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Prevalence of faecal indicator organisms and human bacterial pathogens in bivalves from Maputo Bay, Mozambique.

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Photo: Betty Kronkvist

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“Förekomst av fekala indikatororganismer och humant bakteriella patogener i musslor från Maputo Bay, Moçambique”

Abstract

This paper describes a pilot study of the faecal contamination of the water in Maputo Bay, Maputo, Mozambique, concentrated in shellfish. Shellfish like clams are an important protein source for the inhabitant. Clams are filter-feeding shellfish and as so concentrate microorganisms from the water reservoir. During a period of two months, clams from the local market and from gatherers were analysed for faecal contamination (coliform bacteria) and presence of *Salmonella* spp. Two different collection sites were chosen according to the assumptive pollution degree and when the high tide prevented gatherers from harvesting, clams were bought at the local market.

The bacterial concentration in clams was analysed directly after collection, and growth in room temperature was analysed by leaving the clam samples over night. The results from the direct analyses show that many of the samples contained high concentration of coliform bacteria (up to $1,7 \times 10^5$ bacteria per 100 g clam meat). The bacterial concentration in ten out of the twelve samples exceeded the allowed limit for human consumption. The results from the analyses after 24 hours storage in room temperature, were not significant. In six out of ten samples, the bacterial concentration was higher after the storage. In three samples the concentration was lower after the storage and in one sample, the concentration was the same as before. *Salmonella* spp. was detected in five of the twenty-eight clam samples originated from the three sites, both before and after storage.

Further analyses, i.e. bacterial identification, antibiotic resistance pattern and possible pathogenicity were performed in Sweden. The antibiotic resistance of the *E. coli* strains were in general sensitive to the eleven antibiotics tested. However, ten of eleven strains showed low sensibility against Ampicillin and Cefadroxil. One *E. coli* strain was resistant to

Ampicillin and one strain to Cefadroxil. Six of the eleven strains showed low sensibility against Ceftacidime. The five detected *Salmonella* strains were tested for antibiotic resistance of nine different antibiotics. The sensitivity against these antibiotics was generally high, but the five strains showed low sensitivity against Cefadroxil. One of the five detected strains was resistant to Ampicillin while the other four showed low sensitivity. None of the strains showed resistance against Chloramphenicol.

The results show that the detected strains were rather harmless and none of the strains carried the virulence genes tested (haemolytic and aerobactin genes).

Table of Contents

1. Introduction	5
1.1 Aim with this study	5
1.2 Indicator organisms	6
1.2.1 <i>Escherichia coli</i>	7
1.2.2 <i>Salmonella</i> spp.	7
1.3 Different clam species	7
1.4 Antibiotic resistance	8
1.5 Pathogenicity of the detected strains	8
1.6 Why is this study important?	9
2. Material and methods	10
2.1 Area description and sample collection	10
2.2 Sample preparation	11
2.3 Detection and analysis of <i>Escherichia coli</i>	11
2.4 Detection and analysis of <i>Salmonella</i> spp.	12
2.5 Test for antibiotic resistance	12
2.6 API-20 [®] , coliform identification	12
2.7 Multiplex-PCR	13
2.8 Blood agar plates	13
3. Results	14
3.1 MPN	14
3.2 Coliform identification	19
3.3 Antibiotic resistance, <i>E. coli</i>	19
3.4 Identification of <i>Salmonella</i> spp.	20
3.5 Antibiotic resistance, <i>Salmonella</i> spp.	21
3.6 Virulence gene detection	21
4. Discussion	22

Acknowledgement

References

Appendix

1. Introduction

1.1 Aim with this study

Mozambique is a sub-Saharan country situated on the southeast coast of the African continent, with 2400 km of coastline to the Indian Ocean. The country is one of the poorest in the world with an average income per capita of about \$200 per year. Mozambique is a former Portuguese colony and after the independence in 1975, decades of civil war have marked the country. The democracy is considered to be stable since 1994. Technical and financial support in building a stable infrastructure, government and commerce is provided by international help. The industrial base of the country is very small and the greatest part is found in the outskirts of Maputo, where South African and Australian companies invested and built an aluminium smelter. The investments have been successful, providing the first export products for the country. Thanks to the hydropower station in central Mozambique, energy is abundant and reliable.

In tropical and sub-tropical developing countries, a high percentage of the wastewater is discharged untreated into the ocean where seafood is gathered. This constitutes a major health hazard (Wu 1999). In Maputo, most of the waste areas are situated in residential area and approximately 30% of the buildings are connected to the sewer system. The Maputo storm water system is supplied with the overflow of the Maputo river and some buildings dump their wastewater into the system. Even if the treatment plant is well maintained, it lacks chemical treatment. Some gardeners use water from the settlement ponds to water their crops. Groundwater contamination from pit latrines and storm water effluent is polluting the Maputo Bay to the extent that swimming is inadvisable in many areas. The Ministry of Health tests faecal coliform levels regularly, and there was in 2001 a general ban on consumption of shellfish from the bay. Coping with this problem will require massive capital investment in new sewerage and treatment plant capacities.

As the Mozambican industry is small, only about 50 factories create emission. MOZAL, the aluminium smelter, in the inner part of the Bay is considered to be harmless, even though the inhabitants of Maputo do not agree. New regulation concerning medical waste has been drafted, supporting autoclaving before incineration. In general, the country has strong laws against pollution, but there are no regulations to implement those laws (The Blacksmith Institute 2001).

The agriculture activity along Maputo Bay is low due to the sandy soils with low fertility. Chicken and goat farming are only pastoral activities that are practiced for home economics. As a result, people living next to shores are highly dependent on fishing. A costal area can be contaminated with pathogenic microbes through many different sources. Indeed, a WHO-report from 1987 states that the most clearly identified health risk associated with costal pollution is urban wastewater. Bivalves, like clams, are filter-feeding animals that can concentrate many different types of microorganisms from large volumes of water. The consumers might then be infected by pathogens through ingestion of bivalves harvested in contaminated areas. The most common ways to consume bivalves are either raw or lightly cooked, and since the whole animal is consumed (including the viscera and gills), this significantly contributes to the risk of catching a diarrheic disease. Raw and lightly cooked molluscan shellfish have indeed a long history as vectors of infectious agents and marine biotoxins. Unfortunately, the record in Mozambique is poor due to no federal requirements for reporting gastroenteritis of unspecific nature, and in cases of mild gastroenteritis few victims

ever seek treatment. This means that the data acquired might be misleading and not reflect the true magnitude of the social and economic consequences of the illnesses (Rippey 1994).

1.2 Indicator organisms

Bivalvia are filter-feeding organisms and one kilo (exclusive shell) might filtrate approximately 90 litres of water per hour. The microorganisms in the water reservoir are concentrated in the bivalvia and clams can therefore be used as an indicator of water quality. In order to examine this, microorganisms are generally used that indicate contamination of the water sample. The indicator organism is generally associated with bacteria normally found in the human intestine and might thereby signal a contamination of other potential pathogens, including viruses. One group of indicator organism that is widely used is the coliform group. The bacteria in this group are normally not found in water. The group includes bacteria as *Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter aerogenes*.

E. coli is often used as an indicator organism as it is found in large number in human and other warm-blooded animal intestine. Another reason is that it can easily be cultivated. The presence of *E. coli* in water does not always indicate human faecal input, and methods are needed to identify the source as being human (HS) or non-human (NHS) (Parveen *et al.* 2000). When the enteric bacteria are excreted into the water, they die at a slower rate than pathogenic bacteria as *Salmonella* spp. and *Shigella* spp. and its presence might thereby indicate presence of other pathogens in the water (Madigan *et al.*, 2000). Studies show that bacteria in general are reduced to undetectable levels under depuration of clams, while viruses are reduced at a much lower rate. This indicates that even if the bacterial concentration is low, viruses might be more resistant and more persistent than bacteria (Doré and Lees 1995).

Two different categories of bacterial pathogens from the enteric tract in humans can be found in bivalves. 1) Pathogens circulating in the society that have humans or other terrestrial animals as their main reservoir. These pathogens can reach the marine environment through sewage outlets and temporarily accumulate in bivalves. 2) Marine bacteria like *Vibrio* spp. (*V. cholera*, *V. parahaemolyticus*, *V. vulnificus*), which have the sea as their natural habitat. These bacteria can either cause infection through ingestion of “clean” water or shellfish or be transmitted by faecal contamination of water and food.

1.2.1 *Escherichia coli*

E. coli is an enteric bacteria and a common inhabitant of the mammalian intestine. The characteristics of the rod-shaped bacteria from this gamma *Proteobacteria* group are anaerobic or facultative anaerobic growth, a gram-negative cell wall and that they are non-spore-forming. Some strains of *Escherichia* are highly pathogenic. Diseases caused by *E. coli* include for example diarrhea, dysentery, haemolytic uremic syndrome (kidney failure), bladder infections, pneumonia and meningitis. *E. coli* is commonly implicated in diarrhea in infants. Enteropathogenic strains of *E. coli* are also becoming more frequently implicated in dysentery-like infections and generalized fevers. For example EPEC and EHEC are major causes of infant diarrhea in developing countries (DeVinney *et al.* 1998). EPEC is Enteropathogenic *E. coli* that is responsible for infant diarrhea. EHEC is enterohemorrhagic *E. coli* strain O157:H7 responsible for hemorrhagic colitis. The EHEC toxin is related to *Shigella dysenteriae* and the bacteria have a fimbriae structure that allows the bacteria to adhere to the gastrointestinal cells (Benson 2004). Different sets of virulence genes are the reason to why the strains cause different diseases.

As a result of the different pathogenicity, it is important to identify the *E. coli* strain in order to identify strains causing outbreaks. Before molecular biology, serotyping was the best method for classification. This identification is based on variable bacterial surface molecules. Two types of *E. coli* surface structures form the basis for serological classification system: the O antigen of LPS (O) identifies the serogroup of a strain, and flagella (H) antigen identifies its serotype. In this study, *E. coli* will principally be used as an indicator organism but some additional tests have been performed.

1.2.2 *Salmonella* spp.

Salmonella spp. are generally pathogenic to humans and other warm-blooded animals. The most common diseases are typhoid fever and gastroenteritis. The most important species transmitted by faecal-oral route is *S. typhi*, which causes typhoid fever. After ingestion of contaminated food or water, the bacteria spread from the intestine to the intestinal lymph nodes, to the liver and spleen and further out to multiply in the blood stream. Typhoid fever has a high death rate (up to 50%). Some persons will be colonized by the bacteria without getting any symptoms and these asymptomatic carriers are the main spreaders. The incubation period is long, up to a month after ingestion of the bacteria. However, this illness has been eliminated in most parts of the world as a result of effective wastewater treatment plants.

