Transformation of diclofenac in hydrid biofilm–activated sludge processes

Supplementary data

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1 Compound details, MS$^2$ fragmentation and elucidation of TP structures

Different levels of confidence are given for the structures based on the categorisation proposed by Schymanski et al. (2014). For 4HD, 5HD, DCF-Lactam and DCF-BA, the identity could be confirmed with authentic reference standards. For the other TPs, the structure was postulated based on the exact mass, isotope pattern, retention time, MS$^2$ fragmentation spectrum, the similarities or differences of the MS$^2$ spectrum to that of DCF and other TPs and based on the primary TPs from which they were formed. Software tools that were used to aid structural identification include PeakView and MasterView (Sciex), ChemDoodle (iChemLabs) and ChemCalc.org (Patiny and Borel, 2013).

Table S1: Table of TPs identified in lab-scale experiments with the recorded experimental [M+H]$^+$ mass and retention time.

| Name      | Formula          | Monoisotopic mass (u) | Retention time (min) | Confidence level | Polarity | [M+H]$^+$ mass (u) |
|-----------|------------------|-----------------------|----------------------|------------------|----------|-------------------|
| DCF       | C14H11Cl2NO2     | 295.0167              | 12.74                | pos              |          |                   |
| 5HD       | C14H11Cl2NO3     | 311.0116              | 10.16                | 1                | pos      | 312.0184          |
| 5HDQI     | C14H9Cl2NO3      | 308.9959              | 10.52                | 2                | pos      | 310.0035          |
| DCF-d4    | C14H7D4Cl2NO2    | 299.0418              | 12.71                | pos              |          |                   |
| 4HD       | C14H11Cl2NO3     | 311.0116              | 10.60                | 1                | pos      | 312.0191          |
| 4HDQI     | C14H9Cl2NO3      | 308.9959              | 10.90                | 2                | pos      | 310.0031          |
| DCF-lactam|                 |                       |                      |                  |          |                   |
| DCF-BA    | C14H9Cl2NO2      | 281.0010              | 13.05                | 1                | pos      | 282.0083          |
| TP285     | C12H9Cl2NO3      | 284.9959              | 8.59                 | 3                | pos      | 286.0031          |
| TP287     | C12H11Cl2NO3     | 287.0116              | 7.02                 | 4                | pos      | 288.0191          |
| TP259     | C14H10N2O2Cl     | 259.0400              | 10.10                | 3                | pos      | 260.0467          |
| TP225     | C14H11NO2        | 225.0790              | 9.06                 | 3                | pos      | 226.0856          |
| TP293a    | C14H9Cl2NO2      | 293.0010              | 10.64                | 2                | pos      | 294.0082          |
| TP293b    | C14H9Cl2NO2      | 293.0010              | 11.14                | 2                | pos      | 294.0088          |
| TP391a    | C14H11Cl2NO6S    | 390.9683              | 8.34                 | 2                | neg      | 389.9605          |
| TP391b    | C14H11Cl2NO6S    | 390.9683              | 9.61                 | 2                | neg      | 389.9592          |
| TP297     | C13H9Cl2NO3      | 296.9660              | 11.08                | 2                | pos      | 298.0032          |
| TP273     | C11H9Cl2NO3      | 272.9595              | 7.14                 | 4                | pos      | 274.0030          |
| TP243     | C10H7Cl2NO2      | 242.9854              | 9.34                 | 4                | pos      | 243.9922          |
| TP343a    | C14H11Cl2NO5     | 343.0014              | 7.25                 | 4                | pos      | 344.0088          |
| TP343b    | C14H11Cl2NO5     | 343.0014              | 7.65                 | 4                | pos      | 344.0086          |
| TP275     | C14H10CIN03      | 275.0349              | 9.87                 | 3                | pos      | 276.0422          |
1.1 Diclofenac (DCF)

DCF fragmentation is included for the purposes of comparison. The fragmentation spectrum and corresponding postulated fragment ions are shown in Table S2 and Figure S1.

| Mass/Charge | Intensity |
|-------------|-----------|
| 178.0640    | 2%        |
| 179.0720    | 2%        |
| 180.0801    | 2%        |
| 214.0425    | 100%      |
| 250.0180    | 5%        |

Figure S1: Characterisation of MS$^2$ fragment ions of DCF
1.2 4HD

This TP of DCF was available as a reference standard (level 1 confidence). The relatively simple fragmentation pattern with -CO and -Cl losses is characteristic of secondary TPs formed from 4HD, e.g. TP259 and TP225.

