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Human norovirus occurrence and diversity in the Llobregat river catchment, Spain

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Summary

Human noroviruses (NoV) were quantified and characterized in an 18 month survey conducted along the Llobregat river catchment in Spain. Sample types included freshwater, untreated and treated wastewater and drinking water. High NoV genome copy numbers were reported, reaching up to 10^6 l^{-1} and 10^9 l^{-1} in freshwater and raw sewage respectively. In both types of samples, GII NoV genome copies outnumbered those of GI, although without significance. All samples of semi-treated and treated drinking water were negative for NoV. A clear seasonality of NoV occurrence was observed both in river water and sewage samples, with significantly higher genome copy numbers in the cold than in the warm months period. Mean NoV log reduction rates after biological treatment of sewage were 2.2 and 3.1 for GI and GII respectively. A total of 77 NoV strains isolated in the Llobregat river catchment could be phylogenetically characterized, 44 belonging to GI and 33 to GII. The most prevalent genotype was GI.4, followed by GII.4 and GII.21. Several variants of the pandemic GII.4 strain were detected in the environment, corroborating their circulation among the population.

Introduction

Poor water quality continues to pose a major threat to human health. The World Health Organization declared that diarrhoeal disease alone contributes to an estimated 4.1 % of the total DALY (disability adjusted life years) global burden of disease and is responsible for the deaths of 1.8 million people every year. It was figured that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene and it is mostly concentrated on children in developing countries. However, human enteric viruses become contaminants of the water environment, even in developed communities, since they are excreted by infected individuals in extremely high numbers and current wastewater treatments do not ensure their complete removal (Kaplan *et al*., 1982; Kukkula *et al*., 1999; da Silva *et al*., 2007; Schuster *et al*., 2010).

It is well recognized that the most common viral gastrointestinal illness agents are rotavirus and norovirus (NoV) in the infantile and adult population respectively (Mead *et al*., 1999). However, most well-documented water-borne outbreaks of viral gastroenteritis are related to human NoV (Kukkula *et al*., 1999; Hewitt *et al*., 2007). *Norovirus* is a genus in the family *Caliciviridae*, a group of non-enveloped, icosahedral viruses that have a single-stranded, positive sense RNA genome (Fauquet *et al*., 2005). There are currently five genogroups (G) of NoVs with strains that infect humans found mainly in GI and GII, and to a much lesser extent in GIV (Atmar, 2010).

A study on the prevalence and phylogeny of human NoV strains in the Llobregat river catchment in Catalonia, NE Spain, was conducted from November 2007 to April 2009. This river is the third longest in Catalonia, flowing for 170 km from its source in the pre-Pyrenees mountains to the Mediterranean Sea, and is the source of drinking water for over 5 million inhabitants in municipalities around Barcelona. The Llobregat river receives urban and industrial discharges from more than 30 sewage treatment plants (Cespedes *et al*., 2005).

Results

NoV numbers in samples from the Llobregat river catchment

NoVs were quantified monthly by real-time RT-PCR in samples collected at 12 points in the Llobregat river catchment from November 2007 to April 2009 (Fig. 1 and *Supporting information*) which included freshwater (S1, S2, S3, S4, S7 and S9), urban untreated sewage (S5, S8 and S12), urban treated wastewater (S6), and semi-treated (pre-chlorination, flocculation, decantation, sand filtration, ozonation and carbon filtration; S10) and final (final

