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Developing experimental protocols for chronic irradiation studies on wildlife

R&D Technical Report P3-101/SP2

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The report provides good practice guidance for conducting experiments, where non-human biota are exposed to ionising radiation. The appproach will ensure that results enable to determine dose-response relationships between chronic exposure and biological endpoints.

The report is aimed at directing research by promoting the harmonisation of experimental approaches, and help fill in knowledge gaps.

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Executive summary

The UK has a duty to comply with the EU Birds and Habitats Directives when planning and undertaking its regulatory and operational activities. These European Directives were introduced into UK legislation by the Conservation (Natural Habitats) Regulations 1994, as amended by the Conservation (Natural Habitats) (England) Regulations 2000.

Under these Regulations the Environment Agency has obligations to review all existing authorisations, permits, consents, licences and permissions to ensure that no Agency authorised activity or permission results in an adverse effect, either directly or indirectly, on the integrity of identified European sites.

The Environment Agency is the body responsible for authorising disposals of radioactive material in England and Wales. Research sponsored by the Agency has produced an impact assessment methodology (e.g. [60,61]), which can be used to determine the likelihood of adverse effects of radiation in non-human biota. In addition, the Agency is also participating in the EC-funded FASSET (Framework for ASSessment of Environmental impacT) project (FIGE-CT-2000-00102), which has been collating information on the transfer, dosimetry and effects of ionising radiation on wildlife. However, both studies have identified a number of significant gaps in our scientific knowledge about the effects of ionising radiation on wildlife.

Examination of the scientific literature shows that experiments have not always been conducted in a consistent manner and that the outputs are not always reported in sufficient detail to allow the data to be used for the purposes of impact assessment. This project, therefore, has been undertaken to provide good practice guidance for conducting experiments that expose wildlife to ionising radiation in order to determine dose-effect relationships between exposure and biological endpoints of interest.

The objectives of this project were to:

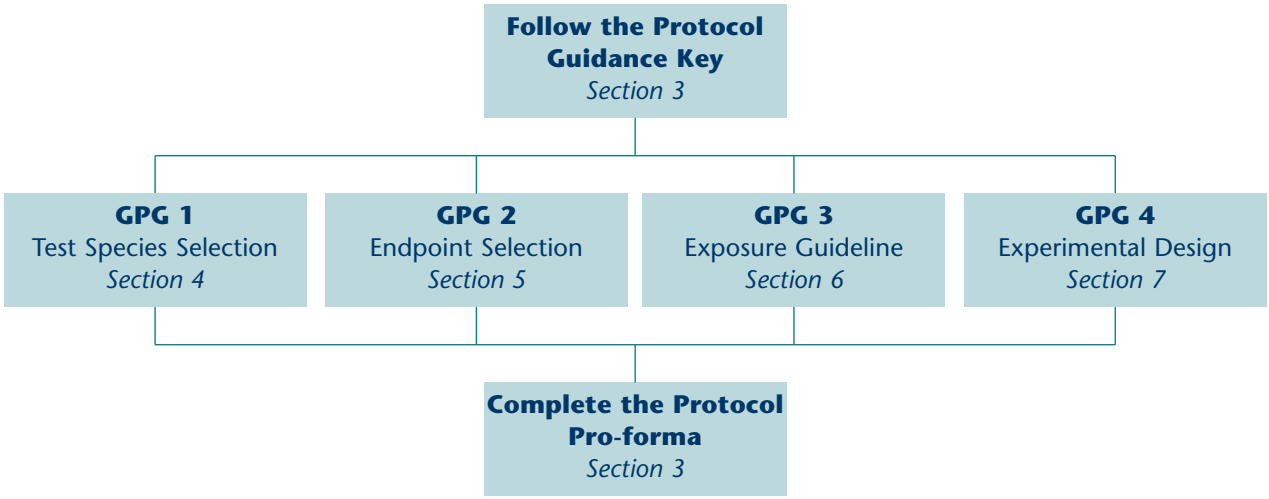
- develop experimental protocols that will enable the derivation of dose-effect relationships, at a chronic level, from a range of wildlife groups;
- consider and advise on good practice for each stage of the design of experiments on the effects of ionising radiation on wildlife;
- provide examples of good experimental design;
- provide information on financial and staffing considerations to be taken into account when planning research;
- identify research priorities from information gaps in the FASSET Radiation Effects Database [219].

It is hoped that this report will facilitate future research on the effects of radiation on wildlife by promoting the harmonisation of experimental approaches, which will compare data between experiments. Furthermore, the guidance makes recommendations on which species should be considered for

conducting laboratory experiments and how they link to the reference organism concept promoted in impact assessment methodologies.

The buidlding of the experimental protocols is sub-divided into four Good Practice Guides (GPGs), each targeting a particular component of the overall protocol; the linkages between these sections are demonstrated in the diagram below.

A key guides users through each of the four GPGs in a systematic manner and prompts them to record their decisions and justifications on the pro-forma provided. The protocol pro-forma forms the basis for the experimental design and indicates what data should be reported so that the experimental data may contribute to future impact assessments.



Approach to follow to set protocols for experiments on wildlife and ionising radiation

The guidance has been produced by reviewing the literature available - but primarily the FASSET Radiation Effects Database (FRED) - on the different wildlife groups that require protection (see Section 5). This review enabled the determination of which species, if any, have been used previously in experiments with ionising radiation. In addition, the use of species representative of particular wildlife groups was considered from a review of ecotoxicology studies.

Following these reviews, species that could be considered for experiments on ionising radiation are suggested (called test species in this report). Information on the husbandry and any particular maintenance requirements is reported where the information is readily available for each recommended test species.

The experimental facilities required for experiments on ionising radiation are considered, together with any practical difficulties that may be encountered (e.g. when considering how to conduct experiments requiring internal exposure). The derivation of appropriate dose rates is discussed, as is the duration of any experiments. Finally, general advice on the statistical design of the experiments is given.

The protocols are primarily aimed at chronic low-level exposure experiments that could be considered similar to the dose rates that may occur under environmental conditions.

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1. Introduction

The UK has a duty to comply with the EU Birds and Habitats Directives when planning and undertaking its regulatory and operational activities. These Directives were introduced into UK legislation by the Conservation (Natural Habitats) Regulations 1994, as amended by the Conservation (Natural Habitats) (England) Regulations 2000.

Under these Regulations, the Environment Agency has obligations to review all existing authorisations, permits, consents, licences and permissions to ensure that no Agency authorised activity or permission results in an adverse effect, either directly or indirectly, on the integrity of identified European sites.

The Environment Agency is the body responsible for authorising disposals of radioactive material in England and Wales. Research sponsored by the Agency has produced an impact assessment methodology (e.g. [60,61]), which can be used to determine the likelihood of adverse effects in non-human biota. In addition, the Agency also participates in the FASSET (Framework for ASSessment of Environmental impacT) project (EC contract FIGE-CT-2000-00102), which has been collating information on the transfer, dosimetry and effects of ionising radiation on non-human biota and ecosystems. However, both studies have identified a number of significant gaps in our scientific knowledge about the effects of ionising radiation on non-human biota.

It is, however, clear from the scientific literature that experiments have not always been conducted in a consistent manner and that the results are not always reported in sufficient detail to allow them to be used for the purposes of impact assessment. This project, therefore, derives good practice guidance for conducting experiments that expose non-human biota to ionising radiation in order to determine dose-effect relationships. A number of experiments are also proposed in order to address current knowledge gaps.

The objectives of this project were to:

- develop experimental protocols that will enable the derivation of dose-effect relationships, at chronic level, from a range of wildlife groups;
- consider and advise on good practice for each stage of designing experiments on the effects of ionising radiation on non-human biota;
- provide examples of good experimental design;
- provide information on financial and staffing considerations to be taken into account when planning research;
- to identify research priorities from information gaps in the FASSET Radiation Effects Database (FRED) [219].

1.1 Background

The protection of humans from ionising radiation is well established, with many years of intensive research and the development of assessment tools to support it. There are many reasons why protecting the environment is less developed, not least because the International Commission on Radiation Protection (ICRP) has always considered the

environment only as a conduit for the transfer of radionuclides to humans.

The disproportionate availability of scientific data on effects to humans compared with non-human biota or wildlife has resulted in the development of generic assessment tools to assess the impact of ionising radiation to wildlife. These tools have been developed on the basis of the best scientific knowledge available. Previous research into the effects of chronic exposure to ionising radiation can be described as being, at best, piecemeal. Future research in this field must, therefore, be targeted at knowledge gaps and be directed to provide maximum benefit for environmental protection.

The need to conduct experiments on the effects of ionising radiation to determine dose-effect relationships led the Agency to commission this project. This required the promotion of a 'common' approach to make both the comparisons of results between experiments and the interpretation of the findings easier.

The information provided in this guidance is by no means exhaustive. Rather, it is as representative as possible of the current level of scientific understanding on the effects of ionising radiation on non-human biota. All experimenters are strongly advised to use this guidance to develop their protocols and then conduct a targeted search of the scientific literature in order to tailor their protocols in light of any advancement in scientific knowledge. An important example of this is the organisms that are recommended for future experimental work (Section 4). These will, no doubt, require revision as work progresses on the currently recommended species.

The collated information on the effects of ionising radiation on non-human species was also compared with the use of Environmental Quality Standards (EQSs) for a number of chemical contaminants. These EQSs are based, in part, on ecotoxicological studies conducted on test species to determine dose-effect relationships. EQSs or environmental criteria are likely to be developed in the future to include radionuclides. In producing research protocols, consideration has therefore been given to identifying test species that are already used for setting EQSs for chemical contaminants and thus that may be suitable for use in setting radionuclide EQSs.

1.2 Structure of the report

A key has been developed to guide users through the decision-making process in order to develop a protocol suitable for the investigation of chronic radiation dose-effect relationships in non-human biota. The various sections of this report describe specific aspects of the experimental design, from the selection of the test species to statistical design. The contents of each section are outlined below.

Section 2 Overview of the approach to follow when producing an experimental protocol for a given wildlife group.

Section 3 A key to guide users through the decision-making process in order to generate a standardised protocol for the experiment. A protocol pro-forma is provided, which users should complete as they progress through the protocol guidance key. The completed protocol pro-forma will contain the basic information for conducting a particular experiment.

Section 4 Test species selection (GPG 1). Appropriate test species for each wildlife group are recommended following a review of the scientific literature on experiments conducted with ionising radiation and in ecotoxicology for different wildlife groups. Husbandry methods are provided for a number of recommended test organisms.

Section 5 Endpoint selection (GPG 2). The choice of biological endpoint for study is considered and examples are given of the different types of specific endpoint under each of four main umbrella endpoints.

Section 6 Exposure guideline (GPG 3). Guidance is given on appropriate methods of internal and external exposure, together with a discussion on how to select suitable dose rate ranges for the experiments in order to ensure that the dose-effect threshold can be identified for any given biological endpoint. The facilities required to conduct experiments on ionising radiation are also considered.

Section 7 Experimental design (GPG 4). The need for an appropriate statistical design to the experiments is discussed.

The report also provides an indication of the financial considerations associated with designing experimental protocols (Section 8) and an example of good experimental design drawn from the

literature (Section 9). Section 9 also includes an example of a protocol that has been produced using this guidance document. Based on the current state of knowledge, the report concludes by suggesting possible research priorities (Section 10).

1.3 Definitions

Users are advised to become familiar with the following terms before reading the report and applying the key.

Feature species — A named species that has been identified as requiring protection in the Agency's EU Habitats Directive and Regulations Process Handbook for Agency Permissions and Activities (2003).

Reference organisms — A series of entities that provide a basis for the estimation of radiation dose rate to a range of organisms, which are typical, or representative, of those in a contaminated environment. These estimates, in turn, provide a basis for assessing the likelihood and degree of radiation effects [122].

Test species — A species that can be used to determine the dose-effect response under experimental conditions. Ideally, these should be biologically similar to both the feature species and reference organisms. They will, therefore, provide data that can be used to assess the impacts from ionising radiation to feature organisms.

The experimental protocols follow the methodology used in previous Agency reports. They rely on the identification of reference organisms to be assessed in order to ensure that the environment as a whole is adequately protected. Test species should, therefore, aim to be representative of the reference organisms and their wildlife group. They can then be employed to predict likely responses of feature species to chronic exposure to ionising radiation in the environment.

1.4 Limitations

Three major categories of radiation exposure can be considered when assessing the effects of ionising radiation on non-human biota:

- Acute radiation exposure: type of exposure received within a short period of time. The term 'acute' is normally used to refer to exposure of sufficiently short duration that the resulting radiation dose to an organism can be treated as having been instantaneous, e.g. less than one hour [60].
- Chronic radiation exposure: exposure that is persistent exposure over time. This is generally continuous exposure of organisms to low concentrations of pollutants [60].
- Transitory radiation exposure: a term that can be used to describe exposures at the interface between acute and chronic. The exposure is too protracted to be defined as acute, but does not persist for many years [60].

Although there are three categories of exposure, **the scope of this work has been restricted to the investigation of chronic radiation exposure.** This is because this category is the least studied but most relevant form of radiation exposure in terms of environmental protection and regulation. Accidental releases of radionuclides may provide acute radiation doses to organisms in the environment, but the majority of biota are more likely to be subjected to prolonged, chronic exposure scenarios [60]. This exposure may result from both 'natural' and 'anthropogenic' sources.

The guidance contains information current at the time of publication. Assessors are strongly advised to carry out a literature search at the time of planning any experiments in order to take into account the latest developments in this field.

2. Development of research protocols

The following sections contain Good Practice Guides (GPGs) that address the four key components of designing research protocols to investigate the effects of radiation on wildlife. The linkages between these sections are shown in Figure 2.1. A key guides users through each section in a systematic manner and prompts them to record their decisions and justification in the protocol pro-forma provided. This pro-forma forms the basis for the experimental design and also indicates what data should be reported in order that the data obtained from the experiment can contribute to any future impact assessments.

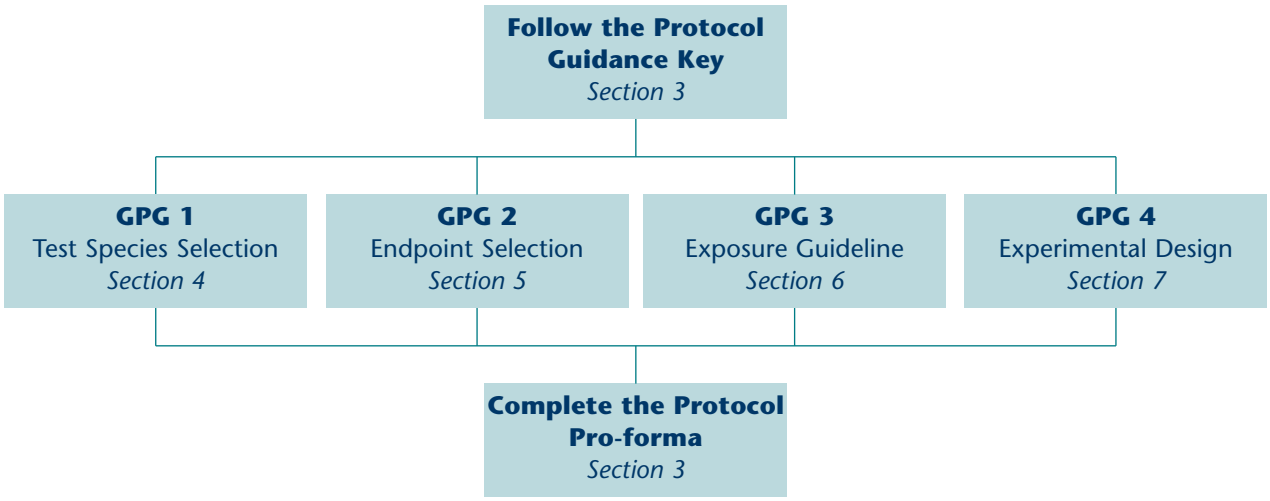

Figure 2.1: Approach to follow to set protocols for experiments on wildlife and ionising radiation

2.1 Protocol guidance key

The protocol guidance key prompts users to make decisions about the protocol they are producing in a systematic manner. It should be used in conjunction with the pro-forma to ensure that each decision, and the justification for that decision, are recorded. Once users have worked through each step of the key and completed the relevant parts of the pro-forma, they will have a document that will form the basis of their experimental design.

2.2 Test species selection (GPG 1)

The proposed test species can be categorised into the following wildlife groups:

- amphibians
- aquatic invertebrates including zooplankton
- aquatic plants
- birds
- crustaceans
- fish
- insects
- mammals
- mosses and lichens
- molluscs
- terrestrial plants
- reptiles
- soil fauna including microflora.

These wildlife groups form the major sub-sections in GPG 1, which includes a description of the test species within each group and their reasons for selection. Full details of the reasons for the inclusion of each wildlife group can be found in FASSET Deliverable 1 [185].

Husbandry considerations for the various species are also included to allow organisms to be bred and maintained, both before and during the experiments, under optimum living conditions. Factors such as diet, light regime, temperature and relative humidity must be regulated carefully to ensure that test organisms used in experiments are in good physical condition. This reduces the likelihood of factors other than the contaminant to which they are exposed during the experiment affecting the specific endpoints under investigation. Standardised husbandry methods also ensure that experiments conducted on the same test organism in different laboratories are comparable.

2.3 Endpoint selection (GPG 2)

GPG 2 describes the four umbrella endpoints identified by FASSET [185] and discusses appropriate specific endpoints. It also gives references to suggested approaches to undertaking the endpoint measurement and details any experimental features that may need to be included in the protocol.

2.4 Exposure guideline (GPG 3)

The prime purpose of any experimental protocol produced using the guidance is to examine the relationship between the radiation dose rate and the effect on the biological endpoint being studied. GPG 3 describes how to determine the dose rates that may be appropriate for a given wildlife group/endpoint combination and reviews the facilities and equipment required to undertake the exposure.

2.5 Experimental design (GPG 4)

GPG 4 provides users with an understanding of the reasons why consideration should be given to the data output from an experiment and the statistical tool(s) that could be used to analyse the data output. This section does not recommend specific statistical techniques, but provides reasons why users should consider statistical data interpretation at the protocol development stage.

3. Protocol guidance key

The guidance for developing experimental protocols provided in this document is intended for use by those wishing to conduct experiments on chronic ionising radiation exposure of non-human biota in order to determine dose-response relationships. Users are recommended to read the document first in its entirety to gain an understanding of the need to focus on particular wildlife groups and the approaches to be adopted.

Thereafter, users are expected to use the document as a reference to guide them through the process of developing their experimental protocol. The objective of this key is to enable users without an in-depth knowledge of the subject to access quickly information and guidance for the development of experimental protocols. Section 9.2 provides a worked example of a protocol that has been developed using this key and the pro-forma provided in this report.

The key guides users through the decision-making process. Users should start at instruction (a) and run through each instruction in turn. The decision selection should be recorded in the protocol pro-forma (Table 3.1); a checklist of items to be included when reporting data from the experiments (Table 3.2) accompanies the pro-forma. Protocol developers can cross-reference this to ensure that their protocol addresses the reporting requirements.

By following the instructions, users will be directed to an appropriate section of text from within the report that will provide them with information and guidance to enable them to make a decision on their experimental design.

The key and GPGs should be used in conjunction with literature searches to enhance the finished experimental protocol.

3.1 Key

Follow the instructions (a)-(l) below and enter your selections into the protocol pro-forma.

(a) Choose the umbrella endpoint you wish to investigate.

	Go to: Page	Section	Table
Mutation	53	5.1.1	5.1 + 5.2
Reproduction	53	5.1.2	5.1 + 5.2
	57	5.2.1	
Mortality	53	5.1.3	5.1 + 5.2
Morbidity	53	5.1.4	5.1 + 5.2
Do not know	52	5	5.1 + 5.2

Note: Users are directed towards consideration of reproduction endpoints because the success of a species depends on its ability to reproduce.

(b) Choose the wildlife group you wish to investigate.

	Go to: Page	Section	Table
Amphibians	23	4.6	4.3a
Aquatic invertebrates including zooplankton	25	4.7	4.4a
Aquatic plants	27	4.8	4.5a
Birds	30	4.9	4.6a
Crustaceans	33	4.10	4.7a
Fish	35	4.11	4.8a

Cont'd	Go to: Page	Section	Table
Insects	39	4.12	4.9a
Mammals	41	4.13	4.10a
Molluscs	42	4.14	4.11a
Mosses & lichens	43	4.15	
Reptiles	44	4.16	4.12a
Soil fauna including terrestrial algae and microflora	44	4.17	4.13a
Terrestrial plants	48	4.18	4.14a
Do not know	18	4	

(c) Establish the conditions in which the chosen species are to be kept.

	Go to: Page	Section	Table
Amphibians	23	4.6	4.3a,b
Aquatic invertebrates including zooplankton	25	4.7	4.4a,b,c,d
Aquatic plants	27	4.8	4.5a,b,c, d,e
Birds	30	4.9	4.6a,b,c,d
Crustaceans	33	4.10	4.7a,b,c
Fish	35	4.11	4.8a,b,c, d,e,f
Insects	39	4.12	4.9a,b
Mammals	41	4.13	4.10a,b
Molluscs	42	4.14	4.11a,b
Mosses & lichens	43	4.15	none
Reptiles	44	4.16	4.12a
Soil fauna including terrestrial algae and microflora	44	4.17	4.13a,b,c
Terrestrial plants	48	4.18	4.14a,b,c

(d) Choose specific endpoint(s) to study.

	Go to: Page	Section	Table
Under the following umbrella endpoints:			
Mutation	53	5.2	5.1 + 5.2
Reproduction	53	5.2	5.1 + 5.2
Morbidity	53	5.2	5.1 + 5.2
Mortality	53	5.2	5.1 + 5.2

Note: Wherever possible, morbidity endpoints should also be incorporated in the experiment as a matter of course.

(e) Choose the type of irradiation to use.

	Go to: Page	Section	Table
Internal (usually alpha or beta radiation)	59	6.3	none
External (usually gamma radiation)	59	6.2	none
Mixed	58	6	none

(f) Decide upon the facilities required.

	Go to: Page	Section	Table
Internal	68	6.7.2	none
External	68	6.7.1	none
Mixed	68	6.7	none

(g) Consider which dose rates to use.

	Go to: Page	Section	Table
Terrestrial plants, mammals & fish	60	6.5.1	6.1 + 6.2 + 6.3
Aquatic invertebrates	63	6.5.2	6.4
Aquatic plants	64	6.5.3	none
Crustaceans & molluscs	64	6.5.4	6.5 + 6.6
Birds, amphibians & reptiles	65	6.5.5	6.7 + 6.8
Soil fauna & insects	66	6.5.6	6.9

(h) Determine whether a screening experiment to determine dose rates is required.

	Go to: Page	Section	Table
Is a pilot study needed?			
	67	6.6	none

Note: If a pilot study is required, one pro-forma should be completed for the pilot study and another for the main study (the latter can summarise the findings of the pilot study).

(i) Decide length of irradiation.

	Go to: Page	Section	Table
Duration	58	6	none

(j) Decide what number of the chosen species is required.

	Go to: Page	Section	Table
Number of replicates	69	7.1	none
Number of individuals	70	7.1.2	none

(k) Decide what statistical tests to use.

	Go to: Page	Section	Table
Statistical requirements	69	7	none

(l) Undertake a further literature review on your chosen species/endpoint.

3.2 The protocol pro-forma

Users should follow the instructions as set out in the key in order to complete the protocol accurately. Table 3.1 provides the blank forms needed by users to set out their own experimental protocol.

All decisions should be justified, either by referring back to the relevant page/section/table of this report or by explaining the decision in the 'Further justification' box provided at the end of the pro-forma. Additional notes can also be recorded at the end of the pro-forma.

