Arch Biol Sci. 2021;73(3):341-351 https://doi.org/10.2298/ABS210321027N

The effects of biocides on the growth of aerophytic green algae (*Chlorella* sp.) isolated from a cave environment

Nataša Nikolić[1,*] Gordana Subakov Simić[1], Igor Golić[2] and Slađana Popović[3]

[1]*University of Belgrade, Faculty of Biology, Department of Algology, Mycology and Lichenology,* Studentski trg 16, 11000 Belgrade, Serbia

[2]*University of Belgrade, Faculty of Biology, Center for Electron Microscopy,* Studentski trg 16, 11000 Belgrade, Serbia

[3]*University of Belgrade, Institute of Chemistry, Technology and Metallurgy, National Institute of the Republic of Serbia, Center of Ecology and Technoeconomics,* Njegoševa 12, 11000 Belgrade, Serbia

***Corresponding author**: nikolic.natasa@outlook.com

Received: March 21, 2021; **Revised**: May 27, 2021; **Accepted**: May 28, 2021; **Published online**: June 22, 2021

Abstract: Lampenflora communities of bacteria, cyanobacteria, algae, mosses and lichens colonize illuminated show-caves as a consequence of disturbances to the caves' ecological equilibrium. These communities have unesthetic impacts and can cause the biodeterioration of limestone. A 15% hydrogen peroxide solution was proposed for use as an ecological agent for the safe removal of these microorganisms. This study tested the effects of three different biocides (hydrogen peroxide, a commercial solution containing salicylic acid and a product containing chlorine dioxide as the active component), on the growth of green algae (*Chlorella* sp.), which are frequently encountered in lampenflora communities. *Chlorella* sp. was treated with the biocides under laboratory condition, and chlorophyll a (Chl *a*) concentrations were measured 1 h, 3 days, 7 days and 10 days after treatment. The change in Chl *a* concentration was compared to the untreated control group at each time point. All three biocides prevented the growth of green algae and the product containing chlorine dioxide appeared to be the most effective growth inhibitor. Hydrogen peroxide is known to suppress lampenflora growth in caves, and further studies on other biocides remain necessary to identify a solution that is both ecologically safe and economically feasible.

Keywords: biofilms; biocides; *Chlorella* sp.; green algae; lampenflora

INTRODUCTION

Caves have served humans as temporary shelters, permanent shelters, sanctuaries and burial grounds since ancient times [1]. In the last 400 years, caves have also become tourist attractions due to their remarkable geological features, beautiful structures and ornaments, streams or waterfalls, or prehistoric drawings [1-3]. Currently, many caves are recognized as geoheritage sites and are protected. However, several of these sites have been adapted for tourists as tourism continues to increase [1].

The deep cave environment is primarily characterized as an oligotrophic habitat, featuring stable temperatures and relative humidity and limited contact with the exterior environment throughout the year [4-9]. This equilibrium can be disrupted and changed when additional energy is introduced to the cave from the outside environment [6]. Caves can be classified into three energetic categories [10]: high-energy caves in which the natural energy flux of the caves outweighs the energy introduced, resulting in no change to the cave environment; moderate-energy caves in which the introduced energy is similar to the natural energy flux of the caves and can influence the cave environment, quickly returning to equilibrium when the outside influence is withdrawn; low-energy caves in which the introduced energy exceeds the natural energy flux of the cave, significantly altering the environment inside the cave. For example, a person with an average temperature of 37°C who walks through a cave emits as much energy as a 200 W light bulb [6]; therefore, a group of 50 to 60 people walking through a cave can raise the ambient temperature by 1 to 2°C

[11]. Such an influence of tourists has been observed in the Altamira Cave in Spain where the estimated effects of tourism on temperature, CO_2 concentration and relative humidity were measured. The presence of tourists increased the temperature in the cave by 2°C, increased the CO_2 concentration from 400 ppm to 1200 ppm and decreased the relative humidity from 90% to 75% [12]. Furthermore, the adaptations made in caves to accommodate tourists, including the construction of pathways and platforms and the introduction of artificial light, can also alter naturally stable temperature and relative humidity [13,14]. The clothes and shoes of tourists can transport spores (which can also be deposited in caves by air, animals, or water), and hair, dry skin and dust can disrupt cave equilibrium and increase the amount of organic matter [6].

All these disturbances and changes can influence the development of a microorganism communities called lampenflora. These communities grow near artificial light as the primary factor for their development. Lampenflora include phototrophic microorganisms (cyanobacteria and algae) [6,14-16], and mosses, lichens and even higher-order plants [14,17]. The most common phototrophs belong to cyanobacteria, Chlorophyta, and Bacillariophyta, but others, such as Xanthophyta, may also be found [14]. Although cyanobacteria are highly adaptable to extreme environmental conditions, they can be easily outcompeted by eukaryotic algae in stable environments, such as those found in caves [14]. Nikolić et al. [8] have reported the presence of Cyanobacteria, Chlorophyta, Bacillariophyta and Xanthophyta in the Podpeć and Stopić caves. While cyanobacteria were dominant at cave entrances, *Chlorella* sp. was recorded in every lampenflora sample [8]. As in other biofilms, phototrophs in lampenflora successfully coexist with bacteria and fungi [3,7]. This community of microorganisms can have negative esthetic impacts (turning the ornaments and structures of caves green, brown or black), and can cause the biodeterioration of rock surfaces. During respiratory processes, microorganisms release CO_2, which can combine with nearby water to produce carbonic acid strong enough to damage the structures in caves [18-20]. Lampenflora may also contain not only bacteria that can cause respiratory or skin infections, but also other microorganisms whose effects on humans have not yet been determined [3,21].

Physical and chemical methods have been applied to remove lampenflora, and recently UV-C radiation was tested under experimental conditions [13,19,22,23]. Physical methods for lampenflora removal generally involve cleaning the speleothems with water and a brush, which can be efficient. However, physical removal can also aggravate the problem by spreading microorganisms to the surrounding areas and causing additional damage to the substrate [13,24,25].

