Running head: Tiered effect assessment approach for fungicides

Is the effect assessment approach for fungicides as laid down in the EFSA Aquatic Guidance Document sufficiently protective for freshwater ecosystems?

Abstract

In Europe, the EFSA Aquatic Guidance Document describes the procedures for the derivation of Regulatory Acceptable Concentrations (RACs) for pesticides in edge-of-field surface waters on the basis of Tier-1 (standard test species), Tier-2 (geometric mean and species sensitivity distributions) and Tier-3 (model ecosystem studies) approaches. In the present study, the protectiveness of such tiered approach was evaluated for fungicides. Acute and chronic RACs for Tier-1 and Tier-2B (species sensitivity distributions) were calculated using toxicity data for standard and additional test species, respectively. Tier-3 RACs based on ecological thresholds (not considering recovery) could be derived for 18 fungicides. This study shows that Tier-1 RACs, in the majority of the cases, are more conservative than RACs calculated based on model ecosystem experiments. However, acute Tier-2B RACs do not show a sufficient protection level when compared with Tier-3 RACs from cosm studies that tested a repeated pulsed exposure regime or when testing relatively persistent compounds. Chronic Tier-2B RACs showed a sufficient protection level, although they could only be evaluated for six compounds. Finally, we evaluated the suitability of the calculated RACs for eight compounds with toxicity data for fungi. The comparison shows that current RACs for individual fungicides, with few exceptions (e.g. tebuconazole), show a sufficient

protection level for structural and functional fungal endpoints. However, more data is needed to extend this comparison to other fungicides with different modes of action.

Keywords

fungicides, ecological risk assessment, aquatic toxicity, laboratory single-species tests, mesocosms

INTRODUCTION

The regulatory aquatic risk assessment scheme that supports the registration of pesticides in the European Union (EU) is based on a tiered approach proposed by the Panel on Plant Protection Products and their Residues (PPR) coordinated by the European Food Safety Authority (EFSA PPR 2013). Each tier is characterized by an exposure assessment, which results in a predicted environmental concentration (PEC), and an effect assessment, which results in a regulatory acceptable concentration (RAC) after the application of an appropriate Assessment Factor (AF) to laboratory or semi-field toxicity data (see Figure 1). By confronting the PEC and the RAC, it is determined whether the risk related with an intended pesticide-use is considered acceptable (PEC < RAC) or non-acceptable (PEC > RAC). The principle of the tiered approach is to start with a simple conservative assessment and to refine the exposure and/or the effect assessment making use of data obtained from more complex and time-consuming experiments (Boesten et al. 2007). The Tier-1 aquatic effect assessment for pesticides, as outlined in the EFSA Aquatic Guidance Document (EFSA PPR 2013), is based on the results of laboratory toxicity tests conducted with a limited number of standard test species. Tier-2 also includes results of laboratory toxicity tests with additional test species, allowing the geometric mean (geomean; Tier-2A) approach or the species sensitivity distribution (SSD; Tier-2B) approach for the calculation of RACs. The experimental Tier-2 studies can be complemented with toxicokinetic-toxicodynamic models to address the risks of time-variable exposures (EFSA PPR, 2018). Tier-3 comprises the use of results from model ecosystem experiments (i.e., micro- and mesocosms). In theory, population-level models can be used to complement results of model ecosystem experiments, but yet no detailed guidance on mechanistic effect models is provided in EFSA documents. In the Tier-3 procedure, the RACs can be derived on the basis of two options: (i) the ecological threshold option

(ETO-RAC), accepting negligible population-level effects only, and (ii) the ecological recovery option (ERO-RAC), accepting some population-level effects under the condition that recovery takes place within a given time frame (EFSA PPR 2013).

In principle, the adequacy of the prospective environmental risk assessment approaches for safeguarding aquatic organisms must be evaluated, e.g. by using results of the most appropriate reference tier. According to the protection goals adopted in the EFSA Aquatic Guidance Document, the ecological entity of plants and animals to protect is the population-level, but in the acute risk assessment vertebrates need to be protected at the individual level to avoid visible suffering and mortality due to direct toxicity (EFSA PPR 2010). Population dynamics in the field (i.e., biomonitoring data) usually are the result of many environmental factors, and consequently may not allow observed effects to be linked to a single active substance but only to multiple stressors (Rico et al. 2016). Consequently, safe threshold concentrations for individual active substances derived from aquatic micro- and mesocosm experiments (based on the ETO), have been used to evaluate the adequacy of the lower tier RACs. Previous studies have evaluated the adequacy of the Tier-1 and Tier-2 approaches, as described in EFSA PPR (2013), for insecticides (Van Wijngaarden et al. 2015; Brock et al. 2016) and herbicides (Van Wijngaarden and Arts, 2018). However, to the best of our knowledge, such an evaluation for fungicides has not yet been performed. Maltby et al. (2009) compared acute HC5 values (Hazardous Concentration for the 5% of species tested) from SSDs with NOECs and LOECs from the most sensitive and relevant population-level endpoint observed in micro-/mesocosm experiments. However, in the EFSA Aquatic Guidance Document, both the criteria to derive a RAC on basis of the SSD approach (Tier-2B) and on basis of the model ecosystem approach (Tier-3) were re-defined, so that a new evaluation is warranted. In addition, the question has been raised as to what extent these RACs

sufficiently protect aquatic fungi populations and communities, and their mediated ecological processes such as organic matter decomposition (Zubrod et al. 2015, 2019; Ittner et al. 2018).

The aim of the present study was to evaluate the adequateness of the experimental effect assessment procedures used in the EU to protect populations of non-target aquatic organisms in edge-of-field surface waters from exposures to a single fungicidal compound. To this end, following the guidance provided by EFSA PPR (2013), acute and chronic Tier-1 and Tier-2 RACs were calculated and compared with Tier-3 ETO-RACs. Tier-2 RACs were only derived on the basis of the SSD approach (Tier-2B), as the adequacy of the geomean approach described in EFSA PPR (2013) is difficult to evaluate for biocidal substances for which a wide array of taxonomic groups of aquatic organisms may be sensitive. This topic will be discussed in a follow-up paper together with a proposal for the implementation of the weight-of-evidence approach in the aquatic effect assessment for fungicides. The present study also compares the derived RACs with available toxicity data for structural and functional endpoints of aquatic fungi. Finally, some recommendations for improving the knowledge that underpins the environmental risk assessment of fungicides are provided.

MATERIALS AND METHODS

Single species toxicity data mining

An initial fungicide list was created (Table 2), which included 18 compounds for which information on ecological threshold levels is available from micro-/mesocosm studies that allowed derivation of Tier-3 ETO-RACs, following the procedures described in the EFSA Aquatic Guidance Document (see section *Derivation of Tier-3 RACs*). Information on the toxicological Mode-of-Action (MoA) to microorganisms for these fungicides was

obtained from the Fungicide Resistance Action Committee (FRAC 2017). CAS numbers were added to each fungicide by cross checking the fungicide names with those contained in the FOOTPRINT database (http://sitem.herts.ac.uk/aeru/iupac/). The CAS number list was used to download all aquatic single-species acute and chronic toxicity data available from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/quick_query.htm). Next, the draft assessment reports (DAR; http://dar.efsa.europa.eu/dar-web/provision) available for the list of selected fungicides were thoroughly searched, as well as the open literature and the toxicity dataset for fungicides collated by Maltby et al. (2009). The single-species toxicity data that was not already contained in the ECOTOX database was added to the dataset. The dataset was managed using Microsoft Excel.

Prior to analysis, the toxicity data reported as parts per billion (ppb) or mol/L were converted to µg/L. Next, only data fulfilling the criteria regarding the measured effect endpoint, the calculated toxicity value and the test duration described in Table 1 were used for further analysis. These criteria were broadly based on the criteria proposed by EFSA PPR (2013) for the selection of standard and non-standard species toxicity data to be used in Tier-2. Comparable selection criteria were also used in the evaluation studies conducted with insecticides (e.g., Brock and Van Wijngaarden 2012; Van Wijngaarden et al. 2015; Brock et al. 2016) and herbicides (Van Wijngaarden and Arts 2017). In case that more than one toxicity value was available for a given taxon, the following rules were applied: (i) the geometric mean was calculated when more than one toxicity value was reported for the same test duration and the same endpoint of a given taxon; (ii) when one taxon was represented by several endpoints, the value corresponding to the most sensitive relevant endpoint included in Table 1 was selected.

