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Analysis of the European baseline survey of norovirus in oysters

European Food Safety Authority (EFSA)

Abstract

The European Commission requested scientific technical assistance for the analysis of a European Union coordinated monitoring programme on the prevalence of norovirus in raw oysters. A total of 2,180 valid samples were taken from production areas and 2,129 from dispatch centres, taken over two consecutive years, ensuring the precision and the confidence desired in the estimation. The prevalence at production areas was estimated to be 34.5% (CI: 30.1–39.1%), while for batches from dispatch centres it was 10.8% (CI: 8.2–14.4%). The analyses show a strong seasonal effect, with higher contamination in the period November to April, as well as lower contamination for Class A areas than other classes. These associations were observed in both production areas and batches from dispatch centres. The results for both genogroups were above the respective limit of quantification (LOQ) in less than 10% of the samples taken. The simple substitution of not-detected and positive samples below the LOQ, by half of the limit of detection and half of the LOQ, respectively, produced estimates of the proportion of samples above or equal to 300 copies per gram (cpg) comparable to the statistical model. The current bacteriological microbiological criteria applicable to live bivalve molluscs might be complemented by a norovirus criterion. The analyses of the substitution approach show that selection of a potential limit within a microbiological criterion close to or lower than the LOQ (for example, less than 300 cpg, given the current test used in this survey) would be difficult to apply. This survey only assessed thresholds from the perspective of the analytical capability and not that of human health risk.

Keywords: norovirus, baseline survey, generalised estimating equation, Bayesian model, oysters

Requestor: European Commission

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Noroviruses (NoV) are known to cause "winter-vomiting disease" or "stomach-flu" referring to their rapid spread in human populations especially during winter months. They are transmitted primarily through the faecal-oral route, either by consumption of contaminated food or water, or by spreading directly from person to person. Many different food items have been associated with NoV outbreaks. Raspberries and oysters have caused several national and international outbreaks.

Bivalve molluscs are a well-documented source of noroviral infection since they have the ability to accumulate and concentrate NoV particles by filtration of water contaminated with faeces. NoV associated with point source human faecal pollution (eg discharges from sewage treatment works) is a persistent problem in coastal waters during the winter months leading to the contamination of bivalve mollusc production areas. Oysters contaminated with NoV pose a particular risk to human health since they are routinely consumed raw.

There is currently no threshold infectivity limit established for NoV as detected by polymerase chain reaction (PCR). The probability of becoming infected increases with the dose but depends also on the characteristics of the organism, the food matrix and the host factors.

Furthermore, the relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR is not a constant, and it is important to realise that the infectious risk associated with low level positive oysters, as determined by real-time PCR, may be overestimated.

In accordance with Article 31 of Regulation (EC) No 178/2002, EFSA is requested to provide scientific and technical assistance on design related to an EU coordinated monitoring programme on the prevalence of NoV in raw oysters, and in particular to:

Prepare a survey protocol for the baseline survey of NoV contamination in oysters in the EU with the objective to:

a) Assess the proportion of EU classified production areas with NoV contamination. The survey should detect at least 1% of representative monitoring points within harvesting areas with > 1,000 total NoV PCR copies per gram with a level of confidence of 95%;
b) Assess the proportion of batches of final product at approved EU dispatch centres with NoV contamination. The survey should be able to detect batches with > 1000 total NoV PCR copies per gram with a level of confidence of 95% and a level of precision of 5% and an expected prevalence of 50%.

Once the data are available (collected) and submitted to the EFSA data collection framework, EFSA is requested to:

c) Assess the proportion of EU classified production areas contaminated with NoV (presence);
d) Assess the proportion of batches of final product at approved EU dispatch centres contaminated with NoV (presence); and
e) Analyse the survey data for NoV in the EU in production areas and at approved dispatch centres including options with regard to establishing a microbiological criterion for NoV in oysters to be harvested and placed on the market.

1.2. Interpretation of the terms of reference

The working group agreed with the Commission that the objective of the survey was to estimate the prevalence of sample units contaminated with NoV for both production areas and batches from dispatch centres to answer terms of reference (c) and (d). The working group proposed to estimate the prevalence for NoV, considering that a sample was contaminated with NoV if either or both of the genogroup results was positive. For this report, potential factors associated with the prevalence of NoV would also be studied.

The baseline survey would be used to assess the prevalence of EU-classified oyster production areas and batches in dispatch centres with NoV contamination. The survey would be used to estimate the prevalence of samples with a number of copies per gram (cpg) of digestive tissues greater than prespecified thresholds (> 1,000 total NoV cpg, as specified in the terms of reference for the design of the baseline survey), in oysters collected from representative monitoring points within production areas and batches of final products in dispatch centres. The potential effect of a set of factors reported in the survey on the observed number of NoV copies in the samples would also be assessed.

In order to address the microbiological criteria (term of reference (e)), the final data analysis would present the proportion of contaminated production areas/batches of final product for a range of NoV copies per gram thresholds (100, 200, 300, 500, 1,000, 5,000, 10,000 and greater than 10,000 cpg) in order to refine the analysis presented in EFSA (2012). In order to achieve this objective, an analysis accounting for the different censoring type would be conducted (left and interval censoring). The obtained distribution would be considered to estimate the prevalence associated with the thresholds previously specified. Potential factors influencing the observed number of copies per gram would also be studied.

1.3. Background information on regulatory risk management

European bivalve mollusc production is subject to a specific regulatory regimen designed to manage particular food-borne risks. In the context of microbial risks arising from human faecal contamination, a key requirement is that production areas must be classified by the coastal competent authorities as Class A, B or C according to the level of *Escherichia coli* present in the molluscs as a marker of faecal pollution. Classification is a general categorisation of the microbial contamination status of production areas and dictates the subsequent requirements for placing molluscs on the market to manage these microbial risks.

With some minor exceptions, bivalve molluscs or their products may only be placed on the market as food for human consumption following handling or processing at a food establishment approved for that purpose. A specific documentary regimen of registration documents for harvested bivalve molluscs denotes status as not yet prepared for human consumption and ensures that details of production area classification status accompany molluscs to the approved establishment handling them. In the case of bivalve molluscs being placed on the market for consumption as live animals, the type of approved establishment in which these must be handled to be prepared as food is a 'dispatch centre'.

While bivalve molluscs may be thermally processed to produce fishery products, the present survey is focused on oyster production and their placing on the market for consumption as live animals. Such placing on the market as live bivalve molluscs may only occur directly (without post-harvest treatment) for those harvested from a Class A area. More contaminated Class B or Class C molluscs may be placed on the market as live animals for direct human consumption only following a decontamination step, either relaying in seawater areas approved for that purpose (B or C), or purification in commercial tanks at an approved purification centre (B). All dispatch of bivalve molluscs for human consumption as live animals may be carried out only by approved dispatch centres and is subject to bacterial microbiological criteria regardless of production area classification, or relaying/purification status (Table 1).

Table 1: Summary of EU classification requirements for live bivalve mollusc production areas, and consequent microbial risk management step required before placing on the market as live bivalve molluscs from an approved dispatch centre

Class	Microbiological classification standard (most probable number per 100 g of flesh and intravalvular fluid)	Microbial risk management required before placing on market as live bivalve molluscs from a dispatch centre
A	At least 80% of samples < 230 *Escherichia coli* Remaining 20% of samples < 700 *E. coli*	None, no decontamination required May be dispatched directly subject to microbiological criteria
B	At least 90 % of samples < 4,600 *E. coli* Remaining 10% < 46,000 *E. coli*	Purification or relaying required to meet microbiological criteria
C	All samples < 46,000 *E. coli*	Relaying for a long period required to meet microbiological criteria
Not classifiable	Any sample > 46,000	

2. Data and methodologies

2.1. Definitions

The EU NoV prevalence (for each genogroup and their combination, defined below) presented in the report are:

- **Genogroup I presence:** Detection of genogroup I in samples tested by RT-PCR.
- **Genogroup II presence:** Detection of genogroup II in samples tested by RT-PCR.
- **Norovirus presence:** A sample was considered to be NoV positive if it was positive for either of the two genogroups, as defined below:

$$Y_{both} = \begin{cases} \text{Positive} & \text{if } Y_I \text{ is positive or } Y_{II} \text{ is positive} \\ \text{Not detected} & \text{otherwise} \end{cases}$$

  Note that other genogroups are not considered in this report.

- **Genogroup quantity present:** in the descriptive analysis of the reported information this quantity refers to results above the limit of quantification (LOQ); to half of the LOQ value when results were reported positive but below the LOQ; and to half of the limit of detection (LOD) when results were reported as not detected. This analysis is referred to as the substitution approach. When statistical models were implemented, survival methodology was used to account for the censored nature of the observations.
- **Raw proportions** of positive samples from production areas or batches at dispatch centres, means the percentage of positive samples in production areas or positive batches at dispatch centres out of all samples taken in production areas or of all batches taken from dispatch centres and does not account for any design aspect (e.g. no weighting, etc.). This might be expressed as percentages (0–100) or proportions (0–1).
- **Apparent prevalence** of NoV, meaning that the prevalence estimates do account for the design of the survey (covering the actual design as well as deviations from it) correcting for under- or over-sampling subpopulations, and using generalised estimating equations (GEE) to account for the clustered/hierarchical nature of the data (see Section 2.3 for further details), but do not account for test characteristics (possible misclassification of the test results). This might be expressed as percentages (0–100) or proportions (0–1). In the report, the term prevalence is used to ease the reading. The apparent prevalence provides an estimate of the NoV prevalence for the population sampled, meaning oyster production areas and batches from dispatch centres in the EU.
- **True prevalence** represents the actual prevalence of the contamination in the population in question, which can be estimated from the apparent prevalence by correcting for misclassification bias due to the imperfect diagnostic test used. The difference between the **apparent** ($\hat{p}$) and the **true** (π) prevalence is defined by Rogan and Gladen (1978) as a function of the sensitivity (Se) and the specificity (Sp) of the diagnostic test used as shown below:

$$\pi = \frac{(\hat{p} + Sp - 1)}{(Se + Sp - 1)}$$

  Sensitivity and specificity values are not currently available for this test; therefore true prevalence was estimated using a range of biologically plausible values for RT-PCR virus detection (EFSA, 2012).

2.2. Data description

Descriptive cross-tables, pie charts, dot plots, histograms and box plots of the variables collected in the baseline survey (EFSA, 2016) are presented. The data description is based on the presence or absence of detectable NoV RNA in the samples taken in production areas, and from batches in dispatch centres. When considering the presence/absence data, raw proportions are presented, which could also be cross-tabulated with another variable collected, such as time period, production area class, etc.

Additionally, descriptive analyses are shown for the quantification of the number of copies per gram reported for both the samples from production areas and for batches in dispatch centres. For this specific analysis, summaries are based on the aforementioned substitution approach. In order to

summarise the information on NoV quantity, the sum of the quantifiable results was used as well as the substituted results.

2.2.1. Sample summary and sample protocol comparison

Sampling took place in 12 Member States between 1 November 2016 and 31 October 2018, in production areas and dispatch centres. Norway also participated in the survey but took samples only from production areas. Each sample from production areas or from dispatch centres consisted of at least 2 g of digestive glands pooled from a minimum of 10 live, undamaged oysters. All samples judged acceptable for laboratory analysis were examined for NoV genogroup I (GI) and NoV genogroup II (GII) (separate tests). The testing was performed according to the published technical specifications for the survey, which was equivalent to ISO 15216-1:2017 'Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR – Part 1: Method for quantification'. In one case, when there was a requirement to test a large number of samples, a two-stage testing method was used involving an initial absence/presence screening performed using the non-quantitative part of the ISO 15216-1:2017 standard, with positive samples subsequently quantified by a central laboratory. Laboratories only carrying out the initial presence/absence screening were trained and supervised by the national reference laboratory (NRL) in that country. All laboratories undertaking quantification of survey samples (11 NRLs and 2 non-NRLs) passed a competence assessment using NoV-contaminated oyster samples organised by the then EU Reference Laboratory (EURL). The assessment of laboratory performance was based on ISO/TS 22117:2010 'Microbiology of food and animal feeding stuffs – Specific requirements and guidance for proficiency testing by inter-laboratory comparison'. Any laboratory returning a questionable or unsatisfactory result was subject to remedial actions in cooperation with the EURL and required to pass a repeat competence assessment.

Data were reported to the EFSA data collection framework (DCF). Validated data were extracted from the EFSA DCF database on 5 April 2019. Tables 2 and 3 describe, for production areas and for dispatch centres, respectively, the planned and achieved sample sizes for each participating country. The samples that needed dilution are not included in Tables 2 and 3, representing 36 samples from production areas and 1 batch sampled from dispatch centers. These samples were not included in the descriptive analysis or in the estimation of the distribution of quantification results, but they were used for estimating norovirus prevalence. Of the total number of 2,275 sample records from the 172 production areas, 41 (1.8%) were not suitable for testing or not available and 54 (2.4%) returned invalid analytical results even after retesting. This is a total of 4.2% missing samples and is below the maximum 10% for missing samples incorporated into the survey design to ensure the planned level of confidence and precision for production areas. Of the total number of 2,362 sample records from the 207 dispatch centres, 188 (7.9%) were not suitable for testing or not available and 45 (1.9%) returned invalid analytical results even after retesting. This is a total of 9.8% missing samples and is below the maximum anticipated 20% for missing samples incorporated as a contingency into the survey design to ensure the planned level of confidence and precision for dispatch centres. Five samples from production areas were reported with valid results only for GI or GII but not for both. The final validated data set included information on 2,180 samples from oysters originating from 172 distinct production areas, with valid results for both GI and GII. From 207 distinct dispatch centres, 2,129 samples of oysters were included, with valid results for both GI and GII. These validated data formed the basis for all subsequent analyses.

Table 2: Achieved sample sizes compared with the planned sample sizes, production areas

Country	Production areas								
	Locations			Samples					
	Planned	Achieved	Difference between achieved and planned	Planned	Total	Valid	Not valid	Not tested or not available	Difference between valid and planned[(a)]
Croatia	2	2	0	24	22	22			−2
Denmark	3	3	0	36	36	36			0
France	74	74	0	888	888	850	27	11	−38
Germany	1	1	0	12	12	12			0
Greece	2	2	0	24	24	23	1		−1
Ireland	22	22	0	264	480	473	7		+209
Italy	7	6	−1	84	72	71		1	−13
Netherlands	5	5	0	60	60	49	11		−11
Portugal	5	5	0	60	60	53		7	−7
Spain	14	14	0	168	167	148	3	16	−20
Sweden	3	3	0	36	35	35			−1
United Kingdom	31	34	+3	372	396	385	5	6	+13
EU	169	171		2,028	2,252	2,157	54	41	
Norway	2	1	−1	24	23	23			−1
Total	171	172		2,052	2,275	2,180	54	41	

(a): '−': under-sampling; '+': over-sampling.

Table 3: Achieved sample sizes compared with the planned sample sizes, dispatch centres

Country	Dispatch centres								
	Locations			Samples					
	Planned	Achieved	Difference between achieved and planned	Planned	Total	Valid	Not valid	Not tested or not available	Difference between valid and planned[(a)]
Croatia	1	1	0	12	11	11			−1
Denmark	1	1	0	12	12	12			0
France	167	176	+9	2,004	2,004	1,793	33	178	−211
Germany	1	1	0	12	12	12			0
Greece	1	1	0	12	11	8		3	−4
Ireland	1	1	0	12	19	19			+7
Italy	9	10	+1	108	108	106	2		−2
Netherlands	3	3	0	36	36	32	3	1	−4
Portugal	2	2	0	24	24	19		5	−5
Spain	6	6	0	72	72	67	4	1	−5
Sweden	1	1	0	12	12	12			0
United Kingdom	4	4	0	48	41	38	3		−10
EU	197	207		2,364	2,362	2,129	45	188	

(a): '−': under-sampling; '+': over-sampling.

Additional descriptive analyses are available in Appendix B. Detailed information on the data model and data elements (variables) used to submit data to the EFSA DCF is in the EFSA scientific report with technical specifications for a European baseline survey of norovirus in oysters (EFSA, 2016). The analysis below focuses primarily on the mandatory variables required for valid data submission.

Figure 1 presents LODs and LOQs (excluding LODs and LOQs for samples tested using diluted RNA due to RT-PCR inhibition) for the 13 laboratories that participated in the survey and undertook quantification of survey samples. Where a one-in-ten dilution of sample RNA was applied, a 10-fold multiplicative factor for both LOD and LOQ values was used. The general principles for the determination of LODs and LOQs were defined by the EURL (Cefas, 2016) and values reported in this baseline for LOD and LOQ are given below.

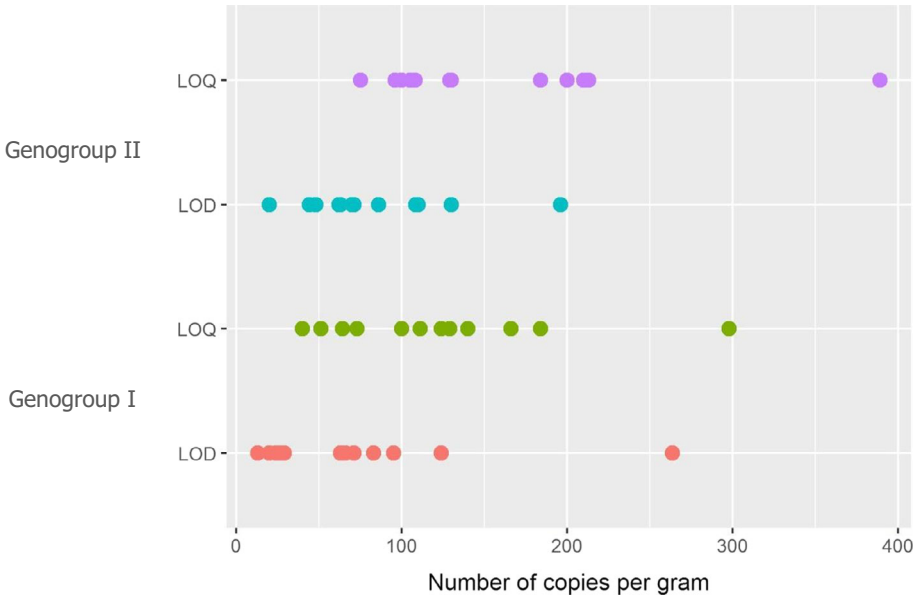

Figure 1: Reported limits (number of copies per gram) of detection (LOD) and limits of quantification (LOQ) that were applied to the results of the quantitative RT-PCR, by genogroup, and by laboratory

For GI, the LOQ ranged between 40 and 298 copies and the LOD between 13 and 264 copies. For GII, the LOQ ranged between 75 and 389 copies and the LOD between 20 and 196 copies. Higher values were reported when a tenfold multiplicative factor was used for reactions where dilution of RNA was required.

The LOD and LOQ were calculated by each laboratory either by using bio-accumulated oysters (considering all extraction steps of the method from the elution to the RT-PCR amplification) or by seeding oyster homogenate with virus (considering all steps after the enzymatic elution). Each laboratory used local oysters which may vary in terms of chemical composition and presence of inhibitors, their laboratory strains of NoV and different RT-PCR machines, all of which might result in variation in the real-time RT-PCR results. Moreover, at low concentrations the standard deviation of real-time RT-PCR varied from 0.2 to 0.6 Log (Persson et al., 2018; Polo et al., 2018).

2.2.2. Description of production area sampling

In the survey, 60% of samples from production areas were taken from Class B areas, 39% from Class A areas and less than 1% from Class C areas (Figure 2).

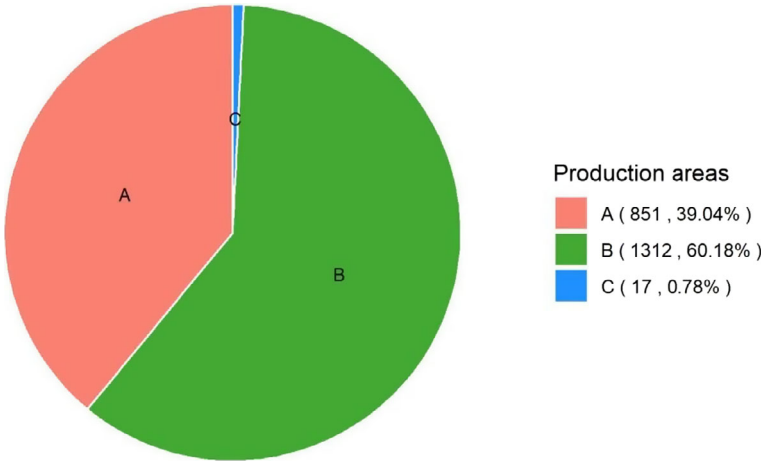

Figure 2: Distribution of the number of oyster samples, by classification of the production areas

The Food and Agriculture Organization (FAO) has defined marine fishing areas globally to support statistical analysis.[1] EU marine waters generally fall within one of two FAO major areas, '27 north-east Atlantic & Baltic Sea' or '37 Mediterranean & Black Sea'. These major areas have been further divided into subareas which can be described in approximate geographical terms. The respective subarea of the FAO major area was reported for each production area included in the survey. Most of the samples were taken from the north-east Atlantic, the Bay of Biscay and the sea surrounding Scotland and Ireland. This reflects relatively well the distribution of commercial oyster production in Europe. Figure 3 shows the subarea where the sample originated from as well as the percentage of samples collected in each subarea. Thirty-four per cent originated from sub area 27.7, 32% from sub area 27.8 and 12% from sub area 27.6.

[1] http://www.fao.org/cwp-on-fishery-statistics/handbook/general-concepts/fishing-areas-for-statistical-purposes/en/

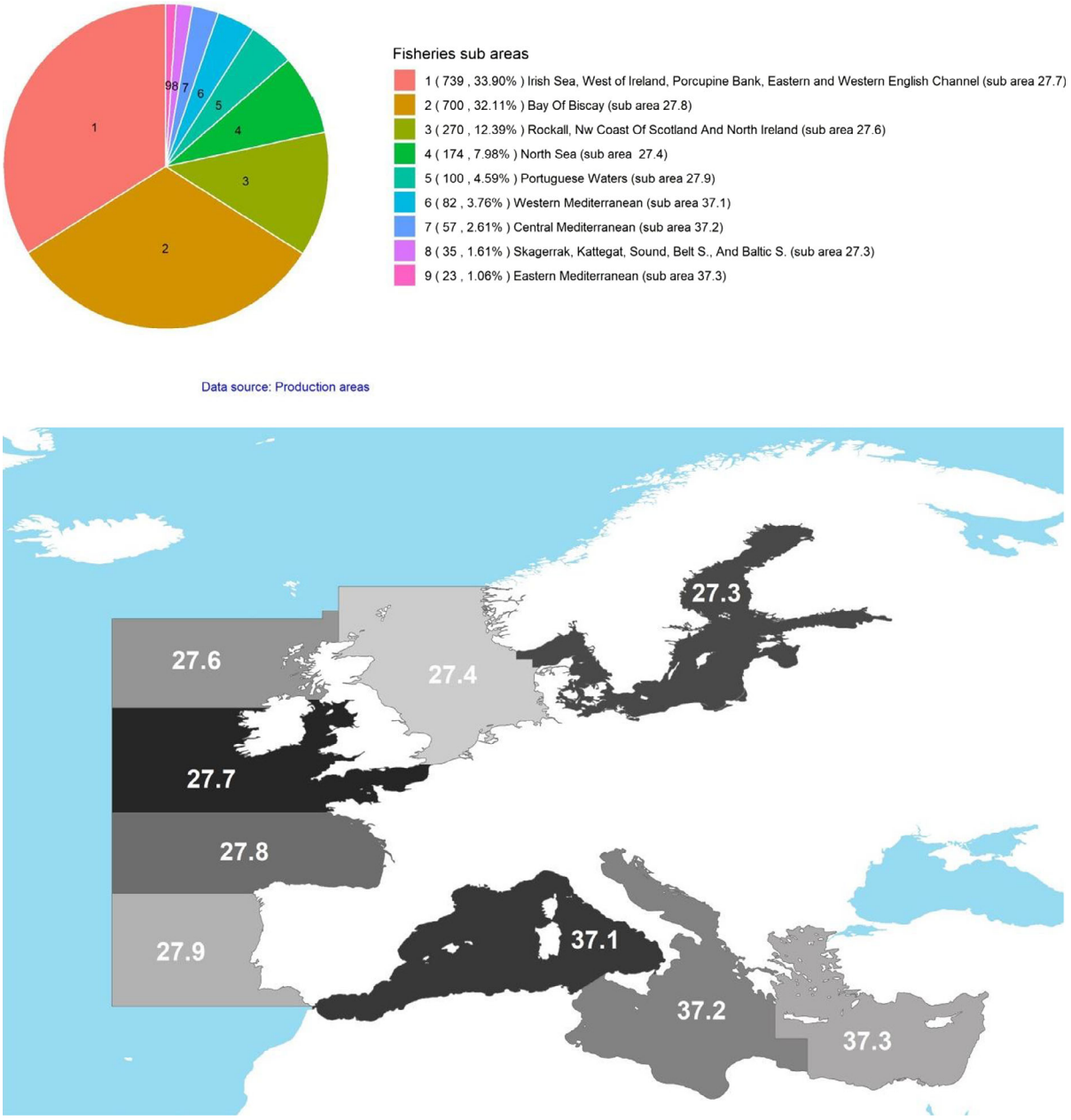

Figure 3: Distribution of the oyster samples, by origin of the sample (fisheries subareas) at the level of sub area and a map showing the location of each subarea

The survey included samples from farmed and wild oysters, 82% and 18%, respectively (see Appendix B, Table B.1). Seventy-two per cent of the sampled oysters were produced in a raised trestle system; 18% were bottom-grown while 9% were suspended from the sea surface (see Appendix B, Figure B.2).

Eighty-five per cent of the samples were from the Pacific cupped oyster (*Crassostrea gigas*, recently renamed *Magallana gigas*), 14% from the European flat oyster (*Ostrea edulis*) and 1% from the Portuguese cupped oyster (*Crassostrea angulata*, recently renamed *Magallana angulata*) (see Appendix B, Table B.2). This corresponds with EU production statistics that report the Pacific cupped as the major production species with 85% of production from France.[2]

Seventy-one per cent of the samples were from intertidal production areas, 27% inshore and 2% offshore (see Appendix B, Table B.3)

[2] https://ec.europa.eu/eurostat/statistics-explained/index.php/Fishery_statistics#Aquaculture_statistics

Eligible oysters were required to be of commercial size. As shown in Figure 4, the mean weight of an oyster in samples from production areas was 93 g. The Portuguese cupped oysters were on average lighter than the Pacific cupped oysters and the European flat oysters.

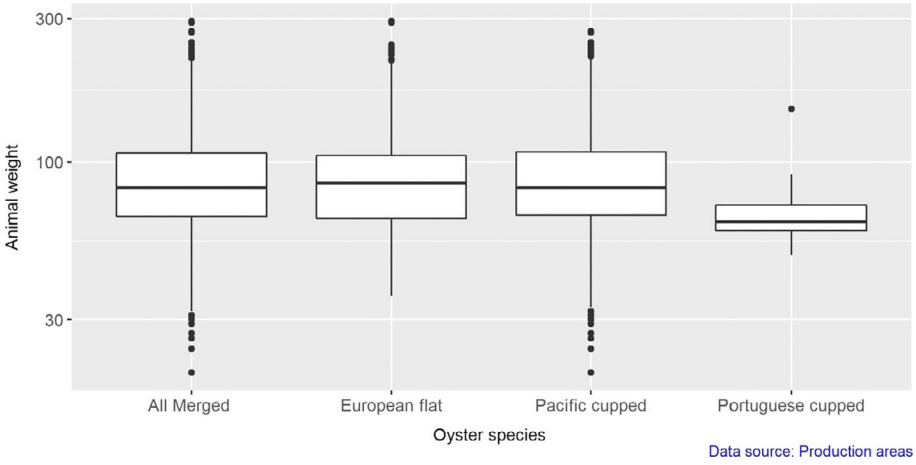

Figure 4: Box plot of mean animal weight in the sample (entire animal including shell in grams), from production areas

2.2.3. Description of dispatch centre sampling

Figure 5 shows the distribution of the production areas that were the source of the sampled batch. In the survey of dispatch centres, 61% of the samples originated from Class A areas, 36% from Class B areas and 2.5% had an unknown origin as to the production area status. This pattern differed from that in production areas. Where a batch comprised oysters from different production areas, those different areas were to be reported (A, B, C or a multiple) but the number of such batches was limited (only eight).

