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Biodegradation of flavonoids – Influences of structural features

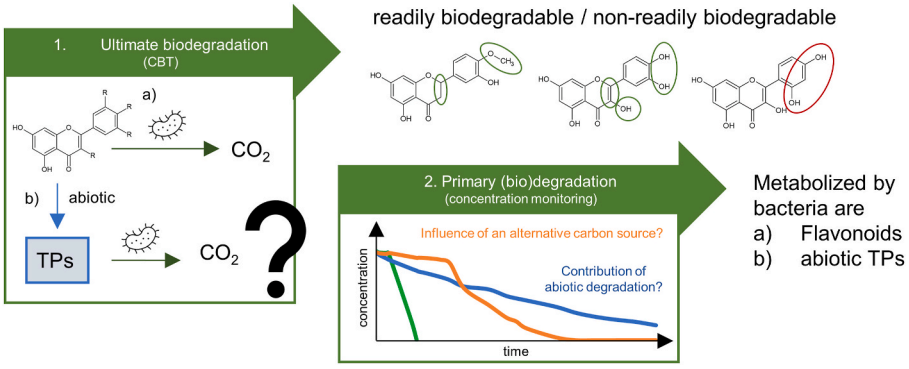
Lena Schnarr [a], Oliver Olsson [a], Klaus Kümmerer [a,b,*]

[a] Institute of Sustainable Chemistry, Leuphana University Lüneburg, Universitätsallee 1, 21335, Lüneburg, Germany
[b] Research and Education, International Sustainable Chemistry Collaborative Centre (ISC3), Universitätsallee 1, 21335, Lüneburg, Germany

HIGHLIGHTS

- Novel biodegradability data of flavonoids provided according to OECD 301 D.
- Insights into the influence of structural features on the biodegradability provided.
- Concentration decrease of 4 flavonoids were monitored with HPLC-UV/vis.
- Degradation at biotic, abiotic, and mixed substrate conditions was compared.
- Flavonoids have a low risk to persistent in the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Flavonoids, a class of natural products with a variety of applications in nutrition, pharmacy and as biopesticides, could substitute more harmful synthetic chemicals that persist in the environment. To gain a better understanding of the biodegradability of flavonoids and the influence of structural features, firstly, the ultimate biodegradation of 19 flavonoids was investigated with the Closed Bottle Test according to the OECD guideline 301 D. Secondly, regarding the fast abiotic degradation reported for several flavonoids with severe concentration decrease within hours and its possible impacts on the processes behind the ultimate biodegradation, primary degradation of 4 selected flavonoids was compared at conditions representing biodegradation, abiotic degradation, and mixed substrates by monitoring the flavonoids' concentrations with HPLC-UV/vis. Our results showed that 17 out of the 19 tested flavonoids were readily biodegradable. Structural features like a hydroxy group at C3, the C2–C3 bond order, a methoxy group in the B ring, and the position of the B ring in regard to the chromene core did not affect biodegradation of the tested flavonoids. Only flavone without any hydroxy groups and morin with an uncommon 2′,4′ pattern of hydroxy groups were non-readily biodegradable. Monitoring the concentration of 4 selected flavonoids by HPLC-UV/vis revealed that biodegradation occurred faster than abiotic degradation at CBT conditions with no other carbon sources present. The presence of an alternative carbon source tends to increase lag phases and decrease biodegradation rates. At this condition, abiotic degradation contributed to the degradation of unstable flavonoids. Overall, as a first tier to assess the environmental fate, our

* Corresponding author.
E-mail addresses: lena.schnarr@stud.leuphana.de (L. Schnarr), oliver.olsson@leuphana.de (O. Olsson), klaus.kuemmerer@leuphana.de (K. Kümmerer).

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results indicate low risks for persistence of most flavonoids. Thus, flavonoids could represent benign substitutes for persistent synthetic chemicals.

1. Introduction

Facing chemical pollution as a major threat to our ecosystem (Persson et al., 2022; Rockström et al., 2009, 2023; Schwarzenbach et al., 2010), the design and use of chemicals has to change towards a sustainable practice (Kümmerer, 2017). If a chemical is required for a certain function, its end-of-life and the environmental fate have to be taken into account early in the design process (Kümmerer, 2017). Natural products could represent benign substitutes of more harmful, synthetic chemicals due to their anticipated non-persistence. While many studies investigated the activity of natural products, less information is available on their environmental biodegradation. However, this knowledge is required to evaluate their potential as benign substitutes and could, furthermore, be useful for the design of non-persistent chemicals in general.

An example of natural products well investigated for their activities but with less knowledge on their biodegradation available are flavonoids. This group of natural products consists of compounds featuring a C6–C3–C6 carbon skeleton forming three rings (A, B and C, see Fig. 2) (Rauter et al., 2018). The C ring is a heterocycle comprising oxygen. Flavonoids, isoflavonoids and neoflavonoids are distinguished based on the alignment of ring B in regard to ring C. Flavonoids are further subdivided into flavones, flavonols, flavanones, flavanonols, flavanols and anthocyanidins according to a combination of structural features at the C ring including a C2–C3 double bond, a hydroxy group at C3 (3-OH) and a keto function at C4 (Figure SI 1). Further modifications like e.g., methylation and glycosylation give rise to the high number (approximately 6000 (Panche et al., 2016)) of different flavonoids.

As secondary metabolites in plants, flavonoids are involved in signaling with symbiotic bacteria, attraction of pollinators, response to UV- or oxidative stress and chemical defense against predators (Falcone Ferreyra et al., 2012; Sugiyama and Yazaki, 2014). Based on these natural functions, flavonoids are investigated for their application as food additives, cosmetic ingredients, pharmaceuticals and as biopesticides (Gonzalez-Paramas et al., 2019; Górniak et al., 2019; Guven et al., 2019; Jucá et al., 2020; Kumar et al., 2023; Maleki et al., 2019; Schnarr et al., 2022; Selin-Rani et al., 2016). However, knowledge on their environmental fate is scarce.

A few studies reported the occurrence of flavonoids in natural water bodies in the ng/L range and up to tens of μg/L at some sites (Erbs et al., 2007; Günthardt et al., 2021; Hoerger et al., 2011; Jarošová et al., 2015; Kolpin et al., 2010; Liang et al., 2023; Nanusha et al., 2020; Yohannes Nanusha et al., 2020, 2021). Most studies investigated isoflavonoids (Erbs et al., 2007; Hoerger et al., 2011; Kolpin et al., 2010; Nanusha et al., 2020) and Jarošová et al. summarized the occurrence of the isoflavonoids biochanin A, daidzein, daidzin, equol, formononetin, genistein, genistin, and glycitein in lakes and rivers in several countries all over the world (Jarošová et al., 2015). Flavonoids were also reported a few times in small streams and rivers in Denmark, Germany and Switzerland (Günthardt et al., 2021; Liang et al., 2023; Yohannes Nanusha et al., 2020, 2021). Most compounds were flavonoid glycosides, but the aglycones quercetin and kaempferol were also among the detected compounds (Yohannes Nanusha et al., 2020). While several sources of the flavonoids were identified (e.g., run-off from agricultural areas or pasture, effluents of fruit processing industries and paper mills (Erbs et al., 2007; Hoerger et al., 2011; Jarošová et al., 2015)), environmental degradation of the detected flavonoids was not investigated.

