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3 **IMPACT OF THE WASTEWATER-MIXING ZONE ON ATTENUATION OF**
4 **PHARMACEUTICALS IN NATURAL WATERS: IMPLICATIONS FOR AN**
5 **IMPACT ZONE INCLUSIVE ENVIRONMENTAL RISK ASSESSMENT**

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12

13 **Abstract**

14 The direct discharge of untreated wastewater has been identified as an important source of
15 environmental contamination by active pharmaceutical ingredients and other 'down-the-
16 drain' chemicals in developing countries. It necessitates the development of an
17 environmental risk assessment approach for the resulting impact zone. This study was
18 designed to investigate the impact of low level of dilution (<10) on the natural attenuation
19 processes of distribution and degradation within the impact zone. Dilution of the untreated
20 wastewater resulted in increased desorption and corresponding environmental
21 concentrations. The presence/absence of the microbial population in the batches affected
22 the degree of sorption depending on the compound charge (i.e. positive or negative),
23 highlighting an experimental technical bias. The degradation half-lives of acebutolol and
24 diclofenac increased with increasing dilution and resulted in higher environmental
25 persistence. The modelling of the biochemical oxygen demand (BOD) allowed an estimate of
26 the temporal end boundary of the impact zone to be predicted as 24 h. Therefore, it was
27 concluded that most of the investigated compounds would persist beyond the end of the
28 impact zone as defined by the return to environmental BOD concentrations. It is proposed
29 that, within environmental risk assessment protocols, the impact zone should be considered
30 as a semi-natural wastewater treatment area in such a way to allow the estimate of
31 environmental concentrations of pharmaceuticals beyond its end.

32 **Keywords**

33 Pharmaceuticals; wastewater; biodegradation; dilution; risk; assessment

34 1. Introduction

35 Wastewater management is an acute challenge in low and low-middle income countries
36 (LLMICs) where increased volumes of wastewater generated by rapid urbanisation have not
37 been matched by upgrades of sewerage infrastructures resulting in direct discharge of
38 untreated wastewater (DDUW) to surface waters (Malik et al., 2015; Nansubuga et al., 2016).
39 This results in serious pollution of a waterway to such a downstream distance that a
40 combination of dilution and biogeochemical processes such as degradation, volatilisation and
41 sorption render the anthropogenic discharges non-toxic. This area downstream of the DDUW
42 has been defined as an “impact zone”, beginning at the DDUW entry point and ending where
43 easily measurable determinants such as biochemical oxygen demand (BOD), ammonia, and
44 dissolved oxygen levels, critical to ecological health, have returned to environmental
45 background concentrations (A.I.S.E./CESIO, 1995; Bagnis et al., 2018a). Whilst DDUW are
46 known to contain high nutrient concentrations and BOD, the presence of emerging
47 contaminants such as active pharmaceutical ingredients (APIs) is of increasing concern
48 (Keller et al., 2014). This applies particularly to LLMICs where from a pharmaceutical point of
49 view, LLMICs are also experiencing an increase in consumption of medicines, as well as
50 relocation of pharmaceutical production plants to these regions (Kookana et al., 2014).

51 The impact zone of DDUW has been linked to higher environmental concentrations of APIs
52 than in high income countries with more advanced wastewater treatment plants (Kookana et
53 al., 2014; Malik et al., 2015) and API concentrations up to mg L^{-1} have been detected in
54 impact zones of LLMICs from Africa to Asia (Ashfaq et al., 2017; Madikizela et al., 2017;
55 Ngumba et al., 2016; Shimizu et al., 2013).

56 To regulate any discharge effectively, it is necessary to be able to define impact zones in
57 order to minimise their extent and plan future wastewater treatment requirements.
58 Furthermore it is necessary to fully understand the fate of chemicals within an impact zone as
59 they are unlikely to behave the same as BOD or ammonia, often used to define such zones.
60 Furthermore, current environmental risk assessment (ERA) guidance for APIs has been
61 developed assuming that sewage is subject to treatment prior to discharge (EMA, 2006), and
62 thus, given that the DDUW does not comply with this assumption, the environmental
63 conditions in the impact zone require a dedicated ERA approach (Bagnis et al., 2018a). This
64 can only be achieved by understanding the mechanisms of natural attenuation that APIs
65 undergo, such as sorption and biodegradation, and the resulting degree of exposure within
66 the impact zone boundaries and beyond.