Since oysters from Class A areas do not require post-harvest treatment, these oysters may be more commercially attractive for food business operators, potentially explaining the finding of these Class A oysters being more frequently found in dispatch centres. Oysters from Class B areas must be subject to purification or relaying before being placed on the market for direct human consumption as live animals. For 2.5% of samples, the production area status was unknown or not reported; this occurred when the official sampler was unable to retrieve all the batch information from the dispatch centre.

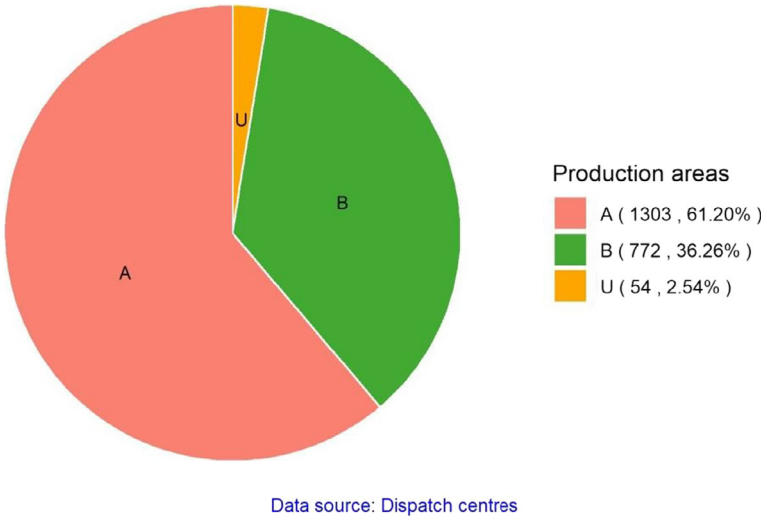

Figure 5: Distribution of the oyster samples from dispatch centres, by classification of the production areas that were the source of the batch

The survey of the dispatch centres included 96% farmed oyster samples and 4% wild oyster samples. In 1% of samples the official sampler was unable to determine whether the oyster was wild or farmed (see Appendix B, Table B.4)

The majority of batches sampled originated from a single production area (99.6%), 0.2% from two areas and 0.1% from three production areas (see Appendix B, Table B.5). The batch sizes showed significant variation (median 250 kg, mean 526 kg, maximum 40,000 kg, see Figure 6.

Ninety-two per cent of the samples from dispatch centres were not repacked, 3% were repacked and for 5% the information reported was stated to be unknown (see Appendix B, Table B.6). The average weight of an oyster from the dispatch centres collected was 82 g, with a median of 75 g and a maximum weight of 406 g (Figure 7). The oysters sampled at dispatch centres were, in general, smaller than those from production areas. This difference in weight could be because oysters sampled from routine monitoring points are not subject to market size preferences and may be maintained for a longer period and are therefore older and larger.

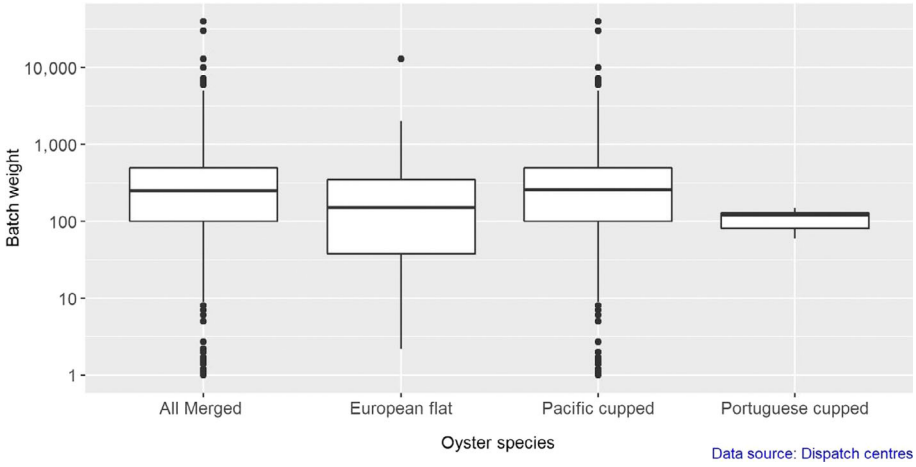

Figure 6: Box plot of overall batch weight, from dispatch centres

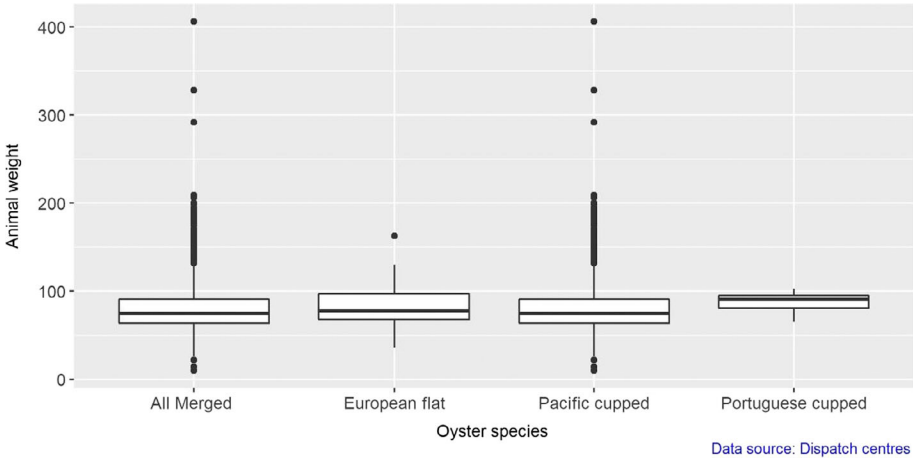

Figure 7: Box plot of mean animal weight in the sample (mass of the entire animal including shell, in grams), from batches in dispatch centres

Ninety-six per cent of the samples from dispatch centres were from the Pacific cupped oyster (*Crassostrea gigas*), 4% from the European flat oyster (*Ostrea edulis*) and 1% from the Portuguese cupped oyster (*Crassostrea angulata*) (see Appendix B, Table B.7).

2.3. Methodologies

Not valid and not tested or not available results were excluded from the analysis, and only valid results were used. In order to investigate the effect of discarding the not valid, not tested and not available results (referred to as missing data), multiple imputations were used, and estimated outcomes were compared with those obtained from the analysis using only valid results.

2.3.1. Estimation of norovirus prevalence including risk factor analysis

The prevalence of NoV infection for the two genogroups was estimated separately as well as for the combination as defined in the previous section. For each outcome, the prevalence with 95% confidence intervals (CI) was estimated. Only the apparent prevalence was investigated and is presented in this report, and no correction has been made for imperfect test sensitivity or specificity.

2.3.1.1. Estimation of prevalence

Prevalence was estimated at the EU level, by GEE considering that outcomes (presence or absence of NoV infection) in oysters from the same production area or dispatch centre are expected to be more alike than those from different production areas or dispatch centres (further details can be found in Appendix A.1).

Generalised estimating equations were used to estimate the overall prevalence for genogroups I and II and for NoV as defined in Section 2.1 for the period sampled. The analysis considered both years as a representation of an average year, thus aggregating the information from the two years to represent the different periods (November–December, January–February, March–April, May–June, July–August, September–October). The model included a restricted cubic spline (Durrleman and Simon, 1989) time effect and several working correlation structures were used in order to evaluate the impact on the estimation of the outcome, i.e. the resulting status for each sample taken, being either positive or not detected for the GI, GII and NoV as defined in Section 2.1. The analyses presented in this report are based on the independent working correlation structure given that prevalence estimated based on different working correlation structures were similar and it is known that estimations based on the independent correlation structure have an appropriate marginal interpretation in the case of informative cluster sizes (Williamson et al., 2003).

Standardised weightings were used in the GEE models to account for a disproportionate stratified sampling design. The survey design used is described in the technical specification (EFSA, 2016). In the design of the survey, Member States were considered as strata, and the proportion of sampled production areas and batches in dispatch centres were defined considering proportional sampling. In order to account for disproportionate sampling of batches in dispatch centres, Member States were asked to provide the number of batches and tonnage dispatched each month during the sampling period (November 2016–October 2018).

- For production areas, weighting was needed due to the potential absence of material to sample (producing missing observations during the sampling period) and to the over-sampling carried out by some Member States. As a result, the proportion of production areas sampled was not constant across Member States. In this case, the ratio between the planned number of samples per Member State and period (n_{ij}^{P}, where i represents the Member State and j the sampling period) and the actual number of samples taken ((n_{ij}^{A}) was used.

$$W_{ij}^{PA} = \frac{\frac{n_{ij}^{P}}{\sum_{i,j} n_{ij}^{P}}}{\frac{n_{ij}^{A}}{\sum_{i,j} n_{ij}^{A}}}$$

- For dispatch centres, the number of dispatch centres to be sampled was defined in the technical specifications (EFSA, 2016) to be proportional to the number of dispatch centres in each Member State. However, the primary objective was to estimate the prevalence of NoV in batches dispatched. When the survey was designed, the number of batches dispatched in each Member State was unknown. In order to appropriately estimate the NoV prevalence in batches dispatched, the ratio between the planned number of batches to be sampled per Member State and period ((m_{ij}^{P}) and the actual number of batches sampled ((m_{ij}^{A}) was multiplied by the ratio between total number of batches dispatched in each Member State and period ((m_{ij}^{T}) and the planned number of batches to be sampled ((m_{ij}^{P}) was used.

$$W_{ij}^{DC} = \frac{\frac{m_{ij}^{P}}{\sum_{i,j} m_{ij}^{P}}}{\frac{m_{ij}^{A}}{\sum_{i,j} m_{ij}^{A}}} \cdot \frac{\frac{m_{ij}^{T}}{\sum_{i,j} m_{ij}^{T}}}{\frac{m_{ij}^{P}}{\sum_{i,j} m_{ij}^{P}}}$$

The information provided did not always contain the number of batches and was not always provided by month, but yearly information was also submitted to EFSA. In order to estimate the number of batches dispatched for each Member State in each period used in the GEE analysis (November–December, January–February, March–April, May–June, July–August, September–October), first the yearly tonnage dispatched was distributed across the months of the year using the information available for the specific Member State in which monthly tonnage was provided, considering the monthly proportions dispatched to build a monthly tonnage for each dispatch centre in each Member State. Once information on tonnage was estimated for each dispatch centre, multiple imputations were used considering year, month and tonnage, to estimate the missing number of batches dispatched for each dispatch centre. In order to estimate the number of batches, 50 multiple imputations using a random forest approach from the mice package (Van Buuren and Groothuis-Oudshoorn, 2011) were made. The use of random forests for multiple imputations has been previously recommended (Cortiñas Abrahantes et al., 2011; Shah et al., 2014), showing better performance than other methods.

In order to evaluate the effect of the missing data, multiple imputation (50 imputations) was used. The imputation of the missing values uses a random forest model to impute the missing response based on sampled location, sampling period and country. These 50 completed data sets were then analysed separately using GEE models with the weighting described above and their estimates were combined using Rubin's rules (Rubin, 1987; Myunghee, 1997).

2.3.1.2. Effect of potential risk factors on the estimation of prevalence

In order to explore the effect of potential risk factors on the probability of being NoV positive (NoV prevalence), several hypotheses were investigated, such as:

1) Prevalence of NoV is higher in productions areas than in dispatched batches.
2) Prevalence of NoV changes depending on the period of the year in both production areas and dispatched batches.
3) Prevalence of NoV is higher in production areas of Class B or C than in those of Class A.
4) The changes in prevalence of GI in production areas throughout the year are less pronounced than those of GII.
5) Prevalence of NoV in production areas differs depending on the production type (i.e. farmed or wild).
6) Prevalence of NoV is higher in larger oysters in production areas and in dispatched batches.
7) Prevalence of NoV in production areas differs depending on the area type (i.e. inshore or intertidal).
8) Prevalence of NoV is not different among different species of oysters in the same production area.
9) Prevalence of NoV in dispatched batches with oysters from Class A areas is lower than in batches with oysters from Class B areas, and that the association is not affected by the presence or absence of conditioning, relaying or purification.

In order to test the hypotheses presented above, GEE models were fitted including the factor to be tested as:

a) Modifying the NoV prevalence time trend by a constant (on the logit scale) for each level of the factor under consideration (additive model):

$$\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Factor}$$

b) The NoV prevalence time trend to be different for each level of the factor included (multiplicative model):

$$\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Factor} + \mathrm{f}(\text{Time Period}) \times \text{Factor}$$

c) No effect of the factor under consideration on the NoV prevalence time trend:

$$\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period})$$

From models a, b and c, the one with the smallest Quasi-Akaike Information Criterion (QIC) (Pan, 2001) was selected. If model c was thus selected, it would indicate that, based on the data, the factor

was not modifying the prevalence of NoV, thus it is not likely to be a risk factor for NoV in oysters (more information can be found in Appendix A.1).

Once all potential risk factors were identified, then all models containing the resulting factors as well as all possible two-way interactions were fitted (more than 500 models considering all potential combinations) and the one with the smallest QIC was selected to model the prevalence of NoV in oysters in production areas and dispatch centres.

Analyses were performed in R (version 3.5.3), using the geepack package to fit the models (Yan, 2002; Yan and Fine, 2004; Højsgaard et al., 2006) and the MuMIn package for model selection (Barton, 2018).

2.3.2. Estimation of the distribution of quantification results

The data set was analysed using a Bayesian framework for estimating the distribution of the sum of the number of copies per gram of GI and GII accounting for censoring. The distribution of the sum of the number of copies per gram was fitted using a lognormal model. Two approaches were undertaken in parallel, a bivariate approach (fitting the lognormal distribution for GI and GII jointly and deriving the sum) and an approach using the direct sum of GI and GII using a lognormal model. Based on the comparison of results of the two approaches, the direct approach was used to estimate the sum from here onwards (see Appendix A.2). The appropriateness of the distributional assumptions was assessed by the Shapiro–Wilk test (Shapiro and Wilk, 1965); this indicated no evidence against the log-normality assumption (results not shown).

It should be highlighted that the percentage of samples with censored results was higher than 90%, and the substitution approach (see Section 2.1) is therefore expected to provide a biased estimation of the distribution of the sum of the number of copies per gram of GI and GII. For this reason, a Bayesian model accounting for censoring and using the lognormal distribution with uninformative priors was developed (see Appendix A.2; Gelman et al., 2013).

Tables 4 and 5 show the lower and upper bounds as well as the number of observations accurately quantified, for both genogroups and for production areas and dispatch centres, respectively. In order to differentiate between not detected and positive below LOQ results, the lower bound for positive below LOQ results was considered to be 10^{-3} (to differentiate between not-detected and positive results below the LOQ, as well as to consider a larger uncertainty for this type of result). The frequency of all the combinations is shown in Tables 4 and 5. In this analysis, specific LOD and LOQ values from each laboratory were used when establishing lower and upper bounds for each of the combinations presented in the tables.

Table 4: Summary information for the data used for modelling the number of copies per gram in production areas based on the data collected in the norovirus baseline survey (excluding in valid samples, not tested or not available samples and samples tested using diluted RNA), EU, 2016–2018

GI	GII	VAL	Lower bound	Upper bound	N
Above LOQ	Above LOQ	GI + GII			138
Above LOQ	Not detected		GI	GI + LOD_GII	29
Above LOQ	Positive below LOQ		GI + 10^{-3}	GI + LOQ_GII	55
Not detected	Above LOQ		GII	LOD_GI + GII	35
Not detected	Not detected			LOD_GI + LOD_GII	1,318
Not detected	Positive below LOQ		10^{-3}	LOD_GI + LOQ_GII	142
Positive below LOQ	Above LOQ		GII + 10^{-3}	LOQ_GI + GII	90
Positive below LOQ	Not detected		10^{-3}	LOQ_GI + LOD_GII	148
Positive below LOQ	Positive below LOQ		2×10^{-3}	LOQ_GI + LOQ_GII	185
TOTAL					2,140

GI: genogroup I; GII: genogroup II; LOD: limit of detection; LOQ: limit of quantification; VAL: reported number of copies per gram in the sample.

Table 5: Summary information for the data used for modelling the number of copies per gram in dispatch centres based on the data collected in the norovirus baseline survey, EU, 2016–2018

GI	GII	VAL	Lower bound	Upper bound	N
Above LOQ	Above LOQ	GI + GII			14
Above LOQ	Not detected		GI	GI + LOD_GII	7
Above LOQ	Positive below LOQ		GI + 10^{-3}	GI + LOQ_GII	13
Not detected	Above LOQ		GII	LOD_GI + GII	12
Not detected	Not detected			LOD_GI + LOD_GII	1,903
Not detected	Positive below LOQ		10^{-3}	LOD_GI + LOQ_GII	72
Positive below LOQ	Above LOQ		GII + 10^{-3}	LOQ_GI + GII	16
Positive below LOQ	Not detected		10^{-3}	LOQ_GI + LOD_GII	47
Positive below LOQ	Positive below LOQ		2×10^{-3}	LOQ_GI + LOQ_GII	45
TOTAL					2,129

GI: genogroup I; GII: genogroup II; LOD: limit of detection; LOQ: limit of quantification; VAL: reported number of copies per gram in the sample.

The Bayesian model included a restricted cubic spline time effect and standardised weightings as described previously in Section 2.3.1.1. The heterogeneity across production areas and dispatch centres was covered by a random intercept (to account for the correlation between samples taken from the same production area or dispatch centre). Only factors found to be significant in the final model for apparent prevalence as described in Section 2.3.1.2 were considered in the multivariate analysis of the quantitative results. For dispatch centres, due to the lack of uncensored data, no risk factor other than the time trend was investigated.

Analyses were performed using R-stan (version 2.18.2) (Carpenter et al., 2017; Stan development team, 2018) and brms package (version 2.8.0) (Bürkner, 2017, 2018) in R (version 3.5.3).

The convergence criterion was assessed and factors were considered significant in the model if the 95% credible interval excluded zero. The probability of exceeding a particular threshold was estimated by posterior predictions (based on 1,000 posterior samples, presented by summary statistics), using the weighted analysis.

For goodness of fit and model comparison, Pareto k samples and leave-one-out (loo) cross-validation were used (Vehtari et al., 2017a,b).

3. Baseline survey results

3.1. Data summary results

3.1.1. Norovirus survey results in production areas

3.1.1.1. Raw percentages for norovirus contamination in samples from production areas

Based on the qualitative laboratory analysis results (Table 6), neither GI nor GII was detected in 61.9% of production area samples. At least one genogroup (GI or GII) was detected in 38.1% of them, so this was the raw proportion of NoV-contaminated oyster samples from production areas in this survey. Both GI and GII were present together in over half (57%) of those samples in which any NoV was detected. Single genogroup contamination was detected in approximately equal proportions of 8.3 and 8.1% of samples, respectively (Table 6).

Table 6: Raw percentages for norovirus contamination in samples from production areas

Presence results for GI – GII	N	%
Not detected – Not detected	1,349	61.9
Not detected – Positive	180	8.3
Positive – Not detected	178	8.1
Positive – Positive	473	21.7
Total	2,180	100.0

GI: genogroup I; GII: genogroup II.

The bulk of results (70%) were below the LOD for individual genogroups, with substantial proportions 18–20% below the LOQ and 10–12% above the LOQ with a quantified value (Table 7). This created the need for active consideration of how to substitute censored quantitative results in the majority (approximately 90%) of the results.

Table 7: Percentages of samples categorised by methodological limits for each genogroup in production areas

Genogroup	Not detected Below LOD (N)	Not detected Below LOD %	Not quantified Below LOQ (N)	Not quantified Below LOQ %	Quantified Above LOQ (N)	Quantified Above LOQ %	Total (N)
GI	1,529	70.1	428	19.6	223	10.2	2,180
GII	1,527	70.1	386	17.7	267	12.3	2,180

GI: genogroup I; GII: genogroup II; LOD: limit of detection; LOQ: limit of quantification.

3.1.1.2. Norovirus quantitative results for samples from production areas

The quantitative results (arithmetic mean, median, mode and geometric mean) taking account of all sample results including the results below the LOD, based on the substitution approach as defined in Section 2.1 is shown in Appendix C, Table C.1. The overall mean number of NoV copies per gram was 337 with a median and mode of 160.

The proportion of results exceeding quantitative thresholds was examined. Sixty-six per cent or two out of three production area samples exceeded 100 cpg for GI and GII combined, while only 5.5% or one in 20 exceeded 1,000 (Table C.7).

In a substantial proportion of samples (approximately 76%), not detected or positive below LOQ results were obtained for GI and/or GII. In these cases, quantification required substitution with LOD/2 or LOQ/2 values as described earlier. Where arbitrary thresholds close to the typical values for LOD and LOQ (e.g. 200 cpg or lower) were applied, a large proportion of samples exceeding the threshold only did so after substituted values were added to any quantified, non-substituted values (i.e. positive above LOQ). For higher arbitrary thresholds (e.g. 300 cpg or higher) in more than 87% of samples exceeding the threshold the sum of quantified GI and GII values alone exceeded the threshold, thus the values used for substitution had no impact on whether the sample was above or below.

Table 8: Proportion of samples from production areas (excluding 35 samples tested with diluted RNA) with quantitative results above arbitrary thresholds, and extent to which substitution of quantitative results contributes to exceeding those thresholds (given in copies per gram, cpg)

Parameter measured	Quantitative threshold (norovirus in cpg)						
	> 100	**> 200**	**> 300**	**> 500**	**> 1,000**	**> 5,000**	**> 10,000**
% of total samples that exceeded threshold	**66.21%**	**17.15%**	**11.92%**	**8.71%**	**5.50%**	**1.19%**	**0.18%**
Total number of samples that exceeded threshold **(N = 2,180)**	**1,444**	**374**	**260**	**190**	**120**	**26**	**4**
Further subdivision of samples that exceed the threshold							
% where both GI and GII results based on substitution approach (not detected or below LOQ), i.e. substitution influences the categorisation as above or below the threshold	75.76%	16.31%	0.00%	0.00%	0.00%	0.00%	0.00%
% with one quantified value, where that value was below the threshold and where the sum with the substituted value from the other genogroup was above the threshold, i.e. substitution influences the categorisation as above or below the threshold	0.42%	9.89%	12.69%	4.74%	1.67%	0.00%	0.00%
% with one quantified value, where that value was above the threshold or two quantified values, where the sum was above the threshold, i.e. substitution does not influence the categorisation as above or below the threshold	23.82%	73.80%	87.31%	95.26%	98.33%	100%	100%

GI: genogroup I; GII: genogroup II.

3.1.1.3. Initial descriptive analysis of prevalence and quantification results

Differences in observed proportion of NoV-contaminated oyster samples and quantification of results from production areas were examined using cross-tables before being investigated further in the modelled risk factor analyses. Specific results for proportion of oyster samples from production areas contaminated with GI or GII are given in Appendix B.

The proportion of NoV-contaminated oyster samples from production area classes A, B and C were 25%, 46% and 65%, respectively, and the mean number of copies per gram detected (in all samples, using the substitution approach as described in Section 2.1) was 143, 459 and 559 (Table 9).

Table 9: Qualitative and quantitative norovirus results in oysters, by status of production area, production areas

Area status	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
A	210	24.7	143	851
B	610	46.5	459	1,312
C	11	64.7	559	17
Total	831	38.1	337	2,180

A winter peak and summer trough was found for NoV in production areas with marked differences observed in positivity and quantitative load of NoV in production area oyster samples during the 2-month periods (2 years of data collapsed, see Table 10). The percentage of NoV contamination was lowest during July–August (17% positive) and highest during January–February (65% positive, also the highest mean cpg at 661), showing an almost fourfold difference in likelihood of positivity, while for the contamination level the lowest period was May–June (111 cpg) with around a sixfold difference between the lowest and highest mean quantity present.

Table 10: Qualitative and quantitative norovirus results in oysters, by two-month period, production areas

Sampling period	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
Jan–Feb	240	64.5	661	372
Mar–Apr	172	46.5	329	370
May–Jun	74	21.2	111	349
Jul–Aug	60	17.3	135	346
Sep–Oct	110	29.9	228	367
Nov–Dec	175	46.5	525	376
Total	831	38.1	337	2,180

Differences were observed in NoV contamination of oyster samples between species of oyster (Table 11). Pacific cupped oysters showed 37% positive with a mean count of 359 cpg; while European flat oysters had a higher proportion of positives (44%) and lower mean count (207 cpg). Very few values were reported for Portuguese cupped oysters.

Table 11: Qualitative and quantitative norovirus results in oysters, by oyster species, production areas

Oyster species	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
European flat oyster	140	44.3	207	316
Oyster, Portuguese cupped	4	33.3	297	12
Pacific cupped oyster	687	37.1	359	1,852
Total	831	38.1	337	2,180

Differences were observed in NoV contamination of oyster samples by production type (different bands of shore proximity, Table 12). The proportion positive and mean quantity were highest from inshore production areas (40%, 424 cpg), while most positive samples originated from intertidal production areas (38%, 310 cpg). Very few values were reported for offshore production areas.

Table 12: Qualitative and quantitative norovirus results in oysters, by type of oyster production area, production areas

Production type	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
Inshore	233	39.8	424	585
Intertidal	589	37.9	310	1,555
Offshore	9	22.5	104	40
Total	831	38.1	337	2,180

Differences were observed in NoV contamination in oysters produced in different systems (Table 13). The proportion positive and mean cpg did not follow similar trends. The lowest positive proportion was among farmed oysters from raised trestle (34%) with a mean quantity of 276 cpg. The highest positive proportion was among farmed bottom-grown oysters (58%) with the lowest mean quantity of 118 cpg. Wild bottom-grown oysters were 53% positive with the highest mean concentration of 590 cpg.

Table 13: Qualitative and quantitative norovirus results in oysters, by production information and production system, production areas

Production	Production system	Sample positive for norovirus		Mean number of copies per gram	Number of samples
		N	%		
Farmed	Bottom-grown	7	58.3	118	12
	Raised trestle	531	33.7	276	1,576
	Suspended from sea surface	89	43.4	335	205
Wild	Bottom-grown	204	52.7	590	387
Total		831	38.1	337	2,180

3.1.2. Norovirus survey results in dispatch centres

3.1.2.1. Raw percentages for norovirus contamination in samples from batches in dispatch centres

Based on the qualitative laboratory analysis results (Table 14), neither GI nor GII was detected in 89.4% of samples from dispatch centres. At least one genogroup (GI or GII) was detected in 10.5% of them, so this was the raw proportion of NoV-contaminated oyster samples from dispatch centres in this survey. Both GI and GII were present together in 39% of those samples in which any NoV was detected. GII was detected more frequently than GI; around 76% of the samples with NoV were positive for GII, while only 61% were positive for GI.

