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Strain-Dependent Norovirus Bioaccumulation in Oysters

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Abstract :

Noroviruses (NoVs) are the main agents of gastroenteritis in humans and the primary pathogens of shellfish-related outbreaks. Some NoV strains bind to shellfish tissues by using carbohydrate structures similar to their human ligands, leading to the hypothesis that such ligands may influence bioaccumulation. This study compares the bioaccumulation efficiencies and tissue distributions in oysters (*Crassostrea gigas*) of three strains from the two principal human norovirus genogroups. Clear differences between strains were observed. The GI.1 strain was the most efficiently concentrated strain. Bioaccumulation specifically occurred in digestive tissues in a dose-dependent manner, and its efficiency paralleled ligand expression, which was highest during the cold months. In comparison, the GII.4 strain was very poorly bioaccumulated and was recovered in almost all tissues without seasonal influence. The GII.3 strain presented an intermediate behavior, without seasonal effect and with less bioaccumulation efficiency than that of the GI.1 strain during the cold months. In addition, the GII.3 strain was transiently concentrated in gills and mantle before being almost specifically accumulated in digestive tissues. Carbohydrate ligand specificities of the strains at least partly explain the strain-dependent bioaccumulation characteristics. In particular, binding to the digestive-tube-specific ligand should contribute to bioaccumulation, whereas we hypothesize that binding to the sialic acid-containing ligand present in all tissues would contribute to retain virus particles in the gills or mantle and lead to rapid destruction.

Keywords : shellfish, norovirus, carbohydrate ligands, bioaccumulation, oysters.

Noroviruses (NoV), are recognized as the leading cause of epidemics of gastroenteritis and an important cause of sporadic cases in both children and adults (13). Members of the *Caliciviridae* family, these small round non-enveloped RNA viruses are highly genetically and antigenically diverse. The genetic classification system, based on relatedness of the complete VP1 capsid protein, currently separates the strains into five recognized genogroups (G), with GI, GII and GIV infecting humans while GIII and GV strains infect cows and mice, respectively (41, 57). In addition, a number of new genogroups have been proposed recently (10, 34, 37). Histoblood group antigen (HBGA) expression is a factor in the genetic resistance of humans to norovirus infection and has been proposed to affect the transmission and epidemiology of noroviruses in human populations (17, 33). HBGAs are complex glycans present on many cell types including red blood cells and vascular endothelial cells as well as some epithelial cells (intestinal, uro-genital and respiratory). Volunteer studies and outbreak analyses indicate that binding to these carbohydrates is required for infection, with many strains infecting only a subset of the population based on their HBGAs expression (15, 20, 25, 27, 28, 48, 49).

NoV ligands have also been identified in oyster tissues and, as observed in humans, variations between NoV strains have been described (30). Oysters are usually grown in coastal waters and thus exposed to waters potentially contaminated by human activities. A better understanding of the role played by oysters in terms of manner of contamination, pathogen persistence and pathogen selection may help improve the sanitary quality of shellfish by promoting new ways of prevention or depuration to increase the consumer's safety. We recently demonstrated that the lack of GIII NoV ligand in oysters induced a lower accumulation compared to human NoV, both in terms of frequency and concentrations, as determined by quantitative real time RT-PCR (*r*RT-PCR) during a field study (56). Likewise, mutant GI VLPs unable to recognize the shared human and oyster ligand had a greatly decreased ability to accumulate in oyster (30). Such observations clearly suggested that specific glycan ligands impact bioaccumulation efficiency and supported our earlier hypothesis based on *in vitro* VLPs binding and bioaccumulation (23).

In order to get a more complete picture of the strain-specific potential for contamination of oysters, the aim of the present study was to obtain quantitative data on viral bioaccumulation in oyster

tissues by one GI and two GII strains using *r*RT-PCR quantification of virus recovery from *in vitro* bioaccumulation experiments performed at several time points during the year. In parallel, measurements of ligand expression were made during the same periods.

MATERIALS AND METHODS.

Virus strains and virus like particles (VLPs). Fecal samples containing GI.1 (Norwalk virus 8FIIa strain, GenBank accession number M87661.1), GII.3 (94% identity in the polymerase and beginning of the capsid coding regions to the GII.3 used for the VLPs, Toronto CAN, GenBank accession number AF247431) and GII.4 (Houston strain, GenBank accession number EU310927) NoVs were used for bio-accumulation experiments. Viral RNAs were extracted from 10% suspensions of stool using the Nuclisens kit (BioMerieux) as recommended by the manufacturer and was eluted in 100 µl of RNAse-free water. Mengovirus strain vMC_0 (kindly provided by A. Bosch, U. Barcelona), was propagated in HeLa cells, and the virus titer was determined as described previously (35).

GI.1 and GII.4 VLPs, produced using plasmids containing open reading frames 2 and 3 of the Norwalk virus strain 8FIIa and the Houston strain, were used for the ELISA as well as their corresponding antibodies produced in rabbits (2, 21). A construct containing the ORF2 of a GII.3 strain (AY247431) and the corresponding antibody was kindly provided by L. Svensson (44).

Reagents. Neoglycoconjugates examined in this study were: H type 1-PAA (Fucα2Galβ3GlcNAcβ-R), H type 3-PAA (Fucα2Galβ3GalNAcα-R), Le^y-PAA (Fucα2Galβ4[Fucα3]GlcNAcβ-R) kindly provided by N. Bovin (Moscow, Russia), Sialyl-Le^x-HSA (NeuAcα2,3Galβ4[Fucα3]GlcNAcβ3Galβ4Glcβ-R), and Sialyl-LNnT-HSA (NeuAcα2,3Galβ4GlcNAcβ3Galβ4Glcβ-R) purchased from Isosep AB (Uppsala, Sweden).

Virus stability in seawater. Fecal samples containing GI.1, GII.3 and GII.4 strains were diluted in seawater in a volume of 3 ml and kept at 12°C. Sampling was performed after 1 and 24 hours. A volume of 1 mL was removed and nucleic acids were extracted directly.

Oyster samples. For all experiments, live oysters (*Crassostrea gigas*) were purchased directly from the same producer (same batch kept over the 6 months study period on special request), and environmental data such as water temperature and salinity were monitored on a daily basis using a Marel smatch TPS (NKE, Hennebont, France). Measured temperature were 15.3 ±0.92, 12.2 ±1.2, 7.7 ±0.6, 8.8 ±1°C in October, November, January and March, respectively. Oysters were immersed the

day after in large tanks of seawater at the laboratory. For all bioaccumulations control tests performed on oysters assayed before bioaccumulation showed no pre-existing GI or GII NoV contamination. After 24 hours of immersion at the designated temperature, oysters were individually checked and only living oysters showing filtration activity were kept.

Bioaccumulations experiments. Natural seawater freshly collected from a single clean area was used for bioaccumulation experiments. The bioaccumulation experiments for all strains and virus concentrations were conducted on the same day for each season. Ten aquariums were filled with three liters of seawater each, nine were artificially contaminated with fecal samples containing GI.1, GII.3 or GII.4 NoVs at three different concentrations, and the last aquarium was kept as a control. Twelve oysters were added to each aquarium and incubated for 24 hours at 12±1°C for the two first bioaccumulation experiments and 8±1°C for the two last ones under oxygenation. Six oysters were harvested after 1 hour and the remaining six after 24 hours. Only six oysters were introduced into the control aquarium and harvesting occurred at 24 hours.

Dissection. Harvested oysters were shucked just after collection and the total weights of the six oyster bodies were determined. Dissections were performed immediately after harvesting and were performed at the same time for each strain by different laboratory members to avoid any delays or differences with subsequent assays. Digestive tissues (DT), gills and mantle were collected from the six oysters, cut in small pieces, mixed carefully to lower individual variation and frozen immediately under 1.5 g separate portions. All recovered tissue weights were recorded.

Shellfish processing. Samples were extracted based on tissue series (i.e. all gills together) on the same day for all experiments. Mengovirus (10^6 $TCID_{50}$) was added to each dissected tissue (1.5 g) before homogenization. The laboratory procedure included tissue homogenization, extraction by vortexing with an equal volume of chloroform:butanol for 30 sec, Cat-Floc T (173 µl per tube) (Calgon, Ellwood City, USA) treatment for 5 min on the bench before centrifugation for 15 min at 13,500 x*g*. The resulting suspension was precipitated with polyethylene glycol 6000 (PEG 6000) (Sigma, St Quentin, France) for one hour at 4°C and centrifuged for 20 min at 11,000 x*g* at 4°C (1).

Nucleic acid extraction and purification. The Nuclisens extraction kit (BioMerieux, Lyon, France) was used following manufacturer's instructions with minor modifications (26). The PEG pellet

was suspended in one ml of RNAse-free H_2O, mixed with the lysis buffer (2 mL), and incubated for 30 min. at 56°C. After a brief centrifugation to eliminate particles (if needed), 50 µL of paramagnetic silica were added and incubated for 10 min at room temperature. All washes were performed using the magnetic ramp and nucleic acids were recovered in 100 µL of elution buffer (BioMerieux, Lyon, France). All extractions were conducted in a short time to avoid freezing before *r*RT-PCR. The remaining nucleic acids were kept frozen (-80°C).

Primers, probes and *r*RT-PCR. For NoV, *r*RT-PCR were conducted using previously described primers and probes targeting the beginning of ORF2, using different sets for GI and GII in separate reactions (26). For mengovirus, primers and probe were previously described (42). The *r*RT-PCR was carried out using the Ultrasens QRT-PCR kit (Invitrogen, France) using adjusted concentrations of primers and TaqMan probes (26).

Five µl of nucleic acid extracts or controls were added per well, for a final total well volume of 25 µL. All samples were analyzed in duplicate undiluted and after 10-fold dilution.

***r*RT-PCR controls and quantification.** 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 background). To be included in the quantitative analysis, all wells had to yield a C_T value ≤ 41 which was considered as the quantification threshold (Q_T).

Extraction efficiency: To estimate loss of viral nucleic acids during extraction, a defined amount of mengovirus was spiked into each sample and the recovery was determined to allow calculation of an extraction efficiency. After extraction of samples seeded with the mengovirus, undiluted and ten-fold diluted extracts were subjected to *r*RT-PCR for mengovirus. The C_T value of the 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 efficiency, using the equation $100e^{-0.6978\Delta CT}$ and expressed in % for each tissue (26).

Quantification: The absence of inhibitors was verified for each sample by comparing undiluted and 10-fold diluted extracts C_T values. Mean C_T value was calculated for each sample and as variations less than one C_T were observed, standard variation was not considered. No adjustment was made for

*r*RT-PCR efficiency as no significant inhibition was observed. The number of RNA copies present in each positive sample was estimated by comparing the C_T value to GI or GII standard curves (*in vitro* transcription plasmids containing nucleotides 146 to 6935 of the Norwalk virus (Genbank M87661), or nucleotide 4191 to 5863 of the Houston virus (Genbank EU310927)). The final concentration was then adjusted based on the volume of nucleic acids analyzed and extraction efficiency and was reported per g of tissue (26). Thus, virus concentration (copies per gram) was determined as follows: Concentration = # copies/5 µl analyzed x 100 µl extract/1.5g tissue x 1/extraction efficiency.

ELISA-based carbohydrate microtiter plate assays. Oysters from the same batch (before bioaccumulation experiments) were prepared as previously described (30). Briefly, digestive tissues, gills and mantle were dissected, homogenized in phosphate-buffered-saline (PBS, pH 7.4) (one third dilution), heated for 10 min at 95°C, centrifuged and the supernatant recovered. After measurement of the protein concentration using a BC assay kit (San Diego, CA), tissue extracts at 40 µg/ml were coated in duplicate onto NUNC Maxisorp immunoplates (ThermoFischer Scientific, Roskilde, Denmark) in carbonate buffer pH 9.6 and were blocked with 10% nonfat dried cow's milk in PBS for 1 h. VLPs (1 µg/mL) were added and incubated for 1 h at 37°C. The respective rabbit anti-VLP antibodies were added, incubated for 1 hour at 37°C anti-rabbit IgG conjugated to horse radish peroxidase (Uptima) was added. Between each step, plates were washed three times with PBS-5% tween 20 (Sigma-Aldrich, France). The enzyme signals were detected with TMB (3,34,5,54-tetramethylbenzidine; BD Bioscience, San Jose, CA), and read at 450 nm with a spectrophotometer (Safire; Tecan). For each tissue negative controls (without VLPs or antibodies) and positive controls (human secretor type A, B and O saliva samples) were included. After validation of the positive and negative controls, OD values obtained for each sample were read and a test ratio sample was determined (OD values of the test sample divided by the OD values of the negative control). A sample was considered as positive if the ratio was ≥2.

Binding of VLPs to immobilized neoglycoconjugates. Oligosaccharides conjugated to either polyacrylamide (PAA) or human serum albumin (HSA) were coated onto NUNC Maxisorp immunoplates as previously described (32). After blocking with 5% defatted dry cow's milk, VLPs at

4 µg/mL (2 x 10^{10} particles per well) were added for 2 hours at 37°C. VLP binding was detected by incubation with the respective anti-GI.1 or anti-GII.4 rabbit antisera diluted at 1/1000 followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins (Uptima, Montluçon, France). The peroxidase substrate TMB (BD Bioscience, San Jose, CA) was used and OD values determined at 450 nm. OD values twice above background were considered positive.

Data calculation and statistical analyses. All concentrations obtained were log transformed and geometric mean titers (GMT) calculated as well as standard deviation (SD). Data were analyzed using StatGraphic software (Sigma Plus, Levallois-Perret, France). Anova tests were used to compare NoV concentrations between samples and significance was declared at a P value of ≤ 0.05.

RESULTS

Virus stability in seawater. Stability of the three viral strains was verified on three separate experiments conducted at the seawater temperature used for bioaccumulation. For each strain, the mean C_T values calculated based on all C_T values obtained (at last 12 values for each) and standard deviations, showed no difference during the time period considered (Table 1). Stability of the VLPs reactivity with rabbit hyperimmune sera corresponding to the GI.1 and GII.4 strains was verified over one day in seawater. For GI.1 VLPs, the OD values after 1 hour and 24 hours were 111% and 85%, respectively, of the OD value at baseline (for an OD value of 1.3 for T0). Similarly, for GII.4 VLPs, the OD values were 115% and 83% after 1 h and 24 hours, respectively, compared to baseline (for an OD value of 0.9 at T0). The lack of change in the C_T values and the small decrease of immune reactivity suggest that the viral particles, used for the bioaccumulation, are equally stable in seawater for at least 24 hours.

Neoconglycoconjugates VLPs binding. Attachment to five neoglycoconjugates of VLPs from the GI.1, GII.3 and GII.4 strains used for the bioaccumulation experiments were compared. The GI.1 VLPs attached preferentially to H type 1 as previously reported (16, 32), whereas the GII.3 and GII.4 attached preferentially to sialyl-Le^x and Le^y and to a smaller extent to the sialylated type 2

precursor, similar to previous observations for the GII.3 strain and for another GII.4 strain (44) (Fig. 1). Thus, binding patterns were quite different between the GI.1 and the GII strains, the latter being characterized by an ability to recognize sialylated structures.

Conditions for the bioaccumulation experiments. Fresh oysters collected from a clean environment were bio-accumulated at. Water salinity was 30.5±1.2 g/L. Three concentrations were assayed for each strain, the highest one being 8.52±0.2, 8.38±0.3, and 8.48±0.33 expressed as $\log_{10}$ RNA copies/L for the GI.1, GII.3 and GII.4 strain respectively. The middle concentration corresponded to a 10-fold dilution and the lowest concentration to a 100-fold dilution. Thus approximately 10^8, 10^7 and 10^6 RNA copies/L were used for the high, middle and low dose, and were introduced into the respective aquaria for the three strains.

GI.1 virus bioaccumulation and VLP binding. The GI.1 virus was bio-accumulated efficiently at all three doses (Fig. 1). A dose dependence response was observed for the three concentrations tested (10^6, 10^7, and 10^8 copies) with final amounts observed in DT of 4.5±0.9, 5.5±0.8 and 6.4±0.7 $\log_{10}$ RNA copies /g, respectively (p= 0.0001, ANOVA test). Concentrations detected in DT were quite similar during the two first experiments conducted in October and November whereas increased concentrations in DT were observed in January and to a lesser extent in March (Fig. 2-A). To assess this difference, relative amounts of virus recovered in DT after 1 or 24 hours were calculated. After 1 hour, less than 1% of virus seeded into seawater was detected for three experiments (October, November, March), but 41% was already concentrated in the DT in January (Fig. 2.B). After 24 h, 5.5 and 1.2% of the seeded virus amounts were detected in the DT in October and November, respectively, and 88 and 27% were detected in the experiments conducted in January and March, respectively. The difference between October/November and January/March was statistically significant (p=0.0004). Concentrations in gills and mantle were quite stable and at least one hundred fold lower than the concentration in DT. For example, for the lowest dose, 1.9±0.8 and 2.0±1 $\log_{10}$ RNA copies/g of gills or mantle respectively were detected after one hour, and 2.0±0.6 and 2.0±0.7 $\log_{10}$ RNA copies/g of gills and mantle respectively after 24 hours. For the highest concentration the

difference after 24 h of bioaccumulation was even larger with 3.2±1 $\log_{10}$ RNA copies/g of gills, compared to 6.4±0.7 $\log_{10}$ RNA copies /g detected in DT.

The GI.1 VLPs capacity to bind to the different tissues was then compared for the four months considered (i.e. October, November, January and March) (Fig. 2-C). These VLPs bound readily to DT with a clear increase in January and March. No binding above the sensitivity threshold was observed for the two other tissues. The distribution of bioaccumulated virus within the different tissues analyzed for the three strains was compared after one hour and 24 hours. Concentrations measured in all three tissues after one hour and in gills and mantle after 24 hours were expressed as a percentage of concentrations measured in DT at 24 hours (Fig. 2-D). The virus was directly accumulated in DT (13%), with only 0.5 and 0.3% of viruses detected after one hour in gills and mantle, respectively. After 24 hours the relative concentrations detected in gills and mantle remained very low (0.15 and 0.12% respectively), showing that the virus specifically accumulated in the DT.

GII.3 virus bioaccumulation and VLP binding. The GII.3 strain was also efficiently bio-accumulated by oysters (Fig. 3). Concentrations detected after 24h in DT were quite similar to those observed for GI.1 (excepted in January) (Fig. 3-A). Unlike with the GI.1 strain, no difference between the four months was observed in terms of bioaccumulation efficiency (p=0.18). After one hour, only 0.1 to 0.5% of the inoculum was detectable in DT (Fig. 3-B). After 24 hours, concentrations in DT increased for all three inocula dosages but never reached more than 4% (0.1 to 4.1%) of the virus dose seeded into seawater (Fig. 3-B). However as for the GI.1 strain, a clear dose impact was observed in recovered DT concentrations (4.2±0.4, 5.1±0.4and 6.4±0.2 $\log_{10}$ RNA copies/g for the three seeded doses assayed respectively) (p= 0.0002).

GII.3 VLPs bound to all three tissues assayed without clear variations between the four months (Fig. 3-C). This is reflected by the observed tissue distributions (Fig. 3-D). If up to 41% of the virus was already detected in DT after one hour, non negligeable proportions were also present in gills (11%) and in the mantle (5%) (Fig. 3-D). However, after 24 hours, the concentrations in these two tissues represented only approximately 0.5% for each of the concentrations detected in DT. Thus, this

strain accumulated in all organs in a transient manner before being concentrated in DT. After one hour, concentrations observed in gills and mantle were approximately 10 times lower than in DT. In the former organs relative concentrations then decreased for the next 23 hours to reach a difference of concentration compared to DT between a hundred or a thousand fold lower. For example, for the lowest dose, 2.3±1.3 and 2.1±1.6 $\log_{10}$ RNA copies/g were detected in gills or mantle respectively after one hour, and 1.9±0.7 and 2.1±0.7 $\log_{10}$ RNA copies/g of gills and mantle respectively after 24 hours. As observed for the GI.1 strain, the difference between virus concentration in gills and DT reached ~3 $\log_{10}$ after 24 hours for the highest dose, with detection of 3.5±0.5 $\log_{10}$ RNA copies/g in gills compared to 6.4±0.2 $\log_{10}$ RNA copies/g in DT.

GII.4 virus bioaccumulation and VLP binding. Surprisingly the GII.4 strain showed very poor bioaccumulation with less than 0.01% of the inocula being concentrated by the oysters (Fig. 4). Even at the higher dose, 10^8 viral particles diluted into seawater, concentrations detected in DT were low with no difference between the four experiments. At variance with the GI.1 strain, the lower efficiency was observed in January (absence of inhibitors was verified, extraction efficiency control was identical to the one observed for the GI.1 or GII.3 experiments, and *r*RT-PCR was repeated several times). The mean concentrations at 24 hours for the four experiments were 2.2±0.3, 2.7±0.6 and 3.5±0.5 $\log_{10}$ RNA copies/g of DT for the three concentrations assayed respectively, showing absence of clear relationship with the seeded doses (p=0.57). These concentrations were quite similar to those detected after one hour (1.7±0.3, 2.0±0.1 and 2.3±0.2 $\log_{10}$ RNA copies/g of DT), showing almost no increase over time, unlike for the two other strains assayed. After one hour, concentrations in gills and mantle were similar to those in DT, unlike for the two other strains. For example for the lowest dose 2.3±1.1 and 2.4±0.2 $\log_{10}$ RNA copies were detected per g of gills and mantle respectively after one hour. After 24 hours concentrations detected were 1.7 and 2.0±0.4 $\log_{10}$ RNA copies/g in gills and mantle respectively. Finally, similar to DT, recovered concentrations in gills or mantle showed no clear dose relationship.

1 As observed for the GII.3 VLPs, the GII.4 VLPs attached to all three tissues except that in October a
2 lower binding to the gills was observed. However, considering the 4 experimentations conducted this
3 showed a clearly different pattern with about 45% of relative viral concentration detected in gills after
4 1h compared to 5.5% detected in DT and 9% in mantle. After 24 hours, gills and mantle tissues still
5 represented 31 and 40%, respectively, of the virus accumulated in DT, indicating that the
6 accumulation of this strain was not organ-specific in addition to being very low, as described above.

DISCUSSION.

Shellfish contamination by infectious agents is classically monitored based on detection of *Escherichia coli* in shellfish tissues (European regulation, 91/492/EC) or fecal coliforms in growing waters (United States National Shellfish Sanitation Program). However shellfish meeting regulation criteria have been implicated in outbreaks, and depuration, efficient at eliminating some bacteria, does not eliminate viruses (7, 29, 43). The observed differences in clearance of bioaccumulated bacteria and viruses raise questions about potential interactions between oysters and viral human pathogens. This is of particular interest for NoVs, the pathogens most frequently involved in shellfish borne outbreaks (4, 7, 40). The finding that NoV-specific ligands exist in shellfish led to the hypothesis that expression of these ligands may influence bioaccumulation, and behavior of these viruses in oyster (23, 30, 51, 56). The ligand for Norwalk virus, the prototype GI strain, was characterized as an A-like carbohydrate structure indistinguishable from human blood group A antigen and whose expression is restricted to the digestive tissues of these animals and shows a clear seasonal variation (23, 30, 50). For the Houston GII.4 strain, we recently demonstrated that the interaction in digestive tissues involved both a sialic acid in α2,3 linkage and an A-like carbohydrate ligand, and that the virus binds to gills and mantle tissue involving the sialic acid-containing ligand exclusively (30). To evaluate the impact of these ligands on NoV bioaccumulation in oysters, three representative strains of NoV GI and GII were compared in terms of efficiency of bioaccumulation, tissue distribution and seasonal influence. Selection of tissues analyzed was based on VLPs binding ability but also on oyster physiology. For feeding activity, oysters pump water over their gills. Suspended particles are captured and passed on to the alimentary tract with some sorting of particles occurring prior to ingestion to help regulate what is presented to the digestive tract. Organs involved in the ingestion and digestion of food and the elimination of feces include the mouth, a short esophagus, stomach, crystalline style sac, digestive diverticula, midgut, rectum and anus (all these tissues dissected and called "digestive tissues" in this study). With the exception of a short section of the rectum, the entire alimentary canal lies within the visceral mass and is completely immobilized by the surrounding connective tissue (included here in mantle tissues). Food is moved from the mouth toward the anus by the strong ciliary activity from

epithelial cells that line the alimentary tract. The digestive gland surrounds the stomach entirely and also part of the intestine. It comprises a series of branched ducts that open into the stomach, and the duct branches serially to terminate in blind-ending tubules, location of the digestion activity (14). Based on these physiological informations, we chose to apply a quantitative approach to three groups of tissues i.e. gills, digestive tissues and mantle.

Environmental conditions have an impact on oyster growth, respiration and nutrient assimilation (8, 31). As these aspects were not considered here, to avoid as much as possible variability due to environmental conditions and to follow the seasonal cycle of oysters, all experiments were performed with the same batch of oysters kept in a clean area during the 6 months of the study. The seawater was collected from the same area, and the aquarium temperature was adapted to the *in situ* measured temperature. The bioaccumulation experiments (the three strains at three concentrations and the control batch) were conducted on the same day for each season, and oysters were dissected at the same time, by different members of the laboratory, to avoid any delay and difference within assays. Similarly, all tissues were then extracted by organs rather than by strains or level of contamination, and *r*RT-PCR were performed in a single experiment, including all negative controls and standard curve. These precautions were important to avoid artifactual experimental variations and to allow safe comparisons of the four experiments.

The first striking observation was that oysters concentrated the three strains with very different efficiencies and tissue distributions. The GI.1 strain was previously shown to bind specifically through an A-like carbohydrate structure to DT but not to other tissues (23, 50). We observed here that it was readily bioaccumulated in DT with less than 1% of the virus detected in other tissues after 1 hour and a 1000-fold difference between gills/mantle and DT after 24 hours, consistent with the lack of a ligand in gills and mantle. The high concentration of GI.1 recovered in DT is also consistent with earlier observations (1, 45). The efficiency of this DT-specific bioaccumulation paralleled the season-dependent expression level of the carbohydrate ligand, strongly arguing in favor of its involvement in the bioaccumulation process. Moreover, we previously observed that GI.1 VLPs bioaccumulated in a manner dependent on this carbohydrate recognition since mutant VLPs that had lost the carbohydrate

ligand-binding property were less well accumulated (30). We also previously observed that in the environment, the ratio between genome copies in oysters and in water was much higher (50 times) for GI strains than for GIII strains that have no carbohydrate ligand in oyster tissues (56). Collectively the previously reported observations and those presented here represent compelling evidence for a major role of the TD-specific carbohydrate ligand in the highly efficient GI.1 strain bioaccumulation in oysters In contrast, the GII.4 strain was poorly bioaccumulated regardless of the month considered and showed a different distribution within shellfish body. Even though a preferential accumulation in DT occurred after 24 hours, after one hour a large percentage of virus was detected in gills and mantle, consistent with the binding to sialic acid in α2,3 linkage previously detected by ELISA and histochemistry (30). Bioaccumulation of GII.4 strains in gills or labial palps of Pacific oysters (*Crassostrea gigas*) (36), or in gills but not in DT of *Crassostrea ariakensis* (53) were reported, suggesting that the behavior of various NoV GII.4 strains may be similar in distinct oyster species, even if the results obtained here are to be considered for the GII.4 Houston strain. Over the last fifteen years, strains of the GII.4 genotype became predominant across human populations (up to 80-90% of clinical cases) and have been responsible for several large outbreaks (3, 6, 47). Despite this high prevalence in human infections and thus large amounts of GII.4 particles discharged in sewage (46), strains of this cluster are not predominant in oyster-related outbreaks (11, 19, 22, 24, 39). Different factors such as viral load in human feces, resistance to sewage treatment, adsorption to different particles may influence the behavior of viral particles (12, 52). However, our observation of very poor accumulation by oysters compared to other NoV strains is concordant with this epidemiological observation.

What is the reason for the weak DT-specific bioaccumulation of GII.4? Assays of water in the bioaccumulation tanks at the end of the two last experiments showed negligible numbers of virus genome copies leftover for all three strains compared to the inocula (data not shown), suggesting that the three strains have almost entirely been captured by the oysters. Although the low GII.4 bioaccumulation might be explained by a lower stability compared to other strains, as previously suggested (9), control experiments showed no decrease in genome copy numbers or capsid immune reactivities in sea water during the short period of time considered (24 hours). However, it should be

noted that detection of RNA by RT-PCR may not correlate with infectivity. Furthermore, the very low bioaccumulation of GII.4 compared to GI.1 was already clearly visible after 1 hour. It is therefore unlikely that the inefficient bioaccumulation of the GII.4 strain can be explained by a lower stability in seawater on such short periods. The binding of the GII.4 strain to gills (and the mantle) may prevent the passage of viral particles to the mouth and thus to DT. Even after one day, and irrespective of the season or concentrations, GII.4 virus persists on the gills (and the mantle) in accordance with the binding detected by ELISA with VLPs (30). Because we were unable to detect residual virus in the seawater after a 24 hour bioaccumulation period (as observed also for the two other strains tested),we hypothesize that binding to gills and mantle through a sialic acid-containing ligand prevents the passage into the digestive tract and is followed by a rapid destruction of the virus by unknown mechanisms that need to be further analyzed and identified.

GII.3 NoVs have a similar ELISA binding pattern to oyster tissues as that observed for the GII.4 strain, with VLPs binding to DT, gills and mantle. However, the bioaccumulation efficiency was much higher compared to that observed for the GII.4 strain. After one hour, as observed for the GII.4 strain, NoV GII.3 is detected in gills and mantle but also in DT. After 24h, gills and mantle tissues displayed concentrations 1000-fold lower than in DT, suggesting that after being transiently retained in the gills, probably due to binding to sialic acid, they are either destroyed, as observed for the GII.4 strain, or they are released to enter the mouth as observed for the GI.1 strain. The release of the gills or mantle might occur if the GII.3 strain has a lower binding affinity for the sialic acid-containing ligand, an aspect that will require further investigation.

GI NoVs represent about 30% of NoV-contaminated shellfish (field or market studies) (5, 12, 54) and this genogroup is also frequently detected in shellfish outbreaks (18, 19, 22, 24, 39, 55). Similarly, NoV GII.3 are frequently reported in shellfish-related outbreaks (11, 19, 38). These data fit with our observation that even after one hour, mimicking an accidental contamination, there is already considerable uptake of GI.1 and GII.3 viruses in oysters. The efficiency of oysters in bioaccumulating GI strains such as Norwalk virus, particularly during winter months (January-March) when oyster consumption is highest in France, may explain why this genogroup of NoVs are so often implicated in shellfish-related outbreaks despite their relatively low frequency of detection in the community. Virus

contamination of oysters can occur in absence of a specific ligand, as observed through a field study with GIII NoV strains (56). However, it is far less efficient than when a digestive tissue-specific ligand is present as in the case of the GI.1 strain.

From a public health perspective, identification of a correlation between ligands in shellfish and bioaccumulation efficiency may help to predict periods of high risk, guide the development of testing protocols that will help to increase the sanitary quality of shellfish put on the market, or even lead to the selection of oyster species that may be less sensitive to NoV contamination.

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1

TABLES

Table 1: Virus stability in seawater

Strain	1 minute[a]	24 hours[a]
GI.1	25.16 ± 1.19	25.32 ± 1.28
GII.3	23.29 ± 0.84	23.53 ± 0.64
GII.4	23.85 ± 1.17	23.55 ± 1.00

[a]: mean Ct values ± standard deviation

FIGURE LEGENDS

Figure 1: Binding of GI.1, GII.3 and GII.4 VLPs to immobilized synthetic oligosaccharides. A panel of neoglycoconjugates was coated on ELISA plates and the binding of GI.1 (white bars), GII.3 (grey bars) and GII.4 (black bars) VLPs was detected as described in the materials and methods section. Results are shown as O.D. 450 nm values from one experiment representative of three independent ones.

Figure 2: GI.1 bioaccumulation and VLPs binding.

A: Virus concentrations measured in DT at 24h bioaccumulation were reported as genome copies (y axis) for the four experiments (October, November, January and March) (x axis). The high dose (triangle) corresponds to 8.52±0.2 expressed as $\log_{10}$ RNA copies/L, medium (square) corresponding to a 10-fold dilution and the lowest (diamond) to 100-fold dilution. Each point represents mean values for 6 oysters.

B: Monthly variations of the bioaccumulation efficiency in digestive tissues: viral bioaccumulation efficiency after one hour (dashed bar) or 24 hours (black bar) (x axis) are expressed as % of virus recovered in DT calculated on virus seeded into seawater and virus detected in DT taking into account recovered weight (y axis) for the four experiments (October ,November , January and March). All values are given above bars.

C: Binding of GI.1 VLPs to extracts of gills (grey bars), mantle (white bars) and digestive tissues (black bars), for the four months (x axis), expressed as signal to noise ratio. The positive threshold is indicated by a dashed line.

D: GI.1 contamination detected in different tissues following bioaccumulation. Results are expressed as mean % values of recovered virus concentrations in the different tissues after 1 hour (dashed bar) and 24 hours (back bar) for the three seeded concentrations. The concentration detected after 24 hours was considered as 100%; actual values are noted above each bar.

Figure 3: GII.3 bioaccumulation and VLPs binding.

A: Virus concentrations measured in DT at 24h bioaccumulation were reported as genome copies (y axis) for the four experiments (October, November, January and March) (x axis). The high dose (triangle) corresponds to 8.38±0.3 expressed as $\log_{10}$ RNA copies/L, medium (square) corresponding to a 10-fold dilution and the lowest (diamond) to 100-fold dilution. Each point represents mean values for 6 oysters.

B: Monthly variations of the bioaccumulation efficiency in digestive tissues: viral bioaccumulation efficiency after one hour (dashed bar) or 24 hours (black bar) (x axis) are expressed as % of virus recovered in DT calculated on virus seeded into seawater and virus detected in DT taking into account recovered weight (y axis) for the four experiments (October ,November , January and March). All values are given above bars.

C: Binding of GII.3 VLPs to extracts of gills (grey bars), mantle (white bars) and digestive tissues (black bars), for the four months (x axis), expressed as signal to noise ratio, the positive threshold is indicated by a dashed line.

D: GII.3 contamination detected in different tissues following bioaccumulation. Results are expressed as mean % values of recovered virus concentrations in the different tissues after 1 hour (dashed bar) and 24 hours (back bar) for the three seeded concentrations. The concentration detected after 24 hours was considered as 100%; acutal values are noted above each bar.

Figure 4: GII.4 bioaccumulation and VLPs binding.

A: Virus concentrations measured in DT at 24h bioaccumulation were reported as genome copies (y axis) for the four experiments (October, November, January and March) (x axis). The high dose (triangle) corresponds to 8.48±0.33 expressed as $\log_{10}$ RNA copies/L, medium (square) corresponding to a 10-fold dilution and the lowest (diamond) to 100-fold dilution. Each point represents mean values for 6 oysters.

B: Binding of GII.4 VLPs to extracts of gills (grey bars), mantle (white bars) and digestive tissues (black bars), for the four months (x axis), expressed as signal to noise ratio, the positive threshold is

indicated by a dashed line.

C: GII.4 contamination detected in different tissues following bioaccumulation. Results are expressed as mean % values of recovered virus concentrations in the different tissues after 1 hour (dashed bar) and 24 hours (back bar) for the three seeded concentrations. The concentration detected after 24 hours was considered as 100%, all values are given above bars.

Figure 1

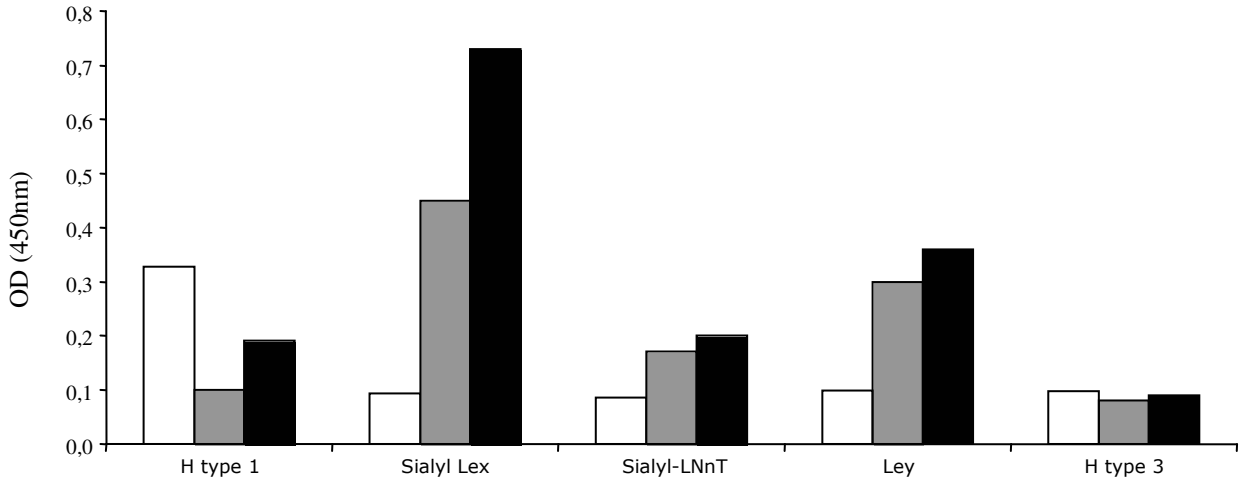

0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
OD (450nm)
H type 1
Sialyl Lex
Sialyl-LNnT
Ley
H type 3

Figure 2

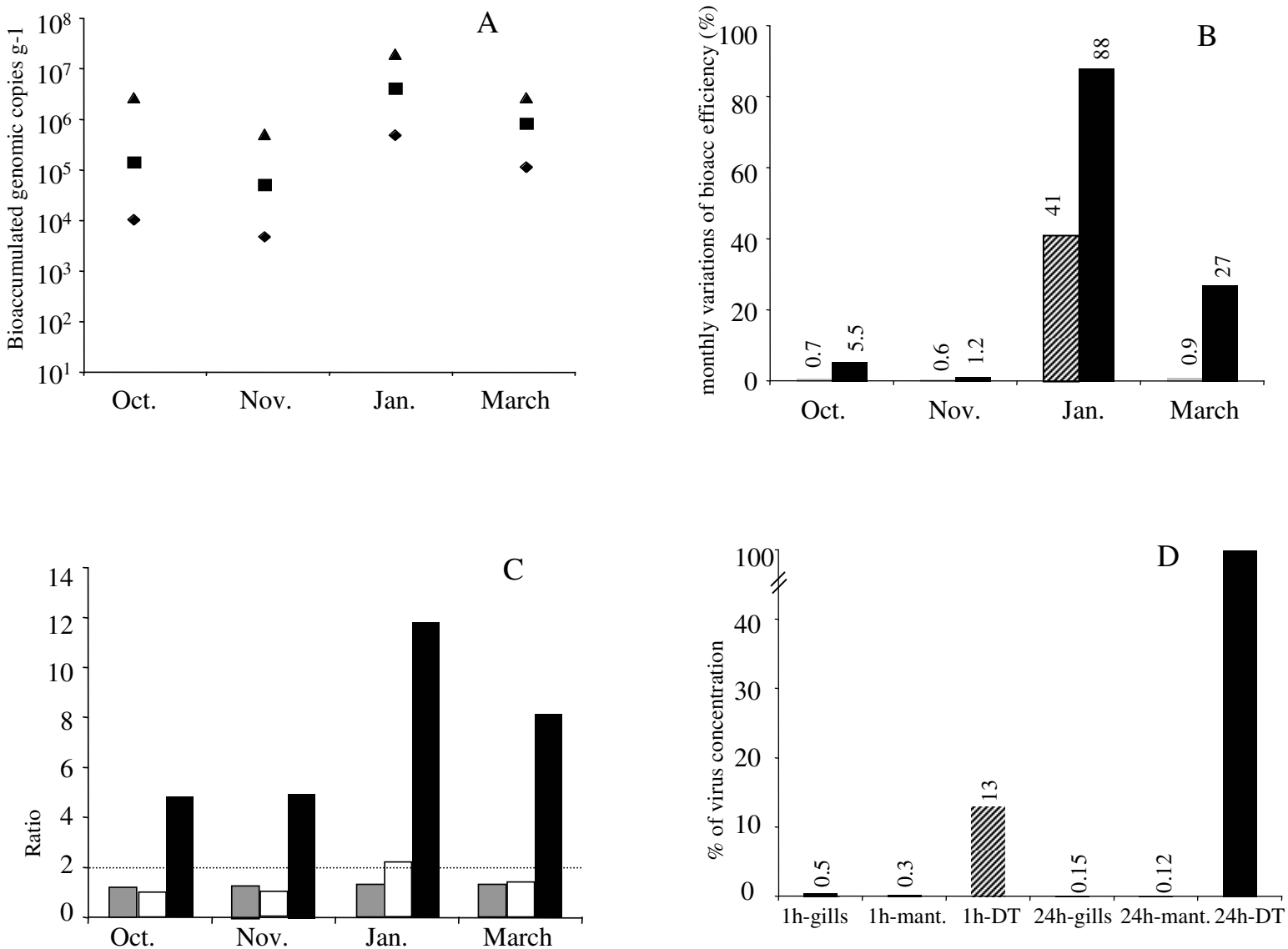

A
Bioaccumulated genomic copies g-1
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Oct.
Nov.
Jan.
March
B
monthly variations of bioacc efficiency (%)
100
80
60
40
20
0
0.7
5.5
0.6
1.2
41
88
0.9
27
Oct.
Nov.
Jan.
March
C
Ratio
14
12
10
8
6
4
2
0
Oct.
Nov.
Jan.
March
D
% of virus concentration
100
40
30
20
10
0
0.5
0.3
13
0.15
0.12
1h-gills
1h-mant.
1h-DT
24h-gills
24h-mant.
24h-DT

Figure 3

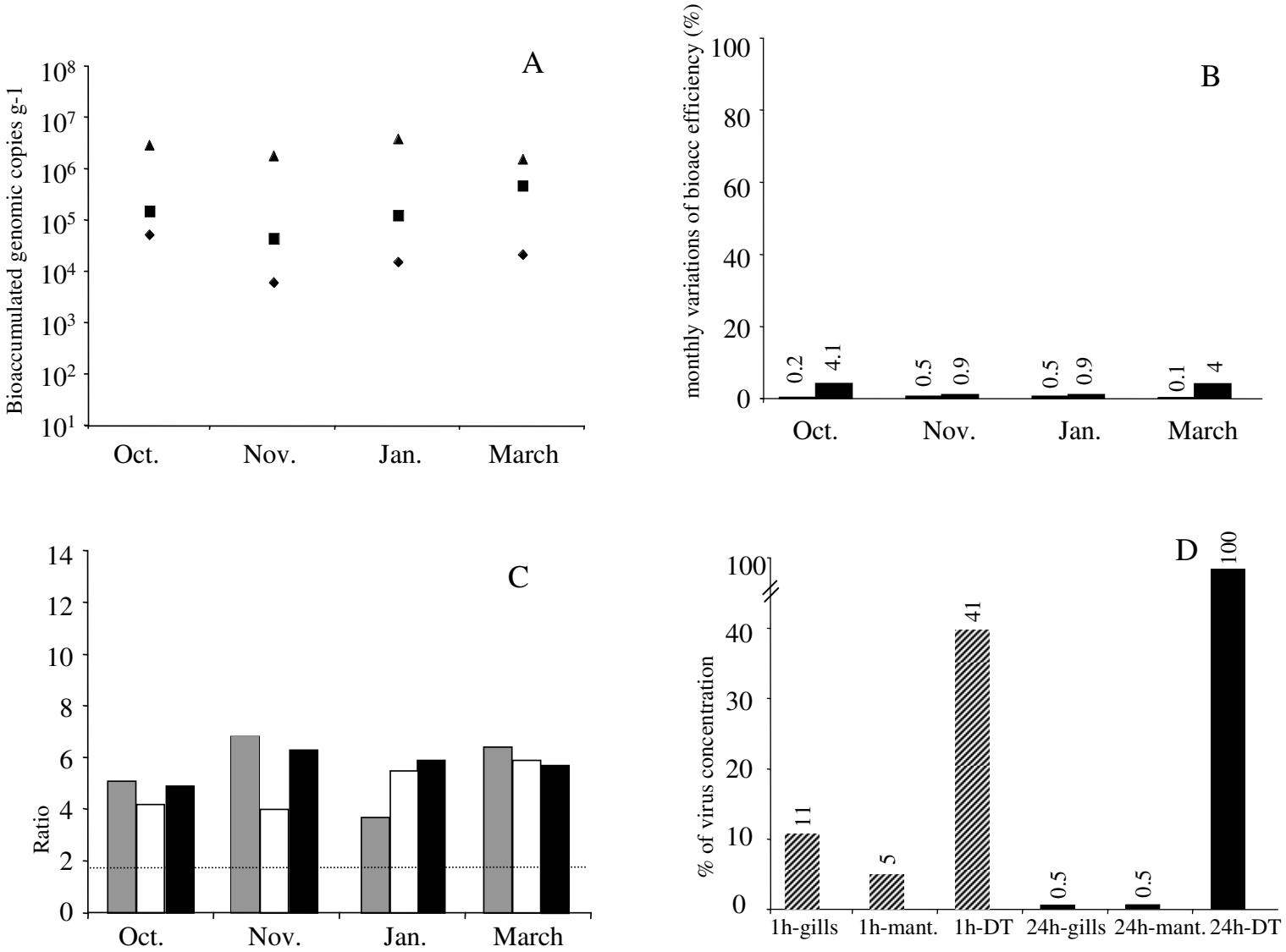
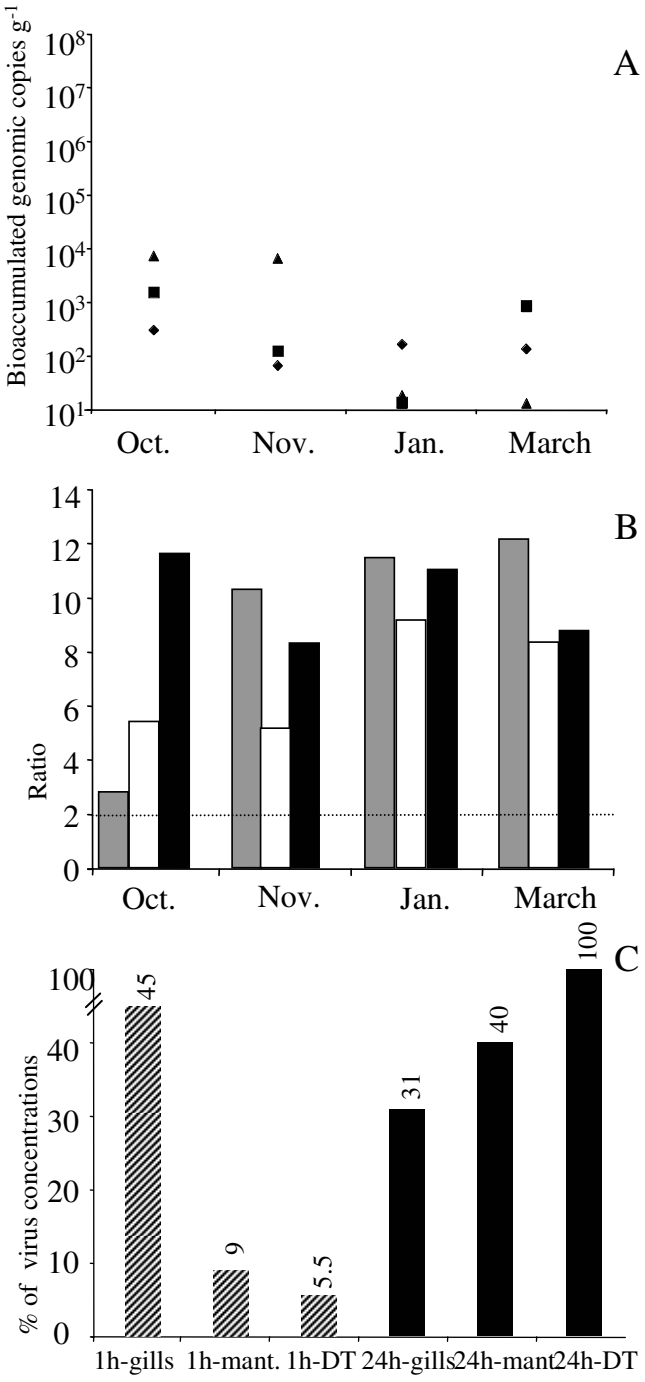

A
Bioaccumulated genomic copies g-1
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Oct.
Nov.
Jan.
March
B
monthly variations of bioacc efficiency (%)
100
80
60
40
20
0
0.2
4.1
0.5
0.9
0.5
0.9
0.1
4
Oct.
Nov.
Jan.
March
C
Ratio
14
12
10
8
6
4
2
0
Oct.
Nov.
Jan.
March
D
% of virus concentration
100
40
30
20
10
0
11
5
41
0.5
0.5
100
1h-gills
1h-mant.
1h-DT
24h-gills
24h-mant.
24h-DT

Figure 4


A
Bioaccumulated genomic copies g-1
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Oct.
Nov.
Jan.
March
B
Ratio
14
12
10
8
6
4
2
0
Oct.
Nov.
Jan.
March
C
% of virus concentrations
100
40
30
20
10
0
45
9
5.5
31
40
100
1h-gills
1h-mant.
1h-DT
24h-gills
24h-mant
24h-DT