



National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Laboratory analysis of environmental samples taken following the reported release of live poliovirus

RIVM Letter report 2015-0032
E. Duizer



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Colophon

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This investigation has been performed by order and for the account of VWS, within the framework of poliovirus exclusion

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Analyse poliomonsters na lozingsincident

Op 2 september 2014 heeft het bedrijf GlaxoSmithKline (GSK), dat vaccins produceert, in België door een menselijke fout 45 liter geconcentreerd poliovirus op het riool geloosd. Via de nabijgelegen rioolwaterzuivering is dit water onder andere in de rivier De Laan terechtgekomen, en vervolgens in de Westerschelde. Er is geen poliovirus aangetoond in de beschikbare monsters. Ook is het poliovirus niet in de onderzochte bevolking verspreid.

Dit blijkt uit onderzoek van het door de WHO erkende referentielaboratorium voor polio van het RIVM. Het onderzoek hier verricht in opdracht van de WHO, omdat België niet over een laboratorium beschikt dat hiervoor geaccrediteerd is. De WHO streeft ernaar om polio uit te roeien. Het onderzoek is ingegeven door het ophanden zijnde oesterseizoen (begin oktober) en het hoge aantal niet-gevaccineerde bewoners van sommige Zeeuwse gemeenten.

Tussen 2 en 18 september zijn monsters bij de rioolwaterzuivering (rwzi) te Rosières verzameld door personeel van GSK en de KU Leuven. Ook zijn monsters van sediment uit de bezinkingsbak van deze rwzi en slibmonsters uit de rivier De Laan genomen. Verder zijn in enkele gemeenten met een lage vaccinatiegraad tussen 30 september en 7 november rioolwatermonsters verzameld.

Daarnaast is berekend dat de lozing vanaf 20 september in de Westerschelde zou kunnen stromen. Aangezien schelpdieren zich voeden door water te filteren, kunnen aanwezige verontreinigingen, zoals poliovirussen, zich ophopen in hun maag-darmkanaal. Op 24 september en 3 en 28 oktober zijn mosselen verzameld in het oostelijke deel van de Westerschelde, daar waar de te verwachte concentratie poliovirussen het hoogst was.

Kernwoorden: poliovirus, lozingsincident, België

Synopsis

Analysis of poliosamples after accidental release

September 2nd, 2014, the vaccine production facility of GlaxoSmithKline (GSK), Rixensart, Belgium, accidentally released 45 liters concentrated poliovirus into the sewage system. After the sewage treatment plant (STP) this water was released into the river Lasne, and subsequently into the Westerschelde. No poliovirus was detected in the samples available and no poliovirus spreading was found in two communities in Zeeland with low vaccination coverage.

This was shown a study by the WHO accredited reference laboratory for polio at the RIVM. This research was conducted in The Netherlands as required by the WHO since Belgium does not have a WHO accredited laboratory for the analysis of poliovirus in environmental samples. WHO aims to eradicate polio. The research is motivated by the impending oyster season (early October) and the high number of unvaccinated residents of some municipalities in Zeeland.

Between 2 and 18 September samples at the sewage treatment (STP) in Rosières collected by staff from GSK and KU Leuven. Also, samples of sediment from the settler of the STP and sludge samples from the river Lasne were analyzed. Furthermore, community sewage samples were collected in some municipalities with low vaccination coverage between 30 September and 7 November.

In addition, it is calculated that from 20 September the discharge could flow in the Westerschelde. Since shellfish feed by filtering water, the presence of impurities such as polio viruses, can accumulate in their gastrointestinal tract. On 24 September and 3 and 28 October, mussels collected in the eastern part of the Westerschelde, where the expected concentration polioviruses was highest, were collected and analyzed.

Keywords: poliovirus, accidental release, Belgium

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Summary

Following the reported release of 10^{13} live poliovirus particles into the environment on September 2, 2014, fourteen wastewater samples, four silt samples and five sediment samples were collected by GSK, and KU Leuven staff. Between September 10 and October 10, these samples were sent to the WHO Specialized Reference Laboratory for polio at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands, where they were analyzed for the presence of poliovirus according to WHO protocols. In addition to these samples, three batches of fifty mussels were collected from the Westerschelde and nineteen sewage samples (1-litre grab samples) were collected between September 24 and November 10, twice a week in Krabbendijke and Stavenisse, two villages in Zeeland with less than 80% polio vaccination coverage.

