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Ecology, conservation and rehabilitation of Black Sea and Pacific common bottlenose dolphin in natural and noogenic environment

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biology

Field of expertise: Zoology-Hydrobiology

Annotation

Batumi - 2018

General description of the work

Topic urgency. Nowadays, in many countries throughout the world, special attention is drawn to the degradation of marine mammals' habitat and the impact of the global climate change on the health and diseases of population, dynamics of reproductive and quantitative changes. While the frequency of marine mammals disease in the world ocean increases, our knowledge of the known and new pathogens in their habitat is limited (Morris et al 2011; Bossart et al., 2003; 2006; Moore, 2005). Infectious epizootics within natural population of cetacean is becoming especially frequent, the main reason of which is microbial contamination of seawater through various pathogens (Wilson et al., 1999). Contamination of coastal water with pathogenic and conditional pathogenic microorganisms and other chemical pollutants create prerequisite for spreading dangerous diseases, as for people so for the wildlife completely. Marine mammals are effective indicators for detection of such pathogens and toxins.

Despite the fact that marine mammals, including dolphins, for a long time represent the target of attention of researchers of various fields, they are not yet fully studied. Scientists of many countries around the world try to explore the mechanisms of dolphins' adaptation to climate and living environment change, which, in the future will enable the humankind to explore seas and oceans more deeply, as one of the most important resources of biodiversity and use it in their own favour without prejudice.

Nowadays the number of marine mammals has considerably decreased through the global anthropogenic impact on the world ocean. Every year tons of persistent organic and inorganic pollutants, phosphorus and nitrogen mineral and organic fertilizers, and various types of superficially active detergents flow into the ocean. Due to excessive and illegal fishing, quantity and area of food base decreases. Sewage and drainage discharge is increasing, which are rich in land animals and people's pathogenic and conditionally pathogenic bacterial flora, some of which are resistant to antibiotics (Schaefer A.M, et al., 2009). Number of bacterial pathogens and opportunistic bacteria (S.*aureus, Stenotropomonas, Brucella, Streptococcus* etc.) are considered cause of dolphins' furunculosis, respiratory diseases, nephritis, cerebral abscess and

other serious pathologies, which, in many cases, results in death of an animal. Virus epizooties of dolphins has become more frequent since 90-ies of the last century. High mortality rate is conditioned by the infections caused by morbillivirus, as in north-west of Atlantic Ocean so in the surrounding areas of Mediterranean Sea and Black Sea. Potential part of environmental factors (pollutant agents, climate change, stresses etc.) in connection with disease frequency and disease severity increase are actively considered by the world's scientists and researchers. However, the study of the above stated complex issues in wildlife is connected with number of complications. Therefore, the animal adapted to the artificial environment, accustomed to cooperation with people enables us to perform constant observation, conduct detailed study of its behavioural and physiological parameters, design and implement efficient methods of prevention, pathogenesis and treatment of disease. Most of the fundamental researches on sea animals these days are performed in artificial conditions created by man.

According to the above stated, the issue is **highly urgent**. The stated researches were carried out on the base of modern dolphinarium of Black Sea flora and fauna educational scientific research centre.

Based on the above, **the aim** of our study is to analyse aspects of ecological condition, protection and conservation of Black Sea and Pacific bottlenose dolphins' natural and artificial habitat and populations living there, considering the modern challenges; detection and study of etiologic agents, development of modern methods and activities for prevention and treatment of disease.

Within the framework of the dissertation with the purpose of achieving the aim, the following **objectives** were set:

- Analysing Black Sea and Pacific bottlenose dolphins' natural habitats condition;
- Perform hydrochemical and microbiological monitoring of Batumi dolphinarium noogenic environment;

- Complex assessment of physiological condition of bottlenose dolphins living in an artificial environment (behaviour, nutrition activity, mobility, haematological indicators);
- Study of the upper respiratory tract microflora of dolphins in noogenic conditions and establishing links with abiotic factors of the environment;
- Collecting and study of infectious/pathological material from fallen/washed ashore animals, by following biosafety norms, for the purpose of determination of possible cause of death;
- revealing etiological agents, identifying and creating collection of sufficient strains of bacteria;
- Study of antibiotics and phage sensitivity of separated bacterial isolates;
- Discussing the possibility of using alternative biological medicine (specific bacteriophages and polyvalent phage medicine) for prevention and treatment of dolphins' infectious diseases.

Scientific novelty:

For the first time in Georgia, the folloving study and analysis of ecological condition, aspects of protection and conservation of Black Sea and Pacific bottlenose dolphin populations living in natural and artificial conditions have been performd; elimination of direct and indirect anthropogenic impact factors on marine mammals and prospects of mitigating "human-dolphin" interaction; main causes and main factors of epizooty and mortality among Black Sea and Pacific bottlenose dolphins in natural and noogenic environment; study of microbial flora of bottlenose dolphins living in noogenic conditions and determination of etiologic role in development of infectious pathologies of dolphins, revealing and identification of agents causing disease; creating collection of strains of bacteria; prospects of developing biological medicine for treatment and prevention of infectious diseases of marine mammals; *ex situ* study and reproduction of marine mammals in Batumi Dolphinarium equipped with ultra-modern systems.

Theoretical and practical significance. Based on the evaluation and analysis of the results of the studies performed in Batumi Dolphinarium the ways and means of improving the habitants' living environment and health status has been revealed, which shall be applied to dolphinarium's rehabilitation and conservation activities.

The results gained through the studies show prospect that bacteriophages and polyvalent phage medicines shall be used on one hand for diagnosing the strains of bacteria and in addition for differentiation of the strains, and on the other hand, for treating bacterial infections, especially in case when the one causing the disease is multi-resistant to the antibiotics and the animal has deteriorated immune status.

Approbation and publications of research results.

Preliminary review of the work was conducted on 20th of July 2017, in the department of biology, at the faculty of natural sciences and health care, in Batumi Shota Rustaveli State University. The research result reports were presented on international forums:

1. International Scientific Conference dedicated to the hundredth anniversary of bacteriophage studies. Tbilisi, Georgia, 26-29 July 2017.
2. International seminars on "Marine mammals' diagnosis and ecology". Batumi, Georgia, 12 -13 October 2017.
3. III international-scientific conference on "Computing/information science, education sciences, teacher education", Batumi, Georgia, 17-19 October 2014.

Three scientific works are published in internationally reviewed science magazines and international conference materials.

Volume and structure of the work: dissertation work covers over 135 pages and comprises of introduction, material review and experimental part, where the research methods and analysis of the research results are given. The work ends with conclusions. In total 132 sources are analysed in the work. The text includes 20 tables, 7 diagrams, 28 photos.

Material review. In the first chapters of the dissertation, the following issues are discussed: current condition of bio ecology, conservation and protection of Black Sea and Pacific bottlenose dolphins (Tursiops truncatus, Tursiops gilli), main causes and main factors of epizooty and mortality among Black Sea and Pacific bottlenose dolphins in natural and noogenic environment.

