ESMIC

Estimation, monitoring and reduction of plastic pollutants in Latvian-Lithuanian Coastal area via innovative tools and awareness raising

Methodology for fast screening of with plastic associated pathogenic microorganisms (D.T1.1.3)

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Content

About the Programme

The Interreg V-A Latvia – Lithuania Cross Border Cooperation Programme 2014- 2020 aims to contribute to the sustainable and cohesive socio-economic development of the Programme regions by helping to make them competitive and attractive for living, working and visiting.

The Estimation, monitoring and reduction of plastic pollutants in Latvian-Lithuanian coastal area via innovative tools and awareness raising (ESMIC) project is funded by the European Union. The total project size is 449 574.89 EUR. Out of them co-funding of European Regional Development Fund is 382 138.64 EUR

Introduction

The *Estimation, monitoring and reduction of plastic pollutants in Latvian-Lithuanian coastal area via innovative tools and awareness raising* (ESMIC) project aims to develop a sustainable, cost-effective framework for plastic litter detection, monitoring and management in marine and coastal environments. One of the key threats to the bathing water quality and health of the common beach goers is the association of the pathogens (Vibrio) with plastic litter.

This report describes the development of a method, that is based on a molecular technique and that allows fast (to one day) identification of with the plastic associated hazardous organisms up to the level of presence/absence. Since the project focuses on *Vibrio* bacteria - that might be transferred to recreational beaches by plastic and are potentially pathogenic microorganisms this methodology addresses two potentially pathogenic *Vibrio* species that have been found in the Lithuanian Baltic Sea coastal waters previously (G. Gyraitė et al., 2020): *V. vulnificus* and *V. cholera.*

In this methodology information is provided how in the plastic samples collected from the beach environment the presence of two potentially pathogenic *Vibrio* species (*V. vulnificus* and *V. cholerae*) can be assessed by molecular-based techniques that rely on polymerase chain reaction (PCR) assay.

Official disclaimer

This report has been produced with the financial assistance of the European Union. The contents of this report are the sole responsibility of Klaipeda University Marine Research Institute and can under no circumstances be regarded as reflecting the position of the European Union.

Methodology

3.1 Principle of the method

Assessment of potentially pathogenic *Vibrio vulnificus* and *Vibrio cholerae* on plastic litter surfaces using polymerase chain reaction (PCR) assay (by conventional PCR method).

3.2 Scope and Application

Plastic litter is known to provide a distinct microbial habitat by supporting microbial biofilms. It also represents a novel mechanism of microbe dispersion, as plastic items easily migrate among coastal and marine habitats (McCormick et al., 2014). Furthermore, plastic items trapped in floating algae wracks can be rapidly washed onshore and be more accessible for beachgoers. The main target of this assessment is potentially pathogenic *V. vulnificus* and *V. cholerae* bacteria found in association with plastic litter. *Vibrio* spp. bacteria are known to be a potential pathogen for human health and are found naturally in surface marine and estuarine ecosystems with preferably warmer (>15°C) and lower salinity (5-25 ppt) waters. The majority of *Vibrio* illnesses usually occur through consumption of raw shellfish or from direct contact with seawater by risk group individuals (Gyraite et al. 2019). Assessment of *Vibrio* spp. in association with plastic litter could be used for research and educational purposes.

3.3 Laboratory safety rules

Warning – detection analysis of potentially pathogenic *Vibrio* spp. should be conducted only in laboratories equipped for this purpose and by experienced microbiologists. Disposal of contaminated material should be regarded with great care.

Further relevant regulations of the Hazardous Substances Ordinance:

- Duty to wear a protective coat;
- Closed footwear;
- Eating, drinking, smoking is prohibited in the laboratory;
- Always use pipetting aids for pipetting (never pipette with the mouth);
- Handle samples in such a way that they are only contained in closed containers;
- Flame-off the inoculation loops, forceps, scissors after contact with site material;
- Place used pipettes in the containers on the table;
- Immediately wipe up any impurities in the working area with a surface disinfectant;
- After working, also treat the table surfaces with surface disinfectant and disinfect the hands, especially between fingers, with a hand disinfectant solution.