Chemicals such as the herbicides diuron, N-3, 4-dichlorophenyl-N9-dimetil urea and atrazine 6-Chloro-N-ethyl-N9-(1methylethyl)-1,3,5-triazine-2,4-diaminehave been used to prevent lampenflora development; however, these methods have been insufficiently successful [13,26]. Although sodium hypochlorite (NaOCl) has been demonstrated to be successful against lampenflora, it can exert environmental pressure by releasing chlorine gas, lowering the pH and eroding calcite [27,28]. A 15% solution of hydrogen peroxide (H_2O_2) is sufficient to destroy lampenflora without causing great harm to the environment [26,28,29]. However, without regular H_2O_2 applications, lampenflora can develop again [27].

UV-C irradiation is a suitable method for limiting algae proliferation because it can damage both their photosynthetic apparatus and DNA [30,31]. UV-C treatments bleach the biofilm by degrading chlorophyll; consequently, the area colonized by phototrophs decreases over time [30,32]. The relationship between UV-C exposure time and physiological response has been studied, and UV-C treatment stronger than 10 kJ m^{-2} was sufficient to kill microorganisms, while low UV-C treatments only inhibited their respiration and photosynthesis [32]. Questions have been raised [31] as to whether UV-C irradiation damages prehistoric drawings, but this type of treatment has been considered to be completely safe [33].

Although several methods have been reported to be capable of eliminating lampenflora, none are ideal, and it is necessary to identify optimal solutions to eliminate lampenflora while protecting the cave environment. Because green algae are quite common in the lampenflora community and cause negative esthetic impacts and biodeterioration, this study explored the effects of different biocides on the green

algae that were most widespread in the cave we studied. The selected biocides were tested under laboratory conditions on cultures of *Chlorella* sp. isolated from lampenflora.

MATERIALS AND METHODS

Locality

Biofilm for algal cultivation was collected in Lazar's Cave (also known as Zlot's Cave), which is located in eastern Serbia (44˚ 01' 44.07" N; 21˚ 57' 44.54" E). The cave has formed in stratified Lower Cretaceous limestone [34]. Hydrologically, Lazar's Cave contains three canal systems: an old dry system, a periodically active system and an active, flooded system [34]. One section of the cave to which paths and artificial light have been added is open to tourists from May to October [35]. The cave structures affected by lampenflora were characterized by the presence of green or dark green biofilms of varying thicknesses.

Sampling

A biofilm from one sampling site was chosen for cultivation, and algal material was directly scraped using a sterile scalpel into test tubes containing solid growth media [36]. From the same sampling site, a small piece of biofilm was taken and observed using a Zeiss Axio Imager M1 light microscope with Axio Vision 4.8 software. The green biofilm was examined at maximum magnification of ×640, and the lampenflora-dominant organism (representative for cultivation) was identified using standard identification keys [37,38].

Preparation of medium for algal cultivation

Before collecting any samples for algal cultivation, BG11 medium was prepared [39]. The pH of 1 L of BG-11 medium was adjusted to a pH approximately 7.5. The medium was then transferred to test tubes and Erlenmeyer flasks to which agar at a concentration of 1.3% to 1.5% was added to solidify the BG-11 medium. After sealing the glassware with plugs made of cotton wool and covering them with aluminum foil, the medium was autoclaved for 15 min at 121°C. After autoclaving, the test tubes were allowed to cool in a slanted position that solidified the agar. The medium in the Erlenmeyer flasks was allowed to cool slightly before pouring into sterile 5.1-cm Petri dishes to solidify. The test tubes containing solid BG11 medium were used for collection of aerophytic algae from the sampling site. Petri dishes with solid BG11 medium were used to maintain algal cultures in the laboratory for experimentation.

Algal cultivation and biocide treatment

Test tubes with the lampenflora-dominant organisms were maintained between 20°C and 22°C, a temperature range found to be optimal for algae growth [40], under a 12 h light:dark cycle. After the algae developed, small samples were transferred from the test tubes to the Petri dishes. The green algae were carefully smeared on the surface of the solid BG11 medium in the Petri dishes and left to grow evenly for 6 weeks. Three biocides, hydrogen peroxide, a commercial solution containing salicylic acid and a product containing chlorine dioxide as the active component, were applied in different volumes and/or concentrations, and their effects on the algae were observed using three replicates for each treatment. The diameter of the Petri dishes was 5.1 cm. The left half of each dish served as the control, while the right half was treated with a biocide (Supplementary Fig. S1). Control biofilms were collected, their chlorophyll *a* (Chl *a*) concentration determined and the solid medium from the control sides of the Petri dishes removed from each dish. Then, the other halves of the Petri dishes were treated with biocides. The liquid biocides were gradually poured from the outside to the inside of the remaining cultured medium in each Petri dish with a 100-1000 µL micropipette for even application and to avoid spreading the biocides over the rest of the dish. The effect of biocide application on the algae was assessed by calculating the percentage reduction in Chl *a* concentration relative to the control after each treatment period.

To determine Chl *a* concentration, the green algae was carefully scraped from each Petri dish with a sterile scalpel, taking care not to lift the solid medium. The samples were then transferred to sterile glass beakers and weighed. Next, 20 mL of boiling 100% ethanol was added. The samples were vigorously homogenized in ethanol for 5 min to extract

chlorophyll and were then filtered. After filtration, the absorbance of the filtrates was measured at 665 nm and 750 nm both before and after acidification. The Chl *a* concentration was calculated based on the modified formula [41]:

$$\mu g\ Chl\ a/\ cm^2 = (A\text{-}Aa)/Kc \times R/(R\text{-}1) \times (10^3 V_e)/(V_s d)$$

$A=A_{665}-A_{750}$ is the absorbance of the extract before acidification; $Aa=A_{665}-A_{750}$ is the absorbance of extract after acidification; V_e is the volume of the extract (mL); Vs is the volume of the filtered sample (ml); Kc=82 L/μg cm is the specific operational spectral absorption coefficient for chlorophyll *a*; d is the path length of the optical cell (cm); R=A/Aa is the ratio A/Aa for a solution of pure chlorophyll *a* that is transferred to pheophytin by acidification; 10^3 is the dimensional factor to fit Ve.

Hydrogen peroxide experiment

Algae cultures were treated with 60 μL (V1) and 120 μL (V2) of a 15% solution of hydrogen peroxide (H_2O_2), which has been suggested to be effective for algal elimination while remaining safe for the substratum [13]. The effects of 15% H_2O_2 were observed 1 h, 3 days, 7 days and 10 days after application. To measure the effect of H_2O_2 after 3 and 7 days, cultures were treated every day until the 3rd and 7th days; to assess the effect after 10 days, the cultures were treated every other day until the 10th day. After the treatments were completed, the percentage change in Chl *a* concentration per each sample was calculated relative to the control.

