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Title:

20 years of fish immunotoxicology – what we know and where we are

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Abstract

Despite frequent field observations of impaired immune response and increased disease incidence in contaminant-exposed wildlife populations, immunotoxic effects are rarely considered in ecotoxicological risk assessment. The aim of the present study was to review the literature on immunotoxic effects of chemicals in fish to quantitatively evaluate (i) which experimental approaches were used to assess immunotoxic effects, (ii) whether immune markers exist to screen for potential immunotoxic activities of chemicals, and (iii) how predictive those parameters are for adverse alterations of fish immunocompetence and disease resistance. A total of 241 publications on fish immunotoxicity were quantitatively analyzed. The main conclusions included: (i) To date, fish immunotoxicology focused mainly on innate immune responses and immunosuppressive effects. (ii) In numerous studies, the experimental conditions are poorly documented, as for instance age or sex of the fish or the rationale for the selected exposure conditions is often missing. (iii) Although a broad variety of parameters were used to assess immunotoxicity, the rationale for the choice of measured parameters was often not given, remaining unclear how they link to the suspected immunotoxic mode of action of the chemicals. (iv) At the current state of knowledge, it is impossible to identify a set of immune parameters that could reliably screen for immunotoxic potentials of chemicals. (v) Similarly, fish immunotoxicologists seem to have insufficient understanding of how and when chemical-induced modulations of molecular / cellular immune changes relate to adverse alterations of fish immunocompetence, although this would be crucial to include immunotoxicity in ecotoxicological risk assessment.

41 # Abbreviations

AhR	arylhydrocarbon receptor
BPA	bisphenol A
COX	cyclooxygenase
CYP1A1	cytochrome P450 family 1 subfamily A member 1
DCF	diclofenac
E2	estradiol
EDCs	endocrine disrupting chemicals / compounds
EE2	ethinylestradiol
ER	estrogen receptor
HAHs	halogenated aromatic hydrocarbons
IFN	interferons
IgM	immunoglobulin M
IHNV	infectious hematopoietic necrosis virus
IL	interleukin
MHC II	major histocompatibility complex class II
MoA	mode of action
NBT	nitro blue tetrazolium
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NP	nonylphenol
PAHs	polycyclic aromatic hydrocarbons
PBDEs	polybrominated biphenyl ethers
PCBs	polychlorinated biphenyls
PPAR	peroxisome proliferator-activated receptor

qRT-PCR	quantitative reverse transcription - polymerase chain reaction
ROS	reactive oxygen species
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDAR assay	T cell dependent antibody response assay
TNF	tumor necrosis factor

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1. Introduction

Numerous chemicals have the capacity to perturb immune structures and functions of exposed organisms and enhance their susceptibility to both infectious and neoplastic diseases. Historically, the awareness that chemicals can interfere with immune function started with early observations that exposure to industrial workplace environments was able to cause immune-mediated lung diseases in humans (Luster 2014). Subsequent research performed during the late 1960s and early 1970s revealed that the immune system is indeed targeted by a broad variety of chemicals and that exposure to these compounds can result in immune dysfunction (Koller 2001). Importantly, the chemically induced immune disruption occurs at lower concentrations than those required to induce commonly measured toxicological endpoints such as lethality (Koller 2001, Luster 2014), which means that immunotoxic effects are not just side effects of general toxicity but a toxic mode of action (MoA) of its own. While laboratory studies demonstrated the potential of environmental chemicals to adversely impact the immune system, epidemiological studies revealed the widespread occurrence of human diseases potentially caused by immunotoxic chemicals (Luster et al. 1993; House & Selgrade 2010). These and other findings led to the recognition of immunotoxicity as an important endpoint in the assessment of chemical toxicity, which gave rise to the development of regulatory guidelines for immunotoxicity testing.

The assessment of immunotoxic effects of chemicals, however, faces a number of challenges. A first challenge is to distinguish between direct chemical effects on the immune system, which result from the interaction of the chemical moiety with molecules of the immune cells, and indirect chemical effects, which may result from a toxicant-induced systemic stress response, and the immunomodulating activity of the stress hormones (Bennett & Wolke 1987; Barton 2002; Levesque et al. 2003; Glaser & Kiecolt-Glaser 2005; Odermatt & Gumy 2008). A second challenge is that chemicals may have no obvious effects in the resting

93 immune system, but may compromise the capacity of the immune system to respond to a
94 pathogen challenge, i.e. the immunotoxic effect becomes visible only in the activated
95 immune system. A third challenge is the interpretation of the toxicant-induced
96 immunomodulation. A toxicant-induced alteration of a molecular or cellular immune
97 parameter does not always lead to an adverse response such as reduced immunocompetence,
98 but it may also represent an immunostimulation (Kimber & Dearman 2002). A fourth
99 challenge in assessing immunotoxic effects is the diversity of potential targets, as well as
100 possible effects. The interactions of toxicants with the immune system can take place at
101 multiple sites within the immune network. The resulting immunomodulations can encompass
102 alterations of immune cell proliferation, differentiation and survival, or alterations in the
103 functioning of the immune organs and cells, which eventually may lead to immunotoxic
104 effects, i.e. adverse changes of immune structures and functions. Immunotoxic effects can
105 involve either immune suppression and increased risk for infectious and malignant diseases,
106 or immune stimulation, which can trigger allergic or autoimmune diseases (Colosio et al.
107 2005; Selgrade et al. 2008). In addition, immunotoxic effects can manifest themselves in the
108 mature immune system as well as in the developing immune system, and they can be both
109 transient and persistent alterations (Dietert 2009; Winans et al. 2011).
110 The immune system represents a highly sophisticated physiological network with varied
111 signal transduction pathways, multiple cellular components and a diversity of mediators and
112 receptors for communication and activation. As a fully dispersed system, present in most
113 tissues and organs, the immune system is readily accessible to toxicants, irrespective of their
114 uptake route, be it *via* respiratory epithelia, skin, or the intestinal system, and during their
115 distribution *via* blood and lymph. Immune organs are particularly exposed to immunotoxic
116 agents due to their intensive vascularization and blood supply. This applies for peripheral
117 immune cells in the blood as well as for resident immune cells in organs like the liver.

Given this complexity of the immune system, a single assay or parameter appears to be hardly sufficient to assess immunomodulating activities and / or immunotoxic effects of chemicals. Instead, to rule immunotoxicity "in" or "out", comprehensive testing panels covering a range of immune assays and endpoints are needed. In fact, for human risk assessment, tiered testing strategies for immunotoxic actions of chemicals have been established, which rely on a suite of immune markers and assays (Burns et al. 1996; Hinton 2000; ICH 2006; Schulte & Ruehl-Fehlert 2006; Luster 2014). First-line assessments encompass hematology, lymphoid organ weights and histopathology, which are routinely assessed in standard tests like repeated dose toxicity studies. The next tier involves functional tests such as the T cell dependent antibody response assay (TDAR assay) which offers the advantage of integrating many of the cellular components of the basic immune response (T cells, B cells, macrophages; Luster et al. 1992; Boverhof et al. 2014; Hartung & Corsini 2013). Follow-up immunotoxicity testing usually relies on a case-by-case design to provide the necessary flexibility in dealing with the diverse effects a chemical may exert on the immune system.

Immunotoxic effects are of relevance not only in human toxicology but also in ecotoxicology. There is increasing recognition that many environmental chemicals impact the immune systems of wildlife animals. Field studies, for instance, have unraveled the frequent association between contaminant exposure and impaired immune functioning of wildlife populations (Luebke et al. 1997; Galloway & Depledge 2001; Acevedo-Whitehouse & Duffus 2009; Morley 2010). Prominent examples include the role of parasite infections in the global decline of amphibian populations, which appear to be favored by toxicant-induced immunosuppression (Kiesecker 2002; Rohr et al. 2008). Another intensively investigated case of wildlife disease with suspected chemical etiology is the distemper virus outbreak in harbor porpoises, which is considered to be related to the bioaccumulation of

143 immunosuppressive polychlorinated biphenyls (PCBs; Beineke et al. 2005; Hall et al. 2006).
144 In line with such field findings, a steadily increasing number of laboratory studies has
145 demonstrated that immune parameters and immunocompetence of both invertebrate and
146 vertebrate wildlife species are responsive to chemical exposure (for reviews see Ross et al.
147 1996; Rice et al. 1996; Keller et al. 2000; Galloway & Depledge 2001; Burnett 2005; Carlson
148 & Zelikoff 2010; Desforges et al. 2016). The toxicant-induced alterations of
149 immunocompetence have consequences for organism fitness and survival as well as for the
150 prevalence and spread of diseases in populations (Arkoosh et al. 1998; Wilson 1999;
151 Springman et al. 2005; Loge et al. 2005; Acevedo-Whitehouse & Duffus 2009; Graham et al.
152 2010). For birds, for instance, it has been shown that changes of non-specific immune
153 parameters reliably predicted changes in survival (Acevedo-Whitehouse & Duffus 2009).
154 Importantly, immunotoxic effects of chemicals can combine with immunomodulating effects
155 of other stressors (Lenihan et al. 1999; Jacobson et al. 2003; Acevedo-Whitehouse & Duffus
156 2009; Segner et al. 2012), and these cumulative effects of multiple stressors may explain why
157 infectious diseases in wildlife populations are increasing at an unprecedented rate (Blaustein
158 et al. 2012) .
159 The discussion above highlights the relevance of immunotoxicity in ecotoxicology. However,
160 in contrast to human toxicology, ecotoxicology currently does not command an agreed set of
161 standard methods or markers informing on immunotoxic actions of chemicals or chemical
162 exposures as they occur in the environment. Given the steadily growing number of
163 ecotoxicological studies addressing immunotoxic effects of chemicals in wildlife animals and
164 / or laboratory test species, a systematic analysis of the existing ecotoxicological literature to
165 identify potential immunotoxicity assays and markers appears to be timely. Thus, within this
166 review, the focus is on studies with teleostean fish, for which a substantial number of
167 immunotoxicological studies has been published since 1995. There already exist several