Table S3: Fragmentation spectrum of 4HD

| Mass/Charge | Intensity |
|-------------|-----------|
| 195.0651    | 4%        |
| 230.0367    | 100%      |
| 266.0137    | 5%        |

Figure S2: Characterisation of MS² fragment ions of 4HD
1.3 5HD

This TP, for which a reference standard was used (level 1 confidence) shows characteristic multiple -CO and -Cl losses similar to DCF and most other TPs. 5HD was found to be an intermediate of nine other TPs in the DCF transformation pathway many of which share a similar fragmentation pattern to 5HD.

Table S4: Fragmentation spectrum of 5HD

| Mass/Charge | Intensity |
|-------------|-----------|
| 168.0791    | 65%       |
| 196.0719    | 50%       |
| 202.0395    | 85%       |
| 238.0223    | 50%       |
| 266.0194    | 35%       |
| 294.0077    | 100%      |

Figure S3: Characterisation of MS$^2$ fragment ions of 5HD
1.4 DCF-lactam

A reference standard of this TP was available (level 1 confidence). The lactam TP shows typical CO and Cl losses as well a characteristic fragment at mass 171.9715, which it shares with other TPs such as TP285 and TP287.

| Mass/Charge   | Intensity |
|---------------|-----------|
| 171.9698      | 26%       |
| 180.0800      | 26%       |
| 208.0751      | 34%       |
| 214.0406      | 100%      |
| 215.0448      | 16%       |
| 243.0429      | 13%       |
| 250.0193      | 5%        |
| 278.0133      | 45%       |

Table S5: Fragmentation spectrum of DCF-Lactam
1.5 DCF-BA

A reference standard of this compound was available (level 1 confidence). The fragmentation pattern is typical of DCF TPs, showing loss of -CO and multiple -Cl losses.

Table S6: Fragmentation spectrum of DCF-BA

| Mass/Charge | Intensity |
|-------------|-----------|
| 166.0655    | 11%       |
| 201.0339    | 16%       |
| 229.0292    | 100%      |
| 263.9979    | 16%       |

Figure S5: Characterisation of MS² fragment ions of DCF-BA
1.6 4HDQI

4HDQI is a known human metabolite of DCF (Poon et al., 2001) and is reported to form by oxidation of 4HD. In this study, 4HDQI was identified by its very similar fragmentation pattern to 4HD and similar retention time (level 2 confidence). However, in incubation experiments of 4HD, 4HDQI was not formed whereas it was formed in incubations of DCF-lactam (and incubations of DCF itself). This might be due to the fast dissipation kinetics of 4HD, which is quickly transformed to other TPs, allowing little oxidation to take place, while DCF-lactam is more stable, allowing the formation of 4HDQI over a different route (e.g. by combined mono-oxygenation and de-amidation).

Table S7: Fragmentation spectrum of 4HDQI

| Mass/Charge | Intensity |
|-------------|-----------|
| 263.9944    | 54%       |
| 229.0288    | 100%      |

Figure S6: Characterisation of MS² fragment ions of 4HDQI

1.7 5HDQI

This TP was previously identified in soil/sediment systems (Gröning et al., 2007) and was identified in this study by the similar fragmentation pattern and retention time to 5HD (level 2 confidence).

Table S8: Fragmentation spectrum of 5HDQI

| Mass/Charge | Intensity |
|-------------|-----------|
| 166.0652    | 100%      |
| 194.0583    | 38%       |
| 201.0334    | 88%       |
| 229.0286    | 35%       |
| 236.0008    | 23%       |
| 263.9964    | 23%       |
| 291.9919    | 81%       |
Figure S7: Characterisation of MS\textsuperscript{2} fragment ions of 5HDQI
1.8 TP285

The characterisation of TP285 was based firstly on the presence of fragment 171.9715, which indicated that ring A was not hydroxylated. TP285 was formed from DCF via 5HD so one oxygen should be located on position 5. The remaining 2 oxygens were considered to be part of a carboxylic acid group which would explain the loss of -CO₂. It was postulated that TP285 was formed as a result of ring-opening of ring B since consecutive -CH₂, -CO and -C₂H₂ losses leading to fragment 171.9715 are indicative of a long-chain structure. According to this structure, the β-keto moiety might be formed via tautomerism. A level 3 confidence is therefore proposed (tentative structure).

