Antibiofilm Activity and Biocorrosion Control by Means of Essential Oil from *Lippiagracilis* Schauer (Verbenaceae) Microemulsion System

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Abstract: In this study the use of the essential oil from a *Lippiagracilis* Schauer micro emulsion system as a biocide and antibiofouling agent upon biofilms as well as the effect of this micro emulsion on the corrosion rate of AISI 1020 carbon steel was investigated. The results showed that a microemulsion type Winsor-IV was efficient in preventing the biofouling formation after 96 hours of contact and inhibited the growth of the sulfate reducing, iron-oxidizing bacteria as well as the fungi forming the biofilms after the 16 days of contact time. The antimicrobial action was likely due to a formation of a protective film.

Keywords: Microbiologically influenced corrosion, Microemulsion, Antibiofouling, Essential oil, *Lippiagracilis* Schauer, Biofilms.

1. INTRODUCTION

Production oil/water mixtures are characterized by a high level of contaminants as well as by the presence of organic and inorganic particles coupled to different types of microorganisms that can build communities known as biofilms. The initial step in the formation of these communities is the accumulation of such particles and cells that follows a sigmoidal standard in space and time. At this point, a synergic mechanism occurs in different types of surfaces leading to corrosion mainlyas a form known as *pitting* (Videla, 2003, Lenhart *et al*., 2014).

Microbiologically influenced corrosion (MIC) plays a key role in many industrial processes (Prasad, 2000), and billion dollars are spent in the United States on maintenance and control of MIC (Gu, 2012). Furthermore, estimates suggest that in tropical countries more than US$ 10 billion a year is spent, mostly by the oil and gas industry, because of direct/operational damage. Additionally, climate conditions such as high temperatures, high water salinity and other ecological characteristics can contribute to an increase on corrosion occurring in these countries.

Different types of microorganisms such as bacteria and fungi occuring in biofilms can favor the MIC due to their metabolism activity or by the production of secondary metabolites. The biofilm development gives biological as well as ecological advantages for the species occurring in these communities where they can colonize different habitats and niches. Additionally, resistance to the external factors such as biocides is increased (Sutherland, 2001; Gambino & Cappitelli, 2016). Thus, the production of extra cellular polymeric substances (EPS) is one of the main strategies used to increase this resistance (Beech *et al*., 2005; Li *et al*., 2007; Hong *et al*., 2012; Baeza *et al*., 2013; Boyle *et al*., 2013; Jin & Guan, 2014).

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In oil and gas companies, the use of biocides to control MIC is quite common. Biocides have been used in different sectors to reduce the occurrence of microorganisms without in habiting the corrosive process itself (Videla & Herrera, 2009). Many types of biocides are currently available in the market including those based on organ ochlorides or gases such as nitrogen. Despite this strategy for MIC control, others methods have been investigated for mitigating environmental issues caused by the biocides as well as for cost reduction. Among these alternative strategies it can be cited the use of plant products such as extracts and essential oils which are being considered largely for their environmental-friendly nature (Nicolaou *et al*., 2012). Additionally, they have lower production costs, proven efficacy and their production can be easily scaled up. The action mechanisms of these natural products are not limited to the elimination of microbial-biofilms but can also induce chemical inhibition such as anodic and capacitive impedance effects. Additionally, they protect the material surface for film development (Abdel-Gaber *et al*., 2011; Loto *et al*., 2011).

Essential oils are volatile compounds of different chemical composition usually rich in antioxidants and with a characteristic smell. They are formed by the secondary metabolism of aromatic plants and show different functions such as insecticide, herbicide and defense against active parasites (Bakkali *et al*., 2008; Viuda-Martos *et al*. 2011). Additionally, antimicrobial activity is mainly due to the phenolic compounds such as flavonoids and terpenoids occurring in these essential oils (Albuquerque *et al*., 2006; Sarikurkcu *et al*., 2010; Viuda-Martos *et al*., 2010; Sarrazin *et al*., 2012; Ferraz *et al*., 2013).

The genus *Lippia* has approximately 250 species known as essential oil producers, which occurs mainly in Central America, tropical Africa and South America. They are common in Brazil, where this genus can be found in the semiarid Northeast region (Terblanché & Kornelius, 1996; Lambert *et al*., 2001; Kunle, 2003; Albuquerque *et al*., 2006;Pimenta *et al*., 2007). *Lippiagracilis* Schauer (Verbenaceae) is a bush popularly known as “alecrim-da-chapada”. It has small branches and leaves rich in glandular trichomes. This plant´s essential oil has been applied in medicinal field for controlling skin diseases and ulcers, respiratory diseases such as sinusitis and bronchitis, influenza as well as antimicrobial and antitumor activities (Pascual *et al*., 2001; Ferraz *et al*., 2013).

In order to enable manipulation as well as aiming to increase the interaction of the oil with its target, the use of microemulsion systems has been proposed in the present study. The microemulsions are isotropic and colorless thermodynamic stable systems of two liquids (usually water and oil) stabilized by a film of surfactants at the oil/water interface. They are characterized by spherical aggregates with diameters lower than 1400Å(Oliveira *et al*., 1997; Oliveira *et al*., 2004). Due to its small diameter, the degree of interaction as well as the molecule thermodynamic stability is increased. Thus, these systems have been used in industrial processes mainly those carried out at high temperature, since it could be kept constant (Langevin, 1988).

Therefore based on the antimicrobial relevance shown by *L. gracilis* Schauer and based on the knowledge that plant products can decrease or even stabilize the corrosion rate of different materials, we investigated the effect of the essential oil of *L. gracilis* Schauer using a microemulsion system on the microbial biofilms by means of a dynamic system. Additionally, its antibiofouling activity as well as AISI 1020carbon steel´s corrosion rate using impedance resistance and electrochemical polarization techniques, gravimetry and surface analysis was assayed. It should be highlighted that AISI 1020 carbon steel was chosen since it is more studied in the literature for the antibiofouling and antimicrobial activities.