S. enterica subspecies *typhimurium* is mainly transmitted by contaminated food. The bacteria colonize the intestinal tract of animals. Symptoms of gastroenteritis appear within 6-24 hours and might cause from a mild to severe bloody diarrhea. The bacteria will be detectable in a patient for up to three months after a diarrhea. In developing countries, the disease occurs and is most severe in children under ten years of age (Ambrus & Ambrus 2004).

1.3 Different clam species

Phylum Mollusca includes aquatic organisms with soft bodies enclosed in a hard shell (made out of CaCO_3). The mantle, a thin sheet of tissue, which encloses the internal organs, secretes the shell. Clams live burrowed in the sand and have enlarged gills that are used both for respiration and filter feeding. They cope with the problem that the sediment enters the ventilating and feeding stream by for example siphons that are tube like mantle formations that stop the sediment to enter around the in and exhalant openings. As the siphons extend up into the free water, they reach water relatively free from sediment.

As clams are frequently exposed to microorganisms their cellular immune system needs to be very efficient. Different microorganisms, such as bacteria, viruses, protozoa and fungi, might infect the clams. The cellular defence mechanisms include phagocytosis and degradation by lytic enzymes (Hernroth 2002).



Meretrix meretrix

photo: www.asahi-net.or.jp/lnzai/hamaquri_e.html



Eumarcia paupercula

Photo:images.conchology.be/label/140000tb/141087.jpg

1.4 Antibiotic resistance

There are different mechanisms of antibiotic resistance.

1. One is restricted access of the antibiotic to the target. One example is the gram-negative bacteria that pump the antibiotic out of the cytoplasm via porins.
2. Another way for the bacteria is to inactivate the antibiotic through hydrolysing or adding chemical groups to the drug
3. Modification of the antibiotic target
4. Failure to activate the antibiotic

Antibiotics that have a broad-spectrum of activity are in general more likely to generate bacterial resistance. The isolated strains from the samples in this study was tested for resistance to the following antibiotics:

β -lactam antibiotics: these share the characteristic structure of β -lactam ring and are potent inhibitors of cell wall synthesis. The enzyme transpeptidase, that normally accomplish the cross linking of two glycan-linked peptide chains, is capable of binding to antibiotics with β -lactam rings. This group of antibiotics includes Ampicillin, Mecillinam, Cefadroxil and Ceftacidime. These antibiotics are active on Gram-positive and Gram-negative bacteria depending on agent and are classified to be broad-spectra antibiotics.

Fluoroquinolones: Ciprofloxacin and Norfloxacin inhibit the action of DNA gyrase. This enzyme normally introduces negative supercoiling of DNA in Bacteria and Archae. The antibiotics are broadly bactericidal.

Aminoglycosids: inhibits the protein synthesis at the 30S ribosomal subunit. Gentamicin and Tobramycin belongs to this group of antibiotics. Normally, they are only used clinically because of serious side effects and bacterial resistance. They are broadly bactericidal.

Trimethoprim: inhibits the growth of bacteria by inhibiting the synthesis of the vitamin folic acid, which is a nucleic acid precursor. This antibiotic is only effective on bacteria as they synthesise their own folic acid while higher animals obtain it from the food.

Chloramphenicol: an aromatic compound that inhibits the elongation step in the protein synthesis by blocking the formation of the peptid bonds. This antibiotic has shown elevated risks for childhood leukaemia and the use is thereby restricted to combat infections for which other antibiotics are ineffective or contraindicated (EPH 2002). As the antibiotic is efficient and cheap it is widely used in developing countries.

Nitrofurantion: a broad-spectrum antibiotic that inhibits enzyme activity and destroys DNA (Madigan *et al.* 2000).

1.5 Pathogenicity of the detected strains

After detection and identification of the strains, pathogenicity was tested using Multiplex-PCR and blood agar plates. Haemolysin genes code for a pore-forming protein that is toxic to blood and intestinal epithelial cells (Johnson 1991). They might cause enteropathy and diarrhea (Elliott *et al.* 1998). The Multiplex-PCR also includes the search for aerobactin genes. As iron is in low concentration in the body, the bacteria compete with the host cells for the accessible iron. The aerobactin is a small molecule with high affinity for iron and an important protein for the success of bacteria growth (Abigail 2002).

1.6 Why is this study important?

In the poorest countries of the world, the most common cause of premature death is gastrointestinal infections due to poor sanitary levels, malaria and tuberculosis (Dgedge *et al.*, 2001). Increasing evidence suggest that malnutrition, both Protein-Energy type Malnutrition (PEM) and essential micronutrient (vitamins, trace minerals, essential amino acids, polyunsaturated fatty acids) type, is the underlying reason for increased susceptibility to infections. On the other hand, certain infectious diseases also cause malnutrition, which results in a vicious cycle (Ambrus & Ambrus 2004). Malnutrition may diminish the immune defence and as shellfish has a high nutrient value, it constitutes an important nutrition source. Clam meat consists of about 13% protein (Jay 2000).

Studies of microbial counting in water are not frequently performed in Africa. Still it is an important issue, due to the poor purification of wastewater. Water samples are normally analysed for faecal contamination at the Ministry of Health. However, the practical work for this study has been performed at the Faculty of Agronomia, University of Eduardo Mondlane. At this department, the main part of the work is focused on marine ecology, but the microbial department is under development.

This screening study of faecal contamination in Maputo Bay is a part of an ongoing SIDA-project, within the SAREK-project (a cooperative project, between the Royal Swedish Academy of Sciences, Kristineberg Marine Research Station in Fiskebäckskil, Kristianstad University and University Eduardo Mondlane in Maputo, Mozambique) with focus on virus, which also contributes to the development of the microbial faculty of the University Eduardo Mondlane. The main purpose of this study is to characterize the presence of faecal coliforms in Maputo Bay, in order to screen the area for faecal contamination and *Salmonella* spp. and indicate the current levels of bacterial pollution. The study also includes analyses of the pathogenicity and antibiotic resistance of the detected bacterial strains. Generally, pathogenic organisms and viruses that may cause different illnesses are more interesting for research due to the pathogenicity. This study might give an indication for further studies on this subject.

2. Material and methods

2.1 Area description

Maputo, the capital of Mozambique, with over a million inhabitants is situated by the Maputo Bay. The bay opens into the Indian Ocean in the northeast. Two rivers discharge into the Bay: the Maputo River and the N'komati River. The study area consists of two different collection locations, Bairro dos Pescadores and Luís Cabral (see map), and one marketplace along the bay. Clams from the local market were analysed when the tide was high and it was impossible for gatherers to harvest. The different sampling sites are located according to the locations used by the students of University Eduardo Mondlane.

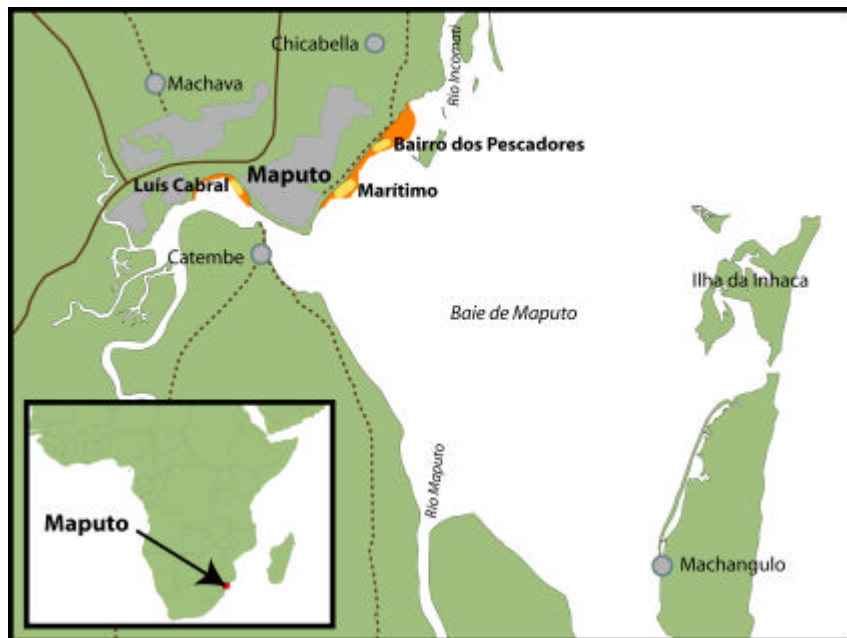


Figure 1 map of collection sites in Maputo Bay

In the course of two months (May and June 2004), clams (*Meretrix meretrix* and *Eumarcia paupercula*) were collected in order to measure the level of coliform contamination. Clams were bought from three different sites, presumed to be of different purity. Clams from the local market were told to be collected at Macaneta and Monthana. Macaneta is by the inhabitant considered to be of least faecal polluted area, Monthana less polluted, Bairro dos Pescadores more polluted and Luís Cabral the most faecal polluted collection site. Clams analysed were of different species according to availability. *Meretrix meretrix* was bought at the market and bought from the gatherers at Luís Cabral. *Eumarcia paupercula* was bought from gatherers at Bairro dos Pescadores. Sellers asserted that the clams at the local market were collected at Macaneta and Montanhana (sites about 20 km north of Maputo), but as the clams were not bought directly from the gatherers, this information cannot be used in the

results. Bairro dos Pescadores, located north of the central Maputo and a lighter bacterial pollution was expected. Luís Cabral was located south of central Maputo, near industrial areas and sewage outlets. More important pollution from the residential areas and industries was expected.

Sea grass meadows in Maputo bay sustain economic activities. Bandeira and Balidy (2003) claim that approximately 100 people visit the sea beds of Bairro dos Pescadores daily for collection of invertebrate. The most commonly found bivalvia was claimed to be *Eumarcia paupercula* and *Meretrix meretrix*. Approximately four thirds of the gatherers were women. The daily revenues for *Meretrix meretrix* reached up to 2 \$US per collector daily.