Experimental part

Material and research methods

Research object. As the research objects 7 viable washed ashore bottlenose dolphins (*Tursiops truncatus ponticus)* placed in Ltd " Black Sea flora and fauna research centre" for rehabilitation and 7 Pacific Ocean bottlenose dolphins (*Tursiops truncatus gilli)* brought from Taiji, Japan were chosen. Therefore, we had unique opportunity for rehabilitation, breeding and research of two subspecies of bottlenose dolphins.

The researches were carried out in dolphinarium of Ltd **"Black** Sea flora and fauna research centre", George Eliava institute of bacteriophage microbiology and virology and on bases of faculty of veterinary medicine, pathobiology department of Utrecht University in Holland.

We have examined 14 bottlenose dolphins of 4-15 age, adapted in noogenic environment, 4 males and 10 females, the number was later increased by three newborn dolphins after reproduction. Furthermore, the dolphins washed ashore on the Black Sea shore in Georgia were also studied.

In accordance with the research aims, regular monitoring on marine mammals' habitat condition were performed by using hydrochemical and microbiological methods. For systematic control of dolphins' health condition, microbiological, physiological, cytological and histological researches were conducted.

Research methodology.

Methods of noogenic environment research. For assessment and optimization of the habitat of the marine mammals (Batumi dolphinarium pools) regular monitoring of chemical and microbiological quality has been carried out in accordance with internationally approved scheme and methodology (DR5000 Spectrophotometer Procedures Manual; ISO/CD 9308-1; ISO7899-2:2000). In particular, water temperature, free chlorine and total chlorine content, redox (ORP), pH, salinity and quantitative content of biogen (Procedures Manual), were controlled through physico-chemical parameters, in the same water samples the following microbiological parameters were determined once a week: total viable count (TVC) 37°C/1ml; TVC 22°C/1ml; total coliform/100ml; colon bacillus/100ml; enterococcus/100ml; St.aureus/100ml; Ps.aeruginosa/100 ml; Proteus spp./100ml; Salmonella/100 ml; Fungi/1ml.

Marine mammals research methods. Visual examination of animal conditions, monitoring of activity and food intake in accordance with dietary were performed on daily basis. Diet was designed for each animal individually, considering their gender, age, season, and breeding, pregnancy and lactation periods.

For assessment of dolphins' health, condition physiological, microbiological, hematologic, cytological and histological methods are used. Animals regularly (predictably) undergo general and biochemical analysis of blood, exhaled air and gastric juice bacteriological and cytological examinations.

Microbiological researches were performed on dolphins' exhaled air and gastric juice samples, and on pathoanatomical materials of washed ashore and fallen animal. Identification of selected isolates were done through various differentiating tests: 3% potassium base test (KOH) ; Cytochrome-oxidase test; catalase test; Glucose oxidation / fermentation test (Hiugh-Leifson in soil); API-20E test-system.

The sensitivity of microorganisms to the antibiotics was studied; disk diffusion method was used for determining isolates' sensitivity to the selected antibiotics.

For determining sensitivity towards bacteriophages, bacterial lawns were being prepared.

Autopsy, sampling and collecting research material. Autopsy, sampling and keeping the research material of washed ashore and/or fallen animal for further bacteriological and histological researches were done in accordance with internationally recognized methods. Recorded material for further histological research was sent in Holland. Received results are described in the dissertation chapter.

Analysis and review of the results

Monitoring of physico-chemical and microbiological parameters of Black Sea bottlenose dolphins habitat

Disease and mortality indicators of marine mammals, including populations of Black Sea dolphins living in wild and noogenic environment, may greatly vary, which depend on origin of the animal, geographical and climate zones, animal density, culture of care etc. Regular monitoring of specific conditions of dolphins' noogenic habitat, including chemical and microbial indicators of water is important for prevention of dolphin diseases including infectious ones. Biological contamination changes the structure of microbial communities, violates the natural biological processes in the sea, and therefore, promotes the accumulation of pathogenic bacteria hazardous for human and hydrobionts in coastal waters. Microbial degree in reservoir is determined not only by water bacteria but also by aloctonic microflora resulted in a biocenosis through biological contamination. The quantitative content of the bacteria of this group, namely, the coliform (Escherichia, Citrobacter, Enterobacter, Klebsiella, etc.) and the faecal streptococcus / enterococcus are the most widely used as sanitary indicators of possible faecal pollution of water reservoirs.

Presented research aimed to perform regular monitoring of chemical and microbiological degree in water of marine mammals habitat study areas (in Batumi dolphinarium tanks). Taking the water samples and their analysis were performed with internationally approved scheme and methodology (Procedures Manual; ISO/CD 9308-1; ISO7899-2:2000). In particular, water temperature, free chlorine and total chlorine content, redox (ORP), pH, salinity and quantitative

content of biogen (Procedures Manual) (see table 1), were controlled through physico-chemical parameters, in the same water samples the following microbiological parameters were determined.

Table 1. List of control parameters of bottlenose dolphins' noogenic habitat's hydrochemical and microbiological monitoring

Hydrochemical parameters	testing frequency	microbiological parameters	testing frequency
water temperature	2 x a day	TMC 37°C/1ml	1 x a week
Free chlorine (ppt)	2 x a day	TMC 22°C/1ml	1 x a week
Common chlorine (ppt)	2 x a day	common coliform /100ml	1 x a week
ORP-potential	2 x a day	E.coli/100ml	1 x a week
pH	2 x a day	Enterococcus /100ml	1 x a week
Salinity (ppt)	1x a week	St.aureus/100 ml	1 x a week
Dissolved oxygen	2x a month	Ps.aeruginosa/100 ml	1 x a week
Nitrites (NO_2^-)	2 x a month	Proteus spp./100ml	1 x a week
Nitrates (NO_3)	2 x a month	Salmonella/100 ml	1 x a week
Biogenic elements (P, N, Phosphates PO_4^{-3}	2 x a month	Fungi/1ml	1 x a week

