IONISING RADIATION-INDUCED DNA DAMAGE RESPONSE IN MARINE MUSSELS, *MYTILUS EDULIS*

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SUMMARY

The effects of ionising radiation (IR) present in aquatic environments have been observed principally in vertebrate species but the potential biological impacts for aquatic invertebrate species are less clear. It is important to determine the influence of IR as a pollutant causing DNA damage in invertebrates at the molecular level since this may serve as an early warning of future population level repercussions.

In this study, the biological effects of the IR as an environemntal contaminant at the molecular level was investigated by studying the induction of DNA damage, measured as mRNA expression of DNA repair genes and comet damage, in experimentally- and environmentally-exposed mussels, *M. edulis*. The experimental exposure consisted of different IR doses (1, 2, 10 and 50 Gy) and sampling at different post-exposure time points (1hr, 4 and 7 days). The environmental exposure was investigated using mussels collected from a contaminated site (Ravenglass Estuary) and a reference site (Brighton Marina). Two new molecular biomarkers were developed and employed. The first involves Rad51, a key protein in resynthesis, catalyzing and transferring of strands between broken sequences and its homologues in double strand break (DSBs) damage. The second biomarker involved a cell cycle checkpoint protein, check point kinase 1 (Chk1). To explore the activation of *Rad51* and *Chk1* mRNA activity as a result of exposure to IR, *Rad51* and *Chk1* mRNA in *M. edulis* were partially isolated and characterized and a quantitative assay developed to measure their expression using real-time PCR. Experimental exposure of *M. edulis* to IR (1, 2, 10 and 50 Gy) resulted in a statistically significant increase in the levels of *Rad51* transcripts. *Chk1* mRNA expression levels, initially investigated in the experimental group, were altered following exposure to IR. In the samples collected from the environment, *Rad51* mRNA expression levels were increased in Ravenglass *M. edulis* gonad samples compared with the reference samples from Brighton Marina. In contrast, *Chk1* transcripts decreased in Ravenglass *M. edulis* gonad samples compared to Brighton samples. The observed effects, and the potential role of both *Rad51* and *Chk1* in the IR DNA damage response of mussels are discussed.

CONTENTS

LIST OF ABBREVIATIONS

^{106}Ru	ruthenium-106
^{131}I	iodine-131
^{134}Cs	caesium-134
^{137}Cs	caesium-137
^{14}C	carbon-14
^{144}Ce	cerium-144
14 MeV	mega-electron volt 14
^{18}F	fluorine-18
^{3}H	hydrogen-3 (tritium)
^{35}S	sulfur-35
^{60}Co	copper-60
^{90}Sr	strontium-90
^{95}Zr	zicronium-95
Am-241	americium-241
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
bp	base pairs
Bq	becquerel
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
C/Kg	coulomb per kilogram
Cm-244	curium-244
dNTPs	deoxynucleotide triphosphates
DDT	dichlorodiphenyldichloroethane

DHJ	double holliday junction
DNA	deoxyribonucleic acid
DSBs	double strand breaks
GBq	gigabecquerel
GSPs	gene specific primers
Gy	gray
H2A	histone 2 A
H2B	histone 2 B
H3	histone 3
H4	histone 4
H2AX	histone
hr	hour
HR	homologous recombination
IAEA	international atomic energy agency
IR	ionising radiation
K-40	potassium-40
kBq	kilobecquerel
Kg	kilogram
LD	lethal dose
MB	megabecquerel
µGy	microgray
mGy	milligray
MN	micronucleus assay
mR	milliroentgens
mRNA	messenger ribonucleic acid

MRX	mre11-rad50-nbs1 complex
mSv	millisievert
NCRPM	national council on radiation protection and measurements
NHEJ	non-homologous end joining
NER	nucleotide excision repair
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
PKcs	protein kinases
Po-210	polonium-210
Po-240	plutonium-240
Pu-238	plutonium-238
Pu-239	plutonium-239
QPCR	quantitative plymerase chain reaction
Ra-226	radium-226
RIFE	radioactivity in food and the environment
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcription polymerase chain reaction
SGS1	small growth supressor1
SDSA	synthesis-dependent strand-annealing
TBT	tributyltin
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
UV-B	ultraviolet-beta

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AUTHOR'S DECLARATION

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Ohoud AlAmri

Chapter 1
Literature Review

1.1. General introduction

As the sphere of human influence continues to expand and include larger and larger aspects of the world's global ecosphere, components within this ecosphere that were once thought to be highly resistant to global change are now becoming ecological concerns. One such component is the global ocean and the impact pollution has had on the enormous variety of life contained within it. The field of environmental toxicology involves the study of stress effects on organisms. Stresses can include physical, chemical and biological. Ionising radiation (IR) is an example of physical stress. Studies may investigate the impacts of stress, such as IR or chemical contaminants, at many levels of biological organization from the molecular or sub-cellular level to the population and community levels. Ideally scientists aim to determine a 'cause-and-effect' relationship that links a specific contaminant or stress to a biological end point that is harmful for an organism. Also ideally, this knowledge is used to decide techniques that may give an early-warning of damage that has not yet become visible but which may have a damaging effect in the future.

Oceanic water pollution takes on many different forms and at present nuclear pollution accounts for only a small amount of oceanic pollutants. While significant amounts of radium, plutonium, and other radioactive materials can cause ecological damage in isolated areas, such as a bay neighbouring a leaking radioactive materials depository, the ocean, as a whole, remains relatively unaffected by the global increase in nuclear materials (Lionetto et al., 2004). Isolated areas of the oceans impacted by radiation sources also include sites of weapons testing (Eisenbud, 1973). Aquatic

environments play an important role in our food chain and in maintaing the balance of the public life and environment due to the enormous occupancy of marine ecosystem on earth. Alteration or Changes in the aquatic environment could affect the biota organisms of marine ecosystem. It is well known that aquatic environment have been receiving several chemical and physical agents that cause harmful impacts (IAEA, 1995; UNSCEAR, 1996, 2006). In the near coastal region, however, aquatic biota are much more likely to be impacted by point sources of radioactive contaminants, mainly from nuclear reprocessing plants situated in estuaries (Table 1.1.1) and other nuclear sites in UK (Fig. 1.1.1).

Table 1.1.1. Levels of IR in the environment of some nuclear sites in the UK (RIFE14, 2008).

Site	**Material**	**Radionuclide**	**Mean Radioactivity Concentration, Bq Kg^{-1}**	**Radiation Dose Rate µGy h^{-1}**	**Total Exposure mSv per year**
Springfield (nuclear power plant, operational)	Sediment	^{137}Cs	31-580	0.073-0.14 sediment	0.16 sediment
	Mussels		0.80		
	Mullet		3.5		
Sellafield (nuclear reprocessing plant, operational)	Soil	^{137}Cs	65	0.1	0.47
		^{60}Co	0.70		
	Plaice	^{14}C	190		
Ravenglass near Sellafield	Cod	^{137}Cs	8.5	0.10-0.17	0.046
	Plaice		3.7		
	Crabs		1.1		
	Winkles		7.8		
	Cockles		4.4		
	Mussels		1.4		
	Sediment		130-330		
Whitehaven near Sellafield	Sediment	^{137}Cs	32	0.10	0.47 Molluscs
Dungeness (nuclear power reactor, operational)	Cod	^{137}Cs	0.20		0.4 direct radiation
	Bass		0.36		
Sizewell (nuclear power reactor)	Oyster	^{137}Cs	0.05-<0.06	0.049-0.068	0.031 direct radiation
	Mussels		<0.14		
Winfrith (nuclear reactor research, decommission 2018)	Oyster	^{137}Cs	<0.10	0.052-0.069	<0.005 sediment
	Cockles		0.07		
	Clams		0.10		
	Oyster	^{60}Co	<0.11		
	Cockles		0.15		
	Clams		<0.11		

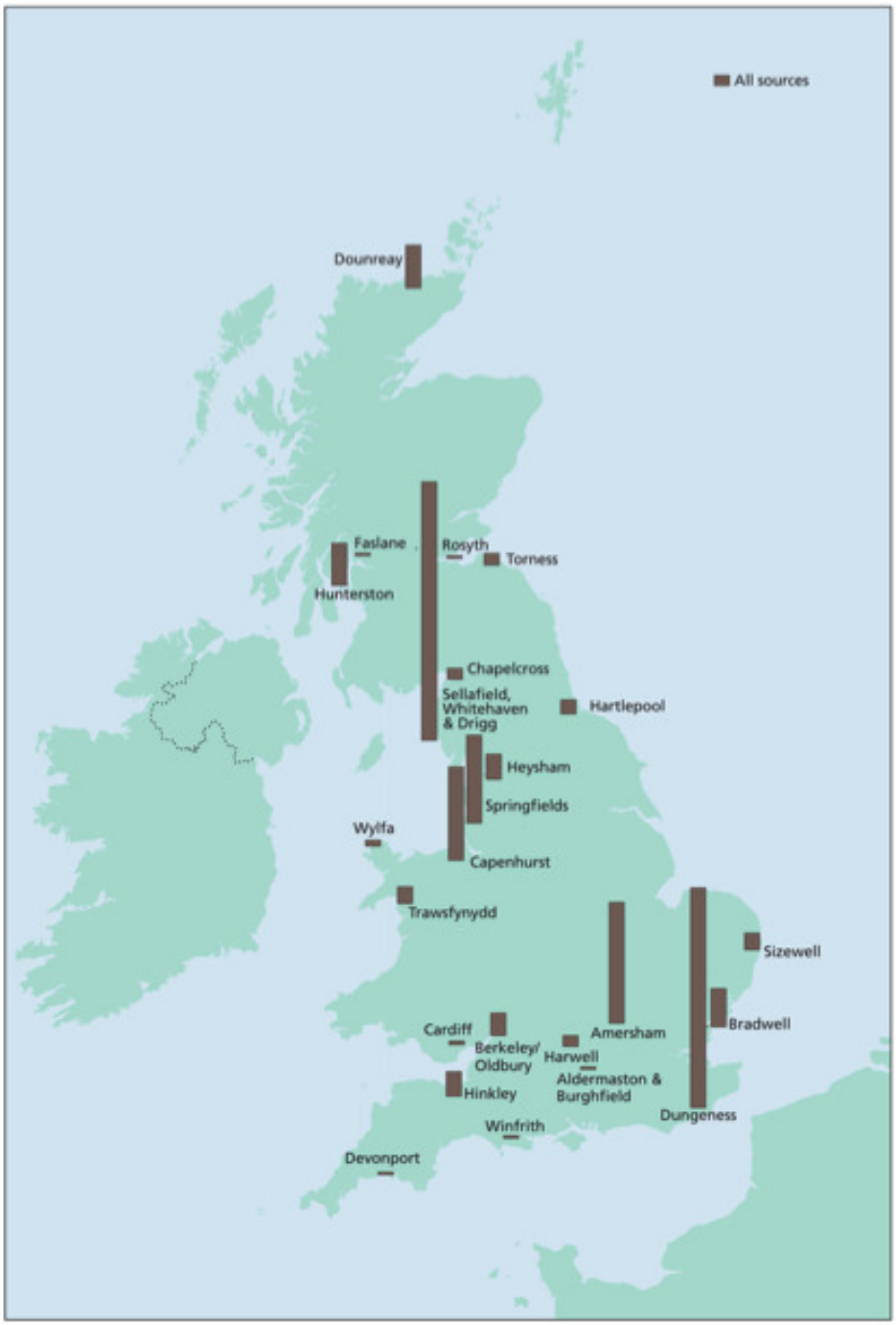


Fig. 1.1.1. Radiation exposure sites in UK from radioactive waste discharges and direct exposure to radiation showing the highest radiation exposure at Sellafield area and Dungeness site (adapted from RIFE 14, 2008).

1.1.1. Sources of radiation in the environment

Exposure to IR may result from background sources as well as radionuclides released during fuel fabrication, the normal operation of nuclear power reactors, nuclear accidents, waste storage sites and past weapons testing. In the U.K. most radiation results from the detonation of nuclear devices and the controlled release of energy by nuclear-power generating plants (Table 1.1.1). For example, discharge ranging from 131 to 1340 Bq Kg^{-1} of Caesium-137 (^{137}Cs) was recorded in Ravenglass mussels (McDonald et al., 1993). Other sources of radiation include spent-fuel reprocessing plants such as that located in Cumbria (Gray et al., 1995), by-products of mining operations (Ahmed, 1981), and experimental research laboratories. In the case of the latter, ^{14}C, ^{18}F and ^{3}H at levels of 870, 353 and 2285 GBq were reported during 2007 in England and Wales (Radioactivity In Food and the Environment 'RIFE'14, 2008). Other sources include hospital discharges such as ^{3}H, ^{14}C, ^{18}F, ^{35}S, ^{131}I and ^{137}Cs gaseous radioactive (RIFE14, 2008).

1.1.2. Caesium-137 (^{137}Cs)

Under normal operation of nuclear power reactors, ^{137}Cs is one of the principal radionuclides present in coolant water of light-water-cooled reactors and it is one of the primary concerns in the environmental studies. ^{137}Cs as a fission product is of ecological concern, it has a high yield from nuclear fission and is one of the major dose-contributing radionuclides in the environment (National Council on Radiation Protection and Measurements 'NCRPM'154, 2006). For example, Sellafield discharges led to estimate dose levels for public health in 2008 of 0.23 mSv of radiation mostly due

to the accumulation of ^{137}Cs (RIFE14, 2008). Individual exposure levels varied according to certain lifestyle habits. Those who consumed shellfish and fish received the highest dose, estimated at 0.6 mSv, (RIFE14, 2008). The levels reported are within the 1 mSv per year considered a safe level for public health exposure (RIFE14, 2008). Interestingly, all the data reported concerning sources of public radiation exposure, including Sellafield, Dounreay, Winfrith, Berkeley, Oldbury, Harwell, Bradwell, Chapelcross, Dungeness, Hinkley Point, Hunterston, Sizewell, Torness, Trawsfynydd, Wylfa, Aldermaston, Devonport, Faslane, Rosyth, Amersham, Cardiff, and Whitehaven ALL cite consumption of contaminated fish and shellfish as the primary source (RIFE14, 2008). Sellafield has released 3.7 X 10^{14} to 5.6 X 10^{15} Bq of ^{137}Cs to the Irish Sea annually (Eisenbud, 1987). Varying amounts of ^{137}Cs were released into the environment during nuclear weapon testing and a number of nuclear accidents such as Windscale in England, Kyshym in Russia and most notably the Chernobyl disaster in Ukraine (Eisenbud, 1987; Leonard et al., 1990). In the UK instance, during October 1957, one of the uranium-reactors was damaged by fire resulting in the release of fission products to the surrounding countryside and the Irish Sea. Radioactivity from the principal isotopes, including ^{137}Cs, released during the fire was estimated at 6.5 to 7.7 X 10^{14} Bq (Eisenbud, 1987). Also in May 1986, another estimated 8 X 10^{19} Bq of radioactivity, including ^{137}Cs, was released from Chernobyl accident-destroyed reactor in Ukraine (Eisenbud, 1987). ^{137}Cs was considered the most significant contaminant because of its high concentration in these fallouts. As of 2005, ^{137}Cs is considered the principal source of radiation in the zone of alienation around the Chernobyl nuclear power plant (NCRPM154, 2006). Due to ^{137}Cs mainly being a fission product, it did not occur in nature prior to extensive nuclear weapons testing. In biota, ^{137}Cs will be distributed throughout the soft tissue of the body. It also binds very firmly to clay

particles in both soil and sediments. In an aquatic system, ^{137}Cs will move from the water compartment to sediments, where it is available to detritivores and bottom feeders. ^{137}Cs concentration factor for molluscs is typically around 100 and 10 (International atomic Energy Agency 'IAEA', 1982; Peterson, 1983). To summarise, ^{137}Cs is of ecological concern in that it has a high yield from nuclear fission and is one of the major dose-contributing radionuclides in the environment. Other radionuclides are also have been released to the environment due to Chernobyl accident (IAEA, 2006), recently Fukushima nuclear accident and they are considered harmful depending on their half lives and exposure period (Table 1.1.2.1).

Table 1.1.2.1. Effects, usage, half lives and radioactive decay of selected isotopes produced in the environment.

Isotope	**Type Decay mode**	**Half lives**	**Production and usage**	**effect**
^{239}Pu plutonium	α emitter	24.110 yrs	Used as nuclear fuel in nuclear reactors and in nuclear weapons.	decreased life spans, diseases of the respiratory tract, and cancer. Health issue with lungs and associated lymph nodes, liver, and bones.
$^{240,241,242}Pu$ plutonium	α and β emitters	6563,14,373 yrs	nuclear fuel used in a thermal reactor, the design of all nuclear power plants.	
^{238}Pu plutonium	α emitter	87.8 yrs	plutonium-producing reactors.	
^{90}Sr strontium	β emitter	28.8 yrs	Nuclear reactors and in nuclear fallout from nuclear tests	Bone cancer or leukemia
^{14}C radiocarbon	β emitter	5,730 ± 40 years	Fossil fuels such as petroleum or coal	Cell damage to cancer
^{210}Po polonium	α emitter	138.376 dys	Nuclear reactor	Cancer deaths
H3 tritium	β emitter	12.32 yrs	Nuclear weapons	health effects: cancer, genetic effects and effects on fetuses.
^{238}U ^{235}U uranium	α emitter	4.47 billion yrs 704 million yrs	Nuclear weapons and nuclear power plants	Renal failure, brain damage, tumors and DNA damage.

^{60}Co cobalt	β and γ emitters	5.27 yrs	As a tracer for cobalt in chemical reactions, sterilization of medical equipment, also as radiation source for medical radiotherapy, industrial radiography, leveling devices and thickness gauges, food irradiation and blood irradiation, and laboratory use.	Cancer to death
^{192}Ir iridium	β and γ emitters	73.83 dys	Industrial radiography and radiotherapy	Skin changes, osteonecrosis and osteomyelitis
^{131}I iodine	β and γ emitters	8 dys	production is from nuclear reactor medical and pharmaceutical	Mutation and death in cell
^{232}Th Thorium	α emitter	14.05 billion yrs	used as fuel in a nuclear reactor, and it is a fertile material, which allows it to be used to produce nuclear fuel in a breeder reactor.	increased risk of cancers of the lung, pancreas, and blood, as lungs and other internal organs, exposure to thorium internally leads to increased risk of liver diseases.
^{40}K Potassium	β emitters	1.3 billion yrs biological half-life 30 days	Potassium-40 is the largest source of natural radioactivity in animals and humans.	cell damage caused by the ionizing radiation, with the general potential for subsequent cancer induction.

1.1.3. Evidence of radiation in the biota from the aquatic environment

Pollution is an on-going problem in all ecosystems. Pollution is the “presence of a foreign substance—organic, inorganic, radiological, or biological—that tends to degrade the quality of the environment so as to create a health hazard” (Moore, 2002).

Public concern over the release of radiation into the environment greatly increased following the disclosure of possible harmful effects to the public from nuclear weapons testing, especially the accident (1979) at the Three Mile Island nuclear-power generating plant near Harrisburg, Pa. USA, and the 1986 explosion at Chernobyl. In the late 1980s, revelations of major pollution problems at U.S. nuclear weapons reactors raised concern again. The medical research field has thus identified IR as a source of pollution for humans. Here we are concerned with the potential impacts on the biota in the aquatic environment. Table 1.1.3.1. summarizes the knowledge regarding levels of IR in different biota.

Table 1.1.3.1. Summary of IR levels reported and induced biological effects observed in marine and terrestrial organisms.

IR source	**Organism**	**Exposure regime**	**Biological effects**	**Reference**
Po-210	*Perna perna*	155 Bq/kg wet weight, 0.02 mGy/d	No increase in micronuclei frequency nor DNA strand breakage	Godoy et al., 2008
Ra-226	*Hediste diversicolor*	30-6600 Bq/kg	Uptake confirmed, no effect on oxygen radical scavenging parameters	Grung et al., 2009
^{137}Cs & tritiated water	*Ophryotrocha diadema*	7.3 Gy/hr	Decrease in number of larvae and eggs produced	Knowles & Greenwood 1997
^{137}Cs	*Neanthes arenaceodentata*	2 Gy 4 Gy	Increase in chromosomal aberrations Decrease in broodsize	Anderson et al., 1990
^{137}Cs	*N. arenaceodentata*	5-10 Gy 0.5 Gy	Decrease in broodsize Increase in embryo mortality	Harrison & Anderson 1994a
^{60}Co	*N. arenaceodentata*	Chronic doses: 0.19-17 mGy/hr; total dose 0.55-54 Gy	Increase in embryo mortality at highest dose. Increased number and % of abnormal embryos	Harrison & Anderson 1994b
Tritiated water	*M. edulis* embryos	0.02-21.14 mGy	Dose dependent increase in sister chromatid exchange between 3.7-370 kBq/ml	Hagger et al., 2005a

			Increase in chromosomal aberrations at 3.7 kBq/ml	
Tritiated water	*M. edulis*	12-485 mGy/hr for 96 hrs	Increase micronuclei frequency and DNA strand breakage	Jha et al., 2005
Am-241, Cm-244, Pu-238, Pu-239, Po-240, ^{137}Cs, K-40, French Coast	*Crassostrea gigas*	Field samples Highest values: 0.5 Bq/kg dry weight ^{137}Cs	No significant difference in mRNA expression of selected stress response genes (heat shock proteins, metallothionein, superoxide dismutase)	Farcy et al., 2007
^{60}Co	*M. edulis*	0.9 Gy/hr 2 Gy/hr	Decrease of gill epithelial cell cilia beat frequency Stopped cilia beats	Karpenko & Ivanovsky 1993
Tritiated water	*M. edulis*	Dose rate at 122 and 79 mGy/hr for 7 and 14 days	bioaccumulation of tritium in foot, gills, digestive gland, mantle, adductor muscle and byssus, significant induction of micronuclei in the haemocytes of mussels	Jaeschke et al., 2011
Gamma rays	*Crepis tectorum*	0.02-20 mR/hr	Chromosome aberrations in root cells	Grinik & Shevchenko 1992
^{60}Co	*Pissum sativum* L. Pea seeds	80-100 Gy	Significant inhibition in growth factor, decreased plant height, water exchange and impacted enzyme activity	Stoeva 2002
^{60}Co	*Cicer arietinum* (Kabuli chickpea)	100-1000 Gy	Increased germination time, decrease germination percentage, decreased shoot length of seedling and root length, higher peroxidase and protease activities and lipid peroxidation contents	Hameed et al., 2008
	Cicer arietinum (Desi chickpea)	400 Gy	Increased peroxidase activity, decreased shoot length of seedling and root length, lowered lipid	

			peroxidation contents, no effect on protein content and protease activity	
γ-irradiation	Human cultured cells	0.5, 2 and 10 Gy, analyzed at different time points	Increased Rad51 and Rad50 nuclear focus formation	Yuan et al., 2003
X-ray	Human cell line	6 Gy	Increase Rad51 protein expression	Chinnaiyan et al., 2005
γ-irradiation	Human cell line	5 and 10 Gy, dose rate of 1.06 Gy/min	Higher doses of radiation induced elevated expression of Rad51 protein	Taghizadeh et al., 2009
γ-irradiation	Rat liver	8-25 Gy	A significant induction of chemokines gene expression	Malik et al., 2010
γ-irradiation	Plaice	0.24 mGy/h for 197 days	Significant reductions in testis due to decreased amounts of sperm	Knowles 1999
γ-irradiation	Rainbow trout	1.87, 3.73 and 9.03 mGy/h for 246 days after fertilization	Significantly lower immune response	Knowles 1992
Tritiated water	*Oryziaslatipes* embryos	9.25-37 MBq/ml	No reduction in hatching rate but reduction in survival of fry was detected in irradiated groups within 1 month after hatching and number of vertebrae decreased	Hyodo-Taguchi & Etoh 1993
^{137}Cs rays		0.44-1.89 Gy/day		

1.1.3.1. Water and sediment IR levels

There have been a number of studies that have quantified the levels of IR in water and sediments. Several studies reported radionuclide discharges, such as ^{137}Cs, ^{60}Co, ^{14}C, ^{90}Sr, derived from nuclear plants into rivers (Hong et al., 1999; Gulliver et al., 2004; Cook et al., 2004; RIFE14, 2008). A very high annual dose, in case of human, from both natural and artificial radionuclides was estimated to be 0.046 mSv in a source of drinking water from Silent Valley, Co Down compare to the mean annual dose (0.028 mSv) of drinking water consumption in the UK (RIFE12, 2006). Sediment-

associated radionuclides are more likely to have impacts in near-shore waters either through direct contact with humans or through uptake by food organisms especially filter-feeding organisms. Among potential depositional sites are beaches, estuaries and their tidal flat areas and open continental shelves. Sediment samples from the Rivacre Brook contained very low but measurable concentrations of technetium-99; also of uranium, which was enhanced above natural levels close to the discharge point (RIFE12, 2006). The highest radioactivity concentration of ^{60}Co, ^{90}Sr, ^{95}Zr, ^{106}Ru, ^{134}Cs, ^{137}Cs and ^{144}Ce in sediments were 25, 330, <6.8, <66, <4.9, 1300 and <8.8 Bq kg^{-1} in Ravenglass, River Mite Estuary, Ravenglass, Ravenglass, Skippool Creek, River Mite Estuary and Ravenglass respectively (RIFE12, 2006). In Sellafield, an increase (0.13 mSV) of gamma dose in intertidal sediments during 2008 was recorded compared to 0.073 mSv during 2007, and this was reportedly due to the increase of gamma dose in the estuarine environment (RIFE14, 2008). Aquatic environments were also impacted by the Chernobyl atomic power plant accident in 1986. Exposure of such organisms may occur externally due to radiation present in water and sediment and the absorption of radionuclides onto the surface of biota, and internally as a consequence of absorption or ingestion.

The IAEA (1976) reported that the annual doses received by marine and freshwater biota from natural sources of radiation are generally less than 5 mGy/year. Nevertheless, a range of γ-radiation doses of 0.5, 2.5 and 10 mGy/day was recommended as a 'safe' population dose depending on the type of species (UNSCEAR, 1996; Environment Canada, 2000). For chronic exposures to radiation, a dose of 40 and 400 μGy/h are reported to produce non-hramful effects on terrestrial and aquatic ecosystems (NCRPM109, 1991c; IAEA, 2003). At the observable effect level, a range of 9.6 to 24 mGy/day was reviewed as the lowest dose range that might produce adverse

effects on aquatic organisms (IAEA, 2003). However, many of freshwater environments that have been studied for radiation effects contain radionuclides at above-background-concentrations. For the most part, these studies have shown the resilience of populations of freshwater biota to doses of less than 10 mGy/day (IAEA, 1976; NCRPM109, 1991c; IAEA, 1992).

1.1.3.2. IR levels reported in aquatic plants

Experimental field studies using ^{137}Cs as an acute and chronic gamma radiation exposures have provided data on effects on natural communities of plants. Experiments have been conducted showing that radiation is mainly a problem when a plant is in the stage of seedling (Table 1.1.1). High doses of radiation can cause seeds to not sprout, grow slowly, lose fertility or develop genetic mutations that can change characteristics of the plant. Most laboratory research on radiation effects on plants has been performed with seeds and seedlings (Xiuzher, 1994; Stoeva et al., 2001; Stoeva, 2002; Hameed et al., 2008). In the most sensitive plant species, the effects of chronic irradiation were noted at dose rates of 1000 to 3000 mGy/hr. It was suggested that chronic dose rates of less than 400 mGy/hr (10 mGy/day) would have effects, although slight, in sensitive plants (United Nations Scientific Committee on the Effects of Atomic Radiation 'UNSCEAR', 1996). They would be unlikely, however, to have significant deleterious effects in the wider range of plants present in natural plant communities (IAEA, 1992). The total internal dose rate was calculated for aquatic plants to be 1.40 rad/year (Blaylock and Witherspoon, 1975). Wood (1987) showed tissue damage, photopigment destruction, reduced growth and low survivorship of sub-canopy kelp sporophytes after exposure to radiation. Photosynthesis was inhibited in phytoplankton, benthic

macroalgae and seagrasses after UV-B irradiation (160 μE/m^2/sec) over periods of 15-30 mins (Larkum and Wood, 1993) and this inhibition is shutting down the photosynthesis, food producing, in the plants by affecting partial reactions of photosynthesis. Recent studies have indicated that radiation can deleteriously affect physiological processes and overall growth in a number of plant species (Tevini, 2000; Rathore et al., 2003; Prasad et al., 2004). In the study of Mishra and Agrawal (2006) a reduction in the photosynthetic pigments and catalase activity of spinach plant (*Spinacia oleracea*) resulted after UV-B radiation exposure.

The use of large gamma sources, such as those used to show changes in plant communities, is a questionable method for demonstrating changes in animal populations and communities because many animals, such as invertebrates, are dependent on the presence of vegetation, which may be destroyed by the radiation. Moreover, radiation doses in the environment are difficult to estimate since this decreases with distance from the source (Krivolutzkii and Pokarzhevski, 1992).

1.1.3.3. Levels and biological effects of IR in aquatic invertebrates

Radiation-induced somatic and genetic effects have been observed in individual organisms following acute exposures in the laboratory (Table 1.1.3.1. for summary) (Templeton et al., 1971; IAEA, 1976; Woodhead, 1984; NCRPM11, 1991b; NCRPM109, 1991c). Around the Chernobyl zone, the soil worm *Aporectodea caliginose*, a diploid species, displayed genetic damage in its male germ cells (chromosome fragments in 20% of the cells), and the population size was smaller in the contaminated zone than in a reference area (Krivolutzki and Pokarzhevski, 1992). Sokolov et al. (1989) reported an increase in dominant lethal mutations in fruit flies

(*Drosophila melanogaster*) collected from an area with a radiation dose of 80.6 mR/hr compared with a reference area. A field experiment conducted by Cooley (1973) in the early 1970s examined the effects of chronic irradiation on the population of an aquatic snail, *Physa heterostropha*. White Oak Lake snails, receiving a dose of 6.5 mGy/day, were found to have a significantly lower number of egg capsules per snail than did snail from the control population (Cooley and Miller, 1971).

In the field, studies have been conducted using animals confined to enclosures and irradiated with chronic doses. For example, populations of three worm species were studied in a lake at the Chernobyl zone and a higher frequency of chromosomal abberations were reported when compared to worms collected from a reference lake, and this was attributed to the low dose rate of IR exposure (IAEA, 1976; Tsytsugina, 1998; Copplestone et al., 2000). In the laboratory, several studies have been reported on the acute response of fishes and invertebrate species (White and Angelovic, 1966; Engel, 1973; Nakatsuchi and Egami, 1981; UNSCEAR, 1996). These report LD_{50} values, the dose lethal to 50% of organisms within 30 or 60 days (Anderson and Harrison, 1986; Harrison and Anderson, 1994; UNSCEAR, 1996). Higher levels of radiation exposure, either acute or chronic, are necessary to show effects on populations of animals (Templeton et al., 1971; Turner, 1975; Whicker and Schultz, 1982; Woodhead, 1984; UNSCEAR, 1996) since lower doses may not bring about an observable effect.

Aquatic environment have long been a cause for ecological concern since the impact within this system has, for many years, not displayed any obvious signs of intense distress, but as researchers have investigated various changes within isolated species, a detrimental pattern has begun to form (Borcherding, 2006). Mussels are a

type of bivalve mollusc (Fig. 1.1.3.3.1) that inhabits various aquatic ecosystems around the world. They are often found in intertidal areas, where they form large beds along the sea floor or colonies attached to underwater cliffs, rocks, or pillars. Mussels are filter feeders. They take in water through a siphon, force the water through their gills, where plankton is captured and digested, and then excrete the waste water through a separate siphon.

Fig. 1.1.3.3.1. Blue mussels distributed around Ravenglass, Cumbria, showing the external characteristic of Sellafield *M. edulis*.

Mussels possess several attributes that recommend them as a suitable indicator organism in environmental monitoring programmes. Due to their sessile nature, wide geographical distribution, large population and high filtering rates, mussels have long been regarded as promising bioindicators and biomonitoring subjects. They demonstrate high accumulation of pollutants, particularly heavy metals (Gardenfors et al., 1988; Hagger et al., 2005b) and radionuclides (Teliitchenko, 1969; McDonald et al., 1993; Valette-Silver and Lauenstein, 1995; Gaso et al., 1995; Alam et al., 1999; Yamada et al., 1999; Burger et al., 2007; The´bault et al., 2008). They are commercially important seafood and the accumulation of radionuclides in their tissues is extremely important for

public health considerations. They are thus considered as an ideal model for use in environmental toxicology (Hart, 2003; Rittschof and McClellan-Green, 2005).

Following the above mentioned mussels advantages, a series of biomonotiring studies from international programmes like “Mussel Watch” (Goldberg, 1975) to smaller scale but nevertheless as importants experiments (Leinio and Lehtonen, 2005) employed *M. edulis* populations to assess the health of the environment in which they thrive.

Molluscs were collected from the Dnieper drainage area and throughout the Kiev administrative region following the Chernobyl nuclear accident (Frantsevich et al., 1996). Radioactivity in shells and soft tissue were found to exceed pre-Chernobyl concentrations by factors as great as three orders of magnitude. The highest recorded concentrations were 4 to 5 MBq/kg in shells of *Lymnaea sp.* and *Planorbarius sp.*. Bivalve mollusc populations of *Anodonta cygnea* appear to be recovering and are actively growing following the radiation insult; however populations of *Dreissena sp.* continue to be decrease (Sokolov et al., 1993). Field studies on the effects of radiation on the marine environment are primarily limited to those that have been conducted in the North Irish Sea. However, pollution has produced noticeable damage to mussel populations through a variety of means. In some areas, chronic pollutant exposure has caused density and diversity reduction in the molluscs (Crowe et al., 2004). The exact nature of DNA damage caused has not yet previously been characterized. From the standpoint of survival of the population, reproduction is the most sensitive indicator of radiation. Chronic exposures of ≤10 mGy are very unlikely to produce measurable deleterious changes in populations or communities of aquatic animals (NCRPM112, 1991a). However most radioactive wastes have half-lives of hundreds to thousands of

years. Surveys of the literature indicate a lack of data on chronic exposures in the environment, especially at the population and community level of organization (Whicker and Schultz, 1982; Woodhead, 1984; NCRPM11, 1991b).

1.1.4. Effect of IR on DNA structure

In the environemnt organisms are exposed to multiple stressors and it is difficult to interpret the biological endpoints caused by which pollutants. Using molecular approaches and studying the impact on DNA is one way to determine cause-effect relationships. Depending on total dose, dose rate, type of radiation, and exposure period, radiation can lead to no observable health effects, genetic changes, physiological changes such as effects on the hemopoietic and reproductive systems, effects on growth and development, or life shortening, including cancer or death (IAEA, 1976; UNSCEAR, 1994; 2006). However, even when effects are not observable, there is a possibility of increased risk of cancer or life shortening. In the laboratory, where most studies have focused on response to acute doses, total dose and dose rates can be closely estimated. The aim of this work is to exploit molecular techniques and changes in the nucleic acids (mRNA expression and DNA damage) to investigate the impact of low doses of radiation in organisms otherwise showing no observable damage.

The environmental effects of exposure to high-level IR have been extensively documented through postwar studies on individuals who were exposed to nuclear radiation in Japan. Some forms of cancer show up immediately, but latent illnesses of radiation exposure have been recorded from 10 to 30 years after exposure (Dobyns and Hyrmer, 1992; Cetta et al., 1997). The effects of exposure to low-level radiation are not yet known. A major concern about this type of exposure is the potential for genetic

damage. Over a 3-year period, recovery of the exposed populations (workers and inhabitants) to Chernobyl accident took place, either by immigration of animals into the area or by a decrease in mortality and lethal genetic effects with time. Many of the studies address accumulation of genetic changes in the resident populations, the consequence of which are presently unknown (Templeton et al., 1971; Krivolutzki and Pokarzhevski, 1992; Zainullin et al., 1992; Sokolov et al., 1993). There are many other radiation biological effects that can cause genomic instability by increasing cell mutations and their offspring mutations or minisatellite mutations meaning inherited germline DNA changes (Ellegren et al., 1997; Kovalchuk et al., 2000; Kovalchuk et al., 2003; Dubrova et al., 2002; Committee Examining Radiation Risks of Internal Emitters 'CERRIE', 2004) and bystander effects which referred to cells next to irradiated cells that could also be damaged (Watson et al., 2000; Xue et al., 2002). All of these events confirmed the influence of radiation at the genetic level, however more advanced research or techniques are recommended.

The sensitivity of molecular techniques allows investigators to document molecular damage in many organisms. IR induces focus formation of DNA repair proteins as a marker of DNA damage and as well as cell cycle checkpoint mechanisms. Molecular damage generally illustrate a sub-lethal endpoint that may present an early warning of potential pollutant's influence, but the consequences of molecular damage to higher levels of biological organization have not been well documented due to the need of distinguishing between radiation biology and radiation ecology (Clements and Kiffney, 1994; Underwood and Peterson, 1988; Forbes and Calow, 1996).

1.2. DNA repair pathways: general and specific to radiation sources

A large number of studies suggests that double-strand breaks (DSBs) induced in DNA by IR are critical lesions, which lack of repair or inaccurate repair can lead to cell death, or cause its transformation to a cancer cell (Khanna and Jackson, 2001; Thacker, 2005). Moreover, low-level IR as 0.5 Gy may induce irreparable lesions in cells (for example, retinal rat cells) which can lead to cell death (Borges et al., 2008). At several cell-cycle checkpoints, the cycle stops if damaged DNA is detected. After DNA damage, cell cycle checkpoints signaling is activated. Checkpoint activation pauses the cell cycle and gives the cell time to repair the damage before continuing to divide. DNA damage checkpoints occur at the G1/S and G2/M boundaries.

Checkpoint activation is controlled by two master kinases, Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) also known as Serine/threonine-protein kinase. ATM responds to DNA double-strand breaks and disruptions in chromatin structure (Bakkenist and Kastan, 2003), whereas ATR primarily responds to stalled replication forks. These kinases phosphorylate downstream targets in a signal transduction cascade, eventually leading to cell cycle arrest. Cells have developed efficient repair mechanisms to remove DSBs and restore integrity of the DNA. DNA repair mechanisms and cellular recovery processes serve to reduce radiation damage. Characterization of these processes is crucial for a complete understanding of the consequences of exposure to radiation, inducing DSBs.

