MENVIPRO
Erasmus Plus Program project
Online Summer School

Microbial Quality of recreational and drinking water reservoirs

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Tbilisi, July 11, 2022

Content of the talk

- Water types: drinking and recreational waters; fresh, marine and brackish water
- Water quality: general statements; Water quality guidelines for major parameters
- Waterborne infections, infectious agents
- Water microbial quality: microbial pollution indicators
- Black Sea coastal zone studies for aquatic pathogens (V.cholerae)
- Diversity of aquatic microbial communities
- Phage abundance and diversity
- Phages as indicators and tracers of microbial pollution

Drinking and recreational water

“Safe and clean water and sanitation is a human right, essential to the full enjoyment of life and all other human rights”

Declaration of the United Nations (UN) General Assembly, 2010

- Drinking water (freshwater)
- Water used in preparation of food
- Irrigation water
- Recreational waters
  - Bathing water (fresh, brackish or marine)

❑ ***Water is vital for life***

- *Makes up 60% of an adult’s body weight.*
- *Main constituent of cells, tissues, and organs, essential part of blood.*
- *Acts as a building material, solvent, participant of enzymatic reactions, transporter of nutrients and wastes, thermoregulator, etc*

Water types and sources

Freshwater

- Freshwater sources: lakes, rivers, and streams, groundwater
- The freshwater salinity is ≤ 0.5 parts per thousand (ppt) % or <1,0 ppt of dissolved matter
- **3% of the earth's water is fresh**, **only 0.5 % is available as a drinking water source**

  (2.5% is locked up in glaciers, polar ice caps, atmosphere, and soil);

Marine water

- Marine habitats include the ocean and salty seas.
- Salinity range 30 – 50 ppt, average 35 ppt (or 3.5%)
- Marine waters cover more than 70% of the surface of the Earth and account for more than 97% of Earth's water supply

• Brackish water

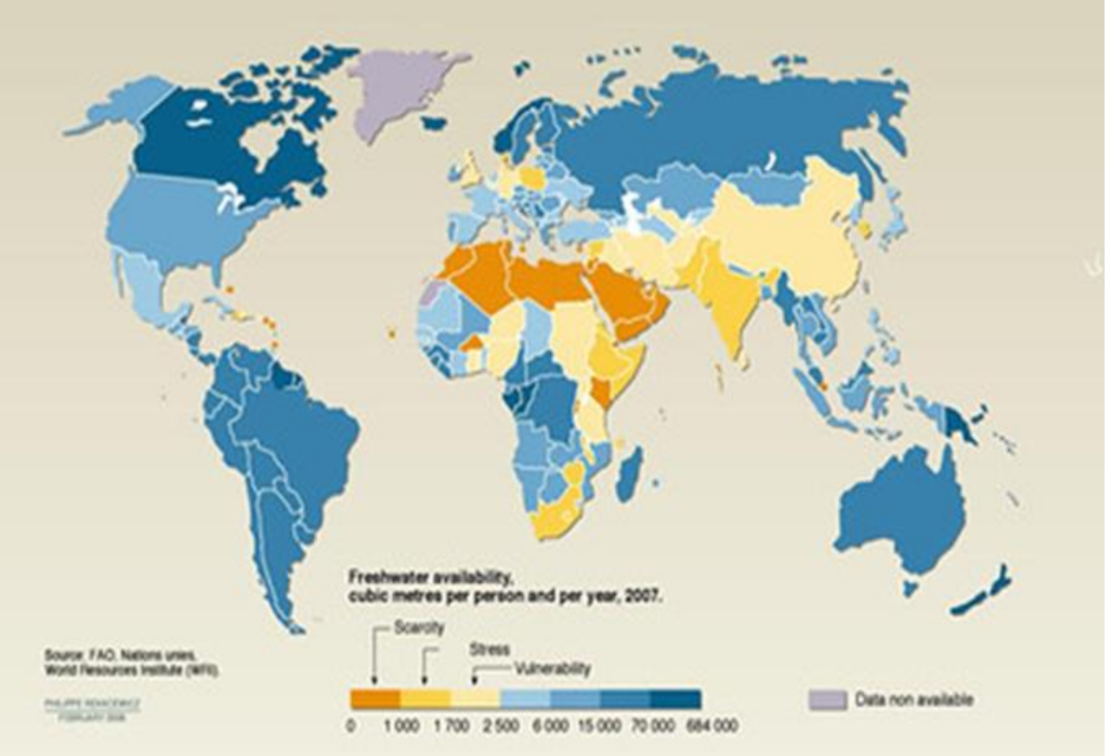
- "Somewhat salty" water (more then freshwater but less then marine)
- Salinity in the range of 0.5 – 30 ppt , may vary depending on environmental factors
- Most commonly found at transitional points where fresh water meets seawater (estuaries), also some natural Lakes and ponds

Drinking water use and availability

❑ Drinking water (potable water) can be obtained from:

- Surface waters, groundwaters,
- precipitation, recycled water source (meeting quality and safety standards)

❑ ***Unequal*** **natural availability of freshwater resources on the earth**


Freshwater availability, cubic metres per person and per year, 2007.

Source: FAO, Nations unies, World Resources Institute (WRI).

Freshwater availability (FAO, 2007)

○ **South Caucasus region**

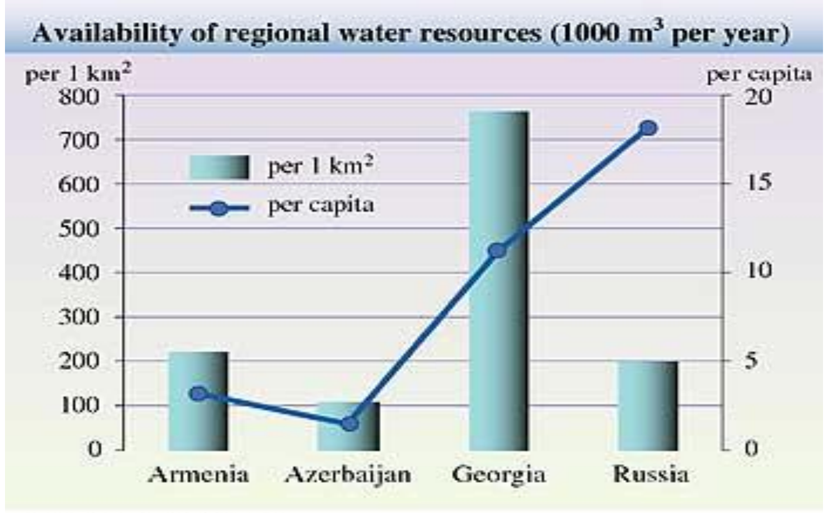
- Georgia is the richest in water resources

➢ **Georgia:**

Total renewable water resources (TRWR) per inhabitant- 11 637 m3/yr

(international water poverty line of 1,000m3 /yr)

Availability of regional water resources (1000 m³ per year)



Source: HDR, Azerbaijan, 1999

Recreational water use

- **Rivers, lakes and coastal marine waters are used for many different recreational purposes**.
- Various physical, chemical, and biological characteristics determine a water body's ability to support recreational activities.
- ***Primary contact recreation***: swimming, diving, surfing, water skiing;

  - direct contact with the water, can be fully immersed and could swallow water.
- ***Secondary contact recreation***: fishing , boating, paddling etc

  - direct contact with the water but are unlikely to swallow it.

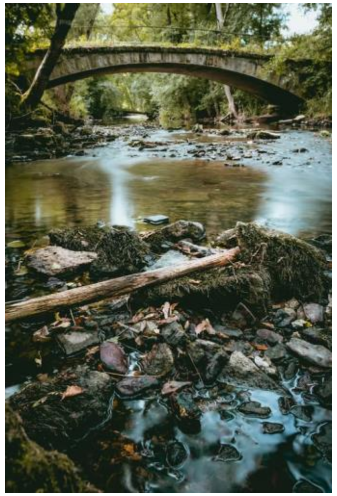
- Potential health hazards vary by water quality and the activity involved.

  - the more contact with the water, the stronger requirements to water quality

- Potential health risks

  - may be exposed to contaminants or pathogens through skin contact during swimming , by swallowing water, through eating recreationally-caught fish

Water pollution



- ☐ Occurs when substances used or spread by humans, enter the water bodies (rivers, lakes, seas, Oceans) and cause harmful effects there
- ▪ Chemical, biological and radioactive pollution
- ☐ **Sources of contaminants.**
- The majority of pollutants and waste (80%) comes from land-based activity
- A wide range of sources contribute chemical and biological contaminants, which affect both human health and ecosystems.
  - Direct discharge from industrial facilities and sewage treatment systems
  - Non-point sources - storm water and sediment runoff, agricultural runoff
  - Intensive land use and land cover (forestry and irrigation practices).
- Marine transportation significantly contributes to pollution level, also

  to introduction of invasive species
- Environmental Stressors can affect habitat and species makeup in a recreational water body (e.g. reduce biodiversity)
- Leading role of weather and climate (Global warming)

Water Pollution and Waterborne Diseases

- Illnesses caused by microorganisms - Bacteria, viruses, protozoa, helminthes - ingested through contaminated water or acquired by contact with contaminated water.
- Large numbers of microorganisms in a body of water generally indicate high nutrient levels in the water:

  - Water contaminated by inflows from sewage systems or from biodegradable industrial organic wastes,

  - Ocean estuaries (fed by rivers) with higher nutrient levels

- Water that moves below the ground's surface undergoes a natural filtering that removes most microorganisms. For this reason, water from springs and deep wells is <u>generally of good quality</u>.
- The most dangerous form of water pollution occurs when feces enter the water supply.

  - different types of pathogenic microorganisms present in urban sewage, and feces of infected humans and animals

Waterborne infections: main causative agents

❑Bacterial infections

- **Dysentery (bacillary) – *Shigella spp., Sh. disenteriae***
- **Campylobacteriosis – *C.jejuni , C. coli***
- ***Cholera – V. cholerae O1, O139***
- ***E.coli infections – E.coli types - ETEC, EHEC, EIEC,***
- **Typhoid fever - *S. typhi***
- **Salmonellosis - *Salmonella enterica (ser. enteritidis, typhimurium)***
- ***Vibrio* illness – *V. parahaemolyticus, V. vulnificus, V. algynolyticus***
- ***Aeromonas* illness – *A. hydrophyla, A. caviae, A. sobria (*Toxicoinfection)**
- **Leptospirosis - *Leptospira interrogans , Leptospira spp.***
- **Legionellosis – *Legionella spp.* (Legionar’s and Pontiac’s disease)-**
- ***Helicobacter* disease – *Helicobacter pylori (* indirect evidence of water transmission)**
- ***F. tularensis, Y. pestis* – Class A bacterial pathogens - high risk of water transmission**

Waterborne infections: main causative agents (cont’d)

❑ **Viral infections**

- **Viral acute gastroenteritis**

Rotavirus, adenovirus, norovirus, coxsackie, echovirus

- **Viral acute hepatitis** - Hep A virus

❑ **Parasitic waterborne infections**

- **Dysentery(amoebic)** - *Entamoeba hystolitica*
- ***Giardiasis* – *G. lamblia***
- **Cryptosporidiosis –** *Cryptosporidium parvum*
- **Shistosomiasis** – *Shistostoma mansoni*
- **Primary amoebic meningoencephalitis** – *Negleria fowleri*
- **Keratatis , meningoencephilitis** - *Acantamoeba spp.*

❑ Some waterborne pathogens, such as *Legionella*, may grow in water

- Some host dependent waterborne pathogens (e.g. noroviruses and *Cryptosporidium*) cannot grow in water, but are able to persist
- Persistence is affected by several factors: temperature, effects of UV/sunlight, pH
- High amounts of biodegradable organic carbon in warm waters, also low residual concentrations of chlorine can support persistence and growth of *V. cholerae, N. fowleri, Legionella, Acantamoeba* in surface and water distribution systems.

Waterborne infections

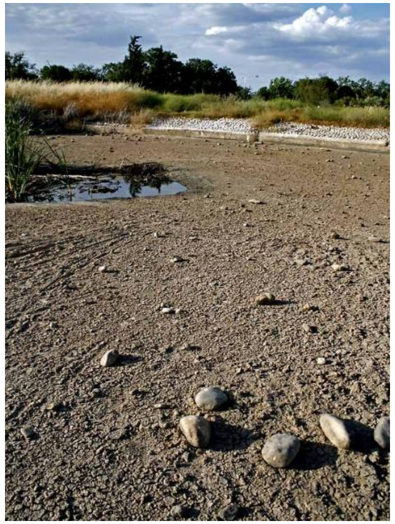
❑ **Waterborne infections may occur**
in the areas /countries experiencing :

- **Freshwater deficiency (arid lands, deserts)**
- **Excess of water (due to flooding)**
- **Natural catastrophes (earthquakes etc)**
- **Extreme poverty**
- **Wars/ethnoconflicts (crowded refugee camps)**

❖ **Global climate change - triggering factor**

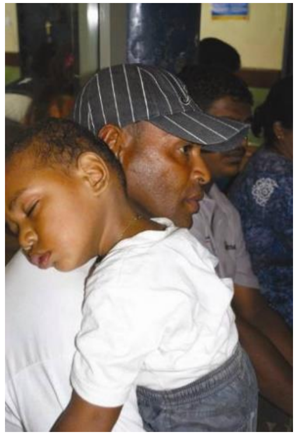
❖ ***The greatest microbial risks are associated with ingestion of water that is contaminated with faeces from humans or animals (including birds).***
WHO, Drinking water guidelines 2017



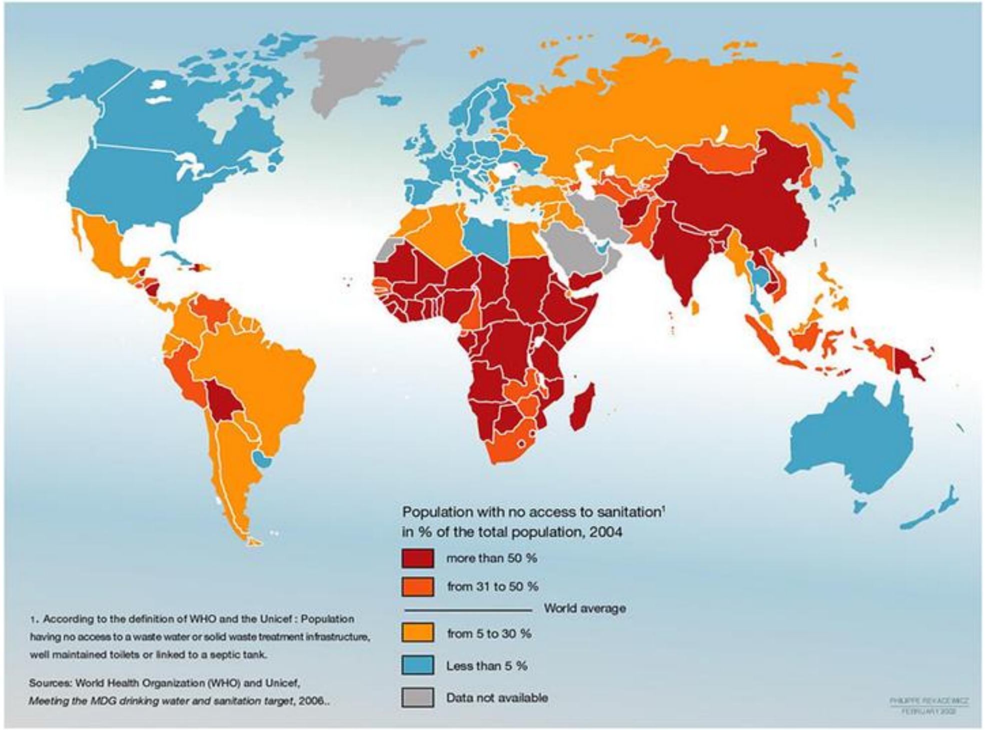
Waterborne diseases

- **Major reason of water borne diseases –**
  Unsafe water supply, poor sanitation and hygiene
- **World-wide around**
  - **1.1 billion people lack access to safe water sources**
  - **2.4 billion have no basic sanitation.**



- **Diarrheal disease**
  ~ 4.1% of global burden of disease
  - **cause of 1.8 million deaths / year (4% of all deaths)**
- **In Southeast Asia and Africa**
  - **up to 8.5% and 7.7% of all deaths respectively**
  - **children under 5 year most affected**

http://www.who.int/water_sanitation_health/diseases/diseasefact



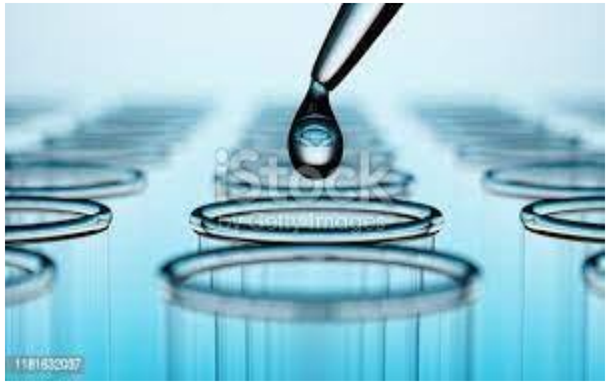
1. According to the definition of WHO and the Unicef : Population having no access to a waste water or solid waste treatment infrastructure, well maintained toilets or linked to a septic tank.

