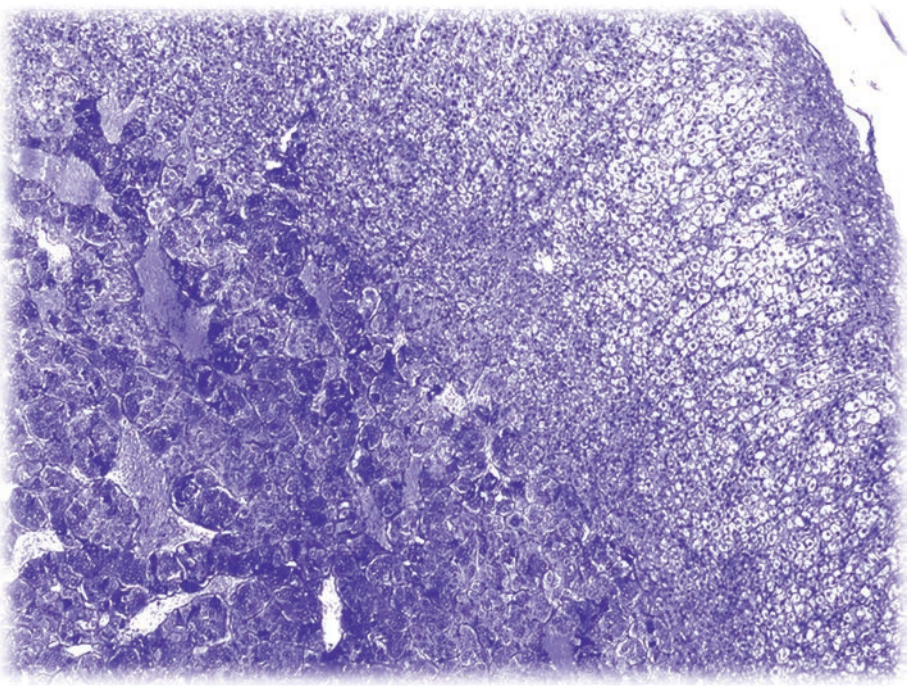
STUDIA UNIVERSITATIS
BABEŞ-BOLYAI



BIOLOGIA

2/2020

STUDIA
UNIVERSITATIS BABEŞ-BOLYAI
BIOLOGIA

2 / 2020
July - December

YEAR **Volume 65 (LXV) 2020**
MONTH **DECEMBER**
ISSUE **2**

PUBLISHED ONLINE: 2020-12-20
PUBLISHED PRINT: 2020-12-30
ISSUE DOI:10.24193/subbbiol.2020.2

STUDIA UNIVERSITATIS BABEŞ-BOLYAI

BIOLOGIA

2

STUDIA UBB EDITORIAL OFFICE: B.P. Hadeu no. 51, 400371 Cluj-Napoca, Romania,
Phone + 40 264 405352, www.studia.ubbcluj.ro

SUMAR – CONTENTS – SOMMAIRE – INHALT

REGULAR ARTICLES

REVIEWS

SHORT COMMUNICATIONS

All authors are responsible for submitting manuscripts in comprehensible US or UK English and ensuring scientific accuracy.

Original picture on front cover: Histological section (5µ thick) of the suprarenalian gland under Fluocinolon acetonid N treatment in male wistar rats (the atrophied cortical zone in the outer part of the gland, and the hypertrophied medullary region in the inner part).
Picture made with a 20x objective © Erika Kis

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 65, 2, 2020 (p. 5-18)
doi:10.24193/subbbiol.2020.2.01

A comparative study of adrenalin and fluocinolon induced oxidative stress in male wistar rats

Erika Kis[1]✉

[1]Babeş-Bolyai University, Faculty of Biology and Geology, Hungarian Department of Biology and Ecology, Cluj-Napoca, Romania;
✉***Corresponding author, E-mail: kiserika2001@yahoo.com.***

Article history: Received 12 August 2020; Revised 2 November 2020; Accepted 15 November 2020; Available online 20 December 2020.

Abstract. Hormone secretion by the hypothalamic-pituitary-adrenocortical (HPA) axis is modulated by multiple factors which include the circadian rhythm, various types of stressors and glucocorticoids. Treatment with synthetic glucocorticoids as e.g. dexamethasone or dermocorticosteroids and repeated immobilization stress, decreases the total body weight gain of animals by disturbing the HPA axis function and accelerating the catabolism of the organism. Synthetic glucocorticoids are widely used as anti-inflammatory and anti-allergic drugs. Neverteheless, their administration may cause side effects in the normal functioning of several organs. Starting from the above findings and from the important physiological roles of the glucocorticoids in the metabolism, we investigated the reactions of the adrenal and thymus, the evolution of the body and organ weight and the level of the free radicals after adrenaline- and fluocinolon stress. In this study, we used electron paramagnetic resonance spectroscopy for the direct detection of free radical content in the organs o f stressed Wistar rats. We followed the changes of the blood glucose level, body weight,structural modification and whole redox state of the rats during adrenaline and Fluocinolon-acetonid N treatment, as endogenous and exogenous sources of elevated glucocoticoid levels. We found a relationship between changes of the redox state and modified homeostasis of the organism, as an effect of elevated glucocorticoid levels. The oxidative stress induced by adrenalin treatment seemed to be an inducer rather than the result of the tissue damage.

Keywords: stress, thymus, adrenal, free radicals.

Introduction

Synthetic glucocorticoids are widely used as anti-inflammatory and anti-allergic drugs (Kis and Crăciun 2006, Adcock and Mumby 2017, Barnes 2017). Nevertheless, their administration may cause side effects in the normal

functioning of several organs. Organisms survive by maintaining equilibrium with their environment. The stress system is critical to this homeostasis. Glucocorticoids modulate the stress response at a molecular level by altering gene expression, transcription, and translation, among other pathways. Glucocorticoids also modulate the growth, reproductive and thyroid axes (Kis and Crăciun 2006). It is well known that glucocorticoids are stress hormones produced in response to many common stressors, such as immobilization, cold and physical overload, and are essential for general adaptive responses. Excessive glucocorticoid secretion or treatment, on the other hand, has been reported to have deleterious effects on the organism - it can induce tissue injury and even cell death (Crăciun *et al.,* 1997, Crăciun *et al*., 1998). Madar and co-workers (1993) have studied the effect of excessive glucocorticoid levels generated through repeated formaldehide stress or Fluocinolon acetonid-N (FC) treatment in rats. Formaldehide was used as an endogenous glucocorticoid inducer, while FC was an exogenous source of the hormone. Both formaldehide- and FC-treated groups showed significant metabolic disorders. The exact mechanism of glucocorticoid-induced cell death is unknown, but several reports indicate that the glucocorticoid-mediated generation of reactive oxygen species (ROS) occurs with the concomitant increase of calcium influx and morphological degeneration and apoptosis of different cells (Landfield and Eldridge 1994, Liu *et al*., 2018, Deng *et al*., 2019). In recent studies elsewhere we have reported that the short-term and long-term percutaneous applications of halogenated glucocorticosteroids in pregnant rats induced changes of thymus oxidative status of dams and newborn animals (Kis and Andras 2017). In immobilization-stressed rats, where glucocorticoid levels were slightly elevated, a marked increase in lipid peroxidation was measured in addition to the inhibition of total body weight gain, showing the involvement of ROS (Yan *et al*., 2000).

Despite numerous investigations, the involvement of free radicals in glucocorticoid-induced alteration is not fully elucidated. In our experiments we followed the changes of body- and some gland weight, the level of free radicals in these glands and the histological aspects of the rat thymus and adrenals during adrenalin (ADR) and Fluocinolon (FC) treatment. We used andrenalin and Fluocinolon-acetonid-N oitment, as endogenous and exogenous sources of elevated glucocorticoid levels. Therefore, the main objective of this study is to determine correlations between glucocorticoid excess and free radical parameters induced by FC and ADR treatment.

Materials and methods

The experiments were carried out in male Wistar rats. The animals were kept under standardized bioclimatic conditions and fed on common rat chow, with water *ad libitum*.

Commercial Fluocinolon-acetonid-N ointment containing 25 mg Fluocinolon-acetonid-N/100 g excipient, was applied topically to the skin at 2 cm2, for five consecutive days, by smearing 50 mg ointment/100 g bw. on the inguinal region, the daily dose of ointment being equal to 12,5 μg/100 g bw. The ADR-treated animals were injected subcutaneously with 0.125 ml of adrenalin (0.1%) solution. The animals were divided into the following groups:

C-control group, untreated animals.
FC-Fluocinolon-acetonid-N-treated animals
ADR- adrenalin-treated group

After 16 hours of fasting and 24 hours following the cessation of treatments, the treated animals together with controls were sacrificed by exsanguination.

Blood samples were obtained from all rats at the end of the experiment and blood glucose levels were measured with Spekol spectrophotometer. The value of the glucose was expressed in mg glucose per 100 ml blood.

The body, adrenals and thymus weights of male rats were measured with an accuracy of 0.00001 g immediately after excision. Before the tissue withered away, they were placed in frozen liquid nitrogen to examine the total level of free radical of the glands. The free radical concentration in frozen samples was measured by electron spin resonance (ESR) spectroscopy. Reduced gluthatione (GSH) and gluthatione disulfide (GSSG) ratio was determined by tissue homogenization (Kis 2012, Schafer and Buettner 2001, Tietze 1969). The experimental data were evaluated using the computer program statistica 99. The mass, ESR signal intensity, antioxidant capacit and GSH/GSSG ratio values showed a non-parametric distribution (Mann-Whitney U test), so the significance levels were determined by parametric t-test. A $p < 0.05$ was considered statistically significant.

The thymus and adrenal gland were fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The fragments were sectioned at the Reichart microtome with a thickness of 7 μ. The staining of glands was carried out by the method of Hurduc and co-workers (Mureşan *et al*., 1974). The histological preparations obtained were examined on the $IORC_4$ optical microscopy.

Results

The effects of adrenalin and Fluocinolon-acetonid-N treatments upon the blood glucose level

In both treated groups we observed a significant increase of blood glucose level (Fig.1), which means that the blood level of the glucocorticoids increases, on the one hand due to the treatment with exogenous glucocorticoid, and on the other hand, due to adrenalin stress. We used glicemy as an indirect indicator for the action of glucocorticoids. We assumed that the high blood glucose level in our experimental protocol was caused by high levels of glucocorticoids.

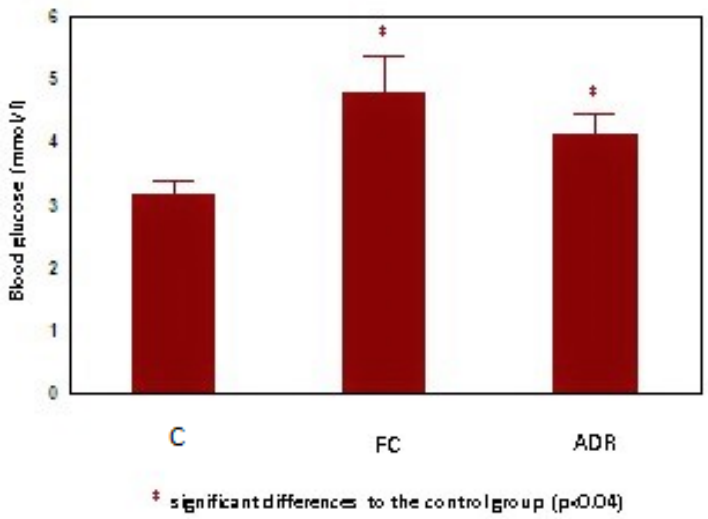


Figure 1. Blood glucose level in controls and treated animals with FC and ADR

The effects of adrenalin and Fluocinolon-acetonid-N treatments upon the body weight

The results presented in fig. 2 show that in our experiment fc treatment and adr stress caused a progressive inhibition of total body weight gain of animals compared to the controls.

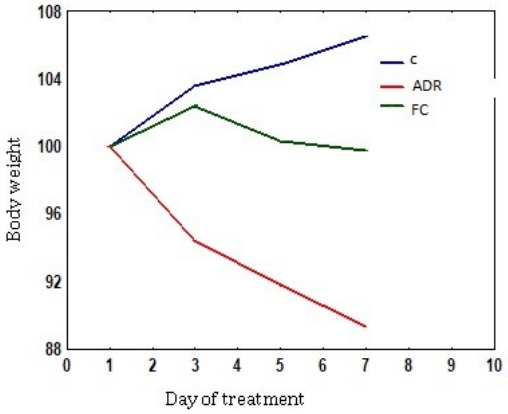


Figure 2. Evolution of the body weight under adrenalin (ADR) and Fluocinolon (FC) treatment in comparation with control.

The Fluocinolon-acetonid-N treatment caused a significant weight loss in contrast with the control group (Fig. 3). However, a significant weight loss in FC group was observed as compared to the ADR treated animals. Body weight loss in ADR-treated group was lower than in the FC-treated one, showing that ADR induces a milder stress than the direct cortisol treatment.

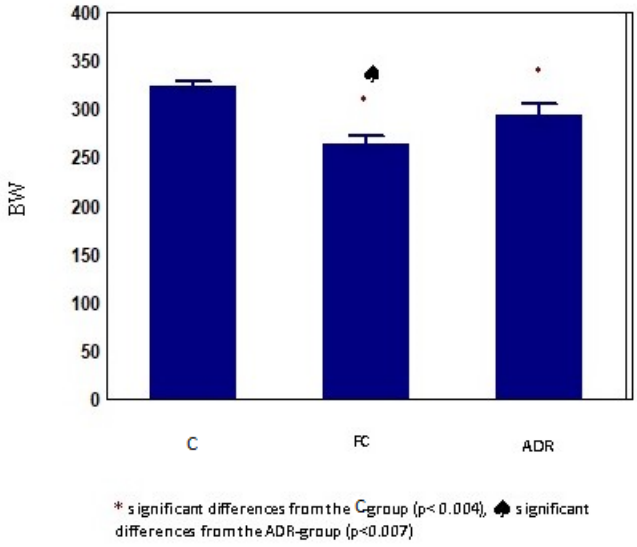


Figure 3. The body weight in the last day of the treatments in control and treated groups

The effects of adrenalin and Fluocinolon-acetonid-N treatments upon the adrenal gland

The relative adrenal weight (Fig. 4.) decreased in FC group comparative with the contol and ADR group. In the ADR group we observed a slight increase of the gland weight. These findings are in compliance with our histological results. The free radical concentration increased considerable in the FC group and decreased in the ADR group compared to controls (Fig. 5).

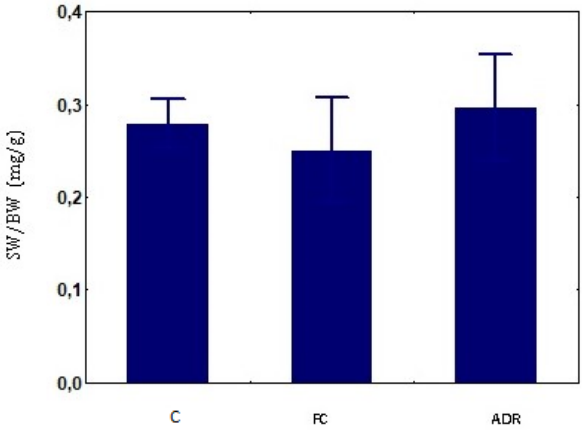


Figure 4. The relative weight of the adrenal gland (SW-suprarenal weight, BW-body weight) in control and treated groups

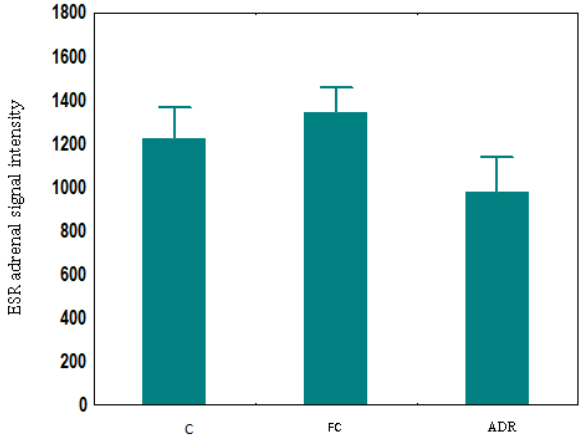


Figure 5. Steady state free radical concentration in adrenal gland under FC and ADR treatment in comparation to the control

Modification of the gland weight and the free radical concentration are in concordance with the results of the microscopical examination (Fig. 6 a). The suprarenal gland of animals treated with Fluocinolon is atrophied in the cortical part (Fig. 6 b), whereas in animals treated with adrenalin we observed the hypertrophy of the gland in the cortical zone (Fig. 6 c). In the FC treated group we observed a hypertrophy of the medullary part of the gland, suggesting a stressful effect of the glucocorticoid treatment.

Spongyocites and balonated cortical cells appeared in this group. In the medullary part we observed congested capillary. In the ADR group we observed dark zone in the cortical part of the gland, suggesting an increased synthetic activity of the cells.

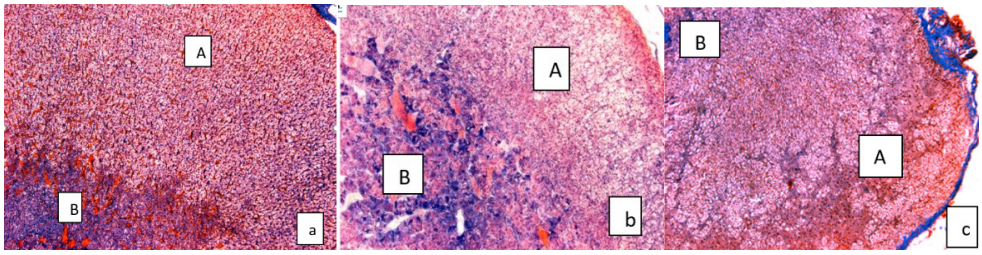


Figure 6. Histological aspect of adrenal gland: a) normal aspect, b) hypertrophy of medullary part (B) and atrophy of the cortical zone (A) in FC group, c) hypertrophy of cortical zone (A) and atrophy of medullary part (B) in ADR group(x 20).

The effects of adrenalin and Fluocinolon-acetonid-N treatments upon the thymus

The relative thymus weight was significantly reduced in FC group (Fig. 7) During the adrenaline treatment we observed a slight decrease of the thymus weight. The steady state free radical concentration increased in FC group in comparison with the control group. These finding are in concordance with the loss of weight in FC group. In ADR group we observed a significant decrease of the steady state free radical concentration in comparison with FC group.

The ratio of GSH/GSSG increased significantly in ADR group as compared with controls (Fig. 8). Modification of steady state free radicals and the GSH/GSSG ratios indicated changes in the redox state of organs of treated animals (Fig. 9).

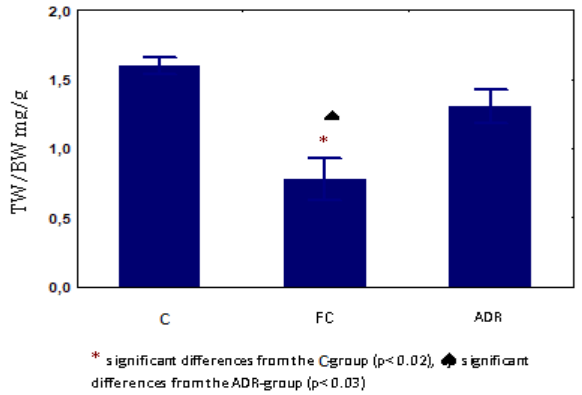


Figure 7. The relative weight of the thymus in control and treated groups

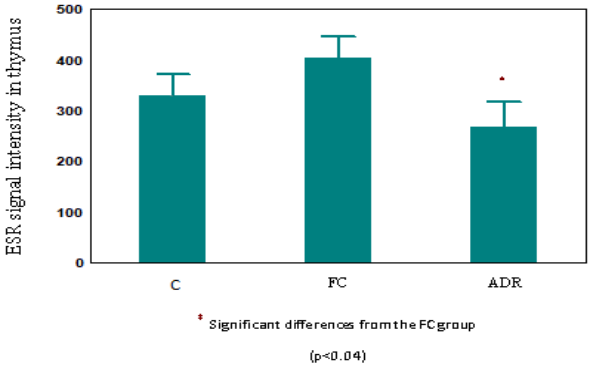


Figure 8. Steady state free radical concentration in the thymus, in control and treated groups

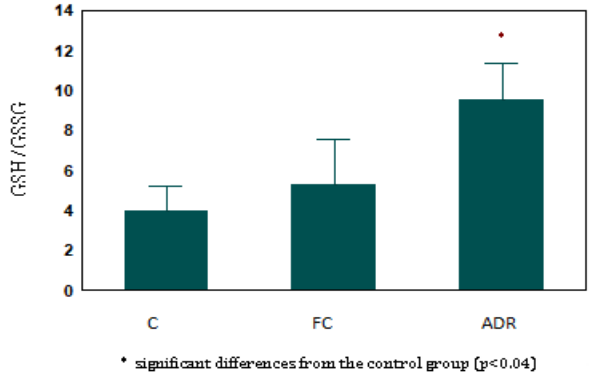


Figure 9. GSH/GSSG in thymus in control and treated groups

The microscopic studies confirm the weight loss of the thymus (Fig. 10 a). Fluocinolon treatment caused the atrophy of the thymus (Fig. 10 b), while in the animals treated with adrenalin the thymus structure hardly differed from the control group (Fig. 10 c). In the FC treated group (Fig. 10 b) we observed a significant rarefication of the corticol and medullary zone of thymic lobule. This means that FC induced an acceleration of thymocites apoptosis.

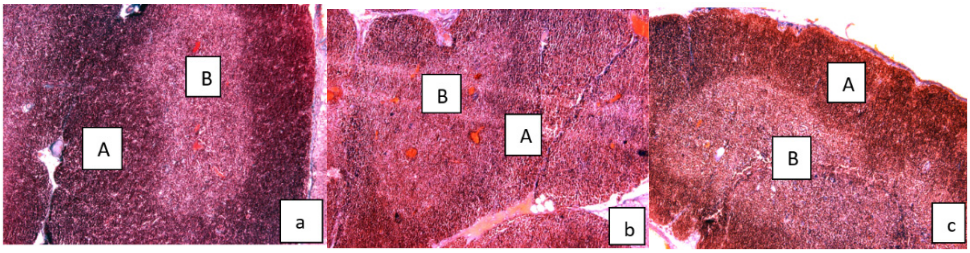


Figure 10. Histological aspects of thymus a) normal structure, A-cortical part of the thymic lobule, B -medullary part of the lobule b) relatively normal aspect of thymus in ADR group, c) moderately altered in FC group (x10)

Discussion

The most frequently studied effect of glucocorticoid application was the impact on blood sugar level. The excess of glucocorticoids increases in liver the storage of glycogen, obstructs the movement of glicogen in the blood and stimulates the glucogenesis (Ivy *et al.*, 2016, Waldron *et al.*, 2013). Glucocorticoids inhibit the uptake and usage of sugar in the muscle and adipose tissues (Gounarides *et al.*, 2008, Lundgren *et al.,* 2004)]. In a long term glucocorticoid application, initial insuline resistance appears at the final stage of diabetes mellitus (Madar *et al.,* 1993). On the basis of these data from the literature corroborated with our experimental data we can conclude that the high level of glucose from the blood is an indicator of the increased level of glucocorticoids. Excessive glucocorticoid level can also activate the HPA axis (Paragliola *et al.,* 2017). Adrenalin has been shown to stimulate both glucose metabolism and H_2O_2 release by macrophages (Costa Rosa *et al.*, 1995).

Excessive exogen glucocorticoid administration inhibits somatotrophic hormone release (Kis *et al.,* 2001) and can cause significant decrease of total body weight [Crăciun *et al.*, 1998, Crăciun *et al.,* 1997, Kis and Crăciun 2006, Orzechowski *et al.*, 2000a). In our work, we used FC ointment as a synthetic source of glucocorticoids and ADR as an endogenous inducer of glucocorticoid production in rats. The observed changes in total body mass and in some organs

weight modification indicate that both exogenously added and ADR-induced glucocorticoid increase induced the activation of catabolic pathways in the rat organism, only ADR turned out to be a milder stressor than FC. A similar situation has been described, where formaldehyde treatment resulted to be less stressful to rats than FC (Madar *et al.,* 1993).

Stress causes disturbances in the homeostasis of the organism, which may lead to tissue injury. Glucocorticoids are widely used as anti-inflammatory drugs because of their immunosuppressive activity (Daley-Yates 2015). Yet, the presence of high glucocorticoid levels can cause secondary side effects such as tissue damage and cell death (Deng *et al*., 2019, Mann *et al*., 2000, Nittoh *et al*., 1998, Orzechowski *et al*., 2000b, Sapolsky *et al*., 1999, 2000).

We have detected a severe degeneration of the thymus in response to FC treatment and adrenal atrophy, although Madar and co-workers (1993) was able to detect adrenal atrophy induced by a two-fold higher dose of FC. To find out whether glucocorticoid-induced tissue damage was related to oxidative stress, we have measured several redox parameters such as steady state level of free radicals and the ratio of GSH/GSSG.

Orzechowski and co-workers (Orzechowski *et al.,* 2000a) described a strong oxidative stress in old and young rats induced by dexamethasone treatment, which was manifested by a significant decrease of blood GSH levels and spleen atrophy. Mild oxidative stress was detected in rats treated with two times lower dose of dexamethasone, as measured by enhanced glutathione peroxidase and catalase activities, as well as reduced thiobarbituric acid reactive substance content in thymus and spleen (Pereira *et al*., 1998, 1999). In every case, oxidative stress was associated with organ atrophy indicating that tissue degeneration may be related to changes in the redox state of the organs. Glutamine oxidation is stimulated by adrenaline, thus providing increased substrate (malate) for NADP (+)-dependent 'malic' enzyme (Madar *et al*., 1993, Pereira *et al*., 1995). In our experiments, ADR treatment resulted in mild thymus atrophy, which was associated with drastically increased GSH/GSSG ratio. Free radical concentration in the organ was not elevated suggesting that the glutathione antioxidant machinery has been induced by pro-oxidants and was able to prevent the development of oxidative stress in the tissue. FC, on the other hand, induced severe thymus atrophy followed by high free radical concentrations. Interestingly, GSH/GSSG ratio remained normal, suggesting that the antioxidant machinery was not induced anymore and was unable to prevent oxidative injury. The changes in the antioxidant defense system of the thymus may be an explanation why this organ is more susceptible to FC treatment. An intensive glucocorticoid treatment or chronic stress can cause changes in the redox state of the lymphoid organs, modifying the activity of antioxidant enzymes and can influence the antibacterial and antiviral defense (Gavan *et al*., 1997, Seiji *et al.,* 1997, Pereira *et al*., 1999).

The fact that the antioxidant system of thymus was induced before the atrophy developed suggests that pro-oxidants are generated at a very early stage of tissue degeneration. By the time organ atrophy fully developed, the induction of GSH synthesis ceased and oxidative stress evolved in thymus and adrenal gland. These observations suggest that oxidative species are present at the very beginning of glucocorticoid-induced tissue injury and may participate in the process of organ degeneration. It will be interesting to investigate whether co-administration of glucocorticoids to rats with different antioxidants could be useful in preventing organ atrophy.

Conclusions

In summary, we can conclude:

Glucocorticoids can induce oxidative stress and lead to tissue injury even at relatively low doses.

Milder and more severe organ atrophies were observed in the thymus and adrenal of ADR and FC treated rats, which was associated with oxidative stress measured in these tissues.

The response to elevated glucocorticoid levels was tissue-dependent, thymus being the most susceptible to injury from among the studied organs.

The oxidative stress takes place in the organs from the very beginning of the atrophic process, suggesting that it is not merely a result but rather an active participant of degeneration.

References

Adcock, I.M., & Mumby, S. (2017). Glucocorticoids, *Handb. Exp. Pharmacol.*, 237: 171-196. doi:10.1007/164_2016_98.

Barnes, P.J. (2017). Glucoccorticosteroids, *Handb. Exp. Pharmacol.*, 237: 93-115. doi: 10.1007/164_2016_62.

