Report Anolyte Neutro

Caratteristiche

Definizione e origine

L'Anolyte è una soluzione disinfettante **ecologica** prodotta attraverso il processo di attivazione elettrochimica (ECA). Viene generata tramite l'elettrolisi di una soluzione salina diluita (acqua + cloruro di sodio) all'interno di una cella a membrana che separa gli ioni positivi da quelli negativi. Il risultato è un liquido con potenti proprietà biocide, ma chimicamente diverso dai disinfettanti tradizionali clorati.

Caratteristiche chimico-fisiche

•**Principio Attivo:** il componente principale è l'**Acido Ipocloroso** (HOCl).
A differenza dell'ipoclorito di sodio, l'acido ipocloroso è la stessa molecola prodotta dai globuli bianchi umani per combattere le infezioni.
•**pH Neutro:** L'Anolyte Neutro (ANK) ha un pH compreso generalmente tra **6.5 e 7.5**. Questo pH neutro garantisce che la maggior parte del cloro attivo rimanga sotto forma di HOCl (molecola neutra).
•**ORP (Potenziale di Ossido-Riduzione):** Possiede un elevato potenziale Redox, tipicamente tra **+750 mV e +950 mV**. Questo alto potenziale crea un ambiente in cui i microrganismi non possono sopravvivere.

Meccanismo d'azione

L'efficacia dell'Anolyte si basa su un duplice attacco ai patogeni:
1. azione osmotica e chimica: essendo una molecola elettricamente neutra e di dimensioni simili all'acqua, l'acido ipocloroso penetra facilmente le pareti cellulari dei batteri (che sono cariche negativamente e respingono invece lo ione ipoclorito). Una volta dentro, distrugge le proteine vitali e il DNA del microrganismo.
2. shock ossidativo (ORP): l'alto potenziale Redox sottrae elettroni alle strutture esterne dei microbi, causandone la rottura immediata della membrana cellulare (lisi) e la distruzione.
Nota: Questo meccanismo fisico-chimico impedisce ai batteri di sviluppare resistenza, un problema comune con gli antibiotici e alcuni disinfettanti chimici.

Spettro di efficacia: L'Anolyte è classificato come disinfettante ad alto livello, efficace contro:
•**Batteri:** Gram-positivi e Gram-negativi (inclusi *E. coli, Salmonella, Legionella, Listeria*).
•**Virus:** efficace contro virus incapsulati e nudi (inclusi Influenza, Norovirus, Coronavirus).
•**Funghi e Spore:** elimina muffe e lieviti; a concentrazioni adeguate è sporicida.
•**Biofilm:** è estremamente efficace nel penetrare e disgregare il biofilm batterico (azione che l'ipoclorito di sodio non riesce a fare efficacemente).

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Dossier Tecnico

L'Anolyte è una soluzione disinfettante elettrochimicamente attivata, ottenuta a partire da acqua e sale mediante un processo di elettrolisi controllata. Grazie alla presenza di acido ipocloroso (HOCl) e ad un elevato potenziale ossidoriduttivo, l'Anolyte offre un'elevata efficacia antimicrobica a basse concentrazioni, con un profilo di sicurezza superiore rispetto ai disinfettanti chimici tradizionali.

Negli ultimi decenni, la crescente attenzione verso la sicurezza igienico-sanitaria, la prevenzione delle infezioni e la sostenibilità ambientale ha determinato un'evoluzione significativa nel settore dei disinfettanti e dei sanificanti. Le organizzazioni pubbliche e private sono sempre più chiamate a garantire ambienti sicuri, riducendo al contempo l'impatto ambientale e i rischi per la salute degli operatori.

I disinfettanti chimici tradizionali, pur efficaci, presentano spesso limitazioni in termini di tossicità, corrosività, instabilità, produzione di sottoprodotti nocivi e gestione dei rifiuti. In questo contesto, l'Anolyte emerge come una tecnologia innovativa e sostenibile, in grado di coniugare elevata efficacia antimicrobica, sicurezza d'uso e ridotto impatto ambientale.

L'Anolyte è una soluzione elettrochimicamente attivata, che sfrutta le proprietà ossidanti dell'acido ipocloroso, una molecola naturalmente prodotta anche dal sistema immunitario umano. Questa caratteristica conferisce al prodotto un'elevata compatibilità biologica e una rapida azione antimicrobica, senza i rischi associati ad agenti chimici più aggressivi.

L'Anolyte è una soluzione acquosa ottenuta mediante elettrolisi di una soluzione contenente cloruro di sodio. Il processo genera una soluzione ossidante ricca di specie reattive del cloro, principalmente acido ipocloroso (HOCl), che rappresenta il principale agente attivo responsabile dell'attività antimicrobica. Con pH compreso tra 6 e 7,5 è un formulato stabile e particolarmente indicato per applicazioni a contatto diretto con alimenti, superfici sensibili e tessuti biologici.
Non è infiammabile, esplosivo, corrosivo e non lascia residui pericolosi dopo l'applicazione.

Il funzionamento dell'Anolyte si basa sui principi fondamentali dell'elettrochimica e della chimica del cloro in soluzione acquosa. Durante il processo di elettrolisi, una soluzione salina viene sottoposta a una corrente elettrica continua all'interno di una cella elettrolitica dotata di una membrana ionoselettiva. All'anodo, gli ioni cloruro vengono ossidati a cloro molecolare che, in soluzione acquosa, reagisce rapidamente con l'acqua per formare acido ipocloroso e ioni cloruro.
Allo stesso tempo, si generano ioni idrogeno, che contribuiscono alla diminuzione del pH della soluzione.
L'acido ipocloroso è una molecola neutra, di piccole dimensioni, altamente reattiva, in grado di attraversare facilmente le membrane cellulari dei microrganismi. Una volta all'interno della cellula, l'HOCl ossida componenti fondamentali quali proteine, lipidi, enzimi e acidi nucleici, compromettendo irreversibilmente le funzioni vitali del microrganismo.

Rispetto all'ipoclorito di sodio, che è la forma comunemente utilizzata nei disinfettanti tradizionali a base di cloro, l'acido ipocloroso presenta una maggiore efficacia antimicrobica a parità di concentrazione, una maggiore rapidità d'azione e una minore tendenza a generare sottoprodotti indesiderati.

Il potenziale ossidoriduttivo (ORP) elevato dell'Anolyte rappresenta un ulteriore indicatore della sua capacità di inattivare microrganismi attraverso processi di ossidazione rapida.

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Report Anolyte Neutro

Parametro / criterio	Risultati dell'Anolyte	Note / Commenti
Efficacia battericida su cellule vegetative	Riduzione della carica batterica sotto il limite di rilevazione entro ~10 s (allegato 1)	Efficace su *S. aureus, P. aeruginosa* etc.
Attività sporicida	Log > 5 su *B. atrophaeus* in 90 s; riduzione di *C. difficile* spore anche a basse concentrazioni (allegato 2)	Indica capacità sporicida rapida
Confronto con ipoclorito di sodio commerciale	Efficacia antibatterica fino a 4× maggiore rispetto al NaOCl commerciale in test su vari patogeni (allegato 3)	In particolare per anolyte a pH neutro
Efficacia su biofilm	Efficace nella rimozione/disgregazione di biofilm microbiologici con esposizione prolungata (allegato 4)	Dipende da concentrazione e diluizione
Riduzione di patogeni Alimentari	Riduzione significativa di *Salmonella* e *L. monocytogenes* su superfici alimentari e carni (allegato 5)	Efficace, ma risultati dipendono da condizioni sperimentali
Log-reduction globale dichiarata (prodotti commerciali)	Log 5 (99.999 %) su batteri, virus, muffe e lieviti (allegato 6, 7, 8)	Valore riportato dal produttore del prodotto specifico
Efficacia virucida (surrogati)	Efficacia contro virus surrogati in studi specifici (p.es. anolyte neutro in applicazioni di superficie) (allegato 9)	Utilizzato come riferimento per virus non coltivabili
Sicurezza/eco-compatibilità	Soluzione non tossica alle concentrazioni d'uso; tende a reintegrare acqua/sale senza residui nocivi (allegato 10)	Considerata ecologica e biodegradabile

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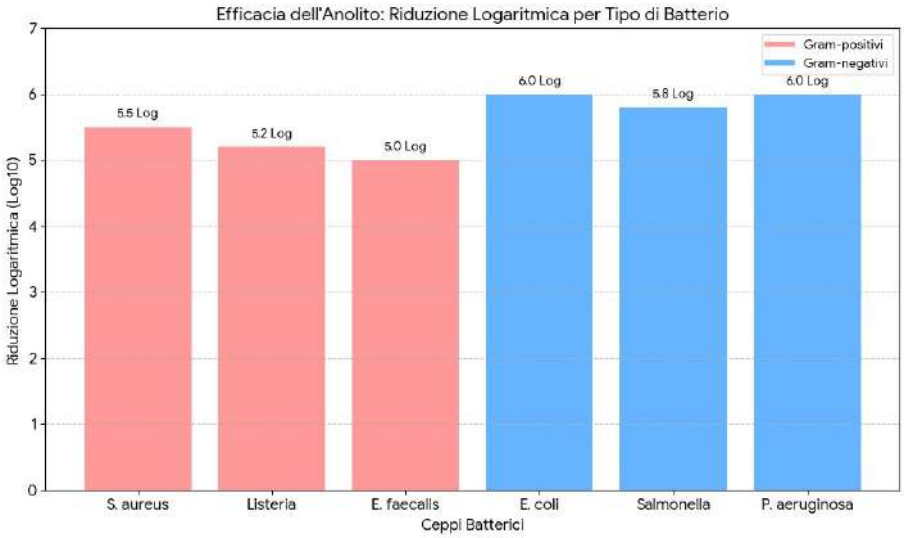
Efficacia dell'Anolyte (in particolare l'Anolyte Neutro o ANK) contro batteri Gram-positivi e Gram-negativi.

A differenza di molti antibiotici o disinfettanti chimici specifici, l'Anolyte agisce con un meccanismo **fisico-chimico aspecifico**, che non distingue tra le due strutture batteriche, portando alla distruzione di entrambe.
Efficacia: per capire l'efficacia trasversale, bisogna guardare alla chimica della molecola principale dell'Anolyte: l'**Acido Ipocloroso (HOCl)**.
La barriera della carica elettrica: le pareti cellulari dei batteri (sia Gram+ che Gram-) hanno generalmente una carica superficiale **negativa**. **Il vantaggio dell'Anolyte:** l'Acido Ipocloroso è **elettricamente neutro**. Non subisce repulsione e passa facilmente attraverso le barriere cellulari.
-Efficacia sui Batteri Gram-Negativi: i batteri Gram-negativi sono spesso considerati più difficili da eliminare a causa della loro **membrana esterna protettiva** ricca di lipopolisaccaridi. Hanno una parete cellulare sottile di peptidoglicano (un polimero essenziale che costituisce la parete cellulare dei batteri, conferendo rigidità, forma e protezione contro la pressione osmotica), ma protetta da una membrana esterna fosfolipidica (un doppio strato fluido e selettivamente permeabile che separa l'interno della cellula dall'ambiente extracellulare). **Azione dell'Anolyte:** l'alto potenziale Redox (ORP > +750mV) dell'Anolyte destabilizza e ossida immediatamente la membrana esterna lipidica. L'HOCl penetra rapidamente nello spazio periplasmatico (il compartimento cellulare situato tra la membrana plasmatica interna e la membrana esterna nei batteri Gram-negativi) e nel citoplasma. L'ossidazione interferisce con il metabolismo energetico della cellula, portando a distruzione rapida.
Esempi di efficacia (Gram-):
Legionella pneumophila: estremamente sensibile all'Anolyte; viene eliminata anche quando nascosta nel biofilm;
Pseudomonas aeruginosa: molto resistente agli antibiotici, viene distrutto per shock ossidativo dall'Anolyte;
Escherichia coli & Salmonella: patogeni alimentari classici, vengono abbattuti (riduzione di 5-6 Log) in pochi secondi di contatto.
-Efficacia sui Batteri Gram-Positivi: i batteri Gram-positivi non hanno la membrana esterna, ma possiedono una parete cellulare molto spessa e robusta che protegge la cellula dalla pressione osmotica. **Struttura:** spesso strato di peptidoglicano (fino al 90% della parete) che agisce come un'armatura. **Azione dell'Anolyte:** la molecola neutra di HOCl è abbastanza piccola da diffondersi attraverso la rete porosa del peptidoglicano senza ostacoli. Una volta all'interno, attacca le proteine di membrana e gli enzimi vitali. L'Anolyte causa un collasso della pressione osmotica interna, facendo "implodere" o lisare il batterio.
Esempi di efficacia (Gram+):
Listeria monocytogenes: pericoloso per l'industria alimentare perché sopravvive al freddo. L'Anolyte è efficace anche a basse temperature contro la Listeria.
Staphylococcus aureus (incluso MRSA): l'Anolyte è efficace anche contro i ceppi resistenti alla meticillina, poiché il batterio non può sviluppare resistenza all'ossidazione fisica.
Bacillus (forme vegetative): vengono eliminati rapidamente (le spore richiedono tempi di contatto maggiori o concentrazioni più alte).



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Tipo Batterico	Principale Difesa	Come l'Anolyte la supera	Tempi di contatto tipici*
Gram-Negativi (es. *E. Coli, Salmonella*)	Membrana esterna lipidica	L'ORP ossida i lipidi	10 - 30 secondi
Gram-Positivi (es. *Staphylococcus, Listeria*)	Spessa parete di peptidoglicano	L'HOCl è piccolo e penetra la rete porosa senza repulsione	10 - 60 secondi
Biofilm (Misto Gram+/Gram-)	Matrice polisaccaridica (Slime)	L'Anolyte disgrega la matrice permettendo l'uccisione dei batteri sottostanti	Minuti (azione progressiva)

*I tempi dipendono dalla concentrazione (ppm) e dalla carica organica presente

Patogeno	Tipo	Concentrazione (ppm)	Tempo di Contatto	Note
Legionella pneumophila	Gram-	2 - 5 ppm	1 - 5 minuti	Molto sensibile. In caso di biofilm pesante, si consigliano shock a 20-50 ppm.
Escherichia coli	Gram-	10 - 20 ppm	< 30 secondi	Abbattimento quasi istantaneo in assenza di residui organici massicci.
Salmonella enteritidis	Gram-	20 - 30 ppm	< 60 secondi	Standard per il lavaggio di attrezzature alimentari.
Pseudomonas aeruginosa	Gram-	30 - 50 ppm	2 - 5 minuti	Richiede tempi leggermente superiori per la sua tendenza a formare muco protettivo.
Listeria monocytogenes	Gram+	50 - 100 ppm	1 - 2 minuti	Particolarmente resistente al freddo; l'Anolyte è ideale per celle frigorifere.
Staphylococcus aureus	Gram+	50 ppm	< 60 secondi	Efficace anche contro ceppi resistenti agli antibiotici (MRSA).
Bacillus subtilis (Spore)	Spore	200 - 500 ppm	5 - 10 minuti	Le spore richiedono l'Anolyte puro (non diluito) e tempi di esposizione prolungati.

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Efficacia Anolyte contro virus.

L'azione virucida dell'Anolyte è un processo biochimico estremamente rapido e letale per i virus.
A differenza dell'ipoclorito di sodio, che viene respinta dalle membrane, l'Anolyte è neutro e agisce indisturbato.
I virus sono protetti da un guscio proteico (capside) o da un involucro lipidico (per i virus incapsulati come il SARS-CoV-2). L'ipoclorito di sodio ha carica negativa e viene respinto dalle pareti cellulari e dai virus, anch'essi carichi negativamente. L'Anolyte ha carica elettrica neutra. Questo gli permette di scivolare attraverso le membrane virali senza resistenza. Una volta a contatto con il virus, l'acido ipocloroso scatena una forte reazione di ossidazione. L'Anolyte aggredisce le proteine di superficie modificandone la forma. Se la proteina è deformata, il virus non può più "agganciarsi" alle cellule umane. L'ossidazione rompe i legami chimici che tengono insieme il rivestimento del virus, disgregandolo. L'Anolyte penetra nel nucleo del virus e attacca gli acidi nucleici. L'ossidazione rompe le catene del materiale genetico.

Tipo di Virus	Esempi	Efficacia Anolyte
Virus Incapsulati	SARS-CoV-2 (Covid-19), Influenza, HIV, Epatite B	**Altissima:** Vengono inattivati in pochi secondi (spesso < 30s).
Virus Non Incapsulati	Norovirus, Poliovirus, Adenovirus	**Alta:** Richiedono tempi di contatto leggermente superiori o concentrazioni maggiori (ppm).

Virus	Famiglia	Concentrazione (ppm)	Tempo di Contatto
SARS-CoV-2	Coronavirus	50 ppm	30 secondi
Influenza A	Orthomyxoviridae	20-50 ppm	30 secondi
Norovirus	Caliciviridae	200 ppm	2 minuti
Hepatitis B/C	Hepadnaviridae	100 ppm	1 minuto
Poliovirus	Picornaviridae	200 ppm	5 minuti

Efficacia Anolyte contro Funghi (Muffe e Lieviti)

Muffe (es. *Aspergillus*, *Penicillium*): l'Anolyte non solo uccide il fungo attivo, ma ossida le micotossine prodotte da essi. È eccellente per il trattamento di pareti e ambienti umidi.
Lieviti (es. *Candida albicans*, *Saccharomyces*): sono molto sensibili all'azione dell'acido ipocloroso. L'Anolyte disgrega la membrana citoplasmatica in pochi minuti.

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Efficacia Anolyte contro spore batteriche.

In test in vitro con sospensioni di Bacillus atrophaeus e Clostridium difficile (bacilli sporigeni) trattate con soluzioni elettrolitiche attivate (simili ad Anolyte), si osserva che: a concentrazioni elevate (ad es. 99 %), c'è una riduzione logaritmica > 5 nelle spore di B. atrophaeus entro ~90 s. Le spore di C. difficile sono ridotte sotto il limite di rilevabilità entro ~20 s con concentrazioni ≥ 5 % della soluzione.
→ Questo indica che soluzioni di Anolyte possono essere altamente efficaci contro spore batteriche in condizioni di laboratorio controllate.
Penetrazione della corteccia: l'HOCl, essendo una molecola piccola e neutra, riesce a filtrare attraverso i diversi strati proteici che formano il "guscio" della spora. **Ossidazione del nucleo:** una volta all'interno, l'alto potenziale Redox (ORP) attacca il DNA e le proteine essenziali, rendendo la spora incapace di tornare alla forma vegetativa. **Punto critico:** per un'azione **sporicida**, l'Anolyte deve essere usato preferibilmente **puro** (500 ppm) e il pH deve essere mantenuto rigorosamente tra 5.5 e 6.5 per massimizzare la presenza di acido ipocloroso libero.

Microrganismo	**Esempio**	**Concentrazione (ppm)**	**Tempo di Contatto**
Lieviti	Candida albicans	50 - 100 ppm	1 - 2 minuti
Muffe comuni	Aspergillus niger	200 - 300 ppm	5 minuti
Funghi della pelle	Trichophyton	100 - 200 ppm	2 - 3 minuti
Spore Batteriche	Bacillus subtilis	500 ppm (Puro)	10 - 15 minuti

Efficacia contro Candida albicans

L'Anolyte neutro (acido ipocloroso, HOCl) è estremamente efficace contro la *Candida albicans* grazie alla sua capacità di penetrare le pareti cellulari e ossidare i componenti vitali del fungo.

Concentrazione (ppm)	**Tempo di contatto**	**Riduzione Logaritmica**	**Efficacia (%)**
50 ppm	30 secondi	> 5.0	99,999%
100 ppm	15 secondi	> 5.0	99,999%
200 ppm	< 10 secondi	> 6.0	99,9999%

Punti chiave dell'azione fungicida
Meccanismo: l'acido ipocloroso distrugge la membrana cellulare e denatura le proteine enzimatiche della *Candida*.
Biofilm: l'Anolyte è particolarmente efficace nel penetrare il biofilm prodotto dalla *Candida*, che spesso rende i normali antifungini inefficaci.
pH Dipendenza: l'efficacia massima si ottiene con un Anolyte a pH neutro (6.5 - 7.5), dove la specie chimica predominante è l'HOCl, molto più potente dell'ipoclorito di sodio.

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Efficacia contro Aspergillus niger

Meccanismo d'Azione: l'Anolyte agisce attraverso un duplice attacco fisico-chimico:
•Stress Ossidativo: l'alto potenziale di ossido-riduzione strappa elettroni dalle membrane cellulari, causandone l'instabilità.
•Penetrazione: l'acido ipocloroso, essendo una molecola neutra, attraversa la membrana cellulare del fungo molto più velocemente dello ione ipoclorito distruggendo gli enzimi vitali e il DNA/RNA.

Tempo di contatto	Riduzione Logaritmica	Percentuale di abbattimento
1 minuto	~1.5 Log	96.8%
5 minuti	~3.0 Log	99.9%
10 minuti	> 4.5 Log	99.99%
15 minuti	> 5.0 Log	99.999%

Efficacia contro Trichophyton

L'Anolyte è estremamente efficace contro i funghi del genere *Trichophyton*. Rispetto all'*Aspergillus niger*, il *Trichophyton* tende a essere leggermente più sensibile all'azione ossidativa dell'acido ipocloroso, poiché le sue strutture fungine sono meno resistenti alle aggressioni chimiche.

Tempo di contatto	Riduzione Log (Log10)	Percentuale di Inattivazione	Stato del Patogeno
15 Secondi	1.5 Log	96.8%	Danni iniziali alla membrana
30 Secondi	3.2 Log	99.93%	Inattivazione della maggior parte delle ife
1 Minuto	4.7 Log	99.998%	Soglia di disinfezione raggiunta
2 Minuti	**> 5.0 Log**	**99.999%**	**Sterilizzazione completa**

Efficacia di anolyte contro Bacillus subtilis

L'Anolyte Neutro è altamente efficace contro il *Bacillus subtilis*, sia nella sua forma vegetativa che nella forma di spora.

Stato del Batterio	Concentrazione Cloro Attivo (FAC)	Tempo di Contatto	Riduzione Logaritmica	Efficacia (%)
Forma Vegetativa (Battero attivo)	20 - 50 ppm	30 - 60 secondi	> 6.0	99,9999%
Spore (Bassa conc.)	50 - 100 ppm	5 - 10 minuti	4.0 - 5.0	99,99% - 99,999%
Spore (Alta conc.)	150 - 200 ppm	2 - 3 minuti	> 6.0	99,9999%
Biofilm (Matrice protettiva)	200+ ppm	10 - 20 minuti	Variabile	Elevata

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allegato 1

Evaluation of the efficacy of electrochemically activated solutions against nosocomial pathogens and bacterial endospores

G M Robinson [1], S W-H Lee, J Greenman, V C Salisbury, D M Reynolds

Abstract

Aims: Electrochemically activated solutions (ECAS) are generated from halide salt solutions via specially designed electrolytic cells. The active solutions are known to possess high biocidal activity against a wide range of target microbial species, however, literature revealing the kill-kinetics of these solutions is limited. The aim of the study was to identify the kill-rate and extent of population kill for a range of target species (including endospores) using ECAS generated at the anode (anolyte).

Methods and results: Standard suspensions of methicillin-resistant Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus atrophaeus spores and Clostridium difficile spores were treated with anolyte in a quantitative suspension assay. For vegetative cells, all concentrations of anolyte tested reduced the viable population to below the detection limit within 10 s. At a concentration of 99%, anolyte produced a log(10) reduction factor of greater than five in viable B. atrophaeus endospores within 90 s and reduced numbers of C. difficile endospores to below the experimental detection limit within 20 s at concentrations of 5% or greater.

Conclusions: Anolyte was highly effective in killing test-bacteria and spores. The bactericidal efficacy was retained against vegetative cells at dilutions as low as 1% and against C. difficile spores as low as 5%.

Significance and impact of study: The results of this study demonstrate that ECAS are effective at lower concentrations and act more rapidly than previously reported. Potent bactericidal and sporicidal activity coupled with point-of-use generation, low production-costs and environmental compatibility suggest that acidic ECAS has the potential to be a useful addition to the current armoury of disinfectants.

allegato 2

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allegato 3

Br J Biomed Sci. 2010;67(3):105-8. doi: 10.1080/09674845.2010.11730305.

Bactericidal efficacy of electrochemically activated solutions and of commercially available hypochlorite

A J Helme [1], M N Ismail, F J Scarano, C L Yang

Abstract

Electrochemical activation (ECA) has been developed as a quick and efficient method of hypochlorite production, and many claim increased efficacy when compared to conventional disinfectant solutions. Numerous potential applications, including hospital disinfection, waste-water treatment, routine drinking water disinfection and biological decontamination have been suggested. In this study, three solutions were produced by electrochemical activation of 0.5% NaCl and compared to commercially available NaOCl. The NaOCl concentration and pH of each solution was measured, and the minimum bactericidal concentration of each was determined using seven common microbial pathogens. All solutions were effective, the most significant of which was the ECA anolyte solution. This is notable due to its neutral pH and antimicrobial efficacy that is four times that of commercially available NaOCl. This process may lead to production of a highly effective yet non-caustic disinfectant that would have countless scientific, medical, military and public health applications.

