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XXIII Ciclo

Norovirus in bivalve molluscs: development of a Real Time RT PCR protocol and its application for a viral contamination monitoring and for a bioaccumulation study

Tutor:

Ch.ma Dott.ssa Tiziana Pepe

Candidata:

Dott. ssa Iole Ventrone

Coordinatore:

Ch.ma Prof.ssa Maria Luisa Cortesi

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1.0 ABSTRACT

The Food Hygiene Package is a body of European Regulations laying down hygiene rules for foodstuffs produced in the EU and non-EU countries exporting to the EU. The pursuit of a high level of protection of human life and health is one of the fundamental objectives of this laws package. Some points of EC Regulations undergo to critical review by the Commission, and particular attention was given to fishery products. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, in Annex I, Chapter 1, lays down the food safety criteria for live bivalve molluscs and live echinoderms, tunicates and gastropods and sets sampling-plans, limits and analytical reference methods only concerning bacterial micro-organisms of *Salmonella* spp. and *Escherichia coli* genera (1).

Epidemiological data of the last years reported the consumption of live bivalve shellfish infected by enteric viruses as common cause of human gastroenteritis. Norovirus resulted the leading cause of all human gastroenteritis outbreaks worldwide.

For this reason, in the first part of Reg. (EC) No 2073/2005, in the whereas at point 12, it's written that the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) issued an opinion on Norwalk-

like viruses (NLVs, noroviruses) on 30-31 January 2002. In that opinion it concluded that the conventional fecal indicators are unreliable for demonstrating the presence or absence of NLVs and that the reliance on fecal bacterial indicator removal for determining shellfish purification times is unsafe practice. So *Salmonella* and *Escherichia coli* can not be used as only indicators of safety criteria in live bivalve molluscs. In particular, at following points we can read that it may be necessary to set harmonized sampling frequencies at Community level, particularly in order to ensure the same level of controls to be performed throughout the Community. Finally it's confirmed that criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently (at point 27) (1).

In response to concerns expressed by EU Regulations and taking into account the latest epidemiological data, the international networks dealing prevention, communication and control of human food borne illness diseases caused by enteric viruses have intensified their researches on norovirus. In the past few years, numerous research projects financed by government departments and international organizations have been implemented in order to get more information on these viral pathogens and check most hazardous areas.

Among the areas traditionally known for a high fish consumption, there are the Southern Italy regions, where mussels are highly appreciated. *Mytilus galloprovincialis* accounts for over 22.4% of annual consumption of fish (data by E.U., Unimar, Ismea e Uniprom), particularly in Campania the mussels consumption was calculated to be about 41 000 tonnes each year. Since ancient times, in most coastal areas the custom of eating raw or undercooked bivalve shellfish remains common. In this way, however, the assumption of norovirus eventually present and bioaccumulated in shellfish makes easier. Noroviruses are highly resistant to adverse environmental conditions and a simple shellfish cleaning or the application of so-called "mild technologies", such as steaming, are unable to eliminate viral contamination.

Among filter feeders bivalve, oysters are the species most common in France. In fact the French coastal areas produce more than 90% of oysters in the EU and France has the historic first for production and consumption of these molluscs (2). Oysters are traditionally eaten raw, still alive, mostly with a few drops of lemon juice. As for *Mytilus galloprovincialis*, oysters can represent an hazard for the occurrence of human food borne illness from enteric viruses.

In the course of my PhD studies, I have dealt with norovirus in bivalve molluscs.

During my first year of PhD study, I concurred to the development of a method for detection of norovirus in live bivalve molluscs working together with researchers of the ISS. The method is based on the use of Real Time RT PCR. During our researches it has been subjected to inner validation by the ISS and it resulted provided of efficiency to research norovirus in live bivalve molluscs. So the Italian Ministry of Health with Note of 24/11/2009 established the one-step Real Time RT-PCR protocol as the official method to research norovirus in live bivalve molluscs (3).

After this period, during the second year of my PhD studies, I worked for a national monitoring to test norovirus presence in bivalve molluscs. The one-step Real Time RT PCR protocol was used again. Studies have been carried out within a project financed by Campania Region. Shellfish collected from harvesting areas and bought at retailers located on the three coastal districts of Naples, Caserta and Salerno have been tested for norovirus presence.

During the last year, my scientific experience has been enriched by a collaboration with IFREMER researchers. IFREMER is one of the French national research centers taking part in FBVE-network. I have worked at the section of the Virology of Laboratory of Microbiology (MIC) sited in Nantes, that was indicated National Reference Laboratory (NRL) for the

control of bacterial and viral contamination of bivalve molluscs in France by the French Ministry of Agriculture and Fisheries. IFREMER has the same functions of the Italian NRL concerning norvirus research and control. During my stage at IFREMER, bioaccumulation physiological activity was analyzed in oysters living in seawater contaminated by *Norovirus* spp. The research was carried out on samples of *Crassostea gigas* coming from different harvesting areas located along the Atlantic coast of Brittany, area known for oysters production worldwide. For bioaccumulation studies classified and quantized strains of norovirus GI.1 and GII.3 were used. At the IFREMER laboratory, the one-step RT Real Time PCR protocol was used and further validated.

2.0 INTRODUCTION

2.1 Norovirus

Noroviruses (NoVs) is one of the most common agents of human viral gastroenteritis and a leading cause of food-borne gastroenteritis worldwide (4).

Norovirus was first recognized as a cause of gastroenteritis in 1972, when it was detected by Dr. Kapikian and colleagues in stool samples collected from infected elementary school students during an outbreak in Norwalk, Ohio, in 1968. For that reason, these viruses were originally called Norwalk - Like virus (5, 6).

The virus particle was identified four years later the Norwalk gastroenteritis outbreak. In fact at first the virus characterization was very difficult because it was impossible using the traditional methods for virus detection. Albert Z. Kapikian bypassed the classical tissue-culture virology approach, which relies on the ability of a virus to infect and produce a change in cells or to infect an animal model. He used a novel approach of “direct virology”, in which the virus particle itself is studied directly as the “center of attention” without the benefit of an in vitro or animal model system. Kapikian used a

technique known as Immune Electron Microscopy (IEM). This method allowed the direct observation of antigen-antibody interaction and it was essential for the recognition and identification of a 27-nm virus-like particle that did not have a distinctive morphology, was low-titered, and was one the smallest viruses known. That and other evidence suggested that this virus-like particle was the etiologic agent of the Norwalk gastroenteritis outbreak. The fastidious 27-nm Norwalk virus is now considered to be the prototype strain of a group of noncultivatable viruses (7).

Norovirus was declared a member of the *Caliciviridae* family of viruses only in 1993 thanks to cloning and sequencing of its genome. *Caliciviridae* family comprises four genera, *Vesivirus* that includes Feline calicivirus, vesicular exanthema of swine virus, and San Miguel sealion virus, *Lagovirus* that includes rabbit hemorrhagic disease virus and European Brown hare syndrome virus, *Sapovirus* (SV), once known as Sapporo-Like virus (SLV) or "typical caliciviruses" and *Norovirus* (8). All viruses of the *Caliciviridae* family are morphologically similar to each other.

Noroviruses are also known as Small Round Structured Viruses (SRSV), in fact the small virions contain RNA molecule in round to hexagonal capsids that are 35-39 nm in diameter, with icosahedral symmetry. The surface structure of the capsid is a regular pattern with distinctive features and 32 cup-shaped depressions, typical of *Caliciviridae* family (Fig. 1).

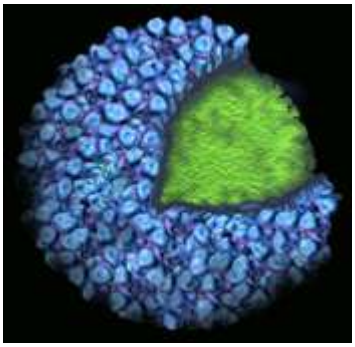

Figure 1: Norovirus spp**.**

Structural analysis of the norovirus capsid showed it is formed of 180 capsid proteins (VP1) arranged to form a T=3 icosahedral virion. Capsid protein has a molecular weight of around 55–60 kDa and is organized into two domains. A protein domain is a part of protein sequence and structure that can evolve its functions, and exists independently of the rest of the protein chain. Domains molecular evolution and instability are responsible of high genetic variability in norovirus.

The capsid surrounds and protects the norovirus genome, a single-stranded, positive-sense RNA molecule of 7.3-7.7 kilobases that is polyadenylated at its 3'end. The genome encodes three open reading frames (ORFs). ORF 1 is the largest (approximately 1700 amino acids) and expressed as a 200-kDa nonstructural polyprotein precursor that is cleaved by the viral 3C-like protease (3CL). The cysteine proteinase yield the mature nonstructural

proteins. ORF1 polyprotein can be divided into at least six functional proteic domains and one of each is RdRp or RNA-dependent RNA polymerase (9). ORF2 (550 amino acids) encodes the 60-kDa major structural capsid protein (VP1) organized into 90 dimers. ORF3 encodes the minor structural capsid protein VP2 a small basic protein of unknown function actually (6) (Fig. 2).

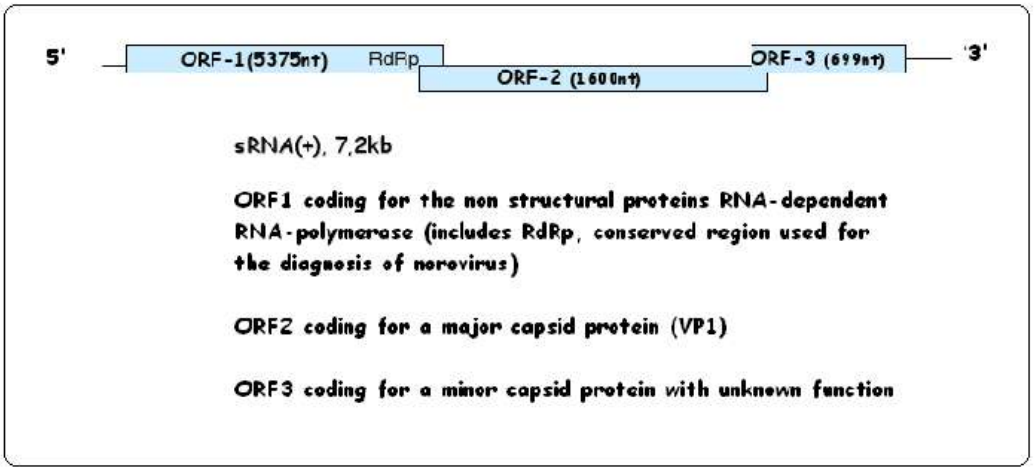

Figure 2: Norovirus genome

The VP1 protein forms two domains: P (protruding, P1 and P2) and S (shell). Most of the cellular interactions and immune recognition features are thought to be located in the P2 sub-domain, which extends above the viral surface and has the most sequence divergence in the genome (10, 11, 12). The capsid protein not only provides shell structure for the virus but also contains cellular receptor binding site(s) and viral phenotype or

serotype determinants. The function of VP2 associates with upregulation of VP1 expression in cis and stabilization of VP1 in the virus structure (13, 14).

Nucleotide sequencing and phylogenetic analysis showed that norovirus genus contains more than 40 different strains that are divided into 5 genogroups (GI to GV) based on sequence similarity. Virus strains belonging to different genogroups have about 51–56% genomic nucleotide similarity, whereas the structural proteins usually differ by more than 50% in capsid amino acid sequence are even more variable strains of different genogroups.

Viruses in genogroups I, II, and IV are primarily human pathogens, although genogroup II contains a porcine-specific virus. Viruses in genogroup III and V infect bovine and murine species, respectively. Norovirus GII has been shown to account for the majority (up to 92%) of reported norovirus human gastroenteritis cases, GI accounts for the large majority of the remaining cases. NoVs GIV was detected rarely (15).

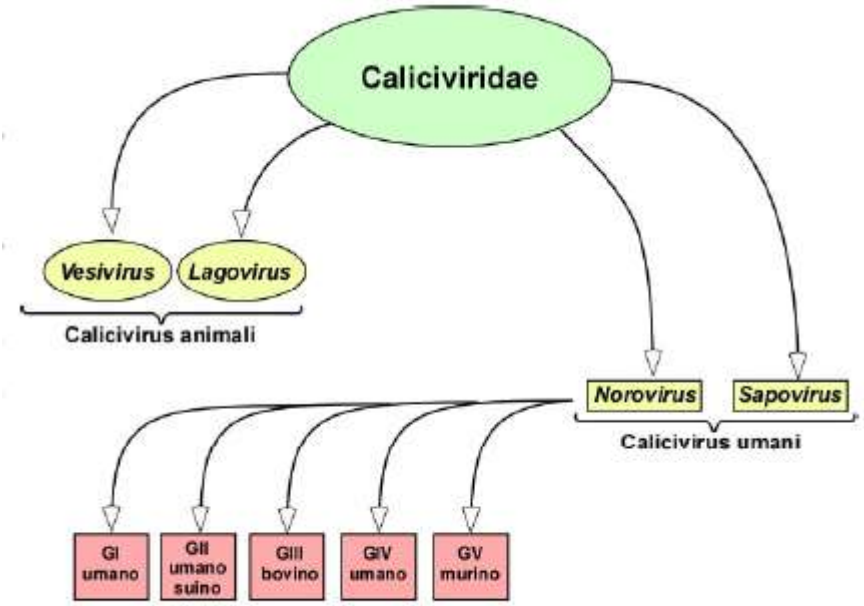

Figure 3: Norovirus spp

Each genogroup is further subdivided into genoclusters based on sequence similarity. Strains in a genotype have 69-97% similarity at the genomic level, whereas the structural proteins generally differ by up to 40% in capsid amino acid sequence.

Noroviruses genogroup I (GI) are subdivided into at least 15 genotypes (GI/1 to GI/15) and genogroup II (GII) viruses into 18 (GII/1 to GII/18). Human noroviruses (NoVs) of genogroup II, genotype 4 (GII.4) are the most common strains detected in outbreaks of acute gastroenteritis worldwide, in fact it causes 70 – 80% of norovirus gastroenteritis outbreaks.

Norwalk virus, Southampton Virus (GI-2), Desert Shield virus (GI-3) and Valletta virus (GI-4) are classified into norovirus genogroup 1 (GI). Hawaii virus (GII-1), Snow Mountain virus and Melksham virus (GII-2), Mexico virus (GII-3) and Grimsby virus (GII-4) are classified into genogroup 2 (GII). Recent evidence indicates that evolution in some genotypes is sufficient to generate mutant clusters that have new ligand-binding characteristics and antigenic properties. In fact, for example, evolution in the GII.4 genotype results in differential receptor binding and novel antigenic features, suggesting that the GII.4 noroviruses are evolving over time, with escape mutants being periodically selected for herd immunity. In addition, similar phylogenetics-based studies of the GII.2 and GII.3 genotypes have suggested analogous patterns of evolution, with evolved clusters that may contain novel innovations that alter viral structure and function. The same work suggests that the GI genogroup of noroviruses has undergone limited evolution with little phenotypic innovation. This is shown in the phylogenetic tree, in which the GI genotypes are less distant from one another compared with the GII genotypes (Fig.4). Actual crystallography studies comparing the structures of multiple GI and GII capsid proteins along with mutagenesis experiments and studies of carbohydrate ligand-binding characteristics should help to elucidate the

differences in the structural constraints between these two genogroups (16).

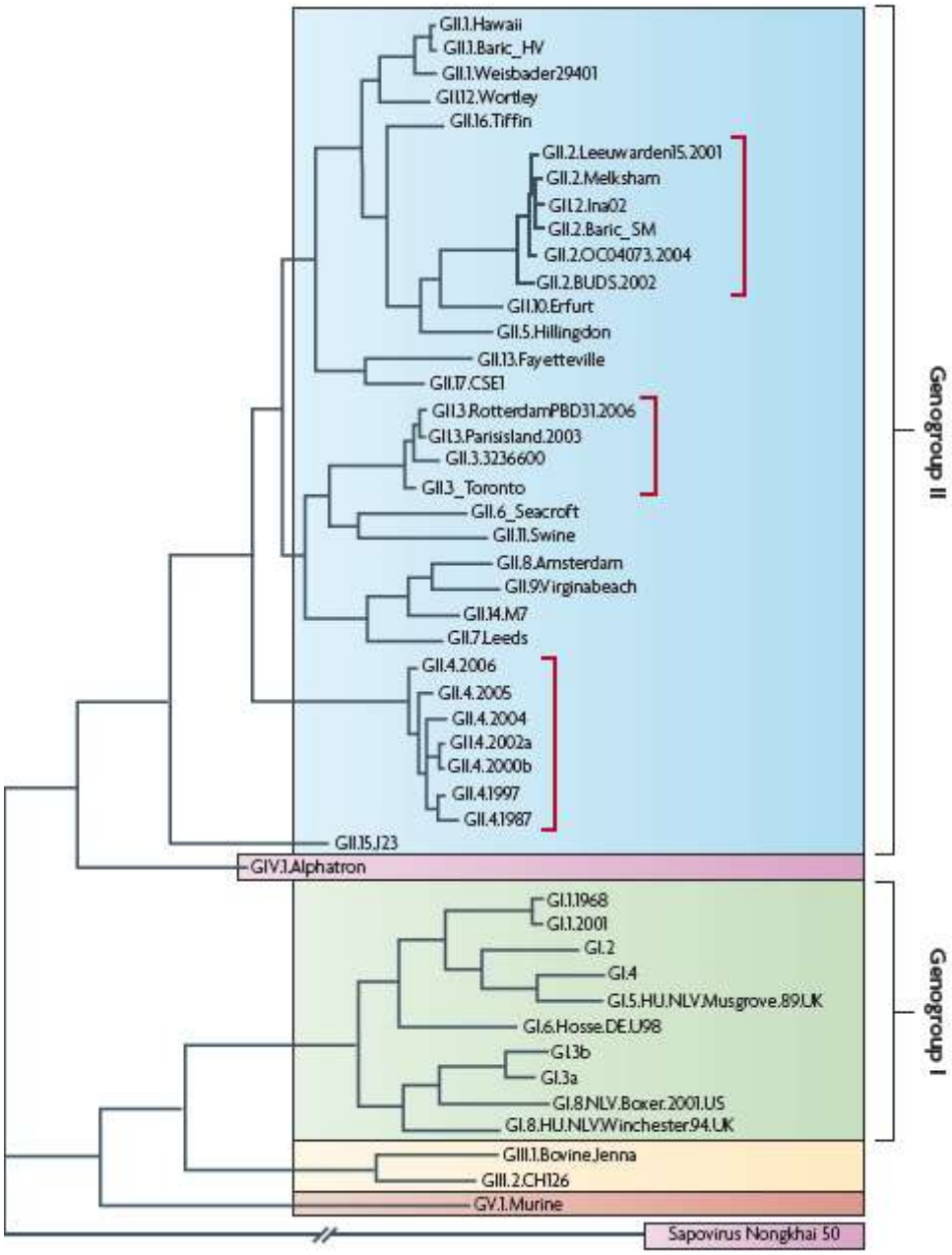

Figure 4 : NoVs GI and GII phylogenetic tree

2.2 Norovirus as cause of gastroenteritis

Gastroenteritis is an inflammation of the digestive tract, particularly the stomach, and large and small intestines. Viral and bacterial gastroenteritis are intestinal infections associated with symptoms of diarrhea, abdominal cramps, nausea, and vomiting. Infectious gastroenteritis may be caused by viruses, bacteria, parasites or biologic toxins.

