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# Bacteria of the Clostridium Genus, Methane and Hydrogen Sulfide in Sulfide Mud of the Taman Peninsula Reservoirs

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**Abstract:** The features of the distribution of the concentrations of hydrogen sulfide, methane and the number of sulfite-reducing clostridia in the mud of the Bugazsky, Kiziltashsky and Vityazevsky limans and Chembursky lake were studied, and their physiological and chemical conditions and levels of anthropogenic loading were assessed. The tendency of reduction of the content of the studied reduced gases from the surface to the lower layers of mud was established, with relatively low concentrations of methane in the limans and abnormally high concentrations in the Chembursky lake, subject to strong anthropogenic influence. It is shown that high concentrations of methane and hydrogen sulphide in mud of water bodies that are planned for use for human treatment, indicate good therapeutic properties of peloids. At the same time, for those same reservoirs (limans) that are simultaneously used for extraction and reproduction of hydrobionts, the presence of high concentrations of reduced gases in the upper layers of bottom sediments and near-bottom water layers, as a rule, will promote the development of pestilence phenomena, which will adversely affect the development of the fishing industry. For reservoirs for both fishery and medical purposes, the high content of reduced gases and the abundance of pathogenic microflora may indicate contamination. The established rectilinear relationships between the number of sulfite-reducing clostridia and the concentrations of methane and hydrogen sulfide indicate the participation of sulfite-reducing clostridia in the processes of methane and hydrogen sulfide formation in the mud of the reservoirs of the Taman Peninsula. Sanitary-microbiological assessment of mud of the studied reservoirs shows that the muds studied cannot be recommended for using with medicinal purposes.

**Keywords:** Taman Peninsula, Clostridium, Total Hydrogen Sulfide, Methane, Therapeutic Muds

## Introduction

The Taman peninsula is situated in the Azov-Black Sea basin and it is one of the most popular balneological resorts in the South of Russia. On its territory there are more than 50 deposits of mud sulphide and associated therapeutic mud with different rates (Stoynov, 1995; Kholopov *et al*., 2003; Fedorov *et al*., 2017). However, if for sulfide therapeutic muds, along with various salts, vitamins, enzymes, hormones and other substances, hydrogen sulphide is an important therapeutic component (Bakhman *et al*., 1965: Fedorov, 2017), then for bottom sediments of reservoirs for fishery purposes, increased concentrations of hydrogen sulphide and other reduced gases are considered as an unfavorable factor (Ivanov, 1988; Fedorov *et al*., 2005; Trubnik *et al*., 2017). Thus, the increased content of hydrogen sulphide and/or methane in bottom sediments, which are usually accompanied by a deficiency in dissolved oxygen in water, is fraught with negative consequences both from an ecological and economic point of view, since they can lead to pestilence phenomena and mass death of fish and other hydrobionts. It should be added that the increase in the number of conditionally pathogenic microflora in the bottom sediments, in particular the sulfite-reducing clostridia, may also lead to the death of hydrobionts leading the benthic lifestyle (Ivanov, 1988; Fedorov *et al*., 2005; Morozova and Fedorov, 2015).

In many countries, including the Russian Federation, sulfite-reducing clostridia are used as sanitary-demonstration microorganisms in the study of therapeutic mud: the detection of spores of sulfide-reducing clostridia indicates a long-standing fecal contamination, and one of the indicators of fresh fecal

contamination of the environment is the high content of vegetative forms of *C. Perfringens* (EHCHiGOS , 1989; Hill *et al*., 1996; Edwards *et al*., 1998; Labinskaya and Volina, 2008; Vierheilig *et al*., 2013). Sulfite-reducing clostridia are also used to assess the effectiveness of drinking water purification from viruses and bacteria (Payment and Franco, 1993; SanPiN, 2001). In addition, interest in sulfite-reducing clostridia is due to the fact that some of their species are causative agents of gas gangrene, botulism, tetanus and other serious diseases (Songer, 1996; McClane *et al*., 2006; Rood, 2006). Taking into account the peculiarities of balneological procedures, during which mud cannot be excluded from the wound surfaces and mucous membranes of the body during applications, sulfite-reducing clostridia represent an extreme danger and can inflict enormous harm to human health, up to a lethal outcome. From these positions, control of the number of sulfide-reducing clostridia in therapeutic muds of water bodies subject to anthropogenic load, in accordance with the EHCHiGOS (1989; MI, 1999) is an actual and important task.