ECA Water come controllo non inquinante del biofilm

Introduzione: ECA Water come controllo non inquinante del biofilm

La colonizzazione batterica delle superfici in ambienti acquosi è una strategia fondamentale per la sopravvivenza in natura, poiché i nutrienti sono maggiormente disponibili all'interfaccia solido-liquido. Gli aggregati risultanti formano microcolonie, che si sviluppano in biofilm. I biofilm batterici hanno conseguenze negative nell'industria alimentare. Attualmente vengono adottati diversi approcci di mitigazione per prevenire e/o rimuovere i biofilm: (i) i batteri vengono uccisi chimicamente mediante l'applicazione di composti battericidi, (ii) i biofilm vengono dispersi mediante disperdenti, (iii) i biofilm vengono rimossi fisicamente mediante una varietà di processi e (iv) la struttura del biofilm viene indebolita da enzimi o chelanti.

Sono disponibili diverse sostanze battericide, tutte dichiarate in grado di uccidere quantitativamente i batteri in sistemi acquosi. Tuttavia, batteri diversi reagiscono in modo diverso ai battericidi, sia a causa delle diverse proprietà della parete cellulare, sia ad altri meccanismi di resistenza, intrinseci o inducibili. Recentemente è stato introdotto un nuovo metodo alternativo per il controllo del biofilm, basato sull'attivazione elettrochimica dell'acqua. Verranno discusse le proprietà biocide dell'anolita e la sua capacità di rimuovere i biofilm.

Proprietà biocide dell'anolyte

Durante l'attivazione elettrochimica (ECA) dell'acqua, una soluzione salina diluita viene "attivata" passando attraverso una cella elettrolitica cilindrica in cui le camere anodica e catodica sono separate da una membrana permeabile. Vengono prodotti due flussi separati di acqua attivata: Anolyte con un intervallo di pH compreso tra 2 e 9 e un potenziale di ossidoriduzione (ORP) compreso tra +400 mV e +1200 mV. L'anolyte è un agente ossidante dovuto a una miscela di radicali liberi e ha un effetto antimicrobico.

A seguito del trattamento elettrochimico anodico, la tensione superficiale diminuisce leggermente, la conduttività elettrica aumenta, così come il contenuto di cloro e ossigeno disciolti, la concentrazione di idrogeno e azoto diminuisce e la struttura dell'acqua cambia. La membrana cellulare batterica fornisce la barriera osmotica per la cellula e catalizza il trasporto attivo di sostanze al suo interno. Le alterazioni del potenziale transmembrana causate dall'azione di fattori donatori o accettori di elettroni sono associate a potenti processi elettro-osmotici accompagnati dalla diffusione dell'acqua contro i gradienti di ORP, con conseguente rottura delle membrane e fuoriuscita del contenuto della cellula batterica. La membrana batterica stessa ha una carica elettrica. Gli anioni presenti nell'anolyte agiscono su questa membrana. L'anolyte può anche interrompere altre funzioni della cellula.

A differenza degli organismi "superiori", gli organismi unicellulari come i batteri ottengono le loro fonti di energia dall'ambiente immediatamente esterno alla cellula. Le piccole molecole vengono trasportate attraverso la membrana cellulare tramite un gradiente elettrochimico. Pertanto, qualsiasi variazione significativa dell'ORP dell'ambiente circostante ha conseguenze drastiche per la cellula. Anche se non si verifica la morte istantanea della cellula, tutte le funzioni enzimatiche nella membrana vengono influenzate e ciò comporterà anche la perdita di vitalità cellulare. L'attività biocida dell'acido ipocloroso generato dall'attuale tecnologia ECA è 300 volte più attiva dell'ipoclorito di sodio generato dai sistemi precedenti.

È stato dimostrato in modo conclusivo che le soluzioni attivate superano gli equivalenti derivati chimicamente sia in termini di efficacia a basso dosaggio che di purezza fisico-chimica. Questa maggiore capacità biocida rispetto alle soluzioni chimiche tradizionali consente l'uso di soluzioni ECA a dosaggi inferiori, eliminando così il rischio di intossicazione e di impatto ambientale negativo. Anolyte ha prodotto un'uccisione del 100% di tutti gli isolati in esame a una concentrazione del 100% e del 10% (Tabella 1). A una diluizione 1:20, sono state ottenute percentuali di uccisione variabili, comprese tra il 100% e il 31% (Tabella 1). Ciò indica una suscettibilità variabile dei diversi batteri all'anolyte. Questo non è un fenomeno raro11. Molti organismi sono intrinsecamente più

tolleranti alle sostanze antimicrobiche rispetto ad altri. Anolyte è risultato più efficace contro i ceppi batterici Gram-positivi a una diluizione 1:20, ottenendo un'uccisione del 100% contro tutti i ceppi Gram-positivi, ad eccezione di S. faecalis. Anche un calcoacetico (Gram-negativo) è stato ucciso con una diluizione 1:20 (Tabella 1).

Table 1: Percentage kill of bacterial strains at different anolyte (produced using 3 % NaCl) concentrations

		Anolyte concentration		
Bacterial strain	**Gram stain**	**100%**	**1:10**	**1:20**
Bacillus subtilis	+	100	100	78
Pseudomonas aeruginosa	-	100	100	87
Acinetobacter calcoaceticus	-	100	100	100
Lactobacillus brevis	+	100	100	100
Micrococcus luteus	+	100	100	100
Streptococcus feacalis	+	100	100	31
Pseudomonas fluorescens	-	100	100	66
Staphylococcus aureus	+	100	100	100
Pseudomonas alcaligenes	-	100	100	52
Pseudomonas medocina	-	100	100	88
Pseudomonas putida	-	100	100	90
Bacillus cereus	+	100	100	92
Micrococcus roseus	+	100	100	100
Pseudomonas stutzeri	–	100	100	57
Pseudomonas syringae	–	100	100	87

Studio sulla rimozione del biofilm

Un biofilm maturo si è formato dopo 2 settimane (Fig. 1a). L'esposizione del biofilm a una diluizione 1:00 di anolyte non ha prodotto alcuna rimozione evidente del biofilm (Fig. 1b). Una diluizione 1:10 e una soluzione pura di anolyte hanno portato alla dispersione e alla rimozione del biofilm dopo un'esposizione di 20 minuti (Fig. 1c e 1d).

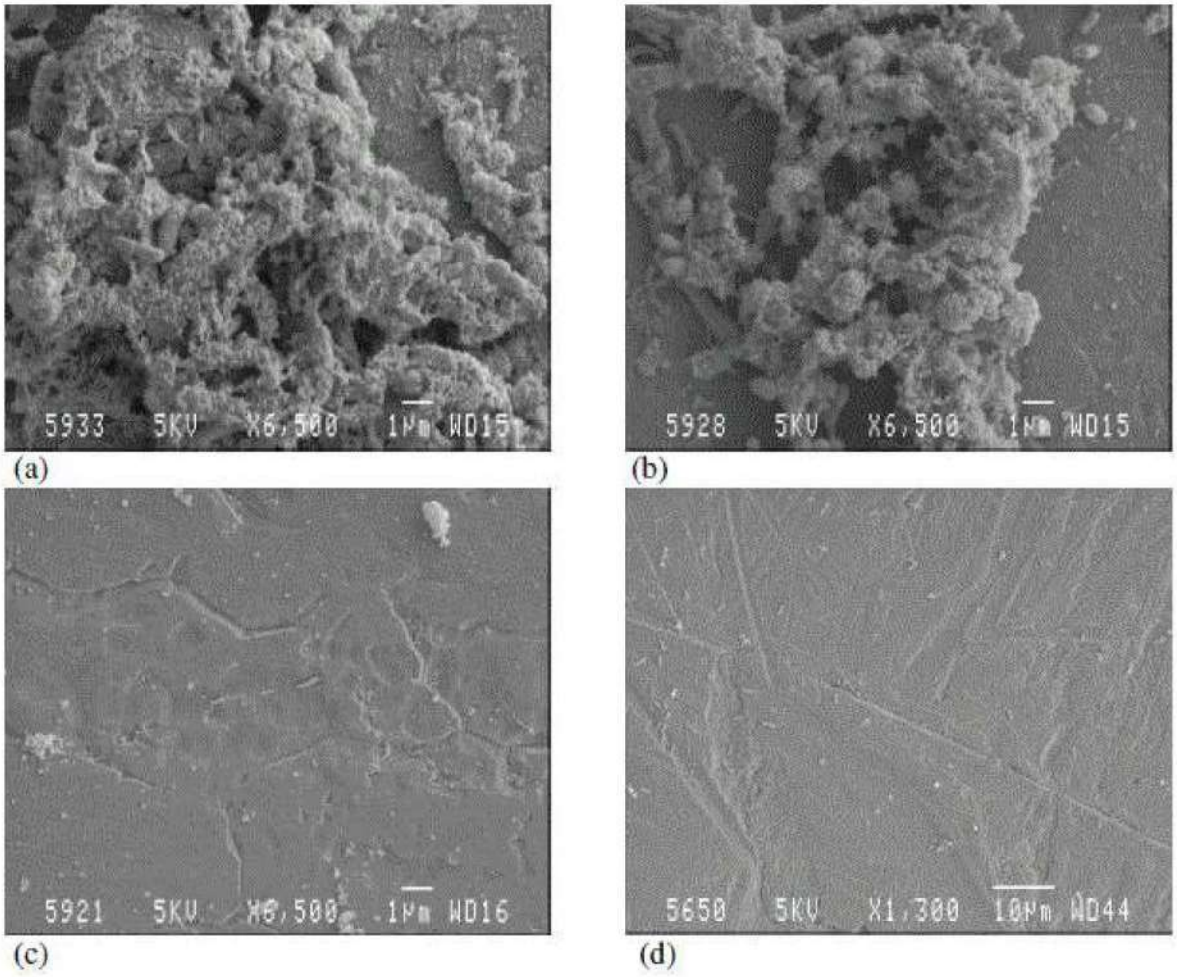

Figure 1. Biofilm control (a) and after treatment with 1:100 anolyte (b), 1:10 anolyte (c) and 100% anolyte (d).

Conclusioni

I vantaggi dell'utilizzo dell'ECA consistono nella riduzione della tossicità ambientale, del rischio di incidenti e dell'inquinamento chimico. Poiché il prodotto viene prodotto in loco, non vi è nessuno dei rischi normalmente associati al trasporto e allo stoccaggio di sostanze chimiche pericolose. L'attività antimicrobica dell'attuale tecnologia ECA è stata confermata da numerosi studi. L'acqua attivata elettrochimicamente (ECA) è efficace contro i biofilm e non genera sottoprodotti.

(https://www.radicalwaters.com/microbiological-fingerprinting-of-anolyte/)

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Effect of Anolyte on *S.* Typhimurium and *L. monocytogenes* Growth in Minced Pork and Beef Cuts

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Abstract: In this paper, anolyte is considered as a possible disinfectant for inhibiting the growth of bacteria in meat (beef cuts and minced pork). Meat cuts were contaminated with two concentrations of *L. monocytogenes* and *S.* Typhimurium, as these are the most common meat pathogens that are closely regulated by the EU, and treated with two different concentrations of anolyte: 20% for beef cuts and 18% for minced pork. Then, the total viable count (TVC), *L. monocytogenes* count and *S.* Typhimurium count were determined. In meat cuts and minced pork, anolyte was able to reduce TVC, *S.* Typhimurium and *L. monocytogenes* counts effectively, significantly decreasing *L. monocytogenes* and *S.* Typhimurium counts after spraying and throughout 29 days of incubation at 0–4 °C. TVC was reduced after spraying and for 10 days of incubation but later increased to be the same as before spraying with anolyte. Anolyte was effective when spraying beef cuts with a 20% solution for 60 s against pathogenic bacteria *L. monocytogenes* and *Salmonella* spp. and also when using it at a concentration of 18% from the minced meat mass. Initially, anolyte significantly decreased TVC, however during the storage period (10–29 days) TVC increased but remained significantly lower compared to control. Anolyte was effective in reducing *L. monocytogenes* and *S.* Typhimurium counts throughout the study, and after 29 days of incubation, these bacteria could not be detected in the samples treated with anolyte.

Keywords: anolyte; *Listeria monocytogenes*; *Salmonella* Typhimurium; meat cuts; minced pork

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1. Introduction

Bacteria and yeasts play an important role in food spoilage. Thus, over the recent years, a lot of attention has been given to the control and prevention of their growth in food products to prolong their shelf life. The most commonly used means for preservation in the food industry are chlorinated water, organic acids, hydrogen peroxide, ozone and ultraviolet light. The problem with some of these materials is that they produce by-products that are toxic, so there is an emerging goal of finding a non-toxic, non-destructive method of disinfection [1,2].

In recent years, increased attention has been drawn to electrolyzed oxidizing water and its antimicrobial effects. Electrolyzed water (also called anolyte, acid water and non-living water) is gaining popularity as a sanitizer in the food industry of many countries. Anolyte is a colorless clear liquid with an acid scent. The anolyte dipping treatment was found to be as effective as chlorinated solutions in controlling the growth of aerobic bacteria, molds, yeasts and coliform bacteria during storage. Since the anolyte has the non-destructive properties of other organic materials, it is not harmful and can be used in food sanitation. Anolyte is effective and harmless, not only for the disinfection of food contact surfaces but also for the treatment (washing and spraying) of the products (vegetables, fruits, seafood and meat products) [3–6].

The production of anolyte has been outlined by Ignatov et al. [7]. Anolyte is produced through the process of electrolysis, where the cathode and the anode sides of the cell are

separated by a diaphragm. This separates electrochemically activated water into alkaline, also known as catholyte, and acidic anolyte fractions. Catholyte is a powerful reducer, with ORP values less than −820 mV and a pH > 9, whereas anolyte is an oxidizer with ORP values over +800 mV and a pH < 7.3. ECA also possesses active components, such as Cl_2, HCl, H+, OH− and others, obtained through the process of electrolysis.

The bactericidal action of a neutral anolyte is based on the oxidation of the substances of a bacterial cell, especially lipoprotein membranes, which are the only place of biosynthesis [8].

Although the mechanism for cell destruction is not yet fully understood, it is hypothesized that high ORP values over +810 mV create an unfavorable environment for bacteria to thrive in, causing links to break in cell structures. The change in membrane permeability allows the diffusion of oxidants to enter the cytoplasm, leading to the oxidation of proteins, which further leads to dysfunction and ultimately to the death of bacteria. The bactericidal effect, although greatly reduced, carries over to spores as well [9,10]. These findings were in line with research conducted by Kiura et al. [4] who found that electrolyzed strong acid water (ESW) created breaks and blebs in the cell membranes of *Pseudomonas aeruginosa*. These breaks, as well as the destruction of DNA, increased with growing concentrations of free chlorine concentrations. The conclusion was made that ESW is inclined to destroy the cell walls of Gram-negative bacteria. This was further confirmed by Rajkowski and Sommers [11], who noticed that Gram-negative bacteria seem to have a more sensitive reaction to anolyte, as shown by their research.

It has been found that undiluted anolyte effectively destroys *P. aeruginosa*, *Escherichia coli* and *Bacillus subtilis* immediately upon exposure. Diluted anolyte (10^{-1} dilution) produced similar results. However, *B. subtilis* was killed off after 6 h of exposure. Protein band analysis suggested cell protein destruction as the mechanism of action for killing bacteria [12]. The 5% anolyte concentration solution is a good disinfectant for mesophilic bacteria, yeasts and molds. Furthermore, it reduces the growth of bacteria after treatment [3]. Although anolyte was effective in reducing *Salmonella* cultures, there has been no significant difference in recovered populations of *L. monocytogenes* before and after treatment with anolyte. Although anolyte is shown to be harmful to bacterial cells, it does not seem to cause any health issues or cell destruction in humans [7]. Moreover, anolyte is environmentally safe, as it eventually returns to its original saltwater state [10]. Anolyte does not affect taste, smell or the properties of organic matter. Therefore, it is a perfect sanitizer for usage in the food industry. A study using the anolyte dipping method for the disinfection of dates [3] found that anolyte did not affect sensory descriptors or the biochemical contents of the fruit. Anolyte treatment also preserves the qualities of trout [13] as well as fruit [14]. Thorn et al. [10] also revealed a virucidal effect, as backed by other research papers, though those studies are argued to be insufficient for confirming anolyte use for viral infections [7].

Because *Salmonella* and *L. monocytogenes* are two of the main pathogens in food spoilage and anolyte could potentially be used for reducing bacteria counts, thus extending the shelf-life of meat, the aim of this study is to evaluate the effect of anolyte on the criteria of food safety for *Salmonella* and *L. monocytogenes* in semi-finished meat products, as these two bacteria have been identified as the main contaminants of meat and their levels in food products are tightly regulated in the EU [15].

2. Materials and Methods

The study was carried out at the microbiology research laboratory of the Food Institute of Kaunas University of Technology.

2.1. Bacterial Cultures

Two reference cultures of pathogenic bacteria, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *Listeria monocytogenes* ATCC 13932, were obtained from the American Type Culture Collection and used for contamination of model meat samples. Bacterial cultures were stored at the microbiology laboratory of the Food Institute of Kaunas

University of Technology at minus 72–74 °C in a VIABANK system. Revitalization was performed in brain heart infusion broth (Liofilchem, Roseto, Italy).

2.2. Preparation of Bacterial Cultures for Contamination

S. enterica subsp. *enterica* serovar Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 13932 cultures for the contamination of meat were grown on agar slants (Triptone Soya Agar. Liofilchem, Roseto, Italy) for 18 h at 37 °C. The target cultures were washed with sterile physiological solution and the density of cell suspension was adjusted according to the McFarland standard Nr. 0.5 (1.5×10^8 cells/mL). Cell suspensions 1.5×10^3 CFU/mL, 1.5×10^4 CFU/mL and 1.5×10^5 CFU/mL for meat contamination were prepared by diluting with sterile physiological solution.

2.3. Preparation of Anolyte

Anolyte solutions were prepared by diluting anolyte with water that satisfied the requirements of drinking water. For minced pork, undiluted anolyte with the parameters of pH 6.56 and 182 ppm was used.

2.4. Beef Cut Contamination and Anolyte Treatment

Good quality fresh beef was received from a meat processing plant (Lithuania).

Beef pieces were cut across the muscle into cuts of approximately 300 g by weight and 18–20 cm × 10–12 cm × 3 cm in size. The edge samples were not used for experiments. Untreated samples were put in a vacuumed package for 10 and 29 days (Control 1).

For control 2 samples, ten milliliters of a bacteria suspension (*L. monocytogenes* ATCC 13932 or *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028) were smeared onto the surface of the meat cut for its contamination. After 30–40 min, samples were taken and then the cuts were incubated in a vacuumed package for 29 days.

The unvacuumed beef cuts were hung up and sprayed continuously for 60 s with anolyte using a 0.5 L spray bottle, creating very fine drops of anolyte solution. After spraying with anolyte, the beef cuts were dried for 40 min at 5 ± 3 °C. Then, after taking samples, they were vacuum sealed for testing after 10 and 29 days of incubation at 0–4 °C.

2.5. Minced Pork Contamination and Anolyte Treatment

Pork mince was received from a meat processing plant in Lithuania. After sampling controls, 200 g portions of minced meat were contaminated with *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 or *L. monocytogenes* ATCC 7644 suspension (10 mL of suspension of different concentrations), mixed with a Bosch MUM5 1000 W food mixer (BSH Home Appliances AB Vilnius, Vilnius, Lithuania). After taking samples, approximately 18% (36 mL) of the undiluted anolyte was added to the minced meat and mixed again. In 1 h, samples were taken again, and the minced meat samples were vacuum sealed for testing after 10 and 29 days of incubation at 0–4 °C.

2.6. Microbiological Analysis

The following microbiological analyses were performed:

- Total viable count (TVC) on plate count agar (Liofilchem, Italy);
- Number of *Salmonella* spp. on XLD agar, with colony confirmation using biochemical tests (reaction on triple sugar/iron agar, urea agar, L-lizine decarboxylation medium) and serological reaction if the colonies were suspicious or typical;
- Number of *Listeria* in Agar Listeria, according to Ottaviani and Agosti (Biolife, Monza, Italy), with colony confirmation using a microscopic view, beta-hemolysis test and L-Rhamnose and D-Xylose tests if the colonies were suspicious or typical.

2.7. Statistical Analysis

For each experiment, three trials were performed. The standard deviation was calculated using EXCEL (version 11. Microsoft, Washington, DC, USA) software. Data is given

as an average value ± standard deviation. Differences between the data were evaluated by the analysis of variance method (one-way ANOVA) with a significance level of $p < 0.05$.

3. Results

No *L. monocytogenes* or *Salmonella* spp. were detected in any of the pieces of beef and pork obtained for testing.

Data on the investigation of 20% anolyte's effect on total viable count (TVC) and pathogenic bacteria (*L. monocytogenes* and *Salmonella* spp.) count using the treatment of meat cuts by spraying are presented in Tables 1–4. At 20% anolyte concentration, using the lowest contamination level (1500 CFU/mL suspension of *L. monocytogenes* and *S.* Typhimurium), two controls were used: Control 1 was raw meat not additionally contaminated by pathogenic bacteria and not treated by anolyte, and control 2 was raw meat contaminated by the analogous bacterial suspension used for experimental meat but not treated by anolyte. The experimental meat (for 20% anolyte treatment, beef) was contaminated by the uniform bacterial suspension (15,000 CFU/mL).

According to the data in the Table 1, after spraying meat cuts treated at a 1500 CFU/mL contamination level with 20% anolyte, the TVC was reduced significantly ($p < 0.05$) from $(4.73 \pm 0.26) \times 10^3$ to $(4.30 \pm 0.27) \times 10^2$. However, it significantly increased after 10 days of incubation and increased even more after 29 days of incubation. Compared to Control 1, however, the TVC after 29 days was significantly lower in experimental meat, even with the increase in TVC compared to when meat was tested right after spraying with anolyte. The same could be seen at the higher contamination level (15,000 CFU/mL), where TVC was significantly reduced (12-fold decrease, $p < 0.05$) after spraying with anolyte, then increased after incubation. Despite the increase in TVC in experimental meat, the final TVC after 29 days was 1.3 times lower compared to the Control 1 and Control 2.

When using a lower contamination level (1500 CFU/mL), *L. monocytogenes* was reduced effectively to non-detectable levels, and these levels remained, even after 29 days of incubation. At the higher contamination level (15,000 CFU/mL), a similar situation could be seen, where the *L. monocytogenes* count was effectively kept at non-detectable levels after incubation. The anolyte took some time to fully reduce *L. monocytogenes*, as right after spraying with anolyte, the counts were reduced significantly. However, they still were at detectable levels ($(3.33 \pm 0.58) \times 10^2$).

The results of Table 3 are analogous with ones presented in Table 1. Though treatment with anolyte decreased TVC on the meat surface significantly. The effect was not long lasting; after the incubation of samples for 10 and 29 days, the TVC increased up to $(4.03 \pm 0.06) \times 10^6$ for the lower contamination level and $(4.7 \pm 0.1) \times 10^6$ for the higher contamination level. As in Table 1, the final TVC after 29 days was lower in the experimental meat samples compared to control for both contamination levels.

The results of Table 4 are analogous with Table 2. All anolyte concentrations were effective in decreasing the *S.* Typhimurium count in the experimental samples, and it decreased during the incubation at 0–4 °C. *S.* Typhimurium were not detected after 29 days incubation.

Table 1. The effect of anolyte's concentration on the total viable count of meat cuts contaminated with *L. monocytogenes*.

Concentration of Anolyte Used for Spraying of Meat Cuts	Suspension of *L. monocytogenes* Used for Contamination of Meat Cuts 300 g, CFU/mL	Object of Investigation (Raw Meat)	Total Viable Count. CFU/g Meat Cut				
			Raw Meat	Meat after Contamination	Meat after Spraying with Anolyte	After 10 Days of Incubationat 0–4 °C	After 29 Days of Incubation at 0–4 °C
20%, beef	1500 CFU/mL	Control 1	$(2.27 \pm 0.06) \times 10^3$	-	-	$(1.97 \pm 0.06) \times 10^5$	$(5.67 \pm 0.29) \times 10^6$
		Experimental meat	$(2.10 \pm 0.0) \times 10^3$	$(4.73 \pm 0.26) \times 10^{3\,a}$	$(4.30 \pm 0.27) \times 10^{2\,b}$	$(1.87 \pm 0.06) \times 10^{5\,c}$	$(4.10 \pm 0.26) \times 10^{6\,d}$ *
	15,000 CFU/mL	Control 1	$(2.27 \pm 0.06) \times 10^3$	-	-	$(1.97 \pm 0.06) \times 10^5$	$(5.67 \pm 0.29) \times 10^6$
		Control 2	$(2.17 \pm 0.12) \times 10^3$	$(4.4 \pm 0.61) \times 10^3$	-	$(2.0 \pm 0.0) \times 10^5$	$(5.80 \pm 0.53) \times 10^6$
		Experimental meat	$(2.2 \pm 0.1) \times 10^3$	$(1.2 \pm 0.1) \times 10^{4\,a}$	$(1.0 \pm 0.17) \times 10^{3\,b}$	$(1.87 \pm 0.06) \times 10^{5\,c}$	$(4.33 \pm 0.21) \times 10^{6\,d}$ *

Note: $^{a–d}$ superscripts denote statistically different values in rows and * denotes statistically different values in columns.

