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CONTENTS

Scientific communications

Notes

Chronicle and information

1871
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Marine Biological Journal
2022, vol. 7, no. 3, pp. 3–16
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EFFECT OF HYPOXIA ON IMMUNE SYSTEM OF BIVALVE MOLLUSCS

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Over the past decades, research on bivalve immune system is focused on studying the effect of environmental factors on the basal status of defense systems. The immune system of bivalves is greatly affected by abiotic factors, and the most significant ones are water temperature, salinity, and level of dissolved oxygen. Hypoxia is widespread in the coastal waters of the World Ocean since the 1950s. Hypoxic zones (with dissolved oxygen concentration < 0.5 mL $O_2 \cdot L^{-1}$) occur in shelf areas for a long time corresponding to the life cycle of many hydrobionts. Being benthic organisms, bivalve molluscs often experience reduced dissolved oxygen concentrations. This group of aquatic invertebrates both plays an important role in aquatic ecosystem functioning and is actively used in aquaculture. The efficiency of bivalve cultivation directly depends on its immune status determining resistance to diseases. The immune system of bivalve molluscs is based on a complex of nonspecific reactions of cellular and humoral components. Hemocytes circulating in the hemolymph are the key effectors of the cellular immune response which, along with the barrier tissues of molluscs, synthesize humoral factors with a wide spectrum of antimicrobial activity. The hemolymph of various bivalve species contains different cell types differing by size, morphology, and granulation of cytoplasm. Most bivalve species have 2 types of hemocytes – granular and agranular ones; those can be subdivided into morphotypes depending on number and color of granules, size of the nucleus, and presence of organelles in the cytoplasm. Granulocytes are considered the main immune cells that perform phagocytosis and (or) encapsulation of infectious agents, as well as their subsequent neutralization by releasing reactive oxygen species, lysing enzymes, and humoral antimicrobial proteins. Moreover, the complex of defense systems includes an antioxidant system which is closely related to mollusc immunity since it neutralizes reactive oxygen species releasing during cellular immune mechanism activation. An excess of these compounds damages mollusc cells by oxidizing proteins, cytoplasmic membrane lipids, and DNA. This article provides data on an oxygen deficiency effect on the cellular and humoral components of the immune system, as well as the tissue antioxidant complex of bivalve molluscs.

Keywords: bivalve molluscs, immunity, hemocytes, hypoxia, antimicrobial proteins, antioxidant enzymes

Deep hypoxia is typical for aquatic ecosystems where oxygen minimum zones are formed (Diaz & Breitburg, 2009). The reasons for hypoxia formation in many aquatic ecosystems are natural, but an increased anthropogenic load on coastal areas of water bodies has led to a significant spread of hypoxia and anoxia in the World Ocean (Gallo & Levin, 2016). At the same time, a sufficient amount of dissolved oxygen is a factor determining survival of organisms in the aquatic environment. Hypoxia

causes significant alterations in the structure of communities resulting in a change in their species composition and transformation in the abundance and biomass of populations (Diaz & Breitburg, 2009). Lack of oxygen greatly affects physiology of benthic macroorganisms (Wang Y. et al., 2012).

Bivalve molluscs are mass representatives of coastal marine water bodies. Many species are cultivated and have a high commercial value (Wijsman et al., 2019). Moreover, molluscs play a key role in marine ecosystem functioning (Hartmann et al., 2016). High biological and economic significance of these organisms determines the interest of researchers in studying the peculiarities of the functioning of their immune system and estimating the effect of negative environmental factors on an ability of the immune system to resist infectious agents of various nature (Anderson, 2001).

The immune defense of bivalves is primarily based on biological barriers (shell and mantle), as well as on nonspecific reactions of innate immunity (Donaghy et al., 2012). Bivalve immunity includes cellular and humoral components (Anderson, 2001). Molluscs have an open circulation system. Hemocytes are the main agents providing the cellular immune response (Donaghy et al., 2012). The mechanisms of the cellular component of the mollusc immune system include phagocytosis and (or) encapsulation of pathogenic microorganisms followed by their destruction by enzymatic cleavage or release of reactive oxygen species (hereinafter ROS) (Pauletto et al., 2014). The humoral response is expressed in affecting pathogens with a complex of molecules, *inter alia* antimicrobial proteins (Rodrigues et al., 2010), C-type lectins (Wang S. et al., 2010), peptidoglycan recognition proteins (Ikuta et al., 2019), and several other compounds (Wootton et al., 2003).

Some authors consider the antioxidant (hereinafter AO) tissue complex as another mechanism of immune defense in molluscs since ROS are produced by hemocytes during the response to pathogens and adverse environmental conditions (Donaghy et al., 2012). In this case, the reduction of oxygen to a superoxide anion results in the occurrence of many highly ROS, *inter alia* hydroperoxides, singlet oxygen, or hydroxyl radicals (Lambert & Brand, 2004). Their excess can damage cellular structures (Valko et al., 2006). However, the balance in ROS production and neutralization is achieved by maintaining a high activity of tissue AO enzymes (catalase, superoxide dismutase, *etc.*) which split hydroperoxides into less active gaseous oxygen and water (Monari et al., 2007). Thus, the internal mechanism of organism protection from the damaging effect of ROS is carried out during the immune system functioning.

Being predominantly bottom-dwelling organisms, molluscs often experience periodic or constant hypoxia; this led to emergence of a wide spectrum of adaptive mechanisms to survive in an environment with a minimum oxygen content (Sokolov et al., 2019). This article provides an overview of modern concepts on the reaction of the cellular and humoral immune response, as well as the response of the AO complex of bivalves to oxygen deficiency.

1. Cellular immune response

1.1. The ratio of hemocyte types and total number of hemocytes in the hemolymph. As known, the cellular composition of the hemolymph and the functional peculiarities of hemocytes are not the same in representatives of bivalve molluscs. Differences in hemocyte classification in different species (and sometimes even in the same one) depend on the method of analysis and the principle underlying the cell classification (Hine, 1999). Summarizing the existing classifications, two main cell types can be distinguished – agranular ones (blast-like cells, agranulocytes, and hyalinocytes) and granular ones (Andreyeva et al., 2019 ; Hine, 1999). Granular cells are more involved in the implementation of the immune

response (Wang W. et al., 2017); therefore, the ratio of cell types in the hemolymph is an important diagnostic indicator of the functional state of the mollusc body. A decrease in the total number of hemocytes or a change in their ratio in the hemolymph can alter the effectiveness of the immune response.

A decrease in the total number of hemocytes under oxygen deficiency in various mollusc species was shown. Specifically, incubation of mussels *Mytilus coruscus* Gould, 1861 for 3 days under hypoxia led to a decrease in the relative number of granular cells in the hemolymph (Sui et al., 2016). In the mussel *Perna perna* (Linnaeus, 1758), incubation in air for 48 hours resulted in a decrease in the number of circulating hemocytes by 73 % (Nogueira et al., 2017). A drop in the total number of hemocytes after incubation under hypoxia was noted in *Perna viridis* (Linnaeus, 1758) (Wang Y. et al., 2011) and *Chamelea gallina* (Linnaeus, 1758) (Matozzo et al., 2005). A decrease in the total number of hemocytes is associated with impaired proliferation, migration of hemocytes to other tissues, and increase in the rate of apoptosis and mortality (Mydlarz et al., 2006). As shown, incubation under oxygen deficiency leads to a rise in the mortality rate of *P. viridis* hemocytes (Wang Y. et al., 2011) and *M. coruscus* hemocytes (Sui et al., 2016). According to other studies, hypoxia can result in a decrease in the total number of hemocytes without an increase in their mortality rate (Nogueira et al., 2017). Interestingly, in *Ch. gallina* subjected to daily hypoxia, after 96 hours of incubation under normoxia, the number of hemocytes recovered to the control level (Matozzo et al., 2005). At the same time, exposure to oxygen deficiency for more than 24 hours led to irreversible changes in the number of hemocytes (Pampanin et al., 2002).

1.2. Phagocytosis. Hemocytes are capable of phagocytizing heterogeneous particles and pathogenic microorganisms penetrating into the mollusc body. The process of phagocytosis includes recognition, binding, and inactivation of the pathogen (Canesi et al., 2002). As a rule, hypoxia results in a suppression of phagocytic activity (Ellis et al., 2011); its decrease was recorded in *P. viridis* after a daily incubation under hypoxia (Wang Y. et al., 2011) and in *Mytilus galloprovincialis* Lamarck, 1819 after 12 and 24 hours of incubation in air (Mosca et al., 2013), as well as in *M. coruscus* (Sui et al., 2016), *Chlamys farreri* (K. H. Jones & Preston, 1904) (Chen J. et al., 2007), and *Ch. gallina* (Matozzo et al., 2005). The changes depended on the incubation duration: the longer hypoxia, the lower intensity of phagocytosis (Sui et al., 2016). A decrease in phagocytic activity during hypoxia is associated with a decrease in the number of hemocytes (Pampanin et al., 2002). Insufficient production of adenosine triphosphate under oxygen deficiency results in a decrease in the ability to migrate and phagocytize heterogeneous particles. At the same time, air incubation of *P. perna* contributed to an increase in phagocytic activity of its hemocytes (Nogueira et al., 2017).

1.3. Spontaneous production of reactive oxygen species. ROS are produced by hemocytes for antimicrobial protection (Anderson, 2001). Typically, granular cells in bivalves are more capable of generating an oxidative burst than agranular ones (Pauletto et al., 2014). As believed, the main source of ROS in hemocytes is mitochondria (Donaghy et al., 2013), and a decrease in ROS production may be caused by inhibition of enzymes involved in the generation of the oxidative burst (Andreyeva et al., 2019). Inhibition of ROS production was registered in *P. viridis* hemocytes (Wang Y. et al., 2011). A decrease in ROS production is associated with the mechanisms of metabolic adjustment with the hypoxia-inducible factor (HIF) involved (Michiels et al., 2002). On the other hand, in *M. galloprovincialis*, short-term hypoxia induces an increase in the ability to produce ROS in agranulocytes and a decrease in granulocytes (Andreyeva et al., 2019). Similar results were obtained by other authors (Chen J. et al., 2007 ; Sui et al., 2016). Probably, in some species, hypoxia induces reorganization of the mitochondrial respiratory chain, and this leads to a rise in ROS production (Chandel et al., 2000).

2. Humoral immunity and response to hypoxia

Bivalve molluscs are endowed with a complex of humoral immune factors that are activated in response to the invasion of pathogenic microorganisms and (or) the negative effect of the environment. The main classes of compounds involved in the humoral immune response of molluscs are antimicrobial proteins, cytokines, complement system factors, AO enzymes, and acute-phase proteins (Rodrigues et al., 2010). Effectors of humoral nonspecific immunity have a wide spectrum of activity against gram-positive and gram-negative bacteria, protozoans, yeasts, fungi, and viruses. One of the first identified and described antimicrobial proteins were those in *Mytilus edulis* Linnaeus, 1758 and *M. galloprovincialis* (Charlet et al., 1996 ; Mitta et al., 2000). In bivalves, defensins are the most common group of antimicrobial proteins; however, other classes of compounds with antimicrobial properties are described as well – histones (Dorrington et al., 2011), lysozymes, *etc.* (Wang Q. et al., 2013). As shown, the lysozyme family in molluscs is represented by a large number of proteins which are mostly expressed in mucosal tissues (mantle, gills, and hepatopancreas) (Wang Q. et al., 2013). Obviously, the spectrum of compounds involved in the humoral immune response of molluscs is much wider than currently known. The research principle is based on the search for analogies with the already described factors of nonspecific immunity in vertebrates; with such an approach, mollusc-specific compounds remain undescribed. The specific role of most of the identified factors of humoral immunity is still unexplored due to complexity in setting up experiments and choosing methods. Most authors, however, agree on the primary role of these compounds in the immune response of molluscs. This assumption is based on rapid changes in the expression levels of humoral factors in response to experimental immunization of hemocytes (Suárez-Ulloa et al., 2013).

Since there is no base of fundamental knowledge on the humoral immunity functioning in bivalves, it is extremely difficult to characterize the degree of negative effect of oxygen deficiency on this part of the immune system. Transcriptomic studies indicate the activation of a whole complex of genes involved in several immune signaling pathways that implement the response to bacterial invasion in molluscs kept under hypoxia (Zhang et al., 2019). As shown, hypoxia has a depressing effect on the humoral immunity of molluscs. The expression level of defensin in the gills of *Brachidontes pharaonis* (P. Fischer, 1870) decreased by 5–20 times after 6 days of exposure to low oxygen concentrations (Parisi et al., 2015). Other representatives of the Mollusca phylum showed significant changes in the expression of immune genes as well. Specifically, in the Pacific abalone, the expression of 6 genes associated with the immune response was inhibited under oxygen deficiency (Shen et al., 2019). On the other hand, incubation of the abalone (*Haliotis discus discus* Reeve, 1846) under hypoxia for 8 hours caused a significant increase in the transcription of proteins involved in the regulation of cytokine activity. This evidences the activation of the latter, as well as of some other proteins involved in the immune response of vertebrates (De Zoysa et al., 2009).

Direct responses of humoral immunity resulting from changes in gene expression remain the subject of discussion, and the results only fix the fact of changes in the expression of humoral immunity factors. The real physiological role of the observed changes is still unexplored. Apparently, such reactions of the immune system are negative since some species showed a decrease in the expression of humoral immunity factors in response to stimulation by pathogenic organisms during incubation under hypoxia (Sun et al., 2016).

3. The effect of hypoxia on the antioxidant complex of bivalves

The AO complex of mollusc is the key system of its nonspecific protection from oxidative stress (hereinafter OS) and to a great extent determines its resistance to the effect of adverse environmental factors (Gostyukhina & Andreenko, 2018 ; Soldatov et al., 2014). Oxygen deficiency is one of the most significant damaging factors that the AO system protects against. Increased release of ROS in molluscs during hypoxia increases the risk of OS (Tomanek, 2015). In molluscs, AO protection is provided by AO enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase (hereinafter CAT, SOD, and GP, respectively), as well as by a number of low molecular weight antioxidants (Livingstone, 2001). Several components of the AO complex not only protect against hypoxia, but also serve as important humoral factors of immune defense. Specifically, the activity of CAT, SOD, and GP correlates well with the immune competence of mollusc cells (Liu et al., 2004 ; Sui et al., 2017). High activity of different parts of the AO system in eurybiont molluscs allows them to withstand a long-term oxygen deficiency (Irato et al., 2007 ; Soldatov et al., 2014), adapt to it, and occupy their own econiche (Dovzhenko, 2006).

3.1. Species specificity in the reactions of the antioxidant complex to hypoxia. Despite the universal, nonspecific nature of the AO complex functioning, it can have specificity depending on species, tissue, evolutionary, and ecological peculiarities of molluscs (Gostyukhina & Andreenko, 2018 ; Dovzhenko, 2006 ; Istomina, 2012 ; Gostyukhina & Andreenko, 2019 ; Livingstone, 2001 ; Soldatov et al., 2014). Thus, under experimental hypoxia and anoxia, three types of reactions were revealed: an increase in the activity of AO enzymes (in species tolerant to hypoxia), a decrease in their activity, and a constancy of the state of the AO complex (Istomina et al., 2011). An increase in the activity of SOD and glutathione reductase was found in *Spisula sachalinensis* (Schrenck, 1862) and *Littorina mandshurica* (Schrenk, 1862) – molluscs experiencing oxygen deficiency due to environmental conditions. Time to time, *S. sachalinensis* burrows into sediments and stays under hypoxia. *L. mandshurica* inhabiting the littoral zone is daily subjected to fluctuations in oxygen level at high and low tides. These reactions are associated with the ability of AO enzymes of these molluscs to respond quickly to an increase in a ROS level. In *S. sachalinensis* hepatopancreas, a decrease in the content of reduced glutathione (hereinafter GSH) was noted as well (Istomina, 2012), which also reflects an active AO role of GSH and a rapid depletion of its resource during hypoxia. In OS-resistant mollusc species, hypoxia increases the activity of AO enzymes, primarily SOD and CAT. Thus, the reaction to short-term and long-term critical hypoxia in the mollusc *Astarte borealis* (Schumacher, 1817) was expressed in the activation of CAT, SOD, and GP (Abele-Oeschger & Oeschger, 1995). In hepatopancreas and gills of the hypoxia-resistant species *Scapharca inaequivalvis* (Bruguière, 1789), high activity of CAT, SOD, and GP was recorded as well (Irato et al., 2007).

For a stenooxybiont species *Patinopecten yessoensis* (Jay, 1857), the activity of AO enzymes decreased which is due to its lower resistance to hypoxia. This is associated with the fact that the mollusc lives under relatively stable conditions and is capable of avoiding hypoxia (Istomina et al., 2011). For the OS-sensitive mollusc *Tapes philippinarum* (A. Adams & Reeve, 1850), a decrease in SOD and CAT activity was shown under hypoxia; this may indicate the OS in tissues (Irato et al., 2007). Moreover, for the scallop *Mizuhopecten yessoensis*, a significant increase in a GSH level – by 6 times – was shown under hypoxia (Istomina, 2012). This reflects the leading role of GSH in protecting OS-sensitive molluscs.

Apparently, the third mode of response – constancy of AO activity – is associated with resistance to oxygen deficiency as well. Lack of significant changes in the AO complex during hypoxia was shown for *Crenomytilus grayanus* (Dunker, 1853) and *Tegula rustica* (Gmelin, 1791) – species inhabiting the sublittoral, leading an attached or sedentary lifestyle, and not being subjected to frequent oxygen deficiency, in contrast to littoral or burrowing molluscs (Istomina et al., 2011). Moreover, the Gray mussel *C. grayanus* is capable of maintaining constant SOD and CAT activity for a long time under oxygen deficiency against the backdrop of a constant level of lipid peroxidation products (Istomina, 2012). Such an AO defense strategy is probably due to an evolutionary adaptation of mytilids to prolonged hypoxia/anoxia (Hicks & McMahon, 2005). However, a decrease in glutathione reductase activity and GSH level was noted (Istomina, 2012). This proves that the main contributors to the AO protection of *C. grayanus* during hypoxia are the key enzymes – SOD and CAT. In the tissues of *Anadara kagoshimensis* (Tokunaga, 1906), even more OS-resistant species than *M. galloprovincialis*, a higher activity of AO enzymes and a significantly increased GSH resource were shown (Gostyukhina & Andreenko, 2018). This gives *A. kagoshimensis* an advantage for living and surviving under hypoxic environmental conditions.

3.2. Sensitivity of individual components of the antioxidant complex to oxygen deficiency. In the response to lack of oxygen, the specificity of the reactions of individual components of the AO complex is recorded as well. First of all, an increase in SOD and CAT activity is detected during hypoxia (Chen J. et al., 2007 ; Chen X. et al., 2014 ; Sui et al., 2017). Out of AO systems, SOD provides the front line of defense – the most significant one (Sui et al., 2017). Under oxygen deficiency, a rapid increase in SOD activity is often recorded – in the scallop *Ch. farreri* hemocytes (Chen J. et al., 2007), in *S. inaequivalvis* hepatopancreas and gills, in the oyster *Pteria penguin* (Röding, 1798) (Gu et al., 2020), in *S. sachalinensis* and *L. mandshurica* (Istomina et al., 2011), and in *M. coruscus* gills and hemolymph (Sui et al., 2017). As a rule, SOD is one of the first to react, but mainly at the initial stages of hypoxia. With further oxygen deficiency, a decrease in enzyme activity is observed; for example, in *Ch. farreri*, a decrease in SOD activity occurred after 7, 14, and 21 days which indicates that prolonged hypoxia can lead to inactivation of the main protective enzymes (Chen J. et al., 2007). Importantly, in a highly resistant to hypoxia bivalve *A. borealis*, a reaction to short-term and long-term critical hypoxia was expressed in the activation of both SOD and CAT and GP (Abele-Oeschger & Oeschger, 1995). This evidences the joint action of different parts of the AO complex, *inter alia* during prolonged lack of oxygen. Apparently, this AO strategy determines high resistance of *A. borealis* to hypoxia.

Increased SOD activity results in a high rate of superoxide anion radical dismutation into H_2O_2, and this stimulates CAT activity which catalyzes the breakdown of H_2O_2 and hydroperoxides and also protects the body from high amounts of hydroxyl radicals (Hermes-Lima, 2004). As shown, CAT activity often increases after a rise in H_2O_2 under OS (Hermes-Lima, 2004). This is consistent with the results of (Sui et al., 2017): for *M. coruscus*, CAT activity increases in response to low oxygen and pH levels.

In some cases, CAT activity decreases under oxygen deficiency in water – for example, in *M. galloprovincialis*. At the same time, other AO enzymes, in particular glutathione transferase (hereinafter GT), can play a more significant role than catalase in protecting mussels from hypoxia (Woo et al., 2013). Such a diversity of responses reflects the species specificity of the reactions of the AO complex during hypoxia and indicates their complexity and variability, as well as the plasticity of the AO system in protecting molluscs from hypoxia/anoxia.

The prevalence of low molecular weight antioxidants, primarily GSH, in the AO defense of molluscs was shown as well. As a rule, this is observed in species that are more sensitive to OS. Thus, in the stenooxybiont species, the scallop *P. yessoensis*, a decrease in the activity of AO enzymes under hypoxia was recorded (Istomina et al., 2011); apparently, this is determined by their lower resistance to oxygen deficiency. However, against this backdrop, a significant increase in a GSH resource is noted in the scallop which ensures the mollusc protection from ROS under conditions of reduced enzyme activity. This can be confirmed by the ability of GSH to inactivate the superoxide anion radical, thus partly duplicating SOD function (Hermes-Lima, 2004). Under hypoxia, such mutual substitution can contribute to effective protection and survival of the mollusc: with an increase in a ROS level, it allows to provide a fast and effective AO response with the help of low molecular weight glutathione without activating SOD (not requiring time and energy) (Gostyukhina & Andreenko, 2018).

An important protective role of GSH is also demonstrated by a comparison of AO reactions of molluscs with different resistance to oxygen deficiency. In a number of tissues of the Japanese scallop *M. yessoensis*, which is sensitive to hypoxia, not only increased activity of AO enzymes was shown, but also a higher GSH resource. In the Gray mussel *C. grayanus*, only an increase in SOD activity in gills was found. This is a species-specific reaction determining the resistance of these species to hypoxia (Belcheva et al., 2016).

3.3. Tissue-specific features of the antioxidant complex under hypoxia. The responses of the AO system of molluscs to hypoxia are tissue-specific as well. As a rule, the highest activity of AO enzymes is detected in hepatopancreas and gills (Gostyukhina & Andreenko, 2018 ; Dovzhenko, 2006), but there are some exceptions. Specifically, for *M. coruscus*, higher CAT, SOD, and GP activity was shown under hypoxia in hemocytes than in gills (Sui et al., 2017). This is associated with a more important role of hemolymph in immune defense. Similar features are registered in other species with lack of oxygen – higher values of CAT activity in hemocytes of *M. galloprovincialis* than in gills (Katsumiti et al., 2015) and higher values of SOD and GT activity in hemocytes of *Venerupis philippinarum* (Adams & Reeve, 1850) (Chen X. et al., 2014). For *A. kagoshimensis*, maximum values of a GSH level and GP activity were recorded in a foot. This proves active participation of GSH in the work of this enzyme as a cofactor; at the same time, this indicates its own active AO role. The content of other low molecular weight antioxidants (glucose, amino acids, and urea) was the highest in hepatopancreas and gills (target organs), and the lowest in a foot (Gostyukhina & Andreenko, 2019). Apparently, these low molecular weight antioxidants contribute much to the AO defense of the mollusc under hypoxia. Other authors came to the same conclusion. For *Anadara* experiencing anoxia and reoxygenation, a conclusion was made on the key role of a low molecular weight link of the AO system in ROS detoxification since low molecular weight antioxidants are less dependent on the intensity of metabolism and energy sources (Istomina et al., 2011).

3.4. Features of the antioxidant system functioning while in the atmosphere. Several studies were devoted to the effect of anoxia on molluscs when exposed to air. Specifically, anoxia in combination with different temperatures in the scallop *Ch. farreri* led to a complex of reactions – an increase in ROS production, a decrease in acid phosphatase activity and in a number of phagocytic hemocytes, and an increase in hemocyte mortality. At the same time, SOD activity did not depend significantly on air temperature but depended on anoxia duration (Chen J. et al., 2007).

Under anoxia in air at +25 °C for 2 hours, SOD activity in scallop hemocyte lysate significantly increased against the backdrop of a simultaneous significant rise in ROS production (Chen J. et al.,

2007). This proves a protective effect of SOD with an increase in an oxidative load under these conditions. In scallops at +5 °C, ROS production was significantly higher than the initial values under this anoxia regime; however, SOD activity in hemocyte lysate remained at the control level (Chen J. et al., 2007). Such reactions testify to the important protective role of SOD under different oxygen deficiency regimes and different temperatures. At the same time, the authors believe that scallops showed a relatively low anoxia tolerance at high temperatures. This is consistent with the opinion of a number of researchers that the effect of anoxic and hypoxic loads can alter the immune reactions of a mollusc and lead to increased susceptibility to diseases (Matozzo et al., 2005 ; Monari et al., 2005 ; Pampanin et al., 2002). Apparently, this type of AO protection is more characteristic of stenooxybiont molluscs.

The leading role of SOD in mollusc protection from hypoxia is also noted for *P. penguin* when exposed to air (Gu et al., 2020). SOD activity increased after exposure to air and then stabilized in oyster hepatopancreas and hemolymph, which resulted in a constant MDA level during 6 and 9 hours of hypoxia. In contrast to SOD activity, CAT and GP activity, as well as total AO capacity, increased rapidly and then gradually decreased after 6 hours of oyster exposure to air. Perhaps, the reason for this was an increase in MDA level in hemolymph after 9 and 12 hours of exposure to hypoxia.

3.5. Reversibility of hypoxia effect on the antioxidant complex during reoxygenation. A number of studies were devoted to the response of the AO system of mollusc to hypoxia and subsequent reoxygenation. In *M. galloprovincialis* gills, under conditions of 48-hour exposure to air followed by 48-hour reoxygenation, an increase in gene expression and a rise in SOD, CAT, and GT activity were observed. A recovery to normoxic levels was registered during reoxygenation (Giannetto et al., 2017). Such reactions reflect a preventive increase in the AO potential of the mollusc to strive the oxidative burst during further reoxygenation.

Under the effect of different hypoxia regimes in *M. galloprovincialis*, a decrease in CAT activity (SOD activity did not change), an increase in GT activity, and its subsequent decrease against the backdrop of a rise in the lipid peroxidation level were established. This reflects the transcriptional stability and selective changes in the genes of individual AO enzymes that protect the mussel under various oxygen deficiency regimes (Woo et al., 2013). Moreover, different reactions were identified for individual isoforms of enzymes. Specifically, under anoxia effect on the immune response of *Ch. gallina* hemocytes, Cu/Zn-SOD activity decreased, while Mn-SOD activity increased significantly. Mn-SOD activity is especially high during reoxygenation which is probably due to high inducibility of this isoform and its important role in protection against ROS when recovering to normoxia (Monari et al., 2005).

Conclusion. The results of recent studies made it possible to significantly deepen and expand knowledge on hypoxia effect on the immune system of molluscs at tissue, cellular, and molecular levels. Further analysis of adaptive mechanisms of mollusc hemocytes will allow assessing and predicting possible negative consequences of hypoxia effect on the cellular component of the immune system. Molecular genetic studies will help in assessing the degree of an oxygen deficiency effect on the humoral component. In turn, the degree of hypoxia effect on the immunity of bivalves will depend on a combination of climatic changes in the environment (global warming, changes in wind characteristics and currents, *etc.*) and on prospects for the use of coastal areas in economic activity. The reactions of the antioxidant complex of bivalve molluscs to hypoxia are species-specific, and so is the immune response. An AO response involves components of both enzymatic and low molecular links of the AO system in various combinations. Changes in AO activity during hypoxia are often caused by preparation for subsequent

reoxygenation and are often reversible. This reflects the functional plasticity of the AO system and its significant role in the defense mechanisms, as well as in the formation of nonspecific immune reactions in the body of bivalves under oxygen deficiency in the environment.

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ВЛИЯНИЕ ДЕФИЦИТА КИСЛОРОДА НА ИММУННУЮ СИСТЕМУ ДВУСТВОРЧАТЫХ МОЛЛЮСКОВ

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В течение последних десятилетий исследования иммунной системы двустворчатых моллюсков сфокусированы на изучении влияния факторов внешней среды на базальный статус защитных систем организма. Иммунная система моллюсков чувствительна к действию абиотических факторов, среди которых наиболее существенны температура, солёность воды и уровень растворённого кислорода. Гипоксия широко распространена в прибрежных водах Мирового океана с 1950-х гг.; гипоксические зоны (с концентрацией кислорода менее 0,5 мл $O_2 \cdot л^{-1}$) сохраняются на шельфе в течение длительного времени, соответствующего продолжительности жизненного цикла многих гидробионтов. Двустворчатые моллюски, являясь бентосными организмами, часто попадают под воздействие пониженной концентрации растворённого кислорода. Данная группа водных беспозвоночных играет важную роль в функционировании водных экосистем, при этом двустворок активно используют для аквакультурного выращивания. Эффективность культивирования этих организмов напрямую зависит от их иммунного статуса, определяющего устойчивость к заболеваниям. Основу иммунной системы двустворчатых моллюсков составляет комплекс неспецифических реакций клеточного и гуморального компонентов. Гемоциты, циркулирующие в гемолимфе, являются ключевыми эффекторами клеточного иммунного ответа, которые, наряду с барьерными тканями моллюсков, осуществляют синтез гуморальных факторов с широким спектром антимикробной активности. Гемолимфа моллюсков различных видов содержит разные типы клеток, которые отличаются по размерам, морфологии и наличию включений в цитоплазме. Большинство видов двустворок имеет два типа гемоцитов — гранулярные и агранулярные гемоциты; они могут подразделяться на морфотипы в зависимости от числа и окраски гранул, размеров ядра и наличия органелл в цитоплазме. Считается, что гранулоциты являются основными иммунными клетками, осуществляющими фагоцитоз и (или) инкапсуляцию инфекционных агентов, а также их последующую нейтрализацию путём выделения активных форм кислорода, лизирующих ферментов и гуморальных антимикробных белков. Также в комплекс защитных систем организма входит антиоксидантная, тесно связанная с иммунитетом моллюсков, поскольку эта система осуществляет нейтрализацию активных форм кислорода, выделяющихся в процессе активации клеточных иммунных механизмов. Избыток этих веществ оказывает повреждающее действие на клетки моллюсков путём окисления белков, липидов цитоплазматической мембраны и ДНК. В настоящем обзоре приведены данные о влиянии недостатка кислорода на клеточный и гуморальный компоненты иммунной системы и на тканевый антиоксидантный комплекс двустворчатых моллюсков.

Ключевые слова: двустворчатые моллюски, иммунитет, гемоциты, гипоксия, антимикробные белки, антиоксидантный комплекс

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STATE OF POPULATION OF *CALANUS EUXINUS* (COPEPODA) IN THE OPEN PELAGIAL AND ON THE SHELF OF THE BLACK SEA NEAR CRIMEA IN AUTUMN 2016

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A copepod *Calanus euxinus* Hulsemann, 1991 is one of the most abundant mesozooplankton species constituting up to 60–80 % of planktonic crustacean biomass in the deeper Black Sea and being the main food component for small pelagic fish. Data on abundance, biomass, age structure, and lipid reserves of *C. euxinus* are required to estimate the state of its population in the open pelagial and on the shelf of the Black Sea. The data were obtained during the 89th cruise of the RV "Professor Vodyanitsky" (30.09.2016–09.10.2016) in the northwestern, central, and northeastern sea (62 stations). Zooplankton was sampled with a Bogorov–Rass net (mouth area of 0.5 m^2; mesh size of 300 μm) by vertical net hauls from the seabed to the surface on the shelf and from the lower border of the oxygen zone to the surface in the deep-sea area. The samples were fixed with 4 % formaldehyde; in the laboratory, the abundance and biomass of all copepodite stages of *C. euxinus* were determined. Wax ester content in the bodies of late copepodite stages and adult specimens was estimated based on the specific oil sac volume (% of the body volume). The relationship between the quantitative species distribution and the habitat depth and macroscale hydrological circulation was revealed. In the deep-sea area, the mean abundance and biomass of *C. euxinus* amounted to (8.3 ± 0.8) thousand ind.·m^{-2} and (7.1 ± 0.7) g·m^{-2}, respectively. On the outer shelf, the abundance and biomass of this species decreased twofold – down to (4.2 ± 1.4) thousand ind.·m^{-2} and (3.3 ± 1.2) g·m^{-2}, respectively. In the deep-sea area, copepodites V, females, and males constituted 91 % of the total abundance and 96 % of the total biomass of the population. On the outer shelf, the ratio of these developmental stages reduced to 67 % and 86 % of the total abundance and biomass, respectively. In the deeper pelagial, the specific oil sac volumes in copepodites V, females, and males [(17.1 ± 0.6), (11.2 ± 0.8), and (11.9 ± 0.5) %, respectively] were twice as high as in the same developmental stages from the outer shelf [(8.1 ± 0.8), (4.7 ± 0.8), and (6.0 ± 0.5) %, respectively] indicating a relation between lipid accumulation in this species and hypoxic conditions of the biotope. Relatively high values of the abundance, biomass, and wax ester content in *C. euxinus* indicate that the population returned to its previous state – the one observed *prior* to expansion of alien ctenophores in the late 1980s and recent climatic changes resulting in a warming of the Black Sea basin.

Keywords: *Calanus euxinus*, abundance, biomass, lipid reserves, Black Sea

Calanus euxinus Hulsemann, 1991 is the most abundant representative of the Black Sea cold-water copepods; it forms 60–80 % of the biomass of planktonic crustaceans in open areas (Anninsky & Timofte, 2009 ; Yuneva et al., 1999) and is the key food source for small pelagic fish (Yuneva et al., 2016). For the Black Sea, the main patterns of the vertical distribution of the *C. euxinus* population are known since the early XX century (Nikinin, 1926), but many issues related to its annual renewal, spatial heterogeneity of the distribution in biotopes, and interannual biomass dynamics require further study.

Quantitative indicators of the state of the *C. euxinus* population in different Black Sea areas are considered in a number of works (Vinogradov et al., 1992 ; Zagorodnyaya et al., 2001 ; Svetlichny & Hubareva, 2011 ; Niermann et al., 1998). In 1980–1990, the biomass of this species in the central sea averaged 7–11 g·m^{-2} (Kovalev, 1996). However, the expansion of the ctenophore *Mnemiopsis leidyi* (A. Agassiz, 1860) in the late 1980s sharply reduced the *C. euxinus* biomass: in the central sea, it decreased to 0.5 and 1.1 g·m^{-2} in 1991 and 1992, respectively (Vinogradov et al., 1999); in the northern sea, it decreased to 4.3 g·m^{-2} in 1993 (Vinogradov et al., 1995). After the sea was invaded by the ctenophore *Beroe ovata* Bruguière, 1789 – the species feeding on planktivorous ctenophores alone – in the late 1990s, the trophic pressure on mesozooplankton by *M. leidyi* significantly decreased (Vinogradov et al., 1999). This resulted in the *C. euxinus* biomass recovery almost to the level of the 1980s (Anninsky & Timofte, 2009). It is unclear to what extent the population of this copepod could have been affected by the factors as follows: recent changes in the ecosystem associated with the effect of climate (Polonskii et al., 2013), phenological deviations in ecology, the reduction of a massive planktivore – the Black Sea sprat *Sprattus sprattus phalericus* (Risso, 1827) (Yuneva et al., 2016), gradual increase in the biomass of the scyphomedusa *Aurelia aurita* (Linnaeus, 1758) in the sea (Anninsky et al., 2019), and structural transformation of the entire community of planktonic gelatinous predators (Anninsky et al., 2020).

For the *C. euxinus* population, an autumn hydrological season is a period characterizing the success of its spring generative renewal, effectiveness of survival and maturation of new generations in summer, and developmental degree of a new generation of spawners (autumn copepodites V, males, and females) for next spring. In autumn, the *C. euxinus* biomass is only slightly lower than its spring maximum values (Vinogradov et al., 1999).

The aim of this work is to assess the current state of the *C. euxinus* population in the open pelagial and on the shelf of the northeastern, central, and northwestern Black Sea. It is of great importance in connection with an annual renewal of the composition, abundance, and biomass of the population.

