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Research Article

PHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN ANTARCTIC AND MESOPHILIC *CHLORELLA VULGARIS* ISOLATES UNDER THE EFFECT OF SANOSIL

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Summary: The impact of various stress factors on the same organisms isolated from different climatic zones is an interesting and little-studied topic. In the present work, the effects of the biocide Sanosil on antarctic and mesophilic isolates of the green alga *Chlorella vulgaris* was estimated. The concentration of 0.15% was found to have an algicidal effect. Changes in the content of photosynthetic pigments, total proteins and total carbohydrates at different concentrations lower than the algicidal were studied. The influence on growth and viability of the algal strains was studied. The results showed a significant inhibition of the biomass accumulation in both isolates with increasing the concentration of Sanosil. However, slight growth stimulation in the *Chlorella vulgaris* antarctic isolate was observed upon treatment with the lowest concentrations of Sanosil (0.01 and 0.03%). This result was in correlation with the viability test. In the mesophilic isolate, the amount of chlorophyll *a* and *b* decreased smoothly after exposure to increasing Sanosil concentrations, while the percent of carotenoids slightly increased at the lowest concentrations applied. In the antarctic isolate there was a slight increase in the quantity of chlorophyll *a* and *b* together with a significant increase of carotenoids content at the lowest biocide concentrations. Application of Sanosil at concentrations higher than 0.03% led to a gradual but significant decrease in pigment content in both isolates. In the mesophilic isolate treated with the lowest concentrations of Sanosil, there was no change in the total protein content, while the amount of carbohydrates was increased by about 8%. However, the same concentrations of Sanosil led to a slight increase in the quantity of total proteins and a decrease in the carbohydrates content in the antarctic isolate. As a result of Sanosil action at concentrations higher than 0.03%, a decrease in the amount of proteins and carbohydrates was observed in both isolates which was most pronounced at the highest concentration applied (0.1%).

Keywords: Antarctic algae; biochemical changes; *Chlorella vulgaris*; degradable biocide.

Abbreviations: DW – dry weight; ROS – reactive oxygen species; TTC – triphenyl tetrazolium chloride.

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INTRODUCTION

Non-toxic and degradable yet potent biocides have become an interesting object of reserch in recent years. Oxidizing agents, notably hydrogen peroxide (H_2O_2), are increasingly used in a number of medical, food and industrial applications as well as environmental ones such as water treatment (Linley et al., 2012). Algae are a very common problem in almost every tank used for various purposes. The presence of algae and bacteria in water reservoirs causes economical problems because they may plug water pipes, tanks and cooling towers. To destroy the harmful microorganisms, it is necessary to find a disinfectant that is harmless for the environment. Sanosil is a disinfectant whose active ingredients are 50% H_2O_2 and 0.05% Ag. The active substance used is H_2O_2, an environment friendly substance. The traces of Ag remaining on the treated surfaces are not visible and non toxic. Oxygen (O_2) separated by H_2O_2 attacks the cell walls of the microorganisms directly and affect photosynthetic activity (Matthijs et al., 2012). Sanosil has a wide range of antibacterial effects (Izadi et al., 2013, Linley et al., 2012). Hydrogen peroxide is extensively used as a biocide, particularly in applications where its decomposition into non-toxic by-products is important. The chemical reaction of O_2 with molecules in the cell walls leads to their destruction. This effect is boosted by Ag ions that bind to the disulfide bonds of certain proteins, both of the reproduction complex as well as the metabolic system of microorganisms, thereby precipitating and deactivating them. Hydrogen peroxide is known to produce a burst of reactive oxygen species (ROS), i.e. a state of oxidative stress in plants (algae) by a mechanism of redox cycling. Oxidative stress is a natural cellular phenomenon in which an organism is subjected to a change in the balance between oxidants and antioxidants in favor of the oxidants (Halliwell and Gutteridge, 1999). These oxidants are generally termed ROS, and include superoxide anion radical and the hydroxyl radical amongst others (Dalton et al., 1999). ROS can be extremely detrimental to cellular viability as they are damaging to DNA, proteins, lipids, and cell membranes (Storz and Imlay, 1999). Nevertheless, their generation is an unavoidable consequence of aerobic life. Aerobic organisms have developed complex antioxidant defence systems to combat the deleterious effects of oxidative stress (Sies, 1993).