Sources: World Health Organization (WHO) and Unicef, *Meeting the MDG drinking water and sanitation target*, 2006..

Population access to sanitation (WHO, 2006)

Water quality parameters

- Water quality determines
  - the health of a water body/water system
  - the possibility to use the water for particular purposes (drinking, recreation, irrigation etc)
- Testing water over a period of time provides with the information about the ongoing changes or trend in the quality of the water;
- **Physical- Chemical Parameters** to be tested:
  - temperature, pH, turbidity, salinity,
  - nutrients: nitrates and phosphates.



- **Assessment of bioindicators**:

  the aquatic macroinvertebrates
- **Water microbial quality parameters**

Drinking water quality regulations and standards

- Apply to the quality of water intended for human consumption
  - to protect human health from adverse effects of any contamination of water
- Apply to all water distribution systems (tap water)
- drinking water from tankers;
- drinking water in bottles or containers;
- water used in the food-processing industry

- **European Drinking Water Directive EC 98/83/** presumes regular monitoring of a total of 48 microbiological, chemical and indicator parameters
- **US EPA** sets legal limits on over 90 contaminants in drinking water.
- **Georgian standards** for drinking water (N 58, 2014) have been set for basic 16 Chemical parameters and 10 Microbial parameters.

https://ec.europa.eu/environment/water/water-drink https://www.epa.gov/dwreginfo/

Indicators of microbial pollution

- ❖ **General Microbial pollution indicators**
- ❖ **Fecal pollution indicators**

- ○ Fecal contamination of water is a serious microbial pollution problem
  - potential for contracting diseases from pathogens
- ○ Impossible to test for many different potential pathogens in every water sample
  - presence of pathogens is determined with indirect evidence
  - testing for an **"indicator"** organism (e.g. coliform bacteria) that come from the same sources as pathogenic organisms.

- <u>Criteria for an indicator organisms</u>:
  - be consistently present in human or animal feces in substantial numbers
  - should also survive in the water at least as well as the pathogens would
  - must be detectable by simple tests not requiring sophisticated approach

❖ No single microbial indicator can predict infectious disease risk consistently in all environments at all times.

General microbial pollution parameters

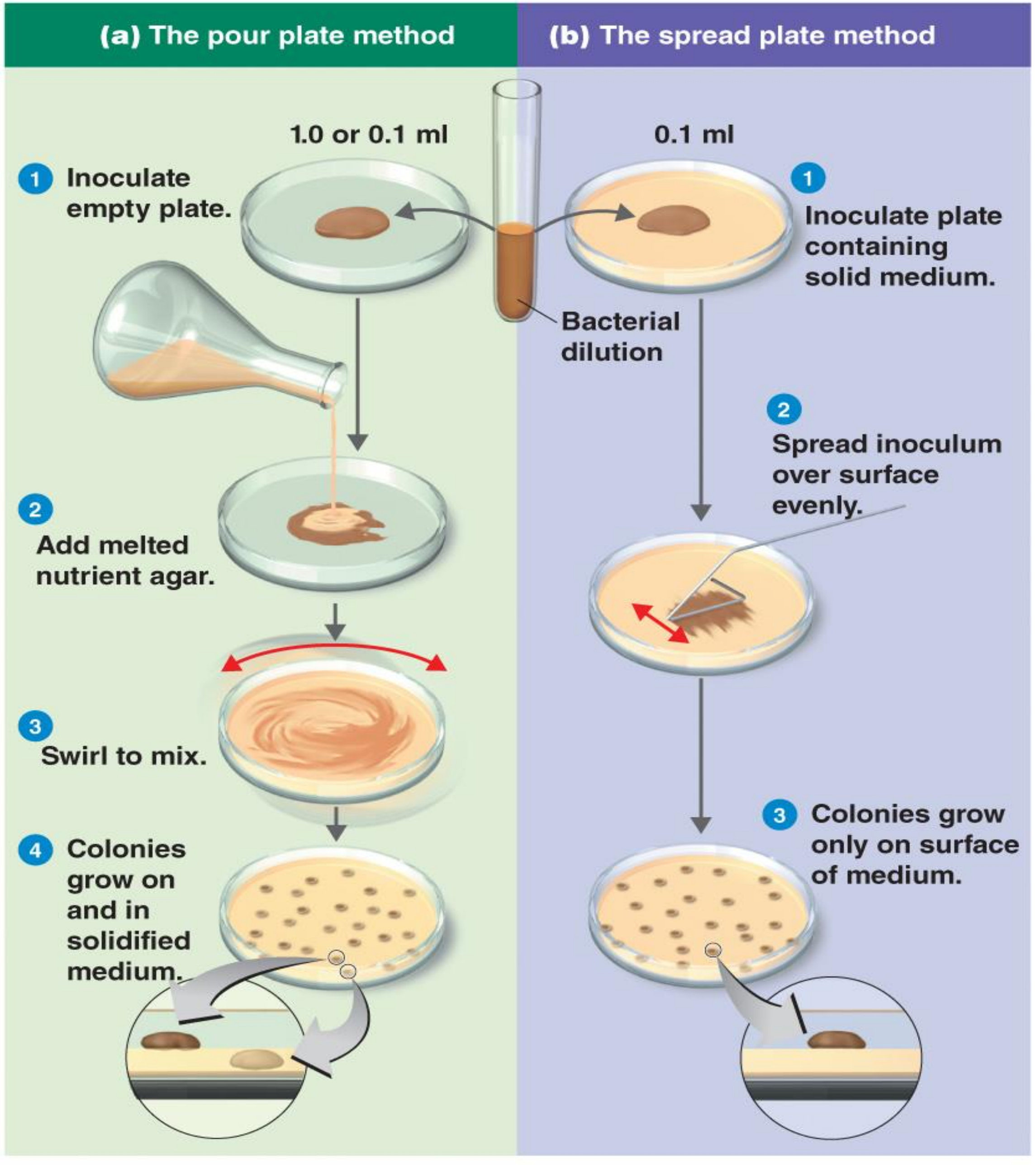
- The total bacteria count (TBC) is **a quantitative estimate of the number of microorganisms present in a sample**.
  - represented by the number of colony-forming units (CFU) per gram (or milliliter) in the sample.

Total viable microbial plate Count (TPC)
↓
Pour plate method

Aerobic viable count plate (APC)
↓
Spread plate method

- TPC and APC are not sufficient indicators of safety , since they do not directly correlate to the presence of pathogens or toxins.

Direct Count Plate



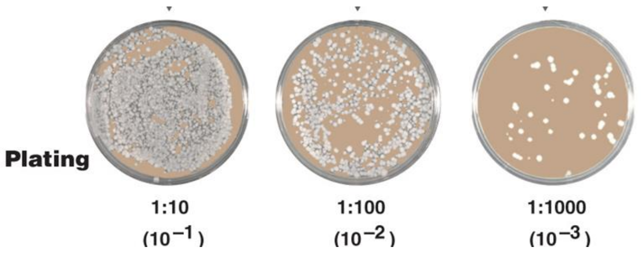
General microbiological parameters of water pollution

❖General Microbial pollution indicators

- Total microbial counts

  Important for natural open reservoirs

- Large numbers of microorganisms in a body of water generally indicate high nutrient levels in the water.
- Water contaminated by inflows from sewage systems or from biodegradable industrial organic wastes is relatively high in bacterial numbers.
- Ocean/sea estuaries have higher nutrient levels and therefore larger microbial populations than other shoreline waters.

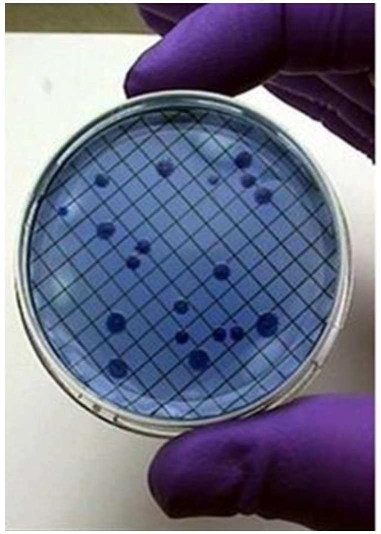


Ranking of water reservoirs by Microbiological pollution parameter

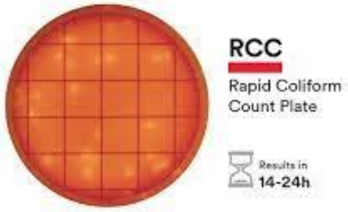
Grade of water pollution	**Total Viable counts**	**Total direct counts (microscopy)**
Very Clean	$n* 10^1$	$n* 10^3$
Clean	$n* 10^2$	$n* 10^4$
Sligtly polluted	$n* 10^3$	$n* 10^5$
Moderately polluted	$n* 10^4$	$n* 10^6$
Polluted	$n* 10^5$	$n* 10^7$
Highly polluted	$n* 10^6$	$n* 10^8$

Golubovsakaya, 1996

Indicators of microbial contamination/pollution



- **Coliforms** - bacteria that are always present in the digestive tracts of animals, including humans, and are found in their wastes, found also in plant and soil material.
- Coliform bacteria are defined as facultatively anaerobic, Gram-negative, non-spore-forming rods that ferment lactose to acid and gas at 35 ± 2 °C within 24 or 48 h.
- Coliform bacteria generally belong to 4 genera of Enterobacteriaceae: *Citrobacter freundii*, *Enterobacter aerogenes orE.cloaceae*, *E. coli*, and *Klebsiella pneumoniae*.
- Coliforms are relatively easy to identify
  - are usually present in larger numbers than more dangerous pathogens
  - respond to the environment, wastewater treatment, and water treatment similarly to many pathogens.
- **Testing for coliform bacteria can be a reasonable indication of whether other pathogenic bacteria are present.**



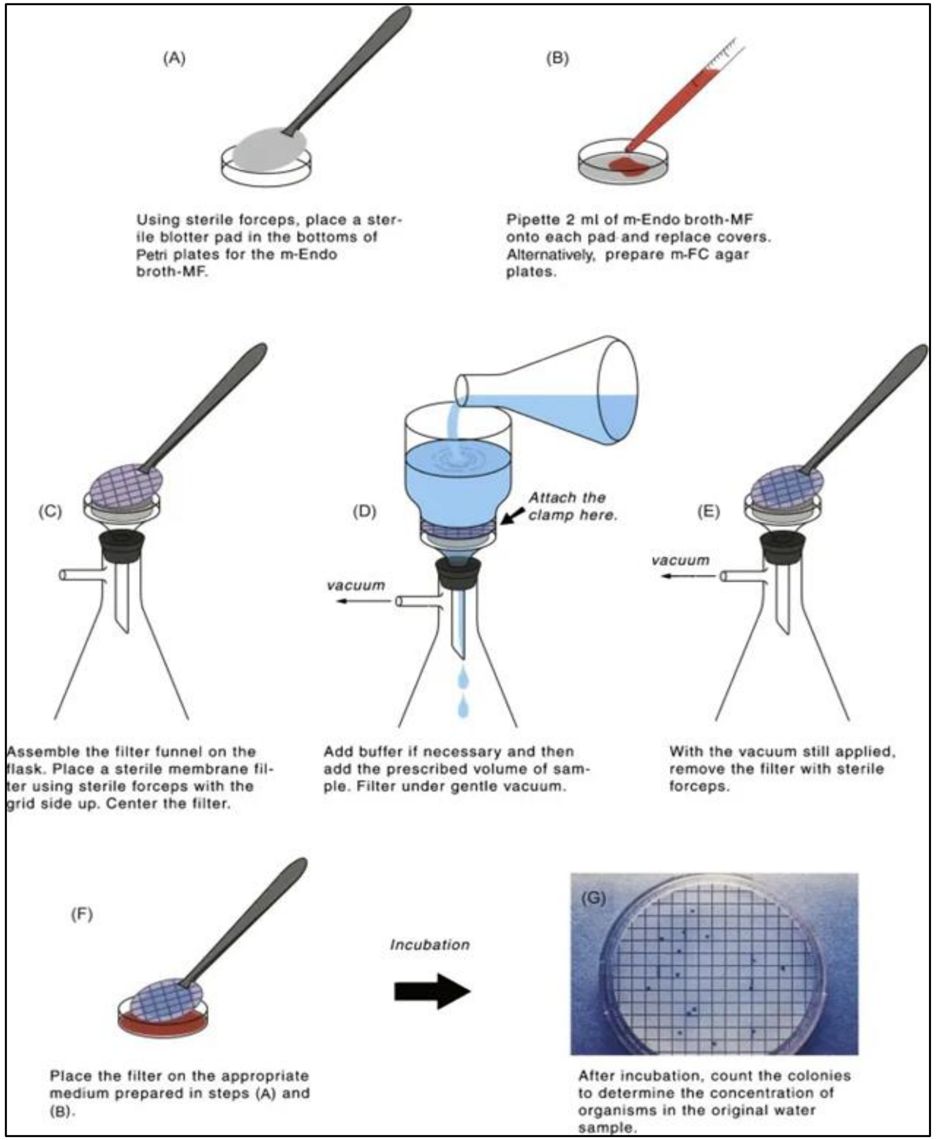
Water microbial quality testing

- **Counting coliform bacteria** in water samples: 0,1-1,0 ml samples are plated on various selective bacteriological media used to detect the coliform bacteria such as: Violet red bile agar, m-Endo agar/broth, M-FC agar/broth, brilliant green bile broth, containing lactose as a primary fermentable sugar. The target bacteria grow well, while the accompanying microbiota is suppressed.

- ***Membrane filtration systems**: used widely in coliform analysis of beverages, drinking,* and *potable water*. Primarily used for testing of coliforms in low-level contaminated water.

  - Samples are passed through a membrane filter of particular pore size (0.45 µm) in a volumes of 10, 100, or 250 ml. The filter is placed in a Petri dish with an appropriate selective medium and incubated at 35 ± 2°C.

  - All developed colonies are counted directly, and the results calculated as the microbial load. Sample volume can be varied to achieve a desirable colony count range (20–60 colonies per filter).



(A) Using sterile forceps, place a sterile blotter pad in the bottoms of Petri plates for the m-Endo broth-MF.

(B) Pipette 2 ml of m-Endo broth-MF onto each pad and replace covers. Alternatively, prepare m-FC agar plates.

(C) Assemble the filter funnel on the flask. Place a sterile membrane filter using sterile forceps with the grid side up. Center the filter.

(D) Add buffer if necessary and then add the prescribed volume of sample. Filter under gentle vacuum.

(E) With the vacuum still applied, remove the filter with sterile forceps.

(F) Place the filter on the appropriate medium prepared in steps (A) and (B).

(G) After incubation, count the colonies to determine the concentration of organisms in the original water sample.

