Environment Agency

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Radiation experiments on aquatic organisms

Science Report – SC030282/SR

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This report is the result of research commissioned and funded by the Environment Agency's Science Programme.

Published by:
Environment Agency, Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol, BS32 4UD
Tel: 01454 624400 Fax: 01454 624409
www.environment-agency.gov.uk

ISBN: 1844324281

Author(s):
W.J. Reynolds, K.S. Leonard, B.P. Lyons, F. Goodsir P. Smedley, J.T. Barry and J.F. Knowles

Dissemination Status:
Publicly available / released to all regions

Keywords:
Fucus vesiculosus, gamma radiation, germination, growth, image analysis, ionising radiation, macroalgae, *Ulva intestinalis*

Research Contractor:
Centre for Environment Fisheries and Aquaculture Science, Lowestoft laboratory, Lowestoft, Suffolk, NR33 OHT
01502 562244

Environment Agency's Project Manager:
David Copplestone, Head Office

Science Project Number:
SC030282

Product Code:
SCHO0605BJCZ-E-P

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Steve Killeen

Head of Science

Executive summary

Background

The Environment Agency is obliged to review all its activities, to ensure that no adverse effects on natural ecosystems are being sanctioned. To do this, it needs to understand how contaminants can affect species and habitats. One potentially serious group of contaminants are those that emit ionising radiation. The Environment Agency funds research to study the effects of radiation on wildlife. This project looks at the effects of gamma radiation on marine organisms, particularly two common species of seaweed: *Fucus vesiculosus* (bladder wrack) and *Ulva intestinalis* (gutweed). The International Commission on Radiological Protection has included a brown seaweed as one of a list of reference animals and plants that should form the basis of a new radiological protection framework for plants and animals (ICRP, 2003). Seaweeds (marine macroalgae) are important components of marine and coastal ecosystems because they are primary producers, and directly support many other species of plant and animal. Up to now, little has been known about the effects of contaminant radiation on these plants. Rather than assessing mortality, this study looks at radiation effects on reproductive capacity, effects that may not be immediately obvious, but could impact on populations in the long term.

Main objectives

There are four objectives. First to conduct a literature review covering radiation effects and methods of studying them, on seaweeds (marine macroalgae) and other potential species of interest. Second to carry out experiments on the effects of gamma radiation on the reproduction of *F. vesiculosus* and *U. intestinalis*. Third, to carry out a second set of experiments on the effects of gamma radiation on the growth of germlings of the two species. Fourth, to assess the data, report the findings and advise on future research directions.

Findings

Germination, or spore development, is a more sensitive indicator of radiation exposure than growth in these species and was adversely affected by gamma radiation in all experiments. In the extreme case, *U. intestinalis* spore development was reduced by 38% after one week's adult exposure, and continued irradiation of the germling to an accumulated dose of 23.04 mGy, at a dose rate of 0.32 $mGyh^{-1}$ (higher than background dose rates).

In *F. vesiculosus*, a two-hour exposure to high gamma radiation (11.79 $mGyh^{-1}$) of receptacles at the time of fertilisation, followed by continuous exposure, gave better germination than just continuous radiation.

In the growth experiments, adult exposure of *U. intestinalis* to 0.32 $mGyh^{-1}$ and above led to significantly smaller offspring at 3 days old than those without radiation exposure. This was true for *F. vesiculosus* at the highest dose rate of 11.79 $mGyh^{-1}$. Post-spawning exposure of *F. vesiculosus* to 0.32 $mGyh^{-1}$ of gamma radiation conferred a growth advantage, possibly due to an effect known as hormesis.

Conclusions

Gamma radiation at dose rates between 0.32 and 11.8 $mGyh^{-1}$ causes clearly observable effects on germination and growth of *F. vesiculosus* and *U. intestinalis.* The two species of macroalgae respond differently. These dose rates are relatively large compared to those expected under environmental conditions.

U. intestinalis may be suitable for future use as an indicator to assess effects of radiation exposure on the environment, because it is more sensitive to low dose radiation than *F. vesiculosus*, especially in its reproductive life history stages. Further work is required to find out if the observed effects would alter populations in the long term, and to establish whether this species is a suitable indicator for protection of the environment.

Contents

1 Introduction

Under the UK Conservation (Natural Habitats) regulations, 1994 and 2000, the Environment Agency is obliged to review all existing authorisations, permits, consents and licenses to ensure that no authorised activity results in an adverse effect on the integrity of identified natural sites. Methods have been derived for making these assessments (Copplestone *et al*., 2001 2003) but in all cases there is a need to understand the relationship between the exposure and the biological or ecological effect on one or more species.

The Environment Agency is actively engaged in research to address this lack of knowledge. It was involved in the European Union Fifth Framework Programme (EURATOM FP5) project: Framework for ASSessment of Environmental impacT (FASSET), part of which collated all known published data on the effects of radiation on plants and animals. Effects were grouped under four umbrella endpoints - morbidity, mortality, mutation and reproductive capacity - and the project found conspicuous data gaps for certain wildlife groups (Woodhead and Zinger, 2003). Now, the Environment Agency is a partner in a new FP6 project, Environmental Risk from Ionising Contaminants: Assessment and Management (ERICA) (www.erica-project.org). ERICA takes a broad approach, integrating scientific, managerial and societal issues concerning the environmental effects of contaminants emitting ionising radiation, with emphasis on biota and ecosystems. To complement this, the Environment Agency is sponsoring specific experimental work. It commissioned the present study to further investigate the impact of ionising radiation on wildlife, in this case on the group of large algae, or macroalgae, also known as seaweeds. This report describes experiments on two species, *Fucus vesiculosus* and *Ulva intestinalis*, investigating radiation effects on their growth and reproduction.

1.1 Why seaweeds?

As primary producers, seaweeds, or marine macroalgae, are an essential component of marine and estuarine ecosystems. They serve as a habitat for many species of animal and plant, providing both food and shelter. Therefore it is important to assess the potential impacts that radiation may have on their biology.

The impacts of ionising radiation on humans and their associated methods of assessment have been well studied and the International Commission on Radiological Protection (ICRP) has used this information to construct the current radiological protection framework for humans. No-one has yet taken the same systematic approach to produce a similar framework for non-human species. ICRP recently set up a new committee to begin the process and has proposed a series of reference animals and plants (RAPs) to be analogous to the reference human used in human radiological protection (ICRP, 2003). One of the proposed RAPs is a brown seaweed. However, relatively little is known about the biological effects of exposure to ionising radiation on any macroalgae. This project helps to address this data gap.

1.2 Objectives

i. Conduct a literature review covering radiation effects and methods of studying them, on potential species of interest.

ii. Conduct radiation experiments at the CEFAS radiation facility on two species of marine algae, to establish **reproductive** effects of radiation.

iii. Conduct radiation experiments at the CEFAS radiation facility on two species of marine algae, to establish the effect of radiation on **growth** of the germlings (young seaweeds).

iv. Assess the data, report the findings and advise on possible further research directions.

1.3 Approach

The review of current literature regarding the effects of radiation upon algae is presented in Appendix 3. It draws information from the FASSET Radiation Effects Database (FRED) and from the extensive literature resource at CEFAS. The review also covers the use of gastropod molluscs for studying the effects of radiation on aquatic organisms, and recommends some directions for future work in this area. The experimental protocols used in this study were designed using information from this review, along with CEFAS's current knowledge of growth and reproduction in macroalgae, and Environment Agency Technical Report P3-101/SP2 (Wood *et al.*, 2003). Wood *et al.* (2003) provided guidance on the development of experiments that conform to good practice, and produce replicable, robust and scientifically valid data fit for evaluating the effects of radiation on non-human species. CEFAS consulted with its in-house statisticians to ensure that validity criteria were met before starting the experimental work. The protocols are detailed in Appendix 2.

The study focuses on two species: *Fucus vesiculosus* and *Ulva intestinalis* (formerly *Enteromorpha intestinalis*) (Hayden *et al.,* 2003). These species were chosen because of their availability, spawning periodicity, ease of maintenance and the published literature that exists about their responses in experimentation.

2 Methods: Chronic radiation experiments with *Fucus vesiculosus* and *Ulva intestinalis*

The experiments were designed in accordance with Good Practice Guidelines laid out in Technical Report P3/101/SP2 (Wood *et al.* 2003). The design process and the detailed protocols are described in Appendix 2.

2.1 Test species

Fucus vesiculosus (common name bladder wrack) (Phaeophyceae) is a brown algae that is common between the tide marks on rocky shores in the UK (Figure 2.1). It is generally found between the mean low water neap (MLWN) and just above the mean high water neap (MHWN) tide marks (Knight, 1950). The distribution of *F. vesiculosus* is dependent on factors such as degree of shore exposure and latitude, and may vary between locations. *F. vesiculosus* exhibits permanent attachment to the substrate and will grow up to 2 m. The species is characterised by thick, leathery branches that exhibit a heavily corticated, thick wall with paired gas bladders along the length of the stipe (Lobban & Harrison, 1997). The plants are dioecious (having male and female plants) and maturity for both sexes is achieved at 15-20 cm. Plants generally live for 3 years with the greatest biomass being achieved in their third year. *F. vesiculosus* reproduces by bearing receptacles at frond tips that either release eggs or biflagellated sperm from conceptacles (depending on whether it is a female or male plant). Development of the receptacles takes 3 months from initiation until the point of gamete release. The species is particularly fecund and may bear over 1,000 receptacles on one plant. Once released from the receptacle, the egg releases a pheromone into the water that attracts the sperm (Derenbach & Gereck, 1980). Post fertilisation the zygote will settle, attach and then germinate. The species is best described as an episodic spawner with reproductive events occurring between mid-winter and late summer (White, 2004).

Figure 2.1*Fucus vesiculosus*

Figure 2.2*Ulva* spp.

Ulva spp. (now considered synonymous with *Enteromorpha* spp. (Hayden *et al*., 2003)) are rapidly growing, opportunistic green macroalgae found on open and sheltered shores,

from fully marine to estuarine and unpolluted to polluted sites (Budd and Pizzola, 2004; Fletcher, 1989) (Figure 2.2). The species is particularly abundant in the summer when large areas of upper shore may be colonised. *U. intestinalis* (common name gutweed) undergoes an isomorphic alternation between haploid gametophytic and diploid sporophytic generations (Budd and Pizzola, 2004; Fletcher, 1989). The sporangia, of which there are gametangia and zoosporangia, are produced on separate plants. The gametophyte produces biflagellate gametes and the sporophyte produces quadriflagellate zoospores. Both the gametes and zoospores are termed swarmers (Fletcher, 1989). Released zoospores are pear-shaped and can be distinguished from the gametes by their larger size and four, terminal, flagella. In addition, the gametes are strongly positively phototactic whereas the zoospores are weakly negatively phototactic and will swim away from a light source and/or settle relatively rapidly. Settled embryos and zoospores develop into male and female sporophyte and gametophyte plants respectively. Zoospores were selected for use in this study, mainly because they settle rapidly, as opposed to gametes that may be persistent swimmers (Fletcher, 1989).

The scientific literature on these two species, *F. vesiculosus* and *U. intestinalis*, was reviewed to assess their suitability for use in radiation experiments (see literature review, Appendix 3). The species were primarily selected because they are important constituents of the coastal and estuarine flora around the British Isles. Indirectly *U. intestinalis* is of some economic importance, as an industry is founded on removing it, among other species of fouling organisms, from boat hulls. Size, spawning frequency and life stage history is known for both species, as they have previously been used in CEFAS experimental protocols (Reynolds *et al*., submitted). Husbandry requirements for adult and germling stages were based on CEFAS' previous experience and the small amount of published information available.

The literature review highlights the paucity of information regarding the effects of radiation on macroalgae. Radiation effects on microalgae have been studied, but effects on macroalgae have only been investigated in *Porphyra* sp. exposed to gamma radiation (Kim, 1985). In that study, decreased survival and subsequent growth of conchospores were observed above a total dose of 200 Gy.

2.2 Endpoint selection

The endpoint refers to the specific measurement to be taken as the result of an experiment, such as the percentage survival, or growth rate of treated plants. Endpoints were selected from under the umbrella of reproductive capacity. This umbrella endpoint is particularly important since successful reproduction is one of the most important factors in ensuring the long-term survival of a population. Effects of radiation on reproductive capacity and growth may not be immediately obvious in the environment, but will impact on populations in the long term. Table A2.2 shows the endpoints selected, and how and when they were measured.

2.3 Exposure guideline

The target dose rates of gamma radiation were 0.1, 0.4, 1.4 and 8.5 $mGyh^{-1}$. These dose rates were based upon previous experiments at the CEFAS radiation facility (Hingston *et al*., 2003). Higher dose rates were not achievable due to the limitations of the gamma sources available for irradiation. An initial dosimetry experiment was carried out to see what was achievable using the experimental system. The results confirmed that the shielding effect of water altered the radiation dose received by the algae (Figure A1.1).

2.4 Experimental design

2.4.1 Irradiation facility and tank set-up

The gamma radiation facility at CEFAS's Lowestoft laboratory consists of ceiling mounted Caesium-137 sources enclosed within lead shielding. Three sources are mounted in an array above each of three benches (bench size 3.1 x 1.5m). Sources over the benches are 3 x 50 GBq, 3 x 25 GBq and 3 x 12.5 GBq. Dose rates at the bench surfaces are approximately 8 $mGyh^{-1}$, 1.3 mGy h^{-1} and 0.4 $mGyh^{-1}$ respectively. The radiation is collimated to give uniform dose rates over the whole bench surface. The radiation dose received is determined by the proximity of organisms to the source. It can be changed by raising or lowering the surface of the bench, elevating the tanks or well plates containing test organisms, or by using lead filters. A control room was identically equipped to the radiation facility, but without the radiation.

In experiments where adult algae were exposed to radiation, they were housed in 200 litre plastic aquaria directly below the gamma radiation sources (Figure 2.3). Three dose rate groups and a control group were set up. Ambient temperature sea water was supplied to the tanks at approximately $1lmin^{-1}$ from the CEFAS Lowestoft laboratory system. The tank set-up was based on a flow through system with siphons draining the tanks once a day to obtain a period of emersion. Light was provided by cool, white fluorescent strip lights, suspended above the tanks, to give a 16-hour photoperiod of 4,000-6,000 lux at the water surface.

Germlings (immature macroalgae) and embryos were kept in plastic multi-well plates (Figure 2.3). Water for the multi-well plate tests was obtained from the CEFAS Burnham on Crouch laboratory. This water, from the Crouch estuary, is obtained at all stages of tide, spun, settled and filtered through 0.2 µm canister filters to remove detritus and fouling organisms. It was used because of its consistent quality.