2.2 Sample collection and preparation

The clams were bought and directly placed in plastic bag during the transport to the laboratory where the analysing started no later than one hour after collection. A total of 12 samples of clams were analysed. Clams that were gapping or in any other way seemed unusable were discarded. The selected clams were opened with a flame sterilised shucking knife and the flesh put into a pre-weight glass cup. Approximately 20 clams, which weights around 50-100 grams were used for each analyse.

2.3 Detection and analysis of *Escherichia coli*

The most probable number (MPN per 100 g) enumeration of coliforms in the samples was determined using the multiple tube method with five tubes and four (five dilutions in one sample) dilutions in MMGB (Mineral Modified Glutamate Broth), according to the CEFAS protocol (Donovan 1998).

After the general sample preparation, 2 ml sterile peptone water was added to each gram of clam meat. The total was put in a blender jar and homogenised at high speed for approximately one minute (4 bursts of 15 s with 5 s break between the bursts). 30 ml of clam homogenate was mixed with 70 ml sterile peptone water to make a 10^{-1} dilution. The 10^{-1} dilution was thoroughly mixed by inversion. 10 ml was then added to 90 ml sterile peptone water to make a 10^{-2} dilution. 10 ml of the 10^{-2} dilution was added to 90 ml sterile peptone water to make a 10^{-3} dilution. Finally, 10 ml of the 10^{-3} dilution was added to 90 ml sterile peptone water to make a 10^{-4} dilution. Bottles with lid were used for dilution to ensure thoroughly mix between the dilution steps. Single and double MMGB was prepared (see appendix). For each sample analysis 300 ml of single MMGB and 100 ml of double MMGB were prepared. The double strength is used to show very low concentrations of *E. coli*. It is important that the media does not limit the bacterial growth. 10 ml of the homogenate was inoculated to the double MMGB and 1 ml of the homogenate was inoculated to the single MMGB. The tubes were incubated in $37\pm 1^\circ\text{C}$ for 24 ± 2 hours. For *E. coli* confirmation, 1 μl of the positive samples was spread onto a TBGA (Tryptone Bile Glucuronide Agar) plate (see appendix) and incubated inverted in $44\pm 1^\circ\text{C}$ for 22 ± 2 hours. For further analysis, colonies from the TBGA plates were picked and put into transport tubes (COPAN) and sent to Sweden, Kristianstad University. The samples were received in Sweden and the content of the transport tubes were spread onto LB-plates (Luria-Bertani medium).

2.4 Detection and analysis of *Salmonella* species

Testing for *Salmonella* was carried out using the Nordic Committee on food analysis (NMKL) method (Wiberg 1998).

After the general sample preparation, the clam meat was put into a small blender jar and homogenised at low speed for approximately one minute (4 bursts of 15 s with 5 s break between the bursts). 25 ml of the homogenised clams was added to 225 ml sterile peptone water and this pre-enrichment dilution was incubated in an incubator at 37°C for approximately 18 hours. 100 µl of the pre-enrichment broth was transferred to five tubes of 10 ml pre-warmed Rappaport-Vassiliadis soy peptone broth. The enrichment was incubated in an incubator at 41,5±0,5 °C for 24±3 hours. 1 µl of the solution from the test tubes was spread onto XLD-agar plates and TSIA-plates. The plates were incubated at 37°C for about 24 hours. For further analysis, colonies from the plates were picked and put into transport tubes and sent to Sweden, Department of Clinic Bacteriology, University of Gothenburg. Half of the clam sample was left in room temperature for twenty-four hours.

2.5 Test for Antibiotic resistance

Bacterial colonies were picked from LB-plates and diluted in 10 ml sterile 0,9% NaCl solution. The solution was spread onto three specific antibiotic resistance plates and 13 different antibiotic test discs (see Table 1) were placed onto the plates. The plates were incubated in 37°C for about 24 hours. The inhibiting growth zones were measured and compared with a table showing the resistance, indeterminate and sensitivity values (Kahlmeter 2004).

Table 1 antibiotics used for testing antibiotic resistance

Ampicillin(AMP)	Cefadroxil (CFR)	Ceftacide (CAZ)	Chloramphenicol (C)
Ciprofloxacin (CIP)	Gentamicin (CN)	Mecillinam (MEL)	Nitrofurantoin (F)
Norfloxacin (NOR)	Trimethoprim (W)	Tobramycin (TOB)	

2.6 API-20[®], coliform identification

In order to identify the samples, API-20E[®] for identification of enteric bacteria (bioMérieux, Inc., Hazelwood, MO) was used. One colony from the antibiotic resistance plates was added to 5 ml sterile H₂O. The solution was added to the strip, containing twenty mini-test tubes and incubated in 37°C for 18 - 24 hours. The coloration reactions were read and the reactions converted to a seven-digit code. The code was compared to a table, provided by bioMérieux, that usually gives the identification (genus and species).

2.7 Multiplex-PCR

To test the virulence genes in the isolated *E. coli* strains, multiplex-PCR was used. This includes two primer sets (listed in Table 2), which amplifies DNA fragments coding for aerobactin and haemolysis.

Table 2 The primers used for detection of pathogen factors by Multiplex-PCR

Gene	Sequences of primers	Positions	Size of the amplicons
drA1	5'-GCCAACTGACGGACGCAGCAC-3'	Dr-321f	229 bp
drA2	5'-CCCCAGCTCCCGACATCGTTTTT-3'	Dr-550r	
iutA1	5'-GGCTGGACATCATGGGAACTGG -3'	aer-851f	301 bp
iutA2	5'-CGTCGGGAACGGGTAGAATCG-3'	aer-1152r	

0,25 µl of each of the four primers and one bacterial colony (approximately 2 µl) were added to 25 µl Master mix (HotStarTaq Master Mix). 22 µl sterile H₂O was added to the solution in order to achieve a total volume of 50 µl. The samples were put into the PCR machine (PTC-100TM, MJ Research, Inc.) using the program in Table 3.

Table 3 Schedule for PCR program

	Temperature	Time
Preparation	94 °C	4 min
Denaturation	94 °C	2 min
Annealing	65 °C	1 min
Extension	72 °C	2 min
Finish	72 °C	3 min
Preservation	4 °C	

10 µl loading buffer (6x) was added to the total volume of the PCR product and loaded onto a 1,5% agarose gel containing 1% Etidium bromide (5 µl/50 ml agarose gel). A ladder (GeneRulerTM 100bp DNA ladder Plus) was used as a reference. The PCR product was separated on agarose gel under 80 V for about 30 min. The gels were visualized under UV-light.

2.8 Blood agar plates

Haemolysis of the *E. coli* strains was also tested on blood agar plates. The *E. coli* colonies were stroke onto each plate and incubated at 37°C for about 24 hours.

3. Results

3.1 MPN

Luís Cabral was the most polluted site. The 21st of June, the clams contained $1,7 \times 10^5$ bacteria per 100 g meat. The lowest bacterial contamination ($1,1 \times 10^2$ bacteria per 100 g clam meat) was recorded the 24th of May in clams bought at the local market. However, June 14th, the clams from the same location contained $9,1 \times 10^4$ bacteria per 100 g clam meat. The average least contaminated site was Bairro dos Pescadores and the highest bacterial concentration from that site was $3,1 \times 10^3$ bacteria per 100 g clam meat. The results from the direct analysis are shown in Table 4 and Figure 2. In Table 4, the results obtained for clams are compared with the limits set by the MPN. Almost every sample (11 out of 12 samples), exceed the limit for “approved” (Category A) areas, categorized according to EU-standards (see appendix).

Table 4 direct analyses of samples according to CEFAS Enumeration of *Escherichia coli* in Molluscan Bivalve Shellfish

Date	Collection site	Clam species	MNP, <i>E. coli</i> /100g meat	Category
May 13 th	Local market	<i>M.meretrix</i>	$3,8 \times 10^4$	C
May 17 th	B. dos Pescadores	<i>E.paupercula</i>	$7,5 \times 10^2$	B
May 18 th	Luís Cabral	<i>M.meretrix</i>	$2,4 \times 10^4$	C
May 24 th	Local market	<i>M.meretrix</i>	$1,1 \times 10^2$	A
June 1 st	Local market	<i>M.meretrix</i>	$2,5 \times 10^3$	B
June 1 st	B. dos Pescadores	<i>E.paupercula</i>	$3,1 \times 10^3$	B
June 7 th	B. dos Pescadores	<i>E.paupercula</i>	$4,0 \times 10^2$	B
June 7 th	Local market	<i>M.meretrix</i>	$2,5 \times 10^3$	B
June 14 th	Local market	<i>M.meretrix</i>	$9,1 \times 10^4$	Prohibited
June 16 th	Luís Cabral	<i>M.meretrix</i>	$3,5 \times 10^4$	C
June 21 st	Luís Cabral	<i>M.meretrix</i>	$1,7 \times 10^5$	Prohibited
June 28 th	Local market	<i>M.meretrix</i>	$1,6 \times 10^4$	C

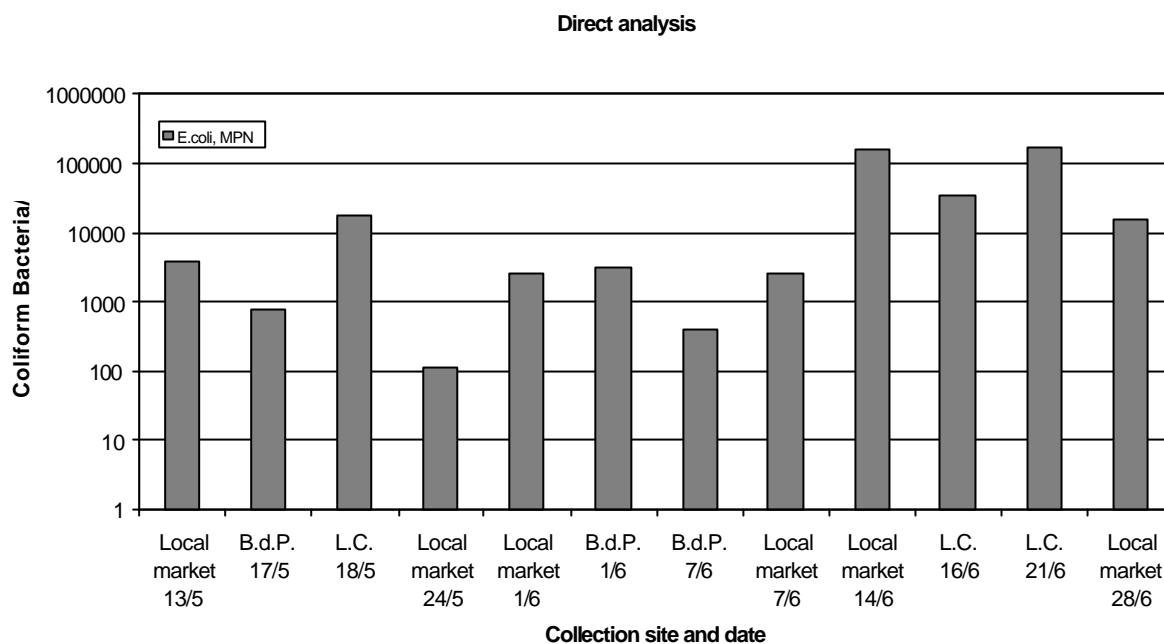


Figure 2 the *E. coli* concentration in clams. B.d.P. is an abbreviation for Bairro dos Pescadores and L.C. for Luís Cabral

Clams from the same sample as were used in the direct analysis, were left at room temperature (20-30°C) for approximately 24 hours. The results of bacterial growth during storage in clams do not show any significant pattern. In six of the samples, the bacterial concentration increased, in three the concentration decreased, and in one the bacterial concentration was the same as before the storage (see Table 5 and Figure 2 through 5).

