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Research Article

Study of the dynamics of the microbial communities in the wedge clam *Donax trunculus* (Linnaeus, 1758) from the Bulgarian aquatory of the Black Sea

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Abstract

In the present work, we investigated the dynamics of the microbial communities in the wedge clam *Donax trunculus* (Linnaeus, 1758) from the Bulgarian coastal waters of the Black Sea. The samples were collected in the period of January 2020 until December 2022 from Arkutino, Ahtopol, Obzor and Tsarevo. The BIOLOG system was used for microbiological determination. In our investigation were isolated the following microorganisms: *Enterococcus cancerogenus*, *Enterococcus hirae*, *Escherichia vulneris*, *Citrobacter farmeri*, *Acinetobacter gyllenbergii*, *Enterococcus hirae*, *Escherichia vulneris*, *Enterobacter cloacae*, *Escherichia hermannii*, *Pseudomonas mendocina*, *Pseudomonas fulva*, *Pseudomonas alcaligenes*, *Pseudomonas putida*, *Acinetobacter johnsonii*, *Acinetobacter gyllenbergii*, *Enterococcus hirae*, *Escherichia vulneris*, *Enterococcus gallinarum*, *Citrobacter sedakii*, *Pseudomonas putida*, *Streptococcus lugdunensis*, *Enterococcus casseliflavus*, *Vibrio cincinnatiensis*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Enterococcus hirae*, *Streptococcus aureus*, *Staphylococcus lugdunensis* and *Enterococcus casseliflavus*. During the winter period, we detected the presence of *Pseudomonas sp.* – *P. alcaligenes*, *P. putida*, and *A. gyllenbergii.* In the autumn months we isolated *C. sedakii*, *C. farmeri*, *A. gyllenbergii*, *A. johnsonii*, *P. fulva* and *E. casseliflavus.* In the spring, *E. cancerogenus*, *E. hirae* and *Pseudomonas mendocina* were found. During the summer, the highest biodiversity of microorganisms - *E. hirae*, *E. vulneris*, *E. cloacae*, *E. gallinarum*, *P. putida*, *V. cincinnatiensis*, *V. alginolyticus*, *V. parahaemolyticus*, *S. aureus*, *E. hermannii* and *S. lugdunensis* were registered. Although our three-year research showed that some species are permanent and others are transient, we tend to accept the conclusion that there is only a transient microbiota in mussels and it changes depending on environmental conditions or is a result of pollution of the Black Sea.

Key words: Bivalves, hydrobiology, microbial identification, molluscs, pathogens, pollutions

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Introduction

The wedge clam *Donax trunculus* (Linnaeus, 1758) is found in the entire region of the Black Sea coast, but according to Fernández-Pérez et al. (2017) its population is extremely high on the territory of the Bulgarian Black Sea aquatory.

The species occurs in the Mediterranean and the Black Seas, and in the Atlantic Ocean from Senegal to the North Atlantic coast of France (Deval 2009). The species inhabits open sandy beaches where it forms thick beds, as the highest density are at depths from 0 to 3 m. It is considered a warm-water temperate species (Bayed and Guillou 1985; Lamine et al. 2020a; Chahouri et al. 2022).

