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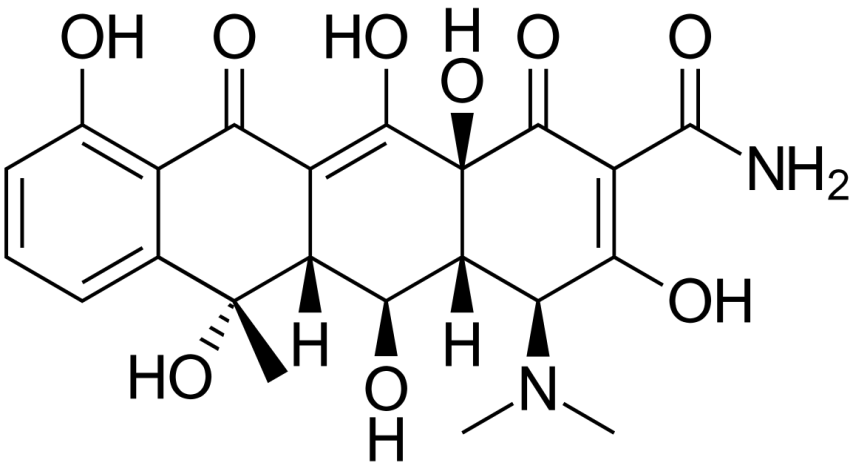
Minor use permit for oxytetracycline for non-salmonid finfish



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Cover image: Oxytetracycline molecule.

Executive Summary

There are no registered or permitted antimicrobial products approved by the Australian Pesticide and Veterinary Medicine Authority (APVMA) for treatment of bacterial infections in finfish. This project developed an application for a minor-use permit (MUP) for the use of oxytetracycline (OTC) to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We used public domain, published and some unpublished data to satisfy the APVMA data requirements to fulfill the requirements of the MUP application for the proposed use pattern. The application was submitted in June 2021.

OTC is the first-choice treatment for bacterial infections in finfish. It is effective, inexpensive and although it has low bioavailability, it is generally palatable to fish at doses that provide tissue concentrations that exceed minimum inhibitory concentrations for target pathogens. This application proposes a treatment range of 50-100 $mg.kg^{-1}$.day for 10 days to effectively treat infections without creating undue environmental effects while countering loss of availability to divalent cation complexing in seawater.

Oxytetracycline complexes with divalent cations in the gut of fish, reducing already low bioavailability. Maximum tissue concentrations are reached 2-48 hours after a single dose. Serum levels typically fit two-compartment kinetic models but OTC is typically excreted unmetabolized. Elimination is reached 4 days after cessation of exposure. Elimination is strongly temperature dependent.

OTC is widely used in fish and broadly regarded as safe although some effects of overdose have been reported, mostly from gavage studies. Studies summarised in the application did detect a few negative effects of overdose or increased duration. Tilapia or snubnose pompano fed 800 $mg.kg^{-1}.day^{-1}$, eight times the maximum dose and more than 10 times the recommended dose of OTC, both showed negative hepatic responses, but these fish recovered after cessation of treatment. Increased duration of treatment did not cause severe negative effects; snubnose pompano treated for 30 days developed only recoverable histopathological changes and no mortality.

Oxytetracycline is an antimicrobial, and antimicrobial resistance (AMR) is a recognised threat to human health. The World Organisation for Animal Health ranks oxytetracycline as an antimicrobial of critical veterinary importance. The Australian Strategic Technical Advisory Committee on Antimicrobial Resistance (ASTAG) considers tetracyclines developed for human use (doxycycline, minocycline) to be of low importance to human health whereas the WHO ranks them as being of Importance to human health. A qualitative microbiological risk assessment is included in the MUP application. It assesses the risk to humans posed by the use of OTC in feed formulations in non-salmonid finfish aquaculture in Australia. It also addresses the potential for OTC to promote resistance in finfish pathogens and/or environmental/commensal organisms that is transferable to human pathogens, and result in resistance to critically important human drugs.

Overall, the likelihood of harm arising from the use of oxytetracycline in non-salmonid finfish species in Australia was considered possible but unlikely, and the risk rating assessed as low. The greatest hazard was selection for tigecycline resistance, which has increased in the last 10 years given the emergence and spread of resistance genes in China among commensal and pathogenic bacteria from humans, animals and the environment. Exposure was considered negligible and impact low, due to conservative regulation of critically important antimicrobials in humans and agriculture in Australia and generally low rates of resistance among Australian Gram-negative pathogens to fluoroquinolones, carbapenems and colistin.

OTC delivered to fish is dispersed in faeces, urine and in dissolved form into the environment. OTC used in a treatment and released into the environment is incorporated into sediments and water. Toxicity of

OTC declines rapidly in sediments, even when no chemical degradation occurs. This project produced environmental toxicity data for OTC for Australian taxa and calculated protective concentrations for 95% of environmental organisms (PC95 values) for OTC using the ANZECC water quality guidelines of 6.4 $mg.L^{-1}$ in freshwater and 9.7 $mg.L^{-1}$ in seawater. Estimations of OTC concentrations released from treatments in pond and marine sea-cage aquaculture have risk quotients <1. Measured OTC concentrations in a treated pond and at a sea-cage site were substantially below predicted environmental concentrations indicating substantial dilution or rapid breakdown with half-lives of <20 d. These data indicate that OTC treatments are environmentally safe in aquaculture where therapeutically justified with maximum concentrations of 1.357 and 3.011 $mg.kg^{-1}$ detected, and that environmental residues deplete to below limits of analytical detection in 3-4 months.

The principal workplace health and safety risk associated with handling OTC is exposure to powder through eye and skin contact, or inhalation. The exposure standard for dust is applicable. Adequate protection can be provided by PPE. Persons who are allergic to tetracyclines or antimicrobials should not handle the product or medicated feed.

Targeted legitimate oxytetracycline administration in the Australian non-salmonid finfish aquaculture industry under the authority of a minor use permit will substantially improve the management of bacterial disease outbreaks given that the majority of pathogenic species causing infection in Australian finfish species remain susceptible.

Australia does not have structured antimicrobial resistance (AMR) information gathering for fish pathogens. Development and implementation of an approach to understanding microbial susceptibility and emergence of AMR at an Australia-wide scale should be prioritised. This would provide evidence of good use practices, inform risks to human health and facilitate reissue of this and other antimicrobial permits.

The data in this report are presented as separate modules that match the APVMA format. The report is immediately available for use for future applications or renewals.

Introduction

There are no Australian Pesticide and Veterinary Medicine Authority (APVMA) registered or permitted antimicrobial products for treatment of bacterial infections in finfish. Several Minor Use Permits (MUPs) have expired, and in some States and Territories off-label prescription by veterinarians is allowed. Consistent, controlled use pursuant to a regulatory approval provides a better basis for protecting fish health, the environment and human health. This project therefore developed an application for an MUP for the use of oxytetracycline (OTC) to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We used public domain, published and some unpublished data to satisfy the APVMA data requirements to fulfill the requirements of the MUP application for the proposed use pattern.

Targeted oxytetracycline administration in the Australian non-salmonid finfish aquaculture industry facilitated by a minor use permit has the potential to substantially improve the management of bacterial disease outbreaks given that the majority of pathogenic species causing infection in Australian finfish species remain susceptible to OTC.

The application was submitted at APVMA via the online portal in June 2021. The APVMA acknowledged receipt in July 2021 and indicated that their assessment would be complete by November 2022.

Objectives

1. Obtain data to satisfy identified gaps and collate available data to satisfy requirements of a minor use permit application.
2. Submit an application for a minor use permit.

Method

To confirm data requirements to complete a minor use permit (MUP) application, we assembled and lodged a request for Pre-Application Assistance (PAA) with the APVMA in December 2018. This PAA included data assembled by the authors outlining the proposed product use. APVMA provided pre-application advice in February 2019.

We used the PAA to collate data types that the APVMA identified as deficient in publicly available information based on the PAA. This comprised primarily the environment module and an antimicrobial resistance risk assessment. Assessments were assembled following the APVMA Data Guidelines. Information on data generation is included in the data modules.

These gaps were addressed by generating and collating data from online data sources, data held from industry use by PIRSA and SARDI, based on the requirements in the APVMA data guidelines. The MUP application was then submitted using the APVMA portal.

Results

Minor Use Permit application modules

This results section comprises documents that were submitted to the APVMA as part of the application for a Minor Use Permit (MUP). Format and headings are prescribed by the APVMA Data Guidelines; as such the sections are intended to stand alone and include substantial repetition and some material may appear in an order that seems unusual for a report in this format.

1.0 Efficacy

1.1 Summary

This application seeks a minor use permit (MUP) for the use of the oxytetracycline product CCD OTC produced by CCD Animal Health (APVMA #52863) to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We are seeking to use public domain, published and confidential unpublished data to satisfy the data requirements for metabolism and kinetics for the proposed use pattern.

OTC is the first choice treatment for bacterial infections in finfish. It is effective, inexpensive and, although it has low bioavailability, it is generally palatable to fish at doses that provide tissue concentrations that exceed minimum inhibitory concentrations (MICs) for target pathogens. This application proposes a treatment range of 50-100 $mg.kg^{-1}.day^{-1}$ for 10 days to effectively treat infections, while countering loss of product in seawater, without creating undue environmental effects, and . Divalent cation complexing reduces OTC availability in seawater, therefore 100 $mg.kg^{-1}.day^{-1}$ is preferred for aquaculture fish in marine environments. This dose is the upper range label dose proposed in this application. OTC is contraindicated where sensitivity testing shows that bacteria are not susceptible to OTC.

1.2 Literature

Oxytetracycline is a broad-spectrum antibiotic with bacteriostatic properties that is active against a wide variety of gram-positive and gram-negative bacteria (Hochstein et al. 1953). As a chemotherapeutant, it can be administered via enteral or parenteral routes with good tissue distribution (Stoffregen et al. 1996). Since its isolation and development in 1950, OTC has become one of the most commonly used antibiotics in aquaculture (Xu and Rogers 1994; Rigos et al. 2004).

Title: Leal et al. (2019) Oxytetracycline in intensive aquaculture: water quality during and after its administration, environmental fate, toxicity and bacterial resistance (efficacy component).

Summary: Efficacy of OTC in finfish aquaculture is summarised.

Methods: This article reviews a broad range of scientific literature.

Results: 75 $mg.kg^{-1}.day^{-1}$ is the recommended effective daily dose of OTC. Higher doses may be required in seawater due to presence of complexing divalent cations. 250 $mg.kg^{-1}.day^{-1}$ is the maximum recommended oral dose; higher doses were assessed as unnecessary and more likely to be associated with poor acceptance of feed by fish. Dose recommendations are included in the draft permit.

Title: Treves-Brown (2000) Tetracyclines (efficacy component of book chapter).

Summary: Efficacy of OTC in finfish aquaculture is summarised.

Methods: This article reviews a broad range of scientific literature.

Results: OTC has been used as a first choice medicine for nearly all bacterial diseases of finfish. It is effective in feed against, for example, *Aeromonas*, *Yersinia*, *Flavobacterium*, *Vibrio*, *Flexibacter* and *Streptococcus*. 75 $mg.kg^{-1}.day^{-1}$ for 10 days is the recommended daily dose. Marine fish require a higher dose than freshwater fish because of complexing of OTC with divalent cations in seawater.

1.3 References

Leal JF, Santos EBH and Esteves VI (2019) Oxytetracycline in intensive aquaculture: water quality during and after its administration, environmental fate, toxicity and bacterial resistance. Reviews in Aquaculture, 11, 1176-1194.

Hochstein FA, Stephens CR, Conover LH, Regna PP, Pasternack R, Brunings KJ and Woodward RB. (1953) The structure of terramycin. Journal of the American Chemical Society, 75, 5455-5475.

Rigos GI, Nengas I, Alexis M, and Athanassopoulou F (2004) Bioavailability of oxytetracycline in sea bass, *Dicentrarchus labrax* (L.). Journal of Fish Diseases, 27, 119-122.

Stoffregen DA, Bowser PR, and Babish JG (1996) Antibacterial chemotherapeutants for finfish aquaculture: a synopsis of laboratory and field efficacy and safety studies. Journal of Aquatic Animal Health, 8, 181–207.

Treves-Brown KM (2000) Tetracyclines. pp 64-82 in: Applied fish pharmacology. Springer, Dordrecht, 309 pp.

Xu D and Rogers WA (1994) Oxytetracycline residue in striped bass muscle. Journal of Aquatic Health, 6, 349-354.

2.0 Metabolism and kinetics

2.1 Summary

This application seeks a minor-use permit (MUP) for the use of CCD OTC to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We are seeking to use public domain, published and confidential unpublished data to satisfy the data requirements for metabolism and kinetics for the proposed use pattern.

Oxytetracycline complexes with divalent cations in the gut of fish, reducing bioavailability. Measured bioavailability is 0.5-15% (Treves-Brown 2000, Rigos et al. 2004). Maximum tissue concentrations are reached 2-48 hours after a single dose and elimination is reached 4 days after cessation of exposure (Ueno et al. 1995). Elimination is temperature dependent; in *Dicentrachus labrax* (European seabass) elimination occurred at 73.5 and 68.7 ml.kg^{-1}.h^{-1} at 13.5 and 22°C, respectively (Rigos et al 2004). Serum levels of OTC typically fit two-compartment kinetic models that normally suit products which are metabolised before excretion, although OTC is typically excreted unmetabolized (Leal et al. 2019).

2.2 Literature

Title: Ueno et al. 1995 Pharmacokinetics and bioavailability of oxytetracycline in cultured yellowtail *Seriola quinqueradiata.*

Summary: Half-life of OTC in serum of the marine fish *S. quinqueradiata* (yellowtail) was assessed at 21 °C. Half-life of OTC in serum was 0.7 h for the distribution phase and 23 h for the elimination phase.

Methods: 40 individual ~640 g yellowtail were anaesthetised and injected with 50 mg.kg^{-1} OTC. Four fish were sampled at each of 20 min, 40 min, 1, 2, 3, 5, 8, 12, 24, 48, 72 and 120 h post administration and serum OTC concentration was assessed by HPLC. Data were fitted to one- two- and three-compartment models in MULTI pharmacokinetic software.

Results: Apparent volume of distribution and total body clearance were 0.49 L.kg^{-1} and 15.1 L.kg^{-1},respectively. The area under the serum concentration time curve and mean residence time were 3310 µg.h.mL^{-1} and 54 h, respectively. Bioavailability of OTC was 0.6 %. Protein binding was 35.6 ± 5.9%. From estimates of bioavailability and elimination, and comparing serum OTC concentrations with minimum inhibitory concentrations of relevant organisms (~2.5 µg.mL^{-1}), a dose of 50 mg.kg^{-1} bw.day^{-1} (standard in Japan in the 1990s) was assessed as too low to be effective against target bacteria in this marine model.

Title: Malvisi et al. (1996) Tissue distribution and residue depletion of oxytetracycline in sea bream (*Sparus aurata*) and sea bass (*Dicentrachus labrax*) after oral administration.

Summary: Concentrations of OTC in serum of the marine fish *S. aurata* (sea bream) treated at 75 mg.kg^{-1} daily for 14 days in a sea cage at 24-28 °C were assessed.

Methods: 200 x sea bream of 50-70 g were held in sea-cages at 19-28°C. Fish were administered 75 mg.kg^{-1} OTC orally using a commercially medicated diet. Fish were sampled and blood, muscle, liver, vertebrae and skin with scales were collected from fish on the 2nd, 5th, 7th, 11th and 15th day

during treatment and on the 10th, 20th, 30th, 40th, 50th and 60th day post treatment. OTC was analysed by HPLC. Data were fitted to a model using a variable non-linear fitting program.

Results: Concentrations peaked on the sixth day of treatment in skin at 7.7 ± 6.71 $\mu g.g^{-1}$ and in liver at 14.65 $\mu g.g^{-1}$. Vertebral concentrations reached a steady state on the 40th day after treatment at 1.73 ± 0.92 $\mu g.g^{-1}$ and persisted at that level to the end of the study at day 60. OTC concentrations were lower in muscle and declined under 0.1 $\mu g.g^{-1}$ 20 days after treatment cessation, with a limit of detection of 0.02 $\mu g.g^{-1}$. No data were provided for *D. labrax* from this study despite its title. This study clearly shows that OTC accumulates in bone.

Title: Rigos et al. (2002) Pharmacokinetics and tissue distribution of oxytetracycline in sea bass *Dicentrachus labrax*, at two water temperatures.

Summary: Half-life of OTC in serum of the marine fish *D. labrax* (European seabass) was assessed.

Methods: 200 x sea bass of ~110 g were randomly assigned to tanks at 13.5°C and 22°C. Fish were administered 40 $mg.kg^{-1}$ OTC intravenously. Fish were sampled at 1, 2, 4, 8, 16, 32, 64 and 128 h post injection, then killed and liver and muscle samples were taken. OTC was analysed by HPLC. Data were fitted to a variety of models and assessed for fit.

Results: The absorption half-life of OTC in sea bass serum was 0.98 h at 13.5°C and 0.192 h at 22°C. Elimination half-life was 69 h at 13.5°C and 9.65 h at 22°C. Apparent volume of distribution and total body clearance were 5.62 $L.kg^{-1}$ at 13.5°C and 2.59 $L.kg^{-1}$ at 22°C. Mean residence time of OTC was 71 h at 13.5°C and 37.7 h at 22°C. Elimination is faster at higher temperatures. The total clearance of OTC was 73.5 $mL.kg^{-1}.h^{-1}$ at 13.5°C and 68.7 $mL.kg^{-1}.h^{-1}$ at 22°C. Liver OTC concentrations were higher than muscle concentrations, but OTC is eliminated from liver more quickly than from muscle.

Title: Yuan et al. (2013) Pharmacokinetics of oxytetracycline in yellow catfish (Pelteobagrus fulvidraco (Richardson, 1846)) with a single and multiple-dose oral administration.

Summary: A pharmacokinetic study of OTC following a single (100 $mg.kg^{-1}$) or a multi-dose (100 $mg.kg^{-1}$ five times daily for 5 days) oral administration was carried out in the freshwater yellow catfish, *Pelteobagrus fulvidraco* at 25 °C.

Methods: 120 g wild caught yellow catfish were given OTC by oral gavage once at 100 $mg.kg^{-1}$. Samples were taken at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 96 and 120 h after oral administration. 120 g wild caught yellow catfish were given OTC by oral gavage at 100 $mg.kg^{-1}$, 5 times per day for 5 days. Samples were taken at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 96 h after oral administration. Blood was collected from each fish. After blood collection, the fish were euthanized and liver, kidney, and skin-on muscle (fillet) samples were collected. OTC was analysed by LC-MS. A one-compartment model was developed for optimal fit for the data.

Results: Following a single (100 $mg.kg^{-1}$) or multi-dose (100 $mg.kg^{-1}$ for 5 days) oral administration of OTC in the freshwater yellow catfish, *Pelteobagrus fulvidraco*, pharmacokinietic data were assessed. For oral administration at 25°C, a one-compartment model was developed. The absorption half-life was 3.92, 1.44, 2.75, and 3.34 h in plasma, muscle, liver, and kidney after the single dose, and 0.35, 0.22, 0.42, 0.32 h in the respective tissues, after the multi-dose. The order of peak concentration was liver > kidney > plasma > muscle, at 3.48 $\mu g.g^{-1}$, 2.90 $\mu g.g^{-1}$, 1.46 $\mu g.mL^{-1}$, and 1.39 $\mu g.mL^{-1}$ after the single dose, and 14.02 $\mu g.g^{-1}$, 8.51 $\mu g.g^{-1}$, 4.17 $\mu g.mL^{-1}$, and 3.84 $\mu g.mL^{-1}$ after the multi-dose, respectively. The elimination half-lives of OTC in plasma, muscle, liver, and kidney were calculated to

be 7.64, 26.29, 19.08, and 10.61 h after the single dose, and 47.54, 70.99, 49.87, and 47.73 h after the multi-dose, respectively. OTC was absorbed faster after the multi-dose than after the single dose, suggesting that OTC could be more effective after multiple doses, albeit with a longer withholding period.

Title: Zhang and Li (2007) Pharmacokinetics and residue elimination of oxytetracycline in grass carp, *Ctenopharyngodon idellus*.

Summary: Grass carp (*Ctenopharyngodon idellus*) in freshwater at 21°C were treated using OTC either once orally by gavage at 100 $mg.kg^{-1}$ or orally with medicated feed at 100 $mg.kg^{-1}.day^{-1}$ for 7 days and pharmacokinetics were assessed.

Methods: 350 6 g grass carp were held in a pond at 21 ± 1°C. Fish were divided into 2 groups; half were administered OTC by oral gavage once at 100 $mg.kg^{-1}$ and the other half were administered OTC in feed at 100 $mg.kg^{-1}.day^{-1}$ for 7 days. Blood was sampled from the fish treated once at 0.5, 1, 2, 4, 8, 12, 24, 36, 48 and 72 h after treatment. Blood, liver, muscle and kidney were taken immediately after the last dose and at 1, 2, 3, 4, 6, 8, 10, 15, 19, 21, 23, 25, 26 and 27 days post-treatment for the fish treated for 7 days. OTC was measured using HPLC. Data were fitted to a pharmacokinetic model.

Results: Grass carp (*Ctenopharyngodon idellus*) in freshwater at 21 ± 1°C were treated using OTC either once orally by gavage at 100 $mg.kg^{-1}$ or orally with medicated feed at 100 $mg.kg^{-1}.day^{-1}$ for 7 days. In serum, the absorption half-life was 1.34 h and the distribution half-life was 5.45 h. The elimination half-life was 83.66 h. The maximum OTC concentration (Cmax) was 4.99 $\mu g.mL^{-1}$ and the time to peak concentration was 5.69 h. Residues in grass carp treated orally for 7 days were highest in kidney and lowest in muscle during OTC-elimination. OTC residues in the muscle of grass carp fell below 0.05 $\mu g.mL^{-1}$ (the detection limit) on day 25. Pharmacokinetic data conformed to a two-compartment model.

2.3 References

Malvisi J, della Roca G, Anfossi P and Giorgetti G (1996) Tissue distribution and residue depletion of oxytetracycline in sea bream (*Sparus aurata*) and sea bass (*Dicentrachus labrax*) after oral administration. Aquaculture 147, 159-168.

Rigos G, Alexis M, Andriopoulou and Nengas I (2002) Pharmacokinetics and tissue distribution of oxytetracycline in sea bass *Dicentrachus labrax*, at two water temperatures. Aquaculture, 210, 59-67.

Ueno R, Uno K and Aoki T (1995) Pharmacokinetics and bioavailability of oxytetracycline in cultured yellowtail *Seriola quinqueradiata.* Diseases in Asian Aquaculture II, 523-531.

Yuan J, Li R-Q, Shi Y, Peng X-Y, Chen X-X and Wu Z-X (2013) Pharmacokinetics of oxytetracycline in yellow catfish (*Pelteobagrus fulvidraco* (Richardson, 1846)) with a single and multiple-dose oral administration. Journal of Applied Ichthyology, 30, 109-113.

Zhang Q and Li X (2007) Pharmacokinetics and residue elimination of oxytetracycline in grass carp, *Ctenopharyngodon idellus*. Aquaculture, 272, 140-145.

3.0 Residues

3.1 Summary

This application seeks a minor-use permit (MUP) for the use of CCD OTC to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We are seeking to use public domain, published and confidential unpublished data to satisfy the data requirements for metabolism and kinetics for the proposed use pattern.

The literature on withholding periods (WHPs) is mixed and confused by inconsistent species, methodology and interpretation. Based on conservative interpretations of published data, we are requesting a 1000 degree day WHP and a 1500 degree day export slaughter interval for OTC in non-salmonid finfish in Australia. Degree days are calculated by multiplying the water temperature in degrees centigrade by the number of days following cessation of treatment e.g. 1000 degree days would represent a WHP of 100 days at 10°C or 50 days at 20°C. The Australian WHP and the Export Slaughter Interval (ESI) are different because the WHP is the period to reliably allow residues to decrease to the Australian Maximum Residue Limit (MRL), whereas the ESI is the period to allow residues to reliably decrease below the limit of detection (LoD) of the most commonly used test. This allows product to be sent to countries which do not have an MRL or use the LoD as the allowable tissue concentration of product.

3.2 Literature

Title: Rigos and Smith (2015) A critical approach on pharmacokinetics, pharmacodynamics, dose optimisation and withdrawal times of oxytetracycline in aquaculture.

Summary: Approaches to setting WHPs for OTC in finfish aquaculture are summarised. WHPs are reviewed and for finfish the periods 13-180 d are outlined.

Methods: This article reviews a broad range of scientific literature.

Results: Mode of administration, target concentration, relevant tissue and statistics are used to describe population distributions. WHPs are summarised for European and North American countries, and range from 30 degree days in Spain to 800 degree days for salmon at <10 °C in Canada. A meta-analysis of all data indicates that 720 degree days is adequate as a WHP for OTC in finfish. Rigos and Smith note that not all species have been investigated and that conservative approaches are appropriate to maintain food safety.

Title: EU Directive 2001/82/EC Directive on the Community Code relating to Veterinary medicinal products

Summary: A general regulatory approach to residues of unregistered products in aquaculture is provided.

Methods: From scientific literature and a statistical review.

Results: The EU concluded that 500 degree days should be the WHP for fish meat for unregistered veterinary medicinal products.

3.3 References

EU (2001) Directive on the Community Code relating to Veterinary medicinal products. Available online. URL: https://ec.europa.eu/health/sites/default/files/files/eudralex/vol-5/dir_2001_82_cons2009/dir_2001_82_cons2009_en.pdf Accessed 30 June 2021.

Rigos G and Smith P (2015) A critical approach on pharmacokinetics, pharmacodynamics, dose optimisation and withdrawal times of oxytetracycline in aquaculture. Reviews in Aquaculture, 7, 77-106.

4.0 Trade

4.1 Summary

This application seeks a minor-use permit (MUP) for the use of CCD OTC to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all bona fide members of the Australian aquaculture industry. We are seeking to use public domain, published and confidential unpublished data to satisfy the data requirements for metabolism and kinetics for the proposed use pattern.

The literature on withholding periods (WHPs) is mixed and confused by inconsistent species, methodology and interpretation. Acceptable/tolerable daily intake, maximum residue limits and other factors vary internationally. We are requesting a 1000 degree day WHP and a 1500 degree day export slaughter interval for OTC in non-salmonid finfish in Australia based on conservative interpretations of published data. The export interval is based on a period over which the residues can be confidently expected to fall below the limit of detection for sensitive tests and below the maximum residue limits (MRLs) of receiving countries.

4.2 Literature

Title: Rigos and Smith (2015) A critical approach on pharmacokinetics, pharmacodynamics, dose optimisation and withdrawal times of oxytetracycline in aquaculture.

Summary: Approaches to setting WHPs for OTC in finfish aquaculture are summarised. WHPs are reviewed and for finfish the periods 13-180 d are outlined. Their meta-analysis of all data indicates that 720 degree days is adequate as a WHP for OTC in finfish. Rigos and Smith note that not all species have been investigated and that conservative approaches are appropriate to maintain food safety.

Methods: This article reviews a broad range of scientific literature.

Results: Mode of administration, target concentration, relevant tissue and statistics used to describe population distributions. WHPs are summarised for European and North American countries, from 30 degree days in Spain to 800 degree days for salmon at <10 °C in Canada.

Title: EU Directive 2001/82/EC Directive on the Community Code relating to Veterinary medicinal products

Summary: A general regulatory approach to residues of unregistered products in aquaculture is provided.

Methods: From scientific literature and a statistical review.

Results: The EU concluded that 500 degree days should be the WHP for fish meat for unregistered veterinary medicinal products.

4.3 References

EU (2001) Directive on the Community Code relating to Veterinary medicinal products. Available online. URL: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32001L0082 Accessed 30 June 2021.

Rigos G and Smith P (2015) A critical approach on pharmacokinetics, pharmacodynamics, dose optimisation and withdrawal times of oxytetracycline in aquaculture. Reviews in Aquaculture, 7, 77-106.

5.0 Workplace health and safety

5.1 Summary

This application seeks a minor-use permit (MUP) for the use of CCD OTC to treat susceptible bacterial diseases in non-salmonid finfish by or under the direction of a registered veterinarian. The intended product users include all *bona fide* members of the Australian aquaculture industry. We are seeking to use public domain, published and confidential unpublished data to satisfy the data requirements for metabolism and kinetics for the proposed use pattern.

The principal risk associated with handling OTC is exposure to powder through eye contact, skin contact or inhalation. The exposure standard for dust is applicable. Adequate protection can be provided by:

- Ensuring persons with tetracycline allergies do not work with OTC
- Eye and face protection are worn
- Skin protection is provided
- Hand protection is provided

PPE need to include:

- Goggles and mask or respirator
- Coveralls or impervious clothing
- Impervious gloves

A Materials Safety Data Sheet (MSDS) for OTC is attached (Appendix 1).

6.0 Environment

6.1 Summary

OTC delivered to fish is dispersed in faeces, urine and in dissolved form into the environment. Environmental residues break down due to chemical processes and exposure to UV light. Over 90% of OTC that is used in fish farms is lost as dissolved product and is dispersed by water currents (Smith 1996). Samuelsen (1989) and Lunestad and Goksøyr (1990) found that OTC in sea water that is subject to average sunlight at around 50° North has a half-life of 30 hours, but in turbid water half-life would be longer. In Australia, with a lower latitude providing the potential for greater ambient light, half-life could be substantially less.

Typically ~2% of OTC used in a sea-cage treatment is incorporated into sediments (Smith 1996), although this figure can be up to 5% (Smith and Samuelsen 1996). Smith and Samuelsen (1996) provided evidence that activity of OTC in sediments decreased over time due to consumption of solids by other animals, leaching into the water column, and inhibition caused by complexing with divalent cations and inorganic compounds in the sediment. Samuelsen *et al*. (1994) showed that the toxicity of OTC to bacteria declined rapidly in sediments, even when no chemical degradation occurred, findings that were supported by Treves-Brown (2000), who estimated that the activity of the portion of OTC that is incorporated into sediments is 100-fold lower than that of the pure product. Most studies on OTC in the environment have been based largely on laboratory experiments, but Coyne *et al*. (1994) investigated the concentration of OTC in the sediment of two cages at a marine fish-farm site, and found that 1.3% of OTC applied is incorporated into sediments. The amount of OTC found in sediments was 1.3 $\mu g.g^{-1}.kg^{-1}.cage^{-1}$ and the product had half-lives of 16 and 13 days at the two sites. OTC is incorporated into the top 5-10 cm of marine sediment but this is site specific based on sediment reworking (Treves-Brown 2000, Coyne *et al.* 1994). Smith and Samuelsen (1996) showed that, in freshwater, OTC in sediment has very little biological activity and that the compound binds to a wider variety of substances than in seawater, forming complexes that have no antimicrobial activity. OTC is lost from sediments by leaching and out-washing. Although these processes can restore antimicrobial activity, the compound is then also susceptible to degradation by UV. Leaching occurs slowly and the concentration of OTC released into the water column in such a manner is negligible (Smith and Samuelsen 1996).

There are important issues about interpreting environmental toxicity values obtained in ecotoxicology studies and extrapolating them to effects from equivalent concentrations of OTC in the real environment. The concentrations required to cause the effects observed in tank trials are likely to be much higher in marine environments, particularly in sediments, than are observed in many tank studies (Pursell *et al*. 1996). This has been interpreted in Ireland as a no observable effect (NOEC) for OTC in sediments accepted under environmental controls of 12.5 $mg.L^{-1}$ (O'Reilly and Smith 2001).

To support this MUP application, we determined concentrations that would protect 95% of species from toxic effects (PC95 values) of OTC (see environmental surveillance and iterative risk assessment sections below). The PC95 calculated were 6.4 $mg.L^{-1}$ in freshwater and 9.7 $mg.L^{-1}$ in seawater. Based on conservative (i.e. a worst-case scenario) data, estimations of OTC concentrations that could be released from treatments in pond aquaculture in South Australia were 0. 76 $mg.L^{-1}$ for water and 1.2 $mg.L^{-1}$ for sediments. In marine sea cage aquaculture, estimations of OTC concentrations released from treatments were 0.0396 $mg.L^{-1}$ for water across the lease and 1.25 $mg.L^{-1}$ for sediments across the lease. For cage specific calculations the OTC concentrations were 0.074 $mg.L^{-1}$ in water and 10.39 $mg.L^{-1}$ in sediment.

For this study, OTC was monitored following treatments in a freshwater pond farm with no exchange or dilution. OTC was also monitored in a sea-cage during the lowest flow period of the year, and with

the start of the monitoring coinciding with neap tides in Spencer Gulf. There, the amplitudes of the main semi-diurnal tide constituents were almost identical and the semi-diurnal tide was therefore virtually absent, resulting in the phenomenon known as a "dodge tide". Maximum concentrations at the sea cage site were similar to those calculated for pond aquaculture but were approximately 10% of calculated values for marine sediment, even given minimal tidal movement. In marine water, OTC could not be detected on days where no treatment was applied and was below the limit of quantification (LoQ) when detected on day 0. Half-life of OTC could not be calculated for marine water because OTC was not detected in samples after day 0. In marine sediment half-life was 15.1-19.4 days. In freshwater, half-life was 24.1 days in sediment and 16.1 days in water. These data show that residues deplete to below limits of analytical detection in 3-4 months and indicate that multiple treatments are environmentally safe where therapeutically justified.