Table 14: Raw percentages for norovirus contamination in samples from dispatch centres

Presence results for GI – GII	N	%
Not detected – Not detected	1,903	89.4
Not detected – Positive	84	4.0
Positive – Not detected	54	2.5
Positive – Positive	88	4.1
Total	2,129	100.00

GI: genogroup I; GII: genogroup II.

The bulk of results (92–93%) were below the LOD for individual genogroups, with 5–6% below the LOQ and 1–2% above the LOQ with a quantified value (Table 15). This created the need for active consideration of how to substitute censored quantitative results in the majority (approximately 98%) of the results.

Table 15: Percentages of samples categorised by methodological limits for each genogroup in dispatch centres

Genogroup	Not detected Below LOD (N)	Not detected Below LOD %	Not quantified Below LOQ (N)	Not quantified Below LOQ %	Quantified Above LOQ (N)	Quantified Above LOQ %	Total (N)
GI	1,987	93.3	108	5.1	34	1.6	2,129
GII	1,957	91.9	130	6.1	42	2.0	2,129

GI: genogroup I; GII: genogroup II; LOD: limit of detection; LOQ: limit of quantification.

3.1.2.2. Norovirus quantification results from dispatch centres

The quantitative results (arithmetic mean, median, mode and geometric mean) taking account of all sample results including the results below the LOD, based on the substitution approach as defined in Section 2.1 is shown in Appendix C, Table C.8 . The overall mean number of NoV copies per gram was 168 with a median and mode of 160.

The proportion of results exceeding quantitative thresholds was examined. Eighty-nine per cent or nearly 9 out of 10 samples from dispatched batches exceeded 100 cpg for GI and GII combined, while only 0.47% or 1 in 50 exceeded 1,000 (Table C.22).

In a substantial proportion of samples (approximately 97%), not detected or positive below LOQ results were obtained for GI and/or GII. In these cases, quantification required substitution with LOD/2 or LOQ/2 values as described earlier. Where arbitrary thresholds close to the typical values for LOD and LOQ (e.g. 200 cpg or lower) were applied, a large proportion of samples exceeding the threshold only did so after substituted values were added to any quantified, non-substituted values (i.e. positive above LOQ.) For higher arbitrary thresholds (e.g. 300 cpg or higher) in more than 81% of samples exceeding the threshold, the sum of quantified GI and GII values alone exceeded the threshold; thus, the values used for substitution had no impact on whether the sample was above or below (Table 16).

Table 16: Proportion of samples from dispatch centres (excluding 1 sample tested with diluted RNA) with quantitative results above arbitrary thresholds, and extent to which substitution of quantitative results contributes to exceeding those thresholds (given in copies per gram, cpg)

Parameter measured	Quantitative threshold (norovirus in cpg)						
	> **100**	> **200**	> **300**	> **500**	> **1,000**	> **5,000**	> **10,000**
% of total samples that exceeded threshold	**89.29%**	**5.59%**	**1.78%**	**1.17%**	**0.47%**	**0%**	**0%**
Total number of samples that exceeded threshold **(N = 2,129)**	**1,901**	**119**	**38**	**25**	**10**	**0**	**0**
Further subdivision of samples that exceed the threshold							
% where both GI and GII results based on substitution approach (not detected or below LOQ), i.e. substitution influences the categorisation as above or below the threshold	96.84%	57.98 %	0.00%	0.00%	0.00%		
% with one quantified value, where that value was below the threshold and where the sum with the substituted value from the other genogroup was above the threshold, i.e. substitution influences the categorisation as above or below the threshold	0.32%	11.76%	18.42%	12.00%	0.00%		
% with one quantified value, where that value was above the threshold or two quantified values, where the sum was above the threshold, i.e. substitution does not influence the categorisation as above or below the threshold	2.84%	30.25%	81.58%	88.00%	100.00%		

GI: genogroup I; GII: genogroup II.

3.1.2.3. Initial descriptive comparison of prevalence and quantification results

Differences in observed prevalence of NoV-contaminated oyster samples and quantification of results from dispatch centre batches were examined using cross-tables before being investigated further in the modelled risk factor analyses. Specific results for proportion of oyster samples from dispatch centre batches contaminated with GI or GII are given in Appendix B.

A winter peak and summer trough was found for NoV in dispatch centre batches, with marked differences observed in positivity and quantitative load of NoV during the two-month periods (two years of data collapsed, see Table 17). Contamination was lowest during July–August (3% positive and mean cpg 147) and highest during January–February (21% positive and mean cpg 204), showing a sevenfold difference in likelihood of positivity and a 1.5-fold difference in mean quantity present.

Table 17: Qualitative and quantitative norovirus results in oysters, by two-month period, dispatch centres

Sampling period	Samples positive for norovirus		Mean number of opies per gram	Number of samples
	N	%		
Jan–Feb	78	21.0	204	373
Mar–Apr	72	19.3	176	374
May–Jun	19	6.1	149	314
Jul–Aug	9	2.9	147	314
Sep–Oct	17	4.6	157	368
Nov–Dec	31	8.1	170	386
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of dispatch centre batches, lowest if sourced from a single production area (10%) and highest when from three production areas (33%) although almost all samples were sourced from a single production area (Table 18).

Table 18: Qualitative and quantitative norovirus results in oysters, by number of production areas that the batches were sourced from, dispatch centres

Number of source production areas	Samples positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
1	199	9.6	168	2,067
2	1	20.0	120	5
3	1	33.3	203	3
Not reported	25	46.3	177	54
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of batches depending on the classification of the production areas from which the oysters originated (Table 19). Batches comprising Class A oysters had the lowest positivity (4%) and quantity (mean 159 cpg) while batches comprising Class B were 20% positive with a mean quantity of 185 cpg.

Table 19: Qualitative and quantitative norovirus results in oysters, by classification of the production areas that were the source of the batch, dispatch centres

Area status	Samples positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
A	48	3.7	159	1,303
B	154	20.0	185	772
Unknown	24	44.4	168	54
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of oyster samples between species of oysters (Table 20). Pacific cupped oysters showed 10% positive with a mean count of 171 cpg; while European flat oysters had a higher proportion of positives (31%) and lower mean count (95 cpg). Very few samples (13) were reported for Portuguese cupped oysters.

Table 20: Qualitative and quantitative norovirus results in oysters, by oyster species, dispatch centres

Oyster species	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
European flat oyster	24	30.8	95	80
Oyster, Portuguese cupped	2	15.4	147	13
Pacific cupped oyster	200	9.8	171	2,036
Total	226	10.6	168	2,129

Quantiles of average oyster weights in the samples collected were estimated to group the samples and the raw proportion of positives of each genogroup and NoV for each group was calculated (see Table 21). Samples with higher oyster weight appeared to be associated with an increasing proportion of positives and an increasing cpg. This was further investigated using the GEE model.

Table 21: Qualitative and quantitative norovirus results in oysters, by mean weight of oysters sampled (in grams) from the batch in dispatch centres

Mean weight of oyster sampled (x)	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
$0 < x \leq 64$	38	7.0	159	544
$64 < x \leq 75$	32	6.0	162	536
$75 < x \leq 91$	47	9.4	167	503
$91 < x \leq 406$	96	18.4	186	523
Not reported	13	56.5	204	23
Total	226	10.7	168	2,129

Quantiles of the batch weights were estimated and the raw proportion of positives of the genogroups and NoV for each groups was calculated (see Table 22). Smaller batch size appeared to be associated with a higher proportion of positives and a higher cpg.

Table 22: Qualitative and quantitative norovirus results in oysters, by weight of the batches sampled (in kg) in dispatch centres

Mean weight of oyster batch (x)	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
$0 < x \leq 100$	97	15.5	183	628
$100 < x \leq 250$	37	8.6	168	433
$250 < x \leq 500$	40	7.6	159	530
$500 < x \leq 40{,}000$	37	7.3	159	510
Not reported	15	53.6	183	28
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of dispatched batches when comparing production types (Table 23). Batches comprising wild oysters had a higher proportion of positives (29%) and a lower mean quantity (106 cpg) than batches comprising farmed oysters (10% and 171 cpg).

Table 23: Qualitative and quantitative norovirus results in oysters, by production information, dispatch centres

Production	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
Farmed	201	9.9	171	2,036
Wild	22	29.0	106	78
Unknown	3	20.0	200	15
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of batches arising from the repacking of previously dispatched oysters (Table 24). Repacked oyster batches had a proportion of NoV-positive samples of 43% and a mean of 169 cpg, while those batches not repacked showed 8% positive and had a mean cpg of 172.

Table 24: Qualitative and quantitative norovirus results in oysters, by production information (repacked or not), dispatch centres

Repacking	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
No	165	8.4	172	1,963
Yes	26	42.6	169	61
Unknown	35	33.3	93.4	105
Total	226	10.6	168	2,129

Differences were observed in the proportions of NoV-positive samples from batches depending on duration of conditioning (Table 25), with the lowest proportion of NoV positives within 1–72 h (5%) and the highest when no conditioning was performed (13%), excluding not reported duration of conditioning.

Table 25: Qualitative and quantitative norovirus results in oysters, by duration (hours) of conditioning, dispatch centres

Duration of conditioning in hours (x)	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
$x = 0$	72	13.1	192	548
$0 < x \leq 72$	26	5.4	164	484
$72 < x \leq 144$	30	5.9	164	509
$144 < x \leq 2{,}400$	26	6.3	167	411
Not reported	72	41.1	124	177
Total	226	10.6	168	2,129

Differences in the observed prevalence of NoV-contaminated oyster samples between different classes of duration of relaying are shown in Table 26.

Table 26: Qualitative and quantitative norovirus results in oysters, by duration (days) of relaying, dispatch centres

Relaying	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
No	147	7.7	174	1,916
Yes	2	33.3	59.4	6
Not reported	77	37.6	118	207
Total	226	10.6	168	2,129

Differences were observed in NoV contamination of batches which had been subject to purification (Table 27), with the lowest proportion positive in the quartile with no purification and highest positivity in the third quartile of purification (performed for less than or equal to 48 hours).

Table 27: Qualitative and quantitative norovirus results in oysters, by duration (hours) of purification, dispatch centres

Duration of purification (x)	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
x = 0	28	2.4	159	1,183
0 < x ≤ 48	49	25.5	173	192
48 < x ≤ 2,400	76	17.2	185	441
Not reported	73	23.3	177	313
Total	226	10.6	168	2,129

From the samples for which purification was reported, 86% originated from oysters that were purified by ambient-temperature water, 13% by actively cooled water and 0.5% by actively heated water (Table 28). Differences were found in NoV contamination depending on the purification temperature of the water used. Active heating or cooling of purification water was associated with a higher proportion of batches positives than ambient purification. Definitions of classes are presented in EFSA (2016) and are not expressed in degrees.

Table 28: Qualitative and quantitative norovirus results in oysters, by thermal status of the water used for purification, dispatch centres

Purification conditions	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
Actively cooled	34	29.6	154	115
Actively heated	3	75.0	360	4
Ambient	118	16.6	189	713
Unknown	71	5.5	157	1,297
Total	226	10.6	168	2,129

Differences were observed in the proportion of positive batches when comparing different classes of water temperature in the purification tank (Table 29); lowest in the warmest water (4%) and highest in the coolest (30%).

Table 29: Qualitative and quantitative norovirus results in oysters, by temperature (Celsius) of water in purification tank, dispatch centres

Purification temperature (x)	Sample positive for norovirus		Mean number of copies per gram	Number of samples
	N	%		
0 ≤ x ≤ 10	62	29.8	202	208
10 < x ≤ 13	50	20.8	181	240
13 < x ≤ 16	36	15.9	202	226
16 < x ≤ 26	6	4.0	151	152
Not reported	72	5.5	157	1,303
Total	226	10.6	168	2,129

3.2. Modelling results

3.2.1. Apparent norovirus prevalence and its trend over time

The GEE model considering the standardised weights described in Section 2.3 has been used to estimate the overall apparent NoV prevalence for production areas and batches in dispatch centres. The apparent prevalence with confidence intervals is shown in Figure 8. It was considerably lower

for batches sampled in dispatch centres than for samples taken in production areas (Figure 8 and Table 30).

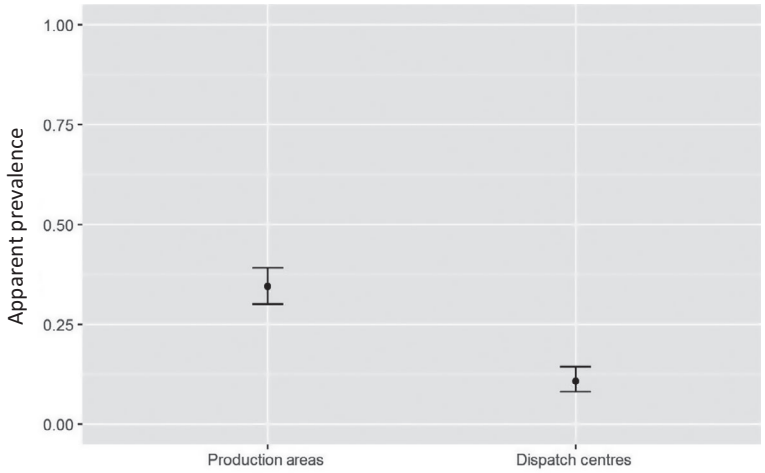

Figure 8: Overall apparent prevalence of norovirus in production areas and batches from dispatch centres

Table 30: Estimate of the overall apparent norovirus prevalence in production areas and batches from dispatch centres and their confidence intervals

	Apparent prevalence	Lower bound	Upper bound
Production areas	0.345	0.301	0.391
Dispatch centres	0.109	0.082	0.144

In order to explore the effect of the diagnostic sensitivity and specificity on the prevalence estimations for NoV, a range of sensitivity values for a set of specificity values was used. The range of values used for the diagnostic characteristics was appropriate given that the diagnostic method used is RT-PCR, in general being highly specific and rather sensitive (EFSA, 2012). Figure 9 shows the effect of the test characteristic on the estimated apparent prevalence for the estimated values in production areas and in batches from dispatch centres. This figure shows that when specificity decreases, the estimated true prevalence is underestimated, but the opposite effect is seen for the sensitivity, with a maximum difference of 3% for batches in dispatch centres and around 8% in production areas. It is important to note that although the exclusion of considerations of sensitivity and specificity from the models will have had an impact on the absolute values for prevalence obtained, the conclusions such as those regarding factors contributing to NoV prevalence will not have been affected.

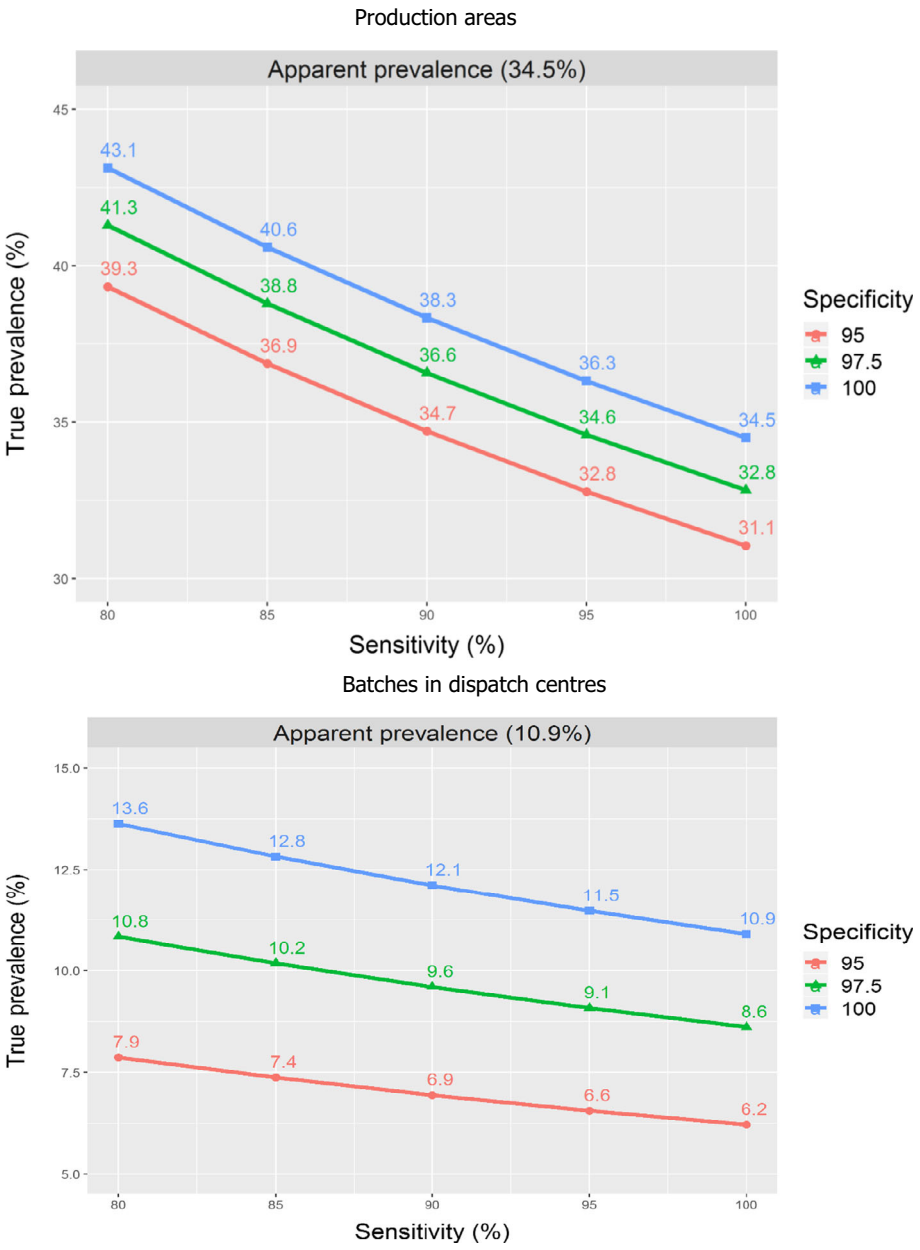

Figure 9: Estimated true prevalence of norovirus given different hypothetical sensitivity and specificity values for the RT-PCR test used to detect norovirus

3.2.1.1. Time trend prevalence for norovirus

The temporal trend was estimated using the GEE model based on a restricted cubic spline to model the prevalence over the year, also including the standardised weighting. The results for production areas and dispatch centres are shown in Figures 10 and 11. The lowest prevalence is found in the period July–August (see Table 31), and the highest in January–February.

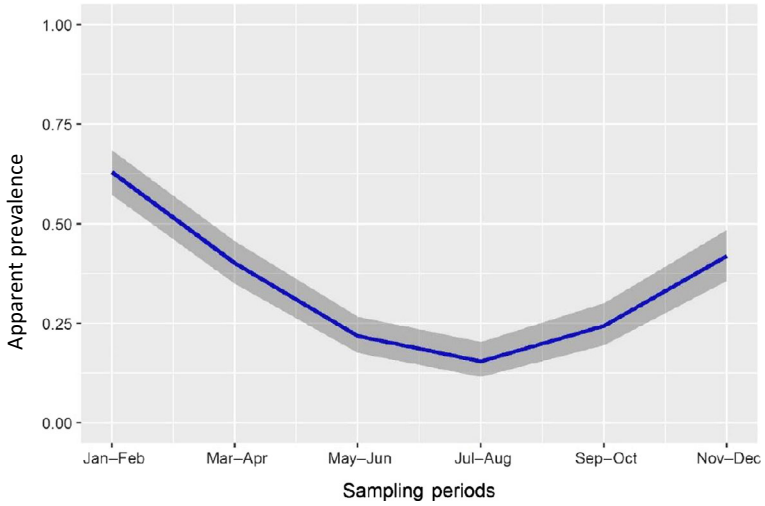

Figure 10: Apparent prevalence trend over a year in production areas and its confidence interval

Table 31: Estimated apparent prevalence of norovirus trend over a year in production areas and confidence interval for each sampling period

Period	Estimated prevalence	Lower bound	Upper bound
Jan–Feb	0.630	0.572	0.684
Mar–Apr	0.401	0.349	0.456
May–Jun	0.218	0.176	0.267
Jul–Aug	0.155	0.116	0.203
Sep–Oct	0.244	0.195	0.301
Nov–Dec	0.419	0.356	0.484

The temporal trend of the apparent prevalence for batches from dispatch centres mirrors that for production areas (Figure 11), although the prevalence levels are smaller in absolute terms (Table 32). However, the increase in prevalence observed in the months November and December in production areas (2.7 times higher in this period compared with the period with the lowest prevalence) is steeper than that observed in batches sampled in dispatch centres (1.7 times).

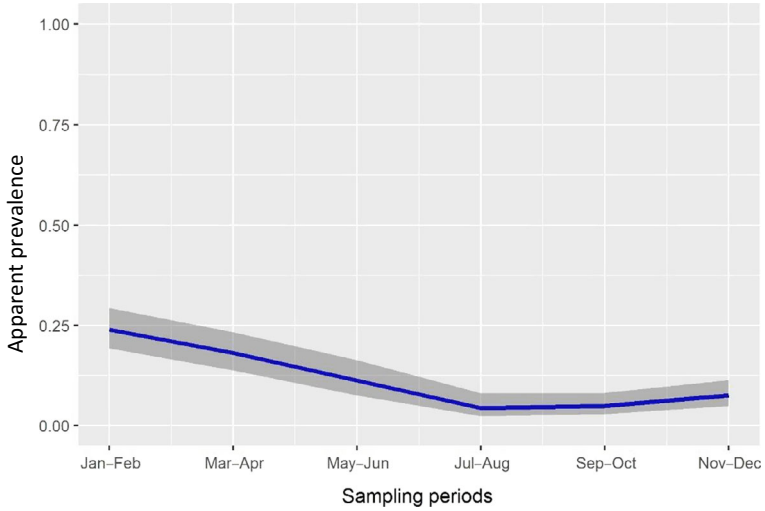

Figure 11: Apparent prevalence of norovirus trend over a year in batches from dispatch centres and its confidence interval

Table 32: Estimated apparent prevalence of norovirus trend over a year in batches in dispatch centres and confidence interval for each sampling period

Period	Apparent prevalence	Lower bound	Upper bound
Jan–Feb	0.239	0.193	0.293
Mar–Apr	0.181	0.138	0.233
May–Jun	0.112	0.076	0.164
Jul–Aug	0.044	0.023	0.081
Sep–Oct	0.049	0.029	0.081
Nov–Dec	0.075	0.049	0.114

3.2.1.2. Multiple imputation to estimate norovirus prevalence

When multiple imputations are used in order to account for missing data, the resulting apparent prevalence time trend for NoV shows a very similar pattern to the one reported in the previous section, where only valid test results were considered for both production areas (see Table 33) and batches from dispatch centres (see Table 34). No significant differences in either the overall trend or the width of the confidence intervals obtained were found. For this reason, analysis of the hypotheses listed in Section 2.3.1.2 and described in the following sections used only valid laboratory results instead of multiple imputations.

Table 33: Apparent prevalence trend over a year in production areas considering multiple imputations of missing values and confidence intervals for each sampling period, together with the apparent prevalence and confidence intervals modelled using only valid results

Period	Multiple imputation estimate	Estimate	Multiple imputation lower bound	Lower bound	Multiple imputation upper bound	Upper bound
Jan–Feb	0.620	0.630	0.561	0.572	0.676	0.684
Mar–Apr	0.395	0.401	0.343	0.349	0.449	0.456
May–Jun	0.218	0.218	0.177	0.176	0.265	0.267
Jul–Aug	0.161	0.155	0.123	0.116	0.209	0.203
Sep–Oct	0.250	0.244	0.201	0.195	0.305	0.301
Nov–Dec	0.415	0.419	0.353	0.356	0.481	0.484

Table 34: Apparent prevalence of norovirus trend over a year in batches from dispatch centres considering multiple imputation of missing values and confidence intervals for each sampling period, together with apparent prevalence and confidence intervals using only valid results

Period	Estimate GEE multiple imputation	GEE estimate	GEE multiple imputation lower bound	GEE lower bound	GEE multiple imputation upper bound	GEE upper bound
Jan–Feb	0.234	0.240	0.188	0.193	0.287	0.293
Mar–Apr	0.173	0.181	0.133	0.138	0.223	0.233
May–Jun	0.109	0.112	0.075	0.076	0.156	0.164
Jul–Aug	0.047	0.044	0.028	0.023	0.081	0.081
Sep–Oct	0.052	0.049	0.033	0.029	0.081	0.082
Nov–Dec	0.075	0.075	0.049	0.049	0.114	0.114

GEE: generalised estimating equations.

3.2.2. Quantification of norovirus in production areas

3.2.2.1. Quantification of norovirus without time effect

The model including weightings as defined in Section 2.3.1.1, censoring considering the different patterns described in Section 2.3.2 and accounting for potential correlation between samples taken from the same production area, results in a mean number of copies per gram of 62 (CI: 27–149). The

convergence of the model run was verified and the fit was evaluated using the Pareto k estimates, which confirm that the model adequately fits the data (values obtained below 0.7, with 2,127 out of 2,140 observations which were below 0.5).

Table 35: Summary statistics of the probability of exceeding a particular threshold considering substitution and a lognormal model

Parameter measured	Quantitative threshold (norovirus in cpg)						
	> 100	> 200	> 300	> 500	> 1,000	> 5,000	> 10,000
% of total samples that exceed threshold based on substitution approach as described in Section 2.1	**66.2**	**17.2**	**11.9**	**8.7**	**5.5**	**1.2**	**0.2**
% of total samples that exceed threshold based on lognormal model as described in Section 2.3.2 (mean and credible intervals)	**19.2 (17.1–21.6)**	**13.3 (11.6–15.0)**	**10.5 (9.0–12.0)**	**7.6 (6.3–8.9)**	**4.7 (3.6–5.7)**	**1.1 (0.6–1.7)**	**0.6 (0.3–1)**

Table 35 shows that the model estimates the percentage of total samples close to the ones in which substitutions were used, when the threshold values are larger than 300. Note that in general, credible intervals for thresholds above 300 contain the raw percentages based on a simple substitution approach. This indicates that simply using the substitution approach might overestimate the proportion of samples above thresholds that are close to or below the LOQ values.

3.2.2.2. Quantification of norovirus and its distribution over time

The temporal trend of norovirus numbers of copies per gram estimated by the fitted model described in Section 2.3.2 is shown in Figure 12. The convergence of the model and the fit was verified. The Pareto k estimates confirm that the model fitted the data adequately (values obtained below 0.7, with 2,084 out of 2,140 observations below 0.5). Based on the loo criteria the model that included temporal trend resulted in a better fit than the one without.

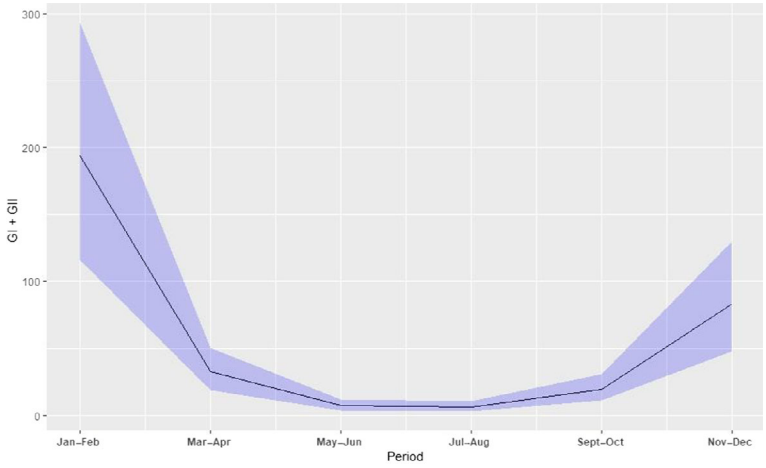

Figure 12: Temporal trend with 95% credible intervals of estimated mean numbers of norovirus copies per gram

The fitted temporal trend is similar to the one presented for the apparent prevalence estimation (see Figure 10), with on average larger numbers of copies per gram in the winter, with the highest number of copies per gram in the January–February period. The credible interval is wider in the winter months than in the summer months.