TABLE 1 ABOUT HERE

Derivation of Tier-1 RACs [a]

According to EFSA PPR (2013), acute Tier-1 RACs were derived from the core dataset, by taking the lowest of (i) *Daphnia magna* 48h-EC50 (immobility) divided by an AF of 100; (ii) *Oncorhynchus mykiss* 96h-LC50 divided by an AF of 100; and (iii) the lowest of *Raphidocelis subcapitata* (synonyms: *Pseudokirchneriella subcapitata* and *Selenastrum capricornitum*) or *Desmodesmus subspicatus* (synonym: *Scenedesmus subspicatus*) 72h to 96 h-EC50 divided by an AF of 10 (Figure 1). For the selection of the algae EC50 values, growth rate was chosen as preferred endpoint, and when not available yield was selected. Regarding the algal test duration, 72h toxicity values were chosen as preference, but 96h were also allowed when 72h values were not available. In the case that the fungicide had an insecticidal mode of action (i.e., toxicity value for *D. magna* was an order of magnitude lower than for the other two species), toxicity data (48h-EC50 immobility, mortality) for a second arthropod species (*Chironomus* spp., preferred, or *Americamysis bahia*) divided by an AF of 100 was also included. In the case the compound had an herbicidal mode of action (i.e., toxicity value for the selected algae species was an order of magnitude lower than the other two), an 72h-EC50 (growth rate preferred over yield) for a non-green algae species (e.g. diatom or blue-green algae) and an 7d-EC50 for *Lemna* sp. divided by an AF of 10 were also chosen. In the case the compound had a piscicidal mode of action (i.e., toxicity value for the *O. mykiss* was an order of magnitude lower than the other two), no further action was taken (EFSA PPR, 2013; Figure 1). The majority of the evaluated fungicides were classified as biocidal, i.e. representatives of different taxonomic groups may be potentially sensitive (Maltby et al., 2009), on the basis of the core acute toxicity dataset and the chronic EC50 values for primary producers. Out of the 18 selected fungicides, three were classified as herbicidal (MoA: two sterol biosynthesis inhibitors and one signal transduction inhibitor); one as

insecticidal (MoA: amino acids and protein synthesis inhibitor); and one as piscicidal (MoA: multi-site contact activity; Table 2).

FIGURE 1 AND TABLE 2 ABOUT HERE

[a] *Footnote: The derivation of Tier-1 RACs, Tier-2B RACs and Tier-3 ETO-RACs in this paper follows the procedure described for RAC derivation in the EFSA Aquatic Guidance Document (EFSA PPR, 2013). These RACs are based on all relevant scientific information that we could obtain from the open and grey literature, including recent literature. Consequently, the RACs we derived may deviate from the values previously published by EFSA or EU Member States in official regulatory documents.*

In line with EFSA PPR (2013), chronic Tier-1 RACs were calculated as the lowest of (i) *D. magna* 21d-EC10 or 21d-NOEC (lowest relevant endpoint in Table 1, usually reproduction); (ii) lowest of *R. subcapitata* or *D. subspicatus* EC50 (using the same endpoint and test duration criteria as described above for the acute assessment); and (iii) the EC10 or NOEC for a standard fish species from an early life stage test or prolonged exposure duration test (lowest of mortality or growth for >21 d), divided by an AF of 10. In the case the compound had an insecticidal mode of action, a chronic EC10 or NOEC for the most sensitive invertebrate species evaluated in the acute assessment (*D. magna*, *Chironomus* spp. or *A. bahia*) was chosen. In the case the compound had an herbicidal mode of action, a 72h-EC50 (growth rate preferred over yield) for a non-green algae species (e.g. diatom *Navicula pelliculosa*) and a 7d-EC50 for *Lemna* sp. were also chosen. In the case the compound had a piscicidal mode of action, no further action was taken.

Derivation of Tier-2B RACs [a]

Acute and chronic SSDs were constructed using the acute and chronic toxicity data for standard and additional test species selected according to the criteria shown in Table 1. SSDs for biocidal fungicides (i.e., one of the Tier-1 test species was not an order of magnitude more sensitive than the two other Tier-1 test species) were constructed by pooling toxicity data for primary producers, invertebrates and fish together, while SSDs for compounds classified as herbicidal, insecticidal or piscicidal in the Tier 1 assessment were at first constructed by only using primary producers, invertebrates and vertebrates (i.e., fish and amphibians) data, respectively. Following the recommendations of EFSA PPR (2013), SSDs were constructed when there were toxicity data available for at least eight different taxa belonging to at least six different families/orders, with the exception of piscicidal compounds, for which five vertebrate toxicity values were set as a minimum

200 requirement (Figure 1). Besides these SSDs, alternative SSDs for biocidal compounds
201 were constructed by considering only toxicity data for primary producers and
202 invertebrates, as they are the taxonomic groups that are usually represented and evaluated
203 in micro-/mesocosms studies, while fish are usually not present in these test systems.

204 SSDs, their corresponding median HC5 values (i.e., Hazardous Concentration for 5% of
205 the species tested; 50% confidence) and the lower limit of the HC5 values (95%
206 confidence) were calculated with the ETX 2.2 software by using a log-normal
207 distribution, as described by Van Vlaardingen et al. (2004). Normality of the distributions
208 was tested with the Anderson-Darling test for datasets with $n \leq 20$, and with the
209 Kolmogorov-Smirnov test for datasets with $n > 20$. The HC5 values were used in the
210 evaluation if the goodness-of-fit was not rejected at the 0.05 level as suggested by EFSA
211 PPR (2013).

212 Acute Tier-2B RACs were derived using EC50 or LC50 data following the procedure
213 described in Figure 1. For compounds classified as biocidal and insecticidal, acute Tier-
214 2B RACs were derived by dividing the median acute HC5 by an AF of 6 (the most strict
215 option in EFSA PPR 2013). For piscicidal fungicides, the acute RACs were derived by
216 dividing the median acute HC5 by an AF of 9. For herbicidal fungicides, and when the
217 SSD is constructed with EC50 values for primary producers only, the RAC is derived by
218 dividing the median HC5 by an AF of 3. Note that according to EFSA PPR (2013) a
219 distinction between an acute HC5 and a chronic HC5 cannot be made for primary
220 producers since in the risk assessment EC50 values are used and the toxicity tests to derive
221 them are considered chronic. For herbicidal fungicides in our dataset, however, there was
222 not enough EC50 data for primary producers to construct a primary producer-specific
223 SSD (Table 2).

For the chronic Tier-2B assessment of biocidal fungicides, SSDs were constructed using selected NOEC or EC10 values (according to Table 1) for primary producers, invertebrates and vertebrates. For compounds classified as insecticidal or piscicidal, there was not enough chronic EC10/NOEC data for the sensitive taxonomic group to construct arthropod- or vertebrate-specific chronic SSDs (Table 2). Chronic Tier-2 RACs were calculated by dividing the median HC5 from an SSD containing chronic toxicity data by an AF of 3 (EFSA PPR, 2013).

Derivation of Tier-3 ETO-RACs [a]

Micro- and mesocosm data were obtained from the open 'grey' literature including DARs (www//dar.efsa.europa.eu/dar-web/provision), RIVM reports (www.rivm.nl/bibliotheek/index-en.html), summary reports of EU member states (e.g.www.ctgb.nl) and scientific papers in the open literature. In addition, available results from industry reports were also used. All micro-/mesocosm studies used in our study to derive a Tier-3 RAC were lentic, so that exposure dynamics may be relatively worst-case, particularly for streams.

Before the derivation of the Tier-3 ETO-RACs, each study was classified into one of the following exposure categories (following Maltby et al. 2009):

1. Short-term pulse exposure: dissipation DT50<1d.
2. Short-term exposure: single application and dissipation DT50 >1d, but < 10d.
3. Medium-term exposure: one of the following options, single application and dissipation DT50>10d, but ≤ 25d; or repeated applications and dissipation DT50>1d, but <10d.
4. Long-term exposure: one of the following options, single application and dissipation DT50>25d; or more or less constant chronic exposure.

248 The effect classes described in EFSA PPR (2013) were used to evaluate the treatment-
249 related responses observed for the most sensitive and relevant endpoint of the micro-
250 /mesocosm study. The effect classes corresponding to the ETO-RAC derivation are:
251 'effect class 1' (i.e., highest test concentration at which a NOEC could be derived for the
252 most sensitive population-level endpoint) and 'effect class 2' (i.e., lowest test
253 concentration with statistically significant, but only slight/transient, effects on an
254 individual sampling occasion for the most sensitive population-level endpoint). An ETO-
255 RAC was derived separately for each fungicide and exposure regime according to the
256 guidance provided by EFSA PPR (2013). Briefly, when only 'effect class 1' values were
257 available, the ETO-RAC was derived by dividing the highest 'effect class 1'
258 concentration by an AF of 2. When only 'effect class 2' values were available, the ETO-
259 RAC was derived by dividing the lowest 'effect class 2' concentration by an AF of 3
260 (Figure 1). When both 'effect class 1 and 2' values were available, the ETO-RAC was
261 derived by dividing the lowest 'effect class 2' value by an AF of 3. When from more than
262 one micro-/mesocosm experiments an 'effect class 1' value was available for the same
263 compound and exposure regime, the geometric mean of these values was used for the
264 ETO-RAC derivation. In the case that more than one 'effect class 2' values was available
265 for the same compound and exposure regime from different studies, the lowest value was
266 chosen for the ETO-RAC derivation. Following this approach, 19 Tier-3 ETO-RAC
267 values could be derived for the 18 different fungicides evaluated (two ETO-RAC values
268 were available for carbendazim based on different exposure categories, Table 2).

269 ***RAC comparison***

270 Acute and chronic Tier-1 RACs, as well as the lowest of the two, were compared with
271 Tier-3 ETO-RAC values, and acute and chronic Tier-2B RACs derived with all aquatic
272 taxa and with only non-vertebrate taxa were compared with Tier-3 ETO-RACs. In the

273 case the lower-tier RACs were found to be less conservative than Tier-3 ETO-RACs,
274 alternative options (different to those proposed in EFSA PPR 2013) were tested such as
275 the increase of the AF or the use of the lower limit of the confidence interval of the HC5.

276 The EFSA Aquatic Guidance Document (EFSA PPR 2013) provides the possibility to
277 use acute laboratory toxicity data for the derivation of acute Tier-2B RACs when the
278 fungicide is expected to result in single and repeated pulsed exposure regimes under field
279 conditions. For this reason we only used micro-/mesocosm experiments characterized by
280 single or pulsed exposure regimes (i.e., exposure categories 1-3) in the evaluation of acute
281 Tier-1 and acute Tier-2B RACs. For the chronic Tier-1 and chronic Tier-2B RACs,
282 comparisons were made with the Tier-3 ETO-RACs derived from micro-/mesocosm
283 experiments that considered all exposure categories (i.e., exposure categories 1-4).

284 Comparisons were illustrated on the basis of scatter plots, in which the 1:1 ratio of both
285 RAC values is indicated (e.g. Figure 2). In such way, cases falling below the 1:1 line
286 indicate that calculated Tier-1 RACs are sufficiently protective (green traffic light sign),
287 whereas cases in which the data points are above the line are insufficiently protective (red
288 traffic light sign).

289 **RESULTS AND DISCUSSION**

290 ***Comparison of Tier-1 and Tier-3 ETO-RACs***

291 The majority of the acute Tier-1 RACs showed a sufficient level of protection when
292 compared with the Tier-3 ETO-RACs (12 out of 14 cases), with the exception of a sterol
293 biosynthesis inhibitor and a respiration inhibitor, which had Tier-1 RACs 2-3 times higher
294 than the Tier-3 ETO-RACs (Figure 2A). Note, however, that the value for the respiration
295 inhibitor (the open diamond in Fig. 2A) concerns a ´smaller than´ Tier-3 ETO-RAC value
296 for the fungicide fentin-acetate (Roessink et al. 2006a, 2006b), a compound that is

297 nowadays banned for use in agriculture in the EU. Regarding the comparisons made with
298 chronic Tier 1 RACs, the majority of cases (15 out of 17) resulted in a sufficient protection
299 level, when excluding the ´smaller than´ Tier-3 ETO RAC value for fentin-acetate (Figure
300 2B). The exceptions were two respiration inhibitors (one of them azoxystrobin; Van
301 Wijngaarden et al. 2014), which had chronic Tier-1 RACs approximately 2 and 4 times
302 higher than the Tier-3 ETO RACs. When comparing the lowest of the acute and chronic
303 Tier-1 RACs, 16 out of the 17 cases (excluding fentin-acetate) resulted in a sufficient
304 protection level (Figure 2C). Again, the exception was azoxystrobin, which was evaluated
305 in a microcosm experiment with a more or less constant exposure regime (Van
306 Wijngaarden et al. 2014; exposure category 4; Figure 2C).

307 *FIGURE 2 ABOUT HERE*

308 The fact that the acute Tier-1 RAC is not protective for fentin-acetate may be related to
309 its high sorption capacity and persistence in the sediment compartment. Roessink et al.
310 (2006a, 2006b) evaluated the fate and effects of this substance in microcosms simulating
311 floodplain lakes. These authors reported water DT50 values of approximately 3 days, and
312 identified some oligochaete, rotifer and mollusk taxa as particularly sensitive to this
313 compound. They attributed their sensitivity to delayed effects from chronic exposure via
314 sediment contact or ingestion of organic matter particles with high accumulated
315 concentrations of the test substance. Since fentin-acetate has a high sediment sorption
316 capacity, this compound must also be evaluated under the low-tier effect assessment
317 proposed in EFSA PPR (2015) for epibenthic and endobenthic organisms. Following such
318 sediment toxicity assessment it is expected that that the corresponding aquatic exposure
319 threshold would have been much lower, probably below the calculated Tier-3 ETO-RAC,
320 however this was not evaluated in this study.

321 that the effect is triggered by the sediment exposure, the compound Subsequently, this
322 toxicity is likely to be covered by the lower-tier sediment toxicity evaluation described in
323 EFSA PPR (2015).

324 In the case of azoxystrobin, Van Wijngaarden et al. (2014) demonstrated that calanoid
325 copepods were highly sensitive to this compound under semi-field conditions using a 42-
326 d constant exposure regime. Maximum population-level effects (NOEC of 1 µg/L on
327 abundance) were observed about 9 days after the start of the exposure. This indicates that
328 these organisms are especially sensitive to this compound under prolonged exposure
329 conditions and that the time needed to show immobility/mortality is quite long.
330 Apparently, populations of these sensitive copepods are not sufficiently protected by the
331 Tier-1 RACs. The high sensitivity of copepods to azoxystrobin was also noted in outdoor
332 brackish water microcosms (Gustafsson et al., 2010), and in indoor freshwater
333 microcosms exposed to another respiration inhibitor: fluazinam (Van Wijngaarden et al.
334 2010). Future studies are needed to evaluate whether this high copepod sensitivity is also
335 the case for other (respiration inhibitor) fungicides. If that is the case, it may be an option
336 to select a copepod species as an additional standard test species for fungicides in the near
337 future.

338 ***Comparison of Tier-2B and Tier-3 ETO-RACs***

339 There was sufficient data to build acute SSDs for 15 fungicides. For four of them the log-
340 normality test was rejected, so HC5 values (and their lower limit; 95% confidence) could
341 only be derived for 11 compounds. However, comparisons of acute Tier-2B RACs with
342 Tier-3 ETO-RACs were only valid for 9 compounds, since for azoxystrobin and
343 kresoxim-methyl Tier-3 ETO-RACs were derived with micro-/mesocosm tests
344 characterized by a more or less constant exposure regime (exposure category 4; Table 2).

345 Acute Tier-2B RACs were generally lower than Tier-3 ETO-RACs (and hence protective)
346 for compounds with exposure category 1 (Figure 3A; all with a multi-site contact activity
347 MoA). However, this was clearly not the case for three compounds characterized by
348 exposure category 3 in micro-/mesocosm tests (Figure 3A). Therefore, alternative acute
349 Tier-2B RAC calculations (not included in EFSA PPR 2013) were derived by applying a
350 larger AF to the median acute HC5 or by using the lower limit of the confidence interval
351 of the HC5. The lowest AFs that resulted in a sufficient protection level for almost all
352 fungicides in acute Tier-2B RAC derivation were: AF of 20 applied to the median acute
353 HC5 (Figure 3C), and AF of 6 applied to the lower limit of the acute HC5 (Figure 3E).
354 Subsequently, these alternative approaches (not yet included in EFSA PPR 2013) may
355 provide sufficiently protective acute Tier-2B RACs for compounds with a moderate water
356 persistence (10 d $\leq$ DT50 $\leq$ 25 d) or for those that are less persistent but characterized by
357 repeated pulse-exposures in edge-of-field freshwater ecosystems.
358
359 The evaluation performed on the basis of acute SSDs constructed excluding vertebrates
360 (mainly fish) slightly improved the situation, with only 3 out of the 8 evaluated cases
361 being not protective (Figure 3 B, D and F). For fentin-acetate, and for two fungicides (one
362 respiration inhibitor and carbendazim) evaluated in micro-/mesocosms with an exposure
363 category 3, the acute Tier-2B RACs were not sufficiently protective (Figure 3B). The
364 application of an AF of 20 to the acute median HC5 or the AF of 6 to the lowest
365 confidence limit of the acute HC5 was also a suitable measure to prevent such situation
366 (Figure 3D and F). Fish has been demonstrated to be less sensitive than invertebrates and
367 primary producers to the majority of fungicide classes, except for some multi-site contact
368 activity compounds with an ethylene bisdithio-carbamate chemical group (Maltby et al.
369 2009). This explains why RACs based on HC5s including fish are less protective.

However, the option to only use non-vertebrate data in the SSD approach can be considered as more realistic as it focuses on the taxonomic groups and endpoints that are usually evaluated in micro-/mesocosm experiments.

The results of this study contrast with those provided by Maltby et al. (2009) who demonstrated that an AF of 3 applied to the median acute HC5, or the lowest confidence limit of the acute HC5, generally suffice for protecting against adverse ecological effects of pesticides (including fungicides) in micro-/mesocosm experiments. Note, however, that the selection criteria of toxicity data to be used in the SSD approach became stricter in the EFSA Aquatic Guidance Document in terms of short-term exposure durations of toxicity data permitted (shorter in EFSA PPR 2013), minimum number of toxicity values (8 in EFSA PPR 2013 *vs* 6 in Maltby et al. 2009), and minimum number of families/orders represented (6 in EFSA PPR 2013 *vs* no minimum number in Maltby et al. 2009). In addition, in EFSA PPR (2013) also the criteria for the conduct and interpretation of micro- and mesocosm tests were sharpened, and an AF (2 – 3) was introduced to derive a Tier-3 ETO RAC from threshold levels (effect classes 1-2) observed in these semi-field tests. Maltby et al. (2009) used the NOEC and LOEC of the most sensitive and relevant population-level endpoints to derive a $NOEC_{eco}$ (= ecosystem-level threshold) from micro-/mesocosm tests. Particularly, these LOEC values also concerned treatment-related responses that, following EFSA PPR (2013), now would be classified as 'effect class 3A', i.e. a short-term treatment-related effect followed by recovery (with an observed deviation from controls during less than 8 weeks). Although these 'effect class 3A' values might be used to derive a Tier-3 ERO-RAC (considering recovery of vulnerable populations), these 'effect class 3A' concentrations cannot be used to derive Tier-3 ETO-RACs according to EFSA PPR (2013).

An observed exceedance of the acute Tier-2B RAC relative to the Tier-3 ETO-RAC does not provide information on the ecological consequences and duration of the effects that may occur at exposure concentrations resembling the Tier-2B RAC. For the substances that were positioned above the 1:1 line in Figures 3A and 3B (except fentin-acetate), concentrations resembling the acute Tier-2B RACs resulted in relatively short-term population-level effects in the micro-/mesocosm experiments. Nevertheless, this study clearly illustrates that the SSD approach as described in EFSA PPR (2013) to derive acute Tier-2B RACs may not be protective for all populations of freshwater organisms, at least when only the ETO is considered and in case of exposure category 3.

FIGURE 3 ABOUT HERE

Considering all relevant taxa, enough chronic toxicity data was available to build SSDs for 8 fungicides, however the goodness-of-fit was rejected for two of them, so comparisons with Tier-3 ETO-RACs were only performed for 6 fungicides (Table 2). The application of an AF of 3 to the derived chronic median HC5 values resulted in a sufficient protection level when compared with the available Tier-3 ETO-RACs. The same assessment with chronic toxicity data for non-vertebrates only allowed the comparison for 4 fungicides, and indicated that the AF of 3 applied to those HC5 values was sufficiently protective as well (Figure 4).

FIGURE 4 ABOUT HERE

Our observation that the chronic Tier-2B RACs for fungicides provide sufficient protection to freshwater organisms in micro-/mesocosm studies (although based on relatively few cases), but not on the basis of acute Tier-2B may be related to several reasons. Most fungicide cases for which the acute Tier-2B was not protective in our

420 calibration exercise concerned micro-/mesocosm studies with medium-term exposure to
421 the test compound (DT50>10d but ≤ 25d). In lentic micro-/mesocosm studies without
422 water flow, this may have caused a relatively long-term, and relatively conservative,
423 exposure regime relative to predicted exposure regimens in edge-field ditches and
424 streams. However, it can be concluded that the treatment-related responses in micro-
425 /mesocosm tests for some moderately persistent fungicides are not covered by short-term
426 acute laboratory toxicity tests with a duration of 2 to 4 days, and the SSD derived from
427 them. The time-to-onset of effects on aquatic organisms may perhaps need more time for
428 slow-acting biocidal fungicides than the 2-4 days considered in acute laboratory toxicity
429 tests with additional test species (see e.g. Brock et al. 2008 for a discussion on incipient
430 toxicity). For most fungicides a wider array of taxonomic groups of aquatic organisms is
431 potentially sensitive than for insecticides (Maltby et al. 2009), and the test duration to
432 reach incipient toxicity for mortality and immobility is poorly investigated for fungicidal
433 compounds and taxonomic groups of invertebrates other than crustaceans and insects. In
434 addition, the particular light, temperature and microbial communities of micro-
435 /mesocosms can influence the formation of fungicide break-down products that are
436 usually not measured and that may have contributed to the observed toxicity (Boudina et
437 al. 2003).
438
439 ***Is the current effect assessment approach protective for aquatic fungi?***
440 In Table 3, freshwater fungi toxicity data (NOECs reported for organic matter
441 decomposition, biomass growth or sporulation/germination of conidia) available for the
442 fungicides evaluated in this study are compared with their lower and higher-tier RACs.
443 Overall, it appears that Tier-1, Tier-2B, and Tier-3 ETO-RACs for the fungicides
444 evaluated, are sufficiently protective for responses of structural and functional fungal

endpoints observed in micro-/mesocosm tests, or in laboratory tests using fungi monocultures. The only exception was azoxystrobin, for which the acute Tier-2B RAC seemed not to be protective for the growth of the oomycete *Pythium* spp. based on an agar plate test. In contrast, the chronic Tier-2B RAC for azoxystrobin (0.2 µg/L) was lower than the laboratory 2d-NOEC for *Pythium* (2 µg/L). Similar findings were reported by Zubrod et al. (2015), who compared Tier-1 RACs for five fungicides with NOEC values for fungal biomass, community composition, species abundance, spore production and leaf decomposition using microcosms incubated with inoculated leaf material. In their study, they found that the Tier-1 RACs for the fungicides studied were generally protective for fungal endpoints, except for tebuconazole for which a chronic Tier-1 RAC was calculated of 1 µg/L (based on toxicity data for *D. magna*). According to their results, exposure to this Tier-1 RAC concentration would result in an increasing microbial leaf decomposition as compared to the control. However, this effect was not observed at higher test concentrations (and neither a decreasing leaf decomposition rate). Zubrod et al. (2015) argued that higher-tier RACs, which are used to assess risks of approximately 20% of fungicides in the European Union, may exceed Tier-1 RACs by a factor of 10. They also speculate that these higher-tier RACs may not be sufficiently protective for fungi.

TABLE 3 ABOUT HERE

In our study, comparisons of higher-tier RACs and fungi functional or structural NOECs were possible for 9 fungicides (representing 5 microbial modes of action). It appeared that for these compounds, even at exposures resembling Tier-3 ETO-RAC concentrations, toxic effects on aquatic fungal structural or functional endpoints will be small (Table 3). This, however, may not be the case for other fungicides for which Tier-3 ETO-RACs

based on the EFSA PPR (2013) methodology could not be derived, such as for tebuconazole and other triazole fungicides.

In an outdoor microcosm study, Dimitrov et al. (2014) did not demonstrate effects on fungal biomass or sediment community structure in systems treated with tebuconazole at the acute HC5 level (238 μg/L, as derived by Maltby et al. 2009 using non-fungi toxicity data). However, this concentration reduced conidia production and altered fungal community composition associated with leaf material, which resulted in a decreased feeding rate of *Gammarus pulex* on exposed leaf material (Dimitrov et al. 2014). This observation for the fungicide tebuconazole is in line with the results of the laboratory study by Zubrod et al. (2015) discussed above. Other laboratory studies have also indicated that triazole fungicides may alter food processing, reduce energy reserves and affect survival of leaf-shredding macroinvertebrates at relatively low concentrations (Bundschuh et al. 2011; Rasmussen et al. 2012; Zubrod et al. 2014, 2015; Feckler et al. 2016). For instance, Bundschuh et al. (2011) noted a preference of *Gammarus fossarum* for control leaf disks as compared to those treated with a concentration as low as 50 μg/L of tebuconazole. Similar findings on decomposer food-chain related endpoints have been reported for some other classes of fungicides, but at relatively high exposure concentrations. Zubrod et al. (2014) assessed the impact of azoxystrobin, carbendazim, cyprodinil, quinoxyfen, and Cu on the feeding rate of *G. fossarum*, and found EC20 values about one order of magnitude higher than the calculated RACs that we have derived for these compounds. The information presented above suggests that the EFSA PPR (2013) approach may be sufficiently protective for decomposer food-chain-related endpoints for most fungicides investigated, except perhaps for triazoles (such as tebuconazole).

CONCLUDING REMARKS AND RECOMMENDATIONS

Several studies that compared the Tier-1 and Tier-2 RACs with Tier-3 ETO-RACs for insecticides (Van Wijngaarden et al. 2015; Brock et al. 2016) and the Tier-1 RACs for herbicides (Van Wijngaarden and Arts 2018) indicate that the EFSA PPR (2013) approach, with few exceptions, offers the required protection level for exposure to individual active ingredients under semi-field conditions, at least when the exposure time tested in the micro-/mesocosms is sufficiently realistic. Cases in which the EFSA PPR (2013) approach has shown a low protection level are mainly related to (i) compounds that have a high sediment sorption capacity and persistence and thus the acute assessment is not sufficiently protective for sediment dwelling or feeding organisms (e.g. pyrethroid insecticides in Brock et al. 2016); (ii) compounds that have shown latency of effects (e.g. benzoylurea insecticides and other insect growth regulators in Van Wijngaarden et al., 2015); and (iii) compounds that have been found to be particularly toxic to non-standard test species (e.g. neonicotinoid insecticides to ephemeropterans in Van Wijngaarden et al., 2015).

In our study we have identified fungicides with high hydrophobicity that require a sediment assessment to complement the acute aquatic one (i.e., fentin-acetate), and compounds that are particularly toxic to copepods (i.e., respiration inhibitor fungicides) which may require further considerations in the acute Tier-1 assessment, e.g. selecting a copepod as an additional standard test species for fungicides. In addition, we have demonstrated that acute Tier-2B RACs are not protective for some compounds that have moderate persistence under semi-field conditions (DT50>10d and <25d), demanding a larger AF to extrapolate the acute HC5, stricter guidance with respect to the toxicity data to include in the SSD (e.g. considering incipient toxicity), or an evaluation with chronic SSDs. The chronic Tier-2B RAC for all compounds evaluated shows a sufficient protection level, but the observation is based on a very limited number of cases.

519 Overall this study shows that the number of adequate fungicide micro-/mesocosm studies
520 available for the lower-tier RAC calibration is relatively low as compared to insecticides,
521 partly because the risk may in some cases be triggered by fish in the lower tiers so that a
522 micro-/mesocosm study with a focus on treatment-related responses of primary producers
523 and invertebrates becomes less relevant. Further research is needed to evaluate the EFSA
524 PPR (2013) approach with a larger number of micro-/mesocosm studies conducted with
525 a wider array of fungicidal compounds differing in toxic mode-of-action, including
526 studies performed under lotic conditions. Attention must be given to sterol biosynthesis
527 compounds, which dominate the EU market together with the multi-site contact
528 compounds (EUROSTAT 2017). In particular, further information is required on the
529 relationship between exposure regime of individual fungicides in edge-of-field surface
530 waters and the protectiveness of the Tier-2B RAC. In addition, it must be taken into
531 account that the evaluation of the tiered approach conducted in this study on the basis of
532 micro-/mesocosms disregards potential direct and indirect effects on fish populations.
533 Therefore, further studies are needed to evaluate the risk of fungicides to fish, particularly
534 for those fungicide classes that are clearly more toxic to them (multi-site contact activity
535 compounds with an ethylene bisdithio-carbamate chemical group, Maltby et al. 2009).
536 This might be done by using validated toxicokinetic-toxicodynamic models (see e.g.
537 EFSA PPR, 2018) and population models. Finally, amphibians should also be further
538 considered as studies have demonstrated effects of strobilurins (respiration inhibitors) on
539 *Bufo cognatus* tadpoles and juveniles at environmentally relevant concentrations (Belden
540 et al. 2010).

541 To the best of our knowledge, very few experimental studies have been performed to
542 assess population- and community-level effects of mixtures of fungicides and other
543 pesticides in general, particularly those that are applied in tank mixtures or that are applied

544 jointly in the same crop and season. Wang et al. (2018) demonstrated synergistic effects
545 of cyprodinil and kresoxim-methyl on zebrafish embryos. Nørgaard and Cedergreen
546 (2010) concluded that some sterol biosynthesis compounds (imidazoles and some
547 triazoles) can enhance the effects of pyrethroid insecticides to *D. magna* when sprayed
548 together in tank mixtures. In a field study, in which 15 fungicides and 4 insecticides were
549 monitored in streams of a German vineyard area, it appeared that the structure of
550 microbial and shredder communities as well as fungal biomass changed along the
551 fungicide toxicity gradient (Fernandez et al. 2015). Therefore, the evaluation of
552 cumulative pesticide-stress under field conditions including fungicides should be further
553 investigated (see e.g. Arts et al. 2006; Focks et al. 2014; Fernandez et al. 2015).

554 So far, fungi or other microorganisms are not included as standard test organisms in the
555 prospective aquatic effect assessment for fungicides. With some exceptions (i.e.,
556 tebuconazole), this study shows that lower- and higher-tier RACs derived according to
557 EFSA PPR (2013) provide a sufficient protection level for most fungal structural and
558 functional endpoints. However, the number of toxicity studies performed with fungicides
559 and aquatic fungi is still very limited, and several modes of action have not yet been
560 properly evaluated (Ittner et al. 2018). Research also indicates that subtle fungal
561 community changes, including alterations in sporulation and germination efficiencies,
562 may alter the palatability of leaf material for macroinvertebrate shredders at
563 concentrations close to regulatory thresholds and at exposure levels monitored in the
564 environment (Zubrod et al. 2015), so further research on the impact of realistic exposure
565 regimes of (mixtures of) fungicidal compounds on the decomposer food-chain is strongly
566 recommended.

567

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TABLES

Table 1. Criteria used for the selection of the toxicity data included in the analysis.

	Vertebrates		Invertebrates		Primary producers
	Acute	**Chronic**	**Acute**	**Chronic**	**Chronic**
Endpoint	LC50	EC10 or NOEC	EC50	EC10 or NOEC	EC50 / EC10 or NOEC
Measured effect	Mortality	Growth rate, development, behaviour, mortality, immobilization	Mortality, immobilization	Growth rate, feeding rate, reproduction, mortality, immobilization	Growth rate (preferred), yield
Test duration (d)	2-4	>21	2-4	>7-21[a] micro/meso-fauna ≥28d macro-invertebrates	3-5 (algae), 7-28 (macrophytes)

[a] It was checked whether for short living organisms such as Rotifera and Nematoda there were EC10 or NOEC values that could be considered chronic (exposure duration higher than 4 days), but there were none.