168 excellent reviews on fish immunotoxicity which consider primarily the mechanisms and
169 adverse consequences of immunotoxic effects in fish (Zeeman & Brindley 1981; Bols et al.
170 2001; Rice 2004; Burnett 2005; Reynaud & Deschaux 2006; Carlson & Zelikoff 2010).
171
172 Scope of the present review:
173 The key question addressed in the present review is, in contrast to these previous reviews,
174 whether fish immunotoxicology is ready to come up with a set of robust and sensitive assays
175 and markers to screen for the immunotoxic potential of environmental chemicals, and
176 whether these screening parameters / assays are predictive of immune dysfunction of the
177 intact fish. To answer this question, we ask (i) which experimental designs and test
178 compound concentrations were used to assess fish immunotoxicity, (ii) which immune
179 markers and assays were employed and how they responded to the toxic exposure, including
180 the question whether there exist toxicant-specific immunotoxic signatures, and (iii) whether
181 *in vitro* and / or *in vivo* screening assays are predictive of changes in the immunocompetence
182 of fish. Methodologically, we performed a quantitative analysis of the literature. For instance,
183 we quantified how often a certain immune marker or immune assay was used; how often it
184 did or did not respond to the chemical exposure, and how often the assay / marker response
185 correlated with an alteration of the fish immunocompetence.
186 Importantly, this review does not discuss the potential use of fish as immunotoxicological
187 model, comparable to the use of species such as zebrafish as model in basic immunological
188 research (e.g. Trede et al. 2004), but this review deals with the ecotoxicological impact of
189 chemicals on the fish immune system. That said it is clear that the discussion will be biased
190 towards the immune parameters which have been measured in fish immunotoxicity studies,
191 and neglects (not because of ignorance but because of the existing bias in the published

192 literature with fish) aspects which are important in human immunotoxicology, for instance,
193 immunotoxic effects on the adaptive immune system.
194

195 2. Material and Methods

196 Terminology

197 *Immunotoxicity*: Immunotoxicity refers to immune dysfunction resulting from exposure to
198 foreign compounds. A chemically induced modulation of an immune parameter is not *per se*
199 adverse, but it may lead to immunosuppression, increased susceptibility to infectious and
200 malignant disease, hypersensitivity / allergy, or autoimmune disease (Kimber & Dearman
201 2002). Immunotoxicology is the study of chemical impacts on the immune system. For the
202 present review of immunotoxicity studies with fish, we analyzed both studies demonstrating
203 adverse effects of chemical exposure on fish immune functions, and studies showing
204 immunomodulating effects without confirming their adversity.

205 *Challenge induced mortality test:* To demonstrate the adversity of a chemical impact on the
206 immune system of fish, the most frequently used experimental approach is the "challenge
207 induced mortality test". In this test, fish are exposed (i) to the suspected immunotoxicant at
208 concentrations that do not induce mortality, (ii) to an acute fish pathogen at doses which
209 induce low to moderate levels of mortality, and (iii) to both the pathogen and the toxicant; the
210 co-exposure is done either serially or simultaneously. Elevated mortalities in the co-exposure
211 compared to the pathogen-only treatment are taken as a proof that the chemical has
212 compromised the immune function of the fish, and thus is an immunotoxicant. We refer to
213 this type of experiment as "challenge induced mortality test".

214 *Chemical exposure*: This was used as short term for both, field exposures in which the fish
215 are confronted with complex chemical mixtures and laboratory experiments where the fish
216 are treated with single substances or defined mixtures of chemicals.

217

218 Data search

219 Primary data searches were performed online on “pubmed“, “sciencedirect / Scopus“ and
220 “Web of Science”. The search terms used were: “fish“, “immunotoxicology”,
221 “immunotoxicity“, “immune“, “innate“, “toxic”, “disease” and combinations thereof. In
222 addition, the reference lists of relevant review articles and the contents of specialized journals
223 such as “Aquatic Toxicology” and “Fish and Shellfish Immunology” were searched. The
224 publication period considered for the search reached from 1995 to 2015. The search yielded
225 an array of publications on chemical impacts on the immune system of fish, including
226 reviews, commentaries and original research articles.

227

228 Quantitative analysis of data from original research articles

229 The main objective of the present review was to evaluate whether, having a look at 20 years
230 of immunotoxicological research on fish, we are in a position to identify robust and sensitive
231 immune parameters and / or assays that are able to screen for immunotoxic potentials of
232 chemicals or chemical groups, and whether these screening parameters / assays are predictive
233 of immune dysfunction of the intact fish. To this end, we decided for a quantitative analysis
234 of the existing literature, meaning that we examined (i) which experimental designs were
235 used how frequently in fish immunotoxicity studies, (ii) which immune markers and assays
236 were employed how frequently, and how often they did respond to specific chemical classes
237 and / or modes of action, and (iii) how often an immune marker / assay response was
238 associated with an altered immunocompetence of the fish. For this quantitative analysis, only
239 original research articles (n = 241) were considered. To enable the reader to easily recognize
240 which publications among all cited articles were included in the quantitative analysis, we
241 provide the list of these 241 articles in Supplement S 1.

For the data analysis, several categorizations were used:

- In a number of publications, one and the same parameter was employed in different experiments, and often, the reaction of immune parameters differed between the experiments. In order to be able to utilize this differentiated information, two different quantitative analyses were performed: (i) the *article-based analysis*: here, the parameter (for instance the fish species, applied methods, chemicals, exposure time, etc.) was counted only once per article, even if it was used in several experiments within the article. Thus, in this case the n-number indicates the number of articles among the 241 articles that have employed a specific parameter; (ii) the *parameter-based analysis*: here, the immune parameter was counted each time it was used, regardless if it was in the same article or in different articles. Thus, in this case the n-number indicates how often the parameter has been used in total. The data of all the 241 reviewed articles (regardless whether *in vitro*, *in vivo* or *ex vivo* studies) were analyzed all together; otherwise it is mentioned specifically in the related paragraph.
- On the basis of functional considerations, individual immune parameters were aggregated into *16 main immune parameter groups*. A list of the groups and the included immune parameters is provided in table 1 and 2. In order to obtain a differentiated picture, we tried to avoid groups comprising too many individual immune parameters. For instance, we did not form a group "hematology" but split it up into groups like "leucocyte counts", "T cells" and "B cells" count. The considered parameters and groups are listed in table 1 for the parameter-based analysis. For the article-based analysis see Supplement S 2.
- Within each of the 16 main groups, the percentage of reported *significant changes of the parameter(s)* was calculated: if, in a given article, the parameter measured showed significant changes (based on the analysis done by the authors of the publication itself), it

was considered as "showed significant change" (regardless whether it was an up- or down-regulation).

As an example: Jin et al. (2010) measured the expression level of the immune-related gene TNF-alpha (tumor necrosis factor-alpha) after exposure to five different chemicals. In the article-based analysis, TNF-alpha would be counted as n = 1. For the parameter-based analysis, in contrast, it would be counted as n = 5. Further, TNF-alpha would be included in the group "cytokines" (Table 1 and 2). Since in the study of Jin et al. (2010), TNF-alpha responded significantly to four out of the five test chemicals, it would be counted as four times "showed significant change" and one time as "no significant change" within the group "cytokines".

Reference list(s):

All articles that were identified by the literature search are included in the reference list of the main text. Hence, the reference list at the end of this text contains reviews, commentaries as well as original research publications on fish immunotoxicology (even before 1995 and after 2015). In addition, the reference list contains articles dealing with the approaches and concepts used in human immunotoxicology and risk assessment. Although it is not the intention of this review to present a sound comparative of mammals / humans and fish immunotoxicological studies, we refer to the mammalian immunotoxicological studies as an example to illustrate possible approaches for immunotoxicity screening and risk assessment.

In contrast, for the quantitative literature analysis (see above) only original research articles on fish immunotoxicology were used. To avoid duplications, reviews were not included in the quantitative data analysis. A total of 241 original research articles dealing with fish immunotoxicity were found for the period from 1995 to 2015. To enable the reader to

immediately see which papers form the basis of the quantitative analysis, they are shown in a separate reference list in the Supplement S 1.

3. Results and Discussion

Between 1995 and 2015, there is a clear upward trend in the yearly number of original research papers dealing with fish immunotoxicity (Figure 1, based on reference list S 1). This increase may reflect the rising interest of both industry and academia in chemicals with specific MoA, including immunomodulating MoAs. Moreover, there appears to be growing awareness of the ecotoxicological relevance of immunotoxic actions of chemicals.

3.1 Which experimental designs were used to assess the immunotoxic actions of chemicals in fish?

The studies used a wide range of experimental designs for the assessment of immunotoxicity in fish (article-based analysis):

Fish species: Four families dominated, Cyprinidae, mainly common carp (*Cyprinus carpio,* 11 % of all used fish species; Figure 2) and zebrafish (*Danio rerio,* 9 %), Salmonidae, mainly rainbow trout (*Oncorhynchus mykiss,* 20 %), Sparidae, mainly gilthead seabream (*Sparus aurata*, 11 %*)*, and Cichlidae, mainly Tilapia (*Tilapia sp*., 6 %).

The use of small laboratory species such as zebrafish (*Danio rerio*) or Japanese medaka (*Oryzias latipes*) for immunotoxicological studies was still limited regarding the reviewed articles, although during the last years the number of articles on small laboratory fish species, especially zebrafish, for immunotoxicity studies has been increasing.

