

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**DRINKING WATER DISINFECTION BY ELECTRO
ACTIVATED WATER AND ITS INACTIVATION MECHANISM ON
BACTERIA**

ÖZGE TÜRKAY
**A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**
DEPARTMENT OF ENVIRONMENTAL ENGINEERING

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THESIS SUPERVISOR
PROF. DR. ANATOLİ DİMOGLO

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2017

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ELEKTRO AKTİF SUYUN İÇME SUYU
DEZENFEKSİYONUNDA KULLANILMASI
VE BAKTERİ İNAKVİTASYON
MEKANİZMASININ BELİRLENMESİ

ÖZGE TÜRKEY
DOKTORA TEZİ
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DANIŞMANI
PROF. DR. ANATOLİ DİMOGLO

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JÜRİ

ÜYE

(TEZ DANIŞMANI) : PROF. DR. Anatoli DİMOGLO

ÜYE

: PROF. DR. Sevil VELİ

ÜYE

: PROF. DR. Melek ÖZKAN

ÜYE

: PROF. DR. Fatma YÜKSEL

ÜYE

: DOÇ. DR. Şeref KESKİN

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun
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SUMMARY

This thesis aims to produce a cost-effective and safe disinfectant agent that is named electro activated water (EAW). With this purpose, two different reactors were designed to generate EAW and the efficacy of generated water as a disinfectant agent was investigated on bacteria. The operation conditions of EAW system was modelled through Response Surface Methodology. The optimum parameters of disinfection process to achieve effective inactivation bacteria was studied. The inactivation efficiency of EAW was evaluated upon measurement of TTC-dehydrogenase activity and flow cytometry analysis. Furthermore, the inactivation mechanism of EAW on bacteria has been evaluated comparing with sodium hypochlorite (NaOCl) and chlorine dioxide (ClO₂). The changes of cellular structure of *Enterococcus faecalis* were presented significantly upon several analyses such as the determination of cell membrane integrity, protein leakage, and the measurement of conductivity, the determination of DNA damage, SDS-PAGE and FTIR analysis. According to the results, electrolysis time is more important than brine concentration and current for oxidative potential of EAW. On the other hand, chlorine concentration of EAW is the most significant factor during disinfection of bacteria. The inactivation mechanism of NaOCl, ClO₂ and EAW was almost similar; however, EAW destroyed the cell structure considerably, since it inactivated the bacteria in less time with more damage.

EAW process was applied for the disinfection of drinking water sample. A cost effective process conditions were determined to obtain complete bacteria removal and provide adequate residual chlorine concentration after 30 minutes of disinfection. Since EAW disinfection process does not cause THM formation, it may be an alternative method for drinking water chlorination.

Key Words: Electro activated water, chlorine, disinfection, drinking water, anolyte, inactivation.

ÖZET

Bu çalışma, elektroaktif su (EAS) adını alan, uygun maliyetli ve tehlikesiz bir dezenfektan üretmeyi amaçlamaktadır. Bu amaçla, EAS üretmek üzere iki farklı reaktör tasarlandı ve üretilen suyun bir dezenfektan olarak etkinliği bakteriler üzerinde araştırıldı. EAS sisteminin çalışma koşulları, Yüzey Yanıt Metodu ile modellendi. Ayrıca, çalışma kapsamında bakterilerin inaktivasyonunu sağlamak için dezenfeksiyon sürecinin optimum parametreleri üzerinde çalışılmıştır. EAS dezenfeksiyon etkisi flow sitometri ve TTC dehidrojenaz aktivitesini ifade eden ölçüm yöntemleri ile incelenmiştir. Ayrıca, EAS'yun bakteri üzerindeki inaktivasyon mekanizması, sodyum hipoklorit (NaOCl) ve klor dioksit (ClO₂) ile karşılaştırılarak değerlendirilmiştir. *Enterococcus faecalis*'in hücresel yapısındaki değişiklikler, hücre zarı bütünlüğünün belirlenmesi için iletkenlik, protein sızıntısı, DNA hasarının saptanması, SDS-PAGE analizi ve FTIR gibi çeşitli analizlere dayanarak sunulmuştur. Elde edilen sonuçlara göre, EAS'yun yüksek oksidasyon potansiyeline sahip olması için elektroliz zamanı, tuz konsantrasyonu ve uygulanan akımdan daha önemlidir. Öte yandan, EAS'yun serbest klor konsantrasyonu bakterilerin dezenfeksiyonunda en önemli faktör olarak belirlenmiştir. NaOCl, ClO₂ ve EAS'yun inaktivasyon mekanizması büyük ölçüde aynı olmasına rağmen, EAS, bakterileri daha çok hasarla daha kısa sürede inaktive ederek hücre yapısını önemli ölçüde tahrip etmiştir.

EAS prosesi, içme suyu dezenfeksiyonu amacıyla uygulanmıştır. Bakterilerin tamamını öldürecek ve 30 dakikalık dezenfeksiyon sonrasında suyun içinde yeterli miktarda kalıntı klor konsantrasyonunu sağlayacak, etkili ve düşük maliyetli proses şartları belirlenmiştir. EAS ile dezenfeksiyon THM oluşumuna sebebiyet vermemesi nedeniyle içme suyu arıtımı için alternatif bir yöntem olabilir. Farklı saklama koşullarında bekletilen EAS'yun stabilitesi test edilmiştir.

Anahtar Kelimeler: Elektroaktif su, klor, dezenfeksiyon, içme suyu, anolit, inaktivasyon.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
ACP	: Atmospheric cold plasma
BHI	: Brain-Heart Infusion
CDC	: Chlorine dioxide concentration
CFU	: Colony forming unit
DBPs	: Disinfection by-products
DES	: Double electrical layer
DMPO	: 5, 5-dimethyl-1-pyrroline-N-oxide
DNA	: Deoxyribo nucleic acid
DO	: Dissolved Oxygen
DOC	: Dissolved Organic Carbon
DRA	: Dehydrogenase relative activity
EAW	: Electro activated water
EDTA	: Ethylenediaminetetraacetic acid
FFC	: Free chlorine concentration
FTIR	: Fourier transform infrared spectroscopy
GTU	: Gebze Technical University
HA	: Humic Acid
HIV	: Human immunodeficiency virus
ICP-OES	: Inductively coupled plasma optical emission spectrometer
LB	: Luria Bertani
LcEW	: Low Concentration Electrolyzed Water
LPS	: Lipopolysaccharides
MDA	: Malondialdehyde
ORP	: Oxidation Reduction Potential
PBS	: Phosphate Buffered Saline
RedOx	: Reduction–oxidation reaction
ROS	: Reactive oxygen species
RSM	: Response Surface Methodology
SAEW	: Strong Acidic Electrolyzed Water

SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STS	: Sodium thiosulphate
TBA	: Thiobarbituric Acid
TCA	: Trichloroacetic acid
TF	: 2,3,5-triphenylformazan
THM	: Trihalomethanes
TTC	: Triphenyl tetrazolium chloride
USEPA	: United States Environmental Protection Agency
UV ₂₅₄	: Ultraviolet Absorbance at 254 nm
WHO	: World Health Organization

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1. INTRODUCTION

The millennium declaration established a goal of halving the proportion of the global population without access to safe water by 2015. One reason for this is that fecal contamination of source and treated water is a persistent, worldwide problem. Sanitation coverage is inadequate in many parts of the world and is likely to persist for the near future. Fecal contamination of source and treated water is further exacerbated by increasing populations, urban growth and expansion, peri-urban settlement and continued and perhaps increasing pollutant transport into ground and surface water due to deforestation, global climate change, recurrent disastrous weather events (hurricanes, cyclones, floods, tsunamis, etc.) and increasing coverage of the earth's surface with impervious materials [1]. The demand for improved water quality and supply of sufficient amount of water to people will increase worldwide.

Over the last century, the cause of mortality and morbidity has decreased via drinking water disinfection. However, 1.2 billion people are still without safe drinking water. There are several sequential units such as settlement, coagulation and filtration before the disinfection process in water treatment. Chlorine based technologies; ozone and UV treatment are widely used disinfection processes worldwide. Although chlorination is an effective process for drinking water disinfection, it has some disadvantages such as taste and odor, ineffectiveness to strong microorganisms (*Cryptosporidium parvum* etc.), formation of carcinogenic disinfection by-products.

Electrochemical disinfection methods have drawn attention as an alternative method. Electrochemical water disinfection is to remove bacterial media in water in the presence of suitable electrodes by passing an electric current through the water. When electric current applies into solution, active species form at the phase boundary between the electrodes and the water. The electric current leads to the electrochemical production of disinfectant species from the water itself (for example ozone) or the species dissolved in the water. Therefore, highly oxidative media with these species provides adequate disinfection effect on deleterious microorganisms [2].

In the motion of environmental aspects, electrochemical disinfection processes can be a good alternative for drinking water disinfection. The usage of electrochemical disinfection techniques can be appropriate for individual treatment systems in the buildings instead of central water treatment plans [3]. Accordingly, electro activated

water (EAW) system as an alternative electrochemical disinfection method is applied for drinking water in this thesis.

1.1. Aim, Contribution and Scope of the Thesis

This thesis aims to evaluate applicability of electrochemical disinfection method to increase access to safe drinking water and sanitation services. This project gives a lot of importance in the development and search of alternative disinfection method to conventional systems. The system is based on electrolysis of brine tap water without adding any other chemical. In this system, due to electrochemical reactions, the physicochemical properties of water can be changed and two different waters with superior properties are produced. Whereas the acidic water, namely EAW, has high oxidation potential, the basic water has negative oxidation-reduction potential. EAW can be used as a disinfection agent with its high chlorine content and a mixture of other oxidant agents.

EAW process was attempted as disinfection methods for drinking water, in this thesis. The reactor configuration for EAW production and the modelling of operation condition of the reactor were conducted. In addition, the determination of optimum application conditions for bacteria inactivation of EAW processes was investigated in this thesis. Besides, the disinfection mechanism on bacteria and drinking water sample was evaluated comprehensively upon various analysis technique.

1.2. Project Management and Plan

Theoretical background of EAW has been given in Section 2. The background information involves general description, characterization and mechanism of the disinfection systems. Literature review has been carried out for EAW applications. The material and methods used during EAW process, as well as the testing protocol for bactericidal activity and inactivation mechanism of disinfectant are given in Section 3. Results are presented in Section 4 including subsequent sections. The optimization of experimental conditions of EAW system is discussed in this section. Then, the bactericidal activity tests and evaluations of inactivation mechanism have been elucidated on indicator microorganisms of drinking water. Besides, the

application of EAW to drinking water sample has been conducted and the results are summarized in this section. The stability test of EAW and the cost analysis of EAW as drinking water treatment system also were given in this section. Finally, the conclusions from the experiments, the recommendations based on the results, and the possible future work are presented in Section 5.

2. THEORITICAL BACKGROUND

2.1. General Description

The uses of technologies to improve microbial quality of household water and thereby reduce waterborne diseases include a number of physical and chemical treatment methods. A series of sequential processes such as coagulation, filtration and settlement are employed to treatment water prior to disinfection process. Ozonation, chlorination and UV treatment are the most widely used disinfection technologies at the present time. Chlorine is favorable chemical process among them due to provide a residual disinfection along primary disinfection. Chlorine can inhibit bacteria in a short time. There are several forms of chlorine such as hypochlorite, chlorine dioxide, organic and inorganic chloramines; however, their effectiveness on microorganisms varies [4].

Electrolysis of sodium chloride solution is the oldest known method to produce hypochlorite solution. In the process of studying the electrolysis, the phenomenon of conservation of the potential energy of the electrode was discovered as a result of significantly changes on the reactivity of the aqueous medium. The conservation of potential energy in a liquid is caused by activation (excitation) of atoms, ions and molecules in the liquid itself, as well as by changing the properties and state of the liquid itself. This phenomenon is called electrochemical activation of liquid media [5].

The change in the rate and selectivity of chemical reactions after electrochemical activation of the water is associated with the accumulation of additional energy in the system and its transition to a metastable state. In this case, the physical and chemical properties of the liquid change substantially, which is observed from the anomalous values of its parameters. The increase in the polarization of the electrode causes greater proportion of excess fluid activation energy. In this case, the solution acquires a certain amount of internal potential energy, changing the kinetics of the reactions [6]. The effect of this excess internal potential energy of a substance is similar to the catalytic impact on the course of the reaction. This is used in the processes of water purification and disinfection, as well as for the conversion of water or dilute solutions of electrolytes into environmentally friendly highly oxidative solutions [7]. The production of EAW is based on this mechanism and can be

generated during the electrolysis of sodium chlorite in a unipolar reactor at the anode side. The electric field strength at the anode (in the so-called double electrical layer - DES) is several million volts per centimeter [8]. The effect of oxidation-reduction destruction and neutralization of toxic substances is accelerated many times due to the formation of highly active components: ozone, atomic oxygen, peroxide compounds, active chlorine compounds, including chlorine dioxide, and short-lived free radicals during electrolysis.

2.2. The Mechanism of Electro Activated Water

The ongoing research on water is still going on in the world. A number of complex physical parameters that are characterized as a complex micro cluster structure located in a non-equilibrium thermodynamic state affect the physiological properties of water. When electric current applies into a saline solution that has high conductivity, electron exchange takes place between electrodes and water as a result of unipolar electrochemical process. Physico-chemical character of water changes due to formation of polarization energy on electrodes during the electrolysis process. In addition, the production of EAW involves electrolysis of a dilute solution of NaCl passing through on electrolysis chamber. Inside the chamber, the brine water is charged and the negative and positive ions of NaCl and H₂O are separated. Positive charge forms at the anode surface through an oxidation process whereas a reduction processes takes place at the cathode. Consequently, two new products form called as catholyte and anolyte. The oxygen gas and many active species such as chlorine, chlorine dioxide and free radicals are being formed in anolyte, while hydrogen gas and sodium hydroxide are being formed in catholyte [9, 10]. The presence of a sufficient number of strong oxidants and free radicals in the anolyte turns it into a solution with strongly pronounced biocidal properties. The catholyte, saturated with reducing agents, acquires a high adsorption-chemical activity, as well as properties of a strong detergent. The properties of the catholyte are pH>11 and ORP <-800 mV, while the properties of the anolyte are pH<2.8, ORP>1100 mV, and free chlorine concentration (FCC) can be between 40 and 1500 ppm [11, 12]. The anolyte, namely EAW, has been used as disinfectant agent because of its high oxidation potential and content of active species [13].

The electrochemical reactions that have been anode and cathode surfaces are given below.

Oxygen gas forms on anode surface by oxidation of water;



Some other reactions can be take place on anode surface according to micro conditions;



If low mineralization occurs in anodic and cathodic side, hydrogen peroxide can decompose by itself;



Hydroxide ions are oxidized into the free radical in the anode region;



The recombination of the radicals after this occurs in a reaction chain;





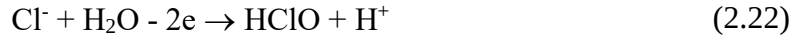
The end products in the reaction are highly active and can be found in anolyte for a long time at low pH (2-4) and high ORP (800-1260 mV). In chlorine-containing solutions, chlorine gas is formed on the anode by oxidation of water;



Some of the chlorine gas is dissolved in water and hypochlorous and hydrogen chloride acids are formed;



The reactions can take place in a different way according to pH and temperature of the solution. Chloride ions are oxidized on the anode surface and give oxygen-containing chlorine compounds;



Some other micro-size reactions may take place in the solution that given Table 2.1.

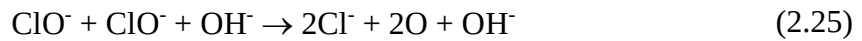
Table 2.1: Possible micro-size reactions on the anode surface.

$\text{HCl} + \text{H}_2\text{O} - 2\text{e} \rightarrow \text{HClO}_2 + 2\text{H}^+$	$\text{ClO}_2 + 6\text{H}_2\text{O} - 10\text{e} \rightarrow 2\text{ClO}_3^- + 12\text{H}^+$
$\text{Cl}_2 + 4\text{H}_2\text{O} - 6\text{e} \rightarrow 2\text{HClO}_2 + 6\text{H}^+$	$\text{Cl}^- + 6\text{OH}^- - 6\text{e} \rightarrow \text{ClO}_3^- + 3\text{H}_2\text{O}$
$\text{Cl}^- + 2\text{H}_2\text{O} - 4\text{e} \rightarrow \text{HClO}_2 + 3\text{H}^+$	$\text{Cl}^- + 2\text{H}_2\text{O} - 5\text{e} \rightarrow \text{ClO}_2 + 4\text{H}^+$
$\text{ClO}^- + 2\text{OH}^- - 2\text{e} \rightarrow \text{ClO}_2^- + \text{H}_2\text{O}$	$\text{Cl}^- + 4\text{OH}^- - 5\text{e} \rightarrow \text{ClO}_2 + 2\text{H}_2\text{O}$
$\text{Cl}^- + 4\text{OH}^- - 4\text{e} \rightarrow \text{ClO}_2^- + 2\text{H}_2\text{O}$	$\text{ClO}_3^- + \text{H}_2\text{O} - 2\text{e} \rightarrow \text{ClO}_4^- + 2\text{H}^+$
$\text{HClO}_2 + \text{H}_2\text{O} - 2\text{e} \rightarrow \text{ClO}_2^- + 3\text{H}^+$	$\text{ClO}_3^- + 2\text{OH}^- - 2\text{e} \rightarrow \text{ClO}_4^- + \text{H}_2\text{O}$
$\text{ClO}_2^- + 2\text{OH}^- - 2\text{e} \rightarrow \text{ClO}_3^- + \text{H}_2\text{O}$	$\text{Cl}_2 + 8\text{H}_2\text{O} - 14\text{e} \rightarrow 2\text{ClO}_4^- + 16\text{H}^+$
$\text{HClO}_2 - \text{e} \rightarrow \text{ClO}_2 + \text{H}^+$	$\text{Cl}^- + 4\text{H}_2\text{O} - 8\text{e} \rightarrow \text{ClO}_4^- + 8\text{H}^+$
$\text{Cl}^- + 3\text{H}_2\text{O} - 6\text{e} \rightarrow \text{ClO}_3^- + 6\text{H}^+$	$\text{Cl}^- + 8\text{OH}^- - 8\text{e} \rightarrow \text{ClO}_4^- + 4\text{H}_2\text{O}$

The anolyte solution features bactericidal agents due to its highly oxidative content. ClO^- , Cl^* , OH^* molecules are the most active free radicals in anolyte. Especially, the presence of HClO and ClO^- in the environment simultaneously is prospective to oxidize the bacterial cell (s RH_2).



Later, O^* , H^+ and Cl^- molecules are formed on the RH_2 biopolymers, after decomposition of ClO^- ...s.. HClO activated complex. The oxygen and OH occur from ClO active radicals;



The reaction chain proceeds with the addition of chlorine atom;



Free radicals and oxygen atom react with RH_2 biopolymers;



Active oxygen species as chemical oxidants inhibit the RedOx ferments of the bacteria cell. With the oxidation of catalytic groups at the center of the cell, hydrogen substrates release from molecules. Thus, the way of protons and electrons to the inhalation system of the cell is inhibited and cell death occurs.

Conversely, reduction reactions take place on the cathode surface as follows;



Hydroxide ion occurred in aqueous media exists free or hydrate forms (H_3O_2^- , H_5O_3^- , H_7O_5^- , H_9O_7^-). Free and hydroxyl radicals are generated after dissociation of hydroxide ion;



Dissolved oxygen in aqueous media is reduced on cathode surface as follows;



The electron passing over the cathode through hydronium ion gives off the free hydrogen radical.



Active substances are formed in the course of this reaction:



In a simplified form, the basic processes occurring in the cell can be represented as follows:

- the oxidation of water at the anode:



- water reduction at the cathode:



- the formation of gaseous chlorine in chloride solutions at the anode:



- the formation of highly active oxidants in the anode cell: Cl_2O , ClO_2 , ClO^- , HClO , $\text{Cl}^\bullet$, $\text{O}_2^\bullet$, O_3 , $\text{HO}_2^\bullet$, $\text{OH}^\bullet$;
- The formation of highly active reducing agents in the cathode cell: OH^- , H_3O_2^- , H_2 , HO_2^- , O_2^- .

In the process of flowing through the anode cell in the reactor, each micro volume of the saline solution touches the anode surface, where it appears in an electric field of high tension. As a result, the solution undergoes a powerful electron-acceptor impact and active substances in the form of strong oxidants are synthesized in it. Most of these compounds cannot exist outside the water, in a different aggregate form. In this respect, the anolyte is unique as to its chemical composition. At the same time, the solution guarantees the absence of dioxins and any xenobiotics, which create the risk of environmental pollution.

2.3. The Characterization of Electro Activated Water

In the process of electrolysis, physicochemical properties of the solution, such as electrical conductivity, the magnitudes of surface tension and density change abruptly, i.e. they decrease in catholyte and increase in anolyte. The adsorption-chemical activity, the dissolving power (in catholyte), so as the extraction ability increase. With unipolar electro processing of solution, high-stable products with alkaline reaction are formed in a diaphragm electrolyser in the cathode zone, while in the anodic zone highly oxidative products with acid reaction are formed. EAW forms at anode side, it consists of free chlorine, super oxide radicals, ozone, and hypochlorite form. EAW becomes metastable form and it does not change unless any energy movement occurs with environment. Besides being in a metastable state, EAW is a non-equilibrium system and, when interacting with the surrounding medium or the target, retains its anomalous properties for a certain period. This may be due to the formation of excited oxidized or reduced forms of substances, free radicals, to the anomalous structure of ion-solvate shells, etc. [14, 15]. Long-living quasi-stable structures in EAW appear near the surface of electrodes in an electric field with a voltage of up to several million volts per cm. As a result, an electron-donating structure

of water near the cathode, and the electron-acceptor structure near the anode are being formed through the rupture of hydrogen bonds. Water with a modified structure forms aqua complexes easily that enhance the reaction activity and catalytic activity of the water-mineral environment.

The main species in EAW is hypochlorite which has 1.482 eV oxidizing potential at 25°C. Molecular or hypochlorite form of chlorine applies to water initially, then both hydrolysis to free chlorine as expressed sum of aqueous molecular chlorine, hypochlorous acid, and hypochlorite ion. Hypochlorous acid is the strongest form of the chlorine compound and has 80 times higher disinfection activity than a same concentration of the hypochlorite acid [16]. pH and temperature notably affects the relative proportion of these forms [17]. On the other hand, the brine concentration is correlated conductivity according to Hsu S.Y., [12]. Similarly, DO ranged between 11.6 and 21.3 mg/L during generation of EAW [18], because oxygen gas is produced at the anode electrode. However, it may not be the exact value due to volatilization of oxygen gas partially [18].

During EAW production, the increment of chlorine concentration causes the decrement of pH value. The reason of pH decrease is formation of chlorine gas, hypochlorous acid and hydrochloric acid in aqueous media [18]. The relationship between pH and the formation of chlorine species has shown in Figure 2.1. Most of chemical processes take place in the desired direction at certain specific pH values, only, which must be maintained constantly. Therefore, pH is one of the main parameter for controlling EAW solutions.

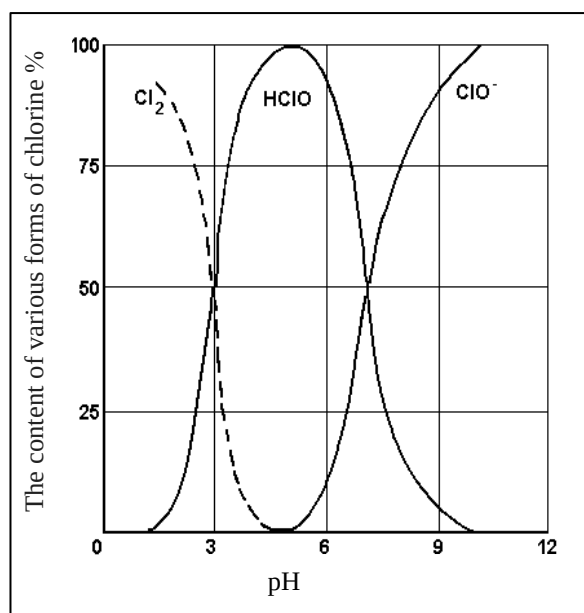


Figure 2.1: The content of various forms of active chlorine in the anolyte, depending on the pH of the solution.

The form of chlorine compounds (OCl^- , Cl_2 or HOCl) present in a sanitizer is majorly affected by pH of the solution. Hypochlorous acid is the most active form of chlorine and is the maximum effective compound at a pH of 5.0-6.5. HOCl will dissociate to hypochlorite ions (OCl^-) at high pH or chlorine gas (Cl_2) at low pH. At a higher pH of ≥ 7.5 , the inactive hypochlorite ion (OCl^-) predominates and very little of the active hypochlorous acid is available (HOCl).

Another parameter for monitoring EAW is the RedOx (reduction–oxidation reaction) potential. This value serves as a measure of the intensity of the oxidation-reduction processes in the system and is determined by the ratio of concentrations for oxidation and reduction sorts of ions forming the given system. In the electro activated aqueous solution, catholyte can take pH value from 7 to 12.8, while RedOx varies from 0 to -960 mV. For anolyte, the pH varies from 7 units up to 1 unit, and the RedOx changes lie between 0 and +1200 mV. As can be seen from the maximum values of the pH and RedOx parameters, the pH of the catholyte corresponds to a practically concentrated alkali solution, and the pH of the anolyte corresponds to the concentration of the acid solution. Nevertheless, both preparations are used to treat living organisms without harming them. This proves one of the properties of activation, that with a low concentration but high activity of ions, the preparations do

not possess the properties of dehydrating of living tissue, but contribute to the destruction of microbes and bacteria.

