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COMPARING TWO SEAWATER TEMPERATURES FOR HUMAN NOROVIRUS DEPURATION FROM OYSTERS.

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Highlights :

- No difference in terms of norovirus depuration was observed when oysters were contaminated through occasional or repeated exposure;
- When purified in seawater at 8°C, the time needed to achieve a one-log decrease of norovirus concentration is around 41 days;
- When purified in seawater at 18°C, the time needed to achieve a one-log decrease of norovirus concentration is around 24 days;
- Warm seawater favors norovirus elimination from oyster digestive tissues, but may increase the risk of bacterial pathogen growth.

Key words:

Oysters, Human norovirus, depuration, water-temperature, shellfish, field-contaminated oysters, T90.

Abstract

Despite regulations set up to monitor the microbiological quality of shellfish in producing areas, shellfish-borne gastroenteritis outbreaks still occur. Indeed, oyster depuration practices that are efficient to eliminate bacteria, fail to eliminate human norovirus from oyster flesh. In order to evaluate the impact of seawater temperature on the elimination of norovirus particles from oysters, large batches of oysters were contaminated using raw sewage containing norovirus and subjected to depuration at 8°C or 18°C. Over the experiment, quantitative RT-qPCR showed a one-log decrease of norovirus (both genogroups combined) genome copies per gram of digestive tissue after 41 days for oysters depurated at 8°C and 24 days at 18°C. The decrease of norovirus (both genogroups combined) in two batches of field-contaminated oysters depurated for two weeks at 18°C was in the same range (21 and 23 days respectively). All experiments showed a difference in genomic decay between the two norovirus genogroups, with norovirus genogroup I being more persistent in March/April compared to April/May.

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1. Introduction

Shellfish have been a significant food source around the world for centuries (Rowan, 2023; Venugopal et al., 2018; Yang et al., 2022). In the 19th century, before the identification of key pathogens, shellfish were suspected of being the vehicle for foodborne outbreaks. The depuration process, consisting of placing shellfish in clean sea waterbefore consumption, was proposed in the early 20th century as a countermeasure (Fabre-Doumergue, 1912; Wells et al., 1916). Shellfish, especially oysters, filter large volumes of water from which they concentrate particles to support their physiological activities. When oysters grown in coastal areas exposed to human sewage contamination, pathogenic bacteria or viruses may be present in the water column and get concentrated in oyster tissues. In recent decades, EU regulations and official sanitary controls have reduced illness outbreaks caused by enteric bacteria in shellfish, but foodborne viruses continue to be implicated in clinical cases linked to oyster consumption (Lucero et al., 2021; Rowan et al., 2023). Oysters are most commonly consumed raw, although in some countries, oysters are steamed, cooked or marinated before consumption, reducing the risk of food-borne outbreaks (EFSA, 2015; Venugopal et al., 2018). However, undercooked oysters have been linked to gastroenteritis outbreaks (Lunestad et al., 2016; McLeod et al., 2017; Rowan, 2023; Yang et al., 2022).

In 2021, human noroviruses (NoVs) were the second most common pathogen after *Salmonella* in terms of the number of identified foodborne outbreaks and cases in Europe, with shellfish, molluscs and crustaceans being the fifth most common food implicated in clinical cases (EFSA, 2022). NoVs are the leading cause of epidemics of acute gastroenteritis and are also recognised as an important cause of sporadic cases in both children and adults (Atmar et al., 2018). These small, round, non-enveloped RNA viruses, which belong to the *Caliciviridae* family, are genetically very diverse. The classification system, which is based on the relatedness of the complete VP1 capsid protein, currently separates the strains into ten recognized genogroups (G) (Chhabra et al., 2019). NoV GI and GII are the two main genogroups infecting humans and are excreted at high concentration in the feces of infected individuals (Atmar et al., 2018). NoVs are very robust and are highly resistant to disinfection treatments, they have been detected at various concentrations in raw and treated wastewater

(Eftim et al., 2017; Sano et al., 2016). Moreover, NoVs are characterized by high infectivity. Only a few genome copies are sufficient to induce disease in volunteers (Atmar et al., 2018; Ge et al., 2023). This finding has been confirmed in oyster-borne outbreaks, where highly contaminated samples are more likely to induce illness in consumers, but some studies have reported that low concentrations are implicated in outbreaks (Lowther et al., 2010; Teunis et al., 2020).

