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Flavonoids as benign substitutes for more harmful synthetic chemicals - effects of flavonoids and their transformation products on algae

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ABSTRACT

Flavonoids, a group of plant secondary metabolites, are seen as chemicals for pharmaceutical, nutraceutical, and pesticidal applications. Due to their natural origin, anticipated low ecotoxicity and non-persistence in the environment, they are considered as benign substitutes for synthetic chemicals. However, data on ecotoxicity is still scarce. Therefore, this study aimed for a better understanding of the bioactivity of flavonoids and thus allowing a better evaluation of their potential as benign substitutes. The growth inhibition of the microalgae *Raphidocelis subcapitata* induced by 13 flavonoids, 10 flavonoid mixtures, and 4 flavonoid transformation products was determined according to the OECD guideline 201. EC_{50} values of flavonoids ranging from 0.7 to 22 mg/L were moderate in comparison to the synthetic algaecides terbutryn and diuron. Mixtures of flavonoids behaved in a dose-addition manner. Moreover, the fate of 11 selected flavonoids during the growth inhibition test was analyzed by analytical methods (HPLC-UV/vis and HPLC-HRMS). Concentration monitoring revealed fast but incomplete degradation of 8 flavonoids. Overall, 25 transformation products of the degrading flavonoids were identified including 5,7-dihydroxychromone. Comparison of structural features indicate that the combination of a methoxy group and a lacking hydroxy group at C3 decreased degradation rates. Growth inhibition of the 4 tested transformation products was weaker than of their respective parent flavonoids. Taken together, the results suggest low ecotoxicity of flavonoids and promote further research towards their utilization as benign substitutes.

1. Introduction

The design of safer chemicals preserving the efficacy of function while reducing the toxicity is one of the principles of green chemistry. In line with this principle, substitution of synthetic chemicals with natural products offers a possibility to prevent environmental damage due to assumed low ecotoxicity and non-persistence of nature derived compounds. To find natural products suitable as benign substitutes, understanding of their bioactivities has a pivotal role for two reasons: Firstly, in finding more benign molecules for

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possible applications and secondly, in assessing ecological impacts on non-target organisms (Harvey et al., 2015; Klaschka, 2016; Lahlou, 2007; Santana-Méridas et al., 2012; Shi et al., 2023).

Flavonoids are a large class of natural products that is widely studied for a variety of applications in the pharmaceutical, health, and food sector and as biopesticides (Gonzalez-Paramas et al., 2019; Górniak et al., 2019; Guven et al., 2019; Juca et al., 2020; Maleki et al., 2019; Schnarr et al., 2022). According to the strict definition of the International Union of Pure and Applied Chemistry (IUPAC), flavonoids are composed of a chromane or chromene with an additional phenyl group (Rauter et al., 2018). The C6–C3–C6 carbon framework forms three rings referred to as A, B and C (Fig. 1). Based on the position of the phenyl group (C2, C3 or C4) compounds are categorized into flavonoids, isoflavonoids, and neoflavonoids. Differences regarding a keto structure at position 4, the number and position of hydroxy groups, methylation, glycosylation, and further modifications give rise to the high structural diversity of flavonoids.

Due to the prevalence of flavonoids in plants, they could be obtained from green biorefinery processes of waste materials as high value products (Alwazeer and Elnasanelkasim, 2023; Banerjee et al., 2017; Günthardt et al., 2021; Zuin et al., 2018). While most research focused on pharmaceutical applications of flavonoids, the last two decades have seen a growing interest in their utilization as biopesticides (Schnarr et al., 2022). Investigations of pesticidal activities mainly focused on bactericidal, fungicidal, insecticidal, and herbicidal activities (Schnarr et al., 2022). In addition to isolated compounds, the utilization of flavonoid containing extracts has been studied by several researchers (Chen et al., 2019; Gómez-Maldonado et al., 2020; Ningombam et al., 2017; Segatto et al., 2022). Interactions of the different constituents in an extract can led to mixture effects, in particular dose addition, synergism, or antagonism. The former two may enhance the activity of the extract in comparison to the isolated compounds. Following this approach, the investigation of mixtures and extracts is of special interest.

In contrast to the pesticidal activities of flavonoids mentioned above, there is much less information available about the effect of flavonoids on algae. However, this is of interest on the one hand to evaluate the proposed application of flavonoids as algaecides (Chen et al., 2019; Li et al., 2020; Xiao et al., 2019; Zhu et al., 2019). On the other hand, regarding the unintended entry in the environment after application as pharmaceutical or pesticide, information on adverse effects on algae as primary producers provide indispensable insights for ecotoxicity evaluation. Previous studies on the effects of flavonoids on algae focused on the control of harmful algae blooms by the marine algae species *Phaeocystis globosa* and the cyanobacterium *Microcystis aeruginosa* (Chen et al., 2019; Huang et al., 2016; Li et al., 2020, 2021; Xiao et al., 2019; Yu et al., 2019; Zhu et al., 2019). The effect of flavonoids on an algae species frequently used for aquatic toxicity assessments was investigated only by D'Abrosca et al., who found adverse effects of 9 flavonoids on the standard test organism *Raphidocelis subcapitata* by determining diameters of non-growth zones in a petri dish assay (D'Abrosca et

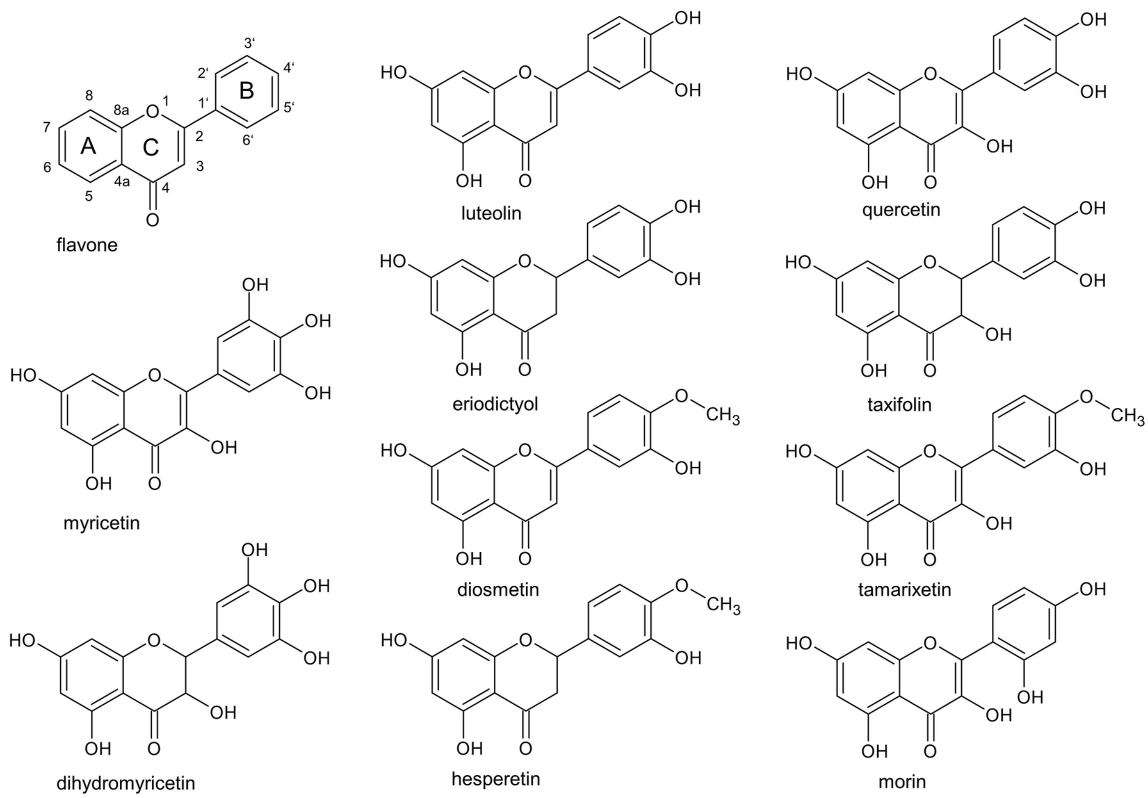


Fig. 1. Structures of the 11 flavonoids selected for a detailed analysis after algae growth inhibition screening of 26 flavonoids in this study. Selected flavonoids differ in the number of hydroxy groups, C2–C3 bond order, presence of a hydroxy group at C3 (3-OH), methoxy groups in the B ring, and positioning of hydroxy groups in the B-ring (quercetin vs morin).

al., 2006). Despite the study of D'Abrosca et al., reporting similar toxicity of the most active flavonoid catechin to the known algaecide $CuSO_4$, the other performed studies (Chen et al., 2019; Huang et al., 2015; Li et al., 2020, 2021; Xiao et al., 2019; Yu et al., 2019; Zhu et al., 2019) on *P. globosa* and *M. aeruginosa* lack positive controls which are needed for a better classification of the observed toxic effects. Furthermore, there has been no investigation on the toxicity of flavonoids according to the standardized guideline 201 of the Organization for Economic Cooperation and Development (OECD) (OECD, 2004). Hence, no data on algae toxicity obtained with standardized test protocols reporting easily comparable parameters like the half maximal effective concentration (EC_{50} value) is currently available for flavonoids. However, this data would provide a solid basis for more comprehensive research towards environmental risks of flavonoids.

Another important aspect that got little attention in the above mentioned studies (Chen et al., 2019; Huang et al., 2015; Li et al., 2020, 2021; Xiao et al., 2019; Yu et al., 2019; Zhu et al., 2019) and investigation of *in vitro* bioactivities of flavonoids in general, is the consideration of the chemical properties of flavonoids. Important properties of flavonoids include the limited solubility in aqueous solution (Plaza et al., 2014), the acidic character (Mezzetti et al., 2011; Musialik et al., 2009), the ability to form metal complexes (Kasprzak et al., 2015), their redox activity, and their oxidative degradation (Sokolová et al., 2016). Hence, observed effects may be attributed not only to the tested flavonoid itself but metal complexes or transformation products (TPs). Decreases in concentration of flavonoids in mammalian cell culture medium (Jun Hu, 2012; Maini et al., 2012; Xiao and Högger, 2015) were previously reported. Xiao et al. proposed structure-stability relationships (Xiao and Högger, 2015): An increasing number of hydroxy groups, a hydroxy group at C3 (3-OH), and a C2–C3 double bond led to shorter half-lives of flavonoids in the cell culture medium Dulbecco's Modified Eagle Medium (DMEM), while glycosylation had the opposite effect. Methylation of mono- or dihydroxyflavonoids decreased the half-lives, while methylation of multihydroxyflavonoids increased the half-lives (Xiao and Högger, 2015). Although the correlation of published antioxidant activity data and degradation rates found by Xiao et al. was weak, the proposed stability-decreasing structural features are in good agreement with structural features enhancing antioxidant activity (Dugas et al., 2000; Furusawa et al., 2005; Plaza et al., 2014; Rice-Evans et al., 1996).