Costa Rosa, L. F., Curi, R., Murphy, C., & Newsholme, P. (1995). Effect of adrenalin and phorbol myristate acetate or bacterial lipopolysaccharide on stimulation of pathways of macrophage glucose, glutamine and O_2 metabolism. Evidence for cyclic AMP-dependent protein kinase mediated inhibition of glucose-6-phosphate dehydrogenase and activation of NADP+dependent 'malic' enzyme, *Biochem. J.*, 310, 709-714.

Crăciun, C., Madar, I., Tarba, C., Frăţilă, S., Ardelean, A., Crăciun, V., & Ilyes, I. (1998). Correlation betwen ultrastructural thymus modification, thymolisis, thymus and blood-serum lipids in response to epicutaneously applied dermacorticosteroids in pubertal rats, In: *Current Problems in Cellular and Molecular Biology*, Crăciun, C., Ardelean, A., (eds.), Risoprint, pp. 218-235.

Crăciun, C., Ardelean, A., Madar, I., Tarba, C., Şildan, N., Crăciun, V., & Fărcaş,T. (1997). Ultrastructural studies of the secondary effets induced at the level of thymus by topic application of Fluocinolon-acetonid-N in prepubertal rats, In: *Current Problems and Techniques in Cellular and Molecular Biology*, Crăciun, C., Ardelean, A. (eds.), Mirton, pp.176-186.

Daley-Yates, P.T. (2015). Inhaled corticosteroids: potency, dose equivalence and therapeutic index, *Br. J. Clin. Pharmacol.*, 80(3): 372-380.

Deng, S., Dai, G., Chen, S., Nie, Z., Zhou, J., Fang, H., & Peng, H. (2019). Dexamethasone induces osteoblast apoptosis through ROS-PI3K/AKT/GSK3β signaling pathway, *Biomed. Pharmacother.*, 110, 602-608.

Gavan, N., Popa, R., Orasan, R., & Maibach, H. (1997). Effect of pericutaneous absorbtion of fluocinolon acetonide on the activity of superoxide dismutase and total antioxidant status in patients with psoriasis, *Skin Pharmacol.*, 10, 178-82.

Gounarides, J.S., Korach-André, M., Killary, K., Argentieri, G., Turner, O., & Laurent, D. (2008). Effect of dexamethasone on glucose tolerance and fat metabolism in a dietinduced obesity mouse model*, Endocrinol.*, 149 (2), 758-766.

Ivy, J.R., Oosthuyzen, W., Peltz, T.S., Howarth, A.R., Hunter, R.W., Dhaun, N., Al-Dujaili, E.A.S., Webb, D.J., Dear, J.W., Flatman, P., & Bailey, A.M. (2016). Glucocorticoids induce nondipping blood pressure by activating the thiazide-sensitive cotransporter, *Hypertension*, 67, 1029-1037.

Kis, E. (2012). Glucocorticoid excess and fetal development in white Wistar rats. *Annals of Romanian Society for Cell Biology*, 17 (2), 82-85

Kis, E., & András, P. (2017). Has the Fluocinolon-acetonid N ointment any effect on the kidneys and the thyroid gland structure and function? *Studia UBB Biologia*, 62(2), 41-52..

Kis, E., & Crăciun, C. (2006). Efecte secundare ale unor glucocoticoizi topici, *Ed. Risoprint*, Cluj-Napoca.

Kis, E., Crăciun, C., Paşca, C., Sandu, V. D., Crăciun, V., & Madar, I. (2001). Comparative studies of the ulrastructure of somatotrope, gonadotrope and corticotrope cells in mature rats treated with topical dermocorticoids, *Studia UBB Biologia*, 46(1), 99-110.

Landfield, P.W., & Eldridge I.C. (1994). The glucocorticoid hyphotesis of age-related hippocampal neurodegeneration: role of dysregulated neuronal calcium, *Ann. N. Y. Acad. Sci.*, 746, 308-321.

Liu, W., Zhao, Z., Na, Y., Meng, C., Wang, J., & Bai, R. (2018). Dexamethasone-induced production of reactive oxygen species promotes apoptosis via endoplasmic reticulum stress and autophagy in MC3T3-E1 cells, *Internat. J. Molec. Medicin*, 41(4), 2028-2036.

Lundgren, M., Burén, J., Ruge, T., Myrnäs, T., & Eriksson, J.W. (2004). glucocorticoids downregulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes, *J. Clin. Endocrinol. Metab.*, 89 (6), 2989-2999.

Madar, I., Şildan, N., & Frecuş, G. (1993). Studiul comparativ al efectului stresului şi tratamentului cu fluocinolon-acetonid asupra unor parametri endocrino-metabolici la şobolani Wistar tineri, *Stiinte şi cercetări biologice, Seria Biol. Anim.,* t. 45(1), 53-59.

Mann, C.L., Hughes, F.M., & Cidlowski, J.A. (2000). Delineation of the signalling pathway involved in glucocorticoid-induced and spontaneous apoptosis of rat thymocytes, *Endocrynol.*, 141:528-538.

Mureşan, E., Gaboreanu, M., Bogdan, A.T., & Baba, A.I. (1974). Tehnici de histologie normală şi patologică, *Ed. Ceres*, Bucureşti, 482pp.

Nittoh, T., Fujimoti, H., Kozumi, Y., Ishihara, K., Mue, S., & Ohuchi, K. (1998). Effects of glucocorticoids on apoptosis of infiltrated eosinophils and neutrophils in rats, *Euro. J. Pharmacol.*, 354, 73-81.

Orzechowski, A J., Grizard, M., Jank, B., Gajkowska, M., Lokociejewska, M., & Godlewskia, M. (2000a). Dexamethasone-mediated regulation of death and differentiation of muscle cells. Is hydrogen peroxide involved in the process? *Reprod. Nutrit. Develop.*, 42,197-216.

Orzechowski, A.P., Ostaszewski, J., Wilczak, M., Jank, B., Balasinska, P., & Wareski, J. F. (2000b). Rats with a glucocorticoid-induced catabolic state, show symptoms of oxidative stress and spleen atrophy: the effect of age and recovery, *J. Vet. Med.,* 49, 256-263.

Paragliola, R.M., Papi, G., Pontecorvi, A., & Corsello, S.M. (2017). Treatment with synthetic glucocorticoids and the hypothalamus-pituitary-adrenal axis. *Internat. J. Mol. Sci.*, 18(10), 2201.

Pereira, B., Costa-Rosa, L.F.B.P., Bechara, E.J.H. , Newsholme, P., & Curi, R. (1998). Changes in TBARs content and superoxid dismutase, catalase and glutathione peroxidase activities in the lymphoid organs and skeletal muscles of adrenodemedullated rats, *Braz. J. Med. Biol. Res.,* 31, 827-833.

Pereira, B., Costa Rica, L.F.B.P., Safi, D.A., Bechara, E.J.H., & Curi, R. (1995). Hormonal regulation of superoxid dismutase, catalase, and glutathione peroxidase activities in rat macrophages, *Biochem. Pharmacol.*, 50 (12): 2093-2098.

Pereira,B., Bechara, E.J.H., Mendoca, J.R., & Curi, R., (1999). Superoxid dismutase, catalase and glutathione peroxidase activities in teh lymphoid organs and skeletal muscles of rats treated with dexamethasone, *Cell Biochem., Funct.*, 17, 15-19.

Sapolsky, R. M. (1999). Glucocorticoids, stress, and their adverse neurological effects: relevance to aging, *Exper. Gerontol.*, 34, 721-732.

Sapolsky, R.M., Romero, L.M., & Munck, A.U. (2000). How do glucocorticoids influence Stress Responses? Integraing, Permissive, Supressive, Stimulatory and Preparative Actions, *Endocr. Rev.*, 21(1), 55-89.

Schafer, Q.F., & Buettner, R.G. (2001). Redox environment of the cell as viewed trought the redox state of the glutathione disulfide/glutathione couple, *Free Radic. Biol. Med.*, 30(11), 1191-1212.

Seiji, M., Yasuaki, N., Eisuke, F., Sato, K.Y., Masanobu, M., & Masayasu, I. (1997). Cold stress induces thymocyte apoptosis in the rat, *Pathophys.*, 213-219.

Tietze, F., (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues, *Ann. Biochem.*, 27, 502-522,1969.

Waldron, N.H., Jones, C.A., Gan, T.J., Allen, T.K., & Habib, A.S. (2013). Impact of perioperative dexamethasone on postoperative analgesia and side-effects: systematic review and meta-analysis, *Br. J. Anaesth.,* 110, 191-200.

Yan, H., Arturo, C., Erdal, G, Philip, A., & Mohammed, K. (2000). Anti-stress effects of dehydroepiandrosterone, *Biochem. Pharmacol.*, 59, 753-762.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 65, 2, 2020 (p. 19-29)
doi:10.24193/subbbiol.2020.2.02

Assessment of enhanced biodegradation potentials of animal wastes on diesel-contaminated soil

Tosan Akiri-Obaroakpo[1]✉, Emmanuel Imarhiagbe[1] and Frederick Ekhaise[2]

[1]University of Benin, Faculty of Life Sciences, Department of Environmental Management and Toxicology, Benin City, Nigeria; [2]University of Benin, Faculty of Life Sciences, Department of Microbiology, Benin City, Nigeria;
✉***Corresponding author, E-mail:*** *mercy.akiri-obaroakpo@uniben.edu.*

Article history: Received 11 February 2020; Revised 16 November 2020; Accepted 4 December 2020; Available online 20 December 2020.

Abstract. Oil spillage is a menace, crippling most oil-producing regions around the globe**.** The aim of this study was to access the role of poultry litter and cow dung in enhancing biodegradation of diesel-contaminated soil. The treatment sets were split into three levels of diesel pollution (50 mL, 100 mL and 150 mL) amended with poultry litters, cow dung and a mixture of both amendments. The microbiological properties**-**and the total petroleum hydrocarbon content was analyzed for a period of six months using the pour plate techniques and Gas Chromatography (GC-FID), respectively. Agarose gel electrophoresis was used for plasmid detection. Mean total heterotrophic bacterial counts ranged between 40.5±0.5 x10^4 cfu^{-1} and 102.0 ±4.0 x10^4 cfu^{-1}, for C_1 (soil with poultry litter and cow dung with 50mL diesel) and Control 2. The mean total hydrocarbonoclastic bacterial counts ranged from 42.0±2.0 x10^4 cfu^{-1} to 66.5±2.5 x10^4 cfu^{-1} for B_1 (soil with cow dung with 50mL diesel) and C_3 (soil with poultry litter and cow dung with 150mL diesel). *Bacillus subtilis* (25.7%) and *Staphylococcus aureus* (4.73%) were reported as the isolates with the highest and least percentage frequency of occurrence. The percentage of diesel oil degradation was highest in C_1 (98.5%) and lowest in Control 1 (31.6%). Plasmid extraction studies carried revealed that two out of the five hydrocarbonoclastic bacteria had both plasmids and chromosomal genes. The result has indicated the enhanced capacity of mixed amendments relative to individual waste treatment used in this study and should be recommended for bioremediation application.

Key words: Diesel oil pollution, animal wastes, total petroleum hydrocarbon, plasmid profile.

Introduction

Oil spill is a term which defines the discharge of liquid petroleum hydrocarbons into the environment and it is a form of pollution. The term oil spill applies mostly to the marine environment where oil is released into the water body (ocean or coastal waters), but it could also occur on land; Adelana *et al.* (2011). This could be through pipeline and tankers corrosion, sabotage, oil production operations and the spill of any oily refuse or waste oil; Adelana *et al.* (2011). The effect of oil spillage on terrestrial environment has damaging impact on crop grown on it (Pepper *et al.,* 1996). The physical properties of floating oil may hinder respiration, photosynthesis or feeding of plants and animals. Bioremediation is the use microorganisms to breakdown contaminant into less harmful compounds (Omotayo *et al*., 2012). In bioremediation, the microorganisms used may be autochthonous to the polluted site or they may be brought from elsewhere to the contaminated site to complement the action of the resident microbial population. Many plasmids carry genes that confer a selective advantage to their hosts in certain environment (De Magalhaes *et al.,* 2008; Mohania *et al.,* 2008). In recent times, organic wastes are being used to stimulate microbial consortium in order to enhance the biodegradation process. Organic wastes contain large amounts of nitrogen because of the presence of high levels of proteins and amino acids. In this study, the aim is to determine the role of poultry litter and cow dung in enhancing biodegradation of diesel-contaminated soil.

Materials and methods

Soil and diesel collection

Soil samples were collected from the Animal and Environmental Biology (AEB) Experimental Garden, Faculty of Life Sciences from a depth of 0-15 cm. The animal wastes, cow dung and poultry litters, were collected from University of Benin Agricultural Farm and the cattle market at Benin Technical College Road, Benin City, Edo state, Nigeria, at coordinates (6° 23'49"N, 5°36'55"E), (6°24'11"N, 5°36'37"E). The petroleum (diesel) used for the experiment was from a filling station in Benin, Edo State, Nigeria.

Treatment set up

Triplicates of eleven samples was set up for the experiment:
Control 1: 2.0 kg of uncontaminated soil only
Control 2: 2.0 kg soil contaminated with 50 mL of diesel

Treatment A_1: 2.0 kg of soil contaminated with 50mL diesel with 100 g of poultry litter; A_2: 2.0 kg of soil contaminated with100 mL diesel with 100 g of poultry litter; A_3: 2.0 kg of soil contaminated with 150mL diesel with 100 g of poultry litter.

Treatment B_1: 2.0 kg of soil contaminated with 50mL diesel with of 100 g of cow dung; B_2: 2.0 kg of soil contaminated with 100 mL diesel with 100 g of cow dung; B_3: 2.0 kg of soil contaminated with 150 mL diesel with 100 g of cow dung.

Treatment C_1: 2.0 kg of soil contaminated with 50 mL diesel with ~~of~~ 100 g of poultry litter and cow dung; C_2: 2.0 kg of soil contaminated with 100 mL diesel with 100 g of poultry and cow dung; C_3: 2.0 kg of soil contaminated with 150mL diesel with 100 g of poultry litter and cow dung.

The experiment was carried out for a period of six months (three months of rainy season and three months of drought). The perforated buckets containing the soil samples were kept in the open but protected from the direct effect of rain. During this period, the soil samples were stirred and the temperature of the soil taken at regular interval. Soil samples were bimonthly collected for analysis.

Enumeration of total heterotrophic and hydrocarbon utilizing bacterial counts

Enumeration of bacterial isolates was carried out on treatments by measuring 10 g of soil sample into 90 mL of distilled water. This mixture was agitated and 1mL was taken from each mixture representing each treatment and was serially diluted to make a ten-fold diluent. Aliquot of 1 mL of the 10^{-4} and 10^{-6} dilutions was seeded unto Nutrient Agar, Mannitol Salt Agar and Mackonkey Agar for the isolation of heterotrophic bacteria. In screening for total hydrocarbon utilizing bacterial counts, vapour phase transfer technique was used (Chikere and Azubuike, 2014). An aliquot of 1mL (10^{-4} and 10^{-6} dilution) of the crude oil soil suspension was seeded onto Bushnell Haas agar, sterile Whatman filter papers soaked in diesel were aseptically placed into the lids of each inoculated Bushnell-Haas Agar plates and incubated at room temperature for 6 days. The colonies, after incubation, were visualized on the agar plates and counted; they are expressed in $cfug^{-1}$; the colonies isolated were further purified by sub-culturing unto agar slant; Sharma, (2009).

Cultural test and Gram stain reaction was carried out on bacterial isolates. Their features were compared with related species from Bergey's Manual of Determinative Bacteriology for bacterial cells, and their identities were confirmed (Holt *et al*., 1994). The biochemical tests of the samples were carried out on: production of catalase, coagulase, citrate utilization, indole and oxidase enzymes. Fermentation of sugars was also examined.

Determination of total petroleum hydrocarbons (TPH)

TPH was analyzed using organic solvent extraction procedures (Onyeonwu, 2000). The TPH was performed using Gas Chromatograph Agilent 6890 Series, with an Agilent FID detector. The percentage of crude oil degraded after six months was determined from the equation:

$$\% \text{ Crude oil degraded} = \frac{\text{Weight of crude oil degraded}}{\text{Original weight of crude oil}} \text{ x } 100$$

Weight of crude oil degraded = Original weight of crude oil – Weight of residual crude oil.

Plasmid DNA isolation

Isolation of hydrocarbonoclastic bacterial plasmid DNA was carried out to ascertain the molecular weight of plasmids based on their movement through agarose gel in comparison with a molecular marker. Plasmid DNA was excised from a bacterial cell by alkali treatment method as described by (Crosa *et al.,* 1994).

Plasmid curing

All isolates that were observed to harbour plasmid were subjected to plasmid curing suggested by Sheikh (2003). The bacterial isolates were cultured in broth medium containing the preferred hydrocarbon in which the bacteria had shown the highest growth. 0.1 mL of the culture was added to 100 mL of nutrient broth containing 1% SDS (Sodium Dodecyl Sulphate). This was incubated at 37^0C for 24 h. Thereafter, the broth was shaken vigorously to homogenize the content and loopfuls of the broth medium were sub-cultured on Nutrient Agar plate and also on Bushnell Haas Agar containing 1% (v/v) diesel. The plates were incubated at 37^0C for 24 h and the colonies counted. Colonies that failed to grow on Bushnell Hass Agar containing 1% (v/v) diesel plates were considered cured.

Statistical analysis*:* Results obtained were computed using Two Way Analysis of Variance (ANOVA) without replication to test the level of significance between the groups of means for the different treatment samples.

Results

The mean total heterotrophic bacterial counts (Table 1) ranged between 40.5±0.5 x10^4 cfu^{-1}and 102.0 ±4.0 x10^4 cfu^{-1}, the minimum being recorded for C_1 in August, 2016 and the maximum Control 2 in December, 2016. The mean total hydrocarbon utilizing bacterial counts ranged between 42.0 ± 2.0 x10^4 cfu^{-1} to 66.5±2.5 x10^4 cfu^{-1}, the minimum being recorded for B_1 in July, 2016 and the maximum C_3 in September, 2016.

Table 1. Mean total heterotrophic bacterial counts (x10^4 cfu^{-1})
(July 2016 – December 2016)

Treatments/ Months	July	August	September	October	November	December	P-value
Control1	65.0±7.0	73±13.0	69.5±2.5	52.5±2.5	54.0±2.0	45.0±3.5	P<0.05[a]
Control2	55.5±0.5	55.5±3.5	51.0±1.0	44.5±1.5	46.5±3.5	40.5±0.5	P<0.05[a]
A_1	72.5±7.5	79.5±9.5	81.0±9.0	77.0±15.0	68.0±1.0	64.0±8.0	P<0.05[a]
A_2	66.5±2.5	73.5±5.5	77.0±5.0	70.0±2.0	64.5±4.5	64.0±8.0	P<0.05[a]
A_3	62.5±2.5	67.0±2.0	72.0±3.0	68.5±3.5	65.5±2.5	62.5±2.5	P<0.05[a]
B_1	64.0±2.0	64.5±2.5	68.0±4.0	65.0±3.0	67.5±0.5	64.5±2.5	P<0.05[a]
B_2	58.0±2.0	61.5±2.5	57.5±5.5	57.5±2.5	56.0±2.0	53.5±1.5	P<0.05[a]
B_3	59.0±1.0	60.5±1.5	58.5±0.5	52.5±2.5	51.0±1.0	51.0±1.0	P<0.05[a]
C_1	98.5±2.5	102.0±4.0	99.5±10.6	94.5±2.5	92.5±2.5	77.0±10.0	P<0.05[a]
C_2	93.0±1.0	93.0±3.0	95.5±0.5	91.5±1.5	88.5±1.5	80.0±2.0	P<0.05[a]
C_3	92.0±2.0	95.0±2.0	93.5±1.5	85.5±1.5	83.0±3.0	79.5±6.5	P<0.05[a]

P < 0.05 – significant difference; 'a' connotes values that are significant
Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Table 2. Mean total hydrocarbon utilizing bacterial counts (x10^4 cfu^{-1})
(July 2016 – December 2016)

Treatments/ Months	July	August	September	October	November	December	P-value
Control1	59.0±1.0	56.0±4.0	56.5±3.5	51.5±1.5	51.5±0.5	46.5±1.5	P>0.05[b]
Control2	50.5±4.5	55.5±3.5	61.5±2.5	59.0±2.0	57.0±1.0	51.5±1.5	P>0.05[b]
A_1	50.5±1.5	56.0±3.0	62.0±2.0	61.0±1.0	59.5±2.5	57.5±2.5	P>0.05[b]
A_2	52.0±3.0	57.5±0.5	63.5±1.5	63.0±0.0	62.0±1.0	57.2±0.5	P>0.05[b]
A_3	53.0±5.0	59.0±1.0	64.0±3.0	63.5±0.5	62.0±3.0	61.0±3.0	P>0.05[b]
B_1	42.0±2.0	51.5±4.5	56.5±0.5	54.5±1.5	50.5±0.5	51.0±2.0	P>0.05[b]

Treatments/ Months	July	August	September	October	November	December	P-value
B_2	43.0±2.0	51.5±3.5	54.0±0.0	60.5±4.5	52.5±0.5	52.5±2.5	P>0.05[b]
B_3	43.0±5.0	53.0±7.0	60.5±2.5	62.0±2.0	57.0±1.0	55.0±1.0	P>0.05[b]
C_1	58.5±0.5	61.5±1.5	62.5±0.5	63.5±2.5	58.5±1.5	53.5±0.5	P>0.05[b]
C_2	60.0±5.0	65.0±3.0	64.5±0.5	63.5±3.5	53.5±1.5	52.0±2.0	P>0.05[b]
C_3	61.5±2.5	65.5±2.5	66.5±2.5	65.0±1.0	64.5±0.5	62.5±2.5	P>0.05[b]

P > 0.05 – No significant difference; 'b' connotes values that are not significant
Keys: control 1: soil only, control 2: soil + 50mL diesel, A1: soil + 100g poultry litter + 50mL diesel, A2: soil + 100g poultry litter + 100mL diesel, A3: soil + 100g poultry litter + 150mL diesel, B1: soil + 100g cow dung + 50mL diesel, B2: soil + 100g cow dung + 100mL diesel, B3: soil + 100g cow dung + 150mL diesel, C1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Seven bacterial species were isolated. Of these heterotrophic bacteria isolated, five isolates were hydrocarbon degrading bacteria. *Bacillus subtilis* (25.7%) was reported to record the highest percentage frequency of occurrence and the least frequency was recorded for *Staphylococcus aureus* (4.73%) (Table 3).

Table 3. Percentage frequency of occurrence of the bacterial isolates

Isolates	Control 1	Control 2	A (A_1,A_2,A_3)	B (B_1,B_2,B_3)	C (C_1,C_2,C_3)	Total
*Bacillus subtilis**	5	7	9	5	12	38 (25.7%)
Bacillus sp*	3	5	6	6	9	29 (19.6%)
Staphylococcus aureus	1	-	1	4	1	7 (4.73%)
Klebsiella sp*	3	3	4	3	6	19 (12.84%)
Escherichia coli	-	-	2	3	5	10 (6.76%)
*Enterobacter aerogenes**	1	1	3	4	6	15 (11.03%)
*Pseudomonas aeruginosa**	2	1	5	2	8	18 (12.16%)
Total	17 (12.50%)	15 (11.03%)	30 (22.06%)	27 (19.85%)	47 (34.82%)	136 (100%)

Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel.

* Hydrocarbon degrading bacteria

Effect of soil amendments on Total Petroleum Hydrocarbon

Table 4 shows the initial and the final TPH utilization with treated soil. Diesel oil degradation was highest in C_1 (98.5%) and lowest in control1 (31.6%).

Table 4. Percentage (%) Total Petroleum Hydrocarbon (TPH) degradation (July 2016 – December 2016)

Treatments	**Cntrl 1**	**Cntrl 2**	**A_1**	**A_2**	**A_3**
Initial TPH (mg/kg)	3.8917	13292.4	11362.3	12140.5	13595.4
Final TPH (mg/kg)	2.6618	8056.41	592.25	848.766	1392.57
% Degradation	31.6	39.3	94.8	93.0	89.8

Treatments	**B_1**	**B_2**	**B_3**	**C_1**	**C_2**	**C_3**	**P-value**
Initial TPH (mg/kg)	11656.7	12193.7	12576.5	11447.1	11558.2	11622.3	P<0.05
Final TPH (mg/kg)	796.067	957.015	1774.65	169.69	270.164	446.83	P< 0.05
% Degradation	93.2	92.2	85.9	98.5	97.7	96.2	P< 0.05

Keys: control 1: soil only, control 2: soil + 50mL diesel, A_1: soil + 100g poultry litter + 50mL diesel, A_2: soil + 100g poultry litter + 100mL diesel, A_3: soil + 100g poultry litter + 150mL diesel, B_1: soil + 100g cow dung + 50mL diesel, B_2: soil + 100g cow dung + 100mL diesel, B_3: soil + 100g cow dung + 150mL diesel, C_1: soil + 50g cow dung + 50g poultry litter + 50mL diesel, C_2: soil + 50g cow dung + 50g poultry litter + 100mL diesel, C_3: soil + 50g cow dung + 50g poultry litter + 150mL diesel

Plasmid profile of bacterial isolates

Figure 1 shows the electrophoretic profile of all five hydrocarbon degrading bacterial isolates having plasmids. The hydrocarbonoclastic bacterial isolates designated B, C, D, E, and F showed positive growth on Bushnell Hass medium before curing, but upon curing, only isolates D and E (*Bacillus subtilis and Enterobacter aerogenes*) had scanty growth. No growth was seen in isolates B, C and F (*Klebsiella* sp., *Pseudomonas aeruginosa* and *Bacillus* sp). Positive growth on media after curing indicates the ability of the isolate to utilize the petroleum present on the media as a sole source of carbon. This enzymatic ability could only be enhanced by the gene that codes for degradation present in the chromosome. No growth indicates that the gene that codes for degradation is not present in the chromosome but in the plasmid which has been cured.

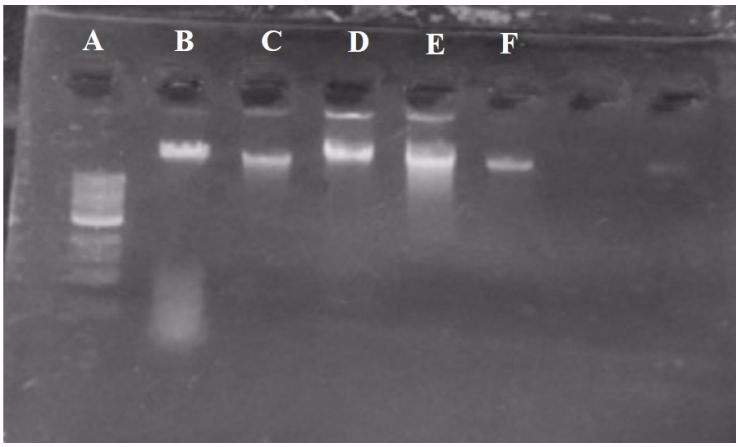


Figure 1. Electrophoretic separation profile plasmid DNAs of isolates before curing (A = 10kb Ladder; B= *Klebsiella* sp.; C=*Pseudomonas aeuginosa* ; D= *Bacillus subtilis*; E=*Enterobacter aerogenes;* F=*Bacillus* sp.) All isolates had plasmids greater than 10kb.

Discussion

Bioremediation is often used in pollution control because of its ecofriendly nature and there are also readily available microbes that effectively carry out the remediation process. The total heterotrophic bacterial counts were in this order; C_1> C_2> C_3> A_1> A_2> A_3> B_1> B_2> B_3> Control 1 > Control 2. High bacterial count observed in the combined amendment could be due to the increased nutrient as a result of the combination of organic wastes.

There was observable increase in the first three months (July-September) and decrease from October to December. This agrees with the work of Stephen *et al*. (2015), who observed that bacterial count reduces with time. This could be attributed to the exhaustion of nutrients with time and also to the fact that bacteria grow well when moisture content is adequate. It was observed that bacterial counts were higher in poultry amended soil than in soil amended with cow dung; this agrees with the report of Obasi *et al.* (2013), which showed a higher bacterial count in poultry litter amended soil than soil amended with cow dung, sawdust and horse manure.