In Europe, depuration has to be applied to shellfish produced in areas of low microbiology quality based on the quantification of *Escherichia coli* in shellfish flesh prior to sale (European Regulation 853/2004/EC). Producers are required to immerse shellfish in clean seawater for short periods of time to reduce the bacterial contamination (FAO, 2009). Short depuration is effective in eliminating bacteria but fail to eliminate NoVs that can persist in shellfish tissues for up to several weeks (McLeod et al., 2017; Rowan, 2023). For many years, different factors have been evaluated to improve the process of NoV elimination from oyster tissues including: salinity, seawater temperature, feeding and relaying into clean oceanic water (McLeod et al., 2017; Richards et al., 1988). Among theses different studies, higher seawater temperatures appear to be the most promising factor that favors NoV reduction as measured by RT-qPCR (Rupnik et al., 2021; Younger et al., 2020).

In the present work, we aimed to evaluate the impact of two water temperatures (8° and 18°C), on the depuration kinetics of NoV from contaminated oysters. These two temperatures were selected on the basis of the winter seawater temperature in north-eastern Atlantic (7 to 10°C) and previous studies (Younger et al., 2020; Doré et al. 1998). Oysters were exposed to high levels of NoVs by immersion in sewage-contaminated seawater in the laboratory. Oysters were depurated over a long period (28 days) in large tanks at an experimental farm. The objectives were to monitor the reduction of NoVs by RT-qPCR (genome copies per g of oyster digestive tissue) and to calculate the time needed to eliminate 90 % of the contamination (T90). In each experiment, to mimic occasional or repeated exposure to contamination, oysters were contaminated either by a single exposure or by multiple consecutive exposures to sewage. In a second phase, field-contaminated oysters were depurated using seawater warmed at 18°C.

2. Materials and methods

2.1 Source of contaminated oysters

In the first part of the study, experiments were performed using laboratory contaminated oysters. Four batches (two batches per experiment) of adult diploid oysters (*Magallana gigas* previously *Crassostrea gigas*) were purchased directly from the same producer (Western France) and control tests were performed before bioaccumulation to verify the absence of pre-existing NoV GI or GII contamination. Natural seawater (75 L) collected from a single clean area was used and spiked with sewage contaminated with NoV. The same raw sewage sample was used for all experiments (200 mL frozen aliquots were prepared from the fresh sewage sample). Each aliquot contained 5 x 10^6 genome copies (GC) for NoV GI and 3 x 10^9 GC for NoV GII.

For both experiment 1 and 2, two batches of oysters were contaminated:

- Repeated contamination (RC): 400 oysters were immersed in seawater and one dose of the NoV positive sewage sample was added daily for five days. This batch received five doses of NoV positive sewage.

- Single contamination (SC): another batch of 400 oysters were immersed in seawater and contaminated only once with the dose of NoV positive sewage (Figure 1).

In the second part of the study (experiments A and B), following environmental contamination events, two batches of 100 NoV field-contaminated oysters (FC) were collected.

2.2 Depuration experiments

2.2.1 Laboratory contaminated oysters

Two experiments were conducted: from March 15 to April 12 (experiment 1) and from April 12 to May 10 (experiment 2). After verification of NoV contamination by RT-qPCR of the two oyster batches (RC and SC), 50 oysters from each batch were randomly selected and kept in the fridge (5 ± 3 °C) for two weeks as controls.

The leftovers were transported under cold conditions (8 ± 2°C) to an experimental farm (one-hour drive). Both batches (RC and SC) were split in two and each half immersed in separate trays within tanks containing 450 L of natural clean seawater (sand filtered) maintained either at 8°C or 18°C (Figure 1). The density was about 1 oyster/L. The water was recirculated continuously at a rate of 25 L/min, and UV treated (254 nm) leading to a dosage of UV of about 18 J/cm^2. Parameters such as temperature, salinity (around 33.5 parts per thousand (ppt)), nitrogen ammonia (comprised between 0.075- 0.1 mg/L) and dissolved oxygen (around 100%) were measured daily and adjusted if needed, including on the weekend. Dead oysters, if any, were removed daily. Samples (15 oysters) were collected from the four trays on days 1, 3, 6, 8, 10, 15, 21 and 28, and on days 8 and 15 for control oysters (kept at 5°C). On day 15, the seawater was removed, tanks were carefully cleaned and 200 L of clean seawater was added, as the number of oysters was reduced to half of the initial amount.

