residual (HOCl) to form nongermicidal N-chloro-compounds (White, 1999). Thus, proteins exert a significant chlorine demand thereby reducing the disinfection effectiveness of the total chlorine residual. This was particularly true in our analyses of the bacteriostatic and fungistatic efficacy of EOWs using a peptide-containing nutrient broth according to DVG guidelines. In these experiments no bactericstatic or fungistatic effectiveness was seen, and it is obvious that the organic N has depleted the disinfection activity. Consistently, the microbicidal activities of EOWs were also completely abolished in our study after addition of 20% BSA to the test solutions. Indeed, the rapid inactivation of the antimicrobial activity following contact with organic matter is the main disadvantage of the application of EOWs (Shimmura et al., 2000; Nakae and Inaba, 2000). Thus, to ensure the efficacy of disinfection procedures using EOW, a thorough precleaning to minimize the organic load present is essential.

Obviously, further studies are required to establish the applicability and safety of EOWs in livestock husbandry. Field trials have to be carried out to determine the effectiveness of EOWs in particular under adverse conditions such as the presence of heavy organic soiling and low temperatures. Moreover, since reactions of HOCl and OCl^- with organic substances are certain to form potentially cytotoxic and genotoxic chlorinated disinfection by-products (DBPs) like trihalomethanes or chlorohydroxyfuranones (Gibbons and Laha, 1999; Knasmüller et al., 1996), the safety of EOWs has to be ascertained prior to application e.g. for continuous disinfection of drinking water in food animal production facilities.