Table 5 comparative direct analyses and analyses of the same samples left at room temperature (20-30 °C) for 24 h

Collection date	Analysis date	Collection site	Clam species	MNP, <i>E. coli</i>
May 17 th	May 17 th	Bairro dos Pescadores	<i>E.paupercula</i>	7,5 x 10 ²
May 17 th	May 18 th	Bairro dos Pescadores	<i>E.paupercula</i>	5,0 x 10 ²
May 24 th	May 24 th	Local market	<i>M.meretrix</i>	1,1 x 10 ²
May 24 th	May 25 th	Local market	<i>M.meretrix</i>	4,0 x 10 ²
June 1 st	June 1 st	Local market	<i>M.meretrix</i>	2,5 x 10 ³
June 1 st	June 2 nd	Local market	<i>M.meretrix</i>	2,0 x 10 ²
June 1 st	June 1 st	Bairro dos Pescadores	<i>E.paupercula</i>	3,1 x 10 ³
June 1 st	June 2 nd	Bairro dos Pescadores	<i>E.paupercula</i>	5,0 x 10 ³
June 7 th	June 7 th	Bairro dos Pescadores	<i>E.paupercula</i>	4,0 x 10 ²
June 7 th	June 8 th	Bairro dos Pescadores	<i>E.paupercula</i>	9,0 x 10 ²
June 7 th	June 7 th	Local market	<i>M.meretrix</i>	2,5 x 10 ³
June 7 th	June 8 th	Local market	<i>M.meretrix</i>	5,0 x 10 ³
June 14 th	June 14 th	Local market	<i>M.meretrix</i>	9,1 x 10 ⁴
June 14 th	June 15 th	Local market	<i>M.meretrix</i>	7,0 x 10 ²
June 16 th	June 16 th	Luís Cabral	<i>M.meretrix</i>	3,5 x 10 ⁴
June 16 th	June 17 th	Luís Cabral	<i>M.meretrix</i>	9,1 x 10 ⁵
June 21 st	June 21 st	Luís Cabral	<i>M.meretrix</i>	1,7 x 10 ⁵
June 21 st	June 22 nd	Luís Cabral	<i>M.meretrix</i>	5,4 x 10 ⁵
June 28 th	June 28 th	Local market	<i>M.meretrix</i>	1,1 x 10 ⁴
June 28 th	June 29 th	Local market	<i>M.meretrix</i>	1,6 x 10 ⁴

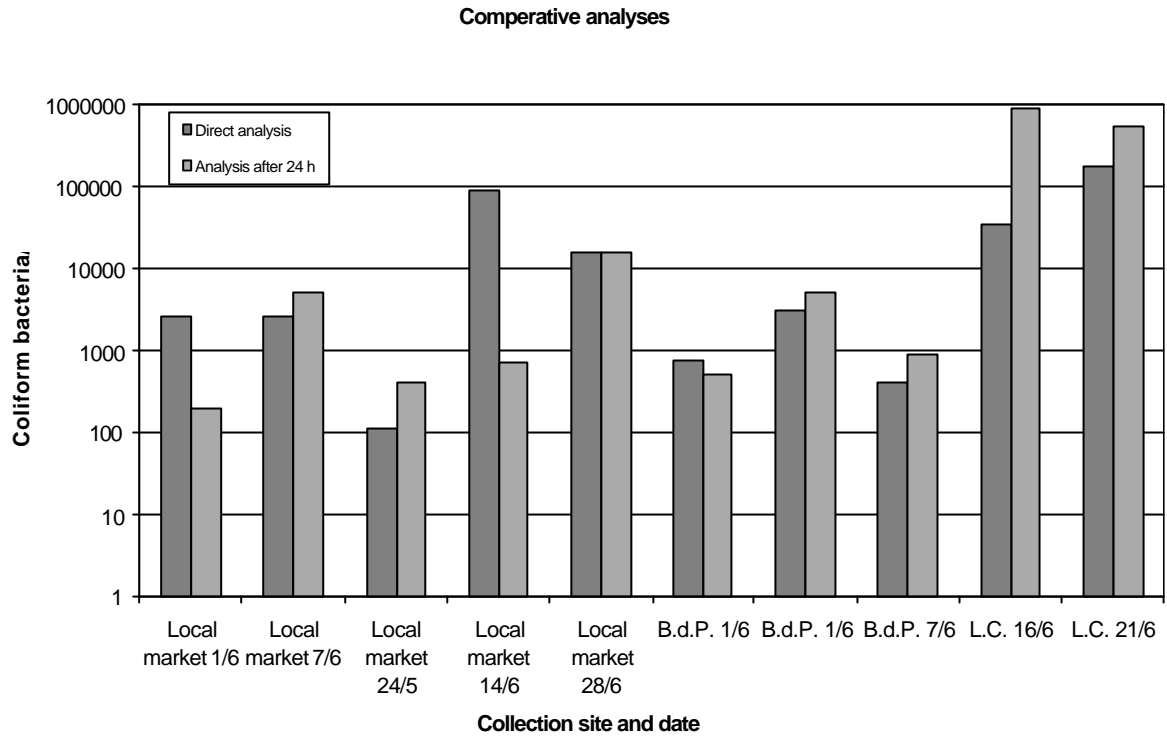


Figure 3 bacterial growth during 24 h at room temperature

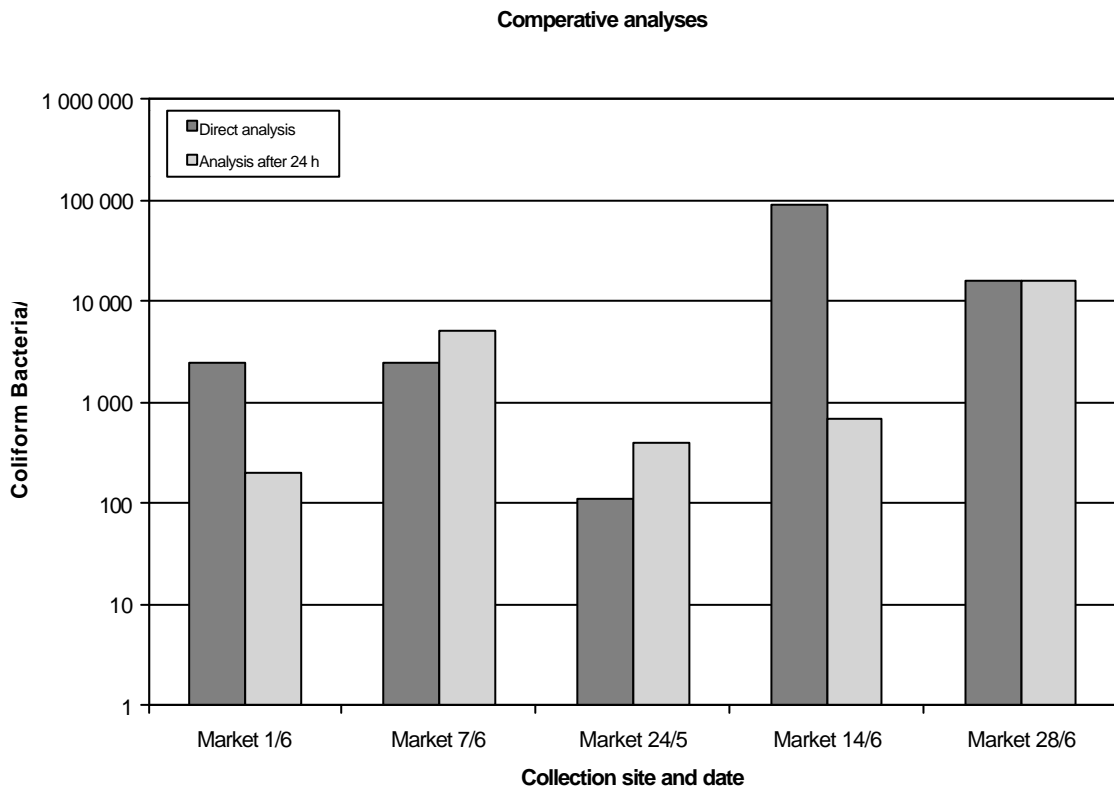


Figure 4 bacterial concentrations in clams from the local market, direct analysis compared with analysis after 24 hours at room temperature

