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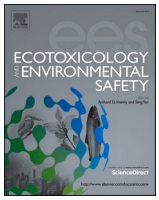

Concentration-response of six marine species to all-*trans*-retinoic acid and its ecological risk to the marine environment

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ABSTRACT

Being a class of vitamin A's main derivatives, retinoic acids (RAs) are important to animals' growth and development. Previous studies demonstrated that exposure of excessive amounts of RAs would lead to malformation and abnormal development in aquatic animals such as amphibians and fishes. Currently, there are only limited toxicity data of RAs available for freshwater species, while those for marine species are seriously lacking. This study aimed to fill such data gap by conducting toxicity tests on six marine species (i.e., one microalga, four invertebrates and one fish) towards the exposure to all-*trans*-RA (at-RA), which is the most widely distributed RA in the environment. Results showed that the embryo of medaka fish *Oryzias melastigma* was the most sensitive towards the exposure of at-RA while the gastropod *Monodonta labio* was the least sensitive. A species sensitivity distribution (SSD) was constructed based on the experimental results generated from the present study. An interim marine-specific predicted no-effect concentration (PNEC) of at-RA was derived at 2300 ng/L. By computing the hazard quotients using the interim marine-specific PNEC and available measured and predicted concentrations of RAs, we found the current levels of RAs posed no immediate risks to the marine environment of Hong Kong. The interim marine-specific PNEC was more than 500-fold of freshwater-specific PNEC (i.e., 3.93 ng/L), indicating that marine species were generally less sensitive than their freshwater counterparts towards RAs. This was the first study to document the concentration-response of various marine species towards at-RA exposure and construct the marine-specific SSD for assessing the ecological risk of at-RA towards the marine environment. Since various forms of RAs and their metabolites often coexist in aquatic environments, further studies should investigate their combined toxicity to an array of marine species of different trophic levels with consideration of chronic exposure scenarios.

1. Introduction

Retinoic acids (RAs), being a class of the major derivatives of vitamin A, naturally exist in aquatic environments and have been widely detected in sewage, seawater, rivers and lakes (Yeung et al., 2020b). RAs are known to be important and essential to animals for a number of vital functions such as reproduction, vision, cell differentiation and immune response (Barua and Furr, 1998; Collins and Mao, 1999; Ross et al., 2000). However, teratogenic effects could be induced on aquatic species upon their exposure to excessive amounts of RAs. Previous studies showed that malformations and abnormal developments were observed in a wide variety of animals, including gastropods, amphibians, fishes and mammals, especially during their developmental stages upon exposure to considerable amounts of RAs (Horiguchi et al., 2008; Jonas et al., 2014; Smutná et al., 2017). Malformations in different parts of amphibians, including but not limited to brain, eyes, tails and hindleg were commonly observed in amphibians with a range of median effect concentrations (EC_{50}s) of all-*trans* RA (at-RA) at 5.0–20.9 μg/L while heart edema, brain and tail tip deformations were usually detected in fish embryos with EC_{50}s of at-RA at 0.10–30 μg/L (Yeung et al., 2020b). Laboratory studies also demonstrated that 9-*cis*-RA (9c-RA) induced imposex (i.e., superimposition of male sexual organs onto females) on the marine gastropod *Reishia clavigera* at 1 μg/g of drained body mass (Horiguchi et al., 2008; Nishikawa et al., 2004).

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Although there were a number of studies investigated the adverse effects of RAs on aquatic species, only six studies, among all works, were conducted on marine species namely *Paralichthys olivaceus* (Haga et al., 2002), *R. clavigera* (Horiguchi et al., 2008; Nishikawa et al., 2004; Zhou et al., 2021), *Crassostrea gigas* (Vogeler et al., 2017) and *Phallusia mammillata* (Groppelli et al., 2007). Details on the adverse effects of RAs on marine species was far from sufficient. Moreover, even with the presence of data of freshwater species, previous studies on the toxicity of RAs largely focused on the concentration-dependent responses of amphibian and fish but not species in other taxonomic groups such as invertebrates (Yeung et al., 2020b), nor species in lower trophic levels such as producers and primary consumers. Hence, the toxicological information was insufficient to construct a representative species sensitivity distribution (SSD), in particular, by the use of toxicity data derived from marine species of different trophic levels, and thus a hazardous concentration for 5% of species and predicted no-effect concentration (PNEC) cannot be meaningfully derived. Without a marine-specific PNEC, the ecological risk assessment of RAs on marine species cannot be fairly and accurately evaluated. It is also difficult to regulate and monitor the levels of RAs in the marine environment as the current marine water quality monitoring system only covers conventional water quality parameters (e.g., EPD, 2020; USEPA, 2021) but not the emerging chemicals of concern such as RAs. Hence, the marine species cannot be reasonably protected at an appropriate threshold level.