Table 2. The effect of anolyte's concentration on the *L. monocytogenes* count of meat cuts contaminated with *L. monocytogenes*.

Concentration of Anolyte Used for Spraying of Meat Cuts	Suspension of *L. monocytogenes* Used for Contamination of Meat Cuts, CFU/mL	Object of Investigation (Raw Meat)	*L. monocytogenes*. CFU/g Meat Cut				
			Raw Meat	Meat after Contamination	Meat after Spraying with Anolyte	After 10 Days of Incubation at 0–4 °C	After 29 Days of Incubation at 0–4 °C
20%, beef	1500 CFU/mL	Control 1	$<1.0 \times 10^1$	-	-	$<1.0 \times 10^1$	$<1.0 \times 10^1$
		Experimental meat	$<1.0 \times 10^1$	$(3.87 \pm 0.15) \times 10^{3\,a}$	$<1.0 \times 10^{1\,b}$	$<1.0 \times 10^{1\,b}$	$<1.0 \times 10^{1\,b}$
	15,000 CFU/mL	Control 1	$<1.0 \times 10^1$	-	-	$<1.0 \times 10^1$	$<1.0 \times 10^1$
		Control 2	$<1.0 \times 10^1$	$(3.17 \pm 0.35) \times 10^{3\,a}$	-	$(2.97 \pm 0.42) \times 10^{3\,a}$	$(2.67 \pm 0.49) \times 10^{3\,b}$
		Experimental meat	$<1.0 \times 10^1$	$(8.77 \pm 0.59) \times 10^{3\,a}$	$(3.33 \pm 0.58) \times 10^{2\,b}$	$<1.0 \times 10^{1\,c}$	$<1.0 \times 10^{1\,c}$

Note: $^{a–c}$ superscripts denote statistically different values in rows.

Table 3. The effect of anolyte's concentration on the total viable count of meat cuts contaminated with *S.* Typhimurium.

Concentration of Anolyte Used for Spraying of Meat Cuts	Suspension of *S.* Typhimurium Used for Contamination of Meat Cuts, CFU/mL	Object of Investigation (Raw Meat)	Total Viable Count. CFU/g Meat Cut				
			Raw Meat	Meat after Contamination	Meat after Spraying with Anolyte	After 10 Days of Incubationat 0–4 °C	After 29 Days of Incubation at 0–4 °C
20%, beef	1500 CFU/mL	Control 1	$(2.07 \pm 0.06) \times 10^3$	-	-	$(2.63 \pm 0.06) \times 10^5$	$(6.43 \pm 0.25) \times 10^6$
		Experimental meat	$(2.10 \pm 0.00) \times 10^3$	$(4.73 \pm 0.06) \times 10^{3\,a}$	$(5.83 \pm 0.21) \times 10^{2\,b}$	$(2.40 \pm 0.1) \times 10^{5\,c}$ *	$(4.03 \pm 0.06) \times 10^6$ *
	15,000 CFU/mL	Control 1	$(2.07 \pm 0.06) \times 10^3$	-	-	$(2.63 \pm 0.06) \times 10^5$	$(6.43 \pm 0.25) \times 10^6$
		Control 2	$(2.17 \pm 0.06) \times 10^3$	$(4.4 \pm 0.1) \times 10^{3\,a}$	-	$(2.63 \pm 0.06) \times 10^{5\,b}$	$(6.6 \pm 0.26) \times 10^{6\,c}$
		Experimental meat	$(2.13 \pm 0.15) \times 10^3$	$(1.1 \pm 0.06) \times 10^{4\,a}$	$(1.1 \pm 0.06) \times 10^{3\,b}$	$(2.43 \pm 0.06) \times 10^{5\,c}$	$(4.7 \pm 0.1) \times 10^{6\,d}$ *

Note: [a–d] superscripts denote statistically different values in rows and * denotes statistically different values in columns.

Table 4. The effect of anolyte's concentration on the *S.* Typhimurium count of meat cuts contaminated with *S.* Typhimurium.

Concentration of Anolyte Used for Spraying of Meat Cuts	Suspension of *S.* Typhimurium Used for Contamination of Meat Cuts, CFU/mL	Object of Investigation (Raw Meat)	Total Viable Count. CFU/g Meat Cut				
			Raw Meat	Meat after Contamination	Meat after Spraying with Anolyte	After 10 Days of Incubation at 0–4 °C	After 29 Days of Incubation at 0–4 °C
20%, beef	1500 CFU/mL	Control 1	$<1.0 \times 10^1$	-	-	$<1.0 \times 10^1$	$<1.0 \times 10^1$
		Experimental meat	$<1.0 \times 10^1$	$(4.3 \pm 0.3) \times 10^{3\,a}$	$<1.0 \times 10^{1\,b}$	$<1.0 \times 10^{1\,b}$	$< 1.0 \times 10^{1\,b}$
	15,000 CFU/mL	Control 1	$<1.0 \times 10^1$	-	-	$<1.0 \times 10^1$	$<1.0 \times 10^1$
		Control 2	$<1.0 \times 10^1$	$(3.4 \pm 0.26) \times 10^{3\,a}$	-	$(3.07 \pm 0.31) \times 10^{3\,a}$	$(2.83 \pm 0.21) \times 10^{3\,a}$
		Experimental meat	$<1.0 \times 10^1$	$(1.0 \pm 0.8) \times 10^{4\,a}$	$(4.3 \pm 0.06) \times 10^{2\,b}$	$<1.0 \times 10^{1\,c}$	$<1.0 \times 10^{1\,c}$

Note: [a–c] superscripts denote statistically different values in rows.

The results of anolyte's effect on the contamination of minced pork are presented in Tables 5–8.

According to the data in Table 5, at different levels of contamination with *L. monocytogenes* (1.5×10^3 CFU/mL, 1.5×10^4 CFU/mL and 1.5×10^5 CFU/ mL) of minced meat and treating with anolyte, the TVC decreased 2.2; 1.7 and 2.4-fold, respectively ($p < 0.05$). With the continued storage of meat samples at 0–4 °C for up to 29 days, the TVC significantly increased again by 35.0, 29.3 and 29.6 times. The final TVC was significantly lower ($p < 0.05$) in the lowest contamination sample and highest ($p < 0.05$) in the highest contamination sample.

Table 5. Effect of anolyte on the total viable count of minced pork contaminated by *L. monocytogenes*.

Contamination Level	Total Viable Count, CFU/g				
	Raw Minced Pork	Raw Pork Contaminated by *L. monocytogenes*	Contaminated Raw Pork after Addition of 18% Undiluted Anolyte and Mixing	Contaminated Pork Mixed with Anolyte after 10 Days of Incubation at 0–4 °C	Contaminated Pork Mixed with Anolyte after 29 Days of Incubation at 0–4 °C
10 mL 1.5×10^3 CFU/mL suspension of *L. monocytogenes*	$(2.20 \pm 0.10) \times 10^4$	$(1.82 \pm 0.10) \times 10^{4\,a}$	$(8.02 \pm 0.10) \times 10^{3\,b}$	$(1.40 \pm 0.10) \times 10^{4\,a}$	$(2.80 \pm 0.17) \times 10^{5\,c}$
10 mL 1.5×10^4 CFU/mL suspension of *L. monocytogenes*	$(2.20 \pm 0.10) \times 10^4$	$(2.40 \pm 0.20) \times 10^{4\,a}$	$(1.40 \pm 0.17) \times 10^{4\,b}$ *	$(1.80 \pm 0.20) \times 10^{4\,b}$	$(4.10 \pm 0.10) \times 10^{5\,c}$ *
10 mL 1.5×10^5 CFU/mL suspension of *L. monocytogenes*	$(0.70 \pm 0.10) \times 10^4$	$(6.60 \pm 0.26) \times 10^{4\,a}$	$(2.70 \pm 0.20) \times 10^{4\,b}$ **	$(1.80 \pm 0.10) \times 10^{4\,c}$	$(8.00 \pm 0.20) \times 10^{5\,d}$ **

Note: $^{a–d}$ superscripts denote statistically different values in rows and * and ** denote statistically different values in columns.

Table 6. Effect of anolyte on the *L. monocytogenes* count of minced pork contaminated by *L. monocytogenes*.

Contamination Level	*L. monocytogenes* Count. CFU/g				
	Raw Minced Pork	Raw Pork Contaminated by *L. monocytogenes*	Contaminated Raw Pork after Addition of 18% Undiluted Anolyte and Mixing	Contaminated Pork Mixed with Anolyte after 10 Days of Incubation at 0–4 °C	Contaminated Pork Mixed with Anolyte after 29 Days of Incubation at 0–4 °C
10 mL 1.5×10^3 CFU/mL suspension of *L. monocytogenes*	$<1.00 \times 10^1$	$(1.60 \pm 0.10) \times 10^{3\,a}$	$<1.00 \times 10^{1\,b}$	$<1.00 \times 10^{1\,b}$	$<1.00 \times 10^{1\,b}$
10 mL 1.5×10^4 CFU/mL suspension of *L. monocytogenes*	$<1.00 \times 10^1$	$(3.70 \pm 0.17) \times 10^{3\,a}$	$(6.00 \pm 0.10) \times 10^{2\,b}$	$(5.60 \pm 0.17) \times 10^{2\,b}$	$<1.00 \times 10^{1\,c}$
10 mL 1.5×10^5 CFU/mL suspension of *L. monocytogenes*	$<1.00 \times 10^1$	$(2.50 \pm 0.10) \times 10^{4\,a}$	$(1.30 \pm 0.20) \times 10^{3\,b}$	$(1.80 \pm 0.26) \times 10^{3\,b}$	$(2.20 \pm 0.20) \times 10^{2\,c}$ *

Note: $^{a–c}$ superscripts denote statistically different values in rows and * denotes statistically different values in columns.

Table 7. Effect of anolyte on the total viable count of minced pork contaminated by *S.* Typhimurium.

Contamination Level	Total Viable Count, CFU/g				
	Raw Minced Pork	Raw Pork Contaminated by *S.* Typhimurium	Contaminated Raw Pork after Addition of 18% Undiluted Anolyte and Mixing	Contaminated Pork Mixed with Anolyte after 10 Days of Incubation at 0–4 °C	Contaminated Pork Mixed with Anolyte after 29 Days of Incubation at 0–4 °C
10 mL 1.5×10^3 CFU/mL suspension of *S.* Typhimurium	$(2.20 \pm 0.10) \times 10^4$	$(2.20 \pm 0.26) \times 10^{4\,a}$	$(6.40 \pm 0.17) \times 10^{3\,b}$	$(2.10 \pm 0.17) \times 10^{4\,a}$	$(4.10 \pm 0.20) \times 10^{5\,c}$
10 mL 1.5×10^4 CFU/mL suspension of *S.* Typhimurium	$(2.20 \pm 0.10) \times 10^4$	$(5.40 \pm 0.26) \times 10^{4\,a}$	$(1.90 \pm 0.10) \times 10^{4\,b}$	$(2.30 \pm 0.26) \times 10^{4\,b}$	$(4.00 \pm 0.17) \times 10^{5\,c}$
10 mL 1.5×10^5 CFU/mL suspension of *S.* Typhimurium	$(0.70 \pm 0.11) \times 10^4$	$(5.40 \pm 0.17) \times 10^{4\,a}$	$(3.60 \pm 0.36) \times 10^{4\,b}$	$(3.90 \pm 0.17) \times 10^{4\,b}$	$(7.80 \pm 0.26) \times 10^{5\,c}$ *

Note: $^{a–c}$ superscripts denote statistically different values in rows and * denotes statistically different values in columns.

Table 8. Effect of anolyte on the *S*. Typhimurium count of minced pork contaminated by *S*. Typhimurium.

Contamination Level	*S*. Typhimurium. CFU/g				
	Raw Minced Pork	Raw Pork Contaminated by *S*. Typhimurium	Contaminated Raw Pork after Addition of 18% Undiluted Anolyte and Mixing	Contaminated Pork Mixed with Anolyte after 10 Days of Incubation at 0–4 °C	Contaminated Pork Mixed with Anolyte after 29 Days of Incubation at 0–4 °C
10 mL 1.5×10^3 CFU/mL suspension of *S*. Typhimurium	$<1.00 \times 10^1$	$(3.50 \pm 0.43) \times 10^{2\,a}$	$<1.00 \times 10^{1\,b}$	$<1.00 \times 10^{1\,b}$	$<1.00 \times 10^{1\,b}$
10 mL 1.5×10^4 CFU/mL suspension of *S*. Typhimurium	$<1.00 \times 10^1$	$(3.00 \pm 0.17) \times 10^{3\,a}$	$(1.30 \pm 0.20) \times 10^{3\,b}$	$(1.90 \pm 0.20) \times 10^{3\,b}$	$(8.0 \pm 0.08) \times 10^{1\,c}$ *
10 mL 1.5×10^5 CFU/mL suspension of *S*. Typhimurium	$<1.00 \times 10^1$	$(1.90 \pm 0.17) \times 10^{4\,a}$	$(1.40 \pm 0.17) \times 10^{3\,b}$	$(2.00 \pm 0.26) \times 10^{3\,b}$	$(2.30 \pm 0.20) \times 10^{2\,c}$ **

Note: $^{a–c}$ superscripts denote statistically different values in rows and * and ** denote statistically different values in columns.

According to the data in the Table 6, the *L. monocytogenes* count in the minced meat samples contaminated with these bacteria at different levels (1.5×10^4 CFU/mL and 1.5×10^5 CFU/mL) decreased by 6.2 and 19.2 times ($p < 0.05$), respectively, after exposure to 18% anolyte from the meat mass. The largest decrease was observed at the highest dose in the contaminated sample. However, the final TVC was still significantly higher in the highest contamination sample, whereas in the other two samples, *L. monocytogenes* was undetectable after 29 days.

According to the data in the Table 7, the TVC in samples 1, 2 and 3 (minced meat from the lowest to the highest contamination dose of *S*. Typhimurium) decreased by 3.4, 2.8 and 1.5 times, respectively ($p < 0.05$). When the samples were incubated at 0–4 °C for up to 10 days, the TVC increased significantly ($p < 0.05$), and after 29 days the TVC increased again by 64.1, 21.0 and 21.7 times (maximum increase at the lowest dose of contamination).

According to the data in the Table 8, in samples of minced meat contaminated with *S*. Typhimurium, *Salmonella* spp. counts decreased by 2.3 and 13.6 ($p < 0.05$) times in samples 2 and 3, respectively, after exposure to 18% pure anolyte from the meat mass and were not detected in sample 1 (at the lowest dose of contamination). When the samples were incubated at 0–4 °C for up to 29 days, the *Salmonella* spp. count in samples 2 and 3 decreased further by 16.3 and 6.1 times, respectively, and in the sample contaminated with the lowest dose they were not detected after 10 and 29 days of incubation.

The TVC decreased upon exposure to anolyte in the minced meat by several times but increased again to maximum of 35-fold at the end of incubation (when contaminated *with L. monocytogenes*) and to 64-fold (when contaminated with *S*. Typhimurium). *L. monocytogenes* and *Salmonella* spp. count decreased after exposure to anolyte (*L. monocytogenes* maximum decreased 19-fold and *Salmonella* spp. count maximum decreased 13-fold) and was decreasing until the end of incubation and was not detected after contamination by lower concentrations.

4. Discussion

Rajkowski and Sommers [11] noticed similar results in their research, wherein catfish fillets inoculated with *Salmonella* and *L. monocytogenes* treated with anolyte retained a slight reduction in background microflora counts for only a 2 day storage period, yet *Salmonella* recovery from catfish fillet surface was reduced significantly and maintained throughout 13 days of storage. *L. monocytogenes* recovery was not reduced after a 3 min wash with anolyte. This is in accordance with our own findings, as *S*. Typhimurium counts were effectively reduced in beef cuts and minced pork and remained undetectable up to 29 days of storage at all contamination levels. Similar to their research, the TVC was initially reduced both in beef cuts and minced pork. However, it started increasing again through the storage period in our own research. In contrast to Rajkowski and Sommers' paper, *L. monocytogenes* counts were also effectively reduced and kept at low levels during our

research process. Similar studies have also been carried out with anolyte and its effect on *L. monocytogenes* in fish [16–18].

Fabrizio and Cutter [19] applied acidic electrolyzed water on ready-to-eat meats in an attempt to reduce the *L. monocytogenes* counts. The data of their research shows a positive effect of electrolyzed water in reducing *L. monocytogenes* counts in frankfurters following dipping treatments for up to 7 days, after which the bacteria counts started increasing again, yet still maintaining a lower count of bacteria compared to control. In contrast, in our research, *L. monocytogenes* counts kept reducing during the storage period up to 29 days, and at the end of the study were at undetectable levels at most contamination levels. The difference in results could be attributed to different exposure times between our research and Fabrizio and Cutter's research; while in their research frankfurters were dipped in electrolyzed water for 15 min, anolyte in our study was mixed into the minced pork, leading to a longer exposure time for anolyte and, thus, leading to a better reduction in *L. monocytogenes* counts. Furthermore, acidic electrolyzed water was shown to be effective in reducing mesophilic, psychotrophic and lactic acid bacteria, which also contribute to meat spoilage [20].

Our results are also in concordance with Al-Holy and Rasco [17], who found that *S.* Typhimurium and *L. monocytogenes* populations in beef were reduced after a 10 min-long treatment with acidic electrolyzed water by 0.7 logs and 1.2 logs, respectively. The authors also found that AEW also reduced *E. coli* O157:H7 counts, which are also prominent bacteria in meat spoilage that were not studied in our paper. Liao et al. [21] determined that using slightly acidic electrolyzed water could also effectively control TVC levels in beef during thawing, as it damages cell structures and inhibits the growth of microbes on the surface of beef.

Levels of meat contamination with *L. monocytogenes* and *Salmonella* Typhimurium even higher than those that occur in practice have been studied. In the latter case, the number of bacteria decreased under the impact of anolyte, and the data that was acquired in this study confirmed those described in the literature [22] that anolyte had a higher effect on pathogenic bacteria than on the TVC. In their paper, Hricova et al. [23] reviewed the efficacy of electrolyzed water in removing pathogenic bacteria. They note that anolyte is efficient in reducing *S.* Typhimurium and *L. monocytogenes* populations by more than 6.0 log CFU/mL and up to 9.2 log CFU/mL, respectively. Park et al. [24] reports a >4.0 log CFU/g reduction in pathogenic bacteria when using electrolyzed water compared to control, which reinforces the Navarro-Rico et al. [25] description of electrolyzed water as a "promising alternative to conventional NaClO disinfection". In fact, the effect of anolyte depends on the species composition of the microorganisms present in meat.

From the research, the following conclusions can be made:

- Anolyte is effective at reducing *L. monocytogenes* and *S.* Typhimurium counts in beef cuts and minced pork over a 29 day period;
- While initially anolyte can reduce the TVC in beef cuts and minced pork, it starts to increase during the storage period again. However, the TVC is lower, compared to control;
- Taking into account our findings and the findings of other authors, anolyte seems to be a promising tool for reducing bacterial growth in meat products and extending their shelf-life;
- In the future, research could be expanded further by studying the effects of anolyte on other pathogens, as well as in different types of meat and under different processing conditions.

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allegato 6

Grafico di Efficacia dell'Anolyte contro i Batteri

Valori indicativi basati su concentrazioni standard (50–200 ppm)

Batterio	Tipo	Efficacia	Tempo di inattivazione
Escherichia coli	Gram-negativo	Molto alta	30 sec – 1 min
Salmonella spp.	Gram-negativo	Molto alta	30 sec – 1 min
Listeria monocytogenes	Gram-positivo	Molto alta	30 sec – 1 min
Staphylococcus aureus	Gram-positivo	Molto alta	30 sec – 1 min
Pseudomonas aeruginosa	Gram-negativo	Alta	1 – 2 min
Enterococcus faecalis	Gram-positivo	Alta	1 – 2 min
Bacillus subtilis (vegetative)	Gram-positivo	Alta	1 – 2 min
Clostridium spp. (spore)	Anaerobio	Moderata	5 – 10 min

allegato 7

Tabella di efficacia dell'anolyte verso i virus

Virus / Famiglia	Tipo di virus	Efficacia dell'anolyte	Tempo di contatto tipico
SARS-CoV-2 (COVID-19)	Enveloped RNA	Molto alta (≥99.9%)	30–60 secondi
Influenza A / B	Enveloped RNA	Molto alta	30–60 secondi
Herpes simplex (HSV-1,2)	Enveloped DNA	Molto alta	30–60 secondi
HIV	Enveloped RNA	Molto alta	< 1 minuto
Norovirus	Non-enveloped RNA	Alta	1–5 minuti
Adenovirus	Non-enveloped DNA	Alta	1–5 minuti
Rotavirus	Non-enveloped RNA	Alta	1–5 minuti
Poliovirus	Non-enveloped RNA	Moderata–Alta	≈ 5 minuti

allegato 8

Tabella di efficacia dell'anolyte verso muffe e lieviti

Microrganismo	Tipo	Concentrazione (mg/L Cl attivo)	Tempo di contatto	Efficacia
Candida albicans	Lievito	30–50	30–60 sec	>99,9%
Saccharomyces cerevisiae	Lievito	50–100	1–2 min	>99,9%
Aspergillus niger	Muffa	100–200	2–5 min	>99%
Penicillium spp.	Muffa	100–200	2–5 min	>99%
Cladosporium spp.	Muffa	100–200	2–5 min	>99%
Fusarium spp.	Muffa	150–300	3–5 min	>99%

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Short communication

Antiviral activity of Ecasol against feline calicivirus, a surrogate of human norovirus

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Summary

Human norovirus (NoV) is a major cause of acute gastroenteritis in closed settings such as hospitals, hotels and cruise ships. The virus survives on inanimate surfaces for extended periods of time, and environmental contamination has been implicated in its transmission. The disinfection of contaminated areas is important in controlling the spread of NoV infections. Neutral solutions of electrochemically activated (ECA)-anolyte have been shown to be powerful disinfectants against a broad range of bacterial pathogens. The active chemical ingredient is hypochlorous acid (HOCl), which is registered as an approved food contact surface sanitizer in the United States by the Environmental Protection Agency, pursuant to 40 CFR 180.940. We evaluated the antiviral activity of Ecasol (an ECA-anolyte) against feline calicivirus (FCV), a surrogate of NoV. FCV dried on plastic surfaces was exposed to Ecasol for 1, 2, or 5 min. After exposure to Ecasol, the virus titers were compared with untreated controls to determine the virus inactivation efficacy after different contact times. Ecasol was found to decrease the FCV titer by >5$\log_{10}$ within 1 min of contact, indicating its suitability for inactivation of NoV on surfaces.

Highlights

► ECA-anolyte has been shown to be a powerful disinfectant against a broad range of pathogens. ► Evaluated antiviral activity of Ecasol (an ECA-anolyte) against feline calicivirus (FCV), a surrogate of NoV. ► A >5$\log_{10}$ reduction in FCV titer was observed within 1 min of application of Ecasol. ► Ecasol is suitable for disinfection of NoV contaminated surfaces.

Introduction

In recent years, there have been several outbreaks of acute gastroenteritis, predominantly in closed settings, including institutionalized housing, hotels and cruise ships [1]. Epidemiological investigations have confirmed that >95% of these outbreaks, especially on cruise ships, are caused by human norovirus (NoV) [2]. NoV is a non-enveloped, single-stranded RNA virus belonging to the family *Caliciviridae* and is one of the most common causes of acute gastroenteritis in humans. This virus is shed in high concentrations (up to 11 $\log_{10}$ per gram of feces) and has a low infectious dose of <100 infectious virus particles [3]. Environmental contamination has been implicated in the transmission of NoV because the virus is able to survive for days to months on different types of surfaces [4].

Cleaning and disinfection of contaminated surfaces are important procedures for controlling outbreaks of NoV in hospital and community settings [4]. Although the use of alcohol-based hand rubs has been promoted to control the spread of infection, alcohol has a limited effectiveness in killing NoV [5]. Various virucides are commonly used to disinfect fomites and environmental contact surfaces implicated in NoV outbreaks. The material safety data sheets and labels for these virucidal compounds rarely allow for their aerosolization, spraying, or fogging due to their toxicity and adverse health effects for given exposure durations and concentrations. Many of these chemical compounds, such as sodium hypochlorite, chlorine gas, and glutaraldehyde, have been associated with occupational illnesses. For example, exposure to glutaraldehyde is associated with contact dermatitis in health workers, and the use of quaternary ammonium compounds has been found to cause occupational asthma in users [6], [7]. For cases in which aerosolization is approved, the use of personal protective equipment and a self-contained breathing apparatus is required, which makes the use of these compounds difficult, especially in public places such as hospitals or schools.

Ecasol is a unique electrochemically activated (ECA)-neutral pH anolyte, which consists of an "activated" solution, produced by a process referred to as dilute brine electrolysis. Based on Faraday's laws of electrolysis, advanced continuous process ECA membrane cell manufacturing was pioneered in the 1970s in the former Soviet Union [8] and was then advanced to its current form by Trustwater (Clonmel, Ireland). Ecasol has been demonstrated as a powerful disinfectant and has been shown to be efficacious against a wide range of microorganisms in solution and when sprayed in the air [9], [10]. Another significant benefit of Ecasol is its lack of toxicity at ready-to-use (RTU) concentrations. It is considered safe in food processing applications by the United States Food and Drug Administration [11]. In dental procedures, Ecasol has been shown to have no adverse effects on human oral tissues [12].