Comperative analyses

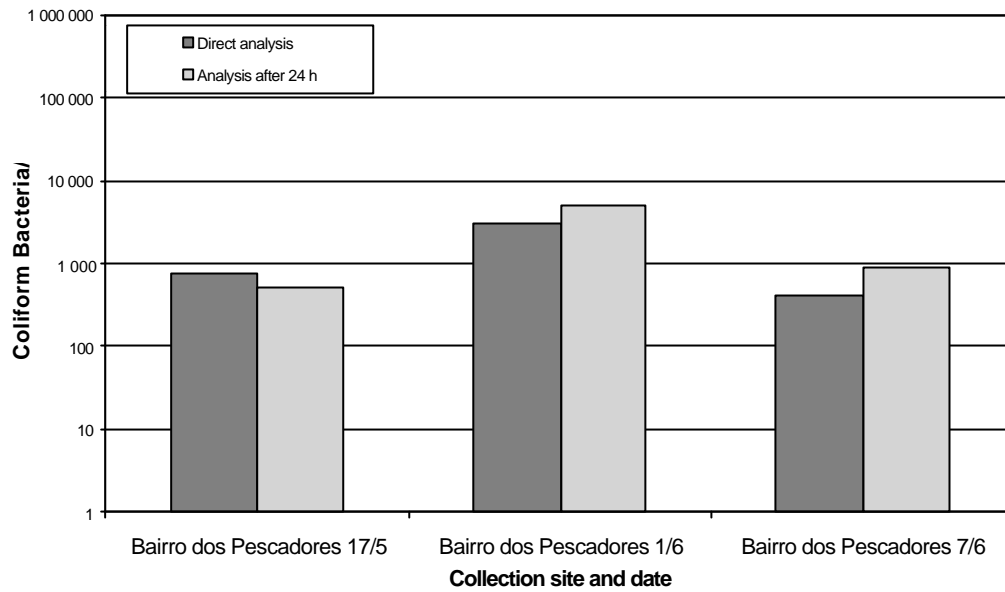


Figure 5 bacterial concentrations in clams from Bairro dos Pescadores, direct analysis compared with analysis after 24 hours at room temperature

Comperative analyses

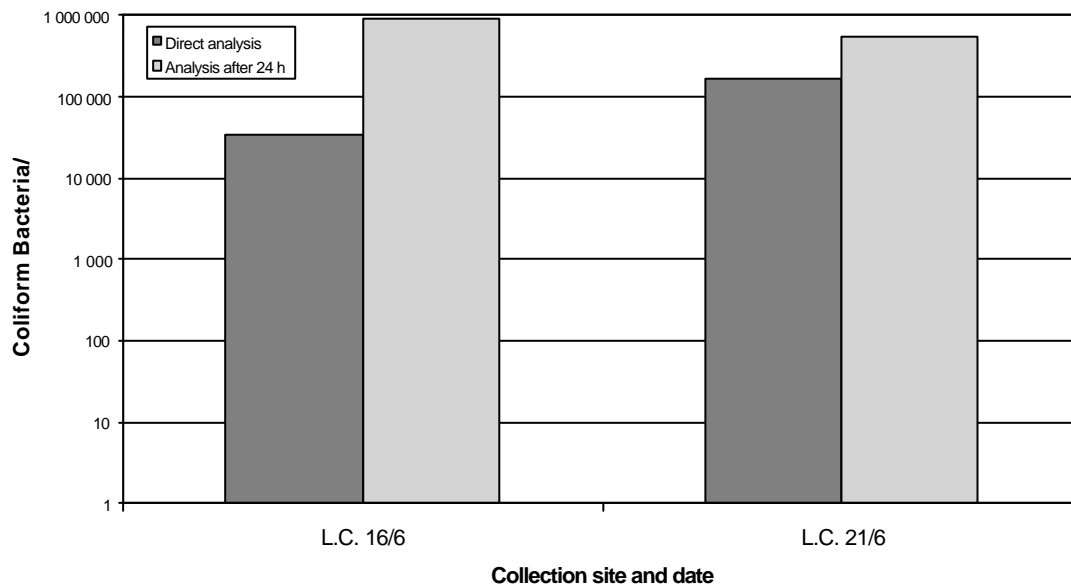


Figure 6 bacterial concentrations in clams from Luís Cabral, direct analysis compared with analysis after 24 hours at room temperature

During the analysis, sterility tests have been performed. With every homogenisation, the blender jar was washed with sterile peptone water of which 1 µl was added to MMGB and the

control sample followed the same procedure as the samples containing clam homogenate. The same pathway was used for sterile control of the *Salmonella* growth. Bacterial growth was not observed in any of the control samples (see appendix).

3.2 Coliform identification

Out of 42 presumptive coliforms isolates from different samples, 37 were successfully identified by API-20[®]. Seven of the samples were heavily contaminated with *Pseudomona* spp. (decomposing bacteria), which inhibited growth of other bacterial species. Twenty-five of the samples were identified as coliform bacteria, i.e. *Escherichia coli*, *Klebsiella pneu.pneumoniae*, *Klebsiella ornithinolytica*, *Klebsiella planticola*, *Enterobacter cloacae* and *Shigella* spp. *Acinetobacter baumannii*, *Flavimonas oryzihabitans*, *Erwinia* spp. and *Serratia marescens* were also identified. The largest species variation was detected in clams from the local market. The lowest variation was detected in clams from the gatherers at Bairro dos Pescadores, the site with presumed lowest pollution level (see Table 6 for site distribution of species).

Table 6 results of identification of samples showing the distribution of bacteria species. B.d.P. is an abbreviation for Bairro dos Pescadores and L.C. for Luís Cabral

Collection site	Bacterial strain	Number	Collection site	Bacterial strain	Number
Market	<i>E. coli 1</i>	3	B.d.P.	<i>E. coli 1</i>	7
	<i>E. coli 2</i>	1		<i>K.pneu.pneumoniae</i>	2
	<i>Klebsiella planticola</i>	4	L.C	<i>E. coli 1</i>	4
	<i>K. ornithinolytica</i>	1		<i>K.pneu.pneumoniae</i>	2
	<i>K.pneu.pneumoniae</i>	1		<i>K. planticola</i>	1
	<i>Aci.baumannii</i>	2		<i>Aci.baumannii</i>	3
	<i>Chryseomonas luteola</i>	1		<i>Erwinia</i> spp.	1
	<i>Serratia marescens</i>	1		<i>Flavi.oryzihabitans</i>	1
	<i>Enterobacter cloacae</i>	1			
<i>Shigella</i>	1				

3.3 Antibiotic resistance, *E. coli*

The *E. coli* strains were tested for antibiotic resistance. The different *E. coli* strains were in general sensitive to most of the tested antibiotics (Table 7). However, the sensitivity for the penicillins, Ampicillin, Ceftacidime and Cefadroxil were low. Fourteen of the strains showed low sensitivity and one strain was resistant against Ampicillin. Six of the fifteen strains reacted indeterminately to Ceftacidime, two strains from each site. Two strains, detected in clams from Luís Carbral, differed from the others. One showed resistance to Cefadroxil and had low sensitivity against Mellicinam. The second strain showed resistance against Ampicillin. The strains sampled from Luís Cabral were in general less sensitive to antibiotics than strains from Bairro dos Pescadores and from the market.

Table 7 results from antibiotic resistance test on *E. coli* strains. B.d.P. is an abbreviation for Bairro dos Pescadores and L.C. for Luís Cabral

Sampling	Bacteria	AMP	C	CAZ	CFR	CIP	CN	F	MEL	NOR	TOB	W
June 1 st Market	<i>E. coli 1</i>	I	S	I	I	S	S	S	S	S	S	S
June 7 th Market	<i>E. coli 1</i>	I	S	I	I	S	S	S	S	S	S	S
June 14 th Market	<i>E. coli 2</i>	I	S	S	I	S	S	S	S	S	S	S
June 28 th Market	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
May 17 th B.d.P.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
May 17 th B.d.P.	<i>E. coli 1</i>	I	S	I	I	S	S	S	S	S	S	S
May 17 th B.d.P.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
June 1 st B.d.P.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
June 1 st B.d.P.	<i>E. coli 1</i>	I	S	I	I	S	S	S	S	S	S	S
June 7 th B.d.P.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
June 7 th B.d.P.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S
June 16 th L.C.	<i>E. coli 1</i>	R	S	I	I	S	S	S	I	S	S	S
June 21 st L.C.	<i>E. coli 1</i>	I	S	S	R	S	S	S	S	S	S	S
June 21 st L.C.	<i>E. coli 1</i>	I	S	I	I	S	S	S	S	S	S	S
June 21 st L.C.	<i>E. coli 1</i>	I	S	S	I	S	S	S	S	S	S	S

3.4 Identification of *Salmonella*

Every clam sample was tested for *Salmonella* spp. Five of the twenty-eight samples tested positive. The five positive samples were from each of the three different sampling sites. *Salmonella* spp. was detected in samples of both clam species. There is no significant pattern between the coliform concentration and *Salmonella* spp. in the samples and *Salmonella* spp. was detected irrespective of harvesting category. The result also shows lack of correlation between detection of *Salmonella* spp. before and after storage. In four out of five cases, *Salmonella* spp. was detected in samples before storage, but not after 24 hours in room temperature in the same sample. In one sample, *Salmonella* spp. was detected exclusively after storage (see Table 8). Table 8 includes a column for coliform concentration that shows that there is no correlation between the coliform concentration and the detection of *Salmonella* spp. Samples with high coliform concentration ($3,5 \times 10^4$) and in samples with low coliform concentration ($4,0 \times 10^2$) tested positive for *Salmonella* spp.

Table 8 results form the *Salmonella* spp. detection. B.d.P. is an abbreviation for Bairro dos Pescadores and L.C. for Luís Cabral

Collection date	Analysis date	Collection site	Clam species	<i>Salmonella</i> spp.	MNP, <i>E. coli</i>
June 1 st	June 1 st	B.d.P.	<i>E.paupercula</i>	Positive	$3,1 \times 10^3$
June 7 th	June 7 th	B.d.P.	<i>E.paupercula</i>	Positive	$4,0 \times 10^2$
June 7 th	June 7 th	Local market	<i>M.meretrix</i>	Positive	$2,5 \times 10^3$
June 14 th	June 15 th	Local market	<i>M.meretrix</i>	Positive	$7,0 \times 10^2$
June 16 th	June 16 th	L.C.	<i>M.meretrix</i>	Positive	$3,5 \times 10^4$

3.5 Antibiotic resistance, *Salmonella* spp.

The detected *Salmonella* strains were sensitivity to the tested antibiotics except for Cefradroxil and Ampicillin, to which the strains showed low sensitivity. One strain, identified from sample bought at Luís Cabral, showed resistance against Ampicillin.

Table 9 results from antibiotic resistance test on *Salmonella* spp. B.d.P. is an abbreviation for Bairro dos Pescadores and L.C. for Luís Cabral.

Sampling	Sampling site	AMP	C	CAZ	CFR	CIP	CN	MEL	NOR	TOB
June 1 st	B.d.P.	I	S	S	I	S	S	S	S	S
June 7 th	B.d.P.	I	S	S	I	S	S	S	S	S
June 7 th	Local Market	I	S	S	I	S	S	S	S	S
June 14 th	Local Market	I	S	S	I	S	S	S	S	S
June 16 th	L.C.	R	S	S	I	S	S	S	S	S

3.6 Virulence gene detection

The results from the Multiplex-PCR show the fifteen *E. coli* strains and four control samples. None of the fifteen tested *E. coli* strains was positive for the virulence genes coding for aerobactin and haemolysin or haemolytic on blood agar plates.

