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Le titre de la thèse

Assessment of viral and metal contamination in molluscs (*Mytilus galloprovincialis* and *Patella rustica)* from Rabat Region

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DEDICATION

I dedicate this work to my father's memory

To my mother

To my sisters, ***Saida*** *and* ***Wafaa***

To my brothers

To my nephews and nieces

To my husband

To my child, ***Hossam***

To my family

For their love, support and encouragement. I can't thank them enough for all sacrifices they have made to help me get to where I am today.

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To my friends

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ABSTRACT

Bivalve and gastropod molluscs have long been recognized as being beneficial to human health and this benefit should be taken into consideration in managing the coastal zone and preserving the marine environmental quality. The consumption of molluscan shellfish contaminated with viruses, bacteria, parasites, metals and pesticides may lead to serious diseases.

This work was done to evaluate the viral and metal contamination of bivalve and gastropod molluscs. Therefore, 288 samples of mussels (*Mytilus galloprovincialis*) and 120 samples of limpets (*Patella rustica*) were collected. The contamination of the bivalve molluscs by enteroviruses was detected by using cell culture, real-time PCR and seroneutralization. Whereas, the contamination of the gastropod molluscs by metallic trace elements such as copper (Cu), chromium (Cr), cadmium (Cd), and lead (Pb) was determined by Atomic Absorption Spectrophotometry (AAS).

The virological study was conducted to detect and type enteroviruses isolated from mussel samples, while chemical analysis was performed to determine the concentration and bioaccumulation of certain essential metals (Cu and Cr) and non-essential metals (Pb and Cd) in the soft tissues of *Patella rustica*.

The sampling sites are polluted by untreated or partially treated domestic wastewater, human waste and substances introduced by human. The results obtained in this study by virological and chemical analysis showed that bivalve and gastropod molluscs in sampling sites are contaminated with enteroviruses and metallic trace elements which may be cause a human health risk. For this reason, it is recommended that the sanitary quality of these molluscs is improved by strengthening the surveillance network of aquatic ecosystems, systematically dosing the chemical substances and performing extensive virological analyses.

Keywords: Contamination assessment, *Mytilus galloprovincialis*, enteroviruses, *Patella rustica*, metallic trace elements, Rabat Region.

RÉSUMÉ

Les mollusques bivalves et gastéropodessont reconnus depuis longtemps comme étant bénéfiques pour la santé humaine. Ces ressources marines doivent être protégées par la gestion et l'utilisation rationnelle des zones côtières et la préservation de la qualité de l'environnement marin. La consommation de mollusques contaminés par des virus, des bactéries, des parasites, des métaux et des pesticides peut entraîner des maladies graves.

Ce travail a été effectué avec un total de 288 échantillons de moules (*Mytilus galloprovincialis*) et 120 échantillons de patelles (*Patella rustica*) pour évaluer la contamination virale et métallique. La contamination de bivalves par les *Entérovirus* a été détectée par culture cellulaire, PCR en temps réel et séroneutralisation. Alors que la contamination de gastéropodes par les éléments traces métalliques y compris le cuivre (Cu), le chrome (Cr), le cadmium (Cd) et le plomb (Pb) a été mesurée par Spectrophotométrie d'Absorption Atomique (SAA). L'étude virologique a été effectuée dans le but de détecter et de typer les *Entérovirus* isolés à partir des échantillons de moules, tandis que l'analyse chimique a été menéepour déterminer la concentration et la bioaccumulation de certains métaux essentiels (Cu et Cr) et non-essentiels (Pb et Cd) dans les tissus mous de *Patella rustica*.

Les sites d'échantillonnages sont pollués par des rejets des eaux usées domestiques non traitées ou partiellement traitées, des déchets rejetés par l'homme et des substances introduites par les activités humaines. Les résultats obtenus dans cette étude par analyse virologique et chimique ont montrés que les mollusques bivalves et gastéropodes sont contaminés par des *Entérovirus* et des éléments trace métalliques et peuvent présenter un risque pour la santé humaine. Pour améliorer la qualité sanitaire de ces mollusques il est indispensable de renforcer le réseau de surveillance des écosystèmes aquatiques, en dosant systématiquement les substances chimiques et en effectuant des analyses virologiques.

Mots-clés: Évaluation de la contamination, *Mytilus galloprovincialis*, *Entérovirus*, *Patella rustica*, éléments traces métalliques, Région de Rabat.

ملخص

منذ فترة طويلة، تعرف الرخويات ذات الصدفتين والقواقع بقيمتها الغذائية باعتبارها مفيدة لصحة الإنسان.ذلك ينبغي حماية هذه الموارد البحرية من خلال التدبير والإستخدام الرشيد للمناطق الساحلية والمحافظة على جودة البيئة البحرية. يمكن أن يؤدي استهلاك الرخويات الملوثة بالفيروسات، البكتيريا، الطفيليات، المعادن والمبيدات الحشرية إلى أمراض خطيرة.

لتقييم التلوث الفيروسي والمعدني للرخويات ذات الصدفتين والقواقع قمنا بجمع 288 عينة من بلح البحر*Mytilus) galloprovincialis*) و 120 عينة من القواقع (*Patella rustica*). وقد تم الكشف عن الفيروسات المعوية المعزولة من الصدفيات بواسطة تقنية زرع الخلايا، تقنية تفاعل البوليميراز التسلسلي اللحظي وتقنية تحديد المصل. في حين تم قياس مستوى تركيز وتراكم بعض المعادن الأساسية (النحاس (Cu) والكروم (Cr)) والغير الأساسية (الرصاص (Pb) والكادميوم (Cd)) ي أنسجة القواقع بواسطة القياس الطيفي للامتصاص الذري.

يرجع تلوث مواقع أخذ العينات إلى صرف المياه العادمة المنزلية غير المعالجة أو المعالجة جزئياً، والمواد التي تنتج عن الأنشطة البشرية. لقد أظهرت النتائج التي تم الحصول عليها في هذه الدراسة عن طريق التحليل الفيروسي والكيميائي أن الرخويات ذات الصدفتين والقواقع الملتقطة من منطقة أخذ العينات ملوثة بالفيروسات المعوية والعناصر المعدنية، وقد تشكل خطراً على صحة الإنسان. لهذا السبب، يوصى بتحسين الجودة الصحية لهذه الرخويات من خلال تعزيز شبكة مراقبة النظم الإيكولوجية المائية، عن طريق قياس منهجي لجرعات المواد الكيميائية (مثل العناصر المعدنية) وإجراء التحليلات الفيروسية (بشكل خاص الفيروسات المعوية).

الكلمات المفتاحية: تقييم التلوث، *Mytilus galloprovincialis*، الفيروس المعوي، *Patella rustica*، العناصر المعدنية، جهة الرباط.

SYNTHÈSE DE RÉSULTATS DE CETTE ÉTUDE

Les mollusques bivalves sont des filtreurs, ils agissent en tant que biofiltres naturels dans l'eau de mer et ils peuvent donc efficacement bioconcentrer, bioaccumuler et amplifier des virus entériques dans leur tissu digestif. Au Maroc, l'analyse de la qualité sanitaire des mollusques n'inclut pas la détection des virus entériques (tel que les *Entérovirus*). Par conséquent, l'objectif de cette étude était donc de détecter la présence des *Entérovirus* dans les moules (*Mytilus galloprovincialis*) recueillies dans trois Sites d'échantillonnage (estuaire de Bouregreg, plage de Yacoub Al Mansour et plage de Harhoura) afin d'obtenir une vue d'ensemble sur la contamination virale en milieu marin. Entre Février 2014 et Février 2015, 288 échantillons ont été collectés et testés; et ce dans le but de déceler la contamination virale à l'aide de la culture cellulaire en utilisant deux lignées cellulaires (les cellules de rhabdomyosarcome humain (RD) et les cellules de souris (L20B)) ainsi que la réaction en chaîne par polymérase en temps réel (PCR en temps réel) à travers la différenciation intratypique (ITD). Les particules des *Entérovirus* ont été extraites et concentrées à partir de l'hépatopancréas des échantillons de moules en utilisant du polyéthylène glycol (PEG) 6000.

Les *Entérovirus* non poliovirus (EVNP) sont des agents infectieux susceptibles de provoquer diverses maladies chez l'homme, telles que le syndrome mains-pieds-bouche, l'angine de poitrine, les maladies respiratoires, les cardiopathies aiguës ou chroniques, la diarrhée, la pancréatite, les hémorragies aiguës et la conjonctivite. Ces virus sont éliminés dans les selles et contaminent ainsi l'environnement marin et les mollusques.

Parmi les 288 échantillons, 216 (75%) ont été révélés positifs par la méthode de culture cellulaire, avec 204 souches d'EVNP (70,8%) et 12 souches de *Poliovirus* Type 1 (4,2%). Selon les procédures recommandées par l'organisation mondiale de la santé (OMS), l'identification antigénique et le sérotypage par séroneutralisation ont été effectuée. Le sérotype de 204 souches d'EVNP a identifié des souches typables (64,7%) et des souches non typables (35,3%) dans le milieu marin. Cependant, la proportion de ces souches non typables confirme la présence de nouveaux sérotypes.

Les résultats obtenus par culture cellulaire et PCR en temps réel ont montré que la consommation des moules contaminés par les rejets des eaux usées domestiques non traitées ou partiellement traitées révélait un risque évident d'infection. Pour cette raison, la présence des *Entérovirus* dans les sites d'échantillonnage représente un danger potentiel pour la santé des consommateurs en provoquant des maladies graves (tel que les gastro-entérites, les hépatites et la poliomyélite).

Les maladies causées par les *Entérovirus* non poliovirus constituent un problème majeur de santé publique. Afin de lutter contre ces maladies et de protéger la santé des consommateurs des mollusques, il est indispensable d'instaurer un système de contrôle et de surveillance virologique de l'environnement marin.

Cette étude a été réalisée également pour déterminer les niveaux de certains métaux, y compris le cuivre (Cu), le chrome (Cr), le cadmium (Cd) et le plomb (Pb) dans les tissus mous des mollusques gastéropodes (*Patella rustica*), en utilisant la spectrophotométrie d'absorption atomique (AAS) après la méthode de minéralisation. Ces échantillons ont été prélevés durant les quatre saisons, cela a été accompli à partir de trois sites différents (plage de Yacoub Al Mansour, plage de Harhoura et plage de Guy ville) situés sur la côte atlantique marocaine. Dans le but d'analyser et d'évaluer la contamination métallique, 120 échantillons de patelles (*Patella rustica*) ont été collectées.

Les résultats de cette recherche montrent que les niveaux de concentration moyenne des éléments métalliques mesurées dans les échantillons de patelles sont les suivantes: au niveau de la plage de Yacoub Al Mansour (Cu: 0,46 à 2,19 µg/g; Cr: 0,60 à 2,21 µg/g; Cd: 0,32 à 1,06 µg/g. et Pb: 0,47-1,30 µg/g), au niveau de la plage de Harhoura: (Cu: 0,81 à 3,17 µg/g; Cr: 0,94-2,5 µg/g; Cd: 0,47-0,95 µg/g et Pb: 0,76-1,42 µg/g) et au niveau de la plage de Guy ville (Cu: 1,24-4,14 µg/g; Cr: 0,87-3,98 µg/g; Cd: 0,56- 1,18 µg/g et Pb: 1,08-2,13 µg/g). Les résultats obtenus indiquent que les concentrations des éléments traces métalliques (ETM) dans les tissus mous de *Patella rustica* étaient réparties différemment au cours de toutes les saisons et à partir de différents sites d'échantillonnage. Les concentrations des éléments traces métalliques (Cu, Cr, Cd et Pb) dans les tissus mous des mollusques gastéropodes (*Patella rustica*) de la côte atlantique marocaine ont été analysées et examinées.

Les relations entre la taille des patelles et les niveaux de métal ont été étudiées par analyse de régression linéaire. Ce travail a été mené pour déterminer la bioaccumulation et les relations de certains métaux essentiels (Cu et Cr) et non essentiels (Pb et Cd) dans les tissus mous de *Patella rustica* en calculant l'analyse de corrélation.

Les résultats de cette étude chimique démontrent que les concentrations en métaux (ng / mg de poids sec) dans les tissus mous de *Patella rustica*, diminuent dans l'ordre suivant: Cu (4.14)> Cr (3.98)> Pb (2.13)> Cd (1.18).

Les résultats de l'analyse de la régression linéaire ont confirmé que, dans tous les échantillons, les relations entre les concentrations de métal et la taille des patelles étaient significatives.

De plus, on a observé que les patelles de grande taille présentent les concentrations les plus élevées de Cu, Cr, Cd et Pb par rapport à celles de petite taille. Cependant, il a été constaté que les *Patella rustica* peuvent être utilisés comme un outil de surveillance de la contamination métallique chez les mollusques gastéropodes car ils sont utilisés comme des bioindicateurs de l'environnement marin.

On peut conclure qu'au Maroc, la prévalence des *Entérovirus* non poliovirus en circulation dans l'environnement marin et le niveau de contamination chimique chez les gastéropodes sont inconnus. Cependant, les données manquent pour évaluer le risque de la contamination virale et métallique chez les mollusques bivalves et gastéropodes, afin de garantir la sécurité alimentaire. Dans ce cadre, notre étude a été réalisée pour enrichir ces bases de données et pour contrôler et surveiller l'état de l'écosystème côtier, dans le but de sensibiliser les consommateurs et de tirer la sonnette d'alarme sur le risque lié à la contamination des bivalves et gastéropodes dans la région de Rabat.

Les résultats de cette étude montrent la présence des *Entérovirus* et des éléments traces métalliques (Cu, Cr, Cd et Pb) à un niveau de concentration très élevé dans toutes les stations étudiées, chose qui représente un danger réel pour la santé des consommateurs des mollusques contaminés.

CONTENT

List of Tables

List of Figures

List of Notations

As	Arsenic	**P**	Phosphorus
bp	base pair	**Pb**	Lead
Ca	Calcium	**$PbCO_3$**	Cerussite
Cd	Cadmium	**PbS**	galena
Cl	Chlorine	**$PbSO_4$**	anglesite
cm^3	cubic centimeter	**pH**	amount of hydrogen ions available
Co	Cobalt		in a solution
Cr	Chromium	**RD**	Human Rabdomyosarcom cells
$Cr(OH)^{2+}$	Chromium (II) Hydroxide	**RSD**	Relative Standard Deviation
$Cr(OH)_3$	Chromium (III) Hydroxide	**s**	second
Cr_2O_3	Chromium oxide	**Sc**	Scandium
CrO_4^{2-}	Chromate Ion	**Se**	Selenium
$Cr_2O_7^{2-}$	Dichromate Ion	**Si**	Silicium
Cu	Cupper	**t/year**	tonnes per year
Hg	Mercury	**UV**	ultraviolet
HNO_3	Nitric acid	**V**	Vanadium
H_2O_2	Oxygenated water	**W**	West
Fe	Iron	**χ^2 test**	khi-deux test
$FeO.Cr_2O_3$	Ferrous chromite	**Zn**	Zinc
K	Potassium	**µg/g**	microgram per gram
I	Iodine	**µg/L**	microgram per liter
L20B	mouse cells	**µL**	microliter
m	meter	**g/kg**	gram per kilogram
Max.	Maximum	**g/L**	gram per liter
Mg	Magnesium	**%**	percentage
min	minute	**°C**	degree celsius
Min.	Minimum		
Mn	Manganese		
Na	Sodium		
Ni	Nickel		

List of Abbreviations and Acronyms

AAS	AtomicAbsorption Spectrophotometry	**IAEA**	International Atomic Energy Agency
ADI	Acceptable Daily Intake	**ISPITS**	Instituts Supérieurs des Professions Infirmières & Techniques de Santé
AdV	Adenovirus	**ITD**	Intratypic Differentiation
AFP	Acute Flaccid Paralysis	**IWMI**	International Water Management Institute
ANOVA	Analysis Of Variance	**JECFA**	Joint Expert Commitee on Food Additives
ATSDR	Agency for Toxic Substances and Disease Registry	**LCD**	Liquid Crystal Display
AV	Astrovirus	**MEM**	Minimum Essential Medium
CDC	Center of Disease Control	**MTE**	Metallic Trace Elements
CPE	Cyto-Pathogenic Effect	**NPEV**	Non *Poliovirus* Enteroviruses
Ct	Cycle threshold	**NoV**	Norovirus
DNA	Deoxyribonucleic Acid	**NCR**	Non-Coding Region
ECC	European Communities Commission	**ORF**	Open Reading Frame
EEC	European Economic Community	**PBS**	Phosphate Buffered Saline
EFSA	European Food Safety Authority	**PCR**	Polymerase Chain Reaction
EV	*Enterovirus*	**PEG**	Polyethylene Glycol
EU	European Union	**PTWI**	Provisional Tolerable Weekly Intakes
FAO	Food and Agriculture Organization	**PV**	*Poliovirus*
FBS	Fetal Bovine Serum	**RNA**	Ribonucleic Acid
FDA	Food and Drug Administration	**RNAi**	Ribonucleic acid interfering
FSR	Faculty of Sciences of Rabat	**RV**	Rotavirus
HAV	Hepatitis A Virus	**SL**	Sabin-like

TDI Tolerable Daily Intake

HEV Hepatitis E Virus

UTRs Untranslated Regions

UNEP United Nations Environment Programme

UNO United Nations Organization

USA United States of America

V/V Volume to Volume

VDVP Vaccine-Derived *Poliovirus*

VP Viral Protein

WHO World Health Organization

GENERAL INTRODUCTION

Marine pollution is an inevitable fact that has been happening for many years in all parts of the world. It results from organic and toxic waste discharged into the seas as a result of human activities (**Moss, 2008; Kacar et al., 2016**).

Morocco has two sea coastlines, the Atlantic ocean and the Mediterranean sea, extending over 3500 km, represented by a Mediterranean facade stretching about 512 km from Tangier to Saidia and an Atlantic facade of 2934 km, ranging from Tangier to Lagouira. The Mediterranean coastline of Morocco is an exceptional and ecologically fragile marine area, which contain an important richness of sea reserves. It is characterized by a large diversity of living aquatic resources which are subjected to natural and anthropic pollution originated by industrial and agricultal activities, and domestic discharges, etc (**Danovaro, 2003; Er-Raiou et al., 2012**).

In most of the world's seas and oceans, there are several classes of potentially toxic contaminants which affect the quality of the marine environment and shellfish products. To mitigate the toxicity of all types of contaminants and to preserve the health of aquatic ecosystems, it is imperative to control and monitor the marine environment against the adverse effects of anthropic activities (**Nakhli and Ghazi, 2008; Gallo et al., 2018**).

The viral and metal contamination (presence of persistent organic and inorganic pollutants) is currently a major problem. Indeed, the levels of contamination in the marine environment, especially in marine organisms such as shellfish represents a potential risk for human consumption (**Riedel et al., 2002; Karouna-Renier et al., 2007; Minerbe et al., 2011**). As a result, the request for analysis is an increase because it is necessary to be able to detect and identify contaminants in the aquatic ecosystem and to quantify them using reliable, sensitive and reproducible methods of analysis (**Law et al., 2015**). In this context, the objective of this study was to evaluate the viral and metal contamination of bivalve and gastropod molluscs (*Mytilus galloprovincialis* and *Patella rustica*) collected from the Moroccan Atlantic coasts (Bouregreg estuary, Yacoub Al Mansour, Harhoura and Guy City coast). For this, we have chosen to asses the contamination level of some metallic trace elements (lead, copper, cadmium, and chromium) and to detect the presence of enteroviruses in the soft tissues of mollusc samples.

In addition, this work is fully in line with the environmental monitoring studies of the marine environment using bioindicators such as molluscs. The detection of the infectious agents and

the monitoring of the concentrations of the toxic substances allow us to identify and know the levels of risks related to the consumption of these contaminated bivalves and gastropods.

This manuscript is organized as follows:

For the first part:

The first and second chapters: focus on the state of knowledge on marine biomonitoring and marine environmental pollution. This is an introduction to general concepts of pollution bioindicators, viral contamination of bivalve molluscs and metal contamination of gastropod molluscs.

The third chapter presents the general and specific objectives of this work.

For the second part:

All chapters are devoted to the detailed description of the study area as well as the completeness of the methods and techniques used from sampling to analysis, with the results and discussions.

Finally, we present the conclusions of this work and propose some research perspectives.

This thesis is structured in two main sections: The first section is devoted to the viral contamination of the marine environment, to detecting and serotyping of enteroviruses isolated from the mussel samples collected at the different sampling sites in this study. The second section is concentrated on chemical contaminants, to assess the contamination level of metals in the soft tissues of the gastropod mollusc and to study the relationship between metallic bioaccumulation in the bioindicators and their size.

This study is the continuation of previous researches dealing with the contamination of the marine environment (mussels, limpets, etc). It allows summarizing the consequences on the quality of shellfish and on the health of the consumer, as well as the scientific arguments already constituted and cited according to the results found.

Currently, there are few environmental studies evaluating viral and metallic contamination of different bivalve and gastropod molluscs from potentially polluted areas in the whole world.

However, each country must establish an environmental quality assessment and monitoring a program based on the results of the environmental contamination risk analysis in order to protect the environment and human health.

The general objective of this project was to detect the viral contamination in bivalve molluscs (*Mytilus galloprovincialis*) and the metal contamination in gastropod molluscs (*Patella rustica*). In order to supplement the available Moroccan databases on environmental contamination by enteroviruses and metallic trace elements. Moreover, this study illustrated the importance of including the routine virological analyses and expanding the list of chemical analyses to all edible molluscs in Morocco.

The specific objectives of this work were:

- ✓ To detect enteroviruses in mussels (*Mytilus galloprovincialis)* collected from three wild populations (Bouregreg estuary, Yacoub Al Mansour and Harhoura coast) in order to get an overview of viral contamination in the marine environment using cell culture and real-time PCR methods;

- ✓ To identify serotypes of enteroviruses that causes contamination of bivalve molluscs (*Mytilus galloprovincialis)* from Moroccan Atlantic coast;

- ✓ To assess the contamination level of metals (Cu, Cr, Cd and Pb) in *Patella rustica* collected from three different locations (Yacoub Al Mansour, Harhoura and Guy ville) of Moroccan Atlantic coast during four seasons by using the standard atomic absorption spectrophotometery (AAS) technique;

- ✓ To determine the relationships between metal (Cd, Cr, Cu and Pb) levels and the size of Moroccan Atlantic coast gastropod species *(Patella rustica*);

- ✓ To evaluate the public health risk associated with consumption of contaminated bivalve and gastropod molluscs.

PART I: BACKGROUND

CHAPTER I: THE MARINE BIOMONITORING & THE POLLUTION OF THIS ENVIRONMENT

A. Bioindicators of the pollution

1. Overview

The marine environment is exposed to various anthropogenic pollutants caused by domestic, agricultural and industrial activities. These discharges alter the communities and affect the organisms that compose them (**D'Adamo et al., 2008; Rao et al., 2007; Ansari and Matondkar, 2014; Bukola et al., 2015**). In order to control the degradation of marine environments, it is essential to develop many tools for assessing and monitoring the quality of the aquatic ecosystem. Therefore, the marine organisms that can be used to identify and quantify the effects of different pollutants on the marine environment are the bioindicators, the biomonitors, and the biomarkers (**Cajaraville et al., 2000; Zhou et al., 2008; Parmar et al., 2016**). Among the various bioindicators which can be utilized to assess the quality of the marine environment, those derived from the benthic macrofauna have many advantages and have been exploited extensively by researches until now. Porifera, echinoderms, corals, marine worms and molluscs are the most commonly used groups of marine organisms because of their specific characteristics such as abundance, size, and sedentary lifestyle (**Linton and Warner, 2003; Magni, 2003; Yusof et al., 2004; Sharma and Rawat, 2009; Dauvin et al., 2010; Bukola et al., 2015**).

The main purpose of assessment and biomonitoring of environmental pollution is to identify the threats and risks posed to marine organisms and human health, related to the ingestion of edible species contaminated. Hence, the great challenge for future generations is to develop environmental assessment methods accessible to all countries and to develop information exchange systems for the monitoring and conservation of coastal marine environments (**Moschino et al., 2016; Vethaak et al., 2017**).

2. Choice of the bioindicators

A bioindicator (biological indicator) is any species or group of species whose function can provide information about the health status of the environment (**Linton and Warner, 2003; Holt and Miller, 2010; Hamza-Chaffai, 2014; Parmar et al., 2016**). We can thus differentiate between negative bioindicators (species that are sensitive to pollutants) and positive bioindicators (species that are resistant to pollutants). We can therefore use a bioindicator to assess the quality of the environment (**Li et al., 2010**).

The criteria that are considered through the selecting of bioindicators are:

- ✓ Sedentary life, easy sampling and simple procedure of identification;
- ✓ Abundance;
- ✓ Wide temporal and spatial distribution;
- ✓ Available throughout the year;
- ✓ Sentinel, stable, sensitive and specific;
- ✓ Capacity to accumulate and concentrate the pollutants;
- ✓ Long enough life;
- ✓ High tolerance for the pollutants analysed;
- ✓ Adequate size to make possible the study of its different soft tissues;
- ✓ Have a specific dose responsiveness to a specific stressor;
- ✓ Have results that are transparent and reproducible **(Gadzała-Kopciuch et al., 2004; Zhou et al., 2008; Parmar et al., 2016)**.

2.1 *Mytilus galloprovincialis*

Marine bivalve molluscs (*Mytilus galloprovincialis*) have been used in our work as bioindicators for the following reasons:

- ✓ They are filter-feeding organism able to accumulate within its soft tissues many of the contaminants;
- ✓ They are widely used as sentinel species in biomonitoring. Indeed, these organisms are indicative of the presence of pollutants in the environment;
- ✓ They have a wide geographical distribution which allows comparisons between different sites;
- ✓ They have a long life for comparing effects and levels of contamination between different stages of development;
- ✓ They offer enough tissue for possible impact research on different biological levels;
- ✓ They have a large abundance which facilitates sample collection;
- ✓ They have a sedentary lifestyle so that the individuals can be directly correlated to the level of pollution of the site (**Casazza et al., 2002; Berthet et al., 2003 ; Serafim et al., 2008; Jovic et al., 2011**).

2.2 *Patella rustica*

The gastropods molluscs more commonly known as snails and slugs such as (*Patella rustica*) are extensively used in monitoring programs in the marine environment for the following reasons:

- ✓ They are abundantly distributed;
- ✓ They are frequently used as sentinel organisms;
- ✓ They accumulate and concentrate pollutants in their soft tissues responding essentially to the fraction present in the environment;
- ✓ They have a sedentary lifestyle;
- ✓ They are easy to collect and maintain in the laboratory;
- ✓ They are available throughout the year;
- ✓ They have an adequate size to make possible the analysis of its different soft tissues;
- ✓ They grow to an accessible ecosystem (**Bu-Olayan and Thomas, 2001; Campanella et al., 2001; Storelli and Marcotrigiano, 2005; Srivastava and Singh, 2018**).

B. The behaviour of the pollutants in the marine environment

Pollution of coastal marine environments is a recognized concern; therefore, the monitoring of the biological effects of pollutants is a real problem. Major sources of pollution are industrial, agricultural and residual waste caused by the rejection of fertilizers, wastewater, pesticides and untreated waste including plastic waste. Several pollutants are collected at the ocean's depths, where they are consumed by small marine organisms and subsequently they are introduced into the food chain. However, the dispersion of these pollutants in the environment is related to some factors such as (the nature of pollutant, the process of their dissemination and their bioaccumulation) (**Okuku et al., 2011; Wilhelmsson and Eriksson-Hagg, 2013; Matoka et al., 2014, Vikas and Dwarakish, 2015; Pawar et al., 2016**). The six properties of pollutants listed below are the most important for predicting the environmental behaviour of a pollutant and they are usually cited in many references:

- Solubility in water;
- Volatility:
- Density;
- Chemical reactivity;
- Biodegradability (**Rhind, 2009; Holt and Miller 2010; Geissen et al., 2015**).

C. Biological pollution: The viral contamination of bivalve molluscs

1. Overview

During feeding, bivalve molluscs (mussels, oysters, and clams) can accumulate pathogenic human enteric viruses when present in a polluted site. These viruses include viral agentscausing paralysis, gastroenteritis, particularly in children (less than 5 years), hepatitis, meningitis, fever, herpangina, hand-foot-and-mouth disease, myocarditis, heart anomalies, rush, pleurodynia, conjunctivitis, respiratory disease, and maybe diabetes (**Bosch et al., 2005; Bosch et al., 2009**; **Rodriguez-Lazaro et al., 2012**; **Bosch et al., 2018**).

Many viruses such as *Enterovirus*, *Rotavirus*, *Norovirus*, *Hepatitis A* and E virus transmitted to humans through the faecal-oral route. They are widely prevalent in the community and the infected individuals can carry many millions of virus particles in their faeces. Consequently, viruses of many types occur in large numbers in sewage **(Vasickova et al., 2005; Bosch et al., 2008; FAO and WHO 2008; Okoh et al., 2010; Rodriguez-Lazaro et al., 2012)**.

For reasons of precautionary consumer protection, molluscs harvesting areas are, therefore, subject to hygiene control. Several authors have required establishing the relationship between the presence of viruses in wastewater and their impact on marine organisms. Moreover, related of these releases, the human viruses were detected in the marine environment (**Van der Poel et al., 2001; Ueki et al., 2005; Le Guyader et al., 2006; Pinon and Vialette, 2018**).

The development of molecular biology was one of the greatest achievements in biological science. Recently, a technological innovation of PCR (Polymerase Chain Reaction), known as Real-Time PCR, has become increasingly important in research laboratories due to its capacity for generating quantitative results. This technique allow rapid and direct detection of viruses in the samples **(Le Guyader et al., 2007)**.

2. Characteristics of enteroviruses

2.1 Classification and structure

The enteroviruses, which are belonging to the genus *Enterovirus* and to the family Picornaviridae, are small and naked viruses with a diameter between 24 and 30 nm (**Figure 1**) Their genome consists of a single positively stranded RNA, which contain about 7500

nucleotides in length. Enteroviruses virions composed of a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTRs), surrounded by an icosahedral capsid formed of 60 capsomers, composed of four structural proteins (VP1 to VP4) (**Figure 2 and Figure 3**) and non-structural proteins (2A to 2C and 3A to 3D) (**Figure 2**). The human enteroviruses are classified into four species (EV-A to EV-D) including more than 100 serotypes. The genus *Enterovirus* combines *Poliovirus* (*Poliovirus* type 1, 2 and 3), *Coxsackievirus* A, Coxsackievirus B, Echovirus, *Enterovirus* A, *Enterovirus* B, *Enterovirus* C, and *Enterovirus* D (**Table 1**). Current *Enterovirus* classification is based on the high nucleotide sequence divergence within the VP1 capsid coding region and it can be identified by comparison of the entire or partial VP1 sequence to a database of prototype strain sequences **(Stanway et al., 2005; Racaniello et al., 2007; Bessaud et al., 2012; Racaniello et al., 2013; Fernandez-Garcia et al., 2017)**.

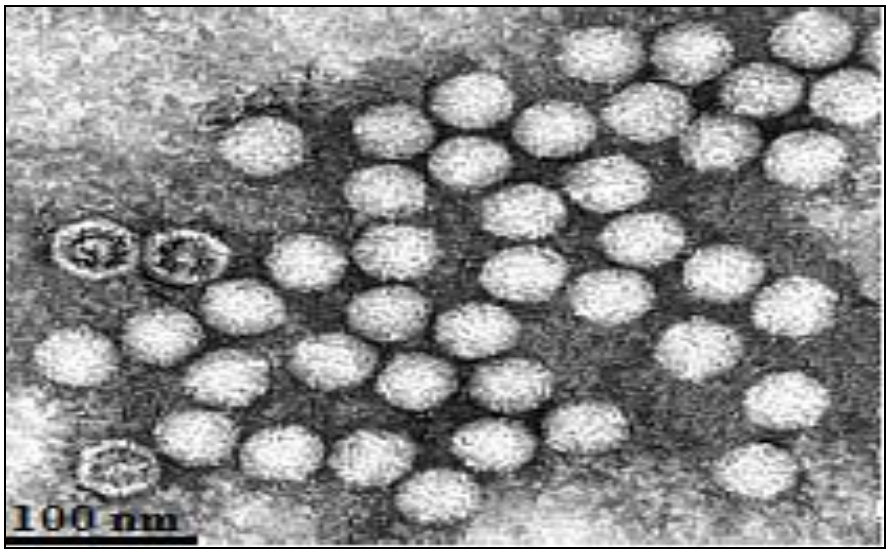


Figure 1: Electron microscopy of *Enterovirus* (CDC, 2005)

Table 1: New molecular classification of *Enterovirus* (Ibrahim et al., 2013)

Genus	Species	Number of serotypes	Serotypes
Enterovirus	*Enterovirus* A	18	*Coxsackievirus* (CV-) A2 to A8, A10, A12, A14, A16 *Enterovirus* (EV-) A71, A76, A89, A90, A91, A114, A119
	Enterovirus B	59	*Coxsackievirus* (CV-) A9, B1 to B6 *Echovirus* (E-) 1 to 7, 9, 11 to 21, 24 to 27, 29 to 33 *Enterovirus* (EV-) B69, B73 to B75, B77 to B88, B93, B97, B101, B106, B107, B110, B111
	Enterovirus C	23	*Poliovirus* (PV-) 1 to 3 *Coxsackievirus* (CV-) A1, A11, A13, A17, A19, A20 to A22, A24 *Enterovirus* (EV-) C95, C96, C99, C102, C104, C105, C109, C113, C116, C117, C118
	Enterovirus D	4	*Enterovirus* (EV-) D68, D70, D94, D111

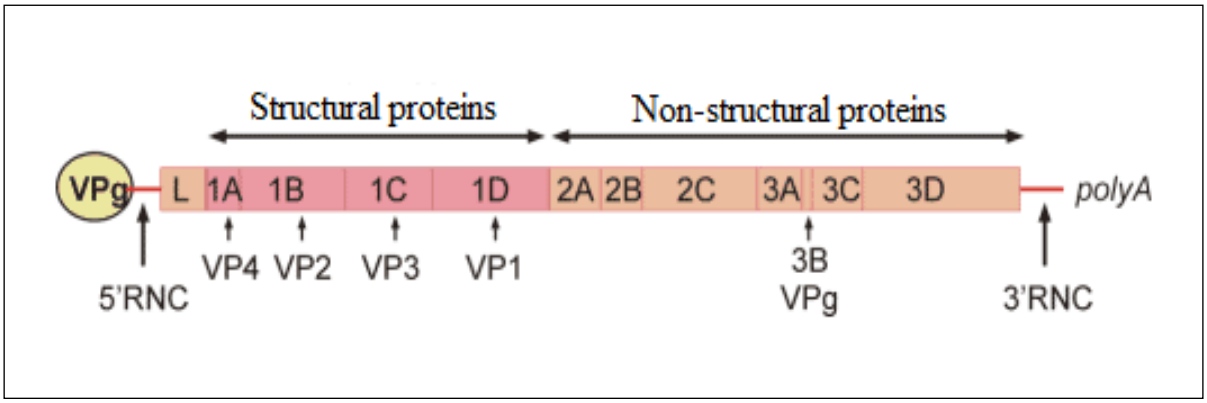


Figure 2: Structural organization of the *Enterovirus* genome (Andreoletti et al., 2009)

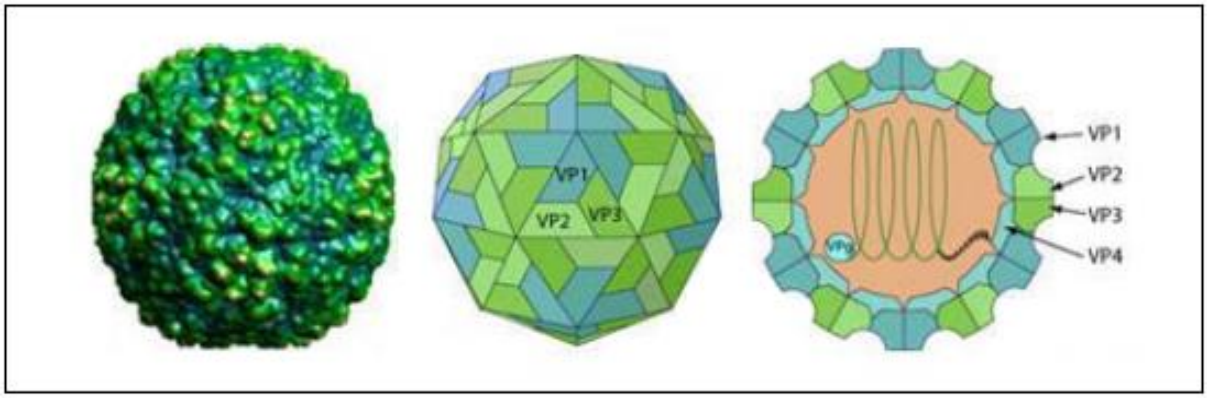


Figure 3: Structural of *Enterovirus* capsid and schematic representation of the structural proteins VP1, VP2, VP3, and VP4 (Plevka et al., 2012)

2.2 Physical and chemical resistance

Enteroviruses like most enteric viruses have evolved stability to adverse environmental conditions, such as changes in temperature, pH, salinity, relative humidity, acidity, salinity, and seasonal variation. Enteroviruses have very good resistance to radiation and ultraviolet (UV) light penetration, which allow survival of these viruses in the environment and facilitate their transmission through multiple environmental routes (such as water, food, and aerosols) (**Vasickova et al., 2010; Betancourt and Shulman, 2017**). Human wastes, such as sewage are the primary source of enteroviruses released into the aquatic environment that subsequently contaminate raw source water for potable supply, bathing water, shellfish culture water, and water used for irrigation (**Rzezutka and Cook, 2004**).