Schmidt et al. (2007) and Macleod et al. (2009) concluded that OTC treatments are environmentally safe in aquaculture where therapeutically justified, and the collated data plus the study data provided here support that conclusion. Environmental residues deplete to below limits of analytical detection in 3-4 months. Where systems with no dilution are treated, water should be retained and held prior to release or diluted on environmental release. In marine systems, dilution is greater than estimated by simple models assuming little water exchange and high deposition near the release point.

6.2 Oxytetracycline environmental surveillance

Summary

OTC was monitored in a freshwater pond system and at a sea cage marine aquaculture site where fish had been treated for bacterial infections. No OTC was detected at either site prior to treatment. OTC in marine water was only detected immediately following application (i.e. Day 0 of post-application monitoring), and while at concentrations above the limit of detection (LoD) (0.002 $mg.kg^{-1}$) it was below the limit of quantification (LoQ) (0.01 $mg.kg^{-1}$). Half-life in marine sediment was 15.1-19.4 d. In freshwater, half-life was 24.1 days in sediment and 16.1 days in water. These data indicate that multiple treatments are environmentally safe where therapeutically justified, and that residues deplete to below limits of analytical detection in 3-4 months.

Methods

A freshwater and a marine site were surveyed for OTC residues following OTC application to treat infections.

Silver Perch (*Bidyanus bidyanus*) in a 0.2 ha freshwater pond were treated with OTC at 75 $mg.kg^{-1}.day^{-1}$ for 10 days to manage an *Aeromonas hydrophila* infection. There were 2 t of fish treated with a total dose of 1.5 kg of OTC. The average water temperature was 17°C. The site is in the Adelaide Hills, has an average depth of 1.8 m and high turbidity with a secchi depth of 0.3-0.5 m. Sediment samples 5 cm deep were taken near the drain at the deepest point in the pond and near the pond edge before treatment, after cessation on the last day of treatment (Day 0) then at 7, 14, 28, 56 and 112 days post treatment. Samples of greater depth could not be obtained because the substrate was hard and not permeable beyond 5 cm. Water samples were taken at the surface at the drain and the pond edge before treatment and after cessation on the last day of treatment (Day 0) then at 7, 14, 28, 56 and 112 days post treatment.

Mulloway (*Argyrosomus japonicus*) in a sea-cage on a marine finfish site were treated with OTC at 100 $mg.kg^{-1}.day^{-1}$ for 10 days to manage a *Vibrio harveyi* septicaemia in November 2003. There were 22 t of fish treated with a total dose of 22 kg of OTC. The average water temperature was 19°C. The site has an average depth of 18 m and low suspended solids, with secchi depth of 5-8 m, and is in

Spencer Gulf. Triplicate sediment samples 10 cm deep were taken under the edge of the cage and 50 m away in the direction of the dominant current before treatment and on the last day of treatment (Day 0) then at 7, 14, 28, 56 and 112 days post treatment. Triplicate water samples were taken at the surface at the cage margin and 50 m away in the direction of the dominant current before treatment and on the last day of treatment (Day 0) then at 7, 14, 28, 56 and 112 days post treatment.

Samples were frozen, transported to The University of South Australia and analysed for OTC based on Yi et al. (2015) using LC-MS. The limits of detection and quantification for sediment were 0.01 $mg.kg^{-1}$ and 0.05 $mg.kg^{-1}$ and for water were 0.002 $mg.kg^{-1}$ and 0.01 $mg.kg^{-1}$ respectively.

Depletion was assessed assuming exponential decay, with half-life calculated from the slope of a linear regression of log concentration over time for each matrix based on Coyne *et al.* (2001). Separate analyses were performed for the marine and freshwater results. No analysis was possible for marine water samples due to a lack of detection after day 0. To determine whether half-life varied between sample location (for both marine and freshwater) and between sample types in freshwater, statistical significance of the interaction terms *site x time* (both models) and *sample type x time* (freshwater model only) in the linear regression was assessed using *F*-tests and an α of 0.05. Analyses were performed in R statistical software (R Core Team 2021).

Results

All samples taken before treatment with OTC did not contain detectable levels of OTC. The marine seawater samples taken on Day 0 at both the cage margin and 50 m away contained OTC below the LoQ but above the LoD. Other seawater samples did not contain OTC above the LoD. Most other samples contained OTC at quantifiable concentrations (Appendices 2, 3).

The model for depletion in freshwater samples showed no significant 3-way interaction of *site x sample type x time* ($F_{1,64}$ = 0.81, p = 0.371), and the two-way interactions *site x time* was also not significant ($F_{1,64}$ = 2.2, p = 0.141), indicating no difference in depletion rate between sites within each sample type. The interaction of *sample type x time* was, however, significant ($F_{1,64}$ = 33, $p < 0.001$), demonstrating a difference in depletion rate between sample types. Freshwater sediments had a half-life of 24.1 days and water had a half-life of 16.1 days (Figure 1).

The model for depletion in marine sediments showed a significant *site x time* interaction ($F_{1,32}$ = 3.096, p = 0.004), indicating a significant difference in the rate of depletion for the sites. Half-life for the near site was 19.4 days, while the far site had a half-life of 15.1 days (Figure 2).

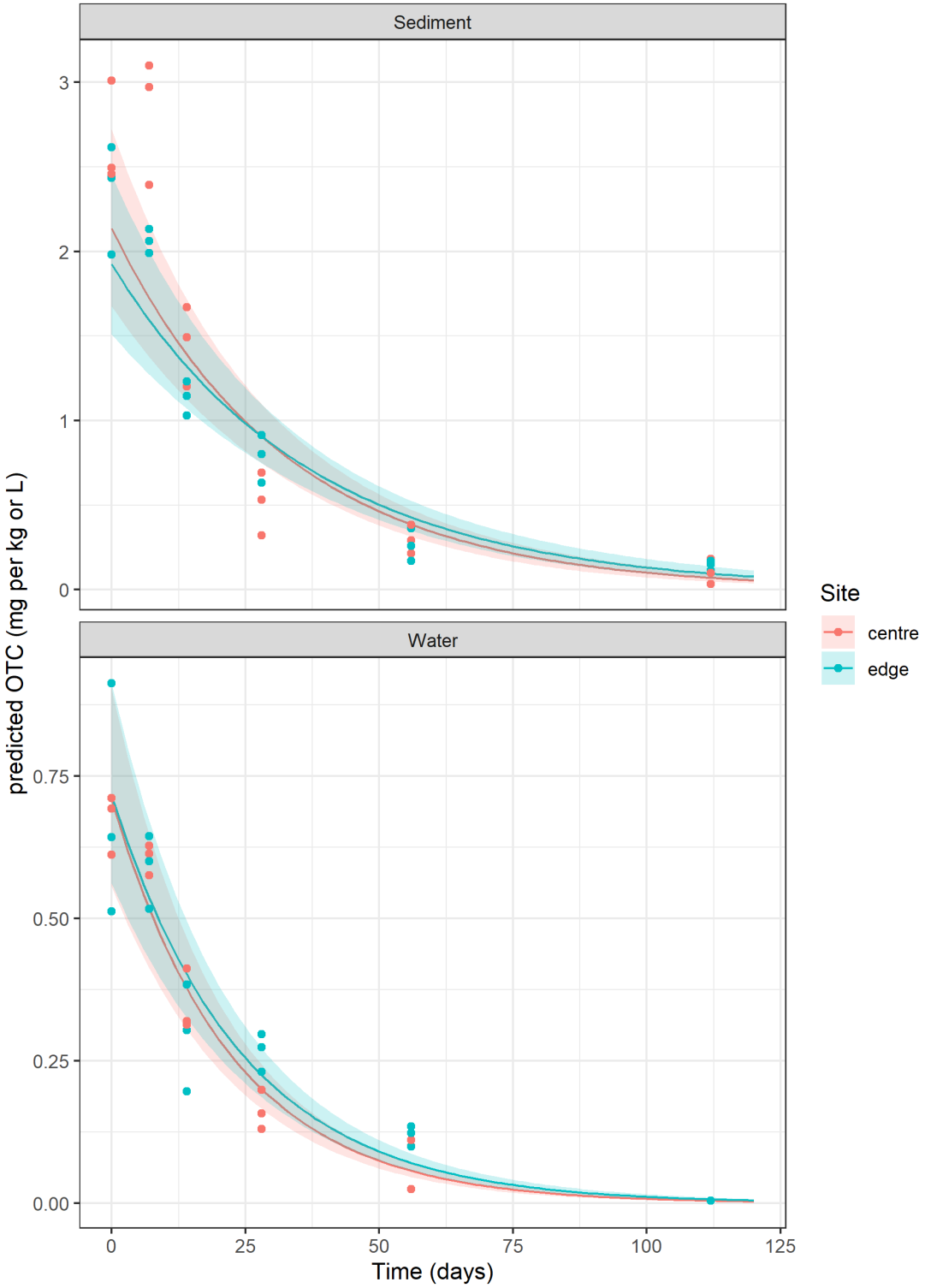


Figure 1. OTC depletion in freshwater sediment and water. Points show data and lines show fitted model with shading indicating 95% confidence intervals. Note different scale between sample types.

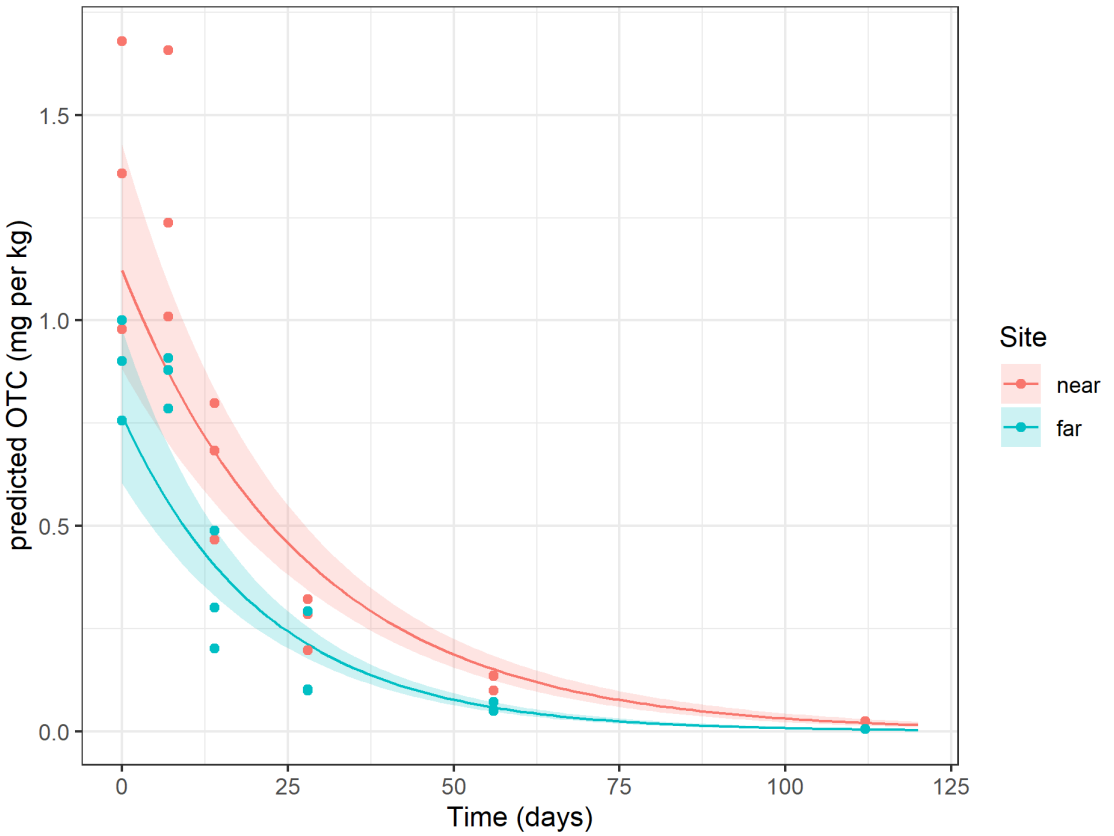


Figure 2. OTC depletion in marine sediments. Points show data and lines show fitted model with shading indicating 95% confidence intervals.

6.3 Iterative risk assessment

Introduction

Environmental risk was assessed based on release scenarios for freshwater and marine aquaculture following the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) Guideline 6 (GL6) and Guideline 38 (GL38) and Lee-Steere (2009), and informed by the environmental surveillance. Risk quotients were calculated based on comparison of PC95 values and estimated environmental concentrations.

Ecotoxicology

Ecotoxicology data were obtained for OTC as outlined in Table 1. Trigger values have been calculated based on these data (see Figures 3, 4, Table 1).

The preferred method for determining concentrations that are safe for release is to use a species sensitivity distribution (SSD) of chronic toxicity test data. BurrliOZ software (CSIRO Environmentrics) was used to determine protective concentrations. A statistical distribution curve is fitted to the data assuming a log-logistic or Burr Type III distribution depending on the size of the data set, and from this the concentration corresponding to the required level of species diversity protection determines the protective concentration. This analysis is based on calculation of a statistical distribution of ecotoxicity data. Values derived from this method are considered highly reliable (ANZECC 2000, Shao 2000).

The ANZECC (2000) method includes a pre-determined level of protection, which is usually for the release concentration to protect 95% of species. ANZECC (2000), however, permits release concentrations for disturbed habitats to provide less than 95% protection, and can be set as low as 80%.

Table 1. Ecotoxicology data used for oxytetracycline protective concentration 95% (PC95) calculation. NOEC indicates the level of no observable effect concentration.

Taxon	**Species**	**Protocol**	**Endpoint**	**NOEC (mg/L)**	**Source**
Freshwater					
Crustacea	*Ceriodaphnia dubia*	US EPA (2002) adapted	Survival	62.5	EcoTox Services*
	Macrobrachium australiense	ESA (2016)	Survival	62.5	EcoTox Services*
Vertebrata	*Melanotaenia splendida* (embryo)	US EPA (2002) adapted	Hatching	125	EcoTox Services*
	Zebra danio (adult)	Isidori et al. (2005)	Survival	1000	Isidori et al. (2005)
Angiosperma	*Lemna disperma*	ASTM (2012) adapted	Growth	125	EcoTox Services*
Marine					
Chlorophyta	*Selenastrum capricornutum*	US EPA (2002) adapted	Growth	62.5	EcoTox Services*
Ochrophyta	*Ecklonia radiata*	Bidwell et al. (1998)	Growth	250	EcoTox Services*
Echinodermata	*Heliocidaris tuberculata* (larva)	ESA (2016)	Development	62.5	EcoTox Services*
Crustacea	*Allorchestes compressa*	US EPA (2002) adapted	96h survival	125	EcoTox Services*
	Penaeus vannamei (mysis 1)	Williams et al (1992)	Immobilisation	160.9	Williams et al (1992)
Mollusca	*Argopecten purpuratus*	Miranda et al. (2013)	Development	4	Miranda et al. (2013)
	Siphonaria australis	Fitzpatrick et al. (2010)	Mortality	200	Fitzpatrick et al. (2010)
Ciliophora	*Euplotes crassus*	Gomiero et al. (2014)	Survival	248.45	Gomiero et al. (2014)
Vertebrata	*Latris lineata*	Battaglene et al. (2006)	Survival	25	Battaglene et al. (2006)
	Sciaenops ocellatus	Denson et al. (2008)	Survival	500	Denson et al. (2008)

*EcoTox Services Australia data obtained in this project (see Appendix 4).

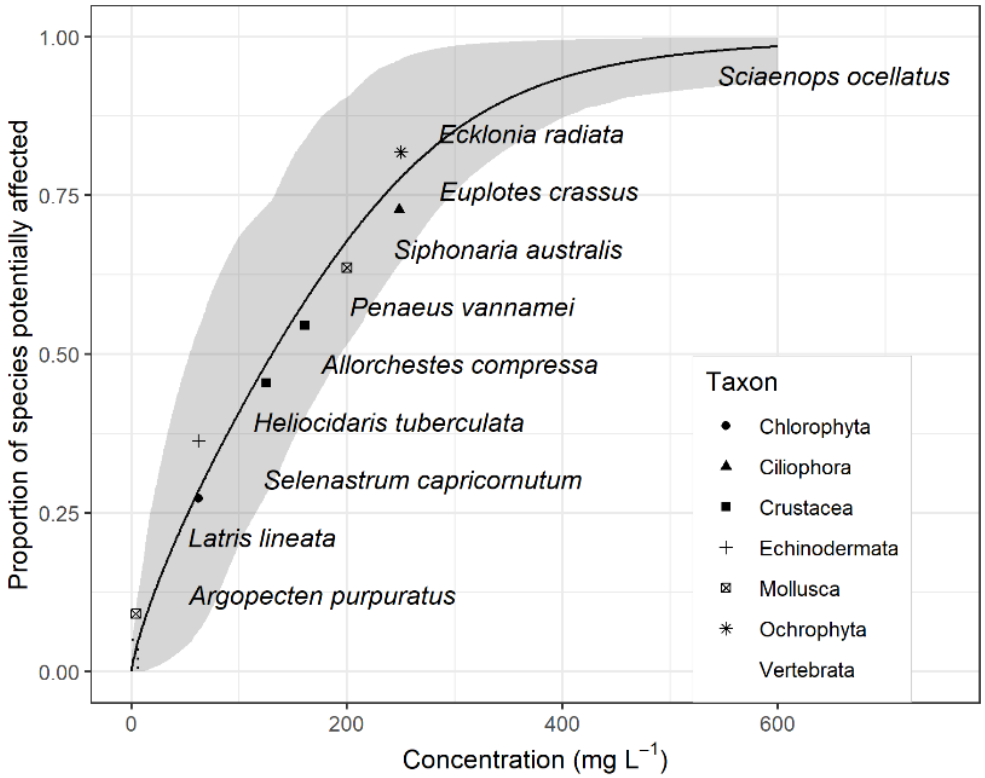


Figure 3. Fitted Burr type III distribution of oxytetracycline toxicity values for marine species.

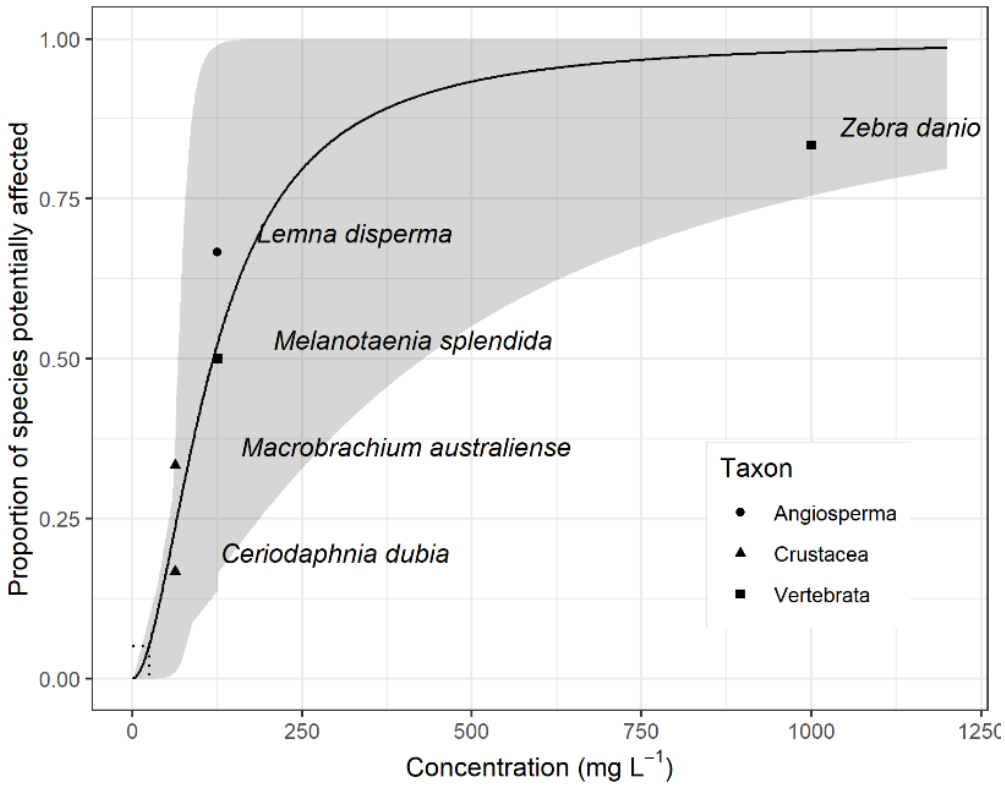


Figure 4. Fitted log-logistic distribution of oxytetracycline toxicity values for freshwater species.

Table 2. Predicted protective concentrations (PCs) based on 95th percentiles of species sensitivity distributions of chronic NOEC data.

Chemical	Environment	Distribution	PC95 (mg L^{-1})	Reliability (ARMCANZ 2000)
Oxytetracycline	Freshwater	log-logistic	6.4	High
Oxytetracycline	Marine	Burr Type III	9.7	High

The PC95 concentrations are high reliability values which can be used to assess the environmental safety of release of oxytetracycline. Given that these values are NOECs derived from chronic exposure data and that the PC95 values are of high reliability, a risk quotient <1 is regarded as acceptable.

Risk quotients

The risk quotient expressed as the predicted concentration as a proportion of the trigger value was calculated for marine and freshwater systems following VICH GL38. Treatment assumptions are outlined in Table 3.

Freshwater pond treatment

- 12 tonnes biomass = 12,000 kg total (biomass based on Department of Agriculture, 2015)
- Dose: 100 $mg.kg^{-1}.d^{-1}$ for 10 days
- Static water system.
- Total OTC delivered to farm = 100 x 12,000 x 10 = 12,000,000 mg
    - 5% OTC distributed to sediment = 600,000 mg
    - 95% OTC distributed to water column = 11,400,000 mg

Pond water

- Total pond water volume = 200 x 50 x 1.5 = 15,000 m^3 = 15,000,000 L
    - Effective dose to water column = 11,400,000 / 15,000,000
    - Dose water = 0. 76 $mg.L^{-1}$
    - Risk quotient: 0.76 / 6.4 = 0. 119
    - **Risk quotient is <1. The overall environmental risk is acceptable**

Sediment

- Total sediment volume (top 5 cm) on lease = 10,000 x 0.05 m^3
    - Sediment volume = 500 m^3 = 500,000 L
    - Dose to the sediment = 600,000 / 500,000
    - Sediment dose = 1.2 $mg.L^{-1}$
    - **Risk quotient**: 1.2 / 6.4 = 0.189
    - **Risk quotient is <1. The overall environmental risk is acceptable**

This calculation illustrates that, even based on conservative safety values, the use of OTC to treat a freshwater pond finfish farm poses an acceptable risk to the environment in the pond, and with any level of dilution or decomposition, is acceptable for discharge.

Table 3. Treatment and environment assumptions for environmental assessment of oxytetracycline for non-salmonid finfish

	System	
Parameter	**Freshwater pond**	**Marine sea cage**
Dose	100 mg^{-1}.kg bw^{-1}.day^{-1}	100 mg^{-1}.kg bw.day^{-1}
Duration	10 days	10 days
Biomass	12 t.ha^{-1}	25 t.ha^{-1} (whole lease)
Maximum biomass	12 t	500 t
Environmental dose	12 kg	25 kg.ha^{-1} per treated cage
Total area	1 ha	20 ha
System shape	20 x 50 x 1.5 m	1,000 x 200 m
Depth	1.5 m	15 m
Total product released	12 kg	500 kg
Sediment proportion OTC	5%	5%
Dissolved proportion OTC	95%	95%
Sediment deposition pattern	Whole footprint	40m radius
Water area	1 ha	20 ha
Average current speed	n/a	0.1 $m.s^{-1}$
Sediment area	1 ha	20 ha
Sediment density	1.6 $kg.L^{-1}$	1.3 $kg.L^{-1}$
Affected sediment depth	5 cm	10 cm
Total sediment volume	500 m^3	20,000,000 m^3
Total sediment mass	800 kg	26,000,000 kg

Marine whole farm treatment – OTC distribution across lease

- 500 tonnes biomass = 500,000 kg total (biomass based on Department of Agriculture, 2015)
- Dose: 100 $mg.kg^{-1}.d^{-1}$ for 10 days
- Assume worst case scenario of "plugging'" of the tidal excursion water volume i.e. the same block of water moves backwards and forwards.
- Tidal excursion (TE) = average current ($m.s^{-1}$) x 60 x 60 x 12 s
  - 0.1 $m.s^{-1}$ x 43,200 s = 4320 m

- Assume conservative 4000 m estimate.
- Total OTC delivered to farm = 100 x 500,000 x 10 = 500,000,000 mg
  - 5% OTC distributed to sediment = 25,000,000 mg
  - 95% OTC distributed to water column = 475,000,000 mg

Water Column

- Total water volume on lease area = 200,000 x 15 = 3,000,000 m^3 = 3,000,000,000 L
  - Effective dose to water column = 475,000,000 / 3,000,000,000
  - Dose water = 0. 158 $mg.L^{-1}$
  - **Risk quotient**: 0.158 / 9.7 = 0.016
  - **Risk quotient is <1. The overall environmental risk is acceptable.**
- Taking tidal excursion into account:
  - Water volume = 4000 x 200 x 15 m^3 = 12,000,000,000 L
  - Effective dose to total water = 475,000,000 / 12,000,000,000
  - Dose total water = 475,000,000 / 12,000,000,000 = 0.0396 $mg.L^{-1}$
  - **Risk quotient**: 0.0396 / 9.7 = 0.0041
  - **Risk quotient is <1. The overall environmental risk is acceptable.**

Sediment

- Total sediment volume (top 10 cm) on lease = 200,000 x 0.1 m^3
  - Sediment Volume on lease = 20,000 m^3 = 20,000,000 L
  - Dose to the sediment = 25,000,000 / 20,000,000
  - Sediment dose = = 1.25 $mg.L^{-1}$
  - **Risk quotient**: 1.25 / 9.7 = 0.129
  - **Risk quotient is <1. The overall environmental risk is acceptable.**

This calculation illustrates that, even based on conservative safety values, the use of OTC to treat an entire marine finfish farm poses an acceptable risk to the environment inside or outside the lease area.

Cage specific calculations

- Pen volume = $\pi r^2 d$
  - Volume of pen = π x $(12.5)^2$ x 12 (water depth 15m, pen clearance 3m)
  - Volume of pen = 5890 m^3
- Biomass fish in pen = Volume of pen x stocking density
  - Biomass = 5890 x 20 = 117,800 kg fish
- Dose of OTC
  - Dose = 117,800 x 100 x 10 = 117,800,000 mg OTC
  - Dose in water = 95% = 111,910,000 mg
  - Dose in sediment = 5% = 5,890,000 mg

Water calculations

- Assume tidal excursion of 4000 m with “plugging” of the water volume
- Assuming distribution limited to surface to seabed along tidal flow of pen volume.
- Tidal excursion volume = $\pi r^2 d$ + (4000 x 25 x d) where d = 15 m
  - Total excursion volume = [π x $(12.5)^2$ x 15] + [4000 x 25 x 15]
  - Total excursion volume = 1,507,363 m^3 = 1,507,363,000 L
- Effective water dose from a single pen = D_{Water} / Tidal Excursion Volume

- Dose water from pen = 111,910,000 / 1,507,363,000 = 0.074 mg/L
- Risk quotient = 0.074 / 9.7 = 0.0076
- **Risk quotient is <1. The overall environmental risk is acceptable.**

Sediment calculations

- Conservative sediment deposition model gives a 40 m radius from the edge of the source at 20 m depth and 30 m radius at 15 m depth (Figure 5).
- Total radius of deposition = 42.5 m
- Volume of sediment receiving OTC = π $(42.5)^2$ x 0.1 = 567.5 m^3 = 567,500 L
- Distributed sediment dose = 5,890,000 mg / 567,500 = 10.39 $mg.L^{-1}$
- Sediment dose = 5,890,000 mg / 567,500 x 1.3 = 7.98 $mg.kg^{-1}$
- Per kg OTC administered, residue of approximately 7 μg $OTC.g^{-1}sediment.kg^{-1}$

- Risk quotient = 7.98 / 9.7 = 0.82
- **Risk quotient is <1. The overall environmental risk is acceptable.**

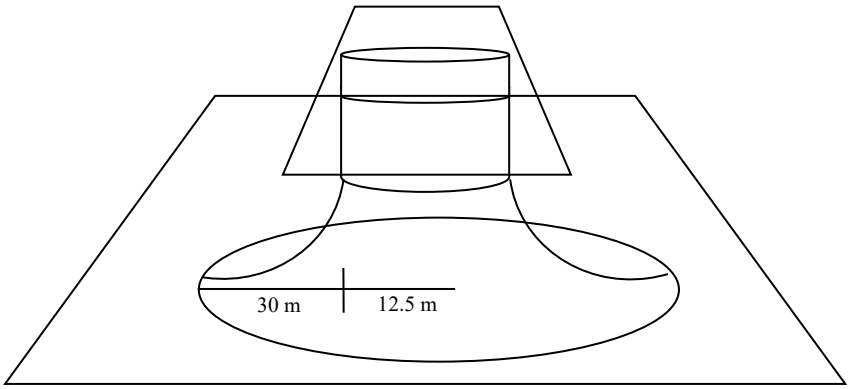


Figure 5. Diagram of sea-cage arrangement outlining assumptions used in the per-cage environmental release calculations.

Comparison of the OTC concentrations derived from the calculations and those measured at a treated aquaculture site indicate that the calculations greatly overestimate the residues in sediment. This overestimation therefore represents another safety factor.

These calculations, combined with the measured OTC concentrations from aquaculture sites illustrate that, based on conservative parameters, the use of OTC to treat a marine finfish pen poses an acceptable and transient risk to the environment even within the immediate aquaculture environment.

6.4 Conclusions

Differences between freshwater and marine results are largely due to differences in aquaculture systems and the environments. Dilution in the dynamic marine environment is most likely responsible for the water concentrations being below the LoD for all but the measurements taken on day 0, which were below the LoQ. Water concentrations in the static freshwater system were higher because of a lack of dilution and had a relatively long half-life of 16.1 days, likely because the OTC bound to divalent cations or adsorbed to suspended clay and organic particles in the water. Sediment half-life in the marine system was approximately 15 days and 24 days in the freshwater system, which is consistent with other environmental data at similar temperatures and in systems with the type of sediments that have available oxygen and high microbial activity.

These data indicate that multiple OTC treatments are environmentally safe in aquaculture where therapeutically justified, and that residues deplete to below limits of analytical detection in 3-4 months. Where systems with no dilution are treated, water should be retained and held prior to release or diluted prior to environmental release.

6.5 References

Coyne R, Smith P, Moriarty C (2001) The fate of oxytetracycline in the marine environment of a salmon cage farm. Marine Environment and Health Series Number 3.

Coyne R, Hiney M, O'Connor B, Kerry J, Cabazon D and Smith P (1994) Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. Aquaculture, 123, 31-42.

Department of Agriculture (2015) Enterprise Manual (2.0) AQUAVETPLAN. Australian Government, Canberra.

Lunestad BT and Goksøyr J (1990) Reduction in the antibacterial effect of oxytetracycline in seawater by complex formation with calcium and magnesium. Diseases of Aquatic Organisms, 9, 67-72.

Macleod C, Eriksen R (2009) A review of the ecological impacts of selected antibiotics and antifoulants currently used in the Tasmanian salmonid farming industry (marine farming phases). FRDC Final Report 2007-246.

O'Reilly A and Smith P (2001) Use of indirect conductimetry to establish predictive no effect concentrations of oxytetracycline and oxolinic acid in aquatic sediments. Aquaculture, 196(1-2), 13-26.

Pursell L, Dineen T, Kerry J, Vaughan S and Smith P (1996) The biological significance of breakpoint concentrations of oxytetracycline in media for the examination of marine sediment microflora. Aquaculture, 145, 21-30.

R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Samuelsen OB (1989) Degradation of oxytetracycline in seawater at two different temperatures and light intensities, and the persistence of oxytetracycline in the sediment from a fish farm. Aquaculture, 83, 7-16.

Samuelsen OB, Lunestad BT, Ervik A and Fjelde S (1994) Stability of antibacterial agents in an artificial marine aquaculture sediment studied under laboratory conditions. Aquaculture, 126, 3-4.

Schmidt LJ, Gaikowski MP, Gingerich WH, Dawson VK, Schreier TM (2007) An environmental assessment of the proposed use of oxytetracycline-medicated feed in freshwater aquaculture. US Geological Survey, Biological Resources Division. 27 July 2007. Available online. URL: https://animaldrugsatfda.fda.gov/adafda/app/search/public/document/downloadEA/156 Accessed 30 June 2021.

Smith P (1996) Is sediment deposition the dominant fate of oxytetracycline used in marine salmonid farms: a review of available evidence. Aquaculture, 146, 3-4.

Smith P and Samuelsen OB (1996) Estimates of the significance of outwashing of oxytetracycline from sediments under Atlantic salmon sea cages. Aquaculture, 144, 17-26.

Treves-Brown KM (2000) Applied Fish Pharmacology. Springer, Dordrecht.

Yi X, Bayen S, Kelly BC, Li X and Zhou Z (2015) Improved detection of multiple environmental antibiotics through an optimized sample extraction strategy in liquid chromatography-mass spectrometry analysis. Analytical and Bioanalytic Chemistry, 407(30), 9071-9083.

7.0 Antimicrobial resistance (AMR) risk assessment

This review is a qualitative microbiological assessment of the risk that the use of in-feed formulations containing oxytetracycline in non-Salmonid finfish aquaculture in Australia would promote resistance in finfish pathogens and/or environmental/commensal organisms that is transferable to human pathogens. The consequence of major importance is that resistance to critically important human drugs could develop through use in fish and be transferred to human pathogens. The World Organisation for Animal Health (OIE) ranks oxytetracycline as a critically important veterinary antimicrobial. The Australian Strategic Technical Advisory Committee on Antimicrobial Resistance (ASTAG) considers tetracyclines developed for human use (doxycycline, minocycline) to be of low importance to human health whereas the WHO ranks them as being of critical importance to human health. The related glycylcycline antimicrobial tigecycline is, however, considered of high importance to human health by ASTAG and the WHO ranks it as critically important. Tigecycline remains critical for the treatment of Gram-negative pathogens that have developed resistance to fluoroquinolones, carbapenems and colistin. Treatment outcomes with tigecycline, however, are not optimal compared with the aforementioned critically important antimicrobials (which are all bactericidal), thus tigecycline is not recommended as a first-line treatment for severe Gram-negative infections in humans. Additionally, the first fully synthetic tetracycline antimicrobials, eravacyline (Xerava®) and omadacycline (Nuzyra®), were approved for use in US patients in 2018 for specific infections including abdominal, skin, soft tissue, and lung infections.

In Australia, tigecycline is used as a reserve agent for both resistant Gram-positive (*Enterococcus*, *Staphylococcus*) and Gram-negative (Enterobacteriales, *Acinetobacter*) pathogen infections. It should be noted, however, that in recent years a number of new antimicrobial agents have been registered for the treatment of resistant Gram-positive infections in humans, providing alternatives to tigecycline. This risk assessment therefore pays particular attention to the likelihood that use of oxytetracycline products in non-Salmonid finfish would promote the selection and dissemination of tigecycline resistance genes, which could potentially be acquired by human Gram-negative pathogens through horizontal gene transfer. Identified tigecycline resistance mechanisms include low level resistance mediated by overexpression of efflux pump genes and high-level resistance associated with possession of *tet*(X) gene variants which encode destructases capable of degrading all tetracycline-like antibiotics.

In determining the likelihood of resistance gene selection, direct cross infection and/or horizontal gene transfer of tigecycline resistance to humans resulting from oxytetracycline use in finfish, consideration was given to current use patterns in the Australian aquaculture industry (mainly metaphylactic to control bacterial disease outbreaks), the population of microorganisms exposed (both within the fish gut and the general environment), the duration of exposure and the concentration of the antimicrobial derivatives following metabolism and excretion. Additionally, oxytetracycline and chlortetracycline are registered for use in other livestock species in Australia and are commonly used drugs in poultry, pigs, sheep and cattle, so evidence was also sought for the selection, maintenance and dissemination of tigecycline resistance genes in these terrestrial food-producing species. Studies reviewed in this risk assessment included published work and grey literature.

As a broad-spectrum, bacteriostatic agent, oxytetracycline is active against the majority of aquaculture pathogens causing infections in non-Salmonid finfish species (particularly *Aeromonas* spp., *Flavobacterium* spp., *Vibrio* spp., *Photobacterium* spp., *Edwardsiella* spp., *Streptococcus iniae, Lactococcus garvieae,* and *Epitheliocystis*). With the possible exception of *Aeromonas hydrophila* and some *Vibrio* species, resistance to oxytetracycline has not been reported in pathogenic bacteria isolated from farmed fish in Australia, but due to a lack of recent data, it is recommended that tetracycline resistance in aquaculture pathogens (particularly members of the Flavobacteriaceae

family) is regularly monitored in future antimicrobial risk (AMR) surveillance programs. Recent unpublished industry AMR project data and a review of veterinary laboratory diagnostic case reports have confirmed variable frequencies of reduced susceptibility to oxytetracycline in Australian aquatic pathogens, but no high-level resistance. Regular monitoring is particularly important given the recent emergence in China of *tet*(X) gene variants (*tet*(X3) and *tet*(X4)) encoding high-level tigecycline resistance, and their location within mobile genetic elements capable of horizontal transmission. Tigecycline resistance genes appear to have been selected internationally by the widespread use of tetracyclines in multiple and/or integrated livestock systems as well as direct human use, but it is highly likely that other antimicrobial selective pressures are also involved, given *tet*(X) genes are often co-located with colistin and carbapenem resistance genes in arrays flanked by transposable elements.

Anecdotal reports of low oxytetracycline minimum inhibitory concentration (MIC) values for Salmonid finfish pathogens in Tasmania are likely to be similar across other fresh and saltwater finfish aquaculture industries within mainland Australia given that oxytetracycline is typically administered sporadically in feed at high dose (approximately 75-100 $mg.kg^{-1}$) for short periods (no more than 10 days) metaphylactically (i.e. administered to both sick and healthy fish within the same cohort) to control outbreaks and prevent disease spread. Given that minor use permits for oxytetracycline administration in finfish have been sporadically available since 2006 (PER9675) and in the Tasmanian salmon industry since 1995 (PER1014) with a maximum residue limit of 0.2 mg/kg, the available susceptibility data indicate the overall absence of significant resistance to oxytetracycline despite reported use for over 26 years**.** There is more likelihood that *tet*(X) would enter Australia in bacteria carried by people (particularly gastrointestinal carriage) who have visited countries where prevalence is high, such as certain regions of mainland China, compared with the risks associated with minimal oxytetracycline interventions in farmed finfish species.

The pharmacokinetics of oxytetracycline in finfish suggest there are large variations in bioavailability between different freshwater and saltwater species, and bioavailability can also be affected by water temperature. This would indicate quite a large potential environmental footprint resulting from oxytetracycline use, with a high proportion of the drug excreted unchanged in faeces from both healthy and sick fish in the same cohort. This potential environmental issue has been noted in several international studies, particularly given the fact that tetracyclines are only slowly degraded in soil and sludge, though recent bioremediation studies have identified specific microbes capable of more rapid degradation. Given the sporadic use patterns described by the Australian finfish aquaculture industry (high doses of minimal duration for treatment of disease outbreaks), however, the push towards improved bacterial disease control through management and efficacious vaccines, and the low overall density of fresh and saltwater aquaculture production in Australia, the environmental footprint is likely to be low, leading to reduced selection pressure. It is recommended, however, that regular sampling is undertaken to measure oxytetracycline levels in aquaculture effluent and confirm the absence of selection pressure leading to tigecycline resistance.

Overall the likelihood of harm arising from the use of oxytetracycline in non-Salmonid finfish species in Australia was considered possible but unlikely with a rating of low risk applied, even though the hazard (selection of tigecycline resistance) is considerably higher given the recent emergence and spread of *tet*(X3) and *tet*(X4) resistance genes in China among both commensal and pathogenic bacteria isolated from humans, animals and the environment. Exposure was, however, considered negligible and impact low, due to conservative regulation of critically important antimicrobials in both humans and agriculture in Australia, and generally low rates of resistance among Australian Gram-negative pathogens to fluoroquinolones, carbapenems and colistin. In addition to having low likelihood of causing harm to humans, targeted oxytetracycline administration in the Australian non-salmonid finfish aquaculture industry, through the extension of a minor use permit has the potential to significantly improve the management of bacterial disease outbreaks, given that the majority of

pathogenic species causing infection in Australian finfish species remain susceptible. Nevertheless, the lack of published Australia-wide data on the antimicrobial susceptibility of common aquaculture pathogens is problematic because the emergence of resistance to oxytetracycline may not be immediately detected without structured AMR information gathering. An approach to understanding microbial susceptibility and emergence of AMR at an Australia-wide scale should be prioritised. The authors of this report will raise the issue with the Subcommittee for Aquatic Animal Health (SCAAH) through the Project Officer employed on "Improving the availability of safe and effective veterinary medicines for Australia's seafood industry" (FRDC: 2020-094).

Antibacterial agent: oxytetracycline
Description of the antibiotic constituent/s of the product
Name and identification of antibiotic
Common name: Oxytetracycline.
Chemical name: (4S,4aR,5S,5aR,6S,12aR)-4-(dimethylamino)-1,5,6,10,11,12a-hexahydroxy-6-methyl-3,12 dioxo-4,4a,5,5a-tetrahydrotetracene-2-carboxamide
Chemical Abstract Services (CAS) registry number: 2058-46-0.

Chemical Structure

The structures of oxytetracycline, related tetracyclines (chlortetracycline and minocycline) and the glycylcycline tigecycline are presented below to demonstrate the main chemical differences that have important effects on pharmacodynamics, metabolism, spectrum of activity and relative vulnerability to resistance mechanisms. The common structural feature of tetracyclines is the linear fused tetracyclic scaffold. Tetracycline (1953), chlortetracycline (1948), and oxytetracycline (1950) represent the first-generation structures and are natural compounds found in actinomycete soil bacteria (*Streptomyces rimosus* for oxytetracycline). Doxycycline (1967) and minocycline (1961) represent the second-generation structures with increased potency and improved pharmacokinetic properties as a result of chemical modifications which include a second dimethylamine residue for minocycline. Further chemical diversification of minocycline through the addition of C9-amino derivatives possessing anamide functionality with a glycine subunit led to the creation of the glycylcyclines, of which tigecycline (1993) is the only FDA-approved (2005) third-generation structure. Tigecycline was primarily developed to be effective against strains of bacteria that had become resistant to the first and second generation tetracyclines via efflux pumps or ribosomal protection. The methylaminecycline omadacycline (2013) and fluorocycline eravacycline (2013) are fourth-generation, totally synthetic structures and both received FDA approval in 2018 (Nelson and Levy 2011; Fang *et al.* 2020). First to fourth-generation tetracyclines will be referred to as the expanded tetracycline class.

Mechanism and type of antimicrobial action

Oxytetracycline inhibits protein synthesis by binding irreversibly to the 30S ribosomal sub-unit, preventing aminoacyl tRNA from binding to the ribosomal acceptor site on the messenger RNA ribosome complex, and thereby preventing protein chain elongation. This results in a bacteriostatic antimicrobial action.

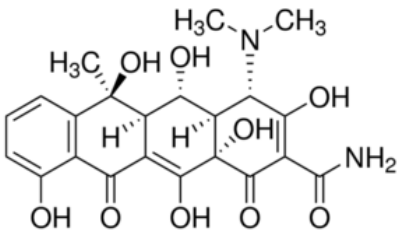
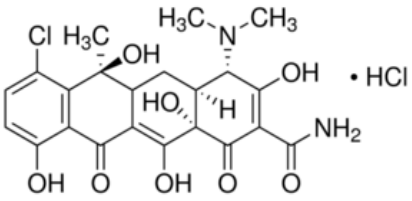
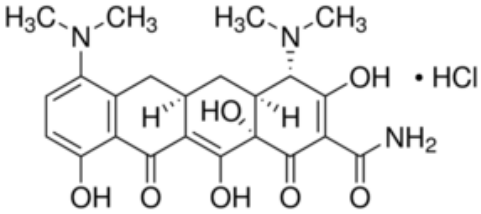
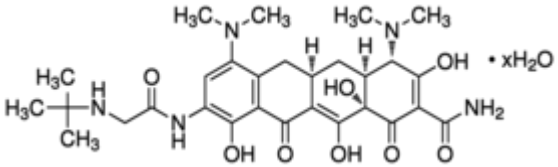
Chemical formula: $C_{22}H_{24}N_2O_9$

Molecular weight: 496.89

Manufacturer's code number and synonyms

Oxytetracycline

Class of antibiotic: Tetracyclines

OXYTETRACYCLINE	CHLORTETRACYCLINE
	
MINOCYCLINE	TIGECYCLINE
	

Antimicrobial activity of the antibiotic

Oxytetracycline is bacteriostatic at recommended use rates and has a broad-spectrum of activity against both Gram-positive and Gram-negative microorganisms including strict anaerobes, spirochaetes, chlamydias, rickettsias, and mycoplasmas. It also has activity against some protozoan pathogens through targeting the bacteria-origin components of the apicoplast.

Antimicrobial spectrum

Oxytetracycline is only approved for use in animals. Formulations (parenteral, oral, in feed, intramammary, foaming pessaries and a topical aerosol) including both oxytetracycline and oxytetracycline hydrochloride are approved for use in poultry, pigs, cattle, sheep, and horses, as well as caged birds, cats and dogs in Australia. Oxytetracycline treats a wide range of bacterial infections in animals caused by Gram-positive and Gram-negative organisms, spirochaetes, rickettsias, chlamydias, and mycoplasmas, including *Actinobacillus pleuropneumoniae, Pasteurella multocida, Streptococcus* spp., *Bacteroides melaninogenicus, Dichelobacter nodosus, Fusobacterium necrophorum, Histophilus somni, Mannheimia haemolytica,* and *Moraxella bovis.*

Post-antibiotic effect and other antimicrobial effects

Post antibiotic effects have not been described for oxytetracycline. However tetracycline and minocycline have a post-antibiotic effect on bacterial growth of 1-3 hours (Athamna *et al.* 2004). A slightly longer post antibiotic effect has been reported for tigecycline (Garrison and Nuemiller 2007, Noviello *et al.* 2008).

Minimum Inhibitory Concentrations for zoonotic pathogens and commensal organisms

The principal aquaculture pathogens that can be acquired topically from fish or shellfish through spine/pincer puncture or open wounds are *Aeromonas hydrophila*, *Edwardsiella tarda*, *Mycobacterium marinum*, *Streptococcus iniae, Vibrio vulnificus* and *V. damsela* (Haenen *et al.* 2013). Due to the vast array of bacteria that can be commensals of fish, MICs for all taxa were not reviewed, though it is important to note that *Erysipelothrix rhusiopathiae*, a commensal of aquaculture species, is an

important zoonotic pathogen. Apart from *A. hydrophila* and some *Vibrio* spp. (see Lehane and Rawlln 2000), there have been no documented Australian case reports of isolates of any of these zoonotic organisms being resistant to the expanded tetracycline class.

Antimicrobial resistance mechanisms and genetics

To date more than 65 individual tetracycline resistance genes have been identified in bacteria. These consist of 34 tetracycline-specific efflux genes, 9 multidrug efflux genes, 13 ribosomal protection genes and 18 tetracycline inactivating genes, the so called "destructor" genes (Fang *et al.* 2020).Tetracycline resistance gene nomenclature is confusing as it is not based on mechanism of action but precedence of discovery. For the majority of genes the *tet* nomenclature is used with either a letter of the alphabet or a numeral in parenthesis (e.g. *tet*(A), *tet*(31)). Further variant diversification is indicated by a numeral following the letter (e.g. *tet*(X4)). Mutations within the 16S rRNA gene may also contribute to tetracycline resistance in many diverse bacteria including *Mycoplasma*. Given the huge diversity of genes, an exhaustive analysis of the various gene families and which bacteria they have been identified in will not be undertaken in this review. The vast majority of genes identified encode resistance to the first- and second-generation tetracyclines. Given the widespread use of tetracyclines in human and veterinary medicine over many years, many pathogenic bacteria carry a high prevalence of resistance genes, particularly gut-based organisms such as *E. coli* that typically share and transfer mobile genetic elements. A remarkably large number of pathogens of humans and animals, however, still remain susceptible to tetracyclines. The relatively recent discovery of tetracycline resistance genes (*tet*(X), *tet*(34), *tet*(37), and *tet*(47-56)) that encode enzymes that inactivate tetracyclines is a cause for genuine concern in medical circles given that the *tet*(X3) and *tet*(X4) variants can also inactivate the third-generation tetracycline tigecycline resulting in high-level resistance, and imparting reduced susceptibility to the fourth-generation tetracyclines.

TETRACYCLINE EFFLUX GENES

Resistance mediated by *tet*(A)-like genes

Tetracycline resistance was first noted in 1953, very soon after the first clinical introduction of tetracycline in human medicine. The widespread adoption of tetracyclines in food-producing animals, particularly for growth promotion, led to the emergence of resistance in zoonotic pathogens such as *Salmonella* and was responsible for the first review governing antibiotic use and abuse in clinical and veterinary medicine, as detailed in Swann *et al.* (1969). In food-producing animals and their immediate environment, identified resistance genes tend to be dominated by elements encoding resistance to sulfonamides and tetracyclines, which may reflect the fact that tetracyclines are slow to degrade. Whilst high concentrations of tetracyclines have been identified in animal manures, agricultural soil and aquatic environments (Fang *et al.* 2020), it is important to note that in urban settings, resistance gene repertoires in both pathogens and commensals tend to reflect those antibiotics used most commonly in clinical practice rather than in animal protein production (Sánchez-Baena *et al.* 2021). Degradation of tetracyclines in aquatic environments has been identified as an important future bioremediation tool to reduce antimicrobial resistance gene (ARG) selection pressure, particularly in aquaculture (Shao and Wu 2020).

There are 6 main classes of specific tetracycline efflux resistance genes identified in extremely diverse groups of bacteria. Horizontal transfer and recombination via integrons, transposons, plasmids and integrative conjugative elements has ensured widespread distribution among both Gram-negative and Gram-positive pathogens. Group 1 encode drug-H+ antiporters which represent the largest group of efflux proteins (Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(J), Tet(Y), Tet(Z), Tet(30), Tet(31), and Tet(33), Tet(39), Tet(41), and Tet(42)) (Thaker *et al.* 2010).

GENERALISED EFFLUX GENES

A range of multidrug-efflux pumps, most often identified in Gram-negative pathogens may use tetracyclines as a substrate. Mutations occurring in the regulatory genes of these membrane bound proteins result in their permanent expression. These include members of the AcrAB-TolC, AdeABC, and MexAB-OprM superfamilies.

Resistance mediated by *acrAB-tolC*-like genes

MarA, the activator protein encoded by the *marRAB* locus, up-regulates efflux of antibiotics, disinfectants and organic solvents via the AcrAB-TolC efflux pump, and down regulates influx through OmpF. This results in low-level resistance to first- and second-generation tetracyclines, but may be important as a stepping stone for the development or acquisition of additional resistance mechanisms (Randall and Woodward 2002)**.**

RIBOSOMAL PROTECTION PROTEINS

Resistance mediated by *tet*(M)-like genes

Ribosomal protection proteins also represent a widely distributed array of tetracycline resistance mechanisms, currently numbering 13 families. Confusingly, these have been given names according to the classical Tet nomenclature and include Tet(M), Tet(O), Tet(Q), Tet(S), Tet(T), Tet(W), Tet(32), Tet(36), Tet(44), and Tet(61), but also may include mosaic genes representing combinations of distinct proteins. Ribosomal protection proteins are believed to have evolved from elongation factor paralogs representing ancient GTPases. Tetracycline resistance is achieved by weakening the interaction of tetracycline and the ribosome with subsequent antibiotic release. These work effectively against first- and second-generation tetracyclines, promoting high-level resistance, but third- and later generation tetracyclines are immune to their action (Randall and Woodward 2002).

GENES ENCODING TETRACYCLINE DESTRUCTASES

The *tet*(X) gene, encoding an enzyme capable of inactivating the expanded tetracycline class, was identified serendipitously in a *Bacteroides fragilis* R-plasmid, and was first described in its native state in *Sphingobacterium* sp. and *Bacteriodes thetaiotaomicron*, though it is possible that these bacteria may have acquired *tet*(X) from another source as it was found inside a transposable element (Thaker *et al.* 2010). In clinical isolates causing infections in humans, *tet*(X) is mainly confined to the Enterobacteriales, Pseudomonadaceae and *Acinetobacter baumannii*, and is frequently associated with overexpression of chromosomal efflux pumps (Randall and Woodward 2002). Tet(X) variants exist in a wide variety of different eco-systems including the human and animal gut, effluent from sewage, animal production facilities, aquaculture systems and hospitals, suggesting that tetracycline use in both humans and animals is the dominant selection and dissemination foci.

Resistance mediated by *tet*(X)-like genes

tet(X) encodes a 388 amino acid flavin-dependent monooxygenase and requires FAD, NADPH, Mg^{2+}, and O_2 for activity. The novel, highly mobile variants *tet*(X3) and *tet*(X4) are particularly noteworthy as they encode high-level resistance to tigecycline together with first- and second-generation tetracyclines and have rapidly dispersed among a wide range of Gram-negative genera. To date, these variants have only been detected in China and Pakistan. Interestingly, a second gene encoding a tetracycline inactivating enzyme Tet(34) was first identified in *Vibrio* sp. isolates from an aquaculture species (yellowtail) (Randall and Woodward 2002), however, no recent studies have documented its occurrence in other fish pathogens.

Occurrence and rate of transfer of antimicrobial resistance genes

Tetracycline resistance genes are located within highly transferrable mobile genetic elements and most conjugation studies have revealed very high rates of plasmid transfer to laboratory adapted recipient strains. This is particularly the case for the recently identified *tet*(X3) and *tet*(X4) genes.

Occurrence of cross-resistance

Cross resistance is defined as resistance to more than one antibiotic or antibiotic class determined by a single mechanism of resistance. While the majority of resistance mechanisms discussed above are tetracycline-dependent, overexpression of efflux pumps and underexpression of major outer membrane proteins affect a variety of antibiotics, disinfectants and heavy metals. As mentioned previously, these are usually responsible for low-level resistance, but are often associated with multidrug-resistant isolates.

Occurrence of co-resistance / co-selection

Co-resistance refers to the presence of several resistance mechanisms in the same microorganism and co-selection refers to the selection of multiple resistance genes when any one gene is selected. The examples above provide many cases of co-resistance and the genetic location of the resistances suggests that co-selection is usual. This is particularly the case for the novel *tet*(X3) and *tet*(X4) variants which have been shown to be co-associated with *mcr-1* (colistin resistance) and $bla_{\text{NDM-1}}$ (carbapenem resistance).

In vitro mutation frequency studies

In vitro mutation frequency studies are not reported. Apart from overexpression of multidrug efflux pumps, which often involve mutations in regulatory genes, the major mechanisms of high-level oxytetracycline resistance are mediated by specific tetracycline efflux genes, ribosomal protection genes, and tetracycline inactivating enzyme genes, with mutation in genes such as the 16S rRNA gene a relatively minor source of resistance.

Other animal studies

No other relevant animal studies have been identified.

7.1 Risk Assessment

Risk Assessment – food-producing animals

Summary of the risk profile

Hazard characterisation

The OIE ranks oxytetracycline as a critically important veterinary antimicrobial. The Australian Strategic Technical Advisory Committee on Antimicrobial Resistance (ASTAG) considers tetracyclines developed for human use (doxycycline, minocycline) to be of Low Importance to human health whereas the WHO ranks them as being of Importance to human health. The related glycylcycline antimicrobial tigecycline, however, is considered of High Importance to human health by ASTAG and the WHO ranks it among the Critically Important to Human Health antimicrobials list. Tigecycline, the first new tetracycline derivative drug in 30 years, was licensed for use by the FDA in 2005 (Livermore 2005). Tigecycline activity is not affected by the numerous efflux pumps encoded by *tet*(A-E) which account for the majority of resistance to earlier generation tetracyclines found in Enterobacteriales and *Acinetobacter* spp. and *tet*(K) in Gram-positive bacteria. Additionally, it will still bind to bacterial ribosomes modified by Tet(M) phenotypes. Tigecycline remains critical for the treatment of Gram-negative pathogens that have developed resistance to fluoroquinolones, carbapenems and colistin. Additionally, the first fully

synthetic tetracycline antimicrobials, eravacyline (Xerava®) and omadacycline (Nuzyra®), were approved for use in US patients in 2018 for specific infections including abdominal, skin, soft tissue, and lung infections but are not yet available in Australia. In Australia, tigecycline is used as a reserve agent for both resistant Gram-positive (*Enterococcus*, *Staphylococcus*) and Gram-negative (Enterobacteriales, *Acinetobacter*) pathogen infections.

The hazard is therefore defined as high-level tigecycline-resistant bacteria and/or genetic elements containing the tetracycline destructase gene variants *tet*(X3) or *tet*(X4) selected by the use of oxytetracycline in mainland Australian freshwater and/or saltwater finfish aquaculture species with the potential to transfer to and cause adverse effects in humans. Section 2 (antimicrobial resistance mechanisms and genetics) summarises current knowledge on the mechanisms of tetracycline and tigecycline resistance, particularly in Gram-negative bacteria. Given the importance of tigecycline to human medicine, the hazard is assessed as Medium.

Exposure characterisation

Oxytetracycline use in the industry is likely to be sporadic in the face of outbreaks of bacterial disease mostly in juvenile fish. Even at maximum forecast use, only 5% of the Australian farmed finfish population is likely be treated in any one year (Matt Landos, unpublished observation). Treatments are authorised by a veterinarian with expertise in fish health and based upon demonstration of a causal pathogen by laboratory culture of appropriately collected samples and antimicrobial susceptibility testing. Oxytetracycline is not used as a prophylactic treatment in the Australian finfish aquaculture industry. Metaphylactic use is required in finfish species during outbreaks, however, because sick fish cannot easily be separated and individually treated, and may also rapidly transmit infection to healthy fish. Oxytetracycline is typically administered at a relatively high concentration of 75 mg/kg for approximately 10 days. Strict maximum residue limits (MRLs) are in place to ensure no antibiotic residues are present in the final product. Oxytetracycline pharmacokinetics suggests large, often species-specific, variations in bioavailability of orally administered oxytetracycline as well as water temperature affects. This suggests that in some finfish species, a large amount of the active ingredient is excreted, leading to potential environmental contamination. Environmental contamination has been identified as a potential issue in several countries where aquaculture is highly concentrated, given that oxytetracycline is only slowly degraded in the environment. In Australia, however, finfish aquaculture farms are widely dispersed. Given antimicrobial use patterns and the low density of the industry, exposure to humans is considered negligible.

Impact characterisation

Impact characterisation is an assessment of infections in susceptible humans caused by bacteria with resistance (or the transfer of the resistance determinants themselves) arising from the use of oxytetracycline in farmed finfish in Australia under the minor use permit. A higher classification is not indicated as within Australia, there are a number of alternative Critically Important Antimicrobial classes (fluoroquinolones, third- and fourth-generation cephalosporins, and carbapenems) that can be used for the treatment of life-threatening Gram-negative infections for which resistance rates are currently low. The severity of the impact of this exposure on susceptible humans is therefore assessed as low.

Assessment of the uncertainty of the data used in risk assessment

There is an absence of recent published Australia-wide data on the antimicrobial susceptibility of Australian finfish aquaculture pathogens to oxytetracycline and the genetic determinants of any resistance identified.

Benefits of use of the antibiotic in Australian animal health

There are significant benefits to aquatic animal health from the continued availability of oxytetracycline, a low ASTAG importance antimicrobial, for the rapid control of outbreaks of bacterial infection in non-salmonid finfish species in Australia. Use is likely to be sporadic, and to occur early in the production phase when young fish are most prone to bacterial infection outbreaks. Oxytetracycline is considered a first line antimicrobial by the Australian Veterinary Association. The main benefits to the finfish aquaculture industry are improved production, improved welfare and business/supply continuity. For example, outbreaks of *Streptococcus iniae* infection in warm water aquaculture (e.g. Bromage *et al.* 1999; Creeper and Buller 2006) have contributed to the failure of at least three barramundi farms in southern Australia, and two in northern Australia (Dr Marty Deveney, unpublished observation). Although erythromycin appears to be more effective in managing *S. iniae* outbreaks than oxytetracycline (Agnew and Barnes 2007), this example illustrates the importance of access to effective antibiotics for aquaculture.

Risk characterisation

Taking into account the combined assessments of hazard, exposure and impact, the probability of disease and treatment failure due to infection in susceptible humans after exposure of humans to tigecycline-resistant bacteria resulting from oxytetracycline use in finfish aquaculture species (or indeed any animal species) in Australia is technically possible but unlikely. Risk is therefore characterized as low. Whilst human exposure is negligible based on current and future aquaculture oxytetracycline use patterns, impact is low, given that alternatives to tigecycline for the treatment of life-threatening infections caused by Gram-negative pathogens (such as bacterial sepsis) exist in Australia due to conservative regulation and prudent use both in humans and animals, antimicrobial stewardship in hospitals and the community, and excellent long-term AMR surveillance. These alternatives include third-generation cephalosporins, fluoroquinolones, carbapenems, and, in extremely rare cases, colistin.

There is more likelihood that *tet*(X) would enter Australia in bacteria carried by people (particularly gastrointestinal carriage) who have visited countries where prevalence is high, such as mainland China, compared with the risks of minimal interventions with oxytetracycline in endemic farmed finfish (both freshwater and saltwater). Increased tetracycline resistance has been observed among pathogens isolated from ornamental fish (Matt Landos, unpublished data) which could be an additional possible oxytetracycline resistance incursion risk from overseas with the potential to become endemic. There may also be a small but currently unquantified risk of entry with imported seafood. Seagulls and other aquatic birds may, in addition, be carriers of multidrug-resistant human sepsis pathogens with critically important antimicrobial resistance genes (Mukerji *et al.* 2019; Mukerji *et al.* 2020). Sea birds are biosecurity intruders at many aquaculture sites within Australia, but typically can be prevented from access to hatchery and juvenile fish facilities where antibiotic use is more common. These latter risks, however, are of limited influence for the minor use permit for oxytetracycline use in farmed finfish because the risk for human transmission is consumption and handling of the seafood product.

Detailed risk assessment - Hazard characterization

The hazard represents the biological agents used in the target animal species with the potential to cause adverse effects in humans. The hazard associated with the use of oxytetracycline is the evolution, selection and transmission of oxytetracycline-resistant bacteria (and/or their transferable resistance genetic elements (*tet*(X3) and/or *tet*(X4) variants) that are also resistant to tigecycline from Australian non-Salmonid finfish species to humans, where they are likely to cause harm.

Expected usage patterns and industry geography

In 2017/2018, the Australian aquaculture industry produced 97,672 tonnes of seafood mainly for local consumption, representing an increased share in gross value of product (GVP) for the entire Australian seafood industry from 29% in 1999–2000 to 44% (Australian Bureau of Agricultural and Resource Economics and Sciences 2018). In any new finfish aquaculture enterprise, bacterial disease issues are encountered early in the life of the operation, which is reflected in the Tasmanian Salmon Industry's antibiotic use figures over a 10-year period, with reductions from a high of 358.5 g of antibiotic/tonne of fish produced in 2006/2007 down to 2.1 g/tonne in 2015/2016 (Figure 6). This reflects both the introduction and availability of effective vaccines for the main endemic fish diseases, together with the fact that antibiotics tend to only be required in juvenile fish early in the production cycle when fish mass is small. Given it is the most commonly prescribed antibiotic in finfish aquaculture, oxytetracycline is estimated to account for approximately 75% of total antibiotic use, with other antibiotics currently used off-label including erythromycin and sulfonamide/trimethoprim combinations.

Salmon, Tuna (ranched in sea cages where they are introduced as 10-20kg fish from the wild fishery and with little or no requirement for antimicrobials), Oysters and Prawns account for approximately 82.3% of Australian aquaculture production; non-Salmonid finfish (e.g. barramundi, trout, kingfish and minor species) are estimated to account for a maximum of 17.7% of production (17,320 tonnes per year). Given a conservative estimate of no more than 5% of fish being treated with an antibiotic in any production cycle, and the figures on antibiotic use from the Salmon industry, it is estimated that assuming a conservative requirement of 5 g oxytetracycline.tonne^{-1} of non-salmonid finfish seafood produced, annual use through the minor-use permit would be in the vicinity of 86.6 kg. By comparison, it was estimated that over 5,500 tonnes of antibiotics were used in the salmon aquaculture industry in Chile over a 10 year period (2008-2018), equivalent to 550 kg per annum (Higuera-Llantén *et al.* 2018).

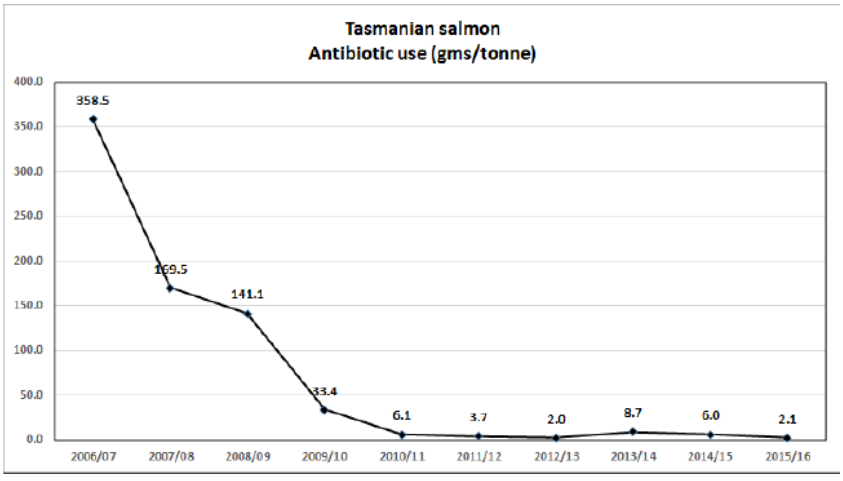


Figure 6. Annual antimicrobial use by the Australian Salmon Industry (2006-2016). Source Tasmanian Salmon Growers Association.

