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Assessment of faecal contamination and the relationship between pathogens and faecal bacterial indicators in an estuarine environment (Seine, France)

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Abstract

The Seine estuary, one of the largest estuaries of the European northwest continental shelf, is subjected to numerous anthropogenic influences. Here we present an assessment of the microbial faecal contamination of the estuary water. The most vulnerable areas were defined on the basis of the fluxes of indicator organisms and the occurrence of *Salmonella* and *Cryptosporidium* sp. and *Giardia* sp. (oo)cysts. The microbial quality of the water changes from upstream to downstream: in the upstream area, contamination by faecal-indicator bacteria and *Salmonella* occurs during periods of high flow; in the urbanized area, mid-way between the uppermost areas of the estuary and its mouth, discharge from a wastewater treatment plant and a tributary degrade water quality; at the estuary mouth, the accumulation of microorganisms attached to particles in the maximum turbidity zone, particularly *Clostridium perfringens* spores and oocysts of *Cryptosporidium*, is accompanied by inputs of ThC and *Escherichia coli* from tributaries. In some areas, significant strong relations are observed between *Salmonella*, (oo)cysts of protozoan, and levels of faecal indicators.

Keywords: Seine estuary; Faecal-indicator bacteria; Microbiological risk assessment; *Salmonella*; *Giardia* sp.; *Cryptosporidium* sp.

1. Introduction

The microbiological quality of coastal and estuarine waters is a major public health concern (Shibata et al., 2004) and is the direct result of human activity. The Seine estuary, located on the Northwest European continental shelf, is greatly influenced by anthropogenic sources. Its watershed of 79,000 km^2 is inhabited by 16 million people, located mainly in urban areas. There also are intense agricultural and industrial activities in the watershed (Guézennec et al., 1999). As a result of these anthropogenic activities, the microbiological quality of the Seine estuary waters is poor (George et al., 2001). This has important socio-economic consequences, and requires an assessment of the microbiological risks associated with consumption of shellfish from the estuary bay and with recreational activities.

In estuaries, as in other natural aquatic systems, levels of faecal-indicator bacteria and enteric pathogens mainly are influenced by point sources (such as discharge of effluents from wastewater treatment plants [WWTP]), and the nature of the watershed. Soil leaching and surface runoff also contribute substantially to the faecal pollution of the water, especially in rural areas (Mawdsley et al., 1995; Lipp et al., 2001; Lemarchand and Lebaron, 2003; Kelsey et al., 2004; George et al., 2004). In estuaries, the survival of faecal microorganisms is influenced by their association with

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particles, which have a complex hydrodynamic behaviour affected by tidal cycles and river flow, and by the presence of a continuous salinity gradient not favourable to the survival of faecal bacteria (Le Hir et al., 2001; Mawdsley et al., 1995; Searcy et al., 2005).

The microbial quality of water currently is monitored by enumerating the levels of faecal-indicator bacteria: thermotolerant coliforms [ThC], *Escherichia coli*, enterococci, and spores of sulphite-reducing anaerobes [SSRA] are used as indicators of the level of faecal inputs and the probability of the presence of pathogens (Davies et al., 1995; Medema et al., 1997; Venczel et al., 1997; Payment et al., 2000). However, some enteric pathogens, such as viruses, protozoa, and some bacteria, have different survivability than faecal-bacterial indicators in aquatic environments (Winfield and Groisman, 2003) because of grazing by protozoa and loss of culturability as they undergo environmental stresses (Barcina et al., 1997; Burkhardt et al., 2000; Colwell and Grimes, 2000; Rozen and Belkin, 2001; Menon et al., 2003). *Cryptosporidium* sp. or *Giardia* sp. (oo)cysts thus are not necessarily correlated with levels of bacterial indicators in river water or in mussels (Hanninnen et al., 2005; Lemarchand and Lebaron, 2003; Gomez-Couso et al., 2005).

Since 1995, the Seine estuary (as defined by Fairbridge, 1980) has been extensively studied within the framework of the "Seine-Aval" multidisciplinary scientific program (see special issue of *Estuaries* (24), 2001; http://seine-aval.crihan.fr). Beyond routine microbiological analysis by the SNS (Service de Navigation de la Seine), a model of the population dynamics of faecal coliform (Garcia-Armisen et al., 2006) and an assessment of microbiological risk associated with recreational uses are being developed (Paul et al., 1995; Lipp et al., 2001; Payment and Pintar, 2006). In Seine estuary water, faecal contamination mainly is dominated by the largest WWTP in Europe, which serves 6.5 million inhabitants and is located 120 km upstream of the upstream limit of the estuary (George et al., 2001). During periods of low flow, however, tributaries to the Seine estuary and effluents of smaller WWTPs located along the estuary are the main sources of faecal pollution (Garcia-Armisen et al., 2005). *Salmonella* frequently have been isolated in Seine estuary water and it has been reported that the three serovars most frequently found were *Salmonella* Typhimurium, *Salmonella* Infantis and *Salmonella* Virchow, which were among the principal serovars isolated in human and animal diagnostic laboratories in France during the same period (1995–2002; Touron et al., 2005).