2.3 Epidemiology and pathways of transmission

Enteroviruses are disseminated globally, they circulate throughout the year in the tropics. In temperate zones, an increase in the diagnosis of enteroviruses infections is observed every year, in summer and autumn. This increase can be observed in May but most often occurs in June and July. In 2017, the summer peak was observed at week 26, followed by a second smaller peak in the fall (**Solomon et al., 2010; Lugo and Krogstad, 2016; Mirand et al., 2018**).

The different enteroviruses serotypes are responsible for a wide spectrum of symptoms, mild or severe: Acute Flaccid Paralysis (AFP), aseptic meningitis, hand-foot-mouth syndrome, angina, respiratory diseases, acute or chronic heart disease, diarrhea, pancreatitis, acute hemorrhagic conjunctivitis, encephalitis,etc (**Andreoletti et al., 2009; Tryfonos et al., 2011**).

The enteroviruses are ubiquitous viruses, they are transmitted via direct contact by ingestion of contaminated shellfishes or fruits and vegetables washed with contaminated water; They also spreaded by drinking contaminated water, by direct contact with contaminated hands (from infected person to others) and also by inhalation of contaminated air (**Figure 4**) (**Blacklow and Greenberg, 2001; Koopmans and Duizer, 2004; Vasickova et al., 2005; Mesquita et al., 2011; Gibson, 2014; Todd and Greig, 2015**).

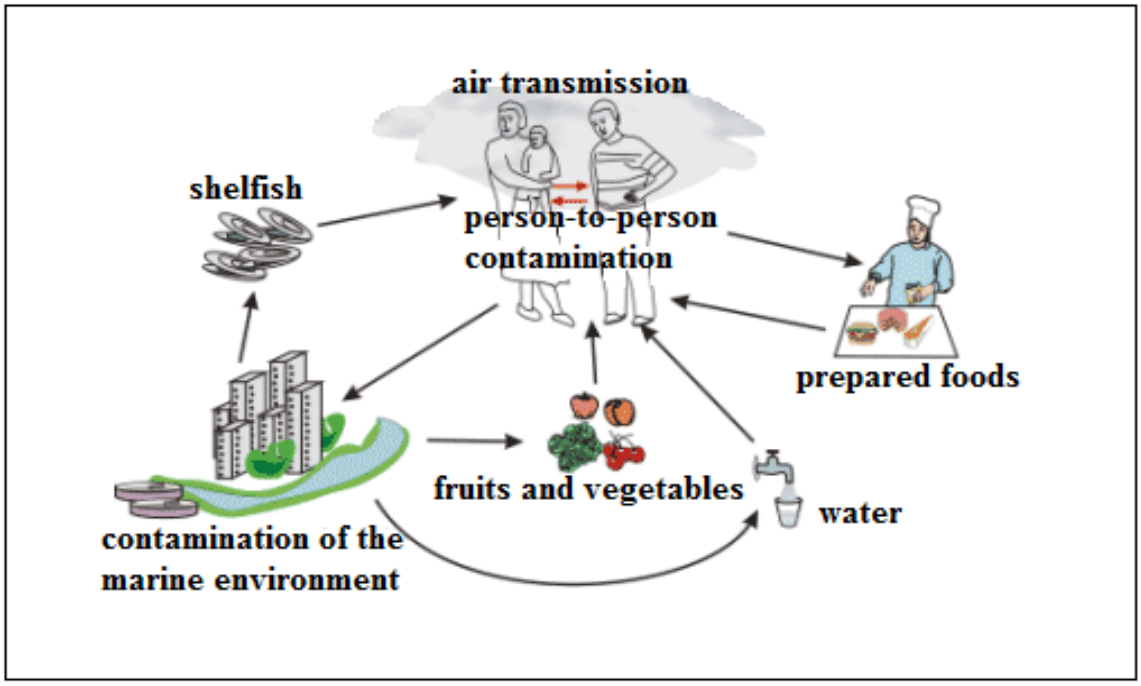


Figure 4: Pathways of enteroviruses transmission (Pothier and Agnello, 2006)

2.4 Strains circulating in the environment

Sewage represents a useful matrix to derive information on circulating enteroviruses in given populations and to describe the enteroviruses epidemiology associated with human disease, also known as environmental surveillance (**Betancourt and Shulman, 2017**).

Almost no data exist on the relationship between strains circulating in the environment and those present in the population (**Miossec et al., 1999; Dimitrov et al., 2016**).

2.5 Pathogenicity

Enteroviruses are common pathogens responsible for very frequent infections estimated at nearly one billion cases each year worldwide **(Palacios et al., 2005; Lugo and Krogstad, 2016; Khediri et al., 2018; Schwartz et al., 2018; Sioofy-Khojine et al., 2018)**.

Like all viruses, enteroviruses are intracellular parasites whose replication depends on their ability to transmit their genome from an infected cell to an uninfected cell. As a result, non-enveloped viruses are able to infect the cell through the cytoplasmic membrane and most enteroviruses use one or more endocytic pathways. The main reason is that endocytotic pathways offer direct access to the cytoplasm through the different cellular barriers encountered during infection **(Conner and Schmid 2003; Smith and Helenius, 2004; Chung et al., 2005; Leveque et al., 2007a)**.

The strain of enterovirus does not consist of a single genotype, but a set of different genomic sequences called "quasi-species", which are responsible for the genetic diversity of a strain and able to cooperate at different pathophysiological stages of the infection. Therefore, genetic diversity is directly related to the pathogenicity of an enteroviruses strain **(Vignuzzi et al., 2006)**.

The resistance of enteroviruses and their rapid spread in a mode that goes beyond simple faecal-oral contamination, especially by aerosols of nasopharyngeal droplets (**Tang et al., 2006; Phyu et al., 2017; Daniel, 2018; Gordon et al., 2018)**. It becomes a major public health problem, even in the so-called developing countries, in the form of summer epidemics **(Antona et al., 2007)**. As well, some enteroviruses serotypes, such as *Enterovirus* 7, are associated with the emergence of specific diseases like foot-hand-mouth syndrome outbreaks, and acute hemorrhagic conjunctivitis associated with an antigen variant of Coxsackievirus A 24 **(Lin et al., 2003; Leveque et al., 2007b)**. Finally, the ongoing eradication of *Poliovirus* leaves a viral ecological niche for the emergence of new enteroviruses, responsible for acute flaccid paralysis, some of which have already begun to be detected and typed. Nowadays, *Poliovirus* type 2 is eradicated but types 1 and 3 still circulate in parts of Nigeria, Pakistan, and Afghanistan. Eradication is underway but not complete **(Junttila et al., 2007; Manor et al., 2014)**. In September 2015, Morocco is declared as a «Polio Free» Zone by the World Health Organization (WHO), because no wild *Poliovirus* strain has been circulated since 1989. However, the risk of importation of the virus poses a real health problem, due to the migratory flows recorded from the still endemic countries **(Idrissi Azzouzi et al., 2015)**.

2.6 Treatment and prophylaxis

Faced with the pathogenic power of enteroviruses, the therapeutic means currently available remain very limited or non-existent in clinical practice **(Barnard, 2006)**. The development of new molecules and antiviral strategies becomes a necessity. It should lead to deepening the knowledge of the infection cycle of the target cell by enteroviruses. These steps represent potential targets for new antiviral strategies, but they may also be able to explain the tissue tropism and the epidemiological characteristics of certain strains or serotypes of enteroviruses **(Nilsson et al., 2008)**.

2.7 Virological diagnosis

Enteroviruses are very stable in the environment so the development of effective detection methods is an important step towards reducing contamination of foods and environment **(Mattison and Bidawid, 2009)**.

The isolation of enteroviruses on cell culture is the reference technique because it is a very delicate method. However, several cell lines need to be seeded and some viruses do not grow (Coxsackievirus A in particular). The diagnosis is oriented by the cytopathic effect, it is confirmed by a monoclonal antibody that recognizes most enteroviruses **(Kittigul et al., 2000; Leggitt and Jaykus, 2000)**.

The performance of molecular detection tools has recently made it possible to detect enteric viruses present at very low concentrations in shellfish, which has made it possible to confirm that this food was a vector of transmission of the disease (**Lees, 2000; Pinto et al., 2004; Le Guyader et al., 2003; Butt et al., 2004; Boxman et al., 2006; Le Guyader et al., 2006**).

The identification of the viral serotype is performed by seroneutralization using antiserum pool (Lim, Benyesh-Melnick pools). However, detection of the enteroviruses genome by PCR is using primers that hybridize conserved of the 5' end Non-Coding Region (NCR) **(Casas and Sunen, 2001)**. It does not perform better than culture but PCR is much easier to use than cell culture **(Le Guyader et al., 2007)**.

3. Bivalve molluscs

The worldwide distribution, biological and ecological importance of molluscs, including bivalves and gastropods, have always been of interest to scientists, as they are located at many levels of the food web, further increasing their role and determining the functioning of ecosystems (**Ridgway et al., 1998; Coen and Bishop, 2015; Fortunato, 2016; Escamilla-Montes et al., 2017)**.

Molluscs are important components for the marine ecosystem and compose the large phylum of invertebrate animals known as the Mollusca. Marine molluscs are filter feeders with a body organized into a muscular foot, a head, a visceral mass containing most of the organ systems, and a fleshy mantle that secretes the calcareous shell. The Mollusca has about 100 000 described species and potentially 100 000 species yet to be described (**Strong et al., 2008**). It is divided into three groups: bivalves, cephalopods, and gastropods (**Haszprunar and Wanninger, 2012; Mark and Kenneth, 2015; Antony, 2017**).

Freshwater molluscs are common animals in lakes and streams. The worldwide diversity is estimated at more than 5000 species. Bivalves number around 1200 species in total, which include 900 mussels (**Graf & Cummings, 2007; Bogan, 2008**).

3.1 Presentation of the species *«Mytilus galloprovincialis»*

Mytilus galloprovincialis are bivalve molluscs; they are among the three species mainly exploited in mussel culture and widely consumed. These are *Mytilus edulis*, *Perna canaliculus* and *Mytilus galloprovincialis* (**Villalba et al., 1997**; **FAO, 2009**).

3.2 Morphology and general anatomy

The shell of mussels has a triangular and elongated with rounded edges. They carry out a variety of functions including support for tissues, protection from predators and against desiccation. The mussel is formed of two symmetrical valves, which joined on the outside, by an elastic ligament (**Figure 5 and 6**). A second adductor muscle located at the posterior pole allows the opening of the animal during its feeding. Inside the shell, we find the mantle and internal organs of the animal (stomach, liver, kidneys, gonad, heart, and mouth) (**Figure 7**). The mussel has a long foot that it can come out through the crack of the shell and use it to move. At the base of this muscular projection is the byssogenous gland allowing the formation of the byssus. The latter consists of an adhesive disc provided with numerous filaments of protein nature very resistant that the animal can break one after the other to move (**Tachet et al., 2000; Moyes and Schulte, 2007; Silverman and Roberto, 2007; Waite, 2017**).

By doing the anatomy of mussels (**Figure 5**), we can know about:

- The external morphology;
- The internal anatomy;
- The reproductive system;
- The distribution;
- The life cycle;
- The feeding behaviour.

Figure 5: Shell appearance of *Mytilus galloprovincialis* and view inside the shells (Lamiot, 2006)

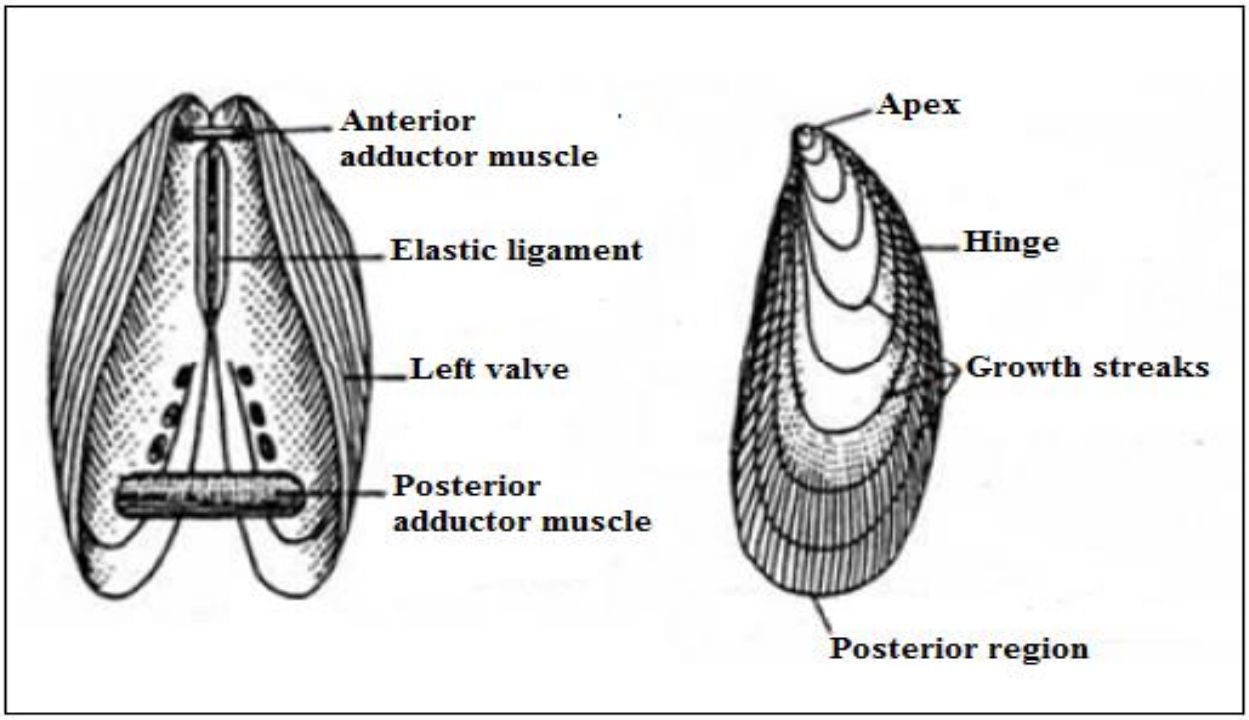


Figure 6: View from the front and the left side of *Mytilus galloprovincialis* (Paiva, 2014)

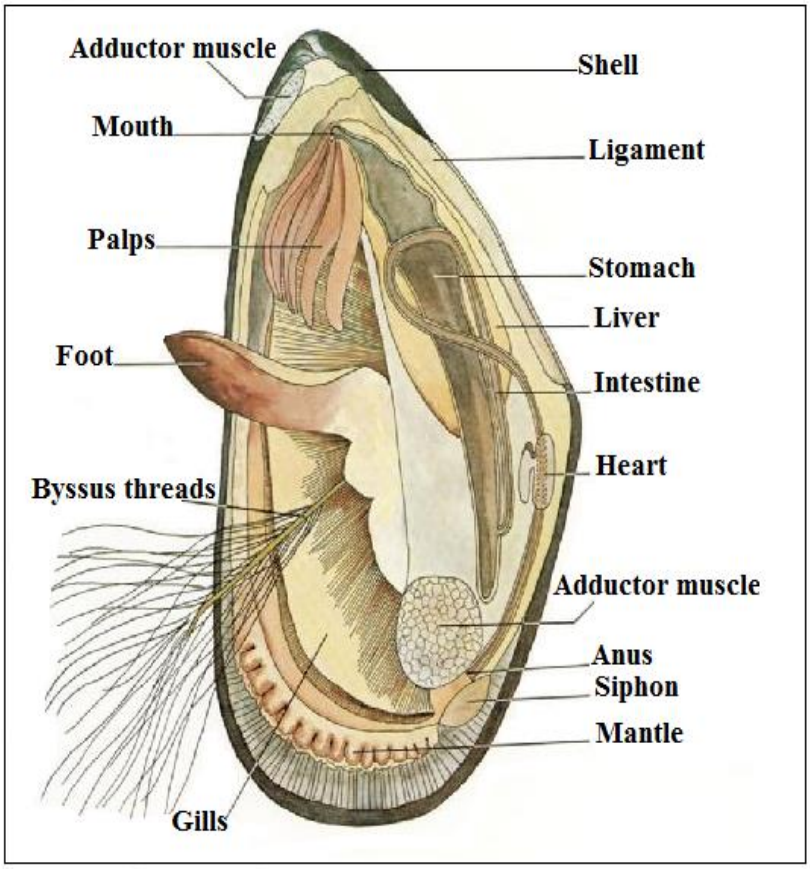


Figure 7: General anatomy of the mussels (Mallet, 2002)

Table 2: Taxonomic rank (Lamarck, 1819)

Realm	*Biota*
Reign	*Animalia*
Under-reign	*Metazoa*
Division	*Eumetazoa*
Under-division	*Bilateralia*
Branch	*Mollusca*
Class	*Bivalvia*
Under-class	*Pteriomorphia*
Order	*Mytilida*
Super-family	*Mytiloidea*
Family	*Mytilidae*
Under-family	*Mytilinae*
Kind	*Mytilus*
Species:	*Mytilus galloprovincialis* (**Lamarck, 1819**)

Two key characteristics of mussels:

- A shell includes two valves which contain the soft body (**Figure 5 and Figure 6**);
- A muscular foot often seen extended from between the two valves, this foot aids the mussels in locomotion, burrowing and positioning in the river bottom (**Figure 5 and Figure 7**).

Species within the genus *Mytilus* include (**Figure 8**):

- ✓ *Mytilus edulis* (**Linnaeus, 1758**) - Edible blue mussel
- ✓ *Mytilus coruscus* (**Gould, 1861**) - Korean mussel
- ✓ *Mytilus chilensis* (**Hupe, 1854**) - Chilean mussel
- ✓ *Mytilus trossulus* (**Gould, 1850**) - Foolish mussel
- ✓ *Mytilus galloprovincialis* (**Lamarck, 1819**) - Mediterranean mussel
- ✓ *Mytilus californianus* (**Conrad, 1837) -** California mussel

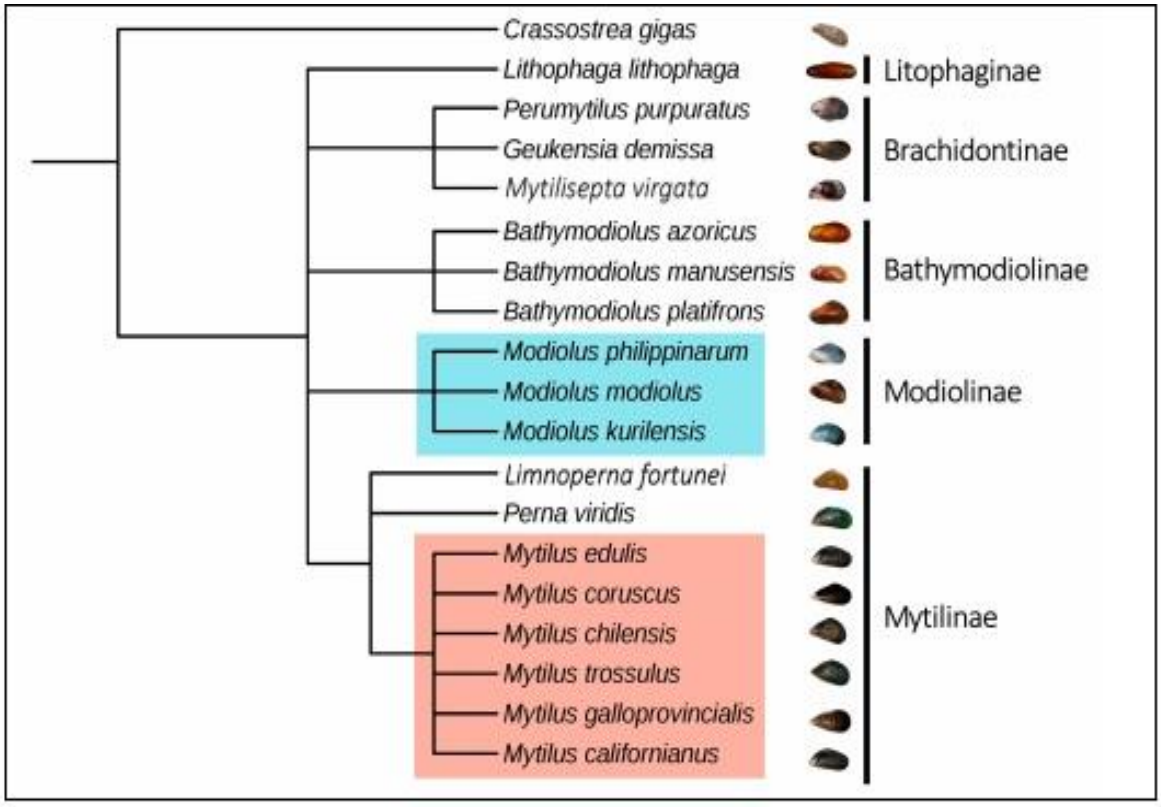


Figure 8: Taxonomic distribution of Mytilinae (Leoni et al., 2017)

3.3 Feeding and Habitat

a. Feeding

Mussels (*Mytilus galloprovincialis*) are filter feeders that feed on phytoplankton or small organic particles by pumping water through enlarged sieve-like gills. This food is directed to the mouth and reaches the stomach where it is crushed by a crystalline stem that releases the digestive juices (**Riisgard et al., 2011**).

Food particles are accumulated on the gill lamellae and are then transported by cilia towards the mouth whilst continually being sorted according to size. The finest elements enter the tubules of the digestive gland or hepatopancreas that surrounds the stomach. However, the rejected elements are eliminated with mucus **(Figure 9)**.

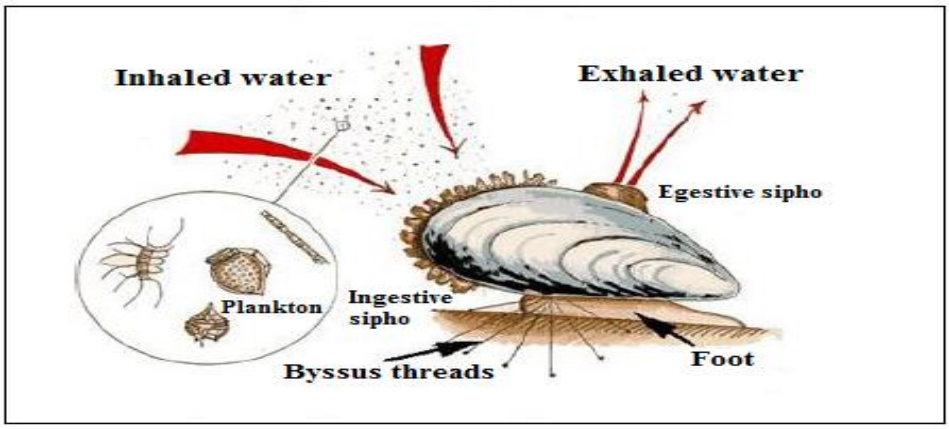


Figure 9: Respiration and nutrition in mussel (Murtaugh, 2017)

b. Habitat

Molluscs are widespread, inhabiting marine, freshwater and terrestrial ecosystems. Globally *Mytilus galloprovincialis* is found on temperate sheltered and exposed rocky shores but is generally absent from heavily silted or sandy areas (**Figure 10**) **(Branch and Steffani, 2004)**. It attaches firmly to rocks by means of strong byssal threads, which are secreted by a mobile foot **(Hammond and Griffiths, 2004)**.

Figure 10: *Mytilus galloprovincialis* collection site

3.4 Geographic distribution

Mytilidae family (Mollusca, Bivalvia) has a wide distribution range from temperate to subarctic coasts of Northern and Southern hemisphere. *Mytilus galloprovincialis* is an important member of this family and known as the Mediterranean mussel (**Branch and Steffani, 2004; Barut et al., 2016**). It is native to the Mediterranean and Atlantic coast of Southern Europe (**Anderson et al., 2002**). **Figure 11** shows the current distribution of *Mytilus* species across the globe **(Astorga et al. 2015**).

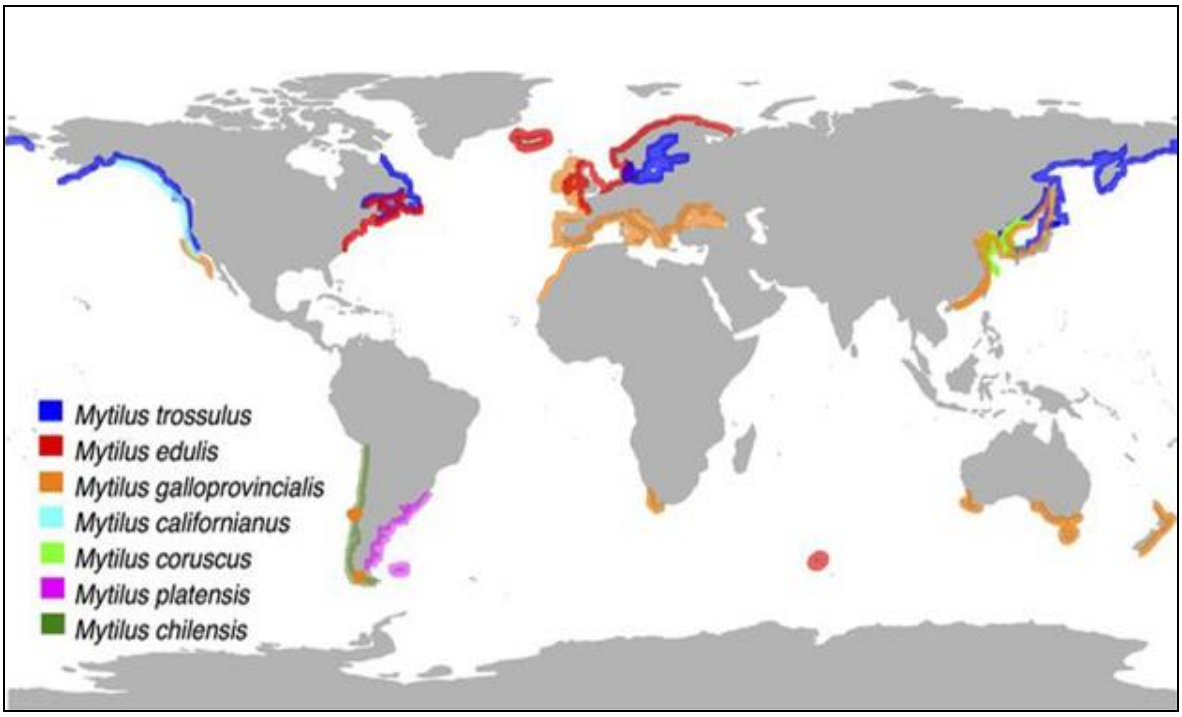


Figure 11: Map of geographic distribution of *Mytilus* species (Astorga et al., 2015)

3.5 Sources and modes of contamination

Enteric viruses are excreted by sick people, but also by healthy carriers, living in the littoral zone of the watershed. These viruses including *Hepatitis* A virus, *Norovirus* and *Enterovirus* are very important in terms of the risk associated with the consumption of shellfish (**Butt et al., 2004; Fong and Lipp, 2005; Le Guyader et al., 2012; Campos and Lees, 2014; Hellmer et al., 2014; Lekshmi et al., 2018**).

According to the concentrations present in the marine environment, marine organisms particularly mussels concentrate contaminants especially viruses. At the international level, contaminant concentrations in living organisms are measured to monitor the marine

environment. Indeed, these bivalves have characteristics that make them good bioindicators widely used in various programs to monitor marine pollution (**Boumhras, 2008**).

In the majority of infection cases, the presence of multiple viral strains has been demonstrated in the shellfish involved, suggesting contamination of shellfish in their marine environment by wastewater (**Kageyama et al., 2004; Gallimore et al., 2005; Boxman, et al., 2006; Le Guyader et al., 2006; Le Guyader al., 2008; Le Guyader et al., 2010**).

3.6 International standards and recommendations

When it comes to food safety, the potential for contamination with pathogenic viruses has been recognized and translated into control programs aimed at reducing the burden of food-borne diseases in many parts of the world. Legislation exists to support countries in these control activities and to advise industries by developing guidelines targeting specific pathogens, commodities or processes (**Koopmans, 2012**).

Environmental virus hazards are increasingly recognized as a cause of illness in all age groups. Calicivirus, Norvirus, Adinovirus, Enterorivus, Renovirus, Hepatitis A virus and Hepatitis B virus are the most common causes of illness because of environmental exposure (**Lazaro et al., 2011**).

D. Chemical pollution: The metal contamination of gastropod molluscs

1. Overview

The monitoring of metal contamination for the marine organisms is preferably based on sedentary species so that the levels of chemical pollution observed are representative of a specific geographical area. Therefore, the bivalves (mussels and oysters, etc) and the gastropods (limpets, etc) have generally been chosen as bioindicators. Molluscs have the ability to bioaccumulate metal contaminants during their life cycle, with a more stable phase between two and three years. The metal contamination in the environment can lead to oxidative stress in organisms (**Hedouin et al., 2011; Ubrihien, 2012**).

2. Characteristics of the metallic trace elements studied

The appellation of metallic trace elements (MTE) refers to metals and metalloids whose content is less than 1g/kg of dry matter in the earth's crust or less than 0.1 g/kg of dry matter in organisms. Scientists preferentially use this appellation after the replacement of the term

"heavy metals". Moreover, this last expression was used wrongly and leads to some confusion in its use. It was one of the conclusions for the Congress on the biogeochemistry of metallic trace elements that took place on 1995 in Paris (**Prost, 1997; Lemiere et al., 2001; Miquel, 2001**). According to the Geneva convention on long-range transboundary air pollution (1979, UNO/EEC), MTE is any metal or metalloid whose density is above 4.5 and which carries risks of toxicity to living organisms (**Merian, 1991**; **Miquel, 2001**). Thus, some elements are trace elements which, at low doses are essential for the development of organisms, but which have toxic characteristics at too high concentrations such as Cu, Cr, Zn, Mn. On the other hand, other elements such as Cd, Hg, and Pb have an unknown metabolic role and present a risk of toxicity even at very low doses. In addition, arsenic and selenium, which are not metals but metalloids, are still included in the classification of heavy metals (**Bourrelier and Berthelin, 1998; Anne and Isabelle, 2005; Marcovecchio et al., 2013**).

The metallic trace elements can be introduced into aquatic ecosystems through natural and anthropogenic processes (**Table 3**) (**Delage, 1999; Anne and Isabelle, 2005**). Once released into the aquatic environment, they are not biodegradable, unlike organic pollutants. They can thus accumulate in the trophic chain and be at concentrations up to 1 million times higher in the top of the food chain. The following elements (arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn)) were proposed in the report of the Academy of Sciences (1998) as a list of trace elements at risk for human health (**Gouzy and Ducos, 2008**).

The main sources of metallic trace elements present in the environment are shown in **Table 4**.

Table 3: Pollution by metallic trace elements (Gentric et al., 2016)

Metallic trace elements	Emission in t/year		Anthropogenic pollution %
	Natural pollution	Anthropogenic pollution	
Cadmium (Cd)	1000	3000	75
Chromium (Cr)	1000	13400	68
Lead (Pb)	19000	116000	86
Copper (Cu)	19000	2150	10
Mercury (Hg)	6000	1100	15
Nickel (Ni)	26000	47000	64
Zinc (Zn)	46000	234000	84

Table 4: Industrial and agricultural sources of metallic trace elements present in the environment (Tchounwou et al., 2012; Al Naggar et al., 2018)

Metallic trace elements	Sources
Mn, Zn, **Cu**, **Pb**, **Cd**, **Cr**, Hg, Ni, As	Batteries, accumulators and electrical devices
Fe**, Pb**, **Cd**, **Cr**, **Cu**, Ni, Hg, Mn, Zn, Sn, Al, As	Pigments and paints
Pb, **Cd**, **Cu**, Ni, As, Mn, Zn, Sn	Alloys and solders
Pb, **Cu**, As, Hg, Sn, Mn, Zn	Biocides (pesticides, fungicides, herbicides, conservatives)
Ni, **Pb**, **Cu**, Hg, Sn	Catalysts
Pb, As, Sn, Mn, Zn	Glass
Cr, **Cu**, **Pb**, **Cd**, Ni, Hg, Al, As, Mn, Zn	Fertilizers
Cd, **Cr**, **Pb**, Sn, Mn, Zn	Plastic
Sn, Hg	Dental products and cosmetics
Cr, Fe, Al	Textile
Fe, Mn, Zn, **Pb**, **Cd**, **Cu**, **Cr**, Ni, V, As	Refineries
Mn, Zn, **Pb**, **Cd, Cu**, Ni, Fe, Hg	Fuel

2.1 Copper (Cu)

Copper is moderately abundant in the earth's crust with an average between 45 µg/g and 70 µg/g (**Figure 12**). It is frequently present in the form of sulphides or oxide (**Alloway, 1995; Grzegorczyk et al., 2014**). Due to its ductility, corrosion resistance and bactericidal and antifungal properties, copper is used in many industrial applications. Metallurgical industries, the electrical industry, anti-fouling paints, fossil fuel combustion and petroleum derivatives such as fuels, household waste incineration are the main anthropogenic sources of copper in the environment (**Auguscik, et al., 2013**; **Peres et al., 2013**).

The baseline concentrations of copper in inland waters are 3 to 5 µg/L on average. It is mainly in the divalent Cu (II) form, whereas the Cu (I) monovalent form is present only at extremely low concentrations since it reacts to form metallic copper and Cu (II) ions. Cu (I) can be produced under reducing conditions, the majority of the compounds then formed are insoluble. Copper in complexed form is associated with inorganic and organic ligands (phenolic acids, peptides, polysaccharides, proteins, humic and fulvic acids). The organically complexed copper is very stable. At least 95% is complexed by natural organic matter in natural waters. The extent of complexation of Cu by organic ligands strongly conditions its bioavailability versus toxicity (**Bruland and Lohan, 2004**).