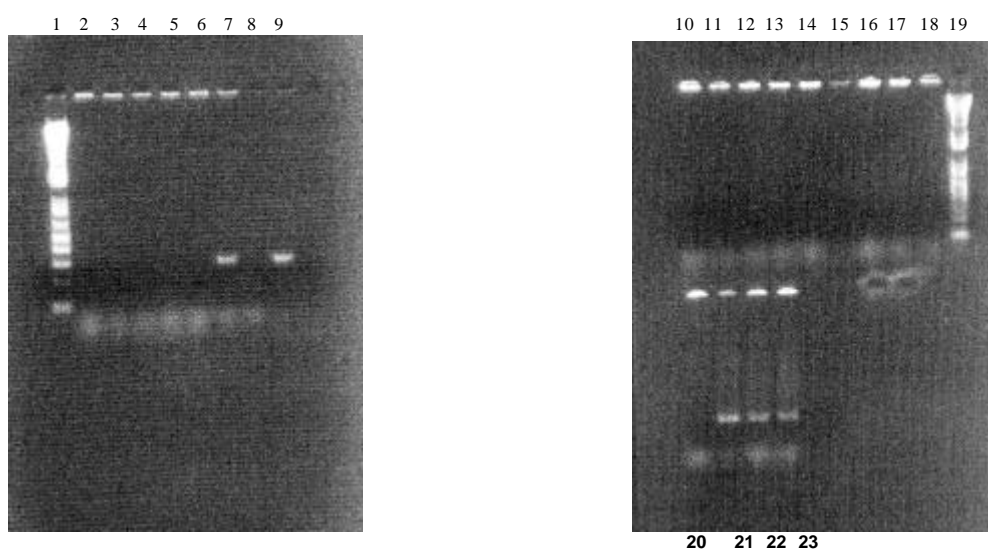


Figure 7 results from the Multiplex-PCR. Lane 1; reference ladder; Lane 2 through 6, isolated *E. coli* strains, Lane 7 through 9; control strains, Lane 10 through 18; isolated *E. coli* strains, Lane 19; reference ladder, Lane 20; isolated *E. coli* strains, Lane 21 through 23; control strains

4. Discussion

The results reported from the three different sampling sites do not show any significant bacterial pattern. Results from samples bought from the market indicates that even if the seller claimed that the clams were collected the same day as they were sold, the bacterial concentration of decomposing bacteria was relatively high. The bacterial strain spectrum in these samples was wide and indicates that clams from the market contained more decomposing bacteria than true faecal contamination. Five samplings were done from the local market, while only three from Bairro dos Pescadores and two from Luís Cabral. This leads to an uneven comparison of the sampling sites.

Some of the samples show that the media used was not specific enough and the sample might have been contaminated by bacterial strains, generally not expected to be detected using this method. In clams bought from gatherers at Bairro dos Pescadores, only indicator organisms were found. As mentioned earlier, these bacteria are specific for human or other warm-blooded animals and indicate a faecal-contamination. Bairro dos Pescadores was expected to be less polluted than Luís Cabral. However, samples from Luís Cabral contained less faecal contaminants, but contained a wider collection of contaminating bacterial species. This collection area is surrounded by different industries and sewage outlets from the city and at the time for sampling, different types of household wastes were found. As mentioned earlier, the great part of the Mozambican industry is situated in Maputo and MOZAL, the aluminium smelter industry is located in the inner part of Maputo Bay, close to the collecting site Luís Cabral. This means that the potential of identifying a broader spectrum of exogenous bacterial species of non-faecal origin in the area, is high.

The results from quantification of bacterial concentration in clams kept at room temperature over night, do not give a significant result of bacterial growth. In some samples, the bacterial concentration increased greatly after 24 hours, as were predicted. However, the concentration decreased in three samples and in one sample, the concentration was the same after storage. The fact that the bacterial number of coliform bacteria was lower after 24 hours growth at room temperature, is probably a result of the high number of *Pseudomonas spp*, a decomposer, in the sample. For the API-20[®] identification, some samples were so heavily contaminated by *Pseudomonas*, that growth of other bacteria became inhibited. Even though the sellers at the local market assured that the clams were fresh, this indicates the contrary.

The reason to the great differences of bacterial concentrations from the same site, one week from the other, might be a result of heavy rainfalls. Even though the rain season occur during the summer (November to April), Maputo experienced a heavy rainfall some days before the sampling of June 16th at Luís Cabral. As filter feeders are indicators over time, the increased bacterial concentration was noticed more than a week after the rainfall, at the sampling of June 21st. Another reason to the high bacterial concentration in clams bought at the market might be the handling and storage at high and favourable temperature for bacterial growth. Other parameters, such as phytoplankton blooms might contribute to increase dissolved organic matter, and thereby availability for bacterial growth (Lizárraga-Partida & Cárdenas 1996).

The results from the bacterial identification show that a large number of the white colonies on the TBGA plates were *E. coli*. If the colonies on the TBGA plates are blue, they are lactose fermentative, and normally >90% of the *E. coli* is lactose fermentative. On the TBGA plates, some of the white and blue colonies grew close together to that extent, that it was difficult to

separate the colonies from each other. This might have failed while transferring colonies from the plates to transport tubes, and the colonies that were supposed to be clean might have been mixed. When the bacterial concentration in the samples was counted, only the blue colonies on the TBGA plates were considered to be *E. coli*, even though the API-20[®] identification showed that a number of the white colonies were *E. coli*. This has previously been shown by others (Hernroth *et al.* 2002). If all the colonies growing on the TBGA plates had been considered as *E. coli*, the bacterial concentration should have been up to seventy times greater in one sample. In other samples the number would have been the same. This might show the insufficiency of the CEFAS method when working in waters different from the cold northern ocean.

Hood *et al.* (2004) have studied the relation among faecal coliforms and *Salmonella* spp. in shellfish. The results from that study indicate that samples negative for faecal coliforms might be a good indicator of the absence of *Salmonella* spp. *Salmonella* spp. were not present in any sample containing less than 230 faecal coliforms per 100g. In accordance to the results from the Hood study, *Salmonella* spp. were not detected in the two samples with a faecal concentration below this value.

One must notify that the bacterial strains are different in Africa from the strains in Europe. The methods used in this study are adjusted for the European marine environment, which might influence the result. *Salmonella* are bacteria that are much more common in the Africans intestines compared to Europeans. For *Salmonella* spp. analyses, XLD-agar plates were used, but unfortunately it was not specific enough. Many different bacterial species grew on the plates and might have inhibited potential *Salmonella* growth. Studies of detection of *Salmonella* spp. in tropical seafood show that more than one enrichment broth is necessary for efficient detection (Sanath *et al.* 2003). In the future, it would be desirable to use another, more well adapted medium for the bacterial flora that is found in tropical waters.

Besides *E. coli* and *Salmonella* strains, nine other species were detected in the samples. Three *Klebsiella* spp. were identified in the samples; *K. ornithinolytica*, *K. planticola* and *K. pneumoniae*. These bacterial species are members of the family enterobacteriaceae. *Klebsiella* have become important pathogens in nosocomial infections (hospital illnesses). It is also ubiquitous in nature and may colonize the skin, pharynx or gastrointestinal tract. However, they may be regarded as normal flora in many parts of the colon, intestinal tract, and in the biliary tract. The bacteria overcome the host immunity by a polysaccharide capsule that is the main determinant of their pathogenicity (©Genoscope). *Klebsiella* spp. is often the most common non-*E. coli* faecal coliform isolated when analysing faecal contamination (Hood *et al.* 1983).

Clams bought at the market showed presence of *Acinetobacter* that belongs to the gamma subdivision of Proteobacteria, Moraxella family. *A. baumannii* (and other related species) is a pathogenic species that in the northern Hemisphere causes nosocomial infections (Guentzel 1996). In tropical countries, the bacteria might be found outside hospitals and cause essentially pneumonias. Its presence in this type of infection can be explained by its very high capacity to acquire antibiotic resistance. The situation is different in tropical countries, where *A. baumannii* is found in community-acquired infections. These infections are essentially pneumonias associated with bacteremia and are found in alcoholic patient (©Genoscope).

Chryseomonas luteola is rarely reports to be a human pathogen, but in some cases the organism affects patients with health or indwelling disorders (Wafae 2004).

Serratia mercenscens is a member of the family *Enterobacteriaceae* in the tribe *Klebsiella*. *Serratia* strains are isolated from water and soil and might also be of human (faecal) origin

(Madigan *et al.* 2000). *S. marcescens* has had a lot of attention lately due to an increasing number of cases of nosocomial infections in the urinary and respiratory tract (Deguzman 2004).

Enterobacter cloacae is a member of the family *Enterobacteriaceae*, closely related to *Klebsiella* and *Serratia*. It is common in the intestinal tract of humans and other warm-blooded animals (Madigan *et al.* 2000). It may be found in different plants, such as cucumbers, radish and sweet corn seeds. This can have usefulness for humans and be used in biological control of plants (Roberts *et al.* 2000).

Flavimonas oryzihabitans is found in soil and thrive in moist environments. It is rarely pathogenic but the cases of described diseases, is mainly in patients with implanted or indwelling materials (Iglesias 2004).

Erwinia spp. was also found in clams from the market. Members of the genus *Erwinia* are primarily plant-pathogenic and plant-associated bacteria. This indicates a contamination from agriculture (Smadja *et al.* 2004).

The antibiotic resistance of the strains shows that they in general are rather sensitive to the antibiotics tested. The *E. coli* strains were resistant or showed a low sensitivity against four of the antibiotics. All of these four antibiotics had a broad-spectra activity and belonged to the β -lactams. Two strains showed antibiotic resistant and both strains were isolated from Luís Cabral. The broad-spectra antibiotics are in general less expensive and might be used for many different kinds of illnesses. Nevertheless, all strains tested for antibiotic were sensitive to Chloramphenicol, a cheap and efficient antibiotic often used in developing countries. As mentioned earlier, this antibiotic may cause hazardous side effects.