Age of fish: Most publications used "juvenile" fish (61 % of the fish were rated as juvenile), while only 12 % were classified as adults, and 9 % as embryos. In 18 % of the studies no information on age of the used fish was given. Given the fact that fish immunocompetence

316 (innate and adaptive) varies with age (Tort et al. 2003; Duffy et al. 2003), precise age / stage
317 information would be important for the interpretation of the experimental findings. Many
318 authors provided information on weight and / or length of fish (59 % on weight, 1 % on
319 length, 17 % on both). However, this information is difficult to link to specific life stages as
320 the length / weight-age relationship varies according to species, feeding regime, fish density,
321 temperature and other factors.
322 Although the use of fish embryos in ecotoxicological research is becoming more and more
323 common (Braunbeck et al. 2014), only 9 % of the use fish in immunotoxicological studies
324 were embryos. This is surprising since, on the one hand, the period of immune system
325 differentiation in early life may represent a window of vulnerability, and, on the other hand,
326 early life stages of fish offer a number of technical advantages. For instance, they allow to
327 examine toxic effects in multiple tissues in parallel (e.g. Zhang et al. 2008), or to visualize -
328 by means of transgenic fish - immunotoxic processes in the live animal (e.g. Fehr et al.
329 2015). Furthermore, since early life stages of fish, in particularly the embryonic stage,
330 possess only innate but no adaptive immunity (e.g. Lam et al. 2004) the role of innate versus
331 adaptive mechanisms in the immunotoxic response can be studied. Finally, the short life
332 cycle of small laboratory fish species such as zebrafish provides the option to study
333 persistent, long-term effects of early life exposure. In human toxicology, developmental
334 immunotoxicity is an increasing concern since it predisposes children for diseases, that have
335 been on the rise in recent decades (e.g. allergic diseases, asthma, autoimmune conditions;
336 Dietert 2009; Collinge et al. 2012). In fish, only few studies addressed persistent
337 immunotoxic effects arising from early life exposure, and those were mainly done with long-
338 lived salmonids (Ottinger & Kaattari 2000; Milston et al. 2003). With the increasing use of
339 fish embryos in toxicological testing the question of developmental immunotoxicology in fish
340 is likely to attract more attention (Liu et al. 2014; Seemann et al. 2015). Depending on the

immune structure or function under consideration, the "sensitive window" is not necessarily restricted to the embryonic stage but may extend into the juvenile stage, as shown for thymus development of the sea bass (*Dicentrarchus labrax*; Seemann et al. 2015).

Sex of fish: Another factor which can influence the outcome of immunotoxicity studies are immunological differences between males and females. Sexual dimorphism is well-known for the mammalian immune system (Nava-Castro et al. 2012), but it seems to be present in fish as well (Segner et al. 2006; Demas et al. 2011). For instance, Hoeger et al. (2005) observed that exposure of rainbow trout to municipal sewage treatment effluents resulted in decreased antibody production and reduced lymphocyte number in sexually mature females but not in males. Similarly, Ye et al. (2012) found sex-specific differences in the response of the complement system of the marine medaka (*Oryzias melastigma*) exposed to a polybrominated flame retardant. These examples show that the fact, that 79 % of the published immunotoxicity studies do not provide information on the sex of the experimental fish, represents a serious information gap.

Exposure duration: Three scenarios were most common (i) exposure over hours (1-24 hours; representing 19 %), (ii) exposure over days (1-7 days; 28 %), or (iii) exposure over weeks (1-4 weeks; 31 %). Long-term studies, with exposure over several months were rare (11 %), as were studies using single pulse exposures, e.g. by injection (5 %). Exposures for less than 1 h were applied in 1 % of the studies, and in 5 % no information on exposure duration was provided. A rationale for the choice of exposure duration was usually not given.

Exposure concentration: A critical parameter in immunotoxicity studies is the selected concentration(s) of the test agent. Structural and functional changes of the immune system occurring at concentrations high enough to induce apical toxic responses such as lethality or cytotoxicity do not necessarily indicate an immunotoxic activity of the test agent, but are likely to represent general toxicity. In order to classify an effect to be immunotoxic, the effect

366 has to occur at concentrations clearly below those required to cause apical toxicity (Koller
367 2001; Luster 2014). Unfortunately, and this is a major drawback in the existing literature on
368 fish immunotoxicity, the vast majority of studies (74 %) does not provide information on how
369 the selected test concentrations relate to general toxicity of the test compound. Some studies
370 (13 %) claim that the immunotoxic effects were tested at "sub-lethal" concentrations
371 however, without providing information on the distance to the lethal concentrations. Only in
372 13 % of the analyzed studies, the test concentrations were defined as a percentage of the
373 concentrations required to induce mortality. Furthermore, we check not only whether the
374 experimental concentrations were reported in relation to general toxicity, but also how many
375 studies controlled for (cyto-) toxic / lethal effect(s): in 62 % (Figure 3), the studies either
376 provided no information at all or, if *in vitro* experiments were conducted, only measured the
377 cell viability after isolation but not after chemical exposure.
378
379 **3.2 Which chemical groups were studied in immunotoxicity studies with fish?**
380 **3.2.1 Hormones / endocrine disrupting compounds (EDCs; 27 %)**
381 Almost one third of the compounds studied in the reviewed articles were hormonally active
382 substances (article-based analysis; for details regarding the chemical grouping see
383 Supplement S 3). These compounds are of high environmental relevance, as the aquatic
384 environment represents a sink for so-called endocrine disrupting compounds (EDCs; Sumpter
385 & Johnson 2005). To date, fish toxicological research on EDCs has mainly focused on their
386 effects on reproduction, whereas little attention was given to possible endocrine-immune
387 interactions (Segner et al. 2012). There exist a few reviews on the influence of endogenous
388 hormones on the fish immune system (Harris & Bird 2000), with a focus on the role of
389 corticosteroids. Concerning environmental EDCs, up to now, mainly compounds with
390 estrogenic, androgenic and thyroidal activities have been investigated (Rice & Xiang 2000;

391 Segner et al. 2006; Milla et al. 2011; Quesada-García et al. 2014). Many immune disorders
392 are rooted in the endocrine system due to the fact that the endocrine and the immune systems
393 are intricately connected, with some of the immune and hormone factors having evolved
394 within the same family of structurally related molecules (Verburg Van Kemenade et al.
395 2009). This intimate relationship ensures the optimal allocation of limited resources among
396 the fitness-associated traits, reproduction and immunity, as it is postulated by the life history
397 theory (Bergman et al. 2013). Thus, inappropriate (in-) activation of selected endocrine
398 pathways by environmental EDCs may also disturb normal immune functioning and may
399 alter disease susceptibility of the organism. In fact, the results reported in the studies
400 analyzed for the present review provide strong evidence that estrogen-active EDCs, such as
401 natural estrogens like estradiol (E2) and xeno-estrogens like nonylphenol (NP) and bisphenol
402 A (BPA) can modulate the immune system and the immunocompetence of fish. Studies
403 analyzing global transcriptomic responses indicate that a significant fraction of immune
404 genes is responsive to treatment by estrogen-active compounds (Wenger et al. 2011; Liarte et
405 al. 2011; Wenger et al. 2012; Burki et al. 2013; Krasnov et al. 2015). While the available data
406 suggests that estrogenic EDCs are able to modulate the innate immune system of fish, the
407 currently available information on the adaptive immune system is still inconclusive (Milla et
408 al. 2011). Likewise, the findings on the consequences of immunomodulation caused by
409 exposure to EDCs on disease resistance of fish are equivocal: e.g. Burki et al. (2013) found
410 that E2-induced changes of immune gene expression were not associated with increased or
411 decreased tolerance of rainbow trout towards the parasite *Tetracapsuloides bryosalmonae*. In
412 contrast, Wenger et al. (2011; 2012) and Shelley et al. (2012) reported enhanced
413 susceptibility of E2- or NP-treated trout towards bacterial pathogens. Finally, Krasnov et al.
414 (2015) observed an increased resistance of estrogen- or androgen-treated salmon against the
415 ectoparasitic salmon louse. Also androgenic EDCs are immunologically active, as reviewed

by Milla et al. (2011), with the main effects being alterations in immune cell proliferation and function, especially of macrophage activity and of genes coding for soluble mediators, complement factors or acute phase proteins. For instance, trenbolone induced alterations mainly of the humoral system and it decreased transcript levels of *rag-1* and *rag-2,* which are involved in the development and differentiation of lymphoid cells (Massart et al. 2015).

The immunomodulating effects of EDCs on the fish immune system are likely to be mediated by estrogen and androgen receptors, which are expressed in piscine immune cells (Slater et al. 1995; Iwanowicz et al. 2009; Casanova-Nakayama et al. 2011; Cabas et al. 2011; Shelley et al. 2013). In a recent *in vitro* study by Yang et al. (2015), the authors provided strong evidence that the estrogen BPA modulates the antibacterial activity of carp macrophages through the estrogen receptor (ER) signaling pathway. This response was concentration-dependent, with concentrations up to 10 μg/L enhancing macrophage activity, whereas higher concentrations induced apoptosis. The fact that the immunomodulatory effects of estrogens are concentration-dependent and that they can cause both immunostimulating and immunosuppressive effects is well documented for mammals, too (Straub 2007). Yang et al. (2015) also showed that the effect of BPA on carp macrophages was mediated not exclusively through the ER pathway but through an interaction with the NFκB pathway (nuclear factor kappa-light-chain-enhancer). More recent findings in mammalian systems corroborate the finding that immunomodulating effects of EDCs are mediated through several signaling pathways. For instance, Rogers et al. (2013) found that BPA exerts its immunological effects *via* the ERs, the arylhydrocarbon receptors (AhRs) and the peroxisome proliferator-activated receptor (PPAR) family. PPARs are also involved in the immunotoxic effects of phthalate esters such as di(2-ethylhexyl)phthalate which are widely used as plasticizer. In mammals, di(2-ethylhexyl)phthalate is known as an important immunotoxicant, which causes inhibition of cell proliferation, inflammation inhibition,