The rate of DNA repair is dependent on many factors, including the cell type, the age of the cell, and the extracellular environment. DNA damage accumulation in the cell or error repair action can lead the cell to enter one of three possible states: 1) an irreversible state of dormancy or ‘senescence’ (Aguirre-Ghiso, 2007), 2) apoptosis or

programmed cell death (Fu et al., 2006; Branzei and Foiani, 2008) or 3) tumor formation due to unorganised cell division (Branzei and Foiani, 2008). The DNA repair mechanism in the cell is critical to maintain the integrity of the cell genome and to preserve normal functioning in the organism. Many genes, such as insulin and insulin-like growth factor, involved in DNA damage repair and protection were having influence on the life span of the organisms (Browner et al., 2004). Mutations introduction in the genomes of the offspring and its consequences influence on the rate of evolution are related to the presence of molecular lesions in the gametes cells (Lynch et al., 1995; Lande, 1998; Jha, 2004; CERRIE, 2004).

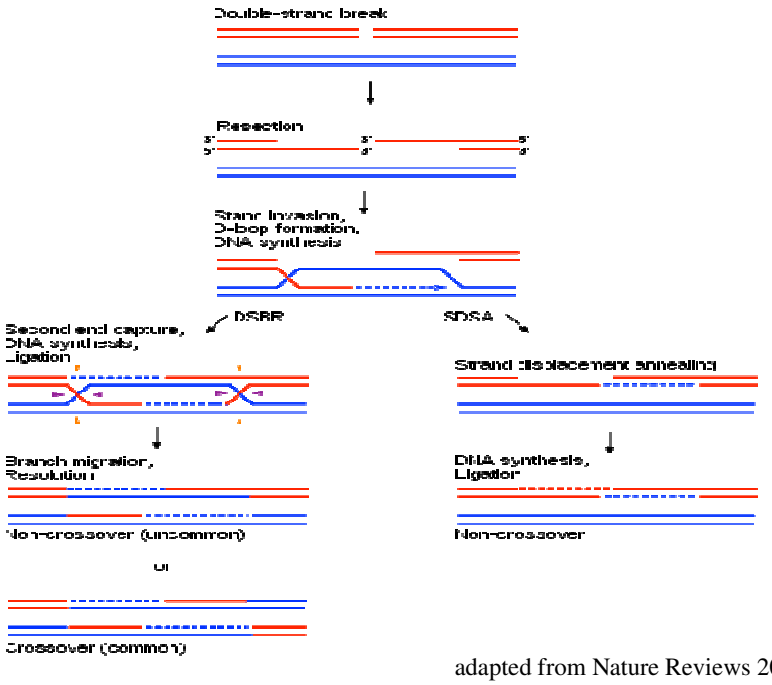
There are a number of DNA damage repair pathways and these are dependent on the type of damage or source of damaging agent. DSBs is the precept cytotoxic lesions caused by IR, however single strand breaks (SSBs) can also be produced by IR. SSBs are formed on one strand of the DNA and repaired by excision repair mechanisms (Caldecott, 2008). On the other hand, DSBs are produced on the two strands of DNA and can be efficiently repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) (Baumann and West, 1998; Sherr, 2004). The mechanisms that regulate these repair pathways throughout the cell cycle vary widely between species (Shrivastav et al., 2008).

1.2.1. Homologous recombination (HR)

HR is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells, which divide by mitosis, to accurately repair DSBs in DNA caused by IR (Griffiths et al., 1999; Lodish et al., 2000). HR appears to be the preferred mechanism

by which DSBs are repaired in lower eukaryotes, such as yeast (Orr-Weaver et al., 1981; Orr-Weaver et al., 1983; Orr-Weaver and Szostak, 1983). HR repairs DNA before the cell enters mitosis (M phase). It occurs during and shortly after DNA replication in the S and G_2 phases of the cell cycle (Alberts et al., 2008). Two models for how HR repairs DSBs in DNA are the double holliday junction model (DHJ) and the synthesis-dependent strand-annealing model (SDSA) (Fig. 1.2.1.1) (Sung and Klein, 2006). After a double-strand break occurs, in humans, a protein complex consisting of Mre11, Rad50 and Nbs1 (MRX) bind to DNA on either side of the break (Daboussi et al., 2002). Next a resection is carried out in two distinct steps mainly is trimming the 5’ ends on either side of the break to create short 3’ overhangs of single-strand DNA then is resection continued by small growth suppressor (Sgs1) and nuclease activity which allows cutting of the single-stranded DNA (Mimitou and Symington, 2009). With the help of several proteins, including Rad51 and Dmc1, binding of the 3’ overhang strand is mediated and consequently nucleoprotein filament start to form. A strand invasion occurs when the filament finds the similar sequence to the 3’ overhang and provides a template, which is identical to the damaged DNA for repair. However, in meiosis, it starts to provide a similar and not identical chromosome (Sung and Klein, 2006). A displacement loop (D-loop) is formed during strand invasion between the invading 3' overhang strand and the homologous chromosome. After strand invasion, a DNA polymerase extends the end of the invading 3' strand by synthesizing new DNA. This changes the D-loop to a cross-shaped structure known as a Holliday junction. Following this, more DNA synthesis occurs on the invading strand (i.e., one of the original 3' overhangs), effectively restoring the strand on the homologous chromosome that was displaced during strand invasion (Thacker, 2005; Sung and Klein, 2006). After the stages of resection, strand

invasion and DNA synthesis, the DSB and SDSA pathways become distinct (Sung and Klein, 2006) (Fig. 1.2.1.1).



adapted from Nature Reviews 2006

Fig. 1.2.1.1. Mechanisms of homologous recombination DNA repair.

Briefly, DSB pathway is unique in that the second 3' overhang (which was not involved in strand invasion) forms a Holliday junction with the homologous chromosome. The double Holliday junctions are then converted into recombination products and results in crossover, though it can sometimes result in non-crossover products (McMahill et al., 2007). The DSB pathway is a likely model of how HR occurs during meiosis while SDSA pathway occurs in cells that divide through mitosis and results in non-crossover products. In this model, the invading 3' strand is extended along the recipient DNA duplex by a DNA polymerase, and is released as the Holliday junction between the donor and recipient DNA molecules slides. The newly synthesized 3' end of the invading strand is then able to anneal to the other 3' overhang in the

damaged chromosome through complementary base pairing. After the strands anneal the SDSA pathway finishes with the resealing, also known as ligation, of any remaining single-stranded gaps (Helleday et al., 2007).

In the pathway of HR, in which *RAD51*, *RAD52*, and *RAD54* appear to be the most essential genes in *S. cerevisiae* for repairing radiation-induced DSBs, human and mouse homologs were readily isolated by preparing primers based on the most conserved regions of these proteins. In eukaryotes, RAD51 is the protein that carries out DSB repair by HR.

1.2.2. Non homologous end joining (NHEJ)

NHEJ is a DNA repair mechanism, which unlike HR does not require a long homologous sequence to guide repair. It is referred as "non-homologous" because the break ends are directly ligated without the need for a homologous template. NHEJ is evolutionarily conserved throughout all kingdoms of life and is the predominant DSBs repair pathway in mammalian cells (Guirouilh-Barbat et al., 2004). NHEJ is predominant in the G_1 phase of the cell cycle, when the cell is growing but not yet ready to divide. It occurs less frequently after the G_1 phase, but maintains at least some activity throughout the cell cycle. NHEJ typically utilizes short homologous DNA sequences called microhomologies to guide repair. These microhomologies are often present in single-stranded overhangs on the ends of DSBs. When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately (Wilson and Lieber, 1999; Budman and Chu, 2005).

1.2.3. Nucleotide Excision Repair (NER) and Base Excision Repair (BER)

NER and BER are important DNA repair mechanisms activated in response of DNA damage caused by several damaging agents including IR (Seeberg et al., 1995; Kuipers et al., 2000). In the case of NER, the severe human diseases is resulted from in-born genetic mutations of NER proteins including Xeroderma pigmentosum and Cockayne's syndrome evidence the importance of this repair mechanism (Friedberg, 2001; McKinnon, 2009). The NER enzymes recognize bulky distortions in the shape of the DNA double helix. Recognition of these distortions leads to the removal of a short single-stranded DNA segment that includes the lesion, creating a single-strand gap in the DNA, which is subsequently filled in by DNA polymerase, which uses the undamaged strand as a template (Sancar, 1996). BER, on the other hand, is a repair system that responds to oxidative DNA damage caused by IR (Seeberg et al., 1995; Chaudhry, 2007). BER mainly function on removing damaged bases that might cause lesions in later stages of DNA replication. The mechanism of BER results short and long patch of DNA strand depending on several factor such as the cell cycle stage (Fortini and Dogliotti, 2007).

1.3. Biomarkers of radiation-induced damage utilized in the medical research field

Approches to estimate or determine the impact of IR can be categorised to physical, biological and clinical dosimetry. Clinical dosimetry refered to nausea, vomiting, blood cell counts, skin reaction and physical dosimetry is refered to dose and other personal dosimetres while biological dosimtery, the interest of this study, is refered to cytogenitic approches such as chromosome abberations (Cabs), fluorescence in situ hybridization (Fish) and micronucleus assay (MN). In the medical field more

DNA damage biomarkers such as DNA mutations, H2AX phosphoryaltion, comet assay, protein levels and gene expressions are used to estimate the impact of IR. However, DNA repair mechanisms and cellular recovery processes serve to reduce radiation damage. Recent technology has made it relatively easy to measure cellular and molecular abnormalities based on such damage and processes. Here we introduce the Comet assay and micronucleus assay as general assays to determine DNA damage.

1.3.1. Comet Assay

The single cell gel electrophoresis assay, which is also known as comet assay is an common and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell (McKelvey-Martin et al, 1993; Wilson et al., 1998; Rank and Jensen, 2003; Jha, 2008; Frenzilli et al., 2009). The resulting image of the comet assay that obtained resembles a "comet" with a distinct head and tail. The head is composed of intact DNA, while the tail consists of damaged (single-strand or DSBs) or broken pieces of DNA. It has since gained in popularity as a standard technique for evaluation of DNA damage/repair (Muller et al., 1996; Kumaravel and Jha, 2006; Jha, 2008), biomonitoring (Kassie et al., 2000; Moller, 2006) and genotoxicity testing (Moller, 2005). In the study of Muller et al. (1994) investigating comet assay in DNA damage and repair on tumour cells after radiation (0.1-10 Gy), they found that the comet tail lengths decreased in the course of time, indicating repair of DNA damage. Also Aka et al. (2004) found an induction of DNA damage in workers exposed to low dose IR using the Comet assay. Garaj-Vrhovac and Zeljezic (2004) estimated the possibility of applying comet assay in the evaluation of DNA damage caused by different gamma radiation doses (0.5, 4 & 10 Gy of ^{60}Co) in human lymphocytes, they found increase in

the tail and the tail moment as the dose increased.

IR can generate free radicals that cause DNA oxidative damage, radiolysis of body water which is considered as an indirect radiation effect. Vanloon et al. (1993) studied induction and repair of DNA SSBs at different stages of hamster spermatogensis treated with IR, and slow repair of base damage in irradiated cells was observed which may influence character of spermatogensis. Moreover, Collins et al. (1995) applied comet assay, on human lymphocytes irradiated with UV-C at a dose rate 4 J/m^2, to detect strand breaks and reported presence of comets with clear tails.

1.3.2. H2AX phosphorylation

DSBs trigger a complex set of responses including cell cycle arrest, relocalization of DNA repair factors and in some cases apoptosis (Morrison et al., 2000). Failure to arrest cellular functions can lead to genomic instability (Thacker, 2005). *H2AX* is one of several genes coding for histone H2A. In humans and other eukaryotes, the DNA is wrapped around histone-groups, consisting of core histones H2A, H2B, H3 and H4. Thus, the H2AX contributes to the histone-formation and therefore the structure of chromatin (Izzo et al., 2008). Phosphorylation of the histone H2AX is one of the first cellular responses to DNA DSBs (Medvedeva et al., 2007). H2AX becomes phosphorylated on serine 139, and then called gamma-H2AX, as a reaction to DNA DSBs (Rogakou et al., 2000). The kinases ATR and DNA-protein kinases (PKcs) are responsible for this phosphorylation, especially ATM (Zakian, 1995; Hoekstra, 1997; Smith and Jackson, 1999; Paull et al., 2000; Bonner et al., 2008) (Fig. 1.3.2.1). The modification can happen accidentally during replication fork collapse or in the response to IR but also during controlled physiological processes such as V(D)J recombination. Gamma-H2AX is a sensitive target for looking at DSBs in cells. The role of the

phosphorylated form of the histone in DNA repair is under discussion but it is known as a first step in the organization of DNA repair. Phosphrylation is the first step in a cell signalling cascade that brings about large number of proteins involved in the repair mechanism including Rad51 and Rad50, DNA repair proteins, and Chk1, cell cycle check point, which are coming to be interest of this study.

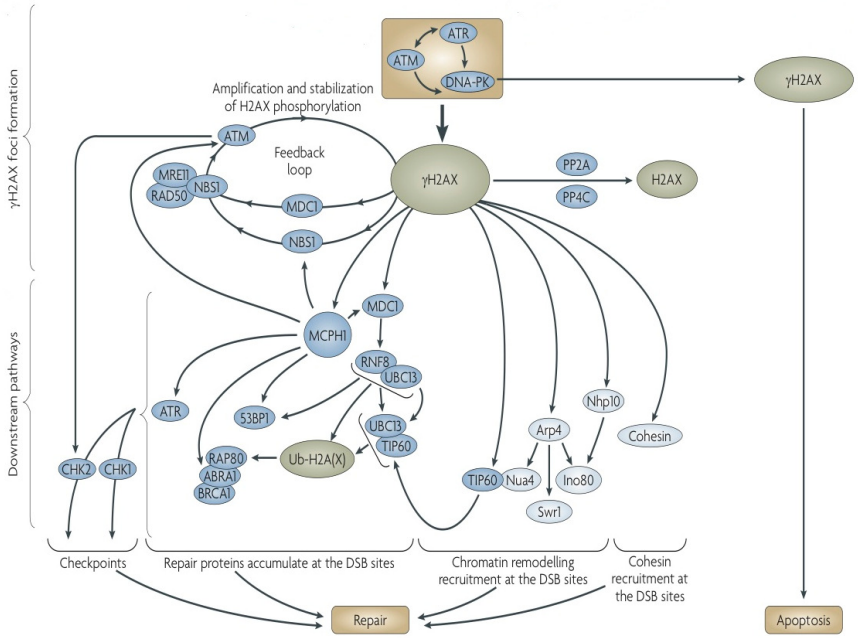


Fig. 1.3.2.1. Mechanism of H2AX phosphorylation, highlighting the role of other protein kinases (adapted from Bonner et al., 2008).

1.3.3. Rad51 phosphorylation

Several proteins known to be involved in DNA repair, Rad51 is a repair protein that assists in the DNA DSBs. Rad51 protein is highly conserved in most eukaryotes, from yeast to humans (Tashiro et al., 2000). Rad51 plays a major role in HR of DNA during DSB repair. In this process, an ATP dependent DNA strand exchange takes place in which a template strand invades base-paired strands of homologous DNA molecules.

Rad51 is involved in the search for homology and strand pairing stages of the process (Thacker, 2005). Unlike other proteins involved in DNA metabolism, the RecA/Rad51 family forms a helical nucleoprotein filament on DNA (Galkin et al., 2006). The structural basis for Rad51 filament formation and its functional mechanism still remain poorly understood. However, recent studies using fluorescent labeled Rad51 (Hilario et al., 2009) has indicated that Rad51 fragments elongate via multiple nucleation events. Rad51 is recruited to DNA repair foci performing a vital role in correcting HR. Haaf et al. (1995) reported that the number of Rad51 foci in fibroblasts subjected to radiation increased, suggesting a role of Rad51 in DNA damage repair and also a potential indicator of such damage.

1.3.4. Chromosomal abberations

Chromosomal abberation is referred to any disruptions or changes in the normal chromosomal content of a cell due to exposure to DNA damaging agent. Several studies determine chromosome abberations following exposure to radiation experimentally or environmentally and reported significant results of chromosome disruption (Brooks et al., 1993; Livingtson et al., 2006). The frequency of chromosome aberrations increases with radiation dose to the cells and serves as an indicator of radiation dose received (UNSCEAR, 1969).

1.4. Biomarkers of radiation-induced damage utilized in the environmental toxicology research field

1.4.1. Comet assay

Environmental exposure to radiation has also been evaluated. The comet assay is an extremely sensitive DNA damage assay that has been used with many freshwater, marine species (Jha et al., 2005; Jha et al., 2006; Frenzilli et al., 2009) and mammals (Miyamae et al., 1998). The Comet assay has already been applied successfully to seeds of several species: different species of beans (Khan et al., 2002a), species (Khan et al., 2002b), kiwi fruit (Jo and Kown, 2006). In the study of Gichner et al. (2000) and Ptacek et al. (2001) investigating gamma irradiation effects on tobacco seedlings, a complete repair of DNA-damage measurable by the Comet assay was observed 24 h after treatment, whereas the yield of somatic mutations manifested in the newly formed leaves increased with the increased dose of irradiation. Other studies assessing the effects of the Chernobyl radiation accident found increased levels of DNA damage and impaired repair capacity (using comet assay) in different types of cells, such as blood cells and thyroid cells, (Plappert et al., 1997; Frenzilli et al., 1998; Hellman et al., 1999; Aroutiounian, 2006). Saghirzadeh et al. (2008) reported a positive strong significant correlation of the DNA damage in nuclei of the root cells of *A. cepa* seeds germinated in the soil of high background radiation areas with Ra-226 specific activity of the soil samples, also the results showed high genotoxicity of radioactively contaminated soils in the Ramsar area of Iran. In aquatic plants, Jiang et al. (2007) indicated general development of the tail in the comet assay image with time of UV irradiation (1, 3, 5 and 7 days of exposure) in *Spirodela polyrhiza*. Sastre et al. (2001) found that damage induced by UV radiation as detected by the comet assay is increasing along with

exposure time (3, 6, 9 and 12 hrs) in *Rhodomonas* sp. In the study of Dietrich et al. (2005) on measuring the effects of UV irradiation on DNA sperm fragmentation, motility and fertilizing ability of *Oncorhynchus mykiss* spermatozoa, a significant increases in DNA strand breaks after UV irradiation for 5 min and clear decrease in the percentage of eyed embryos were reported.

1.4.2. Micronucleus assay (MN)

The micronucleus assay is recognized as one of the most successful and reliable assays for genotoxic carcinogens causing genetic damage. Direct exposure to radiation induces an increase in micronucleus formation (Zhu et al., 2005; Zielinska et al., 2007). A micronucleus is formed during the metaphase/anaphase transition of mitosis.

1.5. Summary

IR pollution has occurred in aquatic environments worldwide and there is sufficient evidence to conclude that radiation-mediated effects have occurred in many species. IR-inducing biological effects have been observed in many organisms following exposures. Low level of radiation can led to no observable effects, however there is a possibility of physiological changes, genetic changes and might lead to increase risk of cancer.

Bivalve molluscs considered as an ideal model for use in environmental toxicology due to the sessile nature, high filtering rate, wide geographical distribution and large population. They demonstrate high accumulation of pollutants, particularly radionuclides (Frantsevich et al., 1996). Mussels have long been regarded as promising bioindicators and biomonitoring subjects.

At the subcellular level, there have been a number of reported effects of proliferative radiation-induced lesions that appear to be specific to IR. Phosphorylation of H2AX after exposure to IR is considered as an early indicator for DNA DSBs and produces foci, which are detectable by immunofluorescence microscopy. The phosphorylated histone H2AX cooperates in repairing the genetic damage. In the DNA damage repair pathways, Rad51 is observed in these foci formations and in line with gamma-H2AX. IR induces focus formation of DNA repair proteins as a marker of DNA damage and as well as checkpoint mechanisms. Rad51 and Chk1 are thus essential proteins in sensing and repairing DNA damage. To date, DNA damage and repair pathways are evaluated by comet assay and detecting foci using the immunofluorescence assay. The development of new technologies such as quantitative polymerase chain reaction (QPCR) can potentially provide a direct cause-effect biomarker of IR exposure-induced DNA damage by utilizing components involved in the initiation of DNA repair pathways.

In the light of above information the hypothesis to be proved in this study are:

(1) H2AX could be used as a potential molecular marker for IR induces effects in mussels.

(2) Rad51 as a promising molecular biomaker for IR inducing DNA damage and involvement in DNA repair pathway of *M. edulis*.

(3) Chk1 role in IR DNA damage induced and DNA repair pathway of *M. edulis*.

1.6. Aims

The overall aim of this work was to assess IR induced biological effects in mussels at the molecular level of organisation by utilising the DNA damage and repair pathway and quantifying specific gene expression analysis as a biomarker of such

damage. For this purpose, mussels, *M. edulis*, were experimentally-exposed to an IR source (different doses) and the following studies performed:

- Isolation and characterization of a fragment of an mRNA involved in the DNA damage (H2AX).
- Isolation and characterization of a fragment of an mRNA involved in the DNA repair pathways (Rad51).
- Isolation and characterization of a fragment of an mRNA involved in the cell cycle checkpoint (Chk1).
- Sequence of events of foci formation sensing DNA damage and repair by comet assay.
- Validation and development of a quantitative assay to measure the expression of the isolated mRNA transcripts.
- Application of the mRNA expression assays experimentally and environmentally IR-exposed to samples.

This project therefore aims initially to identify members of the IR response in mussel including H2AX, Rad51 and Chk1. Establishing mechanisms of action of potential IR can then be used in the future to estimate the nature and the dose of radiation and for predictive risk assessment of environmental pollution. Moreover, the results obtained will also contribute to our existing knowledge on the DNA damage and repair pathways in an invertebrate species. The future aim is to produce a specific molecular biomarker of IR exposure and detrimental biological effect for use in mussel that has been anchored to traditional methods of assessing DNA damage (such as comet assay) and that can be adopted by regulatory authorities to monitor the possible impacts of such contamination sources in the aquatic environment.

It is well known that exposure to chemical and physical pollutants may lead to various negative responses in ecosystems, at different levels or organisation. As a result, several regulatory authorities are continuously monitoring the levels of selected pollutants, as well as their biological effects to provide information on possible hazards. In terms of ionising radiation, there are various regulatory bodies such as International Commission for Radiation Protection (ICRP), the National Commission for Radiation Protection (NCRP) in America, the Atomic Energy Regulatory Board (AERB) in India, UNSCEAR and IAEA. Their aim is to present norms of protection against radiation and dose limits for radiation workers and for the general public. For example, ICRP helps to prevent cancer and other biological effects due to IR exposure by understanding the science of radiation exposure. Also NCRP aim to prevent the occurrence of serious radiation induced acute or chronic effects. Moreover, UNSCEAR (2000) now reports the biological impacts, at the cellular and molecular level, of low doses of radiation, and in doing so, concluding that DNA is the main target for radiation induced cancer.

In Europe, the Oslo and Paris Convention (OSPAR) is also one of the authorities that works on providing a comprehensive and simplified approach to address all sources of pollution, such as nuclear energy, oil and gas extraction, and understand their impacts in the marine environment. They achieved, through their 35 years record, ‘a reduction of discharges from nuclear plants and better ecological quality for a healthy North Sea’ (OSPAR, 2009; 2010). The Department for Environment Food and Rural Affairs (Defra) coordinates with OSPAR in controlling the pollution and protecting the environment. In general, these regulatory bodies support the main role of maintaining a healthy environment through searching, monitoring and reducing the adverse effects of several pollutants including IR.

Chapter 2

Isolation and Characterization of *M.edulis H2AX* mRNA and protein

2.1. INTRODUCTION

Histones are large, alkaline proteins (amino acids) that are considered as among the most important elements of chromatin. Chromatin is the compound that facilitates the compacting form of DNA in the nucleus that makes up chromosomes. Focusing on one of the major and core histones, the DNA that wraps the nucleosome around two copies each of histone proteins, is the H2AX. Histone H2AX is characterized by having a long terminal tail on one end of the amino acid structure. This feature gives its main difference from H2A.

There have been a number of reported effects of radiation-induced lesions that appeared to be specific to IR (Dianov et al., 2001; Ward, 2002; Dutta et al., 2005). Phosphorylation of H2AX after exposure to IR is considered as an early indicator for DSBs and produces foci, which are detectable by immunofluorescence microscopy (Medvedeva et al., 2007). Once the DNA is damaged and its physiology is disturbed in normal cells, the p53 protein or TP53 is activated and can start a cell cycle arrest. The tail of H2AX, also known as the carboxyl terminus, rapidly becomes labelled with phosphate groups that generate species called gamma-H2AX (Bonner et al., 2008). Although it is unclear exactly what gamma-H2AX does following DNA damage, microscopy studies have shown that it is generated in the chromatin flanking a DNA DSB, and that mammalian repair and signaling proteins are recruited to these sites in large numbers. These visible protein accumulations, which can span millions of bases of DNA, are known as "foci". Gamma-H2AX is not required for the initial recruitment of repair factors, but is needed for later foci formation (Celeste et al., 2003). Recently it

was reported that the phosphorylated histone H2AX cooperates in repairing the genetic damage (Bonner et al., 2008) preserving the stability of the cells and preventing the development of tumours.

The Western blot (alternatively, protein immunoblot) is an extremely useful analytical technique that has been used to detect H2AX in a given sample of tissue homogenate or extract (Meng et al., 2005; Hanasoge and Ljungman, 2007; Koike et al., 2008). The technique uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide. The proteins are then transferred to a nitrocellulose membrane, where they are detected using antibodies specific to the target protein (Towbin et al., 1979; Renart et al., 1979). The gonads tissue of *M. edulis* is the organ of choice because of several advantages: active cell division throghout the year, simplicity of tissue identification, isolation and RNA extraction and well know morphology. This chapter presents the isolation, and characterization of *H2AX* mRNA and protein in mussel, *M. edulis*.

2.2. MATERIAL AND METHODS

2.2.1. Animals

Mussels (*M. edulis*) were collected on October 2007 by hand from Brighton, East Sussex, stored on ice and brought directly to the laboratory. The gonads of mature samples were removed and kept in RNAlater (Qiagen Ltd., Crawley, U.K.) at -70°C until further processing.

2.2.2. Total RNA isolation and purification from mussel gonadal tissue

Total RNA was extracted from the tissue using Qiagen RNeasy® (Qiagen Ltd.) reagents. Approximately 30 mg tissue was first disrupted using an Ika Ultra Turrax® T8 homogeniser in 600 µl homogenisation buffer (containing guanidine isothiocyanate and 1% βmercaptoethanol) and left for 2-3 min to digest the tissue. The sample was spun 3 min at 10,000 x *g* and the supernatant transferred into a clean tube. 600 µl of ethanol 70% was added to provide appropriate binding conditions and the sample was then applied to RNeasy spin column, a silica-gel based column, spun 15 sec at 8000 x *g* and the flow-through discarded. To avoid genomic DNA contamination a DNA digestion step was performed by adding 80 µl DNase I and the reaction was incubated at room temperature for 15 min. The column was washed several times with ethanol-based buffers to eliminate contaminants and the flow-through discarded. The column was transferred into a clean tube and eluted by centrifugation for 30 sec at 8000 x *g* with 30 µl nuclease-free water after a 1 min incubation period at room temperature. The procedure was repeated once more with the same 30 µl RNase free water. The sample was stored at -20°C until further processing.

2.2.3. First strand synthesis of cDNA

The SuperScript™ First-Strand Synthesis System from Invitrogen (Invitrogen™ Life Technologies) was used to synthesize first-strand cDNA from total RNA. Up to 2.5 µg total RNA was mixed with 1 µl dNTPs (10 mM), 0.5 µg oligo $(dT)_{12-18}$ and water to 10 µl. The sample was incubated for 5 min at 65°C and then placed on ice for at least 1 min. 4 µl 5x concentrated RT buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µl DTT (Dithiothreitol) 0.1 mM and 1 µl Rnase OUT (40 units/µl) were added to the rest

of the RNA/primer mixture, mixed gently and incubated 2 min at 42°C. 1 µl (50 units/µl) of Super Script™ II reverse transcriptase was added to the reaction, mixed and incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min and placed on ice. The final volume of the reaction was 20 µl. In order to increase the sensitivity of PCR from cDNA, the RNA template from the cDNA:RNA hybrid was removed by digestion with 1 µl RNase H (2 units) for 20 min at 37°C. The sample was stored at -20°C.

2.2.4. Oligonucleotide primer design

The oligonucleotide primers used were designed using aligned fragments of the histone H2AX gene from related species available from GenBank. The fragments were aligned using the computer program ClustalW2, the areas with the greatest homology being used for designing the primers.

2.2.5. Amplification of DNA by the Polymerase Chain Reaction (PCR)

All the reactions were carefully prepared using autoclaved tubes and autoclaved disposable pipette tips in order to avoid contamination of the samples with foreign DNA. The reagents used were aliquoted to prevent degradation by repetitive thawing/freezing cycles. Oligonucleotide primers employed in the reaction were synthesized by Invitrogen Life Technologies and supplied in lyophilised form. In the laboratory, the primers were resuspended in molecular grade depc-treated deionised water to a concentration of 50 µM.

The PCRs performed in order to isolate the H2AX gene in *M. edulis* were carried out in a volume of 50 µl consisting of 200 µM dNTPs, 1x Taq DNA polymerase buffer

(50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, stabilizers and 50% (v/v) glycerol), 0.5-4.5 mM $MgCl_2$, 10-40 µg BSA per reaction, 1.5 µM of each sense and antisense primers and 1.25 units of Platinum *Pfx* Polymerase (Invitrogen™ Life Technologies).

Amplifications were carried out in a Techne Thermal Cycler equipped with a heated lid. Each reaction was optimised in order to create the right conditions for the amplification of the targeted fragment. The oligonucleotide primers used, magnesium ion concentration, BSA concentration and ionic concentration of the buffer were the varied parameters of the reaction. Also the cycling strategy of denaturation, annealing and extension temperatures and duration of the steps were varied.

All reactions were initially denatured at 95°C for 1 min then 30 sec at 95°C denaturation, 30 sec at 45°C annealing and 1 min at 72°C elongation step. The last three steps were repeated 40 times followed by and final extension step of 2 min at 72°C.

Positive and negative controls were set up along side each set of PCR reactions. Negative controls consisted of all components of the PCR reaction excluding the template DNA while the positive controls included the primers 5'-GTGCTCTTGACTGAGTGTCTCG-3' and 5'CGAGGTCCTATTCCATTATTCC-3' for *18s rRNA* gene, which is sequenced for *M. edulis* (GenBank identifier L33448). The former controls were to ensure that there was no contamination while the latter to ensure that the reaction is working, the template DNA is not damaged.

2.2.6. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed using a BRL model H5 horizontal system for submerged gel electrophoresis. 0.8 g agarose (Promega Corporation-analytical grade) was dissolved in 100 ml TBE electrophoresis buffer (45 mM Tris-

borate, 1mM EDTA) (0.8% agarose gel) by boiling in a microwave oven. The solution was cooled to approximate 60°C and ethidium bromide was added to a final concentration of 0.8 µg/ml and mixed thoroughly. The agarose was then poured into the holding tray ensuring that the teeth of the Teflon comb were immersed and allowed to set for approximate 30 min at room temperature prior to removal of the comb and submerging into the electrophoresis buffer in the tank. The samples to be loaded were first mixed with bromophenol blue loading solution (Promega Corporation) to a final concentration of 10% and then loaded into the wells of the gel. A 100 bp molecular weight ladder (Invitrogen Life Technologies) was also loaded into the gel in order to size the DNA fragments. A current of 100V was than applied to the gel and stopped when the dye had migrated an appropriate distance through the gel. Gels were examined on a UV transluminator (UVP, Upland, CA) and photographed using a UP-860 video graphic printer (Sony, USA).

2.2.7. Isolation of DNA fragments from agarose gel slices

The gel areas containing the DNA fragments of interest were excised on the UV transluminator using a clean scalpel. The gel slice was placed into a pre-weighted clean 1.5 ml plastic tube and processed according to the QIAquick Gel Extraction protocol (Qiagen Ltd.). Three gel volumes of QG buffer (containing guanidine thiocyanate and a pH indicator to help maintaining the pH at optimum level) were added over the gel and incubated 10 min at 50°C flicking the tube periodically to dissolve the gel slice. The buffer role is to solubilize the gel slice and to create the binding conditions of the DNA to the QIAquick silica-gel membrane. This step was allowed by the addition of one gel volume of isopropanol, which increases the yield of DNA fragment smaller than 500 bp and bigger than 4 kb. The sample prepared this way was applied to the QIAquick

column and centrifuged 1 min at 10,000 x *g*. 500 µl QG buffer were added to the column and centrifuged 1 min at 10,000 x *g* in order to remove any trace of agarose followed by the addition of 750 µl ethanol-containing PE buffer and centrifuged another 1 min at 10,000 x *g*. The column was subsequently centrifuged for 1 min to eliminate any trace of PE buffer, which might interfere with downstream application and then placed into a clean 1.5 ml tube. To elute the DNA, 30 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was applied to the centre of the membrane, left for 1 min and centrifuged 1 min at 10,000 x *g*. The sample was stored at –20°C.

2.2.8. Quantification of DNA

DNA concentration was determined by a Qubit™ fluorometer (Invitrogen Detection Technologies). The fluorometer measures DNA and RNA concentrations through the use of a dye that becomes fluorescent upon binding to nucleic acids. The concentration data is then generated using a curve-fitting algorithm based on the relationship between two standards used in its calibration.

2.2.9. Addition of A' ends to the DNA fragment

The TA Cloning kit (Invitrogen Life Technologies) used in subsequent steps exploits the nontemplate-dependent activity of *Taq* polymerase that adds a single deoxyadenosine (A) to the 3' end of PCR products. The linearised vector in the kit has a single 3' deoxythimidine (T) residue, which allows PCR inserts to ligate efficiently with the vector. The proofreading polymerase used in our reactions (Platinum *Pfx* polymerase) does not share the same particularity with Taq polymerase leaving blunt-ended PCR products that affect ligation with the vector. We thus attached A' overhangs to our PCR products. The sample was mixed with 2 µl 5x Qiagen A'-addition Master

Mix, mixed gently and incubated 30 min at 37°C. The sample prepared this way was ready for ligation with the pCR 2.1 vector.

2.2.10. Cloning PCR-generated fragments of DNA

The cloning technique allows the separation of different DNA fragments from a mixture and to produce them in large quantities. To achieve this, the DNA was subcloned into bacterial plasmids. The linearized TA plasmid vector pCR®2.1 (Invitrogen Life Technologies) used for the DNA cloning has single 3' deoxythymidine (T) residues and contain the resistance genes to kanamycin and ampicillin as well as the *LacZα* gene.

The DNA fragment, helped by its deoxyadenosine (A) overhangs at the 3' ends added by the *Taq* polymerase, is inserted into the plasmid DNA in the middle of the *LacZα* gene. 3 µl of PCR product processed as described in section 2.2.9 was mixed on ice with 25 ng pCR®2.1 vector, 1 µl 10x ligation buffer (60 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1mg/ml BSA, 70 mM β-mercaptoethanol, 1mM ATP, 20 mM DTT and 10 mM spermidine), 1 µl T4 ligase (4.0 Weiss units/µl) and 4 µl of H_2O. The reaction was incubated overnight at 15°C.

The vectors prepared as above were then ready to be transformed into One Shot® *E. coli* competent cells TOP 10 strain (Invitrogen Life Technologies). 50 µl of frozen One Shot competent cells were thawed on ice and mixed gently with the pipette tip with 2 µl of the ligation reaction. The vial was than incubated for 30 min on ice and then heat shock for exactly 30 sec at 42°C. The vial was then placed again on ice for 2 min. 250 µl S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) at room temperature was added to the reaction and incubated for 2-3 hrs at 37°C into a shaking incubator at 200 rpm. The

culture was plated onto LB agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 15 g/l agar, pH 7.0) containing kanamycin (50 µg/ml) and X-gal in dimethyl formamide (40 µg/ml) and incubated overnight at 37°C. Single white colonies, indicating the presence of the plasmid in the cell because of the kanamycin resistance and disruption of the *LacZα* gene by the insert DNA, were picked using a sterile pipette tip and inoculated into 5 ml of LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing 0.5 mg/ml kanamycin. The cultures were grown overnight into a shaking incubator at 37°C and 200 rpm.

2.2.11. Extraction and purification of plasmid DNA

Plasmid DNA was extracted from *E. coli* cultures using Wizard® Plus Minipreps DNA Purification System (Promega Corporation), which is based on an alkaline lysis of the cells followed by the absorption of plasmid DNA to the resin beads. Approximately 5 ml of the *E. coli* cultures (see 2.2.10) were centrifuged for 2 min at 10,000 x *g* and the supernatant discarded. The pellet was resuspended in 250 µl Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 100 µg/ml RNase A) and mix by inverting 4 times with 250 µl Cell Lysis Solution (0.2 M NaOH, 1% SDS). 10 µl of alkaline protease solution was added and mixed by inverting 4 times, then left in room temperature for 5 min. 350 µl Neutralisation Solution (1.32 M potassium acetate pH 4.8) were then added and mixed by inverting the tube 4 times. The tube was centrifuged 10 min at 10,000 x *g* and the supernatant mixed with 1 ml of resin in Wizard® Miniprep Column attached to a vacuum manifold. The sample was load into the column and washed with 1 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5, 40 mM EDTA, 55% ethanol). The column was removed from the vacuum

manifold and transferred to a microcentrifuge tube and centrifuged for 2 min at 10,000 x *g* to remove any trace of wash solution. The plasmid DNA was eluted in a clean tube with 30-40 µl RNase free water by centrifugation.

2.2.12. Enzymatic digestion of the plasmid DNA using *EcoR I* restriction enzymes

In order to check the size of the insert, the plasmid DNA was digested using *EcoR I* restriction enzyme (Promega Corporation), which recognises two adjacent sites to the inserted fragment. 5 µl of the plasmid DNA sample was gently mixed with 2 µl 10x buffer (900 mM Tris-HCl pH 7.5, 500 mM NaCl and 100 mM $MgCl_2$) , 1 µl *EcoR* I (10 U/µl) and RNase free water to a volume of 20 µl and incubated for 2 hrs at 37°C. The digestion products were analysed on an agarose gel as described in section 2.2.6. and 2.2.7. followed by measuring the DNA as described in section 2.2.8.

2.2.13. Sequencing the potential *H2AX* mRNA-containing sub-clones

Approximately 1 µg plasmid DNA was mixed with a tenth volume of sodium acetate (3 M, pH 5.2) and two volumes of 95% ethanol and left 15 min at –20°C, then centrifuged 10 min at 16,000 x *g*. The supernatant was discarded and the pellet allowed to dry for 15 min at room temperature. The samples were then ready to be sent to Eurofins MWG Operon, Germany for sequencing.