Table 3.1 Pro-forma to be filled in following the protocol guidance key

Pilot experiment/Main experiment (delete as appropriate)					
Key	**Instruction**		**Page**	**Section**	**Table**
a	**Umbrella endpoint of interest** (e.g. reproduction)				
b	**Wildlife group and species**				
c	**Maintenance conditions** (e.g. temperature, light regime, diet)				
d	• Specific endpoint(s) to study (e.g. no. of eggs produced)				
	• Compulsory measurements to record	Weight Length Growth rate No. of mortalities occurring			
e	**Irradiation type** (internal/external/mixed)				
f	**Facilities required** (e.g. Cs-137 source)				
g	**Dose rates to use** (e.g. background, 10, 20, 40, 80, 160, 320, etc. Gy or µGy/h) Where known, also indicate (in the brackets) the total dose received at each dose rate.	Background = Dose rate 1 = ( ) Dose rate 2 = ( ) Dose rate 3 = ( ) Dose rate 4 = ( ) Dose rate 5 = ( )			
h	**Need for a pilot experiment?**	YES/NO			
	• No. of dose rates, including control				
	• No. of individuals per dose rates				
	• Dose/dose rates used				
	• Duration of irradiation				
	• Duration of experiment				
	• Notes/other considerations				

Table 3.1 | cont'd

Key Instruction (cont'd)			Page	Section	Table
i	**Duration of irradiation** (e.g. list daily time period for irradiation - 20 hours)				
	Duration of experiment				
j	**No. of dose rates, including control**				
	No. of individuals per dose rate (e.g. 10)				
k	**Statistical requirements**				
	• Tier 1				
	• Tier 2 (e.g. test used)				
	• Tier 3 (e.g. test used)				
l	**Further literature search conducted?** If yes, then list.	YES/NO			
Further justification of decisions made to complete the pro-forma (to be completed when decisions are not based on information in the guidance document that can be referred to by page/section/table)		(use additional paper if required)			
Notes (general)		(use additional paper if required)			

Table 3.2 | Checklist for data reporting

	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 being studied(s)	☐
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	☐
Dose(s)	☐
No. of individuals per treatment group (including control)	☐
Duration of exposure(s)	☐
Result(s)	☐
Statistical analysis	☐
Any relevant notes	☐
Production of a data sheet to record results	☐
Extra notes/considerations	☐

4. Test species selection (GPG 1)

This Good Practice Guide (GPG 1) aims to guide users through the process of selecting a test species to use in ionising radiation experiments.

The objectives of this GPG are to:

- review scientific literature to identify species from each wildlife group used in studies on the effects of chronic exposure to ionising radiation;
- review scientific literature to identify species from each wildlife group used in studies on the effects of non-radioactive contaminants;
- discuss the positive and negative aspects of using particular species in experimental work;
- make justified recommendations as to appropriate species to use in experimental work on the effects of chronic exposure to ionising radiation on non-human biota.

The use of Environmental Quality Standards (EQSs) in ecotoxicological risk assessment can also help to identify already researched species. Section 4.1 describes the use of EQSs and places EQSs in context for the purpose of ionising radiation experiments.

4.1 EQSs in ecotoxicological risk assessment

Environmental standards are used by regulatory agencies such as the Agency and the Scottish Environment Protection Agency (SEPA) as benchmark concentrations of chemicals which, if exceeded, may result in harm to the environment. Standards are used in authorising releases of chemicals to the environment and the use of chemicals that are applied directly into the environment such as plant protection products. Standards are also used so that concentrations in different environmental media can be compared, e.g. to allow the Best Practicable Environmental Option (BPEO) to be determined for industrial processes that may release chemicals to more than one environmental medium.

Early legislation governing the release of chemicals to the environment covered individual environmental media, such as air or water, with emissions controlled in order to protect human health. The first EU directive to set out a regulatory framework to protect the environment from the discharge of chemicals was the Dangerous Substances Directive (76/464/EEC) [43]. This Directive specified statutory EQSs for 19 List I substances for inland, estuarine and marine waters. The EQS values were concentration limits that should not be exceeded in receiving waters. The List I chemicals were substances considered to be the most harmful to the aquatic environment with reference to their toxicity, persistence and bioaccumulation potential. The Directive also specified a list of other chemicals (List II substances) for which concentrations required control at a national level for specified water uses. These EQS values were specified in 1989 by the then Department of the Environment and Welsh Office [69] for various specified water uses such as abstraction for potable water supply, protection of sensitive aquatic life, protection of other aquatic life and protection of saltwater life.

Further UK legislation aimed to protect the whole environment, rather than considering each environmental medium in isolation. The concept of Integrated Pollution Control (IPC) was introduced in Part I of the Environmental Protection Act 1990. IPC assessed the potential effects of a chemical on the whole environment (including air, water and land), irrespective of the media in which the chemical was originally released. This legislation also introduced the BPEO concept.

Subsequent legislation, including the Environment Act 1995 and the Pollution Prevention and Control Act 1999, further developed the concept of using environmental standards to protect the environment by requiring emission limit values (ELVs) to be calculated for industrial processes to ensure that standards were not exceeded. Their uses also extended to the derivation of financial penalties set by the legal systems.

A variety of environmental standards, with varying legislative powers, have been set by various organisations to protect targets in different environmental media (Table 4.1).

Table 4.1 | Examples of EQSs

Standard		Source
AIR	Expert Panel on Air Quality Standards	[63,64,65,66,67,68]
	EU directives for specific substances	[43,46,47]
	Environment Agency environmental assessment levels	[76]
	Environment Agency citical levels for vegetation damage	[76]
	WHO Air Quality Guidelines for Europe	[214]
	UNECE Protocol Critical Loads	[197]
WATER	List 1 Environmental Quality Standards	[43,69]
	EA Environment Agency Environmental Assessment Levels	[76]
	WHO Drinking Water Guidelines	[215,216]
	EU Freshwater fish Directive	[45]
	EU Shellfish Directive	[44]
	EU directives relating to the protection of targets other than aquatic life, e.g. water intended for abstraction to potable supply	[43,69]
SOIL	EU Directive on use of sewage sludge in agriculture	[46]
	Environment Agency environmental assessment levels for releases to land	[76]

Standards are set within a wide range of objectives in water, air and land. These objectives are not always explicit, but they can include the protection of human health, particular uses of the environment, or other general descriptions such as the structure and function of different ecosystems. The medium for which the highest number of standards has been derived is air, although these standards are predominantly aimed at the protection of human health. The medium with the most standards for environmental protection is water. There are currently few standards available for soil.

There is a general acceptance that a reduction in species diversity is undesirable. However, the tolerable concentration of a chemical is highly site-specific and depends on the complex interactions between the individuals, populations and communities that determine the structure and function of an ecosystem. Generic standards must therefore either be set at a level which is protective for all likely scenarios (and therefore over-protective in some circumstances), or they must provide a compromise level of protection that is adequate for most scenarios. Some organisations, such as the US Environmental Protection Agency (US EPA) and the Dutch authorities have derived standards that aim to protect 95% of species present in an ecosystem. This approach can be problematic if the 5% of species lost include some considered to be of high conservation value. The UK approach is generally more conservative, aiming to protect the most sensitive life stage of the most sensitive species present.

Although the types of EQSs vary, they are generally derived via the five-step process outlined below:

(1) **Objective:** select the general objective, i.e. the use or attribute that is to be protected and the level of protection that the standard is intended to provide. For many standards, this stage is often missing or is not explicitly stated. For many standards, the objectives are likely to be derived at least, in part, by political judgement.

(2) **Target:** select the specific target such as an organism and a response or endpoint to the chemical concerned, which can be used to characterise the general objective. This is generally not straightforward when dealing with the environment, in which the target is initially defined at an ecosystem level. As data are rarely

available at this level, scientific arguments and inferences are required to re-specify the target in terms of communities, then in terms of populations, and ultimately in terms of numbers of individuals in a given species. This sequence will enable the target and endpoint to be specified in terms of an effect concentration affecting n% of a population (EC_n) for a given species, exposure duration and toxicological endpoint.

(3) **Data:** collect data and information relevant to the target and endpoint identified, and apply quality judgements and appropriate manipulation and/or analysis. EQS values are derived from the available toxicity data. Standard approaches require a minimum dataset covering a range of taxa. Standard-setting procedures use laboratory protocols and peer-reviewed quality assurance procedures to judge data reliability. An understanding of the chemistry of the pollutant, its behaviour in the environment, and subsequent likely exposures are also required.

(4) **Derivation:** apply a procedure to convert the data into a standard. This is likely to involve the use of 'safety factors' (sometimes called uncertainty factors) or statistical models. It is unlikely that experimental data will exist for the desired EC_n value. Therefore, the data that are available need to be linked to the desired data. This may involve a number of extrapolation procedures, e.g. linking acute to chronic, interspecies and effect extrapolations. Such extrapolations are also based on either a safety factor approach or on one based on statistical modelling. Both approaches require differences in exposure time, interspecies sensitivities and different toxicological endpoints within the dataset to be addressed. Whichever approach is used, expert judgement is required to take maximum account of the information available on the chemical, organisms and endpoints of concern. The use of expert judgement may make decisions appear arbitrary.

(5) **Validation:** compare the derived standard with real case scenarios and, if necessary, revise it. Where possible, a proposed standard should be validated against field data. This is unlikely to be straightforward, however, due to the complexity of environmental interactions and the lack of integration of monitoring data. Field data may potentially demonstrate the existence of a healthy community at concentrations that exceed the standard. However, unless there is a large amount of reliable data on the margin of error, there will generally be insufficient data to allow the standard to be recalculated. Cost-benefit considerations will also need to be incorporated.

The approach used in the UK to derive EQSs is a pragmatic one based upon detailed scientific assessment of all the data available for the substance. Data considered include laboratory and field toxicity data, sources and releases of the substance to the environment, environmental fate and behaviour (including degradation) and potential bioaccumulation. For a given chemical, the process is as follows.

(1) Identify potential sources for input to the environment.

(2) Determine the environmental fate and behaviour.

(3) Identify the most sensitive organisms and the corresponding lowest credible adverse affect concentration, or the highest no-adverse effect concentration.

(4) Extrapolate these values to a safe concentration under field conditions to derive a preliminary EQS.

(5) Validate the preliminary EQS by comparison with field studies and monitoring data.

(6) If there are any discrepancies, repeat all stages.

(7) Establish the EQS.

Confidence in a derived EQS value is correlated with the size, reliability and relevance of the toxicological dataset from which it is derived. The ultimate aim is to determine the lowest credible concentration at which a significant effect is found and the highest concentration at which no significant effects are found.

All available data should therefore be considered and analysed in order to assess the quality, reliability and relevance to the environment being addressed. There has been considerable discussion as to what constitutes the minimum dataset that can be used to derive EQSs. Most of these discussions refer to the aquatic environment, with some less advanced ones on the terrestrial environment.

Studies on the distribution of species sensitivity suggest that the sensitivity of aquatic organisms follows a lognormal distribution and that a minimum of eight 'no observed effect concentrations' (NOECs) are required. These toxicity data should be from reliable studies and include a mixture of acute and chronic effects on species of algae, crustaceans, insects (freshwater only), non-arthropods (e.g. molluscs) and fish. If the substance under review

(e.g. an insecticide) has a well-known target group or organisms, then these species should be well represented in the dataset. Data on additional factors such as the effect of water quality parameters on toxicity should also be available. If insufficient data are available, a tentative EQS can be derived, which can subsequently be reviewed as additional data become available.

Toxicity data are usually in the form of a concentration reported in the literature from laboratory studies conducted on single species. Extrapolation of this value to a value that will protect the environment normally involves application of one or more safety factors. Safety factors are applied to account for various degrees of uncertainty including extrapolations from acute to chronic effects, laboratory to field conditions, effect concentrations to no-effect concentrations, and test species exposed in isolation to complex aquatic ecosystems. This approach is based on the assumption that, by safeguarding all individuals, the ecosystem structure and subsequent function will be protected. The safety factors used in the UK, were developed by the Organisation for Economic Co-operation and Development (OECD) [146], are 100, 10 and 1 for acute, chronic and field data, respectively. These factors are intended as guidelines and can be altered according to scientific judgement.

The approach to standard setting is more advanced for aquatic media compared with soil. This is mainly due to historical factors, as aquatic organisms were regarded as the ones most at risk from the release of chemicals from industrial processes. There are few standards for chemicals in soil, and the ones that do exist are in response to specific regulations (e.g. those governing the application of sewage sludge to soil and the remediation of contaminated land) and apply only to a small number of chemicals (mainly heavy metals). Regulation of chemicals in soil is set to improve, especially following publication of a report from the Royal Commission on Environmental Protection (RCEP) recommending that soil be accorded equal priority with air and water [166].

Future developments in the derivation of EQSs include characterisation of the variability and uncertainty in the risk assessment process. Current considerations involve the use of probabilistic techniques such as Monte Carlo analysis. Such approaches allow relationships between various factors to be taken into account and also provide the flexibility to investigate the effects of different modelling assumptions. Probabilistic approaches are likely to be included in environmental risk assessments, especially for chemicals with large underlying datasets such as plant protection products.

The data collected from protocols developed using this guidance should be useful for developing future EQSs relating to ionising radiation. Information has already been collated on factors such as husbandry, life span and behaviour for some species, but others are less well studied. It is recognised that species that have been used for setting chemical EQSs may not be the most appropriate to use for EQSs relating to ionising radiation due to considerations such as the way different contaminants behave in the environment. Consideration, however, should be given to the suitability of test species in setting future EQSs.

4.2 Test species

The following sub-sections are designed to:

- guide users through the reasons to focus on a particular wildlife group (Section 4.3);
- the generic considerations when selecting a test species (Section 4.4);
- an indication of the need for appropriate husbandry methods (Section 4.5);
- the identification of species within each wildlife group which may be recommended as appropriate test species for future chronic irradiation experiments (Sections 4.6-4.18).

Further details of the species that have been used in chronic irradiation and/or ecotoxicology experiments are provided for each wildlife group and, where possible, summaries of husbandry methods for recommended test species are provided.

4.3 Wildlife groups for which test species are proposed

The test species proposed can be categorised in the following wildlife groups:

- amphibians
- aquatic invertebrates including zooplankton
- aquatic plants
- birds
- crustaceans
- fish
- insects
- mammals
- mosses and lichens
- molluscs
- terrestrial plants

- reptiles
- soil fauna including microflora.

These wildlife groups form the major sections in GPG 1. It is important to note that the groups of organisms are somewhat artificial in their classification, for example, 'crustaceans' and 'molluscs' have been separated from 'aquatic invertebrates'. The selection of different wildlife groups is consistent with FASSET and the reasoning behind their categories is given in FASSET Deliverable 1 [185]. These wildlife groups have also been used in the development of the FASSET Radiation Effects Database (FRED) [219].

FRED has been extensively used in the production of this report. Users are encouraged to obtain a free copy of the Microsoft® Access database, which can be downloaded from http://www.fasset.org, as a starting point for reviewing literature. FRED was produced as part of FASSET Deliverable 4 on "Radiation Effects on Plants and Animals" [219] and will be referenced in this report as 'FRED'.

4.4 Selecting a test species for experimental work

Some of the main factors to be considered when selecting a test species are given in Table 4.2. Because the list is not exhaustive, species may be recommended which do not apparently fulfil the requirements given in Table 4.2. In particular, species that are of economic or ecological importance may have other features which make them unsuitable for chronic irradiation experiments, e.g. large size or annual breeding. Because rare or threatened species cannot be used as experimental organisms, species more suitable for experimentation can be used provided that they are representative of the organisms in question.

The Animals (Scientific Procedures) Act 1986 requires that all experimental or other scientific procedures carried out in the UK on vertebrates and octopus are part of a programme specified in a project licence and carried out by a person holding an appropriate personal licence from the Home Office. Therefore, it may not be possible to study the species of choice directly (unless approved by the Home Office) and that alternative species will need to be sought.

Table 4.2 Factors to consider when choosing experimental species

Economic importance	Is this organism a food source for humans? Does it have cultural value, e.g. for recreation, sport or tourism?
Ecological importance	Is this organism a key species in the food chain? Does it have a role in ecological processes, e.g. carbon and nutrient cycling?
Size	Consider: (i) laboratory space required (ii) maintenance costs, e.g. of food (iii) amount of radionuclide required (cost, radiological safety).
Breeding frequency	Does the breeding frequency of the organism allow measurement of reproductive endpoints, e.g. continuous or periodic/annual breeding?
Life-stage availability	Early life-stages (seed, seedling, egg, embryo, larva) are often more radiosensitive than adults. The ease with which these stages may be obtained and observed may be important in species choice.
Husbandry	Consider: (i) How time-consuming is the maintenance required (e.g. frequency of cleaning)? (ii) Is it possible to achieve the environmental conditions required by the organism in a laboratory environment (e.g. temperature, light)? (iii) Will the organism thrive in a laboratory environment?
Information	Is detailed information available on the use of the species in experiments (e.g. toxicology, genetics) either from the literature or directly from other scientists?
Availability	Are good quality organisms available from suppliers or from the field, and what is known about the variability of their responses?
Cost	Are the direct costs of the organisms and maintenance costs at a practical level (dependent on most of the factors noted above)?

4.5 Husbandry considerations

Good experimental design should incorporate knowledge of the conditions for maintaining test organisms in the laboratory. Husbandry methods allow organisms to be bred and maintained, both before and during the experiments, under optimum living conditions.

Factors such as diet, light regime, temperature and relative humidity must be regulated carefully to ensure that the test organisms used in the experiments are in good physical condition. This reduces the likelihood of factors other than the contaminant to which they are exposed during the experiment affecting the specific endpoints under investigation. Standardised husbandry methods also ensure that experiments conducted on the same test organism in different laboratories are comparable.

Summary tables of husbandry requirements for organisms recommended for chronic radiation exposure experiments are provided below. The data are not exhaustive and should be used as initial guidance only. The information derives from the references given in the GPG 1 Tables 4.3a-4.14a and provides an indication of the conditions and husbandry requirements of recommended organisms. In many cases, there are several alternative ways of maintaining organisms for experimental purposes. It is recommended that, before attempting any experiments, a review of relevant literature is carried out starting with the references given in the tables in GPG 1.

4.6 Amphibians

Amphibians have a life history that generally includes periods of occupancy in both aquatic and terrestrial ecosystems. Thus, they are likely to be important in assessing the impact of ionising radiation on both ecosystem types. Amphibians are also important because their numbers are declining due to habitat loss caused by human activity. Their egg hatching and early life stages, which may be the most radiosensitive stages in their life cycle, take place in the aquatic environment and this is often followed by a period of terrestrial existence during which the animals become sexually mature. Amphibians also tend to hibernate in terrestrial locations. Therefore, they spend much of their time in close proximity to sediments/soils where radionuclides may become associated. There is also limited evidence to suggest that amphibians may have slow clearance rates for some radionuclides [185].

There appear to be no reports of experiments in which the effects of chronic radiation exposures on amphibians have been examined (Table 4.3a). There are some observations on animals collected from radiation-contaminated areas, but the endpoints examined were usually cytological (e.g. micronuclei), cellular DNA content and abnormalities (e.g. [2,74]). There are several reports of acute radiation exposures, but most of these examined survival or cytological changes and none includes effects on reproductive performance.

Several species have been used in toxicology testing and results from some of these have been used for setting EQSs. However, most tests examined only survival in early pre-metamorphosis life-stages following acute exposure, although some longer exposures (100 days) of frog and toad tadpoles examined survival and metamorphosis [94]. Reproductive endpoints (oogenesis, spermatogenesis) have been examined in adult South African clawed toads fed a low boron diet for 120 days [81,82].

Several studies have examined the use of micronuclei in amphibian blood cells as biomarkers for contaminant damage, including that following acute radiation exposure [117,181,128]. Measurements of the amounts of blood cell DNA and Comet analysis of blood cell nuclei have also been carried out [52,130]. Such tests may provide useful additional information when carried out in parallel with studies on reproductive endpoints during chronic radiation experiments.

The recommendation of species for use in chronic radiation studies has to be made in the absence of any previous studies of this type and with limited observations on reproductive endpoints available from toxicology testing. The South African clawed toad is recommended because it is a standard laboratory animal, which is routinely bred and maintained in many laboratories throughout the world and with a wealth of information available in the literature and on the Internet. It has also been used in studies on reproductive endpoints [81]. Several species of frog have been used in toxicological tests and none seems to have obvious advantages for radiation research. The common British native species, *Rana temporaria*, is available from suppliers. It may be reluctant to breed when first brought into the laboratory and so should be acclimatised long before the breeding season [196].

A table is not given for the common frog (*Rana temporaria*) because details for this species were not obtained. However, a detailed description of all aspects of keeping amphibians in the laboratory is given in the Universities Federation for Animal Welfare (UFAW) Handbook on the care and management of laboratory animals [196].

Table 4.3a Amphibians used in chronic experiments

Chronic radiation experiments		
Species	Ecosystem	Reference
None recorded		
Chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Bullfrog (*Rana catesbeiana*)	All aquatic/ terrestrial	[96]
Common frog (*Rana temporaria*)		[70,91,140]
Common toad (*Bufo arenarum*)		[70]
Fowlers toad (*Bufo fowleri*)		[213]
Frog (*Disco glossus*)		[70]
Frog (*Rana fusca*)		[70]
Frog (*Rana palustris*)		[213]
Frog (*Rana sp.*)		[10,11]
Leopard frog (*Rana pipiens*)		[70,96]
Leopard frog (*Rana pipiens*)		[213]
South African clawed toad (*Xenopus laevis*)		[10,11,16,213]
Toad (*Bufo sp*)		[10,11,96]
Recommended species	**Ecosystem**	**Husbandry method**
		See Table 4.3b
Common frog (*Rana temporaria*)	All aquatic/ terrestrial	[70,89,140]
South African clawed toad (*Xenopus laevis*)		[8,10,11,16,81,82,213]

Table 4.3b Husbandry considerations for South African clawed frog (Xenopus laevis)

Rearing	Adults kept at a density of 4-6 per 1,800 cm^2 of water surface area; frogs paired, one pair per breeding tank for mating and egg laying
Diet	Ground beef liver with multi-vitamin supplement three times a week
Temperature	23±3°C for holding; 21±2°C for breeding
Water	Aerated, preferably well or spring water but dechlorinated tapwater can be used; flow through system preferable, but static can be used; pH 6.5-9.0
Tank size	Large aquaria or raceways of stainless steel or fibreglass; sides opaque and at least 30 cm high; water depth 7-14 cm Breeding tanks are 5-10 gallon glass aquaria with a 1 cm nylon or plastic mesh placed 3 cm from the tank bottom to allow eggs to pass through.
Light regime	12 hours light and 12 hours dark
Notes	Breeding induced by injection of human chorionic gonadotrophin.

4.7 Aquatic invertebrates including zooplankton

As a wildlife group, 'aquatic invertebrates' covers organisms in all three major compartments of the aquatic ecosystem (the sediment, sediment surface and water column). Benthic invertebrates, either within the sediment or on the sediment surface, will receive elevated radiation doses due to the tendency for many radionuclides to become associated with sediments - as described by the partition coefficient (K_d). The invertebrates associated with the benthic component are also an important link in the cycling of nutrients within the system.

Zooplankton are known to bioconcentrate certain radionuclides - particularly alpha emitters [185] - but they are also an important group because many aquatic organisms have zooplanktonic larval forms. Consequently, the protection of this group is extremely important if the populations of aquatic organisms considered within other wildlife groups are to remain unaffected.

The most detailed chronic radiation exposure experiments on aquatic invertebrates have involved the polychaetes, *Neanthes arenaceodentata and Ophryotrocha diadema* [92,114]. The effects of exposure over a lifetime or several generations on reproductive output have been studied in these species. They have also been used in chronic, life-cycle toxicology tests along with other polychaetes. Such tests have also been carried out using midges, tubifex worms and hydroids. Toxicology tests involving other species have generally been with acute exposures and often on larval stages.

Cytological damage caused by radiation and toxic chemicals has been investigated in *N. arenaceodentata* (e.g. [156]), another polychaete *Platynereis dummerili* [108], and midge larvae [23]. If it is possible to carry them out, biomarker tests could be conducted during chronic radiation studies while examining reproductive endpoints.

Although any of the species listed in Table 4.4a may be useful for examining the effects of chronic radiation, those recommended have been used successfully in either radiation studies or chronic toxicology studies. Three methods are well documented for their maintenance and the measurement of endpoints relevant to radiation exposure. The recommended species include members of three different phyla: Coelenterata, Annelida and Arthropoda.