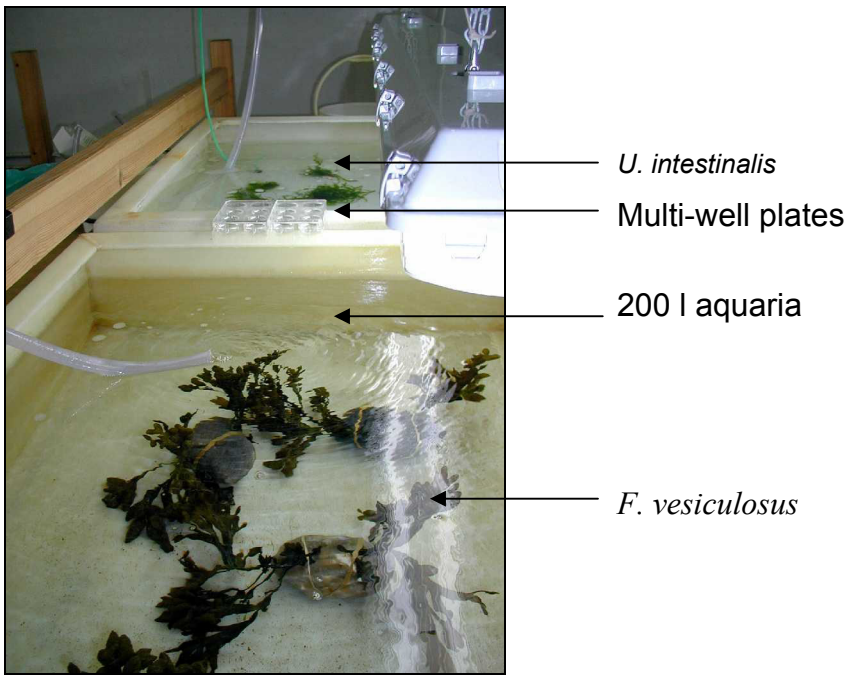

Figure 2.3 Experimental set-up

Tanks containing adult *F. vesiculosus* (foreground) moored on rocks, *U. intestinalis* (background) and well plates containing germlings of both species (between the tanks) in the CEFAS radiation facility.

2.4.1.1 Treatment of organisms

The experiments were designed to compare the effects of different radiation doses with un-irradiated controls and to find out at what dose rate a significant effect occurs.

Table 2.1 Radiation experiments carried out on *F. vesiculosus* (F) and *U. intestinalis* (U)

Expt. No.	Sp.	Length of exposure (days)	Life history stage	Adult stage exposed ?	2 hr high dose exposure ?	Endpoint	Measurement technique
1	F	3, 7, then weekly	Germling	Yes	-	Growth	IA
2	F	3, 7, then weekly	Germling	No	-	Growth	IA
3	U‡	3, 7, then weekly	Germling	Yes	-	Growth	IA
4	U	3, 7, then weekly	Germling	No	-	Growth	IA
5	F	5 days	Embryo	Yes	-	Germination	By eye and IA
6	F	5 days	Embryo	No	-	Germination	By eye and IA
7	F	5 days	Embryo	No	Yes	Germination	By eye and IA
8	U	3 days	Zoospore	Yes	Yes	Growth	By eye and IA
9	U	3 days	Zoospore	Yes	-	Growth	By eye and IA

‡ Measurements were taken weekly until the algae were too large to measure accurately, i.e. overlap of fronds etc.
IA = Image Analysis

The experiments are outlined in Table 2.1. There were four treatments within each experiment – the three different levels of radiation provided by the facility, and one non-irradiated control. All experiments were carried out between July and November 2004. Two series of tests were conducted - germling growth experiments and germination experiments. The growth experiments (experiments 2 to 4 in Table 2.1) tested the effects of radiation on the growth rate of germlings of individuals collected from non-irradiated wild populations. In experiments 1 and 3, adult *F. vesiculosus* and *U. intestinalis* were exposed to the radiation sources and the growth of the progeny of these individuals, at the same radiation dose rates, was measured. In experiments 2 and 4, only the germlings were exposed. Measurements were taken weekly until the algae were too large to measure accurately, due to overlapping fronds.

The germination experiments (experiments 5 to 9 in Table 2.1) tested the effects of radiation on the viability of embryos (*F. vesiculosus*) and zoospores (*U. intestinalis*). In experiments 7 and 8, embryos or zoospores were spawned for a two hour period under a high dose of radiation.

2.4.1.2 Germination experiments

The germination success of *F. vesiculosus* eggs and the initial growth of *U. intestinalis* zoospores were measured. Some previous ecotoxicology data are available for these tests, as detailed in Table A3.2.

F. vesiculosus

The procedure for measuring germination success follows Reynolds *et al*. (submitted). Mature *F. vesiculosus* were obtained from the Crouch Estuary, Essex (OS grid reference TQ 940 960). The swollen receptacles of at least 10 mature algae were collected at low tide and were briefly washed with 0.2 µm filtered sea water (FSW). The receptacles were placed between overlapping sheets of damp blue roll and subject to mild desiccation at 16-18 °C for 24 h in the dark (Gledhill *et al*., 1999) To initiate spawning, the algae were placed into 100 ml of FSW at room temperature (~18°C). This causes non-motile eggs and biflagellated sperm to be released from their respective conceptacles (Thursby & Steele, 1995). The receptacles were left for 2 hours for spawning and fertilisation to occur. After this time, the receptacles and suspending water were gently washed through a 90 µm mesh and then a 20 µm mesh with FSW. The coarser mesh removed any detritus, the finer mesh retained embryos, whilst allowing excess sperm to pass through. The embryos were then re-suspended in 50 ml of FSW and agitated to produce a homogenous suspension. A Sedgewick Rafter cell (Gallenkamp) was used to make estimates of the number of embryos ml^{-1} and to assess the quality of the embryos. For each radiation dose rate, four wells of a multi-well plate, containing FSW, were inoculated with a known volume of embryo suspension using a pipette. The exact number of embryos was counted after inoculation. Well plates were then placed under the radiation sources, and in the control room. After five days, germination success was measured by the presence or absence of a germinal tubule. Germinated embryos are distinguished from unfertilised eggs, or embryos that did not develop, by their smaller and rougher appearance. The number of germinated embryos was counted, and the number of ungerminated eggs was assumed to be the total number of eggs inoculated, minus the number germinated.

U. intestinalis

Fletcher (1989) and Reynolds *et al*. (submitted article) describe a procedure for measuring initial growth of Ulva sp. zoospores that was broadly followed here. Complete plants were collected from the intertidal zone at a site in the Blackwater Estuary, Essex (OS grid reference TL 930 070). The algae were rinsed in 0.1 µm filtered seawater (FSW) and gently wiped with a damp cloth to remove any epibionts and detritus. The algae were placed between overlapping sheets of damp blue roll and subjected to mild desiccation for 24 h at 16-18 °C in a covered chamber in the dark. To initiate spawning, individual algae were placed into 100 ml of 0.1 µm FSW in a 250 ml beaker at room temperature (~18°C), and continuously stirred on a magnetic stirring plate. After two hours, the water in which the algae had been placed was filtered through a 20 µm mesh and the filtrate collected. The filtrate was examined for the presence of zoospores (1ml of the filtrate plus one drop of 4% formalin, observed at 300x magnification). Zoospores were distinguished from gametes by the presence of four terminal flagellae and by their larger pear shape. Spawning gametophytes were thus identified and discarded. Zoospores from all sporophytes were combined in a 1 litre beaker. Zoospores are weakly negatively phototactic and tend to settle to the substrate. The solution was, therefore, mixed with a plunger every five minutes to prevent settlement of zoospores and produce a homogenous suspension. Where the suspension obtained was dark green in colour, the concentration of spores in solution was diluted until faintly green in colour by the addition of FSW. Before transferring the U. intestinalis zoospores to the well plates, a 16 mm diameter coverslip was placed into each well of the plate. The coverslips served as a surface for the embryos to adhere to and grow. Each well was filled with 5ml of FSW,

immediately followed by 50 µl of zoospore suspension. Four replicate wells were assigned to each radiation dose treatment. Well plates were left in the dark for 2 hours while the zoospores settled, then placed under the radiation sources. Germination success was measured after 3 days. For brevity, the term germination will be used to describe the development of the U. intestinalis zoospores, even though it is really a process of development, not germination. Germinated spores were noted as green and either round or oblong (starting to divide into two or more cells), whilst dead cells were white and rounded.

2.4.1.3 Germling growth experiments

F. vesiculosus

Embryos were obtained by the same protocol detailed in section 2.4.2.1. The density of embryos was measured using a Sedgewick Rafter cell and adjusted by the addition of FSW to obtain a final density of approximately 3,000 embryos ml^{-1}. 5 ml of FSW in each test well were inoculated with 20 µl of the homogenised embryo suspension to give a final mean density of approximately 60 *F. vesiculosus* embryos per test well. Embryos were placed into wells in replicates of four per radiation dose. The growth of embryos was measured following the protocol in section 2.4.3.1. Germlings were maintained in water baths except for experiment 2, in which they were maintained on the tank sides. This different position did not affect the physical parameters, although differences in radiation dose rate have been accounted for (see Table A1.1).

U. intestinalis

For U. intestinalis the same procedure as in section 2.4.2.1 was followed for the spawning and test procedure. Measurements of the growing zoospores were made following the protocol in section 2.4.3.2.

2.4.2 Assessment of growth by image analysis

Observations of growth were made using an Olympus IX70 inverted microscope with a motorised x/y stage and Brightfield (Koehler) illumination. Image capture was provided by a Hitachi HV-C20A camera and associated image capture facility (Scope Pro, Media Cybernetics, USA). Algal growth was measured on days 3, 7 and every week thereafter until the algae became too large to measure. Total length of the germlings was measured to the nearest µm using imaging software (Image Pro Plus V.4.1, Media Cybernetics, USA).

Figure 2.4 *F. vesiculosus* germling at 5 days old. 40x magnification.

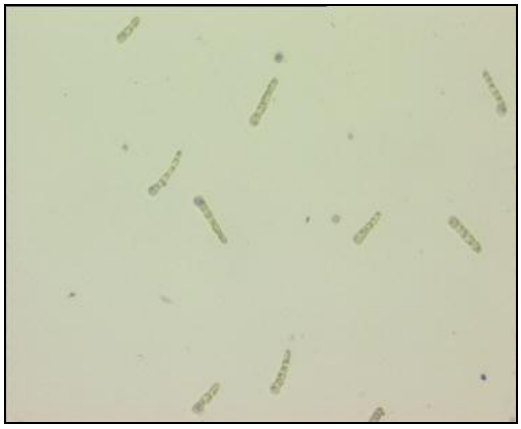

Figure 2.5 *U. intestinalis* germlings at 5 days old. 100x magnification.

2.4.2.1 *F. vesiculosus*

Germlings were viewed at 40x magnification within their multi-well plate wells (Figure 2.4). Shoot length from the first 5 germlings observed was measured to the nearest µm. Five germlings from each of the 4 replicates were measured, giving a total of 20 measurements for each dose rate at each time period. After each measurement, up to 80 % of the seawater was drawn off by pipette and replaced with fresh FSW.

2.4.2.2 *U. intestinalis*

The coverslip was removed from each test well and placed, inverted, onto a drop of FSW on a glass microscope slide. This flattens the vertically growing shoots and aids measurement of their length (Figure 2.5). Germlings were viewed at 100x magnification until they were large enough to measure at 40x magnification. Shoot lengths from the first 5 germlings observed in each replicate were measured to the nearest µm, providing 20 measurements per dose rate at each time period. After each measurement the water was replaced with fresh FSW, in the well and the coverslip placed back into its test well.

2.4.3 Physical parameters

Temperature, light, salinity and pH were measured at regular intervals in all tanks during the experiments. Mean values for these are presented in Tables A1.5 and A1.6.

2.4.4 Dosimetry

Dosimetry was undertaken to set up the system as close to the target dose rates as possible. Radiation dose per unit time was determined using lithium fluoride thermoluminescence. A fixed amount of lithium fluoride was placed in a cryovial (dosimeter), then placed in the tank at a fixed distance from the radiation source. Dosimeters were strategically placed to take into account all possible shielding effects from tanks, fittings and water. A total of 24 thermoluminescent dosimetry (TLD)

measurements were taken, from the surface, mid-water and bottom at 8 different locations in the tank, for each radiation level. Table 2.2 shows that the mean of these values is close to the target radiation dose, in each case. Figure A1.1 shows the spatial differences in radiation dose received. Radiation rates were relatively even across the tanks but quickly attenuated with depth.

Table 2.2. Initial dosimetry to compare target radiation dose rates with measured radiation dose rates under experimental conditions. Mean of 24 TLD measurements for each dose rate, expressed as $mGyh^{-1}$. TLDs were placed in vertical groups of three (surface, mid water and bottom) in 8 locations of the tank.

	Control	Low	Medium	High
Target radiation dose rate	0	0.4	1.4	8.5
Measured radiation dose rate	0	0.30	1.25	7.30

Dosimetry data were then collected for all doses and experimental setups, with TLDs placed in similar positions to the exposed seaweeds within the test container. For adult exposures, TLDs were placed at similar depths to the position of the reproductive tissue. Results are shown in Table A1.1. Where replicate experimental set-ups were used for different experiments further dosimetry was not carried out. This method does not take account of source decay. However, as the half life of caesium-137 is 30.19 years (IAEA TECDOC-619, 1991), radiation dose rates were considered unlikely to change over the course of the study. Total nominal doses received by the algae for each experimental set-up were calculated and are presented in Tables A1.2-A1.4.

2.4.5 Statistical analysis

Statistical analyses were performed to answer a number of questions from the data. For all tests, statistical significance was assigned at $p<0.05$.

2.4.6.1 Germination experiments

Question: Is there a difference between germination rates in control and radiation exposed algae?

The data were first presented as bar charts to identify major changes in germination that may be related to radiation exposure.

Question: At which radiation dose rates can a statistically significant reduction in germination be observed relative to the control values?

The data were binomial (each egg germinates or not), so a standard logistic Generalised Linear Model (McCullagh and Neldet, 1989) was fitted to the data, with radiation dose rate considered as a factor. There are two questions here:

i. Do the four radiation dose rates produced different germination rates?

ii. How does each dose rate compare with the control?

To achieve 1, the deviance in the model was compared with that of the null model, with no factor fitted. This difference was tested against a chi-squared distribution with three degrees of freedom.

To achieve 2, just that part of the data with the control and the relevant treatment (radiation dose rate) was considered. The same test as above was carried out against a chi-squared distribution with one degree of freedom.

2.4.6.2 Growth experiments

Question: At what dose is there a significant dose-related effect on any given day?

All germling lengths in radiation dose rate groups were compared pair-wise with those germlings in the control group: where the mean shoot length was less than that for the control, a one-tailed t-test was performed, based on the mean, logged shoot length.

Question: Are the slopes of growth of the macroalgae significantly different from one another, for the different radiation dose rates?

In this test the control slope of growth was compared against the slope of growth for each radiation dose rate. Growth curves of the form:

log total = a + b(1 – exp(-cday))

were fitted to the growth of the macroalgae at each radiation dose rate.

The growth curve at each radiation dose rate was compared against the control. To make the comparison, two models were fitted. The first model fitted a separate growth equation to the control and the treatment. The Residual Sum of Squares (RSS1) was calculated as the sum of the two Residual Sums of Squares for each equation. The second model fitted a single growth equation to the data for both the treatment and the control. The Residual Sum of Squares (RSS0) was calculated for this single growth equation.

A *p*-value for the comparison was calculated from the statistic:

F = ((RSS0-RSS1)/3) / (RSS1/(nc+nt-3))

where nt is the number of observations of the treatment and nc is the number for the control. Under the null hypothesis of no difference between treatment and control, F has an F-distribution on 3 and (nc+nt-3) degrees of freedom. Significance is reported at the $p<0.05$ level.

Question: At what radiation dose rate was a reduction in length seen relative to the control?

This involves calculating an 'EC_{10}' value: an estimated radiation dose rate at which growth is reduced by 10% relative to the control. EC_{10} values were calculated for day 14 length measurements only, for two reasons. First, it was observed that by day 14 all individuals were actively growing and some way past the initial development stage after fertilisation (*F. vesiculosus*) and settlement (both species). Secondly, any reduction in growth due to such factors as lack of nutrients and competition would become more significant later on in the experiments.

