

Article (refereed) - postprint

Harris, Catherine A.; Scott, Alexander P.; Johnson, Andrew C.; Panter, Grace H.; Sheahan, Dave; Roberts, Mike; Sumpter, John P.. 2014. **Principles of sound ecotoxicology**. *Environmental Science & Technology*, 48 (6). 3100-3111. [10.1021/es4047507](https://doi.org/10.1021/es4047507)

© 2014 American Chemical Society

This version available <http://nora.nerc.ac.uk/507528/>

NERC has developed NORA to enable users to access research outputs wholly or partially funded by NERC. Copyright and other rights for material on this site are retained by the rights owners. Users should read the terms and conditions of use of this material at

<http://nora.nerc.ac.uk/policies.html#access>

This document is the author's final manuscript version of the journal article, incorporating any revisions agreed during the peer review process. Some differences between this and the publisher's version remain. You are advised to consult the publisher's version if you wish to cite from this article.

The definitive version is available at www.acs.org

Contact CEH NORA team at
noraceh@ceh.ac.uk

1 **Principles of Sound Ecotoxicology**

2 Catherine A. Harris ^{*1}, Alexander P. Scott ², Andrew C. Johnson ³, Grace H. Panter ⁴, Dave
3 Sheahan ⁵, Mike Roberts ⁶, John P. Sumpter ¹

4 ¹ Institute for the Environment, Brunel University; ² Cefas (Weymouth Laboratory); ³ CEH,
5 Wallingford; ⁴ Astra Zeneca (Brixham Environmental Laboratory); ⁵ Cefas (Lowestoft
6 Laboratory); ⁶ Defra (Chemicals and Emerging Technologies Division)

7
8 * Corresponding author:

9 Institute for the Environment, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK.

10 Tel: +44 1895 266267

11 Fax: +44 1895 269761

12 E-mail: Catherine.Harris@brunel.ac.uk

13 **Abstract**

14 We have become progressively more concerned about the quality of some published
15 ecotoxicology research. Others have also expressed concern. It is not uncommon for basic, but
16 extremely important, factors to apparently be ignored. For example, exposure concentrations in
17 laboratory experiments are sometimes not measured, and hence there is no evidence that the test
18 organisms were actually exposed to the test substance, let alone at the stated concentrations. To
19 try to improve the quality of ecotoxicology research, we suggest twelve basic principles that
20 should be considered, not at the point of publication of the results, but during the experimental
21 design. These principles range from carefully considering essential aspects of experimental
22 design through to accurately defining the exposure, as well as unbiased analysis and reporting of
23 the results. Although not all principles will apply to all studies, we offer these principles in the
24 hope that they will improve the quality of the science that is available to regulators. Science is an
25 evidence-based discipline and it is important that we and the regulators can trust the evidence
26 presented to us. Significant resources often have to be devoted to refuting the results of poor
27 research when those resources could be utilised more effectively.

28

29 **Introduction**

30 We, and others, have become increasingly concerned that the quality of a significant proportion
31 of ecotoxicological research is not as high as it could, and probably should, be.¹⁻⁴ It is very
32 common nowadays for us to read a scientific article published in a reputable journal and end up
33 thinking “this effect of substance X is surprising”, or even “I find it very difficult to believe that
34 substance X really does cause those effects at those concentrations”. Other scientists have also
35 indicated that they have difficulty deciding what ecotoxicological research is sound and what is
36 not.^{5,6} Indeed, some have already published papers suggesting improvements that could be made
37 to ecotoxicological research.⁷

38 We are not the first people to express concern about the quality of published research, either in
39 our field (ecotoxicology) or any other field. For decades (and possibly hundreds of years),
40 scientists have questioned the merits or demerits of particular pieces of research. Nearly half a
41 century ago, an eminent physician, who was interested in possible links between the incidence of
42 various diseases in people and their exposure to industrial chemicals in their working
43 environments, published a set of criteria that he suggested should be used to support, or refute,
44 reported associations between conditions in the workplace (e.g. exposure to industrial chemicals)
45 and particular diseases.⁸ In other words, he was interested in assessing the quality of research
46 that purported to link substance X with adverse effect Y. More recently, various toxicologists
47 and ecotoxicologists have published updated sets of criteria for quality assessment of published
48 (eco)toxicological studies,⁹⁻¹² mainly as a prerequisite to determining what weight can be placed
49 on a study before it can be used for environmental risk assessment.⁵ The outcomes of these
50 assessments do not inspire much confidence in the existing literature: many influential studies
51 are rated as ‘not reliable’ or ‘unacceptable’;⁶ at least one scientist has gone so far as to suggest
52 that ‘most published research findings are false’.¹³

53 We have no desire to undermine ecotoxicology; on the contrary, our desire is to improve it. We
54 accept that some ecotoxicology, especially fieldwork, can be extremely difficult, if not
55 impossible, to conduct in an ideal way. How, for example, does one obtain a clear-cut answer to
56 a question such as ‘are perfluorochemicals adversely affecting albatrosses?’ in order to
57 determine whether their documented exposure to these extremely persistent pollutants¹⁴ is, or is
58 not, of concern? Nevertheless we still consider that much ecotoxicology, including laboratory-
59 based studies, is not being conducted (or interpreted) as well as it could be. In order to try and
60 improve the situation in the future, we list, then briefly expand upon, the factors we consider

61 most important to defining the quality and usefulness of ecotoxicological research studies. We
62 present a set of principles which, if adhered to, would improve considerably the quality of such
63 research (see Table 1). Our approach is based on the very successful establishment of the
64 principles of Green Chemistry.^{15,16} However, whereas those principles were intended to
65 accomplish the goals of green design and sustainability, ours are perhaps somewhat less
66 ambitious and more practical, and specifically aimed at improving the quality of
67 ecotoxicological research. Our principles also address the issue of reporting results in a balanced
68 manner that reflects the results obtained.

69 Discussing the principles of sound ecotoxicology has necessitated mentioning some examples of
70 what we consider poor ecotoxicology. We have attempted not to be unfair to any individual, or
71 to any particular issue in ecotoxicology, and have tried to provide balance in this article by also
72 mentioning examples of what we consider are good ecotoxicological studies. Most of our
73 examples are in the field of aquatic ecotoxicology and, in particular, endocrine disruption,
74 because this is our area of expertise, but we believe the principles outlined here are relevant to
75 ecotoxicology as a whole.

76

The Principles of Sound Ecotoxicology

1. *Adequate planning and good design of a study are essential*

If the planning stages are not thought through adequately, an entire study could be wasted.

2. *Define the baseline*

When any endpoint is assessed, the 'normal' level of that endpoint in an unexposed organism should be established.

3. *Include appropriate controls*

Solvent controls and positive controls should be used where possible/appropriate. The number of controls should also be considered.

4. *Use appropriate exposure routes and concentrations*

Ensure that the route of exposure is appropriate (e.g. via water or via food) and that the concentrations applied are discussed within the context of concentrations measured in the environment.

5. *Define the exposure*

It is important to measure actual concentrations of the substance/s used, so that the real exposure scenario can be described, rather than a hypothetical one. Further, exposure media should be assessed for common contaminants.

6. *Understand your tools*

Knowledge of the particular test organism and test substance used are vital to generating reproducible results.

7. *Think about statistical analysis of the results when designing an experiment*

The number of exposure concentrations, as well as of target organisms, needs to be carefully considered prior to starting the experiment, in order that the results have sufficient statistical power to provide an answer to the hypothesis being tested.

8. *Consider the dose-response*

Consider the dose-response; any 'unusual' pattern of response needs further analysis and justification.

9. *Repeat the experiment*

This may not be necessary where results are striking and statistical power is strong. However, in general, and particularly where results are unexpected and/or borderline, results must be shown to be repeatable.

10. *Consider confounding factors*

Factors such as temperature, disease, and exposure to multiple substances should be taken into consideration; these may be especially relevant in fieldwork.

11. *Consider the weight of evidence*

Results should be compared with previous studies, e.g. do fieldwork and laboratory studies support each other? Do the effects fit with known mechanism of action of the respective substance/s? Consider the plausibility of the results.

12. *Report findings in an unbiased manner*

Do not over-extrapolate (e.g. from *in vitro* to *in vivo*); be aware of the limitations of the study; don't over-hype a result with very low significance; report negative (i.e. no effect) as well as positive findings.

77

78 *Table 1. A summary of the Principles of Sound Ecotoxicology*

79

80 ***Principle 1: Adequate planning and good design of a study are essential***

81 It can often be the case that studies are undertaken in a hurry without sufficient forethought of
82 the several critical factors involved. The first stage is to define the aim of the experiment. For
83 example, is the aim to define the Lowest Observable Effect Concentration (LOEC) of a
84 particular substance, or is it to establish whether effects might be seen at very low concentrations
85 (equivalent to those seen in the natural environment)? Once the aim is agreed, a great deal of
86 effort needs to go into planning the details of the study. Factors to be considered include: how
87 many substances to investigate in any one study; how many exposure concentrations to use (and
88 how far apart these should be spaced); how many replicates (e.g. tanks in the case of fish) of
89 each concentration; how many subjects (e.g. fish per tank); the physico-chemical properties of
90 the substance to be tested; whether the use of an organic solvent can be avoided; when to sample
91 for chemical analysis; how many endpoints to assess (and which are the most relevant for the
92 substance concerned). In addition, experimental planning needs to incorporate steps that can be
93 taken to avoid operator bias, such as random allocation of animals between treatments and
94 blinded analysis of samples where possible. Trying to achieve too much from a study can be as
95 detrimental to the quality of the results as trying to do too little; a balance must be struck. The
96 planning of a good ecotoxicological study can in some circumstances take longer than the
97 exposure study itself.