The purpose of this work is to study the distribution of hydrogen sulphide, methane, and the abundance of sulfite-reducing clostridia in the mud of a number of limans and one of the lakes of the Taman Peninsula, characterized by different physicochemical conditions and anthropogenic impact levels, as well as an assessment of their sanitary and microbiological condition according to the *clostridium* titer sediments. Since we do not exclude the direct participation of sulfite-reducing clostridia in the generation of hydrogen sulphide and methane (Trubnik *et al*., 2017; Fedorov *et al*., 2016), it is of scientific interest to study the relationship of these reduced gases with sulfite-reducing clostridia in the mud of the studied water bodies. The importance of the work performed, in addition to the fundamental significance, is also due to the special status of the Taman limans, on the one hand, as fishery reservoirs, where the feeding of many fish species of the Azov-Black Sea basin occurs, and on the other hand, as water bodies whose muds are used for self-treatment by tourists at resorts of Krasnodar Region.

## Materials and Methods

During the autumn period, the authors conducted expeditionary studies on the Taman Peninsula, during this expedition data were obtained on the abundance of sulfite-reducing clostridia, the concentrations of methane ($CH_4$), and total hydrogen sulfide ($\Sigma H_2S$) in the bottom sediments of the Bugazsky, Kiziltashsky and Vityazevsky limans and Chembursky lake (Fig. 1).

Sampling of mud was carried out using a specially designed plastic tube with sharp edges and a piston to extrude the core (Fedorov *et al*., 2005).

Determination of methane and hydrogen sulfide content in bottom sediments was carried out by the staff of the Hydrochemistry Institute (Rostov-on-Don) according to generally accepted methods: Methane-vapor-phase gas chromatography method (Fedorov *et al*., 2005; Gar'kusha and Fedorov, 2010), total hydrogen sulfide-photometric method with dimethylparaphenylenediamine (Roshydromet, 2011).


**Fig. 1:** Map of the location of sampling stations (1 - Chembursky Lake; 2 - Vitiazevsky liman; 3 - Kiziltashsky liman; 4 – Bugazsky liman)

Data on the content of methane and hydrogen sulfide in the bottom sediments are given in μg/g wet mass and mg/g wet mass (μg/g and mg/g w. m.), respectively. The quantitative determination vegetative cells of sulfite-reducing clostridia was carried out according to the generally accepted methodology (Labinskaya and Volina, 2008), which does not involve heating the inoculum (in a water bath at 75 (± 5) °C for 15 min), which promotes spore germination. Data on the number of sulfite-reducing clostridia in each layer of bottom sediments were obtained in CFU/g and then transferred to the clostridia titer for the subsequent assessment of the bottom sediments ecological condition.

The clostridia titer is determined from the maximum dilution in which clostridia are found, i.e., the clostridia titer is the lowest amount of bottom sediment, expressed in grams, which contains at least one viable cell of sulfite-reducing clostridia. In addition to the abundance of sulfite-reducing clostridia, concentrations of $CH_4$ and $\Sigma H_2S$ in samples taken from various layers (up to 25 cm) of mud of the investigated reservoirs, measurements the values of Eh and pH in the muds were measured using electrodes of the portable pH meter of the ionomer - "Ecotest 2000". Determination of the number of microorganisms, concentrations of $CH_4$ and $\Sigma H_2S$, as well as of the values of Eh and pH, were performed in 2-fold replication. In addition, the one-way analysis of variance (ANOVA) was carried out for the levels of reduced gases and clostridia in the bottom sediments. The results of the analysis showed that for the above parameters the values of $p<0.01$. Calculations are made with using of the program "STATISTICA". Statistical, graphical processing of results and analyzes of studies was carried out using the MS Excel software package.