Since RAs have conjugated double bonds, various isomers, including at-RA, 9c-RA and 13-*cis*-RA (13c-RA) and their oxidative metabolites, 4-oxo-RAs, often co-exist in the environment. All six forms of RAs are naturally occurring retinoids while at-RA was found to be the most widely distributed form in the environment among them (Herrmann, 1995). This study, therefore, with a special attention to at-RA, aimed to (1) determine toxic effects of at-RA to six marine species of different trophic levels covering algae, invertebrates and fish embryos; (2) construct an SSD to determine an interim marine-specific PNEC based on the toxicity endpoints derived from the present study, and compare the interim marine-specific PNEC with the previously derived freshwater-specific PNEC; and (3) conduct the ecological risk assessment of the six studied RAs in the marine environment based on the interim marine-specific PNEC and the measured and/or predicted environmental concentrations reported in previous studies.

2. Materials and methods

2.1. Chemicals

At-RA (CAS no. 302-79-4) was obtained from Sigma-Aldrich (St. Louis, USA) and used as the test chemical in the study. Stock solution (1000 mg/L) of at-RA was prepared by dissolving at-RA in methanol (HPLC grade, Tedia, Fairfield, OH, USA). Dichloromethane and acetonitrile of HPLC grade were obtained from Tedia (Fairfield, OH, USA).

2.2. Test species

Six marine species were used in the present study including the microalga *Isochrysis galbana*, the copepod *Tigriopus japonicus*, the brine shrimp *Artemia franciscana*, the gastropod *Monodonta labio*, the mussel *Xenostrobus securis*, and the marine medaka fish embryo *Oryzias melastigma*. Both *I. galbana* and *T. japonicus* were cultured in the laboratory of School of Biological Sciences at the University of Hong Kong. Autoclaved f/2 growth medium was used to maintain and culture the microalga (Guillard, 1975) in an environmental chamber (Adaptis A350, Conviron, Canada). The microalga was acclimated to the test condition for four days prior to the experimentation (Roseth et al., 1996). For *T. japonicus*, it was maintained and cultured in filtered artificial seawater with the alga *Chaetoceros gracilis* as food (Lee et al., 2007, 2013). The copepod was acclimated in the test environment for 12 h prior to testing. During the acclimation period, the newly hatched nauplii were collected for the experimentation (OECD, 2004). Table S1 presented the conditions of acclimation and culture of each test species.

Monodonta labio and *X. securis* were collected from Tai Tam and Ma Liu Shui in Hong Kong, respectively. They were acclimated at 25.0 °C ± 0.5 °C with a photoperiod of 12 h: 12 h light: dark in filtered artificial seawater for 14 and 7 days, respectively, prior to experimentation. Light intensity was approximately 100 foot-candles of continuous cool-white fluorescent light. Shell length of both *M. labio* and *X. securis* and shell height of *X. securis* were measured prior to the experimentation.

Cysts of *A. franciscana* and embryos of *O. melastigma* were cultured at the State Key Laboratory of Marine Pollution in City University of Hong Kong. Instar 2–3 stages of *A. franciscana* and fish embryos at 4 h post-fertilization were used for experimentation, respectively.

2.3. Toxicity tests

The 72-h algal growth inhibition test on *I. galbana* and 96-h acute toxicity tests on *T. japonicus, A. franciscana*, and embryos of *O. melastigma* were performed based on the OECD guidelines No. 201, 202 and 236, respectively, with some minor modifications (OECD, 2004, 2011, 2013). The 96-h acute toxicity tests on *M. labio* and *X. securis* were performed based on the experimental conditions of previous studies with some modifications (Astudillo et al., 2017; Wang et al., 2020). Toxicity tests of a duration less than 24 h on microalga and 7 d on fish embryos and invertebrates were classified as acute tests (Warne et al., 2018). All experiments were conducted in a static condition while all test organisms were not fed during the experiment. Methanol was used as solvent control (0.5% methanol in volume) in the experiment. Details of test conditions, treatments for each test species and the number of replications are presented in Table S2. Toxicity tests on animals were considered valid only if the survival of the animals in the control and solvent control groups was greater than 90%.