Copper at low doses is essential for all organisms but becomes toxic at high concentrations. Its toxicity is mainly based on its interactions with the constituents of the cell wall, membrane and cytoplasm (proteins, enzymes, nucleic acids, metabolites, etc). The free form of copper Cu^{2+}, as well as the copper hydroxy-complexes, are the forms most rapidly assimilated by the organisms and therefore the most toxic whereas the forms complexed with the carbonates, with the chlorides or with the organic matter appear much less bioavailable (**He et al., 2001, Huang and Wang 2003, Brooks et al., 2008**).

Figure 12: Copper (https://fr.wikipedia.org/wiki/Cuivre)

2.2 Chromium (Cr)

The appellation "Chromium" comes from the Greek word "chroma" which means "color" and refers to the many colored compounds that make up chromium (**Sueker, 2005)**. Chromium (**Figure 13**) is the 21st most abundant element in the earth's crust with an average concentration of 100 µg/g and an atomic number 24. It is a hard, white and greyish metal with 3 degrees of oxidation (II, III, and VI) which condition its toxicity; Cr (III) is more stable and less toxic than Cr (VI) (**Apte et al., 2005)**. It is present in several ores, the most important being Ferrous chromite ($FeO.Cr_2O_3$). Cr can exist in several oxidation states ranging from -2 to +6, but only trivalent (Cr^{3+}) or hexavalent (Cr^{6+}) compounds are stable to be in the environment at significant amounts. In the rocks, Cr is predominantly trivalent (chromium oxide: Cr_2O_3), unlike hexavalent chromium, which is rare in the natural state and comes mainly from industrial activities.

The main sources of emissions are the metallurgical industry, the refractory stone industry and the chemical industry (chrome plating, tanning, and pigments). Tanneries and dyeing facilities can generate effluents with a high concentration of chromium (**Barnhart, 1997; Belay, 2010; Chowdhury et al., 2015**).

In aquatic environments, trivalent chromium in solution can exist in different forms: Cr^{3+} and $Cr(OH)^{2+}$ in acid medium and in the form of $Cr(OH)^{2+}$, $Cr(OH)_3$ (aq) and $Cr(OH)^-_4$ in a neutral and basic environment. It is poorly soluble and it forms stable complexes with organic or inorganic substrates or hydroxide precipitates. The hexavalent form is, on the contrary, very soluble in water and is found essentially in the form of oxyanions (CrO_4^{2-} and $Cr_2O_7^{2-}$). The presence of chromium (III) or chromium (VI) is controlled by the pH and the oxidation-reduction potential as well as the availability of ligands in the natural environment. Cr (III) would only be present in reduced or highly acidic medium, whereas the conditions of thermodynamic equilibrium impose the almost complete oxidation of Cr (III) in Cr (VI) in the basic pH range (**Johnson, 1990, Ball and Izbicki, 2004**). In the natural environment, only manganese oxides and oxygen are able to oxidize Cr (III) to Cr (VI), whereas reduction is possible in the presence of many reducing agents, such as ferrous iron, sulphides and organic matter (**Naghmush et al., 1994, Sedlak and Chan, 1997; Stanin and Pirnie, 2004**; **Apte et al., 2006; Gorny et al., 2016**).

The toxicity of Cr depends on its total concentration as well as its oxidation state. Chromium (III) is an essential trace mineral involved in the metabolism of glucose,

cholesterol and mammalian fats in contrast to hexavalent compounds which are more toxic because of their high potential for oxidation and the ease with which they cross cell membranes. Chromium toxicity is by ingestion (dizziness, abdominal pain, hemorrhagic diarrhea), skin contact (allergies) and inhalation. Dust of chromic acid or chromate attack the mucous membranes of the nose, mouth and respiratory system and may promote the development of cancer (**Gomez and Callao, 2006**).

Figure 13: Chromium (https://en.wikipedia.org/wiki/Chromium)

2.3 Cadmium (Cd)

Cadmium is a relatively rare element (**Figure 14**) and does not naturally exist in the native state. Cd is extracted from the Zn and Pb ores in which it is in the form of sulphides. Its average concentration in the earth's crust is from 0.15 to 0.20 µg/g (**Senesi et al., 1999; Adriano, 2001**). It is one of the most toxic metal contaminants, mostly from natural and anthropogenic sources. It is used in the manufacture of batteries, in the protection of steel against corrosion, or as a stabilizer for plastics and pigments (**Karlsson et al., 2005**). Cadmium can be dispersed in the air by entrainment of particles from the soil and by volcanic eruptions. However, industrial activities such as refining of non-ferrous metal, combustion of coal and petroleum products, incineration of household waste and steel metallurgy are the main sources of atmospheric rejection. In the water, cadmium comes from natural erosion, soil leaching as well as industrial discharges and the treatment of industrial effluents and mines. The major food groups that contribute to the most Cd exposure are rice and grains, shellfish and seafood, meat including edible offal, and vegetables. A number of studies reported the high Cd contaminated levels in foods from polluted areas (**WHO, 2008; Hajeb et al., 2014; Chunhabundit, 2016**). Cadmium is not essential for the development of

organisms. It competes with calcium for the gill binding sites of fish and molluscs and can thus cause various physiological and enzymatic disturbances in fish, as well as inhibitions of growth in phytoplankton (**Baldisserotto et al., 2005**). It has been shown that Cd could be used in substitution for Zn in case of a deficit in this element in phytoplankton (**Price and Morel, 1990**). The human exposure to Cd compounds may create a serious health problem such as renal dysfunction at urinary cadmium concentration equal to or greater than 0.5 µg/g creatinine (**Chiffoleau, 2001; Rahimzadeh et al., 2017**). Let us recall here the case of the cadmium contamination of the Jintsu river in Japan, by the zinc mines, which was at the origin of the itai-itai disease, whose symptoms are fragility of bone, joint pain, decreased of red blood cell, and kidney disease (**Baque, 2006**).

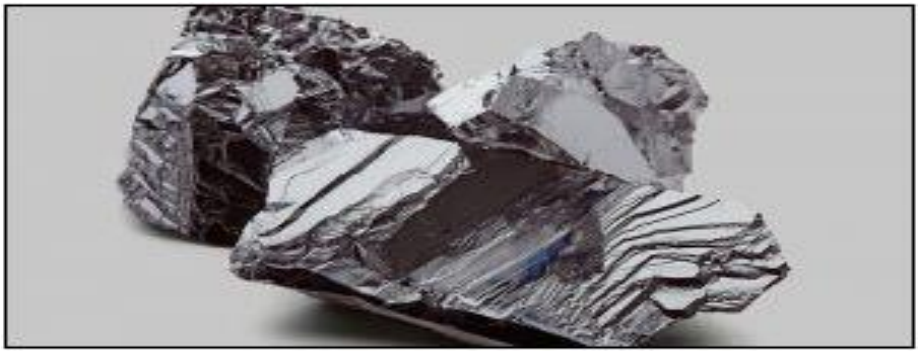


Figure 14: Cadmium (https://www.5nplus.com/fr/tellurure-de-cadmium.html)

2.4 Lead (Pb)

Lead is naturally present in the Earth's crust in the form of ores galena (PbS) (**Figure 15**), cerussite (carbonate «$PbCO_3$») and anglesite (sulphate «$PbSO_4$»). Galena being the first source contains 86.6%. In ore, lead is often associated with other minerals (zinc «Zn», silver «Ag» and copper «Cu») (**WHO, 1995; El Abidi et al., 2000**).

Although lead emissions from fuel have decreased since 2000 through the mandatory use of unleaded fuel (limit value ≤ 0.013 g/L) because lead is considered a pollutant of environment (**ATSDR, 1999)**. Lead has been or is currently used in many industrial activities such as the production of automotive batteries, coating cables, alloys, pigments, steel wire or telephone lines, insulation against noise and vibrations, roofing elements (terraces, balconies). Lead is also used in glassware, especially for crystal production. Some organic lead salts are used in lubricants (**Grousset et al., 1999)**.

In aquatic systems, lead has a high affinity for particles sediments including clays, oxyhydroxides of iron «Fe» and manganese «Mn», sulphides and organic matter (**Shafer et al., 1997; Gurunadha et al., 2008**). It can also associate with carbonates when the medium is poor in organic matter and Fe or Mn oxyhydroxides (**Li et al., 2001**).

Its concentration in uncontaminated waters is very low, not exceeding a few micrograms, and the World Health Organization has set an indicative level of 10 µg/L in surface water for the production of drinking water (**WHO, 2011**).

The toxicity of lead has been known since Roman times. It leads to Pb poisoning, a disease that manifests as anemia, digestive disorders and nervous system involvement. Neurological disorders caused memory loss and disturbances of cognitive and behavioral functions following the changes produced in the brain by lead poisoning. It can also cause delays in development in children, complications in pregnant women, and may cause infertility in the case of prolonged exposure (**Cotran et al., 1990; Wani et al., 2015**).

Figure 15: Lead (http://www.cenelle.fr/mineraux/plomb.html)

3. Toxicity and persistence of metallic trace elements

From a toxicological point of view, not all elements have the same impacts on living organisms. There are elements qualified as essential and others qualified as non-essential. Cd, Pb, Ca, Cl, K, Na, Mg, P, and S are major elements essential to the biological functions of animals and plants. Along with these macro-elements, we find the trace elements (As, Co, Cr, Cu, Fe, I, Mn, Ni, Sc, Se, Si, V, and Zn), which are essential for organisms with very low concentrations (**Tchounwou et al., 2012**).

After the release of metallic trace elements into the environment following their direct use (consumption of nickel and cadmium in Ni-Cd accumulators) or as by-products (release of mercury during the combustion of coal), these elements can be found (in the air, in the waters, in the soils, in the plant and animal organisms, and in the sediments) (**Gouzy and Ducos, 2008**).

Plants, animals, and even humans through the consumption of contaminated food can absorb metallic trace elements (MTE). The drinking water and even air breathed can contain MTE (**Soule et al., 2010**). Some metals such as iron, chromium and copper are needed in small amounts by humans and other animals but in larger amounts, they can cause various health problems **(Markert and Friese, 2000)** (**Table 5**).

Table 5: Toxicity of metallic trace elements to consumers (Boudene, 2000; Miquel, 2001; Andre, 2003)

Metallic trace elements	Acute toxicity	Chronic Toxicity	Mutagenesis
Cadmium (Cd)	Pneumonia, hepato-digestive disorders (vomiting, diarrhea)	Respiratory disorders (bronchitis, emphysema...), renal disorders (albuminuria), anemia, nervous disorders	
Chromium (Cr)	Conjunctivitis, severe lesions of the cornea, ulceration of the nasal mucosa, haemolysis, cystolic hepatitis, dysentery	Eczermatiform dermatitis, chronic rhinitis, laryngitis and pharyngitis, oesophagitis, gastroenteritis ...	Pulmonary cancer
Copper (Cu)	Digestive disorders (vomiting)	Schizophrenic syndrome, liver disorders	
Lead (Pb)	Anemia, nephropathy (with consequent elevation of azotemia, proteinuria ...)	Abdominal pain, colic, nervous disorders (convulsion, hematuric nephritis, lead poisoning)	

4. Bioaccumulation of metallic trace elements

Bioaccumulation is the ability of organisms to absorb and concentrate certain chemicals in all or part of their organisms (living or inert such as the shell of the molluscs). These contaminants pollutants (metallic trace elements or other undesirable toxic substance) possibly rare in the marine environment. This phenomenon occurs when an organism absorbs a contaminant faster than it eliminates it. Bioaccumulation is the process of assimilation and concentration of metals in the body (**Widenfalk, 2002; Abdallah, 2013; Swaleh et al., 2016**).

Assimilation: There are two main routes of exposure to pollutants: the external route, by contact (by air or water, etc) which causes an adsorption phenomenon (the toxic substance remains on the surface), and the internal way by assimilation or absorption. Any absorption of a pollutant is not necessarily dangerous. It depends, on the concentrations of pollutant on the bioavailable soluble fraction. The latter is assimilable and concentrates in certain organs, this is called organotropism (**Ritter et al., 2012**). The assimilation differs according to the metals, the cadmium concentrates almost exclusively in the digestive tract, the liver and the kidneys. Lead diffuses into the skin, muscles, and spine. Mercury, in its organic form, diffuses easily into the nervous system (**Adal and Wiener, 2018**).

Bioaccumulation by the individual: bioconcentration concerns metallic trace elements, more particularly mercury. This metal when it is present in organic form (methylmercury) becomes more toxic for humans. This process of bioaccumulation is expressed as a ratio between the concentration of a contaminant in the tissues of an organism and its concentration in the environment. This ratio is known as a bioaccumulation factor (BAF) (**Widenfalk, 2002; Baeyens et al., 2003; Mann et al., 2011; Ward et al., 2011**).

Contaminants released into the environment by humans, such as pesticides or metallic trace elements, can accumulate in ecosystems and harm the health of living organisms in this environment. These potentially toxic substances are absorbed by organisms and accumulate in muscle tissue. Thus, the simple fact of living in a polluted environment, such as in a stream with a high content of metals, can be fatal for many individuals. There are two types of bioaccumulation: the bioconcentration and the bioamplification or the biomagnification (**Figure 16**) (**Daley, 2013; Hoop, 2013; Daley et al., 2014**).

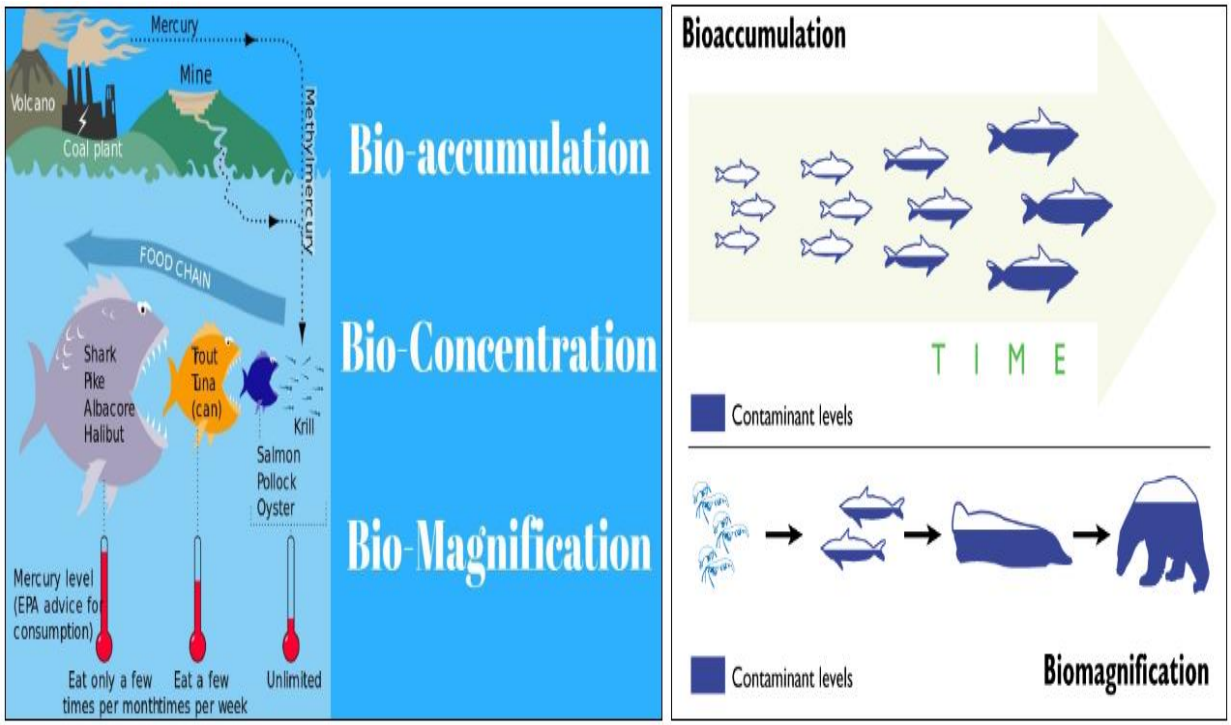


Figure 16: Bioaccumulation, bioconcentration, and biomagnification (Mackay et al., 2018)

5. Bioconcentration of metallic trace elements

Bioconcentration is the tendency of a given substance to accumulate in a living organism at a higher level than the environment by direct capture from that medium such as mercury concentration in fish. This process of bioconcentration is expressed as a ratio between the concentration of the test compound in the environment and the concentration in the body. This ratio is known as a bioconcentration factor (BCF). Living organisms concentrate metals much more than water and air. Transfer analysis highlights a hierarchy among species, ranked according to their proportion to metals. The BCF in fish (4000 to 50 000) is several thousand, or tens of thousands as that of molluscs (5000 to 10 000) and invertebrates (1000 to 5000) (**Widenfalk, 2002; Arnot and Gobas, 2006; Petoumenou et al., 2015**).

Bioconcentration is a form of direct bioaccumulation: there is no *intermedia*te between the contaminant and the living being, since the latter directly absorbs the contaminant that is present in its environment. Filtering aquatic organisms, such as mussels and oysters, filter the water for food. Thus, they absorb a very large amount of contaminants, which accumulate in their body. Eventually, the concentration of contaminants in their system exceeds that of the aquatic environment in which they evolve (**Widenfalk, 2002; Abdallah, 2013; Swaleh et al., 2016**).

6. Bioamplification of metallic trace elements

Bioamplification or biomagnification is a transfer of metals between individuals follow a traditional process called trophic transfer. The pollutant, present in the algae and the microorganisms is ingested by an herbivore, itself prey for a carnivore, and itself prey of a super carnivore, animal or human being. At the end of the chain, we are therefore with a final consumer having bioaccumulated soluble forms of metals. Concentrations increase as one progresses in the food chain. This is the case of lead and especially of mercury in the methylated form. Mercury accumulates at each stage and is concentrated at the end of the food chain, especially in large piscivorous fish (**Bourrinet et al., 2008**).

Biomagnification is a form of indirect bioaccumulation: the absorption of contaminants is through the presence of intermediates. When infested organisms at lower trophic levels are eaten, they will pass the contaminants to their predator. This results in an increase in the concentration of contaminants as we go up in the trophic levels (**Widenfalk, 2002; Garrity, 2009**). Thus, in a contaminated environment, all trophic levels are affected. Producers (first level), drawing nutrients necessary for the transformation of inorganic matter into organic matter, will accumulate the contaminants present in their environment (**Mann et al., 2011**). Primary consumers (second level), in addition to absorbing contaminants living in a polluted environment, will also accumulate pollutants that the producers themselves have absorbed. The same is true for secondary and tertiary consumers (higher levels), all accumulating contaminants previously absorbed by their prey. This phenomenon often means that individuals at the top of the food chain, such as large fish, birds of prey and carnivorous mammals, have a concentration of contaminants that exceeds the threshold of toxicity (**Gray, 2002; Bienfang et al., 2012; Cardwell et al., 2013**).

7. Methods of analysis for the metallic trace elements

7.1 Atomic Absorption Spectrophotometry (AAS)

The levels of some metal including copper (Cu), chromium (Cr), cadmium (Cd) and lead (Pb) were measured in the soft tissues of mollusca gastropoda limpet (*Patella rustica*) by using the Atomic Absorption Spectrophotometry technique with Graphite Furnace (AAS-GF), type (Varian 240 Zeeman) after mineralization method (**Figure 17**). The instrument was calibrated with metal standard solutions (1g/L) prepared by dilution.

The accuracy and precision of this methodology were tested by using a separate comparative study of a standard reference material (**IAEA-MEL, 2016-01-TE**).

Figure 17: Atomic Absorption Spectrophotometry instrument with graphite furnace (AAS-GF)

a. Principle of Atomic Absorption Spectrophotometry

The atoms resulting from thermal dissociation absorb the spectrum emitted by the light source passes through the place of atomization, part of the incident light. The quality of the analysis depends mainly on the atomic concentration with efficient sample atomization. The absorbing medium must contain the highest possible density of atoms in the ground state, while maintaining a proportionality between this concentration and that of the element in the sample under study.

The reading of the sample contents is done directly without treatment. In what follows, we will detail the equipment used.

b. Equipment

The experimental device used in Atomic Absorption Spectrophotometry consists of a source, hollow cathode lamp, a burner, a nebulizer, a monochromator, a detector connected to an amplifier.

- **The hollow cathode lamp**: contains a sealed glass envelope and is provided with a glass or quartz window containing a cylindrical hollow cathode and an anode. The cathode consists of the element that is to be dosed. A high vacuum is created inside the

bulb, which is then filled with a rare gas (argon or neon) under a few mm Hg. When a potential difference of a few hundred volts is applied between the two electrodes, a discharge is established. The rare gas is then ionized and these ions then bombard the cathode, tearing atoms out of it. These atoms are free and are excited by shocks: There is atomic emission of the element constituting the hollow cathode. The particularity of the radiation thus emitted is that it consists of very intense and very fine lines.

- **The oven or burner**: The sample to be analysed is in solution. The latter is sucked by means of a capillary by the nebulizer. Once the sample is injected into the graphite furnace, it undergoes the following physical transformations:
  - ✓ **Evaporation**: An increase in temperature up to 110°C for the removal of water. The flow rate of the carrier gas is 31 cm^3/min.
  - ✓ **Calcination**: A dramatic increase in temperature up to 500°C for the destruction of the rest of the organic matter.
  - ✓ **Atomization**: An increase in temperature up to 2600°C. The elements are in the atomic state and the flow rate of the gas is 0.1 cm^3/min.
- **Monochromator with Detector**: The monochromator will select a particular wavelength of the spectrum of the hollow cathode. For this, we will adjust the position of the network and the slot.

7.2 Another methods

The levels of the metallic trace elements such as lead, copper, chromium, selenium, cadmium, manganese, nickel, and arsenic are determined directly, by using Atomic Absorption Spectrophotometry with graphite furnace (AAS-GF) using the VARIAN model, 240, Zeeman. For iron and zinc contents, Atomic Absorption Spectrophotometry with flame (AAS-F), VARIAN model, 240 was used. Atomic absorption without flame analyses mercury after it has been reduced in solution to generate elemental mercury, otherwise known as cold vapor; the apparatus used is an AAS (VARIAN type) with a hydride generator (**Rodier, 2005**).

8. Gastropod molluscs

The gastropods are the most diverse group of the phylum because they have more than 100 000 existing species and represent approximately 80% of all the molluscs.

8.1 Presentation of the species *« Patella rustica »*

Belonging to the branch of molluscs and the class of gastropods, the Patellidae commonly called "limpets" is the marine organisms whose shell is conical (shaped like hat Chinese) and living on the rocks of the littoral where they constitute dense populations (**Table 6; Figure 18 and Figure 23**). They are edible and have certain bioecological characteristics such as a sedentary lifestyle which place them among the bioindicators species for the study of marine pollution. Among these molluscs, the genus *Patella* which is defined by **Ridgway et al., 1998** as prosobranch gastropods generally very abundant and easily spotted on intertidal rocky shores.

8.2 Morphology and general anatomy

This species (*Patella rustica*) has a thick shell, shaped like a tall, pointed cone. Shell is streaked, often marked with dark interior dark spots with double light rays (**Figure 18 and Figure 21**) (**Gerard, 2005; Harmelin and Bassemayousse, 2008; Hakabe, 2010**).

The limpets are sedentary benthic animals that live on beaten and lit rocks of the mid-littoral stage (**Neal and Skewes, 2004**). They attach themselves very securely in the manner of a very powerful sucker to withstand both desiccation and shock wave (**Boudouresque, 2005**). In soft substrates such as calcareous rock, they dig through acidic secretions cavities called cupules in which they fit to better withstand the onslaught of waves (**Gerard, 2005**).

Although they are sedentary, the limpets move at high tide to feed, and return to their lodging at low tide while marrying perfectly the shape of the rock to reduce the loss of water by evaporation (**Nakhle, 2003**).

The shape of the shell is conical, its base is not exactly circular and its top is quite clearly eccentric and is deported to the side of the head of the animal. This shell is decorated with streaks, more or less marked, radiating from its top. Concentric reliefs, corresponding to stops or disturbances of growth. The edge of the shell is tightly applied to the support, marrying any irregularities, whereas its inside is smooth (**Figure 18 and Figure 21**).

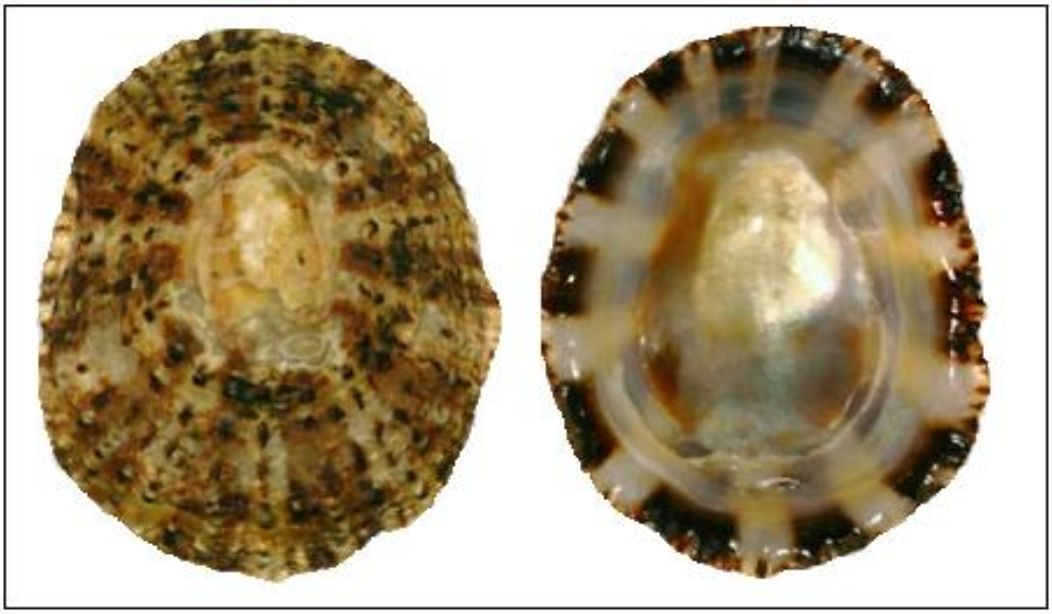


Figure 18: Lateral (left) and ventral (right) views of *Patella rustica* shell

The body of the limpet consists of a head, a pallial cavity, a foot, a coat and a visceral mass (**Le Roux, 2005; Simone and Seabra, 2017**) (**Figure 19 and Figure 20**).

The head is equipped with two large tentacles with tactile function and which may be the seat of the chemical sense. These tentacles carry near their base on the external side two black spots (the eyes). At the end of the head, there is a mouth equipped with an organ called radula which contains many teeth used for feeding (**Figure 22**). This remarkably long organ (about 1.5 times the length of the shell) is folded on itself in a pocket independent of the digestive tract and extends to the right rear part of the body where it can describe a loop.

The pallial cavity or the mantle cavity is a significant part of the anatomy of molluscs, it is the dorsal body wall which covers the visceral mass. It serves as a space for the head and foot when these organs are retracted.

The foot is a rounded and muscular contour used to crawl and fix the animal on supports. The extremely close contact of this organ with the rock is made perfectly tight thanks to a layer of mucus, which contributes to the remarkable strength of the adhesion.

The coat is the place of secretion of the shell located behind the head. It supports and protects the soft parts of molluscs.

The visceral mass consists essentially of:

- The digestive tract which is very long and the digestive gland sectioned to reveal intestines.
- The gonad (male testis″ or female ovary″) is located below the digestive gland.

- The kidneys: the left kidney is of reduced size located to the left of the anus and the right kidney is thin but much extended, spreads on all the right side until the back of the digestive gland.
- The heart is composed of one atrium at the front and one ventricle at the back. It is lodged in a pericardial pocket located between the left kidney and the left anterior pillar of the muscle of the shell.
- The nervous system consists mainly of three pairs of ganglions (brain, pleural and pedal) located on both sides of the anterior digestive tract.

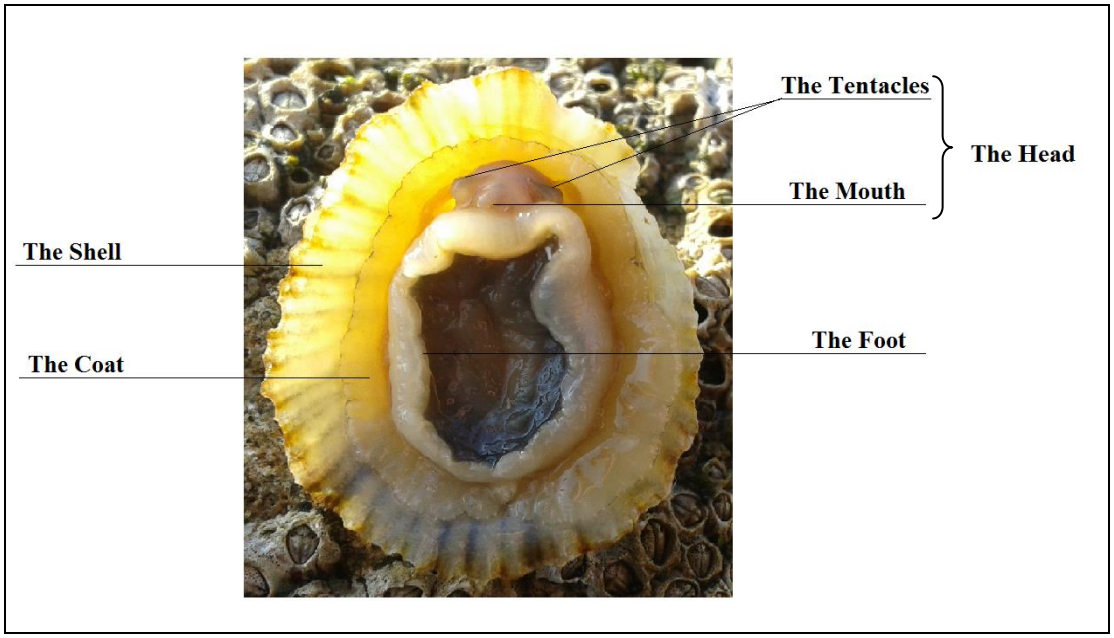


Figure 19: General organization of limpets

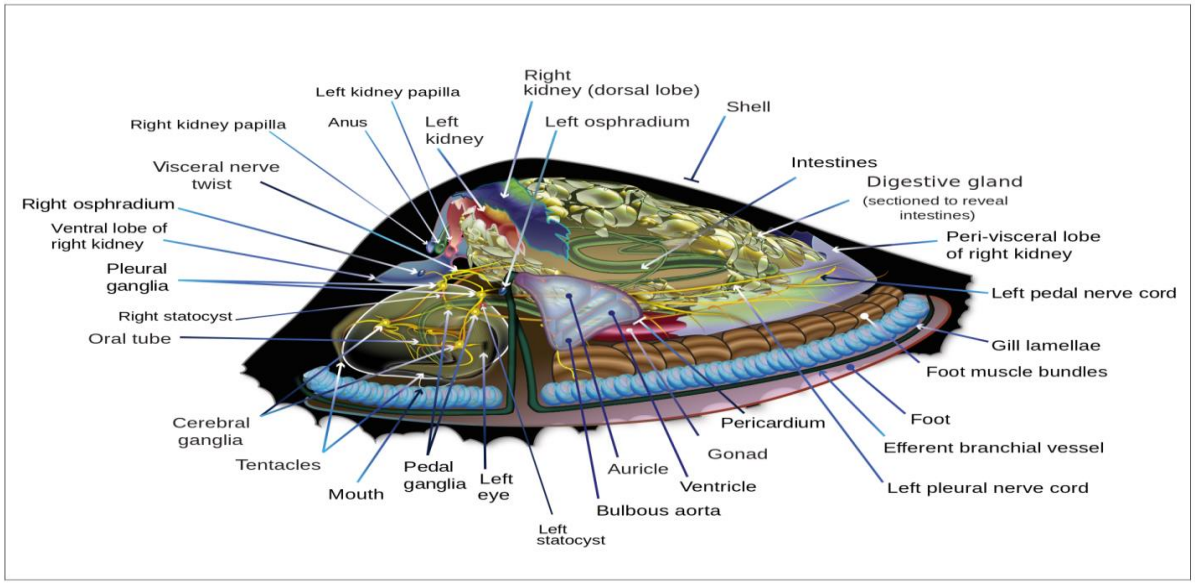


Figure 20: General anatomy of limpets (Oakley, 2013)

The taxonomy of species of the genus *Patella* is very complex and is the subject of an abundant literature. The characters of the shell are very variable and only the study of the soft parts (mainly the radula) allows a sure identification. This makes it difficult to identify limpets in the field because shell characteristics and ecological preferences provide only one indication and should be used with great caution.

The systematic of *Patella rustica* (**Linnaeus, 1758**) is shown in **Table 6**.

Table 6: Taxonomic rank (Linnaeus, 1758)

Realm	*Biota*
Reign	*Animalia*
Under-reign	*Metazoa*
Division	*Eumetazoa*
Under-division	*Bilateralia*
Branch	*Mollusca*
Class	*Gastropoda*
Under-class	*Prosobranchia*
Order	*Patellogastropoda*
Super-family	*Patelloidea*
Family	*Patellidae*
Under-family	*Patellinae*
Kind	*Patella*
Species	*Patella rustica* (**Linnaeus, 1758**)

According to the literature, there are about six species of limpet at the Mediterranean level: *Patella intermedia* (**Murray, 1857**), *Patella rustica* (**Linnaeus, 1758**), *Patella ulyssiponensis* (**Gmelin, 1791**), *Patella vulgata* (**Linnaeus, 1758**), *Patella ferruginea* (**Gmelin, 1791**), and *Patella caerulea* (**Linnaeus, 1758**) (**Figure 21**) (**Nakhle, 2003**; **Vela and Leoni, 2007; Kallouche, 2008; Seddik, 2008**).

