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Quantitative levels of norovirus and hepatitis A virus in bivalve molluscs collected along the food chain in the Netherlands, 2013–2017

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ABSTRACT

Contamination of bivalve molluscs with viruses is well recognized as a food safety risk. A microbiological criterion for norovirus (NoV) and hepatitis A virus (HAV) in shellfish, however, does not exist in the European Union currently. The aim of this study was to evaluate the contamination levels of these viruses for fluctuation over a long period (2013–2017) in oyster ($n = 266$) and mussel samples ($n = 490$) using a method based on ISO/TS 15216-1: 2013. Samples were taken at different points in the food chain, either directly post-harvest, at Dutch dispatch centers or in retail stores, from September until March of each year. Altogether, 53.1% of the mussel and 31.6% of the oyster samples tested positive for NoV RNA. Simultaneous presence of NoV GI and GII RNA was observed in 31.6% of mussel and 10.2% of oyster samples. Contamination levels in NoV positive mussel samples collected post-harvest from B-areas were significantly higher than in those collected post-harvest from A-areas, or at dispatch centers or retail stores. Levels in oysters from dispatch were significantly lower than those collected in retail stores. Ready for sale mussels and oysters contained 2.04 and 1.76 mean $\log_{10}$ transformed NoV genome copies/gram (gc/g), respectively. GII levels were at a constant level in ready for sale mussels throughout all sampling periods in the study. This seemed to be true for oysters as well. HAV RNA was detected in only one of the tested mussel samples ($n = 392$) (typed HAV 1A) and in none of the tested oyster samples ($n = 228$). Critical evaluation of NoV and HAV levels in shellfish can be of help for risk assessment and risk management actions.

1. Introduction

Outbreaks of gastro-enteritis and hepatitis A have been associated with the consumption of bivalve molluscs, like oysters and mussels (Bellou et al., 2013). Bivalve molluscs are filter feeding aquatic organisms that bioaccumulate norovirus (NoV) and hepatitis A virus (HAV) particles from surrounding water that may be fecally contaminated by sewage wastewater overflow, direct release of sewage (Doyle et al., 2004; Guillois-Bécel et al., 2009; Maalouf et al., 2010a; Simmons et al., 2001), leakage from septic tanks (Stafford et al., 1997) or defecation directly into production areas (Berg et al., 2000; Kohn et al., 1995; McDonnell, 2011; McIntyre et al., 2012). This is a public health risk as oysters and mussels are often consumed raw or undercooked.

Current hygienic measures for the production and post-harvest treatment of bivalves molluscs in Europe are based on monitoring and reduction of the fecal indicator organism *E. coli*. By European regulation (Regulation (EC) No 627/2019, article 53, 54 and 55, 2019), shellfish production areas are labeled as Class A, B or C according to the levels of *E. coli* present in shellfish meat. For Class A production areas the most probable number (MPN) per 100 g of flesh and intravalvular fluid should be <230 *E. coli* for at least 80% of the samples and <700 *E. coli* for the remaining 20% of the samples, whereas for Class B areas, this MPN should be <4600 *E. coli* for at least 90% of the samples, and <46,000 *E. coli* for the remaining 10% of the samples. For Class C all samples should have a MPN below 46,000 *E. coli*. Class A derived shellfish are fit for direct and raw consumption, whereas shellfish harvested from class B production areas must be depurated or relayed before putting on the market for raw consumption. Alternatively, molluscs should be heat-treated (Regulation (EC) No 853/2004 of the European Parliament and the Council, 2004). Depuration entails submitting the shellfish to prolonged exposure with clean seawater, commonly treated with chlorine, ultraviolet light or ozone. This process has been shown very effective for indicator organism *E. coli* (Power and Collins, 1990). The rate of reduction of viruses in shellfish is, however, considerably less and