98 Another factor which should be considered at this point is that adequate recording and
99 documentation, not just of the outcome but of all the procedures undertaken along the way, are
100 essential. If any queries arise during or after an experiment, researchers must be able to back up
101 every step of their working, in order to be able to defend and, if necessary, correct what they
102 have done. Furthermore, adequate information should be provided to enable others to repeat the
103 study in full. We would not go so far as to say that all laboratories should follow ‘Good
104 Laboratory Practice’ (GLP) guidelines, although we could learn much from these principles.
105 Instead, we consider it sufficient to work to the spirit of GLP. For example, researchers should
106 be prepared to share their raw data, (perhaps through a link to an appropriate database if the files
107 are too large to be included as Supplementary Information), in addition to retaining a full report
108 of how the study was designed, conducted and analysed in order to allow adequate interpretation
109 of the results. Such steps were amongst those described in a recent editorial announcement in
110 Nature,³ a journal which is also recognising the problems faced by the recent spate of
111 publications of unreliable data, and now, along with many other journals, stipulates that

112 scientists should deposit large datasets in an approved database prior to publication of the
113 manuscript.

114 We cannot stress enough that good planning and management of an ecotoxicological study is
115 vital for a successful outcome.

116 ***Principle 2: Define the baseline***

117 To discriminate between exposed and unexposed test organisms, toxicological studies usually
118 measure one or more biomarker or sublethal effect that occurs in response to substance
119 exposure. Studies may include organisms sampled from wild populations or, in a laboratory
120 context, the use of standard test species. Whatever the origin of the animals, it is important to
121 characterise the natural variability in parameters or endpoints that form the basis of the
122 investigation (in order to be able to design experiments that are sufficiently sensitive to
123 discriminate real effects). In mammalian multi-generation studies, inter-laboratory variability in
124 negative control data has been intensively studied to improve the sensitivity of the test
125 methods.¹⁷ Several fish species have also been the subject of detailed study to ensure that the
126 experimental design is matched to the reproductive biology of the species used (e.g. the fathead
127 minnow [*Pimephales promelas*] and zebrafish [*Danio rerio*]).^{18,19} Essentially, if one of the
128 endpoints in an exposure study is, for example, a plasma hormone concentration, it is important
129 to know what are the ‘normal’ changes that occur within and between individuals over time.
130 Plasma concentrations of sex steroid hormones in particular are highly dependent on the state of
131 maturity of the gonads and thus show strong seasonal fluctuations. These need to be taken into
132 account when planning and subsequently interpreting studies.

133 Another problematic area in the study of chemical effects on reproductive biology is sex
134 differentiation. It is essential that there is a good understanding of the most sensitive period for
135 this parameter for the species being studied, so that this can be taken account of in the
136 experimental design, and exposure can be focussed on key windows – although it is
137 acknowledged that there are in the zebrafish, in particular, contrasting views on the exact timing
138 of sex differentiation, as discussed by Segner.²⁰

139 Studies on the reproduction of molluscs have raised a number of disagreements with, for
140 example, respect to whether or not substances such as Bisphenol-A (BPA) at very low
141 concentrations increase fecundity in the ramshorn snail (*Marisa cornuarietis*).²¹ Benstead *et al*

142 demonstrated the importance of establishing baseline seasonal fecundity patterns before
143 investigating the effects of endocrine disrupting compounds on gastropod molluscs.²² In that
144 study, a clear correlation between number of eggs laid and photoperiod was established in the
145 reference group, with a subsequent steep decline in egg production following the summer
146 solstice. Although the effect reported in that paper (an extended reproductive season in snails
147 exposed to 17 β -estradiol [E2]) was observed to be a trend rather than being significantly
148 different from the reference group at any one timepoint, the establishment of the baseline
149 reproductive performance pattern of these snails is clearly important in determining whether or
150 not estrogenic substances can impact on snail reproduction, and will provide useful background
151 information for future research in this field.

152 Ultimately, the environmental significance of the results of a study can be better interpreted
153 when there is a good understanding of baseline conditions.

154 ***Principle 3: Include appropriate controls***

155 In theory, this is a relatively easy objective to achieve, at least in terms of laboratory exposure
156 studies (perhaps less so in fieldwork situations). There are four main points that need to be
157 considered:

158 **a)** Use appropriate ‘negative’ controls

159 Negative controls are those where no treatment is administered, and hence no response is
160 expected. A scenario where particular thought should be given to the nature of the negative
161 control is that where solvents are used to dissolve substances that are relatively insoluble in
162 water, and thus the concentrated stocks require an organic solvent (such as ethanol, methanol,
163 dimethylformamide, acetone) to deliver the substance to the exposure medium. It has been
164 shown that such solvents can affect various endpoints in exposed organisms, even when used at
165 low concentrations.²³ Hence it is imperative to minimise the use of solvents and also to include a
166 control in which organisms are exposed to the same concentration of solvent as in the substance
167 treatments. Crucially, these ‘solvent controls’ (as opposed to the ‘dilution water controls’) must
168 also be used for comparison with the substance treatments when it comes to the statistical
169 analysis of the results.

170 It has to be acknowledged that negative controls can be more difficult to implement properly in
171 fieldwork, since there may simply not be any pristine sites available with which to compare

172 organisms from exposed sites. An example of this would be the work undertaken by Jobling *et al*
173 (see also Iwanowicz *et al*),^{24,25} where a small proportion of male roach (*Rutilus rutilus*) at
174 supposedly clean sites (i.e. not exposed to Waste Water Treatment Plant (WWTP) effluents)
175 were found to be intersex (albeit mildly so). The likely reason is that these sites are not as clean
176 as we think (or hope) they are, and are likely often subject to diffuse pollution sources. These
177 sites are nonetheless a useful source of reference values and scientists can use a measure of the
178 relative contamination between sites (even if this is as simple as whether the site is upstream or
179 downstream of an effluent outfall) to judge the influence of such contamination on the level of
180 intersex in the fish under investigation.

181 **b)** Use a positive control where appropriate and/or available.

182 The use of a positive control with known levels of activity may not always be possible, but can
183 be incredibly helpful in the interpretation of ecotoxicological data when implemented. One
184 example of this is in endocrine disruption work, where the apparent hormonal activity of a
185 substance is being investigated. For example, some synthetic estrogen mimics are very weak in
186 comparison to the natural steroid hormone, E2, or the synthetic steroid, ethinylestradiol (EE2). If
187 we compare the estrogenic potency of parabens *in vivo* with a control group, they are certainly
188 estrogenically active. However, in comparison with a positive control, such as E2, these
189 substances have been shown to be only weakly estrogenic both in mammals and in fish.^{26,27} Thus
190 a positive control allows us to put the results into perspective, as well as verifying that the
191 bioassay (i.e. test procedure) is actually working properly.

192 **c)** Consider the number of controls.

193 Part of the reason for including controls in experiments is to establish the degree of variability in
194 the responses of the test animals. Hence if an insufficient number of control subjects is used,
195 then an inaccurate assessment of variability may be made and consequently the comparison with
196 the treated subjects will be made on false assumptions. One example of a study which has
197 demonstrated the importance of a robust experimental design including sufficient numbers of
198 controls has been reported by Owen *et al*.²⁸ The authors initially found an effect of clofibrac acid
199 on the growth rate and condition of juvenile rainbow trout, but an expanded version of the study
200 (using an increased number of control animals, spread over four tanks) did not repeat their
201 original findings. Specifically, the effect observed in the original study was because the
202 relatively small number of control fish (n=8) were exceptional and outperformed normal
203 expectations, further highlighting the need for appropriate controls.

204 **d)** Use the appropriate type of control.
205 This advice refers to the fact that researchers must be aware that any bias introduced into the
206 'selection' or handling of control subjects is unacceptable. That is, the control organisms should
207 be the same sex, age, of a similar size, from the same population as those in the treated groups,
208 and definitively not pre-selected for desirable features which make them reliable controls.
209 Further, all controls must be handled in the same way as treatment groups with respect to factors
210 such as disturbance, food, and experimental conditions (such as light and temperature). If one
211 tank requires cleaning, for example, all tanks should be cleaned.

212 ***Principle 4: Use appropriate exposure routes and concentrations***

213 It is probably true to say that the weakest aspect of many ecotoxicological papers concerns
214 exposure to the test substance(s). Most ecotoxicologists have their main training in biology, not
215 chemistry. Ideally, ecotoxicologists should confer with environmental chemists and modellers
216 before any experiments are designed. Some of the main issues to consider regarding exposure
217 are examined in this and the next principle.

218 **a)** What is the most environmentally-relevant route of exposure?
219 Before exposing an organism to a substance in a laboratory experiment, it is wise to consider the
220 most appropriate route of exposure. For aquatic organisms this is likely to be either via the water
221 or the diet, depending in part on the hydrophobicity of the test substance as well as the behaviour
222 and feeding characteristics of the organism concerned. In the wild, exposure to strongly
223 hydrophobic chemicals may occur primarily via the diet; in which case, this route of exposure
224 should be used, if at all practical. The toxicity of a substance can vary depending on the route of
225 exposure.^{29,30,31} In the terrestrial environment, exposure via diet is very common, and is often the
226 main route of exposure to substances: recall the devastating effects that pesticides had on birds
227 of prey in the 1950s and beyond.³² In contrast, injecting any organism with a test substance (in
228 the context of ecotoxicological studies) is wholly unrealistic and should be avoided, as it cannot
229 shed any light on the real environmental exposure of wild animals.

230 **b)** What is meant by an 'environmentally relevant concentration'?
231 The concept of an 'environmentally relevant concentration' is clearly important in
232 ecotoxicology, as it allows us to judge whether a substance is not merely a hazard but actually
233 poses a risk. Since 1991, the phrase 'environmentally relevant concentrations' has appeared in

234 the title, or abstract, of 1675 papers according to the Web of Science (accessed January 2013).
235 Unfortunately, because there is no clear definition of the phrase ‘environmentally relevant
236 concentration’, the whole issue can be dangerously misleading. This problem can be illustrated
237 by considering the following issues.

