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Natural Products as Benign Substitutes for Synthetic Chemicals - the Example of Flavonoids

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Abstract

Chemicals improve health and living standards of humans while at the same time causing severe environmental pollution. Therefore, the aim of this thesis was to explore the potential of flavonoids, a class of natural products studied for a variety of applications, to substitute persistent and harmful synthetic chemicals in line with the Benign by Design concept. This overarching goal was pursued by performing research on three important aspects of a potential benign substitute. Firstly, as example of the functionality, the application of flavonoids as biopesticides was investigated with a systematic literature review. Secondly, the environmental effects and fate of several flavonoids were explored by investigating their ecotoxicity based on algae growth inhibition and their environmental biodegradability. The applied standard tests according to Organization for Economic Co-operation and Development (OECD) guideline 201 and 301 D were accompanied by further analytical methods including High Performance Liquid Chromatography coupled with an ultraviolet and visible light detector (HPLC-UV/vis) or coupled with High Resolution Mass Spectrometry (HPLC-HR-MS) to gain a better understanding of the processes taking place during these tests e.g. abiotic degradation and the formation of transformation products.

The results of the systematic literature review revealed that many different pesticidal activities of flavonoids were investigated, but more cohesive knowledge, e.g. on the effectiveness of flavonoid-based biopesticides and their target spectrum is required. Regarding the environmental effects and fate of a chemical, the observed moderate algae growth inhibition of flavonoids and of the identified abiotic transformation products as well as the readily biodegradability of most tested flavonoids support their utilization as benign substitutes with low ecotoxicity and non-persistence in the environment. Additionally, this thesis highlights the importance to consider the limited abiotic stability of many flavonoids and identified it as one of the major challenges for the application of flavonoids in e.g. agriculture or pharmacy. However, this thesis showed that structural features known to enhance the abiotic stability (lacking hydroxy group at C3, C2-C3 single bond, and O-methylation) do not hinder

environmental biodegradation. Therefore, these structural features can be used to select or design flavonoids with sufficient abiotic stability for application while being readily biodegradable. These obtained new insights show that selected flavonoids have the potential to substitute persistent and harmful chemicals. Among the tested flavonoids, hesperetin was identified as the most promising candidate. All in all, this thesis showed that a combination of algae growth inhibition and environmental biodegradability screening is a suitable approach to apply the Benign by Design concept to natural products. The chosen approach can be used to investigate the environmental effects and fate of other natural products, but small improvements of the experimental setup could be made. Generally, this thesis further promotes the idea to use natural products as substitutes for synthetic chemicals in order to tackle environmental pollution.

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I would like to express my deepest gratitude to Prof. Dr. Klaus Kümmerer for the opportunity to learn more about and do a doctoral thesis in the field of Sustainable Chemistry. I am thankful for his supervision, his feedback and a working environment encouraging discussions and allowing room for mistakes. Special thanks go to Prof. Dr. Carolin Floeter and Prof. Dr. Daniel Pleissner for their examination of my doctoral thesis. Additionally, I am also very grateful for the support of Dr. Oliver Ollson that went beyond his professional guidance including a lot of help structuring publications and also involved encouragement in difficult situations.

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List of Abbreviations

CBT	Closed Bottle Test
EC_{50}	half maximal effective concentration
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
HPLC	High Performance Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of detection
LOQ	Limit of Quantification
NPs	Natural products
OECD	Organization for Economic Co-operation and Development
ThOD	Theoretical oxygen demand
TPs	Transformation products
UNEP	United Nation Environment Program
UV/vis	ultraviolet/ visible light
3-OH	hydroxy group at C3 of a flavonoid

1 Introduction

1.1 Chemical Pollution as a Major Threat to Human Health and Ecosystems

Humans have learned to shape nature's elementary building blocks into molecules, macromolecules, materials, and products at will. In the form of metallurgy, pottery, glass production, dying and tanning, chemical practices and their impact on human lives reach back to the 5th millennium (B.C.). Today synthetic chemicals and chemical products play indispensable roles in our everyday lives fulfilling many functions and increasing life quality. More than 350 000 individual organic and inorganic chemicals as well as mixtures of chemicals have been globally registered for production and use[1]. Furthermore, the production volume of chemicals is increasing[2]. However, in addition to the advantages of chemicals, it is uttermost important to consider the fate of the chemicals after their use and possible negative impacts on human health and the environment. To prevent and reduce the entry of chemicals into the environment, waste and effluents are treated. However, these end-of-pipe measures have limitations and are challenged by the amount and diversity of chemicals[3]. Not all compounds are removed by these treatments or transformation products (TPs) can be formed by incomplete degradation[3,4]. Moreover, chemicals used in agriculture such as pesticides and veterinary pharmaceuticals enter the environment untreated via diffuse sources[5,6]. Another emerging source of chemical emissions is the leaching of biocides used to avoid microorganism growth on facades from buildings[7–9].

Despite current efforts focusing on end-of-pipe measures, chemical pollution accumulated to one of the major challenges humankind is faced with. It has the potential to cause severe ecosystem problems on a global scale and, thus, negatively affect vital earth system processes that sustain human life[10]. Based on the increasing annual production and releases of chemicals at a pace that outstrips the global capacity for assessment and monitoring, Persson *et al.*

estimated that the critical threshold of chemical pollution (planetary boundary of novel entities[a]) is exceeded[10].

1.2 Green and Sustainable Chemistry

In 1998, made efforts to transform chemistry and tackle environmental pollution were manifested in the 12 principles of Green Chemistry[11] (see Annex **Figure A1**). These principles focus on efficiency, waste and hazard prevention, as well as on obtaining chemicals from renewable feedstocks. Later on, the concept of Sustainable Chemistry was developed[12–14]. The societal importance of these two scientific concepts is reflected by their incorporation into political frameworks, e.g., the Green and Sustainable Chemistry Framework Manual of the United Nations Environmental Program (UNEP), and the Chemicals Strategy for Sustainability - Towards a Toxic-Free Environment as part of the European Green Deal[15,16]. However, understandings of Sustainable Chemistry vary. In this thesis, Sustainable Chemistry is understood as a guiding principle that, highlighting reduction and prevention, starts with the question if a chemical is necessary to fulfil a certain need[17]. Only if no non-chemical alternative is available, a green chemical designed for safety and degradability, obtained from renewable resources in a benign synthesis should be used. Following a holistic, systematic approach, Sustainable Chemistry takes the whole life cycle of a chemical and all involved stakeholders into account, including social aspects e.g., fair and safe working conditions, as well as economic aspects e.g., new business models such as chemical leasing. Over the last decades a lot of research was dedicated to Green Chemistry and Sustainable Chemistry. One major aspect is the design of benign chemicals. The Benign by Design concept demands to balance the stability for application of a chemicals and its complete degradation in the environment to

[a] novel entities: “New substances, new forms of existing substances, and modified life forms that have the potential for unwanted geophysical and/or biological effects including chemicals and other new types of engineered materials or organisms not previously known to the Earth system, as well as naturally occurring elements (for example, heavy metals) mobilized by anthropogenic activities.”[107]

water, carbon dioxide and inorganic salts (mineralization)[18]. These considerations should be incorporated as early in the design process as possible[18]. Research showing the feasibility of the Benign by Design concept is crucial for its wide implementation in the chemical industry. A few studies showing the successful re-design of chemicals exist[19,20]. However, the Benign by Design concept was not applied to natural products yet.

1.3 Natural Products as Benign Substitutes

Regarding the design of benign chemicals, nature could be a source of inspiration. Not only humans produce and use chemicals in their everyday life, also plants, fungi, animals, bacteria, and archaea produce chemicals, called natural products (NPs), that are necessary for primary or secondary functions. Beyond energy metabolism, growth, reproduction, and structure (primary metabolism), NPs are involved in e.g. communication and chemical defense against predators (secondary metabolism).

Historically but still today, NPs play a role in the discovery of drugs and pesticides. In drug discovery, the interest in NPs is based on their bioactivity and the diversity in structural scaffolds but their possible advantages of environmental biodegradability is not considered[21,22]. By contrast, research towards the utilization of NPs as biopesticides is driven among other reasons by the motivation to find more environmentally friendly active substances[23,24]. However, can it be assumed that natural products are harmless to non-target organisms and readily biodegradable in the environment? Referring to the known toxicity towards humans of several NPs, Hansen *et al.* stated: “We are picky with what we eat and drink - for good reasons”[25]. Whereas knowledge on the toxicity of several NPs is available, data on the environmental effects and fate is largely missing[25,26]. However, knowledge on the ecotoxicity and environmental biodegradability of NPs could offer valuable contributions to Green Chemistry and the design of benign chemicals. Firstly, to evaluate the potential of NPs to substitute persistent and harmful synthetic chemicals. And secondly, to provide knowledge for the design of biodegradable chemicals in general. Therefore, this thesis sets out to explore the

possibilities for applications and the environmental effects and fate of a class of natural products – the flavonoids.

1.3.1 Flavonoids

Among the diversity of NPs, the class of flavonoids was selected for investigation in this thesis for two reasons. Firstly, they are common in the plant kingdom and could be obtained from biorefinery of e.g. agricultural or food waste[27]. The second reason is the wide range of possible applications of flavonoids[28].

According to the strict International Union of Pure and Applied Chemistry (IUPAC) definition, flavonoids are characterized by the following features[29]: They consist of a C6-C3-C6 carbon skeleton forming three rings (A, B, and C), one of which is a heterocycle with oxygen. Based on the position of the phenyl moiety (B-ring) in regard to the A- and C-ring core of the molecule, this group of NPs is distinguished into flavonoids, isoflavonoids, and neoflavonoids (**Figure 1**). Flavonoids are further divided into six subgroups (flavones (f1), flavonols (f2), anthocyanidins (f3), flavanones (f4), flavanonols (f5), and flavanols (f6)) depending on structural features such as a C2-C3 double bond, a hydroxy group at C3 (3-OH), and a keto function at C4 or a combination of them (**Figure 1**). Next to varying hydroxy group patterns, O-methylation and glycosylation are common modifications of flavonoids. Approximately 6 000 different flavonoids were identified so far[28].

Natural functions of flavonoids in plants include signaling with symbiotic bacteria, pigmentation of flowers to attract pollinators, response to UV or oxidative stress and chemical defense against predators[30,31]. Based on the secondly mentioned natural functions, flavonoids have historically been used as dyes[32]. Furthermore, flavonoids were extensively investigated for their antioxidant activity based on their radical scavenging ability and for a variety of biological activities based on enzyme inhibition and influencing of intracellular signaling pathways[33–35]. The different biological activities of flavonoids could be exploited for food conservation, cosmetic, pharmaceutical or pesticidal applications[28,36–39].

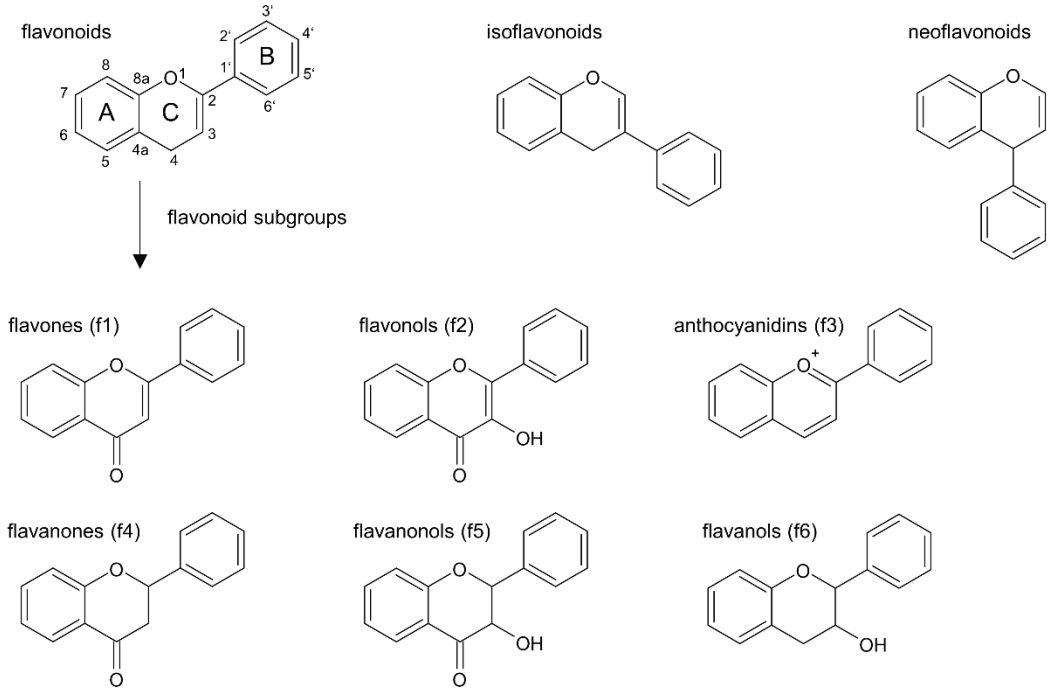

Figure 1: Overview of the different flavonoid types and subgroups.

The flavonoid basic structure is shown with numbering of C-atoms and labeling of the three different rings. The different flavonoid subgroups are marked with an identification code (letters and numbers in brackets) for a better recognition throughout the thesis. Modified figure based on figure 1 of publication 1 (see List of Publications, page 61).

In addition to their activity, the stability[b] is important for possible applications. Flavonoids are known to react with oxygen. Therefore, oxidative degradation of flavonoids during scientific experiments and possible applications has to be considered. Concentration decreases of flavonoids in mammalian cell culture medium were previously observed[40,41]. Therefore, the monitoring of the concentration of the tested flavonoids during scientific experiments is essential to obtain more knowledge about the stability of flavonoids at the test conditions and for the interpretation of the results. Furthermore, possible contributions of TPs formed during the degradation of instable flavonoids to the observed effects have to be kept in mind and investigated. Insights into the influence of certain structural features on the stability or the formation of certain TPs could help to select flavonoids with balanced activity and stability.

[b] In this thesis, the term stability is used to describe if a substance's concentration remains constant under certain experimental conditions during the duration of the experiment.

2 Structure and Objectives

The main goal of this thesis was to evaluate the potential of flavonoids as benign substitutes for synthetic chemicals. Applying the Benign by Design concept to a class of natural products for the first time, it set out to investigate the function and the environmental effects and fate of several flavonoids (**Figure 2**).

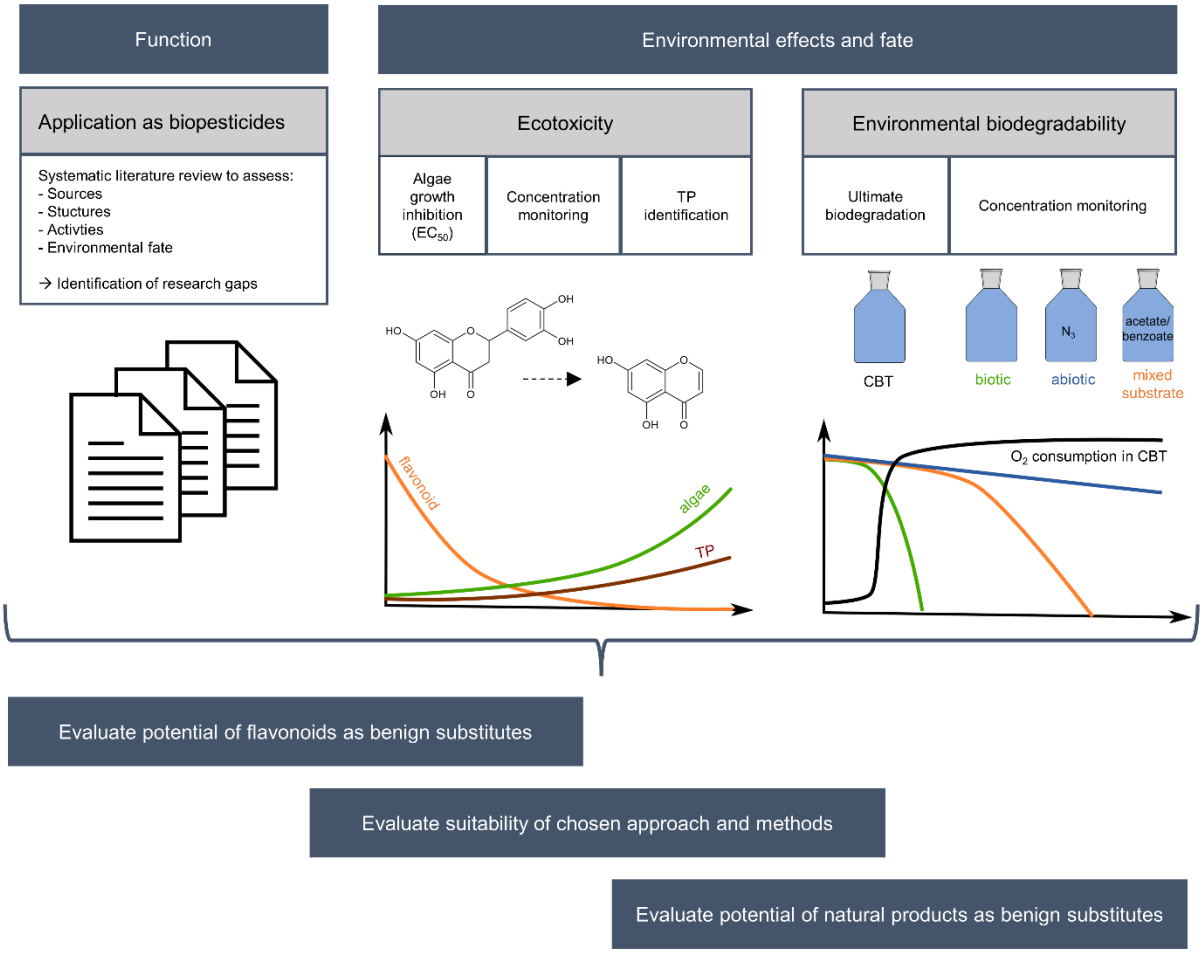

Figure 2: Overview of the aims of this thesis.

Based on the research on the application of flavonoids as biopesticides and their environmental effects and fate, in particular ecotoxicity based on algae growth inhibition and environmental biodegradability, the potential of flavonoids as benign substitutes will be evaluated. Furthermore, the transferability of the used approach and methods, and the general potential of NPs as benign substitutes will be evaluated. Additionally, an overview of the performed studies and experiments regarding the three aspects application, ecotoxicity, and environmental biodegradability is provided.

In Chapter 3-5, the performed research and the obtained results are presented. Regarding the function, this thesis focuses on the possible application of flavonoids as biopesticides (**Figure 2**). Aiming to obtain a better overview on existing knowledge about biopesticidal applications, this thesis attempts to analyze thematically appropriate literature quantitatively and to identify research gaps in this field. Regarding the environmental effects and fate, this thesis sets out to investigate the ecotoxicity based on algae growth inhibition and the environmental biodegradability of a selection of flavonoids according to guidelines of the Organization for Economic Co-operation and Development (OECD) (**Figure 2**). For the experiments, a set of 26 flavonoids was chosen based on structural features in order to investigate the influence of these features on algae growth inhibition and environmental biodegradability (**Figure 3**).

Going beyond the quantification of the algae growth inhibition and the classification as readily biodegradable, this thesis aims to understand the processes that occur during algae growth inhibition and environmental biodegradation tests by investigation possible abiotic degradation and TP formation with High Performance Liquid Chromatography coupled with ultraviolet/visible light detection (HPLC-UV/vis) or High Resolution Mass Spectrometry (HPLC-HR-MS) (**Figure 2**). More details are provided in section 4.1 and 5.1.

Based on the newly obtained knowledge on possible applications, the ecotoxicity, the environmental biodegradability, and abiotic stability, this thesis then aims to evaluate and discuss, firstly, the potential of flavonoids as benign substitutes, secondly, the suitability of the chosen approach and methods to find benign substitutes, and, thirdly, the potential of NPs as benign substitutes in general (Chapter 6-8).

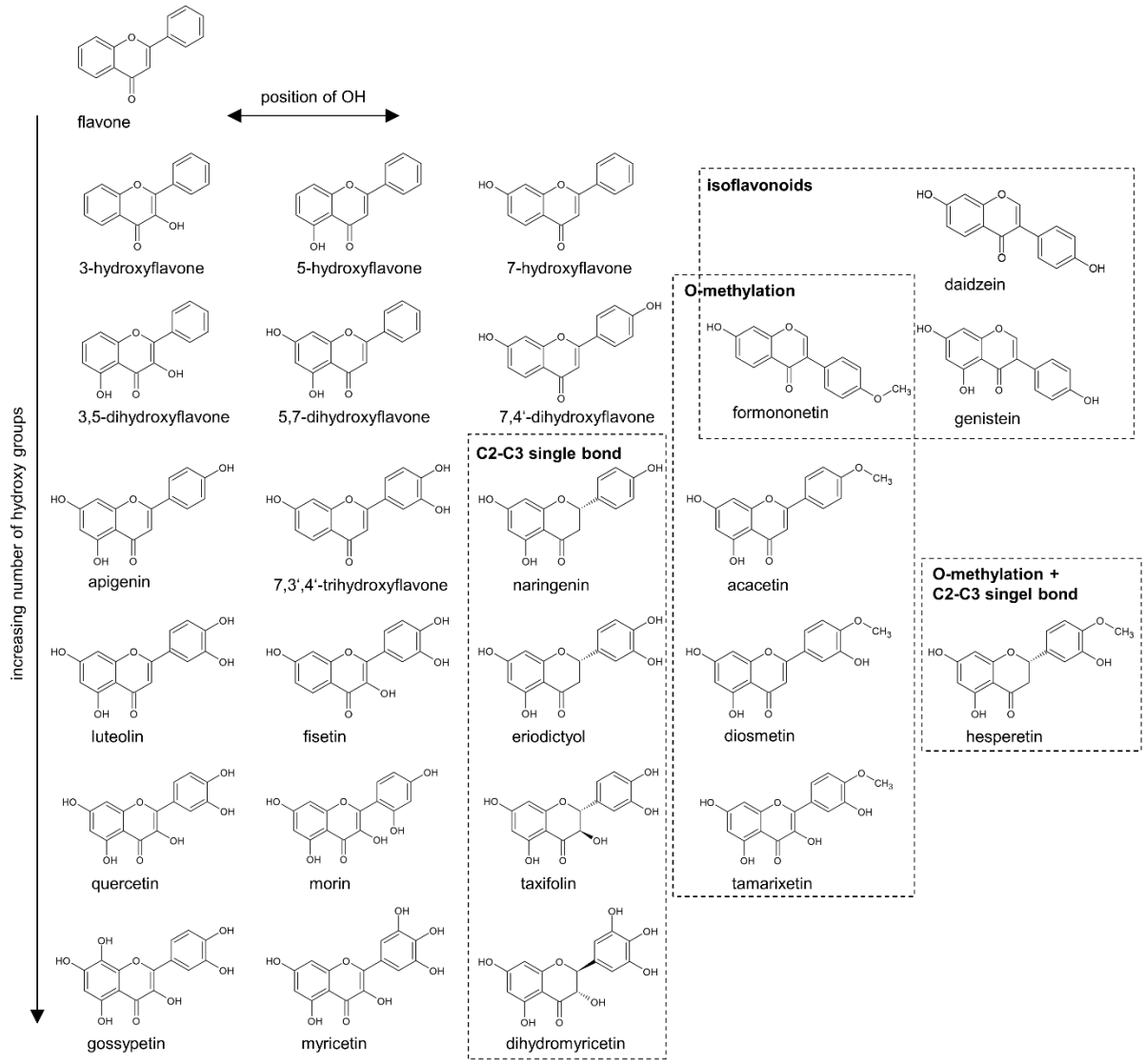

***Figure 3: The 26 flavonoids selected for investigation based on structural features*.**

Each flavonoid is presented with its trivial name. Differences in structural features include the number and position of hydroxy groups, the C2-C3 bond order, O-methylation, a combination of the former two features, and the position of ring B (flavonoids vs. isoflavonoids). However, not all flavonoids could be examined with every method due to low water solubility.

3 The Application of Flavonoids as Biopesticides

3.1 Problem Statement

Many studies explored the activities of flavonoids including their antioxidant, pharmaceutical, and pesticidal activities. Whereas several publications review and summarize antioxidant and pharmaceutical activities[33,34,36,42,43], an overview about pesticidal activities and applications is missing. In line with the goal to design benign chemicals obtained from renewable resources which degrade in the environment, the motivation to investigate flavonoids as possible biopesticides is driven by the search for environmentally friendly alternatives to conventional pesticides[23,44]. Hence, this thesis aims to not only provide an overview of which flavonoids and which pesticidal activities were investigated so far, but also sets out to assess to what extent sustainability aspects including the sources and the environmental fate of investigated flavonoids are considered in publications dealing with pesticidal activity of flavonoids.

3.2 Method

A systematic literature review was conducted that enabled a quantitative analysis of four aspects of the life cycle of flavonoids used as biopesticides. A detailed description of the literature screening and data collection is given in publication 1. In brief, a systematic literature screening was conducted using a combination of specific search terms (flavon*, biocid*, biopesticid*, pesticid*, herbicid*, fungicide*, insecticide*, alg*cid*, and harmful algae bloom*). Publications were selected for analysis from the results if a certain flavonoid that naturally occurs in plants was investigated for a pesticidal activity. Hence, publications that investigated pharmaceutical activities, only measured the total flavonoid content, or investigated synthetically modified flavonoids were excluded. Based on these criteria, the literature screening yielded a pool of 201 research articles investigating the application of flavonoids as biopesticides. From these publications several information on the aspects “sources, structures, activity, and environmental fate” was withdrawn:

- Sources:
    - Were the flavonoids purchased or isolated?
    - Which plant species were used as sources of the isolated flavonoids?
    - Was the used plant material a waste resource?
    - Were pure compounds or extracts investigated?
- Structures:
    - Which flavonoids were investigated?
- Activity:
    - Which activity was investigated and which target organisms were used?
    - What kind of application was intended for the biopesticides?
- Environmental fate:
    - Was data or information on the environment fate provided or cited?

The information obtained for these different aspects were analyzed quantitatively. The results of this assessment are summarized in the following section.

3.3 Results and Discussion

Four different aspects of the utilization of flavonoids as biopesticides were analyzed quantitatively. Firstly, the assessment of the sources of flavonoids revealed that most studies (83 %) used isolated compounds or flavonoid-containing extracts of plant materials instead of purchased, pure compounds. Interestingly, only 10.6 % of the investigated sources of flavonoid-containing plant extracts or isolated flavonoids were waste materials such as orange peels or rice husks (**Figure 4**). Despite the huge potential of agricultural and food waste as source of high-value chemicals[27], the assessment of the actual sources of flavonoids investigated for application as biopesticides revealed a current underutilization of this feedstock. Therefore, the systematic literature review identified an important research gap that could promote the investigation of waste materials as sources of flavonoids in future studies.

Secondly, the assessment revealed that 281 different flavonoids were investigated for pesticidal activities either as isolated compound or as constituent of an extract. Whereas this represents a high number and extensive research effort, the investigated flavonoids embody only a fraction of the more than 6 000 different flavonoids[28]. Hence, bioprospection work of flavonoids has only just begun. Among the 281 different flavonoids identified in the systematic literature review, luteolin, apigenin, quercetin, and kaempferol and their glycosylated or O-methylated derivatives were identified as the most studied flavonoids (**Figure 4**). The identification of frequently investigated compounds can help to select candidates for further comprehensive studies. These studies could aim for a detailed overview on all different activities of one certain flavonoid which was beyond the scope of this study.

Thirdly, the assessment of activities revealed that flavonoids were investigated for diverse pesticidal activities including insecticidal, fungicidal, herbicidal, bactericidal, and algicidal activities. Possible applications ranged from crop protection in the field and post-harvest to controlling harmful algae blooms and antifouling agents. In total 260 different target organisms including plants, insects, fungi, bacteria, algae, nematodes, molluscs, and barnacles were investigated (**Figure 4**). This high variety of different target organisms raises questions regarding the target-specificity of flavonoids. However, this topic is referred to by only 17 % of all considered studies in their results and discussion sections. Therefore, the assessment revealed that data on the target-specificity of flavonoids is lacking and further research is required. The indicated broad target spectrum also enhances risks for non-target organisms e.g. pollinators or earth worms at agricultural sites or organisms in surrounding water bodies affected by pesticide-containing run-offs like algae. Thus, assessing the ecotoxicity of flavonoids is of crucial importance.

Furthermore, some studies compared the effect of the tested flavonoid or flavonoid-containing extract to a conventional pesticide. The results ranged from lesser to superior activity of the tested flavonoids and flavonoid-containing extracts compared to the conventional pesticides[45–48]. Thus, a holistic evaluation of the effectiveness of flavonoids is needed.

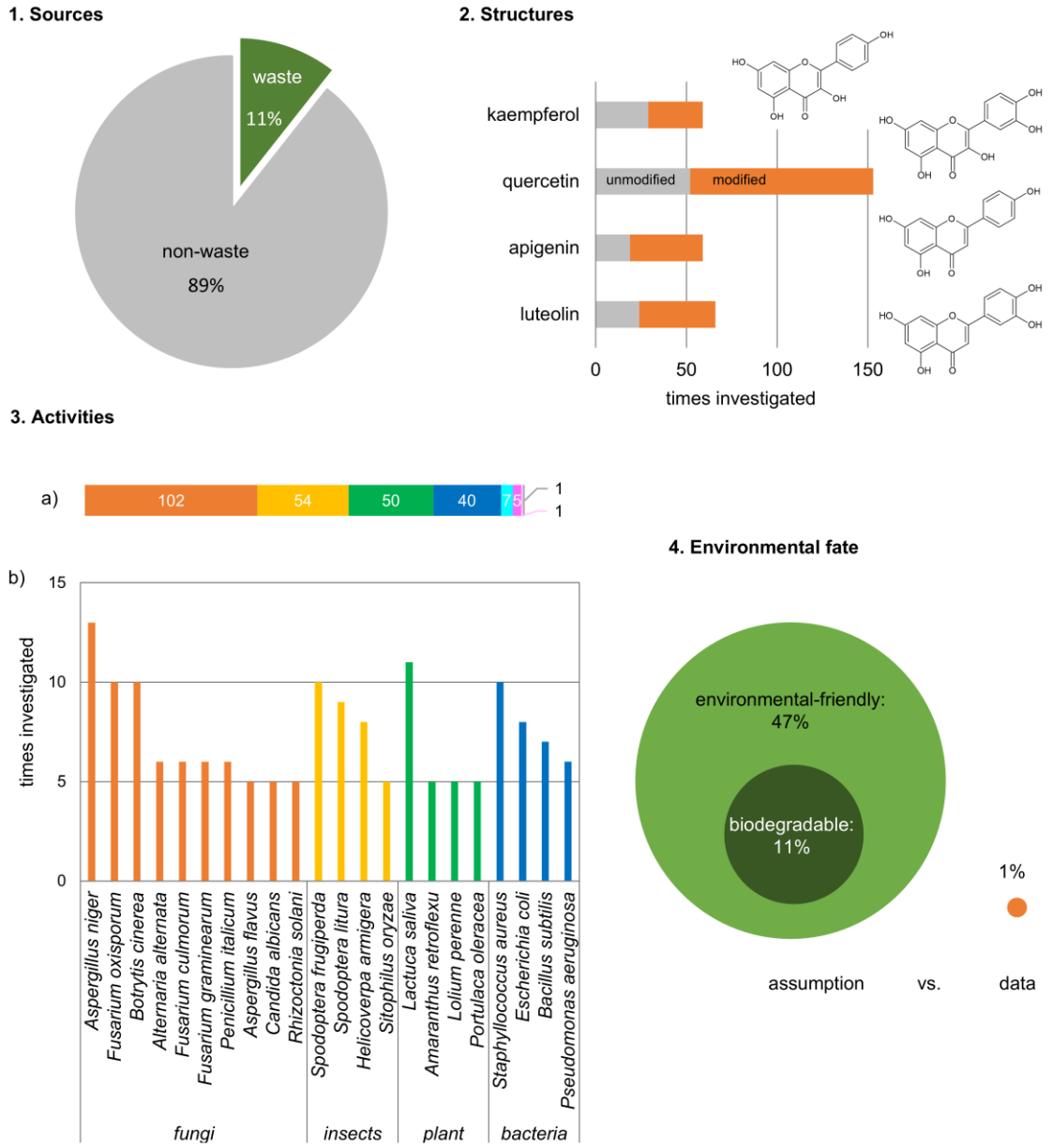

Figure 4: Results of the systematic literature review:

***1. Sources**: The percentage of studies that used waste materials e.g. orange peels as source of the tested flavonoid is shown in comparison to the studies using non-waste sources. **2. Structures**: The four most frequently studied flavonoids and how often they have been studied (count) are presented. In addition to these four flavonoids, the count of flavonoids with the same core structure but small modifications including O-methylation or glycosylation is shown. **3. Activities:** a) The number of different target organisms per taxonomic group is given: fungi (orange), insects (yellow), plants (green), bacteria (blue), algae and cyanobacteria (cyan), molluscs (pink), nematodes (light pink), and barnacles (grey). b) Most frequently invested species are shown. **4. Environmental fate:** Schematic representation of the percentage of studies that assumed environmental friendliness or stated it as a motivation to study flavonoids, including a fraction that specifically stated biodegradability as a wanted or assumed property. By contrast, the percentage of studies providing or citing data on the environmental fate is shown. Graphs 1 to 4 represent modified (1-3) and new graphs (4) according to the figures (3,5, and 7) and data of publication 1.*

Fourthly, environmental friendliness was mentioned as a motivation to investigate flavonoids as biopesticides in 47 % of the studies and biodegradability is an assumed or wanted property in 11 %. However, only 1 % of the studies (2 studies) provided citations or data regarding the environmental fate of flavonoids (**Figure 4**). An additional literature search showed that scattered information on the environmental fate of a few flavonoids is available[49–52]. These studies indicated a low risk for the persistence of flavonoids in the environment. However, a systematic investigation of the environmental fate of flavonoids including first steps like environmental biodegradability testing with standardized methods is missing.