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requires extended depuration times. Meta-analysis of published depuration experiments suggests that the process requires more than nine days to achieve a tenfold reduction in NoV and HAV load (McLeod et al., 2017), while exposure to even a minute dose of NoV, possibly below 100 genome copies, may lead to illness (EFSA, 2012; Teunis et al., 2008). Though the risk of infection and of acute symptoms may vary between individuals and NoV strains (Teunis et al., 2020). A systematic review over 32 years revealed 359 shellfish related virus outbreaks world-wide, of which 83.7% involved NoV and 12.8% HAV (Bellou et al., 2013). Despite the risk of viral infection associated with the consumption of raw and undercooked shellfish, a food safety criterion for NoV in shellfish does not exist at this time. The recently reported European baseline survey on the presence of NoV RNA in oysters (EFSA, 2019) reported an estimated NoV prevalence of 34.5% (CI: 30.1–39.1%) in oysters in EU production areas and an estimated prevalence of 10.9% (CI: 8.2–14.4%) in oysters from EU dispatch centers, though a high percentage of these bivalves were contaminated with only low quantities of norovirus. Viral analyses of bivalve molluscs collected along the food chain is part of a monitoring program of the Netherlands Food and Consumer Product Safety Authority (NVWA). As approximately three-quarters of the NoV cases and outbreaks are occurring in cool months (Ahmed et al., 2013; Kroneman et al., 2008), and the most relevant months for high NoV levels in bivalve molluscs are during autumn and winter (Lowther et al., 2012b), sampling for viral analyses in this monitoring takes place from September until March. Besides oysters, the program also included mussels that are consumed frequently in the Netherlands and are heavily traded internationally. The aim of this study was to evaluate the levels of NoV genogroup I (GI) and genogroup II (GII) and HAV RNA in both types of animals over a five-year period (2013–2017) following ISO/TS 15216-1:2013 with minor modifications. This allowed a multi-year evaluation of fluctuations of the viral load in shellfish sampled in the Netherlands. Moreover, samples were taken along the production chain to determine viral contamination at several steps in the process. For this, oysters and mussels grown in class A or B production areas were sampled directly after being brought ashore (henceforth called 'post-harvest') or were collected from the sorting and packaging line at dispatch centers or at point of sale in retail stores. Critical evaluation of NoV and HAV levels in shellfish can be of help for risk assessment and risk management actions.

2. Material and methods

2.1. Sampling

Sampling of bivalve molluscs for viral analyses was performed from 2013 to 2017 by Inspectors of the Netherlands Food and Consumer Product Safety Authority (NVWA) to collect monitoring data as part of the NVWA monitoring program. Sampling numbers and locations varied upon availability of inspectors and NoV epidemiology (sampling from September until March, information in Supplementary Table 1).

Bivalve molluscs were collected at Dutch dispatch centers from sorting and packaging lines ($n = 247$ mussel and $n = 211$ oyster samples.) Consumer-packed products were also collected at retail stores in the Netherlands throughout this period ($n = 124$ mussel and $n = 42$ oyster samples). In addition, in 2015 and 2016, upon arrival in the Netherlands, the NVWA collected samples randomly from post-harvest batches (bags of 10–20 metric tonnes in weight) of unprocessed bivalve molluscs harvested in A ($n = 38$ mussel, $n = 8$ oyster batches) or B ($n = 81$ mussel, $n = 5$ oyster batches) class areas in United Kingdom, Ireland, Germany, France or Denmark. The samples from these bags were collected before further processing by Dutch companies. Each oyster sample consisted of at least 10 animals, the large majority of samples being *Crassostrea gigas*. Each mussel sample consisted of at least 30 animals; all were *Mytilus edulis*. Samples were kept refrigerated during transport until delivery at the Laboratory of Wageningen Food Safety Research. 540 batches were sampled in duplicate. One of the

Table 1
Presence of NoV RNA in mussel and oyster samples sampled along the food chain.

		No.	NoV positive[a]			
			GI (%)	GII (%)	GI + GII (%)	Total (%)
NoV qualitative analyses in mussels						
Post-harvest (A)	2015	14	7 (50)	4 (28.6)	3 (21.4)	8 (57.1)
	2016	24	4 (16.7)	5 (20.8)	1 (4.2)	8 (33.3)
	Sum:	*38*	*11 (28.9)*	*9 (23.7)*	*4 (10.5)*	*16 (42.1)*
Post-harvest (B)	2015	39	31 (79.5)	32 (82.1)	29 (74.4)	34 (87.2)
	2016	42	27 (64.3)	39 (92.9)	27 (64.3)	39 (92.9)
	Sum:	*81*	*58 (71.6)*	*71 (87.7)*	*56 (69.1)*	*73 (90.1)*
Dispatch centers	2013	60	19 (31.7)	20 (33.3)	16 (26.7)	23 (38.3)
	2014	74	25 (33.8)	18 (24.3)	14 (18.9)	29 (39.2)
	2015	44	20 (45.5)	19 (43.2)	18 (40.9)	21 (47.7)
	2016	31	12 (38.7)	19 (61.3)	12 (38.7)	19 (61.3)
	2017	38	8 (21.1)	23 (60.5)	7 (18.4)	23 (60.5)
	Sum:	*247*	*84 (34)*	*99 (40.1)*	*67 (27.1)*	*115 (46.6)*
Retail	2013	38	4 (10.5)	0 (0)	0 (0)	4 (10.5)
	2015	33	11 (33.3)	13 (39.4)	9 (27.3)	15 (45.5)
	2016	30	10 (33.3)	21 (70)	9 (30)	21 (70)
	2017	23	11 (47.8)	14 (60.9)	10 (43.5)	16 (69.6)
	Sum:	*124*	*36 (29)*	*48 (38.7)*	*28 (22.6)*	*56 (45.2)*
Total		*490*	*189 (38.6)*	*227 (46.3)*	*155 (31.6)*	*260 (53.1)*
NoV qualitative analyses in oysters						
Post-harvest (A)	2015	5	2 (40)	0 (0)	0 (0)	2 (40)
	2016	3	0 (0)	1 (33.3)	0 (0)	1 (33.3)
	Sum:	*8*	*2 (25)*	*1 (12.5)*	*0 (0)*	*3 (37.5)*
Post-harvest (B)	2015	2	2 (100)	1 (50)	1 (50)	2 (100)
	2016	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)
	Sum:	*5*	*3 (60)*	*2 (40)*	*2 (40)*	*3 (60)*
Dispatch centers	2013	38	8 (21.1)	5 (13.2)	2 (5.3)	11 (28.9)
	2014	66	8 (12.1)	12 (18.2)	7 (10.6)	13 (19.7)
	2015	32	9 (28.1)	1 (3.1)	1 (3.1)	9 (28.1)
	2016	43	12 (27.9)	23 (53.5)	8 (18.6)	27 (62.8)
	2017	32	1 (3.1)	6 (18.8)	0 (0)	7 (21.9)
	Sum:	*211*	*38 (18)*	*47 (22.3)*	*18 (8.5)*	*67 (31.8)*
Retail	2016	17	2 (11.8)	3 (17.6)	2 (11.8)	3 (17.6)
	2017	25	5 (20)	8 (32)	5 (20)	8 (32)
	Sum:	*42*	*7 (16.7)*	*11 (26.2)*	*7 (16.7)*	*11 (26.2)*
Total		*266*	*50 (18.8)*	*61 (22.9)*	*27 (10.2)*	*84 (31.6)*