2. MATERIAL AND METHODS

2.1. Chemical

Nonionic surfactant polyoxyethylenesorbitan monooleate (Tween 80), chemical formula ($C_{64}H_{124}O_{26}$) and molar mass 1310 g/mol, was acquired from Sigma-Aldrich (MO/USA). Deionized water was used in the microemulsion systems and was prepared using a Milli-Q system.

2.2. Harvesting of *Lippiagracilis* Schauer, Essential oil Extraction and Chemical Characterization

Samples of *L. Gracilis* Schauer were harvested during a period of drought in the city of Mossoró (5° 11′ 17″ South and 37° 20′ 39″North) in Rio Grande do Norte state, Brazil. The plant taxonomic characterization was carried out to compare the samples to an example deposited in exsiccate at Dárdano de Andrade herbarium in the Federal Rural Semiarid University (UFERSA) located at the above cited city. The record number was 12514.

The essential oil extraction process was carried out viahydro distillation using the *Clevenger* system coupled to a glass balloon. After the extraction process, the yield was calculated, and the oil was stored at 4ºC for further studies. Samples of essential oil of *L. gracilis* Schauer were used for chemical characterization in which the oil composition was determined by gas chromatography coupled to mass spectrometry Hewlett-Packard CG/MS (CG: 5890 SERIES II/ CG-MS: MSD 5971). Identification was performed by searching the library from the equipment and comparing the retention time with those obtained by the co-injection of the oil together with linear hydrocarbon (C11 - C24) calculated according Van den Dool & Kratz (1963). For comparison, other libraries and the literature were also searched.

A CG assay was carried out in a Hewlett-Packard 5890 SERIES II CG chromatographer coupled to a flame ionization detector (FID) and a silica capillary column (J & W Scientific DB-5). The column temperature was 35ºC and hydrogen (H_2) was used as the carrier at a flow-rate of 1.0 mL/min. GC/MS for the oil was carried out using aHewlett-Packard GC/MS (GC: 5890 SERIES II/ GC-MS: MSD 5971) under the conditions as cited above,except for changing the carrier to helium (He). The injected oil volume was 1.0 mL of a 1/100 ethyl acetate diluted solution.

2.2. Microemulsion System

The microemulsion determination for the essential oil of *L. gracilis* Schauer and Winsor regions was carried out according to the methodology proposed by Dantas *et al*. (2002). In this case, Tween 80 (80% v/v) was used as a surfactant, and the oil phase (25 $\mu g.L^{-1}$essential oil) was used as a co-surfactant. The aqueous phase (sterilized deionized water) was at a ratio of C/T = 1.

The Winsor regions were obtained by the determination of active material (surfactant + co-surfactant) at the aqueous phase (AP) and oil phase (OP) by mass titrations. The active material was initially titrated by adding the aqueous solution until the maximum solubility point was reached. This was followed by a change in the system appearance. Next, a point was prepared with a known composition in the monophase region in the pseudo ternary system (T point), considered the titrant of all other points in the binary system: aqueous phase plus oil phase (AP+OP) andco-surfactant (C)/surfactant (T) plus oil phase. The limits for solubility curves of Winsor regions in the microemulsion system were determined by a mass balance.

2.3. Antimicrobial Activity

2.3.1. Total Plankton Microorganisms

The detection of total plankton microorganisms consisted in verifying the presence or absence of five different types of microorganisms: aerobic, anaerobic, sulfate-reducing (SRB) and iron-reducing (IRB) bacteria as well as fungi. In the case of bacteria, 1.0 mL of production water from a ship tank was added separately to flasks containing 10 mL of different culture media. A total of three repetitions were performed for each type of bacterium studied. For fungi, the same water quantity was added to Petri dishes followed by an addition of Sabouraud Agar medium which was performed for a total of three repetitions. The plates were incubated in BODfor 24h at 30º C.

The culture media were established according to the type of the specific microorganism. For the SRB, the modified Postgate E medium was used (0.5g KH_2PO_4, 1.0g NH_4Cl, 1.0g Na_2SO_4, 0.67g $CaCl_2.2H_2O$, 1.68g $MgCl_2.6H_2O$, 0.5g $FeSO_4.7H_2O$, 5.0g NaCl, 1.0gascorbic acid, 1.0g yeast extract, 7.0 mL sodium lactate (50% m/v), 4.0 mL resazurinsolution (0.025% m/v) per liter of medium). For the anaerobic bacteria assay, the fluid medium to tioglycolate was used (5.0g yeast extract, 15.0g tryptone, 55.0g glucose, 0.5g sodium tioglycolate, 2.5g sodium chloride, 0.5g L-cystine, 0.001g resazurin, 0.75g agar). For aerobic bacteria, a nutrient broth medium was used (10.0g Lab-Lenco Powder, 10.0g peptone and 5.0g NaCl). For the IRB the citrate ferric medium was used (0.5g $(NH_4)_2SO_4$, 0.5g $NaNO_3$, 0.5g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.134g $CaCl_2.2H_2O$, 10.0g ammonium ferric citrate, 15.0g agar) and the Sabouraudagar medium was used for fungi. Each medium was sterilized after preparation, and the pH was adjusted if necessary. The culture media for anaerobic microorganisms were deaerated using nitrogen gas (N_2). Inoculation was carried out in BOD at30ºC using different times. For aerobic bacteria and fungi, the incubation was conducted for 48h, while for IRB, it was conducted for 14 days and 28 days for both anaerobic and SRB.

