Investigating an avian source of shellfish faecal contamination in the Thau lagoon (the Mediterranean, France)

Derolez Valérie [1], Serais Ophélie [1], Caprais Marie-Paule [2], Le Saux Jean-Claude [2], Messiaen Grégory [1]

[1] Ifremer, Laboratoire Environnement Ressources, BP171 - 34203 Sète cedex, France.

[2] Ifremer, Département Environnement, Microbiologie et Phycotoxines, BP 70 - 29280 Plouzané, France.

Microbes regularly contaminate Thau lagoon shellfish. Between 1997 and 2007, 18% of the analyses carried out in dry weather recorded more than 230 *Escherichia coli* / 100 g of bivalves (Ifremer French microbial monitoring network), leading the administration to restrict the shellfish harvest area, including suspension of production. This study aims to determine, the contribution of seabirds, which roost at night on the Thau lagoon shellfish growing frames, to shellfish faecal pollution in dry weather.

Data were collected in 2008 from both the drainage area (pollution input) and the lagoon (water quality and microbial monitoring, seabird counts) in dry weather. The data showed that, with no watershed input or lagoon resurgence, microbial contaminants were present in shellfish under oyster farming structures used by seabirds, particularly at night, as roosting areas. Shellfish contamination levels were higher under roosting areas than under control structures. Initial results of F+ specific RNA bacteriophage genotyping in shellfish samples suggested contamination of animal origin.

Keywords: Escherichia coli, avian source, shellfish, faecal contamination, Thau lagoon, seabird, bacteriophage, microbial source tracking, dry weather.

Introduction

With up to 13 000 tons of oysters and 2 500 tons of mussels marketed every year, the Thau lagoon is the main Mediterranean shellfish harvesting area. Due to frequent faecal pollution, it is a "class B" area, according to European sanitary regulations (Anon., 2004); shellfish thus have to be depurated before they are marketed. Between 1997 and 2007, 18% of the analyses carried out during dry weather showed more than 230 Escherichia coli / 100 g of bivalves (Ifremer French microbial monitoring network), leading the

administration to introduce restrictive measures in the contaminated shellfish harvesting areas, including temporarily suspending production.

An inventory of Escherichia coli sources, compiled in 2007 for the Thau drainage area, showed that private wastewater treatment facilities, avian populations and industries may constitute the main pollution sources in dry weather.

Many studies have shown the effect of seabirds on the microbial degradation of water quality (Edge et al., 2007; McLellan et al., 2003; Levesque et al., 2000; Benton et al., 1983). This study aims to clarify the contribution of seabirds roosting at night on the Thau lagoon shellfish growing frames to shellfish faecal pollution in dry weather.

1. Materials and methods

Thau is a 75 km² lagoon in the South of France, with a drainage area of 230 km², connected to the Mediterranean Sea via two outlets (Figure 1). In three shellfish farming zones (Bouzigues, Mèze and Marseillan), oysters and mussels are fixed to ropes of between 3 and 10 m in length, suspended under breeding structures made of wood or metal. The Thau lagoon and its drainage area were monitored in dry weather over four periods in 2008. The Marseillan zone was monitored on three occasions (referred to as periods): 1) 14th and 15th of March; 2) 27th and 28th of March and 3) 30th and 31st of July. The zone of Bouzigues was monitored for the last period: 4) 26th and 27th of August.

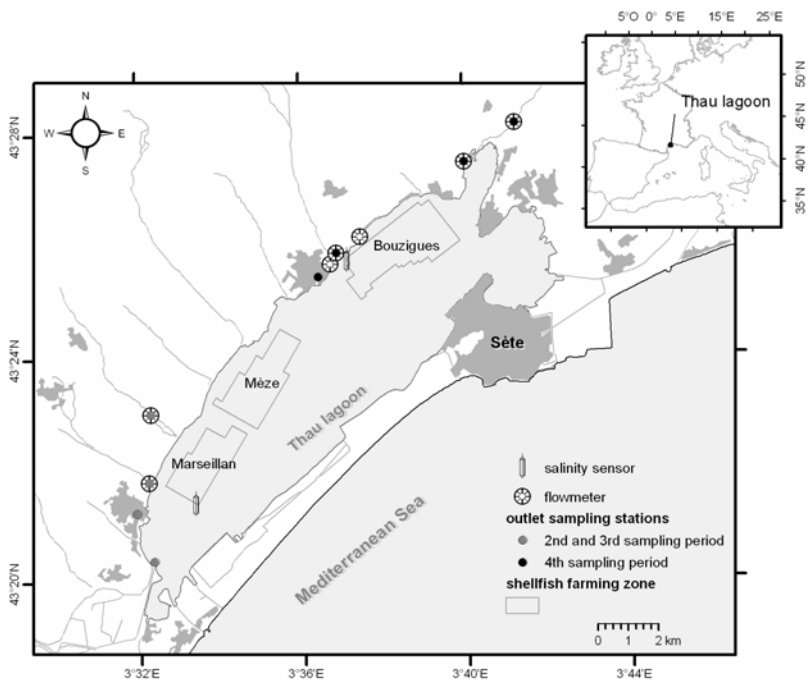

Figure 1. Thau lagoon and its three shellfish farming zones: Bouzigues, Mèze and Marseillan. Location of salinity sensors, flowmeters and water sampling sites on the main drainage area outlets.

1.1. Bird counts

At dusk, seabirds roosting on shellfish structures in areas of interest were counted by two teams of three people, circulating in boats between the shellfish farming structures. Species were identified with the naked eye, or in some cases with the help of binoculars. The shellfish structures were observed 2 hours before to 30 min after sunset for the purpose of counting and identification.

1.2. Sampling

Five rain gauges, located in the drainage area, collected rainfall data. The seven main tributaries were equipped with flowmeters to monitor possible drainage area input (Figure 1). Salinity of the lagoon was monitored at the surface and the bottom of the water column by two high-frequency sensors (TPS35 NKE-MICREL) in the zones of Marseillan and Bouzigues.

For periods 2, 3 and 4: in the afternoon, before the bird counts, samples of water were collected at the outlets of the main potential faecal sources (Figure 1). Water and oyster (*Crassostrea gigas*) samples were collected under the shellfish structures, using a sampling strategy with 12, 15 and 11 initial surface sampling stations respectively (-1 m below the surface). For 1, 2 and 3 respectively of these sampling stations, samples were also collected at depth (+1 m off the bottom). Samples were collected at depth targeted shellfish structures where large groups of seabirds were observed.

For each of the four periods, the day following the bird counts, water and oyster samples were collected at dawn under shellfish breeding structures. These were defined as “roosting structures” if more than 30 seabirds were counted in an area of radius 100 m from the sampling location, or “control structures” if less than 30 birds were counted in an equivalent area around the sampling location. Additional samples were collected from the initial sampling stations for periods 2, 3 and 4. The total number of samples collected at the surface during the four periods under the “roosting structures” was 34 for both water and oysters, with 7, 6, 12 and 9 samples respectively for each period. For 12 of these stations, samples were also collected at depth and targeted shellfish structures where large groups of seabirds were observed (1, 2, 3 and 6 samples respectively). Under the “control structures”, both water and shellfish were collected only at the surface. A total of 35 samples, with 7, 10, 11 and 7 samples were collected respectively for the four sampling periods.

In addition, fresh seabird droppings were collected from the Thau drainage area and lagoon from February to August 2008, using sterile swabs. A total of 14 samples were collected, 13 from yellow legged-gulls (*Larus michahellis*), and 1 from great cormorant (*Phalacrocorax carbo*).

All samples were kept in cool boxes for transportation to the laboratory. Water samples from the drainage area and the lagoon were collected in 500 ml sterile bottles and kept at temperatures between 1 and 4 °C. For shellfish samples, 12 oysters were collected in plastic bags for microbial analyses and kept at temperatures between 2 and 15 °C. Samples of droppings were kept at temperatures between 1 and 4 °C. They were frozen prior to analysis.

1.3. Microbial analyses

Levels of *Escherichia coli* (*E. coli*) were analysed in all the water and shellfish samples collected during the sampling periods. Water samples were analysed using the NF EN ISO 9308-3 standard method, *i.e*. the Most Probable Number (MPN), scaled down for inoculation into liquid culture medium (Afnor, 1999). Samples of oysters were analysed using the NF V08-106 standard method (Afnor, 2002). This is an indirect method of estimating *Escherichia coli* in live bivalves using a biosensor to measure impedance. For each sample, about six oysters were washed, scrubbed under clean running water and opened with a sterile shucking knife. Approximately 100 g of flesh and shell liquor diluted 1:3 with tryptone salt water SW were homogenised in a Waring blender. The samples were diluted, inoculated into selective media and incubated at 44 °C in a Bac Trac 4300 (Sy-Lab, Neupurkersdorf, Austria).

F-specific RNA bacteriophage (FRNAPH) analyses were carried out at various times on shellfish collected during periods 1 and 2, and on seabird dropping samples (Caprais *et al*., 2009) to identify whether contamination was of human or animal origin. FRNAPH counts were performed using the double agar layer method, ISO NF 10705-1 (ISO, 1995). FRNAPH genotyping was performed by transferring lysis plaques onto positively charged nylon membranes for 30 min at 4 °C (Schaper and Jofre, 2000). Nucleic acids on the membranes were denatured by neutralised washings and fixed with UV light. Genotyping was performed when the number of bacteriophages isolated exceeded 20 plaque forming units (PFU) / 100 g of bivalves (Gourmelon *et al*., 2007). Hybridisation of nucleic acids was performed with nucleic acid probes from genogroups I to IV (Beekwilder *et al*., 1996).

1.4. Data analyses

To analyse the effect of seabirds on water and shellfish quality, XLSTAT 2008.7.02 software was used, to perform nonparametric statistical analyses using the Mann-Whitney U-test for unpaired samples and the Wilcoxon signed rank test. A p value of <0.05 was considered to be significant.

2. Results

During the four sampling periods, rainfall was low (<12 mm on the four previous days), *Escherichia coli* input from the drainage area was negligible (<10^{11} *E. coli*.day^{-1}), and no lagoon water freshening was detected; contamination by freshwater input could thus be excluded.

At dusk, large single species groups of seabirds were observed on the shellfish-rearing structures. During the four sampling periods, a total of 600 to 1100 yellow-legged gulls (*Larus michahellis*), 330 to 2200 common black-headed gulls (*Larus ridibundus*) and Mediterranean gulls (*Larus melanocephalus*), 60 terns (*Sterna sandvicensis*) and 150 great cormorants (*Phalacrocorax carbo*) were roosting in the Marseillan area, and about 4 400 yellow-legged gulls and 1000 common black-headed gulls in the Bouzigues area (Fig 2).

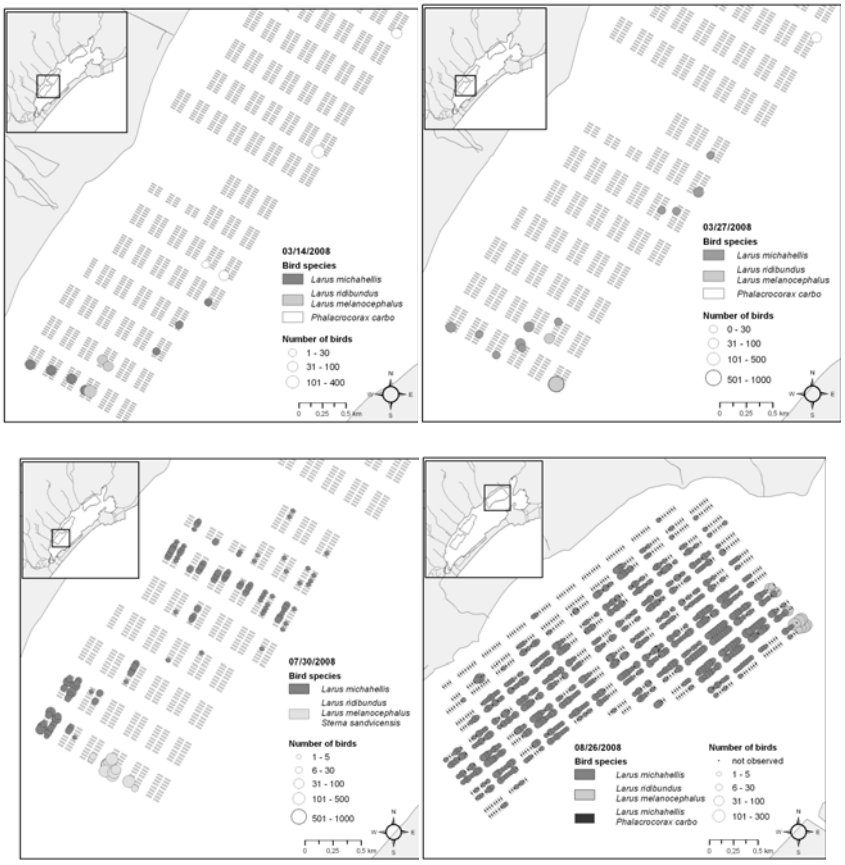

Figure 2. Location of flocks of birds in the Marseillan zone: (nights of) top left: 14 - 15 March (period 1), top right: 27 - 28 March (period 2), bottom left: 30 - 31 July 2008 (period 3); and in the zone of Bouzigues (bottom right) night of 26 - 27 August 2008 (period 4).

Regarding sampling periods 2, 3 and 4, the numbers of *E. coli* in lagoon shellfish (N=36) and water (N=38) samples collected at the surface from the roosting and control

structures at the same sampling stations were significantly higher after nocturnal bird resting than before the arrival of the seabirds (Wilcoxon signed rank test, $p = 0.012$).

The results of microbial analyses performed on oysters collected under roosting structures at dawn, both from the surface and at depth, did not reveal any difference in levels of *E. coli* (Wilcoxon signed rank test: $p = 0.944$, N=13), for the four sampling periods combined. There was also no difference between the water samples collected from the surface and at depth ($p = 0.389$, N=13).

The combined results for the four periods showed that the levels of *E. coli* in shellfish samples collected at dawn, from the surface under roosting structures (N=33) were significantly higher than in those collected under control structures (N=35) (Mann-Whitney test, $p = 0.024$) (

Figure *3*A). However, there was no significant difference between *E. coli* levels in water samples collected from the surface under roosting structures (N=36) and under control structures (N=35, $p = 0.903$).

Regarding periods 1 and 2, the levels of F-specific RNA bacteriophage in shellfish samples collected at dawn from the surface under roosting structures (N=11) were significantly higher than in those collected under control structures (N=18) (Mann-Withney test, $p = 0.001$) (

Figure *3*B). FRNAPH genotyping was performed on 21/43 shellfish samples, collected at the surface, at dawn on 15th and 28th of March. Group I was most frequently detected of the four genogroups (56 to 100%), indicating faecal contamination of animal origin (Hsu *et al.*, 1995 ; Noble *et al.*, 2003 ; Long *et al.*, 2005). Only 3 of 14 seabird dropping samples contained enough FRNAPH isolates to allow genotyping. Human-specific genogroup II was found in two samples and genogroup I was the only one found in the third sample.

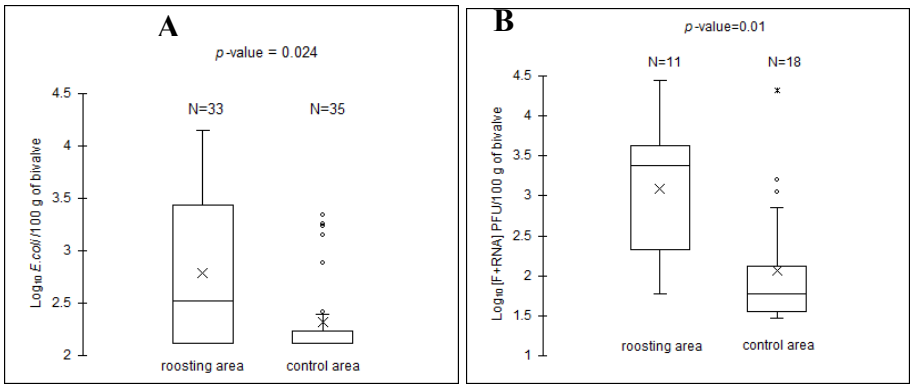

Figure 3. Escherichia coli *(A) and F+ specific RNA bacteriophage counts (B) in shellfish samples collected at dawn from surface, under roosting and control areas. Results in (A) are from all four periods and in (B) from the first two. Numbers of samples (N) and p-values calculated using the Mann-Whitney test are indicated.*

3. Discussion

At dusk, we observed large flocks of seabirds on the Thau lagoon shellfish growing structures. They rested overnight until dawn. To our knowledge, there is no information in the literature on daily loads of faecal coliforms (FC) from terns, great cormorants, mediterranean gulls and yellow-legged gulls. However, Gould and Fletcher (1978) studied the daily loads for black-headed gulls and herring gulls, whose weight is similar to that of yellow-legged gulls. They found values of 3×10^8 and 18×10^8 FC.day^{-1} respectively, similar to loads of 19×10^8 FC.day^{-1} for humans determined by Geldreich (1966). The nocturnal faecal coliform load to which shellfish were directly exposed during the sampling periods was assessed using Gould and Fletcher (1978) data together with results of numbers of birds counted and their presence overnight on the shellfish structures for between 9.5 and 12.5 hours, depending on the sampling period. This load was estimated at between 1.6×10^{11} and 7.9×10^{11} FC per night, which is high for a pollution source with direct access to the shellfish farming area. It is also higher than the level of drainage area input.

In dry weather and with no drainage area input, the roosting of seabirds on the shellfish growing structures significantly increased the level of faecal contamination in shellfish samples, with concentrations exceeding 4 600 *E. coli* / 100 g of bivalves in several

samples. There was no difference in the level of *E. coli* contamination between water samples collected at the surface or at depth. The same was true for shellfish samples collected at the surface or at depth.

Results from F-specific RNA phage genotyping indicated a dominance of the typical genogroup of animal origin, but they were not conclusive. Other Microbiological Source Tracking methods, such as *Catellicoccus marimammalium* analysis in seabird faeces and shellfish are needed to confirm the avian origin (Lu *et al*., 2008).

Seabirds are known to host and transmit human pathogens such as *Salmonella* spp. (Duarte *et al*., 2002; Palmgren *et al*., 2006) or *Campylobacter* spp. (Levesque *et al*., 2000; Dobbin *et al*., 2005). Monthly bird counts are required to estimate the exposure of shellfish to bird inputs in Thau lagoon, to assess the health risks related to shellfish consumption by humans. Tests for pathogenic microorganisms in both bird faeces and shellfish must be carried out to assess health hazards. This information should provide support for a reduction in the restrictions on the Thau lagoon shellfish harvesting areas.

Conclusion

The results from four periods of sampling carried out in the Thau lagoon in dry weather showed that high faecal contamination levels in shellfish were reached, with no significant drainage area input. At dusk, large populations of seabirds were observed on shellfish structures. *Escherichia coli* and F+ specific RNA bacteriophage levels were significantly higher under roosting structures than under the control structures.

Initial results of FRNAPH genotyping in shellfish suggested contamination of animal origin. However, the notion that an avian source contributes to shellfish contamination during dry weather needs confirmation using Microbial Source Tracking methods in seabird droppings and shellfish.

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