The bacterial counts enumerated from the uncontaminated soil (Control 1) were above those from soil incorporated with diesel (Control 2). This difference in bacterial counts could be attributed to the incorporation of diesel to control 2 which reduced the capacity of the bacterial population that could not utilize hydrocarbon as carbon source for growth. Similar were the findings of Stephen *et al,* (2015), which observed higher bacterial counts in oil-free soil than in oil-

polluted soil. There was significant difference ($P < 0.05$) in heterotrophic bacterial and fungal counts between July to December, 2016 indicating that moisture and nutrients influenced microbial growth. The reverse was the case in total petroleum hydrocarbon utilizing bacterial counts (TPHUB), as the bacteria were able to utilize the hydrocarbon as sole source of carbon and energy and thus increased in number as the volume of hydrocarbon increased. There was significant difference in bacterial counts from July to December, 2016. This finding corroborates with the results of Adebusoye *et al.* (2007), who reported an increase in TPHUC; although there was no significant difference ($P> 0.05$) between THBC and TPHBC.

It was observed that soil amended with poultry litters and cow dung had greater percentage degradation than the individual waste treatments. Although poultry litter amended soil had greater percentage degradation than that of soil amended with cow dung. This is in agreement with earlier reports by Tanee and Kinako (2008) who reported a marked decrease in the total hydrocarbon content of amended crude oil polluted soils relative to the control soils. Higher loss of TPH was evident in the combined compost amendment followed by poultry litter amended soil and then cow dung amended soil. This corroborates the findings of Umar *et al*. (2012), who reported that bioremediated soil using cow dung and chicken droppings have high removal rate of TPH compared to control soil.

The result of the electrophoretic profile of plasmid DNAs for the five hydrocarbonoclastic bacteria (*Klebsiella* sp., *Bacillus subtilis*, *Bacillus* sp.*, Enterobacter aerogenes* and *Pseudomonas aeruginosa*) showed plasmids greater than 10kb. *Enterobacter aerogenes* and *Bacillus subtilis* revealed scanty growth after curing. The ability of the cured isolates to grow in crude oil medium thereby retaining their ability to degrade crude oil have been earlier reported by John and Okpokwasili (2012) and Akpes *et al.* (2013), These findings correlate with work done by Akpes *et al.* (2013) whose plasmid-cured isolates retained the ability to degrade crude oil. This observation suggests, however, that some bacteria isolates possess both plasmid and chromosomal DNAs that code for the degradation of crude oil. Since these isolates were cured of their plasmids, it means that the genes that possess the enzyme for degradation were encoded in both the plasmid and the chromosome.

Conclusions

This study has shown that organic wastes play key roles in stimulating microorganisms in order to achieve enhanced biodegradation. The combined compost amendment had better percentage degradation and should be recommended for bioremediation processes. Poultry litters offered better degradation potentials than cow dung and would be preferred when one is faced to choose between either of them.

More studies should be carried out on hydrocarbon degrading bacteria whose gene that codes for the enzymes for degradation are located in both chromosomes and plasmids. It is believed that these groups of bacteria will be most effective in carrying out the remediation process. The Federal Government by way of encouragement should give financial support to scientists so as to fast track the cleanup process of oil-contaminated rivers and soil in some part of Nigeria, especially in Niger-Delta region.

References

Adebusoye, S.A., Ilori, M. O., Amund, O. O., Teniola, O.D., & Olatope, S.O. (2007). Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. *World Journal of Microbiology and Biotechnology* 23:1149-1159.

Adelana, S.O, Adeosun, T.A, Adesina, A.O., & Ojuroye, M.O. (2011). Environmental pollution and remediation: challenges and management of oil spillage in the Nigerian coastal area. *American Journal of Scientific and Industrial Research (AJSIR)* 2(6)**:** 834-845.

Akpes, A.R., Ekundayo, A.O., & Esumeh, F.I. (2013). Degra.dation of crude oil by bacteria: A role for plasmid-borne genes. *Global Journal of Science Frontier Research Biological Science,* 13(6):20-26..

Chikere, C.B., & Azubuike, C.C. (2014). Characterization of hydrocarbon utilizing fungi from hydrocarbon polluted sediments and water *Nigerian Journal of Biotechnology,* 27:49-54.

Crosa, J.H., Tolmasky, M.E., Actis, L.A., & Falkow, S. (1994). *Plasmids*. In: Gerhardt, P., Murray, R.G.E., Wood, W.A., Kreig. N.R., editors. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, 365-386.

De Magalhaes, J.T, Uetanabaro, P.T., & Moraes, C.A. (2008). Identification of Lactobacillus UFV H2B2 (Proboitic strain) using DNA-DNA hybridization. *Brazilian Journal of Microbiology*, 39:524-546.

Holt, J.G., Kreig, N. R., Sneath, P.H.A., Stanley, J.T., & Williams, S.T. (1994). *Bergey's Manual of Determinative Bacteriology.* 9th edition. Lippincott. Williams and Wilkins, Baltimore, USA. 787pp.

John, R.C., & Okpokwasili, G.C. (2012). Crude Oil-Degradation And Plasmid Profile Of Nitrifying Bacteria Isolated From Oil-Impacted Mangrove Sediment in the Niger Delta of Nigeria. *Bulletin of Environmental Contamination and Toxicology* 88:1020–1026

Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O. & Yadav, H. (2008). Molecular approachhes for identification and chacterization of Lactic acid bacteria. *Journal of Digestive Discussion,* 9:190-198.

Obasi, N.A., Eze, E., Anyanwu, D.I., & Okorie, U.C. (2013). Effects of organic manures on the physicochemical properties of crude oil polluted soils. *African Journal of Biochemistry Research,* **7**(6):67-75.

Omotayo, A.E., Ojo, O.Y., & Amund O.O. (2012). Crude oil degradation by microorganisms in soil compost. *Research Journal of Microbiology,* (4):209-218.

Onyeonwu R.O. (2000). *Manual for Waste/Wastewater; Soil Sediment, Plant and Fish Analysis*. Macgill Environmental Research Laboratory Manual. Benin City. 81pp.

Pepper, I. L., & Gerba, C. P. (2004). *Environmental Microbiology: A laboratory Manual.* 2nd edition*.* Elsevier Academic Press, London. 226pp.

Sharma, P. (2009). *Manual of Microbiology, Tools and Techniques*. Ane books. Pvt. Ltd. New Delhi, 405pp.

Sheikh, A.R., Afsheen A, Sadia, K., & Abdu, W. (2003). Plasmid borne antibiotic resistance factors among indigenous Klebsiella. *Pakistan Journal of Biological Science,* 35(2): 243–248

Stephen, E., Okwute, L.O., & Okai, A.I. (2015). Bioremediation of mechanic workshop polluted soil amended with poultry litter. *Bioscience Research in Today's World,* 1(1):77-83.

Tanee, F.B.G., & Kinako, P.D.S. (2008). Comparative Studies of Biostimulation and phytoremediation in the mitigation of crude oil toxicity in tropical soil. *Journal of Applied Science and Environmental Management,* 12(2):143-147.

Umar, A.F., Tahir, F., Larkin, M., Oyawoye, O.M., Musa, B. L., Yerima, M.B., & Agbo, E.B. (2012). *In-situ* biostimulatory effect of selected organic wastes on bacterial atrazine biodegradation. *Advances in Microbiology,* 2:587-592.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 65, 2, 2020 (p. 31-67)
doi:10.24193/subbbiol.2020.2.03

Pseudomonas aeruginosa at the dawn of a post-antibiotic era: clinical significance, resistance mechanisms, novel antibiotics and alternative treatments

Orsolya Kovács[1] and Endre Jakab[1,2]✉

[1]Babeş-Bolyai University, Faculty of Biology and Geology, Hungarian Department of Biology and Ecology, Cluj-Napoca, Romania; [2]Centre for Systems Biology, Biodiversity and Bioresources, Babeş-Bolyai University, Cluj-Napoca, Romania;
✉***Corresponding author, E-mail: endre.jakab@ubbcluj.ro.***

Article history: Received 8 September 2020; Revised 11 November 2020; Accepted 3 December 2020; Available online 20 December 2020

Abstract. Since their discovery, antibiotics have helped treat diseases prior to which many were untreatable, saving millions of lives. However, due to the overuse of antibiotics in medicine and agriculture, the advent of resistant strains of bacteria followed shortly after. The current antibiotic resistance crisis is bringing humanity closer to a post-antibiotic era, when all the advancements made by modern medicine could easily be reversed. *Pseudomonas aeruginosa* is a Gram-negative, rod-shaped bacterium, ubiquitous owing to its minimal nutritional and growth requirements. *P. aeruginosa* is one of the pathogens included in the priority list of the WHO, being assessed as critical due to its high antimicrobial resistance, leaving only a few effective treatment options to combat it. As an opportunistic pathogen, *P. aeruginosa* establishes infection in immunocompromised patients, primarily in hospital settings. In order to initiate infection, it requires several virulence factors that mediate the invasion of the pathogen into host cells. Owing to the multiple resistance mechanisms of *P. aeruginosa*, it has developed resistance to most classes of antibiotics. Due to its increased resistance, treating *P. aeruginosa* infections is a great challenge for clinicians. Several β-lactam/β-lactamase combinations have been approved and are available as treatment options, which overall show high efficacy against *P. aeruginosa*. Moreover, novel antibiotics are currently in development as possible antipseudomonal agents, including a *Pseudomonas*-specific formulation. In addition, new strategies such as bacteriophage therapy, pyocins or the inhibition of the quorum sensing system are being investigated for the treatment of *P. aeruginosa* infections.

Keywords: antibiotic resistance, *Pseudomonas aeruginosa*, *Pseudomonas* infections, resistance mechanisms, novel antibiotics.

The antibiotic resistance crisis

Antibiotic resistance is one of the biggest threats to global health. In 2013, the Centres for Disease Control and Prevention (CDC) published the first Antibiotic Resistance Threat Report which contains the following warning: "simply using antibiotics creates resistance" - sounding the alarm on the danger brought by the increasing number of antibiotic-resistant pathogens (CDC, 2013). According to the most recent report by the European Antimicrobial Resistance Surveillance Network (EARS-Net), more than 670,000 infections occur each year in the EU/EEA which result in approximately 33,000 deaths as a direct consequence of being infected by an antibiotic-resistant pathogen (ECDC, 2019). By 2050, an estimated of 10 million deaths per year would occur as a result of acquiring an infection by a resistant pathogen (Banin *et al.*, 2017).

The discovery of Penicillin

In the 20th century, the introduction of the first antibiotic into commercial use has changed the course of medicine. In 1928, Alexander Fleming discovered that a substance produced by the *Penicillium* mold inhibits the growth of *Staphylococcus aureus*. He later named this inhibitor substance penicillin. Norman Heatley, Ernst Chain and Howard Florey continued Fleming's research and in 1944, during wartime England, they started mass-producing penicillin to treat wounded soldiers (Landecker, 2016). By conducting researches on the effects of the antibiotic, it has been revealed that it can be used for the treatment of bacterial infections. At that time, penicillin was considered a 'miracle drug' for its lack of side effects and for treating diseases that were untreatable prior to its discovery (Landecker, 2016).

Before the mass production of penicillin, the death rate of *Staphylococcus aureus* infections was estimated at 80% (Landecker, 2016). With the introduction of antibiotics, the number of infections and death rates dropped significantly, saving the lives of millions of people (Landecker, 2016; Banin *et al*., 2017).

The life expectancy of the population has also increased significantly with the use of antibiotics. In 1920, the life span of the people living in the United States was estimated at 56.4 years, whereas nowadays is estimated at 80 years (Ventola, 2019). Furthermore, in countries with poor sanitation, antibiotics decreased the mortality and morbidity rate caused by poverty-related infections (Rossolini *et al*., 2014).

Despite the major advancement of medicine brought by the discovery of penicillin, shortly after its introduction penicillin-resistant strains of bacteria were detected (Landecker, 2016).

Causes of antibiotic resistance

Antibiotic resistance occurs when a drug fails to inhibit the growth of bacteria. Therefore, bacteria become resistant and are able to multiply even in the presence of antibiotics (Zaman *et al*., 2017).

The origin of antibiotic resistance is twofold. On one hand, sequencing the genome of ancient microorganisms revealed that antibiotics and antibiotic resistance were already present in soil bacteria long before their clinical use started in the 20th century (Landecker, 2016). Soil bacteria produce antibiotics in order to interact and compete with other microorganisms for space to live in. It has been found that *Vibrio cholera* from 19th century Philadelphia had shown signs of resistance by developing efflux mechanisms (Perry *et al.*, 2016). Moreover, the 30,000 years old permafrost in the Canadian High North preserved DNA samples that show beta-lactam, glycopeptide and tetracycline resistance encoded in the genes of microorganisms (Perry *et al*., 2016). On the other hand, even though antibiotic resistance has been present for a long time, the introduction of antibiotics into medicine and agriculture by humans significantly increased the spread of resistance, causing the crisis we are facing today (Landecker, 2016). Comparing clinical specimens from the beginning of the 20th century to samples collected recently, researchers have found that the frequency of resistance genes present on plasmids has increased over time (Landecker, 2016).

It has been found that the frequency and fast distribution of antibiotic resistance is strongly correlated with the scale of antibiotic consumption of the last 80 years (Landecker, 2016; Zaman *et al*., 2017). In 1945, Alexander Fleming predicted that humanity will abuse antibiotics leading to great consequences (Landecker, 2016). The overuse of antibiotics plays a major role in the advent of resistant strains of bacteria. Incorrectly prescribed antibiotics are one of the contributing factors to the misuse, which results in patients unnecessarily receiving a high dose of broad spectrum antibiotics, for a long period of time (Ventola, 2019). Treating viral infections with antibiotics has also exacerbated the problem, especially in countries where prescriptions are not required to obtain antibiotics (Fair and Tor, 2014; CDC, 2017). Even though antibiotic prescriptions for symptoms of sore throats, colds, sinusitis decreased over time, approximately 50% of antibiotic prescriptions in the US are still unnecessary (CDC, 2017; Ventola, 2019). Patients not respecting the full course of prescribed antibiotics can also aggravate the situation. By leaving bacteria intact these can acquire multiple resistance genes over time, becoming resistant to several classes of antibiotics (Zaman *et al*., 2017).

The extensive use of antibiotics in agriculture has also led to an increase in resistance. In 1949, researchers discovered that waste products of antibiotic production could be used to promote animal growth (Landecker, 2016). Shortly after, food supplements with antibiotics entered the market promising faster growth and less disease for the livestock. Food animals grew to a larger size in a shorter period of time, while receiving the same amount of food as before (Landecker, 2016). An estimated of 80% of antibiotics sold in the US are used in agriculture (Ventola, 2019). Researchers have demonstrated that by consuming animals treated with antibiotics, the drug could easily transfer to humans too, which in turn led to the development of antibiotic-resistant bacteria in their intestinal flora (Ventola, 2019). Moreover, up to 90% of antibiotics given to farm animals is excreted into their nearby environment, driving further the spread of antibiotic resistance (Ventola, 2019). In 2003, the Food and Drug Administration (FDA) banned the use of antibiotics as a means to promote animal growth in Europe, but in several countries this is still an ongoing problem (Fair and Tor, 2014).

Another contributing factor has been the stalling of new antibiotic development by the pharmaceutical industry, leaving only a few effective options to treat resistant bacteria (Ventola, 2019). Half of the antibiotics that are in use today were discovered and developed up until the 1940's and 1960's. Several pharmaceutical companies have abandoned antibiotic development, others merged into one another reducing the number of research groups, moreover, academic research in this area has decreased significantly due to economic constraints (Ventola, 2019). Due to the fact that antibiotics are used only for a short period of time and result in fast recovery, it is not as profitable to pharmaceutical companies to invest in their development as it is in the case of drugs used in the treatment of chronic diseases (Ventola, 2019).

In recent months, the coronavirus pandemic has also lead to an increase in antibiotic use, accelerating the threat of global antibiotic resistance (Nature Editorial, 2020). Despite the fact that COVID-19 is caused by a virus, patients admitted in hospital units are given antibiotics to treat or prevent secondary bacterial infections (Khan, 2020).

On July 9, 2020 the AMR Action Fund has been launched in order to take action and respond to the pressing antibiotic resistance crisis (Bott and Holland, 2020). More than 20 leading biopharmaceutical companies have invested US$1 billion so far in the clinical development of new antibiotics to combat resistant pathogens (Jones and Holland, 2020). The AMR Action Fund aims to develop 2-4 new antibiotics by 2030 (Bott and Holland, 2020).

Important bacterial pathogens

Several Gram-positive and Gram-negative bacteria are responsible for causing serious infections in humans. In the 1990's, with the rise of methicillin- and vancomycin-resistant strains of *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae* and multidrug resistant *Clostridium difficile*, Gram-positive bacteria represented a major public health threat (ECDC, 2019). Even though these bacteria are still widespread, recent EARS-Net (European Antimicrobial Resistance Surveillance Network) data show that their resistance has decreased or optimized over time (ECDC, 2019). During the last decade, Gram-positive bacteria were the focus of new antibiotic development leading to the appearance of several new strains of resistant tuberculosis and strains of resistant Gram-negative bacteria, which are harder to fight due to their complex outer membrane (Fair and Tor, 2014).

In 2017, the World Health Organization (WHO) published a global priority pathogen list (Fig. 1), with the purpose of promoting the research and development of new antibiotics (WHO, 2017). The list contains 12 species of bacteria that are classified based on their level of resistance (critical, high, medium). Ten criteria were chosen to group these pathogens in their respective category, assessed by 70 experts with different backgrounds (Tacconelli *et al*., 2018).

Pseudomonas aeruginosa is one of the bacteria among *Acinetobacter baumannii* and members of the Enterobacteriaceae family classified in the critical, priority 1 category. These bacteria pose a major threat to public health with the life-threatening infections they cause, due to the very few effective treatment options available. Novel antibiotics are in urgent demand to combat these pathogens.

Pseudomonas aeruginosa

Morphology

Pseudomonas aeruginosa is a Gram-negative, rod-shaped bacterium of the *Pseudomonadaceae* family, measuring 0.5-0.8 μm by 1.5-3.0 μm. (Neves *et al.*, 2014; Planet, 2017). Similarly to the other members of the *Pseudomonas* genus, *P. aeruginosa* can commonly be found in nature, soil, water, in humans, even on the surface of plants and sometimes of animals too (Todar, 2004). Almost all of its strains move by the means of one polar flagellum (Todar, 2004; Neves *et al*., 2014). By forming biofilms, *P. aeruginosa* is able to colonize various surfaces and is protected from the activity of several antimicrobial agents (Neves *et al*., 2014).

Figure 1. Global priority pathogen list (WHO, 2017)

From a clinical point of view, *P. aeruginosa* has a major significance due to its increasing antibiotic resistance, making the life-threatening conditions it causes very challenging to treat (Todar, 2004). *P. aeruginosa* is an opportunistic pathogen, exploiting the host's defense mechanisms in order to initiate infection, affecting especially immunocompromised patients in healthcare units (Todar, 2004).

P. aeruginosa is an obligate aerobe bacterium, but it can also thrive in environments that lack oxygen by using arginine and nitrate as a final electron

acceptor, allowing it to achieve anaerobic growth too (Neves *et al*., 2014). *P. aeruginosa* is able to metabolize a variety of substances, has basic nutritional requirements for growth and is able to multiply at as high as 42°C (Todar, 2004; Neves *et al*., 2014). All of these factors allow *P. aeruginosa* to adapt to environments hardly tolerated by other microorganisms.

Its name, *Pseudomonas aeruginosa*, comes from the Greek *pseudo* meaning "false" and from the Latin *monas* which means "single unit", meanwhile the name *aeruginosa* means "copper rust" in Latin (Neves *et al*., 2014). Throughout history, *P. aeruginosa* has received different names based on the blue-green color of its cultures. At first it was named *Bacillus pyocyaneus*, which encompasses the two main characteristics of *P. aeruginosa*: its rod-shape and the distinct color of the cultures which is due the pigments it produces (Lister *et al*., 2009).

In 1882, French scientist Carle Gessard was the first to report this bacterium, after successfully isolating it from the wound infections of patients whose bandages had turned to a blueish-green color (Lister *et al*., 2009). He published his findings in a scientific study entitled "On the Blue and Green Coloration of Bandages". This study showed that *P. aeruginosa* produces water-soluble pigments which are fluorescing in a blue-green color under a specific light (Neves *et al*., 2014). Most colonies of *P. aeruginosa* are able to produce two pigments: pyocyanin, which is soluble in water and chloroform, giving colonies a blue color; and pyoverdin, soluble only in water, giving a yellow-green color to the cultures (Neves *et al*., 2014). In addition, by producing other pigments like pyorubrin or pyomelanin, *P. aeruginosa* colonies can turn to a red or brown color (Neves *et al*., 2014). During growth, the colonies produces a grape-like odor (Neves *et al*., 2014).

Epidemiology of Pseudomonas aeruginosa infections

P. aeruginosa can initiate infections on any part of the human body**.** The colonization rate varies and is specific to different sites: skin (0-12%), nasal mucosa (0-3.3%), throat (0-6.6%) and stool (2.6-24%) (Morrison and Wenzel, 1984). The prevalence of colonization in a hospital setting can be up to 50%, especially in the case of patients whose skin barrier and mucosa have been compromised due to severe burns, surgery, mechanical ventilation or catheters (Lister *et al*., 2009). Patients who are suffering from a significant underlying disease have a bigger chance of developing serious infections (Golemi-Kotra, 2008).

A recent report from the US classifies *P. aeruginosa* as the sixth most common nosocomial pathogen and the second most common cause of ventilator-associated pneumonia (VAP) *(*Nguyen *et al*., 2018). *P. aeruginosa* is accountable for 10-15% of nosocomial infections worldwide (Strateva and Yordanov, 2009),

17% of pneumonia cases, 7% of urinary tract infections and 8% of surgery site infections (Skariyachan *et al*., 2018). Due to its intrinsic resistance to antibiotics, treating infections caused by *P. aeruginosa* is a real challenge to health officials (Strateva and Yordanov, 2009).

Clinical features of Pseudomonas aeruginosa infections

When *P. aeruginosa* colonizes the endocardium of the host, it can lead to endocarditis, the infection of heart valves, which can result in heart failure and the destruction of the heart valves (Golemi-Kotra, 2008).

Respiratory infections caused by *P. aeruginosa* affect mostly patients who have compromised lower respiratory tracts and their immune system is weakened due to a recent transplant, HIV infection, neutropenia or cancer (Sadikot *et al*., 2005; Golemi-Kotra, 2008). Lung infections result in the highest mortality rates (Fujitani *et al*., 2011). Bacteremic *P. aeruginosa* pneumonia can be characterized by necrosis and the invasion of the blood vessels, nonbacteremic pneumonia on the other hand shows signs of hemorrhage, microabscesses and focal necrosis (Fujitani *et al*., 2011). When *P. aeruginosa* invades the upper respiratory tracts, it can cause community-acquired pneumonia (CAP), which is thought to be the cause of cystic fibrosis in children and chronic lung infection (Fujitani *et al*., 2011). A more common syndrome associated with *P. aeruginosa* lung infection is hospital-acquired pneumonia (HAP) (Fujitani *et al*., 2011).

Immunocompromised patients are also susceptible to bacteremia and sepsis. Nearly 25% of bacteremia occurring in hospitals is due to *P. aeruginosa* infections (Golemi-Kotra, 2008). Analyzing the wound infections of burn victims, it has been observed that during the first week of hospitalization the frequency of *P. aeruginosa* colonization increases significantly (Driscoll *et* al., 2007). Patients undergoing transplants are at greater risk of developing bacteremia compared with patients who are not requiring such a n intervention (Driscoll *et al*., 2007).

P. aeruginosa can also initiate infection in the central nervous system (Golemi-Kotra, 2008). These infections can lead to meningitis or brain abscesses. The pathogen invades the brain from the inner-ear or para-nasal sinus, but it can also get to the infection site as a consequence of head traumas, surgery or during invasive diagnostic procedures. Moreover, it can transfer to its final destination from a different infection site (Golemi-Kotra, 2008).

Infections of the skin caused by *P. aeruginosa*, such as folliculitis, dermatitis, otitis externa, are related to burn injuries or other types of infections (Pál, 2013). More severe forms of ear infections can occur in elderly patients who develop face paralysis, hearing deficiencies as a consequence of acquiring *P. aeruginosa* infections, which in some cases can be fatal (Golemi-Kotra, 2008).

P. aeruginosa can also lead to severe eye infections. *P. aeruginosa* is accountable for the majority of bacterial keratitis cases, which can be attributed to improper contact lens use (Golemi-Kotra, 2008). In severe cases, this can result in the degradation of the entire eye (Golemi-Kotra, 2008).

Urinary tract infections (UTIs) are the most prevalent hospital-acquired diseases that affect humans (Mittal *et al*., 2009). Catheterization of the urinary tract predisposes patients to infections by different pathogens, *P. aeruginosa* accounting for 7-10% of the cases (Mittal *et al*., 2009; Lamas Ferreiro *et al*., 2017).

Virulence factors produced by *Pseudomonas aeruginosa*

Several factors contribute to the success of *P. aeruginosa* to initiate infection: its versatile metabolism, the intrinsic and acquired antibiotic resistance mechanisms, its ability to form biofilms and the expression of virulence factors (Balasubramanian *et al*., 2013).

Adhesins

An important first step in establishing infection is the adhesion to the host tissues. *P. aeruginosa* can attach to a variety of surfaces which the pathogen uses as a means to colonize, multiply and exploit for resource acquisition (Wu *et al*., 2014). In the case of *P. aeruginosa*, adhesion takes place with the help of cell-surface components: type IV pili, flagella and the LPS (Wu *et al*., 2014).

Type IV Pili-Mediated Adhesion

The surface of *P. aeruginosa* is covered with type IV pili, which are flexible and retractable filaments having pilin components and measuring 5.2 nm in diameter and 2.5 μm in length (Wu *et al*., 2014). Type IV pili play an important role in biofilm formation, in the movement and signaling processes of the pathogen (Wu *et al*., 2014). It has been demonstrated that pili promote adhesion to host cells in 90% of the cases, the virulence of *P. aeruginosa* decreasing significantly in the event of pili malfunction (Wu *et al*., 2014).

Flagella-Mediated Adhesion

P. aeruginosa moves by the means of a polar flagellum having a FliC flagellin as its main component (Wu *et al*., 2014). The flagellum has a role in motility and biofilm formation, thus, in the event of its deterioration a decrease in pathogenicity can be observed (Wu *et al*., 2014).

The flagellum has several receptors. The main adhesion point of *P. aeruginosa* is represented by mucin, located on the host cell surface (Wu *et al*., 2014). In addition, it has been found that an asialo GM1 receptor on the host

epithelial cells could also play a role in binding the pathogen (De Bentzmann *et al*., 1996). Flagellin can also attach to a Toll-like receptor 5 (TLR5), which plays a role in activating the immune system (Hayashi *et al*., 2001; Wu *et al*., 2014).

Lipopolysaccharide-Mediated Adhesion

The lipopolysaccharide (LPS) is a surface structure, a complex glycolipid, that comprises a major part of the outer membrane of Gram-negative bacteria, including *P. aeruginosa* (Al-Wrafy *et al*., 2017).

The lipopolysaccharide of *P. aeruginosa* is composed of three domains: lipid A anchored in the membrane, the core oligosaccharide and O-polysaccharide (Al-Wrafy *et al*., 2017). Factors such as susceptible patients, the structure of lipid A and the modifications in the O-specific polysaccharide all lead to an increase in the lipopolysaccharide mediated pathogenicity of the bacterium (Al-Wrafy *et al*., 2017).

The core oligosaccharide of the lipopolysaccharide promotes the attachment of *P. aeruginosa* to the CFTR receptor (Cystic Fibrosis Transmembrane Conductance Regulator) of epithelial cells (Wu *et al*., 2014). The CFTR protein assures that the epithelial cell membranes are covered with a moderate amount of mucus (Wu *et al*., 2014). In case of a malfunction of the CFTR, the overload of mucus creates an ideal environment for the pathogen to thrive (Wu *et al*., 2014).