The shellfish are exposed to diseases caused by various bacteria, which can cause a mass extinction of the species. It was detected that the cause of outbreaks of diseases in bivalves is related to conditional pathogens, i.e. free-living pathogenic bacteria which, under favourable conditions, can cause diseases. This poses a serious risk to humans as consumers of bivalve species. Pathogenic bacteria can enter into the clams directly from seawater, from the microalgae they feed on, but also as a result of anthropogenic pollution of the environment. For *D. trunculus*, various studies indicate it importance for the assessment and monitoring of the ecological conditions of the sandy beaches (Moukrim et al. 2004; Idardare et al. 2008; Nadir et al. 2015; Lamine et al. 2020b). *D. trunculus* can be used as an indicator species to understand population dynamics and to interpret variation in various biological parameters used as biomarkers of pollution. The dynamics of the microbial population of *D. trunculus* in the Black Sea Region have not been studied and there are no data concerning the microbial communities inhabiting the bivalves. Our study contributes to the study of microbiota of the Black Sea. It should be linked to additional long-term studies to provide basic information for the development of strategies for the protection and monitoring of the Black Sea coastal ecosystem. In a previous study, the microbial variation in the Arkutino Region was described (Ibryamova et al. 2022a). Our results demonstrated the presence of bacterial species of genera *Pseudomonas*, *Enterococcus*, *Escherichia*, *Citrobacter* and *Acinetobacer* in wedge clams *Donax trunculus* (Linnaeus, 1758). In the present study, only the species *D. trunculus* was investigated, as it was the only one found in the surveyed area. We found that the concentrations of *E. vulneris* exceed by 190 times the maximum available values according to Ordinance No. 4/20.10.2000. Increased concentrations of coliforms in the summer indicate a seasonal worsening of the conditions of the seawater as a consequence of anthropogenic activity. The main goal of the present work was to investigate the dynamics of microbial communities in the wedge clam *D. trunculus* (Linnaeus, 1758) from the Bulgarian Black Sea aquatory.

Materials and methods

Place and duration of the study

The study was conducted at the Department of Biology, University of Shumen, Bulgaria, from January 2020 until December 2022. The samples were collected from the regions of Mussel farms – Arkutino, Ahtopol, Obzor and Tsarevo (Fig. 1).

Collection of samples

After collection of three subsamples (each of about 1 kg), the mussels were refrigerated (4 °C) and transported to the laboratory for further immediate analysis, without freezing the specimens. In this study, we examined wedge clams of similar size, weight and shape to ensure maximal uniformity in the applied

Figure 1. Sampling locations at the Black Sea coast.

methods (Duquesne et al. 2004). The average length of mussels used in the study was 2.2 ± 0.43 cm. The mussels used for the analysis were collected monthly, throughout the three-year period. The collection of samples was carried out by trawling by JSC "Black Sea Fishing", Burgas.

Microbiological analyses

The mussels were scrubbed free of dirt, washed in hypochlorite solution (20 mg l^{-1}), rinsed with sterile distilled water and shucked with a sterile knife. The whole soft tissues of the mussel's liquor samples (about 100 g) were homogenised.

Faecal coliforms (FC) were enumerated through five tubes per dilution most probable number (MPN) series (Ignatova-Ivanova et al. 2018). After 3 h at 37 °C plus 21 h at 44 °C, gas positive tubes were recorded for FC. From each of the FC gas positive tubes, 0.1 ml were transferred in tubes with 10 ml of Tryptone Water (Oxoid, Basingstoke, UK) and then incubated for 24 h at 44 °C. *E. coli* were enumerated by MacConkey agar (Merck, Darmstadt, Germany). The plates were incubated aerobically at 35–37 °C for 18–24 hours. *E. coli* grows matte dark pink to tile-red colonies, surrounded by an opaque area due to the precipitation of salts in this environment. *Pseudomonas* sp. were enumerated by Cetrimide Agar (Merck KGaA, 64271 Darmstadt, Germany). Lactic acid bacteria (LAB) were isolated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia). The strains were cultured overnight (16–18 h) on MRS at 37 °C and in limited oxygen (tubes or Petri dishes with the strains were incubated in plastic bags, which limited the oxygen content). When determining the number of isolated species of microorganisms, the number of cells in 1 ml of sample was calculated, after which the percentage of the total number of microorganisms in the sample was calculated for each isolated species.