Fitting models to the growth plots is difficult because there are not enough dose rate groups for a good fit to the data. This was a limitation of the facility since more radiation dose rate groups could not be accommodated. Simple linear regression models were therefore fitted to three of the experiments and a quadratic model to the fourth (due to the nature of the data). The EC_{10} is defined as the level at which the length at dose rate 0 is reduced by 10%.

Confidence intervals for the EC_{10} estimates were found by using one hundred Monte-Carlo simulations from the fitted model assuming a normal error structure and a variance estimated from the original data and fitted model.

3 Results and observations

3.1 Germination experiments

3.1.1 *F. vesiculosus* germination and development of zoospores in *U. intestinalis*

Figure 3.1 shows that percentage germination of *F. vesiculosus* and percentage development of *U. intestinalis* zoospores were both negatively affected by increased radiation dose rate. *F. vesiculosus* germination success was least affected by the radiation when the embryos were spawned under a 2 hour period of high dose rate radiation (total dose of 23.58 mGy). Germination success in this species was most affected by the radiation without any pre-exposure of the adults. *U. intestinalis* appeared to have a greater reduction in germination success, with adult exposure, at low radiation dose rates (Experiment 9) than *F. vesiculosus* (Experiment 5). A 2 hour high dose rate of radiation (with adult pre-exposure)(Experiment 8) gave markedly reduced germination success compared with adult pre-exposure alone.

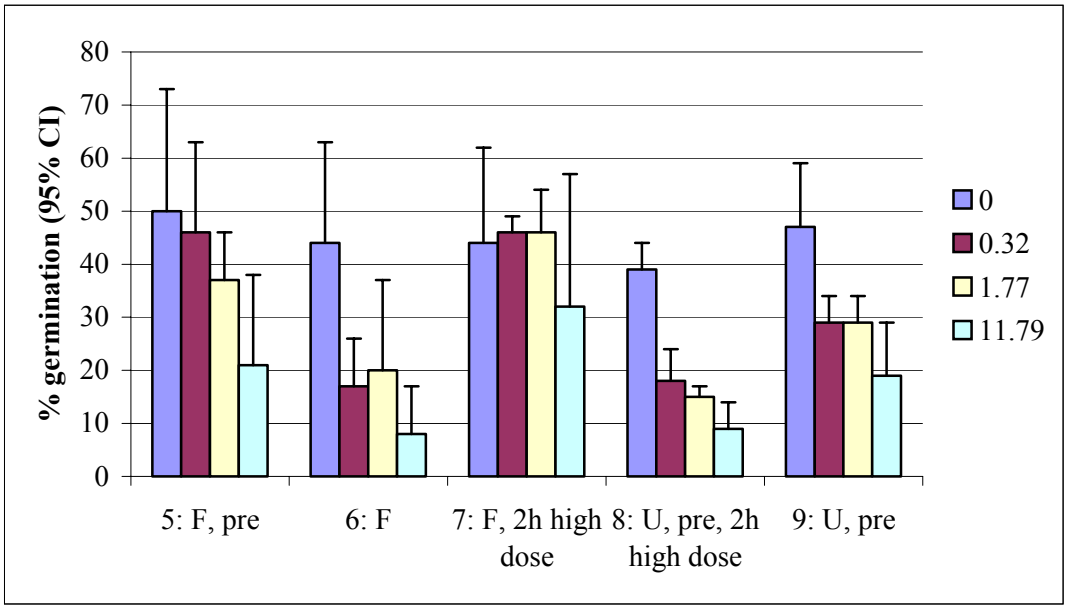

Figure 3.1 Percentage germination of *F. vesiculosus* and percentage development of *U. intestinalis* zoospores at different rates of radiation exposure Radiation dose given in $mGyh^{-1}$, as measured by TLD. Headings on x axis denote experiment number, species (F = *F. vesiculosus*, U = *U. intestinalis*) and whether there was pre-exposure of the adults. See Table 2.1 for details of experiments.

3.1.2 *F. vesiculosus* germination and development of zoospores in *U. intestinalis* – statistical comparisons with the control

P-values for the statistical tests of the effect of radiation dose rate on germination of *F. vesiculosus* and percentage development of *U. intestinalis* are shown in Table 3.1. Overall, the effect of radiation exposure on germination success was highly significant in

both species. However, *F. vesiculosus* did not show a statistically significant difference at the lowest radiation dose rates after pre-exposure of adults (experiment 5), nor at the low and middle radiation dose rates for embryos that received a 2 h pre-exposure (during spawning) of high radiation dose (experiment 7).

Table 3.1 *P*-values from statistical comparisons of control groups with radiation dose rate groups for each germination experiment. See Table 2.1 for experiment details. See Table A1.4 for details of total radiation dose received. Significance at $p<0.05$ is indicated.

	Experiment number				
	5	6	7	8	9
Overall	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-3}$
Low dose	0.49	$< 10^{-3}$	0.14	$< 10^{-3}$	$< 10^{-3}$
Medium dose	$< 10^{-3}$	$< 10^{-3}$	0.26	$< 10^{-3}$	$< 10^{-3}$
High dose	$< 10^{-3}$	$< 10^{-3}$	0.001	$< 10^{-3}$	$< 10^{-3}$

3.2 Growth experiments

In general, the results of these experiments are presented in tabular form here, and as graphs in Appendix 1.

3.2.1 *F. vesiculosus* growth experiments

3.2.1.1 *F. vesiculosus* zinc sulphate positive control

Previous ecotoxicological experiments on *F. vesiculosus* at the CEFAS laboratory have used zinc sulphate as a positive control. Zinc sulphate positive controls were therefore run alongside the growth experiments on *F. vesiculosus*, as a quality control procedure, to bring these experiments in line with previous work. The results are shown in Table 3.2. A dose-dependent decrease in growth for the four highest zinc concentrations was observed (Figure 3.2), while growth values for the lowest four concentrations are above the control values. For concentrations >0.32 mgl^{-1} growth was significantly different to the control on all but one occasion (Table 3.2). An EC_{50} of 8.18 mgl^{-1} was calculated for day 14 of growth (omitting values where an increase in growth was observed due to a nutrient hormesis effect). This is slightly above the 14 day EC_{50} of 1.52 mg l^{-1} (95% confidence interval 1.05-5.73) calculated by Reynolds *et al*. (paper submitted). The difference is likely to be explained by the higher temperature in experiments in the present study relative to those of Reynolds *et al*. (paper submitted).

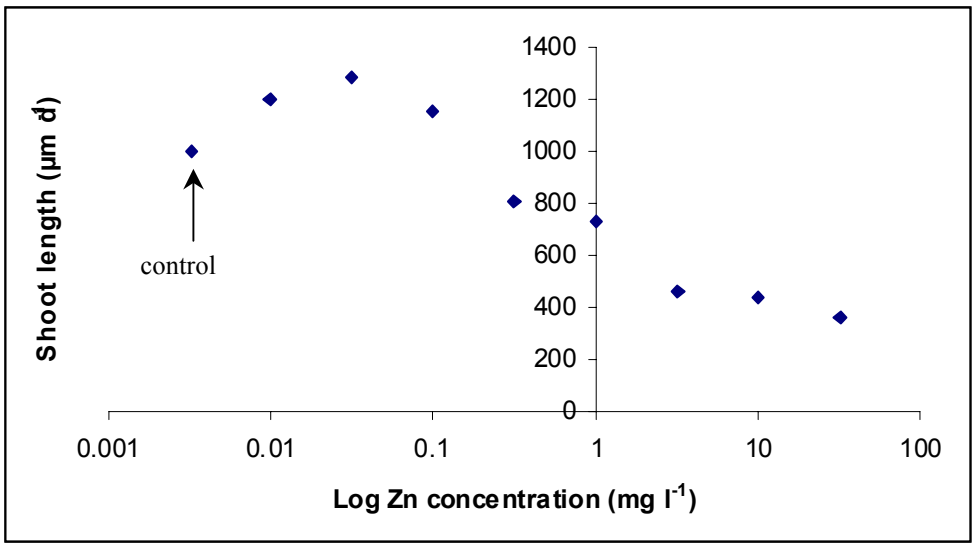

Figure 3.2 Mean shoot length of *F. vesiculosus* germlings against log zinc concentration on day 14. Points represent the means of 20 individuals measured. Control (0) concentration has been graphed as 1/3 of the lowest concentration due to the logarithmic scale.

Table 3.2 Mean shoot lengths (µm) of *F. vesiculosus* germlings exposed to zinc Where shoot length was significantly different from the control, *p*-values are presented in brackets.

Concentration zinc mgl^{-1}	Day 3	Day 7	Day 14	Day 21	Day 28
0	237.6	515.9	1002.0	1282.8	1557.5
0.01	308.2	699.3	1197.8	1283.0	1630.8
0.032	293.4	628.7	1285.6	1823.7	-
0.1	275.6	607.5	1156.9	1645.3	1752.1
0.32	273.8	520.7	806.9**	1201.2	2037.4
1	211.1	521.2	731.8***	973.7*	987.2***
3.2	200.3*	419.0**	465.4***	493.7**	515.7***
10	214.6	381.3***	434.7***	424.8*	420.1***
32	190.1**	315.3***	380.8***	353.9*	325.3***

3.2.1.2 *F. vesiculosus* germling growth at different radiation exposures (experiment 2)

No shoot lengths in any of the radiation dose rate groups were found to be significantly less than the controls (Table 3.3). These results are illustrated in Figure A1.2. It shows how the low dose rates of radiation confer a growth advantage i.e. a longer shoot length.

Table 3.3 Mean shoot lengths (µm) of *F. vesiculosus* germlings exposed to radiation for 28 days, dose rate was measured using TLDs. For this test germlings were maintained on the tank sides (Table A1.1) None of the tests showed significant differences.

Radiation dose rate $mGyh^{-1}$	Day 3	Day 7	Day 14	Day 21	Day 28
0	237.6	515.9	1002.0	1282.8	1557.5
0.32	297.8	649.0	1245.7	2028.5	2693.9
1.53	298.9	771.8	1368.4	2062.8	2096.7
9.23	310.3	747.6	1277.2	1603.1	1894.3
65.33	291.5	617.9	1051.2	1511.1	1460.3

Comparison of the slopes of the graphs with the controls showed that the growth of all radiation dose rate groups was significantly different from the controls (Table 3.4). However, consulting the graphed data shows that growth in exposed groups was significantly greater than in the controls.

Table 3.4 Comparison of the growth curves of *F. vesiculosus* germlings exposed to radiation with control

Radiation dose rate $mGyh^{-1}$	0.32	1.53	9.23	65.33
p-value	<0.001	0.002	<0.001	<0.001

3.2.1.3 *F. vesiculosus* germling growth at different radiation exposures with adult radiation pre-exposure for 1 week (experiment 1, see Table 2.1)

Only the highest radiation dose rate group was found to have significantly shorter shoot length than the controls on day 3 (Table 3.5). Although the highest dose rate group had the greatest number of significant differences from controls for shoot length there is no clear relationship between radiation dose rate and shoot length (see Figure A1.3). Relative growth of germlings appears to fall after day 35.

Table 3.5 Mean shoot length (µm) of *F. vesiculosus* germlings exposed to radiation for 50 days after adult radiation pre-exposure for 1 week. Where shoot length was significantly different from the control, *p*-values are presented.

Radiation dose rate, $mGyh^{-1}$	Day 3	Day 7	Day 14	Day 21	Day 29	Day 35	Day 43	Day 50
0	324.7	393.1	1587.4	3007.4	2480.7	2969.8	2810.1	2158.6
0.32	249.8	381.2	1384.5	1805.1**	2762.1	2440.5*	2085.7	2164.9
1.77	238.3	321.0	1376.0	3213.9	2700.0	2934.5	1919.6*	1264.7**
11.79	169.5*	282.6	825.8**	1812.9**	2702.5	2548.2	2752.3	1706.8*

Comparison of the slopes of the graphs with the controls showed that the growth rates of all radiation dose rate groups were significantly different from the controls (Table 3.6).

Table 3.6. Comparison of the growth curves of *F. vesiculosus* germlings exposed to radiation with pre-exposure of the adults for 1 week, with control

Radiation dose rate $mGyh^{-1}$	0.32	1.77	11.79
p-value	0.014	<0.001	<0.001

3.2.2 *U. intestinalis* growth experiments

3.2.2.1 *U. intestinalis* germling growth at different radiation exposures (experiment 4)

The data show that *U. intestinalis* germlings were significantly shorter than controls by day 35 at the middle radiation dose rate (1.77 $mGyh^{-1}$) and by day 7 at the highest radiation dose rate (Table 3.7). However, values were not significantly different from control in the highest dose rate for day 14 and 28. There were no significant differences from the control at the lowest radiation dose rate. By day 42 the mean shoot length of germlings in the highest exposure group was less than half that of the controls (see Figure A1.4).

Table 3.7 Mean shoot lengths (µm) of *U. intestinalis* germlings exposed to radiation for 42 days. Where shoot length was significantly different from the control, *p*-values are presented.

Radiation dose rate $mGyh^{-1}$	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Control	171.0	263.6	343.8	293.1	482.3	884.4
0.32	136.6	282.5	282.7	378.7	392.8	677.9
1.77	142.7	225.6	288.3	278.3	327.2**	444.6***
11.79	101.7***	222.5	235.7**	251.8	373.1*	427.4***

Comparison of the slopes of the graphs with the control showed that the growth of the lowest (0.32 $mGyh^{-1}$) radiation dose rate group was not significantly different from the controls (Table 3.8). However, growth of the other two radiation dose rate groups was significantly slower than the controls.

Table 3.8. Comparison of the growth curves of *U. intestinalis* germlings exposed to radiation for 42 days, with control.

Radiation dose rate, $mGyh^{-1}$	0.32	1.77	11.79
p-value	0.58	<0.001	<0.001

3.2.2.2 *U. intestinalis* germling growth at different radiation exposures with adult radiation pre-exposure for 1 week (experiment 3)

The highest radiation dose rate gave the most significant differences against control values on the days measured (Table 3.9). Interestingly, all those that received a pre-exposure had shoot lengths approximately a third of the size of those in the controls at day 3. However, all pre-exposure day 3 germlings were much the same length,

irrespective of radiation dose rate. By day 50 mean shoot length in the highest dose rate group was almost half that of the control group (1082.5 vs. 1850.5µm). Measurement of pre-exposure germlings gave lengths over four times those of germlings without the adult pre-exposure on day 35 comparisons (see Figures A1.4 and A1.5).

Table 3.9. Mean shoot lengths (µm) of *U. intestinalis* germlings exposed to radiation for 50 days after adult radiation pre-exposure for 1 week. Where shoot length was significantly different from the control, *p*-values are presented.

Radiation dose rate $mGyh^{-1}$	Day 3	Day 7	Day 14	Day 21	Day 29	Day 35	Day 43	Day 50
0	164.4	No data	631.4	964.6	1114.6	2186.0	2470.6	1850.5
0.32	53.0***	216.3	489.7	704.1	1273.1	2555.9	2200.5	1810.6
1.77	62.0***	188.6	708.2	528.3**	1245.5	2523.7	1603.3*	1793.9*
11.79	57.4***	106.4	408.0*	567.9**	667.8***	1120.7	1327.9***	1082.5***

Comparison of the slopes of the graphs with the controls showed that the growth of all radiation dose rate groups were significantly different from the control (Table 3.10).

Table 3.10 Comparison of the growth curves of *U. intestinalis* germlings exposed to radiation with pre-exposure of the adults, with control.

