Visualizing ecotoxicology's extrapolation dilemma:

A discussion of case study data for 17α-ethynylestradiol (EE2) and implications for Ecological Risk Assessment

by

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ABSTRACT

Predicting and preventing negative environmental impacts from exposure to human-related chemicals stressors is necessary for long-term preservation of global biodiversity and ecosystem function. Anticipating the effects of chemical pollutants, however, remains a pervasive challenge for ecotoxicologist and environmental risk assessors who must rely on controversial extrapolation methods to extend toxicity predictions from a handful of short-term lab studies for standard test organisms to long-term chronic effects for entire ecosystems. Using data from US EPAs ECOTOX database for a synthetic estrogen, 17α-ethynylestradiol (EE2), I illustrate these extrapolation challenges and discuss potential implications for ecological risk assessment and environmental management. Analyses with EE2 data show there is limited opportunity to compare or validate effects across lab and field toxicology studies. Endpoints and effect concentrations for EE2 do not vary predictably within species or among related species, and well-studied test organisms tend to have a lower minimum and overall wider range of effect concentrations than less-studied organisms. Additionally, results from a multi-year whole-ecosystem field experiment demonstrates how species sensitivity distribution curves do not account for interspecific interactions and may therefore result in an underestimation of ecosystem level effects.

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GLOSSARY

ECx	Effective concentration for X% of organisms
EE2	17α-ethynylestradiol
ERA	Environmental Risk Assessment
LOEC/LOEL	Lowest observe effect concentration/level – the lowest test concentration which results in an effect that is statistically different from the control effect
MATC	Maximum acceptable toxicant concentration – the geometric mean between the LOEC/L and NOEC/L values
NOEC/NOEL	No observed effect concentration/level – the highest test concentration which results in an effect that is not statistically different from the control effect
SSD	Species Sensitivity Distribution
US EPA	United States Environmental Protection Agency
HC5	Hazardous concentration for 5% of species

1. INTRODUCTION

Human civilization has introduced novel chemical stressors to the natural environment. Predicting and preventing negative environmental impacts from exposure to these chemicals stressors is necessary for long-term preservation of global biodiversity and ecosystem function. The Ecological Risk Assessment (ERA) framework was developed in the 1980s and 90s by the US Environmental Protection Agency (US EPA) as a systematic process for determining the determine the "likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors" (US EPA, 1992). Early proposals of the ERA process included an emphasis on probabilistic outcomes of specific effects and expressed the necessity of considering impacts of chemical stressors at multiple levels of ecological organization including individual, community, and ecosystem levels (Suter II et al., 2003). However, this probabilistic, ecology-focused approach was ostensibly unconducive to regulatory decision-making, and so a framework similar to the already established Human Risk Assessment framework was adopted which primarily focuses on predicting individual survival and reproduction outcomes.

Today, the majority of ERA effect characterization's do not consider effects of chemical stressors on communities or ecosystems (Dearfield et al., 2005; Suter II et al., 2003). The process instead relies on extrapolating from molecular-level effects for a small number of tested species to determine a Predicted No Effect Concentration which aims to be protective of all species in the management system. This dependence on short term, lab-based, sub organism-effect toxicity studies from only a few standard test species, however, increases uncertainty and exacerbates the challenge of extrapolating to long-term population-level impacts.

The increasing use of short-term, standardized lab studies stems from the desire for reproducible results and low cost, rapid data generation. Time and resource constraints can be partially attributed to the high demand for chemical risk assessment. Currently, there are over 160 million chemicals registered by the Chemical Abstracts Service (CAS) which maintains a near comprehensive database of chemical substances (CAS, 2020). The US EPA has a registry of over 85,000 chemical substances and receives new chemical submissions each day which require human and ecological risk assessment screening or testing within a 90-day period (US EPA, 2019). The US EPA has 290 chemical substances currently under review and thousands more remaining untested due to the grandfathering clause of the Toxic Substances Control Act of 1976 which exempted all chemicals currently used in production from testing (US EPA, 2017). If screening or risk assessment testing is not completed within the 90-day window, then

the chemical is allowed to be put into production. There is, therefore, a time constraint. Faster risk assessment process means reducing the backlog and preventing potentially harmful new chemicals from being produced once the US EPAs 90-day testing window expires. Standardized, short-term lab studies with standard test organisms can save the regulatory agencies time and money, but these time and money saving measures can also make ecotoxicology's extrapolation dilemma even more daunting.

Therefore, most environmental risk assessors have access to only a handful of standardized, short-term lab tests from a few standard test organisms which most likely does not include the most sensitive species or effects. In risk assessments, standard "uncertainty factors" or "assessment factors" are used similar to a unit conversion factor to adjust for the uncertainty or to extrapolation from short term data to long-term effects. Species Sensitivity Distributions (SSD) are another popular extrapolation strategy used by risk assessors to predict the percent of total species affected at different chemical concentration based on the available toxicity data. Frequently, an HC5 threshold, or Hazardous concentration for 5% of species, is considered an acceptable level of risk. Use of defensible extrapolation methods can help to screen out chemicals that do not require additional testing, eliminate unnecessary animal testing, and lower time and resource costs. If not validated, however, these extrapolation factors can lead to over- or underestimation of potential risks resulting in unnecessary time and resource use or inadequate environmental protection, respectively (Forbes & Calow, 2002; Rohr et al., 2016). Additionally, neither of these extrapolation methods (extrapolation factors or SSDs) are capable of anticipating indirect effects resulting from interspecific interactions or food web effects.

Since the birth of ecotoxicology and ERAs, many researchers have questioned the "ecological realism" of risk assessment approaches (Chapman, 2002; Forbes & Calow, 2002; Rohr et al., 2016; Vighi & Villa, 2013). The bias towards the generation of short-term lab studies increases uncertainty and requires more extrapolation to anticipate ecologically relevant effects. Researchers have criticized the use of indefensible extrapolation factors (Forbes & Calow, 2002; Rohr et al., 2016) and the use of unvalidated sub-organism level effects (aka biomarkers) in environmental risk assessment (Holdway, 1996). Current methods in ERA may be standardized, reproducible, and cost effective, but they are still unable to capture the "ecological reality" of the impacts of environmental contaminants on higher levels of organization. By relying on simple extrapolation methods and ignoring intraspecific interactions we can achieve a simple answer to our risk assessment questions, but is it the right answer?

Using a case study approach, the following analysis illustrates the multidimensional extrapolation challenge faced by ERAs using a comprehensive set of aquatic toxicity data from US EPA's ECOTOX database. This analysis uses 3,437 ECOTOX aquatic toxicity records for a synthetic estrogen, 17α-ethynylestradiol (EE2), to (1) illustrate these extrapolation challenges that result from study design and biological variability, (2) explore how these challenges can impact decision-making based on species sensitivity distribution curves, and (3) synthesize key takeaways and future directions for related inquiries into ERA extrapolation and ecological realism.

1.1 Case Study: What is EE2?

All vertebrates and some insects synthesize their own estrogen hormones. EE2 is a synthetic estrogen mimic estimated to be between 20 to 600 times as potent as biogenic forms of estrogen (estrone (E1), 17β-estradiol (E2), and estriol (E2)) (Laurenson et al., 2014). EE2 is the active ingredient in modern estrogen contraceptive pills and human estrogen therapy treatments and is also used as a veterinary growth enhancer (Aris et al., 2014). EE2 excreted by humans and other animals travels into wastewater systems but is not fully removed by wastewater treatment processes. Fish aquaculture industries use EE2 to raise female-only fish populations; release of untreated wastewater from these operations also presents a concerning source of EE2 input to the environment (Aris et al., 2014). Consequently, EE2 has been detected in wastewater effluents, with higher concentrations in urban areas and near intensive agricultural or aquaculture operations (Aris et al., 2014). Due to its continuous use across multiple sectors, EE2 is continuously released to the environment resulting in relatively consistent, low level chronic exposures which may be high enough to cause effects in aquatic environments (Corcoran et al., 2010).

Once in the environment, EE2 can accumulate in sediments (Matozzo et al., 2008) can bioaccumulate in organisms. There is also evidence that EE2 may biomagnify at higher trophic levels within a foodweb (Dussault et al., 2009). EE2 is a powerful endocrine disruptor which can cause adverse effects in aquatic organisms at very low (ng/L) levels. Well-documented effects of EE2 exposure in fish include altered sex ratios, vitellogenin production in males, decreased fecundity, and effects on growth and survival very low (ng/L) environmental concentrations (Aris et al., 2014). EE2 is not the only endocrine disrupting chemical present into aquatic systems due to anthropogenic pollution, and publications from around the globe have documented toxicity effects of estrogen disrupting compounds in wild fish populations in the UK (Allen et al.,

1999; Jobling et al., 1998), USA (Folmar L C et al., 1996), France (Minier et al., 2000), Italy (Viganò et al., 2001), The Netherlands (Vethaak et al., 2002), Germany (Gercken & Sordyl, 2002), Japan (Hassanin et al., 2002), and Canada (Aravindakshan et al., 2004).