2.3.2. Activity of the L. Gracilis *Schauer Essential Oil Microemulsion System over the Microbial Biofilms*

The carbon steel 1020 AISI biocoupons (7.0 x 3.5 cm) were used for studying both antibiofouling and antimicrobial activities. They underwent a previous treatment to remove impurities that were identified and

were then added to the dynamic system. Sonication treatment was performed (Ultra Cleaner 1600A® - Skymen) using dichloroethylene for 15 minutes, followed by washing with ethanol and acetone. Next, deionized water was used to rinse the coupons followed by drying. After drying, the coupons were sonicated again for 15 minutes, and the process was repeated.

The dynamic system was built to simulate the operational conditions of a field pipeline with similar biofilm formation. The system was made from acrylic and had a device to fix the biocoupons as well as to allow water circulation. The biocoupons were placed tangentially in the water stream direction to simulate the pipeline wall. The water circulation was carried out using a peristaltic pump at a flow-rate of 500 mL/h. The coupons were withdrawn from the system after seven days, fixed separately to a nylon wire and submitted to treatments of different concentrations of *L. gracilis* Schauer essential oil in Erlenmeyer flasks that were shaken for 50 rpm at 25º C. The contact time of the biofilms with the essential oil microemulsion was up to 16 days. Dimethyl sulfoxide (25.0 µL) was used as a negative control and 25 mg glutaraldehyde was used as positive control.

For studying the biocide activity, the microemulsion was put in contact to the biocoupons and each four days two biocoupons were withdrawn and scraped separately for microbial density counting. The pour plate technique was used to quantify the cells, and the results are shown as colony forming units (CFU) for the fungi and most probable number (MPN) for bacteria. Additionally, fluorescence microscopy (λ=510 nm) was used to quantify the biofilm cell density using the method proposed by Würth *et al*. (2013) with modification. In this case, after removal of the biofilms that had been previouslykept in contact with the microemulsion, the cell density was assayed after cultivation in specific medium depending on the microorganism (fungi or bacteria). A run blank (with no microemulsion addition) was used as a control. The results are shown as relative fluorescence relative unit (RFU).

A completely randomized design was used to investigate the influence of the biofilms' exposure time to the microemulsion of the *L. gracilis* Schauer essential oil using three repetitions for each treatment. For analysis, the data were submitted to analysis of variance (ANOVA) and mean separate evaluation by Tukey test (p<0.05). The statistical analysis was performed using the software for variance analysis system (SISVAR 5.6, *University of Lavras* (UFLA) – Minas Gerais/Brazil).

2.3.3. Scanning Electron Microscopy (SEM)

The SEM was carried considering each contact time for the microemulsion of the essential oil to the biocopouns. The coupons were prepared using a 1.0% glutaraldehyde solution (consisting of 280.0µL glutaraldehyde (25%) with 6.72mL deionized water). The solution was added to tubes of 15 mL in which the coupons were inserted and left for 4 h. After this contact time, the coupons were withdrawn and put in contact with the following concentrations of ethanol:50, 60, 70, 80 and 90%. The coupons were left in contact for 20 minutes with each ethanol solution, sequentially. Finally, the coupons were left for 20 minutes in ethanol and acetone followed by drying. The coupons were methylated, and the critical point was estimated.

2.3.4. Antibiofouling Activity of L. Gracilis *Schauer Essential Oil Microemulsion*

For this assay, cleaned and dried biocoupons previously in contact with the microemulsion were then immersed in the Erlenmyer flask containing 250 mL sterile water before insertion in the dynamic system to observe the efficiency of the microemulsion in avoiding the microbial biofouling formation. The biocoupons were shaken (150 rpm) at a growing contact time with the microemulsion (0, 48 and 96 hours). In this case, a triplicate was used. After the microemulsion contact period, the biocoupons were kept in the dynamic system with forced water circulation for four weeks. After this time, they were withdrawn, and the surface micrographs and the AISI 1020 steel wear were investigated. The corrosion rate by mass loss was assayed according to Equation (2).

2.3. Corrosion Experiments

2.3.1. Electrochemical Analysis

The corrosion experiments were carried out using a conventional electrochemical cell containing NaCl (0.5M) as the corrosive medium and three electrodes. The copper electrode was used as the working electrode, the Ag/AgCl electrode was used as the reference electrode, and the platinum electrode was used as counter-electrode. The efficiency of inhibition corrosion on AISI 1020 carbon steel due to the *L. gracilis* Schauer essential oil microemulsion was assayed by the linear potentiodynamic polarization (LPP) and electrochemical impedance spectroscopy (EIS) techniques using the potentiostat/gavalnostat Autolab® model PG STATE 204(Metrohm) coupled to

NOVA software version 1.11. The steel region exposed to the corrosive medium had a 2.5 cm radius. The polarization curves were obtained by scanning the potential below and above300 mV in relation to the open circuit potential using a rate of 1.0 mV/s by 180 minutes of immersion. The assays for EIS were carried out using the open circuit potential at 25 ºC at a frequency ranging from 10000 Hz to 6 MHZ. The TAFEL straight extrapolation method was used to obtain the curves and the corrosion inhibition efficiency (IE) according to Equation (1):

$$\mathbf{IE}(\%)=\boldsymbol{Icorr}-\boldsymbol{Icorr(inh)}\boldsymbol{Icorr} \quad \text{Equation (1)}$$

where I_{corr} and $I_{corr(inh)}$ are the AISI 1020 carbon steel currents in the absence and presence of microemulsion, respectively.

2.3.2. Gravimetric Assays

The same biocoupons used for the biocide activity after contact with the microemulsion were used to evaluate the mass loss by gravimetry for different contact periods (0, 8, 16 days). The specimens were withdrawn from the dynamic system every four days thus after pickling of microbial biofilms they were washed using acetone and water followed by drying with hot air. The mass loss was calculated according to Equation 2:

$$\mathbf{CR}=\boldsymbol{87.6WdAt} \quad \text{Equation (2)}$$

where CR is the corrosion rate, W is the mass loss(mg), d is the biocoupon density (g/cm^3), A is the biocoupon exposed area (cm^2) and *t* is the exposure time (h).

