International Journal of Food Microbiology
September 2013, Volume 166 (2), Pages 244–248
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.07.022

Norovirus contamination on French marketed oysters

Julien Schaeffer[a], Jean-Claude Le Saux[a], Monica Lora[a, 1], Robert L. Atmar[b], Françoise S. Le Guyader[a, *]

[a] IFREMER, Laboratoire de Microbiologie, Nantes, France
[b] Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States

[1] Present address: INSERM, U892, Université de Nantes, France.

*: Corresponding author : Françoise S. Le Guyader, tel.: + 33 240374052 ; fax: + 33 240374027 ;
email address : sleguyad@ifremer.fr

Abstract:

Contaminated shellfish have been implicated in gastroenteritis outbreaks in different countries. As no regulation has been set up yet regarding viral contamination of food, very few data are available on the prevalence of contaminated products on the market. This study presents data obtained from oysters collected on the French market in one producing area over a 16 month period of time. Noroviruses were detected in 9% of samples with a seasonal impact and influence of climatic events. Contamination levels were low and, surprisingly, oysters sampled directly from the producer were found to have less contamination than oysters from supermarkets.

Highlights

► Noroviruses were detected in 9% of oyster samples collected on the French market ► Norovirus contamination in oysters depends on the season and climatic events ► Norovirus concentration was low in oyster tissues ► Oysters collected directly from producers were less contaminated than oysters collected in supermarkets.

Keywords: Oyster ; Market ; Norovirus ; Quantification ; Calicivirus ; Prevalence

1. Introduction

Shellfish were identified as a vector for human enteric pathogen transmission more than 150 years ago. Consumption of either raw or undercooked shellfish can lead to transmission of disease as human pathogens can be accumulated during their filter-feeding activity. Contamination of shellfish-growing waters with human sewage was recognized as a contributing cause of outbreaks, leading to the development of bacteriological criteria to assess the impact of sewage on shellfish and shellfish-growing waters. The adoption of regulations to specify acceptable levels of bacterial enteric pathogens in shellfish tissues (European regulation 54/2004/EC) or in waters where shellfish are grown (United States Sanitation Program) led to the classification of production areas and thus to a decrease in the number of bacterial outbreaks associated with shellfish consumption (Iwamoto et al., 2010). However, as viral contamination is not yet controlled, gastroenteritis outbreaks linked to shellfish consumption still occur. Among the diverse array of human enteric viruses shed into the environment, noroviruses (NoVs) are currently the most common cause of infection associated with shellfish consumption. *Norovirus* is a genus in the family *Caliciviridae*. The genus is divided into six genogroups, three of which infect humans (GI, II and IV) (Atmar, 2010). NoV GII strains are responsible for almost 90% of clinical cases (with a high prevalence of the GII.4 cluster), while the other 10% are caused by GI strains; GIV strains are recognized infrequently (Atmar, 2010). NoV infection causes gastroenteritis characterized by the symptoms of vomiting and diarrhea, and infected persons, including those asymptomatically infected, shed a high concentration of viral particles in their stools (Atmar et al., 2008). NoVs are non-enveloped viruses, are very stable in the environment, and can thus contaminate rivers or coastal waters that support shellfish growth (Perez-Sautu et al., 2012; Yang et al., 2012). As a consequence, they have been detected in shellfish in a number of countries (Diez-Valcarce et al., 2012; Rajko-Nenow et al., 2012; Suffredini et al., 2012; Terio et al., 2010; Woods and Burkhardt, 2010).

The present study was conducted to evaluate the prevalence of these viruses in French oysters available on the market. Oyster production is an important industry in France, involving about 3 120

