Black Sea Microbial Water Quality Assessment via Next Generation Sequencing-Based Approaches

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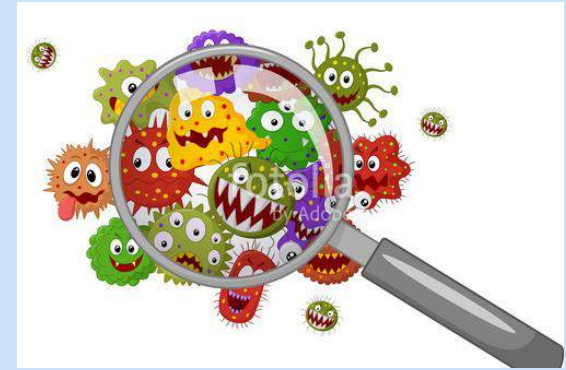National Institute for Marine Research and Development "Grigore Antipa", Constanta, Romania
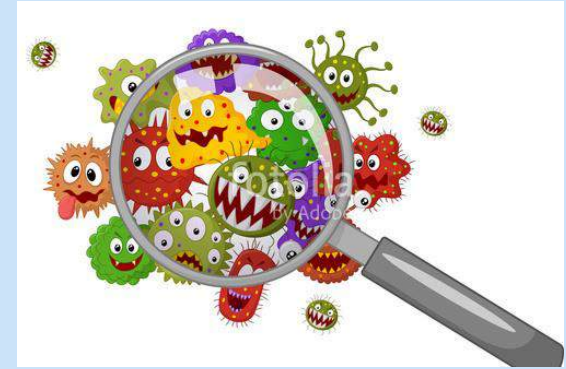
Marine bacterial community

Non-pathogenic species

## Pathogenic species:
- indigenous
- externally introduced

Marine pathogenic bacteria

– most are found in coastal marine habitats

– free-floating through the seawater column (planktonic) and attached to different surfaces (marine animals, phytoplankton, zooplankton, sediments and detritus)

– can cause a broad spectrum of human and animal infectious diseases

– affect marine ecosystem functioning and health status

– are detected by both cultivation-based and cultivation-independent (metagenomics) approaches

Microbial contamination in coastal marine environment is a worldwide phenomenon not yet well understood …

The Black Sea …
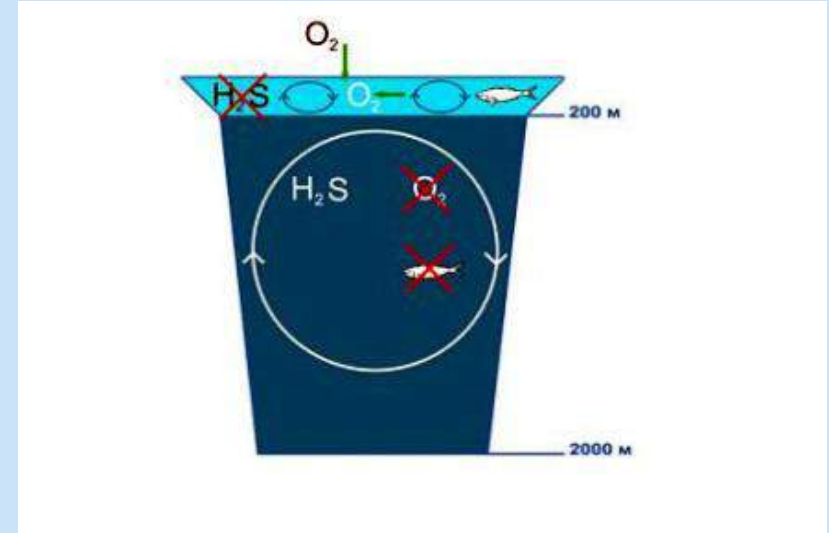
- the most isolated sea from the World Ocean (connected to the Oceans via the Mediterranean Sea and the Sea of Azov)
- very vulnerable to pressure from land based human activity
- its health is equally dependent from the coastal and non-coastal states of its basin
- the thin upper layer of marine water (up to 150 m) supports the unique biological life in this sea
- provides specific environment (*unique geomorphological structure* and *specific hydrochemical conditions)* for many species of non-pathogenic and pathogenic bacteria
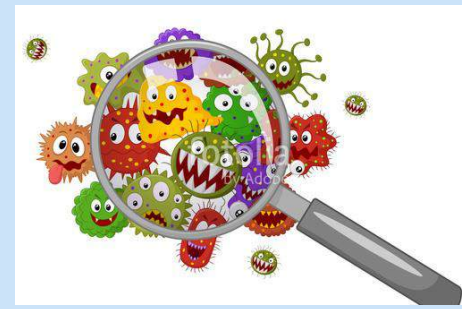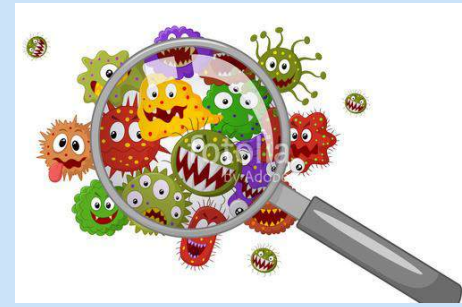
The Black Sea pathogenic microbes