Table 4.4a Aquatic invertebrates and zooplankton used in chronic experiments

Chronic radiation experiments		
Species	Ecosystem	Reference
Ascidian (*Mogula manhattensis*)	Saltwater	FRED
Coral (*coelenterate*) (*Astrangia danae*)	Saltwater	FRED
Midge (*insect larvae*) (*Chironimus tentans*)	Freshwater	FRED
Polychaete worm (*Ophryotrocha diadema*)	Saltwater	FRED
Polychaete worm (*Neanthes arenaceodentata*)	Saltwater	FRED
Chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Caddisfly larva (*Chimarra marginata*)	Freshwater	[70]
Caddisfly larva (*Hydropsyche exocella*)	Freshwater	[70]
Coelenterate (hydroid) (*Laomedea flexuosa*)	Saltwater	[110,210]
Coelenterate (hydroid) (*Eirene vidula*)	Saltwater	[110,210]
Diving beetle (*Dytiscus sp.*)	Freshwater	[140]
Mayfly larva (*Hexagenia spp.*)	Freshwater/Sediment	[8,10,11,18,104]
Mayfly larva (*Baetis sp.*)	Freshwater	[58]
Mayfly larva (*Ephemerella sp.*)	Freshwater	[58]
Midge larva (*Chironomys tentans*)	Freshwater/Sediment	[8,10,11,18,104]
Oligochaete (*Tubifex tubifex*)	Freshwater/Sediment	[8,10,11,18,104]
Oligochaete (*Lumbriculus variegatus*)	Freshwater/Sediment	[104]
Planarian (*Dugesia tigrina*)	Freshwater	[58]
Polychaete worm (*Ophryotrocha diadema*)	Saltwater	[8,10,14,210]
Polychaete worm (*Neanthes arenaceodentata*)	Saltwater/Sediment	[8,10,14,167,210]
Polychaete worm (*Capitella capitata*)	Saltwater/Sediment	[8,10,14,167,210]
Polychaete worm (*Dinophilus gyrociliatus*)	Saltwater	[8,10,14,210]
Polychaete worm (*Ctenodilus serratus*)	Saltwater	[167,210]
Polychaete worm (*Nereis virens*)	Saltwater/Sediment	[8,10,11,18,104]
Polychaete worm (*Arenicola cristata*)	Saltwater	[210]
Polychaete worm (*Arenicola marina*)	Saltwater	[210]
Polychaete worm (*Platynereis dumerilli* (larva))	Saltwater	[108]
Rotifer (*Brachionis calyciflorus*)	Freshwater	[7,58,213]
Rotifer (*Brachionis rubens*)	Freshwater	[58]
Rotifer (*Brachionis plicatilis*)	Saltwater/Estuarine	[58]
Rotifer (*Philodina acuticornis*)	Freshwater	[70]
Stonefly larva (*Pteronarcys sp.*)	Freshwater	[58,140]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.5b-d
Coelenterate (hydroid) (*Laomedea flexuosa* or *Eirene vidula*)	Saltwater	[110,210]
Midge larva (*Chironomys tentans*)	Freshwater/estuarine	[8,10,11,18,104]
Polychaete worm (*Ophryotrocha diadema*)	Saltwater	[114,167]

Table 4.4b Husbandry considerations for hydroid (*Laomedea flexuosa* or *Eirene viridula*)

Rearing	*E. viridula* reared as free-floating colonies. *L. flexuosa* reared as attached colonies to glass plates. *E. viridula* attached by nylon thread for experiments.
Maintenance	Colonies of *E. viridula* rejuvenated by cutting; *L. flexuosa* by removing part of colony and attaching to a new glass plate.
Diet	*Artemia* larvae twice a week; colonies transferred to new tank 1 hour after feeding (removal from excreta and excess *Artemia*); *L. flexuosa* fed daily during experiments
Temperature	20°C
Tank size	2 l glass aquaria containing good quality, filtered (0.15 µm) aerated seawater. Colonies attached on glass plates placed in tank.
Light regime	Culture in total darkness

Table 4.4c Husbandry considerations for midge (Chironomus tentans)

Rearing	12 larvae per experimental chamber
Maintenance	Check and feed daily.
Diet	1.5 ml TetraFin™ (goldfish food) per tank/day
Temperature	23±1°C
Tank size	Experimental chamber: 300 ml lipless beaker with screened ports to allow water overflow; 100 ml sediment and 175 ml water; change water twice daily (12 hours apart) or continuous flow
Light regime	16 hours light and 8 hours dark; 100-1,000 lux

Table 4.4d Husbandry considerations for polychaete worm (Ophryotrocha diadema)

Rearing	Stock: 40-50 worms per tank; experimental ~6 worms per dish
Maintenance	Stock: change every 5-6 weeks (new tanks with ~50 worms); feed once a week. Experimental: change at egg counting, 1-2 times per week; feed after changing
Diet	Spinach - finely divided in a blender or by scraping from frozen block
Temperature	18-20°C
Salinity	~32‰
Tank size	Stock: 4 l tank containing 2.5 l aerated seawater Experimental: Petri dish 50 x 10 mm containing ~10 ml seawater
Light regime	12 hours light and 12 hours dark; not of great importance

4.8 Aquatic plants

Aquatic plants, including phytoplankton, are the primary producers in aquatic ecosystems. Other trophic levels depend on the energy fixed by these organisms, so anything that negatively impacts on aquatic plants is likely to have effects within the food chain. It is therefore important to ensure that these primary producers are protected adequately. Aquatic plants are also known to bioconcentrate certain radionuclides [185].

The aquatic plant species used in chronic radiation experiments are shown in Table 4.5a. The number of laboratory studies in which chronic radiation exposure of aquatic plants (i.e. algae and cyanobacteria) has been considered is very limited for doses or dose rates relevant for natural ecosystems. Only one species, the cyanobacteria *Syenechococcus lividus*, has been studied for chronic low dose gamma irradiation [54,55]. This irradiation was shown to have an inhibitory or stimulatory effect depending on the growth phase of the cells exposed.

The number of aquatic plant species used in ecotoxicological testing is fairly small (Table 4.5a). Species recommended for use in chronic, long-term radiation experiments are also given. Because of the lack of information on radiation effects on aquatic plants, the species recommended are all standard

ecotoxicity test species. While these species have not been used in radiation experiments, they are used extensively by OECD for ecotoxicological testing of a range of contaminants [153,154]. There is thus much information on the culturing conditions, inhibition responses and variability of responses.

Ecotoxicity tests have shown that there is a wide variation in sensitivity to pollutants between different species and groups of aquatic plants and algae. It is, therefore, recommended that a range of species representing the different groups is studied, as it is possible that different species will vary widely in response to irradiation. Two aquatic plant species, a freshwater green alga and a cyanobacteria, are therefore recommended. Other species listed in Table 4.5a for ecotoxicity tests could also be used [153,154].

Endpoints studied have focused on morbidity or mortality using inhibition of growth and algal biomass density. However, finding the most appropriate endpoint for single cells/cell cultures is not straightforward. For instance, reduced cell growth after irradiation may be due to both inactivation of cell division and increased death of cells. Endpoints for mutation and reproduction have not been used in chronic studies; therefore, the recommendations Table 4.5a are made using information from acute studies.

Table 4.5a Aquatic plants used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Cyanobacteria		
Syenchococcus lividus	Freshwater	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Green algae		
Pseudokirchneriella subcapitata	Freshwater	[154]
Scenedesmus subspicatus	Freshwater	[154]
Diatoms		
Navicula pelliculosa	Freshwater	[154]
Cyanobacteria		
Anabaena flos-aquae	Freshwater	[154]
Synechococcus leopoldensis	Freshwater	[154]
Aquatic plants		
Fat duckweed (*Lemna gibba*)	Freshwater	[153]
Common duckweed (*Lemna minor*)	Freshwater	[153]
Lemna aequinoctialis	Freshwater	[153]
Lemna major	Freshwater	[153]
Lemna paucicostata	Freshwater	[153]
Lemna perpusilla	Freshwater	[153]
Ivy-leaved duckweed (*Lemna trisulca*)	Freshwater	[153]
Lemna valdiviana	Freshwater	[153]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.5b-e.
Green algae		
Anabaena flos-aquae	Freshwater	[153]
Cyanobacteria		
Pseudokirchneriella subcapitata	Freshwater	[153]
Aquatic plants		
Common duckweed (*Lemna minor*)	Freshwater	[154]
Fat duckweed (*Lemna gibba*)	Freshwater	[154]

Table 4.5b Husbandry considerations for *Anabaena flos-aquae*

Maintenance	Stock: cultures should be grown in conical flasks in growth media and sub-cultured weekly under the conditions described in this table. When not in regular use, cultures can be streaked on agar slopes and sub-cultured twice a month.
Diet	Growth medium designed for freshwater algae and cyanobacteria, e.g. TG 201 media (OECD) or AAP media (US EPA) should be used [154].
Temperature	21-24°C
Tank size	Glass flasks of sufficient volume to obtain a surface volume ratio of 0.15 cm^2/ml, e.g. 250 ml conical flask containing 100 ml media
Light regime	Surface of the cultures should receive continuous and uniform fluorescent illumination of 'cool-white' or 'daylight' type. Light intensity should be 60-120 $\mu E/m^2/s$ measured in the PAR range 400-700 nm.
Notes	Full details of culturing conditions and growth media are given in [154].

Table 4.5c Husbandry considerations for *Lemna gibba*

Maintenance	Stock: cultures can be held under reduced illumination and temperature (4-10°C). Under normal conditions, monthly sub-culturing of stock cultures is advised.
	Experimental: plants should be obtained from a culture collection or another laboratory, and grown in the same medium used for testing for 8 weeks prior to use.
Diet	Growth medium designed for *Lemna gibba* should be used [153].
Temperature	24 ± 2°C
Tank size	Minimum depth 20 mm, minimum volume 100 ml
Light regime	Plants should be grown under a continuous light source with photosynthetically-active radiation 400-700 nm (6,500-10,000 lux).
Notes	Full details of culturing conditions and growth media are given in [153].

Table 4.5d Husbandry considerations for *Lemna minor*

Maintenance	Stock: cultures can be held under reduced illumination and temperature (4-10°C) and sub-cultured three times a month. Under normal conditions and temperature, monthly sub-culturing of stock cultures is advised.
	Experimental: plants should be obtained from a culture collection or other laboratory and grown in the same medium used for testing for 8 weeks prior to use.
Diet	Growth medium designed for *Lemna minor* should be used [153].
Temperature	24 ± 2°C
Tank size	Minimum depth 20 mm, minimum volume 100 ml
Light regime	Plants should be grown under a continuous light source with photosynthetically-active radiation 400-700 nm (6,500-10,000 lux).
Notes	Full details of culturing conditions and growth media are given in [153].

Table 4.5e Husbandry considerations for *Pseudokirchineriella subcapitata*

Maintenance	Stock: cultures should be grown in conical flasks in growth media and sub-cultured weekly under the conditions described in this table. When not in regular use, cultures can be streaked on agar slopes and sub-cultured twice a month.
Diet	Growth medium designed for freshwater algae and cyanobacteria, e.g. TG 201 media (OECD) or AAP media (US EPA) should be used [154].
Temperature	21-24°C
Tank size	Glass flasks of sufficient volume to obtain a surface volume ratio of 0.15 cm^2/ml, e.g. 250 ml conical flask containing 100 ml media
Light regime	Surface of the cultures should receive continuous and uniform fluorescent illumination of 'cool-white' or 'daylight' type. Light intensity should be 60-120 $\mu E/m^2/s$ measured in the PAR range 400-700 nm.
Notes	Full details of culturing conditions and growth media are given in [154]. Cultures can be obtained from the Culture Collection of Algae and Protozoa (CCAP).

4.9 Birds

Birds are present in both terrestrial and aquatic ecosystems, and many species inhabit both at different stages of their lives. Further importance is placed on birds due to UK and European environmental protection legislation. However, little is known about the way in which radionuclides are accumulated in many of the bird species requiring protection under the Habitats Directive and the way in which chronic doses may affect them [61].

Table 4.6a lists the species of birds used in chronic radiation and chemical ecotoxicity experiments, and the recommended species for the development of the experimental protocols.

Several species have been used in radiation exposure experiments. The following species have been used in field studies looking at nesting success, growth, radiosensitivity of embryos and gonadal development: barn swallow (*Hirundo rustica*); northern bobwhite quail (*Colinus virginianus*); and the house wren (*Troglodytes aedon*) ([135,136,225]. Laboratory studies have investigated body and gonadal weights, numbers of oocytes in female bird ovaries, numbers of spermatogonia in male bird testes and survival rates in the barred rock chicken and the blackheaded gull [138,157].

Fewer bird species appear to have been used in standard chronic or life-cycle toxicology tests. These include the Japanese quail, mallard, northern bobwhite quail and pigeon. The Japanese quail, northern bobwhite and the mallard are recommended for long-term chronic radiation experiments due to the extensive data available and their ease of maintenance in a laboratory environment.

Japanese quail (*Coturnix spp.*) is the standard toxicology test species for avian toxicology tests and there is a record in the FRED for *Coturnix* sp. in a field-based chronic irradiation study. The *Japanese* quail (*Coturnix coturnix japonica*) has been recommended by both the OECD [150] and the US EPA [201] for avian reproduction toxicity tests. It is therefore recommended as a suitable test species for chronic irradiation studies.

The northern bobwhite quail has also been recommended as a test species for reproduction toxicity tests by both the OECD [150] and the US EPA [201]. There is only one record of a chronic radiation study on this species in the FRED and that study was field-based rather than laboratory-based. As the northern bobwhite is a US EPA preferred test species, chemical toxicity data are already available along with detailed information on endpoint measurements and assessments. Using this species as a test species for chronic irradiation studies will increase our understanding of the effects of ionising radiation on terrestrial birds and the data will complement the chemical data that already exist for this species.

Both the Japanese quail and the northern bobwhite quail are terrestrial birds. Te third recommended test species is therefore one that is present in the aquatic ecosystem. The mallard (*Anas platyrhynchos*) is not listed in the FRED as having been the subject of chronic radiation exposure studies, but this species is the preferred test species for chemical toxicity tests proposed by the US EPA [201,203]. Consequently, there is likely to be considerable data for this species with respect to non-radioactive contaminants.

Table 4.6a Birds used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
American robin (*Turdus migratorius*)	Terrestrial	FRED
Barn swallow (*Hirundo rustica*)	Terrestrial	FRED
Black-headed gull (*Larus ridibundus*)	Terrestrial	FRED
Bluebird (*Sialia sialis*)	Terrestrial	FRED
Brown-headed cowbird (*Molothrus ater*)	Terrestrial	FRED
Chicken	Terrestrial	FRED
Common flicker (*Colaptes auratus*)	Terrestrial	FRED
Coturnix quail (*Coturnix sp.*)	Terrestrial	FRED
Gray jay (*Perisoreus canadensis*)	Terrestrial	FRED
Great crested flycatcher (*Myiarchus crinitus*)	Terrestrial	FRED
Hermit thrush (*Catharus guttata*)	Terrestrial	FRED
House wren (*Troglodytes aedon*)	Terrestrial	FRED
Lincolns sparrow (*Melospiza lincolnii*)	Terrestrial	FRED
Northern bobwhite quail (*Colinus virginianus*)	Terrestrial	FRED
Ovenbird (*Seiurus aurocapillus*)	Terrestrial	FRED
Ring-necked pheasant (*Phasianus colchicus*)	Terrestrial	FRED
Swamp sparrow (*Melospiza georgiana*)	Terrestrial	FRED
Swallow (*Iridoprocne bicolor* or *Tachycineta bicolor*)	Terrestrial	FRED
Tufted titmouse (*Parus bicolor*)	Terrestrial	FRED
Weaver finch (*Quelea quelea*)	Terrestrial	FRED
White crowned sparrow (*Zonotrichia leucophrys*)	Terrestrial	FRED
White leghorn (*Gallus domesticus*)	Terrestrial	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Japanese quail (*Coturnix coturnix japonica*)	Terrestrial	[150,203]
Mallard (*Anas platyrhynchos*)	Terrestrial	[201,203]
Northern bobwhite quail (*Colinus virginianus*)	Terrestrial	[150,201,203]
Pigeon (*Columba livia*)	Terrestrial	[203]
Red-legged partridge (*Alectoris rufa*)	Terrestrial	[203]
Ring-necked pheasant (*Phasianus colchicus*)	Terrestrial	[203]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.6b-d
Japanese quail (*Coturnix coturnix japonica*)	Terrestrial	
Northern bobwhite quail (*Colinus virginianus*)	Terrestrial	
Mallard (*Anas platyrhynchos*)	Terrestrial	
Chicken	Terrestrial	

Table 4.6b Husbandry considerations for bobwhite quail (*Colinus virginianus*)

Rearing/growing	Eggs are candled to check for abnormalities and fine cracks. The remaining eggs are equilibrated to room temperature and set in an incubator (37.5-37.8°C at 50-70% humidity). Fertility and embryo viability are checked by candling after 11 days. After 20-21 days, the eggs are transferred to a hatcher and candled again. Hatching should occur after 24-25 days. Chicks should be dry after hatching before they are removed from the hatcher. Chicks may be housed together.
Maintenance	Adult birds should be housed in pairs (one female and one male), though pen mate aggression can occur. Diet and drinking water should be provided *ad libitum*.
Diet	27-29% crude protein; 3.0-5.0% crude fibre; 2.5-7.0% crude fat; 2.6-3.6% calcium; 0.9-1.1% phosphorous
Temperature	15-27°C
Relative humidity	40-80%
Cage/tank size	Minimum floor area 1,000 cm^3 (500 cm^3 per bird [201,203])
Light regime	8 hours light and 16 hours dark
Notes	14 day acclimatisation period required for acquired adult birds. It is recommended that pens/cages are not stacked upon each other unless the test substance is the same in each pen.

Table 4.6c Husbandry considerations for Japanese quail (*Coturnix coturnix japonica*)

Rearing/growing	Eggs are candled to check for abnormalities and fine cracks. The remaining eggs are equilibrated to room temperature and set in an incubator (37.5-37.8°C at 50-70% humidity). Fertility and embryo viability is checked by candling after 8 days. After 15-16 days, the eggs are transferred to a hatcher and candled again. Hatching should occur after 17-18 days. Chicks should be dry after hatching before they are removed from the hatcher. Chicks may be housed together.
Maintenance	Adult birds should be housed in pairs (one female and one male), though pen mate aggression can occur. Diet and clean drinking water should be provided *ad libitum*.
Diet	A standard commercial game bird feed containing 27-29% crude protein, 3.5-5.0% crude fibre, 2.5-7.0% crude fat, 2.6-3.6% calcium and 0.9-1.1% phosphorous.
Temperature	15-27°C
Relative humidity	40-80%
Cage/tank size	Minimum floor area 1,000 cm^3 (500 cm^3 per bird [201,203])
Light regime	8 hours light and 16 hours dark

Table 4.6d Husbandry considerations for mallard (*Anas platyrhynchos*)

Rearing/growing	Eggs are candled to check for abnormalities and fine cracks. The remaining eggs are equilibrated to room temperature and set in an incubator (37.5-37.8°C at 50-70% humidity). Fertility and embryo viability is checked by candling after 14 days. After 21 days, the eggs are transferred to a hatcher and candled again. Hatching should occur by day 24. Chicks should be dry after hatching before they are removed from the hatcher. Chicks may be housed together.
Maintenance	Diet and clean drinking water should be provided *ad libitum*.
Diet	Standard commercial duck bird feed
Temperature	15-27°C
Relative humidity	45-70%
Cage/tank size	Minimum floor area 2,000 cm^3 (1,000 cm^3 per bird [201,203])
Light regime	8 hours light and 16 hours dark
Notes	Prone to regurgitating doses. 14 day acclimatisation period required for acquired adult birds. It is recommended that pens/cages are not stacked upon each other unless the test substance is the same in each pen.

4.10 Crustaceans

Both crustaceans and molluscs (Section 4.14) are known to accumulate certain radionuclides, although the concentration factors associated with the accumulation of particular radionuclides often vary between the two groups. Crustaceans, for example, are known to accumulate ^{99}Tc more effectively than molluscs, but for ^{239}Pu the reverse is true [185]. The intimate association of crustaceans and molluscs with sediments brings them into contact with the key radionuclide sink of the aquatic ecosystem.

There are few reported studies on the effects of chronic radiation exposure on crustaceans. The most comprehensive involved exposure of populations of the cladoceran *Daphnia pulex* [132,133], while exposures of young blue crab, *Callinectes sapidus* [56], and larval goose barnacles, *Pollicipes polymerus* [1], have also been carried out. Effects of acute radiation exposures have been examined in a greater number of species, but mainly with mortality as an endpoint (refer to the FRED for detailed information about individual experiments).

Several species have been used in chronic toxicology tests and data from some of these have been used in developing EQSs. Species of the freshwater *Daphnia* (especially *D. magna, D. pulex*) have been widely used for acute and chronic exposure studies and the US EPA [201] and OECD [149] require them as experimental animals. Methods for maintaining and testing them have been standardised and compared in ring tests [24]. Estuarine and marine species used in toxicological studies include copepods, shrimps and mysids [13]. The calanoid copepod *Acartia tonsa* [210] and the harpacticoid copepod *Tisbe battagliai* [100] have both been used successfully in life-cycle studies. *Mysidopsis bahia* is the most commonly used marine crustacean for acute and chronic tests in the USA [210] and, although not native to European waters, it is also used in toxicity tests in the UK and Europe.

Limited biomarker tests have been conducted on crustaceans. However, the Comet assay has been used successfully to examine DNA damage in grass shrimp embryos [125] and *Daphnia magna* [62] following exposure to a variety of toxicants.

Species recommended for use in chronic, long-term radiation experiments are given in Table 4.7a. It should be stressed that many of the species previously used in radiation or toxicology tests may prove to be useful experimental models for long-term irradiation. However, the recommended species have already been shown to have particular qualities, which will increase the likelihood of experimental success. They have all been used in long-term chronic toxicological tests where reproductive endpoints were examined. All species are of relatively small size and can be kept in large numbers in limited laboratory space. They breed continuously under the right conditions and thus reproductive output can be readily measured. There appears to be no chronic toxicological studies involving reproductive endpoints on larger, economically important, decapod species (e.g. crab and lobster), probably because of the difficulties involved in keeping these animals from early stages through to adulthood. However, methods for rearing larval crabs through several stages of development are available [25].

Table 4.7a Crustaceans used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Blue crab (*Callinectes sapidus*)	Saltwater	FRED
Goose barnacle (*Polliceps polymerus*)	Saltwater	FRED
Water flea (*Daphnia pulex*)	Saltwater	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Amphipod (*Gammarus lacustris*)	Freshwater	[6,58]
Amphipod (*G. fasciatus*)	Freshwater	[6,58]
Amphipod (*G. pseudolimnaeus*)	Freshwater	[5,58,140]
Amphipod (*Hyallela azteca*)	Freshwater/sediment	[8,12,18,104]
Amphipod (*Diporeia sp.*)	Freshwater/sediment	[8,12,18,104]
Amphipod (*Gammarus pseudolimnaeus*)	Freshwater	[140]
Amphipods (*Grandiriella lutosa*)	Estuarine	[53,70]
Blue crab (*Callinectes sapidus*)	Saltwater	[70]
Copepod (*Acartia tonsa*)	Saltwater	[95,210,211]
Copepod (*Tisbe battagliai*)	Saltwater	[100]
Copepod (*Tigriopus brevicornis*)	Saltwater	[124]
Daphnid (*Ceriodaphnia dubia*)	Freshwater	[58]
Freshwater crab (*Barytelphusa querini*)	Freshwater	[70]
Grass shrimp (*Palaemonetes spp.*)	Saltwater	[8,12,18,34,210]
Lobster (*Homarus americanus*)	Saltwater	[70]
Mysid (*Mysidopsis bahia*)	Saltwater	[8,12,18,210]
Water flea (*Daphnia pulex (magna)*)	Freshwater	[8,12,18,58,149]
Water flea (*Moinodaphnia macleayi* and *G.ligorum* (mixed))	Freshwater	[140]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.7b & c.
Copepod (*Tisbe battagliai*)	Saltwater	[100]
Grass shrimp (*Palaemonetes spp.*)	Saltwater	[8,12,18,34,210]
Mysid (*Mysidopsis bahia*)	Saltwater	[8,12,18,58]
Water flea (*Daphnia pulex*)	Saltwater	[8,12,18,58,149]

Table 4.7b Husbandry considerations for copepod (*Tisbe bataglia*)

Rearing	Experiment: larval and adults reared individually in microplates.
Maintenance	Experiment: static system with renewal of water in wells three times a week
Diet	Stock: mixed algae (Isochrysis galbana, Rhodomonas reticulata) 5 x 10^7 cells/ml
	Experiment: *I. galbana* 5 x 10^7 and *R. reticulata* 3 x 10^7 cells/ml
Temperature	20±1°C
Water	~35‰, 0.2 µm filtered natural seawater
Tank size	Experiment: 24 well microplates with 2 ml seawater/well for developing stages and 12 well plates with 5 ml /well for adults
Light regime	16 hours light and 8 hours dark

Table 4.7c Husbandry considerations for water flea (*Daphnia pulex*)

Rearing	Experiment: daily count of live and dead young and adults to give numbers per live adult
Maintenance	Experiment: 10 less than 24-hours old animals per tank
Diet	Alga *Selenastrum capricornutum*, yeast-trout chow-cerophyl suspension; continuous feeding at 0.1-0.2 mg organic carbon/daphnia/day.
Temperature	20±2°C
Water	Freshwater: 17 volume additions per 24 hours
Tank size	100 mm diameter crystallising dishes with 300 ml water
Light regime	16 hours light and 8 hours dark
Notes	Life-cycle test is usually for 21 days.