- All processed culture containers and other approaches used by the participants have to be marked with Experiment number and other abbreviations.

3.4 Sampling Handling

Materials checklist:

- Sterile gloves
- Sterile plastic bags
- Cooling elements
- Refrigerator

Using gloves collect plastic samples from the desired environment (water, sand, algae wrack) and put them into a sterile plastic bag. Keep samples in the refrigerator and carry them to the laboratory within 4 hours of collection.

3.4 Sample preparation

Materials checklist:

- Autoclaved seawater
- Ethanol
- Tweezers
- Scissors
- Lighter
- Bunsen burner
- Meter stick
- 5 mL (blue cap) tubes from DNA PowerWater® or PowerSoil® Isolation Kit

In the laboratory, rinse separate macro-litter pieces with sterile seawater, remove residual debris and non-attached items. Cut plastic fragments of approximately 10 cm^2 from macro-litter items using sterile scissors and put them into a 5 mL (blue cap) tube from the PowerWater® or PowerSoil® DNA Isolation Kit and store plastic samples at -20 °C until further analysis or proceed immediately.

3.5 DNA extraction

Materials checklist:

- Pipettes (1000 µl, 100 µl, 10 µl)
- Pipette tips (sterile)
- Eppendorf tubes stand
- Sterile Eppendorf tubes (1.5 ml and 2.0 ml; PCR-clean)
- Tweezers
- Alcohol, lighter
- Ice in polystyrene vessel
- Centrifuge
- Horizontal shaker
- Thermal bath

Extract genomic DNA from plastic samples using PowerWater® or PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc., Carlsbad, CA) following manufacturer's instructions. Store aliquots at −20°C for short time and at −80°C for long time conservation.

3.6 Conventional PCR for *V. vulnificus* and *V. cholerae* presence/absence identification

Materials checklist:

- Pipettes (1000 µl, 100 µl, 10 µl)
- Pipette tips (sterile)
- Eppendorf tubes stand
- Sterile Eppendorf tubes (1.5 ml and 2.0 ml; PCR-clean)
- Sterile PCR (0.2 mL) 8-strip tubes and caps
- PCR reagents (industrial master mix's, see below)
- PCR thermocycler
- Primers of interest
- Known positives

3.6.1 General instructions for PCR

The solutions, tubes and pipette tips used for the PCR amplification must be and must remain sterile and DNA-free to avoid contamination by foreign DNA. Keep in mind that any contamination, however small it is in proportion to the rest of the sample DNA, will also be exponentially amplified by the PCR reaction. To confirm the absence of contamination in your PCR's, you must always have a DNA negative control, which consists of a reaction mixture without any DNA template (i.e. instead of adding 0.4-5 µL of DNA, you will add 0.4-5 µL of PCR water).

It is also necessary that all solutions and tubes would be free of DNases and RNases to prevent digestion of the nucleic acids in the reaction mixture. A positive control will allow you to determine that the PCR reaction was successful. This positive control consists of a reaction mix; to which you will add a different DNA template on which the PCR is known to work (i.e. *V. vulnificus* (CCM 2838, CECT 520), *V. cholerae* (CECT 514)).

Sometimes the DNA sample concentration might be too high, or the extract may contain inhibiting factors. To ensure a successful PCR you might have to dilute your DNA template in PCR water (usually 1:10 to 1:100).

3.6.2 Primers

The choice of primer sets that you use for the PCR reaction depends on which region of the DNA you want to amplify. To investigate the presence of *V. vulnificus* use vvhA gene[1], which amplifies a fragment of 519 bp, specific to *V. vulnificus.* For *V. cholerae* identification use prVC[2] gene which amplifies a fragment in 295-310 bp range. The target region used for the detection of vvhA and prVC is based on previously published and validated data (Table 1) and is also described in detail in ISO 2187211:2017.