No infectious poliovirus was detected using culture methods and no poliovirus RNA was detected using molecular methods in any of the samples tested. We therefore conclude that the reported release of 10^{13} infectious poliovirus particles has not resulted in detectable levels of poliovirus in any of the samples from Belgium and the Netherlands taken after the incident and that silent transmission of poliovirus in two Dutch communities with low polio vaccination coverage could not be demonstrated.

1 Introduction

On September 6, 2014, the Belgium authorities reported to ECDC the accidental release of 45 litres of concentrated live polio virus solution on September 2 at Rixensart, Belgium by the pharmaceutical company GlaxoSmithKline (GSK). The concentrated solution was estimated to contain 1013 infectious polioviruses type 3 particles (Saukett strain). The solution was released into the sewage system and conducted directly to a water-treatment plant in Rosières and subsequently, following standard treatment, into the river Lasne. The river Lasne is affluent of the river Dyle which is affluent of the Escaut/Scheldt river which flows into the Westerschelde (the Netherlands) and subsequently into the North Sea.

The Belgium's High Council of Public Health stated that the risk of infection for the population exposed to the contaminated water is extremely low due to the high level of dilution and the high polio vaccination coverage (95%) in Belgium. However, the vaccination coverage in some Dutch communities along the Westerschelde is less than 90% and the inactivated polio vaccination (IPV) offered in Belgium and the Netherlands does protect against disease but not against infection. Silent circulation of the virus upon infection of persons can therefore not be excluded and unvaccinated persons are at risk for disease. Additionally, the poliovirus can be concentrated in shellfish due to filter feeding of shellfish. The shellfish harvesting season was about to start in the Netherlands the first week of October 2014.



Figure 1: Map of part of Belgium and the Netherlands. A= Antwerpen, Belgium, B = GSK, Rixensart, Belgium, C = mussels sampling site, Kloosterzande, the Netherlands, D = sewage sampling site Krabbendijke, the Netherlands, E = sewage sampling site Stavenisse, the Netherlands. The blue line represents the water flow from Rixensart to Antwerpen.

Immediately following the reported release, wastewater and silt samples were collected by GSK and KU Leuven. Non-concentrated water samples were analyzed by GSK and KU Leuven and no poliovirus was detected. The method of analyzes by GSK is not known. KU Leuven performed the WHO RT-PCR protocol according to Nix et al.,(2006) for the molecular detection of enterovirus RNA on non-concentrated samples. Under these conditions, the detection limit of this method is approximately 10^4 PCR Detectable Units/L.

In the absence of a WHO accredited Polio Laboratory for detection of polioviruses in environmental samples in Belgium, it was decided to send all samples available to the WHO Specialized Reference Laboratory for polio in the Netherlands (National Institute for Public Health and the Environment, Centre for Infectious Diseases Control, Laboratory for Infectious Diseases and Screening) for analysis with WHO- accredited methods.

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Materials & Methods

Samples and samplings sites

Samples were collected by GSK or KU Leuven staff and sent to RIVM for analysis after storage at 4°C for variable times (1 night to 6 weeks) between collection and analysis at the RIVM. The following samples were analyzed: water from the sewage system at the GSK plant, influent and effluent from the sewage treatment plant (STP) in Rosières, sediment from the quiescent basin of the STP and silt at the entry or exit from the STP at different dates (table 1). Several samples were tested twice since they were received by the RIVM at different days. Sediment was removed daily from the STP. Sediment samples from the first days after the incident (September 2- 5) were not available for analysis. Samples 12-23 are replicates of 4 silt samples (8-11) collected on 9/9/2014.