4.11 Fish

The bioaccumulation of radionuclides in fish is of interest from both an environmental protection and a human protection point of view. Aquatic ecosystems support both benthic and pelagic fish, so different species are exposed to ionising radiation in different ways and to different extents. Fish are an important group within aquatic foodwebs, and the inter-specific variation in adaptations and behaviours makes fish important at a number of trophic levels.

The fish species used in previous chronic radiation experiments are shown in Table 4.8a. The experiments providing data most relevant to possible radiation damage to fish populations are those using guppy (e.g. [74]) and zebrafish (e.g. [111]). These involved radiation exposure for a year or more, including during development to sexual maturity and throughout much of the period of sexual activity. Some experiments with medaka were also over relatively long periods and examined effects on gonad development [101].

Many species have been used in toxicological testing (e.g. [137]) and some of the most common are listed in Table 4.8a. Most of the species used in chronic radiation studies are found in US waters and none are native to the UK. However, some (e.g. fathead minnow, zebrafish) are commonly used in UK laboratories.

A range of biomarker tests has been carried out successfully on fish, e.g. micronucleus test [40,97], cytological damage [163] and Comet assay [21].

Species recommended for use in chronic, long-term radiation experiments are also given in Table 4.8a, as data support their selection. It should be stressed that many of the other species used in radiation or toxicology tests might also prove useful experimental models for long-term irradiation.

Although fathead and sheepshead minnows have not been used in radiation experiments, they are both used extensively for examining acute and toxic effects of pollutants and are given as a principal test species by the US EPA [206,207]. All the species recommended in this guidance are also recommended by the OECD [147,148] for use in toxicity testing. Only one species, the sheepshead minnow, inhabits saltwater, while the others are freshwater; only the rainbow trout is found naturally in British waters. However, it is considered that they are all representative of fish likely to be found in or around Britain and results should be applicable to UK species.

All recommended species can be obtained from scientific suppliers or fish hatcheries and maintained and bred easily in the laboratory. Details on husbandry are referenced in Table 4.8a. With the exception of rainbow trout (which only breeds annually), the recommended species can be bred continuously in the laboratory enabling irradiation of all life-stages to be carried out from an early age and reproductive activity to be measured readily. They are relatively small and can be kept in quite large numbers in limited space. Rainbow trout must be maintained in flowing water and this would probably make exposure to radionuclides via water difficult. In this circumstance, spiked food pellets could be used to deliver the radionuclides.

Table 4.8a Crustaceans used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Atlantic salmon (*Salmo salar*)	Freshwater	FRED
Bighead (*Aristichthys nobilis*)	Freshwater	FRED
Brown trout (*Salmo trutta*)	Freshwater	FRED
Carp (*Cyprinus carpio*)	Freshwater	FRED
Chinook salmon (*Onchorhynchus tschawytscha*)	Freshwater	FRED
Coho salmon (*Onchorhynchus kistuch*)	Freshwater	FRED
Eelpout (*Zoarces viviparus*)	Saltwater/brackish	FRED
Fathead minnow (*Pimephales promelas*)	Freshwater	FRED
Goby (*Chasmichthys glosus*)	Saltwater	FRED
Goldfish (*Carassius auratus*)	Freshwater	FRED
Guppy (*Poecilia reticulata; Lebistes reticulatus*)	Freshwater	FRED
Loach (*Misgurnis fossilis*)	Freshwater	FRED
Medaka (*Oryzias latipes*)	Freshwater	FRED
Mosquitofish (*Gambusia affinis*)	Freshwater	FRED
Northern pike (*Esox lucius*)	Freshwater	FRED
Pinfish (*Lagodon rhomboides*)	Freshwater	FRED
Plaice (*Pleuronectes platessa*)	Saltwater	FRED
Rainbow trout (*Onchorhyncus mykiss; S. gairdneri, S irideus*)	Freshwater	FRED
Trout (*Salvelinus leprechini*)	Freshwater	FRED
Whitefish (*Coregonus peled*)	Freshwater	FRED
Zebrafish (*Danio rerio*)	Freshwater	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Three-spine stickleback (*Gasterosteus aculeatus*)	Freshwater/saltwater	[58]
Bluegill (*Lepomus macrochirus*)	Freshwater	[58]
Bluegill (*Lepomus macrochirus*)	Freshwater	[58]
Brook trout (*Salvelinus fontinalis*)	Freshwater	[58,134]
Carp (*Cyprinus carpio*)	Freshwater	[58,172]
Coho salmon (*Onchorhynchus kistuch*)	Freshwater	[58]
English sole (*Parophrys vetulus*)	Saltwater	[210]
Fathead minnow (*Pimephales promelas*)	Freshwater	[58,121]
Flounder (*Paralichthys sp.*)	Saltwater	[210]
Goldfish (*Carassius auratus*)	Freshwater	[58]
Guppy (*Poecilia reticulata*)	Freshwater	[58]
Herring (*Clupea harengus*)	Saltwater	[210]
Longnose killifish (*Fundulus similis*)	Saltwater	[210]
Mummichog, killifish (*Fundulus heteroclitus*)	Saltwater	[210]
Northern pike (*Esox lucius*)	Freshwater	[58]
Pinfish (*Lagodon rhomboides*)	Saltwater	[210]
Rainbow trout (*Onchorhyncus mykiss*)	Freshwater	[58]
Sheepshead minnow (*Cyprinodon variegatus*)	Saltwater	[90,210]
Shiner perch (*Cymatogaster aggregata*)	Saltwater	[210]
Silverside (*Menidia sp.*)	Saltwater	[210]
Spot (*Leiostomus xanthurus*)	Saltwater	[210]

Table 4.8a Cont'd

Starry flounder (*Platichthys stellatus*)	Saltwater	[210]
Whitesucker (*Catostomus commersoni*)	Freshwater	[58]
Zebrafish (*Danio rerio*)	Freshwater	[58,169,208]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.8b-f.
Fathead minnow (*Pimephales promelas*)	Freshwater	[155]
Guppy (*Poecilia reticulata*)	Freshwater	[218]
Rainbow trout (*Onchorhyncus mykiss*)	Freshwater	[112]
Sheepshead minnow (*Cyprinodon variegatus*)	Saltwater	[210]
Zebrafish (*Danio rerio*)	Freshwater	[111,212]

Table 4.8b Husbandry considerations for fathead minnow (*Pimephales promelas*)

Rearing	Five fish (each 1 inch long) per tank (three females, two males); eggs attached to spawning sites removed and kept in aerated flowing water to hatch, and then reared in separate tanks.
Maintenance	Remove uneaten food and faeces as necessary.
Diet	Promin™ fish pellets daily, supplemented with frozen brine shrimp; larvae fed newly hatched brine shrimp twice daily.
Temperature	Allow to vary with season: Dec-Mar 15°C ; Jun-Sept 24-26°C.
Water	Continuous flow system; carbon filtered tap or spring water; dechlorinated; UV-sterilised, 5 µm filtered; 9 l tank receiving 50 ml/min; 45 l tank receiving 225 ml/min; pH 7.1-8.4
Tank size	Larvae/juveniles: 25 per 9 l tank. Adults: 50 fish per 45 l tank, reduced to four pairs (tank subdivided to give four compartments) each with a polyethylene breeding tile
Light regime	10 h 45 m at hatch; 14 h 15 m at 139 days post hatch; 15 h 45 m at 155-261 days post hatch (spawning period); 13 h 30 m post spawning period 490-680 lux

Table 4.8c Husbandry considerations for guppy (*Poecilia reticulata*)

Rearing	Experiment: pairs reared together from 3 days with re-adjustment to male/female when sexes identifiable at ~50 days.
Maintenance	Inspection twice daily and once daily at weekends. Plastic 'weed' for offspring to hide in. Offspring counted and removed to separate aquaria as soon as they are observed.
Diet	Dried flake food supplemented with brine shrimp nauplii daily.
Temperature	22-24.5°C
Water	Tap water allowed to stand and equilibrate to temperature.
Tank size	2 l aquaria containing 1.5 l of water
Light regime	Not given
Notes	Guppies are livebearers.

Table 4.8d Husbandry considerations for rainbow trout (*Onchorhynchus mykiss*)

Rearing	Eggs in trays with hatched fish passing through holes into the main tank.
Maintenance	Check twice daily (once daily at weekends). Flowing water keeps tanks clear; may be turned off temporarily and any faeces, etc. removed by siphoning. Check chlorine levels regularly (e.g. weekly); must be below ~0.1 mg/l.
Diet	Trout feed, pellet size and amount depending on fish size.
Temperature	Allowed to vary during year: minimum 8°C and maximum 18°C.
Water	All stages require well-aerated, dechlorinated, running water.
Tank size	Depends on fish size, e.g. 50 young adult (~200 days old) fish in 450 l tank.
Light regime	As natural

Table 4.8e Husbandry considerations for sheepshead minnow (*Cyprinodon variegates*)

Rearing	Mating with two males and three females per spawning chamber; embryos and fry reared in special chambers.
Maintenance	Clean aquaria regularly using siphon with a mesh over the end to prevent fish being siphoned off.
Diet	Fry: live brine shrimp nauplii 2-3 times per day. Juveniles and adults: dry fish food (e.g. TetraMin®) twice daily supplemented with frozen brine shrimp.
Temperature	30±1°C
Water	Salinity ≥15 ‰, filtered 15 µm. Flow through to give 90% replacement in 8-12 hours.
Tank size	Experiment: 45 x 90 x 26 cm with a water depth of 19 cm. Spawning chambers 30 x 35 x 22 cm for mating fish must prevent them eating any eggs (e.g. have a screen to allow eggs to fall through).
Light regime	12 hours light and 12 hours dark

Table 4.8f Husbandry considerations for zebrafish (*Danio rerio*)

Rearing	Fry raised in 1.5 l tanks but with only 1-2 cm depth of water initially (until yolk sac absorbed) and then in tanks containing ~50 fish in 1 l water. At 12 weeks reduced to ten fish per tank; at 20 weeks, one pair per experimental tank.
Maintenance	Pairs allowed to breed once a week. Eggs removed for counting and fish transferred to clean tanks and water once per week. Fish observed daily at feeding.
Diet	Fry fed fry food (ZM Systems Ltd) and brine shrimp nauplii. Adults fed tropical fish flake twice a day (once a day at weekends) supplemented with live or frozen *Artemia*.
Temperature	26-28°C
Water	Tapwater purified by reverse osmosis and adjusted to 550 µS using Instant Ocean® salts. Static system with water changed once a week.
Tank size	Experimental tanks: 1.5 l containing ~1 l water. Tanks have grid bottoms to stop parents eating eggs.
Light regime	12 hours light and 12 hours dark

4.12 Insects

Insects, particularly insect larvae, are very important in freshwater ecosystems because they form a major component of the freshwater benthic biomass [185]. Consequently, they are a key component of the aquatic foodweb and must be effectively protected to avoid perturbations in other parts of the foodweb. Insects are a common group in many ecosystems.

Laboratory and controlled field experiments investigating chronic radiation exposure to insects have involved ants (*Formica integra*), aphids (*Myzocallis discolor*), bark beetles (*Ips grandicollis*), wasps (*Dahlbominus fuscipennis*) and the yellow fever mosquito (*Aedes aegypti*) [19,29,30,88,221]. Behavioural changes, population changes, developmental success and eye colour mutations have been investigated.

The insects used in chemical toxicity tests are restricted to a few key test species, especially when compared with experiments on pesticides against target species. For chronic or life-cycle toxicology tests, the honeybee is recommended by both the OECD [152] and the US EPA [202]. The fruit fly, mosquito, biting and non-biting midges are also recommended by the US EPA [205].

As a result, the mosquito is recommended as a test species for chronic irradiation studies. Not only does it occupy a particular niche in the ecosystem as a blood-feeding insect, the mosquito (particularly *Aedes aegypti*) has also been used in chronic irradiation laboratory studies [88]. In addition, there is a wealth of scientific information in terms of ecotoxicological testing of mosquitoes and in the mosquito's role as a vector for tropical diseases.

The honeybee (*Apis mellifera*) has also been selected as a suitable test species. It is representative of pollinators and, like the mosquito, it occupies a particular ecosystem niche as a social insect. It has been used in chronic ecotoxicological tests and is a valid test species for use in acute toxicity tests. Chronic feeding experiments have also been undertaken to assess the long-term adverse effects of the pesticide imidacloprid on honeybee colonies. Endpoints considered were death, feeding activity, wax/comb production, breeding performance and colony vitality [175].

There appears to have been no irradiation studies using bees. One benefit of studying irradiation-induced reproductive effects in bees is the genetic homogeneity of drones. This strengthens the statistical assessment of any observed abnormalities by reducing the influence of genetic variation. For the purposes of conducting low-cost experiments, however, the mosquito takes priority because experiments concerned with reproduction endpoints would require hive maintenance and the irradiation of queen bees before the establishment of the hives.

While it is strongly recommended that the two species mentioned above are used in further studies, a possible third species, *Drosophila melanogaster* (fruit fly), is also proposed. *Drosophila* has been used in ecotoxicology studies and extensively in genetic studies assessing the inheritance of characteristics between generations. Like *Apis mellifera*, it has also been used in chronic feeding experiments investigating dietary exposure to the organophosphate insecticide chlorpyrifos. The endpoints evaluated concerned different developmental stages of the fly, e.g. survivorship, hatchability, emergence, fecundity, fertility and reproductive performance [143].

Note: data have been extracted from the FRED using its classifications. Some insects have also been found in the 'soil fauna' group. Refer to Section 4.17 for further details.

Table 4.9a Insects used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Ants (*Formica integra*)	Terrestrial	FRED
Aphids (*Myzocallis discolor*)	Terrestrial	FRED
Arthropod	Terrestrial	FRED
Bark beetle (*Ips grandicollis*)	Terrestrial	FRED
Leafminer (*Cameraria hamadryadella*)	Terrestrial	FRED
Termite (*Nasulitermes costalis*)	Terrestrial	FRED
Wasp (*Dahlbominus fuscipennis*)	Terrestrial	FRED
Western harvester ant (*Pogonomyrmex occidentalis*)	Terrestrial	FRED
Yellow feaver mosquito (*Aedes aegypti*)	Terrestrial	FRED
Dahlbominus sp.	Terrestrial	FRED
Habrobracon juglandis	Terrestrial	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Biting midges (*Ceratopogonidae*)	Terrestrial	[205]
Black flies (*Simuliidae*)	Terrestrial	[205]
Fruit fly (*Drosophila melanogaster*)	Terrestrial	[205]
Honey bee (*Apis mellifera*)	Terrestrial	[202,205]
Mosquitoes (*Culicidae*)	Terrestrial	[205]
Non-biting midges (*Chironomidae*)	Terrestrial	[205]
Phantom midges (*Chaoboridae*)	Terrestrial	[205]
Recommended species	**Ecosystem**	**Husbandry method**
		See Table 4.9b.
Fruit fly (*Drosophila melanogaster*)	Terrestrial	
Honey bee (*Apis mellifera*)	Terrestrial	
Mosquitoes (*Culicidae*)	Terrestrial	

Table 4.9b Husbandry considerations for honey bee (*Apis mellifera*)

Maintenance	Sugar/water mix should be available *ad libitum*.
Diet	50% sugar/water (purified or distilled) solution
Temperature	25-35°C
Relative humidity	50-80%
Cage/tank size	Small test chambers
Light regime	Kept in dark except during doses and observations.
Notes	Tests should be conducted on worker bees 1-7 days old. No acclimation period is necessary.

4.13 Mammals

Mammals are found in both aquatic and terrestrial ecosystems, but the majority of studies into the effects of ionising radiation have focused on terrestrial mammals. This is also true of studies into the toxic effects of non-radioactive contaminants with the ubiquitous 'laboratory rat' being a common laboratory test organism.

An extensive range of mammals has been used in chronic radiation studies. These studies have encompassed survival rates in guinea pigs and sheep [35,129], chromosomal aberrations in reindeer [168] and tumour studies in beagles [39]. A wider range of endpoints (e.g. percentage mortality, biochemical parameters, seminal vesicle weight, body weight, micronuclei and number of spermatogonia cells) has been studied in the rat [9,59,123,159,160]. In addition, mutation frequencies, lymphoid tumours, expanded single tandem repeat (ESTR) mutation rate, micronuclei and percentage sterility have been studied in the mouse [9,73,127,139,171].

Mammalian species used in ecotoxicology and standard toxicity testing are the rabbit, rat, dog, goat, guinea pig, monkey and Chinese hamster.

The mouse and rat are recommended for further chronic radiation studies because they are standard laboratory animals which are bred routinely and maintained in laboratories worldwide. Both species have copious entries in the FRED, having been the subject of a number of both chronic and acute radiation exposure studies. Mice and rats are representative of small mammals, a group of animals ubiquitous in terrestrial ecosystems worldwide.

Table 4.10a Mammals used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Bank vole (*Clethrionomys glareolous*)	Terrestrial	FRED
Beagle	Terrestrial	FRED
Dog	Terrestrial	FRED
Guinea pig	Terrestrial	FRED
Kangaroo rats (*Perognathus spp., Dipodomys spp.*)	Terrestrial	FRED
House mouse (*Mus musculus*)	Terrestrial	FRED
Mouse	Terrestrial	FRED
Pig	Terrestrial	FRED
Rat	Terrestrial	FRED
Reindeer (*Rangifer tarandus*)	Terrestrial	FRED
Sheep	Terrestrial	FRED
Spanish goat	Terrestrial	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Chinese hamster		
Dog		
Goat		
Monkey		
Pig		
Rabbit		
Rat		
Recommended species	Ecosystem	Husbandry method
		See Table 4.10b.
Mouse		
Rat		

Table 4.10b | Husbandry considerations for rat

Maintenance	Commercial pellets and tap water *ad libitum*
Diet	Commercial pellets; sawdust can serve as bedding material.
Temperature	18-26°C
Relative humidity	40-70% humidity
Cage/tank size	Standard laboratory rat cages
Light regime	12 hours light and 12 hours dark
Notes	Acclimation period of a minimum of 14 days

4.14 Molluscs

Only two species of mollusc have been examined following chronic exposure to gamma radiation over long periods. Effects on predominantly reproductive endpoints were examined in freshwater snails, *Physa heterostropha* [57], while the effects of chronic exposure on growth and survival were studied in juvenile clams, *Mercenaria mercenaria* [20]. In addition larval oysters, *Crassostrea gigas*, have been used in a study of developmental endpoints [145]. A further eight species have been examined for effects of acute radiation exposures (see the FRED for more information).

Only a limited number of species have been used in toxicology tests on molluscs and exposures were usually acute (see Table 4.11a). In addition, it is not possible to carry out whole life-span studies of economically important bivalves such as oysters or mussels in the laboratory without considerable difficulty and expense. However, some long exposure experiments have been carried out. For instance, adult oysters (*Ostrea edulis*) were exposed to tributyltin leachates for a period of 75 days and the effects on the gonads examined [188]. In a similar manner, it was possible to examine the effects of a 16-week exposure of adult slipper limpets (*Crepidula fornicata*) to methyl mercury on their reproductive output and larval settlement [187]. It has been possible to culture a small bivalve, the coot clam (*Mulinia lateralis*), through a complete life-span in the laboratory [38]. The mean life span of this bivalve is around 60 days, making it an attractive choice as an experimental organism. However, it is not a native British species, coming from the eastern coast of North America. The freshwater snail, *P. heterostropha*, used in chronic radiation studies (see above) is also not native to Britain. However, it seems that *Lymnaea stagnalis*, a common British freshwater pond snail, would be a suitable equivalent experimental animal, and can be kept and bred readily in the laboratory [191,209].

Mussels have a sessile habit and, as filter feeders, they pass large volumes of seawater across their gills. This has lead to their use as sentinel organisms, which give an indication of contaminant chemical concentrations (especially metals) in the water [142]. They have also been used for biomarker studies with endpoints such as micronucleus induction measured as an indication of possible genotoxic damage from environmental contaminants [32].

None of the species recommended for chronic radiation studies in Table 4.11a have been used previously in such experiments, but they have been chosen for their suitability based on the factors noted above. Slipper limpets and pond snails are readily obtained in Britain. The coot clam is not a native to the UK, but is included because of its advantages for complete life-span exposure experiments. However, it must be noted that it naturally burrows in sand and adults must be cultured in this medium. This means that attempts to expose adults to radionuclides in the water will be complicated by adsorption on sand. If applicable, radionuclides could be delivered by spiked algal food.

Table 4.11a Mammals used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Clam (*Mercenaria mercenaria*)	Saltwater	FRED
Oyster (larvae) (*Crassostrea gigas*)	Saltwater	FRED
Snail (*Physa heterostropha*)	Freshwater/saltwater	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Brown mussel (*Perna perna*)	Saltwater	[70]
Mussel (*Mytilus edulis*)	Saltwater	[70]
Oyster (*Crassostrea gigas*)	Freshwater	[210]
Oyster (*Crassostrea virginica*)	Freshwater	[210]
Pond snail (*Lymnaea stagnalis*)	Freshwater	[209]
Snail (*Amnicola limnosa*)	Freshwater	[58]
Snail (*Lymnaea luteola*)	Freshwater	[140]
Snail (*Physa heterostropha*)	Freshwater	[58]
Snail (*Physa integra*)	Freshwater	[58]
Recommended species	**Ecosystem**	**Husbandry method**
		See Table 4.11b.
Pond snail (*Lymnaea stagnalis*)	Freshwater	

Table 4.11b Husbandry considerations for pond snail (*Lymnaea stagnalis*)

Rearing	Two snails per litre in a 10 l tank; eggs reared in a Petri dish on agar; larvae in a 2 l tank with 1 cm water and 1 cm added per week (water not changed); transfer to 10 l tank at 6 weeks.
Maintenance	Feed 2-3 times per week; remove left over feed as required; change water and clean tank once per week.
Diet	Green lettuce (main veins removed for larvae)
Temperature	21±2°C; 25±1°C for egg incubation
Water	Tap water; pH8±0.2; aerated for adults and juveniles
Tank size	Adults in 10 l tank; eggs in Petri dish; then larvae in 2 l tanks
Light regime	Moderate illumination
Notes	Egg laying stimulated by change of fresh water.

4.15 Mosses and lichens

Mosses and lichens have been considered as potential test organisms because they are primary producers and both groups are common throughout many types of terrestrial ecosystem.

There have been no studies of chronic or acute irradiation effects on mosses and laboratory studies would be difficult to perform. There is also no protocol for ecotoxicity testing using mosses, so this group has been excluded from further discussion within this good practice guidance.

Two papers were found in the FRED reporting studies on the effects of chronic field exposure on lichen diversity [28,222], but these studies are not relevant for this good practice guide. No other information on laboratory experiments using lichens was found. This group is therefore also excluded from further discussion.

4.16 Reptiles

Reptiles are found in both terrestrial and aquatic ecosystems and, like amphibians, some have a life-cycle where they spend periods of time in both. Reptiles are considered in environmental legislation and command protection, particularly in the UK, due to their conservation status.

Few experiments in which the effects of chronic radiation exposures on reptiles have been reported. There are some observations on animals collected from radiation-contaminated areas and one laboratory-based study [22], but the endpoints examined were cytological, e.g. micronuclei, cellular DNA content and chromosome abnormalities.

No reports are available from standard toxicology testing using reptiles as a recommended test species. As a result, it is not possible to recommend a species for this wildlife group.