Table 1. Primers for Conventional PCR analyses

	Primer sequences	Base pairs (bp)	Resource
Vibrio vulnificus	vvhA_785F: 5'-CCGCGGTACAGGTTGGCGCA-3'	519	Han and Ge, 2010
	vvhA_1303R: 5'-CGCCACCCACTTTCGGGCC-3'		
Vibrio cholerae	prVC_F: 5'-TTAAGCSTTTTCRCTGAGAATG-3'	295-310	Chun et al. 1999
	prVC_R: 5'-AGTCACTTAACCATACAACCCG-3'		

Consider the number of reactions you will need:

[1] vvhA is with virulence-associated gene encoding hemolysin

[2] amplifies a portion of the intergenic spacer region between the 16S and 23S rRNA genes and is specific for *V. cholerae*

- 3 sample replicates at 3 different dilutions (1, 1:10, 1:100)
- 1 positive control
- 1 negative control
- 1 extraction blank
- Total of 12 samples (always best to account for pipetting errors, so prepare a master mix of 15 samples)

3.6.3 Recommendations for PCR master mix

PCR master mix can be prepared using separate reagents needed for PCR reaction; however, we recommend using industrial master mix's dedicated for genetic analyses with environmental samples containing various inhibitors, etc. Examples of industrial master mixes would be VWR Red Taq DNA Polymerase (https://uk.vwr.com/assetsvc/asset/en_GB/id/12367507/contents) or Platinum™ Green Hot Start PCR Master Mix (https://www.thermofisher.com/order/catalog/product/13001012).

- Prepare master mix following manufacturer's instructions;
- Pour master mix into 8-strip PCR tubes and add DNA/positive/extraction blank;
- Run PCR program according to the gene-specific annealing temperature (Table 2).

Table 2. Protocol for PCR program

Denaturation	95 °C	5 min	1x
Denaturation	94°C	45 s	30x
Annealing	**68 °C for vvha gene** **55 °C for prVC gene**	45 s	
Elongation	72 °C	1 min 30 s	
End-elongation	72 °C	10 min	1x
	4 °C	∞	

3.7 Agarose gel electrophoresis

Materials checklist:

- Pipettes (1000 µl, 100 µl, 10 µl)
- Pipette tips (sterile)
- Microwave
- 100-200 mL glass bottle
- Scale
- Agarose
- TAE buffer
- SYBR® Safe dye
- Agarose gel special trays and combs
- Electrophoresis bath and basic power supply
- UV lightbox/gel visualization system

Agarose gel electrophoresis enables DNA fragments from 200 bp to 50,000 bp separate the molecules from each other based on size and charge. For this purpose, the DNA is introduced into an agarose gel and an electric field is applied. After completion of gel electrophoresis, the separated DNA fragments in the gel can be made visible by staining with an intercalating fluorescent dye (here: SYBR® Safe DNA Gel Stain) as a band pattern.

3.7.1 Preparation of agarose gel

- Cast agarose gels in a special Plexiglas tray. In order to be able to pour the gel, these sides must first be sealed with spacers. Place the plastic comb over one end and in the middle of the tray, so that its teeth have a distance of approx. 1 mm (thickness of a slide) to the bottom of the tray. In this way, the finished gel is given small pockets at one end and in the middle, which are used for PCR amplification (Figure 1).
- Heat the agarose (i.e. 1.2 % (w/v) agarose in 1xTAE buffer) in a microwave until a clear, streak-free solution is obtained.
- Before casting the gel, add diluted SYBR® Safe to the dissolved agarose and swirl to mix and then pour hot (touchable) agarose into the prepared tray.
- After about 20 minutes the gel solidifies and the combs can be pulled out carefully (!).

- Finally, the adhesive tape strips are removed. If the gel is not used immediately, it must be protected from drying out in a closed container filled with 1 x TAE buffer.