Table S9: Fragmentation spectrum of TP285

| Mass/Charge | Intensity |
|-------------|-----------|
| 171.9676    | 26%       |
| 197.9856    | 19%       |
| 225.982     | 25%       |
| 239.9951    | 58%       |
| 242.0145    | 100%      |

Figure S8: Characterisation of MS² fragment ions of TP285
1.9 TP287

TP287 has a mass difference of only +2H compared to TP285 and also shows fragment 171.9715, the presence of which indicated that ring A is not hydroxylated. Structural characterisation of TP287 was not possible due to many possible structures on the right side of the molecule. The structure of ring A and the elemental composition of the rest of the molecule could be determined (level 4 confidence).

Table S10: Fragmentation spectrum of TP287

| Mass/Charge | Intensity |
|-------------|-----------|
| 132.9596    | 17%       |
| 159.9697    | 98%       |
| 164.0264    | 21%       |
| 171.9707    | 59%       |
| 187.9648    | 21%       |
| 200.0054    | 14%       |
| 227.9990    | 100%      |

Figure S9: Characterisation of MS$^2$ fragment ions of TP287
The mass of TP259 and the isotopic pattern indicate it has one chloride and two oxygen atoms. Since it is formed from DCF via 4HD, it is assumed that the 4'-position (ring A) is hydroxylated. The fragmentation pattern is very similar to DCF-lactam showing consecutive -CO and -Cl losses, it is therefore postulated that TP259 has a lactam structure, which also accounts for the second oxygen. To account for the extra hydrogen a reductive dechlorination at the 2'-position is postulated (level 3).

### Table S11: Fragmentation spectrum of TP259

| Mass/Charge | Intensity |
|-------------|-----------|
| 132.0451    | 12%       |
| 168.0807    | 30%       |
| 180.0799    | 15%       |
| 196.0747    | 100%      |
| 225.0781    | 21%       |

Figure S10: Characterisation of MS² fragment ions of TP259
1.11 TP225

The mass and isotopic pattern of TP225 indicate that no Cl atom is present in the molecule. The similar fragmentation pattern to TP259 suggest this compound is the result of a second reductive dechlorination, which also accounts for the extra hydrogen. Therefore, a level 3 confidence is given for the structure.

Table S12: Fragmentation spectrum of TP225

| Mass/Charge   | Intensity |
|---------------|-----------|
| 120.0453      | 59%       |
| 132.0440      | 69%       |
| 180.0801      | 86%       |
| 183.0634      | 43%       |
| 196.0745      | 100%      |
| 198.0912      | 53%       |

Figure S11: Characterisation of MS$^2$ fragment ions of TP225
1.12 TP293a

This TP was formed from both DCF-lactam and 5HD. Due to the parent TPs and the fragmentation pattern, the structure can be given at level 2 confidence. Several isomers of this TP were detected, with similar retention times and MS$^2$ spectra. It is postulated that these are formed by hydroxylation of ring B at different positions.

Table S13: Fragmentation spectrum of TP293a

| Mass/Charge | Intensity |
|-------------|-----------|
| 266.0128    | 40%       |
| 238.0187    | 40%       |
| 202.0415    | 60%       |
| 168.0817    | 100%      |

Figure S12: Characterisation of MS$^2$ fragment ions of TP293a
1.13 TP293b

This TP is an isomer of TP293a but is formed from 4HD or DCF-lactam and not formed from 5HD and was previously detected WWTP effluents, where it was identified as a human metabolite of DCF (Stültten et al., 2008). It has a different MS² spectrum since ring A is hydroxylated. A level 2 confidence is given for this structure.

| Mass/Charge | Intensity |
|-------------|-----------|
| 230.0366    | 100%      |
| 224.0696    | 50%       |
| 132.0437    | 45%       |

Figure S13: Characterisation of MS² fragment ions of TP293b.
1.14 TP391a

This DCF-TP is formed via 5HD. The molecular formula and characteristic -SO\textsubscript{3} loss indicate that this TP is the result of sulfate conjugation of 5HD. Due to the observed fragments, and in comparison to similar sulfate conjugation reactions observed at aromatic hydroxy groups in activated sludge (Jewell et al., 2014), it is postulated the conjugation occurs at the hydroxylated 5-position (ring B). Thus, level 2 confidence is given for the structure.