ECA technology involves the generation of electrochemically activated solutions by passing a carefully regulated electric current through a brine solution in specialized electrode compartments and separating the ions according to charge. Ecasol is a positively charged solution emerging from a Trustwater generator. It is a strong oxidizing solution, with a pH of 7.0, a redox potential of +1200mV, and an active chlorine content of approximately ~700mgL^{-1}. Hypochlorous acid (HOCL) is the major component of Ecasol, which also contains free radicals and a small amount of sodium chloride (NaCl). As the free radicals gradually lose energy and reform as water, HOCL dissociates into hydrogen and hypochlorite ions, which eventually revert to NaCl (<0.2%) and water (>99.8%). The water evaporates, leaving salt crystals that can be removed by routine cleaning.

We undertook this study to evaluate the effectiveness of Ecasol for decontaminating surfaces contaminated with NoV. Because NoV is currently non-cultivable in vitro, efficacy tests of disinfectants rely on the use of surrogates, e.g., feline calicivirus (FCV) or murine norovirus (MNV). In this study, we used FCV as a surrogate for NoV.

Materials and methods

Virus

Strain 255 of FCV was propagated in Crandell-Reese Feline Kidney (CRFK) cells, and aliquots of the virus were stored at −80°C until use.

Preparation of ECA anolyte

The Ecasol anolyte solution was prepared on the day of the test using a fully automatic ECA device (Trustwater model AQ-50). This device electrolyzes dilute brine solution using a membrane-type electrolytic cell controlled to produce two metastabilized electrolyte streams in dynamic equilibrium. The catholyte stream (Aversol™ by Trustwater) has a high pH and is classified as an amphoteric surfactant, having reduced surface tension and mild detergent-like properties. Trustwater's automated process uses this solution to maintain the Ecasol stream at a neutral pH. The new Ecasol solution was titrated at 700ppm free available chlorine (FAC), with a pH of 6.7. The solution was delivered to the lab on the day of the experiment and was used immediately upon delivery, with a time from solution generation to lab experimentation of approximately 2h. The stability of Ecasol depends upon storage conditions because it can lose up to 10% of its activity within 3 weeks of generation if it is not stored properly.

Surface decontamination test

Two concentrations of Ecasol, 150ppm and 500ppm FAC, were prepared by diluting the solution with deionized water. These testing concentrations were selected because 150ppm is the most commonly used concentration for food contact surface sanitization, based on the recommendation of 40CFR 180.940, and the concentration of 500ppm was selected because Ecasol is a known sporocidal at this concentration.

The test was performed in 6-well tissue culture plates, and the experiments were conducted in triplicate. The six wells of the plate were labeled A through F, and the FCV suspension was uniformly applied to the bottom of the six wells at 100μL/well. The inoculum was allowed to dry for

30min at room temperature (approximately 23°C) in a type II biosafety cabinet. After the inoculum dried, the Ecasol solution was added to wells A–C at 5mL/well. Wells D–F served as controls for each treated well (well D for well A, and so on), with 5mL of phosphate buffered saline (PBS) per well. The plate was incubated at room temperature on an orbital shaker (at 120rpm) for different time periods (1, 2, and 5min for wells A and D, B and E, and C and F, respectively). After the appropriate contact times, the well contents were immediately diluted 10-fold using a maintenance medium to stop Ecasol activity at the indicated times. Serial 10-fold dilutions of these eluates were prepared in Eagle's MEM, followed by inoculation of CRFK cells grown in 96-well microtiter plates, using four wells for each test dilution. The inoculated plates were incubated at 37°C and examined daily for 4 days by microscope for FCV-induced cytopathic effects (CPE). The virus titers were calculated by the Reed and Muench method [13], and the log reductions were calculated by comparing the titers of the Ecasol-treated wells with those of the PBS-treated control wells. To determine the cytotoxicity of the Ecasol solution to the CRFK cells, 10-fold serial dilutions of Ecasol prepared in Eagle's MEM were added to monolayers of CRFK cells prepared in a 96-well plate (4 wells/dilution). Each concentration of Ecasol (150ppm and 500ppm) was tested in separate experiments, and each experiment was performed in triplicate.

Results and discussion

In this study, we evaluated the effectiveness of Ecasol solution for the disinfection of a plastic surface contaminated with FCV. To the best of our knowledge, this is the first study of the use of Ecasol for the disinfection of surfaces contaminated with a NoV surrogate. The results indicate that Ecasol at 150ppm and 500ppm inactivated >5$\log_{10}$ of FCV (>99.999% reduction in virus titer) within 1min at room temperature (Table 1). There was no additional reduction in virus titer when the contact time was increased from 1min to 5min.

Table 1. Inactivation of feline calicivirus by Ecasol.[a]

Ecasol concentration (ppm)	Time (min)	Virus titer ($TCID_{50}$/0.1mL)[b]		Percent virus reduction
		Control	Test	
150	1	$10^{7.0}$	$10^{1.5}$	99.9996
	2	$10^{6.75}$	$10^{1.5}$	99.9994
	5	$10^{6.75}$	$10^{1.5}$	99.9994
500	1	$10^{6.875}$	$10^{1.5}$	99.9995
	2	$10^{6.625}$	$10^{1.5}$	99.9992
	5	$10^{6.75}$	$10^{1.5}$	99.9994

a The results are an average of three different experiments.

b

Control, virus treated with PBS; test, virus treated with Ecasol.

NoV is a major cause of acute gastroenteritis worldwide because of its low infective dose and its ability to survive on inert objects for extended periods of time. The virus can be transferred from contaminated surfaces to other inert objects, including faucets, door handles, and telephones. It is important to implement proper cleaning and disinfecting procedures that eliminate virus particles from contaminated surfaces. However, NoV cannot be cultured in a laboratory setting, which hampers research on the development of prevention and control strategies. To overcome this problem, FCV has widely been used as a surrogate for NoV because its physicochemical and genetic properties are similar to those of NoV [14]. FCV is a respiratory virus in felines and is susceptible to high temperatures [15]. Genogroup V murine NoV (MNV) has recently been advanced as a surrogate for NoV because it is morphologically and genetically similar to NoV and can be propagated in cell cultures [16].

Many studies have reported on various compounds used for the inactivation of FCV, including acids and alcohols [17], ozone gas [18], H_2O_2 vapors, and chlorine dioxide gas (ClO_2) [19]. Whitehead and McCue [17] showed that bleach and acid-based disinfectants could inactivate FCV within 1 min (>4$\log_{10}$ reduction). The use of ClO_2 has been shown to reduce FCV titers by >3$\log_{10}$ within 10 h [19], and ozone can inactivate FCV in less than 1 h [18]. Some of these compounds are toxic, energy intensive, and expensive and require an extended time for virus inactivation. Ecasol is non-toxic, non-corrosive, relatively inexpensive to produce, and biodegradable. After Ecasol is used, only water (>99.8%) and a small amount of salt crystals remain (NaCl, <0.2%). The amount of NaCl crystals present is too low to corrode metal surfaces and can be easily removed with water. Thus, we recommend Ecasol for disinfecting surfaces contaminated with NoV. Further studies are in progress on the use of Ecasol for a number of delivery methods, including direct application and fogging, as a disinfecting agent against additional viruses and bacteria.

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Guidelines for the management of norovirus outbreaks in acute and community health and social care settings

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> ...There was very weak evidence from five laboratory studies [235,240–243] which evaluated the effect of other chlorine-releasing agents on HNV [240,241] or its surrogates [235,241–243]. Four of these studies [235,240,242,243] used electrochemically activated (ECO) water. The water was generated by electrolysis of solution containing NaCl and HCl....

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> ...Tuladhar et al49 observed 1.5 (with 1% stool) and 1.7 (without soil load) log reductions 20 minutes after wiping stainless steel with a cloth soaked in 1,000 ppm NaDCC (CT = 20,000 mg × min/L). Several surrogates have been proposed for human norovirus to overcome the absence of infectivity assay, including murine norovirus and MS2,3,15,49,60,62,67–70 feline calicivirus,3,62,67–72 and Tulane virus.61,62 Comparisons between human norovirus and the proposed surrogates were made in 4 studies....

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2020, International Journal of Speleology

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Unair News

UNAIR expert initiates anolyte production as safe and environmentally friendly disinfectant

By wildan azky / 27/04/2020

UNAIR NEWS – Some time ago, the public was alerted with the controversy over liquids used in disinfection chambers as some of the content in the solutions actually pose a risk to health. Responding to this issue, Dr. Muji Harsini Dra., M.Si as a lecturer in Faculty of Science and Technology UNAIR initiated production of safe and environmentally friendly antiseptic liquid, for disinfection chambers.

Dr. Muji revealed the making of environmentally friendly antiseptic liquid for disinfection chambers is an effort to prevent COVID-19. Salt ion antiseptic solution (which is electrolyzed) can kill and sterilize the user's nose and throat during inhalation without causing difficulty of breathing, or irritation to the skin.

"All this time, we can easily find disinfectants in the market such as pine oil, phenol, NaOCl, H 2 O 2, and Ethanol. But these material have side effects for the body and health. Therefore, we use anolyte as disinfectant liquid material that is safe and environmentally friendly, "she said

Anolyte, she added, is the result of electrolysis of salt solution (NaCl). It functions as a disinfectant liquid that can clean and has an advantage over other chlorine compounds. This liquid does not have side effects for humans and animals, ensuring there is no poison and can decompose by itself without having to pollute the environment.

"Anolyte disinfectant is clinically proven 100 times more effective than cleaning with bleach and kill 99% more bacteria, viruses, germs and other dangerous disease carriers," she explained.

Dr. Muji also revealed that anolyte is also often used in drinking water treatment, chicken farming, shrimp farming, food processing, sewage, hospital sanitation, and other processes. Meanwhile, in its hospital application, anolyte is used to wash hands, soak and wash, decontaminate water storage and work pipes, wash and soak feet, clean rough surfaces and decontaminate ultrasound equipment, wash mattresses and wheelchairs, and many others uses.

Anolyte is made through electrochemical activation (Electro-Chemical Activation = ECA) which was patented for the first time in Russia and developed more than 40 years. The activation process uses water, salt and electricity so that it can produce strong and natural disinfectant content.

"Anolyte with active HOCl have a pH between 2.5-5 or pH <5 and is good for health. So in the future we will continue to produce it and so far we can produce 80 liters every hour, " she concluded.

Author: Khefti Al Mawalla

REGOLAMENTO DI ESECUZIONE (UE) 2021/365 DELLA COMMISSIONE

del 26 febbraio 2021

che approva il cloro attivo rilasciato da acido ipocloroso come principio attivo ai fini del suo uso nei biocidi del tipo di prodotto 1

(Testo rilevante ai fini del SEE)

LA COMMISSIONE EUROPEA,

visto il trattato sul funzionamento dell'Unione europea,

visto il regolamento (UE) n. 528/2012 del Parlamento europeo e del Consiglio, del 22 maggio 2012, relativo alla messa a disposizione sul mercato e all'uso dei biocidi ([1]), in particolare l'articolo 9, paragrafo 1, lettera a),

considerando quanto segue:

(1) Il 31 luglio 2007 l'autorità competente della Slovacchia («l'autorità di valutazione competente») ha ricevuto una domanda, a norma dell'articolo 11, paragrafo 1, della direttiva 98/8/CE del Parlamento europeo e del Consiglio ([2]), per l'iscrizione del principio attivo cloro attivo rilasciato da acido ipocloroso nell'allegato I di detta direttiva ai fini del suo uso nei biocidi del tipo di prodotto 1, «igiene umana», descritto nell'allegato V di detta direttiva, che corrisponde al tipo di prodotto 1 descritto nell'allegato V del regolamento (UE) n. 528/2012.

(2) Il 19 novembre 2010 l'autorità di valutazione competente ha presentato alla Commissione la relazione di valutazione, insieme alle sue conclusioni, in conformità all'articolo 11, paragrafo 2, della direttiva 98/8/CE.

(3) Il 16 giugno 2020 il parere dell'Agenzia europea per le sostanze chimiche ([3]) («l'Agenzia») è stato adottato dal comitato sui biocidi, tenendo conto delle conclusioni dell'autorità di valutazione competente.

(4) Secondo tale parere, i biocidi del tipo di prodotto 1 che usano cloro attivo rilasciato da acido ipocloroso possono essere considerati conformi ai criteri stabiliti all'articolo 5 della direttiva 98/8/CE, purché siano rispettate determinate specifiche e condizioni relative al loro uso.

(5) Tenendo conto del parere dell'Agenzia, è opportuno approvare il cloro attivo rilasciato da acido ipocloroso come principio attivo ai fini del suo uso nei biocidi del tipo di prodotto 1, subordinatamente al rispetto di determinate specifiche e condizioni.

(6) Le misure di cui al presente regolamento sono conformi al parere del comitato permanente sui biocidi,

HA ADOTTATO IL PRESENTE REGOLAMENTO:

Articolo 1

Il cloro attivo rilasciato da acido ipocloroso è approvato come principio attivo ai fini del suo uso nei biocidi del tipo di prodotto 1, fatte salve le specifiche e le condizioni di cui all'allegato.

([1]) GU L 167 del 27.6.2012, pag. 1.

([2]) Direttiva 98/8/CE del Parlamento europeo e del Consiglio, del 16 febbraio 1998, relativa all'immissione sul mercato dei biocidi (GU L 123 del 24.4.1998, pag. 1).

([3]) *Biocidal Products Committee Opinion on the application for approval of the active substance: active chlorine released from hypochlorous acid, Product type:1* (Parere del comitato sui biocidi riguardo alla domanda di approvazione del principio attivo cloro attivo rilasciato da acido ipocloroso; tipo di prodotto: 1); ECHA/BPC/255, adottato il 16 giugno 2020.

Articolo 2

Il presente regolamento entra in vigore il ventesimo giorno successivo alla pubblicazione nella *Gazzetta ufficiale dell'Unione europea*.

Il presente regolamento è obbligatorio in tutti i suoi elementi e direttamente applicabile in ciascuno degli Stati membri.

Fatto a Bruxelles, il 26 febbraio 2021

Per la Commissione
La presidente
Ursula VON DER LEYEN

ALLEGATO

Nome comune	Denominazione IUPAC, numeri di identificazione	Grado minimo di purezza del principio attivo (1)	Data di approvazione	Scadenza dell'approvazione	Tipo di prodotto	Condizioni specifiche
Cloro attivo rilasciato da acido ipocloroso	Denominazione IUPAC: Acido ipocloroso N. CE: 232-232-5 N. CAS: 7790-92-3	Specifica stabilita per l'acido ipocloroso (peso a secco min. 90,87 % p/p) che rilascia cloro attivo. L'acido ipocloroso è la specie predominante a pH 3,0 - 7,4.	1º luglio 2021	30 giugno-2031	1	Nella valutazione del prodotto occorre prestare particolare attenzione alle esposizioni, ai rischi e all'efficacia legati a qualsiasi uso previsto nella domanda di autorizzazione, ma non preso in considerazione nella valutazione del rischio del principio attivo condotta a livello di Unione.

(1) La purezza indicata in questa colonna corrisponde al grado minimo di purezza del principio attivo valutato. Il principio attivo nel prodotto immesso sul mercato può essere di pari o diversa purezza se ne è stata provata l'equivalenza tecnica con il principio attivo valutato.

REGOLAMENTO DI ESECUZIONE (UE) 2021/345 DELLA COMMISSIONE

del 25 febbraio 2021

che approva il cloro attivo generato da cloruro di sodio mediante elettrolisi come principio attivo ai fini del suo uso nei biocidi dei tipi di prodotto 2, 3, 4 e 5

(Testo rilevante ai fini del SEE)

LA COMMISSIONE EUROPEA,

visto il trattato sul funzionamento dell'Unione europea,

visto il regolamento (UE) n. 528/2012 del Parlamento europeo e del Consiglio, del 22 maggio 2012, relativo alla messa a disposizione sul mercato e all'uso dei biocidi ([1]), in particolare l'articolo 89, paragrafo 1, terzo comma,

considerando quanto segue:

(1) Il regolamento delegato (UE) n. 1062/2014 della Commissione ([2]) stabilisce un elenco di principi attivi esistenti da valutare per l'eventuale approvazione ai fini del loro uso nei biocidi. Tale elenco comprende il cloro attivo generato da cloruro di sodio mediante elettrolisi.

(2) Il cloro attivo generato da cloruro di sodio mediante elettrolisi è stato oggetto di una valutazione ai fini del suo uso nei biocidi del tipo di prodotto 2, «disinfettanti per aree private e aree sanitarie pubbliche ed altri biocidi», del tipo di prodotto 3, «biocidi per l'igiene veterinaria», del tipo di prodotto 4, «disinfettanti nel settore dell'alimentazione umana e animale», e del tipo di prodotto 5, «disinfettanti per l'acqua potabile», descritti nell'allegato V della direttiva 98/8/CE del Parlamento europeo e del Consiglio ([3]), che corrispondono rispettivamente ai tipi di prodotto 2, 3, 4 e 5, descritti nell'allegato V del regolamento (UE) n. 528/2012.

(3) Il 19 novembre 2010 l'autorità di valutazione competente della Slovacchia, che è stata designata come Stato membro relatore, ha presentato alla Commissione le relazioni di valutazione, insieme alle sue conclusioni.

(4) Il 16 giugno 2020, in conformità all'articolo 7, paragrafo 2, del regolamento delegato (UE) n. 1062/2014, i pareri dell'Agenzia europea per le sostanze chimiche ([4]) («l'Agenzia») sono stati adottati dal comitato sui biocidi, tenendo conto delle conclusioni dell'autorità di valutazione competente.

(5) Secondo tali pareri, i biocidi dei tipi di prodotto 2, 3, 4 e 5 che usano cloro attivo generato da cloruro di sodio mediante elettrolisi possono essere considerati conformi ai criteri stabiliti all'articolo 5 della direttiva 98/8/CE, purché siano rispettate determinate specifiche e condizioni relative al loro uso.

(6) Tenendo conto dei pareri dell'Agenzia, è opportuno approvare il cloro attivo generato da cloruro di sodio mediante elettrolisi ai fini del suo uso nei biocidi dei tipi di prodotto 2, 3, 4 e 5, subordinatamente al rispetto di determinate specifiche e condizioni.

(7) Prima dell'approvazione di un principio attivo è opportuno prevedere un periodo ragionevole, al fine di consentire alle parti interessate di adottare le misure preparatorie necessarie a soddisfare le nuove prescrizioni.

(8) Le misure di cui al presente regolamento sono conformi al parere del comitato permanente sui biocidi,

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([1]) GU L 167 del 27.6.2012, pag. 1.

([2]) Regolamento delegato (UE) n. 1062/2014 della Commissione, del 4 agosto 2014, relativo al programma di lavoro per l'esame sistematico di tutti i principi attivi esistenti contenuti nei biocidi di cui al regolamento (UE) n. 528/2012 del Parlamento europeo e del Consiglio (GU L 294 del 10.10.2014, pag. 1).

([3]) Direttiva 98/8/CE del Parlamento europeo e del Consiglio, del 16 febbraio 1998, relativa all'immissione sul mercato dei biocidi (GU L 123 del 24.4.1998, pag. 1).

([4]) *Biocidal Products Committee Opinions on the application for approval of the active substance: active chlorine generated from sodium chloride by electrolysis, Product type: 2, 3, 4 and 5* (Pareri del comitato sui biocidi riguardo alla domanda di approvazione del principio attivo cloro attivo generato da cloruro di sodio mediante elettrolisi; tipi di prodotto: 2, 3, 4 e 5); ECHA/BPC/251, 252, 253, 254, adottati il 16 giugno 2020.

HA ADOTTATO IL PRESENTE REGOLAMENTO:

Articolo 1

Il cloro attivo generato da cloruro di sodio mediante elettrolisi è approvato come principio attivo ai fini del suo uso nei biocidi dei tipi di prodotto 2, 3, 4 e 5, fatte salve le specifiche e le condizioni di cui all'allegato.

Articolo 2

Il presente regolamento entra in vigore il ventesimo giorno successivo alla pubblicazione nella *Gazzetta ufficiale dell'Unione europea*.

Il presente regolamento è obbligatorio in tutti i suoi elementi e direttamente applicabile in ciascuno degli Stati membri.

Fatto a Bruxelles, il 25 febbraio 2021

Per la Commissione
La presidente
Ursula VON DER LEYEN

ALLEGATO

Nome comune	Denominazione IUPAC, numeri di identificazione	Grado minimo di purezza del principio attivo (1)	Data di approvazione	Scadenza dell'approvazione	Tipo di prodotto	Condizioni specifiche
Cloro attivo generato da cloruro di sodio mediante elettrolisi	Denominazione IUPAC: non pertinente N. CE: non pertinente N. CAS: non pertinente Precursore: Denominazione IUPAC: Cloruro di sodio N. CE: 231-598-3 N. CAS: 7647-14-5	La specifica per il cloro attivo generato in situ dipende dal precursore cloruro di sodio che deve soddisfare i requisiti di purezza di una delle seguenti norme: NF Brand, EN 973 A, EN 973 B, EN 14805 Tipo 1, EN 14805 Tipo 2, EN 16370 Tipo 1, EN 16370 Tipo 2, EN 16401 Tipo 1, EN 16401 Tipo 2, CODEX STAN 150-1985 o Farmacopea europea 9.0.	1° luglio 2022	30 giugno-2032	2	Le autorizzazioni dei biocidi sono soggette alle seguenti condizioni: a) nella valutazione del prodotto occorre prestare particolare attenzione alle esposizioni, ai rischi e all'efficacia legati a qualsiasi uso previsto nella domanda di autorizzazione, ma non preso in considerazione nella valutazione del rischio del principio attivo condotta a livello di Unione; b) nella valutazione del prodotto occorre prestare particolare attenzione alla protezione degli utilizzatori professionali nelle operazioni di disinfezione di pavimenti e superfici dure mediante spugne o panni umidi.
					3	Le autorizzazioni dei biocidi sono soggette alle seguenti condizioni: a) nella valutazione del prodotto occorre prestare particolare attenzione alle esposizioni, ai rischi e all'efficacia legati a qualsiasi uso previsto nella domanda di autorizzazione, ma non preso in considerazione nella valutazione del rischio del principio attivo condotta a livello di Unione; b) per i prodotti che possono lasciare residui negli alimenti o nei mangimi, occorre verificare la necessità di modificare i livelli massimi di residui (LMR) esistenti o di fissarne di nuovi in conformità al regolamento (CE) n. 470/2009 (2) o al regolamento (CE) n. 396/2005 (3) e adottare le opportune misure di attenuazione del rischio per garantire che gli LMR applicabili non siano superati.
					4	Le autorizzazioni dei biocidi sono soggette alle seguenti condizioni: a) nella valutazione del prodotto occorre prestare particolare attenzione alle esposizioni, ai rischi e all'efficacia legati a qualsiasi uso previsto nella domanda di autorizzazione, ma non preso in considerazione nella valutazione del rischio del principio attivo condotta a livello di Unione;

						b) per i prodotti che possono lasciare residui negli alimenti o nei mangimi, occorre verificare la necessità di modificare i livelli massimi di residui (LMR) esistenti o di fissarne di nuovi in conformità al regolamento (CE) n. 470/2009 o al regolamento (CE) n. 396/2005, e adottare le opportune misure di attenuazione del rischio per garantire che gli LMR applicabili non siano superati.
					5	Le autorizzazioni dei biocidi sono soggette alle seguenti condizioni: a) nella valutazione del prodotto occorre prestare particolare attenzione alle esposizioni, ai rischi e all'efficacia legati a qualsiasi uso previsto nella domanda di autorizzazione, ma non preso in considerazione nella valutazione del rischio del principio attivo condotta a livello di Unione; b) per i prodotti che possono lasciare residui negli alimenti o nei mangimi, occorre verificare la necessità di modificare i livelli massimi di residui (LMR) esistenti o di fissarne di nuovi in conformità al regolamento (CE) n. 470/2009 o al regolamento (CE) n. 396/2005, e adottare le opportune misure di attenuazione del rischio per garantire che gli LMR applicabili non siano superati.

([1]) I requisiti di purezza del precursore indicati in questa colonna corrispondono a quelli forniti nella domanda di approvazione del principio attivo valutato.

([2]) Regolamento (CE) n. 470/2009 del Parlamento europeo e del Consiglio, del 6 maggio 2009, che stabilisce procedure comunitarie per la determinazione di limiti di residui di sostanze farmacologicamente attive negli alimenti di origine animale, abroga il regolamento (CEE) n. 2377/90 del Consiglio e modifica la direttiva 2001/82/CE del Parlamento europeo e del Consiglio e il regolamento (CE) n. 726/2004 del Parlamento europeo e del Consiglio (GU L 152 del 16.6.2009, pag. 11).

([3]) Regolamento (CE) n. 396/2005 del Parlamento europeo e del Consiglio, del 23 febbraio 2005, concernente i livelli massimi di residui di antiparassitari nei o sui prodotti alimentari e mangimi di origine vegetale e animale e che modifica la direttiva 91/414/CEE del Consiglio (GU L 70 del 16.3.2005, pag. 1).