Membrane filtration for determining the coliform count (https://www.sciencedirect.com)

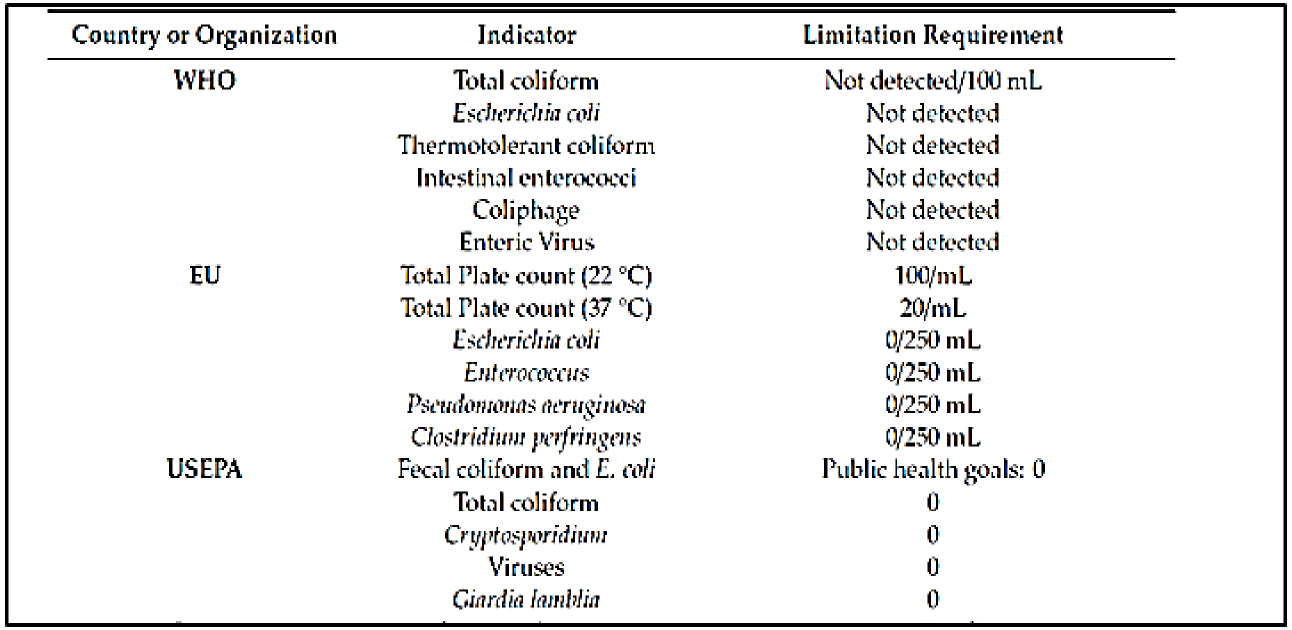
Indicators of water microbial quality

- Water quality standards differ by region and/or country

Drinking water microbial quality
(National standard in Georgia)

Indicator	Limitation requirement
Total plate count (37°C)	20/ml
Total plate count (22°C)	100/ml
Total coliform	0/300ml
E.coli	0/300ml
Fecal Streptococii/Enterococci	0/250ml
Coliphage	0/100ml

Drinking water quality Standards:
WHO, EU and US EPA requirements

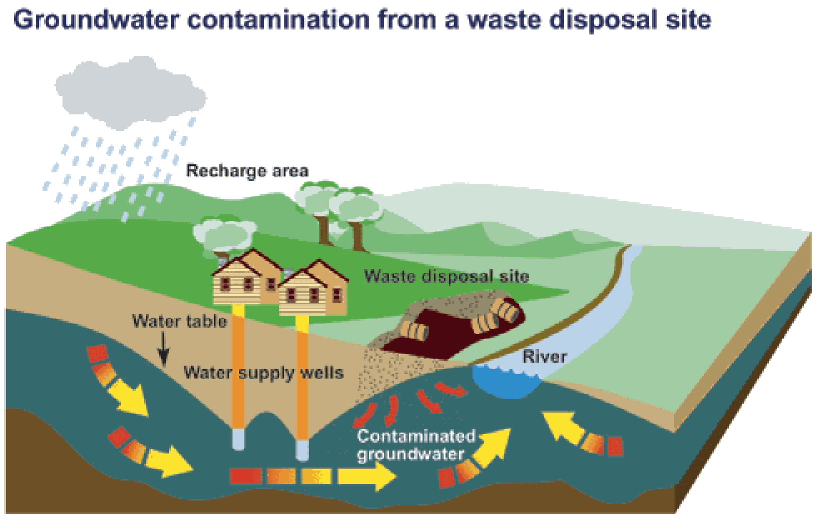


Country or Organization	Indicator	Limitation Requirement
WHO	Total coliform	Not detected/100 mL
	Escherichia coli	Not detected
	Thermotolerant coliform	Not detected
	Intestinal enterococci	Not detected
	Coliphage	Not detected
	Enteric Virus	Not detected
EU	Total Plate count (22 °C)	100/mL
	Total Plate count (37 °C)	20/mL
	Escherichia coli	0/250 mL
	Enterococcus	0/250 mL
	Pseudomonas aeruginosa	0/250 mL
	Clostridium perfringens	0/250 mL
USEPA	Fecal coliform and *E. coli*	Public health goals: 0
	Total coliform	0
	Cryptosporidium	0
	Viruses	0
	Giardia lamblia	0

Sustainability ***2020****, 12, 2249; doi:10.3390/su12062249*

Ground water microbial quality

- Water systems that have ground water sources may be susceptible to fecal contamination, entering from septic systems and possibly containing disease causing pathogens.
- It is important to test for basic microbial parameters for drinking water plus presence of other microorganisms (*Salmonella* spp., *Pseudomonas aeruginosa*, and enteric viruses), also Heterotrophic Plate Count (HPC) at 22 and 37 °C
- Local soil characteristics, especially its filtration capacity, can influence microbiological pollution.
- Climate characteristics, especially rainfall frequency, as well as human activities involving extensive use of water resources, influence the level of groundwater contamination.



Study in Goa, India:
25 groundwater samples were collected from the open dug wells and were analysed for microbiological quality. Most of the samples were found to contain *E. coli, P. aeruginosa, S. aureus* and *Salmonella typhinurium* bacteria.

EPA Ground Water Rule (GWR); O.D.Gigilio, 2016;

Phages as water quality indicators

- **Bacteriophages - viruses of bacteria**

share many properties with human or animal viruses (composition, structure, morphology, or capsid size)

- **Phages** have greater (then bacteria) abundance and persistence in the environment
- Faster replication rate than bacteria
- Circulate through filters (membranes, ultrafiltration).
- Adsorb to surfaces (particles, membranes)
- More resistant to treatment

European Drinking Water Directive (2021) requires monitoring for coliphages as a useful **fecal indicator in the raw source water**

❑**Bacteriophage groups proposed as indicators of fecal (bacterial and viral) contamination**

- **Somatic coliphages** - Infect E.*coli* through bacterial cell wall (e.g. E.coli C)
- **F-specific coliphages** Infect E.*coli* and other enterobacteria (e.g.Salmonella) through the sexual pili encoded by plasmid
- ***Bacteroides fragilis phages-*** Infect *Bacteroides fragilis* through cell wall (HSP40, RYC2056)

Recreational water quality: bathing waters

- Use of Inland and marine waters, coastal areas, swimming pools is increasing worldwide

  Coastal tourism represents ~80% of all tourism

- ❑WHO guidelines assess Public health risk by combining a qualitative sanitary survey and quantitative microbiological data

- Culture-based enumeration of **Fecal indicator bacteria (FIB)** such as *E. coli* or enterococci, or both
  - counts of FIB above a certain benchmark value signaling increased risk to human health.

- WHO recommends analyzing at least 20 water samples during each bathing season of the year for FIB counts

Int J Environ Res Public Health 2021 Jun; 18(11): 5513.

Microbial quality of recreational (bathing) waters

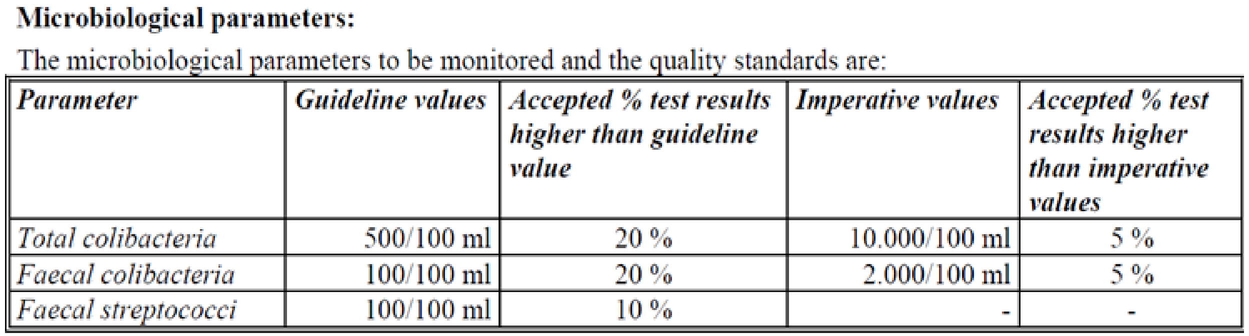
Standard testing for marine bathing water quality (Georgian guidelines)

Microbial indicator	Quantitave allowable values for Surface waters (2nd category –bathing waters)
Mesophylic aerobs and faulatative anaerobs 37ºC 24 hr 22ºC 48 hr	<1000 cfu/ml
Total coliforms	<500 cfu/ 100 ml
Fecal coliforms /E.coli	<100 cfu /100 ml
Fecal Streptococci /Enterococci	<100 cfu/100 ml
Coliphage	<100 pfu./100 ml
Lecitinase positive Staphylococci	< 100 pfu/ml
Pathogens including Salmonella	0 cfu/ml

- Monitoring of bathing waters quality can begin by general indicators and the FIB counts and then additional more specific indicator can be selected from the toolbox.

- **Blue Flag** bathing water quality requirements are based on European Bathing Water Quality Directive
- Monitoring of basic chemical and microbiological parameters
- Regular Water sampling (at least in 18 days)

Blue Flag program recognizes and promotes **swimmable, drinkable, fishable water**.
Provides information if a beach or marina is managed sustainably, has regular water quality monitoring

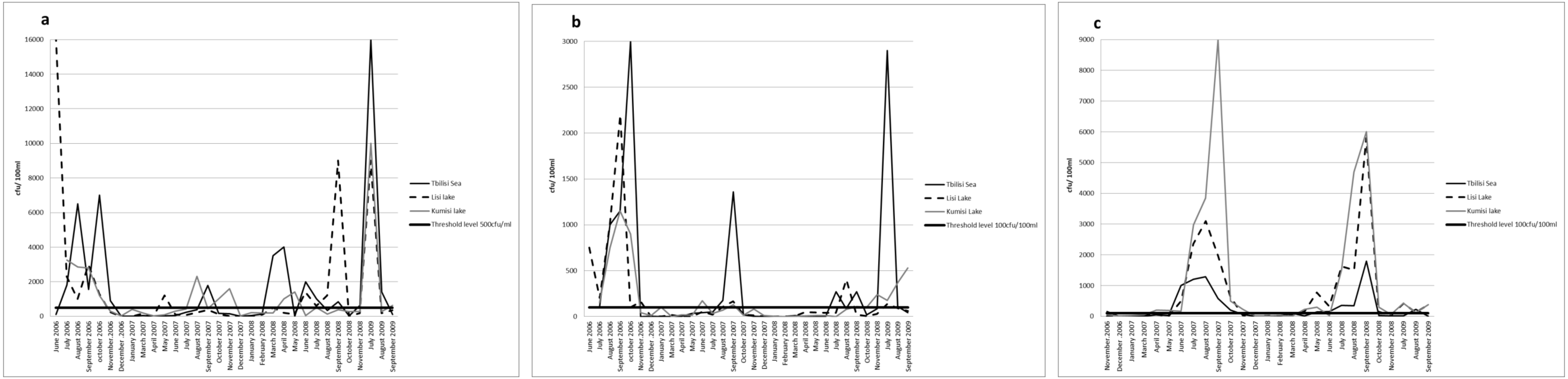


Microbiological parameters:

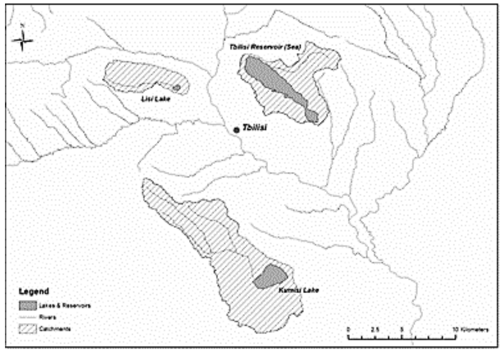
The microbiological parameters to be monitored and the quality standards are:

Parameter	*Guideline values*	*Accepted % test results higher than guideline value*	*Imperative values*	*Accepted % test results higher than imperative values*
Total colibacteria	500/100 ml	20 %	10.000/100 ml	5 %
Faecal colibacteria	100/100 ml	20 %	2.000/100 ml	5 %
Faecal streptococci	100/100 ml	10 %	-	-

Microbial water quality of water reservoirs used for recreation and as a drinking water source (Example)



Microbial quality of water reservoirs in vicinity of Tbilisi, GE Total coliform counts; (b)Fecal coliform counts ;(c)Total enterococci



Tbilisi Sea – Freshwater, Recreation (bathing, sailing) , drinking water source
Lisi lake - Brackish water, recreation (bathing, fishing)
Kumisi Lake- brackish water, recreation, intensive fishing

- Seasonal dynamics
- Different microbial quality paramete

Jaiani et al, JWH, 2013

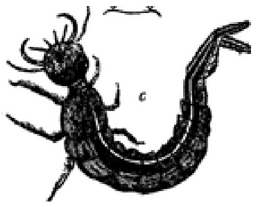
Biomonitoring of water bodies

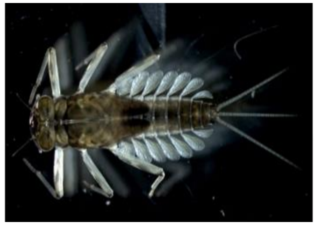
- Biomonitoring is the use of organisms to assess the overall quality of their environment or habitat.
- Macroinvertebrates better reflect the long-term water quality of a site because of their prevalence in aquatic habitats and their differing sensitivities to chemical pollution
- Insects (dragonflies, stoneflies, beetles, midges, and mayflies) are the most common in aquatic systems, living in water as nymphs or larvae. Aquatic worms, leeches, and small crustaceans live entirely in water.
- Most species live in the bottom sediments or or attached to rocks, vegetation etc. Lifespan: few weeks to several years.

❖ *Healthy waterbodies support a diverse population of macroinvertebrates. Samples yielding only pollution tolerant species, a low abundance of organisms, or very little diversity might indicate a degraded waterbody.*

Macroinvertebrate	Sensitive/tolerant
Stonefly	Intolerant
Mayfly	Intolerant
Crayfish	Moderately Tolerant
Leech	Tolerant

www.epa.gov/system/files/documents/


Larva of *Dyticus* (Water-Beetle).