## Results and Discussion

The results of determinations of the number of sulfite-reducing clostridia, physicochemical parameters, as well as methane and total hydrogen sulphide, are presented in Table 1. The studied reservoirs have a similar origin but different formation time, which leaves its imprint on the structure and composition of sediments prevailing in them and as a consequence, on the physicochemical conditions and the intensity of the course of biogeochemical processes.

The mud of the Vitiazevsky liman up to the layer of 10-15 cm are represented by a silt of dark gray color with a large number of sandy particles; the underlying layers are mainly composed of dark gray sand, brightening with depth. On the length of the selected sediment column there is a large number of debris of mollusk shells, with the exception of the upper layer.

The surface layer of the mud of the Kiziltashsky liman differs somewhat from Vityazevsky: the layer (0-7 cm) is composed of dark gray mud with a large number of sandy particles, below this layer there are dense clay-sand deposits of brown color, and from a depth of 20 cm even denser light-brown clay.

Mud of the Bugazsky liman along the entire length of the selected column are stacked with a plastic homogeneous dark gray to black silt with a large amount of plant residues and some admixture of sand particles. In the denser lower layers there are fragments of shells of mollusks. In Chembursky lake, muds are represented by semi-liquid oily silt of dark gray to black color, with a pronounced smell of hydrogen sulfide. In the lower layers of sediments, half-decomposed plant remains are found. It should be noted that the mud of the studied water bodies were previously classified as maritime and marine sulphide muds of saline water bodies (Kholopov *et al*., 2003).

The study of the physicochemical conditions in the muds of the reservoirs of the Taman Peninsula showed a slight variation in the redox potential values (pH), with a very wide range of changes in the values of the hydrogen ion index (Eh). Thus, the pH values in the investigated sediments varied from 7.33 to 8.04 (neutral - weakly alkaline conditions), averaging in the Bugazskysky Liman - 7.72, in the Vitiazevsky Liman - 7.58, in the Kiziltashsky Liman - 7.55 and in Chembursky Lake - 7.57. The most restorative conditions were found in the mud of the Bugazsky liman, where the values of Eh varied from -198 mV in the layer 5-10 cm to -63 mV - in the layer 2-5 cm (average value -111 mV). In the muds of the Kiziltashsky Liman, Eh varied in the range -86 ... -55 mV (average value -67 mV), Vitiazevsky Liman -85 ... -2.5 mV (average value -54 mV), Chembursky Lake - 79 ... + 46 mV average value -15 mV). The tendency of decrease in Eh in bottom sediments in a row is established: clays → sand deposits → mixed sandy-argillaceous deposits → black and dark gray silt.

For mud of all limans, a decrease in the amount of $CH_4$ and $\Sigma H_2S$ from surface to lower layers, at relatively low concentrations of methane and a small range of their variation (from 0.01 to 0.17 μg/g), and an order of magnitude greater variability of hydrogen sulfide concentrations from 0.01 to 1.51 μg/g).

We see a completely different situation in Chembursky lake, the mud of which is characterized as silt, weakly sulphide and slightly salted (Kholopov *et al*., 2003), and is represented by a semi-liquid oily silt of dark gray to black with a pronounced smell of hydrogen sulfide. In this lake maximum concentrations of $CH_4$ and $\Sigma H_2S$, as well as the number of clostridia among the water bodies studied, were recorded. The maximum peaks of $CH_4$ (up to 24.3 μg/g) are set in the upper 0-2 cm layer, while $\Sigma H_2S$ (up to 1.75 mg/g) is in the 10-15 cm layer, which does not correspond to the generally accepted structure of the zonality of microbiological processes, according to which in the brackish reservoirs the zone of active sulfate reduction is located above the active methane generation zone (Lein and Ivanov, 2009;