Secretion systems of *Pseudomonas aeruginosa*

The secretion of different proteins allows *P. aeruginosa* to compete with other microorganisms, but it can also lead to the host manipulation and invasion (Green and Mecsas, 2016). Through secretion systems, pathogens are able to transfer their toxins and exoenzymes in the extracellular matrix or the cytosol of host cells (Green and Mecsas, 2016).

Seven secretion systems of Gram-negative bacteria have been described, from which five can be found in *P. aeruginosa* (Wu *et al*., 2014).

The type I secretion system (T1SS) allows small molecules, such as toxins or antibiotics, to be exported in a one-step process from the bacterial cell (Green and Mecsas, 2016). This systems has three structural components: the ABC transporter found in the inner membrane, a membrane fusion protein (MFP) situated in the membrane and the outer membrane factor (OMF) which forms pores on the surface (Green and Mecsas, 2016). In the case of *P. aeruginosa*, two types of T1SS have been found: a system that secrets AprA protease, an important virulence factor; and one that secrets HasAp which binds to the heme part of hemoglobin, which is thought to play a role in the initial infection and the survival of the pathogen (Wu *et al*., 2014).

The type II secretion system (T2SS) consists of a two-step process in which proteins are secreted (Wu *et al*., 2014). Because T2SS is situated in the outer membrane, another pathway is needed in order to transfer proteins first to the periplasm and only then it can be secreted to the extracellular matrix (Green and Mecsas, 2016). Two T2SS can be found in *P. aeruginosa:* Xcp (extracellular protein) and Hxc (Wu *et al*., 2014). Important virulence factors such as exotoxin A, phospholipase C, LasA, LasB and PrpL can be excreted through T2SS (Wu *et al*., 2014).

Virulence factors are directly injected into host cells through the type III secretion system (T3SS) (Green and Mecsas, 2016). T3SS has three domains: the base complex, the needle and the translocon (Green and Mecsas, 2016). In animal models, T3SS has shown a significant role in the initiation of burn infections, pneumonia and lung infections (Wu *et al*., 2014).

The type V secretion system (T5SS), similarly to the T2SS, uses an additional pathway in order to transfer proteins to the periplasm (Wu *et al*., 2014; Green and Mecsas, 2016). Unlike other secretion systems, T5SS doesn't require a special membrane channel, they secretes proteins to the extracellular matrix by themselves (Green and Mecsas, 2016). EstA, LepB and LepA are among the proteins that are secreted with T3SS (Wu *et al*., 2014).

The most recently discovered structure is the type VI secretion system (T6SS), which can inject proteins directly into host cells through its needle component (Green and Mecsas, 2016). Three T6SS can be found in *P. aeruginosa* (HIS-I, HIS-II, HIS-III), from which one (HIS-II) plays a significant role in initiating infection at the site of epithelial cells (Wu *et al*., 2014).

Toxins and exoenzymes produced by *Pseudomonas aeruginosa*

P. aeruginosa is able to invade tissues through the production of toxins and exoenzymes. By damaging host cell barriers, such as the exopolysaccharides, lipopolysaccharides and the components of the host defense mechanisms, the pathogen can initiate infection with the evasion of the innate immune system.

Exotoxin A

Exotoxin A is a member of the mono-ADP-ribosyltransferases class and is being excreted by the T2SS (Wu *et al*., 2014). As a result of its ADP-ribosyltransferase activity, it can modify and inactivate elongation factor 2 (Eef-2), which leads to the inhibition of protein synthesis, resulting in cell death (Wu *et al*., 2014). Exotoxin A has three domains, which all have different roles in the invasion process: the N terminal-domain (Ia) binds to the receptor, the second domain (II) transports the exotoxin through the membranes and the third domain (III) inactivates elongation factor 2 (Wu *et al*., 2014).

Exoenzymes S, T, U

Four enzymes are secreted by the T3SS: ExoS and ExoT, having ADP-rybosiltransferase and GTPase-activating activity; ExoU, exhibiting lipase activity and the adenylate cyclase ExoY (Wu *et al*., 2014). ExoS plays a role in the transport of vesicles, cell proliferation, differentiation and apoptosis (Wu *et al*., 2014). Its ADP-rybosiltransferase activity has been shown to be correlated with the pathogen's long term survival and dissemination at lung infection sites (Wu *et al*., 2014).

Proteases

The two metalloproteases (elastase and alkaline protease) produced by *P. aeruginosa*, have a role in evading the host immune system, allowing the pathogen to successfully establish infection (Kharazmi, 1991; Golemi-Kotra, 2008). These enzymes interfere with the activity of neutrophils, monocytes, natural killer cells and T cells, that defend the host against bacterial invasions (Kharazmi, 1991). Elastase and alkaline protease have been shown to cleave immunoglobulins G and A, moreover, cytokines such as interleukin-1, interleukin-2, tumor necrosis factor and interferon gamma (Kharazmi, 1991).

Elastases are the most abundant proteases produced by *P. aeruginosa* (Wu *et al*., 2014). They damage components of the extracellular matrix by cleaving elastin, an important element of the lung tissue and blood vessels (Wu *et al*., 2014; Planet, 2017).

Neuraminidases

Neuraminidases are glycoside hydrolase enzymes which cleave terminal neuraminic acids of glycoproteins, glycolipids and gangliosides expressed on the surface of epithelial cells (Ghazaei *et al*., 2010). This results in the increase of gangliosides that lack neuraminic acids, also known as asialo Gm1, which are prevalent in cystic fibrosis lung tissues and serve as receptors of the respiratory tract (Strateva and Mitov, 2011). This enzyme plays an important role in different processes involving colonization, such as attachment to host cells, especially epithelial cells, and invasion (Ghazaei *et al*., 2010; Planet, 2017). Moreover, in early phases of invasion, neuraminidase also promotes biofilm formation (Strateva and Mitov, 2011).

Cytotoxic exoproducts produced by *Pseudomonas aeruginosa*

P. aeruginosa produces three cytotoxic proteins: a cytotoxin and two hemolysins (Strateva and Mitov, 2011). The cytotoxin forms pores in the membranes of immune cells, resulting in cell inactivation (Strateva and Mitov, 2011).

The two hemolysins of *P. aeruginosa,* phospholipase C and rhamnolipid, have a role in breaking down lipids and lecithin, respectively (Golemi-Kotra, 2008; Strateva and Mitov, 2011). Rhamnolipid is a glycolipid surfactant which is thought to solubilize the lung surfactant phospholipids, making it easier for the phospholipase C to cleave them (Strateva and Mitov, 2011). This synergistic activity has been found to have an important role in initiating acute or chronic lung infections (Strateva and Mitov, 2011). In addition, it has been observed that rhamnolipids have functions in mucociliary transport, in the formation of the complex structure of biofilms and in disrupting macrophage activity (Wu *et al*., 2014).

Phospholipase D is a common protein found in eukaryotic cells. By hydrolyzing phospholipids, phospholipase D it generates a signaling lipid, phosphatidic acid (PtdOH), which promotes several cellular processes (Spencer and Brown, 2015).

Iron chelation

Iron chelation is essential for *P. aeruginosa* to initiate infection. Being bound to hemoglobin or ferritin, iron is very limited in the host environment, therefore the pathogen is unable to access it directly (Pál, 2013). In order to sequester iron from the environment, *P. aeruginosa* produces iron chelator molecules, siderophores, to be able to grow under low-iron conditions (Golemi-Kotra, 2008; Al-Wrafy et al., 2017). These siderophores are pyochelin, a pyocyanin derivative, and pyoverdine (Strateva and Mitov, 2011).

Pyocyanin

Pyocyanin, belonging to the class of phenazines, is one of the pigments produced by *P. aeruginosa* and is secreted through the T2SS (Hall *et al*., 2016). The exact function of pyocyanin in the pathogenicity of *P. aeruginosa* is not well understood, but it was shown in high concentration in wounds, urine and sputum samples following *P. aeruginosa* infections (Hall *et al*., 2016). Pyocyanin increases levels of reactive oxygen species inside host cells, causing oxidative stress (Hall *et al*., 2016). Moreover, pyocyanin has shown pro-inflammatory properties by interfering with cytokines, inhibiting interleukin-2 release, decreasing the expression on interleukins on T-cells, decreasing immunoglobulin secretion, all resulting in *P. aeruginosa* evading the host immune system (Hall *et al*., 2016). The oxidative stress and inflammation caused by *P. aeruginosa* infections leads to the damage of the respiratory system (Hall *et al*., 2016; Al-Wrafy *et al*., 2017).

Lectins

The outer membrane of *P. aeruginosa* has two soluble lectins, LecA and LecB, which might contribute to the adhesion process of the pathogen to host cell surfaces (Al-Wrafy *et al*., 2017). In addition, lectins could also have a role in the dissemination of the bacterium at infection sites, affecting the survival and biofilm formation of *P. aeruginosa* (Al-Wrafy *et al*., 2017).

Biofilm formation

P. aeruginosa has been an important model organism in studying bacterial biofilm formation (De Kievit, 2009; Miller *et al*., 2012). This opportunistic pathogen is able to form biofilms on medical equipment, catheters, implants, resulting in severe infections of hospitalized patients (Driscoll *et al*., 2007).

Depending on the conditions of growth, *P. aeruginosa* biofilms can have either complex, 'mushroom-like' architectures or a flat appearance (Klausen *et al*., 2003; Miller *et al*., 2012). Type IV pili and the polar flagellum help the pathogen move in a variety of environments which is essential for generating biofilms (Klausen *et al*., 2003). In the first stage of biofilm formation, planktonic cells reversibly attach to the surface via their flagellum, showing surface associated motility, followed by irreversible attachment and matrix production, and finally the microcolony is formed (Miller *et al*., 2012). Two different types of subpopulations can be observed at this stage: one that keeps exploring the surface and one that has anchored, forming a cap-like structure (Miller *et al*., 2012). When the motile subpopulation moves atop of the attached cells, it creates a cap-like formation, resulting in a mushroom-like architecture (Miller *et al*., 2012). Undergoing maturation, the biofilm forms cavities filled with fluid on the surface of the cap, allowing planktonic cells to detach from the matrix and disperse, presumably forming new microcolonies in a new site (Miller *et al*., 2012).

By producing biofilms, *P. aeruginosa* demonstrates higher antibiotic resistance and is protected from the defense mechanisms of the host, in contrast with planktonic cells that remain susceptible to different antipseudomonal agents (Wu *et al*., 2014). It has been found that *P. aeruginosa* biofilms in cystic fibrosis lungs show higher level of resistance to antibiotic therapies, which has led to the longer survival of the pathogen in the lung tissue (De Kievit, 2009; Wu *et al*., 2014). Moreover, bacterial cells living in proximity to each promotes horizontal gene transfer (De Kievit, 2009).

Biofilms are composed of microcolonies (15%) and of matrix elements (85%) (Skariyachan *et al*., 2018). Extracellular polysaccharides produced by bacteria (exopolysaccharides) comprises of DNA, proteins and polysaccharides (Skariyachan *et al*., 2018).

Exopolysaccharides have an important role in the formation of microcolonies and the architecture of biofilms (De Kievit, 2009; Papp, 2015). *P. aeruginosa* can produce three types of polysaccharides: *pel*, *psl* and alginate (Skariyachan *et al*., 2018). It has been demonstrated that in the lungs of cystic fibrosis patients the mucoid *P. aeruginosa* strains promote oxidative stress and degradation of tissues by attracting immune cells to the site of infection (Skariyachan *et al*., 2018). Alginate overproduction leads to the appearance of mucoid phenotypes of *P. aeruginosa*, which are protected from the host defense mechanisms and show high level of antibiotic resistance (Skariyachan *et al*., 2018). Extracellular DNA also has an important role in generating the structure of biofilms (De Kievit, 2009).

When biofilms mature, cavities on their surface allow the acquisition of nutrients and the secretion of waste products (De Kievit, 2009). It has been elucidated that rhamnolipids are the components that maintain the open structure of these channels (De Kievit, 2009). Moreover, rhamnolipids are important in the development of complex biofilm structures, the detachment of planktonic cells from biofilm cavities and the formation of microcolonies (De Kievit, 2009).

Quorum sensing

Quorum sensing (QS), a cell-to-cell communication mechanism, plays an important role in generating biofilms and in regulating virulence factor production (De Kievit, 2009; Papp, 2015; Al-Wrafy *et al*., 2017). The signal molecules (autoinducers) of quorum sensing systems can be classified in three groups: acylhomoserine lactones (AHLs), oligopeptides and the autoinducer 2 (De Kievit, 2009). In the case of Gram-negative bacteria, autoinducers belong to the AHL family which activate a transcription factor (R protein), resulting in gene expression (De Kievit, 2009). *P. aeruginosa* has two primary AHL quorum sensing systems: Las and Rhl (De Kievit, 2009; Strateva and Mitov, 2011). These systems are generally composed of an acyl-homoserine lacton and a transcriptional activator (Strateva and Mitov, 2011). In addition, a quinolone-based system can also be found in *P. aeruginosa* (Al-Wrafy *et al*., 2017).

Antimicrobial resistance of *Pseudomonas aeruginosa*

Data of EARS-Net collected from 2015 to 2018 shows that in the EU/EEA 32.1% of *P. aeruginosa* isolates were resistant to at least one class of antibiotics, from which the highest population-weighted mean resistance in 2018 was found to be for fluoroquinolones (19.7%), followed by 18.3% of the isolates being resistant to piperacillin ± tazobactam, 14.1% to ceftazidime, 17.2% to carbapenems, and 11.8% of the isolates showing resistance to aminoglycosides (ECDC, 2019). Of all the *P. aeruginosa* isolates tested, 19.2% were resistant to

at least two or more classes of antibiotics (ECDC, 2019). The mean resistance observed to different classes of antibiotics has decreased from 2015 to 2018 (ECDC, 2019). EARS-Net data reported between 2015 and 2018 from Romania show a mean resistance of 49.4% for *P. aeruginosa* isolates, which is an alarming value compared to other EU countries, but this high percentage might be due to Romanian laboratories failing to provide necessary data, misrepresenting the actual state of *P. aeruginosa* resistance in the country (ECDC, 2019).

Extensively drug-resistant (XDR) *P. aeruginosa* strains are susceptible only to two classes of antibiotics, whereas pan-drug resistant strains show resistance to all classes of antibiotics (Horcajada *et al*., 2019). In a large-scale study conducted in Spain, researchers analyzed the resistance of *P. aeruginosa* isolates collected from 51 hospitals. It has been found that 26% of the tested isolates were multidrug-resistant and of those, 65% were extensively drug-resistant strains (Horcajada *et al*., 2019).

Infections caused by *P. aeruginosa* have a high mortality rate due to the fact that the pathogen has an increased resistance to antibiotics, leaving only a few effective options for treatment (Poole, 2011).

Resistance mechanisms of *Pseudomonas aeruginosa*

P. aeruginosa shows intrinsic resistance to most classes of antibiotics due to its less permeable outer membrane, prohibiting antibiotic molecules to enter the bacterial cell (Poole, 2011; Pál, 2013; ECDC, 2019). The acquired resistance mechanisms are a result of mutational changes and the harboring of resistance genes (Poole, 2011). Acquired resistance mechanisms of *P. aeruginosa* include: upregulation of efflux pumps which enable the removal of antimicrobials from bacterial cells, inactivation of antimicrobials by enzymes, such as β-lactamases, and the alteration of the target of antibiotics (Poole, 2011).

Mechanisms of resistance to β-lactam antibiotics

β-lactam antibiotics are one of the most widely used group of antibiotics in the fight against several Gram-negative and Gram-positive bacteria. Penicillins, cephalosporins, carbapenems and monobactams are all part of the β-lactam family, having in common the four-membered β-lactam ring as a core structural feature (Poole, 2004; Pál, 2013).

The mechanism of action of β-lactam antibiotics involves the obstruction of bacterial cell wall synthesis (Donowitz and Mandell, 1988). β-lactams bind to enzymes called penicillin-binding proteins (PBPs), located in the inner bacterial membrane, consequently inhibiting their activity (Cascella, 2019). PBPs facilitate the last steps of biosynthesis of the peptidoglycan layer, an important layer of

the cell wall. β-lactams share a similar structure with a substrate of PBPs, D-alanyl-D-alanine, thus allowing it to bind easily to the mentioned bacterial protein (Zhanel *et al*., 2014). Peptidoglycan cross-linking is prohibited with the binding and inactivation of PBPs, subsequently causing cell lysis. (Donowitz and Mandell, 1988). In the case of *P. aeruginosa*, β-lactams target a variety of PBPs: PBP1b, PBP1c, PBP2, PBP3 and the PBP4 (Zhanel *et al*., 2014).

β-lactamases

A major resistance mechanism of *P. aeruginosa* to β-lactams is the production of β-lactamases, which are hydrolytic enzymes that cleave the amide bond of β-lactam antibiotics (Poole, 2004; Poole, 2011).

P. aeruginosa carries genes for two different β-lactamases: AmpC (class D cephalosporinase) and PoxB (class D oxacillinase) (Poole, 2011). AmpC β-lactamase is strongly correlated with the β-lactam resistance of *P. aeruginosa* clinical isolates (Poole, 2011). The production of AmpC is induced by the presence of certain β-lactams and β-lactamase inhibitors, which in turn contributes to the intrinsic resistance of *P. aeruginosa* to these antibiotics (Poole, 2011; Nguyen *et al*., 2018). The presence of β-lactams result in the increased intracellular level of peptidoglycan components (Nguyen *et al*., 2018). These components bind to the AmpR repressor which subsequently deactivates the repression of the *ampc* gene, leading to the hyper-production of AmpC β-lactamases (Nguyen *et al*., 2018). This mutational derepression of the *ampc* gene is the most important mechanism of resistance to β-lactams (Poole, 2011). AmpC β-lactamases have effect against a few penicillins, cephalosporins and other agents such as ceftazidime and piperacillin-tazobactam (Nguyen *et al*., 2018).

In contrast with the above mentioned endogenous β-lactamases that only confer resistance to narrow-spectrum antipseudomonal agents, acquired β-lactamases contribute to a wider range of resistance to different antimicrobials (Poole, 2011). Acquired β-lactamases include: extended-spectrum β-lactamases (ESBL), which hydrolyze broad-spectrum cephalosporins and monobactams; and carbapenemases which are able to inactivate most β-lactams (Poole, 2011).

Efflux pump systems

From the five efflux pump families described in *P. aeruginosa*, the RND (resistance nodulation division) family plays an important role in the expulsion of antimicrobial agents from the bacterial cell (Poole, 2011). Three RND-type efflux pump systems contribute to the resistance of *P. aeruginosa*: MexAB-OprM, MexCD-OprJ and MexXY-OprM (Poole, 2011; Nguyen *et al*., 2018). These pumps confer resistance to carbapenems, primarily due to the MexAB-OprM pump, and to

non-β-lactam antibiotics (Poole, 2011; Nguyen *et al*., 2018). The MexXY-OprM pump was associated with resistance to cefepime, a fourth generation cephalosporin (Poole, 2011).

Lower permeability

Porin channels are responsible for the acquisition of nutrients, but also represent a way for antipseudomonal agents to enter and damage bacterial cells (Nguyen *et al*., 2018). Compared with other Gram-negative bacteria such as *Escherichia coli,* the outer membrane of *P. aeruginosa* is 92% less permeable (Nguyen *et al*., 2018). In the case of *P. aeruginosa*, OprD is the porin channel responsible for the transport of antibiotics (Nguyen *et al*., 2018). In addition to AmpC β-lactamases, the downregulation of OprD has primary role in the resistance of the pathogen to carbapenems (Poole, 2011; Nguyen *et al*., 2018).

Mechanisms of resistance to fluoroquinolones

Fluoroquinolones are a class of antibiotics used for the treatment of *P. aeruginosa* infections (Poole, 2011). Fluoroquinolones inhibit DNA synthesis by targeting the bacterial enzymes topoisomerase II (gyrase) and topoisomerase IV (Poole, 2011).

Alteration of fluoroquinolones

P. aeruginosa primarily modifies topoisomerase II, one of the main target sites of fluoroquinolones. In the case of highly resistant isolates, an additional mutation can be observed in topoisomerase IV (Poole, 2011; Pál, 2013). Mutational changes occur in the GyrA domain of DNA gyrase and the ParC domain of topoisomerase (Poole, 2011).

Efflux pump systems

Four representatives of the RND efflux pump family contribute to the fluoroquinolone resistance of *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-oprN and MexXY-OprM (Poole, 2011). The expression of *mexAB-OprM* is regulated by three repressors (MexR, NalD, NalC) which all have been shown to contain mutations in fluoroquinolone-resistant *P. aeruginosa* isolates (Poole, 2011). In addition, a mutation in *NfxB* can be observed in the case of MexCD-OprJ, an inactivator mutation occurs in the *mexT* activator of MexEF-oprN and mutations of *mexz* occur in MexXY-OprM (Poole, 2011).

Mechanisms of resistance to aminoglycosides

Since their discovery in the 1940's, aminoglycosides have been one of the most commonly used antibiotics (Forge and Schacht, 2000). Aminoglycoside antibiotics belong to the aminocyclitols family, having amino groups attached to their structural rings (Forge and Schacht, 2000; Pál, 2013). Their mechanism of action consists in binding to the aminoacyl-tRNA recognition site of the 16S rRNA, component of the 30S ribosomal subunit, resulting in the inhibition of polypeptide synthesis and subsequently cell death (Doi *et al*., 2016). Aminoglycosides have a broad-spectrum antibacterial effect, showing bactericidal properties by inhibiting protein synthesis (Forge and Schacht, 2000). Aminoglycoside antibiotics have primarily been used in the treatment of pulmonary infections and cystic fibrosis (Poole, 2005).

Enzymatic alteration of aminoglycosides

Aminoglycosides can be modified and inactivated through different processes, mediated by enzymes such as acetyltransferases, nucleotidyltransferases, phosphoryltransferases (Poole, 2011). Acetyltransferases modify one of the four amino groups of aminoglycosides, resulting in a decreased affinity towards the tRNA binding site of the 30S ribosomal domain (Poole, 2011; Spohn, 2018). By acetylation, *P. aeruginosa* isolates exhibit resistance to gentamicin, tobramycin, amikacin, netilmicin (Poole, 2005). Nucleotidyltransferases are able to inactivate gentamycin and tobramycin and have been found in gentamicin-resistant and tobramycin-resistant *P. aeruginosa* isolates (Poole, 2011). Phosphoryltransferases contribute to the resistance of antipseudomonal agents such as neomycin, kanamycin and streptomycin, that are rarely used for the treatment of *P. aeruginosa* infections (Poole, 2005, 2011).

Efflux pump system

Aminoglycoside resistance of *P. aeruginosa* clinical isolates is conferred by the MexXY-OprM efflux pump, especially in cystic fibrosis isolates (Poole, 2011). The MexXY-OprM is encoded by *mexXY* (regulated by MexZ) and the *oprM* gene (Poole, 2011). It has been shown that mutations occur in *mexZ* in the case of aminoglycoside-resistant *P. aeruginosa*, isolated primarily from cystic fibrosis lungs (Poole, 2005). In addition, other genes might be contributing to the resistance mediated by the efflux system. The mutation of the *parR* gene has been correlated with the expression of important resistance components such as OprD and *mexXY* (Poole, 2011).

Enzymatic alteration of 16S rRNA

Modifying 16S rRNA, the binding site of aminoglycosides, represents an important resistance mechanism of *P. aeruginosa* against aminoglycosides

(Doi *et al*., 2016). Methylation of the 16 rRNA interferes with the binding of aminoglycosides, leading to aminoglycoside-resistant *P. aeruginosa* strains (Poole, 2011). This process in *P. aeruginosa* is mediated by different methylases: RmtA, RmtD, ArmA (Poole, 2011). By altering the binding site of aminoglycosides, *P. aeruginosa* has demonstrated resistance to gentamicin, tobramycin and amikacin (Poole, 2011).

Mechanisms of resistance to polymyxins

Polymyxins (polymyxin B and colistin) are polycationic polypeptides which show strong bactericidal properties (Pál, 2013). They are being used as a last-line treatment for *P. aeruginosa* infections (Al-Wrafy *et al*., 2017). Their bactericidal effect consist in charged-based interaction with the lipopolysaccharide found in the outer membrane of the pathogen (Moffat *et al*., 2019). Lipid A, the endotoxin component of the lipopolysaccharide, is negatively charged owing to the free phosphate groups in its structure, which allows polymyxins to bind to them (Moffat *et al*., 2019). This interaction leads to the destabilization of the lipopolysaccharide and to increased permeability, resulting in more polymyxin uptake (Moffat *et al*., 2019).

P. aeruginosa achieves resistance to polymyxins by altering the initial target of these antibiotics (Olaitan *et al*., 2014; Baron *et al*., 2016; Moffat *et al*., 2019). This process consists in covalently modifying the lipid A moiety of the lipopolysaccharide by adding 4-amino-4-deoxy-L-arabinose (L-Ara4N) to it (Poole, 2011; Olaitan *et al*., 2014). This process is stimulated by the mutations harbored in the two-component regulatory systems (TCSs), the following TCSs being described in *P. aeruginosa*: PmrA/PmrB, PhoP/PhoQ, ParR/ParS, ColR/ColS and CprR/CprS (Olaitan *et al*., 2014).

Biofilm-mediated resistance

P. aeruginosa cells aggregated in microcolonies produce an exopolysaccharide matrix that encapsulates them (Azam and Khan, 2019). This matrix has been found to confer protection to the bacterial cells from the activity of different antipseudomonal agents.

The biofilm-mediated resistance of *P. aeruginosa* involves different mechanisms. First of all, the exopolysaccharide matrix prevents antimicrobials to reach their target by acting as a barrier and limiting their penetration into bacterial cells (Al-Wrafy *et al*., 2017; Azam and Khan, 2019). Not all antibiotics are prohibited in the same way: penicillins are inactivated by the production of β-lactams, prohibiting it from entering the biofilm, whereas aminoglycosides bind to the negatively-charged alginate or interact with the extracellular DNA of the biofilm, resulting in a slow diffusion through the biofilm matrix (Al-Wrafy *et al*., 2017).

Another biofilm-mediated resistance consists in the alteration of the microenvironment of *P. aeruginosa* (Al-Wrafy *et al*., 2017; Azam and Khan, 2019). In contrast with the surface, deeper layers of the biofilms show anaerobic conditions (Al-Wrafy *et al*., 2017). This prohibits aminoglycoside antibiotics, which are proven to be less effective against *P. aeruginosa* biofilms (Al-Wrafy *et al*., 2017).

Bacterial cells growing inside biofilms are metabolically less active due to the limited nutrients and oxygen supply, as a consequence conferring resistance against antibiotics such as β-lactams and aminoglycosides that only target metabolically active cells (Poole, 2011; Al-Wrafy *et al*., 2017).

Another resistance mechanism of the biofilm is related to the presence of persister cell populations, which are highly resistant to antipseudomonal agents (Azam and Khan, 2019). Researchers concluded that whereas the majority of bacterial cells can easily be damaged with antibiotics, certain cells are not susceptible to the effect of these agents, concluding that this persister cell population is responsible for the resistance of the biofilm (Azam and Khan, 2019).

Hypermutator *Pseudomonas aeruginosa* strains

Mutations occurring in the DNA repair mechanism, primarily in the mismatch repair (MMR), have been found to result in highly resistant *P. aeruginosa* isolates, which can be observed in the case of isolates prelavated from chronic diseases (Poole, 2011). It has been found that strains harboring mutations in *mutS, mutL* and *mutU* have a higher resistance than strains lacking these types of mutations (Poole, 2011).

Treatment of *Pseudomonas aeruginosa* infections

Infections caused by multidrug-resistant *P. aeruginosa* strains pose a challenge to health-care officials due to the few available and effective options left for treatment (Hirsch and Tam, 2010). Studies have shown, that the delayed treatment is correlated with higher mortality and morbidity rates and a longer hospital stay for patients suffering from these type of infections (Hirsch and Tam, 2010).