The initial cell density of the marine microalga in test chamber was 10^5 cells/mL which was determined by using a hemocytometer (Neubauer Improved, Precicolor HGB, Germany) and a compound microscope (Olympus CHK, Japan) at 400x magnification. During the test period, algal cell density was determined everyday by sampling 1 mL of algal culture from each replicate, and preserving with 0.01 mL Lugol's solution prior to cell counting (cell counter: Multisizer II, Coulter, Fullerton). For marine animals culturing in a well-plate or beaker, individual well or beaker was considered as an independent replicate.

2.4. Determination of end-points

The calculation of growth inhibition of the marine microalga was based on the OECD guidelines No. 201 (OECD, 2011). Details of calculation refer to Yeung et al. (2020a). The mortality of *T. japonicus, A. franciscana* and *O. melastigma* embryos was determined once every 24 h under a stereomicroscope (Zoom 2000, Leica, Germany). The mortality of *T. japonicus* was defined as its immobility upon a stimulation by a gentle water flow (Kwok and Leung, 2005) while the mortality of *A. franciscana* was observed for its total immobilization when no movement was noted in 10 s of observation (Kalčíková et al., 2012). The mortality of *M. labio* was confirmed by maintaining putatively dead animals in artificial seawater, and those without any sign of movement after 24 h were assumed to be dead (Lam, 1996). The mortality of *X. securis* was determined by the failure of an individual to respond to probing by forceps, and when their shells were open and they were no longer filtering (Astudillo et al., 2017). For *O. melastigma* embryos, its mortality was determined based on four observations, including the coagulation of the embryo, the lack of somites, the lack of detachment of the tail and the lack of heartbeat (Belanger et al., 2013; Braunbeck et al., 2015).

Concentration-response relationships on the test species were constructed using the inhibition and mortality data for marine microalga and marine animals, respectively, against the corresponding measured

concentration of at-RA. Algal growth inhibition was calculated in comparison to the control, while relative mortalities of test animals were presented in the treatment groups with the adjustment based on the mortality of control groups. The median effect concentration (EC_{50}) and lethal concentration (LC_{50}) for marine microalga and marine animals, respectively, were determined using Prism (Version 7.0a, GraphPad, San Diego, CA, USA).

2.5. Chemical analysis of at-RA

The concentrations of at-RA in each replicate were measured before the experiment. Test solutions of 1 mL were collected from each replicate and at-RA was extracted thrice with dichloromethane. The extracts were combined and dried with nitrogen. The extracts were redissolved in acetonitrile for analysis using high-performance liquid chromatography - tandem mass spectrometry. Details of chemical analysis including quality assurance and control can be referred to Zhou et al. (2019b) and Yeung et al. (2021). At-RA can be isomerized instantly in seawater (Zhou et al., 2021), the recovery for total RAs including at-RA and its isomers is 90% ± 9%. The limit of quantitation for at-RA is 0.16 μg/L.

2.6. Species sensitivity distribution

Chronic toxicity endpoints were obtained from acute LC_{50}s of at-RA on marine animals' toxicity test and chronic EC_{50} of at-RA on the 72-h algal growth inhibition test through dividing by an assessment factor of 10 and 5, respectively (Warne et al., 2018). A species sensitivity distribution (SSD) was then constructed by plotting the log measured concentration of at-RA (x-axis) against the rank-assigned centile of converted chronic toxicity endpoints of at-RA (y-axis) obtained from the present study using the USEPA's SSD generator. A hazardous concentration for 5% of species (HC_5) was determined from a log-normal distribution model (Jin et al., 2013; Wheeler et al., 2002a). An interim predicted no-effect concentration (PNEC) was then derived by dividing HC_5 with an assessment factor of 5 (EC, 2003). The SSD of at-RA for marine species constructed in the present study was compared with that for freshwater species reported in Yeung et al. (2020b).

2.7. Ecological risk assessment

Adopting a similar approach of Yeung et al. (2021), hazard quotients (HQs) were computed to assess ecological risks of the studied RAs, in terms of at-RA equivalents (at-RA EQs), in the marine environment of Hong Kong. The selection of Hong Kong only was because no relevant data of measured environmental concentration of at-RA was available in other marine environments or at-RA was not detected in other marine environments (Wu et al., 2010). At-RA EQs were derived from the at-RA equivalency factors (RAEFs), the same values used in Yeung et al. (2020b), i.e., 0.15, 0.04, 3.87, 0.46 and 0.46 for 9c-RA, 13c-RA, at-4-oxo-RA, 9c-4-oxo-RA and 13c-4-oxo-RA, respectively (Wu et al., 2010; Zhen et al., 2009). The measured environmental concentrations (MECs) of RAs and 4-oxo-RAs in seawater from the coastal environment of Hong Kong near the sewage treatment plants (STPs; Zhou et al., 2019b) and the predicted environmental concentrations (PECs) of the six studied RAs in seawater estimated through their concentrations in treated effluent discharged from STPs with the adoption of 10-fold dilution were applied (Yeung et al., 2021). The expression and conversion details of M/PECs refer to Yeung et al. (2021). The computed ecological risk of RAs to marine environment was classified into three tiers: low, medium and high based on the HQ values of $0.01 \leq HQ < 0.1$, $0.1 \leq HQ < 1$ and $HQ \geq 1$, respectively (Hernando et al., 2006).