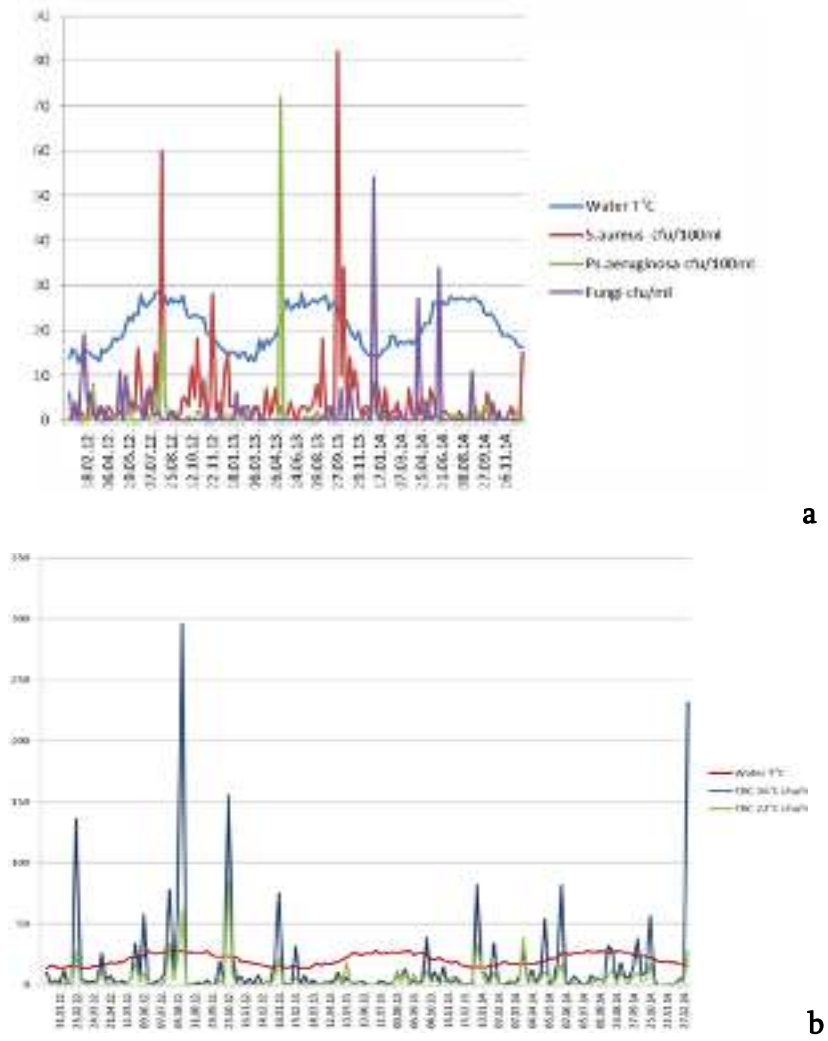
It is known, that the quality of water greatly depends on variation of temperature, free chlorine and potential (Телига, 2012). Our observations have shown that the stated parameters in Batumi dolphinarium tanks during the year were stable and varied within the permissible norm, which is regulated by dolphinarium tanks water supply recycling system, equipped with water mechanical filtration, decontamination (ozonizing, chlorination), and modern high-tech systems for heating and cooling.

In its turn, the controlled physico-chemical parameters of tank water have to provide the stability of microbiological indicators to great extent.

As a result of monitoring carried out in 2012-2014 in researched noogenic environment of marine mammals, it was revealed that dolphins habitat-the majority of regulatory microbiological indicators of water quality tanks correspond to the established norms-

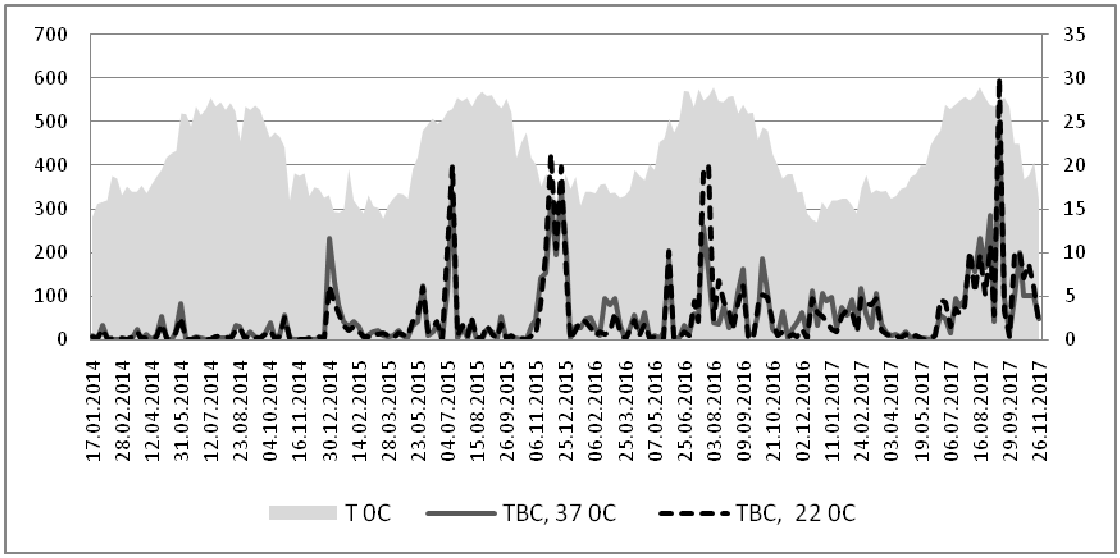
international standards (EAAM, 2009). Total microbial count indicator on 37^{0}C (mesophilic aerobes and facultative anaerobes) exceeded the norm only in a few cases, and on 22^{0}C TMC indicator have not exceeded the permissible range. In case of water faecal contamination indicator the permissible norm was exceeded in 13 cases with respect to total coliforms, as for the excess of e. coli and enterococcus it has been detected more frequently: therefore, in 30 and 35 samples. *S. aureus* was revealed in 89 samples having various physical-chemical parameters, and P*. aeruginosa* – in 39 samples. However, it must be noted that in most cases rates exceeded the permissible norms slightly. As for the fungal flora, it was only detected during minimal bacterial content. Generally, seasonal change was revealed in microbial parameters (see pictures.1, a,b,), which to some extent must be connected with natural (uncontrollable) variation of water temperature, however, it may also have reflected cases of external contamination in water during precipitation, and separate cases of tank water control system's bad maintenance.

Statistical analysis of the collected data was conducted by using the standard computer program Statistical Toolpak for Microsoft Excel 2010. All the correlations stated here has been considered reliable at $P \leq 0.05$ reliability level. Expressed correlation (as positive so negative) has been revealed among number of physico-chemical and microbiological parameters. In particular, reversed correlation has been detected in water, between free chlorine content and total microbial count (r= -0.21), as well as between the numbers of total chlorine and E.coli and S. aureus (consequently, r= -0.16 and r= -0.19). Redox potential (RSP), has also been significantly reverse correlating at temperatures 37°C and 22°C with total microbial count (r=-0.28 and r=-0.33), relatively less (r=-0.18) – with total coliforms and enterococcus number. Much higher positive correlation has been found among separate microbiological rates, such as: TBC (37°C) - TCC (r=0.64), TBC (37°C) – ENT (r=0.39*), E.coli- S. aureus* (r=0.6), *E.coli- P.aeruginosa (*r=0.58) etc.



Picture. 1. Alteration of water microbial parameters in Batumi dolphinarium tanks: **a-** Faecal contamination range; Staphylococcus, pseudomonas and fungal content; **b** – Total microbial count

In the following years (2015-2017) as a result of researches conducted, important stability of physico-chemical and microbiological parameters of noogenic environment of dolphins' habitat. Alteration varied within the permissible range (see picture 2).



Picture 2. Seasonal changes of microbial parameters (TBC 37^{0}C; TBC 22^{0}C) of Batumi dolphinarium tank water in 2014-2017 .

In stated years positive correlations were revealed among most of the water microbial parameters (table 2). The quantity of potential pathogenic bacteria such as S.aureus and P.aeruginosa – is connected with faecal contamination range and total microbial count. Unlike S.aureus, reverse correlation has been determined with P. aeruginosa and enterococcus numbers (table 1). The reason for this is comparatively high resistance of enterococcus towards seawater. Besides, P.aeruginos quantity was the only parameter, which showed direct dependence to temperature. As it was expected, important correlation (table 2) has been detected among faecal contaminator indicators, among total microbial count and the other microbial parameters of water (table 2). Total coliform number is in negative correlation with free chlorine content (r=-0,26 and r =-0,27). Significant negative correlation is noteworthy, as between redox potential (ORP) and total microbial count 37°C (r=-0,2), so between coliform total number (r = -0,26).

