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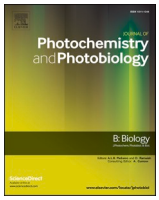

Photoinactivation of sulfate-reducing bacteria using 1,9-dimethyl-methylene blue – DMMB and laser light

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ABSTRACT

Annually, the oil and gas industry faces equipment losses and product quality degradation due to the presence of sulfate-reducing bacteria (SRB). Given the negative impact of SRB, this study evaluates the use of photoinactivation (PI) with zinc chloride double salt of 1,9-Dimethyl-Methylene Blue (DMMB) as a photosensitizer (PS) in varying concentrations and combined with Laser light at different exposures in an SRB consortium. For culture growth, a modified Postgate C medium (without ferrous sulfate) was used, and cell quantification was performed on 100 μL aliquots of the consortium, read on a spectrophotometer (λ600 nm) in an oxygen- and light-free environment at room temperature. Statistical analyses included two-way ANOVA and ANOVA with interaction to separately and jointly evaluate the effects of PS and light in PI. Results indicated microbial activity in all groups, with an antimicrobial inhibition rate exceeding 50 % ($p < 0.05$) for concentrations above 1.5 μg/mL of DMMB. PI efficacy significantly depended on DMMB concentration and light density, achieving a 70.58 % (55.73–70.58, with a mean of 66.71 %) reduction ($p < 0.05$) with 1.5 μg/mL of DMMB and a 70.15 % (65–70.15, with a mean of 68.21 %) reduction with 2.0 μg/mL at an intensity of 21.6 J/cm^2. In conclusion, PI presents a promising alternative to biocides in the oil and gas industry, offering easy application, avoiding bacterial resistance, being environmentally safe, and compatible with other SRB population control techniques.

1. Introduction

Sulfate-reducing bacteria (SRB) are a group of anaerobic microorganisms that use sulfate (SO_4^{2-}) as the final acceptor of electrons, reducing it to hydrogen sulfide (H_2S). It is found in many natural (freshwater sediments and salt marshes) and deep underground environments (oil wells and hydrothermal vents), besides traditional mining wastewater processing facilities [1].

Oil reservoirs are complex environments characterized by abundant organic matter, a temperature range (10–214 °C), pressure, salinity, and anoxic conditions [2]. When present in oil and gas reservoirs, these bacteria are carried into the wells through the injection ducts, where they find ideal conditions for their development [3]. Although most SRBs can grow at temperatures ranging from −5 to 75 °C and can quickly adapt to sudden temperature changes [4]. Under anaerobic conditions, SRB participates in the sulfur cycle, using sulfate as an electron acceptor and a carbon source, mainly from short chains as an energy source, such as lactate, acetate, and even fatty acids [5].

As bioproducts of its metabolism, such as the dissimilative reduction of sulfate, hydrogen sulfides (H_2S) are generated, which lead to the acidification of products and wear of metal surfaces, representing an eminent problem for the oil and gas industry [6], in addition to acidification in reservoirs, other phenomena include the clogging of pipelines that transport liquids, water, and oil, loss of equipment effectiveness, and inefficient heat exchange. Such phenomena can be facilitated or enhanced by microbial biofilms [7,8].

The biofilm formed by SRB on the surface of metals provides for the dissolution of metals, promoting microbiologically influenced corrosion (MIC) [9,10]. During MIC, the concentration of sessile cells increases the kinetics of the anodic and cathodic reactions of the metal/solution,

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increasing the corrosion rate [11].

Biofilm development is related to the microbial protection system, interactions with other microorganisms, and the capture and transport of nutrients by diffusion, as well as physical support and adhesion to surfaces. The formation and accumulation of biofilms in the petroleum industry cause biofouling problems, as well as MIC and biological acidification [12]. Microbial stresses, such as nutrient availability, temperature, and surface conditioning, can activate transition systems from the planktonic phase to life attached to a surface, which can also influence the structural maintenance of the formed biofilm [13].

As this phenomenon occurs in companies across various sectors, it is estimated that approximately 10 to 20 % of corrosion in the oil and gas industry is caused by MIC [14]. Another study conducted by NACE Internacional estimated an expenditure of between 3 and 4.5 % of the global GDP, equivalent to approximately US$ 2.5 trillion [15]. Due to the impact on equipment, such as pipeline failures, equipment damage, and safety risks, annual costs are estimated to run into billions of dollars [16].

Since economic losses in the recovery of this equipment are a significant problem for the industry, alternatives such as using biocides to control SRB have been investigated. However, in addition to their reduced efficiency, biocides can leave harmful residues in the environment due to their low solubility in water. Examples include oxazolidine and thiocarbamates, which accumulate nitrous and sulfureous environmental elements. [17,18]. In addition, recurrent doses are administered with each water injection into the wells to achieve satisfactory results, which increases costs. Furthermore, repeated biocide doses promote resistance, leading to a gradual escalation in dosage with each water injection for oil recovery [19]. There are other challenges to biocides, such as health and safety regulations in some countries that restrict the use of certain biocides, such as Quaternary Ammonium Compounds (QACs) [20].