[a] Categories GI pos and GII pos include all samples that tested GI and GII positive, respectively, irrespective whether RNA of one or both genogroups was present. Category GI + GII pos includes all samples that tested positive for both genogroups. Category Total includes all samples that tested positive for any NoV RNA.

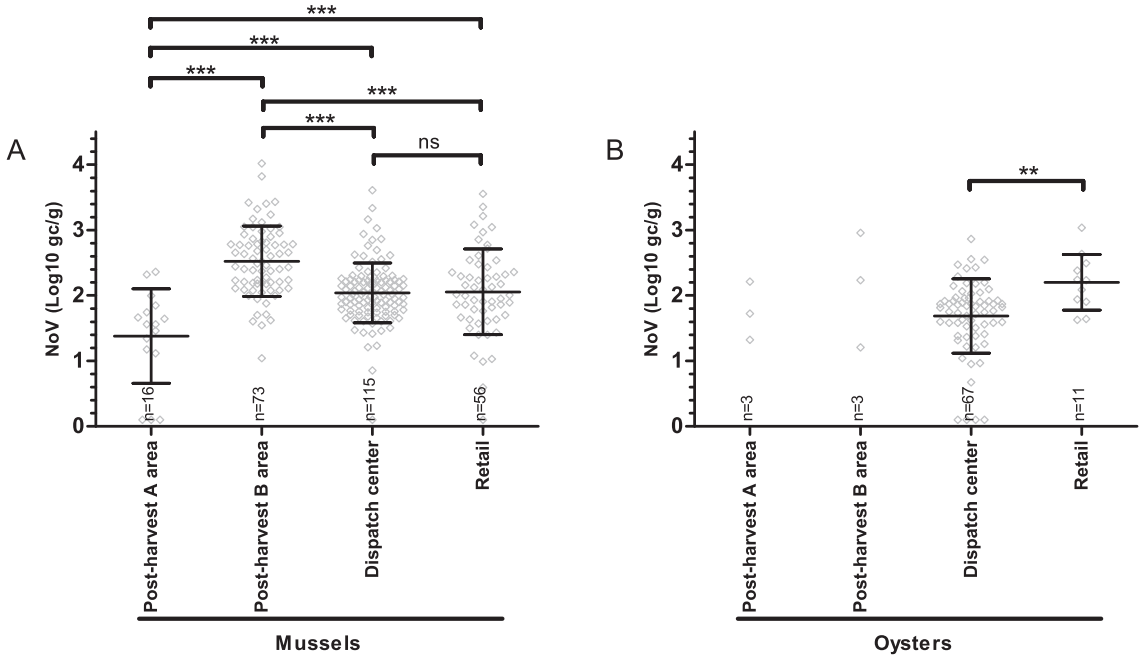

Fig. 1. NoV contamination levels in mussel (A) and oyster (B) samples.
Level for each positive sample is depicted as a dot; population summaries are depicted in plots using mean ± standard deviation; pairwise significance levels are depicted as **($p < .005$), ***($p < .0005$) or ns (no significant difference); n = number of positive samples.