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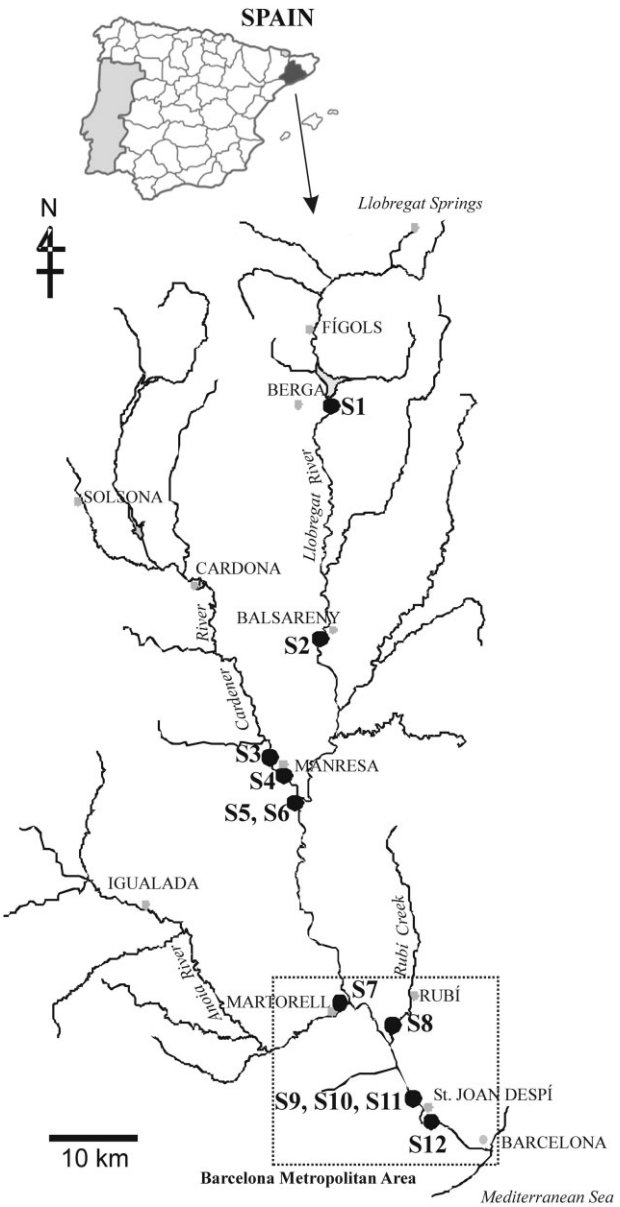

Fig. 1. Location of the sampling sites (S) in the Llobregat river catchment, Spain (see also *Supporting information*).

chlorination, S11) drinking water. The number of NoV genome copies in freshwater per location and month are depicted in Fig. 2. In river water samples, virus titres up to 10^6 genome copies l^{-1} were observed for both genogroups. In freshwater, 75% of the samples were positive for NoV GI and 68.5% for NoV GII. NoV GII numbers in river water were higher than those of GI but the differences were not significant. All samples of semi-treated (S10) and final (S11) drinking water taken at the DWTP were negative for NoV. Data from untreated and treated sewage is shown in Fig. S1 (*Supporting information*). High virus titres were observed in wastewater samples, especially in the raw sewage inflow of the wastewater treatment plant (WWTP) in Manresa (S5), where levels up to 5.9×10^8 and 3.4×10^9 genome copies l^{-1} were reported for GI and GII respectively. In raw and treated sewage, GII genome copies also outnumbered those of GI but again without significance.

Seasonal occurrence of NoV

Figure 3 shows the comparative occurrence of NoV in cold (October–March) and warm (April–September) weather months at the different sampling sites. In river water, both NoV GI and GII genome copy numbers were significantly ($P < 0.05$) higher in the cold than in the warm weather period. In the cold months, GII genome copies significantly ($P < 0.05$) outnumbered those of GI, although the differences were not significant when the comparison in the number of genome copies of both genogroups was performed in the warm months. In raw or treated sewage, GI and GII NoV numbers were significantly ($P < 0.05$) higher in the cold months. GII was again significantly ($P < 0.05$) more prevalent than GI in sewage samples in the cold weather period, while this difference was not significant in the warm period.

NoV removal in a biological wastewater treatment plant

The Manresa WWTP receives each day 53 000 m^3 of sewage corresponding to 85 224 inhabitants. Wastewater at the WWTP is subjected to biological treatment with nitrogen and phosphate removal, through primary sedimentation, activated sludge, anaerobic digestion and band filter dehydration. Figure 4 depicts NoV GI and GII removal after treatment. NoV removal in the WWTP was determined by calculating the $\log_{10}$ (N_t/N_0), where N_0 is the genome copy number in the inflow untreated sewage (S5) and N_t is the genome copy number in the outflow of the treated wastewater (S6). The mean genome copy log reduction for GII was higher than for GI (3.1 ± 0.3 and 2.2 ± 0.4 respectively), but the differences observed were not significant.