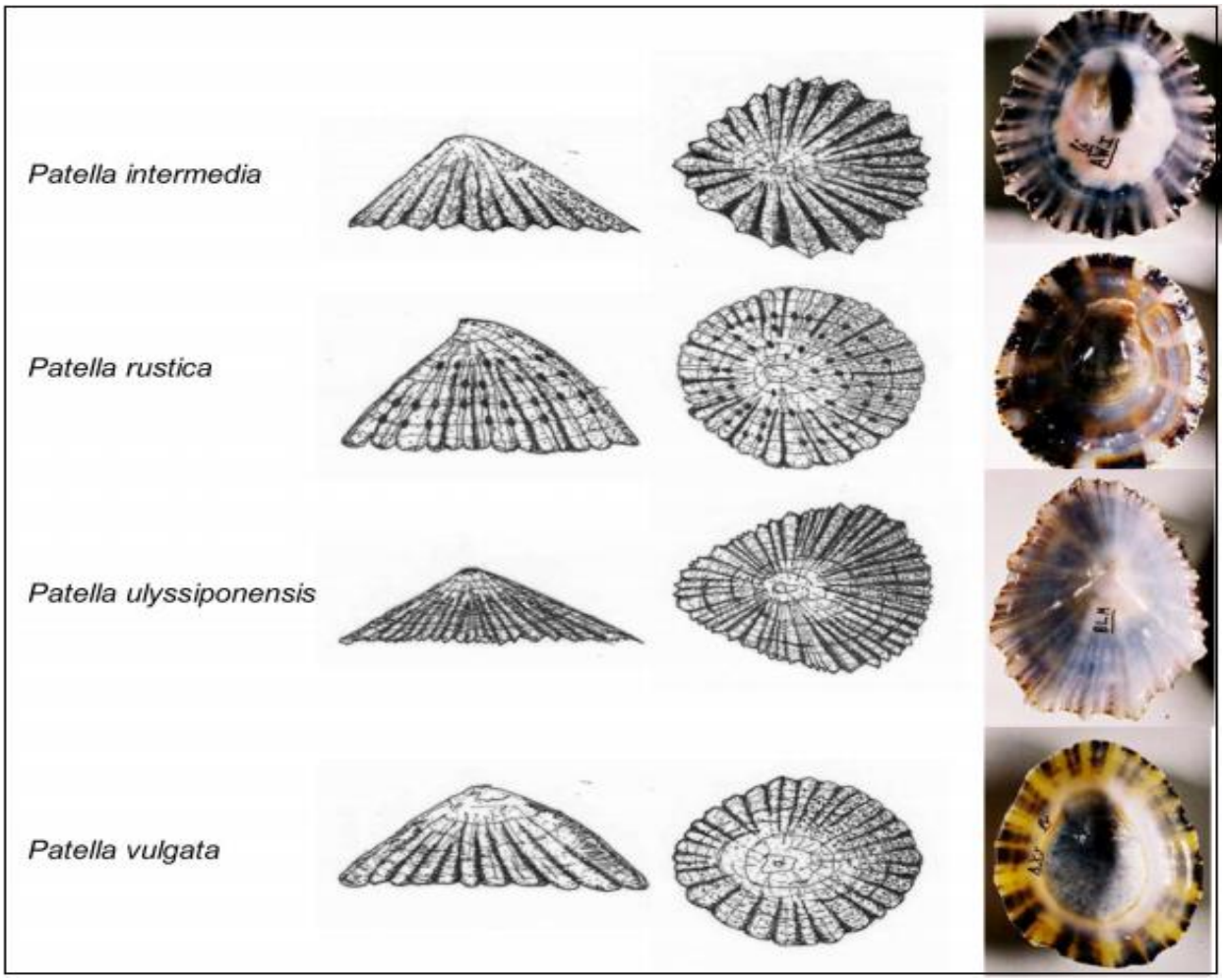


Figure 21: Lateral (left), top (central) and interior (right) views of *P. intermedia*, *P. rustica*, *P.ulyssiponensis* and *P. vulgata* shell (Cabral, 2007)

8.3 Feeding and habitat

a. Feeding

The limpet mainly feeds on the organisms such as the microalgae or debris. It use their teeth to remove from algae rock surfaces by grating their radula on rock surfaces (**Figure 22**) (**Boudouresque, 2005; Rajasekharan, 2015**). This radula, of which all gastropod molluscs are endowed, is composed of teeth arranged symmetrically in rows and actuated by muscles.

It is characterized by three marginal and three lateral teeth, with one of the most external is tricuspid (**Boudouresque, 2013**).

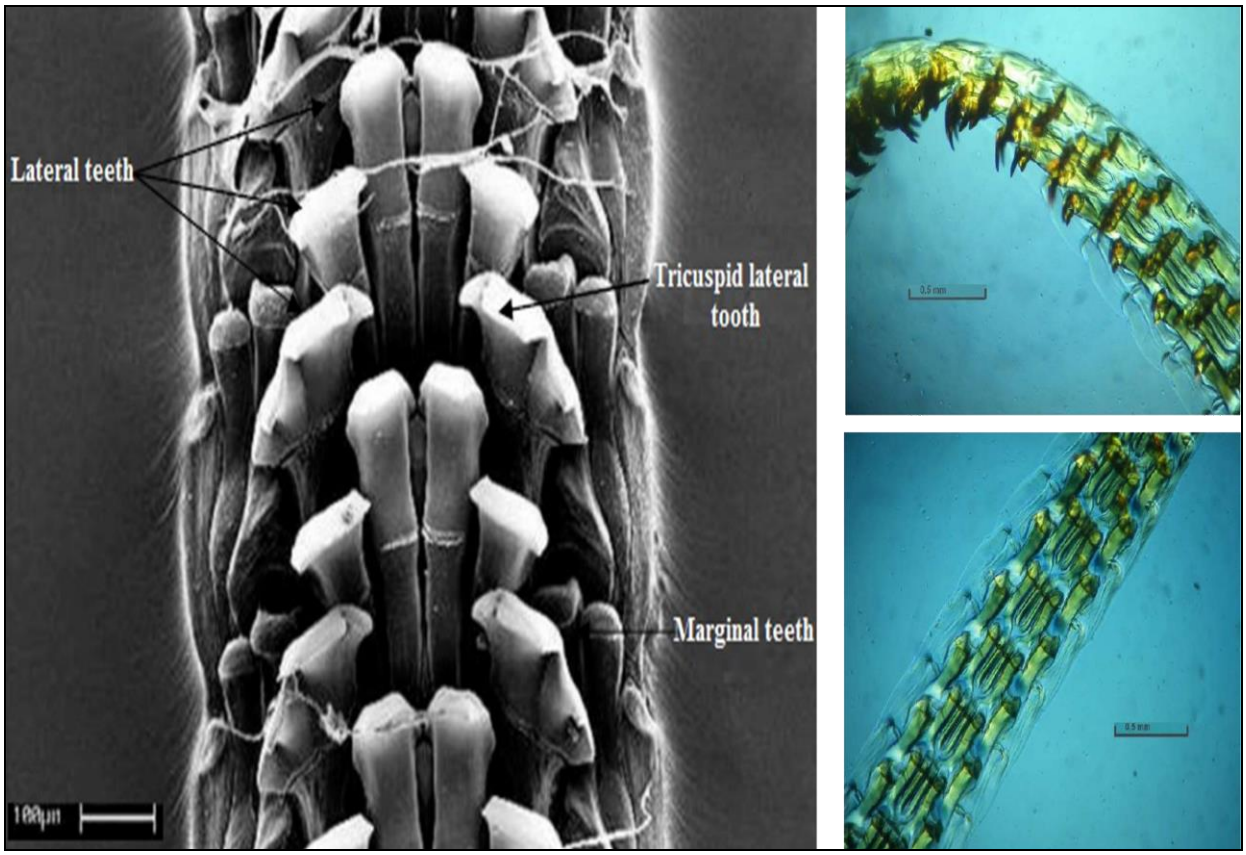


Figure 22: Electron microscopy of radula of limpets (Nakhle, 2003; Le Roux, 2005; Seddik, 2008; Hakabe, 2010)

The limpets can also feed on brown algae such as Fucus and Ascophyllum. They can have a role in the fight against the proliferation of algae on certain rocky coasts of the littoral (**Le Roux, 2005**).

b. Habitat

The limpet is a benthic animal, very common on the upper mediolittoral rocks, from the surface up to 50 cm deep. It is able to live quite high above sea level. For example in Zembra (an island in northern of Tunisia), it was spotted up to 6 m above the water level (**Neal and Skewes, 2004**).

Sedentary, each individual has its own location on the rock. It settles in groups with a large number that can reach several hundred individuals per square meter. It colonizes the rocks of the area beaten by the waves and sometimes finds emerging in the aquatic ecosystem (**Figure 23**) (**Nakhle, 2003**; **Boudouresque, 2005)**.

Figure 23: *Patella rustica* collection site

8.4 Geographic distribution

The sedentary benthic gastropod that lives in the Mediterranean, in the Atlantic and in the North-East of Spain and Portugal (**Figure 24**) is an animal that exists in the lit and unlit rocks of the mediallittotal stage under the upper floor (**Harmelin and Bassemayousse, 2008)**. The limpet resists emersion by trapping water in its pallial cavity and under shell, which is then strongly applied to the rock. It is able to lead a slowed life, withstand long desiccations and significant variations in salinity and temperature. This species is also able to resist to the shocks of the waves (**Boudouresque, 2005**).

Several species of limpets occur in the Mediterranean and the Atlantic sea (**Cretella et al., 1994; Mezali, 2005; Lima et al., 2007**), including *Patella caerulea* (**Linnaeus, 1758**) and *Patella rustica* (**Linnaeus, 1758**), the first of which is endemic to the Mediterranean sea

(**Nakhle, 2003; Lima et al., 2007; Espinosa et al., 2008; Tlig-Zouari et al., 2010; Kallouche, 2011; Belkhodja and Romdhane, 2012**).

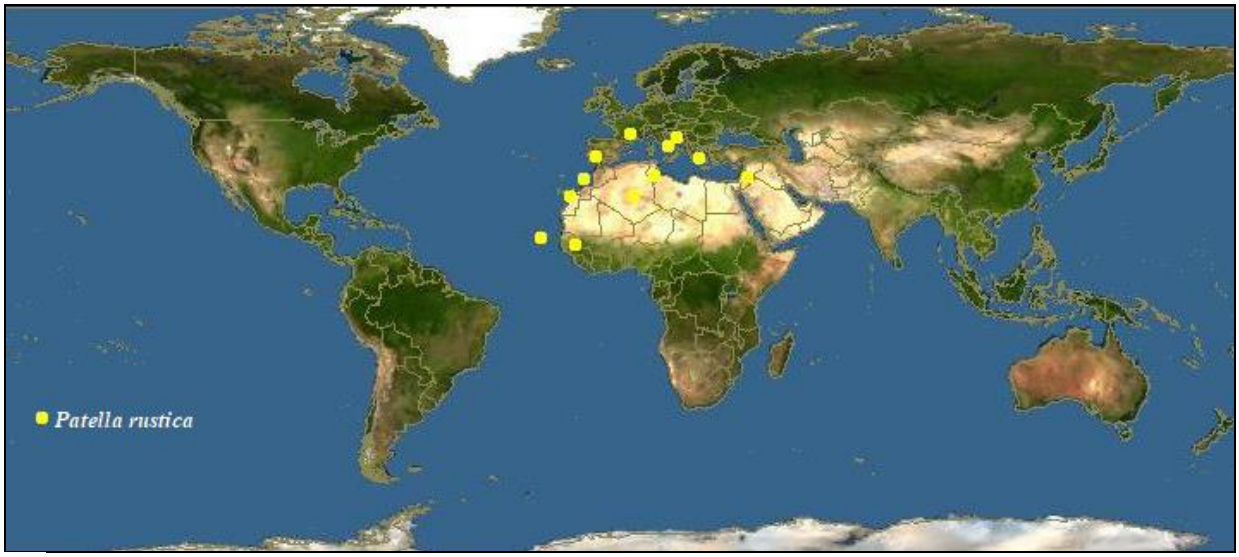


Figure 24: Map of geographic distribution of *Patella rustica* (Bouchet et al., 2015)

8.5 Sources and modes of contamination

Metallic trace elements are present in water, air and soil. Like all ores, they are present in rocks. These nature reserves do not themselves constitute a danger, but the exposure of deposits, erosion and forest fires, water withdrawals or volcanic eruptions, will answer the traces of these elements in the environment. They can become toxic if they are found in sufficient quantities in living organisms (**Grimes et al., 2004; Boutiba, 2006; Kuvarega and Taru, 2008**).

In addition to these natural phenomenon, the human activity, even if it does not create metallic trace elements, contributes to their diffusion in the environment: the effluents of mining extractions, the industrial effluents, the domestic effluents, leaching metals from landfills and solid residues, inputs of metals from rural areas such as metals held in pesticides, atmospheric sources such as fossil fuel combustion, waste incineration, industrial emissions, and petrochemical activities (**Crowley et al., 2003; Gautam et al., 2016; Rathoure et al., 2017**).

8.6 International standards and recommendation

The accumulation of cadmium, copper, chromium and lead metals by gastropod molluscs is related to their environmental concentrations (**Coeurdassier, 2001**). The metals content studied in the soft tissues of *Patella rustica* varied according to their exposure to various sources of pollution (**Modrzewska and Wyszkowski, 2014**).

The limpets are edible, they can be eaten raw or grilled. Its average energy value is 92 calories per 100 grams which is 17 g of protein, 2 g of fat and 1.5 g of carbohydrates (**Mandeville, 2016)**. In order to avoid undesirable health effects as a result of excessive intake of toxicants including toxic metals, international and national scientific organisms such as FAO,WHO, FDA, and EU have used the safety factor approach for establishing acceptable or tolerable intakes of substances that exhibit threshold toxicity. The acceptable daily intake (ADI) or tolerable daily intake (TDI) and provisional tolerable weekly intakes (PTWI) are used to describe safe levels of intake for several toxicants including toxic metals (**Table 7**) (**Kroes and Kozianowski, 2002; da Silva et al., 2005**).

For most kinds of toxicity, it is believed that there is a low dose which no adverse effect will occur. For chemicals that give rise to such toxic effects a tolerable daily intake that is an estimate of the amount of a substance in food, expressed on a body weight basis (mg/kg of body weight) that can be ingested over a lifetime without appreciable health risk **(da Silva et al., 2005)**.

The metallic trace elements have a strong toxicological impact on the consumer of shellfish. It was necessary to regulate the metal content in shellfish for consumption and to control industrial discharges (**Table 8**) (**Mitra et al., 2012; Pandey, 2014; Rajeshkumar and Li, 2018**).

Table 7 : Levels of metallic trace elements allowed in molluscs (ECC, 2006)

Metallic trace elements	Maximum permissible limits (mg/kg wet weight)
Copper (Cu)	5.0
Chromium (Cr)	1.0
Cadmium (Cd)	1.0
Lead (Pb)	1.5

Table 8 : Estimated average daily exposure to metallic trace elements (JECFA, 2006)

Metallic trace elements	Acceptable daily intake (µg/day/person)*
Copper (Cu)	980
Chromium (Cr)	126
Cadmium (Cd)	60
Lead (Pb)	216

* Estimate performed with the assumption of a body weight of 60 Kg

PART II: DETECTION OF VIRAL AND METAL CONTAMINATION IN MOLLUSCS

ABSTRACT

The bivalve shellfish are filter feeders and they act as natural biofilters in seawater and can thus efficiently bioconcentrate and bioaccumulate enteric viruses in their digestive tissue. In Morocco, shellfish sanitary quality analysis does not currently include enteric virus detection. Therefore, the objective of this study was to detect the presence of enteroviruses in mussels (*Mytilus galloprovincialis*) collected from three wild populations (Bouregreg estuary, Yacoub Al Mansour coast and Harhoura coast) in order to get an overview on the viral contamination in the aquatic environment. Between February 2014 and February 2015, two hundred and eighty-eight samples were collected and tested for viral contamination using cell culture and real-time polymerase chain reaction (real-time PCR) for intratypic differentiation (ITD). The results by cell culture and real-time PCR showed that the consumption of mussels originated from a contaminated area revealed a clear risk of infection. For this reason, the presence of enteroviruses in shellfish production area represents a potential health risk by causing serious illnesses (gastroenteritis, hepatitis and poliomyelitis).

Keywords: *Enterovirus*, shellfish, viral contamination, cell culture, real-time PCR.

1. Introduction

The impact of environmental pollution, especially in marine environment, by the transmission of viral infections was suspected in the beginning of the 20th century. However, the propagation of viral diseases has been demonstrated earlier throughout the period from 1940 to 1945 during epidemics of poliomyelitis (**Le Guyader et al., 2014**). The viruses most often transmitted by contamination of the marine water were *Norovirus* (NoV), Hepatitis A virus (HAV), *Hepatitis* E virus (HEV), Adenovirus (AdV), *Astrovirus* (AV), *Rotavirus* (RV) and the *Enterovirus* (EV) (*Poliovirus*, *Coxsackievirus*, *Echovirus*) (**Griffen et al., 2003; Le Guyader et al., 2009**). The diseases associated with enteric viruses are heterogeneous. In addition to poliomyelitis, enteric viruses in the human stool can cause severe acute diseases such as hepatitis, gastroenteritis, meningitis and non-specific febrile illness (**Cristina and Costa-Mattioli, 2007; Gibson, 2014; Shulman et al., 2006**). Therefore, the environmental monitoring can provide an added tool to determine the different viruses circulating in a community (**Pinto et al., 2007; Shulman et al., 2006**).

This is a way to monitor viral transmission in human populations by examining environmental samples in particular from bivalve molluscs (mussels). The mussels were chosen for their wide geographical distribution from temperate to subarctic regions and also because they are filter feeders. These characteristics make them useful bio-indicators (bioaccumulators) to evaluate and monitor the contamination level in aquatic environment (**Formiga-Cruz et al., 2003**). The enteroviruses are one of the most frequently monitored viruses in environmental waterways and are often used as a bioindicator of viral contamination (**Wurtzer et al., 2014**). For this reason, this kind of analysis demonstrates the importance of environmental monitoring by the assessment of enteric virus contamination in selfish. In Morocco, the viral pollution of the environment was the subject of several studies, but no study has been done to search the *Poliovirus* in aquatic environment. The lack of a national monitoring program of enteric viruses was one of the reasons to do this study in order to evaluate the contamination of enteroviruses in mussels collected from potentially polluted areas. The target of this study was to supplement the Moroccan databases available on environmental contamination by *Enterovirus* and illustrate the importance of including routine virological analysis of shellfish in Morocco.

2. Materials and methods

2.1 Samples collection and processing

A total of 288 mussels samples (*Mytilus galloprovincialis*) were collected between February 2014 and February 2015, from three wild population sites that receive domestic waste without previous treatment (Bouregreg estuary, Yacoub Al Mansour and Harhoura coast). The sampling sites were situated mainly in Rabat Region of Morocco (**Figure 25 and Table 9**). This region covers an area of 18,194 km^2, with population nearing 4,581,000. This area belongs to the Mediterranean climate marked by two main seasons softened by oceanic influences. The average temperatures are around 12°C for the colder months (December and January) and 22°C for the warmer months (July to September). The average annual rainfall is more than 550 mm/year. The coastal zone of the region faces various environmental problems (liquid and solid waste) that will destroy coastal quality and threaten the collection of various aquatic products for consumption. The samples were shipped to laboratory on the same day,

in chilled condition. Therefore, they were processed before being stored at -20°C until virological analysis.

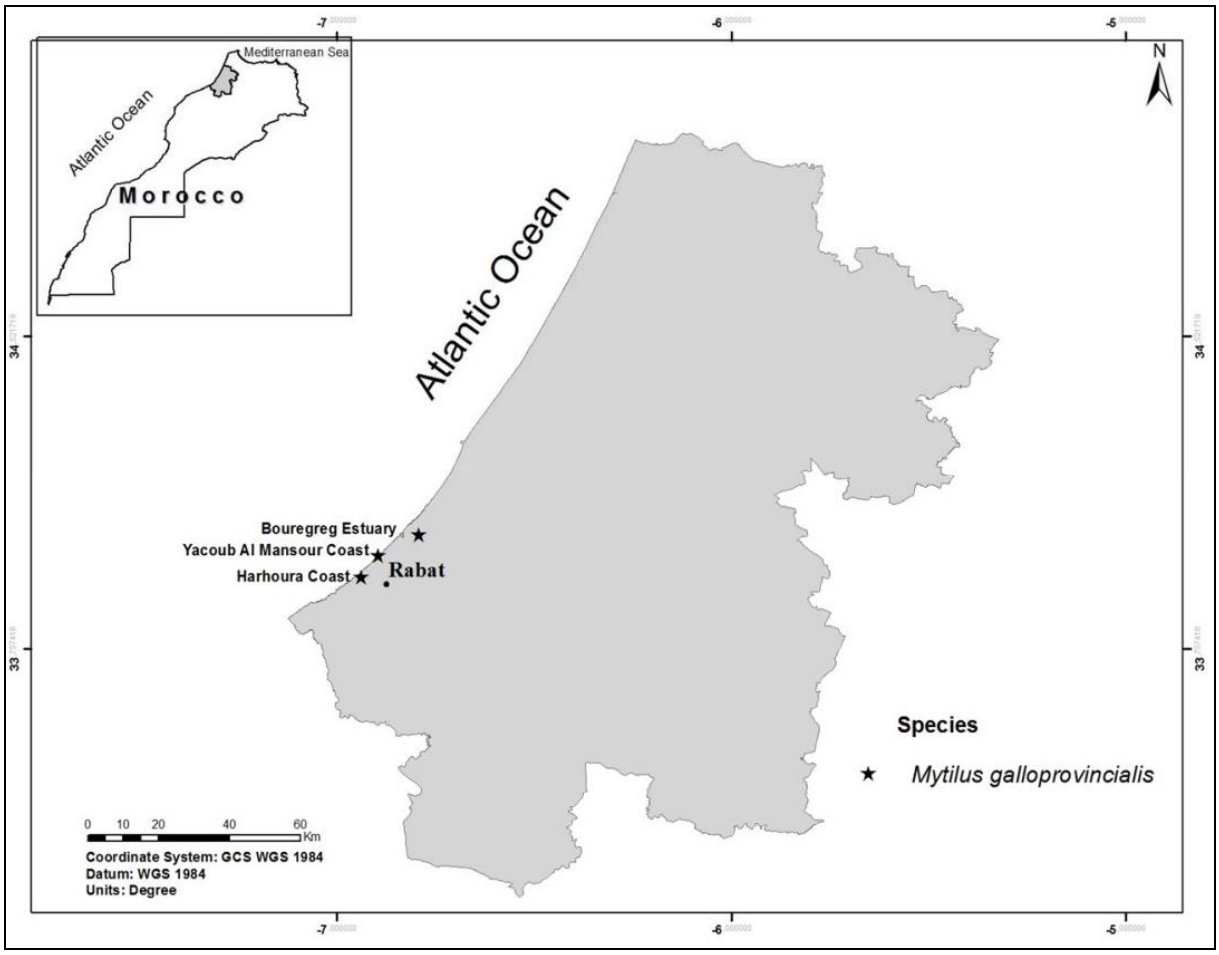


Figure 25 : Map of Morocco coast showing locations of sampling.

Table 9 : Mussels (*Mytilus galloprovincialis*) samples from three wild population of Morocco including geographic coordinates of the location and the size of the samples during the same period.

Specie	Sampling sites	Coordinates	Sample number	Sample date
Mytilus galloprovincialis	Bouregreg estuary	N 34°2′8″; W 6°50′41″	96	February 2014 to February 2015
	Yacoub Al Mansour coast	N 33°59′3″; W 6°53′41″	96	
	Harhoura coast	N 33°57′24″; W 6°55′28″	96	

2.2 Extraction-concentration of virus from samples

The technical laboratory method described by **El-Senousy et al., 2013** was adopted, which is based on the adsorption of the viruses with acid pH and their elution with basic pH, according to the following protocol. The shells were opened in an aseptic way; the digestive system was dissected with a sterile shucking knife, making elimination of inhibitors tissues (polysaccharides, sexual gonads) possible while analysing a larger number of individuals (1.5g of hepatopancreas, weight corresponding to an analysis, represent on average 12 mussels). To extract the viruses, 1.5 g of hepatopancreas were homogenized for 5 to 10 min in a blender. The ground material was subjected to stirring for 15 min at room temperature in the presence of 10 mL of buffer (0.1 M glycine: 0.3 M NaCl) at a pH = 9. After centrifugation of mixture at 10 000 g for 10 min at +4°C, 5 mL of phosphate buffered saline (PBS) at a pH = 7.2 was added to the supernatant, to which polyethylene glycol (PEG 6000 at 50%) was added to a quarter of the final volume to increase the concentration. The pH value was adjusted to 7.2 and the mixture was incubated overnight. The following day, the precipitate formed is recovered by centrifugation at 10 000 g for 30 min at +4°C. The final pellet was resuspended in 5 mL of PBS at pH 7.2. To prevent the contamination of the extracts, it was necessary to add to the mixture 30 µL of antibiotics (Penicillin 10 000 U/mL and Streptomycin 10 000 µg/mL) and 20 µL of Fungizone (250 µg/mL). The viral concentrate was stored at -20°C until used in one of the cell culture or iRNA viral extraction (**El-Senousy et al., 2013**).

2.3 Cell culture

Two cell lines cell culture were used according to the new algorithm (**Figure 26**) (**WHO, 2004b**); the first one was the RD cells line, with 5×10^4 cells/mL, the passage was 255/3; the cells originated from Center of Disease Control (CDC) in Atlanta; they were derived from Human Rabdomyosarcom, sensitive to *Poliovirus* and other non *Poliovirus* enteroviruses (**Figure 27**) and the second one was the L20B cells line, with 5×10^4 cells/mL, the passage was 18/3; cells originated from CDC; cells line results from mouse and have specific receptors to *Poliovirus* and some other non *Poliovirus* enteroviruses such as Coxsackievirus (**Figure 28**) (**Bahri et al., 2005**).

Both cell lines were grown in the minimum essential medium (MEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) in 75 cm^2 plastic flasks. The inoculation of 100 µl of the extract on the two cells line was done in 96 well plates, which were then incubated at 36°C. The reading was made in inverted microscope with LCD display (Life biotechnology, USA) every day to search the appearance of a cyto-pathogenic effect (CPE) evocative of enteroviruses for 10 days (**Sdiri et al., 2004**).

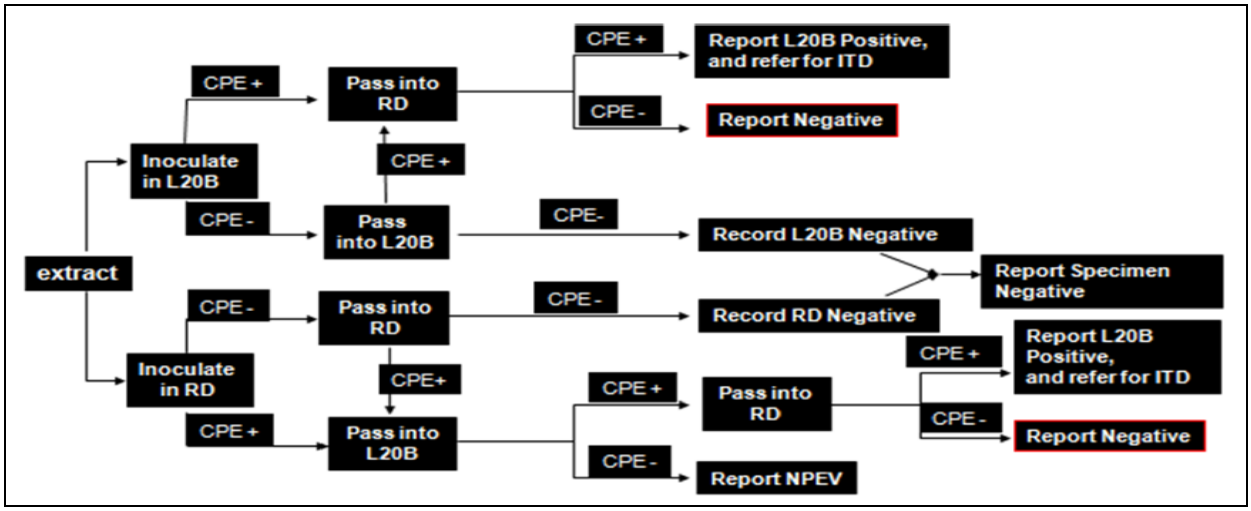


Figure 26: New algorithm for the isolation of enteroviruses (WHO, 2004)

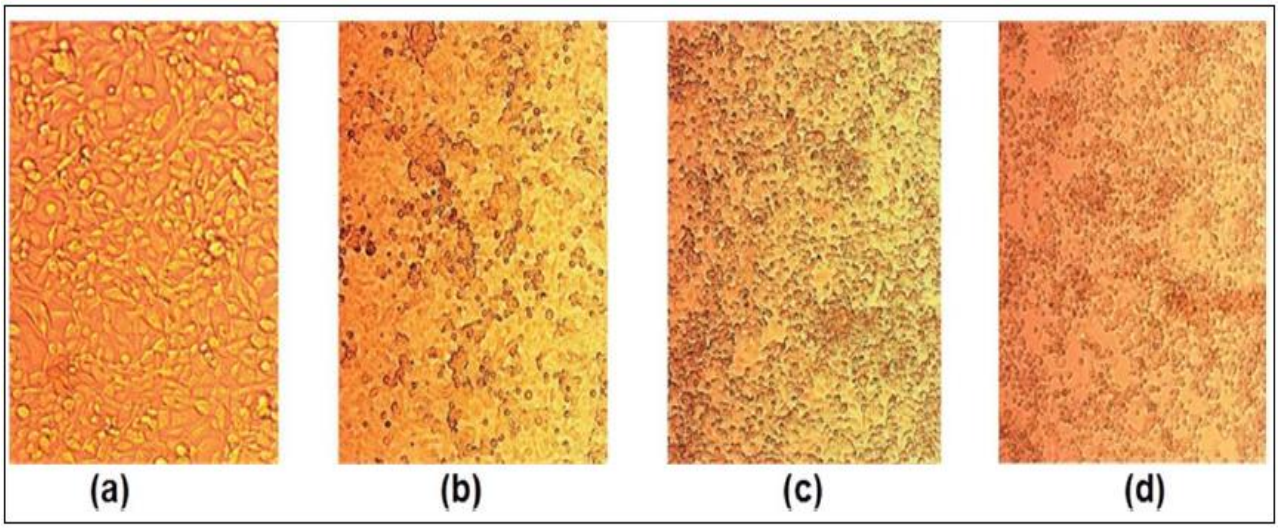


Figure 27: Appearance of inoculated and uninoculated RD cells in inverted microscope with LCD display, (a) RD: uninoculated; (b) RD: CPE=2+; (c) RD: CPE=3+; (d) RD: CPE=4+

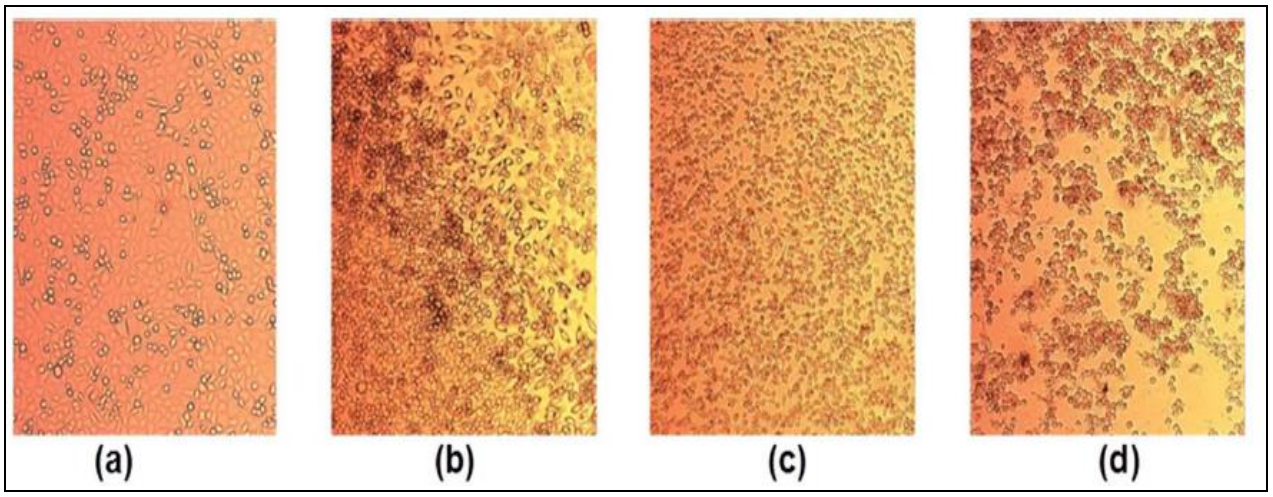


Figure 28: Appearance of inoculated and uninoculated L20B cells in inverted microscope with LCD display, (a) L20B: uninoculated; (b) L20B: CPE=2+; (c) L20B: CPE=3+; (d) L20B: CPE=4+

2.4 Real-time PCR technique

a. Extraction and purification of the viral RNA

The RNA of the viruses for all the positive analysed cultured samples was extracted using a commercial kit (Magmax™ total of the nucleic acids isolation kit, part number AM1840, Termofisher Scientific, USA) according to the supplier instructions.

The RNA viral extraction was carried out using guanidinium thiocyanate, which makes it possible to quickly release the nucleic acid by a chemical lysis and simultaneously

inactivate the nucleases in the matrix of the sample. Afterward, the nucleic acids of the viruses were insulated by purification step using the microspheric magnetic beads, to eliminate substances which could interfere with the real-time PCR reaction. These beads used for the complete binding of the nucleic acid, were washed with absolute isopropanol to eliminate proteins and other contaminants (**Karamoko et al., 2006b**). Finally, the elution buffer used (PBS) made it possible to recover the viral particles that adhered to the surface of the magnetic beads.

b. Detection of the viral genome

After extraction and purification of the viral RNA, the *Enterovirus* was detected according to World Health Organization recommended protocols (**WHO, 2004**), by amplification of the region 5'NTR-3'NTR genome *in vitro*, using specific *Enterovirus* primers (Pan *Enterovirus* (Pan EV), Pan *Poliovirus* (Pan PV), *Poliovirus* Serotype 1 (PV1), *Poliovirus* Serotype 2 (PV2), *Poliovirus* Serotype 3 (PV3), Sabin Multiplex) (**Table 10**) and Sabin primers (Sabin 1 Vaccine-Derived *Poliovirus* (S1 VDVP), Sabin 2 VDVP (S2 VDVP), Sabin 3 VDVP (S3 VDVP) (**Table 11**) and the technique of quantitative RT-qPCR (Applied Biosystems, Termofisher Scientific, USA) . The detection of amplicons generated at each amplification cycle required the use of fluorescent probes (TaqMan®, CDC) hybridizing on specifically amplicon. Quantification of DNA was achieved by the Ct value (Cycle threshold), which corresponds to the number of PCR cycles required for the fluorescence in the sample. This Ct value can be related to an amount of DNA through the use of a standard range or be compared with that of a reference gene. The sample was considered positive if the Ct is less than 30. The real-time PCR reactions were performed in Applied Biosystems 7500 fast real-time PCR System (Applied Biosystems, Termofisher Scientific, USA) as follows: Reverse transcription reaction at 42°C for 45 min, inactivation at 95°C for 3 min followed by 40 cycles of PCR at 95°C for 24 s and 47°C for 30 s, then a 25% speed ramp at 60°C for 24 s. The end point fluorescent data was collected at the end of the 47°C anneal step and the data were captured and analysed by using the 7500 Software v2.0.5 instrument.

Table 10: List of primers and probes for intratypic differentiation (ITD) real-time PCR (WHO, 2004)

Specificity	Primer or probe (Polarity)	Primer or probe sequence (5′-3′)
Pan *Enterovirus*	PCR-1 (A) PCR-2 (S) Pan EV Probe (S)	GCGATTGTCACCATWAGCAGYCA GGCCCCTGAATGCGGCTAATCC **FAM**-CCGACTACTTTGGGWGTCCGTGT-**BHQ1**
Pan *Poliovirus*	Pan PV/ PCR-1 (A) Pan PV/PCR-2 (S) Pan PV/Probe21A (A)	AYRTACATIATYTGRTAIAC CITAITCIMGITTYGAYATG **FAM**-TGRTTNARIGCRTGICCRTTRTT-**BHQ1**
Poliovirus serotype 1	Sero PV1 A (A) Sero PV1, 2S (S) SeroPV1Probe16A (A)	ATCATIYTPTCIARPATYTG TGCGIGAYACIACICAYAT **FAM**-TGICCYAVICCYTGIGMIADYGC-**BHQ1**
Poliovirus serotype 2	SeroPV2 A (A) Sero PV2, 2S (S) SeroPV2Probe5S (S)	AYICCYTCIACIRCICCYTC TGCGIGAYACIACICAYAT **FAM**-CARGARGCIATGCCICARGGIATNGG-**BHQ1**
Poliovirus serotype 3	SeroPV3 A (A) Sero PV3, 2S (S) SeroPV3Probe11S (S)	CCCCIAIPTGRTCRTTIKPRTC AAYCCITCIRTITTYTAYAC **FAM**-CCRTAYGTNGGITTRGCVAAYGC-**BHQ1**
Sabin 1	Sab1/PCR-1 (A) Sab1/PCR-2 (S) Sab1/ Probe (A)	CCACTGGCTTCAGTGTTT AGGTCAGATGCTTGAAAGC **CY5**-TTGCCGCCCCCACCGTTTCACGGA-**BHQ3**
Sabin 2	Sab2/PCR-1 (A) Sab2/PCR-2 (S) Sab2/ Probe (S)	CGGCTTTGTGTCAGGCA CCGTTGAAGGGATTACTAAA **FAM**-ATTGGTTCCCCCGACTTCCACCAAT-**BHQ1**
Sabin 3	Sab3/PCR-1 (A) Sab3/PCR-2 (S) Sab3/ Probe (S)	TTAGTATCAGGTAAGCTATC AGGGCGCCCTAACTTT **ROX**-TCACTCCCGAAGCAACAG- **BHQ2**

Table 11: List of primers and probes for Sabin Vaccine-Derived *Poliovirus* (VDVP) real-time PCR (WHO, 2004).

Primer specificity	**Primer and probe sequences (5′-3′)**	
Sabin 1 VDVP VP1	Sense	CATGCGTGGCCATTATA
	Anti-sense	CAAATTCCATATCAAATCTA
	VP1 probe	**FAM**-CACCAAGAATAAGGATAAGC-**BHQ1**
Sabin 1 VDVP 3D	Sense	GACACTAAGGAAATGCAAAAACTGC
	Anti-sense	ATCGCACCCTACTGCTGA
	3D probe	**ROX**-TCAGTGGCAATGAGAATGGCTTTTGGG-**BHQ2**
Sabin 2 VDVP VP1	Sense	GACATGGAGTTCACTTTTG
	Anti-sense	CTCCGGGTGGTATATAC
	VP1 probe	**FAM**-CATTGATGCAAATAAC-**BHQ1**
Sabin 2 VDVP 3D	Sense	AGGAAATGCGGAGACTCTTA
	Anti-sense	GGATCACAACCAACTGCACT
	3D probe	**ROX**-CTTACCGCTTGTAACATATGT-**BHQ2**
Sabin 3 VDVP VP1	Sense	CATTTACATGAAACCCAAAC
	Anti-sense	TGGTCAAACCTTTCTCAGA
	VP1 probe	**FAM**-TAGGAACAACTTGGAC-**BHQ1**
Sabin 3 VDVP 3D	Sense	CACCAAAGAAATGCAAAGACTTT
	Anti-sense	GGATCGCATCCAACTGCACT
	3D probe	**ROX**-CCTACCATTAGTGACATATGT-**BHQ2**

2.5 Statistical analysis

The statistical analysis using the χ^2 test for proportions was performed using SigmaPlot (version 12) to evaluate the level of pollution between different groups of wild sites. The *P* value superior of 0.05 was considered as non-significant.

3. Results and discussion

From cell culture method of two cell lines RD 255/3 and L20B 18/3, 75% (216/288) of samples showed cyto-pathogenic effect suggestive of *Enterovirus* (**Figure 29**). Therefore, the wild mussel samples were collected from areas with high faecal pollution by domestic wastewater. The virological analysis (cell culture) of these samples displayed that 75% of mussels were contaminated by enteroviruses highlighted, with the predominance in 70.8% (204/288) of non *Poliovirus* enteroviruses (NPEV) from Bouregreg estuary, Yacoub Al Mansour and Harhoura coast, whilst 4.2% (12/288) represented the Sabin strain of *Poliovirus*

type 1 (SL1) from Harhoura coast, which was confirmed by real-time PCR for intratypic differentiation (ITD) (**Figure 30** and **Table 12**). The positivity of enteroviruses by cell culture in wild mussels from Bouregreg estuary was 87.5% (84/96), 75% (72/96) of Yacoub Al Mansour coast and 62.5% (60/96) of Harhoura coast (**Figure 31** and **Table 12**). Consequently, the statistical analysis using the χ^2 test for proportions revealed that differences in these three wild sites were not significant (*P* value > 0.05).

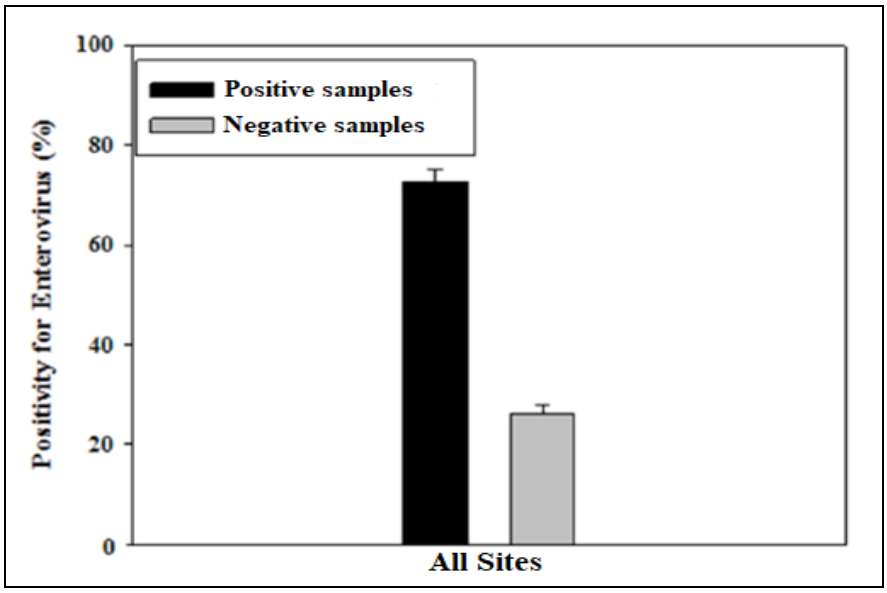


Figure 29: Percentage of positivity for enteroviruses in mussels

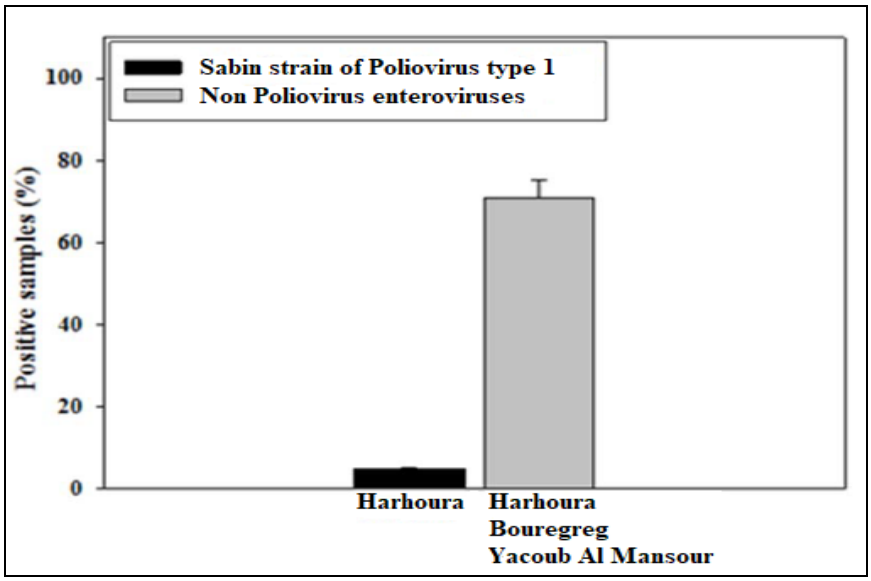


Figure 30: Percentage of positivity for sabin strain of *Poliovirus* type 1 and non *Poliovirus* enteroviruses in mussels

Table 12: Positive and negative samples described by sampling sites and number

Sampling sites	Sample	Sample number	Positivity of enteroviruses	Positive sample	Negative sample
Harhoura coast	M1 to M8	8×12= 96	62.5% (60/96) of enteroviruses	M1, M3, M4, M5, M8	M2, M6, M7
Bouregreg estuary	M9 to M16	8×12= 96	87.5% (84/96) of enteroviruses	M9, M10, M12, M13, M14, M15, M16	M11
Yacoub Al Mansour coast	M17 to M24	8×12= 96	75% (72/96) of enteroviruses	M17, M18, M20, M22, M23, M24	M19, M21
All sites	M1 to M24	96×3= 288	70.8% (204/288) of non *Poliovirus* enteroviruses	M3, M4, M5, M8, M9, M10, M12, M13, M14, M15, M16, M17, M18, M20, M222, M23, M24	
Harhoura coast	M1 to M8	8×12= 96	4.2% (12/288) of Sabin strain of *Poliovirus* type 1	M1	

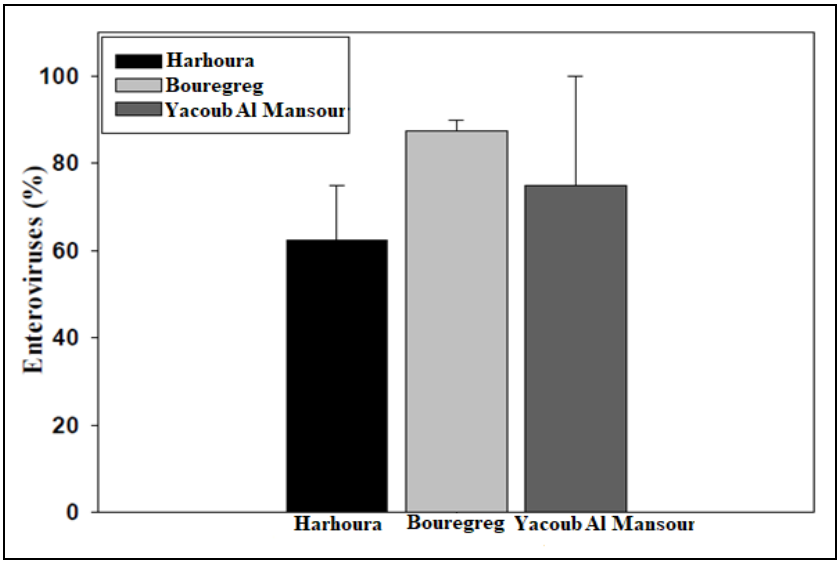


Figure 31 : Comparison of positivity percentages for enteroviruses between three wild population sites of mussels

The enteroviruses are among the infectious agents associated with waterborne diseases faecal-oral transmission reflecting defective hygienic conditions. They can detect asymptomatic infections and multifaceted clinical variable diagnosis depending on the serotype involved (**Avellon et al., 2003; Sutter et al., 2014**). Several studies have shown the involvement of a panel of enteroviruses in the similar events that enhance the acute flaccid paralysis (AFP), in this case, related to different kind of enteroviruses which caused paralysis and suggest a potential threat such as the enteroviruses types 68 and 71 (**Bahri et al., 2005; Delpeyroux et al., 2013; WHO, 2013**). In Morocco, the prevalence of circulating NPEV is unknown, which indicates the need for a deeper investigation of the dissemination in the environment in order to identify them and associate them with clinical manifestations in the country. Thus, the results reported in this study showed a potential health risk to the population. Indeed, the presence of this strain vaccine in the environment could be a source of infection for humans.

The enteroviruses are ubiquitous pathogens present in all regions of the world and able to survive for long periods in the marine environment (**WHO, 2013**). Furthermore, the resistance of these viruses such as acid pH and extreme temperature facilitate their transmission. These properties ensure that enteroviruses are very well dispersed in water surface or wastewater from sewage treatment. The human enteroviruses are not inactivated in the water environment and will therefore often be caught and activated by the filter feeders such mussels (**Benabbes et al., 2013b**). In conclusion, this work gives subsidies to explain the high prevalence of non-polio enteroviruses in the aquatic environment for the countries with low socio-economic and hygienic status. In front of this viral risk, it is necessary to have quick and reliable techniques in order to detect enteric virus from food matrices. Some studies have revealed the presence of Hepatitis A virus (**Karamoko et al., 2006a**), adenovirus (**Karamoko et al., 2005**) and enteroviruses (**Karamoko et al., 2006b**) respectively in 37.5, 15 and 10% of mussel samples in the costal media. Other studies have shown that 88.6% of all mussel analysed samples were contaminated by adenovirus and enteroviruses respectively in 52.3 and 36.3% of the samples from two production areas in Moroccan Mediterranean Coast (**Benabbes et al., 2013b**). Others highlighted the noroviruses contamination in 30% of samples collected in the Mediterranean Sea and the Atlantic Coast (**Benabbes et al., 2013a; Vaillant et al., 2012**). Overall, to control a sanitary quality of bivalve molluscs, the need to evaluate the viral contamination of the aquatic environment during the whole year was

suggested. It was concluded that the results of this research underscore the importance of strengthening the virological quality of bivalve molluscs before their commercialization.

4. Conclusion

The present study highlighted the circulation of a significant number of enteroviruses, with the predominance of non *Poliovirus* enteroviruses (NPEV) and the presence of sabin strain of *Poliovirus* type 1 (SL1). The study also confirmed the absence of statistically significant difference between three wild populations. Therefore, the virological monitoring system of the environment should be reinforced by extending the virological investigation serotyping of NPEV strains, the reinforcement of the prevention against the propagation of enteroviruses (*Poliovirus* and non *Poliovirus* enteroviruses) and the establishment of a monitoring system of these viruses in the aquatic environment.

CHAPTER II: TYPING OF *Enterovirus* IDENTIFIED FROM MOROCCAN MUSSELS (*Mytilus galloprovincialis*) BY SERONEUTRALIZATION

ABSTRACT

Non *Poliovirus* enteroviruses (NPEV) are infectious agents which can determine various illness in human such as hand-foot-mouth syndrome, angina, respiratory diseases, acute or chronic heart disease, diarrhea, pancreatitis, acute hemorrhagic, and conjunctivitis. These viruses are eliminated in the stool and thus contaminate the marine environment and shellfish.

In Morocco, shellfish sanitary quality analysis does not include enteroviruses detection. Therefore, the objective of this study was to detect and to type enteroviruses in 288 mussel samples. Virus particles were extracted and concentrated from mussel samples by using polyethylene glycol 6000, and the presence of enteroviruses was screened by the cell culture method using two cell lines, which are human rhabdomyosarcoma cells (RD) and mouse cells (L20B). Mussel samples (*Mytilus galloprovincialis*) were collected between February 2014 and February 2015 from three wild populations (Bouregreg estuary, Yacoub Al Mansour and Harhoura coast). 216 of 288 samples (75%) were revealed positive by the cell culture method, with 204 strains of NPEV (70.8%) and 12 strains of *Poliovirus* Type 1 (4.2%). According to the procedures recommended by the World Health Organisation (WHO), the antigenic identification by seroneutralization and serotyping has been done. The serotype of 204 NPEV strains has been determined a typable strains (64.7%) and non-typable strains (35.3%) in the marine environment. However, the proportion of untypable strains confirms the presence of new serotypes.

The diseases caused by NPEV constitute an important public health problem. To fight against this human health risk related to viral contamination, it is necessary to have a methodology for the control and virological monitoring of the marine ecosystem.

Keywords: Non *Poliovirus* enteroviruses, *Mytilus galloprovincialis*, marine environment, shellfish, seroneutralization.

1. Introduction

The microbial pollution in the marine environments is a key determinant for the evaluation of the level of viral contamination, with major impacts on the control of the faecal risk for human health. For evaluating the latter risk, different markers have been proposed, including

enteroviruses and adenoviruses (**Hot et al., 2003; Rajtar et al., 2008; Fong and Lipp, 2005; Jung et al., 2014**).

The advantage of enteroviruses as a marker of viral contamination is that certain genotypes are relatively easy to cultivate in cell culture, which is still the reference method for environmental monitoring (**Ehlers et al., 2005; Hematian et al., 2016**). Indeed, typing enteroviruses strains existent in the marine environment may be an important objective, especially to detect the presence of non *Poliovirus* enteroviruses strains and *Poliovirus* strains in areas where these agents are still circulating (**Hovi et al., 2012**).

Non *Poliovirus* enteroviruses (NPEV) circulate in all populations and infection can be associated with a vast range of presentations. In this study, the serotype identification of non *Poliovirus* enteroviruses was done according to the procedures recommended by the World Health Organisation (**WHO, 2004**).

The identification of newly isolated strains by specific neutralization becomes increasingly difficult, as many types of enteroviruses exist in the environment. Seroneutralization tests with composite antiserum pools are very economical in tissue culture and time that the use of pooled antiserum initially is advantageous. The reference method for the laboratory diagnosis of enteroviruses is isolation of the cell culture, followed by serotype identification (**Oberste and Pallansch, 2005; Hambling et al., 2009**).

The lack of a national monitoring program of enteroviruses was one of the reasons to do this study in order to evaluate the viral contamination in mussels collected from potentially polluted areas. The target of this research was to study circulating strains of enteroviruses in the marine environment, in order to supplement the Moroccan databases available on environmental contamination by enteroviruses and to illustrate the importance of including routine virological analysis of shellfish in Morocco.

2. Material and methods

2.1 Study Area

Between February 2014 and February 2015, three sampling sites (Bouregreg estuary, Yacoub Al Mansour and Harhoura coast) located in the Rabat Region of Morocco (**Figure 32**) were chosen for the collection of 288 mussels samples (*Mytilus galloprovincialis*) from wild population sites that receive domestic waste without previous treatment. This region covers an

area of 18.194 km^2, with a population of about 4.581.000. This area belongs to the Mediterranean climate characterized by two main seasons softened by oceanic influences. The average temperatures are approximately 22°C for the warmer months (July to September) and 12°C for the colder months (December and January). Relating to the annual rainfall is in average more than 550 mm/year (**Idrissi Azzouzi et al., 2017a, b**)

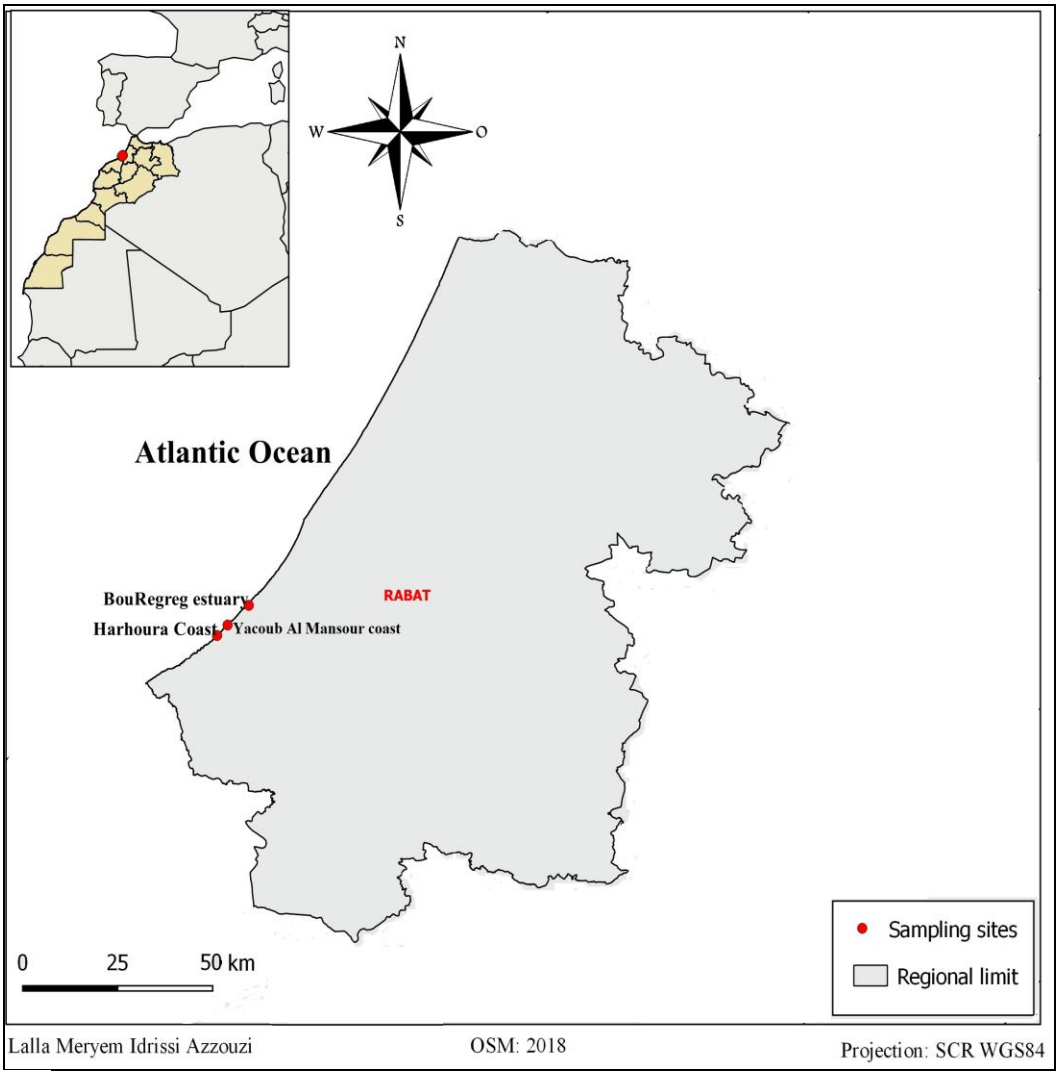


Figure 32: Map of Morocco coast showing the sampling sites

2.2 Samples preparation

The shells were opened aseptically; the digestive system was dissected with a sterile knife allowing the elimination of inhibitors tissues (polysaccharides, sexual gonads). To analyse a larger number of individuals 1.5 g of hepatopancreas were used with a weight corresponding to an analysis, representing on average 12 mussels.

a. Virus recovery from mussels samples

Two hundred and eighty-eight samples of mussels were collected from three stations of Rabat Region in Morocco. Mussels samples (1.5 g of hepatopancreas) were added to 10 mL of buffer (0.1 M glycine: 0.3 M NaCL) at a pH = 9. The mixture was homogenized for 15 min then centrifuged at 10 000 g for 10 min at 4°C. The pellet was resuspended in 5 mL of phosphate buffered saline (PBS) and adjusted to pH = 7.

The mixture was homogenized again and centrifuged at 10 000 g for 30 min at 4°C. The supernatant was used for virus detection.

b. Concentration of virus suspensions

Virus particles recovered from mussel samples by precipitation with polyethylene glycol (PEG) 6000 at 50% as previously described (**El-Senousy et al., 2013; Idrissi Azzouzi et al., 2017a**). In brief, suspensions were mixed with 25% (V⁄V) PEG 6000 and incubated at 4°C overnight. The mixtures were then centrifuged at 10 000 g for 30 min. The final pellet was resuspended in 5 ml of 0.1 M phosphate buffer pH 7.2 and then filtered through a 0.22 µm Millex-GS membrane. To prevent the contamination of the concentrate, it was necessary to add to the mixture 30 µL of antibiotics (Penicillin 10 000 U/mL and Streptomycin 10 000 µg/mL) and 20 µL of Fungizone (250 µg/mL). The suspension was either treated immediately or stored at -20°C until use.

c. Typing of non *Poliovirus* enteroviruses with antiserum pools

Poliovirus strains have been identified by molecular method (real-time PCR) according to the procedures recommended by WHO (**Idrissi Azzouzi et al., 2017a**).

The identification of non *Poliovirus* enteroviruses serotypes (serotyping of NPEV) by the seroneutralization test was done using pools of antiserum prepared and provided by the National Institute of Public Health and the Environment (RIVM) (**WHO, 2011**).

Each box of RIVM enteroviruses typing antiserum contains anti-enterovirus pools A, B, C, D, E, F and G, anti-Coxsackievirus B pool and a trivalent anti-*Poliovirus* pool (**Figure 33**). These pools must be diluted before use. The recommended dilution for all pools is 0.5 mL of each pool is added to 9.5 mL of maintenance medium (minimum essential medium MEM″ with HEPES and 2% FBS). Aliquot pools into clearly labelled cryovials in 1 mL volumes and store at -20°C. Each unknown virus was tested in duplicate against a trivalent-pooled polio

antiserum (PP), a Coxsackievirus B1 to B6 pool (CP), and seven pools against Coxsackievirus A9 and 20 echoviruses (A-G) (**Figure 33 and Table 13**). Non *Poliovirus* enteroviruses that fail to be identified using this antiserum may be in an aggregated form that interferes with the complete neutralization by specific antiserum. Isolates can be retested after emulsification with chloroform (approximately 10% by volume) and separation of the supernatant.

The virus suspension to be used in the seroneutralization tests was prepared by the inoculation of cultures of the specified cells. After inoculation, the cultures were examined daily for cytopathic effect (CPE). Complete destruction of the cells within 3 days is preferable, and if this was not obtained initially, a further passage should be made. When destruction is complete, the cultures are frozen, then rapidly thawed, and harvested. This harvest forms the stock virus suspension for all the seroneutralization tests and is stored at -20°C until required.

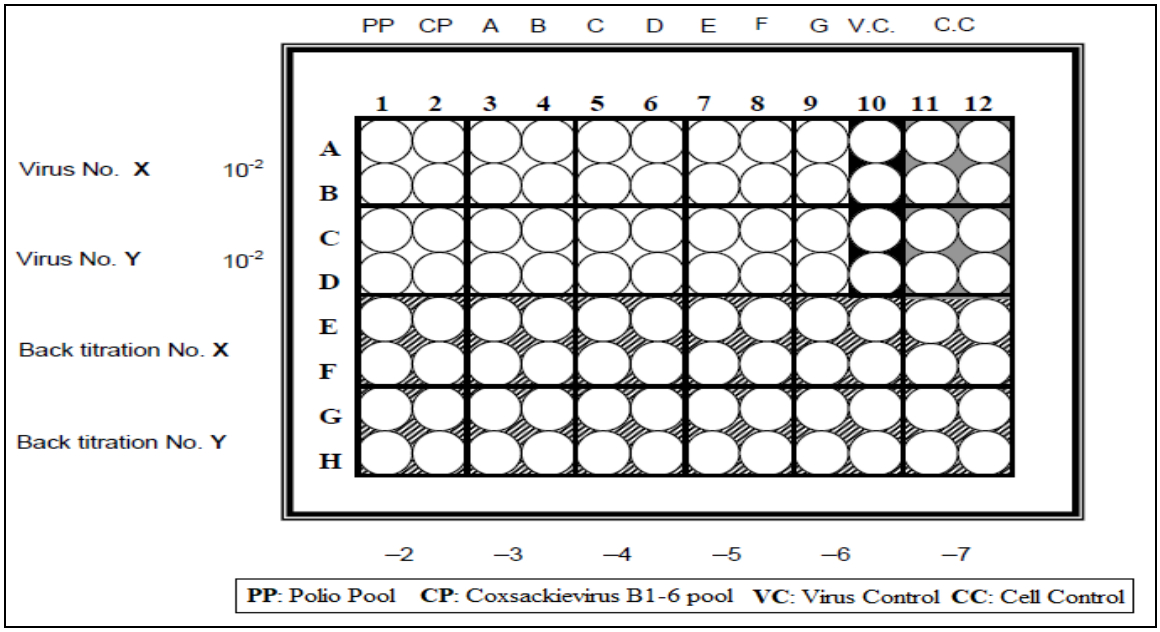


Figure 33: Plate set-up for identification of *Enterovirus* isolates using seroneutralization

Because a large number of viruses makes it impractical to perform individual neutralization tests, these have been pooled in an overlapping scheme that allows many viruses to be identified using as few as nine tests. Interpretation of the results was done with the assistance of a list of the neutralization patterns of individual viruses (**Table 13**).

Table 13: Association of Antiserum pools (A-G) for Non *Poliovirus* enteroviruses typing by seroneutralization

	A	B	C	D	E	F
A		***Echovirus* 4**	***Echovirus* 7**	***Echovirus* 11**	***Echovirus* 14**	***Echovirus* 9**
B	*Echovirus* 4		*Coxsackievirus* A9	***Echovirus* 1**	***Echovirus* 27**	***Echovirus* 3**
C	*Echovirus* 7	*Coxsackievirus* A9		***Echovirus* 21**	***Echovirus* 22**	***Echovirus* 2**
D	*Echovirus* 11	*Echovirus* 1	*Echovirus* 21		***Echovirus* 20**	***Echovirus* 12**
E	*Echovirus* 14	*Echovirus* 27	*Echovirus* 22	*Echovirus* 20		***Echovirus* 33**
F	*Echovirus* 9	*Echovirus* 3	*Echovirus* 2	*Echovirus* 12	*Echovirus* 33	
G	***Echovirus* 6**	***Echovirus* 25**	***Echovirus* 5**	***Echovirus* 30**	***Echovirus* 29**	***Echovirus* 13**

3. Results

The virological analysis (cell culture) of 288 samples collected from Bouregreg estuary, Yacoub Al Mansour and Harhoura coast, showed that 75% of mussels (*Mytilus galloprovincialis*) were contaminated by enteroviruses, with 204 strains (70.8%) of non *Poliovirus* enteroviruses (NPEV) and 12 strains (4.2%) of *Poliovirus* Type 1 (SL1) which was confirmed by real-time PCR using intratypic differentiation (ITD) method.

From the isolates of NPEV were obtained on RD cell lines, 204 of these strains were serotyped by seroneutralization using pools of antiserum, however only 132 strains (64.7%) could be identified, against 72 non-typable strains (35.3%). Among these typable strains 72.7% (96/132) have been determined as *Coxsackievirus* B and 27.3% (36/132) as *Echovirus*. The different serotypes of *Coxsackievirus* B and Echovirus could be identified as 62.5% of *Coxsackievirus* B5 (60 strains), 37.5% of *Coxsackievirus* B3 (36 strains), and 100% of *Echovirus* 6 (36 strains).

4. Discussion

This study revealed the circulation of an important number of typable strains (64.7%) and non-typable strains (35.3%) in the marine environment. However, non-typable strains confirm the presence of new serotypes. A seroneutralization test was used for the identification of enteroviruses in tissue culture with composite antiserum pools. This antiserum with twenty-seven enteroviruses was included in the pools that were used to examine 204 of non *Poliovirus* enteroviruses that consist typed and untyped strains. The results indicate that this method provides a useful screening method for identifying enteroviruses. It has proved to be practicable, time-saving and very economical in tissue culture.

The analysis of viruses in environmental samples is complex. There are a number of issues to consider. Primary isolates of many viruses of interest grow poorly, if at all, in cell culture systems (**Duizer et al., 2004; Straub et al., 2007; Tanaka et al., 2007; Cromeans et al., 2008**). This is further compounded by the fact that major enteric viruses are present in low numbers in the environment, and have been shown to have an infectious dose ranging from 1 to 100 particles. Therefore, a method must concentrate low levels of viral particles and eliminate any inhibitory substances that could interfere with the analytical process (**Brundage and Fitzpatrick 2006; Teunis et al., 2008**). For this reason, it is important to develop methods sensitive enough to detect a single viral particle per sample. In addition, some of the important enteroviruses have a high degree of genetic and antigenic variability (**Kageyama et al., 2004; Matthijnssens et al., 2008; Zheng et al., 2006**). Therefore, to monitor viral contamination in marine environments, the use of molecular techniques targeting certain regions of the genome and the phylogenetic analysis of nucleotide sequences are recommended. These techniques will make it possible to identify new serotypes while ensuring the characterization of non-typable strains. Molecular tests will also, by the determination of recombinant strains explain the genetic evolution of NPEV strains.

The prevention of diseases caused by non *Poliovirus* enteroviruses such as Coxsackievirus B and Echovirus requires the identification of viral contamination sources and the development of effective intervention strategies and decontamination procedures for shellfish and aquatic ecosystem (**Morley, 2010**).

The prevention of diseases caused by non *Poliovirus* enteroviruses such as Coxsackievirus B and Echovirus requires the identification of viral contamination sources and the development of effective intervention strategies and decontamination procedures for shellfish and aquatic ecosystem (**Morley, 2010**).

Comparing the occurrence of viral pathogens in shellfish is difficult since few data are available in the literature and conditions are always different including site conditions, sampling, and detection methods. However, this study can be compared with a previous study conducted to analyse viral contamination in mussel samples collected from sites occasionally impacted with sewage (**Sdiri et al., 2004; Elamri and Aouni, 2005; Elamri et al., 2006; Karamoko et al., 2005; Karamoko et al., 2006a, b; Sdiri et al., 2006; Gabrieli et al., 2007; Bosch et al., 2008; Benabbes et al., 2013a, b; Bou m'handi, 2015; Idrissi Azzouzi et al.,**

2017a). These results allow some conclusions and comments, as many of the collected samples have been found contaminated with human enteric virus particles.

5. Conclusion

The identification of isolated non *Poliovirus* enteroviruses becomes indispensable, as it is important to know the new serotypes associated with diseases. Thus, the surveillance of enteroviruses circulation cannot be limited to the only surveillance of interhuman circulation and should include monitoring of enteroviruses in the marine environment.

The mean number of positive samples in this study is in accordance with data found in the literature, indicating that viral contamination of molluscs is similar among countries investigated which reflects the epidemiological status of the population. Our results confirm that mussels in Morocco were contaminated with several enteroviruses. Therefore, to protect human health worldwide, research should be dedicated to better understand virus circulation and to develop appropriate monitoring in all shellfish producing countries. This will be helpful to understand virus circulation and to improve seafood safety.

CHAPTER III: CONTAMINATION LEVELS OF METALS (Cu, Cr, Cd, and Pb) in *Patella rustica* FROM THE MOROCCAN ATLANTIC COAST

ABSTRACT

This study was carried out to determine the levels of some metal including copper (Cu), chromium (Cr), cadmium (Cd) and lead (Pb) in the soft tissues of mollusca gastropoda limpet (*Patella rustica*), using the standard Atomic Absorption Spectrophotometry (AAS) technique after mineralization method. These samples were collected from three different locations (Yacoub Al Mansour, Harhoura and Guy ville) of Moroccan Atlantic coast during four seasons (winter, spring, summer and autumn). The average ranges of elements concentrations measured in samples were: Yacoub Al Mansour coast (Cu: 0.46- 2.19 µg/g; Cr: 0,60-2,21 µg/g; Cd: 0,32-1,06 µg/g and Pb: 0,47-1,30 µg/g), Harhoura coast: (Cu: 0.81- 3.17 µg/g; Cr: 0,94-2,5 µg/g; Cd: 0,47-0,95 µg/g and Pb: 0,76-1,42 µg/g) and Guy ville coast (Cu: 1.24-4.14 µg/g; Cr: 0,87-3,98 µg/g; Cd: 0,56-1,18 µg/g and Pb: 1,08-2,13 µg/g). During all seasons and from different sampling sites, the results obtained indicate that metals concentration in soft tissues of *Patella rustica* were distributed differently.

Keywords: Contamination assessment, *Patella rustica*, gastropod molluscs, metals, Moroccan Atlantic coast.

1. Introduction

Environmental pollution by metals is constantly increasing. Therefore, the environmental protection has become a currently major concern for every country in the world. The metal elements are considered as critical contaminants in the environment, due to their high potential to enter and transfer in food chains and can be accumulated in the soft tissues of different marine organisms like shellfish. Subsequently, this accumulation establishes a great danger for the human consumption when the concentration exceeds certain threshold of acceptability (**Bradl, 2005; He et al., 2005; Tchounwou et al., 2012**).

The Moroccan Atlantic coast extends for about 3500 km. It has considerable biological diversity and fish production (**Snoussi et al., 2008; Chaibi and Sedrati, 2009**). This coastline is threatened with several types of pollution. Indeed, many chemical pollutants including metal elements and organic contaminants are rejected in this coastal system by industrial, agricultural and anthropogenic activities (**Maanan, 2008; Maanan et al., 2015**). Metals such

as cadmium (Cd), lead (Pb), chromium (Cr), mercury (Hg), copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni) were among these rejections and constitute a group of the most important chemical pollutants. These elements cause a considerable harm to the environment (**Bradl, 2005; Tchounwou et al., 2012; Ben Aakame et al., 2014**).