238 *What is meant by ‘environment’: the sewage effluent, a sewage ditch, or a river?*

239 It is not unknown for scientists to use a value reported in sewage effluent when justifying their
240 experimental concentration as being environmentally relevant to aquatic organisms.^{33,34,35} Some
241 WWTPs discharge into very small streams which are essentially formed from sewage effluent,
242 but could be classed as a water course. However, the vast majority of freshwater aquatic wildlife
243 live in rivers where considerable dilution of the sewage effluent is the norm.

244 *Could the quoted environmental concentration result from an unreliable measurement?*

245 Trying to detect a substance of interest at low and sub ng L⁻¹ concentrations in complex matrices
246 is fraught with difficulties.³⁶ Hence it is also possible that reported exposure concentrations
247 (particularly in the environment) are in error, and require independent verification before they
248 are accepted. For example, the very high (hundreds of ng L⁻¹) concentrations of many sex steroid
249 hormones, particularly EE2, in UK and US streams reported by Aherne and Briggs³⁷ and Kolpin
250 et al³⁸ have proved not to be repeatable.³⁹ Such erroneous reports can have enormous influence
251 on what are, and are not, considered to be ‘environmentally relevant’ concentrations of
252 substances of concern. Hence the need for a broad review of the literature and/or the
253 collaboration with an analytical chemist in studies where necessary.

254 *Might the quoted environmental concentration be accurate but be entirely unrepresentative of
255 the majority of situations encountered by wildlife in time and space?*

256 Occasional very high concentrations can occur in the environment but in terms of probability
257 they are likely to be rare. A good example is the modelling of 11 large US catchments where the
258 50%ile cumulative probability for EE2 was between 0.0008 and 0.01 ng L⁻¹ at mean and low
259 flow, respectively, but there remained in the 99%ile probability a potential for 0.3-1.0 ng L⁻¹
260 being detected. Thus, the vast majority of American aquatic wildlife would be most likely to be
261 exposed to concentrations in the 0.0008-0.01 ng L⁻¹ EE2 range and only a tiny minority to
262 concentrations of ≥ 0.3 ng L⁻¹.³⁹ So whilst some authors might imply that 5 ng L⁻¹ EE2 is
263 environmentally relevant,^{33,35} the overwhelming evidence is that it would be atypical.

264 We should also make it clear that we are not asserting that only environmentally relevant
265 concentrations should be used in ecotoxicological experiments. Indeed, there will be occasions
266 where researchers have to use significantly higher concentrations in order to properly define a
267 LOEC for a substance. The LOEC of a substance is, in fact, far more useful in the regulatory
268 sphere than is a conclusion that no effect occurs at environmentally relevant concentrations,
269 because a LOEC enables the regulators to impose more accurate and meaningful safety limits.
270 Our primary message here is that the explanation of the concentrations selected for a particular
271 study should be comprehensive, and the authors should be open and honest about the context of
272 their results in relation to those concentrations which have been measured (or predicted) in real
273 environmental samples. Thus the derivation of the measured environmental concentration
274 (MEC) and the predicted environmental concentration (PEC) are also key factors here.

275 *Principle 5: Define the exposure*

276 A useful exercise to undertake when considering this principle is to remind ourselves of why we
277 undertake ecotoxicological studies in the first place. The major reason is that we are concerned
278 about the occurrence of certain substances in the environment, and we need to determine
279 whether they are present at concentrations which can be harmful to living organisms. Thus, in
280 conducting such studies, we hope to supplement the database which is used to risk assess
281 environmental contaminants. Such risk assessments will clearly be inaccurate if the
282 concentrations on which they are based are also inaccurate. The two main points to consider
283 within the scope of this principle are outlined below.

284 **a)** The actual amount of exposure substance in the system must be measured
285 It is paramount that an attempt is made to determine the actual concentrations of substance/s
286 present in the test media to which organisms are exposed. This can be done using either
287 analytical chemistry or biological methods of analysis such as immunoassays or receptor binding
288 assays. Which of these methods is more suitable is debatable; however, there is no doubt that
289 without any attempt to measure concentrations of the test substance, the results of the study
290 cannot be fully interpreted. We should also add at this juncture that it is important there is good
291 quality control of analytical chemistry procedures employed, as the data obtained using such
292 methods are of little use if the associated methods have not been properly validated.

293 There are many examples in the literature where no analytical analyses have been performed. In
294 these cases the researchers have no idea whether the concentration to which the organisms are
295 exposed is (for example) 100 ng L⁻¹ or 1 ng L⁻¹, leaving the results wide open to
296 misinterpretation. Many of these studies also involved the use of a static-renewal system, which
297 further increases the risk of unreliable results compared with a flow-through exposure system,
298 hence rendering the measurement of the test substance even more important. Examples of such
299 studies include those undertaken by Oehlmann *et al.*,²¹ whereby prosobranch snails were exposed
300 to octylphenol (OP) and BPA at nominal concentrations ranging from 1 to 100 µg L⁻¹. The
301 authors describe effects being observed ‘at the lowest concentrations’ but it is unclear as to what
302 those concentrations actually were. This is critical information from a risk assessment point of
303 view. Similarly equivocal information has been generated by Lister *et al.*,⁴⁰ Di Poi *et al.*,⁴¹
304 Franzelletti *et al.*⁴² and Guler and Ford;⁴³ these studies tested pharmaceutical products, including
305 fluoxetine, at nominal concentrations as low as 0.3 ng L⁻¹ in static renewal systems; however no
306 measurements of the actual concentrations of the substances that they were testing were
307 performed. We accept that if a significant biological effect is observed in a dose-related manner
308 it might be difficult to argue that something is not present in the water that is causing that
309 response. But this information is of no use to the regulators if it is not known how much of the
310 substance causes that response. In addition, if a response is observed which is unrelated to the
311 concentration of the substance used, or if there is no response at all, it is impossible to provide an
312 accurate interpretation of the data when the exposure concentrations are unknown. There may
313 actually be no effect of the substance concerned at the (nominal) concentration, but it may be
314 that no effect was observed because the chemical was not present in the tanks at anything like
315 the concentrations that were expected. Finally, although technically more problematic, it is
316 particularly important that verification of the actual exposure concentrations is provided when
317 concentrations that are reported to be causing effects are extremely low (i.e. at concentrations
318 similar to those found in the environment).

319 **b)** Potential contaminants in the system should also be monitored, thus providing an
320 accurate profile of all major substances in the test media.

321 It is useful to have some knowledge of potential contaminants in the system. Clearly, not all
322 eventualities can be accounted for, but what is looked for should include the more commonly
323 occurring contaminants, to assess whether they are present at high enough concentrations to be
324 of concern, or whether their presence can be ignored. There may be occasions where
325 contaminants are found in sufficiently high concentrations that they are likely to act as

326 confounding factors in the toxicological assessment. Such a case was reported by Hala *et al*,⁴⁴
327 who discovered butyltin leaching from airline tubing in a flow-through exposure system at
328 concentrations high enough to confer toxic effects on organisms. Conversely, Aoki *et al* reported
329 intermittent detection of diethylhexyl phthalate (DEHP) in a study undertaken to assess the anti-
330 androgenic nature of dibutyl phthalate (DBP) in fish;⁴⁵ however, in this case it was concluded
331 that the DEHP originated from contamination during the extraction/analysis procedure (i.e. not
332 from the tank water itself), and in any case it was present at such low levels as to be negligible in
333 terms of its effect on the fish in this study. It is unlikely that any study which monitors
334 concentrations of DEHP as a contaminant in water would not contain a trace of this chemical,
335 but it is nonetheless wise to determine the concentrations of DEHP present (particularly in
336 phthalate exposure studies) in order that their significance can be assessed. Another potentially
337 problematic situation is where a test substance might be found to be present in the control tank
338 (for example, via cross-contamination, or even due to inadequate cleaning of equipment between
339 studies). Such information could be critical to understanding the results.

340 ***Principle 6: Understand your tools***

341 When using live organisms to try to understand what are often dynamic processes, it is important
342 to try to minimise the variability encountered by having a good understanding of the background
343 of these organisms. For example, the quality of data obtained can be influenced by the age of
344 animals, as well as by the conditions in which they were reared and/or maintained prior to the
345 study. In addition, some species are very difficult to rear in the laboratory (or it is sometimes
346 inappropriate for the particular assay in use) and if wild-caught organisms are used instead, it is
347 vital that the conditions in the environment in which they have been living are well understood.
348 The presence/absence of parasites should also be established. The presence of parasites can
349 affect physiological parameters in animals,^{22,46,47} and if those parameters overlap at all with
350 those being used in a controlled exposure study, the interpretation of data obtained from infected
351 animals can be problematic, to say the least.^{48,49} Parasite infections such as microsporidians can
352 cause gonadal disruption, produce intersex and female-biased populations, as well as affecting
353 secondary sexual characteristics.⁵⁰ Such combinations of changes can be mistaken for changes
354 that result from chemical exposure. Therefore baseline information on the prevalence of
355 parasitism in different species and an awareness of the potential effects ensuing from this are
356 essential considerations in studies undertaken with wild-caught animals.

357 In some mammalian studies, an understanding of the particular strain used in toxicological
358 studies is necessary, as it is well known that some strains are more sensitive than others.⁵¹
359 Likewise, with commonly used fish species, differences in sensitivities occur in the responses to
360 stressors observed between different strains of the same species;^{52,53,54} and Brown et al reported
361 differences in growth and sexual development between inbred and outbred zebrafish,⁵⁵ which
362 can impact on interpretation of data obtained from substance-exposure trials. It is also important
363 to consider the relevance of the species selected in relation to the overall aim of the study.⁷

364 *In vitro* studies may appear to be more reproducible, but they are certainly not immune from
365 variability. For example, the response of different cell lines to the same genotoxic agent can vary
366 widely within and between laboratories. Therefore, the selection of the cell line to be used needs
367 careful consideration;⁵⁶ also, even within the scope of analysing a single protein, different
368 antibody preparations can elicit very different responses.⁵⁷ It is important that researchers are
369 aware of these factors and are able to adequately define the reagents used.