Microbial identification databases for the "BIOLOG" system

The microbial identification was performed by the BIOLOG Microbial Identification System VIO45101AM. The isolated strains were screened on BL4021502

Tryptic Soy Agar (TCA), cultured for 24 hours at 37 °C and then subjected to Gen III plaque identification to identify Gram positive and Gram negative aerobic bacteria. The microscopic pictures were performed using stereomicroscope OPTIKA (Italy) with a DinoEye, Eyepiece camera with 5 megapixels. The photographs were performed by using a Canon EOS 60D camera. The GEN III MicroPlate test panel provides a standardised micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria. BIOLOG's Microbial Identification Systems software (e.g. OmniLog Data Collection) was used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate. The BIOLOG system allows to quickly and accurately identify more than 2900 species of aerobic and anaerobic bacteria, yeasts and fungi. BIOLOG's advanced phenotypic technology provides valuable information for the properties of the strains, in addition to species-level identification. BIOLOG's carbon technology identifies the environment and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" of discrete test reactions performed in a 96-well microplate. The culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared with extensive data-bases (https://www.biolog.com/products-portfolio-overview/microbial-identification).

Results

The isolated species of microorganisms by year and percentage are presented in Figs 2–5. The species *E. cancerogenus*, *E. hirae*, *E. vulneris*, *C. farmeri* and *A. gyllenbergii* were isolated from the Arkutino Region (Fig. 2). The species *E. cancerogenus* was isolated only in 2020 in the month of May. The species *E. hirae* was isolated in all three years, with the highest percentage recorded in 2021 - 60.71% and the lowest in 2020 - 21.25%. This species was isolated during the months of May, June and August. The species *E. vulneris* was isolated in all three years, with the highest percentage reported in 2022 - 20.15% and, in 2020 and 2021, the percentage was 11.69%. The species was isolated

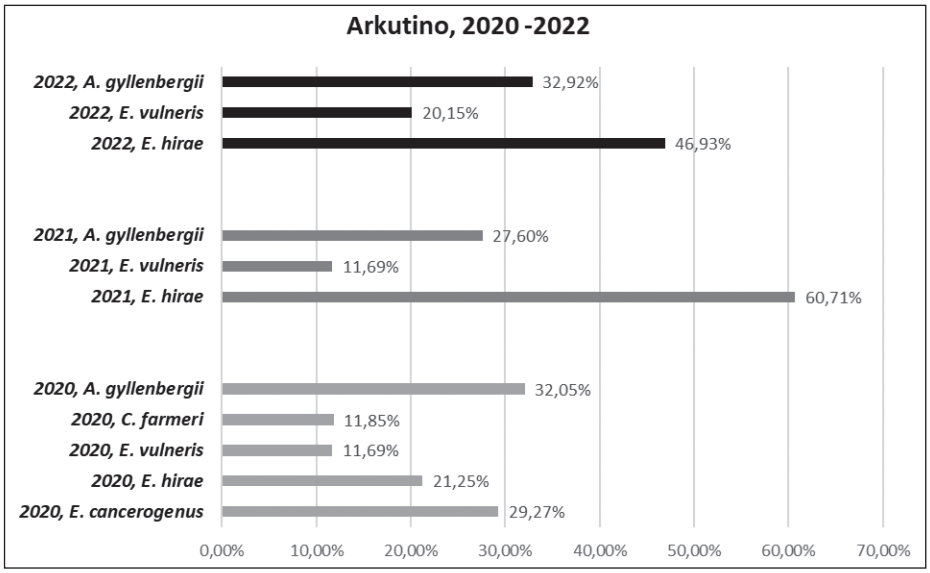

Figure 2. Microbial population dynamics in the Arcutino Region.