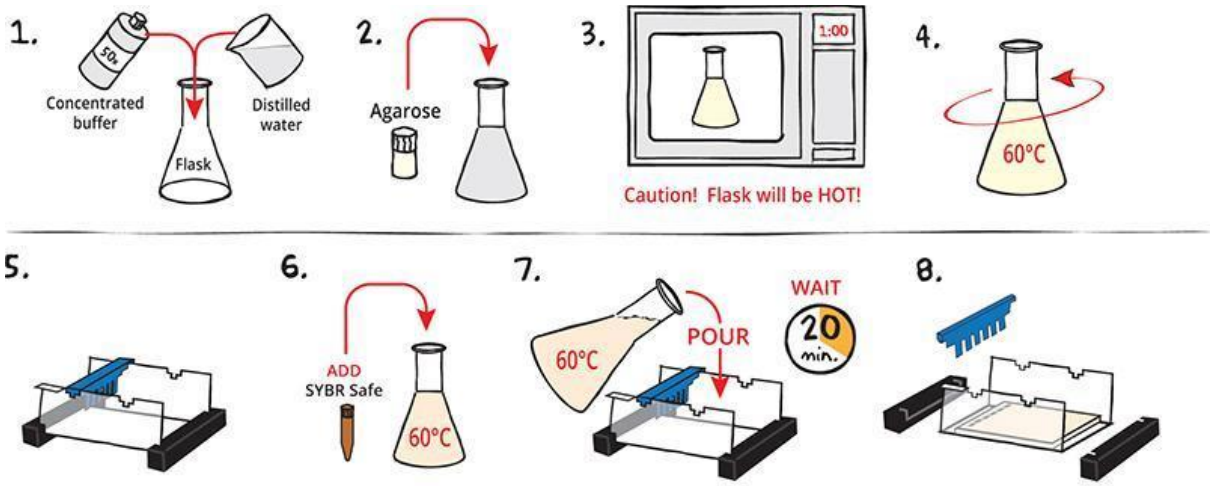

Figure 1. Preparation of agarose gel (https://www.edvotek.com/quick-guide-sybr-safe)

3.7.2 Electrophoresis

- Place the tray with the gel in an electrophoresis chamber (the open ends are placed in field direction).
- Pour 1 x TAE buffer into the tub until the gel is completely immersed.
- Afterwards, pipette each sample (6-10 µL) carefully, precisely and bubble-free into an empty pocket of the gel. A 100 bp ladder is used as standard, which is already equipped with a loading buffer. Load 3 µl of the ladder to the gel.
- Finally, connect a voltage source to the contacts of the electrophoresis chamber (observe correct polarity!). The subsequent electrophoresis is carried out at 100 V for 30 min (optional 110 V for 15 min).
- Analyze the gel in UV light.

3.8 Expression of results

Once you have run DNA samples on an agarose gel and taken a picture, the picture can be saved for later on, to analyse the results and interpret. The main indication that in your sample certain *Vibrio* species were found - are the bands in the agarose gel of a certain length (base pair, Table 1). Important thing is to check if there is no band in the negative control. Few examples are provided further. Potentially enteropathogenic *Vibrio vulnificus* is detected (Figure 2), where vvhA is present in samples coded S1, S3, S4, S6, S7, S8, S10, S11, S13. S14, S15 but not detected in samples coded S2, S5, S9, S12. The presence of bacteria is indicated in a test proportion of x grams or x mL of environmental sample. As for *V. cholerae,* prVC gene is detected in samples S1, S2, S3, S4, except S5 (Figure 3).