Metals are naturally occurring elements in the environment that have a relatively high atomic weight and density compared to water. Their concentrations in the marine environment are the results of both natural sources and anthropogenic activities (**Glasby et al., 2004; Maanan et al., 2004; Singh et al., 2011; Tchounwou et al., 2012; Wani et al., 2017**). The accumulation of metal elements in the aquatic environment affects various organisms (**Baby et al., 2010**). These chemical agents are divided in essential elements that are required to support biological activities and non-essential metals with an unknown biological function. The latter being toxic for organisms when subject to high concentrations, it induces a multiple organ damage even at lower levels of exposure (**Tchounwou et al., 2012; Jaishankar et al., 2014**).

Gastropods molluscs are filter feeders and thus obtain heavy metals from water, food and ingestion of inorganic materials (**Singh et al., 2014**). It is well known that these organisms accumulate metallic and organic pollutants in their soft tissues responding essentially to the fraction existing in the marine environment (**Bergasa et al., 2007**). In this study, gastropods were employed as bioindicator to determine the effect of marine pollution. These organisms were considered as appropriate indicators since they are available all the year, present in almost all coastal areas and easily collected (**Yuzereroglu et al., 2010; Richir and Gobert, 2016**). Linnaeus in 1758 described the taxon *Patella rustica* from the morphological characters in order to identify this species collected from an unknown locality (**Pinto et al., 2010**).

Patella rustica and other gastropods are frequently used as sentinel organisms in monitoring programs in coastal environments due to their ability to accumulate metal elements in their soft tissues (**Bergasa et al., 2007**). In this research, we examined the concentrations level of Cu, Cr, Cd and Pb in the soft tissues of *Patella rustica* during four seasons from three different sites of Moroccan Atlantic coast in Rabat Region.

The aim of this study was to assess the contamination level of metals in the aquatic environment and evaluate the public health risk associated with consumption of contaminated gastropod molluscs.

Data obtained were used in the future to assess the toxicological risk due to the consumption of *Patella rustica*. Thus, four metals (copper, chromium, cadmium and lead) were selected for contamination assessment of *P. rustica*, as well as a comparison between the metals concentration detected in their soft tissues during four seasons.

2. Material and methods

2.1 Study Area

The sampling sites were situated in Rabat Region of Morocco (**Figure 34**). This region covers an area of 18.194 km^2, with a population around 4.581.000. This area belongs to the Mediterranean climate marked by two main seasons softened by oceanic influences. The average temperatures are around 22°C for the warmer months (July to September) and 12°C for the colder months (December and January). Concerning the annual rainfall is in average more than 550 mm/year (**Idrissi Azzouzi et al., 2017**).

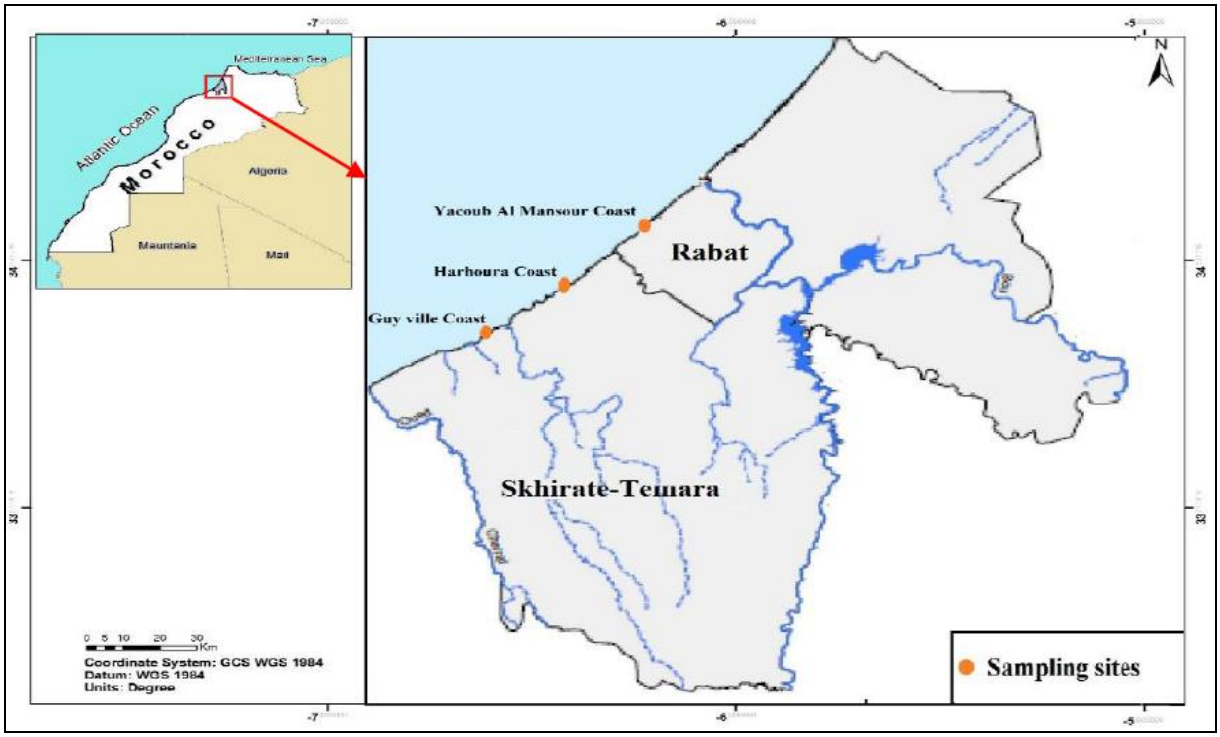


Figure 34: Map of Morocco coast showing the sampling site

2.2 Samples collection

Between February 2015 and February 2016 a total of 120 shellfish of *Patella rustica* common name rustic limpet were collected monthly and seasonally, from three wild populations (Yacoub Al Mansour, Harhoura and Guy ville coast) of Moroccan Atlantic coast in Rabat Region. These locations receive large quantities of untreated or partially treated domestic wastewater.

The samples were collected by scalpel at rocky shores of three different intertidal locations from the Rabat. The specimens collected were an onsite waste, stored ithylene bags and frozen at -20°C until metal analysis was conducted.

2.3 Samples preparation

The sample was partially thawed at room temperature, the soft (edible) parts were dissected separately from the shells using a clean stainless steel scalpel and then washed with deionized water. The samples washed were drained in an incubator at 65°C for 48 h until complete drying and then ground using porcelain mortar. For this purpose, the dried samples were prepared to measure their concentrations.

Several studies have been based on the analysis of heavy metals in the storage organs (liver, kidney, spleen). However, these organs can accumulate very strongly a metal, without being significant in the surrounding environment; therefore, in order to assess the risks incurred by the consumers of gastropod molluscs such as *Patella rustica*, we analysed primarily the edible parts that mean all the soft tissues (**Nakhle, 2003; Santhiya et al., 2013; Rodrigue et al., 2016).**

2.4 Mineralization

In total, ten samples from each location in each season means forty samples in every station. The samples were analysed in order to determine copper (Cu), chromium (Cr), cadmium (Cd), and lead (Pb) in the soft tissues of *Patella rustica*. This determination was carried out after mineralization of the samples.

In the clean digestion bombs containing the soft tissues of *Patella rustica*, 4 mL of nitric acid (HNO_3) 65% (Merck) and 1 mL of oxygenated water (H_2O_2) 30% (Merck) were added. The samples were kept overnight for predigesting, the next day they were placed in sand bath at 120°C for 24 h until the solution became clear. After cooling, we diluted our samples to reach a final volume of 30 ml with ultra-pure water appropriately in the range of standards that were prepared from standard solution of the metals (**Conti et al., 2003; Kelepertzis et al., 2013**).

2.5 Analysis of the metallic trace elements (MTE)

2.5.1 Determination

After dilution, the metal concentrations in the soft tissues of samples were measured by Atomic Absorption Spectrophotometry with graphite furnace (AAS-GF) type (Varian 240 Zeeman) and presented as microgram metal/gram dry weight. The instrument was calibrated with metal standard solutions (1g/L) prepared by dilution (**Cravo et al., 2005; Collado et al., 2006; Nakhle et al., 2006; Bergasa et al., 2007**).

The accuracy and precision of this methodology were tested by using a separate comparative study of a standard reference material (**IAEA-MEL, 2016-01-TE**). The agreement between the results for the reference biological material certified values was satisfactory and proving a good repeatability of the method (**Table 14**). The recovery values of metals analysis were between 85% and 96% and the analytical methods were carried out using triplicate samples.

Table 14 : Analysis of certified reference material (IAEA-MEL, 2016-01-TE): certified values and found values (means ± relative standard deviation) (in µg/g of dry weight)

Metals	Certified	Found
Cu	2,860±0,300	2,490±0,270
Cr	7,830±1,100	7,750±1,090
Cd	0,033±0,004	0,030±0,001
Pb	0,648±0,074	0,587±0,037

2.6 Statistical methods

The concentrations (mean and standard deviation) of heavy metals (Cu, Cr, Cd and Pb) were calculated for overall registered data. The standard deviations refer to the variability within

different replicates. In order to verify the differences in the heavy metal levels in *Patella rustica* during the four seasons a non-parametric statistical method (the Student's t-test) was used for determination of significant differences ($p < 0.05$).

The Mann-Whitney U test was conducted to test the significance of the differences in the metal content between the three sampling sites. The level of significance was set at $P < 0.01$. All analysis was performed using the package R version 3.4.1 software.

3. Results and discussion

In Morocco, the bivalve molluscs such as *Mytilus galloprovincialis* were already used to study the chemical contamination. However, up until now, no study has been undertaken to assess the metal contaminations in the gastropod molluscs (*Patella rustica*). The results obtained showed that the average concentrations of the metals in soft tissues of *Patella rustica* from all stations varied significantly and decreased in the order of [Cu]>[Cr]>[Pb]>[Cd]. The average ranges were as follows: Yacoub Al Mansour coast (Cu: 0.46-2.19 µg/g; Cr: 0,60-2,21 µg/g; Cd: 0,32-1,06 µg/g and Pb: 0,47-1,30 µg/g), Harhoura coast: (Cu: 0.81-3.17 µg/g; Cr: 0,94-2,5 µg/g; Cd: 0,47-0,95 µg/g and Pb: 0,76-1,42 µg/g) and Guy ville coast (Cu: 1.24-4.14 µg/g; Cr: 0,87-3,98 µg/g; Cd: 0,56-1,18 µg/g and Pb: 1,08-2,13 µg/g). From different sampling sites, the results of the present study revealed that metal concentrations in the soft tissues of *Patella rustica* were distributed differently (**Table 15 and Figure 35**).

Table 15: Mean concentrations ± relative standard deviation (RSD) values (µg/g of dry weight) of metals in *Patella rustica* from different stations during four seasons

Elements	Stations	Seasons			
		Spring	Summer	Autumn	Winter
Cu	Yacoub Al Mansour coast	1,18±0,05	1,08±0,03	1,06±0,05	1,18±0,06
	Harhoura coast	1,47±0,02	2,92±0,14	1,01±0,03	1,26±0,02
	Guy ville coast	1,41±0,01	5,51±0,45	1,41±0,04	0,93±0,06
Cr	Yacoub Al Mansour coast	1,26±0,06	1,46±0,04	0,88±0,02	1,02±0,03
	Harhoura coast	1,63±0,07	1,02±0,02	1,56±0,04	1,47±0,02
	Guy ville coast	1,83±0,09	2,96±0,16	1,68±0,06	0,98±0,02
Cd	Yacoub Al Mansour coast	0,43±0,01	0,90±0,04	0,32±0,01	0,40±0,01
	Harhoura coast	0,87±0,03	0,79±0,02	0,56±0,03	0,87±0,03
	Guy ville coast	0,96±0,07	1,07±0,02	0,60±0,01	0,92±0,04
Pb	Yacoub Al Mansour coast	0,50±0,01	1,27±0,01	0,73±0,03	0,86±0,02
	Harhoura coast	0,97±0,03	1,01±0,02	0,93±0,04	0,97±0,05
	Guy ville coast	1,16±0,06	1,97±0,08	1,19±0,07	1,01±0,02

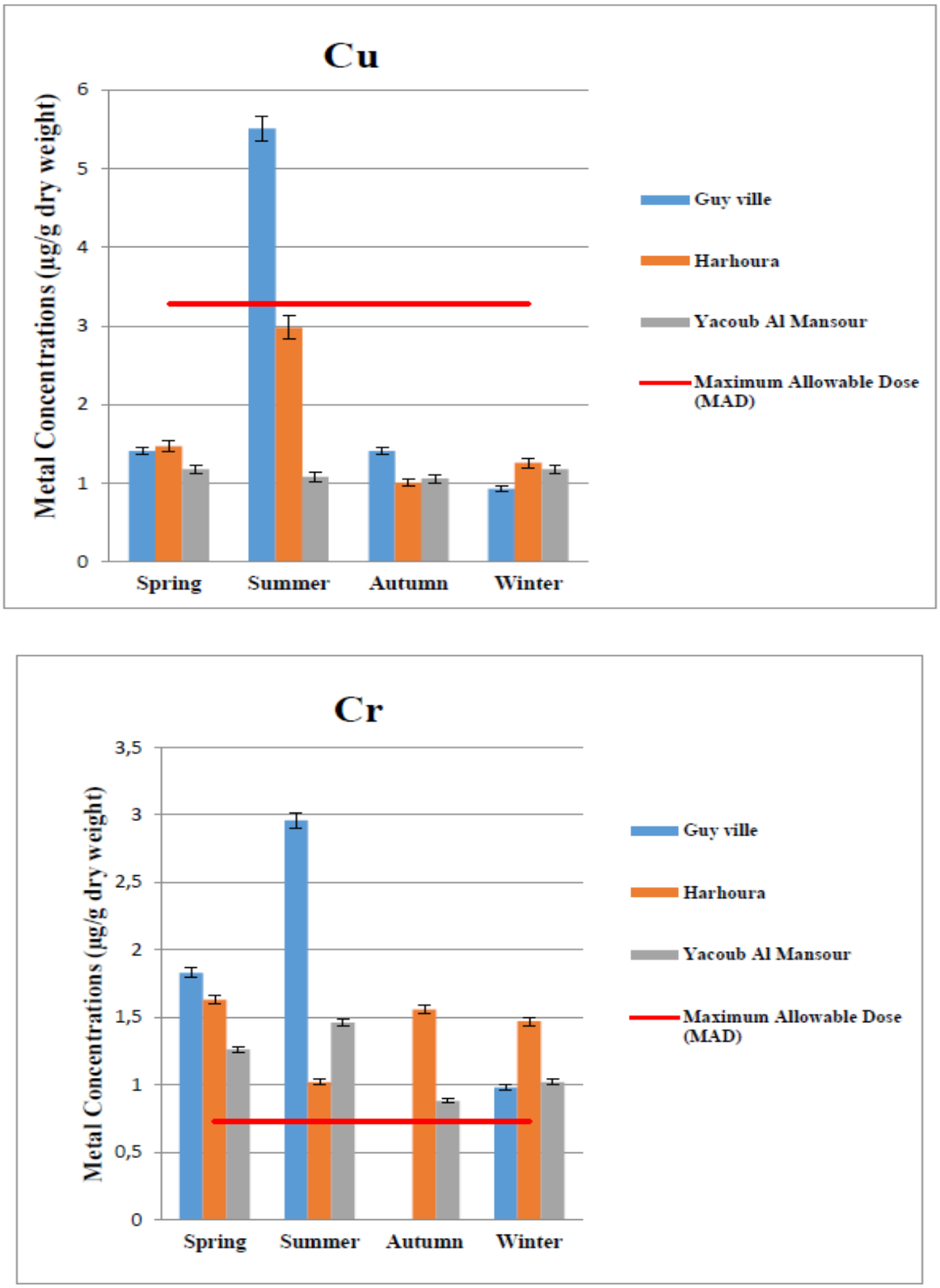
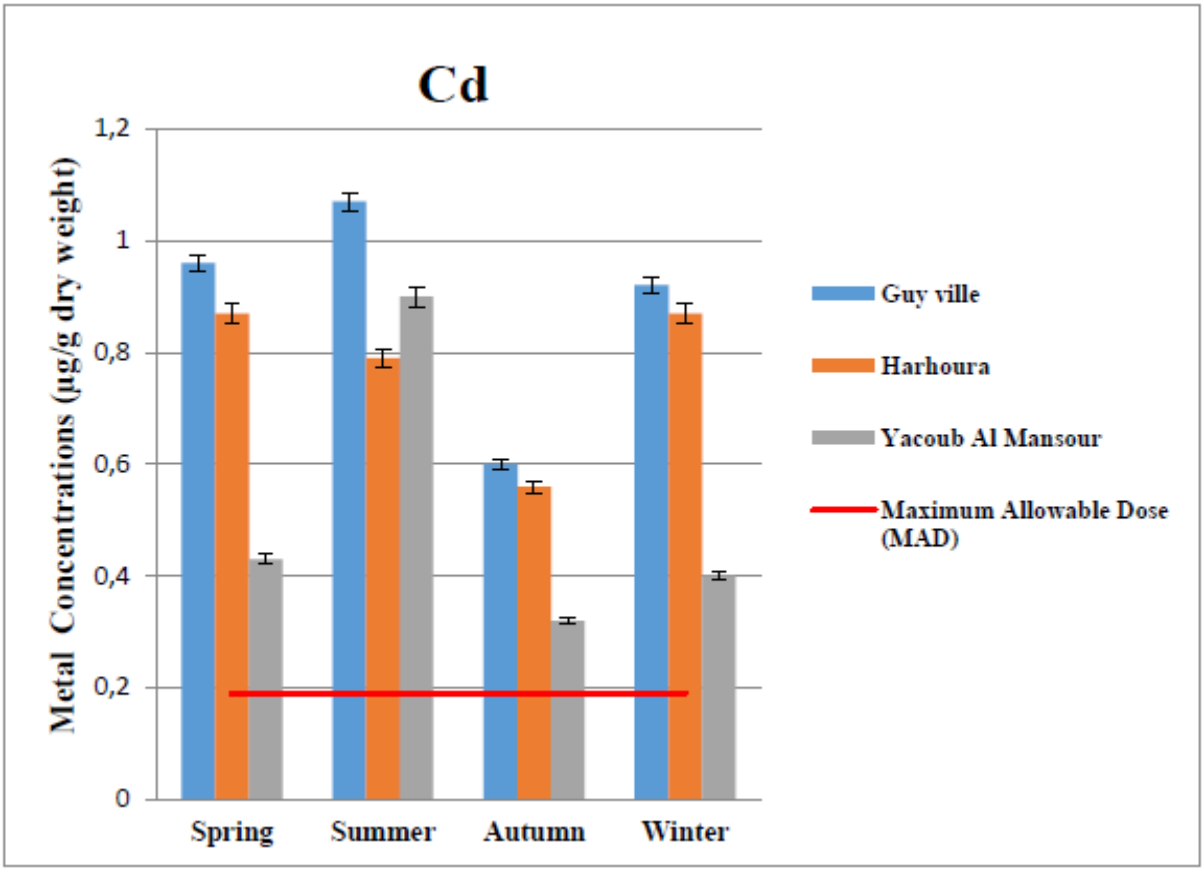


Figure 35 : Mean concentrations (µg/g dry weight) of Cu and Cr in *Patella rustica* at the different sites during four seasons with Maximum Allowable Dose



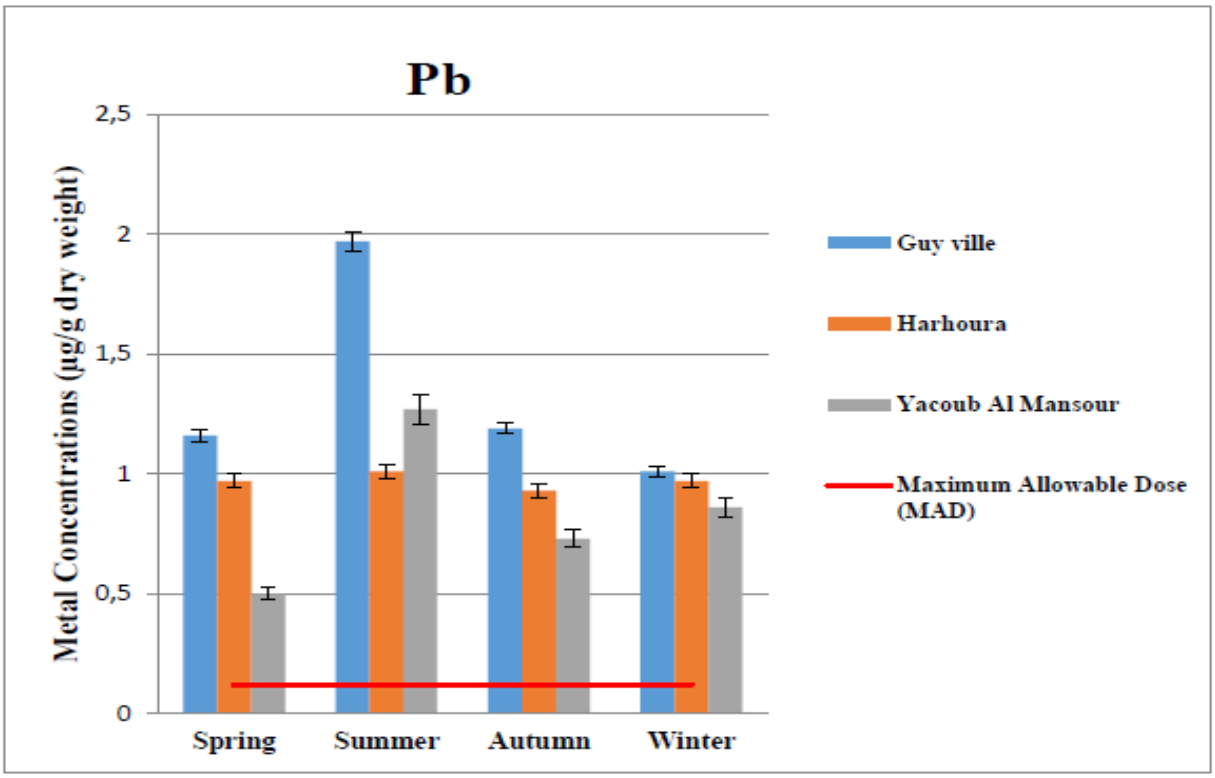


Figure 36 : Mean concentrations (µg/g dry weight) of Cd and Pb in *Patella rustica* at the different sites during four seasons with Maximum Allowable Dose

The mean concentrations (in µg/g dry weight) of copper (Cu), chromium (Cr), cadmium (Cd) and lead (Pb) of the marine gastropod *Patella rustica* at the different sampling sites are summarised in **Table 15** during four seasons. The comparison between metal elements concentrations in the samples from the three stations showed all metal concentrations differed significantly in every season (**Table 15 and Figure 35**).

The ranges of elements concentrations measured (in µg/g dry weight) found in different stations and seasons were: Cu (1,71±0,07 µg/g), Cr (1,48±0,05 µg/g), Cd (0,72±0,03 µg/g) and Pb (1,05±0,04 µg/g).

The seasonal changes for all metal concentrations in the soft tissues of *Patella rustica* from sampling stations were observed in this work. Moreover, the high concentrations of Cu, Cr, Cd and Pb were detected in summer and spring (**Table 15 and Figure 35**). Generally high chemical pollution occurred during the summer and spring months (**Duysak and Ersoy, 2014; Duysak and Azdural, 2017**).

As shown in **Table 15 and Figure 35** the levels of Cu and Cr have been found to be highest in all soft tissues in every season while Cd and Pb have been found at lower levels. Cu and Cr are essential elements and have important roles in growth, cell metabolism, and survival of most animals including gastropod molluscs. Therefore, the relatively high levels of these metals can be attributed to their essentiality (**Mitra et al., 2012**). Other metals have an unuseful role in human physiology such as cadmium, lead, arsenic and mercury (**Tchounwou et al., 2012; Jaishankar et al., 2014**). The results obtained with the Student's t-test showed a significant ($p < 0,05$) variation of the Cu, Cr, Cd, and Pb concentrations in *Patella rustica* during the four seasons.

Metals may be at certain concentrations, toxic to marine organisms even at low levels of exposure and persistent in the aquatic environment. In addition, it can cause diseases, cancers, severe reproductive damage and negatively affect biologically useful metals such as zinc and calcium (**Singh et al., 2011; Tchounwou et al., 2012; Jaishankar et al., 2014**). The recommended maximum allowable dose (MAD) of metals concentration in edible gastropod molluscs (such as *Patella*) as follow (3,28 µg/g for Cu; 0,73 µg/g for Cr; 0,19 µg/g for Cd and 0,12 µg/g for Pb) (**IAEA-407, 2003-09-01**).

The copper (Cu) surplus has been associated with liver damage (**Collado et al., 2006**). The chromium (Cr) is considered as a human mutagen and probably carcinogen. Prolonged exposure to Cr may cause damage to the liver and kidneys (**Velma et al., 2009**). The cadmium (Cd) is known to be toxic even at low concentrations and is also considered as a probably carcinogen; Cd can also affect in bone fracture, arthritis, diabetes, anaemia, hypertension, cardiovascular disease, cirrhosis, reduced fertility, headaches, strokes, kidney dysfunction and even cancer (**Cravo et al., 2005; Collado et al., 2006**). The lead (Pb) is known to be mutagenic and carcinogenic. It induces renal tumours and disturbs the normal functioning of joints, nervous and reproductive systems (**Cravo et al., 2005; Collado et al., 2006**).

In this study, we report the concentrations of Cu, Cr, Cd, and Pb in the soft tissues of Patellidae from three different stations. The results obtained with the Mann-Whitney U test clearly demonstrate a significant ($p < 0,01$) spatial variations of the Cu, Cr, Cd, and Pb concentrations. The highest metal levels were registered in Guy ville coast, followed by Harhoura coast and at last Yacoub Al Mansour coast (**Table 16 and Figure 36**).

Table 16 : Mean concentrations ± relative standard deviation values (µg/g of dry weight) of metals in *Patella rustica* from different sites

Elements	**Stations**		
	Yacoub Al Mansour coast	**Harhoura coast**	**Guy ville coast**
Cu	1,13±0,05	1,67±0,05	2,32±0,11
Cr	1,16±0,04	1,42±0,03	1,86±0,08
Cd	0,51±0,02	0,77±0,03	0,89±0,04
Pb	0,84±0,02	0,97±0,04	1,33±0,06

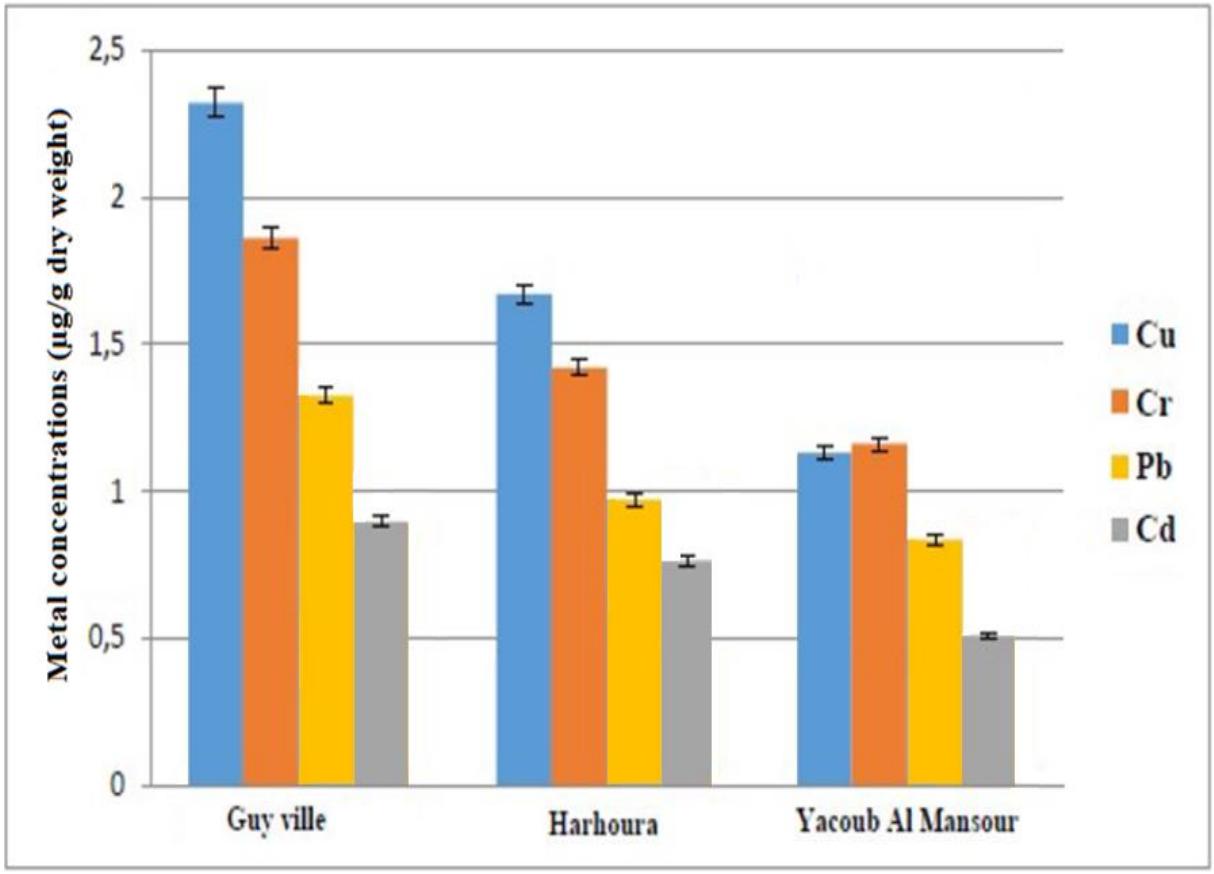


Figure 37: Mean concentrations (µg/g dry weight) of metal elements in *Patella rustica* at the different sites

The limpet *Patella rustica* is filter feeding organism. Therefore, their ability to accumulate metal elements in their soft tissues is well known. This accumulation responding essentially to the fraction of metal present in the aquatic environment gives direct indication of the ecotoxicological level of the pollution.

The comparison of metal concentrations with corresponding values measured in species of *Patella* spp. from various geographical sites is shown in **Table 17**. Such a comparison will additionally provide more useful information on the contamination level of marine ecosystems. The Cu, Cd and Pb concentrations from Yacoub Al Mansour, Harhoura and Guy ville coast of Moroccan Atlantic coast are quite similar to those obtained by **Collado et al. 2006, Bergasa et al., 2007 and Yuzereroglu et al., 2010** from polluted areas, located at Canary Islands (Spain) and Iskenderun Gulf (Turkey). The concentration of copper and chromium fall within the highest available data values in the literature.

Table 17: Comparison of metals concentration (µg/g of dry weight) for *Patella* spp. obtained in this study and from different other geographical areas

Species	Sites	Mean concentrations ± RSD (µg/g dry weight)				References
		Cu	Cr	Cd	Pb	
Patella rustica	Yacoub Al Mansour coast (Morocco)	1,13± 0,05	1,16±0,04	0,51±0,02	0,84±0,02	This work
	Harhoura coast (Morocco)	1,67± 0,05	1,42±0,03	0,77±0,03	0,97±0,04	
	Guy Ville coast (Morocco)	2,32± 0,11	1,86±0,08	0,89±0,04	1,33± 0,06	
Patella rustica and piperata	Canary Islands (Spain)	2.05 ± 0.91	-	0.36 ± 0.26	1.57 ± 1.14	(Bergasa et al., 2007; Collado et al., 2006)
		1.77 ± 0.09	-	0.37 ± 0.05	1.27 ± 0.07	
Patella caerulea	Gulf of Annaba (Algeria)	4,56±0,69	15,88±0,59	-	-	(Boumaza, 2014)
	Iskenderun Gulf (Turkey)	2,31±0,09	-	0,44±0,03	0,30±0,03	(Yuzereroglu et al., 2010)
	Gulf of Suez (Egypt)	6,34±1,96	4,68±1,43	1,38±1,12	6,2±1,75	(Hamed and Emara, 2006)
	Gulf of Gaeta, Tyrrhenian Sea (Italy)	14,3±3,43	0,85±0,23	3,54±0,78	0,95±0,20	(Conti and Cecchetti, 2003)
Patella vulgata	Lebanese Coastal (Lebanon)	-	-	2,16±0,26	1,55±0,34	(Nakhle et al., 2006)
Patella aspera	South coast (Portugal)	6,3±1,72	-	3,5±0,57	-	(Cravo and Bebianno, 2005)

In this work, the presence of high Cu, Cr, Pb and Cd concentrations in limpet samples from Yacoub Al Mansour, Harhoura and Guy ville coast should be attributed to domestic discharges, the waste incineration and the exhaust gas of the vehicles. All these factors may have affected the *Patella rustica*, which indicate the susceptibility of this specie to the chemical pollution in the study area (Moroccan Atlantic coast).

The metal concentrations in limpet depend on both the species and the physiological state of the organism (**Nakhle, 2003**). Indeed, the real mechanism of metals in *Patella* was

complicated and depends on factors directly correlated with weight, sexual cycle, temperature, food abundance and the bioavailability of metals in the environment (**Nakhle, 2003; Storelli and Marcotrigiano, 2005; Bergasa et al., 2009**).

4. Conclusion

Patellidae is commonly consumed seafood in many countries. Therefore, the investigation of metal elements concentrations in the soft tissues of this species may provide useful information on the transfer of potentially toxic elements from aquatic environment. The usefulness of gastropod molluscs as bioindicators for the detection of metal pollution is confirmed. However, the effect of some variables on metal concentrations in gastropod molluscs may be different, according to the sampling period and to the level of metal pollution of the area under investigation.

The results of this study conclude that *Patella rustica* are seriously polluted by metal elements, especially from Cu, Cr, Pb and Cd. The most important source of pollution in these sampling sites was the anthropogenic activities and the road traffic.

The data obtained from this study revealed the necessity to establish a monitoring system to control the consumption of *Patella rustica* from the Moroccan Atlantic coast and to evaluate the toxicological risk related to the contamination of this species.

Patella has a considerable potential as cosmopolitan biomonitors of metals in the aquatic environment. This sedentary species is available in every season all over the coastal area and is easy to sample and identify. Finally, it is recommended that *Patella rustica* are suitable to be used as a successful bioindicator of metal pollution caused by different human activities in this coastal area of the Moroccan Atlantic coast and should be controlled before their consumption.

Further studies of metal levels in gastropod molluscs from different sampling sites of the Moroccan Atlantic coast are required including the investigation of the possible effects of seasonal changes on metal concentrations and distribution.

CHAPTER IV: THE RELATIONSHIPS BETWEEN METAL (Pb, Cu, Cd, and Cr) LEVELS AND THE SIZE OF THE MOROCCAN ATLANTIC COAST GASTROPOD SPECIES (*Patella rustica*)

ABSTRACT

The metal (Pb, Cu, Cd, and Cr) concentrations in the soft tissues of the gastropod mollusc limpet (*Patella rustica*) from the Moroccan Atlantic coast were measured. The relationships between limpets size and metal levels were investigated by linear regression analysis.

This work was conducted to determine the bioaccumulation and relationships of some essential (Cu and Cr) and non-essential (Pb and Cd) metals in the soft tissues of *Patella rustica* by calculating the correlation analysis.

For this study, 120 limpets (*Patella rustica*) were collected from three different locations in the purpose to analyse the levels of metals by using the standard Atomic Absorption Spectrophotometry (AAS). In soft tissues of *Patella rustica*, the metal concentrations (ng/mg dry weight) decrease in the following order: Cu (4.14) > Cr (3.98) > Pb (2.13) > Cd (1.18).