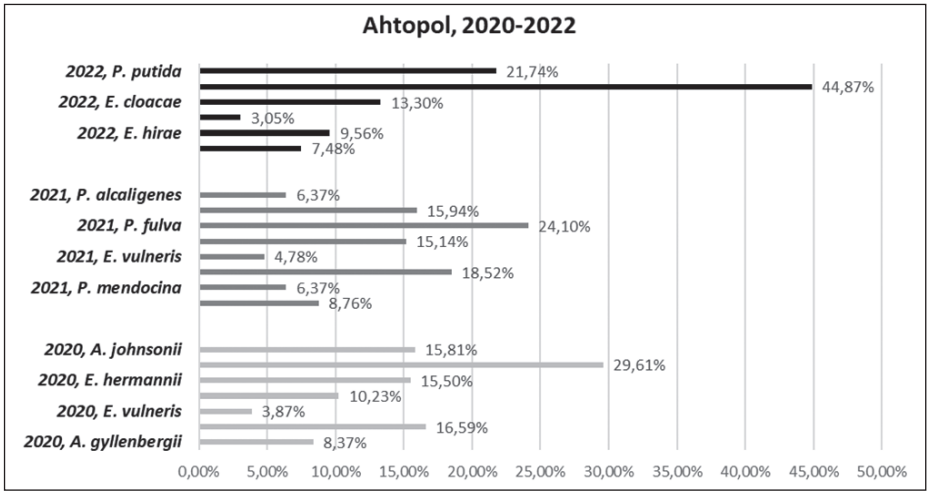

Figure 3. Microbial population dynamics in the region of Ahtopol.

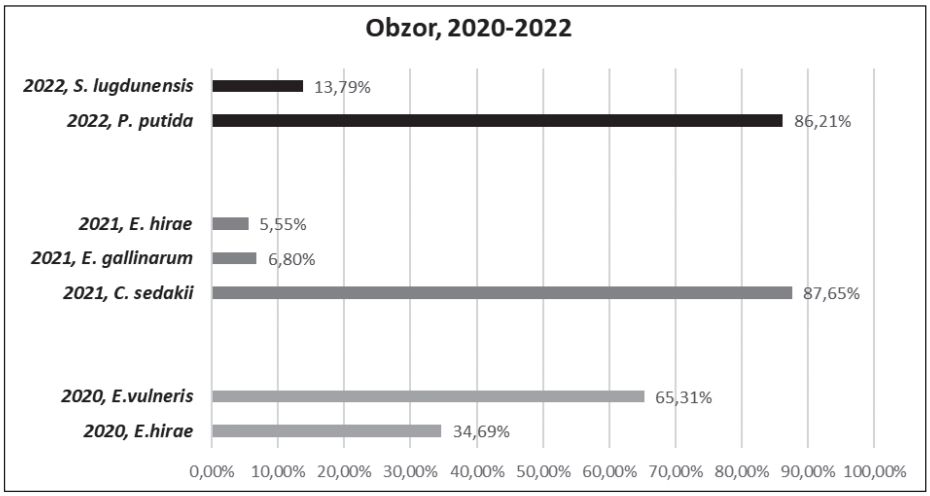

Figure 4. Dynamics of the microbial population in the Obzor Region.

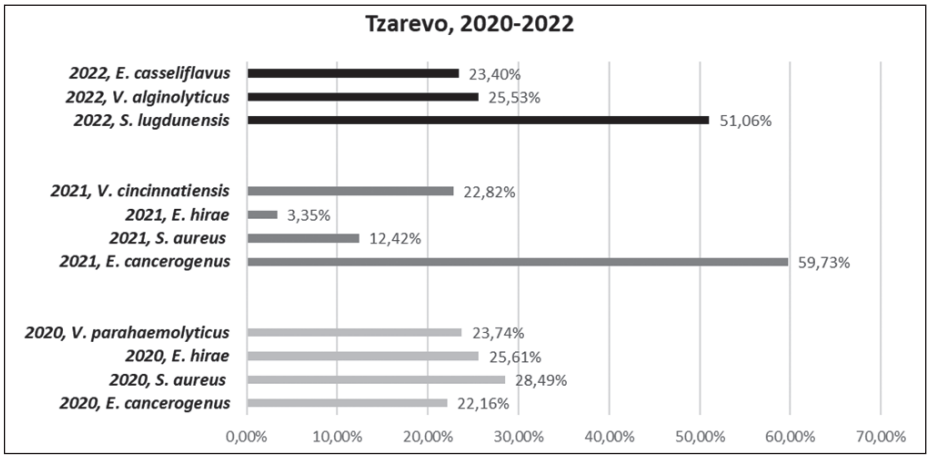

Figure 5. Dynamics of the microbial population in the region of Tsarevo.

in the months of June and August. The species *C. farmeri* was isolated only in 2020 in the month of September. The species *A. gyllenbergii* was isolated in all three years, with the highest percentage reported in 2022, 32.92% and the

lowest in 2021, 27.60%. The species *A. gyllenbergii* was isolated in the month of October.