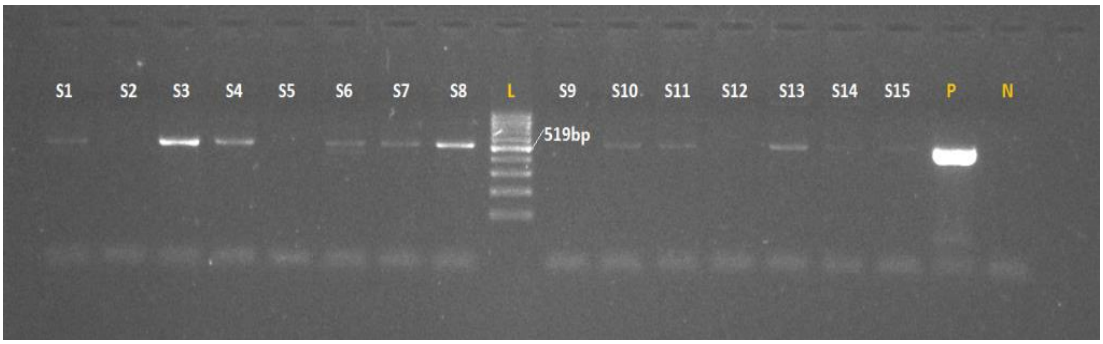

Figure 2. *V. vulnificus* vvhA gene presence/absence (519 bp) identification in an environmental sample (S). P – known positive, N-negative, L – 100 bp ladder.

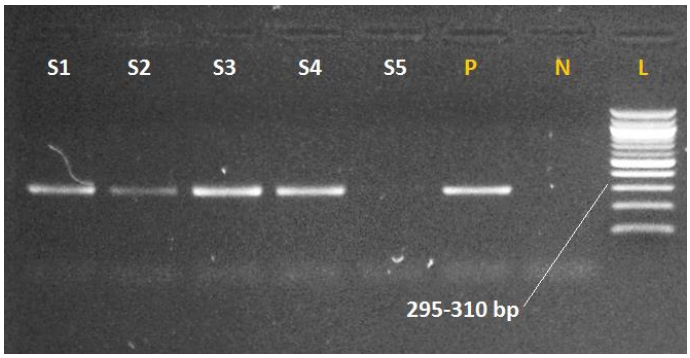

Figure 3. *V. cholerae* prVC gene presence/absence (295-310 bp) identification in an environmental sample (S). P – known positive, N-negative, L – 100 bp ladder.

References

Gyraite G, Kataržytė M, Overlingė D., et al (2020) Skip the dip—avoid the risk? Integrated microbiological water quality assessment in the south-eastern baltic sea coastal waters. Water (Switzerland) 12:1–22. https://doi.org/10.3390/w12113146

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McCormick A, Hoellein TJ, Mason SA, et al (2014) Microplastic is an abundant and distinct microbial habitat in an urban river. Environ Sci Technol 48:11863–11871. https://doi.org/10.1021/es503610r

Annexes

In order to investigate the relative abundance of potentially pathogenic *Vibrio* spp. we recommend using the Real-Time PCR method. However, this method is relatively expensive, and requires a better-equipped laboratory (i.e. Real-Time PCR cycler), as well as laboratory staff trained for such analysis. In case conditions allow performance of Real-Time PCR analysis on *Vibrio* spp. in association with plastic litter, then use the methodology described in Gyraite et al. (2020) and ISO 21872-1:2017 (Table 3).

Table 3. Species-specific primers for Real-Time PCR analysis.

	Primer sequences	**Base pairs (bp)**	**Resource**
Real-time PCR			
Vibrio vulnificus	vvha_F: 5'-GTTTATGGTGAGAACGGTGACA-3'		Campbell et al. 2003
	vvha_R: 5'-TTCTTTATCTAGGCCCCAAACTTG-3'		
	vvha_probe:(FAM)-CCG TTA ACC GAA CCA CCC GCA A-(TAMRA)		
Vibrio cholerae	Pvc-F groEL: 5'-GGTTATCGCTGCGGTAGAAG-3'	117	Fykse et al. 2012
	Pvc-R groEL: 5'-ATG ATG TTG CCC ACG CTA GA-3'		
	groEL-probe: (FAM)-CTGTCTGTACCTTGTGCCGATACTAAAGC-(BBQ)		