The main locations of aquaculture farms in Australian waters are shown in Figure 7. The remote location of many industries is in stark contrast to aquaculture enterprises in China and south east Asia. The estimated 86.6 kg would therefore be widely distributed, sporadic and temporary.

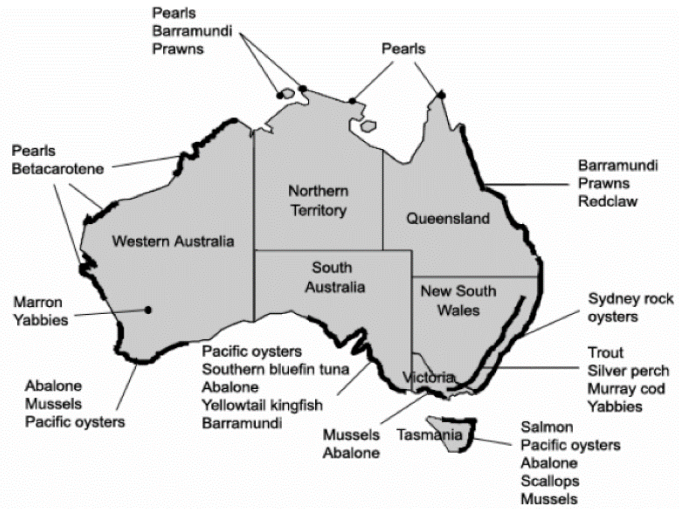


Figure 7. Location of the major aquaculture enterprises in Australian waters. From Productivity Commission Assessing Environmental Regulatory Arrangements for Aquaculture (2004).

Target animal pathogens and potential foodborne pathogens

The main target pathogens in the non-Salmonid finfish species farmed in Australia include *Aeromonas* spp., *Flavobacterium* spp., *Vibrio* spp., *Photobacterium* spp., *Edwardsiella spp.*, *Streptococcus iniae, Lactococcus garvieae* and *Epitheliocystis* sp. Antimicrobial resistance in the main foodborne infections acquired from seafood are typically post-production contaminants during processing (e.g. *Listeria monocytogenes*) and not relevant to this review.

Known mechanisms and genetics of resistance pathways in relevant microorganisms

Initially, low-level resistance to tigecycline was found to be mediated by chromosomal mutations leading to overexpression of efflux pumps (Deng *et al.* 2014). However, in the last 10 years there has been an explosion in the detection and widespread dissemination of *tet*(X) among human and animal pathogens. The *tet*(X) gene encodes a flavin-dependent monooxygenase that inactivates all tetracycline-like antimicrobials, including the new generation fluorocycline and methylaminecycline drugs (Aminov 2021). *tet*(X) was originally described as a rare, chromosomally located gene in *Bacteroides* spp (Livermore 2005), but appears to have its origins in environmental microbiota belonging to the Flavobacteriaceae family, with the suggestion that the widespread use of tetracyclines in agriculture and possibly humans has resulted in its mobilization and rapid dissemination to many Gram-negative pathogens including Enterobacteriales and *Acinetobacter* spp.

Interestingly, the most significant animal pathogen to contain *tet*(X) variants is *Reimerella anatipestifer*, which causes septicaemia in ducks (mainly confined to Asia), and is also a member of the Flavobacteriaceae family. *tet*(X) is the major gene associated with tetracycline resistance in this species, with over 90% of isolates from China resistant to tetracyclines. Recent analysis of a large collection of *R. anatipestifer* isolates from mainland China for *tet*(X) variants confirms this pathogen is a natural reservoir for *tet*(X), with the *R. anatipestifer* chromosome harbouring varied copies of *tet(X)* progenitors (Umar *et al.* 2021).

The *tet*(X3) and *tet*(X4) genes, which are the only variants encoding high-level tigecycline resistance, have recently emerged in mainland China. The highly mobile *tet*(X3) and *tet*(X4) variants, which have greater ability to degrade glycylcycline antibiotics, have been described on a transferrable plasmid also

containing *mcr-1* in commensal/environmental *E. coli* isolates from Chinese pigs, poultry, soil, and dust samples (Sun *et al.* 2019), as well as layer farms, manured soil and lettuce samples (He *et al.* 2021). Resistome studies undertaken on samples from poultry, humans and the general environment of live poultry markets in China have identified *tet*(X3), often associated with *mcr-1*, with the same gene array also identified in human pathogens (Wang *et al.* 2021). Plasmid-mediated tigecycline resistance genes, *tet*(X3) and *tet*(X4) have been described in various *Acinetobacter*, Enterobacteriales and five other bacterial species isolated from animals, meat for consumption, and humans in China (He *et al.* 2021). More recent studies have detected the mobilizable *tet*(X4)-containing plasmids in *E. coli* isolated from poultry and slaughterhouse samples in Pakistan (Mohsin *et al.* 2021) and dual carbapenem/tigecycline resistance plasmids in *Acinetobacter* spp. isolated from farmed waterfowl (i.e. ducks) and their environment in China (Cui *et al.* 2020). A recently published Chinese study (Fu *et al.* 2021) of *tet(X)* variants identified by real-time PCR found that they were more abundant in faecal samples from poultry, compared with pigs and cattle. This study also inferred that veterinary use of tiamulin and florfenicol were more important selection pressures for *tet*(X) variants than tetracyclines themselves.

Sporadic reports of less significant *tet*(X) variants in bacterial isolates from other animal species include its detection in a multidrug resistance (MDR) commensal *E. coli* strain from pigs in Denmark (Herrero-Fresno *et al.* 2016). Analysis of the phylogeny of *tet(X)* variants has suggested that widespread use of tetracyclines (and potentially other co-selecting antimicrobials) in animal production, particularly in China, is likely to be responsible for its dissemination. Tetracyclines are the most used antimicrobials in food-producing animals in most regions of the world and particularly in China. However, it is also noted that *tet*(X) has a high prevalence in human pathogens in developing communities where older generation tetracyclines (doxycycline, minocycline) have been heavily dispensed without prescription, and this may have provided additional selection pressure (Aminov 2021).

Details of the microbial resistance patterns in relevant microorganisms

Reports of *tet(X)* in bacteria isolated from finfish aquaculture systems throughout the world are limited. Resistance mechanisms in two of 36 florfenicol-resistant *Chryseobacterium* spp. isolates (also members of the Flavobacteriaceae) are mildly pathogenic for rainbow trout, and identified the presence of both *floR* and *tet*(X) genes (Michel *et al.* 2005). The genes were located in an array with streptothricin and chloramphenicol resistance genes, suggesting a more ancient rather than recent recombination event. A recent microbiome/resistome study of integrated (duck/fish) and monoculture freshwater aquaculture systems in China found that both *tet*(X) and *mcr* genes were greatly enriched in dual production systems compared with monoculture systems (Xu *et al.* 2020). This would tend to suggest that while *tet*(X) is mainly associated with waterfowl in China, freshwater aquaculture systems, particularly when they are integrated with duck farming, could potentiate its selection. Within the family Flavobacteriaceae, the genus *Flavobacterium* represents an important group of finfish pathogens affecting aquaculture production worldwide (Wahli and Madsen 2018). Tetracycline resistance has been identified in a number of studies of important aquaculture pathogens within this genus (e.g. *F. columnare*) but has usually been mediated by *tet*(A) (Declercq *et al.* 2021). Recent resistome studies of microbiota within aquaculture ponds in mainland China have consistently identified *tet*(X) as a member of the ARG community (Xiong *et al.* 2015; Huang *et al.* 2017; Shen *et al.* 2020).

High-level tigecycline resistance mediated by *tet*(X) variants has been reported in a number of MDR human pathogens internationally, including *E. coli, Klebsiella pneumoniae, Enterobacter* spp., and other members of the Enterobacteriales and Pseudomonadaceae (Fang *et al.* 2020). Whilst the majority of reports come from mainland China, sporadic reports of *tet*(X) variants are noted in Japan, Thailand, North and South America, several countries in Europe including the UK, and several countries in Africa. The Australian Group on Antimicrobial Resistance (AGAR) collates Australia-wide antimicrobial susceptibility data for Gram-negative bacteria causing sepsis and includes tigecycline in

its panel, but to date no high-level resistance has been described. The authors are not aware of any reports of *tet(X)* in clinical or commensal bacterial isolates from animals, humans, or environmental samples in Australia including major aquaculture species and their immediate surroundings.

Studies on the antimicrobial susceptibility of Australian aquaculture pathogens are limited. Akinbowale et al. (2006) subjected 104 aquaculture bacterial isolates (100 Gram-negative and four Gram-positive) to antimicrobial susceptibility testing (AST) for 19 antimicrobial agents using National Committee for Clinical Laboratory Standards (NCCLS) now Clinical and Laboratory Standards Institute (CLSI), human breakpoints for Enterobacteriaceae, which may not be appropriate, particularly with respect to the new CLSI standards available for susceptibility testing of aquaculture pathogens. The isolates included saltwater and freshwater pathogens, environmental and commensal isolates, and were dominated by *Vibrio* spp. (approximately 60% of the 104 isolates) and *Aeromonas* spp. (21%). Approximately 19% of the isolate collection were considered resistant to oxytetracycline (Akinbowale *et al.* 2006). A high proportion of *Vibrio* isolates were resistant to aminopenicillins and first-generation cephalosporins, but few were resistant to oxytetracycline and none to florfenicol or high ASTAG rating antimicrobials. A higher proportion of *Aeromonas* spp. were resistant to oxytetracycline. This study is now 15 years old and it is difficult to determine its significance given the diversity of bacterial species, the use of non-standard techniques for aquaculture pathogen susceptibility testing and the high proportion of environmental isolates such as *Aeromonas* spp. Nevertheless, a follow-up paper (Akinbowale *et al.* 2007) identified resistance mechanisms for most of the oxytetracycline-resistant isolates (mainly *Aeromonas* spp.) by PCR. *tet*(M) (50%) was the most common determinant identified, followed by *tet*(E) (45%), *tet*(A) (35%) and *tet*(D) (15%). Five of the genes were transferable by conjugation to *E. coli*, indicating the potential for horizontal transmission (Akinbowale *et al.* 2007).

Ndi and Barton (2011) investigated the occurrence of class 1 integrons in *Aeromonas* spp. isolates from rainbow trout. Class 1 integrons were detected in 28/90 (31%) isolates, and in addition to sulphonamide (*sul1*) and quaternary ammonia (*qac1*) resistance genes, some integrons contained the streptomycin resistance gene *aadA2* in their variable region. *tet*(C) was also identified in some isolates but was not integron-associated. Ndi and Barton (2012) investigated the occurrence of class 1 integrons in *Pseudomonas* spp. isolated from rainbow trout. Class 1 integrons were detected in 30/129 (23%) isolates and *aadA* streptomycin resistance genes were also detected in nearly half the isolates positive for integrase. The *mexA* multidrug efflux pump gene was detected in 85 isolates and 59/92 isolates tested also were also positive for the cadmium resistance gene *cadA*. It is important to note that these are both environmental organisms with capacity to cause opportunistic infections in finfish and the presence of resistance genes may not be directly related to antimicrobial use in the industry.

There have been few recently published studies on the presence of resistance determinants in potentially pathogenic bacteria isolated from Australian aquaculture finfish and/or their environment. Very few published studies have specifically examined AMR among bacteria isolated from aquaculture species in Australia, although anecdotal reports from the main veterinary diagnostic laboratories supporting the industry suggest that resistance is negligible (unpublished data). As an example, retrospective Tasmanian salmon aquaculture pathogen MIC data (170 isolates representing 16 species) obtained by the Tasmanian Department of Primary Industries, Parks, Water & Environment over a ten year period indicates the majority of isolates with pathogenic significance were not resistant to oxytetracycline, with MIC_{50} values of 1µg/mL and MIC_{90} values of 2µg/mL (Australian Department of Health 2018). Based on a collation of individual diagnostic reports from State Government Veterinary Laboratories, variable reduced susceptibility to oxytetracycline has been observed amongst aquaculture pathogens, but no high-level resistance (unpublished data).

The salmonid antimicrobial susceptibility data are the only data existing in the available literature that confirm the infrequency of oxytetracycline resistance in Australian pathogens that could possibly be extrapolated to other fresh and saltwater finfish species farmed in Australia. These MIC values suggest

a wild type population for the overwhelming majority of salmonid pathogens encountered by the industry, and the success of the introduction of a number of vaccines controlling the main bacterial diseases. These low MIC values are likely to be uniform within the other fresh and saltwater finfish aquaculture industries within mainland Australia given that oxytetracycline is typically administered in the feed at high dose for short periods, metaphylactically (i.e. administered to both sick and healthy fish within the same cohort), to control outbreaks and prevent disease spread. Given a minor use permit for the authorised use of oxytetracycline in the Tasmanian salmon industry with a maximum residue limit of 0.2 mg/kg has existed sporadically since 1995 (Final assessment report; Application A608 2008), the susceptibility data indicate the overall absence of significant resistance to oxytetracycline despite the drug being readily available for use within the last decade and a half.

The putative development rate of resistance *in vitro* is not relevant to high-level tigecycline resistance given the gene is acquired horizontally via plasmids or other mobile genetic elements.

Details of the microbial resistance patterns in relevant microorganisms that have emerged with the use of the product, the antibiotic or related substances

With the exception of *tet*(X3) and *tet*(X4), tetracycline resistance mechanisms described to date affect resistance to doxycycline and minocycline, which are approved for use in humans in Australia. The ASTAG Importance Ratings and Summary of Antibiotic Uses in Humans in Australia states that doxycycline and minocycline are major agents for minor respiratory tract infections and acne, and together have a supportive role in treating pneumonia caused by *Mycoplasma* and *Chlamydia pneumonia* and malaria prophylaxis (doxycycline only). ASTAG rates the importance of these second-generation tetracyclines as low, indicating that there are many alternative antibacterial agents available. Therefore, cross resistance to early generation tetracyclines arising from use of oxytetracycline in finfish aquaculture is unlikely to cause harm to human health given the indications for human use and the availability of additional classes. The hazard associated with this type of resistance is considered negligible.

Low level efflux-mediated resistance to tigecycline caused by overexpression of well recognised efflux pumps with concomitant down regulation of major outer membrane porins has been described in a large number of principally Gram-negative species of bacteria and is not specific to the tetracycline class. The hazard associated with this type of resistance arising from the use of oxytetracycline in finfish species is also considered Negligible.

Inactivation of tetracyclines by *tet*(X3) and *tet*(X4) leading to high-level tigecycline resistance is considered to be the greatest hazard associated with oxytetracycline use in finfish species and has been increasingly identified in multiple animal/human interface environments in China, including mixed aquaculture/water fowl farming systems. Tigecycline is considered to be of high importance to human health by ASTAG, but it is important to note that there are currently many alternatives for life-threatening Gram-positive and Gram-negative infections in Australia, given our current low rates of resistance to critically important antimicrobial classes. High-level tigecycline resistance has been associated with highly transmissible mobile genetic elements that may contain genes imparting resistance to other critically important drug classes. The hazard associated with these new resistance mechanisms may not be just direct tigecycline resistance but could include cross resistance/reduced susceptibility to colistin, carbapenems, fluoroquinolones, and other critically important products due to this co-localisation possibility.

The proposed use of the product and the target animal species

Use of oxytetracycline in farmed Atlantic salmon in Tasmania is already occurring through an APVMA minor use permit that has been in place (on and off) for over 15 years, and oxytetracycline is also being sporadically used under off-label legislation in other Australian aquaculture species for a range of

Gram-positive and Gram-negative aquaculture pathogens. The hazard can be characterized on the basis of the use of the product and the nature of resistance selection within aquaculture pathogens themselves, in addition to commensal bacteria in the fish gut and environmental organisms exposed to oxytetracycline. Even at maximum forecast use, less than 5% of the Australian farmed finfish population will be treated in any one year. Treatments will be authorised by a specialist fish health veterinarian and based upon demonstration of a causal pathogen by laboratory culture of appropriately collected samples and antimicrobial susceptibility testing. Oxytetracycline is never used as a prophylactic treatment in the Australian finfish aquaculture industry. However, metaphylactic use (i.e. mass medication to both sick and healthy fish in the same cohort) is required in finfish species during outbreaks because sick fish cannot be easily separated and individually treated and may rapidly transmit infection to healthy fish.

It is important to note that there are already many registered animal products containing oxytetracycline for the treatment of a broad range of infections in food-producing animals (poultry, pigs, sheep and cattle; both as individual treatments and mass medication, including prophylaxis) in Australia, and no evidence of resistance to tigecycline.

Evidence of *in vitro* cross resistance from overseas data

The emergence and spread of high-level tigecycline resistance and its association with mobile genetic elements containing critically important antimicrobial resistance genes is outlined above.

Potential exposure of gut microbiota

The pharmacokinetics of oxytetracycline have been studied in a number of aquaculture finfish species but most studies have examined intramuscular or intravenous administration and have noted significant differences between species as well as water temperature effects which greatly influence elimination half-life (Grondel *et al.* 1989; Li *et al.* 2015). The recommended oral dose of oxytetracycline varies greatly, but is generally in the range of 75-100 mg/kg or approximately 7.5-10 g of oxytetracycline per 100 kg of fish per day. In studies comparing parenteral with oral delivery, apparent oral bioavailability in rainbow trout was only 5.6% (Björklund and Bylund 1991). Published oral bioavailabilities are generally lower in fresh/cold water species compared with warm/saltwater species, but are still highly variable. For example, a study of oxytetracycline bioavailability in Mediterranean aquaculture finfish species found lower amounts of unabsorbed oxytetracycline in the faeces of sea bass (15–40%) compared with gilthead sea bream (73%) and sharp-snout sea bream (60%) (Rigos *et al.* 2004a). This has led to the conclusion that for marine species, multiday dosing is required to achieve an adequate area under the curve: minimum inhibitory concentration (AUC:MIC) ratios for most pathogens (AUC/MIC is the recommended pharmacodynamic parameter for predicting efficacy of the tetracycline class, and the usual recommended duration of treatment in finfish species is 10 days) (Miller *et al.* 2012). Co-administration of citric acid, however, can double serum concentrations (Akiyama *et al.* 2020). Multiple dosing and low oral bioavailability has led to concerns over the possible environmental consequences of significant quantities of unmetabolised oxytetracycline that can be passed unabsorbed through the body of treated fish and excreted via the faeces into the local aquatic environment (Rigos *et al.* 2004b). In the only fish gut microbiome study undertaken in Australia, the effect of a combination of oxytetracycline, erythromycin and metronidazole was assessed on the gut microbiome of Yellowtail Kingfish (Legrand *et al.* 2020). This treatment resulted in a loss of species diversity and evenness, which did not recover over the 18-day study period. A 6-week treatment of zebrafish with oxytetracycline in their feed resulted in a decrease in intestinal microbial richness (Zhou *et al.* 2018).

These studies demonstrate that the potential exposure of fish gut microbiota resulting from in-feed oxytetracycline is high — i.e. the antimicrobial substance and/or its metabolites in the gastrointestinal

tract are present in concentrations high enough to have an impact on microbial flora after administration.

Gut concentrations of oxytetracycline and its metabolites

Poor oral bioavailability suggests that significant concentrations of oxytetracycline will be present in the gut of treated fish because only a small proportion of oxytetracycline that is consumed is absorbed. In microbiome studies, these have a significant effect on the gut microbiota and a disposition to colonization by oxytetracycline-resistant organisms. No studies have directly linked oxytetracycline levels in the gut of aquaculture finfish species with the presence of *tet*(X) variants encoding high-level tigecycline resistance.

Conclusion

Overall, given the significance and genetic context of these newly described resistance mechanisms, the proposed use pattern and the quantities and distribution of use, the hazard is considered Medium. This is principally based on the significance and ranking of tigecycline as an antibiotic of high importance in Australia, the pharmacokinetics of oxytetracycline in finfish species following oral administration and the relative resistance of tetracyclines to environmental degradation.

Table 4 Hazard posed by development of oxytetracycline resistance associated with use in aquaculture finfish.

	Negligible	**Low**	**Medium**	**High**
Hazard				

Detailed Risk Assessment - Exposure characterization

An exposure characterisation states the amount and frequency of exposure of susceptible humans to antibiotic-resistant microorganisms (or their transferable genetic elements) from animal sources.

Routes of exposure

There are a number of plausible human exposure routes for oxytetracycline-resistant micro-organisms carrying *tet*(X) resistance genes arising in finfish aquaculture species in Australia. These include direct contact with treated fish, their immediate aquatic environment or fish entrails during processing, consumption of fish including potentially raw or improperly cooked product and environmental contamination resulting from increased tetracycline concentrations in effluent. Given the planned infrequency of use, the fact that non-Salmonid aquaculture farms within Australia are widely dispersed and the current lack of detection of tigecycline resistance, these routes likely present a negligible risk of exposure.

Levels of carriage of target pathogens and other relevant micro-organisms in populations of the target animal species

Levels of carriage of the main target pathogens causing mortality in healthy non-Salmonid finfish in Australia are low to negligible, suggesting that human exposure is likely to result from direct contact with sick fish only. In experimental challenge studies, *S. iniae* is cleared 10 days after infection (Bromage and Owens 2002), only low to very low carriage rates have been observed for *Aeromonas*

spp. and *Photobacterium* spp., and for *Vibrio* spp., carriage has only been demonstrated in diseased fish (Pujalte *et al.* 2003; Dong *et al.* 2017).

Potential for direct exposure of those in close contact with treated animals

There is a low to moderate risk of exposure through handling or processing sick fish and/or fish undergoing treatment for a range of aquaculture pathogens, including *S. iniae*, *P. damselae* and *V. harveyi* (see Rivas et al. 2013). These involve entry of the pathogen through knife cuts or hand spike injuries. Whilst infections can be serious and have been reported in Australia, risks of treatment failure due to antibiotic resistance resulting from oxytetracycline use are negligible (Akram *et al.* 2015).

Potential for contamination of food commodities and amplification along the food chain (fish farms, processing plants, retail sale)

While significant microbiota changes and colonization by tetracycline-resistant bacteria are demonstrated in finfish treated with oxytetracycline, no long term studies have been undertaken on the microbiota of processed fish treated with oxytetracycline during production. Given that most fish that are treated are at the juvenile stage and treatment is concluded by 10 days, studies in other livestock species suggest the microbiota within the animal is not permanently altered and should return to pre-treatment levels. This is particularly the case given that tetracyclines are bacteriostatic rather than bactericidal antibiotics. Given that studies of *tet*(X) variants encoding high-level tigecycline resistance are still in their infancy, further information is likely to come from China where studies of *tet*(X) variants are most advanced. Results to date suggest that poultry are a bigger exposure risk than aquaculture species. Australia does not practice integrated farming of ducks and fish, which has been recently shown to be a risk factor for *tet*(X) amplification in China. Exposure risk is therefore negligible.

Efficacy, reliability of Codes of Practice, hazard analysis and critical control points (HACCP) programs relating to contamination

In terms of direct zoonotic transmission of aquaculture pathogens from finfish to humans, diseased fish present a much higher risk to in-contact individuals than healthy fish (Haenen *et al.* 2013). Harvesting only healthy fish results in markedly decreased risk of exposure. In terms of microbiota that may have acquired resistance following use of oxytetracycline, seafood processing procedures adopted in Australia reduce the opportunity for gut microbiota to contaminate the finished product. In a recent Norwegian study of ready-to-eat seafood, sushi had the highest prevalence of pathogenic *Aeromonas* spp. contamination, however, the isolates were all susceptible to tetracycline (Lee *et al.* 2021).

Effectiveness and reliability of process controls to destroy or inhibit micro-organisms

Cooking seafood prior to consumption eliminates any perceived or actual AMR risk but care must be taken to not cross-contaminate raw/uncooked food during preparation. In the case of raw, ready-to-eat product, bacteria within fish muscle will not be eliminated during processing, hence the requirement for harvesting healthy fish only, that do not have evidence of bacterial infection/septicaemia.

Microorganism survival and potential for growth / reduction / dilution in the environment

In several countries, most commonly China, concern has been expressed regarding high density aquaculture systems situated close to urbanized areas and other livestock rearing enterprises (such as duck farms). Combined with high use of oxytetracycline in animals possibly selecting for *tet*(X) variants co-located on mobile genetic elements with other critically important antimicrobial resistance genes, this represents a high exposure risk in those countries. This risk is particularly amplified given the low bioavailability of oxytetracycline in many aquaculture finfish species, and the relatively low inactivation of tetracyclines in the environment. Given the low density of aquaculture farms within Australia, the

often large distances between farms and main urban areas, and the infrequency of use, there is likely to be a significant dilution effect and reduced selection of resistant organisms in the environment.

Probability and extent of human exposure in the general human population (Negligible, Low, Medium, High).

Human exposure to organisms containing high-level tigecycline resistance determinants within the general population in Australia is likely to be negligible. As mentioned previously, *tet*(X) variants are more likely to gain entry to Australia through returned travelers coming from countries with high prevalence rates in animals, animal products, the environment as well as humans. Fish processing workers in Australia represent a slightly higher risk than the general population for zoonotic fish pathogens acquiring resistance genes.

Demonstrated establishment of antibiotic-resistant micro-organisms (of animal origin) in the general human population

As discussed previously, there is no current evidence that *tet*(X3) or *tet*(X4) are present in Australia. Distribution in China and several other countries has been discussed previously.

Factors that influence environmental micro-organism distribution and secondary spread from a point source to a range of susceptible humans (including characterisation, variability, distribution)

Selection and dissemination of *tet*(X) variants in China has been described previously. Compared with other countries, the high proportion of duck farms in China and their integration with other farming enterprises may have been responsible for selection and dissemination of *tet*(X) variants in a range of animal species including other poultry, pigs and aquaculture species. Evidence for duck farms being the primary means of dissemination includes the extremely high proportion of *tet*(X) variants identified in the duck pathogen *Reimerella anatipestifer* (close to 90% of strains) and the higher proportion of *tet*(X) variants identified in mixed farming operations. None of these factors are present in finfish aquaculture farms in Australia. Sea birds continue to be pests in and around Australia's aquaculture facilities. Birds frequent urbanized areas, human sewage plants, refuse sites and other anthropogenically altered sites, and may encounter hospital effluent. Gulls can be colonized with the same multidrug-resistant pathogens that cause severe antimicrobial-resistant infections in humans, including highly virulent *E. coli* ST131 and ST1193 (Mukerji *et al.* 2020). Biosecurity risks are greater in hatcheries than in grow-out, however, where antibiotics are more likely to be used to treat disease outbreaks. By the time fish are moved to areas where they are more likely to encounter seagull faecal matter, antimicrobial treatments are less common and there is a large dilution effect.

Populations of susceptible humans with respect to relevant micro-organisms

Immunosuppressed patients are at higher risk of sepsis. Due to low rates of resistance to critically important antimicrobials in Australia, a range of treatment options are available for Gram-negative sepsis. Tigecycline resistance has not been reported in annual Gram-negative sepsis isolates during AMR surveillance, undertaken by AGAR. Immunosuppressed individuals are already warned against eating ready-to-eat products containing raw meat/seafood.

Probability of spread to susceptible humans (Negligible, Low, Medium, High)

Negligible

Demonstrated establishment of antibiotic-resistant micro-organisms (of animal origin) in susceptible humans

High-level tigecycline resistance has not been reported in Australia.

Table 5 Probability and extent of exposure of susceptible humans to resistant micro-organisms from animal sources (Negligible, Low, Medium, High)

	Negligible	Low	Medium	High
Exposure				

7.2 Detailed Risk Assessment - Impact characterisation

Impact characterisation provides an assessment of infections occurring in susceptible humans caused by bacteria with high-level tigecycline resistance (or the transfer of the resistance determinants themselves) arising from the use of oxytetracycline in animals, but more specifically in non-Salmonid finfish aquaculture species. Whilst oxytetracycline is not used in humans, the majority of identified resistance genes also impart cross resistance to doxycycline and minocycline. These both have a low ASTAG importance rating. Tigecycline has a high ASTAG importance rating and is one of the only available drugs for treating carbapenem-resistant *Acinetobacter* and Enterobacteriale infections worldwide. Therapeutic Guidelines state that data show that outcomes are worse with tigecycline compared with other antimicrobials, however, and it is therefore not recommended as a first-line treatment for severe infection (Alam and Bastakoti 2015). Whilst tigecycline resistance mediated by *tet*(X) variants is increasingly being isolated from animals in China, actual cases of infection in humans caused by strains that are resistant to colistin, carbapenems and tigecycline are still comparatively rare (Wang *et al.* 2018).

Dose-response analysis

The identified hazard is for mobilizable *tet*(X3) and *tet*(X4) gene variants, selected through oxytetracycline use in the aquaculture industry, to be selected and amplified in either: a) fish pathogens; b) gut commensals of treated fish; and/or c) the environment. These genes would then need to be acquired by human pathogens, with the most significant being Gram-negative *E. coli* and *Acinetobacter* spp. causing sepsis and other severe morbidities, particularly those strains that are also co-resistant to other critically important antimicrobials. The genes would need to be co-located on a mobile genetic element, such as a plasmid, to ensure that the clinical isolate acquired resistance to all possible choices for treatment in a single genetic event. In cases of sepsis, empirical treatment would be administered whilst awaiting the results of culture and sensitivity, and poor response to treatment would occur, resulting in increased risk of mortality or co-morbidity. Given tigecycline's importance rating in Australia, it is likely to be used on the basis of culture and susceptibility testing or if there is a poor response to treatment with a carpapenem (Alam and Bastakoti, 2015). Whilst the impact (response) is high, the frequency and magnitude of exposure is negligible.

Severity, morbidity and mortality of antibiotic-resistant diseases

The AGAR provides prevalence rates, antibiotic susceptibility and major resistance genes identified in Gram-negative pathogens causing sepsis in Australia. In the 2019 data, *E. coli* represented approximately 55%, *Klebsiella* spp. approximately 18% and *Acinetobacter baumannii* approximately 0.7% of infections. 30-day all-cause mortality data indicate 12.2% of Gram-negative sepsis infections resulted in death, with the mortality rate for these three pathogens ranging from 10.6-13.4%. Examining *E. coli* in more detail, whilst prevalence of resistance to fluoroquinolones (10.4-20%) and third-generation cephalosporins (7-16.9%) varied between Australian states, resistance to carbapenems peaked at 0.23% in South Australia, indicating extremely low levels of resistance to one

of the most critically important drug classes. Tigecycline resistance rates were not reported, so are considered to approach zero. These data indicate that there is limited risk of tigecycline resistance in the most significant Gram-negative sepsis pathogens causing significant morbidity or mortality in Australian patients.

Expected numbers of infections and deaths

The expected number of infections and deaths in Australia resulting from oxytetracycline use in non-Salmonid finfish species is nil. A very small number of zoonotic infections caused by fish spike or similar injuries are likely to occur each year associated with finfish aquaculture are likely to occur each year and some of these could be fatal (e.g. *S. iniae*, *P. damselae*), but tetracycline class drugs are not used in their treatment in humans, therefore oxytetracycline resistance is unlikely to affect their outcome.

Impact on health and quality of life

Health and quality of life are unlikely to be affected given that tigecycline is likely to be used only in rare cases of carbapenem resistance in Gram-negative blood sepsis pathogens, or specifically on the results of culture and susceptibility testing. Rates of carbapenem resistance in Australia remain very low.

Table 6 Probability of antibiotic-resistant infection development in susceptible humans (negligible, low, medium or high).

	Negligible	**Low**	**Medium**	**High**
Impact				

Assessment of the uncertainty of the data used in the risk assessment

Data on oxyetracycline resistance in aquaculture pathogens in Australia are poor because of limited cases requiring investigation, due to there being few non-Salmonid farmed fish produced and the relative rarity of disease outbreaks in them. A current Fisheries Research and Development Corporation (FRDC) funded project in collaboration with Queensland Department of Agriculture and Fisheries will undertake MIC testing of *S. iniae, E. piscicida,* and *Aeromonas* spp. isolated from farmed barramundi. A detailed background of the Australian Aquaculture industry with respect to antimicrobial use, antimicrobial susceptibility testing and antimicrobial resistance was provided in a recent Department of Health Review of published and grey literature on the presence of antimicrobial resistance in food in Australia and New Zealand (Australian Department of Health 2018). Because the appropriate section of that report was written by the first author, with extensive contributions from Dr Matt Landos and Dr Jeremy Carson, a full excerpt is included below, to provide important information not currently available in other literature sources.

Australian aquaculture production has continued to grow in volume and gross value over the past decade. The sector includes the propagation of over 40 species of aquatic animals including shellfish (e.g. oysters, mussels), a variety of fresh and saltwater fish (e.g. salmon, tuna, barramundi, perch, trout, kingfish, cobia and cod), prawns, abalone and saltwater crocodiles (Hayakijkosol *et al.* 2017).