Fedorov *et al*., 2005) We believe that it is connected with the powerful anthropogenic impact of the city of Anapa on Chembursky lake, as a result of which accumulation of increased quantities occurs in its sediments in the labile organic substance, which neutralizes the competitive relationship between sulfate reductants and methanogens for the possession of nutrient substrates (Fedorov *et al*., 2005; Kuznetsov *et al*., 1985; Winfrey and Zeikus, 1977). Methane formation in such areas can also proceed through noncompetitive reduction reaction molecular hydrogen of methanol, which is not a nutrient substrate for sulfate-reducing bacteria (Fedorov *et al*., 2005; Kuznetsov *et al*., 1985).

It follows that high concentrations of methane and hydrogen sulphide in mud of water bodies cannot be apriori considered as a factor indicative of good therapeutic properties of muds, and even, on the contrary, simultaneous detection of high concentrations of reconstituted gases in the upper layers of mud, as a rule, will be a clear sign of anthropogenic pollution.

Thus, according to the pH and Eh values, the mud of all the investigated reservoirs correspond to the quality of the peloids (with the exception of 0-2 cm of the layer with a positive Eh in Chembursky Lake), whereas in the content of $\Sigma H_2S$ (> 1 mg/g) to the peloids can be attributed only 0-2 cm layer of muds of the Kiziltashsky liman and 0-15 cm layer of Chembursky lake. However, the level and distribution of methane and hydrogen sulphide in the muds of the lake indicates, as shown above, powerful anthropogenic pollution, which limits the possibility of using its mud for medicinal purposes.

The number of sulfite-reducing clostridia in the Kiziltashsky, Vityazevsky and Bugazsky limans varies in the range from 0 to 30 thousand CFU/g (Table 2). In Chembursky lake these values reach 6000 thousand CFU/g and on average 2-3 times higher than the number of microorganisms in the limans.

In the mud of the Vityazevsky liman, the maximum number of microorganisms was recorded in the 2-5 cm layer, the Kiziltashsky Liman in 0-2 and 5-10 cm, the Bugazsky liman 5-10 cm layer. In Chembursky Lake, the maximum number of sulfide-reducing clostridia (6000 CFU/g) is fixed in the 10-15 cm layer of muds, where the maximum concentrations of $\Sigma H_2S$ and one of the highest $CH_4$ contents are also determined. Thus, in the studied reservoirs the maximum peak of the bacterial population is confined both to the surface layer (0-2 and 2-5 cm) of mud, and to their deeper layers (5-10 cm and 10-15 cm).

**Table 1:** Average number of sulfite-reducing clostridia, concentrations of $CH_4$ and $\Sigma H_2S$, as well as pH, Eh in the muds of the studied reservoirs

