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Impact of Xynthia Tempest on Viral Contamination of Shellfish

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Abstract:

Viral contamination in oyster and mussel samples was evaluated after a massive storm with hurricane wind named “Xynthia tempest” destroyed a number of sewage treatment plants in an area harboring many shellfish farms. Although up to 90% of samples were found to be contaminated 2 days after the disaster, detected viral concentrations were low. A 1-month follow-up showed a rapid decrease in the number of positive samples, even for norovirus.

Global climate change, interfering with many complex events, may impact the hydrological cycle, altering mean meteorological measures and increasing the frequency of extreme events (i.e. excessive precipitation, storms, floods or droughts...). Disasters destroy all sub-structures such as ground transportations, roads, sewage networks and sewage treatment plants, leading to microbial contamination in coastal areas. Following hurricanes Katrina and Rita, several investigators evaluated exposure to chemical or microbial contamination originating from human and animal waste, or the broader effects on algal blooms (7, 10, 25). *Vibrio* and *Legionella* concentrations were more abundant shortly after the event, and fecal indicator concentrations in offshore waters returned to pre-hurricane levels within 2 months (27). As shellfish are prone to microbial contamination by filtering sewage contaminated waters, it is important to evaluate microbial quality of shellfish beds after such event, to avoid the introduction of contaminated shellfish on the market.

A massive storm with hurricane force wind, named “Xynthia tempest”, came through France during the night of February 27-28 2010. At 2.30 am, strong wind (140 km/h), important atmospheric pressure variation (up to 2.5 hPa), and a high tide range, caused major destructions in south-western coast of France, with a massive flood reaching more than 4 m of water depth and claimed 51 lives. The impacted area was restricted (about 50 km of coast and two small islands) but the flood damaged most of the sewage pipe network and sewage treatment plants (Figure 1). As many shellfish farms are located in this area, a sanitary alert was raised and shellfish samples were collected. This study reports the follow up of viral contamination in shellfish samples collected in this area over one month.

Oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis*) samples were collected from March 2 to March 29, 2010. Each sample consisted of at least 12 oysters or 24 mussels. *E.coli* analysis was performed on the same samples according to European regulation (2073/2005/EC).

For viral analysis, shellfish were shucked, and stomach and digestive tissues (DT) were removed by dissection and divided into 1.5-g portions. Mengovirus ($2x10^4$ $TCID_{50}$) was added as an external viral control to each sample. Tissues were homogenized, extracted with chloroform-butanol, and treated

with Catfloc-T (Calgon, Ellwood city, PA). Viruses were then concentrated by polyethylene glycol 6000 (Sigma, St Quentin, France) precipitation (3).

Viral nucleic acids (NAs) were extracted with a NucliSens kit (bioMérieux, France), following the manufacturer's instructions, but with extended incubation for 30 min. at 56°C for initial viral lysis. NAs were analyzed immediately of kept frozen at -80°C (15).

NA extracts were screened by real-time RT-PCR (*r*RT-PCR) with previously published primers and probes for Mengovirus (21), norovirus (NoV) (26), sapovirus (SaV) (19), hepatitis A virus (HAV) (5), hepatitis E virus (HEV) (11), Aichivirus (AiV) (14), Enterovirus (EV) (18) and Rotavirus (RV) (20). Positive controls constituted by plasmids (NoV, SaV, HAV), French positive stool (HEV), or cultured viruses (AiV, EV, RV) were included in each run. *r*RT-PCR was performed using RNA Ultrasense One-step (qRT-PCR) System (Invitrogen, France), adjusted concentrations of primers and probes and thermal conditions described previously (15). To avoid possible false negative results due to PCR inhibitors, all samples were analyzed in duplicate by using 5 µl of undiluted or 10-fold-diluted RNA extracts. Negative amplification controls (water) were included in each amplification series and precautions (filter tips and separate rooms) were taken to prevent false-positive results. The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred. To be considered as positive, sample had to yield a C_T value $\leq$ 41. The efficiency of virus extraction procedures was determined for each sample based on Mengovirus recovery (15). For samples presenting an extraction efficiency above 10%, quantification was performed for NoV and SaV considering the NA volume analyzed and weight of DT extracted (1.5g). If the extraction efficiency was less than 10%, extraction was repeated. If the extraction efficiency % was not improved, sample was considered as positive but excluded for quantification.

All concentrations obtained were log transformed, and geometric mean concentrations were calculated. Mean concentrations were compared by using the student *t* test, and a p value of <0.05 was considered significant (Statgraphic centurion XV).

The tempest impacted two production areas located in two bays separated by an island (area 1 and 2, Fig 1B). Twenty-two samples were collected from area 1, and 24 samples from area 2,

representing 28 oyster and 18 mussel samples. On March 2-3, all 8 samples collected from area 1 displayed less than 230 *E.coli*/100g of shellfish meat (class A area according to European regulation 854/2004/EC). Among the 9 samples collected from area 2, the mean concentration was 446 *E.coli*/100 g of shellfish meat, with three samples having less than 230 *E.coli*/100 g of shellfish meat. All samples collected later met European regulation class A requirement, except one sample collected on March 29 from area 2 (240 *E.coli*/100g). The extraction efficiency was considered as acceptable (>10%) for 40 samples and varied from 26% to 40% over the sampling period. Despite repeated extractions, 6 samples showed an extraction efficiency below 10%: one sample collected on March 2 (area 2), one on March 14 (area 1), three on March 18 (all 3 from area 1), and one on March 29 (area 2). Six samples were positive for RV, 7 for EV, 15 for NoV, and 26 for SaV (Table 1). None of the sample was positive for HEV, HAV or AiV. Multiple contaminations were observed more frequently at the beginning of the month, and one sample, collected on March 2 from area 2, was found contaminated by at least 4 different enteric viruses. However, most of samples were contaminated by one type of enteric virus only and the number of samples with concentration lower than the sensitivity threshold of the method (about 50 RNA copies/g of DT) increased over time (Figure 2). The 15 samples positive for NoV were found contaminated by GII strains and none by NoV GI or GIV. More samples were found contaminated on March 2-3 (59%) compared to March 29 (25%), however average concentrations stayed in the same range (Table 1). SaVs were detected in 26 samples. On March 2-3, SaVs were detected in 76% of samples, and in 25% on March 29, with comparable average concentrations (Table 1).

No statistical difference was observed between area 1 and 2, comparing the number of NoV ($p= 0.603$), SaV ($p= 0.393$), EV ($p= 0.157$), or RV ($p=0.429$) positive samples or NoV and SaV concentrations s ($p= 0.958$ and $p= 0.217$ respectively) (Table 2). A large diversity of human enteric viruses may be detected in human sewage, some being frequently detected (for example NoV, RV), and some sporadically, based on local epidemiology (HAV, HEV, AiV) (8, 9, 12, 13, 24). Raw sewage may contain high viral concentrations especially during cold months, period of the winter time gastroenteritis epidemic in many countries (2, 26). Thus, direct discharge of raw water may have an important impact on shellfish contamination (16).

Clearly this tempest had an impact on shellfish quality as two days after the event up to 90% of samples were found contaminated. No sample collected prior to the event was available as this area has never been implicated in a shellfish related outbreak in France, suggesting that such a high number of positive results is unlikely to represent the normal situation. Nevertheless the diversity of viruses detected was low. Controls included in the method made us confident that these samples were truly negative. This observation may be explained by the low prevalence of some viruses in the French population or, in the case of HEV, the absence of pig farms in this area (1, 6, 22, 23). In contrast, NoV and SaV that are frequently detected in French sewage, were detected in the two impacted areas (4, 26).

If the rapid decrease of *E.coli* was expected, it was more surprising to observe that after ten days, only 20 % of samples were found contaminated by two different enteric viruses since viruses are known to persist in oyster tissues for several weeks, particularly NoVs. This may be explained by the low concentration detected per gram of digestive tissues (then reaching rapidly the sensitivity limit of detection of the method) and the fact that only GII strains were detected. During winter epidemic outbreaks, GII.4 strains are the more prevalent strains in human cases suggesting that sewage waters may mostly contain those strains. We previously demonstrated that GII.4 is less efficiently concentrated by oysters (17) and that the decrease of NoV GII concentration in oyster may be faster compared to that of NoV GI (14).

Both bacterial and viral concentrations detected in shellfish tissues were low. Few years ago, an important rainfall event in southern France led to a massive shellfish contamination with high *E.coli* concentrations and a large diversity of human enteric viruses being detected at high concentrations (using the same detection method) (14). However, this contamination occurred in a lagoon, and over a longer period of time. Here, the impacted area was open to the ocean, submitted to marine currents and tide. In addition we may hypothesize that the phenomena abruptness (wind, atmospheric pressure and large volumes of fresh water) stressed the shellfish, hampering their filtration activity for a few hours. These observations highlight the role of environmental parameters that may contribute to the probability of shellfish contamination. Indeed, in case of natural disasters, it is important to react rapidly to protect the consumers but also for shellfish producer's business.

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Figure legend

Figure 1: Map of the impacted area by the Xynthia tempest.

A: satellite observation of the tempest crossing the area on February 28 (http://france.meteofrance.com); B: detailed map of the area destroyed by the tempest (yellow diamond: sewage treatment plants, red dots: shellfish sampling points).

Figure 2: Multiple contaminations observed for shellfish samples over time.

Black bars indicate two or more different enteric viruses detected per sample, gray bars indicate one virus detected per sample; white bars indicate no virus detected. The x axis shows the sampling time and the y axis shows the percentage of positive samples

Fig. 1

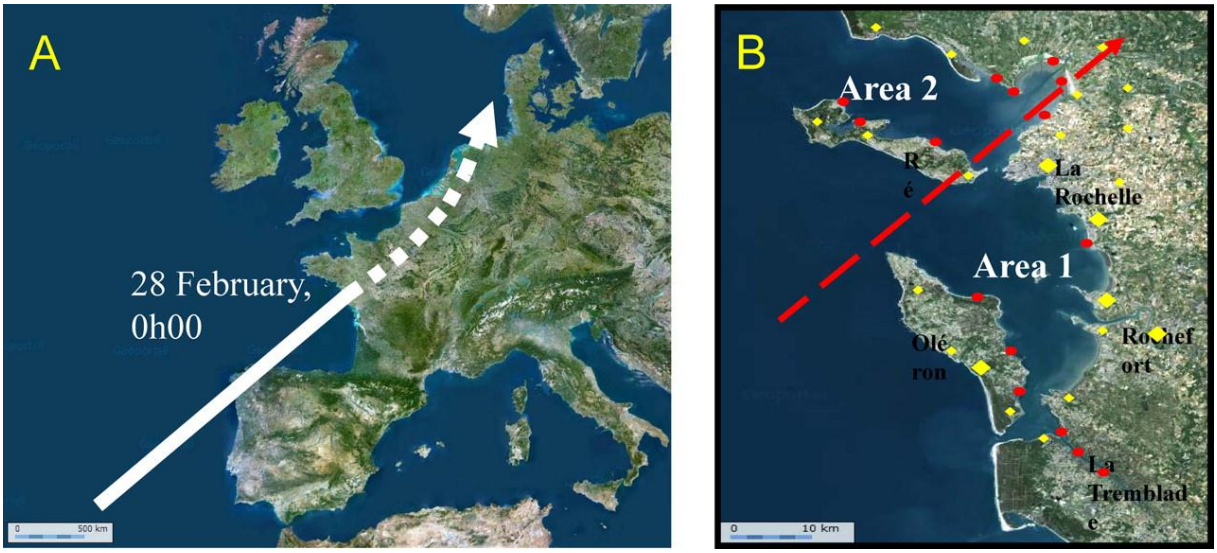
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Fig.2

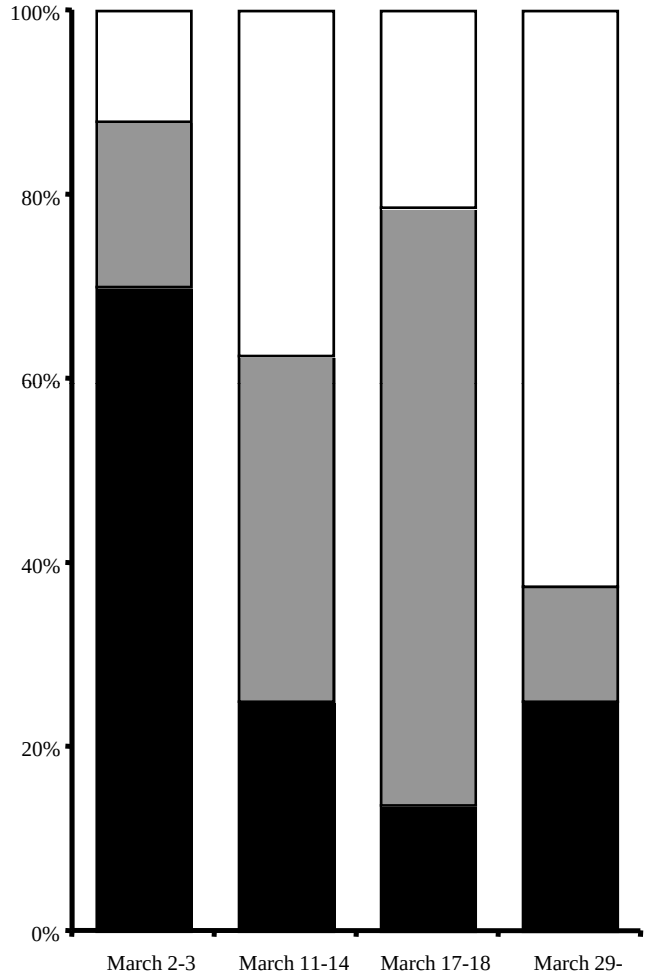

TABLE 1 Detection of human enteric viruses in shellfish samples

Sampling dates in March	No. of samples	Avg extraction efficiency (%)	No. of positive samples and mean concn (RNA copies/g of DT) for each virus				No. of positive samples for each virus				
			NoV		SaV						
			No. of positive samples	Mean concn	No. of positive samples	Mean concn	EV	RV	AiV	HAV	HEV
2 to 3	17	34.91	10	134.86	13[a]	1,976.88	6	3	0	0	0
11 to 14	8	38.25	2	160.09	5[a]	2,978.24	0	1	0	0	0
17 to 18	13	26.35	1[a]		6[b]	1,334.41	1	1	0	0	0
29	8	40.46	2	97.40	2	1,342.28	0	1	0	0	0

[a] One positive sample not considered for quantification due to an extraction efficiency of <10%.
[b] Two positive samples not considered for quantification due to extraction efficiencies of <10%.

TABLE 2 Distribution of viral contamination in the impacted areas

Area	No. of samples	Avg extraction efficiency[a] (%)	No. of positive samples and mean concn (RNA copies/g of DT) for each virus				No. of positive samples for each virus	
			NoV		SaV			
			No. of positive samples	Mean concn	No. of positive samples	Mean concn	EV	RV
1	22	31.98	8[b]	130.75	11[c]	2,707.30	5	2
2	24	32.73	7	161.35	15[b]	1,570.41	2	4

[a] There were four samples from area 1 with extraction efficiencies of <10%. There were two samples from area 2 with extraction efficiencies of <10%.
[b] One positive sample not considered for quantification due to an extraction efficiency of <10%.
[c] Three positive samples not considered for quantification due to extraction efficiencies of <10%.