Due to the known antioxidant activity and reported degradation in cell culture medium, it is important to monitor possible degradation of flavonoids during toxicity tests and to assess the contribution of possibly formed TPs. Knowledge on TPs formed by oxidative degradation is available for aqueous solution (Osman et al., 2008; Ramešová et al., 2012, 2014, 2015, 2012; Sokolová et al., 2012, 2016; Zenkevich and Pushkareva, 2017) but only very limited for cell culture media (Maini et al., 2012). The most studied substance was quercetin. Taxifolin, fistein, luteolin, rhamnezin, galangin, and myricetin were investigated to a lesser extent. Commonly found intermediates were either depsides (Maini et al., 2012; Osman et al., 2008; Sokolová et al., 2016; Zenkevich and Pushkareva, 2017) and/or benzofuranone derivatives (Ramešová et al., 2012, 2014, 2015, 2012; Sokolová et al., 2012, 2016; Zenkevich and Pushkareva, 2017). Sokolová et al. proposed that benzofuranone derivatives are only formed by flavonoids with a 3-OH while degradation via a depside seems to be a common degradation pathway for all flavonoids (including those with 3-OH) (Sokolová et al., 2016). Several benzoic acids were identified as subsequently formed smaller TPs (Maini et al., 2012; Ramešová et al., 2014, 2015; Sokolová et al., 2016; Zenkevich and Pushkareva, 2017). However, information on flavonoid TPs is limited to the 8 mentioned flavonoids, auto-oxidative pathways, and two studies on oxidation via peroxidases (Osman et al., 2008; Osman and Makris, 2011). Currently, there is no data available on the formation of TPs at algae growth inhibition test conditions. Identification of TPs in toxicity tests, however, is crucial for the understanding of their possible contribution to observed effects of degrading flavonoids and increase the reliability of risk assessments.

The aim of this study is to broaden the general understanding of the effect of flavonoids on algae by taking their stability into account. Therefore, we pursued a novel approach by combining an algae growth inhibition test with an analytical assessment of the fate of flavonoids during the toxicity test. As a first step, the toxicity of 26 flavonoids towards the standard test organism *R. subcapitata* was investigated by determining their EC_{50} values in a growth inhibition test according to the OECD guideline 201. This study compared the effect of the tested flavonoids to the two commercial algaecides terbutryn and diuron to provide a reference value for the evaluation of the magnitude of the algae growth inhibition of flavonoids. Exploring possible synergetic effects, this study further tested 10 mixtures containing two to three flavonoids. To enable the testing of a high amount of test substances, a test setup with 24 well plates was established following previous miniaturization approaches (Eisentraeger et al., 2003; Rojickova et al., 1998).

Based on their different growth inhibition of *R. subcapitata* and their structural features, 11 out of the 26 flavonoids, in particular flavone, luteolin, eriodictyol, diosmetin, hesperetin, quercetin, taxifolin, tamarixetin, myricetin, and dihydromyricetin, were chosen for the analytical monitoring (Fig. 1). After preliminary assessment of possible metal coordination and photolysis in the experimental setup, this study set out to follow possible degradation of test substances during the algae growth inhibition test using High Performance Liquid Chromatography coupled with a detector for ultraviolet and visible light (HPLC-UV/vis) and High Performance Liquid Chromatography coupled with High Resolution Mass Spectrometry (HPLC-HRMS). Identified TPs were, if possible, purchased and in analogy to their parent compounds, algae growth inhibition and stability of the pure substances investigated.

2. Material and methods

2.1. Chemicals and solutions

An overview of purchased flavonoids and their vendors is shown in table SI 1. All chemicals and solvents were used without further purification.

Algae culture medium was prepared according to the OECD guideline 201. Stock solutions were prepared by weighting in required amounts of salts (Table SI 2) and dissolving them in ultrapure water. Algae culture medium was prepared by adding 10 mL of stock solution 1, and 1 mL of stock solutions 2, 3, and 4, respectively to 987 mL ultra-pure water. Final concentrations of the constituents are listed in table SI 2. To investigate metal complexation, a sodium carbonate puffer was prepared in analogy to the culture medium but only using stock solution 4. Solutions were sterilized by filtration.

2.2. Algae growth inhibition test

Algae growth inhibition was tested according to OECD guideline 201 with the green freshwater microalgae *Raphidocelis subcapitata* (Culture Collection of Algae at Göttingen University, Germany) (OECD, 2004). *R. subcapitata* stock cultures were cultivated in 250 mL Erlenmeyer flasks containing 100 ml medium prepared according to OECD guideline 201. The pH of culture medium was adjusted to 8.1 ± 0.1. The incubator (AlgaeTron AG 130-ECO, Photo Systems Instruments, Czech Republic) was set to 23 °C ± 2 °C and continuous illumination (100 μE m^{-2} s^{-1}) with cool white LEDs (400–800 nm, Figure SI 6). Cultures were agitated at 120 rpm using a shaker (Unimax 1010, Heidolph Instruments, Germany). For growth inhibition tests, algae cells were harvested after 3–4 days during the exponential growth phase and diluted to a start cell density of 2×10^4 cells/mL.

Following previous miniaturization approaches (Eisentraeger et al., 2003), 24 well plates were used as test vessels and validated in comparison to 250 mL Erlenmeyer flasks. Validation was based on the comparison of EC_{50} values obtained from growth inhibition tests either performed with the 24 well plates or 250 mL Erlenmeyer flasks with the reference compound 3,5-dichlorophenol. 3,5-dichlorophenol stock solutions were prepared in algae culture medium and filtered for sterilization before use (syringe filter Chromafil Xtra RC20/25 0.2 μm, Machery Nagel, Germany). In Erlenmeyer flasks and 24 well plates, the algae culture volumes were 100 and 2 mL, respectively. Culture conditions were similar to stock culture cultivation, only agitation was increased to 150 rpm for 24 well plates. Growth inhibition caused by 3,5-dichlorophenol was measured at 7 different concentrations (final concentration in test vessels: 0.6, 0.9, 1.35, 2, 3, 4.5, and 6.75 mg/L) in both test vessels. Algae growth was followed by measuring chlorophyll *a* fluorescence (excitation filter 460 nm, bandwidth 40 nm; emission filter 680 nm, bandwidth 30 nm) with a microplate reader (Synergy HT, Biotek Instruments). In experiments performed with Erlenmeyer flasks, 2 mL of the algae culture were transferred to 24 well plates before measurement with the plate reader. Measurement intervals were 24 h, the test duration was 72 h. Before measurements, plates were sealed with parafilm and inverted 10 times to ensure homogenous suspension of algae cells in the wells.

After successful validation of the miniaturized test setup, EC_{50} values of the purchased flavonoids, flavonoid mixtures, and the algaecides terbutryn and diuron as positive controls were determined using 24 well plates. Six flavonoids with different toxicity were selected for mixing. In particular the following 1:1 mixtures were tested: luteolin-eriodictyol, luteolin-quercetin, luteolin-morin, luteolin-taxifolin, luteolin-hesperetin, quercetin-morin, quercetin-taxifolin, quercetin-hesperetin, taxifolin-hesperetin, and a 1:1:1 mixture of luteolin, hesperetin, and taxifolin. In addition, some TPs identified in this study or structurally similar substances were purchased and tested for their algae growth inhibition (4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 5,7-dihydroxychromone, and 4-hydroxyphenylglyoxylic acid).

Test substance stock solutions were prepared in dimethylsulfoxid (DMSO) and added to the wells. For mixtures, DMSO stock solutions of the flavonoids were mixed in a 1:1 or 1:1:1 ratio. To reach an equal amount of organic solvent in all wells, DMSO was added to the blank and growth controls (1 % v/v). For each test substance, 6 to 9 concentrations were tested. A concentration range leading to approximately 0–100 % growth inhibition was determined in preliminary experiments. However, concentrations above 100 mg/L were not tested because they exceed threshold values for the classification of acute aquatic toxicity (United Nations, 2009). Each treatment was measured in duplicates. Every test was repeated once yielding 4 replicates of each treatment in total.

Blank corrected fluorescence values were converted into cell densities using a calibration curve obtained by counting cells with a Neubauer counting chamber and measuring the respective fluorescence signal as described in our previous study (Segatto et al., 2022). For a higher precision at low cell densities, these were determined using calibration points 5 000 to 100 000 cells/mL, while the whole calibration ranged to 1.5×10^6 cells/mL. Using the lower range, limits of detection (2 051 cells/mL) and quantification (7 712 cells/mL) were calculated with DINTEST 2000 according to DIN 32645 (result uncertainty: 33.3 %, probability of error: 5 %).

Growth inhibition (%I) based on the growth rate (μ) was calculated according to the equations (1) and (2) below. Dose-response curves (%I vs. log of concentration) were plotted and EC_{50} values obtained by linear regression of selected data points (equation (3)). Each replicate was fitted individually and then an average EC_{50} values was calculated.

$$\mu = (\ln \text{cell density}_{72h} - \ln \text{cell density}_0)/\Delta t \tag{1}$$

$$\%I = (\mu_{control} - \mu_{sample})/\mu_{control} \times 100 \tag{2}$$

$$EC_{50} = 10^{[(50\ \%I\text{-y intercept})/\text{slope}]} \tag{3}$$

In addition to single substances, 1:1 mixtures of two different flavonoids and one 1:1:1 mixture of three flavonoids were investigated. Expected EC_{50} additive values for mixtures assuming a dose addition of substance A and B were calculated according to equation (4) as described previously (Altenburger et al., 2004; Panizzi et al., 2017). For a third substance the equation must be extended with a corresponding term.

$$EC_{50}\ \text{additive} = \text{portion A}/EC_{50}\ \text{A} + \text{portion B} / EC_{50}\ \text{B} \tag{4}$$

2.3. Spectral characterization of the selected flavonoids and algae incubator light source

Investigating possible metal coordination of flavonoids, absorption spectra of flavone, luteolin, eriodictyol, hesperetin, diosmetin, quercetin, taxifolin, morin, tamarixetin, myricetin, and dihydromyricetin (5 mg/L) solved in the algae culture medium and sodium carbonate buffer (algae culture medium without any further metal ions) were recorded with a Nanocolor UV/Vis photometer

(Macherey-Nagel, Düren, Germany) using quartz cuvettes (Hellma, Müllheim, Germany). As described in more detail in the supplement, no coordination of metal ions by the tested flavonoids occurred according to unchanged UV/vis-spectra (see Figure SI 5).

The emission spectrum of the algae incubator light source was recorded with a SpectraWiz spectrometer (Stellar Net Inc, Tampa, Florida, USA) to check spectral overlap between light source and the flavonoids (Figure SI 6). More information can be found in the supplement section 2.3.