Total aquaculture gross value of production in 2015/16 was $1.3 billion, with salmonids the dominant sector contributing $718 million of this value. In 2015/16, 56,300 tonnes of seafood was produced through aquaculture, which was twice as much as that produced during 2005/06 (Mobsby and Koduah 2017). Aquaculture's share of total fishery and aquaculture production value increased from 34% in 2005/06 to 43% in 2015/16 (Australian Bureau of Agricultural and Resource Economics and Sciences 2017). Further investment since 2015/16 is expected to see volumes and value of aquaculture production in Australia continue to grow.

Consumption of seafood among Australians has remained at approximately 15 kg/person/year for the decade leading up to 2015/16 (Australian Bureau of Agricultural and Resource Economics and Sciences 2017). This risk assessment was based on current consumption of seafood by the Australian public. At present, the bulk of seafood is cooked prior to consumption, however, there is a trend for increasing consumption of uncooked seafood, such as sashimi in Japanese restaurants. This change in food preparation has the potential to alter risks for transfer of microbes and their AMR from seafood to humans.

Many of the microbial food safety risks associated with seafood consumption relate to seafood processing and handling, such as listeriosis, rather than the organisms associated with growing the seafood. Antibiotic use is generally very low relative to volumes of production. Where used, antibiotics are for control of clinical diseases. Antimicrobials are not used for growth promotion in Australian aquaculture industries. Antibiotic stewardship within the aquaculture industry is generally good across larger suppliers who utilise industry veterinarians to ensure that usage conforms to appropriate use guidelines, as have been developed for terrestrial food production animals. Practices include ensuring appropriate investigations are made to determine if the cause of disease is bacterial, and using diagnostic laboratories to confirm pathogen identity and carry out antimicrobial sensitivity testing. Some laboratories offer MIC testing, others offer disc diffusion test methods. Given there are no clinical breakpoints available for aquaculture pathogens, epidemiological cut-off values are used as an indication of isolate susceptibility and the development of resistance.

Veterinary advice is also provided on whether husbandry or infrastructure changes could assist in limiting or preventing future bacterial disease outbreaks and thereby avoid the use of antibiotics. Where serious bacterial pathogens emerge, finfish industries are encouraged to invest in vaccine development. FRDC has supported several vaccination projects over the past decade as aquaculture production has expanded, and has recently coinvested with Tasmania's salmon industry in the establishment of the Centre for Aquatic Animal Health and Vaccines.

Antimicrobial use in aquaculture is undertaken with a prescription from a registered veterinarian. Prescriptions include advice on appropriate product withholding periods to ensure products with unacceptable adverse residues are not available for human consumption. Food Standards Australia and New Zealand (FSANZ) has a temporary MRL for oxytetracycline in fish of 0.2mg/kg. No other antimicrobials carry an MRL for any seafood commodity, so from a food safety perspective, antibiotics are not permitted in seafood at levels above the limit of laboratory detection.

There are no fully registered antimicrobial products currently available for use in any of the aquaculture sectors. The salmon industry has a Minor Use Permit for oxytetracycline to control some bacterial diseases. Historically other finfish enterprises have had Minor Use Permits also covering oxytetracycline. The National Aquaculture Council has assisted finfish industries (other than salmon) to get the Minor Use Permit re-issued. For other antibiotics, these are made available through off-label provisions through legislation of state jurisdictions regarding the use of veterinary medicines.

Other than oxytetracycline, antibiotics such as trimethoprim and potentiated sulphonamides are used at times on salmon. The development of locally produced efficacious vaccines for major endemic diseases has led to a marked reduction in antimicrobial usage by the Tasmanian salmon industry

(Carson 2017). For the period 2010-17 antibiotic use was <5 g/t of salmon produced and the industry introduced a self-imposed ban in 2003 on using oxolinic acid.

Antibiotic use is uncommon in the trout farming industry. Where required, it is based on laboratory diagnosis and under the guidance of veterinary prescription. The largest farms participate annually in the National Residue Survey. They have a record of freedom from antibiotic residues in their harvest product.

The Australian Barramundi Farmers' Association members participate in an accreditation and certification scheme that requires antibiotic use to be minimised and where use occurs, it is uniformly under the prescription and guidance of a registered veterinarian. No prophylactic use occurs. The industry participates in national testing schemes for antimicrobials and has demonstrated freedom from residues in its products annually (Anon 2017).

7.3 Benefits of use of the antibiotic in Australian animal health

In finfish aquaculture, oxytetracycline is the most widely used antimicrobial, having a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, excellent distribution into tissues and no safety concerns. The aquaculture industry and the Subcommittee for Aquatic Animal Health have emphasized that access to this product for non-salmonid finfish is a priority. Bacterial diseases in finfish aquaculture are increasingly managed by eradication, maintenance of animals of specified health status, vaccination, biosecurity, and hygiene, but antimicrobial therapy remains vital, in particular for outbreaks of mortality in juvenile fish. Use of oxytetracycline is not intended for prophylactic treatment, but to manage disease outbreaks via metaphylaxis. Use in Australia is sporadic, and the aquaculture industry is committed to Australia's National Antimicrobial Strategy by limiting use to cases of absolute necessity. Appropriate use of antimicrobials cures sick animals and speeds recovery, improving welfare and reducing the spread of infection. A lack of access to an appropriate antimicrobial can cost an individual farm up to $10M, undermine viability and destabilise fish supply for distributors, restaurants and retail consumers.

The beneficiaries of the oxytetracycline minor use permit for non-Salmonid finfish are mainland fish farmers, their veterinary consultants, the seafood distribution and retail sectors, and consumers.

The risk of infection by antibiotic-resistant bacteria is borne mainly by aquaculture and fish processing workers who may acquire wound infections from handling fish and are at risk of acquiring zoonotic infections caused by a variety of fish pathogens. First- or second-generation tetracyclines are not typically used in humans to treat these infections and the risk of acquiring a tigecycline-resistant infection is negligible in these workers. Immunosuppressed individuals are discouraged from consuming raw seafood products and the perceived risk to the healthy seafood consumer of acquiring tigecycline-resistant infections from the use of oxytetracycline in Australian finfish is negligible.

Access to this product is critical for the aquaculture industry, while oxytetracycline is of low priority for human health. Whilst high-level tigecycline-resistant bacteria are increasingly being isolated from mainland China from a variety of sources (animal, human, environment), actual human infection with strains of bacteria that have acquired resistance to all last-line critically important antimicrobials (fluoroquinolones, carbapenems, colistin, as well as tigecycline) are still comparatively rare. Nevertheless, co-location of the genes encoding resistance on mobile genetic elements suggests that acquisition by human pathogens under antimicrobial selection pressure is highly probable in the future. Pigs and poultry represent the main sources of the *tet*(X) variants encoding high-level tigecycline resistance, with aquaculture a minor source more likely to be affected by mixing farming systems (e.g. duck farming and aquaculture). These dual agriculture production systems are not practiced in Australia and to date, no examples of tigecycline resistance have been reported in human or aquaculture pathogens, other food animal or wild animal species, or the environment within Australia. Risk of entry of tigecycline-resistant bacteria into Australia is much more likely to occur from

returned travelers visiting countries where prevalence is high (e.g. mainland China) than arising directly from oxytetracycline use in Australian finfish.

Risk characterization

Given the hazard is classed as Medium, the exposure as Negligible and the impact as Low, the overall likelihood of tigecycline-resistant bacteria arising directly from oxytetracycline use in non-Salmonid infections in aquaculture systems AND affecting human health is low. The degree of risk is far greater (but still relatively low) for humans succumbing to infection by multidrug-resistant phenotypes (e.g. blood sepsis isolates resistant to fluoroquinolones, third generation cephalosporins, carbapenems, and tigecycline) that does not respond to empirical antimicrobial treatments acquired from international travel than arising via selection pressure from oxytetracycline use in finfish species or even terrestrial food-producing animals.

Table 7 Summary of the antimicrobial resistance risk assessment

	Negligible	**Low**	**Medium**	**High**
Hazard				
Exposure				
Impact				
OVERALL RISK				

7.4 Recommendation

Risk is substantially less than the benefit resulting from the approval of an oxytetracycline minor use permit for the industry. Approval of a minor use permit to treat sick non-Salmonid finfish in Australia with oxytetracycline in the feed is not likely to result in any harm to public health. It is recommended, however, that tetracycline resistance be regularly monitored through the collation of diagnostic reports or similar, and any change in MIC or disc diffusion diameter indicating the possible presence of high-level resistance be investigated to identify the putative genes encoding such resistance, thus ensuring that the industry remains free of *tet*(X) genes.

7.5 References

Agnew W, Barnes AC (2007) *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. *Veterinary microbiology* 122, 1-15.

Akinbowale OL, Peng H, Barton M (2006) Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *Journal of applied microbiology* 100, 1103-1113.

Akinbowale OL, Peng H, Barton M (2007) Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *Journal of applied microbiology* 103, 2016-2025.

Akiyama K, Hirazawa N, Hatanaka A (2020) Coadministration of citric acid allows oxytetracycline dose reduction in yellowtail *Seriola quinqueradiata* infected with *Vibrio anguillarum*. *Diseases of Aquatic Organisms* 140, 25-29.

Akram A, Stevens RP, Konecny P (2015) *Photobacterium damselae* and *Vibrio harveyi* hand infection from marine exposure. *Med J Aust* 203, 224-5.

Alam M, Bastakoti B (2015) Therapeutic Guidelines: Antibiotic. Version 15. *Australian prescriber* 38, 137.

Aminov R (2021) Acquisition and Spread of Antimicrobial Resistance: A tet (X) Case Study. *International Journal of Molecular Sciences* 22, 3905.

Anon (2017) Animal product residue testing. Available online. URL: https://www.agriculture.gov.au/ag-farm-food/food/nrs/animal-residue-monitoring Accessed June 30 2021.

Athamna A, Athamna M, Medlej B, Bast D, Rubinstein E (2004) In vitro post-antibiotic effect of fluoroquinolones, macrolides, β-lactams, tetracyclines, vancomycin, clindamycin, linezolid, chloramphenicol, quinupristin/dalfopristin and rifampicin on *Bacillus anthracis*. *Journal of Antimicrobial Chemotherapy* 53, 609-615.

Australian Bureau of Agricultural and Resource Economics and Sciences (2017) Australia's consumption of seafood. Department of Agriculture, Water and the Environment, ABARES.

Australian Bureau of Agricultural and Resource Economics and Sciences (2018) Australian fisheries and aquaculture production 2018. Department of Agriculture, Water and the Environment, ABARES.

Australian Department of Health (2018) Review of published and grey literature on the presence of antimicrobial resistance in food in Australia and New Zealand. University of Adelaide and the New Zealand Food Safety Science and Research Centre.

Björklund H, Bylund G (1991) Comparative pharmacokinetics and bioavailability of oxolinic acid and oxytetracycline in rainbow trout (*Oncorhynchus mykiss*). *Xenobiotica* 21, 1511-1520.

Bromage E, Thomas A, Owens L (1999) Streptococcus iniae, a bacterial infection in barramundi *Lates calcarifer*. *Diseases of aquatic organisms* 36, 177-181.

Bromage Ea, Owens L (2002) Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: effects of different routes of exposure. *Diseases of aquatic organisms* 52, 199-205.

Carson J (2017) Salmonid farming in Tasmania, Health and Disease Management. In 'The 4th Fisheries Research & Development Corporation, Australasian Scientific Conference on Aquatic Animal Health & Biosecurity'. Cairns, Qld

Creeper J, Buller N (2006) An outbreak of Streptococcus iniae in barramundi (*Lates calcarifer*) in freshwater cage culture. *Australian veterinary journal* 84, 408-411.

Cui C-Y, Chen C, Liu B-T, He Q, Wu X-T, Sun R-Y, Zhang Y, Cui Z-H, Guo W-Y, Jia Q-l (2020) Co-occurrence of plasmid-mediated tigecycline and carbapenem resistance in *Acinetobacter* spp. from waterfowls and their neighboring environment. *Antimicrobial agents and chemotherapy* 64, e02502-19.

Declercq AM, Tilleman L, Gansemans Y, De Witte C, Haesebrouck F, Van Nieuwerburgh F, Smet A, Decostere A (2021) Comparative genomics of Flavobacterium columnare unveils novel insights in virulence and antimicrobial resistance mechanisms. *Veterinary research* 52, 1-13.

Deng M, Zhu M-H, Li J-J, Bi S, Sheng Z-K, Hu F-S, Zhang J-J, Chen W, Xue X-W, Sheng J-F (2014) Molecular epidemiology and mechanisms of tigecycline resistance in clinical isolates of *Acinetobacter baumannii* from a Chinese university hospital. *Antimicrobial agents and chemotherapy* 58, 297-303.

Dong H, Techatanakitarnan C, Jindakittikul P, Thaiprayoon A, Taengphu S, Charoensapsri W, Khunrae P, Rattanarojpong T, Senapin S (2017) *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia, *Oreochromis niloticus* (L.). *Journal of Fish Diseases* 40, 1395-1403.

Fang LX, Chen C, Cui CY, Li XP, Zhang Y, Liao XP, Sun J, Liu YH (2020) Emerging High-Level Tigecycline Resistance: Novel Tetracycline Destructases Spread via the Mobile Tet (X). *BioEssays* 42, 2000014.

Final assessment report; Application A608 (2008) Maximum residue limits – oxytetracycline (antibiotic). Available online. URL: https://www.foodstandards.gov.au/code/applications/documents/FAR_A608_MRLs_OTC.pdf Accessed June 30 2021.

Fu Y, Chen Y, Liu D, Yang D, Liu Z, Wang Y, Wang J, Wang X, Xu X, Li X (2021) Abundance of tigecycline resistance genes and association with antibiotic residues in Chinese livestock farms. *Journal of Hazardous Materials* 409, 124921.

Garrison MW, Nuemiller JJ (2007) In vitro activity of tigecycline against quinolone-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. *International journal of antimicrobial agents* 29, 191-196.

Grondel J, Nouws J, Schutte A, Driessens F (1989) Comparative pharmacokinetics of oxytetracycline in rainbow trout (Salmo gairdneri) and African catfish (*Clarias gariepinus*). *Journal of Veterinary Pharmacology and Therapeutics* 12, 157-162.

Haenen O, Evans J, Berthe F (2013) Bacterial infections from aquatic species: potential for and prevention of contact zoonoses. *Revue scientifique et technique (International Office of Epizootics)* 32, 497-507.

Hayakijkosol O, Owens L, Picard J (2017) Case report of bacterial infections in a redclaw crayfish (*Cherax quadricarinatus*) hatchery. *Aquaculture* 475, 1-7.

He T, Wei R-C, Zhang L, Gong L, Zhu L, Gu J, Fu Y-L, Wang Y, Liu D-J, Wang R (2021) Dissemination of the tet (X)-variant genes from layer farms to manure-receiving soil and corresponding lettuce. *Environmental Science & Technology* 55, 1604-1614.

Herrero-Fresno A, Zachariasen C, Hansen MH, Nielsen A, Hendriksen RS, Nielsen SS, Olsen JE (2016) Apramycin treatment affects selection and spread of a multidrug-resistant *Escherichia coli* strain able to colonize the human gut in the intestinal microbiota of pigs. *Veterinary research* 47, 1-10.

Higuera-Llantén S, Vásquez-Ponce F, Barrientos-Espinoza B, Mardones FO, Marshall SH, Olivares-Pacheco J (2018) Extended antibiotic treatment in salmon farms select multiresistant gut bacteria with a high prevalence of antibiotic resistance genes. *PLoS One* 13, e0203641.

Huang L, Xu Y-B, Xu J-X, Ling J-Y, Chen J-L, Zhou J-L, Zheng L, Du Q-P (2017) Antibiotic resistance genes (ARGs) in duck and fish production ponds with integrated or non-integrated mode. *Chemosphere* 168, 1107-1114.

Lee HJ, Hoel S, Lunestad BT, Lerfall J, Jakobsen A (2021) *Aeromonas* spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance. *Journal of Applied Microbiology* 130, 1380-1393.

Legrand TP, Catalano SR, Wos-Oxley ML, Wynne JW, Weyrich LS, Oxley AP (2020) Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota. *Animal Microbiome* 2, 1-16.

Lehane L, Rawlln GT (2000) Topically acquired bacterial zoonoses from fish: a review. *Medical Journal of Australia* 173, 256-259.

Li R-Q, Ren Y-W, Li J, Huang C, Shao J-h, Chen X-X, Wu Z-X (2015) Comparative pharmacokinetics of oxytetracycline in blunt-snout bream (*Megalobrama amblycephala*) with single and multiple-dose oral administration. *Fish physiology and biochemistry* 41, 803-809.

Livermore DM (2005) Tigecycline: what is it, and where should it be used? *Journal of Antimicrobial Chemotherapy* 56, 611-614.

Michel C, Matte-Tailliez O, Kerouault B, Bernardet JF (2005) Resistance pattern and assessment of phenicol agents' minimum inhibitory concentration in multiple drug resistant Chryseobacterium isolates from fish and aquatic habitats. *Journal of applied microbiology* 99, 323-332.

Miller RA, Pelsor FR, Kane AS, Reimschuessel R (2012) Oxytetracycline pharmacokinetics in rainbow trout during and after an orally administered medicated feed regimen. *Journal of aquatic animal health* 24, 121-128.

Mobsby D, Koduah A (2017) Australian fisheries and aquaculture statistics 2016. C ABARES.

Mohsin M, Hassan B, Martins WM, Li R, Abdullah S, Sands K, Walsh TR (2021) Emergence of plasmid-mediated tigecycline resistance tet (X4) gene in Escherichia coli isolated from poultry, food and the environment in South Asia. *Science of The Total Environment* 787, 147613.

Mukerji S, Gunasekera S, Dunlop JN, Stegger M, Jordan D, Laird T, Abraham RJ, Barton M, O'Dea M, Abraham S (2020) Implications of foraging and interspecies interactions of birds for carriage of Escherichia coli strains resistant to critically important antimicrobials. *Applied and environmental microbiology* 86, e01610-20.

Mukerji S, Stegger M, Truswell AV, Laird T, Jordan D, Abraham RJ, Harb A, Barton M, O'Dea M, Abraham S (2019) Resistance to critically important antimicrobials in Australian silver gulls (Chroicocephalus novaehollandiae) and evidence of anthropogenic origins. *Journal of Antimicrobial Chemotherapy* 74, 2566-2574.

Nelson ML, Levy SB (2011) The history of the tetracyclines. *Annals of the New York Academy of Sciences* 1241, 17-32.

Noviello S, Ianniello F, Leone S, Fiore M, Esposito S (2008) In vitro activity of tigecycline: MICs, MBCs, time-kill curves and post-antibiotic effect. *Journal of Chemotherapy* 20, 577-580.

Pujalte M, Sitjà-Bobadilla A, Álvarez-Pellitero P, Garay E (2003) Carriage of potentially fish-pathogenic bacteria in *Sparus aurata* cultured in Mediterranean fish farms. *Diseases of aquatic organisms* 54, 119-126.

Randall L, Woodward MJ (2002) The multiple antibiotic resistance (mar) locus and its significance. *Research in veterinary science* 72, 87-93.

Rigos G, Nengas I, Alexis M, Athanassopoulou F (2004a) Bioavailability of oxytetracycline in sea bass, Dicentrarchus labrax (L.). *Journal of fish diseases* 27, 119-122.

Rigos G, Nengas I, Alexis M, Troisi GM (2004b) Potential drug (oxytetracycline and oxolinic acid) pollution from Mediterranean sparid fish farms. *Aquatic Toxicology* 69, 281-288.

Rivas AJ, Lemos ML, Osorio CR (2013) *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. *Frontiers in microbiology* 4, 283.

Sánchez-Baena AM, Caicedo-Bejarano LD, Chávez-Vivas M (2021) Structure of Bacterial Community with Resistance to Antibiotics in Aquatic Environments. A Systematic Review. *International journal of environmental research and public health* 18, 2348.

Shao S, Wu X (2020) Microbial degradation of tetracycline in the aquatic environment: a review. *Critical Reviews in Biotechnology* 40, 1010-1018.

Shen X, Jin G, Zhao Y, Shao X (2020) Prevalence and distribution analysis of antibiotic resistance genes in a large-scale aquaculture environment. *Science of The Total Environment* 711, 134626.

Sun J, Chen C, Cui C-Y, Zhang Y, Liu X, Cui Z-H, Ma X-Y, Feng Y, Fang L-X, Lian X-L (2019) Plasmid-encoded tet (X) genes that confer high-level tigecycline resistance in Escherichia coli. *Nature microbiology* 4, 1457-1464.

Swann M, Blaxter K, Field K, Hour J, Lucas I, Miller E, Murdoch J, Parsons J, White E (1969) Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and. *Veterinary Medicine. London: Her Majesty's Stationery*.

Thaker M, Spanogiannopoulos P, Wright GD (2010) The tetracycline resistome. *Cellular and Molecular Life Sciences* 67, 419-431.

Umar Z, Chen Q, Tang B, Xu Y, Wang J, Zhang H, Ji K, Jia X, Feng Y (2021) The Poultry Pathogen Riemerella anatipestifer Appears as a Reservoir for Tet (X) Tigecycline Resistance. *Environmental Microbiology*.

Wahli T, Madsen L (2018) Flavobacteria, a never ending threat for fish: a review. *Current Clinical Microbiology Reports* 5, 26-37.

Wang Q, Zhang P, Zhao D, Jiang Y, Zhao F, Wang Y, Li X, Du X, Yu Y (2018) Emergence of tigecycline resistance in Escherichia coli co-producing MCR-1 and NDM-5 during tigecycline salvage treatment. *Infection and drug resistance* 11, 2241.

Wang Y, Lyu N, Liu F, Liu WJ, Bi Y, Zhang Z, Ma S, Cao J, Song X, Wang A (2021) More diversified antibiotic resistance genes in chickens and workers of the live poultry markets. *Environment International* 153, 106534.

Xiong W, Sun Y, Zhang T, Ding X, Li Y, Wang M, Zeng Z (2015) Antibiotics, antibiotic resistance genes, and bacterial community composition in fresh water aquaculture environment in China. *Microbial ecology* 70, 425-432.

Xu C, Lv Z, Shen Y, Liu D, Fu Y, Zhou L, Liu W, Chen K, Ye H, Xia X (2020) Metagenomic insights into differences in environmental resistome profiles between integrated and monoculture aquaculture farms in China. *Environment International* 144, 106005.

Zhou L, Limbu SM, Qiao F, Du Z-Y, Zhang M (2018) Influence of long-term feeding antibiotics on the gut health of zebrafish. *Zebrafish* 15, 340-348.

Discussion and conclusion

An APVMA PAA was assembled and submitted on 13/12/2018, with the assessment notice received on 13/02/19. Further dialogue with APVMA over the following months clarified aspects of the PAA that were unclear and confirmed the data module requirements for the MUP application. The project team commenced collation and review of publicly available, industry and CCD Animal Health held data to complete the data modules required for the MUP application. The draft MUP application was distributed within the project group in June 2021 for review and finalisation prior to submission, to ensure that the permit details aligned with project objectives and industry requirements. The MUP application was submitted on the APVMA online portal on 30/06/2021. Notice was received from the APVMA on 07/07/2021 stating that the MUP application passed preliminary assessment and will be determined under section 112 of the Agvet Code using modules 1.0, 5.3, 6.3, 7.2, 8.3, 10.1 and 11.1. The assessment period for the application was stated as 16 months, with the current application determination date of 07/11/22, noting however that if the APVMA or another prescribed authority makes a request under section 159 of the Agvet Code, the assessment period could be extended.

The project team will continue to monitor the MUP application and provide further information as required by APVMA.

Implications

This project will facilitate improved access to priority chemical products for the non-salmonid finfish aquaculture industries, improve productivity, fish health, survival and welfare. Food safety of seafood products at market will have standards to ensure that maximum residue limits are not exceeded, and prudent use will protect human health through minimising the risk of transfer of AMR genes from fish pathogens to bacteria of human health relevance. This process ensures safe, efficacious, and sustainable use of chemical products within Australian finfish aquaculture industries.

This project collated and assessed data to ensure appropriate use of OTC for industry staff and fish. These data can be used in an ongoing manner to apply for reissue of this and other antimicrobial permits for finfish aquaculture.

Recommendations

Following approval of the MUP it is recommended that additional safety and efficacy, and residue data should be collected to facilitate variations to the MUP conditions and renewal of the MUP. All oxytetracycline use details should be recorded to assist compliance and permitting. Data should follow the APVMA Data Guidelines and concentrate on obtaining safety and residues data for host species and efficacy against pathogens where these have not been assessed.

Further development

Support should be sought to facilitate safe and legal use of a suite of appropriate products for disease control in aquaculture in Australia. OTC is a broad-spectrum antimicrobial and is useful if administered against susceptible pathogens in the framework of the Australian Veterinary Association Guidance for the Rational Use of Antimicrobials framework. Products that specifically target Gram-positive or Gram-negative bacteria should be prioritised and regulatory permits for their use obtained to avoid over reliance on OTC. Access to bacteriocidal products which kill target bacteria rather than bacteriostatic products which prevent bacterial reproduction but do not kill bacteria present at commencement of treatment would facilitate lower doses with lesser environmental effects, more rapid resolution of disease and improved animal welfare outcomes. Ideally a suite of products would be available with .

Extension and Adoption

Communication with relevant individual industry sectors has been ongoing and successful. We have reported the project outcomes to the Subcommittee for Aquatic Animal health (SCAAH) and its Veterinary Medicines Working Group. State and Territory coordinators will be informed by APVMA of the assessment of the MUP and issue of the permit when it is approved.

Industry will be informed about the project and the permit through the industry representative on SCAAH and the Veterinary Medicines Working Group industry representative, veterinarians and government staff.

The project has been communicated to the Freshwater Aquaculture and Barramundi Grower's associations, Cleanseas Pty Ltd and clients of Fiuture Fisheries Veterinary Service, Panaquatic Health Solutions and the Australian Veterinary Association.

Appendix 1 – CCD Animal Health oxytetracycline Safety Data Sheet

Safety Data Sheet

OXYTETRACYCLINE (OXYTET) HCL

1 IDENTIFICATION

Product identifier	Oxytetracycline (Oxytet) HCL
Other means of identification	No information available.
Recommended use	For the water treatment of diseases associated with OTC sensitive organisms in cattle, pigs and poultry.
Supplier Address	CCD Animal Health Unit 2, 84-92 Barnes Street Tamworth NSW 2340
Phone Fax Email	1300 791 009 1300 798 005 ccdsales@ccdanimalhealth.com.au
Emergency phone number	1300 791 009 (Monday to Friday: 8.30 am – 5.00 pm EST) 0487 777 089

2 HAZARD IDENTIFICATION

Classification	Not classified as hazardous.
Signal word	Not applicable.
Hazard statement	Not applicable.
Symbol	Not applicable.
Prevention	Not applicable.
Response	Not applicable.
Storage	Not applicable.
Disposal	Not applicable.

3 COMPOSITION / INFORMATION ON INGREDIENTS

Name of chemical	CAS number	Concentration
Oxytetracycline Hydrochloride	2058-46-0	>95 %
Ingredients determined to be non-hazardous	Not applicable.	Balance

4 FIRST AID MEASURES

Ingestion	Rinse mouth with water. If swallowed, do NOT induce vomiting. Give a glass of water to drink. Never give anything orally to an unconscious person. If vomiting occurs give more water. Seek medical advice.
Eye contact	If in eyes wash out immediately with water. In all cases of eye contamination it is a sensible precaution to seek medical advice.
Skin contact	If skin or hair contact occurs, remove contaminated clothing and flush skin and hair with running water. If swelling, redness, blistering or irritation occurs seek medical assistance.
Inhalation	Remove victim from exposure, avoid becoming a casualty. Remove contaminated clothing and loosen remaining clothing. Allow victim to assume the most comfortable position and keep warm. Keep at rest until fully recovered. Seek medical attention if effects persist.
First aid facilities	If poisoning occurs, contact a doctor or Poison Information Center (phone Australia 131 126, New Zealand 0800 764 766).
Medical attention and special treatment	Treat symptomatically.

5 FIRE FIGHTING MEASURES

Extinguishing equipment	If material is involved in a fire use water fog (or if unavailable fin water spray), foam, dry agent (carbon dioxide, dry chemical powder).

Appendix 2 – Raw data for freshwater environmental OTC monitoring

Water		OTC mg/L				Sediment		OTC mg/kg		
Time (d)	Site	Replicate				Time (d)	Site	Replicate		
		1	2	3				1	2	3
pre tx	1	nd	nd	nd		pre tx	1	nd	nd	nd
pre tx	2	nd	nd	nd		pre tx	2	nd	nd	nd
0	1	0.712	0.693	0.612		0	1	3.011	2.493	2.458
0	2	0.913	0.513	0.643		0	2	1.98	2.434	2.617
7	1	0.576	0.614	0.628		7	1	2.392	3.1	2.971
7	2	0.645	0.517	0.601		7	2	2.132	2.061	1.991
14	1	0.32	0.412	0.314		14	1	1.492	1.201	1.67
14	2	0.304	0.197	0.384		14	2	1.029	1.145	1.232
28	1	0.131	0.158	0.199		28	1	0.531	0.32	0.691
28	2	0.231	0.297	0.274		28	2	0.801	0.913	0.632
56	1	0.101	d NQ	0.111		56	1	0.213	0.291	0.382
56	2	0.1	0.135	0.124		56	2	0.17	0.361	0.258
112	1	nd	nd	nd		112	1	0.032	0.18	0.099
112	2	nd	nd	nd		112	2	0.15	0.112	0.168

loq 0.05 mg/kg sediment lod 0.01

loq 0.01 mg/L water lod 0.002

tx – treatment
loq – limit of quantification
lod – limit of detection

Appendix 3 – Raw data for marine environmental OTC monitoring

Water		OTC mg/L					Sediment		OTC mg/kg			
Time (d)	Site	Replicate					Time (d)	Site	Replicate			
		1	2	3					1	2	3	
pre tx	1	nd	nd	nd	near		pre tx	1	nd	nd	nd	near
pre tx	2	nd	nd	nd	far		pre tx	2	nd	nd	nd	far
0	1	dNQ	dNQ	dNQ	near		0	1	1.357	0.979	1.68	near
0	2	dNQ	dNQ	dNQ	far		0	2	0.756	0.901	1.001	far
7	1	nd	nd	nd	near		7	1	1.01	1.238	1.657	near
7	2	nd	nd	nd	far		7	2	0.879	0.909	0.785	far
14	1	nd	nd	nd	near		14	1	0.467	0.683	0.798	near
14	2	nd	nd	nd	far		14	2	0.201	0.489	0.301	far
28	1	nd	nd	nd	near		28	1	0.285	0.197	0.322	near
28	2	nd	nd	nd	far		28	2	0.102	0.293	0.099	far
56	1	nd	nd	nd	near		56	1	0.134	0.099	0.136	near
56	2	nd	nd	nd	far		56	2	0.052	0.071	0.05	far
112	1	nd	nd	nd	near		112	1	dNQ	dNQ	dNQ	near
112	2	nd	nd	nd	far		112	2	nd	nd	nd	far

loq 0.05 mg/kg sediment lod 0.01

loq 0.01 mg/L water lod 0.002

tx – treatment
loq – limit of quantification
lod – limit of detection

Appendix 4 – Environmental toxicology data

Toxicity Assessment of AFS Oxytet Soluble

South Australian Research and Development Institute

Test Report

December 2020

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ABN 95 619 426 201

Toxicity Test Report: TR1967/1

Accredited for compliance with ISO/IEC 17025 - Testing

Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	48-hr acute toxicity test using the freshwater cladoceran *Ceriodaphnia dubia*
Test Protocol:	ESA SOP 101 (ESA 2017), based on USEPA (2002) and Bailey *et al*. (2000)
Test Temperature:	The test was performed at 25±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with Dilute Mineral Water (DMW) to achieve the test concentrations. A DMW control was tested concurrently with the sample.
Source of Test Organisms:	ESA Laboratory culture
Test Initiated:	25 November 2020 at 1400h

Sample 9837: *AFS*	*Vacant*	*Vacant*
Concentration (mg/L) / **% Unaffected (Mean ± SD)**		
DMW Control: 100 ± 0.0		
31.3: 100 ± 0.0		
62.5: 85.0 ± 10.0		
125: 0.0 ± 0.0		
250: 0.0 ± 0.0		
500: 0.0 ± 0.0		
1000: 0.0 ± 0.0		
48-hr IC10 = 59.9 (57.19-68.43)mg/L		
48-hr EC50 =79.7 (71.33-88.98)mg/L		
NOEC = 62.5mg/L		
LOEC = 125mg/L		

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean % unaffected	≥90.0%	100%	Yes
Reference Toxicant within cusum chart limits	187.2-220.4mg KCl/L	204.91mg KCl/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

Results are based on the samples in the condition as received by ESA.

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NATA Accredited Laboratory Number: 14709

Citations:

Bailey, H.C., Krassoi, R., Elphick, J.R., Mulhall, A., Hunt, P., Tedmanson, L. and Lovell, A. (2000) Application of *Ceriodaphnia cf. dubia* for whole effluent toxicity tests in the Hawkesbury-Nepean watershed, New South Wales, Australia: method development and validation. *Environmental Toxicology and Chemistry* 19:88-93.