441 reduced antibody response, and increased immune cell apoptosis – effects which involve
442 PPARγ signaling. In fish, di(2-ethylhexyl)phthalate is immunoactive, promoting B cell
443 differentiation while suppressing plasmablast expansion, with these effects possibly being
444 mediated through PPAR (Martins et al. 2015). Another receptor pathway which appears to
445 play a role in mediating effects of EDCs on the fish immune system is thyroid signaling *via*
446 thyroid receptors α and β. In fact, the TRs are expressed in fish leukocytes, and,
447 consequently, exposure of fish to thyroid disruptors can modulate transcript levels of diverse
448 immune genes (Quesada-García et al. 2014; 2016).
449 Cortisol and synthetic corticosteroids are well characterized for their immunosuppressive
450 action in fish (Mommsen et al. 1999), and are frequently used as positive controls in
451 immunotoxicity studies. Natural and synthetic corticosteroids reach the aquatic environment
452 *via* effluents of wastewater treatment plants (Kugathas & Sumpter 2011), however, currently
453 it is not well understood whether environmental corticosteroids indeed impact the immune
454 system of exposed fish (Schriks et al. 2010; Kugathas et al. 2013; Macikova et al. 2014;
455 Zhang et al. 2016). In contrast, non-steroidal EDCs were much less intensively investigated
456 and represent only 7 publications among the 241 analyzed articles.
457
458 **3.2.2. Pesticides (18 %)**
459 Pesticides were among the first environmental chemicals to be investigated for their
460 immunomodulatory effects on mammals (Wassermann & Wassermann 1969), and their
461 possible immunological effects on teleost fish attracted early attention (see reviews by
462 Zeeman & Brindley 1981; Dunier & Siwicki 1993). Among the reviewed studies, 18 % of the
463 tested chemicals were pesticides, in particular insecticides and herbicides (article-based
464 analysis). Exposure to pesticides occurs primarily *via* agricultural runoff, and is therefore
465 often periodic due to rainfall occurrence and seasonal changes. This can result in high peak

466 concentrations, especially in smaller creeks flowing through agricultural areas (Moschet et al.
467 2014). The different pesticides that were investigated in the studies reviewed belong
468 primarily to the insecticide groups of organophosphates, organochlorines, and pyrethroids.
469 They have diverse MoAs, but the vast majority of them showed immunomodulating
470 properties when administered to fish. Typically, pesticides affected nonspecific, innate
471 immune parameters such as respiratory burst activity of phagocytes, or leukocyte counts
472 (Harford et al. 2007; Misumi et al. 2008; Kreutz et al. 2011; 2012). The pyrethroids
473 bifenthrin and permethrin, as well as the organophosphate chlorpyrifos, induced changes in
474 the expression of genes that are involved in the immune response of fish, such as IL, TNF or
475 CXC (Eder et al. 2009; Jin et al. 2010; Beggel et al. 2011). In a study with early life stages of
476 zebrafish, Jin et al. (2015) showed that the immunotoxic effects of chlorpryifos occur at
477 sublethal concentrations, in the same concentration range that induces developmental
478 toxicity, neurotoxicity and oxidative stress. Interestingly, sublethal pesticide exposure
479 enhanced susceptibility of fish to pathogens (Clifford et al. 2005; Fatima et al. 2007; Eder et
480 al. 2008). When Clifford et al. (2005) exposed Chinook salmon to either the pyrethroid
481 esfenvalerate, or to infectious hematopoietic necrosis virus (IHNV), they recorded no
482 mortalities. However, the combination of the two agents resulted in 24.1 % mortality.
483 Unfortunately, there is almost no information available on the mechanisms of pesticide action
484 on the fish immune system. Clifford et al. (2015) addressed this question for esfenvalerate
485 and its interaction with IHNV. They found that esfenvalerate, although acting primarily as a
486 neurotoxicant, also decreases the transcription of two early, non-specific anti-viral immune
487 genes (Mx-1 and Vig-8).
488

3.2.3 PCBs / PAHs / other lipophilic organic toxicants (14 %)

Polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and other organic chemicals correspond to 14 % of the compounds studied in the analyzed articles (article-based analysis). These pollutants are known to have diverse toxicological properties. Evidence for the negative impact on fish immune function is relatively abundant: halogenated aromatic hydrocarbons (HAHs) like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and coplanar PCBs are known to induce a wide range of immunological effects in mammals, including thymic atrophy, alterations of cytotoxic T lymphocyte activity or T cell-dependent antibody responses (Selgrade et al. 2008). In fish, a number of studies show that these compounds have immunomodulating activities and increase pathogen susceptibility (Hutchinson et al. 2003; Maule et al. 2005; Iwanowicz et al. 2009).

The polybrominated biphenyl ethers (PBDEs) are another class of immunosuppressive halogenated aromatic xenobiotics. Arkoosh et al. (2010; 2015) observed a dichotomic effect of PBDEs on disease resistance of Chinook salmon: when fish were treated with a concentration reflecting contaminant levels found in stomach contents of wild Chinook salmon, fish were more susceptible to the bacterial pathogen *Vibrio anguillarum* than controls. In contrast, when fish were treated with a 10 times higher PBDE concentration, infectious disease susceptibility was not increased. The reason for this is not yet understood.

Finally, the immunomodulating activities of PAHs in fish are well documented, both from field (Arkoosh et al. 1998) and laboratory studies (Carlson et al. 2004; Reynaud & Deschaux 2005; Bado-Nilles et al. 2009; Hogan et al. 2010; Phalen et al. 2014). PAH effects on lymphocyte proliferation have been described in fish as well (Smith et al. 1999; Carlson et al. 2002). In contrast, Palm et al. (2003) did not find an immunomodulatory effect after exposing Chinook salmon *via* the diet to an environmentally relevant PAH mixture.

A common feature of the immunoactive HAHs and PAHs is that they act as ligands of the AhR. This ligand-activated transcription factor appears to play a central role in their immunomodulating activity. Prominent examples of an AhR-regulated gene include the cytochrome P450-dependent biotransformation enzymes such as CYP1A1. Nevertheless, these enzymes – at least in mammals – appear to be not involved in the immunotoxicity of AhR ligands, since the immunotoxic effects are still present in CYP1A1 knockout models (Selgrade 2007). While for many years, the AhR has been perceived mainly as a regulator of xenobiotic metabolism, there is now increasing attention to its role in regulating immune functions. Recent research has unraveled that the AhR is expressed in a variety of immune cells, and that it has multiple regulatory roles in the immune system of mammals (Marshall & Kerkvliet 2010; Stockinger et al. 2014; Tian et al. 2015). The AhR is strongly involved in the coordination of inflammatory processes, in the differentiation of B cells, of T helper (Th) 1 cells, Th2 cells, Treg cells and Th17 effector cells, as it interacts with the NFκB pathway. It appears that immunotoxic effects of TCDD and related HAHs are mediated *via* these pathways rather than *via* the AhR-dependent activation of biotransformation.

For fish, there is currently little information on the role of the AhR in immune system regulation. Indirect evidence for the presence of an AhR in fish immune cells was provided by Nakayama et al. 2007, who showed that CYP1A can be induced in specific immune cell subpopulations of rainbow trout. However, whether these findings implicate that the immunotoxic effects of HAHs in fish are, comparable to mammals, mediated *via* the immune regulatory function of the AhR, remains to be clarified.

AhR-activating PAHs like benzo(a)pyrene may induce their immunomodulatory effects principally through the same mechanisms as the HAHs, i.e. through the immunoregulatory function of the AhR. However, additional mechanisms appear to play a role in PAH immunotoxicity. PAHs have been shown to disrupt intracellular calcium levels in mammalian

538 and piscine immune cells (Reynaud et al. 2003), and can thereby modify immune cell
539 functioning. Mammalian studies indicated that PAH immunotoxicity can also be caused by
540 PAH metabolites rather than the parent compounds, and this mechanism may also be relevant
541 in fish (Carlson et al. 2002; 2004). The metabolites may reach the immune cells *via* blood
542 circulation from the liver, or they may be generated locally in the immune cells. Immune
543 cells have a limited capacity for metabolizing PAHs and generating reactive metabolites
544 (Carlson et al. 2004; Möller et al. 2014), although the metabolic rates of immune cells are
545 orders of magnitude lower than those of liver cells.
546
547 **3.2.4 Metals (9 %)**
548 The extensive use of metals in industry, agriculture and private households is responsible for
549 the input of different metallic ions and metal organic compounds into the environment.
550 Nevertheless, only 9 % of the compounds investigated by the studies reviewed were metals
551 (article-based analysis). For example, Sanchez Dardon et al. (1999) examined the influence
552 of cadmium, mercuric and zinc chloride, and their mixtures, on the immune system of
553 juvenile rainbow trout. They reported decreased levels of immunoglobulin M (IgM) and
554 changes in lysozyme and phagocytic activity. Changes in oxidative burst activity are
555 frequently reported in response to metal exposure of fish. Recent studies also focused on the
556 immunotoxic properties of metal-nanoparticles. These particles are increasingly used, for
557 instance, in cosmetics and medicine. Due to their small size, nanoparticles may act differently
558 than the "normal-sized" particles, and thus, they could cause unknown effects. And also
559 because of their small size, nanoparticles can be ingested by aquatic animals and even pass
560 through the cell membranes, leading to diverse adverse effects including immunotoxic
561 effects. Bruneau et al. (2013), for instance, showed that quantum dots of cadmium as well as

562 dissolved cadmium ($CdCl_2$) significantly decreased lymphocyte transformation in head
563 kidney lymphocytes of rainbow trout.
564
565 **3.2.5 Pharmaceuticals (4 %)**
566 The input of pharmaceuticals into surface waters can represent a serious hazard for aquatic
567 animals (Overturf et al. 2015). Among the studies analyzed for this review, 4 % of the used
568 chemicals were pharmaceuticals (article-based analysis). As those compounds are designed to
569 be biologically active, and many of the drug targets are phylogenetically conserved, they may
570 impact fish at very low concentrations (Kidd et al. 2007). A prominent example for this
571 aspect is the contraceptive pill ingredient, ethinylestradiol (EE2; an estrogenic EDC), for
572 which a predicted no effect concentration (PNEC) value of 0.35 ng/L has been derived
573 (Caldwell et al. 2008).
574 Probably a considerable fraction of pharmaceuticals detected in the environment are
575 immunoactive, including compounds like the anti-inflammatory steroidal drug,
576 dexamethasone (Lovy et al. 2008; Salas-Leiton et al. 2012) and the non-steroidal drug DCF
577 (Ribas et al. 2015; Mehinto et al. 2010). The presence of immunotoxicity related to
578 pharmaceutical contamination in river systems was evidenced by Khalaf et al. (2009) who
579 showed that pharmaceutical-containing water samples from a river in Sweden induced
580 inflammatory responses in an *in vitro* bioassay with human cells. As many of the drug targets
581 are phylogenetically conserved, these substances could affect these targets in fish as well.
582 Veterinary drugs used for the treatment of fish in fish farms, can also have
583 immunomodulating activities. For instance, the anesthetics benzocaine, MS222 (tricaine
584 methanesulfonate) and quinaldine sulphate were found to suppress immune functions in fish
585 (Ortuño et al. 2002).

586 An interesting case of an immunoactive environmental contaminant is the non-steroidal anti-
587 inflammatory drug DCF. This pharmaceutical is designed as inhibitor of cyclooxygenase
588 (COX) enzymes, which catalyze the synthesis of prostanoids. It is prescribed in human and
589 veterinary medicine to prevent inflammation and to reduce pain. DCF is poorly removed in
590 conventional wastewater treatment plants, and as a consequence, it is widely detected in the
591 aquatic environment and also one of the most import pharmaceutically active compounds
592 (Letzel et al. 2009) where it may affect the immune system of aquatic vertebrates like fish.
593 Surprisingly few studies have addressed the possible immunomodulatory mechanisms of
594 DCF in fish. However, it is known that DCF is taken up by fish, metabolized in the liver, and
595 excreted *via* the bile (Schwaiger et al. 2004; Mehinto et al. 2010). Immune-related effects
596 have been investigated by Mehinto et al. (2010) who showed that environmentally relevant
597 concentrations were able to significantly reduce the transcript levels of *cox1* and *cox2* in
598 rainbow trout. Hoeger et al. (2005) exposed brown trout to DCF (nominal concentrations of
599 0.5, 5.0 and 50 µg/L) and observed that this resulted in reduced hematocrit as well as
600 increased granulocyte accumulation and MHC II (major histocompatibility complex class II)
601 expression indicative of inflammatory processes. In addition, *in vitro* incubations with head
602 kidney macrophages of brown trout revealed that DCF inhibited the synthesis of
603 prostaglandin E_2 (Hoeger et al. 2005). Whether these immune effects are specific to DCF or
604 represent a general response of the fish immune system to non-steroidal anti-inflammatory
605 drugs remains to be shown. What is also lacking to date is a study which shows that
606 environmentally relevant concentrations of DCF are able to increase the susceptibility of fish
607 to pathogens.
608

3.2.6 Field studies (8 %)

Field studies (8 %, article-based analysis) on the immune status of fish were conducted in contaminated environments (e.g. Arkoosh et al. 2001; Hutchinson et al. 2003; Hoeger et al. 2004; Salo et al. 2007; Hébert et al. 2008; Leaver et al. 2010; Connon et al. 2012; Gagne et al. 2013), or some studies exposed fish in the laboratory to complex environmental matrices such as crude oil or oil sands and studied the effects on their immune parameters (Tahir & Secombes 1995; Nakayama et al. 2008; Song et al. 2011; 2012; Bado-Nilles et al. 2011; Leclair et al. 2013). Among the pioneering studies on environmental impacts on the fish immune system were those of Arkoosh et al. (e.g. 1991, 1998, 2001) on migrating Chinook salmon in PAH-contaminated habitats which highlighted that immunotoxic effects are of high relevance in the environment.

3.3 Immune responses of fish to toxicant exposure

3.3.1 Which immune mechanisms of fish were responsive to chemical exposure?

In mammalian immunotoxicology, four major mechanisms are distinguished through which chemicals can cause immune-mediated injury (Selgrade et al. 2008; Luster & Gerberick 2009): immunosuppressive reactions, hypersensitivity or allergic reactions, autoimmune responses, and developmental immunotoxicity. Observations that low molecular weight chemicals can be antigenic and can induce allergic responses were among the very first reports indicating that chemicals can have adverse impacts on the immune system of mammals (Landsteiner & Jacobs 1935). Later it became evident that chemicals can also cause immunosuppressive effects leading to an increased risk of infectious or neoplastic diseases (Selgrade 2007). Immunosuppressive toxicants in mammals include PAHs, HAHs, or heavy metals.

633 For fish, the studies published to date (and briefly discussed above) dealt exclusively with
634 immunosuppressive or –stimulating actions of chemicals. To our knowledge, no report exist
635 describing allergic or autoimmune responses in fish exposed to chemicals. However, it is
636 known that fish are able to mount autoimmune responses, for instance, against the germ cells
637 in the gonads (Secombes et al. 1985; Presslauer et al. 2014), or after vaccination (Koppang et
638 al. 2008). Hence, the absence of reports on autoimmune responses in fish under chemical
639 exposure cannot be explained by the principal absence of the biological mechanism, but the
640 explanation appears to be a technical one: either chemicals with autoimmune / allergic
641 properties have not yet been tested in studies with fish, or there exists insufficient knowledge
642 which parameters to measure for identifying an autoimmune / allergic response in toxicant-
643 exposed fish. This is an important gap in knowledge on fish immunotoxicology which should
644 be addressed in future research.
645
646 **3.3.2 Which parameters were used to assess immunomodulatory effects of chemicals in**
647 **fish?**
648 A broad variety of immune parameters have been used to assess the effects of chemicals on
649 the immune system of fish (Figure 4; full list as Supplement S 2). In total, 1160 individual
650 immune parameters (article-based analysis) were quantified which were used in the 241
651 analyzed publications. While some parameters were only used in a single or in few articles,
652 others parameters were used more frequently: phagocytic activity (representing 17 %),
653 transcript levels of immune-related genes (13 %), respiratory burst activity (10 %), and
654 lysozyme activity (7 %).
655 *Phagocytic activity (17 %):* Phagocytosis plays a key role in the initial defense against
656 pathogens (Castro & Tafalla 2015). Remarkably, in addition to the "classical" phagocytic
657 cells like e.g. granulocytes, piscine B cells also display considerable phagocytic activity (Li

et al. 2006). Methodologically, phagocytic activity is measured mainly as incorporation of fluorescent-labeled beads or (inactivated) bacteria into leukocytes (Shelley et al. 2009; Müller et al. 2009). Both methods enable rapid flow cytometric analysis for determination of the degree of phagocytosis.

Respiratory burst activity (10 %): The respiratory burst activity of phagocytic cells involves the generation of oxyradicals, which then kill pathogens ingested by the phagocytic cells. Methods to measure respiratory burst activity include the reduction of nitro blue tetrazolium (NBT) or chemiluminescence analysis of the reactive oxygen species (ROS; Koellner et al. 2002).

Lysozyme activity (7 %): Lysozyme disrupts the cell walls of bacteria by splitting glycosylic linkages in the peptidoglycan layers. Methodologically, lysozyme activities can be assessed by adding test sera from the study animals to defined bacterial cell suspensions, and then measuring the lysis of the bacterial cells (Alexander & Ingram 1992).

Expression of immune-related genes (13 %): Mostly, cytokines and other soluble immune mediators were measured. Methodologically, the analysis of cytokines and other mediators was usually performed at the mRNA level using quantitative RT-PCR (reverse transcription polymerase chain reaction; microarrays or omics-studies were not included in this quantitative analysis). Immunochemical methods were applied rarely, probably because only few antibodies against immune proteins in fish are available. The most frequently analyzed immune mediators were (Figure 5) (i) interleukins, ILs (representing 24 % of the analyzed genes), (ii) TNF (11 %), (iii) interferons, IFNs (7 %), and (iv) CXC chemokines (5 %). A list of all analyzed genes (*via* qRT-PCR) is available as Supplement S 4.

A few studies applied microarray technology to evaluate the global transcriptomic response of fish to immunotoxicants. Shelley et al. (2012) performed hepatic transcriptomic profiling with rainbow trout exposed for four days to either atrazine or NP. High doses of the toxicants

683 modulated the expression of a number of pathways related to immune system function. Both
684 toxicants down-regulated pathways involved in B cell activation and viral infectivity,
685 whereas complement activation (alternative pathway) and several STAT signaling pathways
686 were differentially regulated, with NP exposure leading to an up-regulation and atrazine
687 exposure leading to a downregulation. These changes of immune gene transcript levels were
688 associated with increased susceptibility of rainbow trout to pathogen infection (*Vibrio*
689 *anguillarum*), both NP and atrazine. Similar results were found for E2 in combination with
690 the pathogen *Yersinia ruckeri* in the study of Wenger et al. (2014). And a number of
691 microarray studies which were not aiming to assess immunotoxicity also found that immune-
692 related genes and pathways were significantly affected by the toxic exposure (Krasnov et al.
693 2005; Williams et al. 2008; Leaver et al. 2010) – indicating again the sensitivity of immune
694 parameters to toxicants. The microarray studies were not included in this quantitative analysis
695 of immune gene responses due to the high number of genes in these studies, they would
696 outweigh the data of PCR-based studies and would introduce a bias.
697
698 Three of the four most frequently measured parameters (phagocytosis, respiratory burst,
699 lysozyme) are constituents of the innate immune system, and even among the measured
700 immune-related genes, the majority of analyzed genes belonged to the innate immune system.
701 Although adaptive immune parameters are responsive to toxic exposure (e.g. Koellner et al.
702 2002; Carlson et al. 2004; Martins et al. 2015), they are rarely considered in fish
703 immunotoxicity studies. Why do fish immunotoxicological studies place so much emphasis
704 on the innate immune system? It may be due to the general believe that the innate immune
705 system is the dominating immune component of fish (Tort et al. 2003; Magnadóttir 2006;
706 Lieschke & Trede 2009). Another reason may be that the majority of studies used naive, i.e.
707 non-infected fish in rather short-term (less than a week) exposures– a scenario which argues

to study immediate innate rather than adaptive responses. Finally, the emphasis on innate parameters may be related to technical constraints such as the limited availability of tools and methods for adaptive immune parameters. For instance, antibodies to identify piscine T cells became available only very recently (Nakanishi et al. 2015).

Methodological considerations may also explain the rare use of histopathology in fish immunotoxicity studies. Histopathological evaluation of immune organs like spleen, thymus, lymph nodes, blood and bone marrow is a central parameter in immunotoxicity risk assessment in mammalian immunotoxicology (Vos et al. 1983; Wester et al. 1994; Krzystyniak et al. 1995). The use of structural changes / histopathology as immunotoxicity marker has already been recommended at an early stage of fish immunotoxicity research (Wester et al. 1994), since the immunopathology provides an important link between the molecular response and the adverse outcome. Nevertheless, until now histopathology has not found widespread use in fish immunotoxicological studies. The bottleneck for a more intensive use of histopathology in fish immunotoxicology may be the limited availability of academic training and expertise in fish histopathology (Feist & Segner 2013; Wolf et al. 2015).

Overall, since most authors do not provide the rationale behind their selection of immune parameters, we can only speculate on the reasons for the preference on the innate immune system, whether this due to the fact that the innate system is indeed the main target of immunotoxic chemicals in fish, or whether it is due to a bias in the parameter selection.

3.3.3 Which immune parameters of fish were responsive to chemical exposure? The search for screening parameters for potentially immunotoxic chemicals

Beyond the question which parameters were used how often in fish immunotoxicity studies, the question is which of these parameters may serve in fish immunotoxicity screening. In

733 many hazard assessment programs, the first tier is a prioritization step in which chemicals are
734 screened for possible toxic activities, which then are confirmed or rejected in the subsequent
735 testing tiers. The screening for potential toxic activities can be done by means of *in silico*, *in*
736 *vitro* and *in vivo* assays. Immune parameters to screen for potential immunotoxicants should
737 ideally be responsive to all known immunotoxicants, independent of their MoA, and they
738 should be non-responsive to non-immunotoxicants.
739 In order to search for possible screening parameters for chemicals with immunotoxic activity
740 to fish, we analyzed how often the parameters summarized in the 16 main groups
741 significantly reacted to chemical treatments (parameter-based analysis, see Material &
742 Methods).
743 *Analysis of all parameters:* Within each of the 16 groups, in at least half of the experiments
744 the measured parameters were responsive to chemical treatment (Figure 6), except the group
745 „T cells“, with 38 % significant responses only. Two groups (non-assigned cellular
746 components (innate) and acute phase proteins) showed 100 % significant responses, which
747 means every time when they were analyzed in an immunotoxicological experiment, they
748 were significantly modulated by the chemical exposure. At a first glance, this result might
749 indicate that the two immune parameter groups are highly suitable as screening markers for
750 potential immunotoxic activity of chemicals. However, since both groups contained only a
751 low number of observations (n = 6 or 8, respectively), it is questionable whether this
752 interpretation holds.
753 A fairly high responsiveness (92 %) was also shown by the group “challenge induced
754 mortality test”. However, it has to be considered that this type of – resource intensive –
755 experiment is mostly done, when information on immunomodulating properties and
756 concentrations of the chemical is already available. In other words: probably a pre-selection
757 has taken place resulting in an overestimation of the responsiveness of the “challenge induced

mortality test" parameter. Moreover, due to resource requirements of the challenge test concerning labor, animals and costs as well as due to ethical considerations, this test is not appropriate as a screening test.

The groups with the largest number of observations were the groups of "cytokines" (n = 540) and "phagocyte functions" (n = 462). As a subset of the group "phagocyte function", the phagocytosis assay (including the parameters "phagocytic activity", "phagocytic index" and "phagocytic capacity"; table 1; parameter-based analysis) was measured 167 times and thus, provided a good data basis for further evaluation. In 71 % of the measurements, the phagocytosis showed a significant chemical-related response, either an up- or down-regulation, while no response was seen in 29 %. In 49 % of the studies, in which phagocytosis showed no response, other measured immune parameters also did not react, while 51 % in contrast displayed a significant chemical-related change. This implies that if the phagocytosis assay would be used as the only screening assay, it might miss about 50 % of potential immunotoxicants.

Analysis of the cytokines: "Cytokines", being the group with the largest number of observations, were employed 540 times as immune parameter among all the 241 immunotoxicity studies with fish analyzed for the present review. This high number is probably related to the fact that the emergence of molecular techniques including qRT-PCR, together with the rapid progress in sequencing of piscine cytokines during the last two decades (Saeij et al. 2003; Secombes et al. 2004; 2011; Zou & Secombes 2011; Zhu et al. 2013; Wang & Secombes 2013) has significantly facilitated the measurement of cytokines. Since cytokines display huge functional diversity and belong to a variety of immune pathways, it is unlikely that they are 100 % responsive. In fact, the group "cytokines" was found to be responsive to toxicant exposure in 66 % of the 540 measurements (parameter-based analysis).

In order to learn on the immunotoxicity screening value of cytokines, it is probably necessary to break down this amorphous group into functionally defined sub-groups such as pro- and anti-inflammatory cytokines. Admittedly, current knowledge of the functional categories of the various cytokines in fish is limited; therefore, only a rather rough classification into five "functional" groups was applied (Figure 7; Table 2). The majority of the analyzed cytokines were pro-inflammatory (n = 275; parameter-based analysis). When quantifying the percentage of significant responses, either up- or downregulation, of the various cytokines to chemical exposure among the five groups, no major differences were found.

Overall, the results of this analysis provide no convincing arguments that a more function-based grouping would improve the identification of markers for immunotoxicity screening. However, since for all groups except the pro-inflammatory cytokines, the n-numbers were low, the results may be largely random-driven.

Analysis of immune parameter responsiveness in relation to chemical MoA: In the analysis above, we asked how responsive the immune parameters to toxicants were in general; we did not discriminate between chemical classes and / or the toxic MoA of the chemicals. However, it is well possible that certain immune parameters are specifically reactive to distinct modes of actions or chemical classes. As a case study (parameter-based analysis) to address this question, we examined all experiments conducted only within the toxicant group of EDCs in general (Figure 8), and only the "estrogen-active EDCs" in particular (Figure 9). These groups were selected since the literature provides comparatively abundant information for them, and, in addition, estrogenic EDCs share a well-defined, common molecular initiating event (i.e. binding and activation of ER). The parameters in the groups "challenge induced mortality test", "leukocytes", "non-assigned humoral components (innate)", "antimicrobial peptides", and "non-assigned humoral components (adaptive)" were 100 % responsive to both EDCs in general and estrogen-active EDCs in particular. Additionally, in case of only

808 the estrogenic EDCs, all parameters of the groups “lysozyme”, “complement” and “non-
809 assigned cellular components (adaptive)” were 100 % reactive to the chemical treatment, too.
810 Nevertheless, the shift from analyzing all chemicals to analyzing only chemicals which share
811 a common mode of action did not drastically alter the responsiveness of the groups. Hence,
812 the mentioned groups seem to be no reliable indicator for the detection of immunotoxic
813 EDCs.
814 However, a biomarker should be supported by mechanism-based rather than correlative
815 relationships (Segner 2011). For mammals, for instance, it is known that certain complement
816 genes possess estrogen-responsive elements in their promoters and therefore their transcript
817 levels can be modulated by estrogenic EDCs; corresponding information, however, is lacking
818 for teleost fish. In conclusion, at the current state of knowledge, there appears to be not one
819 single immune parameter available which would serve as general screening parameter for
820 potential fish immunotoxicants. Given the diversity of the immune system and the
821 immunotoxic MoA, it is likely that rather than a single immune parameter it will need a set of
822 markers to screen chemicals for their potential immunotoxicity to fish. Currently, however, it
823 appears that there is even not sufficient information to decide which set of immune
824 parameters could be used for screening. To advance this field, it would require systematic
825 studies using an array of immune endpoints including parameters of the adaptive immune
826 system, to test an array of chemicals with structural similarities, as well as chemicals with
827 different modes of immunotoxic actions.
828
829 **3.3.4 Relationship between immunomodulating and immunotoxic effects in fish**
830 While chapter 3.3.3 discussed the relationship between chemicals or MoA and the reactivity
831 patterns of fish immune parameters, the present chapter evaluates which immune marker
832 responses are indicative of adverse outcomes, i.e. whether molecular and cellular marker