Table 4.12a | Reptiles used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Desert side-blotched lizard (*Uta stansburiana*)	Terrestrial	[195]
Pond slider	Freshwater	FRED
Slider turtles (*Pseudemys scripta*)	Freshwater	FRED
Snapping turtle (*Chelydra serpentine*)	Freshwater	FRED
Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
None available		
Recommended species	**Ecosystem**	**Husbandry method**
No recommendations made		

4.17 Soil fauna including microflora

This wildlife group describes a wide range of organisms associated with the soil, including bacteria and fungi. The tendency for radionuclides that are released into terrestrial ecosystems to become associated with the soil may result in all organisms within this group being subjected to high radiation doses. These organisms are also common throughout many terrestrial ecosystems. A comprehensive understanding of the effects of ionising radiation within this group is, therefore, applicable to a wide range of terrestrial environmental protection scenarios.

A range of soil fauna has been used in chronic radiation experiments including bacteria, earthworms, beetles, fungi, algae and woodlice. Earthworms, beetles and woodlice have also been used in standard toxicity testing alongside springtails (*Folsomia candida*), centipedes and nematodes. Earthworms, springtails and woodlice are recommended for further chronic radiation studies.

Earthworms have been used in both chronic irradiation studies and ecotoxicology studies, and are viewed as being representative of higher invertebrates [184]. *Eisenia fetida* is recommended as a test species for chronic irradiation studies due to the ease with which it can be obtained and experimented on, and its short life-cycle. This species has also been selected by the American Society for Testing and Materials (ASTM) [17] and the US EPA has used this species for toxicity screening of hazardous waste sites. Much chemical toxicity data exist on *E. fetida*. In addition, there are standard International Standards Organisation (ISO) and OECD protocols [105,151] for conducting ecotoxicology experiments on *E. fetida*, using reproduction as the endpoint [184]. Further laboratory work would facilitate the derivation of dose-effect relationships for this species.

Springtails (collembolans) have been used in toxicity studies since the middle of the 20th century. They are easy to maintain within a laboratory, requiring little space and minimal equipment. They also have a high reproductive rate and short development period (approximately 2-3 weeks). They are thus an extremely useful test species for studies requiring assessment of reproductive endpoints. There are no records in the FRED of springtails being used in chronic ionising radiation exposure experiments, but many species have been used in chemical toxicology

studies. These species include *Folsomia candida*, *Folsomia fimetaria* and Isotoma viradis [184]. The most commonly used is *F. candida* and an ISO guideline [106] exists for this species. It is therefore recommended as a test species for chronic irradiation experiments.

The other recommended springtail species is *I. viradis*. Although the protocol developed by SECOFASE[1] for this species is based around survival rather than reproduction, this species has behavioural characteristics that would make it useful for some types of chronic irradiation test, specifically where the radiation dose is applied by spiking the soil. Spiking the soil by surface watering results in a radionuclide concentration gradient through the soil profile. As *I. viradis* generally resides in the top 1 cm of the soil in an experimental enclosure [184], it is highly unlikely to avoid the elevated radionuclide concentrations of the upper soil layers. This is not necessarily the case for other springtail species.

Woodlice are easy to maintain under laboratory conditions, requiring little maintenance and space. They have been used in both chronic irradiation and ecotoxicology studies, and are a common component of most terrestrial ecosystems. *Porcellio scaber* is proposed as a suitable test species because there is an established method for conducting reproductive endpoint studies on this species [184]. It should, however, be noted that many factors can influence the reproductive success of woodlice. Spurgeon *et al.* [184] recommend using springtails, in preference, on the grounds that they occupy the same trophic level as woodlice and are easier to conduct experiments on.

Algal films growing over the soil surface (aquatic algal species are discussed in Section 4.7) can be cultured in the laboratory on nutrient agar plates. However, some species are microscopic and thus require specialist knowledge. Given that there is little information on chronic irradiation for this group, it is excluded from further discussion.

Some fungi can also be cultured in the laboratory on nutrient agar plates; however, assessments will require specialist knowledge. Given that again there is currently little information on this group, it is excluded from further discussion.

Note: Data have been extracted from the FRED using its classifications. Some 'soil fauna' can also be classified as 'insects'. Refer to Section 4.12 for further details.

[1] SECOFASE (Development, Improvement and Standardisation of Test Systems for Assessing Sublethal Effects of Chemicals on Fauna in the Soil Ecosystem) was a European research project that ran from 1993 to 1996. The project developed protocols for ten soil invertebrate taxa, including earthworms and springtails.

Table 4.13a | Soil fauna, including microflora, used in chronic experiments

Species used in previous chronic radiation experiments		
Species	Ecosystem	Reference
Bacteria		
Actinomycete	Terrestrial	FRED
Bacteria *spp.*	Terrestrial	FRED
Escherichia coli	Aquatic	FRED
Euglena gracilis	Aquatic	FRED
Nitrosomonas	Terrestrial	FRED
Tetrahymena thermophilia	Aquatic	FRED
Beetle		
Carabidae	Terrestrial	FRED
Elateridae	Terrestrial	FRED
Staphylinidae	Terrestrial	FRED
Earthworm		
Dendrobaena octaedra	Terrestrial	FRED
Earthworm	Terrestrial	FRED
Eisenia nordenskioldi	Terrestrial	FRED
Lumbricidae	Terrestrial	FRED
Octolasium lacteum	Terrestrial	FRED
Fly		
Diptera	Terrestrial	FRED
Fungus		
Armillaria	Terrestrial	FRED
Basidiomycetes	Terrestrial	FRED
Lycogala	Terrestrial	FRED
Lycoperdon	Terrestrial	FRED
Scorpion		
Tityus bahiensis	Terrestrial	FRED
Soil algae community		
Botydiopsis	Terrestrial	FRED
Calothrix	Terrestrial	FRED
Chlamydomonas	Terrestrial	FRED
Chlorella	Terrestrial	FRED
Hormidium flacidum	Terrestrial	FRED
Neochloris	Terrestrial	FRED
Nostoc	Terrestrial	FRED
Oocystis	Terrestrial	FRED
Oscillatoria	Terrestrial	FRED
Schizothrix	Terrestrial	FRED
Soil algae *spp.*	Terrestrial	FRED
Stichococcus	Terrestrial	FRED
Tetracystis	Terrestrial	FRED
Tolypothrix	Terrestrial	FRED
Woodlice		
Armadilidium vulgare	Terrestrial	FRED
Oniscus asellus	Terrestrial	FRED
Pocellio scaber	Terrestrial	FRED
Trachelipus wachtleri	Terrestrial	FRED

Table 4.13a Cont'd

Species used in testing chronic exposure to chemicals (ecotoxicity)		
Species	Ecosystem	Reference
Nematode (*Caenorhabditis*)	Terrestrial	[184]
Earthworm (*Eisenia andrei*)	Terrestrial	[184]
Earthworm (*Eisenia fetida*)	Terrestrial	[184]
Earthworm (*Enchytraeus albius*)	Terrestrial	[184]
Springtail (*Folsomia candida*)	Terrestrial	[184]
Springtail (*Folsomia fimetaria*)	Terrestrial	[184]
Springtail (*Isotoma virdis*)	Terrestrial	[184]
Centipede (*Lithobius mutabilis*)	Terrestrial	[184]
Woodlouse (*Porcellio scaber*)	Terrestrial	[184]
Beetle (*Staphylinidae*)	Terrestrial	[184]
Recommended species	**Ecosystem**	**Husbandry method**
		See Tables 4.13b & c.
Earthworm (*Eisenia fetida*)	Terrestrial	[184]
Springtail (*Folsomia candida; Isotoma virdis*)	Terrestrial	[184]
Woodlouse (*Pocellio scaber*)	Terrestrial	[184]

Table 4.13b Husbandry considerations for earthworm (*Eisenia fetida*)

Diet	Air-dried finely ground cow manure
Temperature	20±2°C
Tank size	30 x 20 x 20 cm
Light regime	Controlled light and dark cycles - preferably 16 hours light and 8 hours dark

Table 4.13c Husbandry considerations for woodlouse (*Pocellio scaber*)

Maintenance	Tanks filled to a depth of 15 cm with uncontaminated field maple leaves which have been air-dried at room temperature for 72 hours and then rehydrated at 100% relative humidity for 48 hours.
Diet	Field maple leaves
Temperature	16°C
Water	High humidity required (occasional spraying with distilled water)
Tank size	30 x 20 x 20 cm
Light regime	16 hours light and 8 hours dark
Notes	Tanks covered in polythene sheets

4.18 Terrestrial plants

Terrestrial plants are primary producers in terrestrial ecosystems. If plants are not adequately protected, than all other trophic levels within that ecosystem are likely to be negatively impacted. It is also important to understand the effects of ionising radiation on plants in considerable detail because the majority of plants are permanently exposed to both the air and soil components of a terrestrial ecosystem. Therefore, both plant roots and shoots must be adequately protected.

The terrestrial plant species used in chronic radiation experiments are shown in Table 4.14a. Few laboratory studies have considered the chronic radiation exposure of terrestrial plants due to the logistical difficulties in exposing plants to irradiation for extended time periods. More studies of chronic radiation effects in controlled field conditions have been undertaken. Studies of chronic radiation effects in the field following the Chernobyl and Eastern Ural (Kystym) accidents have not been considered for the purpose of this report, as these are not controlled dose studies.

The chronic laboratory studies that have been undertaken have focused on the germination and growth of a range of conifer species (*Pinus banksiana, Picea mariana, Pinus resinosa, Pinus sylvestris, Picea glauca* and *Pinus strobus* [50,179]) and mutation effects (pollen sterility and chromosome aberrations) in two species, *Hordeum vulgare* [180] and *Viccia cracca* [162]. The controlled field experiments are forest studies focusing on a wider range of plant species, including many tree species and understorey plants. Detailed measurements have generally been conducted on tree species, but there are sparse data for understorey plants. Coniferous species are the most widely studied because of their sensitivity to radiation and their broad distribution in environments with elevated levels of radiation.

Chronic experiments have focused on coniferous tree species with only a few studies of deciduous tree species, agricultural crops and understorey plants. *Pinus spp.* are the most widely studied and have been subject to comprehensive long-term controlled field experiments over 8-10 years (e.g. [182,183]). Studies investigating the effects of radiation on mutation endpoints have focused on chromosome aberrations [36] and DNA damage [116] in agricultural crops.

Plant species have been used in ecotoxicological testing and the most common are summarised in Table 4.14a. The majority of these species are agricultural crops, both monocotyledons and dicotyledons, as the tests were designed to examine the effects of plant protection products used in agriculture. In ecotoxicological testing, 6-10 species (representing a ratio of 1:2 monocotyledons to dicotyledons) are generally used and many of these species would be suitable for experiments using irradiation.

Coniferous trees are, however, excluded from the species recommended in Table 4.14a for use in chronic, long-term radiation experiments. This is because they have not been used in standard ecotoxicity-type work and there are many problems associated with standardising experiments.

Instead, the recommended species are *Brassica rapa* and *Avena sativa*. While they have not been used in radiation experiments, both are used extensively for ecotoxicological testing of a range of contaminants and are given as principal test species by ISO, OECD and ASTM [15,107,152]. There is thus a substantial amount of information on Brassica rapa, which can also be used for a full life-cycle test in a relatively short time-period. It would provide significantly more information than the standard seedling emergence and growth tests. Details of the use and maintenance of these species are also given in Table 4.14a. A full life-cycle test has been designed for *B. rapa*, using a rapid cycling variant [107]. This enables chronic reproductive endpoints to be assessed. There is detailed information available on the growth, inhibition responses and test conditions for Avena sativa.

Ecotoxicity tests have shown that there is wide variation in sensitivity to pollutants between different species of terrestrial plants. It is, therefore, recommended that a range of species representing the different groups is studied, as it is possible that different species will also vary widely in response to radiation. Other species listed in Table 4.14a for ecotoxicity tests could also be used [15,107,152].

Table 4.14a | Terrestrial plants used in chronic experiments

Species used in previous chronic radiation experiments			
Species	Ecosystem	Life Stage	Reference
Controlled laboratory experiments			
Jack pine (*Pinus banksiana*)	Terrestrial		FRED
Barley (*Hordeum vulgare*)	Terrestrial	Seedling	FRED
Pea (*Viccia cracca*)	Terrestrial		FRED
Black spruce (*Picea mariana*)	Terrestrial		FRED
Red pine (*Pinus resinosa*)	Terrestrial		FRED
Scots pine (*Pinus sylvestris*)	Terrestrial		FRED
White spruce (*Picea glauca*)	Terrestrial		FRED
White pine (*Pinus strobus*)	Terrestrial		FRED
Controlled field experiments			
Balsam fir (*Abies balsamea*)	Terrestrial	Mature	FRED
Trembling aspen (*Populus tremuloides*)	Terrestrial	Mature	FRED
Bebb willow (*Salix bebbiana*)	Terrestrial	Mature	FRED
Paper birch (*Betula papyrifera*)	Terrestrial	Mature	FRED
Alder (*Alnus rugosa*)	Terrestrial	Mature	FRED
Yellow sedge (*Carex pensylvanica*)	Terrestrial	Mature	FRED
Buckwheat (*Fagopyrum esculentum*)	Terrestrial	Mature	FRED
Meadow fescue (*Festuca pratensis*)	Terrestrial	Mature	FRED
Black ash (*Fraxinus nigra*)	Terrestrial	Mature	FRED
Black huckleberry (*Gaylussacia baccata*)	Terrestrial	Mature	FRED
Blueberry (*Vaccinium vacillans*)	Terrestrial	Mature	FRED
Barley (*Hordeum vulgare L.*)	Terrestrial	Mature	FRED
Jack pine (*Pinus banksiana*)	Terrestrial	Mature	FRED
Black spruce (*Picea mariana*)	Terrestrial	Mature	FRED
Red pine (*Pinus resinosa*)	Terrestrial	Mature	FRED
Scots pine (*Pinus sylvestris*)	Terrestrial	Mature	FRED
White spruce (*Picea glauca*)	Terrestrial	Mature	FRED
White pine (*Pinus strobus*).	Terrestrial	Mature	FRED
Pitch pine (*Pinus rigida*)	Terrestrial	Mature	FRED
Quercus alba	Terrestrial	Mature	FRED
Quercus coccinea	Terrestrial	Mature	FRED
Quercus ilicifolia	Terrestrial	Mature	FRED
Potato (*Solanum tuberosum*)	Terrestrial	Mature	FRED
Spiderwort (*Tradescantia*)	Terrestrial	Mature	FRED
Wheat (*Triticum monococcum*)	Terrestrial	Mature	FRED
Rye (*Secale cereale*)	Terrestrial	Mature	FRED
Rubus idaeus	Terrestrial	Mature	FRED
Diervilla ionicera	Terrestrial	Mature	FRED
Prunus pensylvanica	Terrestrial	Mature	FRED
Ribes hirtellum	Terrestrial	Mature	FRED
Chimaphila umbellata	Terrestrial	Mature	FRED
Lonicera villosa	Terrestrial	Mature	FRED
Viburnum trilobum	Terrestrial	Mature	FRED

Table 4.14a | Cont'd

Species used in testing chronic exposure to chemicals (ecotoxicity)			
Species	Ecosystem	Life-Stage	Reference
Monotocyteldons			
Oats (*Avena sativa*)	Terrestrial	Seed/seedling	All species referenced in either [15], [107] or [152]
Barley (*Hordeum vulgare*)	Terrestrial	Seed/seedling	
Perrenial ryegrass (*Lolium perenne*)	Terrestrial	Seed/seedling	
Rice (*Oryza sativa*)	Terrestrial	Seed/seedling	
Rye (*Secale cereale*)	Terrestrial	Seed/seedling	
Rye (*Secale viridis*)	Terrestrial	Seed/seedling	
Grain sorghum (*Sorghum bicolour*)	Terrestrial	Seed/seedling	
Shattercane (*Sorghum vulgare*)	Terrestrial	Seed/seedling	
Wheat (*Triticum aestivum*)	Terrestrial	Seed/seedling	
Corn (*Zea mays*)	Terrestrial	Seed/seedling	
Onion (*Allium cepa*)	Terrestrial	Seed/seedling	
Dicotyledons			
Sugarbeet (*Beta vulgaris*)	Terrestrial	Seed/seedling	
Lettuce (*Lactuca sativa*)	Terrestrial	Seed/seedling	
Mustard (*Brassica alba*)	Terrestrial	Seed/seedling	
Chinese cabbage (*Brassica campestris var. chinensis*)	Terrestrial	Seed/seedling	
Oilseed rape (*Brassica napus*)	Terrestrial	Seed/seedling	
Cabbage (*Brassica oleracea*)	Terrestrial	Seed/seedling	
Turnip (*Brassica rapa*)	Terrestrial	Full life-cycle	
Garden cress (*Lepidium sativum*)	Terrestrial	Seed/seedling	
Radish (*Raphanus sativus*)	Terrestrial	Seed/seedling	
Cucumber (*Cucumis sativa*)	Terrestrial	Seed/seedling	
Soybean (*Glycine max*)	Terrestrial	Seed/seedling	
Mung bean (*Phaseolus aureus*)	Terrestrial	Seed/seedling	
Pea (*Pisum sativum*)	Terrestrial	Seed/seedling	
Fenugreek (*Trifolium ornithopodioides*)	Terrestrial	Seed/seedling	
Red clover (*Trifolium pratense*)	Terrestrial	Seed/seedling	
Vetch (*Vicia sativa*)	Terrestrial	Seed/seedling	
Tomato (*Lycopersicon esculentum*)	Terrestrial	Seed/seedling	
Carrot (*Daucus carota*)	Terrestrial	Seed/seedling	
Recommended species	**Ecosystem**	**Life-Stage**	**Husbandry method**
			See Tables 4.14b & c.
Turnip rape (*Brassica rapa*)	Terrestrial	Full life-cycle	[107]
Oat (*Avena sativa*)	Terrestrial	Seedling	[107]

Table 4.14b Husbandry considerations for oats (*Avena sativa*)

Rearing/growing	Test starts from seed so not applicable.
Maintenance	Test starts from seed so not applicable.
Diet	Plants should be watered with de-ionised water, manually or by a wick.
Temperature	23±3°C within the growth chamber
Relative humidity	30-70%
Cage/tank size	Growth chamber size as appropriate to avoid overcrowding of plants; lights should be at least 1 m above the plants.
Light regime	16 hours light and 8 hours dark; illumination intensity of 13,000±2,000 lux
Notes	Detailed method given in [107].

Table 4.14c Husbandry considerations for turnip rape (*Brassica rapa* (rapid cycling))

Rearing/growing	Test starts from seed so not applicable.
Maintenance	Test starts from seed so not applicable.
Diet	Plants should be watered with de-ionised water, manually or by a wick.
Temperature	23±3°C within the growth chamber
Relative humidity	30-70%
Cage/tank size	Growth chamber size as appropriate to avoid overcrowding of plants; lights should be at least 1 m above the plants.
Light regime	16 hours light and 8 hours dark; illumination intensity of 13,000±2,000 lux
Notes	Rapid cycling Brassica rapa can only be obtained from the Carolina Biological Supply Company (http://www.carolina.org). Detailed method given in [107].

5. Endpoint selection (GPG 2)

In order to assess the impact of a stressor (either chemical or radioactive) on a test species, it is necessary to determine the effect that different levels of exposure to that stressor have on particular endpoints.

An endpoint can be defined as *"the final stage of a process, especially the point at which an effect is observed"*[60]. In terms of assessing the effects of chronic exposure to ionising radiation, an endpoint can be more specifically defined as *"the characteristic of the biological unit under investigation that is being assessed in relation to different dose rate regimes"*.

This GPG discusses the selection of specific endpoints for the purposes of experiments to determine dose-effect relationships for particular organisms exposed to ionising radiation. It is hoped that this will help to achieve harmonisation of the approach used for conducting experiments in this field. The aims are to:

- familiarise users with the concept of measuring endpoints to assess the response of a biological unit to a stressor;
- identify specific endpoints appropriate to the range of wildlife groups considered in GPG 1;
- describe some of the techniques that can be used to assess these specific endpoints.

5.1 Umbrella endpoints

To investigate the effects of chronic ionising radiation exposure on a test species, experimenters must identify which aspects or 'characteristics' of that organism they are going to observe during the test. The experimenter may wish to observe such characteristics as the organism's behaviour, physical signs of reduced fitness such as weight loss and increased incidence of disease, reproductive success of the organism and the incidence of mortality within a group of test species. The endpoints to be assessed must be determined at the experimental design stage and appropriate techniques identified to ensure that the assessment approach is replicable and scientifically valid.

Four 'umbrella endpoints' were identified under FASSET [122]:

- Mutation: a change in the genetic material of an organism. This can be spontaneous or induced by chemicals or radiation.
- Reproduction: the formation of new individuals by sexual or non-sexual means (also referred to in FASSET as "reproducive capacity").
- Mortality: the number of deaths in a given period.
- Morbidity: the state of being diseased.

Each of these umbrella endpoints covers a wide range of responses that can be assessed and each one of these responses is referred to as a 'specific endpoint'. For example, the reproduction umbrella endpoint in rats can be assessed using specific endpoints such as gonadal weight, blood levels of sex hormones, spermatogenesis and reproductive organ neoplasia. However, these specific endpoints are not universally applicable to all organisms, e.g. you can not assess spermatogenesis in plants. This Good Practice Guide (GPG 2) aims to help users select appropriate endpoints for their experiments and to provide an indication of the methods that can be used to assess particular endpoints.

5.1.1 Mutation

Mutation may occur at any time and in any part of an organism. As the above definition suggests, mutation can occur spontaneously (as a result of changes in the structure of DNA at cell division), but may also result from the exposure of an organism to mutagens. Mutagens are agents, either radioactive or chemical, that induce mutation in an organism. The physical location and extent of a mutation in an organism will determine its impact on the functioning of that organism.

Mutations, such as benign tumours, are unlikely to reduce the success of an organism other than by creating physical abnormalities that impede movement or reduce the organism's chances of being successful during courtship. However, mutations that occur in gonads, particularly in germ cells, may have profound effects on the reproductive success of individuals. This highlights the fact that the four umbrella endpoints are not mutually exclusive, as reproduction and mutation can both describe the observed impact. Mutations that are not benign, such as malignant tumours, may lead to serious reductions in the overall physical health of an organism. These mutations, therefore, may also be described under the umbrella endpoint of morbidity.

5.1.2 Reproduction

Reproductive endpoints are essentially measures of the reproductive success of an organism. Organisms reproduce either sexually or asexually, so assessing reproductive endpoints generally focuses on measures of the success with which an organism can transmit its genes to its progeny.

It is important to assess this umbrella endpoint when investigating the impacts of ionising radiation on non-human biota because successful environmental protection requires the maintenance of ecosystem function and this is inherently linked to the success of, at a population level, organisms that occupy the different niches within that ecosystem. Therefore, any reduction in reproductive success or fitness that is passed on to the progeny as a result of genetic mutation in the germ cells may be an important effect in terms of ecosystem function.

While there are four umbrella endpoints that may be assessed, this guidance focuses on the development of research protocols to assess reproduction endpoints because successful reproduction is the most important factor in ensuring the long-term survival of a population.

5.1.3 Mortality

The assessment of mortality as an umbrella endpoint is generally restricted to studies of acute toxicity. Chronic exposures to radiation are highly unlikely to result in death. Mortality is therefore only likely to apply as an endpoint in the wild under accident conditions resulting in acute exposure to radiation, including very high levels of >5 Gy or >10,000 µGy/h.

5.1.4 Morbidity

Morbidity relates essentially to the reduction in the physical condition of an organism. The deterioration in physical condition associated with morbidity can cover a wide range of effects, including disadvantageous changes in behaviour.

5.2 Specific endpoints

Umbrella endpoints are the major 'characteristics' of an organism that an experimenter may choose to assess during investigations into the toxic effects of contaminants or radiation exposure. To assess these major characteristics, however, more specific characteristics need to be measured. For example, in an investigation into the reproduction umbrella endpoint, the experimenter could choose to assess one or more specific characteristics such as number of eggs laid, shell thickness, hatching success and survival of hatchlings. These are quantifiable characteristics that relate to the overall umbrella endpoint of reproduction and are termed 'specific endpoints'.

Specific endpoints can therefore be defined as: *"quantifiable characteristics of an organism or its progeny that can be used to investigate the effects of a contaminant on a particular umbrella endpoint"*.

Specific endpoints can be characteristics at any level of biological organisation from the molecular level through to the whole organism level. They can be used for assessing the influence of both radioactive and non-radioactive contaminants. Some examples of specific endpoints for each umbrella endpoint are provided in Table 5.1, together with an indication of which wildlife groups they can be used for.