Table 2. Correlation among various microbial parameters of water 2014-2017
Correlation is considered positive if r> 0,26, sample size n = 45

parameters	Temp. 37°C	temp.22 °C	total coliforms 100ml	E.coli/ 100 ml	Enterococcus/100 ml	Ps.aeruginosa/ 100 ml	St.aureus/ 100 ml

Temperature C 37°C	1,00						
Temperature C 22°C	0,91	1,00					
total coliforms 100 ml	0,80	0,67	1,00				
E.coli/100ml	0,64	0,68	0,54	1,00			
Enterococcus/100ml	0,41	0,32	0,26	0,28	1,00		
Ps.aeruginosa/100ml	0,26	0,38	0,30	0,32	0,19	1,00	
St.aureus/100ml	0,43	0,51	0,39	0,57	0,34	0,60	1,00

Conducted research confirmed the importance of rational management of marine mammals' artificial habitat, as well as, necessity of regular monitoring for maintenance of health status of animals and for protecting them bacterial infections.

Results and analysis of bacterial research of materials taken from marine mammals

Analysis of results of the upper respiratory tract microflora research of the Black Sea and Pacific bottlenose dolphins in noogenic habitat

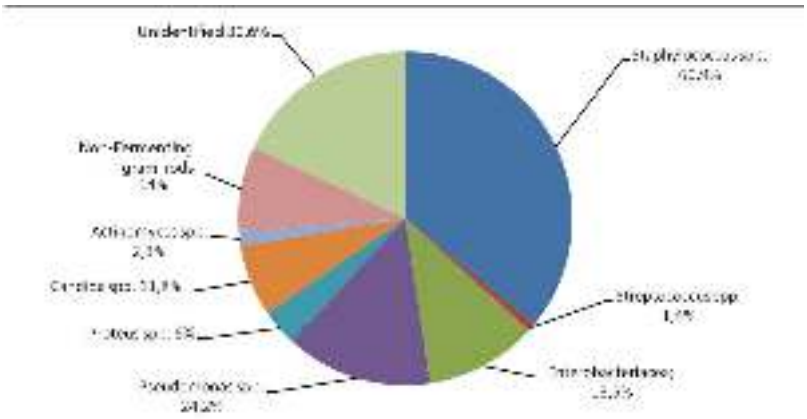
Infectious etiology diseases of upper respiratory tract (URT) represents a serious problem for marine mammals adapted to noogenic conditions. Diagnostics of the stated diseases based on clinical signs is possible only within the process of disease generalization, thus for the purpose of timely diagnostics we performed systematic control of exhaled air, which enabled us to detect the fact of deterioration of immune system and early stage of infectious disease. When assessing clinical condition of adapted dolphins we considered their nutrition and mobility activities. In case of pathology the diseased animal is less active, it often floats on the water surface, moves less, has decreased appetite, sometimes even shows resistance to feeding.

As stated above, one of the most important links in diagnosis of infectious disease represents microbiological analysis of dolphins exhaled air. This enables us not only to reveal the potential pathogen but also to choose efficient antibiotics or other antimicrobial treatment, in order to provide further control of animal treatment process. (Denisenko, 2004).

Study of Black Sea mammals' respiratory tract microflora was being conducted in Batumi dolphinarium in 2012-2017. Retrieving the dolphins' exhaled air material was carried out twice a month, in case of necessity more frequently. In total 1200 samples of exhaled air were examined. Respiratory samples have been collected during six years from 18 adult dolphin. Variability of retrieved sample number in different years was connected with necessity of taking additional samples due to dolphins' health condition. Besides bacteriological and cytological examination of respiratory samples, the animals were undergone general and biochemical blood tests. Exhale air samples retrieved from the diseased dolphins were studied in in details. Bacteriological examinations were carried out by separating pathogens and identifying them. Obtained microbial isolates were studied through morpho-physiological and biochemical indicators and were identified phenotypically.

As a result of conducted researches, it was revealed that in general, the microbial respiratory intake of upper respiratory tract infections in the animals is quite high compared to the number of bacteria in the exhaled air of healthy animals, whereas range of microflora is often virtually identical. In case of infectious processes, the microflora of the respiratory tract (sample of one full exhale) it exceeds 1000 cells, in addition bacterial range is decreased. Most of the screened bacterial isolates reveal the hemolytic activity towards sheep erythrocytes.

The following taxonomic groups of microorganisms are revealed in cultures isolated from the upper respiratory tract of dolphins: gram-negative rods, yeast fungi and actinomycetes. In samples gram-negative rods and gram-positive cocci. The isolated cultures differ with morphological, cultural and biochemical characteristics. During the research period *Staphylococcus, spp.* (60,9%) and *Pseudomonas spp.* (24,2%) were distinguished with the highest frequency screening from the upper respiratory tracts of the researched animals. Average frequency was observed in *Enterobacteriacea* group, non-fermented gram-negative and yeast fungi from *Candida* group. *Proteus spp., Streptococcus spp.* and *Actinomyces spp*. were observed less frequently (picture 6, table.3)

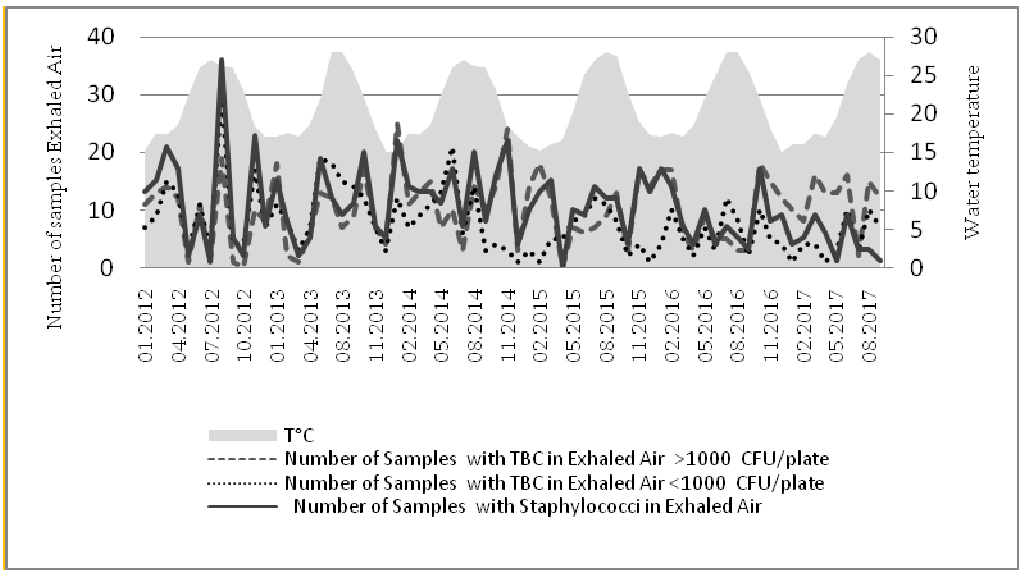


Picture 3. The microflora of the Black Sea and Pacific bottlenose dolphins' upper respiratory tract inhabiting Batumi dolphinarium 2012-2017

Comparative analysis of respiratory and water sampling research results has been conducted during the research period (picture 4).

