ESMIC

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

Report on the pathogenic organisms associated with plastic pollutants

(D. T1 .2. 2)

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1. Introduction

Plastic litter provides 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, previously found in the Lithuanian Baltic Sea coastal waters (G. Gyraitė et al., 2020), 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℃) and lower salinity (5-25 ppt) waters. Most *Vibrio* illnesses usually occur through the 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.

The aim of this deliverable was to test the method developed in the **Deliverable T1.1.3.** "*Methodology for fast screening of with plastic associated pathogenic microorganisms".* This method is based on a molecular technique and allows fast (to one day) identification of with the plastic-associated hazardous organisms to the level of presence/absence.

2. Methodology

Assessment of potentially pathogenic *Vibrio vulnificus* and *Vibrio cholerae* on plastic litter surfaces using polymerase chain reaction (PCR) assay (by conventional PCR method). As indicated in **Deliverable T1.1.3**, this method targets Vibrio-species specific genes *vvhA* for *V. vulnificus* and *prVC* for *V. cholerae*.

Short description of the methodology steps:

1. Using gloves collect plastic samples from the desired environment (water, sand, algae wrack) and put them into a sterile plastic bag (Figure 1). Keep samples in the refrigerator and carry them to the laboratory within 4 hours of collection.
2. 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. Extract genomic DNA from plastic samples using PowerWater® or PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. Store aliquots at −20°C for a short time and at −80°C for long time conservation.
4. Perform PCR and gel electrophoresis according to **Deliverable T1.1.3.**

In the summer of 2021, we also took environmental samples such as wrack from the sandy beach and the water, as well as water and sand samples for the comparison of *V. vulnificus* and *V. cholerae* presence on plastic samples.

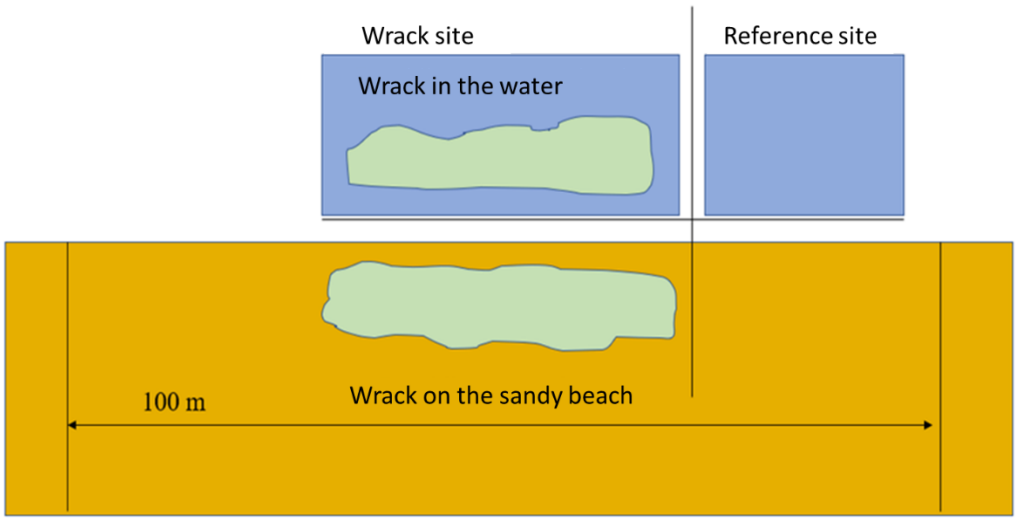

Figure 1. Sampling scheme: a visualization of beach wrack accumulation zone on the coast and in the water; and the reference site without wrack.

3. Results

Sample collections took place in the summer seasons of 2021 and 2022 at four beaches of the Lithuanian Baltic Sea: Šventoji, Palanga, Karklė, and Melnragė. In Melnrage we collected the majority of the plastic samples (n=41). In total, 14 samples were collected in Šventoji, two in Palanga, and none in Karklė (Figure 2).