2.2.2 Field-contaminated oysters

In January, the FC batch was divided into control (20 oysters kept at 5 ± 3 °C) and test samples (80 oysters) submitted to depuration for two weeks using clean seawater at 18°C and changed daily. The density of 1 oyster/L was used to calculate the volume of seawater. Water circulation was facilitated using a pump (Max-Flo II, Hayward, France) without UV treatment. Other conditions such as salinity, pH and oxygenation level were maintained at the same levels as in experiments 1 and 2. Samples (10 oysters) were collected on days 3, 6, 8, 10, 12, and 15, and on days 8 and 15 for control oysters (kept refrigerated at 5°C). In February 2023, the experiment was repeated using the second FC batch. For both experiments, the initial concentration (day 0) was the concentration measured by RT-qPCR when batches arrived in the laboratory.

2.3 Norovirus analysis

All samples were transported to the laboratory within 24-hours in cold conditions. Oysters were opened, the digestive tissues (DT) were dissected, carefully chopped and homogenized before distribution into 2 g aliquots and kept frozen (≤ -20°C) until analysis**.** Three biological replicates (3 x 2 g) were extracted from each sample in different extraction series. The ISO 15216-1 method was applied with the addition of Mengovirus (10^6 $TCID_{50}$) (kindly provided by A. Bosch, University of Barcelona, Spain) as a process control (ISO 15216-1, 2017). Nucleic acid (NA) extractions were carried out using the NucliSens kit (bioMerieux, Lyon, France). An automatic easyMAG extractor (bioMerieux) was used to increase the analysis throughput. NAs were recovered in 100 µL of elution buffer (bioMerieux).

All amplifications were performed using the UltraSens One-Step quantitative RT-qPCR system (Life Technologies, France). For mengovirus, the primers and probe were as described previously (Costafreda et al., 2006). For NoV, QNIF4, NV1LCR primers and NVGG1p probe were used for GI and QNIF2, COG2R primers and QNIFs probe for GII (da Silva et al., 2007; Kageyama et al., 2003; Loisy et al., 2005; Svraka et al., 2007). Each of the 3 biological replicate extracts was analyzed in triplicate using RT-qPCR on 5 µL of undiluted NA, leading to a total of 9 C_T values per target genogroup per sample. Standard curves based on double stranded DNA (gBlocks, IDT USA) corresponding to nucleotides 4484 to 5668 of the GI.1 Norwalk virus (Genbank M87661) or nucleotides 4217 to 5355 of the GII.4 Houston virus (Genbank EU310927), were included in each run, each concentration was tested in duplicate.

Two negative controls (one negative extraction control and one negative amplification control) were included in each amplification series and filter tips and dedicated rooms were used. After completion of all runs, and verification that all standard curves showed amplification efficiencies between 90 and 110%, average values were calculated for each point of the standard curves to calculate the uncertainty of quantification for NoV GI (0.22 Log) and GII (0.21 Log).

For each biological replicate, the mean C_T value was calculated based on the 3 C_T values obtained. Using the mean C_T value and the standard curves, the concentration of GC of NoV was back-calculated based on the volume of NA, the volume of proteinase K supernatant recovered and the weight of DT, and expressed as GC per g of DT. Each biological replicate was quantified separately. The geometric mean concentration across replicates was then calculated for NoV GI and GII. NoV concentrations were expressed either per genogroup or as total NoV concentration (sum of NoV GI and GII) for the T90 calculation.

For FC oysters, when the detected concentration was under the limit of quantification of the method, a substituted value of half the LOQ was assigned (62 and 195 GC/g of DT for NoV GI and GII respectively) (EFSA, 2019).

2.4 Data analysis

Statistical analysis and data plotting were performed using R Studio v3.6. Due to variations in initial NoV oyster concentrations after bioaccumulation experiments, the concentrations (N) were normalized by dividing the measured concentrations for each sampling time by the initial concentration (N0) after Log transformation. ANOVA was performed to determine whether the factors (temperature and duration of depuration) have an

effect on GI and GII NoV depuration. The T90, time required for a decimal reduction of the initial NoV (GI+GII) concentration, was calculated using the coefficients of the ANOVA model, and considering all data obtained for each temperature without distinction of single or repeated contamination.

