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Derivation of an Aquatic Predicted No-Effect Concentration for the Synthetic Hormone, 17α-Ethinyl Estradiol

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17α-Ethinyl estradiol (EE2) is a synthetic estrogen widely used in combination with other steroid hormones in oral contraceptives and in the contraceptive patch. EE2 has been detected in sewage treatment plant effluents in the low nanogram -per-liter range and occasionally in surface waters in the U.S., U.K., Canada, Brazil, Germany, and elsewhere. The mode of action is receptor-mediated, and estrogen receptors exist in mammals and other vertebrates. A large number of studies on the effects of EE2 on aquatic organisms exist. One hundred English language studies published between 1994 and 2007, one as yet unpublished study, and findings published in conference proceedings (in German) were compared to published data quality criteria to identify the most relevant studies for deriving a predicted no-effect concentration (PNEC). Reproduction in fish was identified as the most sensitive end point in aquatic species. A species sensitivity distribution was constructed using no observed effect concentrations (NOECs) for reproductive effects from 39 papers in 26 species, resulting in a median hazardous concentration at which 5% of the species tested are affected ($HC_{5,50}$) of 0.35 ng/L. After comparing this $HC_{5,50}$ to all of the laboratory and field-derived toxicity information available for EE2, we recommend using 0.35 ng/L as the PNEC for EE2 in surface water. This PNEC is below 95% of the existing NOECs for effects on reproduction and is also below virtually all of the NOECs for vitellogenin induction in the key fish reproduction studies.

INTRODUCTION

17α-Ethinyl estradiol (EE2) is a synthetic estrogen used in combination with other steroid hormones in oral contraceptives and in the contraceptive patch. EE2 is only partially metabolized and incompletely removed by sewage treatment plants (STPs) and, therefore not surprisingly, has been detected in sewage treatment plant effluents in the low nanogram-per-liter range and occasionally in surface waters in the U.S., U.K., Canada, Brazil, Germany, and elsewhere (*1*−*6*). EE2 is not the only compound with estrogenic activity detected in STP effluents (*7*−*11*) although available data suggest natural and synthetic hormones represent the majority of the estrogenic activity in many effluents (*12, 13*). Its relatively high relative biological activity (*14*−*18*) has led to a great deal of research on EE2, providing a far greater body of toxicity data than is available for most compounds.

Estrogens in Whole Effluents: Effects in Fish Populations. Purdom et al. (*19*) are often cited as one of the first papers to show that effluents can be estrogenic to fish by inducing the synthesis of vitellogenin (VTG). Now many papers report that a number of fish species respond to nanogram per liter (ng/L) concentrations of EE2 by synthesizing VTG (*19*−*23*). Although laboratory studies exist in which exposure to EE2 causes an increase in VTG in male (and also female) fish and coincides with observed adverse reproductive effects, concluding that EE2 in natural settings might also be causing reproductive effects is not possible because, as noted above, other compounds in effluents also have estrogenic activity. Indeed, determining the environmental relevance of effects from trace levels of EE2 in effluents requires an aquatic risk assessment that focuses specifically on the potential effects of EE2. Key to the conduct of such an assessment is determining the allowable concentration of EE2 in surface water, also referred to as the predicted no effect concentration (PNEC).

Why the Usual Approaches To Derive a PNEC Are Not Viable for EE2. A variety of methods have been proposed to derive PNECs. Many of these assume that limited toxicity data are available for a compound and derive a PNEC using various types of uncertainty or assessment factors. For example, if only acute data are available, the European

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Commission Technical Guidance Document (TGD) (*24*) allows for derivation of a PNEC by applying an assessment factor of 1000 to the lowest acute toxicity value. Abundant acute toxicity data are available for EE2. A median lethal concentration (i.e., 96-h LC50) of 1.6 mg/L was reported for rainbow trout (*25*), and a 96-h LC50 of 1.7 mg/L was reported in zebrafish (*26*). Toxicity to algae was evaluated in an OECD 201 guideline study that examined effects on growth rate and biomass in *Desmodesmus subspicatus*. The 72-h median effective concentration affecting growth (ErC50) was 0.46 mg/L for growth rate, the median effective concentration affecting biomass (EbC50) was 0.13 mg/L, and the NOEC for both end points was <0.1 mg/L (*27*). In a study conducted in *Scenedesmus subspicatus* to German (DIN) standards, a 72-h EC50 of 0.84 mg/L and EC10 of 0.054 mg/L were reported with biomass as the end point (Kopf 1997). *Daphnia magna* had a 24-h LC50 of 5.7 mg/L (*28*) and a 48-h LC50 of 6.4 mg/L (*25*). Jaser et al. (*29*) reported 24-h LC50s for *Ceriodaphnia reticulata* (1.8 mg/L) and *Sida crystalline* (>4.1 mg/L). Acute studies in eight other species reported LC50 values between 0.2 and 9.5 mg/L (data not presented). On the basis of available acute toxicity data, the three baseset taxa (fish, crustaceans, and algae) appeared to be about equally sensitive to EE2, with median effective concentrations of 0.13 to 6.4 mg/L. Using the TGD approach, the resulting PNEC, 0.13 μg/L, although likely to prevent lethality during chronic exposure, is 100 or more times greater than concentrations associated with adverse reproductive effects (see below) and, thus, is not protective of documented effects.