54 farms distributed mainly on the Channel and Atlantic Ocean coasts. One area, located on the Atlantic
55 coast, was selected for this study.
56
57 **2. Materials and methods**
58
59 2.1 Sampling
60 The study was performed in the southwestern coastal area of France (Vendée area). Sampling was
61 performed by the veterinary service directly at a farm's shop or at farmer's markets (both referred to
62 as producers), from packers (wholesalers) and at supermarkets. The sampling scheme aimed to cover
63 selling places in the Vendée area. A total of 388 samples was collected and analyzed between
64 February 2010 and May 2011 (16 months). The sampling scheme was set up to collect 12 samples per
65 month from May to October and a maximum of 40 samples per month from November to April (an
66 average of 36 samples was collected per month during this latter period). The sampling was based
67 upon products available on the market such that small producers were sampled less frequently, as they
68 did not have products to sell each time the sampling was performed. When sampling, the officer
69 completed a questionnaire indicating the sampling place, name of the producer, original oyster
70 growing area, and date and relocation area, if available.
71
72 2.2 Oyster processing
73 Each sample contained at least 12 oysters and was sent to the laboratory refrigerated (4°C) within 24 h
74 of collection. On the day of arrival in the laboratory, the 12 oysters were washed, shucked, and their
75 total weight recorded. The stomach and digestive diverticula (DT) were removed by dissection,
76 pooled, divided into 1.5 g portions (representing approximately 3 oysters) and frozen at -20°C. For
77 analysis, DTs were inoculated with 10^6 genomic copies of mengovirus (MgV) (kindly provided by A.
78 Bosch, University of Barcelona, Spain), homogenized, extracted with chloroform-butanol, and
79 precipitated with Cat-floc (Calgon, Ellwood City, PA, USA), followed by polyethylene glycol 6000
80 (Sigma, St Quentin, France) precipitation (Atmar et al., 1995). For viral nucleic acid (NA) extraction,
81 the PEG pellet was suspended in 1 ml of RNAse-free H_2O, mixed with the NucliSENS lysis buffer (2

ml) (bioMerieux, Lyon, France), and incubated for 30 min at 56°C (Le Guyader et al., 2009). The purification steps were performed with an automatic easyMag extractor (bioMerieux) using a NucliSENS kit (bioMerieux). The NA was suspended in 100 µl of RNase-free water and analyzed immediately or kept frozen (-80°C).

2.3 Real-time RT-PCR (RT-qPCR)

Amplifications were carried out using the Ultrasens quantitative RT-PCR kit (Invitrogen, Saint-Aubin, France), using previously published cycling conditions, primers, and probes for NoV and MgV (Pinto et al., 2009; Sima et al., 2011). Amplifications were performed on undiluted extracts or ten-fold diluted extracts. Precautions, such as the use of isolated rooms for various steps and filter tips on the micropipettes, were taken to prevent false positive results. Two negative amplification controls (water) were included in each amplification series and no more than seven samples were analyzed in each RT-qPCR assay.

The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred (i.e., when fluorescence became distinguishable from the background) and only samples for which wells yielded a C_T value < 41 were considered as positive (the positive threshold (P_T)) based upon our prior experience (Le Guyader et al., 2009).

2.4 Extraction efficiency

After the extraction of samples seeded with the mengovirus, undiluted and ten-fold diluted extracts were subjected to RT-qPCR for MgV. The C_T value of a sample was compared to the C_T value of the positive control used in the extraction series, and to a standard curve made by end-point dilution. This difference (ΔC_T) was used to determine the extraction and amplification efficiency, using the equation $100e^{-0.6978\Delta CT}$, and expressed as a percentage for each sample (Le Guyader et al., 2009). If the extraction efficiency was under 10%, the extraction was repeated. If after the second extraction, the extraction efficiency was still under 10% and greater than 1%, then the sample was analyzed for NoV but not considered for quantification.

2.5 NoV quantification

The limit of quantification is estimated to be 70 RNA copies/g DT (Le Guyader et al., 2009). For samples with a C_T value between 39 and 41, a concentration of 35 RNA copies/g of DT (half of the limit of quantitation) was assigned. Only samples for which wells yielded C_T value < 39, (the quantification threshold (Q_T)), and an extraction efficiency >10% were included in quantitative analysis. Standard curves based on *in vitro* transcription plasmids containing nucleotides 146 to 6935 of the GI.1 Norwalk virus (Genbank M87661), nucleotides 4191 to 5863 of the GII.4 Houston virus (Genbank EU310927), or nucleotides 667 to 967 of the GIV.1 Fort Lauderdale virus (Genbank AF414426.1) were included on each run. The number of RNA copies in each positive sample was estimated by comparing the C_T value with the corresponding standard curve, and the concentration was then back-calculated based on the volume of NA. The NoV concentration was reported as per g of DT and per oyster based on DT weight recovered, without or with extraction efficiency correction.

2.6 Statistical analysis.

Categorical data (including frequency of positivity by year and by producer) were compared using a Fisher exact test. A *P* value of <0.05 was considered significant (Statgraphics Centurion XV).