The current data available on the microbial pathogens in the Black Sea region *is still limited*

For decades, the assessments of quality of the Black Sea marine environment is performed at *national level monitoring programs* under the Bathing Water Directives 2006/7/EC, 76/160 EEC and the Shellfish Water Directive 2006/113/EC, in most of the countries in the region

Intestinal enterococci and *Escherichia coli* for bathing waters, as well as total fecal coliforms, *Escherichia coli* and *Salmonella spp.* for designated shellfish growing areas are determined, on *culture-based enumeration and detection of fecal indicator bacteria (FIB)*

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FIB Limitations:

- monitoring for FIB does not provide insights on the possible occurrence of other microbial contaminants (e.g. pathogens).

- FIB culture-based methods are prone to false-negative results that arise from the failure to resuscitate viable but non-cultivable cells
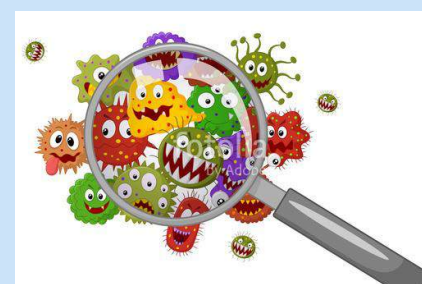
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Cultivation – independent molecular biology tools

PCR-based molecular methods

- cultivation-independent molecular methods based on direct detection of nucleic acids or amplification of targeted genes using polymerase chain reaction (PCR) were developed to avoid some problems associated with culture-based methods (FIBs)

- permit detection and quantification of specific pathogenic bacteria

- such methods target DNA extracted from environmental samples which is subsequently subjected to analysis for the presence or abundance of genes from indicator species or pathogens of interest.

- allow for more fine-scale taxonomic identification by using the small subunit ribosomal RNA gene (e.g. 16S rRNA gene for bacteria) – one of the most frequently used target genes for molecular analysis, due to its ubiquity in all organisms and sequence structure

- PCR-based detection and quantification of organisms in environmental DNA has proven useful for enumeration of sewage indicators and one DNA-based method is approved for quantification of the fecal indicator *Enterococcus* by the US Environmental Protection Agency (USEPA, 2009, 2013)

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Existing data generated by PCR-based molecular methods

Literature review indicates data on *microbial pathogens of the most predominant cultured fish species* at the Black Sea obtained in recent years by using a *combination of classical cultivation methods and traditional molecular techniques* (PCR technique and Sanger sequencing)

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High-throughput sequencing approach

- also known as next-generation sequencing (NGS)

- is a powerful new tool for obtaining information about nucleic acids (allows massively parallel analysis of DNA/RNA sequence information from PCR amplicons or environmental nucleic acids)

- allows faster and more accurate species identification

- decreases dependence on cultivation and morphological taxonomic expertise

- includes the earlier 454 pyrosequencing (Roche), the better optimized Illumina (Solexa), the Ion Torrent and single molecule real-time sequencing (SMRT) technology, and the recent improved Illumina MiSeq

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High-throughput sequencing approach – FINDINGS:

- Information derived from DNA NGS sequencing *is not included yet* in the current Black Sea status assessment programs

- There is widespread recognition of the importance of this approach, and an *increasing number of regional projects generate sequence-based bacterial community diversity inventory (e.g. MARCY-BS-ERA NET, EMBLAS I, II, Plus – UN/UNDP)*

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NGS sequencing approach – FINDINGS:

The *first study to assess the presence and diversity of the pathogenic bacterial community in the inshore and offshore Black Sea waters* by means of a mining DNA next-generation-sequencing dataset approach **shown an unprecedented diversity of pathogenic bacteria dominated** by *Actinobacteria*, *alpha-proteobacteria* and *gamma-proteobacteria*, which varied in relative abundance between species and regions (*Stoica et al., 2018*).