3. Results

3.1 Experiment conditions and controls

For all experiments, the seawater parameters such as salinity (32.9 ± 1 ppt at 8°C and 34.6 ± 1.8 ppt at 18°C), pH (8.1 ± 0.1 at 8°C and 7.9 ± 0.1 at 18°C), dissolved oxygen (102.1 ±1.8 at 8°C and 101.6 ± 2.5 at 18°C) and temperature (8.1°C ± 0.4°C and 18°C± 0.6°C) were kept stable including when the water was changed on day 15. For the laboratory-contaminated oyster depuration experiments (RC and SC batches), a UV treatment was applied to the water circulating in the tanks, to avoid any increase in total marine flora (including *Vibrio spp*). For the field-contaminated oyster (FC batches) experiments, as UV was not available, water was changed everyday using clean seawater prewarmed at 18°C. The extraction efficiency coefficients were all correct (> 1%) as recommended in the ISO 15216-1 protocol.

For laboratory contaminated oysters, the initial NoV concentrations (GI + GII) (day 0) in oyster DT were similar for the two experiments (Table 1). As expected, oysters exposed to repeated contamination (five doses of sewage inoculum, representing 1.35 % of the total volume of seawater) presented higher NoV concentrations compared to oysters exposed to a single contamination dose (single contamination with sewage representing 0.27% of the total volume of seawater).

Control oysters refrigerated at 5°C did not show a decrease in NoV concentrations: after 15 days, concentrations were similar to those obtained at T0 (Table 1). This was also verified for the field-contaminated oysters, the increase observed for experiment B could be attributed to the variability of oyster contamination and falls within the uncertainty of the quantification.

3.2 *Experiment 1*

For this experiment, performed in March-April, NoV concentrations (GI+GII) in RC oysters decreased by half a Log when depurated at 8°C and by one-Log at 18°C (Table 1). Thus, after 8 days, 43% and 80% of the initial NoV contamination were eliminated for oysters depurated at 8° and 18°C respectively. For SC oysters, there was little reduction in NoV concentration after depuration at 8°C and a half-Log reduction in concentration was observed at 18°C (Table 1). In other terms, for oysters depurated at 8°C only 18% of the NoV initially present was eliminated after 8 days, and 52% at 18°C. Comparisons were made on the normalised values expressed as Log N/N0 to evaluate differences in depuration between genogroups and to avoid bias due to variations in concentration on day 0 (Figure 2). The decrease in concentration was mainly observed for NoV GII, for which time of depuration ($p<0.001$) and water temperature ($p<0.001$) were significant factors for both single and repeated scenarios (Figure 2). This was not the case for NoV GI concentration, which did not show significant decrease in oysters over the 28 days at either temperature.

3.3 Experiment 2

Experiment 2 was carried out in April-May and the reduction of NoV reached one-Log for oysters depurated at both 18°C and 8°C with the exception for oysters exposed to a single contamination event and depurated at 8°C for which concentrations decreased less than one-Log (Table 1). For both genogroups a decreasing trend was observed. After 8 days, in RC oysters, 72% of the NoVs were eliminated when depurated at 8°C and 82% when depurated at 18°C. For SC oysters, the NoV concentrations were reduced by about 44% in oysters depurated in water at 8°C and by 72% in water at 18°C. However, in comparison with the first experiment, opposite results were obtained with a faster and significant decrease in NoV GI concentration compared to NoV GII for SC ($p < 0.001$) and RC ($p < 0.01$). The NoV GI concentrations decreased more rapidly than the NoV GII concentrations for the two seawater temperatures and the two batches (Figure 2). In both RC and SC oysters, the decrease in NoVconcentration cannot be explained by temperature alone (not significant in ANOVA), but rather by the interaction between the two variables, temperature and genogroups, since an interaction effect between these two variables was found to be significant ($p<0.001$).

3.4 Experiments A and B

During the following winter (January and February), we identified two batches of oysters from production areas that were contaminated by both NoV GI and GII. Analyses showed concentrations of 2.1 to 2.6 x 10^2 GC/g of DT for NoV GI, and 3.7 to 7.8 x 10^2 GC/g of DT for NoV GII in the two FC batches respectively (Table 2). These two FC batches were immersed in seawater pre-warmed at 18°C. For both experiments, NoV GI was detectable over the two weeks of depuration, with a weak signal under the limit of quantification for experiment A. For NoV GII, concentrations decreased to under the limit of detection, with no detectable signal after 2 weeks in both experiments. Considering the initial concentrations of total NoV (GI + GII), after one week of depuration, a reduction of 32% was observed for experiment A and 64% for experiment B. After two weeks, reduction rates were around 89% and 85% for experiment A and B respectively (Table 2).