Table S15: Fragmentation spectrum of TP391a

| Mass/Charge | Intensity |
|-------------|-----------|
| 79.9572     | 100%      |
| 185.9972    | 29%       |
| 309.9933    | 42%       |
| 345.9676    | 78%       |

Figure S14: Characterisation of MS\textsuperscript{2} fragment ions of TP391a.
1.15 TP391b

This TP is formed from 4HD similarly to TP391a. It is postulated the conjugation occurs at the 4′-position (ring A). Level 2 confidence is given for the structure.

| Mass/Charge | Intensity |
|-------------|-----------|
| 310.0047    | 30%       |
| 266.0127    | 100%      |
| 166.0639    | 30%       |

Table S16: Fragmentation spectrum of TP391b

Figure S15: Characterisation of MS2 fragment ions of TP391b.
1.16 TP243

This DCF-TP is formed via 5HD and has a similar fragmentation pattern to TP285 and TP287, sharing, for example, the fragment 171.9713. This fragment is again an indication that ring A is not hydroxylated. The two oxygen atoms from the formula are thus likely to be on the right side of the molecule. Due to the ambiguous fragmentation pattern, a tentative structure cannot be suggested (level 4). However, the low number of carbons on the right side of the molecule suggest that this TP has lost ring B due to ring opening reactions.

Table S17: Fragmentation spectrum of TP243

| Mass/Charge | Intensity |
|-------------|-----------|
| 216.0006    | 25%       |
| 197.9881    | 25%       |
| 187.9664    | 25%       |
| 171.9713    | 5%        |
| 159.9712    | 100%      |
| 132.9608    | 25%       |

![Chemical structures](image)

Figure S16: Characterisation of MS² fragment ions of TP243
This TP was formed from both 4HD and DCF-BA and to a minor extent 5HD. The molecular formula indicates it is formed as a result of the combination of reactions which lead to the parent TPs, i.e. a hydroxylation and a decarboxylation followed by oxidation to carboxylic acid. Two isomers of this compound are formed with retention times 10.68 min. and 11.08 min., but both with identical fragmentation patterns. It is expected that these are the result of the hydroxylations at different ring positions, i.e. 4′-, or 5-position. The isomer at 10.68 is only formed via 5HD, whereas the isomer at 11.08 is only formed via 4HD and both isomers are formed via DCF-BA. Level 2 confidence is given for this structure.

Table S18: Fragmentation spectrum of TP297

| Mass/Charge | Intensity |
|-------------|-----------|
| 153.0562    | 8%        |
| 182.0601    | 13%       |
| 217.0291    | 17%       |
| 245.0236    | 100%      |
| 279.9942    | 19%       |

Figure S17: Characterisation of MS² fragment ions of TP297
1.18 TP273

This DCF-TP is formed via 5HD and, as with TP243, TP285 and TP287 has a fragmentation pattern indicating ring A is not hydroxylated (fragment with mass 171.9718). The fragmentation pattern indicates several -CO losses however a unambiguous structure cannot be postulated for the right side of the molecule (level 4 confidence).

Table S19: Fragmentation spectrum of TP273

| Mass/Charge | Intensity |
|-------------|-----------|
| 164.0262    | 55%       |
| 171.9718    | 100%      |
| 200.0017    | 35%       |
| 227.9985    | 75%       |

Figure S18: Characterisation of MS² fragment ions of TP273
1.19 TP343a

This TP of DCF and 5HD has a formula with +2O in comparison to 5HD, suggesting that oxidative reactions took place. A characteristic fragment at 159.9731 indicates that ring A is likely not hydroxylated. The remaining fragments show multiple -H₂O and -CO losses (parent mass → 326.0028 → 298.0026 → 270.0039 → 251.9964 → 224.0014) indicating a structure with multiple hydroxy carboxyl groups. However an clear structure can not be postulated from the fragments (level 4).

Table S20: Fragmentation spectrum of TP343b

| Mass/Charge | Intensity |
|-------------|-----------|
| 159.9731    | 20%       |
| 217.0294    | 30%       |
| 224.0014    | 15%       |
| 251.9964    | 100%      |
| 270.0039    | 25%       |
| 298.0026    | 60%       |
| 326.0028    | 15%       |

Figure S19: Characterisation of MS² fragment ion of TP343a

Monoisotopic Mass = 344.0092 u
Molecular Formula = C14H12Cl2NOS+

Monoisotopic Mass = 159.9715 u
Molecular Formula = C6H4Cl2N+
1.20 TP343b

This DCF-TP is formed via 5HD and has a formula with +2O in comparison to 5HD, suggesting that oxidative reactions took place. Although the characteristic fragment at mass 171.9715 was not observed, a similar mass at 173.9834 is again an indication that ring A is not hydroxylated. The other fragments do not allow a full characterisation of the right side of the molecule (level 4). However, multiple -CO$_2$ losses, e.g. from parent mass $\rightarrow$ 298.0063 $\rightarrow$ 254.0122, suggest the presence of carboxylic acid groups. This compound has a similar retention time ($\delta$ 0.5 min) to the structural isomer TP343a, however these TPs do not have similar fragmentation patterns.

| Table S21: Fragmentation spectrum of TP343b |
|--------------------------------------------|
| Mass/Charge | Intensity |
| 173.9834    | 10%       |
| 177.0338    | 35%       |
| 190.0415    | 35%       |
| 204.0208    | 15%       |
| 218.0365    | 55%       |
| 237.9802    | 20%       |
| 254.0122    | 60%       |
| 283.9882    | 100%      |
| 298.0063    | 10%       |

Figure S20: Characterisation of MS$^2$ fragment ions of TP343b

Monoisotopic Mass = 344.0092 u
Molecular Formula = C14H12C2NO5+

Monoisotopic Mass = 173.9872 u
Molecular Formula = C7H6Cl2N+
TP275 was formed via 4HD. The molecular formula indicated a loss of Cl and one H. The fragmentation spectrum was similar to 4HD and 4HDQI showing CO, H$_2$O, Cl and HCl losses. Since other reductive dechlorinations were observed, forming TPs 259 and 225, it was postulated that this TP was also formed through reductive dechlorination. To account for the loss of H an oxidation via dehydrogenation was proposed, similar to the reactions forming 4HDQI and 5HDQI. It was unclear if TP275 was formed directly from 4HD or via TP259. In the later case the reaction would be equivalent to the formation of 4HDQI from DCF-lactam (confidence level 3).

| Mass/Charge | Intensity |
|-------------|-----------|
| 140.0524    | 15%       |
| 167.0717    | 100%      |
| 194.0606    | 15%       |
| 202.0426    | 20%       |
| 230.0372    | 30%       |

Figure S21: Characterisation of MS$^2$ fragment ion of TP275
2 Additional figures and tables

Figure S22: Transformation reactions of DCF (spike concentration 5 µg/L) in incubation experiments inoculated with carriers from WWTP Bad Ragaz.
Figure S23: DCF transformation showing sum of TPs and DCF concentration at each time point (spike concentration 5 µg/L) in incubation experiments inoculated with carriers from WWTP-BR (Bad Ragaz, hydrid-MBBR).

Table S23: TPs formed from primary DCF TPs

| Parent       | TPs              |
|--------------|------------------|
| DCF-Lactam   | DCF-BA 4HDQI TP293b TP285 |
| 4HD          | TP293b TP259 TP225 TP275 TP391b TP297 |
| 5HD          | 5HDQI TP287 TP285 TP343a TP343b TP391a TP273a TP243 TP293a |
| DCF-BA       | TP297 TP285 TP287 |
Table S24: DCF-TPs detected during lab-scale experiments with carriers from WWTP Klippan

| DCF-lactam | DCF-BA | TP285 |
|------------|--------|-------|
| Retention time (min) | 12.42 | 12.88 | 8.44 |
| Retention time of standard (min) | 12.39 | 12.89 | 8.62^a |
| [M + H]^+ mass (u) | 278.0131 | 282.0072 | 286.0027 |
| Calculated mass (u) | 278.0134 | 282.0083 | 286.0032 |
| MS^2 Spectrum | 166.0649 |
| | 214.0445 | 201.0349 | 242.0151 |
| | | 229.0297 |

^aStandard not available, retention time was compared to a previous lab-scale incubation experiment using carriers from WWTP Bad Ragaz.

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