Figure 2. Sampling map

In total, 57 plastic samples were collected at the four beaches in the summer seasons of 2021 and 2022. 20 plastic samples were collected from the wrack accumulation zone (16 from the wrack accumulation and plastic items floating in the water), while 21 – from the reference site on the sandy beach.

In total, *V. vulnificus vvhA* gene was found in 50.87% (n=29) of the samples, while *V. cholerae prVC* gene – in 38.59% (n=22). 62% of positive samples had both genes. *V. vulnificus* vvha gene was found in 50% of the plastic samples collected in the wrack on the beach, 75% from the plastic samples collected from the wrack in the water and 33.33% from the reference site at the sandy beach. The highest proportion of *V. vulnificus* and *V. cholerae* on the plastic was observed in July (75% and 83 %), less in August (57% and 42%).

V. vulnificus vvhA gene was found in 41.46% (n=17) of the total (n=41) plastic samples collected in Melnragė site. The highest percentage (72.72%) of the *Vibrio* positive samples was found on the plastic pieces collected from wrack in the water, while the lowest (20%) on the plastic from the reference site.

In total, 80% (n=4) of the plastic pieces collected in the wrack from the water in Šventoji showed positive for *V. vulnificus vvha* gene. In Palanga, two plastic pieces were taken and tested for vvhA gene presence, and both of them were positive (Table S 1).

V. cholerae prVC gene was found in 38.59% (n=22) of the total plastic samples. *V. cholerae-specific* gene prVC was found in 55% (n=11) of the plastic samples collected in the wrack accumulated on the sandy beach, while from the wrack floating in the water it was present only in 37.5% of the samples (n=6). In 23.80% (n=5) of the samples, the *V.cholerae prVC* gene was found on the plastic collected at the reference site of the sandy beach. *V. cholerae prVc* gene was found in 29.26% (n=12) of the total plastic samples collected in the Melnrage site. The highest percentage 46.66% (n=7) of positive samples were on plastics collected from the wrack on the beaches, while 27.27% (n=3) and 13.33% (n=2) from plastics in wrack from the water and reference site, respectively. Three out of four plastic samples collected from wrack on the beach were positive for *prVC.* In Palanga, two plastic pieces were taken and tested for prVC gene presence, and both of them were positive (Table S 1).

Almost two times higher percentage of positive *V. vulnificus* and *V. cholerae* samples was obtained from plastics collected at the wrack accumulation site compared to the reference site.

Table 1. V. vulnificus vvhA and V. cholerae prVC gene presence on the plastic samples from various environments: wrack on the beach and in the water and from the reference site at the beach (without wrack).

Plastic sample collected from:	**Total no. of samples (n)**	***V. vulnificus vvhA Presence (%)***	***V. cholerae prVC Presence (%)***
Wrack on the sandy beach	20	10 (50%)	11 (55%)
Wrack in the water	16	12 (75%)	6 (37.5%)
Reference site on the sandy beach	21	7 (33.33%)	5 (23.80%)

V. vulnificus and _V. cholerae_ tendencies in environmental samples.

The *vvha* gene of *V. vulnificus* was detected in all investigated beaches throughout the study, except for Melnragė beach in September (54.2% of all samples). *V. cholerae prVC* gene was detected on all beaches: Šventoji, Palanga and Karklė beaches were recorded in July. In August, it was observed in Šventoja and Melnragė. In September, it was recorded only in Melnragė (37.5% of all samples).

V. vulnificus mainly was found in water and sand samples collected at the wrack accumulation sites (88.9% and 40% of samples, respectively). The *V. vulnificus vvha* gene was detectable in 70% of wrack samples and only 30% of the sand samples collected at the reference site. *V. cholerae* was found most abundantly in water and sand samples at the wrack accumulation sites (55.6% and 40%, respectively) and detected in 60% of the wrack samples (Figure 3).

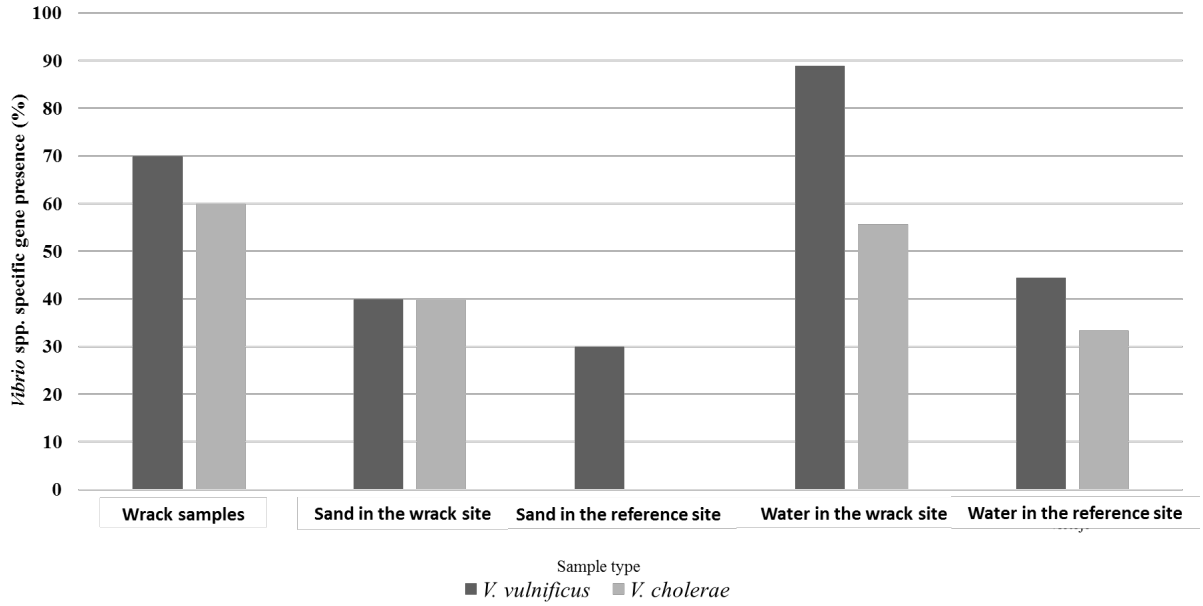

Figure 3. V. vulnificus vvhA and V. cholerae prVC presence in environmental samples (not on the plastic)

Comparison of *V. vulnificus vvhA* and *V. cholerae prVC* gene presence on plastic and in environmental samples

In four out of seven sampling campaigns performed in 2021, *V. vulnificus vvhA* gene was found on plastics when it also occurred in the environmental samples (either wrack, water, or sand). On two occasions in Melnrage, the *vvhA* gene was found in the water with floating wrack samples but not on plastics taken from the same environment. Once, *V. vulnificus* was obtained on the plastics from the water with floating water, but it was present neither in water nor wrack samples from the same spot (Table 2).

During four out of seven sampling campaigns performed in 2021, *V. cholerae prVC* gene was found on plastics when it also occurred in the environmental samples (either wrack, water or sand). On two different sampling events in Melnrage, the *prVC* gene were not found in an environmental sample nor on plastics. Once, *V. cholerae* was found in the wrack sample but not on plastics (Table 2).

Table 2. Comparison of V. vulnificus vvhA and V. cholerae prVC gene presence on the plastic samples and in the environmental samples

Date	Sampling site	Plastic from	vvhA +	prVC +	<--->	Environmental samples	vvhA +	prVC +
2021 07 07	Šventoji	wrack on the coast	+	+		wrack	+	+
2021 07 07	Šventoji	wrack in the water	+	+		water with floating wrack	+	+
2021 07 07	Šventoji	ref on the coast	+	+		sand at the ref site		
2021 08 27	Šventoji	wrack on the coast	+	+		wrack	+	+
2021 08 27	Šventoji	wrack in the water				water with floating wrack	+	+
2021 08 27	Šventoji	ref on the coast	+	+		sand at the ref site	+	
2021 06 18	Melnragė	wrack on the coast				wrack		
2021 06 18	Melnragė	wrack in the water				water with floating wrack	+	
2021 06 18	Melnragė	ref on the coast				sand at the ref site		
2021 08 10	Melnragė	wrack on the coast	+			wrack	+	+
2021 08 10	Melnragė	wrack in the water	+	+		water with floating wrack	+	+
2021 08 10	Melnragė	ref on the coast				sand at the ref site		
2021 09 16	Melnragė	wrack on the coast				wrack		+
2021 09 16	Melnragė	wrack in the water	+			water with floating wrack		
2021 09 16	Melnragė	ref on the coast				sand at the ref site		
2021 07 28	Palanga	wrack on the coast	+	+		wrack	+	+
2021 07 28	Palanga	ref on the coast	+	+		water with floating wrack		
2021 06 02	Melnragė	wrack on the coast				sand at the ref site		
2021 06 02	Melnragė	wrack in the water				water with floating wrack	+	
2021 06 02	Melnragė	ref on the coast				sand at the ref site		

Conclusions

1. Half of the plastic items harbored *V. vulnificus* genes, less often *V. cholera* genes and more than half of positive samples had both genes at once.
2. The highest proportion of vibrio-positive plastic was found in July and August when the water temperature reached 20-23° C.
3. The highest proportion of *V. vulnificus* positive plastic items were found in water affected by wrack, less in the wrack on the beach, and least on the clean beach. In the areas affected by the wrack, if *Vibrio* were found on plastic, they were also found in the environment. For the clean beaches, there were cases when *Vibrio* was found on plastic but not in the sand, thus suggesting that *Vibrio* or its DNA in unsuitable conditions such as dry sand can survive for longer periods if attached to the plastic.
4. Salinity and temperature are the main parameters shaping the presence of potentially pathogenic *Vibrio.* LAT-LIT coast is in similar salinity and temperature regime, thus, the situation described for the Lithuanian coast is relevant to the neighboring coast.

Recommendations

First of all, the municipalities should be aware that beaches on the Lithuanian and Latvian Baltic Sea coast are suitable for the presence of the potentially pathogenic *Vibrio* during the recreational season. Thus, municipalities should inform beachgoers about potential threats and avoidance measures related to the *Vibrio*.

Plastic is one of the environments where *Vibrio* might be found. The beach wrack supports the *Vibrio* bacterial survival on the plastics, but this is not the only environment where it might be found. Potentially pathogenic *Vibrio* species might be found in the water, in the wrack, or on the sand affected by the wrack. Thus, the wrack should be removed from the beach to improve water quality. If this is not possible, an effort should be made to inform the persons who collect the amber in the wrack about the measures that should be taken to avoid exposure to the potential pathogens (personal protective equipment e.g. with disposable gloves, hand disinfectant liquid, protections wounds from direct contact with the environment).

References

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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