2.4. Analysis of the fate of selected flavonoids during the algae growth inhibition test

The 11 flavonoids (Fig. 1) myricetin, dihydromyricetin, quercetin, morin, tamarixetin, taxifolin, luteolin, eriodictyol, diosmetin, hesperetin, and flavone were selected for a more detailed analysis based on their structural features and different degree of growth inhibition. Concentration changes of flavonoids (initial concentration 5 mg/L) and the formation of TPs during the algae growth inhibition test were investigated. In addition to the sampling for concentration monitoring, the algae growth inhibition at 5 mg/L was determined. However, to exclude influences of the volume withdrawal on the algae growth and fluorescence measurements, the well plate contained identical replicates for sample collection and for fluorescence measurements. Moreover, to investigate whether *R. subcapitata* had an influence on the concentration of the flavonoids, control tests without algae cells were performed. Each condition was measured in duplicates per test and the tests were repeated once yielding 4 replicates of each test substance and conditions in total. Samples (75 μL) for concentration determination were collected at the time points 0, 24, 48, and 72 h and stored at −20 °C until the HPLC analysis.

2.4.1. Concentration determination of flavonoids with HPLC-UV/vis

To determine the concentration of selected flavonoids during the algae growth inhibition and control tests, collected samples were analyzed with a HPLC system (Prominence-HPLC-UV-vis&PDA, Shimadzu, Duisburg, Germany) coupled with a UV/vis and a Photodiode Array detector (PDA).

The calibration curves of 9 flavonoids were recorded in triplicates relating the measured peak area to the flavonoids' concentration (data not shown). Calibration of myricetin and dihydromyricetin was not feasible due to fast degradation. Wavelengths chosen for quantification of the flavonoids are shown in table SI 3. Limits of detection and quantification (LOD/LOQ) were calculated using data points 0.08–5 mg/L with DINTEST 2000 as described in section 2.2 (Table SI 3).

Collected samples were injected (50 μL) into the HPLC without further treatment, algae cells were restrained by the precolumn and hence, flavonoids solved in the algae culture medium were detected. The HPLC system was equipped with a phenyl-hexyl column (Nucleodur Phenyl-Hexyl, 125 mm length, 3 mm diameter, 3 μm particle size, Macherey-Nagel) with respective precolumn. The oven temperature was set to 35 °C. Water with 0.1 % formic acid (A) and methanol (B) were used as mobile phases at a flow rate of 0.5 mL min^{-1}. The gradient was as follows: 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. Data analysis was performed with the software Labsolution (Shimadzu). Figures of chromatograms and spectra were generated with the software Inkscape.

2.4.2. Characterization of TPs with HPLC-UV/Vis and HPLC-High Resolution Mass spectrometry (HPLC-HRMS)

To investigate the formation of TPs of the degrading flavonoids during the algae growth inhibition and control tests, obtained HPLC-UV/vis data from experiments in the previous section were analyzed in regard of newly formed signals. Retention times and absorption maxima of major new peaks were determined and individual peak areas at a wavelength of either 260, 280 or 290 nm were used to monitor the relative course of TPs.

Reference standards (3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 5,7-dihydroxychromone, phloroglucinol) were measured using HPLC-UV/vis as described in section 2.4.1. Comparing retention time and absorption spectrum, preliminary identification of some TPs was performed.

Furthermore, structure elucidation of the TPs of the degrading flavonoids was performed using liquid chromatography-high resolution mass spectrometry (LC-HRMS). The LC-HRMS consisted of a Thermo Scientific Q Exactive Focus Orbitrap LC-MS/MS System with a heated electrospray ionization (H-ESI) source (Thermo Scientific, Dreieich, Germany). The same chromatographic column and method as described in section 2.4.1 were used for the LC-HRMS analyses. Injection volume was reduced to 20 μL at the Orbitrap system. ESI source and mass spectrometer settings are given in the supplement (Table SI 4). In addition to samples from the algae growth inhibition test, controls without algae cells were analyzed to confirm the formation of the same TPs in the absence of microorganisms. Moreover, available reference standards (same as above) were analyzed by the LC-HRMS system.

Structure elucidation was based on empirical ion formula, ring double bond equivalents (RDB), fragmentation ions if available, and comparison to reference standards if available (matching of retention time, ion formula and fragmentation pattern). In addition, parent compound information, and the experimental context were taken into account.

3. Results and discussion

3.1. Validation of the 24 well plate-high throughput test set up

A high throughput algae growth inhibition test using 24 well plates was established in this study. Testing of 3,5-dichlorophenol resulted in EC_{50} values of 2.89 ± 0.04 mg/L for Erlenmeyer flasks and 2.88 ± 0.01 mg/L for 24 well plates. This excellent agreement demonstrates the suitability of 24 well plates as test vessels for this algae growth inhibition test (Figure SI 1, Table SI 5). Furthermore, the values derived in this study are in good agreement with other published EC_{50} values for 3,5-dichlorophenol tested against *R. subcapitata* ranging from 1.8 to 3.6 mg/L (Arensberg et al., 1995; Aruoja et al., 2011; Comber et al., 1995). This agreement was reached despite differences in e.g., used test vessels (20 mL glass vials (Arensberg et al., 1995)), method of cell density determination (electronic particle counting (Arensberg et al., 1995; Comber et al., 1995) or optical density at 682 nm (Aruoja et al., 2011)) or fitting procedures (Arensberg et al., 1995; Aruoja et al., 2011; Comber et al., 1995).

Both, the successful validation in this study and the agreement with results for 3,5-dichlorophenol of other studies, demonstrated the suitability of the 24 well plates as vessels for algae growth inhibition tests. These results are in support of previous findings and add to the conclusion that miniaturized algae growth inhibition test are a viable alternative to more cost- and labor-intensive test setups using large glass vessels (Blaise et al., 2018; Eisentraeger et al., 2003; Rojickova et al., 1998). Hence, the miniaturized test set up with 24 well plates was chosen for the investigation of the flavonoids, flavonoid mixtures, and TPs.

3.2. Algae growth inhibition of flavonoids

Using the validated algae growth inhibition test set up with 24 well plates, the growth inhibition of *R. subcapitata* by 26 flavonoids was investigated and compared to 3,5-dichlorophenol and the algaecides terbutryn and diuron (Table 1). For 13 flavonoids, EC_{50} values could be derived (according to equation (3)).

Dose response curves of these flavonoids are shown in the Supplement (Figure SI 2 and 3). For the other 13 flavonoids the EC_{50} values were beyond the solubility of these substances. The obtained EC_{50} values were in the range of 0.7–22 mg/L (2.4–73 μM). The two most toxic compounds eriodictyol and luteolin display high structural similarity differing only in the bond order of the C2–C3 bond (Fig. 1). Among the most toxic flavonoids were also 3,5-dihydroxyflavone, flavone, and gossypetin. These flavonoids differ strongly in their number of hydroxy groups (0–6). The relative high growth inhibition of flavone despite its rather large structural deviation (lack of hydroxy groups) from eriodictyol and luteolin suggests a different mode of action. Overall, no trends relating structural features to higher growth inhibition was evident. However, observed growth inhibition effects could not solely be attributed to the parent compound (flavonoids) but be influenced by the degradation of the flavonoids and formation of TPs.

The EC_{50} values of 3,5-dichlorophenol was in the same range as the EC_{50} values of the tested flavonoids. Thus, their toxicity is similar. In comparison with terbutryn and diuron, the most toxic flavonoids showed weaker growth inhibition by two orders of magnitude (Table 1). This clear difference in the growth inhibition to commercial algaecides strongly indicates limited opportunities of flavonoids as algicides. Furthermore, almost half of the tested flavonoids precipitated in the algae growth inhibition test above concentrations between 1 and 25 mg/L at which only −10 to 42 % growth inhibition was reached (Table 1). Especially, 7-hydroxyflavone, chrysin, 4′,7-dihydroxyflavone, apigenin, acacetin, formononetin, and daidzein with growth inhibition below 20 %

Table 1
Growth inhibition of R. subcapitata treated with flavonoids expressed as EC_{50} values (n = 4) with standard deviation (SD). Toxicity of flavonoids is compared to the two algaecides terbutryn and diuron as well as the reference substance 3,5-dichlorophenol. EC_{50} values are given in mg/L and in μM. For several flavonoids the EC_{50} values could not be determined because of limited solubility. In these cases, EC_{50} > x and the growth inhibition at the highest soluble concentration are presented. Flavonoids are arranged according to decreasing toxicity if EC_{50} values could be derived. The other flavonoids are arranged according to increasing number of free hydroxy groups.

	substance	EC_{50} [mg/L]	standard deviation	EC_{50} [μM]	standard deviation
algizides	terbutryn	0.005	4×10^{-4}	0.019	0.002
	diuron	0.023	0.003	0.023	0.003
reference compound	3,5-dichlorophenol	2.88	0.01	17.7	0.08
flavonoids	eriodictyol	0.7	0.08	2.4	0.3
	luteolin	0.94	0.08	3.3	0.3
	gossypetin	1.4	0.23	4.4	0.7
	flavone	1.91	0.08	8.6	0.4
	3,5-dihydroxyflavone	2.2	0.8	9	3
	7,3′,4′-trihydroxyflavone	2.7	0.4	10	1.5
	quercetin	2.9	0.2	9.6	0.6
	myricetin	3.7	0.6	12	2
	morin	4.8	0.3	16	1
	fisetin	5.4	0.5	19	2
	dihydromyricetin	6.2	0.5	19	2
	taxifolin	8.1	0.5	27	2
	hesperetin	22.2	0.6	73	2
	3-hydroxyflavone	EC_{50} > 0.63 / 20 % ± 1		> 2.6	
	5-hydroxyflavone	EC_{50} > 5 / 27 % ± 1		> 21	
	7-hydroxyflavone	EC_{50} > 5 / 14 % ± 4		> 21	
	chrysin	EC_{50} > 1 / 14 % ± 3		> 3.9	
	4′,7-dihydroxyflavone	EC_{50} > 1 / -10 % ± 3		> 3.9	
	apigenin	EC_{50} > 5 / 11 % ± 7		> 18.5	
	acacetin	EC_{50} > 1 / 17 % ± 3		> 3.5	
	naringenin	EC_{50} > 25 / 26 % ± 2		> 92	
	diosmetin	EC_{50} > 10 / 42 % ± 2		> 33	
	tamarixetin	EC_{50} > 10 / 30 % ± 3		> 32	
iso-flavonoids	formononetin	EC_{50} > 1 / 15 % ± 3		> 3.7	
	genistein	EC_{50} > 5 / 39 % ± 3		> 19	
	daidzein	EC_{50} > 10 / 12 % ± 3		> 39	

at the highest soluble concentration showed very low toxicity towards *R. subcapitata*. 4',7-dihydroxyflavone even stimulated algae growth at a concentration of 1 mg/L which might be due to hormesis (stimulating effect due to low exposure to a toxicant) (Calabrese and Baldwin, 1997). Thus, limited solubility in aqueous media and the concomitant low exposure can represent limiting factors regarding the toxicity of flavonoids.

The results of this study add to those of D'Abrosca et al. (D'Abrosca et al., 2006) who reported adverse effects of 9 flavonoids (chrysoeriol, tricin, luteolin, kaempferol, quercetin, three kaempferol glycosides, and catechin) on *R. subcapitata* determined by measuring diameters of no-growth zones in a petri dish assay at concentrations between 0.5 and 3 μM. Yet, severe differences in the used methods and determined parameters hinder a direct comparison. EC_{50} values are frequently reported parameters of growth inhibition. Thus, the EC_{50} values of flavonoids towards *R. subcapitata* reported for the first time by this study can be helpful for comparing the algae growth inhibition of flavonoids with previous and future studies.

The range of EC_{50} values from 2.4 to 73 μM obtained for *R. subcapitata* in this study is similar to EC_{50} values between 0.2 and 11 μM obtained for 19 flavonoids tested against the marine algae *Phaeocystis globosa* (Xiao et al., 2019). Additionally, the EC_{50} values of 74 μM of luteolin-7-glucuronide tested against *P. globosa* is in agreement with this range (Zhu et al., 2019). Eight of the flavonoids tested by Xiao et al. (quercetin, flavone, myricetin, luteolin, chyrsin, apigenin, formononetin, 5-hydroxyflavone) were also investigated in the current study. EC_{50} values of chyrsin, apigenin, and 5-hydroxyflavone found by Xiao et al. were below the solubility limits found in this study, while formononetin appeared to be soluble at higher concentrations at the test conditions used in the study of Xiao et al. The case of formononetin pointed out that solubility and hence bioavailability can differ in different culture media. Therefore, solubility should be monitored to avoid false results.

In contrast to the order of toxicity of flavonoids in our study, luteolin was among the weakest and quercetin the strongest growth inhibitor in the study of Xiao et al. (2019). Hence *R. subcapitata* and *P. globosa* seem to differ in susceptibility towards certain flavonoids.

Previously reported EC_{50} values of 1.8, 6.5–24, and 12 μM for the flavonoids 5,4′-dihydroxyflavone, luteolin, and kaempferol against the cyanobacterium *M. aeruginosa* are also within the magnitude of the EC_{50} values found in our study (Huang et al., 2015; Li et al., 2020, 2021). Huang et al. were able to determine an EC_{50} value of 14 μM for apigenin, while this flavonoid showed growth inhibition of 11 % at the highest soluble concentration (18.5 μM) in this study (Huang et al., 2015). Hence, *M. aeruginosa* seems to be more susceptible to apigenin than *R. subcapitata*. In addition, quercetin was reported to cause > 99 % growth inhibition of *M. aeruginosa* at a concentration of 23 μM (Chen et al., 2019).

Determination of EC_{50} values differs within the mentioned studies and in comparison to the method used here, too. The main difference to the aforementioned studies is that the growth inhibition was based on biomass yield instead of growth rate, which was used in this study. Determination of growth inhibition based on biomass yield leads to a stronger influence of the test duration on the obtained results (Nyholm, 1985). The test duration in the cited studies varies between 4 and 14 days and is hence longer than the duration of 3 days of our algae growth inhibition test. Despite these differences in methods, limited number of tested substances and different susceptibility of tested algae species towards certain flavonoids, the general similarity of the magnitude of EC_{50} values for *R. subcapitata*, *P. globosa* and *M. aeruginosa* discussed above suggests that growth inhibition caused by flavonoids is comparable between different algae species. Nevertheless, this should be verified by investigating several algae species in one study and identifying a possible common mechanism of action.

Several of the above-mentioned studies evaluate the algicidal activity as strong or suggest the application of flavonoids as algaecides to control harmful blooms of *P. globosa* or *M. aeruginosa* (Chen et al., 2019; Li et al., 2020; Xiao et al., 2019; Zhu et al., 2019). However, these evaluations were made without adequate comparison to established algaecides. Therefore, effectiveness of flavonoids as algaecides needs to be proven by further experiments. The difference in EC_{50} values by two orders of magnitude between the most toxic flavonoids and the commercial algaecides terbutryn and diuron found in this study does not support the previous suggestions. Reported EC_{50} values indicate that flavonoids have to be applied in a mg/L concentration range to act as an algaecide (Chen et al., 2019; Li et al., 2020; Xiao et al., 2019; Zhu et al., 2019). It should be critically reflected if the toxicity of individual flavonoid is high enough for effective application as algaecide.

3.3. Algae growth inhibition of flavonoid mixtures

In addition to testing single substances, the influence of mixing two to three flavonoids on the growth inhibition of *R. subcapitata* was investigated. Dose-response curves are shown in the supplement (Figure SI 4). The experimentally obtained EC_{50} values of nine 1:1 mixtures of two flavonoids and one 1:1:1 mixture of three flavonoids were in a range of 0.8–12 mg/L (Fig. 2 and Table SI 6). The eriodictyol-luteolin mixture was the most and the hesperetin-taxifolin mixture the least toxic. The most toxic mixtures (luteolin-eriodictyol, luteolin-taxifolin) have similar EC_{50} values to the most toxic single flavonoids eriodictyol and luteolin.

To identify possible synergistic or antagonistic effects, expected EC_{50} values for dose-addition as calculated according to equation (4) were compared to the experimentally derived values (Fig. 2). Deviations from the expected values for dose addition indicate synergistic or antagonist effects. Half of the mixtures displayed a very good agreement of expected and experimentally derived EC_{50} values. Four mixtures (luteolin-taxifolin, luteolin-hesperetin, taxifolin-hesperetin, luteolin-hesperetin-taxifolin) showed slightly lower EC_{50} values than calculated. The luteolin-morin mixture had a slightly higher EC_{50} value than expected. These findings indicate that mixtures of 2–3 flavonoids behave according to the dose addition model. This is consistent with the finding of Li et al. showing an additive effect of luteolin and kaempferol (Li et al., 2021). Further in line with this, a mixture of hyperoside and the xanthonoid mangiferin showed similar algae growth inhibition as the single substances (Segatto et al., 2022). However, in the same study, it was also shown that a mango waste ethanolic extract with hyperoside and mangiferin among the main components showed notably higher growth inhibition than the single substances and their 1:1 mixture. Thus, mixing of 2–3 flavonoids does not enhance the toxic-

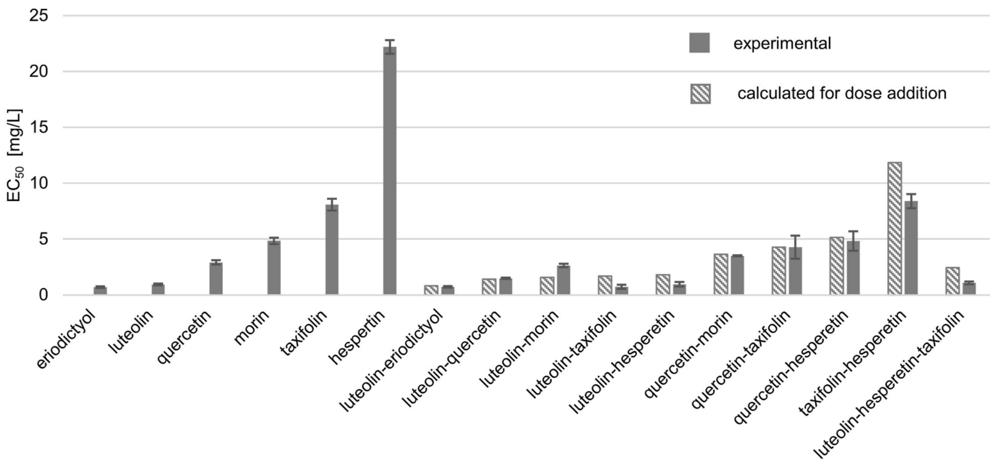


Fig. 2. Comparison of experimentally derived EC_{50} values of single compounds and mixtures of two to three different flavonoids (grey) to theoretical, calculated EC_{50} values of mixtures assuming an additive effect (striped). Average values with standard deviation displayed as error bars (SD, n = 4) of experimentally derived results are given.

ity, but utilization of more complex extracts may lead to an increase in activity (Gómez-Maldonado et al., 2020; Kaab et al., 2020; Kanetis et al., 2017). While this might be favorable for the application as pesticide, utilization of mixtures may also increase environmental risks.

3.4. Degradation of selected flavonoids during the algae growth inhibition test

Initial spectral characterization of the 11 selected flavonoids indicated firstly that no metal coordination occurred (Figure SI 5). Secondly, direct photolysis of eriodictyol, hesperetin, taxifolin, dihydromyricetin, and flavone was excluded because absorption spectra of these flavonoids and the emission spectrum of the light source do not overlap (Figure SI 6). Due to the minimal spectral overlap of the other flavonoids (luteolin, quercetin, tamarixetin, myricetin, morin, and diosmetin), low photon flux, low exposure to irradiation and because of shielding by the algae cells, we assume that direct photolysis has a negligible impact on these flavonoids during the algae growth inhibition test. Hence, (auto)oxidation is the main cause of possible degradation. More information can be found in the supplement section 2.3.

On this basis, analysis of algae growth inhibition test samples revealed that the concentration of flavone (105 % ± 5) and diosmetin (100 % ± 10) remained constant over 3 days (Fig. 3). Hesperetin showed a minor decrease in concentration to 92 % ± 1 of the initial 5 mg/L. The other 8 flavonoids were below limit of quantification or detection (LOQ/LOD) at the end of the test (Fig. 3, LOQ/LOD see Table SI 3). Myricetin and dihydromyricetin were not present in the analyzed sample of day 0 (Figure SI 7 and 8). Quercetin and tamarixetin were below the limit of detection and the limit of quantification at day 1, respectively (Fig. 3). This rather fast degradation accounts for the higher deviations in the determined concentrations on day 0 in comparison to other tested flavonoids since small temporal delays in the sampling and HPLC-measurement procedures influenced the measured signal to a greater extent than for slower degrading flavonoids. The concentration of luteolin was below LOD at day 2, while the concentration of morin was below LOQ at day 2. The concentrations of eriodictyol and taxifolin were still quantifiable at day 2 but below LOQ at day 3. According to these results the velocity of degradation showed the following trend: myricetin = dihydromyricetin > quercetin > tamarixetin > luteolin > morin > eriodictyol = taxifolin > hesperetin > diosmetin = flavone.