The AISI 1020 carbon steel density was 7.86 g/cm^{3}according to NACE-TM (2000). The corrosion rates were calculated in mm/year. The test was performed in triplicate for each experiment at room temperature, and the observed differences were found to be lower than 5%. An analysis of variance was carried out using the different microemulsion contact time. The positive control used only the biocoupon and distilled water, while a0.5 M NaCl solution was used as the negative control.

3. RESULTS AND DISCUSSION

3.1. Chemical Characterization of *L. Gracilis* Schauer Essential Oil

The chemical characterization of *L. gracilis* Schauer essential oil showed an expressive concentration of carvacrol (approximately 50.0%) but with a lower quantity of thymol (approximately 0.59%), as seen in Table **1**. The total oil yield was 1.74%.It should be noted that these two molecules are known for their antimicrobial activities as commented by Costa *et al*. (2001), Botelho *et al*. (2007), Silva *et al*. (2013) and Bitu *et al*., (2014). Changes in the chemical components and the essential oil yield can be influenced by many factors. Specifically for the chemical composition of *L. gracilis* Schauer essential oil, the plant age, environment, genetics, presence or absence of parasites, type of soil and season have been reported to alter significantly the carvacrol and thymol composition (Zein *et al*. 2012), directly influencing the microbial activity. There is evidence that synergism occurs between these two molecules that

Table 1: Chemical Composition of *L. gracilis* Schauer Essential Oil Obtained by GC/MS. * Retention Index

Compound	*R.I	Total (%)
Hexenol<(4Z)->	871	1.46
Thujene<α->	924	1.80
Pinene<α->	932	0.44
Pinene<β->	974	0.40
Myrcene	988	4.29
Terpinene<α->	1014	1.68
Cymene<O->	1022	4.50
Sylvestrene	1025	0.46
Ocymene<(E)-β->	1044	0.33
Terpinene<γ->	1054	14.97
Sabinene Hidrate <cis->	1065	0.45
Terpineolene	1086	1.14
Epoxymyrcene<6,7->	1090	0.14
Linalool	1095	0.70
Menthatriene	1108	0.03
Terpinen-4-ol	1174	4.67
Thymol, methylether	1232	0.59
Carvacrol,methylether	1241	0.56
Carvacrol	1298	49.51
Thymolacetate	1349	0.01
Carvacrolacetate	1370	0.14
Total Chemical Class		
Benzenoides	-	55.90
Monoterpenoides	-	33.80
Sesquiterpenoides	-	10.2
Total		**99.9**

increases the degree of activity in relation to the microorganisms. However, the presence of the others oil components such as *y*-cymene e *y*-terpinenecan also influence the *L*. *Gracilis* Schauer antimicrobial activity (Pascual *et al*., 2001). A fluctuation was observed in the essential oil chemical composition, that could be explained by the influence of the drought season, in which the plants were harvested, as it is characteristic of the region they were harvested. The hydric stress can affect the plant metabolism, changing the pathway as well as altering the secondary metabolites. Furthermore, the hydric stress confers heat resistance and decreases in the water loss (Stitt, 2013).

3.2. Tests for Planktonic Microorganisms Detection

All target microorganisms were detected in the samples of water after different incubation times in the culture media. For aerobic bacteria and fungi the best result was obtained after 24h of incubation. For SRB the best result was obtained after 14 days of incubation in which an iron precipitate could be observed. For anaerobic and SRB, the best results were obtained after 28 days of incubation. The presence of SRB was detected according to the methodology proposed by Lowe *et al*. (2004): in this case, the SRB was evidenced by the color change in the medium, as it changed from pink (control) to a darker hue due to the production of FeS. For the anaerobic bacteria, it was assumed that a medium cloudy appearance was evidence of the presence of these microorganisms. Table **2** shows an estimate of the number of bacteria for a sample of production water of ship's ballast.

Table 2: MPN for Bacteria Existing in the Ballast Ship Tank

Microorganism	MPN of Cells (cells/100mL)
Total aerobic bacteria	3.5×10^5
Total anaerobic bacteria	8.0×10^5
Sulfate reducing bacteria (SRB)	3.5×10^5
Iron oxidizing bacteria (IOB)	2.0×10^5

3.3. Microemulsion System

A rheological study was carried out to classify the microemulsion system of essential oil of *L*. *gracilis* Schauerby performing a scanning in the shear rate from 0 to 1000 s^{-1}at30 ºC. As seen in Figure **S1** in the supplementary material. There was a change in the regime from laminar to turbulent after $200s^{-1}$. This can be observed by the change in the slope, in Figure **S1**. Therefore, the shear stress was defined between 0 and $200s^{-1}$. In this region, the graph of shear stress versus shear rate was again plotted as shown in Figure **S2**, indicating that the fluid behavior was Newtonian with R^2 equal to 0.9992(Kumar & Mittal, 1999). The viscosity stability of the microemulsion system was also determined in relation to the shear stress for the same range between 0 and $200s^{-1}$. There was an initial viscosity reduction reaching the minimal value where it was kept constant, as shown in Figure **S3**. The drop in diameter was also calculated and reached 93.20 nm at the point: 0.4% T, OP = 0.08% andAP = 99.52%, as shown in Figure **S4**. The drop in diameter as well as the visual phase characteristic allowed us to classify the system as a Winsor IV type, which is considered a homogeneous or monophasic system (Winsor, 1948).

3.4. Biocide Activity for the *L*. *Gracilis* Schauer Essential Oil Microemulsion System

The microemulsion of *L*. *gracilis* Schauer essential oil showed biocide activity. During *in vitro* trials different behaviors were observed ($p<0.05$) among the microorganisms in relation to the contact time with the microemulsion. According to analysis of variance to the quantification values of the five microorganisms in relation to the different contact times (0, 8 and16 days) the coefficient of variation (CV) was 27.5.Additionally, there was a significant difference among the contact times of microemulsion of *L*. *gracilis* Schauer essential oil, the type of microorganisms and the interaction among these factors ($p<0.05$).