| Layer, cm | Eh, мВ | pH | SRC, thousand CFU/g | Methane, μg/g w. m. | Total hydrogen sulfide, mg/g w. m. |
|---|---|---|---|---|---|
| Chembursky lake (Station 1) | | | | | |
| 0-2 | +46.0 | 7.51 | 400 | 22.0 | 1.12 |
| 2-5 | -25.0 | 7.64 | 900 | 6.01 | 1.16 |
| 5-10 | -79.0 | 7.59 | 3000 | 11.6 | 1.11 |
| 10-15 | -19.5 | 7.47 | 6000 | 13.2 | 1.56 |
| 15-20 | -11.5 | 7.55 | 200 | 9.78 | 0.64 |
| 20-25 | -3.0 | 7.64 | 3000 | 7.63 | 0.69 |
| Vityazevsky liman (Station 2) | | | | | |
| 0-2 | -84.8 | 7.56 | 1 | 0.01 | 0.57 |
| 2-5 | -67.0 | 7.54 | 20 | 0.02 | 0.50 |
| 5-10 | -84.6 | 7.69 | 1 | 0.01 | 0.27 |
| 10-15 | -49.5 | 7.60 | 1 | 0.01 | 0.35 |
| 15-20 | -33.7 | 7.75 | 2 | 0.01 | 0.07 |
| 20-25 | -2.5 | 7.33 | 2 | 0.01 | 0.01 |
| Kiziltashsky liman (Station 3) | | | | | |
| 0-2 | -68.5 | 7.46 | 20 | 0.03 | 1.44 |
| 2-5 | -54.5 | 7.48 | 4.5 | 0.02 | 0.22 |
| 5-10 | -59.8 | 7.61 | 17 | 0.04 | 0.23 |
| 10-15 | -59.8 | 7.57 | 2 | 0.02 | 0.10 |
| 15-20 | -76.0 | 7.61 | 1.5 | 0.02 | 0.20 |
| 20-25 | -85.5 | 7.56 | 0 | 0.04 | 0.01 |
| Bugazsky Liman (Station 4) | | | | | |
| 0-2 | -76.5 | 7.76 | 2 | 0.13 | 0.35 |
| 2-5 | -62.6 | 8.04 | 2 | 0.07 | 0.37 |
| 5-10 | -104.7 | 7.83 | 30 | 0.02 | 0.12 |
| 10-15 | -198.0 | 7.60 | 2 | 0.03 | 0.27 |
| 15-20 | -101.0 | 7.51 | 2 | 0.03 | 0.19 |
| 20-25 | -125.0 | 7.59 | 1 | 0.03 | 0.16 |

**Table 2:** Assessment of the sanitary-microbiological condition according to the titre of clostridia of mud of the studied reservoirs of the Taman Peninsula

| Reservois | Layer, cm | SRC, thousand CFU/g | Clostridium titre | Category of muds |
|---|---|---|---|---|
| 1) Chembursky Lake | 0-2 | 400.0 | 0.00001 | Highly polluted |
| | 2-5 | 900.0 | 0.00001 | Highly polluted |
| | 5-10 | 3000.0 | 0.000001 | Highly polluted |
| | 10-15 | 6000.0 | 0.000001 | Highly polluted |
| | 15-20 | 200.0 | 0.00001 | Highly polluted |
| | 20-25 | 3000.0 | 0.000001 | Highly polluted |
| 2) Vityazevsky Liman | 0-2 | 1.0 | 0.001 | Polluted |
| | 2-5 | 20.0 | 0.0001 | Polluted |
| | 5-10 | 1.0 | 0.001 | Polluted |
| | 10-15 | 1.0 | 0.001 | Polluted |
| | 15-20 | 2.0 | 0.001 | Polluted |
| | 20-25 | 2.0 | 0.001 | Polluted |
| 3) Kiziltashsky Liman | 0-2 | 20.0 | 0.0001 | Polluted |
| | 2-5 | 4.5 | 0.001 | Polluted |
| | 5-10 | 17.0 | 0.0001 | Polluted |
| | 10-15 | 2.0 | 0.001 | Polluted |
| | 15-20 | 1.5 | 0.001 | Polluted |
| | 20-25 | 0.0 | 0 | Clear |
| 4) Bugazsky Liman | 0-2 | 2.0 | 0.001 | Polluted |
| | 2-5 | 2.0 | 0.001 | Polluted |
| | 5-10 | 30.0 | 0.0001 | Polluted |
| | 10-15 | 2.0 | 0.001 | Polluted |
| | 15-20 | 2.0 | 0.001 | Polluted |
| | 20-25 | 1.0 | 0.001 | Polluted |