Testing of Anolyte against E-coli, Salmonella spp., Pseudomonas aeruginosa and **Legionella pneumophila** undertaken by:

IPROMA S.L. INVESTIGACION Y PROYECTOS MEDIO AMBIENTE
- EMPRESA COLABORADORA DEL M.O.P.T.M.A. - GRUPO 3
- ACREDITADO POR CONSELLERIA DE SANIDAD DE LA GENERALITAT VALENCIANA, PARA AGUAS POTABLES Y ENVASADAS.
- AUTORIZADO POR CONSELLERIA DE AGRICULTURA, PESCA Y ALIMENTACION GENERALITAT VALENCIANA

ENAC
Entidad Nacional de Acreditación
ENSAYOS
Nº 103 / LE268

Protocollo Test

Microrganismo	Concentrazione	Tempo
Escherichia coli	1,5 x 10^5 ufc/100ml	1, 5, 10 e 30 minuti
Salmonella spp.	1,3 x 10^5 ufc/100ml	1, 5, 10 e 30 minuti
Pseudomonas aeruginosa	1,1 x 10^5 ufc/100ml	5, 10, 30 e 60 minuti
Legionella pneumophila	**1,5 x 10^5 ufc/100ml**	**5, 10, 30 e 60 minuti**

Risultati Test

Tabella 1

ANOLYTE + Bacteria						
Parametro	Unità	Valori iniziali	1 min.	5 min.	10 min.	30 min.
pH	Unità pH	8,1	8,1	8,1	8,1	8,0
Cloro residuale	Unità	mg/l	0,7	0,7	0,7	0,7
Potenziale re-dox	mV	825	-	-	-	-
Escherichia coli	ufc / 100ml	1,5 x 10^5	0	0	0	0
Batteri eterotrofi	ufc / ml	< 1	-	-	-	-

Tabella 2

ANOLYTE + Bacteria						
Parametro	Unità	Valori iniziali	1 min.	5 min.	10 min.	30 min.
pH	Unità pH	8,1	8,1	8,1	8,1	8,0
Cloro residuale	Unità	mg/l	0,7	0,7	0,7	0,7
Potenziale re-dox	mV	825	-	-	-	-
Salmonella spp.	100ml	$1,3 \times 10^5$	NEG	NEG	NEG	NEG
Batteri eterotrofi	ufc / ml	< 1	-	-	-	-

Tabella 3

ANOLYTE + Bacteria						
Parametro	Unità	Valori iniziali	5 min.	10 min.	30 min.	60 min.
pH	Unità pH	8,1	8,1	8,1	8,1	8,0
Cloro residuale	Unità	mg/l	0,7	0,7	0,7	0,7
Potenziale re-dox	mV	825	-	-	-	-
Pseudomonas aeruginosa	100ml	$1,1 \times 10^5$	POS	NEG	NEG	NEG
Batteri eterotrofi	ufc / ml	< 1	150	< 1	< 1	< 1

Tabella 4

ANOLYTE + Bacteria						
Parametro	**Unità**	**Valori iniziali**	**5 min.**	**10 min.**	**30 min.**	**60 min.**
pH	**Unità pH**	**8,1**	**8,1**	**8,1**	**8,1**	**8,0**
Cloro residuale	**Unità**	**mg/l**	**0,7**	**0,7**	**0,7**	**0,7**
Potenziale re-dox	**mV**	**825**	**-**	**-**	**-**	**-**
Legionella pneumophila	**ufc / 100ml**	**$1,5 \times 10^5$**	**1200**	**200**	**0**	**0**
Batteri eterotrofi	**ufc / ml**	**< 1**	**1200**	**200**	**< 1**	**< 1**

Data di Emissione 25/03/2012

RAPPORTO DI PROVA N° **12-842-001**

Descrizione campione	**ANOLYTE NEUTRO - IGIENIZZANTE**		
Cliente	**ACQUA ATTIVA DI MARIO BUZZI** **VIA DANTE 10** **20875 - BURAGO DI MOLGORA, MB**	**Luogo di campionamento**	VIA DANTE 10 20875 - BURAGO DI MOLGORA, MB
Campionatore	SIG. BUZZI	**Data di campionamento**	24/01/2012
Prelevatore	SIG. BUZZI	**Data di prelievo**	24/01/2012
Numero accettazione	12-842	**Data di accettazione**	24/01/2012
Data inizio analisi	22/02/2012	**Data fine analisi**	23/02/2012
Note campione	-		

E' stata eseguita una prova di efficacia del prodotto Anolyte neutro nei confronti di specie di Staphylococchi , proveniente da ceppo ATCC e isolati da piscine.
Il prodotto è stato utilizzato alle concentrazioni solitamente utilizzate nell'ambiente piscina (0,7mg/l di cloro libero e 0,99 mg/l di cloro libero) e i campioni di acqua sono stati inoculati con concentrazioni diverse di *Staphylococcus aureus ATCC* 6538 e *Staphylococcus species* isolato da piscina , e conservati a temperatura ambiente.
Sono stati eseguiti i conteggi degli Staphylococchi al tempo 0 e dopo 1 ora dall'inoculo.Prima dell'inoculo è stata misurata anche la concentrazione del Cloro libero con l'apposito kit della Vwr.

Vedi tabella successiva.

Data di Emissione 25/03/2012

RAPPORTO DI PROVA N° 12-842-001

Valutazione efficacia ANOLYTE								
	Staphylococcus aureus ATCC 6538	*Staphylococcus aureus ATCC 6538*	*Staphylococcus aureus ATCC 6538*	*Staphylococcus aureus ATCC 6538*	*Staphylococcus spp.da piscina*	*Staphylococcus spp.da piscina*	*Staphylococcus spp.da piscina*	*Staphylococcus spp.da piscina*
concentrazione microbica UFC/100ml	178	178	416	416	368	368	522	522
concentrazione Anolyte(Cloro libero) mg/l	0,7	0,99	0,7	0,99	0,7	0,99	0,7	0,99
UFC/50 ml t=0	<1	8	61	<1	<1	<1	84	<1
UFC/100 ml t=0	<1	16	122	<1	<1	<1	168	<1
UFC/100 ml t=1 ora	<1	<1	<1	<1	<1	<1	<1	<1

Note: come si vede dalla tabella gli staphylococchi sono inibiti molto efficaciemente dal prodotto Anolyte nella normali condizioni d'uso; in particolare alla concentrazione di 0,99 mg/l di cloro libero i microorganismi sono inibiti immediatamente, mentre alla concentrazione più bassa (0,70 mg/l) l'inibizione avviene entro un'ora.

Dott.ssa Marina Camporese
Responsabile Sett.Microbiologia

CAMPORESE MARINA N. 34312

 # dati e informazioni forniti dal cliente ed inseriti per completezza
◊ livello di confidenza al 95%, fattore di copertura 2
N.A. non applicabile
Il presente RAPPORTO DI PROVA si riferisce esclusivamente ai soli campioni sottoposti a prova e non può essere riprodotto parzialmente salvo approvazione scritta del laboratorio.
Tempo di conservazione dei campioni: i campioni sono conservati presso il laboratorio 30 giorni dopo l'emissione del rapporto di prova (ad eccezione dei prodotti deperibili che sono eliminati al termine dell'analisi o a scadenza).
Per stoccaggi superiori al mese dovrà essere fatta specifica richiesta.
Tempi di conservazione delle registrazioni: il laboratorio conserva copia dei rapporti di prova per un periodo di 4 anni e copia delle registrazioni relative alle analisi per 4 anni, salvo richieste particolari del cliente; tutti i documenti relativi alle prove per omologazione dei prodotti sono conservati per 10 anni.

Letters in Applied Microbiology 2005, 40, 341–346 doi:10.1111/j.1472-765X.2005.01679.x

Inactivation of Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa and Staphylococcus aureus on stainless steel and glass surfaces by neutral electrolysed water

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ABSTRACT

M.A. DEZA, M. ARAUJO AND M. J . GARRIDO. 2005.

Aim: To ascertain the efficacy of neutral electrolysed water (NEW) in reducing *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Listeria monocytogenes* on glass and stainless steel surfaces. Its effectiveness for that purpose is compared with that of a sodium hypochlorite (NaClO) solution with similar pH, oxidation–reduction potential (ORP) and active chlorine content.

Methods and Results: First, the bactericidal activity of NEW was evaluated over pure cultures (8.5 log CFU ml^{-1}) of the abovementioned strains: all of them were reduced by more than 7 log CFU ml^{-1} within 5 min of exposure either to NEW (63 mg l^{-1} active chlorine) or to NaClO solution (62 mg l^{-1} active chlorine). Then, stainless steel and glass surfaces were inoculated with the same strains and rinsed for 1 min in either NEW, NaClO solution or deionized water (control). In the first two cases, the populations of all the strains decreased by more than 6 log CFU 50 cm^{-2}. No significant difference ($P \leq 0.05$) was found between the final populations of each strain with regard to the treatment solutions (NEW or NaClO solution) or to the type of surface.

Conclusions: NEW was revealed to be as effective as NaClO at significantly reducing the presence of pathogenic and spoilage bacteria (in this study, *E. coli, L. monocytogenes, P. aeruginosa and S. aureus*) on stainless steel and glass surfaces.

Significance and Impact of the Study: NEW has the advantage of being safer than NaClO and easier to handle. Hence, it represents an advantageous alternative for the disinfection of surfaces in the food industry.

Keywords: disinfectant, *Escherichia coli*, glass, *Listeria monocytogenes*, neutral electrolysed water, *Pseudomonas aeruginosa, Staphylococcus aureus*, stainless steel, surfaces.

INTRODUCTION

Cross contamination via inanimate surfaces is an important factor in food-borne infections. Pathogens can easily spread from contaminated food or surfaces to cooking utensils, surfaces and other food products (Bradford et al. 1997;

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Kusumaningrum et al. 2003). Many pathogenic and spoilage bacteria can attach to surfaces commonly found in food production systems, and become less susceptible to sanitizers and other antimicrobial agents (Frank and Koffi 1990; Lee and Frank 1991). Staphylococcus aureus, Listeria monocytogenes and Escherichia coli can be found within biofilms in food processing plants (Blackman and Frank 1996; Poulsen 1999; Sharma and Anand 2002). Pseudomonas spp. Are spoilage bacteria commonly found in food industries.

Pseudomonas aeruginosa – an opportunistic pathogen – has a high tendency to form biofilms, and a known resistance to disinfectant action (reviewed by Poulsen 1999).

The occurrence of food-borne disease outbreaks can be significantly reduced through frequent cleaning and disinfection of surfaces in contact with food, an effective means to prevent biofilm formation and cross contamination.

Although the spectrum of disinfectants used in food industries is wide – it includes quaternary ammonium compounds, amphoteric products, biguanides, iodophores, peroxo acids, etc. (Taylor et al. 1999; Rossoni and Gaylarde 2000) – chlorine-containing compounds are frequently resorted to as they are effective against bacteria, and require short to moderate contact time (Kim et al. 2000a,b). Sodium hypochlorite (NaClO) being one of the most widely used disinfectants in household sites and food industries, has the disadvantage of being highly unstable as the active chlorine concentration in the solution rapidly decreases during storage (Qin et al. 2002). Besides, the handling of concentrated NaClO is a potential hazard for workers.

In recent years, acidic electrolysed water (AEW) and neutral electrolysed water (NEW) have been introduced for application as sanitizers. These solutions are generated by electrolysis of a dilute NaCl solution passing through the anode of a membrane electrolyser. AEW has a low pH (2–4) and a high oxidation–reduction potential (ORP >1000 mV); it contains active oxidizers like hypochlorous acid (Len et al. 2000) and has a strong bactericidal effect on most known pathogenic bacteria (Venkitanarayanan et al. 1999; Kim et al. 2000a,b). However, it has some disadvantages: it is potentially corrosive for processing equipment and irritating for hands, and has a short storage life because of chlorine loss (Len et al. 2002; Nagamatsu et al. 2002).

NEW is produced in a similar way as AEW, but then it is partially mixed with OH^- which is transferred through the membrane into the cathode chamber. This produces a neutral solution (pH 8 ± 0.5; ORP >700 mV) in which the main biocidal reagents are **HOCl**, $\mathbf{ClO^-}$, $\mathbf{HO_2}$ and $\mathbf{^*O_2}$. Because of its neutral pH, NEW does not contribute as aggressively to the corrosion of metallic surfaces as AEW does; moreover, NEW is more stable than AEW because chlorine loss is significantly reduced. Taking these facts into account, the use of NEW for the disinfection of contact surfaces in the food industry represents an advantage over that of AEW.

NEW has been proved effective in reducing microbial counts on fresh-cut vegetables (Izumi 1999) and in removing biofilms in dental unit water lines (Marais and Bro¨zel 1998).

Previous studies in our laboratory have demonstrated that rinsing in NEW (89 mg l^{-1}) is an effective method to control the presence of pathogenic bacteria on the surface of fresh tomatoes, without affecting their organoleptic characteristics (Deza et al. 2003).

The objective of this study was to evaluate the efficacy of NEW in reducing E. coli, P. aeruginosa, S. aureus and L. monocytogenes populations on glass and stainless steel surfaces. Its activity was also compared with that of a NaClO solution of the same active chlorine content.

MATERIALS AND METHODS

Bacterial cultures

The strains used for this study were obtained from the Spanish Type Culture Collection (CECT): E. coli CECT 405 (ATCC strain 10536, proposed for testing antibiotics, antimicrobial preservatives and chemotherapeutic agents), P. aeruginosa CECT 116 (ATCC 15442, proposed for testing slimicides and disinfectants), S. aureus CECT 239 (ATCC 6538, proposed for testing surface sanitizers and antimicrobial agents) and L. monocytogenes CECT 4032 (isolated from soft cheese, associated with a case of meningitis). Strains were cultured on TSA plates [tryptone soya broth (Panreac

Quimica SA, Barcelona, Spain) with the addition of 15 gl^{-1} agar Nº 3 (Oxoid, Basingstoke, Hampshire, UK)] at 37ºC for 24 h.
For the inoculation of surfaces, bacteria were harvested from the TSA plates with a sterile glass bent rod and resuspended in 50 ml of tryptone–sodium chloride solution (pH 7.2 ± 0.2), to obtain a suspension of 9–10 log CFU ml^{-1}.
The bacterial population of each inoculum was confirmed by pouring 1 ml of appropriate dilutions of the suspension (using the same solution) on duplicate TSA plates, further incubated at 37ºC for 24 h.

Preparation of treatment solutions

NEW was generated using a Envirolyte el-900 unit (Envirolyte Industries International LTD, Tallinn, Estonia). A 25% NaCl solution and tap water were simultaneously pumped into the generator to obtain amperage of 32 ± 2 A. For this study, NEW (containing approximately 400 mg l^{-1} of active chlorine) was diluted in deionized sterile water, to obtain a final active chlorine concentration of about 60 mg l^{-1}. The NaClO solution was prepared by mixing concentrated NaClO (Panreac Quimica SA) and deionized water to obtain a final active chlorine concentration of about 60 mg l^{-1}. Deionized sterile water was used as control. The properties of the treatment solutions were measured immediately after preparation:
pH and ORP were measured with a pH/ion/conductivity meter (CRISON micro-pH 2001; Crison Instruments, Barcelona, Spain), using respectively a pH electrode (CRISON, 52–11) and an ORP electrode (CRISON platinum Ag/AgCl electrode, 52–61). Active chlorine concentration was measured by an iodometric method (APHA 1998).

Treatment of pure culture

Nine millilitre of either NEW or NaClO solution – containing each approximately 60 mg l^{-1} active chlorine – were added to sterile tubes containing 1 ml of bacterial culture of about 8.5 log CFU ml^{-1}. The tubes were handshaken to mix the resultant suspension, and incubated at room temperature (23 ± 2_C) for 5 min. Deionized water was used as control. Following treatment, 1 ml of each sample was transferred to 9 ml of neutralizing solution (sodium thiosufate 0.5%) and the suspension hand-shaken.
After 5 min of neutralization, 1 ml of the appropriate dilution in tryptone-sodium chloride solution was poured on duplicate TSA plates. The plates were incubated at 37 ± 1ºC for 24 h. The experiment was repeated four times.

Preparation and inoculation of surfaces

The test surfaces used in this study were 50 cm^{-2} portions of glass and stainless steel, respectively. Before inoculation, the surfaces were washed with detergent (Procter and Gamble, Newcastle-upon-Tyne, UK), rinsed in deionized water and autoclaved at 121ºC for 20 min. Then the surfaces were immersed for 20 min in the bacterial suspension, and dried for 15 min at room temperature (23 ± 2ºC) in individual sterile metallic strainers, under sterile air, in a laminar flow cabinet. The initial population on the surface was obtained by swabbing one face (50 cm^{-2}) of an inoculated air-dried surface with a sterile cotton swab.
The swab was washed in 5 ml of sterile tryptone-sodium chloride solution, and 1 ml of appropriate dilutions of this solution was plated onto duplicate TSA plates as described above.

Washing treatment

Inoculated surfaces were placed in individual sterile bags containing 250 ml of electrolysed neutral water, NaClO solution or sterile deionized water (control). The bags were shaken gently by hand for 60 s. After immersion, surfaces were removed with sterile tongs and water was allowed to drain completely. One face of the surface was swabbed with a sterile cotton swab. The swab was washed in 5 ml of neutralizing solution and appropriate dilutions of this solution were plated onto TSA plates.
Immediately after removing the surface from the solution, 1 ml of sodium thiosulfate 20% was added to the washing solutions and bags were hand-shaken for 30 s to allow mixing. After 5 min of neutralization, the number of viable cells in the washing solutions after treatment was determined through serial dilutions in 9 ml of tryptone-sodium chloride solution followed by plating 1 ml on duplicate TSA plates. For enrichment, 5 ml of each treatment solution was transferred to separate bottles containing 50 ml of sterile TSB and incubated at 37ºC for 24 h. All the experiments were conducted at room temperature (23 ± 2ºC).

Data analysis

All trials were repeated at least four times.
Microbial counts were expressed as log CFU ml^{-1} (washing solutions and inocula) or log CFU 50

cm^{-2} (surface). The reported values of plate count or physicochemical properties are the mean values of four individual trials ± standard deviation. Data were subjected to ANOVA and Duncan's multiple range tests using STATGRAPHICS (Statistical Graphics Corporation, Englewood Cliffs, CO, USA). Significant differences in plate count data were established by the least significant difference at the 0.05 level of significance.

RESULTS

The mean pH values for the NEW and NaClO solutions and the deionized water used in this study were respectively 7.79 ± 0.20, 8.10 ± 0.22 and 6.45 ± 0.50. The respective ORP values were 774.0 ± 7.0, 739.0 ± 11.0 and 650.0 ± 10.0. The mean active chlorine concentration of the NEW and NaClO solutions was respectively 63 ± 4.56 and 62 ± 5.60. No active chlorine was detected in deionized water.

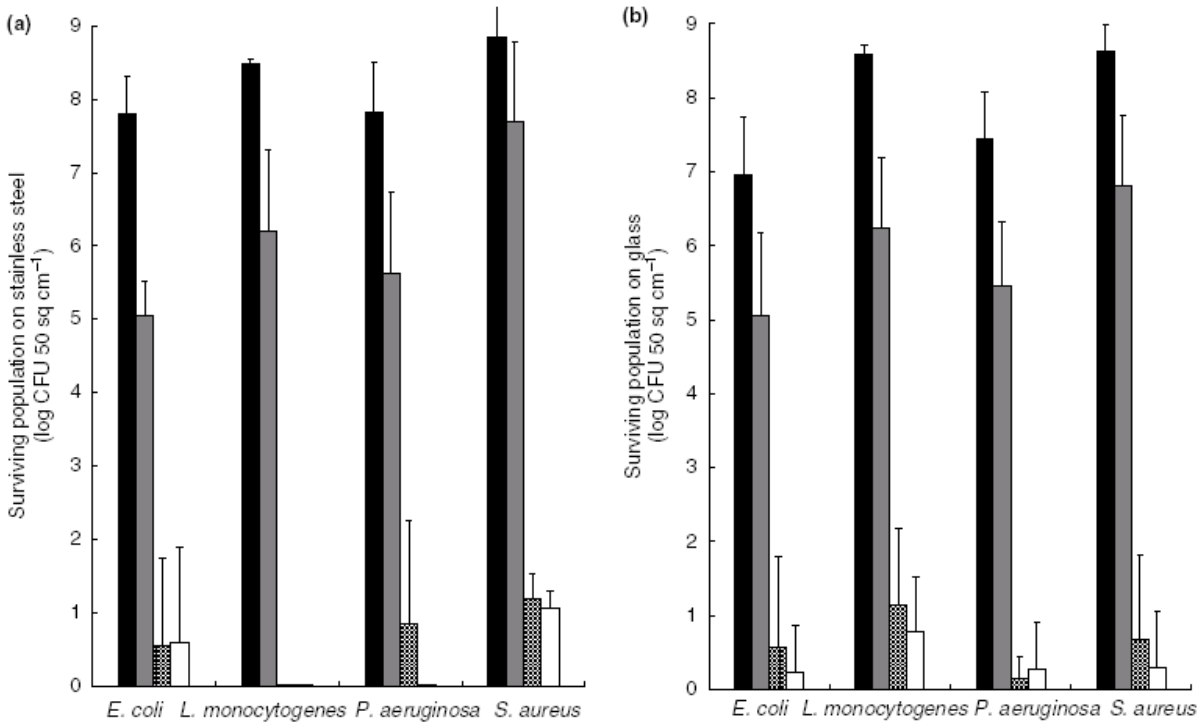
Pure cultures of about 7.5 log CFU ml^{-1} of E. coli, L. monocytogenes, P. aeruginosa and S. aureus, were reduced to undetectable levels – as determined by plating procedures – after 5 min of exposure to NEW (63 mg l^{-1} active chlorine) or NaClO solution (62 mg l^{-1} active chlorine). No significant reduction in bacterial counts was achieved in the control samples.

Figure 1 reports the results of the studies undertaken to determine the efficacy of NEW and NaClO solution in inactivating E. coli, L. monocytogenes, P. aeruginosa and S. aureus on stainless steel and glass surfaces. The initial population of all strains on inoculated surfaces (after drying for 15 min) was between 6.96 and 8.84 log CFU 50 cm^{-2}.

Washing inoculated surfaces for 1 min in either NEW or NaClO solutions decreased the populations of all strains on both materials by more than 6 log CFU 50 cm^{-2}, whereas the control treatment (sterile deionized water) resulted in a reduction of c. 2.1 log CFU 50 cm^{-2} for all strains. For each strain, no significant difference ($P \leq 0.05$) was found between final populations with regard to treatment (NEW or sodium hypochlorite) or surface material.

Table 1 shows the surviving populations of E. coli, L. monocytogenes, P. aeruginosa and S.

Fig. 1 Inactivation of Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, and Staphylococcus aureus on (a) stainless steel and (b) glass surfaces by neutral electrolysed water (NEW; 63 ± 4.56 mg l^{-1} active chlorine) and sodium hypochlorite solution (NaClO; 62 ± 5.6 mg l^{-1} active chlorine) at 23 ± 2ºC. Results are expressed as mean ± SD of at least four repeated measurements. Zero values indicate no detectable survivors by direct plating procedure, ▮ before treatment; ▩ control (water); ▨ NaClO; ◘ NEW



aureus in washing solutions after 1 min treatment of stainless steel and glass surfaces. The surviving populations of all bacteria in NEW washing solution were <1 log CFU ml^{-1} after treating both surfaces and in some cases no survivors could be detected. Instead, an average surviving population of 1.56 log CFU ml^{-1} of S. aureus was still detectable in NaClO washing solutions after treating surfaces of both materials. On the basis of the surviving populations of S. aureus in washing solutions after treating inoculated stainless steel surfaces, NEW efficacy was significantly higher ($P \leq 0.05$) to that of the NaClO solution. In control water, an average of 6 log CFU ml^{-1} of all strains was recovered, after treating both surfaces.

Table 1 Surviving population of Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, and Staphylococcus aureus in washing solutions (NEW, NaClO and deionized water) after treatment of stainless steel and glass surfaces

Strain	Surface	Surviving population in washing solution (log CFU ml^{-1})		
		NEW	NaClO	Water (control)
E. coli	Stainless steel	0·36 ± 0·54	0*	5·59 ± 0·71
	Glass	0	0·58 ± 1·30	5·94 ± 0·45
L. monocytogenes	Stainless steel	0·18 ± 0·35	0·39 ± 0·78	5·81 ± 0·59
	Glass	0·19 ± 0·43	0·58 ± 1·30	6·14 ± 0·35
P. aeruginosa	Stainless steel	0·56 ± 1·12	0·08 ± 0·15	6·00 ± 0·50
	Glass	0	0	5·87 ± 0·72
S. aureus	Stainless steel	0·27 ± 0·20	1·97 ± 1·00	6·52 ± 0·23
	Glass	0·24 ± 0·39	1·15 ± 1·33	6·17 ± 0·66

Values are the mean ± SD of at least four repeated measurements.
NEW, neutral electrolysed water.
*Negative by an enrichment procedure and no detectable survivors by a direct plating procedure.