370 Knowledge of the test substance is equally important. A confirmation of this knowledge should
371 be communicated to the reader by simple means such as stating its purity and CAS number. A
372 discussion of the impact of impurities on the interpretation of data obtained in an *in vitro*
373 estrogen assay was presented by Beresford *et al*,⁵⁸ and has also been recognised by Harris *et al*,⁵⁹
374 who found that two different preparations of a phthalate presented very different estrogenic
375 profiles as a result of one of these preparations having been supplemented with BPA. Some
376 substances consist of different isomers which can have very different biological activities. For
377 example, branched chain isomers of alkylphenolic compounds (such as 4-NP and 4-OP) induce
378 estrogenic effects in fish, mammals and *in vitro* assays, in contrast to the straight chain isomer of
379 the corresponding compound (4-n-NP and 4-n-OP) which are not estrogenic.^{60,61} Hence the
380 inadvertent use of the linear isomer of this substance in an ecotoxicology study could lead to
381 erroneous conclusions of inactivity (as was the case, for example in Moore *et al*).⁶²

382 ***Principle 7: Think about statistical analysis of the results when designing an***
383 ***experiment***

384 The importance of appropriate statistical analysis cannot be overemphasised.⁴ It is crucial that
385 we are able to draw robust conclusions, and that we are able to justify them. In the case of an
386 inappropriate statistical approach being used, an entire study can be undermined and, at worst,

387 misleading conclusions can be drawn. It may be necessary, particularly in some of the more
388 complex analyses required, to enlist the help of professional statisticians. Different statistical
389 approaches exist, the use of which are dependent on the aims of the study in question. These
390 approaches range from testing methods to identify significant effect responses (e.g. to establish a
391 no observable effect concentration [NOEC]); through empirical regression modelling (e.g. to
392 estimate effect or benchmark concentrations); to complex biological modelling (e.g. DEBTOX).
393 Although criticised by many statisticians,⁶³ the NOEC (i.e. the tested concentration just below
394 the LOEC (lowest concentration that produced a significant response)) is still the most
395 commonly used toxicity descriptor. This is derived by statistical testing approaches which
396 assume ‘no effect’ (null hypothesis) and estimate the likelihood that an observed effect happened
397 by chance alone (i.e. not statistically significant) or that it was unlikely to be due to chance alone
398 (statistically significant).

399 Power analysis can be conducted to determine the size of a sample needed to reject a null
400 hypothesis at given error rates, or it can be used to estimate, at given data variation and sample
401 size, the minimal effect size that can be detected as statistically significant. This effect size
402 defines the statistical detection limit which is always present in the data (also called ‘minimal
403 detectable significant difference’). Thus, an *a priori* power analysis can enable the scientist to
404 design a study such that the sample size is high enough to provide reliable answers to the
405 question posed, whilst not being so high that valuable resources are wasted. Nowadays, software
406 packages exist which allow power and sample size calculation without the need to contact a
407 professional statistician, at least for simple study designs. Recommended maximal error rates are
408 usually $\alpha=5\%$ and $\beta=20\%$,⁶⁴ meaning that the minimal power is 80%, i.e. we would identify an
409 effect above the detection limit in 4 out of 5 studies. Another parameter needed for the power
410 calculation is an estimate about the most likely data variation, which can be derived either from
411 previous studies or other historical data sources that are considered comparable to the
412 researchers’ own testing environment. So called ‘range-finding’ studies are often key to
413 providing initial basic information.

414 An example of power analysis is given in table 2. This illustrates the issues involved with
415 assessing the number of individuals required to produce an experiment which will offer a
416 reasonable degree of power in the analysis. Two types of data have been assessed (the data used
417 here are not real, but are derived from real exposure scenarios). The first is where the endpoint
418 assessed is plasma E2 concentration in fish. The response of this parameter can be extremely low

419 (the maximum difference in mean plasma E2 concentration shown here was 2.2 ng ml⁻¹). The
420 second scenario is where the response can be in several orders of magnitude (e.g. plasma
421 vitellogenin concentration). In both cases a high and an intermediate effect detection limit are
422 shown; in the case of plasma vitellogenin a ‘low’ response is also shown. The standard deviation
423 (relative to the mean) is usually lower across individuals exposed to a high level of treatment
424 than it is in the intermediate treatment group. What the information provided in Table 2
425 illustrates is that where the effect size is (or is expected to be) lower, more individuals are
426 required to detect this size as significant at given error rates. Consequently, if the degree of
427 change in a given endpoint is very small, providing robust evidence of any change can be
428 challenging; where the degree of change is far greater, detecting a change in response to a
429 stressor is much easier. In addition, the higher the variability observed within any treatment
430 group, the more individuals are required.

431 Where good baseline (control) data are available, scientists will be able to determine the
432 variability within control groups and use this to aid the experimental design. For example,
433 extensive data sets have been published on the variability of a variety of reproductive and
434 endocrinological parameters in fathead minnows,^{18,65} which are extremely useful to researchers
435 designing experiments using reproductive endpoints in these fish. Furthermore, Paull and
436 colleagues considered that the level of inconsistency in reproductive success between breeding
437 colonies of zebrafish maintained in the laboratory was so high that a minimum of six replicates
438 per chemical treatment is necessary to discriminate a 40% change in egg output of females and
439 sperm quality (in terms of motility) in male zebrafish (at $\alpha=5\%$).¹⁹

440 To conclude, it is important to remember that (i) error rates (and therefore a (controlled)
441 uncertainty) are always present in our conclusions; (ii) statistical significance should not be
442 confused with biological significance; (iii) “no effects” cannot be identified by statistics; and (iv)
443 if one reaches the conclusion to accept a hypothesis, it does not mean that it is proven, it means
444 that the hypothesis is supported given current data.

445 More detailed guidance on statistical approaches used in standard ecotoxicology studies can be
446 found in the OECD Testing and Assessment guidelines.^{63,64}

447

Endpoint	Treatment	Mean	Average or worst-case scenario standard deviation	Number of individuals required to give 80% power
Plasma E2 concentration (ng ml ⁻¹)	Control	3.84		
	Low	2.7	average	17
			worst-case	53
	High	1.62	average	4
			worst-case	7
	Plasma vitellogenin concentration (ng ml ⁻¹)	Control	54	
Low		85	average	16
			worst-case	30
Medium		40000	average	3
			worst-case	5
High		350000	average	2
	worst-case		2	

448

449 *Table 2. The number of individuals required to provide data with a power of 0.8 and an α*
450 *(probability of error) value of 0.05 in particular exposure scenarios. These a priori analyses,*
451 *using log₁₀ values of hypothetical data, were conducted using the statistical package ‘G*Power’.*
452 *A ‘low’ response example is not given for the endpoint of plasma E2 because the overall range*
453 *of response is far smaller here than it is for the vitellogenin response.*

454 ***Principle 8: Consider the dose-response***

455 In order to be able to deduce the dose-response of a substance (and hence put the results into any
456 kind of environmental context), at least three concentrations need to be tested. A recent example
457 of a study which does not report a full dose-response was published in Science,⁶⁶ where only two
458 concentrations of the drug (oxazepam) were tested. Data from just one or two concentrations
459 alone will be of little use in the regulatory field.

460 Secondly, we think that, in almost all cases, the relationship between dose and response should
461 be regularly incremental (or decremental) – i.e. for each increase in dose, there should be a
462 graded increase (or decrease) in response. This produces a ‘monotonic’ dose-response curve.
463 Good examples of monotonic curves are those involving estrogen stimulation of vitellogenin
464 production in fish and androgen stimulation of spiggin production in the stickleback
465 (*Gasterosteus aculeatus*).^{67,68} A key outcome of bioassays with monotonic curves (providing
466 they can be consistently repeated) is that it is possible to accurately calculate the LOEC and the
467 NOEC (or NOAEL) of compounds. These are very important for accurate ecological risk
468 assessments.

469 There are numerous examples (many hundreds) of published dose-response curves in the field of
470 ecotoxicology that are ‘non-monotonic’.⁶⁹ These cover a whole range of shapes such as flat, U-
471 shaped, J-shaped and inverted U, as well as many that are irregular (or ‘multinodal’). When it
472 comes to the interpretation of non-monotonic dose-response curves, a rift has developed between
473 ecotoxicologists. In the view of Vandenberg and co-workers, non-monotonic curves form
474 compelling evidence that low doses of compounds (in many cases well below the current
475 NOAEL) are able to trigger effects that regulators do not currently take into account.⁶⁹ However,
476 there are others who, while conceding that non-monotonic (especially inverted-U-shaped) curves
477 are not unlikely to occur in some circumstances, are of the opinion that many of the non-
478 monotonic relationships that have been reported can equally be ascribed to either poor
479 experimental design and/or technique, or to the action of confounding factors. The gold test of
480 whether a non-monotonic dose-relationship is a real phenomenon (as with other scientific
481 endeavours) should be whether it can be reproduced consistently. Vandenberg *et al* appear,
482 surprisingly, to argue that this is an unfair requirement in the field of low-dose effects, due to
483 such effects tending to be more dependent on factors such as place, time, operators, strain of
484 animal etc. than high dose effects. This view is obviously one that is open to debate.

485 We do accept that a dose-response relationship may, after further research, turn out to be
486 genuinely non-sigmoidal (especially one that has a regular U or inverted-U shape). In such cases
487 the burden of proof is on the researchers who report such data to, firstly, show that the
488 phenomenon is repeatable and secondly, at some stage in the research process, to explain and, if
489 possible, prove the underlying mechanism that causes the effect. Even if these two objectives
490 can be achieved, there is still a major problem with using results from bioassays that have
491 generated non-sigmoidal dose-response curves to guide environmental safety thresholds.