Measurable EE2 concentrations in water bodies, sediment, and/or biota have been reported in at least 12 countries, but concentrations are highly variable ranging from below the device detection limit to 34 ng/L (Aris et al., 2014; Laurenson et al., 2014). Data is sparse because routine testing is not required, and detection limits of instruments may prevent capturing biologically-relevant concentrations which can occur at sub-nanogram per liter concentrations. Currently environmental EE2 concentration are unregulated globally, but several countries including the US are considering setting a water quality standard for aquatic environments due to the concern for ecosystem health, particularly fish populations.

2. METHODS

2.1. Data

All data used in this analysis are publicly available through the United States Environmental Protection Agency's (US EPA) ECOTOX online database. Information on limitations and criteria for inclusion in the ECOTOX database can be accessed online at https://cfpub.epa.gov/ecotox/. All "Aquatic" records for "17alpha-Ethinylestradiol" (CAS# 57636) were downloaded from the ECOTOX web interface on September 30, 2019. This included 4,392 total records. All data wrangling, analysis, and visualization was completed using R (version 3.6.2).The data were filtered to remove studies using dietary exposures (186 records removed) because dietary concentrations expressed as EE2 mass per mass of organism body weight could not be converted EE2 mass per volume of water. For records which did not report a mean concentration (n = 244), the average of the minimum and maximum reported concentrations was used as a proxy for mean concentration. A similar approach was used for study duration when mean study length was not reported (n = 250). For this study, we are only interested in sublethal endpoints, and so all lethal endpoints (e.g. LC50) as well as bioconcentration/bioaccumulation factors were excluded from the dataset (135 additional records removed). The most common endpoints were No Observed Effect Concentration (NOEC) (n = 1671) and Lowest Observed Effect Concentration (LOEC) (n = 1,144) which together made up about 82 percent of the total entries (Table 1). After filtering, 3,437 records from 292 publications and 78 species remained.

Table 1. Summary of endpoints used in analysis from the US EPAs ECOTOX database for 17α-ethynylestradiol (EE2).

Endpoint	*No. of records*
NOEC	1,671
LOEC	1,144
LOEL	284
NOEL	265
EC50	34
EC10	25
EC90	6
MATC	4
EC20	3
EC100	1
Total	3437

2.2. Species Sensitivity Distribution (SSD) Analysis

Code for creating the species sensitivity distribution and HC5 analysis was adapted from Szöcs (2015). First, the lowest effect concentration for each species was selected using the same dataset described above resulting in a single effect concentration for each of the 77 species in the database (Appendix 1). Next, each species was assigned a rank between 0 and 1 according to their effect concentration. Each species was plotted with the most sensitive effect concentration on the x axis and the ranking, or fraction of species affected, on the y axis. Next, a lognormal cumulative probability function was fitted to the points to predict the total percent of species affected at each concentration of EE2. The HC5 (Hazardous Concentration 5%) is defined by the concentration of EE2 at which the resulting log-normal curve reaches 5% of all species affected. To calculate the Predicted No Effect Concentration (PNEC), an assessment factor of 1 was applied to the HC5 due to the high number of species and study types included in the SSD analysis.

3. VISUALIZING EXTRAPOLATION CHALLENGES OVER MULTIPLE DIMENSIONS

3.1. Acute toxicity studies require more extrapolation to anticipate real-world effects than long-term studies conducted at environmentally-relevant study concentrations

For an environmental pollutant like EE2 which may be present at low concentrations for long periods of time, research studies conducted over a significant portion of an organism's lifespan (often called sub-chronic exposure) will require less extrapolation to estimate the long-term effects of real-world exposure. However, sub-chronic experiments require more time and money than short term lab toxicity testing and therefore are much less common. This bias towards shorter study durations and acute toxicity testing is reflected in the EE2 ECOTOX records.

Study length differed significantly by test location (Kruskal-Wallis, $p < 0.001$; Fig 1A). Lab studies include a wide range of study lengths from just over 2 minutes to 730 days with a median study length of 21 days. Artificial field and natural field studies have significantly longer study lengths than lab studies, and natural field studies are longer than artificial field studies (Pairwise Wilcox test, a $p < 0.01$). Median study length for artificial field studies was slightly longer than lab studies at 42 days, and median study length for natural field studies is 365 days. Using the US EPAs definition of chronic exposure for non-human animals, then only 18.4% of lab studies considered chronic exposure durations (US EPA, 2011).

Effect concentrations also differed significantly across test location (Kruskal-Wallis, $p < 0.001$; Fig 1B). Concentrations of EE2 used in lab studies span a full 10 orders of magnitude from 0.03 ng/L to 1×10^9 ng/L with a median of 15.5 ng/L. Natural field studies, however, used only a very narrow range of EE2 concentrations between 4.8 ng/L and 6.3 ng/L, and artificial field studies were somewhere in between with concentrations ranging from 20 ng/L to 12.8×10^4 ng/L. Median concentrations differ significantly by test location (Pairwise Wilcox test, all $p < 0.001$). Test concentrations and test duration are interdependent—if tests must be run in a shorter period of time, then higher (less environmentally relevant) test concentrations must be used. So it is no surprise that the lab studies also tended to use less environmentally relevant testing concentration than the long-term field studies. In this analysis, 58.1% of lab studies were conducted at environmentally relevant concentrations (as defined by the maximum measured aquatic concentrations of EE2 reported in Laurenson et al., 2014).

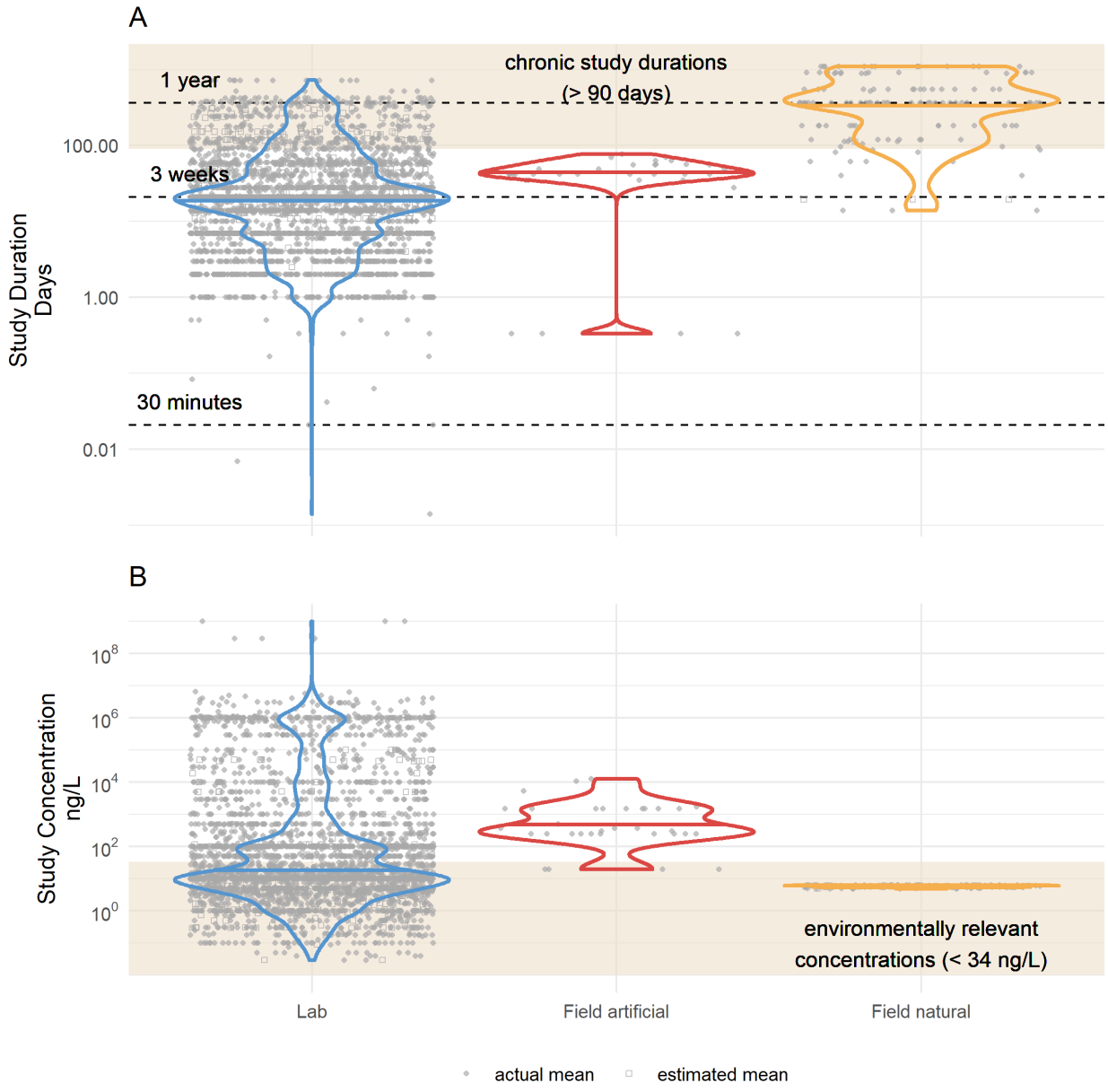

Fig 1. Distribution of mean study durations (A) and study concentrations (B) of all aquatic toxicology records for EE2 in the ECOTOX database. Wider sections of the violin plots correspond to a greater density of records at that y axis value, and the horizontal line indicates the median study length/concentration. Each point represents a single record in the database corresponding to a unique combination of published study, test species, test concentration, effect measurement, and endpoint. Reported and estimated means are shown as filled and open symbols, respectively.