One of the parameters for monitoring the anolyte EAW is the concentration of “active” chlorine. Relative to its magnitude, it is judged on the possibility of using the preparation as a disinfectant solution. The concentration of “active” chlorine varies from 50 mg / l to several grams, depending on the design of the EAW reactor, the magnitude of the current and the mineralization of the solution. Related to working condition of the EAW reactor, two type of anolyte as strong acidic (SAEW) and slightly acidic (LcEW) can be produced [19].

The exact mechanism of action of SAEW against microorganisms is not yet fully understood. The collection of Cl_2 , HOCl and OCl^- ions present in SAEW is referred to as the free chlorine concentration (FCC). Varying reasons have been put forward on the antimicrobial activity of SAEW; some researchers believe it is due to the presence of chlorine species while others have implicated the low pH for its activity. Some few studies have implied that its high ORP is responsible for this activity. Overall, the known antimicrobial properties of SAEW are in being a strong bactericidal, virucidal and moderate fungicidal sanitizer. SAEW is known to have strong bactericidal effect on most known pathogenic bacteria [20].

LcEW has a nearly neutral pH (6.2-6.5) could be produced in an electrolytic cell without membrane by using dilute salt. In the process, chloride ions and water molecules become free chlorine (Cl_2 , HOCl and OCl). Among this chlorine species, HOCl has the highest biocidal character and has the highest concentration (approx. 95%) proportionally at that pH value. LcEW has attracted much interest because it contains low free chlorine, has less Cl_2 gas emissions, has low corrosive effects on surfaces, and is a highly effective antimicrobial agent with limited phytotoxic effects compared to hypochlorous acid derivatives [20]. Therefore, LcEW has been considered as a medical disinfectant due to the corrosive effects of strong acid water [21].

If we consider water as the most inert biochemical substratum and take its energy characteristics as the reference point, we obtain a scale on which biological substrates are distributed in the decreasing order of ionization potentials. This means a decrease in the energy necessary for detaching electrons from orbitals of these compounds. The total energy E that is liberated or expended during electronic transitions is determined from the formula:

$$E = I_d - E_a + d \quad (2.38)$$

Where, I_d is the ionization energy of the compound giving electrons (donor compound), E_a is the energy liberated upon transition of the electron to the free orbital of the reducible acceptor compound, eV, d is the increment depending on the chemical modification of the acceptor.

In carrying out oxidation-reduction reactions between pairs of substances, some of which are electron donors, or reducing agents, and others are their acceptors, or oxidizers (the pairs are also referred to as redox pairs), the transfer of a definite number of electrons (n -electrons = $n\bar{e}$) happens, and the energy E is spent. Accordingly, the potential ϕ for the transfer of one electron is determined by:

$$\phi = E/n, V \quad (2.39)$$

Where: ϕ - potential of this redox pair, referred to the number of electronic transfers.

The term RedOx, according to its meaning, corresponds to the concept of “electrochemical potential”, which denotes “the level of free energy of the system relative to the number of moles of matter in the system”. By definition, the “electrochemical potential”, which is equivalent to the measure of the free energy of biochemical reactions (ΔG) that is necessary for detaching electrons from donor compounds and then attaching them to the chemical acceptor, is calculated from the Gibbs formula.

$$\Delta G = n \cdot F \cdot \Delta\phi \quad (2.40)$$

where, n is the number of transferred electrons, F is the Faraday number and $\Delta\phi$ is the difference between the potentials of electron transfer from the donor compound to the acceptor one.

RedOx corresponds to the work on the transfer of electrons from the oxidized element or compound to the reducible one or from the reducing agent to the oxidizer, the concentrations of which are denoted by the symbols $[Red]$ and $[Ox]$ [22].

Stationary RedOx reflects the donor or acceptor properties of the tested living system relative to NVE (normal hydrogen electrode). Stationary RedOx for the concrete redox pairs is determined by the Nernst formula:

$$\varphi_{\text{CT}} = \varphi_o + (2,303 \cdot R \cdot T \cdot \lg([Ox] / [Red])) / n \cdot F ; \text{ pH}=7,0\text{-const} \quad (2.41)$$

where: φ_o is the standard RedOx of this redox pair at pH = 7.0, at 1 atm and 25 ° C, R is the Boltzmann gas constant, T is the absolute temperature, °K, n is the number of electrons transferred, F is the Faraday number.

$$\varphi_{\text{CT}} = \varphi_o + (0,06 \cdot \lg([Ox]/[Red]))/n \quad (2.42)$$

As can be seen, for calculations under normal conditions it is sufficient to know the concentration of the oxidized and reduced forms of the substance. Figure 2.2 shows [23] the dynamics of the RedOx potential and electromotive force changes for the samples of distilled and infusion solutions. As seen in Figure 2.2, solutions containing salt retain high values of these parameters during the first 7 hours.

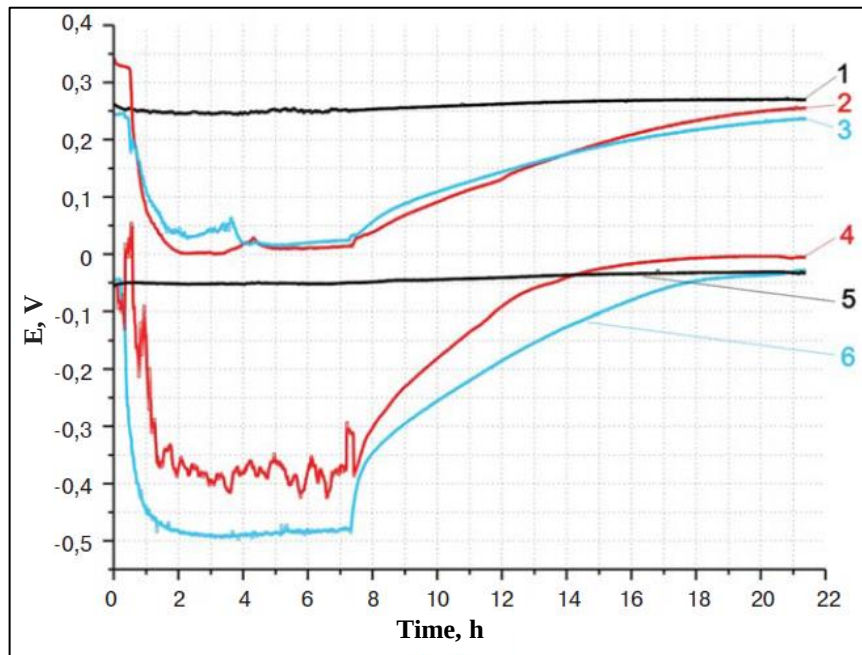


Figure 2.2: The changes in the RedOx potential (1-3) and φ (4-6) for samples of distilled water (1, 5) and infusion solution (2, 3, 4, 6).

The anomalous values of pH and RedOx potential for contactly and non-contactly activated water are explained simply enough by the formation of stable high-energy resonant microclusters from oscillating dipoles (water molecules, OH- ...) near the anode and cathode [18]. The alternating electromagnetic field formed by two dipoles, oscillating synchronously in anti-phase, has a narrow spectrum of frequencies (resonance effect) and rapidly decreases with distance.

Consequently, the efficiency of unipolar electrical processing is determined by the difference in pH and oxidation-reduction (RedOx) potential of the initial and treated liquids, and by the time of physico-chemical relaxation of the system.

2.4. Biological Activity of Electro Activated Water

Almost all biological systems contain molecular structures with separated charges, the electric field strength between which reaches $10^3 - 10^5$ V/cm. It is proved that these fields determine charge transfer in biological systems and control individual stages of complex biochemical transformations. EAW affects the functional properties of electroactive components of biological systems, changes the speed and even the direction of oxidation-reduction processes in the system.

The activity of electrons is the most important characteristic of the internal environment of the organism, since it is directly related to the fundamental processes of life activity. Under the influence of the electric field of the double electrical layer (DEL), the structural network of hydrogen bonds weakens, water molecules acquire releasement, that facilitates the assimilation of such activated in the electric field water of the cells of living organisms and accelerates the removal of biological system.

According to T.M. Lotts [23], microorganisms of different types and groups live and reproduce on nutrient media only in certain ranges of RedOx values, depending on the acid-alkaline characteristics of these media. In the pH range of nutrient media from 3 to 8, compatible RedOx range is between 600 and 800 mV for the life of microorganisms. Bacteria with increased acid resistance, surviving on media with pH = 2-3, do not give growth at RedOx < 400-550 mV, and at RedOx > 900-1050 mV. Microorganisms adapted to moderately acidic media (pH 4-5) survive in the RedOx range from 100-200 mV, up to 900-950 mV. In neutral media (pH 7-8), microorganisms are viable in RedOx from (-130) - (-30) mV, up to 700-820 mV. On

alkalized media (pH = 9-10), the range of values of RedOx that is compatible with the life of bacteria, is between (-120) mV, and (-50) mV. Thus, with the example of microbial cells (Prokaryote), it can be seen that deviations of RedOx towards reducing (negative) or oxidative (positive) values do not in themselves make the medium “alive” or “dead”. Obviously, for various biochemical processes and physiological functions, there is an optimum RedOx. Therefore, at the extreme values of RedOx (positive or negative), bacteria are inactivated. Consequently, the vital activity of individual biological systems (molecular, intracellular, cellular, tissue or macroscopic) becomes impossible at some values of ORP.

2.5. The Use of Electro Activated Water

EAW technology has been widely used in agriculture [24, 25], medical sterilization [26-28], food safety [25, 29-32], cleaning of kitchen staff [33-35] as well as in many other areas [36, 37]. EAW was proved effective in reducing the number of pathogenic bacteria on the surface of vegetables [38, 39] and fruits [40, 41]. Venkitanarayan et al. [42] in their study observed that EAW was able to destroy all microorganisms within 5 minutes. Suzuki et al. [43] demonstrated the existence of an inhibitory effect of EAW on growth and aflatoxin production of *Aspergillus parasiticus*. Other fungi, *Candida albicans* inactivated with EAW efficiently [44]. Okull and Laborde [40] proved that EAW brings about a reduction of more than 4 logs in *Penicillium expansum* live spore population. EAW achieved complete inactivation *Escherichia coli* (*E. coli*) O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* species in a short time [42]. Morita et al. [45] have also described through their experiments the ability of EAW to reduce the viral load of HIV-1 (Human Immunodeficiency Virus-1) and antigenicity of HBV (Hepatitis B virus).

2.5.1. The Application on Food Industry

Rahman et al. [20] compared low concentration electrolyzed water (LcEW) and strong acidic electrolyzed water (SAEW) in their report. Whereas, (LcEW) has normal pH (6.2) and low ORP (500-520 mV), SAEW has low pH (2.7) and high ORP (1100-1120 mV). The efficiency of both electrolyzed water on foodborne pathogens

(*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium*) were determined upon different waiting times (1, 3, 5, 7 and 10 min), pH values (2.5, 4.0, 5.0, 6.0 and 9.0) and temperatures (4, 15, 23, 35 and 50 °C). Increase in waiting time caused lower log reduction in each bacterium due to decrease in FAC. Furthermore, as the pH increased towards the basic side, inactivation of microorganisms showed a decrease. Higher temperatures accelerated log reduction of bacteria. The application of LcEW is a more appropriate sanitizing agent on the grounds that it has neutral pH, minimum time and low current requirement. Whereas, the most resistant pathogen was *Escherichia coli* O157:H7, *Staphylococcus aureus* showed lowest reduction against disinfection.

Kim et al. [16] examined the foodborne pathogen i.e. *E. coli*, *L. monocytogenes*, *B. cereus*, inactivation efficacy of two commercially available EAW and their chemically modified waters equivalents. It looked at the effect of individual residual properties i.e. pH, ORP and residual chlorine on microbial inactivation. One of the water, ROXEO, with a higher chlorine content 56 mg/L had a quick inactivation effect on both Gram + and Gram – bacteria compare to another water JAWEO with a lower chlorine content 10 mg/L. Similar results were also observed for chemically modified solutions with higher chlorine content compared to those with lower chlorine content. *B. cereus* spores were the most resilient bacteria against inactivation but all bacteria were inactivated after 120s of treatment.

2.5.2. The Application on Medical Service

The disinfection potential of electrolysis on tap water in a clinical setting was evaluated. The concentration of free chlorine increased relative to time and current during electrolysis. The free chlorine concentration was a maximum of about 30 ppm. Electrolysis produced heat and heating the water was thought to have an additional bactericidal effect. The maximum temperature obtained was 37 °C. Electrolysis could disinfect tap water infected with *P. aeruginosa*, *E. coli*, *L. pneumophila* and *S. aureus* by electrolysis at 20-30 mA for 5 min. However, a spore-forming bacterium, *Bacillus subtilis*, was resistant to total sterilization at a high suspension but completely inhibited at a low concentration. After electrolysis of a bacterial suspension at 10⁶ CFU/ml for 5 min between 0 and 50 mA, only two logarithmic

reductions of bacterial cells were achieved, however no total bacteria inhibition was noticed. Morphological changes were observed from electrolyzed *P. aeuroginosa* such as the destruction of the cell wall and aggregation in the peripheral cell wall. Yet, no degeneration of the genomic DNA of the electrolyzed bacteria was observed indicating a functioning DNA polymerase. These studies showed the ability to generate available free chlorine from small amounts of chloride, and that free chlorine can be reactivated, retained from low concentrations of chloride normally present in tap water and the electrolyzed tap water used for medical disinfection [46].

2.5.3. The Application on Agriculture

EOW with a pH of 2.6, ORP of 1150 mV and FCC of 50 ppm was used to treat alfalfa seeds and sprouts inoculated with *E. coli* O157:H7 for a period of 64 minutes. Generally bacterial reductions increased with increase in treatment time and the specific percentage reductions in bacterial load of treated seeds were between 38.2% and 97.1% (0.22-1.56 log CFU/g) while the reductions for treated sprouts were between 91.1% and 99.8% (1.05-2.72 log CFU/g) [24].

2.5.4. The Application on Fishery Industry

The disinfectant potential of acidic and alkaline EAW in washing and decontaminating fish fillets infected with *E. coli* O157:H7 and *L. monocytogenes* Scott A was studied [11]. After 64 min of treatment, acidic EAW achieved reductions 1.07 and 1.12 log₁₀ CFU/g for *E. coli* O157:H7 and *L. monocytogenes* Scott A respectively. Similarly, the treatment with alkaline EAW (EAW-C) for 17.5-30 min followed by acidic EAW resulted in significant reductions in *E. coli* O157:H7 and *L. monocytogenes* Scott A, 95.3% and 91.9% respectively. Moreover, a developed response surface model showed correlations (R^2) 0.52 and 0.77 between predicted and actual experimental log₁₀ reduction for *E. coli* O157:H7 and *L. monocytogenes* Scott A.

EAW was used as a bactericidal agent against microorganisms on seafood and platforms of fish retailers [30]. Tilapia fish samples were inoculated with cultured *Escherichia coli* and *Vibrio parahaemolyticus* and later soaked into EAW

(pH= 2.47±0.02, ORP=1159±4 mV, FCC=120±4 ppm) for a period of 10 minutes. Additionally, platform surfaces of retailers were also treated for 10 minutes with EAW solutions (50, 100, 200 ppm). EAW achieved significant bacterial reduction within this short time (i.e. 0.76 and 2.61 log CFU/cm² reduction on *E. coli* and *V. parahaemolyticus* respectively). Significant reductions in bacterial counts were also observed following application of EAW on ceramic tiles of platform surfaces.

2.5.5. The Application on Specific Bacteria

Selkon [47] investigated the effect of EAW (Sterilox, including of 144 mg/L FCC, pH 5.0-6.5, ORP> 950 mV) against to a wide range of bacteria such as *Mycobacterium tuberculosis*, *Mycobacterium avium-intracellulare*, *Mycobacterium chelonae*, *Escherichia coli* (including type 0157), *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Bacillus subtilis* var *niger* spores, methicillin-resistant *Staphylococcus aureus*, *Candida albicans*, poliovirus type 2 and human immunodeficiency virus HIV-1. EAW was significantly effective to destroy *Mycobacterium* species and *Bacillus subtilis* spores that were the most resistant microorganisms among all strains. Furthermore, they compared the EAW efficiency in the presence of organic substances (horse or calf serum) in the media. The loading of organics decreased the efficiency of EAW.

Sterilox EAW which had same character that of above report was used to destroy *Clostridium difficile* spores, *Helicobacter pylori*, vancomycin resistant *Enterococcus* species, *Candida albicans* and many of *Mycobacterium* species [48]. *C.diffile* was completely inactivated with EAW in 10 minutes. However, in the presence of organic substance (horse serum) log reduction was decreased. EAW destroyed *Helicobacter pylori*, *Candida albicans* and all *Mycobacterium* species even in the presence horse serum in 2 minutes. Even at a dilution of 1:10, EAW did not lose its disinfectant activity.

Kerwick and Reddy [49] investigated the effectiveness of electrochemical disinfection technology via a direct electrolyser system (Zappi™). In the system, three types of electrolytes (sodium chloride, sodium sulphate and sodium dihydrogen phosphate) were used to understand the effect of the composition of an electrolyte. Bacteria (*E. coli* or bacteriophage MS2) mixed with electrolyte solution

was placed into Zappi™ for a direct electrochemical disinfection process. Bacteria reduction was observed against time during 120 minutes of application. Although chloride-containing electrolyte was the most effective agent, sulphate and phosphate containing electrolytes also inactivated the bacteria. It was concluded that, other oxidant agents such as free radicals featured in the mechanism of electrochemical disinfection [50].

This study [19] examined the bactericidal properties of two types of EAW as LcEW and SAEW, one containing low NaCl and free-chlorine concentrations and another one containing higher NaCl and Free-chlorine concentrations, respectively. Although both waters were effective against *Mycobacteria* and spores of *Bacillus subtilis*, SAEW was strongly effective inhibiting the bacteria within five minutes of contact. Additionally, the study also tried to dissect the bactericidal mechanism of LcEW by examining the morphological and biochemical characteristics of *Pseudomonas aeruginosa* treated with LcEW. The inactivated bacteria revealed LcEW also inhibited blebs and breaks in its outer membrane and an essential enzyme, nitrate reductase. They showed that EAW targets Gram-negative bacteria based on their morphological findings.

Antibacterial and antiviral activities of a novel commercial EAW were tested [51] on a wide range of microorganisms including the Human Immunodeficiency Virus-1(HIV-1) and Adenoviral vector type-5. The new EAW had a neutral pH range of 6.2-7.8, low free chlorine concentration (51-85 ppm) and an estimated increased shelf life of one year. An 8 log reduction was achieved for all the bacterial samples after just 30s of treatment while the log reductions in the viral load of HIV-1 and Adenoviral type 5 were >4 and >3, respectively after 5 minutes of treatment. It was concluded that, EAW is an ecologically safe disinfectant because of its non-flammable and non-toxic properties.

Many factors affect the destruction of microorganisms such as disinfectant dose and contact time. Furthermore, the type of bacteria is very important to determine the most effective disinfectant, because the inactivation mechanism can be different according to the type of microorganism. Therefore, the ability of EAW to inactivate different types of microorganisms such as Gram-positive and Gram-negative bacteria has been investigated deeply in the literature [15, 52-55]. However, the most studies have been focused on optimizing disinfectant doses and contact time that are necessary to control pathogens by means of public health. In recent years, the studies tend to

evaluation of the destruction mechanism [56-64]. The inactivation mechanism of disinfectant agents on several type of bacteria was compiled in Table 2.2.

Table 2.2: The inactivation mechanism of different disinfectant agents on numerous bacteria.

Disinfectant Agent	Bacteria	Proposed Mechanism	Reference
Free chlorine	<i>E.coli</i>	Diffuses into the cell and attacks to intracellular components including enzyme with no change on permeability and surface structure of the cell	[60]
Chlorine dioxide	<i>E.coli</i>	Causes both surface damage and inner component degradation releasing total proteins. Enzyme degradation occurs slower than free chlorine	
Ozone	<i>E.coli</i>	Causes significantly cell surface damage, later, degrades intracellular components with a modest efficient	
UV	<i>E.coli</i>	Inactivates intracellular DNA by direct photochemical damage with no or little surface damage.	
EAW	Spores of <i>Bacillus subtilis</i> var. <i>niger</i>	On one hand causes DNA, protein, K ⁺ leakage and increases conductivity of the supernatant by destroying membrane structure of spores and on the other hands lows down the cellular metabolism by decreasing their dehydrogenase activities. Cell death occurs by the cumulative effect of these factors.	[64]
Slightly acidic electrolyzed water (SAEW)	<i>S. aureus</i>	Disrupts the permeability of cell membrane by damaging membrane proteins, enters the cells and causes forming of partly agglutinated cellular inclusions. Also, leads to a reduction in metabolism of the cells by disruption of dehydrogenases and other certain enzymes in anabolism and respiration reactions.	[61]
NaClO	<i>S. aureus</i>	HClO and ClO ⁻ lead to conformational changes in proteins and destroy the native structure of the enzymes because of direct reaction and/or forming stable N-Cl bonds with them. Because of strong oxidizing capacity, HClO could oxidize certain enzymes of the cells such as dehydrogenases and enzymes responsible for respiration.	

Table 2.2: Continuation of the table.

HCl	<i>S. aureus</i>	Has negative effects on the transcription process, RNA itself and certain cellular proteins. After treatment, cells remain alive but are sensitive to many antimicrobial agents (sub lethal injury).	
EAW	<i>S. aureus</i> <i>E. coli</i>	EAW enters the cells by damaging cell wall of both Gram positive and Gram negative cells and leads to leakage of cellular components and ions such as protein, DNA, K^+ that damages ionic balance of inner and outer environment of the cell. Also, destroys dehydrogenase activities because of negative influence on the metabolic enzyme active sites especially enzymes take place in the anabolism and respiration reactions. It could also affect translation process negatively because of the direct reactions with protein monomers, amino acids.	[26]
Atmospheric cold plasma (ACP)	<i>S. aureus</i>	Thick peptidoglycan layer can be destroyed by generated ROS (reactive oxygen species) (including ozone) by the way of breaking C-O, C-N and C-C bonds. Because of the loosened cell wall, ROS enter the cell and harm intracellular molecules such as DNA. Cells die because of the increased intracellular destruction.	[58]
Atmospheric cold plasma (ACP)	<i>E. coli</i>	Generated ROS (including ozone) can cause damage on both peptidoglycan layer and the outer membrane (LPS) because of the direct reactions with C-O, C-N and C-C bonds of these molecules. A high amount of leakage is the result of the damage on the outer membrane caused by peroxidation of lipopolysaccharides. Main cause of cell death is severe damage of the cell wall and membrane resulting leakage of cellular components and ions such as K^+ .	

As seen in Table 2.2, the destruction mechanism of disinfectants on microorganisms may differ according to type of disinfectant agent. For instance, chlorine kills pathogens such as bacteria and viruses by breaking the chemical bonds in their molecules and reactive oxygen radicals possess an unpaired electron, which makes them highly reactive and thereby able to damage all macromolecules, including lipids, proteins and nucleic acids. Ozone oxidizes all essential components (enzymes, proteins, DNA, RNA) by cracking the cell membrane, so it causes lysis the cells whose membrane is damaged during this process. The inactivation mechanism of chlorine dioxide includes both chlorine and ozone mechanisms [60].

3. MATERIALS AND METHODS

3.1. Design Principles of Batch Electro Activated Water System

The batch system consists of a circular EAW reactor and power supply. The reactor involves a graphite anode, stainless steel cathode and an ion-permeable membrane between anolyte and catholyte side. Figure 3.1 shows schematic representation of the electrode batch EAW system.

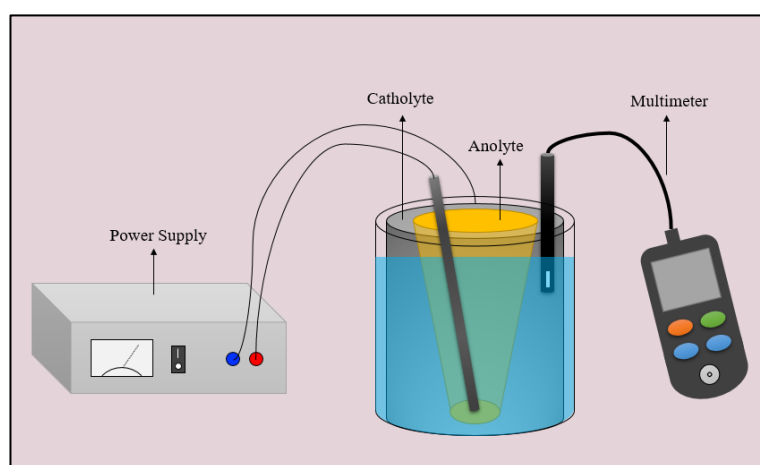


Figure 3.1: Schematic representation of the batch EAW system.

EAW reactor has 250 mL of EAW production capacity through a single batch run. It has 15 cm of diameter and 17 cm of height. To produce EAW, the brine solution is added into reactor and power supply is operated during desired reaction time. EAW is collected from anodic side of the reactor after electrolyzing finished.

3.2. Design Principles of Continuous Electro Activated Water System

A continuous EAW system consists of an electrode module, power supply, brine tank solution, peristaltic pump, measurement cell, and catholyte and EAW storage tanks (See Figure 3.2).

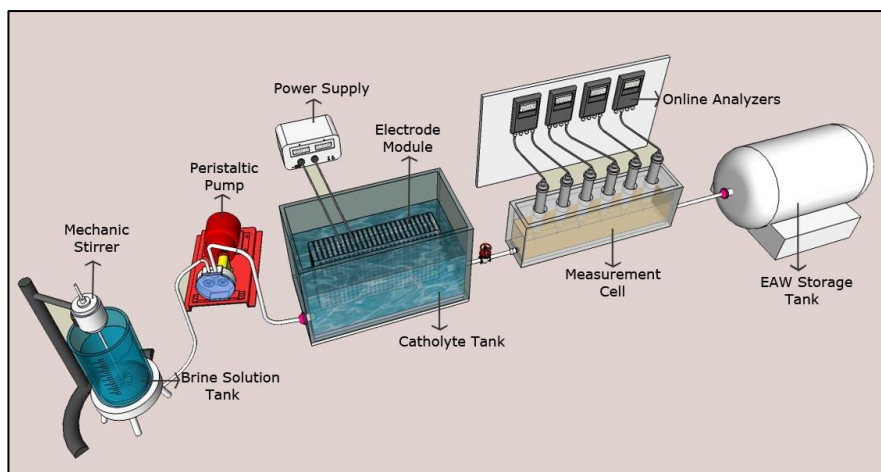


Figure 3.2: Schematic representation of the continuous EAW system.