The concentration decreases in controls without algae showed the same degradation behavior as their counterparts with algae present in the test (Fig. 3). Since the algae test is performed under sterile conditions, bacterial degradation can be excluded. Therefore, we demonstrated for the first time that the degradation of flavonoids at algae growth inhibition test conditions is caused by abiotic processes and not biological ones e.g., biodegradation by the algae cells. This direct comparison of degradation with and without the test organism adds to findings of Maini et al. and Xiao et al. These studies found out that flavonoids degrade in cell culture media (Maini et al., 2012; Xiao and Högger, 2015). They were, however, performed in the absence of a test organism. Our findings of abiotic degradation further support oxidation as the major degradation mechanism.

The results presented in this section show that flavone, diosmetin and hesperetin are the least reactive flavonoids at the conditions of the algae growth inhibition test. Interestingly, two of this three compounds (diosmetin, hesperetin) carry a methoxy group at the B ring indicating that this could be a structural feature lowering the reactivity. This is consistent with increased half-lives of isorhamnetin and kaempferide in comparison to their non-methylated counterparts quercetin and kaempferol in DMEM cell culture medium (Xiao and Högger, 2015). In line with this, tamarixetin (also carrying a methoxy group) was found slightly more stable than quercetin in our study, but still degraded below LOD within 2 days. In comparison to tamarixetin, diosmetin, and hesperetin both lack the 3-OH. The influence of a 3-OH was investigated previously in regard to degradation rates and antioxidant activity. Concurrent with decreasing half-lives (Xiao and Högger, 2015), this structural feature was also found to increase the antioxidant activity of flavonoids (Rice-Evans et al., 1996). Thus, the 3-OH group could counteract the effect of the methoxy group in tamarixetin. The fast degradation of quercetin and taxifolin, also carrying a 3-OH, is in further support of the reactivity enhancing effect of a 3-OH. In comparison to their

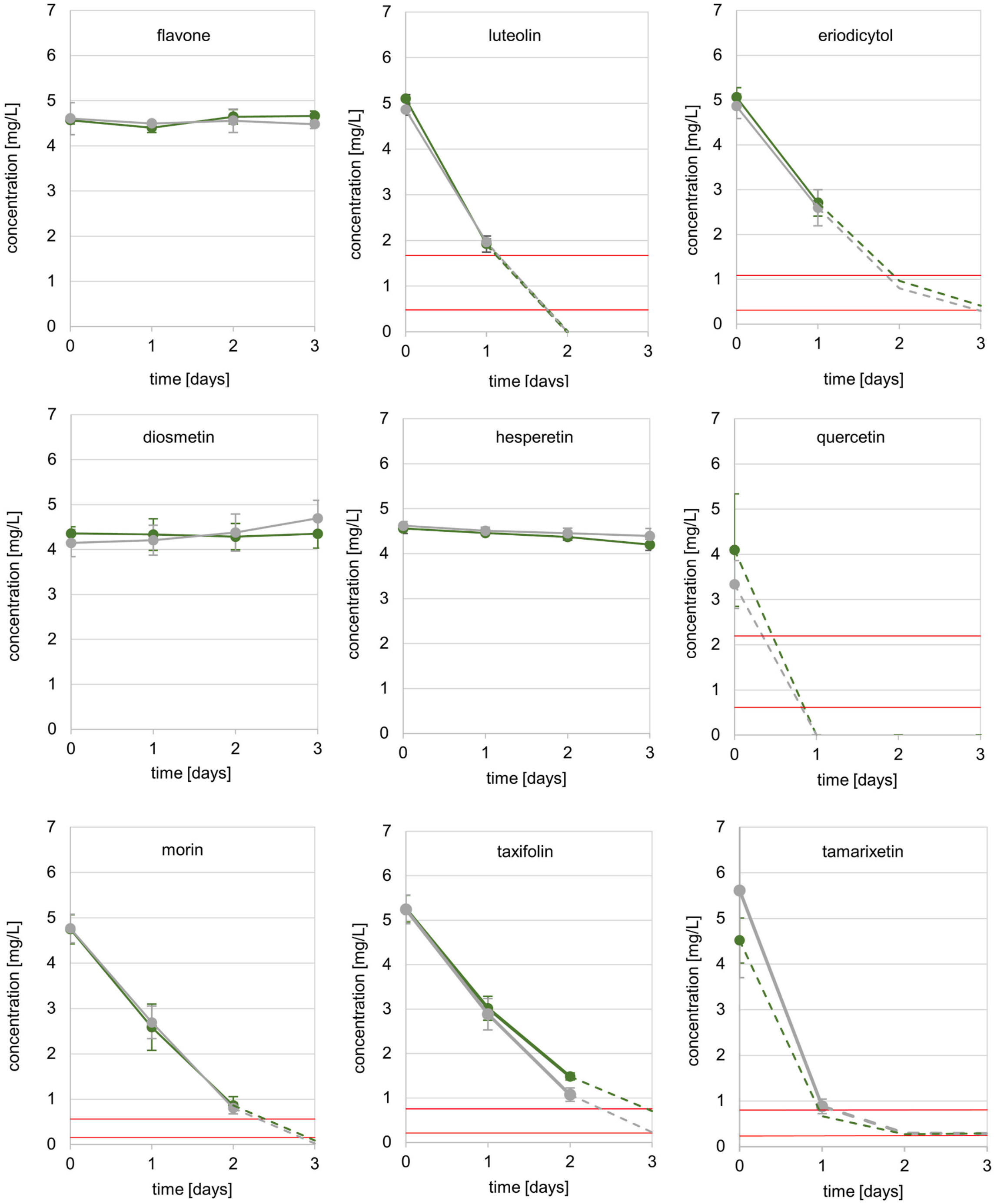


Fig. 3. Concentration development of the flavonoids flavone, luteolin, eriodictyol, diosmetin, hesperetin, quercetin, morin, taxifolin und tamarixetin during the algae growth inhibition test. Samples with algae (green), controls without algae (grey). Start concentration 5 mg/L. Data points represent average values of 4 replicates with standard deviations (SD) displayed as error bars, lines are visual guides and are displayed as dotted lines if the subsequent data point was below limit of detection or quantification shown as red lines.

counterparts lacking the 3-OH, quercetin (below LOD after 24 h) degrades faster than luteolin (below LOD after 48 h), while the concentration of taxifolin and eriodictyol showed a similar decrease. The slower degradation of taxifolin and eriodictyol could be due to a C2–C3 single bond. This structural feature was reported to increase degradation half-lives (Xiao and Högger, 2015) and decrease antioxidant activity (Plaza et al., 2014). Furthermore, the very fast degradation of myricetin and dihydromyricetin supports the proposed rule that an increasing number of hydroxy groups increases degradation rates (Maini et al., 2012; Xiao and Högger, 2015).

In summary, the combination of a methoxy group and a lacking 3-OH reduced degradation rates, while a C2–C3 single bond was not sufficient to hinder degradation of flavonoids over three days under the algae test conditions (Fig. 4).

3.5. Influence of the degradation of flavonoids on their toxicity

The results in section 3.4 showed that 8 out of the 11 tested flavonoids degrade fast during the algae growth inhibition test. At a starting concentration of 5 mg/L as used in the degradation study, these 8 flavonoids showed very different algae growth inhibition (Fig. 5). Tamarixetin was the least toxic with 10 % ± 2 growth inhibition, followed by dihydromyricetin (24 % ± 8) < taxifolin (44 % ± 5) < morin (56 % ± 3) ~ myricetin (57 % ± 4) < quercetin (76 % ± 1) < eriodictyol (95 % ± 5). The three flavonoids which concentrations stayed above 90 % of the initial concentration during the test period of 3 days also showed low to high toxicity. Hesperetin was the least toxic with 7 % ± 1 growth inhibition, followed by diosmetin (37 % ± 5) and flavone with 100 %. Therefore, a trend correlating degradation to the toxicity was not evident (Fig. 5). As mentioned in the previous section, other studies investigating the toxicity of flavonoids towards algae did not monitor concentration changes of the test substances. However, we highly recommend this to provide higher data quality and reliability.

3.6. Identification of flavonoid TPs

HPLC-UV/vis analysis of the algae growth inhibition test samples gave first indications of TPs and their relative course (supplement section 2.5). Additionally performed HPLC-HRMS analysis revealed the presence of in total 25 TPs. The highest number of TPs had morin with 12, followed by taxifolin with 11, eriodictyol with 8, quercetin with 7, tamarixetin with 6, dihydromyricetin with 5, luteolin with 4 and myricetin with 3. Several times, the same TPs were found for more than one flavonoid parent compound.

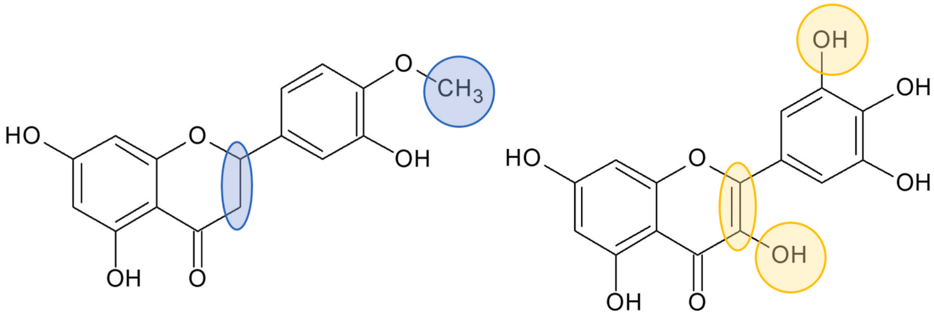


Fig. 4. Proposed general rules describing the influence of structural features on the degradation rate of flavonoids at the conditions of the algae growth inhibition test. Structural features highlighted in blue decrease degradation rates (methoxy groups, C2–C3 single bond). Structural features highlighted in yellow increase degradation rates (high number of free hydroxy groups, 3-OH, C2–C3 double bond).

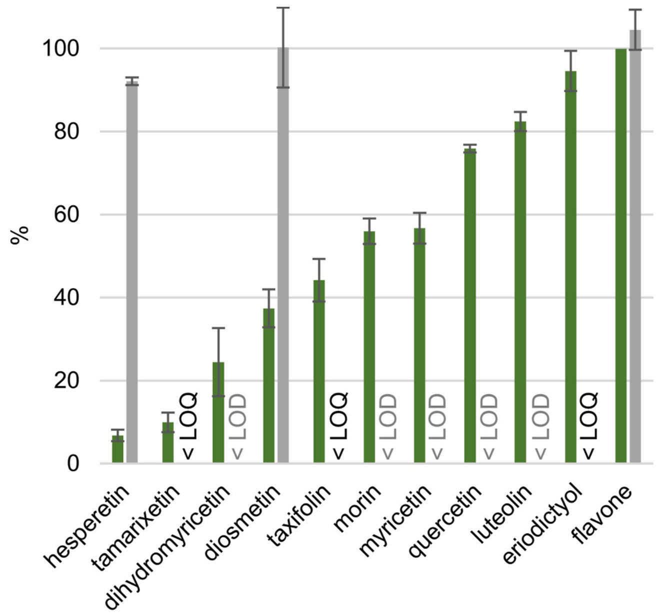


Fig. 5. Comparison of % algae growth inhibition (green) and % remaining concentration (grey) of the 11 selected flavonoids after 3 days. Starting concentration of flavonoids = 5 mg/L. Data points represent average values with standard deviation displayed as error bars (n = 4). Concentrations of tamarixetin, taxifolin, and eriodictyol were below limit of quantification (LOQ). Concentrations of dihydromyricetin, morin, myricetin, quercetin, and luteolin were below limit of detection (LOD).

Proposed structures of the TPs of the different flavonoids are shown in Table 2. Graphical representation of the proposed degradation pathway of each flavonoid are shown in the supplement (Figure SI 11- 18). The confidence of structure elucidation is expressed in 5 levels according to Schymanski et al. (level 1: confirmed structures, level 2: probable structure, level 3: tentative candidate, level 4: unequivocal formula, level 5: exact mass) (Schymanski et al., 2014).

Confirmed structures (level 1) on the basis of MS, MS2, retention time (rt), comparison to a reference standard and partially verification with HPLC-UV/vis data are 2,4,6-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 5,7-dihydroxychromone and 4-hydroxybenzoic acid. 2,4,6-trihydroxybenzoic acid was found to be a TP of all 8 tested flavonoids. It was detected by UV/vis in samples of tamarixetin, quercetin, and morin (Figure SI 9, Table SI 7). This substance was previously reported as an oxidative degradation product of quercetin and taxifolin (Sokolová et al., 2016). In the same study, TPs of luteolin were identified but in contrast to our study, 2,4,6-trihydroxybenzoic acid was not among the found structures. This might be due to the different experimental conditions including the solvent (aqueous potassium hydroxide solution vs. algae culture medium) and time points of sampling (≤ 24h vs. 72 h). 3,4-Dihydroxybenzoic acid was detected in eriodictyol, taxifolin, and quercetin samples. For quercetin, this was confirmed with UV/vis detection (Figure SI 9, Table SI 7). This substance was previously reported as a TP of quercetin, luteolin, and taxifolin (Sokolová et al., 2016). 5,7-Dihydroxychromone was found in eriodictyol, dihydromyricetin, and taxifolin samples. No fragments in the MS2 scans were found in measurements of the reference standard and in the samples of this compound. For eriodictyol, the occurrence of 5,7-dihydroxychromone was confirmed by HPLC-UV/vis (Figure SI 9, Table SI 7). This compound was identified as a TP of flavonoids for the first time in this study.

4-Hydroxybenzoic acid was detected in samples of myricetin, quercetin, morin, taxifolin, and luteolin. This was confirmed with UV/vis data only for morin samples, but the signal intensity was very low (Figure SI 9, Table SI 7). 4-Hydroxybenzoic acid was not reported as a TP of a flavonoid before. Additionally, phloroglucinol was identified as a TP of morin by matching exact mass and retention time to a reference standard in the MS analysis. Due to an early elution from the column, analysis by UV/vis detection was aggravated. Yet, this compound was only proposed as biodegradation product of flavonoids (Braune et al., 2001; Cooper and Rao, 1995). Thus, the current study provides first evidence that phloroglucinol could also be formed abiotically from certain flavonoids.

Based on MS and MS2 data as well as parent compound information (level 2), 8 further structures were proposed. In analogy to the detection of 3,4-dihydroxybenzoic acid in quercetin samples, a 153 *m/z* ion with the same fragmentation pattern was detected in morin samples at 6.4 min. Since the only structural difference in the parent flavonoids is the hydroxy group pattern in the B-ring, it is standing to reason that the TP found in morin is 2,4-dihydroxybenzoic acid (Figure SI 13 and 15). 2,4-Dihydroxybenzoic acid was also found as a TP of morin treated with an enzyme-homogenate (Osman and Makris, 2011). TP-347 and TP-319 of tamarixetin are in line with an oxidation pathway leading to a depside as proposed by Sokolová et al. (2016) (Figures SI 16). As mentioned in the introduction, degradation via depsides is an alternative degradation pathway to degradation via a benzofuranone derivative (Fig. 6) (Sokolová et al., 2016). According to Sokolová et al. a 3-OH and a C2–C3 double bond are required structural features for the degradation via a benzofuranone derivative (Sokolová et al., 2016). In disagreement with these conditions, such a derivative was found for taxifolin (TP-317.1). However, it might be possible that taxifolin is oxidized to quercetin which subsequently transforms into TP-317.1 (Figures SI 14). Quercetin samples did not contain a TP with this *m/z* value indicating that it degraded fast. A benzofuranone derivative was proposed for morin with less confidence (TP-317.2). Additionally, isomers for the benzofuranone derivative of taxifolin and morin were proposed (TP-317.3, TP-317.4). The general similarities of the results of this study to previously reported depsides and benzofuranone derivatives support the idea of a general degradation pathway of flavonols and flavones via these intermediates independently of the kind of aqueous solvent (Maini et al., 2012; Ramešová et al., 2012, 2014, 2015; Sokolová et al., 2012; Zenkevich and Pushkareva, 2017) (Fig. 6).

TP-181.1 of quercetin and morin is most likely 1-benzofuranone-2,3,4,6-tetrol. It was not reported previously but could be built from hydrolysis of a benzofuranone derivative (Fig. 6). Likewise, TP-197 is most likely (2,4,6-trihydroxyphenyl)(oxo)acetic acid. It could be derived from oxidation and hydrolysis of TP-181.1. TP-197 was previously found as a TP of quercetin, fisetin, and taxifolin, but not in luteolin, whose TPs were also investigated (Sokolová et al., 2016; Zenkevich and Pushkareva, 2017). In this current study, TP-197 was found in 6 of the 8 tested flavonoids. The two exceptions are luteolin and eriodictyol, the only two fast degrading compounds lacking a 3-OH. Therefore, (2,4,6-trihydroxyphenyl)(oxo)acetic acid seems to be an ubiquitous TP of flavonoids carrying a 3-OH (Fig. 6).

TP-193 is most likely 2,5,7-trihydroxy-4H-chromen-4-one and was found in luteolin, eriodictyol, and dihydromyricetin samples. It was reported as a TP of luteolin previously (Sokolová et al., 2016). The similar TP-195 was found in eriodictyol as well, but also in quercetin, taxifolin, and tamarixetin. It might be 2,5,7-trihydroxy-2,3-dihydro-4H-1-benzopyran-4-one. Additionally, the eriodictyol samples contained a signal (TP-285.1) matching the retention time and exact mass of luteolin. Although MS2 data was lacking for confirmation, an oxidation of the C2–C3 single bond in eriodictyol to a double bond resulting in the structure of luteolin is likely (Figure SI 18).

There were 10 potential TPs for which the structure remains tentative due to lacking MS2 data. Three were already mentioned above (TP-317.2-0.4). TP-285.2 of eriodictyol could be the chinone derivative resulting from the oxidation of the catechol moiety in eriodictyol (Figure SI 18). In the morin samples two $C_{15}H_{11}O_7$ isomers were detected, and possible structures proposed (TP-303.1-2). The building of these substances from morin requires a reductive addition of hydrogen. TP-271 found in eriodictyol, taxifolin, and morin samples could be a flavonoid with a C2–C3 single bond and three hydroxy groups but their position is unclear. A possible isomer is shown in Table 2. Analogously, TP-269 found only in luteolin could be a flavonoid with a C2–C3 double bond and three hydroxy groups. TP-211 was found in morin and tamarixetin samples. It is the third TP comprising a C9 skeleton, but other than TP-195 und TP193 it has an additionally hydroxy group. These 5 TPs (TP-303.1-2, TP-271, TP-269, TP-211) are neither in agreement with the established degradation pathway via depsides and/or benzofuranone derivatives nor with the here proposed formation of 5,7-

Table 2
Overview of proposed TPs. Characteristics as the retention time (rt), the m/z value (negative mode) and ion formula of the molecular ion, as well as the m/z values and ion formulars of fragmentation ions are given for each TP. Confidence of the structure elucidation is expressed by Schymanski levels 1-5 for each TP in regard of the corresponding parent flavonoid individually (Schymanski et al., 2014). Level 1: confirmed structures, level 2: probable structure, level 3: tentative candidate, level 4: unequivocal formula, level 5: exact mass. N.d. is stated when the TP was not detected. Deviations from Schymanski criteria are shown by letters in superscript: [a] lacking MS2 data, either due to low signal intensity of precursor ion, no fragmentation of precursor ion took place, or fragments had m/z < 70 Da.

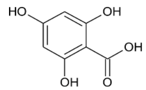
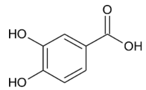
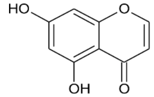
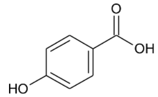
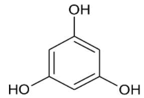
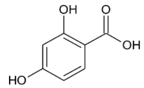
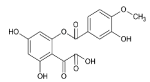
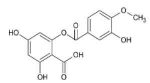
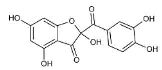
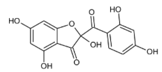
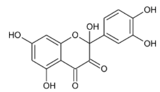
#	Name	RT [MIN]	M/Z	Formula [M − -H]	Fragmentation**M/Z AND (FORMULA)**	Myricetin	Dihydro-myricetin	Quercetin	Morin	Taxifolin	Tama-rixetin	Luteolin	Eriodictyol	Proposed structure
1	2,4,6-Trihydroxybenzoic acid*	7.8	169.0137	C7H5O5	151 (C7H3O4), 125 (C6H5O3)	1	1	1	1	1	1	1[a]	1[a]	
2	3,4-Dihydroxy-benzoic acid	4.6	153.0188	C7H5O4	109 (C6H5O2)	n.d.	n.d.	1	n.d.	1[a]	n.d.	n.d.	1[a]	
3	5,7-Dihydroxy-chromeone	7.6	177.0188	C9H5O4		n.d.	1[a]	n.d.	n.d.	1[a]	n.d.	n.d.	1[a]	
4	4-Hydroxy-benzoic acid	5.6	137.0242	C7H5O3	93	1[a]	n.d.	1	1	1	n.d.	1[a]	n.d.	
5	Phloro-glucinol	2.6	125.0234	C6H5O3		n.d.	n.d.	n.d.	1[a]	n.d.	n.d.	n.d.	n.d.	
6	2,4-Dihydroxy-benzoic acid	6.4	153.0181	C7H5O4	109 (C6H5O2)	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	n.d.	
7	TP-347	11.2	347.0405	C16H11O9	275; 179; 151	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.	
8	TP-319	8.1	319.0455	C15H11O8	169	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.	
9	TP-317.1	7.7	317.0298	C15H9O8	299, 179, 137	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.	n.d.	
10	TP-317.2	7.2	317.0298	C15H9O8	–-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
11	TP-317.3	8.2	317.0298	C15H9O8	–-	n.d.	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	