2.2.14. Western Blotting

2.2.14.1. Samples and preparing whole cell extracts with fully solubilized chromatin

Mussels were irradiated at different doses (5, 10, 25, 50 and 100 Gy of ^{137}Cs, dose rate 0.125 Gy/sec, at 9°C) in 50 ml conical polypropylene sterile tubes in the

presence of seawater. After that, using glass beads, 30 mg fresh *M. edulis* gonad tissue was ground in 400 µl of nuclease digestion buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.0 mM CaCl2, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 1 mM DTT, 0.1% (v/v) Triton X-100) containing 100 U/mL Micrococcal Nuclease (to digest chromatin), 0.1 µM Microcystin-LR (a phosphatase inhibitor) and 1X protease inhibitor cocktail (Sigma-Aldrich, U.K).

The samples were incubated (together with glass beads) at 30^{o}C for 30-45 min. This allows the nuclease time to digest insoluble chromatin. An equal volume of solubilization buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 1 mM DTT, 0.1% (v/v) Triton X-100, 2% (v/v) NP-40, 2% (v/v) Triton X-100, 600 mM NaCl) was added, ground/vortexed briefly and then centrifuged 5000 rpm for 2 min to remove glass beads. After recovering the supernatant, the extract was sonicated twice, at 5 sec per disruption, and finally centrifuged at 10,000 x *g* for 10 min. The final supernatant should contain all cytoplasmic, nucleoplasmic and the majority of chromatin proteins, including histones.

2.2.14.2. Identification of H2AX protein using immunoblotting technique

To immunoblot for the very small histones (~15 kDa), samples were loaded on 17.5% acrylamide SDS PAGE gels (4.4 ml 30% acrylamide, 0.275 ml 2% Bis-acrylamide, 2.8 ml 1M Tris pH 8.8, 37.5 µl 20% SDS, 7.5 µl TEMED, 30 µl 10% APS). Two 0.75 mm thick gels were prepared from stacking gel (85 ml 30% acrylamide, 17.5 ml 2% Bis-acrylamide, 62.4 ml 1M Tris pH 6.8, dH_2O to 500 ml, in dark at 4^{o}C). For every gel, aliquot 1 ml of the stacking gel mixture for polymerization. To polymerize, for every 1 ml of stacking mix, 10 µl 20% SDS, 5 µl 10% APS and 5 µl TEMED were

added and left to polymerize for 3-5 min. The comb was removed as soon as the solution polymerized, flushing with water and the gel then used straight away. Samples and marker were loaded on the gel and run at 110V for 10 min followed by increasing the voltage to 150V for 60 min. After that, the two 0.75 mm gels were transferred to 0.2-micron nitrocellulose membrane using electroblot buffer (48 mM Tris-base, 39 mM glycine, 20% (v/v) methanol) at 100V for 60 min.

2.2.14.3. The H2AX antibody binding reaction

The nitrocellulose was washed later with Ponceau-S stain (5% acetic acid + drop of Ponceau-S dye) for 30 sec followed by water wash and TBS buffer for few seconds (24.2 g of Tris-base, 292.2 g of NaCl, up to 1 L of dH_2O, pH 7.5) to clear the dye off. After that, nitrocellulose membrane was blocked in 25% dry milk in 10 mM TBS-T (150 mM NaCl, and 20% Tween, pH 7.5) for 30 min at room temperature.

The nitrocellulose membrane was probed with anti-gamma H2AX (gamma-H2A-X-phospho-S139-antibody, Abcam Plc.) at 4^oC for overnight, then washed and rotate twice with TBS-T for 5-10 min. The membrane was incubated with 2° mouse antibodies (13858-014, Life Technologies, Inc.) diluted in 5% milk in TBS buffer for 60 min at room temperature. After that, washing the membrane three times with TBS-T buffer was applied then ECL reagent (Pierce ECL Western Blotting substrate from Thermo Scientific) was added to for 1 min. After washing, the membrane was exposed on film (Amersham Hyperfilm ECL, from GE Healthcare, Buckinghamshire, U.K.) using (Compact X4, Xograph Imaging Systems).

2.3. RESULTS

2.3.1. Isolation of total RNA from *M. edulis* gonads

The use of extraction method described in section 2.2.2. provided a high yield of good quality total RNA (A260 : A280 = 2.097). About 30 mg of tissue yielded 1421.2 µg/ml total RNA.

2.3.2. Oligonucleotide primers obtained

The first set of H2AX degenerate primers (H2AXf, H2AXr1, H2AXr2) designed aligning the H2AX protein sequence from different species (see Table 2.3.2.1 and Fig. 2.3.2.1) proved to be unsuccessful. To reduce the degeneracy of the primers a second set of primers was designed (SpecF, SpecR) using the cDNA sequences of *M. edulis, Mytilus trossulus, Mytilus galloprovincialis* and *Mytilus calfornianus* (GenBank identifiers AY267757, AY267758, AY267755 and AY267759) instead of the protein sequences (see Table 2.3.2.1 and Fig. 2.3.2.2). This successful approach with species-specific primers was used to isolate the *H2AX* mRNA in *M. edulis*.

Table 2.3.2.1. Oligonucleotide sequences used as primers for the amplification of *H2AX* mRNA (where N=A+C+T+G, R=A+G, Y=C+T, M=A+C, S=C+G, W=A+T, D=A+T+G)

	Primer name	**Primer sequence**	**Species used (GenBank identifier)**	**TM°C**	**%GC**
Forward primer	H2AXf	GTB GGB GCN GGN GCD CCV GTB TAY	*Danio rerio (XP_001342899)* *Xenopus tropicalis (NP_001015968)* *M. edulis (CAD37821)* *Rattus norvegicus (NP_001102761)* *Homo sapiens (NP_002096)*	60°C	68
	SpecF	AGG ACG AGG AAA AGG AGG AA	*M. edulis (AY267757)* *M. trossulus (AY267758)* *M. galloprovincialis (AY267755)* *M. calfornianus (AY267759)*	47°C	50

Reverse primers	H2AXr1	YTT GTT SAR YTC YTC RTC RTT	*D. rerio* (XP_001342899) *X. tropicalis* (NP_001015968) *M. edulis* (CAD37821) *R. norvegicus* (NP_001102761) *H. sapiens* (NP_002096)	43°C	38
	H2AXr2	VAC RCC DCC YTG VGC RAT VGT BAC	*D. rerio* (XP_001342899) *X. tropicalis* (NP_001015968) *M. edulis* (CAD37821) *R. norvegicus* (NP_001102761) *H. sapiens* (NP_002096)	57°C	60
	SpecR	TTT CCT GCC AAC TCC AAA AC	*M. edulis* (AY267757) *M. trossulus (AY267758)* *M. galloprovincialis (AY267755)* *M. calfornianus (AY267759)*	45°C	45

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D.rerio          MFSIVEKTNICKPMVTSFQHKNCSLIKMSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHR 60
M.edulis         ---------------------------MSGRGK-GGKAKAKAKSRSSRAGLQFPVGRIHR 32
H.sapiens        ---------------------------MSGRGKTGGKARAKAKSRSSRAGLQFPVGRVHR 33
R.norvegicus     ---------------------------MSGRGKTGGKARAKAKSRSSRAGLQFPVGRVHR 33
X.tropicalis     ---------------------------MSGRGKTGGKTRAKAKTRSSRAGLQFPVGRVHR 33
                                             ****** ***::****:**************:**

                          H2AXf                                        H2AXr1
D.rerio          LLRKGNYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDEE 120
M.edulis         LLRKGNYAERVGAGAPVYLAAVLEYLAAEVLELAGNAARDNKKSRIIPRHLQLAIRNDEE 92
H.sapiens        LLRKGHYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEE 93
R.norvegicus     LLRKGHYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAIRNDEE 93
X.tropicalis     LLRKGNYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDEE 93
                 *****:********************:**:**************:**********:*****

                          H2AXr2
D.rerio          LNKLLGGVTIAQGGVLPNIQAVLLPKKT------------EKAAKK---- 154
M.edulis         LNKLLSGVTIAQGGVLPNIQAVLLPKKT------------QKAAK----- 125
H.sapiens        LNKLLGGVTIAQGGVLPNIQAVLLPKKTSATVGPKAPSGGKKATQASQEY 143
R.norvegicus     LNKLLGGVTIAQGGVLPNIQAVLLPKKTSATVGPKAPAGGKKASQASQEY 143
X.tropicalis     LNKLLGGVTIAQGGVLPNIQTVLLPKKTSAAPTATGKSSGKKSSQQSQEY 143
                 *****.**************:*******           :*:::
```

Fig. 2.3.2.1. ClustalW2 multiple sequence alignment of the deduced amino acid sequences of the H2AX of different species showing the designed degenerated primers. Asterix denotes homology.

```
M.edulis                 TACTACCTGGAAGAAGCGATGATTTGATTGGTTTAGAACTGAAACATCTTTCAATCCGTT 60
M.galloprovincialis      TACTACCTGGAAGAAGCGATGATTTGATTGGTTTAGAACTGAAACATCTTTCAATCCGTT 60
M.trossulus              TACTACCTGGAAGAAGCGATGATTTGATTGGTTTAGAACTGAAACATCTTTCAATCCGTT 60
M.californianus          -----------GGCCGCGGGAATTCGATTG------------------------------ 19
H.sapines                ---------------------------------------------------------- 
R.norvegicus             ---------------------------------------------------------- 
X.tropicalis             ---------------------------------------------------------- 
D.rerio                  ----------ATGTTTTCCATAGTAGAAAA------------------------------ 20

M.edulis                 TTGCGGGTATAAATAGTAAACTACCACCTCTTGGGGTAATCATTGTTTATACTTGTTCA- 119
M.galloprovincialis      TTGCGGGTATAAATAGTAAACTACCACCTCTTGGGGTAATCATTGTTTATACTTGTTCA- 119
M.trossulus              TTGCGGGTATAAATAGTAAAATACCACCTAACTGGGTAATCATTGTTTATACTTGTTCA- 119
M.californianus          -------------------------ACCTCTTGGGGTAATCATTGTTTATACTTGTTCAG 54
H.sapines                ---------------------------------------------------------- 
R.norvegicus             ---------------------------------------------------------- 
X.tropicalis             ---------------------------------------------------------- 
D.rerio                  ----------------------AACAAATATTTGTAAACCAATGGTTACTTCCT-TCCAA 57
```

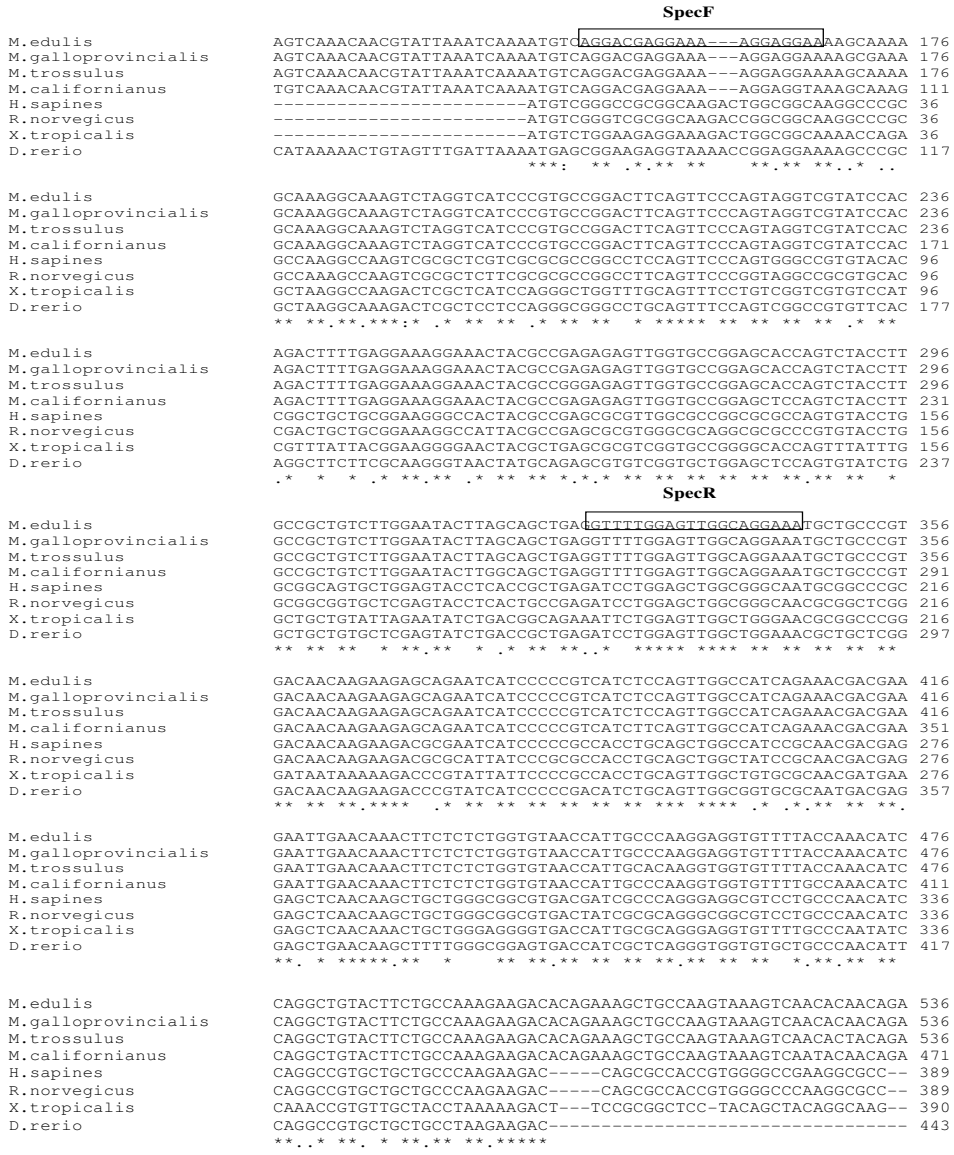

Fig. 2.3.2.2. ClustalW2 multiple sequence alignment of the nucleotides sequences of the *H2AX* of different invertebrate and vertebrate species and the specific designed primers. Asterix denotes homology.

2.3.3. *H2AX* mRNA amplification using mussel cDNA template

Several PCRs were conducted in order to isolate the *H2AX* mRNA fragment from *M. edulis*. Different combinations of the designed primers (Table 2.3.2.1) were used in

reactions while other parameters were also varied (see 2.2.5). Generally, most of the reactions either yielded no product or the products obtained, after sequencing, were revealed not to be the product of interest.

The successful isolation of the *H2AX* mRNA was carried out in a volume of 50 µl consisting of 200 µM dNTPs, 1x Taq DNA polymerase buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, stabilizers and 50% (v/v) glycerol), 0.5-4.5 mM $MgCl_2$, 10-40 µg BSA, 1.5 µM of upstream speceific primer (SpecF) 5'–AGG-ACG-AGG-AAA-AGG-AGG-AA-3' and downstream specific primer (SpecR) 5'-TTT-CCT-GCC-AAC-TCC-AAA-AC-3' and 1.25 units of Platinum *Pfx* Polymerase. For the PCR, the reaction was initially denatured at 95°C for 1 min then 30 sec at 95°C denaturation, 30 sec at 45°C annealing and 1 min at 72°C elongation step. The last three steps were repeated 40 times followed by and final extension step of 2 min at 72°C. After the PCR a fragment of the expected size, 197 bp, was visualized in the agarose gel (Fig. 2.3.3.1).

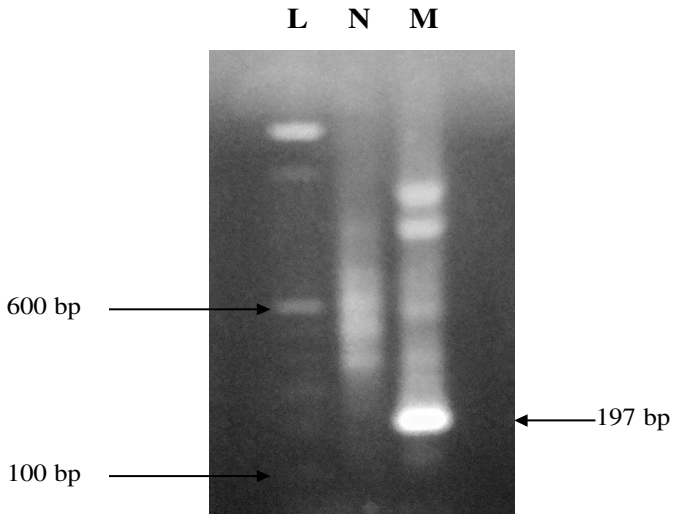

Fig. 2.3.3.1. Ethidium bromide stained 0.8% agarose gel displaying PCR amplification products obtained using *M. edulis* cDNA as a template and the primer pairs SpecF/SpecR (expected product size – 197 bp). Lane L is the molecular size ladder and lane N is the negative control to ensure the reaction was not contaminated. Lane M is the *M. edulis* sample.

2.3.4. Subcloning of PCR-generated DNA fragments

The 197 bp fragment was excised from the agarose gel and extracted from the gel slice (section 2.2.7). The DNA was quantified (section 2.2.8), adenine overhangs were subsequently added to the DNA fragments (section 2.2.9). The DNA fragment was sub-cloned into pCR®2.1 plasmid DNA and transformed into competent *E. coli* cells (section 2.2.10). The colonies were grown in large number and plasmid DNA extracted as described in section 2.2.11. In order to check for the identity of the inserted fragment, 3 µl plasmid DNA was restriction digested with *EcoRI* enzyme (section 2.2.12) and run on an agarose gel. A total number of 8 colonies were picked and analysed for the presence of the desired DNA fragment. All of the colonies contained the 197 bp fragment. On the agarose gel, the fragments are bigger because the *EcoRI* sites does not coincide with the insertion point, the difference being of about 15 nucleotides.

2.3.5. Sequencing the isolated DNA fragments

All of the plasmids containing the fragment of interest were sent for sequencing (section 2.2.13). The BLAST algorithm confirmed the identity of the isolated fragment as a part of a putative *H2AX* mRNA. There was 100% homology between the isolated fragment and *M. edulis* (GenBank identifier AY267757) *H2AX* sequence. Also, a 74%, 73% and 71% similarity with *D. rerio*, *X. tropicalis* and *H. sapiens* homologs respectively.

5’...aggacgaggaaaaggaggaaaagcaaaagcaaaggcaaagtctaggtcatcccgtgccggacttcagttcccagta
ggtcgtatccacagacttttgaggaaaggaaactacgccgagagagttggtgccggagcaccagtctaccttgccgctgtct
tggaatacttagcagctgaggttttggagttggcaggaaa..3’

Fig. 2.3.5.1. Nucleotide sequence of the *M. edulis* putative *H2AX* fragment isolated.

2.3.6. Western blotting using a 2^{o}mouse-specific H2AX antibody

The control and irradiated mussels were immunoblotted with gamma-H2AX-antibody and compared to control and irradiated mammals. Before applying the antibodies, the nitrocellulose membrane showed presence of loads of proteins in irradiated mussels compared to control mussel (Fig. 2.3.6.1). However, after immunoblotting with gamma-H2AX, there were no observations for antibodies reaction in mussels compared to irradiated mammal sample (Fig. 2.3.6.2).

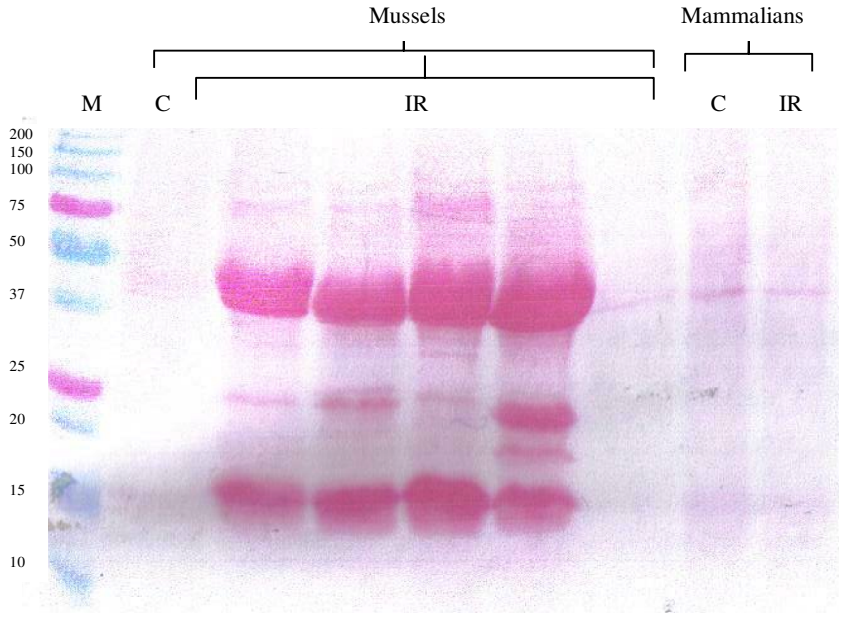

Fig. 2.3.6.1. Nitrocellulose membrane displaying the proteins obtained using control (C), irradiated (IR) mussels and control, irradiated mammalians showing presence possibility of H2AX in mussl samples. Lane M is the protein marker.

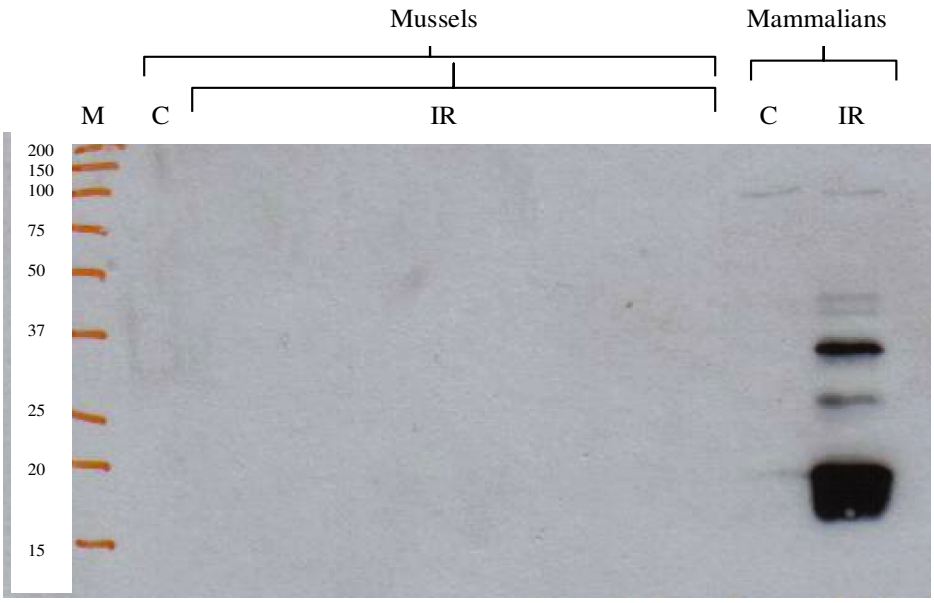

Fig. 2.3.6.2. Film displaying the result obtained using control (C), irradiated (IR) mussels and control, irradiated mammalians showing no interaction between mussel samples and the H2AX antibody. Lane M is the protein marker.

2.4. DISCUSSION

The aim of this section was, firstly, to isolate and characterize the *H2AX* gene from the marine mussel *M. edulis* using real-time PCR technique. Specific primers successfully amplified a *M. edulis* partial *H2AX* cDNA sequence encoding a putative 26 amino acid protein (AAP94676). The deduced amino acids showed 100% similarity with *M. edulis* in gene bank (AY267757) and above 70% similarity to several vertebrate species (Fig. 2.3.2.1) and 99-100% with other mussel species.

The second aim was to isolate and characterize the H2AX protein from *M. edulis*. Application of the western blotting technique, using mammalian gamma-H2AX antibodies, on control and irradiated mussels showed no H2AX phosphorylation compared to a positive control irradiated mammal sample. Yet the phosphorylation of H2AX occurs at amino acid S139 in human and most vertebrate species (Kinner et al.,

2008). This amino acid residue is not present in the predicted protein using the *M. edulis* sequence (AY267757) (Fig. 2.3.2.1) and this may explain why no cross reactivity occurred using the mammalian antibody for this H2AX epitope. H2AX is conserved in that many of the human H2AX antibodies are reported to work with yeast cells and insect cells (*Drosophila*, for example) (Rogakou et al., 1999; Madigan et al., 2002), but no evidence of binding was observed using mussels in this study. It is possible that the particular antibody used in this study might be one of the ones that has less broad specificity, possibly because it included more human amino acids sequence on either side of the protein S139 main epitope. Further work would likely be aimed at testing a number of the other antibodies available for mammalian H2AX or designing new antibody specific to mussels in particular. Moreover, further sequencing of the *M. edulis* *H2AX* have to continue to achieve the complete sequence, which might include the phosphorylated site. Conservation overall of the *H2AX* gene sequence in mussel suggests that a mammalian antibody should find an epitope if enough are tried.

Chapter 3

Isolation and Characterization of *M. edulis Rad51* mRNA

3.1. INTRODUCTION

Rad51 is a DNA repair protein involved in DNA DSB damage and repair. DSBs are introduced into DNA by factors including IR (Morrison et al., 2000). RAD51 forms one of these ends into a presynaptic filament, which seeks out a sequence homologous to (ie. same as) the damaged DNA on the neighbouring chromatid. The filament introduces itself into the intact strands and opens a D loop, which the broken strands then use as templates to repair their sequence (Fig. 3.1.1) (Gerton and Hawley, 2005).

A DSB, where both backbones of the DNA double helix are broken by external factors like radiation occurs approximately 10 times per cell division; the cell's need for highly accurate repair is therefore constant.

In eukaryotes, RAD51 is the protein that carries out DSB repair by HR. It works with several other proteins, which cooperate in the RAD51 complex.

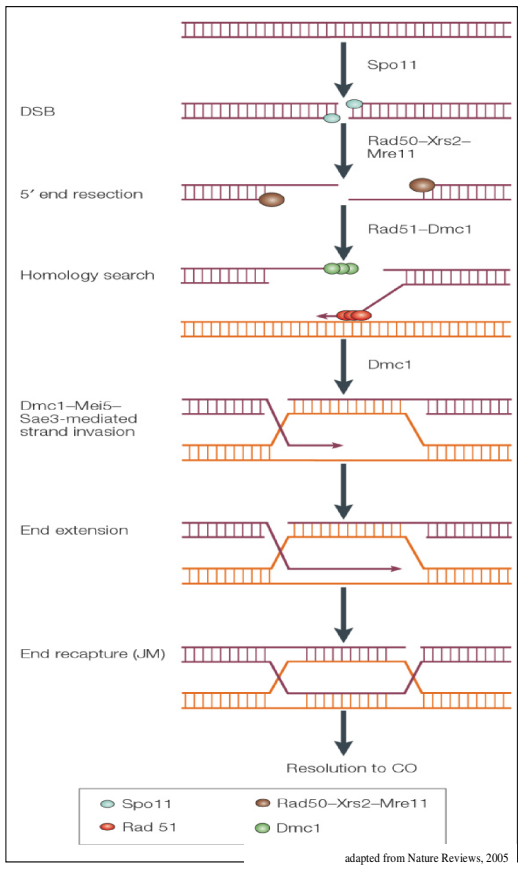

Fig. 3.1.1. Role of Rad51 in DNA DSB-HR repair pathway.

Rad51-DMC1-radA,B is a group of recombinases that includes the eukaryotic proteins RAD51, RAD55/57 and the meiosis-specific protein DMC1, and the archaeal proteins radA and radB. They are closely related to the bacterial RecA group. Rad51 proteins catalyze a similar recombination reaction as RecA, using ATP-dependent DNA binding activity and a DNA-dependent ATPase. However, this reaction is less efficient and requires accessory proteins such as RAD55/57. Rad51-DMC1-radA,B is a member of the superfamily P-loop NTPase, P-loop containing Nucleoside Triphosphate Hydrolases (Shinohara and Ogawa, 1999; Gasior et al., 2001; Pellegrini et al., 2002; Wiese et al., 2006).

Rad51 has been isolated in many vertebrate species such as rodent and human (Strausberg et al., 2002; Cartwright et al., 1998) and invertebrate including fruit fly, nematode and zebra mussel (Akaboshi et al., 1994; Rinaldo et al., 1998; Lamers et al., 2002) (Table 3.1.1.).

Table 3.1.1. A summary showed some details of Rad51 in some vertebrates and invertebrates species.

Phylum	**Species**	**Accession ID**	**Name**
Arthropoda	*D. melanogaster*	BAA04580	Rad51, spn-A, spn-B
“	*Nasonia vitripennis*	NP_001154949	RecA homolog RAD51
“	*Lepeophtheirus salmonis*	ADD24297	RAD51 homolog 1
“	*Caligus clemensi*	ACO14764	RAD51 homolog 1
“	*Bombyx mori*	NP_001037484	Rad51 homolog 1
Nematoda	*Loa loa*	XP_003146628	Rad51
“	*Caenorhabditis elegans*	AAD10194	Rad51
“	*Brugia malayi*	EDP34081	Rad51 homolog
“	*Trichinella spiralis*	EFV57314	Rad51
Mollusca	*Dressina polymorpha*	AAM44815	Rad51
Chordata	*D. rerio*	NP_998371	Rad51 homolog1

"	*Salmo salar*	NP_001134027	Rad51 homolog A
"	*Oreochromis niloticus*	BAD98461	RecA homolog rad51
"	*Esox lucius*	ACO14034	RAD51 homolog 4
"	*Hypophthalmichthys molitrix*	ADF97633	RAD51 4-like
"	*X. laevis*	AAI08487	RAD51
"	*Gallus gallus*	NP_990504	RAD51 homolog 1
"	*Oryctolagus cuniculus*	AAC28561	Rad51
"	*M. musculus*	BAA02718	Rad51
"	*R. norvegicus*	NP_001102674	RAD51 homolog 1
"	*H. sapiens*	CAG38796	Rad51

Rad51 mRNA expression is used in vertebrates as a biomarker in response of IR (Tashiro et al., 2000; Bishay et al., 2001; Chinnaiyan et al., 2005). Pathways of DNA damage repair, HR repair and NHEJ, include Rad51 as a key protein in re-synthesis, catalyzing and transferring, strands between broken sequences and its homologues in DSBs damage (Collis et al., 2001; Rollinson et al., 2007). Studies using mouse, chicken & other mammalian cells have shown that inefficient repair or mis-repair of DNA damage can lead to genomic instability (Sonoda et al., 1998; Thompson and Schild, 1999; Difilippantonio et al., 2000; Zhao et al., 2007). This relationship between DNA repair pathway and *Rad51* mRNA expression can therefore potentially be adapted as a biomarker of radioactive isotope contamination of the aquatic (or indeed, any) environment.

This chapter presents the isolation and characterization of a member of the IR response, the *Rad51* mRNA from the marine mussel *M. edulis*.

3.2. MATERIALS AND METHODS

3.2.1. Animals

The mussels (*M. edulis*) were collected by hand from concrete groins on Brighton beach (U.K.) (50°49′ longitude and 0°8′ latitude), stored on ice and brought in the lab in the same day. The gonads were removed, wrapped in tin foil and snap frozen in liquid nitrogen. The dissecting work was done in the cold room to lower the activity of proteases and nucleases. The samples were then stored at -80°C until further processing.

3.2.2. Total RNA isolation and purification from mussel gonadal tissue

Total RNA was extracted from the tissue using RNA isolation® (Roche) reagent. Approximately 30 mg tissue was first disrupted using glass beads (Sigma) in 400 µl lysis buffer (containing guanidine thiocyanate) and centrifuged at 4°C for 40 sec to homogenate the tissue. The sample was spun 2 min at 16,250 x *g* and the supernatant transferred into a clean tube. 200 µl absolute ethanol was added to provide appropriate binding conditions and the sample was then applied to a silica-gel based column, spun 30 sec at 16,250 x *g* and the flow-through discarded. To avoid genomic DNA contamination, DNase digestion was performed by adding (10 µl of DNase working solution +90 µl DNase digestion buffer) and left at room temperature for 15 min. The column was washed several times with ethanol-based buffers to eliminate the contaminants and the flow-through discarded. The column was transferred into a clean tube and eluted by centrifugation for 1 min at 8000 x *g* with 40 µl elution buffer (RNase free water) after a 1 min incubation period at room temperature. The procedure was repeated once more with the same 40 µl RNase free water. The sample was stored at -20°C until further processing.

3.2.3. First strand synthesis of cDNA

The Transcriptor First-Strand cDNA Synthesis System from Roche (Roche) was used to synthesize first-strand cDNA from total RNA. Up to 1 µg total RNA was mixed with 2 µl 600 pmol random hexamer and water to 13 µl. The sample was incubated for 10 min at 65°C and then placed on ice for at least 1 min. 4 µl 5x concentrated TRT reaction buffer (250 mM Tris-HCl (pH 8.5), 150 mM KCl, 40 mM $MgCl_2$), 0.5 µl Protector RNase Inhibitor (20 mM Hepes-KOH, 50 mM KCl, 8 mM dithiothreitol, 50% glycerol (v/v)) (40 units/µl), 2 µl dNTPs (10 mM) and 0.5 µl Transcriptor Reverse Transcriptase (20 units/µl) were added to the rest of the RNA/primer mixture, mixed gently and incubated 10 min at 25°C. Then incubated for 60 min at 50°C. The reaction was terminated at 85°C for 5 min and placed on ice. The final volume of the reaction was 20 µl.

3.2.4. Oligonucleotide primer design

The oligonucleotide primers used were designed using aligned fragments of *Rad51* mRNA from related species available on GenBank (Table 3.2.4.1).

Table 3.2.4.1. Rad51 Protein accession numbers in different species.

Species	**Protein ID**
H. sapiens	BAA02962.1
Xenopus laevis	NP_001081236.1
D. rerio	NP_998371.1
D. polymorpha	AAM44815.1

The fragments were aligned using the computer program CLUSTALW (Fig. 3.2.4.1), the areas with the greatest homology being used for designing the primers.