MATERIAL AND METHODS

Mesozooplankton, *inter alia* juvenile and adult specimens of *C. euxinus*, was sampled at 62 stations in the northeastern, central, and western Black Sea in the deeper epipelagial, on the outer shelf (depths 50–200 m), and on the inner shelf (depth < 50 m) during the 89th cruise of the RV "Professor Vodyanitsky" (30.09.2016–09.10.2016) (Fig. 1). Sampling was carried out with a Bogorov–Rass net (hereinafter BR net) (mouth area of 0.5 m^2; mesh size of 300 μm) by vertical net hauls from the seabed or the lower border of the oxygen zone (according to the CTD Sea-Bird 911plus, σ_t = 16.2) to the surface. To compare the catchability of all size–age stages of *C. euxinus*, parallel hauls were carried out with the BR net and the Juday net (mouth area of 0.1 m^2; mesh size of 112 μm) at two stations. Samples were fixed with 4 % formaldehyde; the composition and abundance of copepods were determined in the laboratory by examining zooplankton in a Bogorov chamber under a microscope. The individual wet weight of copepodites and mature *C. euxinus* (WW, mg) was calculated by the formula:

$$WW = 0.58 \times l \times d^2 \times \rho\,,$$

where l and d are the length and width of the cephalothorax, respectively, mm;

ρ is the mean body density, g·cm^{-3} (Svetlichny & Hubareva, 2011).

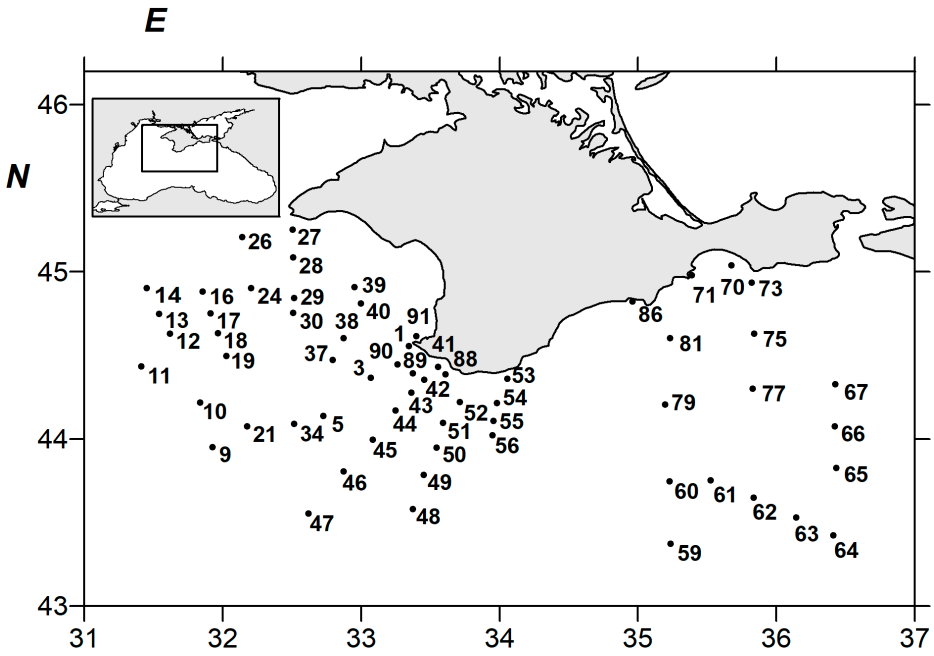

Fig. 1. Map of sampling survey (with station numbers identified) during the 89th cruise of the RV "Professor Vodyanitsky" in the Black Sea (September–October 2016)

The amount of reserve oil accumulated by the older stages of *C. euxinus* was estimated from the specific oil sac volume (Svetlichny & Hubareva, 2011). The oil sac volume (V_{sac}) was quantified according to the formula:

$$V_{sac} = \pi \times l_{sac} \times d_{sac}^2/6\,,$$

where l_{sac} and d_{sac} are the length and width of the oil sac, respectively, mm.

The body volume of copepodites, males, and females (V_b, mm^3) was calculated as follows:

$$V_b = k \times l_{pr} \times d_{pr}^2\,,$$

where l_{pr} and d_{pr} are the length and width of the cephalothorax, respectively, mm;

k is the empirical coefficient equal to 0.64 for males and 0.58 for copepodites and females (Svetlichny et al., 2009).

The data were processed statistically using Grapher 3 and Surfer 8 software for Microsoft Windows. The means were compared applying the Student's *t*-test. Mean values are presented with the standard error.

RESULTS

The results of 10 parallel hauls with the BR net and the Juday net in different sea areas are supplemented by similar data from previous years (Anninsky & Timofte, 2009) and shown in Fig. 2. The analysis of the material indicates that two nets captured early and middle copepodite developmental stages (I–IV) of *C. euxinus* with almost the same efficiency, while the BR net turned out to be more effective in capturing copepodites V. This may be due to lower filtration resistance of the BR net, as well as lower probability of its avoidance by the older copepodite stages and adult copepods.

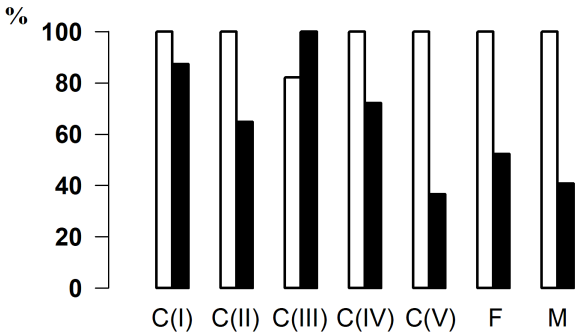

Fig. 2. Comparative catching efficiency for *Calanus euxinus* by the Juday net (black bars) and the Bogorov–Rass net (white bars) in 10 parallel vertical hauls in the Black Sea in September–October 2005 and 2016

The distribution of the *C. euxinus* abundance and biomass in September–October 2016 (Fig. 3) clearly shows a dependence on a macroscale hydrological circulation. The densest accumulations of individuals of this species (up to 21 thousand ind.·m^{-2} and 18.9 g·m^{-2} at stations 29, 63, and 79) were found on the periphery of the Eastern cyclonic gyre and in the core of the anticyclonic eddy west of Crimea (Sevastopol anticyclone). In the central Eastern gyre, there were fewer copepods. In the deep-sea area, the mean abundance and biomass of *C. euxinus* were (8.3 ± 0.8) thousand ind.·m^{-2} and (7.1 ± 0.7) g·m^{-2}, respectively. When moving from the open sea to the outer shelf, the abundance reliably decreased from (8.3 ± 0.8) to (4.2 ± 1.4) thousand ind.·m^{-2}, and the biomass reduced from (7.1 ± 0.7) to (3.3 ± 1.2) g·m^{-2}. On the inner shelf, due to single occurrence of the older copepodite stages, the abundance of *C. euxinus* amounted to only (0.10 ± 0.04) thousand ind.·m^{-2}, and the biomass was (0.09 ± 0.03) g·m^{-2}.

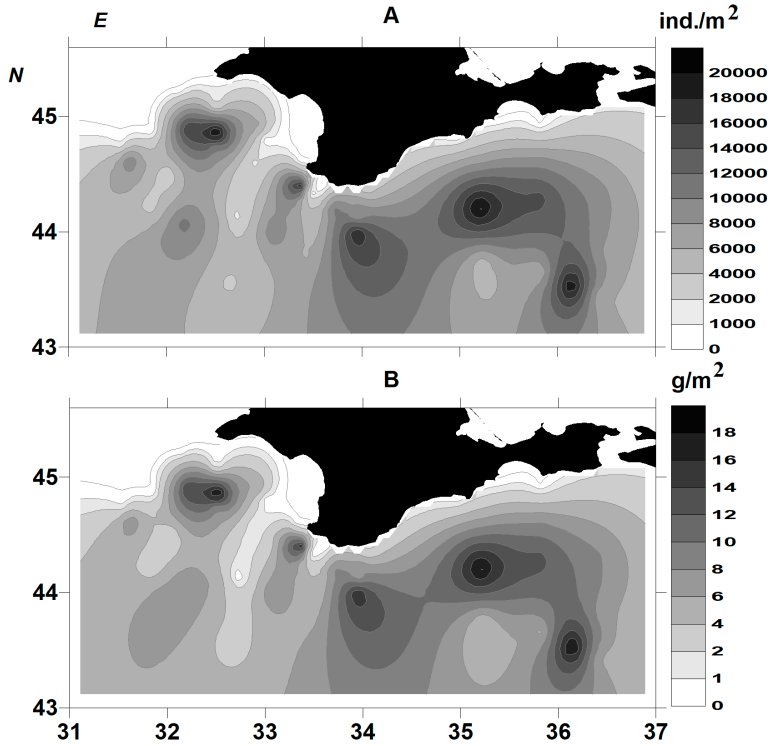

Fig. 3. *Calanus euxinus* abundance (A) and biomass (B) in the northeastern, central, and western Black Sea in September–October 2016

In the deep-sea area, with the lowering of the lower border of the oxygen zone (σ_t = 16.2) from 100–125 to 151–180 m, the *C. euxinus* abundance first increased ($p < 0.05$) from (7.3 ± 0.9) to (10.5 ± 1.1) thousand ind.·m^{-2} (with the depth reaching 126–150 m) and then decreased ($p > 0.05$) to (8.6 ± 2.1) thousand ind.·m^{-2} (with the lower border of the oxygen zone being at a depth of 151–180 m) (Fig. 4). The biomass varied in a similar way: the value first increased ($p < 0.05$) from (6.2 ± 0.8) to (9.02 ± 1.02) g·m^{-2} (at intermediate values of the lower border of the oxygen zone) and then decreased ($p > 0.05$) to (7.3 ± 1.9) g·m^{-2} (with the lowering of the lower border of the copepod biotope to 151–180 m). According to the data obtained, the *C. euxinus* abundance and biomass significantly increase ($p < 0.05$) in the direction from the central areas of cyclonic gyres to their borders. However, quantitative changes in the abundance and biomass of this species inhabiting cyclonic and anticyclonic circulation areas are generally insignificant.

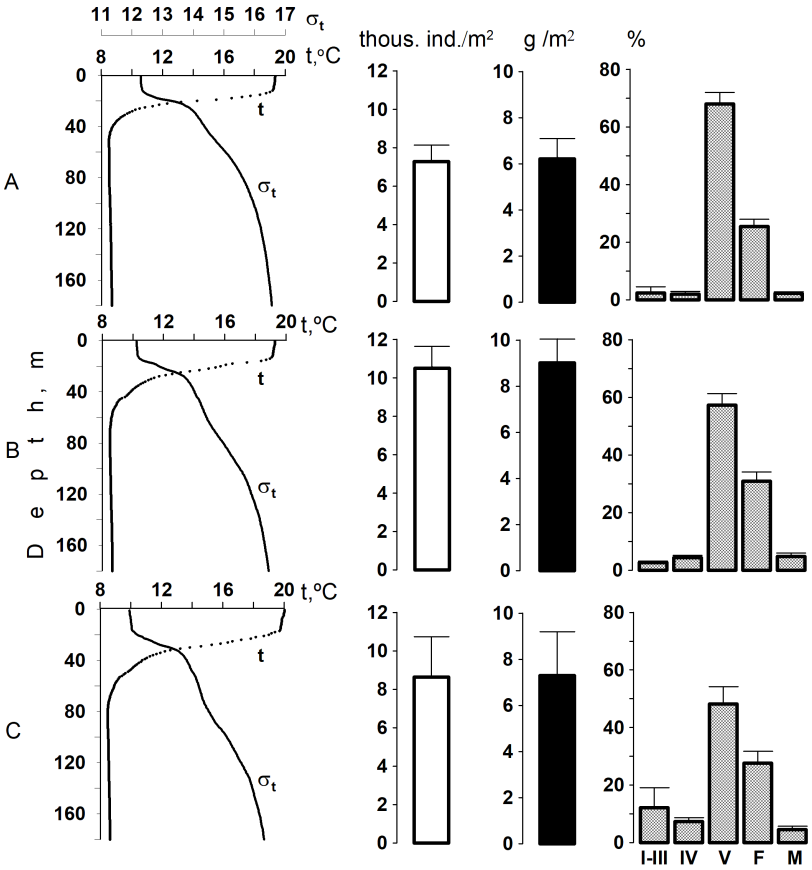

Fig. 4. Total abundance (thousand ind.·m^{-2}), biomass (g·m^{-2}), and age structure (% of total abundance) in the *Calanus euxinus* population in relation to seawater temperature (t, °C) and density (σ_t) in the upper epipelagial of the Black Sea (0–180 m). The lower border of the oxygen zone (σ_t = 16.2) is as follows: 100–125 m (A); 126–150 m (B); 151–180 m (C)

Specific changes in the structure of the *C. euxinus* population depending on the lower border of the oxygen zone indicate as follows: such variations in the abundance and biomass of crustaceans are caused by the redistribution of age stages with water masses of the surface epipelagial. Centrifugal flows in the area of cyclonic gyres displace to the periphery primarily copepodites I–IV inhabiting surface layers and adults. Copepodites V inhabiting a hypoxic biotope are affected to a lesser extent. In the area of anticyclonic eddies, on the contrary, centripetal flows can capture to a greater extent the younger copepodites inhabiting the near-surface water layer, as well as females and males. In this case, copepodites V either disperse or partially die. With the lowering of the lower border of the oxygen zone from 100–125 to 126–150 and 151–180 m, the ratio of copepodites I–III increased from (2.4 ± 1.9) to (2.6 ± 0.4) and (12.2 ± 6.8) %, respectively, and the ratio of copepodites IV

rose from (1.9 ± 0.9) to (4.3 ± 0.6) and (7.4 ± 1.2) %, respectively. With the lowering of the lower border of the oxygen zone, the ratio of copepodites V decreased from (67.9 ± 3.9) to (57.4 ± 3.7) and (48.2 ± 5.8) %, respectively; the ratio of adults remained almost at the same level – (25.5 ± 2.3), (30.9 ± 3.0), and (27.6 ± 4.0) % for females and (2.2 ± 0.4), (4.8 ± 1.0), and (4.6 ± 1.0) % for males.

The structure of the *C. euxinus* population changed similarly in the direction from the deep-sea area to the outer and inner shelf (Fig. 5). The total numerical prevalence of copepodites V [(58.0 ± 2.8) %] and females [(28.0 ± 1.8) %] in the open sea weakened on the shelf [36–40 % and 18–23 %, respectively], and the ratio of younger age stages, on the contrary, rose. On the shelf, it averaged 14–15 % for copepodites I–III and 24–25 % for copepodites IV. At the same time, no significant differences were found in the age structure of the *C. euxinus* populations from the outer and inner shelf.

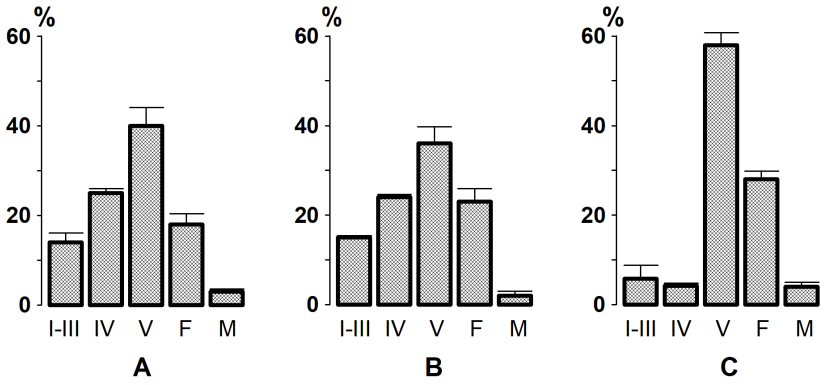

Fig. 5. Age structure of the *Calanus euxinus* population (% of total abundance) on the inner (A) and outer (B) shelf and in the deeper pelagial (C) of the Black Sea

The specific oil sac volume in copepodites V, females, and males of *C. euxinus* in the deep-sea area averaged (17.1 ± 0.6), (11.2 ± 0.8), and (11.9 ± 0.5) %, respectively (Fig. 6). On the outer shelf, the amount of wax esters accumulated by copepods decreased twofold– down to (8.1 ± 0.8) % of the body volume in copepodites V, (4.7 ± 0.8) % in females, and (6.0 ± 0.5) % in males.

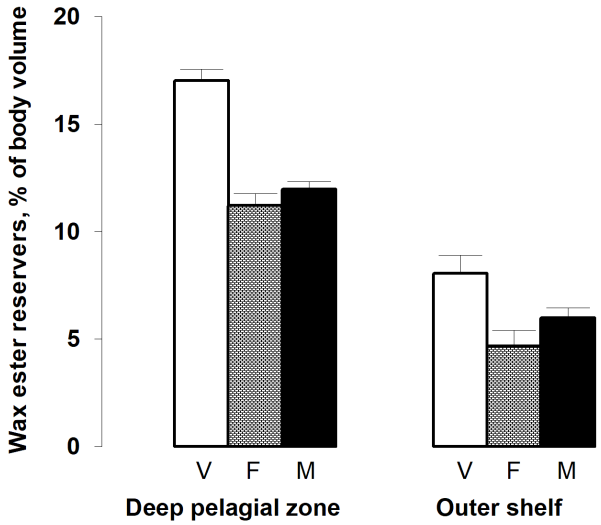

Fig. 6. Mean specific oil sac volume (% of the body volume) of *Calanus euxinus* in the deeper pelagial and on the outer shelf of the Black Sea in September–October 2016 (V denotes copepodites V; F, females; M, males)

DISCUSSION

Analysis of the quantitative characteristics of the state of the *C. euxinus* population in 2016, as well as similar data from previous years (Anninsky & Timofte, 2009 ; Arashkevich et al., 2002 ; Vinogradov et al., 1995 ; Zagorodnyaya et al., 2001 ; Kovalev, 1996 ; Svetlichny & Hubareva, 2014 ; Arashkevich et al., 2014 ; Vinogradov et al., 1999) did not allow revealing any interannual variability in the abundance and biomass for this species in the sea over the past decades. With the existing probability of such a dynamics, the range of interannual fluctuations in the copepod abundance and biomass (under predator pressure and effect of climatic factors) seemed to exceed significantly the possible limits of long-term changes in the population.

Specifically, the mean biomass of the species in deep-sea central [(6.5 ± 1.1) g·m^{-2}] and north-eastern [(8.9 ± 1.3) g·m^{-2}] areas in 2016 was comparable with the corresponding data of 1999 [9.7 g·m^{-2}] (Arashkevich et al., 2002). In the western deep-sea, the *C. euxinus* biomass [(5.9 ± 1.2) g·m^{-2}] was the same [$p > 0.05$] as in October 2005 [(6.2 ± 1.1) g·m^{-2}] (Anninsky & Timofte, 2009). Interestingly, in October 2010, its biomass in these areas (2.8 g·m^{-2}) (Svetlichny & Hubareva, 2014) was at least two times lower than in 2005 and 2016. The mean *C. euxinus* abundance varied in the same way: the value reached (9.9 ± 1.8) thousand ind.·m^{-2} in 2005 (Anninsky & Timofte, 2009) and (7.3 ± 1.3) thousand ind.·m^{-2} in 2016, but decreased to 3.9 thousand ind.·m^{-2} in 2010 (Svetlichny & Hubareva, 2014). On the outer shelf of the central sea, the *C. euxinus* abundance in autumn 2016 [(4.3 ± 1.9) thousand ind.·m^{-2}] on average exceeded the corresponding value for October 2005 [(2.5 ± 0.49) thousand ind.·m^{-2}], but the difference was statistically insignificant [$p > 0.05$]. If such changes did occur in the copepod population, they could not be associated with the interannual dynamics of the biomass of gelatinous macroplankton in general or its representatives. Importantly, despite the fact that the biomass of *M. leidyi* decreased in the sea (from (76 ± 22) g·m^{-2} in 2005 to (48 ± 11) g·m^{-2} in 2016), the biomass of another planktivorous ctenophore – *Pleurobrachia pileus* (O. F. Müller, 1776) – increased over these years (from (22 ± 4) to (45 ± 4) g·m^{-2}, respectively). Moreover, during this period, the biomass of the jellyfish *A. aurita* increased as well, at least by 5 times – from (44 ± 15) to (260 ± 72) g·m^{-2} (Anninsky et al., 2019). Apparently, the annual reproduction of the *C. euxinus* population in the sea is affected not so much by predators as by climatic factors. Specifically, a relation was found between the *C. euxinus* biomass and an increase in water temperature in April ($r^2 = 0.61$; $p < 0.01$) and May ($r^2 = 0.51$; $p < 0.01$) (Anninsky et al., 2020). Importantly, the water temperature in April and May 2016 was on average 0.5 and 2.2 °C higher, respectively, than in the same months of 2005. Therefore, it is more likely that an increase in the copepod abundance and biomass also occurred between 2005 and 2016.

The total prevalence of copepodites V and adults in the deep-sea area for most of the year (Arashkevich et al., 2014 ; Besiktepe, 2001 ; Svetlichny et al., 2009) indicates that the deep-sea hypoxic biotope is important for the development of the *C. euxinus* population: in a certain way, it regulates the accumulation of lipids in the body of copepods (Isinibilir et al., 2009 ; Yuneva et al., 1999). In the deeper pelagial in 2016, the older copepodite stages of the species had significant reserves of wax esters required for the completion of metamorphosis, sexual maturation, and generative production. The decrease in content of reserve lipids in females, males, and copepodites V in the direction from the open sea to the outer and inner shelf reflects the general patterns of formation of oil reserves in the Black Sea population of *C. euxinus*. Daily vertical migrations of the older copepodite stages into deep hypoxic layers can significantly reduce energy expenditure and increase the efficiency

of lipid accumulation (Svetlichny et al., 2006). In the shallow water area in the absence of hypoxia, copepods accumulate fewer reserve lipids; however, due to higher temperature in the biotope, they can develop faster (Svetlichny & Hubareva, 2014).

The formation of oil reserves in *C. euxinus* begins at copepodite stages III and IV, when the mean oil sac volume does not exceed 1–2 % of the body volume. Then, it gradually increases and averages 16–17 % of the body volume in copepodites V (Svetlichny & Hubareva, 2011). In autumn 2016, the specific oil sac volume in copepodites V averaged (17.1 ± 0.6) %; at some deep-sea stations, it reached 20 % of the body volume. Such a high content of reserve lipids for *C. euxinus* is comparable with oil content recorded in April 2003 in the southwestern sea during the bloom of the alga *Proboscia alata* (Brightwell) Sundström, 1986 (16–22 %) (Svetlichny et al., 2009). Similar values of the specific oil sac volume in copepodites V [(16.1 ± 7.6) %] were obtained in October 2005 in the western sea (Svetlichny & Hubareva, 2014). Thus, both in terms of quantitative indicators and content of reserve lipid in the body of copepods, the *C. euxinus* population in the Black Sea in the autumn 2016 was in a more developed state than in the years of the pelagic ecosystem recovery (early 2000s), after the uncontrolled effect of *M. leidyi* at the late XX century.

Conclusion. The data obtained indicate that spatial distribution of *Calanus euxinus* in the northeastern, central, and western Black Sea in September–October 2016 was heterogeneous and depended on the habitat depth and macroscale hydrological circulation. In the deep-sea area, the copepod abundance and biomass were almost twice as high as the values for this species on the outer shelf; this is due to peculiarities of the hydrodynamics of the Main Black Sea Current, cyclonic gyres, and anticyclonic eddies. In the *C. euxinus* population of the deeper pelagial, copepodites V prevailed; with females and males, those accounted for up to 91 % of the total abundance and 96 % of the total biomass of the species. On the outer and inner shelf, the ratio of the older copepodite stages naturally decreased. Copepodites V, females, and males inhabiting deep-sea area had two times more reserve lipids than the older copepodite stages on the shelf; this is due to specific patterns of lipid accumulation in this species. Relatively high values of the abundance, biomass, and wax ester content in *C. euxinus* indicate that the population returned to its previous state – the one observed *prior* to expansion of the alien ctenophores in the late 1980s and recent climatic changes resulting in a warming of the Black Sea basin.

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СОСТОЯНИЕ ПОПУЛЯЦИИ *CALANUS EUXINUS* (COPEPODA) В ОТКРЫТОЙ ПЕЛАГИАЛИ И ЗОНЕ КРЫМСКОГО ШЕЛЬФА ЧЁРНОГО МОРЯ ОСЕНЬЮ 2016 Г.

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Копепода *Calanus euxinus* Hulsemann, 1991 — один из наиболее массовых видов мезозоопланктона Чёрного моря, образующий в глубоководных районах 60–80 % биомассы планктонных ракообразных и составляющий здесь основу рациона мелких пелагических рыб. Данные о численности, биомассе, возрастной структуре и жировых запасах *C. euxinus* необходимы для оценки состояния его популяции в открытой пелагиали и шельфовой зоне Чёрного моря. С этой целью в 89-м рейсе НИС «Профессор Водяницкий» (30 сентября — 9 октября 2016 г.) проведены исследования в северо-западных, центральных и северо-восточных районах моря (62 станции). Пробы мезозоопланктона отбирали сетью Богорова — Расса (площадь входного отверстия — 0,5 м^2; ячея — 300 мкм) методом тотальных вертикальных ловов от дна до поверхности моря в области мелководного шельфа и от нижней границы кислородной зоны до поверхности моря в глубоководной части. Пробы фиксировали 4%-ным раствором формалина, численность и биомассу всех копеподитных стадий *C. euxinus* определяли в лабораторных условиях. Содержание восков в теле старших копеподитов и половозрелых особей *C. euxinus* оценивали по удельному объёму жирового мешка (относительно объёма тела). Выявлена зависимость количественного распределения вида от глубины биотопа и макромасштабной циркуляции водных масс в море.

В глубоководной части моря средняя численность *C. euxinus* составляла (8,3 ± 0,8) тыс. экз.·м$^{-2}$, биомасса — (7,1 ± 0,7) г·м$^{-2}$. На внешнем шельфе численность и биомасса вида снижались вдвое — до (4,2 ± 1,4) тыс. экз.·м$^{-2}$ и (3,3 ± 1,2) г·м$^{-2}$ соответственно. В глубоководных районах копеподиты V стадии вместе с самками и самцами составляли 91 % численности и 96 % биомассы популяции. На внешнем шельфе доля этих возрастных стадий сокращалась до 67 % численности и 86 % биомассы. В районах глубоководной пелагиали удельный объём жирового мешка у V копеподитов, самок и самцов [(17,1 ± 0,6), (11,2 ± 0,8) и (11,9 ± 0,5) % соответственно] был вдвое выше, чем у этих же возрастных стадий на внешнем шельфе [(8,1 ± 0,8), (4,7 ± 0,8) и (6,0 ± 0,5) % соответственно], что указывает на зависимость между накоплением липидных резервов у данного вида и гипоксическими условиями в биотопе. Сравнительно высокие величи́ны численности, биомассы и содержания восков у *C. euxinus* свидетельствуют о том, что его популяция практически вернулась к прежнему состоянию (наблюдавшемуся до экспансии гребневиков-вселенцев в конце 1980-х гг. и последних климатических изменений, которые привели к потеплению в бассейне Чёрного моря).

Ключевые слова: *Calanus euxinus*, численность, биомасса, резервные липиды, Чёрное море

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NEW REPORTS OF SUCTORIAN CILIATES (CILIOPHORA, SUCTOREA) EPIBIONT ON HALACARID MITES AND A HARPACTICOID COPEPOD FROM TÜRKIYE

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Suctorian ciliates are common epibionts on marine and freshwater invertebrates. In the present study, three epibiont suctorian ciliate species, viz. *Praethecacineta halacari* Schulz, 1933, *Thecacineta calix* (Schroder, 1907), and *Thecacineta cothurnioides* Collin, 1909, are reported. Hence, *P. halacari* was observed on the ventral side of the idiosoma and legs of halacarid mite *Copidognathus brachystomus* Viets, 1940 and ventral side of *Copidognathus tabellio* (Trouessart, 1894). *T. calix* was reported on halacarid mite *Maracarus gracilipes* (Trouessart, 1889) – a new host species for the ciliate. *T. cothurnioides* was found on two different harpacticoid copepod specimens. The species *T. cothurnioides* is recorded from Turkish coast for the first time. *T. calix* is reported from Antalya for the first time. Finding of *P. halacari* is the first record for Izmir area. The data on distribution of all registered suctorian species are provided as well.

Keywords: epibiont, suctorian ciliate, halacarid mite, harpacticoid copepod, host, Mediterranean Sea, Türkiye

Suctorian ciliates are common epibionts on marine and freshwater invertebrates such as copepods, cladocerans, nematodes, kinorhynchs, tanaids, and halacarid and hydrachnid mites (Dovgal et al., 2009a ; Durucan, 2019). In Türkiye, the first epibiont marine suctorian ciliate was reported by Durucan and Boyacı (2019) who registered *Praethecacineta halacari* Schulz, 1933 on *Copidognathus venustus* Bartsch, 1977 collected from Antalya. After that, Durucan *et al.* (2019) reported *Paracineta irregularis* Dons, 1927 on a halacarid mite (*Rhombognathus* sp.) from the Sea of Marmara. Recently, *Thecacineta calix* (Schroder, 1907) was recorded as epibiont on a harpacticoid copepod from the Aegean Sea of Türkiye (Fethiye-Muğla) for the first time from this country (Durucan, 2019).

The paper presents the first report of *Thecacineta cothurnioides* Collin, 1909 from Türkiye. *T. calix* is reported for the first time from a halacarid mite *Maracarus gracilipes* (Trouessart, 1889), and at the same time this record is the first for Antalya. Previously found *P. halacari* is reported here for different halacarid hosts – *Copidognathus brachystomus* Viets, 1940 and *Copidognathus tabellio* (Trouessart, 1894) – and location of Izmir.

MATERIAL AND METHODS

Sediment was sampled by snorkeling at locality from Antalya (Kundu) (36.848686°N, 30.831607°E) (fine sand, 2-m depth) (22 July, 2020) and Izmir (Urla–Karantina Island) (*Pinctada radiata* (Leach, 1814), 0–1-m depth) (Fig. 1). Then, sediment samples were sieved in 100 μm in the laboratory under a binocular microscope (Nikon SMZ-10). The light microscopy (Nikon Eclipse E400) micrographs were taken with a camera phone. Halacarid mites and harpacticoid specimens inhabited by ciliates were placed in Hoyer's medium and kept in the collection of the first author (F. Durucan).

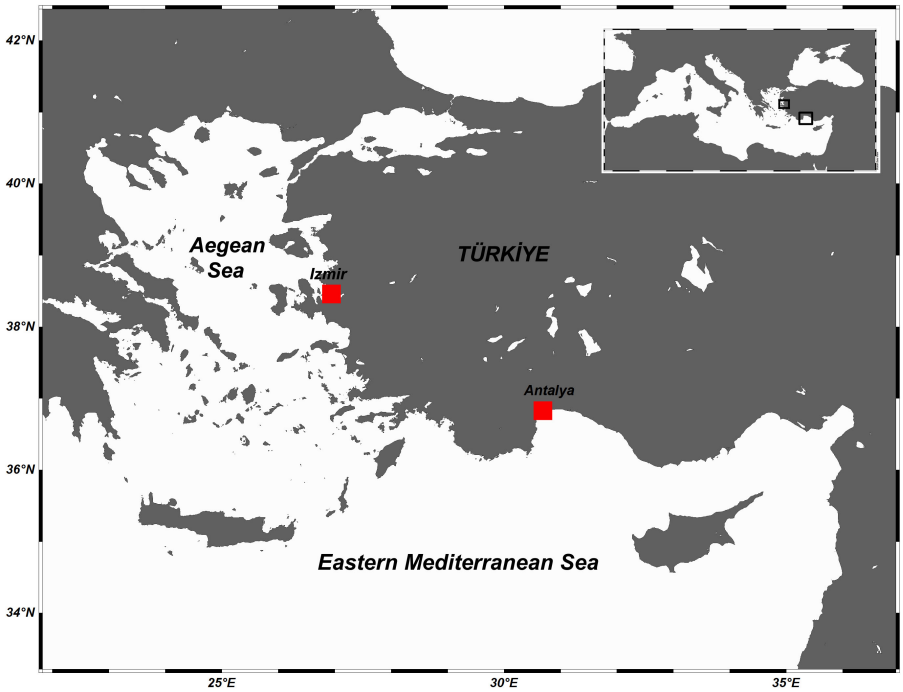

Fig. 1. Studied areas in Türkiye with the sampling stations indicated [source of the map is (Schlitzer, 2022)]

RESULTS AND DISCUSSION

Class Suctorea Claparede & Lachmann, 1859
Subclass Exogenia Collin, 1912
Order Metacinetida Jankowski, 1978
Family Praethecacinetidae Dovgal, 1996
***Praethecacineta halacari* Schulz, 1933**

Material examined. Numerous epibiont ciliates were observed on *C. brachystomus*. Mostly, those were found as attached ventral side of the idiosoma and legs (Fig. 2A). Four ciliates were registered as attached ventral side of another *C. brachystomus* (Fig. 2B). More than ten *P. halacari* were observed on ventral side of *C. tabellio* (Fig. 2C). Length of *P. halacari* lorica was approximately 50–60 μm; width of lorica was 20–25 μm.

Distribution. *P. halacari* is widely distributed species, specific to halacarid mites. *P. halacari* was previously recorded from various species of halacarid mites and different areas worldwide (Chatterjee et al., 2018 ; Durucan & Boyacı, 2019). The species was firstly reported near the Norwegian

coast (Tromsø, type locality) from unidentified halacarids. Subsequent finds were in the Atlantic coast of Brazil, Caspian Sea, Pulau Bedukang (Brunei), Bulgaria, Nova Scotia (Canada), Norfolk (England), Tromsø (Norway), Kiel (Germany), Goa (India), Western Australia, He-Ping-Dao (Taiwan), Matemwe and Zanzibar (Tanzania), Gdańsk (Poland), Singapore, Albufeira (Portugal), Crimea (Russia) (Chatterjee et al., 2018 ; Dovgal et al., 2009a ; Dovgal, 2013), and Antalya and Izmir (Türkiye) (present report).

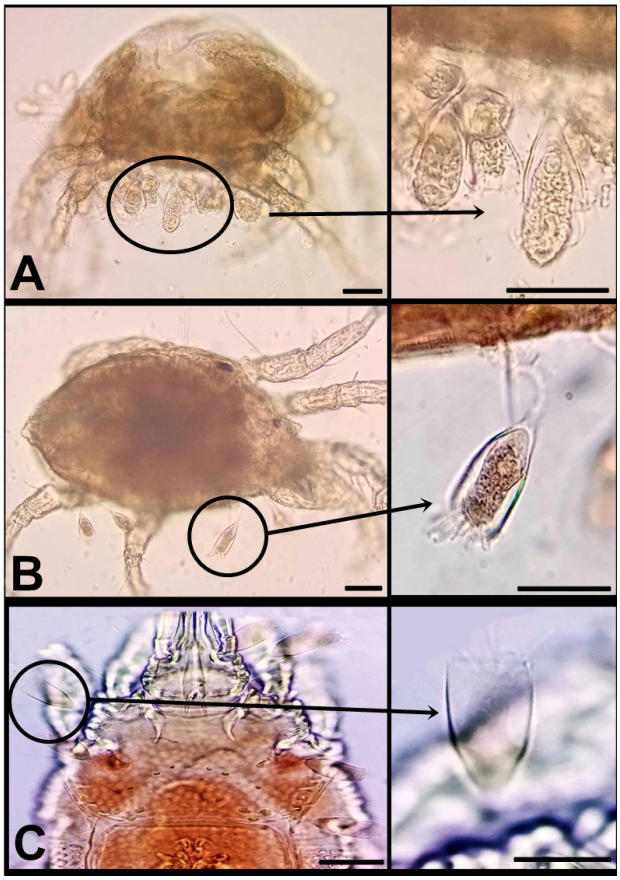

Fig. 2. Ventral views of three different *Copidognathus* specimens infected with *Praethecacineta halacari*: A, B, *Copidognathus brachystomus*; C, *Copidognathus tabellio* (scale bars are 50 μm)

Order Vermigemmida Jankowski, 1973
Family Thecacinetidae Matthes, 1956
Genus *Thecacineta* Collin, 1909
***Thecacineta calix* (Schroder, 1907)**

Material examined. In total, 27 halacarid specimens (14 females and 13 males) were identified as *M. gracilipes*. Out of them, 18 individuals (9 females and 9 males) were found as inhabited by numerous individuals of the species *T. calix* from the sampling area of Antalya. Those were attached to ventral side of idiosoma and gnathosoma. The ciliates were also attached to legs laterally and ventrally (Fig. 3). The suctorian lorica surface was covered with characteristic for the species annular ridges (7–8). Length of lorica was 50–60 μm; width of lorica was 20–25 μm.