Considering the challenges of antimicrobial resistance, photoinactivation (PI) has been used to control the growth of microorganisms. This technique combines a non-toxic photosensitizer (PS) with visible, non-ionizing light, with an effective wavelength to excite the PS to the triplet state. This photochemical reaction allows the generation of reactive oxygen species (ROS), such as singlet oxygen and superoxide, which are highly toxic molecules for cells [21,22]. This mechanism induces the generation of ROS, leading to damage to lipid membranes by altering permeability, oxidizing macromolecules such as proteins, lipids, and nucleic acids, or modifying metabolic activities, ultimately resulting in cell death through one of these pathways [23,24].

Several antimicrobial methods have been tested to inactivate different groups of SRB. Notably, this study's innovative strategy involves the application of PI in a microbial consortium, introducing a technique that has not been previously used in an SRB consortium. PI has the advantage of being able to be applied numerous times without side effects and generating microbial resistance [25,26].

Phenothiazines are hydrophilic, photosensitive compounds capable of generating ROS when photoactivated, with their effectiveness determined by the photophysical and photochemical properties of their monomers [27]. They can form dimers in the presence of inorganic salts, and the methylation of methylene blue, which results in the formation of PS, 1,9-Dimethyl-Methylene Blue double salt of zinc chloride (DMMB), enhances their photodynamic capacity due to the increased lipophilicity compared to methylene blue, a crucial property for intracellular incorporation [28].

Given SRB's negative impact on the oil industry, developing an effective and safe bactericidal treatment is necessary. Therefore, this study aimed to evaluate the potential of PI, using the DMMB as a photosensitizer at different concentrations in combination with Laser light at varying exposures, to analyze the antimicrobial activity in an SRB consortium.

2. Materials and methods

2.1. Bacterial consortium and growth conditions

The bacterial consortium used in this study is part of the microbiota present in produced water collected in wells located in the oil extraction field in the Recôncavo Baiano Basin – Brazil. They were previously identified by Rosário [29] and provided by the LABEM- Laboratory of Biology and Ecology of Microorganisms - Federal University of Bahia (UFBA).

Specimens cryopreserved in an ultra-freezer (Thermo Electron Corporation, Bartlesville, OK - USA) were left at room temperature inside the anaerobic chamber (Bactron VI®, Shellab, Sheldon Manufacturing Inc., USA). After thawing, the specimens were inoculated in 10 mL pharmaceutical penicillin bottles with a rubber cap and properly sealed to avoid oxygen-containing modified Postgate C® medium for bacterial growth. The original composition of the Postgate C medium includes heptahydrate ferrous sulfate ($FeSO_4{\cdot}7H_2O$), which acts as a growth indicator, leading to the darkening of the medium and complicating spectrophotometric measurements of cell concentration. Therefore, a modified culture medium was prepared, omitting the ferrous sulfate. The Postgate C® medium [30,31] modified by Rosario [29] with the following composition was used for SRB culture enrichment: NaCl – 15 g; KH_2PO_4–0.5 g; NH_4Cl – 1.0 g; Na_2SO_4–1.0 g; $CaCl_2$–1.0 g; $MgCl_2.6H_2O$ – 1.83 g; yeast extract – 1.0 g; ascorbic acid – 0.1 g; sodium thioglycolate - 0.130 g; sodium citrate – 6.38 g; sodium lactate 1.75 mL; resazurin 0.025 (m/v) – 4 mL and $FeSO_4.7H_2O$ – 0.5 g. The pH was adjusted to 7.5–8.0 by adding the appropriate amount of hydrochloric acid (HCl) before the solution was homogenized by agitation and autoclaved at 121 °C for 30 min. Subsequently, 10 % (1 mL) of the SRB inoculum was transferred to new sterile pharmaceutical penicillin bottles containing 9 mL of modified Postgate C® medium for bacterial growth. The bottles were sealed with rubber caps to prevent oxygen exposure and incubated at a constant temperature of 37 °C for bacterial growth. After inoculation, the cultures were incubated in a Biochemical Oxygen Demand (B. O.D.) incubator (TE-391/1® TECNAL, Brazil) for 30 h at 37 °C.

2.2. Experimental procedure

All PI procedures were carried out under aseptic conditions in an anaerobic chamber (Bactron VI®, Shellab, Sheldon Manufacturing Inc., USA), with no exposure to ambient light, at room temperature (23 °C), and without humidity. The chamber atmosphere was composed of 80 % nitrogen (N), 10 % carbon dioxide (CO_2), and 10 % hydrogen (H). At each stage of the experiment, 100 μL aliquots of the suspension were added to the wells in 96-well plates (Falcon®, BD Lab., Franklin Lakes, NJ, USA); all the experiments were performed in triplicate and analyzed in a spectrophotometer (SpectraMax 190®, Molecular Device, California, USA) at λ600 nm as a standard of the Optical Density (OD) in SRB to determine the cell quantity in correlation to absorbance [32].

2.3. Evaluation of the toxicity of DMMB on the SRB consortium

The photosensitizer (PS) (DMMB, Sigma-Aldrich, St. Louis, MO, USA) absorbs λ_{max} 649 nm and has a molar mass of 347.905 g/mol. To avoid contamination, the photosensitizer was diluted using sterile distilled water and filtrated using a 0.22 μm membrane (Kasvi, Pará—Brazil). The culture was exposed to 0.050, 0.250, 0.500, 0.750, 1.0, 1.5, and 2.0 μg/mL to evaluate the toxicity.