```
H.sapiens       MAMQMQLEANADTSVEEESFGPQPISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKEL 60
X.laevis        MAMQAHYEAEAT---EEEHFGPQAISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKEL 57
D.polymorpha    ------------------------------------------------------------
D.rerio         MRNASRVEVEAEVE-EEENFGPQPVSRLEQSGISSSDIKKLEDGGFHTVEAVAYAPKKEL 59


H.sapiens       INIKGISEAKADKILAEAAKLVPMGFTTATEFHQRRSEIIQITTGSKELDKLLQGGIETG 120
X.laevis        LNIKGISEAKAEKILAEAAKLVPMGFTTATEFHQRRSEIIQISTGSKELDKLLQGGVETG 117
D.polymorpha    ------------------------------------------------------------
D.rerio         LNIKGISEAKADKILTEAAKMVPMGFTTATEFHQRRAEIIQISTGSKELDKLLQGGIETG 119


H.sapiens       SITEMFGEFRTGKTQICHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYGL 180
X.laevis        SITEMFGEFRTGKTQLCHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYGL 177
D.polymorpha    ----------TGKTQICHTLAVTCQLPIDMGGGEGKCLYIDTEGTFRPERLLAVSERYGL 50
D.rerio         SITEMFGEFRTGKTQLCHTLAVTCQLPIDQGGGEGKAMYIDTEGTFRPERLLAVAERYGL 179
                     *****:************ ******.:****************:*****


H.sapiens       SGSDVLDNVAYARAFNTDHQTQLLYQASAMMVESRYALLIVDSATALYRTDYSGRGELSA 240
X.laevis        SGSDVLDNVAYARAFNTDHQTQLLYQASAMMAESRYALLIVDSATALYRTDYSGRGELSA 237
D.polymorpha    SGSDVLDNVAYARAYNSDHQSQLLIQAAAMMAESRYALLVVDSATALYRTDYSGRGELAA 110
D.rerio         VGSDVLDNVAYARAFNTDHQTQLLYQASAMMTESRYALLIVDSATALYRTDYSGRGELSA 239
                 ************:*:***:*** **:***.*******:******************:*


H.sapiens       RQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTRL 300
X.laevis        RQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTRL 297
D.polymorpha    RQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADP----------------- 153
D.rerio         RQGHLGRFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADPKKPIGGNILAHASTTRL 299
                ** **.*********************************:***


H.sapiens       YLRKGRGETRICQIYDSPCLPEAEAMFAINADGVGDAKD 339
X.laevis        YLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 336
D.polymorpha    ----------------------------------------
D.rerio         YLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 338
```

Fig. 3.2.4.1. ClustalW2 multiple sequence alignment of the deduced amino acid sequences of the Rad51 of different species. Asterix denotes homology.

To reduce the degeneracy, further primers (Rad51F1, Rad51F2, Rad51F3, Rad51R1, Rad51R2, Rad51R3 and Rad51R4) were designed using the cDNA sequence of *D. polymorpha* (GenBank Identifier No. AF508221) instead of the protein sequence (Table 3.2.4.2 and Fig. 3.2.4.2). This approach with species-specific primers was used to isolate the *Rad51* mRNA in *M. edulis*.

Table 3.2.4.2. Oligonucleotide sequences used as primers for the amplification of *Rad51* mRNA.

Primer name		Primer sequence	TM°C	%GC
Forward	Rad51F1	TGT CAC ACT CTG GCA GTC ACC TG	54°C	56
	Rad51F2	TAC ATC GAC ACA GAA GGC AC	47°C	50
	Rad51F3	TAC TCT GGT AGA GGG GAG CT	49°C	55
Reverse	Rad51R1	AGC TCC CCT CTA CCA GAG TA	49°C	55
	Rad51R2	ACC ACG GCA ACA CCA AAC TC	49°C	55
	Rad51R3	GCC ACC ACCTGG TTT GTG AT	49°C	55
	Rad51R4	GGG GTC TGC AGA AAA CAT GGC	51°C	57

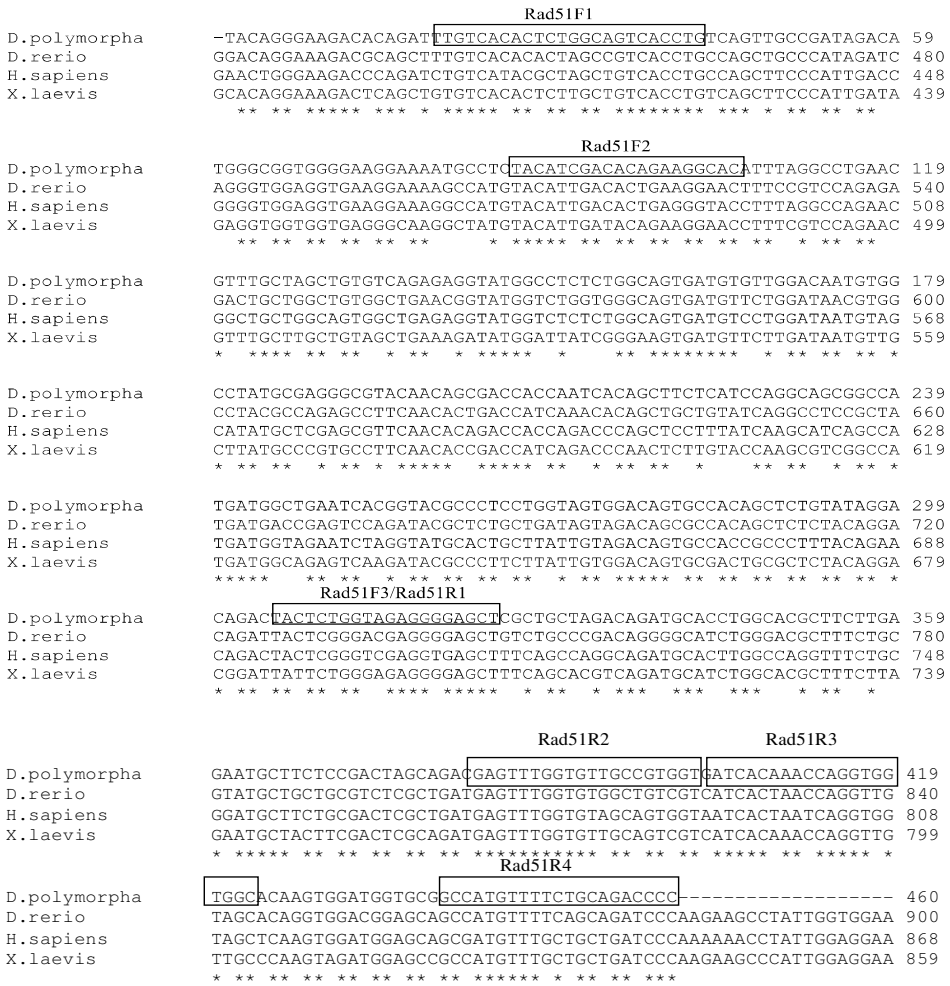

Fig. 3.2.4.2. ClustalW2 multiple sequence alignment of the nucleotides sequences of the *Rad51* of different invertebrate and vertebrate species and the primers designed. Asterix denotes homology.

3.2.5. Amplification of DNA by RT-PCR

All the reactions were carefully prepared using autoclaved tubes and autoclaved disposable pipette tips in order to avoid contamination of the samples with foreign DNA. The reagents used were aliquoted to prevent degradation by repetitive thawing/freezing cycles. Oligonucleotide primers employed in the reaction were synthesized by Invitrogen Life Technologies and supplied in lyophilised form. In the laboratory, the primers were resuspended in molecular grade deionised water to a concentration of 50 μM.

The standard PCRs performed in order to isolate the *Rad51* mRNA in *M. edulis* were carried out in a volume of 25 μl consisting of 200 μM dNTPs, 10x Advantage 2 PCR Buffer (40 mM Tricine-KOH pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 μg/ml BSA, 0.005% Tween 20, 0.005% Nonidet-P40), 10-40 μg BSA per reaction, 1.5 μM of each sense and antisense primers and 0.5 μl 50x Advantage 2 Polymerase Mix (Clontech).

Amplifications were carried out in a Techne Thermal Cycler equipped with a heated lid. All reactions were initially denatured at 95°C for 1 min then 15 sec at 95°C denaturation, 15 sec at 48°C annealing and 1 min at 68°C elongation step. The last three steps were repeated 35 times followed by final step of holding at 4°C. Positive and negative controls were set up along side each set of PCR reactions. Negative controls consisted of all components of the PCR reaction excluding the template DNA while the positive controls were the reactions the primers for ribosomal gene *18s*, which is sequenced for *M. edulis*. The former control was to ensure that there was no contamination, while the latter was to ensure that the reaction is working, the template DNA is not damaged.

For each amplification, an optimisation exercise was carried out in order to create the right conditions for the amplification of the targeted fragment. The oligonucleotide primers used, magnesium ion concentration, BSA concentration and ionic concentration of the buffer were the varied parameters of the reaction. Also the cycling strategy of denaturation, annealing and extension temperatures and duration of the steps were varied.

3.2.6. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed as described in section 2.2.6.

3.2.7. Isolation of DNA fragments from agarose gel slices

The gel areas containing the DNA fragments of interest were excised on the UV transluminator using a clean scalpel. The gel slice was placed into a pre-weighted clean 1.5 ml plastic tube and processed according to the QIAquick Gel Extraction protocol (Qiagen Ltd.) described in section 2.2.7.

3.2.8. DNA cleaning

The PCR reaction at the end of the amplification is composed of a mixture of DNA fragments, residual oligonucleotide primers, unincorporated nucleotides and different salts and enzymes that were required for the amplification process. In order to make our subsequent steps more efficient and specific, some of these ingredients were removed. The excess nucleotides, primers and any DNA fragment under 100 bp were removed using NucleoSpin Extract II PCR clean-up and Gel extraction Protocol (Macherey-Nagel). Briefly, volumes of NT buffer were added, mixed gently then applied to a silica membrane and centrifuged at 11,000 x *g* for 1 min. 600 µl NT3 buffer

were added to the column and centrifuged 1 min at 11,000 x *g* in order to remove any trace of "unwanted" material. The column was subsequently centrifuged for 2 min and then placed into a clean 1.5 ml tube. To elute the DNA, 15-50 µl NE (5mM Tris-HCl, pH 8.5) was applied to the centre of the membrane, left for 1 min and centrifuged 1 min at 11,000 x *g*. The sample was stored at –20°C.

3.2.9. Cloning PCR-generated fragments of DNA

The cloning technique allows us to separate different DNA fragments from a mixture and produce them in large quantities. To achieve this, we followed the DNA cloning method that uses bacterial plasmids. The pGEM®-T Easy plasmid vector (Promega Corporation) is used for the DNA cloning and has single 3' deoxythymidine (T) residues and contain the resistance genes to ampicillin as well as the *LacZα* gene.

The DNA fragment, helped by its deoxyadenosine (A) overhangs at the 3' ends, is inserted into the plasmid DNA in the middle of the *LacZα* gene. 3 µl of PCR product purified as described in section 3.2.8 or processed as in section 3.2.7 were mixed on ice with 50 ng pGEM®-T vector, 5 µl 2X ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP and 10% polyethylene glycol) and 1 µl T4 DNA ligase (3.0 Weiss units/µl). The reaction was incubated overnight at 4°C.

The vectors prepared as above were then ready to be transformed into JM109 *E. coli* competent cells strain, High Efficiency (Promega). 50 µl of frozen JM109 competent cells were thawed on ice and mixed gently with the pipette tip with 2 µl of the ligation reaction. The vial was than incubated for 20 min on ice and then heat shock for exactly 45 sec at 42°C. The vial was then placed again on ice. 950 µl S.O.C. medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the reaction and incubated for 2-3 hr at

37°C into a shaking incubator at 150 rpm. The culture was plated onto LB agar plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 15 g/l agar, pH 7.0) containing kanamycin (50 µg/ml) and X-gal in dimethyl formamide (40 µg/ml) and incubated overnight at 37°C. Single white colonies, indicating the presence of the plasmid in the cell because of the kanamycin resistance and disruption of the *LacZα* gene by the insert DNA, were picked using a sterile pipette tip and inoculated into 400-500 µl of LB broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) containing 0.5 mg/ml ampicillin. The cultures were grown overnight into a shaking incubator at 37°C and 200 rpm.

3.2.10. Extraction and purification of plasmid DNA

Plasmid DNA was extracted from *E. coli* cultures using NucleoSpin Plasmid DNA Purification Protocol (Macherey-Nagel), which is based on, an alkaline lysis of the cells followed by the absorption of plasmid DNA to the resin beads. Approximately 1-5 ml of the *E. coli* cultures (see 3.2.9) was centrifuged for 2 min at 10,000 x *g* and the supernatant discarded. The pellet was resuspended in 250 µl Cell Resuspension Solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 100 µg/ml RNase A) and mixed by inverting 4 times with 250 µl Cell Lysis Solution (0.2 M NaOH, 1% SDS). 10 µl of alkaline protease solution was added and mixed by inverting 4 times, then left in room temperature for 5 min. 350 µl Neutralisation Solution (1.32 M potassium acetate pH 4.8) was then added and mixed by inverting the tube 4 times. The tube was centrifuged 10 min at 10,000 x *g* and the supernatant mixed with 1 ml of resin in Wizard® Miniprep Column attached to a vacuum manifold. The sample was load into the column and washed with 1 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5, 40 mM EDTA, 55% ethanol). The column was removed from the vacuum

manifold and transferred to a micro centrifuge tube and centrifuged for 2 min at 10,000 x *g* to remove any trace of wash solution. The plasmid DNA was eluted in a clean tube with 50 µl RNase free water by centrifugation and stored at –20°C.

3.2.11. Enzymatic digestion of the plasmid DNA using EcoR I restriction enzymes

In order to determine the size of the insert, the plasmid DNA was digested using *Eco*R I restriction enzyme (Promega Corporation) as described in section 2.2.12. The digestion products were analysed on an agarose gel as described in section 2.2.6.

3.2.12. Sequencing the potential *Rad51* containing sub-clones

Approximately 1 µg plasmid DNA was processed as described in section 2.2.13.

3.2.13. RACE Rapid amplification of cDNA ends

The SMART ^{TM} RACE cDNA Amplification Kit (Takara Bio, Clontech) provides a method for performing both 5'- and 3'- rapid amplification of cDNA ends (RACE), allowing the isolation of the complete sequence of the target transcript (Fig. 3.2.13.1).

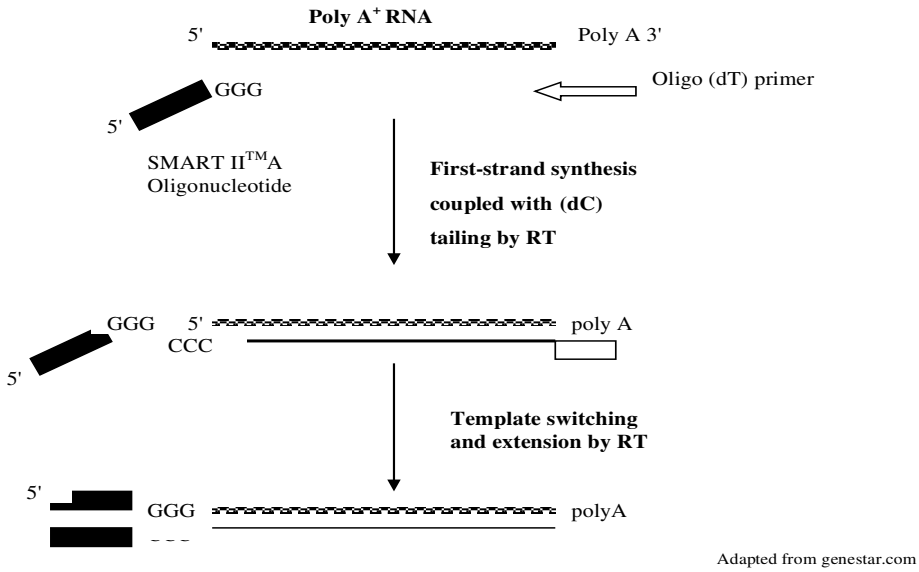

Fig. 3.2.13.1. Mechanism of SMART cDNA synthesis. First-strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The SMART II A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for MMLV RT.

Following reverse transcription, the first-strand cDNA is used directly in 5' and 3' RACE PCR reactions. The gene specific primers designed for these reactions should be at least 23-28 nucleotides in length, have high melting points (≥65^{o}C best result are obtained with melting points ≥70^{o}C), have a GC content of 50-70% and have an area of overlap to act as a positive control for PCR reactions. Longer primers with greater melting points greater than 70^{o}C give a better amplification in RACE PCR. Location of gene specific primers giving the best results should be chosen to give a product of 2kb or less (Fig. 3.2.13.2).

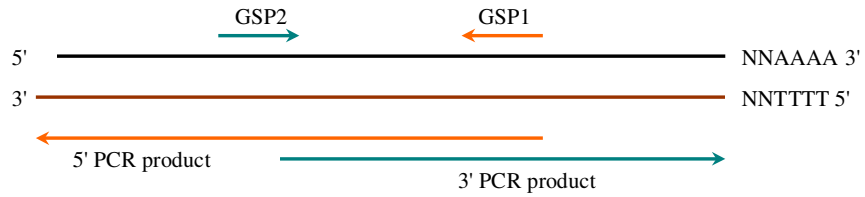

Fig. 3.2.13.2. Illustrating the relationship of the gene specific primers (GSPs) to the cDNA template. The gene specific primers designed will produce overlapping products. This overlap allows the gene specific primers together to give a positive control reaction in the PCR.

3.2.13.1. RACE first strand cDNA Synthesis

Using two 0.2 ml PCR tubes, >200 ng of total RNA was added to two separate 10 µl reactions, of 3' and 5' first strand synthesis. In the 5' RACE ready cDNA tube, 3 µl of RNA sample, 1 µl of 5'-CDS primer, 1 µl SMART II A oligonucleotide and 5 µl of sterile H_2O were added. To the 3' RACE ready cDNA tube, 3 µl of RNA sample, 1 µl of 3'-CDS primer (Table 3.2.13.1.1) and 5 µl of sterile H_2O were added. The content of both tubes were mixed briefly, spun and incubated at 70^{o}C for 2 min in a thermal cycler. They were then cooled on ice for a 2 min and to each reaction tube was added 2 µl of 5X first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 30 mM $MgCl_2$), 1 µl DTT 20 mM, 1 µl dNTP mix 10 mM and 1 µl of PowerScript Reverse Transcriptase. The contents of each tube were mixed by pipetting, spun and incubated at 42^{o}C for 1.5 hr in a thermal cycler. To dilute the first-strand reaction product before use 20-100 µl of Tricine-EDTA ($C_6H_{13}NO_5$-$C_{10}H_{16}N_2O_8$) buffer was added.

Table 3.2.13.1.1. RACE primer details (Clontech)

Component	Conc^{n} ! M	Sequence 5'-3'
SMART II A Oligonucleotide	10	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3' RACE CDS primer	10	AAGCAGTGGTATCAACGCAGAGTAC(T)30V N*
5' RACE CDS primer	10	(T)25V N*
10 x Universal	0.4	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
Primer Mix A	0.2	CTAATACGACTCACTATAGGGC
GSP 1 (Invitrogen)	0.5	GCTGCTCCATCTACCTGTGCTACAACCTG
GSP 2 (Invitrogen)	0.5	GGGTGGAGGTGAAGGAAAAGCTTTA

* N = A, C, G or T; V = A, G, or C

3.2.13.2. Amplification of RACE cDNA

Gene specific primers were designed for the 5' and 3' RACE reactions. Sufficient master mix was prepared for all PCR reactions plus half, to ensure for sufficient volume. The same master mix was used for both 5' and 3' RACE reactions. For each 50 µl reaction the following reagents were mixed:

Master Mix 34.5 µl PCR grade water
5 µl 10x Advantage 2 PCR Buffer
1 µl dNTP Mix (10 mM)
1 µl 50x Advantage 2 Polymerase Mix
41.5 µl Total volume (mixed gently and centrifuged)

For 5' RACE the PCR reactions were prepared as shown in (Table 3.2.13.2.1).

Table 3.2.13.2.1. The component for the 5' RACE PCR reaction.

Component	GPS 1 only (- Control)	UPM only (- Control)	5' RACE Sample
5' RACE Ready cDNA	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	-	5 µl	5 µl
GSP 1 (10mM)	1 µl	-	1 µl
H_2O	5 µl	1 µl	-
Master Mix	41.5 µl	41.5 µl	41.5 µl

For 3' RACE the PCR reactions were prepared as shown in (Table 3.2.13.2.2).

Table 3.2.13.2.2. The component for the 3' RACE PCR reaction.

Component	GPS 2 only (- Control)	UPM only (- Control)	3' RACE Sample
3' RACE Ready cDNA	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	-	5 µl	5 µl
GSP 2 (10mM)	1 µl	-	1 µl
H_2O	5 µl	1 µl	-
Master Mix	41.5 µl	41.5 µl	41.5 µl

Three steps PCR programme (Techne) was used in amplifying 5' and 3' RACE cDNA starting with activation the enzyme with an initial "Hot start" of 95°C for 1 min, followed by:

a) 5 cycles: 94°C, 30"	b) 5 cycles: 94°C, 30"	c) 25 cycles: 94°C, 30"
70°C, 30"	65°C, 30"	60°C, 30"
72°C, 1'	72°C, 1 '	72°C, 1'

Formatted: French (France)

The reaction was incubated at 72°C for 2 min as a final extension and then maintained after completion at 4°C and stored at -20°C. PCR products were analysed and separated by gel electrophoresis.

3.3. RESULTS

3.3.1. Isolation of total RNA from *M. edulis* gonads

The use of extraction method described in section 3.2.2 provided a high yield of good quality total RNA (A260 : A280 = 2.097). About 30 mg of tissue yielded 1421.2 µg/ml total RNA.

3.3.2. *Rad51* mRNA amplification from *M. edulis*

Several PCRs were conducted in order to isolate the *Rad51* mRNA fragment from *M. edulis*. Different combinations of the designed primers (Table 3.2.4.2 and Fig. 3.2.4.2) were used in reactions while other parameters were also varied (see 3.2.5). Using the template cDNA prepared with the specific forward primer Rad51F1 and the reverse primer Rad51R4, yielded a product of the expected size of 441 bp (Fig. 3.3.2.1).

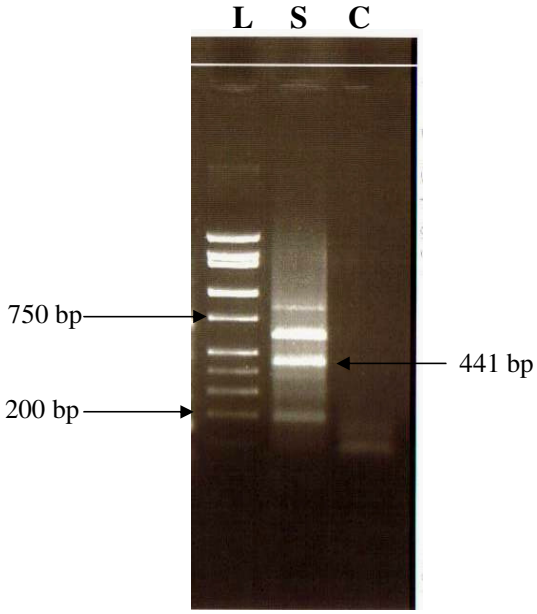

Fig. 3.3.2.1. Ethidium bromide stained 0.8 % agarose gel displaying PCR amplification products obtained using *M. edulis* cDNA as a template and the primer pair (expected product size – 441 bp). Lane L is the molecular size ladder, lane C is the negative control and lane S is the cDNA sample.

3.3.3. Sequencing the isolated DNA fragments

The sequencing results showed that the putative *Rad51* 441 bp fragment was similar to that reported in other species. The BLAST algorithm confirmed the deduced identity of the isolated fragment as a part of the *Rad51* gene (Fig. 3.3.3.1). The ClustalW programme showed 77% similarity between the isolated fragment in *M. edulis* and *H. sapiens Rad51* sequence and a range of similarity between 72-75% with *D. rerio*, *X. laevis* and *D. polymorpha* species.

```
D.polymorpha      -TACAGGGAAGACACAGATTTGTCACACTCTGGCAGTCACCTGTCAGTTGCCGATAGACA 59
D.rerio           GGACAGGAAAGACGCAGCTTTGTCACACACTAGCCGTCACCTGCCAGCTGCCCATAGATC 480
X.laevis          GCACAGGAAAGACTCAGCTGTGTCACACTCTTGCTGTCACCTGTCAGCTTCCCATTGATA 439
M.edulis          ----------------------------------------------TCAGCTTCCTATAGATA 17
H.sapiens         GAACTGGGAAGACCCAGATCTGTCATACGCTAGCTGTCACCTGCCAGCTTCCCATTGACC 448
                                                                *** * ** ** **

D.polymorpha      TGGGCGGTGGGGAAGGAAAATGCCTCTACATCGACACAGAAGGCACATTTAGGCCTGAAC 119
D.rerio           AGGGTGGAGGTGAAGGAAAAGCCATGTACATTGACACTGAAGGAACTTTCCGTCCAGAGA 540
X.laevis          GAGGTGGTGGTGAGGGCAAGGCTATGTACATTGATACAGAAGGAACCTTTCGTCCAGAAC 499
M.edulis          TGGGTGGAGGTGAAGGAAAAGCTTTATACATTGATTCAGAGGGAACATTTAGACCAGAAA 77
H.sapiens         GGGGTGGAGGTGAAGGAAAGGCCATGTACATTGACACTGAGGGTACCTTTAGGCCAGAAC 508
                    ** ** ** ** ** **     * ***** **  * ** ** ** **  * ** **
```

```
D.polymorpha      GTTTGCTAGCTGTGTCAGAGAGGTATGGCCTCTCTGGCAGTGATGTGTTGGACAATGTGG 179
D.rerio           GACTGCTGGCTGTGGCTGAACGGTATGGTCTGGTGGGCAGTGATGTTCTGGATAACGTGG 600
X.laevis          GTTTGCTTGCTGTAGCTGAAAGATATGGATTATCGGGAAGTGATGTTCTTGATAATGTTG 559
M.edulis          GATTGTTAGCTGTTGCTGAAAGGTATGGTTTATCTGGAAGTGATGTTTTAGACAATGTAG 137
H.sapiens         GGCTGCTGGCAGTGGCTGAGAGGTATGGTCTCTCTGGCAGTGATGTCCTGGATAATGTAG 568
                  *  ** * ** **  * **  * *****  *    ** ********  * ** ** ** *

D.polymorpha      CCTATGCGAGGGCGTACAACAGCGACCACCAATCACAGCTTCTCATCCAGGCAGCGGCCA 239
D.rerio           CCTACGCCAGAGCCTTCAACACTGACCATCAAACACAGCTGCTGTATCAGGCCTCCGCTA 660
X.laevis          CTTATGCCCGTGCCTTCAACACCGACCATCAGACCCAACTCTTGTACCAAGCGTCGGCCA 619
M.edulis          CTTATGCTAGAGCCTACAATAGTGATCACCAAACCCAGCTGTTGGTACAGGCTGCTGCAA 197
H.sapiens         CATATGCTCGAGCGTTCAACACAGACCACCAGACCCAGCTCCTTTATCAAGCATCAGCCA 628
                  * ** **  * ** * *** *  ** ** **  * ** **  *    ** **  * ** *

D.polymorpha      TGATGGCTGAATCACGGTACGCCCTCCTGGTAGTGGACAGTGCCACAGCTCTGTATAGGA 299
D.rerio           TGATGACCGAGTCCAGATACGCTCTGCTGATAGTAGACAGCGCCACAGCTCTCTACAGGA 720
X.laevis          TGATGGCAGAGTCAAGATACGCCCTTCTTATTGTGGACAGTGCGACTGCGCTCTACAGGA 679
M.edulis          TGATGTCAGAATCTAGGTATGCTTTGTTGATAGTAGACAGTGCTACCTCTCTCTACAGAA 257
H.sapiens         TGATGGTAGAATCTAGGTATGCACTGCTTATTGTAGACAGTGCCACCGCCCTTTACAGAA 688
                  *****   ** **  * ** **  *  *  * ** ***** ** **  * ** ** ** *

D.polymorpha      CAGACTACTCTGGTAGAGGGGAGCTCGCTGCTAGACAGATGCACCTGGCACGCTTCTTGA 359
D.rerio           CAGATTACTCGGGACGAGGGGAGCTGTCTGCCCGACAGGGGCATCTGGGACGCTTTCTGC 780
X.laevis          CGGATTATTCTGGGAGAGGGGAGCTTTCAGCACGTCAGATGCATCTGGCACGCTTTCTTA 739
M.edulis          CAGATTATTCAGGTCGAGGAGAATTGTCAGCGAGACAAATGCATTTAGCCAGATTTCTGA 317
H.sapiens         CAGACTACTCGGGTCGAGGTGAGCTTTCAGCCAGGCAGATGCACTTGGCCAGGTTTCTGC 748
                  * ** ** ** **  **** **  *  * **  * **   ***  * *   * **  *

D.polymorpha      GAATGCTTCTCCGACTAGCAGACGAGTTTGGTGTTGCCGTGGTGATCACAAACCAGGTGG 419
D.rerio           GTATGCTGCTGCGTCTCGCTGATGAGTTTGGTGTGGCTGTCGTCATCACTAACCAGGTTG 840
X.laevis          GAATGCTACTTCGACTCGCAGATGAGTTTGGTGTTGCAGTCGTCATCACAAACCAGGTTG 799
M.edulis          GAATGTTGTTGAGATTAGCTGATGAGTATGGAGTAGCAGTGGTAATCACTAATCAGGTTG 377
H.sapiens         GGATGCTTCTGCGACTCGCTGATGAGTTTGGTGTAGCAGTGGTAATCACTAATCAGGTGG 808
                  * *** *  *  *  * ** ** **** *** ** ** ** ** ***** ** ***** *

D.polymorpha      TGGCACAAGTGGATGGTGCGGCCATGTTTTCTGCAGACCCC------------------- 460
D.rerio           TAGCACAGGTGGACGGAGCAGCCATGTTTTCAGCAGATCCCAAGAAGCCTATTGGTGGAA 900
X.laevis          TTGCCCAAGTAGATGGAGCCGCCATGTTTGCTGCTGATCCCAAGAAGCCCATTGGAGGAA 859
M.edulis          TAGCACAGGTAGATGGAGCAGCA---------------------------------- 400
H.sapiens         TAGCTCAAGTGGATGGAGCAGCGATGTTTGCTGCTGATCCCAAAAAACCTATTGGAGGAA 868
                  * ** ** ** ** ** ** **
```

Fig. 3.3.3.1. An alignment of the isolated *Rad51* fragment from *M. edulis* with *Rad51* in different invertebrate and vertebrate species showed high homology. Asterix denotes homology.

3.3.4. *Rad51* amplification using mussel 5' and 3' RACE cDNA template

Several PCRs were conducted in order to isolate the remainder of the *Rad51* mRNA from *M. edulis*. 5' and 3' RACE cDNA were prepared with the GSP1 and GSP2 and a smear was observed including a product of a size 800bp obtained in 5' RACE PCR (Fig. 3.3.4.1).

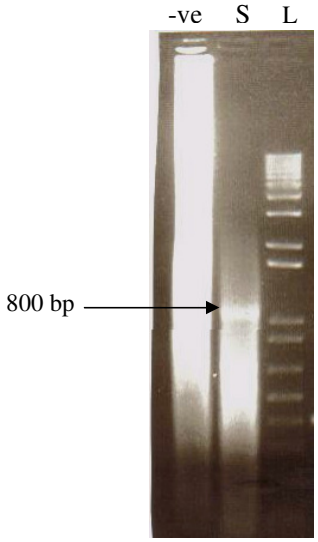

Fig. 3.3.4.1. Ethidium bromide stained 0.8 % agarose gel displaying RACE PCR amplification product, lane S, obtained using *M. edulis* 5' RACE cDNA as a template and the gene specific primer GSP 1(a product size – 800 bp). Lane L is the molecular size ladder.

3.3.5. Characterization of the 5' RACE *Rad51* fragment

The sequencing results showed that the putative *Rad51* 800 bp fragment was similar to the *Rad51* sequence in other species. The BLAST algorithm confirmed the identity of the isolated fragment as a part of the *Rad51* gene. Specifically, there was 70% similarity with *D. polymorpha* and *D. rerio* species and a 69% with *H. sapiens* and *X. laevis* species (Fig. 3.3.5.1).

```
H.sapiens          ATGGCAATGCAG---ATGCAGCTTGAAGCAAATGCAGATACTTCAGTGGAAGAAGAAAGC 57
X.laevis           ATGGCCATGCAA---GCGCACTATGAAGC---CGAAGCCACC------GAGGAAGAGCAT 48
D.polymorpha       ------------------------------------------------------------
D.rerio            ATGAGGAACGCA---TCCCGGGTGGAGGTGGAGGCAGAAGTG---GAGGAAGAGGAGAAT 54
M.edulis           ATGGCAATGCAACAATCTCGTCAACAAGCCTCAGCACAAGCAGAAGAAACTGAAGAAACC 60

H.sapiens          TTTGGCCCACAACCCATTTCACGGTTAGAGCAGTGTGGCATAAATGCCAACGATGTGAAG 117
X.laevis           TTTGGACCACAGGCAATATCCAGATTAGAGCAATGTGGGATAAATGCAAATGACGTCAAG 108
D.polymorpha       ------------------------------------------------------------
D.rerio            TTTGGGCCACAACCAGTTTCCCGCCTAGAGCAAAGTGGCATCAGCAGCAGTGACATTAAG 114
M.edulis           TTTGGACCATTGCCCTTAAAGCAATTAGAGGCAAATGGTATTGGTGCATCAGATATAAAG 120

H.sapiens          AAATTGGAAGAAGCTGGATTCCATACTGTGGAGGCTGTTGCCTATGCGCCAAAGAAGGAG 177
X.laevis           AAACTGGAGGAGGCCGGGTTCCACACAGTAGAAGCAGTGGCTTATGCTCCAAAGAAGGAA 168
D.polymorpha       ------------------------------------------------------------
D.rerio            AAGCTGGAAGATGGTGGTTTCCATACTGTAGAAGCCGTCGCATATGCACCCAAGAAAGAA 174
M.edulis           AAGCTAGAAGAAGCTGGTTACTTCACAGTAGAGGCAGTGGCATATGCACCAAAGAAGAGT 180
```

```
H.sapiens         CTAATAAATATTAAGGGAATTAGTGAAGCCAAAGCTGATAAAATTCTGGCTGAGGCAGCT 237
X.laevis          CTGCTCAATATAAAAGGCATCAGTGAGGCTAAAGCTGAAAAAATCCTAGCAGAAGCTGCC 228
D.polymorpha      ------------------------------------------------------------
D.rerio           CTGCTGAATATTAAAGGAATTAGCGAAGCCAAAGCTGACAAGATCCTGACAGAAGCTGCT 234
M.edulis          CTTTTAGTTATCAAAGGAATCAGTGGAGCTAAAGCTGATAAGATATTGGCAGAAGCTGCT 240

H.sapiens         AAATTAGTTCCAATGGGTTTCACCACTGCAACTGAATTCCACCAAAGGCGGTCAGAGATC 297
X.laevis          AAACTGGTTCCCATGGGATTTACTACAGCCACAGAGTTTCACCAGAGACGCTCTGAAATA 288
D.polymorpha      ------------------------------------------------------------
D.rerio           AAAATGGTTCCCATGGGCTTCACCACAGCGACTGAGTTTCACCAGCGCAGAGCCGAAATC 294
M.edulis          AAACTGGTACCTATGGGTTTCACAACAGCAACAGAATTTCATCAGAAAAGATCAGAAATT 300

H.sapiens         ATACAGATTACTACTGGCTCCAAAGAGCTTGACAAACTACTTCAAGGTGGAATTGAGACT 357
X.laevis          ATACAGATCAGTACAGGTTCCAAGGAGCTTGACAAGCTTCTCCAAGGGGGCGTTGAAACT 348
D.polymorpha      ------------------------------------------------------------
D.rerio           ATCCAGATCTCCACAGGATCTAAAGAGCTGGATAAACTCCTGCAGGGAGGAATCGAGACA 354
M.edulis          ATTCAAATCACAACTGGTTCTAAAGAGTTGGATAAACTATTGCAAGGTGGCATTGAGACT 360

H.sapiens         GGATCTATCACAGAAATGTTTGGAGAATTCCGAACTGGGAAGACCCAGATCTGTCATACG 417
X.laevis          GGTTCCATCACAGAGATGTTCGGTGAGTTTCGCACAGGAAAGACTCAGCTGTGTCACACT 408
D.polymorpha      ------------------------------TACAGGGAAGACACAGATTTGTCACACT 28
D.rerio           GGATCCATTACGGAGATGTTTGGAGAGTTTCGGACAGGAAAGACGCAGCTTTGTCACACA 414
M.edulis          GGGTCAATTACAGAAATATTTGGAGAGTTTAGGACAGGTAAAACACAGCTGACCCACACA 420
                                                  ** ** ** ** *** *    ** **

H.sapiens         CTAGCTGTCACCTGCCAGCTTCCCATTGACCGGGGTGGAGGTGAAGGAAAGGCCATGTAC 477
X.laevis          CTTGCTGTCACCTGTCAGCTTCCCATTGATAGAGGTGGTGGTGAGGGCAAGGCTATGTAC 468
D.polymorpha      CTGGCAGTCACCTGTCAGTTGCCGATAGACATGGGCGGTGGGGAAGGAAAATGCCTCTAC 88
D.rerio           CTAGCCGTCACCTGCCAGCTGCCCATAGATCAGGGTGGAGGTGAAGGAAAAGCCATGTAC 474
M.edulis          TTGGCAGTTACCTGTCAGCTTCCTATAGATATGGGTGGAGGTGAAGGAAAAGCTTTATAC 480
                   * ** ** ***** *** * ** ** **    ** ** ** ** ** **     * ***

H.sapiens         ATTGACACTGAGGGTACCTTTAGGCCAGAACGGCTGCTGGCAGTGGCTGAGAGGTATGGT 537
X.laevis          ATTGATACAGAAGGAACCTTTCGTCCAGAACGTTTGCTTGCTGTAGCTGAAAGATATGGA 528
D.polymorpha      ATCGACACAGAAGGCACATTTAGGCCTGAACGTTTGCTAGCTGTGTCAGAGAGGTATGGC 148
D.rerio           ATTGACACTGAAGGAACTTTCCGTCCAGAGAGACTGCTGGCTGTGGCTGAACGGTATGGT 534
M.edulis          ATTGATTCTGAGGGAACATTTAGACCAGAAAGATTGTTAGCTGTTGCTGAAAGGTATGGT 540
                  ** **  * ** ** ** **  * ** **  *  ** * ** **  * **  * *****

H.sapiens         CTCTCTGGCAGTGATGTCCTGGATAATGTAGCATATGCTCGAGCGTTCAACACAGACCAC 597
X.laevis          TTATCGGGAAGTGATGTTCTTGATAATGTTGCTTATGCCCGTGCCTTCAACACCGACCAT 588
D.polymorpha      CTCTCTGGCAGTGATGTGTTGGACAATGTGGCCTATGCGAGGGCGTACAACAGCGACCAC 208
D.rerio           CTGGTGGGCAGTGATGTTCTGGATAACGTGGCCTACGCCAGAGCCTTCAACACTGACCAT 594
M.edulis          TTATCTGGAAGTGATGTTTTAGACAATGTAGCTTATGCTAGAGCCTACAATAGTGATCAC 600
                   *    ** ********  * ** ** ** ** ** **  * ** * *** *  ** **

H.sapiens         CAGACCCAGCTCCTTTATCAAGCATCAGCCATGATGGTAGAATCTAGGTATGCACTGCTT 657
X.laevis          CAGACCCAACTCTTGTACCAAGCGTCGGCCATGATGGCAGAGTCAAGATACGCCCTTCTT 648
D.polymorpha      CAATCACAGCTTCTCATCCAGGCAGCGGCCATGATGGCTGAATCACGGTACGCCCTCCTG 268
D.rerio           CAAACACAGCTGCTGTATCAGGCCTCCGCTATGATGACCGAGTCCAGATACGCTCTGCTG 654
M.edulis          CAAACCCAGCTGTTGGTACAGGCTGCTGCAATGATGTCAGAATCTAGGTATGCTTTGTTG 660
                  **  * ** **  *    ** **  * ** ******   ** **  * ** **  *  *

H.sapiens         ATTGTAGACAGTGCCACCGCCCTTTACAGAACAGACTACTCGGGTCGAGGTGAGCTTTCA 717
X.laevis          ATTGTGGACAGTGCGACTGCGCTCTACAGGACGGATTATTCTGGGAGAGGGGAGCTTTCA 708
D.polymorpha      GTAGTGGACAGTGCCACAGCTCTGTATAGGACAGACTACTCTGGTAGAGGGGAGCTCGCT 328
D.rerio           ATAGTAGACAGCGCCACAGCTCTCTACAGGACAGATTACTCGGGACGAGGGGAGCTGTCT 714
M.edulis          ATAGTAGACAGTGCTACCTCTCTCTACAGAACAGATTATTCAGGTCGAGGAGAATTATCA 720
                   * ** ***** ** **  * ** ** ** ** ** ** ** **  **** **  *  *

H.sapiens         GCCAGGCAGATGCACTTGGCCAGGTTTCTGCGGATGCTTCTGCGACTCGCTGATGAGTTT 777
X.laevis          GCACGTCAGATGCATCTGGCACGCTTTCTTAGAATGCTACTTCGACTCGCAGATGAGTTT 768
D.polymorpha      GCTAGACAGATGCACCTGGCACGCTTCTTGAGAATGCTTCTCCGACTAGCAGACGAGTTT 388
D.rerio           GCCCGACAGGGGCATCTGGGACGCTTTCTGCGTATGCTGCTGCGTCTCGCTGATGAGTTT 774
M.edulis          GCTAGACAAGTGCATTTAGCCAGATTTCTGAGAATGTTGTTGAGATTAGCTGATGAGTAT 780
                  **  * **   ***  * *   * **  *  * *** *  *  *  * ** ** **** *
```

```
H.sapiens         GGTGTAGCAGTGGTAATCACTAATCAGGTGGTAGCTCAAGTGGATGGAGCAGCGATGTTT 837
X.laevis          GGTGTTGCAGTCGTCATCACAAACCAGGTTGTTGCCCAAGTAGATGGAGCCGCCATGTTT 828
D.polymorpha      GGTGTTGCCGTGGTGATCACAAACCAGGTGGTGGCACAAGTGGATGGTGCGGCCATGTTT 448
D.rerio           GGTGTGGCTGTCGTCATCACTAACCAGGTTGTAGCACAGGTGGACGGAGCAGCCATGTTT 834
M.edulis          GGAGTAGCAGTGGTAATCACTAATCAGGTTGTAGCACAGGTAGATGGAGCAGCAAGCC-- 838
                  ** ** ** ** ** ***** ** ***** ** ** ** ** ** ** ** ** *

H.sapiens         GCTGCTGATCCCAAAAAACCTATTGGAGGAAATATCATCGCCCATGCATCAACAACCAGA 897
X.laevis          GCTGCTGATCCCAAGAAGCCCATTGGAGGAAATATTATAGCACATGCATCAACTACACGG 888
D.polymorpha      TCTGCAGACCCC-------------------------------------------------- 460
D.rerio           TCAGCAGATCCCAAGAAGCCTATTGGTGGAAATATTCTGGCACACGCATCAACTACACGG 894
M.edulis          ------------------------------------------------------------

H.sapiens         TTGTATCTGAGGAAAGGAAGAGGGGAAACCAGAATCTGCAAAATCTACGACTCTCCCTGT 957
X.laevis          TTATATCTGAGGAAAGGCCGCGGTGAAACGCGTATCTGCAAAATCTACGACTCCCCCTGC 948
D.polymorpha      ------------------------------------------------------------
D.rerio           TTATACCTTAGGAAAGGCAGAGGTGAGACGAGGATATGTAAGATCTATGACTCTCCGTGT 954
M.edulis          ------------------------------------------------------------

H.sapiens         CTTCCTGAAGCTGAAGCTATGTTCGCCATTAATGCAGATGGAGTGGGAGATGCCAAAGAC 1017
X.laevis          CTCCCCGAAGCAGAGGCTATGTTTGCAATTAATGCTGATGGAGTGGGAGATGCCAAGGAC 1008
D.polymorpha      ------------------------------------------------------------
D.rerio           TTACCAGAGGCCGAGGCCATGTTTGCCATTAATGCTGATGGAGTGGGAGATGCTAAAGAC 1014
M.edulis          ------------------------------------------------------------

H.sapiens         TGA 1020
X.laevis          TGA 1011
D.polymorpha      ---
D.rerio           TGA 1017
M.edulis          ---
```

Fig. 3.3.5.1. An alignment of the isolated RACE *Rad51* nucleotide from *M. edulis* represents the homology with different invertebrate and vertebrate species. Asterix denotes homology.

Translation of the fragment of the isolated *M. edulis* RACE *Rad51* fragment and alignment with published Rad51 sequences using ClustalW, showed 87% similarity between the *M. edulis* and *D. polymorpha* Rad51 sequence and a range of similarity between 81-83% with the *H. sapiens*, *D. rerio* and *X. laevis* species (Fig. 3.3.5.2).

```
H.sapiens         MAM-QMQLEANADTSVEEESFGPQPISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKE 59
X.laevis          MAM-QAHYEAEAT---EEEHFGPQAISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKE 56
D.rerio           MRN-ASRVEVEAEVE-EEENFGPQPVSRLEQSGISSSDIKKLEDGGFHTVEAVAYAPKKE 58
D.polymorpha      ------------------------------------------------------------
M.edulis          MAMQQSRQQASAQAEETEETFGPLPLKQLEANGIGASDIKKLEEAGYFTVEAVAYAPKKS 60


H.sapiens         LINIKGISEAKADKILAEAAKLVPMGFTTATEFHQRRSEIIQITTGSKELDKLLQGGIET 119
X.laevis          LLNIKGISEAKAEKILAEAAKLVPMGFTTATEFHQRRSEIIQISTGSKELDKLLQGGVET 116
D.rerio           LLNIKGISEAKADKILTEAAKMVPMGFTTATEFHQRRAEIIQISTGSKELDKLLQGGIET 118
D.polymorpha      ------------------------------------------------------------
M.edulis          LLVIKGISGAKADKILAEAAKLVPMGFTTATEFHQKRSEIIQITTGSKELDKLLQGGIET 120


H.sapiens         GSITEMFGEFRTGKTQICHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYG 179
X.laevis          GSITEMFGEFRTGKTQLCHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYG 176
D.rerio           GSITEMFGEFRTGKTQLCHTLAVTCQLPIDQGGGEGKAMYIDTEGTFRPERLLAVAERYG 178
D.polymorpha      -----------TGKTQICHTLAVTCQLPIDMGGGEGKCLYIDTEGTFRPERLLAVSERYG 49
M.edulis          GSITEIFGEFRTGKTQLTHTLAVTCQLPIDMGGGEGKALYIDSEGTFRPERLLAVAERYG 180
                            *****: ************ ******.:***:************:****
```

```
H.sapiens         LSGSDVLDNVAYARAFNTDHQTQLLYQASAMMVESRYALLIVDSATALYRTDYSGRGELS 239
X.laevis          LSGSDVLDNVAYARAFNTDHQTQLLYQASAMMAESRYALLIVDSATALYRTDYSGRGELS 236
D.rerio           LVGSDVLDNVAYARAFNTDHQTQLLYQASAMMTESRYALLIVDSATALYRTDYSGRGELS 238
D.polymorpha      LSGSDVLDNVAYARAYNSDHQSQLLIQAAAMMAESRYALLVVDSATALYRTDYSGRGELA 109
M.edulis          LSGSDVLDNVAYARAYNSDHQTQLLVQAAAMMSESRYALLIVDSATSLYRTDYSGRGELS 240
                  * *************:*:***:*** **:*** *******:*****:************:

H.sapiens         ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTR 299
X.laevis          ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTR 296
D.rerio           ARQGHLGRFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADPKKPIGGNILAHASTTR 298
D.polymorpha      ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADP---------------- 153
M.edulis          ARQVHLARFLRMLLRLADEYGVAVVITNQVVAQVDGAAS--------------------- 279
                  *** **.************:******************

H.sapiens         LYLRKGRGETRICQIYDSPCLPEAEAMFAINADGVGDAKD 339
X.laevis          LYLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 336
D.rerio           LYLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 338
D.polymorpha      ----------------------------------------
M.edulis          ----------------------------------------
```

Fig. 3.3.5.2. An alignment of the predicted *M. edulis* Rad51 protein with Rad51 of different vertebrate and invertebrate species represents high homology. Asterix denotes homology.

The sequence was submitted to GenBank database and can be retrieved using accession number **FJ518826** (Fig. 3.3.5.3).

5′...ctagagcggcatgtgagccagcatatagatgtcacctagacctcacggttacctagtccgctagtgggaatttgacgatacatcaacagaaacg
ctaggcagatacgcagctggtacgatcgatcatagtacgcggcagttgctgatcgctagcagtgtatcacgcagagtacgcggggtatatgtggcgga
cgtctgattttagattgtaacatgtgaaactttacaaatgactaaatagtttactaaacacagagaataaactcgacctctatagatatacttttaattta
aagatagctagacaagaagaagcgtgttggattaagtgtacaggctacagccagtaaattttcagatctttatcatcttcacgtttaacatggcaatgc
aacaatctcgtcaacaagcctcagcacaagcagaagaaactgaagaaacctttggaccattgcccttaaagcaattagaggcaaatggtattggtgc
atcagatataaagaagctagaagaagctggttacttcacagtagaggcagtggcatatgcaccaaagaagagtcttttagttatcaaaggaatcagt
ggagctaaagctgataagatattggcagaagctgctaaactggtacctatgggtttcacaacagcaacagaatttcatcagaaaagatcagaaatta
ttcaaatcacaactggttctaaagagttggataaactattgcaaggtggcattgagactgggtcaattacagaaatatttggagagtttaggacaggta
aaacacagctgacccacacattggcagttacctgtcagcttcctatagatatgggtggaggtgaaggaaaagctttatacattgattctgagggaaca
tttagaccagaaagattgttagctgttgctgaaaggtatggtttatctggaagtgatgttttagacaatgtagcttatgctagagcctacaatagtgatc
accaaacccagctgttggtacaggctgctgcaatgatgtcagaatctaggtatgctttgttgatagtagacagtgctacctctctctacagaacagatta
ttcaggtcgaggagaattatcagctagacaagtgcatttagccagatttctgagaatgttgttgagattagctgatgagtatggagtagcagtggtaat
cactaatcaggttgtagcacaggtagatggagcagcaagcc...3′

Fig. 3.3.5.3. Nucleotide sequence of the *M. edulis* putative *Rad51* fragment isolated.

3.4. DISCUSSION

The aim of this section was to isolate and sequence the *Rad51* cDNA from the blue mussel *M. edulis* using rael-time PCR techniques. After several attempts, a *M. edulis* partial *Rad51* cDNA sequence was amplified encoding a putative 279 amino acid protein (**FJ518826**). The BLAST algorithm confirmed the identity of the isolated fragment as a part of a putative RAD51 (Fig. 3.3.5.2). It shares 87% similarity with

Rad51 in *D. polymorpha* and 83% with the human. The sequence also shares 81% similarity with the *Rad51* of *D. rerio*, *X. laevis*.

The deduced amino acid sequence is part of a conserved area, the putative ATP binding domains that contains the conserved Walker A (GEFRTGKT) and Walker B (LLIVD) motifs, characteristic of a P-loop NTPase superfamily (Thompson and Schild, 1999; Shin et al., 2003) (Fig. 3.4.1). The Walker A and B motifs bind the beta-gamma phosphate moiety of the bound nucleotide (typically ATP or GTP) and the Mg2+ cation, respectively. The P-loop NTPases are involved in diverse cellular functions, and they can be divided into two major structural classes: the KG (kinase-GTPase) class which includes Ras-like GTPases and the additional strand catalytic E (ASCE) class which includes ATPase Binding Cassette (ABC) 4Fe-4S iron sulfur cluster binding proteins of NifH family, RecA-like F1-ATPases, and ATPases Associated with a wide variety of Activities (AAA). Also included is a diverse set of nucleotide/nucleoside kinase families. More conservation of amino acids were predicted to mediate Rad51 filament formation in RecA-like recombinases, the Breast Cancer 2 susceptibility protein (BRCA2) interacts with RAD51 at residues phe 86 and ala 89 in *H. sapiens* (Pellegrini et al., 2002).

```
H.sapiens         MAM-QMQLEANADTSVEEESFGPQPISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKE 59
X.laevis          MAM-QAHYEAEAT---EEEHFGPQAISRLEQCGINANDVKKLEEAGFHTVEAVAYAPKKE 56
D.rerio           MRN-ASRVEVEAEVE-EEENFGPQPVSRLEQSGISSSDIKKLEDGGFHTVEAVAYAPKKE 58
D.polymorpha      ------------------------------------------------------------
M.edulis          MAMQQSRQQASAQAEETEETFGPLPLKQLEANGIGASDIKKLEEAGYFTVEAVAYAPKKS 60

H.sapiens         LINIKGISEAKADKILAEAAKLVPMGFTTATEFHQRRSEIIQITTGSKELDKLLQGGIET 119
X.laevis          LLNIKGISEAKAEKILAEAAKLVPMGFTTATEFHQRRSEIIQISTGSKELDKLLQGGVET 116
D.rerio           LLNIKGISEAKADKILTEAAKMVPMGFTTATEFHQRRAEIIQISTGSKELDKLLQGGIET 118
D.polymorpha      ------------------------------------------------------------
M.edulis          LLVIKGISGAKADKILAEAAKLVPMGFTTATEFHQKRSEIIQITTGSKELDKLLQGGIET 120
```

```
H.sapiens         GSITEMFGEFRTGKTQICHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYG 179
X.laevis          GSITEMFGEFRTGKTQLCHTLAVTCQLPIDRGGGEGKAMYIDTEGTFRPERLLAVAERYG 176
D.rerio           GSITEMFGEFRTGKTQLCHTLAVTCQLPIDQGGGEGKAMYIDTEGTFRPERLLAVAERYG 178
D.polymorpha      -----------TGKTQICHTLAVTCQLPIDMGGGEGKCLYIDTEGTFRPERLLAVSERYG 49
M.edulis          GSITEIFGEFRTGKTQLTHTLAVTCQLPIDMGGGEGKALYIDSEGTFRPERLLAVAERYG 180
                            *****: ************ ******.:***:************:****

H.sapiens         LSGSDVLDNVAYARAFNTDHQTQLLYQASAMMVESRYALLIVDSATALYRTDYSGRGELS 239
X.laevis          LSGSDVLDNVAYARAFNTDHQTQLLYQASAMMAESRYALLIVDSATALYRTDYSGRGELS 236
D.rerio           LVGSDVLDNVAYARAFNTDHQTQLLYQASAMMTESRYALLIVDSATALYRTDYSGRGELS 238
D.polymorpha      LSGSDVLDNVAYARAYNSDHQSQLLIQAAAMMAESRYALLVVDSATALYRTDYSGRGELA 109
M.edulis          LSGSDVLDNVAYARAYNSDHQTQLLVQAAAMMSESRYALLIVDSATSLYRTDYSGRGELS 240
                  * *************:*:***:*** **:*** *******:*****:************:

H.sapiens         ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTR 299
X.laevis          ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFAADPKKPIGGNIIAHASTTR 296
D.rerio           ARQGHLGRFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADPKKPIGGNILAHASTTR 298
D.polymorpha      ARQMHLARFLRMLLRLADEFGVAVVITNQVVAQVDGAAMFSADP---------------- 153
M.edulis          ARQVHLARFLRMLLRLADEYGVAVVITNQVVAQVDGAAS--------------------- 279
                  *** **.************:******************

H.sapiens         LYLRKGRGETRICQIYDSPCLPEAEAMFAINADGVGDAKD 339
X.laevis          LYLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 336
D.rerio           LYLRKGRGETRICKIYDSPCLPEAEAMFAINADGVGDAKD 338
D.polymorpha      ----------------------------------------
M.edulis          ----------------------------------------
```

Fig. 3.4.1. Multiple sequence alignment of the deduced amino acid sequence of Rad51 *M. edulis* (GenBank Accession no. **FJ518826**) and other available Rad51 sequences. Prediction of conserved domains was conducted using (www.ncbi.CDD), **Bold** is for ATP binding domain, *Italic* is for Walker A motif domain, underline is for Walker B motif domain and grey shadowed is for multimer breast cancer repeat complex (BRC) interface. Asterix denotes homology.

In summary, the work presented in this chapter show the isolation of a fragment of the *Rad51* cDNA from *M. edulis*. Using this sequence information it is now possible to develop an assay of *RAD51* mRNA expression to determine its role in the cells response to external damaging DSB agents such as IR.

Chapter 4

Isolation and Characterization of *M. edulis Chk1* mRNA

4.1. INTRODUCTION

Cell cycle checkpoints are control mechanisms that ensure the fidelity of cell division in eukaryotic cells. An important function of many checkpoints is to assess DNA damage, which is detected by sensor mechanisms. When damage is found, the checkpoint uses a signal mechanism to stall the cell cycle until repairs are made. All the checkpoints that assess DNA damage appear to utilize the same sensor-signal-effector mechanism. In response to irradiation, most yeast cells will arrest the cell cycle, repair the damage, and then continue. A cell that cannot repair the damage will arrest and may enter the apoptosis process (Dewey et al., 1995). A cell that can repair the damage but can't arrest will go on to divide, with lethal consequences (Elledge, 1996; Kastan and Bartek, 2004).

In most species blocking mitosis in response to damaged DNA occurs through inhibiting activation of the cyclic dependent kinase Cdc2, which regulates entry into mitosis. Cell cycle checkpoint kinase 1 (Chk1) acts downstream of ATM/ATR kinase to play an important role in DNA damage checkpoint control, embryonic development and tumour suppression (Liu et al., 2000, Sorensen et al., 2005). Activation of Chk1 involves phosphorylation of Ser317 and Ser345 and occurs in response to blocked DNA replication and certain forms of genotoxic stress (Zhao and Piwnica-Worms, 2001). Chk1 exerts it checkpoint mechanism on the cell cycle by regulating the cdc25 family of phosphatases. Chk1 phosphorylation of cdc25A targets it for proteolysis and inhibits its activity (Chen et al., 2003). Activated Chk1 can inactivate cdc25C via phosphorylation

at Ser216, blocking the activation of cdc2 and transition into mitosis (Zeng et al., 1998) (Fig. 4.1.1). Chk1 belongs to Serine/Threonine protein kinases (S-TKc), which is a member of the superfamily (PKc-like Super-family). The enzymatic activity of these protein kinases is controlled by phosphorylation of specific residues in the activation segment of the catalytic domain.

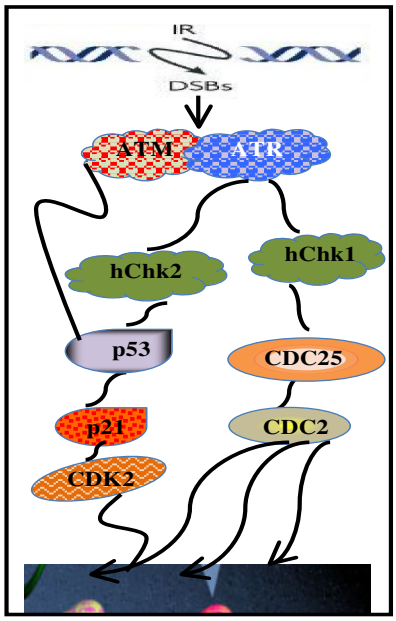

Fig. 4.1.1. Role of Chk1 in cell cycle and DNA damage response.

Chk1 has been isolated from several vertebrate species such as amphibians and mammals (Sanchez et al., 1997; Kumagai et al., 1998; Kudoh et al., 2001; Zachos et al., 2003; Zimin et al., 2009), also from a few invertebrate species including nematodes and arthropods (Adams et al., 2000; Kamath et al., 2003) (Table 4.1.1).

Table 4.1.1. A summary of Chk1 homologs isolated from vertebrate and invertebrate species.

Phylum	Species	Accession ID	Name
Arthropoda	*D. melanogaster*	NP_723987	grapes, isoform D
Nematoda	*Caenorhabditis elegans*	AAA93318	serine/threonine kinase
"	*Trichinella spiralis*	EFV50610	serine/threonine-protein kinase Chk1
Chordata	*D. rerio*	NP_956487	serine/threonine-protein kinase Chk1
"	*X. laevis*	NP_001082039 NP_001082040	serine/threonine-protein kinase Chk1
"	*G. gallus*	Q8AYC9	Serine/threonine-protein kinase Chk1
"	*Bos taurus*	NP_001091492 XP_591405	serine/threonine-protein kinase Chk1
"	*Pan troglodytes*	XP_001146525	CHK1 checkpoint homolog isoform 7
"	*M. musculus*	AAC53334	Chk1
"	*R. norvegicus*	Q91ZN7	Serine/threonine-protein kinase Chk1
"	*H. sapiens*	AAC51736	Chk1

Chk1 mRNA expression is used in yeast as a biomarker in response of IR (Watson et al., 2004). In other studies, cell cycle checkpoint mechanisms, including Chk1, are used as an important kinase activity in sensing DSB damage (Peng and Lin, 2011). Studies using mammalian cells have shown that errors in cell cycle checkpoints can lead to genomic instability (Deng, 2006; Shen, 2011). This relationship between cell cycle checkpoints and DNA repair with the concept of mRNA expression can therefore be considered a future aim to achieve a sensitive biomarker of IR exposure in the aquatic environment.

This chapter presents the isolation and characterization of the cell cycle checkpoint kinase, *Chk1,* mRNA from the marine mussel *M. edulis*.

4.2. MATERIALS AND METHODS

4.2.1. Animals

The mussels (*M. edulis*) were collected and processed as described in section 3.2.1.

4.2.2. Total RNA isolation and purification from mussel gonadal tissue

The total RNA extraction from mussel gonads was carried out following the protocol described in section 3.2.2 and stored at -20^{o}C until further processing.

4.2.3. Quantification of total RNA

RNA concentration was determined by a Qubit^{TM} fluorometer (Invitrogen Detection Technologies) (see section 2.2.8).

4.2.4. First strand synthesis of cDNA

cDNA was synthesised from DNase treated total RNA following the protocol described in section 3.2.3 and stored at –20^{o}C.

4.2.5. Oligonucleotide primer design

The oligonucleotide primers used were designed using aligned fragments of *Chk1* mRNA from related species available on GenBank (Table 4.2.5.1).

Table 4.2.5.1. Chk1 Protein accession numbers in different species.

Species	Protein ID
H. sapiens	AF016582.1
X. laevis	AB019218.1
D. rerio	NM_200193.1
X. tropicalis	CR848200.2

The *Chk1* sequences available in GenBank were aligned using the computer program CLUSTALW, and the areas with the greatest homology were used for designing primers. The primers (Chk1F1, Chk1F2, Chk1R) were designed aligning the Chk1 protein sequence from different organisms and based on areas of homology (Fig. 4.2.5.1).

```
                                Chk1F1          Chk1F2
X.laevis          MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
X.tropicalis      MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
H.sapiens         MAVPFVEDWDLVQTLGEGAYGEVQLAVNRVTEEAVAVKIVDMKRAVDCPENIKKEICINK 60
D.rerio           MAVPFVKDWDVVQTLGEGAYGEVRLLVNKKTEEAVAVKVVDMAKAKDCIENVKKEVCICK 60
                  ******:***:************:* **: ********:*** :* ** **:***:** :

X.laevis          MLSHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
X.tropicalis      MLNHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
H.sapiens         MLNHENVVKFYGHRREGNIQYLFLEYCSGGELFDRIEPDIGMPEPDAQRFFHQLMAGVVY 120
D.rerio           MLSHPNIVRFFG---------------------------------------------------- 72
                  **.* *:*:*:*                                          Chk1R1

X.laevis          LHSIGITHRDIKPENLLLDERDQLKISDFGLATVFRHNGKERLLSKMCGTLPYVAPELIK 180
X.tropicalis      LHSIGITHRDIKPENLLLDDRDHLKISDFGLATVFRHNAKERLLNKMCGTLPYVAPELIK 180
H.sapiens         LHGIGITHRDIKPENLLLDERDNLKISDFGLATVFRYNNRERLLNKMCGTLPYVAPELLK 180
D.rerio           -HSVGITHRDIKPENILLDDKDNLKISDFGLATMFRHRGRERALNRLCGTLPYVAPELMS 131
                   *.:***********:***::*:**********:**:. :** *.::***********:.

X.laevis          SRAFHADPVDVWSCGIVLTAMLAGELPWDQPNEVCQEYCDWKEKNHYL--TKKISATLLA 238
X.tropicalis      SRAFNAEP---------------------------------------------------- 188
H.sapiens         RREFHAEPVDVWSCGIVLTAMLAGELPWDQPSDSCQEYSDWKEKKTYLNPWKKIDSAPLA 240
D.rerio           RSSFNAQPADTWACGIVLTAMLAGELPWDQPSENCQEYLDWLERKTYLTPWKKIDAVPLS 191
                     *:*:*
```

Fig. 4.2.5.1. ClustalW2 multiple sequence alignment of the deduced amino acid sequences of the Chk1 of different species showing the designed degenerated primers. Asterix denotes homology.

The designed primers were degenerate as a consequence of the redundancy in the codification of amino acids (Table 4.2.5.2).

Table 4.2.5.2. Oligonucleotide sequences used as primers for the amplification of *Chk1* mRNA.

Primer name		**Primer sequence**	**TM°C**	**%GC**
Forward	Chk1F1	GGR GAR GGD GCM TAT GGA GAR	52°C	58
	Chk1F2	GAA GAR GCD GTV GCR GTG	47°C	61
Reverse	Chk1R1	GGD GCA ACA TAK GGH ARR GTW CC	52°C	52

4.2.6. Amplification of cDNA by RT-PCR

The standard PCRs performed in order to isolate the *Chk1* mRNA in *M. edulis* were carried out as described in section 3.2.5. Amplifications were carried out in a Techne Thermal Cycler equipped with a heated lid. All reactions were initially denatured at 95°C for 1 min then 15 sec at 95°C denaturation, 15 sec at 55°C annealing and 1 min at 68°C elongation step. The last three steps were repeated 35 times followed by a final elongation step for 10 sec at 70°C then final step of holding at 4°C.

4.2.7. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed as described in section 2.2.6.

4.2.8. Isolation of DNA fragments from agarose gel slices

The gel areas containing the DNA fragments of interest were excised on the UV transluminator using a clean scalpel. The gel slice was placed into a pre-weighted clean 1.5 ml plastic tube and processed according to the NucleoSpin Extract II PCR clean-up and Gel Extraction protocol (Macherey-Nagel). The buffer role is in solubilisation of the gel slice and in creating the binding conditions of the DNA to the NucleoSpin Extract II Columns silica-gel membrane. This step was allowed by the addition of 200 µl Buffer NT (containing guanidine thiocyanate) to each 100 mg of agarose gel and

incubation of 10 min at 50°C flicking the tube periodically to dissolve the gel slice. After that transfer the dissolved gel to NucleoSpin Extract II Columns silica-gel membrane and centrifuge for 1 min at 11,000 x *g*. 600 µl NT3 buffer (containing chaotropic salt) were added to the column and centrifuged 1 min at 11,000 x *g* in order to remove any trace of agarose. The column was subsequently centrifuged for 2 min to eliminate any trace of NT3 buffer that might interfere with downstream application and then placed into a clean 1.5 ml tube. To elute the DNA, 15-50 µl NE (5 mM Tris-HCl, pH 8.5) was applied to the centre of the membrane, left for 1 min and centrifuged 1 min at 11,000 x *g*. The sample was stored at –20°C.

4.2.9. Cloning PCR-generated fragments of DNA

The linearized TA plasmid vector pCR®2.1 (Invitrogen Life Technologies) used for the DNA cloning has single 3' deoxythymidine (T) residues and contain the resistance genes to kanamycin and ampicillin as well as the *LacZα* gene. 7 µl of extracted gel processed as in section 4.2.8 were mixed on ice with 25 ng pCR2.1 vector, 1 µl 10X ligation buffer (60 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml BSA, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM DTT and 10 mM spermidine) and 1 µl T4 ligase (4.0 Weiss units/µl). The reaction was incubated overnight at 14°C.

The vectors prepared as above were then ready to be transformed into JM109 *E. coli* competent cells strain, High Efficiency (Promega). 30 µl of frozen JM109 competent cells were thawed on ice and mixed gently with the pipette tip with 2 µl of the ligation reaction. The vial was than incubated for 20 min on ice and then heat shock for exactly 45 sec at 42°C. The vial was then placed again on ice. 250 µl S.O.C.

medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the reaction and incubated for 2-3 hrs at 37°C into a shaking incubator at 150 rpm. The culture was plated onto LB agar plates (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 15 g/l agar, pH 7.0) containing kanamycin (50 µg/ml) and X-gal in dimethyl formamide (40 µg/ml) and incubated overnight at 37°C. Single white colonies, indicating the presence of the plasmid in the cell because of the kanamycin resistance and disruption of the *LacZα* gene by the insert DNA, were picked using a sterile pipette tip and inoculated into 400-500 µl of LB broth (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.0) containing 0.5 mg/ml kanamycin. The cultures were grown overnight into a shaking incubator at 37°C and 200 rpm.

4.2.10. Sequencing the potential *Chk1* gene-containing sub-clones

Separate PCRs were performed for each one of the cultures using T7 and M13-Reverse priming sites that allowed the amplification of the plasmid with the sequence inserted.

Amplifications were carried out in a Piko Thermal Cycler (Finnzymes Instruments) consisting of 200 µM dNTPs, 0.75 units of Taq DNA Polymerase (Fisher Scientific, Leicestershire, U.K.), 3 µl of Fisher 10x Buffer A, 8 µM of each sense (T7) and antisense primers (M13-Reverse) and nuclease-free water to a final volume of 30 µl. All reactions were initially denatured at 95°C for 2 min then 30 sec at 95°C denaturation, 30 sec at 55°C annealing and a 1 min at 72°C elongation step. The last three steps were repeated 30 times followed by a final extension step of 5 min at 72°C. A negative control was set up along side each set of PCR reactions consisting of all

components of the PCR reaction excluding the template DNA, to ensure that there was no contamination.

An agarose gel electrophoresis (see section 2.2.6) was used to run 6 µl of the PCR product together with a 100 bp molecular weight ladder (Invitrogen Life Technologies) to check the size of the fragments. The DNA from the PCR products of the sub-clones that contained the inserts was purified using a NucleoSpin® Extract II kit (Macherey-Nagel GmbH & Co. KG**,** Düren, Germany). In order to do that, one volume of PCR product was mixed with 2 volumes of Binding Buffer NT (containing the chaotropic salt guanidine thiocyanate). The sample was placed on a NucleSpin® Extract II silica membrane and centrifuged 1 min at 11,000 x g enabling the DNA to bind to the membrane. Salts and soluble macromolecular components were removed by a wash with 600 µl of ethanolic Wash Buffer NT3 and centrifuged 1 min at 11,000 x g. The column was centrifuged for 2 min at 11,000 x g to remove any residual ethanol from the Wash Buffer and then placed into a clean 1.5 ml tube. Pure DNA was finally eluted under low ionic strength conditions with Elution Buffer NE (5 mM Tris/HCl, pH 8.5).

DNA concentration was determined by a Qubit™ fluorometer (see section 2.2.8). Approximately 15 µl of 11 ng/µl purified DNA was sent to Eurofins MWG Operon Company for sequencing.

4.2.11. Extraction and purification of plasmid DNA

Plasmid DNA was extracted from *E. coli* cultures as described in section 3.2.10.

4.2.12. Amplification of RACE cDNA

Synthesis of RACE cDNA was performed as described in section 3.2.13.1 followed by the amplification of RACE cDNA as described in section 3.2.13.2. The

degenerated primers Chk1F1 and Chk1F2 were used to get the rest of *Chk1*. Three steps PCR programme (Techne) was used in amplifying 5' and 3' RACE cDNA starting with activation the enzyme with an initial "Hot start" of 95°C for 1 min, followed by:

a) 5 cycles: 94°C, 30"	b) 5 cycles: 94°C, 30"	c) 27 cycles: 94°C, 30"
68°C, 30"	60°C, 30"	55°C, 30"
72°C, 1'	72°C, 1 '	72°C, 1'

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The reaction was incubated at 72°C for 2 min as a final extension and then maintained after completion at 4°C and stored at -20°C. PCR products were analysed and separated by gel electrophoresis as described in section 4.2.8 and finally sending for sequencing.

4.3. RESULTS

4.3.1. Isolation of total RNA from *M. edulis* gonads

The extraction method described in section 3.2.2 provided a high yield of total RNA with concentration of approximately 1 µg/ml.

4.3.2. *Chk1* mRNA amplification from *M. edulis*

Several PCRs were conducted in order to isolate the *Chk1* mRNA fragment from *M. edulis*. Different combinations of the designed primers (Table 4.2.5.1) were used in reactions while other parameters were also varied (see 4.2.6). Using the template cDNA prepared with the forward primer Chk1F1 and the reverse primer Chk1R, yielded a product of the expected size of around 490 bp (Fig. 4.3.2.1).

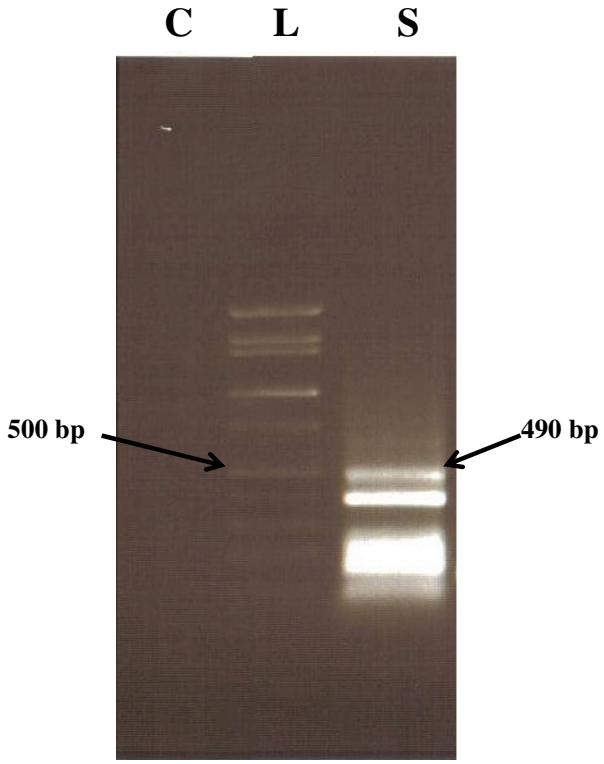

Fig. 4.3.2.1. Ethidium bromide stained 0.8 % agarose gel displaying PCR amplification products obtained using *M. edulis* cDNA as a template and the primer pair (expected product size – 490 bp). Lane L is the molecular size ladder, lane C is the negative control and lane S is the cDNA sample.

4.3.3. Sequencing the isolated DNA fragments

The sequencing results showed that the putative *Chk1* 490 bp fragment was similar to that reported in other species. The BLAST algorithm confirmed the deduced identity of the isolated fragment as a part of the *Chk1* gene (Fig. 4.3.3.1). The ClustalW programme showed 66% similarity between the isolated fragment in *M. edulis* and both *H. sapiens and X. tropicalis Chk1* sequences, also shares 65% similarity with *X. laevis* and 21% with *D. rerio*.

```
X.tropicalis      ATGGCAGTTCCATTTGTCGAAGACTGGGATCTTGTCCAGACTCTTGGGGAAGGGGCATAT 60
M.edulis          ----------------------------------------CTTGGGGAGGGAGCCTAT 18
H.sapiens         ATGGCAGTGCCCTTTGTGGAAGACTGGGACTTGGTGCAAACCCTGGGAGAAGGTGCCTAT 60
X.laevis          ATGGCAGTTCCGTTTGTTGAAGACTGGGATCTGGTCCAGACTCTTGGAGAGGGGGCATAT 60
D.rerio           ATGGCTGTGCCTTTTGTTAAAGACTGGGATGTGGTACAAACTCTTGGAGAGGGAGCATAT 60
                                                          ** ** ** ** ** ***

X.tropicalis      GGAGAAGTGCAGCTGGCAGTAAACCGGAAAACAGAAGAAGCAGTAGCAGTGAAGATTGTG 120
M.edulis          GGAGAAGTTAAACTTGCAGTAAATACTGATACCCAGGAAGCTGTAGCTGTTAAAATTATA 78
H.sapiens         GGAGAAGTTCAACTTGCTGTGAATAGAGTAACTGAAGAAGCAGTCGCAGTGAAGATTGTA 120
X.laevis          GGAGAAGTGCAGCTGGCAGTGAACCGGAAAACTGAAGAAGCGGTAGCAGTGAAGATTGTG 120
D.rerio           GGAGAGGTGCGACTGCTGGTCAACAAGAAAACAGAAGAGGCTGTGGCGGTGAAAGTTGTG 120
                  ***** **    **    ** **       **  * ** ** ** ** ** **  ** *

X.tropicalis      GACATGACACGTGCAGCTGATTGCCCAGAAAACATCAAAAAGGAGATTTGTATTAATAGG 180
M.edulis          AACCTAGAGAAAACAGCATCTGCAGCAGAAAATGTCAGGAAAGAGGTTTGTGTTCACAAC 138
H.sapiens         GATATGAAGCGTGCCGTAGACTGTCCAGAAAATATTAAGAAAGAGATCTGTATCAATAAA 180
X.laevis          GACATGACACGTGCAGCTGATTGCCCAGAAAACATCAAAAAGGAGATCTGTATCAATAGG 180
D.rerio           GACATGGCAAAAGCCAAGGATTGCATCGAGAATGTGAAGAAGGAGGTCTGCATATGCAAG 180
                   *  *        *             ** **  * *  ** *** * **  *    *

X.tropicalis      ATGCTTAATCACACAAATATTGTAAGATTTTATGGACAT--CGACGGGAAGGAAACATTC 238
M.edulis          ATGTTGAATCATGAGAGAGTTATCAAGTATTACGGTTCA--CGTAAAGATAAAAAGATCC 196
H.sapiens         ATGCTAAATCATGAAAATGTAGTAAAATTCTATGGTCAC--AGGAGAGAAGGCAATATCC 238
X.laevis          ATGCTCAGTCACACAAATATTGTAAGATTTTATGGACAT--CGAAGGGAAGGCAACATTC 238
D.rerio           ATGCTTTCACACCCCAACATTGTACGTTTCTTTGGGCACAGTGTTGGGATTACACATCGT 240
                  *** *    **    *   *  *    *  *  **       *    **    *

X.tropicalis      AGTACCTCTTTCTGGAGTATTGT----CGAGGTGGTGAGCTCTTTGATCGCATAGAACCT 294
M.edulis          AGTATTTATTTCTTGAGTATGCT----AGTGGTGGAGAGTTGTTTGATAGAATTGAGCCA 252
H.sapiens         AATATTTATTTCTGGAGTACTGT----AGTGGAGGAGAGCTTTTTGACAGAATAGAGCCA 294
X.laevis          AGTACCTCTTTCTGGAGTATTGT----CGAGGTGGTGAGCTCTTTGATCGCATAGAGCCT 294
D.rerio           GACATAAAGCCTGAGAATATTCTTCTTGATGATAAAGATAATCTGAAGATCTCTGACTTT 300
                     *          ** **   *       *     **     *  *       **

X.tropicalis      GAT---GTTGGAATGCCTGAGCAAGATGCACAAAAAT---TTTTTCAGCAGCTAATTGCT 348
M.edulis          GAT---GCAGGTATGCCACAACTTGAAGCCAACAAAT---TCTTTAAACAGTTGTTAGCA 306
H.sapiens         GAC---ATAGGCATGCCTGAACCAGATGCTCAGAGAT---TCTTCCATCAACTCATGGCA 348
X.laevis          GAT---GTTGGAATGCCTGAGCAAGATGCACAGAAAT---TTTTTCAGCAACTGATTGCT 348
D.rerio           GGCCTGGCTACCATGTTCAGGCACCGTGGCCGTGAGCGAGCTTTGAACCGTCTGTGTGGT 360
                  *           ***      *     *              **  * *   *    *

X.tropicalis      GGT--GTGGAATACCTGCACAGCATTGGAATAACGCACAGA----GATATTAAGCCTG-- 400
M.edulis          GGA--GTTGAATATTTACATACAAAAGGAGTGACTCACAGA----GACCTTAAGCCTG-- 358
H.sapiens         GGG--GTGGTTTATCTGCATGGTATTGGAATAACTCACAGG----GATATTAAACCAG-- 400
X.laevis          GGT--GTGGAATACCTGCACAGCATTGGAATAACTCACAGA----GATATCAAGCCTG-- 400
D.rerio           ACTCTGCCCTATGTTGCCCCAGAGTTGATGTCACGCTCATCTTTTAACGCTCAGCCTGCG 420
                       *     *     *         *   * ** * **       *     * ** *

X.tropicalis      AGAACTTACTCTTAGATGACCGAGATCACCTGAAAATCTCTGACTTTGGTTTGGCAACAG 460
M.edulis          AAAATTTACTTTTGGATGACTTTGATAATTTAAAGGTATCGGACTTTGGTCTAGCCACTG 418
H.sapiens         AAAATCTTCTGTTGGATGAAAGGGATAACCTCAAAATCTCAGACTTTGGCTTGGCAACAG 460
X.laevis          AGAACTTGCTTTTAGATGAACGAGATCAGCTGAAAATCTCTGACTTTGGTTTAGCAACGG 460
D.rerio           GACACTTGGGCTTGTGGCATTGTGCTCACTGCAA--TGTTAGCTGGAGAGTTACCCTGGG 478
                     *  *    **     *    * * *    **  * *  *     *   *  *    *

X.tropicalis      TGTTCCGACACAATGCGAAAGAAAGACTTTTAAACAAGATGTGTGGAAC--CCTACCCTA 518
M.edulis          TGTTCCGATACCAAGGCAGGGAGAGAATGCTGGAGAAATGTTGTGGAAC--CCTACCATA 476
H.sapiens         TATTTCGGTATAATAATCGTGAGCGTTTGTTGAACAAGATGTGTGGTAC--TTTACCATA 518
X.laevis          TATTCAGACACAATGGCAAAGAAAGACTTTTAAGCAAGATGTGTGGAAC--CCTTCCCTA 518
D.rerio           ATCAGCCGAGTGAAAACTGTCAGGAATATTTGGACTGGCTGGAAAGAAAGACCTACCTTA 538
                        *        *        *            * *     * ** **

X.tropicalis      TGTTGCACCAGAACTGATTAAGTCCAGAGCCTTTAATGCAGAGCCTGAGAAACGGCTACC 578
M.edulis          TGTTGCCCC---------------------------------------------------- 485
H.sapiens         TGTTGCTCCAGAACTTCTGAAGAGAAGAGAATTTCATGCAGAACCAGTTGATGTTTGGTC 578
X.laevis          TGTTGCACCAGAACTGATTAAATCCAGGGCCTTTCATGCCGACCCAGTGGATGTGTGGTC 578
D.rerio           CACCCTGGAAGAA--AATTGATGCGGTACCCCTTAGTCTGTTGTCTAAGATATTACTGCA 596
```

Fig. 4.3.3.1. An alignment of the isolated *Chk1* fragment from *M. edulis* represents high homology with *Chk1* in different vertebrate species. Asterix denotes homology.

4.3.4. *Chk1* amplification using mussel 5' and 3' RACE cDNA template

Several PCRs were conducted in order to isolate the remainder of the *Chk1* mRNA from *M. edulis*. 3' RACE cDNA was amplified with the Chk1F1 and Chk1F2 and several bands were observed including a product of a size 744 bp and 800 bp obtained in 3' RACE PCR (Fig. 4.3.4.1).

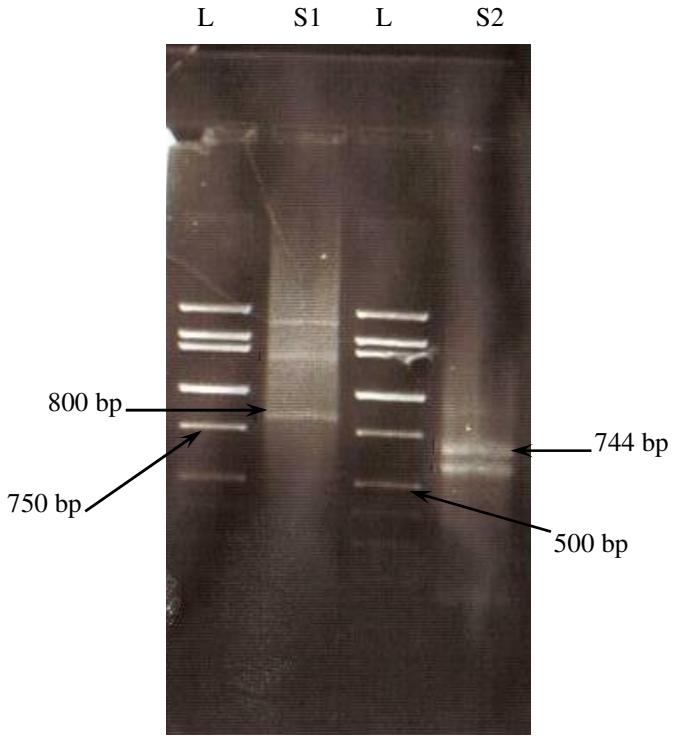

Fig. 4.3.4.1. Ethidium bromide stained 0.8 % agarose gel displaying RACE PCR amplification product, lane S1 and S2, obtained using *M. edulis* 3' RACE cDNA as a template and the degenerated primers Chk1F1 and Chk1F2 respectively (a product size 744-800 bp). Lane L is the molecular size ladder.

4.3.5. Characterization of the 3' RACE *Chk1* fragment

The sequencing results showed that the putative *Chk1* 744 bp fragment was similar to the *Chk1* sequence in other species. The BLAST algorithm confirmed the identity of the isolated fragment as a part of the *Chk1* gene. Specifically, there was 63%

similarity with *H. sapiens*, *X. laevis* and *X. tropicalis* and only 34% similarity with *D. rerio* (Fig. 4.3.5.1).