Mussels were collected in the Westerschelde east of Kruiningen (Kloosterzande) at September 24, and October 3 and 28. Directly after collection the mussels were transported to RIVM and processed on the same day mainly as described in the international standard for the detection of viruses in foods (Anonymous, 2012). Each sampling date, 50 mussels were collected and the digestive tracts of 5 mussels were pooled for further processing, independent of the weight.

Between September and November 10, sewage samples (1 L grab samples) were collected twice weekly in the villages of Krabbendijke and Stavenisse, both communities with a vaccination coverage for poliovirus <80%.

Sample concentration and sample extraction

The water samples were concentrated 10 to 200 times to a volume of 1-3 mL by ultrafiltration using Amicon ultrafiltration membranes PM10 in Amicon stirred ultrafiltration cells at 50-75 psi pressure, at 4°C. When the target volume of 1-3 mL was reached, the pressure was released and the membrane rinsed with a 2 mL pipet to resuspend the viruses. The concentrated fraction was collected in 15 mL tubes and stored until processing at -20°C. The sediment and silt samples were treated as described by Skrabber et al., 2009.

Virus culture for detection of infectious polioviruses

The concentrated and pretreated samples were extracted with chloroform (30% v/v, to remove bacteria and enveloped viruses) and subsequently inoculated (3x100 µL) on 3-7 day old L20b cells in roller tubes for detection of infectious polioviruses. L20b is a mouse cell line expressing the gene for the human cellular receptor for poliovirus. These cells support isolation of polioviruses 1, 2 and 3 and only a limited number of other human viruses. The samples from the last shipment (samples 28 to 36), and the sewage samples from Krabbendijke en Stavenisse were also inoculated on Rd and Ht-29 cells (3x100 µL for each cell type). These cell lines support isolation of wide range of human enteric viruses including most enteroviruses, thus the polioviruses as well (Duizer et al., 2013). The inoculated tubes were incubated at 37 °C and 3 rpm. Cytopathic effect (CPE) was monitored by light microscopy every working day following inoculation for at least 7 days.

RNA extraction and PCR for detection of poliovirus RNA

Viral RNA was extracted from 200 µL concentrated water samples, extracted sediment or cell cultures showing CPE using the MagNAPure LC total nucleic acid isolation kit with a MagNAPure LC instrument as described before (van der Sanden et al., 2008). EV RNA was amplified (PCR 1 in Table 1) using the semi nested RT-PCR as described before (Nix et al., 2006) with a cultured stock of Enterovirus 71 as positive control. Additionally, a subset of samples, including all samples positive in the snRT-PCR, was analyzed by Intravacc using a RT-PCR specific for poliovirus type 3 Saukett strains G/H (PCR 2 in table 1) according to Nijst et al., (2013).

Estimation of poliovirus concentration in the STP effluent and sediment
For the estimation of the virus concentration in the effluent of the STP we used data provided by GSK, the commissioner of the STP and data and models from: Schijven & Hassanizadeh, Schijven et al., Lodder and de Roda Husman. GSK reported to have released 1013 infectious polioviruses, the STP effluent flow was 530 000 L/h and the virus load was reduced between 0.7 and 2 log₁₀ by the STP, assuming primary and secondary treatment only.

The effluent samples analyzed were stored for 10-13 days (samples 2 and 4) or approximately 40 days (samples 31, 33 and 35) prior to analysis. Based on literature data on poliovirus inactivation (Bertrand et al., 2012) we can assume a maximum of 1log₁₀ reduction for samples 2 and 4, and 2log₁₀ reduction for samples 31, 33 and 35 between collection and analysis. The estimated limits of detection for effluent samples at the time of analysis were calculated by conversion of the exclusion limit to polioviruses per litre and multiplying by the expected level of inactivation.

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Results

Virus culture for detection of infectious polioviruses

No infectious poliovirus was detected using culture on L20b cells. All data are presented in tables 1 and 2. Samples 5, 6 and 7 (4a, STP, quiescent basin: this was one grab sample that was split in a water phase and solid phase in our lab) were cytotoxic for the L20b cells and required a second passage. In Table 1 the concentration factors, volumes analyzed and detection limit of the culture method (1 infectious poliovirus per 300 µL pretreated sample) were used to estimate the limit of detection in the original samples (interpretation column) and thus the volumes or mass for which the presence of infectious poliovirus was excluded.