In summary, the review gave important insights into which flavonoids and which pesticidal activities were studied, but also revealed the need for further research on sustainable sources and the environmental effects and fate including e.g. the investigation of the ecotoxicity and of the environmental biodegradation of flavonoids.

3.4 Conclusion

The conducted systematic literature review was the first to provide an overview on the existing research towards the utilization of flavonoids as biopesticides. Major findings were a total number of 281 different investigated flavonoids and a broad target spectrum of the class of flavonoids encompassing plants, insects, fungi, bacteria, and further organisms. The systematic literature review did not lead to the identification of flavonoids that are most promising for biopesticidal applications but offered an important basis for future research aiming at the evaluation of the effectiveness of flavonoids as biopesticides. Furthermore, it revealed that more knowledge on the sustainable production and the environmental effects and fate including ecotoxicity and environmental biodegradability is needed. The identification of these research gaps helps to direct future research towards a more holistic knowledge base for the evaluation of flavonoids as substitutes for persistent and harmful synthetic pesticides.

4 The Ecotoxicity of Flavonoids

4.1 Problem Statement

Knowledge of the ecotoxicity of chemicals in general but most importantly of chemicals with applications leading to an untreated entry into the environment (e.g. pesticides applied on fields) is essential to comprehend impacts on non-target organisms and assess environmental risks. The assessment of the ecotoxicity is required for the registration and use of most chemicals e.g. in accordance with the European regulations for chemicals[53]. Yet, as identified in the systematic literature review, information on the ecotoxicity of flavonoids is largely missing.

Algae as primary producers play an important role in ecosystems and, hence, growth assays with unicellular algae are an established tool in the ecotoxicity assessment of chemicals. Some research on the effects of flavonoids on algae was performed[54–56], however, it focused on the control of cyanobacteria and the red tide algae *Phaeocystis globose* that can form harmful blooms[55–62]. Only D'Abrosca *et al.* used the green fresh water algae *Raphidocelis subcapitata,* a species recommended by the OECD guideline 201, to investigate the effect of an extract and eight identified flavonoids on algae growth in a petri dish assay[54,63]. To date, no data on algae growth inhibition caused by flavonoids obtained with a standardized test protocol reporting half maximal effective concentration (EC_{50} values) is available. Such data, however, would provide important first insights into the ecotoxicity of flavonoids.

Due to the possible oxidative degradation of flavonoids, it is important to monitor the concentration of tested flavonoids for a better understanding and evaluation of the observed effects in ecotoxicity tests. However, none of the above-mentioned studies monitored the flavonoids' concentration during the performed tests.

Addressing these research gaps, this thesis aims to investigate the algae growth inhibition induced by 26 different flavonoids using a standard test according to the OECD guideline 201 with the recommended species *R. subcapitata*. To explore possible synergistic effects, mixtures containing two or three different flavonoids were tested. Additionally, the study set out

to monitor the concentration of selected flavonoids and to identify TPs of instable flavonoids. To shed light upon the contribution of TPs to the observed effects, algae growth inhibition of commercially available TPs was investigated, too.

4.2 Method

4.2.1 Algae Growth Inhibition Testing

An algae growth inhibition test according to the OECD guideline 201 with *R. subcapitata* as test organism was used to determine the EC_{50} values of the test substances based on the growth rate[63]. To enable a higher throughput, a miniaturized test set up with 24 well plates was successfully established. Algae growth was followed by measuring the chlorophyll a fluorescence at 24 h intervals for a test duration of 72 h. The test substances included 26 flavonoids selected based on structural features (**Figure 3**), the two conventional algicides diuron and terbutryn as positive controls, the reference compound 3,5-dichlorophenol, and a few identified TPs or structurally related compounds that were commercially available (4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 5,7-dihydroxychromone, and 4-hydroxyphenylglyoxylic acid). Furthermore, 10 different 1:1 mixtures of two to three flavonoids were tested. The expected EC_{50} values for dose addition were calculated according to equation 1 and compared to the experimentally derived EC_{50} values.

$$EC_{50}\ additive = portion\ A / EC_{50}\ A + portion\ B / EC_{50}\ B \qquad (1)$$

More details on the algae growth inhibition test are provided in publication 2.

4.2.2 Concentration Monitoring and TP Identification

Flavone, luteolin, eriodictyol, diosmetin, hesperetin, quercetin, morin, taxifolin, tamarixetin, myricetin, and dihydromyricetin were chosen for the analytical monitoring based on their solubility, their different growth inhibition of *R. subcapitata* and their structural features. The concentration of these flavonoids was quantified using a HPLC-UV/vis method at the time points 0, 24, 48, and 72 h. In addition to samples with algae cells, abiotic controls without algae cells were assessed to confirm that possible degradation is abiotic and not influenced by the algae cells. The structure of TPs was elucidated with HPLC-HR-MS. More details on the analytical methods are provided in publication 2.

4.3 Results and Discussion

4.3.1 Algae Growth Inhibition of Flavonoids

The results showed moderate algae growth inhibition of the selected flavonoids with EC_{50} values ranging from 0.7 – 22 mg/L (2.4 – 73 µM) (**Table 1**). However, the EC_{50} values of several flavonoids could not be determined due to the limited aqueous solubility of these substances (**Table 1** and **Table A 1**).

Table 1: Growth inhibition of R. subcapitata treated with flavonoids expressed as EC_{50} values (n=4) with standard deviation (SD).

*The toxicity of the flavonoids is compared to the two algicides 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 (see also **Table A 1**). 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. Modified table based on table 1 of publication 2. Data on the growth rates is shown in the annex table A 3.*

	substance	EC_{50} [mg/L]	standard deviation	EC_{50} [µM]	standard deviation
algicides	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
diosmetin	EC_{50} > 10 / 42 % ± 2		> 33	
tamarixetin	EC_{50} > 10 / 30 % ± 3		> 32	

The EC_{50} values obtained for mixtures were in the same range as for the single flavonoids (**Table 2**). Comparing experimentally derived EC_{50} values of the flavonoid mixtures with expected EC_{50} values calculated based on dose addition strongly indicated that these mixtures behaved according to dose addition. Hence, the toxicity of a simple flavonoid mixture does not seem to be enhanced by synergistic effects. However, a more complex mixture may increase the toxicity. Segatto *et al*. showed that a mango waste extract containing among others the flavonoid hyperoside and the xanthonoid mangiferin inhibited the growth of *R. subcapitata* much stronger compared to hyperoside, mangiferin, and a mangiferin-hyperoside 1:1 mixture[64].

Table 2: Overview of theoretical and experimentally derived EC_{50} values of flavonoid mixtures.

Average values with standard deviation (SD, n=4) of experimentally derived results are given. The table is based on data presented in figure 2 of publication 2.

mixture	**EC_{50} [mg/L] (calculated based on dose addition)**	**EC_{50} [mg/L] (experimentally derived)**
luteolin-eriodictyol	0.81	0.72 ± 0.07
luteolin-quercetin	1.42	1.10 ± 0.09
luteolin-morin	1.57	2.64 ± 0.16
luteolin-taxifolin	1.68	0.73 ± 0.18
luteolin-hesperetin	1.8	0.95 ± 0.21
quercetin-morin	3.63	3.50 ± 0.5
quercetin-taxifolin	4.28	4.5 ± 1
quercetin-hesperetin	5.15	4.0 ± 0.7
taxifolin-hesperetin	11.85	8.40 ± 0.63
luteolin-hesperetin-taxifolin	2.45	1.08 ± 0.12

Regarding the three categories for acute aquatic hazards by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (category 1: $EC_{50} \leq 1$mg/L, category 2: $EC_{50} > 1 \leq 10$ mg/L, and category 3: $EC_{50} > 10 \leq 100$ mg/L), most successfully tested flavonoids and their mixtures were in category 2. Hesperetin, the least toxic substance, and the taxifolin-hesperetin mixture were in category 3. The most toxic flavonoids eriodictyol and luteolin as well as their mixture were in category 1. Yet, compared to the conventional algicides terbutryn and diuron, the most active flavonoids were less toxic by two orders of magnitude.

The effects on algae and cyanobacteria of some flavonoids also investigated in this thesis were studied previously. However, eriodictyol, gossypetin, 3,5-dihydroxyflavone, 7,3',4'-trihydroxyflaveone, morin, fisetin, dihydromyricetin, taxifolin, hesperetin, diosmetin, and tamarixetin were investigated for the first time in this thesis. The obtained results for the growth inhibition of *R. subcapitata* are in a general agreement with the magnitude of growth inhibition induced by flavonoids in the algae *P. globose* and the cyanobacterium *Microcystis aeruginosa,* although the comparison is hindered by differences in the EC_{50} determination and the lack of reference compounds or positive controls in the respective studies[55,56,61,65].

From the obtained EC_{50} values it can be deduced that flavonoids entering the aquatic environment in concentrations of approximately 1 mg/L and higher might acutely endanger algae and other organisms. However, reported environmental concentrations of flavonoids in natural water bodies ranged from ng/L range up to tens of µg/L at some sites[66–74]. At these low concentrations flavonoids could contribute to the mixture of other naturally occurring chemicals and anthropogenic pollutants that might combine to toxic effects[75]. Yet, for a comprehensive estimation of the environmental risks, the stability of the flavonoids needs to be considered, too.

4.3.2 Degradation of Flavonoids during the Algae Growth Inhibition Test

The performed concentration monitoring of the 11 selected flavonoids during the algae growth inhibition test provided interesting insights towards a better understanding of the observed effects (**Figure 5**). Only flavone and diosmetin showed a constant concentration over the whole test duration (105 % ± 5 and 100 % ± 10 of the initial 5 mg/L remaining after 72 h.) Hesperetin showed a minor concentration decrease to 92 % ± 1 of the initial 5 mg/L after 72 h. The other eight flavonoids (eriodictyol, luteolin, quercetin, morin, taxifolin, tamarixetin, myricetin, and dihydromyricetin) were either below the limit of detection or quantification (LOD, LOQ) at the end of the test. Based on the different time periods until the concentrations dropped below LOQ or LOD, the stability of the tested flavonoids showed the following order: myricetin = dihydromyricetin < quercetin < tamarixetin < luteolin < morin < eriodictyol = taxifolin < hesperetin < diosmetin = flavone. Controls without algae showed the same degradation behavior, therefore, the algae are not involved in the degradation of the tested flavonoids (**Figure 5**). Thus, the flavonoids degraded abiotically. Due to the aerobic test conditions and the known reactivity of flavonoids with oxygen, it is probable that oxidative degradation of the flavonoids occurred[76].

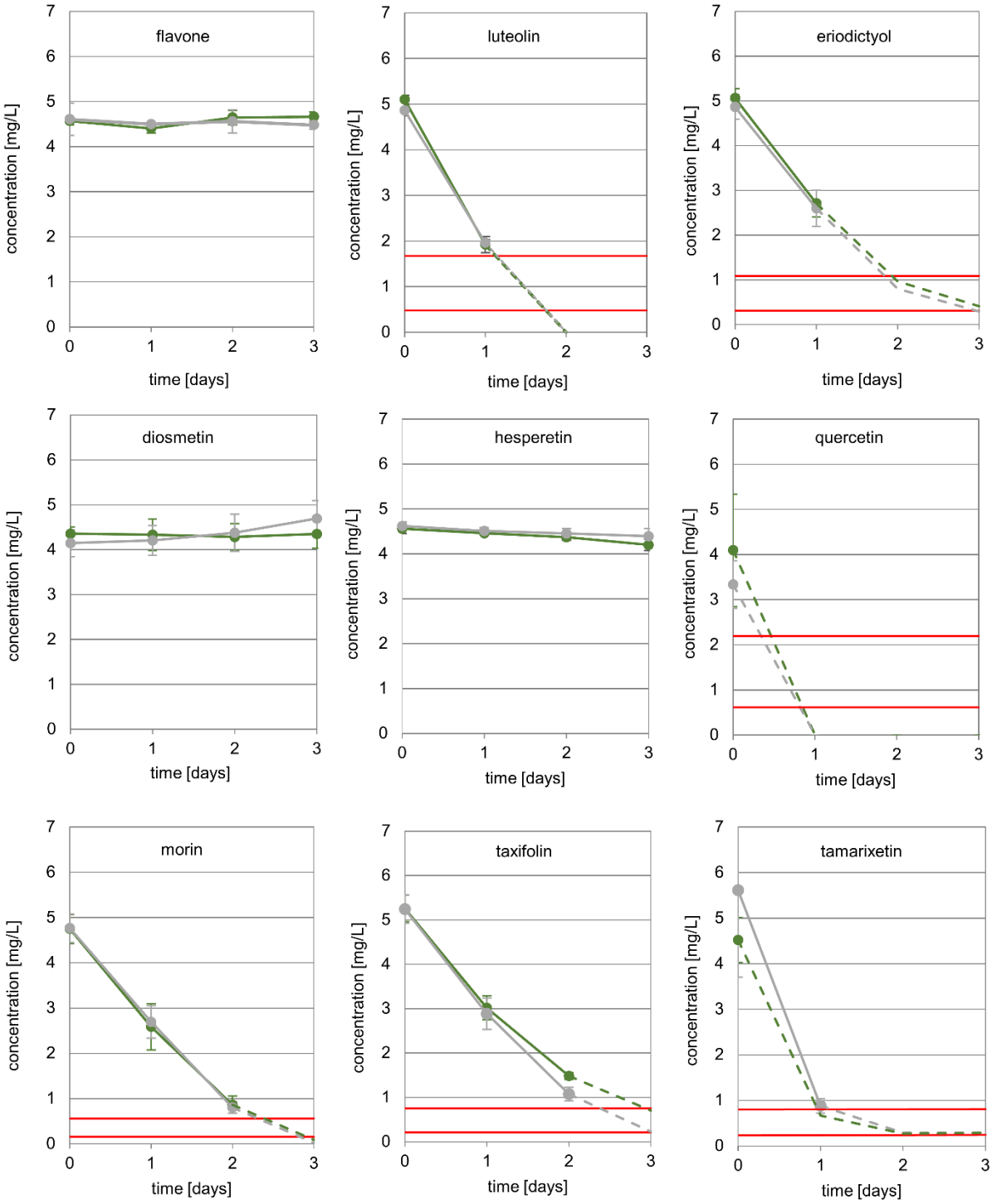

Figure 5: Concentration development of the flavonoids flavone, luteolin, eriodictyol, diosmetin, hesperetin, quercetin, morin, taxifolin, and tamarixetin during the algae growth inhibition test.

Samples with algae (green), controls without algae (grey). Start concentration: 5 mg/L. Data points represent average values of four 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. The figure is taken from publication 2.

Moreover, these observations are in good agreement with previous results on the influence of certain structural features on the antioxidant activity and the stability in cell culture medium[40,77,78]. The comparison of three flavonoid pairs only differing in the O-methylation of one hydroxy group in the B-ring showed that this feature enhances the stability (**Table 3**). A stabilizing effect is also visible for a C2-C3 single bond when comparing taxifolin to quercetin and eriodictyol to luteolin, whereas hesperetin and diosmetin showed a comparable high stability (for structures see **Figure 3**). A 3-OH group as a destabilizing structural feature was supported by the faster concentration decrease of quercetin compared to luteolin and tamarixetin to diosmetin, whereas taxifolin and eriodictyol showed a similar concentration decrease. The fast degradation of myricetin and dihydromyricetin is in line with a high number of hydroxy groups as a destabilizing factor[40,41].

Table 3: Overview of the influence of certain structural features on the stability of flavonoids at the algae growth inhibition test conditions.

The table is based on data of publication 2.

structural feature	**effect on stability**	**comparison of flavonoids differing only in the specific structural feature**
methoxy group in B-ring	enhances stability ↑	diosmetin >. luteolin hesperetin > eriodictyol tamarixetin > quercetin
C2-C3 single bond	enhances stability ↑	taxifolin > quercetin eriodictyol > luteolin hesperetin ~ diosmetin
3-OH	decreases stability ↓	quercetin < luteolin tamarixetin < diosmetin taxifolin ~ eriodictyol
high number of hydroxy groups	decreases stability ↓	myricetin < quercetin dihydromyricetin < taxifolin

4.3.3 Formation and Contribution of TPs

Due to the degradation of several flavonoids during the algae growth inhibition test, the observed growth inhibition may not be linked to the parent compound directly. Therefore, it was investigated whether and which TPs of the eight instable flavonoids are formed during the algae growth inhibition test. Identified TPs were purchased if possible and investigated for their algae growth inhibition as single compounds.

In total, 25 TPs were identified (see Annex **Table A 2**). Of these TPs, 15 were identified for the first time including 5,7-dihydroxychromone (TP-177). Most TPs are in line with the oxidative degradation pathway in aqueous solution via depsides or benzofuranone derivatives proposed by Sokolová *et al.*[76]. Based on the identified TPs, this pathway was extended to the formation of TP-181.1, TP-197, 2,4,6-trihydroxybenzoic acid (TP-169), and possible dihydroxybenzoic acids (**Figure 6**). Moreover, three rules for TP formation were proposed based on the comparison of the eight tested flavonoids:

1. 2,4,6-trihydroxybenzoic acid (TP-169) seems to be a universal TP of all flavonoids.
2. A 3-OH group is required for the formation of TP-197.
3. 5,7-dihydroxychromone (TP-177) appears to be only formed by flavonoids with a C2-C3 single bond (flavanones (f4) and flavanonols (f5)).

Next to 2,4,6-trihydroxybenzoic acid (TP-169), the degradation of the flavanone (f4) eriodictyol and the flavanonols (f5) taxifolin and dihydromyricetin resulted in TPs that were also formed during the degradation of flavones (f1) and flavonols (f2), in particular 3,4-dihydroxybenzoic acid (TP-153.1) and TP-197 (**Table A 2)**. By contrast, 5,7-dihydroxychromone (TP-177) was only formed by flavanones (f4) and flavanonols (f5). Thus, it might be possible, that flavanones (f4) and flavanonols (f5) degrade via two different pathways. One pathway leads to the formation of 5,7-dihydroxychromone, whereas the other pathway begins with an oxidation of the flavanones (f4) and flavanonols (f5) to their flavone (f1) and flavonol (f2) counterparts and subsequent degradation (**Figure 6**). However, the abiotic degradation of flavanones (f4) and flavanonols (f5) is not well studied. Only Sokolová *et al.* identified some TPs including 3,4-

dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid that formed during the degradation of taxifolin, however, they did not detect 5,7-dihydroxychromone[76]. Hence, further research is needed to establish abiotic degradation pathways of flavanones (f4) and flavanonols (f5).

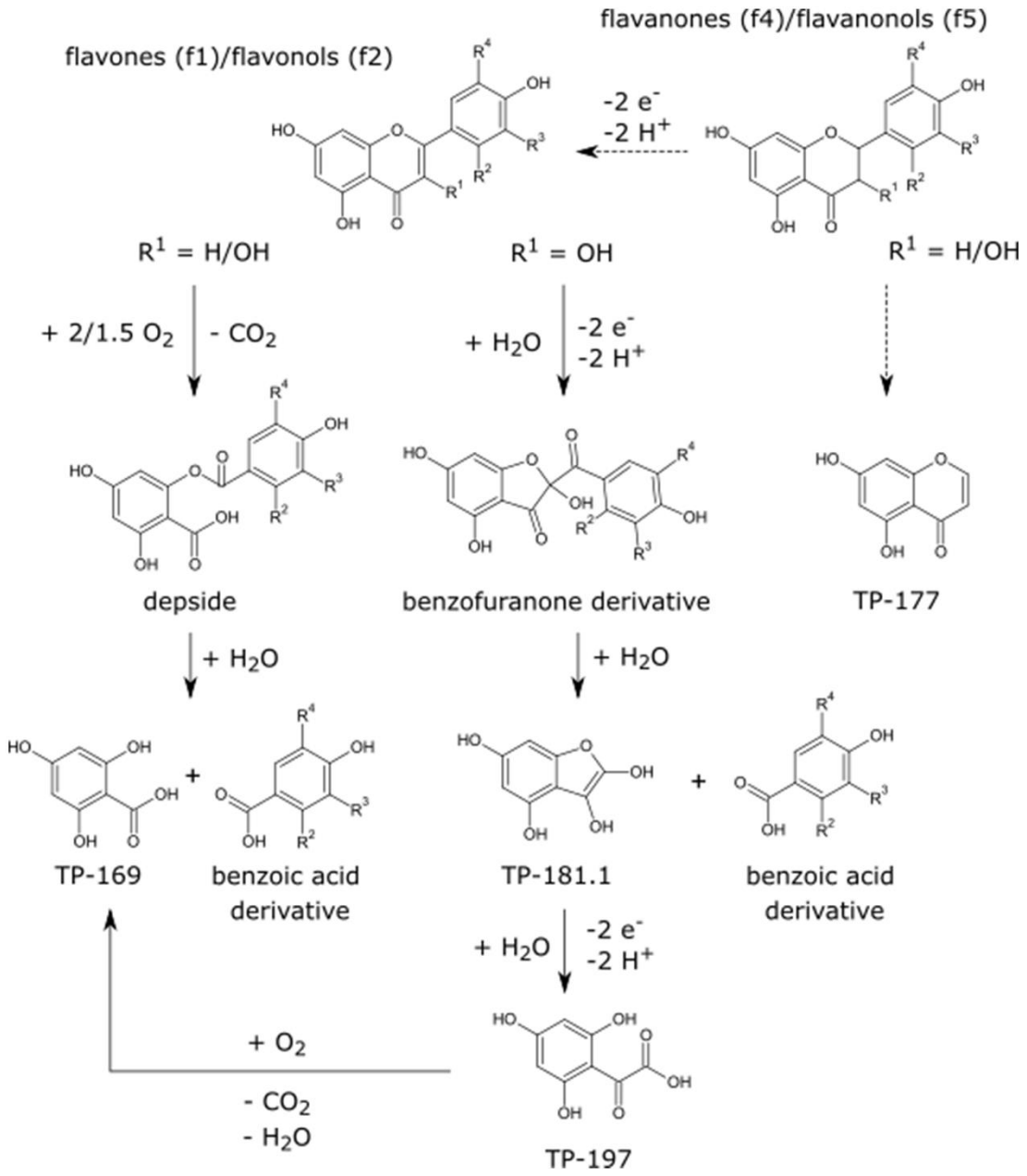

***Figure 6: Proposed degradation pathways of flavonoids**.*

Differences in the degradation of flavones (f1) and flavonols (f2) to the degradation of flavanones (f4) and flavanonols (f5) are shown. 5,7-dihydroxychromone (TP-177) is only formed during the degradation of flavanones (f4) and flavanonols (f5). However, flavanones (f4) and flavanonols (f5) also degrade into similar TPs than flavones (f1) and flavonols (f2). Thus, an oxidation of the flavanones (f4) and flavanonols (f5) to the corresponding flavones (f1) and flavonols (f2) was proposed in this study. Modified figure according to figure 6 of publication 2.

Of the identified TPs, 4-hydroxybenzoic acid (TP-137.1), 3,4-dihydroxybenzoic acid (TP-153.1), 2,4,6-trihydroxybenzoic acid (TP-169), and 5,7-dihydroxychromone (TP-177) were purchased as well as 4-hydroxyphenylglyoxylic acid due to its oxo-acid structural feature also present in TP-197. All tested TPs and 4-hydroxyphenylglyoxylic acid were less toxic than their corresponding parent compounds (**Table 4**). However, for instable flavonoids it is probable that the observed effect was caused by a mixture of the parent flavonoid and occurring TPs.

Not all of the tested TPs were stable during the 72 h-duration of the algae growth inhibition test. The concentration of 3,4-dihydroxybenzoic acid (TP-153.1) and 2,4,6-trihydroxybenzoic acid (TP-169) decreased notably within 72 h to 19 % and 57 % of the initial 30 µM, respectively. In contrast, 4-hydroxybenzoic acid (TP-137.1) and 5,7-dihydroxychromone (TP-177) were stable and thus possibly represent end products of abiotic degradation (**Table 4**).

Table 4: Algae growth inhibition of identified TPs and 4-hydroxyphenylglyoxylic acid.

EC_{50} values (average value, n=4 with standard deviation (SD)) are 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. Additionally, the percentage of the remaining concentration after 72 h is presented based on the measured peak areas of the HPLC-UV/vis analysis, initial concentration of each substance = 30 µM. Modified table based on table 3 of publication 3.

substance	**EC_{50} [mg/L]**	**EC_{50} [µM]**	**%($area_{day3}$/ $area_{day0}$)**
5,7-dihydroxychromone (TP-177)	6.1 ± 0.4	34 ± 2	108 ± 2
3,4-dihydroxybenzoic acid (TP-153.1)	12 ± 2	78 ± 10	19 ± 3
4-hydroxybenzoic acid (TP-137.1)	89 ± 2	641 ± 12	106 ± 6
	%Inhibition at 100 mg/L		
2,4,6-trihydroxybenzoic acid (TP-169)	20 % ± 7		57 ± 5
4-hydroxyphenylglyoxylic acid	23 % ± 7		109 ± 13

4.4 Conclusion

The obtained results regarding the algae growth inhibition, the stability, and the formation of TPs of several flavonoids vitally contributed to a more holistic understanding of the potential environmental risks of flavonoids. The results emphasize the importance to monitor test substances' concentrations during activity tests, to identify TPs and to investigate their contribution to observed effects.

On the one hand, flavonoids such as eriodictyol and luteolin can induce adverse effects on algae. However, these flavonoids degrade fast and formed TPs are less toxic. This suggests low environmental risks. On the other hand, the rather stable hesperetin showed the lowest growth inhibition. Thus, also suggesting low environmental risks. Only flavone which showed a high stability and comparably high toxicity seems unsuited as a benign substitute for a conventional pesticide or pharmaceutical. Therefore, further efforts to find benign substitutes for synthetic chemicals could focus on investigating the effects of the here identified most promising candidate hesperetin on other organisms to exclude negative environmental impacts from its application e.g. as a biopesticide.

5 The Environmental Biodegradability of Flavonoids

5.1 Problem Statement

In addition to low ecotoxicity, benign chemicals should not persist in the environment but degrade fast and completely to inorganic compounds e.g. CO_2 (mineralization). This can be achieved through the biodegradation of the chemical. As pointed out in the systematic literature review, only little information on the environmental fate of flavonoids is known. In addition to a few reports on the occurrence of flavonoids in natural water bodies and the elucidation of biodegradation pathways by different soil bacteria, only two studies investigated biodegradation kinetics of the flavonoid naringenin and the isoflavonoids formononetin and biochanin A in soil[50,52,74,79,80,66–73]. Currently, data on the biodegradation of flavonoids in the aquatic phase is lacking. Therefore, investigating the ultimate biodegradation of flavonoids with a standardized biodegradability test could substantially contribute to fill this research gap and to evaluate the potential of flavonoids as benign substitutes. The Closed Bottle Test (CBT, OECD 301 D) is seen as the gold standard when screening for readily biodegradable chemicals within the frame of the Benign by Design concept[81]. Therefore, the aim of this thesis was to assess the ultimate biodegradation of 19 flavonoids with the Closed Bottle Test as a basis for evaluating the potential of flavonoids as benign substitutes. Beyond that, this thesis set out to look into the processes taking place during the CBT. In particular, due to the reported fast abiotic degradation of several flavonoids[40,82], it was investigated whether the flavonoids themselves or abiotic TPs that are possibly formed during the lag phase are biodegraded. Moreover, the influence of an alternative carbon source on the degradation processes of flavonoids was investigated. Therefore, the primary degradation of four selected flavonoids was monitored at conditions representing biodegradation, abiotic degradation, and degradation during the presence of an alternative carbon source.

5.2 Methods

The CBT was performed according to the OECD guideline 301 D[83]. The inoculum consisted of sewage effluent. Oxygen consumption of the test substances was measured with a fiber-optic oxygen meter. Biodegradation was expressed as the ratio of the real oxygen consumption to the theoretical oxygen demand (ThOD) in percent.

The selection of flavonoids to be tested was made with the aim of investigating the influence of certain structural features including the C2-C3 bond order, a methoxy group in the B-ring, a 3-OH group, and the number (0 to 5) and position of hydroxy groups. Additionally, three isoflavonoids were tested to investigate the influence of the position of the B-ring in regard to the A- and C-ring core.

Luteolin, eriodictyol, diosmetin, and hesperetin were chosen based on structural features and solubility for the monitoring of the primary degradation with HPLC-UV/vis at three different conditions reflecting biodegradation (inoculum and test substance), abiotic degradation (inoculum, test substance, and azide), and degradation at mixed substrate (inoculum, test substance, and either acetate or benzoate) conditions. The experimental set up was very similar to the CBT but the initial concentrations were increased to 5 mg/L and samples were taken regularly over the test period of 28 days. More details are provided in publication 3.

5.3 Results and Discussion

5.3.1 Ultimate Biodegradation according to the CBT

Of the 19 tested flavonoids, 17 showed an oxygen consumption over 60 % of the ThOD and, therefore, were classified as readily biodegradable (**Figure 7**). Only flavone and morin showed no or a minor oxygen consumption and, hence, were classified as non-readily biodegradable.

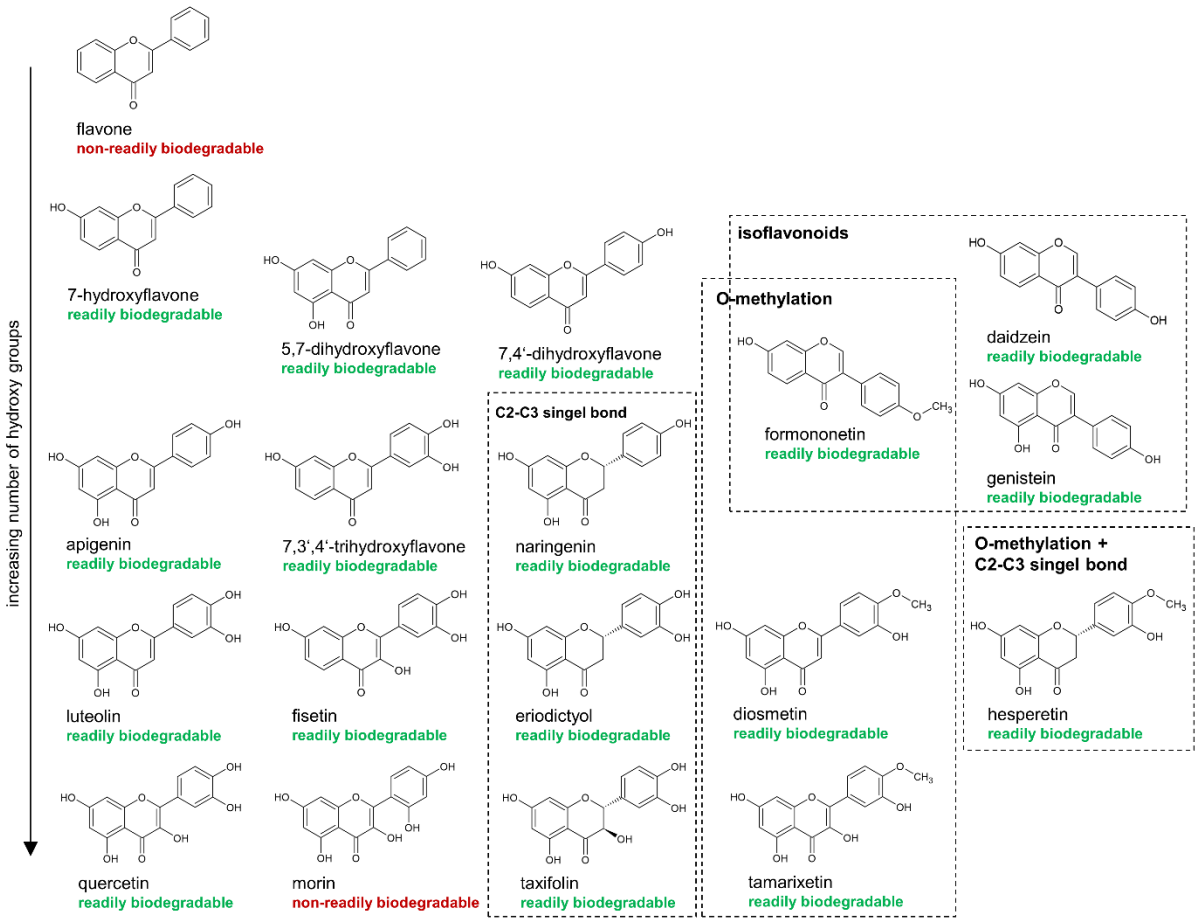

Figure 7: Readily biodegradability test results (OECD 301 D/ CBT) of the 19 tested flavonoids.

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. Modified figure based on figure 2 of publication 3.