The most important pathogens responsible of toxinfection disease are *Listeria monocytogenes*, *Campylobacter jejuni* and *coli*, verocitotoxic *Escherichia spp*., *Salmonella enteriditis* and *typhimurium*, *Yersinia enterocolitica*. There were 76 million cases of food related illness in the USA per year and in a retrospective survey it was estimated that over five million people per year in the UK suffered from acute gastroenteritis which they ascribed to contaminated food. Gastroenteric diseases are caused by the "food-borne pathogens" (17). In 2000 in UK and Wales occurred 1,3 millions cases of food-borne diseases and 480 deaths brought on pathogens as *Campylobacter*, *Salmonella* and *Listeria monocytogenes* (18). Until few years ago *Salmonella* spp. was the most common bacterial cause of human gastroenteritis with more than one million of patients each year. At present *Campylobacter* spp. is the main food-borne pathogens and it records more

gastroenteritis cases than the episodes collected by *Salmonella* spp. and *Shigella* spp. together.

Viruses are considered within emerging pathogens responsible of human gastroenteritis, so the increasing spread of enteric viruses is an emerging problem. In later years many scientists are involved solving that problem all over the world (19).

Human enteric viruses as causes of foodborne disease can be classified into three main groups:

– Gastroenteritis viruses;

– Hepatitis viruses carried by intestine;

– Viruses increasing in human intestine and they cause diseases after migration to others anatomic organs, as the SNC or the liver.

Sapovirus (SaV), *Hepatitis A virus* (HAV), *Astrovirus*, *Rotavirus* and *Norovirus* (NoV) are viruses responsible for human direct gastroenteritis. Norovirus differs from other agents of gastroenteritis because it causes disease in adults (teenagers and above), thus NoVs are the most significant diarrheal virus in terms of working/education days lost. Secondly, it induces a high level of explosive projectile vomiting that may be the first obvious sign of infection. Finally, although there are probably multiple serotypes of NoVs, immunity to all seems to be short-lived. Thus individuals may be protected for only a few months following an infection before they become

infective once more by the same virus. It is now thought likely that they lack the cell-surface receptor (a carbohydrate antigen) to which the virus must bind to initiate infection (20). During last years data suggest that blood group secretor status may be susceptibility alleles for some, but not all, norovirus infections in humans. These are just some recently proposed mechanisms for the ways in which the Human norovirus may select for its human host.

As for all the agents of gastrointestinal disease, the main portal of entry for infection is the oral route. Norovirus have a low infectious dose from 10 to 100 virus particles, meaning that only a few infectious virus particles can cause infection, and environmental contamination may prolong outbreaks. Surfaces, serving dishes or containers, utensils, and food handled by ill persons who are not practicing adequate personal hygiene before preparing food may also contribute to illness. Virions are acid-stable, enabling them to pass the gastric barrier and get the small intestine, where they can multiply. Noroviruses fix on intestinal mucosa and their incubation period, the time between catching the virus and developing symptoms, is very short, usually between 24 and 48 hours.

Recent studies indicate that susceptibility to NoVs infection is associated with ABH histo-blood types, gut-expressed carbohydrates, and strain

preferences norovirus infection is associated with ABO blood type and VLPs representative of both major genogroups GI and GII bind histo-blood group antigens on gastroduodenal epithelial cells and in saliva of individuals with secretor phenotypes. Human norovirus does have a binding specificity within the human species, however. ABH and Lewis histo-blood group antigens are carbohydrate epitopes present throughout many tissue of the human body. The type 1 and 3 chain ABH histo-blood group antigens are present on mucosal epithelial cell surfaces and in salivary secretions, with variations in the carbohydrates in different individuals based on their secretor status and blood type. Recent observations suggest that norovirus likely attaches to either H types 1 or 3 present on gastroduodenal epithelial cells (6).

Noroviruses are transmitted primarily through the fecal-oral route, either by consumption of fecally contaminated food or water or by direct person-to-person spread. Environmental and fomite contamination may also act as a source of infection. Good evidence exists for transmission due to aerosolization of vomitus that presumably results in droplets contaminating surfaces or entering the oral mucosa and being swallowed. Norovirus can spread very easily touching surfaces (such as toilet seats, bed rails, furniture) that have virus on them. During outbreaks, several modes of transmission have been documented as for example, initial foodborne

transmission in a restaurant, followed by secondary person-to-person transmission to household contacts. In more or less closed communities, such as hospitals, restaurants, schools, and hotels, fast-moving widespread of outbreaks may occur.

After infection, norovirus incubation period is 24-72 hours. This is the time between catching the virus and developing symptoms. Norovirus cause an acute gastroenteritis characterized by a sudden onset of nausea as first symptom, which is followed by projectile vomiting and watery diarrhea. Some people may also have a raised temperature (over 38º C), headaches, stomach cramps and aching limbs. Symptoms normally last from 12 to 60 hours but most people make a full recovery within two to three days. There are no long-lasting effects from having a norovirus infection. However in Holland it is reported a norovirus gastroenteritis outbreak where virus was detected up to 20 days after infection in 10% of cases (21).

Norovirus illness is usually a short-term, self-limiting disease in people who are otherwise healthy. No hospitalization or medical advice are required. But, the infection can cause severe vomiting and diarrhea and this can lead to dehydration (loss of too much water from the body), that can lead to serious problems young children, the elderly, and people with other illnesses are most at risk for dehydration. Severe dehydration may require hospitalization for treatment with intravenous fluids. Thus it is important to

prevent dehydration during norovirus illness. Before the medical advice, the best way to protect against dehydration is to drink plenty of liquids; the most helpful fluids for this purpose are oral rehydration fluids (22).

No vaccine is currently available to prevent NoVs disease in humans and there is no drug to treat people who are infected with the virus. Antibiotic drugs will not help for norovirus infection, because they fight against bacteria not viruses. The infection can only be treated with drugs that act on the symptoms of the disease. In addition as previously mentioned, the immunity to norovirus is usually incomplete, only temporary and probably not extended to different genotypes. That is because there are numerous norovirus viral strain with different antigenic properties and it exists an inherited predisposition to infection: individuals with blood type O are more often infected, while blood types B and AB can confer partial protection against symptomatic infection.

The main infection sources are people infected with norovirus. They are contagious from the moment they begin feeling ill to at least 3 days after recovery. The virus may be shed (discharged from the body through vomit or stool) for 2 weeks or more after recovery, although it is unclear whether the virus shed during this time is infectious. During the infection peak one gram of feces may contain up to 10,000,000 viral particles, and a single

episode of vomiting may contaminate the environment with 30,000,000 viral particles. Viral shedding continues over 72 hours after symptoms end.

Releasing into the open air, norovirus can persist active and vital for a long time: noroviruses are highly resistant to adverse environmental conditions. They can survive chlorination (in concentrations up to 10 ppm) and temperatures ranging from below 0 °C to 60 °C and higher. Norovirus keeps at acid pH of 2.7 and at disinfectant treatment with ether-ethanol and common detergents. A recent study regarding the persistence of human noroviruses in waters showed that norovirus genome may persist 1-3 months in different types of water (mineral, tap water and river).

All these characteristics are responsible of high contagious ability of noroviruses and explain the difficult decontamination of surfaces and food and the cosmopolitan distribution of the virus.

In addition, the high environmental resistance of NoVs, the high number of particles released and the low infectious dose clarify the increase of gastroenteritis outbreaks caused by norovirus registered during last years (23).

2.3 Epidemiology and control of Norovirus

Since the Eighties, market globalization led to a gradual intensification of relations and exchanges worldwide in all fields. Main effect was a strong economic and cultural convergence among the countries of the world, but also a greater chance that diseases typical of certain ethnic groups spread beyond the borders. Monitoring and control of infection are the most important ways to limit this problem. Epidemiological information exchanging among countries and a rapid circulation of informations are necessary. Epidemiological data must be collected, provided and disclosed using reliable and standard scientific criteria. Beside to the continuous circulation of the most common bacterial pathogens, epidemiological data show that gastroenteritis viruses, especially norovirus, are multiplying and spreading. Since the first norovirus identification in 1972, they caused many infection outbreaks in all countries around the world. At first, informations about the virus were few and unclear because there were many difficulties associated with inadequate diagnostic techniques. Nowadays virus data are larger thanks to the spreading of innovative methods (7, 23).

The first remarkable references on norovirus infections epidemiology are dated from the early Nineties. In fact the CDC (Centers for Disease Control and Prevention) in the United States recorded more than 8000 outbreaks characterized by symptoms of gastrointestinal type in the population from 1991 al 2000. The number of foodborne outbreaks ranged from 411 outbreaks in 1992 to 1,414 in 2000, and increased markedly in 1998, when the reporting system was changed. In fact a more sensitive and simpler assays were developed to detect NoVs by identifying viral RNA. NoVs gastroenteritis outbreaks were larger than bacterial ones: median persons affected was 25 versus 15. The number of NoVs confirmed outbreaks increased markedly from 11 outbreaks in 1996 to 164 in 2000 (12% of all reported outbreaks). Comparing these data with those of the entire decade examined, the proportion of NoVs confirmed outbreaks increased from 1% in 1991 to 12% in 2000. This increasing was registered in the 11 states of U.S.A. that adopted the new RT-PCR method for the routine detection of NoVs. Particularly PCR was applied in outbreaks in which specimens tested negative for common bacteria. When RT-PCR was used, a NoVs was identified as the etiologic agent in 93% of outbreaks of nonbacterial gastroenteritis submitted for testing to CDC from 1997 to 2000. However, this selection was of specimens from outbreaks of illness characteristic of

viral infection, and they usually have already tested negative for bacteria. The 36 states of U.S.A. that didn't use RT-PCR in their national reference laboratories, reported either few or no norovirus outbreaks. However, underreporting of disease caused by norovirus is a current problem, since only 17 (34%) of 50 state public health laboratories in the United States tested for NoVs (24).

In Bahia state, Brazil, during June–July of 2006 NoVs was identified as the principal etiologic agent in an acute gastroenteritis outbreak involving more than one hundred people. Approximately 42% of the patients were hospitalized with clinical symptoms of strong abdominal pain, vomiting, diarrhea and, occasionally, high fever. Results showed that out of the total of 127 fecal samples analyzed in this outbreak, 80 were positive for norovirus presence (63%). Again the application of RT-PCR molecular techniques has allowed the identification of the virus. Comparing viral genome examined with sequences of reference strains derived from Genbank it was possible identify norovirus genogroups and genotypes involved during Brazilian outbreak. Phylogenetic analysis of sequence data showed that all NoVs infections were caused by the GII.4 strain, which was responsible for 72.5% of all NoVs-positive cases (58/80). Other detected NoVs strains included GII.3 (7/80; 8,8%) (25).

These data confirm the above statement that the GII genogroup of norovirus, especially those classified in genotype GII.4, are responsible for most cases of gastroenteritis in humans.

Data collected through researches and surveillance networks of viral gastroenteritis in European countries, reported for norovirus outbreaks a trend similar to that described for Americas.

European epidemiological data from 1995 to 2002 registered a substantial increase in norovirus outbreaks in all countries except Spain. The sensitivity of diagnostics is thought to have increased during these years and it has permitted noting a norovirus infection increasing. It has coincided with the detection and emergence of norovirus genotype GII4 in Europe, too.

In Netherlands the diagnostic and reference laboratory at the National Institute for Public Health and the Environment in Bilthoven, collected all norovirus outbreaks from 1995 to 2000 and noted a striking increase of norovirus infections. Identification was done by RT-PCR method that permitted the observation of an increase (about 128%) compared with the results of the last two decades. In fact from the previous high of 68 cases registered in 1996, it has passed to 155 episodes in 2002. Dutch population showed an increase in norovirus outbreaks during last years, collected an increase of 53%. In total, genotypic analysis was carried out on 153

outbreaks and 134 (88%) of these resulted belonging to norovirus genogroup GII4.

In Germany, norovirus gastroenteritis trends were drawn based on the number of outbreaks investigated by the molecular virology laboratory at the Robert Koch Institute in Berlin. In 2002, there were 161 norovirus outbreaks diagnosed by RT-PCR, a 94% increase from the previous peak of 83 outbreaks in 1999. In England and Wales, since 1995, there have been 2324 reports of laboratory confirmed outbreaks of norovirus infection captured by the national database for the two countries. In 2002, 614 norovirus outbreaks were reported, a 77% increase from the previous peak of 347 outbreaks in 1995. RT-PCR analysis amplified norovirus genogroup II4 in 232 samples, corresponding to 66% of all outbreaks reported.

Like Germany and the Netherlands, a norovirus outbreak peak was noted also in Northern European countries as Denmark, Finland and Sweden that are all nations with long standing (>5 years) and consistent sources of surveillance data. They have reported epidemiological data similar to the others countries described above, so they registered a high number of cases of norovirus outbreaks between 1995 and the early years of the new millennium, with a peak again in 2002. Spain was the only country where fewer outbreaks of norovirus were reported to the national centre at Instituto

de Salud Carlos III in 2002 than in previous years. This may be artefactual because of small numbers of reports (Table 1) (26).

	2002	Previous yearly maximum (1995–2001)	Change from previous maximum
Denmark*	698	250	+179%
England and Wales†	614	347	+77%
Finland*	745	229	+225%
Germany†	161	83	+94%
Hungary†	116	42	+176%
Slovenia*	402	232	+73%
Spain*‡	83	89	–7%
Sweden*	1175	489	+140%
Netherlands†	155	68	+128%

*From reports of individual samples. †From reports of outbreaks. ‡Based on 10 months of data, Jan–Oct, 2002.

Table 1: Norovirus outbreaks in 2002 compared with previous peak year in nine European countries

In France norovirus surveillance has been in place since 1999 and no historical data were available for comparison with others European countries. Concerning Italian epidemiological data, our country has the same French situation. In Italy a national norovirus gastroenteritis notification system was created only in 2007, after issuing of a Recommendation by Italian Ministry of Health (27). Therefore, studies on this emerging pathogen increased only during last years in Italy and few epidemiological data on norovirus outbreaks are available until now.

Actually there are many international networks dedicated to prevention, communication and control of human infective disease. The most important are the CDCs (Centers for Disease Control and Prevention), organizations

that operate on a global. CDCs are link to many others satellite agencies including ECDC (European Centers for Disease Control and Prevention) that work in Europe to protect human health, through health promotion, prevention of disease, injury and disability, and preparedness for new health threats. They involved with the most important epidemic diseases, especially. ECDC publish periodically Eurosurveillance, the European scientific online journal devoted to the epidemiology, surveillance, prevention and control of infective communicable diseases.

Since the increase of gastroenteritis outbreaks caused by norovirus registered during last years, international organizations that deal exclusively in the control of viral gastroenteritis have emerged. One of these organizations is Noronet, an informal network of scientists sharing virological, epidemiological and molecular data on norovirus (Fig. 5).

Figure 5: NoroNet logo

The first aim of Noronet is to enlarge the knowledge on geographical and temporal trends in the emergence and spread of NoVs variants, thus limiting

the impact and scale of future NoVs epidemics. There is also a system of rapid communication through e-mail alerts that send the information to all members of the network. For example, if there is a gastroenteritis outbreak sustained from a new variant of unknown norovirus, it will be possible to limit impact, dissemination and any consequential damage caused by norovirus outbreak using all information stored on NoroNet. A second aim is the design of a well founded standardized nomenclature for existing and emerging NoVs genotypes and variants or sub-lineages. Thanks to studies on viral genome, a norovirus genomic library is born. It can be consulted at all times by members of the network to acquire more precise information on the spread of epidemics. Virologists and epidemiologists from around the world take part to NoroNet and each member country has indicated one or more reference laboratories for norovirus detection.

All the NoroNet European countries (Germany, France, Netherland, England, Wales, Hungary and Sweden) Russia more, are FBVE-Network members.

FBVE net (Food-borne Viruses in Europe) is a network supported by European Commission, which thirteen European countries are members. It works since 1999, sharing surveillance and research data on enteric virus infections, focusing mainly on norovirus. Twenty six research centers of the thirteen member countries (UK, Netherland, Denmark, Finland, France,

Germany, Hungary, Ireland, Italy, Norway, Slovenia, Spain and Sweden) take part to FBVE Network (Fig. 6).