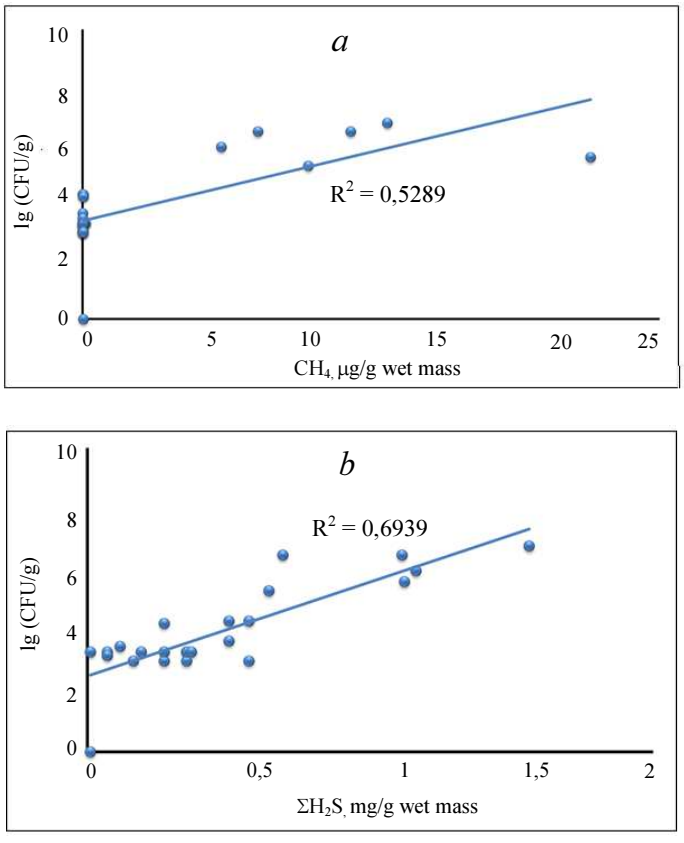


**Fig. 2:** Dependences between the number of sulfide-reducing clostridia and the concentrations of $CH_4$ (a) and $\Sigma H_2S$ (b) in the mud of the studied reservoirs of the Taman Peninsula

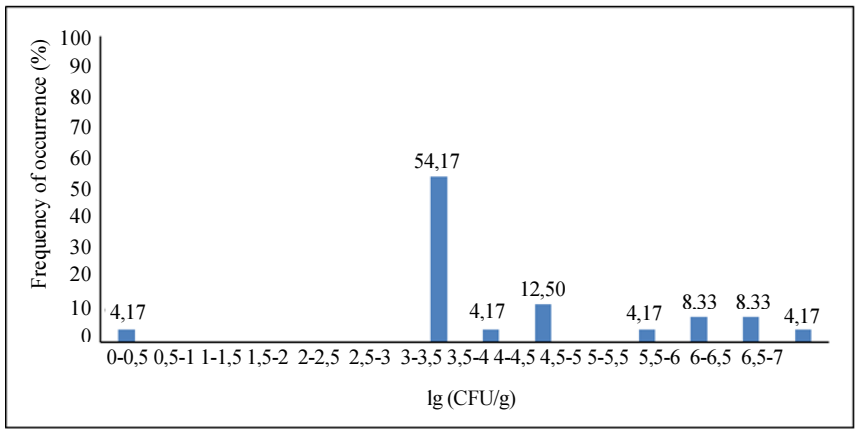


**Fig. 3:** Frequency of occurrence of ranges of the number of sulfite-reducing clostridia in mud of the studied reservoirs of the Taman Peninsula

It is known that methane and hydrogen sulfide are produced during the flow in bottom sediments complex metabolic processes associated with degradation of organic matter involving sulfite-reducing bacteria and then using the resulting metabolites with methanogens. The fact that sulfite-reducing clostridia can participate in the formation of methane and hydrogen sulphide in mud of aquatic ecosystems is very interesting from a fundamental point of view. This, in addition to the results of previously conducted field investigations of sediment ponds and streams of the Eastern Donbass and laboratory experiments (Trubnik *et al*., 2017), indirectly evidenced by the results of this work. Thus, building a regression between the regeneration gases and the number of sulfite-reducing clostridia in mud reservoirs Taman Peninsula showed (Fig. 2) is statistically significant (Lakin, 1990) and reliable connection between the numbers clostridium and the concentrations of methane (correlation coefficient $r = 0.73$, $p<0.01$) and $\Sigma H_2S$ (correlation coefficient $r = 0.83$, $p<0.01$).