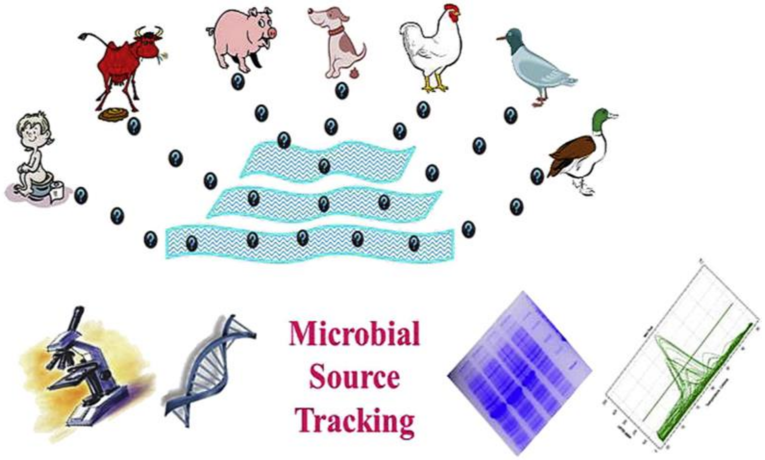

Mayfly (water insect)

Microbial source tracking in Aquatic systems

- **Microbial source tracking (MST)** – To identify source /origin of microbial contamination, primarily fecal pollution in drinking and environmental waters
- Use of fecal bacteria, characteristic for particular host humans or various animals

  - Culture-based and DNA –based methods (PCR)

  - DNA- fingerprinting (REP –PCR, PFGE)



https://ars.els-cdn.com/

Tracers

- Chemical markers (e.g. dyes) as tracers
- **Bacteriophage-based methodology**

  - Phages specific to *E. coli* , *Bacteroides*, other (e.g. *Staphylococcus*)

- Narrow or broad spectrum phages with specific hosts
- Somatic coliphages and RNA-containing male-specific phages

Main principles for selection tracer phages and construction of specific tracing systems

- High specificity between a bacteriophage and its host bacterium.
- Resistance of phages towards environmental factors (UV irradiation, free chlorine, temperature, pH etc), affecting their properties and livability.
- Virulent phages (to avoid horizontal gene transfer)

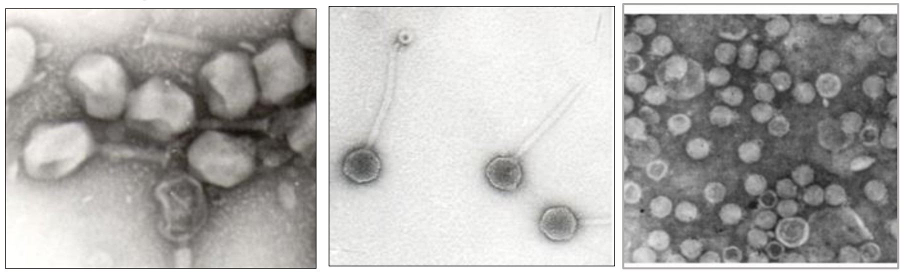
☐ **Properties of the tracer phages resemble those of infectious agents (pathogenic bacteria and viruses).**

☐ **Friendly to the environment: phage is only harmful to a specific bacterial host(s)**

✓ **The water course can be traced over long distances (>20 km);**

✓ **The low detection limit for phages is comparable to that for chemicals;**

Phage tracers (Eliava collection)



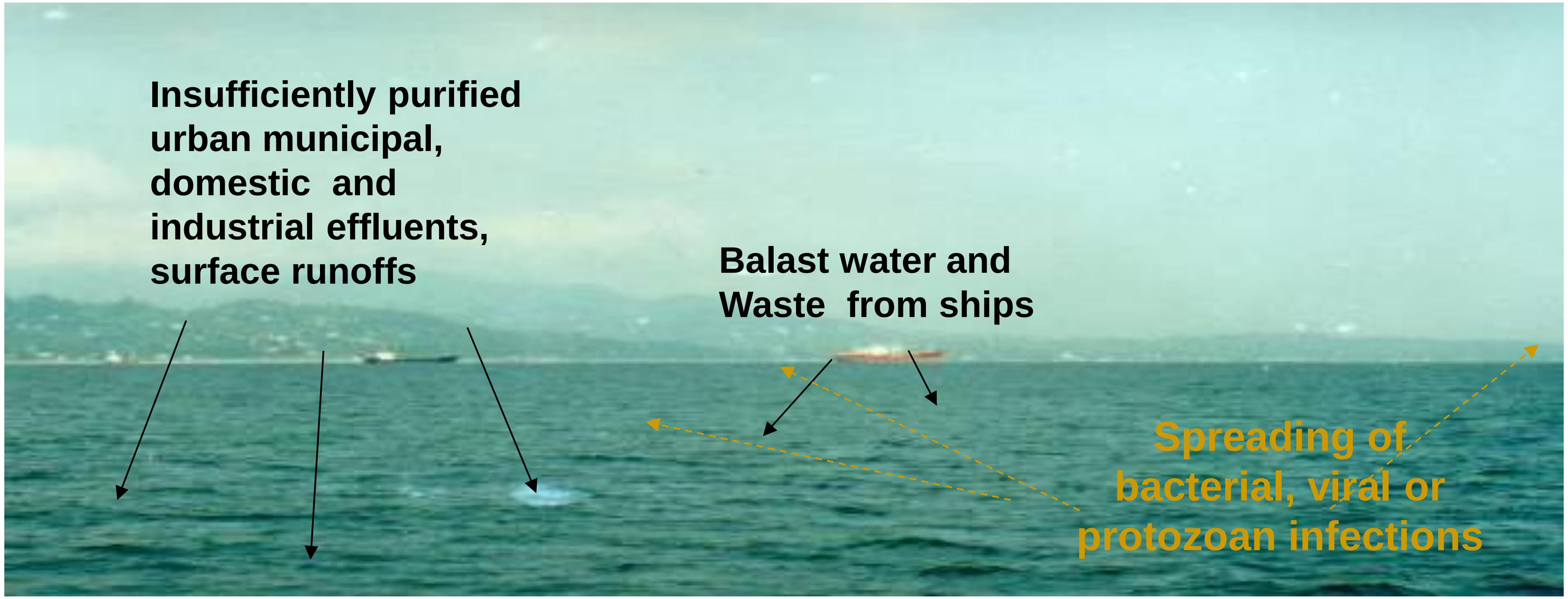
Un phage Cb phage Sd phage

❖ Rapid and simple detection of tracer phages: plating on specific phage resistant mutant strains

Phage	Strain Ph^S	Strain Ph^R	Strains Ph^S+ Ph^R	Results
Ph-tracer	**Clear Zone (+)**	**No zone (-)**	**Turbid zone (±)**	**Positive**
Ph – stranger A	**Clear Zone (+)**	**Clear Zone (+)**	**Clear Zone (+)**	**Negative**
Ph-stranger B	**No Zone (-)**	**No Zone (-)**	**No Zone (-)**	**Negative**

T.Chanishvili et al, 1988

Phage Tracing in water environment: Studies in Batumi aquatoria



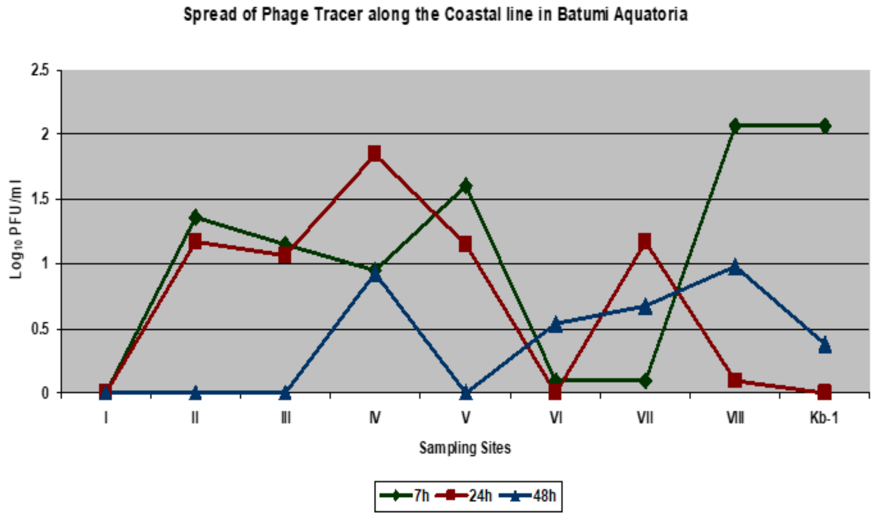
Insufficiently purified urban municipal, domestic and industrial effluents, surface runoffs

Balast water and Waste from ships

Spreading of bacterial, viral or protozoan infections

(1987-1996)
I.Chkonia et al, 1988; 1997; Janelidze, 2009

- ❑ Phage tracers were used to determine possibility of infection spread, contamination source and its transmission ways
  - Risk assessment of swimming associated infections
  - Impact of ballast waters from ships
  - Evaluation of the effectiveness of waste water treatment plants
  - Examination of several sources of pollution using different tracer phages

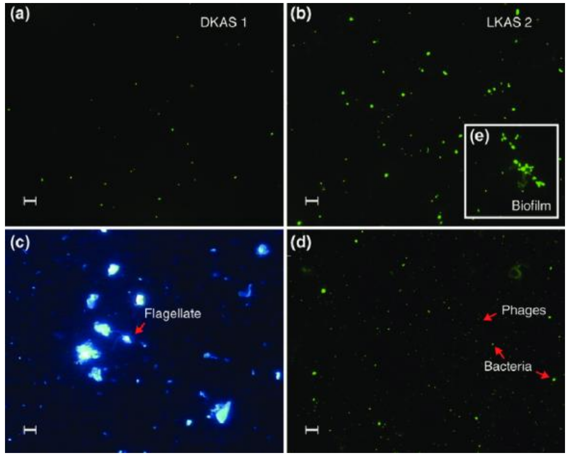


Microbial communities in water ecosystems

- **Aquatic microbial community**: an *assemblage* of co-occurring, and potentially interacting microbes, present in a defined (aquatic) habitat and time.

  Bacteria, viruses, eukaryotic microorganisms
- High structural, functional and genetic diversity

  \- Modern studies based on Metagenomic sequencing
- Different composition of microbial communities in
  - the freshwater and marine systems,
  - in water column and in sediments



Epifluorescence microscopy of microbial communities in spring water(Savio et al, 2018)

- Common Microflora in the water (culturable bacteria):

Vibrio, Aeromonas, Acinetobacter*, Moraxella,* ***Pseudomonas,***
Flavobacterium-Cytophaga,Synechococcus, Bacteroides etc.

- Influence of Increased nutrient's input caused by agriculture, urbanization and industry

  \- climate change

  \- distinct groups of aquatic microbes may flourish

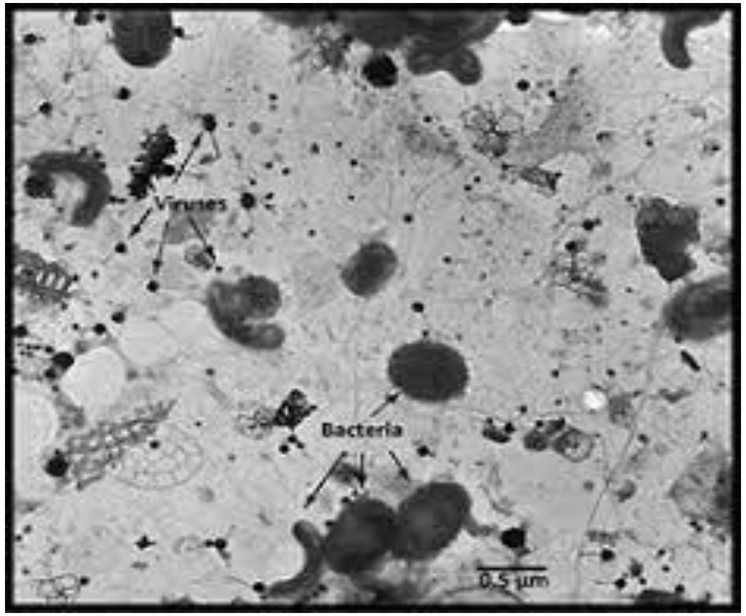
  (cyanobacterial bloom, increased Vibrio counts etc)

Ecology of aquatic viruses

- Total viral abundance in aquatic ecosystems is 1 magnitude higher than total prokaryotic abundance
  - Estimated 10^{29-31} virus-like particles (VLPs)
- Mainly composed of phages, also partly eukaryotic (algal) viruses.
- Typically 10^4 - 10^8 ml^{-1} (VLP) ml^{-1} in water column

  10^7 –10^{10} VLP per gram of dry sediment
- Viral abundance is higher in freshwater lakes and estuaries compared to marine environments
- Freshwater and marine viruses are genetically distinct
- Seasonal cycles of viral abundance, especially in lakes

- Influence of physical - chemical factors (temperature, salinity, nutrients) and particulate organic C and N on prokaryotic and viral abundance



Abundance of VLPs in the stony corals ecosystem

Bacterial groups with higher growth rates may lead to higher abundances of their specific viruses (phages)

Phages - indicators(also predictive or retrospective) of presence of corresponding bacteria

Ecology and epidemiology of Aquatic Pathogens and their viruses : Black Sea Studies

❑ **The Black Sea**

Largely landlocked Sea, Brackish water
(typical salinity 18 ppt in coastal zones);
Largest anoxic water body

- Problems:
  - Accumulation of various pollutants ,
  - Increasing air and sea water temperature,
  - frequent heavy rainfalls, storms and flooding
  - Reduction of populations of fish and mussels,
  - Increased microbial pollution
  - Possibility of spread aquatic pathogens
  - Outbreaks of diahhreal diseases in summer season

Satellite view of the Black Sea

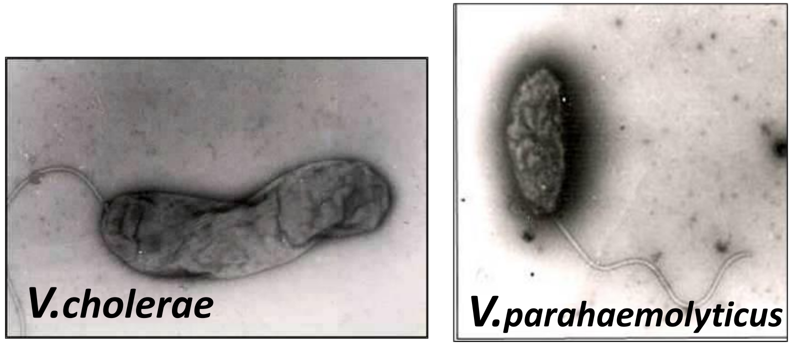
**Climate change strategy in Adjara. UNDP in Georgia. 2013*

Ecology and epidemiology of Aquatic Pathogens and their viruses : Black Sea Studies

V. cholerae and other pathogenic vibrios

- Vibrios are primarily aquatic bacteria.
- 12 *Vibrio* spp. are known human pathogens most important are **V. *cholerae*, *V. parahaemolyticus*, and *V. vulnificus***.

- Species distribution depends on sodium concentration and water temperature.
- Vibrios are very common in marine and estuarine environments, living free or on the surfaces and in intestines of marine animals.
- Species with a low sodium requirement (V. *cholerae, V. mimicus, V. vulnificus)* are also found in freshwater and brackishwater habitats



TEM JEM100 SX(Jeol).

- **Disease Cholera** – severe gastroenteritis
  - caused by *V.cholerae O1* and *O139*
  - potential for pandemic spread
  - Reportable disease
- *V.parahemolyticus* –Mild to severe gastroenteritis (sea-food related)
- *V.vulnificus* – gastroenteritis, Necrotizing fasciitis, septicemia

❖ Regular monitoring and surveillance, rapid detections and identification of pathogenic Vibrios is important public health issue

Studies in the Black Sea Coastal Zone of Georgia

Seasonal monitoring in the Black Sea Coastal zone

- Studies conducted in 3 series:
  2006-2009 (***DTRA GG13***)
  2015- 2016 (*CRDF 6209*)
  2018- 2020 (SRNSF 17-442)

- *On site measurement of physical – chemical parameters (temperature, DO, salinity, pH, TDS)*
- *Microbiological analysis of water and sediment samples*
- *Phytoplankton and zooplankton enuration and speciation*

- Sampling at 4 points

Chorokhi Estuary	**41^{0} 36’ 176’ N**	**41^{0} 34’ 108’ E**
Batumi Bulvard	**41 ^{0}38’ 552’ N**	**41^{0} 37’ 617’ E**
Green cape	41^{0} 44 142’ N	41^{0} 41 480’ E
Supsa Estuary	42^{0} 00’ 773’ N	41^{0} 45’ 059’ E

Microbiological investigation of Black Sea water samples

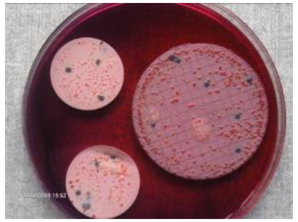
- **Bacteriological tests for water quality**
  - Aquatic microbial parameters (TBC, AC, HPC, Chl a)
  - Fecal Pollution indicators (TCC, FCC, ENT)- membrane filtration technique

- **Abundance of *Vibrio spp*. (TVC)**
  isolation of *Vibrio spp*. bacteria from water & plankton samples
- **Characterization and identification of *Vibrio spp*. isolates**
  - 13 phenotypic parameters
  - PCR identification/confirmation

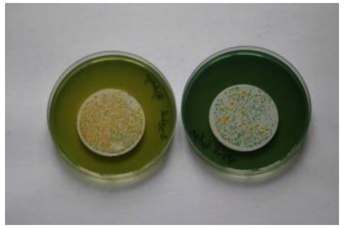
☐ **Direct detection of *Vibrio spp***

- Determination of the full spectrum of Vibrio genomes in the water samples (total bacterial DNA's)
- "Tiger" Platform - Multilocus PCR / ionization electro spray- mass spectrometry (PCR/ESI-MS) (USAMRIID, USA)