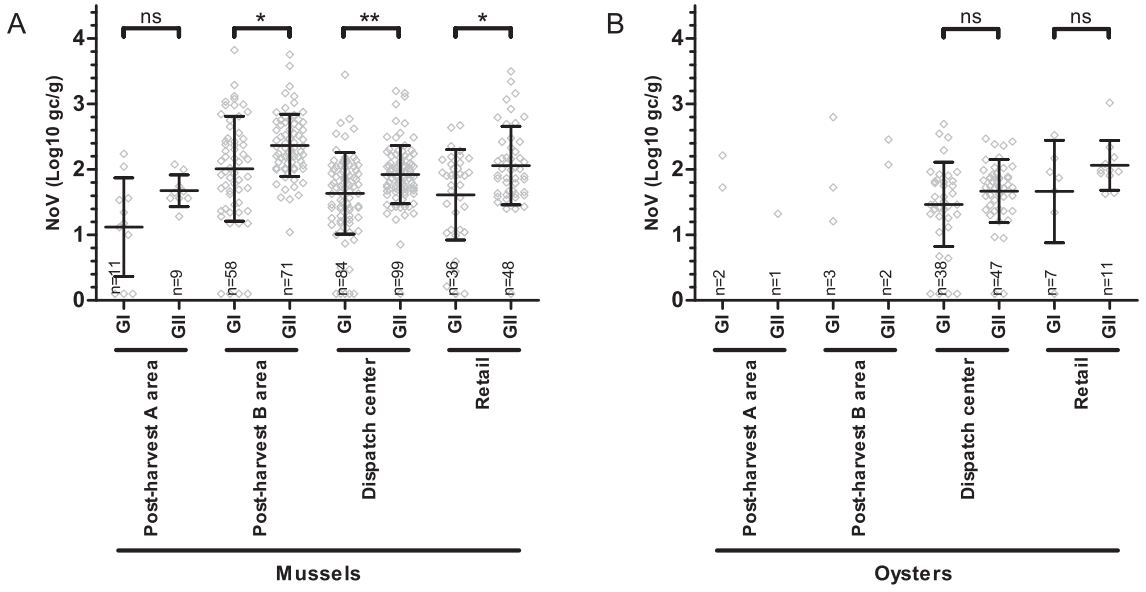

Fig. 2. NoV GI and NoV GII contamination levels in mussel (A) and oyster (B) samples.
Level for each positive sample is depicted as a dot; population summaries are depicted in plots using mean ± standard deviation; pairwise significance levels are depicted as *($p < .05$), **($p < .005$) or ns (no significant difference), n = number of positive samples.

duplicates was stored frozen at −20 °C until viral analyses, the other was stored refrigerated until testing within 36 h of sampling for microbiological quality by means of the *E. coli* most probable number (MPN) method according to ISO 16649-3.

2.2. Sample extraction

All samples were prepared and extracted according to ISO/TS 15216-1:2013, with minor modification. In brief, a subsample of 10 or more individual animals was shucked and the digestive tissue was isolated by dissection and finely chopped with a razor blade. Two grams of the chopped tissues were incubated with 10 µl of feline calicivirus (FCV) (2×10^3 TCID50) (kindly provided by E. Duizer, RIVM, the Netherlands) as process control virus for 20 min at room temperature, a minor modification of the ISO/TS 15216–1:2013, to allow attachment of the virus to the matrix. Subsequently, 2 ml of proteinase K solution (30 U/mg; Sigma) was added to the tissue and the mixture was incubated on a thermoshaker for 1 h at 37 °C (200 rpm), followed by incubation at 60 °C for 15 min, and clarification at 3000 x g for 5 min at 4 °C, deviating from the current ISO 15216-1:2017, as no specification of temperature was prescribed in ISO/TS 15216-1:2013. The volume of the supernatant, the proteinase K extract, was recorded, collected and stored at −20 °C until

nucleic acid extraction.

2.3. Nucleic acid extraction

For nucleic acid extraction, 500 µl of the proteinase K extract was added to 2 ml of Nuclisense lysis buffer (BioMérieux) and subsequently extraction was performed using the Nuclisense Magnetic extraction kit according the manufactures instructions. Nucleic acids were eluted in 100 µl elution buffer included in the kit.

2.4. Detection and quantification of NoV and HAV RNA

RNA of NoV GI, NoV GII and HAV was detected by RT-qPCRs using oligonucleotides as suggested in ISO/TS 15216-1:2013. The oligonucleotides used were QNIF4, NV1LCR and NVGG1p for NoV GI (Da Silva et al., 2007; Svraka et al., 2007), QNIF2, COG2R and QNIFS for NoV GII (Loisy et al., 2005; Kageyama et al., 2003), HAV68, HAV240 and HAV150 for HAV (Costafreda et al., 2006). Oligonucleotides used for the detection of process control virus FCV were FCV-F, FCV-R, and FCV-P for FCV (Lowther et al., 2008). All one-step RT-qPCR reactions were performed after in-house optimization for the CFX96 platform (BioRad). The RNA Ultrasense one-step qRT-PCR system kit (ThermoFisher) was used with 5 µl of nucleic acid preparation in a total reaction volume of 25 µl.