Radiation dose rate $mGyh^{-1}$	0.32	1.77	11.79
p-value	<0.001	0.002	<0.001

3.2.3 Calculation of EC_{10} values for day 14 length measurement

For experiments 1, 3 and 4 (see Table 2.1) EC_{10} values were calculated by simple linear regression (Table 3.11). However, for experiment 2 (*F. vesiculosus*, no pre-exposure) EC_{10} values could not be calculated due to the apparent lack of reduction in growth (a quadratic model had to be fitted to the data). The data show that the lowest EC_{10} estimate (0.203 $mGyh^{-1}$), where growth is most adversely affected by radiation, was for *F. vesiculosus* with pre-exposure of the adults.

Table 3.11 EC_{10} estimates and confidence limits for growth of *F. vesiculosus* (F) and *U. intestinalis* (U) germlings exposed to gamma radiation. EC_{10} values (the estimated dose rate at which growth is reduced by 10%) are based on growth at day 14, and given in $mGyh^{-1}$

Expt.	Sp.	Adult stage exposed?	EC10	Lower CL	Upper CL
1	F	Yes	0.203	0.096	0.672
2	F	No	-	-	-
3	U	Yes	1.254	-	-
4	U	No	3.960	-	-

3.3 Contaminant analysis of algal collection sites

To see whether the macroalgae had been exposed to other non-radioactive contaminants, a water sample was collected from each location where the adults or seawater were collected for use in the experiments (Blackwater estuary, Crouch estuary and Lowestoft laboratory seawater). Analyses for a range of contaminants were performed by the Environment Agency's National Laboratory Service. The results are provided in Table A1.7.

The sampled waters show very low levels of polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs), with nearly all results below the limit of detection. Heavy metal results are consistently low, although the Blackwater Estuary showed higher levels of As, Cr, Cu, Pb, Ni and Zn compared with the other water samples. All results are within the normal range of concentrations observed from the aquatic monitoring programmes (e.g. Marine Pollution Monitoring Management Group, 1998).

4 Discussion

4.1 Germination of *F. vesiculosus* and development of *U. intestinalis* zoospores with radiation exposure

All five germination experiments showed that germination was adversely affected by radiation exposure. *F. vesiculosus* was very sensitive to radiation exposure when the embryos alone were exposed. When the adult macroalgae were pre-exposed to a week of radiation before spawning, the germination of their progeny showed *F. vesiculosus* was less sensitive to radiation and statistically less sensitive than *U. intestinalis* zoospores. It is possible that the conceptacles of *F. vesiculosus*, within which gametes develop, provide some shielding to the effects of the radiation. *U. intestinalis* zoospores may be more sensitive to radiation because they lack such physical protection.

The results of experiment 7 (*F. vesiculosus* germination test with two hour high dose exposure to radiation during spawning) were markedly different from other results. At the medium level of radiation (total dose 212.4 mGy), germination was not significantly different from the controls. It appears that a short high dose exposure, at the point of spawning, confers some kind of protection to subsequent germination during radiation exposure. The result of experiment 5 (*F. vesiculosus* germination with adult pre-exposure), where levels of germination in the control and the low dose radiation group were not significantly different may be another example of this phenomenon.

A two hour high radiation dose administered to *U. intestinalis* at the point of spawning led to markedly reduced germination success, compared to those that did not have the high radiation. It is possible that this high dose of radiation causes irreparable damage to cellular mechanisms in this species.

Germination was measured in *U. intestinalis* after 3 days and in *F. vesiculosus* after five days. Therefore, the *F. vesiculosus* embryos received a substantially greater total dose of radiation than the *U. intestinalis* zoospores (see Table A1.4). This suggests that overall germination in *U. intestinalis* may be a more sensitive indicator of gamma radiation exposure than germination in *F. vesiculosus*.

4.2 Growth of *F. vesiculosus* and *U. intestinalis* germlings with radiation exposure

F. vesiculosus

Growth of *F. vesiculosus* germlings without adult pre-exposure to radiation was not significantly less than the controls on any given day (experiment 2). However, when the slopes of growth were compared, significant differences were observed. With low radiation exposure (0.32 $mGyh^{-1}$), growth was greater than the controls. After 28 days exposure, the germlings given 0.32 $mGyh^{-1}$ were 58% longer. Mean lengths of germlings were only shorter than controls in the highest (65.33 $mGyh^{-1}$) radiation dose rate group. This apparent increase in growth with an increase in gamma radiation may be an example of hormesis (Luckey, 1980). Hormesis means the stimulating effect of a small dose of a substance that at larger doses is toxic. Various mechanisms for radiation hormesis have been postulated. In plants, the induction of various growth factors has been suggested (Upton, 2001). Sagan (1991) describes two proposed mechanisms for hormesis. One is

a cellular response to potential injury (production of antioxidants and efficiency of DNA repair mechanisms); the other is 'cell-replacement repair' or 'altruistic cell suicide', leading to an increase in the production of new cells (Sagan, 1991).

In contrast, growth in *F. vesiculosus* germlings spawned from pre-exposed adults was not increased at the lowest radiation dose rate (or in any of the other dose rate groups). Radiation reduced the length of resultant germlings measured on day 3, although the difference was only statistically significant for the high (11.79 $mGyh^{-1}$) dose rate, where germlings were over 50% shorter than controls. Why pre-exposure of adults and therefore gametes to radiation removes the growth advantage from low radiation, leading to poorer growth in the resultant progeny, is not known. It is also paradoxical, given that pre-exposure of adults led to improved germination success relative to non-pre-exposed adults. It seems that the life history stage at which radiation exposure occurs is important.

U. intestinalis

Unlike *F. vesiculosus*, *U. intestinalis* germlings did not show any increased growth following radiation exposure. At the low dose rate (0.32 $mGyh^{-1}$), no significant differences were found between the length of control group germlings and those treated with radiation. However, germlings in the dose rate groups of 1.77 $mGyh^{-1}$ and above showed significantly less growth when compared to the controls.

When adult *U. intestinalis* were pre-exposed to radiation, the subsequent growth of the germlings was at first significantly reduced by radiation in all dose rate groups. At day 3, the mean length of germlings given the low (0.32 $mGyh^{-1}$) dose rate group was 32% of the size of controls. This suggests that pre-exposure of the adults to radiation at dose rates of 0.32 $mGyh^{-1}$ and above gives rise to zoospores that cannot initially develop at the normal rate. At the end of the 50 day experiment, germlings from the two higher radiation exposed groups were still significantly different from the controls, but those in the low group showed similar results. Comparison of adult pre-exposed groups and those without pre-exposure shows that measurements on day 35 are very different. Shoot lengths on day 35 are at least four times greater in those groups that received pre-exposure. This suggests that, although exposure of the adults initially gives rise to smaller individuals, they are ultimately faster growing than their non-pre-exposed counterparts. However, this difference is not seen when the data are normalised to the controls to compare relative lengths (data in Figure 3.1 shows data not normalised to controls). The apparent differences in shoot length between the experiments may instead be due to the time of year, since the two experiments were carried out a couple of months apart.

4.3 EC_{10} values for growth

The lowest EC_{10} values were derived for *F. vesiculosus* germlings after adult pre-exposure. This indicates that this species has greater sensitivity to radiation after adult pre-exposure than *U. intestinalis*. Without the adult pre-exposure, *U. intestinalis* was much more sensitive to radiation than *F. vesiculosus*, since the latter species showed an increase in growth with increased radiation dose. The EC values should be interpreted with caution due to the low number of dose rates groups that were measured and the lack of scoping studies to determine at approximately which dose rates of radiation EC_{10} values would be found.

4.4 General discussion

The results in the germination experiments indicate that pre-exposure of *F. vesiculosus* adults resulted in greater germination success of eggs (than other exposure regimes)

compared with adults that were not exposed. Yet, pre-exposure of adults in the growth experiments was found to result in insignificant differences with the controls and the non pre-exposed adults. The question then arises: how can a pre-exposure of the adults give increased germination success y*et al*so have the apparent effect of poorer growth? Firstly, life history stage may be an important factor. Secondly, it was noted that the adult pre-exposed germlings that were measured after 3 days were, in general, smaller than the controls. It appears that this may have been as a result of the radiation since the same was noted in *U. intestinalis*. It is possible that changes to future growth are made at this stage, which was then carried forward throughout the experimental period.

Comparing the size of *U. intestinalis* germlings between adult pre-exposure and non pre-exposure experiments on day 35 shows a marked difference in size. On first observation it might appear pre-exposure to radiation confers a growth advantage i.e. pre-exposure germlings grow more vigorously than non pre-exposed. However, this apparent difference is not seen when the data are normalised to the controls to compare relative lengths (data in figure 3.1 shows this data non-normalised to controls). These apparent differences in shoot length between the experiments may be due to spawning time of year since the two experiments were carried out a couple of months apart.

The dose rates used in this study are higher than those likely to be observed in the environment. Estimated absorbed dose rates to marine organisms have been calculated by Brown *et al.* (2004). The total absorbed dose rate to marine macroalgae was estimated at 0.04 μGyh^{-1} with a range of 0.04-0.27 μGyh^{-1}. These values are well below the lowest dose rates used in this study. Here the mean low dose rate of adults in experimental tanks was 0.30 $mGyh^{-1}$, 1000 times greater than even the highest rate in the estimated environmental range. However, Brown *et al.* (2004) did not consider the length of emersion time (time out of the water at low tide) that macroalgae may experience. In their calculation, exposure of marine macroalgae to radiation is assumed to be through either sediment or water. Yet *F. vesiculosus* is found at mid-tide level and may spend around 50% of its life out of the water. This study has demonstrated that the shielding effect of water dramatically reduces the received dose of gamma radiation (see Figure A1.1). Consideration of this important factor could significantly increase the dose rate received by the macroalgae. Also, the data presented by Brown *et al*. (2004) are mainly from alpha emitters whereas this study was concerned with the effects of gamma radiation.

Figures A1.2-A1.5 show growth of the germlings of both species. These have been presented up to day 35. Beyond this day it was found that relative growth appeared to drop (best illustrated in Table 3.9). This is most likely to be due to a lack of nutrients for the growing germlings. Even though the water was changed each time measurements were made, it is thought that the germlings reach a size when they require more nutrients than can be provided from the water within the well plate. Although not presented in this report, a scoping study using nutrient medias within the well plates, was carried out. They were found to give such accelerated growth of the germlings of both species that measurements using image analysis were not possible, due to overlapping fronds. In addition, the use of nutrient media gave rise to large colonies of bacteria that could have interfered with the results. Future experiments, without nutrient media, would be best restricted to these first 35 days of development.

4.5 Conclusions

- Germination of macroalgal germlings is adversely affected by gamma radiation. *U. intestinalis* germination was reduced by 38% after a total dose of 23.04 mGy, at a dose rate of 0.32 $mGyh^{-1}$.

- A short (two hour) high dose of radiation (11.79 $mGyh^{-1}$) to *F. vesiculosus* receptacles at the point of fertilization, followed by radiation exposure gave better germination than just radiation exposure post-fertilisation.
- Germination and other reproductive endpoints are a more sensitive indicator of radiation exposure than growth.
- Continual radiation to *F. vesiculosus* post-spawning appears to confer a growth advantage at dose rates around 0.32 $mGyh^{-1}$.
- Adult exposure of *U. intestinalis* to gamma radiation (>0.32 $mGyh^{-1}$) gives rise to significantly smaller germlings at 3 days old than were found in the controls. This is also true for *F. vesiculosus* at higher dose rates (11.79 $mGyh^{-1}$).

5 Recommendations

- *Ulva intestinalis* is recommended as a species suitable for further research into the effects of radiation exposure. The species is a sensitive marker of low dose radiation exposure, particularly when considering the reproductive life history stages.
- Future research should focus on whether observed endpoints are significant at the population level. Although it has been demonstrated that *U. intestinalis* is sensitive to gamma radiation exposure, it remains to be seen whether the effects of radiation exposure in the laboratory are relevant as indicators of potential population effects in the field.
- Further experiments should include transgenerational studies.
- Further research is required to demonstrate the applicability of the techniques developed in this project in the field. For example are there influences of radiation in the environment that give rise to localised tolerance in the species? Tolerance to other contaminants has been demonstrated in other studies of *Ulva* sp. (Goodman *et al*., 1976).

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List of abbreviations

CEFAS: Centre for Environment Fisheries and Aquaculture Science

EC_x: Environmental concentration at which x % of individuals were affected

FSW: Filtered sea water

FRED: FASSET Radiation Effects Database

FASSET: European Union Fifth Framework Programme (FP5) project (Framework for ASSessment of Environmental impacT)

GPG: Good practice guides in development of experimental protocol (e.g. Wood *et al.*, 2003)

Gy: Unit of Gray – see Glossary

LD_{50}: Dose that kills 50% of individuals

R&D: Research and development

RAP: Recommended animals and plants

TLD: Thermoluminescent dosimeter

Glossary

Apical hairs: The elongation of cells at the apical tips.

Carpospores: Derived from fruiting spores.

Conceptacle: A flask shaped cavity in a thallus, opening to the outside by a small pore, and containing reproductive structures.

Conchospores: Produced by the cochocelis in gametophytic plants of *Porphyra*.

Dioecious: Having the sexes in separate individuals.

Diploid: Possessing two sets of chromosomes, one set from each parent.

Fucoid: Of the genera *Fucus***.**

Gametangia: Any cell or organ in which gametes are formed.

Gametophyte: The haploid generation in plants that exhibit alternation of generations.

Germling: A young algal plant**.**

Gray: Quantity of energy imparted by ionising radiation to a unit mass of biological tissue. This is the SI unit of absorbed dose: 1 Gy = 100 rad**.**

Haploid: Possessing half the number of chromosomes in the somatic tissue, or one set (characteristic of germ cells).

Hormesis: The stimulating effect of a small dose of a substance that at larger doses is toxic.

Isomorphic: Morphologically similar.

Karyological: Associated with the cell nucleus.

Receptacle: Structure on which reproductive organs are borne; in *Fucus* this is the swollen tip of the thallus.

Rhizoid: Outgrowth from an alga, attaching to or growing into the substrate and providing anchorage and, possibly, absorption.

Sporangia: A hollowed, walled, structure in which spores are produced.

Sporophyte: The diploid generation of plants showing alternation of generations, which produces haploid spores by meiosis that develop to give the gametophyte.