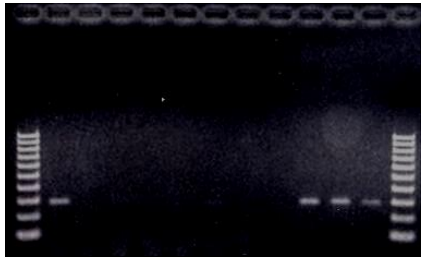
Whitehouse et al, AEM, 2011
Kokashvili et al, Front. Microb., 2015



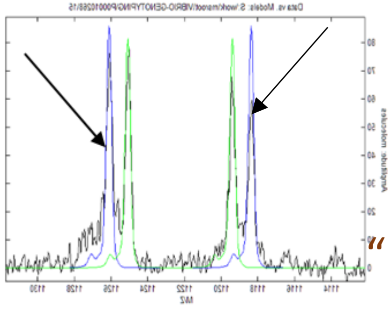
M-FC agar for TCC and FCC



TBCS agar for Vibrio isolation

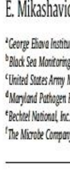


V. cholerae **specific (ITS) PCR on *Vibrio* from Lisi Lake**

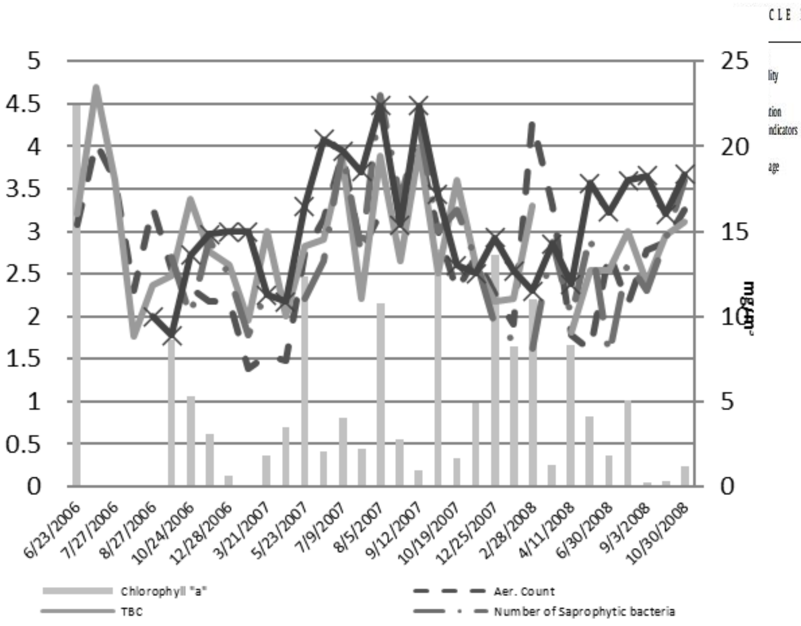


"Tiger" Platform

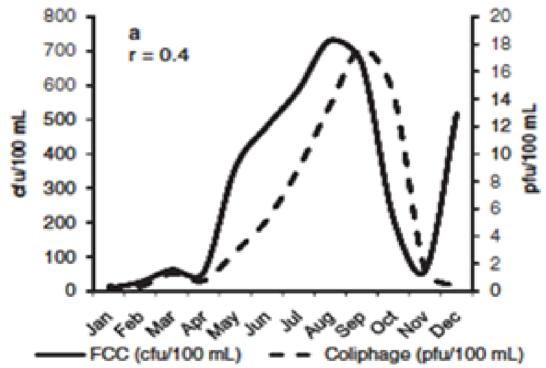
Microbial indices of Water quality in the Black Sea coast of Georgia

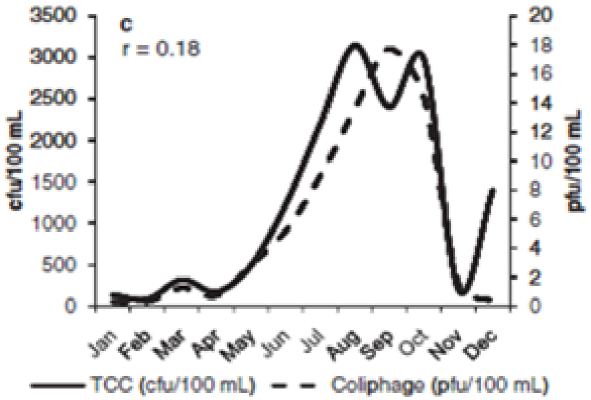


- Seasonal variability of majority of water microbial quality indices
- Increased pollution level at all sites in warm season
- Higher levels of microbial contamination in estuaries (Chorokhi and Supsa sites)
- Aquatic microbial parameters (TBC, AC, HPC), also Fecal pollution indicators (TCC, FCC, Ent) and somatic coliphages showed positive dependence on temperature
- Abundance of indicator somatic coliphages in sea water - correlation with TBC, TCC and FCC.



Microbial parameters in the Supsa estuary





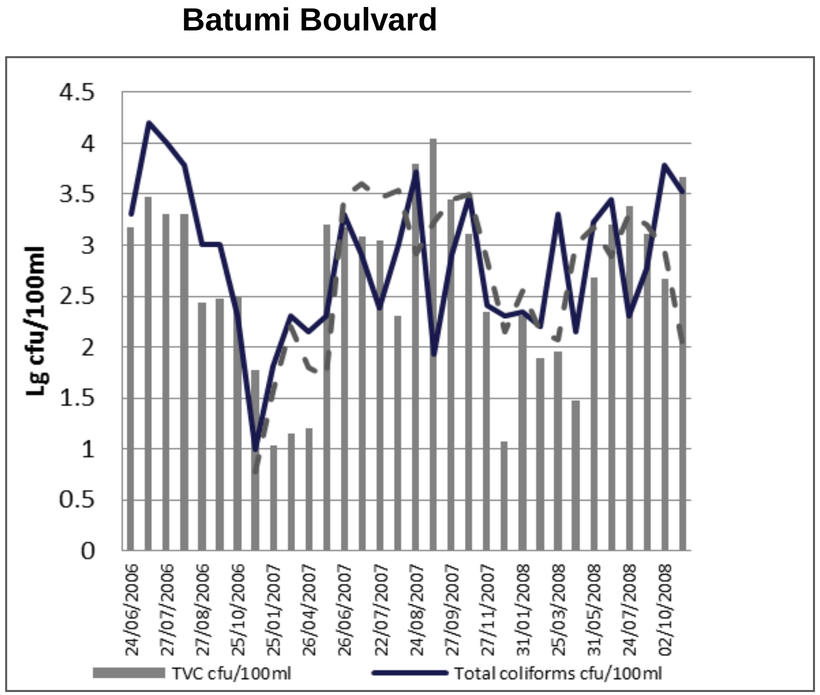
Janelidze et al., 2011. Mar. Poll. Bul.

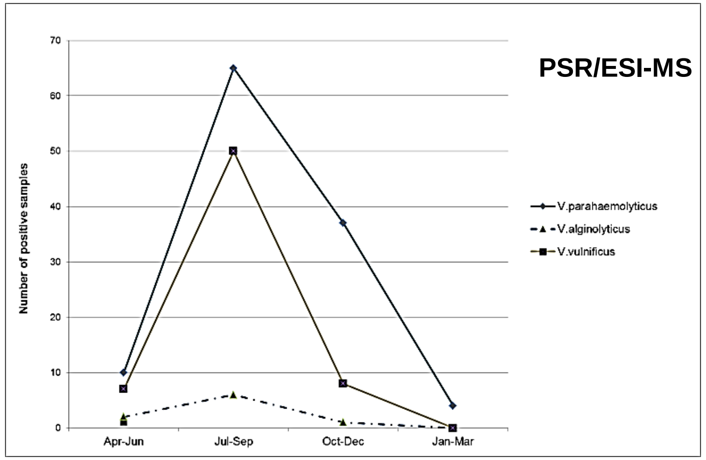
Abundance of vibrios in the Black Sea coastal waters of Georgia

- **Culture and PCR/ESI-MS:**

  Abundance of vibrios (Total vibrio counts -TVC) correlated with the elevated microbial pollution level in warm seasons

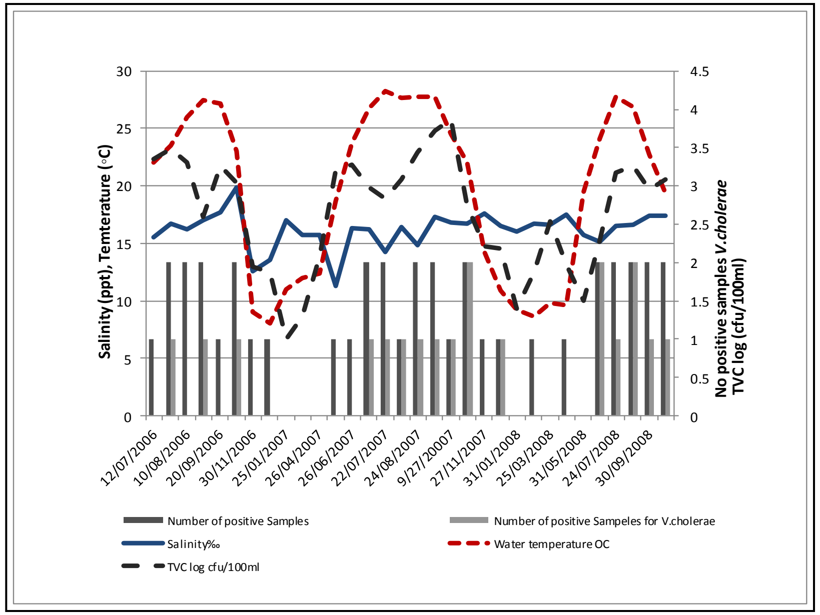
- Total vibrio counts (TVC) as well as presence of *Vibrio spp*. genomes at all sampling sites correlated with the water temperature





- **PCR/ESI-MS:**

  95% of samples positive for *Vibrio spp*. were collected in warm months with peak of isolation in July – September



Human pathogenic vibrios in the Black Sea coastal zone of Georgia

☐ **11** clinically important *Vibrio* species revealed by culture (up to 500 isolates)

- ***V. paraheamolyticus***
- ***V.cholerae***
- ***V.vulnificus***
- ***V.alginolyticus***
- ***V.harvey***
- ***V.metchnikovii***
- ***V.fluvialis***
- ***V.holisae***
- ***V.cincinatensis***

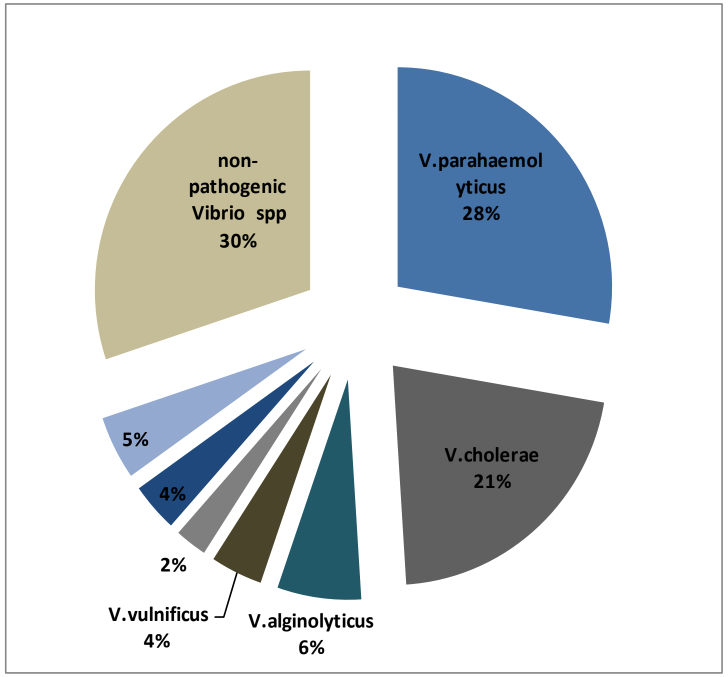
☐ **9** pathogenic ***Vibrio spp****.* detected by PCR/ESI-MS

- Leading Vibrio species

V. parahaemolyticus
V. cholerae
V. vulnificus

- *V. parahameolyticus- dominant species at Green Cape and Batumi Boulevard sites (17,0-18,0 ppt)*

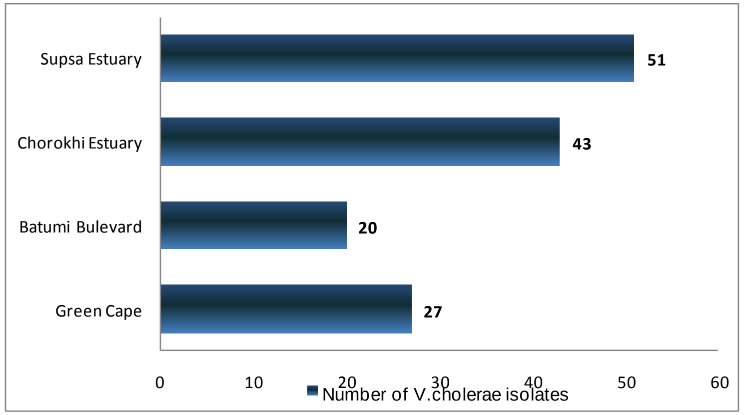
- *V.choleare* and *V.vulnificus* –prevalent in estuaries (4,0-9,4 ppt)



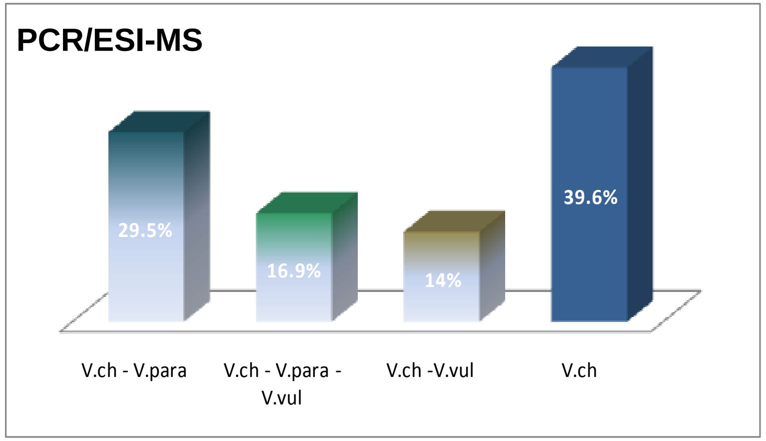
Percent Distribution of *Vibrio spp*. isolates from the Black Sea

Correlation between salinity and the abundance and *Vibrio* species composition at the Black Sea sites.