3. Results and discussion

3.1. Toxicity of at-RA

The 72-h EC_{50} of at-RA to the marine microalga *I. galbana* was 1.3 mg/L while the 96-h LC_{50}s of at-RA to the copepod *T. japonicus*, the brine shrimp *A. franciscana*, the gastropod *M. labio*, the mussel *X. securis* and the fish embryo *O. melastigma*, were 1.1, 0.90, 2.1, 1.4 and 0.11 mg/L, respectively (Fig. 1; Table 1). Mortalities of control and solvent control groups of all test animals were below 10% which fulfilled the requirement of a valid toxicity experiment (OECD, 2004). All test species were relatively insensitive to at-RA for acute exposures since mortalities were only observed at the higher levels of at-RA. It might be because at-RA supports various biological functions and development in organisms and thus certain levels of at-RA would be actually vital but not toxic to them (Collins and Mao, 1999).

The fish embryos *O. melastigma* were found to be the most sensitive towards at-RA among the six test species in the present study, which was consistent with the finding of a previous study that the embryo of zebrafish *Danio rerio* was more sensitive than other species, such as embryos of amphibians *Xenopus tropicalis*, *X. laevis*, *Rana septentrionalis* and *R. clamitans* (Yeung et al., 2020b). The growth of *I. galbana* was only slightly inhibited by the exposure of at-RA, indicating that the microalga was relatively less sensitive to at-RA exposure. Previous studies showed that cyanobacteria and algae could produce RAs, suggesting that cyanobacteria and algae could be one of the sources of RAs contributing to the aquatic environment (Priebojová et al., 2018; Wu et al., 2012; Zhou et al., 2021). Hence, it would be reasonable that the test microalgal species was not very sensitive to at-RA because they were adapted in their cells with considerable amount of RAs. It was not surprising to find that the gastropod *M. labio* was the most tolerant species to at-RA because this species could be responsive to stimuli by closing its operculum to prevent the pollutants to enter. Similar protective behavior was observed in freshwater species exposed to toxicants (Kamble and Kamble, 2014). The closure of operculum might help *M. labio* to reduce their exposure of at-RA. We also observed that the bivalve *X. securis* was tolerant to at-RA as it is a species which could adapt a wide range of environmental conditions such as thermal and salinity stress (Astudillo et al., 2017; Morton and Leung, 2015). Previous studies found that this species could survive and reproduce in high abundance in inner Tolo Harbour and Deep Bay where these places were known to be highly polluted with nutrients and various chemical contaminants in Hong Kong (Lau et al., 2018; Morton and Leung, 2015), implying that this bivalve species was tolerant to high levels of contaminants and able to survive in eutrophic waters.

RAs are sensitive to environmental conditions, such as heat and light, due to the presence of conjugated bonds such that different forms and isomers often co-exist in the environment (van Breemen et al., 1998). At-RA is the focus of this study because it is the most widely distributed form in the environment (Herrmann, 1995). Previous study reported that only 30% of at-RA was detected in the artificial seawater even it was extracted immediately (Zhou et al., 2021). Other forms of RAs, i.e., 9c-RA, 13c-RA, at-4-oxo-RA, 9c-4-oxo-RA and 13c-4-oxo-RA, were detected at various amount in seawater samples, implying that at-RA could be isomerized and metabolized instantly in seawater (Zhou et al., 2021). However, the information on the transformation and degradation of various forms of RAs in seawater was scarce. Further study on the speciation and degradation of at-RA in seawater is warranted to better understand the exposure of at-RA to marine species.