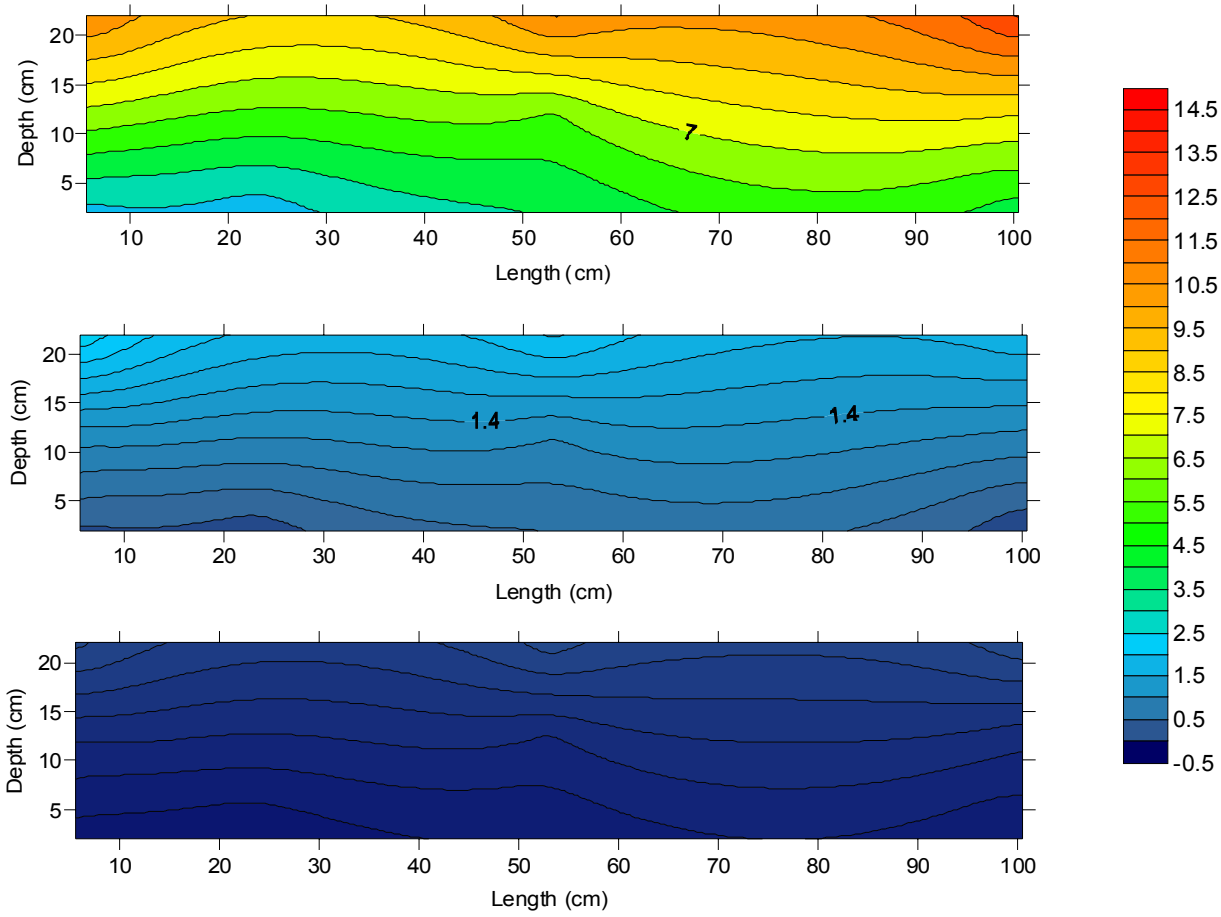
Stipe: A stalk.

Zoospore: A motile asexual (i.e. not a gamete) reproductive cell, swimming by means of one to several flagella.

I

Appendix A1: Charts and Figures

Figure A1.1. Dosimetry charts
Charts show tank profiles at high, medium and low doses. The bottom of the tank is represented by 0 depth on the y-axis. Radiation profiles have been developed using nine



thermo luminescent dosimeters placed at intervals in the xy plane. Values are in $mGyh^{-1}$.

Table A1.1. Dosimetry data for experimental setups, measured by thermo-luminescent dosimeters (TLDs). Means and standard deviations are given in $mGyh^{-1}$. n = the number of TLDs analysed per treatment group.

Experimental setup	n	Control	Low	Medium	High	V. high
Well plates on tank sides	4	0	0.32 ±0.02	1.53 ±0.09	9.23 ±0.58	65.33 ±4.30
Well plates in water baths	11	0	0.32 ±0.04	1.77 ±0.25	11.79 ±2.47	-
Adult exposures	24	0	0.30 ±0.12	1.25 ±0.65	7.30 ±4.99	-

Tables A1.2-A1.4 Total radiation dose received by macrophytes over the duration of experiments, based upon TLD measurements.

Mean cumulative doses are presented in mGy. Dose rates are those given in Table A1.1.

Table A1.2 Experiment 1 (see Table 2.1 for details of experiments).

F. vesiculosus with adult pre-exposure for 1 week. Germlings in multiwell plates on the

	Control	Low	Medium	High	V. High
Adult pre- exposure	0	50.4	210.0	1226.40	-
Day 3	0	23.04	110.16	664.56	4703.76
Day 7	0	53.76	257.04	1550.64	10,975.44
Day 14	0	107.52	514.08	3101.28	21,950.88
Day 21	0	161.28	771.12	4651.92	32,926.32

Table A1.3 Experiments 2-4

F. vesiculosus and *U. intestinalis* germling exposure. Germlings, in well plates, in water baths within tanks to give a constant temperature regime.

	Control	Low	Medium	High
Adult pre-exposure	0	50.4	210.0	1226.40
Day 3	0	23.04	127.44	848.88
Day 7	0	53.76	297.36	1980.72
Day 14	0	107.52	594.72	3961.44
Day 21	0	161.28	892.08	5942.16

Table A1.4 Experiments 5-9.

F. vesiculosus embryo and *U. intestinalis* zoospore exposures. Embryos, in well plates, in water baths within tanks to give a constant temperature regime.

	Control	Low	Medium	High
Adult pre-exposure	0	50.4	210.0	1226.40
Day 3 *U. intestinalis*	0	23.04	127.44	848.88
Day 5 *F. vesiculosus*	0	38.4	212.4	1414.8

Table A1.5. Mean physical parameters for growth experiments of both *F. vesiculosus* and *U. intestinalis*

Mean Temperature °C				
Air	In flow	Lux	Salinity ppt	pH
23.20	Ambient	3594	30.9	7.3

Table A1.6. Mean physical parameters for germination experiments for both *F. vesiculosus* and *U. intestinalis*

Mean Temperature °C				
Air	In flow	Lux	Salinity ppt	pH
20.15	Ambient	2172	31.7	6.5

Table A1.7 Results of non-radioactive contaminant analysis of seawater

All results shown in μgl^{-}

Determinand	Sampling site Blackwater Estuary	Crouch Estuary	Lowestoft Laboratory
Heavy metals			
Arsenic	16.1	1.94	2.08
Boron	3670	3730	3260
Cadmium	<0.10	<0.10	<0.10
Chromium	31.4	0.976	0.66
Copper	16.1	8.04	13.1
Lead	28.8	2.46	0.97
Nickel	24.1	3.46	2.19
Selenium	<1.00	<1.00	<1.00
Zinc	70.5	10.3	4.57
PCBs			
PCB congener 028	<0.01	<0.015	<0.005
PCB congener 052	<0.01	<0.015	<0.005
PCB congener 101	<0.01	<0.015	<0.005
PCB congener 118	<0.01	<0.015	<0.005
PCB congener 138	<0.01	<0.015	<0.005
PCB congener 153	<0.01	<0.015	<0.005
PCB congener 180	<0.01	<0.015	<0.005
ICES7	<0.07	<0.105	<0.035
PAHs			
Acenapthene	<0.02	<0.02	<0.02
Acenapthylene	<0.02	<0.02	<0.02
Anthracene	<0.02	<0.02	<0.02
Benz[a]-anthracene	<0.02	<0.02	<0.02
Benzo-[a]-pyrene	<0.02	<0.02	<0.02
Benzo-[b]-fluoranthene	<0.02	<0.02	<0.02
Benzo-[e]-pyrene	<0.02	<0.02	<0.02
Benzo-[ghi]-perylene	0.04	<0.02	0.02
Benzo-[k]-fluoranthene	<0.02	<0.02	<0.02
Chrysene	<0.02	<0.02	<0.02
Dibenz-[a,h]-anthracene	<0.02	<0.02	<0.02
Fluoranthene	<0.02	<0.02	<0.02
Fluorene	<0.02	<0.02	<0.02
Indeno-[1,2,3-cd]-pyrene	<0.02	<0.02	<0.02
Perylene	<0.02	<0.02	<0.02
Phenanthrene	<0.02	<0.02	<0.02
Pyrene	<0.02	<0.02	<0.02
Naphthalene	0.20	<0.20	<0.20

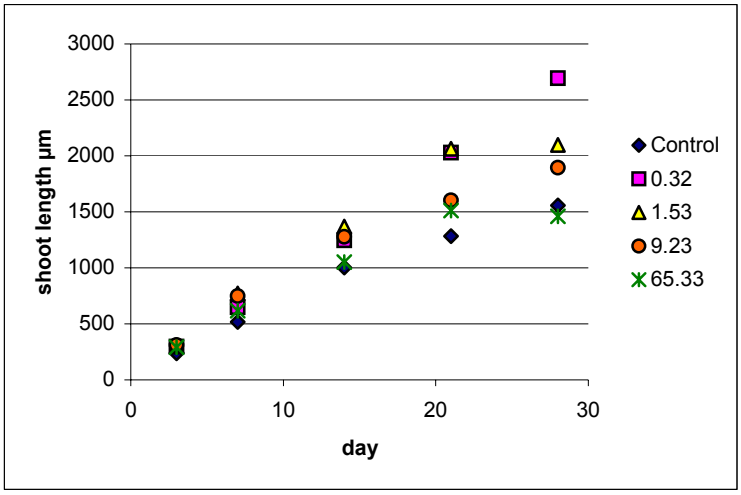


Figure A1.2. Mean shoot lengths of *F. vesiculosus* germlings exposed to gamma radiation. Radiation dose rates presented in $mGyh^{-1}$.

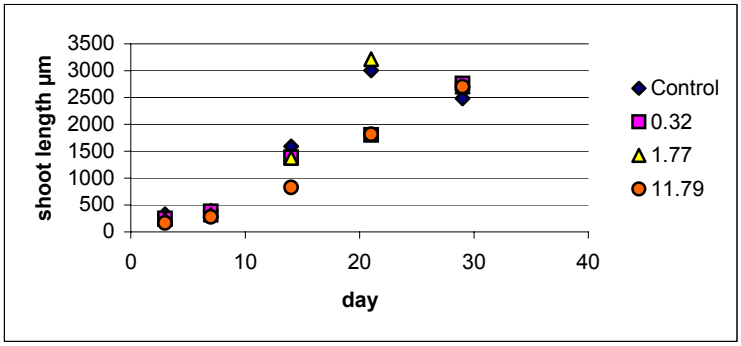


Figure A1.3 Mean shoot lengths of *F. vesiculosus* germlings exposed to gamma radiation after adult gamma radiation pre-exposure for 1 week. Radiation dose rates presented in $mGyh^{-1}$.

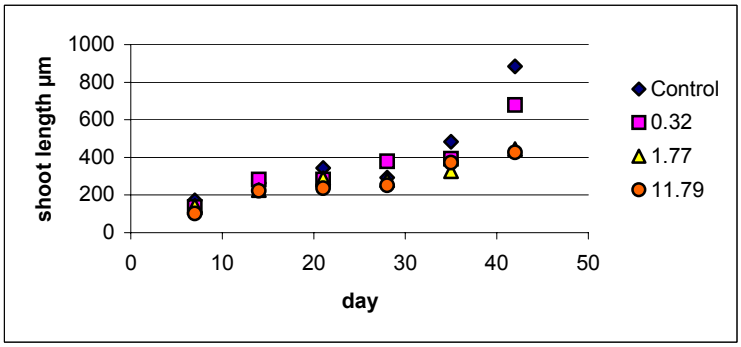


Figure A1.4 Mean shoot lengths of *U. intestinalis* germlings exposed to gamma radiation. Radiation dose rates presented in $mGyh^{-1}$.

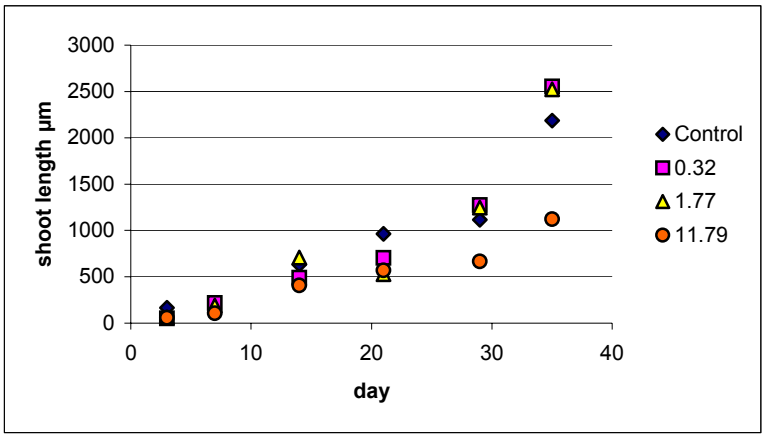


Figure A1.5 Mean shoot lengths of *U. intestinalis* germlings exposed to gamma radiation after adult gamma radiation pre-exposure for 1 week. Radiation dose rates presented in $mGyh^{-1}$.

Appendix A2: Experimental protocols

The process of developing protocols was based on R&D Technical Report P3/101/TR-SP2 (Wood *et al*., 2003) with reference to the FASSET radiation effects database (FRED), current literature and the Burnham CEFAS statistician involved with the project.

The experimental protocols detailed below are divided into the four Good Practice Guides (GPGs) described by Wood *et al.* (2003). Each targets a particular component of the overall protocol. Table A2.1 summarises these four key components of the present study, in the form of a pro-forma that records decisions and provides their justification. This pro-forma forms the basis of the experimental design.

Test species selection (GPG 1)

This section deals with the test species selected for testing seaweeds (macroalgae). Macroalgae are categorised as aquatic plants in the Wood *et al.* (2003) guidelines. The species suggested in the initial tender - *Fucus vesiculosus* and *Ulva intestinalis* were reviewed (see literature review).

Endpoint selection (GPG 2)

Endpoints were selected, including those detailed in the original Environment Agency specification (Table A2.2). These were endpoints that come under the umbrella endpoint of reproduction.

Exposure guideline (GPG 3)

The radiation dose rates were selected as detailed in the original Environment Agency specification for the project. Initial dosimetry was undertaken to confirm what was achievable by the experimental system to be used (Table A2.3). The results confirmed that the shielding effect of water would impact upon the dose rates received by the algae (Figure A1.1). In the case of *F. vesiculosus*, the fronds that contain the reproductive stages are generally those that float and so would receive a dose rate equivalent to that at upper and mid-water. The fronds of *U. intestinalis* that produce zoospores are, in general, at the tips. Since these maintain an upright position in the water they will receive a dose rate equivalent to that at mid-water. Mean values of radiation dose rates at the different depths within the tanks were considered to be appropriate for use in describing the exposure regimes.

Experimental design (GPG 4)

The project focuses on deriving dose rate-effect relationships i.e. comparing effects of treatments (radiation dose rates) with the unirradiated controls to discover at what dose rate a significant effect occurs.

Two separate series of tests were conducted. The first involved the exposure of adult *F. vesiculosus* and *U. intestinalis* to the radiation sources at a number of different doses and the measurement of growth of the resultant progeny. The second set of tests investigated the effects of radiation on the germlings of individuals collected from wild populations (Table 2.1). These experiments were not dependent on one another and were conducted separately. However, the same set-up, including lighting rigs and experimental radiation facility was used for both sets of experiments.

The experimental facility was limited in the amount of space that could be afforded to the radiation exposure of macroalgae in large tanks. Since the two species were maintained separately, it was only possible to irradiate two tanks of each species, per dose rate.

From a statistical viewpoint, it would have been useful to have previous data, to give estimates of standard deviations from which power and sample size calculations could have been made. Some information on growth rates of the species used was available from CEFAS (based on similar experiments). However, the experiments that were proposed were new and little data were available. The current experiments will be useful in the design of future studies.

Figures A2.1 and A2.2 show, diagrammatically, how the growth and reproduction tests on the progeny were conducted. These methods were based upon CEFAS standard operating procedures used for toxicity tests on the two species.

The endpoints (GPG 2) were reviewed to ensure that robust data for analysis could be obtained. The statistical procedures that were applied to the data are detailed in the statistics section of the main project report (section 2.4.6).