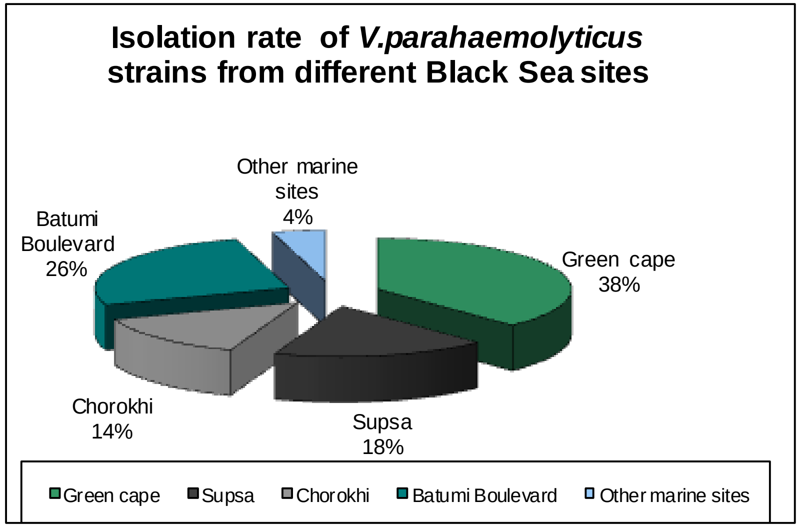
Abundance and Distribution of *V. cholerae* and *V.parahaemolyticus* in the Black Sea coastal zone



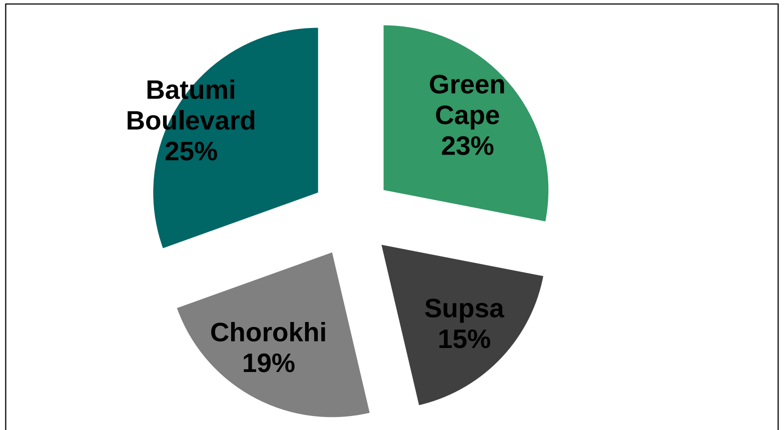
Distribution of *V. cholerae* isolates by sampling site



Percent distribution of samples positive for V. *cholerae* and its combination with other vibrios



Isolation rate of *V.parahaemolyticus* strains from different Black Sea sites



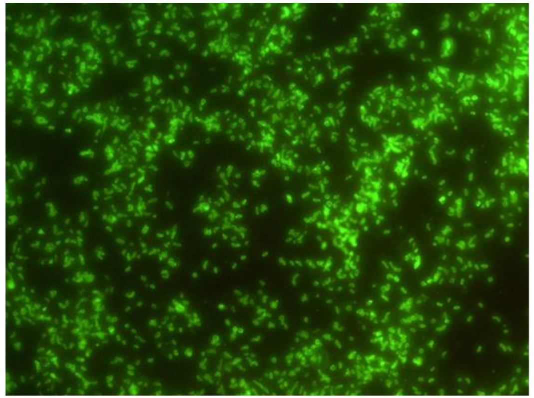
Number of Positve samples for *V.parahaemolyticus* by PCR/ESI-MS

Epidemic serotypes of V. cholerae in the Black Sea coastal zone

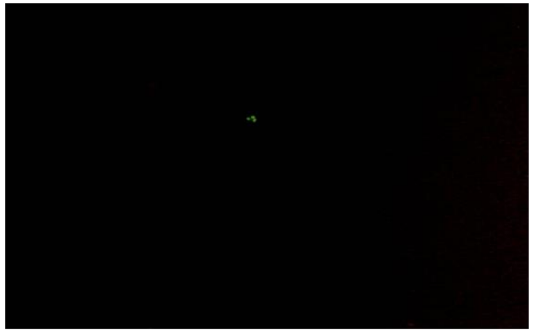
Direct detection of epidemic serotypes of *Vibrio* cholerae in water samples

- Direct Fluorescent Antibody (DFA) Assay

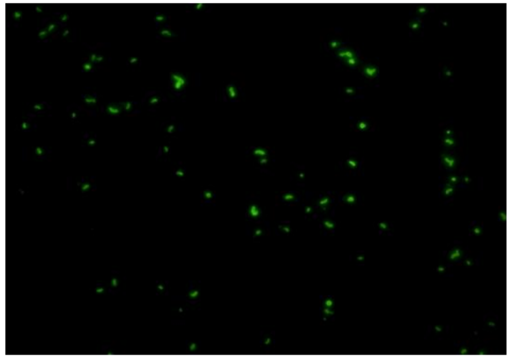
V. cholerae O1 positive DFA sample - concentrated water, Green cape July 2008



V. cholerae O1 positive DFA sample - enriched water, Green cape in July 2008

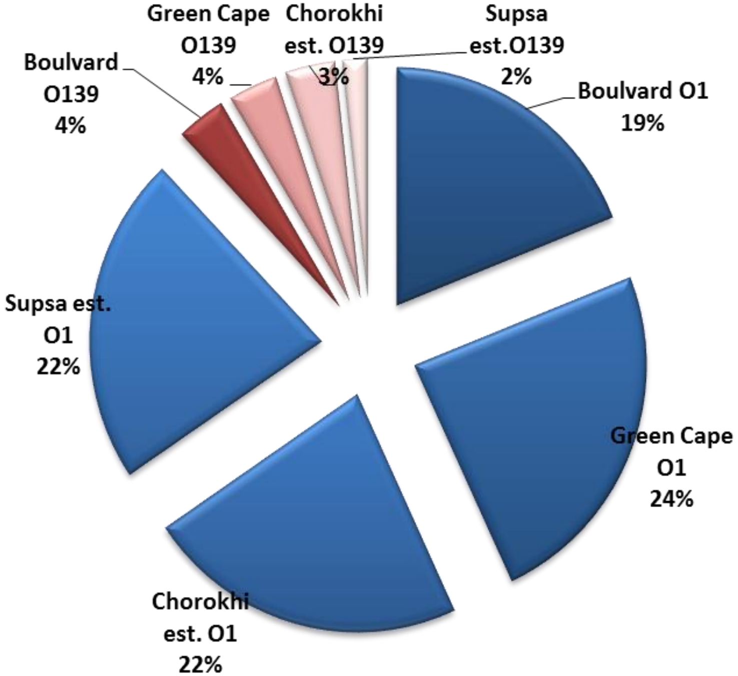


V. cholerae O139 positive DFA concentrated water, Supsa Estuary August 2007



V. cholerae O1 positive DFA sample - enriched water, Chrohki estuary, July 2008

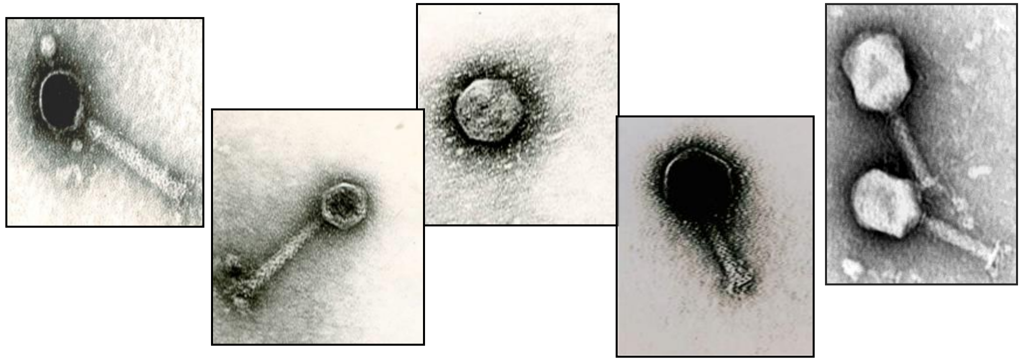
- 134 Black Sea water and plankton samples were examined by DFA assay and 45 were shown to be positive for *V. cholerae* O1.
- Tentative positive signals for V. cholerae O139 were detected only in a few concentrated water samples .



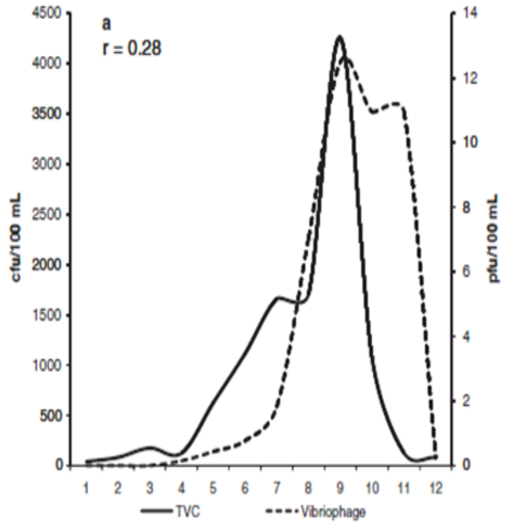
Distribution of DFA positive samples by sampling site

Phages active to autochtonous bacteria in the Black Sea coastal waters of Georgia

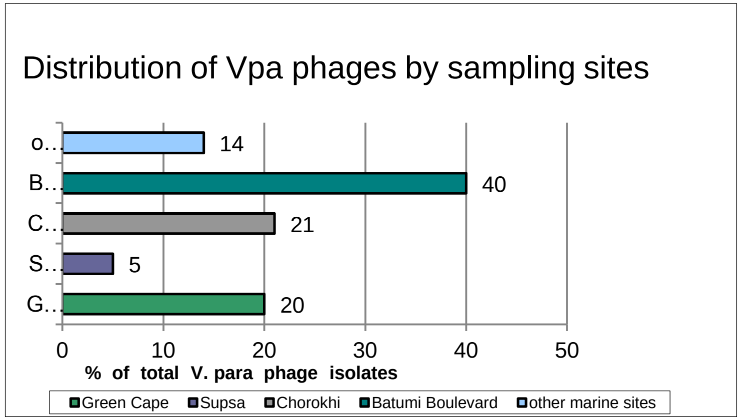
- Aquatic phages - indicators for presence of corresponding bacteria



Vibrio- and *Aeromonas*- specific phages (TEM)



- *V.parahaemolyticus* - specific phage isolation dynamics showed seasonal character and followed the fluctuations of the host bacteria

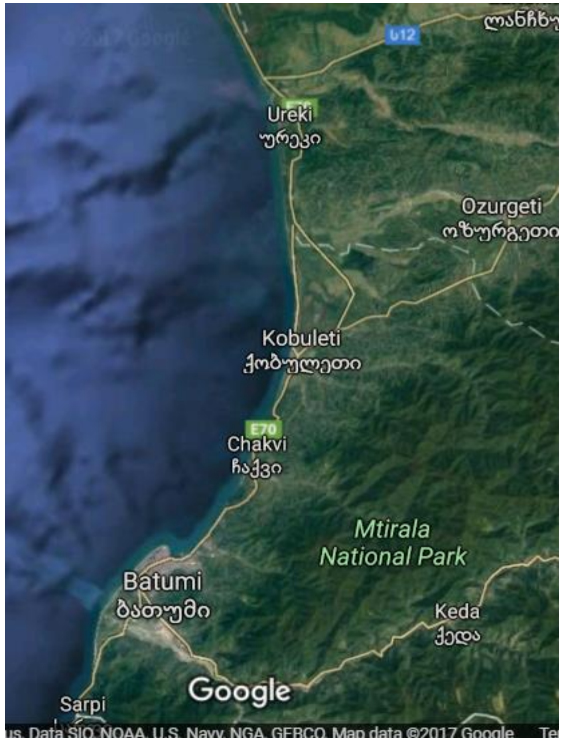


The Phage pool in the Black Sea environment – valuable resource of candidate phages for therapeutic preparations

Major findings of the study in the Black Sea coastal zone (Project GG-13, 2006-2009)

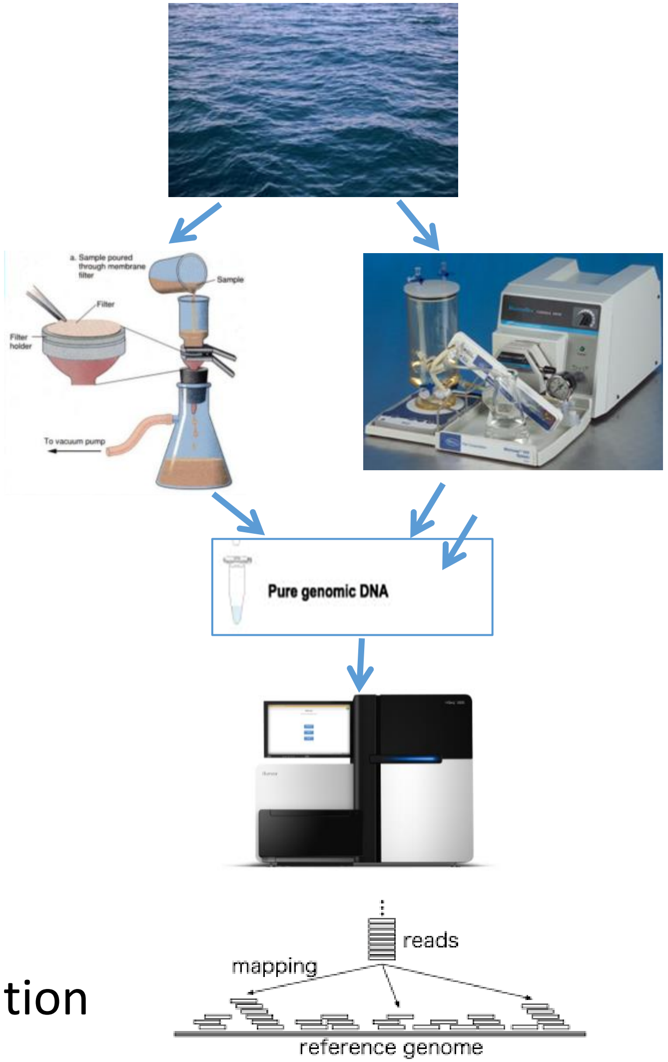
- Pathogenic *Vibrio* species are abundant and diverse in the Georgian coastal waters of the Black Sea.
- Water temperature was shown to be major environmental factor positively correlated with abundance of *Vibrio spp*;
- Eleven *Vibrio* species are present, with prevalence of *V. parahaemolyticus; V.choleare and V. vulnificus* are abundant in estuaries*;* These three major pathogens coexist in the Black Sea environment at the sites with moderate salinity.
- Presence of epidemic serotypes of *V.cholerae 01* and serovariants of *V. parahaemolyticus* O3:K6 pandemic strains in the Black Sea Coastal waters indicate significant public health threat.

Microbial communities in the Black Sea (Georgian coastal zone) -2015-2017



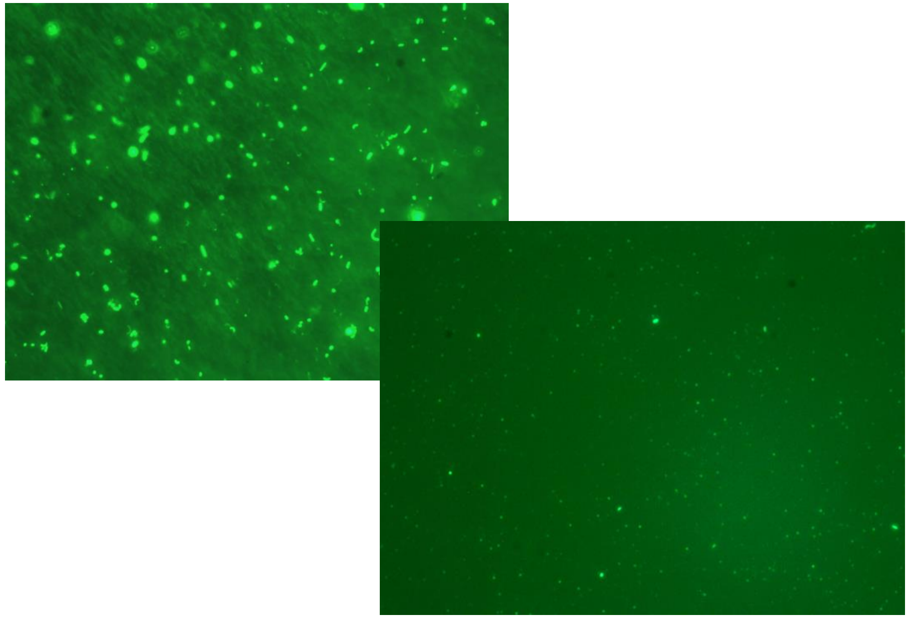
Metagenomic approach

Sampling- Concentration – DNA
Extraction- Sequencing - Annotation



Project CRDF 6209

Counting prokaryotes, protists, virioplankton



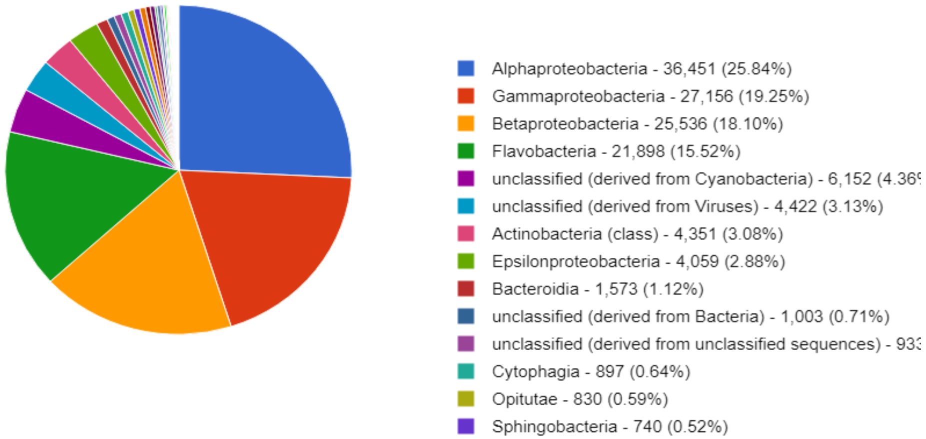
- ✓ Epifluorescent microscopy
  Zeiss Axioscope 40
- ✓ Syber green staining
- ✓ Diversity of viruses by TEM