Providing first hints on the environmental fate, a degradation pathway for flavonoids and isoflavonoids by soil bacteria was proposed based on investigations of quercetin, luteolin, apigenin, naringenin, 7,4′-dihydroxyflavone, genistein, and daidzein (Pillai and Swarup, 2002; Rao et al., 1991; Rao and Cooper, 1994; Cooper, 2004). Accordingly, flavonoids and isoflavonoids are degraded to benzoic acids e.g. 2,4,6-trihydroxybenzoic acid or 2,4-dihydroxybenzoic acid. Supposedly, these degradation products are channeled into the β-ketoadipate pathway and finally enter the citric acid cycle (Cooper, 2004; Rao and Cooper, 1994). Hence, this pathway suggests ultimate biodegradation of flavonoids. Furthermore, Barz provided some evidence for substrate specificity of certain bacteria towards flavonoids by investigating primary biodegradation of 34 flavonoids and isoflavonoids by a bacterium isolated from *Cicer arietinum* roots (Barz, 1970). The isolated bacterial strain was capable of degrading several isoflavonoids and flavonols with common hydroxylation patterns but tested flavanones as well as isoflavonoids and flavonols with hydroxy groups at position 2′ and 8 were not degraded (Barz, 1970). These studies suggest that most flavonoids are biodegraded in the environment. However, they are based on experiments with cell cultures of single bacterial strains occurring in soil and known for or isolated based on their ability to degrade flavonoids (Rao et al., 1991; Rao and Cooper, 1994). Although proving the biodegradability of flavonoids, only limited information on the environmental fate of flavonoids and the degradation kinetics in the environment can be derived from these studies. So far, the primary biodegradation of only a few flavonoids including naringenin, formononetin, biochanin A and isovitexin-6″-*O*-b-D-glucopyranoside in soil was investigated (Kathryn Barto and Cipollini, 2009; Ozan et al., 1997; Shaw and Hooker, 2008). Primary degradation half-lives of isovitexin-6″-*O*-b-D-glucopyranoside in non-sterile soil were 11.2 and 8.9 h at 5 and 25 °C respectively (Kathryn Barto and Cipollini, 2009). The concentration of formononetin and biochanin A in non-sterile soil dropped to 60% and 20% of the initial concentration within 15 days (Ozan et al., 1997). In the study of Shaw and Hooker, the degradation of formononetin in non-sterile soil was faster with a reduction to 4.5% of the initial concentration within 72 h. Also, naringengin degraded to less than 1% of the initial concentration withhin 96 h in this study. Despite these first insights, a systematic investigation of the biodegradation of flavonoids with a standard biodegradability test and information on biodegradation in the aquatic phase are missing.

Readily biodegradability tests like the Closed Bottle Test (OECD 301 D) offer a stringent first tier to assess the fate of chemicals in the aquatic environment. Closed Bottle Test (CBT) results are, therefore, important for environmental risk assessment and for the design of benign chemicals (Leder et al., 2015). Although criticized for lacking knowledge on the bacteria in the inoculum, higher required concentration than usually present in the environment, limited reproducibility, the inability to reflect complex environmental conditions, especially in regard of the diversity of bacteria and the lack of alternative carbon sources, the fulfillment of the biodegradation criteria (oxygen demand in the test vessel $>$ 60% of Theoretical Oxygen Demand (ThOD)) is a good indicator for rapid and ultimate biodegradation in the environment (Kowalczyk et al., 2015).

Next to these indications for the biodegradability of flavonoids in soil, abiotic degradation of flavonoids was reported (Plaza et al., 2014; Sokolová et al., 2016; Xiao and Högger, 2015). Investigating the oxidation of luteolin and quercetin in aqueous solution, rate constants of 0.000183 s^{-1} (half-live = 63 min) and 0.00285 s^{-1} (half-live = 4 min) were determined (Ramešová et al., 2012). Additionally, a stability study of several flavonoids in cell culture medium reported substantial concentration decreases of quercetin, luteolin, kaempferol, baicalein, isorhamnetin, myricetin, and fisetin within 3 h, while other flavonoids including chrysin, apigenin, hesperetin, genistein and daidzein were more stable (Xiao and Högger, 2015). Based on these differences, structure-stability relationships were established (Xiao and Högger,

2015, Jun Hu, 2012; and Maini et al., 2012): Structural features increasing the degradation rate include an increasing number of hydroxy groups, a hydroxy group at C3, and a C2–C3 double bond. On the other hand, a C2–C3 single bond, glycosylation, and the methylation of multihydroxyflavonoids decrease degradation rates. Considering the reported fast abiotic degradation of certain flavonoids, abiotic degradation might occur during the lag phase of readily biodegradability tests. Thus, if only the ultimate biodegradation e.g. via oxygen consumption is measured, no distinction can be made between the biodegradation of the flavonoid itself and an abiotic formation of TPs, which are in turn biodegraded.

This study set out to deepen the understanding of the biodegradation of flavonoids as foundation for their utilization as benign substitutes of more harmful synthetic chemicals and the design of biodegradable chemicals (Fig. 1). For the first time, the ultimate biodegradation of 19 flavonoids was investigated with a CBT according to OECD guideline 301 D to provide standardized data on readily biodegradability. The set of flavonoids was selected based on structural features to enable an investigation of their influence on the biodegradation. The chosen flavonoids differ in the number (0–5) and positions of hydroxy groups (e.g., 3-OH, ortho or meta positioning in the B ring), the bond order of the C2–C3 bond, a methoxy group, and the position of the B ring in regard to the A and C ring core (isoflavonoid vs flavonoid) (Fig. 2). Addressing the critique of limited reproducibility of the results, the CBT was repeated with 9 randomly chosen flavonoids with a newly collected inoculum to investigate the reproducibility of the results.

Furthermore, for a better understanding of the processes in the CBT, the primary biodegradation of 4 selected flavonoids (luteolin, eriodictyol, diosmetin and hesperetin) was investigated by a more time-consuming concentration monitoring with HPLC-UV/vis at three different conditions representing biodegradation, abiotic degradation, and mixed substrates (alternative carbon source present) (Fig. 1). Comparing the primary biodegradation rate to the abiotic degradation rate might shed light on the question, whether the flavonoids itself or abiotic TPs formed during the lag phase are biodegraded. Additionally, the influence of an alternative carbon source as present in the toxicity controls in the CBT on primary biodegradation was investigated. The 4 selected flavonoids represent a group with the same core structure with hydroxy groups at position 5, 7, 3′ and 4′ but differ in regard of the C2–C3 bond order and O-methylation (Fig. 2). Hence, the influence of these two structural features on the primary degradation at the different conditions could be investigated. Furthermore, flavonoids with 4 hydroxy groups may be interesting for applications because they represent a compromise in solubility and stability.

2. Materials and methods

2.1. Chemicals and solvents

An overview of purchased flavonoids and their vendors is shown in the supplementary information (Table SI 1). All chemicals and solvents were used without further purification.