3.5 T90 calculation

In order to approach real-life conditions, i.e. with different oysters in terms of contamination levels, or even the period of contamination (e.g. late winter/early spring), all data obtained for single and repeated contamination experiments for both genogroups were compiled to evaluate the impact of water temperature and depuration duration (Figure 3). Over the 28 days, the NoV concentrations (considering GI and GII separately) decreased slowly, with a significant impact of depuration time ($p< 0.001$) and water temperature ($p<0.001$). The T90 (number of days needed to achieve a one-Log reduction of NoV concentration) calculated using the ANOVA model coefficients was 41 days when depuration was performed at 8°C, and 24 days when depuration was performed at 18°C. However, if we consider the T90 calculated for each experiment and contamination conditions, values varied from 26 to 77 days at 8°C and from 18 to 54 days at 18°C including field-contaminated oysters (Table 3). The T90 also varied according to experiment, with a shorter T90 for experiment 2 (April-May) than for experiment 1 (March-April).

4. Discussion

In Europe, despite regulations and efforts to prevent coastal contamination by human sewage, oyster contamination by NoVs remains a major public health and economic challenge. A European survey conducted over two years showed a NoV prevalence of 10.8 % in dispatch centers based on genomic detection (EFSA, 2019). The seasonal effect, with a marked winter peak in NoV prevalence was also highlighted in the same study. This peak can be explained by the epidemiology of NoVs with a higher prevalence in the human population during the winter months, leading to higher viral concentrations in human sewage compared to summer, a higher stability of viral particles in cold water, and also by the fact that oysters are more likely to concentrate and retain NoVs during cold months (Maalouf et al., 2011). Indeed, some specific affinities between oyster tissues and NoV through HGBA-like glycan expression may explain the long persistence of NoV in oyster tissues (Le Guyader et al., 2006; Tian et al., 2007). The identification of a large range of N-glycans that share the structure of the HBGA type A structures in *Magallana gigas* but also *Ostrea edulis,* confirmed the binding of NoV to oyster tissues (Auger et al., 2023). These ligands probably explain why NoV persists in oyster DT after contamination events (Batistini et al., 2021, McLeod et al., 2017; Richards, 1988; Rowan, 2023, Ueki et al., 2007).

The reduction in NoV genome concentrations during depuration is small. Changes in concentration may be difficult to assess given the variability of contamination among individual oyster and the limitations of RT-qPCR especially for low concentrations close to the limit of quantification. To overcome the potential uncertainty associated with quantifying low concentrations, we contaminated oysters at higher concentrations than those usually found in field-contaminated oysters (EFSA, 2019, Li et al., 2023). Some important quality criteria such as the extraction efficiency, absence of inhibitors and quantitative controls for the qPCR reactions are included in the ISO 15216-1 method. However, the translation of C_T values into genomic copies can introduce some uncertainties linked to standard curves used in each run (ISO 15216-1, 2017). Beside the ISO criteria (verified for each extraction), quality criteria for the validatation of these curves and biological triplicate extractions provide additional assurances with regards to quantification, and were applied here (Drouaz et al., 2015; Le Mennec et al., 2017).

In the literature, data on virus elimination in oysters varies and comparisons are difficult due to the heterogeneity of the conditions used in the different studies and associated data, e.g. strains used, condition of contamination (producing area or laboratory), oyster species, or seawater (Richards, 1988; McLeod et al., 2017; Rowan, 2023; Yang et al., 2022). The number of days to achieve a one-log reduction using different surrogates can range from less than one day using a feline calicivirus (Ueki et al., 2007), to 20 days for phages (Dore et al., 1998) or about 15 days for Tulane virus (Drouaz et al., 2015; Polo et al., 2018). Using a mathematical model, Qin et al. (Qin et al., 2022) studied the dynamics of contamination and depuration of two groups of viruses, one group that can infect and replicate in oysters such as ostreid herpesvirus and one group that included NoV, that does not replicate and does not have adverse effects on oysters. For the latter group, it was found that in addition to taking into account the physiological status of the oysters, it is important to promote high filtration rates by increasing water temperature to achieve high efficiency of depuration (Qin et al., 2022). Another model suggests that NoV sequestration in tissues (which is not taken into account by standard approaches to NoV detection) may have an impact on the time required for the observation of significant NoV elimination (McMenemy et al. 2023). These authors