Molecular epidemiology of human NoV in the Llobregat samples

Figure 5 shows the phylogenetic characterization of all the strains that could be characterized in the present study. A total of 147 samples (all NoV positive samples) were analysed for sequence determination. Seventy-seven sequences were obtained from 50 samples, 44 belonging to GI and 33 belonging to GII. The occurrence of multiple genotypes was observed in all positive samples with the exception of freshwater samples collected at S1 site, located in the upstream part of the

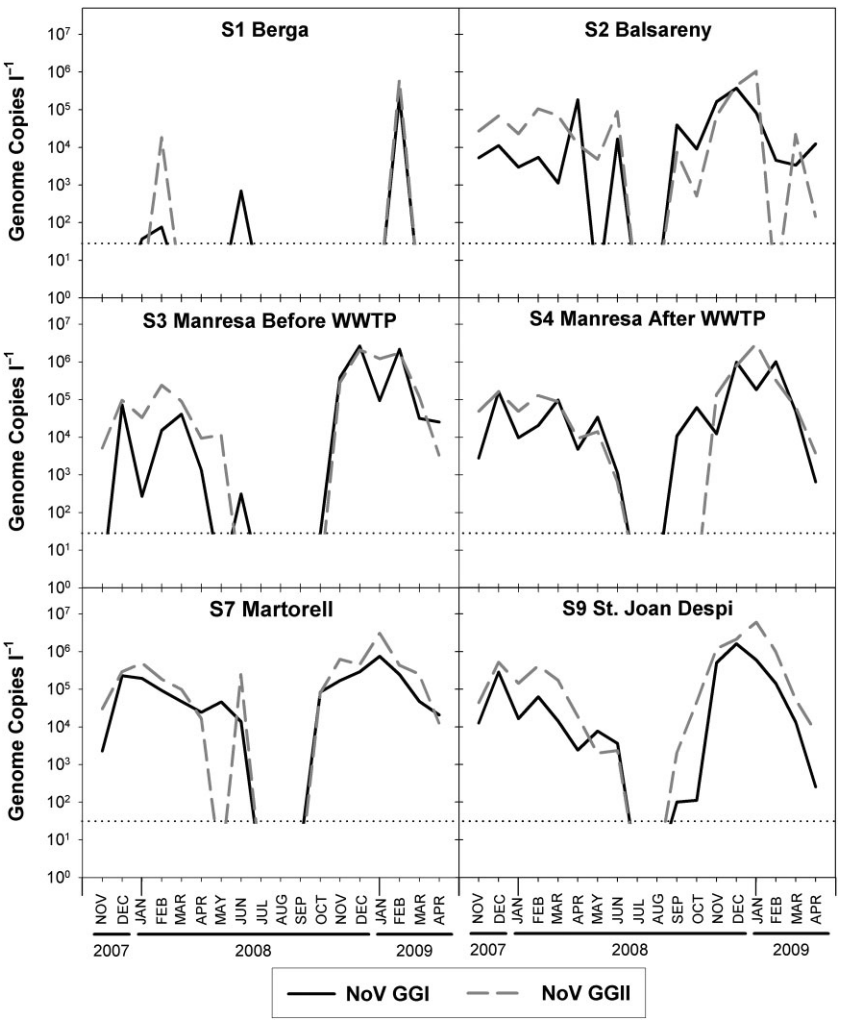

Fig. 2. Quantification of NoV genome copies in freshwater samples from the Llobregat river catchment (see Fig. 1 for sampling site location). Dotted lines indicate the quantification limit of the NoV gene, which is 54 copies l^{-1}.