3. Results

3.1 Sampling

Samples were collected from 42 different locations based on market availability: 12 of these were sampled once, 19 between two and 16 times (134 samples, average of 7 samples collected per location), and 11 more than 16 times (242 samples average of 22 samples) (Table 1). The sites sampled most frequently corresponded to large producers or packers or supermarkets. Among all samples, 149 were produced locally (southwestern coast) and 72 in the western coast (Brittany-Normandy). The data sheet describing the production locale could not be completed for the remaining 167 samples (e.g., in supermarkets) because the sanitation label only contained information about the last distributor. One hundred five samples were produced in good quality areas (<230 *Escherichia*

coli/ 100 g of shellfish tissues, class A area, EU regulation). Three were put on the market within 2 days and the other 102 samples were relocated to aerated basins with seawater changed twice a day (tidal events) located just in front of farms; these areas were classified as potentially contaminated area (class B, EU regulation). The other 283 samples were produced in class B area (283 samples) and underwent depuration. Depuration time was available for only 26 samples (average time of 6.5 days).

3.2 Extraction efficiencies

One sample, collected in April 2010, had an unacceptable extraction efficiency (less than 1%) despite repeated extraction and was excluded from the study (this sample was negative for NoV but could be a false negative). Sixteen additional samples showed poor extraction efficiency (mean = 7 ± 2%). The majority of the samples (95.8%) had good extraction efficiency (mean = 39 ± 21%) (Table 2). Absence of inhibition was verified for all samples by dilution of nucleic acid.

3.3 NoV detection

Among the 387 samples, 352 (91%) were NoV-negative and 35 (9 %) were contaminated with NoV (Table 2). The average extraction efficiency of samples negative for NoV (40%) was comparable to the average extraction efficiency obtained with NoV positive samples (35%). One positive sample (collected in a supermarket) had a poor extraction efficiency and was considered as positive but not quantified, the 15 other samples were negative. GI NoV was detected in two samples (under the limit of quantification), GII NoV in 28 samples (14 under the limit of quantification), and GIV NoV was not detected. Four samples were contaminated by both GI and GII NoVs (two samples collected in packers and two directly from producer farms).

The sampling frequency was based on the amount of shellfish present on the market and thus some large producers were sampled more frequently than others. No statistical difference ($P = 0.07$) was observed between producers sampled less than 16 times (8 positive samples/145 samples) compared to producers sampled more frequently (27 positive samples/242 samples). Among these, only one producer had no positive samples, and four producers had at least 14% of their samples positive for NoV (Table 1).

NoVs were detected during the winter and early spring months in both years, with the exception of one sample that was NoV-positive in September 2010 (Figure 1). A higher frequency of NoV detection was observed among samples collected in February-March 2010 compared to those collected in February-March 2011 (21/77 vs. 7/76, respectively, $P = 0.0059$) (Figure 1).

Among the 82 samples collected from supermarkets, 14 were positive for NoV whereas only seven of 164 samples collected directly from producers were NoV positive ($P = 0.0013$). Among the 140 samples collected from packing companies, 14 were found to be contaminated by NoVs (Table 2).

3.4 NoV quantification

For all positive samples, the mean extraction efficiency was about 33%, with no difference between samples from supermarkets, packers or producers. Sixteen samples had a C_T value under the Q_T, estimated to be less than 70 RNA copies / g DT. The other samples had concentrations between 93 and 220 RNAc /g DT, when extraction efficiency was not taken into account, or between 256 and 738 RNAc/g DT when corrected for extraction efficiency. To further estimate oyster contamination, NoV quantification was estimated per oyster, with a concentration of 35 RNAc/g DT assigned to positive samples under the limit of quantification (Table 3). Geometric mean concentrations varied from 27 to 245 RNAc/oyster considering uncorrected or corrected numbers.