Table 2. Selected fungicides for which micro-/mesocosm data was available, with their MoA and classification (IN: insecticidal; BIO: biocidal; PIS: piscicidal; HERB: herbicidal). The 'x' indicates the availability of data to calculate a RAC in the different tiers; '-' indicates that not enough data was available to calculate a RAC; '*' indicates that enough data was available but the log-normality test of the SSD was rejected at the 0.05 level, so a Tier-2B RAC value could not be calculated. The Tier-3 RAC exposure categories refer to those described in the section *Derivation of Tier-3 RACs.*

MoA, compound	Type	Tier 1 Acute RAC	Tier 1 Chronic RAC	Tier 2B Acute RAC		Tier 2B Chronic RAC		Tier 3 RAC	Open literature references for micro-/mesocosm data (not reported reference means that the data is confidential)
				All taxa	Non-vertebrates	All taxa	Non-vertebrates	Exposure category	
Amino acids and protein synthesis									
Cyprodinil	IN	x[a]	x[a]	x	-	-	-	3	
Cytoskeleton and motor proteins									
Carbendazim	BIO	x[b]	x[c]	x	x	*	-	3,4	Cuppen et al. 2000; Van den Brink et al. 2000; Slijkerman et al. 2004
Multi-site contact activity									
Chlorothalonil	BIO	x[c]	x[b]	x	x	x	x	1	
Mancozeb	BIO	x[d]	x[e]	*	x	-	-	3	
Metiram	BIO	x[c]	x[b]	x	x	x	x	1	Lin et al. 2012
Tolylfluanid	PIS	x[c]	x[c]	x	-	-	-	1	EC 2003
Respiration									
Azoxystrobin	BIO	x[b]	x[f]	x	x	x	*	4	Van Wijngaarden et al. 2014
Fentin acetate	BIO	x[c]	x[c]	x	x	-	-	2	Roessink et al. 2006 a,b
Fluazinam	BIO	x[c]	x[e]	*	*	-	-	3	Van Wijngaarden et al. 2010
Kresoxim-methyl	BIO	x[b]	x[c]	x	x	x	-	4	
Picoxystrobin	BIO	x[b]	x[b]	x	x	-	-	3	
Trifloxystrobin	BIO	x[b]	x[b]	x	x	-	-	3	
Signal transduction									
Fludioxonil	HERB	x[g]	x[c]	-	-	-	-	4	Yin et al. 2018
Sterol biosynthesis in membranes									
Fenpropidin	HERB	x[g]	x[g]	-	-	-	-	3	
Prochloraz	BIO	x[c]	x[b]	x	-	-	-	3	
Spiroxamine	HERB	x[h]	x[h]	-	-	-	-	3	EC 2009
Unclear									
Copper	BIO	x[b]	x[c]	*	*	x	x	4	EC 2007
Pentachlorophenol	BIO	x[c]	x[c]	*	x	x	x	3	Willis et al. 2004

Tier 1 RAC based on toxicity data for: [a] *Americamysis bahia* (crustacean); [b] *Daphnia magna* (crustacean); [c] *Oncorhynchus mykiss* (fish); [d] *Raphidocelis subcapitata* (green algae); [e] *Pimephales promelas* (fish); [f] *Danio rerio* (fish); [g] *Desmodesmus subspicatus* (green algae); [h] *Skeletonema costatum* (diatom).

767 **Table 3.** Literature data on the effects of fungicides on freshwater fungi and calculated RACs[1].

Fungicide name and RACs (µg/L)	Test	Type	Endpoint	Taxonomic division	Taxon	NOEC (µg/L)	Reference
Azoxystrobin	Litter	F	OM	AS, BA, OO	Community	≥60	Gustafsson et al. 2010
Tier 1 acute RAC: 1.6	Leaf	F	OM	AS, BA, OO	Community	20	Zubrod et al. 2015
Tier 1 chronic RAC: 2.0	Leaf	S	BIOM	AS, BA, OO	Community	100	Zubrod et al. 2015
Tier 2B acute RAC: 5.8 - 11.5	Pure	S	BIOM	AS	*Trichoderma hamatum*	460	Dijksterhuis et al. 2011
Tier 2B chronic RAC: 0.2	Pure	S	BIOM	AS	*Fusarium sporotrichioides*	29	Dijksterhuis et al. 2011
Tier 3 ETO RAC: 0.5 (Exp. Cat. 4)	Pure	S	BIOM	AS	*Helicoon richonis*	>5000	Dijksterhuis et al. 2011
	Pure	S	BIOM	AS	*Helicodendron tubulosum*	>5000	Dijksterhuis et al. 2011
	Pure	S	BIOM	BA	*Cryptococcus flavescens*	460	Dijksterhuis et al. 2011
	Pure	S	BIOM	OO	*Pythium* spp	2	Dijksterhuis et al. 2011
	Pure	S	BIOM	ZY	*Mucor hiemalis*	230	Dijksterhuis et al. 2011
Carbendazim	Litter	F	OM	AS, BA	Community	100	Cuppen et al. 2000
Tier 1 acute RAC: 1.3	Leaf	F	OM	AS, BA	Community	35	Zubrod et al. 2015
Tier 1 chronic RAC: 0.6	Leaf	S	BIOM	AS, BA	Community	≥1715	Zubrod et al. 2015
Tier 2B acute RAC: 4.6 – 9.2	Leaf	S	GER	AS, BA	Community	1000	Chandrashekar and Kaveriappa 1994
Tier 2B chronic RAC: -	Pure	S	BIOM	AS	*Trichoderma hamatum*	260	Dijksterhuis et al. 2011
Tier 3 ETO RAC: 1.7 (Exp. Cat. 3) –	Pure	S	BIOM	AS	*Fusarium sporotrichioides*	1000	Dijksterhuis et al. 2011
1.3 (Exp. Cat. 4)	Pure	S	BIOM	BA	*Cryptococcus flavescens*	8200	Dijksterhuis et al. 2011
	Pure	S	BIOM	OO	*Pythium spp*	≥5000	Dijksterhuis et al. 2011
	Pure	S	BIOM	ZY	*Mucor hiemalis*	≥8200	Dijksterhuis et al. 2011
Chlorothalonil	Pure	S	BIOM	AS	*Trichoderma hamatum*	≥260	Dijksterhuis et al. 2011
Tier 1 acute RAC: 0.3	Pure	S	BIOM	AS	*Fusarium sporotrichioides*	≥260	Dijksterhuis et al. 2011
Tier 1 chronic RAC: 0.06	Pure	S	BIOM	BA	*Cryptococcus flavescens*	≥260	Dijksterhuis et al. 2011
Tier 2B acute RAC: 1.7 – 3.3	Pure	S	BIOM	OO	*Pythium* spp	≥200	Dijksterhuis et al. 2011
Tier 2B chronic RAC: 0.3	Pure	S	BIOM	ZY	*Mucor hiemalis*	≥260	Dijksterhuis et al. 2011
Tier 3 ETO RAC: Confidential							
Copper	Leaf	S	BIOM	AS, OO	Community structure	<1271	Duarte et al. 2008
Tier 1 acute RAC: 1.4	Leaf	F	OM	AS, OO	Community	1271	Duarte et al. 2008
Tier 1 chronic RAC: 2.6	Leaf	S	GER	AS, OO	Community	1271	Duarte et al. 2008
Tier 2B acute RAC: -	Pure	S	BIOM	OO	*Halophytophthora vesicula*	1000	Leaño and Pang 2010
Tier 2B chronic RAC: 0.9	Pure	S	GER	OO	*Halophytophthora vesicula*	<1000	Leaño and Pang 2010
Tier 3 ETO RAC: 3.9 (Exp. Cat. 4)	Pure	S	BIOM	OO	*Halophytophthora elongata*	10000	Leaño and Pang 2010
	Pure	S	GER	OO	*Halophytophthora elongata*	<1000	Leaño and Pang 2010
	Pure	S	BIOM	OO	*Halophytophthora spinosa var. lobata*	1000	Leaño and Pang 2010

	Pure	S	GER	OO	*Halophytophthora spinosa var. lobata*	1000	Leaño and Pang 2010
	Pure	S	BIOM	OO	*Halophytophthora sp.*	10000	Leaño and Pang 2010
	Pure	S	GER	OO	*Halophytophthora sp.*	>100000	Leaño and Pang 2010
	Pure	S	BIOM	AS	*Heliscus submersus*	156000	Azevedo and Cássio 2010
	Pure	S	BIOM	AS	*Tricladium chaetocladium*	121333	Azevedo and Cássio 2010
	Pure	S	BIOM	AS	*Varicosporium elodeae*	19063	Azevedo and Cássio 2010
	Pure	S	BIOM	AS	*Ypsilina graminea*	34667	Azevedo and Cássio 2010
Cyprodinil Tier 1 acute RAC: 0.08 Tier 1 chronic RAC: 0.2 Tier 2B acute RAC: 3.0 – 5.9 Tier 2B chronic RAC: - Tier 3 ETO RAC: Confidential	Leaf	F	OM	AS	Community	40	Zubrod et al. 2015
	Leaf	S	BIOM	AS	Community	8	Zubrod et al. 2015
Fluazinam Tier 1 acute RAC: 0.6 Tier 1 chronic RAC: 0.3 Tier 2B acute RAC: - Tier 2B chronic RAC: - Tier 3 ETO RAC: 0.8 (Exp. Cat. 3)	Litter	F	BIOM	AS, BA, OO	Community	50	Van Wijngaarden et al. 2010
	Pure	S	BIOM	AS	*Trichoderma hamatum*	60	Dijksterhuis et al. 2011
	Pure	S	BIOM	AS	*Fusarium sporotrichioides*	60	Dijksterhuis et al. 2011
	Pure	S	BIOM	BA	*Cryptococcus flavescens*	60	Dijksterhuis et al. 2011
	Pure	S	BIOM	OO	*Pythium* spp	100	Dijksterhuis et al. 2011
	Pure	S	BIOM	ZY	*Mucor hiemalis*	60	Dijksterhuis et al. 2011
Mancozeb Tier 1 acute RAC: 4.4 Tier 1 chronic RAC: 0.2 Tier 2B acute RAC: - Tier 2B chronic RAC: - Tier 3 ETO RAC: Confidential	Leaf	S	GER	AS, OO	Community	1000	Chandrashekar and Kaveriappa 1994
Metiram Tier 1 acute RAC: 2.8 Tier 1 chronic RAC: 0.4 Tier 2B acute RAC: 8.1 – 16.2 Tier 2B chronic RAC: 2.3 Tier 3 ETO RAC: 12 (Exp. Cat 1)	Litter	S	BIOM	AS	*Anguillospora longissima, Tetracladium setigerum*	≥324	Lin et al. 2012
Tolylfluanid Tier 1 acute RAC: 0.3 Tier 1 chronic RAC: 1.0 Tier 2B acute RAC: 1.5 Tier 2B chronic RAC: - Tier 3 ETO RAC: 5	Litter	F	OM	AS, OO	Community	≥214	EC 2003

[1] Tier-2B RACs are calculated with the HC5 derived from an SSD with all aquatic taxa divided by an AF of 6 and 3 in the acute assessment, and an AF of 3 in the chronic assessment, except for tolyfluanid (piscicidal) for which an AF of 9 was used in the acute assessment. Tier-3 ETO-RACs are obtained for the exposure categories indicated in Table 2. Values from confidential reports are not provided.
Litter: litter bags in microcosms; Leaf: laboratory microcosms with a focus on leaf-decomposition and associated microbes; Pure: pure culture, usually in agar plates; S: structural; F: functional; OM: Organic matter decomposition; BIOM: biomass (growth); GER: sporulation and germination of conidia; AS: ascomycetes; BA: basidiomycetes; OO: oomycetes; ZY: zygomicota.

FIGURES

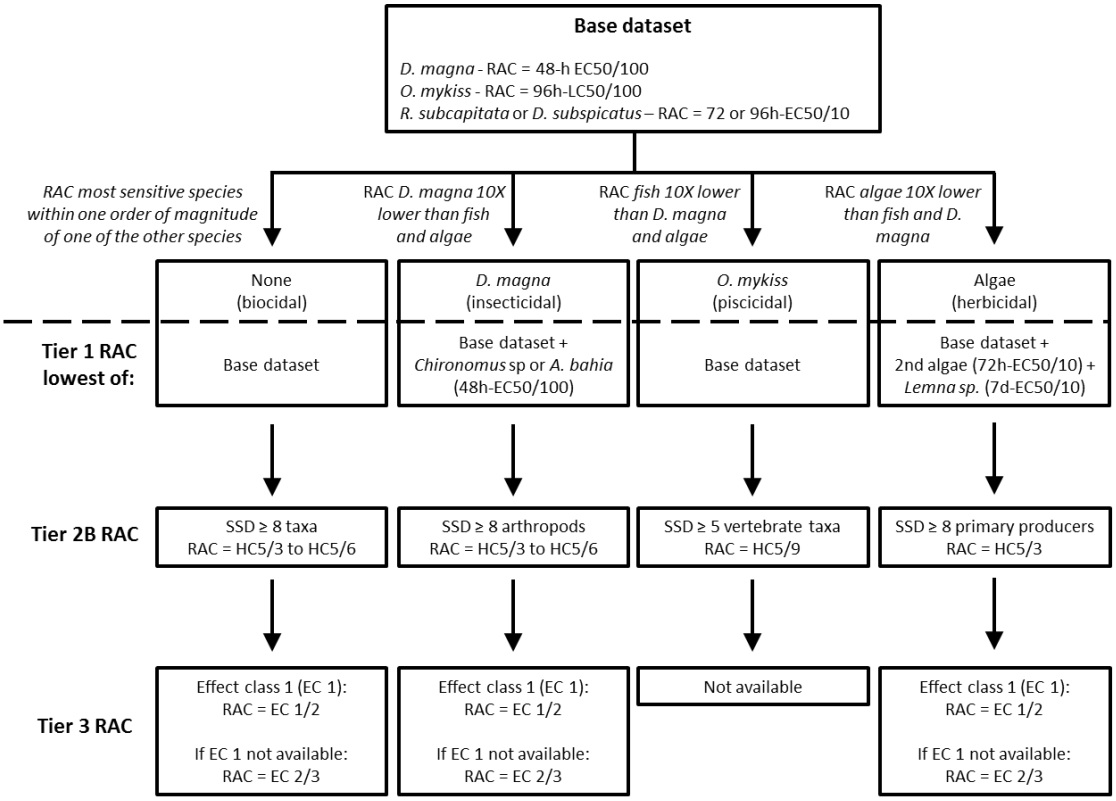


Figure 1. Decision scheme used to calculate acute Tier-1, Tier-2B and Tier-3 RACs according to EFSA PPR (2013). Note that for fungicides with biocidal mode of action, the acute Tier-2B RAC was only derived when a minimum of 8 different taxa belonging to at least 6 different families/orders were available. For fungicides with insecticidal mode of action, the SSD was at first instance based on at least 8 arthropods, but when toxicity data for non-arthropod taxa were available and were found to be sensitive (below the largest toxicity value for arthropods) they were also included in the SSD. When the latter was the case the SSD had to meet the requirement of at least 8 different taxa belonging to at least 6 different families/orders.