catchment (Fig. 5) and mainly in the winter period. Among the GI strains, nine different genotypes were detected (GI.2, GI.3, GI.4, GI.5, GI.6, GI.7, GI.8, GI.NA2 and GI.NA3), while seven genotypes (GII.3, GII.4, GII.6, GII.7, GII.13, GII.14 and GII.21) were detected belonging to GII. The most prevalent genotype was GI.4, which was isolated from January 2008 to February 2009 in almost all sampling sites, accounting for 41.5% (32/77) of all isolated strains. GII.4 was also highly prevalent, being detected from January to April 2008 and from October to December of the same year, and also in almost all sampling sites, accounting for 16.9% (13/77) of all isolated strains. GII.21 was the third most prevalent strain, accounting for 15.6% (12/77). Several variants of genotype GII.4 were detected: 2002CN (three sequences), 2006a (one sequence), 2006b (four sequences) and two non-assigned sequences that were 99% identical to strain E3020 isolated in 2008 in Dijon, France (G. Belliot, M. Estienney, A.H. Kamel, K. Ambert-Balay and P. Pothier, unpublished).

Discussion

Few studies with quantitative data for either GI or GII of human NoV in freshwater are available and data comparing GI and GII numbers in this type of sample are scarce; most studies have focused on the presence or absence of GI and GII, or at utmost on semiquantitative determination of NoV (Haramoto *et al*., 2005; Lodder and Roda Husman, 2005; Westrell *et al*., 2006; Miagostovich *et al*., 2008; Kitajima *et al*., 2010; Lodder *et al*., 2010). Levels of 0.22 to 177 PCR detectable units l^{-1} of NoV RNA have been recently reported in source water for drinking water production in the Netherlands (Lodder *et al*., 2010). In the present study, higher numbers (up to 10^6 genome copies l^{-1}) were detected in Llobregat river, a source of drinking water for the Barcelona urban area.

In sewage, NoV genome copy numbers are also higher than those previously reported in France (da Silva *et al*., 2007), England (Laverick *et al*., 2004), the Netherlands (Lodder and Roda Husman, 2005),

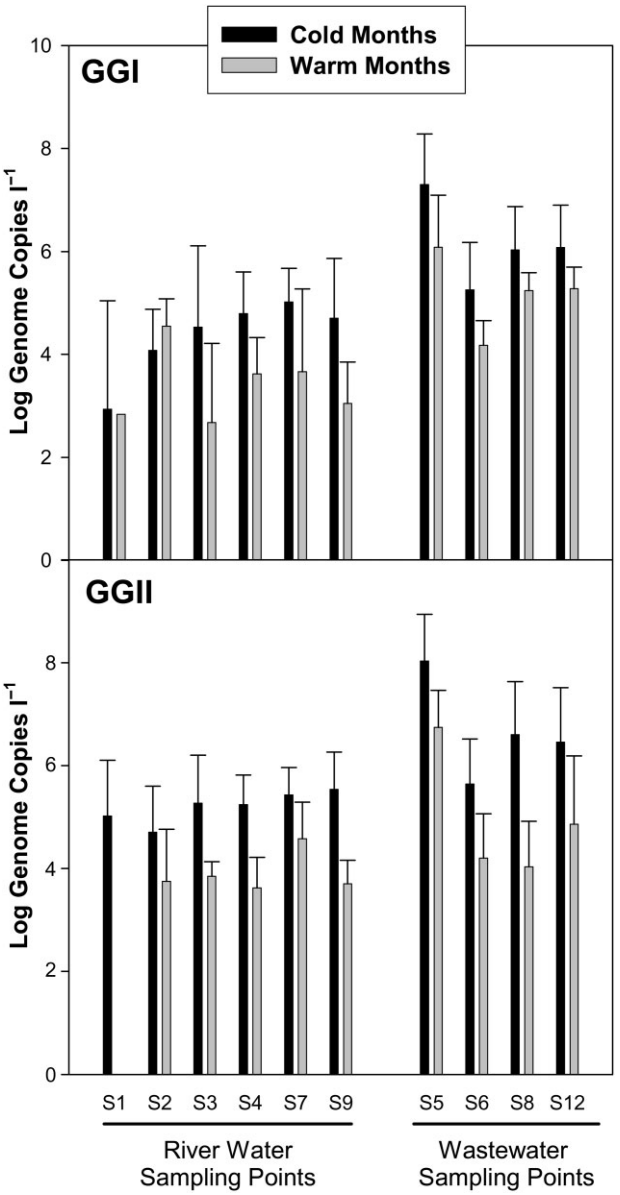

Fig. 3. Comparative occurrence of NoV GI and GII in cold (October–March) and warm (April–September) weather months in the different sampling sites.

Germany (Pusch *et al*., 2005) or Japan (Katayama *et al*., 2008). In the present study, real-time RT-PCR was employed and raw numbers of NoV genome copies (Tables S8 and S9) were corrected taking in consideration the nucleic acid extraction efficiencies and enzyme efficiencies as described elsewhere (Costafreda *et al*., 2006; Le Guyader *et al*., 2009; Pinto *et al*., 2009), thus providing a more accurate estimation of NoV genome copy numbers. This correction and the use of real-time RT-PCR that has been recently reported (Liu *et al*., 2010) that may be 1.1–1.6 $\log_{10}$ more sensitive than endpoint titration RT-PCR (employed in most aforementioned studies) when applied to the same sample, may account for the higher NoV detected in the present study in river water and sewage samples.

A seasonal distribution of NoV occurrence in river water and sewage was observed, with significantly ($P < 0.05$) higher NoV numbers in the cold weather period. Similar findings in river water (Kitajima *et al*., 2010) and sewage (Katayama *et al*., 2008) have been described in the literature. This seasonality may reflect the better stability of the virus in the cold period. Additionally, it is well known that NoV infections in the population dramatically decline in the summer months in most parts of the world although NoV continue to circulate throughout the year (Mounts *et al*., 2000; Dowell, 2001; Kroneman *et al*., 2008; Lopman *et al*., 2008).

GII accounts for the majority of reported NoV gastroenteritis cases (Lopman *et al*., 2004), and faecal load is also higher for GII than for GI (Chan *et al*., 2006). In the present study, GII was found in higher numbers than GI, both in river water and in sewage, although only in the cold months the differences were significant ($P < 0.05$). Moreover, in the present study GI removal after treatment at the WWTP was lower than that of GII. However, the differences between GI and GII genome copy reduction after treatment were not significant ($P < 0.01$).

Overall, GI NoV are more frequently involved in outbreaks transmitted by food or water than GII and this observation is considered to be a consequence of the higher resistance of the former (Kageyama *et al*., 2004; Blanton *et al*., 2006; Le Guyader *et al*., 2006). Nevertheless and in spite of the scarce information on the environmental stability of human NoV strains (Duizer *et al*., 2004; da Silva *et al*., 2007), a different behaviour in their specific biological interactions points to differences at the capsid surface level between NoV GI and GII (Tan and Jiang, 2005; Maalouf *et al*., 2010) that could account for a differential environmental stability.

Phylogenetic analysis of NoV strains provides relevant public health information. Several studies reported a higher frequency of GI NoV strains in treated effluent compared to GII strains (van den Berg *et al*. 2005; Myrmel *et al*. 2006; La Rosa *et al*. 2007; da Silva *et al*., 2007). Interestingly, in our study 7 out of 44 typed GI strains (16%) were found in treated sewage, while only 2 out of 33 GII typed strains (6%) were detected in treated sewage. GI strains in treated sewage belonged to GI.4 (5), GI.2 (1) and GI.NA2 (1), while the two GII strains in treated sewage were GII.21.

Of note, the most abundant strains detected in the present study (GI.4, GII.4 and GII.21) were also reported in river water samples in Japan (Kitajima *et al*., 2010) and in urban surface waters in Singapore (Aw *et al*., 2009). GI.4 has been recently described to be frequently involved in foodborne outbreaks (Verhoef *et al*., 2010).

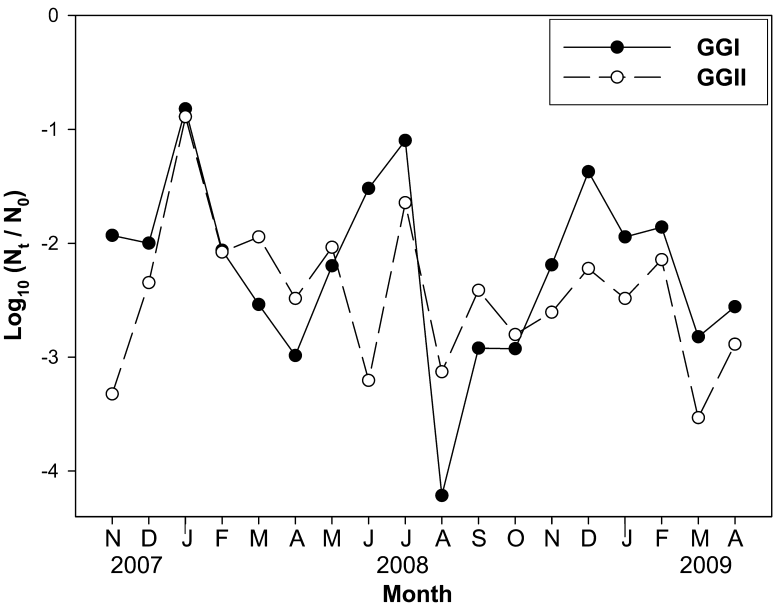

Fig. 4. NoV removal by wastewater treatment at the Manresa WWTP, expressed as $\log_{10}$ (N_t/N_0), where N_0 is the genome copy number in the inflow untreated sewage (S5) and N_t is the genome copy number in the outflow of the treated wastewater (S6).

NoV GII.4 genotype has been pandemically implicated in the past decade in both outbreaks and sporadic cases (Siebenga *et al*., 2009; 2010). Several GII.4 variants (2002CN, 2006a, 2006b and two non-assigned sequences 99% identical to E3020) arisen from this period have been detected in the present study, evidencing their circulation among the population.

Human NoV are abundant in environmental waters receiving urban pollution. Research on the incidence, behaviour and epidemiology of NoV in the environment provides valuable information on the efficiency of wastewater treatments to remove viral pathogens, the risk of infection derived from their occurrence in drinking water sources and an overview of the genotypes circulating among the population, including viruses causing both symptomatic and asymptomatic infections.

Experimental procedures

Sampling strategy

Different types of samples were collected on the first week of each month from November 2007 to April 2009, from 12 sites in the Llobregat river catchment (Fig. 1 and *Supporting information*): freshwater (S1, S2, S3, S4, S7 and S9), urban untreated sewage (S5, S8 and S12), urban treated wastewater (S6), and semi-treated (flocculation, sand filtration, ozonation and carbon filtration, S10) and final (as before, plus chlorination, S11) drinking water. Data on conductivity and bacteriological determinations in representative samples from the same sites are shown in Tables S1–S6 of the *Supporting information*.

Virus concentration

Viruses were concentrated from all types of water except raw sewage by filtration of 10-l samples through positively charged glass wool [Ouest Isol, France (Lambertini *et al*., 2008; Kiulia *et al*., 2010)]. Viruses were eluted twice with 50 ml glycine-beef extract buffer, pH 9.5, and the 100 ml eluate was further concentrated by polyethylene glycol (PEG) precipitation (Ueki *et al*., 2007). The resulting pellet was resuspended in 10–20 ml of PBS, pH 7.4, and stored at –80°C. Viruses were recovered from 600 ml untreated sewage samples into a final volume of 24 ml by PEG precipitation (Ueki *et al*., 2007). Viral RNA was extracted from 500 µl aliquots of the concentrates using the NucliSens miniMAG magnetic system (BioMérieux) to a final volume of 100 µl.

Norovirus detection

A standardized one-step real-time TaqMan RT-PCR using previously described primers and probes (Table S7, *Supporting information*) was employed for the detection of human NoVs of GI (da Silva *et al*., 2007; Svraka *et al*., 2007) and GII (Kageyama *et al*., 2003; Loisy *et al*., 2005). Virus/nucleic acid extraction and enzyme efficiencies were monitored as described elsewhere (Costafreda *et al*., 2006; Le Guyader *et al*., 2009; Pinto *et al*., 2009), and used to estimate the actual genome copy numbers from the raw genome numbers measured by real-time RT-PCR in duplicate.