For quantification, each sample was run against a serial dilution (10^1 to 10^5 genome copies/µl) of a linearized plasmid containing the amplification fragment of the target virus (plasmids with NoV GI and GII sequence were kindly provided by J. Lowther, Cefas, UK, and plasmid with HAV sequence was kindly provided by A. Bosch, Univ. Barcelona, Spain). Samples were considered positive when amplification plots of the real time signals showed an S-shaped curve. Results for individual samples in genome copies per gram (gc/g) were $\log_{10}$ transformed for parametric statistical analysis, descriptive statistics and visualization. Concentrations less than 1.25 gc/g were set to 0.1 $\log_{10}$ gc/g.

2.5. Quality assurance controls

Viral extraction efficiency was calculated in ratio to the FCV process control virus that was added before extraction. Virus recovery was ≥1% for all samples included in this study. Each series of virus extractions consisted of negative extraction control samples in between each set of three samples that were run through all stages of the analytical process. Water controls and positive target RNA template controls were included in each PCR run. Each sample was also tested for PCR inhibition using ssRNA standard in an external reaction. None of the samples showed inhibition compared to water controls at a delta Cq threshold of more than 2.

2.6. Determination of limit of detection and limit of quantification

The limits of detection (LOD95) and quantification (LOQ) for NoV GI, NoV GII and HAV RNA in oysters were determined as described in the guidelines (CEFAS, 2016) as prepared for the EFSA baseline study (EFSA, 2019). The LOD95 was determined at 18 and 61 genome copies per gram (gc/g) for NoV GI.2 and GII.4 (fecal samples kindly provided by H. Vennema, RIVM, the Netherlands) and 53 gc/g for HAV (ATCC VR-2092). The LOQ was determined at 30, 61 and 135 gc/g, respectively. Dot plots and descriptive statistics include all quantitative data, also those below the LOD95 as indicative measurements to avoid an artificial increase of the mean.

2.7. Statistical analysis

Statistical comparison was performed using unpaired two-tailed *t*-tests or in the case of multiple comparison using one-way ANOVA with Bonferroni correction. Population summaries are depicted using mean and standard deviation of the $\log_{10}$ transformed data. Statistical analysis was performed for data included in Figs. 1, 2 and 3 where population size $n \geq 7$. Significance levels are depicted as *($p < .05$), **($p < .005$) or ***($p < .0005$).

3. Results

3.1. Detection of NoV GI and GII RNA in mussel and oyster samples

In total, 490 mussel and 266 oyster samples were collected along the food chain and analyzed for the presence of NoV RNA. The presence of NoV GI and/or NoV GII RNA in these shellfish are summarized in Table 1. Over the duration of the study 53.1% of the mussel samples tested positive for NoV RNA with 31.6% of all samples showing simultaneous presence of both NoV GI as well as NoV GII. In oysters, NoV RNA was detected in 31.6% of the samples with simultaneous presence of NoV GI and GII RNA in 10.2% of samples. The percentage of mussel samples that tested positive for NoV GI and/or GII RNA was significantly

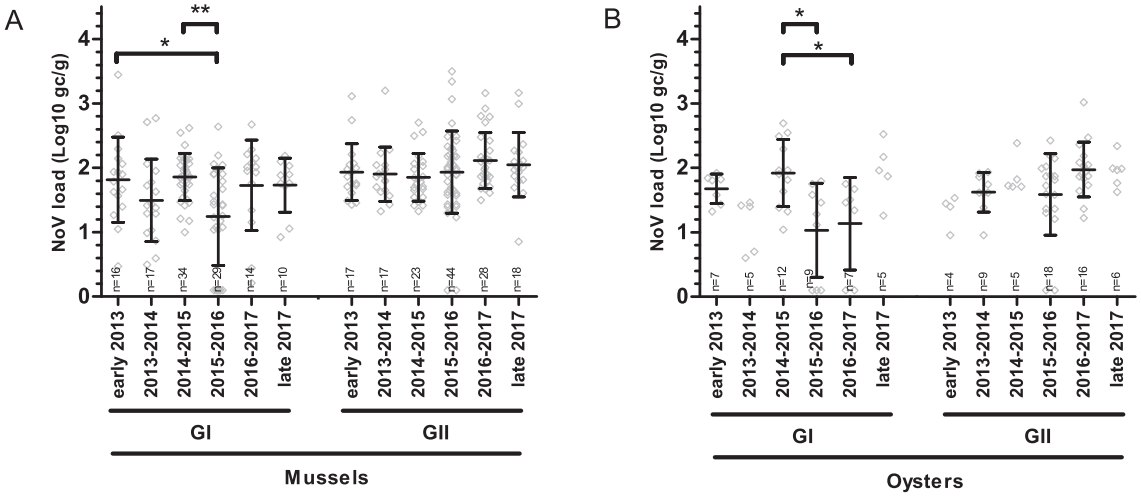

Fig. 3. NoV GI and GII contamination levels in mussel (A) and oyster (B) samples grouped per yearly NoV winter season.
Level for each positive sample is depicted as a dot; population summaries are depicted in plots using mean ± standard deviation. All pairwise comparisons where $n \geq 7$ are performed within genogroup for mussels and oysters, lack of pairwise significant difference not indicated. Significance levels are depicted as *($p < .05$) or **($p < .005$), n = number of positive samples.

higher for undepurated post-harvest class B samples than for samples that were ready for sale, collected either at dispatch centers or in retail stores ($p < .0005$). Compared to oyster samples, a significantly higher fraction of post-harvest mussel samples from class B areas as well as dispatch center and retail stage mussel samples was contaminated with either one or both of the NoV genogroup viruses ($p < .05$). The proportion of NoV GII contaminated mussels sampled at the dispatch center and retail level seemed to increase throughout the years that were sampled.

3.2. Levels of NoV contamination in mussels and oysters

For a better understanding of the total NoV contamination in the samples, quantitative analyses were performed. The NoV GI and GII contamination levels varied greatly in individual samples, ranging from non-detectable level to over 4000 genome copies per gram of digestive tissue (gc/g). Copy numbers were $\log_{10}$ transformed prior to analysis (Fig. 1, Supplementary Table 2). For mussels, the highest levels of NoV contamination were detected in post-harvest samples from class B areas. The mean $\log_{10}$ contamination level in these samples (2.52 ± 0.54 $\log_{10}$ gc/g) was significantly ($p < .0005$) higher than the mean $\log_{10}$ level in post-harvest samples taken from A-classified areas (1.38 ± 0.72 $\log_{10}$ gc/g), as well as those observed in samples collected at dispatch centers (2.04 ± 0.46 mean $\log_{10}$ gc/g) and retail stores (2.06 ± 0.65 mean $\log_{10}$ gc/g) ($p < .0005$). Furthermore, the mean $\log_{10}$ contamination level in mussels collected post-harvest from class A areas was significantly lower than in batches collected post-harvest from class B areas, at the dispatch center and retail level. Performing these comparisons for samples collected in the years 2015–2016 only yielded the same statistical outcome (data not shown). The quantitative findings mirror the trend seen in the qualitative results for mussels (Table 1) with a large proportion of mussels sampled post-harvest from class B areas being contaminated compared to the other sampling locations.

The majority of oyster samples was collected at dispatch centers and retail shops. Contrary to the observation in mussels, the mean $\log_{10}$ contamination level in oysters sampled at the dispatch center (1.69 ± 0.57 mean $\log_{10}$ gc/g) was significantly different from those collected in retail stores (2.20 ± 0.43 mean $\log_{10}$ gc/g) (Fig. 1B). The small number of post-harvest oyster samples in the study did not allow for further statistical analyses.

Analytical quality parameters were comparable between the two types of shellfish, with virus recovery at 68% (95% CI: 61–74) for mussels and 58% (95% CI: 51–64) for oysters. The overall mean $\log_{10}$ for NoV RNA in mussels was significantly higher compared to oysters at dispatch center ($p < .0005$) but similar to oysters at retail stores ($p = .48$).

3.3. Comparison between GI and GII contamination levels

NoV GI and NoV GII were depicted separately to demonstrate the relative contribution of each genogroup to the contamination level in the samples (Fig. 2, Supplementary Table 3). In mussels, the mean $\log_{10}$ level of NoV GII contamination was significantly higher than that observed for NoV GI when sampled post-harvest from B-classified areas ($p < .05$) (2.01 ± 0.80 for GI, 2.37 ± 0.47 for GII), at dispatch centers ($p < .005$) (1.63 ± 0.62 for GI, 1.92 ± 0.44 for GII) and in retail stores ($p < .05$) (1.61 ± 0.69 for GI, 2.06 ± 0.60 for GII), but not for mussel samples collected post-harvest from A-classified areas (1.12 ± 0.75 for GI, 1.67 ± 0.24 for GII) (Fig. 2A).

In contrast to mussels, no significant differences were found between the levels of NoV GI and GII in oyster samples collected at the dispatch center (1.47 ± 0.64 for GI, 1.67 ± 0.48 for GII) or in retail stores (1.62 ± 0.78 for GI, 2.06 ± 0.38 for GII). The other sampling locations did not allow for statistical comparison due to the limited numbers of NoV positive oysters.