Abundance of bacteria
– 10^5 -10^6 cell ml^{-1}
Abundance of phages
– 10^6 -10^7 cell ml^{-1}

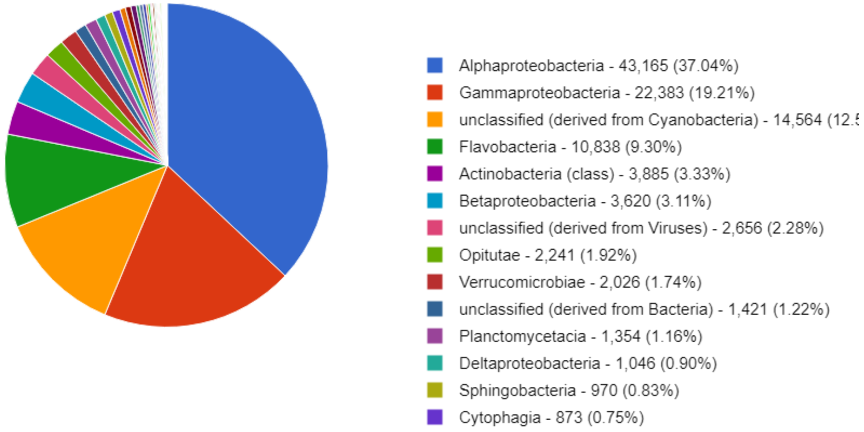
The Black Sea Microbial Diversity studies (2015-2017)

- **Aphaproteobacteria** dominated at all 4 sites, followed by
  - Gammaproteobacteria, - Betaproteobacteria, - Flavobacteria, - Actinobacteria ,

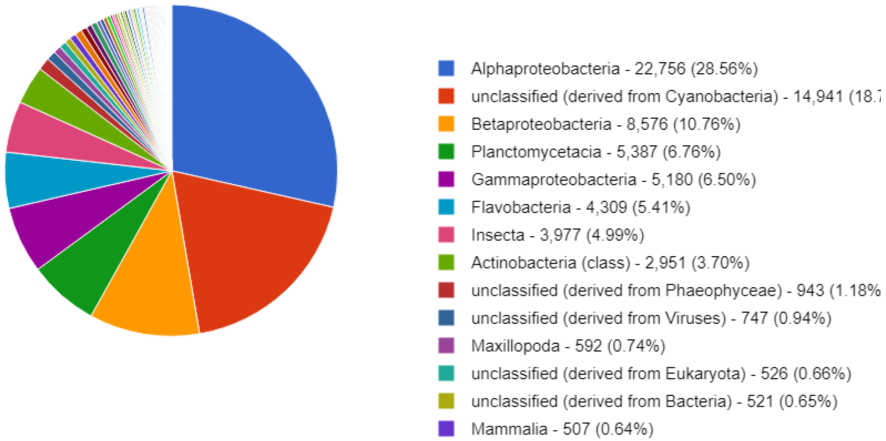
Supsa Estuary



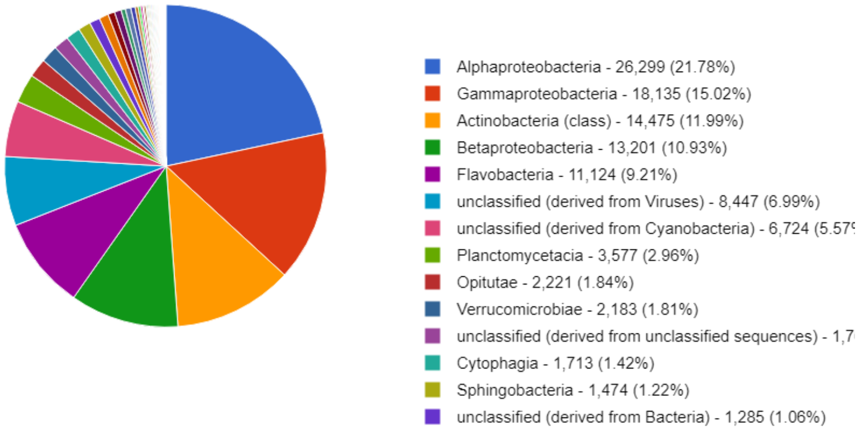
Batumi Boulevard



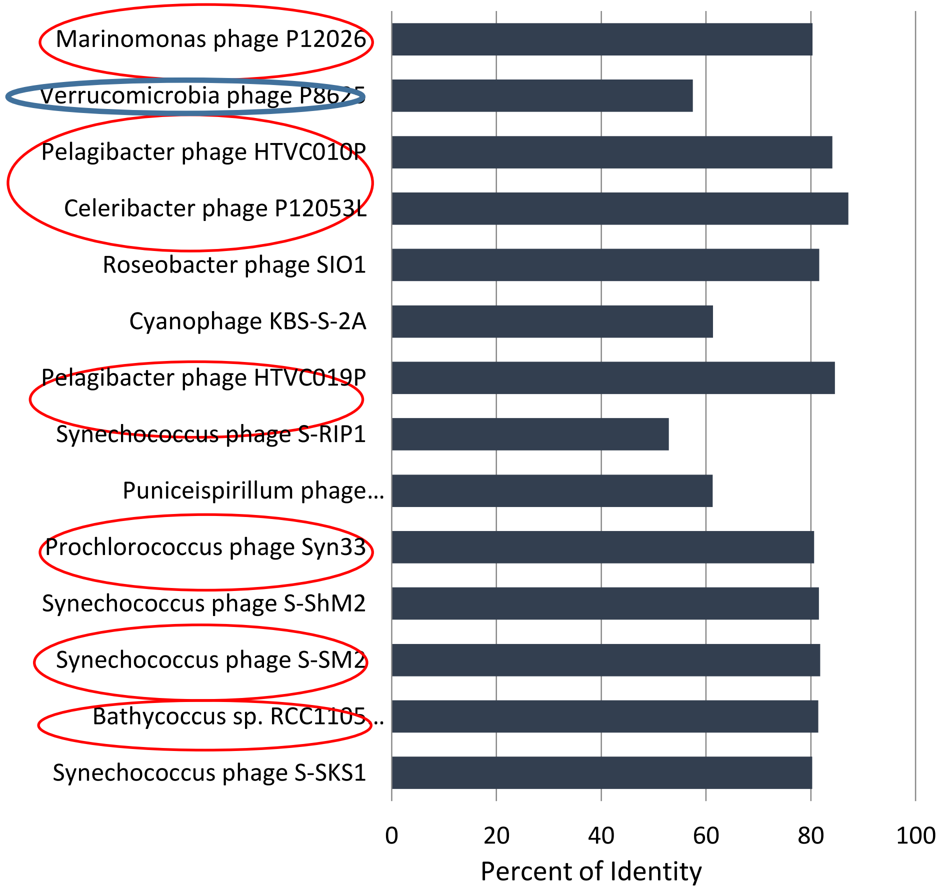
Green Cape



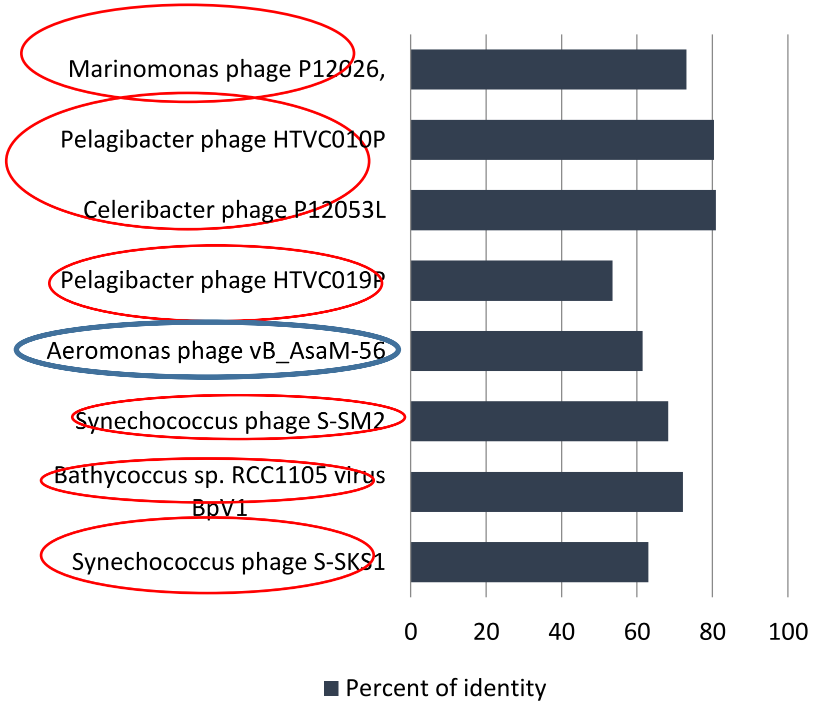
Chorokhi Estuary



The Black Sea (Georgian coast) virome (2015-17)

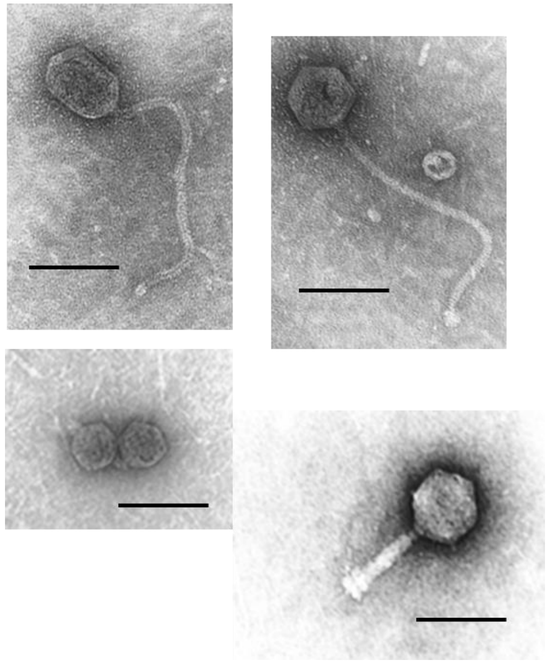


Green Cape



Batumi Boulvard

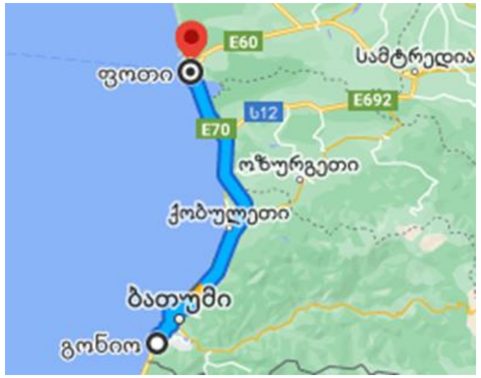
The Black Sea virome – Phage morphology



Batumi Boulvard

Microbial Diversity in the Georgian Coastal Area of the Black Sea (2018-2020)

Whole Genome Metagenomic Sequencing



Sampling sites: **Poti** and **Gonio** (distance 80 km)

- Prokaryotic and viral communities of Poti and Gonio coastal waters were studied
- Metagenomic DNA from collected from water concentrated samples were sequenced on the Illumina Miseq platform and taxonomic and functional profiles were obtained.
- Metagenomics analyses identified the content of microbial communities
- Temporal and spatial variations in microbial communities of the Black Sea coastal waters were shown

Prokaryotic community

- *Proteobacteria*, *Cyanobacteria*, *Actinobacteria* and *Firmicutes* found to be the most dominant phyla in Georgian coastal waters

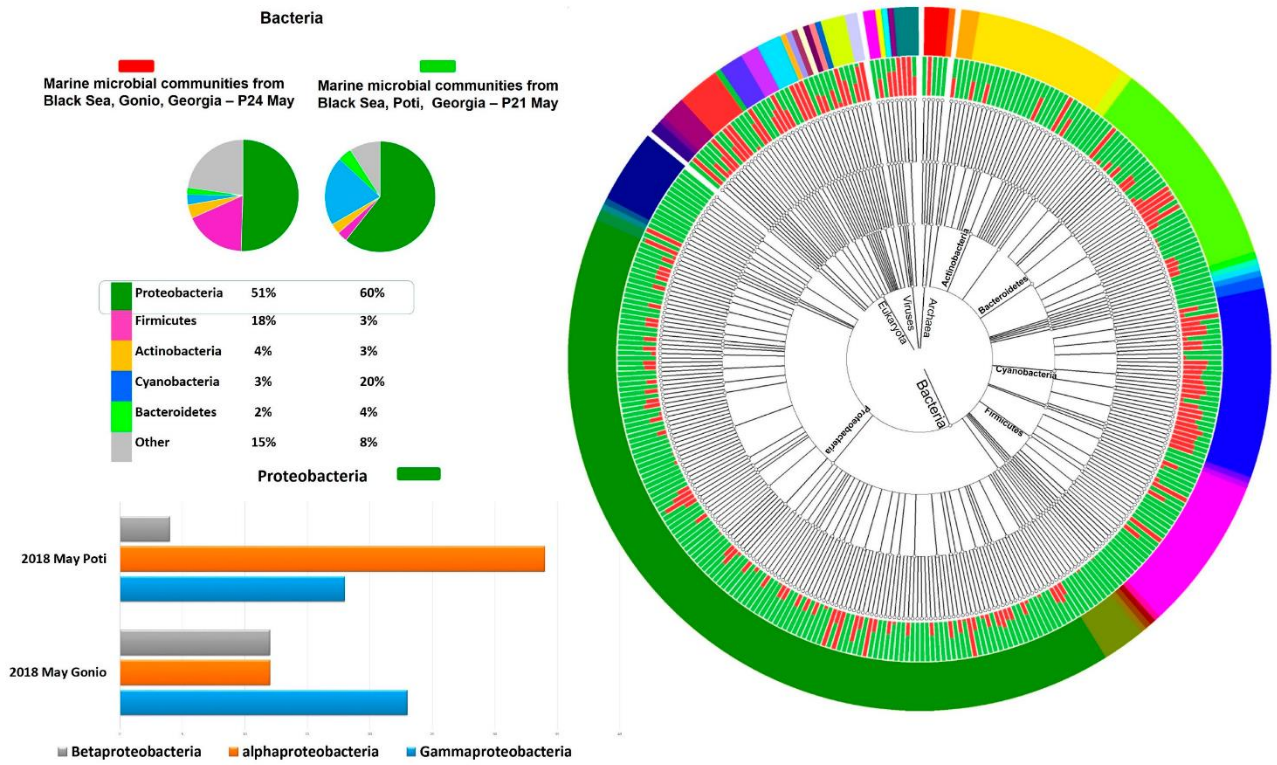


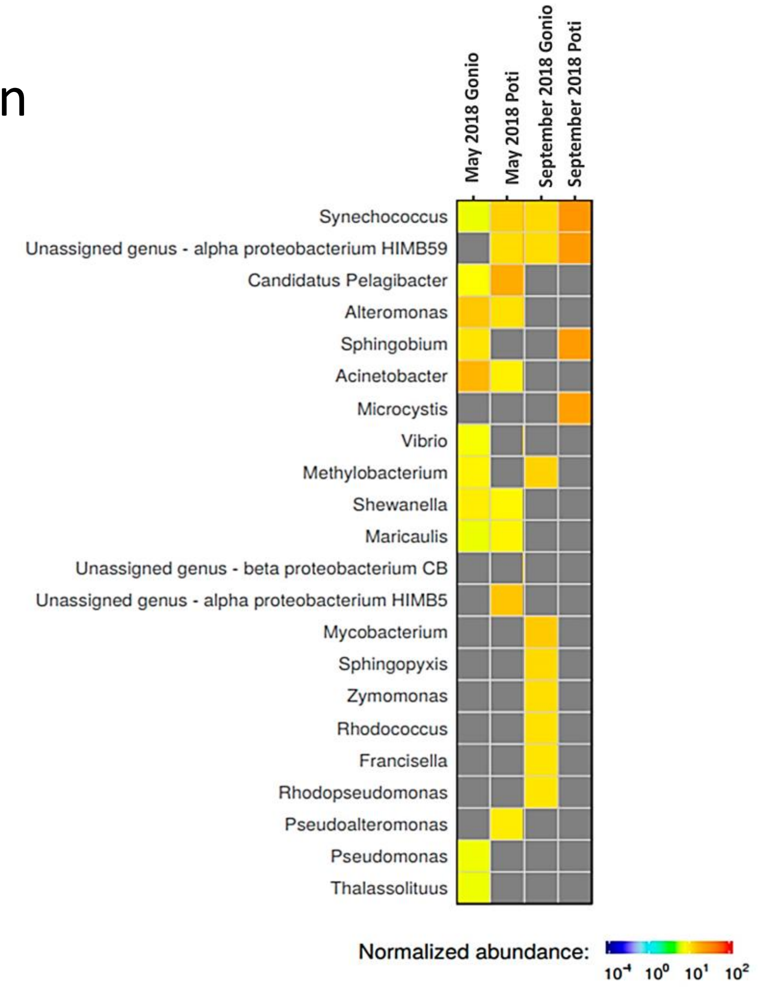
Figure 1. Taxonomic composition of Poti and Gonio metagenomes in May 2018 obtained by alignment of prokaryotic sequence reads to GOTTCHA database.