The 19 tested flavonoids provided interesting insights into the influence of structural features on the biodegradation of flavonoids (**Table 5**). The results demonstrate that at least one hydroxy group is required for biodegradation because of the non-readily-biodegradability of flavone. All tested flavonoids except morin with one to five hydroxy groups were biodegraded

despite differences in structural features including the positions of hydroxy groups, the lack or presence of a 3-OH, O-methylation, the C2-C3 bond order, and the position of the B-ring (**Table 5**). The structural peculiarity of morin is the hydroxy group at position 2' resulting in a meta orientation of the two hydroxy groups in the B-ring, whereas the other flavonoids with two hydroxy groups in the B-ring display a 3',4' pattern. This structural difference could have an impact on the reactivity of morin in at least two ways. Firstly, analogous to the comparison of resorcinol (1,3-benzendiol) with catechol (1,2-benzendiol), morin might not oxidize as easily as its isomer quercetin and other flavonoids with a 3',4' pattern in the B-ring due to the impossibility to form a chinone structure. Thus, this difference in the redox behavior might affect biodegradation. Secondly, quantum chemical calculations of morin and its isomer quercetin revealed that in contrast to quercetin favoring a planar geometry, morin favors a non-planar geometry that might hinder biodegradation sterically[84–86].

The observed biodegradability of most tested flavonoids in the aquatic phase is in good agreement with the previously observed biodegradation of apigenin, naringenin, 7,4'-dihydroxyflavone, luteolin, quercetin, daidzein, genistein, and formononetin in soil or by soil bacteria[50,80,87]. The other tested flavonoids were investigated for the first time.

Table 5: Overview of the influence of structural features on the biodegradation of flavonoids.

Modified table based on table 1 of publication 3.

structural feature	**influence on biodegradation**	**considered flavonoids / flavonoid pairs differing only in the corresponding struct. feature**
number of hydroxy groups	• no hydroxy groups – non-readily biodegradable	• flavone
	• 1-5 hydroxy groups – biodegradable (but one exception)	• all except morin
position of hydroxy groups	• OH at position 3,5,3', and 4' – no influence on biodegradation	• 5,7-dihydroxyflavone – 7,4'-dihydroxyflavone • apigenin – 7,3',4'-trihydroxyflavone • luteolin - fisetin
	• OH at position 2' – can hinder biodegradation	• morin - quercetin
presence or lack of 3-OH	• no influence on biodegradation	• quercetin – luteolin • taxifolin - eriodictyol • tamarixetin - diosmetin
methoxy group	• no influence on biodegradation	• diosmetin – luteolin • hesperetin - eriodictyol • tamarixetin - quercetin
C2-C3 bond order	• no influence on biodegradation	• apigenin – naringenin • luteolin – eriodictyol • diosmetin – hesperetin • quercetin – taxifolin
position of B-ring (C2 vs. C3)	• no influence on biodegradation	• 7,4'-dihydroxyflavone - genistein • apigenin - daidzein

5.3.2 Primary Degradation of Four Selected Flavonoids

The primary degradation at three different conditions representing biodegradation, abiotic degradation, and mixed substrates (alternative carbon source present) was monitored to gain a better understanding of the degradation processes occurring during the CBT. Comparing the concentration decrease of the four selected flavonoids in biodegradation and abiotic degradation samples revealed that biodegradation occurred faster than abiotic degradation (**Figure 8 a and b**). Still, eriodictyol, luteolin, and diosmetin showed severe concentration decreases in the abiotic samples (**Figure 8 b)**. By contrast, the concentration of hesperetin remained constant in the abiotic samples over 28 days demonstrating the stability of this compound. While the measurement of the oxygen consumption in the CBT does not provide information on whether the flavonoids were biodegraded or fast abiotic degradation occurred and the formed abiotic TPs were biodegraded under oxygen consumption, the faster concentration decrease of the flavonoids in the biodegradation samples measured by HPLC-UV/vis strongly indicates that the flavonoids themselves were biodegraded.

In line with carbon catabolite repression[88–90], the presence of acetate or benzoate as alternative carbon source for the bacteria tends to delay and decrease the biodegradation rates of the tested flavonoids to an extent that abiotic degradation of instable flavonoids might be the major cause of concentration decreases at mixed substrate conditions (**Figure 8 c**).

Substances that are classified as readily biodegradable in the CBT are believed to also biodegrade fast in the environment due to the higher abundance and diversity of bacteria usually present there. However, the limited abiotic stability and slower biodegradation in the presence of other carbon sources suggest that biodegradation may not be the most common degradation pathway of instable flavonoids in the environment. Instead, incomplete abiotic degradation may occur. However, known TPs of abiotic degradation such as benzoic acids are also intermediates of the proposed degradation pathway of flavonoids by bacteria[76,79,80]. Thus, abiotic TPs may be further degraded by bacteria in the environment. Therefore, it is very likely that flavonoids do not persist in the environment.

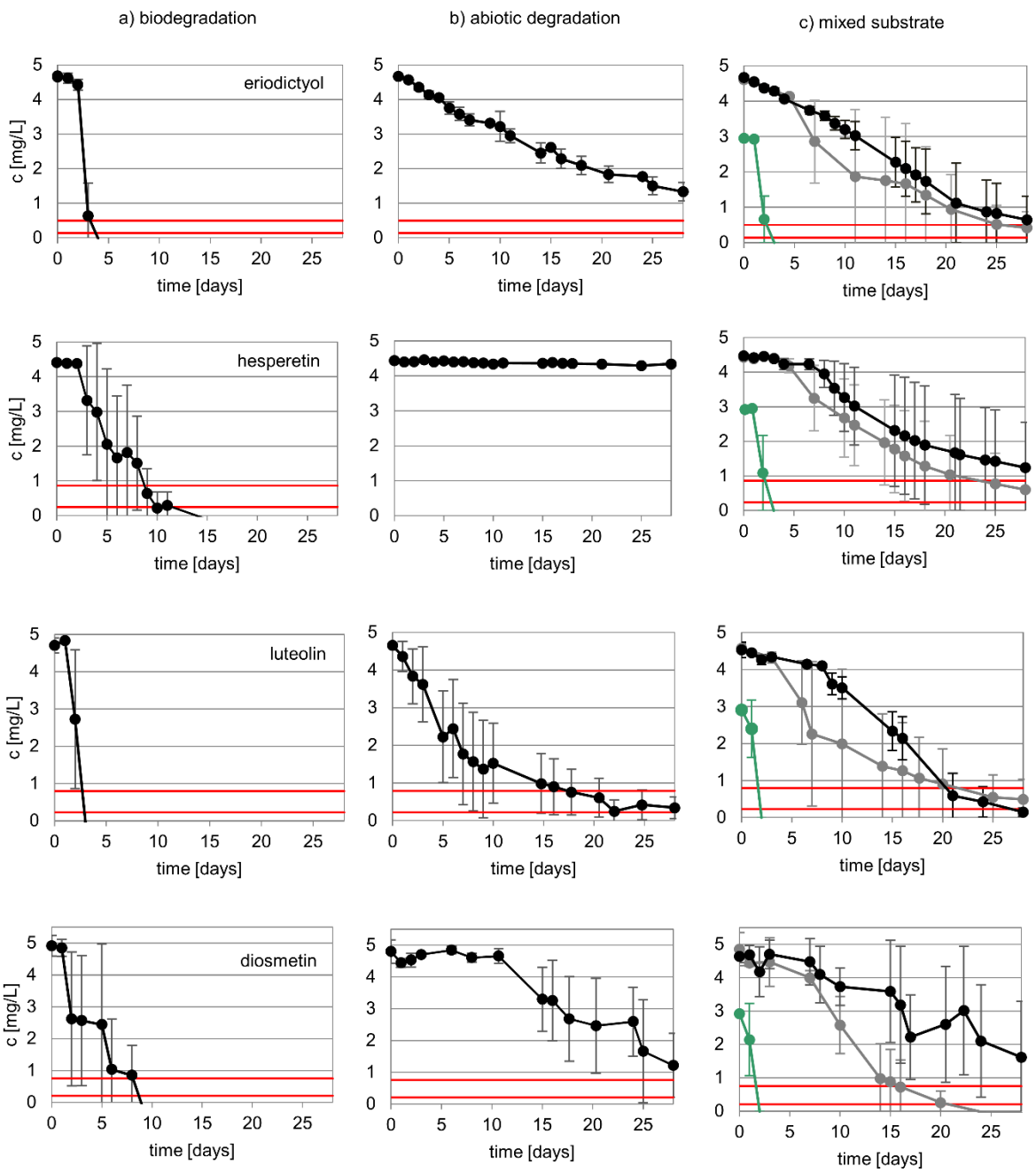

Figure 8: Concentration development of the flavonoids eriodictyol, hesperetin, luteolin, and diosmetin during biodegradability testing.

a) biodegradation conditions (test substance + inoculum), b) abiotic conditions (test substance + inoculum + azide) and c) mixed substrate conditions (test substance + inoculum + acetate/benzoate), flavonoid concentration in the presence of acetate is shown in grey, flavonoid concentration in the presence of benzoate is shown in black, benzoate itself is shown in green. Datapoints represent average values with standard deviations (n=3, n=2 for samples with acetate and benzoate, respectively). Details on individual replicates are shown in Figure A 3. The figure corresponds to figure 4 of publication 3.

5.4 Conclusion

The performed study provided knowledge on the environmental biodegradation of flavonoids in the aquatic phase for the first time. It revealed that whereas the majority of the tested flavonoids (17 out of 19) was readily biodegradable in the CBT, the complete lack of hydroxy groups and a 2',4' pattern of hydroxy groups in the B-ring can hinder biodegradation. Monitoring the primary degradation of four flavonoids further showed that, firstly, biodegradation was faster than abiotic degradation at CBT conditions, and secondly, that the presence of an alternative carbon source tends to decrease the flavonoids' biodegradation to an extent that abiotic degradation contributed substantially to the concentration decreases of instable flavonoids. Only hesperetin was stable during the abiotic conditions. This gained insights provide an important orientation for the selection and design of flavonoids that balance abiotic stability required for application and biodegradability in the environment. Whereas several flavonoids fulfil the criteria of biodegradability, hesperetin seems to be the most suited as a benign substitute due to its high abiotic stability.

6 Evaluating the Potential of Flavonoids as Benign Substitutes

This thesis explored the potential of flavonoids, as an important example of natural products, to substitute persistent and harmful synthetic chemicals. To evaluate the potential of flavonoids as benign substitutes, possible applications as well as the environmental effects and fate were investigated.

6.1 Possible Applications of Flavonoids

A plethora of studies is available that investigated different biological activities of flavonoids and suggested many applications in e.g. pharmacy and agriculture. However, identifying which flavonoids are most effective and suited for which applications remains challenging. For instance, the performed systematic literature research provided an important first overview on pesticidal activities of flavonoids, but also revealed that a more cohesive understanding of the pesticidal activities of flavonoids is needed to find flavonoids that are most effective. At the same time, effectiveness is not the only criteria of a benign substitute. The earlier inclusion of data on the environmental fate of flavonoids could help to limit comprehensive studies on the activity and effectiveness of flavonoids to those representatives of this class of NPs that are e.g. biodegradable in the environment.

6.2 Abiotic Stability and the Influence of Structural Features

The concentration monitoring results during the algae growth inhibition and abiotic controls of the biodegradation tests made evident that the application of flavonoids as pharmaceuticals or biopesticides might be hindered by their low abiotic stability. Accordingly, one challenge to use flavonoids as benign substitutes for synthetic chemicals lies in finding a flavonoid that is stable enough for application while having low impacts on non-target organisms and being biodegradable in the environment.

Concentration monitoring during the algae growth inhibition test revealed that flavone, diosmetin and hesperetin were the most stable under these conditions whereas the concentration of the other eight tested flavonoids including luteolin and eriodictyol decreased substantially within 3 days. Comparing these results to the concentration monitoring of luteolin, eriodictyol, diosmetin and hesperetin in the abiotic controls of the biodegradation testing revealed similarities but also differences. Luteolin and eriodictyol were also instable in these abiotic controls, however their degradation was faster under the conditions of the algae growth inhibition test. Hesperetin showed a minor concentration decrease during the algae growth inhibition test but was stable over 28 days in the abiotic controls of the biodegradation testing. A concentration decrease of diosmetin was observed in the abiotic controls of the biodegradation testing but not during the algae growth inhibition test. However, this concentration decrease was notable only after 10 days and, therefore, this alleged difference to the stability in the algae growth inhibition test might be due to the different test durations. Aside from diosmetin, the presented differences in the degradation rates of luteolin, eriodictyol, and hesperetin have in common that the degradation was faster under the conditions of the algae growth inhibition test. The experimental conditions in the algae growth inhibition test and the abiotic controls of the biodegradation testing differ in pH (8.1 vs 7.4), mineral content in the medium (e.g. Fe^{3+} and Cu^{2+} concentrations), and slightly in temperature (23 vs 20 °C), which could influence the degradation rate of the flavonoids. The faster degradation at the algae growth inhibition test at a higher pH is consistent with a previously reported increase of the degradation rate of quercetin with increasing pH[91]. A possible explanation for the decreased stability at higher pH is the deprotonation of flavonoids that can facilitate oxidation[92,93]. Furthermore, the higher temperature at the algae growth inhibition test also facilitates a faster degradation. Despite the differences in degradation rates under different conditions, the results demonstrate that certain structural features such as a C2-C3 single bond and O-methylation increase the stability of flavonoids. The good agreement between previous and obtained data on structural features that increase or decrease the abiotic stability suggest that the observed

structure-stability relationships (**Table 3**) represent general rules. Accordingly, structural features can be used to tune the abiotic stability of flavonoids.

6.3 Selection of the Most Promising Flavonoids based on Abiotic Stability, Algae Growth Inhibition and Biodegradability

Based on the tunability of the abiotic stability by structural features, the question arises how these features influence algae growth inhibition and biodegradation and if an abiotically stable but non-ecotoxic and environmentally biodegradable flavonoid can be designed or selected from the pool of existing flavonoids.

A direct link between structural features and observed algae growth inhibition is not possible due to the fast degradation of most tested flavonoids and the likely contribution of formed TPs to the observed toxicity. Still the obtained EC_{50} values can be used to select promising candidates from the pool of tested flavonoids. Regarding biodegradation, only two structural features, the lack of any hydroxy groups (flavone) and a 2',4' pattern of hydroxy groups in the B-ring (morin), were found to have hindered biodegradation. Therefore, structural features known to enhance abiotic stability (lacking 3-OH, C2-C3 single bond, O-methylation) can be used to select abiotically more stable but readily biodegradable flavonoids. Hesperetin exhibits each of these three structural features and is , therefore, the most promising candidate among the tested flavonoids. It not only showed high abiotic stability while being biodegradable, but it also exhibited the lowest toxicity towards algae (**Figure 9**). Flavone, which is also stable, was non-readily biodegradable and had comparable strong adverse effects on *R. subcapitata*. Therefore, it is ruled out as a benign substitute. Other flavonoids with structural features enhancing the abiotic stability that might be suited as benign substitutes due to observed comparable low adverse effects on algae and readily biodegradability are taxifolin, diosmetin, and tamarixetin. The limited stability of these flavonoids could be circumvented by encapsulation[94].

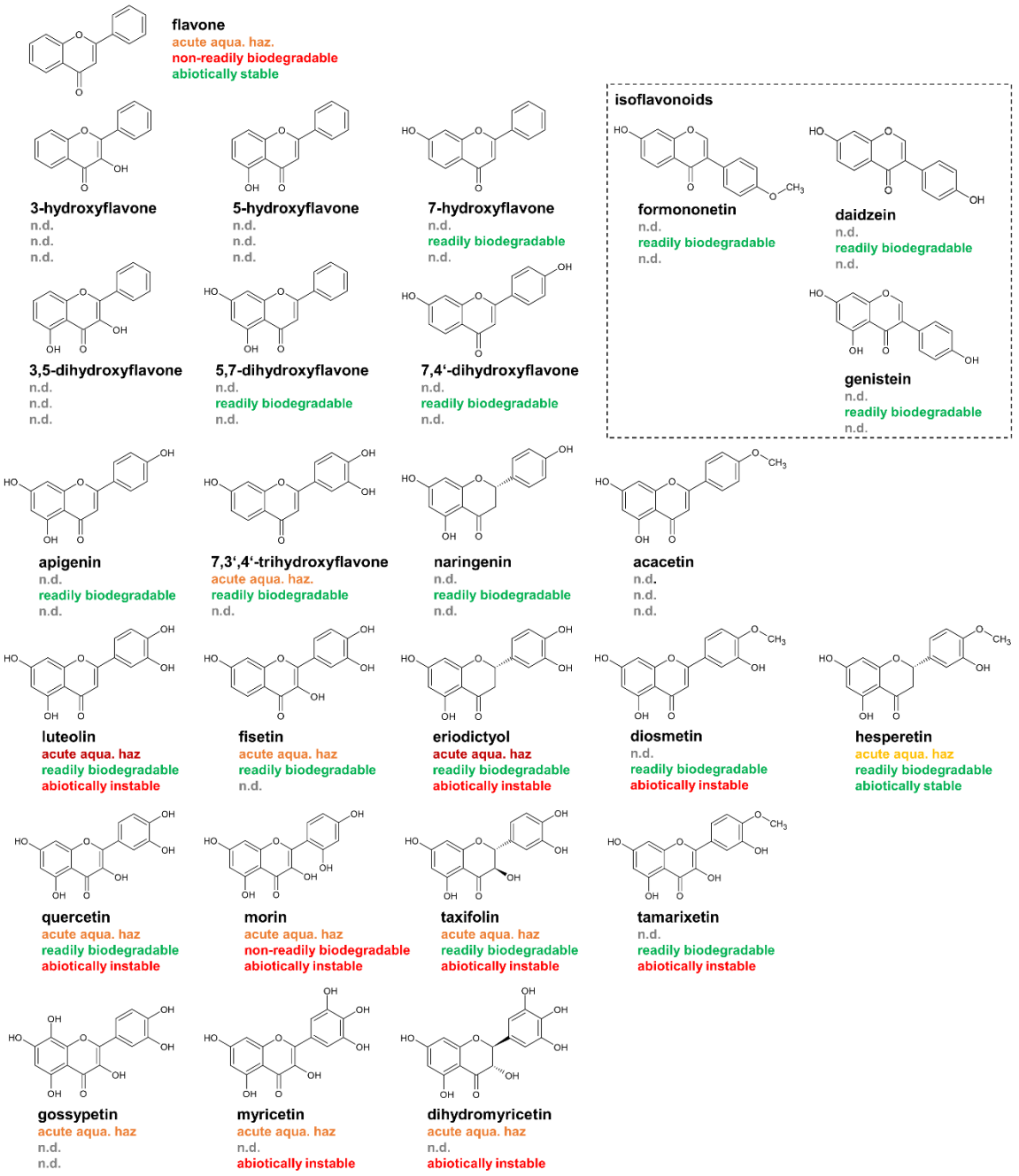

Figure 9: Overview of algae growth inhibition, biodegradability, and abiotic stability of flavonoids.

The results of the algae growth inhibition test are expressed in the corresponding acute aquatic hazard categories: red= category 1: EC_{50} ≤ 1mg/L, orange = category 2: EC_{50} > 1 ≤ 10 mg/L and yellow = category 3: EC_{50} > 10 ≤ 100 mg/L). If the EC_{50} value could not be determined due to low solubility of the flavonoids n.d. (not determined) is stated. The results of the CBT are expressed as either readily biodegradable (ThOD > 60 %) or non-readily biodegradable. A few flavonoids could not be tested in the CBT due to low solubility, in these cases n.d. is stated. According to the concentration monitoring during the algae growth inhibition test and/or the abiotic controls of the biodegradation testing flavonoids are categorized as either abiotically stable or instable. N.d. is stated if no concentration monitoring was performed. This figure summarizes data of publication 2 and 3.

Overall, the obtained data on algae growth inhibition and environmental biodegradability of flavonoids contribute to closing the research gap on the environmental effects and fate of NPs. The results showed that differences in the ecotoxicity and the biodegradability between individual flavonoids exist. Therefore, not all flavonoids represent benign substitutes and candidates most suited as substitutes for persistent and harmful synthetic chemicals need to be carefully selected. However, more knowledge on the ecotoxicity e.g. adverse effects on Daphnia species, fish or earthworms, and the environmental fate e.g. linking the demonstrated readily biodegradability to the occurrence of flavonoids in soils and water bodies is required to fully understand possible environmental risks of flavonoids used as biopesticides or pharmaceuticals.

6.3.1 Applications of the Most Promising Candidate Hesperetin

Hesperetin is by far the most promising candidate among the tested flavonoids to substitute synthetic chemicals. In addition to its indicated low ecotoxicity and environmental biodegradability, it can be obtained from waste sources e.g. orange peels[95]. However, the systematic literature review revealed that the application of hesperetin as a biopesticide is not well studied. Only Chormova *et al*. tested the ability of hesperetin to inhibit a xyloglucan endotransglucosylase, an enzyme involved in the construction of the cell wall in plants[96]. But the results demonstrated that hesperetin is not suited as an inhibitor for this enzyme. However, many more applications as biopesticides could be investigated. Regarding pharmaceutical applications, recent research suggested the use of hesperetin to treat diabetes[97] or cancer[98]. The current indication of low environmental risks encourages further studies towards the utilization of hesperetin a benign pharmaceutical or biopesticide.

7 Finding Benign Substitutes – Evaluation of the Conducted Approach

The conducted literature review and the performed experiments vitally increased the knowledge about flavonoids. Particularly, the algae growth inhibition screening with the miniaturized 24 well plate test setup and the CBT to assess the readily biodegradability provided sound data for the identification of flavonoids with potentially low environmental risks, that are, from this perspective, suited as benign substitutes for harmful, synthetic chemicals. Additionally, the successful identification of hesperetin as promising candidate for benign substitution demonstrated the suitability of a combined algae growth inhibition and biodegradability screening as an important step towards the selection of benign substitutes. A possible limitation of this approach is the requirement of a sufficient water solubility of the test substances. For a few of the flavonoids selected for investigation, neither the CBT nor the determination of EC_{50} value of algae growth inhibition was feasible. Whereas an EC_{50} value that is larger than the water solubility of a test substances indicates low toxicity and hence, provides some information, a CBT with a precipitated test substance does not yield valid results and an alternative biodegradation test should be used. Still, the performed experiments are a good approach to assess first tiers of the environmental effects and fate of most test substances. However, with the experiences gained in this thesis, a few adjustments could be made. Whereas the performed concentration monitoring helped to obtain a better understanding of the processes taking place during the algae growth inhibition and biodegradation tests, a simpler approach to assess the abiotic stability could be used. For example, a buffer solution with a less complex composition could be utilized instead of cell culture medium with various trace elements. The search for a benign substitute should start with a screening for sufficient abiotic stability since no test organisms are required for such tests. On this basis, only the algae growth inhibition and biodegradability of sufficiently stable candidates need to be tested. The obtained data on algae growth inhibition and biodegradability could then be used to further narrow down the pool of possible candidates

whose activity and application need to be investigated. In the case of flavonoids, the insights gained on the environmental effects and fate are a very useful orientation for scientists to select substances which could be further developed towards benign pesticides from the many different flavonoids that have been tested for their pesticidal activity.

8 Discussing the Idea of Natural Products as Benign Substitutes

Applying the Benign by Design concept for the first time to a class of NPs, this thesis provided important knowledge on the environmental effects and fate of flavonoids, that supports the assumptions of low ecotoxicity and non-persistence on which their investigations as biopesticides are often based. Additionally, the successful identification of a promising candidate for benign substitution and the transferability of the performed screening approach to other NPs, promote the idea of NPs as benign substitutes. The screening approach derived in this thesis could be used to assess the environmental effects and fate of other NPs. This could help to address the general research gap on the environmental effects and fate of NPs[25,26] and to find other NPs that are suited as benign substitutes. Although biodegradability testing results of most NPs might be positive as expected, this data could be very useful for the improvement of biodegradability prediction tools as they need trainings sets not only with non-biodegradable but also with biodegradable substances. Additionally, more information on the ecotoxicity of NPs other than flavonoids is needed. So far, only scattered information on the ecotoxicity of other NPs is available in the literature. The plant-derived antimalaria drug artemisinin showed adverse effects on earthworms, lettuce, and *R. subcapitata* (EC_{50} = 0.24 mg/L ± 0.01)[99]. A pyrethrum extract used as bio-insecticide showed acute toxicity towards *Daphnia magna*, unbalanced the oxidative metabolism, and induced neurotoxic effects[100]. An azadirachtin-based biopesticide showed adverse effects on fish[101]. These adverse effects of NPs on non-target organisms align with the concern on the broad target spectrum of flavonoids and show the need to carefully evaluate the environmental risks of NPs. Particularly, the environmental impacts of the application of NPs in large scales and the concomitant entry of large amounts of NPs into environmental compartments exceeding their natural occurrence, e.g. by frequent application at an agricultural site, need to be researched holistically. An industrialized utilization of NPs could also lead to the entry of certain NPs into areas where this

NP does not occur naturally and where possible environmental impacts therefore may differ in comparison to ecosystems used to the occurrence of this certain NP .

In addition to the environmental effects and fate of a benign substitute, its production needs to be considered and aligned with sustainability[c]. The current underutilization of waste as a source of flavonoids as identified in the systematic literature review showed the need to further deepen the understanding of Sustainable Chemistry in the scientific community. Whereas the utilization of NPs aligns easily with the 5th principle of Green Chemistry to use renewable resources, the actual feedstock has to be chosen carefully for an actual contribution to sustainability. Obtaining NPs for the substitution of fossil-based chemicals in biorefinery processes on an industrial scale requires large amounts of biomass. Increased production or harvest of biomass can have severe negative impacts on the environment and humans including among others land-use changes, land grabbing, and biodiversity loss[102,103]. For a sustainable development, the extraction of the biomass must not exceed regenerative rates of (agro)ecosystems, and all stakeholders involved e.g. local farmers and indigenous people must benefit[102]. An alternative to the utilization of (agricultural) waste materials to obtain NPs could be their biotechnological production. A promising approach that combats extensive land needs and the often small amount of the wanted NPs in the plant material is the production of NPs in genetically engineered cyanobacteria[104,105]. This was also suggested for the production of flavonoids[105].

In regard of these concerns, the key element of Sustainable Chemistry becomes evident: To avoid social and environmental conflicts arising with the need for large amounts of biomass, resource-intensive holistic risk assessments and monitoring, and potential environmental risks due to the massive use of NPs, a sustainable society should preferably reduce the amount of chemicals and find non-chemical solutions e.g. disease prevention.

[c] The concept of sustainability is a normative goal including equity, sufficiency, freedom and self-determination, well-being of all humans, and responsibility for the future[108].

9 Conclusion

This thesis set out to evaluate the potential of flavonoids to substitute synthetic chemicals. A major finding of this thesis is the low environmental risk emanating from the use of most tested flavonoids due to low abiotic stability, moderate algae growth inhibition and readily biodegradability. Hence, selected flavonoids might be benign substitutes for synthetic chemicals that can be obtained from renewable sources, are non-toxic, and non-persistent in the environment. The obtained, experiment-based insights into the environmental effects and fate of flavonoids after their application, are an important basis for the further development of flavonoid-based biopesticides and pharmaceuticals. Especially, hesperetin was identified as promising candidate that should be investigated further.

Furthermore, this study showed that a combination of algae growth inhibition and biodegradability screening is a suited approach to apply the Benign by Design concept to NPs. This study emphasizes the role NPs could potentially play in Green Chemistry by showing that besides studying them for new modes of actions, important insights on biodegradability and ecotoxicity can be obtained. Using NPs or derived knowledge on their biodegradability and low ecotoxicity to design benign substitutes for persistent synthetic chemicals is one approach to tackle environmental pollution in line with the EU’s Chemicals Strategy for Sustainability Towards a Toxic-Free Environment and the UNEP Green and Sustainable Chemistry Framework Manual.

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List of Publications

Publications for the Cumulative Dissertation

Publication 1: Schnarr, L., Segatto, M. L., Olsson, O., Zuin, V. G., & Kümmerer, K. Flavonoids as biopesticides–Systematic assessment of sources, structures, activities and environmental fate. *Science of the Total Environment* ***824,*** 153781 (2022)

Publication 2: Schnarr, L., Olsson, O., Ohls, S., Webersinn, J., Mauch, T., & Kümmerer, K. Flavonoids as benign substitutes for more harmful synthetic chemicals - effects of flavonoids and their transformation products on algae. *Sustainable Chemistry and Pharmacy* **38**, 101473 (2024)

Publication 3: Schnarr, L., Olsson, O. & Kümmerer, K. Biodegradation of flavonoids – Influences of structural features. *Chemosphere* **359**, 142234 (2024).

Further Publications in scientific journals

- Linke, F., Olsson, O., Preusser, F., Kümmerer, K., Schnarr, L., Bork, M., & Lange, J. Sources and pathways of biocides and their transformation products in urban storm water infrastructure of a 2 ha urban district. *Hydrology and earth system sciences,* ***25***, 4495-4512 (2021).
- Linke, F., Olsson, O., Schnarr, L., Kümmerer, K., Preusser, F., Bork, M., Leistert, H. & Lange, J. Discharge and fate of biocide residuals to ephemeral stormwater retention pond sediments. *Hydrology Research,* ***53***, 1441-1453 (2022)
- Segatto, M. L., Schnarr, L., Olsson, O., Kümmerer, K., & Zuin, V. G. Ionic liquids vs. ethanol as extraction media of algicidal compounds from mango processing waste. *Frontiers in Chemistry*, ***10***, 986987 (2022).

Conferences contributions

- Schnarr, L., Segatto, M.L., Olsson, O., Zuin, V.G., Kümmerer, K. "Sources, applications and environmental fate of flavonoids used as biopesticides" *18th International Conference on Renewable Resources and Biorefineries*, 1-3. June 2022, Burges, Belgium (Poster)
- Schnarr, L., Olsson, O., & Kümmerer, K. “Insights into ecotoxicity of flavonoids and their mixtures” *IUPAC International Conference on Green Chemistry (9th ICGC),* 5-9. September, Athens, Greece (Oral presentation)
- Schnarr, L., Olsson, O., Kümmerer, K. „Vermeidung von Bioziden in Fassadenmaterialien mittels Nachhaltiger Chemie“ Closing Event NAVEBGO Projekt, 21. June 2022, Freiburg, Germany (Oral presentation + Poster)
- Schnarr, L., Olsson, O., Kümmerer, K. „Naturstoffe als Ersatz für konventionelle Biozide – Untersucht am Beispiel der Flavonoide“ Closing Event NAVEBGO Projekt, 21. June 2022, Freiburg, Germany (Oral presentation + Poster)

Annex

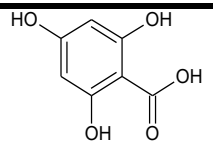
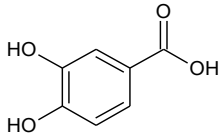
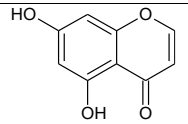
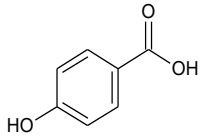
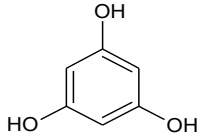
Table A 1: Growth inhibition of *R. subcapitata* induced by flavonoids with low water solubility.

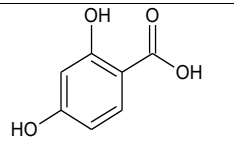
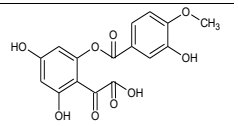
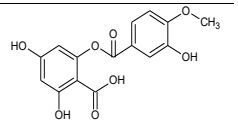
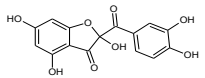
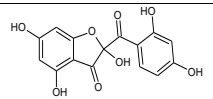
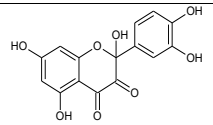
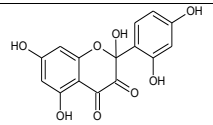
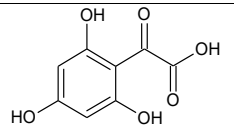
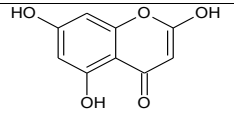
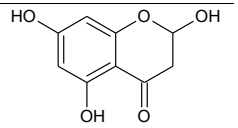
EC_{50} > x and the percentage of growth inhibition at the highest soluble concentration are presented. The table is based on table 1 of publication 2.