An empirical therapy is defined as treatment initiation in the absence or prior to having laboratory results assessing the profile of the pathogen. Treating *P. aeruginosa* bloodstream infections with the appropriate empirical therapy requires the administration of antipseudomonal agents in a 24 hour period after bacterial isolates have exhibited susceptibility to the antimicrobials in blood cultures (Hirsch and Tam, 2010). Four different studies have found that the inappropriate empirical therapies result in a higher mortality rate, compared

with the group of patients receiving appropriate medication in case of acquiring *P. aeruginosa* bloodstream infections (Hirsch and Tam, 2010). A study has shown that delaying treatment for over 52 hours has increased the mortality rate greater than twofold compared with patients whose antibiotic therapies were initiated in 52 hours (Hirsch and Tam, 2010). These studies have shed light on the importance of the importance of promptly initiating appropriate empirical therapy in case of *P. aeruginosa* bloodstream infections. In addition, *P. aeruginosa* biofilms in early stages of development exhibit an increased susceptibility to antimicrobials, which also supports the importance of timely treatment (Ciofu and Tolker-Nielsen, 2019).

Due to the fact that *P. aeruginosa* acquires resistance to more classes of antibiotics in a short period of time, the infections caused by it result in higher mortality rates leading to poorer patient outcomes (Hirsch and Tam, 2010).

A potential treatment option could represent the combined administration of antibiotics, which could have several benefits compared with the administration of only one pseudomonal agent. First of all, it would lead to an increased bactericidal effect, slowing the progression of infections (Hirsch and Tam, 2010). Moreover, owing to the extended antibacterial spectrum of the combined antibiotics, it could lead to a better outcome in targeting and combating the pathogen (Hirsch and Tam, 2010). Studies comparing monotherapies with combined therapies have shown results in favor of the latter. The appropriate and inappropriate empirical therapies both had higher mortality rates compared with combined therapies (Hirsch and Tam, 2010). In addition, it has been found that monotherapies had a bigger chance of leading to inappropriate empirical therapies (Hirsch and Tam, 2010). A conflict of results has also been found when a study has demonstrated that patients suffering from sepsis showed the same outcomes both in the case of combined therapy and monotherapy (Hirsch and Tam, 2010).

Approved novel antipseudomonal antibiotics

Ceftazidime/avibactam

Ceftazidime is an older, third-generation antipseudomonal cephalosporin re-issued in 2015, targeting primarily the penicillin-binding proteins (PBPs) of *P. aeruginosa* (Nguyen *et al*., 2018; O'Neall *et al*., 2020). Avibactam is a non-β-lactam β-lactamase inhibitor, which binds reversibly to the active serine site of β-lactamases (Nguyen *et al*., 2018; Stone *et al*., 2018). Avibactam has an extended spectrum activity inhibiting class A, class C and few representatives of the class D β-lactamases, but has no activity against metallo-β-lactamases (Nguyen *et al*., 2018). In addition, avibactam protects ceftazidime from being degraded by β-lactamases, thus enhancing its activity (Stone *et al*., 2018).

Several *in vitro* studies have demonstrated the efficacy of ceftazidime/avibactam in the treatment of MDR and XDR *P. aeruginosa* infections, the inhibition rate varying between 66.1% and 86.5% (Horcajada *et al*., 2019). A different study has shown that ceftazidime/avibactam inhibited 92.4% of the 5,716 *P. aeruginosa* isolates tested (Horcajada *et al*., 2019). A study conducted between 2012 and 2014 has found that from the 7,062 *P. aeruginosa* strains analyzed, 562 (8%) has shown resistance to the antibiotic, mainly owing to the production of metallo-β-lactamases (Horcajada *et al*., 2019). Moreover, it has been found that the main mechanism of resistance of *P. aeruginosa* is due to the expulsion of the antibiotic through efflux pumps (Horcajada *et al*., 2019). Further clinical experiments are needed in order to assess the exact efficacy of ceftazidime/avibactam in treating *P. aeruginosa* infections.

Ceftolozane/Tazobactam

Ceftolozane/tazobactam is a new β-lactam/β-lactamase inhibitor, approved by the FDA in 2014 and subsequently approved by the European Medicines Agency (EMA) in 2015 as a treatment option for complicated urinary tract infections (cUTIs) and complicated intra-abdominal infections (cIAIs) (Haidar *et al*., 2017; Maraolo *et al*., 2020). Since then, it has been proposed for the treatment of *P. aeruginosa* related hospital-acquired pneumonia and ventilator-associated pneumonia, approved by the FDA in 2019 (Maraolo *et al*., 2020). Ceftolozane/tazobactam showed promising effects against MDR and XDR *P. aeruginosa* strains.

Ceftolozane is a fifth-generation cephalosporin, belonging to the class of β-lactams (O'Neall *et al*., 2020). It binds and inhibits the PBPs, interfering with cell wall synthesis which leads to cell lysis and death (Zhanel *et al*., 2014). Ceftolozane has an increased stability to AmpC β-lactamases and a high penetration rate compared with older generation cephalosporins (O'Neall *et al*., 2020). Tazobactam, a sulfone derivative of penicillanic acid, acts as a β-lactamase inhibitor (Zhanel *et al*., 2014). In contrast with the complex formed by the β-lactam, the complex created by tazobactam at the active site of β-lactamases results in a slower hydrolysis (Zhanel *et al*., 2014). Tazobactam inhibits class A β-lactamases, extended spectrum β-lactamases and a few class C β-lactamases (Zhanel *et al*., 2014).

Several studies have found that *P. aeruginosa* exhibited between 55% and 96.6% susceptibility to ceftolozane/tazobactam (Horcajada *et al*., 2019). It is considered the most active antipseudomonal agent, proven to be up to 20-25% more effective than the other available antibiotics of the class of β-lactams (Horcajada *et al*., 2019; Maraolo *et al*., 2020). Some studies have shown 4-14% of resistance to ceftolozane/avibactam in *P. aeruginosa* isolates (Horcajada *et al*.,

2019). More *in vivo* studies are required to evaluate the efficacy of ceftazidime/avibactam in treating *P. aeruginosa* infections.

Imipenem-Cilastatin-Relebactam (IMI-REL)

Imipenem-cilastatin-relebactam, sold under the name Recarbio, has been approved by the FDA in July 2019 for the treatment of complicated urinary tract infections and intra-abdominal infection (Smith *et al*., 2020). Imipenem is a carbapenem, and similarly to the other representatives of the β-lactams, it inhibits bacterial cell wall synthesis by binding to PBPs (Smith *et al*., 2020). Imipenem is a derivative of thienamycin, a substrate for renal dehydropeptidase-1 (DHP-1), thus it is necessary to combine it with the DHP-1 inhibitor cilastatin in order to prevent its inactivation (Smith *et al*., 2020). Relebactam is a new β-lactamase inhibitor that potentiates the activity of the β-lactam component and shows similarity to avibactam (Smith *et al*., 2020).

In vitro studies have found that 94.4% of *P. aeruginosa* isolates exhibited susceptibility to IMI-REL (Horcajada *et al*., 2019). However, *P. aeruginosa* strains resistant to IMI-REL demonstrated an overall of 80% susceptibility (Smith *et al*., 2020).

Cefiderocol

Cefiderocol is a novel siderophore cephalosporin, proved to be effective against multidrug-resistant Gram-negative bacteria (Horcajada *et al*., 2019).

Owing to the catechol moiety of cefiderocol, it can easily sequester iron from the environment, which is essential for bacterial growth (Sato and Yamawaki, 2019). Cefiderocol mimics natural siderophores produced by bacteria, thus is able to bind and exploit the outer membrane iron transporter of the pathogen, evading the bacterial resistance mechanisms (Sato and Yamawaki, 2019). In the case of *P. aeruginosa*, cefiderocol uses a specific membrane transporter, PiuA, to access the periplasmic space (Sato and Yamawaki, 2019). In order to exhibit its antibacterial activity, the complex formed by the siderophore moiety and the antibiotic dissociates in the periplasmic space (Sato and Yamawaki, 2019). Cefiderocol, similarly to other β-lactams, binds and inactivates PBPs, resulting in cell death (Nguyen *et al*., 2018).

In vivo studies conducted on mouse lung models have demonstrated the efficacy of cefiderocol, showing bactericidal properties against *P. aeruginosa* (Sato and Yamawaki, 2019). It has been observed that mutations occur in the iron transport system as a mechanism of resistance to cefiderocol, but further studies are needed to elucidate the potential of cefiderocol in the emergence of resistance (Sato and Yamawaki, 2019).

Fosfomycin

Discovered in 1969, fosfomycin is an old antibiotic, a derivative of a phosphoric acid found in *Streptomyces spp.* (Falagas *et al*., 2016; Falagas *et al*., 2019). In the past, it has been primarily used in the treatment of uncomplicated urinary tract infections (Falagas *et al*., 2016). Owing to the urgent need of finding novel strategies to combat multidrug-resistant bacteria, fosfomycin re-emerged recently as a possible treatment option, primarily in combination with other antibiotics (Falagas *et al*., 2016). Its structure is composed of a phosphonic acid group and an epoxide group, the latter important in its biological activity (Falagas *et al*., 2019). Fosfomycin has two commercially available oral forms (fosfomycin tromethamine and fosfomycin calcium) and an intravenous form (fosfomycin disodium) (Falagas *et al*., 2016).

Fosfomycin is a phosphoenolpyruvate analog, having bactericidal properties (Pál, 2013). Due to its low molecular weight, it can easily penetrate Gram-negative bacterial cells through the pores of the outer membrane, or in the case of *P. aeruginosa* it can also actively enter through transporters (Borisova *et al*., 2014). In the early stages of bacterial cell wall synthesis, fosfomycin inhibits the formation of *N*-acetylmuramic acid, more precisely the activity of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) (Falagas *et al*., 2016). MurA catalyzes the transfer of the enolpyruvyl group of phosphoenolpyruvate to the UDP-*N*-acetylglucosamine, essential in the synthesis of the peptidoglycan layer (Falagas *et al*., 2016). By binding to the active site of MurA, fosfomycin inactivates this enzyme, which results in the damage of the peptidoglycan layer of the cell wall, leading to cell lysis and death (Falagas *et al*., 2019).

P. aeruginosa is intrinsically resistant to fosfomycin, due to its ability to use a salvage pathway in peptidoglycan synthesis, other than the one targeted by fosfomycin (Borisova *et al*., 2014). This pathway allows the recycling of the peptidoglycan rather than being *de novo* synthesized (Falagas *et al*., 2016). Multidrug-resistant *P. aeruginosa* has intrinsic resistance to fosfomycin when used in monotherapies, nonetheless it has been demonstrated that administering it in combination with other antibiotics may provide an effective treatment option (Dijkmans *et al*., 2017; Horcajada *et al*., 2019). Fosfomycin has been found to exhibit synergistic effect with aztreonam, cefepime, meropenem, imipenem, amikacin, ceftazidime, gentamycin, ciprofloxacin (Dijkmans *et al*., 2017).

Novel antibiotics in clinical development

The latest report of the Pew Charitable Trust organization from April 2020, contains 41 antibiotics that are currently in clinical development. Of the antibiotics currently in the pipeline, 18 have the potential of treating pathogens for

which there is an urgent need of novel antimicrobials. Several antibiotics in phase I (Tab. 1) and phase III clinical trials (Tab. 2) are being investigated for their potential antipseudomonal activity (The Pew Charitable Trusts, 2014).

Table 1. Antibiotics in Phase I clinical trials for the treatment of *P. aeruginosa* infections

Antibiotic name	Drug class	Target	Indication(s)
spr 206	Polymyxin	Outer membrane	cUTI, HAP, VAP
Cefepime/Zidebactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase	cUTI, HAP, VAP
Meropenem/Nacubactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase/ PBP2	cUTI

Abbreviations: cUTI – complicated urinary tract infections, HAP – hospital-acquired pneumonia, PBP – penicillin-binding proteins, VAP – ventilator-associated pneumonia

Murepavadin (POL7080)

Macrocyclic peptides targeting the components of the outer membrane protein are under investigation in efforts to fight resistance of Gram-negative bacteria to last-resort antibiotics (Mclaughlin and Donk, 2020). By modifying protegrin-1, a cationic peptide, researchers developed the peptidomimetic murepavadin (Romano *et al*., 2019; Mclaughlin and Donk, 2020). It has been found, that murepavadin specifically targets LptD, a component of the outer membrane of *P. aeruginosa* (Romano *et al*., 2019). LptD is a transport protein which plays a role in the biogenesis of the LPS by inserting it in the outer membrane (Storek *et al*., 2019). Thus, inhibitors of LptD such as murepavadin interfere with the formation of the outer membrane, leading to growth inhibition through a nonlytic mechanism (Romano *et al*., 2019; Storek *et al*., 2019). *P. aeruginosa* shows high resistance to murepavadin by harboring mutations in *pmrB*, the sensor of the PmrA-PmrB TCS (Romano *et al*., 2019).

In a large-scale study, murepavadin showed high efficacy against *P. aeruginosa*, inhibiting 98.7% of the 1,219 isolates tested (Sader *et al*., 2018). Moreover, murepavadin exhibited higher activity than polymyxins, being 4- to 8- fold more active than colistin and polymyxin B (Sader *et al*., 2018). However, clinical trials evaluating the safety and efficacy of intravenous murepavadin were stopped as of July 2019, due to the high incidence of renal failure observed in patients undergoing murepavadin therapy for lower respiratory

tract infections caused by *P. aeruginosa* (Tümmler, 2019). After the unsuccessful phase III clinical trials, intravenous formulation of murepavadin has been taken back to preclinical stages (Mclaughlin and Donk, 2020). The aerosolized form of murepavadin, however, is still in development.

Table 2. Antibiotics in Phase III clinical trials for the treatment of *P. aeruginosa* infections

Antibiotic name	Drug class	Target	Indication(s)
Cefepime/Taniborbactam	β-lactam + β-lactamase inhibitor	PBP β-lactamase	cUTI
Ceftobiprole	β-lactam	PBP	acute skin infections, CAP, HAP
Tebipenem/tebipenem pivoxil hydrobromide	β-lactam	PBP	cUTI

Abbreviations: CAP – community-acquired pneumonia, cUTI – complicated urinary tract infections, HAP – hospital-acquired pneumonia, PBP – penicillin-binding proteins.

Alternative antipseudomonal strategies

Bacteriophage therapy

Bacteriophages are viruses capable of infecting only bacteria *(Pang et al*., 2019). In search for alternative strategies to combat MDR and XDR *P. aeruginosa* infections, bacteriophages first described in 1915 have resurged recently as a potential treatment option owing to the several advantages they possess (Chanishvili and Aminov, 2019). Bacteriophage therapy demonstrates high specificity by targeting only a specific bacterium, whereas antibiotics exhibit a broad-spectrum activity, consequently affecting the normal microflora of the host (Chanishvili and Aminov, 2019). Moreover, by replicating at the site of infection, bacteriophages show an increased local antibacterial activity (Debarbieux *et al*., 2010; Chanishvili and Aminov, 2019). Other advantages of bacteriophages include: little to no side effects; phages can show efficacy in the treatment of biofilms; they adapt to the resistant strains owing to their coevolution with the bacterial host; being the most ubiquitous entities, their discovery and development as antibacterial therapies is inexpensive; their administration unlikely results in inflammatory responses of the immune system (Debarbieux *et al*., 2010).

Phage-resistance in bacteria has already been observed a century ago when researchers described bacterial regrowth following bacteriophage therapy (Oechslin, 2018). One of the resistance mechanisms of bacteria is the occurrence of spontaneous mutation, affecting the phage receptors located in the bacterial outer membrane (Oechslin, 2018). These phage receptors are represented by components of the cell wall (polysaccharides, outer membrane proteins, type IV pili) that are important virulence factors of *P. aeruginosa* (Oechslin, 2018). Thus, with the emergence of phage-resistance, a trade-off cost has been observed: phage-resistant bacteria become less virulent due to alterations of the cell wall-associated virulence factors (Oechslin, 2018; Mangalea and Duerkop, 2020).

An effective way of getting a successful outcome out of bacteriophage therapy would be the combined administration of phages with antibiotics (Oechslin, 2018). Considering the fact that phage-resistance comes with costs to bacteria, targeting specific receptors of *P. aeruginosa* such as the MexAB and MexXY-OprM efflux pumps with bacteriophages could open up other possibilities for antimicrobials to enter bacterial cells, thus restoring susceptibility of the pathogen to antibiotics (Oechslin, 2018; Mangalea and Duerkop, 2020). In the case of *P. aeruginosa,* the administration of phages with streptomycin or ceftazidime showed promising results in combating infections (Oechslin, 2018; Mangalea and Duerkop, 2020).

Another important aspect is the synergy of bacteriophages with the host immune system. *In vivo* studies of *P. aeruginosa* pneumonia using a mouse model showed that phage therapy failed in mice carrying immune defects due to the emergence of phage-resistant strains (Oechslin, 2018). This observation elucidated the fact that nor bacteriophages, nor the innate immune system is effective enough on its own to fight resistant bacteria (Mangalea and Duerkop, 2020).

Different approaches of addressing phage-resistance have been proposed for a successful bacteriophage therapy: phage cocktails and personalized therapy (Oechslin, 2018). By extending the host spectrum of bacteriophages, phage cocktails have demonstrated a promising empirical treatment (Oechslin, 2018). Despite the higher costs associated with the development of personalized phage therapy, this strategy would represent a better way of tackling the emergence of resistance by specifically targeting the pathogen in question (Oechslin, 2018). During bacteriophage therapy, monitoring phage-resistance by adapting phage composition is a key of achieving the best outcomes (Oechslin, 2018).

Pyocins

Pyocins are antimicrobial compounds produced by bacteria for competitive purposes (Redero *et al*., 2018; Oluyombo *et al*., 2019). Of the three types of pyocins produced by *P. aeruginosa*, R-pyocins have been found to have the

highest activity against competitors of the same species (Redero *et al*., 2018). R-pyocins have a phage origin, structurally and genetically being related to the tail of P2 and lambda bacteriophages (Redero *et al*., 2018; Oluyombo *et al*., 2019). After successfully binding to the receptors located on the LPS, pyocins cause the depolarization of the membrane, subsequently leading to cell lysis and death (Redero *et al*., 2018; Oluyombo *et al*., 2019).

Pyocins used as a therapeutic strategy against *P. aeruginosa* have been found to show advantages in contrast with traditional antibiotic therapies. R-pyocins have a narrow-spectrum activity, having little effect on the normal microflora (Redero *et al*., 2018). Moreover, owing to the fact that R-pyocins have no genetic material, they cannot drive the emergence of antibiotic-resistance through the horizontal-transfer of resistance genes (Redero *et al*., 2018). Resistance to pyocins is mediated by the modification of their binding site, the LPS of bacterial cells (Redero *et al*., 2018).

Studies have found a 80% susceptibility rate of *P. aeruginosa* isolates collected from cystic fibrosis lungs, whereas only 50% susceptibility was found in the case of isolates analyzed from blood stream infection (Redero *et al*., 2018).

Alternative strategies for eradicating biofilms

Biofilms produced by *P. aeruginosa* are highly refractory to treatment, making their eradication a great challenge (Maurice *et al*., 2018). Due to the high antibiotic resistance of the pathogen, several promising alternative therapies are under investigation.

Biofilms often develop on medical equipment, leading to severe *P. aeruginosa* infections. A strategy to prohibit biofilm development would be engineering materials that prevent the adhesion of the pathogen to these surfaces (Maurice *et al*., 2018). This would require the implementation of molecules having high antimicrobial resistance onto the material. In a large study, endotracheal tube coated with silver hydrogel showed promising results in preventing *P. aeruginosa* infections and had better patient outcomes (Maurice *et al*., 2018).

Another strategy to approach biofilm treatment would be the inhibition of quorum sensing systems, that have a major role in biofilm formation (Maurice *et al*., 2018). Studies have found several quorum sensing inhibitors that significantly reduced biofilm mass, decreasing the pathogenicity of *P. aeruginosa* (Maurice *et al*., 2018). Halogenated furanones isolated from the macroalga *Delisea pulchra,* and the subsequently developed synthetic furanones exhibited promising QS inhibitor properties and therapeutic effects against *P. aeruginosa* (Maurice *et al*., 2018; Chang *et al*., 2019). Natural compounds such as patulin from *Penicillium*, iberin from horseradish, ellagic acid from *Terminalia chebula*

and eugenol from clove extract have also been investigated for their QS inhibitor properties, but future clinical investigation is still needed to confirm their antipseudomonal efficacy (Maurice *et al*., 2018).

Quorum quenching enzymes (acylases, lactonases) which target AHLs have also been the focus of studies due to their promising effect on biofilms (Maurice *et al*., 2018). Furthermore, host response modulation and inhibition of c-di-GMP signaling could also represent potential strategies in eradicating *P. aeruginosa* biofilms (Maurice *et al*., 2018).

Experimental studies have concluded that fosfomycin administered alone or in combination with other antibiotics is able to penetrate and destroy biofilms (Falagas *et al*., 2016). It has been found that the combination of fosfomycin with prulifloxacin resulted in the eradication of *P. aeruginosa* biofilm layers in rat models (Falagas *et al*., 2016).

Conclusions

Evaluated as a top priority pathogen, *Pseudomonas aeruginosa* has been a subject of concern due to its rapidly emerging resistance to almost all classes of antibiotics. *P. aeruginosa* is the sixth most common nosocomial pathogen, being accountable for 10-15% of hospital-acquired infections worldwide. *P. aeruginosa* can establish infections in any part of the human body, causing severe conditions such as hospital-acquired pneumonia, community-acquired pneumonia, complicated urinary tract infections and complicated intra-abdominal infections, primarily in immunocompromised patients. The successful invasion into host cells is achieved by the arsenal of virulence factors the pathogen possesses, which also provide protection in evading the immune system.

The various resistance mechanisms acquired by *P. aeruginosa* have led to the emergence of multidrug-resistant and extensively drug-resistant strains. The removal of antimicrobials by efflux pumps, the inactivation of the antibiotics by beta-lactamases or the alteration of the target of the antibiotics all contribute to the high resistance demonstrated by *P. aeruginosa*. Moreover, by forming biofilms the pathogen has additional protective layers due to the hardly penetrable matrix.

The majority of traditional antibiotics have lost their efficacy over time due to the pathogen's increased resistance. In recent years, however, several novel antibiotics have been approved for the treatment of *P. aeruginosa* infections. β-lactam/β-lactamase inhibitor combinations such as ceftazidime/avibactam, ceftolozane/tazobactam, imipenem-cilastatin-relebactam have all shown promising results in treating *P. aeruginosa* infections. Cefiderocol, the siderophore cephalosporin, has also shown strong bactericidal properties by

exploiting the iron transport system of *P. aeruginosa*. In addition, an old antibiotic, fosfomycin, has been recently approved as a possible treatment option, primarily in combination with other antibiotics. Furthermore, several new antibiotics are currently in the pipeline in first or late-stage clinical trials, including the *Pseudomonas*-specific murepavadin. Owing to their several advantages, various alternative strategies are also under investigation.

However, due to the rapid development of multidrug-resistant and extensively drug-resistant strains of *P. aeruginosa*, the above-mentioned novel antibiotics could soon be proven ineffective.

Over the last decade, untreatable infections have become alarmingly frequent with the rise of antibiotic resistant strains of bacteria, to the extent to which clinicians might soon be left with no effective treatment options available. In order to halt the post-antibiotic era we are headed to, there are several courses of actions that can be taken to prevent and stop the spread of resistance: educating the population about the appropriate use of antibiotics, improving antibiotic prescription by healthcare providers, preventing environmental contamination by properly disposing antibiotics and ultimately promoting the research and development of novel antibiotics to combat emerging pathogens.

References

Al-Wrafy, F., Brzozowska, E., Górska, S., & Gamian, A. (2017). Pathogenic factors of *Pseudomonas aeruginosa* - the role of biofilm in pathogenicity and as a target for phage therapy. *Postepy Higieny i Medycyny Doswiadczalnej*, *71*(February), 78–91. doi:10.5604/01.3001.0010.3792

Azam, M. W., & Khan, A. U. (2019). Updates on the pathogenicity status of *Pseudomonas aeruginosa*. *Drug Discovery Today*, *24*(1), 350–359. doi:10.1016/j.drudis.2018.07.003

Balasubramanian, D., Schneper, L., Kumari, H., & Mathee, K. (2013). A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. *Nucleic Acids Res*, *41*(1), 1–20. doi:10.1093/nar/gks1039

Banin, E., Hughes, D., & Kuipers, O. P. (2017). Editorial: Bacterial pathogens, antibiotics and antibiotic resistance. *FEMS Microbiol Rev*, *41*(3), 450–452. doi:10.1093/femsre/fux016

Baron, S., Hadjadj, L., Rolain, J. M., & Olaitan, A. O. (2016). Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents*, *48*(6), 583–591. doi:10.1016/j.ijantimicag.2016.06.023

Borisova, M., Gisin, J., & Mayer, C. (2014). Blocking Peptidoglycan Recycling in *Pseudomonas aeruginosa* Attenuates Intrinsic Resistance to Fosfomycin. *Microb Drug Resist*, *20*(3), 231–237. doi:10.1089/mdr.2014.0036

Bott, M., & Holland, S. (2020). AMR Action Fund. Retrieved from https://amractionfund.com/

Cascella, P. N. (2019). *Beta lactam antibiotics*. *StatPearls*. Treasure Island (FL): StatPearls Publishing. doi:10.1016/j.actpha.2016.06.001

CDC. (2013). *Antibiotic Resitance Threats in the United States, 2013*. doi:/10.1016/j.medmal.2007.05.006

CDC. (2017). *Antibiotic Use in the United States, 2017: Progress and Opportunities*. *US Department of Health and Human Services*. Atlanta, GA: US Department of Health and Human Services. Retrieved from https://www.cdc.gov/antibiotic-use/stewardship-report/pdf/stewardship-report.pdf

Chang, Y., Wang, P. C., Ma, H. M., Chen, S. Y., Fu, Y. H., Liu, Y. Y., Wang, X., Yu, G. C., Huang, T., Hibbs, D. E., Zhou, H. B., Chen, W. M., Lin, J., Wang, C., Zheng, J. X., Sun, P. H. (2019). Design, synthesis and evaluation of halogenated furanone derivatives as quorum sensing inhibitors in *Pseudomonas aeruginosa*. *Eur J of Pharm Sci*, *140*(August), 105058. doi:10.1016/j.ejps.2019.105058

Chanishvili, N., & Aminov, R. (2019). Bacteriophage therapy: Coping with the growing antibiotic resistance problem. *Microbiol Aust*, *40*(1), 5–7. doi:10.1071/MA19011

Ciofu, O., & Tolker-Nielsen, T. (2019). Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-How P. aeruginosa can escape antibiotics. *Front Microbiol*, *10*(MAY). doi:10.3389/fmicb.2019.00913

De Bentzmann, S., Roger, P., Dupuit, F., Bajolet-Laudtnat, O., Fuchey, C., Plotkowski, M. C., & Puchelle, E. (1996). Asialo GM1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect Immun*, *64*(5), 1582–1588. doi:10.1128/iai.64.5.1582-1588.1996

De Kievit, T. R. (2009). Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol*, *11*(2), 279–288. doi:10.1111/j.1462-2920.2008.01792.x

Debarbieux, L., Leduc, D., Maura, D., Morello, E., Criscuolo, A., Grossi, O., Balloy, V., Touqui, L. (2010). Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. *JID*, *201*(7), 1096–1104. doi:10.1086/651135

Dijkmans, A. C., Zacarías, N. V. O., Burggraaf, J., Mouton, J. W., Wilms, E. B., van Nieuwkoop, C., Touw, D. J., Stevens, J., Kamerling, I. M. C. (2017). Fosfomycin: Pharmacological, clinical and future perspectives. *Antibiotics*, *6*(4), 1–17. doi:10.3390/antibiotics6040024

Doi, Y., Wachino, J. I., & Arakawa, Y. (2016). Aminoglycoside Resistance: The Emergence of Acquired 16S Ribosomal RNA Methyltransferases. *Infect Dis Clin N Am*, *30*(2), 523–537. doi:10.1016/j.idc.2016.02.011

Donowitz, G. R., & Mandell, G. L. (1988). Beta-lactam antibiotics. *N Engl J Med*, *318*, 419–426.