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Table 2 (*continued*)

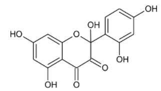
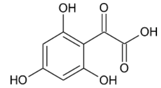
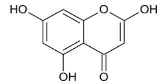
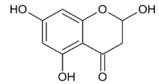
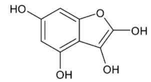
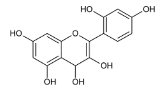
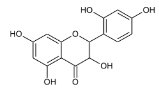
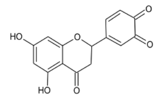
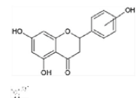
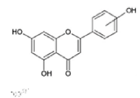
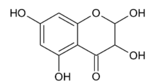
#	Name	RT [MIN]	M/Z	Formula [M − -H]	Fragmentation**M/Z AND (FORMULA)**	Myricetin	Dihydro-myricetin	Quercetin	Morin	Taxifolin	Tama-rixetin	Luteolin	Eriodictyol	Proposed structure
12	TP-317.4	7.9	317.0298	C15H9O8	–-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
13	TP-197	5.8	197.0082	C8H5O6	153, 151, 125	2	2	2	2	2	2	n.d.	n.d.	
14	TP-193	8.5	193.0135	C9H5O5	149	n.d.	2	n.d.	n.d.	n.d.	n.d.	2	2	
15	TP-195	6.5	195.0289	C9H7O5	167, 123	n.d.	n.d.	2[a]	n.d.	2[a]	2	n.d.	2	
16	TP-181.1	6.6	181.0131	C8H5O5	137, 109	n.d.	n.d.	2	2	n.d.	n.d.	n.d.	n.d.	
17	Luteolin (TP-285.1)	8.7	285.0400	C15H9O6	–-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	parent flavonoid detected	2[a]	
18	TP-303.1	7.1	303.0505	C15H11O7	–-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
19	TP-303.2	8.6	303.0505	C15H11O7	–-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
20	TP-285.2	8.4	285.0400	C15H9O6	–-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	
21	TP-271	8.8	271.0605	C15H11O5	–--	n.d.	n.d.	n.d.	3	3	n.d.	n.d.	3	
22	TP-269	9.1	269.0450	C15H9O5	–-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	n.d.	
23	TP-211	6.7	211.024	C9H7O6	–-	n.d.	n.d.	n.d.	3	n.d.	3	n.d.	n.d.	

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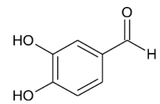
Table 2 (*continued*)

#	Name	RT [MIN]	M/Z	Formula [M − -H]	Fragmentation**M/Z AND (FORMULA)**	Myricetin	Dihydro-myricetin	Quercetin	Morin	Taxifolin	Tama-rixetin	Luteolin	Eriodictyol	Proposed structure
24	TP-137	5.4	137.0234	C7H5O3	–-	n.d.	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	
25	TP-181.2	4.5	181.0131	C8H5O5	–-	n.d.	4	4	n.d.	4	n.d.	n.d.	n.d.	

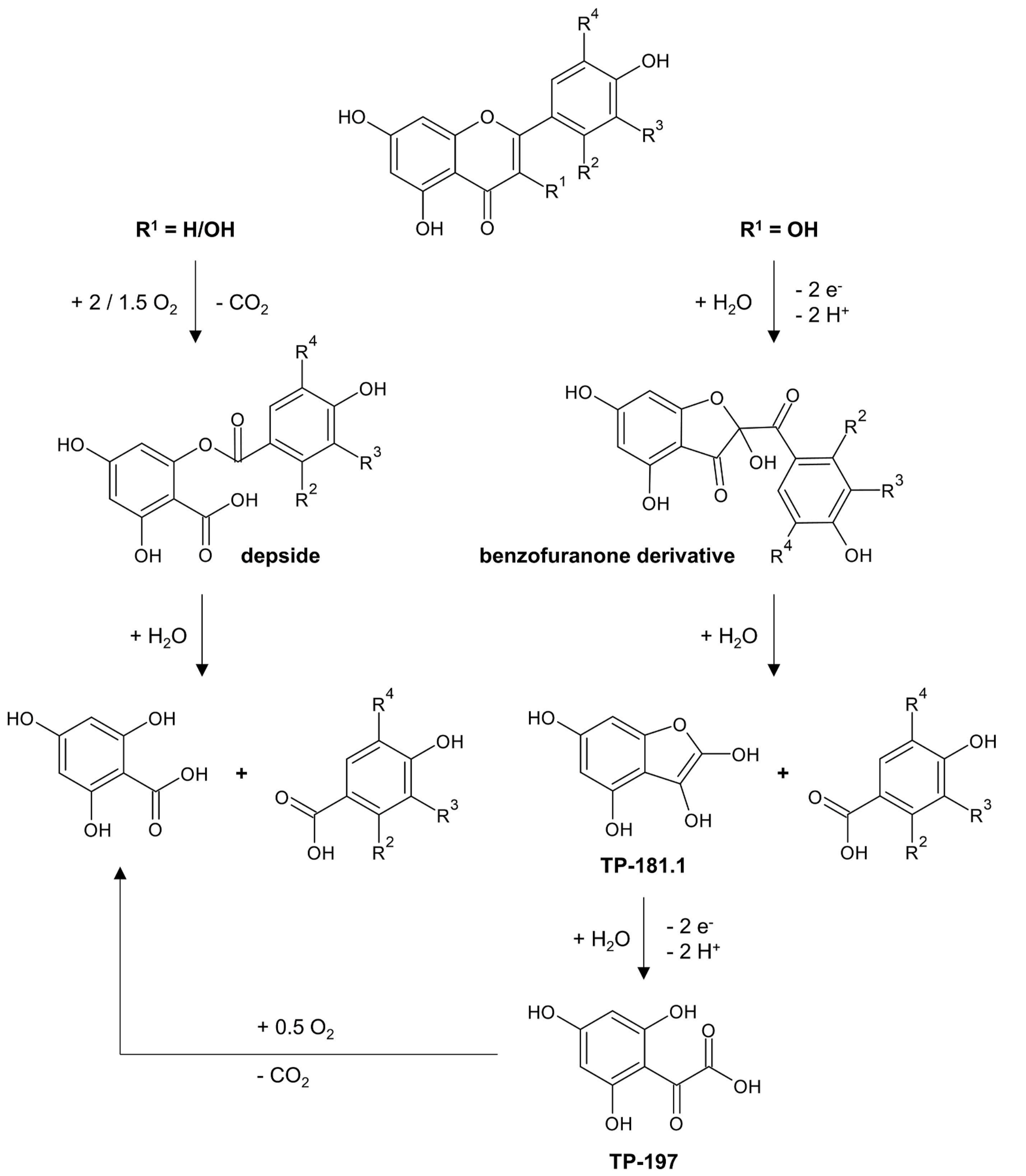


Fig. 6. Pathway of oxidative degradation of flavonols and flavones either via a depside or a benzofuranone derivative to benzoic acid derivatives based on the pathway suggested by Sokolová et al., (2016).

dihydroxychromone from flavonoids with a C2–C3 single bond (Figures SI 14-18)). Thus, these results raise the question if further reactions pathways exist. However, a detailed elucidation of all possible degradation pathways is beyond the scope of this study.

An isomer of 4-hydroxybenzoic acid was found in the taxifolin samples. Retention time differed from the reference standards 4- and 3-hydroxybenzoic acid. In line with Sokolová et al., we propose 3,4-dihydroxybenzaldehyde as a tentative structure (Sokolová et al., 2016).

In addition, one MS signal for which no structure could be proposed based on the obtained information was detected. An isomer of TP-181.2 was found at a retention time of 4.5 min in samples of dihydromyricetin, quercetin, and taxifolin.

These results obtained from measurement of algae growth inhibition test samples were in very good agreement with control samples without algae cells. All signals of the TPs except one were found in samples with and without algae. 4-hydroxybenzoic acid was not detected in controls of luteolin and myricetin. However, the signal intensity was low in samples with algae of these compounds and slight differences in concentration may lead to the missing of this signal in the control samples. The occurrence of the same TPs in samples with and without algae is further supporting our results that the flavonoids degraded abiotically and, hence, the found TPs represent abiotic TPs.

In summary, this study confirmed and extended knowledge on the flavonoid TPs formed under oxidative conditions in aqueous solution. In this study, TPs of eriodictyol, tamarixetin, and dihydromyricetin were identified for the first time. Furthermore, this is the first study that investigates degradation of flavonoids and formation of TPs during an algae growth inhibition test. Identification of some TPs with very high confidence (level 1) was feasible because flavonoids partially degrade to known phenol and chromone derivatives which are available as reference standard. Most TPs identified at the algae growth inhibition test conditions are in agreement with previous studies on the oxidative degradation of flavonoids and found TPs (Osman et al., 2008; Sokolová et al., 2016; Zenkevich and Pushkareva, 2017). Thus, this study adds to the evidence that degradation via depsides and/or benzofuranone derivatives is the major abiotic degradation pathway of flavonoids. Beyond the proposed degradation pathway by Sokolová et al. (2016), we suggested a continuative degradation pathway for the formation of (2,4,6-trihydroxyphenyl)(oxo)acetic acid (TP-197), 2,4,6-trihydroxybenzoic acid, and dihydroxybenzoic acids (Fig. 6). Moreover, 5,7-dihydroxychromone was identified as a TP of certain flavonoids for the first time. Based on the comparison of the 8 tested flavonoids, three rules for TP formation could be derived. Firstly, 2,4,6-trihydroxybenzoic acid seems to be a universal TP of all flavonoids. Secondly, a 3-OH groups seems necessary for the formation of (2,4,6-trihydroxyphenyl)(oxo)acetic acid (TP-197). Thirdly, 5,7-dihydroxychromone seems to be only formed by flavonoids with a C2–C3 single bond.

3.7. Fate of selected flavonoid TPs during the algae growth inhibition test

Identified TPs were, if possible, purchased as pure substance and subjected to the algae growth inhibition test. In addition to 2,4,6-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and 5,7-dihydroxychromone, 4-hydroxyphenylglyoxylic acid was tested due to its oxo-acid structural feature also present in TP-197. The least toxic substance together with 4-hydroxyphenylglyoxylic was 2,4,6-trihydroxybenzoic acid, the common TP of all tested flavonoids (Table 3).

Both substances led to a growth inhibition about 20 % at a concentration of 100 mg/L. Due to the very low toxicity, EC_{50} values were not determined. The toxicity of the other substances was higher with EC_{50} values ranging from 641 to 34 μM (89–6.1 mg/L). 4-Hydroxybenzoic acid with an EC_{50} value of 641 μM is far less toxic than all tested flavonoids. It is very unlikely that it contributes to the observed growth inhibition because it could not have been generated from the flavonoid parent compound concentration in quantities close to its EC_{50} value. 3,4-Dihydroxybenzoic acid is less toxic than its parent flavonoids, respectively taxifolin, morin, myricetin, quercetin, and luteolin by a factor of 3–24. It must be noted that the validity criteria of a maximal pH shift of 1.5 units was violated when high concentration of benzoic acids were tested. Not optimal growth conditions could, therefore, influence the results. 5,7-Dihydroxychromone, the most toxic of the tested TPs, is less toxic than its parent flavonoids eriodictyol by a factor of 14 and slightly less toxic than its parent flavonoids taxifolin and dihydromyricetin by a factor of 1.3 and 1.8, respectively.

Based on the assumption that only 5,7-diyhdroxychromone gave rise to the peak at 8.7 min in the UV/vis-Chromatogram of eriodictyol (Figure SI 9), the concentration of this compound at the end of the algae growth inhibition test was 1.6 μM. At this concentration, no adverse effect on the growth of *R. subcapitata* is expected. The lowest concentration tested for the dose-response curve was 2.2 μM leading to growth inhibition of −2.4 % ± 1.6 (SI Fig. 19).

These findings strongly indicate that the growth inhibition effect of the single tested TPs is not strong enough to cause the toxicity observed for flavonoids that degrade during the algae growth inhibition test. Although not all identified TPs could be tested, due to their broad structural similarity to either the parent flavonoid or tested TPs as well as their low concentrations, it seems unlikely that a single TP is responsible for the observed toxicity. Therefore, it can be assumed that the toxicity of degrading flavonoids is caused by a mixture of the flavonoid parent compound and several formed TPs. These findings correspond to rising concerns in environmental risk assessment on the toxicity of mixtures of synthetic chemicals and their TPs (Boxall et al., 2004; Escher and Fenner, 2011; Kortenkamp and Faust, 2018; Neuwoehner et al., 2010).

Furthermore, analysis of peak areas of purchased TPs during the algae growth inhibition test revealed a decrease of 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid (Table 3). On the contrary, 4-hydroxybenzoic acid and 5,7-dihydroxychromone could be end products since their concentration was constant during the test period of 3 days. Interestingly, the concentration of 4-hydroxyphenylglyoxylic acid was also constant strongly indicating that no decarboxylation took place.

This study is the first to report algae growth inhibition induced by 5,7-dihydroxychromone and 4-hydroxyphenylglyoxylic acid. More knowledge is available for benzoic acids. The observed toxicity of benzoic acids in this study differs from previously reported data. Lee et al. found 2,4,6-trihydroxybenzoic acid the most toxic in comparison to 20 other benzoic acids with an EC_{50} value of 1.93 mg/L (Lee and Chen, 2009). In contrast, 2,4,6-trihydroxybenzoic acid was among the least toxic substances in this study with only 20 % growth inhibition at 100 mg/L. EC_{50} value reported for 3,4-dihydroxybenzoic acid by Lee et al. and Kamaya et al. are 726 and 6 mg/L, respectively (Kamaya et al., 2004; Lee and Chen, 2009). The EC_{50} value of 12 mg/L obtained in this study is close to the

Table 3
Algae growth inhibition of identified TPs and 4-hydroxyphenylglyoxylic acid. EC_{50} values (average value, n = 4 with standard deviation (SD)) is given in mg/L and μM. %Inhibition (average value, n = 4 with standard deviation (SD)) at 100 mg/L is given for substances with EC_{50} values > 100 mg/L.

	EC_{50}-Wert [mg/L] / %Inhibition at 100 mg/L	EC_{50}-Wert [μM]	%($area_{day3}$/$area_{day0}$)
4-hydroxybenzoic acid	89 ± 2	641 ± 12	106 ± 6
3,4-dihydroxybenzoic acid	12 ± 2	78 ± 10	19 ± 3
2,4,6-trihydroxybenzoic acid	20 % ± 7		57 ± 5
5,7-Dihydroxychromone	6.1 ± 0.4	34 ± 2	108 ± 2
4-hydroxyphenylglyoxylic acid	23 % ± 7		109 ± 13

result of Kamaya et al. These studies reported EC_{50} values for 4-hydroxybenzoic acid as 355 and 1367 mg/L (Kamaya et al., 2004; Lee and Chen, 2009). The low growth inhibition observed for 4-hydroxybenzoic acid in this study is supporting these findings.

Differences in the toxicity of benzoic acid may be due to differences in pH, nutrient content of the culture media and different degradation rates of the benzoic acid at the different test conditions. Firstly, influences of the pH on the toxicity of benzoic acids towards algae was reported previously (Lee and Chen, 2009). The current study was performed at pH 8.1 while Lee et al. used pH 6.5 and Kamaya et al. used pH 7.45. Based on the hypothesis that deprotonated, ionic species are less toxic because their membrane permeability is decreased, less toxicity can be expected for higher pH values (Muccini et al., 1999; Zhao et al., 1996, 1998). The ratio of deprotonated and protonated species of 2,4,6-trihydroxybenzoic acid and 3,4-dihyroxybenzoic acid for the pH values of the different studies is shown in the supplement (Table SI 8).

The high amount of deprotonated 2,4,6-trihydroxybenzoic acid may be the reason for its low toxicity observed in this study. Accordingly, the weaker acid 3,4-dihydroxybenzoic acid was found more toxic in this study. The slightly higher toxicity of 3,4-dihydroxybenzoic acid found by Kamaya et al. is further in line with this explanation since at pH 7.5 the base-acid ratio is smaller than at pH 8.1. Differences to the study of Lee et al. cannot be explained by differences in the pH. Secondly, nutrient content can have an effect on toxicity (Kamaya et al., 2004). OECD medium and the algae culture medium of the Environmental Protection Agency (EPA) used by Lee et al. and Kamaya et al. differ in regard of nutrient concentrations and form of nitrogen source. In addition, manganese ions are not added to the EPA medium (USEPA, 2012). Thirdly, degradation of test substances could further limit the exposure time. Lee et al. did not monitor the concentration of the benzoic acids during their toxicity test (duration = 2 days). Kamaya et al. found the concentration of benzoic acids to be constant during the test (duration = 3 days). This result is in disagreement with the decreases of concentrations of 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid observed in this study. However, degradation could also be influenced by medium constitution and pH. In summary, the current study adds to knowledge on benzoic acid by reporting EC_{50} values obtained according to the OECD guideline 201 and provides further evidence that their toxicity depends on pH and medium composition.

3.8. Evaluation of ecotoxicity

The chosen combination of an algae growth inhibition test with HPLC-UV/vis and HPLC-HRMS provided new insights on the effect of flavonoids on algae growth. The miniaturized algae growth inhibition test proved to be a robust method for high throughput-determination of EC_{50} values. However, the testing of flavonoids is limited due to their low solubility at the algae growth inhibition test conditions. Still, it was possible to determine EC_{50} values of 13 out of 26 selected flavonoids.

Due to the lacking efficacy, flavonoids seem to be unsuited as algaecides. On the other hand, this also suggests a low ecotoxicity. However, toxicity data towards other test organisms such as daphnia species is needed. So far, only isoflavonoids caught attention of environmental scientists due to their estrogenic activity. Adverse effect of some isoflavonoids on fish were summarized by Jarošová et al. (2015). Nevertheless, they concluded that reported environmental concentrations in surface water are lower than calculated lowest observable effect concentrations except for contaminated sites close to e.g., fruit processing industry or paper mills (Jarošová et al., 2015). Isoflavonoids tested in the current study precipitated in the algae culture medium. It is, therefore, likely that they would not reach toxic concentrations in the environment.

This current study went beyond the investigation of toxicity and studied the fate of the flavonoids at the toxicity test conditions. In a first step, we provided evidence that no metal complexes are formed by the flavonoids and metals of the algae culture medium. Still, it is possible that flavonoid metal complexes form in the environment and change the toxicity (Kasprzak et al., 2015; Panhwar and Memon, 2011; Wang et al., 1992). Secondly, the concentration of most flavonoids during the test could be measured easily with HPLC-UV/vis, a standard technique to investigate flavonoids (Szultka et al., 2013). The observed degradation of 8 of the 11 selected flavonoids emphasizes the importance to monitor the concentration of flavonoids during bioassays to gain a better understanding of the found effects. Based on the low growth inhibition of four identified TPs and a structural closely related compound, which could be purchased and tested as single substances, we hypothesize that the observed growth inhibition of degrading flavonoids is caused by the mixture of the flavonoid parent compound and formed TPs. To develop a better understanding of the growth inhibition of algae by flavonoids, additional studies are needed to identify molecular targets of flavonoids and their TPs. Additionally, it remains to be investigated if slowly degrading flavonoids like flavone, hesperetin, and diosmetin have a different mode of action than fast degrading flavonoids.

The fast degradation of most flavonoids observed in this study and the low toxicity of formed TPs suggest low environmental risks. However, since environmental conditions may severely differ from the algae growth inhibition test in e.g., availability of metal ions, oxygen concentration and exposure to sunlight further research is required to fully understand impacts of flavonoids in the environment.

4. Conclusions

This study set out to deepen the understanding of adverse effects on algae induced by flavonoids to allow a better evaluation of their potential as benign substitutes for synthetic chemicals. By combining an algae growth inhibition test with further analytical investigation of the fate of 11 selected flavonoids at the test conditions, this study provides new insights into flavonoids' toxicity towards algae, their instability, and the contribution of TPs. This study was the first to determine EC_{50} values of flavonoids according to the OECD guideline 201 and to compare the obtained results to the commercial algaecides terbutryn and diuron. The observed moderate toxicity of tested flavonoids towards *R. subcapitata* provides a solid, standardized knowledge foundation for further ecotoxicity assessment.

Adding to the very limited data on flavonoids stability in cell culture media (Maini et al., 2012; Xiao and Högger, 2015), this study found 8 of 11 tested flavonoids to degrade fast and identified in total 25 different TPs. These results confirmed and extended knowledge on structural features which influence degradation rates and the formation of certain TPs.

Due to the moderate toxicity, fast degradation and the found lower toxicity of TPs, flavonoids may provide substitutes with reduced ecotoxicity for more harmful synthetic chemicals. Still, an understanding of the mechanism of action of flavonoids at the molecular level and information on the environmental fate are needed for a holistic risk assessment.

The methodical approach of this study can be transferred to all kinds of bioactivity tests with flavonoids and be useful to establish structure-activity relationships that take the stability into account. The variety of structural features present in flavonoids could be exploited to tune their properties. Slowly degrading flavonoids with low toxicity towards algae, like hesperetin, may be promising candidates for pharmaceuticals with low ecotoxicity.

CRediT authorship contribution statement

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

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.scp.2024.101473.

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