ESA (2017) *SOP 101 – Acute toxicity test using Ceriodaphnia dubia*. Issue No. 10. Ecotox Services Australasia, Sydney, New South Wales.

USEPA (2002) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. 4th Ed.* United States Environmental Protection Agency, Office of Water, Washington DC.

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Toxicity Test Report: TR1967/2

Client:	Sardi	**ESA Job #:**	PR1967
	2 Hamra Avenue West Beach	**Date Sampled:**	10 November 2020
	SA 5024	**Date Received:**	16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	96-hr acute toxicity test using the freshwater shrimp *Macrobrachium australiense*
Test Protocol:	ESA SOP 123 (ESA 2016)
Test Temperature:	The test was performed at 25±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with dilute mineral water (DMW) to achieve the test concentrations. A DMW control was tested concurrently with the sample.
Source of Test Organisms:	Hatchery reared, QLD
Test Initiated:	25 November 2020 at 1730h

Sample 9837: *AFS* **Concentration (mg/L)**	**% Unaffected (Mean ± SD)**	*Vacant*	*Vacant*
DMW Control	95.0 ± 10.0		
31.3	95.0 ± 10.0		
62.5	95.0 ± 10.0		
125	40.0 ± 16.3 *		
250	0.0 ± 0.0		
500	0.0 ± 0.0		
1000	0.0 ± 0.0		
96-hr IC10 = 79.7mg/L**			
96-hr EC50 =118.3 (101.55-137.92)mg/L			
NOEC = 62.5mg/L			
LOEC = 125mg/L			

*Significantly lower percentage of unaffected shrimp compared with the DMW Control (Steel's Many-One Rank Test, 1-tailed, P=0.05)
**The 95% Confidence limits are not reliable

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean % unaffected	≥90.0%	95.0%	Yes
Reference Toxicant within cusum chart limits	35.8-412.6µg Cu/L	111.24µg Cu/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

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Toxicity Test Report: TR1967/2

Citations:

ESA (2016) SOP 123 *–Acute Toxicity Test Using Freshwater Shrimp.* Issue No 4. Ecotox Services Australasia, Sydney, NSW

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Toxicity Test Report: TR1967/3

Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	Rainbowfish embryo hatching test using *Melanotaenia splendida splendida*
Test Protocol:	ESA SOP 126 (2016), based on USEPA (2002), but adapted for use with native rainbowfish
Test Temperature:	The test was performed at 25±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with dilute mineral water (DMW) to achieve the test concentrations. A DMW control was tested concurrently with the sample.
Source of Test Organisms:	ESA Laboratory culture
Test Initiated:	25 November 2020 at 1830h

Sample 9837: *AFS* **Concentration (mg/L)**	**% Unaffected (Mean ± SD)**	*Vacant*	*Vacant*
DMW Control	85.0 ± 19.2		
31.3	90.0 ± 11.6		
62.5	85.0 ± 10.0		
125	70.0 ± 20.0		
250	0.0 ± 0.0		
500	0.0 ± 0.0		
1000	0.0 ± 0.0		
12-d IC10 = 87.2 mg/L*			
12-d EC50 =150.9 (131.92-172.56)mg/L			
NOEC = 125mg/L			
LOEC = 250mg/L			

* The 95% Confidence Limits are not reliable.

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean % unaffected	≥80.0%	85.0%	Yes
Reference Toxicant within cusum chart limit	26.1-153.3µg Cu/L	77.5µg Cu/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

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Toxicity Test Report: TR1967/3

Citations:

ESA (2016) *SOP 126- Rainbowfish Embryo Hatching Test*. Issue N°6. Ecotox Services Australasia, Sydney NSW

USEPA (2002) *Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms.4th Ed.* United States Environmental Protection Agency, Office of Water, Washington DC.

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Toxicity Test Report: TR1967/4

Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	7-day Growth inhibition of the freshwater aquatic duckweed *Lemna disperma*
Test Protocol:	ESA SOP 112 (ESA 2016), based on ASTM (2012)
Test Temperature:	The test was performed at 25±2°C.
Deviations from Protocol:	Test volume reduced from 100ml to 15ml; Fronds per replicate reduced form 12-16 to 3; Replicates per treatment increased from 3 to 4.
Comments on Solution Preparation:	The sample was serially diluted with Swedish standard medium (SIS) to achieve the test concentrations. A SIS control was tested concurrently with the sample.
Source of Test Organisms:	ESA Laboratory culture
Test Initiated:	25 November 2020 at 1900h

Sample 9837: *AFS*	*Vacant*	*Vacant*
Concentration (mg/L) / **Specific Growth Rate (Mean ± SD)**		
SIS Control 0.31 ± 0.01		
31.3 0.31 ± 0.01		
62.5 0.31 ± 0.01		
125 0.31 ± 0.01		
250 0.23 ± 0.02 *		
500 0.00 ± 0.00		
1000 0.00 ± 0.00		
7 day IC10 = 174.8 (146.74-198.68)mg/L **7 day IC50 = 333.07 (307.55-352.43)mg/L** **NOEC = 125mg/L** **LOEC = 250mg/L**		

*Significantly lower specific growth rate compared with the SIS Control (Dunnett's Test, 1-tailed, P=0.05)

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control specific growth rate	>0.275	0.31	Yes
Reference Toxicant within cusum chart limits	3.2-4.2g KCl/L	4.5g KCl/L	No

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

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Toxicity Test Report: TR1967/4

Citations:

ESA (2016) *SOP 112 – Duckweed Growth Inhibition Test.* Issue No. 7. Ecotox Services Australasia, Sydney NSW

ASTM (2012) Designation E1415. Standard Guide for Conducting Static Toxicity Tests with *Lemna gibba* G3

Toxicity Test Report: TR1967/5

Client:	Sardi	**ESA Job #:**	PR1967
	2 Hamra Avenue West Beach	**Date Sampled:**	10 November 2020
	SA 5024	**Date Received:**	16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	72-hr microalgal growth inhibition test using the green alga *Selenastrum capricornutum*
Test Protocol:	ESA SOP 103 (ESA 2016), based on USEPA (2002)
Test Temperature:	The test was performed at 25±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with USEPA media to achieve the test concentrations. A USEPA control was tested concurrently with the sample.
Source of Test Organisms:	ESA Laboratory culture, originally sourced from CSIRO Microalgal Supply Service, TAS
Test Initiated:	25 November 2020 at 1800h

Controls

Control Treatment	**Cell Yield x10^4 cells/mL (Mean ± SD)**
USEPA Control	21.5 ± 1.3
Colour ctrl 250mg/L	21.9 ± 0.7
Colour ctrl 500mg/L	18.3 ± 1.4 *
Colour ctrl 1000mg/L	17.7 ± 2.2 *

Sample 9837: *AFS*

Concentrations (mg/L)	**Cell Yield x10^4 cells/mL (Mean ± SD)**
USEPA Control	21.5 ± 1.3
31.3	21.2 ± 1.2
62.5	22.7 ± 1.1
125	14.4 ± 2.4**
250	10.2 ± 2.7**
500	2.2 ± 1.8**
1000	0.6 ± 0.8**

72-hr IC10 = 80.9 (72.47-96.16)mg/L
72-hr IC50 = 228.3 (161.87-316.55)mg/L
NOEC = 62.5mg/L
LOEC = 125mg/L

*Significantly lower cell yield compared with the USEPA Control (Heteroscedastic t Test, 1-tailed, P=0.05)
**Significantly lower cell yield compared with the USEPA Control (Bonferroni Test, 1-tailed, P=0.05)

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean cell density	≥16.0x10^4 cells/mL	22.5x10^4 cells/mL	Yes
Control coefficient of variation	<20%	6.0%	Yes
Reference Toxicant within cusum chart limits	2.0-5.5g KCl/L	3.2g KCl/L	Yes

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Toxicity Test Report: TR1967/5

Results are based on the samples in the condition as received by ESA.

Citations:

ESA (2016) *ESA SOP 103 – Green Alga, Selenastrum capricornutum, Growth Test*. Issue No 11. Ecotox Services Australasia, Sydney, NSW.

USEPA (2002) *Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms*. Fourth Edition. EPA-821-R-02-013. United States Environmental Protection Agency, Office of Research and Development, Washington DC, USA,

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Toxicity Test Report: TR1967/6

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Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	72-hr sea urchin larval development test using *Heliocidaris tuberculata*
Test Protocol:	ESA SOP 105 (ESA 2016), based on APHA (1998), Simon and Laginestra (1996) and Doyle *et al*. (2003)
Test Temperature:	The test was performed at 20±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with filtered seawater (FSW) to achieve the test concentrations. A FSW control was tested concurrently with the sample.
Source of Test Organisms:	Field collected from South Maroubra, NSW.
Test Initiated:	27 November 2020 at 1730h

Sample 9837: *AFS* **Concentration (mg/L)**	**% Normal larvae (Mean ± SD)**	*Vacant*	*Vacant*
FSW Control	94.8 ± 1.3		
31.3	94.8 ± 1.7		
62.5	94.8 ± 2.8		
125	36.8 ± 7.2 *		
250	0.0 ± 0.0		
500	0.0 ± 0.0		
1000	0.0 ± 0.0		
72-hr IC10 = 78.7 (71.53-82.12)mg/L **72-hr EC50 = 115.7 (111.81-119.62)mg/L** **NOEC = 62.5mg/L** **LOEC = 125mg/L**			

*Significantly lower percentage of normally developed larvae compared with the FSW Control (Dunnett's Test, 1-tailed, P=0.05)

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean % normal larvae	≥70.0%	94.8%	Yes
Reference Toxicant within cusum chart limits	10.6-12.0µg Cu/L	11.28µg Cu/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

Results are based on the samples in the condition as received by ESA.

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Citations:

APHA (1998) Method 8810 D. Echinoderm Embryo Development Test. In Standard Methods for the Examination of Water and Wastewater, 20th Ed. American Public Health Association, American Water Works Association and the Water Environment Federation, USA.

Doyle, C.J., Pablo, F., Lim, R.P. and Hyne, R.V. (2003) Assessment of metal toxicity in sediment pore water from Lake Macquarie, Australia. *Arch. Environ. Contam. Toxicology*, 44(3): 343-350.

ESA (2016) *ESA SOP 105 - Sea Urchin Larval Development Test*. Issue No. 11. Ecotox Services Australasia, Sydney NSW.

Simon, J. and Laginestra, E.(1997) Bioassay for testing sublethal toxicity in effluents, using gametes of sea urchin *Heliocidaris tuberculata*. National Pulp Mills Research Program Technical Report No. 20. CSIRO, Canberra, ACT.

Toxicity Test Report: TR1967/7

Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	96-hr acute toxicity test using the amphipod *Allorchestes compressa*
Test Protocol:	ESA SOP 108 (ESA 2017), based on USEPA (2002) and Department of Transport and Communications (1990)
Test Temperature:	The test was performed at 20±1°C.
Deviations from Protocol:	Nil
Comments on Solution Preparation:	The sample was serially diluted with filtered seawater (FSW) to achieve the test concentrations. A FSW control was tested concurrently with the sample.
Source of Test Organisms:	In-house culture, originally sourced from Queenscliff, VIC
Test Initiated:	27 November 2020 at 1800h

Sample 9837: *AFS*	*Vacant*	*Vacant*
Concentration (mg/L) / **% Unaffected (Mean ± SD)**		
FSW Control: 100 ± 0.0		
31.3: 100 ± 0.0		
62.5: 100 ± 0.0		
125: 100 ± 0.0		
250: 40.0 ± 16.3 *		
500: 0.0 ± 0.0		
1000: 0.0 ± 0.0		
96-hr IC10 = 207.3 (200.0-218.8)mg/L **96-hr EC50 = 233.3 (200.39-271.51)mg/L** **NOEC = 125mg/L** **LOEC = 250mg/L**		

*Significantly lower percent unaffected compared with the FSW Control (Steel's Many-One Rank Test, 1-tailed, P=0.05)

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
Control mean % unaffected	≥90.0%	100%	Yes
Reference Toxicant within cusum chart limits	0.6-10.1mg SDS/L	3.5mg SDS/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

Results are based on the samples in the condition as received by ESA.

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Citations:

Department of Transport and Communications (1990) Guidelines for Acceptance of Oil Spill Dispersants in Australian Waters. Pollution Prevention Section, Department of Transport and Communications, Canberra ACT.

ESA (2017) SOP 108 – *Amphipod Acute Toxicity Test*. Issue No 10. Ecotox Services Australia, Sydney, NSW.

USEPA (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fifth Edition. United States Environmental Protection Agency, Office of Research and Development, Washington DC, EPA/600/4-90/027F.

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Toxicity Test Report: TR1967/8

Client:	Sardi 2 Hamra Avenue West Beach SA 5024	**ESA Job #:** **Date Sampled:** **Date Received:**	PR1967 10 November 2020 16 November 2020
Attention:	Marty Deveney	**Sampled By:**	Client
Client Ref:	Not Supplied	**ESA Quote #:**	PL1967_q01

Lab ID No.:	**Sample Name:**	**Sample Description:**
9837	AFS Oxytet Soluble	Chemical sample, received at room temperature in apparent good condition.

Test Performed:	14-day macroalgal growth test using *Ecklonia radiata*
Test Protocol:	ESA SOP 116 (ESA 2014), based on Bidwell *et al*. (1998) and Burridge *et al.* (1999)
Test Temperature:	The test was performed at 18±1°C.
Deviations from Protocol:	Test extended from 72 hours to 14 days to encompass growth endpoint.
Comments on Solution Preparation:	The sample was serially diluted with filtered seawater (FSW) to achieve the test concentrations. A FSW was tested concurrently with the sample.
Source of Test Organisms:	Field collected from Mercury Passage, TAS
Test Initiated:	27 November 2020 at 1630h

Sample 9837: *AFS*	*Vacant*	*Vacant*
Concentration (mg/L) / **Gametophyte Length, µm (Mean ± SD)**		
FSW Control: 22.5 ± 0.3		
31.3: 22.6 ± 0.3		
62.5: 23.2 ± 0.2		
125: 22.7 ± 0.4		
250: 22.3 ± 0.2		
500: 8.8 ± 0.4 *		
1000: 0.0 ± 0.0		
14-day IC10 = 284.0 (278.6-288.05)mg/L **14-day IC50 = 451.8 (445.74-459.87)mg/L** **NOEC = 250mg/L** **LOEC = 500mg/L**		

*Significantly lower gametophyte length compared with the FSW Control (Steel's Many-One Rank Test, 1-tailed, P=0.05)

QA/QC Parameter	**Criterion**	**This Test**	**Criterion met?**
14-d Reference Toxicant within cusum chart limits	90.3-1157.0µg Cu/L	841.8µg Cu/L	Yes

Test Report Authorised by: Dr Rick Krassoi, Director on 22 December 2020

Results are based on the samples in the condition as received by ESA. This document shall not be reproduced except in full.

Citations:

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Unit 27, 2 Chaplin Drive
Lane Cove NSW 2066 Australia
www.ecotox.com.au
ECOTOX SERVICES AUSTRALIA PTY LIMITED
ABN 95 619 426 201

Toxicity Test Report: TR1967/8

Bidwell, J. R., Wheeler, K. W., & Burridge, T. R. (1998). Toxicant effects on the zoospore stage of the marine maroalga Ecklonia radiata (Phaeophyta:Laminariales). *Marine Ecology Progress Series.Vol 163* , 259-265.

Burridge, T. R., Karistanios, M., & Bidwell, J. (1999). The use of aquatic macrophyte ecotoxicological assays inmonitoring coastal effluent discharges in southern Australia. *Marine Pollution Bulletin. Vol 39* , 1-12.

ESA (2014) *SOP 116 – Macroalgal Germination Success Test.* Issue No. 13. Ecotox Services Australasia, Sydney NSW

Chain-of-Custody Documentation

Chain-of-Custody / Service Request Form

Datasheet ID: 601.1
Last Revised: 20 September 2018

Customer: Marty Deveney, SARDI
Contact Name:
Phone: (08) 8429 0742 Email: Marty.deveney@sa.gov.au (please provide an email address for sample receipt notification)
Sampled by:

Ship To: R. KRASSOI
Attention: Ecotox Serv. Aust.

Sample Date (day/month /year)	Sample Time	Sample Name (exactly as written on the sample vessel)	Sample Method (eg. Grab, composite etc.)	Number and Volume of Containers (eg 2 x 1L)	Tests Requested (See reverse for guidance)						Comments / Instructions
					As per PR1967-901						**Note that testing will be delayed if an incomplete chain of custody is received** • Additional treatment of samples (i.e. spiking) • Sub-contracted services (i.e. chemical analyses) • Dilutions required (if different than 100% down to 6.25%) • Sample holding time restriction (if applicable) • Sample used for litigation (if applicable) *Note: An MSDS must be attached if Available* **ESA Project Number: PR** 1967
Room temp											
9837 10/11/20		AFS Oxytet Soluble	Bag	1 x 500g	✓						

1) Released By:	Date:	2) Received By:	Date: 16/11/20	3) Released By:	Date:	4) Received By:	Date:
Of:	Time:	Of: ESA.	Time: 1130	Of:	Time:	Of:	Time:

Note that the chain-of-custody documentation will provide definitive information on the tests to be performed.

Ecotox Services Australia . Unit 27, 2 Chaplin Drive, Lane Cove NSW 2066 AUSTRALIA
Phone: 61 2 9420-9481 Fax 61 2 9420-9484 info@ecotox.com.au

Statistical Printouts for the Acute Test with *Ceriodaphnia dubia*

Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	1.0000	1.0000	1.0000	1.0000
31.3	1.0000	1.0000	1.0000	1.0000
62.5	1.0000	0.8000	0.8000	0.8000
125	0.0000	0.0000	0.0000	0.0000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

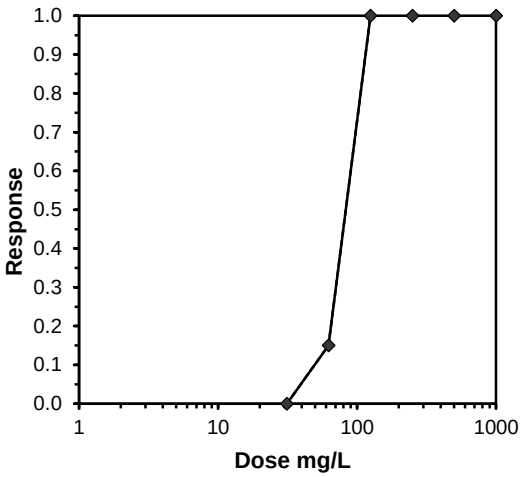
			Transform: Arcsin Square Root					Rank	1-Tailed	Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Resp	Number
DMW Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4			0	20
31.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	11.00	0	20
62.5	0.8500	0.8500	1.1667	1.1071	1.3453	10.206	4	12.00	11.00	3	20
125	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.633513	0.859	2.297825	7.088889
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	62.5	125	88.38835	
Treatments vs DMW Control				

Trimmed Spearman-Karber

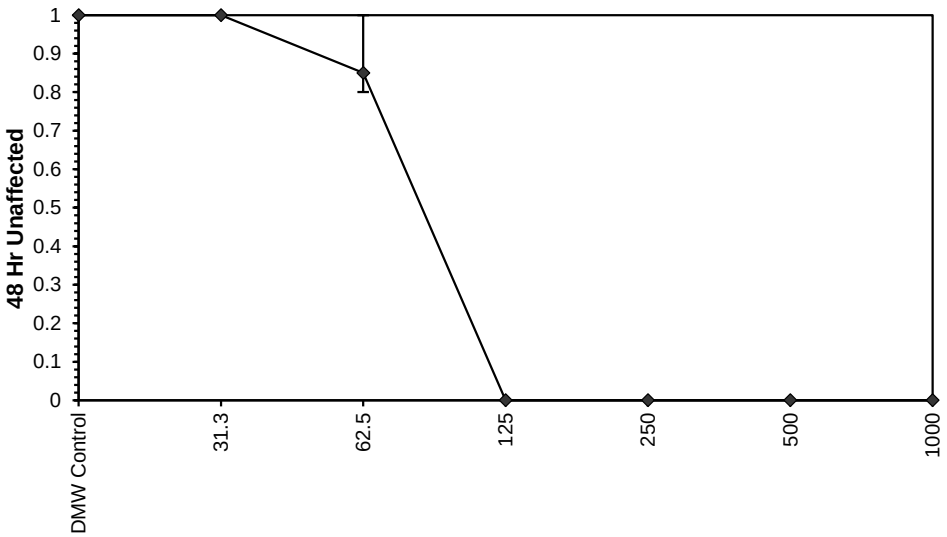
Trim Level	EC50	95% CL	
0.0%	79.670	71.331	88.983
5.0%	81.410	71.525	92.661
10.0%	82.653	69.877	97.765
20.0%	83.145	77.013	89.764
Auto-0.0%	79.670	71.331	88.983



Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Dose-Response Plot



Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% un-immobilised	100.00	100.00	100.00	0.00	0.00	4
31.3		100.00	100.00	100.00	0.00	0.00	4
62.5		85.00	80.00	100.00	10.00	3.72	4
125		0.00	0.00	0.00	0.00		4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	DO %	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1
DMW Control	Cond uS/cm	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1

Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	1.0000	1.0000	1.0000	1.0000
31.3	1.0000	1.0000	1.0000	1.0000
62.5	1.0000	0.8000	0.8000	0.8000
125	0.0000	0.0000	0.0000	0.0000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

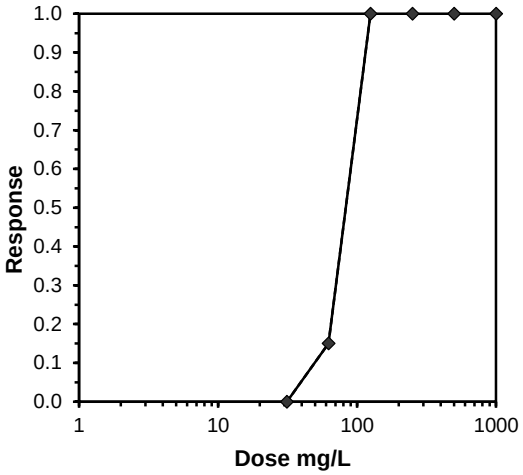
			Transform: Arcsin Square Root					Rank	1-Tailed	Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Mean	N-Mean
DMW Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4			1.0000	1.0000
31.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	11.00	1.0000	1.0000
62.5	0.8500	0.8500	1.1667	1.1071	1.3453	10.206	4	12.00	11.00	0.8500	0.8500
125	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.633513	0.859	2.297825	7.088889
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	62.5	125	88.38835	
Treatments vs DMW Control				

Log-Logit Interpolation (200 Resamples)

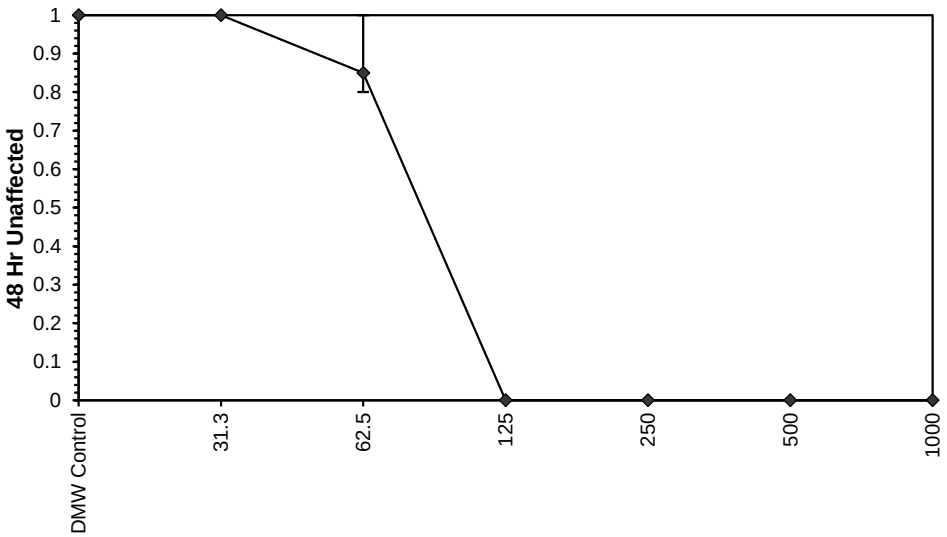
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	55.920	2.693	53.651	66.448	3.6819
IC10	59.898	2.594	57.186	68.432	3.0482
IC15	62.500	2.344	59.488	69.672	3.4979
IC20	63.900	2.176	61.660	70.989	4.1121
IC25	65.079	2.152	62.860	72.095	4.0906
IC40	68.010	2.087	65.847	74.829	4.0394
IC50	69.785	2.045	67.658	76.475	4.0096



Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Dose-Response Plot



Ceriodaphnia Acute Toxicity Test-48 Hr Unaffected

Start Date:	25/11/2020 14:00	Test ID:	PR1967/01	Sample ID:	AFS
End Date:	27/11/2020 14:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 101	Test Species:	CD-Ceriodaphnia dubia
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% un-immobilised	100.00	100.00	100.00	0.00	0.00	4
31.3		100.00	100.00	100.00	0.00	0.00	4
62.5		85.00	80.00	100.00	10.00	3.72	4
125		0.00	0.00	0.00	0.00		4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	DO %	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1
DMW Control	Cond uS/cm	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1

Statistical Printouts for the Acute Freshwater Shrimp Toxicity Test

Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	1.0000	1.0000	1.0000	0.8000
31.3	0.8000	1.0000	1.0000	1.0000
62.5	1.0000	1.0000	0.8000	1.0000
125	0.4000	0.6000	0.2000	0.4000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

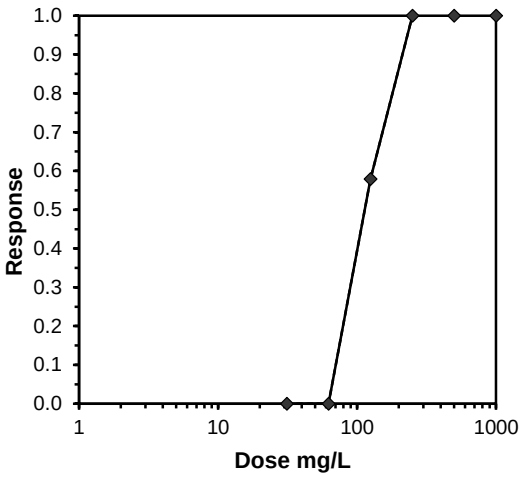
			Transform: Arcsin Square Root					Rank	1-Tailed	Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Resp	Number
DMW Control	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4			1	20
31.3	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4	18.00	10.00	1	20
62.5	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4	18.00	10.00	1	20
*125	0.4000	0.4211	0.6798	0.4636	0.8861	25.383	4	10.00	10.00	12	20
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.780734	0.887	-0.72342	-0.29792
Bartlett's Test indicates equal variances (p = 0.90)	0.605558	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	62.5	125	88.38835	
Treatments vs DMW Control				

Trimmed Spearman-Karber

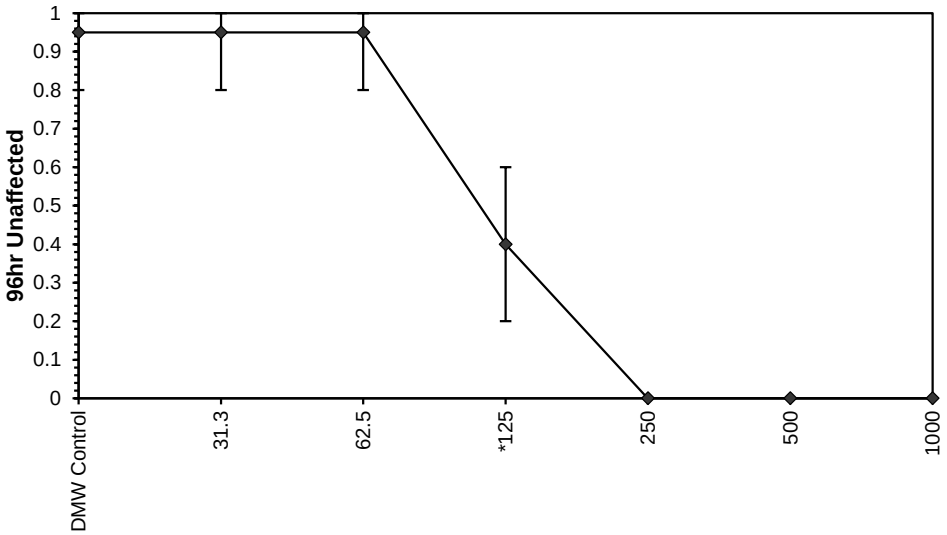
Trim Level	EC50	95% CL	
0.0%	118.34	101.55	137.92
5.0%	117.70	99.35	139.44
10.0%	117.06	96.93	141.38
20.0%	115.82	90.97	147.47
Auto-0.0%	118.34	101.55	137.92



Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Dose-Response Plot



Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% Unaffected	95.00	80.00	100.00	10.00	3.33	4
31.3		95.00	80.00	100.00	10.00	3.33	4
62.5		95.00	80.00	100.00	10.00	3.33	4
125		40.00	20.00	60.00	16.33	10.10	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	Cond uS/cm	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1
DMW Control	DO %	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1

Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	1.0000	1.0000	1.0000	0.8000
31.3	0.8000	1.0000	1.0000	1.0000
62.5	1.0000	1.0000	0.8000	1.0000
125	0.4000	0.6000	0.2000	0.4000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

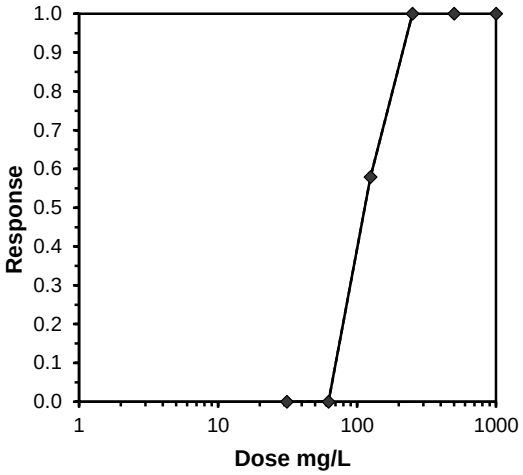
			Transform: Arcsin Square Root					Rank	1-Tailed	Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Mean	N-Mean
DMW Control	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4			0.9500	1.0000
31.3	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4	18.00	10.00	0.9500	1.0000
62.5	0.9500	1.0000	1.2857	1.1071	1.3453	9.261	4	18.00	10.00	0.9500	1.0000
*125	0.4000	0.4211	0.6798	0.4636	0.8861	25.383	4	10.00	10.00	0.4000	0.4211
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.780734	0.887	-0.72342	-0.29792
Bartlett's Test indicates equal variances (p = 0.90)	0.605558	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	62.5	125	88.38835	
Treatments vs DMW Control				

Log-Logit Interpolation (200 Resamples)

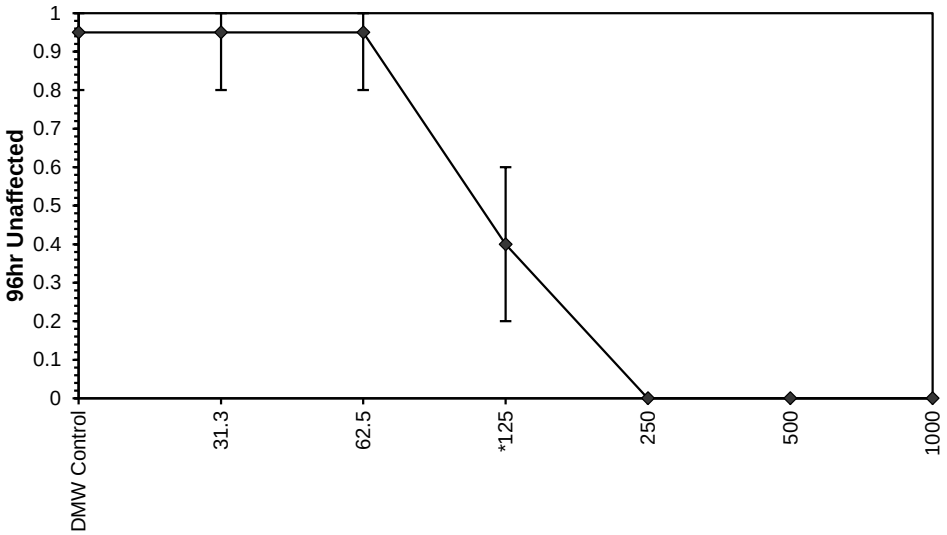
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	72.56	16.95	0.00	81.19	-1.4286
IC10	79.67	10.26	29.06	89.31	-1.5814
IC15	85.49	7.61	48.71	94.87	-0.2279
IC20	90.61	7.14	60.52	101.48	-0.1917
IC25	95.32	6.87	66.12	109.31	-0.1803
IC40	108.47	6.92	85.37	130.14	0.0880
IC50	117.37	6.86	94.58	134.07	-0.2794



Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Dose-Response Plot



Freshwater Shrimp Acute Toxicity Test-96hr Unaffected

Start Date:	25/11/2020 17:30	Test ID:	PR1967/02	Sample ID:	AFS
End Date:	29/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 123	Test Species:	MD-Macrobrachium australiense
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% Unaffected	95.00	80.00	100.00	10.00	3.33	4
31.3		95.00	80.00	100.00	10.00	3.33	4
62.5		95.00	80.00	100.00	10.00	3.33	4
125		40.00	20.00	60.00	16.33	10.10	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	Cond uS/cm	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1
DMW Control	DO %	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1

Statistical Printouts for the Rainbowfish Embryonic Development and Post-hatch Survival Tests

Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	0.8000	0.6000	1.0000	1.0000
31.3	0.8000	0.8000	1.0000	1.0000
62.5	0.8000	0.8000	1.0000	0.8000
125	0.6000	0.6000	0.6000	1.0000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

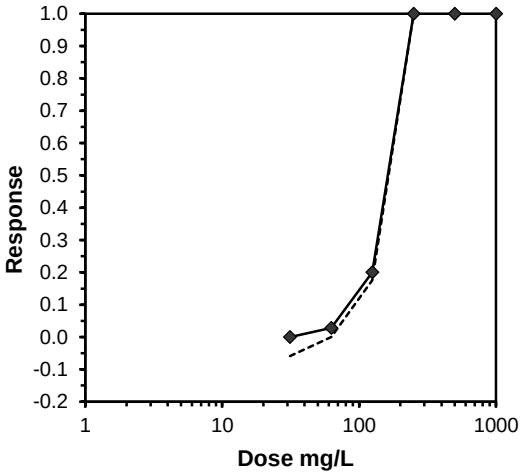
			Transform: Arcsin Square Root					1-Tailed			Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
DMW Control	0.8500	1.0000	1.1709	0.8861	1.3453	18.840	4				3	20
31.3	0.9000	1.0588	1.2262	1.1071	1.3453	11.212	4	-0.426	2.290	0.2969	2	20
62.5	0.8500	1.0000	1.1667	1.1071	1.3453	10.206	4	0.033	2.290	0.2969	3	20
125	0.7000	0.8235	1.0009	0.8861	1.3453	22.940	4	1.312	2.290	0.2969	6	20
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				20	20
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				20	20
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				20	20

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.903524	0.887	0.494016	-0.27871
Bartlett's Test indicates equal variances (p = 0.65)	1.630962	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs DMW Control	125	250	176.7767		0.260267	0.306752	0.037936	0.033617	0.376496	3, 12

Trimmed Spearman-Karber

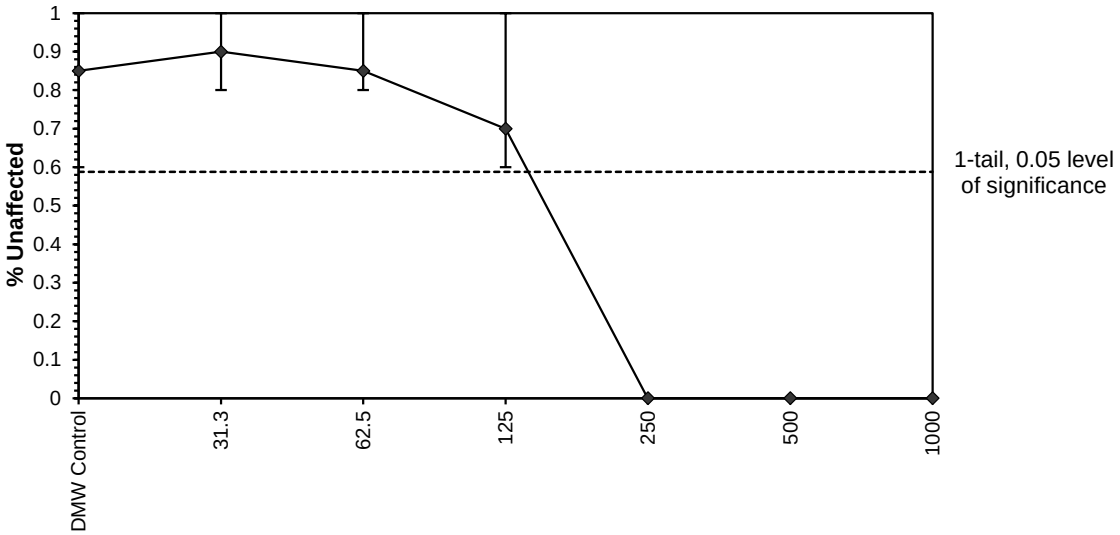
Trim Level	EC50	95% CL	
0.0%	150.88	131.92	172.56
5.0%	155.79	135.40	179.26
10.0%	158.92	134.40	187.91
20.0%	162.10	147.14	178.59
Auto-0.0%	150.88	131.92	172.56



Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Dose-Response Plot



Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% Unaffected	85.00	60.00	100.00	19.15	5.15	4
31.3		90.00	80.00	100.00	11.55	3.78	4
62.5		85.00	80.00	100.00	10.00	3.72	4
125		70.00	60.00	100.00	20.00	6.39	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	Conductivity (uS/cm)	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1
DMW Control	DO (% sat)	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1

Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Conc-mg/L	1	2	3	4
DMW Control	0.8000	0.6000	1.0000	1.0000
31.3	0.8000	0.8000	1.0000	1.0000
62.5	0.8000	0.8000	1.0000	0.8000
125	0.6000	0.6000	0.6000	1.0000
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

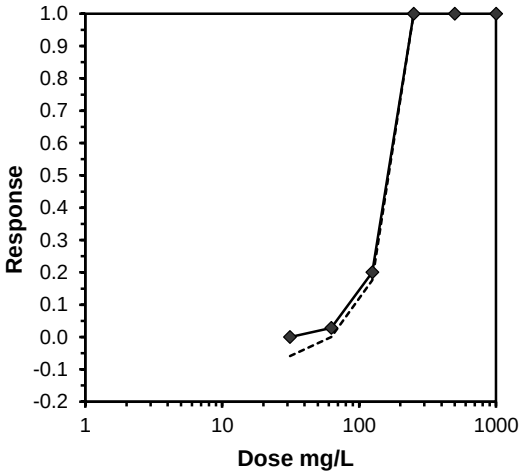
			Transform: Arcsin Square Root					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
DMW Control	0.8500	1.0000	1.1709	0.8861	1.3453	18.840	4				0.8750	1.0000
31.3	0.9000	1.0588	1.2262	1.1071	1.3453	11.212	4	-0.426	2.290	0.2969	0.8750	1.0000
62.5	0.8500	1.0000	1.1667	1.1071	1.3453	10.206	4	0.033	2.290	0.2969	0.8500	0.9714
125	0.7000	0.8235	1.0009	0.8861	1.3453	22.940	4	1.312	2.290	0.2969	0.7000	0.8000
250	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				0.0000	0.0000
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				0.0000	0.0000
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4				0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.903524	0.887	0.494016	-0.27871
Bartlett's Test indicates equal variances (p = 0.65)	1.630962	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs DMW Control	125	250	176.7767		0.260267	0.306752	0.037936	0.033617	0.376496	3, 12

Log-Logit Interpolation (200 Resamples)

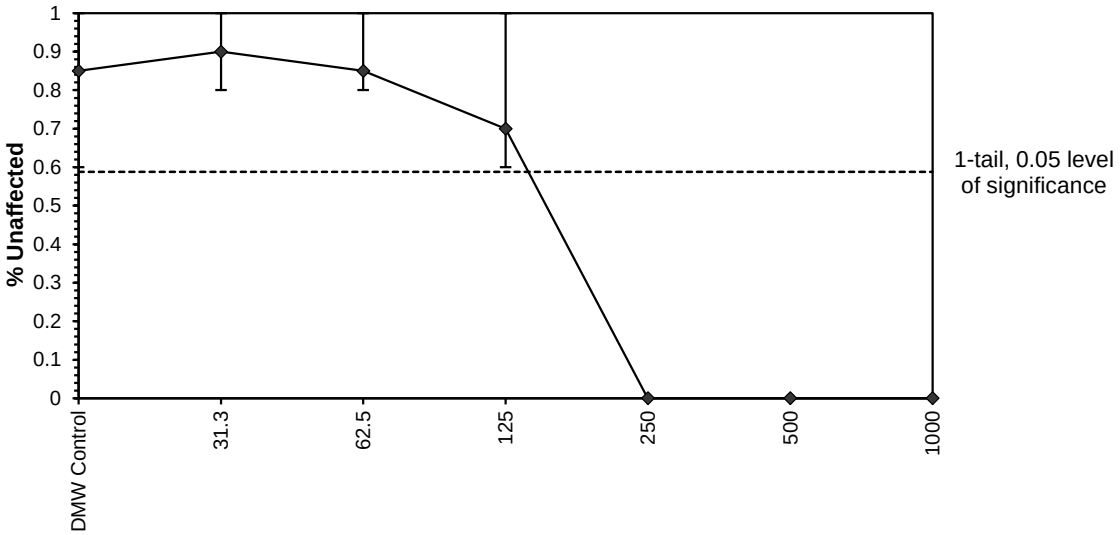
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	69.76	30.71	0.00	160.96	0.1952
IC10	87.15	29.61	0.00	153.13	-0.1598
IC15	105.46	24.09	22.48	144.87	-0.3619
IC20	125.00	18.63	43.01	135.56	-0.6636
IC25	126.74	13.34	64.31	137.34	-0.9346
IC40	131.62	3.69	121.91	142.88	0.3563
IC50	134.85	3.58	125.76	146.26	0.3859



Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Dose-Response Plot



Fish Embryonic Development-% Unaffected

Start Date:	25/11/2020 18:30	Test ID:	PR1967/05	Sample ID:	AFS
End Date:	7/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 126	Test Species:	MS-Melanotaenia splendida
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
DMW Control	% Unaffected	85.00	60.00	100.00	19.15	5.15	4
31.3		90.00	80.00	100.00	11.55	3.78	4
62.5		85.00	80.00	100.00	10.00	3.72	4
125		70.00	60.00	100.00	20.00	6.39	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
DMW Control	pH	8.10	8.10	8.10	0.00	0.00	1
31.3		8.00	8.00	8.00	0.00	0.00	1
62.5		7.70	7.70	7.70	0.00	0.00	1
125		7.20	7.20	7.20	0.00	0.00	1
250		6.70	6.70	6.70	0.00	0.00	1
500		4.20	4.20	4.20	0.00	0.00	1
1000		3.10	3.10	3.10	0.00	0.00	1
DMW Control	Conductivity (uS/cm)	176.00	176.00	176.00	0.00	0.00	1
31.3		179.00	179.00	179.00	0.00	0.00	1
62.5		176.00	176.00	176.00	0.00	0.00	1
125		177.00	177.00	177.00	0.00	0.00	1
250		179.00	179.00	179.00	0.00	0.00	1
500		181.00	181.00	181.00	0.00	0.00	1
1000		185.00	185.00	185.00	0.00	0.00	1
DMW Control	DO (% sat)	99.60	99.60	99.60	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		98.10	98.10	98.10	0.00	0.00	1
125		97.80	97.80	97.80	0.00	0.00	1
250		97.00	97.00	97.00	0.00	0.00	1
500		96.10	96.10	96.10	0.00	0.00	1
1000		70.90	70.90	70.90	0.00	0.00	1

Statistical Printouts for the Duckweed Growth Inhibition Tests

Duckweed Growth Inhibtion Test-Specific Growth Rate

Start Date:	25/11/2020 19:00	Test ID:	PR1967/06	Sample ID:	AFS
End Date:	2/12/2020 19:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 112	Test Species:	LD-Lemna disperma
Comments:					

Conc-mg/L	1	2	3	4
SIS Control	0.2971	0.3191	0.3085	0.3191
31.3	0.2971	0.3085	0.3191	0.3139
62.5	0.2971	0.3241	0.3085	0.3191
125	0.3139	0.3029	0.3241	0.3085
250	0.2391	0.2095	0.2637	0.2201
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

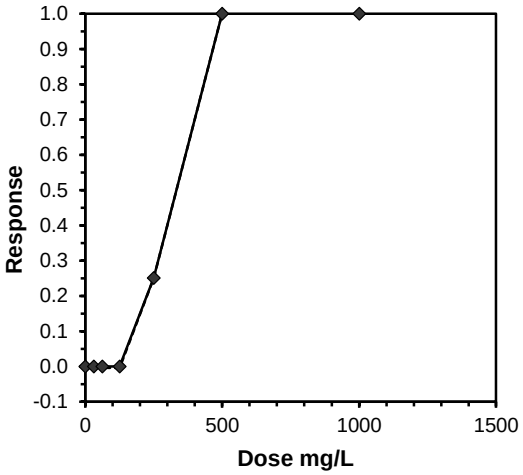
			Transform: Untransformed					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
SIS Control	0.3109	1.0000	0.3109	0.2971	0.3191	3.379	4				0.3113	1.0000
31.3	0.3096	0.9958	0.3096	0.2971	0.3191	3.045	4	0.130	2.360	0.0235	0.3113	1.0000
62.5	0.3122	1.0040	0.3122	0.2971	0.3241	3.843	4	-0.126	2.360	0.0235	0.3113	1.0000
125	0.3123	1.0045	0.3123	0.3029	0.3241	2.891	4	-0.142	2.360	0.0235	0.3113	1.0000
*250	0.2331	0.7497	0.2331	0.2095	0.2637	10.213	4	7.817	2.360	0.0235	0.2331	0.7488
500	0.0000	0.0000	0.0000	0.0000	0.0000	0.000	4				0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000	0.0000	0.000	4				0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.963933	0.905	0.300169	0.58403
Bartlett's Test indicates equal variances (p = 0.39)	4.094777	13.2767		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs SIS Control	125	250	176.7767		0.0235	0.075578	0.004895	0.000198	1.9E-06	4, 15

Linear Interpolation (200 Resamples)

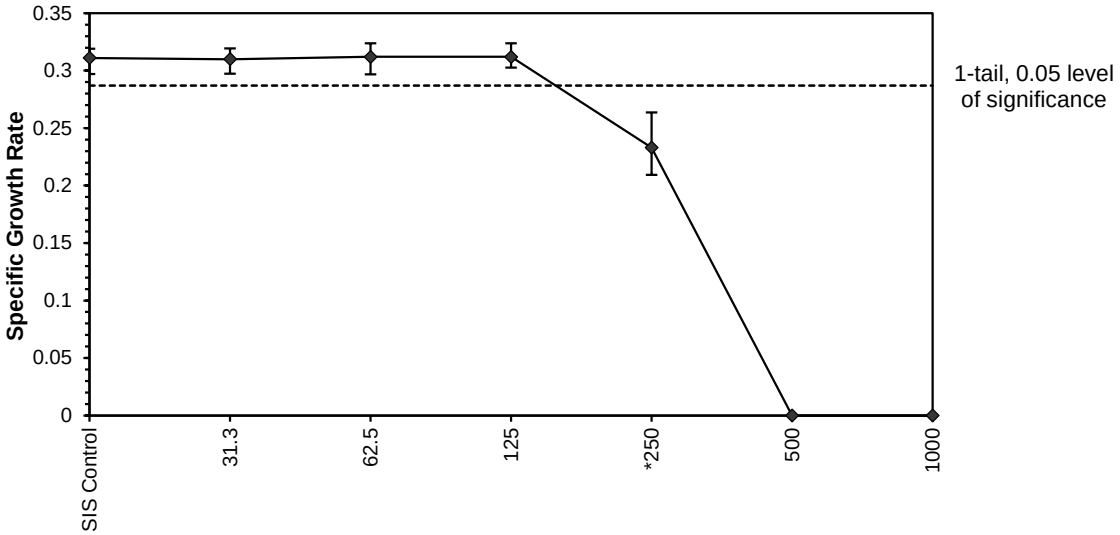
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	149.88	6.17	123.31	159.94	-0.0686
IC10	174.77	8.64	146.74	198.68	0.7387
IC15	199.65	12.03	169.05	235.52	0.9923
IC20	224.54	14.49	187.78	268.92	0.5498
IC25	249.42	13.30	206.34	278.76	-0.0494
IC40	299.69	9.51	269.06	322.92	-0.0336
IC50	333.07	7.93	307.55	352.43	-0.0336



Duckweed Growth Inhibtion Test-Specific Growth Rate

Start Date:	25/11/2020 19:00	Test ID:	PR1967/06	Sample ID:	AFS
End Date:	2/12/2020 19:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 112	Test Species:	LD-Lemna disperma
Comments:					

Dose-Response Plot



Duckweed Growth Inhibtion Test-Specific Growth Rate

Start Date:	25/11/2020 19:00	Test ID:	PR1967/06	Sample ID:	AFS
End Date:	2/12/2020 19:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 112	Test Species:	LD-Lemna disperma
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
SIS Control	Specific growth rate	0.31	0.30	0.32	0.01	32.97	4
31.3		0.31	0.30	0.32	0.01	31.36	4
62.5		0.31	0.30	0.32	0.01	35.09	4
125		0.31	0.30	0.32	0.01	30.42	4
250		0.23	0.21	0.26	0.02	66.19	4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
SIS Control	pH	6.60	6.60	6.60	0.00	0.00	1
31.3		6.60	6.60	6.60	0.00	0.00	1
62.5		6.50	6.50	6.50	0.00	0.00	1
125		6.30	6.30	6.30	0.00	0.00	1
250		5.90	5.90	5.90	0.00	0.00	1
500		4.90	4.90	4.90	0.00	0.00	1
1000		3.00	3.00	3.00	0.00	0.00	1
SIS Control	Cond uS/cm	300.00	300.00	300.00	0.00	0.00	1
31.3		301.00	301.00	301.00	0.00	0.00	1
62.5		299.00	299.00	299.00	0.00	0.00	1
125		299.00	299.00	299.00	0.00	0.00	1
250		302.00	302.00	302.00	0.00	0.00	1
500		299.00	299.00	299.00	0.00	0.00	1
1000		306.00	306.00	306.00	0.00	0.00	1

Statistical Printouts for the *Selenastrum* Growth Inhibition Tests

Microalgal Cell Yield-Cell Yield

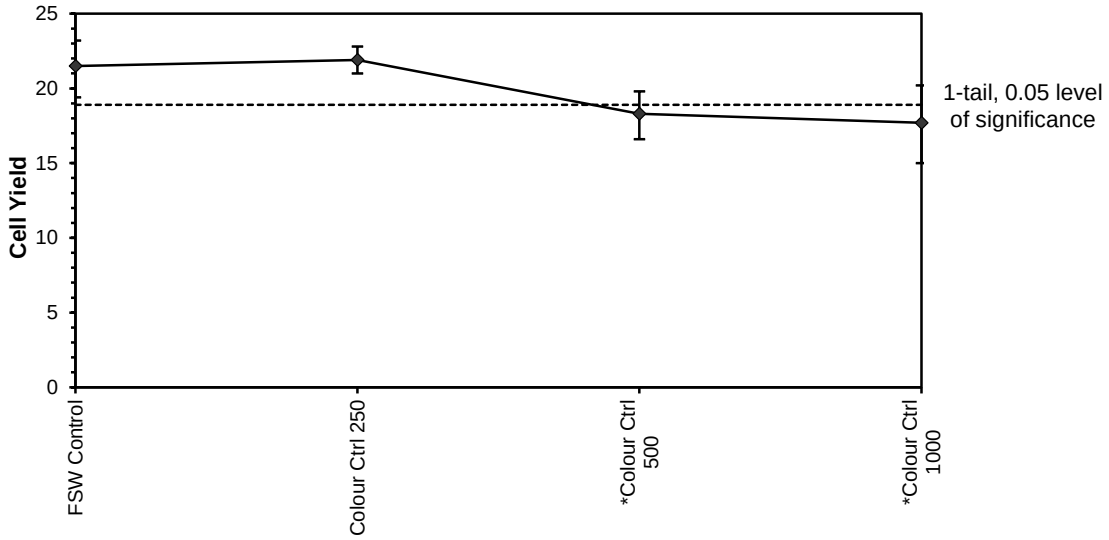
Start Date:	25/11/2020 18:00	Test ID:	PR1967/12			Sample ID:	Controls	
End Date:	28/12/2020 18:00	Lab ID:				Sample Type:	AQ-Aqueous	
Sample Date:		Protocol:	ESA 103			Test Species:	SC-Selenastrum capricornutum	
Comments:								

Conc-	1	2	3	4	5	6	7	8
FSW Control	21.760	21.760	22.760	19.360	20.760	23.160	20.160	21.960
Colour Ctrl 250	22.760	21.760	20.960	21.960				
Colour Ctrl 500	18.960	17.760	16.560	19.760				
Colour Ctrl 1000	17.160	14.960	20.160	18.360				

			Transform: Untransformed					1-Tailed		
Conc-	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD
FSW Control	21.460	1.0000	21.460	19.360	23.160	5.999	8			
Colour Ctrl 250	21.860	1.0186	21.860	20.960	22.760	3.382	4	-0.682	1.833	1.075
*Colour Ctrl 500	18.260	0.8509	18.260	16.560	19.760	7.667	4	3.833	2.015	1.682
Colour Ctrl 1000	17.660	0.8229	17.660	14.960	20.160	12.354	4	3.215	2.132	2.520

Auxiliary Tests	Statistic		Critical		Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.98898		0.905		-0.24268	-0.23441
Bartlett's Test indicates equal variances (p = 0.39)	2.992735		11.34487			
Hypothesis Test (1-tail, 0.05)	**MSDu**	**MSDp**	**MSB**	**MSE**	**F-Prob**	**df**
Heteroscedastic t Test indicates significant differences Treatments vs FSW Control	2.51986	0.117421	21.504	2.0875	5.1E-04	3, 16

Dose-Response Plot



Microalgal Cell Yield-Cell Yield

Start Date:	25/11/2020 18:00	Test ID:	PR1967/12	Sample ID:	Controls
End Date:	28/12/2020 18:00	Lab ID:		Sample Type:	AQ-Aqueous
Sample Date:		Protocol:	ESA 103	Test Species:	SC-Selenastrum capricornutum
Comments:					

Auxiliary Data Summary

Conc-	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	Cell Yield	21.46	19.36	23.16	1.29	5.29	8
Colour Ctrl 250		21.86	20.96	22.76	0.74	3.93	4
Colour Ctrl 500		18.26	16.56	19.76	1.40	6.48	4
Colour Ctrl 1000		17.66	14.96	20.16	2.18	8.36	4
FSW Control	pH	7.80	7.80	7.80	0.00	0.00	1
Colour Ctrl 250		7.80	7.80	7.80	0.00	0.00	1
Colour Ctrl 500		7.80	7.80	7.80	0.00	0.00	1
Colour Ctrl 1000		7.80	7.80	7.80	0.00	0.00	1
FSW Control	Conductivity uS/cm	116.00	116.00	116.00	0.00	0.00	1
Colour Ctrl 250		115.00	115.00	115.00	0.00	0.00	1
Colour Ctrl 500		115.00	115.00	115.00	0.00	0.00	1
Colour Ctrl 1000		115.00	115.00	115.00	0.00	0.00	1

Microalgal Cell Yield-Cell Yield

Start Date:	25/11/2020 18:00	Test ID:	PR1967/09	Sample ID:	AFS
End Date:	28/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 103	Test Species:	SC-Selenastrum capricornutum
Comments:					

Conc-mg/L	1	2	3	4	5	6	7	8
FSW Control	21.760	21.760	22.760	19.360	20.760	23.160	20.160	21.960
31.3	21.760	22.560	19.760	20.760				
62.5	21.760	24.160	22.560	22.160				
125	17.560	12.960	14.760	12.160				
250	7.160	9.760	9.960	13.760				
500	0.000	4.160	2.760	1.760				
1000	0.000	1.760	0.760	0.000				

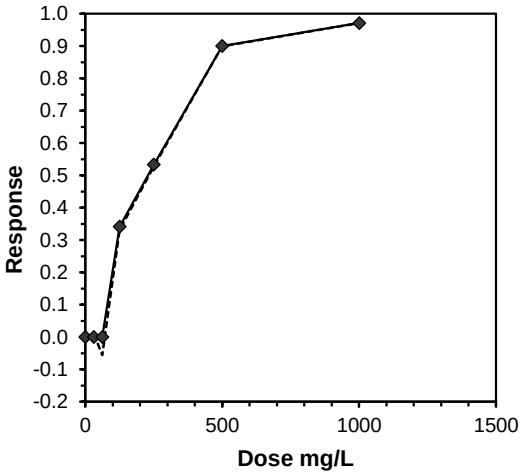
			Transform: Untransformed					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
FSW Control	21.460	1.0000	21.460	19.360	23.160	5.999	8				21.777	1.0000
31.3	21.210	0.9884	21.210	19.760	22.560	5.729	4	0.244	2.566	2.629	21.777	1.0000
62.5	22.660	1.0559	22.660	21.760	24.160	4.642	4	-1.171	2.566	2.629	21.777	1.0000
*125	14.360	0.6692	14.360	12.160	17.560	16.674	4	6.930	2.566	2.629	14.360	0.6594
*250	10.160	0.4734	10.160	7.160	13.760	26.750	4	11.029	2.566	2.629	10.160	0.4666
*500	2.170	0.1011	2.170	0.000	4.160	80.634	4	18.827	2.566	2.629	2.170	0.0997
*1000	0.630	0.0294	0.630	0.000	1.760	132.408	4	20.330	2.566	2.629	0.630	0.0289

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.978702	0.93	0.357704	0.286191
Bartlett's Test indicates equal variances (p = 0.37)	6.462896	16.81189		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test	62.5	125	88.38835		2.629035	0.122508	380.8729	2.799339	7.6E-18	6, 25
Treatments vs FSW Control										

Linear Interpolation (200 Resamples)

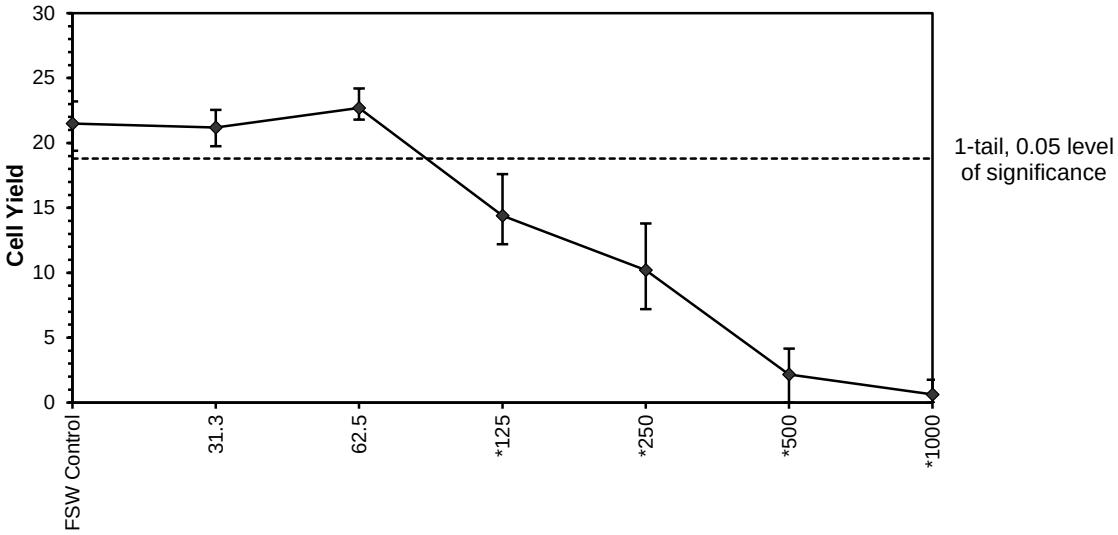
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	71.68	3.77	64.88	79.33	-7.4406
IC10	80.85	3.81	72.47	96.16	1.3086
IC15	90.03	5.48	79.55	112.99	1.5966
IC20	99.20	7.22	86.11	129.82	1.6762
IC25	108.38	9.35	92.99	151.81	1.8664
IC40	163.51	29.38	95.74	259.16	0.7636
IC50	228.32	27.70	161.87	316.55	0.4086



Microalgal Cell Yield-Cell Yield

Start Date:	25/11/2020 18:00	Test ID:	PR1967/09	Sample ID:	AFS
End Date:	28/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 103	Test Species:	SC-Selenastrum capricornutum
Comments:					

Dose-Response Plot



Microalgal Cell Yield-Cell Yield

Start Date:	25/11/2020 18:00	Test ID:	PR1967/09	Sample ID:	AFS
End Date:	28/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 103	Test Species:	SC-Selenastrum capricornutum
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	Cell Yield	21.46	19.36	23.16	1.29	5.29	8
31.3		21.21	19.76	22.56	1.22	5.20	4
62.5		22.66	21.76	24.16	1.05	4.53	4
125		14.36	12.16	17.56	2.39	10.78	4
250		10.16	7.16	13.76	2.72	16.23	4
500		2.17	0.00	4.16	1.75	60.96	4
1000		0.63	0.00	1.76	0.83	144.97	4
FSW Control	pH	7.80	7.80	7.80	0.00	0.00	1
31.3		7.80	7.80	7.80	0.00	0.00	1
62.5		7.80	7.80	7.80	0.00	0.00	1
125		7.10	7.10	7.10	0.00	0.00	1
250		6.30	6.30	6.30	0.00	0.00	1
500		5.00	5.00	5.00	0.00	0.00	1
1000		3.40	3.40	3.40	0.00	0.00	1
FSW Control	Conductivity uS/cm	116.00	116.00	116.00	0.00	0.00	1
31.3		115.00	115.00	115.00	0.00	0.00	1
62.5		119.00	119.00	119.00	0.00	0.00	1
125		113.00	113.00	113.00	0.00	0.00	1
250		114.00	114.00	114.00	0.00	0.00	1
500		119.00	119.00	119.00	0.00	0.00	1
1000		121.00	121.00	121.00	0.00	0.00	1

Statistical Printouts for the Sea Urchin Larval Development Test

Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Conc-mg/L	1	2	3	4
FSW Control	0.9500	0.9600	0.9300	0.9500
31.3	0.9700	0.9300	0.9500	0.9400
62.5	0.9300	0.9800	0.9600	0.9200
125	0.3400	0.4600	0.2900	0.3800
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

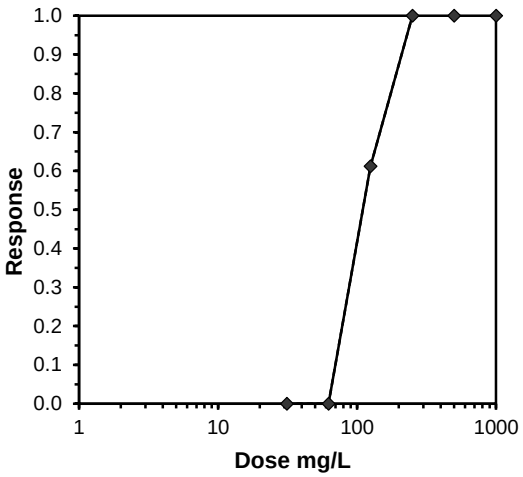
			Transform: Arcsin Square Root					1-Tailed			Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
FSW Control	0.9475	1.0000	1.3408	1.3030	1.3694	2.059	4				21	400
31.3	0.9475	1.0000	1.3421	1.3030	1.3967	3.002	4	-0.034	2.290	0.0898	21	400
62.5	0.9475	1.0000	1.3464	1.2840	1.4289	4.909	4	-0.143	2.290	0.0898	21	400
*125	0.3675	0.3879	0.6502	0.5687	0.7454	11.462	4	17.605	2.290	0.0898	253	400
250	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				400	400
500	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				400	400
1000	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				400	400

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.96948	0.887	0.386752	-0.31959
Bartlett's Test indicates equal variances (p = 0.41)	2.881387	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test Treatments vs FSW Control	62.5	125	88.38835		0.04688	0.049451	0.480095	0.003077	7.1E-10	3, 12

Trimmed Spearman-Karber

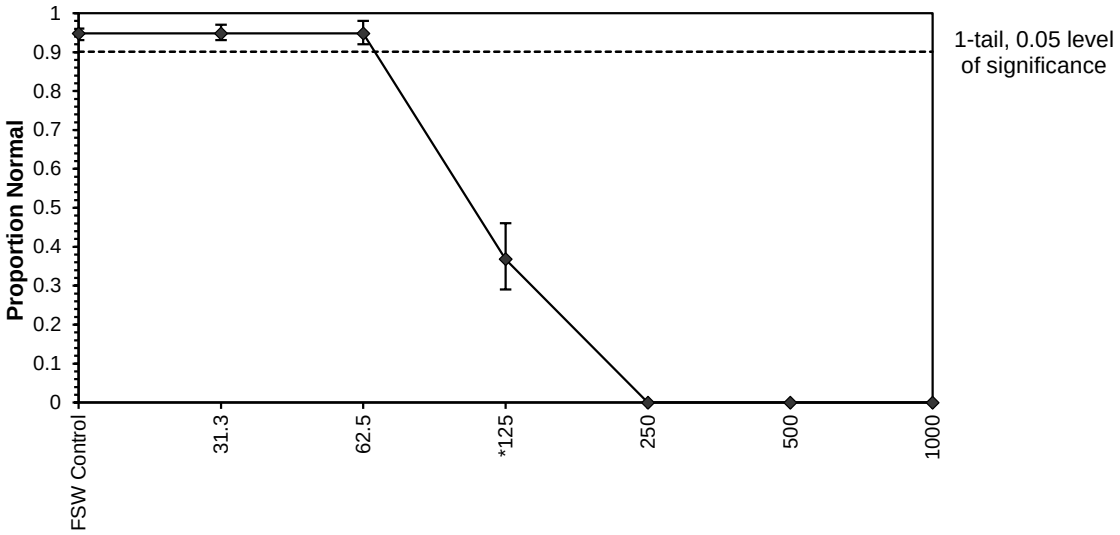
Trim Level	EC50	95% CL	
0.0%	115.65	111.81	119.62
5.0%	114.76	110.56	119.13
10.0%	113.89	109.28	118.70
20.0%	112.23	106.57	118.20
Auto-0.0%	115.65	111.81	119.62



Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Dose-Response Plot



Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Normal	94.75	93.00	96.00	1.26	1.18	4
31.3		94.75	93.00	97.00	1.71	1.38	4
62.5		94.75	92.00	98.00	2.75	1.75	4
125		36.75	29.00	46.00	7.18	7.29	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
FSW Control	pH	8.20	8.20	8.20	0.00	0.00	1
31.3		8.10	8.10	8.10	0.00	0.00	1
62.5		8.00	8.00	8.00	0.00	0.00	1
125		7.60	7.60	7.60	0.00	0.00	1
250		7.10	7.10	7.10	0.00	0.00	1
500		6.30	6.30	6.30	0.00	0.00	1
1000		4.80	4.80	4.80	0.00	0.00	1
FSW Control	Salinity ppt	35.60	35.60	35.60	0.00	0.00	1
31.3		35.60	35.60	35.60	0.00	0.00	1
62.5		35.70	35.70	35.70	0.00	0.00	1
125		35.60	35.60	35.60	0.00	0.00	1
250		35.60	35.60	35.60	0.00	0.00	1
500		35.60	35.60	35.60	0.00	0.00	1
1000		34.80	34.80	34.80	0.00	0.00	1
FSW Control	DO %	101.20	101.20	101.20	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		99.30	99.30	99.30	0.00	0.00	1
125		99.30	99.30	99.30	0.00	0.00	1
250		98.40	98.40	98.40	0.00	0.00	1
500		98.10	98.10	98.10	0.00	0.00	1
1000		96.90	96.90	96.90	0.00	0.00	1

Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Conc-mg/L	1	2	3	4
FSW Control	0.9500	0.9600	0.9300	0.9500
31.3	0.9700	0.9300	0.9500	0.9400
62.5	0.9300	0.9800	0.9600	0.9200
125	0.3400	0.4600	0.2900	0.3800
250	0.0000	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

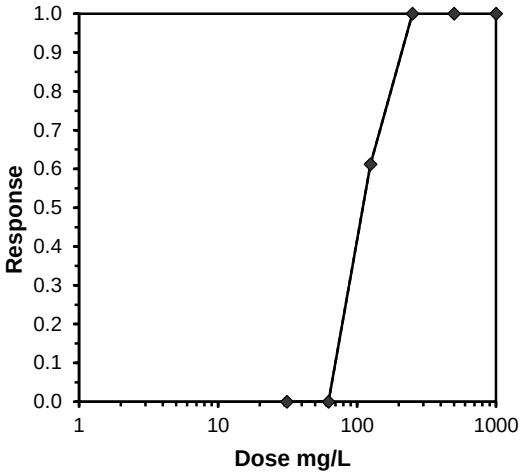
			Transform: Arcsin Square Root					1-Tailed			Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Mean	N-Mean
FSW Control	0.9475	1.0000	1.3408	1.3030	1.3694	2.059	4				0.9475	1.0000
31.3	0.9475	1.0000	1.3421	1.3030	1.3967	3.002	4	-0.034	2.290	0.0898	0.9475	1.0000
62.5	0.9475	1.0000	1.3464	1.2840	1.4289	4.909	4	-0.143	2.290	0.0898	0.9475	1.0000
*125	0.3675	0.3879	0.6502	0.5687	0.7454	11.462	4	17.605	2.290	0.0898	0.3675	0.3879
250	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				0.0000	0.0000
500	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				0.0000	0.0000
1000	0.0000	0.0000	0.0500	0.0500	0.0500	0.000	4				0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.05)	0.96948	0.887	0.386752	-0.31959
Bartlett's Test indicates equal variances (p = 0.41)	2.881387	11.34487		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	62.5	125	88.38835		0.04688	0.049451	0.480095	0.003077	7.1E-10	3, 12
Treatments vs FSW Control										

Log-Logit Interpolation (200 Resamples)

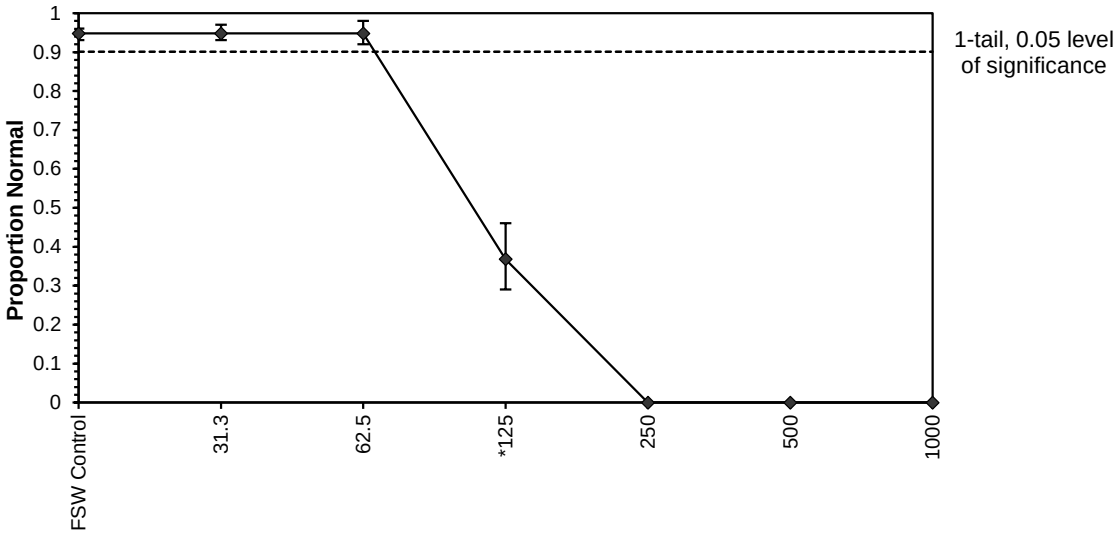
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	71.93	1.66	63.89	74.26	-0.6651
IC10	78.66	1.68	71.53	82.12	-0.3392
IC15	84.18	1.73	77.24	88.43	-0.1121
IC20	89.04	1.81	81.98	93.88	0.0354
IC25	93.52	1.92	86.31	99.22	0.1251
IC40	106.02	2.38	98.10	113.38	0.2167
IC50	114.46	2.82	105.52	123.73	0.2302



Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Dose-Response Plot



Sea Urchin Larval Development Test-Proportion Normal

Start Date:	27/11/2020 17:30	Test ID:	PR1967/10	Sample ID:	AFS
End Date:	30/11/2020 17:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 104	Test Species:	HT-Heliocidaris tuberculata
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Normal	94.75	93.00	96.00	1.26	1.18	4
31.3		94.75	93.00	97.00	1.71	1.38	4
62.5		94.75	92.00	98.00	2.75	1.75	4
125		36.75	29.00	46.00	7.18	7.29	4
250		0.00	0.00	0.00	0.00		4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
FSW Control	pH	8.20	8.20	8.20	0.00	0.00	1
31.3		8.10	8.10	8.10	0.00	0.00	1
62.5		8.00	8.00	8.00	0.00	0.00	1
125		7.60	7.60	7.60	0.00	0.00	1
250		7.10	7.10	7.10	0.00	0.00	1
500		6.30	6.30	6.30	0.00	0.00	1
1000		4.80	4.80	4.80	0.00	0.00	1
FSW Control	Salinity ppt	35.60	35.60	35.60	0.00	0.00	1
31.3		35.60	35.60	35.60	0.00	0.00	1
62.5		35.70	35.70	35.70	0.00	0.00	1
125		35.60	35.60	35.60	0.00	0.00	1
250		35.60	35.60	35.60	0.00	0.00	1
500		35.60	35.60	35.60	0.00	0.00	1
1000		34.80	34.80	34.80	0.00	0.00	1
FSW Control	DO %	101.20	101.20	101.20	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		99.30	99.30	99.30	0.00	0.00	1
125		99.30	99.30	99.30	0.00	0.00	1
250		98.40	98.40	98.40	0.00	0.00	1
500		98.10	98.10	98.10	0.00	0.00	1
1000		96.90	96.90	96.90	0.00	0.00	1

Statistical Printouts for the Acute *Allorchestes* Toxicity Test

Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Conc-mg/L	1	2	3	4
FSW Control	1.0000	1.0000	1.0000	1.0000
31.3	1.0000	1.0000	1.0000	1.0000
62.5	1.0000	1.0000	1.0000	1.0000
125	1.0000	1.0000	1.0000	1.0000
250	0.4000	0.4000	0.2000	0.6000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

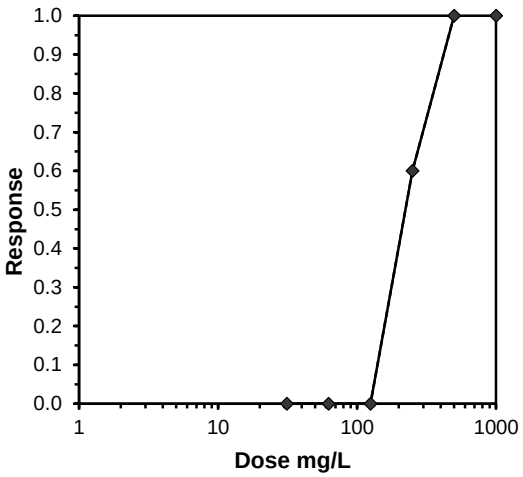
			Transform: Arcsin Square Root					Rank	1-Tailed	Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Resp	Number
FSW Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4			0	20
31.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	0	20
62.5	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	0	20
125	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	0	20
*250	0.4000	0.4000	0.6798	0.4636	0.8861	25.383	4	10.00	10.00	12	20
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			20	20

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.460565	0.905	-0.23934	9.51416
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	125	250	176.7767	
Treatments vs FSW Control				

Trimmed Spearman-Karber

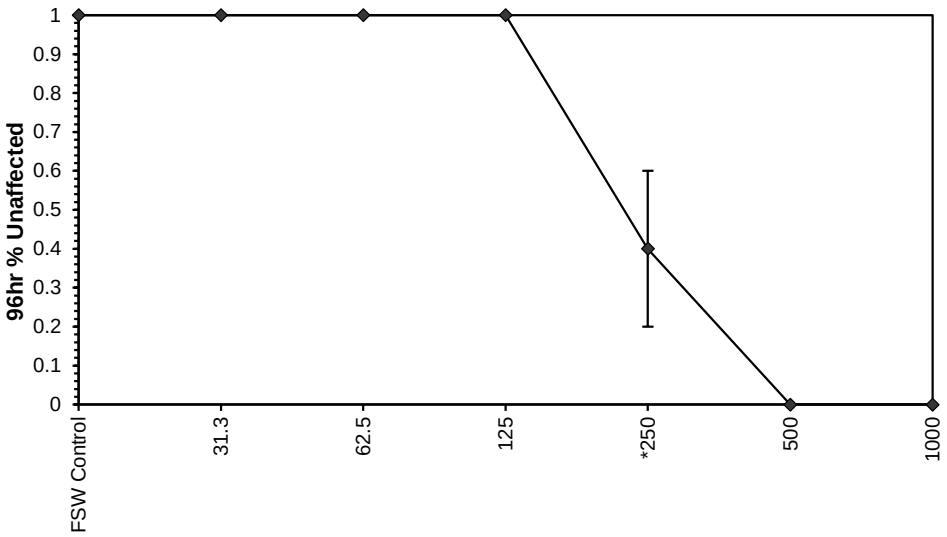
Trim Level	EC50	95% CL	
0.0%	233.26	200.39	271.51
5.0%	231.65	195.83	274.03
10.0%	230.08	190.93	277.26
20.0%	227.05	179.41	287.35
Auto-0.0%	233.26	200.39	271.51



Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Dose-Response Plot



Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Non-immobilised	100.00	100.00	100.00	0.00	0.00	4
31.3		100.00	100.00	100.00	0.00	0.00	4
62.5		100.00	100.00	100.00	0.00	0.00	4
125		100.00	100.00	100.00	0.00	0.00	4
250		40.00	20.00	60.00	16.33	10.10	4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
FSW Control	pH	8.20	8.20	8.20	0.00	0.00	1
31.3		8.10	8.10	8.10	0.00	0.00	1
62.5		8.00	8.00	8.00	0.00	0.00	1
125		7.60	7.60	7.60	0.00	0.00	1
250		7.10	7.10	7.10	0.00	0.00	1
500		6.30	6.30	6.30	0.00	0.00	1
1000		4.80	4.80	4.80	0.00	0.00	1
FSW Control	DO %	35.60	35.60	35.60	0.00	0.00	1
31.3		35.60	35.60	35.60	0.00	0.00	1
62.5		35.70	35.70	35.70	0.00	0.00	1
125		35.60	35.60	35.60	0.00	0.00	1
250		35.60	35.60	35.60	0.00	0.00	1
500		35.60	35.60	35.60	0.00	0.00	1
1000		34.80	34.80	34.80	0.00	0.00	1
FSW Control	Salinity ppt	101.20	101.20	101.20	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		99.30	99.30	99.30	0.00	0.00	1
125		99.30	99.30	99.30	0.00	0.00	1
250		98.40	98.40	98.40	0.00	0.00	1
500		98.10	98.10	98.10	0.00	0.00	1
1000		96.90	96.90	96.90	0.00	0.00	1

Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Conc-mg/L	1	2	3	4
FSW Control	1.0000	1.0000	1.0000	1.0000
31.3	1.0000	1.0000	1.0000	1.0000
62.5	1.0000	1.0000	1.0000	1.0000
125	1.0000	1.0000	1.0000	1.0000
250	0.4000	0.4000	0.2000	0.6000
500	0.0000	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000	0.0000

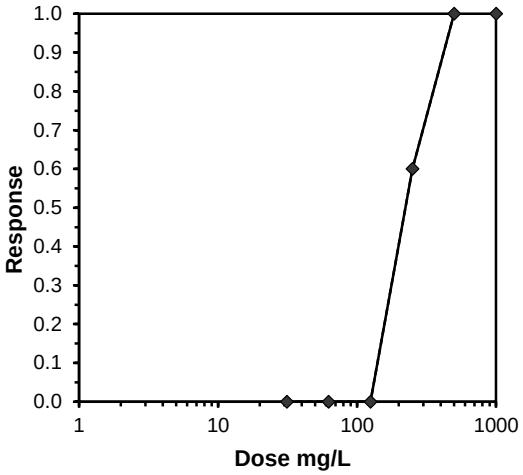
			Transform: Arcsin Square Root					Rank	1-Tailed	Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Mean	N-Mean
FSW Control	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4			1.0000	1.0000
31.3	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	1.0000	1.0000
62.5	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	1.0000	1.0000
125	1.0000	1.0000	1.3453	1.3453	1.3453	0.000	4	18.00	10.00	1.0000	1.0000
*250	0.4000	0.4000	0.6798	0.4636	0.8861	25.383	4	10.00	10.00	0.4000	0.4000
500	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000
1000	0.0000	0.0000	0.2255	0.2255	0.2255	0.000	4			0.0000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.460565	0.905	-0.23934	9.51416
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	125	250	176.7767	
Treatments vs FSW Control				

Log-Logit Interpolation (200 Resamples)

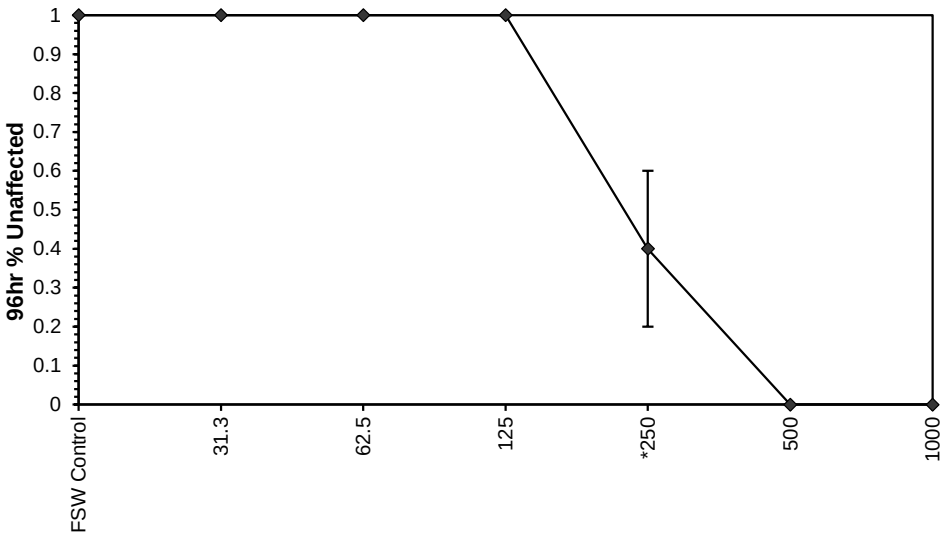
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	196.43	2.66	190.25	206.11	0.3108
IC10	207.29	3.14	200.01	218.75	0.3145
IC15	214.31	3.46	206.29	226.95	0.3168
IC20	219.75	3.72	211.16	233.33	0.3185
IC25	224.35	3.93	215.26	238.73	0.3199
IC40	235.83	4.49	225.47	252.26	0.3233
IC50	242.81	4.82	231.66	260.28	0.3125



Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Dose-Response Plot



Amphipod Acute Toxicity Test-96hr % Unaffected

Start Date:	27/11/2020 18:00	Test ID:	PR1967/11	Sample ID:	AFS
End Date:	1/12/2020 18:00	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 118	Test Species:	AC-Allorchestes compressa
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	% Non-immobilised	100.00	100.00	100.00	0.00	0.00	4
31.3		100.00	100.00	100.00	0.00	0.00	4
62.5		100.00	100.00	100.00	0.00	0.00	4
125		100.00	100.00	100.00	0.00	0.00	4
250		40.00	20.00	60.00	16.33	10.10	4
500		0.00	0.00	0.00	0.00		4
1000		0.00	0.00	0.00	0.00		4
FSW Control	pH	8.20	8.20	8.20	0.00	0.00	1
31.3		8.10	8.10	8.10	0.00	0.00	1
62.5		8.00	8.00	8.00	0.00	0.00	1
125		7.60	7.60	7.60	0.00	0.00	1
250		7.10	7.10	7.10	0.00	0.00	1
500		6.30	6.30	6.30	0.00	0.00	1
1000		4.80	4.80	4.80	0.00	0.00	1
FSW Control	DO %	35.60	35.60	35.60	0.00	0.00	1
31.3		35.60	35.60	35.60	0.00	0.00	1
62.5		35.70	35.70	35.70	0.00	0.00	1
125		35.60	35.60	35.60	0.00	0.00	1
250		35.60	35.60	35.60	0.00	0.00	1
500		35.60	35.60	35.60	0.00	0.00	1
1000		34.80	34.80	34.80	0.00	0.00	1
FSW Control	Salinity ppt	101.20	101.20	101.20	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		99.30	99.30	99.30	0.00	0.00	1
125		99.30	99.30	99.30	0.00	0.00	1
250		98.40	98.40	98.40	0.00	0.00	1
500		98.10	98.10	98.10	0.00	0.00	1
1000		96.90	96.90	96.90	0.00	0.00	1

Statistical Printouts for the kelp *Ecklonia radiata* 14-d Gametophyte Growth Test

Macroalgal Germination Success Test-Gametophyte Length

Start Date:	27/11/2020 16:30	Test ID:	PR1967/14	Sample ID:	AFS
End Date:	11/12/2020 16:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 116	Test Species:	ER-Ecklonia radiata
Comments:					

Conc-mg/L	1	2	3	4
FSW Control	22.661	22.290	22.110	22.800
31.3	22.380	22.500	22.650	22.980
62.5	23.080	23.470	23.040	23.000
125	23.130	22.300	22.520	22.750
250	22.620	22.270	22.220	22.140
500	8.460	8.650	8.630	9.310
1000	0.000	0.000	0.000	0.000

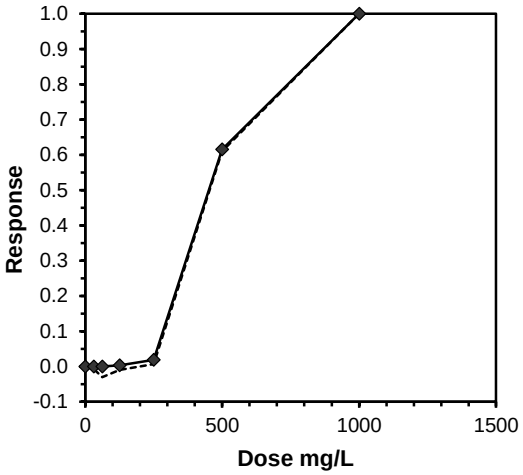
			Transform: Untransformed					Rank	1-Tailed	Isotonic	
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	Sum	Critical	Mean	N-Mean
FSW Control	22.465	1.0000	22.465	22.110	22.800	1.425	4			22.747	1.0000
31.3	22.628	1.0072	22.628	22.380	22.980	1.148	4	20.00	10.00	22.747	1.0000
62.5	23.148	1.0304	23.148	23.000	23.470	0.939	4	26.00	10.00	22.747	1.0000
125	22.675	1.0093	22.675	22.300	23.130	1.564	4	21.00	10.00	22.675	0.9968
250	22.313	0.9932	22.313	22.140	22.620	0.950	4	15.00	10.00	22.313	0.9809
*500	8.763	0.3900	8.763	8.460	9.310	4.278	4	10.00	10.00	8.763	0.3852
1000	0.000	0.0000	0.000	0.000	0.000	0.000	4			0.000	0.0000

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.05)	0.91476	0.916	0.640004	-0.65674
Bartlett's Test indicates equal variances (p = 0.91)	1.559006	15.08627		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Steel's Many-One Rank Test	250	500	353.5534	
Treatments vs FSW Control				

Linear Interpolation (200 Resamples)

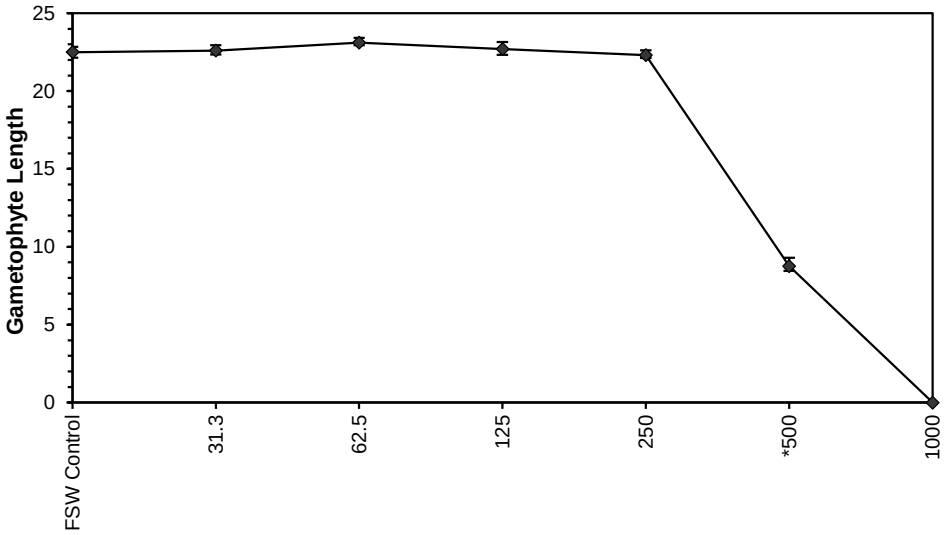
Point	mg/L	SD	95% CL(Exp)		Skew
IC05	262.97	1.80	257.09	267.84	0.2496
IC10	283.96	1.68	278.59	288.46	0.2366
IC15	304.94	1.60	299.89	309.34	0.2093
IC20	325.92	1.57	321.24	330.11	0.1851
IC25	346.91	1.61	342.55	350.96	0.1832
IC40	409.86	1.99	404.52	416.43	0.2922
IC50	451.83	2.41	445.74	459.87	0.3553



Macroalgal Germination Success Test-Gametophyte Length

Start Date:	27/11/2020 16:30	Test ID:	PR1967/14	Sample ID:	AFS
End Date:	11/12/2020 16:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 116	Test Species:	ER-Ecklonia radiata
Comments:					

Dose-Response Plot



Macroalgal Germination Success Test-Gametophyte Length

Start Date:	27/11/2020 16:30	Test ID:	PR1967/14	Sample ID:	AFS
End Date:	11/12/2020 16:30	Lab ID:	9837	Sample Type:	CP-Chemical product
Sample Date:		Protocol:	ESA 116	Test Species:	ER-Ecklonia radiata
Comments:					

Auxiliary Data Summary

Conc-mg/L	Parameter	Mean	Min	Max	SD	CV%	N
FSW Control	Length um	22.47	22.11	22.80	0.32	2.52	4
31.3		22.63	22.38	22.98	0.26	2.25	4
62.5		23.15	23.00	23.47	0.22	2.01	4
125		22.68	22.30	23.13	0.35	2.63	4
250		22.31	22.14	22.62	0.21	2.06	4
500		8.76	8.46	9.31	0.37	6.99	4
1000		0.00	0.00	0.00	0.00		4
FSW Control	pH	8.20	8.20	8.20	0.00	0.00	1
31.3		8.10	8.10	8.10	0.00	0.00	1
62.5		8.00	8.00	8.00	0.00	0.00	1
125		7.60	7.60	7.60	0.00	0.00	1
250		7.10	7.10	7.10	0.00	0.00	1
500		6.30	6.30	6.30	0.00	0.00	1
1000		4.80	4.80	4.80	0.00	0.00	1
FSW Control	Salinity ppt	35.60	35.60	35.60	0.00	0.00	1
31.3		35.60	35.60	35.60	0.00	0.00	1
62.5		35.70	35.70	35.70	0.00	0.00	1
125		35.60	35.60	35.60	0.00	0.00	1
250		35.60	35.60	35.60	0.00	0.00	1
500		35.60	35.60	35.60	0.00	0.00	1
1000		34.80	34.80	34.80	0.00	0.00	1
FSW Control	DO % sat	101.20	101.20	101.20	0.00	0.00	1
31.3		99.50	99.50	99.50	0.00	0.00	1
62.5		94.30	94.30	94.30	0.00	0.00	1
125		99.30	99.30	99.30	0.00	0.00	1
250		98.40	98.40	98.40	0.00	0.00	1
500		98.10	98.10	98.10	0.00	0.00	1
1000		96.90	96.90	96.90	0.00	0.00	1

Appendix 5 – Draft label

PERMIT TO ALLOW SUPPLY AND MINOR USE
OF UNREGISTERED VETERINARY CHEMICAL PRODUCTS
PERMIT NUMBER – PER?????

This permit is issued to the Permit Holder under section 114 of the Agricultural and Veterinary Chemicals Code, scheduled to the Agricultural and Veterinary Chemicals Code Act 1994 (the Agvet Code) in response to an application granted by the APVMA under section 112 of the Agvet Codes of the jurisdictions set out below. This permit allows a Supplier (as indicated) to possess the Products for the purposes of supply and to supply The Products to a person who can use The Products under permit. If this permit were not issued, supply of the Products as specified below would constitute an offence under section 78 of the Agvet Code. This permit also allows a person, as stipulated below, to use The Products in the manner specified in this permit in the designated jurisdictions. This permit also allows the Permit Holder, the Supplier (if not one and the same) and any person stipulated below to claim that The Products can be used in the manner specified in this permit.

THIS PERMIT IS IN FORCE FROM Day Month 2022 TO Day Month 2027.

Permit Holder:
CCD Animal Health
Unit 16, 84–92 Barnes Street
Tamworth NSW 2340

Suppliers authorised by this permit to supply The Products and make claims:
CCD Animal Health
Unit 16, 84–92 Barnes Street
Tamworth NSW 2340

Persons authorised by this permit to use The Product and make claims: *Bona fide* members of the Australian aquaculture industry.

Product to be used under permit:
CCD OTC
Containing: 926 mg/g OXYTETRACYCLINE as its only active constituent.

Directions for Use:
To be used in the treatment of susceptible bacterial infections in non-salmonid finfish (Actinopterygii) under the supervision of a registered veterinary surgeon.

The Attachment to this permit provides guidance on dose rates and treatment protocols.

Withholding Periods:
Meat (fish) – 1000 degree days.

Jurisdiction:
Australia.

CONDITIONS
Supply

The Suppliers authorised by this permit to supply The Products and make claims must supply The Product in a container that complies with Regulation 18(1) and (2) and Regulation 18A to 18I (inclusive) of the Agricultural and Veterinary Chemicals Code Regulations.
The Suppliers authorised by this permit to supply The Products and make claims can only make claims for use of the Products in farmed non-salmonid finfish (Actinopterygii).

Use
Persons who wish to prepare for use and/or use The Products for the purposes specified in this permit must read, or have read to them, the details and conditions of this permit.
THIS PERMIT provides for the use of an unregistered product in accordance with the instructions in the Attachment of this permit, and The Products' Safety Data Sheet.
Any adverse event arising from the use of The Products in fish must be reported to the APVMA's Coordinator, Adverse Experience Reporting Program (phone 02 6210 4792).
The Permit Holder is to monitor the use of CCD OTC in aquaculture overseas and inform the APVMA of any change in status of that use, particularly any incidents of environmental contamination. Also, the Permit Holder is to inform the APVMA of any action taken by overseas regulatory authorities in regard to the use of CCD OTC in aquaculture.

Claim
A person who is authorised by this permit and makes a claim about the use of The Products, can only make a claim consistent with the Directions for Use and the instructions in the Attachment of this permit.

Issued by Australian Pesticides and Veterinary Medicines Authority
Version 1 issued ?/?/??: amended authorized product and updated attached label PER????? Permit Version 1

Attachment
POISON
KEEP OUT OF REACH OF CHILDREN READ SAFETY DIRECTIONS BEFORE OPENING
OXYTETRACYCLINE 92.6%
ACTIVE CONSTITUENTS: 926 mg/g OXYTETRACYCLINE
Used for the treatment of susceptible bacterial infections in non-salmonid finfish (Actinopterygii).

Net contents 500g / 1kg / 25 kg.

Non-Dangerous Goods according to the criteria of the Australian Dangerous Goods Code (ADG Code).

DIRECTIONS FOR USE:

Treat in-feed at 50-250 $mg.kg^{-1}$ bw day^{-1}. Treatment may be repeated if advised by a veterinarian.

WITHHOLDING PERIOD: 1000 degree days.
PROTECTION OF WILDLIFE AND ENVIRONMENT:
DO NOT contaminate streams, rivers, waterways or surface waters with the undiluted chemical or used container.

STORAGE: Store in the closed, original container in a well-ventilated area, below 30°C, away from heat and acids, bases, oxidising agents, heat or sunlight. Store away from other chemicals.

DISPOSAL:
1. Treated Water Management:

Marine Water: Release of treated waters should only occur under conditions likely to lead to adequate dilution/dissipation.

2. Container: Triple rinse into treatment mix. Recycle container if possible otherwise crush dispose of in accordance with site and local regulatory requirements.

Oxytetracycline has a low risk profile and is not a hazardous substance, but appropriate measures should be taken to protect users from exposure. Do not inhale powder. Protective clothing, eyewear and breathing apparatus must be worn at all times when handling powder. When opening the container and using the product, wear gloves, a respirator, protective suit or apron and chemically resistant boots. If the product is on skin, immediately wash area with large volume of water. Observe good hygienic practices while using. Wash personal protective equipment with large volumes of water. Store product in cool well ventilated place. DO NOT expose product to acids, bases, oxidizing agents, heat or UV radiation.

NOT FOR HANDLING BY OPERATORS KNOWN TO HAVE A TETRACYCLINE OR ANTIBIOTIC ALLERGY OR SENSITIVITY.

FIRST AID: If poisoning occurs, contact a doctor or Poisons Information Centre. Phone Australia 131126; New Zealand 0800764766.

Ingestion
- Rinse mouth with water. Do not induce vomiting. Loosen tight clothing such as a collar, tie, belt or waistband.
- If the victim is not breathing, perform mouth-to-mouth resuscitation. Seek immediate medical attention.

Inhalation:
- Remove to fresh air
- Seek medical advice

Eye contact:
- Immediately flush eyes with plenty of water for 15 minutes, holding eyelids open.
- Seek medical advice.

Skin contact:
- remove any contaminated clothing and wash skin with running water.
- If irritation occurs, seek medical advice

Shelf life 2 years
Date of manufacture: