Home Office

NON-TECHNICAL SUMMARY

Chlorine toxicity effects on marine fish species

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- (e) Research aimed at preserving the species of animal subjected to regulated procedures as part of the programme of work

Key words

Chlorine, Toxicity, Marine fish, Antifouling

Animal types	Life stages
marine fish species	Juvenile, Adult

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is required, and should be submitted within 6 months of the licence's revocation date.

Reason for retrospective assessment

This may include reasons from previous versions of this licence.

- Contains severe procedures

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will assess the effects of specific doses (exposure concentration and exposure time) of chlorine, to locally relevant UK marine fish species by mimicking antifouling practices in coastal industries. The results will be presented to industry to aid decisions on a dose that is both fit for purpose whilst considering the health of associated marine fish species.

A retrospective assessment of these aims will be due by 15 April 2030

The PPL holder will be required to disclose:

- Is there a plan for this work to continue under another licence?
- Did the project achieve its aims and if not, why not?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to assess the impact of chemicals that are used by industries in aquatic environments:

- To maintain the UK's high aquatic animal health status.
- To protect aquatic animals from the impacts of chemicals used for antifouling in coastal industries.
- To advise on levels that will minimise harm to aquatic animals.

Although there are data present for the effects of chlorine on many fish species, there are no data available for the doses chosen under this project licence in the species that will be tested. Where chlorine is used as an antifoulant to control biofouling in coastal industries, it is important to provide data to aid decisions on the dose that is both fit for purpose whilst considering the health of associated marine fish species.

What outputs do you think you will see at the end of this project?

- New knowledge on the effect of chlorine on selected marine fish species at doses where this information is unknown.
- Independent science to determine the levels of chlorination for use in industry, to minimise or eliminate adverse effects on marine fish species.
- Advice to industry on potential impacts of chlorine on marine fish species, to aid decisions on antifouling dosing.
- The data will be publicly available through publication of results in a peer reviewed scientific open access journal/open access report.

Who or what will benefit from these outputs, and how?

The industry will benefit from best practice and public perception.

Outputs have the potential to benefit millions of marine fish. The effects of chlorine to specific relevant wild marine fish species at doses used for antifouling will be identified. The results will influence decisions on dosing strategies, used in industry to minimise harm to these species.

How will you look to maximise the outputs of this work?

Dissemination of new knowledge to industry with the aim to publish results in an open access report and a peer reviewed open-access scientific journal.

Where possible, extra samples may be taken to further research aims and to aid associated research. Tissues may be frozen or preserved for future use.

Species and numbers of animals expected to be used

- Other fish:
  - marine fish species: 2170

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The species will be chosen due to (1) their relevance to the potential impact of the industrial antifouling proposals (proximity and quantity); (2) their ecological and commercial importance; (3) lack of previous data; (4) availability for experimental use; and (5) the suitability of the facilities to house and care for the fish.

A species sensitivity distribution review has shown that fish are one of the most sensitive groups to chlorine in marine waters (second to sea urchins). The exposure studies under this licence will be performed on marine fish species that are most likely to be present during stages of chlorine antifouling management within the industrial process. Initially, European sea bass (Dicentrarchus labrax) juveniles (less than 2 years old and around 7 to 30 cm) will be tested. Based on fisheries surveys and data from chlorine exposure tests performed under a previous licence, this species, life stage and size is predicted to be one of the most relevant and commercially important species that is likely to be present where antifouling management will occur and is sensitive to the effects of chlorine. Several fish species are known to be more sensitive to chlorine in their early life stages, juveniles therefore reflect a 'worst case scenario' and limits the requirement to test older species. However, adult fish may be used to confirm the results if results are ambiguous, if older fish of a particular species are identified to be more relevant in the industrial process, or if species specific sensitivities are identified. It is important to understand what effect altering the chlorine dose in industrial antifouling management procedures would have on relevant species and life stages. This data would then be presented to industry to aid decisions on dosing strategies.

Future studies under this 5-year licence will require justification of the marine species selected based on the same criteria and will require approval from the local Animal Welfare and Ethical Review Body before use.

Typically, what will be done to an animal used in your project?

Initially a pilot study will take place to assess the suitability of a metal mesh container for transfer of the fish. Fish will be transferred as they would in the main study using a metal mesh container, (mimicking the screen present at the point of antifouling in the industry), into unchlorinated seawater. Fish appearance and behaviour will be assessed for up to 48 hours.

The main study will mimic potential chlorine antifouling procedures in industry. In the main study, the marine fish species will be transferred in a metal mesh container to a tank/holding container that contains a pre-calculated concentration of chlorine in seawater. The fish will be immersed in chlorine between 0.1 and 0.3 mg/L, (measured as total reactive oxidants), for a maximum of 1 hour. At the end of the exposure, the chlorinated water will receive a rapid dilution with new seawater, to dilute the chlorine to undetectable levels (fish may be tipped from their chlorinated container into a tank of new seawater). When this is not suitable (e.g. due to tank design/size of the fish), the fish will be returned to unchlorinated tanks of seawater from their test tank/holding container by net.

Negative control fish will undergo the same procedure with the addition of reverse osmosis water (used as chlorine diluent, at a volume that will not significantly affect salinity) instead of chlorine.

The effects of chlorine (toxicity) will be assessed for up to 48 hours post-immersion (by frequent observation of appearance and behaviour). Fish will be removed and humanely Schedule 1 killed/killed under procedure using a non-Schedule 1 method (to enable rapid collection of tissues of high quality for analysis of toxicity), if they reach a defined early or humane endpoint throughout the study and all remaining fish after 48 hours.

When multiple tanks of different doses are tested under the same experiment the chlorine exposure will be staggered by at least 24 hours to assess initial acute effects, starting with the tank receiving the

lowest dose. Where the level of harm exceeds previous studies of 2-year-old fish exposed to 0.2 mg/L for 100 minutes, the higher doses will not be tested and the study terminated.

When experiments are performed on different occasions with different batches of fish, previous tests will be considered and the same restrictions will apply - If a dose causes harm over the 48-hour observation period in excess of previous experimental data, (2-year-old fish exposed to 0.2 mg/L for 100 minutes), further studies of higher doses will not be tested on that species at that life stage.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that transfer of the fish via a metal mesh container (within minutes), although smooth mesh, may cause physical damage on the external surfaces of the fish including the skin, eyes and fins. The lifting and movement out of the water are likely to cause mild stress (short term). Should fish reach a defined humane endpoint they will be removed to prevent suffering.

One or more of the following adverse effects may be observed in the appearance or behaviour of the fish post transfer:

- Appearance: Abnormalities can occur in the skin (e.g. bruising, pigmentation colour, loss/increased mucus, raised scales/scale loss, pale patches, pinpoint red marks caused by haemorrhaging), eyes (e.g. cloudy, haemorrhaging), mouth (e.g. haemorrhaging, physical damage), fins (e.g. haemorrhage, splits or tears, injury, drooping, raised or flared,).

- Behaviour: Abnormalities may be observed in feeding behaviour, ventilation (e.g. slow or fast, flaring of the operculum (the flap that covers the gills), gulping at surface, coughing), activity (e.g. lethargy, abnormal swimming), location (e.g. irregular position within the tank, relative to other fish).

It is unknown if the concentrations and duration of exposure of chlorine will have any effect on the animals, and there may be species differences. However, chlorine has been reported to have abnormal effects which can be observed in the appearance and behaviour of fish:

- External appearance: Abnormal skin pigmentation (darkening, lightening, mottled), fin damage, mucus secretion, aggression and/or cannibalism.

- Internal: Gill damage, significant decrease in red blood cells, white blood cells, haemoglobin and haematocrite (PCV%), and an increase in cortisol.

- Behaviour: Loss of equilibrium (abnormal horizontal orientation, abnormal vertical orientation, loss of buoyancy control), abnormal swimming behaviour (hypoactivity, hyperactivity, corkscrew swimming, convulsions, tetany, irritated skin behaviours, abnormal surface distribution/behaviour, abnormal bottom distribution/behaviour, over-reactive to stimulus, under-reactive to stimulus, loss of schooling/shoal behaviour, dense schooling/shoal behaviour), abnormal ventilatory function (hyperventilation, hypoventilation, irregular ventilation, coughing, gulping, head shaking), stress.

A fish showing toxic effects may display one or more of these abnormalities and defined humane endpoints will be used where possible.

Handling effects from netting with a normal fabric net is likely to be minor and short term.

The fish will be held for up to 48 hours post procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The greatest level of severity expected from the effects of chlorine are severe and could lead to mortality. The effect is unknown for the doses we will be giving the fish and is likely to differ between species and size tested.

Fish will be removed where possible before this severity is reached. Defined humane endpoints will be used. Actual severity experienced will be mitigated by timely removal and humane termination if necessary.

- It is expected that 25% of each species will be negative controls and are likely to experience a sub-threshold severity (no adverse effect expected).
- It is expected that 65% of each species might experience a moderate severity.
- Approximately 10% of each species might reach severe severity.

What will happen to animals used in this project?

- Killed

A retrospective assessment of these predicted harms will be due by 15 April 2030

The PPL holder will be required to disclose:

- What harms were caused to the animals, how severe were those harms and how many animals were affected?

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Replacement with another method to determine the effects of the chlorine dose to the fish is not possible. To date, a non-animal model has not been correlated for chlorine toxicity effects on different fish species in the dose range we will be testing. Computer models have not been developed and verified to adequately predict the toxicity effects in live fish. *In vitro* cell culture methods are not

developed enough to represent all species with multiple tissues and cell types. Each species and life stage reacts differently and require complex interrelated metabolic, anatomical mechanisms that cannot currently be adequately replaced with animals at a more immature life stage, species that are less sentient, or animals that have been terminally anaesthetised.

Continual review will ensure replacements are not missed.

Which non-animal alternatives did you consider for use in this project?

- Published data on sequential testing: When sequential testing methods have been employed to a specific species, using a sufficient number of multiple concentrations/doses, a concentration/dose-mortality curve can be applied rather than using the specific species.
- Predictive models.
- Non-animal models e.g. cell culture methods, algae or daphnia.
- Non-protected embryo life stages.

Why were they not suitable?

- Sequential testing using regulated procedures is performed using a 96-hour exposure duration, we have no means of understanding when the mortality occurred within the 96h, therefore doesn’t present the information we are requiring.
- Predictive models have not been sufficiently developed and verified for chlorine toxicity in the species chosen.
- To date, a non-animal model has not been validated to correlate with chlorine toxicity effects on different fish species in the dose range we will be testing. Investment has been made in developing *in vitro* assays with cytotoxicity endpoints, including the rainbow trout RTgill-W1 cell line assay, but currently *in vitro* cell culture methods are not developed enough to represent all species with multiple tissues and cell types. Comparisons have been made between the daphnia reproduction test, and the algae growth inhibition test for many chemicals (but not chlorine) and algae are usually more sensitive than fish. Without the data and correlation on the effects of chlorine in each species compared to algae and daphnia, we cannot be certain they would be representative of the effects in the fish. Each species reacts differently and requires complex interrelated chemical and physical mechanisms that are not currently adequately modelled.
- To date, chemical/chlorine toxicity to embryos has not been validated to correlate with chemical/chlorine toxicity effects on different fish species. Where the Fish Embryo Toxicity and Acute Fish Toxicity have been compared, it has been reported that 22% of substances in the final dataset showed >10 -fold weaker toxicity in fish embryos than in adult fish. Without information on the effects of chlorine at different life stages of each species compared to fish embryos, we cannot be certain they would be representative of later life stages. The fish embryo acute toxicity test does not currently fulfil regulatory acceptance and may underestimate the risk.

A retrospective assessment of replacement will be due by 15 April 2030

The PPL holder will be required to disclose:

- What, if any, non-animal alternatives were used or explored after the project started, and is there anything others can learn from your experience?

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals to be used and minimum number of replicate tanks have been discussed with and approved by our statistician and experienced aquarists. Numbers have been estimated based on the number of experimental units, accounting for three replicates for a single dose, three doses per study with one negative control group per dose, and estimated number of studies that may be required over a 5-year period (6 studies per 5-year period testing species and dose differences). Statistics are based on a minimum of ten fish per tank, however group sizes are guided by the husbandry requirements of the fish (particularly stocking density).

An example of our estimations has been calculated (however, each experiment may differ in the number of doses tested at any one time):

Pre-test: metal mesh container transfer x 10 fish

Immersion with chlorine at three doses (dose = concentration x time), with three replicates of ten fish per dose (= 90 fish)

+ Negative controls, immersion in reverse osmosis water (without chlorine), One replicate of ten fish for each dose (= 30 fish)

x six times over the period of the five-year licence (= 720 fish/five years)

x three to allow for up to 30 fish per tank should husbandry requirements require it (fish = 2,160), although a minimum of ten will be tested when possible. The advice and guidance of the Named Veterinary Surgeon and Named Animal Care and Welfare Officer will be sought and applied.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Tools for best practice have been referred to such as the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines and fish specific guidelines (NORECOPA) and the Experimental Design Assistant (EDA) for design of experiments.

Continual review and improvements will be made where possible in the infrastructure of the facility to enable further reduction of animal numbers with regards to husbandry requirements. An example of a previous improvement at our facility is the design of variable standpipes, which enable stocking density adjustment by changing the tank volume (i.e. tank volume can be reduced to increase the stocking density to one suiting a species rather than using more fish).

A detailed formal study plan is prepared for each study undertaken, and contains study specific objectives and justifications for use, severity of adverse effects and the number of fish required. Study plans are reviewed by the local AWERB, fish husbandry experts and the Statistical Services Group, ensuring that all the 3Rs have been considered and animal use is reduced to the minimum required to achieve the aims of the study.

Juveniles (Less than 2 years old and around 7 to 30 cm) will be the main life stage tested to reflect a 'worst case scenario' and limits the requirement to test older fish.

When multiple tanks of different doses are tested under the same experiment the chlorine exposure will be staggered by at least 24 hours to assess an initial acute effect (toxicity), starting with the tank receiving the lowest dose. Where the level of harm exceeds previous studies of 2-year-old fish exposed to 0.2 mg/L for 100 minutes, the higher doses will not be tested, and the study terminated.

When experiments are performed on different occasions with different batches of fish, previous tests will be considered and the same restrictions will apply - If a dose causes harm in excess of previous experimental data (2-year-old fish exposed to 0.2 mg/L for 100 minutes), over the 48-hour observation period, further studies of higher doses will not be tested on that species at that life stage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A pre-test to assess the suitability of the metal mesh container used to transfer the fish will refine the procedure to be the most suitable design. Should the mesh be too abrasive and cause significant adverse effects which will impact the study alternatives will be sought for the experimental mesh container.

Where possible, extra samples may be taken to aid associated research. Tissues may be frozen or preserved for future use (e.g., control tissue, training purposes, etc).

A retrospective assessment of reduction will be due by 15 April 2030

The PPL holder will be required to disclose:

- How did you minimise the numbers of animals used on your project and is there anything others can learn from your experience?

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare

costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The fish may be produced in our own breeding establishment (from purchased eggs) to ensure disease-free status and high-quality animals. In cases where we need to purchase from fish farms, we typically select sites with an established health history and high standards of biosecurity and animal welfare (including during transportation). Externally sourced fish will be held in quarantine while a health screen takes place to ensure a good health status, and acclimation to our tank conditions, before use in studies.

We will abide by published guidelines and ensure best working practice. Husbandry information (including extra environmental enrichment) is obtained for all species to inform the species-specific needs and ensure they are met. Advice is sought from published literature, experienced Fish Health Inspectors, experienced aquatic facilities staff, the Named Veterinary Surgeon and the Named Animal Care and Welfare Officers.

The study design involves a non-invasive procedure (not requiring the use of sedation, analgesia or general anaesthesia). A pre-test to assess the suitability of the mesh container used to transfer the fish will refine the procedure to be the most suitable design. Exposure to chlorine by immersion will best represent what will happen to the fish during chlorination antifouling in industry. Should the chlorine appear to be intolerable or cause severe adverse effects to an individual fish, defined humane endpoints will be used to prevent pain, suffering, distress or lasting harm. Continual refinement will ensure that if a dose causes harm in a species in excess of previous experimental studies (of 2-year-old fish exposed to 0.2 mg/L for 100 minutes), further tests with a longer duration or higher concentration will not occur on that species at that life stage.

The chlorine exposure studies will first be performed on the lowest dose of chlorine that is relevant to industry based on current practice. Results will then be used as guidance for the requirement to test higher doses.

To find out if the chlorine has any short-term toxic effect on the marine species, we need to observe their appearance and behaviour post immersion. Holding up to 48 hours post immersion will allow observation for any potential acute effects. A non-Schedule 1 kill will allow for samples to be taken of better quality for histology.

Fish under procedure will be monitored via direct visual checks using bespoke in-house recording sheets for abnormalities. It is not possible to maintain large numbers of fish in anaesthesia for the duration of the study and would interfere with mimicking the normal effects of chlorine on the fish. The frequency of direct PIL checks will be at least twice a day and will increase when adverse effects are expected or present, (with checks at least every 2 hours in the first 8 hours and frequency related to the severity and speed of progression of adverse effects after that). Direct visual checks will be supplemented by video observations using underwater cameras mounted within tanks, when possible, to observe behavioural and morphological changes not easily detectable during a normal direct check. The facility has currently over 30 cameras used routinely in disease challenge work. As an aid to

implementing humane endpoints the cameras have been invaluable in our ability to refine severe Protocols.

Why can’t you use animals that are less sentient?

This project licence is specific for the marine fish species of relevance that are exposed to the chlorination process. Assessment of older life stages of the fish will allow for assessment of gill damage, which have direct contact with the chlorine during the exposure period. Fish that are pre-first-feeding are not likely to be present where antifouling management will occur as they would be small enough to pass through the screen.

More immature life stage/less sentient species, for example algae, daphnia, fish embryos or invertebrates, are not a suitable replacement due to the unknown differences between chemical-host interactions compared to the fish, and sensitivity at different life stages.

Currently, there are not enough data and correlations on the effects of chlorine on the fish species compared to algae or daphnia so we cannot be certain they would be representative of the fish. Daphnia and algae are usually more sensitive than fish when comparisons have been made for the daphnia reproduction test, and the algae growth inhibition test.

Fish embryo assays have so far been the most promising potential alternative for predicting fish acute toxicity to chemicals, as a (partial) replacement for fish at later life stages. The broad applicability of the Fish Embryo Acute Toxicity (FET) Test assay has been questioned as there are certain limitations; thus the FET test is not fully accepted as an alternative to the Fish Acute Toxicity test at this point in time.

Where the FET and Fish Acute Toxicity tests have been compared, it has been reported that 22% of substances in the final dataset showed >10 -fold weaker toxicity in fish embryos than in adult fish. It has also been published that fish embryos were less sensitive than larvae or adult stages when chlorine/chemical toxicity was compared. Further assessment when more valid FET data are available would be needed to assess the predictive capacity of the FET given the very limited availability of quality data for inorganic compounds.

In this case the chemical is chlorine which is used as a disinfectant for eggs and can improve survival. Surface disinfection of fertilised fish eggs is widely used in aquaculture to reduce pathogens that may be released from brood fish during spawning. However, chlorine can have severe effects in many species of fish after hatching.

Without data and correlations on the effects of chlorine on the fish species compared to fish embryos, we cannot be certain they would be representative of the fish.

Where possible, when we have eggs in-house (dependant on funding, suppliers and our capabilities rearing different species) we will run a comparative study to help refine future work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To assess the effect of chlorine for each dose, it is important to know if the fish are affected to the point it cannot return to normal (defined early and humane endpoints). As the effects are unknown at the doses that will be included in this protocol, expected adverse effects may be severe in the test species, however, fish will be removed where possible before this severity is reached. Standardised in-house record sheets will be used to record abnormalities in behaviour or appearance over time (where one observation provides a snapshot, while the development and duration of clinical signs are important additional considerations).

A key output from the study will be the quantification of effects allowing for accurate environmental assessment of potential chlorination to ensure that the effects are correctly assessed and where appropriate additional mitigation or offsetting is planned. As such the experiments need to continue for the full duration or until 100% of fish are removed to enable precise mortality/moribundity factors to be derived from the data. However, defined early and humane end points will be used when possible. Frequent monitoring will be undertaken to ensure humane endpoints to avoid mortalities - at least twice a day, increasing in the period during and after treatment and if signs of toxicity are observed. Based upon previous studies, severe effects as a result of exposure are likely to occur within the first 8 hours following initial exposure. Therefore, more frequent monitoring will increase during this period to at least one check every 2 hours.

A camera and video surveillance will be installed in each tank where possible to observe behavioural and morphological changes not easily detectable during a normal direct check and to increase the number of checks (but not replace direct checking) to ensure humane endpoints are met and minimise suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Tools for best practice for design of experiments will be referred to such as: the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines (http://journals.sagepub.com/doi/full/10.1177/0023677217724823); fish specific guidelines (NORECOPA) (https://norecopa.no/prepare); and the Experimental Design Assistant (EDA) (https://www.nc3rs.org.uk/our-portfolio/experimental-design-assistant-eda). Other resources include guidance and publications from the NC3Rs (https://nc3rs.org.uk/3rs-resources) and the CCAC (https://ccac.ca/en/guidelines-and-policies/the-guidelines/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date about advances in the 3Rs by regularly checking information on NC3Rs website, attending scientific meetings, RSPCA meetings/publications, peer reviewed scientific publications, communication with other scientists and obtaining advice from the Named Persons.

By regularly checking the internet we will keep up to date with the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals which are periodically reviewed to incorporate scientific progress, changing regulatory needs, and animal welfare considerations.

As there is an OECD ad hoc group working to address the use of TG 236 (Fish Embryo Acute Toxicity (FET) Test), within the aquatic testing strategy, we will follow that activity as well as the updates to the European Chemicals Agency (ECHA) web page in regard to the potential for adaptation.

We are continually refining systems further updating the infrastructure needed for procedures involving vertebrates (for example, our facility has designed variable standpipes which allow for a range of water volumes, whereas in the past tanks could only be full or half height/we have installed cameras for remote observations and videos).

A retrospective assessment of refinement will be due by 15 April 2030

The PPL holder will be required to disclose:

- With the knowledge you have now, could the choice of animals or model(s) used be improved for future work of this kind? During the project, how did you minimise harm to the animals?