492 ***Principle 9: Repeat the experiment***

493 **a)** Repeat the experiment in own laboratory in the first instance

494 With budgets tight and with scientists who undertake *in vivo* studies always looking to reduce
495 the numbers of animals used, it is understandable that on many occasions a single experiment is
496 cited as producing a particular and significant response pattern. This is especially true the closer
497 the research is to fieldwork (for example, full life-cycle studies and/or mesocosm studies are, for
498 some researchers, too expensive to undertake once, let alone twice). It is also a result of
499 necessary legislation that exists to protect vertebrates used in experimental procedures, which
500 means that researchers have to keep the number of animals used to a minimum. Hence *a priori*
501 power analysis (see Principle 7) is an important tool to inform researchers of the minimum
502 number of animals required to give a sound result in a given study. Furthermore, repeat studies
503 must be justifiable to legislative bodies, and in some cases should include refinements (which
504 aim to improve the robustness of the results obtained). However, all researchers must be aware
505 that it is imperative that where the results are surprising, or especially hard-hitting (for example,
506 a significant response to a very low dose of substance, or a response which contradicts previous
507 studies), the onus is on the researchers concerned to repeat the experiment, in order to verify
508 their conclusions. As is often quoted in the literature, ‘extraordinary claims require extraordinary
509 evidence’.

510 **b)** The importance of independent validation

511 Politicians or risk assessors must take great care when making decisions on the basis of
512 observations that have not been independently confirmed. Unfortunately science funding is
513 usually limited and, also, most scientists and funding bodies prefer to do ‘original research’
514 rather than confirm someone else’s findings. Because of the consequent lack of independently
515 validated studies, people who seek to make decisions on the basis of the scientific literature
516 (such as risk assessors) instead rely heavily on the ‘weight of evidence’ (WoE) approach (i.e.
517 where plausible evidence is built up from fragmented observations from a diverse range of
518 species and approaches); see Principle 11. For example, the majority of us are agreed that in an
519 ideal world we would like to be able to use invertebrates instead of vertebrate organisms in
520 ecotoxicology. In the field of endocrine disruption, for example, molluscs might appear to be the
521 ideal solution. There are at least 200 papers that suggest that the reproductive hormones of
522 molluscs are the same as those of humans. However, as pointed out by Scott,⁷⁰ very few of these

523 studies have ever been properly independently validated (i.e. they have been on different species,
524 with different endpoints, and different experimental designs).

525 Another important reason why one should wait for findings to be independently validated is that
526 ‘to err is human’. It should be safe to assume that any trained scientist (especially one with a
527 good track record in research) should not make mistakes when, for example, working out
528 dilutions and concentrations, making up solutions with defined molarities, or analysing data.
529 However, it is not safe to assume this at all. In fact, the propensity of scientists to make errors
530 appears to be rather high. It was the recognition that mistakes are easily made that was behind
531 the issuance in 2003 of the Joint Code of Practice for Research by the main UK biological
532 research funding bodies.⁷¹ Its major requirement is that scientists should keep accurate and
533 detailed records of all their actions in order that any such errors, if they occur, can be traced and
534 corrected (even post-publication). It is also good practice to have other colleagues cross-
535 checking calculations and/or data analysis, as a form of quality control.

536 The importance of reproducibility was discussed in a recent Nature World View article, in which
537 the author asserts that “reproducibility separates science from mere anecdote”.⁷²

538 ***Principle 10: Consider confounding factors***

539 Confounding factors are those ‘conditions’ present in the test environment which may influence
540 the experimental result in addition to the specific parameter that is being assessed. These may
541 include factors such as variations in temperature, disease and the presence of unexpected
542 substances, amongst others. Although it is not always straightforward, or even possible, to
543 actually quantify the confounding factors present, we must always be aware of their potential
544 influence and be cautious in our interpretation of the results, especially when such factors are
545 known to be present. Fieldwork scenarios, in particular, present a challenging and complex array
546 of confounding factors which may enhance or mask the adverse effects of a chemical or mixture
547 of chemicals. At the very least these must be acknowledged by the authors, and when known,
548 accounted for in the analysis and interpretation of data arising from such studies.

549 As an example of good practice in relation to interpretation of field trials, we point to a study by
550 Burkhardt-Holm et al that dealt with the issue of why fish catches (mainly of trout) have
551 declined very significantly in Switzerland in the last few decades.⁷³ Instead of automatically
552 linking the decline to the existence of estrogens in the aquatic environment (the fashionable

553 explanation at the time), the authors offered eight potential causes, ranging from poor water
554 quality, increased predation (by birds), insufficient food, as well as changes in fisheries
555 management. Each potential cause was discussed, in a very balanced manner, in order to rule
556 them in or out. In the end, the researchers concluded that it is unlikely that the decline in fish
557 stocks has a single cause; instead it is most likely due to a combination of factors (stressors).

558 As an example of bad practice in relation to interpretation of field trial data, we point to a study
559 by Ginebrada et al that implies, in both its title “Environmental risk assessment of
560 pharmaceuticals in rivers: relationships between hazard indexes and aquatic macroinvertebrate
561 diversity indexes in the Llobregat River (NE Spain)” and abstract, that the reason for reduced
562 macroinvertebrate diversity in the studied locations (namely, rivers receiving effluent inputs), is
563 the presence of pharmaceuticals in the effluent discharge.⁷⁴ However, although the
564 concentrations of the selected drugs were found to be correlated to both the density and biomass
565 of macroinvertebrates, it seems inevitable that other properties of the effluents (such as other
566 chemicals that are present in effluents, or the physico-chemical characteristics of the effluents
567 concerned) would also have contributed to this reduction in diversity, and would probably also
568 have shown a correlation. The authors did actually raise this point in the discussion section of
569 the paper, but it should not (in our opinion) have been omitted from the title and the abstract.

570 One final example of a significant confounding factor is parasitic infection (see Principle 6 for
571 further discussion on the impact of parasites on endpoints associated with endocrine disruption).
572 As mentioned above, it is important to acknowledge the potential impact of such phenomena on
573 the outcome of a study, even if the precise relationships are not clear-cut.

574 ***Principle 11: Consider the weight of evidence***

575 The general principle behind assessing the weight of evidence (WoE) concerning the
576 environmental risk posed by a particular substance involves taking all the available information,
577 from whatever source (e.g. field and laboratory; *in vitro* and *in vivo*; ecological and
578 physiological), and judging how well it does, or does not, tell a consistent story.

579 Many papers, especially reviews, refer to the ‘WoE’ for a particular theory, and this is what is
580 used by regulators to determine the risk posed by a particular substance. However, according to
581 Weed,⁷⁵ this term has not been scientifically defined and has been used in the majority of cases
582 in a metaphorical sense (e.g. ‘nine out of ten papers report a positive effect of compound X,

583 therefore surely, reader, you have to accept that compound X is an endocrine disruptor’).
584 However, realising that this usage takes no account of the quality of the papers – and is, in all
585 probability, just a reflection of the prevailing bias in that particular field,⁷⁶ several people in
586 recent years have attempted to develop more focussed methods for quantifying WoE.^{9,77}
587 However, whether this entails ‘weighting’ the studies on the basis of dataset size, or even simply
588 tabulating all the data points (where known) in the literature in an unbiased manner and allowing
589 the reader to make his/her own judgement,^{39,78} all approaches suffer from the same inherent
590 weakness – namely that studies where no effects were observed are very often not published (see
591 Principle 12) and such studies cannot therefore be taken into account.

592 With regards to whether or not the research fits with existing literature, pharmaceuticals provide
593 an excellent example. They have an extremely well defined mechanism of action (at least as far
594 as their activity in humans is concerned). This information can be of immense value both in the
595 design of studies to assess ecotoxicity of pharmaceutical substances, and also in the
596 interpretation of results obtained from such studies, and should be taken into account when
597 assessing the weight of evidence for pharmaceutical substances for which the MOA (and also, in
598 many cases, their potential side effects) is well defined.

599 Despite some potentially difficult areas to negotiate, the WoE approach remains the only way
600 that scientists and policy makers can move forward in the uncertain world of science. A major
601 argument in the philosophy of science is that we can never prove a hypothesis, no matter how
602 many examples are provided, but only falsify it.⁷⁹ At first sight this would appear to keep science
603 in a prison of uncertainty, with nothing able to be proved. However, both Popper⁸⁰ and Hill⁸
604 allowed that where sufficient independently validated supporting evidence existed, the
605 hypothesis could be considered a working hypothesis and a basis for action.

606 ***Principle 12: Report findings in an unbiased manner***

607 Researchers these days are under a great deal of pressure to attract research funding, to deliver a
608 positive outcome to their paymasters and to publish as many papers as possible in high impact
609 journals. We believe that these pressures are behind the increase in papers in which the title and
610 abstract tell one story (often with dramatic claims), while the methods and results tell another
611 (often containing weaknesses in design and/or mundane findings). Aside from the fact that the
612 publication of such papers is an indictment of the peer-review process, we believe that such use

613 of 'spin' is confusing for policy makers, a bad example for young researchers and ultimately
614 gives the profession a bad name. The problems occur when the researchers fail to acknowledge
615 or discuss the weaknesses and/or when they employ hyperbole ('hype') to exaggerate the
616 significance of their findings. A recent example of hype is the paper entitled 'Antidepressants
617 make amphipods see the light' in which the data purporting to show that the organisms
618 concerned move towards the light in response to exposure to fluoxetine is, in our opinion,
619 inconclusive (because although the data from one study show a significant effect, data from the
620 other study reported in the same paper show no such effect).⁴³

621 One of the many reasons why such controversies arise, as proposed by Goldacre,⁸¹ is the
622 'suppression of negative results', a topic also addressed by Knight.⁸² This is the (mostly passive)
623 tendency of researchers to publish only positive results (as negative results do not, except in a
624 few cases, attract research funding or ensure career progression). Goldacre argues, however, that
625 many scientists do not just tend to shy away from negative results, but actually have a bias
626 towards positive evidence, and points to a study that examined the outcome of FDA (Federal
627 Drug Administration) registered clinical trials on a class of antidepressant drugs.⁸³ Thirty seven
628 studies showed a positive effect, of which thirty six were published in peer-reviewed literature.
629 However, there were a nearly equal number of studies (thirty three) that gave negative results; of
630 these, twenty two were not published at all and another eleven were written up and published in
631 a way that implied they had a positive outcome. In the context of endocrine disruption, this
632 tendency for bias towards positive evidence probably explains why scientists, when including
633 negative (i.e. no effect) as well as positive data in their papers, tend to assume that the
634 experiments with the positive results are the 'correct' result, and any negative outcomes are due
635 to unforeseen circumstances – e.g. the experiments with negative outcomes have been variously
636 explained away on the basis that: 'the experiment was not carried out at the right time of year';
637 'the animals were not at the right stage of maturation'; 'the experiment was done at the wrong
638 temperature'; or 'the animals were not of the correct origin'. Although it cannot be denied that
639 there may be a valid explanation for a negative result, we suggest that, without actual hard
640 evidence, there is no *a priori* reason, in any study, to reject the experiments that give negative
641 results and only accept the ones that give positive results. Another reason that controversies
642 often arise in the reporting of ecotoxicological data is that there is no clear definition of what
643 constitutes an 'adverse' effect. Although it is not within the scope of this manuscript to address
644 this issue fully here, the authors recognise that this lack of definition can lead to subjective
645 presentation of data, depending on the personal opinion of the scientist concerned. For example,

646 some think that any alteration in the physiology of organisms, which has been induced by a
647 substance to which that organism would not naturally be exposed, could be considered an
648 adverse effect. On the other hand, others consider that it only becomes an adverse effect once
649 there is an effect on population- or health-related endpoints. Still more may even believe that a
650 reduction in the numbers of an over-crowded population would not necessarily be considered
651 'adverse'. This is perhaps an ethical issue that would be best discussed in another forum.

652 ***Causes and consequences of poor ecotoxicological research***

653 Undoubtedly the most compelling reason for the rush to publish (and never mind the quality), is
654 the fact that scientific research has become increasingly competitive over recent years. This has
655 led to the need for scientists to publish prolifically in order to be able to secure both jobs and
656 further funding. In many cases, quantity appears to rule over quality. The issue of the tendency
657 not to publish 'negative' (no-effect) results may also be a factor here (scientists think that
658 funders and future employers will be less interested in their work if they have not shown a newly
659 discovered sensational effect of substance x on species y); although journal editors also have a
660 duty to encourage the publication of no-effect data arising from well designed and executed
661 studies. There is also evidence that there has been a proliferation of journal output over recent
662 decades,⁸⁴ which may well have led to a dilution of good science with poor (although there are
663 no studies that we know of that have investigated the change in number of ecotoxicological
664 journals in particular over this time). We do agree with the sentiments expressed in a recent
665 'Nature' editorial that the frequently irreproducible data that are published these days are not
666 usually a result of fraud, but of insufficient thoroughness in the analysis and presentation of
667 data.²

668 The potential consequences of unsound ecotoxicology research can be profound.
669 Ecotoxicologists presumably conduct their research because they want to protect wildlife from
670 adverse effects of chemicals that already are, or could in the future be, present in the
671 environment. In other words, they want to improve the environment (or prevent it deteriorating),
672 by researching potentially hazardous chemicals and subsequently reducing chemical pollution in
673 the environment. However, many ecotoxicologists have little or no contact with the people
674 (regulators) who have to act on the results that they publish. Regulators have to assess the degree
675 of risk posed by a substance (based primarily on the published research of ecotoxicologists) and,
676 if necessary, take steps to reduce that risk to an acceptable level. The process of assessing the

677 degree of risk and taking any necessary risk reduction steps (such as setting environmental
678 quality standards, or restricting or even banning the use of a chemical) can often be a very
679 detailed and lengthy one. It often takes a decade or more and, these days, usually occurs at both
680 national and international levels. Hence it costs a great deal of money! Moreover, the funding
681 available for fundamental science to support the data produced by (eco)toxicologists is limited,
682 further hindering progress made by the regulators. In cases where data are published indicating
683 that a particular substance is likely to cause adverse effects to wildlife, it is naturally difficult to
684 change the negative public opinion towards this substance, even when the data concerned
685 emanated from just a single study. The cost of confirming or refuting the results of a poorly
686 designed study can be extremely high; and for fish chronic studies could amount to several
687 hundred thousand US dollars. The cause of protecting the environment itself may suffer as funds
688 are drawn away from studying other more harmful chemicals. In addition, the calculation of
689 Environmental Quality Standards (EQS) involves the evaluation of all studies published on the
690 particular chemical concerned. However, the existence of even one study that shows, for
691 example, that a 100-fold lower EQS should be applied, must be acknowledged by regulators
692 even if the vast majority of studies suggest otherwise. Any inadequacies in study design or
693 inaccuracies in the measurements made could have profound implications for regulators, for the
694 water industry, and ultimately for us as taxpayers, if they lead to a significantly lower acceptable
695 environmental concentration. Furthermore, there are undoubtedly environmental contaminants
696 upon which the regulator should be focussing their attention, and inaccurate data on other (less
697 harmful) substances may mean that their attention is not focussed on the chemicals that really
698 are of environmental concern.

699 In conclusion, ecotoxicologists need to think about the consequences of their research *before*
700 they publish it, and they need to take responsibility for it. This does not mean that results
701 suggesting a substance is of concern should be suppressed, or their publication significantly
702 delayed. Indeed, we embrace the process of publication as a major part of scientific discourse,
703 and its role in facilitating discussion around the subject in hand. But it does mean that scientists
704 have a duty to ensure that their research is sound, and therefore likely to be repeatable, before
705 publishing it. Likewise, readers should be aware that they should always critically appraise the
706 work contained therein, and not take it simply on trust. Scientists also need to give serious
707 consideration to making their raw data publically available, the benefits of which cannot be
708 overstated. Many high quality journals require that such data are deposited in a database prior to
709 publication; those that do not specifically require this do at least encourage authors to share their

710 data on request. Adhering to these guidelines will greatly enhance the trust afforded to individual
711 scientists, and between scientists and policy makers. Transparency and robustness are key
712 elements to a successful scientific outcome.

713 ***Acknowledgements***

714 Many thanks to Luigi Margiotta-Casaluci, Martin Scholze and David Taylor for their comments
715 and insight into the manuscript and the issues surrounding this topic. Andrew Johnson is grateful
716 to CEH for supporting his contribution through science budget funding from NERC.

717 ***References***

- 718 1. Agerstrand, M.; Edvardsson, L.; Ruden, C. Bad reporting or bad science? Systematic data
719 evaluation as a means to improve the use of peer-reviewed studies in risk assessments of
720 chemicals. *Hum. Ecol. Risk Assess.* **2013**, DOI: 10.1080/10807039.2013.854139
- 721 2. Nature Editorial. Must try harder. *Nature* **2012**, 483, 509.
- 722 3. Nature Editorial. Reducing our irreproducibility. *Nature* **2013**, 496, 398.
- 723 4. Vaux, D.L. Know when your numbers are significant. *Nature* **2012**, 492, 180-181.
- 724 5. Küster, A.; Bachmann, J.; Brandt, U.; Ebert, I.; Hickmann, S.; Klein-Goedicke, J.; Maack,
725 G.; Schmitz, S.; Thumm, E.; Rechenberg B. Regulatory demands on data quality for the
726 environmental risk assessment of pharmaceuticals. *Regul.. Toxicol. Pharmacol.* **2009**,
727 55(3), 276-280.
- 728 6. Agerstrand, M.; Küster, A.; Bachmann, J.; Breitholtz, M.; Ebert, I.; Rechenberg B.; Ruden,
729 C. Reporting and evaluation criteria as means towards a transparent use of ecotoxicity data
730 for environmental risk assessment of pharmaceuticals. *Environ. Pollut.* **2011**, 159(10),
731 2487-2492.
- 732 7. Breitholtz, M.; Ruden, C.; Hansson, S.O.; Bengtsson, B-E. Ten challenges for improved
733 ecotoxicological testing in environmental risk assessment. *Ecotox. Environ. Safe.* **2006**, 63,
734 324-335.
- 735 8. Hill A.B. The environment and disease: association or causation? *Proc. Royal Soc. Med.*
736 **1965**, 58, 295-300.

- 737 9. Klimisch, H.J.; Andreae, M.; Tillman, U. A systematic approach for evaluating the quality
738 of experimental toxicological and ecotoxicological data. *Reg. Tox. Pharm.* **1997**, *25*, 1-5.
- 739 10. Durda, J.L.; Preziosi, D.V. Data quality evaluation of toxicological studies used to derive
740 ecotoxicological benchmarks. *Hum. Ecol. Risk Assess.* **2000**, *6*(5), 747-765.
- 741 11. Hobbs, D.A.; Warne, M.,St.J.; Markich, S.J. Evaluation of criteria used to assess the
742 quality of aquatic toxicity data. *Integr. Environ. Assess. Manag.* **2005**, *1*(3), 174-180.
- 743 12. Schneider, K.; Schwarz, M.; Burkholder, I.; Kopp-Schneider, A.; Edler, L.; Kinsner-
744 Ovaskainen, A.; Hartung, T.; Hoffmann, S. "ToxRTool", an new tool to assess the
745 reliability of toxicological data. *Toxicol. Lett.* **2009**, *189*(2), 138-144.
- 746 13. Ioannidis, J.P.A. Why most published research findings are false. *PLOS Med.* **2005**, *2*(8),
747 696-701.
- 748 14. Giesy, J.P; Kannan, K. Global distribution of perfluorooctane sulfonate in wildlife.
749 *Environ. Sci. Technol.* **2001**, *35*(7), 1339-1342.
- 750 15. Anastas, P.T.; Zimmerman, J.B. Design through the 12 principles of green engineering.
751 *Environ. Sci. Technol.* **2003**, *37*(5), 94A-101A.
- 752 16. Anastas, P.T.; Eghbali, N. Green Chemistry: Principles and Practice. *Chem. Soc. Rev.*
753 **2010**, *39*(1), 301-312.
- 754 17. Marty, M.S.; Allen, B.; Chapin, R.E.; Cooper, R.; Daston, G.P.; Flaws, J.A.; Foster,
755 P.M.D.; Makris, S.L.; Mylchreest, E.; Sandler, D.; and Tyl, R.W. Inter-Laboratory
756 Control Data for Reproductive Endpoints Required in the OPPTS 870.3800/OECD 416
757 Reproduction and Fertility Test. *Birth Defects Res. B* **2009**, *86*, 470-489.
- 758 18. Jensen, K.M.; Korte, J.J.; Kahl, M.D.; Pasha, M.S.; Ankley, G.T. Aspects of basic
759 reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*).
760 *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **2001**, *128*, 127-141.
- 761 19. Paull, G.C.; Van Look, K.J.W.; Santos, E.M.; Filby, A.L.; Gray, D.M.; Nash, J.P.; Tyler,
762 C.R.; Variability in measures of reproductive success in laboratory-kept colonies of
763 zebrafish and implications for studies addressing population-level effects of environmental
764 chemicals. *Aquat. Toxicol.* **2008**, *87*, 115-126.

- 765 20. Segner, H. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine
766 disruption. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **2009**, 149, 187–195.
- 767 21. Oehlmann, J.; Schulte-Oehlmann, U.; Tillmann, M.; Markert, B. Effects of endocrine
768 disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part 1.
769 Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology* **2000**, 9, 383–397.
- 770 22. Benstead, R.S.; Baynes, A.; Casey, D.; Routledge, E.J.; Jobling, S. 17 β -Oestradiol may
771 prolong reproduction in seasonally breeding freshwater gastropod molluscs. *Aquat.*
772 *Toxicol.* **2011**, 101, 326–334.
- 773 23. Hutchinson, T.H.; Shillabeer, N.; Winter, M.J.; Pickford, D.B. Acute and chronic effects of
774 carrier solvents in aquatic organisms: A critical review. *Aquat. Toxicol.* **2006**, 76, 69-92.
- 775 24. Jobling S.; Nolan M.; Tyler, C.R.; Brighty, G.; Sumpter, J.P. Widespread sexual disruption
776 in wild fish. *Environ. Sci.Technol.* **1998**, 32(17), 2498-2506.
- 777 25. Iwanowicz, L.R.; Blazer, V.S.; Guy, C.P.; Pinkney, A.E.; Mullican, J.E.; Alvarez, D.A.
778 Reproductive health of Bass in the Potomac, USA, drainage: Part 1. Exploring the effects
779 of proximity to wastewater treatment plant discharge. *Environ. Toxicol. Chem.* **2009**,
780 28(5), 1072-1083.
- 781 26. Routledge, E.J.; Parker, J.; Odum, J.; Ashby, J.; Sumpter, J.P. Some alkyl hydroxy
782 benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **1998**, 153,
783 12-19.
- 784 27. Pedersen, K.L.; Pedersen, S.N.; Christiansen, L.B.; Korsgaard, B.; Bjerregaard, P. The
785 preservatives ethyl-, propyl- and butylparaben are oestrogenic in an *in vivo* fish assay.
786 *Pharmacol. Toxicol.* **2000**, 86, 110-113.
- 787 28. Owen, S.F.; Huggett, D.B.; Hutchinson, T.H.; Hetheridge, M.J.; McCormack, P.; Kinter,
788 L.B.; Ericson, J.F.; Constantine, L.A.; Sumpter, J.P. The value of repeating studies and
789 multiple controls: replicated 28-day growth studies of rainbow trout exposed to clofibric
790 acid. *Environ. Toxicol. Chem.* **2010**, 29(12), 2831-2839.

- 791 29 Klimisch, H-J.; Deckardt, K.; Gembardt, Chr.; Hildebrand, B.; Küttler, K.; Roe, F.J.C.
792 Subchronic inhalation and oral toxicity of n-vinylpyrrolidone-2. Studies in rodents. *Food*
793 *Chem. Toxicol.* **1997**, 35, 1061-1074.
- 794 30 Irving, E.C.; Baird, D.J.; Culp, J.M. Ecotoxicological responses of the mayfly *Baetis*
795 *tricaudatus* to dietary and waterborne cadmium: Implications for toxicity testing. *Environ.*
796 *Toxicol. Chem.* **2003**, 22(5), 1058-1064
- 797 31 Gerhardt, A. Importance of exposure route for behavioural responses in *Lumbriculus*
798 *variegatus* Müller (Oligochaeta: Lumbriculida) in short-term exposures to Pb. *Env. Sci.*
799 *Pollut. Res.* **2007**, 14(6), 430-434.
- 800 32. Ratcliff, D.A. Decrease in eggshell weight in certain birds of prey. *Nature* **1967**, 215, 208-
801 210.
- 802 33. Andrew, M.N.; O'Connor, W.A.; Dunstan, R.H.; MacFarlane, G.R. Exposure to 17 α -
803 ethynylestradiol causes dose and temporally dependent changes in intersex, females and
804 vitellogenin production in the Sydney rock oyster. *Ecotoxicology*. **2010**, 19(8), 1440-1451.
- 805 34. Brion, F.; Tyler, C.R.; Palazzi, X.; Laillet, B.; Porcher, J.M.; Garric, J.; Flammarion, P.
806 Impacts of 17 β -estradiol, including environmentally relevant concentrations, on
807 reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in
808 zebrafish (*Danio rerio*). *Aquat. Toxicol.* **2004**, 68(3), 193-217.
- 809 35. Nash, J.P.; Kime, D.E.; Van der Ven, L.T.M.; Wester, P.W.; Brion, F.; Maack, G.;
810 Stahlschmidt-Allner, P.; Tyler, C.R. Long-term exposure to environmental concentrations
811 of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health*
812 *Perspect.* **2004**, 112(17), 1725-1733.
- 813 36. Hummel, D.; Löffler, D.; Fink, G.; Ternes, T.A. Simultaneous determination of
814 psychoactive drugs and their metabolites in aqueous matrices by liquid chromatography
815 mass spectrometry. *Environ. Sci. Technol.* **2006**, 40, 7321-7328.
- 816 37. Aherne, G.W.; Briggs, R. The relevance of the presence of certain synthetic steroids in the
817 aquatic environment. *J. Pharm. Pharmacol.* **1989**, 41(10), 735-736.

- 818 38. Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.;
819 Buxton, H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in
820 U.S. streams, 1999-2000: a national reconnaissance. *Environ. Sci. Technol.* **2002**, 36,
821 1202-1211.
- 822 39. Hannah, R.; D'Aco, V.J.; Anderson, P.D.; Buzby, M.E.; Caldwell, D.J.; Cunningham,
823 V.L.; Ericson, J.F.; Johnson, A.C.; Parke, N.J.; Samuelian, J.H.; Sumpter, J.P. Exposure
824 assessment of 17 α -ethynylestradiol in surface waters of the United States and Europe.
825 *Environ. Toxicol. Chem.* **2009**, 28(12), 2725-2732.
- 826 40. Lister, A.; Regan, C.; Van Zwol, J.; Van der Kraak, G. Inhibition of egg production in
827 zebrafish by fluoxetine and municipal effluents: A mechanistic evaluation. *Aquat. Toxicol.*
828 **2009**, 95, 320-329.
- 829 41. Di Poi, C.; Darmaillacq, A-S.; Dickel, L.; Boulouard, M.; Bellanger, C. Effects of perinatal
830 exposure to waterborne fluoxetine on memory processing in the cuttlefish *Sepia officinalis*.
831 *Aquat. Toxicol.* **2013**, 132-133, 84-91.
- 832 42. Franzellitti, S.; Buratti, S.; Valbonesi, P.; Fabbri, E. The mode of action (MOA) approach
833 reveals interactive effects of environmental pharmaceuticals on *Mytilus galloprovincialis*.
834 *Aquat. Toxicol.* **2013**, 140-141, 249-256.
- 835 43. Guler, Y.; Ford, A.T. Anti-depressants make amphipods see the light. *Aquat. Toxicol.*
836 **2010**, 99, 397-404.
- 837 44. Hala, D.; Bristeau, S.; Dagnac, T.; Jobling, S. The unexpected sources of organotin
838 contamination in aquatic toxicological laboratory studies. *Aquat. Toxicol.* **2010**, 96, 314-
839 318.
- 840 45. Aoki, K.A.A.; Harris, C.A.; Katsiadaki, I.; Sumpter, J.P. Evidence suggesting that di-n-
841 butyl phthalate has antiandrogenic effects in fish. *Environ. Toxicol. Chem.* **2011**, 30(6),
842 1338-1345.
- 843 46. Geraudie, P.; Boulange-Lecomte, C.; Gerbron, M.; Hinfray, N.; Brion, F.; Minier, C.
844 Endocrine effects of the tapeworm *Ligula intestinalis* in its teleost host, the roach (*Rutilus*
845 *rutilus*). *Parasitology* **2010**, 137, 697-704.