The MPN of the aerobic and anaerobic bacteria was not influence by the contact time with the microemulsion, *i.e*., there was no difference from the positive control, despite the slight drop in the anaerobic cell number after 16 days (Figure **1**). Despite the lack of statistical difference between the aerobic and anaerobic bacteria, we cannot conclude that the oil did not show antimicrobial activity. It is possible that bacteriostatic action occurred, as there was no increase in the number of cells with an increase of the contact time compared to the control. Similar results were reported by Videla *&* Herrera (2004) when testing the effect of *Allium cepa* extract in different concentration using eight contact times (from 5 minutes to 24 hours) over the aerobic bacteria in biofilms, ultimately observing no biocide action.

Different factors can have an influence on the antimicrobial action of these specific bacteria types. The oil concentration in the microemulsion plays an important role in inhibiting the microorganism growth

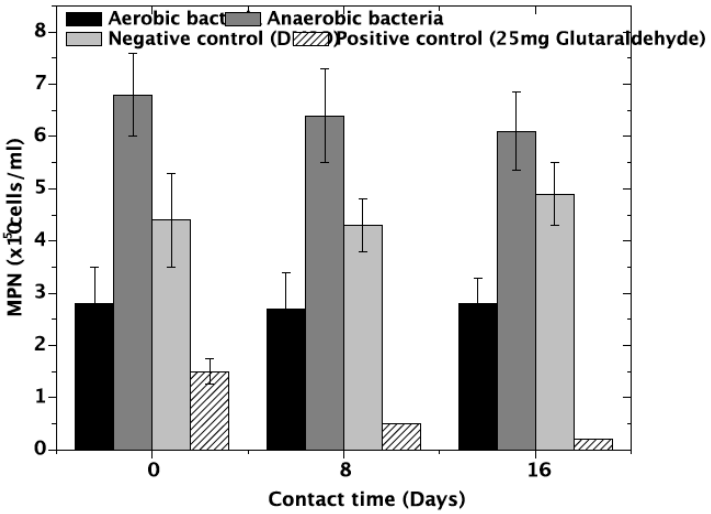


Figure 1: Biocide activity for the *L. gracilis* Schauer essential oil microemulsion on aerobic and anaerobic bacteria. Negative control: biofilm in contact with DMSO. Positive control: 25.0 mg glutaraldehyde.

once higher concentrations can induce higher activity (Tortora *et al*., 2012). Therefore, the use of concentrations higher than 25µg.L^{-1}in the microemulsion system of the *L. gracilis* Schauer essential oil can increase the efficiency due to the possibility of breaking the matrix of extra cellular polymeric substances (EPS) as well as the interaction of the microbial community (Macêdo, 2000). This would then lead to cellular death and a decrease in the bacteria growth rate. Additionally, another important factor for the biocide activity is the location of the different the types of microorganism inside the biofilm, *i.e*., there is often a hierarchy in the community due to the *quorum sensing* as well as the cell type, in which some metabolites from one species can be used by another (Flemming & Wingender, 2010; Boyle *et al*., 2013). For instance, the anaerobic bacteria are located at regions inner inside biofilms after the consumption of oxygen by the aerobic bacteria, thus it becomes more difficult and sometimes derails the activity of different biocides (Javaherdashti *et al*., 2006). This structure occurring in the biofilms is a biological adaptation that hampers or inhibits the access of biocides to certain types of microorganisms (Simões *et al*., 2010). It is probable that the microemulsion of the *L. gracilis* Schauer essential oil was not completely successful to reach completely these bacteria or may be occurred a neutralization of its activity due to its combination with metabolites or another chemical component present in the biofilm derived from its metabolic diversity (Liengen *et al*., 2014). In relation to the aerobic bacteria, a cellular detachment probably occurred and then loss to the medium of active microbial cells due to its location at outer part and peripherical of the biofilm. Additionally, the microemulsion initial contact as well as the disruption of the EPS favored the sessile bacteria, which then derailed the cellular metabolism and remained present in the samples during the quantification analysis.

On the other hand, in the case of fungi, there was a decrease in the CFU in relation to the contact time of *L. gracilis* Schauer essential oil. In this case, 16 days was enough for total inhibition compared to the control,as seen in Figure **2**. It is possible that the *L. gracilis* Schauer essential oil in the microemulsion system worked quite well as an effective biocide for the fungi present in the biofilms, performing its biocide action as reported in the literature (Pascual *et al*., 2001; Albuquerque *et al*., 2006; Melo *et al*., 2013). The *L. gracilis* Schauer essential antifungal action is mainly caused by the presence of carvacrol and thymol substances that can act together or separately over the cellular wall as well as on the ATP synthase, thus acting directly in the microbial metabolism. Other species of fungi, filamentous or not, were probably inhibited by the *L. gracilis* Schauer essential oil action or by the other majority components (Dormans & Deans, 2000; Albuquerque *et al*., 2006). However, it should be noted that especially for the fungi present in the biofilms associated with the MIC, the biocide action of the *Lippiagracilis* Schauer essential oil microemulsion worked very well. Natural products and essential oil of other species reported in the literature were also able to act on fungicorrosion-associated, but they did use a higher concentration than used in present study (Hellio *et al*., 2000).

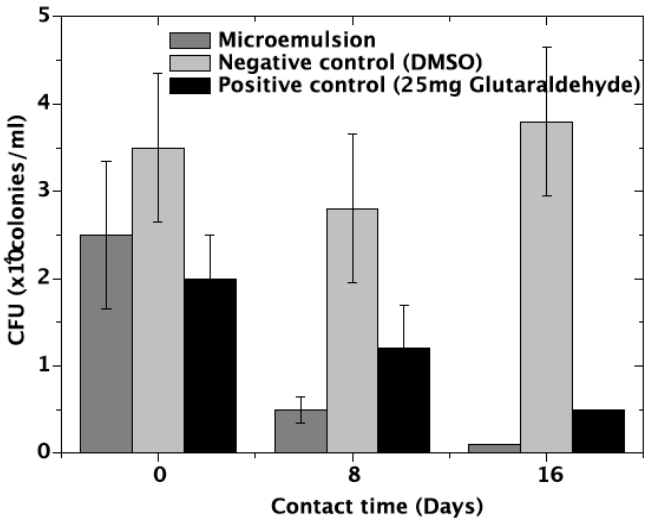


Figure 2: Effect of the *L*. *gracilis* Schauer essential oil microemulsion on CFU. The biofilms were obtained after seven days in the dynamic system. Trials were performed *in vitro* using the AISI 1020 carbon steel. Negative control: biofilm in contact with DMSO. Positive control: 25.0 mg glutaraldehyde.

The rate of iron oxidizing bacteria (IOB) as well as SRB was also reduced proportionally to the contact time due to the *L*. *gracilis* Schauer essential oil microemulsion action (Figure **3**). The IOB proved be more sensitive to the essential oil microemulsion showing full reduction of growth rate after 16 days of contact. The BRS rate was reduced by approximately 0.5 MPN after the final contact time using the essential

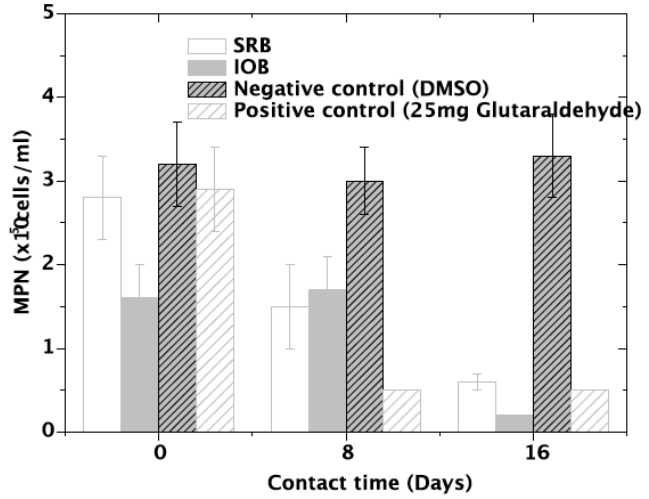


Figure 3: Effect of the *L*. *gracilis* Schauer essential oil microemulsion on MPN for SRB and IOB under different periods of contact: 0, 8 and 16 days. The biofilms were obtained in the dynamic system and runs were performed *in vitro* using AISI 1020 carbon steel. Negative control: biofilm in contact with DMSO. Positive control: 25.0 mg of gluaraldehyde.

oil microemulsion system. It is likely that higher concentrations of essential oil in the microemulsion system could favor a reduction in the SRB rate. It is known that the SRB is more resistant to the biocide action mainly due to the structure of its cellular wall that interferes more with the biocides (Zhang *et al*., 2015). Additionally, another important parameter that could be influenced by the biocide action is the location of the SRB inside the biofilm as well as the total anaerobic bacteria, *i.e*., the fact that they are located more inwards due to the higher CO_2 rate and the reduction of the O_2 concentration. Therefore, it is more difficult for some substances to directly reach the bacteria population, therefore slightly reducing this population or even being totally ineffective.

Others plant-derived substances were able to inhibit the growth of SRB *in vitro* but not by using a microemulsion system. For instance, Oguzie *et al*. (2013) used the ethanol extract of *Capsicum frutescens* showing that it was able to inhibit the growth of *Desulfotomaculum* species by the diffusion method on discs and over the corrosion rate. They reported that substances such as alkaloids, tannins and saponins existing in the *C*. *frutescens* extract acted on the bacteria metabolism, ceasing the growth. Korenblum *et al*. (2013) reported the efficiency of essential oil of *Cymbopogoncitratus* as well as of its majority component citral over the sessile SRB and planktonics. Additionally, the Neem extract (4.0%) was efficient in reducing the sessile SRB growth rate as well as the sulfide and biofilm formation (Bhola *et al*., 2014). Despite the lower concentration *L*. *gracilis* Schauer essential oil used in the emulsion, the efficient biocide action over the biocide can also be observed in Figures **4** and Figure **S4**. The micrographs showed an evident elimination of the microbial biofilms on the AISI 1020 carbon steel surface compared to the control after 16 days of contact to the microemulsion. The quantification using the spectroscopy methods also showed a reduction in the biofilms formation. It is probable that the microemulsion of essential oil reduced the microbial community acting over the EPS, mainly due to the interaction of the essential oil biomolecules with carbohydrates or other substances existing at the EPS structures. It is known that diterpenes can interact strongly with carbohydrates and proteins (Nelson & Cox, 2012) which could have caused the disruption considering the elevated quantity of these molecules in the EPS (Sutherland, 2001; Videla, 2003).

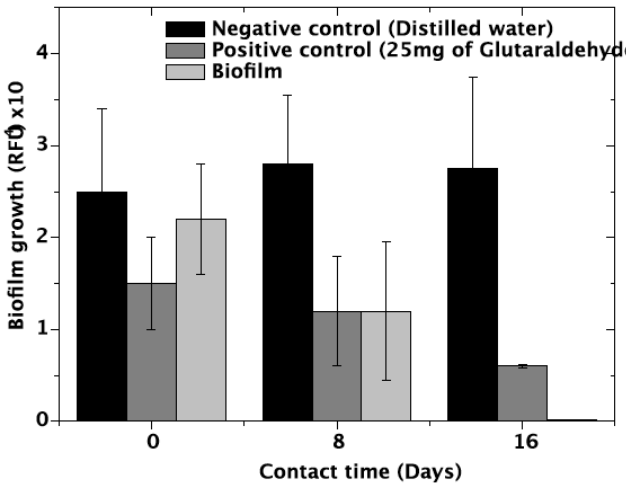
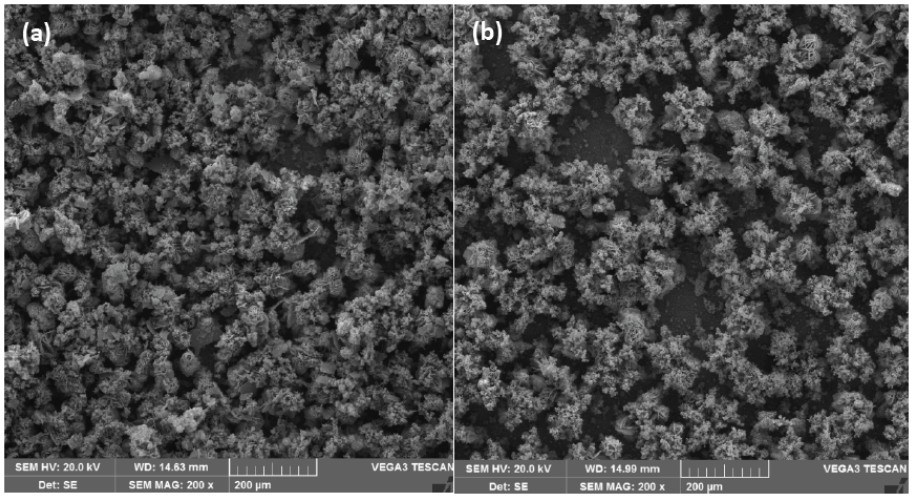


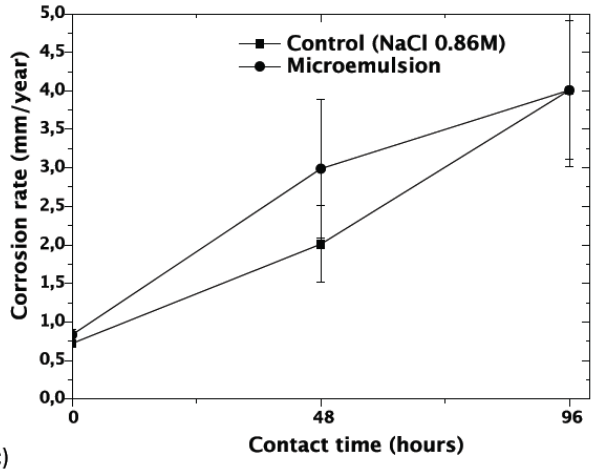
Figure 4: Effect of *L*. *gracilis* Schauer essential oil over biofilm under different contact times. Data are in Relative Fluorecence Unit (RFL) at 510 nm after biofilm pickling.

3.5. Antibioufouling Activity of *L*. *Gracilis* Schauer Essential Oil Microemulsion

The use of the *L*. *gracilis* Schauer essential oil microemulsion as anantibiofouling agent onAISI 1020 carbon steel was proven to be effective at the prevention of biofilm formation after 96 h of contact. However, the microemulsion did not avoid the loss of mass in the material. The micrographs showed the occurrence of corrosion products such as crystals without biofilm formation as compared to the control (Figure **5**). The results obtained by the gravimetry showed a significant difference among the experiments using the microemulsion in relation to the control after 48 h of contact. However, after the final contact period, there was no significant difference in the increase of the corrosion rate (Figure **5c**).

The preventing activity of the microemulsion on the biofilm formation is probably due to a formation of a





(c)

Figure 5: Antibiofouling acitivity of *L*. *gracilis* Schauer essential oil microemulsion: (**a**) micrograph on the AISI 1020 carbon steel biocoupon surface after 0 hours of contact (control) and (**b**) after 96 hours contact (200x). (**c**) corrosion rate for each time assayed (p<0.05).

protective film that is typical of the microemulsion (Paul & Moulik, 2001). It is known that organic substances occurring in the natural products can deposit homogeneously over the material surface (Dantas *et al*., 2002). However, despite the prevention of the biofilm formation, the corrosive process itself could not be avoided. The presence of corrosion products and the increase in the corrosion rate evidenced after only 48 hours suggests that microemulsion can keep the corrosion stable after the initial protective film formation, as reported in the literature. However, it is no longer effective any more probably because of its disruption.

3.6. *L. Gracilis* Schauer Essential oil Anticorrosion Activity in Microemulsion System

The microemulsion of *L. gracilis* Schauer essential oil showed anticorrosion activity for the 1020 AIS carbon steel in a salting medium, compared to the control,as shown using the electrochemical and gravimetry techniques. For LPP experiments, the microemulsion action differed in relation to the control, as shown by the kinetic parameters obtained from the Tafel curves with the current reduction (I_{corr}) and the increase in the electrochemical potential (E_{corr}) (Table **3**). The protective film formation due to the microemulsion compounds adsorption can be seen by the increase in the anode and cathode Tafel´s constants which were more significant than the control. Thus, the microemulsion likely reduced the metalic dissolution at the anode region and hydrogen evolution occurred in the cathode due to the reduction of the contact area. The EIS curves showed that there was a reduction in the current with an increase in the potential due to the action of the *L. gracilis* Schauer essential oil microemulsion system as shown in Figure **S6** (supplementary material). However, only 30% corrosion efficiency was achieved. The electrochemical impedance data showed that indeed there was a difference in the diagram obtained for the microemulsion systems in relation to the control in salt. As shown in Figure **S7** there was no difference between the microemulsion and the control in the Nyquist model, yet the charge transfer resistance (R_{ct}) increased for the former and there was a slight decrease in the electrical double-layer capacitance (C_{dl}) showing the microemulsion at evaluated concentration was weakly adsorbed onto the steel surface. The corrosion inhibition efficiency (IE) was not quite high only 14.0% as calculated from R_{ct} (Table **3**). On the other hand, the gravimetry assays showed that *L. Gracilis* Schauer essential oil microemulsion was able to reduce the corrosion process. Both experiments carried out in the electrochemical cell showed a reduction in the AISI 1020 carbon steel mass loss when in contact to the microemulsion. The reduction of the corrosion rate over the AISI 1020 carbon steel was statistically similar to that of sterilized water (negative control) for almost the contact time ($p < 0.05$) tested, as shown in Figure **7**.