3.2. Species sensitivity distribution of at-RA

Based on the estimated chronic EC values from toxicity endpoints for the six marine species from four taxonomic groups obtained from the present study, a species sensitivity distribution (SSD) was constructed (Fig. 2). The calculated HC_5 was determined to be 0.012 mg/L

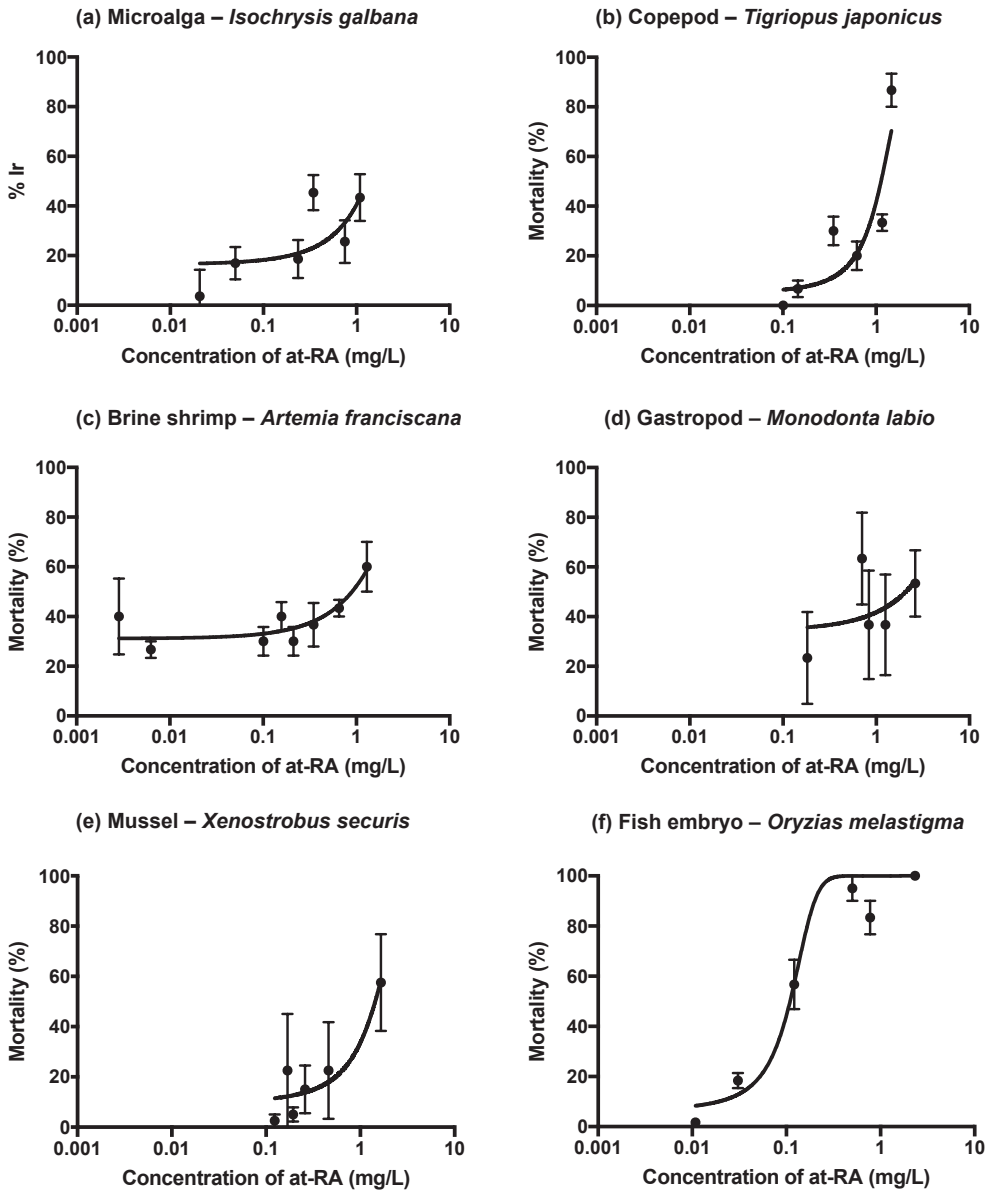

Fig. 1. Concentration-response relationships of measured all-*trans* retinoic acid for (a) the percentage inhibition of growth rate (%Ir) of the marine microalga *Isochrysis galbana* for 72 h ($n = 4$); the relative mortality of (b) the copepod *Trigiopus japonicus* for 96 h ($n = 3$); (c) the brine shrimp *Artemia franciscana* for 96 h ($n = 3$); (d) the marine gastropod *Monodonta labio* for 96 h ($n = 3$); (e) the mussel *Xenostrobus securis* for 96 h ($n = 4$); and (f) the fish embryo *Oryzias melastigma* for 96 h (n = 6) (mean ± SE). Algal growth inhibition was calculated in comparison to the control (a), while relative mortality presented in (b) to (f) was adjusted based on the mortality of the respective control group.

(0.01157 mg/L; 95% confidence interval: 0.002–0.075 mg/L) from the SSD while an interim PNEC for marine organisms was determined to be 2300 ng/L after being corrected by an assessment factor of 5 and rounded up (EC, 2003) (i.e., PNEC = (0.01157 × 1000 × 1000) ng/L ÷ 5 = 2314 ng/L).

Although a few marine toxicity data of RAs were available from the USEPA's ECOTOX database and published literature, including toxic effects on juvenile *P. olivaceus* (Haga et al., 2002), *R. clavigera* (Horiguchi et al., 2008; Nishikawa et al., 2004; Zhou et al., 2021), *C. gigas* (Vogeler et al., 2017) and *P. mammillata* (Groppelli et al., 2007), these data were considered inappropriate to be included in the construction of the SSD of the present study due to the following reasons. First, the effects of RAs were examined through the injection of chemical (i.e., 9c-RA) to *R. clavigera* directly (Horiguchi et al., 2008; Nishikawa et al., 2004). This is different from the present study which focused on waterborne exposure. Secondly, only one or two concentrations were used in previous experiments to reveal the acute effects of RAs on marine species. Although lower jaw deformity in *P. olivaceus* (Haga et al., 2002), abnormal organ and tissue development in *C. gigas* (Vogeler et al., 2017); abnormal pigment of sensory organs in *P. mammillata* (Groppelli et al., 2007) and upregulation of imposex-related genes in *R. clavigera* (Zhou et al., 2021) were observed, only lowest observed effect concentrations (LOECs) or no observed effect concentrations (NOECs) could be derived due to the few concentrations used in their experiments. The use of NOECs and LOECs have been criticized because their derivations greatly depended on the experimental design, especially the chemical concentrations and statistical analysis used in the study (Warne and Van Dam, 2008). Thus, they are not recommended to be used any further to represent the ecotoxicity of chemicals. The use of EC_x to represent the ecotoxicity data was suggested as a better alternative to replace the NOECs and LOECs data (Warne and Van Dam, 2008; Zhou et al., 2019a), and hence EC_x was adopted in the present study. Therefore, the toxicity data of RAs on marine species from ECOTOX database and published literature were not included to ensure consistency of the data used to construct the SSD and its reliability (Wheeler et al., 2002a).

Comparing the PNEC (3.93 ng/L) derived in Yeung et al. (2020b) that was based on acute toxicity tests on five freshwater species from two taxonomic groups, the marine species investigated in the present study were less sensitive to at-RA exposure than the freshwater species (Fig. 3). This agrees with the previously identified phenomenon that

Table 1
Median effect concentration (EC_{50}) or lethal concentration (LC_{50}) from toxicity tests of the measured all-*trans*-retinoic acid on six marine species ($n = 4$ for microalga and mussel, $n = 3$ for copepod, artemia and gastropod; $n = 6$ for fish embryo). Acute-to-chronic ratios were applied to E/LC_{50}s to obtain chronic effect concentrations (i.e., chronic EC values) which were then used to construct species sensitivity distribution.

Taxa	Test species	Endpoint	Control mortality (%)	Solvent control mortality (%)	Concentration-response equation	r^2	E/LC_{50} (SE; mg/L)	Chronic EC value (mg/L)
Alga	*Isochrysis galbana*	Growth inhibition	NA	NA	$y = 100/(1 + 10^{((1.31 - x) * 0.5379)})$	0.22	1.3 (0.4)	0.26
Crustacean	*Tigriopus japonicus* (nauplii)	Mortality	3.3	3.3	$y = 100/(1 + 10^{((1.131 - x) * 1.134)})$	0.75	1.1 (0.1)	0.11
	Artemia franciscana (nauplii)	Mortality	3.3	3.3	$y = 100/(1 + 10^{((0.8985 - x) * 0.3833)})$	0.33	0.90 (0.21)	0.090
Mollusc (Gastropod)	*Monodonta labio*	Mortality	3.3	3.3	$y = 100/(1 + 10^{((2.078 - x) * 0.1348)})$	0.045	2.1 (1.6)	0.21
Mollusc (Bivalve)	*Xenostrobus securis*	Mortality	0.0	2.5	$y = 100/(1 + 10^{((1.44 - x) * 0.6736)})$	0.28	1.4 (0.3)	0.14
Fish	*Oryzias melastigma* (embryo)	Mortality	5.0	5.0	$y = 100/(1 + 10^{((0.1095 - x) * 10.55)})$	0.86	0.11 (0.01)	0.011

NA, not applicable
y, % of affected test population
x, log-transformed at-RA
SE, standard error

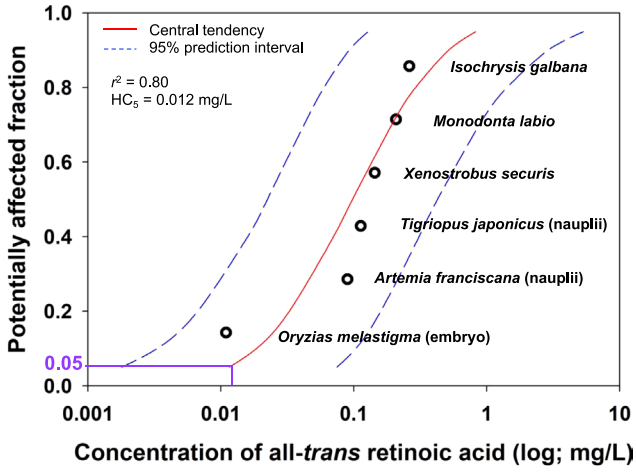

Fig. 2. Species sensitivity distribution of the all-*trans* retinoic acid constructed based on the toxicity results on six species from the present study after applying acute-to-chronic ratios of 10 and 5 on marine animals and marine microalga, respectively (See Table 1 for the detailed toxicity values).

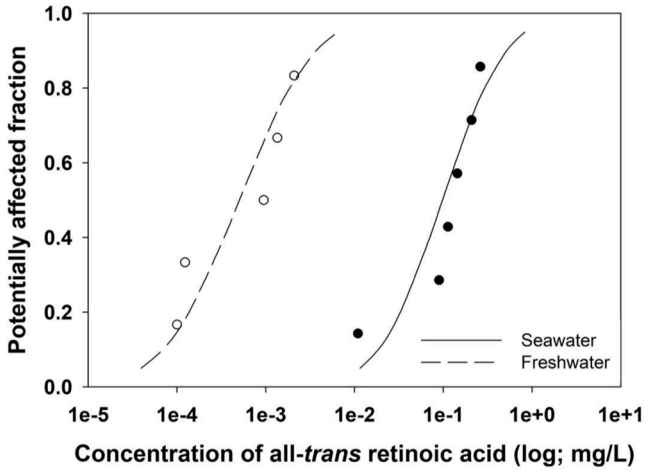

Fig. 3. Species sensitivity distributions for all-*trans* retinoic acid on the data for freshwater (dotted line; obtained from previous literature) and seawater (solid line; obtained from the present study) species, with the application of acute-to-chronic ratios of 10 and 5 on aquatic animals and microalga, respectively.

freshwater species were generally more sensitive than the marine counterparts when exposing to chemicals such as metals and ammonia (Leung et al., 2001; Wheeler et al., 2002b). It implied that the freshwater-specific PNEC of at-RA was more conservative than the interim marine-specific PNEC. It should also be noted that there were only five species of two taxonomic groups, i.e., amphibian and fish, in the freshwater SSD whereas there were six species of four taxonomic groups in the marine SSD. The marine SSD covered a wider spectrum in terms of taxonomic groups as well as species. This case was uncommon because there were generally more toxicity data for freshwater species than those of marine species, and risk assessment of chemical pollutants was more generally conducted on freshwater species (Wheeler et al., 2002b). Although there could be many factors affecting the SSDs of freshwater and seawater species, the wider coverage of taxonomic groups and the higher number of species in each taxonomic group would certainly contribute to a more representative and robust SSD. Therefore, more toxicity data on both marine and freshwater species at different taxonomic groups are warranted to enrich the databases and enable a more reliable comparison. Given the limited taxonomic representation especially in the freshwater SSD, the result of the current preliminary comparison (i.e., freshwater species more sensitive than marine species) should be viewed with caution.

3.3. Environmental implications

In the present study, toxicity tests of at-RA were conducted on six marine species to fill the data gap of toxicity of RAs towards marine organisms. The ecological risk of the six studied RAs to marine organisms was also assessed based on the available data. All HQs determined for seawater and effluent discharges from STPs were less than 1, with a range between 0.00040 and 0.0029 (Table 2). According to the risk ranking criteria (Hernando et al., 2006), there were no immediate risks in the seawater near the STPs and along the coasts of Hong Kong which were comparable with previous studies where the computed risks were low to medium (Yeung et al., 2021; Zhou et al., 2019b). One point should be noted that the interim marine-specific PNEC was used to assess the ecological risk in the present study whereas only a freshwater-specific PNEC was available to conduct ecological risk assessment in previous studies (i.e., Yeung et al., 2021; Zhou et al., 2019b).

However, more taxonomic groups besides the four included in this study could be further evaluated to enrich and consolidate the SSD. It

Table 2
Hazard quotients (HQs) for retinoic acids and their metabolites (expressed as at-RA EQs) detected in marine environments and predicted with an assumption of dilution factor of 10 based on effluents of sewage in Hong Kong.

Country	Location	M/PEC (at-RA EQs; ng/L)	HQs	Risk classification	References
China	Hong Kong – Coastal waters	3.6–6.7	0.0016–0.0029	Little	Zhou et al. (2019b)
	Hong Kong – Effluent discharged from sewage treatment plants	0.93–2.4	0.00040–0.0011	Little	Yeung et al. (2021)

MEC, measured environmental concentration.
PEC, predicted environmental concentration.
at-RA EQs, at-RA equivalents are the sum of the concentration of six RAs and 4-oxo-RAs detected in the study multiplied by their respective all-trans retinoic acid equivalent factor which was developed to normalize the concentration of each compound to an equivalent concentration of at-RA.

also remained unclear how the species would respond to low or environmentally relevant concentrations of various RAs and 4-oxo-RAs for prolonged chronic exposure. Therefore, more marine species should be included as test species in the future studies. Long-term effects of the studied RAs posed to marine species, such as full life-cycle or multi-generation studies, are also warranted to enrich the toxicity database. Moreover, further investigations on revealing the mode-of-toxic action and toxic mechanism of at-RA and other retinoic acids would be useful for improving our understanding of their toxicity on marine species.

Assumptions were made for conducting the ecological risk assessment of RAs in the present study. Firstly, all toxicity experiments in the present study were conducted under controlled environmental conditions or optimal conditions, including fixed temperature and salinity (Versteeg et al., 1999) and only single species and single chemical were tested at one time. Laboratory-based toxicity experiments were able to ensure the consistency of experiments conducted among different species, and reproducible result at different trophic levels for ease of comparison and derivation of SSD (Leung et al., 2014). Yet, conditions could be much more complex in the natural environment where other chemicals and microorganisms present in natural seawater would affect the fate and speciation of at-RA. The actual toxicity of RAs towards marine species may be underestimated as there could be additive and/or synergistic effects of at-RA with other chemicals, e.g., triphenyltin, present in the seawater (Leung et al., 2014; Zhou et al., 2021). Natural microorganisms present in natural seawater may accelerate the degradation rate of at-RA as the possible presence of biodegradation, and oxidation and isomerization due to complicated naturally environmental conditions (van Breemen et al., 1998). Therefore, further studies on the combined effect of at-RA with other chemicals and the degradation and fate of at-RA in both artificial seawater and natural seawater are warranted to better estimate the actual toxicity of RAs towards marine species.

Secondly, a dilution factor of 10 was applied (EC, 2003) to the treated effluent discharged from STPs to estimate the concentrations of the six chemicals in seawater and for the assessment of the ecological risks of the six studied RAs to marine organisms (Yeung et al., 2021). Previous study demonstrated that the STPs in Hong Kong could only partially remove of the six studied RAs and thus it was believed that the effluent of STPs was one of the major sources of these compounds into the marine environment (Yeung et al., 2021; Zhou et al., 2019b, 2020). Besides treated effluent from STPs, there were also other sources of concerned retinoids contributing the marine environment (Yeung et al., 2021). Monitoring the background concentration and evaluating ecological risks of RAs and 4-oxo-RAs both spatially and seasonally are thus required to further elucidate their potential impacts to the marine environment.

4. Conclusions

The present study filled the gap of insufficient toxicity data of marine species towards the exposure of at-RA. The study revealed the toxicity effects on six marine species from four major taxonomic groups. Of the six species, the embryo of medaka fish *Oryzias melastigma* was the most sensitive with LC_{50} of 0.11 mg/L while the gastropod *Monodonta labio* was the least sensitive with LC_{50} of 2.1 mg/L. Based on the species sensitivity distribution (SSD) constructed for marine species to the exposure of at-RA in this study, the HC_5 was determined to be 0.012 mg/L while the interim PNEC was 2300 ng/L. This derived interim marine-specific PNEC was greater than the freshwater-specific PNEC, concluding that the freshwater species was more sensitive than marine species towards RAs. By evaluating the hazard quotients, no immediate risks were indicated for the concerned RAs to the coastal marine ecosystem in Hong Kong. Further investigations on adverse effects of RAs and their metabolites on more marine species from diverse taxonomic groups are needed to better understand the environmental risk of this group of chemicals in the marine environment.

CRediT authorship contribution statement

Katie W. Y. Yeung: Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Racliffe W. S. Lai:** Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Guang-Jie Zhou:** Methodology, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Kenneth M.Y. Leung:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113455.

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