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NGS sequencing approach – FINDINGS:

Black Sea pathogenic bacterial diversity at different trophic conditions :

Among the major bacterial phyla containing pathogenic species, *Actinobacteria* and *alpha-proteobacteria* were the most common in estuarine waters, whereas *gamma-proteobacteria* dominated in the coastal Black Sea (Figure 2). *Mycobacterium* and *Erythrobacter*, containing pathogenic species, formed the most abundant genera of microbial assemblage from the Danube estuarine waters .

Vibrio and *Aeromonas*, formed a diverse and dynamic genera of pathogenic microbial assemblages from the Romanian coastal waters. Two gamma-proteobacterial pathogenic strains related to *Aeromonas* (*Aeromonas spp.* and *Aeromonas veronii bv. veronii*) were found to constitute a relatively large percentage of the total bacterial rRNA contribution
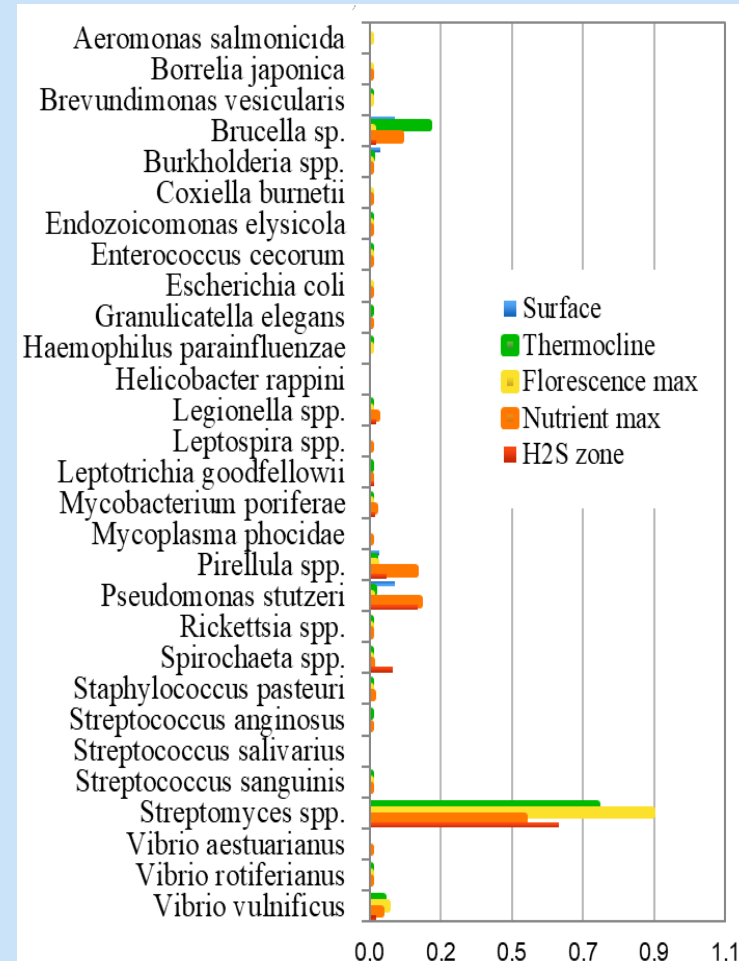
The Black Sea pathogenic microbes

NGS sequencing approach – FINDINGS:

Occurrence of pathogens in offshore Black Sea :

NGS sequences matching pathogenic species were surprisingly found in the offshore waters collected during EMBLAS project in late May – early June 2016.

A total of 29 taxa harboring potentially pathogenic species were identified at different water column depths ranging from the surface to the anoxic H_2S zone of the Black Sea.

The pathogenic bacterial communities observed in the offshore waters were dominated by *Streptomyces spp., Brucella sp.,* and *Pseudomonas stutzeri.*These microorganisms had previously reported in the various inshore regions of the world seas, mostly in water samples taken closest to the coast. **No information is available on the occurrence of pathogenic bacteria in open marine waters.**

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High-throughput sequencing approach – FINDINGS:

– The bacterial OTUs obtained by NGS Illumina sequencing showed that high-throughput tools are needed to decipher the pathogen bacterial diversity of the Black Sea.

– In order for NGS to become a useful tool for Black Sea water quality monitoring purposes, long term sequence data collection and management will be crucial in establishing databases that are important for inter-laboratory data comparison and comparative metagenomics studies.