Table A2.1 Completed pro-forma, based on Wood *et al.* (2003)

Main Experiment					
Key Instruction			**Page**	**Section**	**Table**
a	Umbrella endpoint of interest	Reproduction	53	5.1.2	5.2
b	Wildlife group and species (*Fucus* selected as in original specification)	Aquatic plants *Fucus vesiculosus* *Ulva intestinalis*	27	4.8	4.5a
c	Maintenance conditions	8-10 individuals per tank. Check daily. Temperature at ambient. 200 l tanks with flow through, aerated, water system (>1 $lmin^{-1}$). Lighting: PAR 400-700nm (6,500-10,000 lux) at daylight regime.	27	4.8	4.5b,c,d,e
d	Specific endpoints	(see Table A2.2, below)	53	5.2	5.1+5.2
e	Irradiation type	Gamma radiation source: ^{137}Cs 3 dose rates of gamma radiation and a control. Initial dosimetry shown in Table A2.3.	59	6.2	none
f	Facilities required	CEFAS laboratory, Lowestoft radiation facility	68	6.7.1	none
g	Dose rates to use	See Table A2.3. These have been specified in the original specification and project tender.	64	6.5.3	none
h	Need for a pilot experiment	No. Pilot experiments are suggested by document P3-101/SP2. However, bioassay of compounds using the species *Fucus* and *Ulva* are regularly carried out at the CEFAS Burnham laboratory. Such studies are similar in design to this	67	6.6	none

		radiation study. Other issues e.g. experimental design have been discussed with the CEFAS statistician.			
i	Duration of irradiation Duration of experiment	24h day^{-1}. Adult experiments were to be 2-4 months exposure (depending on spawning) with >28 days exposure of progeny – Subsequently this was not possible due to husbandry difficulties e.g. lighting, therefore exposure of adults was reduced to 1 week. Progeny experiments to be >28 days exposure	58	6	None
j	No. of dose rates, including control No. of individuals per dose rate	4 8-10 individuals per species per tank. 2 tanks per dose containing separate species. Some differences between species due to reproductive strategies. See statistical requirements below	69 70	7.1 7.1.2	none
k	Statistical requirements				
	Tier 1 Tier 2 Tier 3	Graphical representation of data - visual inspection of data Summarise data using descriptive statistics. This will be applied to endpoints e.g. percentage germination. Statistical analysis. Outputs from Tier 1 and 2 combined for appropriate analysis (see section 2.4.6 of main report for full statistical analysis).			
l	Further literature search conducted	YES			
Further justification of decisions made to complete the pro-forma		None needed			

Table A2.2 Specific endpoints section of pro-forma from Wood *et al.* (2003)

Reproduction		
Specific endpoint	Description of technique	Reference
Viability of progeny	Zoospores (*U. intestinalis*) and embryos (*F. vesiculosus*) placed in well plates and their viability noted after 3 and 5 days respectively	Fletcher, 1989; Scanlan & Wilkinson, 1987; Reynolds *et al.* (submitted)
Growth of progeny	Growth of macroalgae (above) measured by image analysis on days 0 (initial spore/egg diameter), 7, 14, 21, 28, 42, until fronds obscured view.	Reynolds *et al.* (submitted)

Table A2.3 Initial dosimetry of tanks in radiation experiments

Mean dose rates taken from 5 dosimeters per tank positioned at the water surface and mid water column. Exposure set up consists of two 200L flow through tanks per exposure level. Values are expressed in $mGyh^{-1}$.

	Low	Medium	High
Water surface	0.41	1.99	11.60
Mid water	0.22	1.11	6.43

Table A2.4 Checklist for reporting data for radiation experiments on *F. vesiculosus* and *U. intestinalis*

	Tick Box
Authors	✓
Article title	✓
Reference details	✓
Keywords	✓
Type of study (laboratory, field, controlled field)	✓
Radiation type (alpha, beta, gamma or mixed)	✓
Exposure type (internal, external, mixed)	✓
Ecosystem	✓
Wildlife group	✓
Species name (latin and common)	✓
Source of organisms (supplier)	✓
Life-stage of organisms	✓
Maintenance of organisms prior to and during the experiments	✓
Umbrella endpoint(s)	✓
Specific endpoint(s) being studied	✓
Frequency and timing of specific endpoint measurements	✓
Dose rate(s)	✓
Notes on how the dose or dose rate was calculated	✓
Activity concentrations for internal exposures	NA
Dose(s)	✓
No. of individuals per treatment group (including control)	✓
Duration of exposure(s)	✓
Result(s)	✓
Statistical analysis	✓
Any relevant notes	None
Production of a data sheet to record results	✓

Figure A2.1 Flow chart of radiation experiments measuring the endpoint of germination using *Fucus vesiculosus* and *Ulva intestinalis.*

Key:

Blue arrows = Exposure group
Yellow arrows = Exposure group with adult pre-exposure
Orange arrows = Exposure group with high dose at spawning

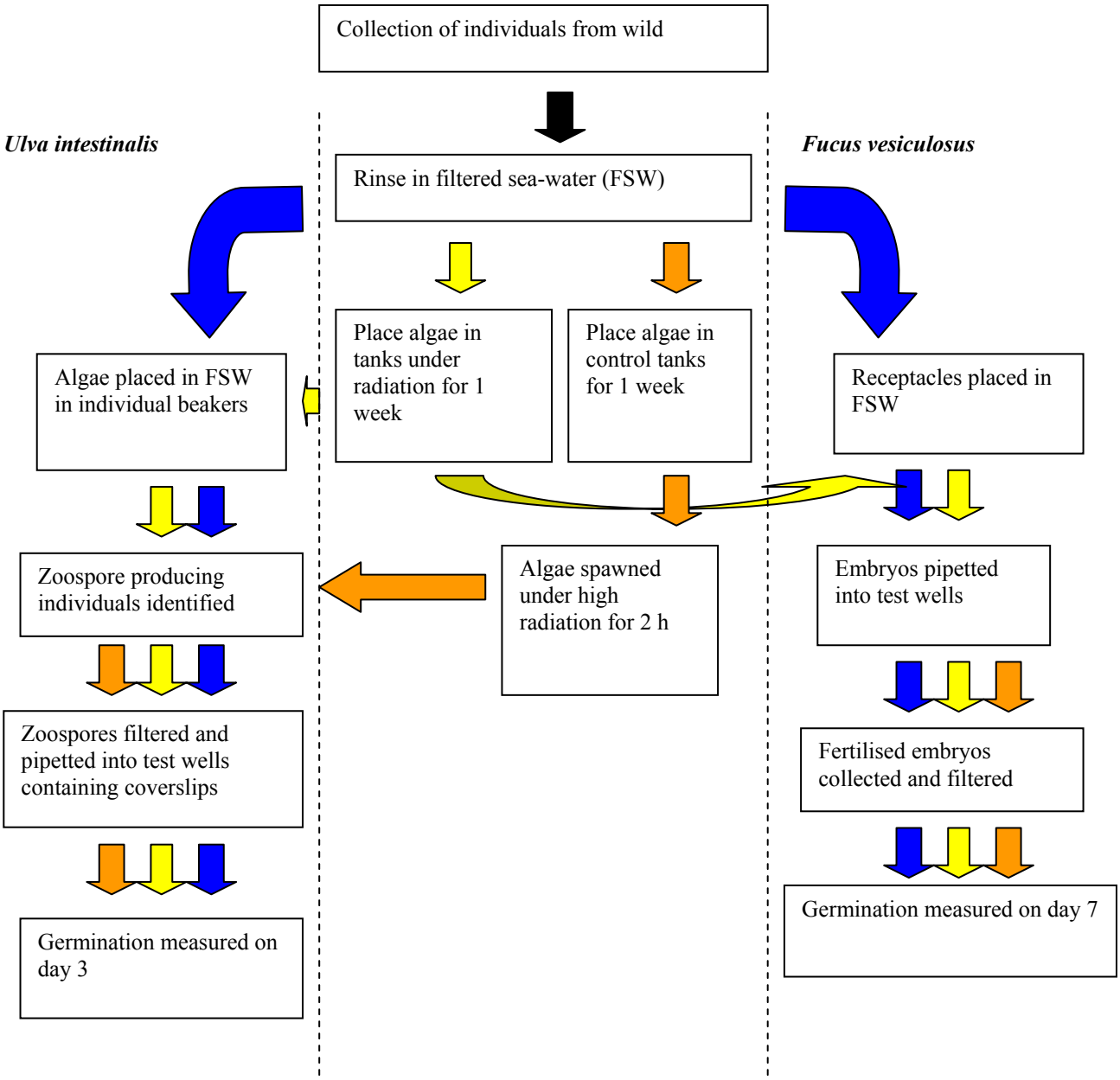
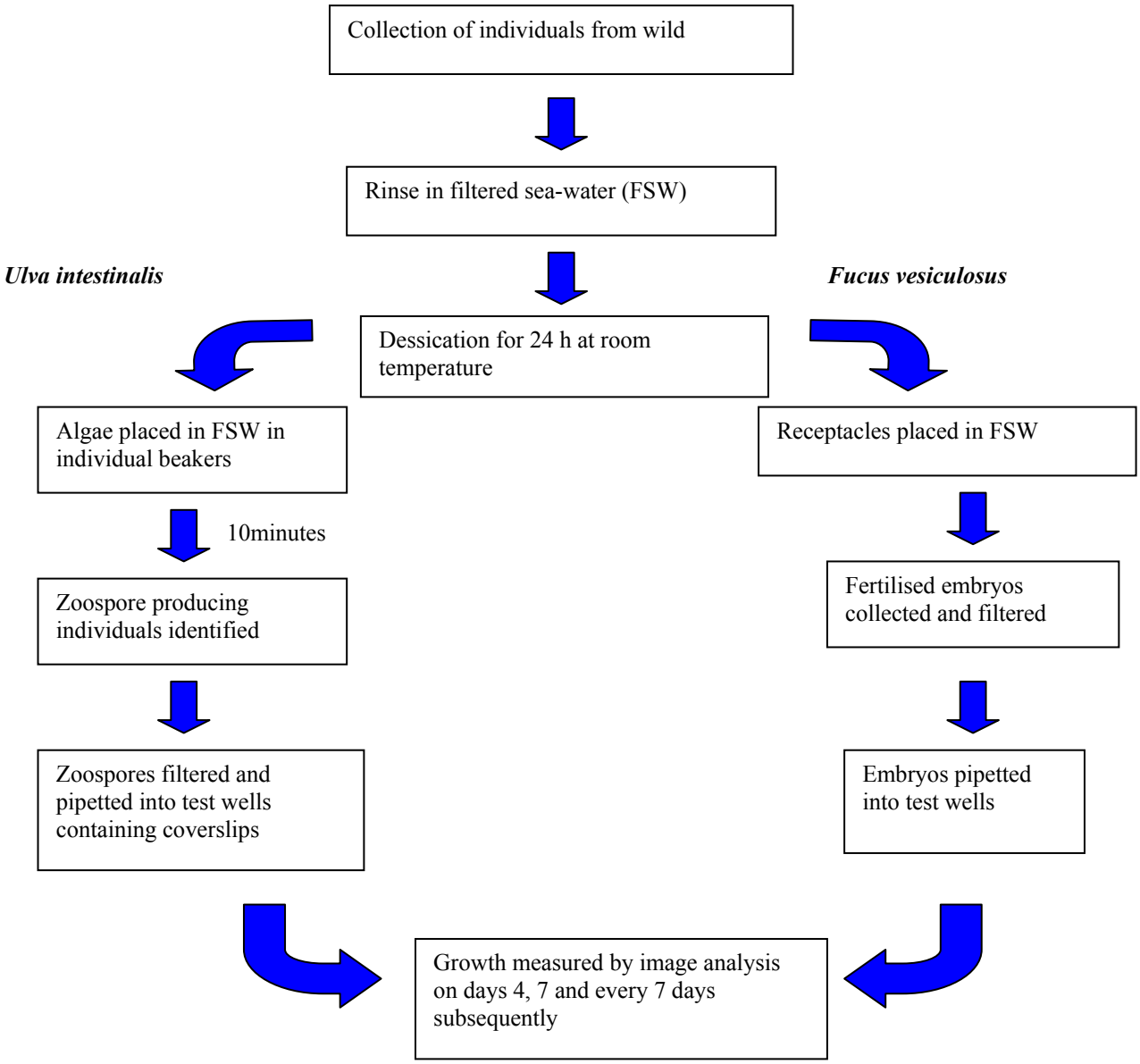


Figure A2.2 Flow chart of radiation experiments measuring the endpoint of growth using *Fucus vesiculosus* and *Ulva intestinalis*



Appendix A3: Literature review of radiation experiments on aquatic organisms

Introduction and Background

The purpose of this literature review is to inform the design of the current study (project SC030282). It will also be useful to those carrying out further radiological research on chronic exposures of macroalgae or developing experimental procedures using gastropod molluscs. It includes information and references gathered from the FASSET Radiation Effects Database (FRED), current literature, CEFAS sources and personal communications.

The impacts of ionising radiation on humans and their methods of assessment have been well studied. This has not been the case for other species. The International Commission on Radiation Protection (ICRP) has previously considered that protection of the environment should be seen in human terms, rather than in terms of the environment itself (Environment Agency, 2003; ICRP, 2003). The Environment Agency commissioned this study of marine macroalgae as part of its research and development into impacts of radiation on non-human species.

This review draws attention to two species of macroalgae: *Fucus vesiculosus* and *Ulva intestinalis*. These species have been extensively studied at the CEFAS laboratories for use in toxicity testing (contracts A1035, AE0255, Reynolds *et al.*, submitted). Other species were considered for radiological testing; however, the two detailed above were selected because their husbandry requirements are understood and it is known to be possible to obtain data on biologically significant endpoints that are relevant in an ecotoxicological context (see Table A3.1). They are relevant species for environmental studies. Both are common to many coasts and estuaries of the UK. *U. intestinalis* is also a widely studied organism due to its importance as a fouling species of boat hulls.

Methodologies and data on the use of *F. vesiculosus* and *U. intestinalis* in ecotoxicological studies are already available (Reynolds *et al*., submitted), demonstrating reproductive and growth endpoints. Reproductive endpoints are significant in the recruitment of individuals to the population. Information regarding their use therefore helps environmental managers wishing to undertake informed radiological risk assessments involving marine macroalgae. This literature review also includes information on the potential use of gastropods in chronic radiation studies, focusing on reproductive endpoints.

The review is divided into four sections: 1) previous radiation research using algae; 2) the use of macroalgae in toxicity testing; 3) species, habit and methodology for radiation experiments on *F. vesiculosus* and *U. intestinalis* and 4) chronic radiation studies using gastropod molluscs.

For ease of comparison, units of radiation absorbed dose (rad) and exposure (roentgen) have been converted to the SI unit Grays (Gy). In compiling the review the following definitions and conversions have been used:

- Roentgen (R) - Unit of gamma radiation for which the resulting ionisation liberates 2.58-4 CKg-1 in air. For the purposes of this review 1 R is comparable to 1 rad.
- Rad (rad) - Former unit of absorbed radiation dose, equal to 0.01 Jkg-1 of the absorbing medium.

- Gray (Gy)- Quantity of energy imparted by ionising radiation to a unit mass of biological tissue. This is the SI unit of absorbed dose. 1 Gy = 100 rad
-
- . 1) Previous radiation research using macroalgae as test species

Table A3.1 presents a summary of research articles where algae have been used in radiation tests. Very little information has been published on the use of marine macroalgae in radiological testing. Research articles concerning the effects of ionising radiation on aquatic plants are mainly confined to freshwater species. Wood *et al.* (2003) states: 'The number of laboratory studies in which chronic radiation exposure of aquatic plants (i.e. including algae and cyanobacteria) has been considered is very limited for doses or dose rates relevant for natural ecosystems.'

