

POLISH JOURNAL OF ECOLOGY (Pol. J. Ecol.)	57	3	547-554	2009
--	----	---	---------	------

Short research contribution

Maciej WALCZAK*, Maria SWIONTEK BRZEZINSKA

Department of Environmental Microbiology and Biotechnology,
Institute of Ecology and Environment Protection, Nicolaus Copernicus University
Gagarina 9, 87-100 Toruń, Poland

*e-mail: walczak@biol.uni.torun.pl (*corresponding author*)

THE IMPACT OF UV MEDIATED HYDROGEN PEROXIDE ON CULTURABLE BACTERIA IN THE SURFACE MICROLAYER OF EUTROPHIC LAKE

ABSTRACT: Hydrogen peroxide (H₂O₂) formation in surface waters is initiated by the absorption of sunlight by dissolved organic matter (DOM). The fraction of the DOM pool that interacts with sunlight, referred to as chromophoric dissolved organic matter, impacts the optical properties of surface waters. Second source of H₂O₂ is wet and dry deposition of photogenerated substance in the atmosphere and biological production.

The study examined the concentration of hydrogen peroxide in water from the surface microlayer (SM) (<100 µm) and subsurface water (SSW) (25 cm) in the typical eutrophic (TOC 5–15 mg dm⁻³; chlorophyll 5–26 µg dm⁻³, water transparency 0.6–1.0 m) lake as well as the impact of this compound on occurrence and survivorship of catalase-positive and catalase-negative bacteria isolated and cultured on the TSA medium (Difco). The experimental H₂O₂ concentrations ranged between 500–5000 nM. The concentration of H₂O₂ in analyzed water samples clearly increased in day-time hours and was different in May, July and October. The highest natural concentration of H₂O₂ (700 nM) was observed in SM water in summer in afternoon hours. During that period, 100% of bacterial populations found in SM water produced catalase. The experiments confirmed that environmental concentrations of H₂O₂ caused no considerable decrease in survivorship of culturable bacteria, while concentrations exceeding 1000 nM were lethal for the majority of catalase-

negative bacteria, but not for catalase-positive bacteria.

KEY WORDS: surface microlayer, hydrogen peroxide, bacteria, catalase

Surface microlayer constitutes an external, very thin (usually about 100 µm but it depends of the sampling device) layer of a water body formed at the air-water interphase.

Surface microlayer is maintained by adhesion forces generated by the intermolecular attraction and surface tension in the air-water boundary. These phenomena are responsible for accumulation of organic and inorganic compounds in that layer (lipids, sugars, proteins, humic substances, inorganic nitrogen and phosphorus) (Maki 1993, Hillbricht-Ilkowska and Kostrzewska Szlakowska 2004). Surface microlayer also contains an increased abundance of bacteria, called bacterioneuston (Donderski *et al.* 1999, Maki and Remsen 1989). Due to the environment they inhabit, bacterioneuston are more exposed to stressful ecological factors than microorganisms living in the water column. Potentially harmful factors, such as toxic organic substances, heavy metals, and intense solar radiation containing UV, which in turn induces formation of reactive oxygen species

(ROS), play an important role in struggle for survival and growth of these organisms. Reactive oxygen species, such as: singlet oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^{\bullet-}$), hydroxyl radical ($\bullet\text{OH}$), and hydrogen peroxide (H_2O_2) are generated in water in the presence of UV radiation, causing photooxidation of dissolved organic carbon (DOC). Subsequently, ROS participate in biogeochemical and biological processes (Scully *et al.* 2003).

Hydrogen peroxide is characterized by lower reactivity in comparison to other ROS; however, it undergoes reaction much easier than molecular oxygen (Bartosz 2004). Hydrogen peroxide primarily acts as an oxidant of compounds with which it reacts; the compound may easily penetrate the cell membrane and react with all major components of a cell, damaging it, interfering with its functioning, and causing its death (Cantoni *et al.* 1992).

The concentration of H_2O_2 in an aquatic environment depends on intensity of UV radiation, concentration of DOC, and the rate of its (primarily biological) decomposition (Petasne and Zika 1997). The concentrations of H_2O_2 in water observed in previous studies vary considerably ranging from a dozen or so to over a thousand nanomols per liter (Cooper *et al.* 1988, O'Sullivan *et al.* 2005). Those results depended on the type of water (fresh or sea water), latitude, or sampling depth. However, it was demonstrated in all studies that concentration of H_2O_2 in a given water body depends most strongly on the depth. Concentration of H_2O_2 is always higher in surface water than in water collected at greater depths (Price *et al.* 1998).

The purpose of this study was to determine concentration of H_2O_2 in surface microlayer water (collected several hundred μm below the surface) and the impact of this compound on occurrence and survivorship of culturable bacteria isolated from that layer.

The study analyzed strains of heterotrophic bacteria isolated from surface microlayer (SM) and subsurface water (SSW). The water was collected from lake Brzeźno (53°57,5'N, 17°48,6'E) located in the forested area, "Bory Tucholskie" National Park in North Poland.. The lake is located 139.8 m a.s.l, area – 71.6 ha, maximum depth – 9.7 m. It is a eutrophic lake: content of total organic carbon (TOC)

ranging from 5 to 15 mg dm^{-3} , chlorophyll 5–26 mg dm^{-3} and water transparency 0.6–1.0 m. Water samples were collected in at least 10 replications during day-night periods in May, July and October from two stations in the pelagic zone. The SM samples were collected using Garrett (1965) technique with a Plexiglas plate gathering 150 mm layer of water. SSW samples were collected from the depth of *ca.* 25 cm with an automatic pump. The samples were transferred to sterile glass containers.

Concentration of H_2O_2 was measured triplicate, immediately after the samples were collected with the fluorometric method (Cooper *et al.* 1988) using fluorometer Hitachi F-2500.

Light and UVB intensities were measured with a Solar Light (Solar Light Co.) meter equipped with appropriate probes. The measure was made continuously during one hour, presented data of UVB radiation are average values of this measure. The temperature and pH of water was measured with an Elmetron meter.

Bacterial strains were isolated through surface inoculation from the volume of 0.1 cm^3 from appropriately diluted samples in three parallel replicates. Inoculation was carried out on TSA medium (Difco). Following 6-day incubation at 20°C, formed colonies were enumerated and their number was adjusted to abundance of bacteria in analyzed water; subsequently, a representative collection of bacterial strains (not less than 100 strains) were isolated and inoculated onto TSA slants.

In order to determine the catalase-positive bacteria, the isolated bacterial strains were inoculated onto TSA slants. The bacteria colonies that developed on the medium were smeared into 3% solution of hydrogen peroxide.

If a bacterial strain produced active catalase, froth was formed in the test tube with added solution of hydrogen peroxide. This phenomenon is a result of breakdown of H_2O_2 by catalase, which is accompanied by release of free oxygen. Liberation of oxygen was considered a positive result, which meant that a given strain was catalase-positive. In contrast, the absence of froth indicated that a given strain was catalase-negative.

In order to prepare bacterial suspension, isolated bacteria were cultured for 3 days at 20°C in 50 ml of liquid TSB (Difco) medium. Subsequently, the cultures were centrifuged for 5 min at 10,000 rpm, at 10°C. Following this procedure, supernatant was removed and bacterial pellet was resuspended in 10 ml of sterile solution of physiological salt. Optical density of bacterial suspension of every strain was adjusted to the same value of 0.5, using sterile solution of physiological salt as diluter. Four 9 ml subsamples were taken from each bacterial strain suspension and placed in sterile 50 ml flasks.

In order to determine the impact of H₂O₂ on survivorship of bacteria the following substances were added to the prepared bacterial suspension (9 ml in 50 ml flask):

- 1) 1 ml of sterile physiological salt – control,
- 2) 1 ml of H₂O₂ solution (concentration of 5000 nM), the final concentration of H₂O₂ equaled 500 nM (environmental concentration),
- 3) 1 ml of H₂O₂ solution (concentration of 10 000 nM), the final concentration of H₂O₂ equaled 1000 nM,
- 4) 1 ml of H₂O₂ solution (concentration of 50 000 nM), the final concentration of H₂O₂ equaled 5000 nM (extreme concentration),

The prepared bacterial suspensions containing H₂O₂ were incubated in a shaker for 30 min at 20°C.

In order to determine the survivorship of bacterial strains, the analyzed suspensions were diluted in TSB medium containing 0.2% sodium thiosulfate as a neutralizer of H₂O₂ and then plated on TSA. Following 6-day incubation at 20°C, developed colonies (CFU) were enumerated and adjusted to the abundance of bacteria in 1ml of tested suspension. Effects of H₂O₂ treatment were expressed as the percentage of surviving cells relative to the cell number in the control bacterial suspension according to the formula:

$$SI = \frac{Nc - Nn}{Nc} \times 100\% \quad (1)$$

where:

SI – survivorship index (%),

Nc – abundance of bacteria in the control

Nn – abundance of bacteria in tested suspension for a specific H₂O₂ concentration.

The CFUs represented viable cells that either were undamaged by the H₂O₂ treatment or suffered damage but were capable of repair, replication, and colony formation.

Determination of impact of H₂O₂ on survivorship of bacteria were investigated in three parallel replicates.

Statistical analyses were done using program STATISTICA 6.0. Analysis of Variance (ANOVA) was the primary statistical method used in calculations.

Recorded temperatures were characteristic of inland water bodies located in this climatic zone (Table 1). Values of pH in all testing stations were very similar (8.0–8.2) across the entire research period. The observed differences in intensity of visible light and UVB radiation between SM and SSW were related to radiation absorption by substances present in water.

The concentration of H₂O₂ increased significantly in periods with strong solar radiation (Fig. 1). In summer, during the strongest insolation, its value reached 700 nM in SM and 300 nM in SSW. In spring, in the presence of solar radiation, the concentration of H₂O₂ did not exceed 300 nM in SM and 150 nM in SSW. In autumn, the highest value observed in SM and SSW equaled 200 nM and 150 nM, respectively.

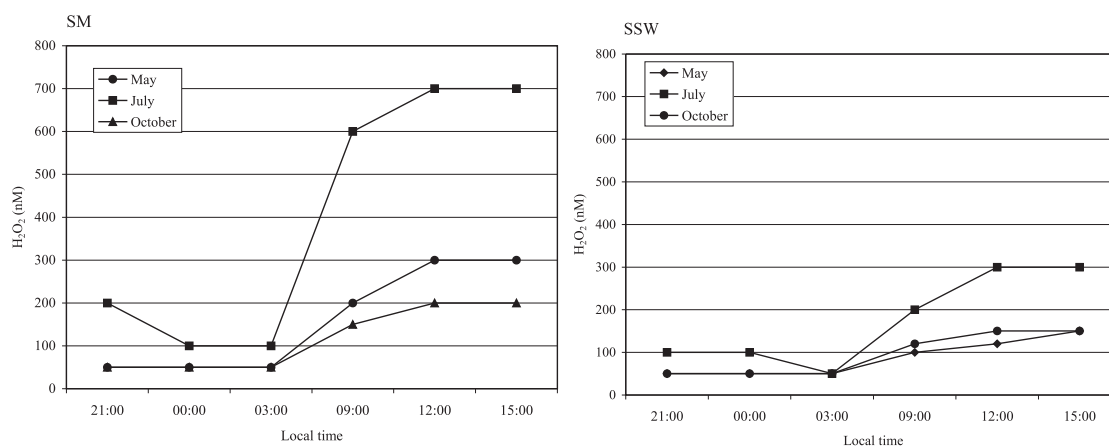
At night, concentrations of H₂O₂ were significantly lower with similar values for SM and SSW. In spring and autumn, the concentration of H₂O₂ at night equaled 50 nM in both MP and SSW. In summer, its value in SM ranged from 100 to 200 nM, and in SSW, from 50 to 100 nM (Fig. 1).

Variance analysis demonstrated significant relationships between H₂O₂ concentration and light intensity and UVB radiation ($P < 0.05$).

In spring and summer the abundance of heterotrophic bacteria capable to forming colonies was significant higher in SM than in SSW (Table 2). Also, in SM these bacteria were significantly more abundant at night than during day. In general, the percentage of catalase-positive bacteria in water samples was always higher in SM than in SSW. In the presence of solar radiation, the percentage of strains producing catalase was higher than in samples collected at night irrespective of the season and analyzed water layer. In SM samples collected in summer during the solar radiation, percentage of catalase-positive

Table 1. Physicochemical parameters of water in surface microlayer (SM – around 100 μm) samples and in subsurface (SSW – 25 cm) samples.

Local time	SM					SSW				
	Light (klx)	UVB ($\mu\text{W cm}^{-2}$)	H ₂ O ₂ (nM)	Temp. ($^{\circ}\text{C}$)	pH	Light (klx)	UVB ($\mu\text{W cm}^{-2}$)	H ₂ O ₂ (nM)	Temp. ($^{\circ}\text{C}$)	pH
May										
9.00	25.0	8.5	200	16.8	7.9	12.8	1.8	100	16.8	7.9
12.00	46.6	9.0	300	18.7	8.1	23.3	2.1	120	18.9	8.1
15.00	52.0	10.2	300	17.2	8.1	27.5	2.2	150	17.3	8.1
21.00	0.6	0.0	50	17.1	8.2	0.0	0.0	50	17.2	8.2
24.00	0.0	0.0	50	16.9	8.1	0.0	0.0	50	17.0	8.1
3.00	0.0	0.0	50	16.6	8.1	0.0	0.0	50	16.7	8.1
July										
9.00	90.0	12.7	600	20.1	8.2	30.0	2.5	200	20.2	8.2
12.00	193.5	17.5	700	21.0	8.2	45.8	4.8	300	20.9	8.2
15.00	170.0	16.4	700	21.6	8.1	23.7	4.3	300	20.9	8.2
21.00	0.1	0.0	200	21.2	8.4	0.0	0.0	100	21.4	8.4
24.00	0.0	0.0	100	20.9	8.3	0.0	0.0	100	21.1	8.3
3.00	0.0	0.0	100	20.8	8.2	0.0	0.0	50	20.9	8.2
October										
9.00	12.9	1.1	150	16.2	8.0	5.1	0.8	120	16.1	8.0
12.00	21.0	1.9	200	17.0	8.2	6.5	0.8	150	17.1	8.2
15.00	11.8	0.9	200	16.7	8.3	2.0	0.2	150	16.9	8.3
21.00	0.0	0.0	50	16.1	8.1	0.0	0.0	50	16.0	8.1
24.00	0.0	0.0	50	16.0	7.9	0.0	0.0	50	16.1	7.9
3.00	0.0	0.0	50	15.9	7.8	0.0	0.0	50	16.0	7.9

Fig. 1. Concentration of H₂O₂ in surface microlayer (SM – about 100 μm) and in subsurface water (SSW – 25 cm) in an eutrophic lake.

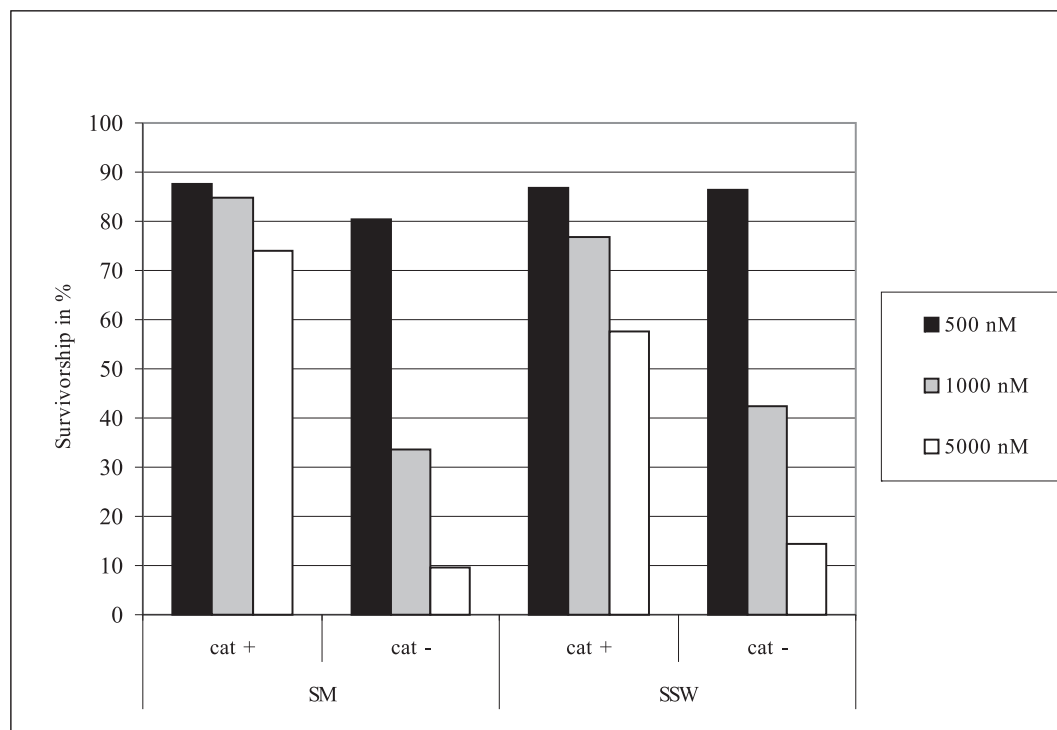


Fig. 2. The impact of different concentration of H₂O₂ on survivorship (formula 1) of catalase positive (cat+) and negative (cat-) bacteria from surface microlayer (SM – about 100 μ m) and subsurface water (SSW – 25 cm)

surfaces. Therefore, photooxidation reactions of organic matter present in the SM layer may be quite efficient. The quantity of radiation reaching the SM is subject to specific periods (night/day, summer/winter). These periods are responsible for relevant fluctuations in concentration of ROS in that layer. During intensive insolation, the SM layer may constitute an extreme environment for many aquatic organisms. In SM during strong solar radiation concentration of hydrogen peroxide reached 700 nM. This relatively high value corresponds with data describing inland water bodies, for which the concentrations of H₂O₂ are known to range mostly from 100 to 1000 nM (Price *et al.* 1998). According to this study, the concentration of H₂O₂ varied with depth despite the fact that the difference was only 25 cm. Price *et al.* (1998) observed the highest concentrations of H₂O₂ (154 nM) at the depth of 20 cm in marine conditions. In contrast, at 80 m, the concentration of this compound did not exceed 13 nM. The values of H₂O₂ concentration presented in Herut *et*

al. (1998) study were exceptionally low with 25 nM in the surface water and below 20 nM several meters deeper. Cooper *et al.* (1988) observed in marine conditions high concentration of H₂O₂ – 500 nM at the surface and 400 nM at the depth of 10 m.

This study analyzed the aquatic environment also from a seasonal perspective, and the results demonstrated that the largest amount of H₂O₂ was produced in summer, much smaller production was observed in autumn. This variability was associated with intensity of solar radiation in a given season. The highest concentrations of H₂O₂ occurred during day time, irrespective of season and depth. This result is related to photochemical synthesis of this compound, which occurs only under photic conditions (Scully *et al.* 2003).

Studies describing the impact of H₂O₂ on occurrence and survivorship of catalase-positive bacteria demonstrated that their percentage in bacterial population is significantly higher in SM than in SSW. This difference

was particularly prominent in summer, during day time in the presence of photochemically produced H_2O_2 . At that time, all bacterial strains collected in SM water produced catalase. At the same time, in subsurface water, catalase-positive strains constituted 65%. Hundred percent share of catalase-positive bacteria in SM water during day time may indicate that concentrations of hydrogen peroxide and other ROS are at that time so high that they constitute a selective factor of bacterial population. According to O'Sullivan *et al.* (2005), during an intensive solar radiation, photochemical production of H_2O_2 in water ranges from 280 to 1130 nM per hour. In Lake Ontario, production of H_2O_2 reached the level of 695–2120 nM per hour (Scully *et al.* 1996). Under these conditions, bacteria producing catalase had a higher chance of survival under oxidative stress, and this characteristic could promote their growth in this environment. On the other hand, the high percentage of catalase-producing bacteria in SM water provided an explanation for the rapid decrease in H_2O_2 concentration after solar radiation terminated. It is clearly evident from Petasne and Zika's (1997) study that decomposition of H_2O_2 in aquatic environment is caused by bacterial catalase. Other studies reported that „micro-particle organic matter” (probably bacterioplankton) was responsible for a rapid breakdown of H_2O_2 after solar radiation terminated (Herut *et al.* 1998, Cooper and Lean 1989).

The conjecture that H_2O_2 generated in water may constitute a selective factor for a bacterial population was confirmed by other results presented in this study.

The analysis of the impact of H_2O_2 on survivorship of bacteria isolated from SM and SSW demonstrated that the concentration of 500 nM caused no substantial decrease in bacterial survivorship. An increase in H_2O_2 concentration to 1000 nM considerably reduced survivorship of catalase-negative bacteria, whereas, the same concentration lowered the survivorship of catalase-positive bacteria only slightly. Further increase in H_2O_2 concentration to the extreme value (5000 nM) in comparison to the control drastically reduced survivorship of catalase-negative bacteria and also considerably reduced survivorship of catalase-positive bacteria. Elkins *et al.*

(1999) obtained similar results for the H_2O_2 concentration of 5000 nM. In the cited study, survivorship of catalase-negative bacteria reached only the level of ca. 10%, while under these conditions, catalase-positive bacteria were characterized by significantly higher survivorship. In contrast, during *in situ* studies, it was demonstrated that such low concentration of H_2O_2 as 50 nM was responsible for a noticeable inhibition of bacterial secondary production, while the concentration of 100 nM inhibited this production by 40% (Xenopoulos and Bird 1997). The same study demonstrated that addition of catalase to bacterial suspension containing H_2O_2 neutralized the inhibition effect.

As opposed to the presented results and aforesaid studies, Corin *et al.* (1998) did not observe inhibition of bacterial growth during culturing on mediums containing humic substances exposed to UV radiation. Hydrogen peroxide is produced under these conditions, which should have inhibited the growth of bacteria. But, those authors did not monitor concentration of H_2O_2 , and therefore, the presented results cannot be unambiguously interpreted.

Presented results demonstrated that concentrations of H_2O_2 in SM water were sufficiently high to result in selection of the bacterial population. Under the conditions of intensive solar radiation, when H_2O_2 concentrations are at the highest levels, catalase-positive organisms may constitute the majority or even 100% of bacterial populations. On the other hand, this may also indicate that a substantial part of bacterioneuston became adapted to such specific conditions, or that during the oxidative stress, some bacteria migrated to deeper layers of water column.

Unfortunately, no information on occurrence of H_2O_2 in a strict-sense surface microlayer of water bodies was found in available literature. It is known that in SM water, humic substances constitute the main part of DOC (Maki 1993, Hillbricht-Ilkowska and Kostrzewska Szlakowska 2004, Kostrzewska-Sztrakowska 2005). This layer in a given water body is exposed to the highest levels of UV radiation, which constitute the favorable conditions for H_2O_2 production.

REFERENCES

- Bartosz G. 2004 – Druga twarz tlenu [Second face of oxygen] – PWN, Warszawa. (in Polish).
- Cantoni O.P., Sestili A., Guidarelli P., Giacomoni U., Cattabeni F. 1992 – Effects of L-histidine on hydrogen peroxide – induced DNA damage and cytotoxicity in cultured mammalian cells – *Mol. Pharmacol.* 41: 969 – 974.
- Cooper W.J., Zika R.G., Petasne R.G., Plane J.M. 1988 – Photochemical formation of H₂O₂ in natural waters exposed to sunlight – *Environ. Sci. Technol.* 22: 1156–1160.
- Cooper W.J., Lean D.R.S. 1989 – Hydrogen peroxide concentration in a northern lake: photochemical formation and diel variability – *Environ. Sci. Technol.* 23: 1425–1428.
- Corin N., Backlund P., Wiklund T. 1998 – Bacterial growth in humic waters exposed to UV radiation and simulated sunlight – *Chemosphere*, 36: 1947–1958.
- Donderski W., Walczak M., Mudryk Z., Kobylński M. 1999 – Neustonic bacteria number, biomass and taxonomy – *Pol. J. Environ. Stud.* 8: 137–141
- Elkins J.G., Hassett D.J., Stewart P.S., Schweizer H.P., McDermott T.R. 1999 – Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide – *App. Environ. Microbiol.* 65: 4594–4600.
- Garrett W.P. 1965 – Collection of slick-forming materials from the sea surface – *Limnol. Oceanogr.* 10: 602 – 605.
- Herut B., Shonam-Frider E., Kress N., Fiedler U., Angel D.L. 1998 – Hydrogen peroxide production rates in clean and polluted coastal marine waters of the Mediterranean, Red and Baltic seas – *Mar. Poll. Bull.* 36: 994–1003.
- Hillbricht-Ilkowska A., Kostrzewska-Szlakowska I. 2004 – Surface microlayer in lakes of different trophic status: nutrients concentration and accumulation – *Pol. J. Ecol.* 52: 461–478.
- Kostrzewska-Szlakowska I. 2005 – Surface microlayer in lakes of different trophic status: dissolved organic matter and microbial community – *Pol. J. Ecol.* 53: 341–351.
- Maki J.S. 1993 – The air-water interface as an extreme environment (In: *Aquatic Microbiology: An ecological approach*, Ed. T.F. Edgcombe) – Blackwell Scientific Publications, Boston, pp. 409–440.
- Maki J.S., Remsen C.C. 1989 – Examination of a freshwater surface microlayer for diel changes in the bacterioneuston – *Hydrobiologia*, 182: 25 – 34.
- O’Sullivan D.W., Neale P.J., Coffin R.B., Boyd T.J., Osburn C.L. 2005 – Photochemical production of hydrogen peroxide and methylhydroperoxide in coastal waters – *Mar. Chem.* 97: 14–33.
- Petasne R.G., Zika R.G. 1997 – Hydrogen peroxide lifetimes in south Florida coastal and offshore waters – *Mar. Chem.* 56: 215–225.
- Price D., Mantoura R.F., Worsfold P.J. 1998 – Shipboard determination of hydrogen peroxide in the western Mediterranean sea using flow injection with chemiluminescence detection – *Anal. Chem. Acta*, 371: 205–215.
- Scully N.M., Cooper W.J., Tranvik L.J. 2003 – Photochemical effects on microbial activity in natural waters: the interaction of reactive oxygen species and dissolved organic matter – *FEMS Microbiol. Ecol.* 46: 353–357.
- Scully N.M., Lean D.R.S., McQueen D.J., Cooper W.J. 1996 – Hydrogen peroxide formation: The interaction of ultraviolet radiation and dissolved organic carbon in lake waters along a 43 75°N gradient – *Limnol. Oceanogr.* 41: 540–548.
- Xenopoulos M.A., Bird F.B. 1997 – Effect of acute exposure to hydrogen peroxide on the production of phytoplankton and bacterioplankton in mesohumic lake – *Photochem. Photobiol.* 66: 471–478.

Received after revision January 2009