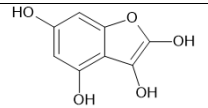
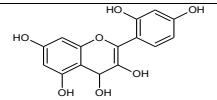
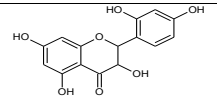
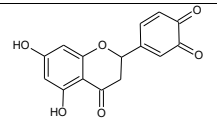
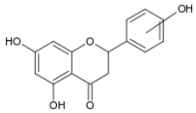
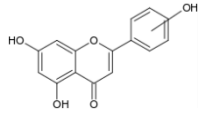
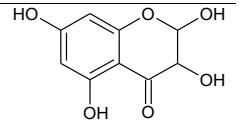
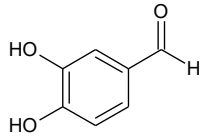
	substance	EC_{50} [mg/L]	EC_{50} [µM]
flavonoids	3-hydroxyflavone	EC_{50} > 0.63 / 20 % ± 1	> 2.6
	5-hydroxyflavone	EC_{50} > 5 / 27 % ± 1	> 21
	7-hydroxyflavone	EC_{50} > 5 / 14 % ± 4	> 21
	chrysin	EC_{50} > 1 / 14 % ± 3	> 3.9
	4',7-dihydroxyflavone	EC_{50} > 1 / -10 % ± 3	> 3.9
	apigenin	EC_{50} > 5 / 11 % ± 7	> 18.5
	acacetin	EC_{50} > 1 / 17 % ± 3	> 3.5
	naringenin	EC_{50} > 25 / 26 % ± 2	> 92
	tamarixetin	EC_{50} > 10 / 30 % ± 3	> 32
isoflavonoids	formononetin	EC_{50} > 1 / 15 % ± 3	> 3.7
	genistein	EC_{50} > 5 / 39 % ± 3	> 19
	daidzein	EC_{50} > 10 / 12 % ± 3	> 39

Table A 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[106]. 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 in the sample of a flavonoid. 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. Modified table based on table 2 of publication 2

name	**rt [min]**	**m/z**	**formula [M-H]**	**fragmentation** m/z and (formula)	**myricetin**	**dihydro-myricetin**	**quercetin**	**morin**	**taxifolin**	**tama-rixetin**	**luteolin**	**erio-dictyol**	**proposed structure**
TP-169 (2,4,6-trihydroxy-benzoic acid)	7.8	169.0137	C7H5O5	151 (C7H3O4), 125 (C6H5O3)	1	1	1	1	1	1	1[a]	1[a]	
TP-153.1 (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]	
TP-177 (5,7-dihydroxy-chromone)	7.6	177.0188	C9H5O4		n.d.	1[a]	n.d.	n.d.	1[a]	n.d.	n.d.	1[a]	
TP-137.1 (4-hydroxy-benzoic acid)	5.6	137.0242	C7H5O3	93	1[a]	n.d.	1	1	1	n.d.	1[a]	n.d.	
TP-125 (phloroglucinol)	2.6	125.0234	C6H5O3		n.d.	n.d.	n.d.	1[a]	n.d.	n.d.	n.d.	n.d.	

TP-153.2 (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.	
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.	
TP-319	8.1	319.0455	C15H11O8	169	n.d.	n.d.	n.d.	n.d.	n.d.	2	n.d.	n.d.	
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.	
TP-317.2	7.2	317.0298	C15H9O8	-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
TP-317.3	8.2	317.0298	C15H9O8	-	n.d.	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	
TP-317.4	7.9	317.0298	C15H9O8	-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
TP-197	5.8	197.0082	C8H5O6	153, 151, 125	2	2	2	2	2	2	n.d.	n.d.	
TP-193	8.5	193.0135	C9H5O5	149	n.d.	2	n.d.	n.d.	n.d.	n.d.	2	2	
TP-195	6.5	195.0289	C9H7O5	167, 123	n.d.	n.d.	2[a]	n.d.	2[a]	2	n.d.	2	

TP-181.1	6.6	181.0131	C8H5O5	137, 109	n.d.	n.d.	2	2	n.d.	n.d.	n.d.	n.d.	
TP-285.1 (luteolin)	8.7	285.0400	C15H9O6	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	parent flavonoid detected	2[a]	
TP-303.1	7.1	303.0505	C15H11O7	-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
TP-303.2	8.6	303.0505	C15H11O7	-	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
TP-285.2	8.4	285.0400	C15H9O6	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	
TP-271	8.8	271.0605	C15H11O5	--	n.d.	n.d.	n.d.	3	3	n.d.	n.d.	3	
TP-269	9.1	269.0450	C15H9O5	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3	n.d.	
TP-211	6.7	211.024	C9H7O6	-	n.d.	n.d.	n.d.	3	n.d.	3	n.d.	n.d.	
TP-137.2	5.4	137.0234	C7H5O3	-	n.d.	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	
TP-181.2	4.5	181.0131	C8H5O5	-	n.d.	4	4	n.d.	4	n.d.	n.d.	n.d.	

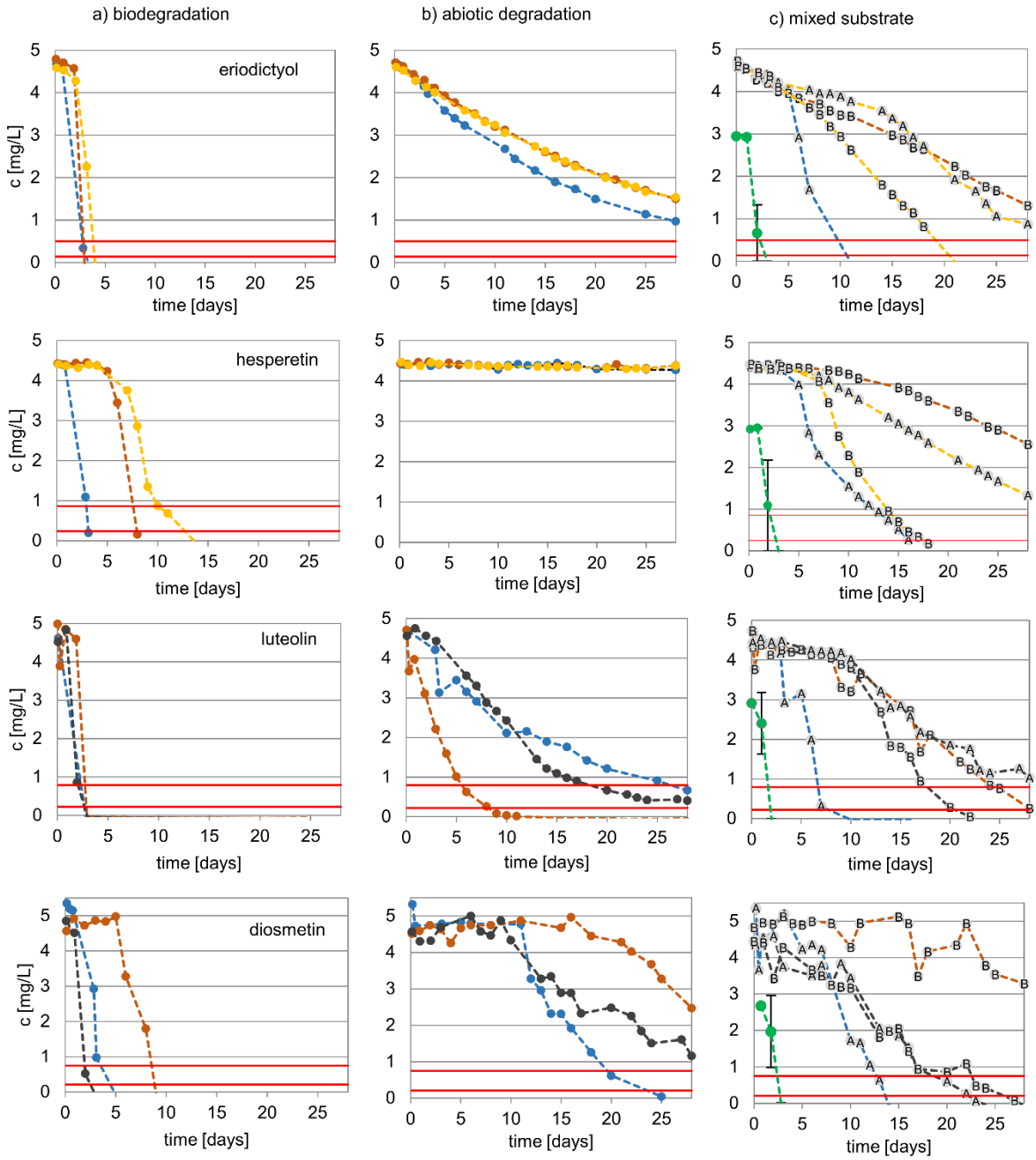


Figure A 3: Concentration development of the individual replicates of the four flavonoids eriodictyol, hesperetin, luteolin, and diosmetin in the biodegradation (a), abiotic (b) and mixed substrate (c) samples*. Colors indicate which replicates were run parallel (blue = first run, orange = second run, yellow= third run, and black = fourth run). In mixed substrate samples, the letter A indicates samples containing acetate, the letter B indicates samples containing benzoate. Concentration of benzoate in the mixed substrate samples is shown in green (data points are average values (n=2) with standard deviation (SD)). The figure is taken from the supporting information of publication 3.*

Table A 3: Overview of growth rates of R.subcapitata in the miniaturized (24 well plate) algae growth inhibition test. *For treatments with chemicals the number of replicates is four. Four growth controls the number of replicates is 16.*

substance	concentration [mg/L]	growth rate [day^{-1}]	deviation
terbutryn	100	-0.10	0.09
	20	0.01	0.09
	10	0.13	0.06
	5	0.55	0.09
	2.5	1.17	0.01
	1.25	1.28	0.02
	0.625	1.32	0.01
	0.3125	1.31	0.02
growth control	-	1.23	0.03
diuron	1	-0.07	0.06
	0.5	0.01	0.03
	0.25	0.11	0.02
	0.125	0.20	0.01
	0.0625	0.38	0.03
	0.0313	0.90	0.02
	0.01	1.10	0.02
	0.005	1.11	0.09
	0.0025	-0.07	0.06
growth control	-	1.18	0.11
3,5-dichlorophenol	6.75	< 0	
	4.5	< 0	
	3	0.71	0.011
	2	1.13	0.007
	1.35	1.24	0.016
	0.9	1.29	0.037
	0.6	1.33	0.026
growth control	-	1.34	0.030
eriodictyol	10	0.09	0.05
	5	0.07	0.06

	2.5	0.40	0.05
	1	0.49	0.06
	0.5	0.54	0.05
	0.25	0.87	0.04
	0.1	1.22	0.08
	0.05	1.25	0.09
	0.01	1.26	0.07
growth control	-	1.22	0.07
	20	≤ 0	
	10	0.04	0.05
	5	0.23	0.03
	2.5	0.39	0.02
luteolin	1.25	0.44	0.03
	0.625	0.84	0.15
	0.1	1.29	0.05
	0.01	1.29	0.02
	0.001	1.33	0.01
growth control		1.3	0.04
	20	≤ 0	
	10	≤ 0	
	5	0.40	0.02
	2.5	0.31	0.02
gossypetin	1.25	0.54	0.01
	0.625	1.01	0.00
	0.3125	1.22	0.03
	0.1	1.33	0.03
	0.01	1.25	0.03
growth control	-	1.22	0.07
	7.5	≤ 0	
	5.0	≤ 0	
	3.3	≤ 0	
flavone	2.2	0.38	0.03
	1.5	0.94	0.02
	1.0	1.15	0.04

	0.5	1.31	0.01
	0.1	1.31	0.06
	0.01	1.33	0.02
growth control	-	1.23	0.03
	10	0.25	0.16
	5	0.29	0.12
	2.5	0.49	0.14
	1.25	0.94	0.11
3,5-dihydroxyflavone	0.63	1.13	0.03
	0.31	1.23	0.01
	0.16	1.42	0.01
	0.08	1.42	0.03
	0.04	1.36	0.03
growth control	-	1.31	0.05
	20	0.16	0.05
	10	0.11	0.07
	5	0.35	0.01
	2.5	0.59	0.07
7,3',4'-trihydroxyflavone	1.25	1.00	0.07
	0.625	1.22	0.02
	0.3125	1.34	0.02
	0.1	1.36	0.01
	0.01	1.34	0.02
growth control	-	1.25	0.04
	10	≤ 0	
	7.5	0.1	0.2
	5	0.3	0.1
	2.5	0.8	0.0
quercetin	1.25	1.1	0.1
	0.625	1.2	0.1
	0.1	1.2	0.1
	0.01	1.3	0.1
growth control	-	1.25	0.05
myricetin	10	≤ 0	

	7.5	0.14	0.10
	5	0.53	0.04
	2.5	0.83	0.10
	1.25	1.01	0.06
	0.625	1.17	0.03
	0.1	1.32	0.01
growth control	-	1.23	0.03
morin	50	0.27	0.02
	25	0.30	0.05
	12.5	0.27	0.03
	6.25	0.52	0.04
	3.13	0.80	0.09
	1.56	1.09	0.07
	0.78	1.23	0.03
	0.39	1.33	0.02
growth control	-	1.26	0.05
fisetin	20	≤ 0	
	10	0.25	0.03
	5	0.82	0.16
	2.5	1.11	0.09
	1.25	1.17	0.03
	0.625	1.23	0.04
	0.1	1.32	0.06
	0.01	1.24	0.05
	0.001	1.25	0.05
growth control	-	1.32	0.04
dihydromyricetin	20	≤ 0	
	10	0.19	0.06
	5	0.95	0.13
	2.5	1.20	0.07
	1.25	1.22	0.05
	0.625	1.27	0.01
	0.1	1.35	0.02
growth control	-	1.25	0.05

taxifolin	50	0.23	0.08
	25	0.43	0.03
	12.5	0.51	0.03
	6.25	0.76	0.05
	3.125	0.99	0.05
	1.5625	1.14	0.04
growth control	-	1.34	0.07
hesperetin	50	0.02	0.02
	25	0.66	0.03
	12.5	0.98	0.06
	6.25	1.22	0.02
	3.125	1.26	0.02
	1.5625	1.28	0.04
growth control	-	1.28	0.07

Publication 1

Lena Schnarr, Mateus L. Segatto, Oliver Olsson, Vânia G. Zuin, Klaus Kümmerer

Flavonoids as biopesticides – Systematic assessment of sources, structures, activities and environmental fate

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Review

Flavonoids as biopesticides – Systematic assessment of sources, structures, activities and environmental fate

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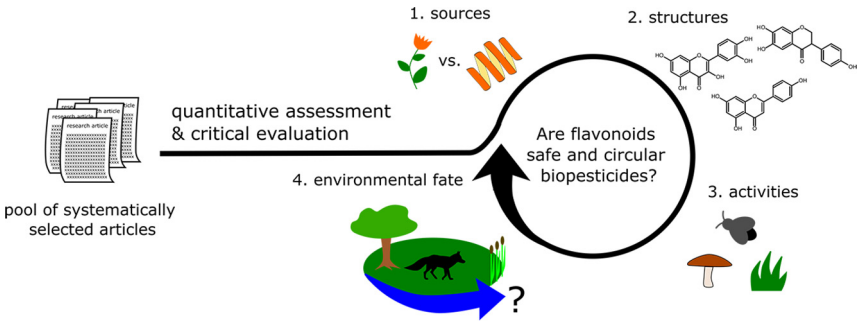
HIGHLIGHTS

- Advantages of flavonoids applied as biopesticides were critically evaluated.
- Systematic assessment of sources, structures, pesticidal activities and fate
- Waste as source of flavonoid-containing extract is currently underrepresented.
- More data is needed on the environmental fate of flavonoids to ensure safe use.

GRAPHICAL ABSTRACT



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ABSTRACT

Biopesticides obtained from renewable resources and associated with biodegradability have the potential to address resource limitations and environmental pollution, often caused by many conventional pesticides, due to the facility of natural products to run in natural nutrient cycles. Flavonoids are considered benign substitutes for pesticides, however, little comprehensive information of their pesticidal activities and critical evaluation of their associated advantages is available. Therefore, this systematic review assessed sources, structures, activities and the environmental fate of flavonoids on a basis of 201 selected publications. We identified 281 different flavonoids that were investigated for their pesticidal activity as either a pure compound or a flavonoid-containing extract, with quercetin, kaempferol, apigenin, luteolin and their glycosides as the most studied compounds. Agricultural or food waste, a potential sustainable source for flavonoids, represent 10.6% of the plant sources of flavonoids within these studies, showing the currently underutilization of these preferable feedstocks. Analysis of pesticidal activities and target organisms revealed a broad target spectrum for the class of flavonoids, including fungi, insects, plants, bacteria, algae, nematodes, molluscs and barnacles. Little information is available on the environmental fate and biodegradation of flavonoids, and a connection to studies investigating pesticidal activities is largely missing. Emerging from these findings is the need for comprehensive understanding of flavonoids pesticidal activities with emphasis on structural features that influence activity and target specificity to avoid risks for non-target organisms. Only if the target spectrum and environmental fate of a potential biopesticide are known it can serve as a benign substitute. Then, flavonoids can be integrated in a valorization process of agricultural and food waste shifting the extract-produce-consume linear chain to a more circular economy.

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Contents

1. Introduction

Environmental pollution by chemicals increasingly threatens human health, water and soil resources. On this matter, one of the roles of sustainable chemistry is the design of chemicals for circularity. Ideally, chemicals are obtained from renewable resources, fulfill their function effectively, and mineralize completely after a suitable lifetime so that they re-enter natural nutrient cycles.

A major aspect of the development and growth of humankind in the last centuries is the agricultural use of fertilizers and pesticides, the latter being one of the main contributors to environmental pollution. Pesticides are products containing active substances used in agricultural and other fields of applications (e.g. preservation, hygiene), which are used with the intention of "preventing, destroying, repelling, or mitigating any pest" (U.S. Code - U.S. Government Publishing Office, 2013). In the latter fields of application, the active ingredient is also referred to as biocide (European Union, 2012). Conventional pesticides and biocides have been increasingly banned from several countries due to follow up environmental problems. They are most often present in the environment longer than necessary to assert their desired impact on target organisms. Furthermore, they are often incompletely mineralized in the environment and may result in toxic and persistent transformation products (TPs) (Fenner et al., 2013; Kümmerer et al., 2018; Postigo and Barceló, 2015). Therefore, both parent compounds and TPs are polluting soil, water and food resources, causing a shortage of clean water, non-polluted soils and non-contaminated food resources.

Pesticides are needed which are stable enough for agricultural application, but they should not be persistent or form stable TPs. Instead, organic pesticides should be completely mineralized. This can be achieved by biodegradation via micro-organisms that utilize them as carbon or energy source resulting in a transformation of the compound to CO_2. Consequently, TPs with unknown risks are avoided and re-entry to the carbon cycle enabled. Such pesticides are key for a future greener and more sustainable agriculture.

Biopesticides are often recalled as viable alternatives to conventional active substances (Ben Mrid et al., 2021). The term "biopesticide" is defined broadly and includes three main categories: microbial, plant-incorporated and biochemical (Marrone, 2019). Biochemical biopesticides are in the focus of this review and comprise natural products with pesticidal activity. In contrast to synthetic pesticides, they are obtained from renewable resources and are associated with modes of action not yet exploited for pesticides, sublethal effects, target specificity, low toxicity to non-target organisms and non-persistence (Czaja et al., 2015; Marrone, 2019; Seiber et al., 2014). The latter is concomitant with less stability which can also be seen as a disadvantage e.g., if frequent reapplications are necessary. The biopesticide's chemical stability therefore needs to balance requirements for applications and non-persistence. Furthermore, the broadness of the target spectrum is discussed ambiguously. While target specificity is mentioned among the advantages, a small target spectrum is considered as a disadvantage by Cooping and Menn. Other disadvantages can include slower speed of kill and lower efficiency (Copping and Menn, 2000; Wilson et al., 2013). The production of biochemical biopesticides at a commercial scale can be further hindered by limited availability of large amounts of the source material and its varying composition (Smith et al., 2021). Therefore, instead of biopesticides from rare plants, the usage of active substances isolated from agro-industrial and food residues, aligned with greener extraction techniques, is seen as a potential strategy in a circular economy context (Zuin, 2016), adding financial value to the food supply chain with a low addition of its environmental impact.

Frequently found in these resources are flavonoids (Ahmad et al., 2020; Banerjee et al., 2017), one of the main groups of bioactive natural products. Flavonoids are a ubiquitous class of secondary metabolites comprising several thousand individual compounds. They exhibit a phenyl-substituted propylbenzene core structure with a C6-C3-C6 carbon skeleton. The group of flavonoids is further divided into 6 subgroups based on structural features of their core structure (Fig. 1). Further modifications such as methoxylation and glycosylation give rise to the structural diversity of these secondary metabolites and contribute to the broad range of biological activities. In plants, flavonoids are involved in stress response including UV-, oxidative and herbivore protection, attraction of pollinators, and signalling with symbiotic bacteria (Falcone Ferreyra et al., 2012; Sugiyama and Yazaki, 2014). Beyond that, flavonoids are associated with beneficial effects on human health and research has been conducted towards their pharmaceutical utilization. Bioactivities encompass anti-cancer, anti-inflammatory, cardioprotective, anti-viral, anti-bacterial and anti-fungal properties attributed to the anti-oxidant and radical scavenging ability of flavonoids, as well as the interaction with enzymes and the modulation of gene expression and intracellular signalling cascades (Gonzalez-Paramas et al., 2019; Górniak et al., 2019; Jucá et al., 2020; Montenegro-Landívar et al., 2021).

While many reviews with summarized information on pharmaceutical activities (Farhadi et al., 2019; Gonzalez-Paramas et al., 2019; Górniak et al., 2019; Jucá et al., 2020; Panche et al., 2016) and nutritional value of flavonoids incorporated in food (Guven et al., 2019; Maleki et al., 2019) are at hand, this is not the case for pesticidal activities. Although the number of studies investigating the pesticidal effects of flavonoids and plant extracts containing flavonoids is high, there is no overview available providing condensed and critically evaluated knowledge about the different activities and applications. First approaches in the field of biologically-derived pesticidal substances – including, but not specific to flavonoids – were conducted by Santana-Méridas et al. (2012) and Mouden et al. (2017). The former gave an overview of the potential of agricultural and food waste as sources of bioactive products. One of the conclusions is the current underutilization of agricultural residues for the production of bioactive compounds with pesticidal activities and the need for bioprospecting work to overcome this shortcoming, as also pointed out by Isman (2020). Mouden's study emphasizes the potential of flavonoids as biopesticides because they are natural food ingredients and residues stemming from their application will probably pose no risk to humans and the environment. The insecticidal effects of a few flavonoids are summarized in this publication as well. Hikal et al. (2017) reviews

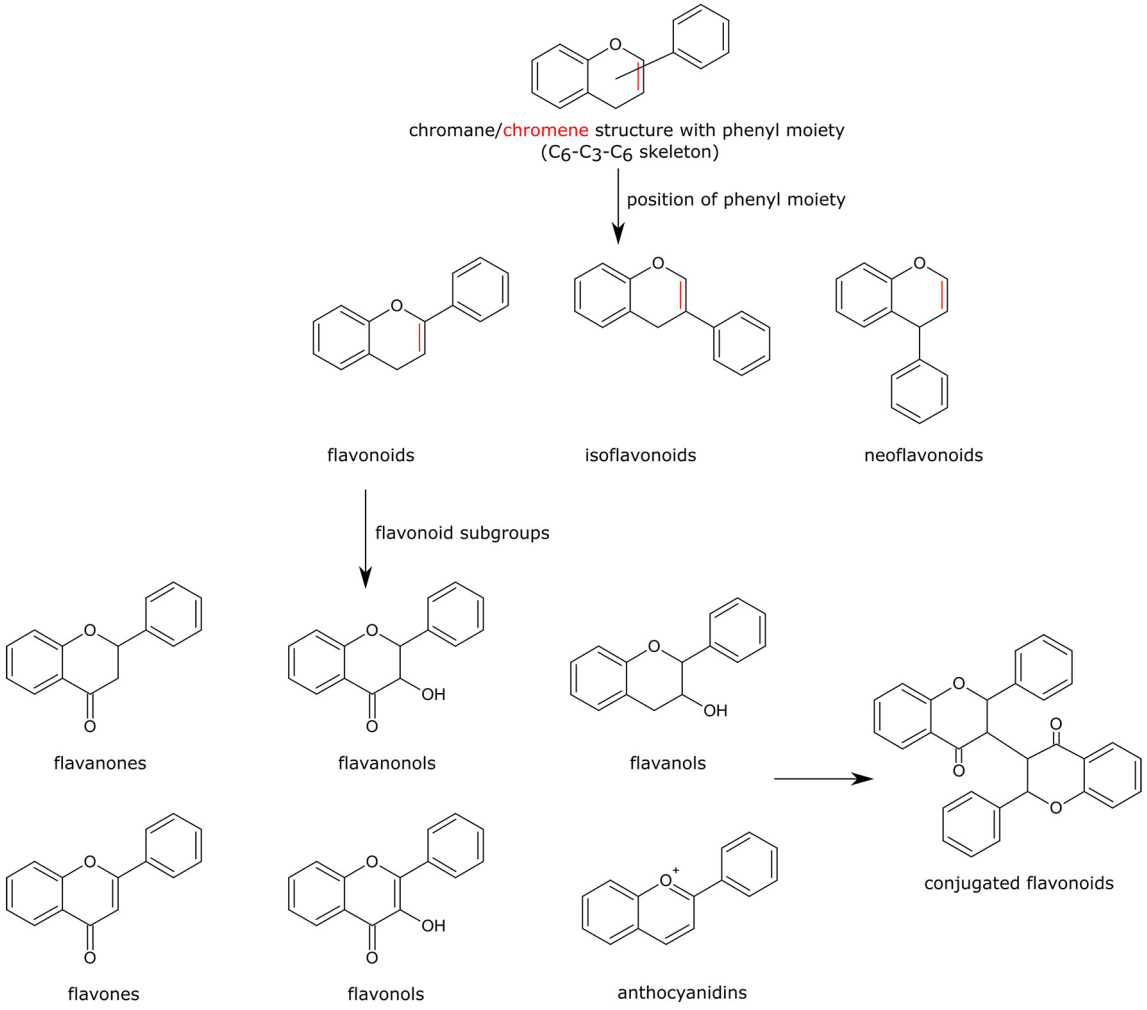

Fig. 1. Skeleton structure of flavonoids and subgroup classification. The skeleton structure either comprises a chromane or a chromene with an additional double bond (red).

botanical insecticides, flavonoids being one of the main classes included, and emphasize different action mechanisms of biopesticides in general.

In contrast to the many reviews focussing on pharmaceutical activities of flavonoids, the objective of this review is to evaluate the potential of flavonoids as green and circular substitutes for synthetic pesticides by looking at different aspects of the whole life cycle of these compounds including their source materials, activities and the environmental fate. Associated advantages of natural products used as biopesticides in particular their production from renewable source, target specificity and their biodegradability are critically examined for the class of flavonoids. Following a systematic approach, this study set out to identify the different structures of pesticidal active flavonoids, their plant sources and to give an overview of all the different pesticidal activities and target organisms. On this basis, target specificity and environmental fate are discussed.

2. Method

A systematic literature search was conducted in order to reach the proposed objectives. Elsevier's Scopus and Clarivate Analytics' Web of Science (core collection) databases were used to search for research and review publications between 1970 and 2021. A third database e.g., SciFinder was not accessed to keep the workload to a manageable level. The search was conducted in May 2021. The results for each database were obtained crossing the term "flavon*" with different application types: biocid*, biopesticid*, pesticid*, herbicid*, fungicide*, insecticide*, alg*cid* and harmful algae bloom*. The target fields on which the search tools identified the inserted terms were "keywords" for Scopus and "topics" (title, abstract and keywords) for Web of Science. In a three-step approach, studies were selected that matched the objectives of this review using certain exclusion criteria (Fig. 2). In step A, duplicates were removed. In step B, review publications were separated. The remaining publications were evaluated on the basis of their titles and abstracts, removing publications with pharmaceutical contexts, changes of flavonoid content in plants due to pesticide treatment or that were otherwise out of scope. In step C, publications were evaluated on the basis of the full text. At this level, only publications that elucidated structures of investigated flavonoids either by MS, NMR or a chromatographic method (UV–vis detector) using a reference substance were included. Identified compounds were counted when the flavonoids were in accordance with the strict IUPAC definition, excluding chalcones, aurones, pterocarpans and rotenoids (Rauter et al., 2018). Due to the focus on plant-derived flavonoids, synthetically-modified substances that therefore not occur naturally and compounds obtained from bacteria or fungi were excluded.

A full list with all accepted articles which are the basis of this analysis can be found in Supplementary Material 1. From the accepted publications information regarding the following eight categories were extracted: source plants; type of activity tested; target species; purpose of application (crop protection, household, etc.); form of application (crude extract or isolated compound); the flavonoids tested or the major flavonoids identified in a tested extract; target specificity or selectivity study (present or not); and degradation or environmental fate discussion or study (present or not). Assessing the sources, special attention was paid to agricultural or processing plant waste materials which were identified if the text clearly expressed that the studied plant part is considered a residue and/or if they are waste

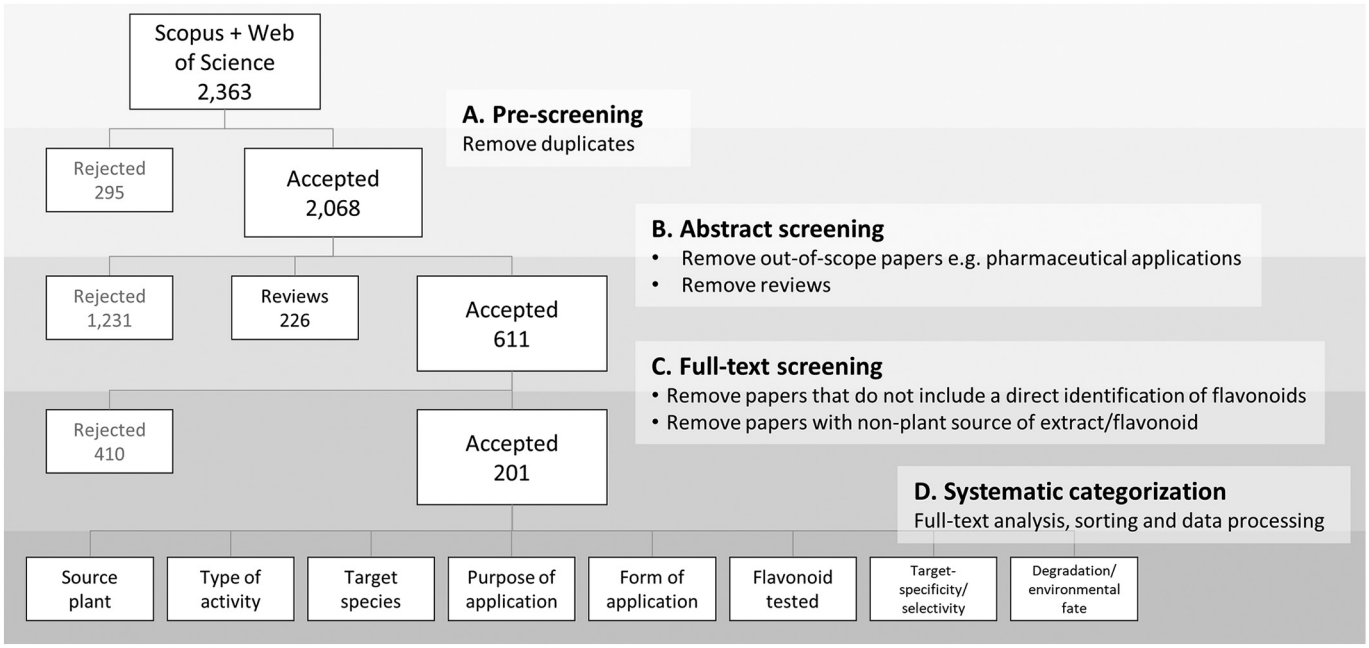

Fig. 2. Scheme of systematic search, screening and categorization steps.

fractions of known commercial products (e.g. fruit peels, wood chips, etc.). Regarding the target species, all mentioned species were included without consideration of the extent of the effect (weak – strong) due to the number of investigated species, deviating methods and endpoints. However, if explicitly mentioned as non-target, these organisms were not included. To identify publications dealing with selectivity/specificity and environmental fate, the full texts were searched for the keyword stems selectiv*, specific*, non*target, broad, field experiment, degrad*, and environment. Publications were only counted if the context was in line with the intended topic. For every category, identified publications were used for quantitative statements regarding each topic. Furthermore, interesting studies are highlighted, or information of several publications is presented in a summarized manner. The outcomes of this assessment and systematic categorization are described in the following sections.

3. Literature data assessment

The search of the databases Scopus and Web of Science for flavonoids with pesticidal activity resulted in 2363 publications (Fig. 2). Overlap between both databases was small with only 295 duplicates. The 2068 publications were screened for relevant studies according to strict inclusion and exclusion criteria as described in the method section. In screening step B, abstracts of the works were analysed to exclude the ones that were out of the scope of this review, remaining only publications that reported at least one biological activity for at least one flavonoid, or an extract containing a flavonoid. Superficial investigation of the remaining 611 publications (exc. reviews) showed that, although not explicitly described in the title, abstract or keywords of the publications, a numerous fraction of the publications dealt with pharmaceutical related applications, which were not desired in this work. Furthermore, it was observed that many publications had indirect analysis of flavonoids, such as Total Flavonoid Content (TFC). As one of the objectives of this review is to analyse the main flavonoids structures found in plants tested for their pesticidal activity, the absence of specific identification of the major flavonoid compounds present in the plants extracts and fractions was a crucial parameter for removing these publications.

410 publications were excluded in the subsequent full text screening (step C) leaving only studies with plant-derived flavonoids or extracts containing them with a clear structural characterization of the compounds that fit the IUPAC definition of flavonoids. Identification of flavonoids and comparison of compounds from different publications was hindered by the usage of different nomenclature (systematic, semi-systematic, trivial) for the same structures. We therefore advocate to state CAS numbers in future research articles.