The species *E. hirae*, *E. vulneris*, *E. cloacae*, *E. hermannii*, *P. mendocina*, *P. fulva*, *P. alcaligenes*, *P. putida*, *A. johnsonii* and *A. gyllenbergii* were isolated from the Ahtopol Region (Fig. 3). The species *E. hirae* was isolated in all three years, in the month of June. The highest percentage was reported in 2021 - 18.52% and the lowest percentage in 2022 - 9.56%. The species *E. vulneris* was isolated in all three years, in the months of June and July. The highest percentage was reported in 2021 - 4.78% and the lowest in 2022 - 3.05%. The species *E. cloacae* was isolated in all three years, in the month of August. The highest percentage was reported in 2021 - 15.14% and the lowest in 2020 - 10.23%. The species *E. hermannii* was isolated in 2020 and 2022, in the months of August and September. The highest percentage was reported in 2022 - 44.87%. The species *P. mendocina* was isolated only in 2021, in the month of May. The species *P. fulva* was isolated only in 2021, in the month of September. The species *P. alcaligenes* was isolated in 2020 and 2021, in the months of November and December. In 2020, its percentage was significantly higher 29.61% compared to 6.37% in 2021. The species *P. putida* was isolated in 2021 and 2022, in the month of December. In 2021, the amount was 15.94% and in 2022 - 21.74%. The species *A. johnsonii* was isolated only in 2020, in the month of October. The species *A. gyllenbergii* was isolated in all three years, in the month of February. The percentage of the species is between 7.5 and 8.7% of the total sample.

The species *E. hirae*, *E. vulneris*, *E. gallinarum*, *C. sedakii*, *P. putida* and *S. lugdunensis* were isolated from the Obzor Region (Fig. 4). The species *E. hirae* was isolated in 2020 - 34.69% and 2021 - 5.55%, in the months of July and November. The species *E. vulneris* was isolated only in 2020 in the month of July at a relatively high percentage of 65.31% of the total sample. The species *E. gallinarum* was isolated only in 2021 in the month of July at a relatively low percentage of 6.80% of the total sample. The species *C. sedakii* was isolated only in 2021 in the month of September at a relatively high percentage of 87.65% of the total sample. The species *P. putida* was isolated only in 2022 in the month of August at a relatively high percentage of 86.21% of the total sample. The species *S. lugdunensis* was isolated only in 2022 in the month of August at a relatively low percentage of 13.79% of the total sample.

The species *E. casseliflavus*, *Vibrio cincinnatiensis*, *V. alginolyticus*, *V. parahaemolyticus*, *E. hirae*, *S. aureus*, *S. lugdunensis* and *E. casseliflavus* were isolated from the Tsarevo Region (Fig. 5). The species *E. casseliflavus* was isolated only in 2022, in the month of September at 23.40% of the total sample. The species *V. cincinnatiensis* was isolated only in 2021, in the month of August at 22.82% of the total sample. The species *V. alginolyticus* was isolated only in 2022, in the month of August at 25.53% of the total sample (Fig. 6a). The species *V. parahaemolyticus* was isolated only in 2020, in the month of August at 25.53% of the total sample (Fig. 6b). The species *E. hirae* was isolated only in 2020, in the month of August at 25.53% of the total sample. The species *S. aureus* was isolated only in 2021, in the month of August at 12.42% of the total sample. The species *S. lugdunensis* was isolated only in 2022, in the month of August at 51.06% of the total sample. The species *E. casseliflavus* was isolated only in 2022, in the month of September at 23.40% of the total sample.