3.4. Stable presence of NoV GII in ready for sale bivalve molluscs over time

Year-to-year fluctuations of viral levels in ready for sale products were investigated (Supplementary Fig. 1). To this end, data of dispatch center and retail samples were added together, as no industrial process is applied to the dispatch samples before sale. Furthermore, rather than following the calendar year, samples collected from September until March were grouped, as NoV epidemiology has been described with a peak in the autumn and winter months (Ahmed et al., 2013). In this way four complete sets (2013–2014; 2014–2015, 2015–2016 and 2016–2017) and two incomplete sets (January–March 2013 and September–December 2017) were created for NoV GI and GII contamination levels throughout the food chain (Fig. 3; Supplementary Fig. 1). For statistical significance, all cross-comparisons were made within the genogroups for either mollusc species, with the exception of some years for the oyster category due to a limited sample size ($n < 7$). This analysis revealed remarkedly consistent levels for NoV GII in bivalve molluscs on the market throughout the periods that were sampled, with mean $\log_{10}$ of 1.96 ± 0.50 gc/g for mussels and mean $\log_{10}$ of 1.74 ± 0.49 gc/g for oysters. The GI contamination load in mussels was slightly lower in the period of 2015–2016 compared to categories 2014–2015 and early 2013. In oysters, the GI contamination load in the autumn/winter of 2014–2015 was slightly increased compared to 2015–2016 as well as 2016–2017.

3.5. Detection of HAV RNA in bivalve molluscs

A total of 228 oyster and 392 mussel samples (all samples from 2014 to 2017) were tested for the presence of HAV RNA. Only one mussel sample tested positive (0.3%) and none of the oyster samples tested positive. The HAV RNA containing mussel sample had been collected post-harvest from a class B area located in close vicinity to a densely populated area in Western Europe in February 2015. The HAV contamination level in these post-harvest mussels was quantified at 3.70 $\log_{10}$ gc/g, whereas levels for NoV GI and NoV GII were 3.82 $\log_{10}$ gc/g and 3.58 $\log_{10}$ gc/g, respectively. Despite this highly viral contamination, the MPN measurement for *E. coli* was at a moderate level of 130 per 100 g. The HAV strain (VWA-M15-50) was sequenced using the HAVnet typing protocol (https://www.rivm.nl/documenten/typing-protocol-havnet-vp1p2a) and typed as HAV 1A (Kroneman et al., 2011). In HAVnet database (up to March 2020), only one case with onset of illness in February/March 2015 matched for 99.8% (459/460 nucleotides) with the VWA-M15–50 stain (GenBank Acc. MT677938), but unfortunately no information was available whether the case had consumed bivalve molluscs (pers. comm. Dr. J. Enkelmann and Dr. J. Wenzel).

3.6. Correlation NoV with E. coli most probable number

540 samples were subjected to both viral as well as microbiological analyses. The *E. coli* most probable number in a sample correlated poorly with total NoV levels in bivalves from post-harvest class B samples (*pearson's* $R^2 < 0.1$ at $p = .6$). Dispatch center and retail level samples contained very low numbers of viable *E. coli* (Supplementary Table 5), although these samples often still were contaminated with NoV (Fig. 1).

4. Discussion

In the present study quantitative levels of NoV RNA have been assessed in mussels and oysters collected along the food chain in the Netherlands. To date, this is the first multi-year study to determine the level of NoV contamination in Western European oysters as well as mussels including samples taken post-harvest, at dispatch centers and in retail stores. The detection and quantification method used in the study according to ISO/TS 15216-1:2013 was unchanged during the study and was applied under accreditation of the Dutch Accreditation Council,

allowing to study fluctuations of NoV contamination over time. This revealed that the level of GII contamination in the samples was remarkedly constant (Fig. 3), despite the year-to-year variation in number of NoV GII outbreaks (Allen et al., 2014; Lopman et al., 2003). For assessment of the risk associated with the consumption of raw and undercooked shellfish, quantitative analyses provide more detailed insight than qualitative measurements and could be a selection tool for risk management actions, like informing industries or searching for causes for incidental high contamination or control thereof. As microbiological criteria for NoV in bivalve molluscs have not been set, regulatory actions are limited.

In the Netherlands, oysters and mussels are both cultured as well as imported from surrounding European harvesting areas for consumption and for export. Compared to the earlier report(s) on prevalence of NoV contamination in bivalve molluscs in the Netherlands (Boxman et al., 2006; Lodder-Verschoor et al., 2005), the present study is much more comprehensive in terms of sample size and over a much broader sampling period. Moreover, the applied method was based on ISO/TS 15216-1:2013 for quantitative analyses of NoV GI and NoV GII. The latter allows for better direct comparison to other or future prevalence studies. Although most data was collected from consumer ready samples, this study additionally incorporates oysters and mussels directly after harvest.

For the purpose of comparison of quantitative NoV findings in shellfish, studies were selected for samples with defined sampling and viral analyses according to either ISO/TS 15216-1:2013 or ISO 15216-1:2017. In two studies from the UK, geometric mean levels were 121 gc/g in 2012 (Lowther et al., 2012a) in oysters from UK production areas and 87 gc/g in oysters collected in retail in UK in winter months (Lowther et al., 2018) compared to geometric mean levels of 48 respectively 159 gc/g in oysters collected at the dispatch center and retail in the Netherlands in the present study. Contrastingly, in an Italian study markedly higher geometric mean levels of NoV were detected in post-harvest mussel samples from class B areas (1800 gc/g) and in post-harvest oyster samples from class B areas (2000 gc/g) (Suffredini et al., 2014), compared to geometric mean level of 333 gc/g and 135 gc/g in the present study. In a large scale NoV baseline study comprising over 2000 samples of European oysters only, the average NoV RNA copy numbers in post-harvest oyster samples from class B areas and from dispatch center oysters were calculated at an arithmetic mean level of 459 gc/g and 168 gc/g respectively (EFSA, 2019) compared to the arithmetic mean levels of 365 and 89 gc/g in the present study. Taken together, the $\log_{10}$ mean NoV genome copy numbers reported in this study for *ready for sale* oysters and mussels passing through the Netherlands were of the same order of magnitude compared to levels founds in most other European countries. Despite the similarities in methods for extraction, detection and quantification of NoV GI and GII in individual samples in the above-mentioned studies, outcomes were expressed differently. ISO/TS 15216–1 focuses on test results for individual samples only. Inter-study comparison would benefit from clear specifications on how to handle datasets in terms of calculation with values below LOQ and LOD, the use of log transformation and whether to apply arithmetic or geometric means.

The poor correlation between E. coli MPN and NoV gc/g in shellfish on a sample-by-sample basis corresponded to other studies (Younger et al., 2018). Generally, higher loads of NoV GII were measured compared to NoV GI in both oyster and mussel samples. This finding was consistent across the food chain, post-harvest, dispatch center and retail, and during the years 2013 to 2017, as well as findings in the EU oysters baseline study (EFSA, 2019). The greater proportion of NoV GII copy numbers in shellfish corresponds with the much higher prevalence of NoV GII amongst the human population compared to NoV GI (Qi et al., 2018). Conversely, shellfish-borne outbreaks are marked by a relatively high frequency of GI strains (Le Guyader et al., 2012). This suggests that shellfish act as a more potent reservoir for the transmission of norovirus GI compared to GII (Yu et al., 2015), possibly owing to an increased stability or slower clearance of GI from shellfish in a cold seawater environment (Maalouf et al., 2010b).

Heat treatment can be applied to reduce the load of infectious virus particles in food (Croci et al., 2005). Where oysters on the European market are generally consumed raw, mussels are most often heated before consumption. Heating at 90 °C effectively reduced the load of FRNA bacteriophage in mussels by 7 $\log_{10}$ after four minutes (Flannery et al., 2014). Modeling on multiple HAV inactivation studies demonstrates complete inactivation of 5 $\log_{10}$ genome copies after 90 s of heat treatment at 90 °C (EFSA BIOHAZ Panel, 2015). However, the specific effect of boiling temperatures on NoV GI and GII infectivity in shellfish matrix is yet to be determined (Croci et al., 2012). Stem cell derived human intestinal organoids pose a promising system to demonstrate the infectivity of human NoV particles from shellfish extracts, but several challenges remain to be solved before the cell culture technique can be routinely implemented (Estes et al., 2019).

As proof of NoV infectivity in bivalves remains challenging, and not enough data are available to reliably predict illness from measured NoV levels in bivalve shellfish, no enforcement criterion has yet been established. Further complicating matters, LOQ levels in participating laboratories in the European baseline study for NoV in raw oysters were variable. In that study, most laboratories were able to achieve an LOQ of 300 gc/g for both genogroups. Application of the half-LOQ substitution approach when a putative threshold would be set below or close to laboratory LOQ would lead to a substantial increase of market rejection in those laboratories with a suboptimal LOQ (EFSA, 2019). Had a monitoring threshold at 300 gc/g been in place at the moment of this study, this would have resulted in market rejection of 2.4% of the oyster samples tested (6/253), and if applied to mussels 7.3% (27/371), though the latter would mostly have been consumed after heat treatment.

The low HAV prevalence in the bivalves reflects the current low incidence in the local Western European population, the Netherlands, United Kingdom, Ireland, Germany, Denmark and France (ECDC, 2016). Despite this, one should be aware of clusters of cases living near harvesting areas in periods of sewage system overflow. Sewage overflow can temporarily spike the waters and lead to contaminated bivalve molluscs. This has been reported recently for two clusters of Dutch HAV cases that were linked to consumption of mussels harvested in Western Europe, even though consumers had boiled the mussels prior to consumption (Boxman et al., 2016) or to the HAV outbreak due to consumption of locally harvested oysters in Australia (Conaty et al., 2000). Given the short shelf life of the fresh products, end-product testing is currently not fast enough to take products from the market to protect the consumer. Therefore, the focus should be on prevention of fecal contamination of the production areas. Future development of rapid and point-of-need diagnostic tests could facilitate the transition towards safety testing that directly benefits consumers (Batule et al., 2018).

In summary, this study provides insight in the quantitative levels of NoV in oysters and mussels, over a long time period, from harvest to retail. Quantitative data can be leveraged for the development of improved risk assessment models for local markets.

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Declaration of competing interest

None.

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