- 846 47. Trubiroha, A.; Kroupova, H.; Wuertz, S.; Frank, S.N.; Sures, B.; Kloas, W. Naturally-
847 induced endocrine disruption by the parasite *Ligula intestinalis* (Cestoda) in roach (*Rutilus*
848 *rutilus*). *Gen. Comp. Endocrinol.* **2010**, 166, 234-240.
- 849 48. Jobling, S.; Tyler, C.R. Endocrine disruption, parasites and pollutants in wild freshwater
850 fish. *Parasitology* **2003**, 126, S103-S108.
- 851 49. Sures, B. How parasitism and pollution affect the physiological homeostasis of aquatic
852 hosts. *J. Helminthol.* **2006**, 80, 151-157.
- 853 50. Ford, A.T.; Fernandes, T.F. Letter to the Editor: Better the devil you know? A
854 precautionary approach to using amphipods and daphnids in endocrine disruptor studies.
855 *Environ. Toxicol. Chem.* **2005**, 24(5), 1019–1021.
- 856 51. National Toxicology Program (NTP). (2001). Final report of the endocrine disruptors low
857 dose peer review panel. In Endocrine Disruptors Low Dose Peer Review. Raleigh, NC.
858 Available at: <http://ntp.niehs.nih.gov/ntp/htdocs/liason/LowDosePeerFinalRpt.pdf>.
859 Accessed May 23, 2013.
- 860 52. Loucks, E.; Carven, M.J. Strain-dependent effects of developmental ethanol exposure in
861 zebrafish. *Neurotoxicol. Teratol.* **2004**, 26(6), 745-755.
- 862 53. Soeffker, M.; Stevens, J.R.; Tyler, C.R.. Comparative breeding and behavioural responses
863 to ethinylestradiol exposure in wild and laboratory maintained zebrafish (*Danio rerio*)
864 populations. *Environ. Sci. Technol.* **2012**, 46(20), 11377-11383.
- 865 54. Vignet, C.; Begout, M-L.; Pean, S.; Lyphout, L.; Leguay, D.; Cousin, X. Systematic
866 screening of behavioural responses in two zebrafish strains. *Zebrafish* **2013**, 10(3), 365-
867 375.
- 868 55. Brown, A.R.; Bickley, L.K.; Ryan, T.A.; Paull, G.C.; Hamilton, P.B.; Owen, S.F.; Sharpe,
869 A.D.; Tyler, C.R. Differences in sexual development in inbred and outbred zebrafish
870 (*Danio rerio*) and implications for chemical testing. *Aquat. Toxicol.* **2012**, 112-113, 27-38.
- 871 56. Nehls, S.; Segner, H. Detection of DNA damage in two cell lines from rainbow trout,
872 RTG-2 and RTL-W1, using the comet assay. *Environ. Toxicol.* **2001**, 16, 321-329.

- 873 57. Fenske, M.; van Aerle, R.; Brack, S.; Tyler, C.R.; Segner, H. Development and validation
874 of a homologous zebrafish (*Danio rerio* Hamilton-Buchanan) vitellogenin enzyme-linked
875 immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals.
876 *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **2001**, 129, 217-232.
- 877 58. Beresford, N.; Routledge, E.J.; Harris, C.A.; Sumpter, J.P. Issues arising when interpreting
878 results from an in vitro assay for estrogenic activity. *Toxicol. Appl. Pharmacol.* **2000**,
879 162(1), 22-33.
- 880 59. Harris, C.A.; Henttu, P.; Parker, M.G.; Sumpter, J.P. The estrogenic activity of phthalate
881 esters in vitro. *Environ. Health Perspect.* **1997**, 105(8), 802-811.
- 882 60. Pedersen, S.N.; Christiansen, L.B.; Pedersen, K.L.; Korsgaard, B.; Bjerregaard, P. *In vivo*
883 estrogenic activity of branched and linear alkylphenols in rainbow trout (*Oncorhynchus*
884 *mykiss*). *Sci. Total Environ.* **1999**, 233(1-3), 89-96.
- 885 61. Odum, J.; Lefevre, P.A.; Tittensor, S.; Paton, D.; Routledge, E.J.; Beresford, N.A.;
886 Sumpter, J.P.; Ashby, J. The rodent uterotrophic assay: Critical protocol features, studies
887 with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regul. Toxicol.*
888 *Pharmacol.* **1997**, 25(2), 176-188.
- 889 62. Moore, A.; Scott, A.P.; Lower, N.; Katsiadaki, I.; Greenwood, L. The effects of 4-
890 nonylphenol and atrazine on Atlantic salmon (*Salmo salar* L) smolts. *Aquaculture* **2003**,
891 222, 253-263.
- 892 63. OECD 1998. OECD Series on Testing and Assessment Number 10. Report of the OECD
893 workshop on statistical analysis of aquatic toxicity data. [Accessed via:
894 [http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.h](http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.htm)
895 [tm](http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.htm) ;15-10-2013]
- 896 64. OECD 2006. OECD Series on Testing and Assessment Number 54. Current approaches in
897 the statistical analysis of ecotoxicity data: a guidance to application. [Accessed via:
898 [http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.h](http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.htm)
899 [tm](http://www.oecd.org/env/ehs/testing/seriesontestingandassessmentpublicationsbynumber.htm) ;15-10-2013]
- 900 65. Watanabe, K.H.; Jensen, K.M.; Orlando, E.F.; Ankley, G.T. What is normal? A
901 characterization of the values and variability in reproductive endpoints of the fathead

- 902 minnow, *Pimephales promelas*. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **2007**,
903 146, 348–356
- 904 66. Brodin, T.; Fick, J.; Jonsson, M.; Klaminder, J. Dilute concentrations of a psychiatric drug
905 alter behavior of fish from natural populations. *Science* **2013**, 339(6121), 814-815.
- 906 67. Sumpter, J. P.; Jobling, S. (1995). Vitellogenesis as a biomarker for oestrogenic
907 contamination of the aquatic environment. *Environ. Health Perspect.* **1995**, 103 (Suppl. 7),
908 173-178.
- 909 68. Katsiadaki, I.; Morris, S.; Squires, C.; Hurst, M. R.; James, J. D.; Scott, A. P. A sensitive,
910 in vivo test for the detection of environmental antiandrogens, using the three-spined
911 stickleback (*Gasterosteus aculeatus*). *Environ. Health Perspect.* **2006**, 114(Suppl. 1), 115-
912 121.
- 913 69. Vandenberg, L.N.; Colborn, T.; Hayes, T.B.; Heindel, J.J.; Jacobs, D.R., Jr.; Lee, D-H.;
914 Shioda, T.; Soto, A.M.; vom Saal, F.S.; Welshons, W.V.; Zoeller, R.T.; Myers, J.P.
915 Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose
916 responses. *Endocr. Rev.* **2012**, 33(3), 378-455.
- 917 70. Scott, A.P. Do mollusks use vertebrate sex steroids as reproductive hormones? II. Critical
918 review of the evidence that steroids have biological effects. *Steroids* **2013**, 78(2), 268-281.
- 919 71. Joint Code of Practice for Research. Issued by BBSRC; DEFRA; FSA; NERC (UK). **2003**.
920 [Accessed via:[http://www.bbsrc.ac.uk/organisation/policies/position/policy/joint-code-of-](http://www.bbsrc.ac.uk/organisation/policies/position/policy/joint-code-of-practice-for-research.aspx)
921 [practice-for-research.aspx](http://www.bbsrc.ac.uk/organisation/policies/position/policy/joint-code-of-practice-for-research.aspx) ; 15-10-13]
- 922 72. Russell, J. F. If a job is worth doing, it is worth doing twice. *Nature* **2013**, 496, 7.
- 923 73. Burkhardt-Holm, P.; Giger, W.; Guttinger, H.; Ochsenbein, U.; Peter, A.; Scheurer, K.;
924 Segner, H.; Staub, E.; Suter, M.J.F. Where have all the fish gone? *Environ. Sci. Technol.*
925 **2005**, 39(21), 441A-447A.
- 926 74. Ginebrada, A.; Muñoz, I.; López de Alda, M.; Brix, R.; López-Doval, J.; Barceló, D.
927 Environmental risk assessment of pharmaceuticals in rivers: Relationships between hazard
928 indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE
929 Spain). *Environ. Int.* **2010**, 36, 153-162.

- 930 75. Weed, D.L. Weight of evidence: A review of concept and methods. *Risk Analysis* **2005**,
931 25(6), 1545-1557.
- 932 76. Ioannidis, J.P.A. Contradicted and initially stronger effects in highly cited clinical
933 research. *JAMA-J. Am. Med. Assoc.* **2005**, 294(2), 218-228.
- 934 77. Brown, R. P. Greer, R.D. Mihaich, E.M. and Guiney. P.D. A Critical Review of the
935 Scientific Literature on Potential Endocrine-Mediated Effects in Fish and Wildlife.
936 *Ecotox. Environ. Safe.* **2001**, 49, 17-25
- 937 78. Carlsen, E.; Giwercman, A.; Keiding, N.; Skakkebaek, N.E. Evidence for decreasing
938 quality of semen during past 50 years. *BMJ* **1992**, 305, 609-613
- 939 79. Popper, K.R. The Logic of Scientific Discovery (translation of *Logik der Forschung*, first
940 published in 1935). Taylor & Francis e-Library, **2005**, ISBN 0-203-99462-0 Master e-
941 book. 545 pp.
- 942 80. Popper, K.R. Conjectures and Refutations: The Growth of Scientific Knowledge.
943 Routledge, London, **1963**, ISBN 0-415-28594-1. 431 pp.
- 944 81. Goldacre, B. Bad Science. Publ. Fourth Estate (London); **2009**, ISBN: 978-0-00-728487-0.
945 370 pp.
- 946 82. Knight, J. Null and void. *Nature* **2003**, 422, 554-555.
- 947 83. Turner, E. H.; Matthews, A. M.; Linardatos, E.; Tell, R. A.; Rosenthal, R. Selective
948 publication of antidepressant trials and its influence on apparent efficacy. *N. Engl. J. Med.*
949 **2008**, 358, 252-260.
- 950 84. Larsen, P-O.; von Ins, M. The rate of growth in scientific publication and the decline in
951 coverage provided by Science Citation Index. *Scientometrics* **2010**, 84(3), 575–603.