Commercial algaecide experiment

Algae cultures were treated with 250 μL of commercial algaecide containing 2 mg/g salicylic acid at a concentration of 0.1 mL/L. The effects of this algaecide were observed after 1 h, 3 days, 7 days and 10 days as described for H_2O_2.

Chlorine dioxide experiment

Algae cultures were treated with 250 μL of chlorine dioxide solution at concentrations of 0.1, 0.05 and 0.01 mL/L. The product containing chlorine dioxide consisted of two components: component A, containing sodium chlorite and additives, and component B, containing sodium bisulfate and additives. Components A and B were added to 1 L of distilled water to produce a 0.4% chlorine dioxide solution, which was then diluted further to create solutions of the selected test concentrations. The effects of this solution were observed after 1 h, 3 days, 7 days and 10 days as described for H_2O_2.

CLSM method

The effects of the chlorine dioxide treatment on green algae cultures were also examined using confocal laser microscopy (CLSM). Microscope observations of biocide effectiveness were made after 1 h, 3 days, 7 days and 10 days. Random 5×5 mm sections of biofilm were excised and mounted on glass slides with Mowiol mounting medium, taking care to place the biofilms onto the coverslips. Confocal images were acquired on a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany), using a 63×/1.4 oil-immersion objective. Autofluorescence was performed with a 543 nm HeNe laser and emission wavelengths of 590 to 790 nm. Images were acquired at 1024×1024 pixels with a scanning speed of 400 Hz.

Statistical analysis

To assess whether significant differences exist among the applied biocides and to determine the effects of biocide application duration, one-way analysis of variance (ANOVA) was performed using the statistical package XLSTAT [42]. Principal component analysis (PCA) was used to illustrate the relationship between the applied biocides (response variables) and time (supplementary variables), using Canoco 5 [43].

RESULTS

The preliminary lampenflora investigation revealed the dominance of green coccal algae (Chlorophyta), and the identification keys [37,38] confirmed the presence of *Chlorella* sp. The genus *Chlorella* belongs to the phylum Chlorophyta. Its cells are spherical, (Supplementary Fig. S2), subspherical or ellipsoid and solitary or forming clusters [37]. The chloroplast is usually single, parietal, cup-, band- or trough-shaped and

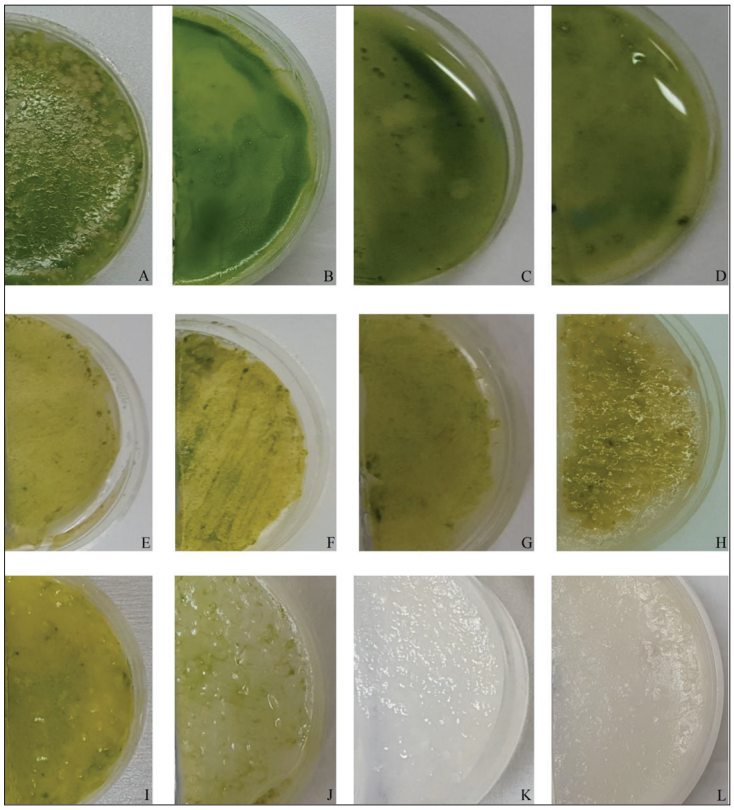

Fig. 1. Effect of treatment with biocides on *Chlorella* sp.: algaecide based on salicylic acid 250 µL (**A** – 1 h, **B** – 3 days, **C** – 7 days, **D** – 10 days); hydrogen peroxide 60 µL (**E** – 1 h, **F** – 3 days, **G** – 7 days, **H** – 10 days), hydrogen peroxide 120 µL (**I** – 1 h, **J** – 3 days, K – 7 days, **L** – 10 days).

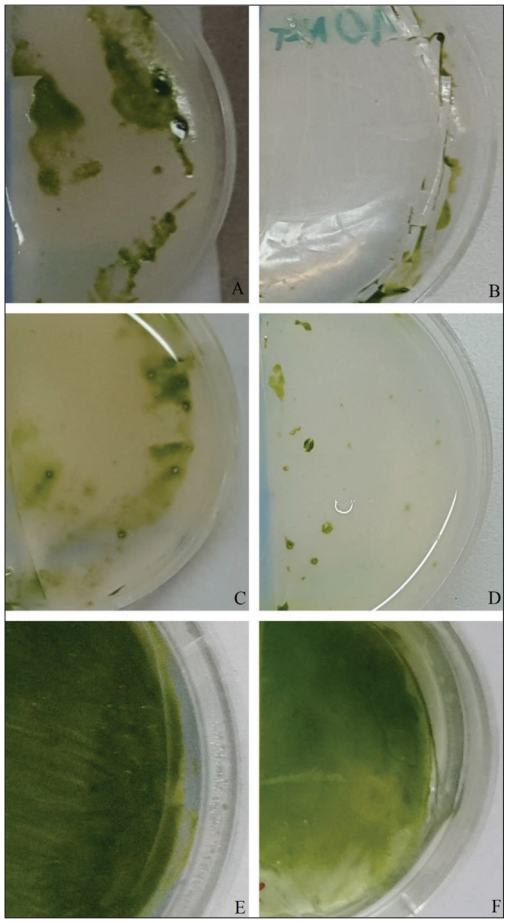

Fig. 2. Effect of treatment with different concentrations of chlorine dioxide on the *Chlorella* sp.: 0.1 mL/L (**A** – 3 days, **B** – 10 days); 0.05 mL/L (**C** – 3 days, **D** – 10 days); and 0.01 mL/L (**E** – 3 days, F – 7 days).

also pyrenoid. Reproduction is asexual by autospores released via disruption of the wall of the mother cell [38]. This genus is widely distributed, being present in freshwater, marine water, soil and subaerial environments [37,38]. *Chlorella* sp. was dominant in the sampled biofilm and was suitable for cultivation in the laboratory (Supplementary Fig. S2).