Natural and artificial field studies which use environmentally relevant exposure concentrations and long study durations offer an opportunity to measure the effects on organisms in more “realistic” scenarios, and thus may offer an opportunity to validate biomarkers or extrapolation factors applied to the lab toxicity results. However, in the case of EE2, there is little opportunity for direct comparisons due to the lack of overlap between lab and field test organisms and effect measurements. Only five species were tested in both lab studies and field scenarios: Order Cladocera (water flea), *Lithobates clamitans ssp.clamitans* (bronze frog), *Lithobates pipi*ens (leopard frog), *Salvelinus namaycush* (lake trout), and *Pimphales promelas* (fathead minnow). The lack of overlapping study concentrations, study length, and limited number of endpoints and effect measurements for field studies prevents direct comparisons of toxicological effects across lab and field studies and therefore prevents the validation of lab results using field data.

Without the opportunity to make direct comparisons of effects across lab and field studies, it is also impossible to validate extrapolation factors applied in risk assessments to extrapolate from lab to field or from acute to chronic exposures. In general, lab studies have the advantage of generating more data in a shorter amount of time, and lab studies have the ability to test a wider range of concentrations. However, the lack of overlap and comparability of lab and field test studies prevents us from validating lab results or from developing evidence-based extrapolation factors to convert from short-term lab studies to chronic, real-world effects.

3.2. *Reliance on molecular biomarker-level effect measurements increases extrapolation needed to predict functional effects at higher levels of biological organization*

In general, effects that measure a change in population abundance or reproduction are considered more “ecologically relevant” than those that measure changes at lower levels of organization like organ, cell, or molecular level changes (Rohr et al., 2016). Ideally, biomarkers are intended to be indicators along the mechanistic pathway from molecular change to functional change that affects organism behavior, survival, or reproduction; however, many biomarkers have not yet been mechanistically linked to population level effects, for example, through Adverse Outcome Pathways (Forbes et al., 2006; Rohr et al., 2016). Forbes (2006) argues that a change at the molecular or biochemical level could be a quick or early indication of harm but could also result in a false positive leading to unnecessary additional testing or overly conservative protection. Therefore, unvalidated biomarkers that have not been

conclusively linked to effects at higher levels of biological organization must be used with caution in risk assessment decision-making.

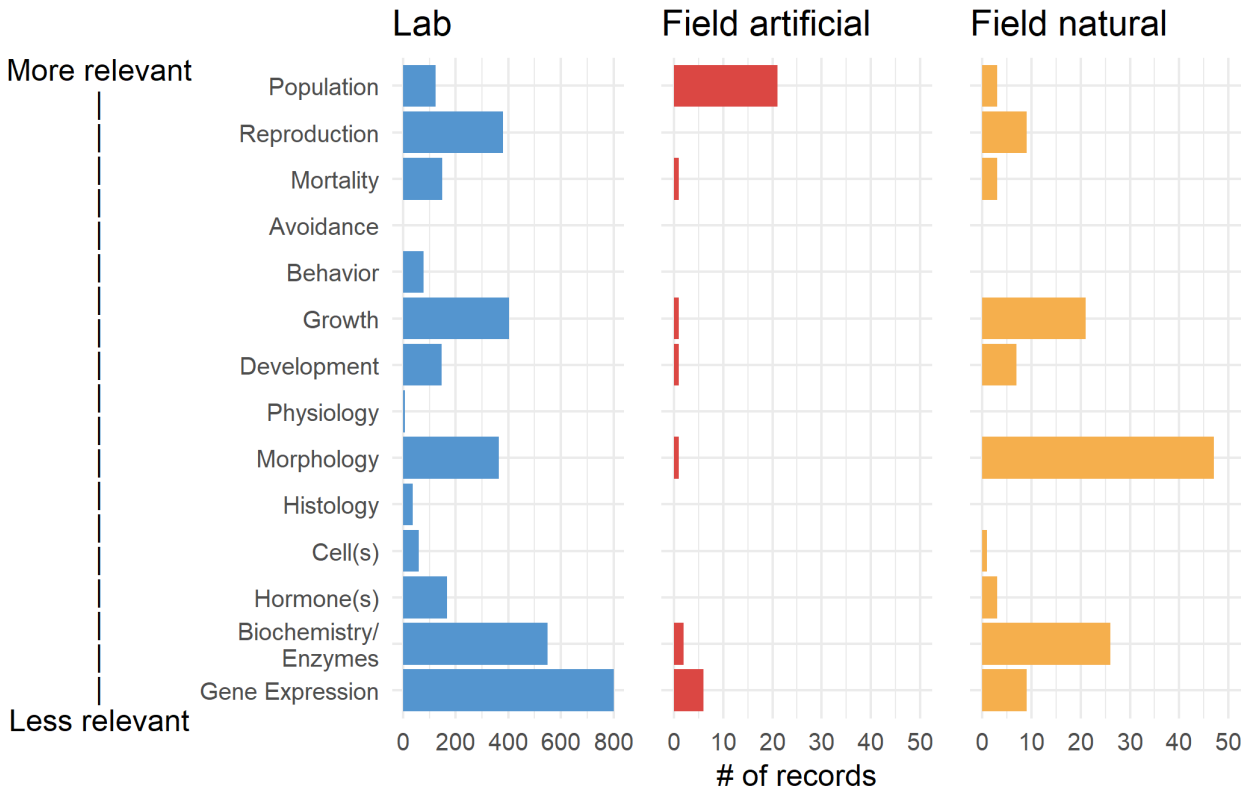

Fig 2. Figure represents the total counts for each toxicological effect categories for EE2 records included in the ECOTOX database separated by test location. Effects on the y axis are arranged in order from “most ecologically relevant” to “least ecologically relevant” based on conceptual figure in Rohr et al. (2016).

In the current study, 40.5% of all records of EE2 were for molecular and biochemical-level effects (Fig 2) whereas population-level effects (population and reproduction categories) make up only 15.6% of the records. Field studies proportionally tend to report more ecologically relevant effects including morphology, growth, and population level effects than lab studies, but these records are far outnumbered by lab studies (notice difference in x axis scales). Published EE2 studies to date demonstrate the strong preference towards less ecologically-relevant effects.

3.3. A lack of statistical differences between endpoints makes extrapolating between endpoints or among different effects mathematically challenging

Variability at the molecular, individual, and species level makes converting between different effects or endpoints even within the same species quite challenging (Forbes & Calow, 2002). Additionally, the use of endpoints like NOEC, NOEL, LOEC, and LOEL have frequently been condemned by the ecotoxicology community since the 1990s (Warne & van Dam, 2008). These common toxicology endpoints are criticized for their misleading names ("No effect level" does not really mean "no effect"), for being highly dependent on the research study design, and for being the result of incorrect application of statistical tests (Warne & van Dam, 2008). Despite these criticisms, these endpoints remain the most commonly reported toxicity endpoints in ERAs.

NOEC, NOEL, LOEC, and LOEL endpoints comprise nearly 98% of the records in the EE2 data used in this study (Table 1). Endpoints concentrations span several orders of magnitude in the top five most studied species even within a single species or effect category (Fig. 3). Just considering the most studied species in the dataset, *Danio rerio*, the only effect category statistically different distributions between NOEC, LOEC, NOEL, and LOEL concentrations was Biochemistry and Enzymes (Dunn Test, $p < 0.01$). Mortality effect category included differences in the distribution of NOEC and LOEL but all other endpoints were statistically similar (Dunn Test, $p < 0.001$). For the next most tested species, *Pimephales promelas*, Biochemistry and Enzymes and Morphology both had different distributions of concentrations for NOEL and LOEC (Dunn Test, $p < 0.05$), but all other endpoint comparisons were statistically similar across all other effect categories.

The lack of statistical differences in endpoint distributions even within a single species makes defensible mathematical conversions between endpoints quite difficult. In risk assessments, ratio-based extrapolation factors are often used to convert between LOEL/Cs to NOEL/Cs, similar to unit conversion factors. For example, a chronic LOEC for a certain effect may be divided by 10 to estimate the chronic NOEC for that effect. The lack of statistical difference between effects and endpoints in this study, however, does not support the use of simple extrapolation factors to convert between endpoints.