Several RD and Ht-29 cell cultures showed CPE after inoculation with samples 28-36 and the mussel samples and ECHOviruses type 3,11 and 25 were typed in the cultured suspensions.

PCR for detection of poliovirus RNA in the Belgium sewage and river samples

No poliovirus RNA was detected using the snEV-RT-PCR or the poliovirus type 3 Saukett strain specific RT-PCR. In the molecular methods small volumes of sample are analyzed (20 µL of pretreated samples for the snEV-RT-PCR and 2 µL for the poliovirus 3 specific PCR) resulting in poliovirus RNA exclusion in only about 7% of the volumes (mass) estimated for exclusion by cell culture methods.

In 19 samples of the samples from Belgium that were analysed by the snEV-RT-PCR, a non-polio-enterovirus was detected. ECHOvirus type 3, 9 and 11(twice) and a Coxsackievirus type A6 could be identified. In 14 of the 19 snEV-RT-PCR positive samples the enterovirus could not be sequenced.

Estimation of poliovirus concentration in the STP effluent and sediment
The estimated virus concentration in the effluent of the STP was: 8×10^3 to 170×10^3 polioviruses/L for September 3; 3×10^3 to 57×10^3 for September 4; 1×10^3 to 20×10^3 for September 5; and 300 to 6×10^3 for September 6. The estimated limits of detection for effluent samples at the time of analysis was calculated to be 500 infectious poliovirus in samples 2 (September 3) and 4 (September 5); 400 for sample 31 (September 2-3) and 700 for samples 33 (September 4) and 35 (September 5).

Estimates for poliovirus concentrations expected in the effluent were made assuming first and secondary sewage treatment only. If however also membrane ultrafiltration was performed on 100% of the treated water, an additional 4log₁₀ reduction would have been achieved by the STP, and consequently poliovirus concentrations in the effluent would peak at September 3rd at 6 per liter and decrease after that. A more likely scenario with approximately 50% of the water treated by membrane ultrafiltration would result in only a 0.3log₁₀ reduction; this is neglected in the calculations.

The poliovirus concentration on September 3 in the quiescent basin was estimated to be from 8×10^3 to 170×10^3 per liter water; well above the

limit of detection of 700 polioviruses per liter at the date of analysis (samples 28 and 29).

Samples collected at September 6 (samples 6 and 7) were estimated to contain $>10^2$ infectious polioviruses per gram sediment at the time of sampling and consequently >10 PV per gram sediment at the time of analysis.

Sewage water surveillance

All sewage samples collected in Stavenisse and Krabbendijke were

negative for poliovirus but positive for other enteroviruses (table 3).

ECHOvirus type 18 was found in 4 out of 9 samples from Krabbedijke while ECHOvirus type 20 was found in 6 out of 10 from Stavenisse.

Other enteroviruses found were Coxsackieviruses type A2 and type B5 and ECHOviruses types 3, 6 and 11. Polioviruses were not detected in sewage samples either, taken in the same period for regular surveillance for exclusion of poliovirus circulation in the Dutch Bible belt (data not shown).

4

Conclusions

No infectious poliovirus was detected using culture methods and no poliovirus RNA was detected using molecular methods in any of the samples tested. Based on all calculations, after release of 10¹³ infectious polioviruses into the sewage system and to the STP, at least the STP basin samples (sample numbers 5, 6, 7 and 28,29) and the effluent samples from September 3, 4 and 5 (sample numbers 2, 4, 31, 33, 35) were expected to contain more viruses than the detection limits. We therefore conclude that the reported release of 10¹³ infectious poliovirus particles has not resulted in detectable levels of poliovirus in any of the samples from Belgium and the Netherlands taken after the incident. We also did not detect any poliovirus in the 3 batches of 50 mussels collected. No signs for poliovirus circulation in the Dutch Bible belt were found. The reported release of poliovirus type 3 Saukett strain by GSK did not result in poliovirus circulation in the Netherlands in the period September 2nd – November 7th, 2014.