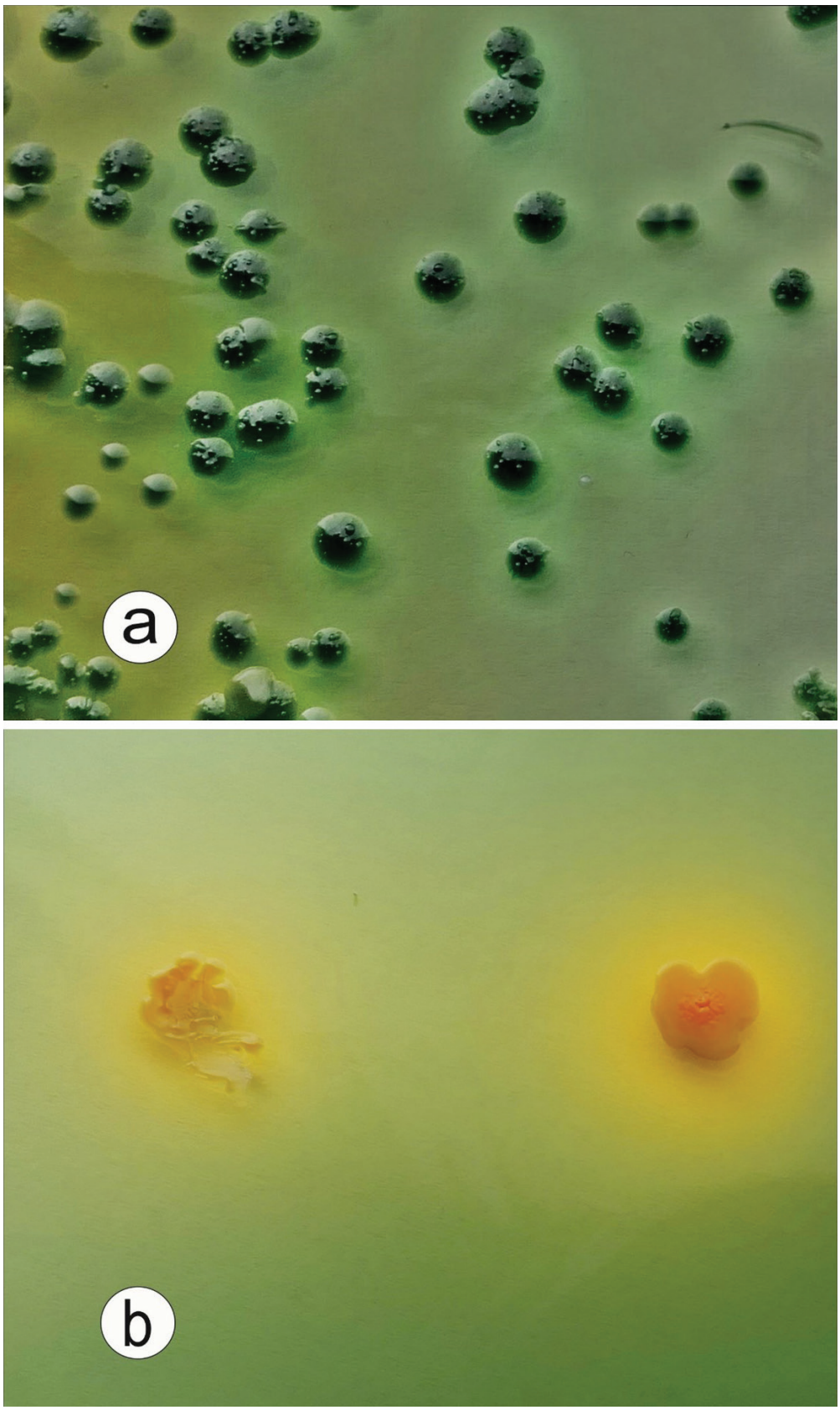

Figure 6. Microscope picture of the colonies of the isolated species *a*) *Vibrio alginolyticus* and b) *Vibrio parahaemolyticus.* The picture was taken using the microscope OPTIKA (Italy) and DinoEye, Eyepiece camera, USB, 1.3 megapixsel, up to 5 megapixels.