Distribution. The worldwide distribution is characteristic for *T. calix* which is reported as an epibiont on nematodes, copepods, and halacarid mites from the Atlantic, Pacific, Antarctic, and Indian oceans, from the intertidal area to the deep sea (Chatterjee et al., 2019b). The species was firstly reported on the coast of Kerguelen Islands and Island of Heard (Antarctica, type locality),

Kiel Bay (Germany), Tarva (Norway), Koprino Harbor and Quatsino Sound (Pacific coast of Canada), Tierra Del Fuego, Falkland Islands, the Adriatic Sea, the Mediterranean Sea, Veracruz (Mexico), Odesa, Sevastopol (the Black Sea), Siladeu and Nias islands (Indonesia), North Sea, Hokkaido (Japan), Near Andaman & Nicobar Islands, He-Ping-Dao (Taiwan), Piran Bay (Slovenia), East Saint John (U. S. Virgin Islands), Caja de Muertos Island, Buoy, La Parguera (Puerto Rico), Pulau Bedukang (Brunei), Southwest Bay of Bengal, Tamil Nadu (India), Brittany (France), Suvadiva Atoll (Maldives), Northern Caspian Sea, Angria Bank, Arabian Sea (Chatterjee et al., 2019a, b, 2020a, b ; Dovgal, 2013 ; Panigrahi et al., 2015), Muğla (Türkiye) (Durucan, 2019), and Antalya (Türkiye) (present report).

Other hosts. *T. calix* was observed on many different halacarid mites. But in this study, we report for the first time the ciliate species on many specimens of *M. gracilipes*. The latter is a new host species for *T. calix*. The ciliate species were also reported from several species of nematodes, copepods, *etc.* (Chatterjee et al., 2019b, 2020a ; Dovgal, 2013).

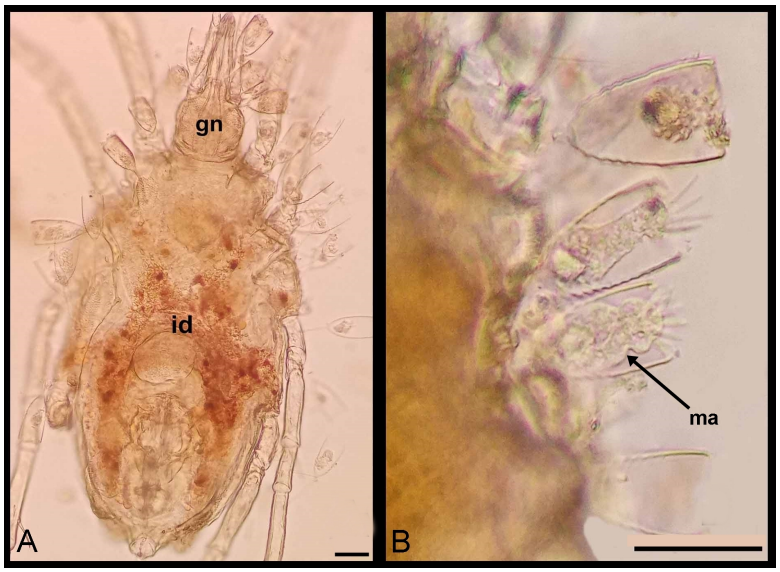

Fig. 3. Specimen of *Maracarus gracilipes* infected by *Thecacineta calix*: A, total view; B, magnificated view (gn, gnathosoma; id, idiosoma; ma, macronucleus) (scale bars are 50 μm)

Thecacineta cothurnioides Collin, 1909

Material examined. In total, two harpacticoid copepod specimens were observed inhabited by *T. cothurnioides* (Fig. 4A, B). Out of them, one was infected with single individual, while another one was inhabited with eleven individuals (Fig. 4C, D).

Distribution. The species was firstly reported on harpacticoid copepod from Banyuls-sur-Mer at the Mediterranean coast of France (type locality) (Dovgal et al., 2009b). Next, *T. cothurnioides* was reported on nematodes from Ratnagiri, Rushikulya, and Sundarbans (India) (Chatterjee et al., 2019a), as well as on nematodes from the Maldivian Archipelago (Baldrighi et al., 2020). Finally, it was registered in the Mediterranean Sea near Antalya (Türkiye) (present report).

Other hosts. Besides harpacticoid copepods [*Cletodes longicaudatus* (Boeck, 1872)], this species was reported from nematodes – *Tricoma* sp., *Chromaspirina* sp., *Chromaspirina parapontica* Luc & De Coninck, 1959, and *Paradesmodora* sp. (Baldrighi et al., 2020 ; Chatterjee et al., 2019a ; Dovgal et al., 2009a).

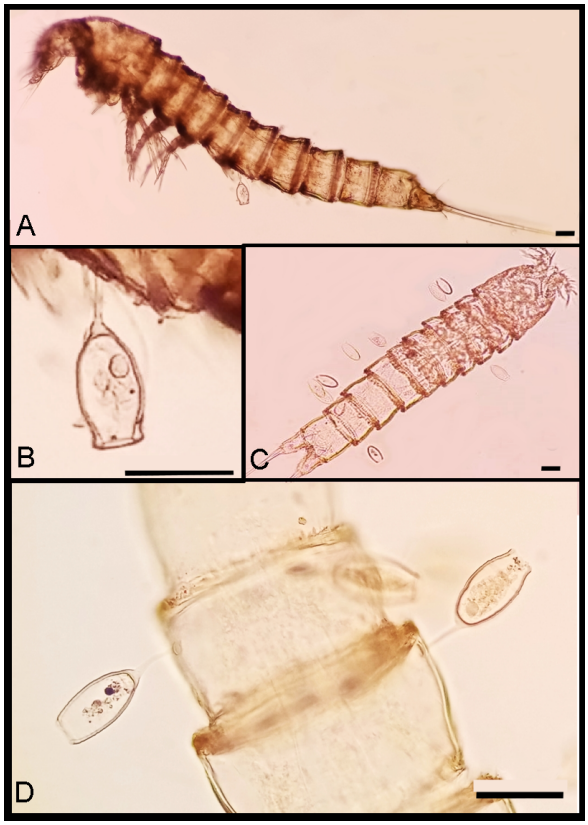

Fig. 4. Distribution of *Thecacineta cothurnioides* on harpacticoid copepod body: A, B, lateral view; C, D, ventral view (scale bars are 50 μm)

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НОВЫЕ НАХОДКИ ЭПИБИОНТНЫХ СУКТОРИЙ (CILIOPHORA, SUCTOREA) НА КЛЕЩАХ-ГАЛАКАРИДАХ И ГАРПАКТИКОИДАХ С ТУРЕЦКОГО ПОБЕРЕЖЬЯ

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Суктории — широко распространённая группа эпибионтных инфузорий, обитающих на представителях многих таксонов морских и пресноводных беспозвоночных. В статье приведены данные о новых находках трёх видов эпибионтных сукторий. *Praethecacineta halacari* Schulz, 1933 обнаружена на вентральной поверхности идиосомы и на ногах галакаридного клеща *Copidognathus brachystomus* Viets, 1940, а также на вентральной стороне тела *Copidognathus tabellio* (Trouessart, 1894). *Thecacineta calix* (Schroder, 1907) отмечена на поверхности клеща *Maracarus gracilipes* (Trouessart, 1889) — нового хозяина для этой инфузории. *Thecacineta cothurnioides* Collin, 1909 зарегистрирована на поверхности тела двух особей гарпактикоид. Это первая находка *T. cothurnioides* у побережья Турции. *T. calix* впервые отмечена в окрестностях Антальи, а *P. halacari* впервые зарегистрирована в окрестностях Измира. В статье приведены данные о распространении всех найденных видов.

Ключевые слова: эпибионт, суктория, галакаридный клещ, гарпактикоида, хозяин, Средиземное море, Турция

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HISTORY OF DISPERSION OF THE NORTH AMERICAN POLYCHAETE *MARENZELLERIA NEGLECTA* SIKORSKI & BICK, 2004 (ANNELIDA: SPIONIDAE) IN THE NORTHEASTERN SEA OF AZOV

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In the early 2010s, the alien polychaete worm *Marenzelleria neglecta* Sikorski & Bick, 2004 invaded the Sea of Azov. In few years, the species has widely spread over the desalinated sea area. Moreover, it was recorded in the Don delta and in the Sea of Azov–Kuban estuaries. This alien species formed a stable and numerous colony localized in the northeastern Sea of Azov; the history of this formation is traced based on material of complex hydrobiological and hydrological surveys of 2010–2020. The colony of this species developed against the backdrop of an increase in water salinity. Obviously, this factor had a decisive effect on the invasive process. An outbreak of abundance observed in the western Taganrog Bay in 2012 and 2013 was followed by a sharp decrease in abundance – down to complete absence of this polychaete worm in the samples. A drop in abundance was accompanied by a reduction of its range and a shift in the core of abundance towards sea areas with the lowest salinity. To date, there is a stable *M. neglecta* population in the central and eastern Taganrog Bay. Changes in the structure of prevalence in benthic communities during invasion were analyzed. As shown, the ratio of alien polychaetes in the periods of their mass development reached 92 % of the total abundance of benthos at individual stations.

Keywords: Polychaeta, alien species, benthic communities, macrozoobenthos, estuaries, Sea of Azov

A polychaete worm *Marenzelleria neglecta* Sikorski & Bick, 2004 is native to coastal and estuarine ecosystems of North America (Sikorski & Bick, 2004). Since the mid-1980s, it actively spreads in the northern seas of Eurasia – the Baltic and North ones (*Marenzelleria neglecta*, 2021). *Marenzelleria* species are quite difficult to identify morphologically. Initially, *M. neglecta* was not considered in the Baltic Sea, and the first polychaetes were identified as *Marenzelleria viridis* (Verrill, 1873). After the genus revision, those were assigned to a new species – *M. neglecta*. Later, two more *Marenzelleria* representatives – *M. viridis* and *M. arctia* (Chamberlin, 1920) – were found in the Baltic (Michalek, 2012). At present, *Marenzelleria* spp. group is considered as the most successful one out of the invaders to the Baltic Sea (Maximov, 2011 ; Zettler et al., 2002). The invasion of these worms significantly affected the structure of benthic and planktonic biocenoses (Ezhova et al., 2005 ; Kotta et al., 2006 ; Maximov, 2011 ; Zmudzinski et al., 1993). In the Gulf of Finland, their bioturbation and bioirrigation activity resulted in alterations in the entire ecosystem (Maksimov, 2018).

Being connected with the Sea of Azov by a network of canals and forming a single transport system, the Baltic could become a secondary donor area for *M. neglecta* invasion into the Sea of Azov basin (Boltachova & Lisitskaya, 2019). For the first time, *Marenzelleria* spp. was recorded there in 2014 (Syomin et al., 2016b). Later, adult worms were found at several stations in the Taganrog Bay and the Don delta, and larvae were registered in the bay plankton (Syomin et al., 2016a, b). In one of the first reports, high dispersion of the invader in the upper Taganrog Bay was highlighted: the occurrence of worms reached 90–100 % (Syomin et al., 2016a). Based on the specimens found, two morphotypes were described corresponding to characteristics of two species – *M. arctia* and *M. neglecta*. Further studies, with genetic analysis methods involved, showed as follows: in the Sea of Azov, there is only one *Marenzelleria* species – *M. neglecta* (Syomin et al., 2017).

In the AzNIIRKh samples, representatives of the family Spionidae, new for the Sea of Azov, were recorded in 2010. Those spionids differed morphologically from other, known representatives of the family, but were not identified. Subsequent processing of the material revealed that the registered polychaetes correspond to the described morphotypes of the genus *Marenzelleria*. Since 2016, alien spionids were confidently diagnosed as *Marenzelleria* sp.; later, as *M. neglecta*. Thus, the available material of surveys allows to trace *M. neglecta* invasion in the Sea of Azov since 2010. Our own data, as well as information on the invader occurrence in the Sea of Azov (Boltachova & Lisitskaya, 2019 ; Bulysheva et al., 2020 ; Syomin et al., 2016a ; Frolenko & Maltseva, 2017 ; Syomin et al., 2016b, 2017), show that the species formed a stable colony in the water area with a dynamic salinity regime.

In the Sea of Azov, salinity averages 11–12 ‰. In the Taganrog Bay, which is characterized by the maximum spatial heterogeneity related to the effect of the Don runoff and the nature of water circulation, salinity varies within 1–9 ‰ (Ekologicheskii atlas Azovskogo morya, 2011). Interannual fluctuations in the sea salinity are irregular: desalinization periods of varying duration are replaced by salinization periods. In the sea, interannual fluctuations in salinity can reach 1 ‰; in the Taganrog Bay, the range is even higher – up to 3.6 ‰ (Gidrometeorologiya, 1991). Since 2007, a steady increase in the sea salinity was observed. In 2010–2020, the mean salinity in the open sea increased from 11.5 to 15.0 ‰, and in the Taganrog Bay, from 8.5 to 11.0 ‰.

The aim of this work is to describe the history of *M. neglecta* colony formation in the northeastern Sea of Azov, to determine the role of abiotic environmental factors in this process, and to assess the current state of the invader population in the Sea of Azov basin.

MATERIAL AND METHODS

Complex surveys in the Sea of Azov were performed according to the standard grid of stations applied in AzNIIRKh since 1952 (Metody rybokhozyaistvennykh, 2005). Annually, 1–4 cruises on the RV were carried out. The article presents the results of the summer survey in 2010, when alien spionids were revealed in the Sea of Azov for the first time, as well as the results of autumn surveys in 2012–2020. Based on them, interannual dynamics of *M. neglecta* abundance is analyzed (Table 1). Sampling was carried out with a Petersen grab with a capture area of 0.1 m^2, in duplicate. The material was processed according to the methodological recommendations (Metody rybokhozyaistvennykh, 2005). Benthos was washed through sieves with a filtration mesh diameter of 5.0 and 0.3 mm (upper and lower sieve, respectively). As a fixative, we used 4 % neutralized formalin or 76 % ethanol with formalin added to prevent maceration of worm tissues.

Table 1. Number of sampling stations carried out in the northeastern Sea of Azov

Month and year	Number of sampling stations	Month and year	Number of sampling stations
July 2010	21	October 2016	17
October 2011	25	September–October 2017	17
October 2012	21	September–October 2018	17
October 2013	16	October 2019	17
October 2014	21	October 2020	18
October 2015	17	**In total**	**207**

Benthic samples were analyzed under a binocular. With high abundance of juvenile worms, a sample was taken, and the individuals were counted in a Bogorov chamber. When analyzing the species population structure, two indicators of specific abundance were used – mean abundance and ecological abundance. Mean abundance (number of worms *per* unit area) was calculated considering all stations. Ecological abundance (abundance in a colony) was determined as the number of worms *per* unit of habitat, *i. e.*, excluding stations with zero values.

To determine salinity, samples were taken at 18 standard stations with a Niskin bathometer: in the bay and in the sea at depths less than 7 m, at two horizons (surface and bottom layer); at depths over 7 m, at three horizons (surface, 5 m, and bottom layer). Maps were generated with Surfer v15. The data were statistically processed using the PAST software (Hammer, 2012).

RESULTS

Spatial distribution. For the first time, alien polychaetes were recorded in samples in July 2010. The colony of worms occupied the eastern Sea of Azov and the western Taganrog Bay (Fig. 1). The core of the population, with the abundance reaching 6,000 ind.·m^{-2}, was localized in the silted shell rocks of the Yeleninsky banks. There, salinity was of 11.5 ‰.

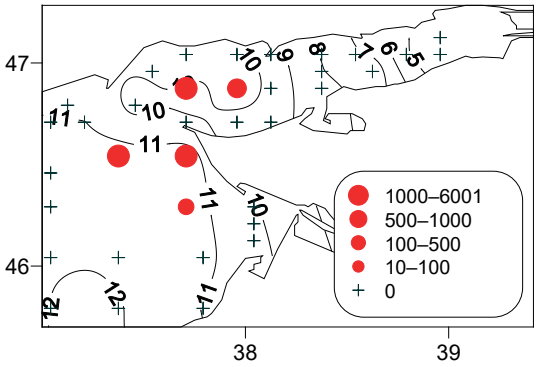

Fig. 1. *Marenzelleria neglecta* abundance, ind.·m^{-2}, in the northeastern Sea of Azov in July 2010 (isolines indicate salinity, ‰)

Since 2012, *M. neglecta* was regularly recorded in almost all surveys, and the polychaete colony occupied the estuarine area of the open sea and most of the Taganrog Bay (Fig. 2). The eastern boundary of the range, in comparison with that of the summer 2010, shifted towards east and ran along the line connecting the Beglitskaya Spit with Port-Katon. The core of the population, with the abundance of worms reaching 55,175 ind.·m^{-2}, was localized in the western bay. Mean water salinity in the western Taganrog Bay was 9.9 ‰. At the Yasensky Bay mouth, the abundance of worms was of 250 ind.·m^{-2}.

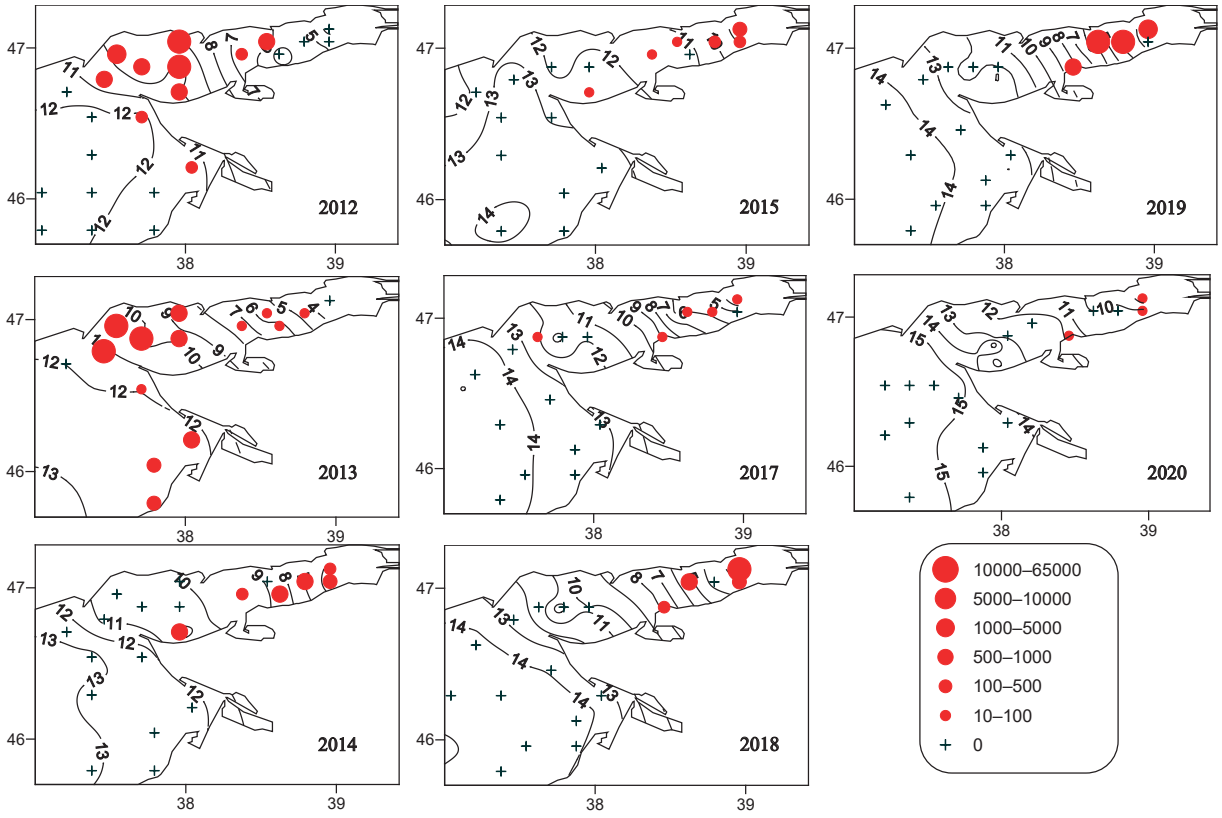

Fig. 2. *Marenzelleria neglecta* abundance, ind.·m^{-2}, in the northeastern Sea of Azov in 2012–2020 in autumn (isolines indicate salinity, ‰)

In 2013, there were no significant changes in the spatial distribution of polychaetes. The main colony still occupied the estuarine sea area and the bay; the core was localized in the western bay (water salinity of 10.2 ‰); and the abundance remained high – up to 61,500 ind.·m^{-2}. The eastern boundary of the range shifted even further: the worms were found in the section Taganrog – the Semibalki village. The water area covered by the eastern aggregation increased, and this aggregation moved southward. Its maximum abundance was of 482 ind.·m^{-2}. Since 2014, changes in the spatial structure were recorded, and a drop in the population abundance was registered. The range of polychaetes began to decrease, and the species was no longer found in the considered sea area (salinity was of 12.3–12.9 ‰). The core of the colony shifted to the central Taganrog Bay (9.1 ‰), and maximum abundance decreased by an order of magnitude – down to 4,620 ind.·m^{-2}.

In 2015, the core of the polychaete aggregation shifted even further – to the eastern bay (9.1 ‰), and maximum abundance decreased to 640 ind.·m^{-2}. Salinity in the central Taganrog Bay reached 11.3 ‰, in the western bay, 12.5 ‰, and in the open sea, 13.3 ‰. In 2016, no polychaetes were found in the Sea of Azov. Salinity in the eastern Taganrog Bay, where the core of the aggregation was localized earlier, dropped to 3.5 ‰. In the central area, the value was 6.9 ‰, in the western area, 12.6 ‰, and in the open sea, 13.8 ‰. In 2017, single worms were recorded in the western Taganrog Bay (12.1 ‰); the main colony, with the abundance up to 640 ind.·m^{-2}, was registered on the border of the eastern and central Taganrog Bay (water salinity of 4.7 and 7.5 ‰, respectively).

In 2018–2020, worms completely disappeared from the western area (11.2–13.7 ‰). The range of the invader was limited to the central and eastern Taganrog Bay. In 2018, the core of the aggregation, with the abundance up to 1,890 ind.·m^{-2}, was found in the eastern bay (3.2 ‰). In 2019,

the maximum (11,000 ind.·m^{-2}) was recorded in the central area, with its salinity reaching 7.0 ‰. In 2020, the abundance of worms decreased sharply: the maximum was 235 ind.·m^{-2}, and the species mainly occupied the eastern Taganrog Bay (9.7 ‰).

Dynamics of quantitative indicators. In 2012 and 2013, the mean abundance of *M. neglecta* was high – 4,628 and 7,084 ind.·m^{-2}, respectively. High values were recorded for ecological abundance as well – 9,719 and 8,720 ind.·m^{-2}, respectively. The next two years, 2014 and 2015, were characterized by a decrease in both indicators by an order of magnitude (Fig. 3). In 2017, mean and ecological abundance of polychaetes remained at a low level – 98 and 334 ind.·m^{-2}, respectively. Then, the population abundance began to increase gradually. By 2018, the mean abundance of worms was 274 ind.·m^{-2}; by 2019, the value reached 1,050 ind.·m^{-2}. A more noticeable growth was observed within the colony: in 2018, ecological abundance was 933 ind.·m^{-2}; by 2019, it was 4,464 ind.·m^{-2}. In 2020, the lowest values for the entire period were registered – the mean abundance of 16 ind.·m^{-2} and ecological abundance of 98 ind.·m^{-2}.

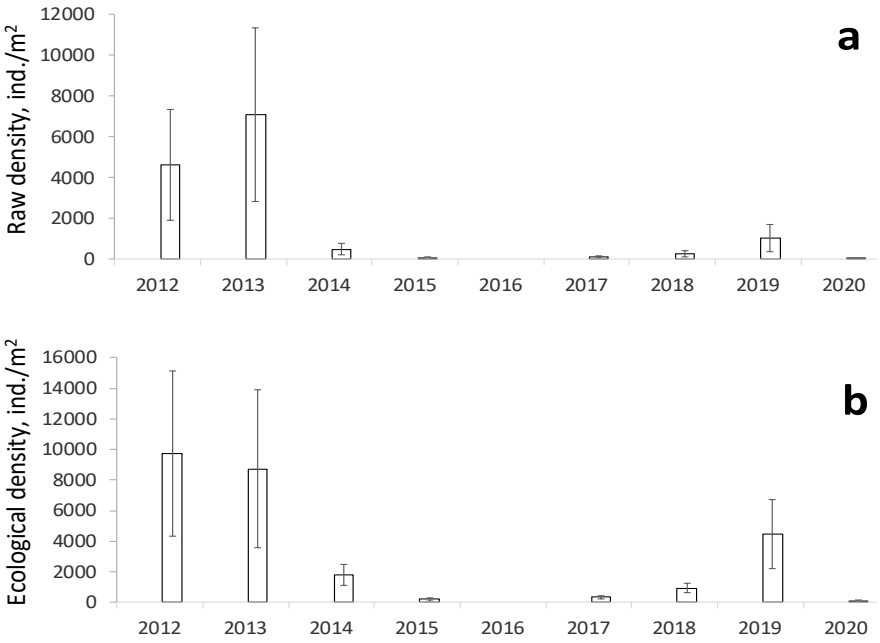

Fig. 3. Dynamics of mean abundance (number of individuals *per* unit area) (a) and ecological abundance (number of individuals *per* unit of habitat) (b) of *Marenzelleria neglecta* in the northeastern Sea of Azov in 2010–2020. Error bars indicate standard error

The structure of prevalence in communities. The main outbreak of the invader abundance was recorded in the western Taganrog Bay. There, *prior* to its beginning (in 2010 and 2011), mass and common benthic species – those with frequency of occurrence ≥ 50 % – were oligochaetes, two polychaetes (*Alitta succinea* (Leuckart, 1847) and *Polydora cornuta* Bosc, 1802), gastropods *Hydrobia* spp., and bivalves *Cerastoderma glaucum* (Bruguière, 1789). The total abundance of macrobenthos in the area averaged 8,953 ind.·m^{-2} in 2010 and 13,358 ind.·m^{-2} in 2011. The listed species and groups accounted for more than 90 % of the total population abundance. In 2012 and 2013, the polychaete *M. neglecta* was registered at all stations. Out of other representatives of the benthic fauna, *A. succinea*, *Hydrobia* spp., and oligochaete worms maintained high frequency of occurrence. The abundance of benthos increased threefold and amounted to 33,848 and 38,944 ind.·m^{-2} in 2012 and 2013, respectively.

In 2012, *M. neglecta* accounted for an average of 38 % of the total abundance in the western area; in 2013, the value was 58 %. During this period, the ratio of the invader in the communities reached 92 % of the total abundance at some stations (Fig. 4).

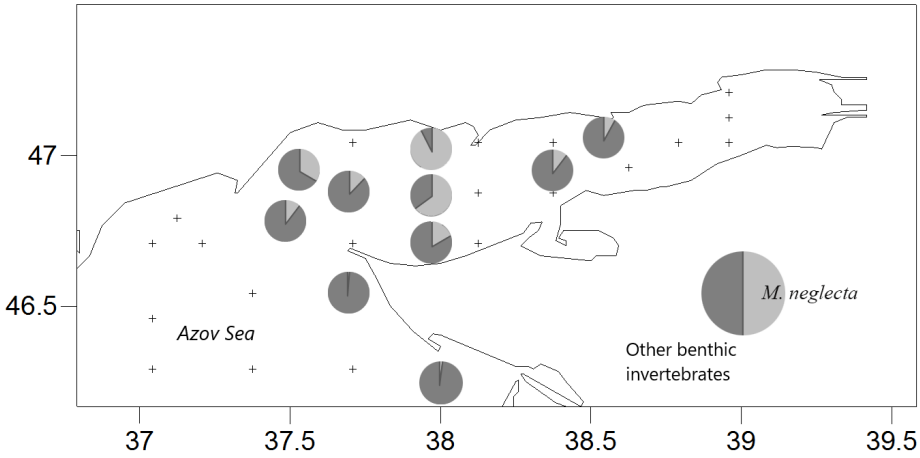

Fig. 4. *Marenzelleria neglecta* ratio in the total abundance of zoobenthos in the Sea of Azov in autumn 2012

In the central and eastern Taganrog Bay, *M. neglecta* was found in mass since 2014. *Prior* to its invasion, oligochaetes, *A. succinea*, and the polychaete *Hediste diversicolor* (O. F. Müller, 1776) prevailed in the bottom communities of this water area. In the eastern Taganrog Bay, the relict polychaetes *Hypaniola kowalewskii* (Grimm in Annenkova, 1927), cumaceans *Pterocuma pectinatum* (Sowinsky, 1893), and insect larvae of the family Chironomidae had high frequency of occurrence. Together, these groups accounted for 88 % of the total abundance of benthic fauna in the central Taganrog Bay and 97 % in its eastern area. In the central area in 2010–2013, the mean abundance of benthos varied from 2,591 to 8,825 ind.·m^{-2}. In 2014, this indicator did not change significantly (3,320 ind.·m^{-2}). *M. neglecta* ratio in the community averaged 31 %. In 2010–2013 in the eastern Taganrog Bay, mean abundance varied from 5,310 to 26,995 ind.·m^{-2}. In 2014, it decreased to 2,393 ind.·m^{-2} due to a drop in the abundance of oligochaetes. *M. neglecta* ratio reached 36 %.

DISCUSSION

M. neglecta expansion into the coastal waters of continental Europe has been studied in detail (Ezhova et al., 2005 ; Maximov, 2011 ; Norkko et al., 1993 ; Zettler et al., 2002 ; Zmudzinski et al., 1993). The polychaete invaded the southern Baltic around the mid-1980s and rapidly distributed in the coastal sea areas; the species was recorded in the Netherlands, Germany, Poland, Russia, Lithuania, Latvia, and Estonia (*Marenzelleria neglecta*, 2021). In the early 1990s, *M. neglecta* reached the coast of Sweden where it was recorded at the Gulf of Finland mouth. In 1990–1993, it invaded the eastern Gulf of Finland and the southern Bothnian Bay. By 2000, the species distributed throughout the entire Gulf of Finland, up to the freshwater Neva Bay.

High rate of worm dispersion is due to its long larval stage. As shown, the pelagic stage of *M. neglecta* at +20 °C lasts for 4–5 weeks; at a lower temperature (+5 °C), the process can last for 2.5–3 months (Bochert, 1997). Juvenile polychaetes are also known to be highly motile and capable of staying in plankton (Dauer et al., 1980, 1982, cited from: Bochert et al., 1996). This provides the species with additional opportunities when colonizing new water areas.

In the Sea of Azov, worm dispersion was very rapid as well. In 3–4 years after the first record of polychaetes at the Taganrog Bay mouth, high-abundant aggregations of *M. neglecta* were registered throughout the northeastern sea, *inter alia* in the bay. According to both literature data and our own observations, *M. neglecta* was also found in other sea areas, up to the Kerch Strait (Boltachova & Lisitskaya, 2019 ; Bulysheva et al., 2020 ; Frolenko & Maltseva, 2017). In 2014, the invader was registered in the Don delta. In 2016 and 2017, worms were regularly recorded at a considerable distance from the mouth – in the upper Mokraya Kalancha channel (Zhivoglyadova & Elfimova, 2021). *M. neglecta* colonies were observed in the Azov estuaries of the Krasnodar Region. In 2015, an aggregation of polychaetes, with the abundance of 160 ind.·m^{-2}, was found in the Akhtarsky estuary.

As shown, with no restrictions on food resource which is typical for eutrophic water areas, polychaetes are capable of increasing their abundance rapidly. This is facilitated by high fecundity of *M. neglecta* (10–40 thousand of eggs *per* ind.) and early maturity which can be reached already during the first year of life (Bochert & Bick, 1995).

In the Sea of Azov, high abundance of the population (5–7 thousand ind.·m^{-2}) was revealed in 2012 and 2013. Comparable values were registered in some areas of the Baltic. Specifically, in the Darss-Zingst lagoons (Germany), a few years after the first record of *M. neglecta* population, there was an outbreak which resulted in a sharp increase of the abundance – from several hundreds to 5,000 ind.·m^{-2} – and a subsequent maximum of 10,000 ind.·m^{-2} (Zettler et al., 2002). An exponential increase in *M. neglecta* abundance and its high values were observed in the Vistula Lagoon as well: in 1988–1994, the abundance of polychaetes reached 5–7 thousand ind.·m^{-2} (Ezhova & Spirido, 2005).

Thus, at the initial stage of invasion, the reasons for the active development of *M. neglecta* population in the Sea of Azov seemed to be high trophic status of the water body and favorable salinity conditions. In 2010–2013 in the western Taganrog Bay, where polychaete colonies with the highest abundance were recoded, salinity averaged 9.7–10.0 ‰; in the northeastern sea, 11.4–12.2 ‰. Apparently, further dispersion of the population was determined by the dynamics of this factor.

The upper optimum limit for the polychaete is 10 ‰ (Sikorski & Bick, 2004). Worms were no longer found in the northeastern sea at mean salinity of 12.9 ‰ (2014); in the western Taganrog Bay, the abundance of colonies decreased by almost an order of magnitude with a rise in salinity to 12.5 ‰ (2015). Then, the population almost completely shifted to freshened areas of the bay – the central and eastern ones, with mean salinity of 10.2 and 7.6 ‰, respectively. Apparently, the eastern Taganrog Bay has unfavorable conditions for the species reproduction. The experiment showed that at salinity < 5 ‰, the survival of larvae is problematic: those cannot complete their development and switch to a benthic lifestyle (Bochert, 1997). According to our data, water salinity in the eastern area in different years varied from 1.4 to 9.1 ‰. Out of the entire observation period, salinity in the eastern Taganrog Bay was higher than the isohaline of 5 ‰ only in 2014 and 2015, and this should have contributed to successful reproduction of the species.

Conclusion. High salinity currently recorded in the Sea of Azov seems to limit the dispersion and to inhibit the large-scale invasion of *Marenzelleria neglecta* there. Despite the facts of worm registration throughout the entire water body, *M. neglecta* forms aggregations with the high abundance only in its desalinated areas. For this species, the optimum of water salinity varies between 7–12 ‰. Since 2017, a stable polychaete population exists within the central and eastern Taganrog Bay. At the same time, significant fluctuations in quantitative indicators are still observed in the colony. Apparently, the main reason for it is a dynamic salinity regime: the species reproduction depends

on the hydrological situation. Evidently, further *M. neglecta* development in the Sea of Azov will be controlled by water salinity. Long-term positive trend of its values allows to assess the situation for the species development as unfavorable.

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ИСТОРИЯ ОСВОЕНИЯ СЕВЕРОАМЕРИКАНСКОЙ ПОЛИХЕТОЙ *MARENZELLERIA NEGLECTA* SIKORSKI & BICK, 2004 (ANNELIDA: SPIONIDAE) СЕВЕРО-ВОСТОЧНОЙ ЧАСТИ АЗОВСКОГО МОРЯ

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В начале 2010-х гг. чужеродная полихета *Marenzelleria neglecta* Sikorski & Bick, 2004 вторглась в бассейн Азовского моря. За несколько лет вид широко расселился по опреснённой акватории моря, а также был отмечен в дельте Дона и в азово-кубанских лиманах.

История формирования чужеродным видом устойчивого и многочисленного поселения, локализованного в северо-восточной части моря, прослежена по материалам комплексных гидробиологических и гидрологических съёмок 2010–2020 гг. Развитие популяции вселенца в водоёме-реципиенте происходило на фоне увеличения его солёности. Очевидно, этот фактор оказал решающее влияние на инвазионный процесс. За вспышкой численности, наблюдавшейся в западной части Таганрогского залива в 2012 и 2013 гг., последовало резкое уменьшение показателей обилия, вплоть до полного отсутствия полихет в пробах. Снижение численности червей сопровождалось сокращением ареала и смещением ядра плотности в наиболее распреснённые районы моря. В настоящее время постоянное поселение *M. neglecta* существует в границах центрального и восточного районов Таганрогского залива. Проанализировано изменение структуры доминирования в донных сообществах в ходе инвазии. Показано, что доля чужеродных полихет в периоды их массового развития на отдельных станциях достигала 92 % общей численности бентоса.

Ключевые слова: Polychaeta, чужеродные виды, донные сообщества, макрозообентос, эстуарии, Азовское море

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DYNAMICS OF FREE AMINO ACIDS IN THE BROWN ALGA *FUCUS VESICULOSUS* LINNAEUS, 1753 FROM THE BARENTS SEA THROUGHOUT THE YEAR

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Free amino acids (FAA) are a significant biochemical component of any cell. Their composition and content depend on physiological state, abiotic environmental factors, and a developmental phase of the organism. Their functions in plants are very diverse; those include participation in both the synthesis of proteins and other compounds and the adaptation to adverse environmental conditions. Information on the FAA dynamics is of key importance for understanding their role in formation of algae resistance to varying environmental factors. The aim of this study is to determine the FAA content in the brown alga *Fucus vesiculosus* and its seasonal changes, as well as to reveal the dependence on environmental factors and the alga developmental phase. The alga for research was sampled in the Kola Bay littoral (the Barents Sea) during low tide once a month from December 2015 to December 2016. The middle part of the thallus was used for the study. The FAA qualitative and quantitative composition was determined by high-performance liquid chromatography. The FAA qualitative composition did not change throughout the year; in the FAA pool, glutamic and aspartic acids, alanine, and proline prevailed. The FAA content varied throughout the year; the maximum amount was recorded in spring–summer. The FAA content depended on external environmental factors. The correlations were determined between the content of individual FAA and air temperature, water temperature, and salinity. The FAA dynamics in different developmental phases of *F. vesiculosus* was associated with processes occurring in the alga; it is affected by growth rate, cell metabolic activity, photosynthesis rate, and generative development. Each phase was characterized by its own dynamics of the FAA content. Based on the dynamics of the FAA concentration in *F. vesiculosus*, correspondences were found with the developmental phases – dormancy, growth activation, growth, and storage. Free glutamate and aspartate may act as one of the reserve sources of organic nitrogen in this alga. Apparently, the transport of organic forms of nitrogen in *F. vesiculosus* thallus is carried out by glutamate, aspartate, alanine, and proline.

Keywords: *Fucus vesiculosus*, free amino acids, seasonal changes, developmental phases, temperature, salinity, Barents Sea

Amino acids are biochemical compounds necessary for the life of any organism. In the organism, those are in two states – bound and free. Free amino acids (hereinafter FAA) are involved in the construction of protein and peptide molecules, as well as in the synthesis of nitrogenous and nitrogen-free compounds – nucleotides, phytohormones, vitamins, alkaloids, betaines, pigments, polyphenols, *etc.* (Hildebrandt et al., 2015 ; Parthasarathy et al., 2018 ; Rhodes & Hanson, 1993 ; Zrenner et al., 2006). Moreover, FAA act as signaling molecules (Lam et al., 1998 ; Oliveira & Coruzzi, 1999), are involved in plant adaptation to varying environmental conditions (Galili & Höfgen, 2002 ; Stewart & Larher, 1980), and serve as antioxidants, osmoregulators, and cryoprotectants (Harris & Logan, 2018 ; Jackson & Seppelt, 1995 ;

Stewart & Larher, 1980 ; Trovato et al., 2008). In algae, as well as in higher plants, FAA participate in the transport of organic nitrogen along the thallus; those can act as reserve sources of nitrogen and be accumulated for further use in growth and development during a period of its low content in the environment (Diouris, 1989 ; Naldi & Wheeler, 1999 ; Schmitz & Srivastava, 1979). FAA are involved in many metabolic processes in plants and indicate the physiological state of an organism.

Fucus vesiculosus Linnaeus, 1753 is one of the most common algal species in the Barents Sea. Recently, much attention has been paid to the study of its physiological peculiarities as a model object for investigating mechanisms of algal adaptation to high latitude conditions (Makarov et al., 2010 ; Ryzhik, 2016 ; Ryzhik et al., 2021 ; Tropin et al., 2003). However, data on the FAA content are scarce and mainly obtained by single or duplicate algal sampling aimed at determining the possibility of their use as raw material for various process industries (Repina, 2005 ; Klindukh & Obluchinskaya, 2018 ; Maehre et al., 2014 ; Mouritsen et al., 2019 ; Peinado et al., 2014). At the same time, changes in the FAA composition and content depending on external environmental factors and the plant developmental phase are almost not described. Moreover, there is a lack of material on FAA significance and role for algae themselves. These data are of key importance for understanding the FAA participation in formation of adaptation and in maintaining organism stability in varying environmental conditions.

The aim of the work is to determine the FAA content in the brown alga *F. vesiculosus* and to reveal its seasonal changes and dependence on environmental factors and the alga developmental phase.

MATERIAL AND METHODS

To study the seasonal dynamics of the FAA composition and content in the brown alga *F. vesiculosus*, the material was sampled monthly from December 2015 to December 2016 in the Kola Bay littoral (the Barents Sea) during low tide (the Abram-Mys area; 68°58′N, 33°01′E). Alga samples were fertile, with 7–10 dichotomous branches. Simultaneously, water and air temperatures were measured with a mercury thermometer (TL-4, Russia), and water salinity was measured with a salinity refractometer (RHS-10ATC, China). For the study, the middle part of the thallus was used (the 4^{th} and 5^{th} dichotomous branches) – as the most mature and active part. In the alga (5–6 thalli), a part of the thallus was separated, cut, and fixed with 96 % ethanol. Fixed samples were stored in a dark and cool place in sealed test tubes.

FAA were extracted from the samples with 70 % ethanol heated up to +60…+70 °C: the alcohol extract was poured off, and the alga was grinded in a mortar with glass sand and then poured with 7 mL of hot 70 % ethanol. The alga was infused with constant stirring for 1 h, and the mixture was centrifuged for 5 min at 3,000 rpm to separate the precipitate. The extract was poured into an evaporating cup, and the precipitate was refilled with hot ethanol. The extraction process was repeated 3 times. The resulting extracts were combined and evaporated to dryness on a water bath. The precipitate was dissolved in 10 mL of distilled water and centrifuged for 10 min at 5,000 rpm; the purification was carried out by ion-exchange chromatography on a KU-2-8 cation exchanger (Metody, 1975). The dry precipitate obtained after purification was dissolved in a small amount of distilled water and used to determine FAA.

The FAA composition and content were analyzed according to the standard method on a Shimadzu LC-20AD Prominence liquid chromatograph (Japan) with a Shimadzu SPD-M20A Prominence photodiode array detector and 250 × 4.6 mm Supelco C18 chromatographic column, 5 μm (the USA) (Rudenko et al., 2010). The measurement was carried out in two parallel samples in duplicate (n = 4). Data on the FAA content are presented as “mean value ± standard deviation.”

The dry matter content in the samples was determined in duplicate according to the standard method: an algal sample with a raw weight of about 1 g was dried to a constant weight for a day at a temperature of +100…+105 °C (GOST 26185-84, 2004).

Using the one-way analysis of variance (ANOVA), the effects of the season, air temperature, water temperature, and water salinity on the FAA content in the alga were determined. Applying the Pearson correlation coefficient, the relationship between the FAA content and salinity, air temperature, and water temperature was identified. To establish significant differences in the FAA content in the alga in different seasons of the year, the Tukey–Kramer multiple comparison test was used. The data were statistically processed at a significance level of $p \leq 0.05$. Data processing and calculations were carried out in Microsoft Excel 2010, NCSS 2004, and PAST v3.22.

RESULTS

At the *F. vesiculosus* sampling site, data on variability of environmental factors were obtained (Table 1). From spring to early autumn, water salinity did not exceed 20 ‰; the lowest values were recorded in May and June 2016. In winter months and in October, it varied within 20–30 ‰. The obtained data on the dynamics of coastal water salinity are typical for the southern bend of the Kola Bay (Kola Bay, 2009). The highest water and air temperatures were recorded in June–August in the Abram-Mys area. Minimum values of water temperature were registered in January–March, and of air temperature, in November–April.

Table 1. Values of some environmental factors during alga sampling

Month and year	Water salinity, ‰	Water temperature, °C	Air temperature, °C
December 2015	19.5	+3.1	–1.2
January 2016	30	–1.5	–30
February 2016	25	–0.6	–4.4
March 2016	18.5	–0.1	–3.8
April 2016	17	+1.3	–1.6
May 2016	7	+7.6	+15.3
June 2016	7.5	+14.2	+18.9
July 2016	12.5	+12.4	+13.8
August 2016	15	+11.1	+11.7
September 2016	17	+9.5	+10.7
October 2016	25	+4.9	+3.2
November 2016	17	+2.4	–1.6
December 2016	20	+2.3	+0.5

Throughout the year, 20 FAA were identified in *F. vesiculosus* thallus (Fig. 1, Table 2). The main part of the FAA pool was represented by aspartic acid (aspartate), glutamic acid (glutamate), alanine, and proline. The prevailing amino acid did not change throughout the year. Glutamic acid remained prevailing one in the FAA composition – 33.9–70.6 % of the total amount of FAA and, accordingly, determined the nature of the change in the total amount of FAA throughout the year. It was followed by aspartic acid, alanine, and proline. In general, the concentrations of other FAA throughout the year did not exceed 2 %. The content of methionine and hydroxyproline in the FAA pool of *F. vesiculosus* was the lowest – less than 0.009 mg·g^{-1} dry weight.

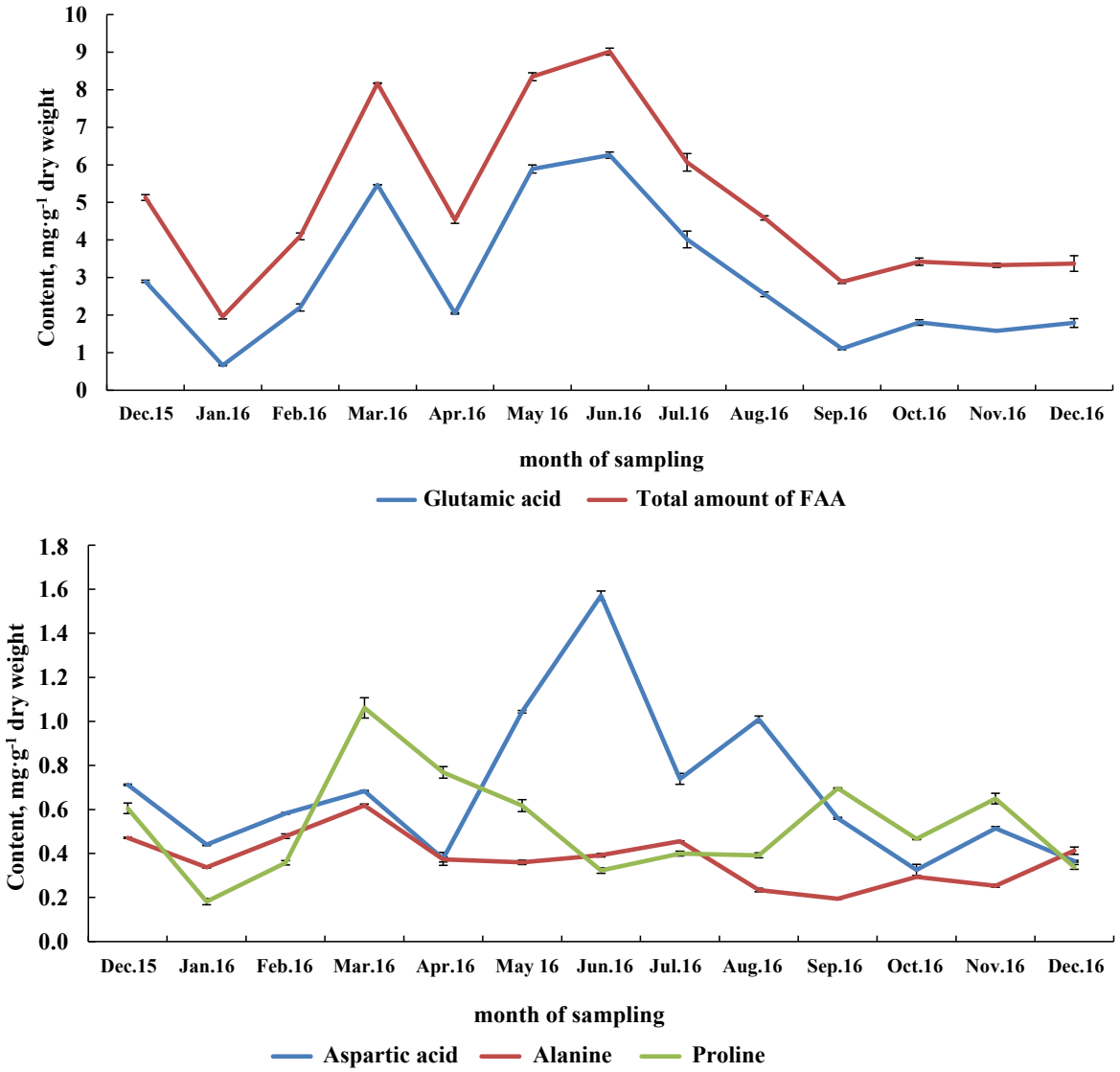

Fig. 1. Dynamics of concentration of dominant free amino acids and their sum in *Fucus vesiculosus* (mean value ± standard deviation; $n = 4$) throughout the year

The mean concentration of 12 FAA in *F. vesiculosus* varied significantly depending on the season (spring, summer, winter, and autumn). At the same time, the concentrations of valine, glycine, leucine, isoleucine, serine, tyrosine, and phenylalanine did not differ significantly between seasons, despite considerable differences in their content in each month of the year (Tables 2 and 3). In spring and/or summer, the concentrations of aspartate, hydroxyproline, histidine, glutamate, methionine, and cystine + cysteine were higher than in autumn and winter. In other FAA, the highest mean concentrations *per* season were recorded not only in spring and summer, but also in autumn or winter.

Analyzing the annual dynamics of the FAA concentration in *F. vesiculosus*, one can distinguish winter–spring, spring–summer, summer–autumn, and autumn–winter periods. In the winter–spring period (from January to March), there was a gradual rise in the FAA concentration in the alga thalli (Fig. 1). The content of all prevailing amino acids increased: glutamate, by 8.2 times; aspartate, by 1.6 times; alanine, by 1.8 times; and proline, by 5.9 times. Alanine and proline concentrations had the maximum values for the entire observation period.

Table 2. Concentration of free amino acids in *Fucus vesiculosus* (mean value ± standard deviation; $n = 4$), mg·g^{-1} dry weight

Amino acid	2015	2016											
	December	January	February	March	April	May	June	July	August	September	October	November	December
Arginine	0.027 ± 0.001	0.016 ± 0.001	0.023 ± 0.0005	0.037 ± 0.001	0.013 ± 0.001	0.040 ± 0.002	0.049 ± 0.002	0.027 ± 0.0003	0.043 ± 0.004	0.066 ± 0.001	0.030 ± 0.001	0.041 ± 0.002	0.034 ± 0.002
Valine	0.042 ± 0.0005	0.020 ± 0.0001	0.024 ± 0.0003	0.031 ± 0.001	0.034 ± 0.003	0.034 ± 0.002	0.041 ± 0.0004	0.030 ± 0.002	0.026 ± 0.001	0.022 ± 0.001	0.032 ± 0.002	0.024 ± 0.001	0.025 ± 0.002
Hydroxyproline	0.002 ± 0.0002	0.002 ± 0.0001	0.003 ± 0.0002	0.005 ± 0.001	0.007 ± 0.0001	0.009 ± 0.0004	0.007 ± 0.00004	0.002 ± 0.0001	0.005 ± 0.0003	0.003 ± 0.00004	0.003 ± 0.00004	0.005 ± 0.00005	0.004 ± 0.0001
Histidine	0.028 ± 0.002	0.033 ± 0.0004	0.052 ± 0.002	0.062 ± 0.001	0.020 ± 0.001	0.064 ± 0.002	0.077 ± 0.001	0.041 ± 0.002	0.058 ± 0.002	0.045 ± 0.002	0.011 ± 0.001	0.052 ± 0.002	0.048 ± 0.006
Glycine	0.048 ± 0.001	0.017 ± 0.001	0.023 ± 0.001	0.020 ± 0.001	0.041 ± 0.003	0.027 ± 0.002	0.033 ± 0.002	0.033 ± 0.001	0.024 ± 0.002	0.021 ± 0.0002	0.031 ± 0.003	0.021 ± 0.001	0.025 ± 0.001
Isoleucine	0.021 ± 0.001	0.009 ± 0.0001	0.008 ± 0.0001	0.008 ± 0.0003	0.013 ± 0.0003	0.010 ± 0.0005	0.012 ± 0.0004	0.012 ± 0.001	0.013 ± 0.0001	0.009 ± 0.0002	0.014 ± 0.001	0.009 ± 0.0005	0.011 ± 0.0001
Leucine	0.019 ± 0.001	0.008 ± 0.0002	0.009 ± 0.0001	0.006 ± 0.0003	0.022 ± 0.001	0.008 ± 0.0001	0.009 ± 0.0003	0.014 ± 0.0001	0.014 ± 0.002	0.006 ± 0.0003	0.020 ± 0.001	0.008 ± 0.0005	0.015 ± 0.0001
Lysine	0.023 ± 0.0003	0.015 ± 0.0001	0.011 ± 0.0003	0.006 ± 0.0002	0.007 ± 0.001	0.012 ± 0.001	0.014 ± 0.0003	0.010 ± 0.001	0.020 ± 0.001	0.005 ± 0.00001	0.019 ± 0.0001	0.008 ± 0.001	0.011 ± 0.0001
Methionine	0.002 ± 0.0002	0.002 ± 0.0002	0.003 ± 0.0005	0.001 ± 0.0002	0.004 ± 0.00004	0.003 ± 0.001	0.005 ± 0.0002	0.003 ± 0.0001	0.004 ± 0.00003	0.002 ± 0.000002	0.003 ± 0.0002	0.002 ± 0.00003	0.002 ± 0.0001
Serine	0.081 ± 0.0002	0.052 ± 0.001	0.052 ± 0.003	0.054 ± 0.002	0.056 ± 0.003	0.056 ± 0.003	0.066 ± 0.002	0.058 ± 0.0005	0.048 ± 0.001	0.048 ± 0.002	0.056 ± 0.002	0.059 ± 0.001	0.057 ± 0.002
Threonine	0.045 ± 0.001	0.022 ± 0.001	0.022 ± 0.0002	0.021 ± 0.002	0.049 ± 0.001	0.025 ± 0.002	0.039 ± 0.0003	0.043 ± 0.002	0.031 ± 0.001	0.033 ± 0.0002	0.044 ± 0.002	0.036 ± 0.001	0.026 ± 0.001
Tyrosine	0.024 ± 0.001	0.015 ± 0.0001	0.017 ± 0.0001	0.005 ± 0.0001	0.064 ± 0.003	0.008 ± 0.0003	0.009 ± 0.001	0.022 ± 0.001	0.017 ± 0.001	0.007 ± 0.0001	0.035 ± 0.0005	0.006 ± 0.0002	0.018 ± 0.003
Tryptophan	0.017 ± 0.001	0.014 ± 0.001	0.014 ± 0.001	0.013 ± 0.001	0.004 ± 0.0003	0.008 ± 0.0003	0.013 ± 0.0001	0.009 ± 0.0001	0.017 ± 0.0001	0.013 ± 0.0001	0.014 ± 0.0001	0.012 ± 0.0001	0.012 ± 0.0003
Phenylalanine	0.039 ± 0.001	0.081 ± 0.002	0.153 ± 0.003	0.024 ± 0.001	0.370 ± 0.032	0.040 ± 0.0005	0.020 ± 0.001	0.101 ± 0.009	0.036 ± 0.003	0.016 ± 0.001	0.149 ± 0.005	0.015 ± 0.001	0.124 ± 0.019
Cystine + cysteine	0.025 ± 0.002	0.031 ± 0.002	0.060 ± 0.004	0.043 ± 0.004	0.265 ± 0.015	0.090 ± 0.004	0.072 ± 0.001	0.051 ± 0.005	0.040 ± 0.002	0.032 ± 0.001	0.070 ± 0.005	0.030 ± 0.004	0.057 ± 0.010

Table 3. Mean concentration of free amino acids in different seasons and results of ANOVA of the influence of the sampling season on the FAA content ($n = 44$)

Amino acid	$F_{(3, 40)}$	p	Mean concentration *per* season, mg·g^{-1} dry weight			
			Winter	Spring	Summer	Autumn
Alanine	10.92	≤ 0.0001	0.425*	0.450*	0.361*	0.247
Arginine	7.69	0.0004	0.025	0.030	0.040*	0.046*
Aspartic acid	11.40	≤ 0.0001	0.525	0.701	1.106*	0.467
Valine	2.26	0.096	0.028	0.033	0.032	0.026
Histidine	3.40	0.027	0.040	0.049*	0.059*	0.036
Glycine	0.57	0.639	0.028	0.029	0.030	0.024
Glutamic acid	16.71	≤ 0.0001	1.889	4.468*	4.278*	1.499
Isoleucine	1.24	0.309	0.012	0.010	0.012	0.011
Leucine	0.20	0.898	0.013	0.012	0.012	0.011
Lysine	4.82	0.006	0.015*	0.008	0.015*	0.011
Methionine	9.30	≤ 0.0001	0.002	0.003	0.004*	0.002
Proline	23.61	≤ 0.0001	0.371	0.816*	0.371	0.604*
Serine	0.73	0.538	0.060	0.055	0.057	0.054
Threonine	2.95	0.040	0.029	0.032	0.038*	0.038*
Tyrosine	0.39	0.764	0.019	0.026	0.016	0.016
Tryptophan	6.49	0.001	0.014*	0.008	0.013*	0.013*
Phenylalanine	1.29	0.291	0.099	0.144	0.053	0.060
Cystine + cysteine	5.93	0.002	0.043	0.133*	0.054	0.044
Hydroxyproline	15.60	≤ 0.0001	0.003	0.007*	0.005	0.004
Total amount of FAA	19.03	≤ 0.0001	3.640	7.015*	6.556*	3.212

Note: * denotes the highest values. Differences in the mean FAA concentration for the season were determined according to the Tukey–Kramer multiple comparisons test ($n = 44$; $DF = 40$; $\alpha = 0.05$).

In the spring–summer period (from April to June), significant changes in the FAA concentration in *F. vesiculosus* were observed. In April, the content of prevailing amino acids (glutamic acid, alanine, aspartate, and proline) decreased, as well as the content of arginine, histidine, and tryptophan. The concentration of phenylalanine, threonine, cystine + cysteine, glycine, tyrosine, leucine, isoleucine, and methionine increased (Fig. 1, Table 2). In May and June, FAA were accumulated. During this period, the concentrations of glutamate and aspartate in the studied *F. vesiculosus* samples increased and reached the highest values – (6.261 ± 0.083) and (1.571 ± 0.021) mg·g^{-1} dry weight, respectively. The content of alanine and proline decreased; by June, their levels were equal to winter ones.

The summer–autumn period (from July to September) was characterized by a drop in the content of the total amount of FAA and most of individual FAA. Specifically, compared with the values in early summer, the concentrations of glutamate, aspartate, and alanine decreased by 2–5.6 times, while the content of proline, on the contrary, increased by 2.2 times.

In the autumn–winter period (from October to December), the FAA concentration in *F. vesiculosus* was low. In October, the total amount of FAA increased compared to that in September; it remained stable until December – within a range from (3.327 ± 0.056) to (3.421 ± 0.098) mg·g^{-1} dry weight. In January, the total amount of FAA in alga thalli decreased and reached its lowest values – (1.959 ± 0.061) mg·g^{-1} dry weight. The content of glutamate varied in a similar way during this period reaching the minimum values for the year – (0.664 ± 0.013) mg·g^{-1} dry weight (Fig. 1). By December, the concentration of alanine increased by 2.1 times compared to the value in September; in January, it decreased by 1.2 times. In December–January, there was a gradual decrease in the content of both aspartic acid and proline.

DISCUSSION

In *F. vesiculosus*, three developmental phases are distinguished throughout the year – the dormancy phase (autumn–winter), the growth phase (spring–early summer), and the storage phase (summer) (Kuznetsov & Schoschina, 2003 ; Ryzhik, 2016). Analyzing the nature of the FAA content changes in the middle part of *F. vesiculosus* thallus obtained in this work, it is possible to distinguish the fourth developmental phase by dividing the growth phase into two – the growth activation phase (winter–spring) and the actual growth phase (spring–summer). Other phases coincide in time with those distinguished earlier. *F. vesiculosus* developmental phases differ in the cell metabolic activity, intensity of photosynthesis, growth rate, and generative development. These alterations are aimed at performing certain processes in different seasons: in summer and autumn, at reproduction and preparation for winter; in spring, at the thallus growth; and in winter, at a rest and adaptation to adverse environmental conditions.

The **dormancy phase** of *F. vesiculosus* covers the autumn–winter period. At this time, the alga is characterized by a minimum growth rate (September–February), as well as by formation, laying, and slow development of reproductive structures (October–February) (Kuznetsov & Schoschina, 2003 ; Makarov et al., 1995). The cell metabolic activity gradually decreases in autumn and is minimal in winter. In late January and early February, physiological processes become more active, and the intensity of photosynthesis increases (Kuznetsov & Schoschina, 2003 ; Ryzhik, 2007, 2016). For the FAA content in September–January, low concentrations of glutamic acid, aspartate, and alanine are characteristic. This is due to low intensity of metabolism and photosynthesis and a lack of need for FAA as structural elements. By changes in FAA, this period can be characterized as the dormancy phase as well.

Since January, the concentrations of glutamate, alanine, aspartate, and proline increase. For this time period, a rise in the intensity of the cell metabolic activity and photosynthesis in *F. vesiculosus* was revealed (Kuznetsov & Schoschina, 2003 ; Ryzhik, 2016). Apparently, a rise in the FAA content is related to the preparation for the intensive alga growth since FAA are involved in building proteins. Probably, the FAA stock at the early growth period will contribute to a more intensive growth of the alga thallus area under low environmental temperatures. The nature of the FAA change in January–March coincides with the alga preparation for the growth period and transition from the dormancy phase to the growth one. This period can be considered as the **growth activation phase**.

The **growth phase** of *F. vesiculosus* lasts from March to June and is characterized by a maximum growth rate of the alga thalli, intensive development of receptacles, and maximum values of the intensity of photosynthesis (April–May) (Kuznetsov & Schoschina, 2003 ; Makarov et al., 1995). During this period, there is a significant rise in the cell metabolic activity indicating active growth processes (Ryzhik, 2016). Plant growth is associated with a considerable need for nitrogen – a part of the amino acids and, subsequently, of proteins required for building new cells. In *F. vesiculosus*, the meristematic tissue is located in the apical areas of the thallus. As shown for *Fucus* species, photoassimilates, *inter alia* FAA, are transported to apical areas of the thallus from its middle part (Diouris, 1989 ; Diouris & Floc'h, 1984). In brown algae, the rate of photoassimilate outflow into the growth area depends on a growth rate of the thallus (Lüning et al., 1973). As revealed, the beginning of intensive growth and, as a result, the FAA outflow from the middle part of the thallus into the apical areas depends on environmental temperature and can shift closer to summer in algae growing under lower temperatures (Klindukh & Obluchinskaya, 2018). Apparently, a sharp decrease in the FAA content in April results from the onset of the intensive growth and an outflow of accumulated FAA from the middle part of the thallus

to the apical areas. The main participants of redistribution and transport of nitrogen along *F. vesiculosus* thallus seem to be glutamic and aspartic acids, as well as proline and alanine. In brown algae, glutamate, aspartate, alanine, serine, and glycine are known to act as a transport form of nitrogen along the thallus (Diouris, 1989 ; Schmitz et al., 1972 ; Schmitz & Srivastava, 1979). In May and June, the content of glutamate and aspartate increased significantly after a decrease in April, despite the continued growth of the alga thalli. This may indicate a gradual decrease in a growth rate and a drop in a need for amino acids. Moreover, this can be considered as an adaptation to the summer nitrogen deficiency in the environment.

The algae of the southern bend of the Kola Bay do not experience a lack of nitrogen throughout the year, but the competition for nitrogen increases greatly in late spring and in summer, and this results in a decrease in its concentration in water (Kola Bay, 1997). In the thallus of the Barents Sea *F. vesiculosus*, nitrogen is mostly represented by organic compounds of a protein nature (Barashkov et al., 1966). As shown, algae are able to accumulate FAA at high concentrations of inorganic forms of nitrogen in water and to use them as reserve forms of nitrogen during its low concentrations (Angell et al., 2014 ; Naldi & Wheeler, 1999 ; Park et al., 2013). This provides high growth rates and a possibility of forming a larger number of reproductive cells (spores and gametes).

The **storage phase** in *F. vesiculosus* begins in July and lasts until September. During this period, the alga completes its vegetative development; release of reproductive cells occurs, as well as restructuring of metabolism and growth processes towards preparation for winter (Kuznetsov & Schoschina, 2003 ; Ryzhik, 2016). In August, the second peak in dynamics of the cell metabolic activity is recorded (Ryzhik, 2016), and the content of dry matter, alginates, and fucoidan increases (Obluchinskaya et al., 2002). In the storage phase, the FAA content in *F. vesiculosus* decreases. Out of prevailing amino acids, a drop was revealed in glutamate, aspartate, and alanine. The concentration of proline, on the contrary, slightly increased. During this period, FAA can be spent on the processes of growth and maturation of reproductive cells. Apparently, in the considered developmental phase, the FAA synthesis and protein formation in *F. vesiculosus* slow down; this may be due to a need for accumulating reserve substances and the orientation of metabolism towards the synthesis of carbohydrates rather than nitrogen-containing substances. The decrease in the FAA content is also associated with the use of internal reserves of amino acids during this period since the content of inorganic forms of nitrogen in the environment decreases (Kola Bay, 1997). A slight rise in proline in the storage phase can be caused by temperature fluctuations and periodic desalination due to precipitation during low tides. Proline is known to be involved in osmoregulation processes; it contributes to plant resistance to low temperatures (Munns, 2005 ; Naidu et al., 1991 ; Trovato et al., 2008). An experiment with the green alga *Ulva pertusa* Kjellman, 1897 showed that significant temperature fluctuations stimulate an increase in the content of free proline and slow down the plant growth (Wang Q. et al., 2007).

The seasonal dynamics of the FAA content in *F. vesiculosus* is similar to that in the White Sea *Fucus* sp., the Far Eastern *Laminaria japonica* Areschoug, 1851, the red alga *Gracilaria vermiculophylla* (Ohmi) Papenfuss, 1967 from the coast of France, the Antarctic brown alga *Ascoseira mirabilis* Skottsberg, 1907, and the green alga *Prasiola crispa* (Lightfoot) Kützing, 1843 (Krupnova, 2002 ; Repina, 2005 ; Gomez & Wiencke, 1998 ; Jackson & Seppelt, 1995 ; Surget et al., 2017). The listed algae are also characterized by prevalence of glutamate, aspartate, alanine, proline, threonine, glycine, and taurine in the FAA pool. In the Scottish *Palmaria palmata* (Linnaeus) F. Weber & D. Mohr, 1805, algae from the southern coast of the Mediterranean Sea, and the Sea of Japan brown

alga *Sargassum fusiforme* (Harvey) Setchell, 1931, the reverse trend of the FAA accumulation was observed – high content in the autumn–winter period and low content during spring–summer (Khaleafa et al., 1982 ; Mohsen et al., 1975 ; Morgan et al., 1980 ; Nagahisa et al., 1994). Seasonal differences in the accumulation and decrease of FAA in algae depend on both external environmental factors and a direction of metabolic processes in a certain developmental phase. The FAA accumulation in autumn–winter may be related to the occurrence of vegetative or generative cycle of algae development during this period, as well as to participation of amino acids in the protection of cellular structures under sub-zero temperatures (Jackson & Seppelt, 1995 ; Morgan et al., 1980 ; Nagahisa et al., 1994).

The onset of the developmental phases is largely determined by external factors. Low water and air temperatures, as well as cloudiness reducing irradiance, delay the beginning of the vegetation period in spring. Importantly, a delay in the beginning of intensive vegetation due to low water temperatures results in a shift in the release of reproductive cells in summer (Kuznetsov & Schoschina, 2003).

Changes in the FAA content in *F. vesiculosus* were affected by both the general direction of metabolic processes in a certain developmental phase and environmental conditions. The one-way analysis of variance revealed the effect of seawater salinity, air temperature, and water temperature on the FAA content in *F. vesiculosus* thallus. The correlation coefficients have a positive linear relationship between water and air temperatures and the content of arginine, aspartate, glutamate, and methionine. Those have a negative linear relationship between the salinity of seawater and the content of arginine, aspartate, histidine, glutamate, and hydroxyproline. The total amount of FAA is also linearly dependent on air temperature and water salinity (Table 4).

Table 4. Values of the Pearson correlation coefficient between the concentration of free amino acids and external environmental conditions ($n = 44$; $p \leq 0.05$)

Amino acid	Water temperature, °C	Air temperature, °C	Water salinity, ‰
Arginine	**0.60**	**0.62**	**–0.52**
Aspartic acid	**0.66**	**0.58**	**–0.71**
Histidine	0.33	0.39	**–0.55**
Glutamic acid	0.41	**0.56**	**–0.73**
Methionine	**0.56**	0.49	–0.41
Hydroxyproline	0.26	0.46	**–0.71**
Total amount of FAA	0.40	**0.57**	**–0.74**

Note: values in bold indicate medium to high correlation between variables.

For most algae of the Barents Sea, the optimal growth temperature is +10…+15 °C (Voskoboinikov et al., 2015). In general, the content of most FAA and their total amount in *F. vesiculosus* is higher at temperatures optimal for growth (Tables 2 and 4, Fig. 1). The exception is March, with high FAA content at low temperatures. This is probably due to the FAA accumulation in the middle part of the thallus *prior* to the beginning of growth and their use as structural substances during the intensive growth. An increase in the FAA content within a certain temperature range indicates that the intensity of the synthesis of these compounds is higher than the rates of their catabolism. These ranges vary for different algal species. Specifically, in the green alga *Ulva fasciata* Delile, 1813, the highest FAA content was recorded at the optimal growth temperature of +25 °C (Mohsen et al., 1973). In *U. pertusa*, a rise in water temperature, optimal for the growth, by 10 °C resulted in a 2.2-fold increase in the FAA content (Kakinuma et al., 2006). In the Antarctic green alga *P. crispa* and the northern Atlantic red alga

Mastocarpus stellatus (Stackhouse) Guiry, 1984, the content of free proline increases during cold periods (Harris & Logan, 2018 ; Jackson & Seppelt, 1995). In these species, the amount of free proline significantly rises as the environmental temperature drops below the freezing point of the cytoplasm in cells: for *P. crispa* and *M. stellatus*, free proline acts as a cryoprotectant and increases resistance to freezing. In *F. vesiculosus*, with a significant drop in environmental temperature in January 2016, the content of FAA, *inter alia* free proline, decreased (Table 1, Fig. 1). Thus, FAA are not directly involved in the protection of the alga from sub-zero temperatures, but those may be precursors in the synthesis of cryoprotectants during cold season.

The highest concentrations of proline and alanine were recorded in March. This is probably due to the fact that these amino acids not only are accumulated as structural components for building proteins but are involved in protective reactions of algae in response to adverse external factors. In autumn and spring, an increase in the concentration of proline and alanine may be related to fluctuations in environmental temperature and irradiance. Low temperatures combined with high irradiance can contribute to an increase in the content of reactive oxygen species. Proline and alanine are capable of acting as antioxidants. As known, free proline in plant cells is involved in inactivation of reactive oxygen species which are formed under various stress effects (Kaul et al., 2008 ; Matysik et al., 2002 ; Saradhi et al., 1995).

Negative correlation between the concentrations of glutamate, aspartate, arginine, histidine, and hydroxyproline and water salinity indicates that this factor has a positive effect on the accumulation of these amino acids under low salinity. Lack of a linear relationship between the concentrations of other FAA and water salinity evidences that it is not a decisive factor for their content. Its effect is manifested only within a complex of environmental factors. According to previous studies on *F. vesiculosus* from natural populations, the content of free proline and other FAA depends on water salinity in the species habitats. The concentration of free proline increased in the algae thalli from low salinity areas (Klindukh et al., 2011). A spring decrease in water salinity had different effects on the FAA content in different parts of the thallus (Klindukh & Obluchinskaya, 2018).

Changes in the FAA content in algae in response to a decrease in water salinity largely depend on the species peculiarities and on the duration of the effect. Specifically, in *U. pertusa* and *Pyropia haitanensis* (T. J. Chang & B. F. Zheng) N. Kikuchi & M. Miyata, 2011, exposure to low-salinity seawater resulted in a decrease in the content of free proline; in *Gracilaria corticata* (J. Agardh) J. Agardh, 1852, on the contrary, such an exposure caused a 2-fold rise (Kakinuma et al., 2006 ; Kumar et al., 2010 ; Wang W. et al., 2020). The content of other free amino acids in algae changes during low salinity as well. The effect of water of 4 ‰ led to a decrease in the content of prevailing FAA in *Ectocarpus siliculosus* (Dillwyn) Lyngbye, 1819; at the same time, the concentration of aromatic amino acids and branched-chain amino acids increased (Dittami et al., 2011). In *Cladophora vagabunda* (Linnaeus) Hoek, 1963, reduction of habitat salinity caused a rise in glutamate and lysine and a drop in aspartate, threonine, valine, arginine, glycine, and histidine (Rani, 2007).

F. vesiculosus is found on the Barents Sea coast both in areas with oceanic salinity and in gulfs and bays with constant low salinity (Malavenda & Voskoboinikov, 2008). Waters with a salinity of 25.5–34 ‰ and 17 ‰ are considered optimal for growth of algae from marine and brackish-water populations, respectively (Voskoboinikov et al., 2015). *F. vesiculosus* inhabiting the littoral in the Abram-Mys area is constantly exposed to low salinity. For this alga, water of 15–20 ‰ is optimal for growth and has no stressful effect on metabolism.

During the period of minimum water salinity recorded, the content of most FAA and their total amount in *F. vesiculosus* thalli was the highest throughout the year. In late spring and early summer, the FAA accumulation occurs due to the storage of nitrogen required for growth and reproduction in the summer season when its content in the environment decreases. Apparently, a severe drop in seawater salinity can contribute to an increase in the FAA content during this period reducing the intensity of protein synthesis and, accordingly, affecting the intensity of alga growth, as well as contributing to a rise in amino acid biosynthesis. Reduced salinity is known to slow down a growth rate of length and mass of *F. vesiculosus* thalli and to cause an increase in the amount of individual total amino acids in the alga (Munda & Garrasi, 1978 ; Nygard & Dring, 2008).

Conclusion. The qualitative composition of free amino acids in *F. vesiculosus* did not change throughout the year. Glutamic and aspartic acids, alanine, and proline prevailed in the FAA pool. Changes in the FAA concentration have a clearly pronounced annual dynamics which coincides with the main developmental phases of the alga. Higher concentrations are typical for most FAA in the spring–summer period, and lower content is typical in autumn–winter. The FAA dynamics in different developmental phases of *F. vesiculosus* was associated with processes occurring in the alga; it is affected by growth rate, cell metabolic activity, photosynthesis rate, and generative development. Each developmental phase is characterized by its own dynamics of the FAA content. Based on the analysis of the FAA concentration, as well as literature data on the dynamics of other physiological parameters (growth rate and cell metabolic activity), the following phases of *F. vesiculosus* development were distinguished: dormancy, growth activation, growth, and storage. An analysis of the effect of environmental factors on the FAA content allowed to assume the participation of FAA in the formation of the alga adaptation to fluctuations in salinity and temperature of the environment. Apparently, in the brown alga, glutamic and aspartic acids, which are accumulated in the middle part of the thallus in May–June, act as one of the reserve organic forms of nitrogen. Probably, the transport of organic forms of nitrogen in *F. vesiculosus* thallus is carried out by glutamate, aspartate, alanine, and proline.

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ДИНАМИКА СОДЕРЖАНИЯ СВОБОДНЫХ АМИНОКИСЛОТ В БУРОЙ ВОДОРОСЛИ *FUCUS VESICULOSUS* LINNAEUS, 1753 БАРЕНЦЕВА МОРЯ В ТЕЧЕНИЕ ГОДА

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Свободные аминокислоты (САК) являются важными биохимическими соединениями любой клетки. Их состав и содержание зависят от физиологического состояния, абиотических факторов среды и фазы развития организма. Функции САК в растениях очень разнообразны и включают участие не только в синтезе белков и других соединений, но и в адаптации водорослей к неблагоприятным условиям среды. Сведения о динамике САК важны для понимания их роли в формировании устойчивости водорослей к меняющимся факторам среды. Цель данного исследования — определить содержание САК в бурой водоросли *Fucus vesiculosus*

и их сезонные изменения и выявить зависимости от факторов среды и фазы развития фукуса. Водоросли для изучения собирали на литорали Кольского залива Баренцева моря в период отлива раз в месяц с декабря 2015 г. по декабрь 2016 г. Для исследования использовали среднюю часть таллома. Качественный и количественный состав САК определяли методом высокоэффективной жидкостной хроматографии. Качественный состав САК в течение года не изменялся; доминирующими в пуле САК были глутаминовая и аспарагиновая кислоты, аланин и пролин. Содержание САК изменялось в течение года; максимальное количество отмечено в весенне-летний период. Содержание САК зависело от внешних факторов среды. Определены корреляционные зависимости между концентрациями отдельных САК и температурой воздуха, температурой и солёностью воды. Динамика САК в разные фазы развития фукуса связана с происходящими в водорослях процессами; на неё влияют скорость роста, клеточная метаболическая активность, скорость фотосинтеза и генеративное развитие. Для каждой из фаз развития характерна своя динамика содержания САК. На основании динамики концентрации САК у фукуса найдены соответствия с фазами развития (покоя, активации роста, роста, накопления запасных веществ). В качестве одного из резервных источников органического азота у фукуса, возможно, выступают свободный глутамат и аспартат. Транспорт органических форм азота в талломе фукуса, вероятно, осуществляется за счёт глутамата, аспартата, аланина и пролина.

Ключевые слова: *Fucus vesiculosus*, свободные аминокислоты, сезонные изменения, фазы развития, температура, солёность, Баренцево море

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TRICHOPLAX SP. H2 CULTIVATION AND REGENERATION FROM BODY FRAGMENTS AND DISSOCIATED CELL AGGREGATES: OUTLOOK FOR GENETIC MODIFICATION

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Trichoplax sp. H2, a simple multicellular animal cultivated in the laboratory, was studied with the aim of its further genetic modification. The idea here is to introduce genetic information into a cell suspension after dissociation of the *Trichoplax* body into single cells, followed by their aggregation and regeneration of the resulting agglomerates into a viable animal. 1. We analyzed the dynamics of the *Trichoplax* growth in Petri dishes on *Tetraselmis marina* algal mats. Specimens were uniform on the exponential growth stage. 2. Trichoplaxes were cut radially in a post-traumatic regeneration research, and the regeneration of the obtained parts was investigated under a microscope. Growth and reproduction rate of animals on nutrient mats were determined that decreased as the animals had been cut. The missing part of the *Trichoplax* body was replaced by remodeling of remaining cells. 3. The animals after a vital staining were dissociated into single cells in a medium with no divalent cations. Pear-shaped or rounded cells were identified, as well as epithelial cells with flagella maintaining motion activity for more than 12 hours. 4. *Trichoplax* plates were disintegrated in the presence of 10 μM amlodipine to quantify a cell population using flow cytometry. As estimated, *Trichoplax* (0.5–1 mm in size) consists of approximately 10,000 cells. 5. Treatment of animals with 10 % BSA (Bovine Serum Albumin) during various exposure intervals suggests a hypothesis on the existence of totipotent cells at the periphery of the *Trichoplax* body, probably in the rim. 6. In the course of reparative regeneration experiments, we achieved *Trichoplax* dissociation into single cells with 0.1 % BSA treatment and the following recreation of the viable organisms by centrifugation of a cell suspension and subsequent dispersion of a large pellet into fragments up to 0.1 mm *prior* to plating multicellular aggregates on nutrient mats. 7. The development of the aggregates was accompanied by active motion of cells and epithelialization of the surface, which resulted in cell growth, formation of a plate, and further vegetative division of *Trichoplax*. As assumed, the artificial stage of a single cell in a line of asexual reproductions allows to introduce foreign genetic information into *Trichoplax*, for example, in order to study the signal processing, organization, and functioning of this multicellular organism. Transgenesis, which is based on the dissociation of an animal body into single cells, could be applied to other organisms with high regenerative potential.

Keywords: *Trichoplax*, Placozoa, post-traumatic and reparative regeneration, cell dissociation and aggregation, cellular engineering, methods of transgenesis

Trichoplax belongs to the Placozoa phylum and is considered one of the simplest multicellular animals (Seravin & Gudkov, 2005 ; Schulze, 1883, 1891). It is a flat invertebrate with an asymmetric body 0.2–2.0 mm in diameter and 25 μm thick; it is found throughout tropical seas (Eitel & Schierwater, 2010 ; Pearse & Voigt, 2007). Trichoplax has no muscle cells and neurons; it consists of three cell layers. The animal adheres to a substrate with the help of microvilli and slides over the surface due to beating of the ventral epithelium cilia and observed rhythmic contractions of the dorsal epithelium under the effect of stellate fibre cells of the middle layer, performing amoeboid movement (Armon et al., 2018 ; Eitel et al., 2013 ; Pearse & Voigt, 2007 ; Schierwater et al., 2009 ; Smith et al., 2019). Two possible feeding types are described for trichoplax – external and internal. With external feeding, the animal crawls onto large prey (*e. g.*, an algal agglomerate) and adheres closely to a substrate forming a kind of food cavity, into which it releases secretions dissolving the food. Then, this food is absorbed by trichoplax during the clathrin-mediated endocytosis (Smith et al., 2015, 2019). Also, trichoplax is capable of picking up small prey (single algae) with cilia and moving it to the dorsal side; there, it is phagocytosed by cells of the middle layer temporarily exposed on the surface (Wenderoth, 1986).

Interestingly, the animal with such a primitive body structure contains genes that are responsible for functions of the immune and nervous systems in highly organized animals (Kamm et al., 2019 ; Varoqueaux et al., 2018). *Trichoplax* sp. H2 has a 94.88-Mb genome with 12,225 genes identified (Kamm et al., 2018). The mitochondrial genome of trichoplax is the largest among all Metazoa; it is a 43-Kb circular DNA (Dellaporta et al., 2006). Six main cell types were described for *Trichoplax adhaerens* H1, using electron microscopy (Table 1). Additional cell types were characterized in *Hoilungia* sp. H4 based on confocal microscopy and differential staining of H4 strain, and the membrane potential of mitochondria was measured (Romanova, 2019).

Table 1. *Trichoplax* prevailing cells types identified by electron and confocal microscopy using specific antibodies (Smith et al., 2014)

Position	Type	Description	Function	Content
Lower layer	Ventral epithelial cells	Small cells with a cilium and microvilli, ventrodorsally elongated; numerous inclusions and vesicles	Active sliding on the surface and adherence to a substrate, absorption, pinocytosis	72 % of all trichoplax cells
	Lipophilic cells	Large cells without cilia; those form contacts with fibre cells and are absent in the edge 20-μm area; one large inclusion with lipid content	Secretion of digestive enzymes	11 % of cells
	Gland cells	Medium-sized, located in the edge area, with a cilium and microvilli; granules of various shapes and colors	Secretion of neurotransmitters	3 % of cells
Middle layer	Fibre cells	Tetraploid cells with outgrowths; various inclusions, intracellular symbiotic bacteria and food odds	Mechanical changes in the body shape, phagocytosis, digestion	4 % of cells
	Crystalline cells	Cells contain crystals ~2 μm in size, contact with fibre cells, do not come to the surface	Possibly, acting as statocysts	< 0.2 % of cells in an animal body
Upper layer	Dorsal epithelial cells	Small T-shaped cells with sensory cilium; intracellular granules	Protective, sensory, contractile	9 % of cells

The most representative cells in the trichoplax body are ventral epithelial cells; those provide adherence to the surface *via* microvilli, sliding with the help of cilia, and absorption of digested food (Table 1). Lipophilic cells are also located in the lower epithelium; those contain a large lipid inclusion. The outer edge of animal body, or the peripheral belt (~20 μm), consists of two types of epithelial cells – dorsal and ventral ones; there are no fibre cells here. There are gland cells on the periphery of the trichoplax plate. Those express such neurospecific proteins, as syntaxin 1, synaptobrevin, and SNAP25. Moreover, neurosecretory cells contain FMRF-amide and other neuropeptides (Mayorova et al., 2019 ; Senatore et al., 2017 ; Smith et al., 2014). Various types of peptidergic cells are recorded not on the periphery alone: those are also located concentrically towards the center in both epithelia (Varoqueaux et al., 2018). Fibre cells are tetraploid, contain symbionts, and are responsible for body shape change, phagocytosis, and digestion (Gruber-Vodicka et al., 2019). Some cells have crystalline inclusions of aragonite. Apparently, they play the role of a vestibular apparatus allowing trichoplax to navigate through environment, turn over, and adhere to a substrate directly on the ventral side (Mayorova et al., 2018 ; Smith et al., 2014). Dorsal epithelial cells are located in the upper layer and have one sensory cilium. Assumedly, there are other, less representative or yet unidentified cell types (Sebé-Pedrós et al., 2018). For example, unusual cells – looking like shiny balls – were described in individuals from natural environment; there are no such cells in cultivated animal lines (Grell & Ruthmann, 1991 ; Syed & Schierwater, 2002). Trichoplaxes are known to deter potential predators, possibly *via* the release of toxins by specialized cells (Jackson & Buss, 2009 ; Pearse & Voigt, 2007). As assumed, stem cells can be located in the peripheral belt, which ensure the growth of an animal on its outer edge (Albertini et al., 2019).

Trichoplaxes reproduce mainly by body binary fission or budding with the participation of “spherical buds” (Kamm et al., 2018 ; Thiemann & Ruthmann, 1991, 1988). In Placozoa, the formation of gonocytes is usually observed in aging cultures; oocytes are extremely difficult to detect (Grell, 1972, 1971 ; Grell & Benwitz, 1974). Spermatozoa have not yet been described (Grell & Benwitz, 1981). Embryos degrade after cleavage for unknown reasons under laboratory conditions; the development of embryos is studied up to the stage of 64–128 cells (Eitel et al., 2011). The life span of individual cells in the animal body is also unknown. Stem cells and their niches have not been found. As assumed, all trichoplax cells are capable of reverse differentiation because an animal can regenerate after dissection into small fragments and even from individual cells (Ruthmann & Terwelp, 1979). However, regenerative morphogenesis experiments showed partial cell differentiation. Specifically, fragments of the middle of the plate do not regenerate. When connecting the belt and the central part of the animal body, redundant material is rejected (Schwartz, 1984). As shown, there are small cells along the edge of the trichoplax body in which the *Trox-2* gene is expressed. Apparently, those are multipotent stem cells since the suppression of the *Trox-2* expression by antisense oligonucleotides or by RNA interference stops trichoplax growth and regeneration (Jakob et al., 2004).

Since genome sequences for several trichoplax strains became available (Dellaporta et al., 2006 ; Kamm et al., 2018 ; Signorovitch et al., 2007 ; Srivastava et al., 2008), it is possible now to manipulate them (for example, by turning off one or another gene) and analyze what changes this would lead to (Hardy et al., 2010). On the other hand, reporter genes encoding fluorescent proteins are often used to identify individual cells and their descendants (Currie et al., 2016). This approach allows to label cells by transgenic mRNA and to study the spatiotemporal distribution of cells in population under a fluorescence microscope, separate them applying fluorescence-activated cell sorting (FACS), and investigate

transcriptomes of individual cells by scRNA-seq analysis (Lush et al., 2019). The molecular genetic study of communication between cells is of great interest in case of trichoplax: it is the cause of the formation of multicellular ensembles and systemic behavior (Kuznetsov et al., 2020b).

Unfortunately, limited data on the fine structure, poor knowledge of the reproductive cycle of trichoplax, and lack of comprehensive information on the dynamics of animal growth and physiology during cultivation hinder work on its genetic modification and reverse genetics. Due to a lack of methods for genetic modification of trichoplax organism, researchers are limited to the analysis of genetically unmarked cells (Moroz et al., 2020 ; Romanova et al., 2020 ; Sebé-Pedrós et al., 2018) and heterologous gene expression (Elkhatib et al., 2019 ; Smith et al., 2017). It should be noted, methods for the introduction of genetic information, such as electroporation and lipofection (except for ballistic transfection) (Sambrook & Russell, 2001), are focused on the manipulation of cells in culture, whether they are prokaryotes or eukaryotes. In case of multicellular organisms with a sexual reproduction, transgenesis occurs at the single cell stage, for example, by injecting DNA into the zygote (Transgenesis Techniques, 2009). Therefore, it is necessary to develop a special method of transgenesis for trichoplaxes, which are not capable of sexual reproduction under laboratory conditions but reproduce vegetatively.

Our key aim was to study the regenerative abilities of *Trichoplax* sp. H2 for the cellular and genetic engineering of this animal.

MATERIAL AND METHODS

Cultivation. *Trichoplax* sp. H2 strain was used for experiments. Every time, 15 animals were placed with a micropipette in a Petri dish 90 mm in diameter and cultivated at a temperature of +25 °C and pH 7.8–8.0. Unicellular green alga *Tetraselmis marina* (Cienkowski) R. E. Norris, Hori & Chihara, 1980 was used as a food source (Kuznetsov et al., 2020b). Artificial seawater (hereinafter ASW) with a salinity of 35 ‰ was changed every 5–7 days. Trichoplaxes were transferred onto a fresh algal mat every 3–5 weeks. Animals were kept in the “starvation” mode the day before the beginning of the experiment: we placed them in a dish with ASW without alga.

Microscopy and image analysis. Animals were investigated under Zeiss Primo Star or Zeiss Stemi 305 microscope with a built-in camera at 8× and 40× magnification. The images were analyzed applying the ImageJ package (https://imagej.nih.gov/ij/). The contrast threshold was selected to separate the images from the background noise. The areas of trichoplax plates were measured. Individual cells and their agglomerates were studied under an inverted Nikon Eclipse Ts2R microscope with DIC optics at up to 600× magnification.

Vital staining. The samples of individual animals or their parts were sequentially rinsed in two drops of ASW. To the second drop, 20 μL of 0.01 % neutral red solution (Sigma-Aldrich, USA) was added; it was exposed for 10 min at room temperature and then rinsed again in two drops of ASW for 30 min.

Microsurgery. We took large animals for the experiments, at least 1 mm in diameter, which changed the behavioral strategy to “waiting” and flattened on a substrate after the active seek for food. For this purpose, trichoplaxes were placed into a Petri dish with a plastic substrate and ASW for about 60 min – until their transition to a resting state occurred. Individual animals in this state were dissected into radial parts. Medial incisions were made with a scalpel under a Zeiss Stemi 305 microscope at 8× magnification. To investigate the ability of trichoplax parts to grow and reproduce, the animals were dissected into 2, 4, or 8 radial lobes; 10 parts were sown on *T. marina* algal mats – with the condition of 1 fragment from an individual. The post-traumatic regeneration was directly observed for 3–4 hours

under a Nikon Eclipse Ts2R microscope. Photo and video recording was carried out at various time intervals.

Dissociation into individual cells. From 50 to 150 individuals, 0.5–1.0 mm in size and of the same age, were taken *prior* to the stationary phase; this corresponded to 2–3 weeks after inoculation. Those were twice rinsed in ASW for 30 min and transferred into 300-μL wells. For dissociation of trichoplax plates into separate cells, bovine serum albumin (hereinafter BSA) (Sigma, USA) at concentrations of 10 and 0.1 %, 10 μM amlodipine (Teva, Russia), or 3.5 % NaCl was used with an exposure of 15 to 90 min. Trichoplaxes were kept in ASW with 0.1 % BSA for 15 min at room temperature, and the medium was intensively stirred for the last 5 min – until a homogeneous cell suspension was obtained.

Flow cytometry. Cytomics™ FC 500 flow cytometer (Beckman Coulter, USA) equipped with an argon laser with a wavelength of 488 nm was used to study the efficiency of trichoplax dissociation into individual cells and to assess animals' abundance and size. Cytometric data were processed applying Flowing Software v2.5.0 (www.flowingsoftware.com, Perttu Terho, University of Turku, Finland). Total abundance of individual cells and their aggregates was determined in unstained samples by gating a cell population on 2-parameter light scattering cytograms (forward scatter, FS, and side scatter, SS), as well as after staining the samples with SYBR Green I (Molecular Probes, USA). The final dilution was 10^{-4} in each sample. Staining was carried out in the dark for 30 min just before cytometry. The stained samples were analyzed using FS and SYBR Green I fluorescence in the green area of the spectrum (FL1 channel, 525 nm). Cell concentration was calculated from a sample flow rate (60 μL·min^{-1}), time, and abundance of cells registered during this interval (60 s). Measurements were controlled and calibrated using fluorescent microspheres (Beckman Coulter, USA) with a size of 1.0, 4.2, and 10.7 μm. Cell sizes (L, μm) were determined based on the FS channel data as an equivalent spherical diameter (ESD), the volume of which is equal to a cell volume regardless of its morphology.

Cell reaggregation and reparative regeneration. A resulting cell suspension was immediately placed on nutrient mats in the first series of experiments. In the second series, the homogenate was transferred into a 1.5-mL microtube, and cells were rinsed three times with 1 mL of ASW by sedimentation on an FVL-2400 Combi-Spin microcentrifuge (BioSan, Latvia) for 2 min. At the final stage, centrifugation lasted for 5 min and was followed by a pellet dispersion on a vortex for 2 sec. Mini-aggregates of cells up to 100 μm in size were sown on algal mats for further trichoplax regeneration, growth and reproduction.

The research was carried out during the year. In total, 14 series of experiments were performed with BSA and amlodipine in various modifications; control tests with 3–5 dishes in a separate experiment were carried out. The data in the article are given as "mean ± standard deviation."

RESULTS

Animal growth. *Trichoplax* sp. H2 were in a state of adaptation to new conditions for a week after sowing on algal mats in Petri dishes. After a lag phase (up to 7–10 days), the animals began to grow and then began to divide, with the formation of an area of stretching and thinning between the cells, which was followed by rupture. The exponential phase occurred, characterized by uniform morphology of the animals. The subsequent logarithmic phase of the trichoplax culture was accompanied by slight changes in the population size (Fig. 1a), and the biomass gain occurred stepwise (Fig. 1b). Deceleration of the culture growth with the transition to the stationary phase and subsequent shrinking of some animals was recorded on the 20th day; the beginning of the terminal phase was registered on the 25th day.

For further cultivation, 10 intact animals were placed into dishes with fresh algal mats. As before, there was an exponential increase in abundance after a 7-day pause (Fig. 1c), with a certain slowdown in the biomass gain at the late stage (Fig. 1d) accompanied by a temporary decrease in animals' size.

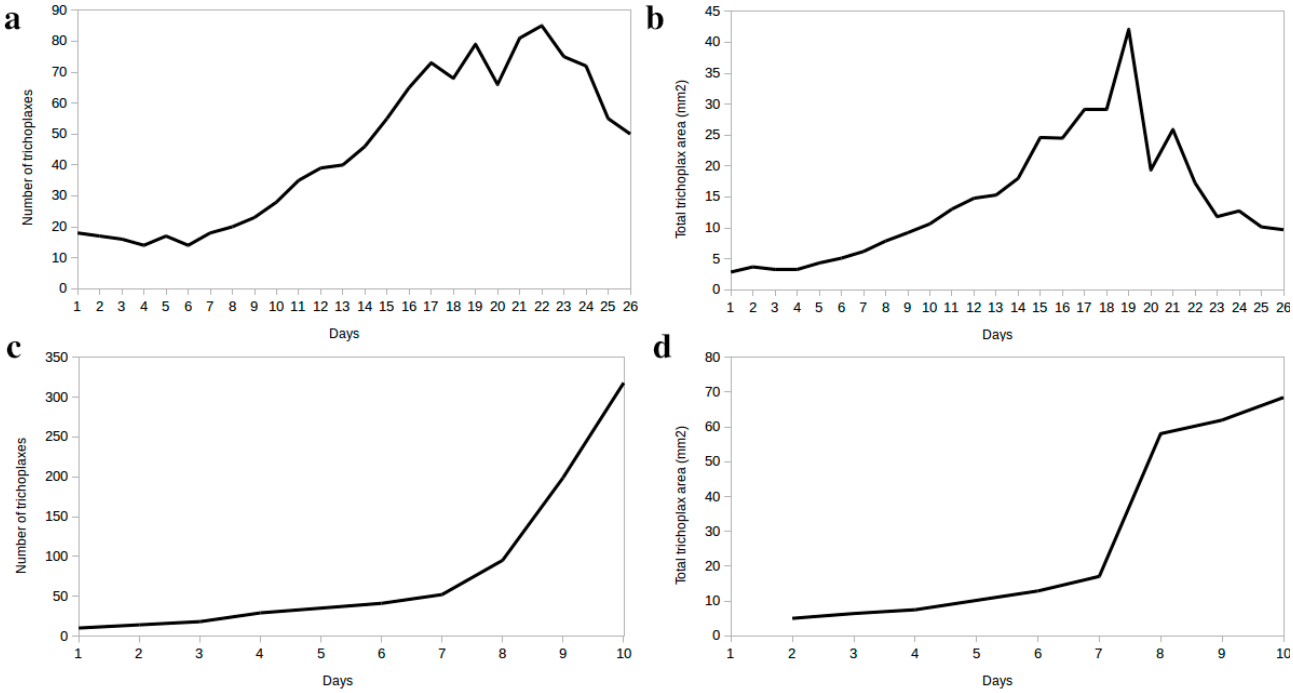

Fig. 1. *Trichoplax* sp. H2 culture growth: a, b, the long-term cultivation; c, d, the following short-term cultivation. Total surface area of animals is in mm^2 (b, d)

Feeding behavior of trichoplaxes. With the excess of food, the animals rested on an algal mat (Fig. 2a). As a mat was consumed, trichoplaxes slowly moved to a new spot abounding in food. In case the animals were sown in ASW without alga, they began to move actively seeking for food (Fig. 2b). After several tens of minutes of unsuccessful seek, the animals calmed down and switched to the "waiting" mode (Fig. 2c); a thin peripheral rim was formed, and trichoplaxes were adhered closely to a substrate as the movement of cells inside their bodies slowed down. The addition of microalga next to an animal stimulated its positive taxis in the direction of food and its subsequent consumption. The addition of a small volume of medium from the old culture or the placement of dead alga near trichoplax, on the contrary, caused an escape reaction.

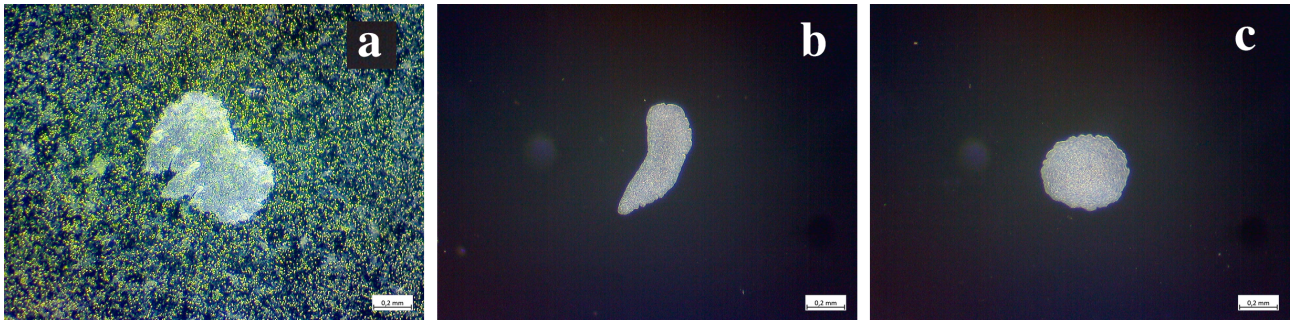

Fig. 2. *Trichoplax* in various physiological states: a, animal resting on an algal mat; b, *Trichoplax* in motion; c, motionless animal. Magnification 40 times

Microdissection of animals. After dissecting trichoplax into two parts (Fig. 3a), the halves began to regenerate into an intact individual and healed the wound in 1 hour (Fig. 3b, c). Dissected animal stopped moving, remained flattened on a substrate, and curled up isolating the damaged area from the environment. The injured part of the plate is characterized by the absence of cilia and belt while its native edge forms a belt consisting of small dark cells with clearly visible mobile cilia. The internal content of the plate was very dynamic, in contrast to the wound edge. Trichoplax permanently changed its shape but did not rise above the surface – just remained adhered closely to a substrate. The belt on the side opposite to the wound somewhat thickened in height, became darker, and adhered closely to a substrate fixing the animal in such a way that it performed amoeboid movement but could not tear itself away from the adherence site (see Supplementary 1: https://marine-biology.ru/mbj/article/view/353). The wound healing lasted about 30 min (Fig. 3a) and ended with deep invagination of the belt towards the central part of the plate in 60 min after bisection (Fig. 3b) (see Supplementary 2: https://marine-biology.ru/mbj/article/view/353). It took about an hour for further repair leading to an alignment of the edge of the plate (Fig. 3c, d). Animals no longer adhered to the surface with the opposite side; trichoplaxes began to rotate. After 3 hours or more, the edge of the plate in the damaged area was rounded, an intact belt was formed, and the scar inside the plate was resorbed. All cells inside the trichoplax plate began to move in a coordinated manner, the plate acquired the initial plasticity, and the animal got the ability to move progressively.

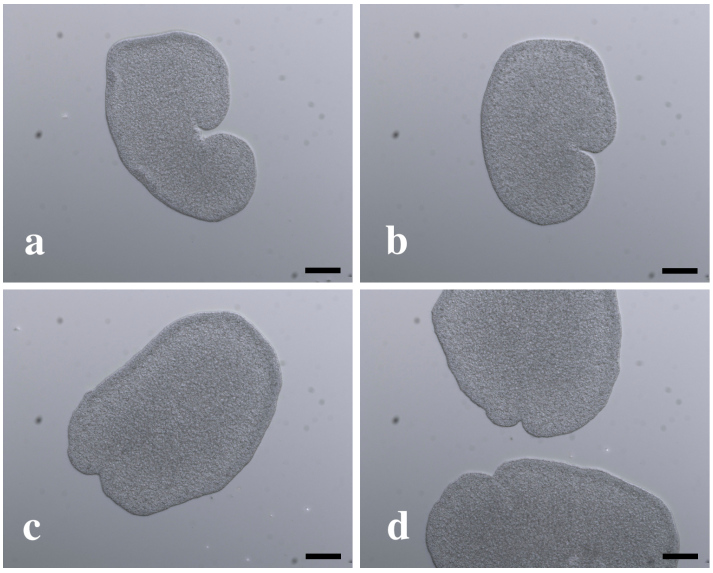

Fig. 3. Repair of a wound area in half of *Trichoplax* sp. H2 within 30 min (a), 60 min (b), and 120 min (c, d). Magnification 400 times. Scale bars are 50 μm

When trichoplax was cut into 4 parts and, especially, into 8 parts, the initial recovery proceeded longer (up to 60–120 min) because the wound area exceeded the undamaged one. Interestingly, to restore the wound area with the help of remaining small cells of the belt of the plate, the animal had to thicken (Fig. 4b), form folds, and even reject some large cells from the central part of the plate. A dissection of the animal resulted in the immobilization of the plate in the damaged area: trichoplax lost its ability to move progressively, performed rhythmic movements in one plane accompanied by cell restructuring and leading to a decrease in the wound area, and later began to rotate. As the wound was healed, the edge of the plate flattened out, and the animal regained its mobility.

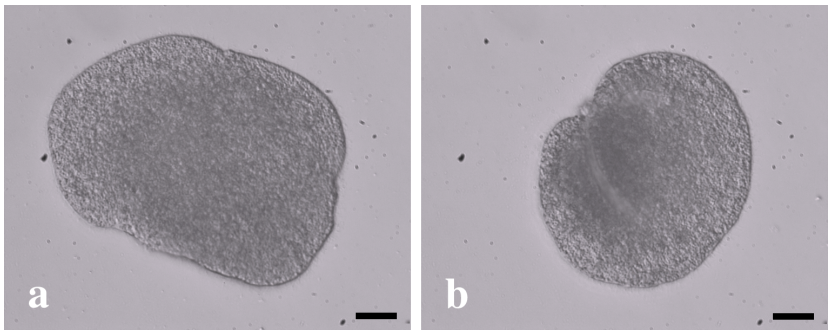

Fig. 4. Repair of a wound area in 1/8 of *Trichoplax* sp. H2 specimen: a, small intact animal as a positive control; b, healing of 1/8 of a large animal within 1–2 hours. Magnification 400 times. Scale bars are 50 μm

In two weeks after sowing 10 halves and 10 quarters of trichoplax on an algal mat, 40 intact individuals were found in each of two dishes (Table 2). Animals were located separately on and under alga, adhered closely to a substrate, were inactive, and had a typical morphology with smooth edges and corresponding plate sizes (about 1 mm). This result indicates that trichoplax halves and quarters have approximately the same potential for recovery over a long period of time in the presence of a food source. Fewer individuals regenerated out of 1/8 of trichoplax, since only some of them were capable of giving rise to full-fledged animals. Nevertheless, all recovered individuals had a reproductive potential: a 4–5-fold increase in the population size *per* week was recorded.

Table 2. *Trichoplax* sp. H2 regeneration after microdissection of the plate

Weeks	Abundance of regenerating animals			
	Whole part	1/2 part	1/4 part	1/8 part
2	42	40	40	10
3	> 200	> 200	> 200	51
4	> 200	> 200	> 200	> 200

Dissociation of animals into individual cells. As a result of placing trichoplax in a medium without divalent cations (3.5 % NaCl), animals' bodies gradually dissociated into individual cells (Fig. 5b). There were mostly rounded cells in 60 min; those performed erratic movements due to cilia beating even the next day. Immobile pear-shaped cells were revealed as well but in a much smaller abundance, because the pear-shaped cells turned into spherical ones with prolonged incubation.

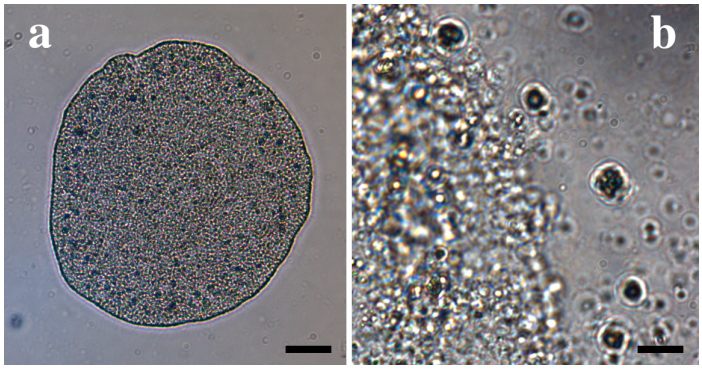

Fig. 5. *Trichoplax* plate stained with neutral red: a, intact animal, magnification 200 times, scale bar is 75 μm; b, animal during dissociation with 3.5 % NaCl solution, magnification 600 times, scale bar is 25 μm

Evaluation of individual cells and their aggregates using a flow cytometer. The analysis of trichoplax cells in unstained samples (Fig. 6a, d) was restricted by the presence in the medium of suspended particles of a comparable size. This problem was especially felt when samples were treated with 3.5 % NaCl; to a lesser extent, with 0.1 % BSA (Fig. 6a). The formation of different-sized cell aggregates after dissociation of the trichoplax body did not allow obtaining a compact cluster on cytograms: it had a core of individual cells (IC) and a plume of cell aggregates (CC) (see Fig. 6a, d).

The efficiency of cell dissociation was estimated by the ratio of individual cells in the total number of recorded objects. The value varied within 60–76 % [(68 ± 8) %]. In terms of this indicator, there was no statistically significant difference between the treatment with 0.1 % BSA and 10 μM amlodipine. Cell abundance in one organism ranged 7,000 to 12,000; the value may be underestimated since the efficiency of tissue dissociation did not exceed 80 %.

Staining of nucleic acids with SYBR Green I fluorochrome facilitated the identification of trichoplax cells on cytograms (TR gates in Fig. 6b, e) and gave more accurate estimations of cell abundance and size based on the gating of their populations (Fig. 6c, f). According to calibration measurements, mean size (ESD) of individual trichoplax cells was of (3.5 ± 0.4) μm; there was no significant difference between the samples with BSA and amlodipine.

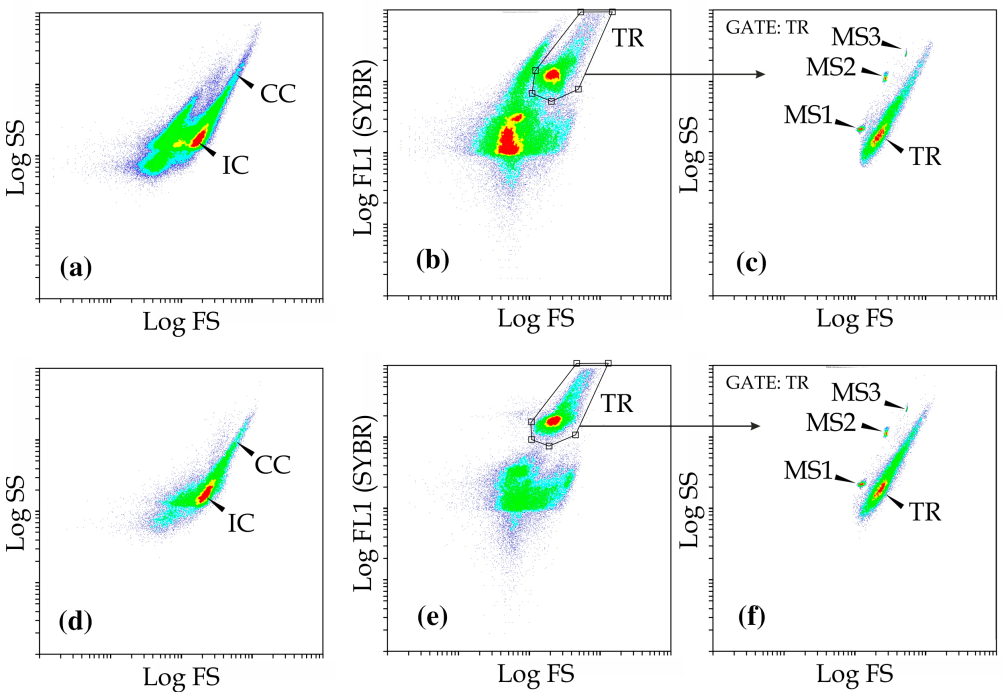

Fig. 6. Flow cytometry of dissociated *Trichoplax* cells with no staining (a, d) and after their staining with SYBR Green I fluorochrome (b, c, e, f). Treatment with 0.1 % BSA (a, b, c) and 10 μM amlodipine (d, e, f). Single *Trichoplax* cells (IC), their aggregates (CC), gating of *Trichoplax* cells in stained samples (TR), and calibration microspheres of 1.0 μm (MS1), 4.2 μm (MS2), and 10.7 μm (MS3) are marked. Data from the TR gate are given on two graphs (c, f)

Regeneration of animals after treatment with 10 % BSA. It was necessary to find out whether trichoplaxes are able to regenerate from fragments of less than 1/8 of the body. To obtain such small fragments, we applied trichoplax plate dissociation technique with BSA. In total, 50 individuals were involved in the experiment, and BSA was added in a concentration up to 10 %. The animals were kept for 15, 45, and 90 min at room temperature; the suspension of animal fragments was removed and sown

on nutrient mats for growth. The use of BSA instead of a scalpel did not allow to obtain decreasing fragments with saving of axes – such as sectors of 1/16, 1/32, *etc.* Instead, random fragments of animals' bodies were obtained. Thus, the viable trichoplaxes were found on mats after only 3 weeks of cultivation. Interestingly, the animals were of different size – from very small to large, about 1 mm. Some individuals remained in close contact with each other after division.

The sizes of trichoplax plate fragments after 90 min of treatment were significantly smaller than after 15 min of incubation. The longer the exposure to 10 % BSA, the lower the ability of trichoplax to reparative regeneration, growth, and reproduction was observed. Specifically, 83, 38, and 1 animal were found in dishes in 3 weeks of cultivation; 333, 220, and 4 intact organisms in 4 weeks of cultivation (after exposure to BSA for 15, 45, and 90 min, respectively) (Table 3). In the latter case, three out of four regenerating animals were registered only in 4 weeks of cultivation, and the reproduction of animals was slow.

The obtained result shows that the lasting of trichoplax disassembling into fragments with 10 % BSA negatively affects the repair of experimental animals. Despite the fact of body fragments differ significantly in structure after microsurgery and BSA treatment, the efficiency of trichoplax recovery after 15 min of incubation with 10 % BSA is comparable to animal regeneration from 1/8 parts (Table 2). However, reparative regeneration of animals and further reproduction required almost one month of cultivation on algal mats after 90 min of treatment with 10 % BSA. Interestingly, the recovered individuals gave rise to a new population which reached a maximum of 182 animals in the second month of culture growth and was characterized with the gradual death of trichoplax by the third month (Table 3).

Table 3. *Trichoplax* sp. H2 regeneration after 10 % BSA treatment depending on incubation period

Weeks	Abundance of regenerating animals		
	15 min	45 min	90 min
3	83	38	1
4	333	220	4
5	> 400	> 400	8
6	> 400	> 400	18
7	> 400	> 400	47
8	> 400	> 400	182

Restoration of animals from an aglomerate of individual cells. Are trichoplaxes capable of recovering after complete dissociation into individual cells? The animals did not grow in case of sowing a suspension of such cells on a nutrient mat. However, we achieved the formation of viable animals after centrifuging a cell suspension, dispersing a pellet, and sowing cell aggregates on mats. Specifically, 74 individuals were recorded in 1 week of cultivation on an algal mat in case of 0.1 % BSA for dissociation, and 2 animals were registered in case of 10 μM amlodipine; then these animals successfully reproduced reaching the value of 380 in 4 weeks (Table 4). In contrast, the treatment of trichoplaxes with 3.5 % NaCl negatively affected their subsequent regeneration.

The experiments were consistently reproduced, and abundance of regenerating trichoplaxes directly depended on abundance of animals taken. Specifically, 5, 74, and 93 individuals grew when we involved 50, 100, and 150 trichoplaxes, respectively, in case of 0.1 % BSA. So, a gain in trichoplax quantity increased the individual cell concentration in suspension, and this positively affected the number of fragments after pellet's disaggregation and, ultimately, the ratio of recovered animals.

Table 4. *Trichoplax* sp. H2 regeneration from aggregates of single cells after dissociation with various reagents

Weeks	Abundance of regenerating animals		
	0.1 % BSA	10 μM amlodipine	3.5 % NaCl
1	74	2	0
2	> 400	25	0
3	> 400	236	0
4	> 400	380	0
5	> 400	> 400	0

Over time, new cell aggregations appeared on algal mats; those grew on a substrate, acquired a typical trichoplax morphology and abilities to move, external feeding, and division (Fig. 7a, b). Trichoplaxes regenerated from cell aggregates asynchronously and formed different-sized colonies with uneven edges (interestingly, initially large aggregates developed faster than small ones). Then these swarms rounded turning into mobile animals, grew up to their usual size (about 1 mm), and began to divide by thinning and stretching – like intact individuals. If animals regenerated from the cell aggregates after centrifugation of a cell suspension and subsequent pellet dispersion without food in ASW, then, initially random, shapeless, and chaotic trichoplax cell aggregates were rearranged due to regenerative morphogenesis into rounded structures similar to “spherical buds”: in those, small epithelial cells with mobile cilia are settled on the surface while large cells are located inside (Fig. 7c).

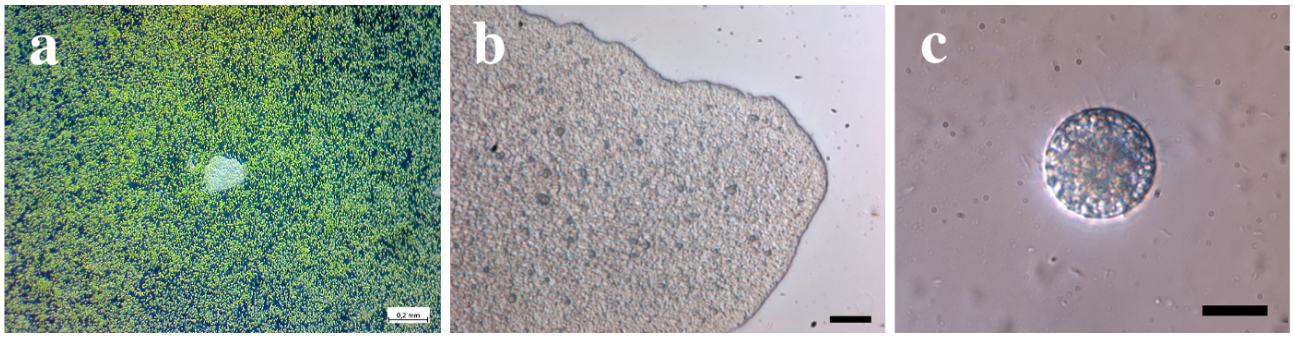

Fig. 7. Regenerative morphogenesis of *Trichoplax* sp. H2: a, 7-day animal formed on an algal mat, magnification 40 times, scale bar is 0.2 mm; b, *Trichoplax* from a 6-week culture, with small cells in the rim and cilia on the periphery, magnification 400 times, scale bar is 50 μm; c, 7-day animal regenerated in ASW with no food source, magnification 2×400 times, scale bar is 100 μm

The dynamics of recovery, subsequent growth, and reproduction of trichoplaxes on nutrient mats varied in experiments and depended on age and state of the selected animals (Fig. 8). The best results were obtained on trichoplaxes taken at the exponential phase. In contrast to intact animals with a lag phase duration of about 1 week (Fig. 1c, d), experimental animals were characterized by the fact that either agglomerates did not regenerate or this stage was delayed up to two or more weeks. However, this was followed by the exponential and logarithmic phases; then, there were the stationary phase (in the second month of the culture existence) and slow death (starting from the sixth week after sowing). The death of the culture was stated in a decrease in the total biomass including a drop in trichoplax abundance in Petri dishes and animals’ shrinking.

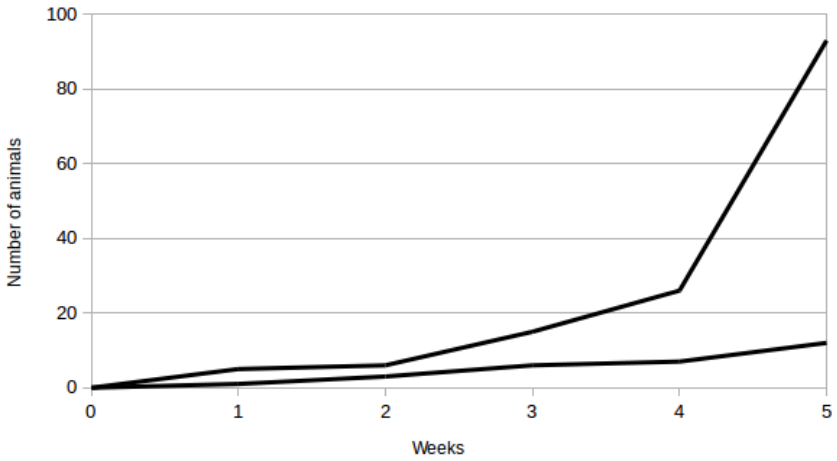

Fig. 8. Dynamics of *Trichoplax* sp. H2 regeneration after dissociation with 0.1 % BSA. Two independent experiments with animal growth in separate Petri dishes

DISCUSSION

The employment of a limited number of model organisms, such as *Escherichia coli* (Migula, 1895), *Caenorhabditis elegans* (Maupas, 1900), *Drosophila melanogaster* Meigen, 1830, *Mus musculus* Linnaeus, 1758, *etc.*, representing different taxonomic groups, allowed biologists to focus their attention on studying the mechanisms of life and led to an understanding of biological processes at the molecular level; moreover, this allows to modify living objects (Sommer, 2009). Out of hydrobionts, in addition to *Danio rerio* (Hamilton, 1822), researchers are interested in new model organisms – *Hydra vulgaris* Pallas, 1766 and *Nematostella vectensis* Stephenson, 1935 (Layden et al., 2016), *Ciona intestinalis* (Linnaeus, 1767) (Liu et al., 2006), *Paracentrotus lividus* (Lamarck, 1816) (Gildor et al., 2016), *etc.* One of them is *Trichoplax* sp. This unique multicellular animal without a nervous system belongs to basal Metazoa (Heyland et al., 2014). Thereby, it is interesting to know the way its functioning is coordinated. At the same time, this organism remains difficult for both developmental biology and molecular genetics study due to poor knowledge of its life cycle and regenerative abilities (Eitel et al., 2011 ; Kamm et al., 2018), implicit symmetry, uncertainty of the body plan, and expression of the corresponding genes (DuBuc et al., 2019 ; Schwartz, 1984 ; Zuccolotto-Arellano & Cuervo-González, 2020), as well as due to a supposed absence of stem cells because of predominant vegetative reproduction (Ruthmann, 1977).

The work with individual trichoplax cells is the basis for studies by methods of molecular genetics. As known, the ability for genetic transformation in bacteria and for genetic transfection in eukaryotic cells in culture is associated with the exponential growth phase characterized by a maximum number of mitoses (Sambrook & Russell, 2001). For this reason, we paid considerable attention to obtaining individual trichoplax cells and their further aggregation to restore reproductive animals. Interestingly, trichoplax growth in Petri dishes differs much from its cultivation in an aquarium (Pearse, 1989).

Under our conditions, the crucial factors were pH stability in the medium and periodic change of ASW allowing to remove trichoplax waste products. Acidification of the medium and water change affected the shape of a growth curve which was manifested in appearance of steps (Fig. 1); this hinders identification of the beginning of the transition to the stationary phase. Consumption of alga by animals

eventually led to a culture death. Such behavior of trichoplaxes, as seeking for food and fixing and flattening on a substrate, as well as feeding behavior, served as a criterion for the physiological state of animals. The most uniform individuals were registered at the exponential phase of growth.

To remember, the ability to manipulate individual cells and regenerate after dissociation was first demonstrated in the early XX century for sponges (Galtsoff, 1925 ; Wilson, 1910, 1907). Hydra regeneration was discovered by A. Trembley even earlier – in the middle of the XVIII century (Lenhoff & Lenhoff, 1986). The regenerative abilities of *Trichoplax adhaerens* are widely studied since the second half of the XX century (Kuhl & Kuhl, 1963, 1966). *Trichoplax* sp. H2 showed high regenerative potential and tissue plasticity in our experiments when it was dissected radially into several parts. The dissected animals remained adhered to a substrate but curled up to minimize the damaged area and surround the wound with healthy tissue. Trichoplaxes recovered much more slowly from 1/4 and, especially, from 1/8 part, than from 1/2 part due to lower ratio of the intact surface area to the wound area. Epithelial cells with a cilium maintaining motor activity for at least 12 hours prevailed among the individual cells obtained after the dissociation of the trichoplax plate. However, their rounded shape did not correspond to that of cells that make up the animal body (Smith et al., 2014) which may result from the absence of neighbors or the effect of the osmotic pressure of the environment. Immobile pear-shaped cells, presumably derivatives of lipophilic cells, were found as well. However, it was impossible to detect less representative cell types under a light microscope. Therefore, the entire pool of available cells was used in the course of reparative regeneration experiments in hope of natural selection of poorly differentiated cells during regenerative morphogenesis. This is confirmed by the rejection of some material which, apparently, contained differentiated epithelial cells and cells of the middle of the plate (Schwartz, 1984). The total abundance of cells in 0.5–1.0-mm animal was rounded up to 10,000 according to flow cytometry data; the value is in agreement with the results of electron microscopy where cell abundance in 1-mm trichoplax was 50,000 (Smith et al., 2014).

Experiments on trichoplax dissociation with 10 % BSA for different time intervals showed that animals lose their ability to recover in accordance to the duration of exposure; apparently, this occurs because of the predominant loss of peripheral cells and is consistent with the results of trichoplax incubation in a calcium-free medium. This evidences in favor of the existence of totipotent cells in the belt of the trichoplax plate that disagrees with the data on sponges where most cells are capable of movement and transdifferentiation (Bond, 1992 ; Harris, 1987). Dissociation of the trichoplax plate into individual cells with 10 μM amlodipine is more effective than with 0.1 % BSA but has the opposite effect on the restoration of animals after centrifugation of a cell suspension. The difference found may be due to different mechanisms of trichoplax tissue dissociation. Specifically, BSA is capable of binding calcium ions and blocking receptors on a cell surface (Kuznetsov et al., 2020b) while amlodipine disrupts calcium channels (Kuznetsov et al., 2020a). The use of a calcium-free medium did not result in a restoration of animals, as in other experiments (Ruthmann & Terwelp, 1979).

The results of our experiments suggest that hypothetical stem cells of trichoplax are located on the periphery in the edge belt of the plate but are not able to proliferate and differentiate independently, without contact with neighboring cells and without active morphogenetic movements that is consistent with the assumption in (Albertini et al., 2019). This is confirmed by cell reconfiguration within several days in the absence of food from shapeless cell aggregates into pronounced spherical bodies with a flat epithelium and large internal cells (Thiemann & Ruthmann, 1991, 1988) but is not supported by self-assembly from individual cells, as described in (Ruthmann & Terwelp, 1979).

On the other hand, the development of multicellular aggregates on algal mats, their subsequent regeneration, and growth of experimental trichoplaxes recapitulated the development of intact animals in culture with a 1–2-week delay.

Conclusion. The widely represented cell types of trichoplax disguise the possible existence of totipotent cells and obstruct their search. However, the system of stem cell selection used by us during the assembling of dissociated cells – centrifugation and further pellet dispersion followed by germination of the obtained aggregates – can be useful and/or critical when working with competent cells for genetic transfection. This assumption requires additional research. The future work on trichoplax cell suspension can involve existing methods of transfection – lipofection and electroporation – using species-specific DNA constructs: this will allow studying and modifying the mechanisms of cell signaling, functioning, and organization of this ancient multicellular organism. In a broader aspect, the transgenesis and genome editing method based on the dissociation of tissue into individual cells can be applied to other hydrobionts with a high regenerative potential – sponges, cnidarias, and planarians.

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КУЛЬТИВИРОВАНИЕ И РЕГЕНЕРАЦИЯ ТРИХОПЛАКСА *TRICHOPLAX* SP. H2 ИЗ ФРАГМЕНТОВ ТЕЛА И АГРЕГАТОВ ДИССОЦИИРОВАННЫХ КЛЕТОК: ПЕРСПЕКТИВЫ ГЕНЕТИЧЕСКОЙ МОДИФИКАЦИИ

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Выполнены исследования на культивируемом в лаборатории простейшем многоклеточном животном *Trichoplax* sp. H2 с целью дальнейшей генетической модификации этого организма. Предлагается вводить генетическую информацию в суспензию клеток после диссоциации тела трихоплакса на отдельные клетки с последующей их агрегацией и регенерацией полученных агломератов в жизнеспособное животное. С этой целью мы исследовали динамику роста трихоплаксов в чашках Петри на матах из одноклеточной водоросли *Tetraselmis marina*. Особи были однородны на стадии экспоненциального роста. В экспериментах по посттравматической регенерации разрезали подопытных животных радиально и исследовали восстановление полученных частей под микроскопом. Оценивали интенсивность роста и размножения трихоплаксов на водорослевых матах — показатели, ухудшавшиеся по мере измельчения животных. Обнаружено, что утраченная часть тела трихоплакса замещается за счёт ремоделинга оставшихся клеток. После витальной окраски животных подвергали диссоциации на отдельные клетки в среде, лишённой двухвалентных катионов. Идентифицированы клетки грушевидной или округлой формы и клетки эпителия со жгутиками, которые сохраняли двигательную активность более 12 ч. Для количественной оценки популяции клеток с помощью проточной цитометрии пластинки трихоплаксов дезинтегрировали при добавлении 10 мкМ амлодипина. Показано, что трихоплакс размером 0,5–1,0 мм состоит примерно из 10 000 клеток. Обработка животных 10%-ным бычьим сывороточным альбумином (БСА) в течение различных промежутков

времени свидетельствует в пользу существования тотипотентных клеток на периферии трихоплакса, вероятно в пояске пластинки. В экспериментах по репаративной регенерации удалось добиться диссоциации трихоплаксов на отдельные клетки при обработке 0,1%-ным БСА, а затем воссоздать живые организмы путём центрифугирования суспензии клеток и последующего диспергирования крупного осадка на фрагменты до 0,1 мм перед высевом многоклеточных агрегатов на питательные маты. Развитие этих агрегатов сопровождалось активными движениями клеток и эпителизацией поверхности, что приводило к увеличению клеточной массы, формированию пластинки, росту и дальнейшему вегетативному делению трихоплаксов. Предполагается, что пребывание экспериментальных животных на искусственной стадии одиночной клетки в ряду бесполых размножений позволит интродуцировать в трихоплакса чужеродную генетическую информацию, например с целью изучения сигнальных систем, организации и функционирования этого многоклеточного организма. Трансгенез, основанный на диссоциации тела животного на отдельные клетки, возможно, будет применим и к другим организмам, обладающим высоким регенеративным потенциалом.

Ключевые слова: трихоплакс, пластинчатые, посттравматическая и репаративная регенерация, диссоциация и агрегация клеток, клеточная инженерия, методы трансгенеза

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PRESERVATION OF BIOLOGICAL DIVERSITY BY CRYOPRESERVATION METHODS: EXPERIENCE OF THE SOUTHERN SCIENTIFIC CENTER OF THE RAS

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One of the promising directions for increasing animal genetic diversity is the formation of cryobanks and long-term storage of reproductive cells in liquid nitrogen. Methods of sperm cryopreservation are known for more than 200 fish species. The resistance to sperm cryodamage in different fish species varies dramatically. There is no unified cryopreservation technique for fish since the habitats vary greatly for different species. In Russia, cryopreserved sperm is currently used extremely insufficiently in aquaculture, but the practice dictates the need for widespread use of cryosperm to solve the problems of producing high-quality fish seed material and for breeding work. The formation of cryobanks is very relevant due to extensive development of aquaculture. Providing commercial and farm enterprises with elite genetic material capable of reproduction at any time of the year will allow not only to set up a biotechnological process, but also to eliminate inbreeding.

Keywords: cryobank, cryopreservation, quality assessment, mobility

Currently, marine fish resources are depleted as they are affected by anthropogenic factors, many of which have an irreversible impact on inland waters (Balykin & Khodorevskaya, 2021). At the same time, the number of commercially important fish species has decreased so much that the question arises of forming broodstocks: those are capable of restoring the normal functioning of natural populations of these fish, maintaining their genetic diversity, and intensifying commercial aquaculture. It will reduce the pressure on wild populations which are significantly undermined by fishing. This is possible only when the formation of artificial populations and commercial aquaculture are based on genetic principles allowing to reduce the risk of a significant depletion of the gene pool for restored populations and to grow fish with high values of commercially important traits. However, formation of broodstocks on fish farms and their management should involve the same principles as the well-being of natural populations: their basis is maintenance of the optimal level of genetic diversity. Cryopreservation is one of the methods of reproductive biology directly related to the preservation of bioresources with the possibility of subsequent restoration of their reproductive functions. In the literature, the term “cryopreservation” usually refers to the storage of biological objects at liquid nitrogen temperature (–196 °C), and the process is considered effective only if the cells or tissues are completely viable after thawing (Amstislavsky et al., 2014).

History of cryopreservation. The first to put forward the idea of freezing reproductive cells was the Italian physician P. Mantegazza. In 1866, he published a monograph on the preservation of the ability of bull and stallion ejaculate to fertilize after its freezing down to −15 °C and subsequent thawing. At the late XIX century, the scientific foundations of cryobiology were laid by the Russian scientist P. Bakhmetyev who studied peculiarities of hypothermia in insects and anabiosis in bats. The French biologist P. Becquerel (1904–1936) and the Austrian scientist P. Rahm (1919–1924) revealed the ability of various organisms (microorganisms and invertebrates), as well as seeds and spores, to tolerate deep freezing (down to −269 and −271 °C, *i. e.*, to temperatures close to absolute zero) in the dried state. It was proved later that some animals and plants survive when the water they contain is frozen. In our country, the first experiments on freezing farm animal spermatozoa were carried out by the prominent Russian biologist I. Ivanov. In 1907, he showed that stallion sperm restored its fertility after freezing down to −15 °C and subsequent thawing. In 1947, I. Sokolovskaya, V. Milovanov, and I. Smirnov obtained offspring from insemination of females with thawed rabbit spermatozoa previously stored at −78 °C. Studies of A. Smith and Ch. Polge were of great importance as well: in 1949, these researchers were the first to propose to use glycerol for cryopreservation. Preservation of sperm fertility after freezing–thawing was shown for 16 mammalian species, 2 mollusc species, 5 bird species, 6 echinoderm species, and 1 amphibian species (Ponomareva et al., 2017a).

The first successful reproducible results of fish spermatozoa cryopreservation were obtained for the herring (Blaxter, 1953). The results of sperm cryopreservation for several sturgeon species – the Beluga sturgeon *Huso huso* Linnaeus, 1758, the sterlet *Acipenser ruthenus* Linnaeus, 1758, the kaluga *Huso dauricus* (Georgi, 1775), and the hybrid *H. huso* × *A. ruthenus* – were obtained for the first time by I. Burtsev and E. Serebryakova (1969). The first possibility of salmon sperm preservation was demonstrated on the example of the chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792); its sperm previously stored in liquid nitrogen for seven days showed a fertilization rate of 77.7 % (Ott & Horton, 1971). The first to obtain good results of using cryopreserved sperm to fertilize bighead and silver carp eggs was A. Sin (1974). In 1976, when using cryosperm of the common carp *Cyprinus carpio* Linnaeus, 1758, the rate of fertilized eggs was 11 % (Pavlovici & Vlad, 1976). There is a positive experience of using cryopreserved sperm to restore and maintain the population structure of the salmon in Iceland, Norway, and Canada. Commercial cryobanks operate in the USA, Norway, Japan, and France.

Prospects for creating a cryobank. Methods of sperm cryopreservation are known for more than 200 fish species. In different species, the resistance to sperm cryodamage varies dramatically. There is no unified cryopreservation method for fish since the habitats of different species (marine, freshwater, anadromous, sedentary, and non-migratory ones) vary greatly. For marine fish resistant to high osmotic water pressure, it is easy to obtain good rates of spermatozoa survival after cryopreservation; for freshwater and anadromous species, it is necessary to search for cryoprotective media (Asturiano et al., 2017 ; Maisse, 1996 ; Martínez-Páramo et al., 2017). So far, experiments on cryopreservation of spermatozoa and somatic cells have been carried out on more than 30 species of marine fish (Cabrita et al., 2010 ; Mauger et al., 2006 ; Suquet et al., 2000). The rate of spermatozoa that survive cryopreservation and are active after it is much higher in marine fish species (80–90 %) than in freshwater ones (40–50 %) (Scott & Baynes, 1980).

In Russia, cryopreserved sperm is currently used extremely insufficiently in aquaculture. However, practical possibilities allow to widely apply cryotechnologies for reproduction of high-quality fish seed material and for breeding work. Considering extensive development of aquaculture, the creation of a cryobank is very promising and relevant. Commercial and farm enterprises will be provided with elite genetic material capable of reproducing regardless of presence of males; the farms will be able both to set up a biotechnological process and to eliminate inbreeding (Savushkina, 1999 ; Cabrita et al., 2015 ; Zhang, 2018).

Creation of a cryobank allows:

1. To preserve the genetic information of rare, endangered, and commercially important animal species in liquid nitrogen for decades. Storage of frozen cells at −196 °C is possible up to 50 years or even longer without the formation of much abnormal DNA sections.
2. To transport genetic material to an area of population reduction or extinction in order to restore the species.
3. To provide opportunities for breeding and genetic work.
4. To form and maintain a genetic collection of various hydrobiont species.

When designing and constructing fish farms, the presence of a regional cryobank should be provided: this will greatly facilitate the work of enterprises in the future. The annual renewal of broodstocks will contribute to “infusion of fresh blood” and rejuvenation of the herd. Moreover, this will allow minimizing the number of males on farms. The cost of sampling and storing genetic material is five times lower than the cost of fish food. The operation of a cryobank can contribute to development of aquaculture in the regions.

Differences of the cryobank-reproducer from existing analogues are as follows:

1. The cryobank-reproducer allows both to store genetic material and to provide fish farms with the required amount of sperm at a convenient time.
2. The exchange of cryopreserved sperm between fish farms will result in an increase in genetic diversity and, consequently, a rise in the quality of juveniles. The exchange of native sperm is not always possible since the timing of spawning activities varies on different enterprises. Moreover, with a significant distance between the farms, there is a problem of quality loss during transportation.
3. Sperm left on fish farms after fertilization can be stored frozen in the cryobank-reproducer and used later.
4. Sperm samples in the cryobank allow farms to reduce the number of males in the broodstocks. This results in reducing the cost of maintaining fish or replacing some males by females aimed at obtaining more juveniles or food caviar.
5. The applying of cryopreservation methods for fish sperm with a high survival rate after freezing–thawing makes it possible to obtain physiologically high-grade offspring.

To ensure the operation of a cryobank, legal regulation is required: this enables the purchase of material from fish farms and the use of cryosperm there.

When forming cryobanks of male reproductive cells, it is important to store high-quality material. Knowledge of specific morphophysiological peculiarities of fish reproductive cells will help in developing more effective cryopreservation methods. Those will consider the need to combine penetrating and non-penetrating cryoprotectants, osmotically active compounds, and antifreezes. Moreover, those will consider the inclusion of cell membrane stabilizers and antioxidants in the media. All this will provide reliable protection of fish sperm from cryodamage during freezing–thawing and optimize

all stages of cryopreservation. The scale of the described problem is determined by the coverage by studies of a large group of commercially important, native, unique, and endangered fish species; those can be used to save endangered fish. In breeding, cryopreserved sperm can serve as a source of the gene pool.

The cryobank of the Southern Scientific Center of the RAS. Researchers of the SSC RAS work on cryopreservation of reproductive cells of rare and endangered fish species of the southern seas of Russia since 2004. The key aim of our investigation is to optimize the process of fish sperm cryopreservation by selecting optimal cryoprotectors and reducing their negative effect on cells. During cryopreservation, crystallization of intracellular and extracellular water occurs, and membranes of germ cells are destroyed; this leads to their death. To prevent cell damage, cryoprotectors and membrane stabilizers are used. Various stimulation methods (chemical, mechanical, magnetic, *etc.*) contribute to a better penetration of protectors into cells. Electrical stimulation is one of the promising directions in cryopreservation work.

The first experiments were carried out with sperm of the Russian sturgeon *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833 obtained from the Bertyulsky sturgeon hatchery (the Astrakhan Region). In research on cryopreservation, sperm with activity ranks 4 and 5 according to the G. Persov scale was used (Persov, 1953). For cryoprotection, we used Stein medium (NaCl, KCl, $NaHCO_3$, glucose, 12.5 % egg yolk, and 12.5 % DMSO) and the cryomedium developed by us (NaCl, KCl, $NaHCO_3$, $CaCl_2$, mannitol, sucrose, 10 % egg yolk, and 10 % DMSO). Freezing was carried out according to the method of L. Tsvetkova and S. Savushkina (1997). A high survival rate was registered – up to 85 %; this value is higher than that of sperm frozen in the developed cryomedium and then thawed. In the experiments, the optimal parameters of the electrical signal were established, at which the survival rate and time of spermatozoa activity increase. Those are frequency of 20 Hz and amplitude of 150 mV. When exposed to an electrical signal for 1 min, thawed sperm of better quality was obtained according to both parameters. Specifically, sperm survival of the Russian surgeon accounted for 50 %, and lifetime was of 290 s; the values for the stellate sturgeon were 56 % and 693 s, respectively.

Since 2007, the researchers work with the white salmon (inconnu) *Stenodus leucichthys* (Güldenstädt, 1772). Its eggs were fertilized with sperm stored for two years in liquid nitrogen. Exposure to electric current during the equilibration and removal of the protector during cell thawing increase the survival rate of germ cells of the sturgeon by 1.4–1.6 times. When using electrical stimulation at the equilibration stage, membrane permeability rises; cryoprotectors penetrate into cells and prevent cryodamage. The survival of spermatozoa with the use of electrical stimulation after thawing increases compared to the survival of sperm frozen by the traditional method (90 % and 60 %, respectively). Sperm of such a high quality can be recommended for artificial insemination of eggs. When carrying out experiments on the insemination of eggs with thawed sperm, the success of fertilization was 80–96 % for the Russian sturgeon and 64–84 % for the stellate sturgeon. The fertilization of the same batches of eggs at a sturgeon hatchery reached 75–80 %. The obtained results indicated high quality of cryopreserved sperm (Bogatyreva, 2010 ; Krasilnikova, 2015 ; Krasilnikova & Tikhomirov, 2018 ; Ponomareva et al., 2017b).

Thus, it was established that deep freezing of the Russian sturgeon sperm and its storage in liquid nitrogen at −196 °C for two years do not adversely affect the quality of thawed sperm, embryonic development of fish larvae and juveniles, and their morphometric parameters. Therefore, the use of thawed germ cells for artificial insemination of eggs is advisable in the lack of producers at sturgeon hatcheries.

Together with the colleagues from the Institute of Cell Biophysics, Russian Academy of Sciences, we developed a method for reducing the low-temperature jump during crystallization of cryoprotective solutions in order to increase the thawed cell integrity after cryopreservation. The core is as follows: in the method which involves freezing of the cryosolution with biomaterial in liquid nitrogen, *prior* to the operation of the freezing of the cryosolution with cells of living organisms, the solution is remotely affected by ultrasonic radiation with a frequency of 0.50–10 MHz (Patent 2540598 RF, 2015). The dependence between the volume of frozen material and survival after thawing was recorded (Krasilnikova & Tikhomirov, 2014a); the possibility of freezing seminal fluid on grids in the form of a thin film was described (Krasilnikova & Tikhomirov, 2014b). Moreover, the effectiveness was established of reducing the volume of toxic substances in the composition of the cryoprotective medium for spermatozoa of sturgeon species; this, in turn, reduced the toxic effect of the latter on the object and led to an increase in the lifetime of thawed cells (Krasilnikova & Tikhomirov, 2015). The obtained results make it possible to recommend the adjustment of the concentration of penetrating protectors in the cryoprotective solution depending on the amount of intracellular water to increase the survival of male reproductive cells after a double temperature shock.

The sperm bank of sturgeon and other fish species has been replenished in the cryobank of the SSC RAS since 2006. All reproductive cells are frozen according to technological methods developed by the researchers of the center. The material is sampled on fish farms of Astrakhan, Volgograd, and Rostov regions; this enables the exchange of genetic material within the Southern Federal District of Russia (Table 1).

Table 1. Collection of fish reproductive cells in the cryobank of the Southern Scientific Center of the RAS

Species	Number of samples
Russian sturgeon *Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833	398
Siberian sturgeon *Acipenser baerii* Brandt, 1869 (the Lena River population)	224
Stellate sturgeon *Acipenser stellatus* Pallas, 1771	38
Ship sturgeon *Acipenser nudiventris* Lovetsky, 1828	196
Hybrid *Huso huso* Linnaeus, 1758 × *Acipenser ruthenus* Linnaeus, 1758	125
Beluga sturgeon *Huso huso* Linnaeus, 1758	105
Sterlet *Acipenser ruthenus* Linnaeus, 1758	337
Paddlefish *Polyodon spathula* (Walbaum, 1792)	20
Amur sturgeon *Acipenser schrenckii* Brandt, 1869	50
White salmon (inconnu) *Stenodus leucichthys leucichthys* (Güldenstädt, 1772)	140

The preserved genetic material can be used to fill the shortage of producers and to adjust existing technologies for the artificial reproduction of rare and endangered fish species. Thus, cryopreservation of male reproductive cells is a key direction in the strategy for the preservation of genetic biodiversity and in the development of fisheries and aquaculture.

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СОХРАНЕНИЕ БИОЛОГИЧЕСКОГО РАЗНООБРАЗИЯ МЕТОДАМИ КРИОКОНСЕРВАЦИИ: ОПЫТ ЮЖНОГО НАУЧНОГО ЦЕНТРА РАН

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Одним из перспективных направлений увеличения генетического разнообразия животных является формирование криобанков и долгосрочное хранение репродуктивных клеток в жидком азоте. Известны методы криоконсервации спермы более чем 200 видов рыб. Устойчивость к криоповреждениям спермы у разных видов рыб различается кардинально. Единой методики криоконсервации для рыб нет, так как среда обитания имеет значительные различия для разных видов. В аквакультуре России криоконсервированная сперма в настоящее время используется недостаточно, однако практика диктует необходимость широкого применения криоспермы для решения проблем производства качественного рыбопосадочного материала и для селекционно-племенной работы. В связи с широким развитием аквакультуры создание криобанка является весьма актуальным. Обеспечение товарных и фермерских хозяйств элитным генетическим материалом, способным к воспроизводству в любое время года, позволит не только наладить биотехнологический процесс, но и исключить инбридинг.

Ключевые слова: криобанк, криоконсервация, оценка качества, подвижность

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BENTHIC ALGAE COMMUNITIES OF CORAL REEFS IN THE SANYA BAY (HAINAN ISLAND, CHINA) IN SITES HEAVILY POLLUTED WITH NUTRIENTS AND THEIR CHANGES AFTER THE POLLUTION SOURCE ELIMINATION

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It was previously found that extremely high concentrations of nutrients in seawater in the polluted area of a fish farm on the Luhuitou Peninsula (the Sanya Bay) cause a significant reduction in species diversity and abundance of low-productive annual and perennial red and brown algae, as well as an increase in number and biomass of highly productive green algae. In 2017–2019, for the first time, we studied changes in the number and structure of benthic algal communities over a range of tidal zones in the Sanya Bay after the pollution source elimination – the fish farm liquidation. It was shown that a decrease in the concentration of dissolved inorganic nitrogen (DIN) (from ~ 20 to 2.5 μM) and orthophosphates (from 5.0 to 0.2 μM) in seawater significantly altered diversity, species composition, and structure of benthic algal communities. One and half years after the pollution source elimination, the main indicators of the flora became, on average, close to those of the moderately polluted areas of the Sanya Bay.

Keywords: seaweeds, Hainan Island, China, eutrophication, restoration

Species diversity and floristic ratios of main algal groups vary between clean and nutrient-polluted areas, as reported in previous studies (Lapointe et al., 2005a, b ; Morand & Briand, 1996 ; Morand & Merceron, 2004). As shown in our earlier investigations, the Sanya Bay is polluted with nutrients derived from urban wastewater and waste of mariculture farms. In seawater around reefs, mean concentrations of dissolved inorganic nitrogen (hereinafter DIN) and orthophosphates are 3.3 and 0.33 μM, respectively (Li, 2011). On oceanic atolls of Australia, French Polynesia, and other tropical regions, the contents of these substances in seawater are within ranges of 0.10–0.11 and 0.03–0.06 μM, respectively (Charpy et al., 1998 ; Charpy-Roubaud & Charpy, 1994 ; Furnas et al., 1997). Meanwhile, our previous research (Titlyanov et al., 2011, 2018) revealed that diversity and composition of macroalgal species, as well as their seasonal shifts, in the Sanya Bay are likely to be similar to those of relatively clean, unpolluted areas of the Indo-Pacific Ocean.

We assumed that mean seawater pollution by dissolved forms of nitrogen and phosphorus was not high enough to cause serious changes in the marine flora of the Sanya Bay. In this regard, we continued our investigations on the benthic flora in extremely polluted coastal areas subjected to extensive discharge from a grouper fish farm (Li et al., 2021, 2016). This farm covered an area of ~ 3,500 m^2. The volume of effluents directly discharged into surrounding waters of Luhuitou reef was about 4,000 tons·$year^{-1}$. According to the data obtained in 2013–2016 (Li et al., 2016), the mean value of DIN was ~ 190 μM at the grouper farm outlet, with a range ~ 30 to ~ 700 μM. However, the value significantly decreased (down to ~ 20 μM) in intertidal and upper subtidal zones opposite the outlet and reduced down to ~ 9 μM at 100 m from the outlet (in front of the Marine Biological Station). The content of phosphates decreased from ~ 10 μM at the outlet to ~ 3 μM in the area opposite the outlet and to ~ 0.2 μM opposite the Marine Biological Station. Our previous works showed that heavily polluted areas significantly differ from moderately polluted ones in terms of floral diversity, species composition, taxonomic composition, and structure of algal communities (Li et al., 2021, 2016).

In October 2017, this fish farm was liquidated, and we had a unique opportunity to trace the dynamic restoration of the marine flora on coral reef damaged by the farm discharges. In our earlier work (Li et al., 2021), we documented a significant increase in species diversity, as well as a change in the composition of main taxonomic groups and life forms of the benthic flora in the investigated coastal area 1.5 years after the fish farm liquidation. In the present work, we aimed at studying possible changes in number and structure of benthic algal communities on the Luhuitou Peninsula coast after the elimination of the fish farm – the key source of extreme pollution for the vicinity.

MATERIAL AND METHODS

Study sites and conditions. Investigations were carried out at Luhuitou fringing reef, the Sanya Bay, Hainan Island, China. Hainan Island (Fig. 1) is located in the subtropical northern periphery of the Indo-Pacific Ocean, in the South China Sea. Main coastal ecosystems of Hainan Island shallow waters are those of coral reefs – one of the most well-known fringing reefs in China. However, almost 80 % of the fringing reefs along Hainan Island coastline were damaged because of intensive human activities in the 1970s–1990s – fishing with dynamite and coral mining for lime and construction. Recently, eutrophication of Hainan coastal waters, particularly in the shallow gulfs, increased due to growing tourist flow, hotel construction along the coast, and mariculture in coastal ponds and pools with wastes draining into the sea (Titlyanov et al., 2011).

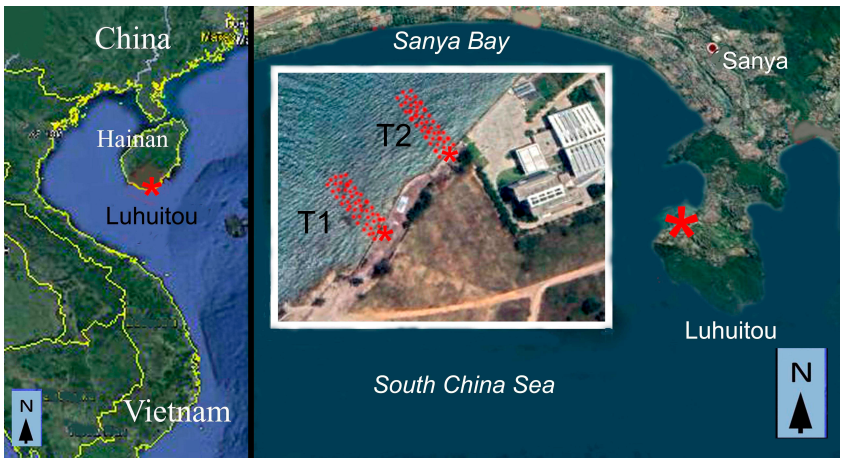

Fig. 1. Study sites on Hainan Island: T1, transect 1, opposite the former outlet of wastewater from the mariculture farm (ponds); T2, transect 2, located at the distance of 100 m from the transect 1

Sampling time and sites. Algae were sampled at the late dry season in March 2017 (while there was the fish farm), March 2018 (0.5 years after the fish farm elimination), and March 2019 (1.5 years after the elimination). In the study area, the dry season lasts from December-January to March-April. The main meteorological and hydrological characteristics of the study area during the dry seasons are given in Table 1.

Table 1. Concentrations of DIN and orthophosphates (μM) in the upper subtidal zone in the study sites at high tide on the first day of algal sampling in 2018 and 2019; * denotes mean data for 4 years (Li et al., 2016)

Transect	2013–2016*		2018		2019	
	DIN*	PO_4*	DIN	PO_4	DIN	PO_4
T1	31.3 ± 17.6	4.7 ± 3.1	2.65 ± 0.26	0.24 ± 0.03	3.05 ± 0.73	0.19 ± 0.01
T2	7.1 ± 2.2	1.0 ± 0.2	2.18 ± 0.34	0.19 ± 0.02	2.35 ± 0.86	0.19 ± 0.02

Algae were sampled on foot or *via* snorkeling from a depth of 0–2 m during low tides, along two transects from the upper intertidal to the upper subtidal zone (Figs 1, 2). Transects were laid perpendicular to a shore, and algae were sampled along these transects within the bottom area of 20–30 m × 50–70 m.

Transect 1 (hereinafter T1) was laid from the fish farm outlet; transect 2 (hereinafter T2) was located at the distance of ~ 100 m (along shoreline) from T1. Samples were taken from all the substrate types. To study the species composition of the benthic flora and taxonomic composition of algal communities, we used the methods of algal sampling and material processing described in (Titlyanov et al., 2019).

Along the transects, in each tidal zone, algal turf communities (with thalli less than 5 cm in height), crust algae, and large upright-growing algae (with thalli more than 5 cm in height) were visually identified. These communities were photographed at a right angle. In communities of algal turf and crust algae, samples were taken from three randomly selected areas, with each area of ~ 100 cm^2. In communities of upright-growing algae, samples were taken from three areas as well, with each ranging 0.5–1.0 m^2. Samples were taken from all the selected algal communities – in at least three quadrats from each community. A total of 54 macrophyte communities and blue-green algae were found; out of them, 162 samples were taken and analyzed; and out of them, 170 species of macrophytes and 13 species of blue-green algae were recorded (Li et al., 2021).

Sampling was carried out from the upper intertidal to the upper subtidal zone from all the substrate types [tidal zones were divided according to (Perestenko, 1980)]. At the investigated sites, the upper intertidal zone consisted of a sloping shore (2–3 m in width), with hard substrates composed of stones and dead coral fragments of various shapes and sizes tossed by storms. The sloping shore of the middle intertidal zone (~ 10 m in width) mainly consisted of flat carbonate patches interspersed with coral debris and stones. The lower intertidal zone (~ 15 m in width) was primarily composed of dead colonies of massive and branching corals interspersed with sand and small fragments of dead branching corals. The upper subtidal zone consisted of a sloping shore (~ 50 m in width) mainly composed of dead and live colonies of massive and branching corals interspersed with sand, stones, and dead coral fragments of various shapes and sizes.

Marine algae sampling, conservation, and identification. Sampling was carried out at each site from each tidal zone. Abundance was visually determined based on photographs of analyzed quadrats – by estimating the mean substrate surface area occupied by algae. The following indicators of abundance were used: rare sighting, found only one-two times with the relative substrata coverage

less than 10 %; common, recorded in most quadrats with the relative substrata coverage 10 to 50 %; and abundant, registered in communities with the relative substrata coverage 50 to 100 %. For the communities, dominance was also visually determined and defined as follows: monodominant, with one algal species occupying more than 50 % of the surface area; bidominant, with two species occupying more than 50 % of the surface area; and polydominant, with more than two species predominating.

Algae sampled from different communities were stored in separate plastic bags placed in a refrigerator for a processing time. Freshly sampled material was identified using monographic publications, results of floristic studies, and systematic articles indicated in (Titlyanova et al., 2014). The systematics and nomenclature followed (AlgaeBase, 2021). Hierarchical classification of the phylum Rhodophyta (hereinafter Rh) was carried out according to (Saunders & Hommersand, 2004). The classification system of phyla Chlorophyta (hereinafter Ch) and Ochrophyta (hereinafter Ph) followed (Tsuda, 2003, 2006). The collections of both macrophytes and their epiphytes were preserved as dried herbarium specimens and deposited in the herbarium at A. V. Zhirmunsky National Scientific Center of Marine Biology FEB RAS, Institute of Marine Biology (Vladivostok, Russian Federation).

Nutrient analysis. For nutrient analysis, bottom water samples were taken along T1 and T2 areas in the upper subtidal zones during high tide on the first day of algal sampling, immediately filtered through pre-weighed glass-fiber filters (Whatman GF/F, 47 mm), and frozen at –20 °C. DIN (NH_4, NO_3, and NO_2) and orthophosphates (PO_4) were photometrically analyzed using an autoanalyzer (model Skalar San Plus).

RESULTS

Differences in the number and structure of algal communities at variously polluted sites in March 2017. In the spring of 2017, under conditions of constant water discharge from cultivation ponds of the fish farm in the study area, differences were found in the number and structure of algal communities formed in shallow waters opposite the outlet (T1, Fig. 2A) and at the distance of 100 m from the outlet (T2, Fig. 3A).

In the upper intertidal zone along the T1 area, monodominant communities – those of *Wilsonosiphonia howei* (Hollenberg) D. Bustamante, Won & T. O. Cho, 2017 (Rh) (Fig. 2B) and *Cladophoropsis fasciculata* (Kjellman) Wille, 1910 (Ch) (Fig. 2C) – predominated. Moreover, in the T1 area, there were monodominant communities of common green algae *Ulva prolifera* O. F. Müller, 1778 and *Ulva clathrata* (Roth) C. Agardh, 1811; monodominant community of a brown crust alga *Neoralfsia expansa* (J. Agardh) P.-E. Lim & H. Kawai ex Cormaci & G. Furnari, 2012; and bidominant turf communities of *C. fasciculata* (Ch) + *W. howei* (Rh) and *U. prolifera* (Ch) + *W. howei* (Rh). Out of the species forming the communities, *Centroceras clavulatum* (C. Agardh) Montagne, 1846, *Gelidium pusillum* (Stackhouse) Le Jolis, 1863 (Rh), *Siphonogramen abbreviatum* (W. J. Gilbert) I. A. Abbott & Huisman, 2004, and *Rhizoclonium riparium* (Roth) Harvey, 1849 (Ch) were commonly found.

In the middle intertidal zone in the T1 area, monodominant communities – those of a green alga *Ulva flexuosa* Wulfen, 1803 (Fig. 2D), the red crust alga *Hildenbrandia rubra* (Sommerfelt) Meneghini, 1841, and the crustose brown alga *Ralfsia verrucosa* (Areschoug) Areschoug, 1845 (Fig. 2E) – predominated on a rocky bottom. Algal turf community of a red fine filamentous alga *C. clavulatum* (Fig. 2F) dominated on dead coral remnants. Here, the rest parts of silt-covered hard coral colonies were occupied by a monodominant community of a blue-green alga (hereinafter Cy) *Lyngbya majuscula* Harvey ex Gomont, 1892; the lower great part of hard substratum was overgrown with a bidominant community of green algae *Ulva lactuca* Linnaeus, 1753 + *Ulva fasciata* Delile, 1813.

In the lower intertidal zone along the T1 area, the surfaces of dead coral blocks were overgrown by a monodominant community of a red turf-forming alga *C. clavulatum*, with accompanying species *Acanthophora muscoides* (Linnaeus) Bory de Saint-Vincent, 1843, *Hypnea pannosa* J. Agardh, 1847, *Hypnea spinella* (C. Agardh) Kützing, 1847, *Spyridia filamentosa* (Wulfen) Harvey, 1833 (Rh), and *Caulerpa racemosa* (Forsskål) J. Agardh, 1873 (Ch) (Fig. 2G). This community occupied 90 % of substratum. Out of the algal turf, *Sargassum polycystum* (C. Agardh), 1924 (Ph), *Bryopsis pennata* J. V. Lamouroux, 1809, *U. lactuca* (Ch), and live colonies of massive hard corals were commonly found (Fig. 2H).

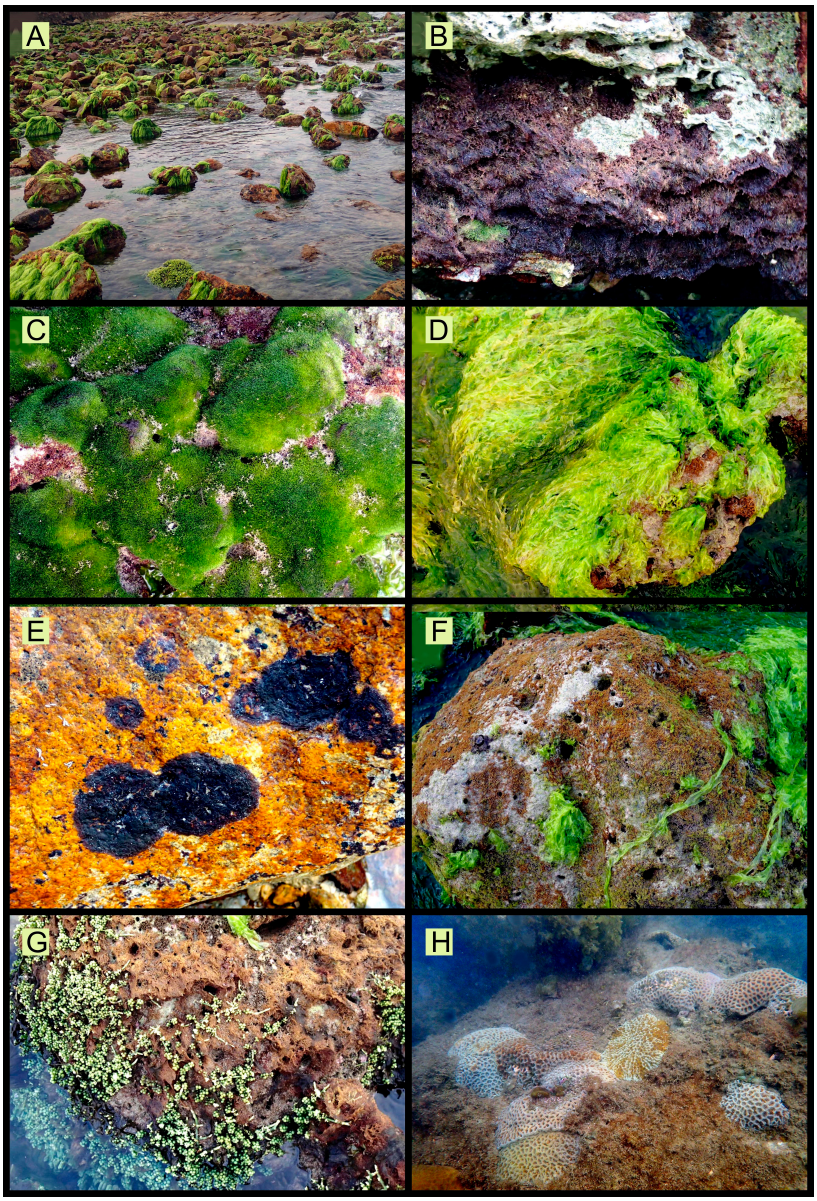

Fig. 2. Algal communities in the T1 area (heavily polluted site) in March 2017. A, the middle intertidal zone, the outlet area; B, the upper intertidal, monodominant community of a red alga *Wilsonosiphonia howei*; C, the upper intertidal, monodominant community of a green alga *Cladophoropsis fasciculata*; D, the middle intertidal, monodominant community of a green alga *Ulva flexuosa*; E, the middle intertidal, bidominant community of a red crust alga *Hildenbrandia rubra* and a brown crust alga *Ralfsia verrucosa*; F, the middle intertidal, monodominant community of the red alga *Centroceras clavulatum*; G, the lower intertidal, polydominant community of *C. clavulatum* with accompanying species *Acanthophora muscoides*, *Hypnea pannosa*, *Hypnea spinella*, *Spyridia filamentosa* (Rh), and *Caulerpa racemosa* (Ch); H, the upper subtidal, polydominant community of *C. clavulatum*, *H. pannosa*, *Hypnea valentiae*, *Jania adhaerens* (Rh), and *C. racemosa* (Ch) among young colonies of massive hermatypic corals

In the upper intertidal zone along the T2 area (Fig. 3A), at the same time, only three monodominant communities – those of *U. prolifera* (Ch) (Fig. 3B), *W. howei* (Rh) (Fig. 3C), and *N. expansa* (Ph) (Fig. 3D) – were common. Out of the species forming the communities, *C. fasciculata*, *U. clathrata* (Ch), and *C. clavulatum* (Rh) were found as well.

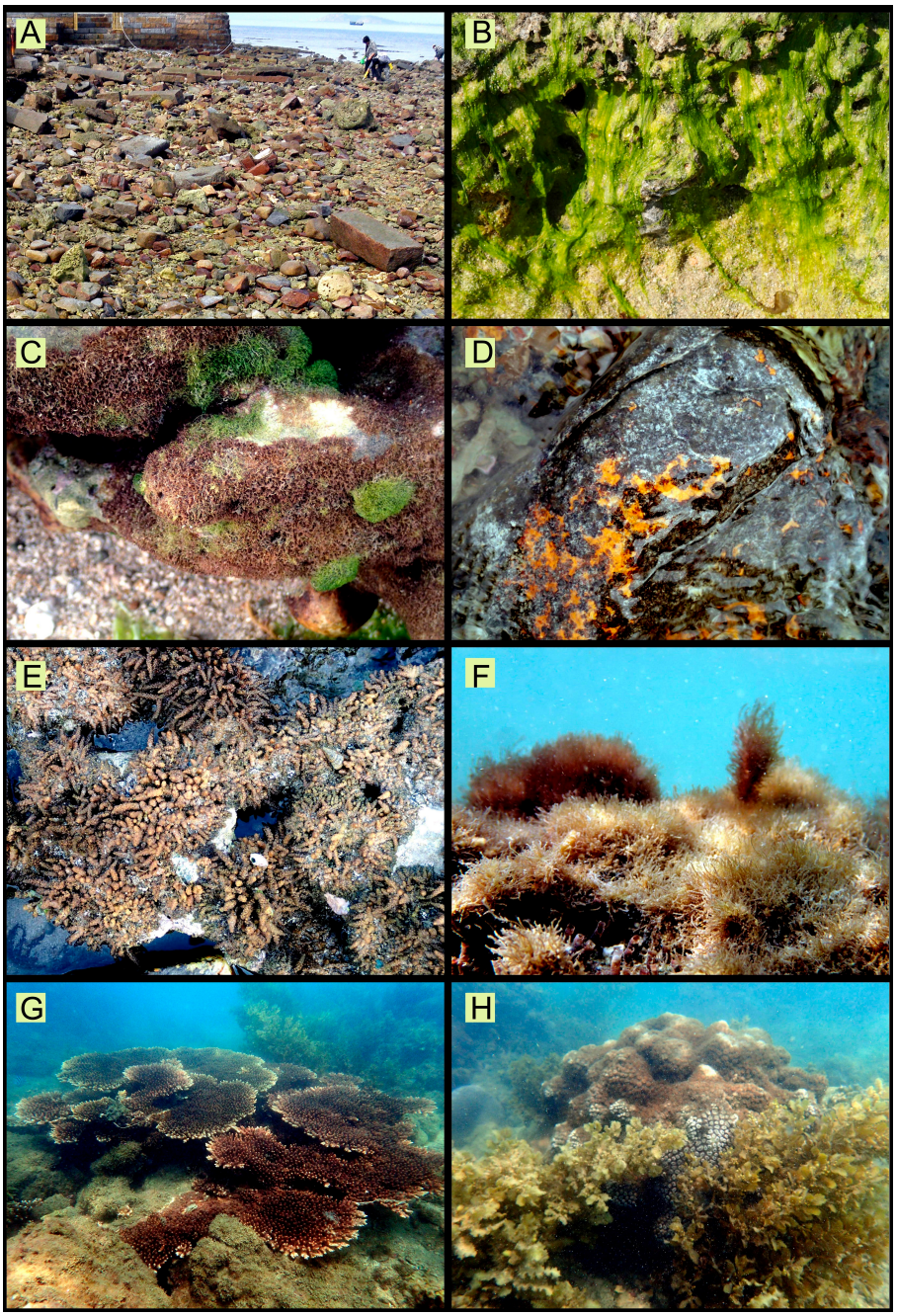

Fig. 3. Algal communities along the T2 area (moderately polluted area) in March 2017. A, the upper intertidal zone near the outlet at low tide; B, the upper intertidal, monodominant community of *Ulva flexuosa* (Ch); C, the upper intertidal, bidominant community of *Wilsonosiphonia howei* (Rh) + *Cladophoropsis fasciculata* (Ch); D, the middle intertidal, bidominant community of the red crust alga *Hildenbrandia rubra* and the brown crust alga *Ralfsia verrucosa*; E, the middle intertidal, mosaic polydominant community with a dominance of *Palisada perforata*, *Centroceras clavulatum*, and *Gelidiella bornetii* (Rh); F, the middle intertidal, polydominant turf community with a mosaic dominance of *Amphiroa fragilissima*, *C. clavulatum*, and *Jania adhaerens* (Rh); G, the upper subtidal, hermatypic corals and polydominant community of *C. clavulatum* (Rh) with accompanying species; H, lower intertidal to upper subtidal, with *Sargassum ilicifolium*, *S. polycystum*, and *S. sanyaense* (Ph) forming dense bed

In the middle intertidal zone along the T2 area, a mosaic polydominant community predominated occupying mainly a hard base of a flat carbonate substrate (Fig. 3E); the following species dominated – *Palisada perforata* (Bory) K. W. Nam, 2007, *C. clavulatum*, *Gelidiella bornetii* (Weber-van Bosse) Feldmann & C. Hamel, 1934 (Rh), *L. majuscula* (Cy), and *Lobophora variegata* (J. V. Lamouroux) Womersley ex Oliveira, 1977 (Ph) – growing on vertical surfaces of reef bases and coral blocks. Monodominant communities of the red alga *H. rubra* and the brown alga *R. verrucosa* occupied rocky substratum (as in the T1 area).

In the lower intertidal zone along the T2 area, a mosaic polydominant community of turf-forming algae overgrew dead coral blocks (Fig. 3F), with a mosaic dominance of *Amphiroa fragilissima* (Linnaeus) J. V. Lamouroux, 1816, *C. clavulatum*, *S. filamentosa*, *Hypnea valentiae* (Turner) Montagne, 1841, *Jania adhaerens* J. V. Lamouroux, 1816 (Rh), *Padina minor* Yamada, 1925 (Ph), and *Dictyosphaeria cavernosa* (Forsskål) Børgesen, 1932 (Ch). The green alga *C. racemosa* represented an often-overgrowing polydominant community of algal turf occupying silt- and sand-covered hard substrata. Upright-growing brown algae with large thalli of genera *Dictyota*, *Padina*, *Sargassum*, and *Turbinaria* were commonly found in the communities and on free substrata.

In the upper subtidal zone along the T2 area, hard substrata were occupied by hermatypic corals with coverage of ~ 50 %, and the rest surface of carbonate reef basis was overgrown by algal communities, primarily by polydominant mosaic algal turf communities with the following dominant species: *C. clavulatum*, *H. pannosa*, *H. valentiae*, *J. adhaerens*, and *S. filamentosa* (Rh) (Fig. 3G). A monodominant community of the green alga *C. racemosa* occupied ~ 10 % of the sand-covered hard substratum (coral reef base). *Sargassum ilicifolium* (Turner) C. Agardh, 1820, *S. polycystum*, and *Sargassum sanyaense* Tseng & Lu, 1997 (Ph) formed dense bed from the low intertidal zone to the upper subtidal zone (Fig. 3H).

Dynamic changes in the structure of algal communities in variously polluted sites after cessation of the discharge of waste from the fish farm. The transect 1, 2018. Six months after the fish farm liquidation, significant changes occurred in the structure and diversity of algal communities (Fig. 4).

In the upper intertidal zone, vertical walls of rocky boulders were partially occupied by monodominant communities (as in 2017) – those of *U. prolifera* (Ch) and *R. verrucosa* (Ph). Small niches of a stone retaining wall were overgrown by a new community – the red alga *Bostrychia tenella* (J. V. Lamouroux) J. Agardh, 1863 and the green alga *S. abbreviatum* with accompanying *R. riparium* (Fig. 4B).

The red alga *W. howei* which formed a dense monodominant community in these niches earlier was rare. Among epiphytes, *R. riparium* (Ch) and *Hydrolithon farinosum* (J. V. Lamouroux) D. Penrose & Y. M. Chamberlain, 1993 (Rh) dominated, as well as blue-green algae *Chroococcus turgidus* (Kützing) Nägeli, 1849 and *Stanieria sphaerica* (Setchell & N. L. Gardner) Anagnostidis & Pantazidou, 1991.

In the middle intertidal zone along the T1 area, stones were occupied by monodominant crust communities of *H. rubra* (Rh) and *N. expansa* (Ph) (as in 2017). Fossil reef base was overgrown by a community of the blue-green alga *L. majuscula* formed in 2017. A polydominant community of algal turf – with a dominance of *Millerella pannosa* (Feldmann) G. H. Boo & L. Le Gall, 2016 (Rh), *R. riparium*, and *U. clathrata* (Ch) (Fig. 4C) – covered remnants of massive coral colonies. Here, we also found *C. fasciculata*, *Chaetomorpha linum* (O. F. Müller) Kützing, 1845 (Ch), and *Coleofasciculus chthonoplastes* (Thuret ex Gomont) M. Siegesmund, J. R. Johansen & T. Friedl, 2008 (Cy), as well as epiphytes – *Erythrotrichia carnea* (Thuret ex Gomont) M. Siegesmund, J. R. Johansen & T. Friedl, 1883 (Rh) and *Myrionema strangulans* Greville, 1827 (Ph). On some flat rocks, a monodominant community of *W. howei* (Rh) was registered (Fig. 4D).

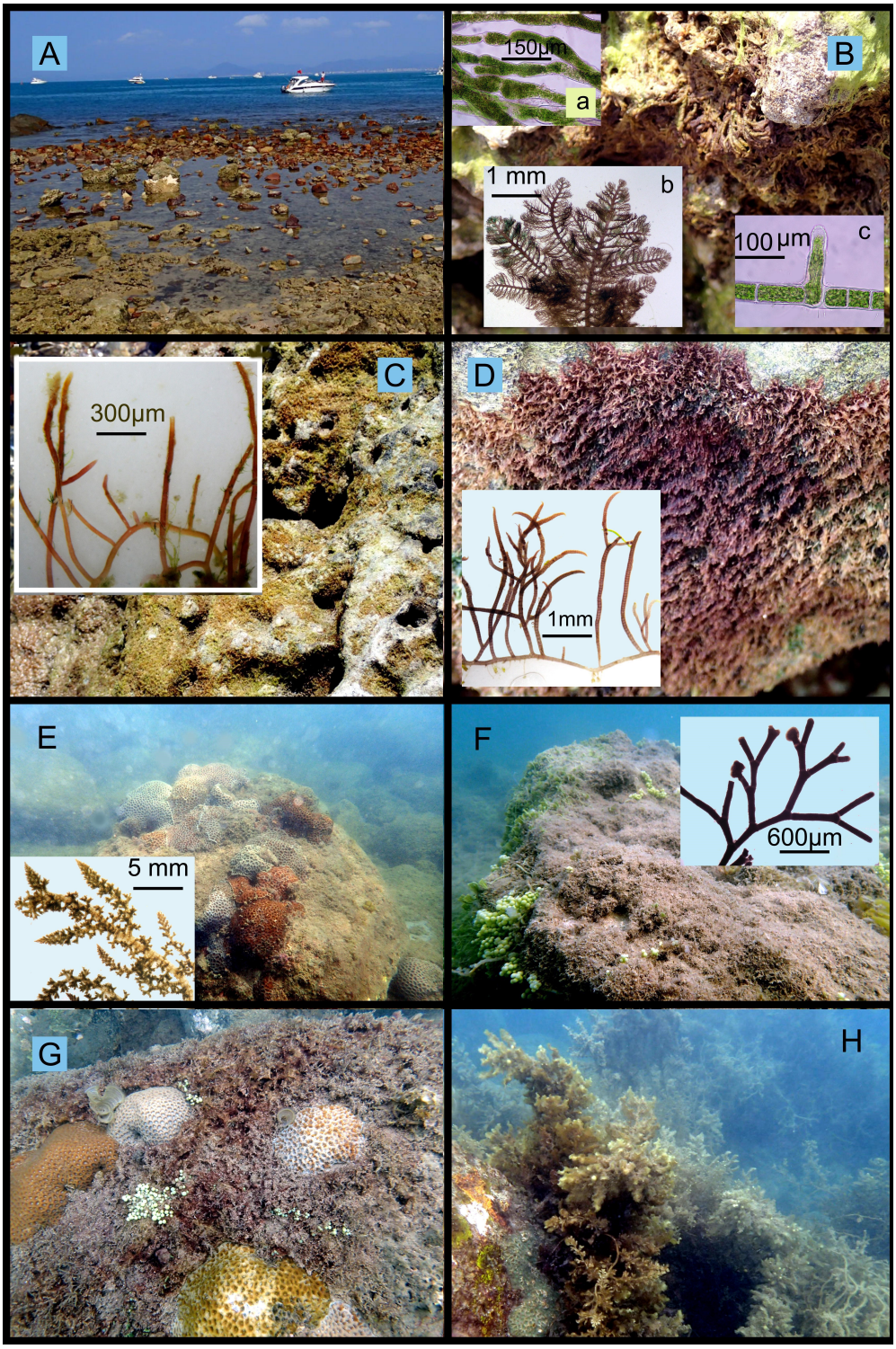

Fig. 4. Algal communities along the T1 area in March 2018. A, the middle intertidal zone opposite the former outlet of the fish farm; B, the upper intertidal, bidominant community of *Siphonogramen abbreviatum* (Ch) (insert **a**) + *Bostrychia tenella* (Rh) (insert **b**), with an epiphytic alga *Rhizoclonium riparium* (Ch) (insert **c**); C, the middle intertidal, polydominant community with a dominance of the red alga *Millerella pannosa* (insert) and green algae *Ulva clathrata* and *R. riparium*; D, the middle intertidal, monodominant community of *Wilsonosiphonia howei* (Rh); E and F, the lower intertidal, polydominant community with a dominance of *Tolypiocladia glomerulata* (E, insert), *Jania adhaerens* (F, insert), *Centroceras clavulatum*, and *Gelidium pusillum* var. *cylindricum* (Rh); G, the upper subtidal, polydominant community of algal turf with a dominance of *J. adhaerens*, *C. clavulatum*, *Asparagopsis taxiformis*, and *T. glomerulata* (Rh); H, the upper subtidal, *Sargassum polycystum* (Ph) thickets

In the lower intertidal zone along the T1 area, the remnants of coral colonies were overgrown by a polydominant community (as in 2017), but with other species predominating – *Tolypiocladia glomerulata* (C. Agardh) F. Schmitz, 1897, *J. adhaerens*, *C. clavulatum*, and *Gelidium pusillum* var. *cylindricum* W. R. Taylor, 1945 – and with common species accompanying – *M. pannosa*, *H. spinella*, *Melanothamnus ferulaceus* (Suhr ex J. Agardh) Diaz-Tapia & Maggs, 2017, *Caulacanthus ustulatus* (Mertens ex Turner) Kützing, 1843 (Rh), *Sphacelaria rigidula* Kützing, 1843 (Ph), and *C. linum* (Ch) (Fig. 4E, F). Out of the species forming the community, *R. verrucosa*, *Sphacelaria novae-hollandiae* Sonder, 1845, *P. minor*, and *S. polycystum* (Ph) were common.

In the upper subtidal zone along the T1 area in 2018, as in 2017, a polydominant algal turf community dominated, with different composition of dominating species [*J. adhaerens*, *C. clavulatum*, *Asparagopsis taxiformis* (Delile) Trevisan de Saint-Léon, 1845, and *T. glomerulata*], as well as accompanying species of epilithic algae [*Peyssonnelia rubra* (Greville) J. Agardh, 1851 and *S. filamentosa*] and epiphytes [*Herposiphonia tenella* (C. Agardh) Ambronn, 1880, *Gayliella mazoyerae* T. O. Cho, Fredericq & Hommersand, 2008, *Melanothamnus savatieri* (Hariot) Díaz-Tapia & Maggs, 2017, and *Wrangelia argus* (Montagne) Montagne, 1856 (Rh)] (Fig. 4G). Out of the species forming the community, *S. polycystum* (Ph) and *C. racemosa* (Ch) were common (Fig. 4H).

Transect 1, 2019. In the spring of 2019, 1.5 years after the fish farm elimination, some alterations in the marine flora were detected in the intertidal and upper subtidal zones compared with the spring of 2018.

In the upper intertidal zone, rocky boulders, as always, were occupied by a monodominant community of the crust alga *N. expansa* (Ph). In niches of these boulders, a bidominant community of *W. howei* (with the blue-green epiphytic alga *C. chthonoplastes*) + *B. tenella* (Rh) and a bidominant community of *P. howei* (Rh) + *C. fasciculata* (Ch) [with accompanying *Bostrychia* sp. (Rh), *Rhizoclonium grande* Børgesen, 1935 (Ch), *S. abbreviatum* (Ch), and *Ceramium camouii* E. Y. Dawson, 1944 (Rh)] dominated. Moreover, the fossil reef base was covered with black film composed of blue-green algae – *Kyrtuthrix maculans* (Gomont) I. Umezaki, 1958, *C. chthonoplastes*, *Scytonematopsis crustacea* (Thuret ex Bornet & Flahault) Kováli k & Komárek, 1988, and *C. turgidus*.

In the middle intertidal zone, some alterations were recorded as well. The fossil carbonate base was covered by a dense mat of blue-green algae, with a dominance of *Lyngbya sordida* Gomont, 1892, *Lyngbya martensiana* Meneghini ex Gomont, 1892, and *K. maculans*. In a polydominant community of algal turf, the composition of dominant species changed as well. There, dominant species were *M. pannosa*, *P. howei* (Rh), *C. fasciculata*, and *R. grande* (Ch). Common algal species were *P. minor* (Ph), *Ceratodictyon intricatum* (C. Agardh) R. E. Norris, 1987, and *Jania capillacea* Harvey, 1853 (Rh), as well as an epiphyte *S. crustacea* and accompanying blue-green algae *C. chthonoplastes* and *K. maculans*.

In the lower intertidal zone, a polydominant algal turf community was enriched with new dominants species [*C. ustulatus* (Rh) and *S. novae-hollandiae* (Ph)] and with accompanying ones [*J. adhaerens*, *Herposiphonia secunda* (C. Agardh) Ambronn, 1880, *S. filamentosa*, *Pterocladiella caerulescens* (Kützing) Santelices & Hommersand, 1997, *H. spinella* (Rh), *S. rigidula*, *L. variegata* (Ph), *Anadyomene wrightii* Harvey ex J. E. Gray, 1866, and *C. racemosa* (Ch)]. Out of epiphytes, the most common ones were *E. carnea*, *Sahlingia subintegra* (Rosenvinge) Kornmann, 1989, *Acrochaetium microscopicum* (Nägeli ex Kützing) Nägeli, 1858, *H. farinosum*, *Ceramium aduncum* Nakamura, 1950, *Ceramium cimbricum*

H. E. Petersen, 1924, *Ceramium vagans* P. C. Silva, 1987, *G. mazoyerae*, and *M. ferulaceus* (Rh). Out of the algal turf, *Padina australis* Hauck, 1887 and *S. polycystum* (Ph) were the species forming upright-growing communities on remnants of coral colonies.

In the upper subtidal zone, a mosaic polydominant algal turf community occupied all substrata between colonies of live corals. *J. adhaerens*, *T. glomerulata*, *H. spinella* (Rh), and *C. racemosa* (Ch) were the main dominant species. *P. australis*, *S. polycystum*, and *S. sanyaense* (Ph) were common ones. The richness and species composition of epiphytes in the upper subtidal zone were similar to those of the lower intertidal zone.

Transect 2, 2018 and 2019. Alterations in the marine flora along the T2 area were registered only in the structure of polydominant communities. The composition of dominant and accompanying species changed only partially. The diversity and structure of mono- and bidominant communities remained the same as in 2017.

In 2018, the composition of dominant species changed in polydominant communities in the middle intertidal and upper subtidal zones. Specifically, in the middle intertidal zone, dominant species – *P. perforata* (Rh), *L. variegata* (Ph), and *C. racemosa* (Ch) – were not found, while *H. pannosa*, *H. spinella* (Rh), and *Caulerpa sertularioides* (S. G. Gmelin) M. Howe, 1905 (Ch) appeared. In the upper subtidal zone, dominant species – *A. fragilissima*, *J. adhaerens*, and *H. valentiae* (Rh) – were not registered (as it was before), while *J. capillacea*, *H. secunda*, *P. caerulescens* (Rh), and communities of upright-growing *S. sanyaense* and *P. australis* (Ph) appeared.

In 2019, insignificant changes in the flora along T2 were recorded only in the composition of dominant species in polydominant communities.

DISCUSSION

Adaptation of the coral reef ecosystem to moderate and extremely high nutrient concentrations. Earlier, we showed that DIN and orthophosphate levels in seawater of Luhuitou and Xiaodong Hai reefs (as most likely across all the Sanya Bay) are higher (3–5-fold and 10-fold, respectively) than those in clean waters of insular coral reefs (Titlyanov et al., 2011). About the same DIN and orthophosphate levels were noted as threshold concentrations for degradation of coral reefs resulting from eutrophication and subsequent macroalgal blooms at Kaneohe Bay in Hawaii, fringing reefs of Barbados, and inshore reefs within the lagoons of the Great Barrier Reef (Bell, 1992 ; Done, 1929 ; Hughes, 1994 ; Lapointe et al., 1997 ; Lapointe, 1997 ; Smith et al., 1981). In coral reefs, the concentrations of nutrients above the threshold ones are reported to induce growth and accumulation of biomass by frondose macroalgae provoking superabundant macroalgal blooms. Evidently, reefs exposed to chronic nutrient enrichment increase their primary productivity which can be mainly attributed to expansion of macroalgae.

Our previous floristic surveys at the Sanya Bay (Titlyanov et al., 2011, 2019) showed that this site is occupied by algal communities and species typical for healthy coral reefs. At the same time, bloom of green benthic macroalgae was observed in a few local areas of the Sanya Bay coast (Li et al., 2016). Moreover, Luhuitou reef is characterized by a high species diversity of hermatypic corals; among them, there are branching corals of genera *Acropora* and *Pocillopora* – indicators of healthy reefs (Fong & Paul, 2011 ; Littler et al., 2006 ; McManus & Polsenberg, 2004 ; Raffaelli et al., 1998 ; Rosenberg, 1985). In our opinion, the Luhuitou coral reef ecosystem in most sites of the coast has adapted to conditions of increased (moderate) nutrient concentration. It is currently stable; there are no signs of degradation,

except for spots with heavy pollution, for example, the area of water flow from fish ponds. In the latter case, corals could lose their competitive ability in the struggle for the substrate and give way to highly productive algal species, and the coral reef might eventually turn into a "plant reef". However, our monitoring studies of the benthic flora in the area of constant heavy pollution by nutrients (2012–2017) did not reveal signs of ongoing degradation of the coral reef (alterations either in diversity or species composition of macrophytes and mass species of hermatypic corals) and its turning into a "plant reef" (Li et al., 2021, 2016). This gives reason to assume that the ecosystem of coral reefs can adapt to extremely high concentrations of nutrients. The main adaptive changes in the ecosystem to heavy pollution could be summarized as follows:

1) biomass of green algae in the upper and middle intertidal zones (exposed to air at low tide) and brown algae in the submerged zone (lower intertidal) increased significantly (by several times);
2) species diversity of green algae in the upper and middle intertidal zones increased, while species diversity of brown and red algae in the submerged zone decreased;
3) number of mono- and bidominant communities of algae in the upper intertidal zone increased, while polydominant communities in the middle intertidal zone disappeared;
4) in the communities of the upper and middle intertidal zones, absolute and relative numbers of dominant species in green algal communities increased, while the number of dominant red algae decreased over a range of tidal zones;
5) in polydominant communities, the species composition of both dominant and accompanying species changed.

Some of the listed above changes in the flora that occurred during nutrient water pollution were previously known, such as accumulation of green algal biomass (Fong & Paul, 2011 ; Lapointe, 1997 ; Littler et al., 2006 ; Raffaelli et al., 1998 ; Rosenberg, 1985) and increase in diversity (Li et al., 2021), while other alterations were recorded for the first time. The significance of these changes representing the ecosystem homeostasis could be ascertained only with a further long-term study of the reef ecosystem under conditions of heavy pollution.

Changes in the ecosystem under sharp decrease in nutrient concentration from heavy to moderate. In (Li et al., 2021), it is shown as follows: after the fish farm liquidation, concentration of nutrients in seawater opposite the outlet (T1) and at 100 m from it (T2) dropped by more than an order of magnitude; it is almost equal to the mean value for the Sanya Bay (Li, 2011 ; Li et al., 2016). At the same time, the values of indicators of other major environmental factors in 2017, 2018, and 2019 did not differ significantly.

In parallel, the following main alterations in the flora were recorded during the transition from heavy to moderate water enrichment with nutrients: for a year and a half, the taxonomic composition changed, the relative number of red algae increased, and the relative number of green algae decreased. The maximum similarity of the flora for T1 and T2 areas increased after the farm liquidation by 18 % and reached the value of 82 %. These alterations occurred mainly due to enrichment of the local benthic flora with unproductive annual species of red algae and depletion of highly productive species of green algae – ephemeral filamentous and membrane forms. These changes in species and taxonomic composition of the flora resulted in a decrease in the number of dominant species (mainly green o nes) and a sharp (even 6 months after the farm elimination) decrease in the mass of vegetation cover (Li et al., 2021).

As shown in this paper, within a year and a half after the fish farm liquidation, changes occurred in the number and structure of algal communities. Specifically, in the intertidal zone, the number of monodominant and bidominant algal turf communities decreased; in the middle intertidal zone, a polydominant algal turf community was formed; and in the lower intertidal and upper subtidal zones, the composition of dominant and accompanying species partially changed.

Along the T2 area (moderate pollution), alterations in the marine flora for 1.5 years were barely noticeable, and the only significant interannual change was registered in the composition of dominant and accompanying species in polydominant communities. Nature and dynamics of changes in the benthic flora along T1 give reason to talk about the adaptation of the ecosystem to new conditions of mineral nutrition by establishing homeostasis.

Conclusion. Our current findings once again confirmed our previously obtained data that the benthic flora in the Sanya Bay greatly varies in diversity, species composition, taxonomic composition, and the structure of algal communities in variously polluted coastal areas. Extremely high concentrations of nutrients in seawater near the outlet of polluted wastewater caused significant depletion in species diversity and abundance of unproductive annual and perennial red and brown algae, as well as enrichment of highly productive green species with opportunistic and often ephemeral algae. For the first time, we showed that a sharp decrease in nutrient concentration near the fish farm one year and a half after its liquidation resulted in a partial-to-complete restoration of macroalgal species diversity. We assumed that coral reef ecosystems on Hainan Island in areas with various (even extreme) nutrient pollution adapted to these conditions.

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СООБЩЕСТВА БЕНТОСНЫХ ВОДОРОСЛЕЙ КОРАЛЛОВЫХ РИФОВ ЗАЛИВА САНЬЯ (ОСТРОВ ХАЙНАНЬ, КИТАЙ) В МЕСТАХ, СИЛЬНО ЗАГРЯЗНЁННЫХ БИОГЕННЫМИ ВЕЩЕСТВАМИ, И ИХ ИЗМЕНЕНИЯ ПОСЛЕ УСТРАНЕНИЯ ИСТОЧНИКА ЗАГРЯЗНЕНИЯ

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Ранее установлено, что экстремально высокие концентрации биогенных веществ в морской воде в районе стока загрязнённых вод рыбной фермы на полуострове Лухуэйтоу (залив Санья) приводят к значительному сокращению видового разнообразия и обилия низкопродуктивных одно- и многолетних красных и бурых водорослей, а также к увеличению количества и биомассы высокопродуктивных зелёных водорослей. С 2017 по 2019 г. впервые были изучены изменения в количестве и структуре донных водорослевых сообществ в приливно-отливных зонах залива Санья после устранения источника загрязнения — ликвидации рыбной фермы. Показано, что снижение концентрации растворённого неорганического азота (DIN) (с ~ 20 до 2,5 μM) и ортофосфатов (с 5,0 до 0,2 μM) в морской воде существенно изменило разнообразие, видовой состав и структуру бентосных водорослевых сообществ. Через 1,5 года после ликвидации источника загрязнения основные показатели флоры стали близки в среднем к таковым умеренно загрязнённых участков залива Санья.

Ключевые слова: водоросли, остров Хайнань, Китай, эвтрофикация, восстановление

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COMPETITIVE ADVANTAGES OF THE DIATOM *SKELETONEMA COSTATUM* CLEVE, 1873 IN THE BLACK SEA IN THE WINTER–SPRING PERIOD

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Individual physiological features of the *Skeletonema costatum* vegetation under low light intensity and low temperature are described; these peculiarities allow the species to prevail in the Black Sea phytoplankton in winter and early spring. This marine diatom is characterized by high growth efficiency under light-limiting conditions (0.13 $day^{-1}\cdot(\mu E\cdot m^{-2}\cdot s^{-1})^{-1}$) which indicates an increase in the specific growth rate of the alga with a rise in light intensity by 1 $\mu E\cdot m^{-2}\cdot s^{-1}$. Moreover, the species is characterized by low values of the light intensity saturating the growth – 12 $\mu E\cdot m^{-2}\cdot s^{-1}$ at +5 °C and 18 $\mu E\cdot m^{-2}\cdot s^{-1}$ at +10 °C. At +5…+10 °C, *S. costatum* growth rate is about 2 times higher than that of other representatives of the Black Sea phytoplankton in the winter–spring period. This diatom shows increased sensitivity to high light intensity: at +10 °C, photoinhibition of microalgae growth is observed under light intensity above 120 $\mu E\cdot m^{-2}\cdot s^{-1}$.

Keywords: diatoms, *Skeletonema costatum*, light intensity, temperature

The diatom *Skeletonema costatum* Cleve, 1873 is one of the prevailing representatives of the Black Sea phytoplankton in the winter–spring period when, according to field observations, its bloom occurs in coastal areas. A study of the species diversity of microalgae in Crimean coastal waters carried out by M. Senicheva in 1983–2006 showed as follows: in years with colder winter (temperature below +8 °C) with intense convective mixing of water, *S. costatum* contribution to the total phytoplankton biomass can reach 95–98 %. The species development peaks in early spring (February–March); during these months, the temperature minimum (+6…+8 °C) is recorded, as well as the maximum concentration of mineral salts. In years with warmer winter (+8…+12 °C) and less intense convective mixing of water, the species diversity of algae increases significantly, but *S. costatum* remains one of the prevailing species (Senicheva, 2008). According to Yu. Bryantseva (2008), in January and February 2004–2006, *S. costatum* contribution to the total phytoplankton abundance in the Sevastopol Bay varied within 89–94 %.

Apparently, *S. costatum* prevalence under low light intensity and low temperature is due to certain competitive advantages of this diatom over other phytoplankton representatives: those allow the species to prevail in sea during winter and early spring. The results of our own complex experimental

studies [their methodological aspects are presented in (Akimov & Solomonova, 2019 ; Shoman & Akimov, 2012, 2015)] allowed to identify several individual physiological features of the species during cultivation under low light intensity and low temperature:

1. *S. costatum* is characterized by high growth efficiency (α) under light-limiting conditions – 0.13 $day^{-1}\cdot(\mu E\cdot m^{-2}\cdot s^{-1})^{-1}$. This parameter is not temperature-dependent within a range of +8…+20 °C. The described value reflects an increase in algal specific growth rate under light limitation with a rise in the light intensity by 1 $\mu E\cdot m^{-2}\cdot s^{-1}$. According to the results of one of the recent reviews (Edwards et al., 2015), the growth efficiency of different phytoplankton representatives under light limitation varies within 0.001–0.1 $day^{-1}\cdot(\mu E\cdot m^{-2}\cdot s^{-1})^{-1}$; at the same time, the prevailing ratio of the α values in diatoms covers a range of 0.015–0.03 $day^{-1}\cdot(\mu E\cdot m^{-2}\cdot s^{-1})^{-1}$. Considering that, the value of *S. costatum* growth efficiency is comparable with the maximums recorded in microalgae in total.
2. *S. costatum* has lower values of the light intensity saturating the growth (Ik) than other diatom species. Thus, at a temperature of +5 °C, the Ik value for *S. costatum* was 12 $\mu E\cdot m^{-2}\cdot s^{-1}$, and at +10 °C, it was 18 $\mu E\cdot m^{-2}\cdot s^{-1}$. At +15 °C, the light dependence of the growth rate reached the plateau under the light intensity of 24 $\mu E\cdot m^{-2}\cdot s^{-1}$. For comparison: for *Phaeodactylum tricornutum* Bohlin, 1897 and *Nitzschia* sp. No. 3, the Ik values under similar growth conditions at +10 °C were 40 and 33 $\mu E\cdot m^{-2}\cdot s^{-1}$, respectively (Shoman & Akimov, 2012). According to literature data, the saturation of the growth rate for diatoms at the optimal temperature (+18…+22 °C) is recorded at an average level of 84 $\mu E\cdot m^{-2}\cdot s^{-1}$ (Richardson et al., 1983). Having data on approximately a twofold decrease in the Ik value at +10 °C, we can conclude that it decreases to 40 $\mu E\cdot m^{-2}\cdot s^{-1}$. The minimum values of photosynthetically active radiation incident on the sea surface in January–February are 4–5 $E\cdot m^{-2}\cdot day^{-1}$. Due to a large extent of the upper quasi-homogeneous layer (about 30 m) during late winter, the mean light intensity there does not exceed 2 $E\cdot m^{-2}\cdot day^{-1}$ (≈ 25 $\mu E\cdot m^{-2}\cdot s^{-1}$) (Finenko et al., 2018). Thereby, in January–February, phytoplankton exists under conditions of a temperature minimum, low light intensity, and extended upper quasi-homogeneous layer.
3. *S. costatum* is characterized by a high growth rate at low temperatures. Specifically, at +5…+10 °C, its growth rate is approximately twice as high as that of other representatives of the diatom complex of the winter–spring succession in the Black Sea phytoplankton. So, the experiments showed as follows: within the specified temperature range, other growth conditions being equal, *S. costatum* specific growth rate is 0.9–1.5 day^{-1}, while in *Chaetoceros curvisetus* Cleve, 1889, *Cylindrotheca closterium* (Ehrenberg) Reimann & J. C. Lewin, 1964, *Thalassiosira parva* Proschkina-Lavrenko, 1955, and *Ditylum brightwellii* (T. West) Grunow, 1885, this value is 0.3–0.8 day^{-1} (Akimov & Solomonova, 2019).
4. *S. costatum* shows an increased sensitivity to high light intensity. Under the conditions of a laboratory experiment at a temperature of +15 °C, light inhibition of algal growth begins to manifest itself at a light intensity above 140 $\mu E\cdot m^{-2}\cdot s^{-1}$, and at +10 °C, above 120 $\mu E\cdot m^{-2}\cdot s^{-1}$. As known, *S. costatum* development peaks in early spring (Bryantseva, 2008). In April, a shift in growth conditions (an increase in photosynthetically active radiation, rise in temperature, and beginning of a temperature water stratification) results in a change in the species composition of the phytoplankton community and a significant increase in its diversity. During the winter–spring succession in the Black Sea phytoplankton in years with warmer spring, cold-loving small-celled diatom species are replaced in April–May by more thermophilic ones – *Chaetoceros curvisetus*, *Chaetoceros affinis* Lauder, 1864,

Pseudo-nitzschia delicatissima (Cleve) Heiden, 1928, *Proboscia alata* (Brightwell) Sundström, 1986, and *Dactyliosolen fragilissimus* (Bergon) Hasle, 1996. At the same time, in years with colder spring, *S. costatum* can prevail in plankton until late May (Senicheva, 2008). Thus, the combination of light intensity conditions and temperature conditions observed in the Black Sea in April is unfavorable for *S. costatum*: it results in a significant decrease in the algal growth rate and, apparently, is one of the reasons for the replacement of *S. costatum* by other algae species in mid-spring.

Conclusion. Main competitive advantages of *Skeletonema costatum* during vegetation under low light intensity and low temperature are high growth efficiency (0.13 $day^{-1}\cdot(\mu E\cdot m^{-2}\cdot s^{-1})^{-1}$), low values of the light intensity saturating the growth (12 $\mu E\cdot m^{-2}\cdot s^{-1}$ at +5 °C and 18 $\mu E\cdot m^{-2}\cdot s^{-1}$ at +10 °C), and high specific growth rate at low temperature (0.9–1.5 day^{-1} at +5…+10 °C). Along with low competition, this creates the most favorable conditions for *S. costatum* development in the Black Sea in winter and early spring.

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КОНКУРЕНТНЫЕ ПРЕИМУЩЕСТВА ДИАТОМОВОЙ ВОДОРОСЛИ *SKELETONEMA COSTATUM* CLEVE, 1873 В ЧЁРНОМ МОРЕ В ЗИМНЕ-ВЕСЕННИЙ ПЕРИОД

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Описаны индивидуальные физиологические особенности вегетации морской диатомовой микроводоросли *Skeletonema costatum* в условиях низкой освещённости и низкой температуры, позволяющие ей занимать доминирующую позицию в фитопланктоне Чёрного моря в зимний и ранневесенний период. Показано, что для *S. costatum* характерна высокая эффективность роста в условиях светового лимитирования (0,13 сут$^{-1}$·(мкЭ·м$^{-2}$·с$^{-1}$)$^{-1}$), отражающая увеличение удельной скорости роста водорослей при повышении интенсивности света на 1 мкЭ·м$^{-2}$·с$^{-1}$, а также низкие значения насыщающей рост интенсивности света (12 мкЭ·м$^{-2}$·с$^{-1}$ при температуре +5 °С и 18 мкЭ·м$^{-2}$·с$^{-1}$ при +10 °С). При +5…+10 °С скорость роста *S. costatum* примерно в 2 раза выше, чем у других представителей фитопланктона Чёрного моря в зимне-весенний период. Для *S. costatum* характерна повышенная чувствительность к свету высокой интенсивности: при +10 °С фотоингибирование роста микроводоросли отмечено при интенсивности света выше 120 мкЭ·м$^{-2}$·с$^{-1}$.

Ключевые слова: диатомовые водоросли, *Skeletonema costatum*, интенсивность света, температура

1871
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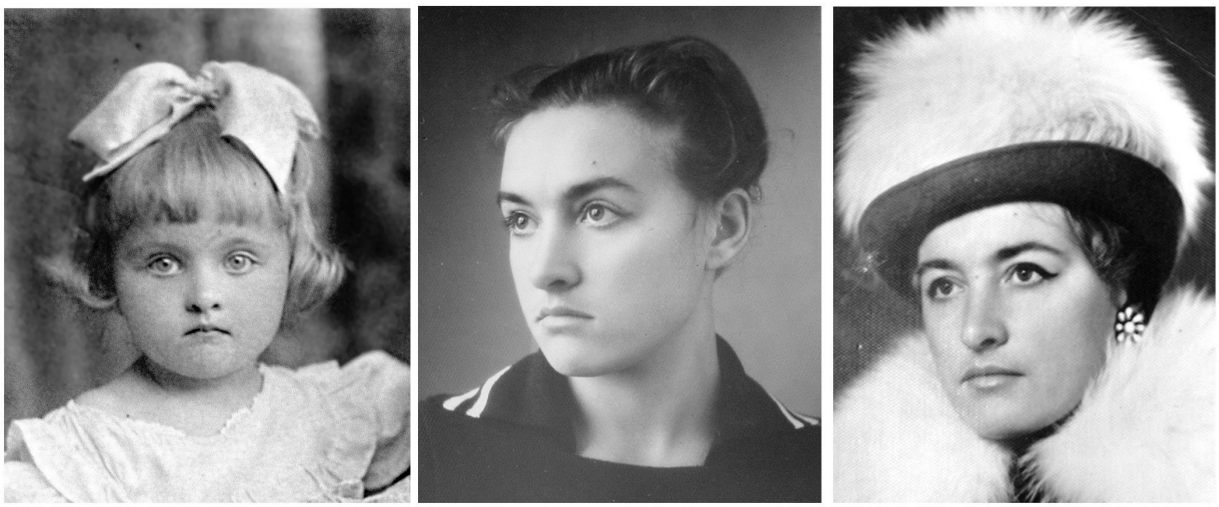
CHRONICLE AND INFORMATION

ON THE 85TH ANNIVERSARY OF THE WORLD-FAMOUS PARASITOLOGIST – PROFESSOR ALBINA GAEVSKAYA

Albina Gaevskaya (maiden name Yatsevich) was born on 21 August, 1937, in Sevastopol. She was a very beautiful child; perhaps, this was facilitated by the mixing of blood of representatives of different nations – Poles, Lithuanians, Italians, Ukrainians, Moldovans, Kazakhs, and Russians. As a child, Albina often fought with boys and gained her small victories. She always considered it necessary to defend her point of view, and back then, it was the most understandable way for her.

Since her childhood, Albina was fond of geography, dreamed of traveling, bought all available books of the "Travelling. Adventures. Fantasy" series, went sailing (in her home archive, there are certificates of honor for prizes in competitions of various levels – from local to All-Union ones), and dreamed of becoming a sea captain. In those years, girls were not admitted to nautical schools, and she entered the geography faculty of the Crimean Pedagogical Institute in Simferopol. Almost every Saturday after lectures, and sometimes instead of them, Albina rushed to Sevastopol to spend Sunday at the yacht club. Sailing is a fascinating and romantic, but unsafe activity. Once, while sailing on the "Druzhba" yacht, a guy did not pick the mainsail sheets at the turn; when transferring the boom to the other side, those sheets flew up and spun around Albina's neck. Pearl beads which wrapped her neck in three rows took the whole blow of the involuntary "noose". Pearls scattered, and a scar remained on the skin for a long time.



Albina Gaevskaya at the age of 3, 21, and 30

When Albina was in her third year, the geography faculty of her institute was transformed into the natural geography faculty – with the study of biological disciplines added. Fate decreed that Albina who initially had no inclination towards biology did not work a day as a geographer. After university, she worked as a biology teacher in one of Donetsk schools for a year; there, she got married and took her husband's last name – Dolgikh. In 1962, she got into the parasitology sector of the Sevastopol Biological Station as an intern and worked for free. Albina started her scientific career like a good soldier who plans to become a general. For Valentina Nikolaeva who headed the parasitology sector, she translated scientific material from English, made drawings for articles, catalogued publications for index cards, and carried out sample preparation. At the same time, she read parasitological literature. Already having a two-month-old son, she entered the PhD graduate school. Her supervisor was a well-known specialist in helminths, head of the zoology department of the Crimean Pedagogical Institute, professor Semen Delyamure. She presented her PhD thesis "Trematoda Larvae: Parasites of Molluscs of the Black Sea Crimean Coast" (1965) even before graduating, being assigned to the Institute of Biology of the Southern Seas.

A few years later, being divorced, Albina met her second love in Jūrmala, got married, and took her husband's last name. Under the last name Gaevskaya, she lives to this day. She decided to stay in the Baltics and worked in the Atlantic branch of the All-Union Research Institute of Fisheries and Oceanography (Kaliningrad) since 1971. There, she organized the parasitology department. In 1986, after the death of her husband and defense of her D. Sc. dissertation "Parasites of Fish of the Northeastern Atlantic: Fauna, Ecology, and Peculiarities of Formation" (1985), she was invited to IBSS by Alla Morozova, its director. Here, professor Gaevskaya organized the ecological parasitology department and headed it for 25 years.

Her personal life is vividly reflected in her wonderful offspring: she has 3 sons, 7 grandchildren, and 4 great-grandchildren. Dreams of travelling came true in expeditions along the Black Sea coast of Crimea, Caucasus, and Odessa estuaries. Participation in scientific conferences and symposiums allowed visiting many European regions of the USSR (from Murmansk to Tbilisi) and foreign countries (Germany, then Czechoslovakia, Turkey, Poland, Hungary, and the UK).

Over the decades of fruitful scientific activity, she has published more than 380 papers, *inter alia* 30 monographs and 5 patents. "Parasitology and Pathology of Fishes: Encyclopedic Glossary–Reference Book" (2003, 2004, and 2006), two-volume "Parasites and Diseases of Fishes in the Black Sea and the Sea of Azov" (2012 and 2013), and three-volume "World of Human Parasites" (2015, 2016, and 2017) are unique: there are no similar ones in Russia.

The scientific range of marine parasitology issues studied by A. Gaevskaya is very wide. She contributed much to taxonomy of parasites of marine fish and invertebrates (14 genera and 1 subfamily are described, as well as more than 100 parasite species new to science – trematodes, monogeneans, myxosporeans, and crustaceans). Resulting from her research, the data were expanded on ranges of hundreds of parasite species of various systematic groups, their parasite–host complexes, and peculiarities of parasitic system formation and functioning taking into account biogeographic history of water bodies and effect of environmental factors, as well as systematic position, biology, and ecology of hosts representing various areas of the World Ocean.

She was the first to carry out a complete inventory of the parasite fauna of fish in the northeastern Atlantic – with the compilation of lists of parasites of all taxa indicating their hosts and areas of discovery; those are 1,035 species of 423 genera of 153 families. The role of different fish classes

was revealed in the origin and formation of several large taxa of parasites in this area; the role of squids in the ocean trophic–parasitic system was shown. As established, parasitism formation of the overwhelming majority of groups of marine parasites is associated with bottom fish, primarily those of the shelf, while depth and pelagic colonization is of a secondary nature. For the first time, a method of vertical zonality of ecological groups was proposed for the analysis of fish parasite fauna in marine areas, which makes it possible to combine the issues of the genesis of the fauna and its modern distribution. Albina Gaevskaya's theoretical works contributed much to development of ideas on the role of parasites in the transformation of matter and energy, as well as their stabilizing function in ecosystems.

The results of her research are of great practical significance. She showed that parasites can serve as indicators of various aspects of fish biology, help in identifying stock units of commercial fish, and be markers of anthropogenic load on marine ecosystems. In her publications, much attention is paid to epizootiological significance of parasites of fish and invertebrates. A concept for mariculture development is proposed which substantiates the need for including parasitological work as an integral element of biotechnology for cultivation of marine organisms. A theoretical model was developed of parasitic system formation and functioning under conditions of artificial reefs.

In her honor, colleagues from India, the UK, Russia, and Ukraine named a trematode genus – *Gaevskajatrema* Gibson & Bray, 1982 – and 15 parasite species of different taxonomic groups. This is a recognition of her great contribution to world science.

At the initiative of A. Gaevskaya, "Marine Ecological Journal" was created at IBSS; in 2002–2014, she was its scientific editor. She was the editor-in-chief of the "Marine Biological Journal" (2016), deputy editor-in-chief of the "Sea Ecology" proceedings (1997–2010), and scientific editor of more than 10 collective monographs. For many years, she headed the specialized dissertation council in hydrobiology and was the deputy chairman of IBSS academic council.

Albina Gaevskaya is the Honored Worker of Science and Technology of Crimea, Academician of the Crimean Academy of Sciences, laureate of the Schmalhausen Prize for achievements in zoology, laureate of the State Prize of Ukraine in Science and Technology, and laureate of the City Forum "Public Recognition" (Sevastopol). She was awarded the commemorative medals of Academician K. Skryabin and Academician E. Pavlovsky, the Vernadsky Medal, and other medals, diplomas, and letters of thanks from the State Duma of the Russian Federation, the Verkhovna Rada of Ukraine, the Council of Ministers of Crimea, the Presidium of the National Academy of Sciences of Ukraine, the Crimean Academy of Sciences, the Sevastopol City Administration, the Governor of Sevastopol, the Leninsky District Administration of Sevastopol, and the Sevastopol Regional Branch of the Union of Women of Russia.

Under A. Gaevskaya's supervision, 13 PhD theses were successfully defended. Her students work in Sevastopol, Kaliningrad, Moscow, Murmansk, Odessa, Dnipro, and Bila Tserkva. To date, Albina Gaevskaya is actively involved in Sevastopol public life being a member of the social movement "For Our Hero City Sevastopol" council.

On behalf of all students, colleagues, and friends, we congratulate our dear hero of the day on a wonderful date and wish her many years of life in joy and happiness with good health!

Leading researcher of IBSS ecological parasitology department, PhD
V. M. Yurakhno

К 85-ЛЕТИЮ ПАРАЗИТОЛОГА С МИРОВЫМ ИМЕНЕМ — ПРОФЕССОРА АЛЬБИНЫ ВИТОЛЬДОВНЫ ГАЕВСКОЙ

21 августа 2022 г. свой юбилей отметила известный паразитолог — профессор Альбина Витольдовна Гаевская. Она описала 1 подсемейство, 14 родов и свыше 100 новых видов морских паразитов, стала автором более чем 380 научных работ, в том числе 30 монографий и 5 патентов. Под руководством А. В. Гаевской защищено 13 кандидатских диссертаций.

1871
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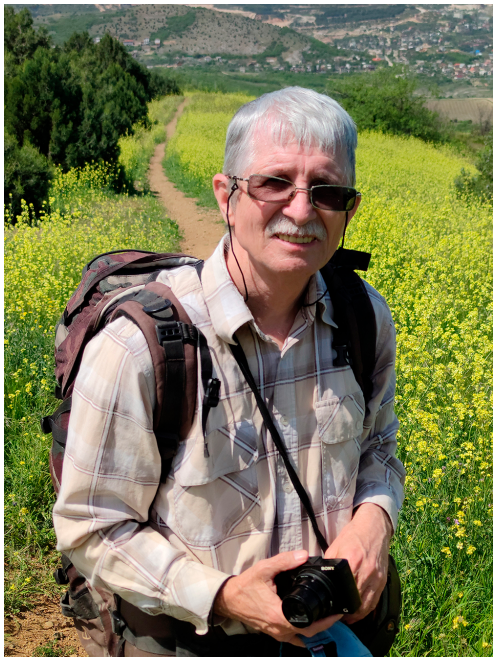
ON THE ANNIVERSARY OF D. SC. ALEXANDER PRAZUKIN



On 1 September, 2022, D. Sc. Alexander Prazukin celebrates his 70th birthday – the leading researcher of IBSS laboratory of extreme ecosystems.

A fifth-grader Sasha Prazukin who was keen on biology first came to IBSS in 1965. There, in the club "Dolphin" formed by the institute's employees, he began his scientific path with the study of ichthyology and zoobenthos. Four years later, he met the prominent scientist Kirill Khailov. A. Prazukin remains a faithful student and follower of Khailov's scientific ideas.

After graduating from the Odesa University, he came to work at IBSS in 1977. He started as a laboratory assistant, and now he is a leading researcher. During these decades, he published about 200 scientific papers, 7 monographs, *inter alia* 5 authored ones. His research covers many areas: functional morphology of multicellular algae; spatial organization of phytosystems; and biological and ecological aspects of phytosystem functioning in coastal shallow waters and lakes of different salinity. Alexander Prazukin developed the issues of the hierarchical organization of aquatic bioinert phytosystems; general methods for estimating habitable space in phytosystems of different organization levels; methods for determining photosynthetic activity of plant mats, *etc.* At all the stages of his scientific activity, he is interested in the practical application of knowledge – the multi-purpose use of seaweed biomass, principles of constructing artificial reefs, *etc.*

In 1991, A. Prazukin defended his PhD thesis in hydrobiology "Structural and Functional Changes in the Black Sea *Cystoseira* Under Eutrophication (Hierarchical Approach)." In 2013, he defended his D. Sc. dissertation in ecology "Structural, Functional, and Environmental Organization of Terrestrial and Aquatic Phytosystems in the South of Ukraine."

He is a member of editorial boards of several scientific journals ("Marine Biological Journal", "Bulletin of MSTU", and "Ecological Safety of Coastal and Shelf Zones of Sea") and a member of the specialized dissertation council in hydrobiology at IBSS. He is a deputy chairman of the Crimean Branch of Russian Hydrobiological Society of the RAS and a member of the Russian Botanical Society of the RAS.

Over the years, scientific activity of Alexander Prazukin does not decrease. In the last five years, along with the implementation of the state research assignment, he headed the RFBR project. Moreover, he remains the executor of the Russian Science Foundation grant. He generously shares his knowledge with young people who are as curious as he was. For 15 years, he gave lecture courses "Fundamentals of Ecology" and "Natural Science" at the Sevastopol Branch of the Ushinsky OSPI.

Alexander Prazukin has already done a lot, but he has many scientific plans. Colleagues from IBSS wish him good health, inspiration, and implementation of all his ideas!

К ЮБИЛЕЮ ДОКТОРА БИОЛОГИЧЕСКИХ НАУК АЛЕКСАНДРА ВАСИЛЬЕВИЧА ПРАЗУКИНА

1 сентября 2022 г. исполнилось 70 лет Александру Васильевичу Празукину — д. б. н., в. н. с. лаборатории экстремальных экосистем ФИЦ ИнБЮМ. А. В. Празукин — автор около 200 публикаций, член редколлегий нескольких научных журналов и заместитель председателя Крымского отделения Гидробиологического общества при РАН.

1871
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A FEW PAGES FROM THE LIFE OF VITALY GIRAGOSOV (ANTI-OBITUARY)

Why “anti-obituary”? Because even though we’ll never hear his “Hi!” again, it is absurd to say or write that Vitaly Giragosov is no longer with us on this sinful Earth. The word “obituary” does not fit with him in any way – from his name meaning “vital” to all our vivid memories and thoughts about him.

Different people have different associations, different impressions, different memory, and different recollections. Anybody who was acquainted with Vitaly could tell something unique – something that arose in the dialogue with him. It did not matter whether it was the conversation on a research subject, about fish, birds, or plants, about expeditions and trips, about books read or films watched, about childhood, or adulthood, and whether the conversation was serious, or comic. Anyone would say that it is very interesting with him and never boring – you always learn something new and always look forward to talk next time. Never getting into any squabbles and gossip, he was capable of maintaining relations even with very difficult and, possibly, unpleasant persons. In any situation, he was the most reliable companion and a stronghold in everything. He had an exclusively man character, completely lacking brutality; he was a person of exceptional decency, exceptional erudition, and exceptional modesty. His research was thorough and reliable – just like he was. Not being personally acquainted with him, not knowing about his sportive past but having only read his articles, one might think that he was a pedant and a bookworm. Oh, yes, he was an avid reader and knew a lot. However, his life was full of sport, competitions, and freedom of movement from childhood. During school-time, he explored all abandoned coal heaps around his native city Stakhanov, and he bred all kinds of living creatures at his native house; during summer-time, he learned more and more about the sea life in Gelendzhik.

Seems, the childhood of this Soviet boy in many ways turned out to be similar to the childhood of the English boy Gerald Durrell on the island of Corfu (see “My Family and Other Animals”). Fate led both to become the naturalists. From the beginning, the fate directed Vitaly to the faculty of biology of the Kuban University, and gave him an opportunity to explore the Kuban and the Caucasus. Then, the fate beckoned him to enter the PhD graduate

school at IBSS and, with the PhD thesis theme of studying myctophids of the World Ocean, promised him expeditions to far seas and oceans, but he was mysteriously not allowed to any oceanic cruise. And he studied the life of myctophids based of conserved material so thoroughly, and wrote such a unique PhD thesis that the Institute of Hydrobiology where he defended his work wanted to award him (but could not do it because of bureaucracy) a doctoral degree.

He managed to travel to distant countries a little. In 2001, at the University of the Sea in Mexico, he was a unique professor and the only expert in fish reproduction. Study of oceanic myctophids' age by otoliths increments led him to get acquainted with a well-known specialist – Dr. Tomasz Linkowski, the director of the National Marine Fisheries Research Institute in Gdynia. In 2009, he invited Vitaly to study the collection of the Black Sea turbot otoliths on unique specialized equipment. That's all his travels to distant lands.

But he came back to the love of his youth, the most faithful one – to birds – and devoted a lot of time to studying waterfowl migratory birds in Sevastopol area. He turned out to be a unique specialist – an ornithologist this time. Over past ten years, he discovered many peculiarities in the hidden life of these birds just in the vicinity of our city. He took hundreds of great pictures and presented them in a fascinating and professional way online and on TV to countless people. He captivated people with his passion and helped them to fall in love with these free creatures.

On 20 February, 2022, he suddenly flew away from our world. Maybe too tired from many things, he left early. His soul is free now, and can be anywhere. May soar in the sky with birds or explore the ocean depths. And for all of us, it still seems that he joked and once will suddenly come in, or call, and will say that he was late.

We all miss him very much.

But you can turn on the video "Long Way of Marine Expeditions" (https://media.marine-research.ru/items/show/1610) and listen for ten minutes to his wonderful voice which tells, perhaps, you personally: "Why do people study the Ocean?"

Leading researcher of IBSS aquaculture and marine pharmacology department, PhD
A. N. Khanaychenko

НЕСКОЛЬКО СТРАНИЦ ИЗ ЖИЗНИ ВИТАЛИЯ ЕВГЕНЬЕВИЧА ГИРАГОСОВА (АНТИНЕКРОЛОГ)

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