To assess the microbial concentration exposed to DMMB, the experiment procedure described in item 2.1.2 was used.

2.4. Evaluation of the effect of different laser exposures on the SRB consortium

The light source was a Laser (TF Premier Plus ®, MMO Optics, São

Paulo-Brazil, λ660 nm, spot of 3mm^2, 100 mW). The Laser emission parameters are described in Table 1. Carrying out a scanning application using Eq. (1) to determine the exposure [33]:

$$\text{ed}\ (\text{j/cm}^2) = \frac{\text{P (W)} \times \text{T (s)}}{\text{A } (cm^2)} \quad (1)$$

Where ed. is the exposure, P is the power, T is the irradiation time, and A is the irradiated area.

The irradiation was carried out at an angle of 90° in an anaerobic environment without ambient light at room temperature (23 °C) free from humidity in the anaerobic chamber (Fig. 1). Temperature and humidity were controlled and monitored during the experiments. The microbial concentration exposed to the Laser was evaluated as described in item 2.1.2.

2.5. Photoinactivation

To perform the Photoinactivation, DMMB at concentrations of 1.0, 1.5, and 2.0 μg/mL were chosen, and each one was associated with the exposures described in Table 1 and pre-irradiation time of five minutes in the dark at room temperature. After pre-irradiation, the flasks were exposed to the light source at the time described in Table 1. Posteriorly, aliquots of 100 μL of the suspension were added to each well of the 96-well plate (item 2.1.2) (Falcon®, BD Lab., Franklin Lakes, NJ, USA) and analyzed the Optical Density (OD_{600}) on a spectrophotometer (SpectraMax190®, Molecular Device, California, USA). The following groups were tested: control and PI groups (PI 8 J/cm^2 + 1.0 μg/mL DMMB, 10 J/cm^2 + 1.0 μg/mL DMMB, 12 J/cm^2 + 1.0 μg/mL DMMB, 14.4 J/cm^2 + 1.0 μg/mL DMMB, 21.6 J/cm^2 + 1.0 μg/mL DMMB, 8 J/cm^2 + 1.5 μg/mL DMMB, 10 J/cm^2 + 1.5 μg/mL DMMB, 12 J/cm^2 + 1.5 μg/mL DMMB, 14.4 J/cm^2 + 1.5 μg/mL DMMB, 21.6 J/cm^2 + 1.5 μg/mL DMMB, 8 J/cm^2 + 2.0 μg/mL DMMB, 10 J/cm^2 + 2.0 μg/mL DMMB, 12 J/cm^2 + 2.0 μg/mL DMMB, 14.4 J/cm^2 + 2.0 μg/mL DMMB and 21.6 J/cm^2 + 2.0 μg/mL DMMB).

2.6. Statistical analysis

The results obtained through Optical Density (OD_{600}) were converted into cells/mL using the McFarland [34] scale. The equation developed by Edington [35] was used to calculate the percentage of inhibition achieved in the experiment. Multivariate analyses were conducted using two-way ANOVA without replication, two-way ANOVA with interaction, and multiple comparisons with the Tukey test, all performed using GraphPad Prism® software (San Diego-CA, USA). For all analyses, $p < 0.05$ values were considered statistically significant.

3. Results

3.1. Toxicity of DMMB on the SRB Consortium

The results indicated that DMMB alone does not cause significant microbial reduction compared to the control group at concentrations of 0.050, 0.250, 0.500, and 0.750 μg/mL. However, the concentrations of 1.0, 1.5, and 2.0 μg/mL caused microbial reduction of 2.83, 3.70, and 9.72 %, respectively (Fig. 2). For this reason, the last three concentrations that statically showed some levels of microbial reduction were used in the experiment.

Table 1
Irradiation time for Laser Light in the bacterial consortium.

Exposure	Time/min
8 J/cm^2	6.5
10 J/cm^2	8.1
12 J/cm^2	9.8
14.4 J/cm^2	11.75
21.6 J/cm^2	17.6

3.2. Effect of different laser exposures on the SRB consortium

The exposures evaluated (8, 10, 12, 14.4, and 21.6 J/cm^2) showed that there was no microbial inactivation when compared to the control group (Fig. 3). The results show that light alone cannot reduce the consortium, reinforcing the need for a PS molecule that interacts with it.

3.3. Effect of photoinactivation on the SRB consortium

Using 1.0 μg/mL of DMMB in all exposures reduced the microbial population from 39.39 to 48.13 % (with a mean of 43.51 %). At the concentration of 1.5 μg/mL, microbial reduction ranged from 55.73 and 70.58 % (mean of 66.71 %), and using 2.0 μg/mL of DMMB, microbial inactivation ranged from 65 to 70.15 % (mean of 68.21 %), when compared to the control group (Fig. 4). PI show us that when combined with PS and Laser, it brings expected microbial reduction. We can see this range of expected reduction when comparing with item 3.1.1, which only had a 9.72 % reduction using DMMB in the highest concentration (2.0 μg/mL), whereas using Laser resulted in no reduction as described in item 3.1.2. This reduction of PI is illustrated in (Fig. 5), and the groups tested are described in item 2.1.5. This means that the higher the PS concentration and light density, the more desired effect of PS on the consortium.

The analysis of the effect of PI on the tested groups showed that concentrations lower than 1.0 μg/mL of PS were not as effective when compared to the control group and to the groups with concentrations higher than 1.0 μg/mL. The values considered for the analysis correspond to the $p < 0.05$(****) present in all groups compared to the control (Fig. 5). While at the concentration of 1.0 μg/mL of PS, the values varied when compared to the other two groups, which at concentrations of 1.5 μg/mL and 2.0 μg/mL of PS showed little variation. Thus, we can highlight that the best concentration is ≥1.5 μg/mL in all light exposures used in these tests, performing a reduction of more than 50 % of the consortium population.

The concentration of 1.0 μg/mL DMMB significantly differs from the concentrations of 1.5 μg/mL and 2.0 μg/mL ($p < 0.05$) used in the PI. However, no significant difference was found between the 1.5 μg/mL and 2.0 μg/mL concentrations, as both groups exhibited similar antimicrobial effects.

3.4. Analyses ANOVA

To analyze the response variable of both factors, PS and light intensity, independently in the microbial reduction of the consortium, a two-way ANOVA without replication was used (Table 2), where significant data are indicated by $p < 0.05$ for the PI.

To assess whether there is an interaction between the increase of both variables in the antimicrobial reduction using PI, a two-way ANOVA with interaction was performed (Table 3), where significant data are indicated by $p < 0.05$.

To determine whether there is a significant difference in the number of cells reduced in the consortium when using different DMMB concentrations, the PS groups were compared, and statistical significance was assessed at $p < 0.05$. The concentration of 1.0 μg/mL significantly differs from the concentrations of 1.5 μg/mL and 2.0 μg/mL ($p < 0.05$) used in the PI. However, no significant difference was found between the 1.5 μg/mL and 2.0 μg/mL concentrations, as both exhibited similar antimicrobial effects (Fig. 6).

In terms of the reduction in cell count per mL, the concentrations of 1.0 μg/mL, 1.5 μg/mL, and 2.0 μg/mL showed promising and expected results, as higher concentrations are anticipated to result in greater reductions.

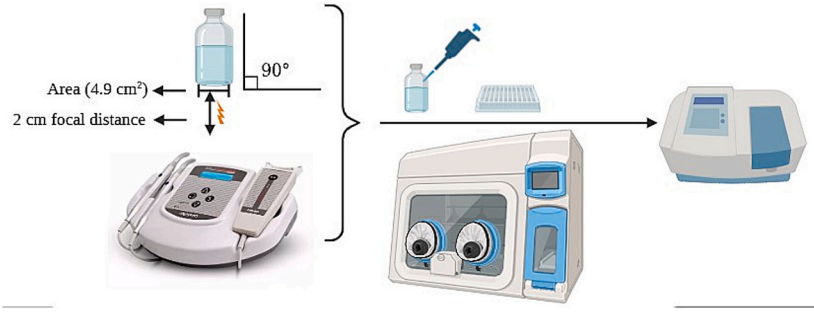

Fig. 1. Diagram of the irradiation of the Samples. The irradiated area corresponded to the location of the bottom of the flask (4.9 cm^2).

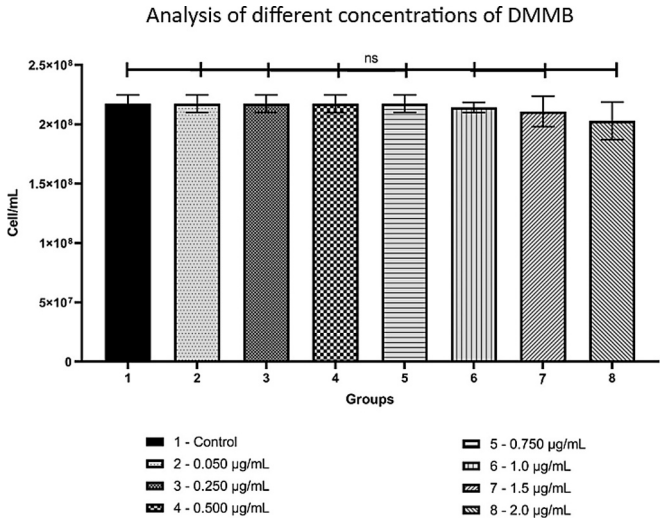

Fig. 2. Analysis of different concentrations of DMMB without Laser light application showing no major lethality (ns: no significant).

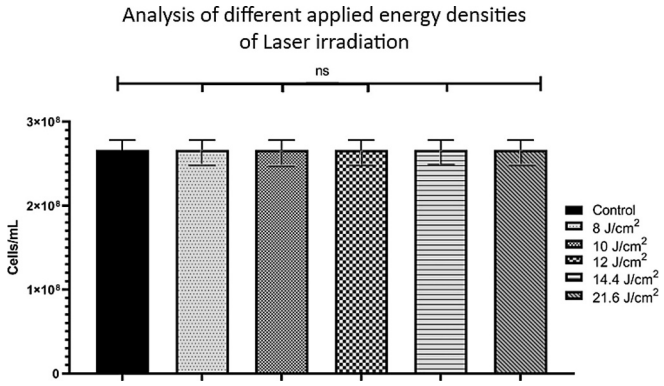

Fig. 3. Analysis of different applied exposures of Laser irradiation (ns: no significant).

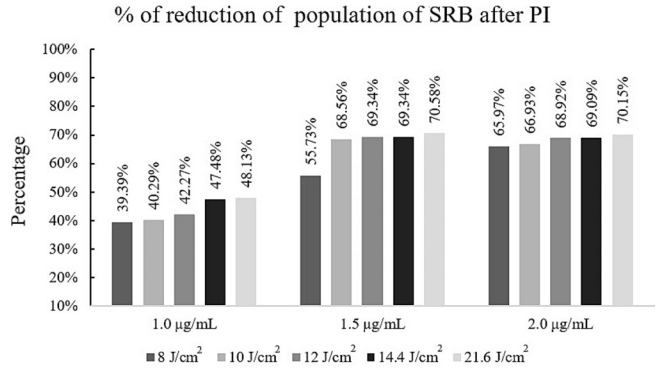

Fig. 4. Analysis of the reduction in % of tested groups after applying PI in the SRB consortium.

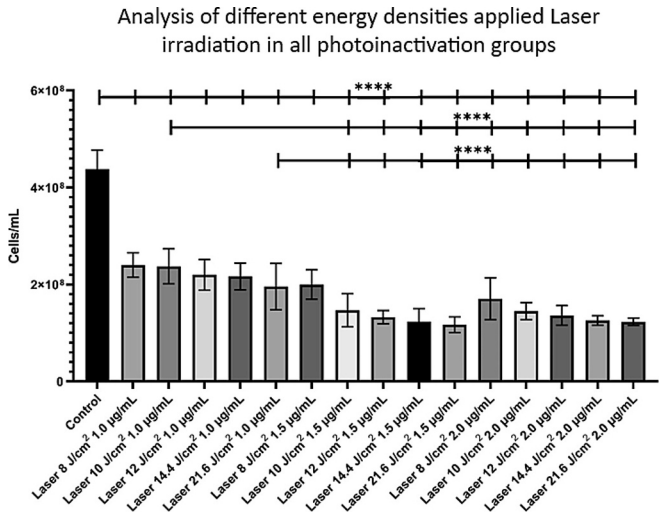

Fig. 5. Analysis of different Laser exposures in all photoinactivation groups. The values denoting significance between the compared groups correspond to the $p < 0.05$ (****).

4. Discussion

In flow systems such as the petroleum industry, adding biocide does not eliminate microorganisms by increasing proliferation with each addition of water [36]. Usually, high dosages of biocides are required in oil and gas fields to treat biofilms; this is due to the various defence mechanisms of SRB [37]. SRB natural defences are impacted by the availability of nutrients, fluid flow, and some toxic substances, such as biocides, which can induce cellular stress, resulting in a morphological protection mechanism, making the cell group on a surface form biofilm [38].

Other studies have already shown resistance to applying biocides and that the remaining cells on biofilm can withstand concentrations 500–5000 times higher than the concentration required of planktonic cells [39,40]. The most common biocides in the treatment of produced water are glutaraldehyde (GA), 2,2-dibromo-2-cyanoacetamide (DBNPA), Tetrakis (hydroxymethyl) phosphonium sulfate (THPS), and compounds known as benzalkonium chloride [37].

Scientific research evidences the search for a more economically viable, sustainable, and environmentally safe alternative that goes

Table 2
Two-way ANOVA without replication of the Laser light action at all its intensities and the photosensitizer (PS) DMMB at all concentrations.

Source of Variation	ss	df	Ms	F	*p*-Value	Critical F
Laser Ligth (Intensity)	6.10E+15	4	1.52E+15	1.15E+01	0.002101	3.84E+00
PS (concentrations)	2.13E+16	2	1.06E+16	8.07E+01	4.98E-06	4.46E+00
Residual	1.06E+15	8	1.32E+14			
Total	2.85E+16	14				

Table 3
Two-way ANOVA with interaction.

Source of Variation	ss	df	Ms	F	p-Value	Critical F
Intensity (I)	4.24E+17	4	1.06E+17	4.03E+29	2.67E-260	2.93
Concentrations (C)	7.11E+16	2	3.55E+16	1.35E+29	2.55E-254	3.55
Interactions (I*C)	2.12E+18	8	2.65E+17	1.01E+30	2.95E-265	2.51
Residual	4.73E-12	18	2.63E-13			
Total	2.615E+18	32				

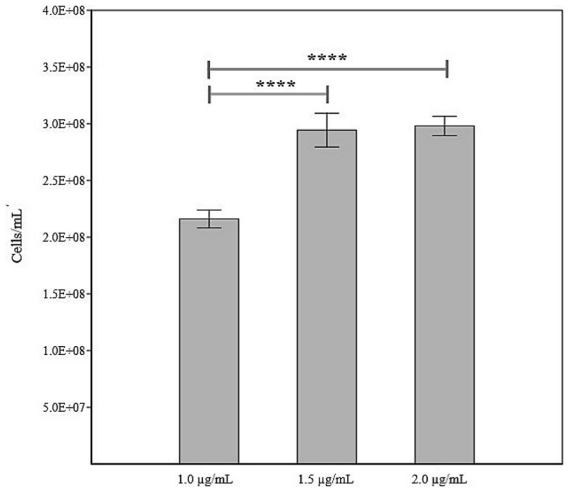

Fig. 6. Comparison of cell reduction (cells/mL) between the groups receiving PI at DMMB concentrations ($p < 0.05$****).

beyond conventional biocides by adding other compounds to enhance the action of the treatment in sessile or planktonic cells. The study done by Lavania et al. [41] tested cow urine for the control of *Desulfovibrio vulgaris* and *Desulfovibrio gigas*. The study concluded that a significant reduction in these bacteria required 90 days of adding urine to the microorganism culture. The authors compared four other chemical biocides that control SRB: THPS, BTAC (benzyl trimethyl Ammonium Chloride), glutaraldehyde, and formaldehyde. The authors reached a 3 $\times$ 10^2 CFU/mL result in planktonic cells compared to the control group of 7 $\times$ 10^6 CFU/mL. Biofilm-forming cells reached a 9 $\times$ 10^1 CFU/mL result compared to the 8 $\times$ 10^5 CFU/mL control group. The concentrations of each biocide and cow urine added to the tubes in this study were 10, 20, 50, 70, 100, and 200 mg/L. The results indicated cow urine's effectiveness in reducing planktonic and biofilm populations (1 to 6 log units) in 90 days.

Renewing treatment cycles with more biocides, including THPS, GA, and formaldehyde, is common in oil and gas systems. THPS in high concentrations increases the availability of sulfate, which aids the metabolism of these bacteria and the formation of barium sulfate, thus reinforcing the biofilm layers [42]. Also, it is unsuitable for systems with a pH > 8, behaving as a Fe^{2+} chelator, reducing its effect as a biocide, which ultimately adds higher concentrations in systems with a high FeS content [43]. The availability of dissolved Fe^{2+} in the medium interacts with the biocide, reducing the rate of uptake by the target cells. Additionally, in the long term, THPS is less efficient in eliminating the populations in produced water, as the mechanism of action is fast and not long-lasting [44].

That is why GA and THPS required concentrations above 500 mg/L^{-1} to exert bacteriostatic effects on consortia of produced water [45]. Regarding the use of GA studies performed by Pereira et al. [46], this compound is moderately toxic to aquatic organisms in a range of 3.6 mg/L to 31.3 mg/L for the species tested, especially Zebrafish (*Danio rerio*). The same study shows that microcrustaceans are more sensitive to glutaraldehyde concentrations below 3.6 mg/L.

Meanwhile, in *Escherichia coli,* tolerance to GA increases due to the overexpression of ALDH genes involved in lipid biosynthesis when exposed to this biocide, which may contribute to biofilm resistance [47,48]. Thus far, there are glimpses of the effectiveness of several biocides used in produce water. However, due to the environment's complexity and the cells metabolism, predicting their total compromise in controlling natural populations is challenging. The formation of biofilm remains a significant limitation and focus for current treatments. In addition, the viability of microorganisms is not always affected when exposed to biocides, as they interact with the extracellular component and the environment [49].

In evaluating the GA's ability to inhibit cell growth, a dose of 50 ppm was necessary to delay the development of planktonic SRB cells in the Postgate C medium by 143-h [50]. On the other hand, the combination of GA (30 ppm) and ethylene diamine disuccinate (EDDS) (2000 ppm) delayed the stationary phase of SRB by 212-h. In contrast, the GA alone (30 ppm) is ineffective, losing the ability to inhibit the growth of sessile cells on the fifth day in batch systems [51].

Our study presents the first application of an unexplored methodology using photoinactivation with DMMB in a consortium inoculated in a medium simulating produced water required only 17.6 min to achieve over 70.58 % microbial inactivation applying 1.5 µg/mL, showing a slight percentage difference from the 2.0 µg/mL concentration, which achieved a value of 70.15 %. Additionally, this approach provided rapid results compared to other methods that typically require hours, days, weeks, or even months to control planktonic cells, often involving frequent, high-dose biocide treatments. The action of photoinactivation depends on several factors to achieve the expected result, such as the interaction between different microorganisms and the photosensitizer, its concentration and class of microorganisms, whether they are bacteria (Gram-positive or Gram-negative), fungi, or viruses. For this technique, the critical parameters for interaction include the relative solubility in water and lipids of PS, the ionization constant and light interactions characteristics, and the efficiency of triplet excited state formation and singlet oxygen production [52]. Using a light source and a photosensitizer has become an alternative technique for eradicating multiresistant microorganisms, and researchers are focusing on compounds related to effective photosensitizers that are easy to manage and act [53].

In metallic surfaces, SRB metabolism allows these metals to dissolve and remain in the environment, eroding the surface and releasing them, generating the MIC. In this way, the SRB can use the metal electrons in their electron chain to reduce sulfate. When the surface loses metal ions, there is an interaction between the surfaces in the extracellular layer since metal ions cannot readily diffuse into the SRB [5]. Some studies show that heavy metals generally compete and are replaced by essential ions used by the cell structure. This mechanism blocks some functional groups of macromolecules, causing damage to the integrity of cellular enzymes [54]. This allows a better understanding of the better uptake of the PS into the cell since DMMB is composed of zinc, thereby enabling better interaction of the PS with the surface of the membrane. This allows cell penetration, and therefore, photoinactivation would occur when light is applied, considering that the cytochrome chain provides electrons to reduce metal ions [55]. Having two methyl groups in its molecule means that the DMMB is retained in the cell more prolonged than other dyes of the same family, which increases its participation in photoinactivation. This binding shows specificity to the teichoic acid in the bacterial membrane; it also has a hydrophobic characteristic that allows a more significant cellular interaction, preferring to diffuse into the cell and resulting in chromophoric methylation [56,57].