5

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nr	Sample	Type of sample	Sampling date	date received at RIVM	quantity	treatment	Results			non-polio-enterovirus result	Interpretation
						Cult on L20B cells	PCR 1	PCR 2			
1	influent	water	3/9/2014	16/9/2014	250 mL	conc to < 1 mL	negative	negative	<1 PV per 20 mL water	positive	positive
2	effluent	water	3/9/2014	16/9/2014	250 mL	conc to < 1 mL	negative	negative	<1 PV per 20 mL water	negative	negative
3	influent	water	5/9/2014	16/9/2014	250 mL	conc to < 1 mL	negative	negative	<1 PV per 20 mL water	negative	negative
4	effluent	water	5/9/2014	16/9/2014	250 mL	conc to < 1 mL	negative	negative	<1 PV per 20 mL water	negative	negative
5	4a, STP, quiescent basin	water	6/9/2014	16/9/2014	0.5 L	conc to < 5 mL	negative	negative	<1 PV per 150 mL water	negative	negative
6	4a, STP, quiescent basin	sediment				sediment extracted	negative	negative	<1 PV per gram sediment	negative	negative
7	4a, STP, quiescent basin	sediment				sediment extracted	negative	negative	<1 PV per gram sediment	negative	negative
8	STP entry 1	silt	9/9/2014	10/9/2014	silt extracted	negative	negative	negative	<1 PV per gram silt	negative	negative
9	STP entry 2	silt	9/9/2014	10/9/2014	silt extracted	negative	negative	negative	<1 PV per gram silt	positive	positive
10	STP Exit 1	silt	9/9/2014	10/9/2014	silt extracted	negative	positive	positive	<1 PV per gram silt	positive	positive
11	STP Exit 2	silt	9/9/2014	10/9/2014	silt extracted	negative	positive	positive	<1 PV per gram silt	positive	positive
12	STP entry 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
13	STP entry 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
14	STP Exit 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
15	STP Exit 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
16	STP entry 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
17	STP entry 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
18	STP Exit 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	positive	positive
19	STP Exit 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	positive	positive
20	STP entry 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
21	STP entry 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	negative	negative
22	STP Exit 1	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	positive	positive
23	STP Exit 2	silt	9/9/2014	10/9/2014	silt extracted	negative	ND	ND	<1 PV per gram silt	positive	positive
24	STP, quiescent basin , sediment	sediment	18/9/2014	19/9/2014	2 L	sediment extracted	negative	ND	<1 PV per gram sediment	positive	positive
25	STP sediment	sediment	18/9/2015	19/9/2015	2 L	sediment extracted	negative	ND	<1 PV per gram sediment	positive	positive
26	STP sediment	sediment	18/9/2016	19/9/2016	2 L	sediment extracted	negative	ND	<1 PV per gram sediment	negative	negative
27	STP sediment	sediment	18/9/2017	19/9/2017	2 L	sediment extracted	negative	ND	<1 PV per gram sediment	negative	negative
28	STP, quiescent basin 1, water	water	3/9/2014	10/10/2014	500 mL	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
29	STP, quiescent basin 2, water	water	3/9/2014	10/10/2014	500	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
30	influent#	water	2/9/2014	10/10/2014	900	conc to < 3 mL	negative	negative	<1 PV per 300 mL water	positive	positive
31	effluent#	water	2/9/2014	10/10/2014	900	conc to < 3 mL	negative	negative	<1 PV per 300 mL water	positive	positive
32	influent	water	4/9/2014	10/10/2014	500	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
33	effluent	water	4/9/2014	10/10/2014	500	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
34	influent	water	5/9/2014	10/10/2014	400	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
35	effluent	water	5/9/2014	10/10/2014	400	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive
36	GSK plant#	water	2/9/2014	10/10/2014	500	conc to < 3 mL	negative	negative	<1 PV per 150 mL water	positive	positive

Table 1: Sample list and analytical results from samples collected by GSK and KU Leuven.

stp = sewage treatment plant, PCR 1 = semi nested RT-PCR, WHO protocol for enteroviruses (Nix et al., 2006), performed by RIVM, IDS-VIR, WHO specialized reference laboratory for polio, PCR 2 = RT-PCR specific for poliovirus type 3 Saukett strains G/H (Nijst et al., 2013), performed by Intravacc. silt = sediment taken at the entry or exit of the STP, sediment is taken form the basin of STP, ND = not determined

: samples 30, 31 and 36 were pooled samples collected over a 24 h period (2/9/2014 - 3/9/2014).