Consideration of Mode of Action. An evaluation of the mode of action (MOA) of EE2 reveals why extrapolating acute data using an assessment factor of 1000 is not protective of reproductive effects. Estrogens are sex hormones with a receptor-mediated MOA (*30*). EE2 is specifically designed to impede the normal reproductive function in humans via an estrogen receptor-mediated MOA and would be expected to produce effects in aquatic organisms known to have estrogen receptors similar to those observed in mammalian systems (*31*). Thus, it is possible for concentrations of EE2 below a PNEC derived from a combination of acute toxicity results and default assessment factors to cause potential reproductive effects in fish if the concentration of EE2 is sufficiently high to induce receptor-mediated effects.

Another line of evidence providing an alert that a compound acts via a specific MOA is variation in the acute to chronic ratio (ACR) between taxa. In the case of EE2, ACRs based upon reproductive effects can be calculated for a range of taxa, some with and some without the estrogen receptor (*31, 32*). Fish are the only taxa in the aquatic toxicity baseset that have estrogen receptors. It is currently unclear whether invertebrates possess a functional estrogen receptor. Studies on various species of mollusks have suggested that this group possesses an estrogen receptor (*33*−*36*). However, this receptor does not appear to bind estradiol and, unlike the vertebrate ER, is constitutively active. Other researchers have chosen to call this mollusk receptor an estrogen-related receptor (ERR). Currently, the role of this receptor is unknown. If this receptor does not bind estradiol, it is unlikely to bind EE2, and hence, its relevance to any (possible) effects of EE2 on invertebrates seems likely to be minimal. Daphnia have an ecdysteroid receptor, but no analogous receptor has been identified in algae. The lethality to reproduction ACRs for daphnia and algae are 570 and 16, respectively. In another invertebrate, *Nitocra spinipes*, there were no effects on mortality, larval development rate, fecundity, or sex ratio from long-term (18-d) exposure of this species to 0.05 mg/L EE2, which was a NOEC, resulting in an ACR of 10 (*37*). However, the growing number of longer-term studies in multiple fish species show larger ACRs that correlate with the presence of the estrogen receptor in the organism. Multiple studies, including full life cycle (FLC) fish studies, have demonstrated reproductive effects in fish to be the most sensitive biologically relevant end point for exposure to EE2 in the aquatic environment, with corresponding ACRs of over 1 million for reproductive effects as compared to acute lethality (*27, 38*−*42*). Thus, the studies that examined reproductive end points were considered to be the key studies to derive the PNEC.

In this paper, we review the published aquatic toxicity data on EE2 and conclude reproduction is the critical end point, which is consistent with EE2's mode of action, that is, binding to estrogen receptors. Further, fish were identified as the most sensitive species for reproduction effects. Given the relative abundance of reproductive effects studies investigating EE2 and the recommendation of the TGD to use a species sensitivity distribution (SSD) to derive a PNEC (*24*), we derived a PNEC by constructing a species SSD from the NOECs reported in the reproduction studies.

METHODS USED TO DERIVE A PNEC FOR EE2

Sources of Data. The Pharmaceutical Research and Manufacturers of America (P*h*RMA) compiled and summarized the peer-reviewed English language literature in the Pharmaceutical Assessment, Characterization, and Transport (P*h*ACT) database. All available papers investigating EE2 and published through mid-2007 were screened, and relevant papers containing aquatic toxicity data were reviewed in detail for data on reproductive effects. Published proceedings from a German conference that presented data on EE2 were also evaluated. In addition, the U.K. Environment Agency (*43*) provided results from a yet to be published study conducted in roach (*Rutilus rutilus*). Once studies reporting on reproductive effects were identified, they needed to be evaluated and ranked on the basis of their quality.

Selection Criteria. Evaluating and ranking toxicity data requires expert judgment. The use of specified data quality criteria ensures that an effect or end point (in the case of EE2, reproductive effects) is consistently characterized. Ranking the study also provides a relative scale that can be used to justifiably select one or more results from several conflicting results to properly describe an effect or end point. Therefore, the existing studies with reproductive data were reviewed to assess the quality of data and rank the reliability of data with regard to relevance and adequacy according to Klimisch et al. (*44*), who proposed four ranking categories. Further, those studies using only one exposure concentration or excessively high exposure concentrations were excluded from the analysis, with one exception (*40*), discussed below.

For purposes of ranking data, the terms reliability, adequacy, and relevance are defined as follows (*44*):

Reliability considers the completeness of the reported test methodology in comparison to accepted, standardized methodology. For data to be considered reliable, the experimental procedure and results must be properly described as well as sufficiently clear and plausible and must support the findings.

Relevance considers whether the data, the test procedures, or both are appropriate to assess the reported effect or end point.

Adequacy considers the usefulness of the data. When there is more than one set of data for an effect or end point, the most reliable and relevant data are used to describe the effect or end point.

Four categories of reliability are proposed by Klimisch (*44*):

Reliable without restriction (code 1): studies described in the literature or reports that were conducted according to, or on the basis of, generally valid or international or national accepted testing guidelines (preferably performed according

to GLP), or studies in which all parameters described are closely related/comparable to a guideline method.

Reliable with restrictions (code 2): studies described in the literature or reports that may not be performed according to GLP, and not all test parameters comply with a specific testing guideline, but are sufficient to accept the data. This includes investigations that cannot be subsumed under a testing guideline(s), but that are nevertheless well-documented and scientifically acceptable.