Microbial Diversity in the Georgian Coastal Area of the Black Sea Whole Genome Metagenomic Sequencing (Cont’d)

Prokaryotic community

- ☐ Gonio and Poti waters showed a different microbial composition
- ☐ Higher Alpha (species) diversity in Poti area

- ➢ Poti samples were dominated by Cyanobacteria (26%), followed by Alphaproteobacteria (22.5%), Gammaproteobacteria (15%), Betaproteobacteria (7.5%), Actinobacteria (3.5%), and Flavobacteria (2%).
  Genera:*Synechococcus*, *Alteromonas*, *Pseudoalteromonas*, *Acinetobacter*, *Maricaulis*, *Candidatus Pelagibacter, Shewanella* etc
- ➢ *Gonio* samples were dominated Proteobacteria (51%),, and Firmicutes (18%) in May, and Actinobacteria (48%) and Proteobacteria (28%) in September. Cyanobacteria were less prevalent in Gonio (average 5%). Among Proteobacteria gammaproteobacteria prevailed.
  Genera: *Acinetobacter, Alteromonas, Sphingobium, Candidatus Pelagibacter, Vibrio, Pseudomonas, Mycobacterium etc*



Normalized abundances of microorganisms at the genus level in Poti and Gonio water samples in May and September 2018.

Microbial Diversity in the Georgian Coastal Area of the Black Sea Whole Genome Metagenomic Sequencing (2018-2020)

☐ **Caudoviricetes** - dominant class of viruses under the double-stranded DNA (dsDNA) virus phylum;
- Myoviridae and Podoviridae - dominant viral families
- Significant portion - unclassified /unknown viruses

- Viral communities of Gonio

✓ Spring, 2018
- dominated by Synechococcus (44%) and Pelagibacter (42%)

✓ Autumn, 2018
- exclusively dominated by Synechococcus phages (94%)

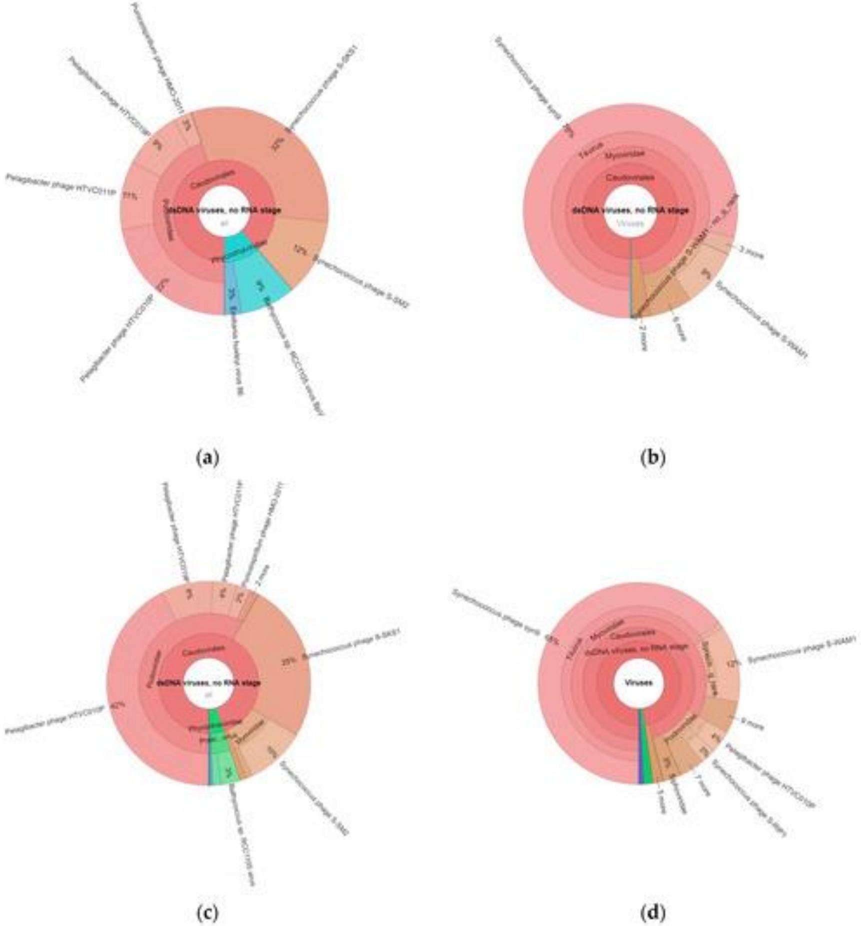
- Viral communities of Poti

✓ Spring, 2018
- dominated by *Pelagibacter* phages (56%), followed by *Synechococcus* (35%)

✓ Autumn, 2018
- *Synechoccocus* phages became more prevalent (77%)

- Phages *Aeromonas, Pseudomonas, Acinetobacter, Vibrio, Klebsiella, Erwinia, Ralstonia, Escherichia, Salmonella,* and *Campylobacter*

Minor groups, affecting human and animal pathogens



Taxonomic composition of viruses in the Black Sea viral metagenomes. (**a**) Gonio May 2018; (**b**) Gonio Sept. 2018; (**c**) Poti May 2018; (**d**) Poti Sept. 2018.

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

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Microbial water quality of the Georgian coastal zone of the Black Sea

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ARTICLE INFO

Keywords:
Water quality
Black Sea
Fecal pollution
Microbial indicators
Vibrios
Bacteriophage

ABSTRACT

From 2006 to 2008, microbial water quality was monitored along the Georgian coast of
Temperature, pH, salinity, and dissolved oxygen were measured, along with a variety of aq
parameters, including heterotrophic plate count (HPC), total culturable bacterial count (T
rophyll a (Chl-a) concentration. Total and fecal coliforms and total enterococci counts w
indicators of fecal pollution. *Vibrio* bacteria, and *Escherichia coli*- and *Vibrio*-specific bacte
isolated and enumerated to determine their relationships to standard marine pollution i
Persistent microbial pollution was observed, particularly in the summer months, with
contamination in estuaries. Microbial indicators generally showed seasonal dependence,
temperature may influence bacterial dynamics in this environment. No correlation

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2010, p. 1996–2001
0099-2240/10/$12.00 doi:10.1128/AEM.01919-09
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Vol. 76, No. 6

Identification of Pathogenic *Vibrio* Species by Multilocus PCR-Electrospray Ionization Mass Spectrometry and Its Application to Aquatic Environments of the Former Soviet Republic of Georgia▽†

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Received 10 August 2009/Accepted 4 January 2010

The Ibis T5000 is a novel diagnostic platform that couples PCR and mass spectrometry. In this study, we developed an assay that can identify all known pathogenic *Vibrio* species and field-tested it using natural water samples from both freshwater lakes and the Georgian coastal zone of the Black Sea. Of the 278 total water samples screened, 9 different *Vibrio* species were detected, 114 (41%) samples were positive for *V. cholerae*, and 5 (0.8%) samples were positive for the cholera toxin A gene (*ctxA*). All *ctxA*-positive samples were from two freshwater lakes, and no *ctxA*-positive samples from any of the Black Sea sites were detected.

The genus *Vibrio*, within the family *Vibrionaceae*, is a diverse group of Gram-negative bacteria found exclusively in the aquatic environment. Important pathogenic members include *Vibrio cholerae*, the causative agent of cholera, and *Vibrio parahaemolyticus* and *Vibrio vulnificus*, which have been implicated in diarrhea, septicemia, and wound infections (5). The Ibis T5000 uses electrospray ionization-mass spectrometry to analyze the products of broad-range PCR (PCR-electrospray ionization-mass spectrometry [PCR/ESI-MS]) and is designed to rapidly detect and identify emerging pathogens and biothreat agents without prior knowledge of a pathogen's nucleic acid sequence (4, 10).

supplemental material). All primers used in the assay are listed in Table 2. All PCRs were performed in 96-well microtiter plates as described previously (1). A Bruker Daltonics microTOF (Billerica, MA) mass spectrometer was used for analyzing the purified DNA as described previously (3, 7). The presence of *V. cholerae* was confirmed by using species-specific intergenic spacer region (ISR) PCR primers (2). For detection of the cholera toxin A gene (*ctxA*), PCR was performed as described by Rivera et al. (9). For confirmation of the *Vibrio* population data obtained with the PCR/ESI-MS assay, an environmental clone library was con-

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frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 February 2014
doi: 10.3389/fmicb.2014.00045

Molecular diversity and predictability of *Vibrio parahaemolyticus* along the Georgian coastal zone of the Black Sea

Bradd J. Haley[1], Tamar Kokashvili[2], Ana Tskshvediani[2], Nino Janelidze[2], Nino Mitaishvili[2], Christopher J. Grim[1,3], Guillaume Constantin de Magny[4], Arlene J. Chen[1], Elisa Taviani[1], vili[2], Marina Tediashvili[2], Chris A. Whitehouse[5], Rita R. Colwell[1,3,6,7] and Anwar Huq[1,8]*

Vibrio parahaemolyticus is a leading cause of seafood
autochthonous member of marine and estuarine env
seventy strains of *V. parahaemolyticus* were isolate
collected along the Georgian coast of the Black
collection. All isolated strains were tested for pres
of strains were serotyped and tested for additional
Twenty-six serotypes, five of which are clinically re
170 isolates were negative for *tdh*, *trh*, and the Ka
the GS-PCR sequence and 27 the 850 bp sequenc
strains. The *V. parahaemolyticus* population in the
genomically heterogeneous by rep-PCR and the sero
with rep-PCR genomic diversity. Statistical modeling
V. parahaemolyticus as a function of water tempe
observed for Green Cape site samples (Percent of tot
demonstrate a diverse population of *V. parahaemoly*
carry pandemic markers, with increased water temp
abundance of *V. parahaemolyticus*.

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frontiers in Public Health

ORIGINAL RESEARCH
published: 13 October 2015
doi: 10.3389/fpubh.2015.00232

OPEN ACCESS

Edited by:
Nathan Wolfe, Metabiota, USA

Reviewed by:
Li Xu, Cornell University, USA
Carla Pruzzo, University of Genova, Italy

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Specialty section:
This article was submitted to Infectious Diseases, a section of the journal Frontiers in Public Health

Received: 16 June 2015
Accepted: 28 September 2015
Published: 13 October 2015

Citation:
Kokashvili T, Whitehouse CA, Tskhvediani A, Grim CJ, Elbakidze T, Mitaishvili N, Janelidze N, Jaiani E,

Occurrence and diversity of clinically important *Vibrio* species in the aquatic environment of Georgia

*Tamar Kokashvili[1], Chris A. Whitehouse[2†], Ana Tskhvediani[1], Christopher J. Grim[3,4‡], Tinatin Elbakidze[1], Nino Mitaishvili[1‡], Nino Janelidze[1], Ekaterine Jaiani[1], Bradd J. Haley[4], Nino Lashkhi[1], Anwar Huq[4], Rita R. Colwell[3,4] and Marina Tediashvili[1]**

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Among the more than 70 different *Vibrio* species inhabiting marin
freshwater ecosystems, 12 are recognized as human pathogens. The
climate of the Black Sea coastal area and inland regions of Georgi
favorable environment for various *Vibrio* species. From 2006 to 200
ecology, and diversity of clinically important *Vibrio* species were studie
tions in Georgia and across seasons. Over a 33-month period, 1,595
isolates were collected from the Black Sea (*n* = 657) and freshwater l
(*n* = 938). Screening of a subset of 440 concentrated and enriched
PCR-electrospray ionization/mass spectrometry (PCR-ESI/MS) detec
of DNA from eight clinically important *Vibrio* species: *V. cholerae*, *V. pa*
vulnificus, *V. mimicus*, *V. alginolyticus*, *V. harveyi*, *V. metschnikovii*, and
Almost 90% of PCR/ESI-MS samples positive for *Vibrio* species we
June through November. Three important human-pathogenic *Vibrio* sp
V. parahaemolyticus, and *V. vulnificus*) were detected in 62.8, 37.8, a
ples testing positive for *Vibrios*, respectively. The results of these acti
natural reservoirs for human-pathogenic *Vibrios* exist in Georgian aqu
Water temperature at all sampling sites was positively correlated with
clinically important *Vibrio* spp. (except *V. metschnikovii*), and salinity w
species composition at particular Black Sea sites as well as inland res

Keywords: aquatic environment, Black Sea, lakes, infection, Vibrios, conventional cu diversity

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marine drugs

MDPI

Article

Microbial Diversity and Phage–Host Interactions in the Georgian Coastal Area of the Black Sea Revealed by Whole Genome Metagenomic Sequencing

Ekaterine Jaiani [1,*], Ia Kusradze [1], Tamar Kokashvili [1], Natia Geliashvili [1], Nino Janelidze [1], Adam Kotorashvili [2], Nato Kotaria [2], Archil Guchmanidze [3], Marina Tediashvili [1] and David Prangishvili [4,5]

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Received: 9 October 2020; Accepted: 30 October 2020; Published: 14 November 2020

Abstract: Viruses have the greatest abundance and highest genetic diversity in marine ecosystems. The interactions between viruses and their hosts is one of the hot spots of marine ecology. Besides their important role in various ecosystems, viruses, especially bacteriophages and their gene pool, are of enormous interest for the development of new gene products with high innovation value. Various studies have been conducted in diverse ecosystems to understand microbial diversity and phage–host interactions; however, the Black Sea, especially the Eastern coastal area, remains among the least studied ecosystems in this regard. This study was aimed at to fill this gap by analyzing microbial diversity and bacteriophage–host interactions in the waters of Eastern Black Sea using a metagenomic approach. To this end, prokaryotic and viral metagenomic DNA from two sampling sites, Poti and Gonio, were sequenced on the Illumina Miseq platform and taxonomic and functional profiles of the metagenomes were obtained using various bioinformatics tools. Our metagenomics analyses allowed us to identify the microbial communities, with *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, and *Firmicutes* found to be the most dominant bacterial phyla and *Synechococcus* and *Candidatus Pelagibacter* phages found to be the most dominant viral groups in the Black Sea. As minor groups, putative phages specific to human pathogens were identified in the metagenomes. We also characterized interactions between the phages and prokaryotic communities by determining clustered regularly interspaced short palindromic repeats (CRISPR), prophage-like

Publications on the Black Sea microbial pollution, Vibrio abundance and speciation, Microbial community (metagenome analysis)

THANK YOU!

Acknowledgement

Laboratory of Microbial Ecology
Eliava Institute of Bacteriophages

Eliava Institute

Dr. Ekaterine Jaiani

Collaborators :
University of Maryland
Michigan State University
National Center for Disease control (NCDC)