Driscoll, J. A., Brody, S. L., & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs*, *67*(3), 351–368. doi:10.2165/00003495-200767030-00003

ECDC. (2019). *Surveillance of antimicrobial resistance in Europe 2018*. *Surveillance Report*. doi:/10290035994

Fair, R. J., & Tor, Y. (2014). Perspectives in Medicinal Chemistry Antibiotics and Bacterial Resistance in the 21st Century. *Perspect Medicin Chem*, *6*, 25–64. doi:10.4137/PMC.S14459.Received

Falagas, M. E., Athanasaki, F., Voulgaris, G. L., Triarides, N. A., & Vardakas, K. Z. (2019). Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. *Int J Antimicrob Agents*, *53*(1), 22–28. doi:10.1016/j.ijantimicag.2018.09.013

Falagas, M. E., Vouloumanou, E. K., Samonis, G., & Vardakas, K. Z. (2016). Fosfomycin. *Clin Microbiol Rev*, *29*(2), 321–347. doi:10.1128/CMR.00068-15.Address

Forge, A., & Schacht, J. (2000). Aminoglycoside antibiotics. *Audiol Neurootol*, *5*, 3–22. doi:10.1002/9783527678679.dg00403

Fujitani, S., Sun, H. Y., Yu, V. L., & Weingarten, J. A. (2011). Pneumonia due to *Pseudomonas aeruginosa*: Part I: Epidemiology, clinical diagnosis, and source. *Chest*, *139*(4), 909–919. doi:10.1378/chest.10-0166

Ghazaei, C., Ahmadi, M., & Jazani, N. H. (2010). Detection of neuraminidase activity in *Pseudomonas aeruginosa* PAO1. *Iran J Basic Med Sci*, *13*(3), 69–75. doi:10.22038/ijbms.2010.5087

Golemi-Kotra, D. (2008). *Pseudomonas* infections. *XPharm*, 1–8. doi:10.1016/B978-008055232-3.63828-0

Green, E. R., & Mecsas, J. (2016). Bacterial Secretion Systems: An Overview. *Microbiol Spectr*, *4*(1). doi:10.1128/microbiolspec.vmbf-0012-2015

Haidar, G., Philips, N. J., Shields, R. K., Snyder, D., Cheng, S., Potoski, B. A., Doi, Y., Hao, B., Press, E. G., Cooper, V. S., Clancy, C. J., Nguyen, M. H. (2017). Ceftolozane-Tazobactam for the Treatment of Multidrug-Resistant *Pseudomonas aeruginosa* Infections: Clinical Effectiveness and Evolution of Resistance. *Clin Infect Dis*, *65*(1), 110–120. doi:10.1093/cid/cix182

Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A. J., Forbes, A., Perkins, A. V., Davey, A. K., Chess-Williams, R., Kiefel, M. J., Arora, D., Grant, G. D. (2016). Cellular effects of pyocyanin, a secreted virulence factor of *Pseudomonas aeruginosa*. *Toxins*, *8*(8), 1–14. doi:10.3390/toxins8080236

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature*, *410*(6832), 1099–1103. doi:10.1038/35074106

Hirsch, E. B., & Tam, V. H. (2010). Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Rev Pharmacoecon Outcomes Res*, *10*(4), 441–451. doi:10.1586/erp.10.49

Horcajada, J., Milagro Montero, Oliver, A., Sorlí, L., Sònia Luque, Gómez-Zorrilla, S., Benito, N., Grau, S. (2019). Epidemiology and Treatment of Multidrug-Resistant and Extensively Drug-Resistant *Pseudomonas aeruginosa* infections. *Clin Microbiol Rev*, *32*(4), 1–52.

Jones, A., & Holland, S. (2020). New AMR Action Fund steps in to save collapsing antibiotic pipeline with pharmaceutical industry investment of US$1 billion – IFPMA. *International Federation of Pharmaceutical Manufacturers & Associations*, (July), 20–22. Retrieved from https://www.ifpma.org/resource-centre/new-amr-action-fund-steps-in-to-save-collapsing-antibiotic-pipeline/?linkId=100000013447572

Khan, A. (2020). Doctor's Note: How COVID-19 is increasing antibiotic resistance | Coronavirus pandemic | Al Jazeera. Retrieved November 7, 2020, from https://www.aljazeera.com/indepth/features/doctor-note-covid-19-increasing-antibiotic-resistance-200608151154225.html?fbclid=IwAR2B8BgK42ZUQOhRWzDNgVlvrztn85bjhlpWk-z6_UX57xQWugEA15n6mqU

Kharazmi, A. (1991). Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. *Immunol Lett*, *30*, 201–205.

Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jørgensen, A., Molin, S., & Tolker-Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol*, *48*(6), 1511–1524. doi:10.1046/j.1365-2958.2003.03525.x

Lamas Ferreiro, J. L., Álvarez Otero, J., González González, L., Novoa Lamazares, L., Arca Blanco A., Bermúdez Sanjurjo, J. R., Rodríguez Conde, I., Fernández Soneira, M., de la Fuente Aguado, J. (2017). *Pseudomonas aeruginosa* urinary tract infections in hospitalized patients: Mortality and prognostic factors. *PLoS ONE*, *12*(5), 1–13.

Landecker, H. (2016). Antibiotic Resistance and the Biology of History. *Body Soc*, *22*(4), 19–52. doi:10.1177/1357034X14561341

Lister, P. D., Wolter, D. J., & Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev*, *22*(4), 582–610. doi:10.1128/CMR.00040-09

Mangalea, M. R., & Duerkop, B. A. (2020). Fitness trade-offs resulting from bacteriophage resistance potentiate synergistic antibacterial strategies. *Infect Immun*, *88*(7), 1–15. doi:10.1128/IAI.00926-19

Maraolo, A. E., Mazzitelli, M., Trecarichi, E. M., Buonomo, A. R., Torti, C., & Gentile, I. (2020). Ceftolozane/tazobactam for difficult-to-treat *Pseudomonas aeruginosa* infections: A systematic review of its efficacy and safety for off-label indications. *J Antimicrob Agents*, *55*(3), 105891. doi:10.1016/j.ijantimicag.2020.105891

Maurice, N. M., Bedi, B., & Sadikot, R. T. (2018). *Pseudomonas aeruginosa* biofilms: Host response and clinical implications in lung infections. *Am J Respir Cell Mol Biol*, *58*(4), 428–439. doi:10.1165/rcmb.2017-0321TR

Mclaughlin, M. I., & Donk, W. A. Van Der. (2020). The Fellowship of the Rings: Macrocyclic Antibiotic Peptides Reveal an Anti-Gram-Negative Target. *Biochemistry*, *59*(343–345), 2019–2021. doi:10.1021/acs.biochem.9b01086

Miller, J. K., Badawy, H. T., Clemons, C., Kreider, K. L., Wilber, P., Milsted, A., & Young, G. (2012). Development of the *Pseudomonas aeruginosa* mushroom morphology and cavity formation by iron-starvation: A mathematical modeling study. *J Theor Biol*, *308*, 68–78. doi:10.1016/j.jtbi.2012.05.029

Mittal, R., Aggarwal, S., Sharma, S., Chhibber, S., & Harjai, K. (2009). Urinary tract infections caused by *Pseudomonas aeruginosa*: A minireview. *J Infect Public Health*, *2*(3), 101–111. doi:10.1016/j.jiph.2009.08.003

Moffat, J. H., Harper, M., & Boyce, J. D. (2019). Polymyxin Antibiotics: From Laboratory Bench to Bedside. In *Springer Nature Switzerland* (Vol. 1145, pp. 1–8). doi:10.1007/978-3-030-16373-0

Morrison, A. J., & Wenzel, R. P. (1984). Epidemiology of infections due to *Pseudomonas aeruginosa*. *Rev Infect Dis*, *6 Suppl 3*(October). doi:10.1093/clinids/6.supplement_3.s627

Nature Editorial. (2020). Antimicrobial resistance in the age of COVID-19. *Nat Microbiol*, *5*(6), 779. doi:10.1038/s41564-020-0739-4

Neves, P. R., McCulloch, J. A., Mamizuka, E. M., & Lincopan, N. (2014). *Pseudomonas*: *Pseudomonas aeruginosa*. *Encyclopedia of Food Microbiology: Second Edition*, *3*, 253–260. doi:10.1016/B978-0-12-384730-0.00283-4

Nguyen, L., Garcia, J., Gruenberg, K., & MacDougall, C. (2018). Multidrug-Resistant *Pseudomonas* Infections: Hard to Treat, But Hope on the Horizon? *Curr Infect Dis Rep*, *20*(8). doi:10.1007/s11908-018-0629-6

O'Neall, D., Juhász, E., Tóth, Á., Urbán, E., Szabó, J., Melegh, Sz., Katona, K., Kristóf, K. (2020). Ceftazidime–avibactam and ceftolozane–tazobactam susceptibility of multidrug resistant *Pseudomonas aeruginosa* strains in Hungary. *Acta Microbiol Immunol Hung*, *67*(1), 61–65. doi:10.1556/030.2020.01152

Oechslin, F. (2018). Resistance development to bacteriophages occurring during bacteriophage therapy. *Viruses*, *10*(7). doi:10.3390/v10070351

Olaitan, A. O., Morand, S., & Rolain, J. M. (2014). Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. *Front Microbiol*, *5*(NOV), 1–18. doi:10.3389/fmicb.2014.00643

Oluyombo, O., Penfold, C. N., & Diggle, S. P. (2019). Competition in biofilms between cystic fibrosis isolates of *Pseudomonas aeruginosa* is shaped by R-pyocins. *MBio*, *10*(1), 1–13. doi:10.1128/mBio.01828-18

Pál, T. (2013). *Az Orvosi Mikrobiológia Tankönyve*. Budapest: Medicina Könyvkiadó.

Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J., & Cheng, Z. (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol Adv*, *37*(1), 177–192. doi:10.1016/j.biotechadv.2018.11.013

Papp, J. (2015). *Orvosi Mikrobiológia (jegyzet)*. Kolozsvár.

Perry, J., Waglechner, N., & Wright, G. (2016). The prehistory of antibiotic resistance. *Cold Spring Harb Perspect Med*, *6*(6). doi:10.1101/cshperspect.a025197

Planet, P. J. (2017). *Pseudomonas aeruginosa*. *Principles and Practice of Pediatric Infectious Diseases* (Fifth Edit). Elsevier Inc. doi:10.1016/B978-0-323-40181-4.00155-9

Poole, K. (2004). Resistance to β-lactam antibiotics. *Cell Mol Life Sci*, *61*(17), 2200–2223. doi:10.1007/s00018-004-4060-9

Poole, K. (2005). Aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemoter*, *49*(2), 479–487. doi:10.1128/AAC.49.2.479-487.2005

Poole, K. (2011). *Pseudomonas aeruginosa*: Resistance to the max. *Front Microbiol*, *2*(APR), 1–13. doi:10.3389/fmicb.2011.00065

Redero, M., López-Causapé, C., Aznar, J., Oliver, A., Blázquez, J., & Prieto, A. I. (2018). Susceptibility to R-pyocins of *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients. *J Antimicrob Chemother*, *73*(10), 2770–2776. doi:10.1093/jac/dky261

Romano, K. P., Warrier, T., Poulsen, B. E., Nguyen, P. H., Loftis, A. R., Saebi, A., Pentelute, B. L., Hung, D. T. (2019). Mutations in pmrB Confer Cross-Resistance between the LptD Inhibitor POL7080 and Colistin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*, *63*(9), 1–6. doi:10.1128/AAC.00511-19

Rossolini, G. M., Arena, F., Pecile, P., & Pollini, S. (2014). Update on the antibiotic resistance crisis. *Curr Opin Pharmacol*, *18*, 56–60. doi:10.1016/j.coph.2014.09.006

Sader, H. S., Dale, G. E., Rhomberg, P. R., & Flamm, R. K. (2018). Antimicrobial activity of murepavadin tested against clinical isolates of *Pseudomonas aeruginosa* from the United States, Europe, and China. *Antimicrob Agents Chemother*, *62*(7), 1–6. doi:10.1128/AAC.00311-18

Sadikot, R. T., Blackwell, T. S., Christman, J. W., & Prince, A. S. (2005). Pathogen-Host Interactions in *Pseudomonas aeruginosa* Pneumonia RESPIRATORY INFECTIONS. *Am J Respir Crit Care Med*, *171*(11), 1209–1223. doi:10.1164/rccm.200408-1044SO

Sato, T., & Yamawaki, K. (2019). Cefiderocol: Discovery, Chemistry, and *in Vivo* Profiles of a Novel Siderophore Cephalosporin. *Clin Infect Dis*, *69*(Suppl 7), S538–S543. doi:10.1093/cid/ciz826

Skariyachan, S., Sridhar, V. S., Packirisamy, S., Kumargowda, S. T., & Challapilli, S. B. (2018). Recent perspectives on the molecular basis of biofilm formation by *Pseudomonas aeruginosa* and approaches for treatment and biofilm dispersal. *Folia Microbiol*, *63*(4), 413–432. doi:10.1007/s12223-018-0585-4

Smith, J. R., Rybak, J. M., & Claeys, K. C. (2020). Imipenem-Cilastatin-Relebactam: A Novel β-Lactam–β-Lactamase Inhibitor Combination for the Treatment of Multidrug-Resistant Gram-Negative Infections. *Pharmacotherapy*, *40*(4), 343–356. doi:10.1002/phar.2378

Spencer, C., & Brown, H. A. (2015). Biochemical characterization of a *Pseudomonas aeruginosa* phospholipase d. *Biochemistry*, *54*(5), 1208–1218. doi:10.1021/bi501291t

Spohn, R. (2018). A bakteriális antibiotikum rezisztencia de novo evolúciója és járulékos következményei Ph . D . értekezés.

Stone, G. G., Newell, P., Gasink, L. B., Broadhurst, H., Wardman, A., Yates, K., Chen, Zhangjing, Song, J., Chow, J. W. (2018). Clinical activity of ceftazidime/avibactam against MDR Enterobacteriaceae and *Pseudomonas aeruginosa*: pooled data from the ceftazidime/avibactam Phase III clinical trial programme. *J Antimicrob Chemother*, *73*(June), 2519–2523. doi:10.1093/jac/dky204

Storek, K. M., Chan, J., Vij, R., Chiang, N., Lin, Z., Bevers, J., Koth, C. M., Vernes, J. M., Meng, Y.G., Yin, J., Wallweber, H., Dalmas, O., Shriver, S., Tam, C., Schneider, K., Seshasayee, D., Nakamura, G., Smith, P. A., Payandeh, J., Koerber, J. T., Comps-Agrar, L., Rutherford, S. T. (2019). Massive antibody discovery used to probe structure–function relationships of the essential outer membrane protein lptd. *ELife*, *8*, 1–20. doi:10.7554/eLife.46258.001

Strateva, T., & Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections. *Ann Microbiol*, *61*(4), 717–732. doi:10.1007/s13213-011-0273-y

Strateva, T., & Yordanov, D. (2009). *Pseudomonas aeruginosa* - A phenomenon of bacterial resistance. *J Med Microbiol*, *58*(9), 1133–1148. doi:10.1099/jmm.0.009142-0

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Oullette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, Nalini, Theuretzbacher, U., Magrini, N. and the WHO Pathogen Priority List Working Group. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*, *18*(3), 318–327. doi:10.1016/S1473-3099(17)30753-3

The Pew Charitable Trusts. (2014). Antibiotics Currently in Clinical Development. Retrieved from http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development

Todar, K. (2004). Todar's online textbook of bacteriology. Retrieved from http://www.textbookofbacteriology.net/pseudomonas.html

Tümmler, B. (2019). Emerging therapies against infections with *Pseudomonas aeruginosa* [version 1; peer review: 2 approved]. *F1000Research*, *8*, 1–14. doi:10.12688/f1000research.19509.1

Ventola, L. C. (2019). The Antibiotic Resistance Crisis Part 1: Causes and Threats. *PT*, *40*(4), 277–283. doi:10.24911/ijmdc.51-1549060699

WHO. (2017). *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. doi:/10.4103/jms.jms_25_17

Wu, W., Jin, Y., Bai, F., & Jin, S. (2014). *Pseudomonas aeruginosa*. *Molecular Medical Microbiology: Second Edition* (Vol. 2–3). Elsevier Ltd. doi:10.1016/B978-0-12-397169-2.00041-X

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., & Hossain, N. (2017). A Review on Antibiotic Resistance: Alarm Bells are Ringing. *Cureus*, *9*(6). doi:10.7759/cureus.1403

Zhanel, G. G., Chung, P., Adam, H., Zelenitsky, S., Denisuik, A., Schweizer, F., Lagacé-Wiens, P. R. S., Rubinstein, E., Gin, A. S., Walkty, A., Hoban, D. J., Lynch, J. P., Karlowsky, J. A. (2014). Ceftolozane/tazobactam: A novel cephalosporin/β-lactamase inhibitor combination with activity against multidrug-resistant gram-negative bacilli. *Drugs*, *74*(1), 31–51. doi:10.1007/s40265-013-0168-2

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 65, 2, 2020 (p. 69-86)
doi:10.24193/subbbiol.2020.2.04

Current perspectives on the remediation methods of marine plastic pollution: a review

Andreea Lup[1], Mihai Gorea[1], Denisa Bruhs[1], Oana Căpriţă[1]✉ and Patricia Vajda[1]

[1]*Babeş-Bolyai University, Faculty of Biology and Geology, Cluj-Napoca, Romania;*
✉***Corresponding author, E-mail: andreeaoana.k@gmail.com.***

Article history: Received 26 September 2020; Revised 20 November 2020; Accepted 3 December 2020; Available online 20 December 2020.

Abstract. Plastic debris represents a contemporary point of concern for the marine environment, being discharged into the ocean at an alarming scale. However, the quantity of waste that is found in the ocean is unknown. Where does this waste come from, and where does it end up are questions that scientists and researchers are still trying to accurately answer. The majority of plastic products that make their way into the ocean come mainly from human activities. Most of them land on beaches, and eventually find their way into the ocean, being washed away by waves and tides. To assess the impact of these pollutants that are found in the marine environment, it is necessary to determine the concentration of the chemicals accumulating in the biomass, and the effects they cause. There are numerous biological effects which lead to many obvious diseases in marine species. Also, these harmful effects determine changes in community structure, the modification of the habitat and local or complete extinction of many aquatic species. This review aims to lay out the present situation of the marine environment, and the effects of the pollution caused by industrialization and urbanization. Different types of remediation approaches have been discussed, such as physical remediation techniques. Besides that, the role of numerous bacteria and fungi that are capable of breaking down these chemicals that surround us, has been highlighted and point at some of the bioremediation technologies that are currently available.

Keywords: plastic, pollution, marine environment, microorgansims, bioremediation.

Introduction

Taking into account the current situation in which the Planet is found, one can clearly observe the rapid degradation of the environment of marine organisms due to the massive pollution of the oceans. This matter has profound

implications, both ecological and ethical, as well as economic and medical (Iñiguez *et al.*, 2016). The global marine ecosystem is at risk, including its major biodiversity and its unique realm. Ever since humans became a global driving force, constantly growing, and demanding for industrialization and globalization, natural environments such as the high seas have been deeply disturbed (Alava, 2019). Marine litter is considered to be any type of manufactured or processed material that is discarded or abandoned and that persists for a long time in the environment (Iñiguez *et al*., 2016). The debris that is most commonly found in the marine environment is represented by glass, paper, plastic, and metal. Besides these, chemical waste and oil spills are also worth being mentioned and taken into consideration when addressing the rising concern regarding the pollution and therefore, the destruction of the marine environment. Plastic represents the main pollutant on our Planet; more than 380 million tonnes of plastic is annually produced, which eventually ends up in the ocean. The rate of total plastic waste reaching the ocean is about 3% (Jambeck *et al.*, 2015). There are two categories of plastic: macroplastic – plastic with the diameter larger than 0.5 cm, and microplastic – particles smaller than 0.5 cm (Lebreton *et al.*, 2019). Macroplastics are synthetic materials that last for a long time in the environment, which can be physically broken down into smaller pieces (microplastics), under the influence of sun rays and solar radiation (Moore, 2008). This review aims to present a clear image of the current situation regarding ocean plastic pollution and the measures that are used at the moment to reduce the ongoing harm. Even though different approaches have been described and applied so far, these including physical, chemical and biological methods, each one of them still has its limitations. Therefore, we aim to present their advantages and disadvantages, and also bring new perspectives on the remediation methods that are currently employed.

The amount of plastic waste that reaches the oceans

Plastic accounts for about 80% – 85% of marine litter; this percentage is increasing annually, as a result of the rising global consumption (Auta *et al*., 2017). Based on the *National Oceanic and Atmospheric Administration* (Lippiatt *et al.*, 2013), at least 24 types of macroplastics have been determined, that currently float on the surface of the oceans. These are mainly represented by bags (for shopping and garbage), followed by food wrappers, disposable bottles (water and soft drinks), bottle caps, cleaning products packaging, personal care products, toys, toothbrushes, etc. (Lippiatt *et al*., 2013). Different items were discovered per transect, around 217 macroplastics, where 1.15 m^{-2} and 91 of these being represented by food packaging. Unsurprisingly, the highest volume was taken up

by empty bottles (Lippiatt *et al*., 2013). The large majority of macroplastics (around 82 million tonnes), originates from land regions, mostly beaches. Most of them come from the last 15 years; still, a substantial amount is older, showing that plastic can persist for several decades without degrading (Lebreton *et al*., 2019). A well-known hypothesis is that the macroplastic is continuously fragmented under the action of physical (eg. sunlight), chemical and biological (microorganism) factors into smaller pieces, called microplastic (Lusher *et al*., 2014; Reisser *et al*., 2015). The microplastic is coming from two main sources: the primary microplastics are those that are manufactured specifically for industrial applications and domestic products, such as cosmetics, cleaning products, pharmaceuticals, or resin pellets used in the plastics industry; the secondary ones are formed following the decomposition of macroplastics (Solomon and Palanisami, 2016; Sharma and Chatterjee, 2017; Lu *et al*., 2019).

Ocean's most affected areas

The Pacific Ocean Pollution

In oceans, massive circular current systems accumulate plastic waste in garbage *islands* at significant distances from land. In the northern part of the Pacific Ocean, in the midst of circular currents, such an island accumulates– the Great Pacific Garbage Patch (GPGP), which is reported to be a solid, continental form, entirely made of garbage. GPGP is one of the largest offshore plastic accumulation zones and is estimated to be bigger than Texas, possibly twice the size of the southern US's state (Lebreton *et al*., 2018). This Pacific trash vortex is made up of the Western Garbage Island, located near Japan and the Eastern Garbage Island, located between the states of Hawaii and California. The amount of debris within the GPGP accumulates because of the persistence of the pollutants in the environment and its reduced biodegradation capability (Morét-Ferguson *et al*., 2010; Philp, 2013). Researchers have shown that there are inter annual and seasonal variations in that specific location (Chen *et al*., 2018).

In 2001, the team of researchers led by oceanographer Moore C. found that in some areas of the patch, the concentration of plastic had already reached one million particles km^{-2}. Thus, there is a quantity of 335.000 pieces of plastic km^{-2} with an average weight of 5 kg km^{-2} (Ryan *et al*., 2009). Specific characteristics of the GPGP suggest that only certain types of plastic have the ability to persist on the surface of the ocean for a long time, accumulating and forming plastic patches in the ocean. It is known that plastic pollution is growing exponentially in the Pacific trash vortex, and at a much faster rate than in the surrounding waters, which means that the mass inflow is significantly higher than the outflow. The mass of plastics floating on the surface of the oceans is mostly

mega- and macroplastic, and it is difficult to estimate how long it takes for them to degrade into smaller pieces, eventually disappearing from the surface of the water by sinking in the ocean (Lebreton *et al*., 2018). The existence of macroplastics that date back to the 70s, 80s and 90s, compared to more recent samples, suggests that some specific types of plastic (with a high volume-surface ratio, and in the presence of low wind) persist and accumulate in the GPGP (Brandon *et al*., 2016).

The Atlantic Ocean

The Atlantic Ocean is probably the second most polluted ocean after the Pacific, consisting of two main areas where the debris is accumulating (Morét-Ferguson *et al*., 2010). These are located in the North Atlantic Ocean, as well as in the South Atlantic Ocean, being represented by garbage patches that are similar to the GPGP (Lusher *et al*., 2014). It comprises different types of plastics that vary in size and form, such as the ones found in the GPGP (Lusher *et al*., 2014). Researcher Wilcox C. and his team have shown that the amount of plastic in the North Atlantic Ocean is growing over time, estimating that in 2010 alone it increased by 506,000 tonnes. They also suggested the fact that the abundance of garbage increases in the ocean due to the development of the industry and the never-ending use of plastic (Wilcox *et al*., 2019).

At the opposite pole, the garbage patch from the South Atlantic Ocean is located between South America and southern Africa (Ryan, 2014). Currently, it is considered that the South Atlantic Ocean garbage patch is substantially smaller, and more dispersed than the one in the North Atlantic Ocean since it is located between two continents that are still developing, and that have lower consumption rates. However, new studies claim that the eddies caused by ocean currents are interconnected globally, also stating that much of the waste will eventually end up in the GPGP (Van Sebille *et al*., 2012).

Causes and effects of plastic waste discharges into the oceans

This type of debris has a great impact on the marine environment, affecting the living organisms and causing their movement from one geographical area to another, where the living conditions are not optimal for them to thrive. Usually, these are represented by sessile and mobile organisms, including algae, invertebrates, fish, even iguanas, which have been observed floating on marine waste, becoming the main prey of plastic (Barboza *et al*., 2019). Over half of the macro items contain at least one hazardous component in their consistency. Macroplastic is the product of oil refining and its properties are enhanced using additives that are accountable for strengthening and softening the material.

Chemicals contained in plastic, like bisphenol-A and nonylphenol, are potential pests for the marine environment. These two additives are known to be harmful to the human endocrine system, which has led to the conclusion that they could have the same effects on several species. These chemicals disrupt the endocrine system by acting on estrogenic and androgenic hormones, thus stopping the development of organisms. It has been observed that macroplastics contain heavy metals, chemicals and pesticides, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (Rios *et al*., 2010).

Marine and mangrove sediment have the ability to accumulate microplastics, these being considered to be settling tanks for these types of particles. Multiple studies have shown that microplastics can be bioaccumulated by the phytoplankton and ingested by the zooplankton and other organisms (invertebrates, fish, turtles, mammals), serving as vectors for the transport of pathogens, adsorbing and accumulating toxic substances (bisphenol A, ethers of polybrominated diphenyl, DDT) which can be subsequently transferred through the food chain (Solomon *et al*., 2016; Sharma and Chatterjee, 2017). Microplastics are easily accessible to a wide range of marine organisms due to their small size, often being confused with food and being ingested (Lu *et al*.,2019). However, there are also cases in which certain organisms target them. Several studies have shown that microplastics present a great health risk for the organisms that consume them, causing pathological stress, a false sensation of satiety, complications in the reproductive process, blockage of enzymatic processes, reducing growth rates and oxidative stress. Microplastics have the potential to cause cancer as well, lowering the immune response and causing malformations in animals, including humans. Consumption of microplastics can cause physical diseases, as well as chemical imbalances. The first developed as a follow up of the attachment of polymers to the surface externalities of the consumers, impeding mobility, and clogging the digestive tract. The latter includes inflammation, liver stress, accumulation of lipids in the liver and diminished development, thus, developing lipid and energetic metabolism disorders (Solomon *et al*., 2016; Auta *et al*., 2017; Sharma and Chatterjee, 2017; Lu *et al*., 2019). Once in the body, the microplastic can interact with the gut microbiota, causing several negative effects such as inflammation of the intestines and metabolism disorders. The microbiota and the immune system are connected; therefore, once the microbiota changes due to the accumulation of microplastics, various imbalances and diseases will be highly possible to develop in the host organism (Lu *et al*., 2019). Accumulation of microplastics can also take place through the ventilation process performed by the gills, microplastics being therefore bioaccumulated. These plastic particles endanger the life of fish, the mortality rate to those who have not yet reached maturity and have consumed plastic particles, growing considerably (Auta *et al*., 2017).

Remediation methods for macro- and microplastic pollution

Due to the exaggerated increase in plastic pollution, different countries have made huge efforts in order to develop innovative technologies that reduce, and have the potential to even overcome the enormous waste problem (Othman *et al*., 2020). When it comes to the remediation of plastic polluted areas, commonly used methods are the physical and the biological ones.