2.2. Aerobic biodegradation testing (OECD 301 D)

Aerobic biodegradation was determined using the Closed Bottle Test (CBT) according to the OECD guideline 301 D (OECD, 1992) as described previously (Grabitz et al., 2020). In brief, the mineral medium (pH buffered at 7.4) was prepared and inoculated with 2 drops of filtered (retention range: 5–8 µm) sewage effluent per liter medium. The sewage effluent was obtained from a domestic sewage treatment plant, size class 5. Stock solutions of test compounds were prepared in DMSO due to limited solubility of the test compounds at high concentrations in aqueous medium. In addition to the samples with test substances, a blank control (mineral medium with inoculum), a quality control containing acetate and a toxicity control containing acetate and the test substance were carried along. Samples and controls contained 1% (v/v) DMSO. The Theoretical Oxygen Demand (ThOD) for each test substance was 5 mg/L. Accordingly, the theoretical oxygen demand in toxicity controls was 10 mg/L. Actual oxygen concentration in the test vessels was monitored with a fibre-optic oxygen meter (Fibox 3, PreSens GmbH) for 28 days. The temperature was controlled at 20 ± 2 °C. Per run, duplicates of each sample and control were measured. To check reproducibility of the results, an additional independent CBT was performed

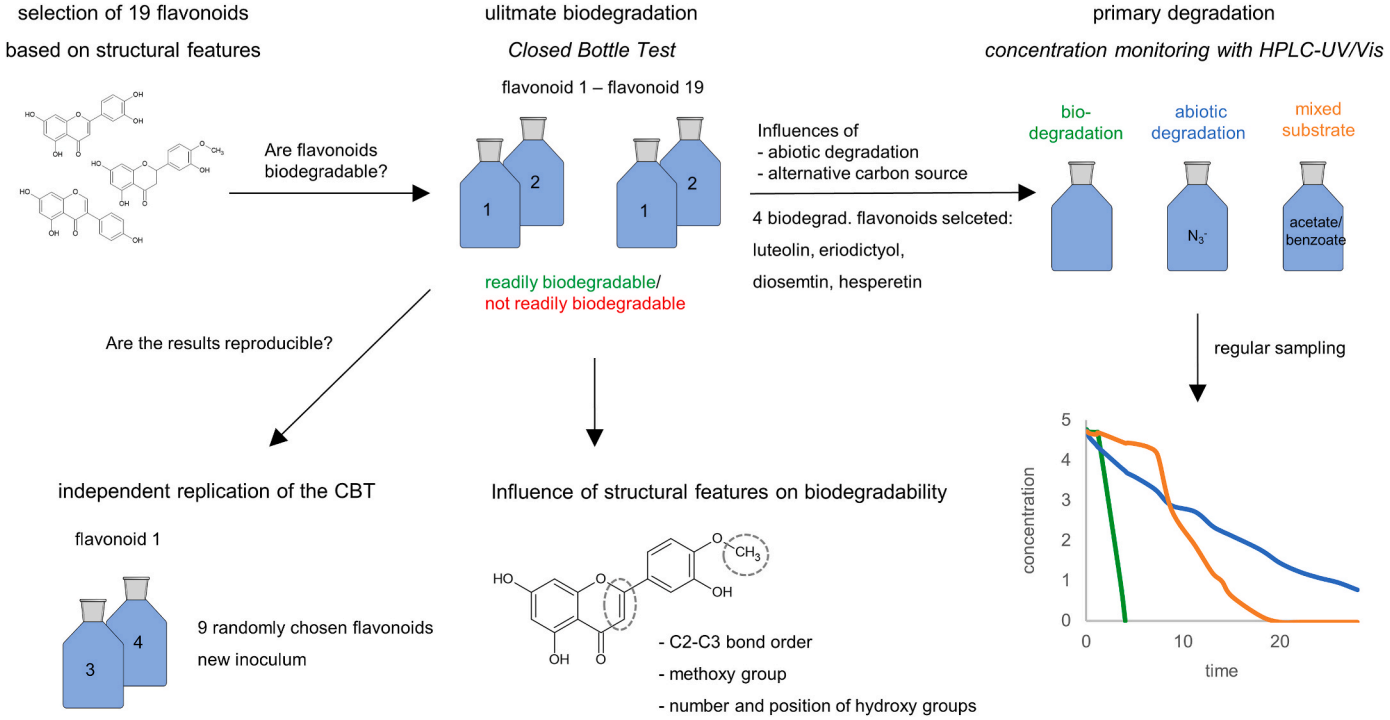

Fig. 1. Overview of study design and performed experiments.

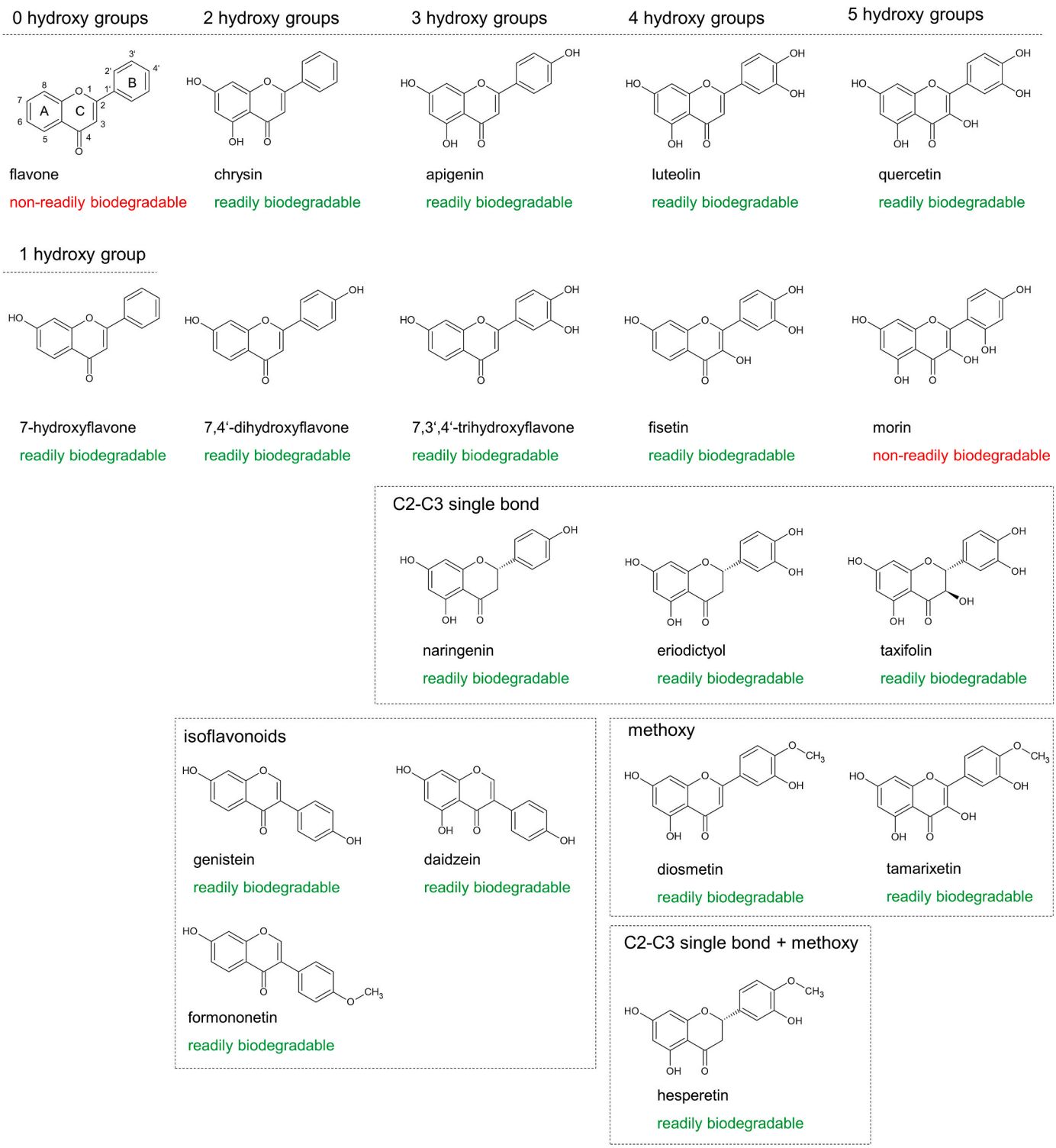

Fig. 2. Readily biodegradability tests results (OECD 301 D – CBT) of the 19 flavonoids. Flavonoids are arranged according to the number and position of hydroxy groups and further structural features to compare their influence on the biodegradation. O-methylated flavonoids are in the same column as their non-methylated counterparts. Below the substance name the CBT result is displayed as either readily biodegradable or non-readily biodegradable. For more details see Table SI 3.