```
X.laevis          ATGGCAGTTCCGTTTGTTGAAGACTGGGATCTGGTCCAGACTCTTGGAGAGGGGGCATAT 60
X.tropicalis      ATGGCAGTTCCGTTTGTTGAAGACTGGGATCTGGTCCAGACTCTTGGAGAGGGGGCATAT 60
D.rerio           ATGGCTGTGCCTTTTGTTAAAGACTGGGATGTGGTACAAACTCTTGGAGAGGGAGCATAT 60
M.edulis          ---------------------------------------------CTTGGGGAGGGAGCCTAT 18
H.sapiens         ATGGCAGTGCCCTTTGTGGAAGACTGGGACTTGGTGCAAACCCTGGGAGAAGGTGCCTAT 60
                                                               ** ** ** ** ** ***

X.laevis          GGAGAAGTGCAGCTGGCAGTGAACCGGAAAACTGAAGAAGCGGTAGCAGTGAAGATTGTG 120
X.tropicalis      GGAGAAGTGCAGCTGGCAGTGAACCGGAAAACTGAAGAAGCGGTAGCAGTGAAGATTGTG 120
D.rerio           GGAGAGGTGCGACTGCTGGTCAACAAGAAAACAGAAGAGGCTGTGGCGGTGAAAGTTGTG 120
M.edulis          GGAGAAGTTAAACTTGCAGTAAATACTGATACCCAGGAAGCTGTAGCTGTTAAAATTATA 78
H.sapiens         GGAGAAGTTCAACTTGCTGTGAATAGAGTAACTGAAGAAGCAGTCGCAGTGAAGATTGTA 120
                  ***** **    **    ** **       **  * ** ** ** ** ** **  ** *

X.laevis          GACATGACACGTGCAGCTGATTGCCCAGAAAACATCAAAAAGGAGATCTGTATCAATAGG 180
X.tropicalis      GACATGACACGTGCAGCTGATTGCCCAGAAAACATCAAAAAGGAGATCTGTATCAATAGG 180
D.rerio           GACATGGCAAAAGCCAAGGATTGCATCGAGAATGTGAAGAAGGAGGTCTGCATATGCAAG 180
M.edulis          AACCTAGAGAAAACAGCATCTGCAGCAGAAAATGTCAGGAAAGAGGTTTGTGTTCACAAC 138
H.sapiens         GATATGAAGCGTGCCGTAGACTGTCCAGAAAATATTAAGAAAGAGATCTGTATCAATAAA 180
                   *  *        *            ** **  * *  ** *** * **  *    *

X.laevis          ATGCTCAGTCACACAAATATTGTAAGATTTTATGGACATCGAAGGGAAGGCAACATTCAG 240
X.tropicalis      ATGCTCAGTCACACAAATATTGTAAGATTTTATGGACATCGAAGGGAAGGCAACATTCAG 240
D.rerio           ATGCTTTCACACCCCAACATTGTACGTTTCTTTGG-------------------------- 215
M.edulis          ATGTTGAATCATGAGAGAGTTATCAAGTATTACGGTTCACGTAAAGATAAAAAGATCCAG 198
H.sapiens         ATGCTAAATCATGAAAATGTAGTAAAATTCTATGGTCACAGGAGAGAAGGCAATATCCAA 240
                  *** *    **    *   *  *    *  *  **

X.laevis          TACCTCTTTCTGGAGTATTGTCGAGGTGGTGAGCTCTTTGATCGCATAGAGCCTGATGTT 300
X.tropicalis      TACCTCTTTCTGGAGTATTGTCGAGGTGGTGAGCTCTTTGATCGCATAGAGCCTGATGTT 300
D.rerio           ------------------------------------------------------------
M.edulis          TATTTATTTCTTGAGTATGCTAGTGGTGGAGAGTTGTTTGATAGAATTGAGCCAGATGCA 258
H.sapiens         TATTTATTTCTGGAGTACTGTAGTGGAGGAGAGCTTTTTGACAGAATAGAGCCAGACATA 300

X.laevis          GGAATGCCTGAGCAAGATGCACAGAAATTTTTTCAGCAACTGATTGCTGGTGTGGAATAC 360
X.tropicalis      GGAATGCCTGAGCAAGATGCACAGAAATTTTTTCAGCAACTGATTGCTGGTGTGGAATAC 360
D.rerio           ------------------------------------------------------------
M.edulis          GGTATGCCACAACTTGAAGCCAACAAATTCTTTAAACAGTTGTTAGCAGGAGTTGAATAT 318
H.sapiens         GGCATGCCTGAACCAGATGCTCAGAGATTCTTCCATCAACTCATGGCAGGGGTGGTTTAT 360

X.laevis          CTGCACAGCATTGGAATAACTCACAGAGATATCAAGCCTGAGAACTTGCTTTTAGATGAA 420
X.tropicalis      CTGCACAGCATTGGAATAACTCACAGAGATATCAAGCCTGAGAACTTGCTTTTAGATGAA 420
D.rerio           --GCACAGTGTTGGGATTACACATCGTGACATAAAGCCTGAGAATATTCTTCTTGATGAT 273
M.edulis          TTACATACAAAAGGAGTGACTCACAGAGACCTTAAGCCTGAAAATTTACTTTTGGATGAC 378
H.sapiens         CTGCATGGTATTGGAATAACTCACAGGGATATTAAACCAGAAAATCTTCTGTTGGATGAA 420
                     **       **  * ** **  * **  * ** ** ** **  * **  * *****

X.laevis          CGAGATCAGCTGAAAATCTCTGACTTTGGTTTAGCAACGGTATTCAGACACAATGGCAAA 480
X.tropicalis      CGAGATCAGCTGAAAATCTCTGACTTTGGTTTAGCAACGGTATTCAGACACAATGGCAAA 480
D.rerio           AAAGATAATCTGAAGATCTCTGACTTTGGCCTGGCTACCATGTTCAGGCACCGTGGCCGT 333
M.edulis          TTTGATAATTTAAAGGTATCGGACTTTGGTCTAGCCACTGTGTTCCGATACCAAGGCAGG 438
H.sapiens         AGGGATAACCTCAAAATCTCAGACTTTGGCTTGGCAACAGTATTTCGGTATAATAATCGT 480
                     *** *  * **  * ** ********  * ** **  * **  *  *

X.laevis          GAAAGACTTTTAAGCAAGATGTGTGGAACCCTTCCCTATGTTGCACCAGAACTGATTAAA 540
X.tropicalis      GAAAGACTTTTAAGCAAGATGTGTGGAACCCTTCCCTATGTTGCACCAGAACTGATTAAA 540
D.rerio           GAGCGAGCTTTGAACCGTCTGTGTGGTACTCTGCCCTATGTTGCCCCAGAGTTGATGTCA 393
M.edulis          GAGAGAATGCTGGAGAAATGTTGTGGAACCCTACCATATGTTGCCCCTGAGGTGCTGTCA 498
H.sapiens         GAGCGTTTGTTGAACAAGATGTGTGGTACTTTACCATATGTTGCTCCAGAACTTCTGAAG 540
                  **  *     *          ***** **  * ** ******** ** **  *  *
```

```
X.laevis          TCCAGGGCCTTTCATGCCGACCCAGTGGATGTGTGGTCATGTGGAATTGTGCTGACTGCC 600
X.tropicalis      TCCAGGGCCTTTCATGCCGACCCAGTGGATGTGTGGTCATGTGGAATTGTGCTGACTGCC 600
D.rerio           CGCTCATCTTTTAACGCTCAGCCTGCGGACACTTGGGCTTGTGGCATTGTGCTCACTGCA 453
M.edulis          AGGCAACCATATCATGCTGAGCCAGCTGATATCTGGTCATGTGCCATTATACTGGTAGCC 558
H.sapiens         AGAAGAGAATTTCATGCAGAACCAGTTGATGTTTGGTCCTGTGGAATAGTACTTACTGCA 600
                           * * * **  * ** *  **    *** * ****  **  * **    **

X.laevis          ATGTTAGCAGGAGAGTTACCATGGGATCAACCAAACGAAGTATGCCAGGAGTATTGTGAT 660
X.tropicalis      ATGTTAGCAGGAGAGTTACCATGGGATCAACCAAACGAAGTATGCCAGGAGTATTGTGAT 660
D.rerio           ATGTTAGCTGGAGAGTTACCCTGGGATCAGCCGAGTGAAAACTGTCAGGAATATTTGGAC 513
M.edulis          ATGTTGGCTGGAGAACTCCCTTGGGATGAACCAAATTATGGCTGTCAAGAATATTGTAAT 618
H.sapiens         ATGCTCGCTGGAGAATTGCCATGGGACCAACCCAGTGACAGCTGTCAGGAGTATTCTGAC 660
                  *** * ** *****  * ** *****  * ** *   *    ** ** ** ****   *

X.laevis          TGGAAGGAAAAAAAATCATTATCTCACT------AAAAAAATTAGTGCTACCCTTCTTGCA 714
X.tropicalis      TGGAAGGAAAAAAATCATTATCTCACT------AAAAAAATTAGTGCTACCCTTCTTGCA 714
D.rerio           TGGCTGGAAAGAAAGACCTACCTTACACCCTGGAAGAAAATTGATGCGGTACCCCTTAGT 573
M.edulis          TGGAAGGACTGTAAAATAACCCTGTCTCCTTGGAATAAAGTAGACAACCTAGCTTTGTCA 678
H.sapiens         TGGAAAGAAAAAAAAACATACCTCAACCCTTGGAAAAAAATCGATTCTGCTCCTCTAGCT 720
                  ***   **    **       **          ** *** *             *

X.laevis          TTGCTGGGTAAAATG-TTAACAGAAAATCCACAAAGCAGAATCACTATTCCAGACATAAA 773
X.tropicalis      TTGCTGGGTAAAATG-TTAACAGAAAATCCACAAAGCAGAATCACTATTCCAGACATAAA 773
D.rerio           CTGTTGTCTAAGATA-TTACTGCACAATCCAGAAGACAGGTTCACCATTCCTGAAATTAA 632
M.edulis          CTACTAAAAAAAGTTGCTGGTAGAATATCCAGAGAAGAGATATACAATTCAACAAGTTAT 738
H.sapiens         CTGCTGCATAAAATC-TTAGTTGAGAATCCATCAGCAAGAATTACCATTCCAGACATCAA 779
                   *  *    **  *   *     *  *****      **    ** ****   *  * *

X.laevis          GAAGGACCGTTGGTTTACAGAAATAATCAAAAAAGGACTTAAGAGAAGCCGCGTTATCTC 833
X.tropicalis      GAAGGACCGTTGGTTTACAGAAATAATCAAAAAAGGACTTAAGAGAAGCCGCGTTATCTC 833
D.rerio           GAAACACCGCTGGTTTAGCAGAAGTTTCAAATCAGCAGTACAACGTCAGGGCATCACACC 692
M.edulis          ATCTCA---------------------------------------------------- 744
H.sapiens         AAAAGATAGATGGTACAACAAACCCCTCAAGAAAGGGGCAAAAAGGCCCCGAGTCACTTC 839
                        *
```

Fig. 4.3.5.1. An alignment of the isolated RACE *Chk1* nucleotide from *M. edulis* represents the homology with different vertebrate species. Asterix denotes homology.

Translating the fragment of the isolated *M. edulis* RACE *Chk1* into predicted amino acids and alignment of the resulting Chk1 sequence using ClustalW, showed 61% similarity between the isolated *M. edulis* sequence and the *H. sapiens* Chk1 sequence. Also, it showed 57%, 48% and 44% similarity with the *X. laevis*, *X. tropicalis* and *D. rerio* sequences respectively (Fig. 4.3.5.2).

```
X.laevis          MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
X.tropicalis      MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
H.sapiens         MAVPFVEDWDLVQTLGEGAYGEVQLAVNRVTEEAVAVKIVDMKRAVDCPENIKKEICINK 60
D.rerio           MAVPFVKDWDVVQTLGEGAYGEVRLLVNKKTEEAVAVKVVDMAKAKDCIENVKKEVCICK 60
M.edulis          --------------LGEGAYGEVKLAVNTDTQEAVAVKIINLEKTASAAENVRKEVCVHN 46
                                *********:* **  *:******:::: :: .. **::**:*: .

X.laevis          MLSHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
X.tropicalis      MLNHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
H.sapiens         MLNHENVVKFYGHRREGNIQYLFLEYCSGGELFDRIEPDIGMPEPDAQRFFHQLMAGVVY 120
D.rerio           MLSHPNIVRFFG---------------------------------------------- 72
M.edulis          MLNHERVIKYYGSRKDKKIQYLFLEYASGGELFDRIEPDAGMPQLEANKFFKQLLAGVEY 106
                  **.* .::::::*
```

```
X.laevis          LHSIGITHRDIKPENLLLDERDQLKISDFGLATVFRHNGKERLLSKMCGTLPYVAPELIK 180
X.tropicalis      LHSIGITHRDIKPENLLLDDRDHLKISDFGLATVFRHNAKERLLNKMCGTLPYVAPELIK 180
H.sapiens         LHGIGITHRDIKPENLLLDERDNLKISDFGLATVFRYNNRERLLNKMCGTLPYVAPELLK 180
D.rerio           -HSVGITHRDIKPENILLDDKDNLKISDFGLATMFRHRGRERALNRLCGTLPYVAPELMS 131
M.edulis          LHTKGVTHRDLKPENLLLDDFDNLKVSDFGLATVFRYQGRERMLEKCCGTLPYVAPEVLS 166
                   *  *:****:****:***: *:**:*******:**:. :** *.: **********::.

X.laevis          SRAFHADPVDVWSCGIVLTAMLAGELPWDQPNEVCQEYCDWKEKNHYL--TKKISATLLA 238
X.tropicalis      SRAFNAEP---------------------------------------------------- 188
H.sapiens         RREFHAEPVDVWSCGIVLTAMLAGELPWDQPSDSCQEYSDWKEKKTYLNPWKKIDSAPLA 240
D.rerio           RSSFNAQPADTWACGIVLTAMLAGELPWDQPSENCQEYLDWLERKTYLTPWKKIDAVPLS 191
M.edulis          RQPYHAEPADIWSCAIILVAMLAGELPWDEPNYGCQEYCNWKDCKITLSPWNKVD----- 221
                     ::*:*
```

Fig. 4.3.5.2. An alignment of the predicted *M. edulis* Chk1 protein showed homology with Chk1 of different vertebrate species. Asterix denotes homology.

The sequence was submitted to GenBank database and can be retrieved using accession number **GU812861** (Fig. 4.3.5.3).

5'…cttggggagggagcctatggagaagttaaacttgcagtaaatactgatacccaggaagctgtagctgttaaaattataaa
cctagagaaaacagcatctgcagcagaaaatgtcaggaaagaggtttgtgttcacaacatgttgaatcatgagagagttatcaa
gtattacggttcacgtaaagataaaaagatccagtatttatttcttgagtatgctagtggtggagagttgtttgatagaattgagcca
gatgcaggtatgccacaacttgaagccaacaaattctttaaacagttgttagcaggagttgaatatttacatacaaaaggagtga
ctcacagagaccttaagcctgaaaatttacttttggatgactttgataatttaaaggtatcggactttggtctagccactgtgttccg
ataccaaggcagggagagaatgctggagaaatgttgtggaaccctaccatatgttgcccctgaggtgctgtcaaggcaaccat
atcatgctgagccagctgatatctggtcatgtgccattatactggtagccatgttggctggagaactcccttgggatgaaccaaat
tatggctgtcaagaatattgtaattggaaggactgtaaaataaccctgtctccttggaataaagtagacaacctagctttgtcacta
ctaaaaaaagttgctggtagaatatccagagaagagatatacaattcaacaagttatatctca...3'

Fig. 4.3.5.3. Nucleotide sequence of the *M. edulis* putative *Chk1* fragment isolated.

4.4. DISCUSSION

The aim of this section was to isolate and characterise the *Chk1* cDNA sequence from the blue mussel *M. edulis* using real-time techniques. After several attempts, a *M. edulis* partial *Chk1* cDNA sequence was amplified encoding a putative 221 amino acid protein (**GU812861**). The BLAST algorithm confirmed the identity of the isolated fragment as a part of a putative Chk1 (Fig. 4.3.5.2). It shares 61% similarity with *Chk1* in *H. sapiens* and 57% with the *X. laevis*. The sequence also shares 48% and 44% similarity with the *Chk1* of *X. tropicalis* and *D. rerio* respectively.

The deduced amino acid sequence isolated is part of a conserved area, the catalytic and activation loop (A-loop) also called T-loop, putative ATP and substrate-binding pocket (Fig. 4.4.1), which is characteristic of protein kinases catalytic (PKsc) like-superfamily (Chen et al., 2000; Ventura and Maioli, 2001). Another conserved area, asparagine (N135) residue that is reported required for kinase activity (Kumagai et al., 1998). Also, tyrosine (Y20) present in human Chk1 phosphorylation of which inhibits Cdc2 activity (Krek and Nigg, 1991; Parker and Piwnica-Worms, 1992).

PKs regulate many cellular processes including proliferation, division, differentiation, motility, survival, metabolism, cell-cycle progression, cytoskeletal rearrangement, immunity, and neuronal functions. Many kinases are implicated in the development of various human diseases including different types of cancer (Lahiry et al., 2010). The protein kinase superfamily is mainly composed of the catalytic domains of serine/threonine-specific and tyrosine-specific protein kinases. It also includes RIO kinases, which are typical serine protein kinases, aminoglycoside phosphotransferases, and choline kinases. These proteins catalyse the transfer of the gamma-phosphoryl group from ATP to hydroxyl groups in specific substrates such as serine, threonine, or tyrosine residues of proteins. Majority of protein phosphorylation, about 95%, occurs on serine residues while only 1% occurs on tyrosine residues. Protein phosphorylation is a mechanism by which a wide variety of cellular proteins, such as enzymes and membrane channels, are reversibly regulated in response to certain stimuli.

```
X.laevis      MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
X.tropicalis  MAVPFVEDWDLVQTLGEGAYGEVQLAVNRKTEEAVAVKIVDMTRAADCPENIKKEICINR 60
H.sapiens     MAVPFVEDWDLVQTLGEGAYGEVQLAVNRVTEEAVAVKIVDMKRAVDCPENIKKEICINK 60
D.rerio       MAVPFVKDWDVVQTLGEGAYGEVRLLVNKKTEEAVAVKVVDMAKAKDCIENVKKEVCICK 60
M.edulis      --------------LGEGAYGEVKLAVNTDTQEAVAVKIINLEKTASAAENVRKEVCVHN 46
                            *********:* **  *:******:::: :: .. **::**:*: .

X.laevis     MLSHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
X.tropicalis MLNHTNIVRFYGHRREGNIQYLFLEYCRGGELFDRIEPDVGMPEQDAQKFFQQLIAGVEY 120
H.sapiens    MLNHENVVKFYGHRREGNIQYLFLEYCSGGELFDRIEPDIGMPEPDAQRFFHQLMAGVVY 120
D.rerio      MLSHPNIVRFFG------------------------------------------------ 72
M.edulis     MLNHERVIKYYGSRKDKKIQYLFLEYASGGELFDRIEPDAGMPQLEANKFFKQLLAGVEY 106
             **.* .::::::*

X.laevis     LHSIGITHRDIKPENLLLDERDQLKISDFGLATVFRHNGKERLLSKMCGTLPYVAPELIK 180
X.tropicalis LHSIGITHRDIKPENLLLDDRDHLKISDFGLATVFRHNAKERLLNKMCGTLPYVAPELIK 180
H.sapiens    LHGIGITHRDIKPENLLLDERDNLKISDFGLATVFRYNNRERLLNKMCGTLPYVAPELLK 180
D.rerio      -HSVGITHRDIKPENILLDDKDNLKISDFGLATMFRHRGRERALNRLCGTLPYVAPELMS 131
M.edulis     LHTKGVTHRDLKPENLLLDDFDNLKVSDFGLATVFRYQGRERMLEKCCGTLPYVAPEVLS 166
              *  *:****:****:***: *:**:*******:**:. :** *.: **********::.
```

Fig. 4.4.1. Multiple sequence alignment of the deduced amino acid sequence of Chk1 *M. edulis* (GenBank Accession no. **GU812861**) and other available Chk1 sequences. Prediction of conserved domains was conducted using (www.ncbi.CDD), **Bold** is for ATP binding domain, *Italic* is for Active site domain, underline is for Activation loop domain and grey shadowed is for the substrate binding domain. Asterix denotes homology.

Finally, the work presented in this chapter show the isolation of a fragment of the *Chk1* cDNA from *M. edulis*. With the sequence information it is now possible to develop an assay using *Chk1* mRNA expression to determine its role in the cell cycle checkpoints and the relationship to DNA damaged by IR.

Chapter 5

Real-time PCR Method Development and Validation for the Quantification of *Rad51* and *Chk1* mRNA expression in *M. edulis*

5.1. INTRODUCTION

The objective of this work was to develop and validate a quantitative real-time PCR method for the mRNA expression analysis of the putative *Rad51* and *Chk1* genes isolated as described in Chapters 3 and 4 in *M. edulis*.

The information for the synthesis of all proteins in an organism is coded in the genomic DNA in the form of genes. The process of transcription transfers the information of a gene into mRNA, which is translated into proteins in the ribosomes. Therefore, the quantification of mRNA can be used to assess expression levels of a particular gene. Real-time PCR can quantify alterations in RNA concentrations that were previously undetectable using earlier techniques such as gel-based end-point detection RT-PCR or RNase protection assays (Wang and Brown, 1999). The real-time PCR technique relies upon the detection and quantification of a target gene expression by using a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. In our case, the reporter is the double-strand DNA (dsDNA)-specific dye SYBR Green that binds to double stranded cDNA and upon excitation, emits light or fluorescence signal. The advantages of SYBR Green method are that it is inexpensive, easy to use and sensitive but it has one limitation in that it can bind to any dsDNA in the reaction including primer dimers or non-specific products. Consequently, the oligonucleotide primers should be specific, should not form primer-dimers or hairpins and all genomic DNA is digested as part of the method.

The quantification of the target gene can be measured in an absolute way, the amount of the nucleic acid is determined using external standards (such as a standard curve), or in relative way, the ratio between the amount of target molecule and a reference molecule within the same sample is calculated. In order to control the variability introduced by the real-time PCR technique and assure accurate results, a reference gene that is assumed to have equal levels of expression in each experimental sample can be used. The reference gene chosen in this study is the one encoding the information for the synthesis of *18s rRNA* a non-coding type of RNA that constitutes the small subunit of a ribosome. The use of *18s rRNA* as internal standard is recommended for its constant and independent expression in a variety of experimental conditions including IR (Thellin et al., 1999; Venier et al., 2006; Banda et al., 2008). Herein, we employ a relative quantification method where the target genes expression have been normalized to a reference gene (*18s rRNA*), and its levels relative to the gene expression of a non-treated sample (Livak and Schmittgen, 2001).

5.2. MATERIALS AND METHODS

5.2.1. Total RNA isolation

The total RNA extraction from mussel gonads was carried out following the protocol described in section 3.2.2 and stored at –20^{o}C until further processing.

5.2.2. First strand synthesis of cDNA for real-time PCR

cDNA was synthesised from DNase treated total RNA following the protocol described in section 3.2.3 and stored at –20^{o}C.

5.2.3. Oligonucleotide primer design

The target gene specific primers were designed by Invitrogen Custom Primers (Invitrogen, U.K.) using the *Rad51* and *Chk1 M. edulis* sequence described in section 3.3.5 and 4.3.5 (GenBank Accession numbers **FJ518826** and **GU812861**). The primers were supplied in lyophilised form as forward and reverse pairs (e18sF-e18sR, nqRad51F-nqRad51R and qChk1F-qChk1R) (Table 5.3.1.1).

5.2.4. Primer optimization

To ensure the efficient and accurate quantification of the target template, real-time PCR assays should be optimized. Assays are first optimized by evaluating primer concentrations. To do that, three concentrations with equimolar amounts of each primer were tested: 100 nM, 300 nM and 600 nM. The amount of template added was the same in all the samples in the optimization exercise. All the samples were run in duplicates. The ideal primer pair should yield the lowest average Ct value as well as presenting a dissociation curve that shows a single product. The Ct value is calculated using a threshold level of fluorescence set above the background but within the linear phase of amplification. The cycle number at which an amplification plot crosses this threshold fluorescence level is called the Ct or threshold cycle.

5.2.5. Assay performance

Following primer optimization, in order to test the efficiency, precision and sensitivity of the real-time PCR reaction, a standard curve was performed using a serial dilution of a positive template. In this case, a two-fold dilution series starting with 1:10 diluted cDNA and consisting of six points was generated in triplicates. To obtain the

standard curve, the Ct values of the serial dilution of the positive template were plotted against the cDNA dilution.

5.2.6. Amplification using real-time PCR

The real-time PCRs were carried out in a total volume of 20 µl consisting of 10 µl of Precision 2 x real-time PCR Master Mix, 4 µl of the cDNA template diluted from the samples prepared as described in section 3.2.3, 1 µl of each forward and reverse primers and 4 µl of PCR-grade water. The Precision 2 x real-time PCR Master Mix contained 2 x reaction buffer, 0.025 U/µl Taq Polymerase, 5 mM $MgCl_2$, dNTP mix (200 µM of each dNTP), ROX (passive reference dye) and SYBR Green.

Amplifications were carried out in a Mx3005P Real-Time PCR System (Stratagene) which includes a built-in thermal cycler equipped with a heated lid, a Quartz-Tungsten Halogen lamp to excite fluorescence, photomultiplier tubes for high-sensitive detection and Mx3005P real-time quantitative detection software.

All samples were analysed in duplicate. All reactions were initially denatured at 50^{o}C for 2 min then at 95^{o}C for 10 min followed by a three-step protocol of 40 cycles of denaturation at 95^{o}C for 10 sec and annealing at 60^{o}C for 1 min then extension step at 72^{o}C for 1 min. In order to test the specificity of the primers the products were slowly melted starting with 1 min at 95^{o}C followed by 30 sec at 55^{o}C and 30 sec at 95^{o}C and the products analysed in the melting or dissociation curve (plotting fluorescence versus temperature). The temperature at which a DNA molecule melts depends on its length and sequence, therefore if the PCR product consists of molecules of the same sequence a single peak will be detected. A negative control was set up along side each set of PCR reactions consisting of all components of the PCR reaction excluding the template DNA.

5.2.7. Confirmation of the identity of the products formed

In order to confirm the identity of the obtained amplicons, the amplification reactions were run on an agarose gel (section 2.2.6). Subsequently, the bands were excised and isolated as described in section 4.2.8. The DNA was then cloned into a pCR®2.1 vector (section 4.2.9), transformed into *E. coli* competent cells and sequenced (section 4.2.10).

5.2.8. Quantification of the gene expression and validation of the quantitation method

A relative quantitation method was chosen to analyse changes in gene expression of the target gene in the treatment group compared to a control sample. The results were normalized with a reference gene (*18s rRNA).* The method used to calculate the relative change values was the comparative ΔCt method using the formula $RQ = 2^{-\Delta Ct}$ where $\Delta Ct = Ct_{,Rad51 \text{ or } Chk1} - Ct_{,18s}$ (RQ=relative quantitation) (Livak and Schmittgen, 2001).

To apply this method, the efficiencies of the target gene and reference gene, established via a standard curve, must be approximately equal (in the 5% range) and close to 100%. A method for assessing if the two amplicons have the same efficiency is to look at how ΔCt varies with the template dilution (Livak and Schmittgen, 2001). To do that, the average Ct for both *Rad51* or *Chk1* and *18s rRNA* and the ΔCt ($Ct_{,Rad51 \text{ or } Chk1} - Ct_{,18s}$) was determined. The data were fit using least-squares linear regression analysis. The absolute value of the slope of the plot cDNA dilution versus ΔCt should be < 0.1.

The relative expression data was analysed using SPSS 15.0 for Windows and tested for normality using the Kolmogorov-Smirnov test and the Q-Q plots. All the data was not normally distributed and therefore differences between the groups were determined using the non-parametric test Kruskall Wallis. In order to check where the

differences occurred, pair-wise comparisons were performed by Mann-Whitney *U* tests. In order to avoid inflation of type I error rates, Bonferroni corrections were performed by using a critical value for significance of 0.05 divided by the number of tests conducted.

5.3. RESULTS

5.3.1. cDNA synthesis and gene specific primers design

The cDNA concentrations of all the samples (control and irradiated) were 41 ng/µl. The primer pairs designed to investigate *Rad51*, *Chk1* and *18s rRNA* expression were 19-25 nucleotide long with G-C content between 40-60% for an annealing temperature close to 60^{o}C (Table 5.3.1.1). The length of the amplicons was 121 bp, 106 bp and 114 bp for *Rad51*, *Chk1* and *18s rRNA* respectively.

Table 5.3.1.1. Oligonucleotide sequences used as primers for the amplification of *Rad51*, *Chk1* and *18s rRNA* genes.

Primer name		**Primer sequence**
***18s rRNA* primers**		
Forward	e18sF	CAT TAG TCA AGA ACG AAA GTC AGA G
Reverse	e18sR	GCC TGC CGA GTC ATT GAA G
***Rad51* primers**		
Forward	nqRad51F	TGG CAT AGA GAC TGG GTC AA
Reverse	nqRad51R	CCT TCA CCT CCA CCC ATA TC
***Chk1* primers**		
Forward	nqChk1F	CTT GGG GAG GGA GCC TAT GGA G
Reverse	nqChk1R	CTC TTT CCT GAC ATT TTC TG

5.3.2. Oligonucleotide primer optimization

In order to determine the optimal primer concentration of the *Rad51*, *Chk1* and *18s rRNA* primers, different concentrations of equimolar forward and reverse primers were used. The Ct values obtained with different concentrations of the primers are presented in Table 5.3.2.1. In the case of the *Rad51* fragment, only the 600 nM and 600 nM primer pairs gave single products when analysed in the dissociation curve. Based on that, the primer pair with a lowest Ct value (600 nM) was chosen and the same was applied on *Chk1*. For *18s rRNA* all the concentrations generated single products, but only the 200 nM and the 600 nM primer pairs decreased the formation of primer-dimers. For that reason, and choosing the lowest Ct value of the two, the 600 nM primer pair was selected for future amplifications.

Table 5.3.2.1. Ct values of the real-time amplifications using different primer concentrations.

PRIMERS/CONCENTRATION	**100 nM**	**200 nM**	**300 nM**	**600 nM**
18sF-18sR	19.91	16.89	-----	15.81
Rad51F-Rad51R	37.57	-----	29.92	28.98
Chk1F-Chk1R	no Ct	-----	38.12	35.34

5.3.3. Standard curves for analysis of assay performance

In order to evaluate the overall performance of the real-time PCR reaction, a standard curve was generated for each gene. After amplification, the Cts for each standard dilution were determined and plotted against the initial template dilution. The amplification of *18s rRNA* serial dilution generated a satandard curve with an efficiency of amplification of 95.3% (Fig. 5.3.3.1). The slope of the line of best fit determines the efficiency of a reaction using the equation $E = 10^{(-1/slope)} - 1$. The linearity of the assay, denoted by the R squared (RSq or R^2) was 0.995. A value close to 1 implies a linear

range and that the efficiency of the reaction is consistent at varying template concentrations (sensitivity). It also indicates agreement between replicates (precision).

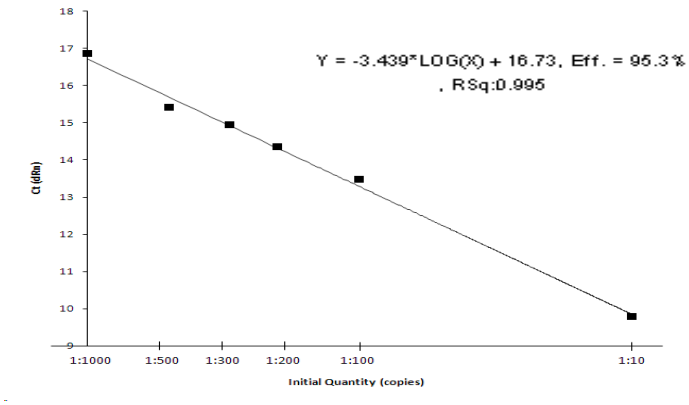

Fig. 5.3.3.1. Standard curve generated from *18s rRNA* amplification data.

For the *Rad51* cDNA serial dilution, the amplification was linear with a regression coefficient of 0.997 and an amplification efficiency of 98.4% (Fig. 5.3.3.2).

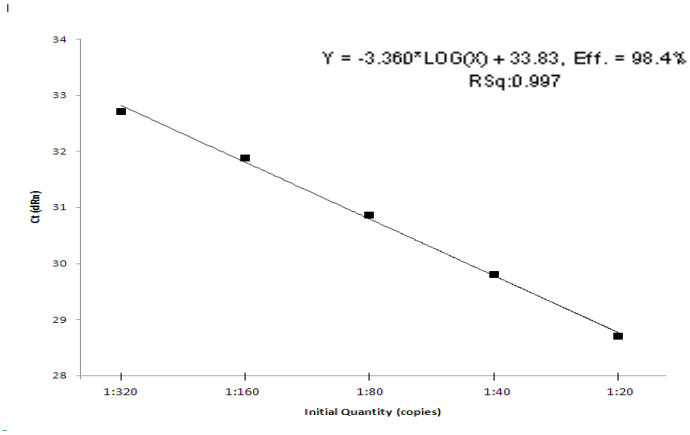

Fig. 5.3.3.2. Standard curve generated from *Rad51* amplification data.

For the *Chk1* cDNA serial dilution, the amplification was linear with a regression coefficient of 0.994 and an amplification efficiency of 95.0% (Fig. 5.3.3.3).

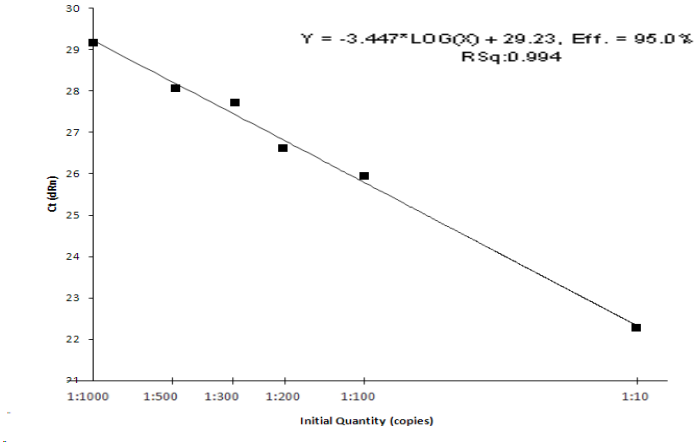

Fig. 5.3.3.3. Standard curve generated from *Chk1* amplification data.

5.3.4. Real-time amplification using mussel cDNA

After the primer optimization and the assay performance evaluation, the next step was to employ the primers at the optimised concentration (600 nM) with *M. edulis* cDNA from an experimental sample set. The cDNA was diluted at the concentrations tested during the standard curve exercise (1:20) for *Rad51*, (1:10) for *Chk1* and (1:320) for *18s*. The amplification of the reference gene *18s rRNA* generated a single product with a melting temperature of 80^{o}C (Fig. 5.3.4.1a). *M. edulis* cDNA amplified with the *Rad51* primer pair yielded a single product (Fig. 5.3.4.1b) at a melting temperature of 77.5^{o}C and the *Chk1* primer pair also yielded a single product (Fig. 5.3.4.1c) at a melting temperature of 76^{o}C. The “no template control” for all target genes did not record any amplification.

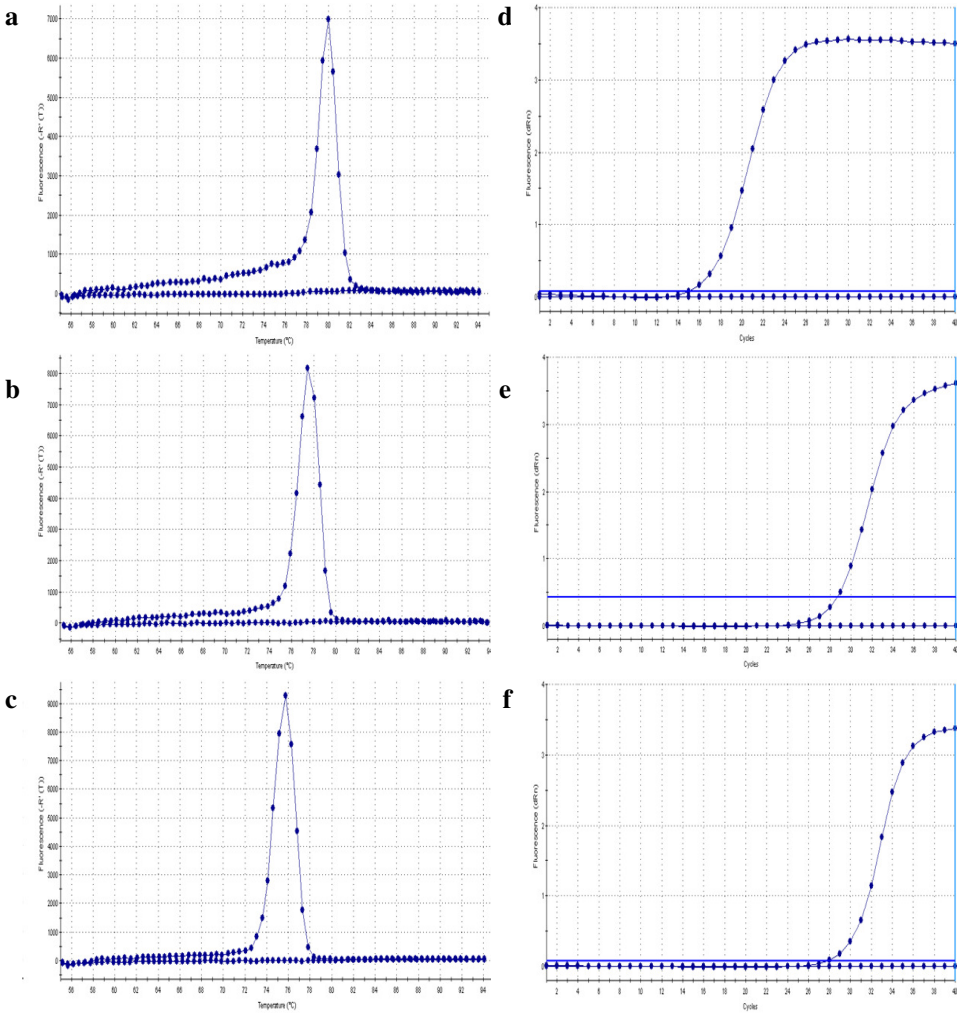

Fig. 5.3.4.1. Dissociation curve of the real-time amplification of *M. edulis 18s rRNA* (a), *Rad51* (b) and *Chk1* (c). Real-time PCR amplification of *18s rRNA* (d), *Rad51* (e) and *Chk1* (f).

5.3.5. Confirmation of the identity of the products formed

After cloning, the sequences obtained confirmed the identity of the real-time PCR generated fragments as *Rad51*, *Chk1* and *18s rRNA*.

5.4. DISCUSSION

Real-time PCR is a popular method for characterizing target gene expression patterns in different organisms under differing conditions. The simplicity of the method combined with its high sensitivity and specificity makes it a powerful technique for the quantification of several mRNA expression levels at once. This chapter describes the development of a quantitative method to measure the *Rad51* and *Chk1 RNA* levels in the mussel *M. edulis* using the real-time PCR technique. This methodology can be applied to the study of the putative *Rad51* and *Chk1* mRNA expression levels in mussels experimentally-exposed or environmentally-exposed to different levels of IR.

The template preparation is a crucial step for a successful identification of target gene expression profiles. Any significant DNA contamination will result in an inaccurate RNA quantification. A DNase treatment of the RNA samples and a DNase removal step was added prior to the reverse transcription. The priming strategy used for the reverse transcription was using random hexamers. The advantage of using random hexamers instead of oligo-d(T) is that they do not require the presence of a polyA sequence allowing the synthesis of cDNA from all RNA species not just mRNA. Therefore, *18s rRNA* could not be reverse transcribed using oligo-d(T) primed cDNA synthesis.

The reverse transcription step is also critical in that different enzymes will have different sensitivity and specificity (Bustin, 2002) and that the efficiency of each reaction can vary considerably. In our method, the use of a gene (*18s rRNA*) that is equally expressed in all the samples as a reference or "housekeeping" gene will normalize any differences in the efficiency of the reverse transcription. The use of *18s rRNA* has been previously recommended (Thellin et al., 1999) but different experimental conditions make some "housekeeping genes" to vary considerably

(Schmittgen and Zakrajsek, 2000). As a result, the choice of a reference gene should be determined based on the exposure condition (Radonic et al., 2004; Arukwe, 2006). Previous studies have shown that the levels of expression of *18s rRNA* in organisms exposed to radiation (Banda et al., 2008; Wilson et al., 2010) are kept constant and consequently it can be used under similar experimental conditions as a housekeeping gene.

A reference dye (ROX) was also included in the master mix in the amplification reactions. The reference dye is always present at the same concentration in all the samples and should normalize the fluorescence signal of the reporter dye (SYBR Green). The use of a DNA binding technology, in our case SYBR Green, is very flexible because the same dye can be used with any pair of primers for any target. The main disadvantage of this technique is that because it binds to any double stranded DNA and not a specific sequence is prone to false positives (Wong and Medrano, 2005). For this reason, the design and the concentration optimization of the oligonucleotide primes for the amplification of the target and housekeeping gene is the major challenging step when performing a real-time PCR experiment. The primers were selected using the data available from other species. In general, the resultant amplicon should be between 100-300 bp in length and the length of each primer between 15-30 bp. The 5' and 3' ends should not contain many guanines or cytosines together to prevent the primers folding on themselves and to avoid G/C clamps.

The concentration of the primers is also a prerequisite for a successful amplification as a low primer concentration could become a limiting factor during the amplification reaction and a high primer concentration can increase the formation of non-specific products and primer-dimer formation. The primer optimization was carried out testing several dilutions of equimolar primer pair concentrations. The primers

chosen were those that provided the best compromise of low Ct values, reduced primer-dimer formation and specific amplification. The presence of non-specific products can be detected by performing a melting curve analysis (also known as dissociation curve). As the melting temperature of a product is sequence-specific, the presence of a single homogeneous melt peak for all the samples will confirm specific amplification (Ririe et al., 1997). The amplification of mussel cDNA resulted in the formation of a single product. In order to ensure that the products formed belonged to our gene of interest and housekeeping gene they were also run in an agarose gel and cloned confirming the products expected and therefore the specificity of the primers.

The accuracy of real-time PCR experiments is dependent on PCR efficiencies of both the gene of interest and the gene used as a reference. If the efficiency of the reaction is 100% the amount of template is being doubled in each cycle. Ideally, the efficiencies of the standards and targets should be between 90% and 110% and within 5% (typical run-to-run variance) of each other. To calculate efficiencies, a serial dilution of cDNA templates is performed, and the slope of the line of best fit of the standard curve is directly correlated with it using a formula equivalent to a calculated 90-110% efficiency. The *Rad51*, *Chk1* and *18s rRNA* standard curves showed efficiencies in the amplification reaction close to 100% and within 3% of each other, confirming that the efficiency of both genes (target and normalizer) were similar and the suitability of the use of the comparative Ct method for the relative quantitation of *Rad51* and *Chk1* mRNA expression.

Quantification of RNA transcription by real-time PCR can be either relative or absolute. Absolute quantification, also known as standard curve method, requires the construction of an absolute standard curve that produces a linear relationship between Ct and known initial amounts of cDNA. The determination of the copy numbers of

RNA transcripts of unknowns is based then on their Ct values (Heid et al., 1996; Liu and Saint, 2002). Nevertheless, the generation of reliable standard material precisely quantified is very time consuming and the amplification efficiencies of the target cDNA and the cDNA used in the calibration curve have to be identical. In relative quantitation, changes in gene expression are compared to an external standard and/or a reference sample. There are many mathematical models to calculate the gene expression from relative quantitation assays (Wong and Medrano, 2005). The comparative Ct methods ("delta Ct" and "delta-delta Ct") are based on the comparison of the distinct cycle differences (Livak, 1997; Livak and Schmittgen, 2001). The main disadvantage of the comparative Ct methods is that they assume equal efficiencies (calculated from a standard curve) of target and reference genes. An efficiency corrected method that accounts for the differences in amplification efficiencies of the target and reference genes has been developed (Pfaffl, 2001). The main disadvantage of these methods is that they do not take into account run-to-run variances. For more precise results, averages of efficiencies should be taken running different standard curves at separate times.

In summary, herein we have validated and developed a quantitative real-time PCR method for the mRNA expression analysis of the putative *Rad51* and *Chk1* genes, relative to a robust reference gene. The method has utility in determining quantative differences in mRNA expression of these target genes in mussels with differing IR exposure histories.

Chapter 6

Experimental Induction of *Rad51* and *Chk1* mRNA Expression in *M. edulis*

6.1. INTRODUCTION

It has been confirmed in the literature that IR induces DNA damage, specifically DSBs, and to repair such damage checkpoints are involved in sensing and controlling the cell cycle allowing the DNA repair (Bishay et al., 2001; Bahassi et al., 2008; Shen, 2011; Peng and Lin, 2011). As described in the previous chapters Rad51 and Chk1 expression and IR impacts have previously been investigated in several vertebrate species as well as some fungi (Collis et al., 2001; Chinnaiyan et al., 2005; Watson et al., 2004).

To recap, DNA damage checkpoint pathways function to delay the eukaryotic cell cycle in response to DNA damage induced by IR, thus providing an opportunity for DNA repair. ATM and ATR are highly conserved kinases; their activation is related to DNA damage, which leads to cell cycle arrest through a number of effector kinases molecules including Chk1 (Wright et al., 1998; Nyberg et al., 2002). Chk1 is involved in two IR-induced G1/S and G2 checkpoints in mammalian cells, human cells analyzed at 12 hrs after 10 Gy dose of IR treatment showed 70-80% of all cell types were in arrest state at G_2 (Liu et al., 2000). There is also considerable evidence that IR-induced S-phase checkpoint signaling is targeting degradation of Cdc25A, a tyrosine phosphatase that contributes to activation of Cdk2 (cyclin-dependent kinase 2) (Zhao and Piwnica-Worms, 2001; Sorensen et al., 2003). In the study of Hu et al. (2005), Chk1 kinase protein activity was over-activated following a 4 Gy dose of IR in mouse epithelial cells. It should be noted that 4 Gy is a very high dose in that levels involved in

a single environmental exposure are at the levels ranged between 0.049 and 0.17 μGy/hr (RIFE14, 2008). Chk1 protein levels have thus been examined in many irradiated cells from different sources including fruit fly, *Xenopus* and mouse (Fogarty et al., 1997; Guo et al., 2000; Weiss et al., 2002; Wang et al., 2002; Gatei et al., 2003; Hu et al., 2005). Here, we employ *Chk1* mRNA expression to investigate IR response in the commonly used marine bioindicator species, blue mussel, *M. edulis*.

Rad51 expression levels have been assessed in organisms exposed to DNA damaging agents including radiation in the environment or medical therapy (Yuan et al., 2003; Taghizadeh et al., 2009; Wang et al., 2010). Studies have shown that the Rad51 protein level is increased after radiation treatment. For instance, Chinnaiyan et al. (2005) showed an increase in Rad51 protein expression following 10 and 24 hrs after radiation (X-ray) treatment. Moreover, they observed accumulation of cells in G_2 phase and reduction of number of cells in S phase after 24 hrs post exposure to 6 Gy of X-ray. Du et al. (2010) indicated a correlation between the reduced Rad51 protein level and increased radiosensitivity to gamma radiation. To date, only medical studies have focused on *Rad51* gene expression levels. For instance, Tsai et al. (2010) reported an increase in the expression of Rad51 by a noticeable increasing in *Rad51* mRNA and protein stability after treatment with gemcitabine, a clinical treatment for lung cancer patients. It is also suggested in the literature that an increase in *Rad51* mRNA expression is associated with higher risk of tumour relapse, distant metastases and worst overall survival (Barbano et al., 2010). Although high expression of Rad51 is associated with enhanced resistance to DNA damage induced by chemicals and/or IR however, to date there has not been any real-time qPCR assay developed for the study of *Rad51* gene expression in an invertebrate species.

In order to apply the developed method and to study the RNA transcription of the partially isolated *Rad51* and *Chk1* genes, their expression was analyzed in mussels following experimentally-controlled exposure to different levels of IR. The aim of this work was thus to determine if the *Rad51* and *Chk1* mRNA expressions, using the sequences isolated in chapters 3 and 4, are altered in the gonads of mussels experimentally-exposed to different doses of IR.

6.2. MATERIALS AND METHODS

6.2.1. Mussel collection

Mussels were collected at low tide near Brighton marina (50^{o} 48' longitude and 0^{o} 5' latitude) in September 2010, kept in seawater and immediately brought to the laboratory. Seawater temperature was 9^{o}C, conductivity 54 mS/cm^2 and dissolved oxygen 10 mg/l. 90 mussels (size 4.25±0.65 cm) were placed randomly in each of two large glass tanks with 60 l of artificial seawater (InstantOcean, Sarrebourg, France) at a light regime of 12 hrs light/12 hrs dark. The temperature of the water was kept at 9^{o}C by heaters, dissolved oxygen at 10 mg/l by aerators and the conductivity at 50 mS/cm^2. The mussels were kept for a period of 7 days and the water was renewed every 48 hrs throughout all the experiment.

6.2.2. Experimental IR exposure

The mussels were divided into three groups: the first group contained 50 mussels and were exposed to ^{137}Cs at different doses (0, 1, 2, 10 and 50 Gy, dose rate 0.125 Gy/sec, n=10 at each dose) and dissected 1 hr after exposure. The second group contained 20 mussels exposed to the same source at dose 0 and 2 Gy, n=10 and dissected 96 hrs (4 days) after exposure. The third group comprised 20 mussels also

exposed as group two but dissected 168 hrs (7 days) after exposure. The samples were exposed to radiation in 50 ml conical polypropylene sterile tubes in the presence of seawater.

The size (4.25±0.65) of every individual was recorded, the gonads were dissected and submerged in RNAlater (Qiagen Ltd., U.K.) and stored at -20^{o}C for further processing in molecular analysis.

6.2.3. Total RNA isolation and First strand synthesis of cDNA for real-time PCR

Approximately 20 mg of RNAlater preserved gonadal tissues were extracted using NucleoSpin® RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) reagents and manufacturers protocol. The samples were disrupted by adding 600 µl of homogenisation lysis buffer (containing large amounts of chaotropic ions, guanidine thiocyanate and 1% β mercaptoethanol). 20 mg of gonadal tissue was first disrupted using glass beads (Sigma) in 600 µl of homogenisation buffer and left for a few minutes to digest the tissue. Samples were centrifuged at 4°C for 40 sec to homogenate the tissue after that spun 3 min at 11,000 x *g* and the supernatant transferred into a clean tube. 600 µl of ethanol 70% was added to provide appropriate binding conditions and the sample was then applied to a silica-gel based column, spun 30 sec at 11,000 x *g* and the flow-through discarded. To avoid genomic DNA contamination a DNA digestion step was performed by adding 95 µl a DNase reaction mixture containing 10% of RNase-free rDNase in rDNase reaction buffer to the column and the reaction was incubated at room temperature for 15 min. The column was washed several times with ethanol-based buffers to eliminate the contaminants and the flow-through discarded. The column was then transferred into a clean tube and eluted by centrifugation for 1 min at 11,000 x *g* with 30 µl nuclease-free water after a 1 min incubation period at room

temperature. The procedure was repeated once more with another 30 µl RNase-free water. The samples were stored at -20^{o}C until further processing. The total RNA samples were reverse transcribed into cDNA (section 3.2.3) and stored at -20°C.

6.2.4. *Rad51* and *Chk1* mRNA expression in mussel gonad tissue samples

The expression of the *Rad51* and *Chk1* mRNA was analysed using real-time PCR as described in sections 5.2.6 and 5.2.8. For the analysis of the putative *Rad51* and *Chk1* mRNA expressions in *M. edulis,* relative gene quantitation was expressed in relation to the expression of a housekeeping gene, *18s rRNA*, and the ΔCt method was employed to quantify the expression of each control and exposure groups of mussels (section 6.2.2).

The relative expression data was analyzed using SPSS 15.0 for Windows and tested for normality using the Kolmogorov-Smirnov test and the Q-Q plots. All the data was not normally distributed and therefore differences between the different doses were determined by the non-parametric test Kruskall Wallis. In order to check where the differences occurred, pair-wise comparisons of the stages were performed by Mann-Whitney *U* tests. In order to avoid inflation of type I error rates, Bonferroni corrections were performed by using a critical value for significance of 0.05 divided by the number of tests conducted.

6.3. RESULTS

6.3.1. *Rad51* mRNA expression in mussel gonads exposed to IR

An increasing trend in *Rad51* mRNA expression was observed in all exposed mussels, however, only at the highest dose of radiation (50 Gy) was the increase statistically significant compared to the control group (Fig. 6.3.1.1). After 4 and 7 days

of exposure a highly significant increase in the Rad51 expression was observed in comparison to the control of each group C 4 d and C 7 d respectively (Fig. 6.3.1.1).

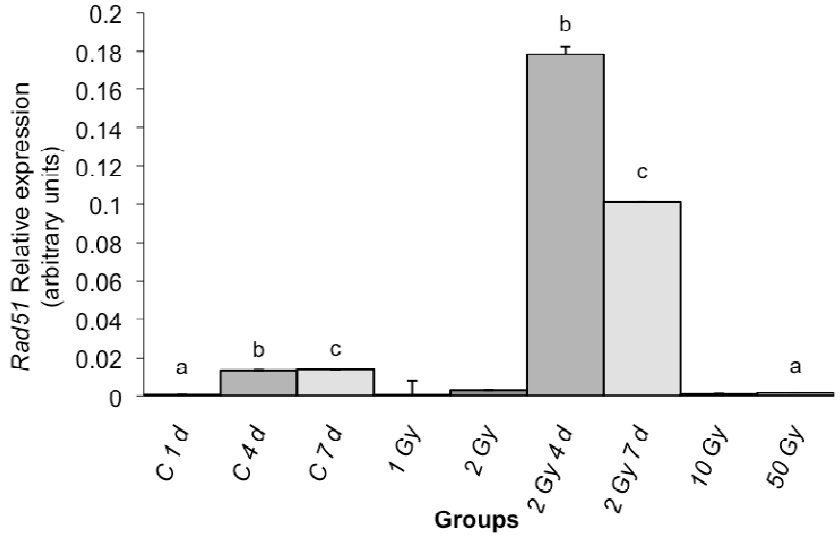

Fig. 6.3.1.1. *Rad51* mRNA expression in gonad of mussels exposed to different doses of IR (1, 2, 10 and 50 Gy), sampled at different time points (1 d= the same day, 4 d= 4 days and 7 d= 7 days after exposure) and control groups (C). The figure shows relative *Rad51* mRNA expression to *18s rRNA* mRNA expression. Mean data are plotted ± standard error of the mean (SEM). Asterix indicates significance at a Bonferroni corrected $p<0.05$ compared to controls.

6.3.2. *Chk1* mRNA expression in mussel gonads exposed to IR

Changes in *Chk1* relative mRNA expression were observed in irradiated mussels compared with control mussels (Fig. 6.3.2.1). In the first group, *Chk1* mRNA expression has increased significantly in most of the irradiated mussel groups 1, 2 and 10 Gy compared to control samples (Fig. 6.3.2.1). In the second time course group, irradiated mussels after 4 days of exposure showed a decrease in the *Chk1* mRNA expression compared to non irradiated mussels but this was not statistically significant. An increase of *Chk1* mRNA expression was observed in the irradiated mussels after 7 days of exposure compared to control, however, again, this was not statistically significant (Fig. 6.3.2.1).

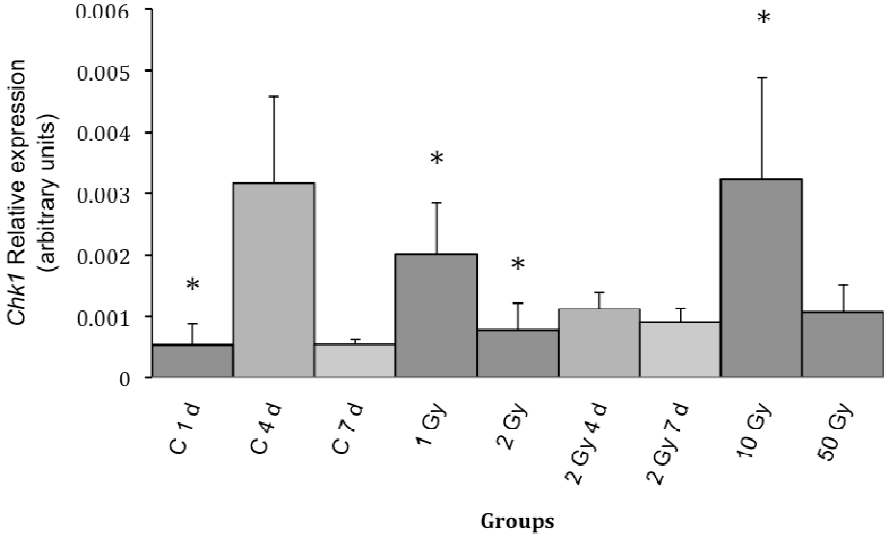

Fig. 6.3.2.1. *Chk1* mRNA expression in gonad of mussels exposed to different doses of IR (1, 2, 10 and 50 Gy), sampled at different time points (1 d= the same day, 4 d= 4 days and 7 d= 7 days after exposure) and control groups (C). The figure shows relative *Chk1* mRNA expression to *18s rRNA* mRNA expression. Mean data are plotted ± standard error of the mean (SEM). Asterix indicates significance at a Bonferroni corrected $p<0.05$ compared to controls.

6.4. DISCUSSION

The objective of this work was to determine if *Rad51* and *Chk1* mRNA expression would be induced in *M. edulis* individuals exposed to different doses of IR by using the real-time qPCR method developed earlier. In order to determine if these changes were correlated between the cell cycle related to DNA repair in the gonads and variations due to radiation impact, the study was carried out at different doses of IR and at different points of time after radiation. Here we found that *Rad51* mRNA expression levels was increased though only at high dose level or following a 4 day period after exposure to lower radiation doses.

The damage caused in DNA after exposure to radiation has been extensively studied. DNA repair, specifically HR is a critical pathway to recover DNA damage after

radiation exposure. So, the levels of the *Rad51* mRNA studied in the first group showed changes between the control and irradiated mussels but these changes in *Rad51* mRNA were significant only between the control and mussels irradiated with 50 Gy dose. The lack of statistically significant differences in expression of *Rad51* mRNA in the samples from the other different doses of radiation can be due to several reasons including dose of radiation, cellular shape and manner of contact of the tissue (Taghizadeh et al., 2009). However, in a time dependent manner (4 and 7 days of exposure to 2 Gy dose of ^{137}Cs), there were significant increases in the levels of *Rad51* mRNA expression between the control and the irradiated group. These results are in agreements with studies showing increasing Rad51 expression in mouse and human after radiation exposure and also confirmed that the expression of *Rad51* mRNA levels increase in a time-dependent manner (Yuan et al., 2003; Chinnaiyan et al., 2005; Taghizadeh et al., 2009).

Since pathways of DNA damage repair include Rad51 as a key protein in re-synthesis, catalyzing and transferring, strands between broken sequences and its homologues in DSBs damage (Rollinson et al., 2007), it could be possible that overexpression of *mRad51* mRNA can results in an increase in spontaneous recombination between intrachromosomal repeat sequences which has previously been reported in mammalian cells (Vispé et al., 1998; Arnaudeau et al., 1999; Huang et al., 1999). Watson et al. (2004) also reported that a functional homologue of Rad51 was found to be inducing in response to IR in fission yeast. As discussed in chapter 4, since the *Rad51* gene isolated contained some of the important domains of the protein, it is likely that the gene codes for a functional protein in the mussel.

The level of *Chk1* mRNA expression in gonads of mussels exposed to different doses of radiation also showed a significant increase. Similar results, albeit a different

mechanism of activation, were observed with irradiated mammalian cells within 30 min post-IR, showing induction of phosphorylated Chk1 (Gatei et al., 2003).

In vertebrates, there is evidence that induction of Rad51 and Chk1 are an IR DNA damage-induced event. For instance, radiation was capable of increasing Chk1 protein levels in the human cells and a rise in ATM or ATR levels (Liu et al., 2000) and *Rad51* mRNA levels and protein abundance increased in human osteosarcoma cell after radiation or drug treatment (Du et al., 2010). The γ-irradiation-induced Rad51 focus formation increased significantly during cell cycle progression, with the highest induction at the S and G2/M phases (Yuan et al., 2003). In addition, it was noted that G2 phase in the cell cycle is more sensitive to radiation than other phases, which conclude that the cell cycle could change the sensitivity of the cell to radiation (Pawlik and Keyomarsi, 2004). Yao et al. (2007) reported that depletion of Chk1 siRNA leads to a loss of Rad51 protein in human leukemia cells. Moreover, Chk1 siRNA treatment prevented radiation-induced Rad51 focus formation (Bahassi et al., 2008). Much less information is available, however, on the correlation between DNA repair and checkpoints in invertebrates.

A further point for discussion relates the higher levels of *Rad51* mRNA in mussels to the levels of *Chk1* mRNA. Relevantly, deficient Chk1 cells leads to a loss of Rad51 localization to nuclear foci in response to replication arrest (Bahassi et al., 2008). Also cells lacking Chk2 showed a defect in Rad51 localization in response of DNA DSBs indicating that each of these kinases may contribute somewhat differently to the formation of Rad51 nucleoprotein filaments depending on the type of DNA damage incurred by the cells (Bahassi et al., 2008).

In summary, the exposure of *M. edulis* to IR increased the level of putative *Rad51* mRNA expression in the experiment but not in all different doses. The explanation for

the lack of repression in the first experiment could relate to the observation that the individual variances were different. In addition, the *Rad51* mRNA levels associated with the repair pathway, regulation by ATM or ATR indirectly, and *Chk1* levels reported in this study were all in agreement with changes levels of Rad51 and Chk1 activity of other vertebrate reported in the literature. Therefore, the possibility that the isolated *Rad51* and *Chk1* genes characterized, might act as a future DNA-damage biomarker in the aquatic environment.

Chapter 7

Environmentally-induced DNA Damage and Induction of *Rad51* and *Chk1* mRNA Expression in *M. edulis*

7.1. INTRODUCTION

Most of the attention focused on IR pollution stems from nuclear weapons used during the Second World War in Japan and the accident at Chernobyl in Russia. In 1945, the United States exploded two nuclear weapons in a military operation on Hiroshima and Nagasaki, Japan. Survivors of the cities still experience higher than normal cancer rates (Muirhead, 2003). In 1986, a Russian nuclear plant at Chernobyl leaked high amounts of radiation pollution into the surrounding area (Delfanti et al., 2006). Such products, which include ^{58}Co, ^{137}Cs, ^{238}Pu, ^{241}Am, ^{65}Zn and ^{110}Ag, are also occasionally present, albeit at significantly lower levels, in close proximity to nuclear facilities (Clifton et al., 1983). The´bault et al. (2008), for instance, found a strong relationship between the concentration of ^{137}Cs measured in mussels, *M. galloprovincialis*, and the distance of their sampling locations from the Chernobyl Nuclear Power Plant.

IR as a pollutant causes both primary and secondary damage. Primary damage has a direct identifiable impact on the environment, and secondary damage is considered as minor perturbations in the delicate balance of the biological food web, detectable only over long time periods (Muirhead, 2003; Yamada et al., 1999; Burger et al., 2007). Secondary damage and also sometimes referred to as chronic damage in the literature, may range from mild tissue irritation or immune suppression to an increase in the formation of carcinogenic cells (Cardis et al., 2006). As discussed in chapter 1 , IR can damage DNA by breaking the double strands, by cross-linking different DNA strands

(Fig. 1.2.1.1.), and by cross-linking DNA and proteins (Gebicki and Gebicki, 1999). As such the damage produced by IR is more complex, with localized areas of DNA molecules with multiple and complex lesions consisting of a combination of base damage and single-strand breaks and DSBs (Ward, 1995; Nikjoo et al., 2001). These complex lesions are less easily repaired with fidelity than are more simple forms of DNA damage (Jeggo, 1998). Damage to DNA can lead to cancers, birth defects, and even death (Tallarico et al., 2004; Roos and Kaina, 2006). However, cells have biochemical repair systems that can reverse some of the damaging biological effects of low-level exposures to radioactivity (Ward, 2002; Rothkamm and Lobrich, 2003; O'Driscoll and Jeggo, 2006). This allows the body to better tolerate radiation that is delivered at a low dose rate, such as over a longer period of time. If radiation damages DNA and the cell cannot repair itself, then cancer may become an increasing risk (Cardis et al., 2006). Yet all organisms are exposed to IR in extremely small doses throughout their life from natural sources (Meli et al., 2008). The biological effects of such small doses over such a long time are difficult to measure, and are essentially unknown at present. There is, however, a theoretical possibility that the small amount of radioactivity released into the environment by normally operating nuclear power plants and by previous atmospheric testing of nuclear weapons, has slightly increased the incidence of certain cancers in human populations (Cardis et al., 2006; NRC, 2006).

IR biological effects have been measured using a number of different ways in several organisms from the environment. For instance, Krivolutzkii and Pokarzhevski (1992) reported a difference in population numbers of resident groups (earthworms, beetles and their larvae) between the contaminated (30 km zone surrounding Chernobyl) and a control site 70 km away. Also for Orbatid mites, radioactive fallout observed to affect early stages macrofauna, particularly earthworms. Moreover, deterioration was

reported in the condition of the colonies (population, community reduction and growth factor) of the bivalves *Anodonta cygnea* and *D. polymorpha* after the Chernobyl accident, particularly the latter (Skolov et al., 1993). In these studies investigating bivalve species, it was believed that before Chernobyl accident there were a number of genetic effects induced by chronic radiation exposure at dose rate of 0.1 rad/year and above 40 rad/year (Skolov et al., 1993). However, this study refers to genetic effect as disturbances in the number and structure of chromosomes, different species mutations but does not describe methodology (Skolov et al., 1993). Combined, these studies showed that IR at certain dose levels affects organisms, terrestrial and aquatic.

In a controlled laboratory exposure environment, studies of Anderson and Harrison (1986) and Anderson et al. (1990) involving measured radiation doses, reported several biological effects on aquatic organisms. With increasing radiation dose these effects included: an increase of chromosomal aberrations, a decrease in fecundity, an increase in the number of mitotic cell delay and an increase in the possibility cell death occurring during interphase (Anderson and Harrison, 1986; Anderson et al., 1990). Survivorship of irradiated worms was also observed to differ with life stage, sex, and reproductive condition (Anderson et al., 1990; Krivolutzkii and Pokarzhevski, 1992). In a different experimental exposure, NCRPM109 (1991c) reported that the number of egg capsules was reduced in the pond snail *Physa* at doses of 6.5 mGy/day. However, overall egg numbers only decreased at doses of 240–1200 mGy/day. Also, birth rates in *Daphnia* drop at doses of more than 4600 mGy/day (Blaylock et al., 1993). Thomas and Liber (2001) showed that the equivalent doses to *Chironomus tentans* and *Hyalella azteca* in Horseshoe Pond, Canada (540–560 mGy/year) surpassed the lowest reproductive dose limit of 360 mGy/year. These laboratory experimental approaches add weight to the environmental exposure data, that indeed, IR exposure at certain dose

levels can induce biological effect in organisms.

Several studies have focused on DNA damage using comet assay, which is a sensitive technique for the detection of DNA damage, in the aquatic species at different irradiated polluted sites. For instance, Sugg et al. (1996) associated elevation of strand breaks in catfish with ^{137}Cs exposure in a cooling pond contaminated from the Chernobyl nuclear power plant. It is also possible to distinguish different degrees of comet tail fluorescence resulting from different doses of UV radiation (Gedik et al., 1992; Villela et al., 2006). In the study of Grazeffe et al. (2008), the comet assay was employed using the snail, *Biomphalaria glabrata,* following exposure to radiation doses of 50 Gy and 100 Gy. The results showed an excessively low number of cells that prevent the analysis. Jha et al. (2005) also observed that following exposure to tritium resulted in the induction of DNA damage as increasing dose in mussel haemocytes.

The aim of this study was to determine the extent of DNA damage, using comet assay, and induction of *Rad51* and/or *Chk1* mRNA expression in gonad tissue of *M. edulis* collected from the environment at two sites: one in the vicinity of a nuclear processing plant (in Ravenglass Estuary) and one at a reference site (in Brighton Marina).

7.2. MATERIALS AND METHODS

7.2.1. Mussel collection

Mussels were collected at low tide in Ravenglass near Sellafield nuclear reprocessing plant (54^{o} 21' longitude and -3^{o} 24' latitude) on July 2010, kept in seawater and brought to the laboratory of Plymouth University. Another group of mussels were collected from the same 'clean' site (Brighton Marina) described in chapter 6 and used as reference samples. Brighton and Ravenglass mussels were placed

each in two large glass tanks with 60 l of artificial seawater (Instant Ocean, Sarrebourg, France) at a light regime of 12 hrs light/12 hrs dark. The temperature of the water was kept at 9^{o}C by heaters, dissolved oxygen at 10 mg/l by aerators and the conductivity at 50 mS/cm^{2}. The mussels were kept for a period of 2 days. On day 2, 200-400 µl of haemolymph was withdrawn from the posterior adductor muscle with a needle of 1 ml syringe after the size of every individual was recorded (4.7±0.5 cm and 5.55±0.35 cm for Brighton and Ravenglass respectively). The haemolymph was added to phosphate buffer saline, PBS (8 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, 0.27 g KH_2PO_4, pH 7.4) directly on ice. Mussels were dissected, gonads kept in 1.5 ml of RNALater (Qiagen Ltd., U.K.) and stored at -70^{o}C for molecular analysis.

7.2.2. Comet assay

This work was conducted at University of Plymouth with generous guidance of Ph. Yanan Di. For the slide preparation, Super-frost slides were coated with 1.5% Normal Melting Agarose (NMA) and left to air dry at least 24 hrs before the comet assay. The haemolymph cell suspension was centrifuged at 2.4 x *g* for 2 min and the supernatant was discarded and replaced with 200 µl 0.75% low melting point agarose (LMA). The mixture was then applied to the pre-coated slides as two drops of 100 µl. Coverslips were placed over each drop and gels were allowed to set at 4°C for 1 hr. When the gels had solidified to form duplicated microgels, coverslips were gently removed and the slides were immersed for 1 hr in cold (4°C) lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% N-Lauroyl-sarcosine, 1% Triton X 100, 10% DMSO, pH 10). After the lysis period, slides were placed in a horizontal electrophoresis unit containing freshly prepared electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH>13). The DNA was allowed to unwind for 30 min before

electrophoresis at 25 volts, 260 mA for 30 min. The slides were removed from the electrophoresis tank and gently immersed in neutralization buffer (0.4 M Tris base, pH 7.5) for 5 min and this step was repeated three times followed by rinsing with distilled water for 10 min and left it to dry for overnight. Finally, to visualize comets, 40 µl of ethidium bromide (20 µg/ml) stain was applied to each gel. Cells were randomly selected and measured by video capture and image analysis using Komet 5.0 software (Kinetic Imaging, Liverpool, U.K.) with 50 cells scored per microgel. % DNA in head and tail were determined.

7.2.3. Total RNA isolation and first strand synthesis of cDNA for real-time PCR

Approximately 20 mg of RNAlater preserved gonadal tissues were extracted using RNA isolation® (Roche) reagents as described in section 3.2.2. cDNA was synthesised using Transcriptor High-fidelity cDNA Synthesis System reagents supplied by Roche (Roche Diagnostics Ltd, Burgess Hill, U.K.). Up to 50 ng/µl total RNA was mixed with 2 µl 600 pmol random hexamer and water to a final volume of 11.4 µl. The sample was incubated for 10 min at 65°C and then placed on ice for 1 min. 4 µl of 5x concentrated TRT reaction buffer (250 mM Tris-HCl pH 8.5, 150 mM KCl, 40 mM $MgCl_2$), 0.5 µl Protector RNase Inhibitor (20 mM Hepes-KOH, 50 mM KCl, 8 mM dithiothreitol, 50% glycerol (v/v)) (40 units/µl), 2 µl dNTPs (10 mM) and 1.1 µl Transcriptor High Fidelity Reverse Transcriptase (20 units/µl) were added to the rest of the RNA/primer mixture, mixed gently and incubated for 30 min at 55°C. A final incubation of 5 min at 85°C was carried out and then the reaction was placed on ice. The final volume of the reaction was 20 µl.

7.2.4. *Rad51* and *Chk1* mRNA expression in mussel gonad tissue samples

The expression of the *Rad51* and *Chk1* mRNA was analysed using real-time PCR as described in sections 5.2.6 and 5.2.8. For the analysis of the putative *Rad51* and *Chk1* mRNA expressions in *M. edulis,* relative mRNA quantitation was expressed in relation to the expression of a housekeeping gene, *18s rRNA*, and the ΔCt method was employed to quantify the expression of each control and exposure group of mussels (section 7.2.1).

The relative expression data was analyzed using SPSS 15.0 for Windows and tested for normality using the Kolmogorov-Smirnov test and the Q-Q plots. All the data was not normally distributed and therefore differences between the different doses were determined by the non-parametric test Kruskall Wallis. In order to check where the differences occurred, pair-wise comparisons of the stages were performed by Mann-Whitney *U* tests. In order to avoid inflation of type I error rates, Bonferroni corrections were performed by using a critical value for significance of 0.05 divided by the number of tests conducted.

7.3. RESULTS

7.3.1. Comet assay

Following electrophoresis the presence of strand breaks allows fragments of DNA to move from the core toward the anode, resulting in the classical comet formation (Singh et al., 1988). With the increasing amount of damage, more DNA migrates into the tail region and its quantified in terms of increased flourescence in the tail region and tail length. The percentage of DNA in the tail region (tail % DNA) was used as the criterion for quantifying DNA strand breakage (Anderson et al., 1994). Head % DNA, tail length and tail moment, a product of tail DNA and length, are also reported. Control

cells (prepared from mussels collected from the reference site at Brighton Marina) consisted of nucleotid core with zero or minimal DNA migration into the tail region (Fig. 7.3.1.1a). While the Ravenglass cells (Fig. 7.3.1.1b) showed a noticable difference in DNA head and tail shape, decrease in the DNA head and formation of DNA tail were observed.

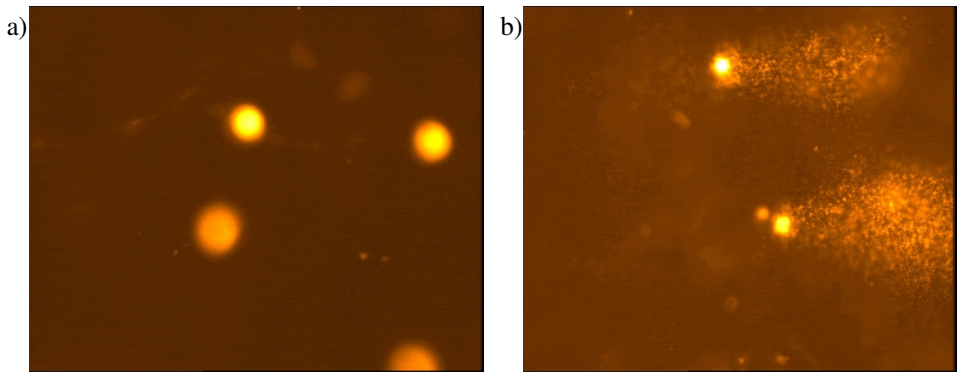

Fig. 7.3.1.1. Typical comets showing no DNA damage in (a) reference (Brighton Marina) and observable DNA tail damage in (b) Ravenglass mussel haemocytes.

A statistically significant decrease in the head DNA % of Ravenglass mussels compared to a reference site was observed (Fig. 7.3.1.2a). While a high significant increase was observed in the tail DNA % (Fig. 7.3.1.2b) and olive tail % (Fig. 7.3.1.2c) of Ravenglass mussels in comparison to Brighton reference mussels.

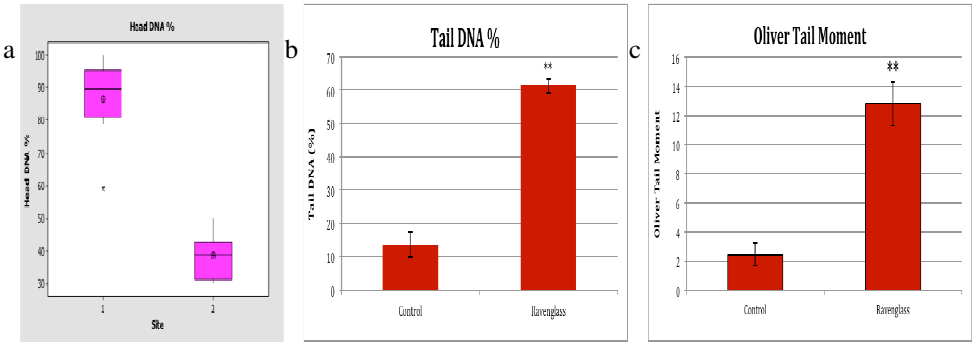

Fig. 7.3.1.2. DNA damage measured in haemocytes of Control (1) and Ravenglass (2) mussels using the Comet assay (a) head DNA % (b) tail DNA % and (c) olive tail moment. The values are means ± SEM. Asterix indicates a statistically significant difference from the control, $p<0.01$).

7.3.2. *Rad51* mRNA expression in mussel gonads sampled from two environmental sites

A statistically significant increase in *Rad51* mRNA expression was observed in gonad tissue isolated from Ravenglass mussels compared to reference mussels (Fig. 7.3.2.1).

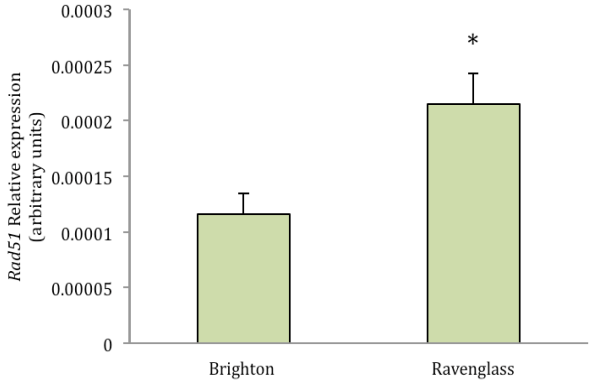

Fig. 7.3.2.1. *Rad51* mRNA expression in gonad tissues from *M. edulis* sampled at Ravenglass and Brighton illustrating significant increase in Ravenglass mussels compare to Brighton. The figure shows relative *Rad51* mRNA expression to *18s rRNA* mRNA expression. Mean data are plotted ± standard error of the mean (SEM). Asterix indicates significance at a Bonferroni corrected $p<0.05$ compared to controls.

7.3.3. *Chk1* mRNA expression in mussel gonads sampled from two environmental sites

Chk1 mRNA relative expression decreased significantly in gonad tissues isolated from Ravenglass mussels compared with control mussels (Fig. 7.3.3.1).

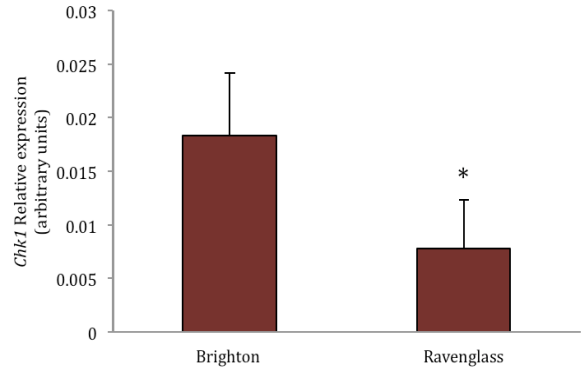

Fig. 7.3.3.1. *Chk1* mRNA expression in gonad tissues from *M. edulis* sampled at Ravenglass and Brighton illustrating significant reduction in Ravenglass mussels compare to Brighton. The figure shows relative *Chk1* mRNA expression to *18s rRNA* mRNA expression. Mean data are plotted ± standard error of the mean (SEM). Asterix indicates significance at a Bonferroni corrected $p<0.05$ compared to controls.

7.3.4. Radionuclide levels in sediment and mussels of two environmental sites

Radionuclide concentrations were kindly provided by Prof. Cundy (at University of Brighton) and his University of Southampton collaborators. This included artificial and natural radionuclide concentrations in sediment and mussel tissue samples also collected from Brighton Marina and Ravenglass Estuary (Table 7.3.4.1). As seen in Table 7.3.4.1, radionuclide concentrations are generally low at both sites, however the Ravenglass sediment samples showed higher concentration in most of the isotopes compared to Brighton. Also in the mussel tissue samples, ^{238}Pu, ^{239,240}Pu and ^{241}Am showed higher concentrations in individuals collected from Ravenglass compared to Brighton. While the remaining isotopes showed lower concentrations in Ravenglass mussels compared to Brighton mussels.

Table 7.3.4.1. Anthropogenic radionuclide concentrations at Brighton Marina (BM) and Ravenglass Estuary (RE).

Source		Anthropogenic Radionuclide (Bq/kg dry)								
		^{210}Po	^{238}Pu	^{239,240}Pu	^{90}Sr	^{241}Am	^{137}Cs	^{60}Co	^{155}Eu	^{65}Zn
Sediment	BM	3.1±0.8	<0.2	0.17±0.09	0.019±10	<0.9	<0.6	<0.9	<200	<2
	RE	4.9±0.7	15±2	83±8	35±12	170±10	36±20	<0.8	<300	<2
Mussels	BM	76±18	<0.1	<0.1	31±15	<4	<6	<9	<800	<20
	RE	64±15	3.7±0.5	19±2	27±16	34±3	5.1±1.6	<5	<600	<10

Also, natural radionuclide concentrations were measured in sediment and mussel tissue samples at both sites (Table 7.3.4.2). Higher concentrations of natural radionuclides (^{228}Ac, ^{40}K, ^{210,212,214}Pb, ^{226}Ra, ^{234}Th and ^{234}U) were observed in Ravenglass sediment samples compared with those from Brighton. However, mussel tissue samples showed lower concentration of natural radionuclides (^{228}Ac, ^{210,212,214}Pb, ^{226}Ra, ^{234}Th and ^{235}U) in Ravenglass when compared to Brighton, and only ^{40}K showed higher concentration in Ravenglass samples than Brighton samples.

Table 7.3.4.2. Natural radionuclide concentrations in Brighton Marina (BM) and Ravenglass Esturay (RE).

Source		Natural Radionuclide (Bq/kg dry)							
		^{228}Ac	^{40}K	^{210}Pb	^{212}Pb	^{214}Pb	^{226}Ra	^{234}Th	^{235}U
Sediment	BM	<3	16±8	<8	2.8±0.5	3.4± 0.5	<10	5± 1.8	0.6± 0.3
	RE	7.2±1.5	240±20	<20	9.6±0.8	7.5±0.7	<20	<10	0.8± 0.3
Mussels	BM	<30	<200	<40	18±3	<20	<60	<50	<10
	RE	<20	220±50	<20	5.3±1.9	<9	<40	<30	<8

7.4. DISCUSSION

The objective of this work was to determine the extent of DNA damage, if any, at two sites: one an IR-contaminated site, Ravenglass Estuary, and the other a reference site at Brighton Marina, using *Rad51* and *Chk1* mRNA expression induction in *M. edulis* and an established technique (comet analysis).

At the cellular level, the comet analysis showed significant damage in the DNA with a decrease in the head DNA % and an increase in the tail DNA % and oliver tail values for the Ravenglass mussels compared to the reference site (Brighton Marina) (Fig. 7.3.1.2). These results are in concordance with other published work in the literature. For instance, similar genetic damage in the embryo larval stages of mussels exposed to radiation (tritiated water at dose of 0.02-21.41 mGy) has previously been reported (Hagger et al., 2005a). In the study of Jha et al. (2005), mussel haemocytes treated with low doses (<500 μGy/hr) of tritiated water, showed DNA fragmentation and micronuclei formation. Moreover, it was also concluded that the tritium accumulation differed in different tissues of mussels treated with a dose range of 12-485 μGy/hr for 96 hrs. However, Grazeffe et al. (2008) treated snails, *Biomphalaria glabrata*, with high doses 2.5, 5, 10 and 20 Gy of ^{60}Co gamma-radiation and obtained comet results showing either small or nonexistent heads and large diffuse tails, which were consequently referred to by the authors as ‘dead cells’.

The levels of the *Rad51* mRNA studied in the Ravenglass mussels showed a statistically significant increase compared to the reference site at Brighton Marina (Fig. 7.3.2.1). The elevation of *Rad51* mRNA expression suggests that the cell's DNA repair mechanism has been triggered. This work represents the first work application of *Rad51* mRNA expression in a mollusc and in an environment setting. In the laboratory, similar results have been observed in fission yeast, *Schizosaccharomyces pombe, Rad51* mRNA expression was induced following exposure to IR (500 Gy) (Watson et al., 2004). In the study of Yuan et al. (2003), it was reported that IR (at a dose level of 10 Gy) induced Rad51 nuclear focus formation significantly particularly at the S and G2/M phases of the cell cycle. Moreover, an increase of Rad51 protein expression was reported in cultured cells following exposure to 6 Gy of X-ray radiation (Chinnaiyan et al., 2005). Organisms with enhanced DNA repair systems, such as *Deinococcus radiodurans,* the most radiation-resistant known organism, exhibit remarkable resistance to the double-strand break-inducing effects of radioactivity, likely due to enhanced efficiency of DNA repair and especially NHEJ (Kobayashi et al., 2004). These observations of elevated *Rad51* mRNA expression provide evidence that Rad51 likely plays a similar role in DNA repair in invertebrate species including mussels. Moreover, these results provide additional evidence that changes in genetic structure of *M. edulis* exposed to a genotoxicant (radiation) can be detected at the DNA level.

The level of *Chk1* mRNA expression in gonad tissues of Ravenglass mussels showed a statistically significant decrease in comparison to reference mussels sampled from Brighton Marina (Fig. 7.3.3.1). Similar to these results, Gatei et al. (2003) concluded a decrease in Chk1 phosphorylation activity following exposure to 6 Gy of IR for 30 min. In contrast, Watson et al. (2004) reported that IR does not alter mRNA levels of checkpoints genes, *rad3*, *chk1* and *cds1*. The behaviour and significance of

Chk1 mRNA expression following exposure to IR is therefore less clear and further work is needed to clarify this. It is important to note that *Chk1* mRNA expression, as well as that of other cell cycle checkpoints, are not specific to IR and maybe induced or inhibited by many different agents present in the environment (Bi et al., 2006; Shiromizu et al., 2006; Caino et al., 2007).

Several studies investigated radionuclide concentration in many organisms from different environmental locations in the world. For instance, in Amchitka island (Alaska) Octopus showed high concentration of ^{137}Cs (0.262 ± 0.029 Bq/kg) while mussels showed high concentration of ^{234,238}U (0.844 ± 0.804 Bq/kg, 0.730 ± 0.646 Bq/kg respectively) (Burger et al., 2007). Much lower values were obtained in mussels sampled from two different locations in Bangladesh, the ^{137}Cs and ^{134}Cs were reported to be under the detection limit (0.024 Bq/kg and 0.076 Bq/kg) (Alam et al., 1999). However, the concentration factor of ^{226}Ra, ^{232}Th and ^{238}U was higher in both *Perna viridis* and *Modiolus striatulus* mussel's shell in comparison to the tissue. Higher radionuclide concentrations were reported in Rhône River, France, which is known as Europe's biggest concentration of nuclear power plants, ^{137}Cs concentration was estimated to reach 100 GBq/yr and 2000 Bq/m^2 in water and soil respectively, also $^{239+240}$Pu and ^{238}Pu concentrations reached 50 Bq/m^2 and 1.5 Bq/m^2 in soil (Eyrolle et al., 2005). Also in Italy, radionuclide concentrations were measured at seven different sites between north and south Marche, using *M. galloprovincialis* and the mean total uranium and ^{210}Po activity was recorded at 2.34 Bq/kg and 149 Bq/kg respectively (Meli et al., 2008). Further radionuclide concentrations were measured at different sites in the UK (Table 1.1.1). In relation to these published levels of radionuclides, the data provided showed a relatively high concentration of radionuclides in Ravenglass mussel tissues (^{238}Pu at 3.7 Bq/kg, ^{239,240}Pu at 19 Bq/kg, ^{241}Am at 34 Bq/kg) compared to

Brighton mussels (<0.1 Bq/kg, <0.1 Bq/kg, <4 Bq/kg respectively). Moreover, radionuclide concentrations in Ravenglass mussels showed higher levels (^{210}Po at 64 Bq/kg, ^{60}Co at <5 Bq/kg, ^{155}Eu at <600 Bq/kg, ^{65}Zn at <10 Bq/kg) than Ravenglass sediment samples (4.9 Bq/kg, <0.8 Bq/kg, <300 Bq/kg, <2 Bq/kg respectively). Also, the concentration of ^{137}Cs radionuclides in Ravenglass mussel tissues (5.1 Bq/kg) noticed to be higher than these reported previously in Bangladesh and Alaska (Alam et al., 1999; Burger et al., 2007). ^{210}Po concentration, in contrast, showed lower levels in Ravenglass mussel tissues (64 Bq/kg) compared to levels reported in *M. galloprovincialis* (149 Bq/kg) sampled in Italy (Meli et al., 2008). However, in the study of Yamada et al. (1999) determining different radionuclide concentrations in several species of bivalve along the Japanese coast, ^{239,240}Pu and ^{137}Cs concentration levels were reported as significantly lower (0.8-6.1 mBq/kg wet weight and 47-62 mBq/kg wet weight respectively) compared to the levels of Ravenglass mussel tissues. Moreover, along the coastal region of the Baltic and Mediterranean Seas, 60 locations were investigated for ^{137}Cs concentration level using *M. galloprovincialis* (The´bault et al., 2008), and a range of values were reported in different European countries (0.01-0.03 Bq/kg wet weight in France, 0.008-<0.05 Bq/kg wet weight in Italy, 0.01-0.077 Bq/kg wet in Spain, 0.7-1.5 Bq/kg wet in Ukraine), which were all lower than ^{137}Cs concentration level reported in Ravenglass mussel tissues in this study. In the UK, radionuclide concentration levels reported in RIFE14 (2008) were lower in comparison with this study. For instance, concentrations of ^{241}Am, ^{137}Cs, ^{60}Co and ^{155}Eu of Ravenglass mussel tissues in the current study were higher (34 Bq/kg, 5.1 Bq/kg, <5 Bq/kg, <600 Bq/kg respectively) compared to these reported (12 Bq/kg, 1.5 Bq/kg, 1.3 Bq/kg, <0.16 Bq/kg respectively) in RIFE14 (2008). This trend is consistent with the concentrations also recently reported in RIFE15 (2009).

Despite these low IR concentrations, relative to EU dose limits, detected in mussel tissues from the Ravenglass site, the comet analysis and *Rad51* mRNA levels indicate that the organisms are indeed impacted. In agreement with this, Jha et al. (2005) reported that *M. edulis* treated with low doses of tritium (3.7-147 MBq/l equivalent to 12 to 485 μGy/h) showed induction of DNA damage, micronuclei and increasing activity concentration in different tissue starting with gut followed by the gill, mantle, muscle and the lowest concentration was observed in faeces and pseudo-faeces. Using *Rad51* mRNA expression and MN assay on blood samples, Bishay et al. (2001) also reported a significant correlation ship between the induction of MN and *Rad51* mRNA expression following exposure to radiation at dose of 0.5 and 2 Gy. Moreover, Harrison and Anderson (1994b) who studied the effects of life time exposure to IR on the polychaete worm, *N. arenaceodentata,* reported a significant decrease in live embryos and an increase in abnormal embryos.

In this study, the mussels collected from the impacted environmental sampling site at Ravenglass have been chronically exposed to relatively low doses of IR, yet show a significant increase of DNA damage detected at the cellular level using the comet assay and also at the molecular level using the *Rad51* mRNA expression qPCR method. The possible role of Rad51 and essential kinases in the DNA repair mechanism in the invertebrate, *M. edulis*, based on predicted homology of sequence with the vertebrate counterparts, is shown in Fig. 7.4.1.

In previous chapter, it was observed that a range of experimentally-induced IR doses, resulted in a significant increase in *Rad51* and *Chk1* mRNA expression levels, and which forces a consideration of the potential effects of IR on *M. edulis* at the molecular level. These results are in agreement with the data of Anderson et al. (1990) in which *N. arenaceodentata* treated with one of four different radiation doses (2, 4, 8, and 16 Gy) induced detrimental reproduction and genetic damage (increase chromosomal aberrations) impacts.

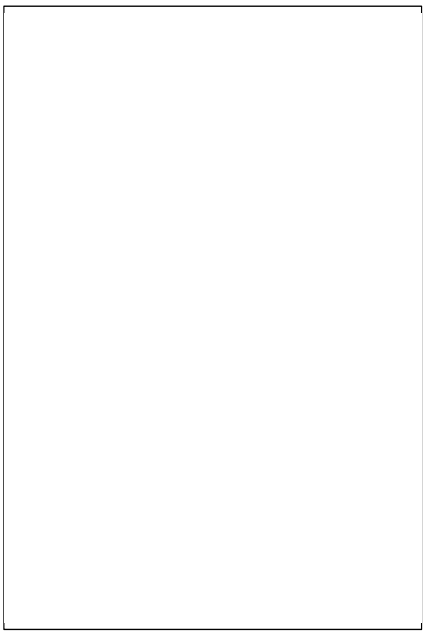

Fig. 7.4.1. Simplified diagram of Rad51 actions and possible DNA repair mechanism in invertebrate.

In the environmental sampling analysis, the radionuclide concentration values (Table 7.3.4.1) included β and γ radionuclides. γ-emitters are believed to be more biologically harmful than β-emitters such as tritium (Jha et al., 2005). However, it has been suggested that the biological influences of β-radiation could be higher in some aquatic invertebrates than mammalian (Straume and Carsten, 1993) due to the fact of high ionization of β-emitters per unit of tissue volume. In both cases, chronic exposure to either γ or β leads to reduction in the reproductive function of marine environment (Knowles and Greenwood, 1997). The biological effects of deposited radionuclides depend on many factors mostly on the activity, biodistribution and removal rates of the radionuclide, which in turn depends on its chemical form. Also, another factor may be the chemical toxicity of the deposited material. The amount of injury caused by a

radioactive isotope depends on its physical half-life, process or time of absorption and excretion by the organism.

In summary, many studies of the harmful effects of radiation have been reported (Templeton et al., 1971; IAEA, 1976; Anderson and Harrison, 1986; Sokolov et al., 1989; Anderson et al., 1990; NCRPM109, 1991c; Abramov et al., 1992; IAEA, 1992; Zainullin et al., 1992; Sokolov et al., 1993; Harrison and Anderson, 1994a,b; Zdzienicka, 1995; Sugg et al., 1996; UNSCEAR, 1996; Neel, 1998; Theodorakis and Shugart, 1998; Sastre et al., 2001; Stoeva et al., 2001; Stoeva, 2002; Aka et al., 2004; Tallarico et al., 2004; Hagger et al., 2005a; Jha et al., 2005; Jo and Kwon, 2006; NRC, 2006; UNSCEAR, 2006; Hameed et al., 2008; Saghirzadeh et al., 2008; Seaver et al., 2009). Here, we have observed cellular and molecular indications of DNA damage in mussels sampled from a site impacted by chronic, yet relatively low level, IR.

Chapter 8

Summary and Conclusion

8.1. SUMMARY

IR pollution is a pressing environmental concern for international and national regulatory authorities, tasked with monitoring the levels of contaminants in the environment as well as the health of organisms living in the environment, and the public. The handling and use of radioactive materials, the design and operation of nuclear power plants are likely to become more of an issue, particularly after the Fukushima accident in Japan that is now considered to be the second largest nuclear accident after the Chernobyl disaster. Aquatic environments are vulnerable to biological impacts by radioactive contaminants, as evidenced by a large number of studies that have confirmed the presence levels of IR in water, sediments and aquatic biota in the aquatic environment (Harrison and Anderson, 1994a,b; Cook et al., 2004; Gulliver et al., 2004; Hagger et al., 2005a; Jha et al., 2005; Arnaud et al., 2006; RIFE12, 2006; Burger et al., 2007; Farcy et al., 2007; Godoy et al., 2008; The´bault et al., 2008; RIFE14, 2008; Grung et al., 2009; RIFE15, 2009).

The aim of this project was to establish whether IR can affect mussels at the molecular level by developing a molecular biomarker in *M. edulis* specific to double strand DNA damage and repair pathways, while also anchoring the new technique to an established sub-cellular analytical technique that detects DNA damage through the use of comet assay. The novel molecular biomarker was initially to be developed using experimentally-exposed mussel samples and then applied to the environment.

Initially, an extraction and PCR methodology was developed to isolate *Rad51* and *Chk1* mRNA sequences from normal *M. edulis* tissues. A qPCR was then developed and

employed using mussel samples that had been exposed to experimental (to elicit a response) and environmentally-relevant doses of IR. A partial fragment of a *Rad51* gene (involved in vertebrates in the DNA repair) and *Chk1* gene were isolated from the marine mussel, and a quantitative assay to measure their expressions was developed. To validate the assay, the response following experimental and environmental exposure to IR was assessed.

A molecular analysis of a gene involved in the pathway (targeted molecular approach) should provide more information about the action of IR within the organism. To do that, a partial DNA fragment of 837 bp of a *Rad51* gene was isolated and characterized using primers designed from several different vertebrate and invertebrate species including *D. polymorpha* Rad51. The deduced amino acid sequence was homologous to more than 80% of the entire mRNA sequence of the *Rad51* gene in vertebrates (Fig. 3.4.1). The fragment isolated from *M. edulis* had between 83%-87% similarity with the corresponding area of *Rad51* sequences in vertebrate and zebra mussel. All amino acid residues shown to be important for the ATP binding domain (Walker A, B motifs) and multimer BRC interface were present in the isolated *Rad51* sequence (Thompson and Schild, 1999; Shin et al., 2003; Wiese et al., 2006).

Another molecular target involved in DNA damage and repair was also analysed, Chk1, which is an essential kinase that plays an important role in cell cycle checkpoints (Liu et al., 2000). In order to investigate Chk1, a partial DNA fragment of 744 bp of a *Chk1* gene was isolated and characterized using primers designed from several different vertebrate species. The deduced amino acid sequence corresponded to approximately two thirds of the entire mRNA sequence of the *Chk1* gene in vertebrates (Fig. 4.4.1). The fragment isolated from *M. edulis* had a range of 44%-57% similarity with the corresponding area of *Chk1* sequences in vertebrate. All amino acid residues shown to

be important for the for ATP binding, Activation loop, Catalytic loop, kinase activity and for the substrate binding are present in the *Chk1* (Krek and Nigg, 1991; Parker and Piwnica-Worms, 1992; Kumagai et al., 1998; Chen et al., 2000).

The partial fragments of the mussel *Rad51* and *Chk1* genes isolated were used to quantify *Rad51* and *Chk1* mRNA expression using a real time PCR technique and DNA-specific dye SYBR Green as a fluorescent reporter. The values obtained from the fluorescence signal were normalized with a housekeeping gene, *18s rRNA*, which is equally expressed in all the samples (Arenz et al., 2007; Banda et al., 2008). The oligonucleotides designed for the amplification of *Rad51* and *Chk1* were highly specific, as confirmed for the presence of a single homogeneous melt peak of each for all the samples and the cloning of the fragments obtained (sections 5.3.4 and 5.3.5). The efficiencies of the amplification, for the housekeeping gene, *Rad51* and *Chk1*, were close to 100% and within 5% with each other (section 5.3.3) and therefore confirmed the suitability of the use of the comparative Ct method for the relative quantification of *Rad51* and *Chk1* mRNA expression.

Several proteins are involved in the DNA damage response and repair pathways particularly HR. H2AX phosphorylation has been applied to many studies due to its important role as a biomarker in response to DSB (Celeste et al., 2003; Kinner et al., 2008; Medvedeva et al., 2007) and recently involving in the DNA repair (Paul et al., 2000; Hanasoge and Ljungman, 2007). Another essential DNA repair protein suggested to involve with H2AX phosphorylation is Mre11-Rad50-Nbs1 complex (MRX) which is a protein complex recognizes DNA damage and rapidly relocates to DSB sites and forms nuclear foci (Paull and Lee, 2005; Yuan and Chen, 2010). Another protein play important role in preventing single stranded DNA from winding back on it self at DSB site is replication protein A (RPA) and its function leads to ease the way for Rad51

repairing DNA (Golub et al., 1998; Mimitou and Symington, 2009; Peng and Lin, 2011). These proteins can act as early warning molecular biomarkers of DNA DSB damage.

M. edulis individuals, collected in September 2010 from Brighton Marina and exposed to a range of experimental dose levels of IR, were screened using the developed assay to assess the levels of expression of the putative *Rad51* and *Chk1* genes. Experimental exposure of *M. edulis* to ^{137}Cs (1, 2, 10 and 50 Gy) resulted in an increase in the levels of *Rad51* transcripts, but only statistically significantly at 50 Gy (sections 6.3.1). In a time dependent manner (using mussel exposure groups analysed on 4 and 7 days following exposure to 2 Gy of ^{137}Cs), the *Rad51* mRNA expression increased significantly, similarly to other studies reported in the medical literature (Yuan et al., 2003; Chinnaiyan et al., 2005; Taghizadeh et al., 2009). For the expression of *Chk1*, a significant increase resulted following exposure to 1, 2 and 10 Gy of IR. While no such studies using samples from the environment on *Chk1* mRNA expression have been reported in the literature as yet, similar results have reported on using Chk1 phosphorylation as an indication of increased activity (Gatei et al., 2003).

For the analysis of mussel tissues collected from environmental sampling sites, radionuclide concentrations were measured in sediment samples and mussel tissues collected from an impacted site at Ravenglass Estuary and a reference site at Brighton Marina (Table 7.3.4.1 and Table 7.3.4.2). These concentrations were compared with previous radionuclide levels reported around the world. In the mussel samples collected from the two environmental sites, comet analysis showed highly significant DNA damage in Ravenglass *M. edulis* haemolymph compared to samples from Brighton Marina (Fig. 7.3.1.2). This finding is in agreement with previous studies where low level IR doses apparently induce DNA damage measured using comet assay (Hagger et

al., 2005a; Jha et al., 2005). At the molecular level, increased *Rad51* mRNA expression was observed in Ravenglass mussel tissue samples compared to Brighton mussels (Fig. 7.3.2.1). Similarly to our results, levels of *Rad51* mRNA and protein have been found to be higher following IR treatment in different species including yeast (Bishay et al., 2001; Watson et al., 2004; Chinnaiyan et al., 2005). These results highlight the impact of IR at the cellular and molecular level in an invertebrate species and suggest that *Rad51* could act as an IR-specific molecular biomarker for inclusion in environmental biomonitoring studies.

Currently, there is disagreement among scientists about whether there is a threshold dose for radiation causing damage to organisms, Cohen (2008) discussed the no-threshold theory and conclude that the risks of low radiation dose may be zero or even negative. Other scientists believe that biological repair systems can fix the biological damage caused by low doses of radiation (Mitchel, 2007; Cuttler and Pollycove, 2009). However, these scientists claim that the low doses of radiation are not harmful. In toxicology, this opinion could be referred to 'Radiation Hormesis', which is a theory of chronic low doses of IR being beneficial stimulating repair mechanisms (Calabrese and Baldwin, 2003; Feinendegen, 2005; Cuttler and Pollycove, 2009). Much of the studies on radiation hormesis relates to plants, fungi, algae, protozoans, insects, and no mammalian vertebrates (Calabrese and Baldwin 2000). It was reported that low dose of radiation might be beneficial and cause stimulatory responses such as accelerate growth rate in young, increase reproductive ability, extend life sapan, and other stimulatory effects on the immune system (UNSCEAR, 1994). Other studies were mentioned in UNSCEAR (1994) reporting that chronic exposure to low doses of radiation followed by a single challenge dose showed reduction in chromatid abberation and sister chromatid exchange compared to a control group that receives only the

challenge dose (Olivieri et al., 1984). These responses have been referred to as the 'adaptive response', meaning that the effective response remains for several hours after exposure (UNSCEAR, 1994; Bonner, 2003).

In summary, relatively low-level IR apparently causes an induction of DNA damage (as measured using the comet assay) and triggers at least one DNA repair mechanism (*Rad51* mRNA expression) in mussels.

8.2. CONCLUSIONS

1. Published evidence in the scientific literature has already confirmed that DNA damage is present in aquatic biota following IR exposure in the environment. Herein, a molecular biomarker Rad51, DNA repair protein, was investigated in *M. edulis* following IR exposure. An 837 bp fragment of a *Rad51* gene was isolated from mussel gonads using RT-PCR and degenerate primers designed. The deduced amino acid sequence is part of the ATP binding domain of Rad51 and shares 80% similarity with Rad51 in vertebrate species. The isolated fragment features the amino acid residues important for the ATP binding activity, further supporting the identity of the fragment as part of the *Rad51* gene.
2. Cell cycle checkpoints are also essential in the DNA damage response pathways. *Chk1* was also investigated in *M. edulis* following IR exposure. A 744 bp fragment of a *Chk1* gene was isolated from mussel gonads using RT-PCR and degenerate primers designed. The deduced amino acid sequence is part of the ATP and substrate binding domain of Chk1 and shares 44% to 57% similarity with Chk1 in vertebrate species. The isolated fragment features the amino acid residues important for the ATP and substrate binding activity, further supporting the identity of the mussel fragment as part of the *Chk1* gene.

3. A real-time PCR based assay was developed to quantify the expression of the novel *Rad51* and *Chk1* genes. It was optimised to provide a high degree of specificity and subsequently used to measure *Rad51* and *Chk1* mRNA levels in mussel samples experimentally-exposed to different levels of IR.
4. The expression of *Rad51* mRNA studied in experimentally exposed mussels increased in IR dose groups (1, 2, 10 and 50 Gy) relative to the control samples. However, there was only a statistically significant increase in *Rad51* mRNA expression at 50 Gy dose compared to control group. In terms of time course, *Rad51* mRNA expression significantly increased after 4 and 7 days following a dose of 2 Gy compared with control samples. For *Chk1*, a significant increase in mRNA levels was detected in mussels exposed to 1, 2 and 10 Gy of IR. There were no significant changes in the levels of *Chk1* mRNA expression between the control and the irradiated (2 Gy) group analysed on 4 and 7 days.
5. In the samples collected from two environment sample sites (IR impacted and reference), the haemolymph from mussels collected from the IR impacted site at Ravenglass Estuary showed statistically significant DNA damage compared to mussels sampled from the reference site at Brighton Marina. At the molecular level, *Rad51* mRNA expression was significantly higher in tissue samples of mussels sampled at the IR-impacted Ravenglass site compared to mussel samples from the reference site at Brighton Marina. In contrast, a reduction on *Chk1* mRNA was observed in mussels collected from Ravenglass compared to mussels from the reference site. The radionuclide analytical data provided by Prof Cundy (Brighton University) confirmed that the sediments and mussel tissues at Ravenglass Estuary were elevated in a number of radionuclide concentrations compared to the reference site.

6. These results present evidence of sub-cellular, molecular level IR impact in the aquatic invertebrate, *M. edulis*. *Rad51* mRNA expression may provide a potential biomarker of IR-inducing DNA-DSBs. In conducting this work, we have also increased our understanding of the DNA damage response and DNA repair mechanisms in an aquatic invertebrate species and this may lead to the discovery of new early warning biomarkers that can be used as tools for biomonitoring of pollution effects in the environment.
7. There are a number of limitations inherent in this investigation. One is that the sample saize is relatively small and that ideally a larger study would be performed to gain more statistical power. Also, ideally, a lower experimental exposure dose, and greater range of dose level, should be employed to determine a dose response relationship.

8.3. FUTURE WORK

Further experiments could be targeted towards:

1. Molecular analysis, and employment of the *Rad51* mRNA expression, of mussels exposed to lower levels of IR. At present there is a hypothesis that very low level IR exposure has no effect, and a concept of a threshold. This assay is very sensitive, as shown by the results of mussels collected from Ravenglass Estuary where radionuclide concentrations are below EU statutory limits, would allow scientists to determine if chronic low level exposure has detrimental effects not previously measured.
2. The sequencing and investigating of proteins involved in DNA repair in mussel. The results obtained would provide a better understanding of the DNA repair mechanism in mussels and pathways involved in DNA damage response. This would recognise and

address the possible issue that not all DNA damage is detrimental since it may be repaired before any long term repercussions occur.

3. Sequence the complete *Chk1* gene in *M. edulis* and other possible proteins to confirm Chk1 role in cell cycle, study expression patterns and enzyme activity following different IR exposure regimes. By analysing the expression of the novel *Chk1* RNA, as well as other proteins involved in cell cycle checkpoints we could be able to clarify if the RNA transcript of the novel *Chk1* is likely to play a functional role in DNA damage response.

4. Sequence biomarkers such as Rad52, replication protein A (RPA) and Rad55/57 genes in *M. edulis* and other possible DNA repair proteins, study expression patterns following different exposure conditions. By analysing the expression of other novel biomarkers, a clear view of DNA repair pathways will be achieved in invertebrate.

5. Apply the same methodology for the study of DNA damage in different invertebrate species, including terrestrial indicator organisms, which are also exposed to sources of IR. The results could enhance our understanding of the DNA repair mechanisms between invertebrate species, hence, gaining insight into the extent to which it is possible to extrapolate between species.

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