Physical methods

Considering the fact that we live in a world that is run by high technologies, it is surprising to see that, despite the horrifying environmental conditions, almost no robotic research has been run to expand a process that involves identification and collection of the waste materials, followed by its sorting, both at a macro- and micro-scale (Rojas, 2018). Another step that should be also taken into consideration is related to the possible sale of recycled plastic (van Giezen and Wiegmans, 2020). The few trash cleaning systems and robots that have been developed so far, can be categorized as static or dynamic systems (Fig.1). The first type, which is stationary, is divided into autonomous and mechanical systems, while the latter, that has the ability to move around, is either an autonomous system or a computational one. Besides these, there is also another alternative that integrates both systems. To put it more into context, when it comes to a static trash cleaning system, India and the US seem to be the leading parties. The Asian country developed a static-autonomous system that is environmentally friendly, being powered by solar energy. The marine debris is collected using a conveyor. The US, on the other hand, used the river's current energy, which is another environmentally friendly alternative to collect the floating trash. The system used fits into the static-mechanical category (Othman *et al*., 2020).

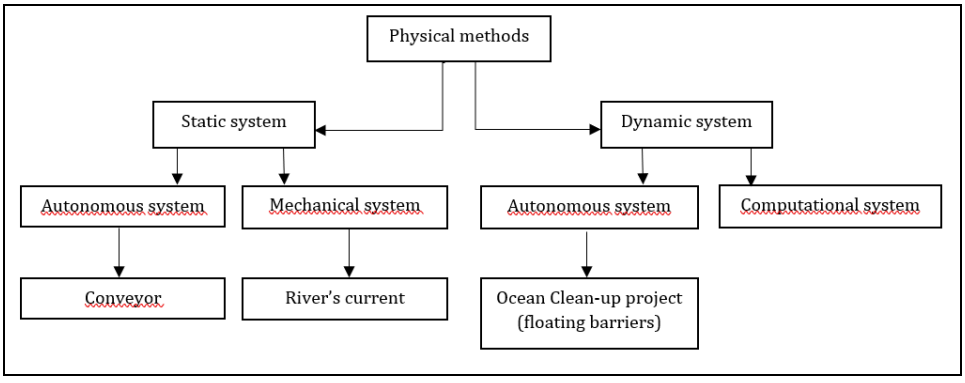


Figure 1. Physical methods for combating ocean plastic pollution.

When it comes to a dynamic-autonomous system, one of the most popular at the moment is The Ocean Cleanup project, which dates back to 2013. This system is currently deployed in the Pacific Ocean, where the greatest garbage patch is found (Slat, 2014; Hohn *et al.*, 2020). One of its main goals is to clean the ocean, while also protecting the environment, and its marine organisms, so that none of the wildlife could turn into a bycatch. Besides that, a well-thought design would include a low carbon-footprint of all the steps taken, from the construction to the supply chain processes (van Giezen and Wiegmans, 2020). The Ocean Cleanup project set the goal to clean the GPGP in the upcoming 20 years. Despite their high hopes, researchers such as Hohn and his team came to the conclusion that the surface plastic found in the entire ocean will be reduced by only 0.09% of the total amount by 2150. Following their model, even if deploying multiple cleaning systems into the oceans (around 200), the plastic debris would still be reduced by only 5,21% by 2150 (44,900 Mg of plastic out of 860,000 Mg). Their assessment took into consideration that the cleaning system assumingly works without failure, and that the plastic is homogeneous distributed (Hohn *et al.*, 2020). The system consists of an array of booms (floating barriers) and platforms. These are moored to the ocean floor and are able to capture the floating plastic particles, while marine organisms that are neutral remain underneath the boom, in the water flow. Turning this concept into reality, buoyant plastic can be efficiently removed from the seawater, following three phases. In the initial phase, plastic particles and pellets are trapped in the frontal area of the floating barriers. These come from the main flow of the ocean, and end up into the almost still water, located in the front of the barriers. Following the second phase, plastic particles and fragments accumulate, moving along the boom, following the path towards the platform. At the same time, new plastic waste is being continuously retained into the stream. In the final stage, the plastic flow that comes from both sides of the system meets in a central area, in front of the collection platform. Due to the increased concentration of debris, an efficient collection of floating plastics is possible (Slat, 2014). When it comes to dynamic-computational systems, the technology is not so advanced; therefore, these do not operate great garbage patches. Most of them are used to clean areas closer to the shore such as rivers, deltas, or seaports. Examples of such systems are Buddy catamaran (UK), Trash skimmers (US), Trash robot (US), etc. (Othman *et al*., 2020).

Considering the fact that The Ocean Cleanup project is among the most discussed at present, being also the only one that aims to operate on such a large scale, it is worth to be presented more in-depth, focusing on the advantages, disadvantages, opportunities, and threats that are associated with it. The Ocean Cleanup proposes the first large-scale marine plastic removal project *in situ*

(Morrison *et al*., 2019). The concept is based on passive cleaning, ocean currents favouring the accumulation of plastic around certain areas of the platform, subsequently being collected. However, the system does not work in line with initial expectations at the moment, as much of the waste is not retained, and does not reach the collection points. There are also concerns about how these platforms affect marine life due to the size of the equipment, and the lack of accurate data regarding the species that live in the North Pacific Ocean. Although the organization claims the fact that the wildlife is not affected, a survey conducted by experts in marine biology reports a major concern about the possibility of marine animals being affected or even dying as a result of their interaction with the platform equipment. One of the main concerns was regarding neustons, that thrive on the surface of the water (Helm, 2019). Thus, compared to other plastic removal alternatives, The Ocean Cleanup project possesses a potential danger to marine wildlife. Microplastics, as well as plastic debris that reached the ocean floor, cannot be caught by The Ocean Cleanup system. Besides that, the system cannot be considered as an approach that aims to reduce the enormous production of plastic, being only a post-consumer intervention, compared to various campaigns and projects that aim to prevent the use of plastic (Morrison *et al*., 2019). Even though there are drawbacks, the CEO and founder of the Ocean Cleanup project, Slat and his team are constantly improving the system based on the results that they get, following the multiple tests employed in the past years, concluding that an accurate ocean clean up is a complex process which involves years of work and assessments (Slat, 2018). However, the citizens participate in clean-ups and surprinsingly they can create immediate results and permanent changes in their local areas. These should serve as catalysts for major changes in people's behaviour and also encourage adoption of practices that can have a great effect on the remediation of the problem (Kiernan, 2009). Education is very important to reduce plastic waste and excesive pollution. This can change people's attitude and knowledge toward plastic waste and its management. There are also information campaigns that support and promote this type of education, such as The Ocean Cleanup project (Chow *et al*., 2017).

Beach cleanup represents a key approach used to reduce the marine and coastal environment pollution, such as plastic pollution. For example, Ocean Conservancy, organizes international coastal cleanups, as well as waterway and ocean cleanups every year involving many volunteers. However, it is impossible to identify how to organize them best and where and when to carry them out because the effects of these beach cleanups are not quantitatively well understood (Kataoka, 2015). It must be noted that the process of cleaning beaches requires good management methods, funds and adequate human resources (Krelling *et al*.,

2017). This process may be done using specialized machinery such as sand cleaning machines that rake or sift the sand, or other chemicals such as oil dispersants (Frampton, 2010). Beach cleaning may be done by civic organizations, professional companies, the military or volunteers such as the Marine Conservation Society. There are two types of beach cleaning: mechanical and manual. Mechanical cleaning is defined as the removal of organic material or litter, which relies on the work of automatic or push machineries that rake or sieve the superficial layer of sand (Zielinski *et al*., 2019). Manual cleaning involves individuals picking up litter exclusively by hand. The combined use of manual and mechanical cleaning represents an approach which is effective and environmentally sound (Stelling-Wood *et al*., 2016).

Biological methods (bioremediation)

Bioremediation is a greener solution in comparison to the traditional physical and chemical approaches, being a cost-effective method, that is less destructive for the environment (Fig.2) (Adzigbli *et al*., 2018; Baniasadi and Mousavi, 2018; Junusmin *et al*., 2019). The microbial degradation of organic compounds is a biochemical process that implies the uptake of polymers. These polymers are referred to as degradation products that are processed by living microorganisms (MO). MO such as fungi and bacteria are involved in the degradation of the regular, synthetic plastic, as well as of the bioplastic. MO can degrade plastic differently depending on the area that the material is found. Plastic is split in nature aerobically, while the one present in landfills and sediments undergoes an anaerobic degradation, plastic in soil and compost being degraded partly aerobically (Ishigaki *et al*., 2004). Aerobic degradation, also known as aerobic respiration, is one of the most important factors in the decrease of contaminants in hazardous waste sites. The oxygen is used by aerobic MO as an acceptor electron. The MO break down organic substances into components with smaller molecular mass (Priyanka, 2011). Anaerobic biodegradation is the decomposition of organic compounds in the absence of oxygen. Some anaerobic bacteria use sulphate, manganese, nitrate, iron and CO_2 as their electron acceptors, to break down organic chemicals into smaller compounds. MO are not able to carry polymers directly through their external membrane to the cell organelles where most of the cell's biochemical processes take place, as long as the molecular mass of the polymer is large, and has no solubility in contact with water. In order for them to use carbon as an energy source, MO have developed a strategy in which they remove extracellular enzymes that cause the depolarization and decomposition of polymers, to be transported inside the cell (Gu, 2003). Also, the biodegradation of polymers to occur, MO must attach to the surface of polymers, eventually developing and growing by degrading the polymer, and

using it as a source of carbon. MO can attach to the surface of polymers as long as the membrane is hydrophilic. During primary degradation, enzymes secreted by the organisms cause the main chain to split, leading to the formation of fragments with a low molecular mass, such as oligomers, dimers or monomers. These compounds with a low molecular mass are still used by MO as a source of carbon (Premraj and Doble, 2005).

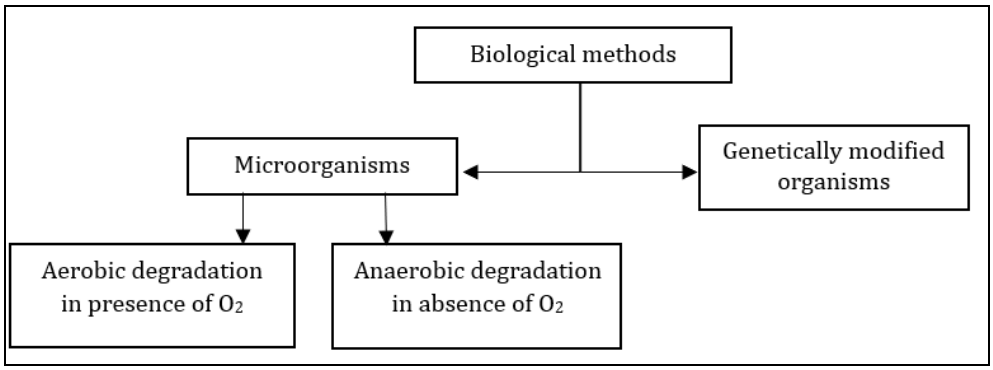


Figure 2. Degradation of ocean plastic using biological methods.

Using MO in the process of natural degradation of plastic has the advantage that these are able to resist under various conditions, such as withstanding extremely high or extremely low temperatures, and thriving in oceans at different depths. So far, a number of studies have shown the potential of plastic degrading bacteria, such as some that are capable of carrying the process below 4°C. Still, the main problem is often the identification of isolated MO that can carry such processes to an end result (Cameron *et al*., 2012). However, plastic degradation by MO is a slow process, because of their natural adaptation, which takes a long time, and therefore, the disposal of plastic into the oceans becomes irreversible (Debroas *et al*., 2017).

Genetically modified organisms (GMOs) are able to remove different types of pollutants such as plastics from the environment and to reduce the toxicity of those elements as well (Saxena *et al*., 2020). Polyethylene terephthalate (PET) is a synthetic polymer that has an unprecedented resistance to degradation, lasting centuries in the ecosystem. Recent studies have led to the discovery of a new bacterium called *Ideonella sakaiensis* 201-F6, that has the ability to grow using PET as an energy and carbon source, by producing an enzyme called PETase. The resulted products from degadation reactions are mono(2-hydroxyethyl) terephthalic acid (MHET) as a primary product and a smaller amount of terephthalic acid (TPA) and bis(2-hydroxyethyl)-TPA. MHET is then converted

into two monomers, ethylene glycol (EG) and TPA, by a second enzyme called MGETase. (Austin *et al*., 2018). Despite the low solubility and stability of the enzyme, scientists succeeded in producing an active extracellular *Is*PETase. The enzyme will be used to generate a new *E. coli* strain, capable of accumulation and degradation of PET in its culture medium (Seo *et al*., 2019). The discovery of an enzyme that can break down such a resistant substrate as PET and the fact that the process takes place in only a few days, which is significantly less than the time that is naturally required for the biodegradation, raises the hopes for future success in using GMOs for plastic biodegradation (Carrington, 2018). Besides bacteria, scientists study other organisms, such as mealworms – *Tenebrio molitor* or moths – *Galleria mellonella* to which they can improve possible plastic degradation abilities, through different genetic engineering techniques (Yang *et al.*, 2015).

GMOs are created through various genetic engineering techniques with the main purpose of significantly improving the degradation rate of plastics and other pollutants. Many of these techniques are currently known, and used either to improve the expression of the enzymes found in the natural MO that are responsible for biodegradation in the environment, or to create MO that have a specific and efficient degradation rate for only one type of pollutant, by inserting a gene of interest that accelerates their performance. The discovery of the genes and metabolic pathways involved in biodegradation offers the possibility of creating GMOs that have a faster and a more efficient degradation activity, do not produce secondary pollution, eliminate the need to transport the plastic waste and the damaged environment can easily recover once the pollutants are removed (Liu *et al*., 2019). The biggest issue associated with the use of GMOs for the biodegradation of pollutants is the horizontal gene transfer to other species present in the ecosystem. This is the main reason why these cannot be currently released in the natural environment (Saxena *et al*., 2020). At the moment, there are no clear laws regarding the use of GMOs in bioremediation schemes, and the acceptance of their use is very low, especially in Europe. Therefore, detailed studies are required to assess the risks, which is a time-consuming process and which may lead to results not as satisfying as expected. Many studies and research are required in order to prove the lack of long-term risk to the environment, so that GMOs could be eventually used outside the laboratory as well (Janssen and Stucki, 2020).

Legal regulation to prevent the plastic pollution in the ocean

For several years, the marine environment has been considered by some states as the ideal landfill for a different range of waste products. In this context, an international regulation regarding ocean dumping had to be put in place. A large number of instruments at regional, national and international levels have

been adopted to tackle marine pollution problems. These instruments comprise regulations, conventions, action plans, agreements, strategies, guidelines and programs. They contain some management measures that may be either voluntary or compulsory. Firstly, a convention was signed in London on 13 November 1972 (Farnelli and Tanzi, 2017), the purpose of which was to prevent marine pollution by discharging waste or other materials that could endanger human health and cause major damage to living marine organisms that are considered to be valuable resources, or could harm in any way the legitimate use of the high seas. Based on this convention, the parties shall take all possible measures to prevent marine pollution (Birchenough and The Hague, 2020).

Third United Nations Conference

The third United Nations Conference on the *Law of the Sea* was adopted in 1982 and is considered to be a true *Constitution of the Oceans*. It is also one of the most important treaties in the field, because it codifies the international law of the sea, so that, with regard to aspects of marine pollution, Article 194 provides that states must take into account measures to prevent, reduce and control pollution of the marine environment from any cause, regardless of its origin. Moreover, Article 197 obliges states to cooperate at regional or global level, directly or through any competent international organizations, to develop international standards and practices that comply with the provisions of the convention, in order to protect and conserve the resources of the seas and oceans (Nordquist, 2011).

International Instruments

United Nations Convention on the Law of the Sea (UNCLOS)

The UNCLOS is one amongst the foremost important agreements associated with the employment of the oceans. It introduces a wide regime for the law of the ocean by managing aspects of the marine environment such as environmental control, research projects, geopolitical delimitations, technology, economic activities and also the regulation of debates referring to ocean matters (Roberts, 2006).

Council Directive 2007/71/EC

Regulations are imposed regarding waste from ships and boats, so as to stop it from being dumped over the edge. Therefore, the directive requires ship captains to dispose the waste in reception centers in European ports before leaving it. Offshore of the ships that have not unloaded their waste is also prohibited (Carpenter, 2017).

UNEP Regional Sea Programme

The UNEP Regional Sea Programme and the Global Programme of Action (GPA) started working together in 2003 on the development of a general Initiative on Marine Litter (UNEP, 2011). The main activities include: reviewing the status of marine pollution within the region, organizing meetings of experts on marine pollution and national authorities, preparing regional action plans for proper management of marine litter, and also participating in a clean-up day within the International Coastal Cleanup Campaign (Jeftic *et al.*,, 2009).

National instruments

US Marine Debris Program

The Marine Debris Program (MDP) is an important national program to analyze and solve the issues that stem from marine debris, so as to guard and conserve the nation's marine environment, natural resources, economy, industries and also the people. It offers a holistic approach to marine pollution, and was established by the Marine Debris Research, Prevention and Reduction Act of 2006 (MDRPRA) (Lippiatt *et al*., 2013). The MDP has sponsored a large number of programs, including Fishing for Energy, monitoring and assessment projects, international coastal clean-ups, and also collaborated with UNEP to provide technical assistance to some countries in the Caribbean region (Barry, 2010).

Regional instruments

EU Initiatives on Land-Based Waste Management

The EU incorporates a wide selection of initiatives on land-based waste management, which can have a major impact on the quantity of waste in the marine environment. As an example, the Packaging Waste Directive outlines a variety of requirements to scale back the impact of packaging waste in the environment. It contains provisions on the prevention of packaging waste, on the re-use of packaging, and on the recovery and recycling of packaging waste (Interwies *et al*., 2013).

EU Marine Strategy Framework Directive

A large number of initiatives exist to approach marine debris in the EU. Among them, perhaps the foremost relevant may be the Marine Strategy Framework Directive (MSFD), the environmental pillar of the EU Integrated Maritime Policy. This directive is actually an integral policy instrument for the protection of the ocean environment for the European Community, following an adaptive ecosystem-based, and integrated approach to the management of human activities (Galgani *et al*., 2013).

Other significant legislations to marine pollution could have an important impact on the volume of waste in the oceans. For instance, The Beaches Environmental Assessment and Coastal Health Act aims to scale back the chance of diseases to users of the coastal recreation waters (Assessment, 2000).

The summary description of regional, national and international instruments approaching marine pollution can be a representative image of some of the most relevant methods. Because of the absence of a universal nature act, the level of international regulation development concerning the marine pollution mostly from land-based sources depends especially on the level of regional legislation development, also as on national legal institutions associated with the regulation of seas and oceans area, river basin pollution, the use and therefore the disposal of waste. Currently, most of the coastal states have already adopted the most relevant legislation in order to control marine pollution from land.

Conclusions

As scientists, we must have a vital role in transparently describing the scale of environmental hazards and what should be done in order to prevent them. The global implications of plastic pollution, coupled with the effects of other pollutants, are distressing. The current influx of wastes to the coastal regions of the seas and oceans is damaging and has a detrimental effect on many marine species. Progressive and dynamic pollution of the aquatic ecosystems may lead to a tragic deterioration of a significant part of marine resources. Such a decline might not be reversed for a large number of generations and will have a profound and lasting impact on the future of humanity. International collaboration is required in order to clean up all types of debris on the ocean and to reduce the major source of ocean microplastics. Research is also required to develop different strategies for *in situ* biodegradation of macro- and microplastics. While today research offers reason for faith and hope, future research should determine whether microbial genes involved in plastics degradation have begun to expand in the marine environment. When pollution management operations are not sufficient to overcome impacts and reach reduction targets in a reasonable time, social mobilization is an important ally to engage and motivate the general public and stakeholders to implement pollution solutions through behavioural change, social learning, and community-based conservation actions. The main focus must be on converting the way we live more sustainable by adjusting our over-consumptive lifestyles, rather than a narrower focus on sustainable consumer choices. We must renovate the way we live instead of only tweaking the choices that we make.

Acknowledgements. This paper was developed during the course Biotechnologies for recycling of waste products, at the Faculty of Biology and Geology, Babeș-Bolyai University, under the guidance of PhD. student Dumitrana Iordache.

References

Adzigbli, L., & Yuewen, D. (2018). Assessing the impact of oil spills on marine organisms. *J. Oceanogr. Mar. Res.*, *6*(179), 2.

Alava, J. J. (2019). Ocean pollution and warming oceans: toward ocean solutions and natural marine bioremediation. In *Predicting Future Oceans* (pp. 495-518). Elsevier.

Assessment, B. E. (2000). Coastal Health Act, 33 USC 1251. Public Law, 106-284.

Austin, H. P., Allen, M. D., Donohoe, B. S., Rorrer, N. A., Kearns, F. L., Silveira, R. L., Pollard, C. B., Dominick, G., Duman, R., Omari, K. E., Wagner, A., Michener, W. E., Amore, A., Skaf, M. S., Crowley, M. F., Thorne A. W., Johnson C. W., Woodcock H. L., McGeehan J. E., Beckham G. T., & Mykhaylyk, V. (2018). Characterization and engineering of a plastic-degrading aromatic polyesterase. *Proc. Natl. Acad. Sci. U.S.A.*, *115*(19), E4350-E4357.

Auta, H. S., Emenike, C. U., & Fauziah, S. H. (2017). Distribution and importance of microplastics in the marine environment: a review of the sources, fate, effects, and potential solutions. *Environ Int*, *102*, 165-176.

Baniasadi, M., & Mousavi, S. M. (2018). A comprehensive review on the bioremediation of oil spills. In Microbial Action on Hydrocarbons (pp. 223-254). Springer, Singapore.

Barboza, L. G. A., Cózar, A., Gimenez, B. C., Barros, T. L., Kershaw, P. J., & Guilhermino, L. (2019). Macroplastics pollution in the marine environment. In World seas: *An environmental evaluation* (pp. 305-328). Academic Press.

Barry, T. (2010). Fishing for energy: A public-private partnership approach to preventing and reducing derelict fishing gear. *Marine debris prevention projects and activities in the Republic of Korea and United States: A compilation of project summary reports*, 41-50.

Birchenough, A., & Haag, F. (2020). The London Convention and London Protocol and Their Expanding Mandate. *Ocean Yearb.*, *34*(1), 255-278.

Brandon, J., Goldstein, M., & Ohman, M. D. (2016). Long-term aging and degradation of microplastic particles: comparing in situ oceanic and experimental weathering patterns. *Mar. Pollut. Bull.*, *110*(1), 299-308.

Cameron, K. A., Hodson, A. J., & Osborn, A. M. (2012). Structure and diversity of bacterial, eukaryotic and archaeal communities in glacial cryoconite holes from the Arctic and the Antarctic. *FEMS Microbiol. Ecol.*, *82*(2), 254-267.

Carpenter, A. (2017). Ship-Source Pollution as an Environmental Crime.

Chen, Q., Reisser, J., Cunsolo, S., Kwadijk, C., Kotterman, M., Proietti, M., Slat B., Ferrari, F. F., Schwarz, A., Levivier, A., Hollert, H., Koelmans, A. A., & Yin, D. (2018). Pollutants in plastics within the north Pacific subtropical gyre. *Environ. Sci. Technol.*, *52*(2), 446-456.

Chow, C. F., So, W. M. W., Cheung, T. Y., & Yeung, S. K. D. (2017). Plastic waste problem and education for plastic waste management. In Emerging practices in scholarship of learning and teaching in a digital era. Springer, Singapore.

Debroas, D., Mone, A., & Ter Halle, A. (2017). Plastics in the North Atlantic garbage patch: a boat-microbe for hitchhikers and plastic degraders. *Sci. Total Environ.*, *599*, 1222-1232.

Farnelli, G. M., & Tanzi, A. (2017). Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter 1972 and 1996 Protocol. In *Elgar Encyclopedia of Environmental Law* (pp. 175-183). Edward Elgar Publishing Limited.

Frampton, A. P. (2010). A review of amenity beach management. *J. Coast. Res.*, *26*(6), 1112-1122.

Galgani, F., Hanke, G., Werner, S. D. V. L., & De Vrees, L. (2013). Marine litter within the European marine strategy framework directive. *ICES J. Mar. Sci.*, *70*(6), 1055-1064.

Gu, J. D. (2003). Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. *Int. Biodeterior. Biodegradation*, *52*(2), 69-91.

Helm, R. R. The ocean cleanup project could destroy the Neuston. The Atlantic. Feb 2019.

Hohn, S., Acevedo-Trejos, E., Abrams, J. F., de Moura, J. F., Spranz, R., & Merico, A. (2020). The long-term legacy of plastic mass production. *Sci. Total Environ.*, *746*, 141115.

Iñiguez, M. E., Conesa, J. A., & Fullana, A. (2016). Marine debris occurrence and treatment: A review. *Renew. Sust. Energ. Rev.*, *64*, 394-402.

Interwies, E., Görlitz, S., Stöfen, A., Cools, J., Van Breusegem, W., Werner, S., & de Vrees, L. (2013). Issue paper to the international conference on prevention and management of marine litter in European seas.

Ishigaki, T., Sugano, W., Nakanishi, A., Tateda, M., Ike, M., & Fujita, M. (2004). The degradability of biodegradable plastics in aerobic and anaerobic waste landfill model reactors. *Chemosphere*, *54*(3), 225-233.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan R., & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771.

Janssen, D. B., & Stucki, G. (2020). Perspectives of genetically engineered microbes for groundwater bioremediation. *Environ. Sci. Process. Impacts*, *22*(3), 487-499.

Jeftic L., Seba S., & Ellik A. (2009). Marine Litter: A Global Challenge. [Accessed September 16, 2020]. www.unep.org/regionalseas.

Junusmin, K. I., Manurung, B. S., & Darmayati, Y. (2019, November). Bioremediation of oil-contaminated sediment by hydrocarbonoclastic bacterial consortium immobilized in different types of carrier. In *AIP Conference Proceedings* (Vol. 2175, No. 1, p. 020056). AIP Publishing LLC.

Kataoka T., & Hinata H., (2015). Evaluation of beach cleanup effects using linear system analysis. *Mar. Pollut. Bull.*, *91*(1), 73–81.

Kiernan I. (2009). Clean Up the World. Sydney, N.S.W. [Accessed September 19, 2020]. https://www.un.org/esa/earthsummit/cleanup.htm.

Krelling A. P., Williams A. T., & Turra A., (2017). Differences in perception and reaction of tourist groups to beach marine debris that can influence a loss of tourism revenue in coastal areas. *Mar Policy* , *85*, 87–99.

Lebreton, L., Egger, M., & Slat, B. (2019). A global mass budget for positively buoyant macroplastic debris in the ocean. *Sci. Rep.*, *9*(1), 1-10.

Lebreton, L., Slat, B., Ferrari, F., Sainte-Rose, B., Aitken, J., Marthouse, R., Hajbane, S., Cunsolo, S., Schwarz, A., Levivier, A., Debeljak, P., Maral, H., Schoeneich-Argent, R., Brambini, R., Reisser, J., & Noble, K. (2018). Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. *Sci. Rep.*, *8*(1), 1-15.

Lippiatt, S., Opfer, S., & Arthur, C. (2013). Marine debris monitoring and assessment: recommendations for monitoring debris trends in the marine environment.

Lippiatt, S., Opfer, S., and Arthur, C. (2013). Marine debris monitoring and assessment. NOAA Technical Memorandum NOS-OR&R-46.

Liu, L., Bilal, M., Duan, X., & Iqbal, H. M. (2019). Mitigation of environmental pollution by genetically engineered bacteria—Current challenges and future perspectives. *Sci. Total Environ.*, *667*, 444-454.

Lu, L., Luo, T., Zhao, Y., Cai, C., Fu, Z., & Jin, Y. (2019). Interaction between microplastics and microorganism as well as gut microbiota: A consideration on environmental animal and human health. *Sci. Total Environ.*, *667*, 94-100.

Lusher, A. L., Burke, A., O'Connor, I., & Officer, R. (2014). Microplastic pollution in the Northeast Atlantic Ocean: validated and opportunistic sampling. *Mar. Pollut. Bull*., *88*(1-2), 325-333.

Moore, C. J. (2008). Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. *Environ. Res*., *108*(2), 131-139.