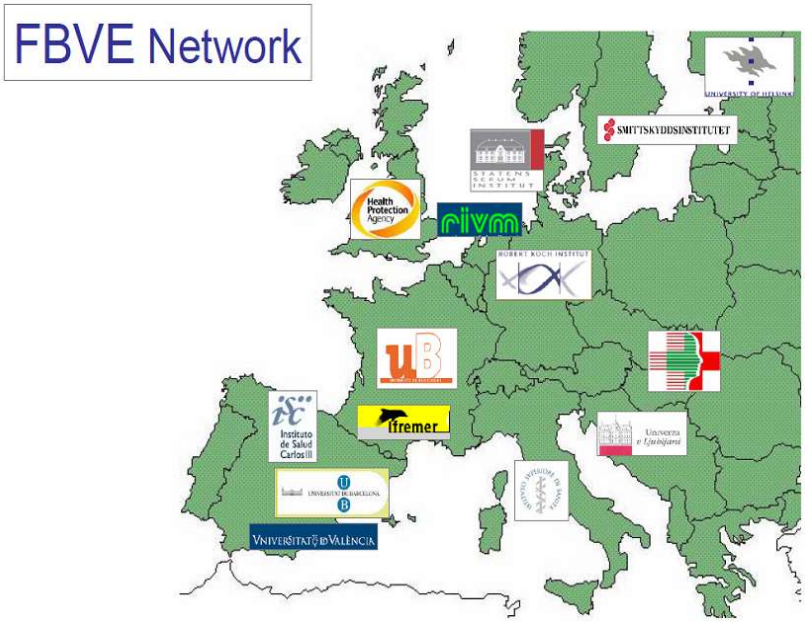

Figure 6 : FBVE network member countries and research centers

FBVE is structured in the same way as the other international networks described above. In fact FBVE has a well organized computerized network that member countries use to communicate quickly alert situations. In addition, since 1999 the FBVE network maintains a joint database accessible via the Internet, in which all members enter outbreaks, partly with sequence information. Database is password protected. The norovirus sequences can be searched and compared to new sequences by the network

members at the sequence database (participants only). Besides these password protected databases the FBVE network maintains a public database for quicktyping. Part of the activities of the FBVE network has been financed by the EU through different projects; one of these was FBVE-Net Project (2000-2004). In this project the importance of enteric viruses as causes of illness across Europe was studied, with a special focus on multinational outbreaks of infection with Norwalk-like viruses and Hepatitis A virus. Novel, standardized, rapid methods for virus detection and typing were being developed to be used in all participating laboratories. The network was used as a framework for a rapid, prepublication exchange of epidemiological, virological and molecular diagnostic data. The high-risk foods and major transmission routes of foodborne viral infections in the different countries and between countries were determined and the pattern of diversity within and between countries was described, and potential pandemic strains were identified at the onset.

Thanks to monitoring of European area, a large increase in norovirus outbreaks in Hungary and Germany was reported by Foodborne Viruses in Europe network (FBVE) to European national health authorities during last years of the twentieth century. Subsequently, an email was sent to all FBVE members requesting a current overview on norovirus outbreaks in their

country, and asking for preliminary reports on genotyping. Data obtained by FBVE-Network are in Table 2.

FBVE members	**Outbreaks / cases in 2002**	**Outbreaks / cases in 2003**	**Outbreaks / cases in 2004**	**Outbreaks / cases in 2005**	**Outbreaks / cases in 2006**	**Outbreaks/ cases in (2002 to 2006)**
Germany	216	0	0	2019	3156	5391
Spain	18	6	4	11	15	54
Finland	75	4	16	20	14	129
France	103	72	10	69	58	312
England	795	219	301	357	221	1893
Hungary	111	85	63	68	104	431
Ireland	0	0	31	53	152	236
Italy	2	2	4	6	5	19
Holland	150	52	124	93	219	638
Norway	0	0	0	25	29	54
Sweden	15	7	9	19	28	78
Slovenia	22	10	8	24	22	86
TOT Outbreak each year	1523	464	592	2777	4074	9430

Table 2: Norovirus outbreaks registered from 2002 to 2006 in FBVE countries members

Thirteen country authorities participate in the FBVE network. Nine of the 11 country authorities which replied to the survey indicated an increase in norovirus activity (outbreaks and/or case reports) in 2006 compared with 2004 and 2005. In some countries, no increase in norovirus activity was

found. The norovirus isolates, causing 108 outbreaks which started in October and November 2006, have been characterized and 87 (81%) belonged to genotype GII. 4, which has been the predominant genotype in recent years (28).

As showed in Figure 6, the Istituto Superiore di Sanità (ISS), sited in Rome, is the FBVE-network National Reference Laboratory for Italy. The official controls and of monitoring programs are performed by National Centre for Food Quality and Risk Assessment (N.C.F.Q.R.A.) of the ISS. In 2002 Italian Ministry of Health selected this laboratory as National Reference Laboratory (NRL) for viral contamination in live bivalve molluscs, too. The N.C.F.Q.R.A. works on Hepatitis A virus (HAV) and on norovirus especially. As NRL, the ISS coordinate activities of peripheral laboratories which carry out controls for viral contamination in live bivalve molluscs. Moreover ISS works together with the competent authorities to organize a monitoring system for bacterial and viral contamination and to dispose comparative tests on microbiological parameters between different Peripheral national laboratories. Finally, the Istituto Superiore di Sanità cooperates and spreads informations coming from the Community Reference Laboratory, the "Centre for Environment, Fisheries and Aquaculture Science" (CEFAS) - Weymouth - United Kingdom.

IFREMER (French Research Institute for Exploitation of the Sea) cooperates with CEFAS, too. IFREMER is one of the French national research centers taking part in FBVE-network (Fig. 6). It has the same functions of the Italian NRL concerning norovirus research and control. In particular, the section of the Virology of Laboratory of Microbiology (MIC) sited in Nantes at one of the five regional centers of IFREMER, works as National Reference Laboratory (NRL) for the control of bacterial and viral contamination of bivalve molluscs. It was indicated NRL by the French Ministry of Agriculture and Fisheries, since January 23, 2003. Like the ISS for Italy, IFREMER aims to ensure a high level of safety from bacterial and viral contamination of shellfish, and to carry out norovirus monitoring and experimental studies.

For both the Community Reference Laboratories, norovirus researches and studies are carried out on bivalve molluscs. Oysters and mussels consumed raw or undercooked have represented and still represent the main source of contamination for humans. Since January 2010, 334 cases of norovirus gastroenteritis were registered in five European countries (United Kingdom, Norway, France, Sweden and Denmark) and the most was showed linking to bivalve molluscs consumption (20).

2.4 Bivalve molluscs

Phylum Mollusca (Linnaeus, 1758) is the second phylum in the Kingdom Animalia for number of described species, after Arthropoda. There are about 140,000 species of living molluscs, very different in form and anatomy. They are classified into eight Classes: Aplacophora, Bivalvia, Caudofoveata, Cephalopoda, Gastropoda, Monoplacophora, Polyplacophora, Scaphopoda.

Currently the generic term "shellfish" is used to indicate the three most important Classes:

- Bivalvia
- Cephalopoda
- Gastropoda

The Class most common in trade is Bivalvia, knowk as Pelecypoda, too. It includes about 20.000 species living both in sea water (about 600 were present in the Mediterranean sea), than river and lake water.

Bivalvia dimensions are normally measured on the major axis of shell and they can range from one millimeter to over one meter (as for *Tridacna gigas*). The Class includes mussels, clams, cockles, razor clams, oysters,

clams, shellfish and many other bivalves commonly found on tables all over the world.

According to the study of fossils, it seems that molluscs have appeared on Earth before the Cambrian (about 570 million years ago), but there are no traces of earlier Paleozoic era in any field. They are probably formed by a strain that had characteristics typical of Annelida Class; it is believed that the ancestor of Phylum Mollusca was a worm-like animal, formed from the shell flakes scattered on the back, similar to those of current Caudofoveata and Aplacophora Classes. The Molluscs formed soon the mantle, the dorsal wall covering the back and sides of the body. The surface of the mantle produced the shell, hardy and limestone, under which animals can protect in emergency situations. Once gained that protection, worm-like molluscs have adapted to live in the mud and algae and corals. Thanks to their shell, they have colonized rocky or stony surfaces lived by animals and plants, that represent molluscs feed.

In a second time, thanks to the efficient physiological organization and flexibility of anatomical organization, the Molluscs adapted and colonized various different environments. Mollusca Bivalvia have settled in the seabed where they can filter microorganisms in suspension on water competing with Brachiopoda and Tunicata; Gastropoda pteropoda colonized pelagic waters competing with Coelenterata, Ctenophora and Crustacea.

Cephalopoda populated slowly both ocean waters and the seabed, competing with the Fishes. Mollusca Bivalvia and Gastropoda colonized lake basins and wetlands, so the lung-provided Gastropoda appeared inshore together with Annelida, Arthropoda and the Vertebrates (29).

Molluscs are highly diverse, not only in size and in anatomical structure, but also in behaviour and in habitat. However, all molluscs have some constant characteristic elements. Its body is generally composed of the shell and the fleshy, living part. The fleshy parts of a mollusc can be further divided into the foot and the visceral mass. The foot is a muscular organ, adapted to different purposes in different classes. The foot carries a pair of statocysts, which act as balance sensors. In gastropods, it secretes mucus as a lubricant to aid movement. In forms that have only a top shell, such as limpets, the foot acts as a sucker attaching the animal to a hard surface, and the vertical muscles clamp the shell down over it; in other molluscs, the vertical muscles pull the foot and other exposed soft parts into the shell. In bivalves, the foot is adapted for burrowing into the sediment; in cephalopods it is used for jet propulsion, and the tentacles and arms are derived from the foot. The visceral mass, or visceropallium, is the soft, non-muscular metabolic region of the mollusc and includes the organs for digestion, circulation, reproduction, and respiration. It also includes two external flaps

of tissue called the mantle, which secretes the calcareous shell and encloses a mantle cavity.

The mantle (also known by the Latin word “pallium”) is a significant part of the anatomy of molluscs: it is the dorsal body wall which covers the visceral mass and usually protrudes in the form of flaps well beyond the visceral mass itself. The mantle cavity is a central feature of molluscan biology. This cavity is formed by the mantle skirt, a double fold of mantle which encloses a water space. The fluid in the mantle cavity, which in aquatic mollusks is continually replaced with water from the outside, carries away excess water, ions and wastes, and helps circulate nutrients and oxygen. Another structure unique to molluscs, found in most groups, except bivalves and a few others, is the radula. In most forms the radula is a rasping organ near the mouth variously modified for special feeding techniques. The mantle and the radula found in Mollusca and nowhere else in the animal kingdom. Finally a characteristic element of Mollusca are the large gills, that play an important role in both respiration and feeding. Each gill consists of a double fold of tissue suspended in the mantle cavity. Each gill fold is a lamella. The lamellae of each gill (left and right) join ventrally to form a food groove and dorsally to form a chamber, which carries water posteriorly to the excurrent siphon. The gills contain blood vessels and obtain some oxygen from the incoming water currents.

The dorsal surface of the mantle, in more advanced Mollusca classes, secretes proteins for the mollusc shell. Moreover, the mantle repairs and maintains the shell. Any injuries or abnormal conditions of the mantle are usually reflected in the shape and form and even color of the shell. When the animal encounters harsh conditions that limit its food supply, or otherwise cause it to become dormant for a while, the mantle often ceases to produce the shell substance. When conditions improve again and the mantle resumes its task, a "growth line" is produced. The growth line is a fine transverse line marking the growth of the shell and it is formed by deposition of crystals of calcium carbonate on a protein structure called "conchiolin". This protein, together the perlucin, is secreted by the mantle to form the hard shell which encloses, supports and protects the soft parts of the molluscs. Shells are made of calcium carbonate embedded in an organic matrix secreted by the mantle. The organic constituent is mainly made up of polysaccharides and glycoproteins. Its composition may vary widely, in fact some molluscs employ a wide range of chitin-control genes to create their matrix, whereas others just express one, suggesting that the role of chitin in the shell framework is highly variable. This organic framework controls the formation of calcium carbonate crystals, and dictates when and where crystals start and stop growing, and how fast they expand. It even controls

the polymorph of the crystal deposited, controlling positioning and elongation of crystals and preventing their growth where appropriate.

The periostracum, the outermost organic layer, is secreted by the inner surface of the outer mantle fold at the mantle margin. It is a substrate upon which calcium carbonate can be deposited by the outer surface of the outer mantle fold. The number of calcareous layers in the shell (in addition to the periostracum), the composition of those layers (aragonite or aragonite and calcite), and the arrangements of these deposits (e.g. in sheet, or foliate) is characteristic for different group of bivalves. The inner layer (ipostracum) is called also pearly, because it is responsible for pearls generations. Pigments cause shell colours, while shell iridescence is caused by refraction of light as it passes through layers of calcite and aragonite. Shelled creatures use colour in camouflage, protecting their tissue from harmful light, and to harden the shell structure and eliminate harmful chemicals from their bodies. True pigments or biochromes derived by metabolic products ingested by molluscs as part of their diet and can also give shells their colour. Shell form and complexity are an obvious adaptation to the environment where molluscs live. For example, shells with spiral have low stability in the presence of powerful waves, shells thick or swollen and with a narrow opening, typical of Bivalvia, protect shellfish from predators. Further defense ways are shell mimetic colours molluscs and algae coating.

Scaphopoda shells are conical and curved in a planispiral way, hollow and open at both ends. The opening at the larger end is the main or anterior aperture of the shell, the smaller opening is known as the apical aperture. The mantle of a Scaphopod is entirely within the shell. The foot extends from the larger end of the shell, and is used to burrow through the substrate. The scaphopod positions itself head down in the substrate, with the apical end of the shell (at the rear of the animal's body) projecting up into the water. Water enters the mantle cavity through the apical aperture, and it is wafted along the body surface by cilia. There are no gills and the entire surface of the mantle cavity absorbs oxygen from the water. Unlike most other molluscs, there is no continuous flow of water with a separate exhalant stream. Instead, dexoygenated water is expelled rapidly back through the apical aperture through muscular action. Some others mollusc species, as the Pectinidae family, have a hardy adductor muscle which allows opening and sudden closing of valves. It causes a water jet that permits jump movements. The Cephalopods (“head-foot”) have as primary method of movement the jet propulsion. Oxygenated water is taken into the mantle cavity to the gills and through muscular contraction of this cavity, the spent water is expelled through the hyponome, created by a fold in the mantle. Motion of the cephalopods is usually backward as water is forced

out anteriorly through the hyponome, but direction can be controlled somewhat by pointing it in different directions. In some more evoluted molluscs the mantle contributes to the movement together with foot and valves muscles activity.

Mollusc body is inner the shell. Body wall has three layers: cuticle, epidermis, muscle layers. The cuticle not only forms the girdle, which bears calcified spines or spicules, but also extends between the shell plates. The principal part of the cuticle consists largely of mucopolysaccharide material but there is also a thin discrete inner region which is similar chemically to the periostracum of other molluscs. The epidermis has one layer of cuboidal or columnar cells, including cuticle-secreting cells, and large secretive cells producing spicules or epidermal papillae with excretory function. Gland cells that secrete mucus, which in mollusks has a variety of important uses, such as locomotion, food entrapment, and prevention of water loss. Epidermal cells include also epidermal papillae, for a sensory reception function. Muscle tissue is found in the body wall, and is particularly plentiful in the foot, which is used for locomotion in most molluscs (although some swim and some are sedentary), and in the mantle in species with reduced shells. Muscles are separated by a basal membrane and consist generally of three layers of smooth muscle fibers: an outer circle, a diagonal average and an internal longitudinal.

Mantle is the dorsal tissue layer of mollusc body. During its development, the mantle forms a mantle cavity containing the gills or ctenidi and that receives fecal material and the metabolites of reproductive and excretory systems. Mantle folds cover the shells placed sideways and they are fused in some taxa and prolonged to form tube-like siphons. One siphon carries water to the mantle cavity (the inhalant siphon) and one from it (the exhalent siphon); in many taxa they are fused but the water streams remain separate. Filtered water comes in contact with ctenida (or comb gills), so that they can extract food materials and provide for gas exchanges. In Cephalopoda these functions are carried out by mantle muscles. In fact there isn't a shell, so the mantle can contract and expand bringing water in its cavity. Filtering water comes into contact with ctenida, anus, genital pores and excretory systems from which it is pushed out through the siphon. These movements contribute to molluscs locomotion.

Bivalves mantle forms a thin membrane surrounding the body and it has two lobes secreting the two valves. Shell is usually symmetric and it has formed by two part, the right and left valves. The shell valves are held together at the animal's dorsum by the ligament, which is composed of the tensilium and resilium. The ligament opens the shell (Fig. 7).

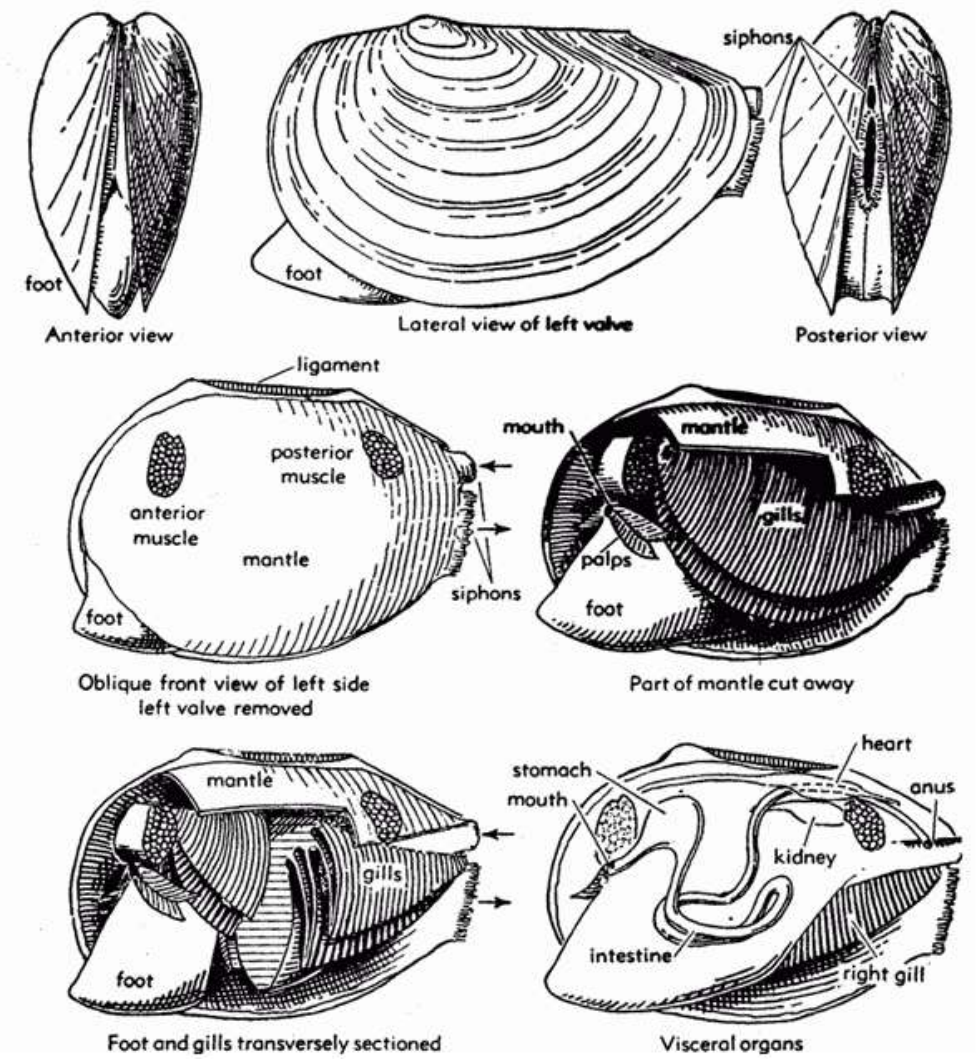

Figure 7 : Bivalve anatomy

Middorsally, the elastic ligaments creates the opening thrust that operates against the closing action of the adductor muscles. The ligament typically develops either externally (parivincular) or internally (alivincular) but comprises outer lamellar, and inner fibrous, layers secreted by the mantle crest. The ligament type is generally characteristic of each bivalve group.