The Electrode module is made of Plexiglas material with the dimensions of 25x20x5 cm. Graphite material was used for both anode and cathode with the dimensions of 20x15x0.2 cm. In order to get the uniform water distribution, there were 35 holes on the electrodes, 1cm² in diameter on the surface of graphite plate (See Figure 3.3). The holes are located within equal distance.

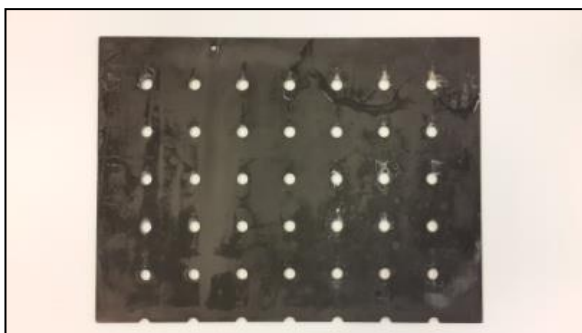


Figure 3.3: Graphite plate as anode or cathode electrode.

An ion-permeable diaphragm was located between the electrodes and the space between the electrodes and the diaphragm was five mm. EAW was generated from table salt and tap water at room temperature. The brine solution was introduced to the bottom left-hand corner of the module and was electrolyzed while moving through the chamber; it then left from the top right-hand corner of the chamber. A peristaltic pump was used to pump the brine solution into the reactor, and the brine solution was electrolyzed by feeding a direct current into the reactor. A digital power supply (0-20A; 0-40V) was used to provide energy during the experiments. The flow rate was

adjusted to 36L/h, and the system was running in an up-flow continuous mode. The catholyte was collected in the reactor tank, and anolyte was brought in through a flexible pipe. The length of the pipe was 30 cm, and polypropylene was used as the pipe material, which is resistant to acidic solution. A measurement cell with six sensors holders and a sampling holder was located at the end of the pipe. The samples collected from the cell were applied to the bacteria suspensions immediately.

3.3. Cyclic Voltammetry (CV) Studies

CV measurements were performed using an advanced electrochemical system, a computer-controlled potentiostat CH Instruments at scan rate of 200 mV/s. The working electrode was graphite with the area of 0.28 cm². The counter electrode was Pt wire, where Ag|AgCl was used as a reference electrode. The electrode potentials were measured in three-electrode cylindrical glass cell filled with different concentrations of the brine solution (20, 60 and 100 g/L). Before each analysis, the impurities on the electrode surface were removed by emery paper.

3.4. Electro Activated Water Disinfection of Bacteria and Drinking Water

3.4.1. The Preparation of Pure Bacterial Cultures

The bactericidal effect of the EAW was examined after careful preparation of pure cell cultures. When examining the effects of a disinfectant on bacteria it should use at least one Gram negative (*E. coli*) and one Gram positive (*Enterococcus*) bacteria. The selected bacteria are need to be identified in drinking water according to Water Intended for Human Consumption Regulations [65] and National Primary Drinking Water Regulations [66].

E. coli (ATCC 35218) and *E. faecalis* (ATCC 19433) bacterial cultures were used as Gram negative and Gram positive bacteria, respectively. Stock bacteria was cultured in 75 ml Brain-Heart Infusion (BHI) broth in a 500 ml Erlenmeyer and incubated in an incubator shaker overnight at 200 rpm, 37°C. After incubation, the culture was split equally into two 50 ml falcons and centrifuged for 20 minutes at 3500

rpm, +4°C. Supernatant was discarded and the pellets were washed with sterilized dH₂O and again centrifuged for 20 minutes at 3500 rpm, +4°C. Following supernatant allocation, the cells were adjusted to 0.5 McFarland which was equal to 10⁸cfu/ml in 500 ml total volume (in that step sterilized dH₂O was used for dilution). Later on, 15 ml of 0.5 McFarland cells was centrifuged for 20 minutes at 3500 rpm, +4°C. Over again, supernatant was discarded by pipetting carefully. Above previous pellets, fresh 15 ml of 0.5 McFarland cells was added and centrifuged again for 20 minutes at 3500 rpm, +4°C. Supernatant was discarded by pipetting carefully. In that way (two step of centrifugation and removal of supernatant by pipetting), loss of pellet was prevented. In the end, each falcon consisted 30x10⁸ cells (i.e. OD₆₀₀=0.6 for *E. coli* and OD₆₀₀=0.4 for *E. faecalis* cells).

3.4.2. The Preparation of Synthetic Drinking Water

Synthetic drinking water was prepared including bacteria and humic acid. Humic acid (Sigma Aldrich) was used as a natural organic matter source and prepared at a concentration of 1000 mg/L as a stock solution. 1M of NaOH solution was used for quick dissolution. It was diluted to desired concentration before adding bacteria suspension to be ready for the disinfection experiment. NaCl was added as supporting electrolyte and its final concentration in solution was 0.05 M. The characterization of synthetic water sample is given Table 3.1.

Table 3.1: The characterization of synthetic water sample.

Parameter	Value
pH	7,73
Conductivity (ms/cm)	5102
Turbidity (NTU)	1.08
TOC (mg/L)	5,6
UV ₂₅₄ (nm)	0,443
<i>E.coli</i> (cfu/mL)	0,23 x 10 ⁶

3.4.3. The Characterization of Real Drinking Water

Real drinking water was conducted EAW disinfection into continuous system. Two types of water were taken from Ömerli Drinking Water Treatment Plant. The first one is from effluent of slow sand filter (before conventional chlorine disinfection). The other was taken from dam effluent, i.e. aeration influent point. The characterization of the samples is given in Table 3.2.

Table 3.2: The characterization of real water sample.

Parameter	Value	Parameter	Value
<i>Filtration Effluent</i>		<i>Aeration Influent</i>	
pH	7.01	pH	7.49
Conductivity (µs/cm)	314.25	Conductivity (µs/cm)	323.14
Turbidity (NTU)	1.08	Turbidity (NTU)	1.36
DOC (mg/L)	2.489	DOC (mg/L)	3.145
UV ₂₅₄ (nm)	0.026	UV ₂₅₄ (nm)	0.078
Ca ⁺² (mg/L)	0.031	Ca ⁺² (mg/L)	0.086
Mg ⁺² (mg/L)	11.8	Mg ⁺² (mg/L)	13.3
Total Iron (mg/L)	0.02	Total Iron (mg/L)	0.085
THM (ppb)	7	THM (ppb)	18
<i>E.coli</i> (cfu/mL)	0	<i>E.coli</i> (cfu/mL)	240

The treatment plant units consist of pretreatment, pre-chlorination, chemical treatment processes, filtration and post-chlorination units. Due to pre-chlorination, THM concentration as 7 ppb was observed in sample taken from filtration effluent. The samples were tested immediately after collection from treatment plants.

3.4.4. Electro Activated Water Disinfection of Bacteria in Batch Mode

In this study, batch treatment with EAW for bacteria inactivation was conducted with contact times of 30 seconds, 1, 5, 10 and 20 minutes. Subsequently, six tubes (i.e. one for control and the others for samples to be tried) of 30 ml cultures were centrifuged at 3500 rpm for 20 minutes for both cells. The supernatants were discarded

and 10 ml of desired concentration of EAW was added onto the pellets and mixed properly. During treatment time, EAW and the cells media was kept on a shaker to provide proper diffusion of disinfection through bacteria. When treatment time was over, 2 ml of 5 g/L sodium thiosulphate (STS) was added onto the samples to stop disinfectant activity. For control tube, 10 ml of sterile ultra-pure water added instead of EAW but 2 ml of STS was also added like the other samples. After treatment, samples were centrifuged again at 3500 rpm for 20 minutes to separate supernatants and pellets for individual analysis. Supernatants were used for measurement of protein, DNA leakage and conductivity assays whereas pellets were used for measurement of TTC (triphenyl tetrazolium chloride) dehydrogenase relative activity, flow cytometry, lipid peroxidation, and DNA damage and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Logarithmic reduction after EAW treatment was by counting the number of colonies formed by growing on a solid medium. The number of surviving colonies on each plate was counted after a 24 h incubation period. Sterile deionized water was run in parallel as a positive control. Disinfection treatments with chlorine and chlorine dioxide were conducted within same procedures to compare the efficiencies with that of EAW.

3.4.5. Electro Activated Water Disinfection of Drinking Water in Continuous Mode

Drinking water sample including bacteria and humic acid was treated in the continuous EAW system. To compare the effect of EAW on *E. coli* cells in the absence and presence of humic acid as an organic matter, two sets of experiments were conducted. In the first step of the experiments, 250 mg of humic acid was added to 10 L of tap water and dissolved totally for the experiments with humic acid. 1-2 drops of 5% NaOH solution were added to the water to enhance solubility of humic acid. For the experiments without humic acid, this step was missed. Then, NaCl was added to the water at 0.05 M final concentration and dissolved properly. For the second step of the experiments, 1 ml of overnight culture of *E. coli* cells was added to the solution and EAW was started to produce in continuous system at a flow rate of 12.75 L/hour. Five different currents (0.1, 0.3, 0.5, 0.7, 1 A) were tried and for each current, 1 ml of samples were collected at eight different times (0, 1, 3, 5, 7, 10, 20 and 30 minutes). 9 ml of sodium thiosulphate (STS, 0.1 g/L) was added to 1 ml of samples to stop

activity of EAW. From this mixture, appropriate dilutions were performed in 50 ml of sterile ultra-pure water to get countable number of colonies in the agar plates after membrane filtration procedure. All dilutions were passed through the 0.45 pore sized membrane (Sartorius) by using membrane filtration system. Membranes were placed on Endo Agar plates (Sartorius) and incubated at 37 °C overnight for bacterial growth. After incubation period, colonies were counted and agar plates containing 30-300 colonies considered as significant. Log reduction values for each sample were calculated and residual Cl₂ (mg/L) concentration was observed during reaction time. Additionally; UV₂₅₄ (nm), TOC (mg/L) and THM (ppb) values were measured for the experiments with humic acid solution.

3.5. Inactivation Mechanism of Electro Activated Water

3.5.1. The Measurement of TTC Dehydrogenase Relative Activity

Since TTC dehydrogenase enzyme works only in living bacteria, it is typically used for the detection of viability of bacterial cells after treatment with antibacterial molecules. This enzyme produces insoluble red colored TF (2,3,5-triphenylformazan) molecule, by reducing hydrogen acceptor TTC (triphenyl tetrazolium chloride) molecule in the solution Figure 3.4 [67] shows the role of TTC dehydrogenase enzyme during the reduction of TTC.

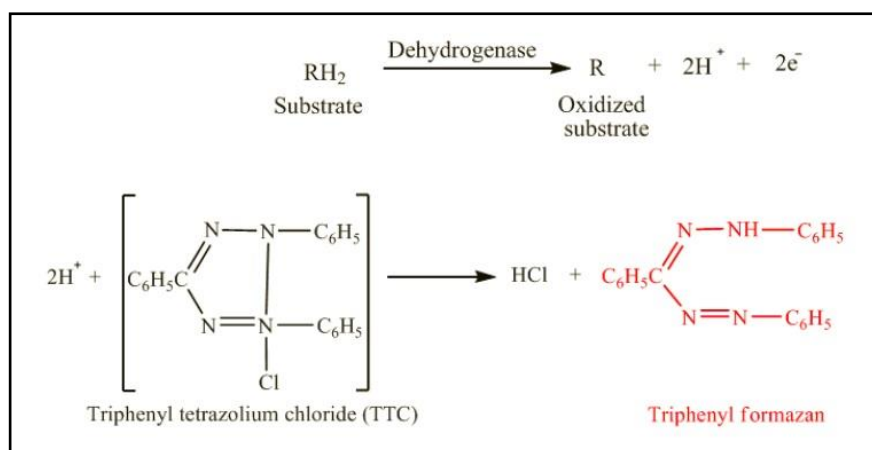


Figure 3.4: The role of TTC dehydrogenase enzyme during the reduction of TTC (2,3,5-triphenyl tetrazolium chloride) to TF (2,3,5-triphenylformazan).

The measurement of absorbance of TF molecule at 485 nm is used for the determination of TTC dehydrogenase enzyme activity. For analysis, pellets of the control and treated samples were solved in 10 ml of Phosphate Buffered Saline (PBS, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄ for 1L, pH:7.4) solution. 2 ml of Tris HCl (1.5 mole/L pH: 8.8) and 2 ml of TTC-glucose solution (is prepared by mixing 4% TTC solution and 0.1 mole/L glucose in equal ratio) were added to the 6 ml of samples. Then, samples were incubated at 37 °C for 2 hours in dark for activation of chromogenic reaction. After incubation, 7.5 ml of toluene and 7.5 ml of glacial acetic acid were added onto the samples, mixed properly and incubated at room temperature for extra 1 hour to separate organic phases. Whereas toluene triggers this separation reaction, glacial acetic acid makes red color of TF more apparent for detection. Upper phase of samples transferred to quartz cuvettes to measure absorbance at 485 nm by using a UV-Vis spectrophotometer (Hach Lange). For calculation of TTC dehydrogenase relative activity (DRA), “DRA (%) = (A_x/A₀) x 100” equation was used [61, 64]. In this equation, A₀ and A_x refers to absorbance value of control sample and treated samples at 485 nm, respectively.

3.5.2. Flow Cytometry

Although flow cytometry technique was used for eukaryotic cells in the beginning, it has been also adapted to enumeration of viable or dead bacterial cells. In this technique, propidium iodide (PI) dye is commonly used for detection of dead cells in a solution due to its character. PI can enter only the cells with damaged membranes whereas live cells are impermeable to this dye due to their intact cell membranes.

After treatment, pellets of the control and treated samples were solved in 10 ml of sterile ultra-pure water. Technique was performed according to the manufacturer’s instructions (BD Biosciences), hereunder; 100 µl of samples were diluted at 1:10 in staining buffer (PBS, 1 mM Ethylenediaminetetraacetic acid (EDTA), 0.01% Tween-20 and 0.1% sodium azide, pH 7.4). After mixing properly, 200 µl of mixtures transferred to new Eppendorf tubes. 5 µl of PI (BD Biosciences) added onto the samples and incubated in dark for 5 minutes at room temperature. After incubation time, samples were analyzed on BD ACCURI C6 (BD Biosciences) flow cytometer. PI dye was excited with 480 nm laser and analyzed with FL2 bandpass filter (585/20).

First, to determine the gate of live bacterial cells, unstained control cells were given to the flow cytometer. Then, stained control and treated samples were analyzed according to this gate. The percentage of PI positive cells was taken as dead cell population.

3.5.3. Lipid Peroxidation

Malondialdehyde (MDA) is produced normally during polyunsaturated fatty acid peroxidation process in the cells. However, the damage of reactive oxygen species (ROS) on the cells leads to lipid peroxidation, which could be detected by increasing amount of MDA. Accordingly, amount of this compound is a marker for oxidative stress of the damaged cells. For detection of MDA level in the cells, TBA (Thiobarbituric Acid) is used as MDA forms a pink colored complex with TBA (See Figure 3.5). MDA amount could be easily detected by measuring absorbance at 532 nm of the samples.

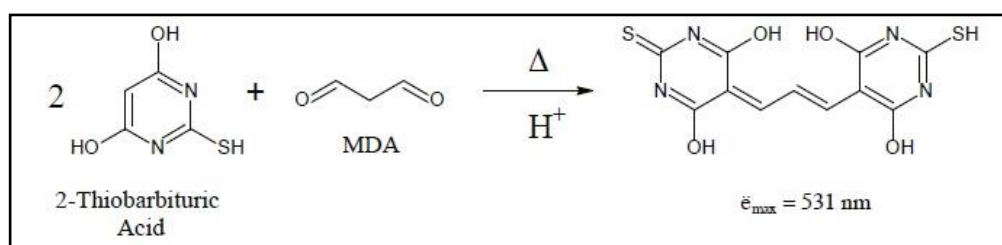


Figure 3.5: The reaction between 2-thiobarbituric Acid (TBA) and Malondialdehyde (MDA) under acidic condition.

After resolving of pellets of the control and treated samples in 10 ml of PBS, 1.5 ml of these samples were mixed with 3 ml of 10% (wt. /vol) trichloroacetic acid (TCA). Mixtures were centrifuged at 4000 rpm for 30 minutes. Then, the supernatants were transferred to new centrifuge tubes and 0.67% (wt. /vol) 4.5 ml of freshly prepared TBA solution added onto them. To activate chromogenic reaction, samples were kept at boiling water for 10 minutes in dark. The absorbance values at 532 nm of the samples were measured by using a UV-Vis spectrophotometer (Hach Lange) after cooling down to room temperature. For quantification of MDA molecule as $\mu\text{g/ml}$, a standard curve was formed by using certain amount of standard MDA molecule (Sigma) after interaction with TBA [60].

3.5.4. The Measurement of Intracellular Protein Leakage

The treated and control samples were centrifuged at 3500 rpm for 20 minutes. 500 µl supernatant of each sample was collected and stained with 500 µl Coomassie brilliant blue G-250 solution for 10 minutes. Then, the leakage of protein was determined according to absorbance measurements at 595 nm using a UV/Vis spectrophotometer by the Bradford Method. The calibration curve was performed using BSA (bovine serum albumin) [60].

3.5.5. The Measurement of DNA Leakage

DNA normally is found inside the living cells and its detection in the supernatant refers to disruption of bacterial cell membrane because of treatment. The supernatants of treated and control samples were used for the measurement of absorbance 260 nm which is used for detection of DNA by using a UV-Vis spectrophotometer (Hach Lange). The concentration of DNA in the supernatants of the samples were calculated based on “OD₂₆₀=1 equals to 40 µg DNA” equation [26].

3.5.6. The Measurement of Conductivity

Not only intracellular organic molecules but also ions could leak to the supernatant because of the negative effects of disinfectants on the cell membrane of bacteria. After releasing these components from cell structure, conductivity increases instinctively. Conductivity measurement of the supernatants of treated and control samples were carried out by using Hach Lange HQ40 multimeter.

3.5.7. DNA Damage

For determination of intracellular damage, the treated and control samples were waited for 24 hours to give enough time for reaction of cell components and reactive oxygen species (ROS). SYBR Green I [2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-

phenyl-quinolinium] (Sigma-Aldrich, S9430) is a dye which is used to determine nucleic acid quantity because of its sensitivity to dsDNA.

To evaluation of DNA damage, cells were destroyed with lysozyme, which has capability of breaking 1-4 bonds between N-acetyl- β -D-glucosamine (NAG) and N-acetyl- β -D-muramic acid (NAM), and lysostaphin, which has capability of breaking polyglycine cross-links present in the peptidoglycan. After that, the treated samples were incubated in 100 μ g/ml lysozyme at 37 °C for 4 hours. In the case that the treated cells are Gram positive, 10 μ g/ml of lysostaphin is also added beside lysozyme before incubation.

Untreated cells with and without digestion were used as positive and negative control groups, respectively. It is considered that the digestion is achieved completely when there was not any bacterial colony on the Petri dish after incubation period. SYBR Green is added on the digested solutions in 1:1 ratio, mixed properly and then incubated at 37 °C for 15 minutes. 200 μ l of the mixture of each sample was taken into 96 well fluorescence microplate wells to measure excitation and emission wavelengths at 485 and 525 nm, respectively [62].

3.5.8. SDS-PAGE Analysis

Overnight cultures of *E. coli* and *E. faecalis* cells were inoculated into fresh Luria Bertani (LB) broth and BHI broth in 1:20 ratio, respectively. Both cultures were grown until OD₆₀₀ value equaled to 3 at 37 °C with agitation (200 rpm). Later, 10 ml of cultures transferred to centrifuge tubes for control and treated samples per time. After centrifugation at 4000 rpm for 15 minutes, supernatants were discarded and pellets were treated with sodium hypo chloride, chlorine dioxide and EAW; for 30 seconds, 1, 5, 10 and 20 minutes in batch cultures.

For control samples, sterile ultra-pure water added on the pellets instead of disinfectant agents. To stop activity of disinfectant agent, 5 g/L STS were added onto the samples. The control and treated samples were centrifuged at 4000 rpm for 15 minutes again and pellets were solved in 1 ml lysis buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM DTT, 1 mM PMSF).

To rupture the cells and acquire intracellular proteins, 5 minutes of sonication (20 seconds “on”, 15 seconds “of” with 20% amplitude) was conducted. Samples were

centrifuged at 11000 rpm for 30 minutes; the supernatants were transferred into new Eppendorf tubes and stored at -20 C until usage. For determination of total protein concentrations of samples, Bradford method was used. According to protein concentrations, volumes of samples were determined to load same amount of proteins to the gel.

After addition of 4X loading dye (2 ml of 1M Tris-HCl (pH 6.8), 0.8 g of SDS, 4 ml of 100% glycerol, 0.4 ml of β - mercaptoethanol, 1 ml of 0.5 M EDTA and 8 mg of bromophenol blue in 10 ml of ultra-pure water), samples were kept at boiling water for 5 minutes. Thereafter, the samples were loaded to the gel and run in the running buffer (14.4 g of glycine, 3 g of Tris and 1 g of SDS in 1 L of ultra-pure water). Coomassie brilliant blue solution (50% methanol, 0.05% Coomassie brilliant blue R-250, 10 % acetic acid and 39.95% ultra-pure water) was used for staining of the gel after electrophoresis. Destaining solution (50% methanol, 10 % acetic acid and 40% ultra-pure water) was used for decolorization of the gel except for the marker and separated intracellular protein bands.

3.5.9. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Perkin-Elmer spectrum 100 Fourier transform infrared spectroscopy (FTIR) with Golden Gate Attenuated Total- internal Reflection (ATR) accessory was used to spectroscopy analyze of treated and control samples. The bacterial pellets were laminated in Petri dishes; dehydrated KBr was added and grounded with glass knob. The scan range was 4000-650 cm^{-1} with a scan number of 4 and scan speed of 0.5 cm s^{-1} .

3.6. Analytical Procedures

Instantaneous measurements of pH, conductivity and ORP were measured by Hach-Lange brand HDQ41 model multimetre probes connected to the reactor. Turbidity was measured using a 2100P ISO turbidimeter (Hach, Germany). Free chlorine concentration was measured by using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method [17]. Standard chlorine solution was purchased from La Motte and the DPD method was verified. Chlorine dioxide concentration was

determined using an analyzer (Krohne- Optisens CL 1100), which has high selectivity due to its membrane-free gold (potentiostatic) electrode sensor. The $\cdot\text{OH}$ radical concentration was analyzed according to terephthalic acid (TA) cumulative protocol [68].

Ultraviolet Absorbance (UV) at 254 nanometer indicates the total concentration of organic compounds that is capable of absorbing ultraviolet illumination in drinking water. Therefore, it signifies changes in the aromatic structure of HA that can be broken down. Alongside UV_{254} , all of the spectroscopic measurements that given in the previous section were carried out according to Standard Method in a spectrophotometer device Hach-Lange brand DR 5000 model. Also, Hach Co. cuvette testing protocols were applied for total iron and hardness measurements.

Dissolved Organic Matter (DOC) is an expression of the organic matter in drinking water. The DOC concentration was analyzed according to Standard Method Section 3510 B [17] in the DOC analyzer (Shimadzu ASI-L). The technique based on nondispersive infrared method through oxidation of organic compound. All samples were filtered through a 0.45- μm Sartorius filter paper prior to analytical measuring. Three-time repetitive test results are presented as mean values.

In the presence of HA, disinfection by chlorine causes formation of DPBs, which are carcinogenic compounds. In addition, it is necessary to measure the potential of EAW in forming chlorinated active agent. Volatile trihalomethanes were analyzed immediately after reaction. Hach Lange Total Trihalomethanes plus Reagent Set was used for measurements.

3.7. Statistical Planning and Modelling by Response Surface Methodology for Optimization

Optimization is an important method used to improve the performance of systems and increase yields of processes without any additional costs. In order to determine optimal operating conditions, a parameter change is observed by keeping other parameters constant in a process referred to as one variable-at-a-time technique. However, the lack of interactive effects between parameters on the process, which makes it unable to predict the complete effects of the parameters, is a major drawback of this technique.

Response Surface Methodology (RSM) can be used for optimization studies to overcome this problem. RSM as a group of mathematical and statistical techniques can be used to generate beneficial mathematical experimental models that define the relationships between responses and variables working either independently or in combination on the processes. The term Response Surface Methodology came up because the resulting perspective of the mathematical model is a graph.

RSM is a statistical and a mathematical tool that is beneficial in developing, improving and optimizing processes where a response of interest is influenced by several variables and the objective is to optimize this response. RSM is applicable in the design, development and formulation of new products as well as improving existing products. This experimental methodology can generate a mathematical model that describes chemical or biochemical processes as well as defining the effects of independent variables on the processes.

Several parameters affect chemical and biochemical processes. However, it is important to select the parameters with major effects since not every effect of all parameters can be identified. Screening experiments help in identifying the independent parameters and this can include the use of factorial designs. Identification of the important parameters is followed by determining the direction of improvements and identifying the levels of the parameters. The success of the optimization process is directly dependent on the selected levels and any wrong levels will result in an unsuccessful optimization.

An optimization study using RSM can be divided into three parts. The first part is the preliminary work and involves determining independent parameters and their levels. The second part deals with selecting the experimental design and the prediction and verification of the model equation. Obtaining the response surface plot and contour plot of the response and determining optimum points is the last part of a study.