7 Figures and tables

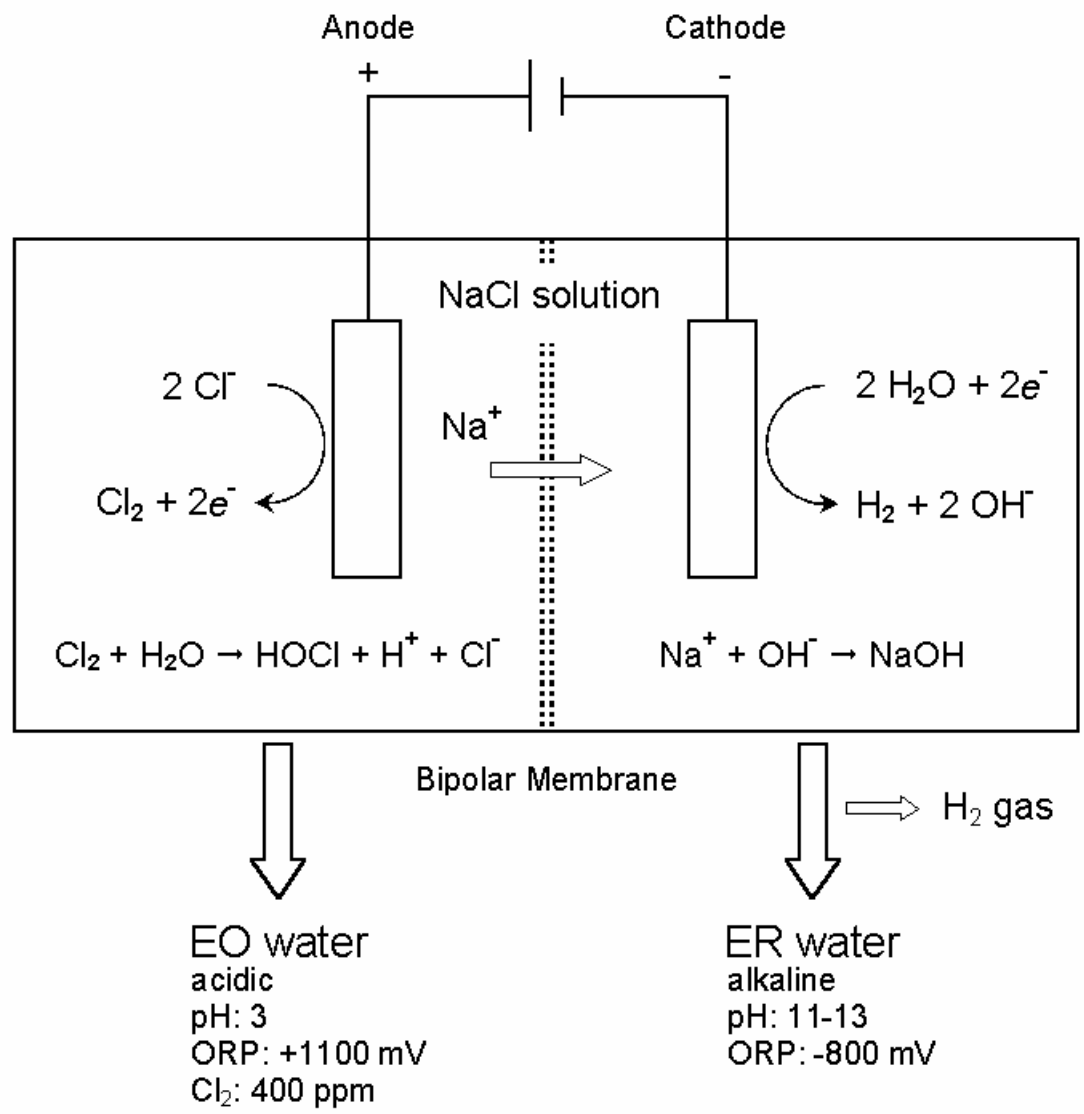

Figure 1. Basic principle of the electrolysis chamber. The anode (+) and cathode (-) are separated by a bipolar membrane which inhibits the migration of hydroxyl ions (OH^-) from the cathode to the anode. The overall chemical reaction during operation of electric current is: $NaCl + H_2O \rightarrow NaOH + ½\ Cl_2 + ½\ H_2$. The principle anode and cathode reactions are given in the figure. At the anode chlorine evolves which hydrolyzes rapidly into hypochlorous acid (HOCl) according to the equation given in the figure. At the cathode, the H^+ ions evolve from H_2O as hydrogen gas, leaving the hydroxyl ion (OH^-) behind the catholyte. The Na^+ ion is free to migrate from the analytic chamber into the catholytic chamber and to join the OH^- ion.

Table 1. Bactericidal and fungicidal efficacy of anode EOW and combined EOW

Test microorganism	Exposition time (min)	Concentration of anode EOW (vol%)						Concentration of combined EOW (vol%)						
		1	5	7.5	10	25	50	1	5	10	20	25	50	80
S. aureus	5	+	+	-	-	-	-	+	+	+	+	+	-	-
	15	+	+	-	-	-	-	+	+	+	+	+	-	-
	30	+	+	-	-	-	-	+	+	+	+	+	-	-
	60	+	+	-	-	-	-	+	+	+	+	+	-	-
P. mirabilis	5	+	+	-	-	-	-	+	+	+	+	+	+	-
	15	+	+	-	-	-	-	+	+	+	+	+	-	-
	30	+	-	-	-	-	-	+	+	+	+	-	-	-
	60	+	-	-	-	-	-	+	+	+	+	-	-	-
M. avium ssp. *avium*	5	+	+	+	+	-	-	+	+	+	+	+	+	+
	15	+	+	+	-	-	-	+	+	+	+	+	+	+
	30	+	+	-	-	-	-	+	+	+	+	+	-	-
	60	+	+	-	-	-	-	+	+	+	-	-	-	-
E. faecium	5	+	+	+	+	-	-	+	+	+	+	+	-	-
	15	+	+	+	-	-	-	+	+	+	+	+	-	-
	30	+	+	+	-	-	-	+	+	+	+	+	-	-
	60	+	+	-	-	-	-	+	+	+	+	+	-	-
P. aeruginosa	5	+	+	+	+	-	-	+	+	+	+	+	+	-
	15	+	+	+	+	-	-	+	+	+	+	+	-	-
	30	+	+	+	-	-	-	+	+	+	+	+	-	-
	60	+	+	-	-	-	-	+	+	+	+	+	-	-
C. albicans	5	+	-	-	-	-	-	+	+	+	+	+	-	-
	15	+	-	-	-	-	-	+	-	-	-	-	-	-
	30	+	-	-	-	-	-	+	-	-	-	-	-	-
	60	+	-	-	-	-	-	+	-	-	-	-	-	-

+, positive for in vitro growth; -, negative for in vitro growth

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