According to our observations, excessive number of microbes in samples of dolphins' exhaled air was only detected in winter and autumn, when the temperature of pools was comparatively low; however, water microbial parameters remained within norm range. Exhaled air samples with low screening capacity prevailed during summer, when water microbial indicators were comparatively high in comparison to permissible norm.

As stated above, upper respiratory tracts microflora was represented by *Staphylococcus spp., Pseudomonas spp.* etc. non-fermenting gram-negative bacillus, enterobacteriaceae, aeromonads, streptococcus, yeast fungi, actinomyces etc. Most of these bacteria are considered opportunistic pathogens that can cause respiratory illness in animals with impaired immunity.



Picture 4. Monthly distribution of exhaled air of various microbial load in relation to dolphins' pool water temperature within 2012-2017 research years.

Correlative connections between physico-chemical and microbial parameters of water and number of bacteria in dolphins' exhaled air were revealed as a result of analysis of conducted researches. Important correlation (r= 0,69) has been detected between samples of high sifting out capacity and those containing staphylococci. Negative correlation has been detected between the samples having high total microbial count, water temperature and ORP (therefore r=-0,36, r=-0,32). It is noteworthy that there have not been identified connection between the quantities of exhaled air samples with increased microbial contamination and water microbial parameters, which may indicate less impact of the latter on respiratory flora of the dolphins living in noogenic conditions. The stated consideration is verified by other researches (Bik et al.,2017).

On the basis of researches it shall be noted that during cold period, due to stressful conditions, protective mechanisms of dolphin at comparatively low temperature are less efficient and not enough for prevention of the colonization and great increase of bacteria in the respiratory tract. Bacterial attack on dolphin is far frequent in winter, which increases their morbidity cases (Кузнецов, 2006). It is interesting that quantity of exhaled air samples containing staphylococcus correlates with free chlorine content (r = 0,301). It is likely that the

use of sodium hypochlorite for decontamination of water shall cause suppression of the microorganisms sensitive towards chloride, which in itself stimulates the growth of more sustainable gram-positive bacteria, namely, the increase of staphylococci (Engelbrecht et al., 1974).

Research of microbial flora of sea mammals in the noogenic environments for determination of the alleged infectious pathology

In recent years the Pacific Bottlenose dolphins *(Tursiops truncatus gilli)* are often found in noogenic environment of different dolphinariums or aquariums. Despite the fact that within the modern conditions,dolphinariums are regularly monitoring the status of living environment, the supply of food and health of their inhabitants, still, these animals are somehow vulnerable to health. There have been many individual cases reported on dolphin's illness and death cases (Song et al, 2017; Venn-Watson et al, 2008.; Romanov et al, 2009;Elfadl, 2017),which can be due to different reasons.

In January 2012, during the study of the inhabitants of Batumi Dolphinarium with the signs of different types and qualities of the diseases, the gastric juice and exhaled air samples were analyzed for revealing the causes of infections. The primary processing and microbiological analysis was conducted here, on the basis of the laboratory of the Black Sea Flora and Fauna Educational Scientific-Research Centre, while the further characterization and identification - study of selected (on quantitative content) isolates according to various parameters (biochemical, cultural, morphological characteristics, differentiation tests, including API identification systems, photosensitivity) and the complex analysis of the resulted data have been done in the laboratory of G. Eliava Institute of Bacteriophages, Microbiology and Virology. 6 out of the 13 bacterial isolates turned out to be the Gram Positive Cocci, bacilli and Coccobacilli and 7 – gram-negative bacilli. Some isolates from gram-positive cocci cultures (N1,5,7) – have been identified as non-hemolytic*Staphylococcus spp.*,supposedly, the isolate *S.epidermidis.* N2 with its microscopic and cultural properties belong to the saccharomycotina-

*Candida spp.*Four isolates (N10,11,12,13) – the gram-positive bacilli are generator of hydrogen sulfide – with a range of morphological and cultural-biochemical properties were included in atypical varieties of *Erysypellothryx ruthiopathie*or*Corynebacterium spp*. (in both cases there is no coexistence of individual biochemical signs). 5 isolates (N3,4,6,8,9) – gram-negative bacilli Ox^{+}- after biochemical characteristics and identifying the selected stamps with API tests (3 selected isolates) belong to *Shewanella (Pseudomomas) putrifaciens.*

It should be mentioned that the majority of the bacteria can usually be met in the aquatic environment, as well in samples taken from the animals. Part of them are pathogens of fish and other vertebrate animals (e.g. *Aeromonas salmonicida,Erysypellothryx ruthiopathie, Shewanella putrifaciens*), while the part belongs to opportunistic pathogens.

In terms of causing the diseases, except the pathogenic factors, the number of bacteria is significant, determination of which was not available on the basis of the original material. From this point of view, it's recommended to strew the primary material on the solid area, by recording the following quantity of the microbial grown. The antibiotic and phago-sensitivity of the isolates under the research have been studied (table N 3, 4). Besides the diagnostic significance of this data, there is a possibility of their use for disease prevention and sanitation of the environment.

Table 3. The antibioticogram of the bacterial isolatesallocated from the inhabitants of the dolphinarium*

N	Sensitivity to antibiotics	1	2	3	4	6	7	8	11	13
1	Am –Ampicillin	R	R	R	R	R	R	R	R	S
2	Er - Erythromycin	I	R	S	I	S	S	S	R	S
3	Amp sulb - Ampicillin/sulbactam	R	R	R	R	R	R	R	R	R
4	Amox Clav - Amoxicillin Clavulanate	R	R	R	I	I	R	R	R	S
5	Cfz - cefazolin (Kefzol)	R	R	R	R	R	R	R	R	R
6	Gen -Gentamicin	S	S	I	S	S	I	S	R	S

7	Cfp - Cefoperazone	R	R	R	R	R	R	R	R	I
8	Cfxt –Cefoxitin	R	R	R	R	I	R	R	R	S
9	Im - Imipenem	S	R	R	S	S	R	R	R	S
10	Mer - Meropenem	S	R	R	S	S	R	I	R	S
11	Mox - Moxifloxacin	R	R	I	I	R	I	S	R	S
12	Gat - Gatifloxacin	R	R	R	R	R	R	S	R	S
13	Clin - Clindamycin	R	R	R	I	I	R	R	R	R
14	Chl - Chloramphenicol	R	R	I	I	S	I	I	R	I
15	Met - Metronidazole	R	R	R	R	R	R	R	R	R
16	Van - Vancomycin	R	R	R	R	S	R	R	S	I
17	Tet - Tetracycline	I	R	R	I	I	R	R	S	S
18	Furz - Furazolidone	R	R	R	R	R	R	R	S	R
19	Cip - Ciprofloxacin	R	R	R	R	I	I	I	S	S
20	Cftz - Ceftazidime	R	R	R	R	R	R	R	S	I
21	Trim - Trimethoprim	S	R	S	I	R	S	R	R	R
22	Bac -Bacitracin	S	R	R	R	R	R	R	R	R
23	Col - Colistin	S	R	R	R	R	R	R	R	R
24	Poly B - Polymyxin B	S	R	R	R	R	R	R	R	R
25	Km - Kanamycin	I	R	R	R	I	R	R	S	S
26	Sulfd - Sulfadiazine	R	R	R	R	R	R	R	R	R

*S -sensitive;I - moderately sensitive;R - resistent

Table 4. The phagosensitivity of the bacterial isolates segregated from the habitants of the dolphinarium*

N	Bacterial isolate	Pheresis	SES	PIO	Intest
1	*Staphylococcus spp.*	cl	cl	cl	cl
2	*Candida spp.*	-	-	-	-
3	Non-fermenting bacilli, probably *pseudomonas*or of related specie	-	-	-	-
4	*Shevanella (Pseudomonas putrifaciens)*	-	-	-	-
5	*Staphylococcus spp.*	-	-	-	-

6	*Shevanella (Pseudomonas putrifaciens)*	cl	cl	cl	cl
7	*Staphylococcus epidermidis*	cl	cl	cl	cl
8	Non-fermenting G- bacilli,*Shevanella (Pseudomonas putrifaciens)*	-	-	-	-
9	Non-fermenting G- bacilli,*Shevanella (Pseudomonas putrifaciens)*	-	-	-	-
10	probably *Erysypellothryxruthioparie* or *Corynebacterium spp.*	-	-	-	-
11	probably *Erysypellothryxruthioparie* or *Corynebacterium spp.*	-	-	-	-
12	probably *Erysypellothryxruthioparie* or *Corynebacterium spp.*	-	-	-	-
13	probably *Erysypellothryxruthioparie* or *Corynebacterium spp.*	-	-	-	-

*cl - High sensitivity;ntv - Moderate sensitivity

The case of acute myocarditis of bacterial etiology in Batumi Dolphinarium.

The case of acute myocarditis of bacterial etiology was met in one of the inhabitants of Batumi Dolphinarium, nicknamed “Kako” which was brought from Japan Island Taiji in 2011.

The inhabitants of Dolphinariumwere kept in a controlled living environment, where the chemical and bacteriological parameters of water quality wereroutinelychecked and adjusted (Tserodze et al.,2016). Such monitoring of the environment, the high quality balanced food, rich in vitamins and minerals ensured adequate health status preservation of the marine mammals in this particular noogenic environment.The regular monitoring of animals’ health status has been performed based on daily observation of qualified veterinarians and marine mammals’ trainers, along with cytological and bacteriological analysis of dolphin’s exhaled air and gastric juice as well as routine blood tests (complete blood analyses and also blood biochemical parameters) once in 3 months

Additional determination of the above mentioned parameters has been performed according to the needs, in particular, if any change in animal's behavioral traits such as passive swimming, rapid breathing, irritating factors and inadequate reaction of the others was identified.

From 2011 to February 2017, "Kako"dolphin's health condition was satisfactory except for one episode that took place on January 31, 2015 when the general blood analysis of the animal showed leukocytosis and some changes in blood biochemical indicators without any notable clinical symptom. However,appropriate antibiotic therapy was initiated and after two weeks blood parameters returned to normal values.

The general blood analysis of the «Kako" dolphin conducted between the period of February -March demonstrated leukocytosis (15.740 k/mkl) with increased neutrophil counts (76%) followed by minor changes in blood biochemical indicators.The value of ESR did not exceed the norm.

Despite the small hematological and bacteriological changes, the behavioral characteristics of the dolphin were satisfactory.However, antibacterial and immunomodulating treatment was initiated to control the potential infectious process.

The next hematological investigation (conducted on28.04.2017) showed the same results: leukocytosis (13.97 k/mkl)with increased neutrophil counts (71%),and still normal biochemical parameters, with only increased bilirubin value (1.1 mg /dl).Since complex medicinal therapy conducted so far didn't produce positive results, that's the prescription was corrected.

Despite the treatment carried out, the animal's blood parameters remained unchanged, although the behavior of the animal did not change substantially. In July, 2017 the dramatic changes were observed in the behavior of the dolphin. Characteristics of the diseased animal behavior have dramatically changed. It was occasionally descended on the bottom of the pool,and showed very weak breathing activity during the water surfacing.

The hematological investigation revealed growing number ofleukocytosis (17.19 k/mkl)also sharply increased erythrocyte sedimentation rate (ESR) (54 mm / h),and reduced hemoglobin value (12.3 g / dl).Despite the complex treatment, it appeared not possible to save the animal, and it died soon.

Histological analysis of pathological material

The autopsy of the dead animal was conducted, aiming macroscopic examination of the internal organs, bacteriological and histological investigation of the internal organs. During the autopsy, there was no significant visual changes in the internal organs, except for the heart. On the left ventricle there was a 4-5 cm diameter white insert, which after the section was found to be anabscess filled with the yellowish-colored creamy-caseous mass. According to the preliminary cause, the dolphin's death was determined as focal purulentmyocarditis (image 5.).

The histopathological examination of the heart sample revealed the acute fibrosis with pyogranulomatous lesions (image 6).

Image. 5. The heart of a dead dolphin with purulent abscess.

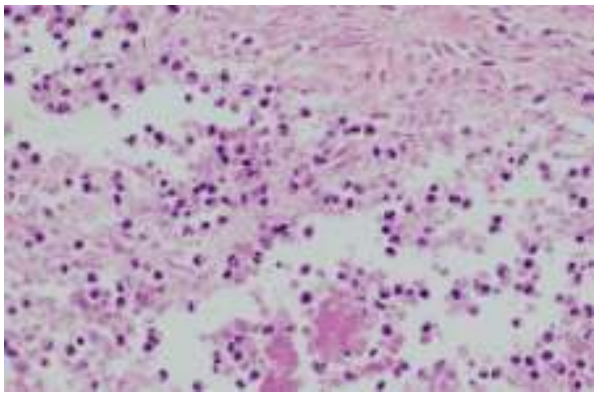


Image.6. Hystopathological study of the heart tissue of dead —Kako‖ dolphin. Microscopy picture of the thin section of heart tissue. (Staining with hematoxylin,eosin and PAS)

In the adjacent myocardium fibroangioblastic tissue with scattered polymorph nucleated granulocytes and macrophages was present.Locally fibrin depositions on the epicardium with hemorrhage in the epicardial fat and thrombosis of an artery. Few macrophages in the tunica media of another artery. Periodic acid–Schiff (PAS) staining was negative for mycotic

elements.The pleura of the lungs showed fibrosis with mild non-suppurative infiltrates. Pulmonary interstitial lymphoplasmocytic and neutrophilic infiltrates with edema and intra-alveolar macrophages was present.

Bacteriological analysis of pathological material

Bacteriological smears from the heart, lung and liver tissues have been sampled. in all three organs of the dolphin, the bacterial abundance was present in the first sector of swab plates moderate in the second sector, and in the third - increase in the form of individual bacterial colonies.Their number in the III sector per plate varied from 10 to 50 colonies. The bacterial growth in the fourth sector of the plate has not been observed.

It should be noted that from the liver and lung tissue samples monocultures were grown. The lung's sample was represented by β- hemolytic, mucoid, 3-4mm in size gray colonies, In case of liver tissue sample cream - yellow, 22,5mm -sized colonies were developed. From hart sample of the dolphin the mixed culture was isolated one isolate was β-hemolytic, 2-3 mm diameter yellowish colonies and another -γ- hemolytic white- cream colonies with D=2-3 mm.

After analyzing the biochemical profile of bacterial isolates allocated from dolphin organs and analysis of the obtained results, The two gram-positive bacterial isolates isolated from the heart were identified as staphylococci, one of these isolates - Isolate N1 belongs to stapylococcus aureus, and the second N2, coagulase-negative staphylococcus group.The bacterial isolate from the liver turned out to be the non-fermenting micro organism *Spingomona spaucimobilis*, which belongs to the nosocomial infections. There is a human opportunistic pathogen and is known for its multiple resistance to various antibiotics.The bacterial isolates from the lung of the dolphin was identified as *E.coli,* without lactose fermentation ability.

The sensitivity of bacterial isolates in relation to various antibiotics has been studied by Kirby-Bauer disk diffusion method. The isolates have shown different sensitivity to 20 tested antibiotics.In staphylococcus, the strains under investigation have been found to be sensitive to

most tested antibiotics. The resistance has been shown to the antibiotic of the Macrolide group – Erythromycin. It is noteworthy that this antibiotic was involved in the treatment scheme of a dolphin for a long period of time,which may have contributed to the development of the resistance to antibiotics in this microbe.

The isolates under investigation turned out to be resistant to the third generation Cephalosporin, however, it should be noted that both of the staphylococcus isolates were sensitive, to the fourth as well as to Cephalosporins of the first generation.

Most important is the fact that both isolates of staphylococcus have shown resistance to Vancomycin. It means that both isolates are dangerous for environment and human health, because the dissemination of such isolates in the environment by using the different mechanisms may cause the proliferation of Vancomycin resistant genes in other microorganisms.

The strain of E. coli was resistant to 9 (45%) tested antibiotic.Based on this results obtained, this isolates may be attributable to bacterial isolates with multiple resistance to antibiotics.

The resistance was noted to some representatives of beta-lactam group antibiotics, macrolides, tetracycline and quinolones, in particular to Ciprofloxacin.The results obtained indicate that this clinically important bacterial isolate and can be considered as a threat to human health.

It was impossible to prepare the bacterial lawn and study its sensitivity towards the antibiotics by using a disk-diffusion method in case of *Spingomonas paucimobili,* as well as in case of Mueller-Hinton.

The sensitivity of these bacterial isolates was studied towards the various commercial and experimental bacteriophages, kept in the collection of G. Eliava Institute of Bacteriophages, Microbiology and Virology (tables 5 and 6).

Table №5. Study of the sensitivity to the *Stapylococcus spp.* bacterial strains allocated from the lungs of the dead dolphin

Bacterial strains	Isolated	Phages								
		Staphylococcal	Phagio	SES	Intest	pheresis	Pio	Experimental staphylophagus Eka 92	Experimental staphylophagus TK 14	Experimental staphylophagus TK5
		1	2	3	4	5	6	7	8	9
Stapylococcus spp……K1	Heart of the dolphin	scl	scl	R	R	R	R	cl	R	R
Stapylococcus aureus …….K2	Heart of the dolphin	+	R	R	R	R	R	cl	R	R

TableN№6. Study of the sensitivity to the *Escherichia coli* bacterial strains allocated from the lungs of the dead dolphin

Bacterial strains	Isolated	Phages							
		Phagio	SES	Intest	Pheresis	PIO	Experimental staphylophagus UN	Experimental staphylophagus DD VI	Experimental staphylophagus T4
		1	2	3	4	5	6	7	8
Escherichia coli K3	Lung of the dolphin	R	ntv	+	R	ntv	scl	Scl	Scl

Selected strains of staphylococci, have shown resistance to most of the phages, however, strong lytic activity was observed using experimental bacteriophage "Eka 92". It should be noted that the multidrug resistant E. coli strain have shown weak and moderate susceptibility to most of the tested phages.

Study of the microbiological flora of material taken from the dolphin washed up on the Black Sea beach.

In May 2014, all attempts of returning back to its natural habitat of the whiteside dolphin, washed up on the beach nearby the fish market, turned out to be futile. Therefore, it was taken to a special tank of the dolphinarium for rehabilitation. Despite the urgent resuscitation procedure, the dolphin died. The pathanatomic autopsy was carried out in accordance with the instructions. The material was taken for further microbiological analysis.In particular, the liver, lung, kidney, large and small intestine tissues were taken.The primary processing of the material and initial bacteriological analysis (primary cultivation and selection of the leading isolates) was carried out in Batumi, in the laboratory of Batumi Dolphinarium, while the further evaluation, study of the standard biochemical, cultural and morphological parameters was done in the laboratory of G. Eliava Institute of Bacteriophages, Microbiology and Virology.

On the basis of the complex analysis of the results obtained, and by using the API systems, the identification of the bacterial isolates was carried out.

5 of the isolated cultures were the gram-positive – cocci, which were expressed during the microscopic research as diplococci, chains or clusters. The part of gram-positive cocci isolates N3,9 were identified as non-hemolytic*Staphylococcus spp.*, probably *S.epidermidis*. The isolate N 2 with its morphological-physiological features belong to *Stomatococcusspp.*(it is also close to *Stomatococcusspp.,* while the isolates N 6 and N 7 were identified as *Enterococcus spp*.

3 strains of the isolates belonged to Ox-positive, gram-negative bacilli.Detailed biochemical characterization and application of API tests allowed us to identify

them:*Shewanella putrifaciens* (isolate N8); *Aeromonas salmonicida*(isolate N10); *Bukholderia cepacia*(isolate N12).

Five isolates Ox-negative gram-negative bacilli with biochemical parameters complied with the representatives of the family of E *Enterobacteriaceae,* among them N 11 and N13 isolates have been identified as E.Coli (approved by API 20 E tests), while the other three isolates N1, N4 ღა N5 with similar biochemical-physiological profile may be equally belonged to *Klebsiella pneumoniae, Enterobacter* spp., *Citrobacter freundii*-ს. N 5 isolate was tested by API 20 E system and was identified as the *Enterobacter cloaceae.*

The resistance of 11 bacterial isolates from the stranded dolphin towards 26 antibiotics and phages of 4 types (pheresis, SES, PIO, Intest), exceeded 70%.

Most of the above mentioned bacteria can usually be found in water environments and samples taken from animals.The part of them are pathogens of fishes and vertebrate animals (e.g., *Aeromonas salmonicida, Shewanella putrifaciens*), while part of opportunistic pathogens (*Bukholderia cepacia*), which can cause serious bacterial infections in the sea mammals.

In terms of disease causing, except the pathogenic factors, the number of bacteria is important, the determination of which on the basis of the primary material, was not possible. Also, we cannot exclude the existence of infectious etiology, which requires special, immunoperative and / or immunofluorescence studies.

The effectiveness of noogenic environmental conditions for the preservation and rehabilitation of dolphins, for ex situ conservation

Recreational and regenerating systems for life of sea inhabitants

The well-organized life maintaining system is crucial for successful and safe conservation of dolphins in the noogonic environment. Its main and primary purpose is to maintainthe high quality water for the inhabitants permanently. In order to achieve this goal, life insurance systems should:

- The quick removal of all colloid and suspicious residuals;

- Disinfection of water without any hazard to inhabitants;
- Removal of toxic components derived from animal wastes by physical, chemical and / or biological means;
- Maintenance of the effective gradient of water temperature and the relevant level of the oxygen dissolved in water;

The following processes are being undertaken during the water processing in life insurance systems:

WATER TREATMENT PROCESS	RESULT OF TREATMENT
Filtration of the low pressure sand	Removal of residuals
Biofiltration	Removal of urine, ammonium chloride and nitrates
Ozonization	Disinfection
deaeration	Removal of excessive ozone
Division	Removal of proteins, fat and superficial active substances
Heating-cooling	Temperature control

The effectiveness of the filtration and regeneration process is significantly affected by the time of recirculation of the total volume of water in the habitats and other auxiliary tanks which is desirable to be as little as possible, which varies within 30 (Quarantine Pool) - 130 minute (Residential Pool) in Batumi Dolphinarium.The maximal disinfect effect of ozonization can be reached through the recirculation of the full amount of water in this regime, as well as the water aesthetically pleasing blue color.

Batumi Dolphinarium includes three residential and one quarantine tanks, the total volume of which consists of 3370 cubic meters, they are equipped with ultra-modern systems for maintenance of the life of inhabitants, including all the above mentioned processes.This, on the one hand, is important for maintaining the normal animal life and immune status of living animals, as well as for elimination of the risk factors, including elimination of life-threatening generalized infections

Conclusions

1. Dolphinarium has identified correlation between the mentioned parameters. The free chlorine content and common microbial number, as well as common chlorine and E.coli and S.aureus have been shown to be of negative correlation. The ORP was significantly negatively correlating on the common microbial number at the temperature of 37°C and 22°C and relatively less –on common coefficients and enterococcal substations;and the The physical-chemical and microbiological monitoring of the water of Batumi gigh positive correlation was observed between individual micro-biological indicators, such as TBC (37°C) - TCC, TBC (37°C) – ENT, E.coli- S. Aureus ⴗ E.coli - P.aeruginosa.
2. The bacterial isolates allocated from the biological materials of the inhabitants of the dolphinarium, as well as from the washed up animals, belong to the gram-positive bacilli, colli, coccobacilli and gram-negative bacilli and characterized by high variety of diversity. Most of them are commonly found in water environments, as well as in samples taken from animals and may be considered as opportunistic pathogens, while the parts of the isolates (*Aeromonas salmonicida, Erysypellothryx ruthiopathie, Shewanella putrifaciens, Burkholderia spp*) belongs to pathogens of fish and other vertebrate animals.
3. The microbiota of upper respiratory tract (URT) of the Black Sea and pacific bottlenose dolphins (Tursiops truncatus ponticus and Tursiops truncatus gilli) was presented with gram-positive cocci, *Staphylococcus spp., Streptococcus spp.)*nad gram-negative, among them non-fermentable bacilla (*Enterobacteriaceae, Pseudomonas spp., Proteus spp.),* as wells as *Actinomyces spp.* and *Candida spp.* The microflora taken from the washed up dolphin consisted of: *Staphylococcus spp.*, *Stomatococcusspp*, *Enterococcus spp.* , *Shewanella putrifaciens, Aeromonas salmonicida, Bukholderia cepacia, E.coli*, *Klebsiella pneumoniae, Enterobacter cloaceae*, *Citrobacter freundii.*
4. The data collected as a result of research has revealed correlation between the physical and chemical and microbial parameters of water and the number of bacteria in the air

exhaled by the dolphin. The significant correlation was observed between the exhaled air of high sowing and the samples containing staphylococcus. The negative correlation has been observed between the highly common microbial numbers, water temperature and ORP.There was no connection between the quantity of the exhaled air with increased microbial contamination and microbial parameters of the water, which should indicate on the impact of the latter on the respiratory flora of dolphins in the noogenic conditions.

5. Investigation of the phagesensitivity of the part of allocated isolates (isolate 39) confirmed their differences with morpho-culture and biochemical properties; 53.8% of strains are sensitive to polyvalent fagicides, which contain active bacteriophages against staphylococci, pseudomonas, enterococcus and intestinal group bacteria; the lysate activity towards the microflora isolated from the dolphins was shown a number of vibriophage and the phage of aeromonas.
6. One of the keys for diagnostics of the infectious diseases, is the microbiological analysis of the air exhaled by the dolphins. It gives not only the possibility of detecting the potential pathogen but the possibility of selecting the effective antibiotics or other antimicrobial means and subsequently controll the process of animal treatment.
7. Specific bacteriophages and polyvalent phage preparation may be used to diagnose bacterial strains, on one hand and for regrouping these strains (for differentiation) as well as for treatment of infections of the bacteriological etymology, on the other hand, especially in cases where the disease-causing is the multi-resistant to antibiotics and the animal has a reduced immune status.

8. The data collected as a result of the conducted investigation that due to the continuous monitoring of hydro-chemical and bacteriological parameters for the efficient operation of water supply recycling system, the utilization of the latest methods of water use, the optimization of water sanitary indicators, the ecological condition, hydrochemical and

bacteriological parameters have provided the good conditions for dolphin inhabitance which is largely ensured with a high level of care, storage and viability of mammals living in the noogenic environment.

9. The systematic research has been demonstrated by the importance of rational management of artificial subsistence of sea mammals, as well as the need for regular monitoring to maintain the status of animal health, successful reproduction and protection against infectious and other diseases.
10. Based on the assessment and analysis of the results of the conducted observations, the ways and means of monitoring improvement were revealed, the special scheme for monitoring the living environment and their health status was developed,which was considered as the basis of the rehabilitation and conservation activity of the dolphinarium.

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The Published Works within Dissertation Work:

1. *T.Tserodze, N.Zobova, D.Jgenti, M.Mgeladze, R. Goradze, E.Jaiani, E. Didebulidze, M.Tediashvili.***Study of Water Hydrochemical and Microbiological Quality in the Noogenic Habitat of the Black Sea Bottlenose Dolphins.** International Journal of Advanced Research (IJAR), 4(9) ISSN: 2320-5407. pp. 67-71, 2016.
2. *T.Tserodze, D. Jgenti, M. Mgeladze, N. Chkheidze, R. Lomidze, I. Gambashidze, N.Janelidze,E. Didebulidze, A. Groene, M. Kik, M.Tediashvili.***Fatal bacterial infection of the heart in captive bottlenose dolphin (Tursiopstruncatusgilli). A case study.** International Journal of Advanced Research (IJAR), 6(1), 795-804, 2018.
3. *T. Tserodze, D. Jgenti, M. Mgeladze, I.Gambashidze, N.Chkheidze, E.Jaiani, E.Didebulidze, N. Janelidze, R. Goradze, M.Tediashvili.*Dynamic changes in the upper respiratory tract microflora of the Black Sea and Pacific Bottlenose dolphins in noogenic habitat: possible influence of environmental conditions.International Journal of AdvancedResearch (IJAR), 2018.