Here we report the results of a study undertaken to assess the faecal contamination of Seine estuary water, to identify the major intra-estuarine faecal sources, and to investigate the relations between the occurrence of *Salmonella*, protozoan parasites (oo)cysts, and levels of four faecal indicators. A geographical distribution of microbiological risk along the estuary was established on the basis of 9 years of data collected by the SNS public survey.

2. Materials and methods

2.1. Study site

The Seine estuary is a macrotidal estuary open to the English Channel and located in the northern part of France (Table 1). The Poses dam at kp 202 (kp is a kilometric unit increasing from upstream to downstream, and set to kp 0 at Pont Marie in central Paris) marks the upstream limit of tidal propagation and thus of the estuary (Fig. 1). In the upper part of the estuary, sedimentation and resuspension of particles occur at 3-h intervals as a result of the influence of the dynamic tide (Guézennec et al., 1999). In the urbanized part of the estuary watershed (kp 243 to kp 260), there is a WWTP serving 550,000 inhabitants (Rouen WWTP, kp 247) that discharges treated effluent into the estuary. The freshwaters of the estuary extend down to Caudebec (kp 310.5) and there is a continuous salinity gradient extending from 15 km upstream of Honfleur (kp 355.8) to Caudebec (Table 1). The mouth of the

Table 1
Characteristics of sampling sites in the Seine estuary

	Station	kp (km)	Number of samples		Salinity	SPM (mg L^{-1})
			SNS[a]	SA[b]	Min–max	Min–max
Upstream input	Poses	202	45	13	0	3.5–86.1
Urban area	Rouen	243	45	8	0	3.7–67.2
	Le Croisset	246.6	45	10	0	0.6–112.9
	Docks	251.3	45	2	0	4.9–87.2
	La Bouille	260	45	11	0	0.8–405.0
Limit of saline intrusion	Caudebec	310.5	45	5	0	1.4–769.4
Mouth of the estuary	Tancarville	337	45	14	0.5–25	32–3 419.2
	Berville	346	n.a.	7	0.5–25	28.0–3 293.5
	Honfleur	355.8	45	13	0.5–25	101.1–3 419.2

n.a., not analysed; SPM, suspended particulate matter.
[a] 1997–2005: ThC, *E. coli* (since 2000), enterococci and *Salmonella* (culturable).
[b] 2001–2005: ThC, enterococci, *C. perfringens* spores and *Salmonella* (nmPCR); 2003–2005: *Giardia* sp. cysts and *Cryptosporidium* sp. oocysts.

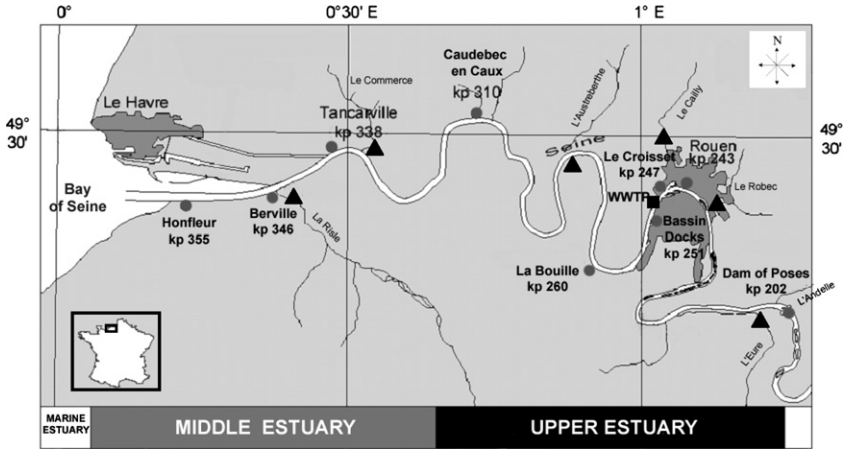

Fig. 1. Map of the Seine estuary (Guézennec et al., 1999) and its seven tributaries: the Risle, Commerce, Austreberthe, Cailly, Robec, Eure, and Andelle rivers. Stations sampled in the Seine estuary are indicated by grey dots, and those sampled in the tributaries by dark triangles. The wastewater treatment plant (WWTP) is indicated by a black square.

estuary (from kp 337 to kp 355.8) is characterized by a zone of high turbidity with suspended particulate concentrations as high 10 g L^{-1}, which results from the mixing of freshwater and seawater (the turbidity maximum); the location of this zone varies as a function of tidal dynamics and river flow rate (Guézennec et al., 1999; Le Hir et al., 2001).