The aim of this study was to determine the algicidal and bactericidal concentration of Sanosil on 9 microalgal and 8 bacterial strains commonly found in nature as well as to study and compare the physiological and biochemical changes in the cells of two *Chlorella vulgaris* isolates - mesophilic and antarctic under the influence of the oxidizing agent.

MATERIALS AND METHODS

Microorganisms

The following bacteria (*Escherihia coli, Serratia macesceus, Bacillus subtilis, Bacillus. cereus, Bacillus megaterium, Aeromonas hydrophyla, Staphylococcus aureus, Oerskovia xanthineolytica*) and algae (*Tribonema sp., Cladophora sp., Oedogonium sp., Navicula sp. Phormidium sp. Oscillatoria sp. Spirogyra sp., Chlorella vulgaris*) were used in the experiments. The isolates of *Chlorella*

vulgaris Beyer (Chlorophyta) unicellular green algae – mesophilic and antarctic were obtained from the Collection of Autotrophic Organisms (CCALA) at the Institute of Botany-Trebon, Chech Republic and used as test objects.

Biocidic effect estimation

Pure bacterial strains were cultivated in meat peptone broth at 30°C for 48h and used for testing the bactericidic effect of Sanosil (Water Treatment Products Ltd, Unit 1, Gilchrist Thomas Indus trial Estate, Blaenavon, Pontypool, Torfaen, NP4 9RL, United Kingdom). Bacterial suspensions were incubated with different concentrations of Sanosil (0.01%, 0.03%, 0.05%, 0.08% and 0.15%) in a thermostate for 48–96h at 30°C. For CFU counts, cells were diluted serially in sterile water and plated on agar plates which were incubated for 24h at 30°C. The number of living cells (colony-forming units, cfu) was count. The colonies counted in the untreated control sample were taken as 100%. To determine the algicidal effect, algal suspensions of the tested species with a density of 3.10^6 cells ml^{-1} were used. The same five concentrations of Sanosil were added. The samples were incubated on luministate with illumination (180 μmol m^{-2} s^{-1}) for 5 days. Cell count was performed using a Burger chamber.

Cultivation conditions and treatment with Sanosil

Antarctic and mesophilic isolates of the green alga *Chlorella vulgaris* were intensively cultivated in laboratory vessels (glass containers of 200 ml) for 5 days until stationary phase of development prior to exposition to Sanosil. The nutrient medium of Setlik Simer modified by Georgiev et al. (1978) was used. Green algal lines were cultured at 26°C with continuous illumination provided by fluorescent lamps (light intensity of 180 μmol m^{-2} s^{-1}) and aeration with 2% CO_2 (Dilov, 1985). Algal cultures were treated with different concentrations of Sanosil (0.01%, 0.03%, 0.05%, 0.08% and 0.1%) for 72 h.

Algal growth

The growth of algae was determined gravimetrically as an absolute dry weight (DW). Ten ml aliquots of the algal suspension were placed in centrifuge tubes and centrifuged after 10 min of treatment with 1 ml of 11.5% CH_3COOH. After the supernatant was discarded, the precipitated cells were dried in a desiccator at 105°C for 20h.

Viability test (triphenyl tetrazolium chloride test)

Two ml algal suspension was placed in centrifuge tubes, centrifuged for 20 min at 4600 rpm, then the supernatant was discarded and 5 ml of 0.6% triphenyl tetrazolium chloride (TTC) in 0.05M Na_2PO_4-KH_2PO_4 buffer, pH 7.4 was added to the precipitated cells. Incubation was carried out in a thermostat at 37°C for 18-20h. After incubation, the cell suspensions were centrifuged and supernatant discarded. Precipitated cells were stirred and poured with 2-3 ml of 95% ethanol, then placed in a boiling water bath for 5 min to extract the water-insoluble formazan. After cooling, the tubes were centrifuged, the supernatant was transferred into measuring tubes and filled to 10 ml with ethanol. The extinction was measured by a spectrophotometer (S-20, Boeco, Germany) at 530 nm (Steponkus

and Lanphear, 1967). The viability was expressed as part of the 100% viability of the untreated algal culture.