The results of the linear regression analysis confirmed that in all samples the relationships between metal concentrations and limpets size were significant. Moreover, the smaller size of limpets showed higher concentrations (ng/mg dry weight) of Cd, Pb, Cu, and Cr than the larger ones. However, it was found that the tissue of *Patella rustica* has the potential to be used as a biomonitoring agent for the metal contamination in gastropod molluscs, as indicated by the significant correlation between metal concentrations (Pb, Cu, Cd, and Cr) in the soft tissues of limpets and their size.

Keywords: Metals, *Patella rustica*, length, weight, bioaccumulation, Moroccan Atlantic coast.

1. Introduction

The term "heavy metals" is often used as a group name for metals that have highly toxic or ecotoxic properties. They are considered as serious contaminants in the environment, due to their high potential to enter and accumulate in food chains (**Duffus 2002; Begum and Sehrin 2013; Kouddane et al. 2016; Hazrat et al. 2019**). Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of

public health significance (**Boyd 2010; Jakimska et al. 2011; Tchounwou et al. 2012; Ayangbenro and Babalola 2017**).

The gastropod mollusc, *Patella rustica* was chosen because they are often considered as a keystone species on rocky shores and several studies have demonstrated that limpet has the ability to accumulate metals in their soft tissues responding essentially to the fraction present in the environment, which is of direct ecotoxicological relevance. Therefore, it is considered as a bioindicator of metal contaminations in the aquatic ecosystems. (**Davies and Hatcher, 1999; Lopez et al., 2003; Battelli, 2016; Jellison et al., 2016**). These gastropods are commonly harvested and consumed by humans around the world. It could possibly be a harmful metal transfer to the food chains (**Wang, 2002; Collado et al., 2006; Bergasa et al., 2007; Vinas et al., 2018**).

The metal concentrations measured in the tissue of molluscs could be used as biomonitors of metal bioavailability and contamination in the coastal marine environment, in which they live. However, the accumulation of metals in the molluscs also get affected by a number of intrinsic such as environmental stress and extrinsic factors such as spawning season and body size (**Davies et al., 2005; Yap et al., 2006; Sarkar et al., 2008; Azizi et al., 2018**). Previous work has revealed that the body size might change the heavy metal uptake due to changes in environmental conditions. Evidently, the size of the organism would affect the bioaccumulation of metals in absorption and excretion rates. Moreover, the effects of the body size on different physiological levels such as filtration and respiration have been reported in molluscs (**Wang, 2002; Jakimska et al., 2011**).

Until now, no study has identified the relationships between metal levels and the size of the Moroccan Atlantic coast gastropod species such as *Patella rustica*. Therefore, the present study was aimed to offer comparative information in understanding the physiological strategies for the accumulation of Pb, Cu, Cd, and Cr in relation to limpets size. Consequently, the objective of this work was to investigate the effects of the length and weight on metal concentrations (ng/mg dry weight) in the soft tissues of *Patella rustica*.

2. Material and methods

2.1 Study area

The sampling locations were situated in Rabat Region of Morocco (**Figure 38**). This region covers an area of 18.194 km^2, with a population of about 4.581.000. This area belongs to the Mediterranean climate characterized by two main seasons softened by oceanic influences. The average temperatures are approximately 22°C for the warmer months (July to September) and 12°C for the colder months (December and January). Relating to the annual rainfall is in average more than 550 mm/year (**Idrissi Azzouzi et al., 2017a, b**).

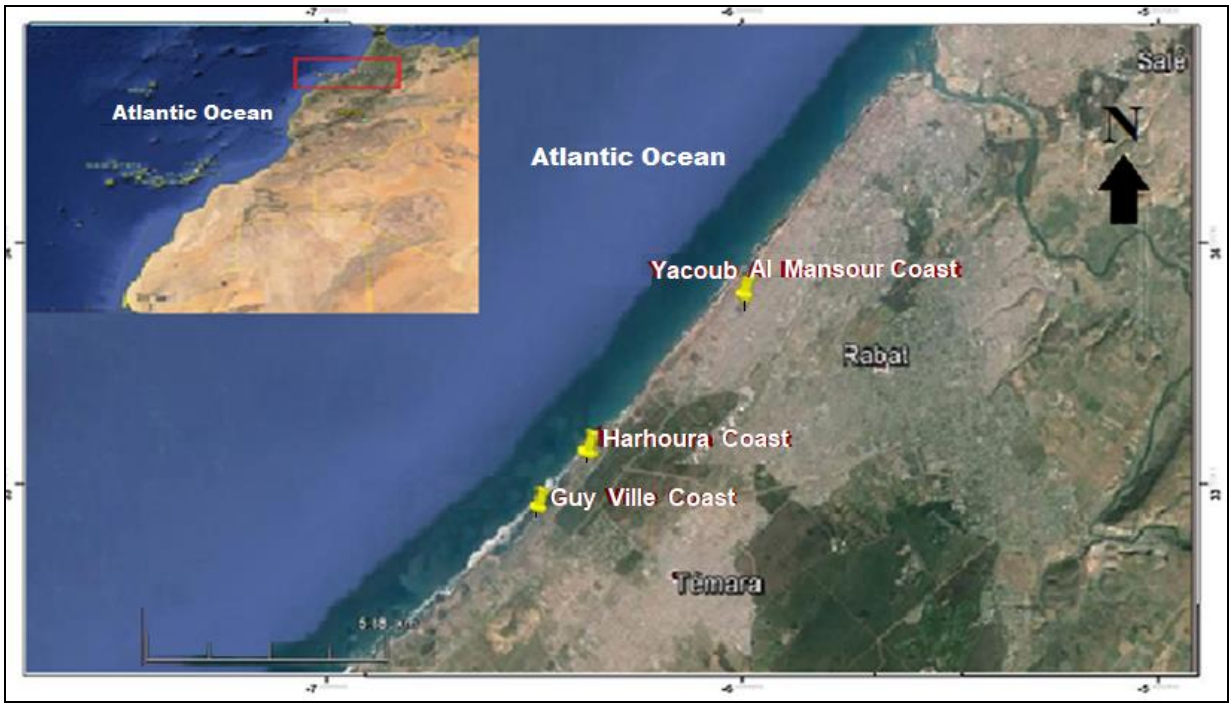


Figure 38: Location of the sampling sites (Yacoub Al Mansour, Harhoura and Guy ville Coast)

2.2 Samples collection

Among February 2015 to February 2016 a total of 120 limpets (*Patella rustica*) with approximate size (0.24-3.59 g tissue dry weight) were collected from three wild populations (Yacoub Al Mansour, Harhoura and Guy ville coast) of Moroccan Atlantic coast in Rabat-Sale-Kenitra region. These sites receive large quantities of untreated or partially treated domestic wastewater.

The limpets were collected by scalpel at rocky shores of three different intertidal locations from the Rabat Region. The specimens collected were stored in polyethylene bags and frozen at -20°C until analysis.

2.3 Samples preparation, analysis and data analysis

In the present study, the breadth, length and height of the shell (**Figure 39**) was measured using a vernier caliper giving a measurement of 1/10th of a millimeter; the soft tissues of samples were weighed after their preparation using a precision balance. The sample preparation and the analysis of the metal concentrations in the soft tissues of limpet were described by **Idrissi Azzouzi et al., 2017b**.

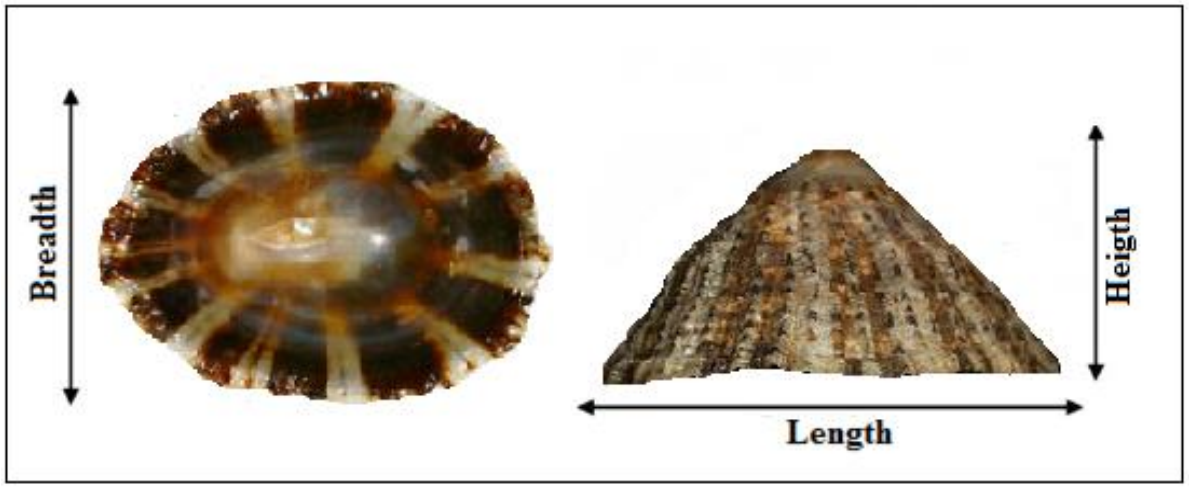


Figure 39 : Shell dimensions of *Patella rustica*: Breadth, Length and Heigth

The data analysis was carried out by means of the statistical package, R version 3.4.1 software. The correlation test was used to check for significant relationships between metal concentrations and limpets size. The level of significance was set at a probability lower than 0.05 ($p < 0.05$). To evaluate significant differences between groups, the Levene test was applied to verify the equality of variances. Subsequently, ANOVA or Kruskal-Wallis test were applied according to the distribution of the data (normal or not, respectively).

3. Results

All biometric relationships performed on *Patella rustica* collected showed a significant correlation. These correlations indicate that the limpets of the Moroccan Atlantic coast are in

biological balance. Therefore, more the coefficient of determination R^2 gets close to 1, the correlation is better and the individuals are in biological balance (**Figure 40**). On the set of the individuals, we find a class of Shell length between 20-44 mm and tissue dry weight between 239-3590 mg (**Table 18**).

Table 18 : Sampling sites of limpet (*Patella rustica*) and their sizes

Site	Shell length (mm) (Min-Max)	Shell breadth (mm) (Min-Max)	Shell height (mm) (Min-Max)	Shell weight (mg) (Min-Max)	Tissue dry weight of limpet (mg) (Min-Max)
Yacoub Al Mansour coast	24-35	18-34	8-16	298-5779	239-2343
Harhoura coast	21-38	15-35	8-15	266-6216	271-2126
Guy ville coast	20-44	18-38	8-18	741-7760	263-3590

The logarithmic relationships between shell length and dry weight of the limpet tissues was determined by using parabolic form of the following equation ($\mathbf{W= aL^n}$, where **W**= Weight (in mg), **L**= Length (in mm), **a** is a constant and **n** an exponent usually between 2.5 and 4.0), this correlation indicates an allometric growth, that is, the length becomes an irrelevant variable in relation to the weight (**Figure 40 a**).

The correlation between shell length and shell weight of the limpet was described using a power regression equation (**Figure 40 b**). Whereas the relation between shell weight and dry weight of limpet tissues is linear (**Figure 40 c**); this assumes that, although the growth of the shell length is slower, its weight remains increasing. The relationships between the shell length on the one hand, its breadth and height on the other hand is linear and highly significant (**Figure 40 d and Figure 40 e**). This means that the shell of limpet (*Patella rustica*) has a conical shape, so its growth in height is the result of an increase in its base (the length-breadth is large or small depending on the diameter of shell).

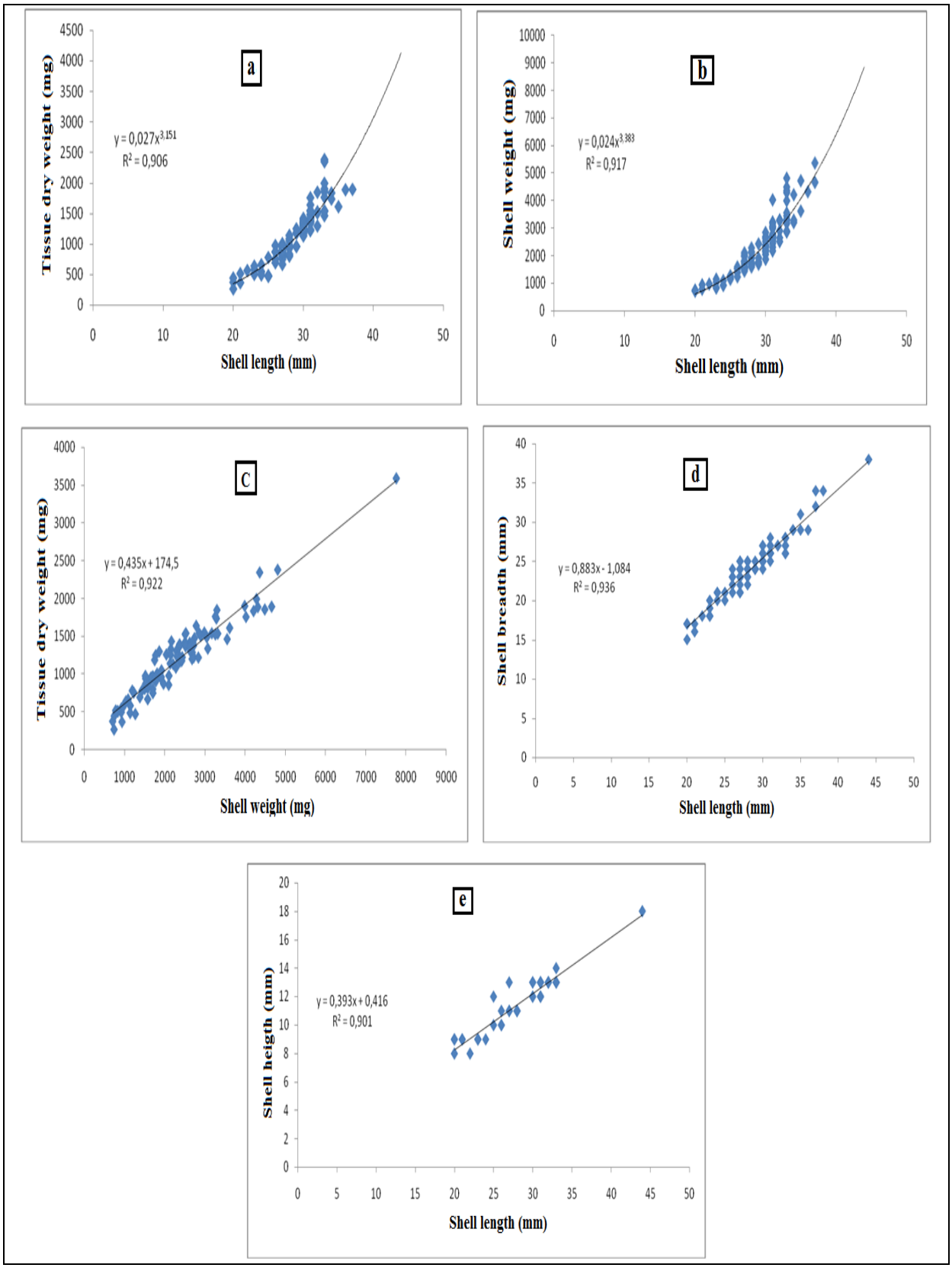


Figure 40: Relationships between all size components of *Patella rustica*

In this study, the results of the linear regression analysis showed that the relationships between metal concentrations and limpets size were significant. Highly significant negative relationships ($p < 0.001$) were found between shell length and concentrations of lead (Pb) and copper (Cu) in the soft tissues of *Patella rustica* (**Figure 41 a and Figure 41 b**). The shell length and concentrations of cadmium (Cd) and chromium (Cr) showed significant positive relationships ($p < 0.05$) (**Figure 41 c and Figure 41 d**).

The results of this study demonstrated that the plotting of the metal content, versus tissue dry weight, gave good straight lines; this indicates that *Patella rustica* presented a different physiological strategy for each metal studied, which is related to the size of limpets. These explain the presence of a significant correlation between bioaccumulation of metals and physiological indices (**Figure 41**).

Growth parameters (length, weight and condition factor) showed a significant relationships with metal concentrations (Pb, Cu, Cd, and Cr) in the soft tissue of samples. Also, the smaller limpets showed higher concentrations (ng/mg dry weight) of Pb, Cu, Cd, and Cr than the larger ones at each site (**Figure 42)**.

The Condition factor (CF) based on the length-weight relationships is often used to express the overall wellbeing of molluscs, this parameter being affected by habitat quality and food availability; It can be calculated according to the following formula: $\mathbf{CF=W/L^{b}\times 100}$, where **W**= Weight, **L**= Length. The exponent **b** is derived from the length-weight relationships **(Bervoets and Blust, 2003; Banerjee et al., 2016**).

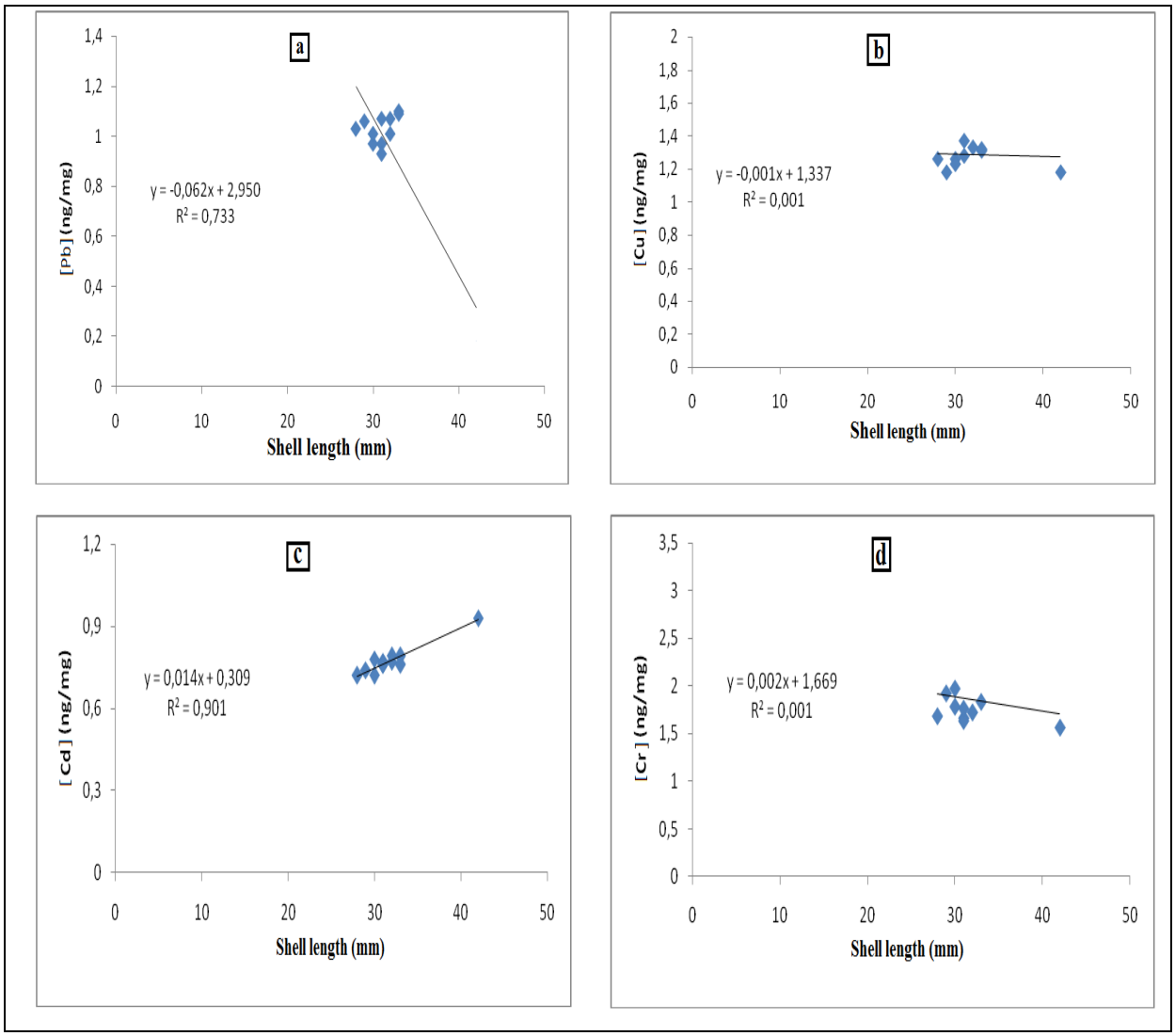


Figure 41: Correlation between shell length and metal (Pb, Cu, Cd, and Cr) concentrations in *Patella rustica*

The metal (Pb, Cu, Cd, and Cr) concentrations in the tissue of limpets studied at different sites show great variations, with the highest concentrations of Cu, Cd and Cr exist in Guy ville coast station. While the concentration of Pb is very important in Harhoura coast station (**Figure 42**).

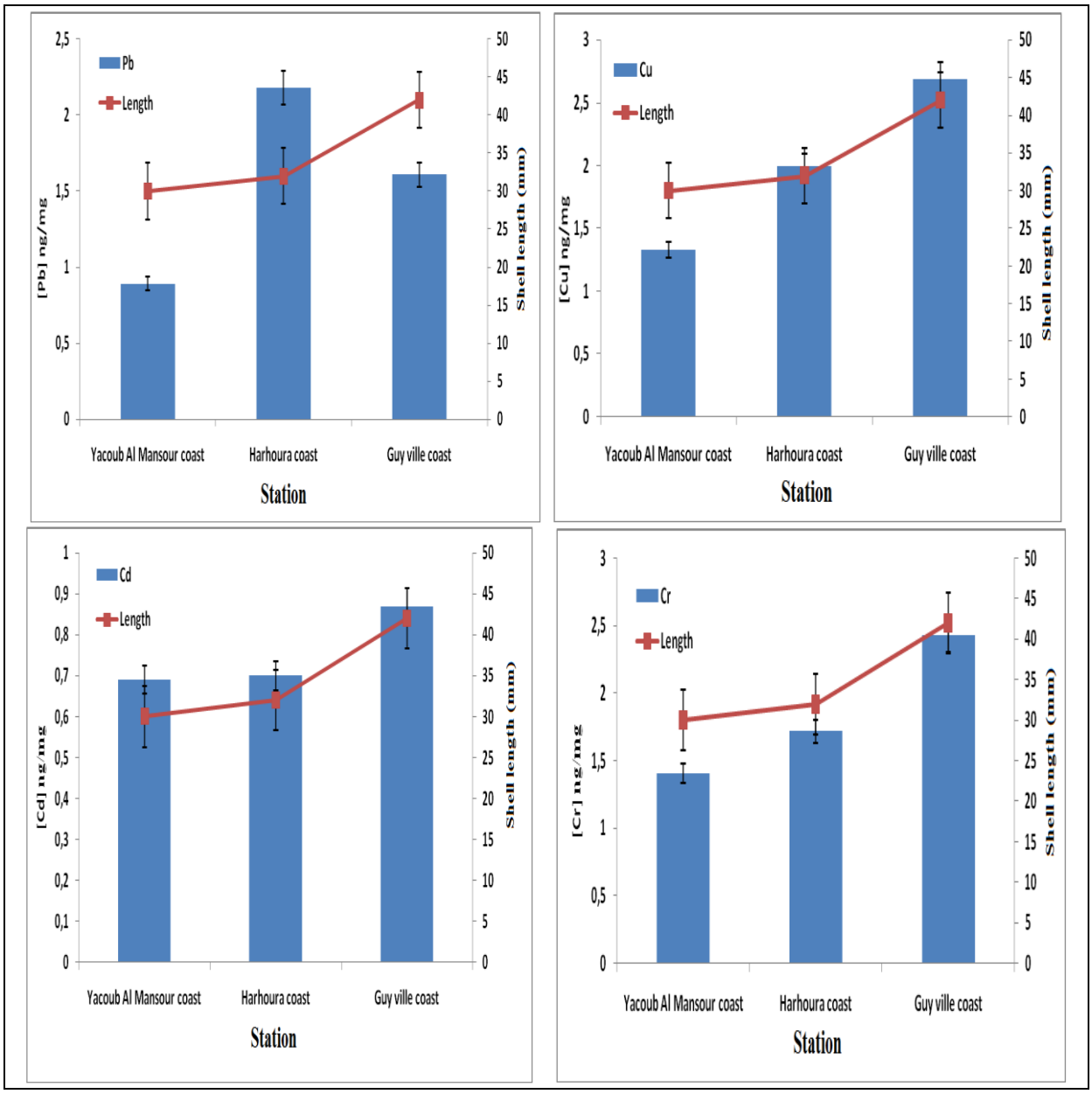


Figure 42: Correlation between shell length and metal (Pb, Cu, Cd, and Cr) concentrations in *Patella rustica*

4. Discussion

In the work of previous studies showed at the level of metal accumulations by the bioindicator there are two groups of metals (**Bervoets and Blust, 2003; Canli and Atli, 2003; Nakhle, 2003; Yi and Zhang, 2012; Banerjee et al., 2016**):

- Cu and Pb, these two metals are influenced by the size of the individual;
- Cd and Cu, these two elements correlated with the level of environmental contamination especially for large size of individual.

Other works showed a considerable difference during all seasons, reproductive cycle and in the different geographical position (**Jenkins and Hartnoll, 2001; Monsefrad et al., 2012; Banerjee et al., 2016**).

The studies of **(Cubadda et al., 2001; Nakhle, 2003)** showed that there is a power-type relationships between the concentration of the metal and the total weight of the individual. The authors suggest that *Patella rustica* functions as a bioindicator of marine pollution but also assume that it has a very high coefficient of concentration of Cd. They linked this fact to the nutritional habits and to the morphological and physiological effects of the species.

This study was mainly aimed to investigate relationships between metal concentrations in the soft tissues of *Patella rustica* and the size of these limpets (generally the length and the weight).

Statistical analysis reveal that metal concentrations in limpet depend on the type of the species and the physiological condition of the organism (**Nakhle 2003; Yap et al. 2009; Idrissi Azzouzi et al. 2017b**). Certainly, the real mechanism of metals bioaccumulation in *Patella* rustica is associated with factors that directly correlated with length, weight, age, sexual cycle, temperature, food abundance (**Nakhle 2003; Storelli and Marcotrigiano 2005; Bergasa 2009; Idrissi Azzouzi et al. 2017b**).

The results obtained from this study indicate that the concentration of metals (Pb, Cu, Cd, and Cr) in *Patella* varies significantly depending on the size of the limpets and the pollution load of each site (**Lopez et al., 2003**; **Nakhle, 2003; Collado et al., 2006; Bergasa et al., 2007**).

High concentrations of Cd in the soft tissues of limpets may be the result of a combination of several factors: the behaviour of the animal in its search for food and the adsorption of metals on the mucus. These metals especially Pb, Cu, Cd, and Cr adsorb on the mucus deposited by the limpets during its dislocation (**Lopez et al., 2003**; **Nakhle, 2003; Collado et al., 2006; Bergasa et al., 2007**).

This mucus fixed on the bedrock and on the bacterial and algal film with high metal concentrations even in non-contaminated environments. The displacement of the limpet on the same location or trail obligates him to feed on microalgae already impregnated with this mucus. The concentration of metals is increased in the new mucus secreted. Therefore, there is an increase in concentration of metals even if the sites are slightly contaminated or uncontaminated (**Lopez et al., 2003**; **Nakhle, 2003; Collado et al., 2006; Bergasa et al., 2007; Ayangbenro and Babalola, 2017**).

5. Conclusion

The present study revealed that smaller size of *Patella rustica* accumulated higher concentrations of Pb, Cd, Cu, and Cr; as well as the relationships between the accumulation of metal and size were confirmed. Therefore, the factor of body size of samples should be considered in all studies.

Based on both the characteristics of gastropod molluscs (bioaccumulators, biomonitors and bioindicators) and the quality of the marine environment, the levels of Pb, Cd, Cu, and Cr observed in the tissue of limpets studied for the three sites (Yacoub Al Mansour, Harhoura and Guy ville coast) should be considered to be an important warning signal.

GENERAL DISCUSSION

For several decades, the damage caused by the various types of pollution in the marine environment has been increasing and reaching high levels. Assessments of the quality of the aquatic ecosystem and its components are an integral part of the protection programs for marine and coastal areas. They provide the opportunity to gather and evaluate the results of scientific research with surveillance practice (**Bertsch, 2010; Paterson et al., 2011; UNEP, 2017; FAO and IWMI, 2017**).

Measured contamination in marine organisms (bioindicators) reflects that of the surrounding aquatic environment (**Holt and Miller, 2010; Li et al., 2010; Hamza-Chaffai, 2014; Parmar et al., 2016**). This study was initiated to update the databases on current levels of various chemical and virological contaminants and indicators in a limited number of exploited species (*Mytilus galloprovincialis* and *Patella rustica*), represent different sites in the Moroccan Atlantic coast. The main objective is to evaluate from the contents two main components: the safety of the products sampled (molluscs) and the quality of the sampling sites (marine environment).

The marine ecosystems remain highly sensitive to the impacts of climate change as they already face several stressors such as overfishing and habitat destruction caused by commercial fishing, coastal redevelopment and pollution (**Keller et al., 2009; Craig, 2012; Rogers, 2013; Corrales et al., 2018)**.

The pollution of marine coastal has increased throughout the worldwide, mainly due to domestic, agricultural, and industrial effluent discharges, atmospheric deposition, oil spills and other wastes and contaminants from shipping. Pollution of the marine environment does not only affect biodiversity as an ecosystem or species but also causes significant socio-economic damage (**Duraisamy and Latha, 2011; Guo, 2017**).

The great diversity of marine resources leads to a very large variation in their nutritional values. Many other factors can still vary these properties within the same species, as is the age, sex, year, location and season (**Bouthir, 2004; De Sherbinin et al., 2007**).

Bivalve molluscs that are exploited by fishing or aquaculture represent wealth in Morocco whose economic stakes are high. These molluscs consist mainly of clams, oysters and mussels. Their exploitation contributes to the national goal of food self-sufficiency and

provides a substantial contribution to foreign exchange annually. Most of Morocco's shellfish production is exported to the European Union. However, the local market, especially the tourism and hotel sector, is also very demanding. Moreover, this activity generates a large number of jobs. For these reasons, shellfish farming is considered by the public authorities as a flourishing activity to support (**Appukkuttan, 2008; FAO, 2016; FAO, 2018)**. In addition, the shellfish farming activity, developed near the coast, is directly conditioned by environmental factors and the quality of the marine environment (**Shumway et al., 2003; Hudson, 2016**).

The bivalve, by their activity of nutrition by filtration, behave like filters of microorganisms, while absorbing the elements necessary to their food, they actively retain and accumulate the free or associated viruses with particulate supports. These data make it possible to possible to recognize that seafood has a high probability of being a preferred agent for spreading viral infections. Among these, hepatitis and gastroenteritis are those for which the epidemiological evidence is most numerous (**Webb, 2000; Dunlop et al., 2005, Mojica and Brussaard, 2014; Huguet, 2017; Mcleod et al., 2017**).

In fact, coastal waters are the receptacle of domestic and agricultural effluent discharges that may contain substances potentially pathogenic for humans. Thus, the microorganisms found in water are concentrated by shellfish and may in some cases constitute a health risk for the consumer. Therefore, foodborne illnesses are a growing public health burden worldwide. The infectious diseases linked to the consumption of raw shellfish like mussels, oysters, cockles and clams have long been identified. Viral foodborne illnesses have emerged as a significant cause of all reported foodborne illnesses. Viral diseases such as hepatitis, acute gastroenteritis, Paralysis and hand-foot-mouth syndrome were the first to be suspected of being linked to the consumption of contaminated shellfish. The majority of viral foodborne illnesses are caused by a few types of viruses including *Hepatitis* A and E (HAV and HEV), *Norovirus* (NoV), *Rotavirus* (RV) and *Enterovirus* (EV) (**Butt et al., 2004; Koopmans, 2012; Le Guyader et al., 2012; Stals, 2012)**. The viruses likely to be encountered in the marine environment are essentially enteric viruses, excreted by humans or animals in their stools. The stool of a person infected with enteric viruses, whether or not they have clinical signs, may contain from 109 to 1011 viral particles per gram of stool. The viruses thus eliminated in the faeces are found in the wastewater which is the first source of the viral contamination, then the human is the secondary receptor of the pathogens carried by the

contaminated seafood and water. The viral concentration in wastewater can reach 105 particles per liter. These quantities vary according to geographical and seasonal factors, but especially according to the socio-economic level and the sanitary conditions of the place considered. More than 130 types of enteric viruses belonging to several family and genus are listed. They can cause various syndromes ranging from gastroenteritis to meningitis (**Cotruvo et al., 2004; Fong and Lipp, 2005; FAO and WHO, 2008**; **Hellmer et al., 2014)**. In 2007, viruses were estimated as being responsible for almost 12% of all reported foodborne outbreaks in the European Union. The European Food Safety Authority reported that this value had increased to 14% by 2012 **(EFSA, 2011; EFSA, 2014)**. In Morocco, the prevalence of circulating NPEV is unknown, which indicates the need for a deeper investigation of the dissemination in the environment in order to identify them and associate them with clinical manifestations in the country. Thus, the results reported in this study showed a potential health risk to the population. Indeed, the presence of this strain vaccine in the environment could be a source of infection for humans.

The typing of *Enterovirus* (EV) is essential to identify the strains responsible for these infections, in order to determine the mode of circulation and transmission specific to each genotype and to identify new variants causing epidemics. EV identification is also needed to look for and differentiate *Poliovirus* strains from other enteroviruses. Moreover, the precise identification of non *Poliovirus* enteroviruses is interesting, in order to progress in the studies of relations between the different serotypes and the pathologies generated. Generally, EV identification has been based on cell culture and seroneutralization of cytopathic effect using polyclonal antiserum. However, no cell line can isolate all types of EV. In addition, The techniques applied in tissue culture are expensive, complex and difficult (**Racaniello et al., 2007; Rhoades et al., 2012; Betancourt and Shulman, 2017; Pogka et al., 2017**).

In this work, the first objective was to detect the presence of enteroviruses in mussels (*Mytilus galloprovincialis*) collected from three wild populations (Bouregreg estuary, Yacoub Al Mansour coast and Harhoura coast) in order to obtain an overview of the viral contamination in the marine environment. The second objective was to type enteroviruses identified in mussel samples in order to study strains of enteroviruses circulating in the sampling sites. This study revealed the circulation of an important number of typable strains (64.7%) and non-typable strains (35.3%) in the marine environment. However, non-typable strains confirm the presence of new serotypes. For this reason, it is important to develop methods sensitive enough to detect a single viral particle per sample. Therefore, to monitor viral contamination

in marine environments, the use of molecular techniques targeting certain regions of the genome and the phylogenetic analysis of nucleotide sequences are recommended.

When enteroviruses are searched in shellfish different problem are encountered. Firstly, the adsorption of the virus at the level of the different organs of the molluscs with a preferential localization at the level of the stomach and the hepatopancreas imposes the use of an extraction method to remove the virus from its support. Moreover, in the absence of viral multiplication, the infection rates infecting the shells are low. These low virus concentrations force us to concentrate viruses and to use particularly sensitive detection techniques. Finally, these shellfish are also environments of complex chemical composition (**Bosch et al., 2009; La Bella et al., 2017)**. This is likely to interfere with the detection system implemented and to prevent viral detection. Thus, the quality of nucleic acid extraction, sensitivity and specificity of the RT-PCR are essential for their highlighting. Several authors have emphasized the importance of molecular biology methods for the detection of enteric viruses in environmental samples. These allow the rapid and specific detection of low or non-culturable viruses, such as *Hepatitis* A virus and caliciviruses. However, the use of this technique poses the problem of false positives (related to the risks of contamination during handling in the laboratory) and false negatives (persistence of inhibitors). The development of internal controls makes it possible to alleviate some of these problems by avoiding the use of viral strains of control (presenting risks of secondary contamination of the samples). Their use also allows the evaluation of the amplification efficiency in each sample. In addition, these internal controls will eventually provide a semi-quantitative estimate of viral contamination (**Jean et al., 2001; Formiga-Cruz et al., 2002; Li et al., 2002; Rodriguez et al., 2009; Perrin et al., 2015; Adefisoye et al., 2016; Forbes et al., 2018)**.