Both volumes of H_2O_2 treatment affected *Chlorella* sp. growth (Fig. 1, Fig. 4). The effect appeared to be gradual; after 10 days, the V1 reduced the concentration of Chl *a* by 86.3% compared with that of the control, whereas after 7 days, *Chlorella* sp. was eliminated by the V2 treatment. Unexpectedly, V1 was more effective than V2 at eliminating green algae after 1 h. An expected trend was observed for V2 in which algal elimination increased with applications performed over longer periods. After 1 h, a small biocidal effect was observed (the Chl *a* concentration decreased by 21.5%, compared to the control). After 3 days, Chl *a* was bleached and almost all algae were eliminated, and after 7 days there was insufficient biomass present to determine Chl *a* concentration (Figs. 1 and 4).

The commercial algaecide based on salicylic acid (Fig. 1) had a medium effect on *Chlorella* sp. growth. After 10 days, the Chl *a* concentration was reduced by 60.4%, after 7 days by 62.2%, after 3 days by 38.3%, and after 1 h by 29.3% compared to the control (Fig. 4).

The results of the chlorine dioxide solution treatment are presented in Figs. 2 and 4. After 1 h, the 0.1 mL/L solution reduced the Chl *a* concentration by 73.6% compared to the control. After 3 days of treatment, the concentration of Chl *a* was reduced by 86.3%, and after 7 days, complete visual bleaching of Chl *a* was achieved. The 0.05 mL/L solution showed that after 1 h the Chl *a* concentration was reduced by 43.3%, and after 3 days by 86.9%. All existing biofilm

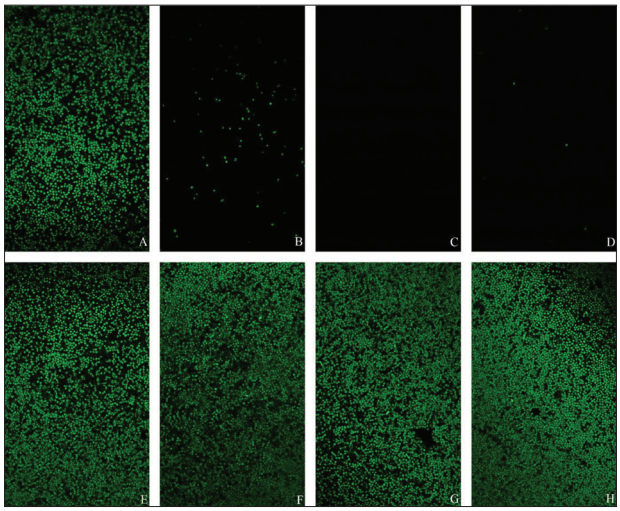

Fig. 3. Culture of *Chlorella* sp. after treatments with different concentrations of chlorine dioxide observed by confocal laser microscopy (CLSM): 0.1 mL/L (**A** – 1 h, **B** – 3 days, **C** – 7 days, **D** - 10 days); 0.01 mL/L (**E** – 1 h, **F** – 3 days, **G** – 7 days, **H** – 10 days).

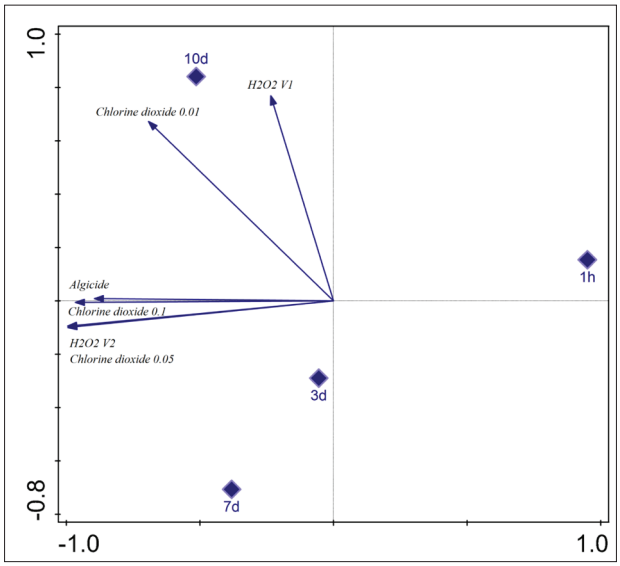

Fig. 5. PCA showing the relationship between applied biocides (vectors) and time (squares).

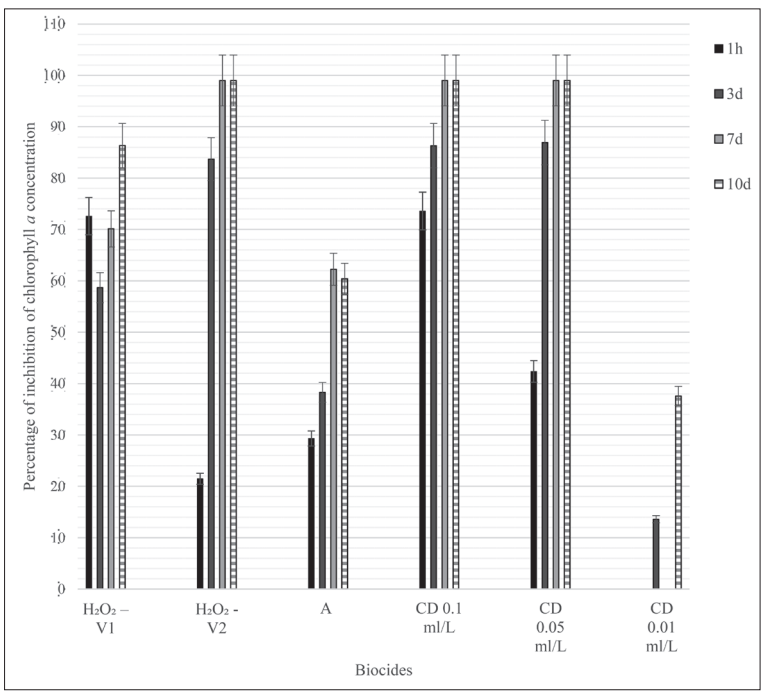

Fig. 4. Effect of biocides on *Chlorella* sp. culture expressed as percentages of inhibition of chlorophyll *a* concentration after 1 h (1h), 3 days (3d), 7 days (7d) and 10 days (10d); biocides: H_2O_2 – hydrogen peroxide (V1 – 60 µL, V2 – 120 µL); A – algaecide based on salicylic acid (250 µL); CD – chlorine dioxide (0.01 mL/L, 0.05 mL/L, 0.1 mL/L).

on the solid medium was eliminated after 7 days of treatment. The 0.01 mL/L chlorine dioxide solution had no significant impact on the *Chlorella* sp. culture, although the strongest effect of this treatment was observed after 10 days. As shown in the CLSM images (Fig. 3), the 0.1 mL/L and 0.01ml/L chlorine dioxide treatments produced different results. The efficacy of the higher concentration could be observed even after 3 days, and after 7 days this concentration caused a bleaching effect so complete that autofluorescence could not be observed. The 0.01 mL/L solution was unstable, and maximum treatment efficacy could not be determined. Additionally, the 0.01 mL/L solution was not equally effective over the whole surface of the Petri dish, therefore the imaging results varied depending to the part of the biofilm that was used for the CLSM observations.

According to one-way ANOVA, the effect of time (duration of biocide application) was significant (P=0.034). The difference in effect between the applied biocide and time was also significant (P=0.005). PCA demonstrated a relationship between the applied biocides (shown as vectors on the ordination diagram) and time (squares). All biocides (Fig. 5) are oriented toward the left side of the ordination diagram and were

predominantly positively correlated with supplementary variables representing their application over longer periods.

DISCUSSION

Biodeterioration processes are undesirable changes that affect cultural and natural heritage sites such as caves. H_2O_2 is a well-known oxidizing agent that is environmentally friendly and does not release harmful byproducts in reactions with organic matter [13,28]. During the oxidation process, oxygen atoms in H_2O_2 attract electrons from the living organism, damaging or breaking the cell membrane [44]. Previous research has suggested that although H_2O_2 has a toxic effect on living organisms in the dark, its effect is magnified in the presence of light, which also affects its mobility through cell membranes and its activity against sensitive organelles, such as chloroplasts [45,46]. H_2O_2 was successful in eliminating cultured lampenflora green algae, an effect that improved with repeated treatments over time. Similar results [28] indicate that a 15% solution of H_2O_2 applied three times over 2 or 3 weeks was sufficient to eliminate the cyanobacteria, algae and mosses that compose lampenflora communities. This experiment found that a treatment volume of 120 µL was more effective than 60 µL, eliminating more than 99% of the biomass after 7 and 10 days because a higher volume H_2O_2 allows for contact with larger numbers of living algal cells. According to several authors [45,47], each species is sensitive to a specific dose of H_2O_2, which can be determined under laboratory conditions. However, microorganism communities are composed of different species and genera; therefore, a single dose could be effective for one species, but not for the others, and some higher doses could negatively affect the environmental equilibria of caves [48]. Gradual treatment with a 15% solution of H_2O_2 has been suggested because it is strong enough to damage the cell membrane or disrupt enzyme activities without negatively affecting the environment [28]. Although treatment with a 15% solution of H_2O_2 eliminates lampenflora, its frequent use can damage carbonate substrates, even more than karst water [28], and it was suggested [26] to wash the substrate 5-30 min after H_2O_2 application. Additionally, it was shown [29] that a 15% H_2O_2 solution is insufficient to eliminate lampenflora (especially mature communities), but a combination of chemical treatment, mechanical cleaning with water and light modification can be very effective.

The commercial algaecide containing salicylic acid was designed to suppress the growth of green algae in freshwater aquariums, and this experiment is the first application of this product to aerophytic green algae originating from lampenflora. The recommended concentration for the treatment in freshwater aquariums is 0.1 mL/L, which was applied. The active component of this algaecide is salicylic acid [49], a natural phenolic compound derived from white willow (*Salix alba* L.), which affects growth, photosynthesis and various enzymatic activities [50,51]. Interestingly, salicylic acid has a positive effect on plants, stimulating their growth, development and sugar production; however, certain doses can produce antibacterial and antifungal effects [51,52]. While this solution was somewhat effective in eliminating *Chlorella* sp., it was less effective than the other biocides tested. After 10 days of treatment, the concentration of Chl *a* had decreased by 60.4% compared with the control, and the best results were achieved after 7 days of treatment. This may result from the daily application of the biocide in 7-day treatments, whereas biocides in 10-day treatments were applied every other day. Additionally, although the treated algae were cultivated on a solid medium, each application of algaecide increased the quantity of liquid in the Petri dishes, which increased the difficulty of scraping the biofilm and may have affected Chl *a* concentrations. The cultured algae in almost every Petri dish were dissolved by the addition of algaecide and liquid was present, which could also have affected the values of Chl *a* obtained after 7 or 10 days of treatment. Applying a 10^{-4} M solution of salicylic acid can stimulate the growth of *Chlorella* strains, including increasing the primary production of sugars and lipids [53], whereas high concentrations of salicylic acid (>10 mg/L) might have inhibitory effects [54,55]. It was demonstrated [55] that applying a 10 mg/L solution of salicylic acid increased lipid production, whereas a double dose had the opposite effect. In contrast to these studies, the algaecide had an inhibitory effect in our experiments, preventing algae growth even when only a low concentration was applied. Salicylic acid can induce stress, increasing the toxicity of ions in algal cells [56]. Thus, although the algaecide based on salicylic acid inhibited the growth of green algae in

laboratory conditions; determining whether it would have the same effect in caves requires further study, especially because its effects on different cave substrates are unknown.

Chlorine dioxide at appropriate concentrations has been shown to have lethal effects on the green algae culture; chlorine dioxide damages the cell membrane and chloroplasts, leading to chlorophyll bleaching and the death of phototrophic organisms. It was suggested [57] that the effectiveness of chlorine dioxide is due to its adsorption by the cell wall, where chlorine dioxide reacts with enzymes containing sulfhydryl groups. In contrast, it was shown [58] that chlorine dioxide inhibits protein synthesis. Although both experiments were performed in bacterial cells, chlorine dioxide is likely to interact with phototrophic microorganisms similarly. Two of the tested concentrations of chlorine dioxide (0.1 mL/ L and 0.05 ml/L) were successful for the elimination of *Chlorella* sp. (Fig. 4), decreasing the Chl *a* concentration by more than 99% compared with the control after 7 days. The 0.01 mL/L chlorine dioxide concentration was unstable and did not satisfactorily suppress green algae growth, although it resulted in a 37% decrease in Chl *a* concentration as compared to the control after 10 days. The 0.1 mL/L chlorine dioxide solution was very efficient but perhaps too strong for application in cave environments as an initial treatment choice. Further study of the 0.05 mL/L chlorine dioxide solution in laboratory conditions as well as in cave environments is necessary. The effects of chlorine dioxide depend on the solution concentration, the presence of organic matter, pH and temperature. Depending on the chlorine dioxide concentration and the length of exposure, a 7-30% reduction of microorganisms can occur within 1 h [58]. It was found [59] that a 0.25 mg/L solution of chlorine dioxide inactivated a strain of *E. coli* in less than 60 s at pH 6.5. The same concentration, under the same conditions, inactivated a strain of *E. coli* in 25 s at pH 8.5 [58]. Although chlorine dioxide is effective against microorganisms at certain concentrations, temperatures and pH values, the effects of chlorine dioxide on the environment, different substrates and different groups of microorganisms remain unclear. Although chlorine dioxide has been demonstrated to be effective under laboratory conditions, caves are sensitive systems and must be treated carefully to avoid unintentional damage.

Caves, as unique geological features, should be preserved and maintained without lampenflora. Thus, preventative measures against lampenflora development need to be considered, such as reducing light intensity, shortening the light exposure time and removing all contamination that supports lampenflora growth [60]: the use of LED lamps is also advised [60]. However, if lampenflora develops, a method for its removal should be applied, and the locations from which it has been removed should be monitored for several years [60]. A safe agent for the removal of lampenflora should be identified. Such an agent should be safe for the cave environments, the organisms that live in caves, as well as humans, and optimally it should also prevent the reappearance of lampenflora long-term.

CONCLUSIONS

Three biocides, hydrogen peroxide, a commercial algaecide based on salicylic acid and developed for use on freshwater green algae, and chlorine dioxide, were examined for their effects on the growth of green algae (*Chlorella* sp.) under laboratory conditions. Different volumes and/or concentrations of each biocide were tested, and the concentration of Chl *a* was measured at 1 h, 3 days, 7 days and 10 days after treatment. Both volumes of H_2O_2 controlled *Chlorella* sp. growth, but a better trend was observed with the larger volume, which almost fully bleached the algae after 3 days. The algaecide based on salicylic acid showed an intermediate effect against algae, reducing the concentration of Chl *a* by 60% after 10 days. Chlorine dioxide solutions were most effective against *Chlorella* sp. growth. The highest concentration of chlorine dioxide (0.1 mL/L) reduced the Chl *a* concentration by 73.6% after only 1 h and completely bleached of Chl *a* after 7 days. The medium concentration of chlorine dioxide (0.05 mL/L) was also effective against algal growth, however, the lowest concentration (0.01 mL/L) was unstable and ineffective. The obvious difference between the lowest and highest concentrations of chlorine dioxide used was further demonstrated using CLSM. While H_2O_2 has been used for the removal of lampenflora in caves, the other tested biocides have never been used in cave environments. Further studies are necessary to evaluate the effects of the tested biocides on cave limestone in laboratory conditions, after which studies in the cave environments should be conducted.

Funding: This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia Grant No. 451-03-9/2021-14/200026, Grant No. 451-03-9/2021-14/ 200178.

Acknowledgements: The authors want to thank family and friends for their support.

Author contributions: All authors performed the investigation, analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

REFERENCES

1. Cigna A, Forti P. Caves: The most important geotouristic feature in the world. Tourism Karst Areas. 2013;6:9-26.
2. Bastian F, Alabouvette C. Lights and shadows on the conservation of a rock art cave: The case of Lascaux Cave. Int J Speleol. 2009;38(1):55-60. https://doi.org/10.5038/1827-806x.38.1.6
3. Jimenez SC. Microbiological and environmental issues in show caves. World J Microbiol Biotechnol. 2012;28:2453-64.
4. Hajdu L. The Flora of Hungarian Caves. Karszt és Barlang. 1977;Spec.Issue:39-42.
5. Abdulin ShR. Effect of Illumination on the Distribution of Phototrophic Organisms in the Entrance Part of the Shul'ganTash Cave. RussJ Eco. 2011;42:249-51.
6. Cigna AA. Tourism and show caves. Zeitschrift für Geomorphologie. 2016;60(2):217-33. https://doi.org/10.1127/zfg_suppl/2016/00305
7. Popović S, Subakov Simić G, Stupar M, Unković N, Krunić O, Savić Ž, Ljaljević Grbić M. Cave biofilms: characterization of phototrophic cyanobacteria and algae and chemotrophic fungi from three caves in Serbia. J C K Studies. 2017;79(1):10-23. https://doi.org/10.4311/2016mb0124
8. Nikolić N, Zarubica N, Gavrilović B, Predojević D, Trbojević I, Subakov Simić G, Popović S. Lampenflora and the entrance biofilm in two show caves: comparison of microbial community, environmental, and biofilm parameters. JCK Studies. 2020;82(2):69-81. https://doi.org/10.4311/2018ex0124
9. Rugnini L, Migliore G, Tasso F, Ellwood NTW, Sprocati AR, Bruno L. Biocidal Activity of Phyto-Derivative Products Used on Phototrophic Biofilms Growing on Stone Surfaces of the Domus Aurea in Rome (Italy). Appl Sci. 2020;10(18):6584. https://doi.org/10.3390/app10186584
10. Heaton T. Caves, a tremendous range in energy environments on Earth. Nat Speleol Society News. 1986;44:301-4.
11. Gillieson SD. Management of caves. In: van Beynen EP, editor. Karst Management. London: Springer; 2011. p. 141-58.
12. De Freitas CR, Littlejohn RN. Cave climate: assessment of heat and moisture exchange. Int J Climatol. 1987;7(6):553-69. https://doi.org/10.1002/joc.3370070604
13. Mulec J, Kosi G. Lampenflora algae and methods of growth control. JCK Studies. 2009;71(2):109-15.
14. Mulec J, Kobešova S. Diversity of Bryophytes in show caves in Slovenia and relation to light intensities. Acta Carsologica. 2010;39(3):587-96. https://doi.org/10.3986/ac.v39i3.86
15. Dobat K. Flore de la lumiéreartificiélle (lampenflora-maladieverte). In: Juberthie C, Decu V, editors. Encyclopaedia Biospeleologica, Tome 2. Moulis-Bucarest: Société de Biospéologie; 1998. p. 1325-35.
16. Mazina SE, Severin AV. Development of a method for rehabilitation of anthropogenically transformed underground ecosystems: The example of the New Athos Cave. Ekol Khim. 2007;16:175-81.
17. Cigna A. The problem of Lampenflora in Show Caves. In: Bella P, Gazík P, editors. Proceedings of the 6th ISCA Congress. Slovakia: Slovak Caves Administration; 2011. p. 201-05.
18. Brunet J, Vidal P, Vouvé J. Concervation de l'Art Rupestre. Paris:UNESCO; 1985. p 107.
    Macedo FM, Miller ZA, Dionísio A, Saiz-Jimenez C. Biodiversity of cyanobacteria and green algae on monument in the Mediterranean Basin: an overview. Microbiology. 2009;155(11):3476-90.
19. https://doi.org/10.1099/mic.0.032508-0
20. Pfendler S, Munch T, Bousta F, Alaoui-Sosse L, Aleya L, Alaoui-Sosse B. Bleaching of biofilm-forming algae induced by UV-C treatment: a preliminary study on chlorophyll degradation and its optimization for an application on cultural heritage. Env Sci Poll Res. 2018;25:14097-105. https://doi.org/10.1007/s11356-018-1654-6
21. Jurado V, Laiz L, Rodriguez-Nava V, Boiron P, Hermosin B, Sanchez-Moral S, Saiz-Jimenez C. Pathogenic and opportunistic microorganisms in caves. Int J Speleol. 2010;39(1):15-24. https://doi.org/10.5038/1827-806x.39.1.2
22. Urzi C, De Leo F. Evaluation of the efficiency of water-repellent and biocide compounds against microbial colonization of mortars. Int Biodeterior Biodegradation. 2007;60(1):25-34. https://doi.org/10.1016/j.ibiod.2006.11.003
23. Bromblet P. Guide "Guide Altération de la Pierre". Association Medistone; 2010. 32 p.
24. Ash J, Ashton K, Bonny A, Dimond P, Hendy C, May B, Nelson C, Wheele D, Williams P. Report on the conservation of Waitomo caves. N Zealand Speleol Bull. 1975;5:373-96.
25. Hazslinszky T. Übersicht der Lampenflorabekämpfung in Ungarn. In: Hazslinszky T, editor. International Conference on Cave Lighting. Budapest: Hungarian Speleological Society; 2002. p. 41-50.
26. Grobbelaar JU. Lithophytic algae: A major threat to the karst formation of show caves. J Appl Phycol. 2000;12(3):309-15.
27. Zelinka J, Hebelka J, Fillo M, Novomesky J. Illumination reconstruction in Slovakian show caves in relation to "lampflora" creation prevention. In: Hazslinszky T, editor. International Conference on Cave Lighting. Budapest: Hungarian Speleological Society; 2002. p. 151-57.
    Faimon J, Stelcl J, Kubesova S, Zimåk J. Environmentally acceptable effect of hydrogen peroxide on cave "lampflora", calcite speleothems and limestones. Enviro Pollution. 2003;122(3):417-22. https://doi.org/10.1016/s0269-7491(02)00309-3

28. Trinh DA, Trinh QH, Tran N, Guinea JG, Mattey D. Eco-friendly remediation of lampenflora on speleothems in tropical karst caves. J C K Studies. 2018;80(1):1–12. https://doi.org/10.4311/2017es0101
29. Borderie F, Laurence A, Naoufal R, Faisl B, Geneviève B. UV-C irradiation as a tool to eradicate algae in caves. Int Biodeterior Biodegradat. 2011;65:579-84. https://doi.org/10.1016/j.ibiod.2011.02.005
30. Borderie F, Tête N, Cailhol D, Alaoui-Sehmer L, Bousta F, Rieffel D, Aleya L, Alaoui-Sossé B. Factors driving epilithic algal colonization in show caves and new insights into combating biofilm development with UV-C treatments. Sci Total Enviro. 2014;484:43-52. https://doi.org/10.1016/j.scitotenv.2014.03.043
31. Pfendler S, Einhorn O, Karimi B, Bousta F, Caihol D, Alaoui-Sossé L, Alaoui-Sossé B, Aleya L. UV-C as an efficient means to combat biofilm formation in show caves: evidence from the La Glacière Cave (France) and laboratory experiments. Environ Sci Pollut Res. 2017;24(31):24611-23. https://doi.org/10.1007/s11356-017-0143-7
32. Pfendler S, Alaoui-Soussé B, Alaoui-Soussé L, Bousta F, Aleya L. Effects of UV-C radiation on Chlorella vulgaris, a biofilm-forming alga. J Applie Phycol. 2018;30:1607-16.
33. https://doi.org/10.1007/s10811-017-1380-3
34. Lazarević R. Kras Dubašnice, Gornjana i Majdanpeka. Beograd: Srpsko geografsko društvo; 1998. 100 p. Serbian.
35. Nikolić N, Popović S, Vidaković D, Subakov Simić G, Krizmanić J. Genus Humidophila from caves in Serbia with an improved detailed description of rare H. brekkaensoides. Arch Biol Sci. 2020;72(2):279-89. https://doi.org/10.2298/abs200228022n
36. Gärtner G, Stoyneva PM, Mancheva DA, Uzunov AB. A new method in collection and cultivation of aerophytic and endolithic algae. Ber Nat Med Verein Innsbruck. 2010;96:27-34.
37. Ettl H, Gärtner G. Syllabus der Boden-, Luft-, und Flechtenalgen. 2nd ed. Berlin: Springer Spectrum; 2014. 773 p. https://doi.org/10.1007/978-3-642-39462-1
38. John DM, Whitton BA, Brook AJ. The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae. Cambridge, UK: Cambridge University Press; 2003. 702 p. https://doi.org/10.1002/aqc.579
39. Rippka R, Deruelles J, Waterbury J, Herdman M, Stanier R. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiology. 1979;111(1):1-61. https://doi.org/10.1099/00221287-111-1-1
40. Aleya L, Dauta A, Reynolds SC. Endogenous regulation of the growth-rate responses of a spring-dwelling strain of freshwater alga, Chlorella minutissima, to light and temperature. Eur J Protistol. 2011;47:239-44. https://doi.org/10.1016/j.ejop.2011.05.003
41. Popović S, Subakov Simić G, Stupar M, Unković N, Predojević D, Jovanović J, Ljaljević Grbić M. Cyanobacteria, algae and microfungi present in biofilm from Božana Cave (Serbia). Int J Speleol. 2015;44(2):141-49. https://doi.org/10.5038/1827-806x.44.2.4
42. 42.Xlstat.com:XLSTAT statistical and data analysis solution [Internet]. New York: Addinsoft; [cited 2020 May 26]. Available from: https://www.xlstat.com.
43. Ter Braak CJF, Šmilauer P. Canoco reference manual and user's guide: software for ordination, version 5.0. Ithaca, USA: Microcomputer Power; 2012. 496 p.
44. Claudi R, Evans DW. Chemical addition strategies for zebra mussel (Dreissena polymorpha) control in once-through service water systems. In: Nalepa TF, Schloesser DW, editors. Zebra Mussels: Biology, Impacts, and Control. Boca Raton: Lewis Publishers; 1993. p. 563-73. https://doi.org/10.1201/b15437-45
45. Draäbkova AM, Admiral W†, Marsälek B‡. Combined Exposure to Hydrogen Peroxide and Lights Selective Effects on Cyanobacteria, Green Algae, and Diatoms. Environ Sci Technol. 2007;41(1):309-14. https://doi.org/10.1021/es060746i
46. Russell AD. Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemotherapy. 2003;52(5):750-63. https://doi.org/10.1093/jac/dkg422
47. Zhou QLL, Huang L, Guo L, Song L. Combining hydrogen peroxide addition with sunlight regulation to control algal blooms. Environ Sci Pollut Res. 2017;25(3):2239-47. https://doi.org/10.1007/s11356-017-0659-x
48. Burson A, Matthijs HCP, de Bruijne W, Talens R, Hoogenboom R, Gerssen A, Visser MP, Stomp M, Steur K, van Scehpinngen Y, Huisman J. Termination of a toxic Alexandrium bloom with hydrogen peroxide. Harmful Algae. 2014;31:125-35. https://doi.org/10.1016/j.hal.2013.10.017
49. Arberg B. Plant growth regulators. Monosubstituted benzoic acid. Swedish J Agricult Res. 1981;11:93-105.
50. Hayat Q, Hayat S, Irfan, M. Ahmad A. Effect of exogenous salicylic acid under changing environment: A review. Environ Experime Bot. 2010;68(1):14-25. https://doi.org/10.1016/j.envexpbot.2009.08.005
51. Yousuf DYM. Effects sprayed solution of salicylic acid to prevent of wilt disease caused by Fussarium oxysporium. J Phys Conf Ser. 2018;1003(1):1-6. https://doi.org/10.1088/1742-6596/1003/1/012001
52. Madan KR, Levitt J. A review of toxity from topical salicylic acid preparations. J Am Acad Dermatol. 2014;70(4):788-92.
53. Czerpak R, Bajguz A, Gromek M, Kozlowksa G, Nowka I. Activity of salicylic acid on the growth and biochemism of Chlorella vulgaris Beijerinck. Acta Phycologie Plantaru. 2002;24:45. https://doi.org/10.1007/s11738-002-0020-x
54. Gao ZQ, Meng CX, Diao YY. The effect of extraneous salicylic acid on astaxanthin accumulation of alga Haematoccus pluvialis. Fish Sci. 2007;26:377-80. Chinese.
55. Wu G, Gao Z, Du H, Lin B, Yan Y, Li G, Guo Y, Fu S, Wei G, Wang M, Cui M, Meng C. The effects of abscisic acid, salicylic acid and jasmonic acid on lipid accumulation in two freshwater Chlorella strains. J Gen Appl Microbiol. 2018;64(1):42-9. https://doi.org/10.2323/jgam.2017.06.001
56. Xu Q, Shi M, Wang S, QingY. Study on the effect of exogenous salicylic acid on algae growth in the environment. E3S Web Conf. 2020;165:02001. https://doi.org/10.1051/e3sconf/202016502001
57. Ingols RS, Ridenourm GM. Chemical properties of chlorine dioxide in water treatment. J Am Water Works Assoc. 1948;40:1207-27. https://doi.org/10.1002/j.1551-8833.1948.tb15069.x

58. Benarde MA, Israel BM, Olivieri VP, Granstrom M. Efficiency of chlorine dioxide as a bactericide. Appl Microbiology. 1965;13(5):776-80. https://doi.org/10.1128/am.13.5.776-780.1965
59. Benarde MA, Snow WB, Olivieri VP, Davidson B. Kinetics and mechanism of bacterial disinfection by chlorine dioxide. Appl Microbiol. 1967;15(2):257-65. https://doi.org/10.1128/am.15.2.257-265.1967
60. Hebelka J. Methodology of lampenflora removal in caves accessible for tourists. Průhonice : Cave Administration of the Czech Republic; 2014. 15 p.

Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Nikolic%20et%20al_6413_Supplementary%20Material.pdf