The results of the assessment of the sanitary and microbiological condition of the mud of the studied reservoirs of the Taman Peninsula by the *clostridium* titre are presented in Table 2.

According to the regulations adopted in the Russian Federation for therapeutic muds of all types (peat, sapropelic, silty mineral, copulative), the *clostridium* titre should be at least 0.1. We have revealed that the number of vegetative cells of sulfite-reducing clostridia in more than 95% of samples of mud exceeds the established norms (EHCHiGOS, 1989; MI, 1999) and the *clostoridium* titre by several orders of magnitude (Fig. 3) as contaminated for the limans, and how heavily polluted - for the Chembursky Lake.

For comparison, we note that in therapeutic muds in lakes Bolshoy Tambukan and Solenoye (Lushnikovsky deposit), which do not experience significant anthropogenic pressure and do not require special restrictions for use (Malchukovsky *et al*., 2012), the clostridium titre is within the norm (Trubnik *et al*., 2017). At the same time, in the bottom sediments of a number of water bodies of the Eastern Donbass, located in the zone of anthropogenic impact, the same high values of clostridium titre (up to 0.000001) are recorded, as well as in the mud of the investigated reservoirs of the Taman Peninsula.

## Conclusion

The processes of methanogenesis and sulfite reduction in "background" and anthropogenically disturbed aquatic ecosystems can proceed according to different scenarios. However, despite the fact that from the point of view of balneology, it is completely unimportant in the course of what reaction hydrogen sulphide or methane was formed, because they are de facto attributed to integral natural therapeutic components. In addition, the simultaneous detection of high concentrations of methane and hydrogen sulfide and clostridium in the upper layers of mud can be a clear indication of the impact of anthropogenic impact on them.

The analysis of the results of the study of the Kiziltashsky, Bugazsky, Vityazevsky limans and Chembursky Lake made it possible to reveal the tendency of a decrease in the content of reduced gases from the surface to the lower layers of mud, with relatively low concentrations of methane in the limans and abnormally high concentrations in the Chembursky lake, subject to strong anthropogenic influences in the city of Anapa. The regression relationships between the number of sulfite-reducing clostridia and the concentrations of methane and hydrogen sulphide, established by us, indirectly indicate the involvement of sulfite-reducing clostridia in the formation of methane and hydrogen sulphide in the mud of the reservoirs of the Taman Peninsula.

If it is viewed from an applied point of view, for the water bodies with sulphide mud studied, the most informative and objective indicator of anthropogenic load is the clostridium titre, which allows you to assess the sanitary-microbiological condition of mud. The results of the sanitary and microbiological assessment of the clostridium titre of the mud of the Vitiazevsky, Kiziltashsky, Bugazsky limans and Chembursky Lake showed that the muds studied cannot be recommended for use for medicinal purposes, since the abundance of the studied pathogenic microflora poses a serious danger to human life and health, if using this mud for treatment. In connection with the results obtained, it becomes necessary to organize more in-depth and extended studies to identify sources of pollution and to clarify the extent of anthropogenic pressure on the water ecosystems of the Taman Peninsula.

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## Author's Contributions

**Yuriy A. Fedorov:** The head of the investigation, designed the research plan and organized the study, coordinated the data-analysis and contributed to the writing of the manuscript.

**Dmitriy N. Gar'kusha:** Contributed in drafting the manuscript and reviewing it critically for significant intellectual content.

**Roman G. Trubnik:** Participated in all experiments, collected the field data and contributed to the writing of the manuscript.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of other authors have read and approved the manuscript and no ethical issues involved.

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