The accepted 201 publications (Supplementary Material 1) present the basis of the performed quantitative analysis. In respect to the quantification of the individual categories often was found that a publication had worked with more than one flavonoid, tested for more than one activity and/or extracted from more than one plant source. Therefore, the term “count” will be used in this work, as referred to the number of times an item has been studied among the 201 publications. Thus, for all categories, the numbers will show a higher number of total counts than the number of analysed publications, as will be observed in the next sections.

During a systematic literature screening not all scope-fitting publications can be found due to variety and differences in keywords, choice of databases, etc., but the carefully selected 201 publications represent a broad basis for the conducted analysis.

4. Source materials and the use of agro-industrial waste

In this section, we present our observations on the data found on the literature scan and a critical view on the use of flavonoids from plant resources, aiming at finding directions for the research and development of greener pesticides and biocides.

Analysis of the plant sources used for the extraction and activity experiments of flavonoids show that, from the 201 selected publications, 35 used only pure compounds without stating a plant extraction or isolation, while 166 publications cited at least one plant species from which flavonoid substances were extracted. From these publications, 209 different species of plants were found, with a total of 235 counts, which shows a diversity of plant species containing flavonoids studied for their bioactivity, either as isolated compounds or crude extracts. The distribution of species among the selected academic publications can tell a lot about how research has been directed in the topic, showing a pattern of “screening” of active flavonoids from different plants, with little concern on the actual applicability of large-volume use of these compounds. While there is importance on finding highly active compounds in different plant species, few publications focus on studying flavonoids from common commercially-explored species, i.e. domesticated species that are easily found in different regions of the planet and are produced in large scales, as well as waste sources generated in agro-

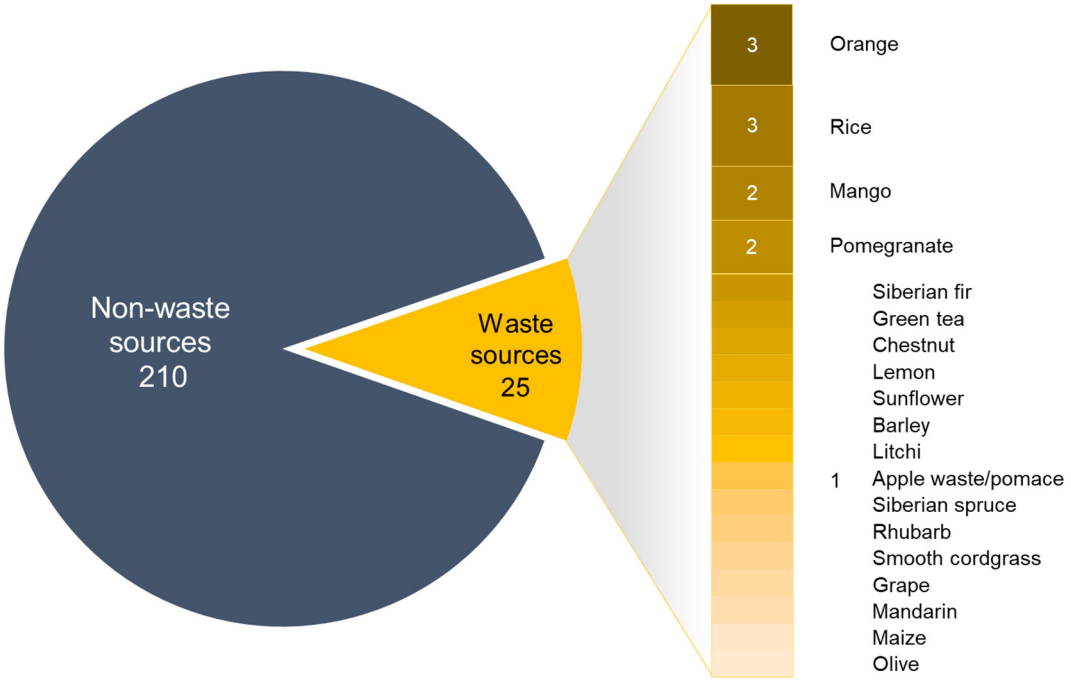

Fig. 3. Analysis of the plant sources identified in the research, detailing all flavonoid-containing waste sources.

industrial and food processing plants. In fact, from 235 counts, only 25 were cited to be related to waste materials, with 19 different species (Fig. 3).

From the works that mentioned waste samples, the most common species studied were orange (*Citrus sinensis*) and rice (*Oryza sativa* L.), two major crops that are responsible for a global production of 79 million tons/year and 755 million tons/year, respectively (FAO, 2019). Fruits and vegetable crops are most commonly found when investigating waste sources of bioactive flavonoids, followed by food-related grains and species related to wood production, such as Siberian fir and Siberian spruce, as can be seen in detail in Fig. 3.

A common residue obtained after harvesting, rice leaves from an allelopathic rice accession were used to obtain tricin (3′,5′-dimethyl tricetin) from a fractioned portion of the methanolic extract (Kong et al., 2004). The flavone was tested against spore germination and weed growth of several different weeds (*E. crus-galli*, *C. iris*, *P. oryzae*, *R. solani* and *C. difformis*), showing effective herbicidal activity, even at higher rates when re-incorporated into the fraction of the extract, suggesting a synergetic matrix effect. Tricin was also tested against the growth of rice, showing no autotoxicity and therefore herbicidal selectivity against the tested weeds.

Orange processing waste is one of the most common sources of studies dealing with agro-industrial residues, as the orange fruit is processed in high volumes across the world to produce orange juice, generating its peels and bagasse as co-products. An interesting biorefinery approach suggests the extraction of the essential oil from the peels, commonly by cold-press or steam distillation, followed by the extraction of functional materials, such as pectin, from the remaining solid residue (Matharu et al., 2016). The essential oil is majorly constituted by D-limonene, which can be used as a green solvent, and other trace components. Vargas et al. (Vargas et al., 1999) used a non-volatile fraction of the orange essential oil to isolate three methoxylated flavones that presented bioactivity against 5 species of fungi. When compared to commercial fungicide benomyl, tangeretin (5,6,7,8,4′-pentamethoxyflavone), quercetogetin (3,5,6,7,3′,4′-Hexamethoxyflavone) and 3,5,6,7,8,3′,4-heptahydroxyflavone, isolated from orange essential oil, showed higher mycelium growth inhibition for *G. candidum* fungi species, although they also presented significant activity to *P. italicum*, *C. gloeosporioides* and *A. parasiticus* at 50 μg/mL.

Generated in large volumes during the processing of mango fruits into food products, mango kernels ethanolic extract was tested for its fungicide activity by Gómez-Maldonado et al. (Gómez-Maldonado et al., 2020). The authors found that the ethanolic extracts had, among other constituents, the flavonols myricetin, quercetin and rutin (quercetin-3-rutinoside). In vitro studies showed superior mycelial growth inhibition and spore germination inhibition of *Colletotrichum brevisporum* when compared to the commercial fungicide thiabendazole. In the same study, in vivo tests in *C. brevisporum*-inoculated mango fruits immersed in the ethanolic extract showed no anthracnose lesions, a common disease in mango fruits caused by the fungi, a superior result when compared to control (sterile water) and thiabendazole-immersed fruits, which showed an average of 4.5 mm lesions after 9 days.

Wood production for household applications, structural materials and paper manufacturing also presents as a large fraction the agro-industrial sector. Wood residues consists mostly of the barks, leaves and residual wood from several types of trees from the Pinaceae (spruce, pine, fir, larch and tsuga), Betulaceae (birch), Salicaceae (aspen) and Myrtaceae (eucalyptus) families. Only one work was classified for citing to deal with wood waste resources, which studied the bactericide activity of water extracts containing the flavonoids quercetin, taxifolin and kaempferol from the barks of Siberian fir and Siberian spruce (Krotova et al., 2019). Besides that, from all publications included in this literature search, it was possible to account for 26 other citations of plants from the families related to wood production, with 23 different species. Analysis of these publications showed that most of the authors worked with waste fractions, such as barks and leaves, which could be considered as initiatives of researches directed to the use of wood residues to produce biopesticides.

The analysis of the main plant sources from which flavonoids and flavonoids extracts have been studied for their pesticidal activity has shown that not many works have been focused on the use of large volume waste streams. In addition to the waste materials found, other agricultural and food processing by-products from asparagus (Fuentes-Alventosa et al., 2013; Rosado-Álvarez et al., 2014; Viera-Alcaide et al., 2020) and winery (Germanó et al., 2021; Moro et al., 2021; Sirohi et al., 2020) are rich in flavonoids and extracts or isolated compounds of these sources could be further investigated for their pesticidal activity. The use of such waste flows has been discussed as an important opportunity to advance new guidelines concerning sustainability, including the development of biorefineries and shifting the extract-produce-consume linear chain of economy to a more

circular economy (Zuin et al., 2018). In the case of agro-industry, the generation of large volumes of residues – containing valuable substances – presents issues regarding their disposal or, in the best scenario, low-value uses such as animal feed and land spreading are adopted (Tuck et al., 2012). The same agroindustry sector can benefit from the use of such wastes to produce green pesticides in a circular approach, as agriculture is one of the most prioritized sectors regarding greener practices and changes towards a sustainable future, with pressing issues to be resolved, especially concerning the use of toxic and persistent pesticides and other harmful agricultural practices. Therefore, an extensive gap in the development of greener pesticides using agro-industrial waste has been spotted for flavonoids, especially for finding consistent feedstocks, improving extraction yield, activity and selectivity of isolated flavonoids or extracts.

5. Flavonoid structures

Data categorization and analysis revealed 281 different flavonoid compounds in total that were investigated as pure compound or constituent of a plant extract for its pesticidal activity among 712 total counts. A full list of the flavonoids with CAS numbers can be found in Supplementary Material 2. Most of the individual flavonoids either belong to the subgroup of flavones or flavonols, with respectively 110 and 84 different structures, followed by flavanones (38), isoflavones (21), conjugated flavonoids (10), flavanols (9) and flavanonols (7). The least represented group was the anthocyanidins, with only 2 compounds. They are popularly considered as their own group apart from flavonoids, as their studies do not often cite their structural relationship with the flavonoids parent group.

To provide a clear but detailed overview about the individual compounds and frequent modifications, we introduce a hierarchic systematization (Fig. 4). At the first level individual compounds are assigned to 8 different subgroups, 6 from the flavonoid subgroups (flavones, flavonols, flavanones, flavanonols, flavanols and anthocyanidin), isoflavones and conjugated flavonoids (as previously shown in Fig. 1). Next, the unmodified structures of each group and their hydroxylated versions are defined as core compounds. Modified core flavonoid compounds are then divided into five categories, namely glycosylated, methoxylated, alkylated, mixtures of the previous groups and rare modifications. For instance, apigetrin, vitexin, acacetin and syzalterin belong to the same core structure represented by the trihydroxy flavone apigenin, as can be seen in Fig. 4. O-glycosylated and methoxylated compounds necessarily must be modified in the same position as a hydroxyl group from the core structure (apigetrin and acacetin), while C-glycosylated and alkylated compounds are modified in the position where there are free carbons in the core structure, as represented by vitexin and syzalterin.

This hierarchic systematization shows the most common flavonoids studied, as well as the frequency of different modifications and if there is a core structure that is persistent among different studies. Overall, a high number of different core structures was observed, with most common subgroups, flavones and flavonols, showing 22 and 21 unique core structures. The main findings on the major core structures and their most common modifications are illustrated in Fig. 5. By far, the most frequently identified and investigated core structure is represented by the flavonol quercetin, being studied 153 times (Fig. 5A). The most common substance found within this core is the unmodified quercetin (52 counts), followed by rutin, a 3-O-rutinoside substituted form of quercetin (45 counts). The core structures represented by the flavones luteolin and apigenin, and the flavonol kaempferol, are found over 50 times each, with their unmodified core compounds being the most common substances studied (luteolin 24; apigenin 19; kaempferol 29), followed by their glycosylated forms. The diastereomers catechin and epicatechin were also found in a significant number of studies, accounting for 26 investigations of the core compounds and 3 other studies of their modified structures.

Fig. 5B illustrates that unmodified core structures are the most commonly found group studied for pesticidal activity of flavonoids, credited for 41% of total counts, followed by glycosylated (27%), methoxylated (22%) and mixed modifications (7%). Alkylated and rare modifications,

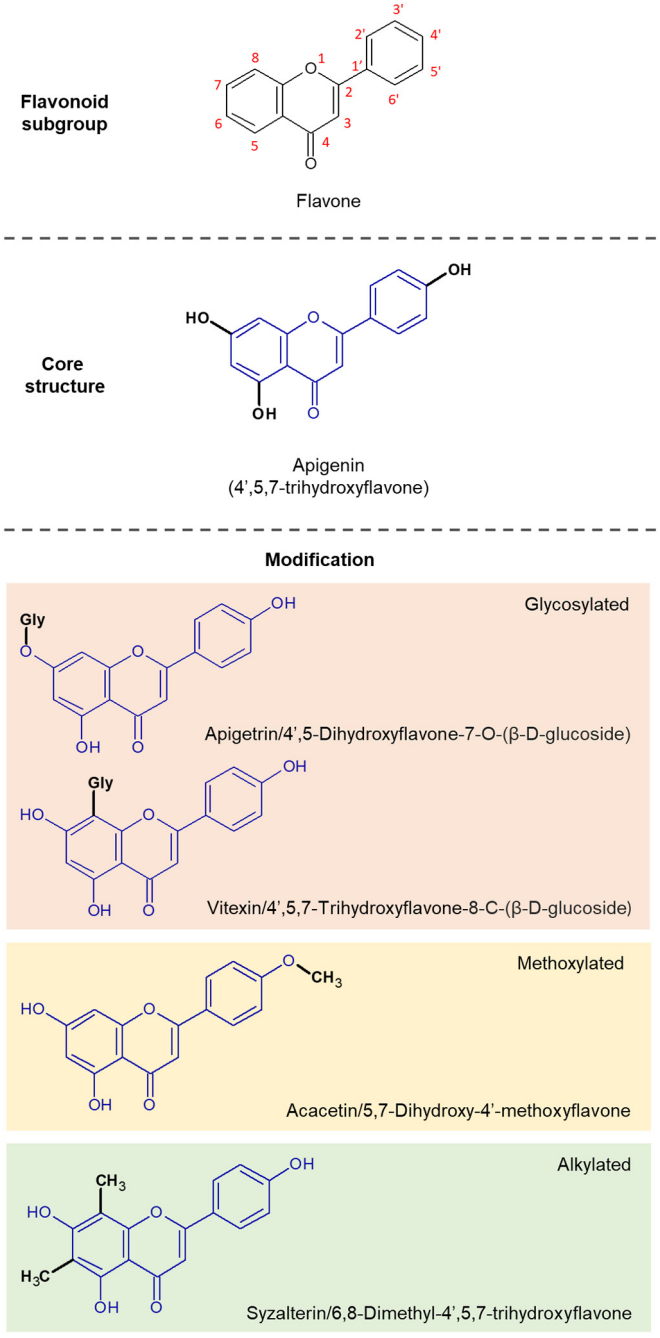

Fig. 4. Example of the hierarchic systematization used to categorize flavonoids grouped into subgroups, core structures and different types of modifications.

such as the presence of amino and sulphate functional groups, were found in only 2% and 1% of the cases, respectively. Although the majority of compounds found had an unmodified core structure, glycosylated and methoxylated modifications had, together, nearly half of the occurrences. Considering the high number of different flavonoids and core backbones, especially for flavones and flavonols, a high diversity of structures is observed. This may also be extended to a wide range of bioactivities tested for these compounds, as addressed in the next section.

The high number of different flavonoid structures is a response to the diversity and complexity of the biosynthesis of secondary metabolites in each plant, resulted from different evolutionary and ecological processes in response to abiotic and biotic stresses and other phenomena (Kessler and Kalske, 2018). Therefore, regarding the diversity of structures found, we have observed that many studies are focused on a general screening

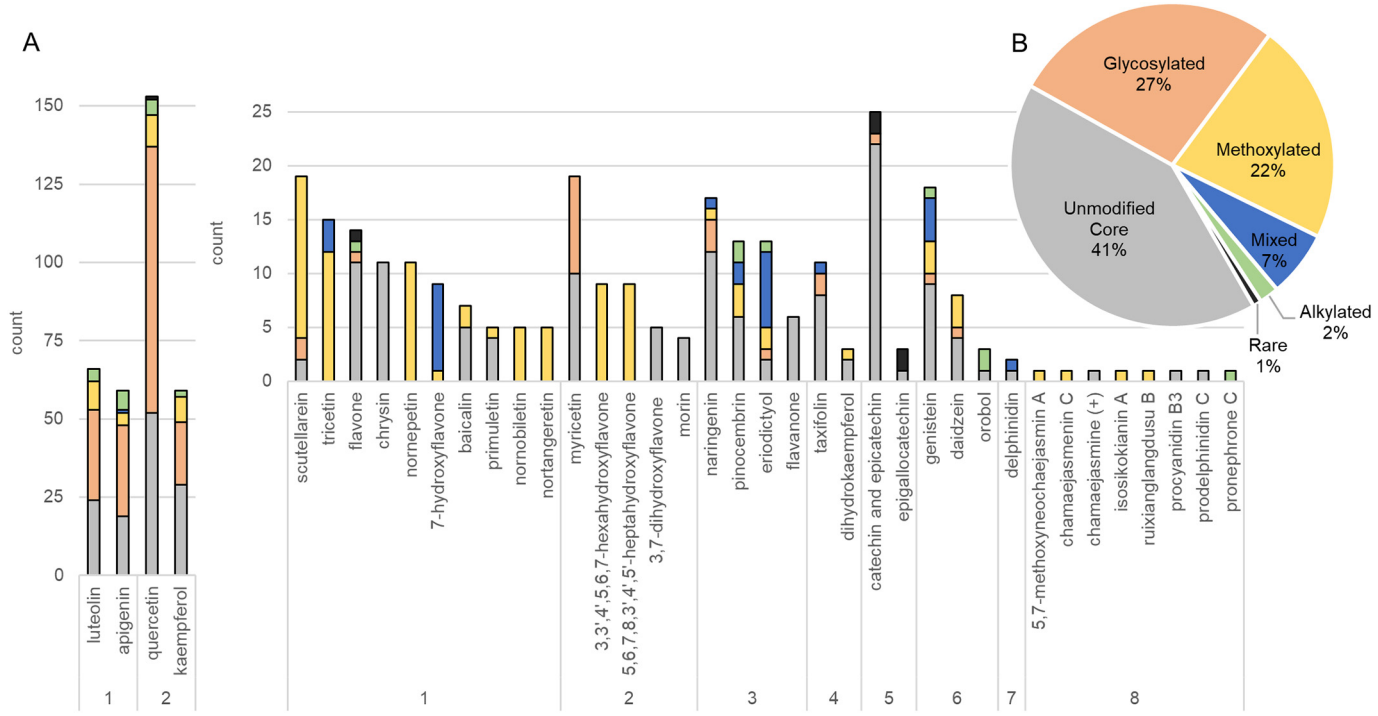

Fig. 5. (A) Count of selected core structures of the different groups (1: flavones, 2: flavonols, 3: flavanones, 4: flavanonols, 5: flavanols, 6: isoflavonoids, 7: anthocyanidins, 8: conjugated flavonoids) and their modified derivatives (grey: unmodified, orange: glycosylated, yellow: methoxylated, blue: mixed, green: alkylated, black: rare). (B) Occurrence of unmodified core flavonoids and the most common modifications, in percentage of total counts.

approach to find highly active compounds, with a high number of different plant species being studied. However, in a more practical approach, flavonoids that are most commonly found in nature should be focused on for further investigations regarding their activity, selectivity and applicability, as high quantities are demanded for biopesticide application in the near future. This extends also for the type of feedstock that could be used for obtaining these extracts or individual compounds, as discussed in the previous section.

6. Pesticide applications and activities

An analysis of the 201 publications was done to identify the different application and activity categories of flavonoid-containing plant extracts and pure or isolated compounds and to examine the development of these topics over time. Research on flavonoids as pesticides goes back to the 1980s and the number of studies have increased strongly since then (Fig. 6). In 39% of the selected studies, flavonoid-containing extracts were investigated. Isolated and purchased pure compounds were investigated in 35%. Studies investigating both, extracts and isolated compounds, are slightly less common, representing 26% of the selected studies. The major application domain of flavonoids or plant-extracts containing them is the protection of crops at fields but also postharvest during storage (Fig. 6A). Additionally, flavonoids were investigated as protective agents against tree diseases and wood preservation. Less common applications like the control of harmful algae blooms, antifouling e.g. to prevent the settlement of barnacles on ships or bacteria in paper mills, and household

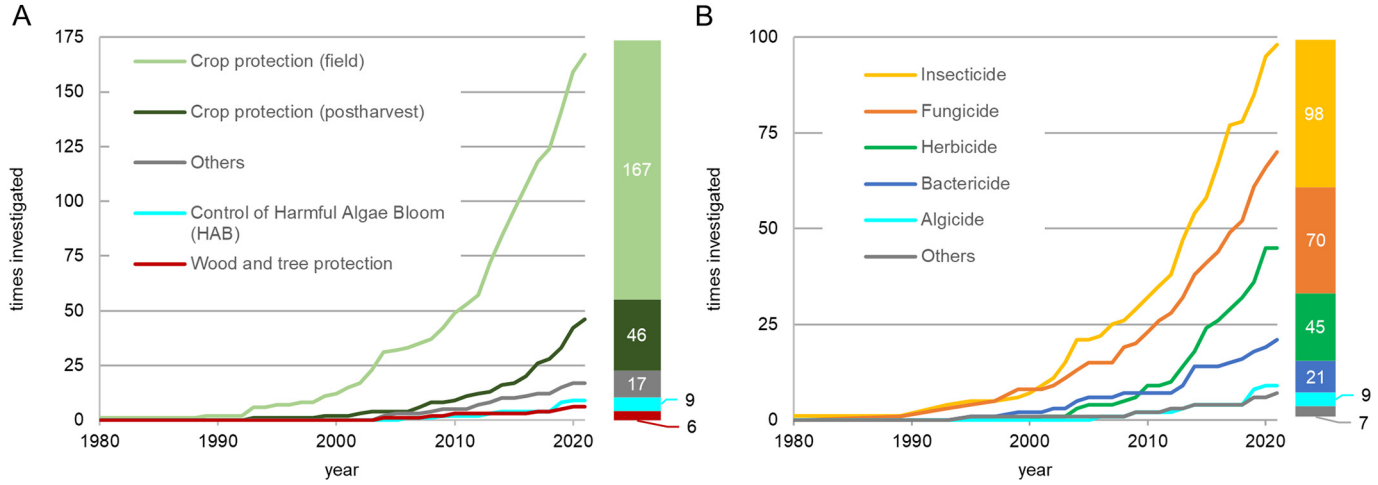

Fig. 6. (A) Time evolution of the categorized intended applications for flavonoids in terms of the number of studies (cumulative) along the time range selected for this analysis (1980–2020) and total counts of the intended applications of flavonoids. "Others" includes antifouling, household biocides, and general pesticide applications. (B) Time evolution of the categorized activities of flavonoids, or extracts containing flavonoids, in terms of the number of studies (cumulative) along the time range selected for this analysis (1980–2020) and total count represented in the bar. "Others" includes nematocidal, molluscicidal and anti-barnacle activity.

biocides show that the application of flavonoids as pesticides can go beyond plant protection products. In 10 studies, the application was not stated clearly or derivable, therefore these publications were grouped as general pesticide application.

These different applications of flavonoids translate into eight different categories of activities. Most investigated are insecticidal, fungicidal and herbicidal effects (Fig. 6B). In 21 of the selected studies, anti-bacterial activity was investigated. More studies on bacteria exists but were out of scope of this review due to their medical application. Rarely investigated activities are anti-algae, anti-nematode, anti-barnacle, and anti-molluscs activities. These findings indicate a broad target-spectrum of flavonoids and flavonoid-containing extracts. To obtain more information on this subject, the investigated species in the selected publications were assessed quantitatively. All mentioned species were taken into account despite the extent of the activity (weak – strong).

In total 260 different species were proposed as target organisms of different flavonoid-containing extracts and single compounds. Tested organisms belong to several different taxonomic groups with fungi, insects, plants and bacteria being the most frequently investigated (Fig. 7). Additionally, algae (including cyanobacteria), nematodes, molluscs and barnacles were studied. While most publications investigated insecticidal activity (Fig. 6), more individual fungi species than insect species were tested (Fig. 7).

Interestingly, different taxonomic groups are not dominated by a few frequently studied organisms. Instead, several organisms (68%) are investigated in only one study. However, frequently studied fungi belonged to genera of *Aspergillus* and *Fusarium*. Additionally, *Botrytis cinerea* and *Alternaria alternate* were investigated often (Fig. 7). These fungi infect plants and crops, especially stored fruits. *A. niger* and *F. culmorum* can also contaminate food by producing mycotoxins.

The most studied insects *Spodoptera frugiperda*, *Spodoptera litura* and *Helicoverpa amigera* are widely distributed major pests of a wide variety of crop plants, while *Sitophilus oryzae* causes high economic losses by feeding on stored grains worldwide. Furthermore, these insects developed resistance to commonly used insecticides such as carbamates and pyrethroids (Hilliou et al., 2021; Riaz et al., 2021). Considering herbicidal effects, the most frequently studied plant is *Lactuca sativa* (lettuce), generally used as standard target species to assess phytotoxicity. Subsequently, the three common weed species *Portulaca oleracea*, *Lolium perenne* and *Amaranthus retroflexus* were each investigated in five studies.

As for the activity tests on bacteria, mostly investigated species include gram positive *Staphylococcus aureaus* and *Bacillus subtilis* and gram negative *Eschericha coli* and *Pseudomonas aeruginosa*.

The 201 selected publications show that intensive research was performed to identify the activities of flavonoid-containing extracts and isolated compounds and their potential application as biopesticides. The 281 different active flavonoids identified are accompanied by the same variety of test organisms. However, while the structural diversity is clearly dominated by a quercetin, luteolin, apigenin, kaempferol and their derivatives the distribution of test organisms is more even without a focus on certain organisms. Most frequently studied is *A. niger* with 13 counts. This variety of potential target species challenges the stated advantage of target specificity attributed to biopesticides (Fig. 7). In 47% of the selected studies, more than one test organism was investigated. Out of these, 19% (18) covered more than one activity category (e.g. bactericidal and fungicidal activity). Especially extracts showed pesticidal activity against test organisms from several taxonomic groups (Aziman et al., 2014; Dane et al., 2016; EL-Hefny et al., 2020; Hajji-Hedfi et al., 2019; Kraśniewska et al., 2014; Rios et al., 2019), but this was also found for isolated flavonoids (Ali et al., 2005; Belofsky et al., 2014; Kong et al., 2004; Zhu et al., 2004). Only two of these 18 studies referred to the topic of target specificity. Zhu et al. pointed out that their results indicate a broad target spectrum including gram-positive and negative bacteria, yeasts and molds (Zhu et al., 2004). Jha et al. noted the importance of further research on the mode of action and selectivity before using natural products as biopesticides (Jha et al., 2014).

In regard of all selected publications, in the introduction of 94 of them (47%), the need for pesticides which don't negatively affect the environment is pointed out, with 17 publications explicitly addressing target specificity that biopesticies may possess as an advantageous property. However, only 35 (17%) publications refer to this topic in their results and discussion or conclusion sections. Six studies said their results indicate a broad target spectrum of investigated compounds or extracts (Balah, 2016; Chormova et al., 2015; Diaz Napal et al., 2009; Franceschini Sarria et al., 2020; Weidenbiirner and Chandra Jha, 1993; Zhu et al., 2004). The study of Chormova being especially interesting since a specific inhibitor of xyloglucan endotransglucosylase, an enzyme common in plants, was investigated. The flavanol (−)-epigallocatechin gallate turned out to be one of the least specific inhibitors and interfered with 8 out of 9 tested plant enzymes (Chormova et al., 2015). On the other hand side, 13 studies conclude that investigated compounds or extracts may represent environmentally safe biopesticides (Batista Ferrreira et al., 2021; Ben Kaab et al., 2020; D'Abrosca et al., 2004; Diaz Napal and Palacios, 2015; El Marsni et al., 2015; Gatto et al., 2016; Georges et al., 2008; Ilk et al., 2017; Kaab et al., 2020; Li et al., 2020; Othman and Latip, 2021; Salih et al., 2017; Vargas et al., 1999). In contrast, Jmii et al. noted that “biopesticides are not less harmless in the environment than synthetic pesticides just because they are natural compounds”(Jmii et al., 2020). In line with this, eight studies point out that more information on the mode of action, specificity, concentration dependency of activities, and environmental safety is needed (Chang et al., 2020; Cui et al., 2019; Hussain and Reigosa, 2014; Jha et al., 2014; Lawrence et al., 2019; Nenaah, 2014; Stavropoulou et al., 2017; Xu et al., 2019). Additionally, Stompor et al. discussed the possibility to influence selectivity by tuning the liphophility of flavonoids by using methoxy groups (Stompor et al., 2015).

Six publications report species-specific results of conducted activity tests (Araniti et al., 2014; Hosni et al., 2013; Pardo-Muras et al., 2020; Pedersen et al., 2015; Rios et al., 2018; Vitalini et al., 2020). For instance, the flavonone linarin isolated from *Zanthoxylum affine* showed specificity towards *L. sativa* (dicotyleous) in pre-emergent assays when compared to the weed *L. perenne* (monocotyleous), while both plants were sensitive towards extracts of *Z. affine* (Rios et al., 2018). Pedersen et al. suggested

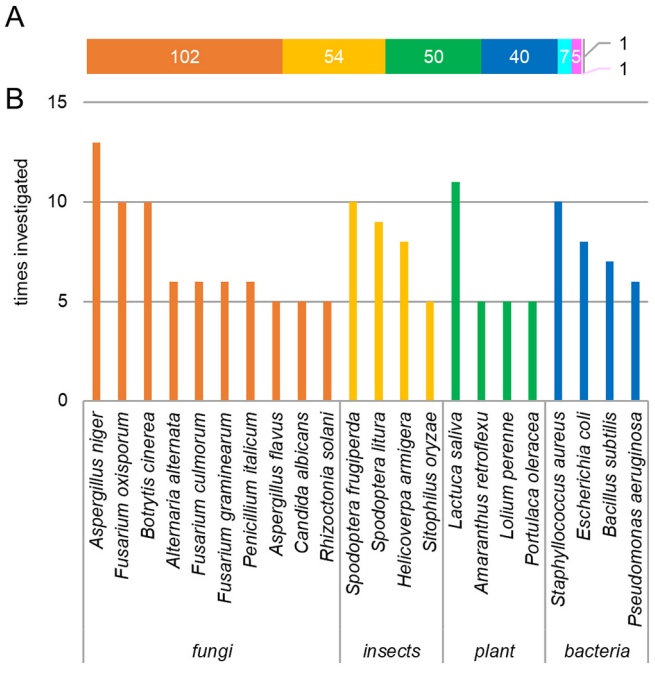

Fig. 7. (A) Number of different species in each taxonomic group. (B) Most frequently (≥5) studied species of each group. Orange: fungi; yellow: insects, green: plants, blue: bacteria; cyan: algae (including cyanobacteria), pink: nematodes, light pink: molluscs; grey: barnacles.

species-specific effects of the isoflavonoid biochanin A based on a comparison of the very weak effects they observed for *Arabidopsis thaliana* to strong growth inhibition of dicotyledonous weeds *Silene noctiflora* and *Geranium molle* observed in a previous study (Pedersen et al., 2015; Shajib et al., 2012). Aqueous extracts of *Chrysanthemum coronarium* containing chlorogenic acid, di-cafeoylquinic acids isomers, and the flavonoids rutin, luteolin, luteolin-7-O-glucoside, myricetin-3-O-galactoside and tricin inhibited seed germination and seedling growth of the two weeds *Sinapis arvensis* (dicotyledonous) and *Phalaris canariensis* (monocotyledonous) but did not affect the crop plants *Triticum durum* (durum wheat, monocotyledonous) and *Zea mays* (maize, dicotyledonous) (Hosni et al., 2013). Furthermore, the authors suggested tricin as the main active compound and that it might be inactive towards species in which it occurs naturally including wheat and maize. This is an interesting aspect in favor of the utilization of agricultural waste as biopesticides.

Similarly, all six studies base their suggestion towards target specificity on the comparison to few selected other organisms. To evaluate target specificity or the lack thereof in more detail, a broader perspective is needed that encompasses data for several organisms including standard organisms used for ecotoxicity assessment.

There are a few additional publications in support of the above indicated trend that dicotyledenous plants are more sensitive to treatments with flavonoids or extracts containing flavonoids in comparison to monocotyledons. Xie et al. showed root and stem growth inhibition of dicotyledonous *Codonopsis pilosula* and *Amaranthus retroflexes* but promoting effects for the monocotyledonous crops *Oryza sativa* and *Triticum aestivum* by luteolin and its 5-O-glucoside (Xie et al., 2018). Germination, stem and root elongation of the monocotyledonous weed *Lolium perenne* was only weakly affected by *Croton ehrenbergii* hexane and methanol extracts containing kaempferol, tiliroside, nicotiflorine and rutin while *Lactuca sativa* was strongly impacted (Rios et al., 2019). In contrast, Jmii et al. found *L. perenne* root and shoot elongation to be more sensitive to treatments with luteolin-7-O-glucoside and apigenin-7-O-glucoside than *L. sativa* (Jmii et al., 2020). In the same study, mono- and dicotyledonous weeds were efficiently suppressed in pot experiments with pre-geminated lettuce (*L. sativa*). Therefore, this trend cannot be generalist for the class of flavonoids and more comprehensive knowledge about compounds and all their different activities is needed.