In this study, we used a consortium previously identified by Rosario [29]. Rosario's sequencing results included ten samples: one of *Desulfovibrio dechloracetivorans*, two of *D. alaskensis*, two were identified as *Thermovirga lienii*, and five were not identified, in addition to the reference bacterium *D. vulgaris*. The genus *Desulfovibrio* is known to be composed of motile, non-spore-forming, Gram-negative bacteria. At the same time, *T. lienii* is a motile, Gram-negative motile cell isolated in a reservoir in the North Sea [58,59]. By using a Gram-negative bacterial consortium, the solubility of PS was expected to enter the cell, either by the natural capacity of the photosensitizer to interact with the membrane or by its hydrophobic capacity since the liquid medium used simulated produced water. When excited, it can generate singlet oxygen having a lifetime in water of approximately 4 μs, covering a diffusion distance of ~220 nm, and this mechanism reflects in an instantaneous action [58,60]. This diffusion time and distance are necessary for singlet oxygen production and for this reactive molecule to interact with the target.

The use of PS in the liquid medium demonstrated an interaction with the microbial consortium (Fig. 2) at higher concentrations within the parameters of this study. Conversely, at concentrations below 1.0 μg/mL, PS showed limited interaction with the cells and did not effectively reduce the consortium. In this context, increasing the PS concentration corresponded with a greater reduction in the bacterial population, even without light exposure, thereby enhancing the reduction effect through PS alone. This shows that cell reduction is a mechanism reflecting PS uptake, as reductions of 2.83 %, 3.70 %, and 9.72 % were observed at PS concentrations of 1.0 μg/mL, 1.5 μg/mL, and 2.0 μg/mL, respectively. Regarding the application of Laser light alone, no reduction in the SRB population was observed (Fig. 3), indicating that light in the red spectrum lacks bactericidal capacity. Therefore, the primary mechanism for controlling the consortium population lies in the concentrations of DMMB, with the light serving to enhance the effect via photoinactivation. Laser light is used due to its unique properties, including monochromaticity, parallel beam propagation, narrow spatial intensity, and coherence [59]. These effects and characteristics allow the light beam to be precisely directed at its target, whether dispersed cells in the medium or cells in biofilm form.

In this study, it was observed that the microbial inactivation rate occurred in all groups, specifically in those that were inoculated with a concentration equal to or greater than 1.5 μg/mL of DMMB in all light exposures, reducing by more than 50 % the cells present in the medium.

In the two-way ANOVA without replication model used to evaluate the antimicrobial reduction of the PS and Laser in PI, it was found that all combinations of light exposures and DMMB concentrations resulted in a significant reduction rate ($p < 0.05$).

The results of other studies show that biocides are focused solely on treating sessile cells, which can take days and weeks to control and reduce the biofilm layers. Using a triple combination of GA (50 ppm), EDDS (1000 ppm), and methanol (15 %), over nine days, the authors were able to entirely remove the sessile cell layer on the carbon steel surface (C108) [52]. To evaluate the biofilm reduction of *D. vulgaris* (ATCC 7757), a combination of THPS (50 ppm) was applied to the sessile cells (10^6 cells cm^{-2}) together with D-tyrosine (1 ppm). After seven days, the presence of *D. vulgaris* (10 cells cm^{-2}) was not detected, while THPS alone (50 ppm) was not effective (10^4 cells cm^{-2}) [61]. Combining THPS (30 ppm), EDDS (500 ppm), and 6.6 ppm of a mixture of D-amino acids in sessile cells (10^7 cells cm^{-2}), they were able to halve the biofilm layer (10^3 cells cm^{-2}) in a period of seven days [62].

Some studies aim to control isolated or multiple SRB populations but face difficulties in achieving significant reductions across all concentrations proposed in their investigations. However, PI proves to be effective by achieving a reduction of more than 50 % in the 1.5 μg/mL and 2.0 μg/mL groups, using all light exposures. This is evident from the two-way ANOVA with interaction analysis, which shows a significant interaction between the factors applied in the study. The *p*-values were very close to zero, with significant interactions observed between light intensities ($p < 2.67E^{-260}$), between FS concentrations ($p < 2.55E^{-254}$), and the interaction of both factors ($p < 2.95E^{-265}$) for PI in this study.

These results reflect a direct interaction between the increase in light intensity and FS concentration to achieve greater antimicrobial activity (Fig. 6). The effects of these variables are dependent on each other, exhibiting a more exponential rather than additive behaviour. This allows for a broader selection of PS and light for PI application, and this experimental model can be adapted effectively to other experimental settings. However, no significant differences were observed between the results obtained for the concentrations of 1.5 μg/mL and 2.0 μg/mL (Fig. 6). Nonetheless, both concentrations showed significant differences ($p < 0.05$) when compared to 1.0 μg/mL of DMMB. Given the relationship between photosensitizer concentrations and light intensities, higher DMMB concentrations are expected to result in greater antimicrobial effects.

Moreover, there are several common errors in the use and selection of biocides, including the lack of laboratory and field testing, choosing the first available option—often the least expensive—or selecting biocides based on contractual agreements with suppliers, budget constraints, or legal restrictions imposed by regulatory agencies [63]. A poorly selected biocide can lead to significant damage and losses for the industry, as well as harm to the environment.