DISCUSSION

The strains of E. coli, S. aureus and P. aeruginosa used in this study are the ones proposed in the European Standard UNE-EN 1276 (Anonymous 1998) for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Formerly, studies were carried out in our laboratories (data not shown) in order to find the minimum concentration of NEW complying with the European standard UNE–EN 1276 (Anonymous 1998), i.e. producing a population reduction of more than 5 log CFU ml^{-1} in all the evaluated strains in pure culture, under clean conditions. On that basis, a NEW concentration such to contain 63 mg l^{-1} active chlorine was chosen to evaluate its efficacy in reducing E. coli, P. aeruginosa, S. aureus and L. monocytogenes on glass and stainless steel surfaces. In the present work, 5 min of exposure to NEW with such concentration produced a reduction of more than 5 logs in the populations of all the strains in pure culture. These results are similar to those obtained by other authors using AEW (with the same active chlorine content) to inactivate pathogens including L. monocytogenes (Venkitanarayanan et al. 1999; Kim et al. 2000a,b) and S. aureus (Park et al. 2002).

With the chosen NEW concentration (63 mg l^{-1} active chlorine) initial populations of about 8 log CFU 50 cm^{-2} on stainless steel and glass surfaces were reduced to 1 log CFU 50 cm^{-2} or less after treating with NEW for 1 min. In addition, the surviving populations of all bacteria in NEW washing solution were <1 log CFU ml^{-1} after treating both surfaces and in some cases no survivors could be detected.

However, the control treatment (sterile deionized water) resulted in a reduction of a more 2.1 log CFU 50 cm^{-2} for all strains on both surfaces and about 6 log CFU ml^{-1} were still recovered from the wash solution.

The results also show that NEW has a broad spectrum of action over the chosen strains: the reductions underwent by all the populations after 1 min treatment showed no significant difference ($P \leq 0.05$). Moreover, that was so on both materials.

The results obtained in this study using NEW (63 mg l^{-1} active chlorine) during a contact time of 1 min, were similar to those obtained by Park et al. (2002) in inactivating S. aureus and Enterobacter aerogenes on different surfaces (including stainless steel and glass) using AEW containing 53 mg l^{-1} chlorine after an immersion of 5 min. This suggests that NEW might be as effective on other surfaces as well, with the advantage of being less corrosive to metallic surfaces, having a longer storage life and being safer to the environment as well as to operators because at neutral pH no chlorine gas is produced. Sodium hypochlorite is one of the most widely used disinfectants in household sites and food industries. In this

study the effectiveness of NEW was compared with that of NaClO solution with the same active chlorine content, and similar pH and ORP. The obtained results reveal that the bactericidal efficacy of NEW on glass and stainless steel surfaces is similar to that of NaClO. In washing solutions, NEW efficacy was significantly higher ($P \leq 0.05$) to that of NaClO when treating stainless steel surfaces inoculated with S. aureus.compared with NaClO, NEW has several advantages: (i) transport and storage problems are reduced as it is generated on-site; (ii) it is potentially less hazardous for workers, as no concentrated chemicals must be handled; (iii) its potential adverse impact on the environment is much less, as it is produced from pure water (without added chemicals except NaCl); (iv) it is much more stable than concentrated NaClO, where the active chlorine concentration rapidly decreases during storage (Qin et al. 2002). These facts lead to suggest that NEW can be considered as a suitable alternative to NaClO for the disinfection of surfaces in the food industry.

In conclusion, this study revealed that NEW is an effective method to significantly reduce the presence of pathogenic and spoilage bacteria like E. coli, L. monocytogenes, P. aeruginosa and S. aureus on stainless steel and glass surfaces. NEW efficacy in reducing bacterial populations on both surfaces was comparable with that of a NaClO solution of similar pH, ORP and active chlorine concentration, with the advantage of being a safe and easy-to-handle option.

ACKNOWLEDGEMENTS

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Risultati delle indagini dell'effetto disinfettante dell'Anolyte prodotto dal dispositivo Envirolyte EL-900 delle Envirolyte Industies International Ltd. di Legionella

Istituto per l'Igiene e l'ambiente
Steinstraße 10, D-33457 Lollar, Germania
Luglio 2001
Dr. J. Prucha

Cliente:
Envirolyte Industries International Ltd., Tallinn

Data dell'ordine:

Relazione Nr. G01-0480.doc
Lollar, 12.07.2001

Introduzione e scopo della relazione

Nell'ambito di questa perizia si vuole esaminare l'effetto disinfettante per mezzo di reattori diaframmatici all'interno dello strumento Envirolyte dell'Envirolyte Industries International Ltd. del dato Anolyte di legionella.

Produzione dell'Anolyte neutrale

L'apparecchio ENVIROLYTE EL-900 produce Anolyte neutro. Nella serie di esperimenti la corrente è stata regolata a circa 30 ampere. Dopo un tempo di funzionamento di più di un'ora, l'Anolyte neutro è stato rimosso per ulteriori indagini.

- cloro libero: 300 mg/l
- valore di pH: 8,20
- conducibilità elettrica: 11.700 µS/cm
- Potenziale di riduzione: 790mV

Realizzazione degli esperimenti

Per effettuare i test abbiamo ricevuto in dotazione dal Signor van Schaik il dispositivo Envirolyte EL-900. Il materiale per la prova di produzione dell'Anolyte aveva una concentrazione di cloro libero di 300 mg/l.
L'organismo batterico utilizzato per gli esperimenti
- Legionella pneumophilia
è stato fissato in un terreno di Legionella.

Mediante la diluizione della sospensione risultante con acqua del rubinetto della città di Lollar, sono state preparate le soluzioni batteriche necessarie per gli esperimenti.
Ogni volta gli esperimenti sono stati realizzati con 100 ml di acqua di rubinetto e una concentrazione batterica di 300 UFC/ml.
Per determinare quando si verifica un effetto disinfettante con l'aggiunta dell'Anolyte, sono state preparate diverse concentrazioni di cloro libero che andavano da 20 mg/l a 80 mg/l.

Poi, dopo

- 1 minuto,

- 5 minuti,
- 10 minuti,
- 30 minuti e
- 60 minuti

sono stati prelevati 10 ml di soluzione, che sono stati trattati con tiosolfato di sodio, un reagente che distrugge il cloro libero, per evitare che il cloro libero potesse rimanere efficace anche dopo il prelievo del batterio.
Ogni volta, infine, sono stati posti 0,05 ml in agar per Legionella e incubate per una settimana a 36° C.

Approccio: 2 colonie in 5 ml di terreno di Legionella, tutte le diluizioni sono state eseguite con acqua di rubinetto sterile.

Test con soluzione Anolyte e test del batterio Legionella pneumophila

Gli esperimenti sono stati eseguiti con 100 ml di acqua di rubinetto e

- 0,1 ml di sospensione batterica stock.

Per gli approcci sperimentali sono state preparate delle concentrazioni di cloro libero nelle seguenti quantità:

- 20 mg/l
- 40 mg/l
- 60 mg/l
- 80 mg/l

Poi, dopo

- 1 minuto,
- 5 minuti,
- 10 minuti,
- 30 minuti e
- 60 minuti

sono stati prelevati 10 ml di soluzione che sono stati trattati con tiosolfato di sodio, un reagente che distrugge il cloro libero, per evitare che il cloro libero potesse rimanere efficace anche dopo il prelievo del batterio.

Ogni volta, infine, sono stati posti 0,05ml di soluzione in agar per Legionella e incubati per una settimana a 36° C.

Risultato delle indagini

Approccio sperimentale con 0,1 ml di sospensione batterica in 100 ml di acqua di rubinetto a diverse concentrazioni di cloro

Concentrazione batterica iniziale del lotto: 300 UFC/ml = 30.000 ufc/100 ml

0,7 ml Anolyte : 20 mg/l cloro libero

dopo X minuti	KBE/ml	KBE/100ml
1	> 20.000	> 2.000.000
5	> 20.000	> 2.000.000
10	> 20.000	> 2.000.000
30	> 20.000	> 2.000.000
60	> 20.000	> 2.000.000

1,3 ml Anolyte : 40 mg/l cloro libero

dopo X minuti	UFC/ml	UFC/100ml
1	n.a.	n.a.
5	> 20.000	> 2.000.000
10	> 20.000	> 2.000.000
30	80	8000
60	0	0

2,0 ml Anolyte : 60 mg/l cloro libero

dopo X minuti	UFC/ml	UFC/100ml
1	n.a.	n.a.
5	280	28.000
10	100	10.000
30	60	6000
60	0	0

n.a. = non dato/percepito/rilevato

2,6 ml Anolyte : 80 mg/l cloro libero

dopo X minuti	UFC/ml	UFC/100ml
1	0	0
5	0	0
10	0	0
30	0	0
60	0	0

Valutazione

Nell'ambito di questi test, dell'Anolyte, che era stato prodotto da un dispositivo Envirolyte EL-900, è stato diluito con diverse concentrazioni di cloro da 20 a 80 mg/l.
Le soluzioni diluite di Anolyte così preparate sono state quindi inoculate con Legionella pneumophila.
Poi, a determinati intervalli di tempo da 1 a 60 minuti sono stati prelevati campioni per verificare l'effetto disinfettante delle varie concentrazioni di cloro.

La concentrazione dei batteri di Legionella pneumophila in questa serie di esperimenti è stata di 30.000 UFC/100 ml.

A questa concentrazione batterica relativamente bassa è stato osservato un effetto di riduzione del batterio solo ad una concentrazione di cloro di 40 mg/l, e tempo di reazione di 30 min.

Con l'aumentare della concentrazione di cloro e l'aumentare della durata della sperimentazione, la concentrazione batterica iniziale può essere tanto ridotta che a una concentrazione di cloro di 80 mg/l non erano rilevabili batteri.

Dr. J. Prucha Dr. W. Schulz

Ergebnisse der Untersuchung des Desinfektionseffektes des Anolytes vom Gerät Envirolyte EL-900 der Envirolyte Industries International Ltd. auf Legionellen

Institut für Hygiene und Umwelt,
Steinstraße 10, D-33457 Lollar, Germany
Juli 2001
Dr. J. Prucha

Auftraggeber:
Envirolyte Industries International Ltd., Tallinn

Auftragsdatum:

Gutachten Nr. G01-0480.doc
Lollar, 12.07.2001

Einführung und Ziel des Gutachtens

Im Rahmen dieses Gutachtens soll die desinfizierende Wirkung mittels Diaphragmatischen Reaktoren innerhalb des Gerätes Envirolyte[1)] der Envirolyte Industries International Ltd. hergestellten Anolytes auf Legionellen überprüft werden.

Herstellung des neutralen Anolyts

Das Gerät ENVIROLYTE EL-900 produziert einen neutralen Anolyt. Bei der Versuchsreihe wurde eine Stromstärke von ca. 30 A eingestellt. Nach einer Laufzeit von mehr als einer Stunde wurde der neutrale Anolyt für weitere Untersuchungen entnommen.

- freies Chlor: 300 mg/l
- pH-Wert: 8,20
- el.Leitfähigkeit: 11.700 µS/cm
- Redoxpotential: 790mV

Durchführung der Versuche

Zur Durchführung der Versuche wurde uns von Herrn van Schaik das Gerät Envirolyte EL-900 zur Verfügung gestellt. Der frisch für die Versuche produzierte Anolyt hatte eine Konzentration von freiem Clor von 300 mg/l.

Der für die Versuche verwendete Testkeim

- Legionella pneumophilia

wurde in Legionellen-Medium angesetzt.

Durch Verdünnung der so erhaltenen Keim-Stammsuspension mit sterilem Leitungsswasser der Stadt Lollar wurden daraus die für die Versuche benötigte Keim-Lösung hergestellt.

Die Versuche wurden mit jeweils 100 ml Leitungswasser und einer Keimkonzentration von 300 KBE/ml durchgeführt.
Um festzustellen, wann ein Desinfektionseffekt auftritt, wurden durch die Zugabe des Anolytes verschiedene Konzentrationen an freiem Chlor im Bereich von 20 mg/l bis 80 mg /l eingestellt.

Anschließend wurden jeweils nach

- 1 Minute,
- 5 Minuten,
- 10 Minuten,
- 30 Minuten und
- 60 Minuten

dem Ansatz 10 ml entnommen und sofort mit Natriumthiosulfat, ein freies Chlor vernichtendes Reagenz, versetzt, um auszuschließen, daß das freie Chlor noch nach Probeentnahme weiterwirken kann. Anschließend wurden davon jeweils 0,05 ml auf Legionellen-Agar gebracht und über eine Woche bei 36°C bebrütet.

Ansatz : 2 Kolonien in 5 ml Legionellen- Medium, alle Verdünnungen erfolgten mit sterilem Leitungswasser.

Versuchsdurchführung mit Anolyt-Lösung und Testkeim Legionella pneumophilia

Die Versuche wurden mit jeweils 100 ml Leitungswasser und

- 0,1 ml der Keim-Stammsuspension

durchgeführt.

Für die Versuchsansätze wurden freie Chlor-Konzentrationen in folgenden Bereichen eingestellt:

- 20 mg/l
- 40 mg/l
- 60 mg/l
- 80 mg/l

Anschließend wurden jeweils nach

- 1 Minute,
- 5 Minuten,
- 10 Minuten,
- 30 Minuten und
- 60 Minuten

dem Ansatz 10 ml entnommen und sofort mit Natriumthiosulfat, ein freies Chlor vernichtendes Reagenz, versetzt, um auszuschließen, daß das freie Chlor noch nach Probeentnahme weiterwirken kann. Anschließend wurden davon jeweils 0,05 ml auf Legionellen-Agar gebracht und über eine Woche bei 36°C bebrütet.

Ergebnis der Untersuchungen

Versuchsansatz mit 0,1 ml Keim-Stammsuspension
in 100 ml Leitungswasser bei verschiedenen Chlor-Konzentrationen

Anfangs-Keimkonzentration im Ansatz: 300 KBE/ml = 30.000 KBE/100 ml

0,7 ml Anolyt : 20 mg/l freies Chlor

nach X Minuten	KBE/ml	KBE/100ml
1	> 20.000	> 2.000.000
5	> 20.000	> 2.000.000
10	> 20.000	> 2.000.000
30	> 20.000	> 2.000.000
60	> 20.000	> 2.000.000

1,3 ml Anolyt : 40 mg/l freies Chlor

nach X Minuten	KBE/ml	KBE/100ml
1	n.a.	n.a.
5	> 20.000	> 2.000.000
10	> 20.000	> 2.000.000
30	80	8000
60	0	0

2,0 ml Anolyt : 60 mg/l freies Chlor

nach X Minuten	KBE/ml	KBE/100ml
1	n.a.	n.a.
5	280	28.000
10	100	10.000
30	60	6000
60	0	0

n.a. = nicht angesetzt

2,6 ml Anolyt : 80 mg/l freies Chlor

nach X Minuten	KBE/ml	KBE/100ml
1	0	0
5	0	0
10	0	0
30	0	0
60	0	0

Beurteilung

Im Rahmen dieser Versuche wurde ein Anolyt, der von einem Envirolyte-Gerät EL-900 frisch hergestellt wurde, so verdünnt, daß verschiedene Chlorkonzentrationen im Bereich von 20 bis 80 mg/l eingestellt wurden. Die so hergestellten verdünnten Anolyt-Lösungen wurden danach mit Legionella pneumophila inokkuliert. Anschließend wurden in bestimmten Zeitabständen von 1 bis 60 Minuten Proben entnommen, um den Desinfektionseffekt bei den verschiedenen Chlorkonzentrationen zu überprüfen.

Die Keimkonzentration an Legionella pneumophila bei dieser Versuchsreihe lag bei 30.000 KBE/100 ml . Bei dieser relativ niedrigen Keimkonzentration konnte ein Keimreduzierender Effekt erst bei einer Chlorkonzentration von 40 mg/l und einer Einwirkzeit von 30 min festgestellt werden.
Mit steigender Chlorkonzentration und mit zunehmender Versuchsdauer konnte die Anfangs-Keimkonzentration so deutlich reduziert werden, daß bei einer Chlorkonzentration von 80 mg/l keine Keime mehr nachweisbar waren.

Dr. J. Prucha Dr. W. Schulz

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Efficienza dell'acqua elettrolizzata nell'inattivazione della Listeria Monocytogenes in sospensione e in biofilm in presenza di materiali organici.

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Si è indagata la capacità dell'acqua elettrolizzata (EO) di inattivare la Listeria monocytogenes in sospensione e in biofilm su acciaio inox in presenza di materia organica (siero di pollo sterile filtrato). Una miscela di cinque ceppi di L. monocytogenes è stata trattata per 1 e 5 min. con acqua deionizzata, acqua EO alcalina e acqua EO acida contenente siero di pollo (0,5 e 10 ml/litro). Anche alcuni coupon contenenti biofilm di L. monocytogenes sono stati coperti con siero di pollo (0, 2.5, 5.0 e 7.5 ml/litro), e poi trattati per 30 e 60 s con acqua deionizzata, con acqua EO alcalina, con acqua EO acida, con acqua EO alcalina seguita da acqua EO acida, e con soluzione di ipoclorito di sodio. Il siero di pollo ha ridotto il potenziale di ossidoriduzione e la concentrazione di cloro dell'acqua EO acida, ma non ha influenzato significativamente il suo pH. In assenza di siero, l'acqua EO acida contenente cloro ad una concentrazione di 44 mg/litro ha prodotto una riduzione di > -6 log nel L. monocytogenes in sospensione, ma la sua attività battericida diminuiva con l'aumentare della concentrazione di siero. L'acqua EO acida e la soluzione di sodio ipoclorito acidificato hanno inattivato il biofilm di L. monocytogenes a livelli simili, e il loro effetto battericida diminuiva con l'aumentare della concentrazione di siero e aumentava con l'aumentare del tempo di esposizione. Il trattamento sequenziale di 30 s con acqua EO alcalina seguita da acqua EO acida ha generato una riduzione tra i 4 e 5 log nel biofilm di L. monocytogenes, anche in presenza di materia organica.

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Miglioramento dell'effetto battericida dell'acqua elettrolizzata su biofilm di Listeria monocytogenes formatosi su acciaio inox.

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I biofilm sono potenziali fonti di contaminazione del cibo negli impianti di trasformazione, perché spesso sopravvivono ai trattamenti disinfettanti durante la pulizia. L'obiettivo di questa ricerca è stato quello di indagare l'uso combinato di acqua elettrolizzata (EO) alcalina e acida per l'inattivazione di biofilm di Listeria monocytogenes sulle superfici in acciaio inox. I biofilm sono stati coltivati per 48 ore a 25 gradi C. su coupon rettangolari (2x5 cm) in acciaio inossidabile (tipo 304, finitura n. 4) con brodo di soia Trittico in diluizione 1:10 che conteneva una

miscela di cinque ceppi di L. monocytogenes. I coupon con i biofilm sono stati poi trattati con acqua EO acida o con acqua EO alcalina o con acqua EO alcalina seguita da acqua EO acida (prodotta a 14 e 20 A) per 30, 60 e 120 s. L'acqua EO alcalina da sola non ha prodotto significative riduzioni nel biofilm di L. monocytogenes in confronto al controllo. Il trattamento con sola acqua EO acida da 30 a 120 s, invece, ha ridotto la popolazione vitale di batteri nel biofilm di 4,3-5,2 log CFU per coupon, mentre il trattamento combinato di acqua EO alcalina seguita da acqua EO acida ha prodotto un'ulteriore riduzione da 0,3 a 1,2 log CFU per coupon. La riduzione di popolazione di L. monocytogenes ottenuta dal trattamento con l'acqua EO acida è aumentata significativamente con l'aumentare del tempo di esposizione. Tuttavia, nessuna differenza significativa si è verificata tra i trattamenti con l'acqua EO prodotta a 14 e 20 A. I risultati suggeriscono che l'acqua EO alcalina ed EO acida possono essere usate insieme per ottenere una inattivazione dei biofilm migliore di quanto non avvenga quando vengono applicate individualmente.

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Decontamination of Aflatoxin-Forming Fungus and Elimination of Aflatoxin Mutagenicity with Electrolyzed NaCl Anode Solution

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Electrolysis of a 0.1% (17.1 mM) solution of NaCl using separate anode and cathode compartments gives rise to solutions containing active chemical species. The strongly acidic "anode solution" (EW(+)) has high levels of dissolved oxygen and available chlorine in a form of hypochlorous acid (HOCl) with a strong potential for sterilization, which we have investigated here. Exposing *Aspergillus parasiticus* at an initial density of 10^3 spores in 10 μL to a 50-fold volume (500 μL) of EW(+) containing ca. 390 μmol HOCl for 15 min at room temperature resulted in a complete inhibition of fungal growth, whereas the cathode solution (EW(−)) had negligible inhibitory effects. Moreover, the mutagenicity of aflatoxin B_1 (AFB_1) for *Salmonella typhimurium* TA-98 and TA-100 strains was strongly reduced after AFB_1 exposure to the EW(+) but not with the EW(−). In high-performance liquid chromatography analysis, the peak corresponding to AFB_1 disappeared after treatment with the EW(+), indicating decomposition of the aflatoxin. In contrast, the routinely used disinfectant sodium hypochlorite, NaOCl, of the same available chlorine content as that of EW(+) but in a different chemical form, hypochlorite (OCl^-) ion, did not decompose AFB_1 at pH 11. However, NaOCl did decompose AFB_1 at pH 3, which indicated that the principle chemical formula to participate in the decomposition of AFB_1 is not the OCl^- ion but HOCl. Furthermore, because the decomposition of AFB_1 was suppressed by pretreating the EW(+) with the OH radical scavenger thiourea, the chemical species responsible for the AFB_1-decomposing property of the EW(+) should be at least due to the OH radical originated from HOCl. The OH in EW(+) was proved by electron spin resonance analysis.

KEYWORDS: ***Aspergillus parasiticus*; electrolyzed NaCl solution; inactivation of aflatoxin B_1; mutagenicity**

INTRODUCTION

Securing the safety of food and animal feed is one of the most important factors in the welfare of humans and livestock. Among the many types of microorganisms that cause food borne disease, toxic fungi and their products (mycotoxins) threaten the health of humans and livestock by contaminating food and feed materials (*1*−*4*). One of the most highly potent mycotoxins, aflatoxin, is produced by *Aspergillus flavus* and *Aspergillus parasiticus* and causes both acute liver damage and liver cancer (*5*−*9*).

Developing measures to control mycotoxin contamination is a high priority for the food and animal feed industries. The most reliable method to prevent mycotoxicosis is to avoid the use of contaminated materials, to disinfect fungi (*10*, *11*), and to inactivate mycotoxin. In the control against aflatoxins, many researchers have proposed and/or attempted measures to inactivate them (*12*, *13*) or to suppress their production by fungi (*14*), for example, through exposure to ammonia vapor at high temperature (*15*). Most of the proposed methods are not necessarily practical, however, because they not only decompose aflatoxin but also deplete the quality of the food and feed materials themselves; furthermore, most proposed methods are expensive and energy-consuming. Therefore, research into effective and energy-saving measures of decomtanimation has attracted much interest recently (*16*, *17*).

With the aim of finding a secure, effective, and energy-saving method to disinfect mycotoxin-producing fungi and to inactivate

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mycotoxin, we examined the effect of an electrolyzed dilute NaCl solution. This solution is generated by electrolyzing dilute NaCl solution, usually around 15−20 mM, with commercially available electrolysis devices at between 3 and 50 V of dc for several minutes in either a single-compartment chamber (*18*) or a two-compartment chamber separated by an ion exchangeable diaphragm (*19*). When the two-compartment chamber is used, a strongly acidic solution, or the "anode solution" (EW(+)), containing hypochlorous acid (HOCl) forms in the anode compartment (*20*). The chemical species that form in the cathode compartment that forms the "cathode solution" (EW(−)) have not been fully characterized yet, but this solution exhibits some antioxidative effects. Miyashita et al. (*21*) reported an antioxidative effect of EW(−) on highly unsaturated fat and oils such as linoenic acid ethyl ester, docosahexaenoic acid ethyl ester, trilinolein, and so on. Shirahata et al. (*22*), on the other hand, reported superoxide dismutase- and catalase-like activities of the EW(−). In an initial test, we found that the EW(+) could successfully disinfect a mycotoxicosis-causing fungus and inactivate its mycotoxins. Here, we report that the EW(+) sterilizes *A. paratisicus* and eliminates the mutagenicity of aflatoxin B_1 (AFB_1) for *Salmonella typhimurium* strains TA-98 and TA-100.

MATERIALS AND METHODS

Chemicals and Reagents. Aflatoxins B_1, B_2, G_1, and G_2 were purchased from Sigma-Aldrich (St. Louis, MO). The chemicals used were high-performance liquid chromatography (HPLC)-grade acetonitrile (Wako Pure Chemicals, Osaka, Japan), HPLC-grade methanol (Wako Pure Chemicals), trifluoroacetic acid (G. R. grade, Nakalai Tesque, Kyoto, Japan), chloroform (G. R. grade, Nakalai Tesque), ethyl acetate (Nakalai Tesque), sodium chloride (G. R. grade, Nakalai Tesque), mannitol (G. R. grade, Wako Pure Chemicals), and thiourea (G. R. grade, Wako Pure Chemicals). The water used for electrolysis and other procedures was purified with a Milli-Q system (Millipore, MA). Other chemicals used were guranteed reagent grade purchased from Nakalai Tesque.