The interest of this study was to evaluate the viral contamination of bivalve molluscs. This control will prevent health risks related to the shellfish consumption and monitor the evolution of the enteroviruses in the marine environment. The establishment in Morocco of virological tools for the control of shellfish will make it possible to better contribute to the improvement of their microbiological quality in order to respond by anticipation of the future requirements of international standards.

Bivalve molluscs feed by filtering large volumes of seawater and accumulating food particles from their environment. When this environment is contaminated by sewage, shellfish will also accumulate human pathogenic bacteria and viruses during filter feeding and pose a health risk

when consumed raw or only lightly cooked (**Robertson, 2008; Bosch et al., 2009; Burge et al., 2016)**. During the past century, various strategies have been established in shellfish growing areas throughout the world to assure the sanitary quality of bivalve molluscs. In order to make these shellfish suitable for consumption, three main commercial treatment processes have been traditionally used. Firstly, heat treatment (cooking) can be used to destroy pathogens before consumption. Secondly, shellfish harvested from polluted areas can be replaced in clean areas (areas free of microbiological contamination) to allow shellfish to cleanse or purge themselves by the continuation of their normal filter-feeding and digestive processes. This process is called relaying or container relaying. Thirdly, the natural cleansing process can be performed in a controlled environment by immersion in tanks of clean seawater to allow sewage contaminants to be purged. This process is called depuration or controlled purification. The relaying and depuration processes, unlike cooking, allow bivalve shellfish to be marketed. This is commercially important for species such as oysters, clams and mussels, which are traditionally eaten raw or lightly cooked prior to consumption (**Pommepuy et al., 2009; Lees et al., 2010; Rice et al., 2015; Baker, 2016)**. However, if the bioaccumulation phenomenon are at the origin of the viral contamination, the process of release of the viral particles allows a natural purification of the seafood when they are placed in unpolluted waters. In these circumstances, it is necessary to be able to determine the virological health status of molluscs delivered for human consumption. To do this, it is essential to have appropriate analysis methodologies. In addition, the risks of pollution must be taking into account in the shellfish growing marine area. Therefore, it is highly desirable to have purification devices that make it possible to rid the molluscs of possible viral contaminants (**FDA, 2009; Lees et al., 2010; Correa et al., 2012)**.

The persistence of viruses in the marine environment is due to this great ability to aggregate or adsorb to suspended matter. It has been shown that viruses persist longer in seawater containing sediments than in seawater alone. The adsorption of viruses on particulate elements gives them physical protection, the virion is stabilized by electrostatic forces and bonds. It is protected from inactivating factors (chemical and physical agents). Taking into account farming conditions, contaminant inputs into coastal seawater and the feeding of molluscs by filtering large volumes of water, it is easy to say that the health status of molluscs dependents closely on the environment in which they live. If they are farmed in a contaminated area, there is a certain risk of contamination of shellfish by pathogenic

microorganisms and may also pose a risk to public health (**Griffin et al., 2003; Gerba and Betancourt, 2017**).

This study presents viral contamination in shellfish from three geographically distinct areas over a one-year follow-up period. The results obtained demonstrate the effectiveness of cell culture and molecular biology techniques for the detection of viral particles in environmental samples. the viruses that were found in the shellfish collected from the sampling sites are enteroviruses. The target in this study was to supplement the Moroccan databases available on environmental contamination by enteroviruses and to illustrate the importance of including routine virological analysis of shellfish in Morocco. These data contribute to the assessment of the viral risk associated with shellfish consumption in Morocco. They provide a first estimate of the levels of viral contamination of shellfish destined, without secondary treatment (purification), for consumption because the risk of secondary contamination of shellfish, during packaging and transport, remains limited. In the absence of viral quantification in shellfish and precise information on the maintenance of the pathogenicity of these viral particles, they make it possible to identify periods of health risks during which additional preventive measures can be envisaged to guarantee the safety shellfish distributed on the markets, such as temporary marketing bans. Other solutions could be selected including intensive purification treatments, provided however to test beforehand the effectiveness. It seems important to continue to develop in the interests of quality assurance, simple methods for the detection of enteric viruses. Research should continue taking into account the interference of new pathogens such as the Hepatitis E virus or the Aichi virus. Globalization facilitating exchanges, it is also necessary to conduct epidemiological studies to collect clinical data.

The chemical contamination by metallic trace elements can affect aquatic life from primary producers. The risk of metallic contamination increases as one goes up through the links of the trophic chain (bioaccumulation or biomagnification phenomenon) (**Gray, 2002; Cardwell et al., 2013**). Metal contamination of the marine environment is most often of human origin, more rarely of natural origin. In aquatic ecosystems, metals can be bioaccumulated in mussels, oysters, shrimp, scampi and fish. Sensitivity to contaminants can vary considerably between marine organisms (**de Astudillo, 2005; Swaleh et al., 2016; Kumar and Weerasooriyagedara, 2018**).

In our study, we are interested to evaluate the level of metal contamination by metallic trace elements (copper, chromium, cadmium, and lead) in the sampling sites (Yacoub Al Mansour, Harhoura and Guy ville coast) at the Moroccan Atlantic coast through a bioindicator (mollusc gastropod "*Patella rustica*").

The risks of contamination at the moment of sampling and analysis are numerous, making the measurements delicate. Many ecologists and environmental scientists have proposed to follow up the international level, the concentrations of contaminants in living organisms in order to monitor the environment. Therefore, marine organisms concentrate contaminants, in relation to the concentrations present in the ecosystem, it is the principle of "quantitative bioindicators" (**Namiesnik, 2001; Borja et al., 2011; Siddig et al., 2016**).

The accumulation of Cd, Cu, Cr and Pb metals in gastropod molluscs is related to their concentrations in the environment, and also to the penetration of metals and their bioaccumulation and bioconcentration capacity of molluscs (**Coeurdassier, 2001**). The content of the species studied in metallic trace elements varies according to their exposure to various sources of pollution **(Modrzewska and Wyszkowski, 2014)**. The metallic elements have a strong toxicological impact on the shellfish and on the consumer. It was necessary to regulate the metal content in seafood for consumption, and also regulated and controlled industrial discharges (**Mitra et al., 2012; Pandey, 2014; Ullah et al., 2017; Rajeshkumar and Li, 2018**). According to the results obtained in this study, the metals are classified as follows, in descending order of specific toxicity: [Cu]>[Cr]>[Pb]>[Cd] (**Table 14**). These results show important contamination by Cd, Pb, Cr, and Cu during all the period of our study.

The levels of copper and chromium have been found to be highest in all soft tissues in every season while cadmium and lead have been found at lower levels (**Table 15 and Figure 35)**. Copper, chromium, iron, and zinc are essential elements and have important roles in biological processes such as growth, cell metabolism, and survival of most animals including gastropod molluscs. Therefore, the relatively high levels of these metals can be attributed to their essentiality (**Ansari et al., 2004; Turkmen et al., 2005; Lafabrie, 2007; Mitra et al., 2012; Duysak and Ersoy, 2014; Komar et al., 2018**). Other metals such as cadmium, lead, arsenic and mercury are considered as non-essential elements. They have a useless role in human physiology and biology and can produce toxic effects for living organisms (**Turkmen et al., 2005; Lafabrie, 2007; Tchounwou et al., 2012; Jaishankar et al., 2014; Komar et**

al., 2018). Overall, cadmium and lead are in critical condition via the environment as their concentrations exceed the baseline average levels for limpets. According to many kinds of research, we noticed that our results are similar to literature results (**Collado et al. 2006, Bergasa et al., 2007 and Yuzereroglu et al., 2010)**.

The study of the interaction between chemical contaminants and biological membranes is of considerable interest for the understanding of the ecotoxicological phenomenon and the interpretation of bioaccumulation and transfers through trophic chains. The bioavailability of contaminants depends on many physical factors (content of organic matter and suspended particles etc), chemical (solubility and reactivity of compounds), biological (pelagic or benthic organisms used, contamination mode etc) (**Borgmann, 2000**; **Widenfalk, 2002; Gourlay-France and Tusseau-Vuillemin, 2013)**.

Trace metals are normal constituents of the environment that can be toxic above a certain threshold (**Kucuksezgin et al., 2006**). Toxic metals at certain concentrations may be harmful to marine organisms even at low levels of exposure and persistent in the aquatic ecosystem. In addition, they can cause diseases, cancers, severe reproductive damage and negatively affect biologically useful metals such as zinc and calcium (**Singh et al., 2011; Tchounwou et al., 2012; Jaishankar et al., 2014; Jitar et al., 2015**). These toxic metals do not all pose the same risks because of their effects on organisms, their chemical, physicochemical and biological properties. Their toxicity is very variable and their impact on the environment very different (**Singh et al., 2011; Wuana and Okieimen, 2011; Tchounwou et al., 2012; Machado et al., 2016**; **Shah, 2017**). The recommended maximum allowable dose (MAD) of metals concentration in edible gastropod molluscs such as *Patella* as follow (3,28 µg/g for Cu; 0,73 µg/g for Cr; 0,19 µg/g for Cd and 0,12 µg/g for Pb) (**IAEA-407, 2003-09-01**).

Pollutants can persist for several years in marine environment where they hold the possibility to affect human health and the ecosystem. Hence, coastal and marine pollution control is necessary to determine and monitor the impacts of anthropological activities on marine and estuarine ecologies (**Mackeviciene et al., 2002**). Metals are pollutants whose harmfulness is related to their speciation and persistence due to their non-degradable nature. They are poorly metabolized (unlike organic pollutants), so the impact of this type of contamination on the human is its relation by the consumption of sea products since some elements could be transferred by bioaccumulation and biomagnifications in the food web and accumulate in

the living organisms (**Wuana and Okieimen, 2011; Lenart-Boron and Boron, 2014; Jaishankar et al., 2014; Ayangbenro and Babalola, 2017; Boukhelf et al., 2018**).

Different species of molluscs show a differential capacity to accumulate pollutants and to monitor the quality of the aquatic ecosystem, in particular, the bivalves and gastropods which retains unwanted substances found in trace amounts in the marine environment (**Ravera, 2001; Salanki et al., 2003)**. The gastropod (*Patella rustica*) was used in this study to evaluate the metal contamination of the sampling sites. The problems that have been identified over the last thirty years of using bioindicators included, on the one hand, biotic effects such as age, size, weight, sex, food abundance and the sexual cycle, and on the other hand abiotic effects like the bioavailability of metals in the environment, the level of organic carbon, temperature, pH, dissolved oxygen and hydrology (**Nakhle, 2003**).

In this work, we report the concentrations of Cu, Cr, Cd and Pb in the soft tissues of *Patella rustica* from three different stations. The results obtained with the Mann-Whitney U test clearly show a significant ($p < 0,01$) spatial variations of the metal concentrations. The highest metal levels were registered in Guy ville coast, followed by Harhoura coast and finally the Yacoub Al Mansour coast (**Table 15 and Figure 35**), which highlights that some sites are more contaminated than others. The presence of high concentrations of Cu, Cr, Pb and Cd in limpet samples from (Yacoub Al Mansour, Harhoura and Guy ville coast) is expected to be attributed to domestic discharges, waste incineration and exhaust gas of the vehicles. All these factors may have affected the *Patella rustica*, indicating the vulnerability of this species to metal pollution in sampling sites of the Moroccan Atlantic coast. The seasonal changes for all metal concentrations in the soft tissues of *Patella rustica* from sampling stations were observed in this study. In addition, the high concentrations of Cu, Cr, Cd and Pb were detected in summer and spring (**Table 15 and Figure 35**). In general, high chemical pollution has occurred during the summer and spring months (**Duysak and Ersoy, 2014; Duysak and Azdural, 2017**).

The gastropod molluscs presented negative correlations between metal concentrations and body weight and size (**Cubadda et al., 2001; Collado et al., 2006)**. The present study revealed that a smaller size of *Patella rustica* accumulated higher concentrations of Pb, Cd, Cu, and Cr; as well as the relationships between the accumulation of metal and size of limpet were confirmed. Therefore, the body size component of the samples is an important factor which should be taken into account in research studies.

Limpets have considerable potential as cosmopolitan biomonitors of metals in the marine environment. These sedentary species are available in every season all over the coastal area and are easy to sample and identify. Finally, it is recommended that the limpets are suitable to be used as a successful bioindicator of metal contamination caused by different human activities in this coastal area of the Moroccan Atlantic coast and must be controlled before their consumption. Further studies of metal levels in gastropod molluscs from different sampling sites are required including the investigation of the possible effects of seasonal changes on metal concentrations and distribution.

The value of the contaminant concentration, measured in the indicator organism, is the result of processes involved at different scales: at the scale of the contaminant (nature of the metal, chemical speciation, bioavailability, etc), at the scale of the receptor organism (membrane properties, pathways of entry, exit routes, life cycle, etc) but also at the scale of the intra and extracellular environment (temperature, trophic conditions, environmental contamination, etc) (**Nakhle, 2003; Storelli and Marcotrigiano, 2005**; **Bergasa et al., 2009**). The study of contamination constantly faces this complexity due to the diversity of abiotic and biotic factors, but especially of their variations and interactions in space and time.

Statistical analysis revealed that metal concentrations in limpet depend on the type of the species and the physiological condition of the organism (**Nakhle, 2003; Yap et al., 2009; Idrissi Azzouzi et al., 2017b**). Certainly, the real mechanism of metals bioaccumulation in *Patella rustica* is associated with factors that directly correlated with length, weight, age, sexual cycle, temperature, food abundance (**Nakhle, 2003; Storelli and Marcotrigiano, 2005; Bergasa et al., 2009; Idrissi Azzouzi et al., 2017b**).

Lead, copper, chromium and cadmium have concentrations at or below the IAEA standard. Despite the presence of certain metals in the sampling sites through bioindicators (gastropod molluscs), efforts will have to be made in the future to protect our coasts from all human activities. These first preliminary results come from our research must be complemented by other studies extended on time and space.

All the results obtained by Atomic Absorption Spectrophotometry assay are statistically processed by different parametric and non-parametric tests in order to analyse the homogeneity between stations (Yacoub Al Mansour, Harhoura and Guy ville coast) and between seasons (summer, autumn, spring and winter) (the Student's t-test), or between several parameters such as stations, (ANOVA test, Mann-Whitney U test), and to represent the biological, physiological, ecological and environmental relationships between metal (Pb,

Cu, Cd, and Cr) concentrations in the soft tissues of *Patella rustica* and limpets size (generally the length and the weight) (Linear regression analysis). The results obtained and argued through biological, ecological and environmental discussions and interpretations.

This analysis carried out on molluscs, in relation with their external environment (marine environment), will make it possible to evaluate the bioaccumulation capacity of selected bioindicators. A discussion of the results and their interpretations will make it possible to verify the choice of the bioindicator species of pollution and to study the relationships between the bioconcentration and the samples size. In the end, a conclusion and general perspectives will make it possible to draw up an assessment of the state of health of the Moroccan littoral, in the matter of metallic pollution generated by the different anthropic activities, and to position this space in a global environment: the Atlantic coast. Monitoring programs and research on metals in marine environmental samples have become widely important due to concerns overaccumulation and toxic effects in marine organisms and to humans through the food chain.

This study provides information that is useful towards the status of metals in the Atlantic Moroccan coast. Dissemination of these findings will be helpful to the main stakeholders or agencies that monitor environmental pollution such as Ministry of the environment and Ministry of Health.

The current knowledge of the metal contamination of marine environments reveals an extreme ecotoxicological complexity, due to the numerous interrelations existing between the abiotic factors of the environment, the biotic factors and the many forms of metal derivatives present in the different compartments of the ecosystems.

From an ecological and health point of view, it becomes necessary to establish a program of monitoring and continuous monitoring of edible molluscs and identify the different sources of viral and metal contamination which can affect the marine environment and the human health. This environmental protection strategy must be based on national regulations and a willingness to apply it through the development of national laws and standards governing domestic, industrial and agricultural discharges and the quality of the receiving environment.

GENERAL CONCLUSION

Although shellfish can generally be considered healthy, safe and nutritious, it can occasionally pose a risk to the consumer. Regulations currently rely on routine monitoring of shellfish for the presence of microorganisms and chemicals contaminants to determine their sanitary quality. However, viral and chemical contamination of molluscs bivalves and gastropods is currently recognized as one of the leading causes of disease. The monitoring of viral contamination is complex and must take into account various factors such as detection methods, technology, social requirements and the sustainable development of aquaculture. Therefore, recent technological advances, particularly in the development of molecular tools, make it possible to search for pathogens directly in the shellfish involved in hatching. The evaluation of seafood products for *Enterovirus*, *Norovirus*, *Hepatitis* A virus and *Hepatitis* E virus for regulatory purposes was recommended by experts from the FAO and WHO working groups. However, mandatory surveillance of the virus may take place in the near future, but additional information including the level of exposure and the infectious dose of the virus is still needed to assess the risks.

An important aspect of monitoring is the sustainable development of the aquatic ecosystem. This evolution is closely linked to the quality of the environment in the shellfish breeding areas. The regulations established for the marine environmental quality and the shellfish growing areas, if properly implemented, should provide guarantees and management tools. There are promising examples of coastal management designed to reduce household and industrial waste discharges, which could help recover aquatic ecosystem quality such as used in conjunction with early warning systems. They could help to ensure the quality of shellfish and increase consumer confidence, thus contributing greatly to the sustainable development of aquaculture. Shellfish have long been recognized as beneficial to human health and this benefit should also be taken into account in coastal zone management and the preservation of marine environmental quality.

FUTURE DIRECTIONS

The results obtained in this research open up some perspectives of study that it would be useful to undertake:

- Study of the contamination of other metallic trace elements (MTE) such as mercury, arsenic, and other viruses such as Norovirus, Hepatitis A virus, Hepatitis E virus, Adenovirus, Astrovirus, and Rotavirus which is very harmful to living beings;
- Spread out this study at all sites in the region, which are exposed to significant sources of metallic traces elements contamination. Such an eventuality would make it possible to map pollution levels, assess environmental impacts and project conservatory measures of regional biodiversity;
- Continue this study taking into account the climatic and the physicochemical parameters, and make several samples for the same site in order to gather the maximum information on the contamination levels;
- Extend this study on the sediments and water, to adopt a strategy of sampling for each type of ecosystem to better identify and track sources and levels of MTE contamination;
- Establish a monitoring network of aquatic ecosystems by metals assay for all bioindicators such as *Pollicipes pollicipes*, especially those at the base of the chain food, and who are responsible for contamination of the food web (lichens, invertebrates and vertebrates, etc);
- Establish severe laws to reduce the sources of viral and metal contamination, which cause a risk to consumer and environmental health;
- Reduce waste discharges in coastal areas. As a result, water quality studies have shown that it is possible to identify the main sources of contamination in coastal areas;
- Establishment of warning systems in developing countries, by creating databases on epidemics linked to environmental contamination. Thus, combined with forecasting information, malfunctions and others. An alert system could lead to real-time assessments of bathing water quality or harvesting areas. The development of new tools for the rapid detection of pathogens can help to collect additional information on the presence of pathogens in wastewater;

- Reduce viral intake. Therefore, the small towns must be equipped with small individual treatment tanks to comply with the regulations. So, the new technologies may be needed to improve the elimination of viruses in wastewater effluents;

- Other factors will also need to be taken into account to protect the consumer and ensure the safety of shellfish on the market.

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ANNEX

List of Scientific Publications

✓ Original articles

1. Benhafid, M., El Omari, N., El Qazoui, M., **Idrissi Azzouzi, M.**, Rguig, A., Filali-Maltouf, A., & Elaouad, R. (2013). **Diversity of *Rotavirus* strains circulating in children under 5 years of age admitted to hospital for acute gastroenteritis in Morocco**. *J. Med. Virol*, 85(2): 354-362.

2. Benhafid, M., El Omari, N., **Idrissi Azzouzi, M.**, Rguig, A., Gentsch, J. R., Parashar, U., & Elaouad, R. (2015). **Effect of Monovalent *Rotavirus* Vaccine on *Rotavirus* Disease Burden and Circulating *Rotavirus* Strains Among Children in Morocco**. *J. Med. Virol*, 87(6): 944-53.

3. **Idrissi Azzouzi, L. M.**, El Qazoui, M., Benhafid, M., El Omari, N., & Oumzil, H. (2015). **Étude de la circulation des *Entérovirus* au Maroc à travers la surveillance virologique des paralysies flasques aiguës de Janvier 2009 à Décembre 2014**. *BINH*, 4: 2-4.

4. **Idrissi Azzouzi, L. M.,** Senouci, S., El Qazoui, M., Oumzil, H., & **Naciri, M.** (2017). **Detection of *Enterovirus* in mussels from Morocco by cell culture and real-time PCR**. *Afr. J. Biotechnol*, 16(34): 1791-1799.

5. **Idrissi Azzouzi, L. M.,** Benaakame, R., Elabidi, A., & **Naciri, M.** (2017). **Contamination levels of metals (Cu, Cr, Cd and Pb) in *Patella rustica* from the Moroccan Atlantic coast**. *IJESE*, 12(10): 2347-2361.

6. **Idrissi Azzouzi, L. M.,** Benaakame, R., Elabidi, A., & **Naciri, M.** (2018). **The relationships between metal (Cd, Pb, Cu and Cr) levels and the size of Moroccan Atlantic coast gastropod species (*Patella rustica*)**. *Environmental Monitoring and Assessment*. Under review.

7. **Idrissi Azzouzi, L. M.,** El Qazoui, M., Oumzil, H., & **Naciri, M.** (2019). **Typing of *Enterovirus* identified from Moroccan mussels (*Mytilus galloprovincialis*) by seroneutralization**. *International Journal of Environmental Research*. Submitted.

8. **Idrissi Azzouzi, L. M.,** Benaakame, R., Elabidi, A., & **Naciri, M.** (2019). **Size-dependant concentrations of metals (Pb, Cu, Cd, and Cr) in Atlantic gastropods (*Patella rustica*)**. *Atlantic Journal of Science.* Submitted.

✓ Oral communications

1. **Lalla Meryem Idrissi Azzouzi**. **La détection des *Entérovirus* dans les mollusques bivalves du Maroc à l'aide de la culture cellulaire**. 4th Doctoriales of the faculty of sciences, organized on February 19, 20 and 21, 2015, Rabat, Morocco.

2. **Lalla Meryem Idrissi Azzouzi**. **Recherche et détection des virus entériques dans les bivalves au Maroc**. International congress organized by microbial biotechnology for development «MICROBIO 3» on October 24, 25 and 26, 2016 at Aventi hotel, Mohammedia, Morocco.

3. **Lalla Meryem Idrissi Azzouzi**. **Changements climatique: Impact de l'acidification des océans sur les mollusques.** Workshop, organized by the superior school of technology on October 27-28, 2016, Essaouira, Morocco.

4. **Lalla Meryem Idrissi Azzouzi**. **Évaluation du danger viral chez des moules (*Mytilus galloprovincialis*) de la côte Atlantique Marocaine.** COP22 international congress, organized by the council of the Moroccan community abroad on November 11-12, 2016 at Mogador Agdal hotel, Marrakech, Morocco.

5. **Lalla Meryem Idrissi Azzouzi**. **Impact des changements climatiques sur la transmission des maladies virales, exemple des virus entériques**. COP22 workshop, organized by the scientific association of the National Institute of Hygiene on November 16, 2016 at the aculty of sciences and technologies, Marrakech, Morocco.

6. **Lalla Meryem Idrissi Azzouzi**. **Évaluation de la bioaccumulation de métaux traces (Cu, Pb et Cd) chez le gastropode, *Patella rustica*, en milieu atlantique Marocaine**. 6th International congress of toxicology, organized by the Moroccan society of clinical and analytical toxicology on december 15, 16 and 17, 2016 at Palm Plaza hotel, Marrakech, Morocco.

7. **Lalla Meryem Idrissi Azzouzi**. **Résultat de la surveillance virologique des paralysies flasques aiguës**. Training workshop on active surveillance of acute flaccid paralysis, organized by the department of epidemiology and disease control on February 21, 22 and 23, 2017, Rabat, Morocco.

8. **Lalla Meryem Idrissi Azzouzi**. **Procédure de la collecte et du transport des prélèvements, cas fortement suspect de poliomyélite et prélèvement chez les contacts**. Training workshop on active surveillance of acute flaccid paralysis, organized by the department of epidemiology and disease control on July 04, 05, 06 and 07, 2017, Rabat, Morocco.

9. **Lalla Meryem Idrissi Azzouzi**. **La détection des *Entérovirus* dans les moules du Maroc par culture cellulaire et PCR en temps réel**. 2nd Franco-Maghrebian days of virology "viral infections: preventive approaches", organized on October 18, 19 and 20, 2017, Marrakech, Morocco.

10. **Lalla Meryem Idrissi Azzouzi**. **Indicateurs de performance de la surveillance épidémiologique au laboratoire des PFA au Maroc**. Evaluation workshop of surveillance activities of acute flaccid paralysis, organized by the department of epidemiology and disease control on July 03-04, 2018, Rabat, Morocco.

11. **Lalla Meryem Idrissi Azzouzi**. **Procédure de la collecte et du transport des prélèvements, cas fortement suspect de poliomyélite et prélèvement chez les contacts**. Training workshop for the staff of university hospital center on surveillance of acute flaccid paralysis, organized by the Department of Epidemiology and Disease Control on July 09-10, 2018, Rabat, Morocco.

12. **Lalla Meryem Idrissi Azzouzi**. **Analyse virologique des cas de paralysie flasque aiguë (PFA).** Training workshop for the FETP on surveillance of Acute Flaccid Paralysis, organized by the department of epidemiology and disease control on December 18-19, 2018, Rabat, Morocco.

✓ Communications posted

1. Maria El Qazoui, Mohamed Benhafid, **Meryem Idrissi Azzouzi**, Nezha El Omari, Rajae El Aouad, **Initiative mondiale d'éradication de la poliomyélite: Rôle de l'Institut National d'Hygiène dans la surveillance des cas de paralysie flasque aiguë au Maroc**. First Franco-Maghrebian days of virology, organized on October 23, 24 and 25, 2013, Marrakech, Morocco.

2. Maria El Qazoui, Nezha El Omari, **Meryem Idrissi Azzouzi**, Saaid Amzazi, Mohamed Benhafid and Rajae El Aouad. **Les *Norovirus*: Principale cause de gastroentérite aiguë chez les enfants de moins de cinq ans au Maroc.** International days of biology "French and European medical biology", organized by the French society of clinical biology on October 8, 9 and 10, 2014, Paris, France.

3. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Étude de la bioaccumulation métallique chez les bioindicateurs (Patellidae) de la côte Atlantique Marocaine**. 4th Doctoriales of the faculty of sciences, organized on February 19, 20 and 21, 2015, Rabat, Morocco.

4. **Lalla Meryem Idrissi Azzouzi**, Samira Senouci, Maria El Qazoui, Hicham Oumzil, Mariam Naciri. **La détection des *Entérovirus* dans les mollusques bivalves du Maroc à l'aide de la culture cellulaire**. 4th Doctoriales of the faculty of sciences, organized on February 19, 20 and 21, 2015, Rabat, Morocco.

5. **Lalla Meryem Idrissi Azzouzi**, Samira Senouci, Maria El Qazoui, Hicham Oumzil, Mariam Naciri. **Évaluation de la contamination virale dans l'environnement: Recherche des *Entérovirus* dans les mollusques bivalves collectés au Maroc**. 6th Scientific days of the CEDoc SVS and 3rd scientific days of AMADOC, organized by the faculty of medicine and pharmacy on March 04, 05, 06 and 07, 2015, Rabat, Morocco (**Award for best communication posted**).

6. **Lalla Meryem Idrissi Azzouzi**, Maria El Qazoui, Hicham Oumzil, Mariam Naciri. **Évaluation de la contamination virale du milieu hydrique: Recherche des *Entérovirus* dans les eaux usées au Maroc**. 2nd Environment day "impact of pollution on the population", organized by the association of youth and environment of the 3rd millennium on May 23, 2015, Kenitra, Morocco.

7. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Évaluation de la bioaccumulation métallique chez des bioindicateurs de la côte Atlantique Marocaine**. 5th Doctoral of the faculty of sciences, organized on March 09, 10 and 11, 2016, Rabat, Morocco.

8. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Étude de la contamination métallique des mollusques gastéropodes (Patellidae) de la côte Atlantique Marocaine**. 7th Scientific days of the CEDoc SVS and 4th scientific days of AMADOC, organized by the faculty of medicine and pharmacy on March 16, 17,18 and 19, 2016, Rabat, Morocco.

9. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Étude de la contamination par les métaux lourds (Cu, Cr, Pb, Cd) des Patellidae prélevés de la côte atlantique Marocaine**. 2nd Doctoral of science and technology days, organized by the faculty of science and technology on April 14, 15 and 16, 2016, Errachidia, Morocco.

10. **Lalla Meryem Idrissi Azzouzi**, Samira Senouci, Maria El Qazoui, Hicham Oumzil, Mariam Naciri. **Assessing viral contamination in the environnement: Search of *Enterovirus* in mussels collected in Morocco**. 2nd International congress "environment and sustainable development", organized by the superior school of technology on April 20-21, 2016, Sale, Morocco.

11. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Évaluation de la pollution métallique dans l'environnement marin (côte Atlantique Marocaine) en utilisant des bioindicateurs**. COP22 (conference of parties) days, organized by the Mohammedia school of engineers on October 10-11, 2016, Rabat, Morocco.

12. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Évaluation de la pollution métallique et affichée dans l'environnement marin: Étude de la qualité des eaux côtières de la côte Atlantique Marocaine en utilisant des bioindicateurs**. COP22 international congress, organized by the council of the Moroccan community abroad on November 11-12, 2016, Mogador Agdal hotel, Marrakech, Morocco.

13. **Lalla Meryem Idrissi Azzouzi**, Samira Senouci, Maria El Qazoui, Hicham Oumzil, Mariam Naciri. **Viral pollution in the mussels: *Enterovirus* detection by cell culture and real-time PCR**. National workshop protection and enhancement of coastal and marine areas: what vision and what actions for integrated and sustainable management? organized by the national center for scientific and technical research on June 27, 2018, Rabat, Morocco.

14. **Lalla Meryem Idrissi Azzouzi**, Rachid Ben Aakame, Abdellah El Abidi, Mariam Naciri. **Heavy metal (Cu, Cr, Cd and Pb) contamination of *Patella rustica* in Moroccan Atlantic coast**. National workshop protection and enhancement of coastal and marine areas: What vision and what actions for integrated and sustainable management? organized by the national center for scientific and technical research on June 27, 2018, Rabat, Morocco.

List of framed Tutorial Project Memories

1. Memory of tutorial project to get the 1st cycle of paramedical studies (section: laboratory technician), entiteled: «**La détection des *Entérovirus* dans les eaux usées par culture cellulaire au Maroc (étude de cas des villes: Khemisset, Tifelt, Bouznika et Benslimane)**», presented at the ISPITS-Rabat, on 16 June 2014 by Chaimae BELKOH, Nissrine EL HASSANY & Najoua BOUROUAHA to the jury composed of Pr. Fatna EL MEHDAOUI, **Dr. Lalla Meryem IDRISSI AZZOUZI**, Dr. Houda EL OUDIYI & Dr. Fatima EL FALAKI.

2. Memory of tutorial project to get the bachelor in science of life, entiteled: «***Entérovirus* circulants au Maroc chez les enfants de moins de 15 ans, durant une période de 2 ans**», presented at the FSR, on 22 June 2015 by Imane EDDOUNIT, to the jury composed of Pr. Mariam NACIRI, **Dr. Lalla Meryem IDRISSI AZZOUZI**, Pr. Laila Medraoui & Pr. Laila Sbabou.

Résumé

Les mollusques bivalves et gastéropodes sont reconnus depuis longtemps comme étant bénéfiques pour la santé humaine. Ces ressources marines doivent être protégées par la gestion et l'utilisation rationnelle des zones côtières et la préservation de la qualité de l'environnement marin. La consommation de mollusques contaminés par des virus, des bactéries, des parasites, des métaux et des pesticides peut entraîner des maladies graves.

Ce travail a été effectué avec un total de 288 échantillons de moules (*Mytilus galloprovincialis*) et 120 échantillons de patelles (*Patella rustica*) pour évaluer la contamination virale et métallique.

La contamination de bivalves par les *Entérovirus* a été détectée par culture cellulaire, PCR en temps réel et séroneutralisation. Alors que la contamination de gastéropodes par les éléments traces métalliques y compris le cuivre (Cu), le chrome (Cr), le cadmium (Cd) et le plomb (Pb) a été mesurée par Spectrophotométrie d'Absorption Atomique (SAA). L'étude virologique a été effectuée dans le but de détecter et de typer les *Entérovirus* isolés à partir des échantillons de moules, tandis que l'analyse chimique a été menée pour déterminer la concentration et la bioaccumulation de certains métaux essentiels (Cu et Cr) et non-essentiels (Pb et Cd) dans les tissus mous de *Patella rustica*.

Les sites d'échantillonnages sont pollués par des rejets des eaux usées domestiques non traitées ou partiellement traitées, des déchets rejetés par l'homme et des substances introduites par les activités humaines. Les résultats obtenus dans cette étude par analyse virologique et chimique ont montrés que les mollusques bivalves et gastéropodes sont contaminés par des *Entérovirus* et des éléments trace métalliques et peuvent présenter un risque pour la santé humaine. Pour améliorer la qualité sanitaire de ces mollusques il est indispensable de renforcer le réseau de surveillance des écosystèmes aquatiques, en dosant systématiquement les substances chimiques et en effectuant des analyses virologiques.

Mots-clefs : Évaluation de la contamination, *Mytilus galloprovincialis*, *Entérovirus*, *Patella rustica*, éléments traces métalliques, Région de Rabat.

Abstract

Bivalve and gastropod molluscs have long been recognized as being beneficial to human health and this benefit should be taken into consideration in managing the coastal zone and preserving the marine environmental quality. The consumption of molluscan shellfish contaminated with viruses, bacteria, parasites, metals and pesticides may lead to serious diseases.

This work was done to evaluate the viral and metal contamination of bivalve and gastropod molluscs. Therefore, 288 samples of mussels (*Mytilus galloprovincialis*) and 120 samples of limpets (*Patella rustica*) were collected.

The contamination of the bivalve molluscs by enteroviruses was detected by using cell culture, real-time PCR and seroneutralization. Whereas, the contamination of the gastropod molluscs by metallic trace elements such as copper (Cu), chromium (Cr), cadmium (Cd), and lead (Pb) was determined by Atomic Absorption Spectrophotometry (AAS). The virological study was conducted to detect and type enteroviruses isolated from mussel samples, while chemical analysis was performed to determine the concentration and bioaccumulation of certain essential metals (Cu and Cr) and non-essential metals (Pb and Cd) in the soft tissues of *Patella rustica*.

The sampling sites are polluted by untreated or partially treated domestic wastewater, human waste and substances introduced by human. The results obtained in this study by virological and chemical analysis showed that bivalve and gastropod molluscs in sampling sites are contaminated with enteroviruses and metallic trace elements which may be cause a human health risk. For this reason, it is recommended that the sanitary quality of these molluscs is improved by strengthening the surveillance network of aquatic ecosystems, systematically dosing the chemical substances and performing extensive virological analyses.

Key Words : Contamination assessment, *Mytilus galloprovincialis,* enteroviruses, *Patella rustica,* metallic trace elements, Rabat Region.