at a different time point with 9 randomly chosen flavonoids yielding 4 replicates for these flavonoids in total. Biodegradation is expressed as measured oxygen consumption in % of ThOD (see equations (1)–(4)). Validity of results is given if the following criteria are fulfilled: oxygen depletion in blank control < 1.5 mg dissolved oxygen per L, differences of extremes of replicate values is less than 20%, and oxygen consumption in toxicity control > 25% ThOD (OECD, 1992). The pass level for the classification as readily biodegradable is an oxygen consumption > 60% of ThOD.

$$O_2 \text{ consumption} = c[O_2] \text{ day } 0 - c[O_2] \text{ day } x \quad (1)$$

$$\text{real consumption} = \text{consumption sample} - \text{consumption blank} \quad (2)$$

$$\%\text{biodegradation} = \%\text{ThOD} = (\text{real consumption} \times 100)/\text{ThOD} \quad (3)$$

$$\%ThOD_{toxicity\ control} = (real\ consumption \times 100)/(ThOD_{sample} + ThOD_{sodium\ acetate}) \quad (4)$$

2.3. Monitoring the concentration of selected flavonoids with HPLC-UV/vis

To investigate possible abiotic degradation and the influence of another carbon source, the concentration of 4 selected flavonoids was monitored at three different conditions representing biodegradation, abiotic degradation, and degradation in the presence of an alternative carbon source (mixed substrates) as in the CBT toxicity control. The four flavonoids eriodictyol, hesperetin, luteolin and diosmetin were chosen for this more detailed investigation. Using well established methods (Szultka et al., 2013), the concentrations of 4 selected flavonoids were monitored over 28 days using a HPLC-UV/vis system equipped with a UV/vis and PDA detector (Prominence-HPLC-UV-vis&PDA, Shimadzu, Duisburg, Germany).

At first, calibration curves of the 4 selected flavonoids correlating the peak area to the concentration were recorded (final concentrations: 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, 7.5 mg/L). Wavelengths used for quantification are shown in Table SI 1. Limit of quantification and detection (LOQ/LOD see Table SI 2) were calculated with DINTEST 2000 according to DIN 32645 (result uncertainty 33.3%, probability of error 5%), a standard statistical method based on linear regression of a calibration curve and confidence intervals (Brüggemann et al., 2010; Kolb et al., 1993). Additionally, a calibration curve of sodium benzoate was recorded at 275 nm, while sodium acetate could not be quantified with HPLC-UV/vis.

In order to compare CBT and HPLC-UV/vis results, concentration monitoring experiments were performed at conditions similar to the CBT. Biodegradation samples contained test substance and inoculum. Abiotic samples contained test substance, inoculum, and sodium azide. Poisoning of the sample with azide to obtain an abiotic control was chosen in order to account for abiotic interaction of the flavonoids with the inoculum e.g. reactions with metal ions or sorption to small organic particles. Azide is a suited poison for aerobic biodegradation tests because it targets the respiration chain of aerobe bacteria. Mixed substrate samples contained test substance, inoculum, and either acetate or benzoate. In addition to acetate used in the toxicity controls in the CBT, benzoate was selected as alternative carbon source due to its detectability with a UV/vis detector and alignment with the OECD guideline 301 D. A total volume of 50 mL of test solutions were transferred in 100 mL brown glass bottles and stored in the dark at 20 °C ± 2. Bottles were opened for the withdrawal of 500 μL samples at time intervals ≥1 day. Gas exchange with the atmosphere was possible ensuring a consistent access to oxygen. Concentration of tested flavonoids was increased to 5 mg/L in comparison to approximately 3 mg/L in the CBT to have initial concentrations with higher distance to LOQ and LOD. Sodium azide in abiotic controls had a concentration of 320 mg/L. Sodium acetate or sodium benzoate had a concentration of 6.4 and 3.0 mg/L, respectively. Biodegradation and abiotic samples were tested in 3 independent runs (n = 3). Mixed substrate samples were tested twice for each alternative carbon source (n = 2). In each run, samples containing the 4 flavonoids were tested in parallel with the same inoculum. However, due to some irregularities in the third run, additional replicates of luteolin and diosmetin were performed (see color coding in Figure SI 2).

Calibration standards and samples were measured at the HPLC-UV/vis using a phenyl-hexyl column (Nucleodur Phenyl-Hexyl, 125 mm length, 3 mm diameter, 3 μm particle size, Macherey-Nagel) with respective pre-column according to the following parameters: 50 μL injection volume; mobile phase A: water with 0.1% formic acid, mobile phase B: methanol; gradient: 0–1 min, 5% B; 1–10 min raise to 100% B, 10–13 min 100% B, 13–15 min drop to 5% B, 15–18 min 5% B; column temperature = 35 °C, flow = 0.5 mL/min.

3. Results and discussion

3.1. Ultimate biodegradation of flavonoids

The biodegradation of 19 flavonoids was investigated with the Closed Bottle Test (OECD 301 D). Based on the measurements meeting all validation criteria, 17 out of the 19 tested flavonoids were classified as readily biodegradable (Fig. 2, Table SI 3). Flavone and morin showed an oxygen consumption lower than 60% of ThOD and were, therefore, classified as non-readily biodegradable (Fig. 2, Table SI 3).

The course of the oxygen consumption of the four flavonoids (luteolin, eriodictyol, diosmetin, hesperetin) investigated in more detail (see section 3.2 and 3.3) and of the two non-readily biodegradable flavonoids is presented in Fig. 3. Samples containing eriodictyol, hesperetin, luteolin and diosmetin showed a fast oxygen consumption up to 50% of the ThOD within 5 days. This course is comparable to the quality control containing acetate, therefore, flavonoids seem to be as easily metabolized as acetate with no long lag phase required for the bacteria to adapt to flavonoids as carbon source. In toxicity controls, the oxygen consumption also exceeded 60% of ThOD demonstrating that the flavonoids did not inhibit the biodegradation of acetate and, moreover, indicating that acetate and the flavonoid are metabolized by the bacteria. After an initial increase in oxygen consumption, toxicity controls of eriodictyol and hesperetin displayed a small plateau lasting 3 days before oxygen consumption rose again (Fig. 3). This suggests that acetate was degraded by the bacteria prior to these flavonoids and the 3 days represent the time needed by the bacteria to adapt their metabolism from acetate to the flavonoid as carbon source (see also section 3.3).