Examples of specific endpoints relating to reproduction are given in Table 5.2. This table does not contain an exhaustive list of potential specific endpoints and users are encouraged to conduct a review of the available literature (particularly the FRED) to determine specific endpoints and techniques that are appropriate to their purpose.

The experiments within this guidance focus on chronic exposures. Therefore mortality should not occur, although experimenters would be expected to record if a particular individual died during the course of a study and, where possible, to determine the cause of death. Morbidity and mutation may both affect an individual but if this does not interfere with successful reproduction, or it occurs in later life after the organism has reproduced, it is much less likely to impact significantly on the population as a whole.

Table 5.1 | Examples of specific endpoints in studies on the effects of contaminants on a range of wildlife

Mutation	Reproduction	Mortality	Morbidity
Seedling emergence ***tp***	Seedling emergence ***tp***	Survival rates ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***	Biomass (shoot, root, plant) ***tp***
Sister chromatid exchange ***aqi, m***	Changes in sex hormones ***f, m***	Life span reduction ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***	Immunocompetence ***f, m***
Comet assay ***a, c, f, m, mc***	Number of dead offspring per litter ***m***		Vertebral abnormalities ***f***
Visual detrimental effects ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***	Visual detrimental effects ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***		Shell length and deposition ***mc***
Mitotic index ***tp***	Seedling growth ***tp***		Plant height ***tp***
Chlorophyll mutation frequency ***tp***	Seed productivity per cone or per plant ***tp***		Leaf length ***tp***
Production of stress proteins ***sf, m***	Pollen viability ***tp***		Number of leaves per plant ***tp***
Frequency of chromosome aberrations ***a, aqi, aqp, b, c, f, l, m mc, r, sf, tp***	Pollen tube growth ***tp***		Photosynthetic rate ***tp***
Frequency of cell aberrations ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***	Length of inflorescence ***tp***		Tumour development ***m***
	Nesting success ***b***		Weight decrease ***a, aqi, aqp, c, f, l, m, mc, r, sf, tp***
	Hatchability success ***b, f, l, mc, sf***		Change in biochemical parameters (e.g. hormone changes) ***m***
	Number of oocytes ***b, f, m***		Soil respiration ***sf***
	Number of spermatogonia ***b, m***		Percentage of substrate used ***sf***
	Average number of eggs laid ***aqi, b, f, i, mc***		Histopathological changes ***aqi, b, c, f, m, mc, r, sf***
	Defective sperm ***m***		Visual detrimental effects ***a, aqi, aqp, b, c, f, l, m, mc, r, sf, tp***
	Mean litter sizes ***m***		
	Morphological and histopathological changes of gametes and gonads ***m, r***		
	Fertilisation success ***f***		
	Altered reproduction rates ***aqi, c, sf***		

Key:
a = amphibians; aqi = aquatic invertebrates; aqp = aquatic plants; b = birds; c = crustaceans; f = fish; i = insects; m = mammals; mc = molluscs; r = reptile; sf = soil fauna; tp = terrestrial plants.

Table 5.2 | Examples of specific endpoints, under reproduction, used to investigate the effects of contaminant exposure on wildlife

	Reproduction	
Specific endpoint	Description of technique	Reference
Seedling emergence	Seeds are planted in soil at 1.5-2 times their depth; 14 days after 50% emergence in the control group; the proportion of seeds which have emerged above the soil is counted.	[107,152]
Seedling growth	Seeds are planted as in the emergence test above, and grown for 14 days following 50% emergence of the control group.	[107,152]
Seed productivity per cone or per plant	Measure the mass or number of seeds produced per plant.	
Pollen viability/sterility	Pollen viability/sterility can be assessed using a variety of staining techniques.	[161,180]
Pollen tube growth	Pollen tube growth is assessed by transferring pollen from anthers to stigmas and placing on a slide with pollen growth media and the stigma. Pollen germination and pollen tube growth are assessed after 2-6 hours.	[174]
Length of inflorescence	Measure the length of each of the plant's inflorescence and calculate the average.	
Nesting success		[33]
Hatchability success	Count the number of live young hatched successfully.	[86,157]
Number of oocytes		[78,138]
Number of spermatogonia	Perform a sperm count.	[131,138]
Average number of eggs laid	Count the number of eggs laid per individual.	[57,87]
Defective sperm		[78]
Mean litter sizes	Count the number of offspring in a litter and work out the mean.	[176]
Morphological and histopathological changes of gametes and gonads	Obtain sections of gonad and gametes; mount, stain and subject to microscopic analysis.	[101,131]
Fertilisation success	Perform a necropsy very early in gestation.	[178]
Altered reproduction rates	Measure the rate of reproduction for a species and note any change in rate.	[132,133,192]
Sister chromatid exchange	Perform differential staining of metaphase cells that have been allowed to incorporate 5-bromo-deoxyuridine.	[93,156]
Number of dead offspring per litter	Count the number of dead offspring present within a litter.	[78]
Visual observed gross abnormalities	Record anything abnormal as seen by the human eye for further investigation.	

5.2.1 Reproduction

As this report focuses on reproduction endpoints, the population rather than the individual is the aim of the protection. In other words, the goal of environmental protection is to ensure the long-term viability of a population through successive generations. One of the many factors that may influence this is the exposure of individuals from that population to ionising radiation.

A reproductive endpoint is the measure of the level of success with which individuals can produce viable offspring. In a laboratory situation, where conditions can be controlled with a high degree of precision and accuracy, the specific reproduction endpoints are restricted to such factors as histopathological changes of gametes and gonads, litter size, pollen viability and sporulation. In field situations, however, reproduction endpoints may be more subtle and are likely to include specific endpoints that, in laboratory situations, would be categorised under morbidity or mutation. This is particularly true in the animal kingdom where many sexually reproducing organisms actively select their mate. For example, while a change of the plumage of a bird in a laboratory situation (where there is a limited number of organisms, which have almost no choice but to mate with each other) may not affect reproductive success, the same change may seriously affect the success of that same bird in the environment.

This argument suggests a cautious approach when applying laboratory-derived data to field scenarios. In addition, it highlights the need for experimenters to be vigilant in their approach to radiation effect studies and, in particular, to ensure that they record any effects on other endpoints as a matter of course. Therefore, the two major points to consider are:

- the application of laboratory data in field situations;
- the importance of recording additional endpoints during laboratory investigations.

The issue of extrapolation to environmental situations is one that is difficult to overcome without conducting field experiments. One approach may be to conduct experiments to determine a particular dose-effect curve in a laboratory situation and then evaluate the results in terms of the type of organism being studied, its method of reproduction and the potential for the influence of co-stressors (e.g. other pollutants in the environment).

The concept of observing additional endpoints has already been highlighted. Experimenters who have an understanding of the ecology and population dynamics of their chosen test species will be at an advantage when identifying other endpoints that should be observed and recorded. However, every experimenter should observe and record details of the following additional specific endpoints as a minimum:

- any differences in physical appearance during the duration of radiation exposure;
- any individuals that die during the course of the experiment;
- number of offspring;
- weight of offspring;
- physical dimensions of offspring (length, width, etc.).

6. Exposure guideline (GPG 3)

This GPG aims to familiarise experimenters with the issues surrounding the use of ionising radiation as a contaminant to which test species are exposed to and to detail the different approaches that can be adopted for exposing organisms to particular dose rates.

The objectives of this section are therefore to:

- explain the concept of dosimetry;
- identify ways of achieving external and internal exposures;
- describe how to address the lack of RBE data for non-mammalian biota;
- advise on the selection of appropriate dose rates for chronic radiation exposure experiments that cause effects for a range of 'organism:specific endpoint' combinations;
- highlight some of the requirements of a radiation facility that are necessary to ensure that experiments are conducted in a safe environment.

Unlike chemical toxicity tests, the organisms and the radiation source do not need to be in direct physical contact. Therefore, depending on the type of radiation, exposure to ionising radiation can be internal or external, and from a source that is in direct physical contact or at a distance from the test species. Accurate dosimetry work is required to determine the dose received by the test species.

The Relative Biological Effect (RBE) (see Section 6.4) of different radiation types must also be considered in experiments using ionising radiation. RBE is well understood in relation to humans and some other mammals, but this is not the case for the rest of the wildlife groups. Suggestions are made on how RBE experiments should be conducted for the different wildlife groups considered in Section 4.

This GPG provides guidance on how to design and undertake experiments to determine dose-effect relationship curves for particular 'organism:specific endpoint' combinations. Such experiments require a particular test species to be exposed to a particular radiation at a range of known dose rates, and the effects on one or more specific endpoints at each dose rate to be observed and recorded.

To produce a dose-effect curve, the radiation dose rates used in the experiment **must** span the threshold at which an effect is observed. This section therefore provides an indication, where information exists, of the dose rate thresholds for particular specific endpoints in each wildlife group. Where this information is unavailable, experimenters are advised to consider a pilot study to establish the dose threshold prior to the main experiment.

The different radionuclides and the radiation types that they emit are not considered in detail in this GPG. This information is readily available in the literature; an overview is given in the Environment Agency's Radionuclides Handbook (R&D Technical Report P3-101/SP1b, 2003).

6.1 Dosimetry

The purpose of this GPG is to facilitate the determination of the relationship between absorbed radiation dose rate to an organism and its effect as measured on a chosen endpoint. An accurate measurement or estimate of the absorbed dose rate is vital. All experiments must, therefore, give an

account of the dosimetric methods used, a clear indication of the dose rate received by each experimental group and, preferably, an estimate of the confidence limits.

Exposure of organisms in the environment is likely to be at relatively low dose rates and over protracted periods, often the whole life-span. Apart from fish [219], experiments examining effects of acute exposures to high radiation doses cannot be extrapolated reliably to the chronic low dose rate situation. Thus only environmentally relevant experiments using chronic, low dose rates are considered in this guidance.

6.2 External irradiation

Exposure to external gamma radiation is conveniently and safely carried out using sealed sources. A range of different gamma-emitting radionuclides could be used as sources, but ^{137}Cs and ^{60}Co are the most common. For internal irradiation, it may be necessary to consider the radiation dose resulting from the progeny of the radionuclide being used.

Dosimetry measurements can be carried out by a variety of methods, e.g. ion chambers and thermoluminescent dosimeters (TLDs). TLDs are particularly useful as they allow the dose rates received at several different places in the radiation field to be measured simultaneously. The TLD must be chosen to allow measurements in the required range (mGy) and read on a suitable TLD reader.

Large organisms will receive different dose rate s to different parts of their body, depending on the distance from the source. Therefore, measurements must be taken at different positions on the organism (e.g. root, stem, growing tip of a plant).

Water and soil have shielding effects on organisms living in them and dose rates should be measured at several positions in the tank/container. For non-mobile organisms (e.g. plant roots), dose rates can be received by different parts in different positions in the soil and the air. For mobile animals (e.g. fish, burrowing animals), a mean dose rate can be calculated. This should take into account the different periods of time spent by the animal at different depths in soil or water. Separate observations on their movements over a period of 24 hours or more may be necessary to allow this.

6.3 Internal irradiation

Results from external irradiation experiments (see Section 6.2) are relevant to internal exposure to most beta-emitters with the exception of tritium, ^{14}C and those nuclides that concentrate in certain organs (e.g. iodine in the thyroid). Results for gamma irradiation are not relevant to alpha-emitters and these must be investigated separately.

An alpha-emitter suggested for experimental studies on animals is ^{210}Po. This is taken up readily by many animals, as it has a relatively high gut transfer factor [41,99]. This means that the amount administered to achieve any given tissue concentration is much lower than for other alpha-emitters (e.g. ^{239}Pu, ^{241}Am) and that the radiological risk to experimenters is also greatly reduced. In addition, ^{210}Po is distributed in all organs of the body, including the gonads, which are of particular interest when measuring effects on reproductive endpoints (see Sections 5.1.2 and 5.2.1).

Internal exposure to ^{210}Po or other alpha-emitters is achieved by administering it in spiked food or water to animals, and in spiked water or soil to plants. Dosimetry requires knowledge of the uptake and distribution of the alpha-emitter within the organism and the equilibrium concentrations reached over a prolonged experimental period. Where suitable mathematical models (e.g. [60,220]) are available, they can be used under equilibrium concentrations to determine dose rates. Such models require a knowledge of the physical dimensions of the experimental organism, which should be measured and recorded at the start of the experiment and periodically thereafter (see Section 5). Pilot experiments will usually be required to establish the relationship between the rate of administration of known amounts/concentrations of alpha-emitter and the resultant equilibrium tissue activity concentrations. The measurement of tissue activity concentration requires the sacrifice of the organism and the chemical isolation of the radionuclide before radiometric counting. This should be carried out by experienced staff using accredited or well-proven methods.

If some simple assumptions are made, tissue dose rates can often be calculated from the activity concentrations. Here is an example using ^{210}Po [42].

If it is assumed that a concentration steady-state exists and that the isotope is distributed uniformly in the tissue, then the alpha radiation dose rate from ^{210}Po in an infinite volume (i.e. much greater than the 60 µm range of alpha particles) of the tissue depends on its concentration in the tissue. This can be

derived using the equation:

$D\alpha\ (\infty) = 5.76 \times 10^{-7} \times E\alpha \times C\ (\mu Gy/h)$

where: $D\alpha\ (\infty)$ is the alpha radiation dose rate to the tissue

$E\alpha$ = 5.4 MeV, the particle energy of ^{210}Po

C = tissue concentration of ^{210}Po in Bq/g

$D\alpha\ (\infty)$ = 3.11 µGy/h (per Bq/g).

The experimental dose rates (see Section 6.5) are achieved by administering the radionuclide at the rate required to achieve the necessary equilibrium concentrations. These concentrations should be checked by measurement at the end of the experiment and, if possible, by planned sacrifice of some individuals from each dose rate group during the test. The organisms used in these internal exposure experiments are likely to be small in size, as the greater amounts of activity required for larger organisms will increase costs greatly and may present unacceptable risks in radiological safety terms.

6.4 Relative Biological Effect (RBE)

Alpha radiation is more effective than gamma radiation at producing biological damage in man and many other mammals, and there is no reason to expect this is not true for other organisms.

The RBE describes the relationship between dose rates of radiation of different types that produce the same endpoint effect. Thus for gamma and alpha radiation:

$$RBE\alpha = \frac{\text{Dose rate of alpha radiation producing a specific damage}}{\text{Dose rate of gamma radiation producing the same damage}}$$

RBEs for beta radiation or other radiation qualities may be obtained in the same way.

However, the RBE_α is likely to be of most significance in the environment. Use of the RBE_α allows the biologically effective dose rate of combined alpha and gamma radiation, which may occur in the environment, to be estimated. It also allows some extrapolation of results from gamma irradiation experiments to alpha radiation exposures for which relatively few results are available. Determination of RBE_α requires experimental exposures to alpha and gamma radiation to be carried out, as described above, under conditions which are as identical as possible. Dose rates producing the same observed effect can then be compared to obtain the RBE_α.

Unfortunately, there are extremely few data available for environmental organisms that are suitable for the determination of RBE. This is probably due to the difficulties encountered in carrying out experimental alpha-irradiation experiments. It is recommended that, whenever alpha-irradiation experiments are carried out, a parallel gamma-irradiation experiment is conducted, if possible, to allow RBE estimation. A number of RBEs for environmental biota have been recommended in the literature (e.g. [60,219]).

6.5 Dose rates and resulting effects in wildlife groups

When determining the range of dose rates to be used in an experiment, it is important to use whatever information is available from previous studies. Information on the effects of exposure to ionising radiation, which is likely to aid the choice of experimental dose rates, is given in Tables 6.1-6.9, which are a composite summary of the information held in the FRED and R&D Publication 128 [60]. The tables should be used with caution as they are only a brief summary of the information available and users are advised to conduct a detailed examination of the literature for all relevant information before commencing an experiment.

6.5.1 Terrestrial plants, mammals and fish

Some data exist for these wildlife groups (see Tables 6.1-6.3). These indicate that a range of effects on a range of endpoints have been observed after exposure at dose rates of <5,000 µGy/h.

Experiments on fish and mammals have predominantly investigated reproductive endpoints. In all groups, external gamma radiation sources have been the main method of irradiation. In view of these results, there would seem to be little need for future experiments to examine dose rates >5,000 µGy/h. Exceptions to this may be non-coniferous plants, as all effects on plants following exposure to dose rates of <5,000 µGy/h have been observed with pine. A number of reviews (e.g. [200]) suggest that, within plants, there is a decreasing radiosensitivity in the order: **coniferous trees** > **deciduous trees** > **shrubs** > **herbaceous plants** > **lichen, bryophytes and fungi.**

Table 6.1 | Effects of different dose rates of chronic ionising radiation on plants

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Pine	100-1,000	Gamma	Reduced trunk growth of mature pine trees	Morbidity	[223]
Pine		Gamma	Death of some conifers; population changes little.	Mortality	[4]
Pine	(1-5) x 10^3	Gamma	Reduced canopy cover of individual conifers; whole canopy remains constant.	Morbidity	[4]
Pine		Gamma	Decreased stem growth of saplings	Morbidity	[3]
Pine		Gamma	Reduced photosynthetic capacity of pines and thus growth	Morbidity	[26]
Pine	(5-10) x 10^3	Gamma	Death of all conifers within 2-3 years	Mortality	[4]
Pine	(10-20) x 10^3	Gamma	Reduced seed production and germination	Reproduction	[204]
Pine		Gamma	Morphological changes in leaves of some plants	Morbidity	[204]
Pine		Gamma	Withered crowns	Morbidity	[204]
Birch		Gamma	Under developed leaves in birch trees	Morbidity	[204]
Herbaceous	>20 x 10^3	Gamma	Reduced reproductive potential of herbaceous species	Reproduction	[200]
Birch		Gamma	Death of birch trees	Mortality	[4,204]
Grasses		Gamma	Death of grasses and forbs	Mortality	[204]
	>100 x 10^3	Gamma	Death of all higher plants	Mortality	[4,204]
Lichen	>1000 x 10^3	Gamma	Reduced diversity of lichen communities after exposure of 1 year	Mortality	[28,222]

Table 6.2 | Effects of different dose rates of chronic ionising radiation on mammals

Species	Dose rate (μGy/h)	Radiation	Description	Endpoint	Reference
Mouse	<100	Alpha	Chromosome damage	Mutation	[177]
		Beta	Reduction in oocyte numbers following in-utero irradiation	Reproduction	[72]
		Gamma	Reduction in numbers of offspring	Reproduction	[126]
		Alpha	Reduction in sperm output by 10% (5-8 months)	Reproduction	[177]
Mouse	100-1,000	Gamma	Decreased germ cell production	Reproduction	[199]
		Alpha	Reduction in oocyte numbers by 20%	Reproduction	[173]
		Gamma	Reduced survival	Mortality	[83]
Rat		Gamma	Reduction in germ cell production following irradiation of embryo	Reproduction	[199]
		Beta	Reduction in offspring oocyte numbers following parental irradiation of parent	Reproduction	[158]
		Beta	Reduction in brain size of offspring following maternal irradiation during early pregnancy.	Morbidity	[37]
Monkey		Beta	Sterility following neonate exposure	Reproduction	[71]
Pig		Gamma	Reduction in gonad weight of offspring	Reproduction	[77]
		Gamma	Reduction in number of germ cells following in-utero exposure	Reproduction	[199]
Dog		Gamma	Sterility	Reproduction	[200]
Mouse	(1-5) x 10^3	Gamma	Increased genetic defects of sperm	Mutation	
		Gamma	Sterility following irradiation during early embryonic development	Reproduction	[31,170]
		Gamma	Reduced life-span following lifetime exposure	Mortality	[198]
Rat		Beta	Reduction in ovary size following irradiation of embryo	Reproduction	[198]
		Beta	Reduction in offspring weight following irradiation during gestation	Morbidity	[198]
Pig		Gamma	Sterile offspring following parental exposure	Reproduction	[77]
		Gamma	Reduction of post natal brain weight	Morbidity	[200]
All	(5-10) x 10^3		No data available		
Mouse	>10 x 10^3	Beta	Embryo mortality	Reproduction	[224]

Table 6.3 | Effects of different dose rates of chronic ionising radiation on fish

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Plaice	<100		Anomalies of reproductive system	Reproduction	[120]
Plaice	100-1,000	Gamma	Decrease sperm production	Reproduction	[113]
Medaka		Gamma	Reduction in testis mass	Reproduction	[101]
Roach		Gamma	Lower fecundity, delayed spawning	Reproduction	[144]
Guppy	(1-5) x 10^3	Gamma	Infertility induced	Reproduction	[164]
Plaice		Gamma	Reduced testis weight and sperm content (at 168 days)	Reproduction	[113]
Eelpout		Gamma	Decrease testis weight/sperm content	Reproduction	[85]
Medaka		Gamma	Reduced fertility	Reproduction	[101]
Medaka		Beta	Severe depletion of spermatogonia (30 days)	Reproduction	[102]
Guppy		Gamma	Fecundity reduced (988 days)	Reproduction	[218]
Guppy		Beta	Reduced male courtship activity (17 days)	Reproduction	[79]
Rainbow trout		Gamma	Reduced immune response	Morbidity	[112]
Medaka	(5-10) x 10^3	Gamma	Depletion of spermatogonia (120 days)	Reproduction	[101]
Medaka			No effect on mortality	Mortality	[101]
Medaka	(10-50) x 10^3	Gamma	Increase in vertebral anomalies	Mutation	[103]
Medaka		Beta	Reduction in larval survival	Reproduction	[103]
Medaka		Beta	No effect on hatching rate	Reproduction	[103]
Medaka		Gamma	No effect on hatching rate	Reproduction	[103]
Guppy		Gamma	Sterility (288 days)	Reproduction	[218]
Guppy	>50 x 10^3	Gamma	No impact on offspring survival following parental irradiation	Mortality	[218]

6.5.2 Aquatic invertebrates

Data exist mainly for marine polychaetes (see Table 6.4). Clear effects on reproductive endpoints have been observed at dose rates of <5,000 µGy/h for gamma radiation and <10 x 10^3 µGy/h for tritium beta radiation. Effects on growth in a sponge colony were also observed after (5-10) x 10^3 µGy/h. For polychaetes and sponges, dose rates below 10 x 10^3 µGy/h and probably below 5 x 10^3 µGy/h should be used in future experiments. For other phyla, a wider range may be necessary (see Section 6.5.4).

Table 6.4 Effects of different dose rates of chronic ionising radiation on aquatic invertebrates

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Marine polychaete	100-1,000	Gamma	Reduced embryo survival (probably due to lethal mutations)	Reproduction	[92]
Marine polychaete	(1-5) x 10^3	Gamma	Reduced breeding performance	Reproduction	[114]
Marine polychaete	(5-10) x 10^3	Beta	No effect on egg production; decreased survival of eggs to larvae	Reproduction	[115]
Marine polychaete		Gamma	Decreased egg production; no effect on larvae survival	Reproduction	[115]
Marine sponge		Gamma	Inhibition of new growth by sponge colony	Morbidity	[217]
Marine polychaete	(10-50) x 10^3	Gamma	Gamete killing; reduced fertilisation success	Reproduction	[92]
Marine polychaete		Gamma	Sterility	Reproduction	[92]

6.5.3 Aquatic plants

Data for these are very limited, with virtually no information on chronic exposures. The lowest dose rates shown to produce sublethal effects on aquatic plants are between 2,000 and 5,000 µGy/h [48].

As a result, no summary table is provided for this wildlife group.

6.5.4 Crustaceans and molluscs

Each of these groups has data indicating effects on larvae after exposure to dose rates of <1,000 µGy/h (see Tables 6.5 and 6.6). Effects on larval goose barnacle (a crustacean) were reported following only <0.1 µGy/h of beta radiation from tritiated seawater while, in oysters, larval abnormalities occurred after exposure to 125 µGy/h of beta radiation from tritiated seawater and 170 µGy/h gamma radiation from ^{65}Zn in seawater [145]. Unfortunately, there are no reliable effects data for any other dose rates below 50 x 10^3 µGy/h. At these higher rates, there were clear effects on reproductive endpoints.