4. Discussion

In France, the largest oyster producer in Europe, shellfish production is an important economic activity, with producing areas distributed along different coasts. Most of the oysters produced are consumed in France, especially in the western coastal area (Secodip-Ofimer, 2007). Oyster farmers sell their produce directly from the farms or at markets to supermarkets or to packing companies. As in other countries, oyster consumers have been infected by NoV. We have identified the conditions leading to oyster contaminations for some outbreaks in France in the past (Le Guyader et al., 2006; 2008; 2010), but at times no particular event was found (unpublished data). This observation raised the question of how prevalent NoVs are in marketed shellfish. No studies on this matter have yet been

194 conducted before in France and there are few published data on market samples (Boxman, 2010;
195 Costantini et al., 2006; Woods and Burkhardt, 2010).
196 An area of production representing 466 producers was selected in southwestern France. Our
197 study sought to evaluate the contamination throughout the year, but sampling was done more
198 frequently during the winter-spring period. This choice was made for several reasons: the probability
199 of NoV levels in sewage is higher during the winter outbreak of gastroenteritis (Phillips et al., 2011;
200 Rohayem, 2009), the higher carbohydrate-ligand expression in oyster tissues during this time increases
201 NoV bioaccumulation (Maalouf et al., 2010, 2011), oyster consumption is higher in this period of the
202 year (Secodip-Ofimer, 2007), and many French-shellfish related disease outbreaks have been observed
203 to occur in February-March (Le Guyader et al., 2006; 2008; 2010).
204 Extraction of human enteric viruses from shellfish involves several steps that depend on the
205 method used but all studies targeted the digestive tissues and analyzed comparable amounts of tissues
206 (1.5 g to 2 g), with eventually an additional step to further concentrate viral particles (Le Guyader et
207 al., 2009; Nishida et al., 2007; Terio et al., 2010; Woods and Burkhardt, 2010; Lowther et al., 2012a).
208 Potential inhibitor persistence, specificity and sensitivity are important parameters that need to be
209 checked when analyzing food or environmental samples with low levels of contamination (Julian and
210 Schwab, 2012). The use of kits for NA extraction and purification and the inclusion of quality controls
211 to verify the extraction efficiency and lack of inhibitors are now commonly used (Pinto et al. 2009; Le
212 Guyader et al., 2009; Nishida et al., 2007; Mesquita et al., 2011; Woods and Burkhardt, 2010;
213 Lowther et al., 2012a). Finally, the use of one-step TaqMan-based RT-qPCR, targeting the ORF1-
214 ORF2 junction region, also constitutes a major improvement for NoV detection and lowering the risk
215 of contamination (Kageyama et al., 2003; Loisy et al. 2005). The number of samples is important for
216 representativity, so we only considered papers reporting NoV prevalence based on at least one
217 hundred analyzed samples. Taking into account these considerations, NoVs have been detected in
218 76.2% of oysters collected in commercial harvesting areas in the UK (Lowther et al., 2012a), in 3.9%
219 of US oysters sold live (Woods and Burkhardt, 2010), and in 5% of oysters in Japan (Nishida et al.,
220 2007). Possible explanations for such large difference in contamination within countries include
221 differences in the efficiency of sewage treatment, different environmental conditions, or different

222 policies regulating oyster production. For example, a recent study from Scotland, reported the
223 presence of HEV in 92% of mussels collected for human consumption that had been grown near a
224 slaughterhouse (Crossan et al., 2012). Both in the US and the UK, a seasonal impact of the
225 contamination was observed (in the Japanese study samples were collected only during winter and the
226 beginning of spring). During our study we confirmed a seasonal effect, but also found a difference
227 between the two winters of the study. This may be explained by the intensity and duration of the
228 winter outbreak in the human community directly impacting the level of NoV in sewage, and also by
229 climatic conditions (www.sentiweb.org)(Grodzki et al., 2012).

230 Some samples of this study were produced in clean areas (class A regarding EC regulation).
231 However, as oyster collection is easier when the tidal coefficients are highest (occurring twice a
232 month), producers collect them at these times and then keep them in front of the farms (classified as B
233 area) in large basin connected to the sea (with sand filtered seawater changed twice a day). Out of the
234 105 samples in the present study that were produced in a good quality (class A) area, only three
235 (negative for NoV) remained in such basins for less than two days. Among the 102 other samples kept
236 for more than two days in these basins, 13 were found positive for NoV, confirming the inadequacy of
237 such practice (Ventrone et al., 2013). It is important to consider oyster farming practices to improve
238 shellfish quality as we know that depuration is not efficient for viral elimination (Richards et al.,
239 2010).