67 Additionally, an important mechanism related to the natural attenuation of chemicals in
68 wastewater is dilution (Keller et al., 2014). Many environmental risk assessment and
69 management strategies are overly reliant on the paradigm that dilution reduces a chemical's
70 concentration such that it positively contributes to the reduction of environmental risk in a
71 directly proportional way. In the current ERA protocol, the default ratio of river flow to
72 treated wastewater is set to 10 (EMA, 2006; European Commission Joint Research Centre,
73 2003). However, despite the conservative nature of the ERA protocol, this dilution factor is
74 overestimated in at least 53 countries, many of which are LLMICs severely affected by DDUW
75 (Keller et al., 2014). Such an assumption may therefore be resulting in under estimates of
76 downstream concentrations of chemicals and given the fact that chemicals are not actually
77 transformed by dilution, this premise is often criticized (Van Breukelen, 2007).

78 A highly important factor often overlooked are the questions as to what the impacts of
79 dilution are on other key processes, such as sorption and biodegradation, which are currently
80 poorly understood but are critical to the derivation of a scientifically robust risk assessment
81 or for use within the development of water quality models (Bagnis et al., 2018a; Hajj-
82 Mohamad et al., 2017).

83 The practicalities of measuring APIs in impact zones of LLMICs with substantial inputs of
84 untreated wastewater are highly challenging. The number of environmental variables are
85 high and sample collection, preservation and analysis is often not possible locally. Therefore,
86 it is necessary to develop and adopt laboratory simulations which provide the opportunity to
87 control important variables such as wastewater quality and characteristics, dilution and API
88 concentration and thus provides the ability to generate vitally important fate data upon
89 which to base future risk assessment approaches.

90 This study focuses on the fate and behaviour of APIs within a simulated impact zone through
91 the recreation of conditions of dissolved organic carbon – DOC (considered a surrogate of
92 BOD), suspended solids and microbiological inoculums on a laboratory scale. Additionally, it
93 investigates the effects of dilution on the main natural attenuation processes controlling the
94 concentration of APIs in the impact zone and beyond, i.e. distribution and biodegradation.
95 Given that there is the potential for some APIs to persist beyond the impact zone defined by
96 parameters such as BOD, for example, owing to either high concentrations discharged or
97 being more recalcitrant than the observed BOD, it is essential to be able to quantify the
98 extent as part of any risk assessment procedure. The overarching aim is to contribute to the
99 development of an approach of environmental risk assessment for APIs within and beyond

100 the impact zone generated by DDUW. Further contributing to the novelty of this research is
101 the development of a simple model based on simulated BOD concentrations (derived from
102 experimentally derived DOC values) to predict API concentrations outside of the BOD derived
103 impact zone, leading to a draft protocol for the assessment of the environmental risk posed
104 by APIs in LLMICs with limited or poor wastewater treatment.

105 **2. Materials and methods**

106 All glassware and plasticware were cleaned prior to use (2 % v/v Decon, \geq 24 h; 10 % v/v HCl,
107 \geq 24 h) and rinsed with ultra-high purity water (UHP) with resistivity above 18.2 M Ω cm
108 (Merck Millipore).

109 2.1. Active pharmaceutical ingredients

110 The APIs chosen for this study were the neutral acetaminophen (ACT), carbamazepine (CBZ),
111 and nevirapine (NVR), the acidic diclofenac (DCF) and valsartan (VLS), and the basic
112 acebutolol (ACE) and amitriptyline (AMI). These compounds represent APIs with different
113 structure and functionalities (neutral, acidic and basic) which are commonly consumed in
114 LLMICs (Bagnis et al., 2018a). The chemicals were purchased at the highest purity available,
115 either from Sigma-Aldrich (acebutolol hydrochloride, amitriptyline hydrochloride, nevirapine,
116 valsartan, and acetaminophen) or Fisher Scientific (carbamazepine and diclofenac sodium
117 salt).

118 2.2. Synthetic wastewater

119 Wastewater composition varies considerably according to both time and location
120 (Tchobanoglous et al., 2003), presenting challenges in its standardisation for experiments
121 (Boeije et al., 1999). Therefore, a surrogate of synthetic wastewater is needed to achieve
122 experimental reproducibility as well as to ensure there are no background API concentrations
123 present. Also, in order to perform an ERA for chemicals, some form of standardization is
124 needed to facilitate inter- and intra-lab replication. Therefore, a synthetic medium is a logical
125 starting point for such complex studies (Bagnis et al., 2018a). The experimental design was
126 informed by OECD standards tests for chemicals in the environment, which are also
127 standardized in some respect, and was optimised by a through critical evaluation of synthetic
128 wastewaters reported in the literature (Bagnis et al., 2018a; O’Flaherty et al., 2013). Thus, a
129 synthetic wastewater (SW) was prepared to achieve controlled and reproducible
130 experimental conditions (Boeije et al., 1999). In this study, sodium azide (NaN_3) was added to
131 sterilized batches, whilst inoculum (5 mL; consisting of fresh wastewater influent collected at
132 the local wastewater treatment plant of South West Water, Plymouth, England, UK) was
133 added to all other batches (OECD, 1992). The batches containing the inoculum were pre-
134 conditioned for 3 days before the experiments began (OECD, 1992).

135 2.3. Experimental approach

136 Dilution factors (DF) of 1, 2, 4 and 10 for untreated SW were prepared in triplicate and a
137 control (500 mL) produced for each DF (Figure 1). The SW was buffered at pH 7.5, which is
138 typical for both wastewater and receiving waters. Inoculum was added to each replicate and
139 left for 72 h before addition of 7 APIs, each to a final concentration of $100 \mu\text{g L}^{-1}$ as a

140 representative concentration for impact zones of LLIMCs (K'oreje et al., 2016, 2012;
141 Madikizela et al., 2017; Ngumba et al., 2016). The flasks were wrapped in aluminium foil to
142 avoid photodegradation and continuously stirred. The temperature was 21 °C during the
143 experimental period. After an equilibration time of approximately 4 hours, batches were
144 diluted with UHP at a DF of 1, 2, 4, and 10. Samples were collected at several time points
145 over 50 days for API and DOC determination. An identical abiotic experiment was performed
146 for DF 1 and 2 only, and run for 20 days to determine the sorption component only and/or
147 hydrolysis in order to quantify loss through biodegradation (Figure 1). This second batch was
148 kept sterile by adding sodium azide (0.02%).

149 2.4. Analytical procedures

150 Samples were collected in 15 mL centrifuge tubes (Fisher Scientific) and centrifuged for 10
151 minutes at 4000 rpm. The supernatant was filtered through 0.7 µm GF/F filters (Whatman) in
152 order to allow for solid phase extraction (SPE) and preconcentration without interference
153 from suspended solids present at high levels in the simulated untreated wastewater. For
154 analysis of DOC, an aliquot was diluted 20 times (owing to the high levels present) acidified
155 (HCl, 6 M) and frozen at -20 °C for preservation prior to analysis (Badr et al., 2003).

156 Concentrations of APIs were measured according to Bagnis et al. (2018). Briefly, aqueous
157 samples were passed through a SPE cartridge for sample clean-up and to optimise analyte
158 resolution, mass detection and quantification. The SPE cartridges (OASIS HLB, 200 mg
159 polymeric sorbent; 6 mL barrel volume; Waters, UK) were activated with 5 mL of methanol
160 (Thermo Fisher Scientific, Optima LC/MS) and 5 mL of UHP. A 5 mL sample aliquot was then
161 loaded onto the cartridge at a flow rate of 3 mL min⁻¹, followed by 1 mL of UHP. The APIs

162 were eluted with 5 mL of methanol amended with formic acid (2 %). The eluent was
163 evaporated to dryness under a gentle stream of nitrogen gas then reconstituted with 1 ml of
164 methanol : UHP at a 1 : 10 ratio.

165 Chromatographic separations were measured by high performance liquid chromatography
166 (HPLC) coupled with a high-resolution mass spectrometry (HR-MS) orbitrap-based system
167 (Thermo Scientific), using a reversed phase column (XBridge BEH C18 2.5 μm 2.1 x 50 mm
168 Column XP, Waters) maintained at 50 °C. A gradient elution programme was employed,
169 starting at 100 % UHP amended with 0.1 % formic acid, progressing to 100 % methanol after
170 5.5 minutes. The parameters used for high resolution mass spectrometric detection are
171 described in Bagnis et al. (2018).

172 The dissolved organic carbon (DOC) analyses were performed using high-temperature
173 catalytic oxidation (TOC-5000A - Shimadzu) according to the method of Badr et al. (2003).

174 2.5. Calculations

175 Biodegradation rates were assumed to follow first-order kinetics (Schwarzenbach et al.,
176 2003). The 50 % dissipation time (DT_{50}) was modelled as a surrogate measure of the
177 biodegradation rate according to Equation 1:

$$DT_{50} = \frac{0.5IC - I}{K} \quad (1)$$

178 Where I is the intercept and IC is the initial concentration after dilution in $\mu\text{g L}^{-1}$ and K is the
179 slope of the equation of the line obtained from the linear trend line of the biodegradation
180 starting at the first sample taken after dilution; the dissipation time is expressed in days.

181 BOD was not directly determined owing to practicalities of sample size and time constraints.
182 However, reliable datasets are available that provide a strong correlation between measured
183 DOC and BOD for untreated municipal wastewater (Comber et al., 2018; Kwak et al., 2013).
184 Based on data from over 100 UK wastewater treatment works crude sewage the following
185 relationship was derived from a DOC vs BOD correlation of $R^2 = 0.86$ (Comber et al., 2018):

$$BOD = \frac{DOC+9.9851}{0.2876} \quad (2)$$

186 3. Results and discussion

187 3.1. Effects of dilution on natural attenuation

188 Three out of the seven APIs investigated, namely ACE, DCF and ACT showed effective
189 biodegradation within the simulated impact zone. The biodegradation rate of ACE and DCF
190 was strongly affected by the dilution, showing an overall increase of persistence (longer half-
191 life) at increased dilution. This inverse correlation is well described by the half-life dissipation
192 time (DT_{50}) of DCF and ACE (Table 1).

193 ACE biodegraded over fifteen days then reached a pseudo-plateau with a much slower rate
194 of biodegradation (Figure 2A). The removal rate at no dilution (DF 1) was approximately 78 %
195 after 14 days. This was similar to Lin et al. (2010) who ascribed the disappearance of the
196 compound to sorption processes. However, based on the tests carried out here and
197 examining the data for the abiotic tests here (Figure 2B), it appears that the sorption of ACE
198 was much less significant than biodegradation (Lin et al., 2010). Lin et al. (2010) suggested
199 that their results were most likely due to experimental artefacts which might explain such
200 difference between the two studies. Nonetheless, the progressive dilution from DF 1 to 10

201 reduced the original concentration of the API, but also the biodegradation rate (Figure 2A)
202 and therefore the removal of ACE from the solution. In this case, the progressive increase of
203 dilution concurrently reduced the degrading microbial biomass present in the system, which
204 might explain the trend. This observation is consistent with previously reported studies for
205 other compounds in continuous input studies (Landa et al., 1994).

206 In the case of DCF the increasing dilution specifically affected its biodegradation profile. In
207 fact, at zero dilution (DF1) the biodegradation curve of DCF showed immediate removal after
208 addition (Figure 2C), whilst a progressive increase in the lag phase prior to biodegradation
209 was observed at DF 2 and 4, lasting respectively 14 and 28 days. At a dilution factor of 10 the
210 DCF concentration remained constant after dilution, for the duration of the whole test (50
211 days), thus rendering the compound highly persistent. It is worth noting that, although DCF is
212 generally described as fairly biodegradable in wastewater treatment works (Joss et al., 2006;
213 Kruglova et al., 2014), in soils (Al-Rajab et al., 2010) and in aqueous environments (Jiskra and
214 Hollender, 2008; Poirier-Larabie et al., 2016), different environmental conditions can lead to
215 varying degrees of persistence (Baena-Nogueras et al., 2017).

216 These data strongly suggest that while dilution decreases the concentration of a chemical in
217 the solution, it can increase its persistence. The latter is most likely caused by the
218 concomitant dilution of the bacterial community and the substrate concentration
219 represented by increasing lag phase of acclimation of the bacteria responsible for the
220 contaminant biodegradation (Maier et al., 2009; Swinnen et al., 2004). Such a lag phase is a
221 measure of the resilience of the degrader to such an environmental stress before returning
222 to the conditions necessary to catabolise the contaminant (Ramadan et al., 1990).

223 Furthermore, it has been shown that such a dilution effect might be related to the dilution of
224 other wastewater components such as salts, which indirectly affect the species lag phase
225 duration prior to degradation of the contaminant (Robinson et al., 2001). The reduced
226 removal from dilution of wastewater influent in a wastewater treatment plant has been
227 reported (Joss et al., 2006) and is considered as a factor to be avoided to increase effluent
228 treatment efficiency.

229 The disappearance of ACT in the abiotic batch was extensive, being 40-50 % within 20 days
230 (Figure 2F), but the degradation rate was much slower than in the combined sorption-
231 biodegradation batch (Figure 2E), where removal by biodegradation occurred in a few hours
232 (< 12 h). As such, dilution did not visibly influence the biodegradation rate of ACT since the
233 latter was faster than the experimental time designed for batch equilibration. In the study of
234 Lin et al. (2010) consumption of $50 \mu\text{g L}^{-1}$ was completed with half-life (DT_{50}) of 2.1 days,
235 compared with 3 days for Yamamoto et al. (2009), and 38 days for Baena-Nogueras et al.
236 (2017).

237 The slower disappearance rate observed in such experiments could be explained by the use
238 of river water, which has a lower dissolved organic matter (DOM) concentration and lower
239 microbial biomass as well as different bacterial communities than untreated wastewater.
240 Such differences highlight the likely variable rates of biodegradation for APIs entering the
241 environment in wastewater as treated effluent or untreated sewage (Tappin et al., 2014,
242 2012).

243 CBZ, NVR, VLS, and AMI were persistent over the 50 days of the experiment (Figure 3A, C, E,
244 G), consistent with their known metabolic stability (Nassar et al., 2004; Siddiqui et al., 2011).

245 The only effective natural attenuation processes occurring in the impact zone for these APIs
246 were sorption and dilution.

247 The neutral species NVR and CBZ have a similar chemical structure which can explain their
248 comparable behaviour (Bagnis et al., 2018b). Their sorption was moderate (10-20 %),
249 consistent with their similar lipophilicity ($\log K_{ow}$ 2.7 and 2.5 for NVR and CBZ, respectively);
250 no further losses were observed in either the biotic and abiotic batches (Figure 3A, B). Whilst
251 sorption and dilution were the main factors controlling attenuation of CBZ and NVR in both
252 biotic and abiotic batches. A positive deviation from the expected concentration occurred
253 after dilution suggesting desorption.

254 The high frequency of detection of NVR in the environment (K'oreje et al., 2016; Madikizela
255 et al., 2017; Ngumba et al., 2016) has been attributed to both its widespread use and to poor
256 removal efficiency in wastewater treatment plants (Madikizela et al., 2017). The work of
257 Vankova et al.(2010) provides the only available information on the biodegradability of NVR
258 and the results of this study were consistent with that study, which suggested high
259 recalcitrance in wastewater treatment works. Due to the almost identical behaviour of CBZ,
260 further investigation to evaluate its applicability as a possible marker for sewage
261 contamination (Lim et al., 2017) is recommended, especially targeted to African countries
262 where it is widely consumed (Madikizela et al., 2017).

263 Little information is available about the natural attenuation of VLS (Bergheim et al., 2014;
264 Mandaric et al., 2019). The distribution of ionisable APIs is controlled by both lipophilicity and
265 charge, expressed by the ionization constant (pK_a). In general, the concentration values for
266 the different dilutions, reported for the ionisable compounds used in this study,

267 corresponded to the expected calculated arithmetical value plus desorption, when the latter
268 occurred, in accordance with the compound lipophilicity and speciation (Bagnis et al., 2018a).
269 The results for VLS contrasted with reported data, which described a relatively fast
270 biodegradation with consequent detection of transformation products such as valsartan acid,
271 de-alkylated valsartan and amino-valsartan (Helbling et al., 2010; Kern et al., 2010). These
272 differences highlight the variability in behaviour of this API with the experimental conditions
273 employed. While only a few studies on the persistence of AMI have been documented, these
274 report high persistence in both water (Baena-Nogueras et al., 2017) and agricultural soils (Li
275 et al., 2013), in agreement with the data from this study (Figure 3G).