suggested a minimum time of 62 h of depuration before analysis. This would allow sufficient time for viral particles to reach the DT, which is the target of the ISO method. In 2010, a study carried out with field contaminated oysters showed that a relaying period in a clean area for two weeks prior to depuration at an elevated temperature (15-17°C) for four days was sufficient to reduce NoVs GII to non-detectable levels (Dore et al., 2010). More recently, a study comparing various factors such as temperature, salinity, light, feeding, and disturbance, concluded that increasing seawater temperature was most effective, but reductions varied by NoV genogroup, with GII being more efficiently depurated than GI (Younger et al., 2020). In Italy, using field-contaminated oysters, a greater reduction of NoV GII was achieved compared to NoV GI after 9 days of depuration in seawater at temperatures ranging from 12 to 19°C, but without significant effect compared to initial concentrations (Batistini, 2021). The different behavior between the two NoV genogroups is confirmed by the data presented here. In our study NoV GI showed stable concentrations during the first experiment (conducted in March-April). However, it was eliminated earlier than NoV GII during the second experiment (performed in April-May) (Figure 2). In the field-contaminated samples (collected in January-February), NoV GI was detected for longer periods than NoV GII, supporting than NoV GII was depurated more rapidly than NoV GI. This confirms the results obtained with the laboratory-contaminated oysters and highlights the possible role of animal physiology and ligand expression (Table 2) (Maalouf et al., 2011; Tian et al., 2007). In all experiments carried out at the end of winter and beginning of spring (January to April), NoV GI persisted for a longer period of time compared to NoV GII, suggesting that oyster biology influences the persistence of different NoV genotypes. In the literature, most of depuration studies have been carried out during the winter months, a period of the year when the probability of finding NoV contaminated oysters is higher. We can hypothesise that in April-May, increased seawater temperature may have an impact on some gene expression and possibly ligand expression (Jones et al., 2019, Maalouf et al. 2010, Auger et al. 2023).

We chose to contaminate oysters with higher concentrations of NoV GII compared to NoV GI to mimic the contamination observed in coastal area. Field-contaminated oysters often show higher concentrations of NoV GII compared to NoV GI, and with a higher strain diversity (Eftim et al., 2017; Ollivier et al., 2022; Sano et al., 2016). In a recent meta-analysis of 75 studies on NoV contamination of oysters, both genogroups were detected worldwide either separately or as a mixture. This suggests that depuration needs to work efficiently for both NoV GI and GII (Li et al., 2023). In addition, to mimic field-contamination, we exposed oysters to repeated contamination events or to a single event, as it may occurr during accidental one-time contamination events (e.g. wastewater treatment plant failure or dysfunction) or through regular discharge of sewage. After single or repeated contaminations, no difference in NoV depuration was observed. This confirms that NoVs can persist for weeks even after an occasional contamination (Le Mennec et al., 2017). To calculate a T90 for the two depuration temperatures, all data were plotted on the same graph. This approach allowed us to confirm the effect of water temperature over time. This was further confirmed by the two experiments carried out on field-contaminated oysters. The T90s obtained were in the same range for all experiments. This was also the case for the field-contaminated oysters, which had much lower concentrations than the laboratory-contaminated oysters. After a depuration period of 6 days at 18°C the NoV concentration decreased 32% for experiment A and 64% for experiment B. These results are comparable to the 60% removal after 5 days of depuration at 18°C data obtained in England (Younger et al., 2020), but longer than the 1 Log reduction obtained after 3 days in Ireland (Rupnik et al., 2021). These two studies confirmed than NoV GI concentrations declined slower than NoV GII.

The need to assess the impact of depuration and the ratio of infectious to non-infectious particles was highlighted in a recent microbial risk assessment study linking NoV concentrations in oysters from the production area to consumer consumption (Hunt et al., 2023). Since the development of human intestinal enteroids for the demonstration of NoV infectivity, some improvements have been made in the understanding of the resistance of these viruses to various treatments including persistence in seawater (Costantini et al., 2018; Desdouits et al., 2022; Ettayebi et al., 2016; Rexin et al., 2024). In a bench-scale experiment, we found that the characteristics of natural seawater can influence NoV infectivity (Desdouits et al., 2022). Therefore, we can assume that oyster tissues also influence NoV behavior. The recent demonstration that infectious NoV can be detected in raw sewage has confirmed the public health impact of sewage discharged into coastal environments (Carmona-Vicente et al., 2024). To conclude, our results confirm previous reports showing that an increase in seawater temperature favours NoV depuration with a significant impact on depuration time but did not completely eliminate viral contaminants, which have a low infectious dose. It is also pertinent to be reminded of the risk of pathogenic bacterial pathogen growth, which is favoured under warmer seawater temperatures. This underscores the importance of preventing the contamination of coastal areas with human sewage, which is undoubtedly the most effective way to protect consumers and producers and ensure the safety of products on the market.