Flavone samples showed no oxygen consumption. In morin samples the oxygen consumption increased steadily over the whole test period reaching 14% of ThOD at day 28. The toxicity controls of flavone and morin showed oxygen consumptions higher than 25% of ThOD demonstrating that flavone and morin had no adverse effect on the bacteria of the inoculum at a concentration of approximately 3 mg/L (Fig. 3). Consequently, the bacteria present in the test were unable to degrade flavone and morin. Thus, flavone and morin were classified as non-readily biodegradable.

To investigate the reproducibility of the CBT results, additional independent replicates of the CBT were performed for 9 randomly chosen flavonoids (flavone, 7-hydroxyflavone, chrysin, 7,4′-dihydroxyflavone, apigenin, eriodicytol, luteolin, hesperetin, daidzein). The results of these replicates yielded the same classification as in the first run demonstrating the good reproducibility of CBT results for flavonoids (Table SI 3). Hence, the tested flavonoids were either readily biodegradable or non-readily biodegradable despite possible differences in the bacterial inoculum of the independent replicates of the CBT. Due to the very good agreement no further additional replicates were run.

Biodegradation of flavonoids in the aquatic phase has not been investigated before. Our results show that the CBT is a suited method to investigate the readily biodegradability of flavonoids with sufficient solubility. Since most of the tested flavonoids were readily biodegraded at the experimental conditions representing a low amount and diversity of bacteria, it is very likely that bacteria able to degrade flavonoids are abundant in the environment, too.

The obtained results for the aquatic phase are in good agreement with previous investigations on the biodegradation of flavonoids by soil bacteria known for their interaction with plants like rhizobia, agrobacteria, and pseudomona species (Barz, 1970; Rao and Cooper, 1994; Shaw and Hooker, 2008). Apigenin, naringenin, 7,4′-dihydroxyflavone, luteolin, quercetin, daidzein, genistein and formononetin that were investigated and found biodegradable in these previous studies with soil bacteria, were also readily biodegradable in the CBT with a bacterial inoculum taken from a sewage treatment plant effluent. The non-readily biodegradable flavonoids flavone and morin were investigated for the first time in this study. Further work could investigate the possible

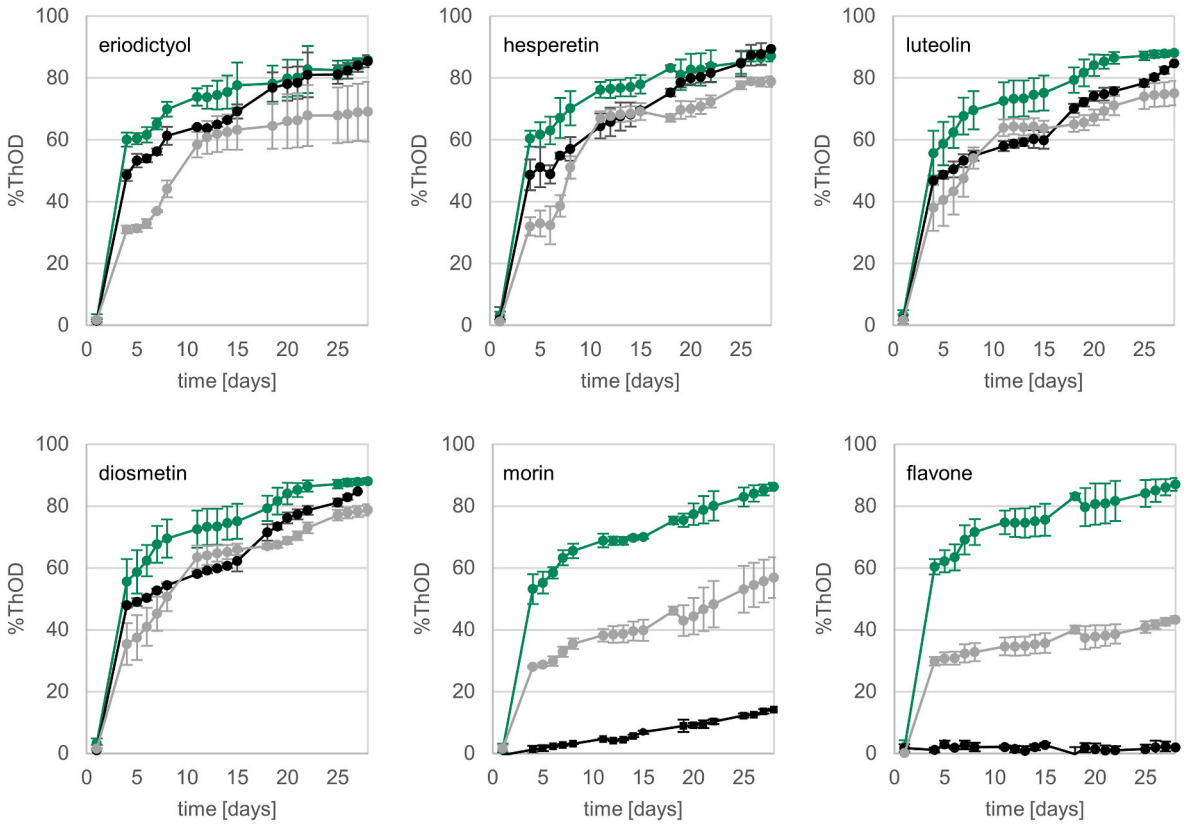

Fig. 3. Course of oxygen consumption in % of Theoretical Oxygen Demand (ThOD) of the 4 flavonoids analyzed in more detail and the two non-readily biodegradable flavonoids over the time period of 28 days. Quality control containing acetate (green), test substance (black) and toxicity control containing test substance and acetate (grey) are displayed. Data points represent average values with standard deviations (SD, n = 4 except morin with n = 2).

biodegradation of these two flavonoids at different conditions e.g. inherent biodegradability tests comprising larger microbial numbers and diversity.

The readily biodegradability of the majority (17 out of 19) of the flavonoids tested in this study and the high reproducibility of the results further support a wide distribution of the ability to degrade flavonoids among bacteria or a wide distribution of certain bacteria able to degrade flavonoids among different environmental compartments.

Comparison of CBT results shed light on the influence of the number and position of hydroxy groups and further structural features on the readily biodegradability of flavonoids (Fig. 2, Table 1). The non-readily biodegradability of flavone demonstrated the need for at least one hydroxy group. Besides the number of hydroxy groups, other structural differences of the tested flavonoids including the positioning of the hydroxy groups, the C2–C3 bond order, a methoxy group in the B ring, and the presence of a 3-OH did not affect their biodegradation (Table 1). Comparing the isoflavonoids daidzein and genistein to their flavonoid counterparts 7,4′-dihydroxyflavone and apigenin revealed that the position of the ring B in regard to the chromene core also had no influence on their biodegradation (Fig. 1). While the tested structural features had no influence on biodegradability, a lacking 3-OH group, a C2–C3 single bond and a methoxy group are known to increase abiotic stability (Plaza et al., 2014; Xiao and Högger, 2015). Therefore, these structural features could be used to design flavonoids that exhibit a sufficient stability for applications but are readily biodegradable.

Out of the 19 tested flavonoids, only flavone and morin were non-readily biodegradable. Flavone occurs naturally e.g., in *Feijoa sellowiana* and *Cipadessa fruticose* (Leite et al., 2010; Ruberto and Tringali, 2004). Its observed non-biodegradability in contrast to the biodegradation of 7-hydroxyflavone is in line with the rule of thumb that hydroxylation enhances biodegradability (Boethling et al., 2007).

Table 1
Overview of the influence of certain structural features of flavonoids on their biodegradation. The structures of the flavonoids can be found in Fig. 2.

structural feature	influence on biodegradation	considered flavonoids
number of hydroxy groups	• no hydroxy groups – non-readily biodegradable • 1–5 hydroxy groups – biodegradable (but one exception)	• flavone • all others except morin
position of hydroxy groups	• no influence on biodegradation	• chyrsin vs. 7,4′-dihydroxyflavone • apigenin vs. 7,3′,4′-trihydroxyflavone • luteolin vs. fisetin
hydroxy groups in position 2′,4′(B-ring)	• can hinder biodegradation	• morin
3-OH	• no influence on biodegradation	• luteolin vs. quercetin • eriodictyol vs. taxifolin • 7,3′,4′-trihydroxyflavone vs. fisetin
methoxy group	• no influence on biodegradation	• luteolin vs. diosmetin • eriodictyol vs. hesperetin • quercetin vs. tamarixetin • genistein vs. formononetin
C2–C3 bond order	• no influence on biodegradation	• luteolin vs. eriodictyol • diosmetin vs. hesperetin • quercetin vs. taxifolin
position of B-ring (C2 vs. C3)	• no influence on biodegradation	• daidzein vs. 7,4′-dihydroxyflavone • genistein vs. apigenin

Looking into the case of morin, its outstanding structural feature is the 2′,4′-meta positioning of the two hydroxy groups in the B ring. The meta positioning influences on the one hand, the geometry and, on the other hand, the redox potential of the flavonoid. Quantum mechanical calculations for morin and its isomer quercetin provided insights into the influence of the meta positioning of hydroxy groups in the B ring on the conformation. While the B ring with hydroxy groups at position 3′ and 4′ in quercetin is able to rotate rather freely around C2–C1′ bond

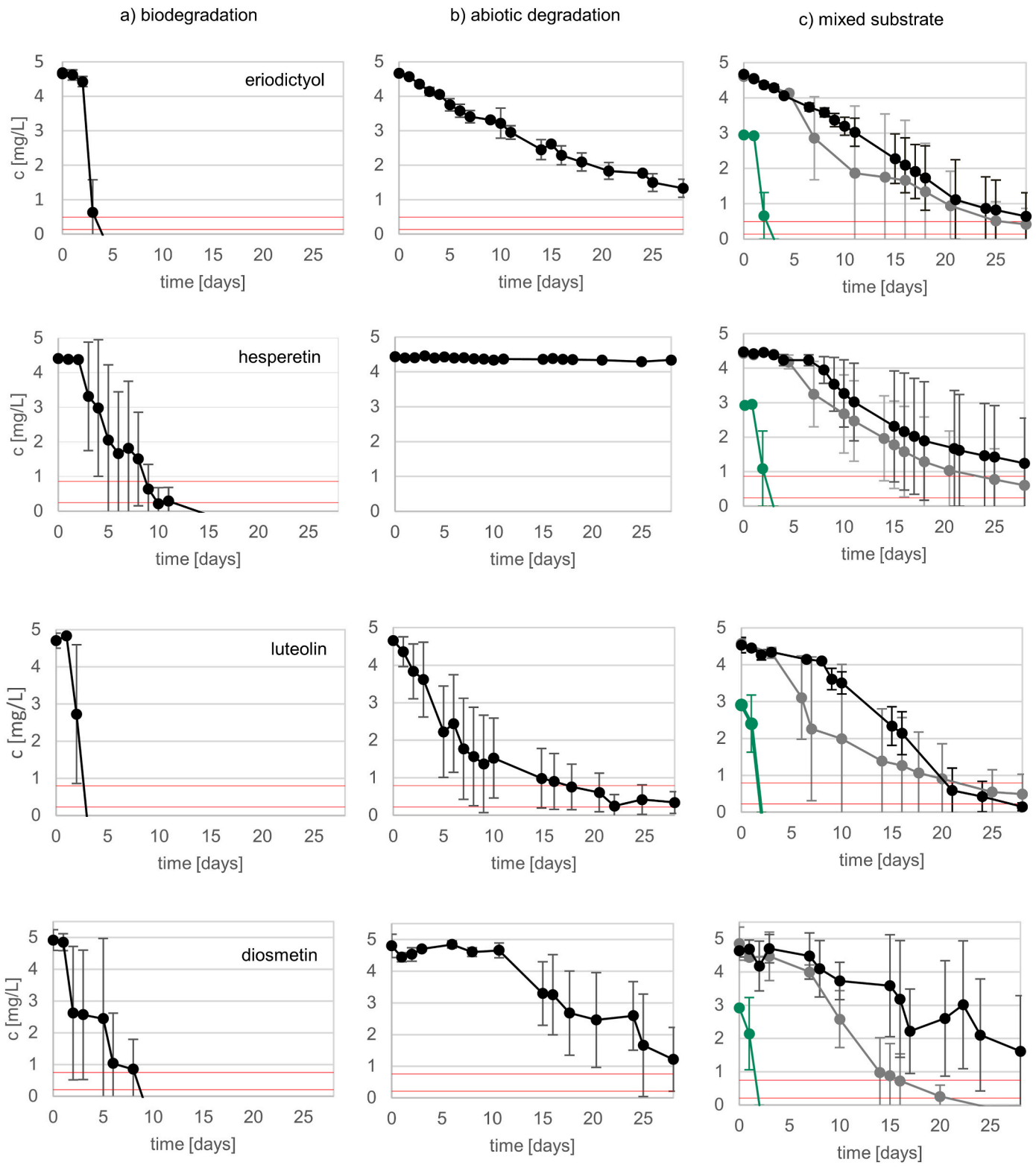

Fig. 4. Course of concentration of the 4 flavonoids eriodictyol, hesperetin, luteolin, and diosmetin in a) biodegradation (test substance + inoculum), b) abiotic (test substance + inoculum + azide) and c) mixed substrate samples (test substance + inoculum + acetate(grey)/benzoate(black)). In addition to the flavonoid concentration in the mixed substrate samples, the concentration of benzoate was measured (green). Datapoints represent average values with standard deviations (n = 3, n = 2 for acetate and benzoate mixed substrate samples, respectively).

(Brovarets and Hovorun, 2019, 2020), this rotation is constrained by the hydroxy group at position 2′ in morin that, therefore, favors a non-planar conformation (Dimitrí C Marković et al., 2013). Computational modeling and the analysis of a crystal structure of morin indicate an intramolecular hydrogen bond between the 3-OH and the 2′OH in this non-planar conformation, however, it has to be kept in mind that these approaches are limited in the considerations of solvent effects and hence, H-bonding in aqueous solutions may differ (Cody and Luft, 1994; Dimitrí C Marković et al., 2013). In line with reported substrate specificity of enzymes for certain constitutional isomers (Kim et al., 2020), a possible explanation for the non-biodegradability of morin may be that the non-planarity sterically hinders the entry or binding to the catalytic center of the required enzyme. Going beyond the comparison of the two isomers morin and quercetin, there are other flavonoids, in particular naringenin, eriodictyol, taxifoin and hesperetin, that exhibit a non-planar conformation due to the saturated C2–C3 bond but were readily biodegradable. However, knowledge on the enzymes involved in the aerobic biodegradation of flavonoids is scarce. A quercetinase, catalyzing the degradation of quercetin to the depside and carbon monoxide, was identified in *Aspergillus* species and two bacterial strains, *Bacillus subtilis* and a *Streptomyces* species (Bowater et al., 2004; Merkens et al., 2007; Tranchimand et al., 2010) These studies indicate substrate specificity of the quercetinase towards flavonols while e.g. luteolin and taxifolin were not degraded. Additionally, conversion of morin by the *Spretomyces* quercetinase was severely slower than quercetin conversion. Further studies are needed that reveal more details on the substrate specificity of the quercetinase and that identify the enzymes involved in the degradation of flavones, flavanonols, and flavanones.