276 It is worth noting that the results of this work showed a different degree of sorption between
277 the biotic and the abiotic experiments (Figure 2 and Figure 3). For instance, the anionic VLS
278 showed approximately 20 % of sorption in the abiotic experiment compared with no sorption
279 under biotic conditions (Figure 3E and F). The same behaviour was observed for the
280 negatively charged DCF (Figure 2C and D). In contrast, the positively charged AMI had lower
281 sorption under abiotic conditions (Figure 3G and H). In fact, at DF 1, 80 % the compound was
282 adsorbed in the biotic batch as opposed to 50 % in the abiotic one. Such behaviour has been
283 ascribed to the difference of specific surface area for ionic exchange determined by the
284 presence/absence of a bacterial biofilm on the sorbent surface (Carlson and Silverstein, 1998;
285 Headley et al., 1998; Wunder et al., 2011). The bacterial extracellular polymeric substances
286 are composed of anionic (e.g. $-\text{COO}^-$, $-\text{SH}^-$, $-\text{SO}_4^-$, HPO_4^-), cationic (e.g. $-\text{NH}_3^+$), and apolar
287 (e.g. aromatic) functional groups, which at the experimental pH (7.5) are predominantly
288 negatively charged (Wunder et al., 2011). This translates to additional surface sites for
289 exchange of positively charged compounds and the repulsion of negatively charged ones;

290 thus, respectively increasing and decreasing sorption. Such differences between the
291 environmentally more realistic biotic system and the artificial abiotic system highlights a
292 technical bias in the experimental evaluation of the distribution of ionisable chemicals, such
293 as APIs.

294 3.2. APIs persistence through the impact zone

295 Data from the experiments was used to model the environmental concentrations of APIs
296 within and beyond the impact zone and to determine its extent. The end of the impact zone
297 is defined as the point at which BOD reaches background environmental levels ($< 1 - 8 \text{ mg L}^{-1}$)
298 ¹⁾ (Schwarzenbach et al., 2003). Therefore, the experimental DOC was measured and used in
299 the mathematical model (2) derived from the linear correlation of DOC and BOD ($R^2=0.86$)
300 obtained from a large set of experimental data for untreated wastewater (Kwak et al., 2013).
301 Through this correlation it was possible to estimate the temporal end boundary of the impact
302 zone.

303 According to the model, BOD decreased most rapidly in the first day (Figure 4), achieving a
304 plateau which constitutes the recalcitrant or slowly degrading organic load present in the
305 mixture (dashed red line in Figure 4) (Dignac et al., 2000). The percentage of refractory DOC
306 in wastewater can be highly variable, but roughly comprised 10-30 % of the initial amount
307 (Dignac et al., 2000; Reynolds, 2002), consistent with the generated data (Figure 4). The
308 recalcitrant DOM could not be excluded from the simple modelling approach adopted in this
309 study and therefore the plateau was considered as the lowest level of observable BOD, and
310 consequently marked the end of the impact zone (A.I.S.E./CESIO, 1995). The end of the
311 impact zone was calculated to be reached in approximately 24 hours irrespective of dilution.

312 The modelled data suggested that six of the seven APIs investigated in this study would
313 persist through the impact zone and occur at the initial observed concentration beyond its
314 end boundary. Furthermore, although ACT was quickly biodegraded, the high rate of input
315 and patterns of use could lead to pseudo-persistence in the natural environment, leading to
316 the occurrence in concerning concentrations ($> 0.01 \mu\text{g L}^{-1}$) beyond the impact zone end
317 boundary.

318 Nonetheless, it must be highlighted that uncertainties are likely to be associated with both
319 the model applied for the calculation of BOD and the calculation of the 24h estimate when
320 applied to a real environmental setting. These uncertainties are related to the environmental
321 complexity of each individual scenario which translate in likely varying DOM composition and
322 consequently variable rates of consumption; therefore, further studies are necessary for the
323 development of a more accurate model.