Table 6.5 Effects of different dose rates of chronic ionising radiation on crustaceans

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Goose barnacle	<100	Beta	Impaired larval development (0.1 µGy/h)	Morbidity	[1]
Daphnia	>50 x 10^3	Gamma	Reduced fecundity; increased mortality (with additional stress of food limitation, 54 x 10^3 µGy/h)	Reproduction Morbidity	[57]
Blue crabs		Gamma	Reduction of growth; increased mortality (290 x 10^3 µGy/h)	Mortality Morbidity	[75]

Table 6.6 Effects of different dose rates of chronic ionising radiation on molluscs

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Oyster	100-1,000	Beta	Increased larval abnormalities	Reproduction	[145]
Oyster		Gamma	Increased larval abnormalities	Reproduction	[145]
Freshwater snail		Mixed	Decreased egg capsules but increased eggs per capsule	Reproduction	[56]
	1,000 to 50 x 10^3	No data			
Freshwater snail	>50 x 10^3	Gamma	Decreases in numbers of egg capsules, eggs and egg hatch rate (100 x 10^3 µGy/h)	Reproduction	[57]
Freshwater snail			Life span reduction (100 x 10^3 µGy/h)	Morbidity	[57]
Clam		Gamma	Decreased survival (160-370 x 10^3 µGy/h)	Mortality	[20]

6.5.5 Birds, amphibians and reptiles

For birds, there is little evidence of effects at <5,000 µGy/h; Table 6.7 contains a brief summary of the information available in R&D Publication 128 [60]. As previously, users are advised to conduct a full literature search to assess the availability of information on the biological effects of ionising radiation on birds.

The only reliable data for chronic radiation effects on amphibians indicate increased chromosome damage in frogs exposed to <100 µGy/h. The very limited data indicate that, for lizards, effects on reproduction and life-span occur after exposure to dose rates <100 µGy/h. Data for both amphibians and reptiles are summarised in Table 6.8.

Table 6.7 Effects of different dose rates of chronic ionising radiation on birds

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Tree swallow	<100	Gamma	No impact on breeding performance, production of fully fledged young, growth or embryonic mortality	Reproduction	[226,227]
	(1-5) x 10^3		No data available		
	(5-10) x 10^3	Gamma	Reduced nesting	Reproduction	[204]
Tree swallow	>10 x 10^3	Gamma	Embryonic mortality	Reproduction	[226]

Table 6.8 Effects of different dose rates of chronic ionising radiation on reptiles and amphibians

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Frog	<100	Beta	Increased chromosome aberration rate 2-10 fold	Mutation	[74]
Lizard	100-1,000	Gamma	Regression of ovaries	Reproduction	[194]
Lizard		Gamma	Induction of sterility in males	Reproduction	[194]
Lizard		Gamma	Impact on maximal life span of some reptile species	Morbidity	[195]
	>1,000		No data available		

6.5.6 Soil fauna and insects

Effects of different dose rates of chronic ionising radiation on soil biota fauna and insects are summarised in Table 6.9.

Data on soil fauna are mainly from field experiments, where soil activity has been increased artificially or due to a nuclear accident (e.g. [193]). Reduced numbers of earthworms were observed after a dose rate of 100 µGy/h of alpha radiation (^{226}Ra) and increased chromosomal damage occurred in scorpions exposed to gamma radiation in this low dose rate range.

Table 6.9 Effects of different dose rates of chronic ionising radiation on soil fauna and insects

Species	Dose rate (µGy/h)	Radiation	Description	Endpoint	Reference
Earthworm	<100	Alpha	Reduced numbers compared with control plots. Smaller, reproductive and histological changes	Mortality Reproduction Morbidity	[118]
Insect larvae		Alpha	Reduced numbers compared with control plots	Morbidity	[118]
Scorpion		Gamma	Increased chromosomal aberrations	Mutation	[186]
Midge		Mixed	Increase in chromosome aberrations	Mutation	[23]
	100-1,000		No data available		
Earthworm	(1-5) x 10^3		Reduced population size	Mortality	[119]
Myriapods Spiders Earthworm	(5-10) x 10^3	Beta	Reduced numbers compared with control plots	Mortality	[193]
Bark beetle	(10-50) x 10^3		Reduced pupal survival	Morbidity	[30]
Soil Invertebrates			Reduced population sizes	Mortality	[118]
Ants	>50 x 10^3		Behavioural changes of colony	Morbidity	[29]

Earthworms showed reduced population size after exposure to <5,000 µGy/h of gamma radiation, while myriapods and spiders also decreased in numbers following exposure to (5-10) x 10^3 µGy/h of beta radiation ($^{90}Sr/^{90}Y$). Given their numbers and ubiquity, there are few reliable data for the effects of chronic radiation on insects.

Chromosomal aberrations were increased in midge larvae exposed to <100 µGy/h of mixed radiation, while a similar dose rate of alpha radiation reduced numbers of insect larvae. Bark beetles exposed to 21 x 10^3 µGy/h of gamma radiation had reduced pupal survival. An ant colony exposed to 100 x 10^3 µGy/h showed behavioural changes, although it was not clear whether this effect was due directly to radiation or to radiation-induced changes in the environment, e.g. plant cover.

6.6 Dose rate ranges in chronic irradiation experiments

In more than half the wildlife groups, data on chronic exposures to radiation are so limited that pilot experiments are recommended to estimate the best dose rate ranges to use in the main experiments. Pilot experiments should use three or four dose rates. An unirradiated control group should always be used in all experiments for comparison purposes.

Pilot experiments are demanding in terms of time and expense, particularly for larger, long-lived organisms. As an alternative, to be used with caution, there may be limited possibilities to extrapolate results from one wildlife group to another; thus, results for mammals could be used as a guide for birds, those for fish as a guide to amphibians, or those for polychaete worms as a guide for other aquatic invertebrates and some soil biota (e.g. oligochaete worms).

Table 6.10 gives an indication of the dose rate ranges that might be used in future experiments.

Table 6.10 | Suggested dose rate ranges for future experiments

Wildlife group	Dose rates for further investigations (µGy/h)			Comment
	Adult organisms	Larval organisms	Chromosome Damage	
Mammals	<1,000		<100	Many data
Reptiles	<1,000			Limited data, but clear indication of effects
Fish	≤5 x 10^3			Many data
Coniferous plants	≤5 x 10^3			Many data
Soil biota	≤5 x 10^3		<100	Few data' alpha-irradiation effect on adult earthworms at <100 µGy/h
Aquatic plants	≤5 x 10^3			Very few data; need pilot experiment
Aquatic invertebrates	≤10 x 10^3	<1,000		Data mainly for polychaetes; other species may need pilot experiment
Non-coniferous plants	≤20 x 10^3			Dose rate range used will vary with plant type
Birds	≤10 x 10^3			Very few data; need for pilot experiment
Crustaceans	≤50 x 10^3	<100		No data (0.1-50) x 10^3 µGy/h; need pilot experiment
Molluscs	≤50 x 10^3	<1,000		No data (1-50) x 10^3 µGy/h; need pilot experiment
Insects	≤50 x 10^3	<100	<100	Very few data; need pilot experiment

The dose rates in the table are based on information found in Section 6.5. The rates given should be used as upper limits with other, lower dose rates descending to about 1,000 µGy/h (or lower depending on the endpoint). The same endpoints as those to be observed in the main experiment should usually be examined in the pilot one.

The life-stage to be irradiated and the endpoint to be examined are of great importance when determining the dose rates to be used. There are several indications that dose rates for larval organisms and studies of chromosome damage may be considerably lower than those for other endpoints measured in adults.

6.7 Facilities required for chronic radiation studies

Any facilities in which exposure to ionising radiation is carried out must comply with the Radioactive Substances Act 1993 (RSA93) [165] and the Ionising Radiation Regulations 1999 [189]. A Certificate of Registration will be required for all radioactive material held and all storage and disposal of radioactive materials. A Radiation Protection Supervisor must be appointed to ensure adequate training and safe working procedures and, where expert advice is needed, a Radiation Protection Adviser must be appointed. As stated in Section 4.4, all experiments using vertebrates require Home Office project licences and the staff involved must hold personal licences.

Any facility being used for irradiation experiments must also, as a minimum, have monitoring equipment for temperature, humidity and lighting so that the environmental conditions for the organisms can be monitored and controlled as necessary. Ideally, it should be possible to control these environmental variables and the monitoring should provide the supporting evidence that this is the case. It is important to eliminate as many potential sources of variation as possible in order to demonstrate that it is exposure to the radiation having an impact. The control group's environmental conditions should be the same as those for the test groups.

6.7.1 External irradiation

Radiation will be supplied from one or more sealed gamma radiation sources. The facility must allow exposure of several different dose rate groups (Section 6.5). The sizes of the sources, as well as the distance of the irradiated organisms from them, will determine the dose rates received. All organisms in a single experimental group must receive the same dose rate. The facility should allow continuous exposures for periods of at least several months.

During experiments, there will be time periods when irradiation is stopped to allow for necessary husbandry, feeding, measurements, etc. Dose rates quoted should be the hourly rates for the periods when irradiation occurred and not averaged over periods when it did not. An estimate of the number of hours per day when irradiation was not occurring should be given and any prolonged periods (e.g. 4 hours or more) when irradiation did not occur should be noted.

The radiation sources must be able to be remotely exposed and returned to a safe position when staff entry is required (e.g. for feeding or watering), and the system should 'fail-to-safe', i.e. if the source/interlocks fail, the source should automatically be returned to a shielded position (unexposed). There should be adequate warning (audible and visual) when sources are exposed and entrance to the facility should not be possible during this time (e.g. by use of an interlock system).

The facility must have walls, floor and ceiling with shielding properties such that, when the sources are exposed, the dose rate at any point outside does not exceed 5 mSv/year or 2.5 µSv/hour. The entrance door will probably need to have a maze system to ensure this. A room allowing experimental conditions (e.g. temperature and lighting) identical or close to those in the radiation facility will be required for an unirradiated control group (e.g. same temperature and light settings).

6.7.2 Internal irradiation

Internal exposure of experimental organisms will require the use of unsealed radiation sources. Experiments will have to be carried out in a radiation-controlled area as defined in IRR99. Unirradiated controls can be maintained in the same controlled area or, if necessary, in another room where conditions close or identical to those in the controlled area can be maintained.

7. Experimental design (GPG 4)

Experimental design before conducting the research is an essential step to ensure that results are reproducible and scientifically robust. Consideration should be given to the data required to test any hypothesis with an appropriate degree of confidence.

This means that all experiments should be conducted after the statistical test that will be used to assess the data has been identified.. The experimental design should also be modified, if necessary, so that the data outputs meet the assumptions of the chosen statistical test.

The aims of this GPG are to provide users with:

- an understanding of the reasons why consideration should be given to the data outputs;
- the need to be familiar with the statistical methods that will be used to analyse and interpret the data following the experiment.

This GPG is not intended to recommend particular statistical tools or to provide a basic statistics tutorial. For this information, users should refer to one of the many statistics textbooks and other resources available.

7.1 Importance of considering statistical analysis when designing experiments

As with all scientific investigations, it is essential that those responsible for the experimental design of chronic irradiation studies are familiar with, and competent at using, a range of statistical tests. Tailoring an experiment at the design stage so that it meets the requirements of a particular statistical test is the best way to ensure that findings can make a valuable contribution to scientific knowledge. Important considerations include the number of dose rates and the number of test organisms necessary to ensure that the appropriate statistical analysis can be conducted.

A common mistake is to enter into an experimental phase without fully considering the requirements of the subsequent statistical analyses. The result tends to be a dataset that has to be forced through a particular type of statistical test, often with too few replicates, measurements or other factors that are key assumptions for that test. This may result in possible trends and relationships being observed graphically, but without the ability to draw conclusions on these with any level of statistical confidence.

7.1.1 Number of dose rates to consider

The number of dose rates selected for an experiment will be linked to the goals of the particular experiment. The protocol developer must consider how they intend to use the data obtained from their experiments. This decision is likely to be based on the specific endpoint being assessed.

Some specific endpoints, e.g. death and sterility, allow the determination of the percentage of organisms affected. Dose rates should include one causing little or no effect (~0%) and one causing an effect in all organisms (100%), with preferably one or more in between.

Other specific endpoints, e.g. reduction in number of eggs or seed and weight, will require the use of a range of dose rates from those that produce effects not significantly different from controls (e.g. $P > 0.95$) to those producing highly significant effects (e.g.

P <0.001), and preferably with a group which is marginally significant (e.g. P <0.05).

Fitting data to a regression model of dose rate versus effect allows the determination of point estimates, e.g. the dose rate producing a 10% reduction in egg or seed production. The number of dose rate groups and of individual organisms in each group should allow reasonably good definition of the regression line such that the confidence limits on point estimates are small enough to be meaningful. Ideally, at least five dose rates should be used in each experiment.

This guidance has been produced with an emphasis on reproductive endpoints for the reasons outlined in Section 5. The use of at least five dose rates will, therefore, be required for most of the experiments developed using this guidance.

7.1.2 Number of test organisms to use

The number of test organisms used will depend on a variety of experimental variables such as the number of dose rates, the number of replicates required for the statistical test chosen, the requirement for interim kills and the number of individuals needed to assess the specific endpoint under investigation. Some of these issues are considered further in Section 5.

7.2 Using statistical analyses

Statistical analyses provide a means by which outputs from experiments (generally as numerical datasets) can be tested in an objective manner to answer a particular 'question' with a given degree of confidence. The 'question' generally takes the form of a statement known as the hypothesis.

In its simplest form, the question is in a yes/no format and two paired statements (hypotheses) are tested to determine which is accepted as being correct. For example, in an experiment on worm reproduction, the two hypotheses may be:

- chronic gamma radiation exposure at a dose rate of 5,000 µGy/h does not cause a reduction in the reproductive success of the earthworm; or
- chronic gamma radiation exposure at a dose rate of 5,000 µGy/h does cause a reduction in the reproductive success of the earthworm.

The first hypothesis, which refers to a 'no effect' situation, is termed the null hypothesis (H_0). The second hypothesis, which refers to an effect being observed, is termed the alternative hypothesis (H_1).

Statistics can also be used to answer other types of questions. These may be about a particular relationship or about the association between samples. For example:

- What is the dose-response relationship exhibited by earthworms exposed to chronic gamma radiation in terms of reproductive output?
- Which stressor or combination of stressors are most closely associated with the reproductive output endpoint in a multi-contaminant study on earthworms using both radioactive and chemical stressors?

In general, the more complex the question, the more complex the statistical test, and the greater the importance of statistical considerations at the experimental design stage. Statistical tests essentially provide the means by which data can be assessed and used as the basis for drawing conclusions in a scientifically justifiable way.

7.3 Interpreting experimental data - a statistical approach

Experimental data are generally collected in a numerical form and, in the case of the laboratory-based experiments discussed in this report, there should be a control group to allow comparisons with the treatment groups (i.e. groups exposed at different dose rates).

The assessment of the experimentally derived data is normally a three-tiered approach, as illustrated in Figure 7.1.

Data are subjected to an initial screen, summarised and then compared to determine whether any differences can be described as significant and at what level of confidence. Reviewing findings in this way develops familiarity with the data before conducting a detailed statistical analysis. This helps to ensure that data are not interpreted incorrectly.

Tier 1 is an important first step in data analysis. Graphical representation of the raw data provides an immediate indication of whether or not there is a clear relationship within the dataset and/or a difference between treatment groups. It also helps to highlight anomalous data.

Contemporary statistical tests often run through thousands of calculations to return a value that demonstrates whether a difference between experimental groups is statistically significant at a particular level of confidence. To permit such calculations to be conducted quickly, a number of

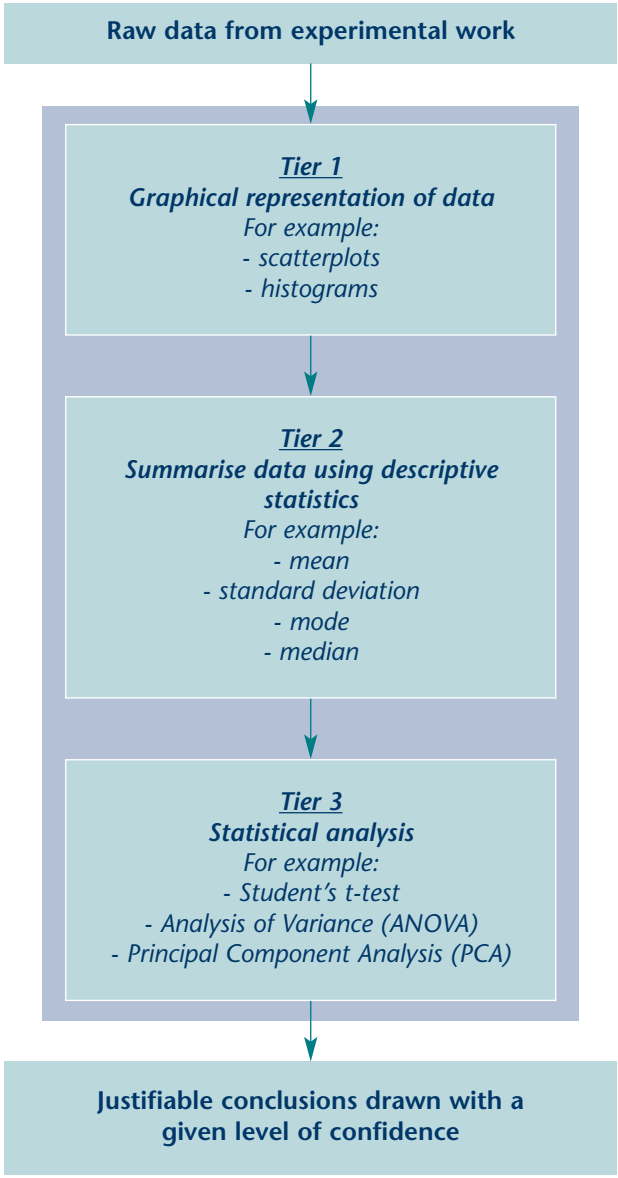

Figure 7.1: Three-tiered approach to data analysis

software packages have been developed to run on a personal computer. Packages currently used include Microsoft® Excel (capable of running most common statistical tests), MINITAB® (http://www.minitab.com) and SPSS® (http://www.spss.com). The latter two are capable of running more complex statistical tests such as Principal Component Analysis (PCA). These programmes have made the process of data interpretation more manageable. However, because the algorithms that form the basis of the statistical tests run in the background, the programmes become 'black boxes' into which data are fed and from which a value is obtained. The person undertaking the analysis thus gains little feel for the way in which the data are being manipulated and has a reduced ability to determine points at which errors may be introduced and/or compounded.

For this reason, visual inspection of raw data in a graphical form is an important step in avoiding anomalous values being introduced into the analysis, which may result in erroneous conclusions being drawn. Graphical assessment of the data also helps to indicate whether the key assumptions of the chosen statistical test are met. Such assumptions often include whether or not the data show a normal distribution and must be met if the outputs from the statistical test are to remain valid.

In Tier 2, descriptive statistics help to provide an additional check for anomalous results and allow the dataset to be summarised and reviewed easily. Descriptive statistics are also useful when determining whether the assumptions of the particular analysis test(s) to be used in Tier 3 are valid. If necessary, the data can be transformed or normalised to reduce the potential for error before the statistical test is applied.

When dealing with a null and alternative hypothesis situation, there are two potential errors:

- Type I error - the null hypothesis is rejected when it is actually true.
- Type II error - the null hypothesis is accepted when it is actually false.

Statistical tests generally take a cautious approach by limiting the probability of making a Type I error. Results of an analysis are then quoted as being significant or not significant at a given confidence level. However, as stated previously, if the original assumptions of the chosen statistical test are not met, the outputs from the analysis may not be valid. Tiers 1 and 2 of the assessment approach therefore complement each other in ensuring that the analysis undertaken in Tier 3 is appropriate and that the outputs are truly justifiable.

Tier 3 is the stage that allows visually determined relationships (via graphical outputs from Tier 1) to be tested to determine their statistical significance. The output from Tiers 1 and 2 should have confirmed that the assumptions of the chosen statistical test are valid for the particular dataset. However, certain assumptions of the main statistical test selected may require additional checking to confirm their validity. This may include tests for factors such as homogeneity of variance and normality of the dataset. Once the validity of the assumptions is confirmed, the analysis can proceed. The type of statistical test used will depend on the questions that are being asked of the data. Any basic statistics

textbook will be able to provide guidance on this. Examples of tests applicable to toxicology experiments include regression, correlation, Analysis of Variance (ANOVA) and Principal Component Analysis (PCA).

Following the application of an appropriate statistical test, conclusions can be drawn and reported. Any report should state the statistical manipulations applied to the data, including any tests conducted to confirm that the data meet the assumptions of the main statistical test and the confidence level at which a significant or not significant conclusion is being made. This ensures that the experimental results are justifiable to the wider scientific community and that any worker can easily follow the data analysis process. The aim of statistics is to reduce the level of subjectivity that influences the conclusions drawn by experimenters.

7.4 Application of statistics in designing experiments to determine dose-response relationships

Experimental design has to take place within the practical and logistical constraints resulting from the choice of a particular species. Unfortunately, even within these constraints, it is not possible to give a simple prescription for an appropriate experimental design in terms of levels of replication and selection of doses. However, it is possible to state the general principles that must be followed in all cases. These principles have been described in many publications (e.g. [51,80,84]).

A key step is to identify and quantify the objectives of the experiment. If these are phrased in terms of estimating some parameter (e.g. LD_{50}), the corresponding objective of the design is usually to provide sufficient replication to ensure that the confidence limits of the estimated parameter are sufficiently narrow. If the experiment results in a statistical test such as comparing the mean response in a control and study group, the objective is for sufficient replication to ensure a high probability that a given difference between the control and study group is statistically significant. This is usually referred to as statistical power analysis, about which there is extensive literature (e.g. [141]) supported by both commercial and free software (e.g. [190]). Users who do not feel confident about applying these techniques should consult a statistician.

When fitting dose-response curves, four design factors that affect the precision of estimates or the power of tests must be considered. These are:

- dose range
- dose spacing
- number of doses
- intensity of replication.

The first two are determined by considering the shape of the dose-response curve. The last two are determined by considering the variability around the dose-response curve. If there is no prior information about the likely shape and variability, a pilot scheme should be carried out. If there is no information about an appropriate dose range, a practical upper bound of, for example 10 mGy/h, could be adopted.

8. Other considerations

There are a number of other deliberations, that must be taken into account, in addition to the technical issues surrounding the design of experimental protocols.

8.1 Staffing and resources

With chronic irradiation experiments, there is often an intense period of activity during the experimental set-up phase, followed by the regular work needed to monitor and maintain the test species. This regular work will often involve part-time working, e.g. 1-2 days per week. However, animals experimented on under Home Office licences must be observed daily, including at weekends. During any interim sampling periods and at the end of the experiment, there will be a significant increase in workload. These points should be considered when planning staffing requirements for a long-term chronic experiment.

Specialist support may be needed on a short-term basis to provide, for example, technical input into the endpoint measurements and suitable dosimetry calculations, if these tasks can not be conducted by available members of the project team.

As mentioned in Section 6.7, the primary resource requirement will be access to a suitable laboratory where the irradiation can be conducted, if necessary, under RSA93, IRR99 and Home Office licensed conditions (depending upon the test species). Access to such facilities may be limited and construction costly. Therefore, thought should be given to maximising the potential for any given facility to undertake multiple experiments at the same time. This will, of course, depend on the species and dose rates being targeted. With appropriate extra shielding, it should be possible to conduct multiple experiments at different dose rates within the same facility.

8.2 Finances

It is impossible to generalise about the overall cost of chronic radiation experiments. Some of the elements that require consideration are indicated below.

If possible, the period of irradiation should include a complete life-span or, if not, a significant part of the period of reproductive maturation and activity. This period will vary considerably depending on the test species involved; its life-span and its breeding cycle.

The number of individual organisms needed to obtain significant results from an experiment may vary considerably. Where there is great uniformity in the dose-rate response of organisms for chosen endpoints, smaller numbers will be required to establish significant differences from an unirradiated control group. It may be necessary to carry out pilot experiments in order to determine inter-organism variability and thus the experimental number of individuals required.

The cost of obtaining organisms will vary greatly. Some may have to be purchased from suppliers, while others may be obtained from other research groups or from the wild. The organisms may require culturing in the laboratory prior to use in the experiments to ensure that the individuals included in the experiment are at a similar life-stage.

The maintenance of some organisms and/or the assessment of some endpoints may demand more staff hours, attendance during out-of-work periods or specialised equipment.

Experiments using internal emitters (alpha and beta) will generally be more expensive than those using external sealed sources (gamma radiation). Pilot experiments will be necessary to establish the relationship between delivered activity (in food, water, soil) and equilibrium concentrations in the organism (and thus the dose rate to it). Radioanalysis will be required to measure radionuclide concentrations. The cost of the radionuclide may be significant and will vary depending on the one used.