Furthermore, morin showed a lower antioxidant activity than quercetin (Kummer et al., 2014; Rice-Evans et al., 1996). This may be because the 3′,4′-ortho substituted B ring is able to from a chinone structure while this is not possible for the 2′,4′-meta substitution in the B ring of morin. Therefore, the lower antioxidant activity of morin may also contribute to its non-biodegradability. To further investigate whether a meta position of hydroxy groups in the B ring hinders biodegradation, the biodegradation of other flavonoids also comprising this structural feature like norartocarpetin and steppogenin should be investigated. However, these two flavonoids are not purchasable, and their synthesis or isolation was beyond the scope of this study.

3.2. Comparison of primary biotic and abiotic degradation of 4 selected flavonoids

The four flavonoids eriodictyol, hesperetin, luteolin, and diosmetin were investigated in more detail to explore if the flavonoids themselves were biodegraded in the CBT or if severe abiotic degradation of the flavonoids occurred during the lag phase leading to the formation of TPs, which are then biodegraded. Monitoring of eriodictyol, hesperetin, luteolin, and diosmetin showed a concentration decrease below LOD in the biodegradation samples (test substance + inoculum) within 4–15 days (Fig. 4a). In abiotic samples (test substance + inoculum + azide), the concentration of hesperetin was constant while abiotic degradation of eriodictyol, luteolin, and diosmetin occurred (Fig. 4b). However, the concentration decrease of these 3 flavonoids was clearly slower than in biodegradation samples (Fig. 4a and b).

In more detail, eriodictyol was degraded after a lag phase of 1–2 days within 2 days in all 3 biodegradation replicates. This extraordinary good reproducibility between independent experiments is reflected by the low standard deviation in Fig. 4. Individual replicates are shown in Figure SI 2. In abiotic samples, the eriodictyol concentration steadily decreased without lag phase to 1.3 ± 0.3 mg/L at day 28.

Hesperetin was degraded after a lag phase of 2 up to 7 days within 2–8 days. Individual replicates are shown in Figure SI 2. Variance between the three independent replicates was higher as for eriodictyol, especially in regard to the duration of the lag phase, reflected by high error bars in Fig. 4a. In abiotic samples, the hesperetin concentration remained constant demonstrating the low reactivity of this flavonoid.

Luteolin was degraded after a lag phase of 1–2 days within 1–3 days in the biodegradation samples (Figure SI 2). In abiotic samples, the concentration decrease was slower than in biodegradation samples but varied between the replicates (Figure SI 2). While two replicates are comparable and luteolin was still detectable at day 28, in one replicate, luteolin concentration was below LOQ at day 6. Variance in abiotic samples may be due to differences in the medium caused by the addition of the inoculum. In addition to bacteria, e.g., metal ions or small organic particles can be transferred may leading to degrading reactions or sorption (Pę;kal et al., 2011; Rothwell et al., 2005). Despite the rather high variance in the abiotic degradation replicates, degradation of luteolin in biodegradation samples is clearly faster than in abiotic samples.

Diosmetin was degraded after a lag phase of 1–4 days within 2–4 days in the biodegradation samples (Figure SI 2). In the abiotic samples, the concentration remains above 4 mg/L until day 10, subsequently concentration decrease fastens but differently. In two of the replicates, the concentration remained above LOQ till day 28, while in the third replicate, concentration is below LOD after day 25. Noteworthy, the abiotic replicates of diosmetin and luteolin which vary the most from the other two replicates were not part of the same parallel run. Thus, the differences were not caused by a variation specific to one run. Despite the high variance in abiotic samples, degradation of diosmetin in biodegradation samples is clearly faster than in abiotic samples.

Comparing the biodegradation of the 4 flavonoids, eriodictyol and luteolin are degraded faster than hesperetin and diosmetin and their results showed higher reproducibility. In all biodegradation replicates of eriodictyol and luteolin, fast degradation occurred after short lag phases (≤2 day). In contrast, lag phases longer than 3 days were only observed for hesperetin and diosmetin. However, both compounds were also degraded after short lag phases (≤2 days) (Figure SI 2). The course of the oxygen consumption during the CBT of hesperetin and diosmetin does not indicate longer lag phases than for the other tested flavonoids (Fig. 3). Variations in biodegradation test results can be attributed to differences in the bacterial inoculum between the independent runs (Kowalczyk et al., 2015). The higher reproducibility in the biodegradation experiments of eriodictyol and luteolin suggests that the ability to degrade these two flavonoids is more widely distributed among bacteria than the ability to degrade diosmetin and hesperetin.

The observed abiotic degradation of eriodictyol, luteolin and diosmetin is consistent with other studies indicating low stability of several flavonoids (Maini et al., 2012; Plaza et al., 2014; Ramešová et al., 2012; Sokolová et al., 2016; Xiao and Högger, 2015). However, at the experimental conditions of the CBT, abiotic degradation was much slower in comparison to the studies Ramešová et al. (2012); Xiao and Högger (2015). Only hesperetin was abiotically stable supporting previous findings (Xiao and Högger, 2015). The constant concentration of hesperetin in the abiotic samples also demonstrated that azide efficiently inhibited bacteria capable of flavonoid biodegradation. The degradation of eriodictyol, luteolin and diosmetin in abiotic samples may be due to reactions with oxygen and the present azide ions. However, nucleophile substitutions or Michael-like reactions of the azide and the flavonoids might be hindered due to the electron-richness of the aromatic rings, the unsuitability of hydroxide ions as leaving groups, and a sterically hindrance of C2 due to the attached phenyl moiety. Hence, oxidation of flavonoids seems to be the major cause of abiotic degradation. Even if reactions with azide contributed to the observed concentration decrease in abiotic controls, the primary biodegradation of flavonoids is much faster than the abiotic degradation. Therefore, the experiments indicate that the flavonoids themselves are metabolized by the bacteria.

3.3. Primary degradation of 4 selected flavonoids in the presence of an alternative carbon source

Concentration decreases of the four selected flavonoids in the

presence of acetate and benzoate as alternative carbon sources were analyzed (mixed substrate samples). Average concentrations of two independent replicates for each alternative carbon sources are shown in Fig. 4c. High error bars reflect that this more complex system gave rise to higher deviations. However, in all mixed substrate samples of the 4 tested flavonoids, degradation was slower than in biodegradation samples. Details in the concentration decreases of each tested flavonoid are highlighted in the following paragraphs.

In all mixed substrate replicates of eriodictyol, eriodictyol was present longer than in the biodegradation samples (Figure SI 2c). Two replicates (A-yellow and B-orange) showed a similar concentration decrease to the abiotic samples indicating that no biodegradation of eriodictyol occurred in these two replicates. In the other two replicates (A-blue and B-yellow) degradation was faster than in abiotic samples indicating that biodegradation occurred in addition to abiotic degradation. However, degradation was slower than in biodegradation samples. Furthermore, these replicates suggest that eriodictyol was only biodegraded after acetate or benzoate were consumed. This is in line with the small plateau in the course of the oxygen consumption in the CBT toxicity control (Fig. 3). Overall, the results show that the presence of an alternative carbon sources delayed biodegradation of eriodictyol on a scale that abiotic degradation largely contributed to observed concentration decreases.

Hesperetin concentration decreased in the mixed substrate samples demonstrating that biodegradation occurred (Fig. 4c). Due to a long lag phase of 7 days in one biodegradation replicate, it remains ambiguous if acetate or benzoate prolonged the lag phase of hesperetin biodegradation. However, benzoate itself was degraded faster than hesperetin and not detectable anymore at day 3. The fast degradation of benzoate suggests that this is the preferred carbon source and hesperetin was degraded subsequently. The course of oxygen consumption in CBT toxicity controls with a small plateau lasting 3 days further supports that hesperetin was only biodegraded after acetate was consumed (Fig. 3). Despite the differences between individual replicates, in all mixed substrate samples, degradation of hesperetin was slower than in biodegradation samples (Figure SI 2).

In all mixed substrate replicates of luteolin, luteolin was present longer than in the biodegradation samples (Figure SI 2c). In 3 of the 4 mixed substrate samples, luteolin concentration decreased comparable to abiotic controls suggesting that abiotic degradation was the main driving force behind the concentration decrease. However, in one acetate replicate (A-blue), degradation of luteolin was clearly faster than in the other mixed substrate samples. This observation indicates that biodegradation of luteolin occurred in this mixed substrate replicate but was slower than in biodegradation samples.

In all mixed substrate replicates of diosmetin, diosmetin was present longer than in the biodegradation samples (Figure SI 2c). Concentration decreases in mixed substrate samples were comparable to abiotic controls. However, mixed substrate samples and abiotic controls show high deviations between the independent runs (Figure SI 2b and c). Additionally, one mixed substrate replicate (B-orange) displayed some irregularities suggesting that additional abiotic processes to oxidation occurred.

In summary, abiotic degradation seems to contribute substantially to the observed concentration decreases of the abiotically unstable flavonoids eriodictyol, luteolin and diosmetin at mixed substrate conditions. In contrast to this indicated high contribution of abiotic degradation in mixed substrate samples, the high oxygen consumption in CBT toxicity controls (eriodictyol: 69% ± 10 (n = 4), luteolin: 75% ± 4 (n = 4), diosmetin: 70% ± 2, (n = 2)) suggest that acetate and the flavonoids were metabolized by the bacteria (Fig. 3). According to the abiotic, oxidative degradation pathway of flavonoids without a 3-OH to depsides and a subsequent hydrolysis to benzoic acids proposed by Sokolová et al. (2016), luteolin and diosmetin abiotically degrade to 2,4,6-trihydroxybenzoci acid and 3,4-dihydroxybenzoic acid or 3-hydroxy-4-methoxybenzoic acid, respectively. A degradation pathway of eriodictyol is not available in the literature. This abiotic degradation of luteolin and diosmetin requires 0.22 g O_2 per g luteolin and 0.21 g O_2 per g diosmetin. The theoretical oxygen demand for an oxidation to CO_2 as expected in biodegradation is much higher with 1.6 and 1.7 g O_2 per g luteolin and diosmetin, respectively. The abiotic oxidation of luteolin and diosmetin as described above would represent 7 and 6 % of the ThOD in the toxicity controls. Hence, it seems reasonable that abiotically unstable flavonoids are oxidized abiotically with a low oxygen demand to transformation products which are then degraded biotically with a higher oxygen demand.

In the mixed substrate sample in which biodegradation of the flavonoids occurred, the presence of acetate or benzoate tents to delay (longer lag phase) the biodegradation and decrease its rates (less steep concentration decreases). This result is in agreement with the phenomenon of carbon catabolite repression that enables bacteria to selectively use the carbon source allowing the most efficient growth (Görke and Stülke, 2008; Markiewicz et al., 2011; Rojo, 2010; Stülke and Hillen, 1999). Alternative carbon sources are available to the bacteria in the environment and, hence, for reactive flavonoids like eriodictyol, luteolin and diosmetin which also degraded abiotically biodegradation may not be the rate determine step at more complex conditions as present in the environment. Instead, abiotic degradation products like 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid might be formed (Sokolová et al., 2016). These are the same products as obtained during biodegradation of flavonoids by soil bacteria which enter the β-ketoadipate pathway and are further metabolized (Cooper, 2004; Rao and Cooper, 1994). Therefore, incomplete abiotic degradation in the environment might produce TPs which are easily biodegraded by bacteria.

4. Conclusion

Although flavonoids are often proposed e.g. as biopesticides due to anticipated non-persistence in the environment, experimental data on the environmental fate of flavonoids is scarce. Addressing this knowledge gap, the aim of this study was to systematically investigate the ultimate biodegradation (CBT) of 19 flavonoids in regard of the influence of common structural features and to investigate the primary biodegradation (concentration monitoring with HPLC-UV/vis) of 4 selected flavonoids in more detail as foundation to evaluate the potential of flavonoids as benign substitutes for persistent synthetic chemicals.

For the first time, ultimate biodegradation of flavonoids was investigated with a standardized readily biodegradability test (OECD 301 D). Since the CBT is a standard method to assess the biodegradability, the obtained data can be useful for the improvement of biodegradability prediction tools.

The obtained insights into the influence of structural features on biodegradation offer important knowledge for the selection of candidates suited as benign substitutes. Balancing abiotic stability and biodegradability, we identified flavonoids with abiotic stability-enhancing structural features including a lacking 3-OH, a C2–C3 single bond, and a methoxy group in the B ring to be suited as benign substitutes. Especially hesperetin that combines these three structural features was readily biodegradable but stable over 28 days in abiotic controls and is, therefore, a promising candidate for application in pharmacy or agriculture.

The comparison of primary biodegradation and abiotic degradation of the four selected flavonoids luteolin, eriodicytol, diosmetin, and hesperetin allowed the conclusion that the flavonoids themselves and not abiotically formed TPs are metabolized by the bacteria during the CBT.

However, the CBT and, thus, the additionally performed concentration monitoring at the same conditions, require higher concentrations of the test substances than usually present in the environment. Hence, in the environment, it is possible that due to low flavonoid concentrations and the availability of other carbon sources, genes involved in the

catabolism of flavonoids are not expressed by the bacteria. Due to carbon catabolite repression and the low stability of several flavonoids, abiotic degradation might be the dominant degradation process for unstable flavonoids in the environment. However, incomplete abiotic degradation in the environment might produce TPs which are easily biodegraded by bacteria. Concludingly, it is likely that flavonoids are non-persistent in the environment. However, more research is needed to fully understand the lifetime and fate of flavonoids in the environment and link the results of this study to the occurrence of flavonoids in natural water bodies.

CRediT authorship contribution statement

Lena Schnarr: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Oliver Olsson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Klaus Kümmerer:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142234.

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