Author contributions

FLG, JO, PG, SP designed the study, MG, JO, SP performed the shellfish analysis, FVH performed the statistical analysis, MP, CS, VF were in charge of the experiments in the experimental farm, all authors contributed to the writting.

Declaration of competing interest

All the authors declare they have no conflicts of interest.

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Table 1: Norovirus (GI+GII) concentrations quantified in oysters exposed to repeated or single contamination during depuration assays at two water temperatures.

		Repeated contaminations		Single contamination	
Temp.	Days	Exp 1.	Exp 2.	Exp 1.	Exp 2.

	0	1.6×10^5	2.2×10^5	2.4×10^4	1.5×10^4
5°	8	1.8×10^5	3.1×10^5	3.2×10^4	1.9×10^4
	15	1.3×10^5	4.9×10^5	4.1×10^4	2.7×10^4
	1	1.5×10^5	2.0×10^5	1.6×10^4	3.2×10^4
	8	8.6×10^4	5.6×10^4	1.3×10^4	1.8×10^4
8°	15	8.3×10^4	7.5×10^4	1.9×10^4	8.5×10^3
	21	4.6×10^4	3.9×10^4	5.6×10^3	7.6×10^3
	28	5.0×10^4	3.7×10^4	1.5×10^4	6.3×10^3
	1	2.4×10^5	1.8×10^5	2.5×10^4	1.7×10^4
	8	4.6×10^4	3.3×10^4	1.2×10^4	4.8×10^3
18°	15	1.5×10^4	3.6×10^4	8.1×10^3	1.2×10^4
	21	2.0×10^4	2.5×10^4	9.2×10^3	1.8×10^3
	28	3.0×10^4	1.2×10^4	6.8×10^3	3.6×10^3

NoV concentrations are expressed in genome copies /g of digestive tissues and calculated as the sum of the geometric mean concentrations for GI and GII, obtained from the three biological replicates. The temperature of 5°C correspond to the control oysters (not submitted to depuration assay).

Table 2: Norovirus concentrations in field-contaminated oysters depurated at 18°C.

Days	Experiment A			Experiment B		
	GI	GII	GI+GII	GI	GII	GI+GII
0	209	378	587	260	738	998
3	62	249	311	216	319	535
6	62	343	405	85	271	356
9	62	503	565	62	525	587
12	62	249	311	126	195	321
15	62	nd	62	153	nd	153
Control	151	640	791	415	1568	1983

NoV concentrations (genome copies/g of digestive tissues) were expressed as the geometric mean concentration from three biological replicates for each genogroup (GI or GII) and as the sum (GI+GII). When the contamination was under the limit of quantification a substituted value half of the LOQ was assigned which correspond to 62 and 195 genome copies/g of DT for NoV GI and GII respectively). Nd: not detected, control: oysters kept for 15 days at 5°C (not submitted to depuration assay).

Table 3: T90 obtained for all experiments

Experiment	Contamination	8°C	18°C
1	Repeated	61	28
	Single	77	54

2	Repeated	30	18
	Single	26	22
A	Field	-	21
B	Field	-	23

-: not assayed

The time expressed in days needed to eliminate 1 Log of NoV (GI+GII) concentrations in term of genome copies/g of digestive tissues was calculated for the different experiments.

Figure legends:

Figure 1. Workflow of the experiments with laboratory or field contaminated oysters and follow up depuration.

For RC and SC experiments, the water recirculates in the two tanks during 4 weeks (water changed once) at a rate of 25 L/min using a pump (cross in a blue circle) and was UV treated before re-introduction into the tank. For the FC experiments, depuration was performed only at 18°C with water changed everyday.

Figure 2. Diminution of norovirus concentrations in oyster digestive tissues at temperature of 8°C and 18°C for the two experiments.