The most suitable biomarker tests may vary with different organisms and the time and equipment needed to carry these out may also vary considerably.

9. Examples of good experimental design and protocol

This guidance aims to help users develop high-quality experimental protocols that can make a significant contribution to knowledge. This section demonstrates good experimental design in practice by examining an investigation into the effects of chronic radiation exposure on fish. It also provides a worked example of how to develop a protocol for experimenting on earthworms.

9.1 Good experimental design in practice

This example of good experimental design has been included to indicate the quality of experiments that are anticipated. The key stages of the experiment are related to the appropriate GPGs to highlight the way in which the guides integrate to form a protocol development tool.

The following sections describe an experiment designed to compare the effects of gamma and alpha radiation on zebrafish, Danio rerio. This experiment takes into account many of the factors necessary for successful radiation exposure experiments described in this guidance. The experimental information is taken from Environment Agency R&D Technical Report P3-053/TR [111].

9.1.1 Test species selection (see Section 4, GPG 1)

Most economically or ecologically important fish species have features that make them unsuitable for long-term laboratory experiments (e.g. large size, difficult to maintain). However, it is apparent from the literature that fish are among the most radiosensitive of the aquatic organisms for which information is currently available [200,220] and all species (freshwater or marine, tropical or temperate) appear to have similar radiosensitivities [101,113,218]. Thus, results for more suitable experimental fishes are likely to be relevant for most species. The zebrafish was chosen as an experimental animal for the following reasons: small size; ease of maintenance; a continuous breeder; relatively short life-span with sexual maturity achieved in a few months; large literature available on husbandry and biology; and ease of obtaining fish from reliable sources. The zebrafish, as with all vertebrates (and octopus) is covered by the UK Animals (Scientific Procedures) Act 1986 and the experiments were carried out under a valid project licence by staff holding appropriate personal licences.

9.1.2 Endpoints (see Section 5, GPG 2)

The main endpoints examined were those of reproductive output. These endpoints are of major importance to the maintenance of the population in the wild and it is populations, rather than individuals, that are generally the object of environmental protection measures.

It has been shown that approximately the same number of eggs per week per female zebrafish is produced when breeding is allowed daily or once per week [27]. Thus for practical reasons (less demand

on staff time), pairs of zebrafish were allowed to breed only once per week.

The following were recorded at each weekly lay opportunity: total number of eggs; number of eggs viable at 24 hours; and number of viable eggs which subsequently hatched successfully. Lay opportunities at which eggs were actually laid were recorded as 'used lay opportunities', and numbers of eggs, etc. per lay opportunity and per used lay opportunity were computed for each pair. In addition to these endpoints, routine measurements of fish length and weight were carried out and histological sections of whole fish or fish gonads were examined.

Although no biomarker tests were carried out in the main experiment, Comet analysis was successfully carried out on cells from irradiated larval zebrafish in a separate additional study. There seems to be no reason why cells from adults could not be similarly examined. However, the relatively small size of zebrafish means that non-fatal sampling of blood or other tissues during an experiment is unlikely to be successful and experiments must include enough fish to permit interim kills if biomarker tests are to be undertaken at times other than only at the end of the experiment.

9.1.3 Exposure (see Section 6, GPG 3)

Gamma-irradiation of zebrafish was achieved easily using ^{137}Cs sealed sources in a radiation facility, which included all the safety features described in Section 6.7. Fish were exposed continuously (except for approximately one hour per day for husbandry and experimental observations) for over one year.

There were three dose rate groups, each group being exposed to a separate array of three ^{137}Cs sources: 7,400 µGy/h; 1,000 µGy/h; and 300 µGy/h. Dosimetry was carried out using lithium fluoride TLDs and took into account the shielding effects of water at different positions within the experimental tanks. The experimental dose rates were chosen, on the basis of previous experience and reports in the literature, to produce severe effects at the highest rate, no effects at the lowest rate and marginal effects at the intermediate rate.

Alpha radiation exposures represented a much greater challenge. The radionuclide ^{210}Po was chosen as the source of alpha radiation as it is well known to accumulate to a high degree in soft tissues, including the gonads, which were a prime target in the experiment [41,42,49]. In addition, it is important in the environment both as a significant component of the natural background and as a contaminant from human activities (e.g. concentrated in wastes from phosphogypsum plants).

^{210}Po was delivered to zebrafish by feeding them meals of spiked *Artemia* larvae (brine shrimp). The alpha radiation dose rate is dependent on the tissue radionuclide concentration and so, in order to obtain alpha radiation exposures at the desired dose rates, preliminary experiments had to be carried out to determine the relationship between the rate of feeding ^{210}Po activity to the zebrafish and the resultant steady-state tissue concentration. The results of pilot experiments allowed the feeding rates required to give the chosen dose rates of 8, 25,185 and 740 µGy/h to be determined.

At the end of the main experiment, however, when the fish had received spiked food for approximately a year, radioanalysis showed ^{210}Po tissue concentrations lower than predicted and the dose rates calculated using these were 9.6, 19, 84 and 215 µGy/h. This suggests that, over a prolonged period, uptake of ^{210}Po decreased or its clearance rate increased in the higher dose rate groups. Thus, in future it will be necessary to allow enough fish in each experimental group so that samples can be taken for radioanalysis during the experiment and any changes in tissue concentration of alpha-emitter, and thus dose rate, can be monitored and taken into account or, possibly, compensated for. Determination of the location of ^{210}Po at the cellular level in zebrafish gonads was carried out using alpha-autoradiography.

The use of sealed and unsealed radiation sources requires authorisation and compliance with IRR99 and RSA93. The experiments on zebrafish were carried out in strict compliance with these. Radioanalysis for ^{210}Po was carried out under the supervision of experienced radioanalysts using well-established protocols. The recovery of ^{210}Po and efficiency of scintillation counting were checked regularly.

An unirradiated control group was treated in the same way and maintained under exactly the same conditions as each of the radiation groups, except that it received no gamma radiation or any ^{210}Po-spiked food. Control and alpha radiation groups were kept in the same constant temperature room, where conditions of temperature and light were kept identical to those in the gamma radiation facility.

9.1.4 Experimental design (see Section 7, GPG 4)

The design of the experiment was greatly facilitated by previous experience of chronic gamma-irradiation experiments with other fish species [113,218]. Prior knowledge of the dose rates likely to produce effects

on the reproductive system and of the variation in response between individual fish allowed gamma radiation dose rates to be chosen with a degree of confidence. Only three experimental groups were used with dose rates which clearly spanned the range from no effect to severe effect (cessation of egg laying).

There was little information available to aid decisions on the alpha radiation dose-rate groups. While pilot experiments were necessary to determine feeding rates of spiked food as noted above, it was not practical to carry out a pilot experiment to examine radiation effects as it would have taken as long as the main experiment. The dose rate groups were chosen on the basis of experience with chronic gamma-irradiation of fish and known RBEs from the literature for alpha radiation compared with gamma radiation. These RBEs are mainly for man and mammals. Although some very high RBEs of 50 to several hundred have been reported [109,173], an RBE of 10-40 was used to determine the desired alpha dose rates to zebrafish of 8-740 µGy/h from the gamma dose rates of 300-7,400 µGy/h. The number of alpha radiation groups was increased to four rather than the three for gamma radiation.

Statistical analysis of the results utilised all of the tiers described in Section 7.3. Results for the measured endpoints were examined by plotting graphs and by calculating values such as mean number of eggs per fish per lay and mean number of eggs hatching for the fish in each experimental group. Comparisons were then made using ANOVA. The analyses showed that none of the alpha-radiation dose rates produced a significant effect on the reproductive endpoints measured.

As noted above, the highest alpha radiation dose rate actually achieved was 215 µGy/h due to the lower than expected tissue concentration of ^{210}Po. It may be that, had the chosen alpha dose rate of 740µGy/h been achieved, an effect would have been observed. Alternatively, it may be that the RBE for reproductive endpoints in zebrafish is less than 10.

9.1.5 Conclusions

While the work described in this example has, for the most part, taken into account those factors required for successful chronic radiation exposure experiments, the comparison between the gamma-irradiations, where there were already many data available, and the alpha-irradiations, where there were very few, is instructive. The desirability of having sufficient numbers of fish in each dose rate group to allow for interim sampling and radioanalysis has already been mentioned.

In situations where there is little information, it may be that, despite the expense of relatively long pilot experiments, their use in narrowing down the experimental dose rates to those of interest (i.e. the effect/no effect boundary) may prove efficient. They would probably allow other problems (e.g. delivery of alpha radionuclide) to be overcome before the main experiment is carried out. Alternatively, a single main experiment where the dose rate groups are spread out over a relatively large range, such that a least one causes significant effects on the endpoint of interest, may prove worthwhile. Even though the dose rate causing an effect and the highest dose rate not causing one may be separated by a large gap, the provision of a range (i.e. from RBE no greater than to RBE no less than) could still be important where no RBE information exists.

9.2 Developing protocols - an example

This section details the approach to follow when deriving a protocol for a chronic irradiation experiment on earthworms. Each stage in the decision-making process is described and the completed pro-forma is presented. Considerations for finances and resources were excluded from this example.

9.2.1 Using the key to complete the pro-forma

The key described in Section 3.1 provides a structure to the decision-making process required to complete the pro-forma. At each stage in the process, the decision is recorded on the pro-forma (Figure 9.1) along with the justification for that decision. The justification in this example is based on the information and recommendations of this report and is, therefore, referenced by page, section and/or table number. Additional justification is shown in the box provided.

(a) Umbrella endpoint of interest

The appropriate umbrella endpoint to use was unknown, so Section 5 (GPG 2) was consulted. This highlighted the importance of reproduction as an endpoint of interest in terms of environmental protection because factors affecting reproduction affect the long-term survival of the population.

Because the experiment being developed in this example is intended to provide data applicable to environmental protection, reproduction was chosen as the most relevant umbrella endpoint.

(b) Choice of wildlife group and species

Reading Section 4.3 and FASSET Deliverable 1 [185] helped to understand the justification behind the inclusion of each test species group. Soil invertebrates were recommended as reference organisms for terrestrial ecosystems, with particular emphasis being placed on earthworms. Section 4.17 described the widespread use of earthworms, particularly *Eisenia fetida*, as a test organism for ecotoxicology studies and highlighted the ease with which this species can be experimented on. It also provided references for ISO and OECD publications [105,151] to assist in developing the protocol as well as a relevant Environment Agency technical report [184].

(c) Maintenance conditions

Table 4.13b provided some information on the husbandry considerations for earthworms. Additional details were available from the ISO and OECD publications [105,151].

(d) Specific endpoints to study

References to Section 5.2, Tables 5.1 and 5.2 helped to understand the types of specific endpoints under reproduction that can be considered. Candidate specific endpoints for soil fauna were identified using Table 5.1. Table 5.2 helped to identify references that contained details on the techniques associated with these endpoints. This information was then crosschecked with the OECD and ISO information to identify the most appropriate specific endpoints to investigate.

(e) Selection of irradiation type

FASSET Deliverable 1 [195] emphasised the fact that worms are highly exposed to external irradiation. It thus seemed appropriate to select this type of radiation exposure to ensure that the results would be environmentally relevant. Section 6.2 confirmed that this was appropriate and achievable given the resources available for the project. External gamma irradiation was selected because, although soil fauna may also be exposed to external beta [185], the safest and most cost-effective approach seemed to be the use of remotely exposed sealed sources in a dedicated irradiation chamber.

(f) Facilities required

The facility available for the experiment already had a ^{137}Cs source in its irradiation chamber, so this was selected for use; as noted in Section 6.2, that ^{137}Cs and ^{60}Co are the most commonly used sources for conducting external gamma irradiation experiments. Section 6.7.1 highlighted additional facility requirements (e.g. a source that will 'fail-to-safe' in the event of a problem), so the facility was checked against these requirements to ensure that it was appropriate for use.

(g) Dose rates to use

Section 6.5.6 suggested that there was evidence of a reduction in earthworm population size at dose rates below 5,000 µGy/h. Five dose rates, in addition to the control, were selected to ensure that there would be sufficient data points to produce a dose-effect curve as described in Section 7. The upper dose rate exceeded 5,000 µGy/h because it is important to ensure that an effect is seen. If an obvious effect is not observed, a reliable dose-effect relationship cannot be derived (Section 6). The background dose rate is to be measured at the time when the experiment is conducted.

(h) Requirement for a pilot experiment

It was decided that no pilot experiment would be required as there is already evidence of an effect below 5,000 µGy/h (as described in Section 6.6).

(i) Duration of irradiation and experiment

It was decided that the duration of the experiment should be determined by the life span of *E. fetida* because the environmental exposure of organisms to chronic doses is normally continuous throughout their lives (as noted in Section 6.1). Reference to the ISO and OECD documentation [105.151] and the Environment Agency technical report [184] revealed that 108 days would be an appropriate duration for the experiment.

(j) Number of replicates and individuals

Through reference to Section 7 and a statistics textbook, it was decided that a minimum of 12 tanks of worms would be used at each dose rate. Histopathological examination was planned at the mid-point of the experiment's duration and this would require an interim kill of half the worms. This would leave six tanks per dose rate to be used for the statistical analysis at the end of the experiment. However, concerns over the potential for unplanned deaths (e.g. due to soil drying out in some of the tanks) led to the number of tanks being increased to 20 per dose rate. This would ensure that, even in the event of some tanks failing, there should still be sufficient material to complete the experiment.

The number of individuals per tank was determined by the size of tanks to be used. The tanks chosen were 30 cm x 20 cm x 20 cm. Reference to the ISO and OECD documentation led to the decision to use six worms per tank.

(k) Statistical analysis

Through reference to Section 7 and a statistics textbook, the importance of selecting the right statistical technique was highlighted at the protocol development stage. In this example, the most appropriate method for analysing the data appeared to be ANOVA. This requires the observations to be distributed normally - a requirement that can be confirmed by graphically assessing the data in Tier 1 of the statistical analysis. The variances of the samples must also be similar, so this will be checked in Tier 2 of the statistical analysis using the F_{max} test. Once it has been confirmed that these requirements are met, ANOVA can be performed in Tier 3 of the statistical analysis.

After determining the statistical method to be used, the protocol on the pro-forma was checked to ensure that the experiment would provide the quantity and type of data necessary to perform the statistical analysis. To ensure that all necessary data are collected accurately, a data recording sheet was developed for use during the experiment (Figure 9.2).

(l) Further literature review

A further literature review was conducted in parallel with the completion of the pro-forma. Additional sources other than this guidance have been inserted in the reference section of the pro-forma.

Figure 9.1 Completed pro-forma for developing a protocol to investigate the effects of chronic irradiation on earthworms

~~**Pilot experiment**~~**/Main experiment (delete as appropriate)**					
Key instruction			**Page**	**Section**	**Table**
a	**Umbrella endpoint of interest** (e.g. reproduction)	Reproduction	53 57	5.1.2 5.2.1	5.1 5.2
b	**Wildlife group and species**	Soil fauna Earthworm (*Eisena fetida*)	44	4.17	4.13a
c	**Maintenance conditions** (e.g. temperature, light regime, diet)	Topsoil and manure, 16L 8D Tank moisture kept at 50%	44	4.17	4.13b
d	• Specific endpoint(s) to study (e.g. no. of eggs produced)	No. of cocoons produced No. of juveniles and viable juveniles	53+ 54	5.2	5.1 + 5.2
	• Compulsory measurements to record	Weight Length Growth rate No. of mortalities occurring			
e	**Irradiation type** (internal/external/mixed)	External gamma	59	6.2	
f	**Facilities required** (e.g. Cs-137 source)	Cs-137 source that 'fails-to safe'	68	6.7.1	
g	**Dose rates to use** (e.g. background, 10, 20, 40, 80, 160, 320, etc. Gy or µGy/h) Where known, also indicate (in the brackets) the total dose received at each dose rate.	Background = to be measured Dose rate 1 = 0.1 mGy/h (0.4 Gy) Dose rate 2 = 0.4 mGy/h (1.6 Gy) Dose rate 3 = 1.5 mGy/h (6 Gy) Dose rate 4 = 4.0 mGy/h (16 Gy) Dose rate 5 = 8.0 mGy/h (33 Gy)	66	6.5.6	
h	**Need for a pilot experiment?**	~~YES~~/NO			
	• No. of dose rates, including control				
	• No. of individuals per dose rates				
	• Dose/dose rates used				

Figure 9.1 | cont'd

Key instruction (cont'd)			Page	Section	Table
	• Duration of irradiation				
	• Duration of experiment				
	• Notes/other considerations				
i	**Duration of irradiation** (e.g. list daily time period for irradiation - 20 hours)	Continual - irradiation only interrupted for assessing endpoints	58	6.1	
	Duration of experiment	108 days	58	6.1	
j	**No. of dose rates, including control**	6 groups (20 tanks per group)	69	7	
	No. of individuals per dose rate (e.g. 10)	6	69	7	
k	**Statistical requirements**		69	7	
	• Tier 1	Graphs. Check data distributed normally.			
	• Tier 2 (e.g. test used)	F_{max}test			
	• Tier 3 (e.g. test used)	ANOVA			
l	**Further literature search conducted?** If yes, then list.	YES/~~NO~~ [105, 151, 184, 185], statistical textbook			
Further justification of decisions made to complete the pro-forma (to be completed when decisions are not based on information in the guidance document that can be referred to by page/section/table)		The dose rates were calculated based on the dimensions of the irradiation facility in conjunction with consulting relevant literature.			
Notes (general)		Endpoints assessed every two weeks for 17 weeks. Week 9: Interim kill of half the worms (six tanks in each dose group); proportion of these worms to be prepared for histopathological analysis. Week 17: Final kill of remaining worms (six tanks in each dose group); proportion of these worms to be prepared for histopathological analysis.			

Figure 9.2 | Data recording sheet for earthworm experiment

Earthworm Experiment Data Recording Sheet							
Date and week no.							
Dose rate							
Tank no.	Average	Average weight (g)	No. of length (mm)	No. of mortalities	No. of offspring cocoons	No. of offspring (no. of viable offspring)	Other observations
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							

10. Research priorities

The EC-funded Framework for Assessment of Environmental Impact (FASSET) project began in November 2000. Its aim is to develop a framework that would enable the environmental impact of radioactive contamination to be assessed in order to achieve appropriate radiation protection for non-human biota. One of the outputs, Deliverable 4 [219], produced the FASSET Radiation Effects Database (FRED).

The FRED contains information collated from over 1,000 references on the effects of radiation on non-human biota. Summaries of each document reviewed are included in the FRED, along with any dose-effect relationship data. The development of this database has enabled gaps in knowledge to be identified and, in particular, areas in which data are insufficient for dosimetric modelling and thus impact assessment.

Targeted research is required in order to address these gaps. This report aims to ensure that targeted research is undertaken in a harmonised way to aid comparison of results between wildlife groups. Some gaps should be addressed as a matter of priority, not least because current environmental protection legislation requires certain species to be adequately protected and the information required to assess whether this is being achieved is lacking [61].

Different species are recommended within each wildlife group for chronic experiments (Section 4). Available information has been reviewed and summarised, and those wildlife groups with little, if any, data on the effects of ionising radiation at chronic environmental exposures are highlighted as priorities for research. Test species are missing for the three wildlife groups - lichens, mosses and reptiles.

Studies of reproductive endpoints are recommended within this report (Section 5) as the assessment requirements are directed at ensuring the integrity of wildlife populations, and not of individuals within those populations. Measures of reproduction will therefore contribute to our understanding of how radiation exposure may affect a population's ability to maintain itself. It should be noted, however, that standard measures of morbidity (growth rate, biomass, etc.) should be undertaken in parallel. Measures of mutation endpoints could also be conducted at the same time, provided sufficient numbers of individuals and hence biological material are available within the experiment.

As mentioned in Section 1, this report has assumed that low-level chronic dose rates will be used in the experiments. Therefore, priority for experiments should be given to low-level chronic exposures. However, experiments should also target the threshold level above which an effect can be observed in order to be able to state when an effect may occur (Section 6).

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Glossary

Absorbed dose
Quantity of energy imparted by ionising radiation to unit mass of matter such as tissue. Unit: Gray; symbol: Gy. 1 Gy = 1 joule per kilogram.

Acute exposure
Exposure received within a short period of time. Normally used to refer to exposure of sufficiently short duration that the resulting dose can be treated as instantaneous (e.g. less than an hour).

Biomarker
A biological response to an environmental pollutant which gives a measure of exposure. The response may be molecular, cellular or whole organism.

Best Practicable Environmental Option (BPEO)
The option, which in the context of releases from a prescribed process, provides the most benefit or least damage to the environment as a whole, at acceptable cost, in the long-term as well as the short-term

Chronic exposure
Exposure persisting in time. Normally used to refer to continuous exposures to low concentrations of pollutants.

DNA
Deoxyribonucleic acid. The compound that controls the structure and function of cells, and is the material of inheritance.

Dose
General term for quantity of ionising radiation.

Dose rate
Dose released over a specified unit of time.

Endpoint
The characteristic of the biological unit under investigation that is being assessed in relation to different dose rate regimes.

Experimental (or specific) endpoints
Quantifiable characteristics of an organism or its progeny that can be used to investigate the effects of a contaminant on a particular umbrella endpoint.

Feature habitat
A named habitat that has been identified as requiring protection in Agency guidance on the Habitats Directive; see Appendix 13 (Habitats and species protected under the Habitats and Birds Directives) of EU Habitats Directive and Regulations Process Handbook for Agency Permissions and Activities (2003).

Feature species
A named species that has been identified as requiring protection in Agency guidance on the Habitats Directive; see Appendix 13 (Habitats and species protected under the Habitats and Birds Directives) of EU Habitats Directive and Regulations Process Handbook for Agency Permissions and Activities (2003).

Fecundity
The number of viable offspring produced by an organism; mature seeds produced, eggs laid, or live offspring delivered, excluding fertilised eggs that have failed to develop.

Fertility
In sexually reproducing plants and animals, it is the number of fertilised eggs produced in a given time.

Habitat
The place in which a plant or animal lives.

Habitats Directive
The abbreviated name for Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. The Directive aims to promote the conservation of certain habitats and species within the European Union.

Ionising radiation
Radiation that produces ionisation in matter. Examples include alpha particles, gamma rays, X-rays and neutrons.

LD_{50}
The dose that causes mortality in 50% of the organisms tested.

Morbidity
The state of being diseased.

Mortality
The number of deaths in a given period.

Mutation
A change in the genetic material of an organism. This can be spontaneous or induced by chemicals or radiation.

Radiation weighting factor (w_r)
w_r values represent the relative biological effectiveness of the different radiation types, relative to X-rays or gamma rays, in producing endpoints of ecological significance.

Radionuclide
An unstable nuclide that emits ionising radiation.

Reference organism
A series of entities that provide a basis for the estimation of radiation dose rate to a range of organisms, which are typical or representative of a contaminated environment. These estimates, in turn, provide a basis for assessing the likelihood and degree of radiation effects.

Relative Biological Effect (RBE)
A relative measure of the effectiveness of different radiation types at inducing a specified health effect, expressed as the inverse ratio of the absorbed doses of two different radiation types that would produce the same degree of a defined biological endpoint.

Reproduction
The formation of new individuals by sexual or non-sexual means.

Risk
A measure of the probability and extent of harm.

Test species
A species that can be used to determine the dose-effect response under experimental conditions. Ideally, these should be biologically similar to both the feature species and reference organisms. They will, therefore, provide data that can be used to assess the impacts from ionising radiation to feature organisms.

Umbrella endpoints
A descriptive term that is used to group biological effects of particular types, e.g. morbidity, mortality, mutation and reproduction.

List of abbreviations

ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
BPEO	Best Practicable Environmental Option
EQS	Environmental Quality Standard
FASSET	Framework for ASSessment of Environmental impacT; EC Fifth Framework Programme (FP5) project, Contract FIGE-CT-2000-00102
FRED	FASSET Radiation Effects Database
GPG	Good Practice Guide
HSE	Health and Safety Executive
IRR99	Ionising Radiations Regulations 1999
NOECs	No Observed Effect Concentrations
OECD	Organisation for Economic Co-operation and Development
PCA	Principal Component Analysis
R&D	Research and Development
RSA	Radioactive Substances Authorisation
RSA93	Radioactive Substances Act 1993
SEPA	Scottish Environment Protection Agency
TLD	Thermoluminescent dosimeter
UNECE	United Nations Economic Commission for Europe
US EPA	US Environmental Protection Agency

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