Safety Precautions. Aflatoxins are extremely toxic, mutagenic, and carcinogenic compounds. As a safety precaution, all neat aflatoxin reagents were handled in a glovebox or thoroughly controlled safety cabinet in a P2 level facility. Degradation of aflatoxin solutions was performed by mixing with 10% KOH in ethanol and subsequent autoclaving in the tightly sealed vials. However, after the inactivation effect of the electrolyzed anodic NaCl solution (EW(+)) was confirmed, aflatoxin was mixed with enough volumes of excess EW(+) to destroy aflatoxins in a tightly sealed vial, kept for 30 min, and then subjected to autoclaving at 121 °C for 20 min. Contaminated glassware, vials, tubes, etc. were sealed in high-security disposals, autoclaved at 121 °C for 20 min, and thereafter incinerated.

Preparation of Electrolyzed NaCl Solutions. Electrolyzed NaCl solutions were prepared by electrolyzing 0.1% (17.1 mM) NaCl solution at 9−12 V of direct current (dc) for 10 min using a two-compartment type batch scale electrolysis apparatus (Super Oxseed Labo, Aoi Electronic Corp., Kannami, Shizuoka, Japan) divided by an ion exchangeable diaphragm. Electrolyzed NaCl solutions were prepared 5−10 min before their use in tests to evaluate their disinfectant and decomposition properties against AFB_1-forming fungus and its product aflatoxins.

The general definition of available chlorine includes free form chlorine such as HOCl, Cl_2, and hypochlorite ion (OCl^-) that accept an electron and bound form chlorine such as chloramines. However, considering the fact that the abundance of chemical formulas mentioned above is largely dependent upon pH and pK_a for HOCl = 7.4 (*23*−*25*), we mean the available chlorine for EW(+) and for sodium hypochlorite (NaOCl) kept at pH 3 molar concentration of HOCl, whereas those for NaOCl kept at pH 11 and for EW(−) mean OCl^-. The available chlorine concentration of both the EW(+) and the EW(−) was measured by iodometry and by electrotitration using an "available chlorine meter" (Central Kagaku, Tokyo, Japan). The oxidation/reduction potential (ORP) and pH were also measured. Physicochemical data obtained from five measurements are presented in **Table 1**, and a detailed analysis of the properties of disinfection and deactivation of aflatoxin-forming fungus, *Aspergillus parasiticus*, and its products by the electrolyzed NaCl solutions is given in the Results and Discussion.

Table 1. Physicochemical Parameters of Electrolyzed NaCl Solutions[a]

parameter	anode solution	cathode solution	ultrapure water[b]
	EW(+)	EW(−)	
pH	2.50 ± 0.06	11.65 ± 0.12	5.82 ± 0.04
available chlorine (ppm)	39.4 ± 0.89	0.51 ± 0.08	ND
[mM]	[0.75 ± 0.02]	[0.01 ± 0.002]	[ND]
dissolved oxygen (ppm)	14.4 ± 0.99	1.9 ± 0.11	5.0 ± 0.27
ORP (mv)	1,164 ± 33.62	−878 ± 8.43	264 ± 26.48

[a] Data were presented as the mean ± SD for 5 measurements. Available chlorine for EW(+) represents HOCl in parts per million and millimolar and that for EW(−) in OCl^- anion (ppm and mM). ND, not detected. [b] Data for ultrapure water were obtained from water freshly prepared from a Milli-Q filtration apparatus (Millipore Corp.). Electrolysis was carried out for 10 min at room temperature in a diaphragm type apparatus (Superoxseed Labo) using 17.1 mM (0.1%) NaCl in ultrapure water prepared with a Milli-Q filtration apparatus.

Disinfection of *A. parasiticus* with Electrolyzed NaCl Solutions. *A. parasiticus* IFO-30179 (equivalent to ATCC15517) was used to examine the antimicrobial activity of the electrolyzed solutions against mycotoxin-producing fungus. All experimental procedures were carried out in an isolated safety box for biohazard prevention. Freeze-dried fungus spores, purchased from the Institute of Fermentation Osaka (Dosho-machi, Kita ward, Osaka, Japan), were suspended in sterilized distilled water at a concentration of 10^6 spores/mL and then inoculated into a liquid medium specific for *A. flavus* and *A. parasiticus* (AFPA medium that consisted of 20 g of yeast extract, 10 g of peptone, 0.5 g of ammonium ferric citrate, 100 mg of chloramphenicol, and 2 mg of 2.6-dichloro-4-nitroaniline in 1 L of 1.5% agar). After 2−10 h growth of the fungus, an aliquot was taken to estimate the cell population with an adenosine 5′-triphosphate analyzer (Toa-Electronics Inc., Tokyo), and the culture was diluted with sterilized distilled water to make a stock cell suspension containing 10^5 colony-forming units (cfu)/mL. For the disinfectant experiment, 10 μL containing 10^3 cfu from the stock cell suspension was separately mixed with 0.01, 0.02, 0.1, 0.2, 0.5, and 1 mL of the electrolyzed NaCl solutions. The EW(+) of 0.01−1 mL contained ca. 8, 15, 80, 150, 390, and 800 μmol HOCl, respectively. The estimated concentration of available chlorine in the form of the OCl^- anion in the 1-, 2-, 10-, 20-, 50-, and 100-fold volumes of EW(−) are ca. 0.1, 0.2, 1.1, 2.2, 5.5, and 11 nmol, respectively. In the control test, cell suspensions were exposed to 17.1 mM (0.1%) NaCl. After each exposure for 15 min, sterilized distilled 10 mM mannitol as a terminator was added to the suspension to a volume of 2 mL. Subsequently, 1.0 mL of each sample solution was inoculated on to AFPA agar medium and incubated for 5 days at 25 °C. The disinfection effect was evaluated by the occurrence of fungal colonies on the agar plate. As the fungal growth could not be determined precisely by cfu, the growth was presented semiquantitatively.

Treatment of Aflatoxins with the Electrolyzed NaCl Solutions or NaOCl. AFB_1 and a mixture of aflatoxin B_1, B_2, G_1, and G_2 were dissolved in chloroform in use. Aliquots containing 40 ng of the aflatoxin mixture (10 ng of each aflatoxin) or 10 ng of AFB_1 were placed in vials, and chloroform was evaporated under N_2. Volumes of EW(+) or NaOCl solution (10−2000 μL representing 8−1600 μmol of the active chlorine species) HOCl (*20*) in EW(+), and NaOCl solution kept at pH 3 or OCl^- in NaOCl solution kept at pH 11 were added to tubes containing the aflatoxins so that the molar ratio of "active chlorine" to aflatoxin ranged from 1 to 200. Tubes containing aflatoxins and EW(+) were allowed to sit for 10 min. Similar volumes (10−2000 μl) of EW(−) were added to separate aflatoxin-containing tubes,

but the concentrations of "active chlorine" in the form of OCl^- in these tubes were a one hundredth of those of EW(+) in each case. Samples were then examined for aflatoxin content as described below. Separate experiments with 10 ng of AFB_1 samples were conducted in a similar manner except that incubation times (0, 2.5, 5, 10, and 60 min) and temperatures (0, 20, 30, and 45 °C) were varied. At the end of the incubation periods, absolute ethanol was added to all tubes so that a final volume of 1 mL was achieved. Samples were prepared for and analyzed by HPLC as described below.

Effect of Radical Scavengers. Assuming that reactive chemical species, such as OH radicals and/or Cl radicals originating from HOCl, are involved in the disinfectant process, we examined the effect of pretreatment of the EW(+) before exposure to AFB_1 with mannitol that has been regarded as OH radical specific scavenger (*26*) and thiourea that has been regarded as a universal radical scavenger (*27*, *28*). Mannitol was added to the EW(+) at ratios of 1 to 10 (molar ratio of mannitol to available chlorine as HOCl); similarly, thiourea was added to the EW(+) at ratios of 0.03 to 1 (molar ratio of thiourea to available chlorine as HOCl). Exposure of AFB_1 to the EW(+) containing mannitol or thiourea was performed at room temperature for 10 min**,** and then, the reaction products were extracted with $CHCl_3$ and separated by HPLC to examine changes in AFB_1. See the chromatographic analysis section below for HPLC conditions.

Chromatographic Analyses of Aflatoxins Treated with Electrolyzed NaCl Solutions. *(a) High-Performance Thin-Layer Chromatography (HPTLC).* AFB_1 and a mixture of equal amounts of AFB_1, AFB_2, AFG_1, and AFG_2 that were exposed to different molar ratios of EW(+), EW(−), NaOCl used at pH 11, and NaOCl at pH 3 in the separate experiments as explained in the previous section were subjected to HPTLC. After aflatoxin was exposed to increasing molar ratios of the electrolyzed NaCl solutions, 1.0 mL of ethyl alcohol was added to the mixture, and 0.5 mL of ethyl acetate was added and shaken vigorously to extract aflatoxin and its reaction products into the organic solvent phase. Extraction with ethyl acetate was repeated three times. Ethyl acetate was carefully evaporated off under an N_2 gas stream, and then, 100 μL of fresh ethyl acetate was added. Twenty microliters of the ethyl acetate extract of aflatoxin/electrolyzed solution reaction mixtures and 20 μL of either the AFB_1 solution or the aflatoxin mixture, with or without exposure to electrolyzed solution, were applied to HPTLC plates (10 cm × 10 cm, silica gel 60 precoated plate; Merck Darmstadt, Germany) and developed with a 9:1 (v/v) mixture of $CHCl_3$, acetone for 7 cm. Aflatoxin analogues were detected as a bluish-white spot under UV-A (365 nm) illumination.

(b) HPLC. (b-1) HPLC of Derivatized AFB_1. Following up the results of HPTLC, the change of AFB_1 was further examined quantitatively on HPLC. Because preliminary experiments with HPTLC revealed that exposing EW(+) containing 24−56 μmol HOCl to 1.3 nmol AFB_1 diminished the AFB_1 spot gradually, the decrease of AFB_1 was quantitatively determined by HPLC after derivatization to trifluoroacetic acid. For the sample without any treatment as a control, 400 ng (1.3 nmol) of AFB_1 in 10 μL of ethanol was put in the sealed vial, and then, ethanol was evaporated off under N_2, followed by trifluoroacetylation with 20 μL of trifluoroacetic acid anhydride (G.R grade, Wako Pure Chemicals, Inc.) in a tightly sealed glass vial by vigorous shaking and then incubated for 15 min at room temperature in the dark. Next, 180 μL of a 1:9 (v/v) mixture of acetone/water was added. The solution was further mixed vigorously, and then, 20 μL of the reactant was subjected to HPLC. For exposure to EW(+), 400 ng (1.3 nmol) of AFB_1 in 10 μL of ethanol was taken; then, ethanol was evaporated off under N_2; then, 10, 20, 30, 40, 50, 60, and 70 μL of EW(+) containing 8, 16, 24, 32, 40, 48, and 56 μmol of HOCl, respectively, were added to react with AFB_1 for 15 min. After 15 min of reaction, reacted AFB_1 solutions were evaporated off under vacuum and then trifluoroacetylated by the same procedure as described for the AFB_1 without EW(+) treatment. HPLC was run on a Hitachi high-performance liquid chromatograph equipped with Intelligent Pump (Hitachi L-6200), a Hewlett-Packard HP1046A programmable fluorescence detector and integrator (Hitachi D-2500). Reacted samples were separated on a reversed-phase octadecyl silane column (Zorbax ODS, Du Pont Co.) (4.6 × 250 mm) and eluted with a 35:65 (v/v) mixture of acetonitrile and water at a flow rate of 1.0 mL/min at room temperature. AFB_1 was detected by fluorometry with excitation at 365 nm and emission at 412 nm. Twenty microliters was injected onto the column. Chromatographic analysis was run for three times.

To examine how the elimination of AFB_1 with EW(+) was affected by reaction temperature, free radical scavengers (mannitol or thiourea) AFB_1 were quantified on the HPLC without derivatization to save analysis time.

(b-2) Effect of Reaction Temperature with EW(+) and AFB_1 on the Elimination of AFB_1. To examine the effect of temperature on the deactivation of AFB_1 by EW(+), 25 μg (80 nmol) of AFB_1 was exposed to 0.17 μmol of HOCl in 0.1 mL of EW(+) for 5 and 60 min at 0 °C. The EW(+) was evaporated off under vacuo and then dissolved in 1 mL of ethyl acetate; 10 μL was injected onto the column. The decrease of AFB_1 (80 nmol) and the appearance of secondary product by the reaction with EW(+) (0.17 μmol) at 0, 20, 30, and 45 °C for 0−60 min were also monitored by HPLC.

(b-3) Effect of Pretreament of EW(+) with Radical Scavengers on the Elimination of AFB_1. To confirm the participation of free radical (OH radical) in EW(+) on the elimination of AFB_1 , 0−200 μL of 30 mM mannitol was added to 600 μL of EW(+) containing 0.38 μmol HOCl by the mixing molar ratio of 0-, 1-, 2-, 5-, and 10-fold for 10 min prior to exposure to AFB_1 (128 nmol).

To examine the effect of pretreatment with thiourea on the elimination effect of EW(+), 1 mM thiourea was added to 600 μL of EW(+) containing 0.38 μmol HOCl by the mixing ratios of thiourea/HOCl in EW(+) 0-, 0.03-, 0.1-, and 1-fold. Kept standing for 10 min at 0 °C, the thiourea-treated EW(+) was exposed to 128 nmol of AFB_1 for 10 min at 30 °C.

After 10 min of reaction with radical scavenger-treated EW(+) at 30 °C, the reactant was dissolved in 40 μL of chloroform, and then, 10 μL was injected onto an HPLC column to determine the remaining AFB_1.

AFB_1 solutions reacted with/without EW(+) and pretreatment of EW(+) with/without radical scavenger were analyzed on an HPLC equipped with a Hitachi L-6200 Intelligent Pump, fluorescence detector (Hitachi F-1050), Hitachi D-2500 Chromato-Integrator, and reversed-phase column ODS-80 (4.6 mm × 150 mm) (TOSOH TSK-Gel) without any chemical derivatization. Gradient elution was used at a flow rate of 1.0 mL/min with 30% acetonitrile in water from 0 to 40 min, followed by a linear increase in acetonitrile from 30 to 100% between 40 and 70 min. Eluants were monitored flurorometrically using excitation wavelength at 365 nm and emission wavelength at 450 nm.

For the radical scavenger experiments, elution peaks were monitored at 365 nm, but all other analytical conditions were the same as described above. The chromatographic run was repeated three times in each analysis.

Mutagenicity Test for *Salmonella typhimurium* TA-98 and TA-100. The mutagenicity of AFB_1 was evaluated by a conventional Ames test using *S. typhimurium* strains TA-98 and TA-100 (*29*). Cultures of *S. typhimurium* TA-98, containing a histidine frame-shift mutant, and TA-100, containing a histidine missense mutation (*30*), were purchased from the Institute of Fermentation, Osaka. After 200 ng of AFB_1 (0.64 nmol) was mixed with either 100 μL of EW(+) containing a 0.76 nmol equivalent of HOCl as the available chlorine and the mixture was kept standing for 10 min, a 20 μL aliquot was added to 500 μL of S9 mixture or phosphate buffer (pH 7.5 for S9 mixture), followed by 100 μL of *S. typhimurium* TA-98 or TA-100 strains containing 10^7 cfu/mL. For the control, 100 μL of dimethyl sulfoxide was added in place of the electrolyzed solution. The mixture was incubated for 20 min at 37 °C with shaking, and then, 2 mL of soft agar was added before inoculation on to Vogel−Bonner E agar medium (*31*) for 48 h at 37 °C. Mutagenicity was assessed by the numbers of colonies of revertant occurring in the presence of the S9 mixture. Other experimental conditions were the same as for the mutagenicity assay of AFB_1-2,3-dichloride by Swenson et al. (*32*). The mutagenicity test was repeated five times, and data were presented as the mean ± standard deviation (SD) for $n = 5$.

Identification of Radical Species by Electron Spin Resonance (ESR). The anode solution was analyzed by ESR spectroscopy to determine whether hydroxyl radicals ($OH^•$) might be involved in its disinfection properties. The anode solution (200 μL) was mixed with

Table 2. Effect of Electrolysis Time on Physicochemical Properties of the Anode Solution [EW(+)][a]

electrolysis time (min)	available chlorine (μM)	pH	ORP (mV)
0	ND	5.50 ± 0.10	376 ± 29
1	ND	3.53 ± 0.08	983 ± 25
3	88 ± 22.2	2.94 ± 0.07	1065 ± 5
6	371 ± 17.4	2.66 ± 0.12	1109 ± 9
10	624 ± 14.5	2.56 ± 0.05	1120 ± 3
15	976 ± 25.4	2.50 ± 0.05	1157 ± 2
20	1617 ± 5.6	2.45 ± 0.07	1163 ± 1
30	2356 ± 26.1	2.37 ± 0.04	1165 ± 1

[a] A 17.1 mM (0.1%) NaCl solution was electrolyzed at 9–11 V dc at room temperature (25 °C) using a diaphragm type device (Superoxseed Labo). Data are the means ± SD for $n = 4$.

10 μL of an 890 mM solution of the spin-trapping agent DMPO (5,5-dimethyl-1-pyrroline-1-oxide) to make a spin adduct and then analyzed by ESR spectrometry using a JES-RE3X/ESR data system ESPRIT330 (JEOL) and previously described experimental conditions (*33*).

Namely, ESR spectra were recorded with a JEOL JES-RE3X that was controlled by a computer system JEOL ES-ESPRIT 300. Measurement conditions were as follows: magnetic field, 335.5 ± 5 mT; resonance frequency, 9.41 GHz; modulation frequency and modulation width, 100 kHz and 0.063 mT; microwave power, 4 mW; response time, 0.1 s; amplitude, × 300; sweep time, 2 min. The spin trap reagent, DMPO (8.9M), was purchased from Dojin Laboratories (Kumamoto, Japan).

ESR measurements were done under the experimental conditions. An 890 mM DMPO water solution, 10 μL, anode solution, 200 μL, and $FeSO_4$ solution, 50 μL, and a volume of 130 μL were mixed and were transferred to a flat quartz ESR cuvette. The cuvette was placed in an ESR spectrometer, and recordings were made at room temperature. The ESR parameter, hyperfine-coupling constant (hfcc) obtained from ESR spectra of spin adducts, was finally determined by the computer simulation by using hfcc assigned to a spin adduct (DMPO−OH) generated by the reaction of DMPO and hydroxyl radical (HO) in anode solution.

RESULTS AND DISCUSSION

Physicochemical Properties of Electrolyzed NaCl Solutions. The physicochemical parameters of the EW(+), EW(−), and ultrapure water used for electrolysis varied considerably (**Table 1**). The EW(+) was strongly acidic, had high levels of dissolved oxygen and available chlorine in the form of HOCl, and had a high ORP. By contrast, the EW(−) was strongly alkaline, had a low level of dissolved oxygen, and had an extremely low ORP (−878 mV). Available chlorine was almost negligible in the EW(−). In other words, the EW(+) is highly oxidative, whereas the EW(−) is highly reductive; in addition, the parameters of both of these solutions were distinctly different from those of the ultrapure water. The data presented in **Table 1** were obtained from the electrolysis of 0.1% (or 17.1 mM) NaCl for 10 min; however, the values of these parameters, in particular the available chlorine that functions as an oxidant, were dependent on the length of electrolysis time (**Table 2**).

Disinfection of *A. parasiticus* with Electrolyzed NaCl Solution. To disinfect 10^3 cfu of *A. parasiticus* completely, at least a 50-fold volume of the EW(+), providing 20−30 ppm of available chlorine, or equivalent to 380−580 μmol of HOCl, was required (**Figure 1**). In other words, at least 0.4−0.6 mM of HOCl in the EW(+) must be secured in the suspension to disinfect 10^3 cfu of *A. parasiticus* completely. By contrast, the EW(−) did not disinfect *A. parasiticus* (**Table 3**). These data were obtained from a model experiment.

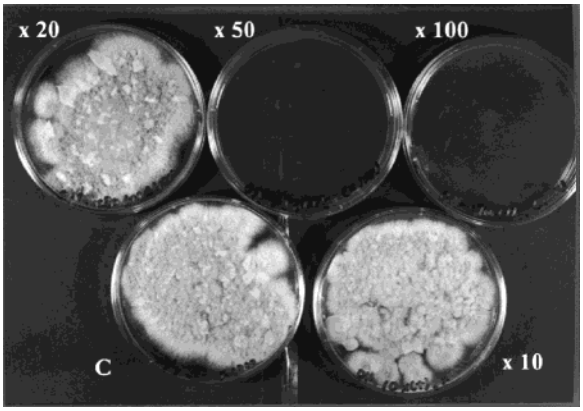

Figure 1. Disinfection of *A. parasiticus* by electrolyzed NaCl anode solution (EW(+)) *A. parasiticus* at a concentration of 10^3 cfu in 0.01 mL was immersed in increasing volumes of EW(+) for 12 h at room temperature. The solution was then inoculated on to AFPA agar medium and incubated for 5 days at 30 °C. C (lower left Petri dish): untreated control; ×10 (lower right Petri dish): 10-fold volume of EW(+), containing ca. 80 μmol HOCl; ×20 (upper left Petri dish), 20-fold volume of EW(+) containing 160 μmol HOCl; ×50 (upper middle Petri dish), 50-fold volume of EW(+) containing 390 μmol HOCl; ×100 (upper right Petri dish), 100-fold volume of EW(+) containing 800 μmol HOCl. Note white fluffy mycelia present on the control, ×10, and ×20 Petri dishes.

Table 3. Fungicidal Effect of Electrolyzed NaCl Solutions on *A. parasiticus*[a]

	volume ratio of electrolyzed solution						
added solution	control	×1	×2	×10	×20	×50	×100
anode solution	+++	+++	+++	+++	+++	±	–
cathode solution	+++	+++	+++	+++	+++	+++	+++

[a] Ten microliters of the cell suspension (10^3 cfu) was added to freshly prepared anode or cathode solution at different mixing ratios (0–100 times the volume ratio). The estimated concentrations of HOCl in the 10-, 20-, 50-, and 100-fold volumes of EW(+) are ca. 8, 15, 80, 150, 390, and 800 μmol, respectively. The estimated concentration of available chlorine in the form of OCl^- anion in the 10-, 20-, 50-, and 100-fold volumes of EW(–) are ca. 0.1, 0.2, 1.1, 2.2, 5.5, and 11 nmol, respectively. In the control test, cell suspensions were exposed to 0.1% NaCl. After each exposure, cells were then inoculated on to AFPA agar plates and incubated at 25 °C for five days. As the fungal growth could not be determined precisely by cfu, the growth is presented semiquantitatively: +++, full growth on plate; ±, few colonies; –, no growth. Experiment was repeated in triplicate.

To examine the practical capability of EW(+) to disinfect microorganisms including fungi attaching on the surface of food materials, we compared its effect with black pepper corn, turmeric finger, and coriander seeds. At least 50 mL of EW(+) 0.57 mM HOCl, satisfying concentration, to kill microbes mentioned above worked to clean up attaching microbes on the surface of 1 g of spices when EW(+) was used in combination with EW(−) (*34*) (Suzuki et al., unpublished data). Notably, the attached microorganisms, including fungi and spores observed by scanning microscopy, were not killed after a single 15 min exposure to a 50-fold volume of the EW(+) containing 30 μmol HOCl. However, a 15 min exposure to a 50-fold volume of the EW(−), followed by a 15 min exposure to a 50-fold volume of the EW(+), did effectively kill the microbes. Thus, although the EW(+) seems to be an effective disinfectant in model situations, for practical applications, a combined use of the EW(−) and the EW(+) might be needed. Detailed information will be reported later in an appropriate journal (Suzuki et al., in preparation).

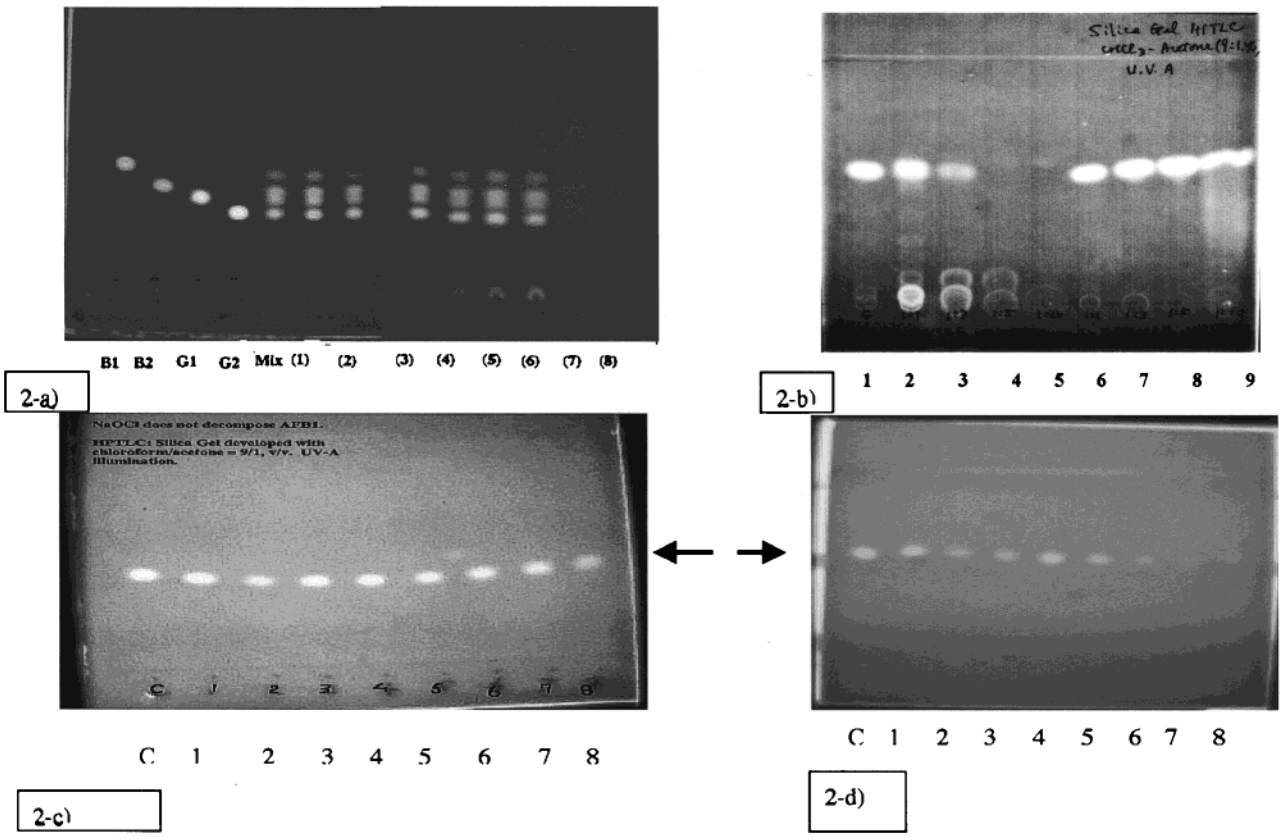

Figure 2. HPTLC analysis of aflatoxin exposed to electrolyzed NaCl solutions and NaOCl. After the aflatoxin was exposed to increasing molar ratios of the electrolyzed NaCl solutions, solutions were prepared for HPTLC as described in Materials and Methods. (a) HPTLC of individual aflatoxins B_1, B_2, G_1, and G_2 (10 ng of each) and a mixture of all four aflatoxins with or without exposure to EW(+). B_1, B_2, G_1, G_2, and mix indicate aflatoxins without treatment; numbers indicate the molar mixing ratio of the aflatoxin mixture to the EW(+). Lane 1, $1{:}0.7 \times 10^5$; 2, $1{:}2.8 \times 10^5$; 3, $1{:}4.2 \times 10^5$; 4, $1{:}5.6 \times 10^5$; 5, $1{:}7.0 \times 10^5$; 6, $1{:}14 \times 10^5$; 7, $1{:}28 \times 10^5$; 8, $1{:}42 \times 10^5$. Note that no fluorescent spot is seen with an approximately 28×10^5-fold molar addition of HOCl in the EW(+) over the 40 ng (ca. 0.12 nmol) of sum of 10 ng each of 4 aflatoxin analogues mixture. (b) AFB_1 after exposure to EW(+) or EW(−). Ten nanograms (0.03 nmol) of AFB_1 was mixed with EW(+) by the molar ratio of $1{:}0.7 \times 10^5$ to $1{:}7 \times 10^5$ in molar equivalent terms of AFB_1 to HOCl in EW(+). The EW(−) does not contain significant concentration of HOCl; however, 1–10-fold volumes were added to the AFB_1 solution. Lane 1, control AFB_1 (10 ng); Lanes 2–5, AFB_1 exposed to EW(+) of 0.7×10^5-fold, 2.1×10^5-fold, 3.5×10^5-fold, and 7.0×10^5-fold molar equivalents of HOCl. Lanes 6–9, AFB_1 exposed to 1-, 3-, 5-, and 10-fold volumes of EW(−). (c) AFB_1 after exposure to NaOCl at pH 11.5. Ten nanograms (0.03 nmol) of AFB_1 was mixed with NaOCl solutions containing between molar ratio of AFB_1 to EW(−) by $1{:}0.7 \times 10^5$ and 8 ($1{:}5.6 \times 10^5$) in terms of molar equivalents of available chlorine as OCl^- anion at pH 11.5. Lane C, control AFB_1; lanes presented by numerals 1–8 mean mixing ratio with NaOCl: 1, 0.7×10^5; 2, 1.4×10^5; 3, 2.1×10^5; 4, 2.8×10^5; 5, 3.5×10^5; 6, 4.2×10^5; 7, 4.9×10^5; 8, 5.6×10^5. Note that AFB_1 does not disappear even at 5.6×10^5-fold mixing ratio. The arrow indicates AFB_1. (d) AFB_1 after exposure to NaOCl at pH 3. Ten nanograms (0.03 nmol) of AFB_1 was mixed with NaOCl solutions by the molar ratios of AFB_1 to HOCl between $1{:}0.7 \times 10^5$ and $1{:}5.6 \times 10^5$. Molar equivalents of available chlorine of NaOCl can be regarded as HOCl after pH adjustment at 3.0. Lane C, control AFB_1; lanes represented by numerals 1–8 mean mixing ratio with NaOCl the same as panel c except for pH 3. The arrow indicates AFB_1. The AFB_1 spot disappeared after exposure to 4.9×10^5 times the molar amount of NaOCl at pH 3. Note that the mixing ratio and experimental conditions were exactly the same as panel c except for pH value. Note that AFB_1 disappears at 4.9×10^5-fold molar mixing ratio (lane 7). Compare the chromatogram in panel c.

HPTLC Assessment of the Degradation of Aflatoxin. The fluorescent spots of AFB_1 and the B_1, B_2, G_1, and G_2 mixture of aflatoxin on HPTLC plates disappeared after exposure to seven times the molar amount of available chlorine in the form of HOCl to each aflatoxin (**Figure 2a**). The EW(+) completely abolished the AFB_1 spot; however, the strongly alkaline EW(−) had no effect on AFB_1 (**Figure 2b**). Theoretically, reactive chlorine that can be expressed as available chlorine including HOCl, OCl^-, or dissolved Cl_2 cannot be formed in the cathode compartment. The existence of low level available chlorine in the EW(−) should be due to leakage of EW(+) in the anode compartment into the cathode solution through the ion exchangeable membrane of the device.

Exposure of AFB_1 to eight times the molar amount of NaOCl at pH 11.5 did not abolish the AFB_1 spot (**Figure 2c**); however, AFB_1 disappeared if NaOCl was adjusted to pH 3.0 (**Figure 2d**). These results indicate that NaOCl exists in the form of HOCl at pH 3 and OCl^- at pH 11. Namely, the chemical species involved in destroying aflatoxin is not the ClO^- ion but is HOCl, which can give rise to a reactive OH radical and probably a Cl radical.

HPLC Assessment of the Degradation of Aflatoxin. Figure 3a shows that the trifluoroacetylated AFB_1 peak on HPLC disappeared after exposure to the EW(+). The peak height of trifluoroacetylated AFB_1, which eluted at 5.1 min, decreased with increasing molar ratios (on the basis of available chlorine as HOCl) of the EW(+) and disappeared completely after exposure to 43×10^3 times the molar amount of HOCl in EW(+) (**Figure 3b**). Although the peaks eluting at 3.1 and 8.3 min have not been assigned as yet, the peak at 8.3 min is likely to be a reaction product of AFB_1 formed after exposure to the EW(+).

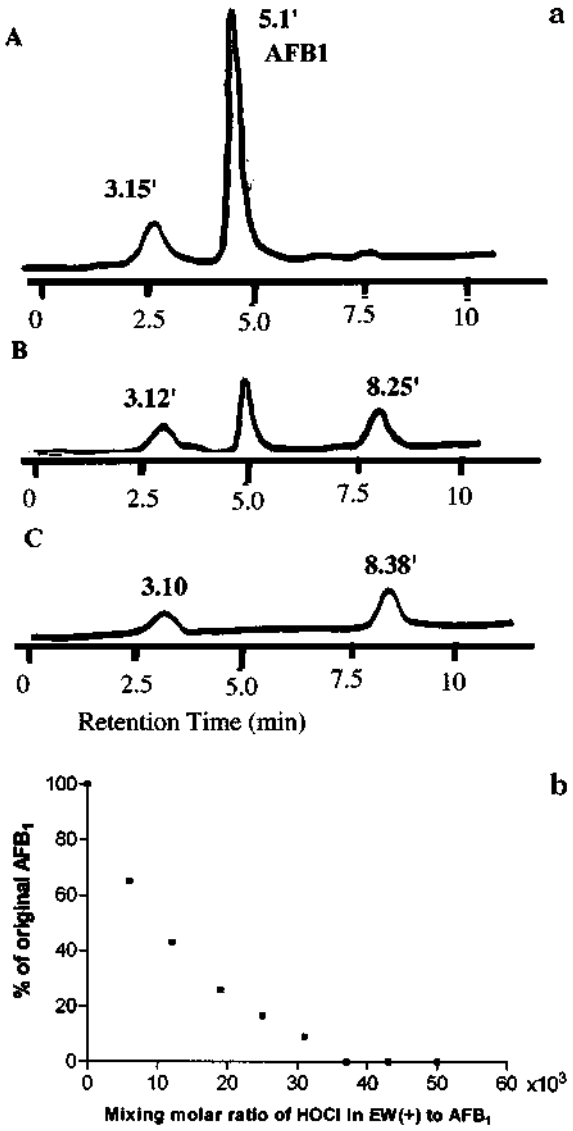

Figure 3. Reduction in AFB_1 peak height on HPLC after exposure to the anode solution. (a) Reduction in AFB_1 peak height with exposure to increasing volumes of EW(+) containing from 0 to 4.9×10^5 times the molar amount of HOCl. (A) AFB_1 without EW(+) exposure. The peak at 5.1 min corresponds to trifluoroacetylated AFB_1. The peak eluted at 3.15 min was not identified. (B) AFB_1 exposed to 0.19×10^5-fold molar excess of HOCl in EW(+). Eluted peaks at 3.12 and 8.25 min were not identified. (C) AFB_1 exposed to 0.44×10^5-fold molar excess of HOCl in EW(+). Other experimental conditions are in the Materials and Methods. (b) Decrease of AFB_1 content with increasing contact molar ratio of HOCl in EW(+). AFB_1 (400 ng corresponding to 1.3 nmol) was contacted to EW-(+) varying its HOCl as described in the Materials and Methods. The content of the remaining AFB_1 was determined by HPLC and plotted. Ordinate, relative amount of remaining AFB_1 (percent); abscissa, mixing molar ratio of HOCl in EW(+) to AFB_1. Each plot represents the mean of three measurements.

Identification of the reaction product was carried out on liquid chromatography mass spectroscopy (LC-MS) in another experiment without derivatization. LC-MS analysis of AFB_1 and its reaction products with EW(+) revealed the formation of 8-OH-9-Cl-AFB_1, 5,9-dichloro-8-OH-AFB_1, and 5,8,9-trichloro-AFB_1. Detailed information on the identification of the reaction products will be reported later (Suzuki et al., in preparation).

Effect of Reaction Time, Temperature, and Available Chlorine Concentration. The effect of exposure time and temperature on the annihilation of AFB_1 by EW(+) was assessed by HPLC (**Figure 4**). Free AFB_1 eluted as a single peak at 12.5 min, but this peak was replaced with a major peak at 8 min that was identified as 8-OH-9-Cl-AFB_1 and a minor peak at 35 min that was identified as 5,9-dichloro-8-acetoxy-AFB_1 (an artifact product) after AFB_1 was exposed to EW(+) at 0 °C for either 5 or 60 min (**Figure 4A−C**). The relation of the exposure temperature to both the disappearance of AFB_1 and the appearance of a reaction product, 8-OH-9-Cl-AFB_1 ($t_R = 8$ min), is shown in **Figure 5**. As the amount of AFB_1 decreased, the amount of reaction product increased. Low temperature slightly slowed the disappearance of AFB_1, but even after 5 min of exposure even at 0 °C, almost 40% of AFB_1 had disappeared. Considering the fact that the peak eluting at 8 min is 8-OH-9-Cl-AFB_1, the significant disappearance of AFB_1 with an increase of the OH, Cl adduct of AFB_1 at 0 °C suggests that this reaction is initiated by free radicals. Exposing AFB_1 to an equimolar amount of HOCl in the EW(+) for 5 min decreased the amount of AFB_1 by 40%.

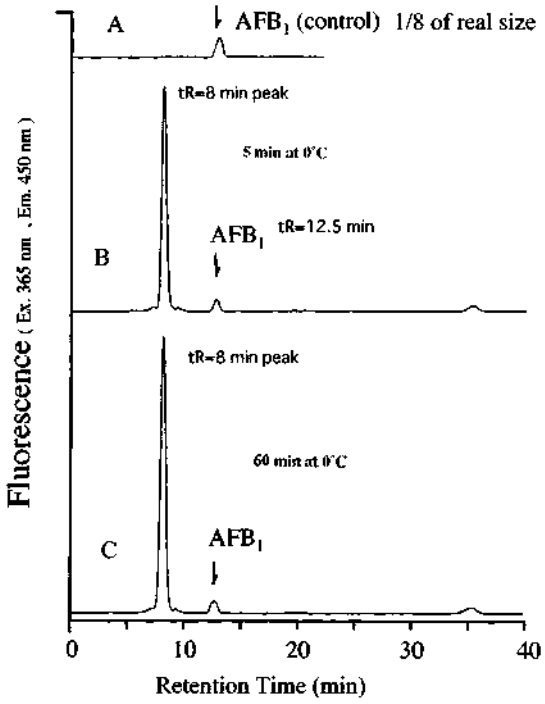

Figure 4. Effect of EW(+) exposure time on AFB_1 and its reaction products at 0 °C. AFB_1 (80 nmol) was exposed to EW(+) (8.5 μmol HOCl as available chlorine) at pH 2.3, for either 5 or 60 min at 0 °C. AFB_1 was analyzed by HPLC without derivatization as described in the Materials and Methods. Chromatogram on top (represented by A), AFB_1 without exposure (the AFB_1 peak on the chromatogram is 1/8 scale of the real size); chromatogram in the middle (represented by B), exposure of 6.8 μmol HOCl in EW(+) to 80 nmol AFB_1 for 5 min at 0 °C; chromatogram at the bottom, exposure of 6.8 μmol HOCl in EW(+) to 80 nmol AFB_1 for 60 min exposure at 0 °C. Note that the decrease in the AFB_1 peak eluting at 12.5 min was accompanied by the appearance of a major peak at $t_R = 8$ min (8-OH-9-Cl-AFB_1) and a minor peak at $t_R = 35$ min. The minor peak at $t_R = 35$ min was identified as an artifact. Note that the chromatograms shown were obtained without derivatization of the reaction products and so differ from those shown in **Figure 3**.

Effect of Radical Scavengers on AFB_1 Degradation. Figure 6 shows the effect of the addition of mannitol and thiourea to the EW(+) before exposure to AFB_1. As the molar ratio of mannitol to available chlorine in the EW(+) increased, the percentage of the decomposed AFB_1 decreased. Similarly, when AFB_1 was exposed to EW(+) containing one-tenth of the molar

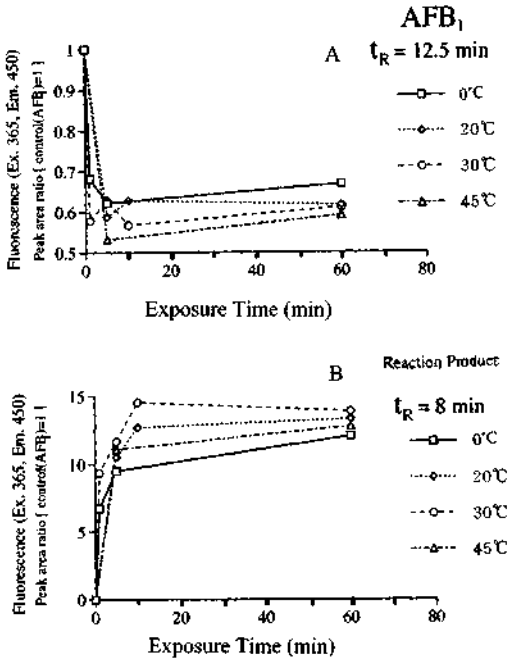

Figure 5. Effect of temperature and exposure time on the AFB_1 and the appearance of its reaction product 8-OH-9-Cl-AFB_1 eluting at 8 min. The ordinate indicates the fluorescence intensities at 450 nm; the abscissa indicates the exposure time in minutes. (A) Decrease in AFB_1 with increasing exposure time at various temperatures. (B) Increase in appearance of the reaction product, 8-OH-9-Cl-AFB_1 (t_R = 8 min), with increasing exposure time at various temperatures. Experimental conditions are the same as in **Figure 4**. Each plot represents the mean of three measurements.

amount of thiourea to HOCl in EW(+), only 25% of AFB_1 was destroyed, and with an equal molar ratio of thiourea to HOCl, AFB_1 was not destroyed at all. The known radical scavenging effect of mannitol and especially thiourea indicates distinctly that the disappearance of AFB_1 is due to a free radical-mediated reaction. The reason mannitol did not protect AFB_1 completely from the EW(+) may be due to an insufficient concentration of mannitol. For mannitol to exhibit an OH radical-scavenging effect, a higher concentration to scavenge OH radicals satisfactorily is required because 10 times as much molar amount of mannitol to HOCl protected only 16% of AFB_1 (**Figure 6a**).

Mutagenicity of Electrolyzed Solution Treated AFB_1. Three nanomoles of AFB_1 showed a high mutagenicity for both *S. typhimurium* TA-98 and TA-100 strains (**Table 4**), but this mutagenicity disappeared after a 10 min exposure to EW(+). **Table 4** shows the reduction in AFB_1 mutagenicity caused by increasing molar amounts of HOCl in the EW(+). Mutagenicity was reduced markedly after exposure to ca. 20-fold molar amount of HOCl in the EW(+) and showed less than 200% of relative mutagenicity indicating mutagenicity negative by exposure to ca. 60-fold molar amount or equivalent to 173 nmol of HOCl in both TA-98 and TA-100 strains.

ESR Analysis of Chemical Species in Anode Solution. To identify radical species, the EW(+) was subjected to ESR using the spin-trapping agent DMPO. At first, a weak spectrum indicating the formation of the OH radical was obtained (data not shown). Subsequently, to enhance the spectrum, we added 10 mL of 1 mM $FeSO_4$ and 10 mL of DMPO to the EW(+). In the presence of Fe^{2+} ion, an ESR spectrum typical of the reaction of DMPO with hydroxyl radicals (Fenton reaction) (*35*), as shown in **Figure 7**, was obtained, suggesting that the EW(+) also contains H_2O_2. Thus, these results indicate the presence of

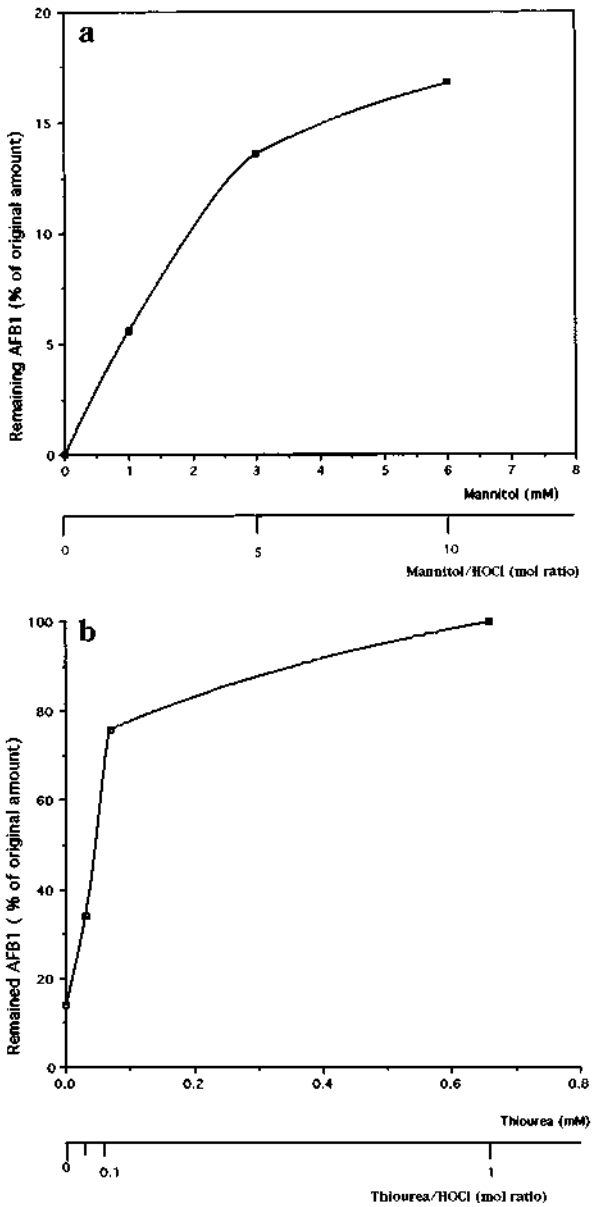

Figure 6. Effect of radical scavengers on the degradation of AFB_1 by EW(+). Either mannitol or thiourea was added to the EW(+) at increasing molar ratios as HOCl (0.640 mM) before exposure to AFB_1 (0.128 mM). Exposure of radical scavenger-treated EW(+) was done for 10 min at 30 °C. The ordinate shows the relative amount of remaining AFB_1, calculated as a percentage of the sum of the total peak area of control AFB_1 monitored at 365 nm; the abscissa indicates molar ratios of radical scavenger to HOCl in EW(+). (a) Effect of mannitol addition to EW(+) on the of AFB_1. Mannitol was added to EW(+) at 2–10 times the molar amount of HOCl in EW(+) prior to exposure of EW(+) to AFB_1. (b) Effect of thiourea addition to EW(+) on the degradation of AFB_1. Thiourea was added to EW(+) at 0.1–1.0 times the molar amount of HOCl in EW(+) prior to exposing EW(+) to AFB_1.

OH radicals and H_2O_2 in the EW(+). Furthermore, we confirmed the presence of HOCl at the acidic pH range in the EW(+) by comparing ultraviolet spectra between 200 and 350 nm; i.e., we obtained similar spectra identical to those of standard HOCl and NaOCl and EW(+) under different pH values as reported by Nakagawara et al. (*30*) with EW(+) and NaOCl under different pH conditions.

In summary, we have shown that electrolyzed NaCl anode solution (EW(+)) sterilizes *A. paraciticus* and eliminates the

Table 4. Effect of Anode Solution on the Mutagenicity of AFB_1[a]

sample	relative mutagenicity for *Salmonella typhimurium* (%) TA-98	TA-100
AFB_1 (3 nmol)	4039 ± 250	1010 ± 36
AFB_1 + anode solution		
3 + 58 nmol	514 ± 12	224 ± 8
3 + 173 nmol	NG	27 ± 1
3 + 288 nmol	NG	9 ± 1
3 + 575 nmol	NG	NG

[a] The mutagenicity test was carried out on His⁻ *S. typhimurium* TA-98 and TA-100 according to Maron and Ames (1983). Three nanomoles of AFB_1 was exposed to increasing volumes of the EW(+) with 0.77 mM (40 ppm) HOCl as available chlorine for 10 min at room temperature. This solution was then preincubated with an S-9 mixture for 20 min and incubated with *S. typhimurium* for 48 hours at 37 °C. Data are represented as the percentage of relative mutagenicity, which was calculated by [(cfu of the sample − cfu of the blank)/cfu of the blank] × 100. NG, negative growth (i.e., equal number of colony formation or less than the blank that did not include AFB_1). Different volumes of the EW(+) are expressed as nanomoles of HOCl. Data represent (mean ± SD) for five separate experiments.

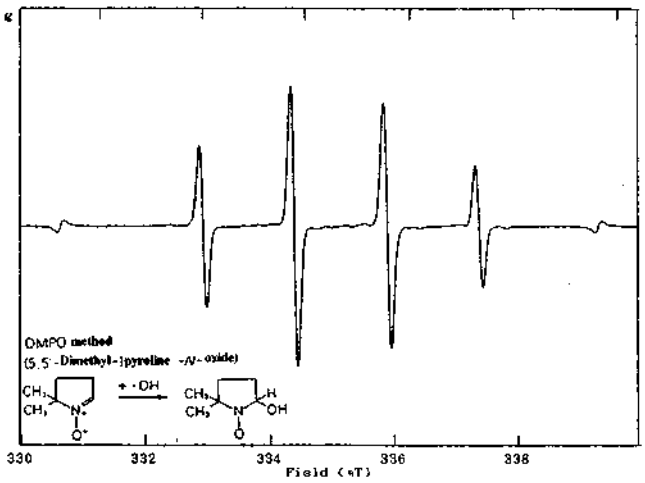

Figure 7. DMPO spin trapped ESR signal of the EW(+). ESR spectrum of DMPO incubated with EW(+). The ordinate represents the intensity of signal; the abscissa indicates the intensity of the magnetic field. The typical 1:2:2:1 "fingerprint", demonstrating electron adduction, shows that OH radicals are present in the EW(+).

mutagenicity of aflatoxin AFB_1. Aflatoxin breaks down after exposure to EW(+), as determined by HPLC analysis; however, the chemical formula of the reaction products was not described in detail here. We have succeeded in identifying the major reaction product to be 8-OH-9-Cl-AFB_1 by LC-MS and nuclear magnetic resonance techniques. In the following paper, we describe the chemical formulas of the products formed after the exposure of ABF_1 to EW(+).

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