240 A large proportion of produced oysters are distributed by packing companies to other parts of
241 France (markets or supermarkets or restaurants) or to other countries. Locally, consumers can buy
242 shellfish directly from producers (on the farm or at a market) or in supermarkets. To our surprise,
243 shellfish sold directly by producers were significantly less frequently contaminated by NoV than
244 shellfish sold in supermarkets. Such a difference is difficult to explain as supermarkets provide few
245 data about the oysters they sell, with no information on the production area. It is not secondary
246 contamination as French regulation forbids the re-immersion of oysters after packaging. We can
247 hypothesize that supermarkets sell oysters from other producing areas (than the ones we studied) or
248 even imported oysters. The main question though is how producers select oysters they want to sell
249 directly. Since direct sales can represent up to 30% of consumed oysters, this point is important to

consider for risk analysis evaluation. Additional data are needed from other producing areas to examine these issues.

NoVs are recognized as very infectious pathogens (Teunis et al., 2008). Here we expressed the NoV concentration per g of DT, as is usually published, but also per oyster. A recent risk analysis study performed on oyster-related disease outbreaks showed a high infectivity for both GI and GII strains, especially for secretor positive phenotype individuals (Thebault et al., 2013). The level detected in these oyster samples may be sufficient to induce gastroenteritis symptoms in consumers. However, low virus concentrations are associated with fewer symptomatic cases and make case identification more difficult (Lowther et al., 2012b). Epidemiologic studies to evaluate the association of levels of NoV contamination with risk of disease are needed.

The recognition that NoVs are everywhere and that food plays an important role in their transmission is now clear (Havelaar et al., 2012; Wobus and Nguyen, 2012). From a public health perspective, the sanitary quality of shellfish for human consumption needs to be improved, just as such a needs has been identified for other food such as fresh produce (Baert et al., 2011). The future CEN/ISO horizontal method will be helpful to estimate the exposure of consumers to contaminated food and, eventually to define an acceptable threshold of virus presence, if such a threshold can be identified. Data collection, large epidemiological studies and further development of risk analysis based on quantitative data will guide changes needed to improve future regulations.

Acknowledgements

This work was supported by the French government (DGAl), 2010-149/19081, program 206-sous action 37, and Public Health Service grant NIH P01 AI057788, Agriculture and Food Research Initiative Competitive grant 2011-68003-30395 from the USDA National Institute of Food and Agriculture, and the John S. Dunn Research Foundation.

The authors thank G. Poiraud (Veterinary service of Vendée area) for all the sampling.

Figure legend:

Figure 1: Monthly frequency of positive samples during the study.

The number of positive samples was expressed in % per month, with numbers of sample collected per month indicated at the top. The * indicates the significant difference observed during the two months February and March 2010 compared to 2011 (P = 0.0059).

References

Atmar, R.L., 2010. Noroviruses: state of the art. Food and Environmental Virology 2, 117-126.

Atmar, R.L., Opekum, A.R., Gilger, M.A., Estes, M.K., Crawford, S.E., Neill, F.H., Graham D.Y., 2008. Norwalk virus shedding after experimental human infection. Emerging and Infectious Disease 14, 1553-1557.

Atmar, R.L., Neill, F.H., Romalde, J.L., Le Guyader, F., Woodley, C.M., Metcalf, T.G., Estes, M.K., 1995. Detection of Norwalk virus and Hepatitis A virus in shellfish tissues with the PCR. Applied and Environmental Microbiology 61, 3014-3018.

Baert, L., Mattison, K., Loisy-Hamon, F., Harlow, J., Martyres, A., Lebeau, B., Stals, A., van Coillie, E., Herman, L., Uyttendaele, M., 2011. Norovirus prevalence in Belgian, Canadian and French produce: a threat to human health? International Journal of Food Microbiology 151, 261-269.

Boxman, I.L.A., 2010. Human enteric viruses occurrence in shellfish from European markets. Food and Environmental Virology 2, 156-168.

Costantini, V., Loisy, F., Joens, L., Le Guyader, F.S., Saif, L.J., 2006. Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. Applied and Environmental Microbiology 72, 1800-1809.

Crossan, C., Baker, P.J., Craft, J., Takeuchi, Y., Dalton, H.R., Scobie, L., 2012. Hepatitis E virus genotype 3 in shellfish, United Kingdom. Emerging and Infectious Disease 18, 2085-2087.

Diez-Valcarce, M., Kokkinos, P., Soderberg, K., Bouwknegt, M., Willems, K., de Roda-Husman, A-M., von Bonsdorff, C-H., Bellou, M., Hernandez, M., Maunula, L., Vantarakis, A., Rodriguez-Lazaro, D., 2012. Occurrence of human enteric viruses in commercial mussels at retail level in three European countries. Food and Environmental Virology 4, 73-80.

306 Grodzki, M., Ollivier, J., Le Saux, J-C., Piquet, J-C., Noyer, M., Le Guyader, F.S., 2012. Impact of
307 Xynthia tempest on viral contamination of shellfish. Applied and Environmental Microbiology 78,
308 3508-3511.
309 Havelaar, A.H., Haagsma, J.A., Mangen, M.-J. J., Kemmeren, J. M., Verhoef, L.P.B., Vijgen, S.M.C.,
310 Wilson, M., Friesema, I.H.M.., Kortbeek, L.M., van Duynhoven, Y.T.H.P., van Pelt, W. 2012.
311 Disease burden of foodborne pathogens in the Netherlands, 2009. International Journal of Food
312 Microbiology 156, 231-238.
313 Iwamoto, M., Ayers, T., Mahon, B.E., Swerdlow, D.L., 2010. Epidemiology of seafood-associated
314 infections in the United States. Clinical Microbiology Review 23, 399-411.
315 Julian, T.R., Schwab, K.J., 2012. Challenges in environmental detection of human viral pathogens.
316 Current Opinion in Virology 2, 78-83.
317 Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F.B., Takeda, N.,
318 Katayama, K., 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on
319 real-time quantitative reverse transcription-PCR. Journal of Clinical Microbiology 41, 154-157.
320 Le Guyader, F.S., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., Doyle, A., Zidane,
321 M., Suffredini, E., Kholi, E., Maddalo, F., Morini, M., Gallay, A., Pommepuy, M., Pothier, P.,
322 Ruggeri, F. M., 2006. Detection of multiple noroviruses associated with an international
323 gastroenteritis outbreak linked to oyster consumption. Journal of Clinical Microbiology 44, 3878-
324 3882.
325 Le Guyader, F.S., Le Saux, J-C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., Giraudon, H.,
326 Delmas, G., Pommepuy, M., Pothier, P., Atmar, R. L. 2008. Aichi virus, norovirus, astrovirus,
327 enterovirus and rotavirus involved in clinical cases from a French oyster-related gastroenteritis
328 outbreak. Journal of Clinical Microbiology 46, 4011-4017.
329 Le Guyader, F.S., Krol, J., Ambert-Balay, K., Ruvoen-Clouet, N., Desaubliaux, B., Parnaudeau, S., Le
330 Saux, J-C., Ponge, A., Pothier, P., Atmar, R. L., Le Pendu, J., 2010. Comprehensive analysis of a
331 norovirus-associated gastroenteritis outbreak, from the environment to the consumer. Journal of
332 Clinical Microbiology 48, 915-920.

333 Le Guyader, F.S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., Atmar, R. L.,
334 2009. Detection and quantification of noroviruses in shellfish. Applied and Environmental
335 Microbiology 74, 618-624.
336 Loisy, F., Atmar, R.L., Guillon, P., Le Cann, P., Pommepuy, M., Le Guyader, S.F., 2005. Real-time
337 RT-PCR for norovirus screening in shellfish. Journal of Virological Methods 123, 1-7.
338 Lowther, J.A., Gustar, N.E., Powell, A.L., Hartnell, R.E., Lees, D.N., 2012a. Two-year systematic
339 study to assess norovirus contamination in oysters from commercial harvesting areas in the United
340 Kingdom. Applied and Environmental Microbiology 78, 5812-5817.
341 Lowther, J.A., Gustar, N.E., Hartnell, R.E., Lees, D.N., 2012b. Comparison of norovirus RNA levels
342 in outbreak-related oysters with background environmental levels. Journal of Food Protection 75,
343 389-393.
344 Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu J., Atmar, R.L., Crawford, S.E., Le Guyader, F.
345 S., 2011. Strain-dependent norovirus bioaccumulation in oysters. Applied and Environmental
346 Microbiology 77, 3189-3196.
347 Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J-C., Atmar, R.L., Le Guyader, F.S., 2010.
348 Norovirus genogroup I and II ligands in oysters: tissue distribution and seasonal variations.
349 Applied and Environmental Microbiology 76, 5621-5630.
350 Mesquita, J.R., Vaz, L., Cerqueira, S., Castilho, F., Santos, R., Monteiro, S., Manso, C.F., Romalde, J.
351 L., Nascimento, M.S.J., 2011. Norovirus, hepatitis A virus and enterovirus presence in shellfish
352 from high quality harvesting areas in Portugal. Food Microbiology 28, 936-941.
353 Nishida, T., Nishio, O., Kato, M., Chuma, T., Kato, H., Iwata, H., Kimura, H., 2007. Genotyping and
354 quantitation of norovirus in oysters from two distinct sea areas in Japan. Microbiology and
355 Immunology 51, 177-184.
356 Perez-Sautu, U., Sano, D., Guix, S., Kasimir, G., Pinto, R.M., Bosch, A., 2012. Human norovirus
357 occurence and diversity in the Llobregat river catchment, Spain. Environmental Microbiology 14,
358 494-502.
359 Phillips, G., Tam, C.C., Rodrigues, L.C., Lopman, B., 2011. Risk factor for symptomatic and
360 asymptomatic norovirus infection in the community. Epidemiology and Infection 139, 1676-1686.

361 Pinto, R.M., Costafreda, M.I., Bosch, A., 2009. Risk assessment in shellfish-borne outbreaks of
362 hepatitis A. Applied and Environmental Microbiology 75, 7350-7355.
363 Rajko-Nenow, P., Kaeveney, S., Flannery, J., O'Flaherty, V., Dore, W., 2012. Characterisation of
364 norovirus contamination in an Irish shellfishery using real-time RT-qPCR and sequencing analysis.
365 International Journal of Food Microbiology 160, 105-112.
366 Richards, G.P., McLeod, C., Le Guyader, F.S., 2010. Processing strategies to inactivate enteric viruses
367 in shellfish. Food and Environmental Virology 2, 183-193.
368 Rohayem, J., 2009. Norovirus seasonality and the potential impact of climate change. Clinical
369 Microbiology and Infection 15, 524-527.
370 Secodip-Ofimer, 2007. Consommation des produits de la pêche et de l'aquaculture. Annual Report,
371 Paris, France, Secodip/Ofimer 1-107.
372 Sima, L.C., Schaeffer, J., Le Saux, J-C., Parnaudeau, S., Elimelech, M., Le Guyader, F.S. 2011.
373 Calicivirus removal in a membrane bioreactor wastewater treatment plant. Applied and
374 Environmental Microbiology 77, 5170-5177.
375 Suffredini, E., Magnabosco, C., Civettini, M., Rosseti, E., Arcangeli, G., Croci, L., 2012. Norovirus
376 contamination in different shellfish species harvested in the same production areas. Journal of
377 Applied Microbiology 113, 686-692.
378 Terio, V., Martella, V., Moschidou, P., Di Pinto, P., Tantillo, G., Buanavoglia, C., 2010. Norovirus in
379 retail shellfish. Food Microbiology 27, 29-32.
380 Teunis, P. F. M., Moe, C. L., Liu, P., Miller, S. E., Lindesmith, L., Baris, R. S., Le Pendu, J.,
381 Calderon, R. L., 2008. Norwalk virus: how infectious is it? Journal of Medical Virology 80, 1468-
382 1476.
383 Thebault, A., Teunis, P. F. M., Le Pendu, J., Le Guyader, F. S., Denis, J-B., 2013. Infectivity of GI
384 and GII noroviruses established from oyster related outbreaks. Epidemics.
385 http://dx.doi.org/10.1016/j.epidem.2012.12.004
386 Ventrone, I., Schaeffer, J., Ollivier, J., Parnaudeau, S., Pepe, T., Le Pendu, J., Le Guyader, F.S., 2013.
387 Chronic or accidental exposure of oysters to norovirus: is there any difference in contamination?
388 Journal of Food Protection 76, 505-509.

389 Wobus, C., Nguyen, T. H., 2012. Viruses are everywhere-what do we do? Current Opinion in
390 Virology 2, 60-62.
391 Woods, J.W., Burkhardt III, W., 2010. Occurrence of norovirus and hepatitis A virus in US oysters.
392 Food and Environmental Virology 2, 176-182.
393 Yang, N., Qi, H., Wong, M.M.L., Wu, R.S.S., Kong, R.Y.C., 2012. Prevalence and diversity of
394 norovirus genogroups I and II in Hong Kong marine waters and detection by real-time PCR.
395 Marine Pollution Bulletin 64, 164-168.
396
397

Table 1: Norovirus detection among frequently sampled sites

Sampling frequency	≤15	≥ 16									
Number of site	31	11									
Sampling site*		A	B	C	D	E	F	G	H	I	J	K
Number of samples	145	16	16	17	17	18	21	21	26	29	29	33
Number of positive samples	8	1	0	1	1	3	3	2	3	3	4	5

* details provided only for frequently sampled sites.

Table 2: Mean extraction efficiencies and norovirus detection.

Sampling site	Number collected	Negative samples		Positive samples		# pos with conc. < 70	# pos with conc. > 70	NoV geometric mean conc/g DT[a]	
		number	Mean extraction efficiency (%)	number	Mean extraction efficiency (%)			uncorrected	corrected
All	387	352	40 ± 20	35	35 ± 15	16	19	187	624
Supermarkets	82	68	35 ± 25	14[bc]	35 ± 20	5	8	220	738
Packers	141	127	35 ± 25	14	30 ± 10	6	8	188	658
Producers	164	157	40 ± 30	7*	35 ± 15	5	2	93	256

[a] calculated for positive samples above the quantification limit of 70 RNAc/g DT,

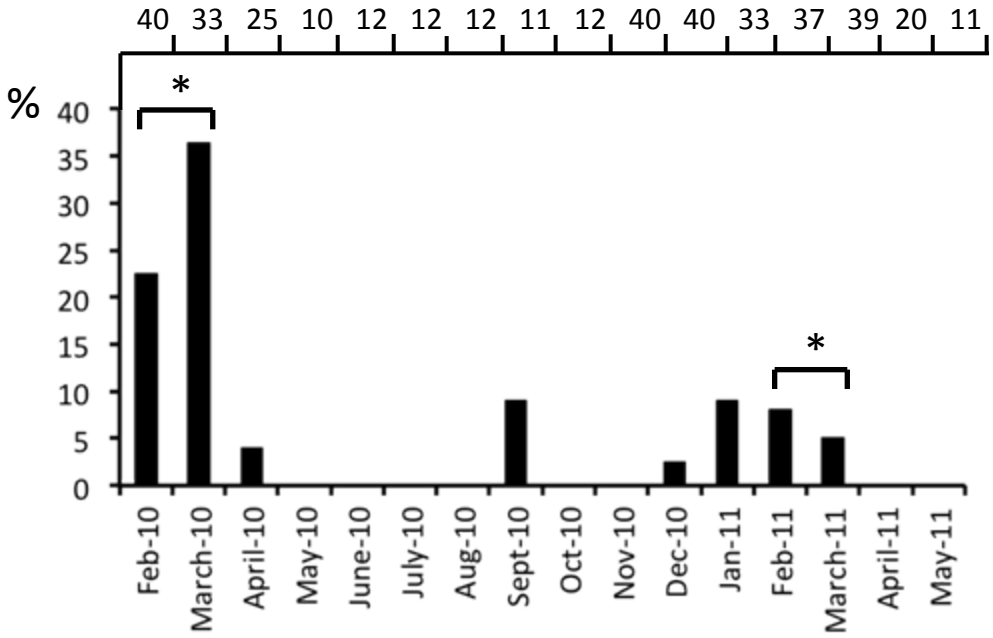
[b] one sample with a low extraction efficiency was not considered for quantification

[c] number of positive samples collected in supermarkets is significantly different (p= 0.0013) from the number of positive samples collected from producers.

Table 3: Norovirus quantification per oyster.

Sampling site	Number of positive samples	Mean flesh weight per oyster (g)	Mean weight of DT per oyster (g)	NoV geometric mean conc per oyster	
				uncorrected	corrected
All	34	11.17	0.68	56	192
Supermarkets	13	10.11	0.69	73	245
Packers	14	8.83	0.66	63	228
Producers	7	11.31	0.70	27	89

Fig. 1


40 33 25 10 12 12 12 11 12 40 40 33 37 39 20 11
%
40
35
30
25
20
15
10
5
0
*
*
Feb-10
March-10
April-10
May-10
June-10
July-10
Aug-10
Sept-10
Oct-10
Nov-10
Dec-10
Jan-11
Feb-11
March-11
April-11
May-11