324 3.3. Implications for an environmental risk assessment approach

325 This work provides novel, robust data that could inform for the future development of an ERA
326 approach for the impact zone with regard to APIs. The data presented here suggests that
327 natural attenuation in the impact zone receiving untreated or poorly-treated wastewaters
328 might not be enough to significantly restrict the environmental burden of APIs within its
329 boundaries. In fact, even at the highest rate of biodegradation, the initial concentration of
330 APIs in the impact zone could be expected beyond its end boundary.

331 According to this investigation, while low levels of dilution (< 10) reduce the API
332 concentration, the rate of biodegradation also slows increasing the risk of environmental
333 persistence and likely occurrence of the API beyond the impact zone boundary. The dilution
334 level of down-the-drain chemicals in surface waters is highly dependent on local hydrological
335 conditions, such as seasonal runoff variability and so this must also be considered in the
336 evaluation of the risk (Keller et al., 2014). Therefore, further studies are necessary to evaluate
337 this important factor contributing to the environmental exposure of APIs in the impact zone.

338 Based on the data presented here, it is possible to propose a possible preliminary ERA
339 approach. In phase 1 of the European ERA a predicted environmental concentration (PEC)
340 above $0.01 \mu\text{g L}^{-1}$ triggers a tier of tests to refine the understanding of the APIs environmental
341 fate (EMA, 2006). Such calculations assume no biodegradation or loss (to sludge or
342 atmosphere) within the wastewater treatment plant. In the case of DDUW the API
343 concentrations would likely exceed such exposure threshold and as a consequence the
344 development of a dedicated phase with the risk assessment is proposed (Figure 5; proposed
345 as phase 2.2). It does not aim to reproduce the current ERA for APIs but is strongly informed
346 by such protocols and compliments it to take account of DDUW. The new phase of the ERA
347 would be parallel to the current phase 2, renamed in Figure 5 as phase 2.1, for the evaluation
348 of the PEC for APIs beyond the impact zone generated by the DDUW. This phase is divided in
349 two parts, tier A and B, as for phase 2.1. Tier A is based on the modelling methodology used
350 in this study and therefore constrains the impact zone at 24 h. In accordance with the
351 guidelines for the ERA, the experimental studies necessary to evaluate environmental fate
352 should be based on protocols issued by the European Commission, the Organization for
353 Economic Co-operation and Development (OECD) or the International Organization for

354 Standardization (ISO) (EMA, 2006). The PEC beyond the impact zone would therefore be used
355 to evaluate the environmental risk using the PNEC calculated as from the actual protocol
356 (EMA, 2006).

357 If a risk is still evident further refinement of the PEC would be necessary in tier B, taking into
358 account parent compounds PEC and PNEC as well as the most relevant metabolic fraction (\geq
359 10 % of amount excreted) (EMA, 2006). In this case, an appropriate model should be
360 developed for the impact zone in the same fashion as SimpleTreat model in EUSES (EMA,
361 2006).

362 The approach proposed here would quantify and manage the risk posed by the
363 environmental occurrence of APIs to LLMICs where dilution occurs at factors below 10, such
364 is the case of 53 countries worldwide.

365 **4. Conclusions**

366 According to the results of this investigation, the dilution significantly affects the
367 biodegradation rate of DCF and ACE and this might be true for other APIs. ACT instead
368 behaves consistently with previously reported data and it is quickly biodegraded in the
369 wastewater solution regardless the degree of dilution. The other compounds here
370 investigated show high persistence along the experimental timescale. The temporal
371 modelling of the extent of the impact zone allow an estimate of its end at about 24 hours
372 after discharge in the environment, without influence by dilution. The model applied to the
373 APIs natural attenuation data of this study shows a persistence beyond the end of the impact

374 zone. Thus, an ERA approach is proposed, considering the impact zone as a semi-natural
375 wastewater treatment area. The extent of the zone may be modelled with the aim of
376 estimating the APIs concentrations beyond its end, after which the traditional ERA protocol
377 can be applied.

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