The surface analysis of the biocoupons pretreated with the microemulsion showed that even for a lower *L. gracilis* Schauer essential oil concentration, there was a reduction in the mass loss, as can be seen in the micrographs in Figure **S8** and Figure **S9**. The corrosive action of the biocoupons in contact with the oil production water is evident (Figure **S8a**). However, after 16 days in contact with the microemulsion, the steel wear is reduced, as shown in Figure **S8b**.

Table 3: Kinetic Parameters Obtained from Polarization Curves for the AISI 1020 Carbon Steel in Salting Solution (0.5 M NaCl) in Presence of *L. gracilis* Schauer Essential Oil Microoemulsion

Substances	b_a (mV/dec)	b_c (mV/dec)	E_{corr} (mV/Ag/AgCl)	I_{corr} (A/cm^2 x10^{-6})	Corrosion Rate (mm/year)	IE (%)
Control	16.846	28.762	-780.73	7.23	0.0120737	-
Microemulsion	119.91	217.18	-751.07	5.10	0.0040430	29.46

Table 4: Impedance Electrochemical Parameters Obtained for the AISI 1020 Carbon Steel in Salting Solution (0.5 M NaCl) in Presence and Absence of *L. gracilis* Schauer Essential Oil

Substances	R_{ct} (Ω cm^2)	Y_0 (µMho cm^{-2})	C_{dl} (µF cm^{-2})	*n* (%)
Control	358.74	1.128	99.31	-
Microemulsion	416.46	3.775	9.25	14.0%

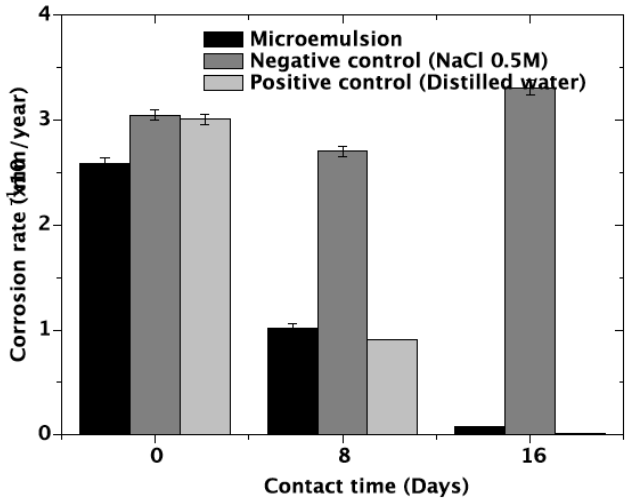


Figure 7: Effect of the *L. gracilis* Schauer essential oil microemulsion over the corrosion rate of AISI 2010 carbon steel. Assays were performed in triplicate for treatment. Negative control: destilled water. Positive control: salting solution (0.086M NaCl) with biofilm.

The use of plant-derived natural products to avoid or reduce the corrosion rate has been currently used as an alternative to the chemical biocides mainly for showing efficacy action as well as being biodegradable. Many studies on the literature prove this product utilization (Abiola & James, 2010; Rocha *et al*., 2010; Felipe *et al*., 2013; Umoren *et al*., 2012; Benahmed *et al*., 2013; Djeddi *et al*., 2015; Muthukrishnan *et al*., 2015). Most substances considered inhibitors of corrosion act similarly to synthetic substances, mainly reducing the electric resistance or the anodic and cathodic reactions on the metal surfaces (Raja & Sethuraman, 2008). Additionally, there are some mechanisms of natural products that are completely dependent of the chemical composition as well as the way these compounds are obtained. Vegetal extracts rich in saponins, alkaloids and tannins are reported to strongly inhibit corrosion by adsorbtion at the metal surface. The essential oils act by forming a protective film over the material, thus interfering in the process of electron loss for the medium and consequently slowing or inhibiting the corrosion (Bouyanzer *et al*., 2010). In our study *L. gracilis* Schauer essential oil microemulsion likely decreased the corrosion on the AISI 1020 carbon steel after the microbial community elimination thus reducing the acid production as well as the by-products of microbial metabolism that increase corrosion, mainly pitting. Additionally, the higher quantity of carvacrol, approximately 50%, might also have influenced the corrosion process helping in the corrosion control (El ouariachi *et al*., 2015). To the best of our knowledge, the use of water circulation to control corrosion is investigated for the first time in the present study. This system showed significant advantage since it provides a higher interaction with the whole biofilm community even at lower oil concentration. Additionally, it has good interaction with the aqueous phase that is used in the most steps of oil processing, thus garnering further interest, considering the industrial scale of this phase.

4. CONCLUSION

The *L. gracilis* Schauer essential oil microemulsion investigated showed good biocide activity against aerobic and total anaerobic bacteria. Additionally, it showed biocide activity *in vitro* at lower concentrations on SRB as well as fungi occurring in biofilms existing on carbon steel. It significantly reduced the microbial growth in only two hours of contact time. The antimicrobial action of the essential oil is probably due to presence of the carvacrol. The corrosion rate of AISI 1020 carbon steel was also controlled by the *L. gracilis* Schauer essential oil microemulsion after 16 days of contact time. In this case, the inhibitory action was due to the formation of a protective film over the carbon steel surface.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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