Based on the screening for the keywords non-target and field experiments, further studies were identified that investigated pests and crop plants or beneficial organisms. These can give important information about the applicability of flavonoid-containing extracts as biopesticides. Selin-Rani et al. demonstrated that the earthworm *Eisenia fetida* was not affected by 250 mg quercetin/kg soil but *Spodoptera litura Fab* has a LC_{50} value of 11 mg/L (Selin-Rani et al., 2016). Furthermore, field and pot experiments with crops including *Triticum aestivum* (wheat), *Solanum lycorpersicum* (tomato) and *Oryza sativa* (rice) infected by fungi (*Puccinia triticina*, *Furuarium oxysporum*, *Rhizoctonia solani*) or the nematode *Meloidogyne javanica* showed reduced disease incidences due to treatments with flavonoid-containing extracts (catechin, epicatechin, epicatchein gallate, quercetin) or plant materials (containing tricin) amended to the soil (Gillmeister et al., 2019; Hajji-Hedfi et al., 2019; Kong et al., 2010). Although no experiments were performed to assess impact of the treatments to the plants without infection, these results suggest no adverse effects on crop plants. On the other hand, *T. aestivum* is used in the wheat coleptile assay for quick assessment of phytotoxicity and strong inhibition was observed for e.g. pectolinargenin, heliannone B, flavone, pentamethoxyflavone and two heptamethoxyflavones at 1000–100 μM (El Marsni et al., 2015; Nebo et al., 2014). Therefore, negative effects of flavonoids on crop plants cannot be excluded and need to be further investigated.

Our quantitative assessment of target species and the highlighted examples show that much information is available, however a higher quality of data, a better overview and connection of existing knowledge is needed about the activities of flavonoids and their mechanisms to better understand structure-activity relationships of this compound class. While the summary of possible target species presented in this section is a first step illustrating the potentially broad target spectrum, comprehensive comparison of activities against different species is hindered by the variety of different endpoints (mortality, growth inhibition, reproduction, repellent activity, physiology and morphology changes) and variations in methods e.g. regarding duration and concentrations. More standardized procedures and easily comparable parameters like EC_{50}/LD_{50} values can contribute to a better understanding of flavonoids broad activities. Comparison of doses needed to obtain certain adverse effects could shed light on species-related differences in sensitivities.

Next to the target spectrum, it is important to consider the form of application. Using extracts instead of isolated compounds can be advantageous in terms of production and effectivity. Although technical possibilities steadily increase the isolation of natural compounds can still be challenging (Bucar et al., 2013). The presence of several compounds in extracts may lead to synergistic effects that enhance the activity (Céspedes et al., 2014; Gillmeister et al., 2019; Gómez-Maldonado et al., 2020; Ningombam et al., 2017). This is also well known in medical applications of plant extracts (Caesar and Cech, 2019). One the other hand side, the exposure to a mixture of several different compounds may enhance risk to environmental and human health. Awareness to the toxicity potential of mixtures needs to be raised (Hernández et al., 2017; Kortenkamp et al., 2019; Kortenkamp and Faust, 2018). Accordingly, a broad target spectrum and complex composition increase the need for knowledge on the environmental fate to protect beneficial and other non-target organisms. Ecotoxicity of plant extracts and isolated flavonoids and their degradation behaviour should be further investigated.

In summary, based on the systematically selected publications the main application purposes, different activities and target organisms were identified and quantified. Whereas the main application of flavonoid-containing extract and isolated compounds is in agriculture, additional fields like wood preservation, the control of harmful algae blooms and antifouling are investigated as well. The categorization of observed activities into eight different groups (insecticidal, fungicidal, herbicidal, bactericidal, algicidal, nematicidal, molluscicidal and anti-barnacle) and the 260 different tested organisms indicate a broad target spectrum of flavonoids. Although, the probable broad target spectrum can be partially attributed to the investigation of extracts, this review shows the need for more comprehensive knowledge on flavonoids several activities. Further research is needed that compares activities of flavonoids towards a variety of test organisms and that takes applied concentrations into account. Without extensive knowledge on selectivity, environmental risks cannot be excluded. Thus, this information is crucial for environmentally safe application of flavonoid-containing extracts and isolated compounds as biopesticides.

7. Environmental fate and biodegradability

It has commonly been assumed that natural products are non-persistent due to their biodegradability (Kumar, 2016; Manda et al., 2020; Marrone, 2019; Sharma et al., 2020). However, being derived from natural sources does not automatically ensure their environmental saftyness (Copping and Menn, 2000). Recently, concerns were risen some phytotoxins may pose environmental risks since they can possess similar physiochemical properties to synthetic pesticides and were detected in soils and surface waters. Therefore, they can reach non-target organisms and contribute to mixture toxicity (Bucheli, 2014; Günthardt et al., 2018).

Biodegradability is assumed or described as a wanted property in 11% of the analysed publications, but investigations or citation of experimental data on abiotic and biotic degradation and environmental fate of flavonoids is very rare. Only in two studies, hydrolysis of genistein and degradation of biochanin A in soil were addressed, respectively (Furbo et al., 2011; Zhou et al., 2009).

Scattered information on half-lives of a few flavonoids is available in the literature and indicates low risks of persistence. Half-lives of genistein, daidzein, formonoetin, biochanin A, quercetin and naringenin in non-sterile water and soil are in the range of 26 to 41 and < 1 to 5 days (Carlsen et al., 2012; Furbo et al., 2011; Hoerger et al., 2009; Shaw and Hooker, 2008). For the two compounds formononetin and kaempferol,

half-lives in soil exceeding 14 days were found (Carlsen et al., 2012; Ozan et al., 1997). Furthermore, degradation pathways of flavonoids by soil bacteria were proposed (Cooper, 2004; Rao and Cooper, 1994).

These indications of short half-lives have to be balanced with stability requirements for agricultural applications. A limitation of the use of biopesticides is a too short durability and the necessity for frequent reapplications (Isman, 2020; Smith et al., 2021). However, encapsulation could be used to overcome this shortcoming (Campos et al., 2019). Combining a biodegradable biopesticide with natural polysaccharides in a sustained release system is a promising strategy. Additionally, active flavonoids can be released from decomposing plant materials (Kong et al., 2010), which may represent another kind of natural encapsulation.

Regarding the increased input accompanied with biopesticides use, knowledge about the environmental fate, especially degradation kinetics and mineralization potential should be extended. As first indicator of safe and circular use, biodegradation within an adequate time period should be assessed using a standardized test (e.g. OECD standard tests). Based on this first indicator, further experiments and field studies should be undertaken to establish extensive knowledge on the environmental fate of flavonoids including degradation pathways, kinetics, possible transformation products and their properties, influences on soil microbiota, threshold concentrations for biotic degradation and mineralization under environmental conditions. This knowledge is prerequisite before flavonoids can be considered as green and circular substitutes of currently used synthetic pesticides. By summarizing investigated flavonoids, this review provides important information to selected flavonoids for future investigations.

8. Conclusion and perspectives

The usage of synthetic pesticides increasingly threatens environmental and human health. Biopesticides, attributed with advantageous properties such as the production from renewable sources, low-toxicity to non-target organisms and biodegradability, are seen as viable alternatives. In regard of these credited advantages, this systematic review evaluated the potential of flavonoids as green and circular substitutes for synthetic pesticides by looking at different aspects of the whole life cycle of these compounds. An analysis of the temporal evolution regarding tested activities and applications of flavonoids on published studies between 1970 and 2021 showed a growing interest over time, especially from the year of 2000 until recent years. Insecticidal and fungicidal activities and crop protection applications were the major findings, with a higher boost of interest in the last decade.

Further analysis showed that only a minority of studies investigated waste parts of commercially-explored plants as sources of active flavonoid-containing extracts or isolated compounds. To avoid conflicts with food production, research needs to shift further towards the exploitation of agricultural waste as source material which could be a cheap, circular and sustainable alternative for the production of green and circular chemicals. Besides that, even though the search parameters were not directed towards this angle, none of the works had tested or mentioned the use of green extraction techniques. The use of such prominent technologies to extract flavonoids from natural resources is considered to be in many cases more efficient in terms of yield, solvent and energy consumption.

Next, an overview of flavonoids identified in the various source materials is given and the flavonols quercetin and kaempferol, the flavones luteolin and apigenin, and their glycosides were identified as the most studied compounds. These compounds are commonly found in a variety of plant species, showing that they could be used as platforms for further investigation of applicability. On the other hand, a diverse range of different flavonoids core structures and modifiers was observed (281 different structures found), as screening approaches for highly active compounds are common practices in the field. A more focused approach should be utilized in order to find viable solutions, as feedstock supply could be an issue when considering a broader applied scenario. The provided overview could be used to select priority compounds for future investigations.

Analysing activities and target organisms, we found indications for a broad target spectrum for the class of flavonoids including insecticidal, fungicidal, herbicidal, bactericidal, algicidal, nematocidal, molluscicidal and anti-barnacle effects. In regards of this potentially very broad target spectrum, further research needs to address structure-activity relationships and concentration-dependencies of activities in more detail to find out if certain features are responsible for differences in sensitivity of certain species to assess the degree of target specificity. This includes the assessment of ecotoxicity of flavonoids and extracts containing them. Finally, little information is available on the environmental fate and biodegradation of flavonoids and a connection to studies investigating pesticidal activities is largely missing. Such information is crucial for a proper assessment of environmental and health risks due to accelerated input when used as biopesticides.

Thus, flavonoids and flavonoid-containing extracts may represent green and circular biopesticides if they are imbedded in biorefinery process of agricultural waste materials, they have verified mineralization and when adverse effects on non-target organisms are excluded. The summarized information in this review provides a good foundation to direct research to close the knowledge gap identified by this systematic approach.

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CRediT authorship contribution statement

The authors have contributed to the review article Flavonoids as Biopesticides – Systematic Assessment of Sources, Structures, Activities and Environmental Fate as follows:

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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.

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Supporting Information of Publication 1

Lena Schnarr, Mateus L. Segatto, Oliver Olsson, Vânia G. Zuin, Klaus Kümmerer

Flavonoids as biopesticides – Systematic assessment of sources, structures, activities and environmental fate

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List of the publications obtained by the systematic literature screening. The analysis of the systematic literature review are based on these 201 publications.

#	authros	title	journal	publication year	DOI
1	Ahmadu, T; Ahmad, K; Ismail, SI; Rashed, O; Asib, N; Omar, D	Antifungal efficacy of Moringa oleifera leaf and seed extracts against Botrytis cinerea causing gray mold disease of tomato (Solanum lycopersicum L.)	Brazilian Journal Of Biology	2021	10.1590/1519-6984.233173
2	Azizian T., Alirezalu A., Hassani A., Bahadori S., Sonboli A.	Phytochemical analysis of selected Nepeta species by HPLC-ESI-MS/MS and GC–MS methods and exploring their antioxidant and antifungal potentials	Journal Of Food Measurement And Characterization	2021	10.1007/s11694-021-00819-8
3	da Silva, WM; Santos, RG; Duarte, GKGF; Cunha, GOS; da Silva, DM; Marchesin, TFR; Bueno, OC; Menezes, ACS	Odontadenia lutea (Apocynaceae) LEAVES: PHYTOCHEMICAL STUDY AND INSECTICIDAL ACTIVITY AGAINST LEAF-CUTTING ANTS Atta sexdens rubropilosa Forel	Quimica Nova	2021	10.21577/0100-4042.20170641
4	Fadda, A; Sarais, G; Lai, C; Sale, L; Mulas, M	Control of postharvest diseases caused by Penicillium spp. with myrtle leaf phenolic extracts: in vitro and in vivo study on mandarin fruit during storage	Journal Of The Science Of Food And Agriculture	2021	10.1002/jsfa.11062
5	Ferrreira, ECB; Nova, ICV; de Almeida, WA; Albuquerque, FMD; Cruz, GD; da Costa, HN; Procopio, TF; da Silva, WAV; Ferreira, MRA; Paiva, PMG; Soares, LAL; Teixeira, AAC; Teixeira, VW; Napoleao, TH; Barros, R; Pontual, EV	Opuntia ficus-indica cladode extract is an embryotoxic, larvicidal, and oviposition-deterrent agent for the diamondback moth, Plutella xylostella	Crop Protection	2021	10.1016/j.cropro.2020.105351
6	Othman S.N., Latip S.N.H.M.	Antifeedant Activity of Peltophorum pterocarpum and Ipomoea aquatica Extracts as Botanical Pesticides against Pomacea canaliculata	Iop Conference Series: Earth And Environmental Science	2021	10.1088/1755-1315/685/1/012028
7	Pham D.Q., Pham H.T., Han J.W., Nguyen T.H., Nguyen H.T., Nguyen T.D., Nguyen T.T.T., Ho C.T., Pham H.M., Vu H.D., Choi G.J., Le Dang Q.	Extracts and metabolites derived from the leaves of Cassia alata L. exhibit in vitro and in vivo antimicrobial activities against fungal and bacterial plant pathogens	Industrial Crops And Products	2021	10.1016/j.indcrop.2021.113465
8	Sarria, ALF; Matos, AP; Volante, AC; Bernardo, AR;	Insecticidal activity of copper (II) complexes with flavanone derivatives	Natural Product Research	2021	10.1080/14786419.2020.1868465

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9	Silva, BN; Cadavez, V; Ferreira-Santos, P; Alves, MJ; Ferreira, ICFR; Barros, L; Teixeira, JA; Gonzales-Barron, U	Chemical Profile and Bioactivities of Extracts from Edible Plants Readily Available in Portugal	Foods	2021	10.3390/foods10030673
10	Badalamenti N., Rosselli S., Zito P., Bruno M.	Phytochemical profile and insecticidal activity of Drimia pancration (Asparagaceae) against adults of Stegobium paniceum (Anobiidae)	Natural Product Research	2020	10.1080/14786419.2020.1729154
11	Ben Kaab, S; Lins, L; Hanafi, M; Rebey, IB; Deleu, M; Fauconnier, ML; Ksouri, R; Jijakli, MH; De Clerck, C	Cynara cardunculus Crude Extract as a Powerful Natural Herbicide and Insight into the Mode of Action of Its Bioactive Molecules	Biomolecules	2020	10.3390/biom10020209
12	Chang, BH; Qiang, B; Li, S; Ullah, H; Hao, K; McNeill, MR; Rajput, A; Raza, A; Huang, XB; Zhang, ZH	Inhibitory effect of genistein and PTP1B on grasshopper Oedaleus asiaticus development	Arthropod-Plant Interactions	2020	10.1007/s11829-020-09757-6
13	Chen, CY; Wan, CP; Peng, X; Chen, JY	A flavonone pinocembroside inhibits Penicillium italicum growth and blue mold development in 'Newhall' navel oranges by targeting membrane damage mechanism	Pesticide Biochemistry And Physiology	2020	10.1016/j.pestbp.2019.11.025
14	de Oliveira, APS; Agra-Neto, AC; Pontual, EV; Lima, TD; Cruz, KCV; de Melo, KR; de Oliveira, AS; Coelho, LCBB; Ferreira, MRA; Soares, LAL; Napoleao, TH; Paiva, PMG	Evaluation of the insecticidal activity of Moringa oleifera seed extract and lectin (WSMoL) against Sitophilus zeamais	Journal Of Stored Products Research	2020	10.1016/j.jspr.2020.101615
15	Elaloui, M; Hamdi, SH; Ghazghazi, H; Ben Nasr, R; Bouslih, E; Ammari, Y; Mediouni, J; Laamouri, A	Characterization of epicathechin contents in theZiziphus spina-christiL. root extracts using LC-MS analyses and their insecticidal potential	Plant Biosystems	2020	10.1080/11263504.2020.1779837
16	El-Hefny M., Salem M.Z.M., Behiry S.I., Ali H.M.	The potential antibacterial and antifungal activities of wood treated withwithania somnifera fruit extract, and the phenolic, caffeine, and flavonoid composition of the extract according to HPLC	Processes	2020	10.3390/pr8010113

17	Golla, SK; Sharma, HC; Rajasekhar, P; Mishra, SP; Jaba, J	Biochemical components of wild relatives of chickpea confer resistance to pod borer, Helicoverpa armigera	Arthropod-Plant Interactions	2020	10.1007/s11829-020-09768-3
18	Gómez-Maldonado D., Lobato-Calleros C., Aguirre-Mandujano E., Leyva-Mir S.G., Robles-Yerena L., Vernon-Carter E.J.	Antifungal activity of mango kernel polyphenols on mango fruit infected by anthracnose	LWT	2020	10.1016/j.lwt.2020.109337
19	Hafeez, M; Qasim, M; Ali, S; Yousaf, HK; Waqas, M; Ali, E; Ahmad, MA; Jan, S; Bashir, MA; Noman, A; Wang, M; Gharmh, HA; Khan, KA	Expression and functional analysis of P450 gene induced tolerance/resistance to lambda-cyhalothrin in quercetin fed larvae of beet armyworm Spodoptera exigua (Hubner)	Saudi Journal Of Biological Sciences	2020	10.1016/j.sjbs.2019.05.005
20	Huang, XB; Lv, SJ; Zhang, ZH; Chang, BH	Phenotypic and Transcriptomic Response of the Grasshopper Oedaleus asiaticus (Orthoptera: Acrididae) to Toxic Rutin	Frontiers In Physiology	2020	10.3389/fphys.2020.00052
21	Hussain, MI; El-Sheikh, MA; Reigosa, MJ	Allelopathic Potential of Aqueous Extract from Acacia melanoxylon R. Br. on Lactuca sativa	Plants-Basel	2020	10.3390/plants9091228
22	Jang, YH; Park, JR; Kim, KM	Antimicrobial Activity of Chrysoeriol 7 and Chochlioquinone 9, White-Backed Planthopper-Resistant Compounds, Against Rice Pathogenic Strains	Biology-Basel	2020	10.3390/biology9110382
23	Jmii G., Molinillo J.M.G., Zorrilla J.G., Haouala R.	Allelopathic activity of Thapsia garganica L. leaves on lettuce and weeds, and identification of the active principles	South African Journal Of Botany	2020	10.1016/j.sajb.2020.02.027
24	Kaab, SB; Rebey, IB; Hanafi, M; Hammi, KM; Smaoui, A; Fauconnier, ML; De Clerck, C; Jijakli, MH; Ksouri, R	Screening of Tunisian plant extracts for herbicidal activity and formulation of a bioherbicide based on Cynara cardunculus	South African Journal Of Botany	2020	10.1016/j.sajb.2019.10.018
25	Kikuta, S	The Cytotoxic Effect of Genistein, a Soybean Isoflavone, against Cultured Tribolium Cells	Insects	2020	10.3390/insects11040241
26	Ladhari, A; Tufano, I; DellaGreca, M	Influence of new effective allelochemicals on the distribution of Cleome arabica L. community in nature	Natural Product Research	2020	10.1080/14786419.2018.1501688
27	Li, JM; Hu, JQ; Cao, LR; Yuan, Y	Growth, physiological responses and microcystin-production/-release dynamics of Microcystis aeruginosa exposed to various luteolin doses	Ecotoxicology And Environmental Safety	2020	10.1016/j.ecoenv.2020.110540

28	Makenzi, AM; Manguro, LOA; Owuor, PO	Phytochemistry and insecticidal activity ofAnnona mucosaleaf extracts againstSitophilus zeamaisandProstephanus truncatus	Journal Of Asian Natural Products Research	2020	10.1080/10286020.2020.1818726
29	Muzzalupo, I; Badolati, G; Chiappetta, A; Picci, N; Muzzalupo, R	In vitro Antifungal Activity of Olive (Olea europaea) Leaf Extracts Loaded in Chitosan Nanoparticles	Frontiers In Bioengineering And Biotechnology	2020	10.3389/fbioe.2020.00151
30	Pardo-Muras, M; Puig, CG; Souto, XC; Pedrol, N	Water-soluble phenolic acids and flavonoids involved in the bioherbicidal potential of Ulex europaeus and Cytisus scoparius	South African Journal Of Botany	2020	10.1016/j.sajb.2020.07.023
31	Scavo, A; Pandino, G; Restuccia, A; Mauromicale, G	Leaf extracts of cultivated cardoon as potential bioherbicide	Scientia Horticulturae	2020	10.1016/j.scienta.2019.109024
32	Shi, GQ; Kang, ZY; Ren, F; Zhou, Y; Guo, PL	Effects of Quercetin on the Growth and Expression of Immune-Pathway-Related Genes in Silkworm (Lepidoptera: Bombycidae)	Journal Of Insect Science	2020	10.1093/jisesa/ieaa124
33	Vitalini, S; Orlando, F; Palmioli, A; Alali, S; Airoldi, C; De Noni, I; Vaglia, V; Bocchi, S; Iriti, M	Different phytotoxic effect of Lolium multiflorumLam. leaves againstEchinochloa oryzoides(Ard.) Fritsch andOriza sativaL.	Environmental Science And Pollution Research	2020	10.1007/s11356-020-09573-8
34	Cui, BY; Huang, XB; Li, S; Hao, K; Chang, BH; Tu, XB; Pang, BP; Zhang, ZH	Quercetin Affects the Growth and Development of the Grasshopper Oedaleus asiaticus (Orthoptera: Acrididae)	Journal Of Economic Entomology	2019	10.1093/jee/toz050
35	Datta R., Kaur A., Saraf I., Singh I.P., Kaur S.	Effect of crude extracts and purified compounds of Alpinia galanga on nutritional physiology of a polyphagous lepidopteran pest, Spodoptera litura (Fabricius)	Ecotoxicology And Environmental Safety	2019	10.1016/j.ecoenv.2018.10.065
36	Duarte, GKGF; Menezes, ACS; Naves, PLF; Bueno, OC; Santos, RG; da Silva, WM	TOXICITY OF Esenbeckia pumila Pohl (Rutaceae) ON Artemia salina AND Atta sexdens rubropilosa	Revista Caatinga	2019	10.1590/1983-21252019v32n111re
37	Elamir, EE; Almadiy, AA; Nenaah, GE; Alabas, AA; Alsaqri, HS	Comparing six mathematical link function models of the antifeedant activity of lesser grain borer exposed to sub-lethal concentrations of some extracts from calotropis procera	Bioengineered	2019	10.1080/21655979.2019.1641399
38	Esposito, T; Celano, R; Pane, C; Piccinelli, AL; Sansone, F; Picerno, P; Zaccardelli, M; Aquino, RP; Mencherini, T	Chestnut (Castanea sativa Miller.) Burs Extracts and Functional Compounds: UHPLC-UV-HRMS Profiling, Antioxidant Activity, and Inhibitory Effects on Phytopathogenic Fungi	Molecules	2019	10.3390/molecules24020302
39	Gao, Q; Shi, YH; Liao, M; Xiao, JJ;	Laboratory and field evaluation of the	Pest Management Science	2019	10.1002/ps.5434

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40	Gillmeister, M; Ballert, S; Raschke, A; Geistlinger, J; Kabrodt, K; Baltruschat, H; Deising, HB; Schellenberg, I	Polyphenols from Rheum Roots Inhibit Growth of Fungal and Oomycete Phytopathogens and Induce Plant Disease Resistance	Plant Disease	2019	10.1094/PDIS-07-18-1168-RE
41	Givi, F; Gholami, M; Massah, A	Application of pomegranate peel extract and essential oil as a safe botanical preservative for the control of postharvest decay caused by Penicillium italicum and Penicillium digitatum on "Satsuma" mandarin	Journal Of Food Safety	2019	10.1111/jfs.12639
42	Glasenapp Y., Catto C., Villa F., Saracchi M., Cappitelli F., Papenbrock J.	Promoting beneficial and inhibiting undesirable biofilm formation with mangrove extracts	International Journal Of Molecular Sciences	2019	10.3390/ijms20143549
43	Hajji-Hedfi, L; Larayedh, A; Hammas, NC; Regaieg, H; Horrigue-Raouani, N	Biological activities and chemical composition of Pistacia lentiscus in controlling Fusarium wilt and root-knot nematode disease complex on tomato	European Journal Of Plant Pathology	2019	10.1007/s10658-019-01770-7
44	Krotova I.V., Gulenkova G.S., Osmolovskaya N.A., Smirnov R.Yu.	Waste management of dark coniferous trees debarking for producing antibacterial preparations	Iop Conference Series: Earth And Environmental Science	2019	10.1088/1755-1315/315/5/052033
45	Lawrence S.A., Burgess E.J., Pairama C., Black A., Patrick W.M., Mitchell I., Perry N.B., Gerth M.L.	Mātauranga-guided screening of New Zealand native plants reveals flavonoids from kānuka (Kunzea robusta) with anti-Phytophthora activity	Journal Of The Royal Society Of New Zealand	2019	10.1080/03036758.2019.1648303
46	Li Y., Wei W., Zhang J., Li G., Gao K.	Structures and antipathogenic fungi activities of flavonoids from pathogen-infected Astragalus adsurgens	Natural Product Research	2019	10.1080/14786419.2017.1413560
47	Maazoun, AM; Hamdi, SH; Belhadj, F; Ben Jemaa, JM; Messaoud, C; Marzouki, MN	Phytochemical profile and insecticidal activity of Agave americana leaf extract towards Sitophilus oryzae (L.) (Coleoptera: Curculionidae)	Environmental Science And Pollution Research	2019	10.1007/s11356-019-05316-6
48	Oleszek, M; Pecio, L; Kozachok, S; Lachowska-Filipiuk, Z; Oszust, K; Frac, M	Phytochemicals of Apple Pomace as Prospect Bio-Fungicide Agents against Mycotoxigenic Fungal Species-In Vitro Experiments	Toxins	2019	10.3390/toxins11060361
49	Rios, MY; Leon-Rivera, I; Rios-Gomez, R; Cordova-Albores, LC; Aguilar-Guadarrama, AB	Phytotoxic and nematicide evaluation of Croton ehrenbergii (Euphorbiaceae)	Pest Management Science	2019	10.1002/ps.5336

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52	Xiao, X; Li, C; Huang, HM; Lee, YP	Inhibition effect of natural flavonoids on red tide alga Phaeocystis globosa and its quantitative structure-activity relationship	Environmental Science And Pollution Research	2019	10.1007/s11356-019-05482-7
53	Xu, CC; Ge, ZW; Li, C; Wan, FH; Xiao, X	Inhibition of harmful algae Phaeocystis globosa and Prorocentrum donghaiense by extracts of coastal invasive plant Spartina alterniflora	Science Of The Total Environment	2019	10.1016/j.scitotenv.2019.133930
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55	Zhu, JY; Xiao, H; Chen, Q; Zhao, M; Sun, D; Duan, SS	Growth Inhibition of Phaeocystis Globosa Induced by Luteolin-7-O-glucuronide from Seagrass Enhalus acoroides	International Journal Of Environmental Research And Public Health	2019	10.3390/ijerph16142615
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196	Pandji, C; Grimm, C; Wray, V; Witte, L; Proksch, P	Insecticidal Constituents From 4 Species Of The Zingiberaceae	Phytochemistry	1993	10.1016/0031-9422(93)80020-S
197	Riveravargas, LI; Schmitthenner, AF; Graham, TI	Soybean Flavonoid Effects On And Metabolism By Phytophthora-Sojae	Phytochemistry	1993	10.1016/0031-9422(93)85219-H
198	Weidenborner, M; Jha, HC	Antifungal Activity Of Flavonoids And Their Mixtures Against Different Fungi Occurring On Grain	Pesticide Science	1993	
199	Wheeler, GS; Slansky, F; Yu, SJ	Fall Armyworm Sensitivity To Flavone - Limited Role Of Constitutive And Induced Detoxifying Enzyme-Activity	Journal Of Chemical Ecology	1993	10.1007/BF00984999
200	Laks, PE; Pruner, MS	Flavonoid Biocides - Structure Activity Relations Of Flavonoid Phytoalexin Analogs	Phytochemistry	1989	10.1016/0031-9422(89)85015-0
201	Elliger C.A., Chan B.C., Waiss Jr. A.C.	Flavonoids as larval growth inhibitors - Structural factors governing toxicity	Naturwissenschaften	1980	10.1007/BF01106595

Asingment of a number to each flavonoid subgroups and conjugated flavonoids.

Group number	**Group name**
1	flavone
2	flavonol
3	flavanone
4	flavanonol
5	flavanol
6	isoflavone
7	conjugated flavonoid
8	anthocyanidin

List of different flavonoid structures investigated for pesticidal activity. Number of counts represent how often this particular flavonoids was investigated.

Group	**Compound**	**CAS**	**Nb. of counts**		
1	3',4'-methylenedioxy-5,7-dimethoxyflavone	89029-12-9	1		
1	3',4' -dihydroxy-5,6,7-trimethoxyflavone	51145-79-0	1		
1	3',4',5,5',7-pentamethoxyflavone	53350-26-8	1		
1	3'-hydroxyflavone	70460-18-3	1		
1	4',5,7-trihydroxy-3',8-dimethoxyflavone	24126-72-5	1		
1	4',5,7-trihydroxy-8-methoxyflavone (4'-Hydroxywogonin)	57096-02-3	2		

1	5,4'-dihydroxyflavone	6665-67-4	2		
1	5,6,7-trihydroxy- 4'-methoxyflavone	6563-66-2	1		
1	5,6-dihydroxy-3',4',7-trimethoxyflavon	25782-23-4	1		
1	5,7,2',3'-Tetrahydroxyflavone	74805-70-2	1		
1	5,7,4’-trihydroxy-6,8-dimethylflavone (Syzalterin)	94451-48-6	1		
1	5,5',7-trimethoxy-3',4' -methylenedioxyflavone	89029-11-8	1		
1	5-demethylnobiletin	2174-59-6	1		
1	5-demethyltangeretin (Gardenin B)	2798-20-1	1		
1	5-hydroxy-3,4′,7-trimethoxyflavone	29080-58-8	1		
1	5-hydroxy-3′,4′,6,7-tetramethoxyflavone	21763-80-4	1		
1	5–hydroxy-3′,4′,7-trimethoxy-flavone	29080-58-8	1		
1	5-hydroxy-7, 4'-dimethoxyflavone-5-O-α-D-arabinopyranosyl-(2""→1''')-O-α-D-rabinopyranosyl-2'''-O-3"", 7""-dimethylnonan-1""-oate	-	1		
1	5-hydroxy-7, 4'-dimethoxyflavone-5-O-β-D-arabinofuranosyl- (2""→1''')-O-β-D-arabinopyranosyl-2'''-O-lanost-5-ene	-	1		
1	5-hydroxy-7,4'-dimethoxy-8-methylflavone	14004-50-3	2		
1	5-Hydroxy-7,4′-dimethoxyflavone	5128-44-9	1		
1	5-Methoxyflavone	42079-78-7	1		
1	5,6,7,8,3′,4′,5′-heptamethoxyflavone (5'-Methoxynobiletin)	6965-36-2	4		
1	6-hydroxyflavone	6665-83-4	3		
1	6-methoxyflavone	26964-24-9	1		
1	6-Methoxyluteolin (Nepetin)	520-11-6	1		
1	6-methylflavone	29976-75-8	1		
1	7,4′-Dihydroxyflavone	2196-14-7	1		
1	7-aminoflavone	15847-18-4	1		
1	7-Hydroxy-3',4'-dimethoxyflavone	33513-36-9	1		
1	7-methoxyflavone	22395-22-8	1		
1	4′-Methylapigenin (Acacetin)	480-44-4	2		
1	acacetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside	-	1		
1	apigenin	520-36-5	19		
1	Apigenin 6,8-di-C-D-lucoside (vicenin-2)	23666-13-9	3		
1	apigenin 6-C[beta-D glucopyranosyl (1-->6) O-beta-D-glucoside] 8-C[beta-D-glucopyranosyl (1-->6) O-beta-D-glucoside]	-	1		
1	Apigenin-6-C-galactoside 8 -arabinoside	1983982-50-8	1		

1	Apigenin 6-C-glucoside-8-C-arabinoside (schaftoside)	51938-32-0	1		
1	apigenin 7-O glucoside	578-74-5	7		
1	apigenin 7-O-neohesperidoside (Rhoifolin)	17306-46-6	1		
1	apigenin 7-O-(6-p-coumaroyl)-glucoside	171367-93-4	1		
1	Apigenin 8-C-glucoside (Vitexin)	3681-93-4	4		
1	Apigenin 8-C-neohesperidoside (Vitexin-2”-O-rhamnoside)	64820-99-1	1		
1	Apigenin malonylglucoside	86546-87-4 or	1		
1	Apigenin-7-O-glucoronide	29741-09-1	1		
1	Apigenin-7-rutinoside (isorhoifolin)	552-57-8	2		
1	Baicalin	491-67-8	5		
1	chrysin	480-40-0	11		
1	Chrysoeriol 8-C-glucoside (Scoparin)	301-16-6	1		
1	cirsilineol	41365-32-6	1		
1	Cirsiliol	34334-69-5	3		
1	cirsimaritin	6601-62-3	3		
1	desmethoxy-centaureidin	22934-99-2	1		
1	diosmetin	520-34-3	2		
1	diosmin	520-34-4	1		
1	eucalyptin	3122-88-1	1		
1	eupalestin (5,6,7,8,3′-pentamethoxy-4′, 5′-methylenedioxyflavone)	73340-44-0	4		
1	eupatorin	855-96-9	1		
1	flavone	525-82-6	11		
1	flavone-7-O-[6-acyl]-glucoside	-	1		
1	Galangustin (3) (5,7-dihydroxy-4',8-dimethoxyflavone) (Bucegin)	65501-87-3	1		
1	gardenin A	21187-73-5	1		
1	Genkwanin	437-64-9	1		
1	glabratephrin (+)	75444-26-7	2		
1	Hispidulin (Dinatin)	1447-88-7	5		
1	hymenoxin	56003-01-1	1		
1	Hypolaetin	27696-41-9	1		
1	isoglabratephrin	1456799-69-1	2		
1	Isoscutellarein (4',5,7,8-Tetrahydroxyflavone)	41440-05-5	1		
1	isovitexin	38953-85-	2		
1	Isovitexin-7-O-beta-D-glucopyranoside (saponarin)	20310-89-8	1		
1	isowogonin	4431-47-4	1		

1	jaceosidin	18085-97-7	1		
1	lanceolatin-A	41689-78-5	2		
1	laxifolin	144049-81-0	1		
1	linarin	480-36-4	1		
1	Luteolin	491-70-3	24		
1	Luteolin 7-glucoside (cynaroside)	5373-11-5	12		
1	luteolin 7-O-rutinoside (Scolimoside)	20633-84-5	3		
1	luteolin-3′,4′-dimethylether-7-O-β-glucoside (5-Hydroxy-3′,4′-dimethoxyflavone 7-O-β-glucoside)	1006899-90-6	1		
1	luteolin-3'-methyl ether (Chrysoeriol)	491-71-4	4		
1	luteolin-3'-xyloside	93078-91-2	1		
1	luteolin-5-O-glucoside (Galuteolin)	20344-46-1	1		
1	Luteolin-6-C-glucoside (Isoorientin/Homoorientin)	4261-42-1	4		
1	Luteolin-7-O-(6"-malonylglucoside)	98767-38-5	1		
1	Luteolin-7-O-glucuronide	29741-10-4	3		
1	Luteolin-8-glucoside (orientin)	28608-75-5	1		
1	Luteolin 4′-O-glucoside	6920-38-3	1		
1	maysin	70255-49-1	2		
1	Maysin 3'-methyl ether (Methoxymaysin)	74158-05-7	1		
1	mosloflavone (5-Hydroxy-6,7-dimethoxyflavone)	740-33-0	1		
1	moslosooflavone	3570-62-5	1		
1	nobiletin (5,6,7,8,3',4'-hexamethoxyflavone)	478-01-3	3		
1	norwogonin	4443-09-8	1		
1	oroxylin A (5,7-Dihydroxy-6-methoxyflavone)	480-11-5	1		
1	pectolinaringenin	520-12-7	4		
1	primuletin (5-hydroxyflavone)	491-78-1	4		
1	Salcolin A	369390-51-2	2		
1	Salcolin B (Tricin 4'-O-(threo-beta-guaiacylglyceryl) ether)	369390-52-3	2		
1	salvigenin (5-Hydroxy-6,7,4′-trimethoxyflavone)	19103-54-9	1		
1	Scutellarein	529-53-4	2		
1	scutellarein-7-O-glucuronide (Scutellarin)	27740-01-8	2		
1	tangeretin (Ponkanetin/ pentamethoxyflavone/ 5,6,7,8,4'-pentamethoxyflavone)	481-53-8	4		
1	tephroapollin-F	1000210-12-7	2		
1	tetramethyl-o-scutellarin (4′,5,6,7-Tetramethoxyflavone)	1168-42-9	1		
1	tricin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone)	520-32-1	6		
1	Tricin 7-O-[2'-O-sinapoyl-β-D-glucuronopyranosyl-(1→2)-O-β -D-	-	1		

	glucuronopyranoside]				
1	Tricin 7-O-[2'-O-feruloyl-β-D-glucuronopyranosyl(1→2)-O-β -D-glucuronopyranoside]	-	1	Flavones	
1	Tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 7-O-β -glucopyranoside	-	1	total count	255
1	wogonin	632-85-9	1	total amount of different structures	110
2	3,3',5,7-Tetrahydroxyflavone	210560-14-8	1		
2	3,5,6,7,3',4'-hexamethoxyflavone	1251-84-9	3		
2	3,5,6,7,8,3',4'-Heptamethoxyflavone	1178-24-1	3		
2	3,5,6,7,8-pentahydroxyflavone	727388-91-2	1		
2	3,5-Dihydroxy-7,4'-dimethoxyflavone	15486-33-6	1		
2	3,6,7,3',4'-pentamethoxyflavone	74514-47-9	1		
2	3,7,4'-trihydroxy-5,6,8-trimethoxyflavone	2305336-23-4	1		
2	3,7-Dihydroxyflavone (7-Hydroxyflavonol)	492-00-2	5		
2	5,3'-dihydroxy-3,7,4'-trimethoxyflavone (ayanin)	572-32-7	1		
2	5,6,4'-trihydroxy-3,7,8-trimethoxyflavone	857823-60-0	1		
2	5,6-dihydroxy-3,7-dimethoxyflavone	73213-67-9	1		
2	5,7,4'-Trihydroxy-3,8-dimethoxyflavone (3,8-Dimethyl-herbacetin)	14965-09-4	1		
2	5-hydroxy-3,6,7,8,4'-pentamethoxyflavone	50439-46-8	1		
2	5-hydroxy-3,6,7,8-tetramethoxyflavone	15249-62-4	2		
2	5-hydroxy-3,7,4'-trimethoxy flavone (Kaempferol 3,7,4'-trimethyl ether/3,7,4'- trimethyl-quercetin)	15486-34-7	1		
2	6,3'-dihydroxy-3,5,7,4'-tetramethoxyflavone	154662-04-1	1		
2	6,4'-dihydroxy-3,7-dimethoxyflavone	1402703-55-2	1		
2	6,4'-dihydroxy-3,5,7-trimethoxyflavone	154662-03-0	1		
2	calycopterin	481-52-7	1		
2	casticin	479-91-4	2		
2	chrysosplenetin	603-56-5	1		
2	Datiscetin	480-15-9	1		
2	5,7-Dihydroxy-3,4'-dimethoxyflavone (Ermanin)	20869-95-8	2		
2	Eupalitin	29536-41-2	2		
2	3,7-dihydroxy 3′,4′-orthodihydroxy flavone (Fisetin)	528-48-3	3		
2	3-hydroxyflavone	577-85-5	3		
2	galangin	548-83-4	3		
2	Gossypetin-8-O-beta-D-glucopyranoside (Gossypin)	652-78-8	1		

2	Icariin	489-32-7	1		
2	isorhamnetin	480-19-3	5		
2	isorhamnetin-3-O-beta-D-(6''-acetyl)-galactopyranoside	-	1		
2	isorhamnetin-3-O-rutinoside	-	1		
2	isorhamnetin-3-O-sophoroside-7-O-rhamnoside	-	1		
2	isorhamnetin-3-O-β-D-glucopyranoside	-	1		
2	isorhamnetin-3-O-β-D-glucopyranosyl-β-D-glucopyranoside -7-O-glucosyl	-	1		
2	Isorhamnetin-3-sulfate	-	1		
2	kaempferide-3-O-β-D-arabino pyranosyl -(2''→1''')-O-β-D-arabinopyrnosyl- 2'''-O-3'''', 7''''-dimethylnonan-1''''-oate (5,7-dihydroxy-4'-methoxy-flavonol-3-O-β-D-arabino-pyranosyl -(2''→1''')-O-β-D-arabinopyrnosyl- 2'''-O-3'''', 7''''-dimethylnonan-1''''-oate)	-	1		
2	kaempferol (3,5,7,4'-tetrahydroxyflavone) (22)	520-18-3	29		
2	kaempferol-3,7-di-O-alpha-rhamnoside (kaempferitrin)	482-38-2	1		
2	5,7,4'-trihydroxy-3-methoxyflavone (kaempferol-3-methylether)	1592-70-7	1		
2	kaempferol-3-O-alpha-L-rhamnopyranosyl-(1→4)-O-alpha-L-rhamnopyranosyl-(1→6)-beta-D-glucopyranoside	-	1		
2	Kaempferol-3-O-beta-galactoside (Trifolin)	23627-87-4	2		
2	kaempferol-3-O-beta-glucopyranosyl(1 -> 2)-O-[alpha-rhamnopyranosyl(1 -> 6)]-beta-galactopyranoside-7-O-alpha-rhamnopyranoside	-	1		
2	kaempferol-3-O-beta-glucopyranosyl(1 -> 2)-O-[alpha-rhamnopyranosyl(1 -> 6)]-beta-glucopyranoside-7-O-alpha-rhamnopyranoside''	-	1		
2	kaempferol-3-O-glucoside	480-10-4	4		
2	Kaempferol-3-O-rutinoside (nicotiflorin)	17650-84-9	3		
2	Kaempferol-3-O-rutinoside-7-O-glucoside	34336-18-0	1		
2	kaempferol-3-O-beta-D-glucopyranosyl-7-O-a-L-rham- nopyranoside	-	1		
2	kaempferol-4'-O-rutinoside	1169835-58-8	1		
2	kaempferol-6-O-[(2E)-3-(4-hydroxyphenyl)prop-2-enoyl]-beta-D-glucopyranoside (tiliroside)	20316-62-5	2		
2	kaempferol-3-O-gentiobioside	22149-35-5	1		

2	kumatakenin (jaranol/5,4'-dihy-droxy-3,7-dimethoxyflavone)	3301-49-3	3		
2	meliternatin	479-78-7	1		
2	morin	480-16-3	4		
2	Myricetin	529-44-2	10		
2	Myricetin-3-O-galactoside	15648-86-9	3		
2	myricetrin-3-O-alpha-L-rhamnoside (myricetrin)	17912-87-7	7		
2	3-Hydroxy-3',4',5,6,7,8-hexamethoxyflavone (natsudaidain)	35154-55-3	1		
2	pachypodol (5,4′-dihydroxy-3,7,3′- trimethoxyflavone)	33708-72-4	1		
2	penduletin (5,4'-dihydroxy-3,6,7-trimethoxyflavone)	569-80-2	1		
2	Pronephrone D	1220519-69-6	1		
2	quercetin	117-39-5	52		
2	Quercetin-3-D-xyloside (Reynoutrin)	549-32-6	1		
2	Quercetin-3-galactoside (hyperoside)	482-36-0	3		
2	quercetin-3-glucoside (not specified)	-	1		
2	quercetin-3-O-(6”-O-trans-p-coumaroyl)- β-D-glucopyranoside	-	1		
2	quercetin-3-O-alpha-D-glucopyranoside	-	1		
2	quercetin-3-O-alpha-L-rhamno-pyranosyl-(1→2)-beta-D-glucopyranoside	-	1		
2	quercetin-3-O-alpha-L-rham-nopyranosyl-(1→4)-O-alpha-L-rhamnopyranosyl-(1→2)-beta-D-glucopyranoside	-	1		
2	Quercetin-3-O-arabinoglucoside (peltatoside)	23284-18-6	1		
2	Quercetin-3-O-beta-arabinofuranose (Guaijaverin)	22255-13-6	1		
2	quercetin-3-O-beta-D-glucoside (isoquercetrin)	482-35-9	10		
2	quercetin-3-O-glucopyranosyl-(1 2)-D-glucopyranoside	-	1		
2	quercetin-3-O-glucuronide (miquelianin)	22688-79-5	2		
2	Quercetin-3-O-robinobioside	52525-35-6	1		
2	Quercetin-3-rutinoside (rutin)	153-18-4	45		
2	quercetin-7-O-galloyl-glucoside	231289-25-1	1		
2	quercetin-7-β-O-diglucoside	-	1		
2	quercitrin (Quercetin 3-rhamnoside) (4)	522-12-3	13		
2	retusin (5-hydroxy-3,7,3′,4′-tetramethoxyflavone)	1245-15-4	2		
2	rhamnetin	90-19-7	1		
2	Robinetin	490-31-3	1	Flavonols	
2	syringetin-3-O-galactoside	55025-56-4	1	total count	283
2	tambulin	571-72-2	1	total amount of different structures	84

3	(2S)-5′-(2-methylbut-3-en-2-yl)-8-(3-methylbut-2-en-1-yl)-5,7,2′,4′-tetrahydroxyflavanone	460345-19-1	1		
3	5,6,7,4'-tetrahydroxyflavanone	479-54-9	1		
3	5,7-dihydroxy-6,8-dimethylflavanone	82297-99-2	1		
3	5,7-dihydroxy-6-methylflavanone	11023-71-5	1		
3	5-hydroxy-7-methoxy-6-methylflavanone	55743-20-9	1		
3	alpinetin	36052-37-6	1		
3	Dihydrooroxylin A	18956-18-8	1		
3	dihydrowogonin	4431-41-8	1		
3	eriocitrin (Eriodictyol-7-O-rutinoside)	13463-28-0	1		
3	Eriodictyol	552-58-9	2		
3	Flavanone (4)	487-26-3	6		
3	heliannone B	193411-11-9	1		
3	heliannone C	193411-12-0	1		
3	hesperetin	520-33-2	1		
3	hesperetin-7-O-alpha-L-rhamnopryanoside-2-beta-D-glucopyranoside (Neohesperidin)	13241-33-3	1		
3	hesperetin-7-O-alpha-rhamnopyranoside-6-O-beta-D-glucopyranoside (hesperidin)	520-26-3	4		
3	homohesperetin	89294-54-2	1		
3	homohesperetin-7-rutinoside	-	1		
3	liquiritigenin (S)	578-86-9	1		
3	malheuran A	1607438-29-8	1		
3	malheuran B	1607438-30-1	1		
3	malheuran C	1607438-31-2	1		
3	malheuran D	1607438-32-3	1		
3	naringenin (4',5,7-trihydroxyflavanone)	480-41-1	14		
3	naringenin 6,8-di-C-glucoside	81446-26-6	1		
3	naringenin-3-O-glucoside (b-D-glucopyranose)	-	1		
3	naringenin trimethyl ether (5,7,4')	38302-15-7	1		
3	naringenin-4'-methoxy-7-pyranoside	-	1		
3	naringenin-7-O-alpha-L-rhamnopryanoside-2-beta-D-glucopyranoside (naringin)	10236-47-2	6		
3	nubatin	873799-84-9	1		
3	nymphaeol C (-)	73676-36-5	1		
3	pinocembrin (S) (2, 3-dihydro-5, 7-dihydroxy-2-phenyl-4H-1-Benzopyran-4-one/dihydrochrysin))	480-39-7	6		
3	pinostrobin	480-37-5	2		
3	pinocembroside	75829-43-5	1		
3	Pronephrone B	1220519-72-1	1		

3	prostratol F	162382-66-3	1	Flavanones	
3	tanariflavanone A	352276-80-3	1	total count	71
3	tanariflavanone B	352276-81-4	1	total amount of different structures	38
4	(2R,3R)-(+)-4',5,7-rimethoxydihydroflavonol (2) (syn=(2R,3R)-3-Hydroxy-5,7-dimethoxy-2-(4-methoxyphenyl)-2,3-dihydro-4H-chromen-4-one))	76792-94-4	1		
4	6-(p-hydroxybenzyl)taxifolin-7-O-D-glucoside (tricuspid)	936637-41-1	1		
4	dihydrokaempferol Aromadendrin)	480-20-6	2		
4	dihydromyricetin (1)	27200-12-0	1		
4	pinobanksin (3, 5, 7-trihydroxy-2-phenyl-4H-1-Benzopyran-4-one)	548-82-3	2	Flavanonols	
4	taxifolin (Dihydroquercetin)	480-18-2	8	total count	17
4	Taxifolin 3-O-rhamnoside (Astilbin)	29838-67-3	2	total amount of different structures	7
5	(2R, 3S)-7,4′-dimethoxy-2′-hydroxyflavanol	2211189-87-4	1		
5	Catechin (both steromers)	154-23-4	18		
5	epicatechin (both steromers)	490-46-0	8		
5	epicatechin gallate	1257-08-5	2		
5	epicatechin(-)-3-O—glucopyranoside (1)	103303-00-0	1		
5	(-)-Epiafzelechin	24808-04-6	1		
5	Epigallocatechin (-)	970-74-1	1	Flavanols	
5	epigallocatechin 3,5-digallate	37484-73-4	1	total count	34
5	epigallochatechin gallate	989-51-5	1	total amount of different structures	9
6	5-hydroxy-3-(4-hydroxyphenyl)pyrano[3,2-g]chromene-4(8H)-one	-	1		
6	7-hydroxy-8,4'-dimethoxyisoflavone (Isoafrormosin)	37816-20-9	1		
6	biochanin A	491-80-5	4		
6	daidzein (4', 7-dihydroxyisoflavone)	486-66-8	4		
6	daidzein-8-C-glycoside (Puerarin)	3681-99-0	1		
6	Formononetin	485-72-3	4		
6	genistein (9)	446-72-0	11		
6	Genistein 7-O-rutinoside (sphaerobioside)	14988-20-6	1		
6	griffonianone E	874340-42-8	1		
6	irilin A	132915-49-2	1		
6	irilin B	132915-50-5	1		
6	irilone	41653-81-0	1		
6	isoflavone	574-12-9	1		
6	lupalbigenin	76754-24-0	1		
6	millewanin G	874303-33-0	1		
6	millewanin H	874303-34-1	1		
6	Orobol (Isoluteolin)	480-23-9	1		
6	osajin	482-53-1	1		

6	scandinone	5233-97-6	1	Isoflavone	
6	7-isopropoxyisoflavone (ipriflavone)	35212-22-7	1	total count	40
6	Warangalone (scandenone (1))	4449-55-2	1	total amount of different structures	21
7	5,7-methoxyneochaejasmin A	-	1		
7	chamaejasmenin C	89595-70-0	1		
7	chamaejasmine (+)	69618-96-8	1		
7	isosikokianin A	1070995-17-3	1		
7	ruixianglangdusu B	447454-49-1	1		
7	epigallocatechin(+)-(2β-O-7, 4β-8)-(+)-catechin (proanthocyanidin)	-	1		
7	epicatechin-(4β-->8)-epicatechin-(4β-->8,2β-->O -->7) (proanthocyanidin)	-	1		
7	Procyanidin B3 (3)	23567-23-9	1	Conjugated Flavonoids	
7	Prodelphinidin C (4)	78392-25-3	1	total count	10
7	Pronephrone C	1220519-70-9	1	total amount of different structures	10
				anthocyanidin	
8	delphinidin	13270-61-6	1	total count	2
8	Petunidin-3-O-glucoside	-	1	total amount of different structures	2
				All groups	
				total count	712
				total amount of different structures	281

Publication 2

Lena Schnarr, Oliver Olsson, Sonia Ohls, Jolanda Webersinn, Tim Mauch, Klaus Kümmerer

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

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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

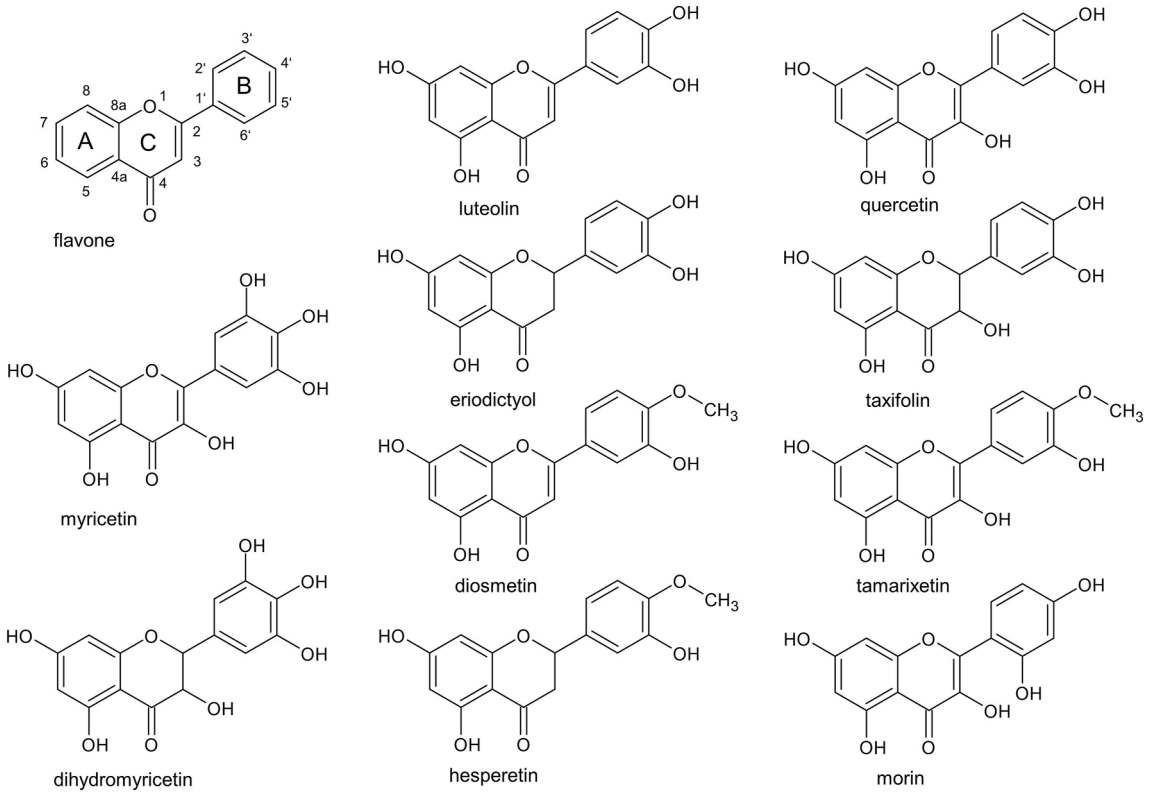


Fig. 1. Structures of the 11 flavonoids selected for a detailed analysis after algae growth inhibition screening of 26 flavonoids in this study. Selected flavonoids differ in the number of hydroxy groups, C2–C3 bond order, presence of a hydroxy group at C3 (3-OH), methoxy groups in the B ring, and positioning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

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

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

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

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.

$$EC_{50}\ \text{additive} = \text{portion A}/EC_{50}\ \text{A} + \text{portion B} / EC_{50}\ \text{B} \quad (4)$$

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-

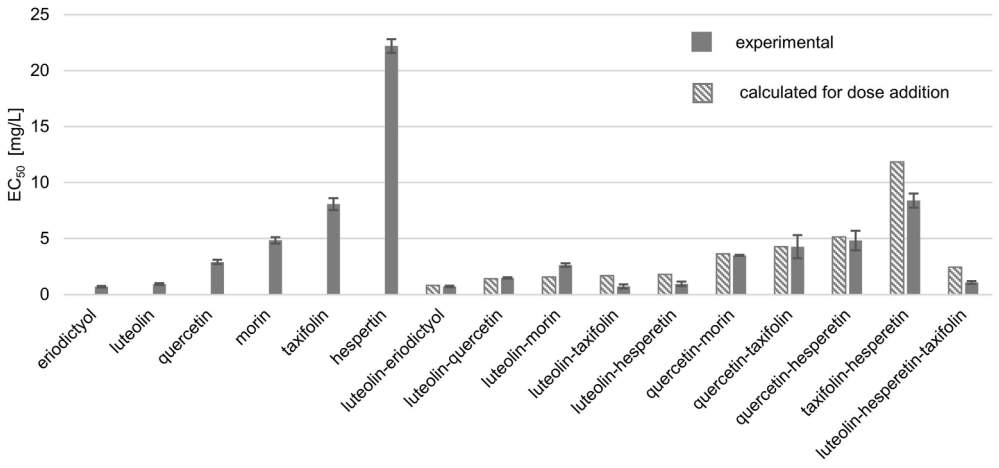


Fig. 2. Comparison of experimentally derived EC_{50} values of single compounds and mixtures of two to three different flavonoids (grey) to theoretical, calculated EC_{50} values of mixtures assuming an additive effect (striped). Average values with standard deviation displayed as error bars (SD, n =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

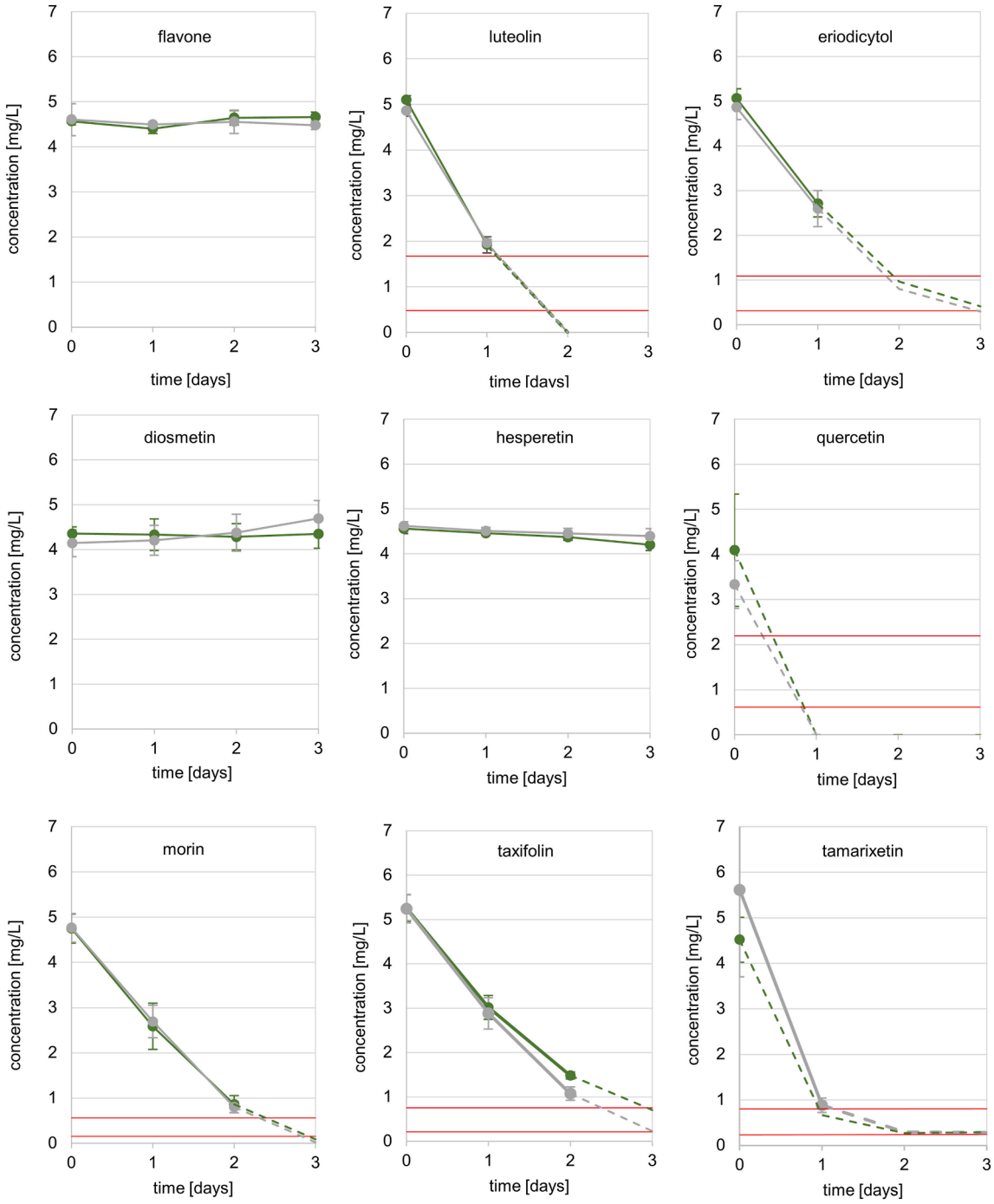


Fig. 3. Concentration development of the flavonoids flavone, luteolin, eriodictyol, diosmetin, hesperetin, quercetin, morin, taxifolin und tamarixetin during the algae growth inhibition test. Samples with algae (green), controls without algae (grey). Start concentration 5 mg/L. Data points represent 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.

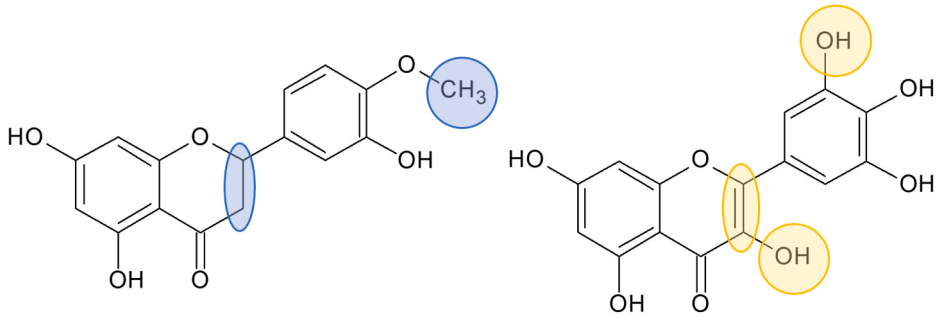


Fig. 4. Proposed general rules describing the influence of structural features on the degradation rate of flavonoids at the conditions of the algae growth inhibition test. Structural features highlighted in blue decrease degradation rates (methoxy groups, C2–C3 single bond). Structural features highlighted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

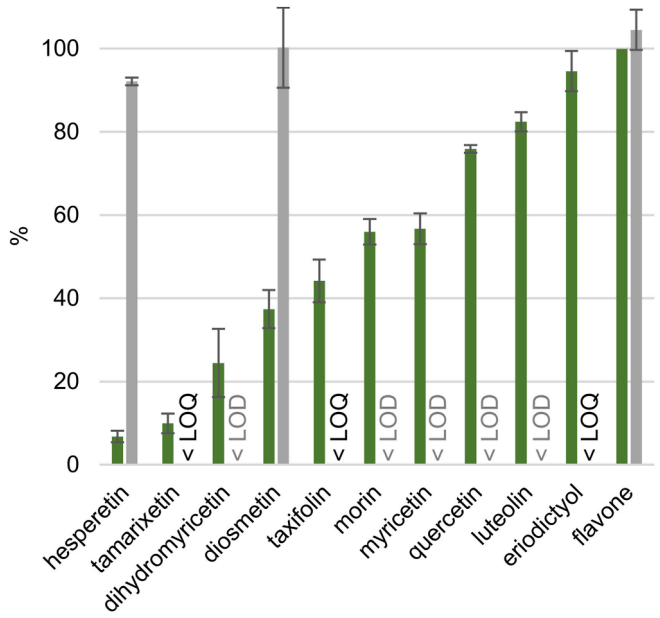


Fig. 5. Comparison of % algae growth inhibition (green) and % remaining concentration (grey) of the 11 selected flavonoids after 3 days. Starting concentration of flavonoids = 5 mg/L. Data points represent average values with standard deviation displayed as error bars (n = 4). Concentrations of tamarixetin, 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 ($\leq$ 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 C15H11O7 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.