Discussion

According to our results, the microbiota found in mussels can be considered in two types. The so-called autochthonous microbiota, which was stable and permanently present, independent of changes in environmental conditions. The allochthonous (transient) microbiota, was entirely dependent on environmental conditions and was related to the change in these conditions. On the basis of our investigation, we can state that the species *E. hirae* (from the end of the spring and during the summer), *E. vulneris* (during the summer) and *A. gyllenbergii* (in the autumn), which were isolated in all three years, represented autochthonous microflora for the Arkutino area. In contrast, the species *E. cancerogenus* (isolated only in May) and *C. farmeri* – detected in September, were considered transitional species. In our previous research (Ibryamova et al. 2022a), we detected a significant increase in the quantity of the coliforms in the region of Arkutino in July 2020, when the quantity of the faecal coliforms was 190 times over the norms prescribed in Ordinance No. 4/20.10.2000 (the number of FC in the inter-shell content should be less than 300 NVB). Two years later, the tendency for a high titter of faecal coliforms was maintained. In 2022, the amount increased by 50%, which, according to the regulation, showed more than 250 times the sanitary accepted amount.

E. vulneris is an opportunistic human pathogen. It was reported primarily in elderly patients and invasive infections have been observed in immunocompromised persons. *E. vulneris* can cause severe diarrhoea and sepsis in infants (Jain et al. 2016). *E. vulneris* has limited clinical reports of human infections worldwide. In humans, *E. vulneris* was originally isolated from infected wounds, along with other bacteria such as *Staphylococcus aureus*, *S. epidermidis*, streptococci, enterococci and *Enterobacter* spp., *Acinetobacter lwoffii* and *Cedecea neteri.* Later, *E. vulneris* was also isolated from other clinical samples, such as faeces, sputum, urine, vaginal and throat swabs, where it was believed to be a coloniser (Jain et al. 2016). Isolation of this species firstly indicates anthropogenic contamination and the consumption of mussels with such a high content of microorganisms of this species can have a serious effect on human health.

The presence of *E. vulneris* was permanent and that species may have become part of the autochthonous microflora as a result of anthropogenic activity and environmental pollution from the hotels and resorts in the area - the species was mainly isolated during the active summer season - from June to August.

The most diverse microorganisms were isolated from the Ahtopol Region. Of these, autochthonous species, detected in all three years were: *E. hirae*, *E. vulneris*, *E. cloacae* and *A. gyllenbergii*. The species *E. hermannii*, *P. mendocina*, *P. fulva*, *P. alcaligenes*, *P. putida* and *A. johnsonii* were considered allochthonous. As in the Arkutino Region, we registered an increase over the allowed amounts of faecal coliforms (under Ordinance No. 4/20.10.2000) during the summer season until the end of September. In the region of Ahtopol, two species of the genus Escherichia sp. were detected – *E. hermannii* and *E. vulneris*. The species of *Pseudomonas* sp. were isolated during the cold months. Presumably, the different species of the genus *Pseudomonas* sp. appeared because of accidents related to changes in the direction of sea currents and variations in the water temperature or pH. However, it can also be speculated that the genus *Pseudomonas* sp. is autochthonous to the mussels *D. trunculus*, as some

species may be allochthonous. Jorquera et al. (2001) suggested that the bivalves contain only "transition" microbiota. We confirm partly such hypothesis for *Acinetobacter* sp. – the genus was autochthonous and some of the species were transient.

In the region of Obzor, where there was no active summer touristic season, only a few species of the microorganisms were isolated. All microorganism species were transient, as there was not a single species that was isolated regularly in all of the three years. Only three species of *Vibrio* sp. were isolated from the region of Tsarevo. We can attribute all three species to the transitional microbiota and connect them to the anthropogenic factors, since they were isolated only in the month of August. All other species were isolated sporadically in all of the three years of our investigation and no time pattern was detected.