Not reliable (code 3): studies described in the literature or reports that include procedures that are in conflict with the proper performance of the test procedure or ascribed test guideline. This includes interferences between the analytical procedure and the test substance, the use of organisms or test systems that are not relevant to the exposure, the use of a test procedure that is not acceptable, and documentation that is not convincing to the expert judge.

Not assignable (code 4): studies described in the literature or reports that do not provide sufficient experimental details and that are only listed in short abstracts or secondary literature.

Data Used To Construct the SSD. The data in Table 1, extracted from 39 papers presenting reproductive data for 26 species (8 of which are fish), contain 52 reproduction NOECs used to construct the SSD. All 39 studies were evaluated against the Klimisch criteria, and the NOECs reported by the authors are considered to be correct and were used for $HC_{5,50}$ derivation, except as noted in the footnotes to Table 1. The reproductive effect NOECs range from 0.3 ng/L (*Danio rerio*, *Pimephales promelas*) to 500 000 ng/L (*Hydra vulgaris*), a difference of 6 orders of magnitude. Table 1 also summarizes the LOECs for reproduction end points and the NOECs for VTG induction reported in the fish studies. Comparison of NOECs across taxa demonstrate that fish are the most sensitive, followed by another vertebrate taxon, amphibians (frogs).

Seven full life cycle fish studies are especially critical. Key aspects and findings of those studies are briefly summarized below along with their respective Klimisch code:

Länge et al., 2001. In a well-designed two-generation full life-cycle (FLC) study with the fathead minnow (*P. promelas*), conducted under a protocol based on the USEPA Fish FLC Standard Evaluation Procedure, Länge and colleagues exposed newly fertilized embryos to five concentrations of EE2 (0.2, 1, 4, 16, and 64 ng/L) under continuous flow-through conditions for 305 days (*27*). Exposure concentrations were verified by 14C-EE2 radiochemistry and supported by radioimmunoassay, and mean measured values were >70% of nominal. For the F0 adults exposed until 301 days post hatch (dph), the NOECs for growth, survival, and reproduction (measured as egg production) were all >1 ng/L. Male fish exposed to 4 ng/L for 56 dph failed to develop normal secondary sexual characteristics, whereas females exposed to this level of EE2 were able to breed when paired with unexposed males. Histological evaluation of control, 0.2 ng, and 1 ng/L exposed fish at 56 dph indicated an approximate female-to-male (F:M) ratio of 50:50, whereas fish exposed at 4 ng/L had a F:M sex ratio of 84:5. No ovatestes were observed in the control fish, but ovatestes were observed in 11% of fish exposed at 4 ng/L. After 172 dph, no testicular tissue was observed in any fish exposed to EE2 at 4 ng/L. The NOEC for VTG induction was 4 ng/L. The authors concluded the overall reproduction NOEC was 1 ng/L. This study was assigned a Klimisch code of 1.

Parrott and Blunt, 2005. Parrott and Blunt (*41*) conducted a FLC exposure study of EE2 in fathead minnows. Fertilized eggs (48 h post fertilization) were exposed to 0.32, 0.96, 3.5, 9.6, and 23 ng/L of EE2 for 150 dph under continuous flow conditions. The fish were observed through the larval, juvenile, and adult stages. Except at the highest concentration, there were minimal effects in juvenile growth through 60 dph. At 60 dph, an increase in the ovipositor index (a female secondary sex characteristic) was observed in female fish exposed to EE2 concentrations of 3.5 ng/L and greater. There were significant decreases in secondary sex characteristics in males exposed to a nominal concentration of 0.96 ng/L and above at 150 dph. Fertilization success (defined as the percent fertilized eggs laid by fish exposed for the entire life cycle) decreased in a dose-related manner (81% in controls, 63% at 0.32 ng/L, 36% at 0.96 ng/L, 0% at 3.5 ng/L and above). Fish exposed to 0.32 and 0.96 ng/L produced more eggs in total than control fish during the breeding period; those exposed to >3.5 ng/L EE2 laid no eggs, and all fish were externally female. The authors report 0.32 ng/L was the NOEC based on decreases in secondary sex characteristics in male fish, but a LOEC for effects on fertilization. From the viewpoint of overall reproductive success, 0.32 ng/L may more readily be viewed as a NOEC than a LOEC, despite the decrease in percent fertilization. Ultimate reproductive success is determined by the number of fertilized eggs produced by each female. When the number of eggs per female is combined with percent fertilization success, it turns out that females exposed to 0.32 ng/L produced more fertilized eggs (130 per female) than control females exposed to only water (64 eggs per female) or water and ethanol (92 eggs per female). The laboratory was not able to measure VTG. A Klimisch code of 2 is assigned because analytical sensitivity did not permit measurement of the two lowest exposure concentrations (nominal concentrations were reported), which are most relevant for determining the NOEC.

Nash et al., 2005. To investigate impacts on reproductive success and mechanisms of disruption, Nash and colleagues (*39*) exposed breeding populations of zebrafish (*D. rerio*) to EE2 over multiple generations under continuous flow-through conditions. Measured mean concentrations were between 90 and 100% of nominal. After 10 days' exposure, there was complete reproductive failure (no egg production) and high mortality (85%) in the F0 group exposed to 50 ng/L. There were no effects on egg production after 40 days' exposure to 0.5 or 5 ng/L, and exposure of the parental F0 generation at 5 ng/L had no impact on reproductive success. The NOEC for VTG induction was 0.5 ng/L. The authors consider 5 ng/L as the NOEC for the F0 group. Conversely, life-long exposure of the F1 group at 5 ng/L resulted in complete reproductive failure in the F1 generation, with no viable eggs in almost 12 000 spawned. Egg production was also reduced in the F1 group (~42−45% of controls). Infertility in the F1 generation after life-long exposure to 5 ng/L EE2 was due to disturbed sexual differentiation, with males having no functional testes and undifferentiated gonads. These F1 males also showed a reduced vitellogenin response when compared with F0 males (NOEC for VTG induction of 5 ng/L), indicating an acclimation to EE2 exposure. Depuration studies found only a partial recovery in reproductive capacity after 5 months. Significantly, even though the F1 males lacked functional testes, they showed male-pattern reproductive behavior, induced the spawning act and competed with healthy males to disrupt fertilization. Although not explicitly stated by the authors, the NOEC for the F1 generation, derived from the data presented, was 0.5 ng/L. This study is assigned a Klimisch code of 1.

Schäfers et al., 2007. Schäfers and colleagues (*42*) conducted partial and FLC studies in zebrafish under continuous flow-through conditions at concentrations of 0.05, 0.28, 1.7, and 10 ng/L EE2. Measured concentrations varied between 80 and 120% of the nominal (0.05, 0.28, and 10 ng/L) concentrations, except for the 1.7 ng/L treatment group, in which 66% of the nominal concentration was found. Fecundity and fertility were evaluated during the partial life-cycle (PLC) exposure of parental (F1) fish exposed from

TABLE 1. Summary of Available Reproductive NOECs for 17α-Ethinyl Estradiol (EE2)

test species	reproductive end point	duration (days)	NOEC (ng EE2/L)	LOEC[a] (ng EE2/L)	VTG NOEC[a] (ng EE2/L)	ref	Klimisch code[a]
D. magna	reproduction	21	387 000[b]			*25*	
D. magna	reproduction	21	100 000[b]			*28*	
S. subspicatus[c]	Biomass	3	54 000			*28*	
D. subspicatus	Biomass	3	<100 000			Länge, 2002	
D. subspicatus	Growth Rate	3	<100 000			Länge, 2002	
Brachionus calyciflorus	No. of females	3	202 000			*52*	
S. crystalline	reproduction	3generations	100 000			*29*	
Ceriodaphnia reticulate	reproduction	3generation	200 000			*29*	
Nicotra spinipes	reproduction	18	50 000			*37*	
Tisbe battagliai	Fecundity, sex ratio, development	21	>100 000[d]			*53*	
Gammarus pulex	Sex ratio, pop size	100	100			*54*	
Hyalella azteca	reproduction	273	100			*55*	
Potamopyrgus antipodarum	Embryo production	63	100			*10*	
Marisa cornuarietis	Imposex, oogenesis	180	50[e]			*56*	
Lymnaea stagnalis	Not specified	21	100			*57*	
L. stagnalis	Egg masses	70	50			*57*	
Chironomus riparius	Emergence, sex ratio, egg prod and viability	30	100[f]			*58*	
Strongylocentrotus purpuratus	Development	4	100			*59*	
Lytechinus anamesus	Not specified	4	100			*59*	
H. vulgaris	Sexual reproduction	42	100 000			*60*	
Xenopus (Silurana) tropicalis larvae	Sex ratio	42	<784[g]			*61*	
Rana pipiens	Gonad differentiation and sex ratio	134−162	<1000[g]			*62*	
Rana sylvatica	Same as above	76	<1000[g]			*62*	
Rana temporaria	Sex ratio	40	2.3[g]	27		*63*	
X. (Silurana) tropicalis	Sex ratio	32	2[g]	20		*63*	
P. promelas	reproduction (F0)	301	1	4	4	*27*	1
P. promelas	reproduction (F1)	28	1	>1	ND	*27*	1
P. promelas	reproduction	150	0.32[h]	1	ND	*41*	2
P. promelas	Ovipositor index	60	1	3.5	ND	*41*	2
P. promelas	Egg fertilization	21	3	10	0.1	*64*	2
P. promelas	Egg production	21	1			*10*	2
D. rerio	reproduction F0	40	5	50	0.5	*39*	1
D. rerio	reproduction F1	210	0.5	5	5	*39*	1
D. rerio	reproduction	42	3		3	*40*	2
D. rerio	Multiple	75	0.05[i]	1.67		*57*	1
D. rerio	Gonad transition	28	1.67	3	ND	*65*	2
D. rerio	Not specified	21	5	10	5	*18*	2
D. rerio	Male gametogenesis	60	1	10	1[j]	*66*	2
D. rerio	Female gametogenesis	60	10		1[j]	*66*	2
D. rerio	Not specified	60	1	10	1	*67*	2
D. rerio	Sex ratio	90	1	10	1	*18*	2
D. rerio	Sex ratio	40	1	2	1	*68*	2
D. rerio	Not specified	28	1			*69*	4
D. rerio	Feminization	21	25		2.5	*70*	2
D. rerio	Sex ratio	60	<10[k]	10	>10	*71*	2
D. rerio	reproduction	75	0.31	1.1		*42*	1
D. rerio	reproduction F0	177	0.31	1.1		*42*	1
D. rerio	reproduction F1	162	0.36	2		*42*	1
O. latipes	reproduction	120−180	2	10	ND	*38*	2
O. latipes	Not specified	21	261	488	32	*72*	2
O. latipes	Feminization	100	10	100	ND	*73*	2
O. latipes	Male	60	1	10		*74*	2
O. latipes	Female	60	10			*74*	2
Cyprinodon variegates	Sex ratio, 14d egg prod; hatch, 7d fry-survival	59	2	20	ND	*75*	2
C. variegates	Same as above	43	20	200	ND	*75*	2
Oncorhynchus mykiss	Semen quality, ivf embryo viability	62	<16[l]	16	ND	*76*	2
O. mykiss	GSI	21	11.2		ND	*77*	4

TABLE 1. Continued

test species	reproductive end point	duration (days)	NOEC (ng EE2/L)	LOEC[a] (ng EE2/L)	VTG NOEC[a] (ng EE2/L)	ref	Klimisch code[a]
Poecilia reticulate	reproduction; sex ratio	108	44	112	ND	*78*	2
R. rutilus	Sex reversal	720	0.3	4	4	*43*	2
Acipenser fulvesens	GSI	25	60			*79*	2

[a] Fish studies only. [b] The geometric mean of 196, 723 ng/L based upon these two studies was used to derive the $HC_{5,50}$. [c] Renamed *D. subspicatus*. A NOEC of <100 000 ng EE2/L for *D. subspicatus* (Länge, 2002 [personal communication, D. Caldwell and R. Länge, unpublished data] was discarded, as it is an unbounded value and lower bounded NOEC of 54 000 ng EE2/L (*28*) is available. [d] For *Tisbe battaglia*, a NOEC of 100 000 ng EE2/L is used and is based on the unbounded LOEC of >100 000 ng EE2/L. [e] For *M. cornuarietis*, the NOEC for "super female" was not used; the NOEC for oogenesis was used, instead. [f] For *C. riparius*, a worst-case NOEC of 100 ng EE2/L is used, based on the unbounded LOEC of >100 ng EE2/L. [g] For amphibians, the relatively high unbounded values of <1000 ng EE2/L for the frogs *R. pipiens* and *R. sylvatica* (*62*) and <784 ng EE2/L for the frog *X. (Silurana) tropicalis* (*61*) were discarded from the data set. Instead, the NOECs of 2.3 ng EE2/L for *R. temporaria* and 2 ng EE2/L for *X. (Silurana) tropicalis* (*63*) were used. [h] For fathead minnow, *P. promelas*, the reported LOEC of 0.32 ng EE2/L (*41*) was considered a NOEC (see discussion in text). [i] The NOEC of 0.05 ng EE2/L for *D. rerio* reported by Segner et al. (*57*) is excluded from the analysis because a subsequent study by this group using intermediate exposure concentrations resulted in NOECs of 0.31 and 0.36 ng/L for the F0 and F1 generations (*42*). [j] VTG from Hill and Janz (*67*). [k] The unbounded NOEC of <10 ng EE2/L for the zebrafish, *D. rerio* (*71*), was not used as multiple (lower) bounded NOECs are available from other studies. [l] The value of <16 ng EE2/L for the rainbow trout, *O. mykiss* (*76*), was discarded because it is an unbounded value and a (lower) bounded value of 11.2 ng EE2/L (*77*) is available.

fertilization to 75 dpf. There were no effects at the two lower concentrations (measured 0.05, 0.31 ng/L). There was delayed spawning and a reduction in fertilization success to 41% of controls at measured exposure concentrations of 1.1 and 9.3 ng/L (the two highest exposure groups). There was no effect on fecundity (number of eggs/female/day) at any concentration. Thus, 0.31 ng/L was the NOEC, and 1.1 ng/L, the LOEC. FLC exposure of parental (F1) fish from fertilization to 177 dpf resulted in the same NOEC and LOEC. However, fecundity was slightly but significantly decreased at 1.1 ng/L, accompanied by a decrease in fertility to 52%. A lack of mating behavior was observed, and no spawning occurred in fish exposed at 9.3 ng/L. After depuration in clean water, fish previously exposed to 9.3 ng/L resumed spawning after about 2.5 months and were observed until 285 dpf. The average egg number/day/female was 21 (comparable to the 1.1 ng/L group); however, fertilization success of the eggs was below 3%. Histological examination indicated all fish in the 9.3 ng/L group displayed gonads with ovarian morphology at 177 dpf. Further, no mature ovaries were found at 177 dpf, whereas after depuration, all ovaries appeared mature, and likewise, testes were mature. Testes appeared normal in all individuals, but in 7 of 20 fish with ovaries, pathological changes were found in the gonads, including enhanced follicular atresia, fibrosis, and macrophage infiltration. Similar pathological changes were found at both time points, at the end of exposure (177 dpf) and after depuration (285 dpf). Mean plasma VTG levels remained elevated in males exposed to 9.3 ng/L, even after 98 days depuration (285 dpf). Life-cycle exposure of the F2 generation was continued at 0.09, 0.36, and 2 ng/L (measured concentrations) for 162 dpf. The two lowest concentrations had no significant effect on time of first spawning, egg production, or fertilization success. First spawning occurred earlier than in the F1 generation, and the average number of eggs per day per female was more than twice as high as in parental fish. At 2 ng/L, all three reproductive parameters were impaired. The NOEC for the F2 generation at 162 dpf was 0.36 ng/L, and the LOEC was 2 ng/L. This study was assigned a Klimisch code of 1.

Fenske et al., 2005. In a single concentration experiment, Fenske et al. (*40*) exposed zebrafish to 3 ng/L of EE2 under continuous flow-through conditions from fertilization until the all-ovary stage of gonad development (i.e., 42 days postfertilization (dpf)) or from fertilization until the reproductive stage (beginning 75 dpf) for a total of 118 dpf). The exposure concentration was confirmed analytically. Although the study design did not permit traditional concentration−response determination, since only one exposure concentration was used, this study further refined the concentration-time relationship for complete feminization of the exposed fish. Early life exposure to 42 dpf led to a lasting induction of plasma VTG in adult females but altered neither the sex ratio nor the reproductive capabilities. FLC exposure to 3 ng/L for 75 days or 118 days resulted in elevated VTG concentrations and caused gonadal feminization in 100% of exposed fish. Effects of FLC exposure were at least partly reversible, and 26% of fish of the previous all-female cohort developed fully differentiated testes after 2 months depuration (176 dpf). These findings suggest that continuous EE2 exposure arrested the developmental transition of the gonads of genetic males from the all-ovary stage to functional testes. Because only one exposure concentration was used, 3 ng/L was the NOEC for the Early Life Stage study (42 days), but the unbounded LOEC for the FLC study (75 and 118 days). Although well-reported, this study is assigned a Klimisch code of 2 because only one exposure concentration was used.

Balch et al., 2004. In a FLC study, male and female medaka (*Oryzias latipes*) were exposed to nominal concentrations of 0.2, 2, or 10 ng/L of EE2 under static-renewal conditions for 120−180 days and then paired with unexposed fish of the opposite sex (*38*). There were no effects on reproductive behavior or mating in the 0.2 or 2 ng/L groups. Testes-ova were observed in 20% of males at 2 ng/L, and 68% of males at 10 ng/L; however, these intersex males were capable of fertilizing the eggs of females. Among the 19 males exposed to 10 ng/L EE2 then placed with unexposed females, 16 did not copulate, and reproductive success was very low. None of the females exposed to 10 ng/L participated in reproductive behavior with unexposed males. After depuration for120 d, there was 75% recovery at 2 ng/L, and 30% at 10 ng/L. The NOEC was 2 ng/L. Vitellogenin was not measured. This study is assigned a Klimisch code of 2 because a static-renewal exposure system was used and only nominal concentrations were reported.

U.K. Environment Agency, 2008. In a study carried out for the U.K. Environment Agency, the feminizing effects of EE2 on roach (*Rutilus rutilus*) were investigated over a 2-year period (exposure of fertilized eggs up to 720 dph). Nominal EE2 concentrations were 0.1, 1 ng/L (measured 0.3 ng/L), and 10 ng/L (4 ng/L measured). Fish were sampled on days 56, 84, 112, 250, 518, and 720 of the exposure and analyzed for gonadal sex development and VTG induction. Fish

exposed to a measured concentration of 4 ng/L developed a female-like gonad morphology, determined by a characteristic shape of the gonad and the presence of two points of attachment of the gonad to the peritoneal wall, forming an ovarian cavity. At later life stages, 4 ng/L was shown definitively to result in an all-female population (as assessed by gonad histology), and stages of ovarian development varied more widely as compared to the control females. This likely reflected the presence of both females and sex-reversed males in this treatment group. At all life stages, VTG was significantly elevated in fish exposed to 4 ng/L as compared to controls. At lower EE2 exposure concentrations, there appeared to be a higher proportion of females in the 0.3 ng/L group compared to controls, but this was not statistically significant. Two males out of a total of 52 histologically confirmed male fish sampled at 720 dph were intersex, one exposed to 0.3 ng/L and the other exposed to nondetectable levels of EE2 (i.e., the 0.1 ng/L nominal exposure group). The significance of this finding is unknown, as there is an occurrence of this condition in a very small proportion of the normal roach population. Gonads of the other fish from the two lowest exposure groups did not differ from controls in terms of the stage of sexual development (for either males or females). Overall, the study showed a long-term LOEC of 4 ng/L, which induced complete gonadal sex reversal in roach, resulting in an all-female population. The corresponding NOEC was 0.3 ng/L based on measured concentrations (*43*). Although not a reproduction study per se, the NOEC and LOEC for feminization of male fish is consistent with the other fish reproduction studies. A Klimisch code of 2 is assigned because reproductive trials were not conducted.

Species Sensitivity Distribution. A species sensitivity distribution (SSD) was constructed using the reproduction study NOECs summarized in Table 1 to determine the hazardous concentration of EE2 at which 5% of all the species tested are affected (referred to as the HC_5). Selecting the fifth percentile of the SSD means that as long as concentrations of EE2 are less than or equal to the HC_5, 95% of the species tested will not display adverse effects associated with EE2 exposure. In this analysis, all NOECs for a single species (each representing a specific exposure period) are compared and used for HC_5 derivation. In those cases for which more than one NOEC is available for the same species and exposure period, the geometric mean is used.

The SSD was constructed by fitting a distribution to the 52 reproduction NOECs (see Table 1). Often, the log-normal distribution (*45, 46*) and the log−logistic distribution (*47*) best fit toxicity data. However, several other techniques can be used to construct a SSD from which to derive hazardous concentration percentiles. These include parametric (e.g., log-normal, Weibull distributions) and nonparametric methods (e.g., use of statistical software packages such as "BestFit"). Both statistical (e.g., Kolmogorov−Smirnov, Anderson−Darling tests) and visual (e.g., Q−Q plots) goodness-of-fit techniques can be used to select the most appropriate distribution function for a data set. To select the most appropriate distribution for a given data set, goodness-of-fit statistics (software BestFit, Palisade Inc.) are used. Preference is given to the Andersen−Darling (A−D) test because it places more emphasis on tail values. This test belongs to the wide class of quadratic statistics measuring vertical discrepancy in a cumulative distribution function-type probability plot and is sensitive to departures of the distributions in the tails (*48*). A critical p value (statistical significance level) of 0.05 was used to determine goodness of fit. A value of the calculated A−D statistic above the 95th percentile of the distribution leads to the rejection of the null hypothesis; that is, the distribution is not a good fit (*49*).

The Weibull distribution (Weibull (0.95, 1.91) + (−0.52)) resulted in the most optimal fit through the tails of the log-transformed reproductive effect NOECs used for the derivation of a PNEC for EE2. This Weibull distribution was subsequently used for the determination of the HC_5. This parameter is derived as the fifth percentile of this Weibull distribution. In the TGD, the 50% confidence interval (or median confidence interval) of the HC_5 is considered in deriving the PNEC. This percentile was calculated by conducting a parametric bootstrap simulation using @RISK (version 3.5, Palisade Corporation). The bootstrap simulation consisted of taking 2000 sets of n (= 52) samples from the fitted distribution (with $n = 52$ = the number of data points used for the determination of the Best Fit distribution), and each time, the fifth percentile is calculated. The resulting distribution of HC_5's is used to calculate the 50% confidence interval and the median value determined ($HC_{5,50}$).

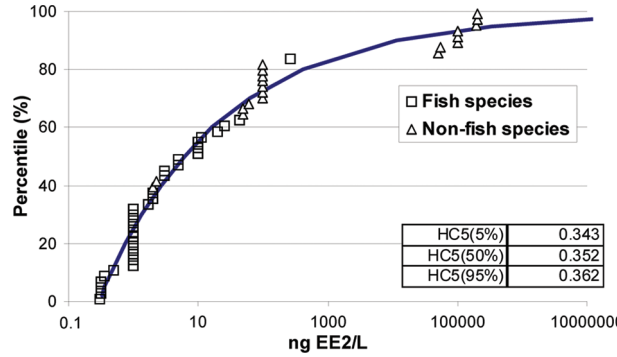

FIGURE 1. Species sensitivity distribution for 17α-ethinyl estradiol, based on all available reproductive NOECs.

The TGD specifies an aquatic plant (e.g., *Lemna*) be part of the data set used for a SSD. However, plants do not contain the estrogen receptor or ERR. In whole effluent toxicity testing, *Lemna* have not been shown to be sensitive to effluents containing estrogenic materials (personal communication, D. Caldwell and D. Heijerick, unpublished test data). Thus, the lack of *Lemna* data is inconsequential to the derivation of the $HC_{5,50}$ for reproductive effects of EE2.

RESULTS AND DISCUSSION

On the basis of the best fit distribution through the reproductive effect NOECs summarized in Table 1, a SSD resulting in an $HC_{5,50\%}$ of 0.35 ng/L was derived (Figure 1). The $HC_{5,50\%}$ changes slightly, depending upon which EE2 concentration is assumed to represent the NOEC from Parrott and Blunt (*41*), the study reporting the lowest effect concentration in fathead minnows. The authors report as an effect a 10−20% decrease in percent fertilization as compared to controls at the lowest tested concentration of 0.32 ng/L (nominal). According to the TGD, a NOEC can be estimated as LOEC/2 when the effect at the LOEC is less than 20%. This would result in a NOEC of 0.16 ng/L. However, because all other bounded NOECs for this species were almost 1 order of magnitude higher than this estimated NOEC (see Table 1) and the overall number of fertilized eggs produced was not decreased at this concentration (see discussion above), the SSD was generated assuming a NOEC of 0.32 ng/L for this study instead of the estimated value of 0.16 ng/L. It is noteworthy that by replacing the value of 0.32 ng/L with 0.16 ng/L, the distribution that best fits the log-transformed data becomes a Gamma distribution with an $HC_{5,50\%}$ of 0.26 ng/L instead of a Weibull distribution. However, the change in the $HC_{5,50\%}$ values derived from these different distributions (i.e., 0.26 ng EE2/L versus 0.35 ng EE2/L) is not very large, indicating the PNECs derived from the reproduction NOEC data converge on similar values.

A potential concern about the protectiveness of using 0.35 ng/L as a PNEC is the consistent finding of a steep

dose−response in the key fish studies. NOECs range between 0.3 (or a calculated value of 0.16) and 1 ng/L, yet 100% feminization occurs at 3 ng/L and above. A SSD derived from the reproduction NOECs allows for a statistical derivation of the $HC_{5,50}$ and, therefore, a PNEC by considering all existing, relevant data for the 26 species that have been studied for reproductive effects. This approach further reduces the uncertainty associated with the fish data by considering data from other chordata demonstrating similar sensitivity to EE2 (e.g., frogs).

Comparison to Key FLC Studies in Fish. The PNEC of 0.35 ng/L can also be compared to the NOECs and LOECs reported in the key fish reproduction studies to document its protectiveness. The PNEC is below the ~1 ng/L LOEC for changes in secondary sex characteristics and decreased egg production in fathead minnows reported by Parrott and Blunt (*41*) that corresponded to a decrease in fertilization success, and the 1 ng/L NOEC reported in the Länge et al. (*27*) FLC study. Further, the PNEC is below the NOEC of 0.5 ng/L in zebrafish reported by Nash et al. (*39*) for the F1 generation. Schäfers and colleagues (*42*) found that 0.31 and 0.36 ng/L were full life cycle NOECs for zebrafish in both the F0 and F1 generations, respectively. The PNEC is also below the NOEC of 2 ng/L for reproductive effects in medaka (*38*). The 2-year study by the U.K. Environment Agency (*43*) in roach that found a NOEC of 0.3 ng/L provides additional evidence that the PNEC is protective.

Comparison to VTG NOECs and LOECs. The FLC fish studies (Table 1) show induction of VTG in the low ng/L range. However, these effects occur at concentrations greater than the PNEC of 0.35 ng/L but generally at concentrations equal to or lower than the LOECs associated with significant reproductive effects (Table 1). Consequently, induction of VTG in male fish can be considered a marker of exposure and not of effect (*23*).

Comparison to Field Studies. Although not considered key studies for PNEC derivation, field studies reporting adverse reproductive effects but employing only one exposure concentration can be used as a bounding comparison. A recent report on the Canadian experimental lake studies is informative in that significant reproductive effects were reported after multiyear exposure at an EE2 concentration of 5−6 ng/L (*50*), which is 15−20 times greater than the PNEC of 0.35 ng/L. These findings are consistent with those observed in laboratory toxicity studies in trout, sand goby, and other fish species that used only a single concentration ≥6 ng/L. Further, these results are predictable from the results of the key fish studies that employed multiple exposure concentrations and evaluated the effects on reproduction.

Comparison to Fish Population Growth Effects. The PNEC of 0.35 ng/L can be compared to the ErC_{20}, the concentration of EE2 estimated to reduce population growth of fathead minnow by 20%, a criterion that serves as a conservative estimate of the no-effects concentration for population growth. The lower 95% confidence limit on the ErC_{20} based on the Länge et al. (*27*) data was calculated to be 0.53 ng/L and is a conservative estimate of the NOEC for population growth (*51*). The PNEC derived from the $HC_{5,50}$ and the multiple FLC reproduction studies is consistent with the NOEC for population growth in this species.

Comparison to Environmental Threshold of No Concern. The PNEC can be compared to a screening environmental threshold of no concern (ETNC) derived by de Wolf et al. (*32*). An ETNC of 0.4 ng/L (0.0004 ug/L) for mode of action 4 (MOA 4) chemicals was derived from evaluation of a data set consisting of 239 chronic toxicity studies conducted on MOA 4 chemicals. MOA 4 chemicals exert aquatic toxicity through a specific mechanism (e.g., by targeting a specific cellular receptor). The data-derived PNEC for EE2 that has a receptor−mediate MOA is nearly identical to the ETNC for MOA-4 chemicals and further supports the ETNC concept.

Robustness of the Proposed PNEC. After consideration of all of the data, including the partial and full life-cycle fish studies, a PNEC of 0.35 ng/L, equal to the $HC_{5,50}$ derived from the SSD, is recommended for EE2 in surface water. This PNEC is expected to be protective for chronic, FLC exposures to EE2. The PNEC is supported by an overwhelming amount of data for EE2 from over 100 studies examining a diversity of effects in a variety of species. These abundant data, including 39 papers reporting on reproductive effects in 26 species, clearly indicate that decreased reproduction in fish is the most sensitive biologically significant effect caused by EE2. Unlike most other compounds, several multigeneration studies have specifically investigated EE2 effects on reproduction in several fish species. Virtually all of these studies have established similar NOECs for reproduction. This similarity in NOEC across several studies and species lends a sense of confidence and certainty to the EE2 reproductive effects data that are absent for most other compounds. Moreover, uncertainty about the PNEC is further reduced by the results of the SSD. The upper and lower 95% confidence intervals of the HC_5 (0.34 and 0.36 ng/L, respectively) suggest that little uncertainty surrounds the $HC_{5,50}$. Even when the assumed NOEC from one of the fish reproduction studies is changed (see discussion above regarding Parrott and Blunt (*41*)) the $HC_{5,50}$ changes by less than 25%, providing further confidence in the robustness of the proposed PNEC. For these reasons, application of an additional assessment factor to the $HC_{5,50}$ to account for uncertainty in the database is not warranted (i.e., an assessment factor of 1 is appropriate) (*24*).

Finally, it is possible that if toxicity studies including several subnanogram dose groups were available, an even more refined and robust PNEC for EE2 than presented here could be derived. For example, it could be that the true NOEC in some of the key fish studies occurs at twice the lowest exposure concentration used in the current experiments. Measuring subnanogram per liter concentrations remains challenging, either to document that such concentrations were achieved and maintained in experiments to establish NOECs, or to determine EE2 concentrations in ambient waters to assess whether the PNEC is exceeded. Thus, there is likely no practical difference between PNECs of 0.35 and 0.5 ng/L. Given the difficulties of working with and analyzing for such ultralow concentrations of EE2, it will likely be some time before a researcher conducts a FLC study using fish exposed to a series of subnanogram EE2 exposures (e.g., 1.0, 0.75, 0.5, 0.25, 0.1, and 0.05 ng/L). If accurate dosing could be measured and maintained, such an experiment would yield important information on the shape of the dose response curve in the critical concentration range below 1 ng/L and could lead to a more refined NOEC, which could in turn lead to a more refined PNEC. However, until such a study is performed, available data supported by multiple analyses suggest that the PNEC of 0.35 ng/L recommended here is robust and protective of biota in surface water.

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