The only articles cited in the report by Wood *et al.* (2003) concern the species *Synechococcus lividus* (cyanobacteria) exposed to chronic low dose gamma irradiation (Conter *et al*., 1983; 1984; 1986). Conter *et al.* (1984; 1986) found that chronic low-level doses of gamma radiation could stimulate the proliferation of *S. lividus* due to the stimulation of certain metabolic pathways. Furthermore, Agrawal (1986), studying the green algae *Stigeoclonium pascheri*, found that acute exposure of gamma radiation at 50 Gy increased the percentage germination of spores. At 50 Gy germination of 84% was observed compared to 73% at 25 Gy and controls of 58%. At 100 Gy germination was reduced to 5%. The author notes that the decline in percentage germination may be due to gamma induced injury to the DNA of cells, but no mechanism for increased germination at lower gamma levels is described. Vedajanani and Sarma (1979) list the nuclear (karyological) effects in *Spirogyra azygospora* for exposure to gamma radiation >200 Gy. These effects range from slight enlargement of nuclei and nucleoli to deformed nuclei and nucleoli. Cytological studies have shown cellular changes in chronic radiation studies. Gamma irradiation (30 Gy and 150 Gy) of the freshwater, green filamentous, algae *Sirogonium sticticum* and *S. melanosporum* caused an increase in the number of giant cells produced, at the highest exposure (Wells & Hoshaw, 1980). Filaments of *S. melanosporum* died after receiving 150 Gy for three months, while *S. sticticum* survived for longer than six months at this exposure.

Using the marine seaweed *Porphyra* sp., Kim (1985) studied the effects of gamma irradiation on the development of conchospores. Total doses of 50, 100, 150 and 200 Gy were applied at a dose rate of 780 $mGymin^{-1}$. After culturing for 5 days, doses of gamma irradiation less than 200 Gy did not affect germination of conchospores. But after 5 days of the highest exposure (200 Gy) germlings withered and the growth of giant cells was induced. Giant cells were observed as abnormal growth of the holdfast, seen as lumpish thalli and callus tissue. The two species of *Porphyra* that were studied (*Porphyra tenera* (Keun-cham-gim) and *Porphyra saga* No. 5) showed different responses to radiation at equivalent doses. For instance, survival rate at 200 Gy was 70% for Keun-cham-gim but only 47% for Saga No. 5 at the same dose. Other responses at the highest doses of radiation included a reduction in growth rate and abnormal branching of the holdfast, lumpish and uncertain form of fronds and abnormally multiplied callus-like tissue. The author concludes that growth is inhibited at higher than 50 Gy and that germlings wither at doses above150 Gy. However, regardless of radiation dose, those germlings that formed and grew liberated neutral spores in a normal way, and all the surviving mature fronds generated carpospores.

Some plants such as brown algae living in seawater can concentrate elements from it. *Fucus vesiculosus* is a species that has been monitored, in the western Irish Sea, since 1988 for Technetium-99 (Smith *et al*., 2001). Monitoring the ^{99}Tc activity concentrations has shown a peak in 1997, approximately two years after the peak in ^{99}Tc discharges from Sellafield. However, this article does not report on the radiation dose or any effects of the ionising radiation upon algae.

Although there are a few standardised ecotoxicological tests using marine macroalgae (Ecklund & Kautsky, 2003), there are none for testing the effects of chronic irradiation. A number of research articles on macroalgae include information on culture conditions, responses and variability (Fletcher, 1989; Machlachlan, 1964; Machlachlan *et al*., 1971; Thursby & Steele, 1995). Species of the genera *Fucus* and *Ulva* fulfil the requirements for a bioassay procedure: they are sensitive, quick and reliable with developed protocols for their husbandry. Through development of experimental protocols suitable tests could be developed for radiation experiments on the two species *Fucus vesiculosus* and *Ulva intestinalis*.

Table A3.1 Summary literature review on the use of algae in radiation research (m = marine, f = freshwater species)

Species (marine / freshwater)	Stage	Exposure	Radiation	Endpoint	Result	Reference
Fucus spiralis (m) *Fucus vesiculosus* (m) *Fucus serratus* (m)	Germling	14 days laboratory	UVR (λ = 280-400nm) UVBR (λ = 280-315nm) Temperature included as a factor	Relative Growth Rate (RGR)	Species specific differences in RGR. Sensitivity dependant on shoreline distribution	Altamirano *et al.* (2003)
Porphyra tenera (m) *Porphyra* Saga No. 5 (m)	Conchospores	1-5 days	Gamma: 0, 50, 100, 150 and 200 Gy total dose @ 780 $mGymin^{-1}$	Germination and growth	Gamma irradiation <200 Gy did not affect germination 200 Gy caused abnormal growth Normal spores from mature thalli were observed on fronds exposed to 10-20 Gy Frond area and wet weight was greatest at dose of 10 Gy Withering to death observed at the highest dose	Kim (1985)
Spirogyra azygospora (f)	Adults	Acute	Gamma: 20, 40, 60, 80, 100, 150, 200, 300, 400, 500, 750, 1000 and 1300 Gy.	Morphological	Fragmentation of filaments into individual cells >200 Gy	Vedajanani and Sarma (1979)
				Cytological	Mitotic delay linearly increased with dose	
				Karyological	Chromosomal erosion and clumping of chromosomes >150-200 Gy	
Sirogonium melanosporum (f) and *Sirogonium sticticum* (f)	Adults	Chronic - Up to 6 months	Gamma: 30 and 150 Gy	Morphological	By days 4 and 13 there were 2% and 26% giant cells respectively at 150 Gy	Wells and Hoshaw (1980)
				Cytological	Reduction in cell division from 13 to 5% after 3 days at 30 Gy	
				Karyological	23% of cells with chromosomal fragments after 3 days at 30 Gy	
Stigeoclonium pascheri (f)	Adults and spores	X-rays and Gamma rays.	25-100 Gy	Germination of spores and survival of vegetative colonies	X-rays between 25-100 Gy and Gamma rays between 25-75 Gy increased the % germination of spores. Germination of vegetative colonies and sporulation was not changed up to 100 Gy of X-rays and 50 Gy of gamma rays	Agrawal (1986)

2) The use of macroalgae in testing - development of protocols

Seaweeds have been used for many years, *in situ*, as indicators for monitoring coastal waters (Levine, 1984). Until 20 years ago, marine macroalgae were thought to be best confined to field tests, due to their large size and requirements for large volumes of seawater. Jensen (1984) describes a method for measuring apical growth of Fucoid species, and considers it the only procedure to show promise for the routine testing of chemicals. However, in recent years several *in vitro* reproduction tests have been developed using macroalgae as test species in ecotoxicological bioassays (Thursby & Steele, 1995). Table A3.2 presents a summary of articles that describe the use of algae in toxicity testing. It demonstrates the range of tests that have been developed. A review of studied toxic effects on macroalgae by Eklund & Kautsky (2003) found that a total of 120 substances have been investigated using 65 macroalgal species (taken from 82 articles, 1959-2000). The *Champia parvula* sexual reproduction test is one bioassay that is widely used and is covered by Environmental Protection Agency (EPA) and American Society for Testing and Materials (ASTM) guidelines (ASTM-1498, 1995; US EPA, 2002). Other reproduction tests on macroalgae that have been used and published include those on Fucoid algae, specifically: *Fucus serratus* (Bird *et al.*, 1978; Creed *et al.*, 1997; Johnston, 1977; Scanlan & Wilkinson,1987), *Fucus vesiculosus* (Reynolds *et al.*, submitted; Wrabel & Peckol, 2000), *Fucus spiralis* (Bond *et al.*, 1999) and *Fucus edentatus* (Steele, 1977). Other species of brown and a number of green algae have also been used in tests: *Laminaria saccharina* (Steele & Hanisak, 1978), *L. digitata*, *L. solidungula*, *Saccorhiza dermatodea*, *Alaria esculenta* (Wiencke *et al.*, 2004), *Enteromorpha sp.* (Scanlan & Wilkinson, 1987; Fletcher, 1989; Reynolds *et al*., submitted and *Ulva lactuca* (Scanlan & Wilkinson, 1989).

The culture and growth of *Fucus* species has been investigated by McLachlan *et al.* (1971) who suggested a protocol for spawning. This protocol forms the basis of the CEFAS standard operating procedure for spawning of *F. vesiculosus*. Ang (1991) found that during such artificial spawnings the number of eggs produced by the species *Fucus distichus* was a mean of 366 per conceptacle.

A previous investigation with *Fucus serratus* estimated percentage fertilisation in eggs by the presence of fluorescence in ultraviolet light, following treatment with an optical brightener (Scanlan & Wilkinson, 1987). However, this method did not produce satisfactory results. In the present study fertilisation success was measured by the presence of a germinal tubule after 5-7 days.

The density of germlings of *Fucus vesiculosus* in experimental chambers has been found to affect their development (Creed, 1996). The authors found that increased densities resulted in depressed growth and a negatively skewed population structure during the first month of newly settled germlings. The limiting factor was thought to be nutrients. In the present study, densities were kept low for these reasons and also for the difficulties of measuring large numbers of embryos.

A recent study investigated the effects of UV radiation on the growth of germlings of three species of *Fucus* (Altamirano *et al.*, 2003). The authors measured the relative growth rates of germlings while dosing with UV radiation. The experimental design used was similar to that used for the present study. However, growth of germlings was restricted to only 14 days. In studies by McLachlan *et al.* (1971) the authors grew several species of *Fucus* embryo beyond their 14th week. This is important for the development of the experimental protocols in the present study since existing CEFAS protocols for this species are only relevant up to 28 days. In particular the use of fortified seawater medium for embryos older than 1 week (Chen, 1969; McLachlan, 1964; McLachlan *et al*., 1971) may be necessary for the growth of macroalgae to more mature stages.

Growth of *Fucus vesiculosus* germlings is characterised by the presence of numerous secondary rhizoids that may be as long as the primary rhizoid (Lobban & Harrison, 1997; McLachlan *et al.*, 1971; Reynolds *et al.*, submitted). Altamirano *et al.* (2003) measured the relative growth rate of *Fucus* spp. germlings using changes in length of the major axis, without taking into account the length of primary and secondary rhizoids, or apical hairs. Similarly, in the present study, growth was measured by the total length of germlings.

On husbandry requirements for *F. vesiculosus*, the findings of Reynolds *et al.* (submitted) concerning light and temperature agree with McLachlan *et al.* (1971). Reynolds *et al.* used cool-white fluorescent lights providing an intensity of 6,000 lux on a 12h light/dark photoperiod. McLachlan *et al.* (1971) found that rapid growth was achieved using the same lighting equipment and an intensity of at least 5,000 lux on a 10- to 16-h photoperiod. Temperature was found to have a substantial effect on growth. McLachlan *et al.* (1971) note that maximum growth rates occur at 13-15°C. This equates well with ambient coastal sea temperatures during the spawning periods.

Little published literature is available for the culture of *Ulva* sp. However, there is a substantial amount of data available on their life cycle (in particular their spawning periodicities) and the use of individuals collected from wild populations in toxicity tests. Christie and Evans (1962) note that the best time to collect *Ulva* spp. material for maximum production of zoospores is during the spring tide period although material suitable for spore tests may be collected at other times. Fletcher (1989) suggests a protocol for the bioassay of *Ulva*, modified by Reynolds *et al.* (submitted), that was considered suitable for use in the present study. No literature exists on growing *Ulva* longer than 28 days (Reynolds *et al*., submitted). However, it is thought that fortified seawater (as with the *Fucus* embryos) may provide a suitable growth medium. Previous experience at the CEFAS laboratories (Reynolds *et al*., submitted) suggests that survival up to 28 days in un-fortified, filtered seawater gives growth that follows a quadratic and linear model in the case of *U. intestinalis* and *F. vesiculosus,* respectively.

Table A3.2 Summary literature review of the use of *Fucus* and *Ulva* (*Enteromorpha*) spp. in toxicity testing

Species	Stage	Exposure	Toxicant	Endpoints	Result	Comments	Ref erence
Many	Egg, germling, adult	Various	Various	Various	NA	Review of toxicity testing using marine macroalgae	Ecklund and Kautsky (2003)
Several marine macroalgae including *Fucus*.	*Fucus*: Zygote	Acute – 48 h	Results for *Fucus* using fuel oil	Germination	% germination reduced with increasing oil contamination	Review of methods available for a range of species and endpoints. Results show *Fucus* sp. are a sensitive indicator of the toxicity of water samples	Steele (1977)
Fucus serratus	Egg - germling	Acute - Chronic 0-20 days	Biocidal (Dodigen v181-1, Dodigen v2861-1, ML-910)	Spermatozoid motility	Non-quantitative. Spermatozoa adversely affected >0. 1 ml^{-1} of biocide	Methodology paper	Scanlan and Wilkinson (1987)
				Percentage fertilisation	Method used not suitable		
				Growth rate of germlings	Growth inversely related to biocide concentration. Effects at >0.1ml^{-1}		
Fucus serratus	Developing zygote	Acute - 96 h	North Sea oils	Fertilisation, settlement (adhesion) and germination	Concentrations of oil >0.1µg/ml prevented development. Concentrations >0.05µg/ml reduced % fertilisation	Short communication paper	Johnston (1977)
Fucus spiralis	Germlings	10 days	Cu^{2+}	Growth	Concentrations >84 nM affected growth	During post exposure in copper free medium recovery of germlings from copper related effects was observed	Bond *et al.* (1999)
Enteromorpha intestinalis	Zoospore and germling	5 days	Cu^{2+}	Germination	Growth inversely related to Cu^{2+} concentration. Effects at >0.01mg l^{-1}	Test developed as a rapid screening method	Fletcher (1989)

3) Species, habit and methodologies for radiation experimentation on Fucus vesiculosus and Ulva intestinalis

Basic descriptions of the biology and ecology of these two species are given in chapter two.

Fucus vesiculosus

Prior to toxicity testing, suitable reproductive individuals must be collected from the wild. The period over which eggs are released from *F. vesiculosus* is protracted. Receptacles that release gametes may be collected over a period of 6 months (Knight, 1950). Research by Knight & Parke (1950) showed that release of gametes was greatest in May and June on the Devon coast. At Port Erin on the Isle of Man, gamete release was found from April until June. However, plants were still found to bear receptacles in July and August. More recently, Altamirano *et al.* (2003) collected fertile *F. vesiculosus* receptacles in December 1998 at Helgoland, Germany (North Sea). These were found to provide viable embryos using the spawning method of McLachlan *et al.* (1971). McLachlan *et al.* (1971) note that for *F. vesiculosus* the greatest numbers of eggs are released during winter and spring, on the southeast coast of Cape Breton Island, near Halifax. However, plants with mature receptacles were found at this location throughout the year. In contrast to the findings of Knight & Parke (1950), Thursby & Steele (1995) state that reproductive plants of the fucoids are more difficult to find in the summer months. It appears that the confusion about spawning times in the literature is due to regional differences. In CEFAS's experience, viable embryos can be gathered during the early summer months and later in the year (September/October) in sufficient numbers for toxicity testing.