In our opinion, allochthonous microbiota can enter mussels as a result of environmental pollution of different origins - natural, due to changes in climate, temperature, salinity, currents or as a result of human activity. Considering that the mussels are the filter of the sea, many microorganisms enter them during feeding. In 1960, Colwell and Liston showed a high percentage of the presence of the species *Pseudomonas* sp., *Vibrio* sp., *Flavobacterium* sp. and *Achromobacter* sp. in the Pacific oyster (*Crassostrea gigas*). Most of the studies since then were concentrated on the pathogens that cause shellfish diseases. The best studied pathogenic species belonged to *Vibrio* sp. For example, *V. tapetis* received special attention since it caused Brown Ring Disease (BRD), the bacterial etiology which is described in adult clams. In addition, the disease caused by it is considered one of the main limiting factors for the colonies of the Manila clams (*Venerupis philippinarum*) and was also detected in cultured clams in Korea (Europe-Borrego et al. 1996; Park et al. 2006). In this regard, the fact of the appearance of *Vibrio* sp. (Fig. 6a, b) in one of the most visited seaside resorts - Tsarevo, is disturbing. This may indicate the spread of mussel disease in this area. In our previous study on the influence of water parameters on the occurrence of transient microbiota in mussels (Ibryamova et al. 2022b), we showed that, as seawater salinity decreases, transient species and faecal coliforms appeared in the probes.

A study by Romalde et al. (2012) demonstrated that the genus *Pseudomonas* sp. is one of the main groups of microbiota in mussels, although some seasonal variations can be observed. Presumably, these variations are related to the environment impacts on the sea water - temperature, pH and water conductivity changes. These authors also stated that wide diversity of *Pseudomonas* species occurred sporadically, which also correlates with our results from the Ahtopol Region.

Previous research on different fish species from the Bulgarian Black Sea aquatory showed that the *Pseudomonas* species are of key importance for the ichthyofauna and are a permanent part of the composition of the fish microbiota (Ibryamova et al. 2022b). In mussels, however, the result was different. Despite the limited amount of data, we suggest that the clam *D. trunculus* does not have a permanent intrinsic microbiota, but only a transient one. The fact that completely different species of microorganisms have been isolated from geographically rather close regions, such as Ahtopol, Arkutino and Tsarevo for a long period of time (3 years) proves that hypothesis. Our results indicate that the anthropogenic factors may impact to a large degree the composition of the microbiota in mussels.

Conclusion

When studying bivalves, it is very important to know their microbiological composition, in order to be able to evaluate the various diseases related to consumption of these organisms by humans. Many species inhabiting the Black Sea feed on wedge clams, which, if infected by a certain type of microorganism, can cause imbalances in the populations. This could prove potentially fatal for the fragile ecology of the Black Sea. Our results regarding the dynamics of the microbial population in *D. trunculus* showed that some species of microbial pathogens were permanent and others were transient. Despite the fact that we detected rather regularly some species of microorganisms, we tend to accept the idea that there is only a transient microbiota in mussels and it changes depending on environmental conditions or may be a result of pollution of the Black Sea. We cannot exclude, however, that *D. trunculus* developed symbiosis with some species of bacteria and use them as a source of vitamins and minerals. Further investigations will verify or disprove this hypothesis.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Sevginar F. Ibryamova, Stephany Toschkova, Darina Ch. Bachvarova, Elitca Stanachkova - microbiological analysis, Radoslav I. Ivanov and Nikolay D. Natchev - delivery of mussels by trawling, Tsveteslava V. Ignatova-Ivanova - writing the article and its concept.

Data availability

All of the data that support the findings of this study are available in the main text. The data underpinning the analysis reported in this paper are deposited at “Data repository” at https://doi.org/10.3897/biorisk.21.111253.

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