Influence of environmental and seasonal factors on microbial contamination levels in clam production areas in southern Tunisia: *Escherichia coli*, *Salmonella* spp., hepatitis A virus and norovirus

This paper (No. 05122018-00142-FR) is a translation of the original French article, which was peer-reviewed, accepted, edited, and corrected by authors before being translated. It has not yet been formatted for printing. It will be published in December 2018 in issue **37** (3) of the *Scientific and Technical Review*.

S. Zormati [(1, 2)], I. Kallel [(2)], H. Sellami [(2, 3)] & R. Gdoura [(2)*]

(1) Centre Régional de Recherches Vétérinaires de Sfax, Route aéroport Km 1,5, B.P. 3000, Sfax, Tunisia

(2) Laboratoire de Recherche de Toxicologie – Microbiologie Environnementale et Santé (LR17ES06), Faculté des Sciences de Sfax, Université de Sfax, B.P. 1171, 3000 Sfax, Tunisia

(3) Laboratoire de Traitement et de Valorisation des Rejets Hydriques (LTVRH), Centre de Recherches et des Technologies des Eaux (CERTE), Université de Carthage, Route touristique de Soliman, B.P. 273-8020, Nabeul, Tunisia

*Corresponding author: gdoura.radhouane@gmail.com

Summary

To study the influence of environmental and biological factors on levels of contamination by *Escherichia coli*, *Salmonella* spp., hepatitis A virus (HAV) and norovirus in clam production areas, an epidemiological study was conducted on 791 samples of live clams (*Ruditapes decussatus*), 539 of which were sent for bacteriological analysis and 252 for detection of norovirus and HAV. These samples were collected in different production areas in the Sfax region of southern Tunisia over four consecutive years, from March 2013 to December 2016. The prevalence of positive samples was 36% for *E. coli*, 11% for *Salmonella*

spp., 19% for norovirus and 3% for HAV. There was a significant correlation between contamination by *E. coli* and by *Salmonella* spp., as well as between contamination by noroviruses and by HAV and between contamination by noroviruses and by *Salmonella* spp. Temperature, the presence of migratory birds and tourism are the main factors affecting microbial contamination levels in bivalve molluscs.

Keywords

Bivalve mollusc – Contamination – Environmental factor – *Escherichia coli* – Hepatitis A virus – Norovirus – *Salmonella* spp.

Introduction

Molluscs filter large quantities of water in order to extract nutrient particles. But pathogenic microorganisms, toxic substances and toxic phytoplankton in the water can also become concentrated in these mollusc organisms and transmitted to consumers. When ingested, either raw or lightly cooked, these shellfish can cause foodborne diseases, the symptoms of which are generally digestive in nature. A study carried out in the United States of America on several surveillance systems showed that 58% of food-related diseases are attributable to viruses, in particular human norovirus and hepatitis A virus (HAV) (1). The latter are commonly associated with cases of gastroenteritis that are linked to consumption of this type of food (2). Noroviruses are the most frequently identified cause of acute, non-bacterial gastroenteritis in all age categories worldwide. Diarrhoeal diseases are one of the main causes of mortality in children under the age of five and there are almost 1.4 billion cases each year, and 1.6 to 2.5 million deaths, largely in developing countries (3, 4, 5). In France, cases of hepatitis A are rare. Contaminated food is involved in 17% of cases, with shellfish suspected in half of them (6). Each year in the United States of America, noroviruses cause an average of 19 million to 21 million cases of gastroenteritis. This results in 56,000 to 71,000 hospitalisations and 570 to 800 deaths, mainly in young children and the elderly (7).

A number of epidemiological studies highlight the role of bivalve molluscs in epidemic episodes of viral hepatitis A. The most severe

epidemic occurred in 1988 in Shanghai (People's Republic of China) with 300,000 cases linked to the consumption of shellfish collected from a contaminated site (8). In Tunisia, hepatitis A remains a common infection, mostly in children. In 2008, Rezig *et al*. (9) reported a very high level of HAV endemicity in Tunisia, as 84% of children had already been infected by the virus within the first five years of life.

Salmonellosis, caused by enterobacteria of the genus *Salmonella*, is one of the most common and widespread foodborne diseases. It has been estimated that several tens of millions of human cases are recorded each year and the disease results in more than 100,000 deaths (10). Shellfish, consumed raw or lightly cooked, can cause outbreaks of food poisoning the symptoms of which are generally digestive in nature (11). Of 520 outbreaks of food poisoning, 51 (9.8%) involved seafood products, of which 13 (2.5%) involved shellfish. Moreover, experts believe that these official statistics represent only 1% to 10% of actual cases of salmonellosis (12, 13).

In 2010, the pathogenic agents responsible for the majority of food-related deaths were *Salmonella* Typhi (52,000 deaths), enteropathogenic *Escherichia coli* (37,000 deaths) and noroviruses (35,000 deaths) (14).

This makes an analytical study of environmental factors critical for assessing the source of contamination of bivalve molluscs by these agents and its impact on consumers.

European regulations stipulate that, to be allowed on the market, live bivalve molluscs cannot contain any *Salmonella* spp., and that levels of *E. coli* contamination must be fairly low (< 230 colony forming units [CFU]/100g of flesh and intervalvular liquid [FIL]).

In Tunisia, the shellfish sector plays a major role in the country's economy. It provides unskilled jobs and, most important of all, it is a significant source of foreign currency, because the majority of Tunisian bivalve mollusc production is exported to Europe.

Collecting clams (*Ruditapes decussatus*) is one of the main fishery and aquaculture activities in Tunisia. The average annual output is in the order of 564 tonnes (15, 16, 17). Local consumption of clams in Tunisia is very limited and more than 99% of production is destined for export to European Union countries (primarily Italy and Spain). However, the sector is as fragile as it is promising, and affected by many factors, the most important of which are disease risks. Because clams feed through a water filtration system, like all bivalve molluscs, they concentrate particles and microorganisms (18). In addition, they play a very important role in pathogen transmission, as people often consume these molluscs raw (18).

This article presents the first environmental study carried out in the Sfax region of southern Tunisia, during which microbiological contamination risk factors for clams were examined over four consecutive years. The objectives of the study were:

– to determine the levels of contamination by *E. coli*, *Salmonella* spp., the hepatitis A virus and noroviruses in clams collected from different sites

– to study the link between environmental, biological and meteorological factors and levels of contamination of clams by *E. coli*, *Salmonella* spp., the hepatitis A virus and noroviruses in production areas.

Materials and methods

Description of the survey

The environmental survey was conducted in collaboration with the following partners:

– The Microbiological Surveillance Network (*Réseau de Surveillance Microbiologique*: REMI), which checks the microbiological quality of live bivalve molluscs every two weeks. The analyses, carried out by the Veterinary Research Institute in Sfax (*Institut de Recherche Vétérinaire de Sfax*: IRVT Sfax), look for *E. coli* and *Salmonella* spp. in the flesh

and intervalvular liquid of shellfish, using the ISO 16649-3:2015 (19) and ISO 6579-1:2017 (20) standards, respectively

– The Virological Surveillance Network (*Réseau de Surveillance Virologique*: REVI), which checks the microbiological quality of live bivalve molluscs every two weeks. The analyses, carried out by the National Institute of Marine Sciences and Technologies, which is located in La Goulette (the port in Tunis, the capital city of Tunisia), detect ribonucleic acids (RNA) of noroviruses in genogroups I and II (GI and GII) and HAV using the real-time reverse transcription polymerase chain reaction (RT-PCR) technique, in accordance with ISO 15216-1:2017 (21).

In total, 791 samples of clams (*R. decussatus*) were collected from different production sites in the Sfax region over a period of four years, from March 2013 to December 2016. Of these, 539 samples were used to search for *E. coli* and *Salmonella* spp., while the remaining 252 were used to detect the RNA of the hepatitis A virus and noroviruses.

A questionnaire was drawn up for all samples with microbial and/or viral contamination (*E. coli* > 230 CFU/100 g FIL, presence of *Salmonella* spp., detection of norovirus RNA and/or HAV). This questionnaire examined the factors associated with microbial contamination during the period in which the shellfish were harvested. Firstly, it was necessary to determine the harvest date of the samples, in order to identify the season. Next, the factors observed during sample collection, such as the temperature, the presence of migratory birds and whether it was the tourist or sheep-shearing season, were identified. Finally, the proximity of shellfish production sites to locations where sea water mixes with waste water and water channelled from wadis (valleys, ravines or channels that are dry except during the rainy season) was examined. These parameters were used to calculate the average positivity, depending on the period, using Student's t-test.

The survey also exploited the positive results (bacterial and/or viral contamination) for each period by calculating the correlation factor between contamination by these different micro-organisms using Pearson's correlation test.

Identification and quantification method for microbial contaminants

The ISO 16649-3:2015 method (19) is the reference technique for the enumeration of *E. coli*. This is a horizontal method for identifying and enumerating *E. coli* β-glucuronidase by culture in a liquid medium (glutamate) and calculating the most probable number (MPN) after incubation at 37°C ± 1°C, then at 44°C ± 1°C on tryptone bile X-glucuronide (TBX) agar.

The ISO 6579-1:2017 method (20) is the reference technique for the identification of salmonella. This horizontal method is used to detect the majority of *Salmonella* serovars. It requires a pre-enrichment phase using buffered peptone water and enrichment on two specific liquid mediums: Rappaport-Vassiliadis Soya (RVS) broth and Müller-Kauffmann Tetrathionate-Novobiocin (MKTTN) broth, followed by isolation on two solid mediums: Xylose-Lysine-Deoxycholate (XLD) agar and Brilliant Green (BG) agar. Where colonies are suspected, they must be identified and confirmed by biochemical and serological analysis.

The samples were analysed as follows: 170 samples were analysed at IRVT Sfax and 230 samples were sent for analysis to a bacteriological analysis laboratory in Italy (Centro Ricerche Marine, Cesenatico).

Identification and quantification method for viral contaminants

The ISO 15216-1:2017 (21) standard describes a method for the quantification of HAV RNA and the norovirus genogroups GI and GII, found in the samples, and is used for testing food (soft fruits, vegetables, leaves, stems, bulbs, bottled water and live bivalve molluscs) and food preparation surfaces.

The method for bivalve molluscs is as follows: a 2-gram sample of the digestive gland is taken from live molluscs and cut into very small pieces. For virus extraction, the pieces are mixed with 10 µl of the process control material, constituted with the Mengo virus.

Immediately after this, 2 ml of a proteinase K solution (30 U/mg) is added. The mixture is incubated in a shaking incubator at 37°C for 60 minutes at 320 rpm, then the mixture is incubated at 60°C for 15 minutes. The resulting solution is centrifuged at 3,000 *g* for 5 minutes, then 500 µl of supernatant containing the viruses is treated to extract and concentrate the viral RNA. Two millilitres of the viral capsid lysis solution composed of guanidine thiocyanate is then added. Following incubation for 10 minutes at ambient temperature and the washing and centrifugation phases, the viral RNA is recovered by selective adsorption on silicon. Each target HAV and the norovirus genogroups GI and GII are detected and quantified using quantitative RT-PCR (qRT-PCR). This kinetic quantification and analysis is based on the real-time detection of a fluorescent signal of intensity proportional to the quantity of the PCR product generated during amplification.

Statistical studies

In order to exploit the results of the survey, a statistical analysis was performed using the SPSS 20.0 software (SPSS, Chicago, Illinois, United States of America). An average-to-average comparison was carried out using Student's t-test. The correlations between values were analysed using the Pearson correlation coefficient. *P*-values lower than 0.05 were considered to be statistically significant.

Results

Influence of environmental, biological and seasonal factors on contamination levels in clam production areas

Contamination by *Escherichia coli*

Out of the 539 clam samples analysed, 194 (36%) had levels of *E. coli* contamination that were above the regulatory limit (> 230 CFU/100 g FIL) (Table I).

Monitoring of *E. coli* contamination over time showed that contamination rose significantly between the summer and winter periods (Fig. 1). The survey found a contamination level of 21% to 23%

in the months of September and October, a period marked by the presence of migratory birds.

A correlation was also observed between 6% of cases of *E. coli* contamination and the location of clam production sites near areas where seawater mixes with water from wadis or waste water, as well as between 2% of the contamination cases recorded during the spring and when wool is washed on the seashore near production areas.

Contamination by *Salmonella* spp.

In total, 59 samples of *R. decussatus* out of the 539 analysed were contaminated with *Salmonella* spp., or around 11% (Table I). The study showed that there was a significant spike of 31% in cases of *Salmonella* contamination in the spring (Fig. 1), which is a period marked by variations in temperature and wind that are conducive to the development of *Salmonella*. A total of 28 of the 59 cases (47.4%) had high levels of *E. coli* and *Salmonella* spp. contamination during two periods of the year: from March to May and from September to November (Fig. 1).

Contamination by noroviruses

The survey showed that 19% of the *R. decussatus* samples (48/252) were contaminated by noroviruses (Table I). Examination of the seasonal pattern of contamination in bivalve molluscs by noroviruses revealed higher levels of contamination during the months of March and April. A lower percentage was observed between the months of August and October (Fig. 1). Typing of the 48 noroviruses detected in the current study found a predominance of genogroup GI compared with genogroup GII (78% *vs* 21.8%).

The results showed that 58.3% (28/48) of samples that were positive for norovirus were also contaminated by *Salmonella* spp. and/or *E. coli*.

Contamination by hepatitis A virus

An HAV contamination level of 3% was found in clams in the samples examined (Table I). The study showed that the pattern of HAV

contamination in bivalve molluscs depends on the season, with a spike in March and another in July (Fig. 1).

Correlations between clam contamination by the agents under study and biotic and abiotic factors

Correlations between levels of clam contamination by the different enteropathogenic microbes under study

The Pearson test was used to analyse the correlation between clam contamination by *E. coli*, *Salmonella* spp., noroviruses and HAV respectively (Table II).

Statistical analyses found a highly significant correlation between contamination by *E. coli* and by *Salmonella* spp. ($p < 0.001$), showing a positive association with a correlation factor of 0.216. A highly significant correlation was also observed between clam contamination by *Salmonella* spp. and by noroviruses ($p < 0.001$). This positive association had a correlation factor of 0.269 (Table II).

A significant association was found between clam contamination by noroviruses and by HAV, with a *p*-value of 0.031 and a correlation coefficient of 0.135.

However, contamination by *E. coli*, by noroviruses and by HAV, showed an inverse correlation, with correlation factors of -0.045 and -0.074, respectively. This correlation is not statistically significant, with *p*-values of 0.481 for noroviruses and 0.245 for HAV.

An analysis of the linear correlation between contamination by HAV and by *E. coli* gave a correlation coefficient *r* of -0.02, i.e. too close to zero, indicating no link between the two pathogenic agents.

No significant correlation was found between the presence of *Salmonella* spp. and HAV ($p = 0.401$), with a correlation coefficient of -0.053 (Table II).

Seasonal variations

Student's t-test was used to analyse variations in levels of clam contamination by *E. coli*, *Salmonella* spp., noroviruses and HAV depending on variations in temperature, represented by four periods of the year: January to March (period 1), April to May (period 2), June to August (period 3) and September to December (period 4).

These analyses found no significant difference between the microbial load of *E. coli* ($p = 0.098$), *Salmonella* spp. ($p = 0.383$), noroviruses ($p = 0.822$) and HAV ($p = 0.495$) between periods 1 and 2 (Table III).

A significant difference was found for *E. coli* ($p = 0.002$) between periods 1 and 3, with a greater load during period 3 (Table III). Conversely, during the same periods, no significant difference was observed for *Salmonella* spp., noroviruses and HAV.

Significant differences in the bacterial load were found between periods 1 and 4 for *E. coli* and *Salmonella* spp., with *p*-values of 0.004 and 0.012 respectively. The bacterial load was greater during period 4.

No difference in the bacterial and viral load was observed between periods 2 and 3.

Contamination of clams by *E. coli* was significantly higher during period 4 compared with period 2 ($p = 0.030$) (Table III). There was no significant difference for the other microorganisms between these two periods.

The average positivity for periods 3 and 4 was significantly higher during period 4 for *E. coli* and *Salmonella* spp., with *p*-values of 0.018 and 0.009 respectively.

No significant difference in the average positivity for the viral load was recorded between the four periods of the year.

Discussion

Bacterial and viral contamination of clams

Contamination by *Escherichia coli* and *Salmonella* spp.

There were several environmental factors conducive to the contamination of clams by *E. coli* and *Salmonella* spp. The first of these factors was the season and, more specifically, the presence of migratory birds (pink flamingos) from September to October. Just like poultry, these birds play host to enterobacteria in their intestines. In 1979, Baylet and Rollin (22) found salmonella in the intestines of pink flamingos and *E. coli*, a classic indicator of faecal pollution of water, in the digestive flora of seagulls in the Camargue (France), which occupy virtually the same ecological niche as pink flamingos (22). These birds gather by the seashore to feed, resulting in their faeces contaminating both the water and marine food products. According to the European Union, the risk of faecal contamination from human and animal sources in bivalve molluscs is determined by the concentration of *E. coli* in samples taken from production areas (23). Birds living in these ecosystems can carry several zoonotic pathogens (viruses, bacteria, parasites, fungi) (24).

There is also a link between faecal contamination levels and the location of sites where seawater mixes with water from wadis or waste water. A Moroccan study in 2014 (25) demonstrated that high reported contamination levels could be explained by the proximity of production sites to the Moulouya wadi, which drains all the contaminants from farming activities throughout the catchment area (poultry farming, beef and sheep farming, etc.), as well as urban waste from inland towns. This could be caused by a malfunctioning waste water treatment plant or sewer system, or by the disposal of inadequately treated water (26).

E. coli contamination was linked to tourist activity during the summer season. A study conducted on a network of coastal lagoons at Ria Formosa (a protected site in Portugal) found high levels of shellfish contamination by *E. coli* following a rise in the tourist population (27).

Levels of shellfish contamination by salmonella coincide with a high level of *E. coli* contamination during two periods of the year (from March to May and from September to November), accounting for 47% of such contamination cases (Fig. 1). In 1997, Monfort *et al*. (28) reported a highly significant link ($p < 0.001$) between the frequency of salmonella isolation and high levels of *E. coli* faecal contamination test bacteria.

Contamination by noroviruses

Low levels of clam contamination by noroviruses in the present study (19%) could be due to the difficulty of detecting these RNA viruses, and the fact that the effectiveness of the RT-PCR method depends on two factors: the effectiveness of nucleic acid extraction and its purity (29). A Tunisian study conducted using the real-time RT-PCR method on bivalve molluscs in the Bizerte lagoon (northern Tunisia) found higher levels of norovirus contamination in shellfish of around 45% (30).

Two norovirus genogroups (GI and GII) were detected in bivalve molluscs. A Tunisian study established a correlation between norovirus strains found in both waste water and shellfish and those found in the stools of patients (31). This study suggests a link between water contamination and infantile diarrhoea (31). The two main genogroups affecting humans are GI and GII (32).

The predominance of GI (78%) over GII (21.8%) in the present study is in line with the results of a study carried out in the Galician estuaries (north-western Spain) to detect norovirus GI and GII in clams. This study showed that genogroup GI was the most widespread (32.1%), followed by genogroup GII (25.6%) (33). In 2012, Flannery *et al*. (34) reported a high concentration of norovirus genogroup GI in waste water. This is consistent with a rise in cases of norovirus genogroup GI infection in the population during this period. However, contrary to the results in the present study, in 2010 Siebenga *et al*. (35) reported that, for several years, the norovirus strains detected belonged mainly to genogroup GII, which represents 70% to 80% of the strains identified in clinical cases (35).

The results of this study of seasonal variations in norovirus confirm those of Flannery *et al*. in 2012 (34). The latter study showed that noroviruses were present in waste water throughout the year, with a significant increase between the months of January and March (34). The study by Green *et al*. in 2001 also corroborates these results (36). They reported seasonal variations in infection, with a high frequency in winter and occasional epidemic spikes in spring and summer.

According to the French Directorate General for Food (DGAL) in 2013 (37), cross-contamination of samples by noroviruses and *E. coli* (the official indicator of microbial faecal contamination) and/or *Salmonella* spp. does not always correlate with the presence of the virus in the environment. In fact, the shellfish found in the environment or in a disposal zone (on the foreshore – a sensitive zone because it is nearer waste water outlets) can concentrate the viruses disposed of in the water in less than an hour and the contamination can remain in the shellfish for eight to ten weeks.

Contamination by the hepatitis A virus

The low levels of HAV contamination in clams (3%) in this study could be due to the presence of inhibitory substances extracted and concentrated in the same way as the RNA viruses. This inhibitory effect is greater the smaller the number of copies of the viral genome in the sample (38). The presence of enzymatic reaction inhibitors can mask the presence of the virus, as reported by Gantzer *et al*. in 1998 (39).

The theory of seasonal HAV contamination in bivalve molluscs was confirmed by Schwab *et al*. in 1998 (40). They reported that bioaccumulation of the virus in shellfish varies according to the shellfish species and their physiological activity, and that the latter depends on the season and seawater temperature.

Correlations between contamination by bacteria and viruses and biotic and abiotic factors

Correlations between levels of clam contamination by the different enteropathogenic microbes

The study results concerning the correlation between contamination by *E. coli* and *Salmonella* spp. are similar to those reported by Monfort *et al*. in 1997 (28). The latter reported a highly significant link ($p < 0.001$) between the frequency of salmonella isolation and an abundance of *E. coli* faecal contamination test bacteria. In 2011, Amri *et al*. (41) also reported a correlation between contamination of clams by *E. coli* and by *Salmonella* spp.

This study found no correlation between the presence of *E. coli* and the presence of noroviruses or HAV. These results agree with those of Amri *et al*. in 2011 (41), who found that shellfish samples contaminated by HAV (31.48%) were negative for *Salmonella* spp. and for *E. coli*.

The use of coliforms as virological water quality indicators has been widely criticised and several studies have shown that there is no quantitative link between the concentration of coliforms and that of enteroviruses (42).

The Pearson test shows a significant link between contamination by noroviruses and the presence of HAV. The results of this study corroborate those of Croci *et al*. in 2007 (43), who reported contamination of marine molluscs and crustaceans by enteric viruses, HAV and noroviruses.

Seasonal variations

Student's t-test was used to study variations in the levels of contamination of clams by *E. coli*, *Salmonella* spp., noroviruses and HAV during four periods, determined according to variation in temperature, the bird migration period and tourist activity. The study of seasonal variations in clam contamination levels revealed that temperature, the presence of migratory birds and tourism are major factors in seawater and shellfish contamination by bacteria and viruses.

A uniform microbial load was found during periods 1 and 2. This could be because the factors conducive to contamination were the same in both periods.

The bacterial load for *E. coli* in clams was greater during period 3. This period covers the tourist season, with increased activity at the seaside and higher enterobacteria levels.

The high bacterial load (for both *E. coli* and *Salmonella* spp.) during period 4 could be attributed to two main factors: the presence of migratory birds (pink flamingos) at the seaside during this period of the year, as they carry pathogenic agents; and variation in temperature. In 2014, Ryu *et al*. (44) demonstrated that migratory birds are important reservoirs and carriers of pathogenic species, in particular enterobacteria. In 1995, Hubálek *et al*. (45) reported that the highest prevalence of *Salmonella* spp. in migratory birds was found during the breeding season and during overwintering of certain urban species, especially those that feed on waste. Temperature is one of the parameters used in models to explain the proliferation of bacteria (46). An experimental study on the development of this abundance of *E. coli* and other pathogenic bacteria of public-health interest, such as *Salmonella enterica* serotype Typhimurium and *Aeromonas hydrophila* in waste water and treated water, showed that temperature is one of the factors affecting variations in bacterial proliferation in aquatic environments (47). In 1972, McFeters and Stuart (48) noted an inverse relationship between the abundance of *E. coli* and a rise in temperature, especially between 5°C and 30°C. *E. coli* survive longer in seawater the lower the temperature (49).

The contamination level, which corresponded to the faecal contamination indicator (*E. coli*), was significantly higher during period 4 than during period 2 (Table III). This supports the theory that temperature affects *E. coli* contamination levels.

The high level of contamination by *Salmonella* spp. and *E. coli* observed during period 3 could be attributed both to high average temperatures during this period, conducive to the proliferation of these two species, and to significant tourist activity (Table III).

As for the viral load, this study found a significant difference in the average rate of positive samples between the four periods of the year. Tunisia enjoys long hours of sunshine throughout the year. Several studies have demonstrated the influence of solar radiation on virus survival rates. Viral inactivation occurs following a photochemical reaction, resulting in alteration of the nucleic acid or other non-nucleic receptors in viruses under the effects of ultraviolet (UV) radiation and light in the visible spectrum (50).

Conclusion

The study of correlations between contamination by different pathogens in clam production areas in the region of Sfax highlighted three highly significant types of correlation: between contamination by *E. coli* and by *Salmonella* spp. ($p < 0.001$); between contamination by noroviruses and by HAV ($p = 0.031$); and between contamination by noroviruses and by *Salmonella* spp. ($p < 0.001$).

A statistical analysis of the different data and correlations between clam contamination by *E. coli*, *Salmonella* spp., HAV and noroviruses on the one hand, and biotic and abiotic factors on the other, confirmed a direct link between the level of contamination in live bivalve molluscs and environmental and seasonal factors in particular.

References

1. Scallan E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M.A., Roy J.L., Jones S.L. & Griffin P.M. (2011). – Foodborne illness acquired in the United States: major pathogens. *Emerg. Infect. Dis.*, **17** (1), 7–15. doi:10.3201/eid1701.P11101.

2. Kosek M., Bern C. & Guerrant R.L. (2003). – The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull. WHO*, **81** (3), 197–204. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC2572419/ (accessed on 9 August 2018).

3. Parashar U.D., Hummelman E.G., Bresee J.S., Miller M.A. & Glass R.I. (2003). – Global illness and deaths caused by rotavirus

disease in children. *Emerg. Infect. Dis.*, **9** (5), 565–572. doi:10.3201/eid0905.020562.

4. Bryce J., Boschi-Pinto C., Shibuya K. & Black R.E. (2005). – WHO estimates of the causes of death in children. *Lancet*, **365** (9465), 1147–1152. doi:10.1016/S0140-6736(05)71877-8.

5. Patel M.M., Hall A.J., Vinjé J. & Parashar U.D. (2009). – Norovirus: a comprehensive review. *J. Clin. Virol.*, **44** (1), 1–8. doi:10.1016/j.jcv.2008.10.009.

6. Miossec L. (1997). – Études épidémiologiques sur l'importance des coquillages en tant que facteur d'exposition de l'hépatite A: approche méthodologique. Rapport interne. Direction de l'Environnement et de l'Aménagement Littoral, Ifremer, Nantes, France, 35 pp. Available at: http://archimer.ifremer.fr/doc/00105/21576/19156.pdf (accessed on 9 August 2018).

7. Hall A.J., Lopman B.A., Payne D.C., Patel M.M., Gastañaduy P.A., Vinjé J. & Parashar U.D. (2013). – Norovirus disease in the United States. *Emerg. Infect. Dis.*, **19** (8), 1198–1205. doi:10.3201/eid1908.130465.

8. Lees D. (2000). – Viruses and bivalve shellfish. *Int. J. Food Microbiol.*, **59** (1–2), 81–116. doi:10.1016/S0168-1605(00)00248-8.

9. Rezig D., Ouneissa R., Mhiri L., Mejri S., Haddad-Boubaker S., Ben Alaya N. & Triki H. (2008). – Séroprévalences des infections à hépatite A et E en Tunisie. *Pathol. Biol.*, **56** (3), 148–153. doi:10.1016/j.patbio.2007.09.026.

10. World Health Organization (WHO) (2018). – Typhoid. Press release. WHO, Geneva. Available at: www.who.int/news-room/fact-sheets/detail/typhoid (accessed on 9 August 2018).

11. Delmas G., Jourdan da Silva N., Pihier N., Weill F.-X., Vaillant V. & de Valk H. (2010). – Les toxi-infections alimentaires collectives en France entre 2006 et 2008. *Bull. Épidémiol. Hebdo.*, **31–**

32, 344–348. Available at: http://opac.invs.sante.fr/doc_num.php?explnum_id=265 (accessed on 9 August 2018).

12. Mossel D.A.A. (1989). – Infections et intoxications alimentaires. *In* Microbiologie: le tube digestif, l'eau et les aliments (H. Leclerc & D.A.A. Mossel, eds.). Quatrième partie, chapitre IV. Éditions Doin, Paris, 389–412.

13. Desenclos J.C., Bouvet P., Pierre V., Brisabois A., Frémy S., Lahelléc C., Grimont F. & Grimont P.A.D. (1996). – Épidémiologie des infections à *Salmonella*: tendances récentes en France et en Europe. *Bull. Soc. Fr. Microbiol.*, **11** (3), 209–215.

14. World Health Organization (WHO) (2015). – World Health Day 2015: from farm to plate, make food safe. WHO, Geneva. Available at: www.who.int/mediacentre/news/releases/2015/food-safety/en/ (accessed on 9 August 2018).

15. Direction Générale des Services Vétérinaires (Tunisia) (1995). – Arrêté du ministre de l'agriculture du 28 novembre 1995, fixant les règles sanitaires régissant la production et la mise sur le marché de mollusques bivalves vivants. *JORT*, **97**, 3 pp. Available at: http://extwprlegs1.fao.org/docs/pdf/tun6780.pdf (accessed on 14 August 2018).

16. Direction Générale des Services Vétérinaires (Tunisia) (2006). – Arrêté du ministre de l'agriculture et des ressources hydrauliques du 2 novembre 2006, modifiant et complétant l'arrêté du ministre de l'Agriculture du 28 novembre 1995 fixant les exigences auxquelles doivent satisfaire les zones de production des mollusques bivalves vivants. *JORT*, **90**, 3920–3921. Available at: www.cnudst.rnrt.tn/jortsrc/2006/2006f/jo0902006.pdf (accessed on 14 August 2018).

17. Direction Générale des Services Vétérinaires (Tunisia) (2009). – Arrêté du ministre de l'agriculture et des ressources hydrauliques du 3 février 2009, complétant l'arrêté du 28 novembre

1995, fixant les exigences auxquelles doivent satisfaire les zones de production des mollusques bivalves vivants. *JORT*, **13**, 512–513. Available at: www.cnudst.rnrt.tn/jortsrc/2009/2009f/jo0132009.pdf (accessed on 14 August 2018).

18. Lee R., Lovatelli A. & Ababouch L. (2010). – Bivalve depuration: fundamental and practical aspects. FAO Fisheries Technical Paper No. 511. Food and Agriculture Organization of the United Nations, Rome, Italy, 155 pp. Available at: www.fao.org/docrep/011/i0201e/i0201e00.htm (accessed on 9 August 2018).

19. International Organization for Standardization (ISO) (2015). – Microbiology of the food chain – horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli*. Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide-glucuronate. ISO, Geneva, Switzerland, 10 pp.

20. International Organization for Standardization (ISO) (2017). – ISO 6579-1:2017: Microbiology of the food chain – horizontal method for the detection, enumeration and serotyping of Salmonella. Part 1: Detection of *Salmonella* spp. ISO, Geneva, Switzerland, 50 pp.

21. International Organization for Standardization (ISO) (2017). – ISO 15216-1:2017: Microbiology of the food chain – horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR. Part 1: Method for quantification. ISO, Geneva, Switzerland, 49 pp.

22. Baylet R. & Rollin P.E. (1979). – *Edwardsiellae tarda* et sp., et *Salmonella typhimurium* chez des oiseaux en Camargue. *Bull. Soc. Pathol. Exot.*, **72** (5–6), 405–410.

23. Lee R.J. & Silk R. (2012). – Sources of variation of *Escherichia coli* concentrations in bivalve molluscs. *J. Water Health*, **11** (1), 78–83. doi:10.2166/wh.2012.114.

24. Janovy J. (1997). – Protozoa, helminths and arthropods of birds. *In* Host–parasite evolution: general principles and avian models (D.H. Clayton & J. Moore, eds). Oxford University Press, Oxford, United Kingdom, 303–337.

25. Belbachir C., Chafi A., Aouniti A. & Khamri M. (2014). – Qualité microbiologique de trois espèces de mollusques bivalves à intérêt commercial récoltées sur la côte méditerranéenne nord-est du Maroc. *J. Mater. Environ. Sci.*, **5** (1), 304–309. Available at: www.jmaterenvironsci.com/Document/vol5/vol5_N1/36-JMES-600a-2014-Chaouki.pdf (accessed on 9 August 2018).

26. Le Guyader F.S., Neill F.H., Dubois E., Bon F., Loisy F., Kohli E., Pommepuy M. & Atmar R.L. (2003). – A semi-quantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. *Int. J. Food Microbiol.*, **87** (1–2), 107–112. doi:10.1016/S0168-1605(03)00058-8.

27. Botelho M.J., Soares F., Matias D. & Vale C. (2015). – Nutrients and clam contamination by *Escherichia coli* in a meso-tidal coastal lagoon: seasonal variation in counter cycle to external sources. *Mar. Pollut. Bull.*, **96** (1–2), 188–196. doi:10.1016/j.marpolbul.2015.05.030.

28. Monfort P., Piclet G., Le Gal D., Raguénès P., Le Naour G., Boulben S. & Le Saux J.C. (1997). – Incidence de *Salmonella* spp. dans les bivalves issus de zones conchylicoles du Finistère (France). Colloque salmonelles et salmonelloses, Ploufragan, France, 20–22 May 1997, 431–433.

29. Loisy F. (2004). – Devenir des virus entériques humains en milieu marin: apport des VLPs (*virus like particles*) pour la purification des coquillages. PhD thesis, School of Pharmacy at Châtenay-Malabry, Paris-Sud University, France, 232 pp. Available at: http://archimer.ifremer.fr/doc/2004/these-327.pdf (accessed on 9 August 2018).

30. Essebai El Amri D. & Aouni M. (2005). – Recherche par

RT-PCR des virus entériques chez des moules (*Mytilus galloprovincialis*) et des palourdes (*Ruditapes decussatus*). *Bull. Inst. Natl Sci. Technol. Mer*, **32**, 93–98. Available at: www.oceandocs.org/handle/1834/3755 (accessed on 9 August 2018).

31. Sdiri-Loulizi K., Gharbi K.H., de Rougemont A., Chouchane S., Sakly N., Ambert-Balay K., Hassine M., Guédiche M.N., Aouni M. & Pothier P. (2008). – Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. *J. Clin. Microbiol.*, **46** (4), 1349–1355. doi:10.1128/JCM.02438-07.

32. Lindesmith L.C., Donaldson E.F., LoBue A.D., Cannon J.L., Zheng D.-P., Vinje J. & Baric R.S. (2008). – Mechanisms of GII.4 norovirus persistence in human populations. *PLoS Med.*, **5** (2), e31, 0269–0290. doi:10.1371/journal.pmed.0050031.

33. Polo D., Varela M.F. & Romalde J.L. (2015). – Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *Int. J. Food Microbiol.*, **193**, 43–50. doi:10.1016/j.ijfoodmicro.2014.10.007.

34. Flannery J., Keaveney S., Rajko-Nenow P., O'Flaherty V. & Doréa W. (2012). – Concentration of norovirus during wastewater treatment and its impact on oyster contamination. *Appl. Environ. Microbiol.*, **78** (9), 3400–3406. doi:10.1128/AEM.07569-11.

35. Siebenga J.J., Lemey P., Kosakovsky Pond S.L., Rambaut A., Vennema H. & Koopmans M. (2010). – Phylodynamic reconstruction reveals norovirus GII.4 epidemic expansions and their molecular determinants. *PLoS Pathog.*, **6** (5), e1000884. doi:10.1371/journal.ppat.1000884.

36. Green K.Y., Chanock R.M. & Kapikian A.Z. (2001). – Human caliciviruses. *In* Fields virology (B.N. Fields, D.M. Knipe, P.M. Howley & D.E. Griffin, eds), 4th Ed. Raven Press, New York, United States of America, 841–874.

37. Ministère de la Transition Écologique et Solidaire (France) (2013). – Note de service DGAL/SDSSA/N2013-8187 du 20 novembre

2013 relative à la contamination des zones de production de coquillages par les norovirusx protocole cadre de gestion. Available at: https://aida.ineris.fr/consultation_document/32006 (accessed on 9 August 2018).

38. Jaykus L.A., De Leon R. & Sobsey M.D. (1996). – A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization. *Appl. Environ. Microbiol.*, **62** (6), 2074–2080. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC167985/ (accessed on 9 August 2018).

39. Gantzer C., Dubois É., Crance J.-M., Billaudel S., Kopecka H., Schwartbrod L., Pommepuy M. & Le Guyader F. (1998). – Devenir des virus entériques en mer et influence des facteurs environnementaux. *Oceanol. Acta*, **21** (6), 983–992. doi:10.1016/S0399-1784(99)80020-6.

40. Schwab K.J., Neill F.H., Estes M.K., Metcalf T.G. & Atmar R.L. (1998). – Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR. *J. Food Protec.*, **61** (12), 1674–1680. doi:10.4315/0362-028X-61.12.1674.

41. Amri I., Hmaïed F., Loisy F., Lebeau B., Barkallah M., Saidi M. & Slim A. (2011). – Détection du virus de l'hépatite A dans les coquillages en Tunisie par *reverse transcription-nested PCR* – recherche de corrélation entre la contamination virale et bactérienne. *Pathol. Biol.*, **59** (4), 217–221. doi:10.1016/j.patbio.2009.10.009.

42. Wheeler D. (1990). – The pollution of beaches by viruses. House of Commons Environment Committee inquiry into the pollution of beaches. Appendices to the minutes of evidence, No. 11, 24–39. Government of the United Kingdom, London, United Kingdom.

43. Croci L., Losio M.N., Suffredini E., Pavoni E., Di Pasquale S., Fallacara F. & Arcangeli G. (2007). – Assessment of human enteric viruses in shellfish from the northern Adriatic Sea. *Int.*

J. Food Microbiol., **114** (2), 252–257. doi:10.1016/j.ijfoodmicro.2006.09.015.

44. Ryu H., Grond K., Verheijen B., Elk M., Buehler D.M. & Santo Domingo J.W. (2014). – Intestinal microbiota and species diversity of *Campylobacter* and *Helicobacter* spp. in migrating shorebirds in Delaware Bay. *Appl. Environ. Microbiol.*, **80** (6), 1838–1847. doi:10.1128/AEM.03793-13.

45. Hubálek Z., Sixl W., Mikuláskova M., Sixl-Voigt B., Thiel W. & Halouzka J. (1995). – *Salmonellae* in gulls and other free-living birds in the Czech Republic. *Centr. Eur. J. Public Hlth*, **3** (1), 21–24. Available at: https://cejph.szu.cz/pdfs/cjp/1995/01/03.pdf (accessed on 9 August 2018).

46. Davey K.R. (1989). – A predictive model for combined temperature and water activity on microbial growth during the growth phase. *J. Appl. Bacteriol.*, **67** (5), 483–488. doi:10.1111/j.1365-2672.1989.tb02519.x.

47. Mezrioui N. & Baleux B. (1992). – Effets de la température, du pH et du rayonnement solaire sur la survie de différentes bactéries d'intérêt sanitaire dans une eau usée épurée par lagunage. *Rev. Sci. Eau*, **5** (4), 573–591. doi:10.7202/705148ar.

48. McFeters G.A. & Stuart D.G. (1972). – Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Appl. Microbiol.*, **24** (5), 805–811. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC380667/ (accessed on 9 August 2018).

49. Vasconcelos G.J. & Swartz R.G. (1976). – Survival of bacteria in seawater using a diffusion chamber apparatus *in situ*. *Appl. Environ. Microbiol.*, **31** (6), 913–920. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC169856/ (accessed on 9 August 2018).

50. Suttle C.A. & Chen F. (1992). – Mechanism and rates of decay of marine viruses in seawater. *Appl. Environ. Microbiol.*, **58** (11),

3721–3729. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC183166/ (accessed on 9 August 2018).

Table I
Levels of contamination by *Escherichia coli*, *Salmonella* spp., noroviruses and hepatitis A virus in clams collected from different production sites in the region of Sfax (Tunisia)

Pathogen concerned	Percentage of contaminated samples
Escherichia coli (> 230 CFU/100 g FIL)	36% (194/539)
Salmonella spp. (present)	11% (59/539)
Norovirus (presence of viral RNA)	19% (48/252)
HAV (presence of viral RNA)	3% (8/252)

CFU: colony forming unit

FIL: flesh and intervalvular liquid

HAV: hepatitis A virus

RNA: ribonucleic acid

Table II

Pearson test study of correlation between contamination of bivalve molluscs by *Escherichia coli*, *Salmonella* spp., noroviruses and hepatitis A virus

	***Salmonella* spp.**			***Escherichia coli***			**Norovirus**			**HAV**		
	E. coli	**Norovirus**	**HAV**	***Salmonella* spp.**	**Norovirus**	**HAV**	***E. coli***	***Salmonella* spp.**	**HAV**	***E. coli***	***Salmonella* spp.**	**Norovirus**
p-value	<0.001	<0.001	0.401	<0.001	0.481	0.245	0.481	<0.001	0.031	0.245	0.401	0.031
Correlation factor	0.216	0.269	-0.053	0.216	-0.045	-0.074	-0.045	0.269	0.135	-0.074	-0.053	0.135

HAV: hepatitis A virus

Table III

Microbial contamination of clams in Tunisia: variations in levels of contamination by *Escherichia coli*, *Salmonella* spp., noroviruses and hepatitis A virus depending on variations in temperature, represented by four periods of the year (calculated using Student's t-test)

	Period 1				Period 2				Period 3			
Period	***E. coli***	***Salmonella* spp.**	**Norovirus**	**HAV**	***E. coli***	***Salmonella* spp.**	**Norovirus**	**HAV**	***E. coli***	***Salmonella* spp.**	**Norovirus**	**HAV**
1	–	–	–	–	–	–	–	–	–	–	–	–
2	0.098	0.0383	0.822	0.495	–	–	–	–	–	–	–	–
3	0.002*	0.374	0.561	0.519	0.096	–	–	–	–	–	–	–
4	0.004*	0.012*	0.454	0.286	0.030*	0.184	0.261	0.178	0.018*	0.009*	1.000	0.062

Period 1: January, February, March
Period 2: April, May
Period 3: June, July, August
Period 4: September, October, November, December
HAV: hepatitis A virus
*: presence of a significant difference

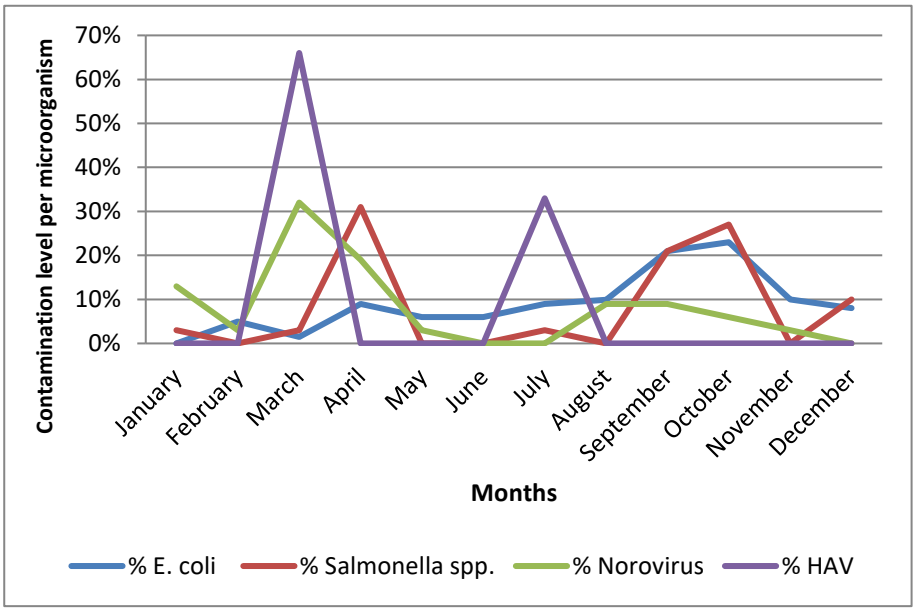

Fig. 1

Study of the seasonal trend in clam contamination by *Escherichia coli*, *Salmonella* spp., noroviruses and hepatitis A virus

HAV: hepatitis A virus