3.2.3. Quantification of norovirus in dispatch centres

3.2.3.1. Quantification of norovirus without time effect

The model including weightings as defined in Section 2.3.1.1, censoring considering the different patterns described in Section 2.3.2 and accounting for a potential correlation between samples taken from the same production area, results in a mean number of copies per gram of 22 (CI: 7–72). The convergence of the model run was verified and the fit was evaluated using the Pareto k estimates, which confirm that the model fits the data adequately (values obtained below 0.7, with 2,114 out of 2,129 observations which were below 0.5).

Table 36: Summary statistics of the probability of exceeding a particular threshold considering substitution and lognormal model

Parameter measured	Quantitative threshold (norovirus in cpg)						
	> 100	> 200	> 300	> 500	> 1,000	> 5,000	> 10,000
% of total samples that exceed threshold based on substitution approach as described in Section 2.1	**89.3**	**5.6**	**1.8**	**1.2**	**0.5**	**0.0**	**0.0**
% of total samples that exceed threshold based on lognormal model as described in Section 2.3.2 (mean and credible intervals)	**8.4 (6.0–11.5)**	**3.8 (2.7–5.2)**	**2.3 (1.5–3.2)**	**1.1 (0.6–1.7)**	**0.4 (0.1–0.8)**	**0.02 (0.00–0.13)**	**0.01 (0.00–0.07)**

Table 36 shows that the model estimates the percentage of total samples close to the ones in which substitutions were used, when the threshold values are larger than 300. Note that in general, credible intervals for thresholds above or equal to 300 contain the raw percentages based on the simple substitution approach. This indicates that simply using a substitution approach might overestimate the proportion of samples above thresholds that are close to or below LOQ values.

3.2.3.2. Quantification of norovirus and its distribution over time

The temporal trend of the fitted model described in Section 2.3.2 (Figure 13), mirrors the trend observed in production areas but not the extent. The convergence of the model was verified, together with the goodness of fit. The Pareto k estimates confirm that the model fitted the data adequately (values obtained below 0.7, with 2,086 out of 2,129 observations below 0.5). Based on the loo criteria, the model including temporal trend resulted in a better fit than the one without.

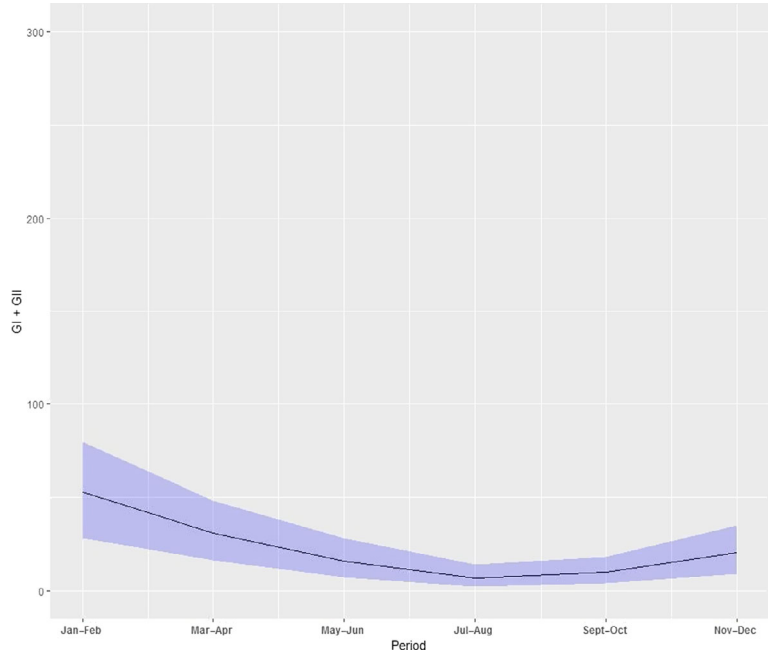

Figure 13: Temporal trend with 95% credible intervals of estimated mean numbers of norovirus copies per gram

The fitted temporal trend is similar to the one presented for the apparent prevalence estimation (see Figure 11), with on average larger numbers of copies per gram in the winter, with the highest number of copies per gram in the January–February period. The credible interval was wider in the winter months than in the summer months.

3.2.4. Factors associated with norovirus

3.2.4.1. Factors associated with norovirus prevalence

In Section 2.3.1.2 a list of hypotheses is provided. Appropriate models were fitted in order to test the specified hypotheses. The first two hypotheses have already been explored in Section 3.2.1, indicating that in general, a higher apparent NoV prevalence is observed in production areas than in batches from dispatch centres. Although a direct comparison is not statistically possible, since this baseline survey was not designed to make such a comparison, it nevertheless provides a representation of the expected prevalence in both parts of the food chain.

The next hypothesis tested related to the comparison of the apparent prevalence in Class A areas with that in other classes (B and C). The three models described in Section 2.3.1.2 were fitted, showing that the additive model $\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class}\right)$ was the best in terms of QIC (see Figure 14) In all sampling periods the apparent prevalence was lower in samples collected from Class A production areas than in samples collected from other classes.

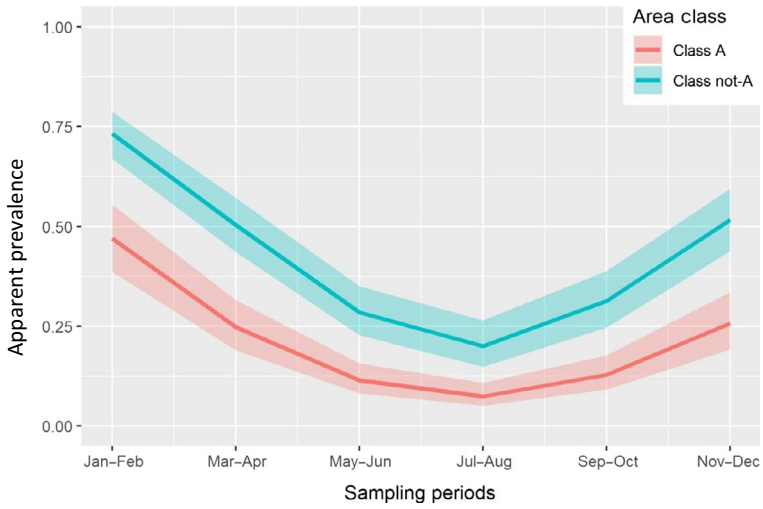

Figure 14: Apparent prevalence of norovirus trend over a year in production areas for the model containing area class, differentiating Class A areas from others

In order to compare the temporal trends between genogroups (GI and GII) and the apparent prevalence, the three models were fitted, with the multiplicative model

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Genogroup} + \mathrm{f}(\text{Time Period}) \times \text{Genogroup}\right)$$

fitting best according to QIC criteria (Figure 15). This model includes the interaction effect, implying that time trends are different between genogroups. In particular, the apparent prevalence of GII is higher than that of GI in the periods January–February and November–December but lower in the periods July–August and September–October.

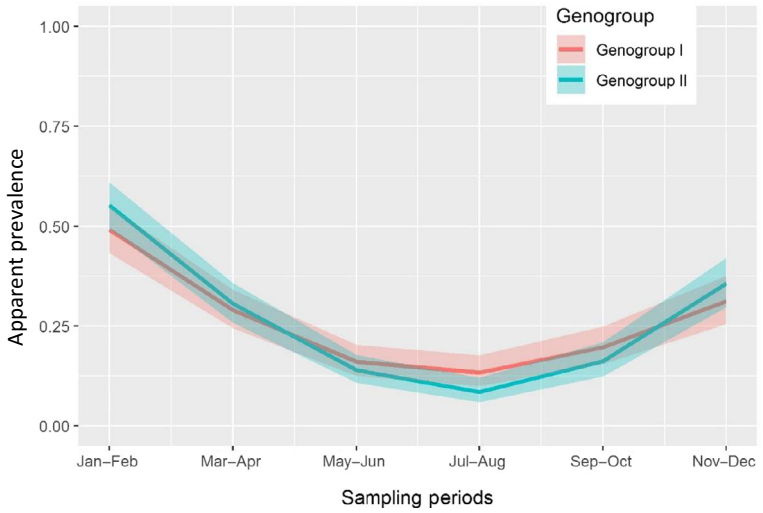

Figure 15: Apparent prevalence of norovirus trend over a year in production areas for each genogroup

Exploring differences between production types, the additive model

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Production Type}\right)$$

produced the best fit. The apparent prevalence of farmed and wild production areas is shown in Figure 16. The apparent prevalence in production areas with wild harvested oysters was higher in all periods than that for farmed oysters.

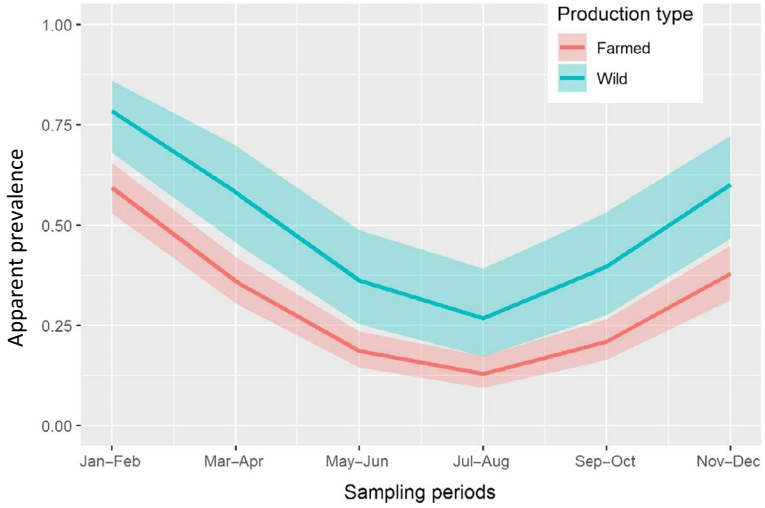

Figure 16: Apparent prevalence of norovirus over a year in farmed and wild production areas

In order to study the effect of animal weight on the apparent NoV prevalence, models for production areas and dispatch centres were fitted. The best fitting model was the additive

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Mean Animal Weight}\right)$$

for production areas and the multiplicative

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Mean Animal Weight} + \mathrm{f}(\text{Time Period}) \times \text{Mean Animal Weight}\right)$$

for dispatch centres. The results of the best models for the mean and the 5th and 95th percentiles of the reported mean animal weights in the samples for production areas and batches in dispatch centres are shown in Figures 17 and 18, respectively.

The apparent prevalence increased with increasing mean animal weights for both production areas and batches from dispatch centres.

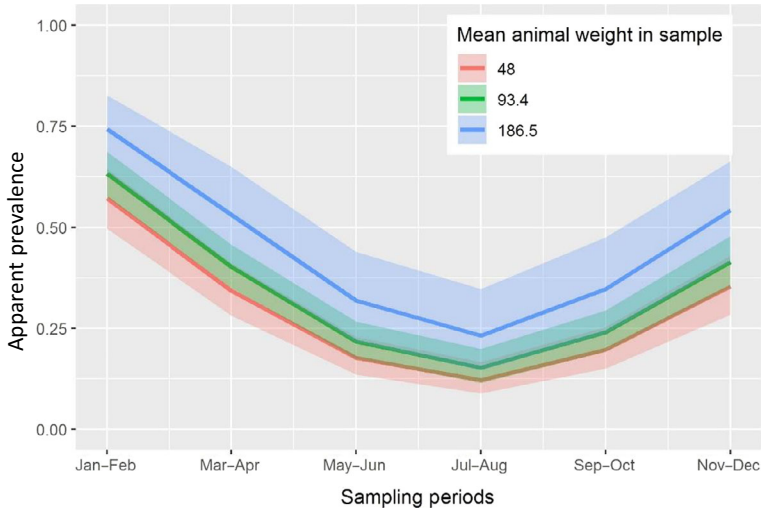

Figure 17: Apparent prevalence of norovirus over a year for different mean oyster weights in the sample taken in production areas

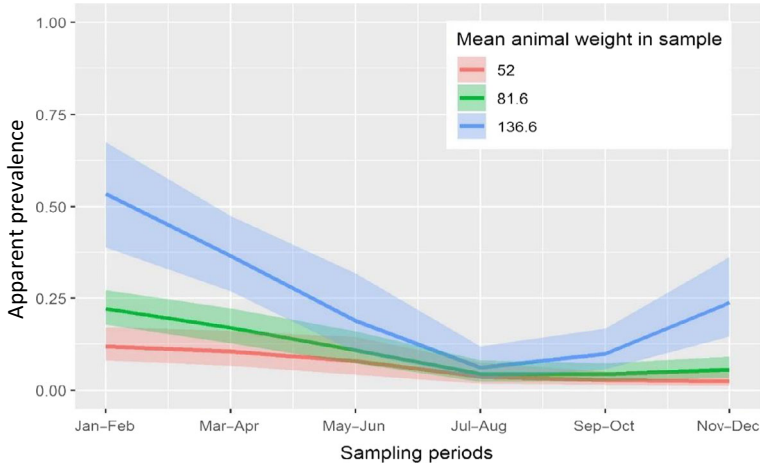

Figure 18: Apparent prevalence of norovirus over a year for different mean oyster weights in the sample taken in batches dispatched in dispatch centres

The production area type (intertidal or inshore; offshore production areas were excluded due to the very small number of samples from this type of production area) was also considered as a potential factor that could show difference in apparent NoV prevalence. The best fitting model, which is shown in Figure 19 was the additive one

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Type}\right)$$

Apparent prevalence was higher in oysters grown inshore than in those grown in intertidal areas.

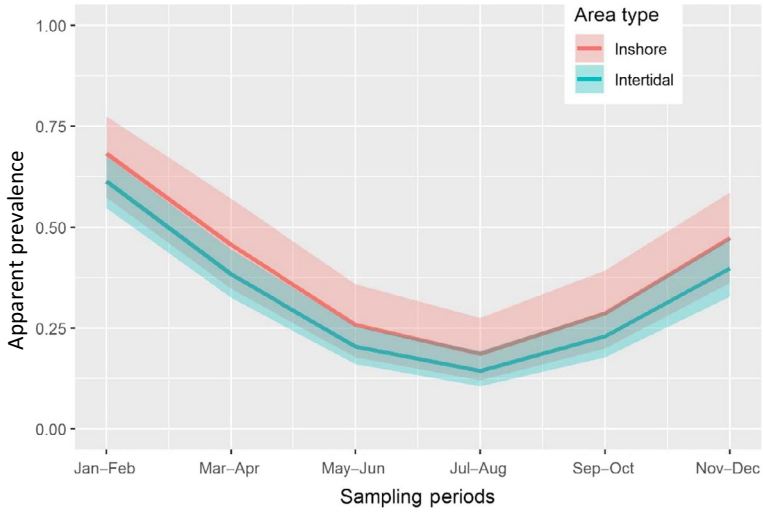

Figure 19: Apparent prevalence of norovirus over a year for inshore and intertidal production areas

The oyster species were also considered as a potential modifying factor on the apparent prevalence of NoV. The additive model

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Oyster Species}\right)$$

best fitted the data (Figure 20). The apparent prevalence was higher for European flat oysters (*Ostrea edulis*) than for Pacific cupped oysters (*Crassostrea gigas*).

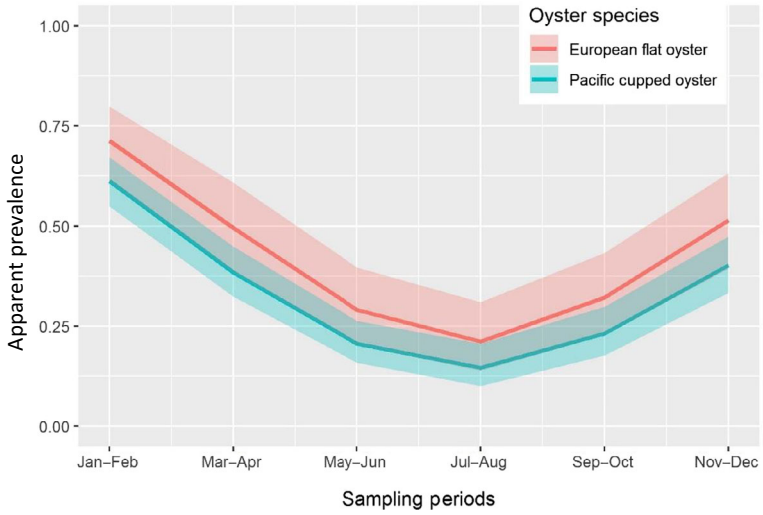

Figure 20: Apparent prevalence of norovirus over a year in production areas with different oyster species

For batches from dispatch centres, the model including the additive effect containing the classification of the production area where the oysters originated

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class}\right)$$

provided the best fit. The apparent prevalence for batches harvested from Class A and Class B areas is shown in Figure 21. These followed the same pattern as for Class A and 'other' samples (Class B or C – note no Class C samples from dispatch centres were taken) from production areas, with a lower apparent prevalence for Class A in both types of samples.

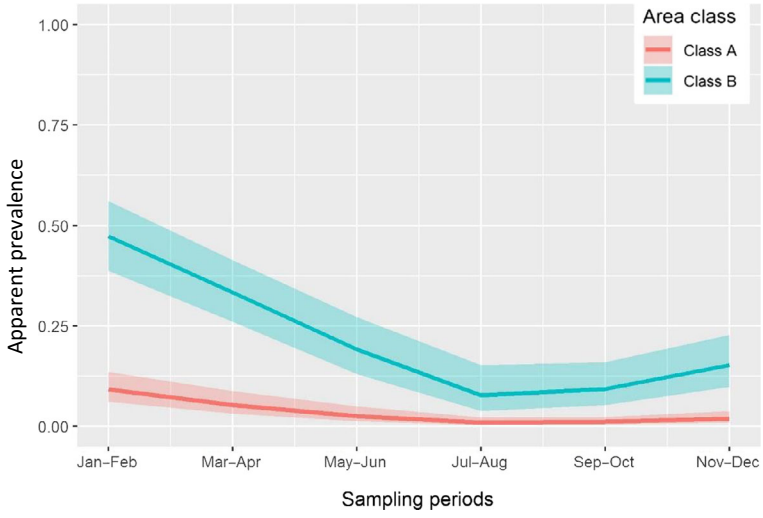

Figure 21: Apparent prevalence of norovirus over a year in batches from dispatch centres originating from Class A and B production areas

In order to assess whether the apparent prevalence for batches from dispatch centres originating in Class A and Class B areas was affected by measures taken in the dispatch centres such as conditioning, or any other intervention (relaying or purification), a model including this factor was also fitted. Figures 22 and 23 show the best fitting models. The best fitting model with conditioning included an interaction of conditioning with the area classification

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class} + \text{Conditioning} + \text{Area Class} \times \text{Conditioning}\right),$$

while the best fitting model with intervention included an interaction term between area classification and time

$$\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class} + \text{Intervention} + \mathrm{f}(\text{Time Period}) \times \text{Area Class}\right).$$

For batches from dispatch centres originating from Class A areas, the apparent prevalence was lower in the presence of conditioning; while for batches originating from Class B areas no difference was seen.

For batches from dispatch centres originating from Class A areas, the apparent prevalence was higher where other interventions (purification and/or relaying) were carried out (all samples from Class B areas were subject to these types of intervention).

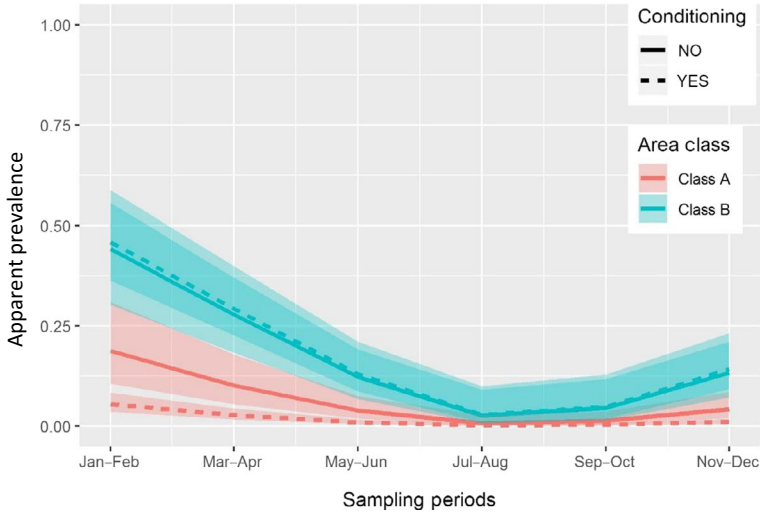

Figure 22: Apparent prevalence of norovirus over a year in batches from dispatch centres originating from Class A and B production areas with or without conditioning

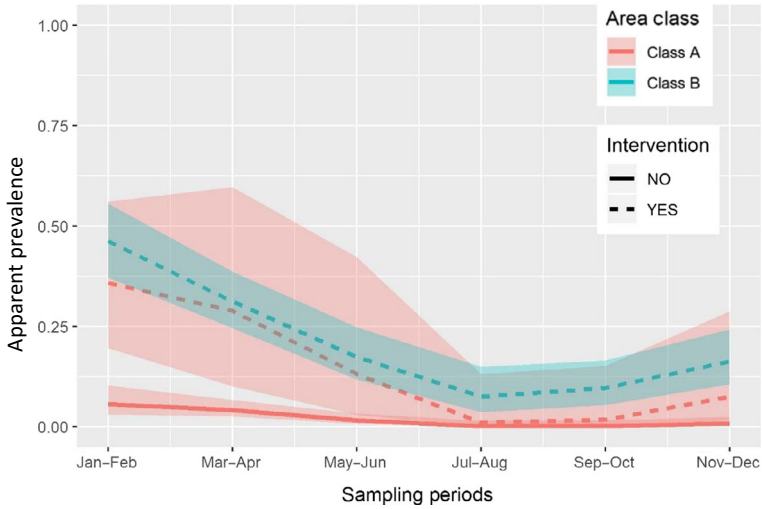

Figure 23: Apparent prevalence of norovirus over a year in batches from dispatch centres originating from Class A and B production areas in combination with other interventions (relaying or purification)

Considering the factors identified above that were associated with differences in the NoV prevalence time trend, a model-building process took place fitting all potential combinations of these factors as well as their two-way interactions for production areas and batches sampled in dispatch centres.

The best model based on the QIC of all models fitted for production areas contained the area classification, the area type, the production type, and the mean weight of the animal, including in addition the interactions between area classification and area type, and area classification and production type. The result of the model is shown in Figure 24. Inshore production areas consistently showed differences between Class A and other classes, but with increased mean weight of the oysters the differences became smaller. For intertidal production types in Class B or C areas, wild oysters show a higher prevalence than farmed oysters, but when the area classification is A, the opposite is observed.

For batches sampled in dispatch centres, the best model contained the area classification, the presence or absence of both conditioning and other interventions (relaying and/or purification), and the mean weight of the animals, including in addition the interactions between area classification and the period of sampling, and the mean weight of the animal and the presence or absence of conditioning, respectively. The result from the best model is shown in Figure 25. The most obvious observation was the difference between oysters coming from Class A or B production areas. Another difference observed was that interventions in general are associated with higher estimated prevalence, consistent with the hypothesis that these interventions take place when the oysters in the batches have higher pre-intervention NoV contamination.

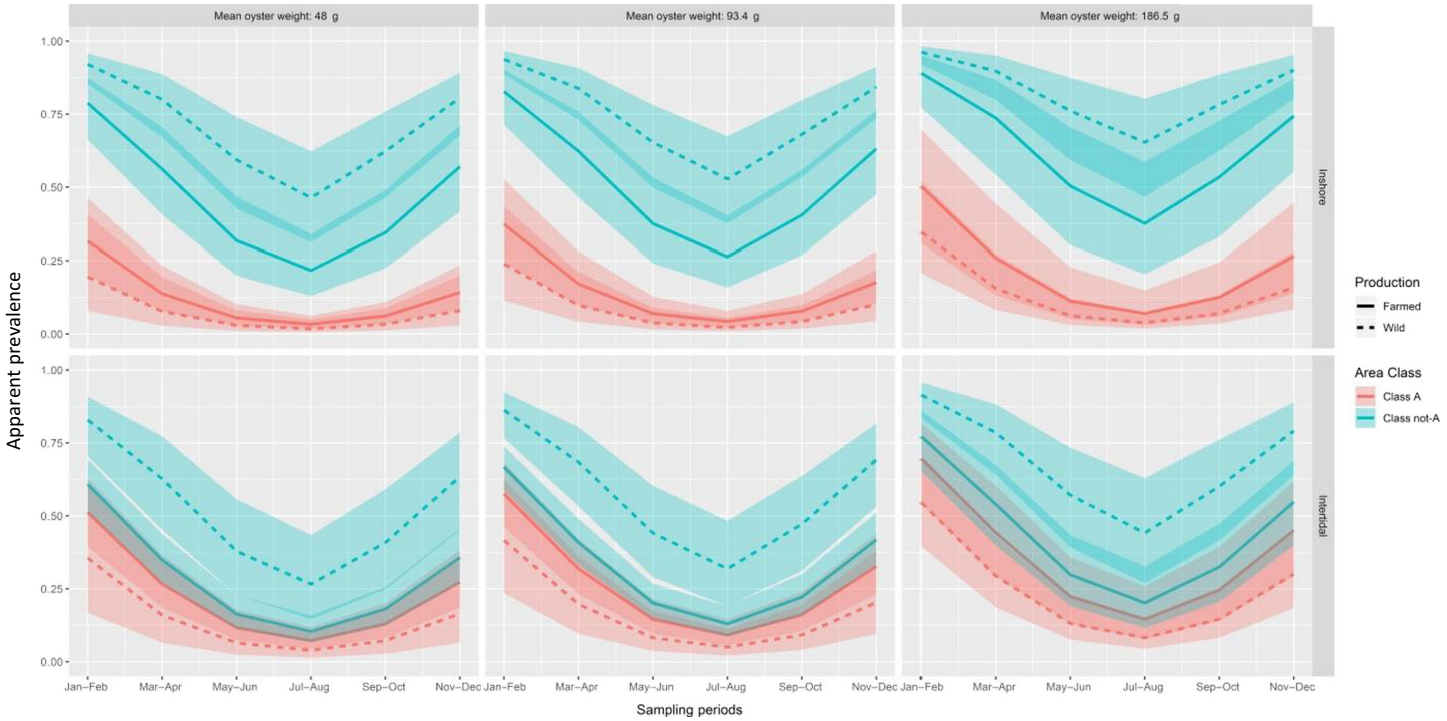

Figure 24: Prevalence of norovirus over a year from Class A and other class production areas in combination with area type, production type and mean weight of oysters sampled

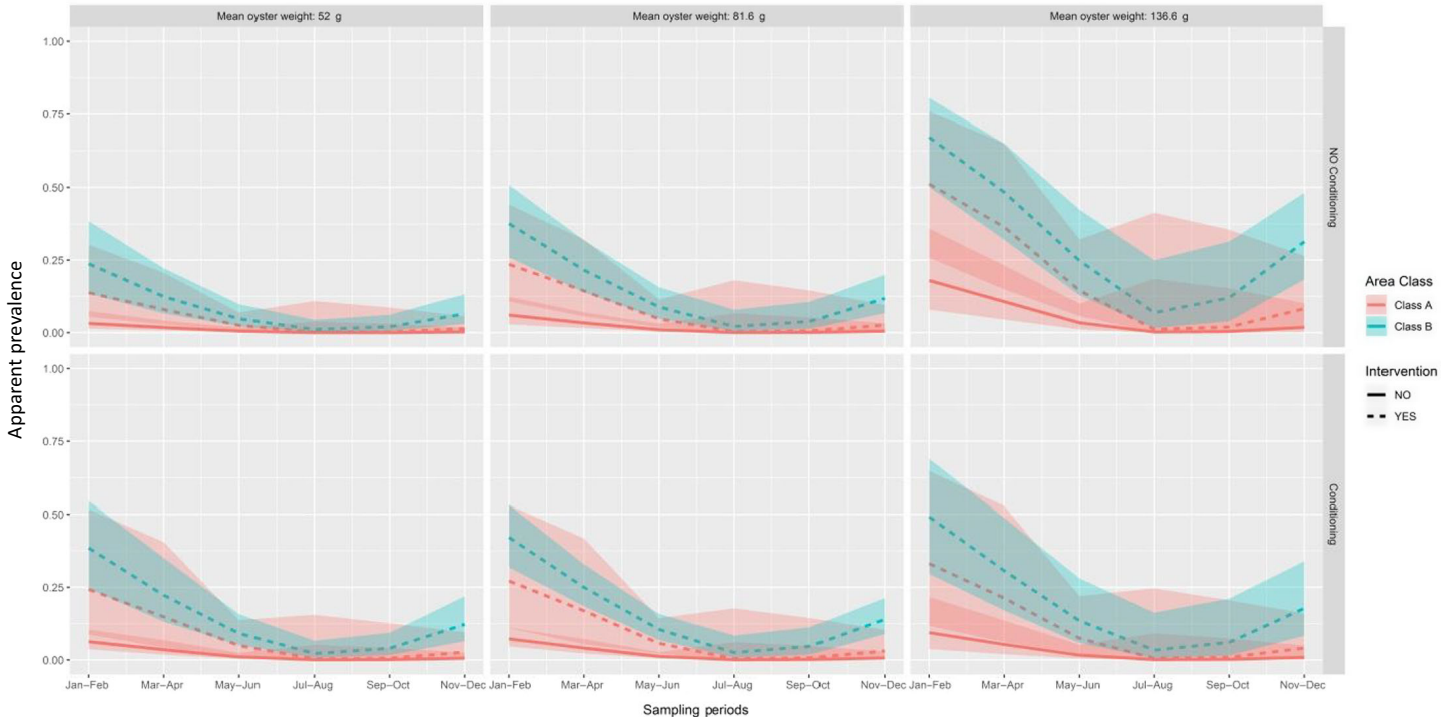

Figure 25: Prevalence of norovirus over a year in batches from dispatch centres originating from Class A and B production areas in combination with conditioning, interventions (relaying and/or purification) and mean weight of oysters sampled

3.2.4.2. Factors associated with the norovirus quantitative results

It was not computationally feasible to perform a full model-building exercise for the quantitative NoV results. Therefore, only those factors identified in the prevalence model (Section 3.2.4.1) were considered in the quantitative model for production areas.