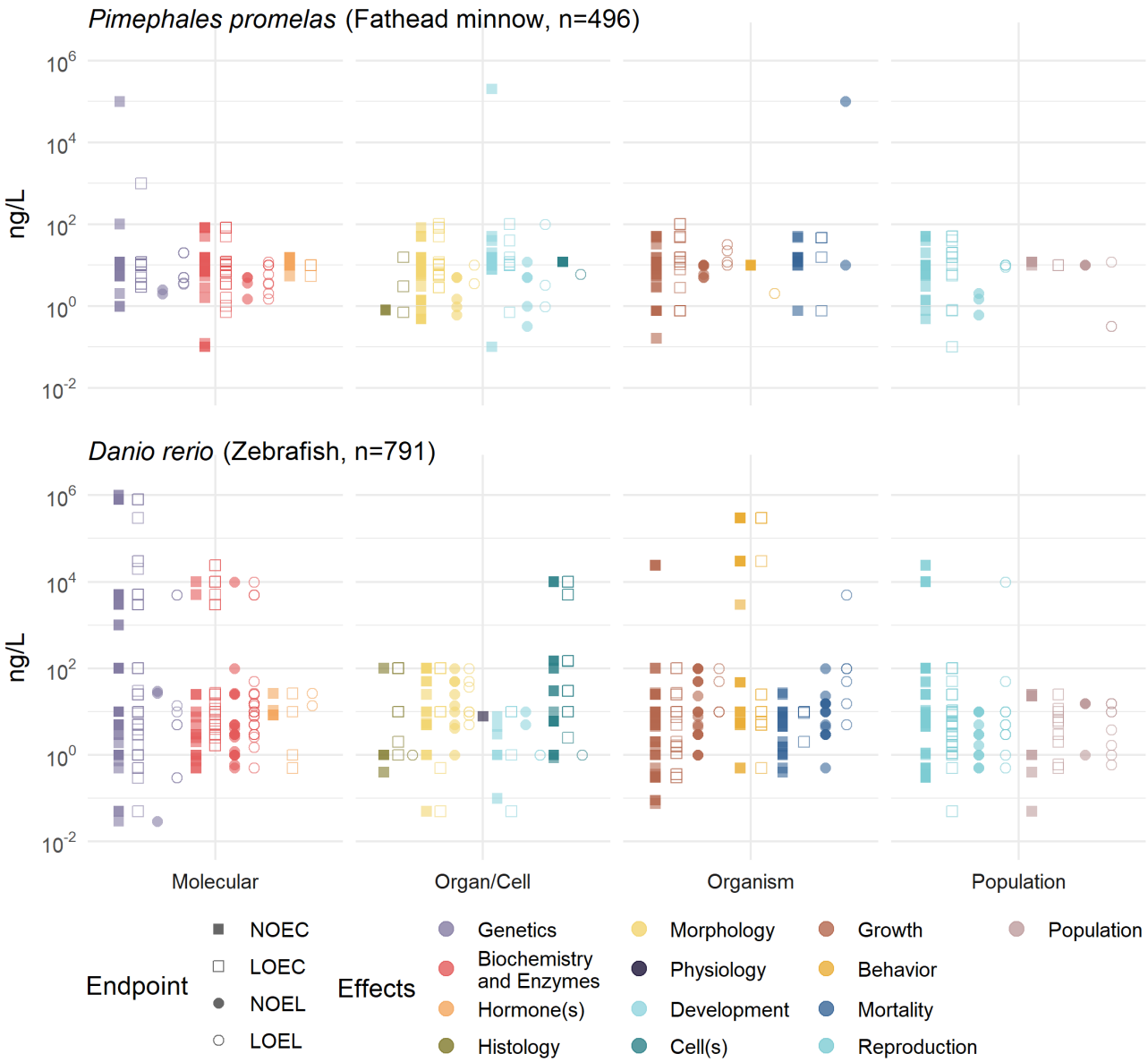

Fig 3. Variability in effects and endpoints for the two most reported species in the ECOTOX database for EE2. Notice the wide variability in concentrations even within a single effect category. Also notice there is no noticeable difference in the distribution of endpoints for each effect.

3.4. The apparent sensitivity of a species may depend on how well studied that species is

Often when data is lacking for a certain species of interest, a substitute species is used to estimate the effects. However, choosing a representative species can be quite challenging when there is high variability in sensitivities even within groups of related species. Considering

only fish species, EE2 effect concentrations vary by more than 7 orders of magnitude. Additionally, the more well-tested species tend to have wider effect concentration ranges and include effects at lower concentrations of EE2 than less-studied species (Fig. 4). For example, if we define the most sensitive species as those with effects measured at the lowest concentrations, then with the exception of *Salmo salar* and *Gasterosteus aculeatus*, fish species with more than 100 records are all located within the top 7 “most sensitive” fish species (Fig 4). This would suggest that the apparent “sensitivity” of a species may depend on how well-tested the species is, and not a true reflection of the real sensitivity of that species. This same trend can be seen in the species sensitivity distribution analysis in Figure 6.

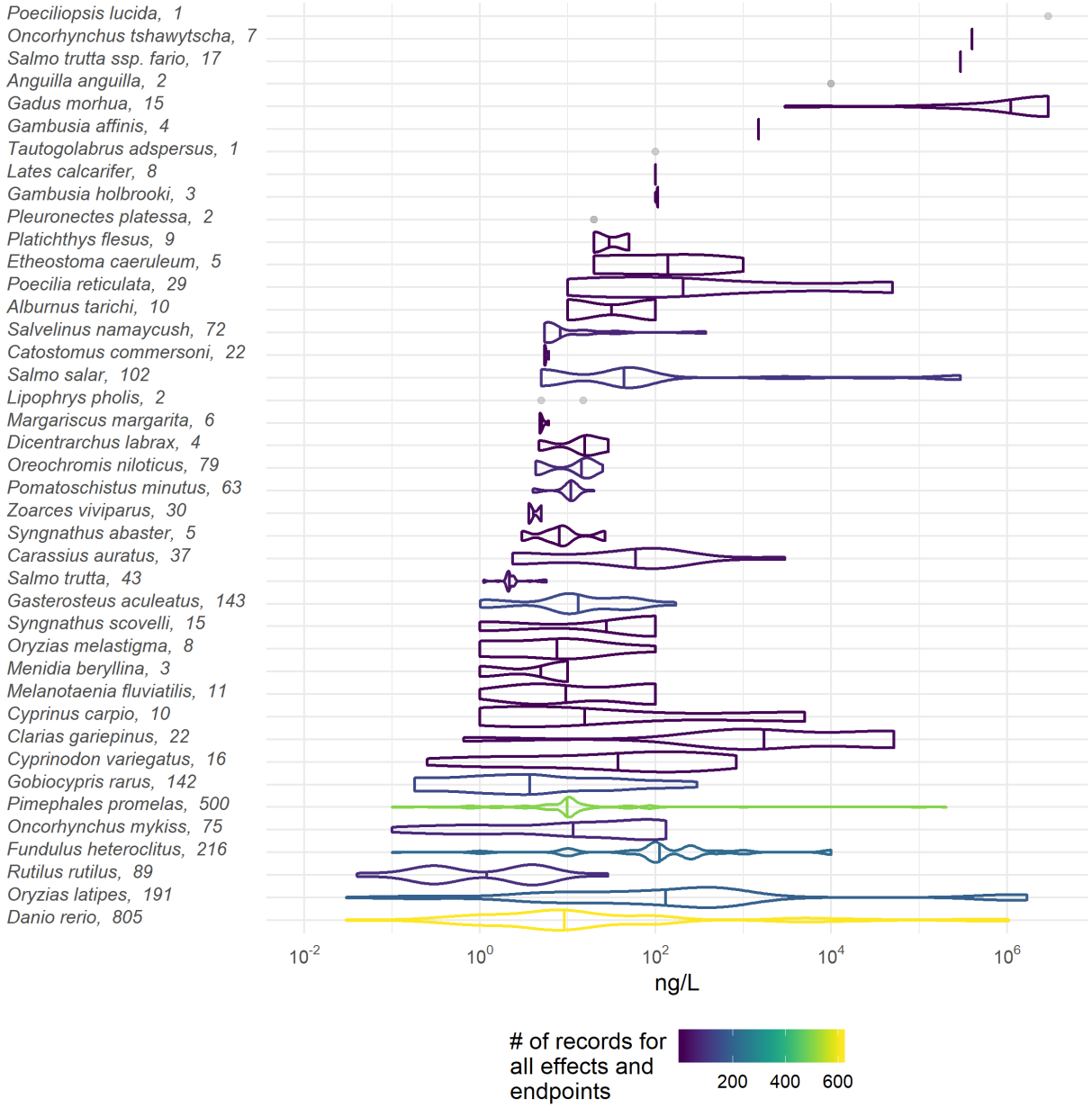

Fig 4. Distribution of EE2 effect concentrations for all species of fish in the ECOTOX database arranged from most sensitive to least sensitive with respect to the minimum concentration. Number following the species name corresponds to the number of records for that species.

3.5. Wide variability within and among species makes it challenging to choose a surrogate species to anticipate effects on an untested species of interest

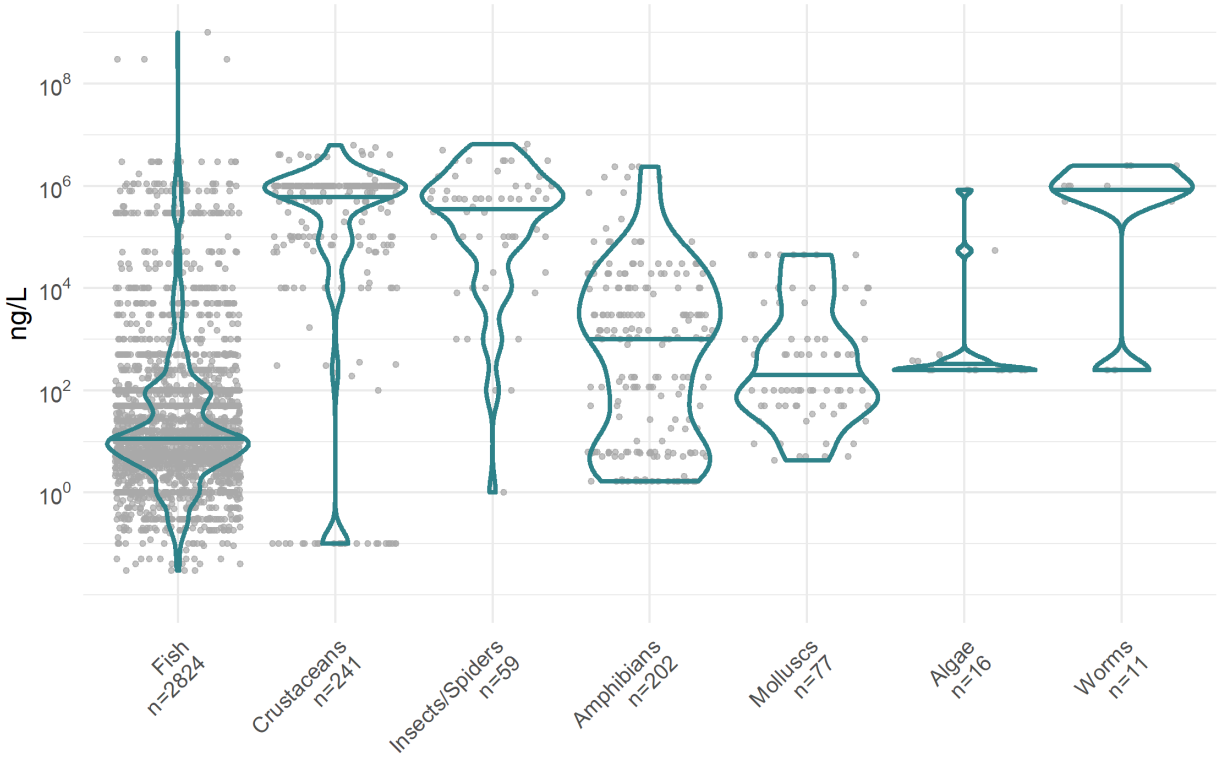

Fig 5. Range of effect concentrations of EE2 reported for in the ECOTOX database by species groups. Notice there is little consistency in the shape of the distributions for each species, which suggests that the range of sensitivities of species is not similar across different species groups.

It is well established that some species are more sensitive than others to the effects of certain toxins, and, in general, certain groups of related species exhibit more similar sensitivity ranges due to their underlying biological similarities. The analysis with EE2, however, shows that there is little conclusive evidence that EE2 effect concentrations also do not vary consistently within or across species groups (Fig 5). For example, concentrations range 11 orders of magnitude for fish and 4 to 8 orders of magnitude for other species groups, and there is also little consistency in the shape of the distribution among species groups (Fig 5). A similar

pattern is seen for species groups as with individual species, where the groups with the most records also tend to have the widest range and also include the most sensitive effect concentrations. Extrapolation between related species may therefore be a challenge due to the unpredictable variability in effect concentrations within and among species.

4. Implications of these extrapolation issues for risk assessment and decision-making

4.1. Species Sensitivity Distribution analysis is dependent on what data is available, how the researcher chooses to summarize that data, and which extrapolation factors are applied

In ERAs, species sensitivity distribution (SSD) curves are often used to extrapolate from known to unknown species and also to derive a Predicted No Effect Concentration (PNEC) which may be used to calculate a risk ratio or establish regulatory standards. SSD-based PNECs are dependent upon which species or studies are included in the analysis, whether the mean, median, or minimum concentrations for each species were chosen for the analysis, and what assessment factors are applied to account for extrapolation or uncertainty. Each of these choices can affect the outcome of the analysis.

An SSD analysis for all 77 species in the EE2 database including both lab- and field-derived data resulted in a PNEC concentration 1.4 to 20 times lower than all five previously published PNECs for EE2 (Fig 6, Table 2, Appendix). I do not make this comparison to suggest that any of the methods described in Table 2 are more or less correct but to demonstrate how predictions can vary by more than an order of magnitude based on the analysis choices of the researcher.

Based on my investigation of the variability in effect concentrations within and among species, it makes sense that the choice of which studies or species to include in an SSD analysis will have a great impact on resulting SSD and HC5. A single species may have effect concentrations ranging 6 orders of magnitude, so including a study which reports more sensitive effects versus a study with less sensitive effects could have a significant impact on an SSD curve derivation. Additionally, lab studies tend to focus on a small handful of standard test organisms which limits the number of species that can be used for an SSD curve approach. And finally, SSD curves do not account for the relative sensitivity of these standard test organisms compared to all other less-tested or un-tested species. This oversight may be potentially

problematic, especially if more well-tested species also frequently appear to be the most sensitive species as demonstrated by the clustering of more well-studied species in Figures 4 and 6.

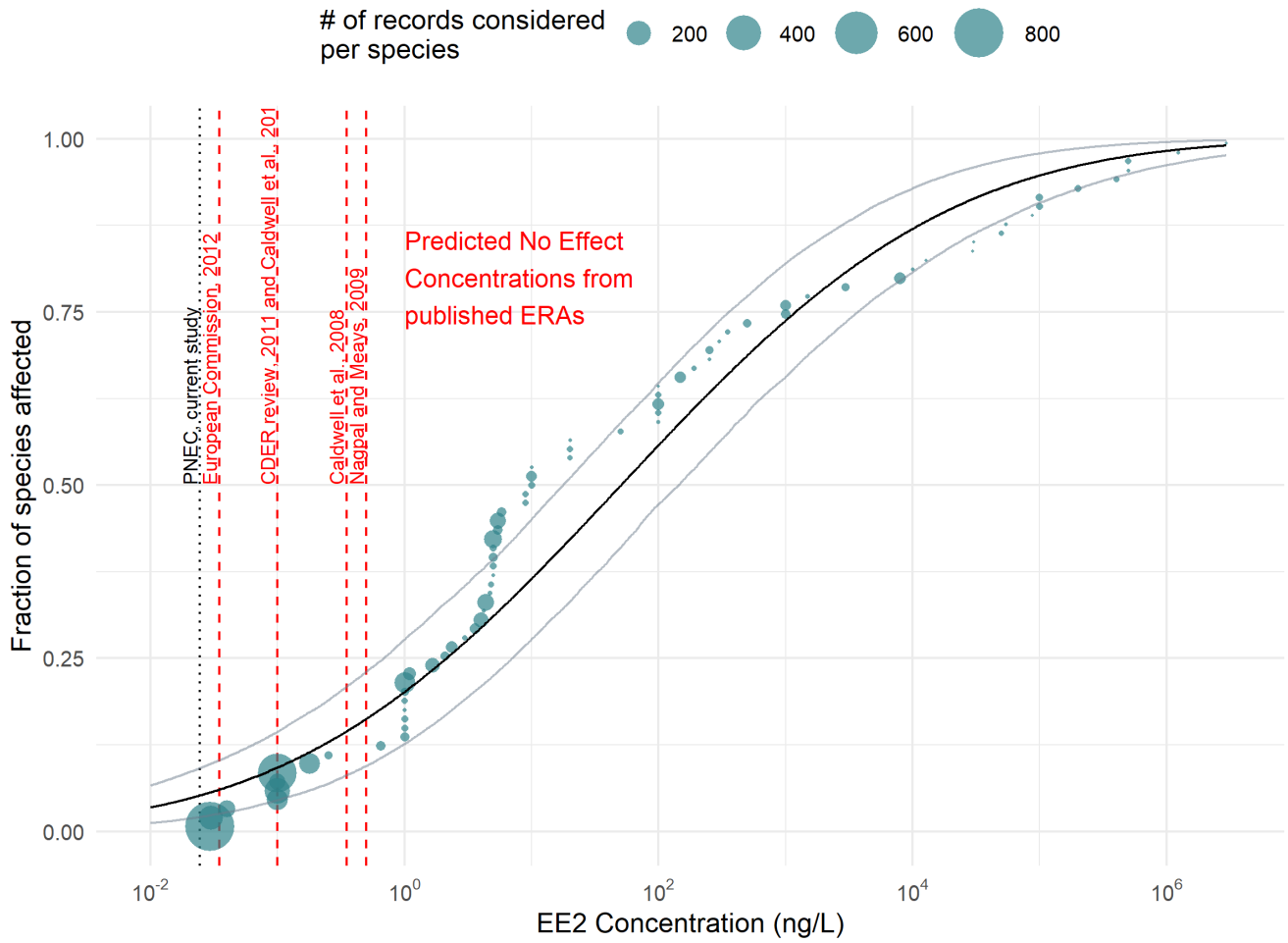

Fig 6. Species sensitivity distribution curve for EE2 derived from the most sensitive endpoints (lowest concentration) for each species in the ECOTOX database. Each point represents a single species with more sensitive species located at the bottom left and less sensitive species at the top right. Black dotted line is the HC5 estimate based on the lognormal curve. Concentrations and sources of PNECs (dashed vertical lines) are listed in Table 2.

Table 2. Predicted No Effect Concentrations (PNECs) for EE2 risk assessment with descriptions of methods or basis for concentrations (quoted text is from Laurenson et al. 2014).

PNEC (ng/L)	Brief description of methods	Source
0.5	"Based on studies reporting a multi-species LOEC of 1.0 ng/L for reproduction and egg production, along with an added AF of 2"	Nagpal and Meays, 2009
0.35	"Based on available chronic toxicity data, no observed effect concentration data (NOEC), and a species sensitivity distribution (SSD) approach, which fits a distribution to the NOECs from available studies across multiple taxa to determine the hazardous concentration of EE2 at which 5% of all the species tested are affected (referred to as the HC5)"	Caldwell et al., 2008
0.1	"Based on a LOEC of 0.19 ng/L that corresponds to a 25% increase in mortality of fertilized zebrafish eggs in a two-generation study (Soares et al., 2009), with an added AF of 2"	Center for Drug Evaluation and Research-commissioned literature review, 2011 (as presented in Laurensen et al, 2014)
0.1	"Used the same SSD methods as Caldwell et al. in 2008 (above), but based on the most current chronic reproductive toxicity data available and argument that the robustness of the data supports an AF of 1"	Caldwell et al., 2012
0.035	"Developed using an SSD approach to obtain an HC5 of 0.07 ng/L, which is less than the above HC5 of 0.1 ng/L due apparently to the use of two different data sets. Also, EC applied an AF of 2, while Caldwell et al. argued that an AF of 1 was sufficient"	European Commission (proposed), 2012
0.025	Used an SSD approach using the lowest effect and endpoint for 77 aquatic species included in the ECOTOX aquatic toxicity database. HC5 of 0.025 ng/L was calculated and AF of 1 was deemed to be sufficient due to the large number of species and studies considered in the analysis.	Current study

4.2. SSD analyses do not capture indirect effects on communities or ecosystems

Current methods in ERA do not allow for extrapolating from individual-level effects to ecosystem level effects. The SSD curve method is commonly used to determine the concentration at which 5% of species is predicted to be affected. However, this method does not consider interspecific interactions like food web effects or keystone species and does not consider metrics for ecosystem function or vulnerability (Vighi & Villa, 2013). An HC5 threshold could theoretically impact considerably more than 5% of species over a multi-year period when considering these indirect and ecosystem level effects.

A strong example of this phenomenon with aquatic EE2 exposure was documented by a multi-year, whole-ecosystem experiment conducted at the Experimental Lakes Area in Ontario,

Canada (Kidd et al., 2007). Exposure to 5-6 ng/L EE2 over for two years caused populations of fathead minnows (*Pimphales promelas*) to collapse due to failure to reproduce and also led to a decrease in slimy sculpin (*Cottus cognatus*) and pearl dace (*Margariscus margarita*) biomass. However, these three species which were directly impacted by EE2 levels in the lake are part of a larger ecosystem within the lake.

All three of these species are small fish which consume algae, zooplankton, and macroinvertebrates and are the dominant prey species for large predator fish in the lake system. This small population crash was followed by a decreased abundance and biomass of lake trout (*Salvelinus namaycush*), and worsening body condition in white suckers (*Catostomus commersonii*) and male lake trout (Kidd et al., 2014). There was no evidence to suggest that lake trout and white suckers were directly affected by EE2 exposure; instead, the researchers hypothesized that these delayed effects were due loss of common prey species. Additionally, they documented an increased biomass of rotifers, crustacean zooplankton (calanoid copepods), and aquatic invertebrates (*Chaoborus* spp., littoral macroinvertebrates), likely due to the decreased predation pressure from the two small fish species which nearly disappeared from the lake. Thus, a population collapse of fathead minnows, an important predator and prey species in the lake, resulted in a trophic cascade affecting several other species and overall foodweb structure and function within the lake.

The SSD approach in ERA would not have predicted the effects seen in the Kidd et al. (2014) study. To illustrate this example, the SSD curve is shown in Figure 7 with four of the study species highlighted in orange. Notice that only the fathead minnow and pearl dace are predicted to be affected at 5 ng/L concentrations of EE2 while lake trout, white suckers, and rotifers are not predicted to be impacted at this concentration using the SSD threshold approach. In the Kidd et al. (2014) study however, the loss of the two more sensitive species resulted in increased biomass of rotifers, and worsening body condition for lake trout and white sucker populations (Kidd et al., 2014).

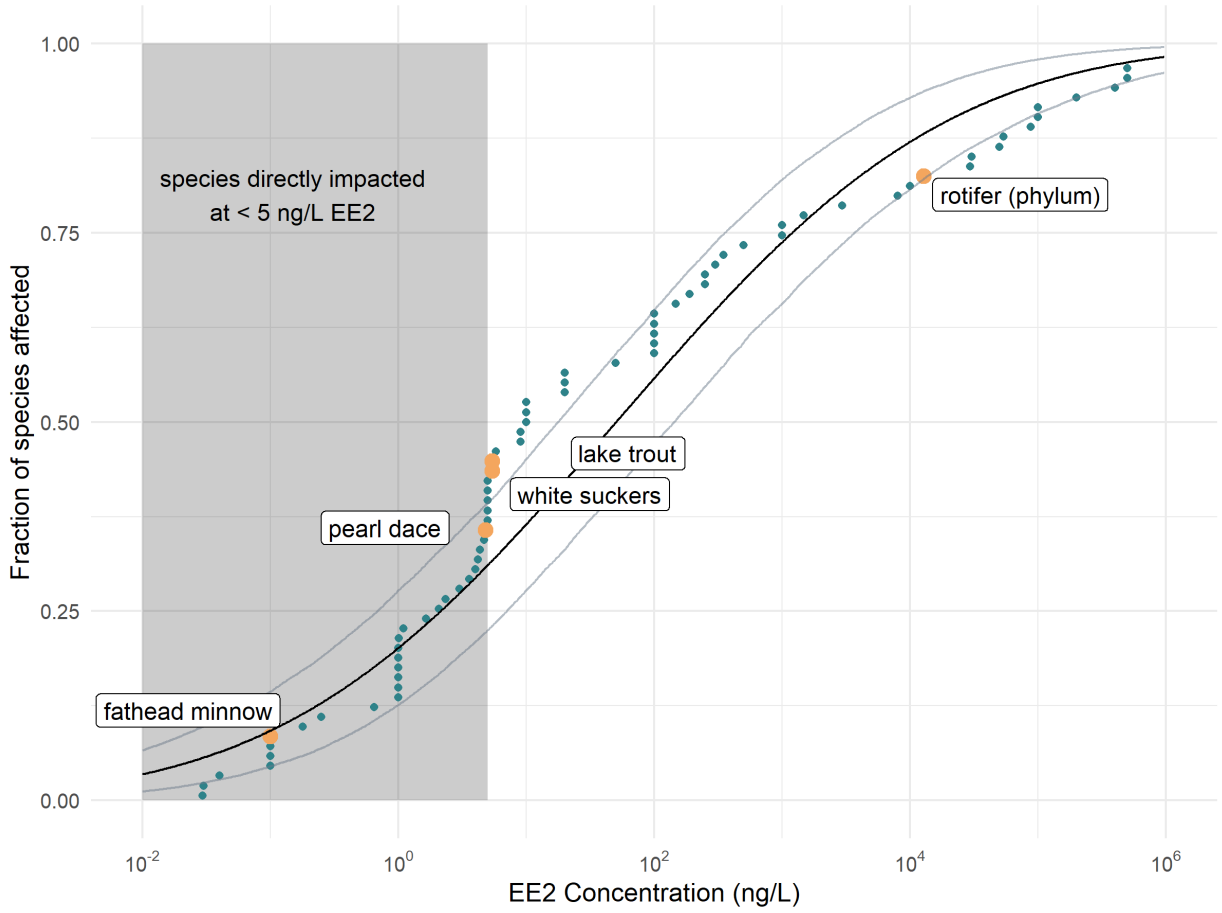

Fig 7. Species Sensitivity Distribution curve for EE2 which highlights species in three different trophic levels.

5. CONCLUSIONS FOR RISK ASSESSORS AND ENVIRONMENTAL MANAGERS

Without robust, evidence-based, and statistically-sound methods of extrapolation, environmental risk assessors and environmental managers will not be able to make decisions to achieve their management goals. Ecotoxicology must grapple with the challenges of both its parent fields—ecology and toxicology. Current day toxicology relies on controversial endpoints like NOEC and LOEC and on short term lab-based studies with a small number of test organism. Using these limited sets of data to extrapolate to other endpoints, effects, untested species, and ecosystem function is a daunting task when confronted with uncertainty introduced by both biological variability and study design.

Lab and field toxicology studies each have their own strengths and weaknesses. Field studies require less extrapolation because they use more ecologically relevant endpoint

measurements and may include some consideration of indirect effects and natural variability of the system. However, field studies are limited by time and cost constraints and a limited range of test concentrations and test species as compared to lab-based studies (Fig 8). Time and resource constraints, toxicology study design choices, innate biological variability, and limitations of current ERA methods combine to create a multi-dimensional extrapolation challenge for ecotoxicologists, risk assessors, and decision-makers alike.

Changes and adaptations are needed to address these challenges to improve the ecological realism of ERA predictions. Possible steps to address some of these issues are discussed below.

5.1. Increase collaboration across field and lab toxicology research.

Field data can be used to better understand the relationship between acute and chronic or multi-generation effects. A coordinated effort should be made to identify opportunities to validate lab-based results (including biomarkers effects) and extrapolation factors using field data. Also, risk assessors could incorporate available field toxicology studies into the risk assessment process.

5.2. Establish mechanistic pathways between biomarkers and ecologically relevant, functional effects.

Due to time and resource constraints, it is unlikely that future ecotoxicology research will be able to directly measure toxicity effects at higher levels of organization on a large scale. And so, future research should focus on establishing scientifically-defensible pathways between lab-measured biomarkers and ecologically-relevant endpoints (Forbes & Calow, 2013). Adverse Outcome Pathways (AOP) are gaining attention as a way to establish mechanistic pathways between structural or biomarker changes and population level effects (Ankley et al., 2010). Toxicokinetic-toxicodynamic models (TKTD) have also been proposed to as a promising tool for mechanistically linking time and space dependent chemical stressors to individual survival (Ashauer et al., 2016). Dynamic energy budget (DEB) models are another modeling tool that can link changes in energy uptake and allocation to survival, growth, and reproduction (Baas et al., 2018). However, an additional step is needed to extended these mechanistic pathways from individual organism survival or reproduction to ecosystem function. Individual-based models (also called agent-based models) have been proposed by some researchers as a flexible mathematical tool which can link individual organism metrics to changes in interspecific

interactions, food web dynamics, and ecosystem function while also considering the effects of time, space, and exposure to simultaneous stressors (Forbes & Calow, 2013).

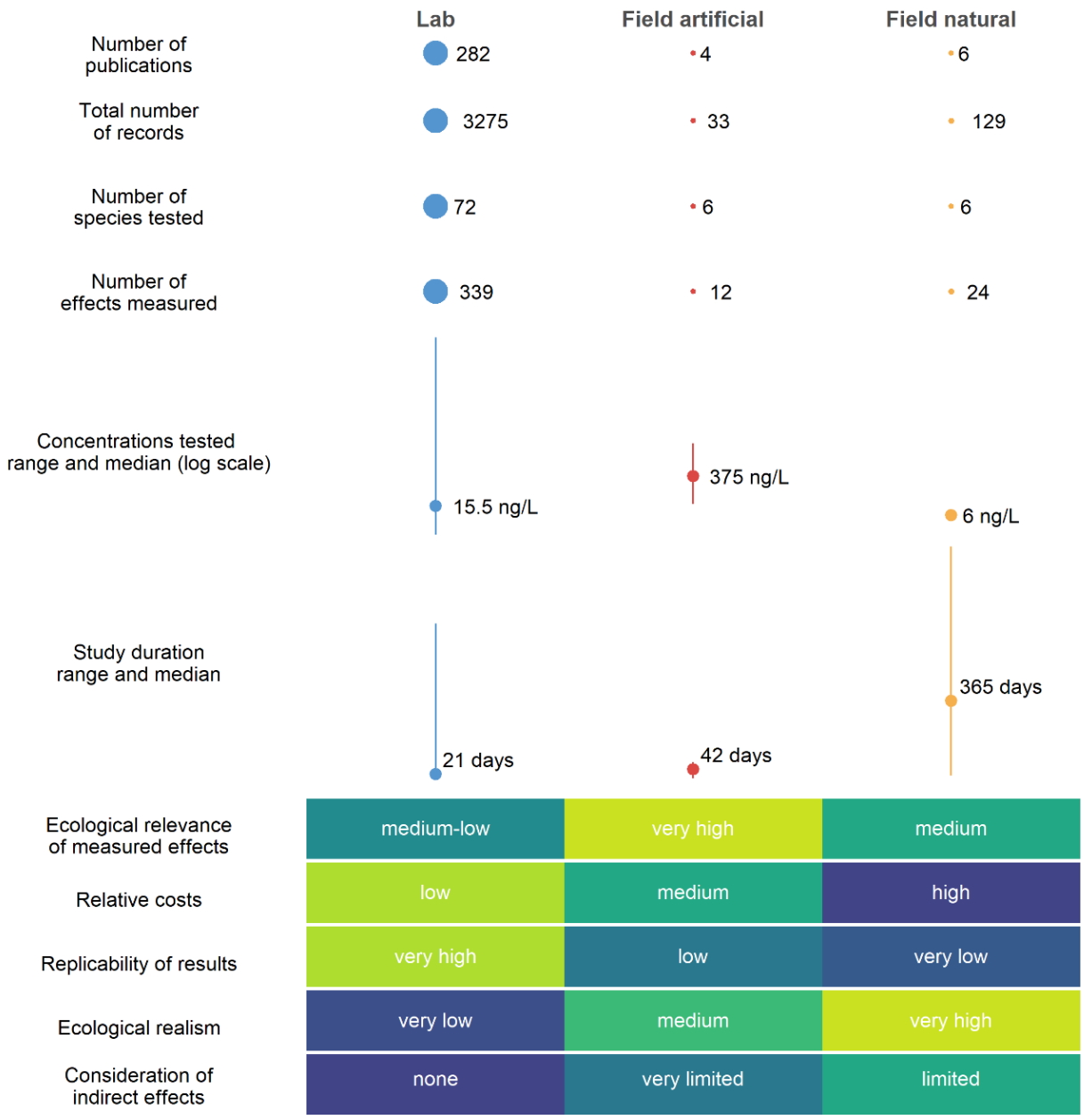

Figure 8. Summary of data included in the current study on EE2 as well as a visual representation of some of the relative strengths and weakness of lab and field studies in toxicology. In the color blocks section, brighter values are more desirable and darker values are less desirable.

5.3. ERAs must evolve to incorporate more sophisticated mechanistic pathways and models to replace simple ratio and threshold-based decision making.

The analyses presented in this paper have shown that simple multiplication or division is not an adequate or reasonable method for extrapolating from lab-based biomarkers to ecologically relevant effects due to the unpredictable and wide variability in effects and endpoints over time, space, and species. Models described in (5.2.) may be better equipped to support sound environmental management decisions because they will be better able to describe the degree and severity of pollution-related stress on ecologically relevant endpoints. Other tools for describing confidence intervals, probability or effects, and severity of effects are needed. Additionally, a consideration of indirect effects resulting from food web cascades or losses of essential species is needed in order to extend protection from individual organism or populations to entire ecosystems or landscapes.

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APPENDIX

Appendix 1. Summary of studies included in the Species Sensitivity Distribution analysis. *See Appendix 2 for source information.

Conc (ng/L)	Species name	Common name	Endpoint	Effect	Effect measurement	Study length (days)	Source *
0.030	*Danio rerio*	Zebra Danio	NOEC	Genetics	Cytochrome P450aromB mRNA	3	1
0.030	*Oryzias latipes*	Japanese Medaka	LOEC	Morphology	Imposex, intersex conditions	97.5	2
0.040	*Rutilus rutilus*	Roach	NOEC	Growth	Length	518	3
0.100	*Daphnia magna*	Water Flea	LOEC	Growth	Length	4	4
0.100	*Fundulus heteroclitus*	Mummichog	NOEC	Reproduction	Vitellogenesis	364	5
0.100	*Oncorhynchus mykiss*	Rainbow Trout	LOEC	Biochemistry	Vitellogenin	10	6
0.100	*Pimephales promelas*	Fathead Minnow	LOEC	Reproduction	Progeny counts/numbers	21	7
0.180	*Gobiocypris rarus*	Chinese Rare Minnow	LOEC	Reproduction	Progeny counts/numbers	180	8
0.250	*Cyprinodon variegatus*	Sheepshead Minnow	NOEC	Histology	Fibrosis	73	9
0.650	*Clarias gariepinus*	Zambezi Barbel	LOEC	Biochemistry	Vitellogenin	7	10
1.000	*Chironomus riparius*	Midge	LOEL	Enzyme(s)	Catalase	1	11
1.000	*Cyprinus carpio*	Common Carp	NOEC	Biochemistry	Vitellogenin	10	6
1.000	*Melanotaenia fluviatilis*	Crimson-Spotted Rainbowfish	NOEC	Genetics	Vitellogenin mRNA	7	12
1.000	*Menidia beryllina*	Inland Silverside	LOEC	Biochemistry	Choriogenin	14	13
1.000	*Oryzias melastigma*	Indian Medaka	NOEC	Genetics	omChgH mRNA	7	14
1.000	*Syngnathus scovelli*	Gulf Pipefish	LOEC	Reproduction	Courtship behavior	10.0417	15
1.010	*Gasterosteus aculeatus*	Threespine Stickleback	LOEC	Genetics	Cyclin B2 mRNA	4	16
1.100	*Salmo trutta*	Brown Trout	LOEC	Biochemistry	Vitellogenin	11	17

1.650	*Xenopus tropicalis*	Clawed Frog	LOEC	Morphology	Perimeter	243.52	18
2.075	*Rana temporaria*	Frog	NOEC	Population	Sex ratio	1	19
2.350	*Carassius auratus*	Goldfish	LOEC	Biochemistry	Vitellogenin	14	20
3.000	*Syngnathus abaster*	Shortsnouted Pipefish	NOEC	Behavior	Movements, number of	7	21
3.600	*Zoarces viviparus*	Viviporous Blenny	LOEC	Histology	Ultrastructural changes	19	22
4.000	*Pomatoschistus minutus*	Sand Goby	LOEC	Reproduction	Pair bonding nesting behavior	15.5	23
4.200	*Mytilus edulis*	Common Bay Mussel,Blue Mussel	LOEC	Genetics	5-Hydroxytryptamine receptor mRNA	10	24
4.340	*Oreochromis niloticus*	Nile Tilapia	LOEC	Genetics	Estrogen receptor alpha mRNA	20	25
4.700	*Dicentrarchus labrax*	Sea Bass	NOEC	Biochemistry	Vitellogenin	14	26
4.800	*Margariscus margarita*	Pearl Dace	LOEL	Morphology	Organ weight in relationship to body weight	882.76	27
5.00	*Lipophrys pholis*	Shanny	NOEC	Genetics	Vitellogenin 2 mRNA	21	28
5.00	*Lithobates septentrionalis*	Mink Frog	LOEC	Growth	Weight	87	29
5.00	*Potamopyrgus antipodarum*	Snail	NOEC	Reproduction	Progeny counts/numbers	63	30
5.00	*Saccostrea glomerata*	Rock Oyster	NOEC	Biochemistry	Vitellogenin	56	31
5.00	*Salmo salar*	Atlantic Salmon	LOEC	Enzyme(s)	Glutathione S-transferase	3	32
5.45	*Catostomus commersoni*	White Sucker	LOEC	Biochemistry	Vitellogenin	1095	33
5.45	*Salvelinus namaycush*	Lake Trout, Siscowet	LOEC	Morphology	Organ weight in relationship to body weight	1095	33
5.80	*Lithobates clamitans ssp. clamitans*	Bronze Frog	LOEC	Morphology	Diameter	119	34
9.00	*Bithynia tentaculata*	Snail	LOEC	Growth	Growth rate	222	35
9.00	*Radix balthica*	Freshwater Snail	LOEC	Growth	Growth rate	116	35

10	*Alburnus tarichi*	Tarek	NOEC	Genetics	Apoptosis, programmed cell death, DNA fragmentation	32	36
10	*Poecilia reticulata*	Guppy	LOEC	Enzyme(s)	Aromatase	13	37
10	*Silurana tropicalis*	Tropical Clawed Frog	NOEC	Histology	Histological changes, general	98	38
20	*Etheostoma caeruleum*	Rainbow Darter	NOEC	Morphology	Imposex, intersex conditions	21	39
20	*Platichthys flesus*	Starry, European Flounder	LOEL	Biochemistry	Vitellogenin	10	40
20	*Pleuronectes platessa*	Plaice, Sand Dab	LOEL	Biochemistry	Vitellogenin	16	41
50	*Rana catesbeiana*	Bullfrog	LOEC	Genetics	Cytochrome P450 aromatase A mRNA	2	42
100	*Gambusia holbrooki*	Eastern Mosquito fish	NOEL	Enzyme(s)	7-Ethoxyresorufin O-deethylase	7	43
100	*Haitia pomilia*	Freshwater Snail	NOEC	Population	Population growth rate	98	44
100	*Hyalella azteca*	Scud	NOEL	Cell(s)	Structural changes	273	45
100	*Lates calcarifer*	White Sea Bass	LOEC	Genetics	Vitellogenin mRNA	2	46
100	*Tautogolabrus adspersus*	Cunner	NOEL	Mortality	Hatch	2	47
148	*Xenopus laevis*	African Clawed Frog	LOEC	Enzyme(s)	Luciferase	4	48
190	*Cladocera*	Water Flea Order	NOEL	Population	Abundance	45	49
250	*Caenorhabditis elegans*	Nematode	NOEL	Growth	Length	3	50
250	*Plankton*	Plankton	LOEC	Population	Population changes, general	63	51
300	*Maxillopoda*	Crustacean Class	NOEL	Population	Abundance	35	52
350	*Gammarus pulex*	Scud	NOEL	Reproduction	Reproductive behavior changes	1.1667	53
500	*Marisa cornuarietis*	Snail	LOEC	Morphology	Imposex, intersex conditions	273.96	54
1000	*Lithobates pipiens*	Leopard Frog	LOEL	Reproduction	Vitellogenesis	124	55
1000	*Lithobates sylvaticus*	Wood Frog	NOEL	Growth	Length	47	55

1482	*Gambusia affinis*	Western Mosquito fish	LOEC	Genetics	Vitellogenin mRNA	35	56
2964	*Gadus morhua*	Atlantic Cod	LOEC	Genetics	Vitellogenin A mRNA	1	57
8000	*Chironomus tentans*	Midge	LOEC	Genetics	Gene expression	1	58
10000	*Anguilla anguilla*	Common Eel	LOEL	Biochemistry	Protein content	9	59
12800	*Rotifera*	Rotifer Phylum	NOEL	Population	Abundance	42	52
29641	*Hemicentrotus pulcherrimus*	Sea Urchin	LOEL	Growth	Diameter	80	60
30300	*Strongylocentrotus purpuratus*	Purple Sea Urchin	EC50	Development	Abnormal	4	61
50000	*Nitocra spinipes*	Harpacticoid Copepod	NOEC	Reproduction	Progeny counts/numbers	16.5	62
54000	*Desmodesmus subspicatus*	Green Algae	EC10	Population	Biomass	3	63
88000	*Acartia tonsa*	Calanoid Copepod	EC50	Development	Slowed, Retarded, Delayed or Non-development	5	64
100000	*Sida crystallina*	Water Flea	NOEL	Development	Age at first reproduction	7	65
100000	*Tisbe battagliai*	Harpacticoid Copepod	LOEC	Reproduction	Fecundity	21	66
200000	*Ceriodaphnia reticulata*	Water Flea	NOEC	Mortality	Survivorship	34	65
400000	*Oncorhynchus tshawytscha*	Chinook Salmon	LOEL	Population	Sex ratio	0.1667	67
500000	*Ceriodaphnia dubia*	Water Flea	NOEC	Mortality	Survival	7	68
500000	*Dugesia japonica*	Flatworm	NOEC	Growth	Limb/ body part regeneration	3	69
1230099	*Brachionus calyciflorus*	Rotifer	EC50	Population	Intrinsic rate of increase	3	70
2964094	*Poeciliopsis lucida*	Topminnow	NOEC	Cell(s)	Viability	1	71

Appendix 2. Source information for Appendix 1.

Source	Author(s)	Title	Journal	Publication year
1	Kazeto,Y., A.R. Place, and J.M. Trant	Effects of Endocrine Disrupting Chemicals on the Expression of CYP19 Genes in Zebrafish (Danio rerio) Juveniles	Aquat. Toxicol.69(1): 25-34	2004
2	Metcalfe,C.D., T.L. Metcalfe, Y. Kiparissis, B.G. Koenig, C. Khan, R.J. Hughes, T.R. Croley, R.E. March, and T. Potter	Estrogenic Potency of Chemicals Detected in Sewage Treatment Plant Effluents as Determined by In Vivo Assays with Japanese Medaka (Oryzias latipes)	Environ. Toxicol. Chem.20(2): 297-308	2001
3	Lange,A., G.C. Paull, T.S. Coe, Y. Katsu, H. Urushitani, T. Iguchi, and C.R. Tyler	Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol	Environ. Sci. Technol.43(4): 1219-1225	2009
4	Dietrich,S., F. Ploessl, F. Bracher, and C. Laforsch	Single and Combined Toxicity of Pharmaceuticals at Environmentally Relevant Concentrations in Daphnia magna - A Multigenerational Study	Chemosphere79(1): 60-66	2010
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