Total proteins

Protein content was determined according to the method of Bradford (1976).

Carbohydrates

Carbohydrates were measured by the method of Antron (Hodge and Hofreiter, 1962).

Pigments

The pigments (chlorophyll *a*, chlorophyll *b*, and carotenoides) were extracted with methanol and determined spectrophotometrically at 665, 650 and 460 nm. Calculations were made using the Mc Kinny formulas (1941).

RESULTS

The antibacterial and antialgal activities of Sanosil were studied after treatment of 9 microalgal and 8 bacterial strains with different concentrations of the agent. Sanosil showed bactericidic and algicidic effects (100% inhibition) at a concentration of 0.15% with the exception of *Serratia marcescens* (30% survival) and *Oerscovia xanthineolytica* (5% survival) (Table 1).

Table 1. Effect of different concentrations of Sanosil on algae and bacteria development inhibition.

Species	Sanosil %					
	Control	0.01%	0.03%	0.05%	0.08%	0.15%
Algae						
Tribonema	0	70	85	95	100	100
Cladophora	0	60	80	90	95	100
Oedogonium	0	50	85	95	100	100
Navicula	0	65	85	95	95	100
Phormidium	0	30	45	90	95	100
Oscillatoria	0	28	60	95	100	100
Spirogyra	0	24	50	95	100	100
Chlorella vulgaris(mesophilic)	0	4	13	65	64	100
Chlorella vulgaris(Antarctic)	0	0	0	63	78	100
Bacteria						
Escherihia coli	0	4	18	50	80	100
Serratia marcescens	0	0	4	10	30	70
Bacillus subtilis	0	4	15	40	90	100
Aeromonas hydrophila	0	30	42	60	100	100
Bacillus cereus	0	6	15	75	90	100
Bacillus megaterium	0	20	44	60	85	100
*Oerskovia xanthineolytic*a	0	22	35	70	95	95
Staphylococcus aureus	0	20	34	60	95	100

Physiological and biochemical studies were carried out to determine the influence of subalgicidic concentrations of Sanosil on the biomass production, cell viabilityty, pigment, protein and carbohydrate contents of the antarctic and mesophilic *Chlorella vulgaris* isolates. The results showed a growth inhibition of mesophilic *Chlorella vulgaris*, which increased with increasing the concentration of the preparation (Figure 1A, B). However, in the case of antarctic *Chlorella*, there was a slight growth stimulation at the lowest Sanosil concentrations (0.01 and 0.03%) and a significant growth inhibition at the higher concentrations applied.

At the highest Sanosil concentrations tested (0.05, 0.08 and 0.1%), an algistatic effect on both *C. vulgaris* isolates was observed. The data on the changes of biomass production correlated well with the established cell viability (Fig. 2).

Visualization of the algal cell viability assay (TTC) is presented in Fig. 3. Red formazan crystals were formed (Fig. 3B) as a result of the enzyme reaction, thus

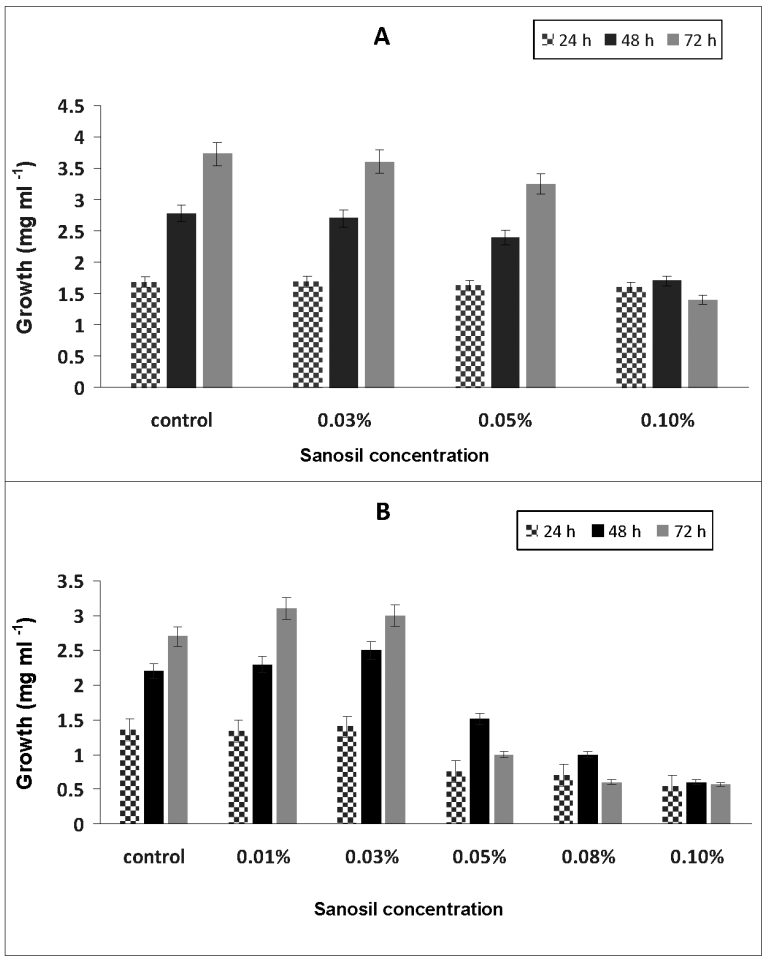

Figure 1. Changes in growth [mg ml^{-1}] in mesophilic (A) and antarctic (B) isolates of *Chlorella vulgaris* at 24, 48 and 72 h following treatment with different concentrations of Sanosil – 0% (control), 0.01%, 0.03%, 0.05%, 0.08%, 0.10%. Data represent means ± SE.

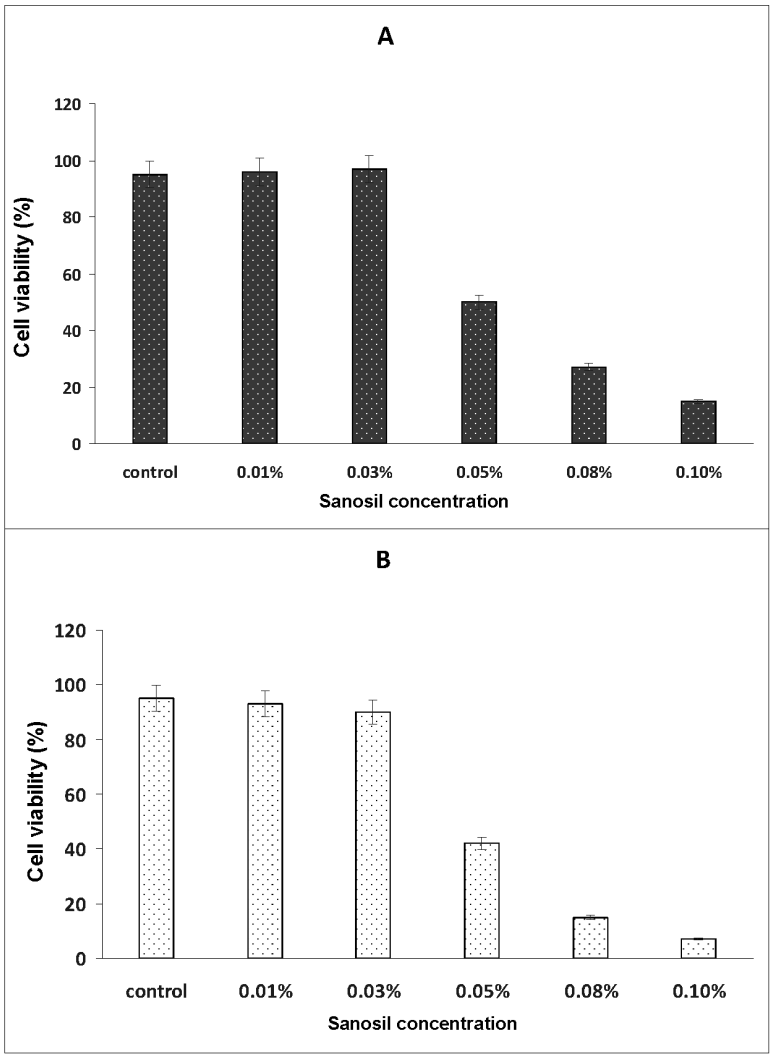

Figure 2. Cell viability [%] of mesophilic (A) and antarctic (B) isolates of *Chlorella vulgaris* after 72-h treatment with different Sanosil concentrations – 0% (control), 0.01%, 0.03%, 0.05%, 0.08%, 0.10%. Data represent means ± SE.

indicating cell viability.

The cellular pigment contents also changed in response to Sanosil treatment. In the cells of the mesophilic isolate, the amounts of chlorophyll *a* and *b* decreased smoothly after exposure to increasing Sanosil concentrations (Fig. 4A). In the antarctic *C. vulgaris* isolate the amounts of chlorophyll *a* and chlorophyll *b* were slightly increased after treatment with Sanosil at concentrations of 0.01% and 0.03%. However, at higher concentrations the content of these pigments was reduced (Fig. 4B). The level of carotenoids in both microalgal strains increased at the two lowest doses of biocide compared to the controls. The application of higher doses led to a significant decrease of these pigments (Fig. 5A, B).

Measurement of protein and carbohydrate contents in the algal cells treated with Sanosil was also carried out

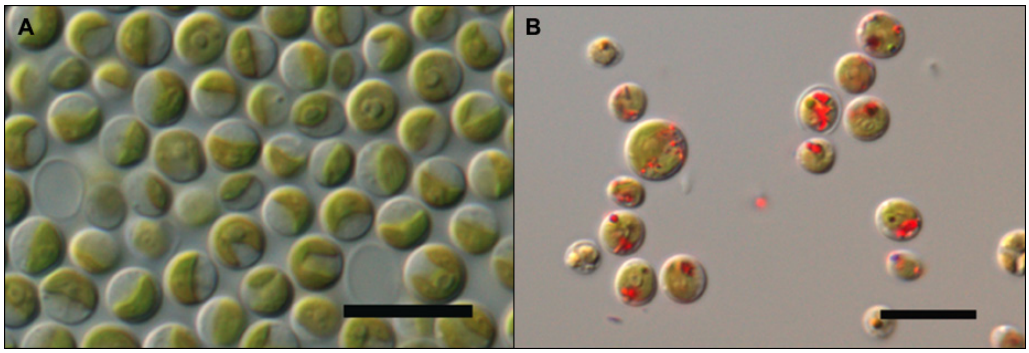

Figure 3. Light microscopy images of *Chlorella vulgaris* (A) and Sanosil treated *Chlorella vulgaris* cells (B) after viability determination by the TTC method. Bar =10 μm.

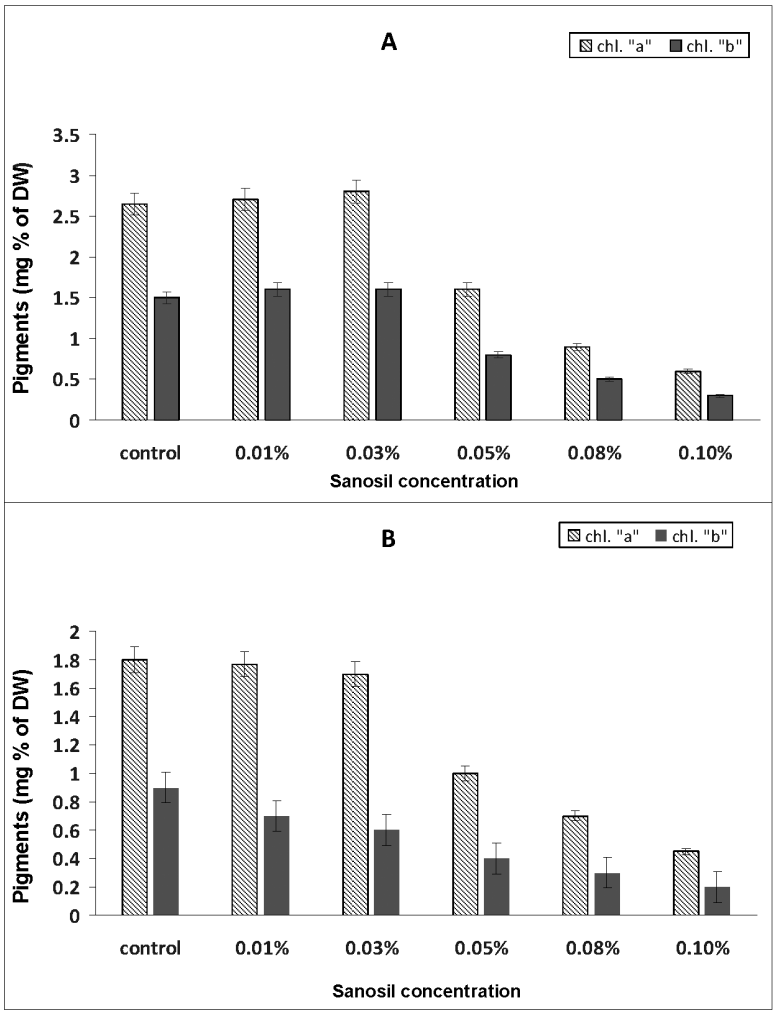

Figure 4. Changes in chlorophyll *a* and chlorophyll *b* content [mg % of DW] in mesophilic (A) and antarctic (B) isolates of *Chlorella vulgaris* after 72-h treatment with different Sanosil concentrations – 0% (control), 0.01%, 0.03%, 0.05%, 0.08%, 0.10%. Data represent means ± SE.

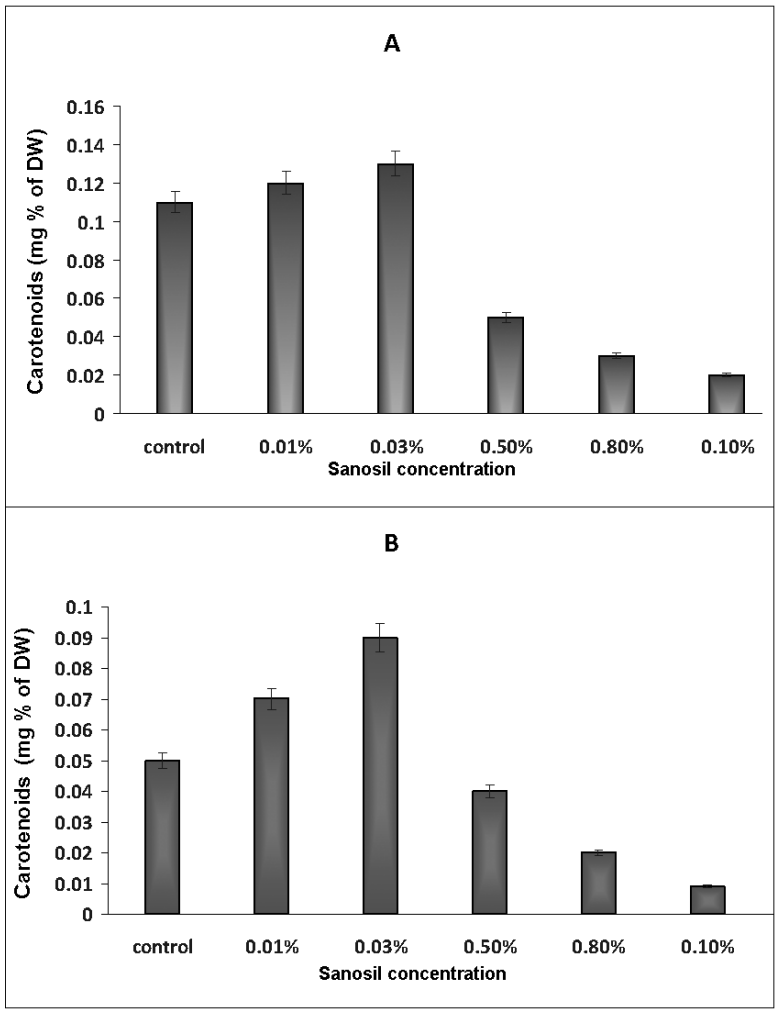

Figure 5. Changes in carotenoids content [mg % of DW] in mesophilic (A) and antarctic (B) isolates of *Chlorella vulgaris* after 72-h treatment with different Sanosil concentrations – 0% (control), 0.01%, 0.03%, 0.05%, 0.08%, 0.10%. Data represent means ± SE.