Norovirus typing

Genotyping of the NoV strains was achieved following a semi-nested RT-PCR protocol using specific primers for GI and GII. Reverse transcription of viral genes in concentrated water samples was performed with the *Expand Reverse Transcriptase* (Roche) using a 100 µM random hexamer stock as reverse primer and following the manufacturer's instructions. The first PCR was conducted with primers COG1F and G1SKR for GI, and COG2F and G2SKR for GII (Kojima *et al*., 2002; Kageyama *et al*., 2003). The second PCR was conducted using primers G1SKF and G1SKR for NoV GI and G2SKF and G2SKR for GII (Kojima *et al*., 2002).

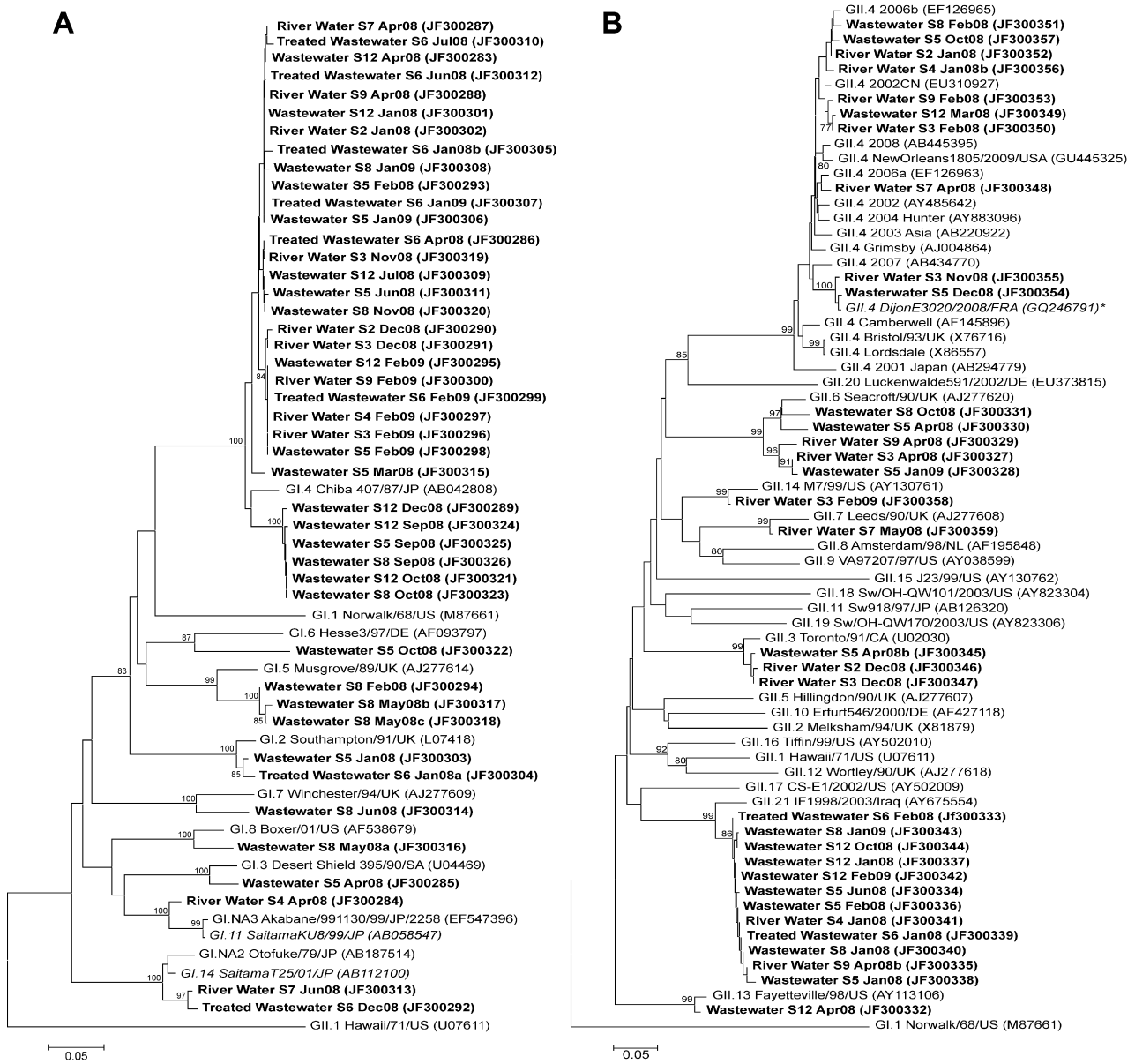

Fig. 5. Phylogenetic analysis of NoVs GI (A) and GII (B) strains isolated in water samples from the Llobregat river basin. The dendrogram was constructed using the neighbour-joining method with distance calculation by the Kimura-2-parameter correction implemented in the MEGA4 program. A boostrap of 100 replicates was performed and values above 75 are shown in the figure. Bold characters indicate NoV sequences obtained in this study. Reference strains for the respective genotypes and GII.4 variants were obtained from the Norovirus genotyping tool (National Institute of Public health and the Environment, the Netherlands, http://www.rivm.nl). For GI.NA genotypes ('non-assigned'), the closest reference set from Kageyama and colleagues (2004) were also included and indicated in italics. Sequences indicated with an asterisk are sequences from the GenBank that were included in the analysis due to their close relationship with isolates sequences in this study.

The size of the semi-nested RT-PCR fragments for NoV GI and GII were 330 bp and 344 bp respectively. Both PCR reactions were conducted using the *Expand HiFi PCR System* (Roche) following the manufacturer's instructions. PCR products were cloned into a BamH1-HincII-digested pGEM cloning plasmid and DNA sequences were determined with at least three clones with the *ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v3.1* (Applied Biosystems) according to the manufacturer's instructions. Sequence analysis was conducted in an *ABI Prism 3700* automatic sequencer (Applied Biosystems, EEUU).

The dendrogram was constructed using the neighbour-joining method with distance calculation by the Kimura-2-parameter correction implemented in the MEGA4 program (Tamura *et al.*, 2007). Reference strains for the respective genotypes and GII.4 variants were obtained through the Norovirus genotyping tool (National Institute of Public health

and the Environment, the Netherlands, http://www.rivm.nl/mpf/norovirus/typingtool). For GI.NA genotypes ('non-assigned'), the closest reference set from Kageyama and colleagues (2004) were also included. The nucleotide sequences of NoV determined in this study were deposited in GenBank under accession numbers JF300283–JF300359.

Statistical methods

A paired Student's *t*-test was applied to ascertain the significance at $P < 0.05$ with the Sigmaplot 8.0 package. Values below the quantification limit were arbitrarily assigned to a value corresponding to this limit number minus one. In the seasonality study, data from all sampling sites of river water (S1, S2, S3, S4, S7 and S9) and sewage (S5, S6, S8 and S12) were grouped for statistical analysis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Quantification of NoV genome copies in raw sewage samples (see Fig. 1 for sampling site location). Dotted lines indicate the quantification limit of the NoV gene, which is 3580 copies l^{-1} for urban untreated sewage samples.
Table S1. Conductivity levels (μmho cm^{-1}) of representative samples at the Llobregat river basin sampling points. Measures were taken on location with a Fluke 87 III digital multimeter (Applied Biosystems).
Table S2. Total coliform levels of representative samples at the Llobregat river basin sampling points.
Table S3. *Escherichia coli* levels of representative samples at the Llobregat river basin sampling points.
Table S4. *Clostridium perfringens* levels of representative samples at the Llobregat river basin sampling points.
Table S5. Enterococci levels of representative samples at the Llobregat river basin sampling points.
Table S6. *Legionella* spp. levels of representative samples at the Llobregat river basin sampling points.
Table S7. Primers used in this study.
Table S8. Mean raw genome copy numbers, RT-PCR enzyme efficiencies and sample extraction efficiencies for NoV GI.
Table S9. Mean raw genome copy numbers, RT-PCR enzyme efficiencies and sample extraction efficiencies for NoV GII.

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