The hinge plate with ligament also possesses interlocking teeth to enforce valve alignment and locking, when closed, to prevent shear. Many variations in teeth structure occur. The outer shell surface may be smooth or entirely striated by grooves parallel, corresponding to the bivalve growth line. The interior surface is always smooth, pearled, and it has the adductor muscles scar. If each valve has a single such scar, the shell is said to be monomyarian. If there are two scars on each valve, the shell is dimyarian. A slender scar often marks the attachment of the mantle edge, and it is called the pallial line or pallial scar.

Bivalves can have the two valves similar, so the shell is said to be equivalve (as in clams and mussels). If valves are dissimilar, molluscs are said inequivalve (for example in scallops). In equivalve shells, the two valves are mirror images of each other and they usually live perpendicular to the substrate. Bivalves with inequivalve shell live lying or merged with a valve at the substrate.

Bivalves head is very small, entirely enclosed within the mantle and shell valves and it isn't in direct contact with the external environment. Cephalic structures including sense organs have been lost, the head having only a mouth and associated labial palps. Bivalves scavengers species have the edges provided with lashes to collect organic debris. Thanks their filtration

system, the palps select organic debris and introduce them to the mouth. Filtering species don't have this system for collect debris and mouth palps receive feeding from gills.

The bivalve foot doesn't have a flat creeping sole but is bladelike (laterally compressed) and pointed for digging. The muscle mainly responsible for movement of the foot are the anterior and posterior pedal retractors. They retract the foot and effect back and forth movements. The foot is extended as blood is pumped into it, and it is prevented from overinflating by concentric rings of circular, oblique and longitudinal fibres, which also help to direct pedal extension and permit fine mobility. During burrowing, the foot is greatly extends anteriorly from between parted shell valves. Taking a grip on the substratum, tipically by dilation of the tip, the pedal retractors pull the shell downward. This is accompanied by sharp closure of the shell valves, forcing water out of the mantle cavity into the burrow, helping to fluidize the sediment, and making movement through it more efficient. So effective is this mechanism that fast burrowers, when removed from the sediment, can swim short distances.

Bivalves produce threads using a byssus gland, located within the organism's foot. Mussels can move slowly by extending a byssal thread, using it as an anchor and then shortening it. The byssus gland is always active during the larval stages, but it may regress or disappear in adult

individuals. When a mussel's foot encounters a crevice, it creates a vacuum chamber by forcing out the air and arching up. The byssus, which is made of keratin, quinone-tanned proteins, and other proteins, is spewed into this chamber (byssus chamber) in liquid form, and bubbles into a sticky foam. By curling its foot into a tube and pumping the foam, the mussel produces sticky threads about the size of a human hair. The mussel then varnishes the threads with another protein, resulting in an adhesive. Byssus is a remarkable adhesive, one that is neither degraded nor deformed by water.

The openings of the mantle through which water enters and exits are inhalant and exhalent siphon, respectively. In Bivalvia living in the sand (clams, cockles), or in tunnels dug into the wood or rock (shipworm), the siphons get longer, especially the inhalant one. In this way the animal's body is protected in deep, but it can continue the filtration of water not commingled with sand or debris for nutrition and breathing.

Near the head, just behind the mouth, there is a pair or more of ganglia and a nerve ring from which two nerve chords arise that reach out through the body. The reproductive methods of molluscs are quite varied. Some species of mollusc are hermaphroditic, while others have separate sexes. The simplest molluscan reproductive system relies on external fertilization, but there are more complex variations (30, 31, 32).

2.5 Molluscs bivalve consumption

Since ancient times bivalve molluscs are considered one of the most appreciated but feared foods. Nowadays shellfish are highly valued by consumers for their nutritional properties. All molluscs have nutrient values similar to each other. The chemical composition in percentage is as follows: water 82,20%; nitrogenous constituents 1,21%; minerals 1,30%; other components 0,04%. Carbohydrates are well represented as glycogen, in an amount ranging from 1% to 3%. Proteins of high biological value constitute about 10% by weight, a quantity less than that found in meat and fish, but comparable to that of eggs; fats are limited (between 1 and 3% by weight) and consist mainly of long-chain polyunsaturated fatty acids. These lipid components are known to play important role in reducing the risk of many degenerative diseases including coronary thrombosis and atherosclerosis. Among their nutrient features, shellfish are an excellent source of vitamin B12 and contain other B vitamins in different amounts. They also contain many minerals, as iodine (essential for the physiology of thyroid), iron (necessary for oxygen binding in hemoglobin in red blood cells), zinc (important for growth and for immune system) and selenium (antioxidant and favoring growth and fertility). Calories in shellfish are very low

(between 70 and 85 calories/100 grams). Molluscs meat has a little quantity of connective tissue and so they are soft and highly digestible. These nutritive features make shellfish suitable for weight loss. Concerning to the excess of cholesterol attributed to certain species of shellfish, especially oysters and mussels, it should be noted that this "dangerousness" can be reduced associating molluscs with low-fat foods. However shellfish are delicious food with high nutritional value and represent a good alternative to meat, eggs and cheese.

In Table 3 it's reported the chemical composition in the main bivalves. Molluscs pattern of consumption is affected by regional food habits. In fact, the consumption of raw shellfish, especially oysters, it's traditional in different areas, in particular on coasts. Since ancient times Romans considered shellfish a real delicacy and they ate especially those coming from Lucrino lake. In literary written by Juvenal, Horace and Petronius there is description of this habit (33, 34).

Chemical composition for 100 g of edible part in the main bivalves			
Composition	**Mussel**	**Oyster**	**Clam**
Edible part %	**32**	**12**	**25**
H_2O g	**82,1**	**85,7**	**82,5**
Proteins g/100	**11,7**	**10,2**	**10,2**
Fats g/100	**2,7**	**0,9**	**2,5**
Cholesterol mg/100 g	**121**	**150**	
Carbohydrates g/100	**3,4**	**5,4**	**2,2**
Vitamins:	***mg/100 g***	***mg/100 g***	***mg/100 g***
Vitamin A	**0,05**	**0,08**	**0,02**
Vitamin B_1	**0,12**	**0,10**	
Vitamin C	**17**	**5**	
Vitamin D	**0,15**	**0,22**	
Vitamin E	**0,2**		
Minerals:	***mg/100 g***	***mg/100 g***	***mg/100 g***
Zinc	**1,87**		
Sodium	**290**	**510**	**36**
Calcium	**88**	**186**	
Iron	**5,8**	**6**	
Phosphorus	**236**	**267**	
Potassium	**320**	**260**	
Chlorine	**455**	**620**	
Copper	**0,09**		**1,2**
Magnesium	**65,7**		

Table 3: Chemical composition for three important bivalves from INRAN (National Research Institute for Food and Nutrition)

2.6 ***Mytilus galloprovincialis***

TAXONOMIC CLASSIFICATION:

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia
Order	Mytiloida
Family	Mytilidae
Genus	Mytilus
Species	*Mytilus galloprovincialis*
Classification	Lamarch, 1819
Common name	mussel

Figure 8 : *Mytilus Galloprovincialis*

Mytilus galloprovincialis (Fig. 8) is a shellfish provided with a dark blue or brown to almost black shell. Shell shape varies by region but it is usually long and tapered. It also tends to grow up to 15cm, although typically only 5-8 cm.The two shells are equal, nearly quadrangular and they are provided with fine concentric transverse growth lines. The outside is black-violet coloured; on one side the rim of the shell ends with a pointed and slightly bent umbo while the other side is rounded.

Inside the shells, visceral mass colour is normally purple-pearled, but it can change according to *Mytilus galloprovincialis* reproductive cycle and sex. Male mussels are usually yellowish-white coloured, while female individuals have a yellow-orange visceral mass. Mantle, visible inside shell, has a violet rim near shells and involves visceral mass (32, 35).

Mytilus galloprovincialis, commonly mussel, is marine benthic species living the North Atlantic ocean and the Mediterranean Sea. Adriatic Mussels can reach 4-6 cm in length in 12 months, the maximum size is about 10 cm. They live fixed on rocks or other objects in shallow-water, joined by the byssus. In mussels, sexual maturity is reached in the first year of life and they are sexually active all the year round with peaks in spring and autumn. *Mytilus galloprovincialis* species have separate sexes and their reproductive system relies on external fertilization. Mussels reproduce by releasing either eggs or sperm directly into the water in a process known as

spawning. During spawning, a mussel may produce up to eight million eggs, each of which is 0.07 mm in diameter. Once fertilization has taken place, a free-swimming larva is formed. The larvae are 'planktonic' meaning they simply drift with the currents. In the past mussels were caught up in natural banks where they spontaneously multiplied or they were harvested on farmed areas of lakes. Nowadays a large part of mussels production is obtained through farming offshore, using semi-floating plants. Fishing on natural banks and on rocks or submerged platforms is still important. Spring and summer, from May to August, are the seasons in which mussels meat is of good quality (36).

In Italy mussels breeding is one of the most important sectors of national aquaculture and it has a long tradition in many coastal regions. Along the Atlantic coasts of the European countries are mainly kept *Mytilus edulis* species, while *Mytilus galloprovincialis* is the mussel species most commonly bred in Mediterranean sea. Mussels farms are developed on coasts of 11 Italian regions, but productions are especially concentrated, in Puglia, Veneto, Emilia Romagna, Friuli and Sardinia, in descending order. These regions account for 80% of national mussels production (data by E.U., Unimar, Ismea e Uniprom).

Campania region has thirty-seven harvested areas that working only for *Mytilus galloprovincialis* production. Many harvested areas are located in Naples district, from the Phlegrean coast to Castellammare di Stabia. In Salerno district there is only one. Some harvesting areas have very ancient origins. In fact, the brackish-water Fusaro Lake, located in the Naples district, was already used by Romans for shellfish farming activities and it is currently one of the most important harvesting areas for *Mytilus galloprovincialis* production (36, 37).

2.7 ***Crassostea gigas***

TAXONOMIC CLASSIFICATION:

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia
Order	Ostreoida
Family	Ostreidae
Genus	Crassostea
Species	Gigas
Classification	Thunberg, 1793
Common name	Pacific Oyster

Figure 9: *Crassostea gigas*

Crassostea gigas (Fig. 9) is one of the largest specie of oysters. It is an estuarine species but can also be found in intertidal and subtidal zones. They prefer to attach to hard or rocky surfaces in shallow or sheltered waters up to 40m but have been known to attach to muddy or sandy areas when the preferred habitat is scarce. It is native to the Pacific coast of Asia and after it has become an introduced species in North America, Australia, Europe, and New Zealand. Therefore, *Crassostea gigas* doesn't develop on Mediterranean sea. Oyster species naturally ranged along the western and southern coasts of Europe and the Mediterranean coast is *Ostrea edulis* commonly known as mud oyster, or edible oyster. It has an oval or pear shaped shell with a rough, scaly surface. The irregular shell has a distinct hooked beak, patterned with delicate foliation. The two halves (valves) of the shell are different shapes, subcircular to circular and inequivalve. Left shell is deeply concave and fixed to the substratum, the right being flat with rougher edges and sitting inside the left acting as a lid. Inner surfaces of both valves are smooth and usually pearly, white or bluish-grey, often with darker blue areas. Valves are held together at their narrow ends by an elastic ligament. No teeth are reported on the hinge. A large central muscle serves to close the valve against the pull of the ligament. The shell is off white, yellowish or cream in colour with light brown or bluish concentric bands on

the right valve. Shell consist of a series of chalky layers which may include laminar and hollow chambers. The hard rough gray shell contains a meat that can vary in color from creamy beige to pale gray, in flavor from salty to bland, and in texture from tender to firm (30, 32).

The shell of *Crassostrea gigas* varies widely with the environment where it is attached and it has an irregular shape. The left valve is slightly convex and the right valve is quite deep and moderately cup shaped. One valve is usually entirely cemented to the substrate. The shells are sculpted with large, irregular, rounded, radial folds with overlapping, concentric lamellae in mature specimens. The oysters have thick rough curly shells that range in colour from silver/gold to white with purple streaks and spots, radiating away from the umbo. The interior of the shell is smooth, pearly white coloured, with a single muscle scar that is sometimes dark, but never purple or black. This particular feature permits to differentiate this species from *Crassostrea virginica*. *Crassostea gigas* small individuals are up to 50-60 mm long, are usually rounded or slightly elongated; mature oysters are regularly stretched in umbo-ventral direction and may reach up to 30 cm (exceptionally 40 cm). However, the average length is 8-15 cm. Visceral mass contains the internal organs, as for all molluscs. External characteristics of oysters don't allow a distinction between two sexes. Pacific oyster has separate sexes, however hermaphrodites sometimes do

exist. The sex of the Pacific oyster can be determined by examining the gonads and can change from year to year normally during the winter months. In certain environmental conditions one sex is favoured over the other. Protandry is favoured in areas of high food abundance and protogyny occurs in areas of low food abundance. In habitats with a high food supply, the sex ratio in the adult population tends to be favoured towards females and areas with low food abundances tend to have a larger proportion of male adults. This species is very fecund with females releasing about 50-200 million eggs in regular intervals (with a rate at 5-10 times a minute) in a single spawning. Once released from the gonads, the eggs move through the suprabranchial chambers (gill), are then pushed through the gill ostia into the mantle chamber and finally they are released in the water forming a small cloud. In males, the sperm is released at the opposite end of the oyster along with the normal exhalent stream of water. A rise in water temperature is thought to be the main cue in the initiation of spawning, as the onset of higher water temperatures in the summer results in earlier spawning in the Pacific oyster. The larvae (named "veliger") of the Pacific oyster are planktotrophic and are about 70 μm at the prodissoconch 1 stage. The larvae move through the water column via the use of a larval foot to find suitable settlement locations. They can spend several weeks at this phase which is

dependent on water temperature, salinity and food supply. Over these weeks, larvae can disperse great distances by water currents before they metamorphose and settle as small spat. Similarly to other oyster species, once the Pacific oyster larvae find a suitable habitat, they attach to it permanently using cement secreted from a gland in their foot. After settlement, the larvae metamorphose into juvenile spat. The growth rate is very rapid in optimum environmental conditions and market size can be achieved in 18 to 30 months. Unharvested Pacific oysters can live up to 30 years (31, 32).

Crassostea gigas is an exotic species, native to the Japanese coast, then introduced on the west coast of the USA in the early twentieth century. In fact United States still remain the largest producers of oysters in the world (60-70% of world production). Harvesting areas are located on the Pacific Ocean coasts, but production has moved to the eastern Pacific coast and in Europe, along the northeastern Atlantic coast, where they found many along the coasts of the British Isles, Portugal and France. In European countries until the middle of last century, oysters were harvested in large natural banks. When they began to reduce, the French, the largest consumers of oysters, had to run for learning techniques of oysters cultivation.

A limited production of *Crassostea gigas* there is along Mediterranean coasts, too. In particular it is developed in Italy, on the Venice area, where *C. gigas* were imported for trade (2, 38).

2.8 Filtration and bioaccumulation of bivalve molluscs

Most species of bivalves are filter feeders. Digestion typically takes place in two phases: extracellular in the stomach and intracellular in the digestive diverticula, opening laterally from the stomach wall (35). Currents of water are drawn into the body through the radula, an anatomical structure used by molluscs as a tongue. It is a minutely toothed, chitinous ribbon, which is typically used for scraping or cutting food before water and food enter the esophagus. Organic particles come into pallial cavity are picked up and caught in the mucus produced by gills. Transport of tiny food particles is effected thanks to the action of numerous cilia, creating an array of tracts and sorting areas within the stomach. The most important organ of extracellular digestion is the cristalline style. It is rotated in its sac by cilia; the head, projecting into the stomach, grinds against a part of the stomach wall lined by a chitinous gastric shield. As it rotates, it dissolves, releasing enzimes and initiating primary extracellular digestion of the mucus-bound food. Products of this process are passed in a fluid suspension into large embayments and thence into the digestive diverticula, where intracellular digestion takes place. Waste material is consolidated in the midgut and

rectum and expelled as firm fecal pellet from an anus opening into the exhalant stream (Fig. 10) (29, 35).

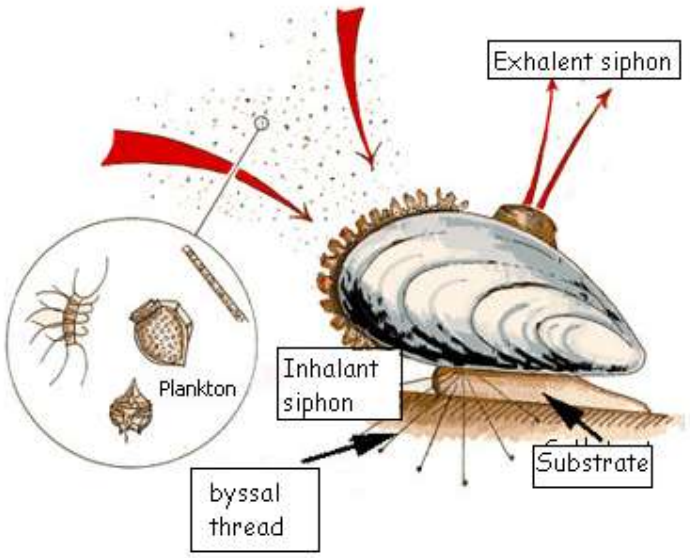

Figure 10 : Bivalves filtration system

This feeding system based on a water filtration system favors the bivalves bioaccumulation of all the suspended particles and substances dissolved in water. Therefore, the nutritional status of shellfish depends to a great extent on environmental conditions, there is a strong relationship between nutrition and environment. The great adaptability of bivalves living in polluted environments means that they can accumulate all the environmental contaminants or pathogens: marine environment conditions mirror molluscs health and finally human health. The seawater contamination with human

sewage sludge carried out health problems that have involved the attention of competent authority. Sewage can carry a large amount of pathogen microorganisms causing infections by oral-fecal transmission. In addition to known microorganisms as *Salmonella*, *Shigella*, *Vibrio cholerae* and to bacteria as *Yersinia* and *Campylobacter* identified recently, seawater are often as carriers of viral particles dangerous from human or animal health (33).

In fact since the 50s, bivalve molluscs have been identified as vehicles of enteric diseases caused by viruses such as Hepatitis A and Norovirus. Despite the improvement of harvesting techniques, viruses represented still an important hazard for human health.

The filtration activity depends on size and species of bivalves and it is also in function of water temperature. In fact mussels can filter about 1.5 liters of water per hour at a temperature of 14° C, the European oysters filter 12 liters at 15° C, while the American one can exceed 18 liters at 20° C. Concerning our seas, during the warmer months, temperature has a positive effect on filtration activity (39, 40).

Therefore, temperature is an important factor that can influence the shellfish bioaccumulation: in ideal experimental conditions, bivalves can reach concentrations up to 10 to 1000 times the pathogens in the water. Viral particles bioaccumulation occurs in the digestive tissues of bivalve

molluscs. Toxins and microorganisms such as bacteria and viruses, especially enteric viruses, are absorbed in the nonspecific form on digestive tissues, there remain adherent after several hours of treatment (2, 34).

Bioaccumulation activity is typical of bivalve molluscs and it is a further hazard for people that eat shellfish entirely, only removing its outer shell. Bioaccumulation means an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the environment. Compounds accumulate in living things any time they are taken up and stored faster than they are broken down (metabolized) or excreted. Bioaccumulation is a normal and essential process for the growth and nurturing of organisms. All animals daily bioaccumulate many vital nutrients, such as vitamins A, D and K, mineral traces, essential fats and amino acids. What concerns toxicologists is the bioaccumulation of substances to levels in the body that can cause harm. Bioaccumulation begins when a chemical passes from the environment into an organism's cells (uptake). Uptake is a complex process which is still not fully understood. Scientists have learned that chemicals tend to move, or diffuse, passively from a place of high concentration to one of low concentration. The force or pressure for diffusion is called the chemical potential, and it works to move a chemical from outside to inside an organism. A number of factors may increase the chemical potential of certain substances, as

lipophilic and hydrophobic factors. The same factors affecting the uptake of a chemical continue to operate inside an organism, hindering a chemical's return to the outer environment. Some chemicals are attracted to certain sites, and by binding to proteins or dissolving in fats, they are temporarily stored. If uptake slows or is not continued, or if the chemical is not very tightly bound in the cell, the body can eventually eliminate the chemical.

A factor affecting bioaccumulation is whether an organism can break down and/or excrete a chemical. The biological breakdown of chemicals is termed metabolism. This ability varies among individual organisms and species and also depends on characteristics of the chemical itself. Therefore, the extent of bioaccumulation depends on the concentration of a chemical in the environment, the amount of chemical coming into an organism from the diet, water, or air, and the time it takes for the organism to acquire the chemical and then excrete, store, and/or degrade it. The nature of the chemical itself, such as its solubility in water and fat, affects its uptake and storage. Water solubility is the ability of a chemical to dissolve in water. Usually, compounds that are highly water soluble have a low potential to bioaccumulate and do not leave water readily to enter the cells of an organism. Once inside, they are easily removed unless the cells have a specific mechanism for retaining them. Many lipophilic chemicals pass into organism's cells through the fatty layer of cell membranes more easily than

water-soluble chemicals. Once inside the organism, these chemicals may move through numerous membranes until they are stored in fatty tissues and begin to accumulate. Equally important is the ability of the organism to degrade and excrete a particular chemical. When exposure ceases, the body gradually metabolizes and excretes the chemical (41).

Key factors determining norovirus bioaccumulation in bivalve molluscs are the environmental conditions, the shellfish active filtration and the high level of viral pollution in water. In particular, viral bioaccumulation is an important hazard for people when bioaccumulation concerns the two species of bivalve molluscs previously described, *Mytilus galloprovincialis* and *Crassostea gigas*, since these products are traditionally eaten raw or slightly cooked.

3.0 COMMUNITY AND NATIONAL LEGISLATION

Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 repeals and replaces the Italian Legislative Decree No 530/92 concerning health regulations applying to the production and marketing of bivalve molluscs for human consumption.

In Annex III, Section VII, Chapter I relates the general requirements for the placing on the market of life bivalve molluscs. They have to come from production areas registered and approved in accordance with EC Regulations; whenever a food business operator moves a batch of live bivalve molluscs between establishments, up to and including the arrival of the batch at a dispatch centre or processing establishment, a registration document must accompany the batch. The registration document must be in at least one official language of the Member State in which the receiving establishment is located and it must contains some informations: the gatherer's identity and address, the date of harvesting, the location of the production area described in as precise detail as is practicable or by a code number, the health status of the production area, the shellfish species and quantity and the destination of the batch. The same indications are required

in the case of a batch of live bivalve molluscs sent from a relaying area or from a purification centre to the others centers registered and approved in accordance with EC Regulations. Food business operators receiving batches must date-stamp the document on receipt of the batch or record the date of receipt in another manner; they must keep a copy of the registration document relating to each batch sent and received for at least twelve months after its dispatch or receipt.

In Chapter II, hygiene requirements for the production and harvesting of live bivalve molluscs are reported. Special attention is given to production areas, in fact they must have fixed locations and boundaries that the competent authority has classified (42).

Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004, Annex II, fixes criteria for the classification of production and relaying areas for live bivalve molluscs. The competent authority must fix the location and boundaries of production and relaying areas that it classifies. Where appropriate, it may do so in cooperation with the food business operator. Three categories areas have been established according to the level of fecal water contamination. The competent authority may classify as being of Class A areas from which live bivalve molluscs may be collected for direct human consumption.

Harvesting areas classified as being of Class B are areas from which live bivalve molluscs may be collected, but placed on the market for human consumption only after treatment in a purification centre or after relaying. Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution Most Probable Number (MPN) test of 4 600 *E.coli* per 100 g of flesh and intravalvular liquid.

The competent authority may classify as being of Class C areas from which live bivalve molluscs may be collected but placed on the market only after relaying over a long period. Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution MPN test of 46 000 *E.coli* per 100 g of flesh and intravalvular liquid (43).

Food business operators may place live bivalve molluscs collected from class A production areas on the market for direct human consumption, only if they have organoleptic characteristics associated with freshness and viability, including shells free of dirt, an adequate response to percussion and normal amounts of intravalvular liquid. Life bivalve molluscs must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the following limits:

- Paralytic Shellfish Poison (PSP), 800 micrograms per kilogram;
- Amnesic Shellfish Poison (ASP), 20 milligrams of domoic acid per kilogram;
- okadaic acid, dinophysistoxins and pectenotoxins together, 160 micrograms of okadaic acid equivalents per kilogram;
- yessotoxins, 1 milligram of yessotoxin equivalent per kilogram;
- azaspiracids, 160 micrograms of azaspiracid equivalents per kilogram.

That standards are laid down in Annex III, Chapter V of Regulation (EC) No 853/2004.

Food business operators may place live bivalve molluscs collected from class B production areas on the market for human consumption only after treatment in a purification centre or after relaying. Molluscs collected from class C production areas may be place on the market for human consumption only after relaying over a long period.

After purification or relaying, live bivalve molluscs from class B or C production areas must meet all of the requirements of Regulation (EC) No 853/2004, Annex III, Chapter V. However, live bivalve molluscs from such areas that have not been submitted for purification or relaying may be sent to a processing establishment, where they must undergo treatment to eliminate pathogenic microorganisms (where appropriate, after removal of

sand, mud or slime in the same or another establishment). The permitted treatment methods are: sterilisation in hermetically sealed containers and heat treatments, involving immersion in boiling water for the period required to raise the internal temperature of the mollusc flesh to not less than 90°C and maintenance of this minimum temperature for a period of not less than 90 seconds, cooking for three to five minutes in an enclosed space where the temperature is between 120 and 160°C and the pressure is between 2 and 5 kg/cm2, followed by shelling and freezing of the flesh to a core temperature of -20°C. Steaming should be under pressure and in an enclosed space.

Further handling must not cause additional contamination or excessive damage to the shells or tissues of the live bivalve molluscs. Food business operators must adequately protect live bivalve molluscs from crushing, abrasion or vibration, not expose live bivalve molluscs to extreme temperatures and not re-immerse live bivalve molluscs in water that could cause additional contamination (42).

Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs establishes the Food safety criteria for live bivalvia molluscs and it fixes only bacteriological limits (for *Salmonella* spp. and *E.coli*). Therefore, Regulation doesn't indicate as safety criteria for molluscs enterical viruses research.

But, in the first part of Reg. (EC) No 2073/2005, in the whereas at point 12, it's written that the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) issued an opinion on Norwalk-like viruses (NLVs, noroviruses) on 30-31 January 2002. In that opinion it concluded that the conventional fecal indicators are unreliable for demonstrating the presence or absence of NLVs and that the reliance on fecal bacterial indicator removal for determining shellfish purification times is unsafe practice. In particular, it's possible read at following points, it may be necessary in certain cases to set harmonized sampling frequencies at Community level, particularly in order to ensure the same level of controls to be performed throughout the Community and finally it's confirmed that criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently (at point 27) (1).

Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, clarifies on the necessity to establish sampling and analysis standard methods complied with Community rules or with internationally recognized rules or protocols. They can be for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation or, in the absence of the above, with other

methods fit for the intended purpose or developed in accordance with scientific protocols (44).

In compliance with EU legislation, Italian Ministry of Health published April 20, 2007 a Recommendation on the research of norovirus in food. In this Recommendation, Ministry marked the ISS as National Reference Laboratory (NRL) for viral contamination in live bivalve molluscs to which sends shellfish samples for the detection of norovirus and enteric viruses in case of alert. However, in order to intensify and spread the controls in all national areas, Italian Ministry of Health selected also other laboratories performing norovirus monitoring activities. That laboratories can carry out norovirus research and control only after appropriate training by ISS personnel authorized to perform the analysis in order to learn the quantitative method (Real Time PCR) used by the Institute (27). The above-mentioned method has been subjected to inner validation by the ISS and it resulted provided of efficiency to research norovirus in live bivalve molluscs as published with Note of the Ministry of Health 24/11/2009 (1).

4.0 ANALYTICAL METHODS FOR NOROVIRUS IDENTIFICATION

During last years, viruses detection was laborious, due to the lack of analytical method sufficiently sensitive, rapid and specific. Especially for noroviruses, it's necessary to consider the high rates of genomic variability which requires the development of a technique able to detect all the known virus serotypes at the same time (45).

The first method used to detect NoVs from stool samples was the direct electron microscopy (Electronical Microscopy, EM) followed by the ImmunoElectro Microscopy, IEM) (7). These tests are still the most widely used analyzing stool samples for the presence of NoVs. EM and IEM allow to identify the viruses only if they are concentrated at least 10^6-10^7 viral particles / mL of stool samples. During norovirus infections you may have a low number of viruses in stool, then IEM tests may give false negative results. Electron microscopy is a laborious technique with a low rate of sensitivity, so it is rarely used for the detection of norovirus from complex food samples. Diagnostic instruments aren't available in all the areas and they have high operating and maintenance costs (46).

Since 1978, tests as RIA (RadioImmuno Assay) and later ELISA (Enzyme Linked Immunosorbent Assay) were widely used for the identification of most common viral strains. Although these assays allowed to analyze a large number of samples, the high specificity of the NoVs antigenic pattern limited its use in epidemiological analysis. ELISA assays resulted good tests to perform a screening of clinical samples suspected of viral gastroenteritis, but they were little used for viral detection in food. In fact these tests give a high rate of false negative results linked to a difficult detection of some circulating strains and to the low sensitivity of assays (8).

Since 1990, the cloning and sequencing of the Norwalk virus complete genome allowed the development of molecular methods which have gradually replaced other diagnostic test for NoVs identification.

During last years, the molecular biology techniques have had a wide application as a method for the analysis of foods of animal origin. Numerous studies have been carried on different foods. These techniques were applied to the detection of pathogenic species, to the species identification in prepared or processed food of animal origin and to the traceability analysis. Many authors used molecular biology and during the first tests, the Polymerase Chain Reaction (PCR) methods were applied (27, 47).

The PCR end-point played an important role in medical diagnostics and for analytical tests in general (48). This method allows the identification of nucleic acids, even if present in traces and caused by minimum or accidental contaminations (up to 0.5%) along the food production chain. However, the end-point PCR is a qualitative test, unable to make a DNA quantitative valuation.

Real Time PCR is a development of the end-point PCR. In fact, Real Time PCR technique is rapid, sensitive and specific and it allowed also the quantification of the starting concentration of a target sequence from a biological sample.

First applications of molecular techniques for norovirus detection were unsatisfying, since the primers were designed on sequences coding for the viral antigens. Many of the viral samples tested positive for norovirus by electron microscopy have not been confirmed by molecular techniques because of the variability antigenic typical of *Norovirus* spp. Since 1994, information on norovirus genetical variability was added to the first genetic knowing, thanks to analysis on additional sequences obtained from the RNA-polymerase region (49).

Genetical variability is still the main obstacle in the development of methods for NoVs detection. Another problem of primary importance for food analysis is the sensitivity of tests. Viruses are usually low concentrated

in foods, then the sensitivity provided by single-round PCR is often not sufficient to obtain the analysis results.

Studies carried out later by nested-PCR were complicated by need to find the most conserved sequences for each strain. In 1989, Ruano et al. proposed booster-PCR method for forensic and population genetic studies, and this technique has since been used for the analysis of food pathogens, too. Booster-PCR is a double-PCR amplification different from the nested-PCR, because it was carried out using the same pair of primers (8).

4.1 Real Time PCR

The best results for norovirus detection from stool, food and environmental samples were obtained using Real Time PCR method. The most important problem is the presence of organic substances which may interfere with the amplification reaction, especially when the food sample is a complex matrix. Hence, it is necessary using specific extraction protocols to avoid the presence of false negative results caused by Real Time PCR inhibitors. In order to limit any false negative results linked to inhibition problems, an internal control is introduced; it is amplified together with viral nucleic acid targets. Another problem for norovirus detection by Real Time PCR is the choice of primers. In fact these viruses are genetically divided into several genogroups and genotypes characterized by unstable antigenical constellation.

The high number of norovirus gastroenteritis has increased scientific interest to this pathogen, hence the Italian Ministry of Health published April 20, 2007 a Recommendation on the research of norovirus in food. Ministry lists all the National Reference Laboratory (NRL) for viral contamination in live bivalve molluscs to which sends shellfish samples for

the detection of norovirus and enteric viruses in case of alert. The C.N.Q.A.R.A. of the ISS was marked, too. The Institute's researchers are also included in the group of experts appointed by the Ministry to elaborate a standard method for the detection of enteric viruses in food. The scientific team has been working since 2007. The application of Real-Time PCR resulted the best, among the techniques studied for norovirus detection in bivalve molluscs (27).

Researchers at ISS worked on a protocol one step Real Time RT-PCR. In the same Real-time RT PCR reaction viral nucleic acid of mengovirus was also amplified and it was used as the extraction internal control.

The Real-Time RT-PCR was carried out using primers and probes reported in Table 4.

The two probes for NoVs GI and GII were labelled with 6-carboxyfluorescein (FAM) at the 5' end and with 6-carboxytetramethylrhodamine (TAMRA). The probes for mengovirus were labelled with 5' 6-carboxyfluorescein (FAM) and 3' MGB (minor groove binder) (0.2 μM) (50).

Real-Time PCR Primers & Probes	
NOROVIRUS GI	
QNIF4 (FW)	**CGC TGG ATG CGN TTC CAT** (50) (N is A, C, G or T)
NV1LCR (REV)	**CCT TAG ACG CCA TCA TCA TTT AC** (15)
NVGG1p (PROBE)	**TGG ACA GGA GAY CGC RAT CT** (15) (Y è C o T e R è A o G)
NOROVIRUS GII	
QNIF2 (FW)	**ATG TTC AGR TGG ATG AGR TTC TCW GA** (51) (R is A or G and W is A or T)
COG2R (REV)	**TCG ACG CCA TCT TCA TTC ACA** (52)
QNIFS (PROBE)	**AGC ACG TGG GAG GGC GAT CG** (51)

Table 4 : Primers and probes

Retrotranscription and one step PCR were performed on ABI Prism 7700 SDS detector (Applied Biosystems) or on MX300P apparatus (Stratagene). Amplification was carried out using Platinum® Quantitative RT-PCR ThermoScriptTM One- step System (Invitrogen, France). Five microliters of nucleic acid extract or control was added to 20 µl of reaction mixture containing 1X thermoScript reaction mix, 900 nM reverse primer, 500 nM forward primer, 250 nM Taqman probe, 1X Rox Reference dye (50X) and 0.5 ThermoScript Plus/Platinum Taq enzyme mixture. The ending total volume was 25 µl for each well. Rox is commonly used as a reference dye in Real-time experiments for normalization of the fluorescence signal of the reporter fluorophore. The reference dye fluoresces at a constant level during

the reaction. Rox Reference dye was used at different concentrations, 1X for ABI Prism detector and 0,1X for MX300P apparatus. RT-PCR conditions were: retrotranscription for 60 min at 55°C and amplification for 5 min at 95°C and 45 cycles of 15 s at 95°C (denaturation), 1 min at 60°C (annealing), and 1 min at 65°C (extension) (Fig. 11).

Mix RT-PCR

(kit Platinum Quantitative RT-PCR ThermoScript One-Step System – INVITROGEN)

- 1x Thermoscript reaction mix
- Primer 1 (500 nmol/L)
- Primer 2 (900 nmol/L)
- Probe (250 nmol/L)
- ROX (1/0,1 x)
- ThermoScript Plus / Platinum Taq (2,5 U)
- H_2O

RNA

Step description		Temperature and time	Number of cycles
RT		55° C 1 h	1
Preheating		95° C 5 min	1
Amplification	Denaturation	95° C 15 sec	45
	Annealing-extention	60° C 1 min 65° C 1 min	

Figure 11: RT- PCR Real Time Protocol

Two negative samples and a positive sample consisting of RNA extracted from Norovirus were used as amplification controls for each reaction. All samples were tested undiluited and in tenfold dilutions and amplification tests were performed in triplicate using the described protocols. Samples were selected as positive when *Ct* (*Cycle threshold*) value was lower than 44 for two times at least.

The One-Step Real Time RT-PCR method has been subjected to inner validation by the ISS and it resulted provided of efficiency to research norovirus in live bivalve molluscs as published with Note of the Ministry of Health 24/11/2009 (3).

After validation, that PCR protocol was used to research norovirus in live bivalve molluscs in the two experiments described below.

5.0 NOROVIRUS DETECTION IN SHELLFISH HARVERSTED AND COMMERCIALIZED IN CAMPANIA REGION

5.1 Shellfish sampling

A total of n. 163 bivalve molluscs were sampled during period July 2007/ April 2010. No 117 samples were *Mytilus galloprovincialis* obtained from 15 different harvesting areas of Campania region, in Naples, Caserta and Salerno districts, sited on the Tyrrhenian sea coasts. Seven harvesting areas were collocated on areas classified as A (product suitable for direct consumption) and eight collocated on B areas (product suitable for consumption after depuration treatment) in accordance with EC Regulation No 854/2004 (43) (Fig. 12).

Figure 12 : Sampled harvesting areas.

Each sampling has carried out in ideal weather conditions and recording environmental (temperature, salinity, dissolved O_2) and meteorological conditions (rains, winds, stream directions). As appropriate, harvesting areas were sampled once, twice or three times.

For each sampling, mussels were taken from the four corners of harvesting area and from center point, when it was possible. During first times, samples were carried out at all points. For the next sampling, the number of points has been reduced, up to a single representative sample of the whole harvesting areas. Each sample was made by a pool of *Mytilus galloprovincialis* coming from upper, middle and bottom of row on harvesting areas corners or center points.

In addition, No 46 samples of bivalve molluscs belonging to different species were also taken. They were purchased from retails sited in the three districts of Naples, Caserta and Salerno. Molluscs belonged to the species are listed in Table 5.

Samples	Species
35 *Mytilus galloprovincialis*	
4 *Ensis minor*	
3 *Chamelea gallina*	
2 *Tapes philippinarum*	
1 *Ostrea edulis*	
1 *Glycymeris glycymeris*	

Table 5: Shellfish samples bought by different retailers in Campania Region

No 35 samples of *Mytilus galloprovincialis* have been bought by different retailers in the Naples, Caserta and Salerno districts. Nineteen store points were regularly registrated in accordance with the Regulation (EC) No 852/2004, while the other sixteen stores were not registrated and sold

unpacked mussels (53). All other samples were purchased from registrated retailers. Only samples of *Tapes philippinarum* and two of three samples of *Chamelea gallina* came from not registrated retailers. The only sample of *Glycymeris glycymeris* has come to our laboratories after a seizure in a restaurant of Naples district.

Mussels taken from harvesting areas and shellfish purchased from different retails were shipped in an insulated box to laboratories of Section of Food Inspection of the Faculty of Veterinary Medicine, Federico II University in Naples. Samples were immediately examined for NoVs contamination.

5.2 Preparation of samples and nucleic acid extraction

Fifteen live individuals were selected at random for each pool and they were washed with clean water to remove mud and debris eventually present. The opening of valves was made aseptically by sterile blade. Digestive tissue was dissected by sterile tweezers and scissors and then it was placed in a Petri dish. Digestive gland was finely chopped with a sterile razor and mixed in Stomacher blender. Tissue was used for the nucleic acids extraction.

An aliquot of 2.0 g of chopped tissue was transferred to a centrifuge tube and 2 ml of proteinase K (0.1 mg/ml) were added for cellular lysis. Solution was vortexed for about 1 min. and incubated at 37°C in a shaking incubator for 60 min. Then solution was placed in a water-bath at 65°C for 15 min to inactivate the enzyme. Finally, samples were centrifuged at 3000 x *g* for 5 min and supernatant (approximately 4.5 ml) was transferred into a tube for the last stages of extraction.

Viral RNA extraction and purification were performed by the Nuclisens extraction kit (BioMerieux) according to the manufacturer's instructions. The kit allows to isolate nucleic acids using a silica-based system patented by Boom. This technology has been widely accepted by the scientific

community as the gold standard method for the isolation of nucleic acids. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms in the presence of this agent (54).

After addition of silica particles, a solution containing mengovirus concentrated 10^8 TCID50/ml was mixed to extraction solution. Mengovirus was used as internal control that allows to validate the efficiency of extraction reaction, i.e. to verify that the reaction was conducted in order to ensure a good recovery of viral RNA from the matrix.

Washing stages carried out through specific solutions of NucliSENS MiniMag extraction kit were performed using the magnetic ramp and permitted to remove any inhibitors of the amplification reaction (Fig. 13).

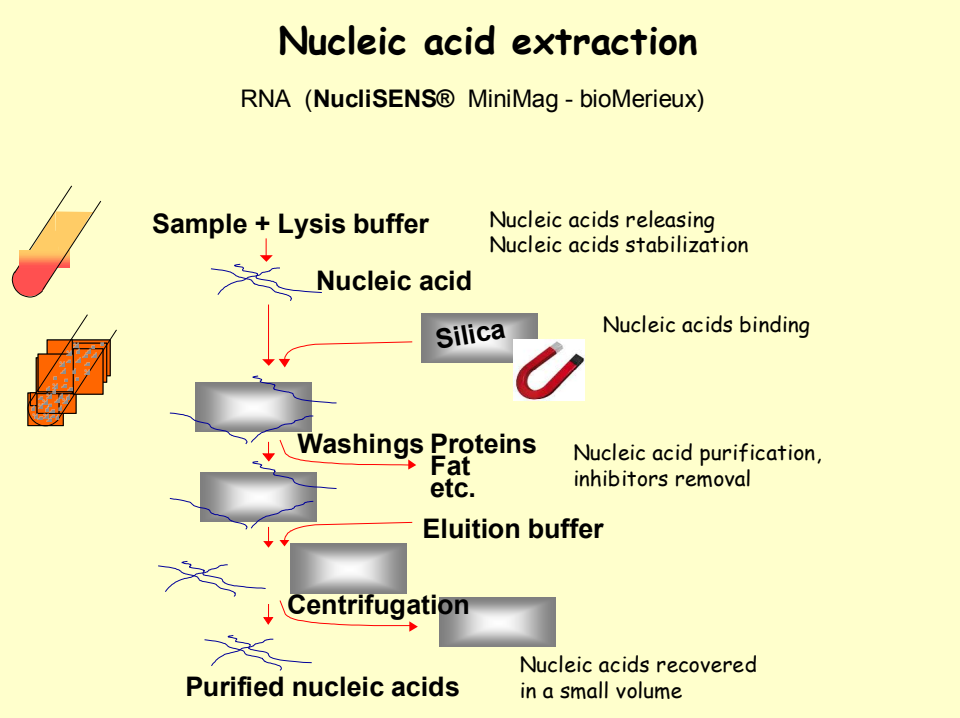

Figure 13 : Nucleic acids extraction

Nucleic acids were recovered in 100 µl of elution buffer (BioMerieux kit) and were transferred in 1.5 mL micro tubes. Finally, they were kept a refrigerator at 4 ° C until they were used for Real Time RT PCR analysis.

5.3 Results

It was always possible to extract good concentrations of viral RNA from samples analyzed. The one-step real-time PCR allowed to amplify RNA of mengovirus internal control, RNA of norovirus GI and GII using specific primers and probes (Table 4) (15).

Mengovirus amplification gave *Cycle threshold* values which showed a good recovery of viral RNA from the matrix.

Amplification runs containing samples were considered acceptable when *Cycle threshold* (*Ct*) value felt in the range defined by *Ct* positive controls and *Ct* negative controls excluded. Figure 14 set out some of the One-Step Real Time RT-PCR results obtained by samples analysis.

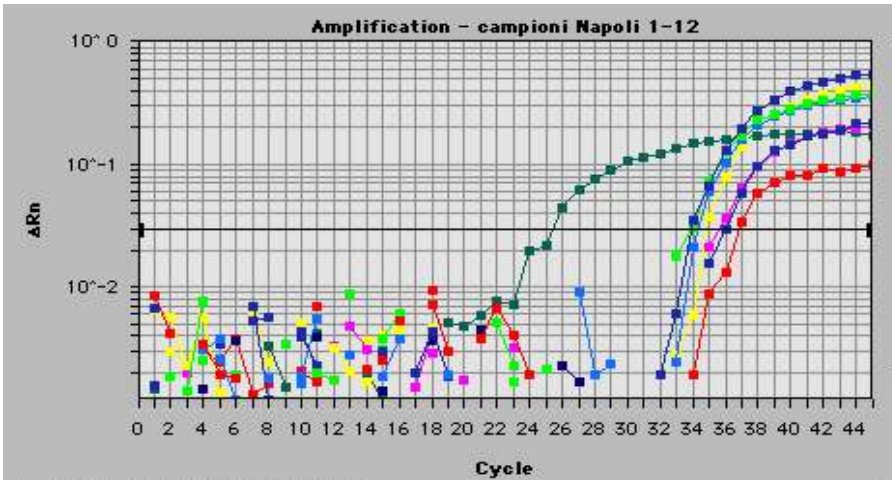

Figure 14 : Norovirus amplification in Real-Time PCR

The figure shows a Cartesian coordinate system: on the y-coordinate (ordinate) the values of different concentrations of norovirus nucleic acid expressed in logarithm are reported, while on the x-coordinate (abscissa) the values of *Ct*, i.e. the first cycle in which the signal exceeds the treshold, are reported. Signals that do not exceed the treshold line represents the value of background emissions. Dark green curve shows the amplification curve of positive control, consisting of RNA extracted from norovirus (*Ct* 25.33).

Besides dark green curve, there are the amplification curves of some samples tested. Here the first amplification cycle, the *Cycle threshold*, is greater than the positive control and less than *Ct* 45. That samples can be judged positive for the norovirus presence.

Negative controls and negative samples didn't show amplification curves under 45 cycles of Real Time RT PCR reaction.

Results obtained by harvesting areas are in Table 6.

Harvesting areas	Mussels sampling: pool coming from the three deeps of rows																
	1° sampling July 2007 / May 2008						2° sampling September 2008 / June 2009						3° sampling November 2009 / March 2010				
Area A	**NW**	**NE**	**SW**	**SE**	**C**		**NW**	**NE**	**SW**	**SE**	**C**		**NW**	**NE**	**SW**	**SE**	**C**
1	-	-	-	-	-		-	-	-	-	-		NS				
2	-	-	-	-	-		-	NS	-	-	+		+				
3	+	+	+	+	NS		-	-	-	-	-		NS				
4	-	-	-	+	-		NS						NS				
5	-	-	-	-	-		-	-	-	-	NS		NS				
6	-	-	+	-	-		NS						NS				
7	-	-	-	-	-		+	+	+	+	+		NS				
Area B	**NW**	**NE**	**SW**	**SE**	**C**		**NW**	**NE**	**SW**	**SE**	**C**		**NW**	**NE**	**SW**	**SE**	**C**
1	+	-	-	+	+		+	+	+	+	+		+	-	+	+	+
2	-	-	-	+	-		+	+	+	+	+		NS				
3	+	+	+	+	NS		+						+				
4	+	+	+	+	NS		+						+				
5	+	+	+	+	NS		NS						NS				
6	NS	+	NS	+	NS		+						+				
7	-	-	-	-	-		NS	+	NS	+	NS		+				
8	+	+	+	+	NS		+						+				

Table 6 : Sampled harvesting areas.

NW=North-West, NE=North-East, SW=South-West, SE= South-East, C=Center,

NS= Not sampled.

Thirteen out fifteen sampled Campania region harvesting areas (86,6%) resulted positive for the presence of NoVs in one of the three samples taken at least. Five positive areas were located in areas classified as A. The eight sampled harvesting areas of B class resulted positive.

Totally, positive shellfish pools were n. 60 out of 117 total (51.3%). All positive samples showed NoVs GII presence and for 29 of these samples (almost 50%) both norovirus genogroups (GI and GII) were observed.

Thirty-three out forty-five (73%) samples bought by retailers of Campania region resulted positive for the presence of NoVs. They were positive for Norovirus GII and more than half of these samples (18 out 33) resulted positive for NoV GI, too. Positive results are showed in Table 7.

Purchased samples	**Positive results for NoV**	
	NoV GII	**NoV GI + NoV GII**
35 *Mytilus galloprovincialis*	**27**	**15**
4 *Ensis minor*	**0**	**0**
3 *Chamelea gallina*	**3**	**2**
2 *Tapes philippinarum*	**2**	**1**
1 *Ostrea edulis*	**1**	**0**
1 *Glycymeris glycymeris*	**1**	**1**
TOT positive samples	**34**	**19**

Table 7: Results for shellfish bought at retails

Only eight samples of *Mytilus galloprovincialis* and the four samples of *Ensis minor* gave negative results.

As for the harvesting areas samples, in more than half of the positive samples (55.8%) from retailers there is the presence of both NoV GI and NoV GII. *Glycymeris glycymeris* sample, come to our laboratories after a seizure in a restaurant of Naples district, resulted contaminated by both noroviruses genogroups, too.

Concerning authorized and regularly registered retailers of *Mytilus galloprovincialis*, 17 out 19 samples showed positive results for norovirus presence. Positive results were obtained by for 10 out 16 not registered stores that sold unpacked mussels.

These results represent only a semi-quantitative value for norovirus presence in shellfish samples. In fact, there aren't scientific references for norovirus standard curve in order to obtain a reference quantitative value.

Observing amplification curves and *Ct* values in shellfish samples, it's possible noting that NoVs GII has registered high contamination values, reaching *Ct* values of 25.83. In addition, when both norovirus genogroups were found in a same sample, NoVs GII registered a *Cycle threshold* value lower than NoVs GI. It confirms the increasing contamination registered for this pathotype all over the world. There were no differences of *Ct* between

samples purchased from authorized and regularly registered retailers and not registered ones.

6.0 NOROVIRUS BIOACCUMULATION IN *CRASSOSTEA GIGAS* SAMPLES

From December 2009 to May 2010, studies on norovirus bioaccumulation in oyster (*Crassostea gigas*) samples have been carried out at the Virology section of IFREMER (Microbiology Laboratory -MIC) located in Nantes (France). Studies aimed to evaluate the ability of *Crassostea gigas* samples to bioaccumulate norovirus in their digestive tissues (DT) based on a quantitative approach.

Experiments were conducted in ideal environmental conditions, with clean sea water and constant temperature. Bioaccumulation metabolic activity of oysters was studied and it was also tested if climatic differences could affect shellfish ability to bioaccumulate.

Bioaccumulation tests were carried out in aquariums filled up with clean sea water experimentally polluted. Norovirus GI.1 and GII.3 strains were used for bioaccumulation experiments. The two genotypes were tested separately and then experiments with a solution containing a cocktail of both viruses were carried out. This was done to evaluate any possible competition between the two different norovirus genotypes during bioaccumulation activity in the oysters.

6.1 Oyster samples

Bioaccumulation studies were carried out on *Crassostea gigas* samples. At the beginning of the study, one large batch of clean oysters was taken and located in a clean area (near Brest) (Fig. 15).

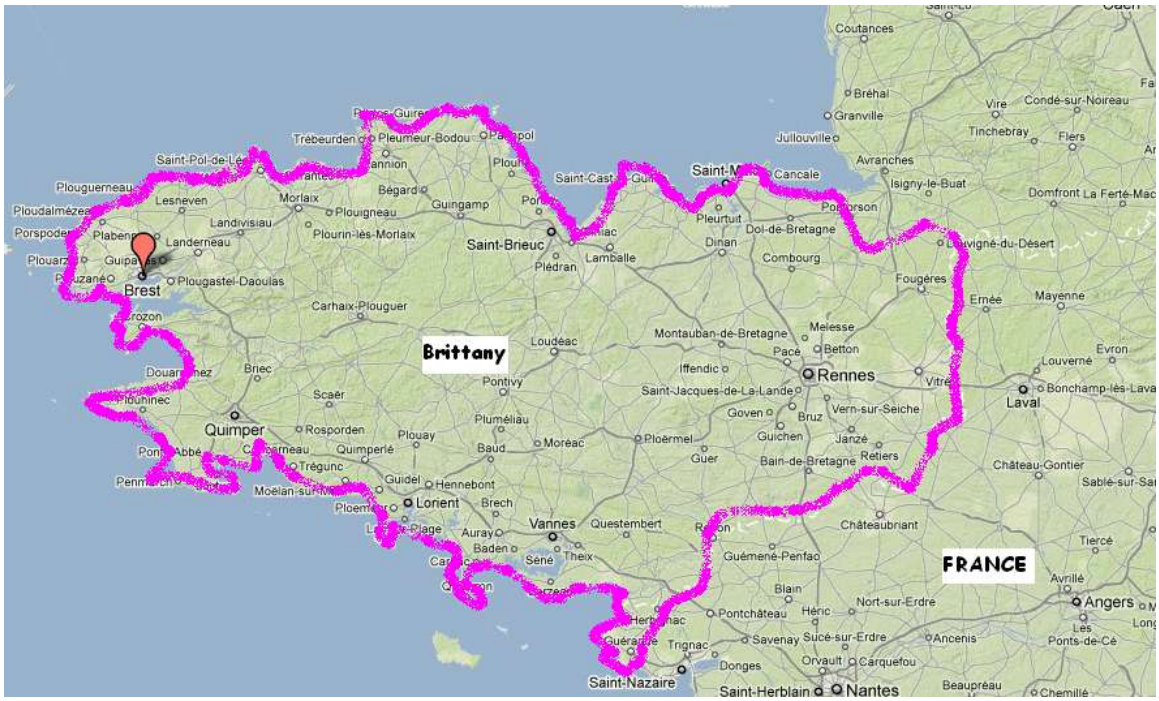

Figure 15 : Brittany region, France. Bay of Brest

On request, one sample was collected and in order to maintain quality and integrity of the animals, *Crassostea gigas* were placed in wooden boxes well packed and filled to avoid any movement or shaking of the oysters during transport. Samples were transported in refrigerated trucks, maintaining the cold chain until arriving to IFREMER. *Crassostea gigas*

arrived at the section of the Virology, Laboratory of Microbiology (MIC) IFREMER, Nantes within 24 hours of collection. Packaging was removed and the oysters were immediately transferred in a refrigerating room at 9± 1°C, where they were placed in aquariums containing clean sea water at a constant temperature of 9 °C.