The reduction of norovirus GI (orange dots and line) and GII (blue dots and line) concentrations after repeated (A) or single (B) contaminations submitted to depuration in seawater at temperature of 8°C or 18°C , was calculated as the ratio of the Log of the concentration measured for each biological replicates (N) obtained for each sampling day divided by the Log of the initial concentration (N0) (Log(N/N0) the two experiments (1 and 2).

Figure 3. Impact of seawater temperature and depuration time on norovirus removal from oyster DT.

The concentration measured for each biological replicates (N) obtained for each sampling day divided by the Log of the initial concentration (N0) (Log(N/N0) in oyster digestive tissues exposed to repeated or single contaminations and depurated in seawater at 8°C (black) or

18°C (red) over 28 days, for the two experiments were compiled. Conditional smoothed regression lines are represented by the grey area.

Figure 1

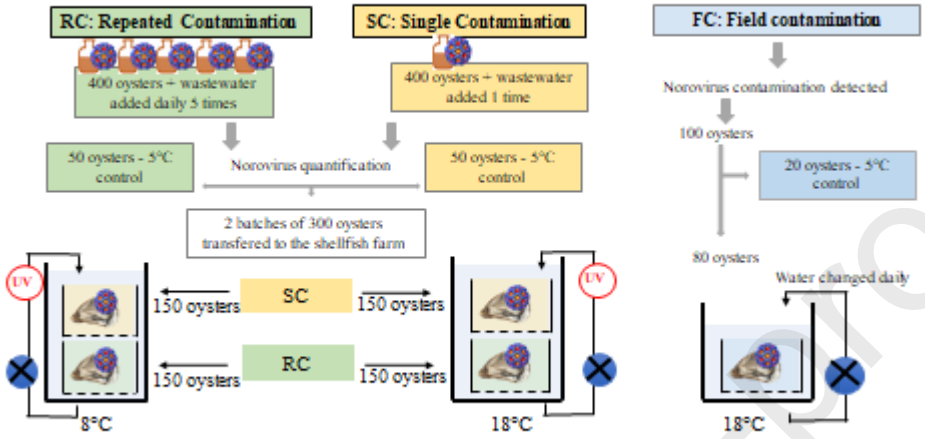


Figure 2

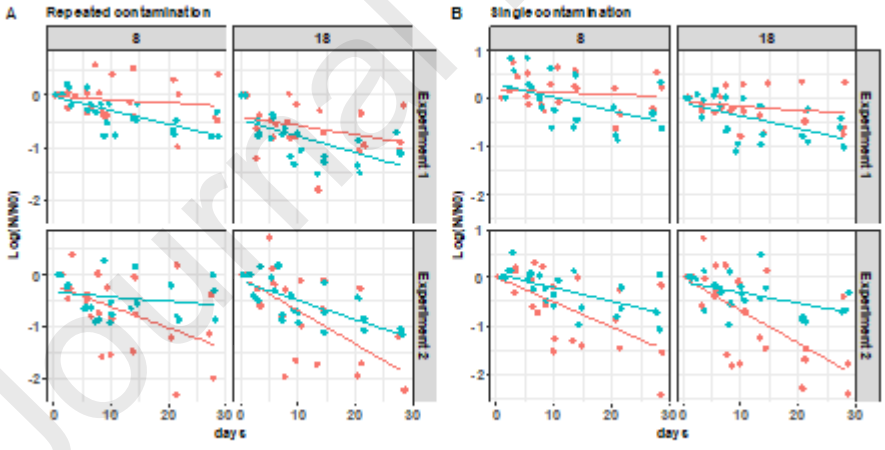
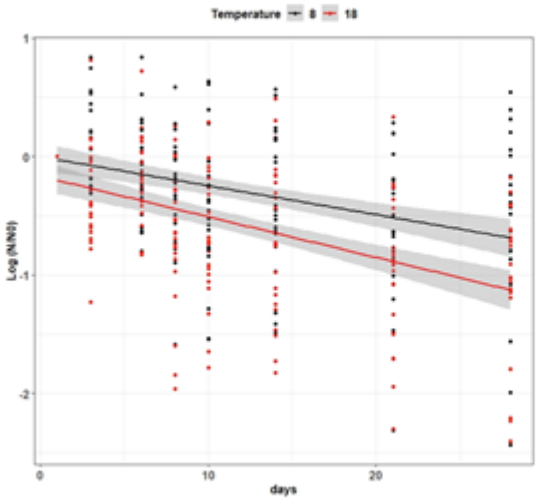


Figure 3



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: