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IMPACT OF γ-IRRADIATION ON BIOFILM-FORMATION BY CORROSION-RELEVANT HETEROTROPHIC BACTERIA

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Abstract. *At nuclear hazard sites, such as the Chernobyl reactor sarcophagus or Fukushima Nuclear Power Plant, radiation is one of the main factors influencing microbial communities including those involved in microbially influenced corrosion (MIC) of metal structures. By studying the impact of radiation on corrosion-relevant bacteria it may be possible in the future to predict changes in MIC. We believe that the composition and function of natural multi-species biofilms will change when exposed to the stress of ionizing radiation. To address this possibility, biofilm formation by Pseudomonas pseudoalcaligenes and Stenotrophomonas maltophilia were studied after exposure to a range of radiation dosages. Altered planktonic cell morphologies and biofilm architectures on submerged glass surfaces were noted 3 – 7 days after low-doasage sub-lethal irradiation (5.3 Gy) of samples at the micro-colony, macro-colony and mature biofilm stages of development. Furthermore, significant differences in the percentage area covered by biofilms and the release of viable planktonic cells was also noted. These observations suggested that exposure, considered as insignificant levels of irradiation, can be enough to alter biofilm formation of corrosion-relevant bacteria. Such low dosage radiation may have significant impact on soil microbial communities in nuclear hazard sites, potentially altering the MIC of exposed metal structures, their stability and service life of underground metal constructions.*

Key words: *Bio-corrosion, biofilm formation, exo-polymeric substances, ionizing radiation, Pseudomonas pseudoalcaligenes, Stenotrophomonas maltophilia*

Introduction. The study of stress factors that impact on whole ecosystems, as well as specific organisms, is particularly relevant today because of growing anthropogenic influences in a wide range of environments. Stress

factors include polluting chemicals such as radionuclides (radioactive isotopes) and the contamination of soil by these is poorly understood, despite having significant impact on land use and communities worldwide (for reviews, see [1–4]. Such contamination reduces microbial population numbers and may also impact on community structure and function [5–8]. Particular concern today is the microbial-induced corrosion (MIC) of underground metal pipes, supports and cladding used for the Chernobyl reactor sarcophagus in northern Ukraine and others constructions (for reviews of MIC see Beech, Sunner, and Hiraoka 2005; Javaherdashti 2011; Lee and Newman 2003; for a review of the Chernobyl accident see [12]. At sites such as Chernobyl, radiation is one of the main factors influencing microbial communities [5]. In order to predict the impact of MIC on the sarcophagus it is necessary to study the influence of radiation on corrosion-associated bacterial communities (metal corrosion, concrete corrosion and other).

Bacteria can form biofilms on almost every natural and artificial surface causing problems for transport systems, industrial plants and structures (for reviews see [13–16]. According to our previous research, MIC occurs when heterotrophic bacteria such as *Pseudomonas pseudoalcaligenes* and *Stenotrophomonas maltophilia* produce slimes and biofilms containing sulphur-cycle bacteria adhere to metal surfaces [17]. Such mixed-species biofilms create anodic zones which promote iron oxidation and metal corrosion [18]. Bacteria encased within biofilms may be more resistant to the effects of ionizing radiation caused by the radioactive decay of nuclides compared to free-swimming ‘planktonic’ cells, and the survival also depends on species and growth status [19]. Doses above 2 kGy are lethal for biofilms [20, 21], and in comparison, relatively low dosages ranging between 0.5 – 5 Gy have been reported for the contaminated Chernobyl site [22]. This raises the possibility that the composition and function of multi-species biofilms may change when exposed to the stress of low-dosage ionizing radiation, resulting in enhanced (or decreased) corrosion activity that may have a critical impact on the survival and functioning of the reactor sarcophagus.

Our aim in this research was to determine the radiation sensitivity of the model bio-corrosion-associated biofilm-forming bacteria *P. pseudoalcaligenes* 109 UKM and *S. maltophilia* 5436 UKM [23]. As well the impact ionizing radiation has on biofilm formation and structure was studied by confocal laser

scanning microscopy (CLSM) and transmission electron microscopy (TEM). This knowledge will help us to better predict the impact of radiation on the bio-corrosion process in sites such as Chernobyl.

Materials and methods

Bacteria, culture conditions and enumeration. *Pseudomonas pseudoalcaligenes* 109 UKM and *Stenotrophomonas maltophilia* 5647 UKM, isolated from a corrosion-related community [24], were cultivated statically in 35 ml flasks containing 20 ml nutrient broth (NB) medium (Himedia, India) at 28 °C. Cells recovered from over-night NB cultures were washed in fresh NB medium (7 000 rpm / 10 min) to provide inocula for survival assays. Biofilms were formed on SDS pre-treated glass slides (10 x 40 mm) placed into flasks inoculated with bacteria and incubated until the micro-colony (1 h), macro-colony (1 day) and mature biofilm (3 day) stages [23] were reached before use. Planktonic cells for cells number determination were obtained directly from the culture, and biofilm-associated cells for microscopy analysis recovered by vortexing slides vigourously in PBS. Total cell counts were determined using a counting chamber with a phase-contrast microscope (MT 4000, MEIJI Tech, Japan) at 400x magnification and viable cell counts determined by dilution in fresh NB, spreading aliquots onto NB plates, and the enumeration of colony forming units (CFU).

Irradiation. Samples were irradiated at 5.3 Gy using a RUM-17 X-ray therapeutic apparatus (Budker Institute of Nuclear Physics (former USSR) at 200 kV and 10 mA, with 0.5 mm Cu and 1.0 mm Al filters, and at 1.3 – 14 kGy using an ILU-6 pulsed linear accelerator (Budker Institute of Nuclear Physics (former USSR) at 2 MeV and 17 mA.

Effect of irradiation on survival. Replicate flasks inoculated with 1 x 10^7 cell/ml washed cells were irradiated at 1.3 – 14 kGy for 20 min. Cultures were then incubated statically for 12 h before dilution and the determination of viable cell counts. Survival was expressed as the percentage of viable cells recovered compared to the no-radiation control, and all experiments were done in triplicate.

Effect of irradiation on cell morphology and biofilm-formation. Replicate flasks containing pre-treated glass slides were inoculated with 1 x 10^7 cell/ml washed cells and incubated to provide micro-colony, macro-colony and mature biofilm samples before irradiation at 5.3 Gy for 20 min. Samples were then assessed 0, 3 and 7 days after irradiation by electron microscopy to

investigate cell morphology, confocal laser scanning microscopy to investigate biofilm-formation and the determination of planktonic cell numbers. All experiments were done in triplicate.

Confocal laser scanning microscopy (CLSM). Glass slides were removed from flasks after irradiation and washed carefully with sterile water, air-dried and then stained with 0.1 % (w/v) Ethidium bromide (EtBr) for 10 min. 200 µl of the anti-fading agent CitiFuor (CitiFuor, USA) was added immediately before microscopic analysis. Images were obtained with a LSM 510 laser scanning module (Carl Zeiss, Germany) coupled to an inverted Zeiss Axiovert 100 MBP microscope using a plan-apochromatic 100x 0.79 oil DIC objective. For each sample 20 images were acquired, and the average surface area covered by biofilms measured using COMSTAT 1 [25].

Transmission electron microscopy (TEM). A modified floating-drop method was used for sample preparation [26], 2013). A 1 ml aliquot of irradiated culture was centrifuged at 10,000 x*g* for 5 s and a 100 µl drop of the re-suspended cell pellet placed on the surface of a piece of Parafilm (Bemis, USA). Cu-coated grids (Formvar, Russia) were placed onto the drop and cells allowed to adhere for 5-10 min before the grids were air-dried without staining. Images were obtained using a Jeol 1400 electron microscope (Jeol, Japan) at 80 kV, with 20 fields of view imaged for each sample.

Results and discussion

Effect of irradiation on the survival of Pseudomonas pseudoalcaligenes 109 UKM and Stenotrophomans maltophilia 5246 UKM cultures. The survival of *P. pseudoalcaligenes* and *S. maltophilia* cultures were assessed after 20 min of irradiation of 1.3 – 14 kGy. It should be mentioned, that D_{10}, the dose that is needed to eradicate 90% of the irradiated population significantly differs for both investigated strains (Figure 1). Although doasages higher that 4 kGy inhibited the growth of both strains, survival was significantly different at lower dosages (e.g. at 1.3 kGy: t-test, $P = 0.0019$) with *P. pseudoalcaligenes* showing greater resistance than *S. maltophilia* (Figure 1). Furthermore, 12 h after irradiation, morphological differences in cell shape were observed for both strains irradiated at 3.7 kGy, and cell lysis noted for those irradiated at ≥ 10 kGy. Increasing evidence of lysis was observed at high dosages where no viable cells were recovered, suggesting that the sublethal radiation-induced damage repair system of *S. maltophilia* [27] and *P. pseudoalcaligenes* are overwhelmed at ≥ 10 kGy.

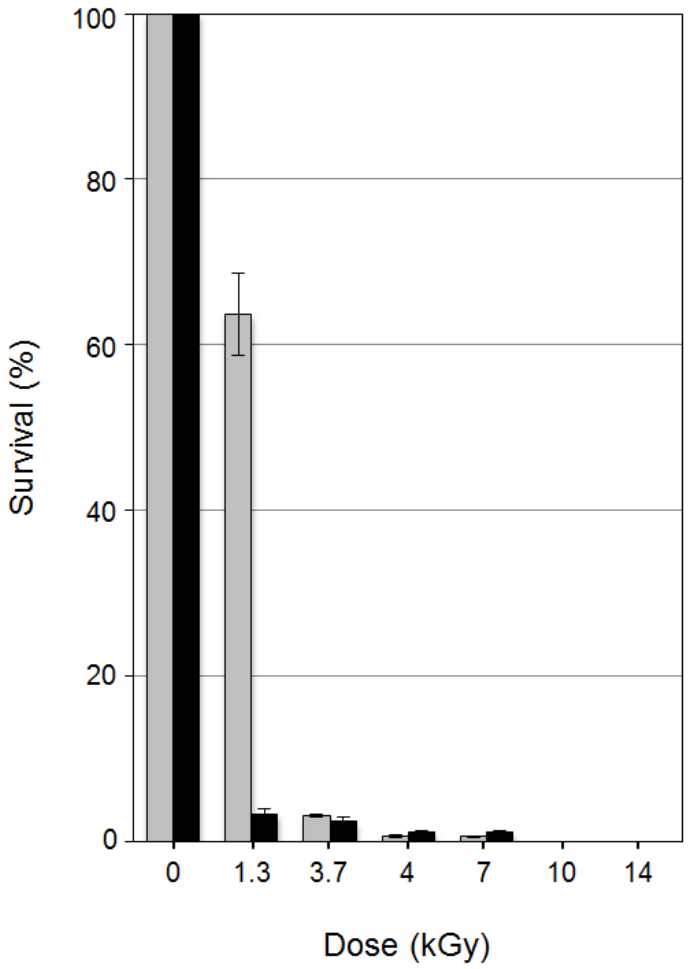

Figure 1. The model bio-corrosion-associated biofilm-forming bacteria, *P. pseudoalcaligenes* 109 UKM and *S. maltophilia* 5436 UKM, respond differently to low doses of irradiation. Shown here are the survival of *P. pseudoalcaligenes* (grey bars) and *S. maltophilia* (black bars) cultures after irradiation with 0 – 14 kGy for 20 min. Means and standard errors are shown (n = 3). The survival of the two strains is significantly different at 1.3 kGy (t-test, P = 0.0019).

Whilst irradiation levels ≥ 10 kGy are hardly observed at nuclear contamination sites, it is significant that irradiation in the range of that reported in soils sampled from underneath the Chernobyl sarcophagus (0.5 – 5 Gy) [22] were found to have an impact on *P. pseudoalcaligenes* and *S. maltophilia* cell morphology and survival. Damaged but viable populations might exhibit altered MIC activities within biofilms compared to unstressed wild-type populations and they might also lead to competitive mutant strains with altered colonisation and biofilm-formation characteristics.

The negative effect of both low- and highlevel radiation is well known [28, 29] but information is lacking on the ecological or evolutionary consequences of human-induced and naturally occurring radiation [30]. It is a common

knowledge, that the main mechanism of the organisms' sensitivity to ionising radiation is the size of genome as a main target for the action of irradiation along with the ability and speed of DNA repair processes [31]. Bacteria are one of the most radioresistant species on the planet – due to luckily combination of the two abovementioned factors.

Yet the range of bacteria ionizing radiation resistances is large [32–34], with a factor of 200 separating the most-resistant from the most-sensitive species [32]). For example, *Deinococcus radiodurans* can survive after high dosages of γ-irradiation (D_{10} is 10 kGy) that induce approximately 100 DNA double-strand breaks per genome, whereas *Shewanella oneidensis* is killed by doses ranged around 0.07 kGy that result in less than 1 double-strand breaks per genome (Daly et. al., 2004). There were reports about extremely radiotolerant species, such as *Rubrobacter radiotolerans* (D_{10} is 11 kGy) [35], *Chroococcidiopsis* spp. (D10 is 5.5 kGy) [34, 36] and others. Therefore, species, which have been described in this study has the medium radiotolerance and can be attributed neither to radioresistant, nor to radiosensitive species.

Influence of irradiation on biofilm formation and the release of planktonic cells. According to the traditional concept of biofilm formation, planktonic cells attach to a submerged surface with cells aggregating to produce micro-colonies, growing into macro-colonies, coalescing to form mature biofilms and releasing planktonic cells to colonise other sites, and during this developmental process, cell behaviour, physiology and gene expression patterns change significantly [13, 37]. It is therefore possible that sub-lethal irradiation causing substantial cellular stress and mutation might impact on this process depending on the relative timing of the exposure to radiation.

The impact of sub-lethal 5.3 Gy irradiation of micro-colonies, macro-colonies and mature biofilms on the subsequent development and growth of biofilms was investigated for *P. pseudoalcaligenes* and *S. maltophilia* using CLSM and TEM. Differences in the appearance of biofilms in exopolymer substances producing, cells distribution was observed for both strains. A series of representative CLSM images of biofilms and TEM images of cells after the exposure to irradiation at the micro-colony biofilm stage are shown in Figures 2 and 3. Irradiated samples formed atypical biofilms with conglomerates and filamentous exopolymer substances strands that were not observed in the non-irradiated controls. TEM images of individual cells

also showed morphological differences between irradiated and non-irradiated control samples.

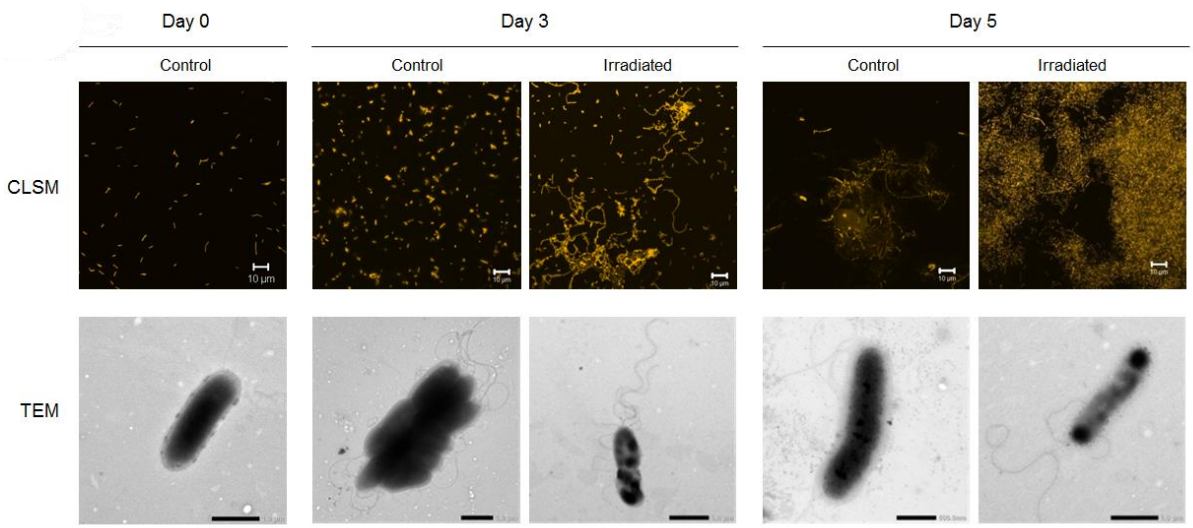

Figure 2. Irradiation at low levels of *P. pseudoalcaligenes* 109 UKM at the micro-colony, macro-colony and mature biofilm stages has a significant impact on subsequent biofilm-formation and cell morphologies. Shown here is a representative series of CLSM images of biofilms (top row) and TEM images of cells (bottom row) at the macro-colony stage (Day 0) and three and seven days after irradiation with 5.3 Gy for 20 min. CLSM scale bars indicate 10 μm and TEM scale bars indicate 0.5 and 1 μm.

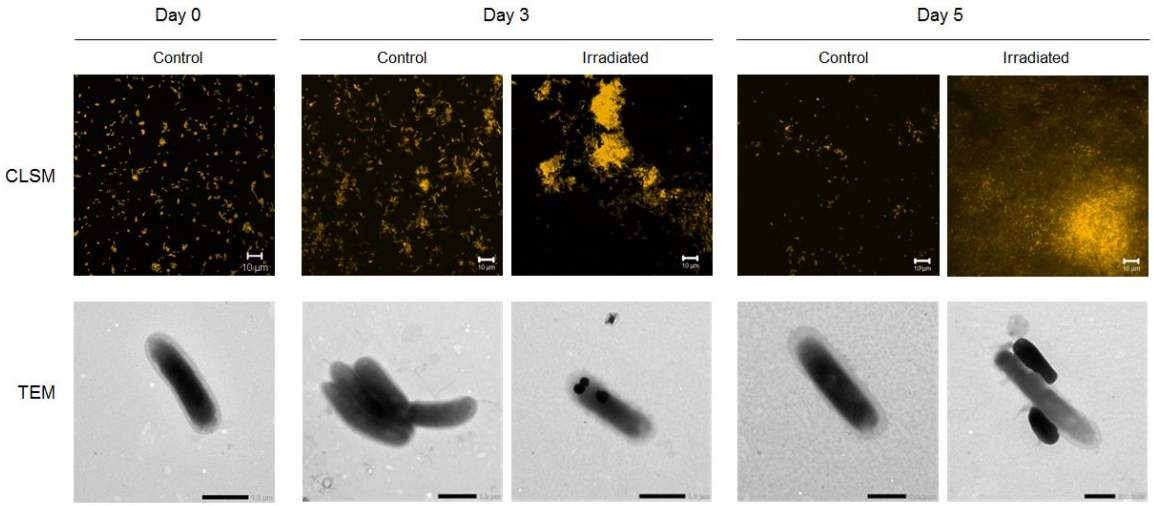

Fig. 3. Irradiation at low levels of *S. maltophilia* 5642 UKM at the micro-colony, macro-colony and mature biofilm stages has a significant impact on subsequent biofilm-formation and cell morphologies. Shown here is a representative series of CLSM images of biofilms (top row) and TEM images of cells (bottom row) at the macro-colony stage (Day 0) and three and seven days after irradiation with 5.3 Gy for 20 min. CLSM scale bars indicate 10 μm and TEM scale bars indicate 0.5 and 1 μm.

Such conglomerates, formed on the underground metal construction by corrosion relevant bacteria, could be more dangerous due to possibility of the metal covering by slime and unpredicted anaerobic zones formation. As showed in [10], hydrogen sulfide produced by anaerobic sulfate reduced bacteria lead to cathode zone formation and metal degradation. Also non-controlled processes could happen under such condition in mentioned dangerous nuclear plants area. Ecologically such biofilm formation changes could cause to forming bacterial community with new interrelationships on the genetic, metabolic communicates levels. Well-known, bacterial communities play a key role in the production and degradation of organic matter, the degradation of many environmental pollutants, and the cycle of nitrogen, sulfur, and many metals. Most of these natural processes require the concerted effort of bacteria with different metabolic capabilities, and it is likely that bacteria residing within biofilm communities carry out many of these complex processes. The microcolonies that constitute the biofilm can be composed of single-species populations or multimember communities of bacteria, depending on the environmental parameters under which they are formed. Numerous conditions, such as surface and interface properties, nutrient availability, the composition of the microbial community and hydrodynamics can affect biofilm structure [14].

Quantitative analyses of CLSM data revealed significant differences in biofilm development for *P. pseudoalcaligenes* and *S. maltophilia* after irradiation of micro-colonies, macro-colonies, and mature biofilm–stage samples (Table A). Seven days after the irradiation of *P. pseudoalcaligenes*, biofilm development from the micro-colony stage (1d) was not significantly affected (t-test, P = 0.543), whereas the biofilm development at the macro-colony stage (3d) was enhanced 5x (P = 0.002). In comparison, further biofilm development by *S. maltophilia* micro-colony–stage samples (1d) was reduced 0.3x (P = 0.001), development by macro-colony–stage samples enhanced 4x (P = 0.001).

Irradiation of micro-colonies, macro-colonies, and mature biofilm–stage samples also had an impact on the release of viable planktonic cells. Although the release of planktonic cells seven days after the irradiation of micro-colony–stage samples was enhanced 3x (t-test, P = 0.015) for *P. pseudoalcaligenes* but reduced 0.3x (P = 0.004) for *S. maltophilia*, the release of cells was unchanged (P = 0.244, 0.433) for macro-colony–stage samples and

reduced 0.6 – 0.5x (P = 0.023, 0.005) for mature biofilm–stage samples for *P. pseudoalcaligenes* and *S. maltophilia*, respectively. These observations further confirm that the timing of exposure to radiation can result in significant differences in the subsequent development of biofilms and release of planktonic cells by these model bio-corrosion-associated biofilm-forming bacteria.

Sites, which were contaminated by radionuclides have their own special needs. After the incident on the Fukushima Nuclear Power Plant Japanese government adopted the decision to collect the upper soil layer from the contaminated territories and deposit it in plastic bags, while the higher activity material will be separated and may be stored in metal and concrete containers [38, 39]. Thus appears the problem of prediction the state of the containers and the calculation of the time, during which it is still possible and safe to use them. In this situation the material of the containers really matters – because there is no doubt that, after the radioactive waste was once deposited, one of the main goals is to prevent the leakage and also to avoid disturbing the containers [38]. One of the factors, which need to be checked before using the material for long-term disposal of the radioactive waste is the amenability to the microbial corrosion and the influence of low for bacteria doses [40] on the intensity of metal and concrete bacterial distraction.

In accordance with lately investigations, exposure doses due to the radionuclide contamination can vary in dozens of times [41] and sometimes its estimation may cause lots of difficulties [42]. That is why it is essential to be as conservative as possible in estimation the risk of protective construction destruction and preventing the sources of contamination to spread in the environment.

Conclusion. This work has investigated the response of two model bio-corrosion-associated biofilm-forming bacteria to low-level dosages of irradiation. *P. pseudoalcaligenes* and *S. maltophilia* where found to have different survival curves, with *P. pseudoalcaligenes* more resistant at lower dosages of 1.3 kGy than *S. maltophilia*. Furthermore, biofilm-formation and the release of viable planktonic cells by both were found to be sensitive to the timing of irradiation during the biofilm-developmental process, suggesting that low-dosage irradiation of soil microbial communities in nuclear hazard sites might result in significantly altered biofilm-formation and the MIC of exposed metal structures.

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ВПЛИВ γ-ОПРОМІНЕННЯ НА ФОРМУВАННЯ БІОПЛІВКИ КОРОЗІЙНИМИ- ГЕТЕРОТРОФНИМИ БАКТЕРІЯМИ

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Анотація. *У місцях з підвищеним вмістом радіонуклідів, таких як саркофаг Чорнобильської АЕС або АЕС Фукусіма, випромінювання є одним з основних факторів, що впливають на мікробні спільноти, включаючи такі, що задіяниі у мікробіологічній корозії (МІС) металевих конструкцій. Вивчаючи вплив іонізуючого випромінювання на корозійно-активні бактерії, в майбутньому можна буде передбачити зміни МІС. Можна припустити, що склад і функції природних багатоскладових біоплівок будуть змінюватися під впливом стресу, викликаного іонізуючим випромінюванням. Для вивчення цієї можливості вивчали формування біоплівки Pseudomonas pseudoalcaligenes та Stenotrophomonas maltophilia під впливу декількох доз опромінення. Змінену морфологію планктонних клітин та архітектуру біоплівки на занурених поверхнях скла відзначали через 3 - 7 днів після сублетального опромінення з низьким вмістом дози (5,3 Гр) на етапах розвитку мікроколонії, макроколонії та*

зрілої біоплівки. Крім того, були відзначені суттєві відмінності у відсотках площі, охопленої біоплівками, та вивільнення життєздатних планктонних клітин. Ці спостереження дозволили припустити, що дози, що розглядаються як незначні, можуть бути достатніми для того, щоб змінити формування біоплівки корозійно-активних бактерій. Таке випромінювання з низькими дозами може мати значний вплив на грунтові мікробні спільноти в місцях ядерної небезпеки, потенційно змінюючи MIC відкритих металевих конструкцій, їх стійкість та термін служби підземних металевих конструкцій.

Ключові слова: *біокорозія, формування біоплівки, екзополімерні речовини, іонізуюче випромінювання, Pseudoomonas pseudoalcaligenes, Stenotrophomonas maltophilia*