The PCR did not indicate any virulence genes. This does not exclude that the strains are pathogenic as there are numerous of different virulence genes. The deficiency of time lead to that just these set of primers were used. However, results from the ongoing SIDA-project show a widely presence of HAV (Hepatit A virus) in mussels from the collection area in Maputo Bay (personal comment Hernroth).

The result showing that *Salmonella* spp. only was found in few samples might indicate the insufficiency of the media and the necessity of better-suited methods. However, the purpose with this study was to screen the diversity of bacteria (and viruses) that could be found in clams from the area and not to develop sophisticated culturing methods.

The studies done on the water quality of Maputo Bay are insufficient and the purification of the sewage water is unsatisfying. By the European standard, this study show that the water in many cases would be classified in Category C (needing purification) or to be Prohibited from clam harvesting (see appendix). As the harvesting is not industrial, it might be difficult to prohibit the gatherers from harvesting. As the results also indicate, the bacteria isolated from the samples tend to be rather harmless with low pathogenicity. This could be investigated in more detail, for example by taking more samples and testing for other virulence genes. Since the time for this study was short, this might be an interesting study to do, when more time, effort and money is available. It would of course be interesting to analyse other filter feeders, such as oysters and other clam species, and the correlation of faecal coliforms and *E. coli* in fresh and stored clams. As indicated from the results in a study performed by Hood *et al.* 1983, the accumulation of microorganisms in different shellfish varies even though the samples are collected in the same area. Oysters seemed to differ most. Results from the same study show that the faecal coliform levels strongly correlates to the storage time and temperature. In future studies, these parameters might be included to see the significance of

bacterial growth in clams. In addition, no chemical or physical parameters, like temperature, pH, oxygen or salinity was measured during our sampling. In the Hood study (1983), showed that no correlation was observed between the indicator bacteria and these parameters.

To work in a laboratory in a developing country, with problems of poorly developed hygiene, incubators and lack of sterility, might influence the results. The enrichment temperature of Rappaport-Vassiliadis-medium is a very important factor and the incubator used in this study was not totally reliable. The laboratory used was most suited for ecological work and not optimal for microbial work. When glassware was used, the sterilisation was performed in an oven in the laboratory. To be sure of the sterility, the glassware was left at $>120\text{ }^{\circ}\text{C}$ for >2 hours. The optimum would be only to use disposable material.

It is important to start the analyses soon as possible. However, in this study, the isolated strains had to be analysed in Sweden, which might, in some cases, give misleading results.

There is no standard time for how long time the clams need to be cooked to kill the bacteria. Normally, *E. coli* dies at 50°C and *Salmonella* spp. at a slightly higher temperature. Thus, it is important that the whole mollusc reaches these minimum temperatures to achieve this. Unfortunately, this may not assure the clams to be harmless as many bacterial strains also produce thermotolerant toxins.

As nutrition deficiency in Mozambique is an important issue, every protein source is important. As the results show, there is not an immediate danger for people in the area to eat clams collected and bought in Maputo Bay and its environment. This is a result of the bacterial flora in Africa, which leads to both a higher immunotolerance against strains circulating in the community and to a tolerance of a higher bacterial concentration in the food. It would be inadvisable for Europeans that visit Maputo to eat clams raw or lightly cooked, since they probably will catch different illnesses.

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References

- Ambrus, Sr. J.L., Ambrus, Jr. J.L. (2004). Nutrition and Infectious Diseases in Developing Countries and Problems of Acquired Immunodeficiency Syndrome. *Experimental Biology and Medicine* 229:464-472
- Balidy, H.J. and Bandeira, S.O. (2003). Sea grass ecological economics in Maputo Bay: sea grass meadows role in invertebrate food security. In: Sustainable management and valuation of sea grass ecosystems in Western Indian Ocean region.
[Internet: http://www.wiomsa.org/download/MASMA/MASMA_Abstracts.pdf]
[Downloaded 04-08-20]
- Benson, D.R. (2004). Chapter: Proteobacteria II. In: Applied/Industrial Microbiology. MCB235/336. [Internet: <http://web.uconn.edu/mcb235/secure/ProteobacteriaII.pdf>]
[Downloaded 04-09-15]
- © The Blacksmith Institute, 2001, Two Park Avenue, 29th floor, New York, NY 10016;
[Internet: <http://www.blacksmithinstitute.org/mozambique.html>] [Downloaded 04-08-05]
- COPAN innovation. Sterile Transport Swab.[Internet: www.copaninnovation.com]
- Deguzman, C. (2004). *Serratia*. Environmental microbiology. BIOL/CEE/CSES 4984- Spring 2004 [Internet: http://soils1.cses.vt.edu/ch/biol_4684/Microbes/Serratia.html]
[Downloaded 04-09-10]
- DeVinney, R., Gauthier, A.Abe., Finnlay, B.B. (1998). Enteropathogenic *E. coli*: a pathogenic that inserts its own receptor into host cells. *Cell and Molecular life sciences* 55, pp. 961-976
- Dgedge, M., Novoa, A., Macassa, G., Sacarlal, J., Black, J., Michaud, C., Cliff, J. (2001). The burden of disease in Maputo City, Mozambique: registered and autopsied deaths in 1994. *Bulletin of the World Health Organization*, 2001, Number 79 p. 546-552
- Donovan, T.J., Gallacher, S., Andrews, N.J., Greenwood, M.H., Graham, J., Russle, J.E., Roberts, R.L. (1998). Modification of the standard method used in the United Kingdom for counting *Escherichia coli* in live bivalve molluscs. *Communicable Disease and Public Health* 1:188-196
- Doré, W. (1999). CEFAS, The Centre for Environment, Fisheries & Aquaculture Science. Weymouth Shellfish Hygiene Standard Operating Procedure, Enumeration of *Escherichia coli* in Molluscan Bivalve Shellfish. SS 028167, Edition 2
- Doré, W.J. & Lees, D.N. (1995). Behavior of *Escherichia coli* and Male-Specific Bacteriophage in Environmentally Contaminated Bivalve Molluscs before and after Depuration. *Applied and Environmental Microbiology*, Aug. 1995, p. 2830-2834
- Elliott, S.J., Srinivas, S., Albert, M.J., Alam, K., Robins-Browne, R.M., Gunzburg, S.T., Mee, B.J., Chang, B.J. (1998). Characterization of the Roles of Hemolysin and Other Toxins in Enteropathy Caused by Alpha-Hemolytic *Escherichia coli* Linked to Human Diarrhea.

Applied and Environmental Microbiology, Infection and Immunity May 1998, Vol. 66, No. 5, p. 2040-2051

Emedicine, instant access to the minds of medicine, [Internet: <http://www.emedicine.com/med/byname/klebsiella-infections.htm>] [Downloaded 04-08-20]

Environmental Health Perspectives (2002). Report on Carcinogens, 10th ed.; U.S. Department of Health and Human Services, Public Health Services, National Toxicology Program, December 2002

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Guentzel, M.N. (1996) Chapter 26: *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter* and *Proteus*. In: Medical Microbiology (4th ed.) Edited by Samuel Brown, The University of Texas Medical Branch at Galveston ISBN: 0-9631172-1-1

Hamer, H.D., Simon, J., Thea, D., Keush, G.T. (1998). Childhood Diarrhea in Sub-Saharan Africa. Child Health Research Project Special Report, Vol. 2, Number 1, April 1998

Hernroth, B.E., Conden-Hansson, A-C., Rehnstam-Holm, A-S, Girones, R. & Allard, A.K. (2002). Environmental Factors Influencing Human Viral Pathogens and Their Potential Indicator Organisms in the Blue Mussel, *Mytilus edulis*: the First Scandinavian Report. *Applied and Environmental Microbiology*, Sept. 2002, p. 4523-4533

Hernroth, B., 2002. Uptake and fate of pathogenic microbes in the blue mussel, *Mytilus edulis*. University of Gothenburg ISBN: 91-628-5200-0

Hood, M.A., Ness, G.E., Blake, N.J. (1983). Relationship Among Fecal Coliforms, *Escherichia coli*, and *Salmonella* spp. in shellfish. *Applied and Environmental Microbiology*, Jan. 1983, p. 122-126

Iglesias, A.V. & Martínez, J.S. (2004). Bronchiectasis Due to *Flavimonas oryzihabitans* in an Immunocompetent Patient. *Archivos De Bronconeumologia*. Vol 40, Issue 8, p. 384-385

Jay, M.J. (2000). Modern food microbiology (6th ed.). pp. 101-112, Aspen Publishers

Johnson, J.R. (1991) Virulence Factors in *Escherichia coli* Urinary Tract Infection. *Clinical Microbiology Reviews*, Jan. 1991. p. 80-128

Kahlmeter, G. The Swedish Reference Group for Antibiotics (SRGA) and its subcommittee on methodology (SRGA-M) [Internet: <http://www.srga.org/>] [Downloaded 04-09-01]

- Lindquist, J. [Internet: <http://www.jlindquist.net/generalmicro/102bactid2.html>] [Downloaded 04-08-17], Department of Bacteriology, University of Wisconsin-Madison
- Lizárraga-Partida, M.L., Cárdenas, G.V. (1996). Influence of Water Circulation on Marine and Faecal Bacteria in a Mussel-Growing Area. *Marine Pollution Bulletin*, Vol. 32, No. 2, pp. 196-201
- Madigan, M.T., Martinko, J.M. and Parker, J. (2000). Brock Biology of Microorganisms (9th ed.) p. 741-771. New Jersey: Prentice-Hall
- Parveen, S., Hodge, N.C., Stall, R.E., Farrah, S.R., Tamplin, M.L. (2000). Phenotypic and genotypic characterization of human and nonhuman *Escherichia coli*. *Wat. Res.* Vol. 35, No. 2, p. 379-386
- Rippey, S.R. (1994). Infectious Diseases Associated with Molluscan Shellfish Consumption. *Clinical Microbiology Reviews*, Oct. 1994, p. 419-425
- Roberts, D.P., Dery, P.D., Yucel, I., Buyer, J.S. (2000). Importance of *pfkA* for Rapid Growth of *Enterobacter cloacae* during Colonization of Crop Seeds. *Appl Environ Microbiol* January;66 (1) p. 87-91
- Salyers, A.A. & Whitt, D.D. (2002). Bacterial Pathogenesis, a molecular approach (2nd ed.) p.422-436. Washington: ASM Press
- Sanath K.H., Sunil, R., Venugopal, M.N., Karunasagar, I., Karunasagar, I. (2003). Detection of *Salmonella* spp. in tropical seafood by polymerase chain reaction. *Int. J. Food Microbiol* 2003 Nov 15;88(1):91-5
- Smadja, B., Latour, S., Burini, F., Chevalier, S., Orange, N. (2004). Thermodependence of growth and enzymatic activities implicated in pathogenicity of two *Erwinia carotovora* subspecies (*Pectobacterium* spp.) *Canadian Journal of Microbiology*, January 2004, vol. 50, no.1, pp. 19-27
- Wafae, C., Alaoui, A.S., Amar, M. (2004). *Chryseomonas luteola* Identified as the Source of Serious Infections in a Moroccan University Hospital. *Journal of Clinical Microbiology*. April; 42 (4) p. 1837-1839
- Wiberg, C. (1999) Nordic Committée on Food Analysis (5th ed.) 71: pp.1-8
- Wu, R.S.S. (1999). Eutrophication, Water Borne Pathogens and Xenobiotic Compounds: Environmental Risks and Challenges. *Marine Pollution Bulletin*. Vol. 39, Nos. 1-12. p. 11-22

Appendix

Sterile peptone water

- 1000 ml peptone water: 1 g Neutralised bacteriological peptone

The media was added to 1000 ml hot distilled water and autoclaved at 121°C±2°C for 15 min.