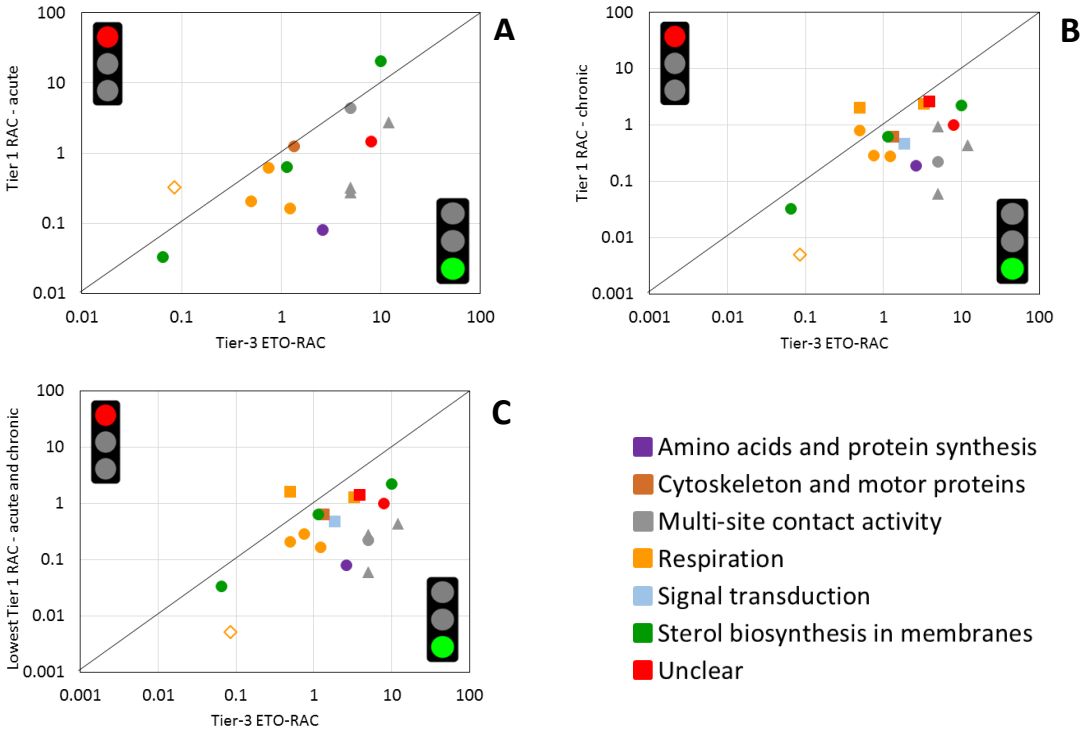


Figure 2. Comparison of Tier-3 ETO-RACs with acute Tier-1 RACs (panel A), chronic Tier-1 RACs (panel B) and the lowest of the acute or chronic Tier-1 RACs (panel C). The symbol type indicate the micro-/mesocosm exposure category: 1. Short-term pulse exposure (triangles); 2. Short-term exposure (diamonds); 3. Medium-term exposure (circles); 4. Long-term exposure (squares). Exposure category 4 is not used for the comparison with acute RACs. The symbol color relates to the mode of action of the compound. The Tier-3 ETO-RAC value of fentin-acetate for which a "lower than" value (< 0.086 µg/L) is available is shown as an empty diamond.

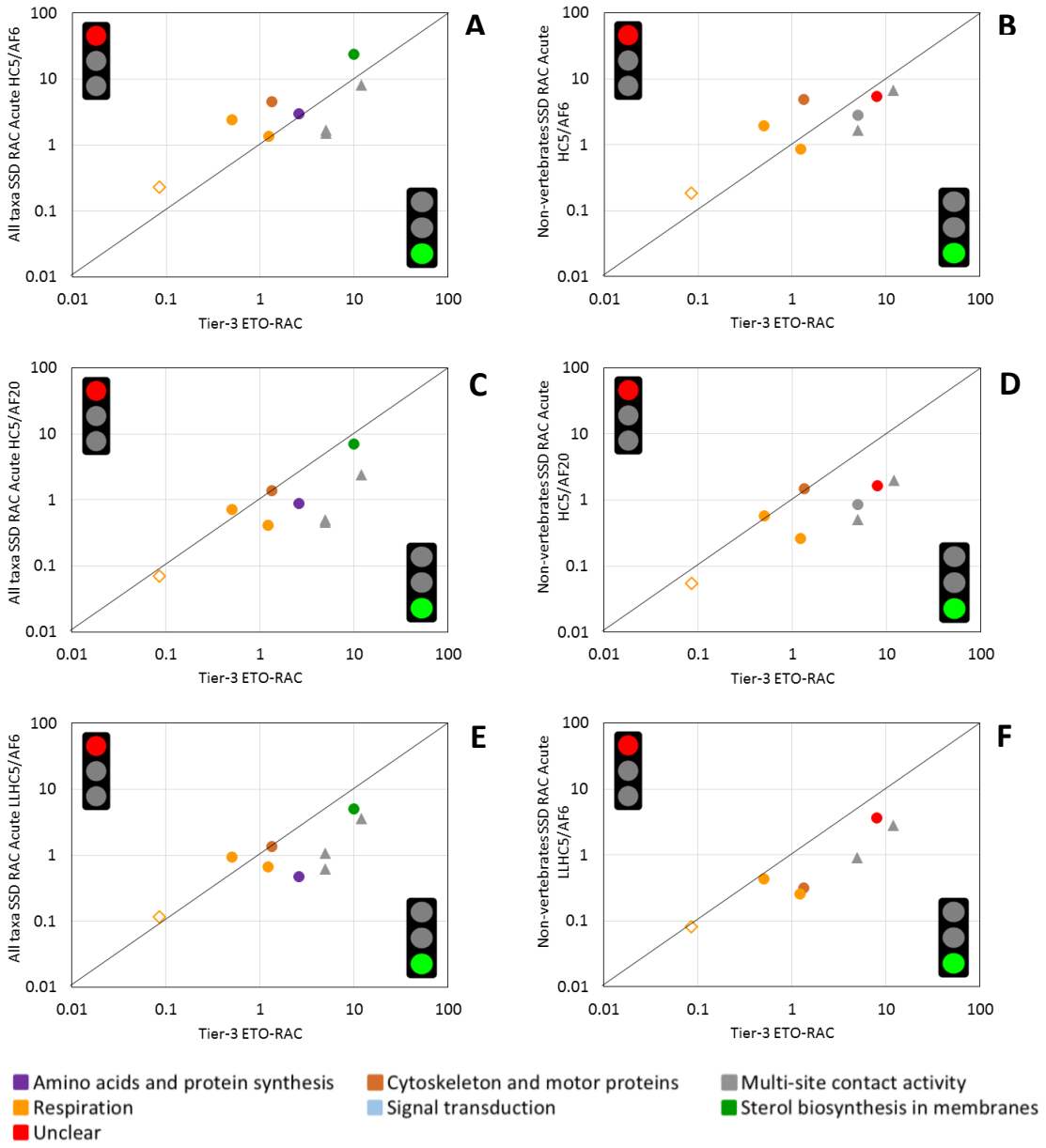
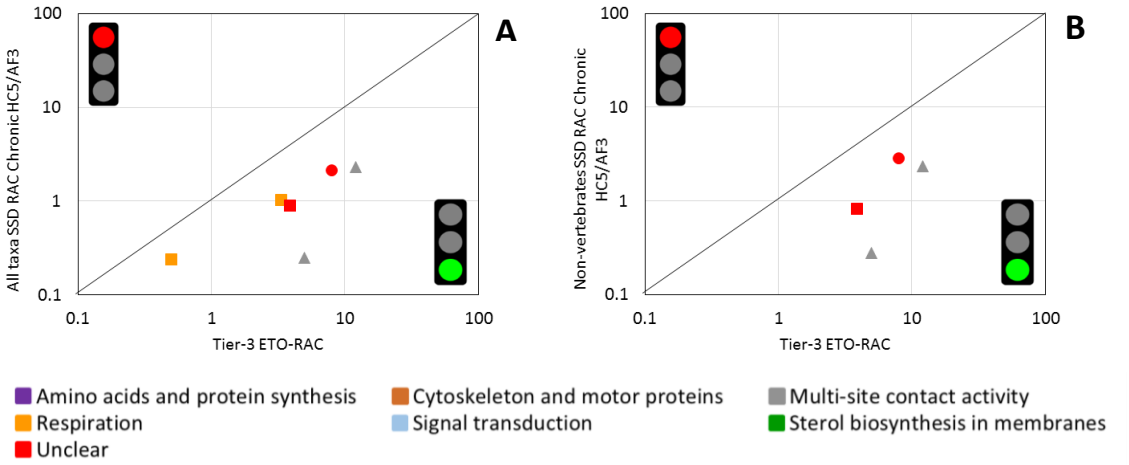


Figure 3. Comparison of Tier-3 ETO-RACs with acute Tier-2B RACs calculated by dividing the HC5 by an AF of 6 and 20, and by taking the lowest limit of the 95% confidence interval of the HC5 divided by an AF of 6. Comparisons are done with SSDs built with all taxa (panels A, C, E) and with non-vertebrate taxa (panels B, D, F). The symbol type indicate the micro-/mesocosm exposure category: 1. Short-term pulse exposure (triangles); 2. Short-term exposure (diamonds); 3. Medium-term exposure (circles). Colors represent different microbial modes of action. Note that the RAC comparisons for fentin-acetate (open diamond) are indicative as the Tier-3 ETO-RACs is only provided as a 'lower than' value (< 0.086 µg/L).



843 **Figure 4.** Comparison of Tier-3 ETO-RACs with chronic Tier-2B RACs calculated by
844 dividing the chronic HC5 by an AF of 3 with all taxa (A) and with non-vertebrate taxa
845 (B). The symbol type indicate the micro-/mesocosm exposure category: 1. Short-term
846 pulse exposure (triangles); 2. Short-term exposure (diamonds); 3. Medium-term exposure
847 (circles); 4. Long-term exposure (squares). Colors represent different microbial modes of
848 action.