Sampling date	Results	Cult on L20B cells	PCR 1	PCR 2	poliovirus result	non-polio-enterovirus result	Interpretation		enterovirus type
24/9/2014	Negative		positive	negative	negative	positive	< 1 PV per mdt		NPEV
24/9/2014	Negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	Negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	Negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	Negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
24/9/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
3/10/2014	negative		positive	negative	negative	positive	< 1 PV per mdt		NPEV
3/10/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
3/10/2014	negative		positive	negative	negative	positive	< 1 PV per mdt		ECHOvirus type 25
3/10/2014	negative		positive	negative	negative	positive	< 1 PV per mdt		NPEV
3/10/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
3/10/2014	negative		positive	negative	negative	positive	< 1 PV per mdt		ECHOvirus type 25
3/10/2014	negative		positive	negative	negative	positive	< 1 PV per mdt		NPEV
3/10/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
3/10/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
3/10/2014	negative		negative	negative	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		positive	ND	negative	positive	< 1 PV per mdt		ECHOvirus type 11
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		
28/10/2014	negative		negative	ND	negative	negative	< 1 PV per mdt		

Table 2: Sample list and analytical results from mussel samples collected from the Westerschelde near Kloosterzande between September 24 and October 28, 2014. All samples represent digestive tracts of 5 mussels pooled. PCR 1 = semi nested RT-PCR, WHO protocol for enteroviruses (Nix et al., 2006), performed by RIVM, IDS-VIR, WHO specialized reference laboratory for polio. PCR 2 = RT-PCR specific for poliovirus type 3 Saukett strains G/H (Nijst et al., 2013), performed by Intravacc.

date	location	poliovirus	enterovirus		EV-sn-RT-	typering
		L20B	RD	Ht-29	PCR	
30-Sep	Krabbendijk	neg	neg	ND	pos	Coxs A2
2-Oct	Krabbendijk	neg	pos	pos	pos	ECHO 18
2-Oct	Stavenisse	neg	pos	pos	pos	ECHO 20
7-Oct	Krabbendijk	neg	pos	pos	pos	ECHO 18
7-Oct	Stavenisse	neg	pos	pos	pos	ECHO 20
10-Oct	Krabbendijk	neg	pos	pos	pos	ECHO 6
10-Oct	Stavenisse	neg	pos	pos	pos	ECHO 20
14-Oct	Krabbendijk	neg	pos	neg	pos	ECHO 18
14-Oct	Stavenisse	neg	pos	pos	pos	ECHO 20
16-Oct	Krabbendijk	neg	pos	neg	pos	ECHO 18
16-Oct	Stavenisse	neg	pos	pos	pos	NPEV
28-Oct	Krabbendijk	neg	pos	pos	pos	Coxs B5
28-Oct	Stavenisse	neg	pos	pos	pos	ECHO 11 mixed
30-Oct	Krabbendijk	neg	pos	pos	pos	Coxs B5
30-Oct	Stavenisse	neg	pos	pos	pos	ECHO 11
4-Nov	Krabbendijk	neg	pos	pos	pos	ECHO 3
4-Nov	Stavenisse	neg	pos	pos	pos	ECHO 20
7-Nov	Krabbendijk	neg	pos	pos	pos	NPEV
7-Nov	Stavenisse	neg	pos	pos	pos	ECHO 20

Table 3: Sample list and analytical results from sewage samples collected in Stavenisse en Krabbendijk. Coxs A2 = coxsackievirus type A2, ECHO # = ECHOvirus type #, NPEV = non-polio Enterovirus.