Modified Mineral Glutamate Broth

- 300 ml **Single MMGB**: 3,42 g Mineral Modified medium base
1,92 g Sodium Glutamate
0,75 g Ammonium Chloride

The ingredients were dissolved in 300 ml hot distilled water and 10 ml was distributed into 15 ml plastic tubes.

- 100 ml **Double MMGB**: 2,27 g Mineral Modified medium base
1,27 g Sodium Glutamate
0,5 g Ammonium Chloride

The ingredients was dissolved into 100 ml hot distilled water and 10 ml distributed into 50 ml plastic tubes.

Tryptone bile glucuronide agar (TBGA)

- 600 ml: 21,9 g TBGA

The TBGA was dissolved in 600 ml boiling distilled water and autoclaved at 121°C ±2°C for 15 min. when the media cooled down to 60°C, 12 ml 1% calcium-glucose solution was added to make 2% agar.

Rappaport-Vassiliadis soy peptone (RSV) broth

- 1000 ml: 26,6 g RSV medium

The medium was suspended in 1000 ml cold distilled water and then heated to dissolve. The broth was sterilised by autoclaving at 115°C for 15 min.

Xylose Lysine Desoxycholate (XLD) agar

- 1000 ml: 55 g XLD medium

The medium was dissolved in 1000 ml cold distilled water, heated to boiling with agitation to completely dissolve. This agar is not supposed to be autoclaved.

Triple Sugar Iron Agar (TSIA)

- 1000 ml: 65 g TSIA

The TSIA was dissolved in 1000 ml boiling distilled water while stirring, for one minute. The agar was then autoclaved at 121°C ±2°C for 15 min.

The TSIA contains meat, yeast, pancreas, proteos, dextrose, lactose, sucrose, iron sulfate, sodium chloride, sodium thioisulfate, agar and phenol red.

API-20[®] Enteric Identification System

A plastic strip holding twenty mini-test tubes is inoculated with a saline suspension of a pure culture (Lindquist 2004). Some tubes are completely filled (why?) and others are covered with mineral oil in order to carry out anaerobic reactions.

- Amino acids tested: arginine, lysine and ornithine
- Decarboxylation

- Carbohydrate tested: glucose, mannitol, inositol, rhamnose, sucrose, melibiose, amygdalin and arabinose
- Hydrogen sulfide production (H₂S) and Gelatine hydrolysis
- Positive reaction for tryptophan deaminase (TDA)

Appendix B: *E. coli* Test Form

Dilution	Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	Local	Collection date	Analysis date	Number
Weight (g)	1	0,1	0,01	0,001	0,0001	0,00001				
Total	5	5	5	4	0	-	B.d.P.	May 13 th	May 13 th	160 000
<i>E. coli</i>	-	4	4	1	-	-	Market (Montanhana)			3 800
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	4	0	0	0	-	B.d.P.	May 17 th	May 17 th	1 300
<i>E. coli</i>	5	3 (1)	-	-	-	-	Gatherers			1 300
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	3	0	1	-	B.d.P.	May 17 th	May 18 th	>18000
<i>E. coli</i>	-	1 (4)	2 (1)	-	-	-	Gatherers			18 000
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	5	0	0	-	L.C.	May 18 th	May 18 th	24 000
<i>E. coli</i>	-	5	5	-	-	-	Gatherers			24 000
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	3	0	0	0	-	B.d.P.	May 24 th	May 24 th	750
<i>E. coli</i>	4 (1)	0 (3)	-	-	-	-	Market (Macaneta)			220
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	5	3	0	0	-	B.d.P.	May 24 th	May 25 th	>18000
<i>E. coli</i>	-	1 (4)	1 (2)	-	-	-	Market (Macaneta)			18 000
<i>Salmonella</i>	Not positive						<i>M.meretrrix</i>			
Total	5	5	1	0	0	-	B.d.P.	June 1 st	June 1 st	3 500
<i>E. coli</i>	-	5	1	-	-	-	Gatherers			3 500
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	4	0	1	-	B.d.P.	June 1 st	June 1 st	16 000
<i>E. coli</i>	-	4 (1)	3 (1)	-	0	-	Market (Montanhana)			16 000
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	5	3	0	0	-	B.d.P.	June 1 st	June 2 nd	>18000
<i>E. coli</i>	-	5	2 (1)	-	-	-	Gatherers			18 000
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	3	2	0	-	B.d.P.	June 1 st	June 2 nd	14 000
<i>E. coli</i>	-	1	0	-	-	-	Market (Montanhana)			-
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	5	1	0	0	-	B.d.P.	June 7 th	June 7 th	3 500
<i>E. coli</i>	-	1 (2)	1	-	-	-	Gatherers			9 00
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	3	0	0	-	B.d.P.	June 7 th	June 7 th	3 500
<i>E. coli</i>	-	4 (1)	3	-	-	-	Market (Montanhana)			3 500
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	5	1	0	0	-	B.d.P.	June 7 th	June 8 th	9 100
<i>E. coli</i>	-	3(2)	1	-	-	-	Gatherers			9 100
<i>Salmonella</i>	Positive						<i>E.paupercula</i>			
Total	5	5	3	0	0	-	B.d.P.	June 7 th	June 8 th	>18000
<i>E. coli</i>	-	5	2 (1)	-	-	-	Market (Montanhana)			18 000
<i>Salmonella</i>	Negative						<i>M.meretrrix</i>			
Total	5	5	5	4	0	-	B.d.P.	June 14 th	June 14 th	160 000
<i>E. coli</i>	-	5	5	3 (1)	-	-	Market (Macaneta)			160 000
<i>Salmonella</i>	Positive						<i>M.meretrrix</i>			
Total	5	5	0	0	0	-	B.d.P.	June 14 th	June 15 th	-
<i>E. coli</i>	-	3	-	-	-	-	Market (Macaneta)			-

<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total		5	5	1	0	-	B.L.C.	June 16 th	June 16 th	35 000
<i>E. coli</i>	5	5	5	1	-	-	Gatherers			35 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total	5	5	5	5	3	-	L.C.	June 16 th	June 17 th	910 000
<i>E. coli</i>	-	5	5	5	3	-	Gatherers			910 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total	5	5	5	5	2	-	B.L.C.	June 21 st	June 21 st	540 000
<i>E. coli</i>	-	5	5	4	1 (1)	-	Gatherers			220 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total	5	5	5	5	2	0	L.C.	June 21 st	June 22 nd	540 000
<i>E. coli</i>	-	5	5	5	2	-	Gatherers			540 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total	5	5	4	0	0	-	B.d.P.	June 28 th	June 28 th	16 000
<i>E. coli</i>	-	3 (2)	4	-	-	-	Market (Macaneta)			11 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			
Total	5	5	5	2	0	-	B.d.P.	June 28 th	June 29 th	54 000
<i>E. coli</i>	-	1 (3)	4 (1)	1 (1)	-	-	Market (Macaneta)			16 000
<i>Salmonella</i>	Positive						<i>M.meretrix</i>			

According to the CEFAS method (Fisheries Research Services, Food Standards Agency, Scotland);

Category A	< 230 <i>E. coli</i> / 100 g	May go for human consumption
Category B	> 230 - < 4 600 <i>E. coli</i> / 100 g	Must be deputed, heat-treated or relayed to meat Category A requirement
Category C	> 4 600 - < 46 000 <i>E. coli</i> / 100 g	Must be relayed for long periods or after intensive purification
Prohibited	> 46 000 <i>E. coli</i> / 100 g	Unsuitable for production

Sterility control:

Date	Control	Results
May 25 th	Two plastic tubes (15 ml)	No growth
May 25 th	Large mixer, sterile peptone water	No growth
May 25 th	Small mixer, sterile peptone water	No growth
June 1 st	Large mixer, sterile peptone water	No growth
June 7 th	Large mixer, sterile peptone water	No growth
June 7 th	Small mixer, sterile peptone water	No growth
June 7 th	Two test tubes (15 ml), plastic	No growth
June 7 th	Two test tubes, glass	No growth
June 8 th	Large mixer, sterile peptone water	No growth
June 8 th	Small mixer, sterile peptone water	No growth
June 14 th	One test tube, glass	No growth
June 15 th	Large mixer, sterile peptone water	No growth
June 15 th	Small mixer, sterile peptone water	No growth
June 16 th	Large mixer, sterile peptone water	No growth
June 16 th	Small mixer, sterile peptone water	No growth
June 17 th	Large mixer, sterile peptone water	No growth
June 17 th	Small mixer, sterile peptone water	No growth
June 21 st	Large mixer, sterile peptone water	No growth
June 21 st	Small mixer, sterile peptone water	No growth
June 22 nd	One test tube, glass	No growth
June 28 th	Large mixer, sterile peptone water	No growth
June 28 th	Small mixer, sterile peptone water	No growth

Date	Control	Results
May 25 th	One test tube (15 ml), plastic	No growth
May 25 th	Small mixer, sterile peptone water	No growth
7/6	One test tube (15 ml), plastic	No growth
15/6	Small mixer, sterile peptone water	No growth
29/6	One test tube, glass	No growth
30/6	One test tube, glass	No growth