2.2. Sampling

The Seine estuary was sampled at nine stations along an upstream/downstream transect of 156 km over and 9-year period. One sampling station is located at the Poses dam (kp 202) at the upstream limit of the estuary; four stations are located in the highly urbanised area (Rouen, kp 243; Le Croisset, kp 246.6; Bassin des Docks, kp 251.3; La Bouille, kp 260); one station is located at Caudebec (kp 310.5), corresponding to the upstream limit of saline intrusion; and three stations are located along the salinity gradient at the estuary mouth (Tancarville, kp 337; Berville, kp 346; Honfleur, kp 355.8).

The microbiological quality of Seine river water (in terms of the occurrence of thermotolerant coliforms [ThC], enterococci, culturable *Salmonella*) was monitored at the nine stations by the SNS 45 times during 1997–2005 (SNS dataset; Table 1), with the exception of *E. coli*, which was enumerated only from 2000 to 2005. In addition, at the same stations, spores of sulphite-reducing anaerobes were enumerated and *Salmonella* was monitored by PCR during 14 of the 45 sampling events (2001–2005) (SA dataset; Table 1). During eight of these (2003–2005), cysts of *Giardia* sp., and oocysts of *Cryptosporidium* sp. were also analyzed.

In April 2002, March 2003, July 2003, February 2004, and March and July 2005, samples were collected from the seven major tributaries of the Seine estuary at stations located as close as possible to the confluence of the tributaries with the Seine estuary but not under the influence of the tide (Table 2). Wastewater samples were collected at the outlet of the treatment plant of Rouen (kp 247). The samples represent integrated daily mean compositions of the wastewater produced. All samples were kept at 4 °C until the microbiological analyses were carried out, which occurred within 8 h.

Table 2
Characteristics of the tributaries of the Seine estuary

Tributary	kp equivalent of the outlets locations (km)	Number of samples	Watersheds surface (km^2)	Flow rate[a] ($m^3\ s^{-1}$) min–max
Andelle	204	4	757	4.12–10.30
Eure	218	4	6032	15.20–36.00
Robec	242	7	152	0.21–0.41
Cailly	246	7	246	1.63–4.30
Austreberthe	278	5	214	1.40–2.50
Commerce	335	5	176	0.62–1.20
Risle	344	6	2315	6.60–17.50

[a] Flow rates corresponding to sampling events were collected by the DIREN (Regional agency for the environment).

2.3. Microbial analysis

2.3.1. Enumeration of faecal-indicator bacteria

ThC and enterococci were enumerated using membrane filtration methods (0.45 μm, HA047, Millipore, Bedford, MA, USA), following standard international methods ISO 9308-1 and ISO 7899-2 (SA and SNS data), respectively. From 2000 to 2004, the enterococci and *E. coli* were enumerated by microplate methods NF ISO 7899-1 and NF ISO 9308-3 (SNS data), respectively. Spores of sulphite-reducing anaerobes were enumerated on TSN agar (AES Laboratoire, Combourg, France) following ISO 6461 standard (SA data).

2.3.2. Salmonella

Salmonella were isolated from 100 mL of filtered water after selective enrichment steps and growth on selective media (ASAP and XLT4, AES Laboratoire, Combourg,

France), following the standard ISO 6340 (since 2000, SNS data). Confirmation of suspected *Salmonella* was carried out by PCR analysis (SA data) or by biochemical tests using the standardized API 20E system (Biomérieux) (SNS data).

DNA extraction from water samples and molecular detection of *Salmonella* was carried out by nested multiplex PCR amplification of a specific 892 bp fragment of the *fliC* gene coding for a phase-one flagellin, as described by Touron et al. (2005).

To compare inputs of *Salmonella* from the different sources of faecal contamination, *Salmonella* fluxes were calculated, taking into account minimum and maximum flow rates and assuming that a positive sample contained at least one culturable unit per 100 mL.

2.3.3. Pathogenic protozoa

For the detection of *Giardia* cysts and *Cryptosporidium* oocysts, 20 L of water were filtered using an Envirocheck cartridge filter (Pall Gelman, Saint Germain en Laye, France). Capsules were eluted and (oo)cysts were extracted from the eluate by immuno-magnetic separation (Dynabeads GC Combo, Dynal, Compiègne, France). The (oo)cysts were labelled by FITC conjugated monoclonal antibodies (Aqua-Glo G/C, Waterborne, Inc., New Orleans, LA, USA), identified, and counted by epifluorescence microscopy following the French standard (AFNOR NF T 90-455) and/or fluoro-flow cytometry as previously described (Vesey et al., 1994; Delaunay et al., 2000).

2.4. Data analysis

All statistical analyses were performed using SAS/STAT® (version 8, SAS Institute Inc., Cary, NC, 1999) and XL-STAT (version 7.5.2, Addinsoft). The water quality of the Seine River was assessed by applying the European directive (2006/7/EC concerning the management of water quality for bathing) for coastal waters and transitional waters. A hierarchical ascendant clustering analysis with average linkage in Euclidean distance was used to classify the sampling stations into similar groups (Everitt, 1993; Gordon, 1996). One-way ANOVA was performed on the groups of stations segregated in the clusters (low/high flow rates) to evaluate the existence of significant spatial differences. A post hoc Tukey–Kramer test then was performed to determine for which groups of aggregated stations these inter-group differences were significant. The data was evaluated to determine if it followed a normal distribution, and a logarithmic transformation was performed if required. A Pearson's chi-square test was used to investigate if *Salmonella* occurrence was associated with geographical distribution. The non-parametric Wilcoxon test was used to investigate the existence of a relation between levels of bacterial faecal indicators and occurrence of *Salmonella*, and the Spearman test was used to investigate relations between levels of faecal indicators and parasite protozoan (oo)cysts for each defined section (upstream estuary, urban area, limit of saline intrusion and mouth of the estuary).

3. Results and discussion

3.1. Spatial distribution of faecal-indicator bacteria in Seine estuary water

The highest levels of faecal indicators were observed along the urbanized section of the estuary (kp 243 to kp 260) (Table 3). The water quality objective was set such

Table 3
Levels of faecal-bacterial indicators in the Seine estuary water

	Station	kp (km)	Thermotolerant coliforms		Enterococci[b]		*Escherichia coli*[c]		*Clostridium perfringens* spores[d]	
			Min–max[a]	Geom. mean	Min–max[a]	Geom. mean (90-percentile)[e]	Min–max[a]	Geom. mean (90-percentile)[e]	Min–max[a]	Geom. mean
Upstream input	Poses	202	30–150,000	1399	<10–46,000	301 (4655)	34–54,130	1051 (10,066)	<20–1450	309
Urban area	Rouen	243	130–43,000	1918	36–6217	528 (2914)	292–23,690	2200 (8292)	<20–1500	296
	Croisset	246.6	930–93,000	3750	57–9300	645 (2649)	350–26,700	2409 (8677)	<20–5500	280
	Docks	251.3	230–43,000	2001	36–4300	465 (1921)	160–16,620	2185 (9806)	n.a.	n.a.
	Bouille	260	30–93,000	2083	15–15,000	414 (2620)	78–65,470	2075 (9046)	<20–5000	338
Limit of saline intrusion	Caudebec	310.5	27–23,000	582	<10–110,000	172 (1048)	12–13,290	400 (2409)	<20–300	54
Mouth of the estuary	Tancarville	337	10–25,000	548	10–2300	115 (596)	30–2472	183 (870)	<20–4500	572
	Berville	346	<10–8000	463	23–600	86 (n.a.)	n.a.	n.a.	<20–20,000	667
	Honfleur	355.8	10–25,425	849	<10–11,454	161 (1132)	36–4300	278 (1235)	<20–10,000	677

Geom. mean: geometric mean; n.a.: not analysed.

[a] Levels are expressed as cfu 100 mL^{-1} or as spores 100 mL^{-1}.

[b] Data for the enterococci take into account both methods (classical and MPN miniaturized).

[c] *E. coli*: data collected from 2000 to 2004 (SNS dataset).

[d] *C. perfringens*: spores detected during the sampling period from 2001 to 2005 (SA dataset).

[e] Based upon a 90-percentile evaluation according to the European directive (2006/7/EC).

Table 4
Inputs of faecal-bacterial indicators by tributaries and WWTP to Seine estuary water

Lateral input	kp equivalent of the outlets locations (km)	Thermotolerant coliforms		Enterococci[b]		*Clostridium perfringens* spores
		Min–max[a]	Geom. mean	Min–max[a]	Geom. mean	Min–max[a]
Andelle	204	7.15×10^{7}–2.05×10^{8}	1.05×10^{8}	1.13×10^{7}–2.06×10^{7}	1.54×10^{7}	n.c.
Eure	218	3.6×10^{6}–5.95×10^{8}	5.13×10^{7}	3.96×10^{6}–1.19×10^{8}	9.57×10^{6}	n.c.–2.31×10^{8}
Robec	242	2.25×10^{8}–1.21×10^{10}	8.48×10^{8}	8×10^{7}–8.77×10^{8}	1.64×10^{8}	n.c.–2.24×10^{7}
Cailly	246	7.34×10^{7}–5.55×10^{8}	1.36×10^{8}	1.26×10^{7}–1.18×10^{9}	7.30×10^{7}	n.c.–1.63×10^{7}
Austreberthe	278	3.63×10^{7}–2.91×10^{8}	7.17×10^{7}	2.78×10^{6}–3.17×10^{7}	1.17×10^{7}	n.c.–8.05×10^{6}
Commerce	335	2.16×10^{7}–2.35×10^{8}	5.83×10^{7}	3.11×10^{6}–3.19×10^{7}	9.09×10^{6}	n.c.–2.61×10^{7}
Risle	344	2.49×10^{7}–1.26×10^{10}	1.03×10^{9}	1.93×10^{6}–1.23×10^{8}	1.72×10^{7}	n.c.–8.15×10^{7}
WWTP	247	2.66×10^{8}–2.32×10^{9}	5.30×10^{8}	3.15×10^{7}–2.04×10^{8}	9.66×10^{7}	n.c.–2.32×10^{8}

SA dataset (2001–2005); n.c., not calculated, numeration was below the detection threshold (see Section 2).
[a] Flow rates of bacteria (cfu s^{-1}) or spores of *C. perfringens* (spores s^{-1}).
[b] Data for the enterococci take in account both methods (membrane filtration and microplate methods).

that the *E. coli* and enterococci 90-percentile level should not exceed 500 cfu 100 mL^{-1} and 185 cfu 100 mL^{-1} (''sufficient'' category), respectively, on the basis of the recent European directive for bathing waters (coastal and transitional waters, 2006/7/EC). The 90th percentile values for *E. coli* and enterococci enumerations (Table 3) were worse than the ''sufficient'' values set out in the European directive and indicated that the quality of water should be classified as ''poor''. The microbial quality of this urbanized zone was mainly influenced by the Robec (kp: 242) and Cailly (kp: 246) tributaries, and the treated effluents from the outfall of a WWTP at kp 247 (Table 4). Because of the action of the tide, the zone upstream from the WWTP is systematically contaminated: water (including suspended sediment and bacteria) moves upstream during flood tide and moves downstream during ebb tide. The input from the Robec did not appear to be related to land use within its watershed, but might be related to effluent from a hospital with a 1250-bed capacity located along the tributary. The high number of antibiotic-resistant *E. coli* strains isolated from the Robec tributary supports this hypothesis (data not shown).

Levels of ThC and enterococci were lower upstream from the Poses dam (kp: 202) than in the urbanized area. However, on occasion levels of faecal-indicator bacteria were higher upstream from the Poses dam than those observed in the urbanized area, and 90th percentiles for *E. coli* and enterococci exceeded the acceptable values of the European directive for bathing waters (Table 3). High levels of faecal bacteria mainly were associated with input from the upstream part of the Seine River under high flow conditions ($\geqslant 500\ m^3\ s^{-1}$), similar to results found in a study of the source of ThC in Seine estuary water (Garcia-Armisen et al., 2005). The Eure River, which has substantial agricultural activity in its watershed, might be an important source of faecal bacterial in this area (Table 4).

Minimal levels of all bacterial indicators were observed at the mouth of the estuary (kp 337 to kp 355.8). The 90th percentile for *E. coli* and enterococci levels in the samples from this section of the estuary still exceeded the values deemed acceptable by the European directive (Table 3). The saline environment along the downstream part of the estuary does not favour the survival of faecal bacteria, particularly ThC and *E. coli* (Davies et al., 1995; Pires Coelho et al., 1999; Menon et al., 2003; Winfield and Groisman, 2003). However, the input of the Risle tributary must be considered as a potential point source of faecal bacteria (Table 4). Unlike the other faecal-bacterial indicators, the number of spores of sulphite-reducing anaerobes increased along the upstream/downstream transect, with maximum values observed at the mouth of the estuary. This is most likely caused by an accumulation of these spores, which seem to have the same hydrodynamic behaviour as particles (Pettibone et al., 1996; Obiri-Danso and Jones, 1999). These spore-forming bacteria can survive for long periods in this type of aquatic environment, mainly because of the greater resistance of the spores to osmotic, light, and temperature stresses (Davies et al., 1995; Burkhardt et al., 2000). They have been reported to occur in the sediment of intertidal mudflats at the mouth of the estuary, and when detected they were consistently in the top 30 cm of sediment. The spores thus might be resuspended periodically into the water column, as has been reported for similar aquatic environments (Deloffre et al., 2005; Desmarais et al., 2002; Obiri-Danso and Jones, 1999).

On the basis of the microbiological results from 2000 to 2005 and comparison to the recent European quality directive for bathing waters in coastal and transitional waters (2006/7/EC), levels of enterococci and *E. coli* in the three sections of the Seine investigated indicate that the water throughout the Seine estuary should be classified as being of poor quality.

3.2. Spatial distribution of bacteriological contamination of Seine estuary water

Using cluster analysis based on an ascendant hierarchy method, individual stations of the estuary were grouped together on the basis of the fluxes of ThC in surface

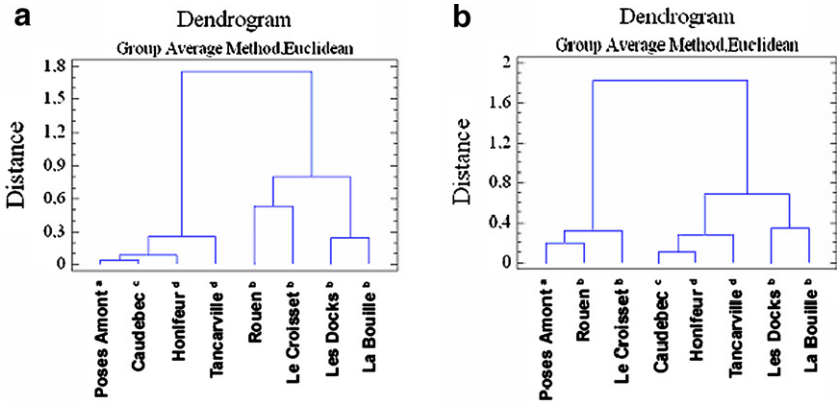

Fig. 2. Distribution of the faecal contamination in the Seine estuary. Cluster analysis of the estuarine sites on the basis of the fluxes of ThC. (a) low flow conditions ($\leqslant 300\ m^3\ s^{-1}$); (b) high flow conditions ($\geqslant 500\ m^3\ s^{-1}$). SNS dataset: 1997–2005: (a) upstream zone; (b) urban area; (c) limit of saline intrusion; (d) mouth of the estuary.

estuarine waters (SNS dataset, Fig. 2). To estimate the influence of the flow rate of the Seine River, the cluster analysis was performed taking into account the discharge values at the upstream limit of the estuary (at the station at the Poses dam). For low flow rates ($\leqslant 300\ m^3\ s^{-1}$), two distinct groups were identified (Fig. 2a): one cluster that corresponds to higher faecal contamination and thus a greater microbiological risk (Harwood et al., 2005), and which includes stations located in the urban area of Rouen (kp 243–260), and a second cluster that corresponds to lower faecal contamination, and which includes stations located at the upstream limit of the estuary (kp 202), Caudebec (kp 310.5), and along the salinity gradient (Tancarville kp 337 to Honfleur kp 355.8). The distribution of the significant clusters observed in cluster analysis was confirmed by the post hoc Tukey–Kramer test ($p < 0.05$) which indicates a spatial significant differences on the groups of stations segregated in the clusters according to the low flow rates. At high flow rates ($\geqslant 500\ m^3\ s^{-1}$), a similar pattern was observed, but the station at the upstream limit of the estuary (the Poses dam) was included in the cluster corresponding to the highest faecal contamination (Fig. 2b). These results indicate the predominance of input from upstream during periods of high flow, whereas it has been shown that the ThC input from the tributaries and the intra-estuarine WWTPs predominates during periods of low flow (Garcia-Armisen et al., 2005).

3.3. Occurrence of *Salmonella* and relation to faecal-bacterial indicators in the Seine estuary

From 2000 to 2005, *Salmonella* was isolated frequently from Seine estuary water and the presence of culturable *Salmonella* in water from the eight stations was significantly different ($p < 0.05$). Occurrence of *Salmonella* was highest in the urban area and the upstream part of the estuary (Table 5). Detection of *Salmonella* by molecular methods during collection of the SA samples indicated chronic contamination of the urban area by these enteric bacteria. At the furthermost upstream station of the estuary, the propor-

Table 5
Occurrence of *Salmonella* and pathogenic protozoan in Seine estuary water

	Station	kp (km)	*Salmonella* (n/N[a])		*Giardia* sp.[b]	*Cryptosporidium* sp.[b]
			Culturable	nmPCR	Geom. mean (n/N[a])	Geom. mean (n/N[a])
Upstream input	Poses	202	5/13	7/13	2.5 (4/8)	5.9 (6/8)
Urban area	Rouen	243	6/8	7/8	2.7 (2/4)	12 (4/4)
	Le Croisset	246.6	6/10	8/10	2.4 (2/4)	7.4 (4/4)
	Docks	251.3	2/2	2/2	1*[c] (1)	1*[c] (1)
	La Bouille	260	10/12	12/12	3.0 (3/5)	3.5 (3/5)
Limit of saline intrusion	Caudebec	310.5	4/5	5/5	2.6 (3/4)	5 (3/4)
Mouth of estuary	Tancarville	337	7/14	8/14	1.3 (1/6)	2.7 (3/6)
	Berville	346	3/7	4/7	2.1 (2/4)	4.5 (4/4)
	Honfleur	355.8	6/13	9/13	2.5 (4/5)	9.7 (6/6)

n.a., not analysed.
1*: zero parasites detected but the value of 1 was added to each result to allow calculation of the geometric means.
[a] *n*, number of positive samples. *N*, number of analysed samples.
[b] Protozoa are given as geometric means in cysts and oocysts per 10 L (SA dataset, 2003–2005).
[c] Only a single value for protozoa.

Table 6
Contamination by culturable *Salmonella* of the water at the estuary stations under different flow rate conditions (SNS dataset)

	Upstream input	Urban area				Limit of saline intrusion	Mouth of estuary	
	Poses (kp 202)	Rouen (kp 243)	Le Croisset (kp 246.6)	Docks (kp 251.3)	La Bouille (kp 260)	Caudebec (kp 310.5)	Tancarville (kp 337)	Honfleur (kp 355.8)
Number of positive samples during high flow conditions n/N (%)	16/18 (88.9)	10/15 (66.6)	12/19 (63.1)	15/19 (78.9)	11/17 (64.7)	13/18 (72.2)	8/16 (50)	6/15 (40%)
Number of positive samples during low flow conditions n/N (%)	1/13 (7.69)	4/11 (36.3)	8/11 (72.7)	8/11 (72.7)	4/11 (36.3)	3/13 (23)	5/13 (38.4)	1/10 (10%)

SNS dataset; high flow conditions $\geqslant 500\ m^3\ s^{-1}$, low flow conditions $\leqslant 300\ m^3\ s^{-1}$; *n*, number of isolates; *N*, number of total samples.

Table 7
Pathogens occurrence in the lateral inputs (SA dataset)

Lateral input	kp equivalent of the outlets locations (km)	*Salmonella* (n/N[a])		*Giardia* sp.[b]	*Cryptosporidium* sp.[b]
		Culturable	nmPCR	Geom. mean (n/N)	Geom. mean (n/N)
Andelle	204	2/4	2/4	1.4 (1/4)	2.3 (2/4)
Eure	218	2/4	3/4	2.5 (3/4)	2.4 (2/4)
Robec	242	6/7	7/7	11 (2/3)	6.0 (3/3)
Cailly	246	3/7	4/7	3.5 (3/4)	2.0 (2/4)
Austreberthe	278	1/5	1/5	2.3 (2/4)	1.3 (1/4)
Commerce	335	1/5	3/5	1.6 (2/4)	2.0 (2/4)
Risle	344	4/6	4/6	1.8 (2/4)	1.7 (2/4)
WWTP	247	3/4	4/4	37.5[c] (1)	6.25[c] (1)

[a] *n*, number of positive samples; *N*, number of total samples.
[b] Parasites are given as geometric means in cysts and oocysts per 10 L (SA dataset, 2003–2005).
[c] Only a single value for protozoa.

tion of water samples containing culturable *Salmonella* was ten times higher when flow rates were high than when they were low (Table 6), indicating the strong influence of the upstream input on the microbial quality of the Seine estuary. In contrast, during periods of low flow, *Salmonella* occurrence was more frequent in the urban area (pk 243–260) than during periods of high flow, particularly at those sites close to the confluence with the highly contaminated Robec River (pk 242). The tributaries most frequently contaminated were the Eure, Risle, and Robec Rivers, and the discharge from the Rouen WWTP (Table 7) was also frequently contaminated. The estimated minimum fluxes were 3×10^6–5×10^6 *Salmonella* per second at the upstream part of the estuary (Poses dam, kp: 202), and the tributaries contributed 2×10^3–3×10^5 *Salmonella* per second. These results were consistent with the cluster analysis of the fluxes of faecal indicators in the Seine estuary, and indicate that the occurrence of *Salmonella* in the urbanized zone reflects local contamination by the discharge of treated effluent from the intra-estuarine WWTP and the Robec River.

Two significant relations ($p < 0.05$) between the levels of faecal-contamination indicators and the presence of culturable *Salmonella* were identified by the non-parametric Wilcoxon test analysis: one at the upstream part of the estuary at Poses, between *Salmonella* and ThC, *E. coli*, and enterococci; and one at the mouth of the estuary at Honfleur, between *Salmonella* and enterococci detected by a culture-based method. This suggests that in the mixed zone of the Seine estuary, which is greatly influenced by salinity, the levels of enterococci are a more reliable indicator of faecal contamination. Unlike at the upstream area and at the mouth of the estuary, no significant relationship between levels of faecal bacterial indicators and *Salmonella* was observed in the urbanized area.

The results of any microbiological analysis are influenced by the complexity of the aquatic environments. The hydrodynamic parameters of the estuary, in particular flow rate, salinity gradient, and tidal cycles, might explain the different relations between faecal-bacterial indicators and pathogens (Mill et al., 2006). In similar aquatic environments, a relation between *Salmonella* and faecal bacterial-indicators was observed only rarely, and occurrence of *Salmonella* mainly was influenced by environmental parameters such as rainfall, salinity, and temperature (Dionisio et al., 2000a,b; Lemarchand and Lebaron, 2003; Martinez-Urtaza et al., 2004). A positive relation between levels of total coliforms, ThC, and enterococci and the presence of *Salmonella* were observed in coastal waters in Spain (Polo et al., 1998, 1999). In coastal and estuarine environments, enterococci have been suggested to be better indicators of faecal pollution and thus microbial risk, because of their better survival in saline waters (Dionisio et al., 2000a; Kamizoulis and Saliba, 2004; Noble et al., 2003; Polo et al., 1998; Prüss, 1998).

3.4. Occurrence of protozoan (oo)cysts and relation to faecal-bacterial indicators

Investigation of the occurrence of the parasites *Giardia* sp. and *Cryptosporidium* sp. were carried out in the Seine estuary and its tributaries from 2003 to 2005 (Tables 5 and 7). At the upstream part of the Seine estuary, *Giardia* sp. were detected in four of eight water samples, with concentrations ranging from 2.4 to 27 cysts $10\,L^{-1}$ and *Cryptosporidium* sp. in six of eight samples with concentrations ranging from 3 to 24 oocysts $10\,L^{-1}$. In this area, significant relations ($p < 0.05$) were observed between *Giardia* sp. and ThC and between *Giardia* sp. and enterococci. These results might be explained by the human faecal origin of *Giardia* sp. In Seine estuary water, the highest levels of cysts were observed in urban areas, where concentrations of *Giardia* sp. ranged from 2 to 23 cysts $10\,L^{-1}$ (7/14 positive) and *Cryptosporidium* sp. from 1.7 to 34 oocysts $10\,L^{-1}$ (11/14 positive). The input of both *Giardia* and *Cryptosporidium* (oo)cysts from the Robec River was substantial, as was that from Rouen WWTP treated effluent (Table 7). However, in this area the only significant relation was observed between *Giardia* sp. and enterococci. In the mouth of estuary, with concentrations of *Giardia* sp. and *Cryptosporidium* sp. ranging from 1.9 to 5 cysts $10\,L^{-1}$ (7/15 positive samples) and from 1.7 to 15.10 L^{-1} oocysts (13/16 positive samples), respectively, significant relations were observed between oocysts and *Clostridium perfringens* spores and between oocysts and enterococci. High levels of oocysts were observed in this area, probably resulting from animal sources transported to the estuary by surface runoff, rather than from humans via WWTP effluents. These results might be explained by the selective filtration of pathogens through soils and the resistance of oocysts and *Clostridium* spores compared to that of faecal indicators, particularly in saline environments (Medema et al., 1997; Rose et al., 2002; Kistemann et al., 2002; Tyrrel and Quinton, 2003). In this study, the high levels of oocysts observed in the high turbidity zone might result from the association of oocysts with suspended particles accumulating at the mouth of the estuary (Searcy et al., 2005). Thus *C. perfringens* spores might be a conservative indicator for *Cryptosporidium* for assessing quality of water, as suggested by Payment et al. (2000).

Our results indicate a general contamination by protozoan (oo)cysts in the waters of the Seine estuary. Levels of (oo)cysts of *Giardia* sp. and *Cryptosporidium* sp. in the Seine estuary and its tributaries, except for the Robec River, are in the same range as those reported in surface water from Canada, the USA, and Europe (Gomez-Couso et al., 2005; Lemarchand and Lebaron, 2003; Payment et al., 2000; Smith and Rose, 1998). (Oo)cysts in the water from the tributaries most likely have a livestock origin resulting from the intensive farming activity in these watersheds, with the exception of the Robec and Cailly rivers, which are in urbanized areas.

4. Conclusion

This study indicates that the microbiological contamination of the estuary waters is the result of contributions from upstream inputs, WWTPs, tributaries, and several non-point pollution sources. However, this study did not take into account the contribution of combined sewer overflows or intense runoff events during high precipitation periods, which might cause substantial local inputs of faecal bacteria. In Seine estuary water, faecal contamination is at levels that might present a public health risk associated with recreational activities (boating and occasional swimming) throughout the river system. Of additional concern is the potential effect of the contamination observed at the mouth of the Seine estuary, including the presence of *Cryptosporidium*, on shellfish beds located in the Bay of Seine. When particle-associated microorganisms reach the estuary mouth they merge into the turbidity maximum, which during high river flows is expelled in the northern channel (Le Hir et al., 2001). A similar problem has been described for similar environments (Gomez-Couso et al., 2005), but has not been investigated here.

This study was carried out to complement a modelling approach being developed as part of the Seine-Aval program to predict the presence of pathogens. This model takes into account the levels of faecal indicators (Garcia-Armisen et al., 2006) and is designed to help local managers assess the microbiological risks of Seine estuary water and determine priorities for sanitation management. On the basis of our results, "higher microbiological risk" and "lower microbiological risk" areas can be defined using the fluxes of indicator organisms and taking into account the occurrence of *Salmonella* and *Giardia* sp. and *Cryptosporidium* sp. contamination. Depending on the zone of the estuary, it might be possible to characterize those factors determining the microbial quality of the water: in the upstream part of the estuary, the most contamination by faecal bacteria and *Salmonella* occurs during periods of high flow; in the urbanised area, high faecal contamination is related to WWTP discharges and a highly contaminated tributary; and at the estuary mouth the accumulation of microorganisms attached to particles in the maximum turbidity zone, particularly *C. perfringens* spores and oocysts of *Cryptosporidium* , is combined with inputs of ThC, *E. coli* from tributaries.

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