Morét-Ferguson, S., Law, K. L., Proskurowski, G., Murphy, E. K., Peacock, E. E., & Reddy, C. M. (2010). The size, mass, and composition of plastic debris in the western North Atlantic Ocean. *Mar. Pollut. Bull*., *60*(10), 1873-1878.

Morrison, E., Shipman, A., Shrestha, S., Squier, E., & Whitney, K. S. (2019). Evaluating The Ocean Cleanup, a Marine Debris Removal Project in the North Pacific Gyre, Using SWOT Analysis. *Case Studies in the Environment*.

Nordquist, M. (Ed.). (2011). *United Nations Convention on the law of the sea 1982, Volume VII: a commentary*. Brill.

Othman, H., Petra, M. I., De Silva, L. C., & Caesarendra, W. (2020, January). Automated trash collector design. In *J. Phys. Conf. Ser.* (Vol. 1444, No. 1, p. 012040). IOP Publishing.

Philp, R. B., (2013). *Ecosystems and Human Health: Toxicology and Environmental Hazards*, Third Edition. CRC Press, 116.

Premraj, R., & Doble, M. (2005). Biodegradation of polymers. *Indian J. Biotechnol.*, *4*(2), 186-193.

Priyanka, N., & Archana, T. (2011). Biodegradability of polythene and plastic by the help of microorganism: a way for brighter future. *J. Environ. Anal. Toxicol.*, *1*(4), 1000111.

Reisser, J. W., Slat, B., Noble, K. D., Plessis, K. D., Epp, M., Proietti, M. C., Sonneville, J., Becker, T., & Pattiaratchi, C. (2015). The vertical distribution of buoyant plastics at sea: an observational study in the North Atlantic Gyre.

Rios, L. M., Jones, P. R., Moore, C., & Narayan, U. V. (2010). Quantitation of persistent organic pollutants adsorbed on plastic debris from the Northern Pacific Gyre's "eastern garbage patch". *J. Environ. Monit*., *12*(12), 2226-2236.

Roberts, J. (2006). *Marine environment protection and biodiversity conservation: the application and future development of the IMO's particularly sensitive sea area concept*. Springer Science & Business Media.

Rojas, J. (2018, December). Plastic Waste is Exponentially Filling our Oceans, but where are the Robots?. In *2018 IEEE Region 10 Humanitarian Technology Conference (R10-HTC)* (pp. 1-6).

Ryan, P. G. (2014). Litter survey detects the South Atlantic 'garbage patch'. *Mar. Pollut. Bull.*, *79*(1-2), 220-224.

Ryan, P. G., Moore, C. J., van Franeker, J. A., & Moloney, C. L. (2009). Monitoring the abundance of plastic debris in the marine environment. *Philos. Trans. R. Soc. Lond., B, Biol. Sci*., *364*(1526), 1999-2012.

Saxena, G., Kishor, R., Saratale, G. D., & Bharagava, R. N. (2020). Genetically modified organisms (GMOs) and their potential in environmental management: constraints, prospects, and challenges. In *Bioremediation of industrial waste for environmental safety* (pp. 1-19). Springer, Singapore.

Seo, H., Kim, S., Son, H. F., Sagong, H. Y., Joo, S., & Kim, K. J. (2019). Production of extracellular PETase from *Ideonella sakaiensis* using sec-dependent signal peptides in E*. coli*. *Biochem. Biophys. Res. Commun.*, *508*(1), 250-255.

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: a short review. *Environ. Sci. Pollut. Res*., *24*(27), 21530-21547.

Slat, B. (2014). *How the oceans can clean themselves: A feasibility study*. Ocean Cleanup.

Slat, B. (2018) *A Peculiar Survey | Updates*. [Accessed 17 September 2020]. https://theoceancleanup.com/updates/a-peculiar-survey.

Solomon, O. O., & Palanisami, T. (2016). Microplastics in the marine environment: current status, assessment methodologies, impacts and solutions. *Journal of Pollution Effects & Control*, 1-13.

Stelling-Wood, T. P., Clark, G. F., & Poore, A. G. (2016). Responses of ghost crabs to habitat modification of urban sandy beaches. *Mar. Environ. Res.*, *116*, 32-40.

*The Guardian (*2018). *Scientists accidentally create mutant enzyme that eats plastic bottles.* [Accessed at 18 September 2020]. https://www.theguardian.com/environment/2018/apr/16/scientists-accidentally-create-mutant-enzyme-that-eats-plastic-bottles.

United Nations Environment Programme. Division of Early Warning, & Assessment. (2011). *UNEP Year Book 2011: Emerging issues in our global environment*. UNEP/Earthprint.

van Giezen, A., & Wiegmans, B. (2020). Spoilt-Ocean Cleanup: Alternative logistics chains to accommodate plastic waste recycling: An economic evaluation. *Transportation Research Interdisciplinary Perspectives*, *5*, 100115.

Van Sebille, E., England, M. H., & Froyland, G. (2012). Origin, dynamics and evolution of ocean garbage patches from observed surface drifters. *Environ. Res. Lett*., *7*(4), 044040.

Wilcox, C., Hardesty, B. D., & Law, K. L. (2019). Abundance of floating plastic particles is increasing in the Western North Atlantic Ocean. *Environ. Sci. Technol*., *54*(2), 790-796.

Yang, Y., Yang, J., Wu, W. M., Zhao, J., Song, Y., Gao, L., Yang, R., & Jiang, L. (2015). Biodegradation and mineralization of polystyrene by plastic-eating mealworms: Part 1. Chemical and physical characterization and isotopic tests. *Environ. Sci. Technol.*, *49*(20), 12080-12086.

Zielinski, S., Botero, C. M., & Yanes, A. (2019). To clean or not to clean? A critical review of beach cleaning methods and impacts. *Mar. Pollut. Bull.*, *139*, 390-401.

STUDIA UNIVERSITATIS BABEŞ-BOLYAI BIOLOGIA, 65, 2, 2020 (p. 87-94)
doi:10.24193/subbbiol.2020.2.05

=== SHORT COMMUNICATION ===

Contributions to the knowledge of amphipod fauna (Crustacea, Amphipoda) from the Danube Delta, Romania

Ana-Maria Purcari[1], Mirela Cîmpean[1,2]✉ and Karina Paula Battes[1,2]

[1]Babeş-Bolyai University, Faculty of Biology and Geology, Department of Taxonomy and Ecology, Cluj-Napoca, Romania, [2]Center for Systems Biology, Biodiversity, and Bioresources, Babeş-Bolyai University, Cluj-Napoca, Romania.
✉***Corresponding author, E-mail: mirela.cimpean@ubbcluj.ro.***

Article history: Received 8 April 2020; Revised 7 December 2020; Accepted 10 December 2020; Available online 20 December 2020.

Abstract. The amphipod species *Pontogammarus maeoticus* (Sovinskij, 1894) was identified in two locations from the Danube Delta, Romania (Sfântu Gheorghe and Sulina beaches) in July 2019. This is an eurybiont species, able to withstand high salinity variations characteristic to mixing fresh and sea waters. The individuals presented a special character in their morphology, a depression on the basis of pereiopod V. The present paper contributes to the knowledge of existing amphipod fauna from the Danube Delta, in the Black Sea coast area.

Keywords: monospecific community, *Pontogammarus maeoticus*, varying intraspecific character.

Introduction

Amphipods (Order Amphipoda, Supraorder Peracarida, Class Malacostraca, Väinölä *et al*., 2008) are a group of crustaceans with key roles in water quality assessment, ecological or ecotoxicological studies (Glazier, 2014). They are considered bioindicators due to their large distribution, their ecological role in the food chain, their susceptibility to pollutants, and due to their effortless reproduction in new environments (Neuparth *et al*., 2002; Alonso *et al*., 2009;

Grabowski *et al*., 2014). From an ecological perspective, amphipods have an important role in aquatic ecosystems as they contribute to nutrient recycling, they participate in the process of water purification and they represent a high-quality source of food for a variety of animals (Muskó, 1990; Väinölä *et al*., 2008; Glazier, 2014).

The Danube Delta is located in the northern part of the Romanian Black Sea coast, being the largest wetland in Europe (Gâștescu and Știucă, 2008), and the newest land formation in Romania. Previous data on the Danube Delta amphipod fauna were included in more extensive research dealing with benthic invertebrate communities (Graf *et al.,* 2008, 2014; Stoica *et al*., 2014; Pavel *et al*., 2017) or multiple biological communities (Cristescu *et al*., 2003; Tudorancea, 2006; Stoica *et al*., 2013).

The present paper reports data on amphipods from two locations in the Danube Delta, near the Black Sea coast: a monospecific community was found in all sampling points, probably due to a highly variable salinity in the seashore habitat near the Danube mouth, suitable only for an eurybiont species.

Materials and methods

Amphipods were collected from two locations in the Danube Delta, Sulina and Sfântu Gheorghe, on the 6th and 9th of July 2019. Sulina beach begins at the homonymous channel discharge point and expands South (INCDPM, 2014). while Sfântu Gheorghe beach is located North from the mouth of the homonymous Danube channel (Savu and Comănescu, 2008).

Seven sampling points (A to G) were considered on Sulina beach (45.0859N, 29.4132E) and 7 points (1 to 7) on Sfântu Gheorghe beach (44.5401N, 29.3724E) (Fig. 1). On both locations, six points were situated on the Black Sea coast, on a 2 km long stretch, and one was situated on the Danube River shore (point G in Sulina and point 7 in Sfântu Gheorghe) (Fig. 1). The qualitative samples were collected using a 300 μm mesh size net and they was preserved in the field in 96 % ethanol. Physico-chemical parameters (Total Dissolved Solids TDS, conductivity and pH) were measured from a total of 6 water samples (3 from Sulina and 3 from Sfântu Gheorghe), using a Hanna HI98194 multiparameter. Taxonomical identifications were performed in the laboratory, using a Nikon SZM 645 dissecting microscope and a Nikon YS100 microscope, and appropriate keys (Cărăuşu *et al*., 1955).

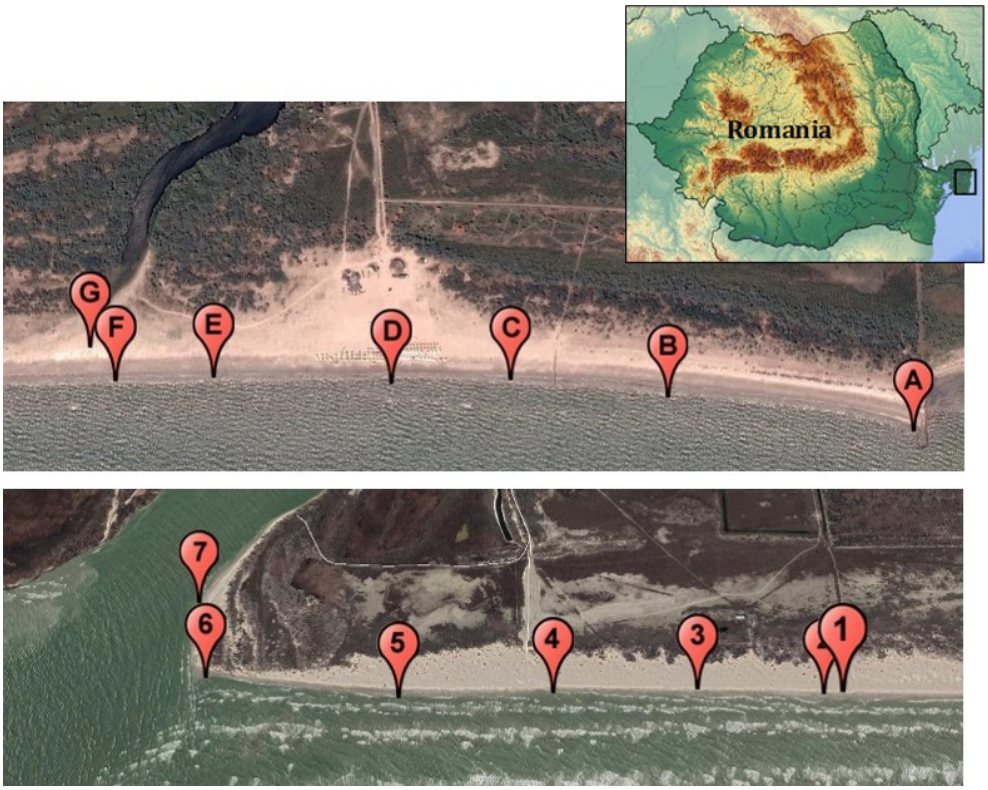


Figure 1. The two sampling locations: Sulina beach (top) – points A to G; and Sfântu Gheorghe beach (bottom) – points 1 to 7 (Image source: Google Earth Pro - 7.3.2.5491).

Results and discussions

Salinity (expressed as TDS) recorded the highest variations between the sampling points (Fig. 2), due to the mixture of sea and freshwater. Lower salinity values at Sfântu Gheorghe sampling points were caused by higher discharge of the Danube compared to Sulina channel (Tockner *et al*., 2009). pH had circumneutral values in both locations, with slight alkaline ones in Sfântu Gheorghe.

A total number of 5442 individuals (2561 from Sulina beach and 2881 from Sfântu Gheorghe beach) were identified (Table 1), adults and juveniles, all belonging to one species, *Pontogammarus maeoticus* (Sovinskij, 1894). This species is part of Family Pontogammaridae, Suborder Senticaudata (Lowry and Myers, 2013, 2017), and represents a Ponto-Caspian endemism (Cristescu *et al*., 2003; Nahavandi *et al*., 2013). *P. maeoticus* distribution comprises the Black,

Caspian, and Azov Seas as well as the Danube, Dniester, Don and Bug Rivers (Barnard and Barnard, 1983; Stock *et al*., 1998). In Romania, *P. maeoticus* is found alongside sandy beaches (Vasile Roaită, Agigea, Mamaia, Midia, Portiţa), around Danube's estuaries (Sacalin Island seashore, Sărături belt), in the Danube Delta, and in Lake Siutghiol (Cărăuşu *et al*., 1955). *P. maeoticus* is an eurybiont species that lives in the sand of fresh, brackish, and marine waters, buried from 0 to 10 cm deep (Cărăuşu *et al*., 1955). *P. maeoticus* individuals were observed to emit bioluminescence in Sfântu Gheorghe and Sulina, both in dead and alive individuals (Copilaş-Ciocianu and Pop, 2020).

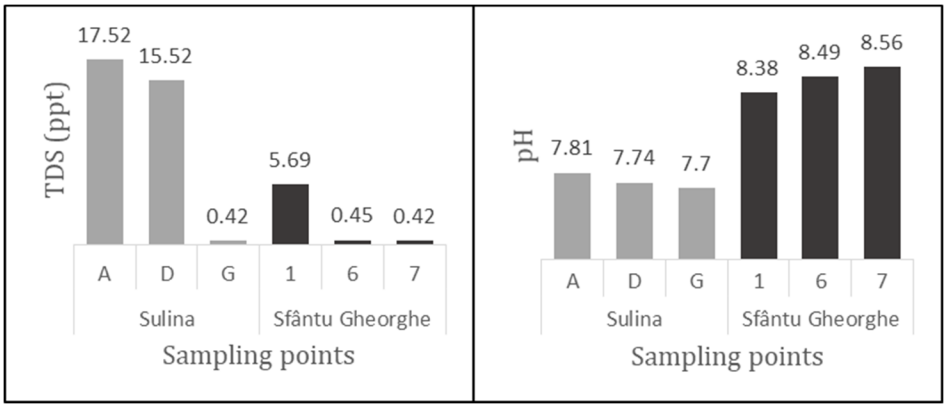


Figure 2 Total Dissolved Solids (TDS) and pH values of water samples from Sulina and Sfântu Gheorghe sampling points

Table 1 Number of collected *Pontogammarus maeoticus* individuals (No. ind.) from each sampling location

Location	Sampling point	No. ind.	Observations
Sulina	A	259	numerous juveniles
	B	638	
	C	599	3 individuals with a depression on the basis of pereiopod V
	D	517	1 individual with a depression on the basis of pereiopod V
	E	251	3 individuals with a depression on the basis of pereiopod V
	F	297	3 individuals with a depression on the basis of pereiopod V
	G	0	
Sfântu Gheorghe	1	810	
	2	161	
	3	898	
	4	192	
	5	202	
	6	595	
	7	23	

The key features of *P. maeoticus* are depicted in Figure 3. The body is robust and has a tall curve (Cărăuşu *et al*., 1955; Kokataş, 2003). Antenna I is slightly shorter than the second one and has a robust peduncle, especially its first segment. The peduncle of antenna II has a comb-like appearance (Fig. 3a). The mandibular palp is characteristic, with long, fine setae on the ventral margins of second and third segments (Fig. 3b). Gnathopods have all segments covered in numerous setae, the propodus has multiple long, curved setae on the ventral side (Fig. 3c). Uropod III has a long endopodite, almost reaching half of the exopodite, with setae on the interior margin. Its exopodite is surrounded by long setae, with two series of external spines (Fig. 3d). The telson is composed of two lobes that have 3-4 apical spines and a pair of fine setae on their surface (Fig. 3e). Pereiopod V has an oval basis; its posterodistal corner is well developed, with short setae on the posterior margin (Fig. 3g) (Cărăuşu *et al*., 1955; Kokataş, 2003). A total number of 10 individuals presented a depression on the posterior margin of the basis of pereiopod V (Fig. 3f). This character appeared on one or both pereiopods. The other specimens did not present such depression, having a relatively plain margin. This feature could be interpreted as a varying intraspecific character.

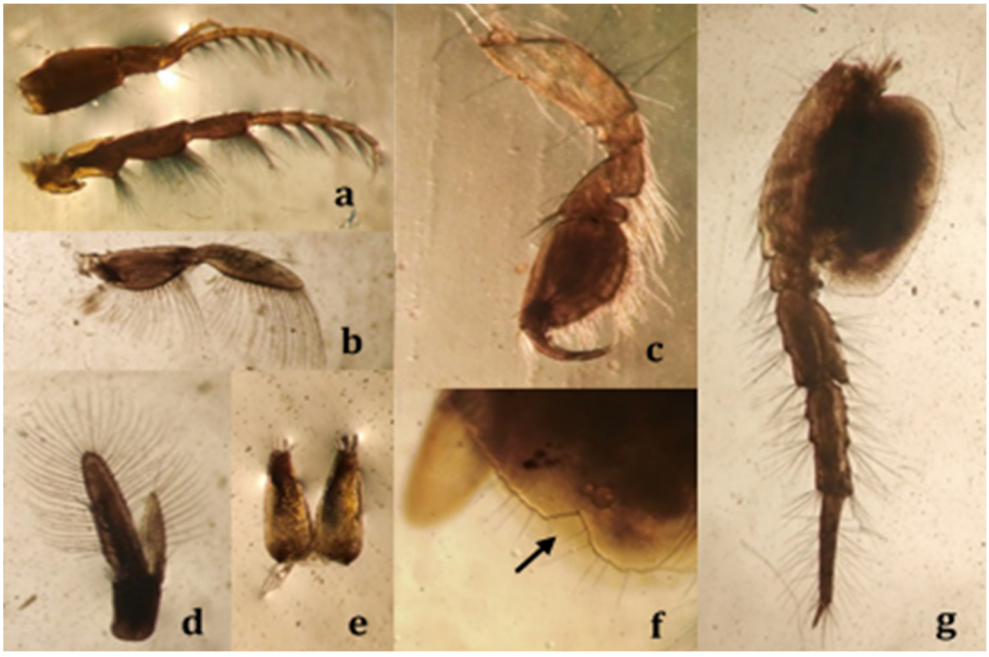


Figure 3. Key identification characters of *Pontogammarus maeoticus* (male): **a.** antennae (I, II); **b.** mandibular palp; **c**. gnathopod II; **d**. uropod III; **e.** telson; **f.** depression on the basis of pereiopod V; **g**. pereiopod V.

The presence of only one species in the samples collected from Sulina and Sfântu Gheorghe disagrees from previous data from the literature. Pavel *et al*. (2017) identified 10 amphipod species in the Sfântu Gheorghe freshwater meanders, with *Chelicorophium curvispinum* (G.O. Sars, 1895) as the most abundant. The Joint Danube Survey reports on macroinvertebrate also cited more than 10 amphipod species in the lower Danube River (Graf *et al*., 2008, 2014). At the confluence of the Danube with the Black Sea, near Sfântu Gheorghe locality, Stoica *et al*. (2013, 2014) found 3 amphipod species that dominated the macroinvertebrate communities in terms of abundance: *C. curvispinum*, *Dikerogammarus villosus* (Sowinsky, 1894) and *D. haemobaphes* (Eichwald, 1841). Cristescu *et al.* (2003) identified *P. maeoticus* in the Black Sea at Sulina and Sfântu Gheorghe, and *Pontogammarus robustoides* (Sars, 1894) in the Danube River at the same locations.

The monospecific amphipod community found in Sulina and Sfântu Gheorghe can only be explained by the variable salinity values caused by the mixing of fresh and seawaters, suitable only for eurybiont species as *P. maeoticus*. This species is known to withstand high salinity and temperature variations (Soldatova, 1986; Casties *et al.,* 2019), and is therefore considered to have the potential of a successful invasive species (Baltazar-Soares *et al.,* 2017). However, the absence of the species in the Danube River mouth should be investigated further.

Conclusions

The present paper found that the amphipod fauna of Sfântu Gheorghe and Sulina beaches consists of only one eurybiont species: *P. maeoticus*. Both adult and juvenile individuals were identified, few presenting a varying intraspecific character on the basis of pereiopod V.

References

Alonso, Á., De Lange, H. J., & Peeters, E. T. H. M. (2009). Development of a feeding behavioural bioassay using the freshwater amphipod *Gammarus pulex* and the Multispecies Freshwater Biomonitor. *Chemosphere, 75*, 341–346.

Baltazar-Soares, M., Paiva, F., Chen, Y., Zhan, A., & Briski, E. (2017). Diversity and distribution of genetic variation in gammarids: Comparing patterns between invasive and non-invasive species. *Ecology and evolution*, 7(19), 7687-7698.

Barnard, J. L., & Barnard, C. M. (1983). Freshwater Amphipoda of the world. 1-2. Hayfield Associates, Mt. Vernon, Virginia.

Casties, I., Clemmesen, C., & Briski, E. (2019). Environmental tolerance of three gammarid species with and without invasion record under current and future global warming scenarios. *Diversity and Distributions*, 25(4), 603-612.

Cărăuşu, S., Dobreanu, E., & Manolache, C. (1955). Fauna Republicii Populare Române, Crustacea, Amphipoda - Forme salmastre şi de apă dulce. 4. [in Romanian]. Ed. Academiei Republicii Populare Române.

Copilaş-Ciocianu, D., & Pop, F.M. (2020) An account of luminescence in the Ponto-Caspian amphipod *Pontogammarus maeoticus* (Sowinskyi, 1894), with an overview of amphipod bioluminescence, *North-Western Journal of Zoology* 2020: e207301

Cristescu, M. E., Hebert, P. D., & Onciu, T. M. (2003). Phylogeography of Ponto-Caspian crustaceans: A benthic–planktonic comparison. *Molecular Ecology*, 12(4), 985-996.

Gâştescu, P., & Ştiucă, R. (2008). Delta Dunării - Rezervaţie a Biosferei. [in Romanian]. Bucureşti: Ed. C.D. Press.

Glazier, D. S. (2014). Amphipoda. Reference Module in Earth Systems and Environmental Sciences, *Elsevier*, pp. 49. doi: 10.1016/B978-0-12-409548-9.09437-9.

Google Earth Pro Version 7.3.2.5491 (2020) Dobruja, Romania. 45°9'24.73"N, 29°39'32.14"E; Available at: http://www.earth.google.com. Accessed 25 September 2020

Grabowski, M., Bacela-Spychalska, K., & Pešić, V. (2014). Reproductive traits and conservation needs of the endemic gammarid *Laurogammarus scutarensis* (Schäferna, 1922) from the Skadar Lake system, Balkan Peninsula. *Limnologica*, *47*, 44-51.

Graf, W., Csányi, B., Leitner, P., Paunović, M., Janeček, B., Šporka, F., Chiriac, G., Stubauer, I., & Ofenböck, T. (2008). Joint Danube Survey 2, Report on macroinvertebrates, ICPDR International Commission for the Protection of the Danube River, www.icpdr.org, pp. 87.

Graf, W., Csányi, B., Leitner, P., Paunović, M., Huber, T., Szekeres, J., Nagy, C., & Borza, P. (2014) Joint Danube Survey 3, Full report report on macroinvertebrates, ICPDR International Commission for the Protection of the Danube River, www.icpdr.org, pp. 87.

INCDPM (Institutul Naţional de Cercetare-Dezvoltare pentru Protecţia Mediului) (2014). Studiu de evaluare adecvată pentru proiectul de realizarea lucrărilor de împrejmuire a obiectivului "Amenajare plajă Sulina". [in Romanian]. pp. 83-93.

Kokataş, A., Katagan, T., Özbek, M., & Sezgin, M. (2003). A New Amphipod for The Turkish Fauna: *Pontogammarus maeoticus* (Sowinsky, 1894*). Crustaceana, 76* (7), 879-884.

Lowry, J. K., & Myers, A. A. (2013). A phylogeny and classification of the Senticaudata subord. nov. (Crustacea: Amphipoda). *Zootaxa, 3610*(1), 1–80.

Lowry, J. K., & Myers, A. A. (2017). A Phylogeny and Classification of the Amphipoda with the establishment of the new order Ingolfiellida (Crustacea: Peracarida). *Zootaxa*, *4265*(1), 1-89.

Muskó, I. B. (1990). Qualitative and quantitative relationships of Amphipoda (Crustacea) living on macrophytes in Lake Balaton (Hungary). *Hydrobiologia, 191*, 269-274.

Nahavandi, N., Ketmaier, V., Plath, M., & Tiedemann, R. (2013). Diversification of Ponto-Caspian aquatic fauna: Morphology and molecules retrieve congruent evolutionary relationships in *Pontogammarus maeoticus* (Amphipoda: Pontogammaridae). *Molecular Phylogenetics and Evolution*, 69(3), 1063-1076.

Neuparth, T., Costa, F. O., & Costa, M. H. (2002). Effects of temperature and salinity on life history of the marine amphipod *Gammarus locusta* - Implications for ecotoxicological testing. *Ecotoxicology, 11*, 61-73.

Pavel, A. B., Duţu, L. & Patriche, N. (2017). The benthic fauna associations from the meanders area of Danube–Sfantu Gheorghe branch, in the period 2016-2017. Geo-Eco-Marina, 23, 233-244.

Savu, M. M., & Comănescu, L. (2008). Studiu geografic complex al comunei Sfântu Gheorghe, judeţul Tulcea. Lucrare de licenţă. [in Romanian]. Universitatea din Bucureşti, Facultatea de Geografie.

Soldatova, I. N. (1986). Eco-physiological properties of Pontogammarus maeoticus (Amphipoda) in a salinity gradient. Marine Biology, 92(1), 115-123.

Stock, J. H., Mirzajani, A. R., Vonk, R., Naderi, S., & Kiabi, B. H. (1998). Limnic and brackish water Amphipoda (Crustacea) from Iran. *Beaufortia, 48* (9), 173-234.

Stoica, C., Stănescu, E., Lucaciu, I., Gheorghe, S., & Nicolau, M. (2013). Influence of global change on biological assemblages in the Danube Delta.

Stoica, C., Gheorghe, S., Petre, J., Lucaciu, I., & Nita-Lazar, M. (2014). Tools for assessing Danube Delta systems with macro invertebrates. Environmental Engineering & Management Journal (EEMJ), 13(9). 2243-2252.

Tockner, K., Uehlinger, U. & Robinson, C. T. (2009). Rivers of Europe. Elsevier Science. pp. 728.

Tudorancea, C. (2006). Benthic fauna of the Danube Delta. In: *Danube Delta, Genesis and Biodiversity*, Tudorancea, C., Tudorancea, M.M. (eds.)., Backhuys Publishers, 211-236.

Väinölä, R., Witt, J. D. S., Grabowski, M., Bradbury, J. H., Jazdzewski, K., & Sket, B. (2008). Global diversity of amphipods (Amphipoda; Crustacea) in freshwater. *Hydrobiologia, 595*, 241–255.