Response surface plot and contour plot are the two ways of visualizing the obtained predicted model equation. The relationship between the response and the independent variables is represented by the theoretical three-dimensional plot, the response surface plot, while the contour plot is a two-dimensional plot display of the surface plot in which lines of constant response are drawn in the plane of the independent variables. The shape of the response surface is displayed by contour plot.

The center of the system of a contour plot may refer to a point of maximum or minimum responses when the contour plot is in the shape of an ellipse or a circle.

Additionally, contours plots may display hyperbolic or parabolic system of the contours whereby the stationary point is neither a maximum nor a minimum point and is called a saddle point. These plots are useful in determining the model fit but do not necessary represent the true behavior of the system. It is also important to note that the contours (or surfaces) are a representation of estimated responses in which the fitted model gives rise to the nature of the system but not the true structure.

There is some of packaged software for RSM. Most of them use second order polynomial model due to its easy application to optimization (See Eq. 3.1).

$$R = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (3.1)$$

where R is the predicted response, X_i and X_j are independent factors, β_o is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient. When the model has three independent factors, Eq. 3.1 becomes;

$$R = \beta_o + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3.2)$$

The obtained regression coefficients can be used to estimate the response using the model equation. However, since most of the time the behavior of the system is unknown, it should be checked whether the model fits to the experimental data. The model adequacy can be verified by a number of techniques. These include; residual analysis, scaling residuals, prediction error sum of squares (PRESS) residuals, and testing of the lack of fit. The coefficient of determination (R^2), which normally explains the overall predictive capability of the model, is calculated from PRESS. However, R^2 is not a measure of the model's accuracy rather it is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model. Therefore, a large value of R^2 does not necessary mean that the regression model is a good one. R^2 can be easily increased by addition of a variable to the model, whether the variable is statistically significant or not. In this regard, models that have large values of R^2 can result in poor predictions of new observations or estimates of the mean response. One should obtain a straight line passing through the origin with angle of 45° [$y = x$] when experimental results are plotted against

model results. However, a line [$y = a + bx$] might be obtained in some cases with a regression analysis of $R^2 = 1.000$ that is very insignificant.

RSM affords a number of merits when compared to classical experimental or optimization methods that test one variable at a time. To start with, large amount of information can be obtained from a small number of RSM experiments. This is in contrast to the time consuming nature of classical methods where a large number of experiments are needed to in order to define the behavior of a system. Moreover, the interaction effect of the independent parameters on the response can be observed through RSM. This is especially true for biochemical processes where the interaction effects of the parameters such as synergism, antagonism and addition are more critical. These effects are easily clarified by the model equation for binary combination of the independent parameters.

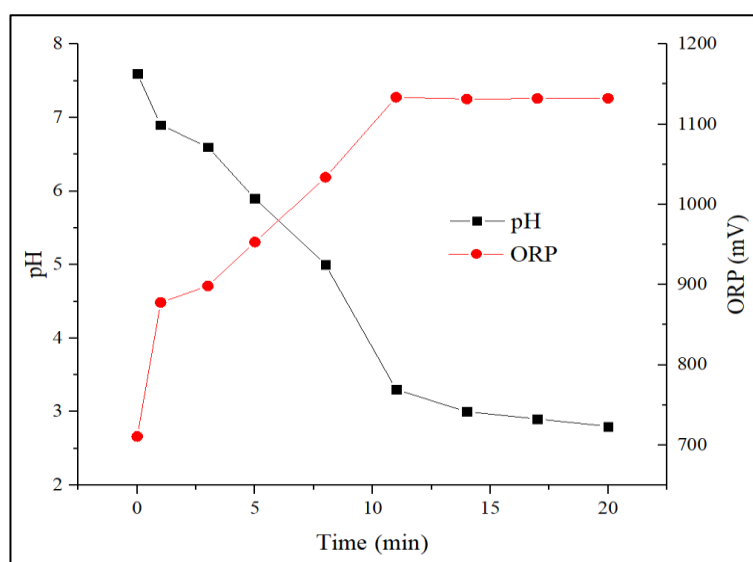
If the system is not easily explained by a second order model a smaller range of independent parameters can be selected. Additionally, the accuracy of the model equation can be increased by working in a narrow range of independent parameters, though this reduces the possibility of determining the stationary point. Thus, preliminary work is very important in determining the range of independent parameters.

Most of the reported RSM studies lack enough preliminary work about the range of independent parameters. Similarly, most of the optimization studies also lack an optimum point because of unsuitable range of independent parameters. The optimum point is defined as the maximum (or minimum) value of the response without the stationary point. However, this type of studies cannot be called optimization studies. In RSM studies where the stationary point is not determined, it is necessary that the aim of the study and the selection of the range of independent parameters are clearly explained. The predicted equation as well as the suitability of the estimated response to the expected result should be verified. In addition, the physical mean of the estimated results should be discussed.

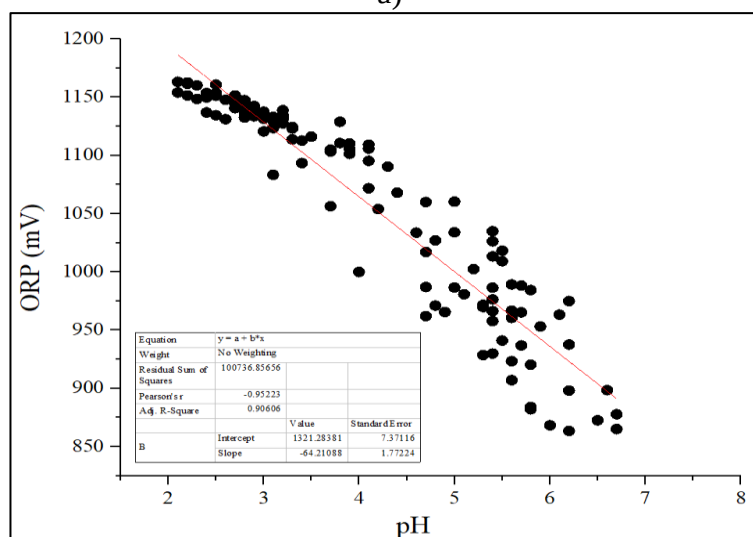
4. EXPERIMENTAL RESULTS AND DISCUSSION

4.1. The Characteristic Behavior of Electro Activated Water

Electro activated water acquires different properties according to production conditions. During electrolysis time, these changes in terms of pH and ORP can be seen as given Figure 4.1.



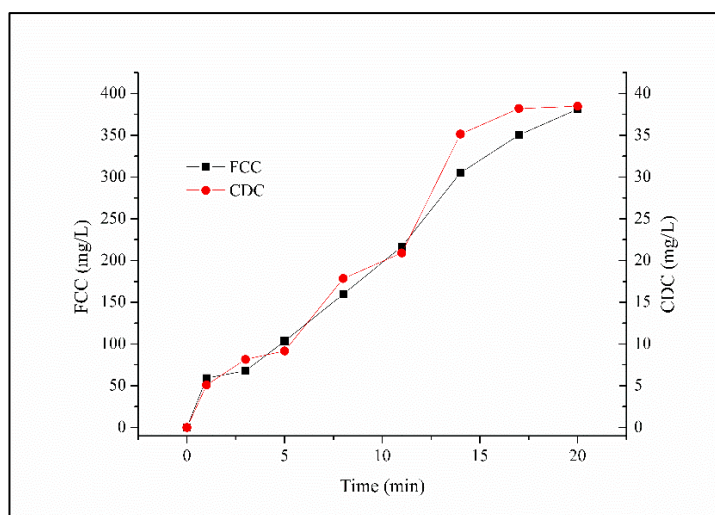
a)



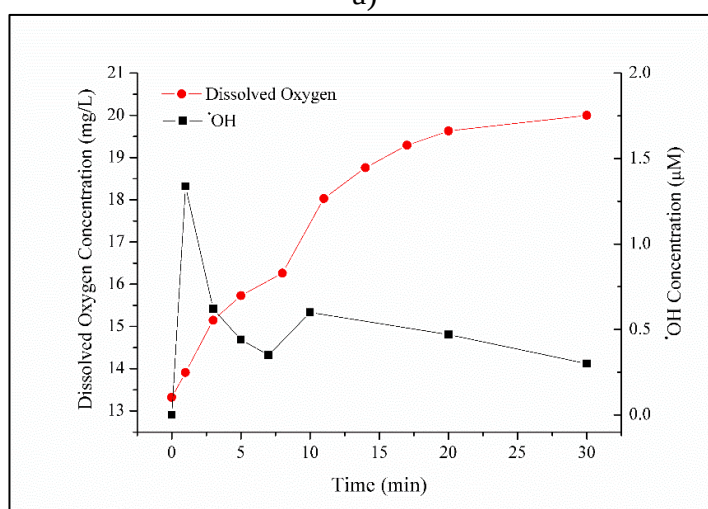
b)

Figure 4.1: The change of EAW parameters a) pH and ORP changes of EAW during electrolysis (temperature 25 °C, applied current 10 ampere, flow rate 36 L/sa, brine concentration 60 mg/L), b) The correlation between pH and ORP (25 °C).

Figure 4.1 a shows pH and ORP values during EAW production process. The increase in electrolysis time caused increase in ORP and decrease in pH value. It takes a certain amount of time to acquire the characteristic feature of EAW. After a while, pH and ORP showed a constant trend. The initial pH of brine solution was 7.5 and then decreased sharply in 10 minutes. After this point, EAW became its strong acidic and highly oxidative form, i.e. SAEW. When ORP value is between 1100-1200 mV, SAEW has antimicrobial activity against bacteria, viruses and some fungi. Figure 4.1. b shows the correlation of pH and ORP. A linear relation can be seen between them with the high Adj- R^2 value as 0.90. Whereas ORP has not changed at low pH, it is more scattering in high pH. Furthermore, other characteristic parameters such as free chlorine, chlorine dioxide, dissolved oxygen and OH radical concentrations are given in Figure 4.2.



a)



b)

Figure 4.2: The change of EAW parameters a) FCC and CDC concentrations, b) Dissolved Oxygen and OH• concentrations during EAW electrolysis (temperature 25 °C, applied current 10 ampere, flow rate 36 L/sa, brine concentration 60 mg/L).

Figure 4.2 a shows free chlorine and chlorine dioxide content of EAW. As given in introduction section, free chlorine is most abundant species in EAW. FCC reached to 375 mg/L in 20 minutes of electrolysis time in given reaction conditions. On the other hand, ClO₂ was produced up to 37 mg/L at that time. Another characteristic behavior was evaluated in terms of dissolved oxygen, as seen in Figure 4.2 b. At anodic side, oxygen molecules are generated during electrolysis and some of oxygen in gas form releases (See Eq.2.1). Dissolved oxygen concentration increased properly during reaction time, and after a while, water is saturated by oxygen. On the other hand, OH• forms in the solution which has been considered as responsible for oxidation power of EAW.

4.2. The Modelling of the Operating Conditions for Continuous Electro Activated Water System

4.2.1. Development of Box- Behnken Design

A number of electrode configurations and materials for the EAW systems have been tried against different microorganisms [69]. The effectiveness of the systems, relative to the reduction of bacteria, viruses, and protozoa, differs from one system to another one. These differences arise due to variable cell configurations, electrolyte concentrations and composition, electrode material, types of microorganisms, and other experimental parameters, such as flow rate, brine concentration and current density [70]. The individual effect of certain parameters such as brine concentration, temperature, and flow rate on the EAW character has been investigated [12, 19, 49]. However, the truer approach is to define interaction among processing parameters and to determine optimum conditions as those of Ezeike and Hung [13] and Hsu [18].

Multiple linear regression model uses the least squares method in optimization step. The statistical analysis of obtained data was run through multiple linear regressions. The second order polynomial model was used for the regression analysis. The analysis of variance (ANOVA) was used to predict statistical parameters. Fisher F-test and Lack of Fit test were used to evaluate statistical significance of the model. Box-Behnken experimental design was employed to evaluate controlling factors (A: Electrolysis time, B: Brine concentration, C: Applied current) for the optimization of the EAW production according to two responses (R_1 : ORP, R_2 : Free Chlorine Concentration). A series of preliminary tests were conducted to determine minimum and maximum level of each factors. Table 4.1 shows the determined levels of each factors.

Table 4.1 The determined levels of each factor.

Factor	Min. -1	Medium 0	Max. +1
A (time)	2	11	20
B (brine conc.)	20	60	100
C (current)	5	7.5	10

Minimal and maximal levels of factors were determined as 2-20 minutes for electrolysis time, 20-100 g/L for brine concentration, and 5-10 ampere for current, respectively. The experimental design for the EAW production including 17 runs with three replicates has been given in Table 4.2.

Table 4.2: Box-Behnken design and actual and predicted values of responses.

Run	A: Electrolysis Time min	B: Brine Conc. mg/L	C: Applied Current Ampere	Actual ORP mV	Predicted ORP mV	Actual FCC mg/L	Predicted FCC mg/L
1	11	60	7.5	1008.30	1072.84	340.70	241.30
2	11	60	7.5	1125.70	1072.84	318.33	241.30
3	20	60	10.0	1140.40	1145.75	605.01	588.35
4	11	60	7.5	1111.50	1072.84	170.69	241.30
5	11	20	10.0	1139.60	1131.07	304.01	292.33
6	11	100	10.0	1132.60	1120.17	606.00	605.30
7	2	20	7.5	946.60	939.52	44.94	27.58
8	20	20	7.5	1111.90	1115.07	38.64	66.98
9	20	100	7.5	1121.10	1128.17	560.79	578.15
10	11	100	5.0	1018.00	1026.52	305.00	316.68
11	2	100	7.5	947.30	944.12	99.99	71.65
12	11	60	7.5	990.40	1072.84	220.18	241.30
13	2	60	5.0	857.90	852.55	45.45	62.11
14	11	60	7.5	1128.30	1072.84	156.62	241.30
15	11	20	5.0	985.50	997.92	73.71	74.41
16	20	60	5.0	1026.20	1010.60	361.58	332.54
17	2	60	10.0	928.60	944.20	283.81	312.85

Table 4.2 shows the summary of experimental planning with responses in terms of ORP and FCC. The actual values for ORP and FCC were the results of experiments; the predicted values were results given by the design programmer.

Figure 4.3 shows the correlation between predicted and actual values of ORP and FCC, respectively. The correlation factors (R^2) were equal to 0.851 and 0.942 for ORP and FCC, respectively, and showed the fitness of the regression models. Adjusted R^2 values (Adj- R^2) showed that there were high correlations between the actual and predicted values of responses (i.e. Adj- R^2 were 0.659 and 0.868 for ORP and FCC).

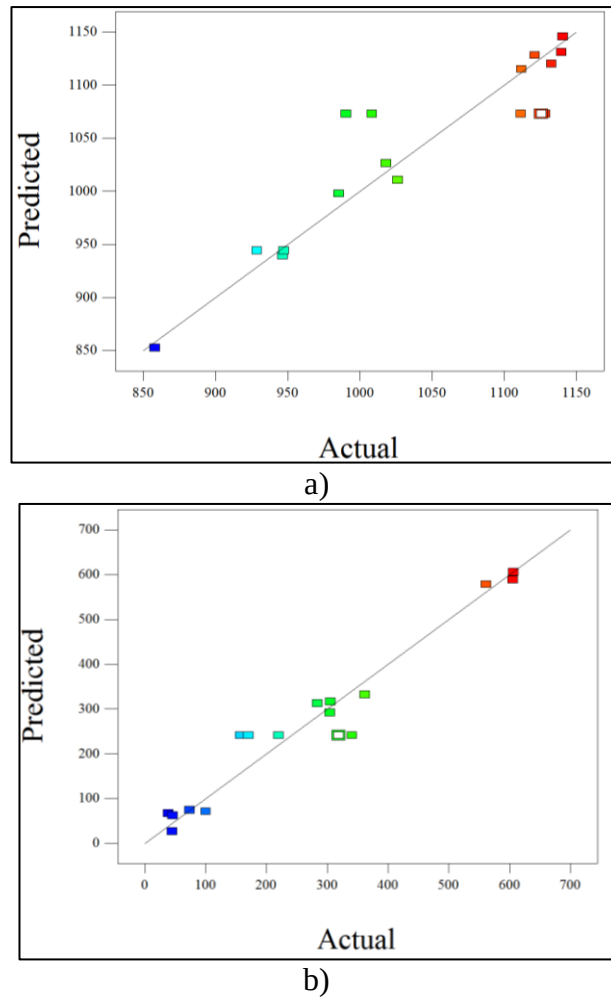


Figure 4.3: The correlation between predicted and actual values of parameters for; a) ORP, b) FCC.

The model F-value (Fisher variation ratio), probability value (Prob>F) and adequate precision are the main indicators showing the significance and adequacy of the employed model. According to F-value and p-value, the applied model for each response was significant at 95% confidence level z ($p < 0.05$), as seen in Table 4.3. p-values of each model were 0.0310 and 0.0015 for ORP and FCC, respectively.

Table 4.3: The analysis of variance for quadratic model of EAW.

Source	Sum of Squares	F Value	p-value Prob > F	
R ₁ , ORP				
Model	1.110E+005	4.44	0.0310	<i>significant</i>
A-Time	64656.08	23.28	0.0019	
B-Brine Conc.	156.65	0.056	0.8191	
C-Current	25719.12	9.26	0.0188	
AB	18.06	6.502E-003	0.9380	
AC	473.06	0.17	0.6922	
BC	390.06	0.14	0.7190	
A ²	15607.07	5.62	0.0496	
B ²	1645.28	0.59	0.4667	
C ²	2361.52	0.85	0.3872	
Residual	19444.70			
Lack of Fit	1118.35	0.081	0.9667	<i>not significant</i>
Pure Error	18326.35			
Cor Total	1.305E+005			
R ₂ , FCC				
Model	5.419E+005	12.72	0.0015	<i>significant</i>
A-Time	1.490E+005	31.47	0.0008	
B-Brine Conc.	1.541E+005	32.56	0.0007	
C-Current	1.283E+005	27.10	0.0012	
AB	54545.60	11.52	0.0115	
AC	6.43	1.357E-003	0.9716	
BC	1249.62	0.26	0.6232	
A ²	3005.27	0.63	0.4518	
B ²	3419.68	0.72	0.4235	
C ²	50369.01	10.64	0.0138	
Residual	33143.18			
Lack of Fit	4725.88	0.22	0.8769	<i>not significant</i>
Pure Error	28417.30			
Cor Total	5.750E+005			

The lack of Fit test indicates mathematical error of the model, and the test is considered as “non-significant”, if the error is large. The lack of Fit value for each response was large enough, that means that experimental data fit the models. Whereas the minimum value of ORP was 857.90 mV, its maximum value was 1140.40 mV. Moreover, FCC could be produced from 38.64 to 606.00 mg/L, according to experimental design in the reactor. The differences between minimum and maximum values of ORP and FCC showed that controlling variables affected the EAW character considerably.

The significance of the interaction among variables indicates that these factors have synergetic effect on the responses. When the Anova analysis is evaluated through individual terms and interactions; the significant model terms (i.e. $p < 0.05$) are A, C, A^2 for ORP and A, B, C, AB, C^2 for FCC. Therefore, none of combined effects dominated the regression model of ORP. However, electrolysis time and brine concentration (AB interaction) have synergetic effect on the regression model of FCC.

In the quadratic model equation (Eq. 3.2) x_1 , x_2 , x_3 were input factors as mentioned before. When design factors (electrolysis time (A), brine concentration (B), applied current (C)) are placed in the Eq.3.2 instead of x_1 , x_2 , and x_3 , respectively, the second-order polynomial equation becomes:

$$\begin{aligned} \text{ORP (mV)} = & 525.94 + 22.54 A - 0.69 B + 80.12 C + \\ & 0.0059 AB + 0.483 AC - 0.09 BC - 0.751 A^2 + 0.012 B^2 - 3.78 C^2 \end{aligned} \quad (4.1)$$

$$\begin{aligned} \text{Free Cl (mg/L)} = & 664.97 + 2.53A + 0.713B - 223.06C + \\ & 0.32 AB + 0.056 AC + 0.176 BC - 0.329 A^2 - 0.017 B^2 + 17.49 C^2 \end{aligned} \quad (4.2)$$

Although the brine concentration does not play important role for ORP, it is quite significant factor for FCC. Individual effects of factor designs are also shown through the perturbation plots in Figure 4.4.

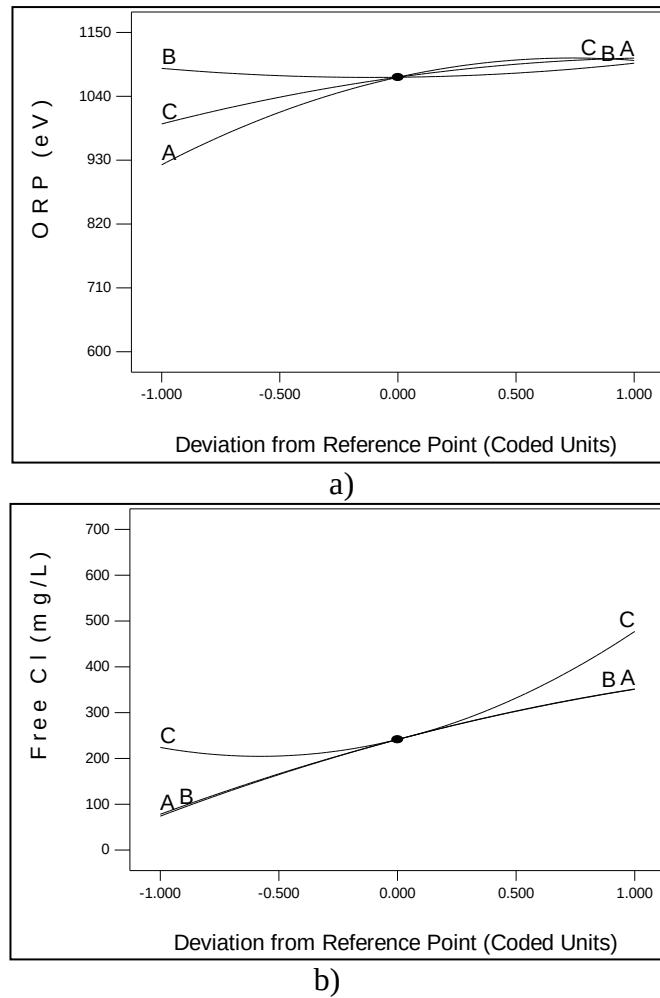


Figure 4.4: Perturbation plots for; a) ORP, b) FCC.

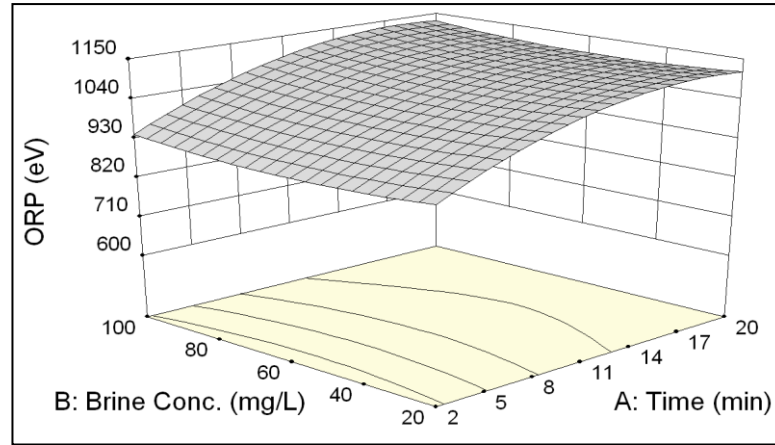
In Figure 4.4, the values of y-axis signify coded units, which were minimal (-1.000), central (0.000) and maximal (+1.000) levels of design factors. The reference (intersection) point of curves denoted 11 minutes of electrolysis time, 60 mg/L of brine concentration and 7.5 ampere. In this point, ORP was 1073 mV, and FCC was 244.5 mg/L. Individual effects of the factors on the ORP value of EAW are shown in Figure 4.2 a. Electrolysis time (A curve) has the highest influence on the ORP value. However, brine concentration (B curve) slightly affected the ORP value. The increase in current affected positively the ORP value of the EAW, as is shown also in Eq. 4.1. However, ORP increased significantly with the progress in the electrolysis time; and an instantaneous retardation towards the maximum point was observed.

Figure 4.4 b shows the perturbation plot for the effect of the factors on FCC. A and B smoothly increased from the minimal level to the maximal level of the design factor. Thus, the electrolysis time and the brine concentration showed similarity as to

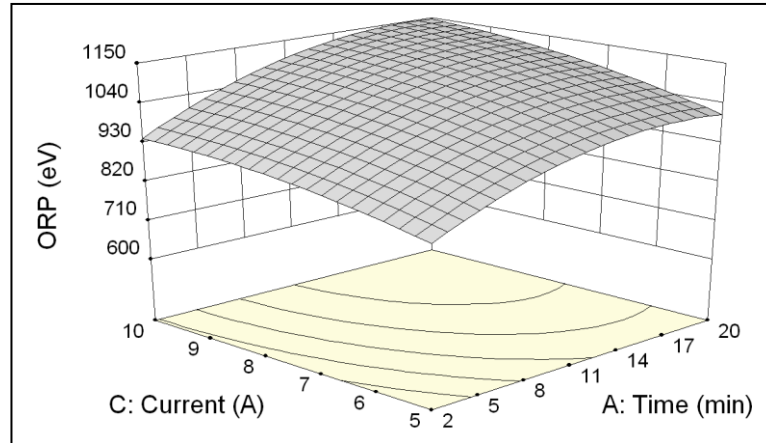
their effect on FCC. However, C curve indicated that applied current decreased before the reference point, and then there was a slight increase after the reference point. This means that the increase of applied current affected the FCC under higher brine concentration and longer electrolysis time.

3D response surface graphics of the regression models are used for the evaluation of the experimental data. Each graph demonstrated the interaction of two factors, as the third factor stayed constant at its central point.