The sea water used for the experiments came from tanks inside the IFREMER laboratories, and it was clean and not infected by virus. In preparation for the tests, water was brought to the temperature of 9 °C and then it was spilled into the aquariums. These precautions were taken to avoid samples undergo sudden temperature changes.

Oysters placed in the aquarium in the refrigerating room at 9± 1°C were let for acclimatation in this system for 24 h to allow them to rehydrate, revitalize and adapt to new environmental conditions. Then samples were transferred to aquariums containing 300 ml of clean seawater per oyster to start bioaccumulation experiments.

During the storage and throughout all the tests, oyster vitality was constantly monitored by visual observation.

6.2 Preparation of norovirus solutions for bioaccumulation experiments

Bioaccumulation experiments were carried out using sea water seeded with viral suspensions of norovirus GI.1 and GII.3. NoVs were extracted from diarrhoeal stools of clinical specimens collected from individuals hospitalized and displaying symptoms characteristic of norovirus infection. Stool samples were tested at IFREMER laboratory and it was possible to confirm the presence of NoVs GI.1 and NoVs GII.3. Later, stool samples were titrated in order to know the viral concentration per gram of stool and the viral solutions for bioaccumulation tests were prepared.

Viral extraction was carried out with NucliSens extraction kit (BioMerieux) using Minimag (BioMerieux) apparatus. Stools were diluted to a final concentration of 10% (weigt/volume) suspension in sterile water. Nucleic acids were extracted from this supension using the same kit and then nucleic acids (NA) were amplified by real-time RT-PCR. NAs were analyzed at various concentrations, pure, one and two log dilutions in sterile water.

During the preliminary studies, bioaccumulation experiments showed that the best results obtained using solutions containing 10^6 virus / g of stool, both for GI.1 and GII.3 strains. Hence, the viral concentration used for the experiments was 10^6 virus / g of stool. For the bioaccumulation experiments

using a cocktail of NoVs, the viral solutions contained both viruses at the same concentration of 10^6 virus / g of stool.

NoV GI.1 and GII.3 suspensions were prepared the first day of each experiment and stored at 4 °C throughout the experiment.

In order to control viral concentrations, for each bioaccumulation experiment, solutions were titrated on the first and the last day of experimentations.

6.3 Bioaccumulation experiments: preliminary tests

During the period from December 2009 to February 2010, two bioaccumulation tests were carried out. They allowed to develop a testing protocol.

The first experimental test lasted 24 hours and it was necessary to check the *Crassostea gigas* bioaccumulation activity of NoV GI.1, GII.3 and GII.4. These three viral genotypes were tested separately, each concentrated 10^6, 10^7 and 10^8 viruses / g of stool. In addition it was carried out a bioaccumulation experimentation using a cocktail solution of NoV GI.1 and GII. 3, both at the same concentration. Viral cocktail was tested at two concentrations of 10^6 and 10^7 virus / g of stool (Table 8).

NOROVIRUS	Viral Concentration
GI.1	10^6 virus / g of stool
GI.1	10^7 virus / g of stool
GI.1	10^8 virus / g of stool
GII.3	10^6 virus / g of stool
GII.3	10^7 virus / g of stool
GII.3	10^8 virus / g of stool
GII.4	10^6 virus / g of stool
GII.4	10^7 virus / g of stool
GII.4	10^8 virus / g of stool
GI.1 + GII.3	10^6 virus / g of stool
GI.1 + GII.3	10^7 virus / g of stool

Table 8 : Norovirus solution concentration for preliminary tests

Bioaccumulation experiment was carried out in refrigerating room at 9± 1° C. Twelve aquariums containing 300 ml of clean sea water for each oyster were used. Water temperature was always at 9± 1°C. For each aquarium, a system of active oxygenation of the seawater was provided, allowing oyster's oxygenation. Oysters were taken at random from the storage aquarium and 12 *Crassostea gigas* individuals were placed in each bioaccumulation aquarium. Sea water of eleven aquariums was contaminated with the corresponding eleven viral solutions (Table 8). Last

aquarium was not seeded by any virus solution, representing the negative control of the experiment.

Six individual oysters were collected after 1 h from each aquarium and the last 6 oysters after 24 h of bioaccumulation. Oysters were immediately transported to the laboratory for analysis.

During the second experiment of preliminary stage, bioaccumulation test was conducted, keeping fixed all environmental parameters set and described above. Viral contamination was performed using NoV GII.3 10^7 virus/g of stool. Bioaccumulation lasted three days. Four aquariums were used: three aquariums were for bioaccumulation tests and the last one was the negative test. Sea water was daily contaminated by a fixed concentration of virus. Water in aquariums was changed every 24 hours by new clean sea water seeded with the viral solutions. Each aquarium was daily emptied, well rinsed and then filled by the same volume of clean sea water. The water temperature inside the aquariums and the environmental temperature of refrigerated room were constantly monitored and kept stable during all the experimentation. Bioaccumulation test was carried out as showed in Figure 16.

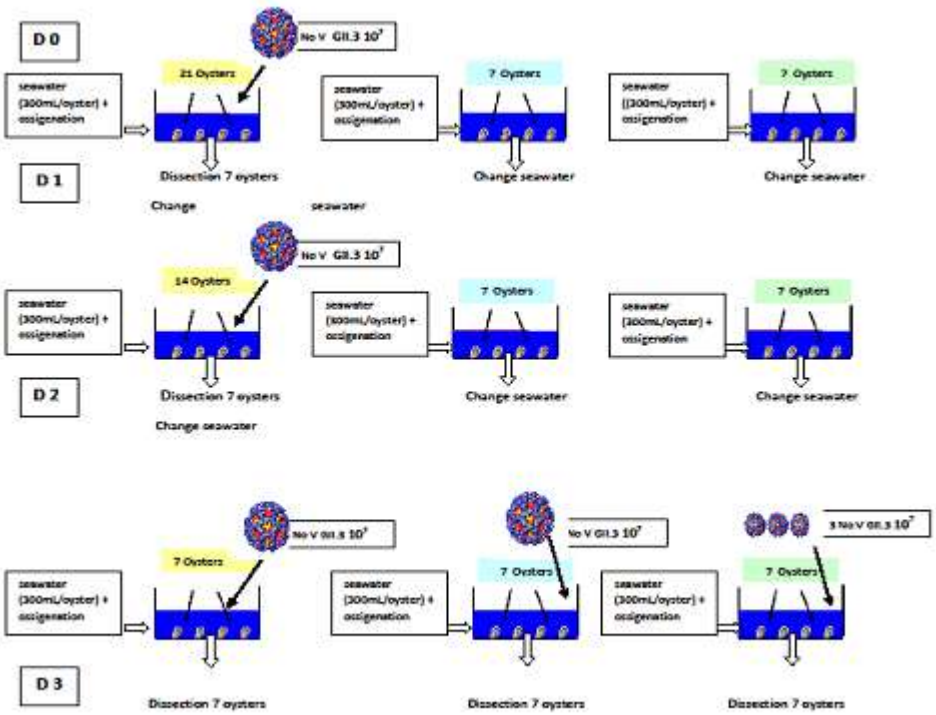

Figure 16: Preliminary bioaccumulation test lasted three days

6.4 Bioaccumulation experiments

From February to May 2010 others experiments were conducted using the two strains NoVsGI.1 and NoVsGII.3. They were individually tested three times (six experiments) and then a cocktail of both viral genotypes was used two times for others bioaccumulation tests.

During the experiments, environmental conditions were the same used for preliminary studies. Parameters were daily checked to ensure that they remained constant throughout the bioaccumulation test. Each of the eight experiments lasted ten days (from D0 to D9) and every time three aquariums (A, B, C) were used. The aquariums simulated molluscs natural conditions of filtration and bioaccumulation in sea water. Only oysters that appeared alive, viable and filtering during the experiment were kept for bioaccumulation tests.

For each test, eight *Crassostea gigas* individuals were used as negative control. They were placed in a clean aquarium.

6.4.1 Bioaccumulation tests using a single NoV genotype

Three bioaccumulation tests were conducted using NoVs GI.1, and three tests were carried out by NoVs GII.3.

In all tests, bioaccumulation studies were conducted as showed in Figure 17. The day D0, 32 *Crassostea gigas* individuals were placed in the aquarium A, 8 samples were put in aquariums B and C, respectively.

Sea water in the aquarium A was contaminated with solution of NoV concentrated 10^6 virus / g of stool. Water was daily changed and replaced with the same volume of clean sea water, after polluted by stool solution containing the same NoV genotype at the same concentration. Starting from the results of previous experiments, it was considered appropriate to pick up samples for analysis with the frequency of D1, D3, D6 and D9. Eight oysters from aquarium A were randomly collected each time and analyzed to study bioaccumulation at different viral concentrations of 10^6virus / g stool, $3x10^6$virus / g stool, $6x10^6$virus / g stool and $9x10^6$virus / g stool.

In aquarium B eight oysters were immersed in the sea water. Samples in this aquarium were studied to establish if the viability and the filtration activity remained constant in oysters from the first to the last day of the experiments. In fact, sea water was contaminated with the viral solution at a concentration of 10^6 virus / g of stool only at day D8.

The same experimental procedure was carried out for the aquarium C. The only difference is that on day eight, a solution containing 9 x 10^6 NoVs / g of feces was seeded. Oysters were analyzed to highlight any possible dose-dependent differences in bioaccumulation, comparing to aquarium B.

Crassostea gigas in aquariums B and C were collected for analysis on day D9, after 24 hours of viral solution addition.

Bioaccumulation test was carried out as showed in Figure 17.

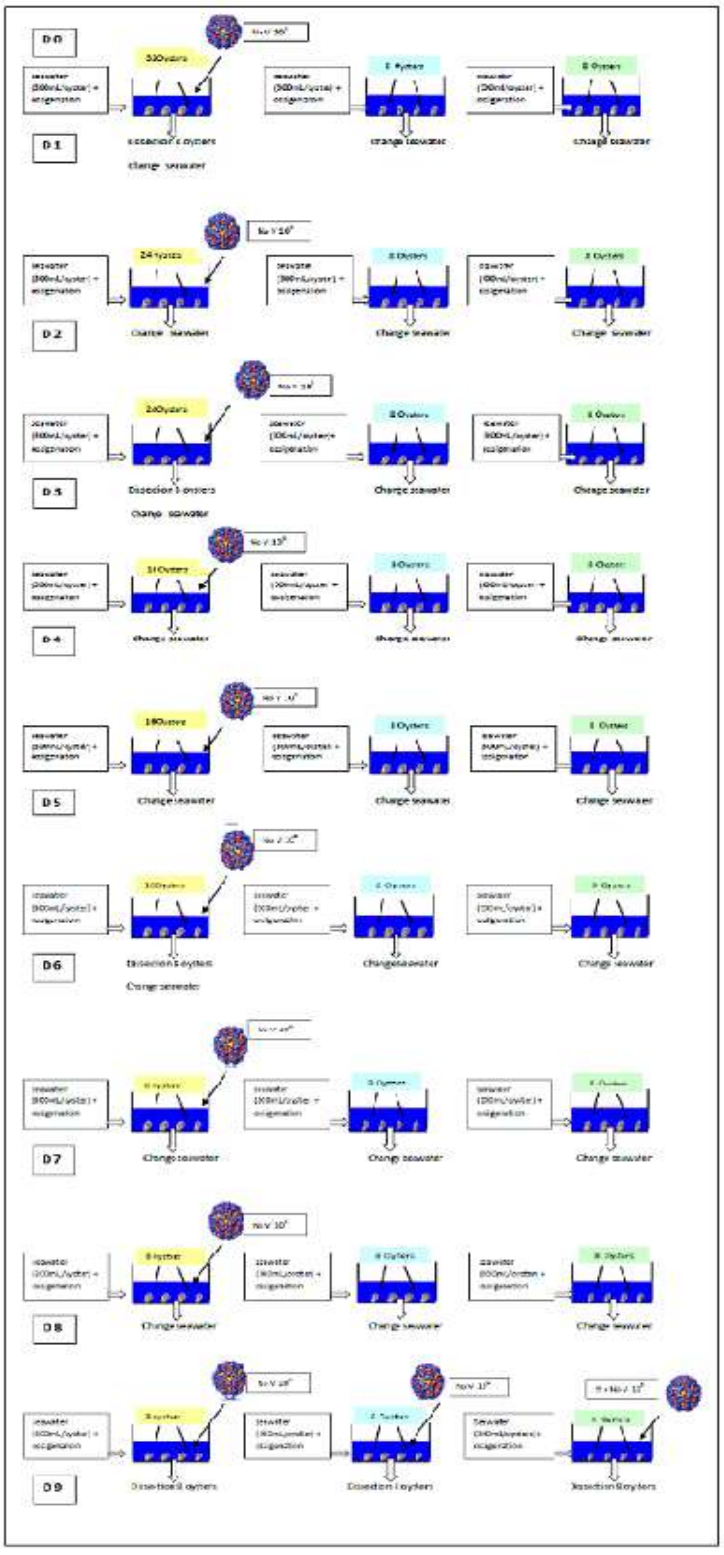

Figure 17: Bioaccumulation tests using a single NoV genotype

6.4.2 Bioaccumulation tests using a NoVsGI.1 and NoVsGII.3 cocktail

Two different experiments were performed with a viral cocktail of NoV GI.1 and GII.3. Sixteen *Crassostea gigas* individuals were placed in each aquarium, A, B and C. Oysters in aquarium A were analyzed to study bioaccumulation at different concentrations of the viral cocktail. Samples in aquariums B and C were tested to value if the presence of both viral genotypes lead to a different bioaccumulation of the two genotypes in shellfish.

Sea water of aquarium A was daily contaminated with the viral cocktail at concentration of 10^6 virus/g of stool, and it was carried out the same experimental procedure used for aquarium A in bioaccumulation tests with single NoVs strain.

In the other two aquariums, every day after 24 hours, water was contaminated with solutions containing NoVs GI.1 for the aquarium B and NoVs GII.3 for the aquarium C, respectively. Viral solutions were titrated 10^6 virus / g of stool for both No strains.

For all aquariums, eight oysters were picked up for analysis at day D1, after 24 hours of viral bioaccumulation. Last eight oysters were collected at the day D9.

6.5 Extraction of viral nucleic acids

Crassostea gigas samples used for NoV bioaccumulation were collected from the aquarium and immediately transported to the laboratory for the analysis. They were opened and it was carried out the dissection of digestive tissues and the viral nucleic acid extraction and purification. For each bioaccumulation test, oysters were taken separately from aquariums A, B and C, avoiding cross-contamination. All samples from the same aquarium were shucked and oyster bodies were placed in sterile Petri dish. Digestive tissues were dissected by sterile tweezers and scissors and then digestive glands were finely chopped with a sterile razor. Tissues were weighed and 8 *Crassostea gigas* individuals of medium-size resulted about an average of 3 - 4 grams of digestive tissue. Digestives tissues were placed in 1.5 ml Eppendorf tubes and stored in a freezer at -20 º C before nucleic acids analysis.

An aliquot of 1.5 g of chopped tissue was taken for the viral nucleic acids extraction. After homogenization of tissues, extraction was carried out using buffers that allowed the viral particles elution (Glycine buffer), the removal of proteins and other residues (Chloroform - Butanol solution), the fats separation (Cat-Floc T solution) and finally the virus concentration (PEG /

NaCl solution). Oysters digestive tissue was placed in a sterile glass tube containing 2 mL of glycine buffer pH 9.5 and 10 µL of a solution containing mengovirus concentrated 10^8 TCID50/ml were added. Mengovirus was used as process control of extraction. Digestive tissues were homogenized using a potter- Eveljhem by and then poured into a sterile 50 mL tube type Falcon. Three mL of glycine buffer pH 9.5 and six ml of chloroform-butanol solution (50:50) were added. The mixture was vortexed for about 30 seconds and 500 µL of a Cat-Floc T solution were added. Samples were afterwards placed at room temperature on a rocker platform for 5 minutes to facilitate mixing. Then tubes were centrifuged at 4 ° C for 15 minutes at 13,500 *g*. The supernatant was recovered by sterile pipette and placed into new 50 ml tube containing 3 mL of PEG / NaCl solution. PEG precipitation was carried out by agitation on rocker platform at 4 ° C for 1 hour. The mixture was centrifuged at 4 ° C for 20 minutes at 11,000 *g* and the supernatant was thrown out. The PEG pellet on the bottom of the tube was suspended in 1 mL of sterile water at 56 ° C.

Viral nucleic acids recovery was performed by NucliSens MiniMag extraction kit (BioMerieux) according to the manufacturer instructions (54). The eluate obtained was transferred to a sterile 1.5 mL microtube and placed in refrigerator at 4 ° C for real time RT PCR analysis.

6.6 Results

During bioaccumulation experiments from December 2009 to May 2010, oysters vitality was daily checked and the mortality rate was very low. Room and water temperatures were monitored, too. Both controls have not showed any variations, rising or decreasing of maximum two degree (from 8°C to 11°C). The same control was done for the system of active oxygen pump inside the aquariums, that results always in working throughout the experiments. Norovirus solutions used for bioaccumulation tests were titrated on the first and last day of each test in order to check a constant viral concentration during the experiment. Titration was performed by Real Time RT- PCR method and it showed always the same viral concentration with less than one log variation from D0 to D9. Results reported in Tables 9 and 10 are expressed in copies of NoVs RNA per gram of digestive tissue analyzed.

GII.3	Copies/g (G 0)	Copies/g (G 9)
16/02/2010	$1{,}8 \times 10^7$	1×10^6
10/03/2010	$1{,}8 \times 10^7$	$6{,}50 \times 10^6$
13/04/2010	$4{,}9 \times 10^6$	$1{,}3 \times 10^6$

GI.1	Copies/g (G 0)	Copies/g (G 9)
10/03/2010	1×10^6	$7{,}1 \times 10^5$
13/04/2010	$6{,}3 \times 10^5$	$3{,}5 \times 10^5$
27/04/2010	$7{,}1 \times 10^5$	$4{,}7 \times 10^5$

Table 9: Results of titration for norovirus GI.1 and GII.3 solutions used in bioaccumulation tests

GI.1+GII.3	Copies/g GI.1 (G0)	Copies/g GI.1 (G 9)	Copies/g GII.3 (G 0)	Copies/g GII.3 (G 9)
27/04/2010	$7{,}1 \times 10^5$	$4{,}7 \times 10^5$	$3{,}7 \times 10^6$	$1{,}5 \times 10^6$
18/05/2010	$2{,}4 \times 10^6$	$1{,}4 \times 10^5$	$2{,}2 \times 10^6$	$1{,}5 \times 10^6$

Table 10: Results of titration for norovirus cocktail (GI.1 + GII.3) solutions used in bioaccumulation tests

Analysis on negative controls gave negative results in all bioaccumulation tests. Therefore it was possible to exclude a starting viral contamination of oysters.

The first bioaccumulation tests were carried out as preliminary tests and they have allowed fixing a protocol for the following experiments. The results of the first test performed to verify the *Crassostea gigas* bioaccumulation activity in presence of Norovirus genotypes GI.1, GII.3 and GII.4 are in Table 11.

GI.1	Copies/g (1 h)	Copies/g (24 h)
10^6/g stool	$1,1x10^5$	$1,8x10^6$
10^7/g stool	$1,7x10^6$	$1,5x10^7$
10^8/g stool	$5,8x10^6$	$3,6x10^7$
Neg.		0

GII.3	Copies/g (1 h)	Copies/g (24 h)
10^6/g stool	$4x10^4$	$1,3x10^5$
10^7/g stool	$5,5x10^5$	$9,4x10^5$
10^8/g stool	$4x10^6$	$3,2x10^7$
Neg.		0

GII.4	Copies/g (1 h)	Copies/g (24 h)
10^6/g stool	$1x10^2$	$1,7x10^2$
10^7/g stool	$1,2x10^2$	0
10^8/g stool	$1,3x10^2$	0
Neg		0

Viral cocktail

GI.1	Copies/g (1 h)	Copies/g (24 h)
10^6/g stool	$9,6x10^3$	$6,5x10^5$
10^7/g stool	$9x10^5$	$1,2x10^6$
Neg		0

GII.3	Copies/g (1 h)	Copies/g (24 h)
10^6/g stool	$1,7x10^4$	$1,7x10^5$
10^7/g stool	$1,4x10^5$	$1,2x10^6$
Neg		0

Table 11: Results of the first bioaccumulation test.

Crassostea gigas samples showed a good bioaccumulation rate for NoV GI.1 and NoV GII.3 one hour after the addition of viral solution in the aquariums. Comparing the results obtained for the two strains, NoV G1.I

was concentrated in oysters digestive tissue with one Log greater rates compared to No GII.3 and GII.4. It was noticed that bioaccumulation activity increased during he experiment, , increasing in both cases of 1 log after 24 h. Concerning the GII.4 strain used in the experiment, bioaccumulation rate was very low. In fact it resulted about 10^2 after 1 hour and then it was zero after 24 hours of bioaccumulation for the concentrations of 10^7 and 10^8 copies virus / gram of stool. The results obtained with the viral cocktail showed that NoV bioaccumulation was about 2 log lower than the viral concentration in the starting solutions, after 1h from the virus addition. Bioaccumulation activity increased of 1 log after 24 h, as with NoVs GI.1 and GII.3 individually tested. *Crassostea gigas* samples didn't show large differences in bioaccumulation activity in the presence of solutions containing viral genotypes used individually or combined in a cocktail.

The second preliminary test lasted three days and *C. gigas* bioaccumulation was conducted using viral solution containing only NoV GII.3 at a concentration of 10^7 viruses per gram of stool (Fig. 16). Results are shown in Table 12 .

GII.3	Aquarium 1	Aquarium 2 (10^7/g feci)	Aquarium 3 (3 x10^7/g feci)	Aquarium Neg
G 1	6,1 x 10^6			0
G 2	1,3 x 10^7			
G 3	8,7 x 10^6	1 x10^7	1,2 x10^7	

Table 12: Results of the second bioaccumulation preliminary test.

Oyster samples from the first aquarium were daily analyzed 24 h after viral addition. They showed an increasing bioaccumulation activity between D1 and D2. During the last day of experiment, a little decrease was detected even after a third addition of virus. Samples placed in the second and in the last aquarium were used to quantify the bioaccumulation activity after 24 h, when NoV GII.3 was concentrated 10^7/g of stool and 3x10^7/g of stool, respectively. Results showed that *Crassostea gigas* were active and filtering until the last day of test, but the bioaccumulation activity was not affected by amount of virus added in aquariums.

Results of last bioaccumulation tests using NoVs genotypes GI.1and GII.3 and using a NoVsGI.1 and NoVsGII.3 cocktail are in Tables 13, 14, 15.

NoVs GI.1 10^6/g stool	10/03/2010			13/04/2010			27/04/2010		
	Aq. A	Aq. B	Aq. C	Aq. A	Aq. B	Aq. C	Aq. A	Aq. B	Aq. C
D1	$1,8x10^3$			$9,5x10^3$			$8,5x10^3$		
D3	$7,5x10^3$			$9,1x10^3$			$4,5x10^4$		
D6	$3,5x10^4$			$4,5x10^4$			$7,1x10^4$		
D9	$6,5x10^4$	$4,5x10^3$	$3,9x10^4$	$3,5x10^4$	$5,4x10^4$	$7,7x10^4$	$9,5x10^4$	$1,0x10^4$	$2,0x10^5$

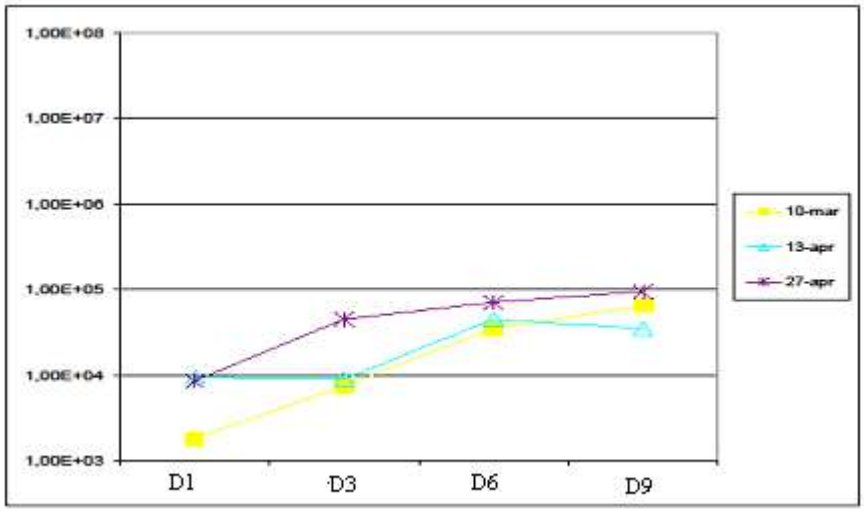

Table 13: Results of last bioaccumulation tests using NoVs genotype GI.1

NoVs GII.3 **10^6/g stool**	16/02/2010			10/03/2010			13/04/2010		
	Aq. A	Aq. B	Aq. C	Aq. A	Aq. B	Aq. C	Aq. A	Aq. B	Aq. C
D1	$1,3x10^6$			$1,2x10^4$			$5,2x10^4$		
D3	$2,8x10^6$			$1,3x10^5$			$3,3x10^5$		
D6	$3,2x10^7$			$1,4x10^5$			$6,0x10^5$		
D9	$9,6x10^6$	$1,1x10^6$	$1,7x10^7$	$3,4x10^5$	$2,1x10^5$	$7,0x10^5$	$4,9x10^5$	$6,1x10^4$	$8,5x10^5$

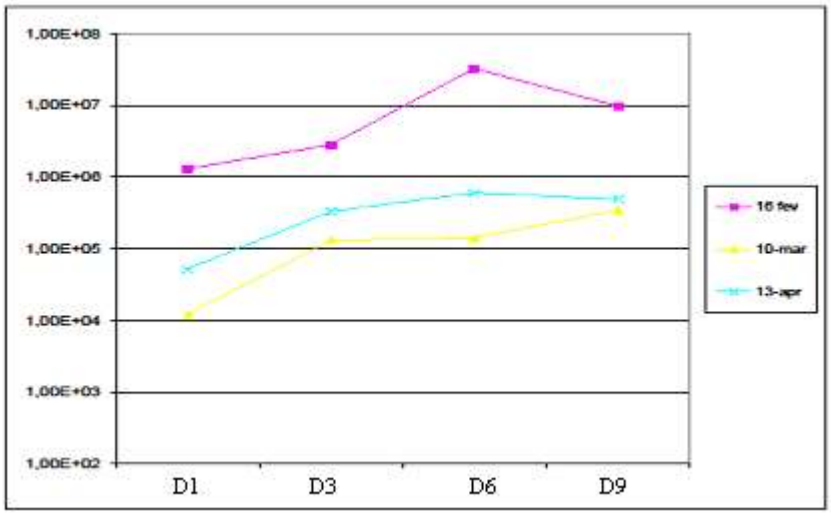

Table 14: Results of last bioaccumulation tests using NoVs genotype GII.3

NoVs GI.1+GII.3 10^6**/g stool**	27/04/2010				18/05/2010			
	Aquarium A		Aquarium B	Aquarium C	Aquarium A		Aquarium B	Aquarium C
	GI.1	GII.3			GI.1	GII.3		
D1	$1,0x10^4$	$2,5x10^4$	$8,5x10^3$	$8,1x10^4$	$1,1x10^4$	$9,5x10^4$	$5,0x10^3$	$6,4x10^5$
D9	$2,0x10^5$	$1,4x10^5$	$9,5x10^4$	$4,0x10^5$	$3,2x10^5$	$2,7x10^6$	$6,0x10^5$	$1,5x10^6$

Table 15: Results of last bioaccumulation tests using NoVs cocktail

Comparing experiments conducted either with NoVs GI.1 or GII.3 strains, it resulted that *Crassostea gigas* samples bioaccumulate genotype GII.3 more than GI.1 one, when there are the same experimental conditions and the same concentration of virus. In fact, there is a difference of about 1 log between the two genotypes. It was possible to observe an increase of 3 logarithms only during an experiment. Larger bioaccumulation of NoVs GII.3 was also confirmed in tests carried out using the viral cocktail.

In the three experiments to value bioaccumulation of Norovirus GI.1 concentrated 10^6/ gram of stool, viral concentrations detected in the digestive tissue of oysters collected from the aquarium A at days D 1, D 3, D 6 and D 9, were similar. They did not differed in the three tests, observed variations being within less than a Log range. During the nine days, virus concentration is constantly grown, with the exception of a little decrease occurred between D 1 and D 3 for an experiment. Bioaccumulation results obtained analyzing oysters from controls B confirmed that filtration activity

is the same until the last day of tests. In addition, environmental conditions of experiments were optimal, promoting a bioaccumulation greater than that observed in oysters from aquariums A at D 1.

Finally, analyzing the samples of aquariums C was possible to verify that bioaccumulation was constant throughout the experiments. In fact, in presence of the same virus concentration, oysters were able to bioaccumulate Norovirus until the last day of experiments in the same amount bioaccumulated from samples of aquarium A during the first 24 hours. For all experiments, except one, oysters have always bioaccumulated Norovirus in concentration greater than concentration recorded from samples collected by aquarium A at the day D9.

7.0 CONCLUSIONS

The C.N.Q.A.R.A. of the Istituto Superiore di Sanità (ISS), in cooperation with us, developed the One-Step Real Time RT-PCR protocol. This molecular method resulted provided of efficiency for norovirus detection in live bivalve molluscs. ISS, selected as National Reference Laboratory (NRL) for viral contamination in live bivalve molluscs, after inner validation, published the quantitative method with Note of the 22/10/2009. Later, Italian Ministry of Health with Note of the 24/11/2009 recommended the Real Time PCR application to research norovirus in live bivalve molluscs (3). The Note from Ministry of Health was a response to European Community requests to revise or establish safety criteria for foods, especially concerning viruses detection.

On 30-31 January 2002, Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) pointed out that the conventional fecal indicators are unreliable for demonstrating the presence or absence of NLVs and the reliance on fecal bacterial indicator removal for determining shellfish purification times is unsafe practice. In particular, it may be necessary to set safety criteria for pathogenic viruses in live bivalve

molluscs. Finally Scientific Committee confirmed that criteria should be established only when analytical methods are developed sufficiently (1).

Concerning the Ministerial Note of the 24/11/2009, only now that the method for norovirus detection has been set at Community level, it will be possible assess the risk from eating molluscs, carrying out sampling plans. It will be possible to revise food safety criteria, too.

For this purpose, One-Step Real Time RT-PCR protocol was applied to research norovirus during the two successive experiments.

Results of analysis carried out on shellfish harvested and commercialized in Campania region showed a high norovirus contamination in a food that is traditionally one of the most consumed in this region (37).

Thirteen out fifteen sampled harvesting areas (86,6%) resulted positive for the presence of NoVs in one of the three samples taken at least.

Norovirus detection in shellfish harvested in seawater classified as A area, confirms that fecal bacteria can not be considered reliable indicators for norovirus presence in these foods, as established by European Community (1).

In harvested areas placed in B areas, norovirus contamination was found in all samples and no significant differences were observed during the different seasons.

On the other hand, norovirus infection is sometimes called "winter vomiting disease", because people often get them during the winter months. That is possible thanks to the high resistance of the virus at low temperatures (23). The indiscriminate presence of norovirus could be caused by a constant and remarkable release at sea by sewage. It is also important to remark the high sensitivity of the molecular method that detect the virus more effectively than the previous analysis methods.

High positivity (73%) was recorded in mussels bought by retailers of Campania region. Only eight samples of *Mytilus galloprovincialis* and all the samples of *Ensis minor* gave negative results. Positive results were obtained by 10 out 16 (more than 50%) not registered stores that sold unpacked *Mytilus galloprovincialis*. This is not a surprising result, since at not registered stores the conditions of sampling, transport, storage and marketing are often carried without respecting the health and hygiene standards.

Concerning authorized and regularly registered *Mytilus galloprovincialis* retailers, 17 out 19 samples showed positive results for Norovirus presence.

The high positivity rate could be as suspect of the habit of using "refresh" of mussels during marketing. In this case, shellfish are re-immersed or sprinkled with water that does not have the microbiological characteristics established by Community legislation.

In addition to the above mentioned causes, norovirus detection in the *Glycymeris glycymeris* sample seized by a popular restaurant located in the Naples district, may be also brought by a contamination occurred during food preparation and linked to the non-hygienic conditions in restaurant kitchens.

In this monitoring experiment, all norovirus detected by Real Time RT PCR in positive bivalve mollusc samples were typed, and all NoVs resulted belonging to GII genogroups. For 29 out 60 positive samples collected by harvesting areas and for 18 out 34 positive samples purchased by retailers were observed both norovirus genogroups (GI and GII). *Glycymeris glycymeris* sample seized by the restaurant also resulted positive for both norovirus genogroups. This result confirms the high circulation of genogroup II in agreement with the world references. The conclusion is that there is no competition between the two norovirus genogroups.

During last experiment, it was possible observed that norovirus solution made for bioaccumulation experiment and stored in refrigerator at 4°C, kept a constant viral concentration from D0 to D9. That confirms the high stability of norovirus at low temperatures.

The physiological activity of filtration in bivalve molluscs, and in particular in *Crassostea gigas*, resulted very active until the last day of

experimentation. In presence of the same virus concentration, oysters were able to bioaccumulate in 24 hours a norovirus amount higher than the quantity of virus gradually bioaccumulated during the nine days of tests.

Oysters have bioaccumulated a norovirus amount proportional to virus quantity dissolved in the seawaters of harvesting areas. This result remarks once again the importance to establish new criteria of classification for seawater in shellfish harvesting areas, setting safety criteria for pathogenic viruses and in particular for norovirus.

The tests carried out to study noroviruses filtration and bioaccumulation in *Crassostea gigas* samples and performed with single viral genotypes, showed that the norovirus GII.3 is the best bioaccumulated pathotype in digestive tissue of mussels (25). Following analysis proved that, adding a constant quantity of norovirus daily, shellfish bioaccumulate in their tissues a virus concentration less than 2 logarithms of the amount in seawater, if they are in ideal environmental conditions.

It is also shown that *Crassostea gigas* samples can filter and bioaccumulate an increasing amount of virus, up to a maximum concentration of about 10^6 after nine days of experimentation.

Norovirus have a low infectious dose from 10 to 100 virus particles (23).

In Brittany it's traditional eating a dish of 6-7 *Crassostea gigas* served raw and dressed only by a few drops of lemon. Considering the above, it may represent a hazard for consumer health.

Results of tests carried out using a norovirus GI.1 e GII.3 cocktail showed that there is no competition between the two viral genotypes, in fact oysters bioaccumulate a same concentration of both norovirus genotypes. Scientific studies confirmed that there are many norovirus viral strains with different antigenic properties and the immunity to norovirus is usually incomplete, only temporary and probably not extended to different genotypes. Thus individuals may be protected for only a few months following infection (6). We can conclude that shellfish represent a greater hazard for the consumer when molluscs are harvested in seawater polluted by both norovirus genotypes.

Results of experiments carried out in the course of my PhD studies confirm the importance to revise the safety criteria for shellfish and to re-establish new depuration times for bivalve molluscs harvested in the different areas. It's also supported that in order to ensure the safety of food, it is necessary to consider all aspects of the food production chain as a continuum, from and including primary production, until sale or delivery of bivalve mollusc at the canteens or restaurants. Each production stage may have a potential impact on food safety. The safety and confidence of consumers are of

paramount importance and they are two principles in Food Hygiene Package of European Regulations.

Regulation (EC) No 178/2002 of the European parliament and of the council laying down the general principles and requirements of food law, introduces the concepts of traceability and transparency. Controls must be made at all stages of production, processing and distribution of the food production chain, not only on the final food product. It's important to recognize hazards, identify critical control points and establish monitoring procedures and safety criteria for each food production chain (55).

In this context it is crucial to apply the H.A.C.C.P. (Hazard Analysis and Critical Control Point) method, focusing on hazards which might result from the stages of harvesting up the marketing and consumption of bivalve molluscs. In fact it's still important consider that these products are traditionally consumed raw or undercooked.

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