Production areas

The model was fitted considering the area classification (A versus other classes), the area type (intertidal versus inshore), production type (farmed versus wild), and the mean weight of the sampled animals, including the interactions between area classification and area type as well as area classification and production type. Mean animal weight was not found to be significant (95% confidence interval includes zero). The convergence of the model was assessed and the fit was evaluated using the Pareto k estimates, which confirmed that the model fitted the data adequately (values obtained below 0.7, with 2,069 out of 2,140 observations which were below 0.5). The model fitted to the quantified NoV results retained the interactions between area classification and area type as well as area classification and production type, given that the credible intervals for the parameters associated with the factor did not contain the value zero. Figure 26 presents the results of the final model by production type and production area class. The predicted marginal effect of area class by period is shown in Figure 26.

Figure 26 shows that Class B and C areas have higher cpg counts and wild production in Class B and C areas also present higher quantitative NoV results. The intertidal/inshore effect shows that intertidal areas for Class B or C areas have a decreased level of norovirus, but the effect is different for Class A areas; intertidal areas are a little more contaminated than inshore Class A areas. The variation between production areas was found to be statistically significant after accounting for other factors, but as interest lies on population estimations, the focus will be on estimates for an average production area. Figure 26 underlines the effect of area class on the level of contamination.

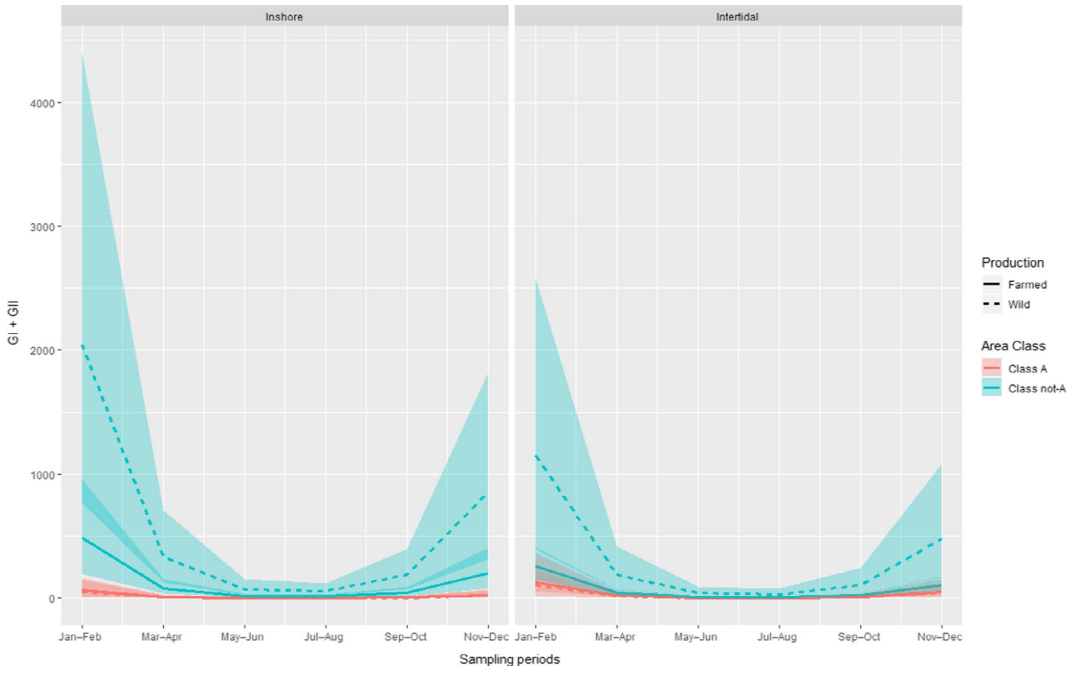

Figure 26: Number of norovirus copies per gram over a year from Class A and other class production areas in combination with area type and production type, shown by area type

Table 37: Summary statistics of the percentage of production areas exceeding a particular threshold considering the lognormal model

Period	Quantitative threshold (norovirus)						
	> 100	> 200	> 300	> 500	> 1,000	> 5,000	> 10,000
All periods pooled (*)	**19.2 (17.1–21.6)**	**13.3 (11.6–15.0)**	**10.5 (9.0–12.0)**	**7.6 (6.3–8.9)**	**4.7 (3.6–5.7)**	**1.1 (0.6–1.7)**	**0.6 (0.3–1)**
Jan–Feb	43.1 (36.7–48.9)	32.7 (27.4–38.3)	27.1 (22.3–31.9)	20.8 (16.7–25.3)	13.8 (10.4–17.5)	4.1 (2.3–6.1)	2.1 (0.8–3.8)
Mar–Apr	19.1 (15.5–23.1)	12.6 (9.4–16.0)	9.6 (6.9–12.7)	6.6 (4.5–9.1)	3.7 (2.0–5.7)	0.7 (0–1.7)	0.3 (0–0.9)
May–Jun	6.8 (4.2–9.8)	3.9 (1.9–6.1)	2.7 (1.0–4.5)	1.6 (0.4–3.3)	0.7 (0–1.9)	0.1 (0–0.6)	0.0 (0.0–0.3)
Jul–Aug	5.7 (3.2–8.5)	3.2 (1.4–5.2)	2.1 (0.6–3.8)	1.2 (0.3–2.6)	0.5 (0–1.5)	0 (0–0.4)	0 (0–0.3)
Sep–Oct	13.9 (10.1–17.8)	8.8 (6.1–12.0)	6.5 (4.1–9.0)	4.3 (2.4–6.6)	2.3 (0.8–3.9)	0.3 (0.0–1.1)	0.1 (0–0.6)
Nov–Dec	29.9 (24.5–35.6)	21.0 (16.7–25.7)	16.6 (12.9–20.8)	11.9 (8.8–15.4)	7.2 (4.8–10.0)	1.7 (0.5–3.1)	0.7 (0–1.8)

*: Not accounting for temporal trend based on the lognormal model as described in Section 2.3.2.

Table 37 shows that percentages of samples above pre-specified thresholds vary across the year, which was already highlighted when the temporal trend was studied. The periods November–December and January–February showed higher percentage of samples above the thresholds than overall, being up to four times higher for the January–February period than overall.

Dispatch centres

Models designed to explore factors associated with the quantitative NoV results will not be fitted given that only 14 samples submitted were quantified above the LOQ for both genogroups, implying that more than 99 % of the sampled batches in dispatch centres are censored. Considering the censoring percentage, any exploration of the factors potentially associated with the quantitative NoV results will be fully model driven, since less than 1% of the batches can be used to assess the fit. For this reason, only the model containing the time effect was used. The resulting percentage of samples from the lognormal model that are expected to be above the pre-specified thresholds are shown in Table 38.

Table 38: Summary statistics of the percentage of batches exceeding a particular threshold considering the lognormal model

Period	Quantitative threshold (norovirus)						
	> 100	> 200	> 300	> 500	> 1,000	> 5,000	> 10,000
All periods pooled (*)	**8.4 (6.0–11.5)**	**3.8 (2.7–5.2)**	**2.3 (1.5–3.2)**	**1.1 (0.6–1.7)**	**0.4 (0.1–0.8)**	**0.0 (0.0–0.1)**	**0.00 (0.0–0.1)**
Jan–Feb	20.2 (13.1–28.7)	10.6 (6.3–15.7)	6.8 (3.7–10.5)	3.6 (1.5–6.2)	1.4 (0.3–3.0)	0.1 (0.0–0.5)	0.0 (0.0–0.5)
Mar–Apr	13 (8.3–18.6)	6.4 (3.4–9.7)	4.0 (1.9–6.5)	2.1 (0.5–4.1)	0.8 (0.0–2.0)	0.0 (0.0–0.6)	0.0 (0.0–0.0)
May–Jun	6.4 (2.7–11.1)	2.6 (0.6–5.2)	1.4 (0.0–3.3)	0.6 (0.0–2.0)	0.2 (0.0–0.9)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
Jul–Aug	2.3 (0.3–5.1)	0.8 (0.0–2.4)	0.4 (0.0–1.7)	0.2 (0.0–1.0)	0.1 (0.0–0.5)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
Sep–Oct	3.6 (1.2–6.5)	1.4 (0.3–2.9)	0.7 (0.0–2.0)	0.3 (0.0–1.2)	0.1 (0.0–0.7)	0.0 (0.0–0.0)	0.0 (0.0–0.0)
Nov–Dec	7.5 (3.7–12.1)	3.2 (1.3–5.8)	1.8 (0.5–3.6)	0.8 (0.0–1.9)	0.3 (0.0–0.9)	0.0 (0.0–0.2)	0.0 (0.0–0.0)

*: Not accounting for temporal trend based on a lognormal model as described in Section 2.3.2.

Table 38 shows that percentages of samples above pre-specified thresholds vary across the year, which was already highlighted when the temporal trend was studied. Here as well, the periods November–December and January–February showed higher percentages of samples above the thresholds than overall, being up to three times higher for the January–February period than overall.

4. Discussion

4.1. Overview and representativeness of survey

This survey targeting NoV in live raw oysters consisted of two separate sub-surveys. It started in November 2016 and ran for a period of 24 months. The baseline survey at production areas was conducted by 12 Member States and Norway, whereas the one at dispatch centres was carried out by the same 12 Member States. This was in accordance with a sampling plan designed to ensure the representativeness of EU production areas and dispatch batches, where specific sample numbers were allocated to Member States in accordance with their contribution to EU production and dispatch.

In general, the sampling design was implemented through the effort of the countries participating in the survey, achieving the target number of samples and not exceeding the missing proportions anticipated at the design stage (less than 10 % for production area samples and less than 20 % for batches from dispatch centres). Descriptive analysis of the data submitted indicates a good geographical spread of production areas across Europe, and included the various production types and species anticipated, consistent with a good representation of European oyster production and dispatch. Over- or under-sampling by individual Member States was considered in the calculation of apparent prevalence through weighting to correct the relative contribution of that Member State's results to the apparent EU prevalence.

4.2. Design and method limitations

The primary aim was to get an accurate view of the contamination of oysters with NoV in Europe both at the primary production stage of animals growing in seawater before harvest, and also at the stage of placing them on the market for human consumption as live bivalve molluscs. The survey was not designed as a comparative study, meaning oysters were not followed from the production area to the dispatch centres, so the results may not be expressed in such continuity terms as, for example, a decrease from production to dispatch, but merely as two separate contamination levels at two separate points, e.g. lower at dispatch than in production. The baseline survey was designed with the aim of estimating the prevalence of NoV in oysters as mentioned before, as well as to evaluate any seasonal effect. It was not specifically designed to study the effect of any other factor on the prevalence of NoV or the number of copies per gram. However, the information submitted was used to explore the association of the prevalence with several factors of interest, such as production type, area class, weight of the oysters, etc. These results should be considered as indicative, and further investigations should be undertaken in order to confirm the findings.

The report deals with the apparent prevalence, as detected using the PCR method prescribed in the technical specifications in order to meet the sensitivity mandate. Had data existed that describe how this method compares to some gold standard test, deriving the true prevalence taking into account imperfect test sensitivity and specificity results might have produced different prevalence figures but would not have altered the factors associated with contamination. The differences observed between production area and dispatch centre contamination would also remain. The temporal trend would also remain unchanged with higher prevalence in winter than summer periods. However, no such gold standard test exists for NoV in oysters, and the apparent prevalence figures reported are the most accurate outcomes that the current survey can provide. An assessment of the potential effect of a range of values for sensitivity and specificity (minimum values of 0.8 and 0.95, respectively) shows that the true prevalence could range from 31.1% to 43.1% for production areas and 6.2 % to 13.6% for batches from dispatch centres, given these assumptions.

The survey followed the technical specifications set out by EFSA, notably applying the real-time RT-PCR-based ISO (15216-1:2017) method to detect and quantify NoV. This methodology can potentially amplify RNA from viable viruses and also RNA from non-viable or lysed viruses. Therefore, the prevalence figures and quantities reported may reflect contamination of samples with both viable and non-viable virus material, with the respective representations unknown. Previous work indicates that there is a potential for naked RNA fragments (non-infectious) to persist in the marine environment; however, the same work showed the negligible potential for such RNA to bioaccumulate in oysters and contribute to the resultant quantitative load (Dancer et al., 2010). Meanwhile, notwithstanding the potential for non-infectious viruses to contribute to the results, NoV quantity in live oysters as determined by PCR methods has been shown to correlate with the likelihood of association with human illness, so the PCR result is relevant to public health (Le Guyader et al., 2010; Lowther et al.,

2010, 2012a; Polo et al., 2016). Some depurated oysters have been implicated in outbreaks (McLeod et al., 2017). As this is the only sensitive method available to detect NoV this was the method used to estimate the dose–response relationship in volunteer studies (Teunis et al., 2008; Atmar et al., 2014). This PCR approach has also been successfully used to analyse shellfish-borne outbreaks showing the same sequence in oysters and infected consumers (Le Guyader et al., 2008, 2010; Rajko-Nenow et al., 2013; Lunestad et al., 2016; Polo et al., 2016; Rasmussen et al., 2016).

4.3. Main findings and public health significance

The mean EU apparent prevalence of NoV contamination of oyster samples from production areas was 34.5% (CI: 30.1–39.1%), and the mean EU apparent prevalence of NoV contamination of batches dispatched by dispatch centres was 10.9% (CI: 8.2–14.4%). Quantitative contamination levels showed a mean of around 337 cpg in production area samples and around half that (168 cpg) in batches from dispatch centres. These findings are consistent with expectations from the literature, indicating a real and ongoing potential for NoV contamination at the pre-harvest stage of oyster production, and for that to contribute to contamination of food placed on the market (FSA UK, 2012; Lowther et al., 2018). As with all bivalve molluscs, oysters filter water to extract food particles, and have a well-described potential for bioaccumulation of NoV present in the water resulting from contamination with human faeces (Lees, 2000). Oysters may be eaten both raw and as entire animals and are therefore one of the food groups recurrently implicated in food-borne transmission of NoV (EFSA and ECDC, 2018).

Transmission of NoV differs to other known viruses; they were the first viruses to show different infection risks depending on host genetics (Le Pendu et al., 2014). One consequence of this is that when an outbreak is identified, a larger number of consumers can be infected without presenting any symptoms, and thus contribute to the virus dispersion. Following an oyster-related outbreak, only sensitive consumers considering the strain detected in the oyster samples have been reported to show signs of illness (Le Guyader et al., 2010). Besides this genetic sensitivity, NoV is among the most infectious agents with low infectious odds, as reported in studies based on volunteers or even when outbreak data were used (Teunis et al., 2008; Thebault et al., 2013; Atmar et al., 2014; Kirby et al., 2015; Van Abel et al., 2017). In the EU, NoV is reported by many Member States (20 in 2017) to cause food-borne and waterborne disease outbreaks (EFSA and ECDC, 2018). Some depurated oysters have been implicated in outbreaks worldwide (McLeod et al., 2017). Another characteristic of NoV is that they are considered fast evolving viruses with an extensive diversity linked to point mutation or recombination (Parra et al., 2017). This helps the virus to escape the immune surveillance of the host and thus protective immunity varies depending on the strain (De Graaf et al., 2016). New recombinants have been linked to large outbreaks in the population and oysters may favour the distribution of emerging strains (Rasmussen et al., 2016), but some more static strains, such as some GI strains, have also been identified and implicated in outbreaks (Parra et al., 2017). In a worldwide study, a higher frequency of GI strains was found in oyster-related outbreaks when compared with other means of transmission, suggesting that oysters may act as a reservoir for NoV in the environment (Yu et al., 2015). An additional reason for this enhanced transmission of GI strains through oyster consumption may be the existence of a specific ligand in oyster digestive tissues (Le Guyader et al., 2012).

The presented findings of a more than one-in-three likelihood of EU oyster production being NoV-contaminated, with such contamination in more than 1-in-10 batches dispatched for human consumption highlights the potential hazard of NoV when producing oysters for consumption as live bivalve molluscs. The public health significance of these findings is difficult to characterise given the limited data on the health risks of different concentrations of NoV in oysters as determined by real-time RT-PCR. Many factors outside the scope of the survey may influence the likelihood of illness, notably strain type(s) of NoV present with different infectivity and herd-immunity to those strains in human consumers. Previous work has demonstrated an increasing risk of infection with increasing levels of NoV as detected by real-time RT-PCR (Lowther et al., 2010, 2012a). The evidence presented indicates that while values below 100 cpg are unlikely to be associated with outbreaks, there is an increased risk of outbreaks where levels exceed 500 cpg (Lowther et al., 2012a). Nevertheless, some oysters with lower concentrations have been implicated in outbreaks (Le Guyader et al., 2008, 2010; Polo et al., 2016). Approximately half of the positive batches (17.15% in production area samples and 5.59% of dispatch batches) had values over 200 cpg; while analogous proportions over 500 cpg were 8.71% and 1.17%, respectively. This extent of contamination could be regarded as a public health

concern, highlighting the particular risks associated with this food production system, supporting the need for active risk management strategies to mitigate NoV risk in this food chain in addition to those currently in place. The primary recommendation of the EFSA norovirus/oyster opinion (EFSA, 2012) was that contamination should be prevented in the first instance, and this might be achieved by ensuring antiviral treatment of human sewage outflows or growing oysters sufficiently distant from human sewage outflows. Oysters are essentially a ready-to-eat food and this baseline survey indicates an extent of human faecal contamination of this food which is a public health concern.

4.4. Laboratory and methodological considerations

Laboratory analyses were performed in accordance with the prescribed methodology, which allowed for either a simultaneous qualitative and quantitative analysis, or an initial qualitative screen followed by quantitative analysis of only screen-positive samples. The survey utilised a method involving two separate real-time RT-PCR assays, designed to detect GI and GII, respectively. At a simple level, NoV GI or GII can be deemed to be present when the fluorescence signal associated with amplification of the PCR target rises above the background. The point in the PCR at which this fluorescence rise occurs in positive samples can then be used to quantify viral RNA in the initial sample; in more concentrated samples the fluorescence rises above background levels earlier in the PCR. Because a separate result is obtained for GI and GII, deriving a quantitative figure for total NoV is complicated by the potential for results pertaining to one or both genogroups to be either not detected (below the LOD) or positive but below the LOQ.

This issue is further complicated by the potential for values for LOD and LOQ to vary across laboratories. Notwithstanding a small number of samples where 10-fold dilution of RNA was necessary to deal with PCR inhibition (where a commensurate 10-fold increase in LOD and LOQ is applied), the range of LODs and LOQs observed may be partly due to the application of somewhat different approaches to determining LODs and LOQs in different laboratories. In some laboratories these values were determined by producing a dilution series of contaminated digestive tissues in uncontaminated tissues. In other laboratories a more easily practicable approach using a dilution series of contaminated tissue homogenate in uncontaminated homogenate was used. In addition, the use of contaminating viruses from different strains may have contributed to the different values for LOD and LOQ reported.

Quantitative analyses were substantially influenced by a large bulk of samples in the present survey having laboratory results with at least one of the two NoV genogroups categorised as either $<$ LOD or $<$ LOQ. It was therefore necessary for the data analysis to consider approaches to assigning quantitative outcomes to these samples in order to assess how such samples might be classified with regard to notional thresholds based on the sum of both genogroups. Consequently, summation exercises require some sort of convention to arrive at an overall cumulative total GI + GII for the number of NoV copies per gram when the result for a particular genogroup is not zero but not reliably quantifiable. The initial data analysis approach was a simple substitution approach where half of the LOD value of a genogroup was assigned to not detected results for that genogroup; while the value of half of the LOQ was assigned for positive samples where the quantity recorded was below the LOQ.

Quantification of low NoV concentrations is a challenging issue even for laboratories used to performing quantification such as the reference laboratories performing this work. The ISO method has greatly improved NoV detection and quantification and facilitates comparison between laboratories. However, some technical issues persist and may lead to variability in quantification at low concentrations (Polo et al., 2018). The variation observed for LOQ or LOD values is inherent in the PCR method, in the oyster samples and in the different reagents and apparatus used. One promising approach that could be used in the future is digital PCR, which might provide greater precision in quantification for samples contaminated at low levels (Polo et al., 2016; Persson et al., 2018; Tan et al., 2018).

4.5. Difference between production area samples and dispatch centres

The survey found a significant difference between the prevalence in production areas and the prevalence in dispatch batches. Dispatch batches were less likely to be contaminated and had lower quantitative levels than the values found in production area samples. This was not a comparative study so this finding should not be interpreted as a decrease, but as a case of different prevalence figures for the two points, with batches dispatched from dispatch centres less contaminated than

production areas. Oyster production including harvesting, either following farming or wild capture, is regarded as primary production, where the resultant products are not yet on the market for human consumption. In order to attain the status of food placed on the market for direct human consumption, oysters should be dispatched from a food establishment approved by the competent authorities as meeting the necessary requirements within its food safety management systems to ensure food safety. The current survey shows a lower prevalence in what is dispatched than what is grown. Also, production areas are not uniform in their size and production volumes and it is possible that European dispatch centres predominantly deal in oysters from areas with the lowest impact of NoV contamination, either by design, or as a result of individual production areas tending to be larger in geographical regions where baseline NoV levels are lower.

One consideration of relevance to the production area survey is the utilisation of the normal *E. coli* classification sampling point for sample collection. This is required by EU guidance to represent the worst-case scenario of faecal contamination (Cefas, 2018), thereby possibly skewing the data towards higher NoV results. The data are also consistent with the hypothesis that the population of oysters selected to be placed on the market for human consumption is a biased subset of the population of oysters in production at any time (growing in the water). This could arise through harvesting strategies actively targeting oysters with a lower NoV risk, arising from a risk-based harvesting strategy such as foregoing of harvesting entirely at known high-risk times or more nuanced risk management strategies at higher risk times involving harvesting only from parts of classified production areas known to present a lower risk. This hypothesis is supported by the finding of more marked steepness in the temporal trend for production area NoV prevalence than was found in dispatch centres. The data are also consistent with a degree of effectiveness of post-harvest pre-dispatch treatment including depuration optimised to manage NoV risk. Overall, these data indicate that it is possible to have moderately high prevalence and quantitative load in production areas without that translating to the same extent of contamination in what is dispatched.

4.6. Seasonality

The survey found a marked seasonality in the contamination of both production areas samples and dispatched batches with the coldest months showing the highest contamination. The overall temporal trend in apparent prevalence for production area samples across the six sampling periods within a calendar year is consistent with that observed in previous surveys (Flannery et al., 2009; EFSA, 2012; Lowther et al., 2012b). Norovirus does not multiply outside of the human intestine, neither in sewage, seawater nor oyster tissues, so the apparent effect of environmental temperature requires active consideration. Higher contamination of oysters may theoretically arise from more bioaccumulation of the NoV that is present in the water, and/or also from greater NoV concentrations in the growing waters and therefore more NoV being filtered.

Considering a hypothesis of different bioaccumulation in the colder months, oysters are a poikilothermic (cold-blooded) animal so the extent of metabolic activity, including mechanical filtration, is influenced by seawater temperature. Rates of both bioaccumulation and clearance are reduced at normal winter temperatures. Therefore, although uptake of viruses from the environment is slower, colder temperatures during the winter result in longer depuration times and prolonged residency of viruses in the oysters' intestinal tracts (Doré et al., 1998; Hernroth and Allard, 2007).

It is also possible to explain this seasonal effect by an increased viral load in the water which is filtered by the oysters during the winter months. Norovirus is one of the few viruses with clear seasonal epidemiology (Ahmed et al., 2013). Lower water temperatures and lower levels of solar irradiation lead to prolonged survival of viruses in the water column during this period (Allwood et al., 2005; Cannon et al., 2006). Norovirus is among the most common causes of acute gastroenteritis in the community in the EU, with a marked winter peak in human incidence, and therefore a winter peak in NoV contamination of human sewage discharged to oyster production areas, (Mounts et al., 2000; Lopman et al., 2003) with consequently normal oyster filtration accumulating more NoV. In such considerations it can be difficult to disentangle the cycle of more NoV in the community from more NoV in the oyster food chain and hence greater risk of human NoV. However, the epidemiology of NoV infection in the human population is entirely distinct from oyster consumption, with oysters acting as just one potential vehicle allowing NoV to be transmitted from one person to another by the faecal–oral route.

The apparent prevalence of GII was higher than for GI in the periods January–February and November–December but lower in the periods July–August and September–October. Higher apparent

prevalence of GII during the winter may reflect the fact that this genogroup is more prevalent in the human population during this period (Siebenga et al., 2009). Meanwhile, the apparent prevalence of GI may remain more constant during the summer as a result of slower clearance of these viruses due to specific binding to oyster tissues (Maalouf et al., 2010). The more pronounced seasonality of GII in oysters than GI in oysters, is therefore consistent with the hypothesis of oyster NoV seasonality arising from the winter peak in human community infections and the resulting greater NoV loads in sewage.