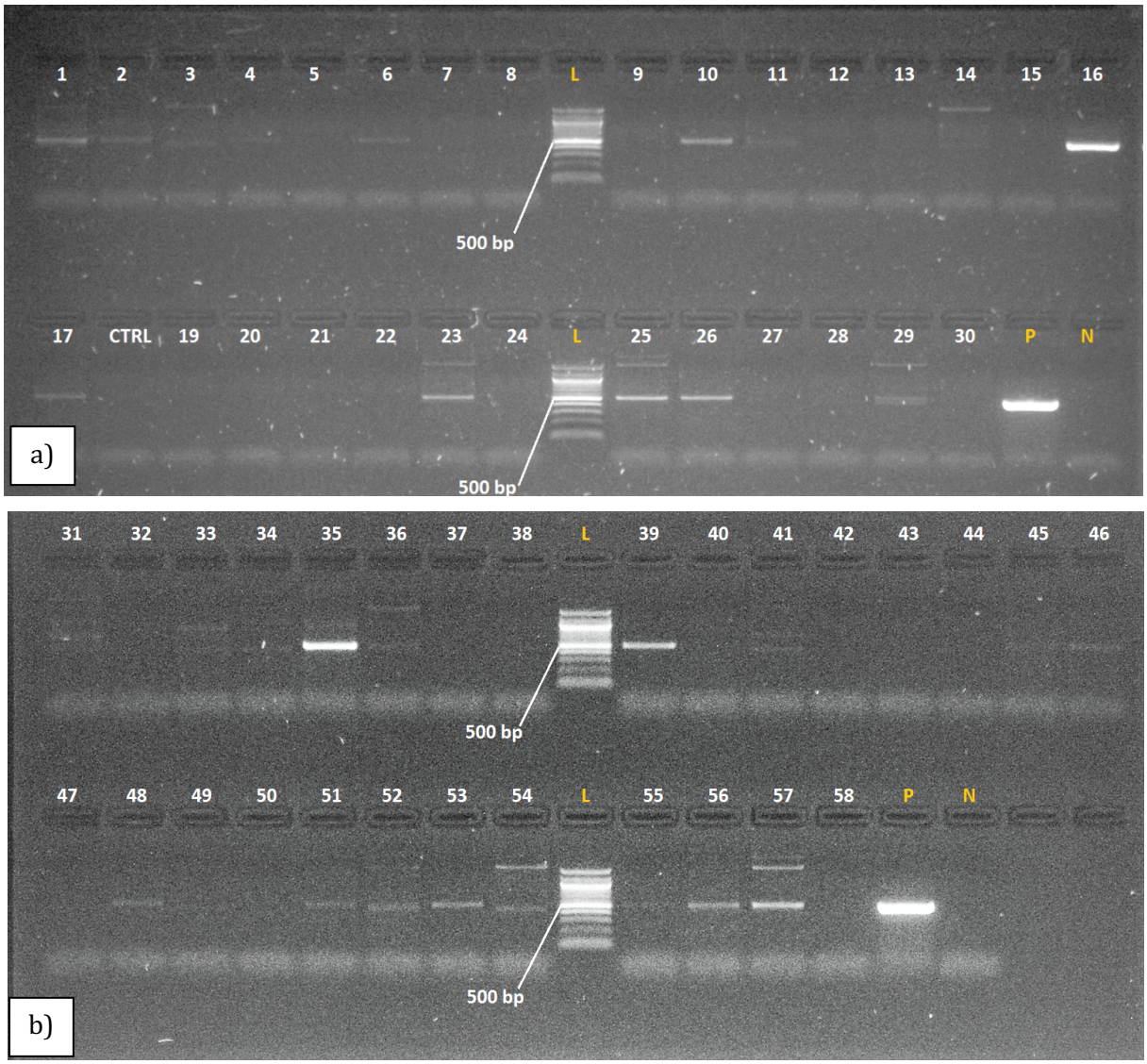

Figure S4. V. vulnificus vvhA gene presence/absence (519 bp) identification on plastic samples (1-58). P – known positive, N-negative, L – 100 bp ladder.

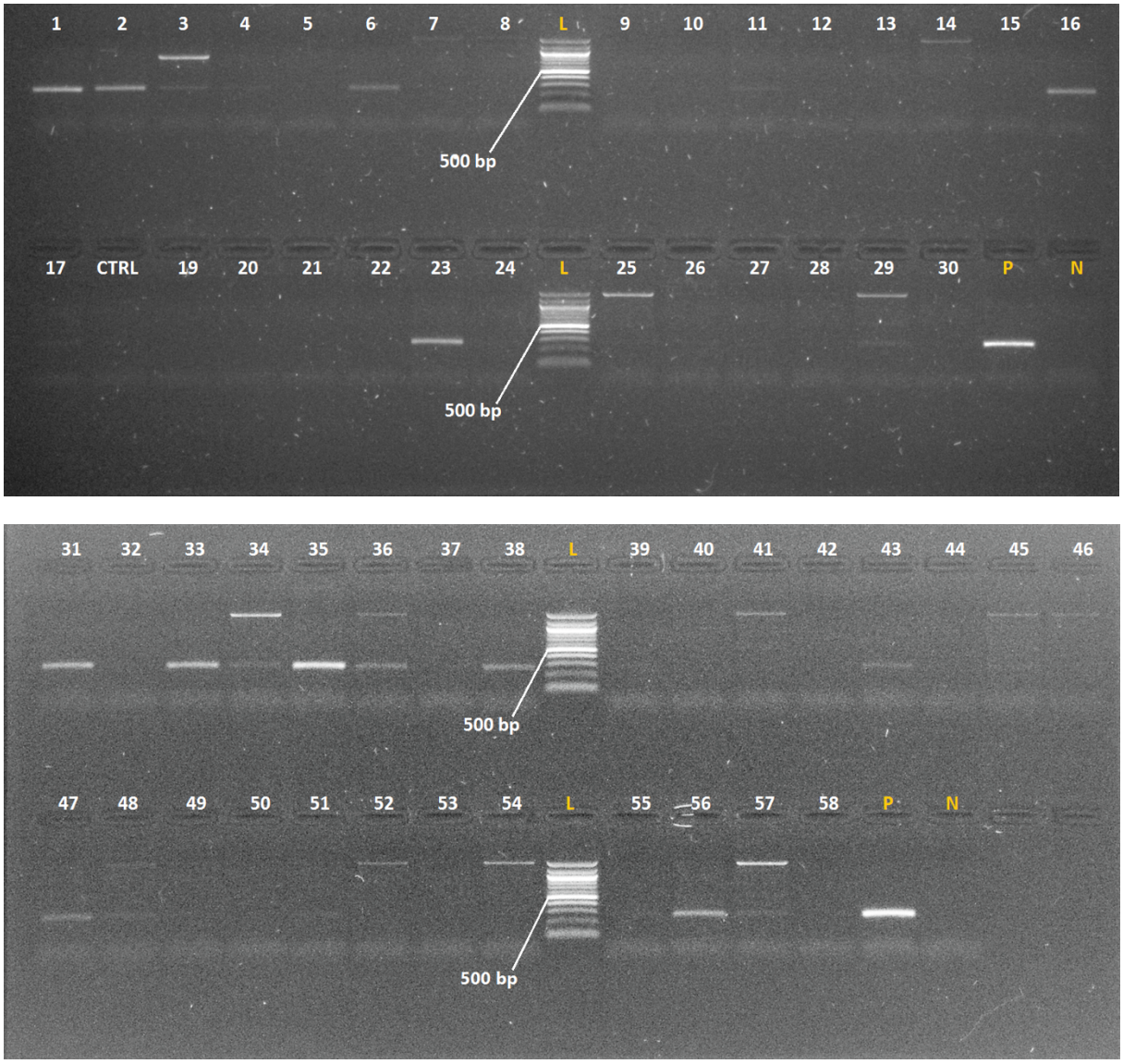

Figure S5. V. cholerae prVC gene presence/absence (295-310 bp) identification on plastic samples (1-58). P – known positive, N-negative, L – 100 bp ladder.

Table S 1. V. vulnificus vvhA and V. cholerae prVC gene presence on the plastic samples from various environments: wrack on the sandy beach and in the water and from the reference site at the sandy beach (without wrack).

Sample no.	Date	Sampling site	Plastic from	Weight (g) of plastic used for extraction	vvhA +	prVC +
1	2021 07 07	Šventoji	wrack on the coast	0,58	+	+
2	2021 07 07	Šventoji	wrack in the water	0,23	+	+
3	2021 07 07	Šventoji	ref on the coast	0,33	+	+
4	2021 08 27	Šventoji	wrack on the coast	0,52	+	+
5	2021 08 27	Šventoji	wrack in the water	0,43	-	-
6	2021 08 27	Šventoji	ref on the coast	0,55	+	+
7	2021 06 18	Melnragė	wrack on the coast	0,55	-	-
8	2021 06 18	Melnragė	wrack in the water	0,36	-	-
9	2021 06 18	Melnragė	ref on the coast	0,46	-	-
10	2021 08 10	Melnragė	wrack on the coast	0,39	+	-
11	2021 08 10	Melnragė	wrack in the water	0,39	+	+
12	2021 08 10	Melnragė	ref on the coast	0,46	-	-
13	2021 09 16	Melnragė	wrack on the coast	0,57	-	-
14	2021 09 16	Melnragė	wrack in the water	0,43	+	-
15	2021 09 16	Melnragė	ref on the coast	0,47	-	-
16	2021 07 28	Palanga	wrack on the coast	0,51	+	+
17	2021 07 28	Palanga	ref on the coast	0,44	+	+
CTRL	extraction control				-	-
19	2021 06 02	Melnragė	wrack on the coast	0,45	-	-
20	2021 06 02	Melnragė	wrack in the water	0,43	-	-
21	2021 06 02	Melnragė	ref on the coast	0,41	-	-
22	2022 08 01	Melnragė	wrack on the coast	0,43	-	-
23	2022 08 01	Melnragė	wrack in the water	0,46	+	+
24	2022 08 01	Melnragė	ref on the coast	0,54	-	-
25	2022 09 01	Melnragė	wrack on the coast	0,42	+	-
26	2022 09 01	Melnragė	wrack in the water	0,51	+	-
27	2022 09 01	Melnragė	ref on the coast	0,43	-	-
28	2022 08 10	Šventoji	wrack on the coast	0,47	-	-
29	2022 08 10	Šventoji	wrack in the water	0,50	+	+
30	2022 08 10	Šventoji	ref on the coast	0,48	-	-
31	2022 07 29	Melnragė	wrack on the coast	0,45	-	+
32	2022 07 29	Melnragė	ref on the coast	0,45	-	-
33	2022 07 30	Melnragė	wrack on the coast	0,46	+	+
34	2022 07 30	Melnragė	ref on the coast	0,5	+	+
35	2022 07 31	Melnragė	wrack on the coast	0,49	+	+
36	2022 07 31	Melnragė	wrack in the water	0,52	+	+
37	2022 07 31	Melnragė	ref on the coast	0,44	-	-
38	2022 08 02	Melnragė	wrack on the coast	0,49	-	+
39	2022 08 02	Melnragė	ref on the coast	0,46	+	-
40	2022 09 03	Melnragė	wrack on the coast	0,47	-	-
41	2022 09 03	Melnragė	wrack in the water	0,46	+	-

42	2022 09 03	Melnragė	ref on the coast	0,47	-	-
43	2022 09 02	Melnragė	wrack on the coast	0,47	-	+
44	2022 09 02	Melnragė	ref on the coast	0,55	-	-
45	2022 09 04	Melnragė	wrack on the coast	0,45	-	-
46	2022 09 04	Melnragė	wrack in the water	0,52	+	-
47	2022 09 04	Melnragė	ref on the coast	0,47	-	+
48	2022 09 05	Melnragė	wrack on the coast	0,44	+	+
49	2022 09 05	Melnragė	wrack in the water	0,46	-	-
50	2022 09 05	Melnragė	ref on the coast	0,32	-	-
51	2022 09 06	Melnragė	wrack on the coast	0,28	+	+
52	2022 09 06	Melnragė	wrack in the water	0,44	+	-
53	2022 09 06	Melnragė	ref on the coast	0,18	+	-
54	2022 08 09	Šventoji	wrack in the water	0,21	+	-
55	2022 08 09	Šventoji	ref on the coast	0,41	+	-
56	2022 08 11	Šventoji	wrack on the coast	0,48	+	+
57	2022 08 11	Šventoji	wrack in the water	0,46	+	+
58	2022 08 11	Šventoji	ref on the coast	0,45	-	-

Table S 2. V. vulnificus vvhA (519 bp) and V. cholerae prVC gene (295-310 bp) presence/absence identification in environmental samples.

Sample no.	Date	Sampling site	Sample type	*vvhA +*	*prVC +*
2	2021 06 02	Melnragė	Water samples (wrack)	+	-
5	2021 06 02	Melnragė	Water samples (ref)	-	-
8	2021 06 02	Melnragė	Wrack sample	-	-
11	2021 06 02	Melnragė	Sand samples (wrack)	-	-
14	2021 06 02	Melnragė	Sand samples (ref)	-	-
16	2021 06 18	Melnragė	Water samples (wrack)	+	-
17	2021 06 18	Melnragė	Water samples (ref)	-	-
18	2021 06 18	Melnragė	Wrack sample	+	-
19	2021 06 18	Melnragė	Sand samples (wrack)	-	-
20	2021 06 18	Melnragė	Sand samples (ref)	+	-
21	2021 07 07	Šventoji	Water samples (wrack)	+	+
22	2021 07 07	Šventoji	Water samples (ref)	-	-
23	2021 07 07	Šventoji	Wrack sample	+	+
24	2021 07 07	Šventoji	Sand samples (wrack)	+	+
25	2021 07 07	Šventoji	Sand samples (ref)	-	-
26	2021 07 21	Karklė	Water samples (wrack)	+	+
27	2021 07 21	Karklė	Water samples (ref)	+	+
28	2021 07 21	Karklė	Wrack sample	+	+
29	2021 07 21	Karklė	Sand samples (wrack)	+	+
30	2021 07 21	Karklė	Sand samples (ref)	-	-
31	2021 07 29	Palanga	Water samples (wrack)	+	+
32	2021 07 29	Palanga	Water samples (ref)	+	+
33	2021 07 29	Palanga	Wrack sample	+	+
34	2021 07 29	Palanga	Sand samples (wrack)	+	+
35	2021 07 29	Palanga	Sand samples (ref)	-	-
36	2021 08 10	Melnragė	Water samples (wrack)	+	+
37	2021 08 10	Melnragė	Water samples (ref)	+	+
38	2021 08 10	Melnragė	Wrack sample	+	+
39	2021 08 10	Melnragė	Sand samples (wrack)	-	+
40	2021 08 10	Melnragė	Sand samples (ref)	-	-
41	2021 08 27	Šventoji	Water samples (wrack)	+	+
42	2021 08 27	Šventoji	Water samples (ref)	+	-
43	2021 08 27	Šventoji	Wrack sample	+	+
44	2021 08 27	Šventoji	Sand samples (wrack)	-	-
45	2021 08 27	Šventoji	Sand samples (ref)	+	-
46	2021 09 16	Melnragė	Water samples (wrack)	-	-
47	2021 09 16	Melnragė	Water samples (ref)	-	-

48	2021 09 16	Melnragė	Wrack sample	-	+
49	2021 09 16	Melnragė	Sand samples (wrack)	-	-
50	2021 09 16	Melnragė	Sand samples (ref)	-	-
51	2021 09 17	Karklė	Wrack sample	-	-
52	2021 09 17	Karklė	Sand samples (wrack)	-	-
53	2021 09 17	Karklė	Sand samples (ref)	-	-
54	2021 09 17	Šventoji	Water samples (wrack)	+	-
55	2021 09 17	Šventoji	Water samples (ref)	-	-
56	2021 09 17	Šventoji	Wrack sample	+	-
57	2021 09 17	Šventoji	Sand samples (wrack)	+	-
58	2021 09 17	Šventoji	Sand samples (ref)	+	-