833 responses are predictive of alterations in immunocompetence and disease susceptibility of the
834 organism. Not any chemically-induced modulation of an immune parameter (e.g. cytokine
835 levels or phagocytic activity) translates into immune dysfunction, but the adversity of the
836 effect needs to be demonstrated (Selgrade 2007). In immunotoxicity studies with fish, the
837 most frequently used parameter to demonstrate that chemical exposure has an adverse effect
838 on immune functioning is the challenge test with pathogens. In this test, fish are exposed to a
839 chemical, in the absence or presence of a pathogen, and if the presence of the chemical alters
840 the susceptibility of the fish towards the pathogen, this is taken as demonstration of the
841 immunotoxicity of the chemical. A broad variety of pathogens was applied in the reviewed
842 studies, probably corresponding to the variety of fish species used. The most commonly
843 applied pathogens for the stimulation of the immune system were *Aeromonas hydrophila* (25
844 %), *Aeromonas salmonicida* (14 %), *Vibrio anguillarum* (15 %), *Yersinia ruckeri* (7 %) and
845 IHNV(7 %; article-based analysis).
846 The effect parameter typically used in those challenge tests is the pathogen-induced
847 mortality. As explained in "Material and Methods" we designate this parameter as "challenge
848 induced mortality test". Among the reviewed studies in which a challenge induced mortality
849 test was employed, 75 % could show an increase of pathogen-induced mortality if the fish
850 were exposed to chemicals. In 17 % of the experiments a decrease and in 8 % no significant
851 differences between exposed and control fish were observed (parameter-based analysis). The
852 challenge induced mortality test, however, is complex, costly, and critical with respect to
853 animal welfare. Thus, it would be advantageous to have molecular or cellular immune marker
854 parameters that can qualitatively or quantitatively predict the consequence for the
855 immunocompetence and disease resistance of the fish. The predictive value of immune
856 parameters for host resistance has been extensively studied in mammalian toxicology (Luster
857 et al. 1993; Germolec 2004). Certain immune marker responses were found that show good

858 correlation with immune functioning of the organism as indicated from challenge tests. For
859 instance, in rodents the plaque forming assay had a 73 % correlation with host resistance
860 (Germolec 2004). The question is whether the quantitative analysis of the fish
861 immunotoxicology literature since 1995 points to molecular or cellular immune markers that
862 might have a predictive value for the immunocompetence of the fish. To this end, the articles
863 were analyzed on correlations between the immune markers and the results of the challenge
864 induced mortality tests. Only for one parameter, phagocytic activity, a trend could be
865 observed: decreased activity in the phagocytosis assay (n=5 out of 8 experiments) was
866 associated with increased mortality, and increased phagocytic activity (n=4 out of 4
867 experiments) was associated with decreased mortality after pathogen challenge (as a subset of
868 the main group "phagocyte function", the "phagocytic activity", "phagocytic index" and
869 "phagocytic capacity", were included in this parameter-based analysis). However, as
870 discussed before, this observation must be interpreted with care, given the high diversity of
871 the database together with the small number of studies. Thus, at this current state of literature
872 data, fish immunotoxicologists seem to have no predictive immunotoxicity markers available.
873
874 **3.3.5 *In vitro* assays to screen for potential immunotoxicants of fish**
875 Chemical regulations increasingly ask for the use of *in vitro* assays rather than *in vivo* tests
876 for hazard assessment. In immunotoxicity assessments, a major drawback of *in vitro* assays is
877 that they do neither reflect systemic interactions within the immune system nor other
878 interactions such as the neuro-endocrine-immune crosstalk. Thus, the complexity of
879 chemical-induced immunotoxicity assessed in whole animal tests is difficult to cover by *in*
880 *vitro* assays. Nevertheless, apart from the value of *in vitro* assays for mechanistic studies
881 (Corsini et al. 2012), they may still be of value for rapid screening of substances for
882 immunotoxic potentials and their prioritization for *in vivo* testing. An example is provided by

883 the T cell-based *in vitro* assays used in human immunotoxicology (Martin et al. 2010). The
884 induction of a T cell response by chemicals, drugs or allergens is the crucial step for the
885 manifestation of allergic diseases and T cell-mediated adverse drug reactions. Therefore, T
886 cell-based assays can serve as an *in vitro* screen to identify compounds that have the
887 capability to interfere with T cells. Importantly, a screening assay may well produce false
888 positives, but it should not produce false negatives.
889 Among the 241 reviewed publications, 54 of the conducted experiments (21 %; parameter-
890 based analysis) were carried out *in vitro*, either alone or in combination with *in vivo* tests. *Ex*
891 *vivo* assays were taken as *in vivo* study for this analysis, because in this approach, the cells
892 are conditioned *in vivo* and only their response capacity is measured outside the animal
893 (Gatta et al. 2001; Dautremepuits et al. 2004; Leclair et al. 2013). The *in vitro* studies
894 included experiments with permanent cell lines or with cells isolated from non-exposed
895 control animals (Bols et al. 2001; Law et al. 2001; Castro et al. 2011).
896 Initially, we examined how often immune parameters of the 16 main groups responded
897 significantly to chemical treatment in the *in vitro* experiments (Figure 10; parameter-based
898 analysis; only *in vitro* conducted experiments were considered for this analysis). Four out of
899 the 16 groups – "non-assigned cellular components (innate)", "antimicrobial peptides",
900 "acute phase proteins" and "B cells" – showed 100 % reactivity. The very low n-numbers
901 (for each of these groups: $n < 5$), however, raise question marks on the robustness of the
902 findings. An important drawback in the analysis of *in vitro* data is that many studies did not
903 determine the cytotoxicity of the chemical exposure (see 3.1 *Exposure concentration*).
904 Therefore, it is not clear whether the observed responses are indeed due to an
905 immunomodulating activity of the test chemicals, or may represent secondary effects of
906 cytotoxicity.

907 In a next step, an *in vitro* / *in vivo* comparison was performed, analyzing those studies that
908 included both, *in vivo* and *in vitro* measurements. An example is provided by the study of
909 Cabas et al. (2012) who compared the immunological effects of EE2, an estrogenic EDC, in
910 the gilthead seabream (*Sparus aurata*) *in vivo* and in isolated head kidney leukocytes *in vitro*.
911 Both approaches classified EE2 to be an immuno-modulator and thus yielded a congruent
912 conclusion, although the responsive immune parameters differed partly between the *in vitro*
913 and the *in vivo* tests. Overall however, the number of studies that allow a comparative *in vitro*
914 / *in vivo* analysis was too low to support any conclusive statement on the suitability of *in vitro*
915 assays to screen for potential immunotoxicants.
916

917 # 4. Conclusions

918 A first conclusion from this quantitative analysis of the fish immunotoxicological literature is
919 that to date fish immunotoxicology mainly **focused on immunosuppressive effects**, but did
920 not consider possible autoimmune or hypersensitivity reactions. This is in sharp contrast to
921 human immunotoxicology, where much emphasis is given to hypersensitizing effects of
922 chemicals (Luster 2014). The fact that there are no reports on chemical-induced immune
923 hypersensitivity / allergy in fish does not necessarily mean that there are not such effects, but
924 may be more related to the fact that currently there is little understanding what an allergic
925 reaction in fish is. This is an important knowledge deficit in fish immunotoxicology which
926 should be to be addressed in future research.
927 Similar to the underrepresentation of studies on autoimmune and allergic responses in fish
928 immunotoxicology, also toxic effects on the adaptive immune system of fish are little studied.
929 This is surprising since adaptive immune parameters such as *lymphocyte assays* are broadly
930 used in human immunotoxicology (Luster et al. 1992; 1993). Protocols for assays measuring
931 adaptive immune parameters in fish are available, for instance, B and T cell lympho-

proliferation assays (Zelikoff 1998; Carlson et al. 2002; Koellner et al. 2002). Thus, it is not necessarily the lack of methods that can explain the under-representation of adaptive immune parameters in fish immunotoxicological studies, but it may be more related to the general belief that fish rely primarily on the innate immune system.

A second conclusion from this literature analysis is that in numerous fish immunotoxicity studies, the **experimental conditions are poorly documented**. For instance, *age and sex* of the experimental fish are often not reported, nor do the studies provide an explanation for the selection of *exposure duration*: why were fish exposed for one day, for one week, or longer. Were these selections based on, e.g. information on the immunotoxic MoA of the test chemical or were they chosen arbitrarily? A particularly important drawback is the fact that often no information is provided on the *ratio of the selected effect concentrations* to test compound concentrations that induce general toxicity such as lethality or cytotoxicity. Therefore, it is often difficult to judge whether an observed effect indeed represents an immunotoxic effect or a side effect of general toxicity. Immunotoxic effects typically occur at concentrations clearly below general toxic levels (Koller 2001; Luster 2014), consequently, information on how the selected test concentration relates to general toxicity is essential. Overall, the *standards in the reporting* of immunotoxicity studies with fish need to be improved to achieve progress in this field, since the number of studies using the same experimental set-up is still too low to be able to compare them properly.

A third conclusion is that although a broad variety of parameters and methods were used to assess the immunotoxic effects of chemicals, many studies give **no rationale for the choice of the measured parameters**. Selection of effect parameters in an immunotoxicity study is not trivial, since there is a plethora of potential target sites and functions for chemical attacks

957 in the immune system (Carlson & Zelikoff 2008; Segner et al. 2012), but exactly for this
958 reason the rationale for the selection needs to be given. Also the purpose of the study needs to
959 be considered – is it a screening study, an adverse effect study or a mechanistic study? In
960 identifying which immune parameters should be measured in an immunotoxicological study,
961 *mechanistic information* is of value. It is not only relevant to select appropriate effect
962 parameters for the immunotoxicity screening, but also to link chemical-induced changes of
963 molecular and cellular immune parameters to responses at the organism level, as it
964 conceptualized in the adverse outcome pathway (AOP) framework (Ankley et al. 2010).
965 However, often mechanistic information is lacking in fish immunotoxicology. In this
966 situation, we may rely on "biological read across" (Rand-Weaver et al. 2013) utilizing
967 mechanistic information from human immunotoxicology. An example of the biological read-
968 cross approach is provided by the study of Martins et al. (2015) who investigated the
969 immunotoxicity of di(2-ethylhexyl)phthalate in rainbow trout. From mammalian studies it
970 was known that this compound has effects on, among other targets, B cells and their
971 maturation. Thus, a number of B cell-related parameters and function tests was selected to
972 check for the immunotoxicity of di(2-ethylhexyl)phthalate in fish. Observed effects included
973 inhibition of kidney B cell proliferation and IgM secretion.
974
975 A fourth conclusion is that fish immunotoxicology does not yet command on a validated set
976 of immune parameters **to screen for the immunotoxic potential of chemicals**. Such
977 parameters should have an integrative nature or they should be located at critical crossing
978 sites in the immune network (Luster & Gerberick 2009; Hartung & Corsini 2013). One of the
979 most frequently used parameters in fish immunotoxicity studies is the phagocytosis assay.
980 Because fish rely strongly on the innate immune system, many authors appear to consider this
981 assay as a kind of screening assay, although, as indicated from this quantitative data analysis,

982 the available evidence to support this claim is equivocal. To advance the establishment of
983 screening parameters in fish immunotoxicology, it will need a more systematic evaluation of
984 candidate markers and assays with respect to responsiveness to diverse chemical groups and /
985 or immunotoxic MoAs, but also with respect to their sensitivity to immunotoxicity in
986 comparison to general toxicity.
987
988 A fifth conclusion from this review is that the understanding of how and when **chemical-**
989 **induced modulations** of molecular and cellular immune changes **relate to alterations of the**
990 **fish immunocompetence** is not well developed yet. Since in ecotoxicology, the adverse
991 outcome at the organism and population level is the relevant endpoint, immunotoxicologists
992 have to place more emphasis on establishing the relation between screening measurements,
993 immune dysfunction and impaired health of the organism. Currently we have too little
994 information on how often immune parameters and assays that might be used for screening,
995 for instance the phagocytosis assay, produce false negatives and false positive results. It is
996 clearly not enough to identify immune parameters that are responsive to a broad variety of
997 immunotoxic chemicals or immunotoxic MoAs, but we also have to know how these
998 parameters relate to adverse immunotoxic outcomes. As evident from the results of the
999 quantitative analysis of the relation between molecular / cellular effects and immunotoxic
1000 outcomes this is clearly an area where fish immunotoxicology has still a long way to go.
1001
1002
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Tables with captions:

Table 1: "Main immune function groups" used for the quantitative literature data analysis. The table shows which immune parameters measured in the reviewed studies (right column) were aggregated into which "main group" (left column). Importantly, the "main groups" contain those parameters that have been measured in the reviewed fish immunotoxicity studies, and therefore do not represent a full coverage of the respective immune functional groups but necessarily remain patchy. Furthermore, the assignment of certain parameters to one or the other group is debatable, also because a number of immune parameters have multi-faceted functions. We tested how much a different group assignment of single parameters would change the overall outcome of the quantitative analysis, and found that the effect is negligible for the purposes of this review. The groups of "non-assigned" components contain miscellaneous parameters difficult to assign to a specific main group. The 16 main groups provided the basis for the parameter-based analysis as shown in Figs 6, 8, 9 and 10.

main groups	**parameters**
challenge induced mortality test	challenge tests with bacteria, viruses and parasites with the purpose of having influence on the mortality of the fish
leukocytes	number of leukocytes, leukocrit, leukocyte apoptosis & proliferation, mitogenic response, blastogenic response
phagocytes	phagocyte number, total granulocytes, total neutrophils, total monocytes, total macrophages, total myeloids, CSF-1R / M-CSFR, NCC
phagocyte function	phagocytic activity & index & capacity, nramp, nrf2, keap1, bactericidal activity, bacterial agglutination, chemotaxis activity (leukocytes), chemiluminescence response of phagocytes / NBT /

	nitroblue tetrazolium assay / ROS / resp. burst assay, NADPH oxidase, peroxidase activity & content, superoxide production, iNOS; nitric oxide (NO) production & release & concentration & synthase activity, reactive nitrogen intermediates & species (RNI & RNS), arg2 / arginase, mpo / myeloperoxidase content
lysozyme	lysozyme number & activity & assay, g-type lysozyme
antimicrobial peptides	elastase 2 / neutrophil elastase, bd / β-defensin, hamp 1 / hepcidin, antiprotease activity, extracellular trap release, degranulation
acute phase proteins	crp / c-reactives protein, saa, a-saa, tcpbp / trout c-polysaccharide binding protein
complement	acp & ach / alternative complement pathway & activity, C3 / complement, factor h & b, nhc / (natural) hemolytic complement, other factors like c1r/s, mbl-2, f2, c9, cfp
non-assigned cellular components (innate)	g-csf / granulocyte colony-stimulating factor, phagocyte migration
non-assigned humoral components (innate)	pannexin 1, TLR9a, mx-protein / mx / mx-1
cytokines	This group contains all parameters mentioned in table 2 (cytokine)
B cells	B cell number & proliferation, surface immunoglobulin-postive leukozytes (sIG+)
T cells	T cell number & proliferation, TCR, CD 4, CD 8
antibodies	Ig (e.g. IgM), AB (antibody) titer, AB secretion; agglutination AB titer
non-assigned cellular components (adaptive)	lymphocyte / lymphoblast transformation & proliferation & index & blastogenesis & mitogenic response, number of lymphocytes

non-assigned humoral components (adaptive)	MHC, MHC I, MHC II, pfc / plaque-forming cell assay & response

Table 2: Assignment of individual cytokines (right column) to cytokine groups (left column). Importantly, the groups do not contain full coverage of the respective cytokine groups but represent those cytokines that have been measured in the reviewed fish immunotoxicity publications. The groups of "non-assigned" components contain miscellaneous parameters difficult to assign to a specific cytokine group. These groups provided the basis for the parameter-based analysis as shown in Fig 7.

cytokine groups	**cytokines**
pro-inflammatory cytokines	IL 1, IL 6, IL 8, IL 17, TNF, COX, PTGS 1&2
anti-inflammatory cytokines	IL 10, IL 14
regulatory factors	NFkB, NFkBia, NFkB-inh., nkapl, RELA, suppression of cytokine signaling; SOCS protein
chemokines	CC-chem, CXC, CXCA, CXCL, CXCL-C1C, CXCL-CLC, CC, CX4C, XC, receptors: CXCR & CCR
non-assigned cytokines	lymphokine production, TGF-beta, IFN & IFN-1 & IFN-alpha & IFN-gamma, ISG15 / interferon stimulated gene 15, IL 12, γIP

Figure captions:

Figure 1: Number of fish immunotoxicology-related publications per year. The data are based on the publications given in Supplement S 1.

Figure 2: Percentage use of different fish species in the reviewed immunotoxicity studies (article-based analysis). The most commonly used species are *O. mykiss* (20 % of all used fish species), *C. carpio* (11 %) and *S. aurata* (11 %).

Figure 3: Provision of general toxicity information in fish immunotoxicity (*in vitro* and *in vivo*) among the 241 reviewed publications; article-based analysis. In 62 % no apical toxicological endpoints (e.g. cytotoxicity *in vitro* and lethality *in vivo*) were determined after chemical treatment. The ratio between general and immunotoxic thresholds remain unclear. In 28 % of the studies, the general toxicity was determined. 10 % relied on literature derived general toxicity values without confirming them experimentally.

Figure 4: Percent measurement of immune parameters in the reviewed studies on fish immunotoxicity; article-based analysis. The total number of parameters was 1160 (= 100 %). A detailed list of the frequency in the use of immune parameters / methods is provided in Supplement S 2. The most commonly used parameters were phagocytic activity (17 % of all used parameters), the expression of immune-related genes (13 %) and the measurement of the respiratory burst activity (10 %).

Figure 5: Percent measurement of immune genes as effect parameters in the reviewed fish immunotoxicity studies. The total number of analyzed genes was set to be 100 %; article-

based analysis. A detailed list of genes analyzed by means of qRT-PCR is provided in Supplement S 4. Predominantly, the interleukins (IL, 24 % among all analyzed genes) were analyzed, followed by the tumor necrosis factor (TNF, 11 %) and the interferons (IFN, 7 %).

Figure 6: The percentage of chemical-induced responses of immune parameters in the 16 main groups. The solid bars indicate how often (in percent) the immune parameters of a main group responded significantly to the chemical treatment. The n-numbers depictured in the bars represent the total number of measurements for the respective main group (*in vitro* and *in vivo*) and were set to be 100 %. For details regarding the grouping see table 1; parameter-based analysis.

Figure 7: The percentage of chemical-induced responses of functional cytokine groups. Each measured cytokine was assigned to one of the five functional groups: pro-inflammatory cytokines (e.g. IL 1), anti-inflammatory cytokines (e.g. IL 14), regulatory factors (e.g. NFκB), chemokines (e.g. CC-chem), and cytokines which are not predominantly associated with one of the previous groups ("non-assigned cytokines"). The n-numbers depictured in the bars represent the total number of measured cytokines included in each group (*in vitro* and *in vivo*) and was set to be 100 %. The solid bars indicate how often (in percent) the immune parameters of a main group responded significantly to the chemical treatment. For details regarding the grouping see table 2; parameter-based analysis.

Figure 8: The percentage of EDC-induced responses of immune parameters in the 16 main groups. The solid bars indicate how often (in percent) the immune parameters of a main group responded significantly to EDC treatment. The n-numbers depictured in the bars represent the total number of measured immune parameters in the respective main group (*in*

vitro and *in vivo*) and was set to be 100 %. For details regarding the grouping see table 1; parameter-based analysis.

Figure 9: The percentage of estrogenic EDC-induced responses of immune parameters in the 16 main groups. The solid bars indicate how often (in percent) the immune parameters of a main group responded significantly to estrogenic EDCs. The n-numbers depictured in the bars represent the total number of measured immune parameters included in the respective main group (*in vitro* and *in vivo*) and was set to be 100 %. For details regarding the grouping see table 1; parameter-based analysis.

Figure 10: The percentage of chemical-induced immune response as determined in *in vitro* conducted exposure experiments. The n-numbers depictured in the bars represent the total number of measured immune parameters of the respective main group (only *in vitro*) and was set to be 100 %. The solid bars indicate how often (in percent) the parameters of *in vitro* conducted experiments significantly responded to the chemical treatment. For details regarding the grouping see table 1; parameter-based analysis.