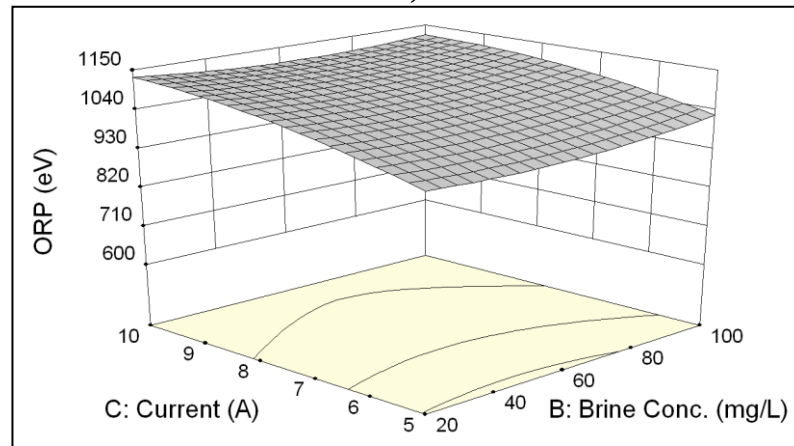
Figure 4.5 a is a function of the brine concentration, and electrolysis time at 7.5 ampere. The increase of time showed same efficacy with the lowest and highest brine concentrations on the ORP value of EAW. ORP was approximately 950 eV at the minimal level of reaction time without noticing the brine concentration. Furthermore, there was a potential difference of 9 eV between 20 and 100 g/L brine concentration at the maximal level of electrolysis time. The perturbation plot had denoted insignificance of the brine concentration, further the combined effect of brine concentration and electrolysis time was not significant ($p(0.9380) > 0.500$), as well. On the other hand, ORP increased noticeably as the electrolysis time and applied current increased, as seen in Figure 4.5 b. The regular spring-shaped contours indicated ORP varied by every different current and time and it reached the maximum of ORP being 1140 eV, eventually. Figure 4.5 c presents the interaction of the applied current and the brine concentration. The sparse contours at the bottom showed that the effect of these factors on ORP value was insignificant. And more affirmingly, the coefficient of BC interaction in Eq. 4.1 was negative.



a)



b)



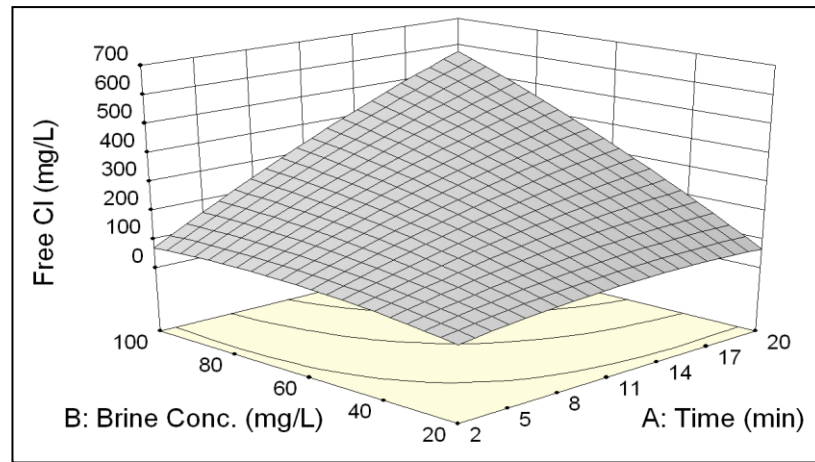
c)

Figure 4.5: Response Surface Graphs of ORP change under designed experimental conditions. The effect of the interaction of; a) brine concentration and electrolysis time on ORP (applied current 7.5 ampere), b) applied current and electrolysis time on ORP (brine concentration 60 mg/L), c) applied current and brine concentration on ORP (electrolysis time 11 minutes).

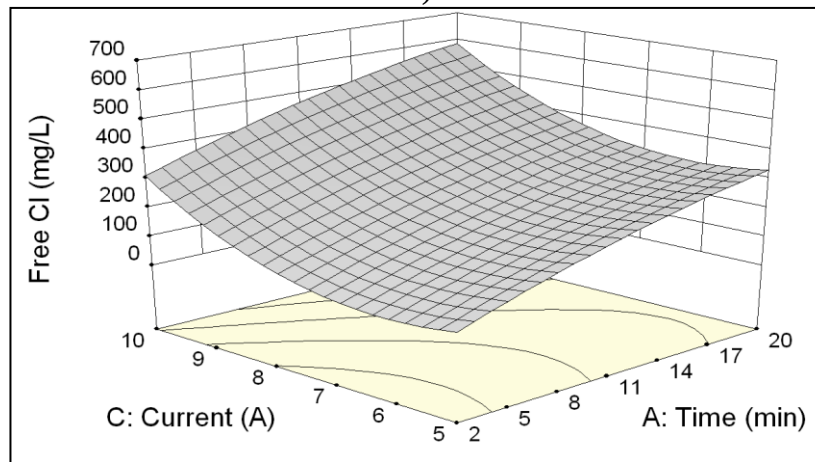
According to Eq.4.2, there was a synergy between the brine concentration and electrolysis time. In Figure 4.6 a, this relationship could be demonstrated by V-shaped surface graphs. The higher brine concentration and the longer electrolysis time resulted

in higher FCC. For example, the usage of 100 mg/L brine solution provided 560.8 mg/L of FCC at the maximum of electrolysis time. Figure 4.6 b presents the interaction among electrolysis time and applied current under the application of 60 mg/L brine concentration. The increase in electrolysis time caused an apparent gradient at all levels of current. However, FCC showed slight decrease through the middle value of current and then it started to increase significantly through the maximum value.

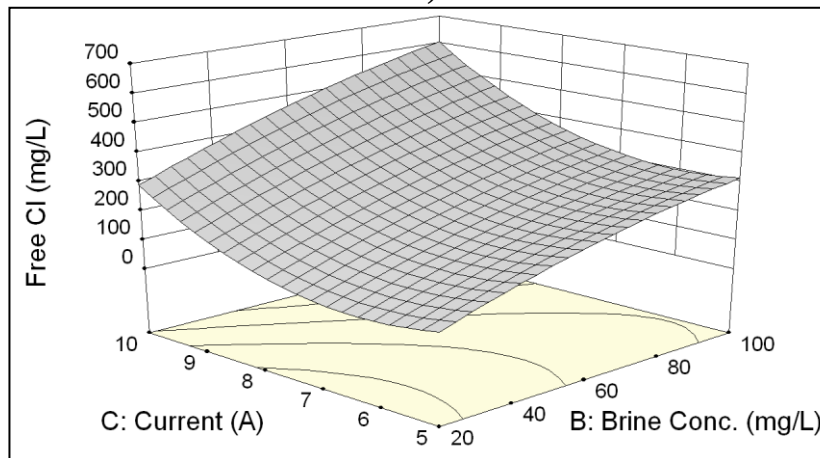
At the application of 5 amperes, FCC increased from 45 mg/L to 361 mg/L during the electrolysis time. Similarly, it increased from 283 mg/L to 580 mg/L at the application of 10 amperes. The effect of brine concentration and applied current that can be seen in Figure 4.6 c, is similar to the effect of applied current and electrolysis time. The increase of FCC relative to electrolysis time and applied current has also been reported in literature [19, 46].



a)



b)



c)

Figure 4.6: Response Surface Graphs of the EAW production under design experiment conditions. The effect of the interaction of; a) brine concentration and electrolysis time on FCC (applied current 7.5 ampere), b) applied current and electrolysis time on FCC (brine concentration 60 mg/L), c) applied current and brine concentration on FCC (electrolysis time 11 minutes).

The EAW production has also been modelled with RSM by other researchers. Ezeike G.O.I. et al. [13] evaluated controlling factors such as voltage, salt

concentration, flow rate and temperature. According to their results, the salt concentration has the highest effect on ORP among all parameters. When the voltage and NaCl concentration increase, both ORP and residual chlorine increase. Hsu S. [18] investigated previously same controlling factors but within a different design. The responses were pH, ORP, conductivity, salinity, dissolved oxygen and chlorine concentration. However, pH and dissolved oxygen were found insignificant ($p>0.05$) according to the Anova analysis. The brine concentration showed highly important effect on the chlorine concentration, whereas the change of temperature was not a significant factor. The increase of flow rate resulted in the lower values of ORP and chlorine concentration, according to both researchers.

4.2.2. Determination of Optimum Conditions for Electro Activated Water Production

EAW is used for different purposes by various users, and commercial devices for the EAW production differ from each other. Here, it is important to get the sufficient concentration of EAW, but not to produce the most concentrated EAW. Therefore, EAW production on demand can be realized according to the usage area.

There are no precisely defined parameters for the EAW generation. However, the brine concentration, current and electrolysis time are the most reported parameters that can significantly affect the character of EAW [12, 18]. Hsu [18] and Ezeike G. et al. [13] have shown that brine concentration was the most critical factor for the chlorine concentration among all factors.

According to our results, the brine concentration was also the most significant factor for FCC, however, other factors also should be considered to produce desired concentration of EAW, because the interactions of brine concentration with other factors were generally positive. The increase of current and electrolysis time together with brine concentration increased FCC. It can be concluded that the higher brine concentration provides more chloride ions and formation of hypochlorous acid (HOCl) and hypochlorite ion (OCl^-). An adequate electrolysis time leads to complete ion transport and new species formation. During electrolysis, the brine solution is separated, and ions are transported through membrane.

Current is another very important parameter in all electrochemical processes. Increase in current provides an efficient electro dialysis process, but it does not mean

that higher current always provides higher efficiencies. The desired value of current should be determined to avoid high-energy consumption. In our case, both electrolysis time and current highly affected the ORP value of the EAW. However, ORP exceeded 1000 mV after the middle values of all factors (i.e. 60 mg/L brine concentration, 11 minutes and 7.5 ampere), then it reached the stable stage. It can be said that current, which is higher than 7.5 ampere, is not necessary for providing higher ORP value.

Two approaches can be developed upon low and high voltage applications to define optimal processing parameters. The optimum values were determined within desirability function [71], as shown in Figure 4.7.

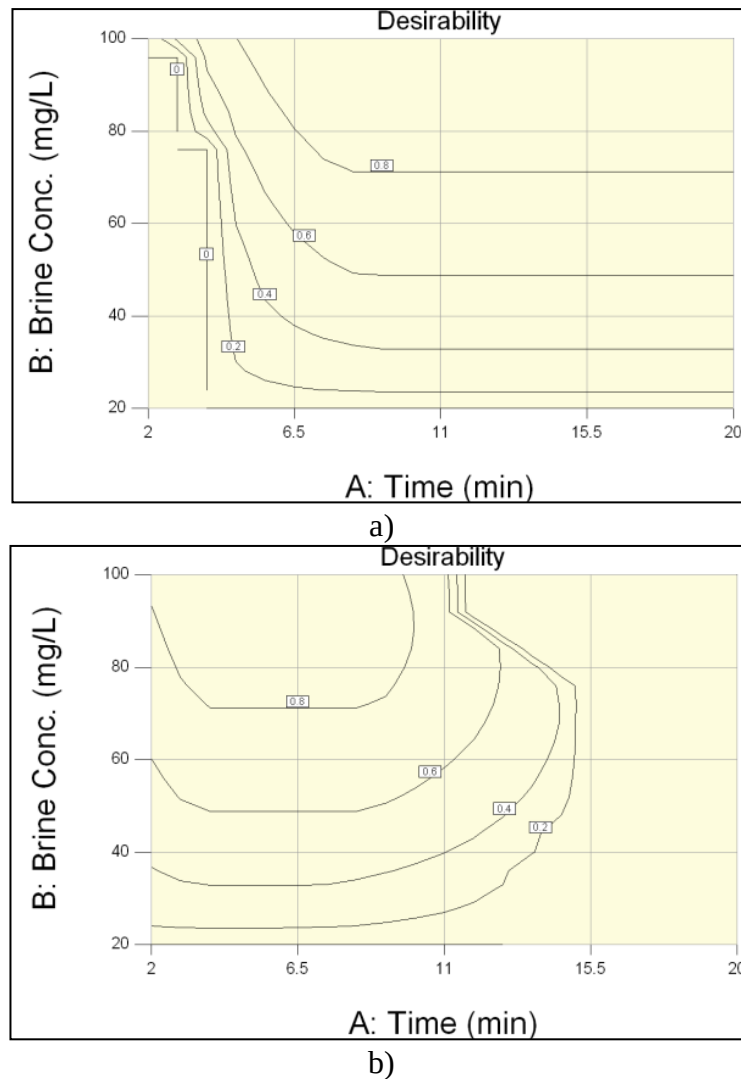


Figure 4.7: Desirability graphs for desired process conditions; a) applied current 5 ampere, b) applied current 10 ampere.

Figure 4.7 a and 4.7 b show the desirability graphs of the interaction of the brine concentration and the electrolysis time with the application of 5 and 10 ampere current, respectively. The optimum values were obtained by choosing “in range” function. Thus, ORP and FCC were chosen as 1000-1140 eV and 30-600 mg/L, respectively. The goals of the two responses were combined into one desirability function. The numerical optimization found a point that maximized the desirability function ($d > 0.8$). From the point of view of economy, EAW could be produced efficiently with the application of 5 amperes. In Figure 4.7 a, the desired operating conditions were achieved in about 6.5 minutes with the brine concentration of 70 mg/L. In this case, the energy consumption by the process was calculated as follows:

$$C_{\text{energy}} = \frac{Uit_{\text{process}}}{V} \quad (4.3)$$

Where U is an average cell potential (V) in the EAW reactor; i is a current (A) (5 ampere) t_{process} is electrolysis time (h) (6.5 min), and V is the volume (m^3) of the reactor (4.875 L in 6.5 min). According to this, 2.78 kWh of energy was needed to produce 1 m^3 EAW, which contains 100 mg/L of FCC. However, same properties of EAW can be produced in shorter electrolysis time with the application of 10 ampere current, as seen in Figure 4.7 b. Additional experiments were performed under the arbitrary conditions to confirm the model adequacy and the results of verification experiments are given in Table 4.4.

Table 4.4: The results of additional experiments under arbitrary conditions for model accuracy within the range of experimental design.

Experimental conditions	ORP (mV)		FCC (mg/L)	
	Predicted	Actual	Predicted	Actual
10 ampere 80 mg/L brine conc. 6.5 minutes	1041.94	1037.66	443.68	438.61
5 ampere 70 mg/L brine conc. 7.5 minutes	960.80	958.72	181.36	178.74
7.5 ampere 100 mg/L brine conc. 10 minutes	1083.64	1084.03	319.16	321.12

As seen from Table 4.4, the predicted values of ORP and FCC fitted the actual values. These results testify that the developed model can be used for prediction of desired operating conditions [72].

4.3. Cyclic Voltammetry (CV) Measurements and the Mechanism of Irreversible Electrochemical Reactions

Low-voltage process of electrolysis for the solution in the study has been carried out under high current intensity. CV graphs (curves) have been used to analyze the dependency on the experiment conditions and the electrolysis products obtained. It should be stressed that any electrochemical reaction being a non-equilibrium process is accompanied with the electron transfer at the electrode-solution border. By this, old bonds break-up and new bonds formation happens in the molecules and ions. By the electrolysis of water solutions, water molecules enter the reaction firstly. Corresponding reactions on the cathode and anode are the following ones:

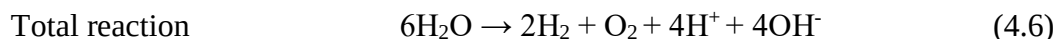
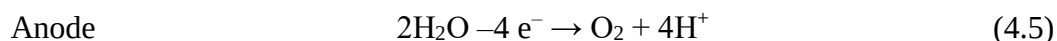
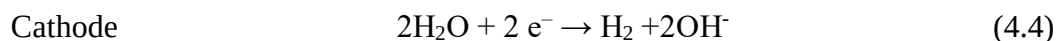


Figure 4.8 shows the effect of brine concentration on the CV behavior of graphite electrode at a scan rate of 200 mV/s using Ag-AgCl as a reference electrode. Polarization curves were measured for the graphite electrodes in NaCl solution at the concentrations of 20, 60 and 100 g/L.

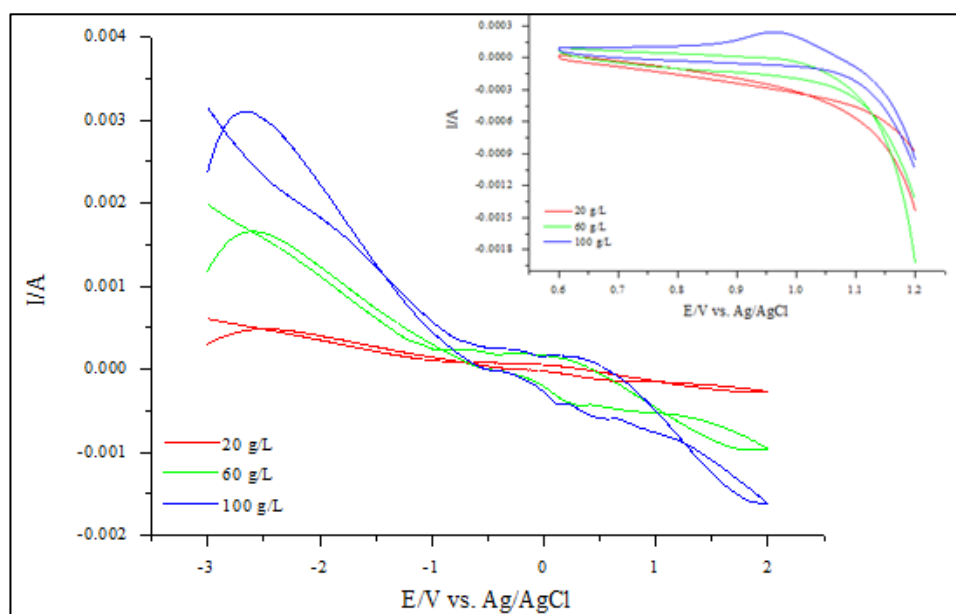


Figure 4.8: Cyclic voltammogram of different concentration of brine solution.

Spectroscopic researches of the water electrolysis show the value of breaking energy for the H-O bond as 1.59 eV. This energy is close to the electron energies for the hydrogen atom (1.51 eV) and oxygen atom (1.53 eV), both electrons being on the third energy level.

Peaks corresponding to the irreversible redox processes may be absent or considerably shifted. As seen from the CV curves, current poorly changes with the potential change at the initial stage (flat site). With the potential growth (1.5 – 2.0 V), a value was reached when the electrochemical reaction began. The increase of intensity caused the growth of ionic conductivity, and ions of hydronium (H_3O^+), coming into

contact with the cathode, dissociated and gave rise to molecular hydrogen and ions of hydroxyl OH^- formation. For other chlorine-containing substances that are present in the activated solution the corresponding reactions are shown in below.

The cyclic voltammograms showed a reduction peak at $E_{pc} = -2.71$ V vs. Ag-AgCl on graphite electrode which was assigned to Na formation. This peak can be seen clearly for 60 and 100 g/L NaCl solutions. Possible reactions at anodic and cathodic side during electrolysis are given in Table 4.5.

Table 4.5: Anodic and cathodic reactions during EAW production.

Equation	E^0_{red}
$\text{ClO}_3^- + 3 \text{H}_2\text{O(l)} + 6 \text{e}^- \rightarrow \text{Cl}^- + 6 \text{OH}^-$	+ 0.62
$\text{ClO}_2^- + 2 \text{H}_2\text{O(l)} + 4 \text{e}^- \rightarrow \text{Cl}^- + 4 \text{OH}^-$	+ 0.76
$\text{ClO}^- + \text{H}_2\text{O(l)} + 2 \text{e}^- \rightarrow \text{Cl}^- + \text{OH}^-$	+ 0.89
$\text{Cl}_2(\text{g}) + 2 \text{e}^- \rightarrow 2\text{Cl}^-$	+ 1.36
$2 \text{ClO}_3^- + 12 \text{H}^+ + 10 \text{e}^- \rightarrow \text{Cl}_2 + 6 \text{H}_2\text{O(l)}$	+ 1.47
$2 \text{HClO} + 2 \text{H}^+ + 2 \text{e}^- \rightarrow \text{Cl}_2(\text{g}) + \text{H}_2\text{O(l)}$	+ 1.63
$\text{H}_2\text{O}_2 + 2 \text{H}^+ + 2 \text{e}^- \rightarrow 2 \text{H}_2\text{O(l)}$	+ 1.77

There was almost no cathodic or anodic peak for 20 g/L NaCl solution. In addition, Cl^- formation can be observed between +0.62 and +0.89 for 60 and 100 g/L NaCl solutions according to the following reactions in Table 4.5. O_2 evolution can be observed at $E_{pa} = +0.08$ V for 60 and 100 g/L NaCl solutions. Between +1.2 and +1.8 V possible reactions at anode also can be seen in Table 4.5. The highest current was obtained in the 100 g/L NaCl solution. CV results promoting to the experimental results obtained in the study for 100 g/L NaCl were found more efficient in the EAW production [72].

4.4. The Modelling of Bacteria Inactivation with Electro Activated Water

4.4.1. Development of Box-Behnken Design

The experimental design for the EAW disinfection including 17 runs with three replicates has been given in Table 4.6.

Table 4.6: Box-Behnken design for EAW disinfection of bacteria.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: FCC mg/L	B: Temp °C	C: Time min	Log Reduction of <i>E. coli</i>	Log Reduction of <i>E. faecalis</i>
1	26	25	9	5.48	4.53
2	26	25	9	4.67	5.15
3	48	35	9	8.0	5.60
4	4	35	9	3.05	0.34
5	26	25	9	5.64	4.23
6	4	25	0.5	2.77	1.24
7	26	15	0.5	4.77	4.31
8	26	15	17.5	5.22	7.0
9	4	25	17.5	3.05	2.02
10	48	25	17.5	8.0	7.0
11	26	35	0.5	3.48	5.52
12	26	35	17.5	8.0	4.58
13	26	25	9	5.15	4.80
14	4	15	9	2.17	1.31
15	48	25	0.5	5.13	5.39
16	26	25	9	5.0	4.08
17	48	15	9	7.0	4.53

The experimental data were analyzed through multiple linear regressions. The regression models resulted in R-Square of 0.96 and 0.98 for *E. coli* and *E. faecalis*, respectively. The quadratic models that were established for *E. coli* and *E. faecalis* inactivation were significant ($p < 0.05$) at 95% confidence level (See Table 4.7).

Table 4.7: The analysis of variance for quadratic model for *E. coli* inactivation.

Source	Sum of Squares	df	Mean Square	F Value	p-value	Prob > F
Model for <i>E. coli</i>	52.85	9	5.87	22.96	0.0002	<i>significant</i>
A-FCC	36.51	1	36.51	142.74	< 0.0001	
B-Temp.	1.42	1	1.42	5.55	0.0506	
C-Time	8.24	1	8.24	32.22	0.0008	
AB	3.600E-003	1	3.600E-003	0.014	0.9089	
AC	1.68	1	1.68	6.56	0.0375	
BC	4.14	1	4.14	16.19	0.0050	
A ²	0.61	1	0.61	2.40	0.1656	
B ²	0.26	1	0.26	1.02	0.3469	
C ²	0.020	1	0.020	0.078	0.7876	
Residual	1.79	7	0.26			
Lack of Fit	1.20	3	0.40	2.68	0.1823	<i>not significant</i>
Pure Error	0.59	4	0.15			
Cor Total	54.64	16				
Model for <i>E. faecalis</i>	58.08	9	6.45	42.01	< 0.0001	<i>significant</i>
A-FCC	38.73	1	38.73	252.14	< 0.0001	
B-Temp.	0.15	1	0.15	0.99	0.3540	
C-Time	2.12	1	2.12	13.81	0.0075	
AB	1.03	1	1.03	6.73	0.0357	
AC	0.17	1	0.17	1.12	0.3260	
BC	3.27	1	3.27	21.32	0.0024	
A ²	9.82	1	9.82	63.97	< 0.0001	
B ²	0.033	1	0.033	0.21	0.6593	
C ²	3.27	1	3.27	21.30	0.0024	
Residual	1.08	7	0.15			
Lack of Fit	0.32	3	0.11	0.57	0.6640	<i>not significant</i>
Pure Error	0.75	4	0.19			
Cor Total	59.15	16				

Lack of fit value for each model was statistically insignificant compared to the calculated probability value. This proved that the models were adequate and fitting for

prediction within the range of parameters employed. According to p-values of parameters, significant variables were determined and placed into the second-order polynomial equation, they became as follows,

$$\text{Log Reduction of } E. coli = +5.07 + 0.10 A - 0.25 C + 0.003 AC + 0.012 B \quad (4.7)$$

$$\begin{aligned} \text{Log Reduction of } E. faecalis = & -0.56 + 0.19 A + 0.078 C + \\ & 0.002 AB - 0.010 BC - 0.003 A^2 + 0.012 C^2 \end{aligned} \quad (4.8)$$

According to Anova Analysis (See Table 4.7), significant model terms were A, C, AC, BC in equation for *E. coli* and A, C, AB, BC, A², C² in equation for *E. faecalis*. These equations were the modified version of the regression models (i.e. the insignificant model terms did not take place in equations). Temperature was an insignificant model term for both bacteria inactivation. Based on Eq.4.7, the other individual effects and the combined effects of FCC and time as well as temperature and time played an important role in the inactivation of *E. coli*. However, temperature and time did not affect *E. faecalis* inactivation. Individual effects of factor designs are also shown through the perturbation plots in Figure 4.9.

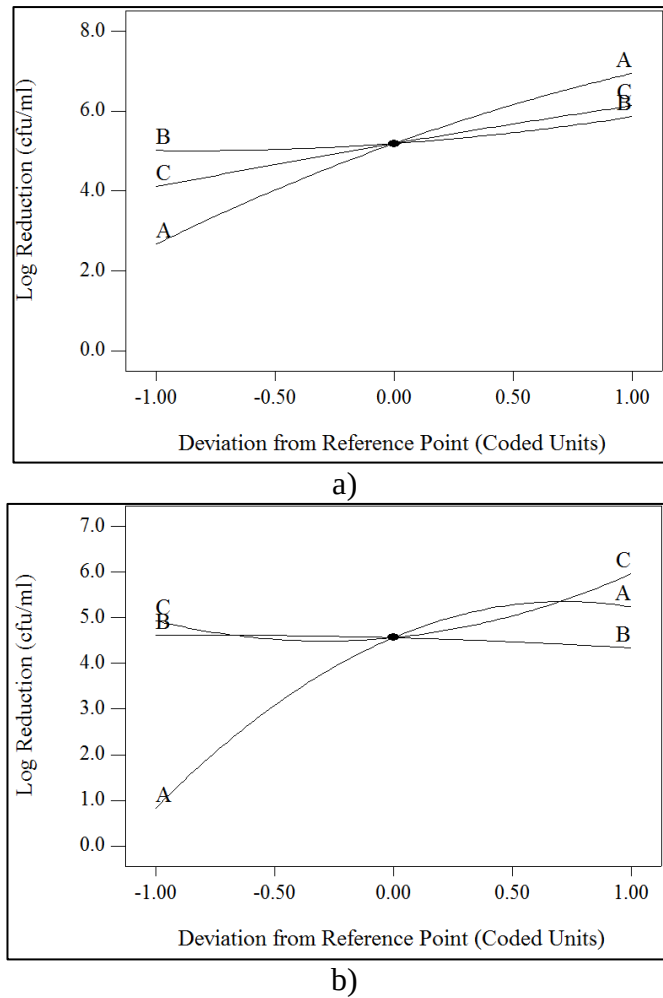


Figure 4.9: Perturbation plot for Log reduction of; a) *E. coli*, b) *E. faecalis* (FCC: 26 mg/L, temperature 25 °C, time 9 min.).

The individual effects of all factors can be compared using perturbation plots. The values of x-axis signify coded units which are minimum (-1.000), central (0.000) and maximum (+1.000) level of design factors. According to Figure 4.9 a, the FCC (curve A) had the highest influence on the Log reduction of *E. coli*, whereas the most ineffective factor was temperature (curve B).

The central point of the curves was denoted as 9 minutes of treatment time; 26 mg/L of FCC and 25 °C of temperature. At this point, Log reduction of *E. coli* was 5.37. Figure 4.9 b shows the perturbation curves of parameters affecting the inactivation of *E. faecalis*. According to these results, FCC also had the most effect on *E. faecalis* inactivation due to the high slope of curve A; on the other hand, the relatively straight slope of curve B implied that temperature was an insignificant parameter as seen in Eq.4.8. Despite an initial decrease in the bending of curve C up

to the central reference point, a steep increase was observed after this point indicating an influence of treatment time on *E. faecalis* inactivation after the reference point.

The response surface graphs of the regression models for *E. coli* inactivation are shown in Figure 4.10. Each subsequent graph demonstrates the interaction between two factors as the third factor stays constant at its central point.

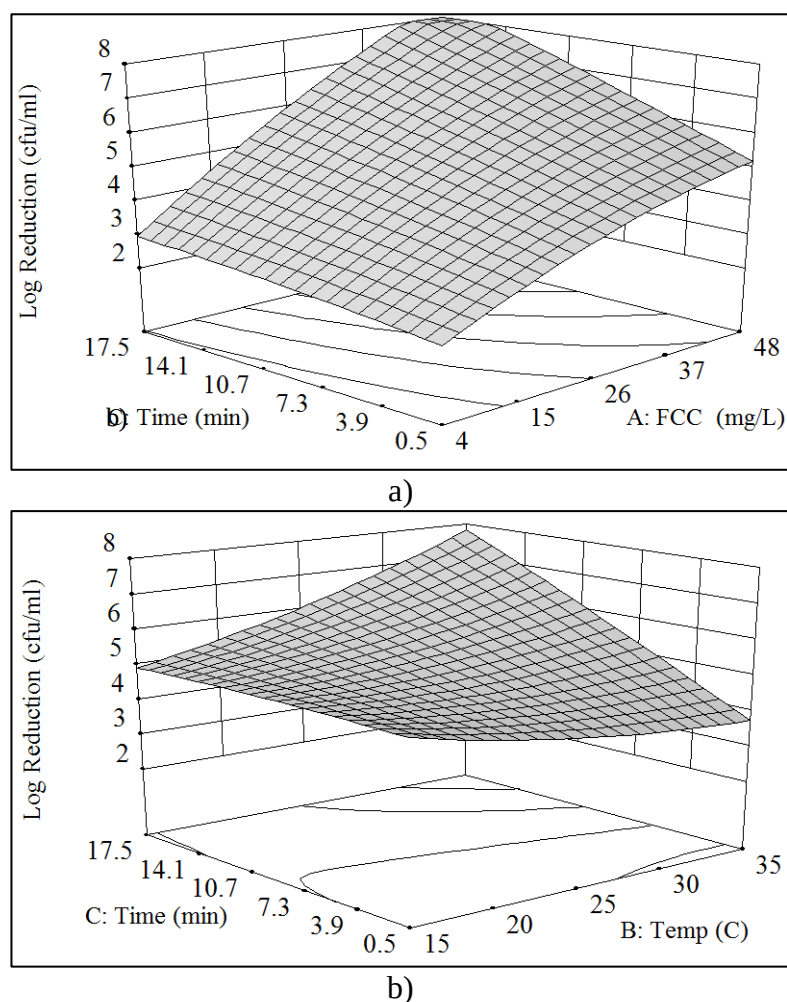


Figure 4.10: 3D Graphs of the EAW treatment of *E.coli* under design experiment conditions. a) FCC and treatment time interaction (temperature 25 °C), b) treatment time and temperature interaction (FCC 26 mg/L).

Figure 4.10 a represents the interaction of FCC and treatment time at 25°C. An increase in FCC resulted in higher Log reduction of *E. coli* with every minute. The longest treatment time with the highest FCC across completely inactivated *E. coli*. Moreover, the shortest treatment time (1 minute) was similarly efficient, as 5.13 Log reductions could be achieved with the same FCC (i.e. 48 mg/L). Even in the worst conditions of the experiment, i.e. 1 minute of treatment time and 5 mg/L of FCC of

EAW, 2.77 Log reductions of *E. coli* were observable. However, treatment time was not a considerable factor concerning the application of low FCC.

According to ANOVA analysis, AB interaction was an insignificant model term, which means that Log reduction of *E. coli* was not affected by interaction of FCC and temperature. That is, the effect of increasing FCC on *E. coli* inactivation was similar under all temperatures. The lowest Log reduction of *E. coli* observed was 2.17, under the application of 4 mg/L of FCC and at 15 °C of EAW. Nonetheless, 7 log reductions were achieved by 48 mg/L of FCC at all temperatures (Data not shown).

A synergetic behavior between temperature and treatment time that influenced the reduction of *E. coli* was observed as shown in Figure 4.10 b. Although the increase in time has a small effect between 15-25 °C, the reduction of *E. coli* significantly increased after 25 °C. Furthermore, the effect of temperature was negative around the minimum amount of treatment time, whereas it was positive upwards 6 minutes of treatment time. More than 5 Log reductions could be achieved after 6 minutes of treatment time at 20 °C and above. The highest Log reduction of *E. coli* obtained was 7.72 with the longest treatment time at 35 °C of EAW.

The response surface graphs of the regression models for *E. faecalis* inactivation are shown in Figure 4.11.

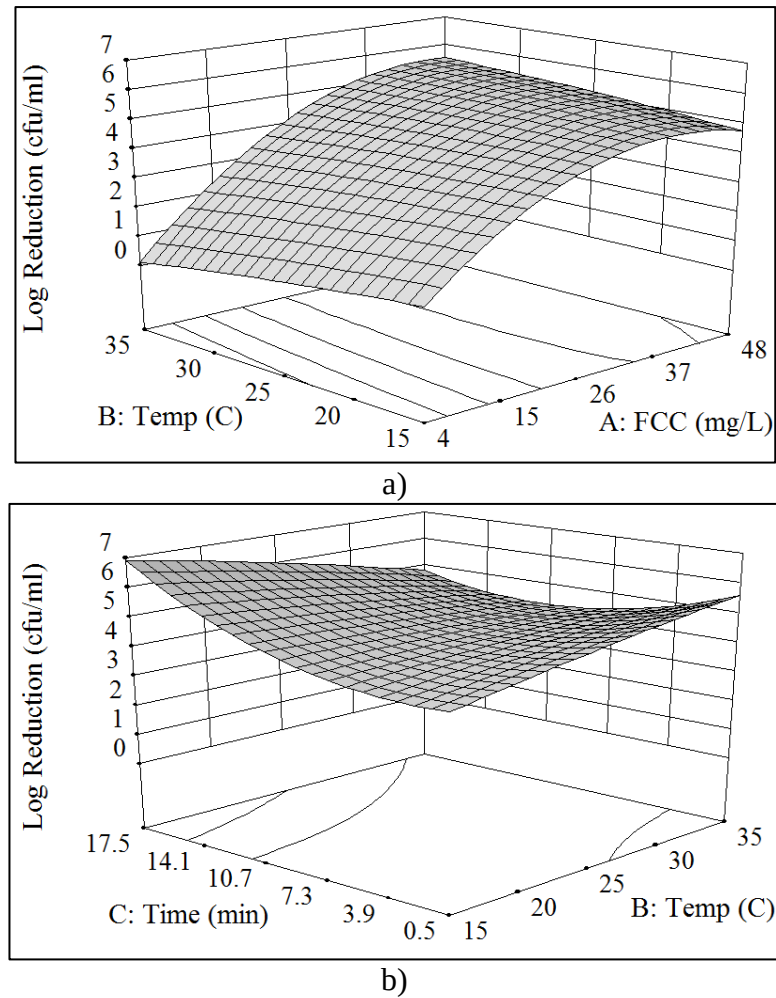


Figure 4.11: 3D Graphs of the EAW treatment of *E. faecalis* under design experiment conditions. a) temperature and FCC interaction (treatment time 9 minutes) b) treatment time and temperature interaction (FCC 26 mg/L).

Figure 4.11 a represents the interaction of FCC with temperature at fixed treatment time of 9 min. Increase in FCC resulted in significant *E. faecalis* inactivation. However, the effect of temperature on FCC was not observed after about 26 mg/L. Interestingly, 1.2 log reductions were obtained with the lowest chlorine concentration between the temperatures 15°C and 35°C. This implied that the inactivation effect of temperature increase is reflected at low chlorine concentration; nonetheless, the mutual interaction effect of these two parameters in *E. faecalis* inactivation is a positive one.

Figure 4.11 b demonstrates the interaction of various treatment times and temperatures at a fixed chlorine concentration of 26 mg/L. Although the coefficient of BC; is negative based on Eq. (4.8), the interaction effect of these two parameters was significant statistically. At short treatment times, while temperature increases,

bacterial inactivation increases, whereas the exact opposite results were observed in longer treatment times.

This situation is clearly seen from the sparse contour lines of Figure 4.11 b. The interaction effect of chlorine concentration and treatment time in *E. faecalis* inactivation was insignificant (Data not shown). There was no significant difference in *E. faecalis* inactivation between the shortest and the longest treatment time at chlorine concentration of 4 mg/L. Increase in treatment time became partly effective at higher chlorine concentrations. At 48 mg/L chlorine concentration of EAW, 0.5 min treatment time resulted in 5.4 log reductions of *E. faecalis* while complete inactivation was observed at 17.5 min treatment time.

Rahman et al. [20] also studied the efficacy of different treatment conditions on microbial inactivation. *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium* were exposed to different treatment times (1, 3, 5, 7 and 10 min), pH values (2.5, 4.0, 5.0, 6.0 and 9.0) and temperatures (4, 15, 23, 35 and 50 °C). As the pH increased towards the basic side, inactivation of microorganisms showed a decrease. Higher temperatures resulted in increased log reduction of bacteria, and this observation is compatible with our experiments. On the other side, as the treatment time increased, lower bacterial log reductions were recorded due to the decrease in chlorine concentration.

In a similar way, Sharma et al. [24] investigated the effect of contact time and current on the inactivation of *E. coli* O157:H7. Increase in treatment time did not have any significant correlation with inhibition of bacterial growth. When we compared our results with these reports, the individual effects of contact time were considerable; however, it became a more significant factor when it interacted with both higher temperature and FCC of EAW. Nonetheless, it had a low effect on log reduction under the application of low FCC and temperature. Similarly, in another study, EAW was applied to inactivate *E. coli* for 1, 5 and 10 minutes resulting in log reductions of 0.45, 1.17 and 1.68 (Log CFU/cm²), respectively [30]. A slight increase in log reduction of *E. coli* was obtained with an increase in time under the condition of 120 mg/L FCC. All of these studies had evaluated the individual effects of application parameters of EAW, on bacteria inactivation. However, when the application parameters are designed by RSM, the interaction between parameters and the main factor for efficiency can be defined. Furthermore, after developing the predictive model, an equation may be given through an established model.

There have been few reports on EAW application on bacteria as part of RSM. Ding et al. studied the inactivation of *Listeria monocytogenes* with EAW. The factors for EAW application were the same as ours but within different ranges of treatment time (1-5 minutes) and FCC (30-70 mg/L). The combined effect of temperature and FCC was highly significant, and resulted in increased log reduction of *L. monocytogenes*. On the other hand, the effect of FCC was more distinct than temperature, as observed in our results.

According to their ANOVA results, the combined effects of treatment time/temperature and treatment time/chlorine concentration were insignificant model terms. Specifically, the effect of treatment time was very low both individually and as a combined behavior. This can be due to the narrow range of treatment time of between 1-5 minutes. In our studies, we chose the maximum amount of treatment time as 18 minutes and therefore could obtain a significant effect of each value of the treatment time with both FCC and temperature. In this respect, it is highly crucial to determine the minimum and maximum levels of independent factors in order to see the efficacy on the response clearly [73].

4.4.2. Determination of Optimum Parameters for Bacteria Inactivation

Figure 4.12 shows the region of optimum operational condition that provides high log reduction of bacteria. Change in temperature did not affect the reduction of both bacteria. In this case, the temperature of EAW may be kept constant at room temperature to obtain optimum inactivation. The desired log reduction levels were chosen as 7 – 8 for *E. coli* and 6 - 7 for *E. faecalis*.

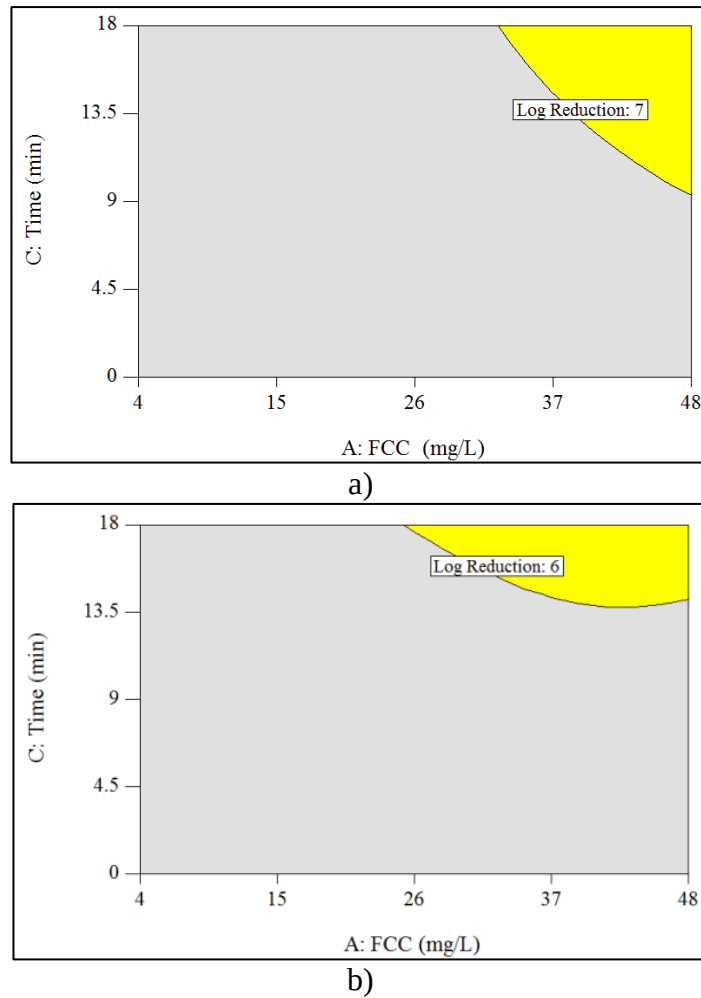


Figure 4.12: Optimal operational conditions to get high Log reduction levels; a) *E.coli*, b) *E. faecalis*.

The yellow portion of the graphs represents the parameters of EAW that can yield high inactivation efficiency, i.e. an efficient *E. coli* removal can be obtained with the application of EAW at 30 mg/L FCC and above, in 9 minutes and longer treatment times. The applied concentration and time were selected based on these graphs for further experiments. For example, 5 log reduction of *E. coli* was acquired with the application of 18 mg/L FCC during 18 minutes. However, FCC was required at 26 mg/L for *E. faecalis* treatment at a similar treatment time. On the other hand, 48 mg/L FCC application for 9 minute provided complete inactivation of *E. coli* but required 18 min for complete reduction of *E. faecalis*.

4.5. Disinfection Mechanism of Electro Activated Water

4.5.1. The Comparison of the Bacteria Inactivation Capacity of Chlorine Based Disinfectants

The bactericidal efficiency of EAW on *E. faecalis* was studied comparatively with other chlorine-based disinfectants as NaOCl and ClO₂. Batch experiments were conducted for each disinfectant agent with a constant concentration within determined contact time. The efficiencies of disinfection processes are reported through TTC dehydrogenase enzyme activation and flow cytometry analysis and the results are given in Figure 4.13.

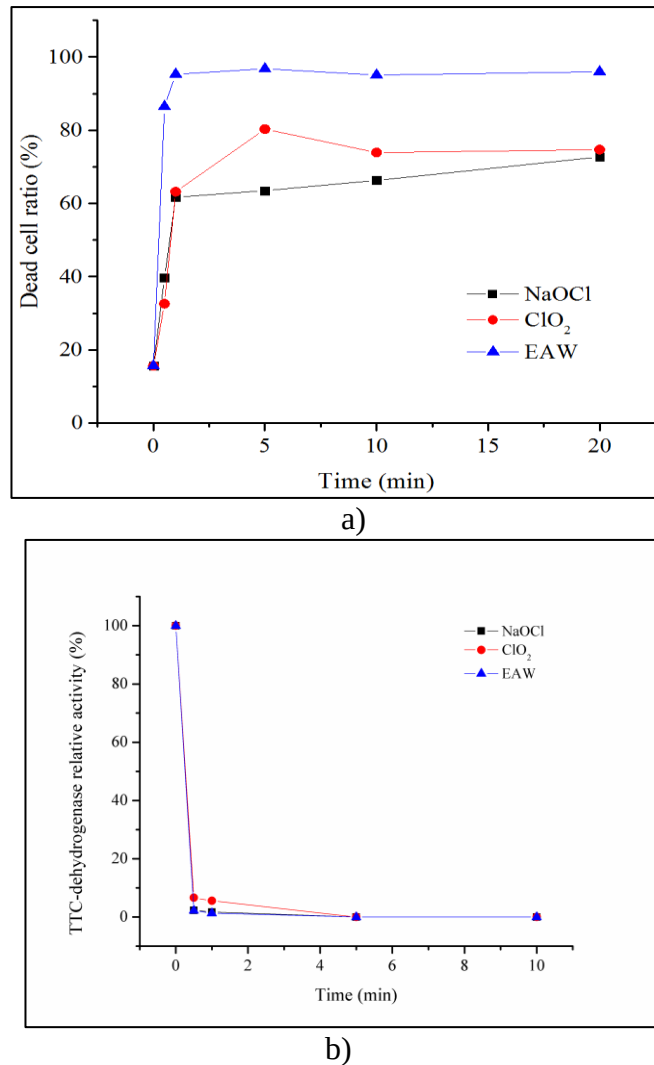


Figure 4.13: a) Dead cell ratio, b) TTC-dehydrogenase relative activity after time-dependent disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants is 1.2 ppm; temperature is 10°C, the initial concentration of the cell culture 3x10⁹cfu/ml).

Flow cytometry provides detection of the enumeration of viable bacteria in a sample. The undamaged membranes of live cells are impermeable to dyes such as propidium iodide (PI). However, PI dye can leak into cells through damaged membranes and thus, informs about death cells in a media. Dead cell ratio of control and treated samples are given in Figure 4.13 a. EAW achieved complete degradation of bacteria within 1 minute. Although EAW inactivated bacteria totally, other disinfectant agents could not achieve complete inactivation. NaOCl and ClO₂ provided almost 70 % of efficiency at the end of the reaction. Figure 4.13 a shows the dehydrogenase relative activity of *E. faecalis* during disinfection processes.

Dehydrogenase is the key enzyme of respiratory chain. The reduction of dehydrogenase activity inhibits respiration and anabolism. Therefore, the relative activity signifies the survival rates of cells. All disinfectant agent showed sharp decline trend of dehydrogenase activity. *E. faecalis* dehydrogenase activity decreased 94.4%, 98.3%, 98.7% after 1-minute treatment of ClO_2 , NaOCl , EAW, respectively. There is no significant difference between disinfection processes in the meaning of dehydrogenase activity. All agents oxidized the dehydrogenase and some other enzymes in respiratory chain.

Disinfection processes could change the electron flow in the cells; thereby cause disruption of intracellular enzyme metabolism. Sulfhydryl groups of enzymes, essential for carbohydrate metabolism in respiration chain, can be oxidized by HClO , in that way microorganisms were inactivated because of inhibition of their glucose oxidation [20, 74]. Additionally, these chlorine compounds, such as ClO^- , HClO has ability to react with enzymes and form N-Cl bonds which disrupt the functional enzymes [75].

4.5.2. Determination of Ion Leakage Caused by Disinfection

The change in conductivity signifies the destruction of the cell membrane permeability. The increase in cell membrane permeability causes ion leakage, therefore conductivity of supernatant of damaged bacteria raises. Disinfectants could harm or degrade transmembrane proteins that are responsible of transporting ions, such as Na^+/K^+ pumps. The conductivity changes of *E. faecalis* after treatment with NaOCl , ClO_2 and EAW are shown in Figure 4.14.

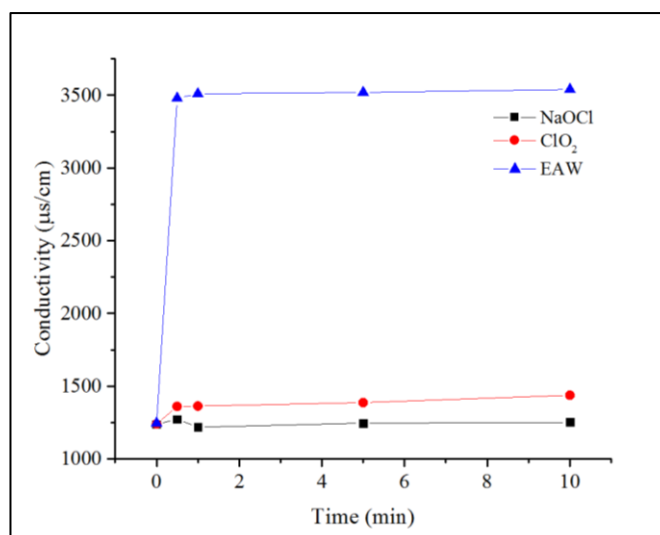


Figure 4.14: The change of conductivity after time-dependent disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants 1.2 ppm, temperature 10 °C, the initial concentration of the cell culture 3x10⁹cfu/ml).

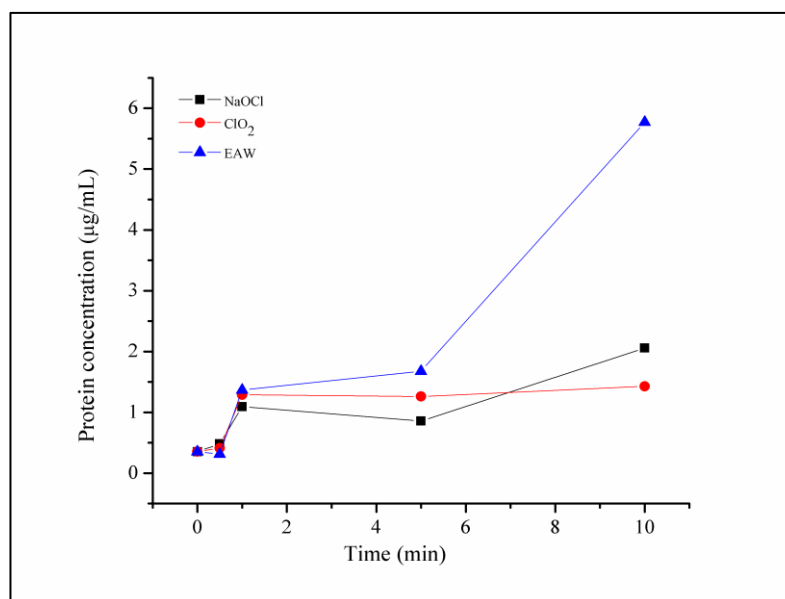
In the first minute of treatment, EAW sharply increased the conductivity from 1238 μs/cm to 3540 μs/cm, and then reached a steady state. In the case of ClO₂, there was a slight increase, while NaOCl almost did not change conductivity levels as seen in Figure 4.14. The sharp increase in conductivity after treatment with EAW indicates the increased cell permeability because of degraded cell membrane. After one minute, leakage reached a steady state since almost all bacteria were inactivated. These results are parallel with the study of Zeng et al. [26] investigated the effect of EAW on *Escherichia coli* and *Staphylococcus aureus*.

In a similar manner with our results, EAW showed a sharp increase in conductivity of both microorganisms and reached a steady state. In the case of NaOCl, there was no significant difference. The reason could be that NaOCl interacted with ions and precipitated. So even NaOCl harmed the cell membrane, conductivity levels did not change. Free radicals and oxidizing agents that only EAW include, damage the cell membrane more than other disinfectants do. This is the reason that EAW has a higher effect on cell membrane permeability [64].

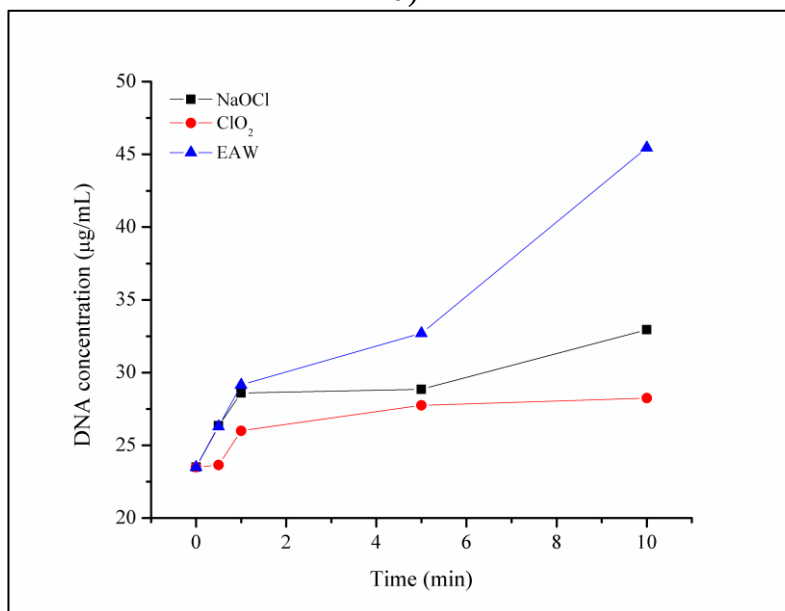
4.5.3. The Measurement of Intracellular Protein and DNA Leakage

DNA and protein leakage are significant indicators for disruption of cell membrane integrity. The release of intracellular material after destruction of cell

membrane can be observed with the measurement of absorbance value at 260 expressing DNA and Bradford analysis for protein leakage. Figure 4.15 a and b shows protein and DNA concentration during disinfection processes, respectively.



a)



b)

Figure 4.15: The change of; a) protein b) DNA concentration after time-dependent disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants 1.2 ppm, temperature 10°C, the initial concentration of the cell culture 3×10^9 cfu/ml).

In Figure 4.15 a, the leakage of protein level is shown. After treatment of NaOCl, protein leakage increased from 0.35 µg/ml to 1.09 µg/ml in first 1 minute, then

decreased to 0.86 µg/ml at the end of 5 minutes. Similarly, ClO₂ treatment also increased the protein leakage in first 1 minute. However, after 1 minute it reached a steady state. In the case of EAW treatment, protein leakage levels exhibited significant and continuous changes until reached to 5.77 µg/ml after treatment for 10 minutes.

In the first minutes, increment in protein leakage levels caused by cell membrane damage through the all disinfection processes. On the other hand, leaked protein concentrations decreased slightly after NaOCl treatment of 5 minutes. The reason of this slight decrease could be that intracellular material including proteins may get solidified after a period of NaOCl treatment. Solidification of intracellular material was also observed in previous studies [64].

Tang and his research group [64] obtained the same result after disinfection process with EAW. Results showed that EAW treatment caused more damages to outer membrane than other disinfectants do. It may be linked with inactive species or oxidizing agents such as free radicals, hydrogen peroxide that EAW includes besides of chlorine including components.

DNA leakage caused by membrane disruption is shown in Figure 4.15 b. Leakage increased within first 1 minute after all disinfection process. After EAW treatment, DNA leakage started from 23 µg/ml then continuously increased and reached to 45.5 µg/ml. On the other hand, NaOCl treatment caused incremental growth in DNA leakage after for 5 minutes while ClO₂ treatment exhibited a steady state profile for 5 minutes.

Results showed that EAW has stronger effect on membrane permeability than other two types of disinfectants. Membrane structures are direct targets of EAW whereas chlorine based disinfectants targets protein and enzyme structures [26]. That may be a reason of the steady state observations. Weak interactions between Na⁺ ion from NaOCl and phosphate group of nucleic acid molecules cause nucleic acid precipitation known as hypochromic effect that cause a decline in the absorbance value of DNA under 260 nm [64, 76]. During an encounter between bacteria and an inhibition agent, small ions (potassium and phosphate) have a tendency to leach out first, before the release of large molecules (DNA and RNA) [57].

4.5.4. Lipid Peroxidation

Because of high lipid content, unsaturated lipids in the cell membrane are one of the main targets of disinfectants [58]. Disinfectant agents' leads to disruption of the cell wall, subsequent leakage of intracellular molecules and ions, then eventually cell death. Accordingly, lipid peroxidation could be used as a marker for detection of cell membrane integrity [75]. MDA is produced during peroxidation process of polyunsaturated phospholipids in lipid membranes. MDA as a most abundant individual aldehyde is used for indication of lipid peroxidation. Figure 4.16 shows MDA concentration during disinfection processes.

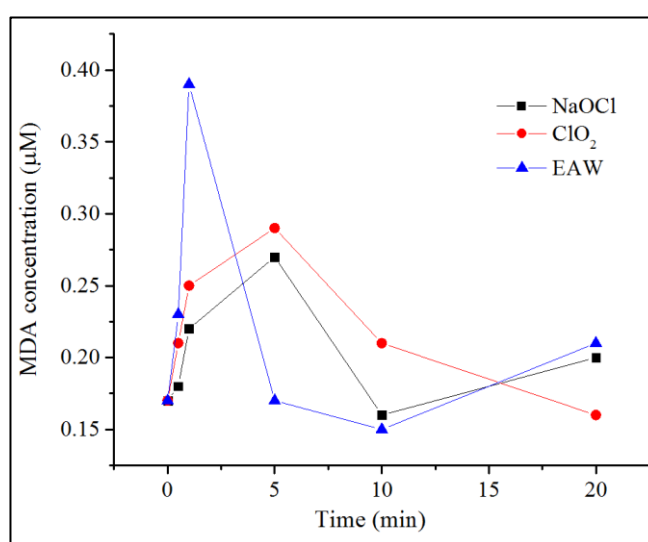


Figure 4.16: Lipid peroxidation after time-dependent disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants 1.2 ppm, temperature 10°C, the initial concentration of the cell culture 3x10⁹cfu/ml).

Due to abundant phospholipid content of cell membrane, disinfectant agents tend to oxidize lipid layer and subsequently disrupt membrane structure. In Figure 4.16, it is shown that lipid peroxidation in *E. faecalis* peaked at just 1 minute but decreased sharply after that time in EAW treatment. Although the maximum MDA concentration was obtained in EAW treatment as 0.38 μM, it just reached almost 0.26 and 0.28 μM in NaOCl and ClO₂ treatment, respectively. It is concluded that, EAW is the most effective disinfectant on lipid peroxidation due to having strong oxidizing agents such as free radicals in addition HOCl.

4.5.5. DNA Damage

SYBR Green I dye binds the dsDNA of bacteria and causes a detectable emission at 532 nm. Due to this feature, it can be used as a marker for DNA damage in control and treated samples.

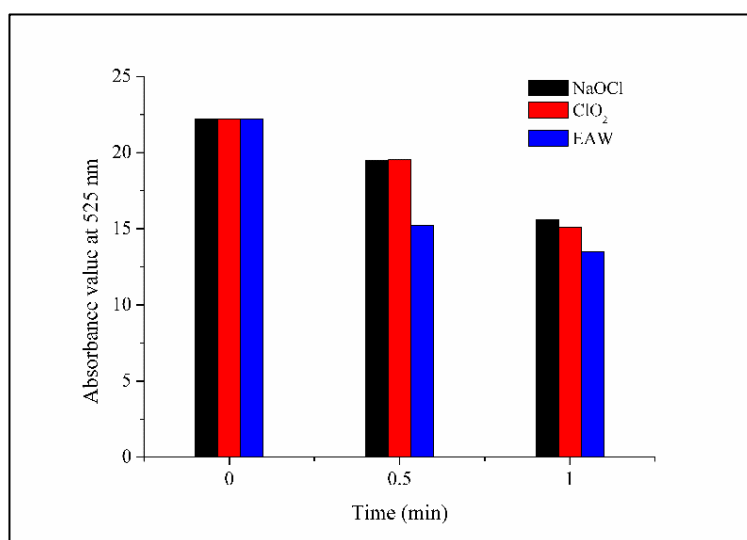


Figure 4.17: DNA damage after time-dependent disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants 1.2 ppm, temperature 10°C, and the initial concentration of the cell culture 3x10⁹ cfu/ml).

As seen in Figure 4.17, EAW provided lower signal level than other disinfectant agents within determined treatment times. This finding signifies further DNA degradation of EAW treated samples. Since EAW contains free radicals apart from the other disinfectants. Free radicals are responsible for DNA damage by breaking DNA strands and causing base alterations.

4.5.6. Protein Degradation

SDS-PAGE analysis reflects intracellular protein degradation. Figure 4.18 shows the changes in protein concentration of samples after disinfection compared to control.

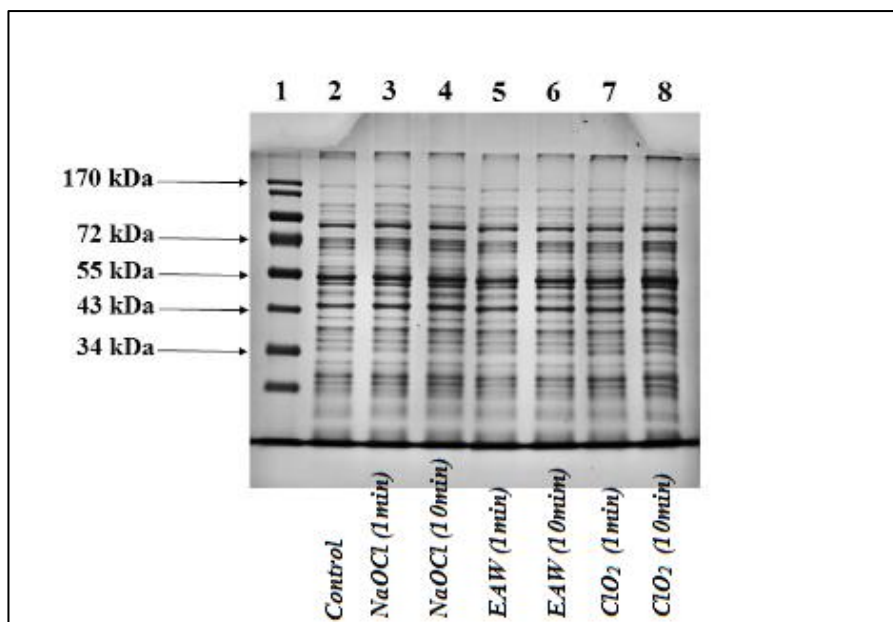


Figure 4.18: Protein degradation after disinfection treatment with NaOCl, ClO₂ and EAW (The concentration of disinfectants 1.2 ppm, temperature 10 °C, and the initial concentration of the cell culture 3x10⁹ cfu/ml).

As seen in Figure 4.18, *E. faecalis* cells were lysed to visualize intracellular proteins by SDS-PAGE after 1 and 10 minutes' treatment with EAW, NaOCl and ClO₂. After sonication, protein amounts were equalized by using Bradford method before loading the gel. Because the results of other analysis indicate cell membrane damage, protein bands at 35, 37 and 47 kDa, which show major cell wall proteins, were targeted. No important difference in the protein profile was detected between control and treated samples.

4.5.7. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

An FTIR spectrum of bacteria provides a better understanding of the structure of cell wall polysaccharide, lipids and proteins [77]. Figure 4.19 shows overlay plots of IR spectrum from 4000 cm⁻¹ to 400 cm⁻¹ for treated and untreated samples of *E. faecalis*. Figure 4.19 shows overlay plots of IR spectrum from 4,000 cm⁻¹ to 400 cm⁻¹ for control and treated samples of *E. faecalis*.

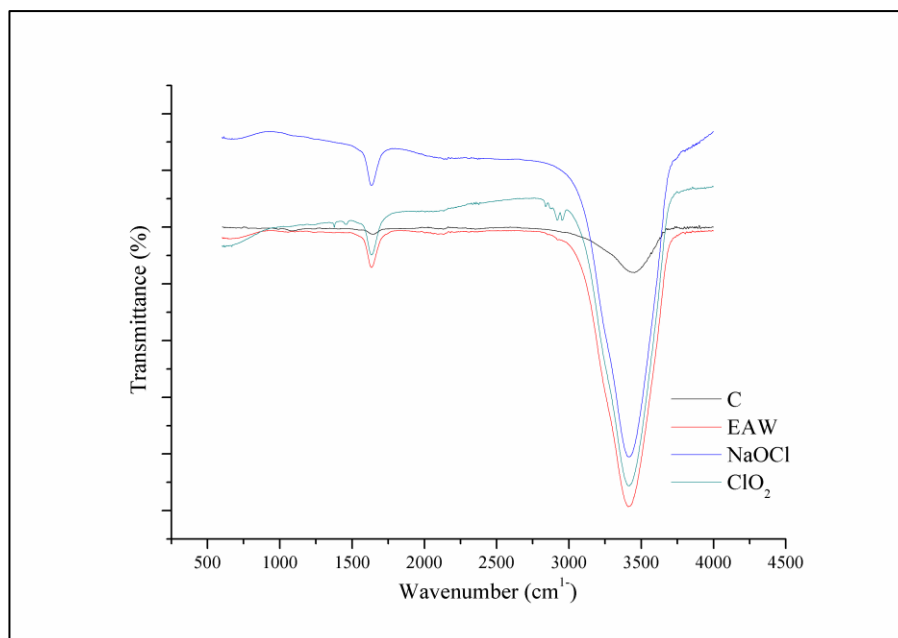


Figure 4.19: FTIR analysis for bacterial cellular component bonds breakage.

All disinfection treatments showed bands at 1632 cm^{-1} due to destruction of proteins by breaking β -pleated sheet and subsequently, water molecules were observed at 3400 cm^{-1} . ClO_2 caused stretching of CH_2 bands and CH_3 groups of fatty acids according to formed vibrations on 2870 cm^{-1} and 2850 cm^{-1} , respectively.

Protein structures were also affected after treatment of ClO_2 from different parts including amide III band and C-H bonds of amino acids that gives bands at 1310 cm^{-1} and 2898 cm^{-1} .

Hypochlorous acid is present in both NaOCl and EAW and performs as an oxidant that is responsible for protein deterioration. HClO destroys certain enzymes in carbohydrate metabolism by oxidizing their sulfhydryl groups. Therefore, glucose oxidation does not occur and bacterial cell kills [16, 20].

4.6. Electro Activated Water Disinfection of Drinking Water

EAW system has been investigated for its utility in water disinfection. For this purpose, in the first step of the study, water sample containing only bacteria was treated in the continuous EAW system to investigate bacteria inactivation capacity of the system. Synthetic drinking water sample containing bacteria and humic acid was prepared in the second step of the study. The initial concentration of bacteria in

solution was adjusted to 0.23×10^6 cfu/mL. Experiments were carried out to determine the current value that killed all of the bacteria and provided sufficient amount of residual chlorine in the drinking water.

Figure 4.20 shows the disinfection performance of EAW at the applied current of 0.1 and 0.3 A. Actually; further experiments were run with the application of 0.5, 0.7 and 1 amperes. However, all the bacteria were inactivated under these conditions in bacteria and in the presence of HA solution (Data not shown).

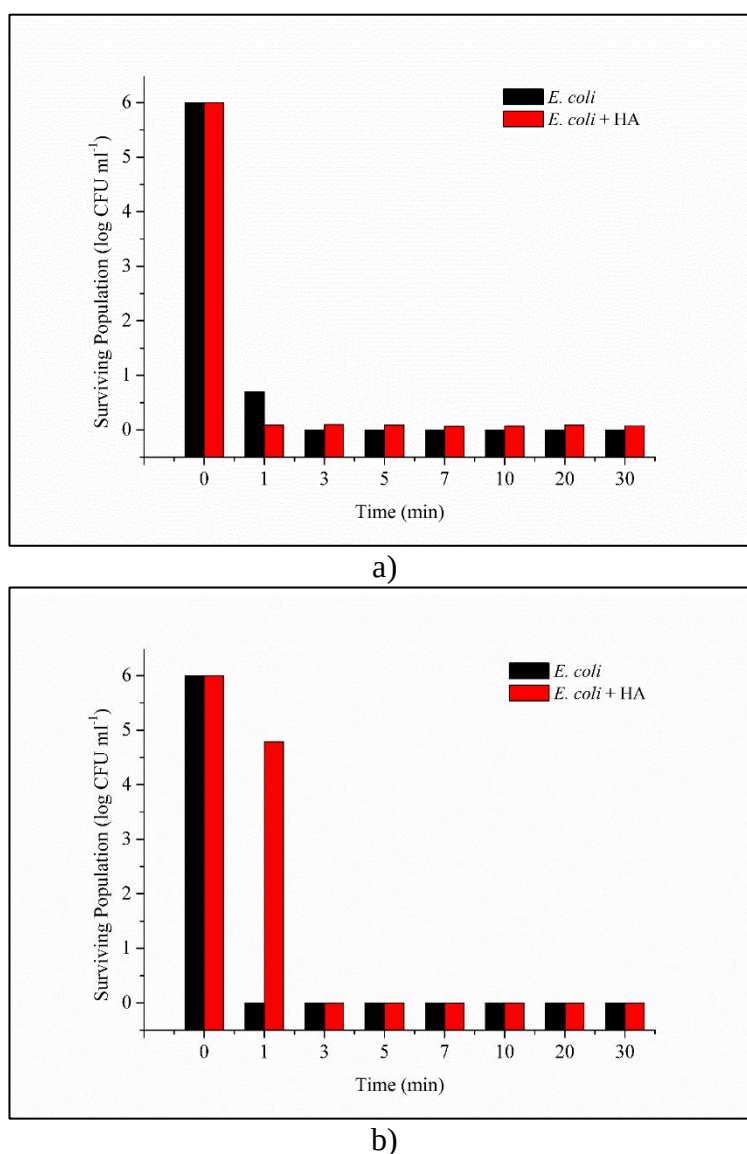
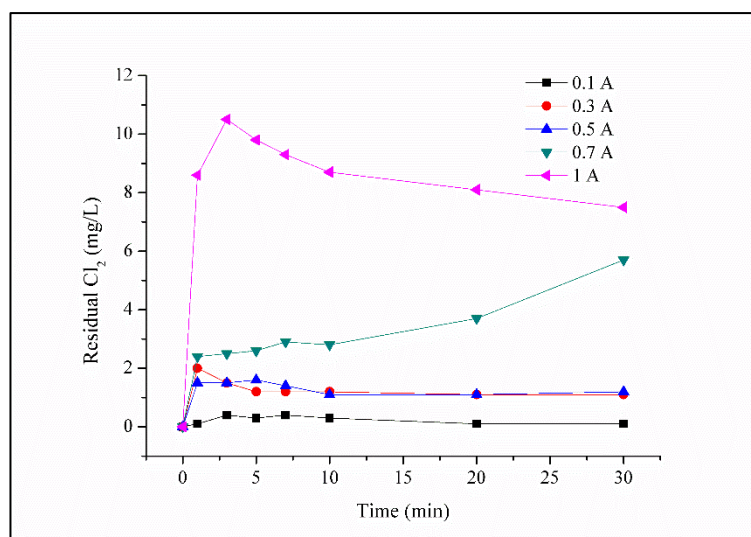


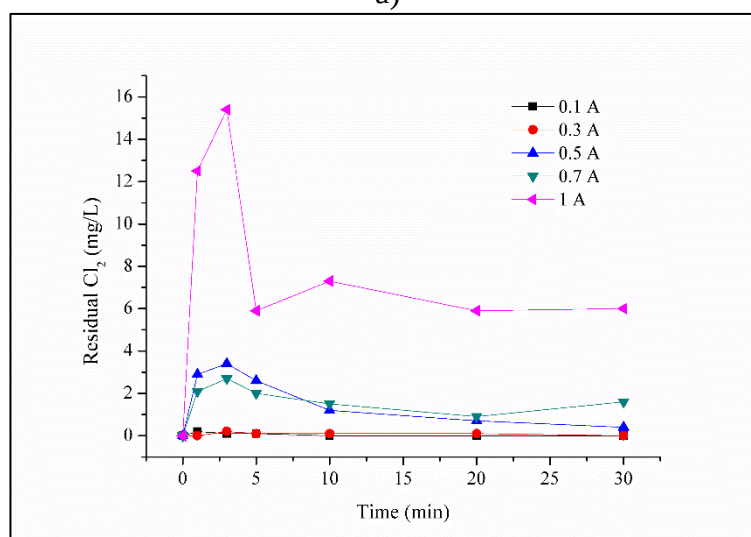
Figure 4.20: Surviving Population during EAW Disinfection with the application of; a) 0.1 A, b) 0.3 A current (Initial bacteria concentration 0.23×10^6 cfu/mL, initial pH 7.73, conductivity 5102 ms/cm, flow rate 12.75 L/h).

At the applied current of 0.1 A, %100 inactivation of *E. coli* was provided with the contact time of 3 min (see Figure 4.20 a). However, in the presence of HA, 100% inactivation of *E. coli* could not be achieved through the entire range of studied contact time. According to researcher [30, 47], in the case of coming across EAW and organic substances, combined available chlorine forms due to occur a reaction between chlorine and organics. In this case, the disinfection effect of EAW decreases because the combined available chlorine has lower inactivation capacity than free chlorine. On the other hand, increasing the applied current to 0.3 A (see Figure 4.20 b), provided complete inactivation of *E. coli* even in the presence of HA at only 3 min of contact time.

The amount of free residual chlorine is an important parameter for drinking water distribution systems. Thus, free residual chlorine concentrations were determined according to different applied current values. Residual Cl_2 concentrations of merely bacteria suspension and bacteria in the presence of humic acid during EAW disinfection are given in Figure 4.21.



a)



b)

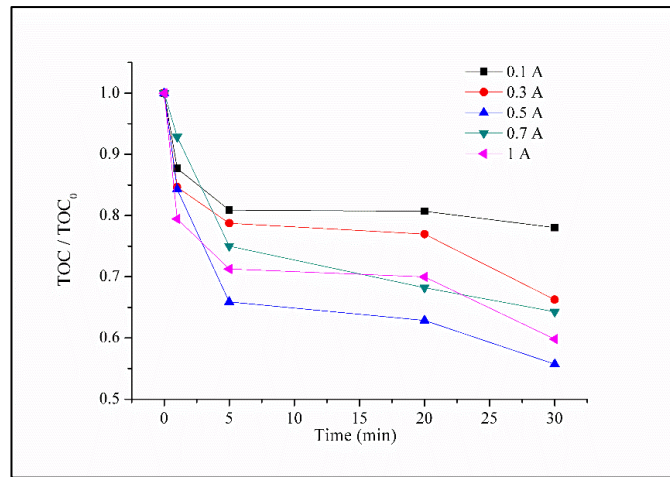
Figure 4.21: Residual Cl_2 concentration during EAW disinfection of; a) merely bacteria suspension, b) bacteria in the presence of humic acid. (Initial bacteria concentration 0.23×10^6 cfu/mL, initial pH 7.73, conductivity 5102 ms/cm, flow rate 12.75 L/h).

In Figure 4.21 a, free residual chlorine concentrations can be seen during *E. coli* inactivation by EAW. At the lowest applied current (0.1 A), there was insufficient free residual chlorine concentration in the media. The applied currents of 0.3 and 0.5 A provided adequate free residual chlorine concentration during disinfection process as the values were around 1 mg/L. However, further increase in the applied current enhanced free residual chlorine concentration significantly during the process. After 30 min of contact time, it reached to 5 and 8 mg/L of free residual chlorine concentration.

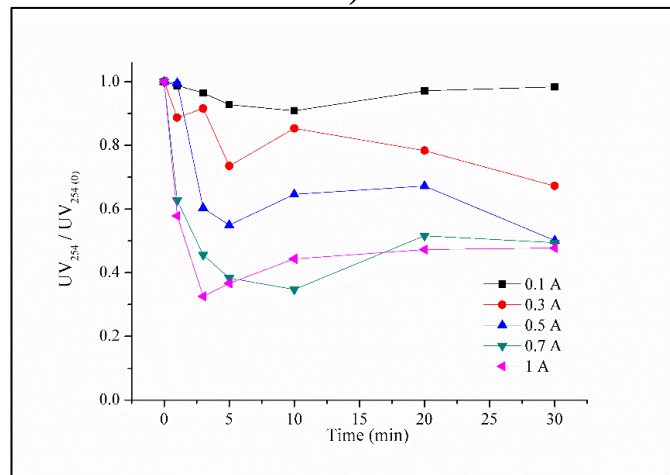
In the presence of HA, there was no free residual chlorine with the applied currents of 0.1 and 0.3 A (see Figure 4.21 b). Increasing the applied current to 0.5 A provided satisfactory free residual chlorine concentration during the process. Further increase in the applied current (0.7 A) resulted free residual chlorine concentration between 1.8-2 mg/L. When the applied current was 1 A, free residual chlorine concentration was up to 15 mg/L at 1 min of process time and then decreased to about 6 mg/L at the end of the process. Consequently, in spite of providing complete inactivation of *E. coli* even in the presence of HA with the applied current of 0.3 A, 0.5 A can be determined the optimum value as providing also satisfactory free residual chlorine concentration.

Humic substances also have high complexing capacity relative to cations of metals because HA comprises ionisable functional groups such as carboxylic, alcoholic, ketonic, phenolic, quinone groups and hydroxyls [78]. Because of the structural complexity, heterogeneous composition and variable chemical content of HA, its determination is quite difficult. Dissolved organic carbon (DOC) and specific ultraviolet absorbance at 254 nanometers (UV_{254}) are the most widely used parameters for the HA characterization. DOC is an expression of the organic matter in drinking water. UV_{254} also indicates the total concentration of organic compounds that are capable of absorbing ultraviolet illumination in drinking water.

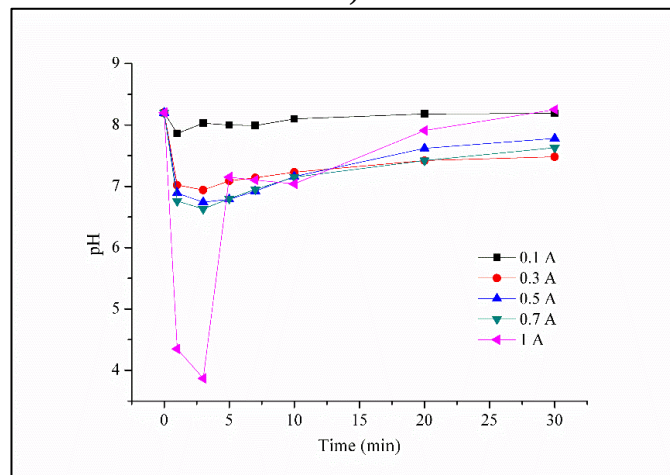
TOC and UV_{254} removal trends and pH changes during electrolysis processes can be seen in Figure 4.22.



a)



b)



c)

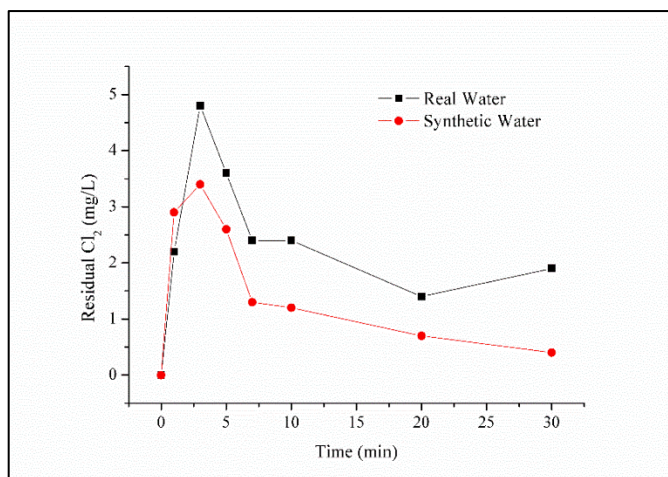
Figure 4.22: a) TOC removal, b) UV absorbance removal, c) pH change during EAW disinfection of bacteria in the presence of humic acid against time. (Initial bacteria concentration 0.2×10^6 cfu/mL, initial pH 8.3, conductivity 5112 ms/cm, flow rate 12.75 L/h, initial TOC 5.6 mg/L, UV₂₅₄ 0.443 nm).

The removal of TOC with EAW increased with increasing applied current up to 0.5 A (Figure 4.22 a). At 30 min of process time, TOC reduced to 4.37, 3.71 and 3.12 mg/L from 5.6 mg/L with the applied currents of 0.1, 0.3 and 0.5 A, respectively. However, further increase in applied current did not provide higher TOC removal rate. 35.7% and 40.2% TOC removal rates were gained at the applied currents of 0.7 and 1 A, respectively.

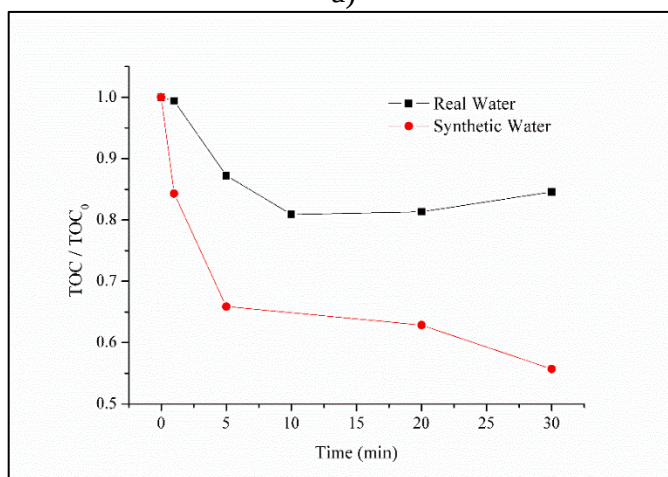
The increase in applied current provided higher UV_{254} removal rates as seen in Figure 4.22 b. The lowest studied applied current showed poor performance on UV_{254} removal as only 2.4% removal was obtained. Further increase in applied current (0.3 A) provided 33.3% removal rate. UV_{254} removal rates ranged between 46.8-53% with the applied currents of 0.5, 0.7 and 1A. UV_{abs} removal showed wavy trend for all applied current values. This can be attributed to the formation of by-products during HA degradation by EAW. As a result, having high Redox potential, EAW can oxidize organic matter, and inactivates bacteria. pH value changed according to chlorine increase or decrease in the solution as seen in Figure 4.22 c.

As a result of a general evaluation, the applied current of 0.5 A is applicable for disinfection of drinking water. Both a complete bacteria inactivation and some TOC degradation were provided in 30 minutes of reaction time. Furthermore, the desired residual concentration was achieved to prevent re-growth of the bacteria in distribution system. Further disinfection experiments for real water were studied with the applied current of 0.5 A.

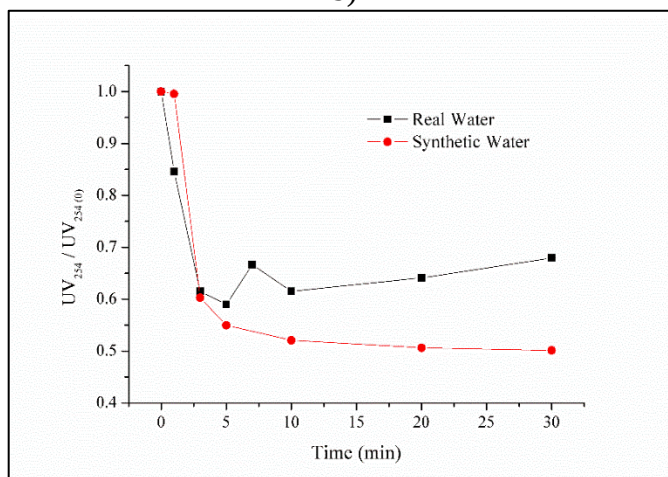
In the third part of the study, EAW disinfection was applied to real water samples. The real water sample was collected from pool located between dam pond and aeration unit (For the character of water sample See Table 3.2). The results are given in comparison with the results of the experiments made with synthetic water. The residual chlorine concentration, TOC and UV_{254} removal can be seen in Figure 4.23.



a)



b)

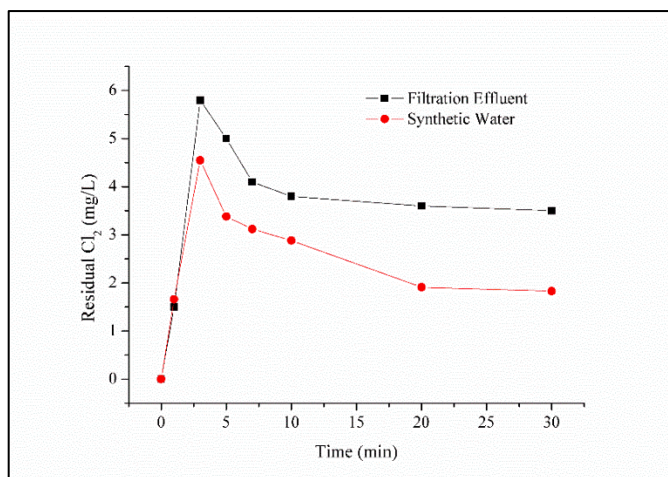


c)

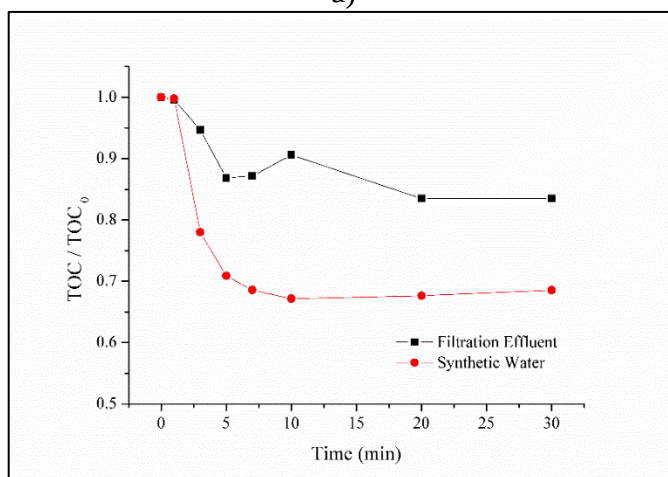
Figure 4.23: a) Residual chlorine concentration, b) TOC concentration, c) UV absorbance removal during EAW disinfection of drinking water sample taken from aeration influent (The applied current 0.5 A, flow rate 12.75 L/h, initial pH 7.49, conductivity 232 $\mu\text{S}/\text{cm}$, initial TOC 3.145 mg/L, UV₂₅₄ 0.078 nm).

The changes of residual chlorine concentration of synthetic and real water samples showed similar trends as seen in Figure 4.23 a. However, more chlorine left in the solution during disinfection of real water in relation to TOC removal. In other words, free chlorine was consumed in disinfection of synthetic drinking water. As seen in Figure 4.23 b, TOC abatement of real water was approximately 30 %, whereas it was 50 % for disinfection of synthetic drinking water in 30 minutes of reaction time. Similarly, higher UV_{254} removal was provided during synthetic drinking water disinfection.

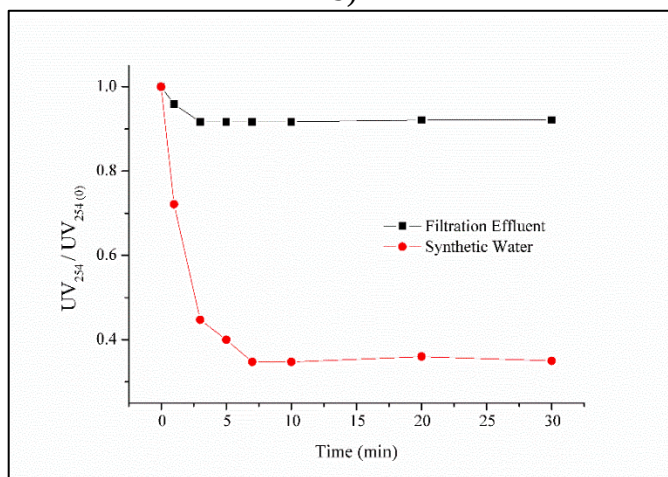
Nevertheless, EAW with its highly oxidative component was effective for real drinking water treatment. Further experiments had been carried out to investigate whether this process can be used instead of conventional chlorination in drinking water treatment plant. Therefore, disinfection process was applied to water sample taken from filtration effluent under same experimental conditions (i.e. 0.5 A of applied current) and synthetic water simulated according to real water values. The results are given in Figure 4.24. In this case, TOC concentration of the synthetic water was lower than that of the dam water.



a)



b)



c)

Figure 4.24: a) Residual chlorine concentration, b) TOC concentration, c) UV absorbance removal during EAW disinfection of drinking water sample taken from filtration effluent (Applied current 0.5 A, flow rate 12.75 L/h, initial pH 7.01, conductivity 5031 ms/cm, initial TOC 2.48 mg/L, UV₂₅₄ 0.026 nm).

EAW disinfection provided more TOC and UV_{254} removal of synthetic water sample as expected. The other component in real water such as inorganics affected the degradation efficiency of EAW. However, the efficiency obtained is the same as the results after conventional chlorination. Here, it is important to ensure that chlorine remains in the water, rather than the removal of organics from the water. When examining Figure 4.24 a, desired residual chlorine was provided at the end of the 30 minutes of disinfection. According to the drinking water supply regulations, the formation of carcinogenic disinfection by-products (DBPs), which threaten human health, needs to be kept under control [79]. During the disinfection process, the use of chlorine may lead to the formation of trihalomethanes and haloacetic acids due to humic substances content of surface water. The formation of THM from the drinking water sample during EAW disinfection is shown in Figure 4.25.

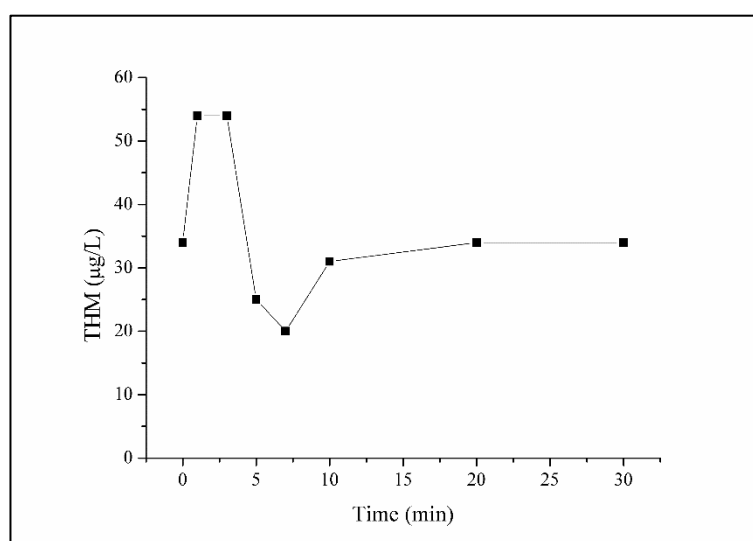


Figure 4.25: The formation of THM during EAW disinfection of drinking water sample taken from filtration effluent (Applied current 5 A, flow rate 12.75 L/h, initial pH 7.01, conductivity 314.25 $\mu\text{s}/\text{cm}$, initial TOC 2.48 mg/L, UV_{254} 0.026 nm).

The THM concentration was measured as 54 $\mu\text{g}/\text{L}$ at 1 min of process time and then decreased up to 20.1 $\mu\text{g}/\text{L}$ until 7 min. The reason for this rapid increase of THM is related to the excess chlorine concentration remaining in the water as seen in Figure 4.24 a. No further decrease was observed with increasing process time and it reached to 30 $\mu\text{g}/\text{L}$ at 10 min of process time. After that, THM concentration was almost stable and reduced to 32 $\mu\text{g}/\text{L}$ concentration level. This suggests that the formation of THM

during EAW process may stay at desirable levels as its allowed concentration is 80 µg/L in drinking water [79].

4.7. The Stability Test of Electro Activated Water

EAW is a mixed liquid with more than one oxidant substance in its content. The properties of EAW can vary over time, as chlorine gas is volatile from EAW constituents, while ozone and free radicals are unstable. The EAW parameters were observed when stored after being produced to evaluate commercial usage of EAW in bottles instead of in-situ usage. Table 4.8 shows the changes of EAW parameters under different storage conditions.

Table 4.8: Stability of acidic EAW.

Time	pH	ORP	FCC	ClO ₂	<i>E. coli</i>
h		mV	mg/L	mg/L	cfu/mL
0 (daylight)	2,9	1131,4	904,5	148,7	No reproduction
4 (daylight)	2,9	1122,7	376,7	123,2	No reproduction
6 (daylight)	4,7	1113,9	258,5	20,2	No reproduction
72 (daylight)	4,9	1101,2	198,1	9,8	No reproduction
1152 (daylight)	5,1	959,2	12,7	0	Reproduction
1152 (dark)	4,0	1104,8	393,1	6,3	No reproduction

Time-dependent degradation of EAW with high chlorine content and low pH was observed. In this experiment, EAW was kept in the glass bottle (in sunlight) and half full of the bottle volume. As seen in Table 4.8, the pH, ORP values of EAW, as well as the concentration of chlorine and chlorine dioxide, vary in part versus time. The increase in pH over time can be interpreted as the passing of chlorine gas through the liquid and into the HOCl form. Minor changes in the ORP indicate that EAW does not lose its polarization energy and is still active. However, even after 72 hours, the ORP value remained quite high. Despite the reduction in chlorine and chlorine dioxide concentrations, all EAW samples destroyed *E. coli* bacteria (10⁸ cfu/mL). Accordingly, ORP value has great importance in the inactivation of bacteria. On the other hand, the changes of parameters of EAW were observed in daylight and darkness

with an 1152 hour waiting period. EAW waited in the daylight for the same time changed anymore and *E. coli* reproduction was observed after treatment with that EAW samples. Consequently, in order to preserve the bactericidal properties of acidic EAW, it should be kept in the dark for a maximum of 1.5 months. The results of stability test of slightly acidic EAW are given in Table 4.9.

Table 4.9: Stability of slightly acidic EAW.

Time	pH	ORP	FCC	ClO ₂	<i>E. coli</i>
h		mV	mg/L	mg/L	cfu/mL
0	6,0	892,3	15,5	2,1	No reproduction
72 (daylight)	6,6	653,2	3,6	0	Reproduction
72 (dark)	6,2	788,1	5,4	0	Reproduction

Slightly acidic EAW with nearly neutral pH and low oxidants concentration exhibits its bactericidal activity immediately usage after being produced. As shown in Table 4.9, keeping this water in the dark is favorable in protecting EAW properties.

4.8. The Cost Analysis of Electro Activated Water Production

The technologies to improve the microbial quality of household water and reduce waterborne disease include a number of physical and chemical treatment methods. Some water treatment and storage systems use chemicals and other media and materials that cannot be easily obtained locally at reasonable cost and require relatively complex and expensive systems and procedures to treat the water. Such systems may be too inaccessible, complex and expensive to employ for treatment and storage of household water in some places and settings.

The production of EAW is a no needed chemical process. The cost of EAW disinfection can be calculated upon investment and operating cost. The initial investment cost determined according to the chemical compounds and materials used in the construction of this reactor are as in Table 4.10.

Table 4.10: The initial investment cost of continuous flow EAW reactor.

Design of Reactor	Pieces / Quantity	Cost (\$)
Plexiglas material	10 pieces	250,00
Check valves and poses	8 pieces	25,00
Montage	Service Procurement	300,00
Electrodes	4 pieces (0.25 \$ / 1 cm ²)	300,00
Membrane	3 pieces	120,00
Total		995,00

This amount may reduce when serial production of the device begins and when EAW production increases per unit volume. On the other hand, the operating cost calculated according to the unit cost of the components required to operate the reactor is as shown in Table 4.11.

Table 4.11: The operating cost of the reactor.

	Unit Cost		Consumption		Total Cost	
Salt	0.15	\$/kg	0.5	kg	0.075	\$
Tap Water	1.26	\$/m ³	0.06	m ³	0.076	\$
Electric current ^{**}	0.0498	\$/kw	0.15	kw	0.00747	\$
Unit cost for 1 lt EAW in the system has 60 lt/h EAW production capacity				0.00263	\$	
Unit cost for EAW disinfection of 1 m ³ drinking water within 1 hour operation time				0.0098	\$	
Unit cost for conventional disinfection of 1 m ³ with a 1.5 mg/L final FCC concentration in drinking water				0.0102	\$	

Since EAW system uses only salt, water, and 150 watts of electricity, it is an inexpensive and clean technology. This technology provides to consumer's savings in water, energy, and chemical expenses, and in turn an instant return on investment. EAW technology has a variety of application areas such as agriculture, medical sterilization, food safety and water treatment. This water generating system can be designed with desired volumes. This way it can be modular in structure. It can be easily integrated in place of the currently used disinfection systems.

5. CONCLUSION

The millennium declaration established a goal of halving the proportion of the global population without access to safe water by 2015. One reason for this is that fecal contamination of source and treated water is a persistent, worldwide problem. Sanitation coverage is inadequate in many parts of the world and is likely to persist for the near future. Fecal contamination of source and treated water is further exacerbated by increasing populations, urban growth and expansion, peri-urban settlement and continued and perhaps increasing pollutant transport into ground and surface water due to deforestation, global climate change, recurrent disastrous weather events (hurricanes, cyclones, floods, tsunamis, etc.) and increasing coverage of the earth's surface with impervious materials. The demand for improved water quality and supply of sufficient amount of water to people will increase worldwide. Therefore, water disinfection is one of the most important public issue to provide high quality drinking water. Chlorine, chlorine dioxide, chloramines and ozone are the frequently used disinfectants. These chemical disinfectants are efficient for killing detrimental microorganisms in drinking water. Re-evaluation of disinfectants containing NaCl as a disinfectant has been ongoing recently due to environmental poisoning concerns.

Since EAW is produced from pure water with the addition of only NaCl, it has the advantage of being less harmful to the environment than many chemical antibacterial treatments. Low pH, high ORP and the relative concentrations of aqueous molecular chlorine, chlorine, hypochlorous acid (HOCl) and hypochlorite ion (OCl⁻) are responsible for the disinfection properties of EAW. These factors depend on the operating condition of EAW generation system. Within this study, the effect of the changes of the operating parameters was discussed and EAW production conditions were optimized. In this content, the effects of brine concentration, electrolysis time, and current on the EAW character were investigated. All the factors as a whole play a significant role, individually or in a combined form. However, brine concentration dominated the EAW character, as seen from cyclic voltammetry graphs. After electrolysis of NaCl, new bonds formation was observed in the solution as molecules (Cl₂, O₂) and ions (OH⁻, Cl⁻). ORP reached the stable stage after the middle values of all factors. However, chlorine concentration increased with the increasing of all factors. Optimal operational conditions were suggested to produce EAW (i.e. ORP is

1000–1140 eV and FCC is 30–600 mg/L) upon the application of different ampere values. It is concluded that, the application of higher ampere provided shorter electrolysis time.

In the second stage of the study, the effects of different operating parameters of EAW on inactivation of bacteria were evaluated by response surface methodology (RSM). The optimal parameters for complete inactivation of the bacteria with EAW treatment were studied through temperature disinfection concentration and contact time. There was no significant effect of temperature on bacteria inactivation. The interaction of free chlorine concentration and treatment time at 25 °C had a synergetic effect on *E. coli* and *E. faecalis* inactivation.

Then, the inactivation mechanism of EAW was investigated comparing with other chlorine-based disinfection agents; sodium hypo chloride (NaOCl) and chlorine dioxide (ClO₂). Wide range of experiments were carried out to investigate underlying mechanisms of EAW, NaOCl and ClO₂ disinfection process on *E. faecalis* in the matter of membrane integrity damages, cell viability, cellular protein degradation and physical damages on cellular components. To detect membrane integrity damages; DNA, protein leakage levels and conductivity were measured.

TTC-dehydrogenase enzyme activity assay, flow cytometry dead bacteria count and serial dilution used to determine cell viability. Cellular protein degradation was observed on SDS-PAGE. Monoaldehyde (MDA) used as an indicator of oxidizing agent induced lipid peroxidation. FTIR analysis was performed to verify physical damages on cellular components including DNA, RNA, protein, lipid molecules. Another physical damage analysis specified to DNA was applied with Syber Green, which forms covalent bond with DNA by intercalating into double strands of DNA. According to this investigation, EAW was determined as the strongest chlorine based disinfectant. EAW targets membrane lipid molecules whereas NaOCl and ClO₂ mostly target membrane proteins and enzymes. Lipids are the major molecules of cellular membranes.

Thus, EAW treatment exhibits effect that is more lethal by causing leakage of either small or large molecules that are all essential for microorganisms, to out of the cell. As a conclusion, all disinfectants inactivated the bacteria by disrupting cell membrane integrity, unbalancing the ion concentration and damaging the cellular components such as DNA, RNA, protein, lipid molecules. However, EAW showed the

most effective disinfection mechanism among three chlorine-based disinfectants by means of inactivating the bacteria in less time with more damage.

Since EAW system uses only salt, water, and 150 watts of electricity, it is an inexpensive and clean technology. Electrolyzed NaCl solution is easily broken down in the environment after use; the EAW compounds can be disposed down the drain without worry. They quickly dissociate back into their original forms – H₂O and NaCl – with zero impact on aquatic life. Other advantages of usage of EAW are not to have a need for transportation and storage harmful chemical and less negative effect on the environment. With all these advantages, EAW has begun to be widely used in agriculture, medical sterilization, and food safety and fishery industry. According to the results presented in this thesis, EAW can be used to meet the requirement for clean water such as;

- As a disinfectant agent in drinking water treatment plants.
- For cleaning of the sanitation system that is contaminated with time and in the prevention of bacterial re-growth.
- As on site treatment technology in packed water treatment units.
- Using in exceptional cases where bacterial contamination cannot be found on the surface or ground water (for prevention of water-borne infectious diseases).
- In cleaning of pipelines of facilities involved in the retail production and selling of water.
- Removal of bacterial contamination in water obtained for reuse.
- In cleaning of all tanks used for keeping and storing water.

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BIOGRAPHY

Özge Türkay has been working as a researcher in Gebze Technical University since 2009. Türkay completed her M.Sc. at Gebze Technical University (2011) and her undergraduate study at Dokuz Eylül University (2008) in Turkey. Türkay is doing her doctoral degree in Graduate School of Natural and Applied Sciences, Department of Environmental Engineering at Gebze Technical University.

APPENDICES

Appendix A: Articles Published During the Thesis Study

Turkay O., Barışçı S., Dimoglo A., (2016), “Assessment of Parameters Influencing the Electro Activated Water Quality”, *Process Safety and Environmental Protection*, 99, 129-136. DOI: 10.1016/j.psep.2015.10.020

Turkay O., Şeker M.G., Ramadhan S.S., Dimoglo A., (2017), “Electro activated water mechanism on *Escherichia coli* and *Enterococcus faecalis* and optimisation of operating parameters”, *Journal of Food Processing and Preservation*, 00:e13345. DOI: 10.1111/jfpp.13345