A variation on the methodology of McLachlan *et al.* (1971) is generally used to spawn fucoids. In brief, ripe receptacles are collected from mature plants and rinsed in deionized water. The receptacles are then placed overnight in a damp petri dish at room temperature. In CEFAS's experience refrigeration of the receptacles also works well. The eggs and sperm are released by re-wetting the receptacles in filtered seawater and are left to fertilise for two hours. Eggs can then be transferred into test treatment vessels for exposure to radiation sources. No assessment of fertilisation success is made at this point in the procedure.

Ulva intestinalis

The methodology used for toxicity testing with *U. intestinalis* is a modification of that of Fletcher (1989). Upon collection, algae are rinsed in filtered seawater and gently wiped with a damp cloth to remove any epibionts and detritus. Algae are placed onto damp paper and subject to mild desiccation for 24 hours at 18°C in a covered chamber in the dark. To initiate spawning individual algae are placed into 100 ml of filtered (0.1 µm) seawater and continuously stirred on a magnetic stirring plate. After two hours the water in which the algae has been placed is filtered through a 20 µm mesh and the filtrate collected. One ml of the filtrate is examined microscopically for the presence of zoospores. The solution is mixed with a plunger every 5 minutes to prevent settlement of zoospores and to produce a homogenous suspension. Aliquots are taken whilst the solution is stirred and added to the test chamber. The zoospores will settle on coverslips that have been previously placed in the test chamber. Algae that have adhered to the coverslip can then be removed, on the coverslip, for measuring growth at defined intervals.

4) Chronic radiation studies using gastropod molluscs

Several studies have investigated the effects of radiation on freshwater and marine gastropods (Table A3.3). Environment Agency Technical Report P3-101/SP2 (2003) is about the development of protocols that deal with chronic radiation exposure. This category of radiation exposure is both the least studied and the most relevant, in terms of environmental protection and regulation (Environment Agency, 2003). In a study of the effects of mercury on the prosobranch mollusc *Crepidula fornicata*, Thain (1984) notes that growth and reproductive capacity are probably the two most important chronic and sub-lethal responses. Such responses are highly important for the survival of a species and therefore form the basis of this review.

A suitable gastropod species for radiation exposure tests is likely to be one where sufficient data exist on husbandry requirements to avoid the need for large pilot studies. In addition, a species where growth or reproductive endpoints have previously been investigated provides a good basis for experimental protocols. Species that fulfil these requirements are reviewed below. Ecotoxicological tests have been developed for certain molluscs to study the effects of compounds on these endpoints. Several such tests have the potential to be modified to investigate the chronic effects of radiation.

Nucella lapillus

Nucella lapillus (dog whelk) is a neogastropod mollusc that has been extensively studied to determine the effects of tributyltin (TBT) exposure (Gibbs *et al.*, 1987; Bryan *et al.*, 1988; Evans *et al.*, 2000). Samples of *N. lapillus* are easy to collect and can be maintained in simple, flow-through, aquaria. *N. lapillus* is a gonochoristic, oviparous species. Spawning occurs throughout the year but is maximal in spring and autumn (Marlin, 2004). Gametogenic development in *N. lapillus* is continuous throughout the year with no 'resting' phase in the cycle (Feare, 1970). Feare (1970) found that for whelks collected from Robin Hood's Bay (Yorkshire) spawning occurs in April and May but a second spawning may also occur in August. No specific investigations into the use of *N. lapillus* in radiation exposure experiments were found. Broom *et al.* (1975) investigated the accumulation of Caesium-137 in littoral organisms, including *N. lapillus*, on the Cumbrian coast. The studies found that Caesium-137 concentrations were greatest in organisms occupying higher trophic levels, such as *N. lapillus*. However, the authors did not comment on the effects of such contamination.

Crepidula fornicata

Crepidula fornicata (slipper limpet) is a prosobranch mollusc that has been cultured for studies of pollution effects. Calabrese & Rhodes (1974) identified *Crepidula* as having the following characteristics: a short generation time; a fecundity of about 6,000 in a single brood release; males and females that can be easily identified; observable embryo development; predictable larval release; large, free-swimming larvae; spat that can be easily grown on. Although breeding can occur between February and October, peak activity occurs in May and June when 80-90% of females spawn. Most females spawn twice in a year, apparently after neap tides. Thain (1984) developed an experimental system for investigating the chronic effects of mercury on *C. fornicata*. The system was designed to measure growth and the reproductive endpoints of fecundity and spawning frequency in spawning pairs of limpets, after exposure through the algal feed. It is relatively simple to construct and allows inspection of the organisms where necessary and measurement of endpoints as required. This protocol is directly applicable to radiation experiments. Greenberger *et al.* (1986) used *Crepidula* larvae to study the effects of x-irradiation on growth and metamorphosis. The authors note that the larvae of this species can be reproducibly reared in the laboratory with high survival. These experiments were

short-term (20 day). Acute exposures of radiation over 20 Gyh^{-1} caused a significant increase in larval mortality.

Littorina littorea

Littorina littorea (edible winkle) is an intertidal mollusc that can breed throughout the year. The length and timing of breeding are dependent on climatic conditions. The sexes are separate and fertilisation is internal. Egg release is synchronised with spring tides. In UK estuaries, the population matures earlier than on the coastline, with maximum spawning occurring in January. *Littorina* is very fecund. A large female produces up to 100,000 eggs. CEFAS has successfully developed an acute (96 hour) water column bioassay using the larvae of *Littorina*. This assay could easily be adapted to assess the effects of ionising radiation on the egg and larval stages.

Littorina littorea has been previously used in laboratory and field studies (Swift *et al.,* 1995a, 1995b) to determine the concentrations and biological half-lives of a number of radioactive elements. The most recent Radioactivity in Food and the Environment (RIFE) report (Environment Agency *et al*., 2003) details mean radioactivity concentration in shellfish for sites in the Irish Sea vicinity. Edible winkles tend to have higher levels of mean radioactivity, per nuclide, than whelks.

Other species

Several laboratory radiation studies have been carried out using freshwater snails (*Physa* spp.) (Cooley, 1973; Fujita & Egami, 1984; Ravera, 1967), but few include marine species (see Table A3.3). A study using the marine snail *Nassarius obsoletus* (Engel & Davis, 1973) found that radiation sensitivities, measured by mortality, followed a biphasic curve, as opposed to the classic sigmoidal curve, after irradiation in excess of 700 Gy (at a dose rate of 31 $Gymin^{-1}$) over a 30 day examination period. They therefore suggest it is not possible to calculate a reliable LD_{50} based on these data. White & Angelovic (1966) plotted LD_{50}s against time after irradiation and the radiation dose using the same species. In contrast to studies by Engel and Davis (1973) they found an almost linear relationship. However, they do not report on the calculation of the individual LD_{50}s that demonstrate the relationship.

Examples of experimental techniques using *Littorina littorea* to assess the effects of radiation upon reproductive endpoints

Based on this review, *Littorina littorea* seems the most promising species for studying the effects of ionising radiation upon gastropod molluscs. Techniques that could be used to assess the impacts of chronic ionizing radiation exposure on reproductive endpoints in *Littorina littorea* are outlined below. They are divided into three broad areas: larval bioassay, COMET assay of sperm and histopathology. It is envisaged that *L. littorea* would be maintained in tidal, flow through tanks and subject to environmentally realistic radiation exposures. Adult *Littorina* should be collected from a clean source and maintained in the laboratory under chronic radiation exposure regimes. Basic parameters including reproductive output, can be assessed throughout the experimentation period.

Larval bioassay

Littorina sp. would be a favourable species in which to study the effects of radiation on larvae, because it produces planktotrophic larvae that are amenable to experimental protocols developed by CEFAS for assessing acute water toxicity. This assay could be amended to study effects of radiation on endpoints such as mortality and sub-lethal endpoints such as those involving image analysis techniques.

Comet assay of Littorina sperm

The single-cell gel electrophoresis assay (the comet assay) was originally described by Ostling and Johanson (1984) as a simple and rapid technique to measure DNA damage. From this technique, the alkaline comet assay, which measures single-strand breaks and alkali-labile sites, and the neutral assay, which measures DNA double-strand breaks, were developed. In the alkaline version, single cells are embedded in agarose [GEL?] and lysed to release their nuclear DNA. Following alkaline electrophoresis and ethidium bromide or Sybr Green staining, the condition of the DNA is assessed using fluorescence microscopy (Fairburn *et al*., 1995; Speit & Hartmann, 1999). If the nuclear DNA is damaged, it is observed as a comet-like image. The assay is used in such diverse applications as genotoxicity testing (both *in vitro* from treated cell cultures, and *ex vivo* from virtually any tissue of experimental animals), bio-monitoring and DNA damage induction and repair studies (Singh *et al*., 1995; Nacci *et al*., 1996; Malyapa *et al*., 1998). This methodology could be transferred to investigate radiation effects upon gastropod sperm with minimal development.

Previous reports have used the comet assay to detect DNA damage induced by low doses of gamma radiation (Singh *et al*., 1995; Malyapa *et al*., 1998). Malyapa *et al.* (1998) used Caesium-137 gamma -rays and delivered absorbed doses of 3, 13 and 50 mGy to mammalian lymphocytes at a dose rate of 65.5 $mGymin^{-1}$. All of these doses produced a statistically significant response. Singh *et al.* (1995) used Technetium-99m to deliver doses of 1.02 and 4.08 mGy in 1 hour to human lymphocytes. Both doses produced a statistically significant response.

The comet assay is also used to determine strand breaks in the DNA of cells taken from a whole organism rather than a single-cell type (Jarvis & Knowles, 2003). Jarvis and Knowles used two-day-old (post-hatching) larvae of zebrafish (*Danio rerio*) that had been exposed to Caesium-137 gamma radiation for 1 or 24 hours at dose rates of 0.4, 1.2 or 7.2 $mGyh^{-1}$. Zebrafish larvae exposed to only 1.2 mGy h^{-1} of gamma radiation for 1 hour showed a statistically significant increase in DNA damage compared to controls.

Histopathology of adults after chronic exposure

Histopathological assessment should be carried out on all major organs of the molluscs: gill, mantle, kidney, digestive gland and gonad. A range of pathologies can be assessed including the presence of lysosomal alterations of the digestive gland, the index of relative abundance of adipogranular (ADG) cells in the connective matrix and the presence of neoplastic and inflammatory lesions. Reproductive status can be assessed using a similar scheme to that set out by Seed (1976). Reproductive development can be tracked through stage 0 (resting), stages 1-4 (developing), stage 5 (ripe) and post-spawning, then back through regressive stages.

Reproductive cells have developed complex and rigorous surveillance systems to detect genome damage (Majno & Joris, 1996). When these cells detect harmful genomic lesions, they undergo apoptosis (cell suicide) to prevent abnormalities being perpetuated in developing gametes (Kuwahara *et al*., 2002). Numerous studies have suggested that increased cell death rate in reproductive organs of various species may be considered an endpoint of exposure to contaminants or other environmental stressors (Russell & Kelly 1982; Van der Meer *et al*., 1992; Hasegawa *et al.*, 1997; Marty, *et al*., 1997; Weber & Janz, 2001). Cells contain complex mechanisms whereby DNA is routinely assessed for any damage that may occur (Majno & Joris, 1996). Once the level of damage has reached a stage whereby abnormalities may be passed on during cell division, the apoptosis programme is initiated and cell suicide takes place. This concept is particularly relevant when used in the context of gonadal germ cells and of abnormalities being passed on to daughter cells during meiosis. A recent study by Kuwahara *et al.* (2002) showed that an increased rate of spermatogonial apoptosis occurs in male fish (*Oryzias latipes*) exposed to gamma radiation. The most sensitive stage during spermatogenesis is in the early differentiating spermatogonia, the immediate descendants of the stem cells.

Other studies using fish exposed to various contaminants, including PAHs, tributyltin (TBT), cadmium, copper and crude oil-contaminated sediments have also demonstrated increased rates of gonadal cell apoptosis (Julliard *et al*., 1996; Xu *et al*., 1996; Marty *et al*., 1997; Weber & Janz, 2001). In one study, apoptosis of gonadal cells was correlated to reproductive impairment in wild stocks of pink salmon (*Oncorhynchus gorbuscha*) up to four years after the Exxon Valdez oil spill (Marty *et al*., 1997). A similar study for molluscs would analyse histological tissue sections using the terminal deoxynucleotide transferase mediated deoxy-UTP nick-end labelling (TUNEL) assay that labels fragmented DNA within intact cells.

Table A3.3 Summary review on the use of molluscs in radiation research

Species	Stage	Exposure	Acute / Chronic	Radiation	Endpoints	Result	Comments	Reference.
Crepidula fornicata	Larvae	20 day experiment	Acute radiation exposure	X-irradiation on Day 0: 5, 10, 20, 50, 100, 200, Gy total dose @ 2000 $mGymin^{-1}$	Mortality, growth (shell length and biomass) and metamorphosis	Dose dependent decrease in growth rate of larval shells >20 Gy. Shell length specific biomass decreased at doses >100 Gy. Mortality increased at doses >20 Gy. Cumulative % of metamorphosis decreased up to 50 Gy Metamorphosis >50 Gy occurred faster than other groups		Greenberger *et al.* (1986)
Physa heterostropha	Adult	Life-long	Chronic	Field study: environmental radiation of approx. 6.5 $mGyd^{-1}$ with a control population	Fecundity	Frequency of egg capsule production decreased, number of eggs per capsule increased in irradiated individuals compared to controls	Suggest irradiated snails compensate for fewer capsules with increased number of eggs per capsule	Cooley (1973)
Physa acuta	Embryos, adults	Up to 80 days	Acute radiation exposure	Gamma: 10, 80, 330, 540, 600 and 1500 Gy @ 0.25-0.5 $Gymin^{-1}$	Mortality, histological changes, fecundity and survival of young	LD_{50} of adult snails 400 Gy LD_{50} of embryos 9-20 Gy 0 and 1 day after oviposition respectively. High radiosensitivity of germ cells in early stages	Relative constitution of germ-cell populations greatly changed by gamma rays of 8 kR.	Fujita and Egami (1984)
Physa acuta	Adult	50 days	Acute radiation exposure	X-rays: total 280, 540, 1100, 2200 Gy @ 10-20 $Gymin^{-1}$	Mortality and fecundity	Significant mortality at 540 Gy, 20 days after irradiation 100% mortality at 2200 Gy,1 day after irradiation Egg capsules and egg		Ravera (1967)

						production reduced by radiation up to 100 Gy. 20 Gy reduced viability and fertility		
Nassarius obsoletus	Adult	Up to 70 days	Acute radiation exposure	Gamma: 0, 200, 300, 400, 500, 600 and 700 Gy @ 3100 $mGymin^{-1}$	Mortality (LD_{50})	Calculation of LD_{50}s not possible due to results giving a biphasic survival curve.	A hypothetical model provides an explanation for the observed response	Engel and Davis (1973)
Nassarius obsoletus	Adult	Up to 80 days		Gamma: 4800 $mGymin^{-1}$	Mortality (LD_{50}) calculated during experiments to show mean lethal dose-time curves.	LD_{50} times range from 15 days at 515 Gy to 50 days at 140 Gy.	Changes in LD_{50}s with radiation dose over time. Several other marine invertebrates included	White *et al.* (1966)
Ilyanassa obsoleta	Eggs	Until cleavage	Acute radiation exposure	X-radiation: 10, 20, 40, 50, 60, 80 and 100 Gy @ 10 $Gymin^{-1}$	Cleavage delay	Cleavage delay (1^{st} cleavage) up to 40 Gy. Above this dose slight increase in delay but >50 Gy lethality before blastulation increases.	Delays in cleavage of further divisions are studied and described.	Cather (1959)

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