4.7. Classification status

The survey showed a significantly lower prevalence of NoV contamination in samples from Class A production areas than other areas (Class B or C), and similarly a significantly lower prevalence of NoV in batches of oysters dispatched from Class A production areas than from other areas. Classification is a concept set out in EU legislation where the competent authorities consider the overall faecal contamination potential of a production area as indicated by a sanitary survey of inputs to the area and *E. coli* concentration in molluscs grown in that area and then award a classification accordingly. A-classified molluscs may be placed directly on the market for consumption as live animals through an approved dispatch centre. B- and C-classified molluscs must be thermally processed or be purified to meet microbiological criteria consistent with Class A *E. coli* status before being placed on the market as live animals.

The findings of the current survey suggest that the use of *E. coli* as a generalised indicator of faecal contamination in European shellfish hygiene regulations provides a useful indication of the likelihood of contamination with NoV, as previously observed (Lowther et al., 2012b; Younger et al., 2018). Therefore, as Class A areas are less prone to faecal contamination, they are expected to exhibit less NoV contamination. However, this survey shows that Class A status is not a guarantee of the absence of NoV contamination. In the present survey, NoV was lower but nevertheless present in dispatched batches both from Class A and Class B purified to meet the Class A *E. coli* standard. Therefore, *E. coli* -based indices of acceptable faecal contamination do not appear sufficient to indicate the extent of NoV contamination.

4.8. Primary production factors

The survey examined the association of different aspects of primary production with NoV contamination both in production areas and in dispatched batches. These were examined at a univariate level and then at a multivariate level.

The apparent prevalence of NoV was generally significantly lower in farmed oysters than wild oysters and this correlated with the trend of lower prevalence in Pacific cupped oysters, which are generally farmed, than in European flat oysters, which are generally wild. While it might be anticipated that wild oysters grow and are harvested further from sources of faecal contamination, a further interaction arises with 'production type' where farming techniques generally involve a growing phase raised higher in the water column than the seabed. Wild oysters grown on the seabed have a higher likelihood of exposure to settled faecal particles and waters not subject to UV irradiation. Those hypotheses are supported by the finding of a lower prevalence in intertidal oysters than oysters from inshore production. Intertidally grown animals have a growing phase with twice-daily exposure above the sea surface, and when submerged, the water filtered by intertidal animals is relatively UV-exposed.

These interrelated findings are further confounded by the size of oysters where the analysis found, for both production areas and dispatched batches, an association of higher prevalence with increasing animal weight. In addition to the potential for older wild oysters to be harvested, larger oysters filter more water and have a greater likelihood of having been grown with access to more organic material to support their development, and therefore to have been able to accumulate viruses over successive winter seasons.

The analytical approach further examined the potential confounding effect that might arise through association of the NoV-associated factors with each other, by developing multivariate models. In the case of production area samples the model validated the earlier finding of significantly lower prevalence in Class A than other (B or C) classifications, but with that difference becoming smaller with increasing oyster size, and also less marked in intertidal than in inshore production.

4.9. Post-harvest factors

The survey provided representative data for prevalence estimation, and the distribution of oyster samples among the levels of several factors was unbalanced, making the analysis of factors challenging owing to sparseness problems. The data analysed post-harvest predispatch interventions designed to reduce microbial contamination, either depuration or relaying, as potential factors associated with NoV contamination. The analysis of the data for Class A oysters, for which such interventions are optional, found a non-significant trend towards higher prevalence in batches dispatched from purified oysters than in batches comprising oysters not subject to this intervention. This finding of an apparent association of an intervention which should at least to some extent lower NoV prevalence may seem non-intuitive. The intuitive hypothesis is one whereby these treatments should always result in some lowering of contamination, but that would not be either proven nor disproven by the current survey in the absence of paired longitudinal comparison pre- and post-treatment in the same or known comparable populations.

Given the association detected in the current study, there is the possibility that interventions actually increase NoV contamination due to poor hygiene practices. However, a potentially more valid hypothesis centres on aversion to intervention due to direct and indirect commercial costs. There would then be a consequent targeting of the intervention, which has some but limited efficacy against NoV, primarily in the case of known higher-contamination status oysters. In this hypothesis, the population of oysters selected for such interventions is therefore a biased population with greater propensity for NoV contamination pretreatment, compared with batches where oysters had not been selected for such an intervention. This would explain the higher prevalence and quantity in batches subject to interventions.

The analysis of interventions also attempted to characterise any association of 'conditioning' on NoV contamination. Conditioning is a concept of re-immersion in water for the purpose of market preparation, removing grit or slime, but not for the purpose of dealing with microbial contamination. While legislation does not impose a time limit on such re-immersion, the survey found a broad range of conditioning times reported, some in the order of time durations more associated with commercial purification protocols. Conditioning could therefore be associated with recontamination or conversely decontamination of oysters, but should by definition be associated with neither. A trend, significant at certain times of the year for Class A oysters, was found in decreased prevalence of NoV in batches derived from conditioned oysters. A relevant consideration is that the process of purification of bivalve molluscs requires validation and approval of the establishment for such an operation, while conditioning requires no such controls, but findings indicate that conditioning might have been used to reduce contamination levels.

Even if NoV concentrations decreased in depurated oysters, a review identified several outbreaks linked to oysters that had been depurated in water at a maximum temperature of 18°C (McLeod et al., 2017).

The above findings are consistent with the EFSA norovirus/oyster opinion (EFSA, 2012) that found that current treatment regimens for products placed live on the market (depuration and relaying) as commonly practised do not effectively reduce NoV in oysters.

4.10. Considering microbiological criterion thresholds

Data analysis considered the probability of exceeding particular thresholds with a view to examining the potential effects of introducing a microbiological criterion either at harvest or at dispatch. A primary consideration in understanding such impact arises from the difference in contamination between production areas and dispatch centres, where more samples from production areas than those from dispatch centres would exceed any particular thresholds. Similarly, any threshold would have a seasonal variation in its impact; with more results exceeding any threshold in the winter than summer months from both production area and dispatch centre samples.

Analysis of the impact of any threshold that is of similar magnitude to the LOD and LOQ values requires active consideration of how to assign a quantity to not detected results and positive below LOQ results for individual genogroups. The so-called substitution approach, where a quantity of half LOD or half LOQ was applied to not detected results and positive below LOQ results for each genogroup, was relatively crude but simple and therefore readily accessible.

In order to explore the robustness of such a simple substitution approach, data analysis also applied appropriate statistical methodologies based on survival methods accounting for censored

observations, modelling the quantities that would have been expected in such results. Data analysis found such a substitution approach made a substantial contribution to exceeding particular thresholds when those putative thresholds were below or close to the LOQ values, but a relatively low contribution to results exceeding thresholds substantially higher than the LOQ value. Comparing the threshold exceedance probabilities, when applying the substitution approach compared to the statistically modelled approach, for data in the present survey as influenced by the LOQs across the various laboratories, the substitution method showed similar impact to the statistically modelled impact for thresholds above or equal to 300 cpg. Thresholds below 300 cpg showed significant divergence between approaches, with the substitution method resulting in many more samples over these lower thresholds than would have been predicted by the model.

5. Conclusions

In this report, the following terms of reference where addressed and the conclusions drawn for each of them are presented in the subsequent sections. The terms of reference are:

c) Assess the proportion of EU-classified production areas contaminated with NoV (presence);
d) Assess the proportion of batches of final product at approved EU dispatch centres contaminated with NoV (presence); and
e) Analyse the survey data for NoV in the EU in production areas and at approved dispatch centres including options with regard to establishing microbiological criteria for NoV in oysters to be harvested and placed on the market.

5.1. Term (c) Prevalence in production areas

- The apparent prevalence of norovirus in EU oyster production areas was estimated to be 34.5% (CI: 30.1–39.1%), meaning that during these two years the probability that any EU production area was contaminated with norovirus from human sewage at any time was 0.345 (CI: 0.301–0.391).Therefore, human faecal contamination is present in oyster production areas.
- Production area contamination including prevalence and quantity present had substantial seasonal variability with peak contamination in the winter months (November–April) and lowest contamination in the summer months. Given that contamination in winter is higher, this implies that any threshold set at primary production areas would have a greater impact during this period.
- Production area prevalence was also significantly lower in Class A areas than in Class B or C areas, supporting a conclusion that the current existing bacteria-based classification system is useful. However, Class A areas were not free from NoV, so additional criteria should be considered when managing NoV risk.

5.2. Term (d) Prevalence in batches from dispatch centres

- The apparent prevalence of NoV in batches of oysters from dispatch centres was 10.9% (CI: 8.2–14.4%), meaning that during these two years the probability that any batch of live oysters dispatched in the EU as a food product suitable for human consumption was contaminated with NoV was 0.109 (CI: 0.082–0.144).
- A more moderate seasonality, mirroring that observed in production areas, was also seen in batches from dispatch centres. This is consistent with the conclusion that contamination of final products arises from NoV contamination of oysters at primary production. Given that contamination in winter is higher, this implies that any threshold set at dispatch centres would have a greater impact during this period.
- It can be concluded that the apparent prevalence in batches from dispatch centres was lower than that for production areas. The reasons for these findings were not identified, due to the design of the survey which was responding to a different aim, estimating prevalence at both production areas and batches at dispatch centres.
- Classification as A was associated with lower NoV contamination, supporting a conclusion that the current existing bacteria-based classification system is useful. However, Class A areas were not free from NoV, so additional criteria should be considered when managing NoV risk. Even though batches containing oysters originating from Class B areas must have been purified or

relayed, contamination remained higher than that observed in batches of oysters originating from Class A areas.

5.3. Term (e) Data analysis around establishing microbiological criteria

- Consideration of options for a microbiological criterion should take into account the point in the oyster production food chain at which it would apply, and it should also account for seasonality.
- In order to deal with not detected or positive below LOQ results (representing more than 90% of the total number of samples) in the determination of a single NoV quantification from the separate results for NoV GI and GII, an approach has been proposed (substituting not detected results with half LOD values and positive results below LOQ with half LOQ values). This approach performed well when the level of NoV contamination was above 300 cpg.
- Selecting a threshold below the maximum of the sum of half of the LOQs for each genogroup could produce a high proportion of samples whose categorisation would be influenced by the substitution method. In this survey, the maximum of the sum was 257 cpg and if the threshold of 100 cpg was applied, substitution influenced the categorisation of up to 87% of the total samples.
- Conversely, when the thresholds exceed the sum of half LOQs for each genogroup, the substitution approach only influences the categorisation of a low proportion of samples. In this survey, if the threshold of 300 cpg was applied, substitution influenced the categorisation of up to 1.5% of the total samples.
- This survey only assessed thresholds from the perspective of the analytical capability and not that of human health risk.

6. Recommendations

The baseline survey has also highlighted several aspects that should be further considered when applying the testing procedure used to analyse the samples and related to the information provided in production areas and dispatch centres. Below are some points to consider:

- Any application of RT-PCR in a regulatory context should be supported by a prescribed method of determining LOQ or LOD, prepared by the EU reference laboratory for food-borne viruses, in order to minimise inter-laboratory variation.
- Conditioning was unexpectedly found to be associated with lower prevalence levels, which indicates that this should be further investigated in order to understand the effect of such practices.
- Control measures for NoV in oysters should include efforts to prevent human faecal contamination in oyster production areas.
- *E. coli* classification of production areas should be maintained. However, in addition, risk of NoV contamination should be considered in the sanitary surveys and ongoing monitoring programmes.
- The current two bacteriological microbiological criteria applicable to bivalve molluscs placed on the market as live food products, (dispatched batches) might be complemented by a NoV criterion for operators of dispatch centres. The monitoring based on this microbiological criterion should consider the strong seasonality effect reported here.
- There should be an explicit obligation to identify NoV as a hazard within the HACCPnnnn of dispatch centres, and purification centres even without a legally established limit.
- Any microbiological criterion should include explicit instructions for how to deal with not detected or positive below LOQ results in the determination of a single NoV quantification from the separate results for NoV GI and GII. Legislative thresholds should be greater than anticipated LOQ values.

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Glossary

Batch	A group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period
Dispatch centres	Any onshore or offshore establishment for the reception, conditioning, washing, cleaning, grading, wrapping and packaging of live bivalve molluscs fit for human consumption. Dispatch centres are approved by the competent authority and assigned a unique approval number
Prevalence, apparent, observed and true	Observed prevalence, apparent prevalence or measured prevalence mean the prevalence estimated on the basis of a diagnostic test used to detect the infection in the given population. In contrast, true prevalence represents the actual prevalence of the infection in the population in question. True prevalence can be estimated from the apparent/observed prevalence by correcting for misclassification bias due to the imperfect diagnostic test used. The discrepancy between the apparent and the true prevalence is a function of the sensitivity and the specificity of the diagnostic test used
Prevalence of norovirus-contaminated oysters at production areas and batches of oysters at dispatch centres	Prevalence of oysters with detectable norovirus RNA
Production areas	Harvesting areas include both production areas and relaying areas. 'Production area' means any sea, estuarine or lagoon area, containing either natural beds of bivalve molluscs or sites used for the cultivation of bivalve molluscs, and from which live bivalve molluscs are taken. 'Relaying area' means any sea, estuarine or lagoon area with boundaries clearly marked and indicated by buoys, posts or any other fixed means, and used exclusively for the natural purification of live bivalve molluscs

Abbreviations

CI	confidence interval
cpg	copies per gram
EURL	EU Reference Laboratory
FAO	Food and Agriculture Organization
GEE	generalised estimating equations
GI	genogroup I
GII	genogroup II
GLMM	generalised linear mixed models
LOD	limit of detection
LOQ	limit of quantification
NoV	noroviruses
NRL	national reference laboratory
PCR	polymerase chain reaction
QIC	Quasi-Akaike Information Criterion
RT-PCR	real-time polymerase chain reaction

Appendix A – Statistical methods

A.1. Methods used to estimate the prevalence

The proportion of positive samples is used to estimate the (apparent) prevalence of norovirus. Like a linear regression model for the mean of a continuous outcome, the logistic regression model makes it possible to estimate the effect of covariates/factors on the prevalence. Is there a significant difference in prevalence for Class A and B production areas? Is prevalence constant over time or are there seasonal effects? Such factors are combined in a linear expression with slopes quantifying the strength of the effects of these factors on the prevalence of norovirus, and exponentiated slopes have the well-known interpretation of odds ratios (e.g. the exponentiated slope for the factor 'production area' reflects, as a ratio, the change in odds for a positive sample when comparing samples from Class A and Class B production area). Estimation and testing of the slopes in a logistic regression model is based on maximum likelihood inference.

The basic logistic regression model has been extended to account for a particular survey design (disproportionate stratified sampling) and for particular correlated/hierarchical data structures (samples collected within Member States). A weighted maximum likelihood analysis accounts for the survey design. To accommodate correlated/hierarchical data in the logistic regression model, there are essentially two different approaches, known as generalised estimating equations (GEE) and generalised linear mixed models (GLMM).

The GEE approach applies exactly the same logistic regression model (the linear expression and the interpretation as odds ratios) as basic logistic regression, but, using a so-called working correlation structure, it corrects the estimates from the basic logistic regression model for the correlated data structure. The point estimates are very close to those of a basic logistic regression, but standard errors are typically larger (a phenomenon also known as overdispersion). Consequently, inferential results (confidence intervals and hypothesis tests) might differ, from very little to substantially according to a lower or higher correlation in the data, respectively.

The GLMM approach extends the basic logistic regression model with so-called random effects, giving each hierarchical unit (Member State) its own logistic regression model. All samples from the same hierarchical unit share the same specific model inducing a correlation between samples from that Member State. As this GLMM approach constructs a model for each Member State, the inference is 'conditional' (specific to each Member State) and not 'marginal' or 'population averaged' (referring to the whole population, as for basic regression and GEE). By integrating across all Member States, however, a GLMM model can also provide marginal results, but this needs an additional, non-trivial step. For more information on logistic regression and its extensions GEE and GLMM, see, for example, Agresti (2019).

As interest here is on estimation and inference on the EU level, the weighted marginal GEE approach has been applied for the part on the estimation and study of factors for the prevalence of norovirus.

A.2. Methods used to estimate the sum of the number of copies per gram of genogroups I and II

The distribution of the sum of the number of copies per gram of GI and GII was estimated while accounting for censoring. The distribution of the sum of the number of copies per gram was fitted using a lognormal model. Similar to the estimation of the prevalence of norovirus, inference for the parameters of the lognormal model can incorporate the effects of covariates/factors and extensions to account for particular survey designs and hierarchical data structures as well. The GEE approach, however, does not follow the principles of Bayesian inference and is not available. Therefore, the study of the quantification of norovirus (the number of copies per gram) was performed in the Bayesian framework with a weighted GLMM approach.

As a preliminary step, two approaches were considered in parallel, a bivariate approach (fitting the joint lognormal distribution for GI and GII jointly and deriving the sum from there) and an approach using the sum of the number of copies per gram of GI and GII directly, using a univariate lognormal model. In the first bivariate approach, a joint lognormal distribution is fitted to all observed pairs (number of copies per gram for GI, number of copies per gram for GII) and the distribution of the sum of the number of copies per gram for GI and GII is derived from this joint, bivariate distribution. So, in this first approach, it is not necessary to define the observed sum of the number of copies per

gram, with all the difficulties of defining such a sum in the presence of censoring for one or for both numbers of copies per gram. In the second, direct approach, the joint distribution is not fitted, but the distribution of the sum is estimated directly using the 'observed' sum according to the definition of the substitution approach. More precisely, the two approaches were compared using the same data set, replacing censored data by half LOD (for not detected results) or half LOQ (for positive below LOQ results), and the observed value for quantified results (as described in Section 2.1). The results obtained from both approaches showed very similar estimates for the mean and variance of the lognormal distribution and consequently for the shape of the distribution.

Based on the finding of this experiment and because interest does not go to the bivariate distribution but rather to the sum of the number of copies per gram of GI and GII, the first approach was no longer applied for further analysis. Moreover, the incorporation of covariate/factor effects and the extension to include random effects accounting for hierarchical data structures is straightforward for the second, direct method, whereas it is rather problematic for the first approach.

Appendix B – Data description

B.1. Production areas

Descriptive analyses of the information collected are presented in this appendix. Figure B.1 displays the number of samples tested by laboratories with different accreditation/validation statuses for the analytical method; 76% of the samples were analysed in accredited laboratories, 19% were analysed in non-accredited laboratories with an internally validated method; and 5% in non-accredited laboratories with a non-internally validated method.

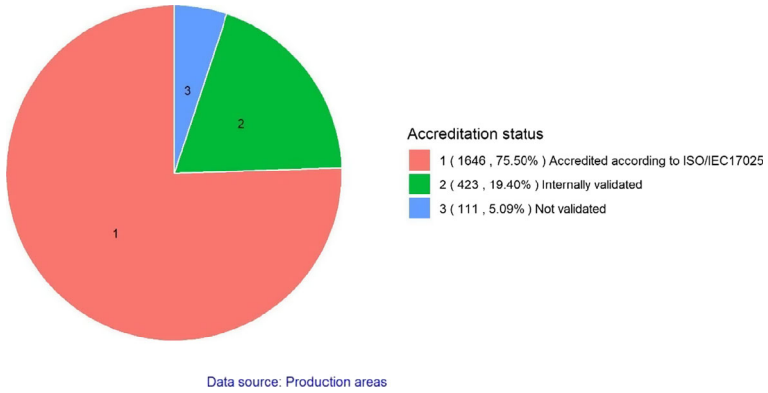

Figure B.1: Number of oyster samples, by accreditation status of the analytical method

Table B.1: Distribution of the oyster production information, in production areas

Production information	N	%
Farmed	1,793	82%
Wild	387	18%
Total	2,180	100%

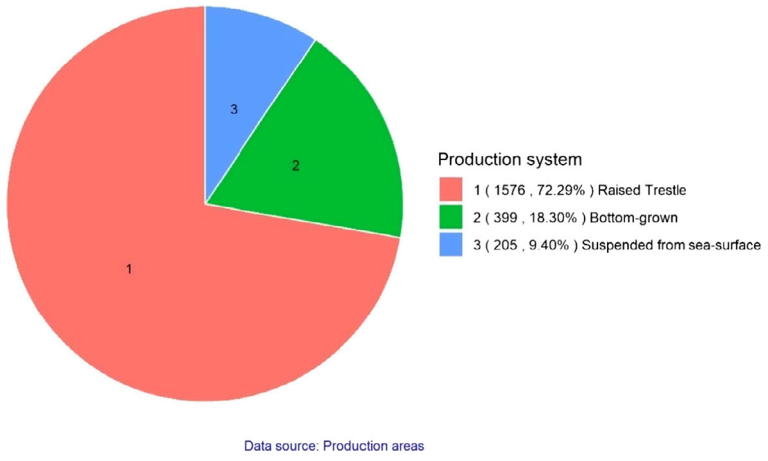

Figure B.2: Distribution of the oyster samples from production areas, by production system

Table B.2: Distribution of the oyster species information, in production areas

Oyster species	N	%
European flat oyster	316	14%
Oyster, Portuguese cupped	12	1%
Pacific cupped oyster	1,852	85%
Total	2,180	100%

Table B.3: Distribution of the type of oyster production areas, in production areas

Production area	N	%
Inshore	585	27%
Intertidal	1,555	71%
Offshore	40	2%
Total	2,180	100%

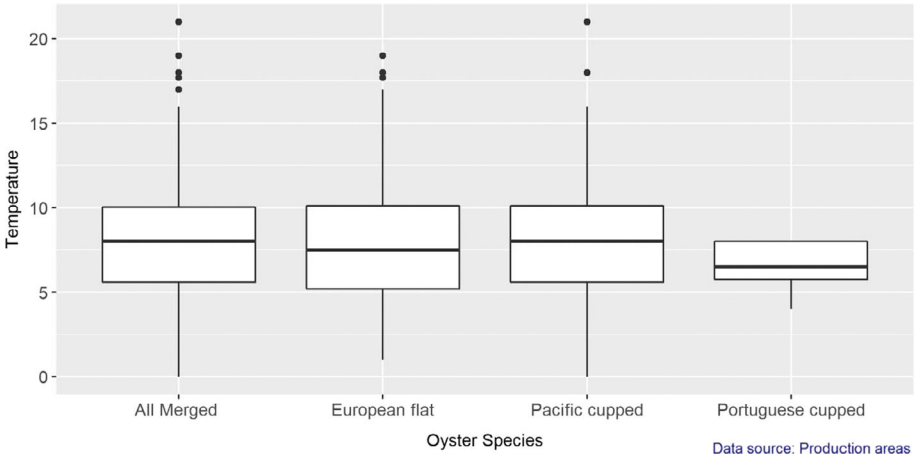

Figure B.3: Box plot of sample temperature at the time of arrival in the laboratory, by oyster species

Figure B.3 displays the sample temperature at time of arrival in the laboratory in degrees Celsius, overall and by oyster species. Values were missing for 35 samples (1.5%). Few samples were reported to have arrived in the laboratory with a temperature above the limit of 15 (N = 15). Considering that the arrival temperature is not expected to affect the results, these sample data were retained.

B.2. Dispatch centres

Figure B.4 displays the accreditation status of the analytical method; 72% of the samples were analysed in accredited laboratories, 26% were analysed in non-accredited laboratories with an internally validated method; and 3% in non-accredited laboratories with a non-internally validated method.

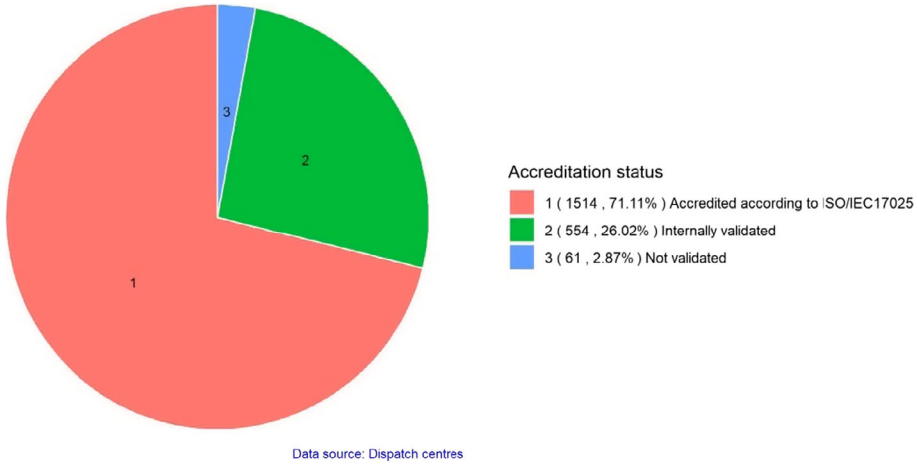

Figure B.4: Number of oyster samples, by accreditation status of the analytical method

Few samples were reported to have arrived in the laboratory with a temperature above the limit of 15 degrees (N = 15). Considering that the arrival temperature is not expected to affect the results, these sample data were retained. Data with missing values (185 samples; 8%) were not used to construct Figure B.5.

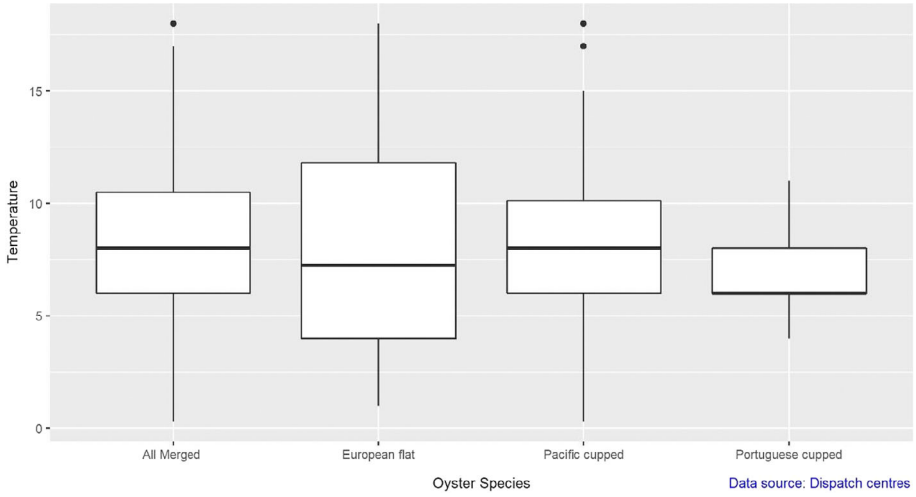

Figure B.5: Box plot of sample temperature at the time of arrival in the laboratory, by oyster species, dispatch centres

Table B.4: Distribution of the oyster production information, in dispatch centres

Production information	N	%
Farmed	2,036	96%
Unknown	15	1%
Wild	78	4%
Total	2,129	100%

Table B.5: Distribution of the number of source production areas, in dispatch centres

Number of source production areas	N	%
1	2,067	97.1%
2	5	0.2%
3	3	0.1%
Not reported	54	2.6%
Total	2,129	100%

Table B.6: Distribution of the oyster production information (repacked or not), in dispatch centres

Repacked	N	%
No	1,963	92%
Unknown	105	5%
Yes	61	3%
Total:	2,129	100%

Table B.7: Distribution of oyster species, in dispatch centres

Oyster species	N	%
European flat oyster	80	4%
Oyster, Portuguese cupped	13	1%
Pacific cupped oyster	2036	96%
Total	2,129	100%

From the 898 samples for which purification was reported to have been carried out, 82% originated from oysters that were purified by ambient-temperature water, 13% by actively cooled water, 4% by unknown reported thermal status of the water used for purification and 0.5% by actively heated water (Figure B.6).

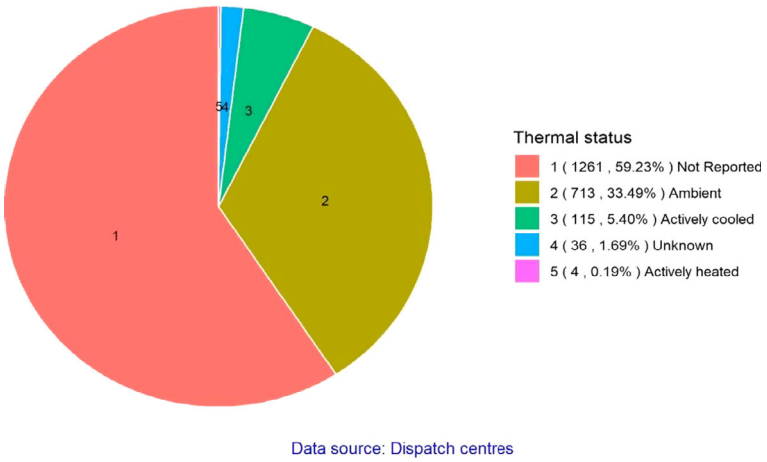

Figure B.6: Thermal status of the water used for purification, dispatch centres

Figure B.7 displays the duration of conditioning (hours) of the batches sampled in dispatch centres, overall and for the three oyster species.

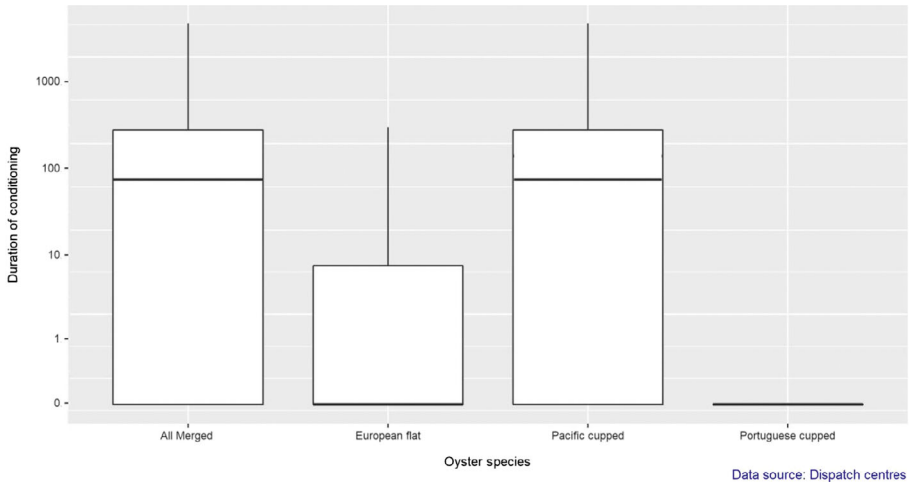

Figure B.7: Box plot of the duration (hours) of conditioning, dispatch centres

Figure B.8 displays the duration of purification (hours) of the batches sampled in dispatch centres, overall and for the three oyster species.

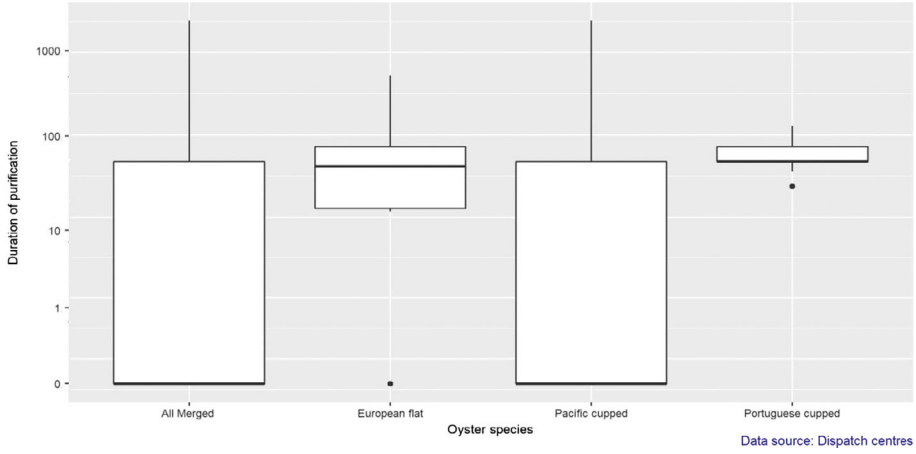

Figure B.8: Box plot of the duration (hours) of purification, dispatch centres

Figure B.9 displays the duration of relaying (hours) of the batches sampled in dispatch centres, overall and for the three oyster species.

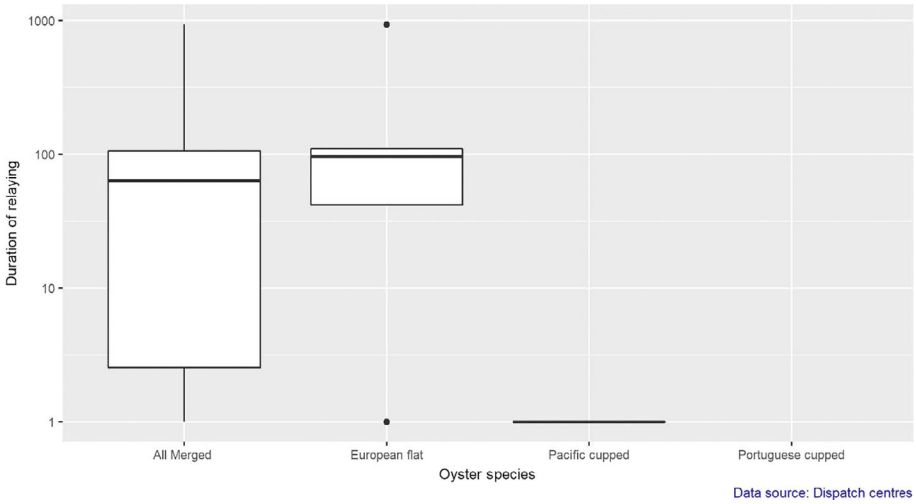

Figure B.9: Box plot of the duration (days) of relaying, dispatch centres

Table B.8: Overall batch origin (production area/s or catch area/s) most representative of origin in terms of quantity (Article 35 (3) of Regulation (EC) 1379/2013), dispatch centres

Country of origin	N	%
Croatia	11	0.5
Denmark	13	0.6
France	1,899	89.0
Greece	8	0.4
Ireland	30	1.4
Italy	5	0.2
Jersey	1	0.1
Netherlands	36	1.7
Portugal	21	1.0
Spain	55	2.6
Sweden	12	0.6
United Kingdom	38	1.8
Total	2,129	100

Figure B.10 displays the temperature used for purification (Celsius) in the water tanks for the batches sampled in dispatch centres

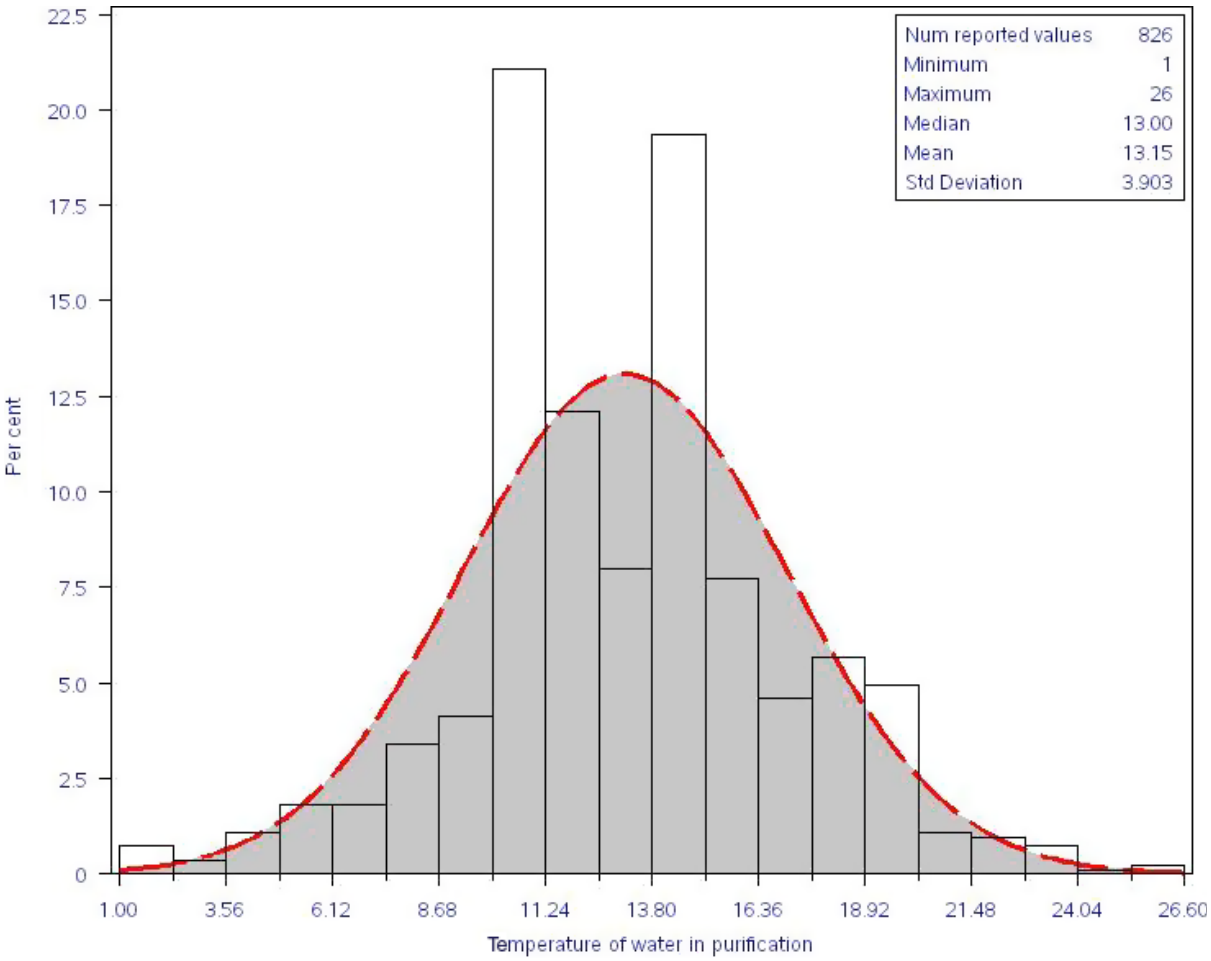

Figure B.10: Temperature of water in purification tank, dispatch centres

Table B.9: Qualitative and quantitative norovirus results in oysters, by temperature (Celsius) of the sample upon arrival in the laboratory, dispatch centres

Arrival temperature	GI		GII		Norovirus		Mean number of copies per gram			Number of samples
	N	%	N	%	N	%	GI	GII	Norovirus	
Q25 = 6	54	7.5	61	8.4	78	10.8	67.9	112	180	723
Q50 = 8	24	4.7	33	6.5	45	8.8	63.4	98.9	162	510
Q75 = 10.5	29	7.9	35	9.6	46	12.6	66.1	104	171	366
Q100 = 18	35	6.6	43	8.1	57	10.8	58.6	97.6	156	530
Total	142	6.7	172	8.1	226	10.6	64.3	104	168	2,129

GI: genogroup I; GII: genogroup II.

No effect of the arrival temperature was observed in terms of apparent prevalence of norovirus contamination (see Table B.9).

Appendix C – Data summary results

In this appendix, some summary statistics are presented for each of the genogroups, considering the quantification results as well as the indicator variable referring to contamination with norovirus for production areas and batches in dispatch centres.

C.1. Norovirus survey results in production areas

Table C.1: Summary statistics of quantitative results, production areas

Genogroup	Results	Central tendency measures			
		Mean	Median	Mode	Geomean
GI	Quantified	726.4	326	105	370.5078
	Below LOQ	63.7	62	50	58.1809
	Not detected	46.0	62	62	35.8983
GII	Quantified	1,270.0	492	208	578.5616
	Below LOQ	97.4	92	92	86.9921
	Not detected	63.9	98	98	48.8513
GI + GII	Norovirus	336.7	160	160	135.2335

LOQ: limit of quantification; GI: genogroup I; GII: genogroup II.

Table C.2: Qualitative and quantitative norovirus results in oysters, by status of production area, production areas

Area status	GI		GII		Norovirus		Mean number of copies per gram			Number of samples
	N	%	N	%	N	%	GI	GII	Norovirus	
A	147	17.3	152	17.9	210	24.7	59.6	83.6	143	851
B	494	37.7	493	37.6	610	46.5	154	305	459	1,312
C	10	58.8	8	47.1	11	64.7	377	182	559	17
Total	651	29.9	653	29.9	831	38.1	119	218	337	2,180

GI: genogroup I; GII: genogroup II.

Table C.3: Qualitative and quantitative norovirus results in oysters, by two-month period, production areas

Sampling period	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Jan–Feb	190	51.1	212	57.0	240	64.5	180	481	661	372
Mar–Apr	138	37.3	133	36.0	172	46.5	99.5	229	329	370
May–Jun	48	13.8	47	13.5	74	21.2	48.5	62.9	111	349
Jul–Aug	49	14.2	36	10.4	60	17.3	61.7	73.2	135	346
Sep–Oct	93	25.3	73	19.9	110	30.0	105	123	228	367
Nov–Dec	133	35.4	152	40.4	175	46.5	210	315	525	376
Total	651	29.9	653	30.0	831	38.1	119	218	337	2,180

GI: genogroup I; GII: genogroup II.

Table C.4: Qualitative and quantitative norovirus results in oysters, by oyster species, production areas

Oyster species	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
European flat oyster	108	34.2	101	32.0	140	44.3	89.3	117	207	316
Oyster, Portuguese cupped	3	25.0	3	25.0	4	33.3	85.2	212	297	12
Pacific cupped oyster	540	29.2	549	29.6	687	37.1	124	235	359	1,852
Total	651	29.9	653	30.0	831	38.1	119	218	337	2,180

GI: genogroup I; GII: genogroup II.

Table C.5: Qualitative and quantitative norovirus results in oysters, by type of oyster production area, production areas

Production type	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Inshore	190	32.5	180	30.8	233	39.8	134	290	424	585
Intertidal	455	29.3	465	29.9	589	37.9	116	194	310	1,555
Offshore	6	15.0	8	20.0	9	22.5	33.8	69.9	104	40
Total	651	29.9	653	30.0	831	38.1	119	218	337	2,180

GI: genogroup I; GII: genogroup II.

Table C.6: Qualitative and quantitative norovirus results in oysters, by production information and production system, production areas

Production	Production system	Number and percentage positive						Mean number of copies per gram			Number of samples
		GI		GII		Norovirus		GI	GII	Norovirus	
		N	%	N	%	N	%				
Farmed	Bottom-grown	6	50.0	4	33.3	7	58.3	63.8	54.3	118	12
	Raised trestle	412	26.1	414	26.3	531	33.7	110	166	276	1,576
	Suspended from sea surface	64	31.2	67	32.7	89	43.4	81.2	254	335	205
Wild	Bottom-grown	169	43.7	168	43.4	204	52.7	176	414	590	387
Total		651	29.9	653	30.0	831	38.1	119	218	337	2,180

GI: genogroup I; GII: genogroup II.

Table C.7: Proportion of norovirus-contaminated oysters, categorising the quantification results considering different thresholds for production areas as well as the classification of results combination considering the substitution approach for not detected (ND) or positive below LOQ results (BL) and the actual observed values for those results above LOQ (P) for the norovirus baseline survey

Norovirus result patterns		Norovirus thresholds												
		> 100		> 200		> 300		> 500		> 1,000		> 10,000		Total
Classification	**Pattern**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**
Both substituted	BL+BL	128	5.87	21	0.96	.	.	.	.	.	.	.	.	186
	BL+ND	82	3.76	.	.	.	.	.	.	.	.	.	.	149
	ND+BL	95	4.36	40	1.83	.	.	.	.	.	.	.	.	144
	ND+ND	789	36.19	.	.	.	.	.	.	.	.	.	.	1,349
Total		1,094	50.18	61	2.80	.	.	.	.	.	.	.	.	1,828
Only one substituted	BL+P	93	4.27	80	3.67	52	2.39	28	1.28	15	0.69	15	0.69	93
	ND+P	36	1.65	30	1.38	26	1.19	17	0.78	7	0.32	7	0.32	36
	P+BL	56	2.57	45	2.06	36	1.65	13	0.60	5	0.23	5	0.23	56
	P+ND	27	1.24	20	0.92	11	0.50	6	0.28	4	0.18	4	0.18	29
Total		212	9.72	175	8.03	125	5.73	64	2.94	31	1.42	31	1.42	214
Observed	P+P	138	6.33	138	6.33	135	6.19	126	5.78	89	4.08	89	4.08	138
Overall total		1,444	66.24	374	17.16	260	11.93	190	8.72	120	5.50	120	5.50	2,180

LOQ: limit of quantification; BL: positive below limit of quantification; ND: not detected; P: above limit of quantification.

C.2. Norovirus survey results in dispatch centres

Table C.8: Summary statistics of quantitative results, dispatch centres

Genogroup	Results	Central tendency measures			
		Mean	Median	Mode	Geomean
GI	Quantified	379.7	199	.	231.9
	Below LOQ	64.1	62	62	61.4
	Not detected	58.8	62	62	55.0
GII	Quantified	596.9	384	188	395.8
	Below LOQ	142.3	194.5	194.5	126.4
	Not detected	91.0	98	98	87.1
GI + GII	Norovirus	168.4	160	160	151.2

LOQ: limit of quantification; GI: genogroup I; GII: genogroup II.

Table C.9: Qualitative and quantitative norovirus results in oysters, by two-month period, dispatch centres

Sampling period	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Jan–Feb	44	11.8	69	18.6	78	20.9	74.2	130	204	373
Mar–Apr	58	15.5	47	12.6	72	19.3	66.6	109	176	374
May–Jun	11	3.5	12	3.8	19	6.1	58.6	90.8	149	314
Jul–Aug	5	1.6	4	1.3	9	2.9	58	89	147	314
Sep–Oct	10	2.7	12	3.3	17	4.6	65.4	91.8	157	368
Nov–Dec	14	3.6	28	7.3	31	8.0	61	109	170	386
Total	142	6.7	172	8.1	226	10.6	64.3	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.10: Qualitative and quantitative norovirus results in oysters, by number of production areas that the batches were sourced from, dispatch centres

Number of source production areas	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
1	128	6.2	149	7.2	199	9.6	64.2	104	168	2,067
2	0	0.0	1	20.0	1	20.0	46.3	73.8	120	5
3	0	0.0	1	33.3	1	33.3	132	71.7	203	3
Not reported	14	25.9	21	38.9	25	46.3	63.4	114	177	54
Total	142	6.7	172	8.1	226	10.6	64.3	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.11: Qualitative and quantitative norovirus results in oysters, by classification of the production areas that were the source of the batch, dispatch centres

Area status	GI		GII		Norovirus		Mean number of copies per gram			Number of samples
	N	%	N	%	N	%	GI	GII	Norovirus	
A	25	1.9	34	2.6	48	3.7	62.3	96.3	159	1,303
B	104	13.5	118	15.3	154	20.0	67.9	117	185	772
Unknown	13	24.1	20	37.0	24	44.4	58.7	109	168	54
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.12: Qualitative and quantitative norovirus results in oysters, by oyster species, dispatch centres

Oyster species	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
European flat oyster	13	16.3	19	23.8	24	30.0	32.6	61.9	93.8	80
Oyster, Portuguese cupped	1	7.7	1	7.7	2	15.4	34	113	147	13
Pacific cupped oyster	128	6.3	152	7.5	200	9.8	65.6	106	171	2036
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.13: Qualitative and quantitative norovirus results in oysters, by mean weight of oysters sampled (in grams) from the batch in dispatch centres

Mean weight of oysters sampled (g)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q25 = 64	24	4.4	23	4.2	38	7.0	61.1	97.8	159	544
Q50 = 75	20	3.7	26	4.9	32	6.0	62	99.6	162	536
Q75 = 91	30	6.0	34	6.8	47	9.3	63.7	103	166	503
Q100 = 406	59	11.3	78	14.9	96	18.4	69.7	116	185	523
Not reported	9	39.1	11	47.8	13	56.5	76	128	204	23
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.14: Qualitative and quantitative norovirus results in oysters, by weight of the batches sampled (in kg) in dispatch centres

Mean weight of batch sampled (kg)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q25 = 100	61	9.7	75	11.9	97	15.5	66.3	117	183	628
Q50 = 250	26	6.0	27	6.2	37	8.6	63.4	105	168	433
Q75 = 500	21	4.0	30	5.7	40	7.6	61.9	97.5	159	530

Mean weight of batch sampled (kg)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q100 = 40,000	25	4.9	27	5.3	37	7.3	65	94.4	159	510
Not reported	9	32.1	13	46.4	15	53.6	63.6	119	183	28
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.15: Qualitative and quantitative norovirus results in oysters, by production information, dispatch centres

Production	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Farmed	130	6.4	150	7.4	201	9.9	65	106	171	2,036
Wild	11	14.1	19	24.4	22	28.2	38.3	65.9	104	78
Unknown	1	6.7	3	20.0	3	20.0	88.1	111	200	15
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.16: Qualitative and quantitative norovirus results in oysters, by production information (repacked or not), dispatch centres

Repacking	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
No	103	5.3	129	6.6	165	8.4	65.3	107	172	1,963
Yes	15	24.6	21	34.4	26	42.6	60.5	108	169	61
Unknown	24	22.9	22	21.0	35	33.3	45.4	48	93.4	105
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.17: Qualitative and quantitative norovirus results in oysters, by duration (hours) of conditioning, dispatch centres

Duration of conditioning (h)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q25 = 0	45	8.2	60	11.0	72	13.1	69.9	122	192	548
Q50 = 72	20	4.1	19	3.9	26	5.4	63.3	101	164	484
Q75 = 144	19	3.7	22	4.3	30	5.9	62.7	102	164	509
Q100 = 2,400	12	2.9	19	4.6	26	6.3	65.9	101	167	411
Not reported	46	26.3	52	29.7	72	41.1	49.8	74.3	124	177
Total	142	6.7	172	8.1	226	10.6	64.3	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.18: Qualitative and quantitative norovirus results in oysters, by duration (days) of relaying, dispatch centres

Duration of relaying (days)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
0	95	5.0	113	5.9	147	7.7	66.2	108	174	1,916
1	0	0	0	0.0	0	0.0	21	29.8	50.8	2
42	1	100	0	0.0	1	100.0	20	24	44	1
96	1	100	1	100.0	1	100.0	44.9	105	150	1
110	0	0	0	0.0	0	0.0	6.5	24	30.5	1
936	0	0.	0	0.0	0	0.0	6.5	24	30.5	1
Not reported	45	22.0	58	28.3	77	37.6	47.5	71.1	119	207
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.19: Qualitative and quantitative norovirus results in oysters, by duration (hours) of purification, dispatch centres

Duration of purification (hours)	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q25 + Q50 = 0	15	1.3	19	1.6	28	2.4	62.7	96.4	159	1,183
Q75 = 48	35	18.2	38	19.8	49	25.5	60.9	112	173	192
Q100 = 2,400	49	11.1	57	12.9	76	17.2	66.6	119	185	441
Not reported	43	13.8	58	18.7	73	23.5	68.9	109	178	313
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.20: Qualitative and quantitative norovirus results in oysters, by thermal status of the water used for purification, dispatch centres

Purification conditions	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Actively	28	23.5	25	21.0	37	31.1	60.9	100	161	119
Ambient	78	10.9	92	12.9	118	16.6	68.9	120	189	713
Unknown	36	2.8	55	4.3	71	5.5	62	95.6	158	1,297
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.21: Qualitative and quantitative norovirus results in oysters, by temperature (Celsius) of water in purification tank, dispatch centres

Purification temperature	Number and percentage positive						Mean number of copies per gram			Number of samples
	GI		GII		Norovirus		GI	GII	Norovirus	
	N	%	N	%	N	%				
Q25 = 10	40	19.2	50	24.0	62	29.8	79.6	122	202	208
Q50 = 13	34	14.2	40	16.7	50	20.8	58	123	181	240
Q75 = 16	25	11.1	23	10.2	36	15.9	74.7	127	202	226
Q100 = 26	4	2.6	4	2.6	6	4.0	58.1	92.7	151	152
Not reported	39	3.0	55	4.2	72	5.5	61.8	95.3	157	1,303
Total	142	6.7	172	8.1	226	10.6	64.2	104	168	2,129

GI: genogroup I; GII: genogroup II.

Table C.22: Proportion of norovirus-contaminated oysters, categorising the quantification results considering different thresholds for batches sampled in dispatch centres as well as the classification of results combination considering the substitution approach for not detected (ND) or positive below LOQ results (BL) and the actual observed values for those results above LOQ (P) for the norovirus baseline survey

Norovirus result patterns		Norovirus thresholds												
		> 100		> 200		> 300		> 500		> 1,000		> 10,000		Total
Classification	**Pattern**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**
Both substituted	BL+BL	43	2.02	28	1.32	.	.	.	.	.	.	.	.	45
	BL+ND	35	1.65	.	.	.	.	.	.	.	.	.	.	47
	ND+BL	50	2.35	41	1.93	.	.	.	.	.	.	.	.	72
	ND+ND	1,713	80.46	.	.	.	.	.	.	.	.	.	.	1903
Total		1,841	86.47	69	3.24	.	.	.	.	.	.	.	.	2,067
Only one substituted	BL+P	16	0.75	16	0.75	11	0.52	5	0.24	3	0.14	3	0.14	16
	ND+P	11	0.52	9	0.42	6	0.28	4	0.19	1	0.05	1	0.05	12
	P+BL	13	0.61	9	0.42	7	0.33	2	0.09	.	.	.	.	13
	P+ND	6	0.28	2	0.09	.	.	.	.	.	.	.	.	7
Total		46	2.16	36	1.69	24	1.13	11	0.52	4	0.19	4	0.19	48
Observed	P+P	14	0.66	14	0.66	14	0.66	14	0.66	6	0.28	6	0.28	14
Overall total		1901	89.29	119	5.59	38	1.78	25	1.17	10	0.47	10	0.47	2,129

LOQ: limit of quantification; BL: positive below limit of quantification; ND: not detected; P: above limit of quantification.

Appendix D – Apparent prevalence estimation results for each genogroup

The result of the analysis for each genogroup considering temporal trend as well as factors associated with norovirus prevalence for production areas sampled as well as for batches sampled in dispatch centres are presented in this appendix.

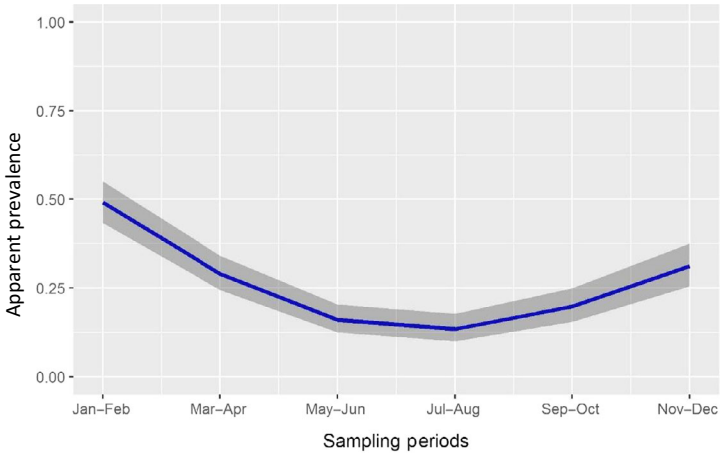

Figure D.1: Estimated apparent prevalence trend for genogroup I in production areas

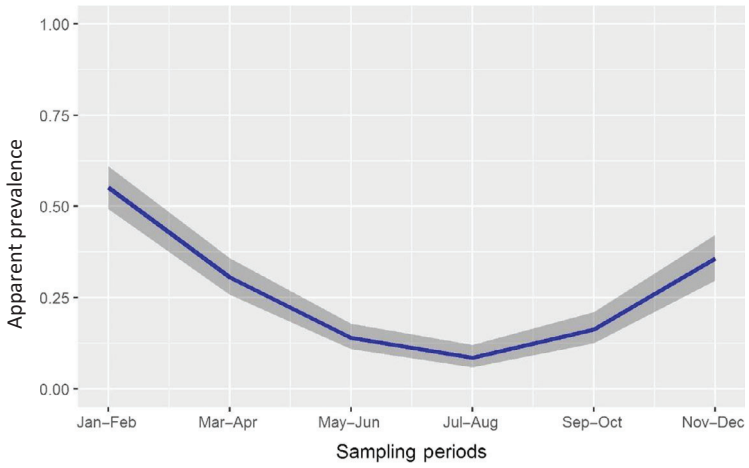

Figure D.2: Estimated apparent prevalence trend for genogroup II in production areas

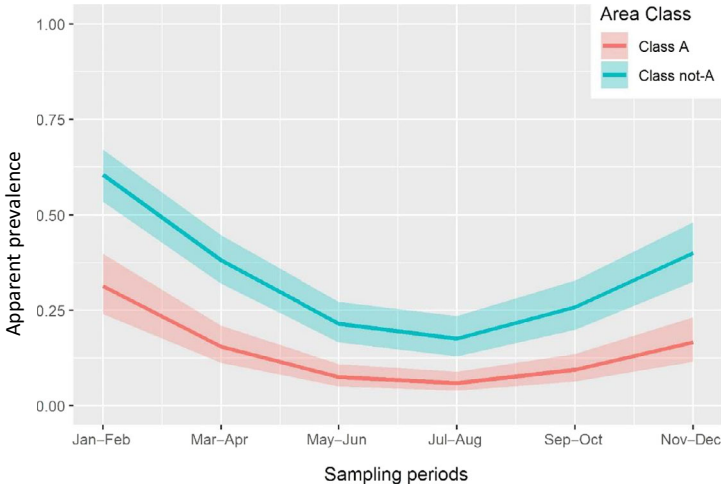

Figure D.3: Estimated apparent prevalence trend for genogroup I comparing Class A with other production areas classes (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Class}\right)$)

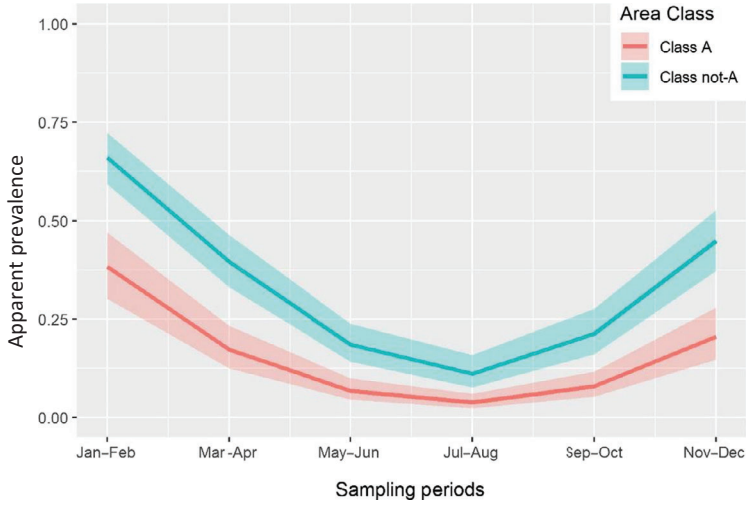

Figure D.4: Estimated apparent prevalence trend for genogroup II comparing Class A with other production areas classes (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Class}\right)$)

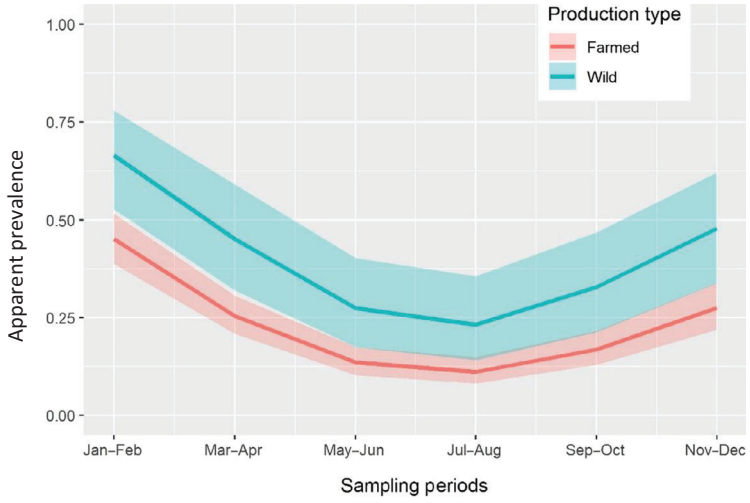

Figure D.5: Estimated apparent prevalence trend for genogroup I comparing farmed and wild production areas (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Production Type}\right)$)

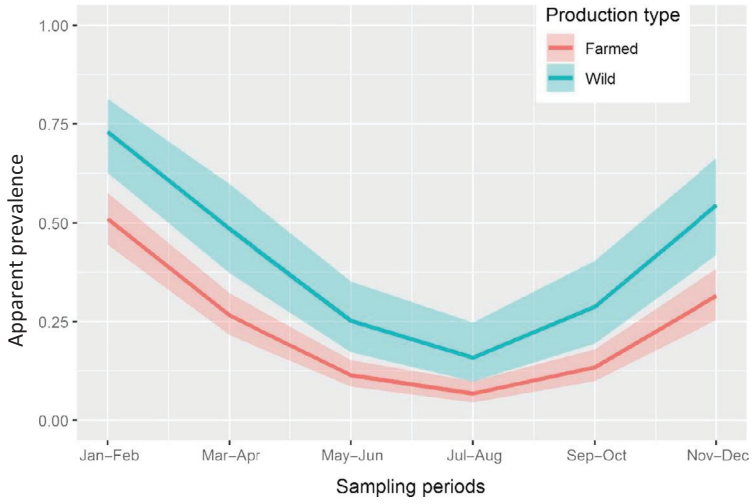

Figure D.6: Estimated apparent prevalence trend for genogroup II comparing farmed and wild production areas (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Production Type}\right)$)

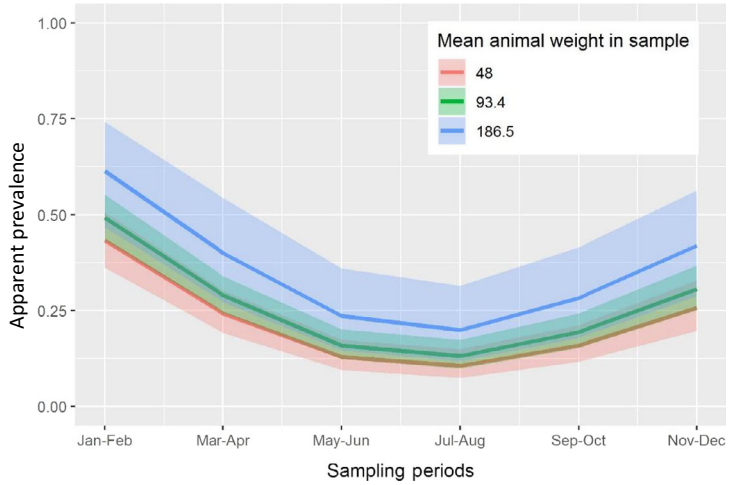

Figure D.7: Estimated apparent prevalence trend for genogroup I in production areas for different mean weight of the animals sampled (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Mean Animal Weight}\right)$)

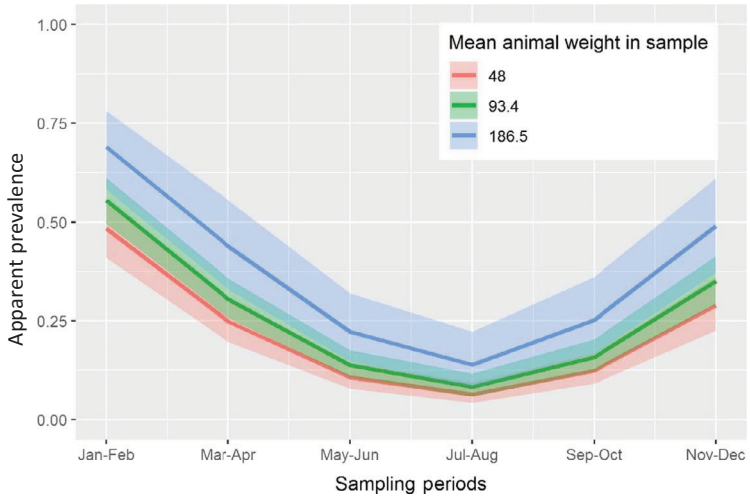

Figure D.8: Estimated apparent prevalence trend for genogroup II in production areas for different mean weight of the animals sampled (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Mean Animal Weight}\right)$)

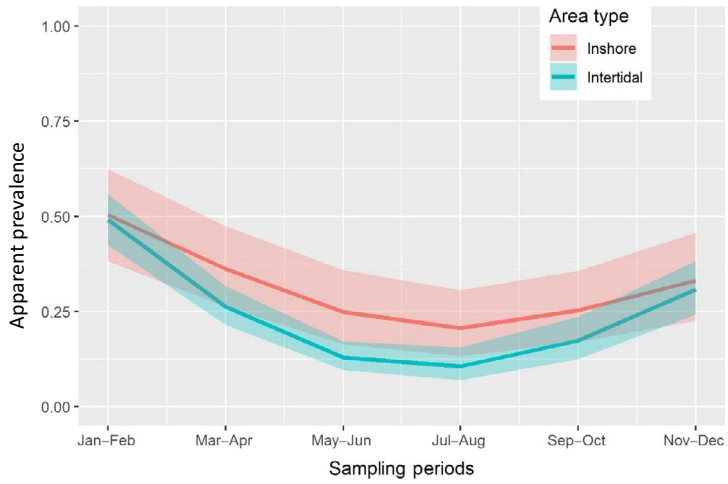

Figure D.9: Estimated apparent prevalence trend for genogroup I comparing inshore and intertidal production areas (multiplicative model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Type} + \text{f}(\text{Time Period}) \times \text{AreaType}\right)$)

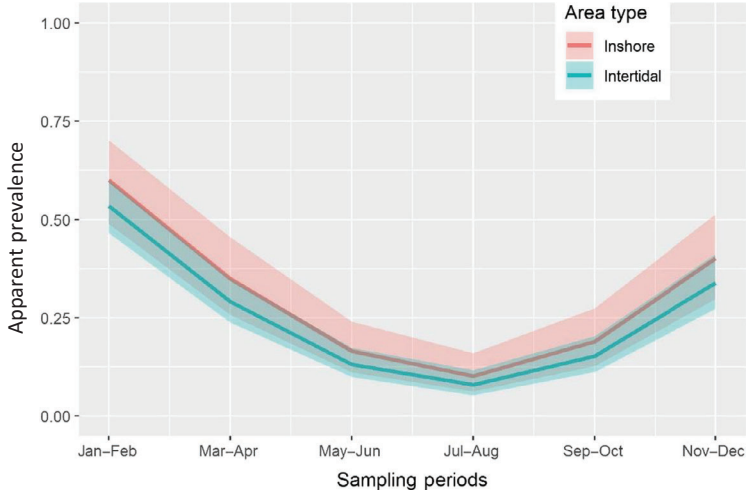

Figure D.10: Estimated apparent prevalence trend for genogroup II comparing inshore and intertidal production areas (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Type}\right)$)

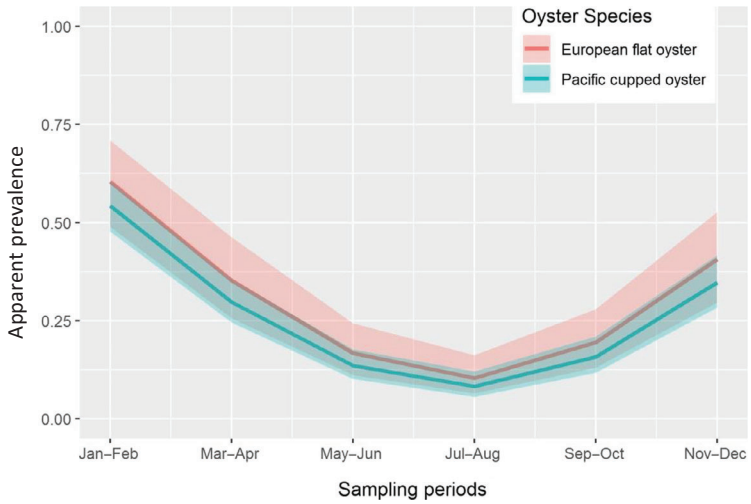

Figure D.11: Estimated apparent prevalence trend for genogroup II comparing the main oyster species sampled in production areas (additive model $\left(\text{Ln}(\frac{\pi}{1-\pi}) = \text{f}(\text{Time Period}) + \text{Oyster Species}\right)$)

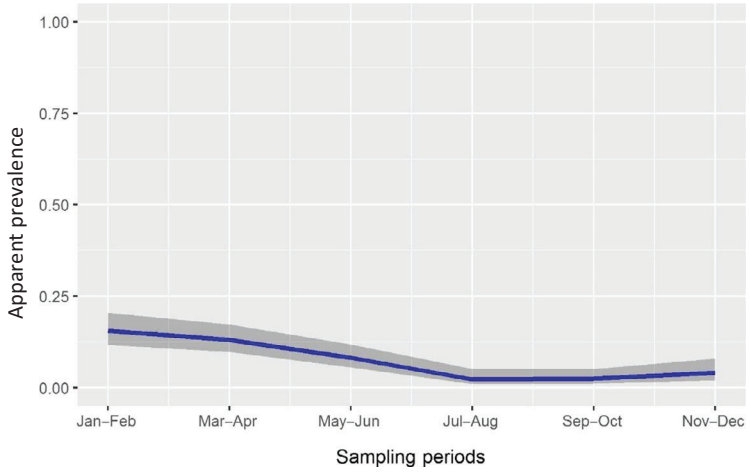

Figure D.12: Estimated apparent prevalence trend for genogroup I in batches sampled in dispatch centres

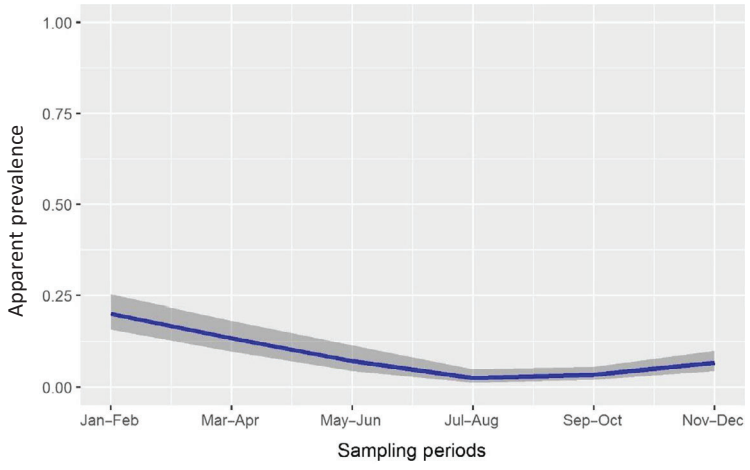

Figure D.13: Estimated apparent prevalence trend for genogroup II in batches sampled in dispatch centres

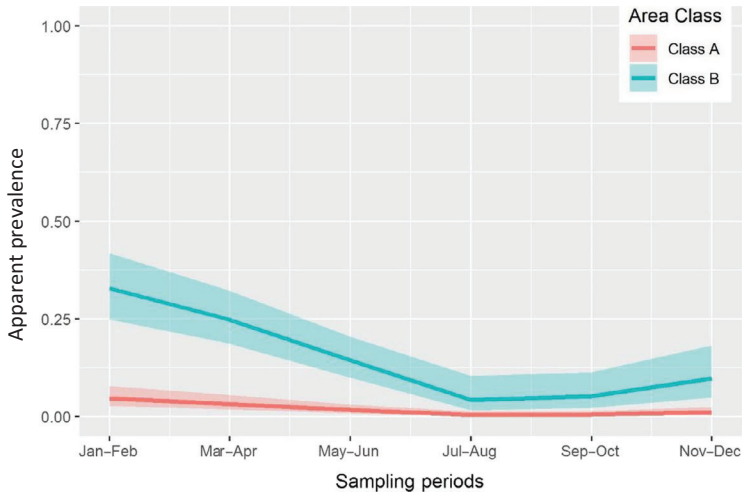

Figure D.14: Estimated apparent prevalence trend for genogroup I in batches sampled in dispatch centres originating from Class A and B production areas (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Class}\right)$)

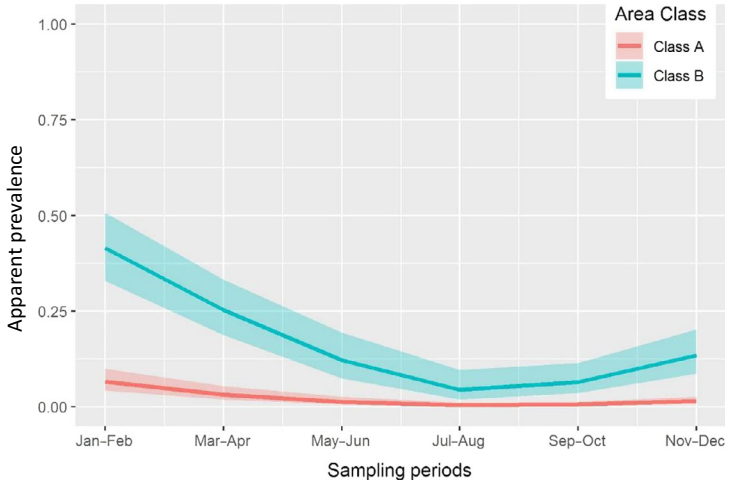

Figure D.15: Estimated apparent prevalence trend for genogroup II in batches sampled in dispatch centres originated from Class A and B production areas (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Area Class}\right)$)

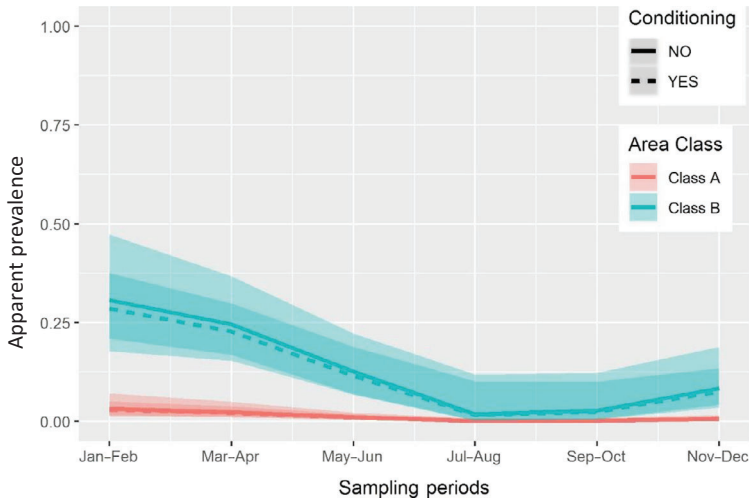

Figure D.16: Estimated apparent prevalence trend for genogroup I in batches sampled in dispatch centres originating from Class A and B production areas, differentiating as to whether or not conditioning was performed (additive model $\left(\text{Ln}(\frac{\pi}{1-\pi}) = \text{f}(\text{Time Period}) + \text{Area Class} + \text{Conditioning}\right)$)

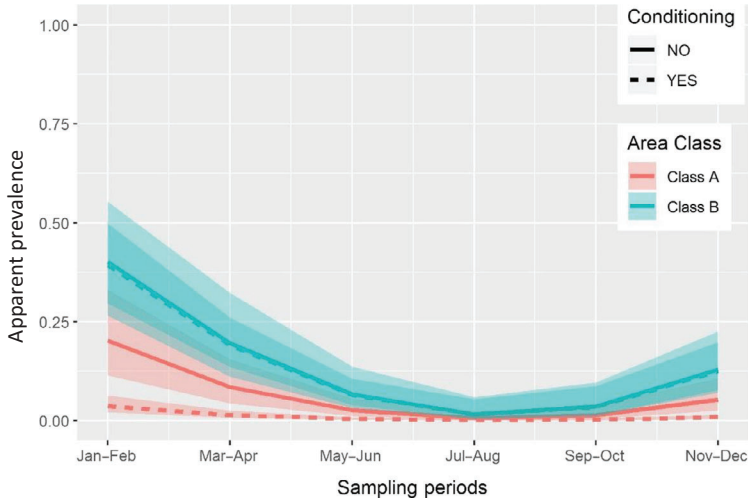

Figure D.17: Estimated apparent prevalence trend for genogroup II in batches sampled in dispatch centres originating from Class A and B production areas, differentiating as to whether or not conditioning was performed (multiplicative model $\left(\text{Ln}(\frac{\pi}{1-\pi}) = \text{f}(\text{Time Period}) + \text{Area Class} + \text{Conditioning} + \text{Area Class} \times \text{Conditioning}\right)$)

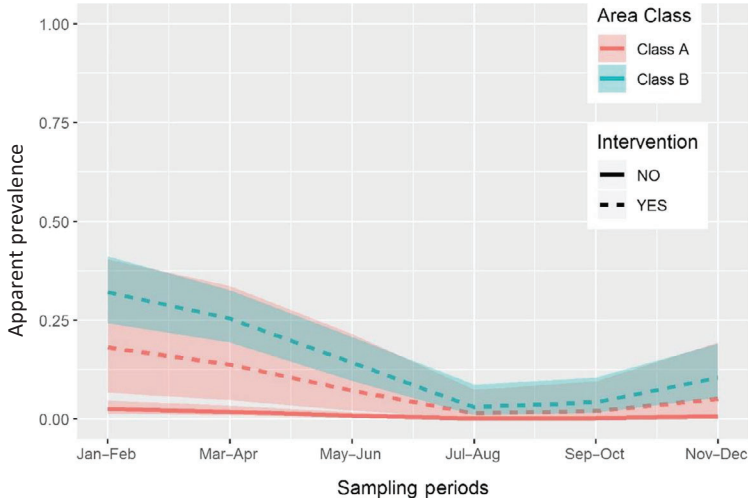

Figure D.18: Estimated apparent prevalence trend for genogroup I in batches sampled in dispatch centres originating from Class A and B production areas, differentiating as to whether or not a treatment (relaying and/or purification) was performed (additive model $\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class} + \text{Intervention}\right)$)

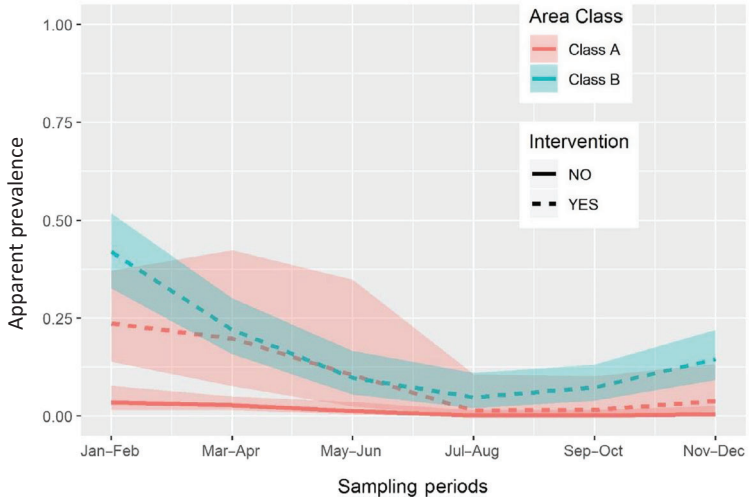

Figure D.19: Estimated apparent prevalence trend for genogroup I in batches sampled in dispatch centres originated from Class A and B production areas, differentiating as to whether or not a treatment (relaying and/or purification) was performed (multiplicative model $\left(\mathrm{Ln}\left(\frac{\pi}{1-\pi}\right) = \mathrm{f}(\text{Time Period}) + \text{Area Class} + \text{Intervention} + \mathrm{f}(\text{Time Period}) \times \text{Area Class}\right)$

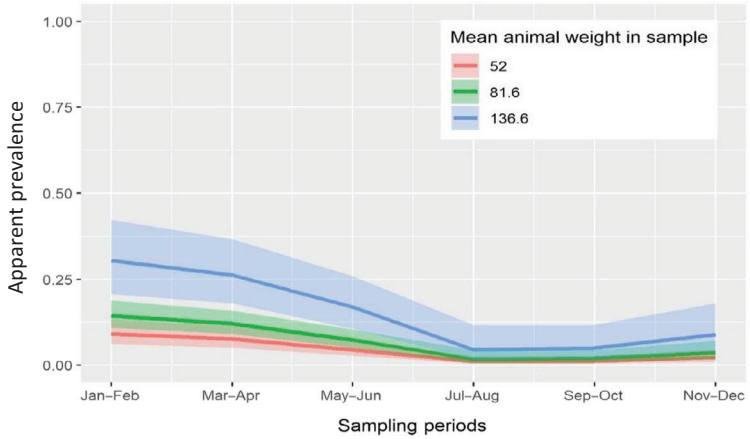

Figure D.20: Estimated apparent prevalence trend for genogroup I in dispatch centres for different mean weights of the animals sampled (additive model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Mean Animal Weight}\right)$)

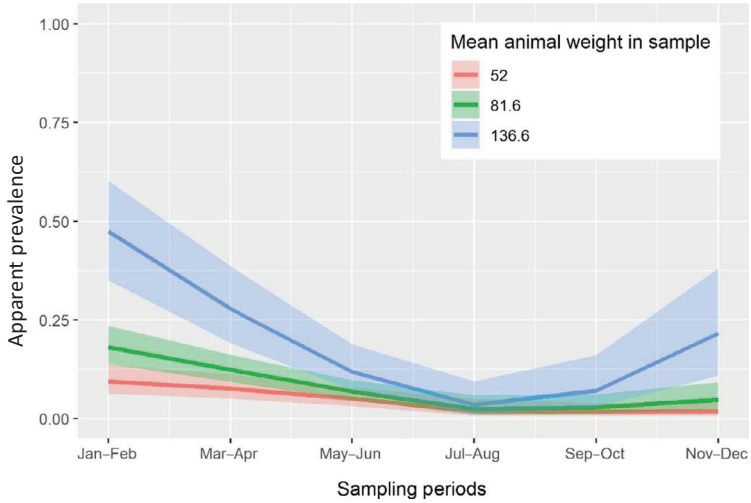

Figure D.21: Estimated apparent prevalence trend for genogroup II in dispatch centres for different mean weights of the animals sampled (multiplicative model $\left(\text{Ln}\left(\frac{\pi}{1-\pi}\right) = \text{f}(\text{Time Period}) + \text{Mean Animal Weight} + \text{f}(\text{Time Period}) \times \text{Mean Animal Weight}\right)$)