According to several studies, most biocides have a rapid effect, and in order for their effect to be permanent, they require injections in batch systems and months of additions to prevent the growth of sessile and planktonic cells. These constant biocide applications may contribute to the development of natural resistance in SRB, following the same mechanism as the use of pharmaceutical drugs in medically important bacteria. However, according to a review study by Kashef et al. [64] and more recently by Rapacka et al. [65], the photoinactivation method utilizes distinct mechanisms of action, which are non-selective, multitarget, and ROS-dependent, and due to these characteristics, bacterial resistance is highly unlikely.

The PS initially acts on the extracellular molecules of the bacteria, potentially destroying their structures, particularly the cytoplasmic membrane in Gram-positive bacteria and both the cytoplasmic and outer membranes in Gram-negative bacteria, leading to cell lysis or enzyme deactivation [66]. This type of interaction may induce an accumulation of PS within the cell, which could cause toxicity and cell death [65]. This cytotoxic effect was observed when PI was applied only at concentrations higher than 1.0 μg/mL, where even isolated concentrations showed antimicrobial activity, and although the reduction did not exceed 10 %, it already demonstrated an initial inhibitory effect. This external accumulation may lead to three well-known mechanisms: (I) external action, (II) intracellular interaction (self-promoted), and (III)

active transport, all of which are effective in both Gram-positive and Gram-negative bacteria [67].

Thus, we can observe that even at concentrations lower than 1.0 μg/mL, antimicrobial activity may still occur, as an important factor is ROS production. Both singlet oxygen and reactive oxygen species can cause damage to bacterial cells [64]. This does not necessarily depend on absorption, as it only requires interaction with the bacterial extracellular molecules and excitation by a light source, which will induce the expected photochemical effects of PI.

One may question that, if photoinactivation requires the formation of ROS and samples were irradiated under anaerobic conditions how could ROS be formed? When a photosensitizer (PS) absorbs light energy, it transitions to an excited state, where it can interact with molecules in the environment and generate reactive oxygen species (ROS) independently of molecular O_2. This process can be summarized as follows: (PS) + Light → (^{1}PS*) or (^{3}PS*). In this excited state, the photosensitizer can participate in oxidation or reduction reactions [68]. There are two primary pathways for ROS generation. In Type I reactions, electron transfer to biological substrates occurs, resulting in the formation of radicals that react with oxygen, such as hydrogen peroxide (H_2O_2), superoxide anions $O^{2\cdot-}$ and hydroxyl radicals ($\cdot OH^-$) [69]. In Type II reactions, energy transfer (photons) produces singlet oxygen ($^1O_2$) [70].

Defined concentrations of reactive species like H_2O_2, $O_2^{\bullet-}$, and •OH determine various cellular responses. These responses can lead to the induction, reduction, or inhibition of growth, as well as the activation of mechanisms related to tolerance, acclimation, or defence against environmental stress [71]. It is known that anaerobic bacteria are not uniformly sensitive to oxygen, as incidental aeration frequently occurs in various habitats [72]. In facultative anaerobes, molecular oxygen can impair metabolism in several ways: by directly quenching radical-based enzymes, oxidizing low-potential enzymatic metal centers, and triggering the rapid formation of $O_2^{\bullet-}$ and H_2O_2 [73].

5. Conclusion

The results of this study indicate that photoinactivation (PI) of the SRB consortium is feasible at all tested PS concentrations and light intensities. When laser light was evaluated independently, no antimicrobial effect was observed. Conversely, at DMMB concentrations above 1.0 μg/mL, a slight reduction in microbial population was observed. Upon applying photoinactivation across all groups, antimicrobial activity was detected at all light exposures and DMMB concentrations, with a minimum reduction of 39.39 % relative to the control. In groups with DMMB concentrations above 1.5 μg/mL, the reduction exceeded 50 %, with reductions of 70.58 % and 70.15 % achieved after 17.6 min in a consortium predominantly composed of planktonic Gram-negative cells, using 1.5 μg/mL and 2.0 μg/mL of DMMB, respectively. When evaluating the interaction between both factors, higher PS concentrations and light intensities increased the PI effect on the consortium. Independently, these factors also contributed to an increased antimicrobial activity rate. Photoinactivation is known for not inducing resistance mechanisms in bacteria, as it relies on ROS production to achieve its phototoxic effects. Thus, PI is a viable alternative for SRB treatment, as it does not induce resistance within the microbial population in wells and is a safe option for aquatic biomes. Additionally, it can be used in combination with other established techniques to reduce the environmental and economic impacts associated with the oil and gas industry.

CRediT authorship contribution statement

Gustavo Vital dos Santos: Investigation. **Hesrom Fernandes Serra Moura:** Investigation. **Pedro Jorge Louro Crugeira:** Writing – review & editing, Investigation. **Anna Paula Lima Teixeira da Silva:** Investigation. **Isabele Cardoso Vieira de Castro:** Writing – original draft. **Wellington Luís Reis Costa:** Investigation. **Paulo Fernando de Almeida:** Methodology, Investigation, Conceptualization. **Antonio Luiz Barbosa Pinheiro:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2025.113103.

Data availability

Data will be made available on request.

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