(Table 2). In the mesophilic isolate treated with the lowest concentrations of Sanosil there was no change in the total protein content, but the amount of carbohydrates increased by about 8%. However, there was a slight increase in the amount of total proteins and a decrease in the carbohydrate content in the antarctic isolate at the same Sanosil concentrations. As a result of Sanosil action at concentrations higher than 0.03%, in both isolates a decrease in the amount of proteins and carbohydrates was observed, which was most pronounced at the highest concentration used (0.1%). The decrease in carbohydrate content was very sharp in the antarctic isolate (from 43% to 34%).

DISCUSSION

The two main components of Sanosil - hydrogen peroxide as an oxidizing agent and silver with its bactericidal and algicidal effects, contribute to the effective

Table 2. Content of total proteins and carbohydrates in algal cells for both lines after treatment with Sanosil.

Concentration of Sanosil [%]	Mesophilic isolate		Antarctic isolate	
	Total proteins [% of DW]	Total carbohydrates [% of DW]	Total proteins [% of DW]	Total carbohydrates [% of DW]
Control	21.00±0.77	34.00±0.72	20.80±0.42	42.80±0.50
0.01	21.05±0.66	36.80±0.65	21.95±0.29	42.20±0.60
0.03	21.10±0.61	36.60±0.47	22.90±0.28	38.90±0.63
0.05	20.20±0.56	36.20±0.37	20.90±0.32	37.00±0.44
0.08	19.00±0.50	30.80±0.41	19.90±0.40	34.80±0.34
0.10	18.80±0.44	30.00±0.37	18.70±0.29	34.00±0.28

action of Sanosil in combating unwanted microbial contamination (Murdoch et al., 2016). This explains its widespread use in wastewater treatment, preventing the problem of developing algae and bacteria in pools. An advantage of Sanosil is its rapid biodegradability, making it environmentally friendly. Hydrogen peroxide decomposes into water and oxygen. Oxygen destroys biofilms and thus allows silver ions to kill the microbes found beneath them. The results of our study revealed the wide spectrum of algicidal and bactericidal effects of Sanosil even at concentration (0.15%) lower than recommended, and confirmed the benefits of its application for ecologically expedient control of the unwanted microorganisms. Furthermore, the impact of subalgicidal concentrations of this oxidizing agent on certain physiological and biochemical traits of antarctic and mesophilic *Chlorella vulgaris* was estabiliched and compared. In studies on algae subjected to stress factors some authors conclude that the main property of all generated types of ROS is to induce oxidative damage to proteins, lipids, photosynthetic pigments and photosynthetic structures (He et al., 2002; Marnett et al., 2003; Breusegem and Dat, 2006). Other researchers suggest that traditionally considered toxic, ROS are also signaling molecules by which plant organisms regulate various physiological processes related to plant protection and stress tolerance (Edreva, 2005). Photosynthesis plays an important role in the lifestyle of photoautotrophic algae, which relies on the absorption of sunlight by chlorophyll molecules in photosystems I and II. Superfluous ROS are produced when the algal cells are under stress, causing growth inhibition, photosynthetic pigments degradation, reduction of protein and carbohydrate content (Zidarova and Pouneva, 2006; He et al., 2015). The data from our study showed a decrease of the biomass and the amount of chorophylls *a* and *b* in the mesophilic strain of *C. vulgaris* after application of Sanosil as an oxidative stress agent proportionally to the concentrations applied. For the antarctic one, it was the same at the higher concentrations (0.05 to 0.1%), but a slight increase was established at lower Sanosil concentrations. Almost the same trend

was observed in the protein content of mesophilic and antarctic *C. vulgaris* in dependence of the Sanosil concentration used.

The comparison between the two isolates revealed that the antarctic isolate appeared to be more tolerant to this type of oxidative stress only when low concentrations of Sanosil were applied (0.01% and 0.03%). This is probably due to the fact that antarctic isolates are exposed to more extreme environmental conditions (Rautenberger and Bischof, 2006). At concentrations above 0.05% however, both *C. vulgaris* strains showed a significant decrease of biomass, protein, carbohydrate and pigment contents as well as cell viability.

The comparative study of the physiological and biochemical parameters of antarctic and mesophilic microalgal strains provides the directions towards further elucidation of the divergence of the species and their defence and adaptive mechanisms depending on the conditions of their environments.

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