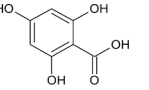
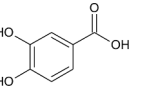
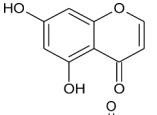
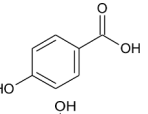
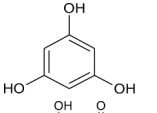
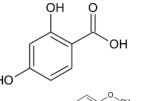
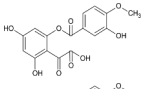
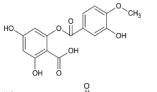
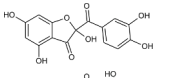
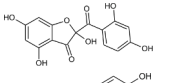
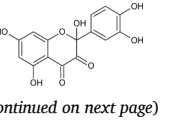
#	Name	RT [MIN]	M/Z	Formula [M − -H]	Fragmentation**M/Z AND (FORMULA)**	Myricetin	Dihydro-myricetin	Quercetin	Morin	Taxifolin	Tama-rixetin	Luteolin	Eriodictyol	Proposed structure
1	2,4,6-Trihydroxybenzoic acid*	7.8	169.0137	C7H5O5	151 (C7H3O4), 125 (C6H5O3)	1	1	1	1	1	1	1[a]	1[a]	
2	3,4-Dihydroxy-benzoic 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*)

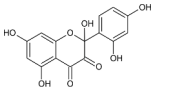
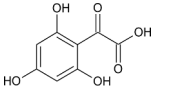
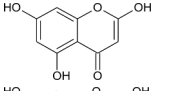
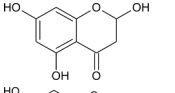
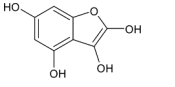
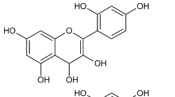
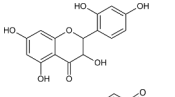
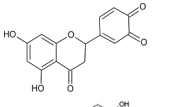
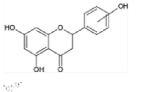
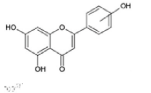
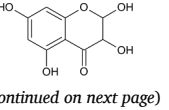
#	Name	RT [MIN]	M/Z	Formula [M − -H]	Fragmentation**M/Z AND (FORMULA)**	Myricetin	Dihydro-myricetin	Quercetin	Morin	Taxifolin	Tama-rixetin	Luteolin	Eriodictyol	Proposed structure
12	TP-317.4	7.9	317.0298	C15H9O8	—	n.d.	n.d.	n.d.	3	n.d.	n.d.	n.d.	n.d.	
13	TP-197	5.8	197.0082	C8H5O6	153, 151, 125	2	2	2	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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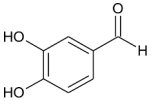
Table 2 (*continued*)

#	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.	

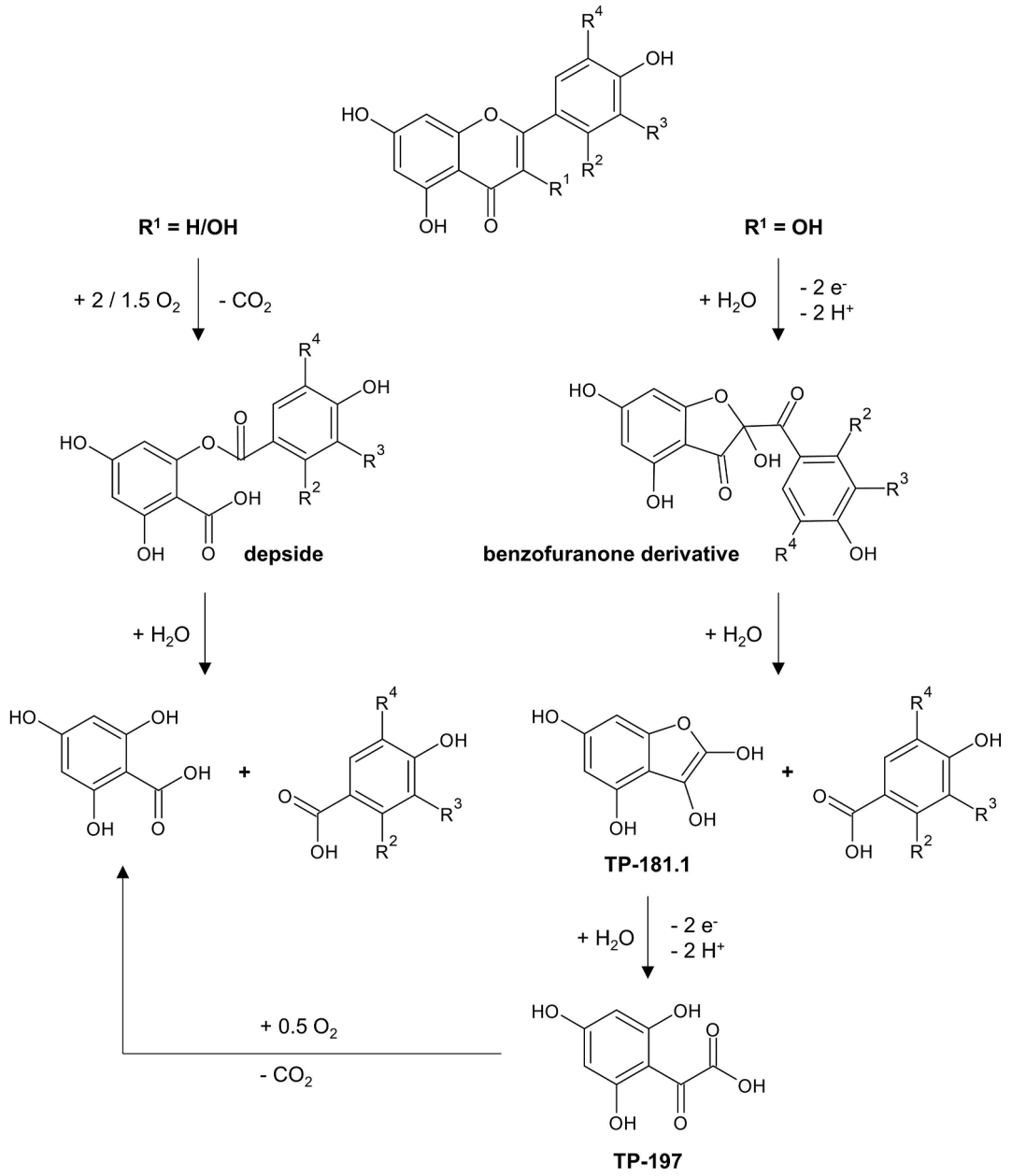


Fig. 6. Pathway of oxidative degradation of flavonols and flavones either via a depside or a benzofuranone derivative to benzoic acid derivatives based on the pathway suggested by Sokolová et al., (2016).

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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Supporting Information of Publication 2

Lena Schnarr, Oliver Olsson, Sonia Ohls, Jolanda Webersinn, Tim Mauch, Klaus Kümmerer

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

Sustainable Chemistry and Pharmacy, 38, 101473 (2024)

Online available at:

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Material and Methods – additional information

Chemicals, Solvents and OECD Algae Culture Medium

List of purchased flavonoids, their CAS numbers, vendors, and purity.

substance	CAS number	vendor	purity
flavone	525-82-6	Sigma Aldrich	>99%
3-hydoxyflavone	577-85-5	abcr chemie	98%
7-hydroxyflavone	6665-86-7	abcr chemie	98%
5-hydroxyflavone (primuletin)	491-78-1	alfa aeser	≥97%
chrysin (5,7-dihydroxyflavone)	480-40-0	abcr chemie	98%
7,4'-dihydroxyflavone	2196-14-7	abcr chemie	97%
3,5-dihydroxyflavone	6665-69-6	abcr chemie	98%
7,3',4'-trihydroxyflavone	2150-11-0	abcr chemie	98%
apigenin	520-36-5	TCI Deutschland GmbH	98%
naringenin	67604-48-2	alfa aeser	≥97%
acacetin	480-44-4	TCI Deutschland GmbH	98%
fisetin	528-48-3	abcr chemie	98%
luteolin	491-70-3	abcr chemie	97%
eriodictyol	552-58-9	ACROS	≥94%
diosmetin	520-34-3	Sigma-Aldrich	≥98%
hesperetin	520-33-2	abcr chemie	97%
morin hydrate (morin)	654055-01-3 (480-16-0)	Sigma Aldrich	not specified
quercetin	117-39-5	Sigma Aldrich	≥95%
taxifolin	480-18-2		95%
tamaritexin	603-61-2	abcr chemie	99%
myricetin	529-44-2	TCI Deutschland GmbH	>97%

dihydromyricetin	27200-12-0	abcr chemie	95%
gossypetin	489-35-0	abcr chemie	not specified
genistein	446-72-0	TCI Deutschland GmbH	98%
formononetin	485-72-3	Sigma Aldrich	>99%
daidzein	486-66-8	Cayman Chemical	>95%
3-hydroxybenzoic acid	99-06-9	TCI Deutschland GmbH	> 99%
4-hydroxybenzoic acid	99-96-7	TCI Deutschland GmbH	> 99%
3,4-dihydroxybenzoic acid	99-50-3	TCI Deutschland GmbH	> 98%
2,4,6-trihydroxybenzoic acid	83-30-7	TCI Deutschland GmbH	not specified
5,7-dihydroxychromone	31721-94-5	abcr chemie	not specified
phloroglucinol	108-73-6	Sigma Aldrich	> 99%
3,5-diphlorophenol	591-35-5	Sigma Aldrich	97%
terbutryn	886-50-0	Sigma Aldrich	analytical standard
diuron	330-54-1	Sigma Aldrich	analytical standard
DMSO		Sigma Aldrich	
methanol		VWR	LC-MS grade

Composition of the OECD medium and its stock solutions.

stock solutions	**nutrient**	**concentration in stock solution**	**final concentration in medium**
Stock solution 1: macro nutrients	NH_4Cl	1.5 g/l	15 mg/l
	$MgCl * 6H_2O$	1.2 g/l	12 mg/l
	$CaCl_2 * 2H_2O$	1.8 g/l	18 mg/l
	$MgSO_4 * 7H_2O$	1.5 g/l	15 mg/l
	KH_2PO_4	0.16 g/l	1.6 mg/l

Stock solution 2: **Fe-EDTA**	$FeCl_3 * 6H_2O$ $NaEDTA * 2H_2O$	64 mg/l 100 mg/l	64 µg/l 100 µg/l
Stock solution 3: **trace elements**	H_3BO_3 $MnCl_2 * 4H_2O$ $ZnCl_2$ $CoCl_2 * 6H_2O$ $CuCl_2 * 2H_2O$ $Na_2MoO_4 * 2H_2O$	185 mg/l 415 mg/l 3 mg/l 1.5 mg/l 0.01 mg/l 7 mg/l[b]	185 µg/l 415 µg/l 3 µg/l 1.5 µg/l 0,01 µg/l 7 µg/l
Stock solution 4: **bicarbonate**	$NaHCO_3$	50 g/l	50 mg/l

Concentration determination with HPLC-UV/vis

Quantification wavelength and limit of detection and quantification of the 9 selected, quantifiable flavonoids analyzed with HPLC-UV/vis.

Flavonoid	**Wavelength for quantification [nm]**	**Limit of detection [mg/L]**	**Limit of quantification [mg/L]**
Luteolin	350	0.48	1.67
Morin	350	0.16	0.56
Taxifolin	290	0.21	0.75
Hesperetin	290	0.24	0.86
Tamarixetin	370	0.23	0.80
Diosmetin	350	0.19	0.69

Eriodictyol	290	0.31	1.09
Quercetin	370	0.61	2.20
Flavone	300	0.35	1.21

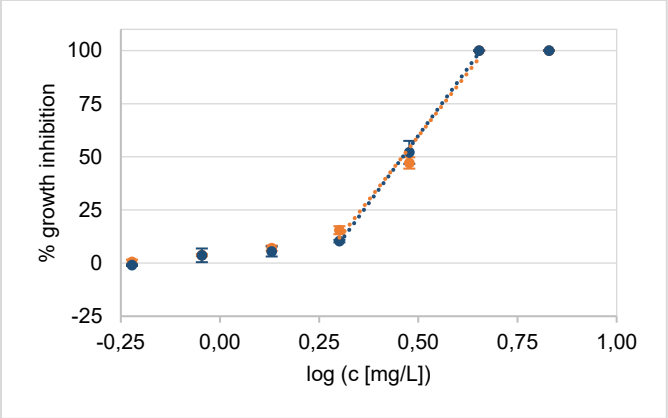
ESI-MS/MS Parameters

Settings of ESI source and mass spectrometer.

sheath gas flow	80
aux gas flow	35
sweep gas flow	3
spray voltage (positive mode)	4 kV
spray voltage (negative mode)	3.5 kV
capillary temperature	260 °C
aux gas heater temperature	390 °C
scan range	50 to 750 m/z
collision energy (stepped)	10, 50, 100

Results – additional information

Validation of algae growth inhibition test in 24 well plates

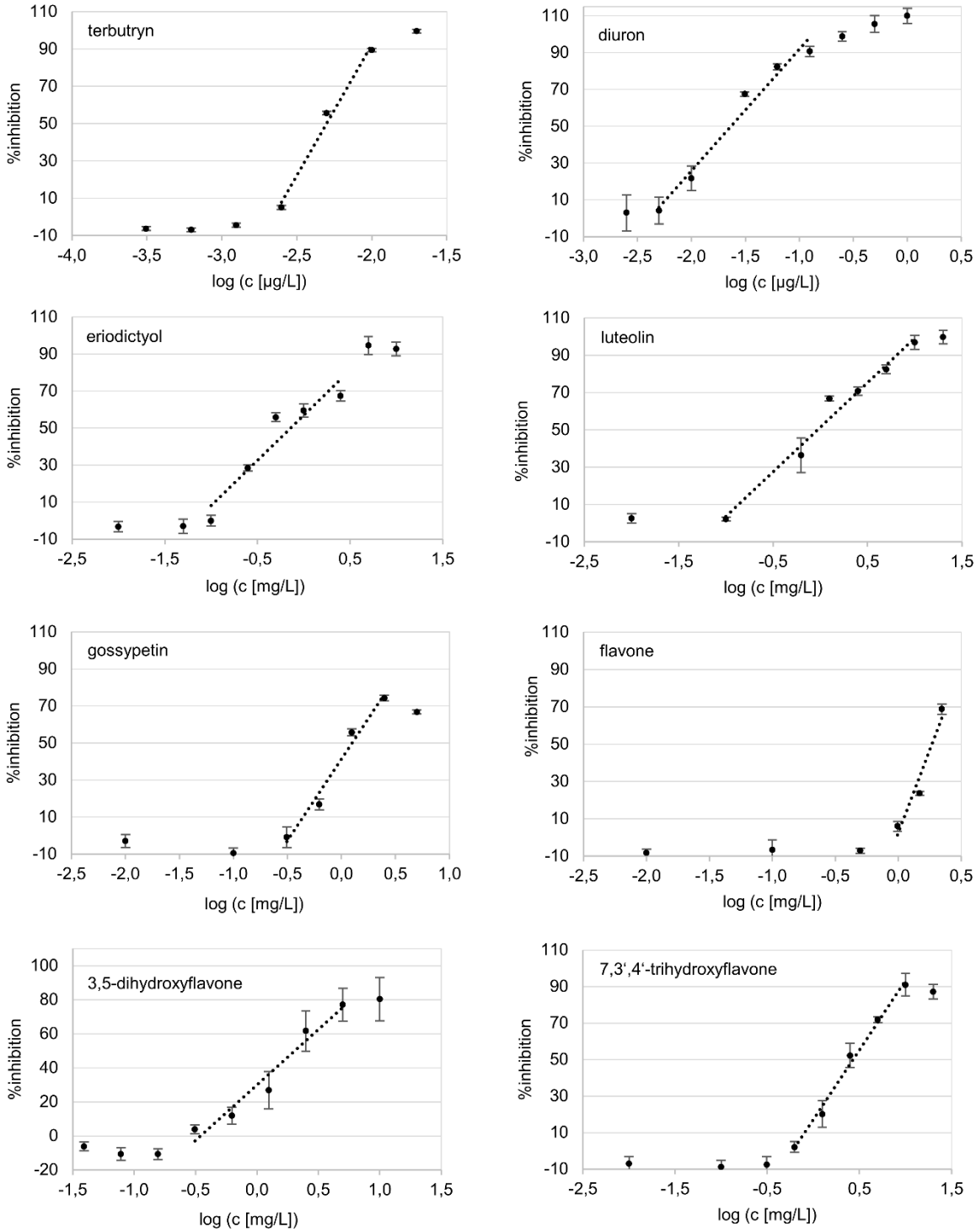


Comparison of dose-response curves for 3,5-dichlorophenol derived using either 250 mL Erlenmeyerflasks (blue, n=3) or 24 well-plates (orange, n=4)). For the highest two concentrations, cell density was below limit of quantification and consequently inhibition was set to 100%. Average values with standard deviation (SD) displayed as error bars are given. Dotted lines represent linear regression used to calculate EC_{50} values.

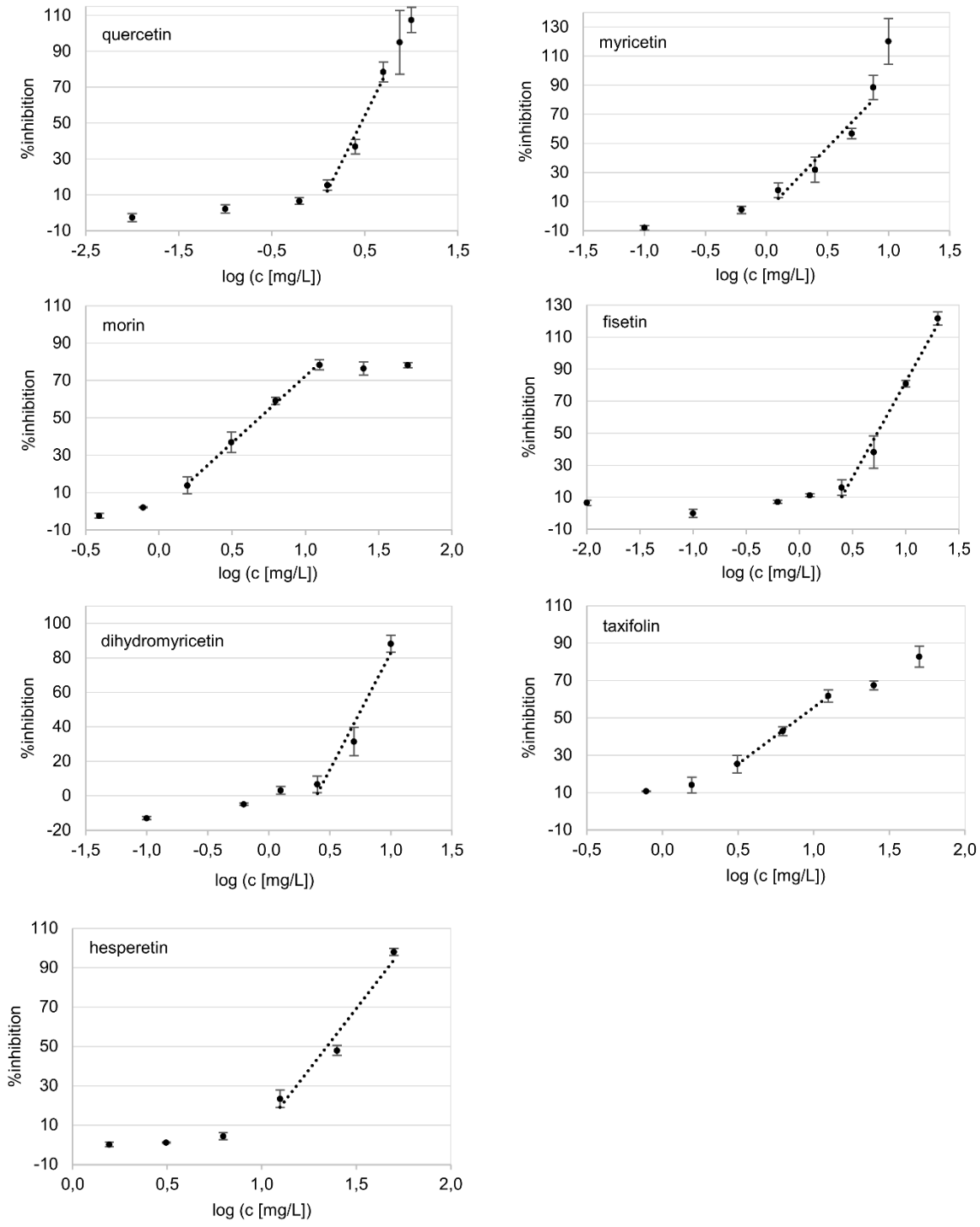
Comparison of EC_{50} values for 3,5-dichlorophenol derived using either 250 mL Erlenmeyerflasks (n=3) or 24 well-plates (n=4). Average values of replicates and standard deviation (SD) are given.

	test vessel	
	250 mL Erlenmeyer flasks	24 well plates
EC_{50} [mg/L]	2.89 ± 0.04	2.88 ± 0.01

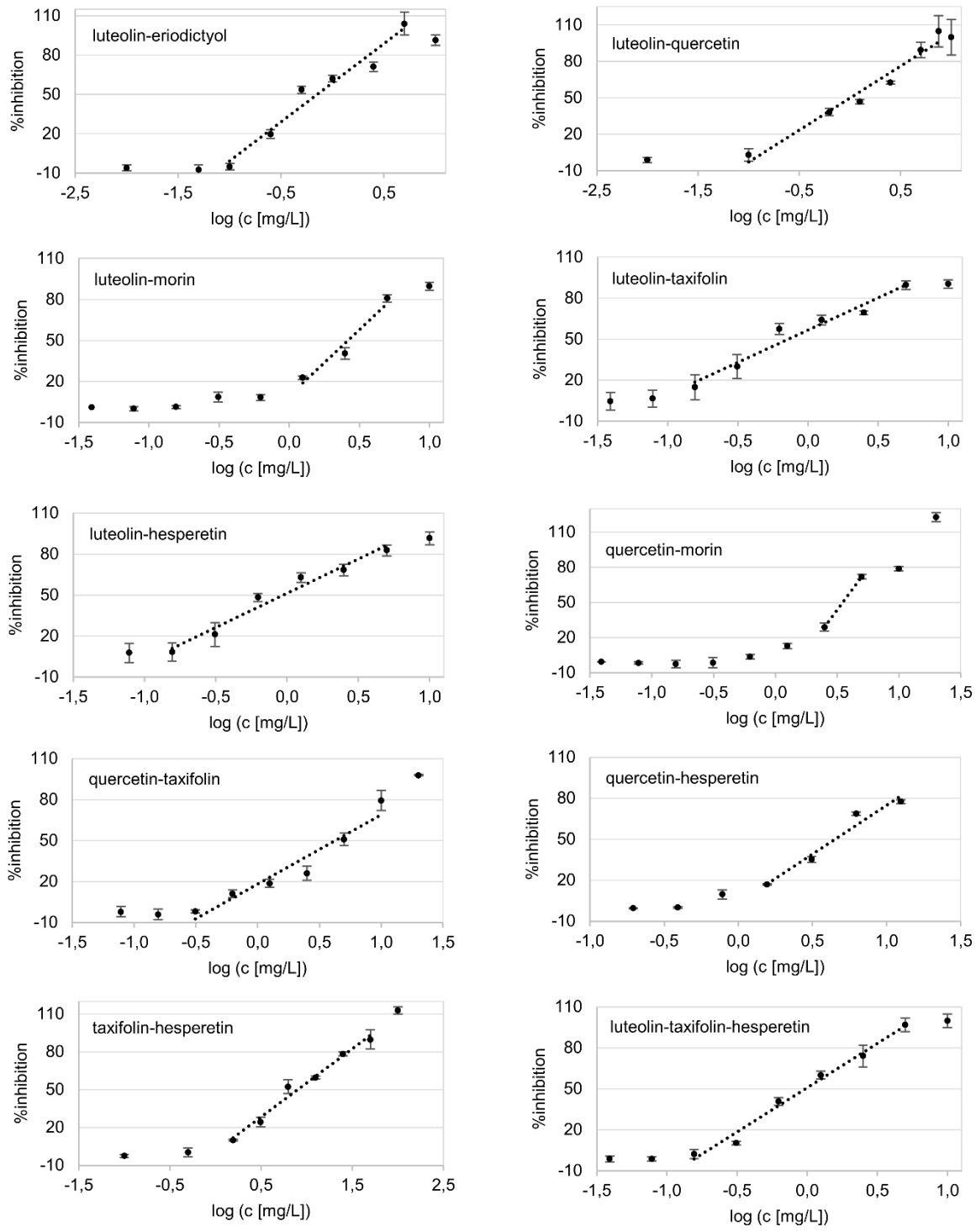
Dose-response curves of flavonoids and additional information on mixtures



Dose-response curves of the two algaecides terbutryn and diuron and 6 flavonoids. Data points represent average values (n=4) with standard deviations (SD) displayed as error bars. Dotted lines represent the linear regression used for EC_{50} value determination.



Dose response curves of 7 flavonoids. Data points represent average values (n=4) with standard deviations (SD) displayed as error bars. Dotted lines represent the linear regression used for EC_{50} value determination.

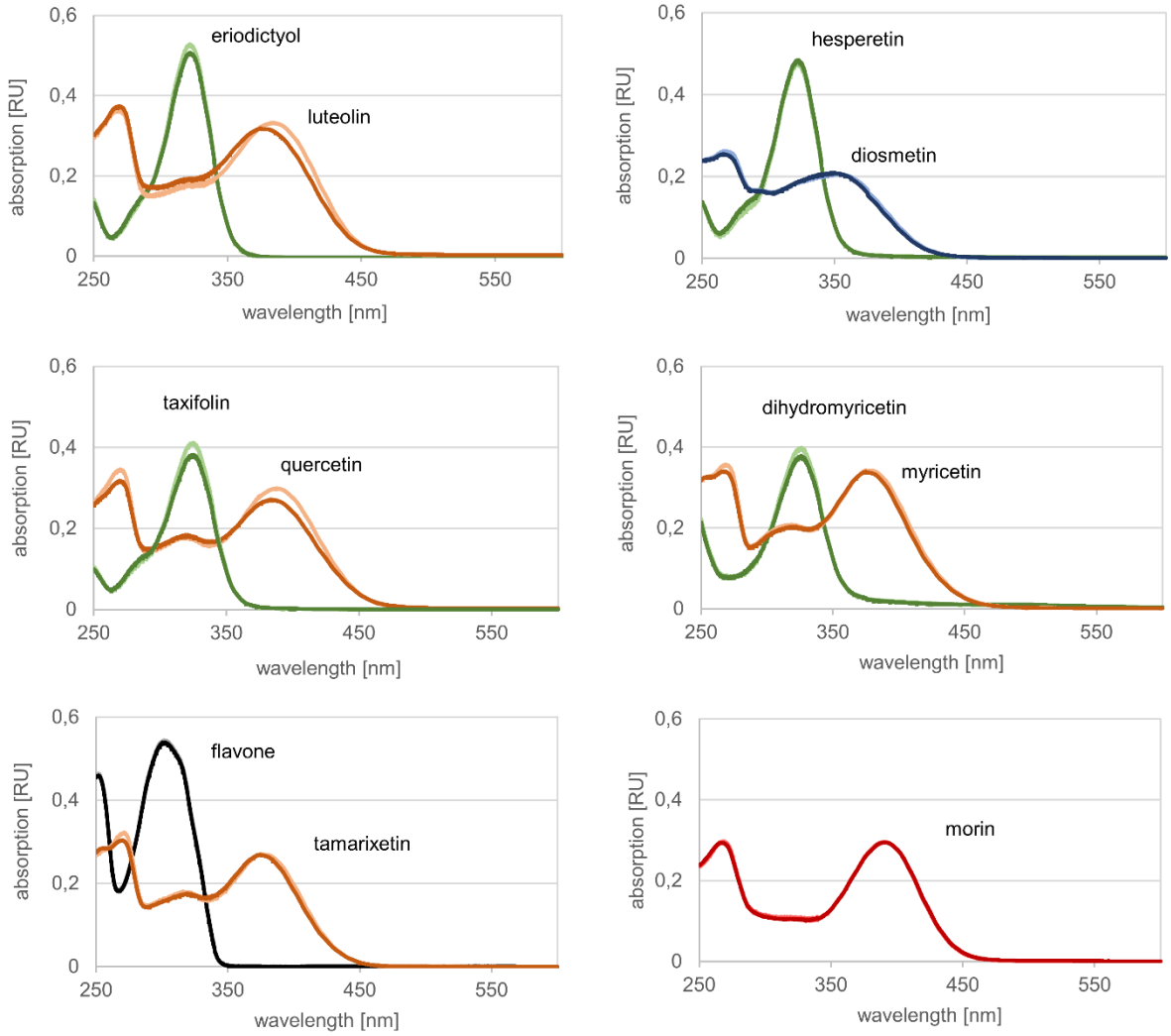


Dose-response curves of 10 flavonoid mixtures. Data points represent average values (n=4) with standard deviations (SD) displayed as error bars. Dotted lines represent the linear regression used for EC_{50} value determination.

Overview of theoretical and experimentally derived EC_{50} values of flavonoid mixtures (n=4). Average values with standard deviation (SD, n=4) of experimentally derived results are given.

mixture	**EC_{50} in mg/L (calculated based on dose addition)**	**EC_{50} in mg/L (experimentally derived)**
luteolin-eriodictyol	0.81	0.72 ± 0.07
luteolin-quercetin	1.42	1.10 ± 0.09
luteolin-morin	1.57	2.64 ± 0.16
luteolin-taxifolin	1.68	0.73 ± 0.18
luteolin-hesperetin	1.8	0.95 ± 0.21
quercetin-morin	3.63	3.50 ± 0.5
quercetin-taxifolin	4.28	4.5 ± 1
quercetin-hesperetin	5.15	4.0 ± 0.7
taxifolin-hesperetin	11.85	8.40 ± 0.63
luteolin-hesperetin-taxifolin	2.45	1.08 ± 0.12

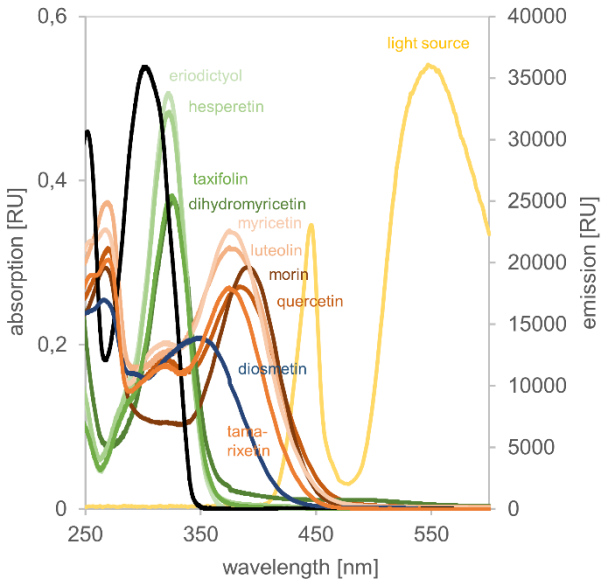
Spectral characterization of flavonoids and incubator light source



Comparison of absorption spectra of 11 selected flavonoids in algae culture medium (darker color shade) and sodium carbonate puffer (lighter color shade). First four graphs show compound pairs that only differ in C2-C3 saturation.

Comparison of the absorption spectra of flavonoids solved in algae culture medium and an equivalent buffer solution without metal ions except sodium (595 µM) revealed that metal ions which are constituents of the algae culture medium like Ca^{2+} (122 µM), Mg^{2+} (59 µM), Fe^{3+} (0.2 µM), Cu^{2+} (6×10^{-5} µM) had no influence on the absorption spectra (SI **Fehler! Verweisquelle konnte nicht gefunden werden.**). Metal complexation was reported to result in a bathochromic shift of the absorption maxima[1,2]. Therefore, our results indicated that at the

conditions of the algae growth inhibition test no metal complexes were formed. Former algae growth inhibition studies did not investigate if metal complexes with the tested flavonoids occurred.



Comparison of absorption spectra of 11 flavonoids to the emission spectrum of the algae incubator light source.

Comparison to the emission spectrum of the light source of the algae incubator to the absorption spectra of flavonoids showed no overlap with the spectra of eriodictyol, hesperetin, taxifolin, dihydromyrcetin, and flavone, while minor overlap with the spectra of luteolin, quercetin, tamarixetin, myricetin, morin and diosmetin in a range of 410 to 460 nm occurred (SI **Fehler! Verweisquelle konnte nicht gefunden werden.**). Therefore, direct photolysis of eriodictyol, hesperetin, taxifolin, dihydromyricetin and flavone can be excluded. The other flavonoids luteolin, quercetin, tamarixetin, myricetin, morin and diosmetin may be subjected to photolysis at the algae growth inhibition test conditions. However, studies that observed photolysis of flavonoids mostly used light sources with emission spectra ranging from 290 to 400 nm[3–5]. Only Chaaban et al. used a climate cell with lighting from 390 to 750 nm[6]. The type of information given on irradiation intensity differs. Thus comparison is hindered[3–6]. Moreover, the algae cells present in the algae growth inhibition test also absorb light and may protect the flavonoids from irradiation. Due to the minimal spectral overlap, low photon flux, probably low

exposure to irradiation, we assume that direct photolysis has a negligible impact on the tested flavonoids during the algae growth inhibition test and auto-oxidation is the main cause of possible degradation.

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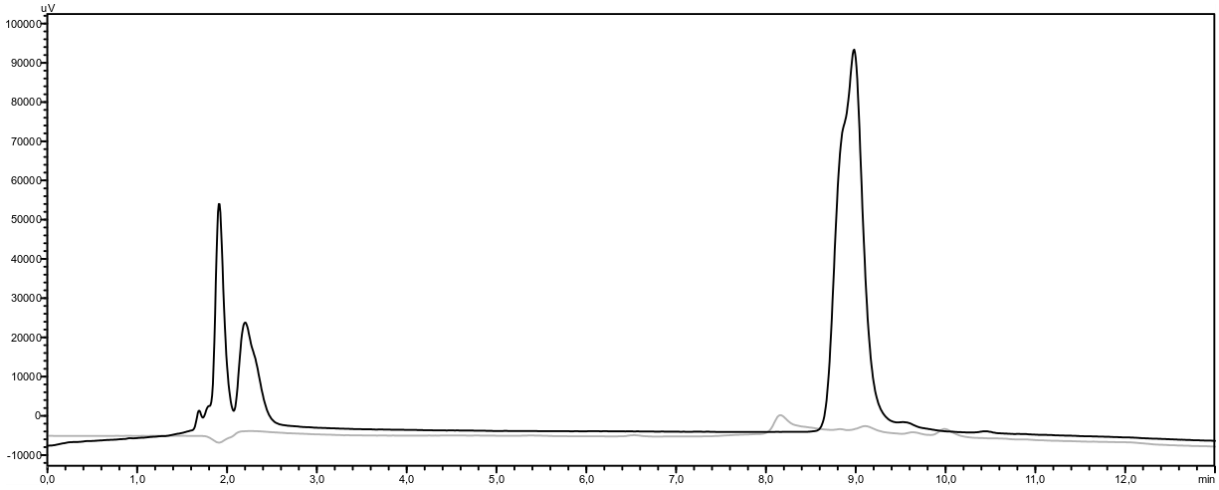
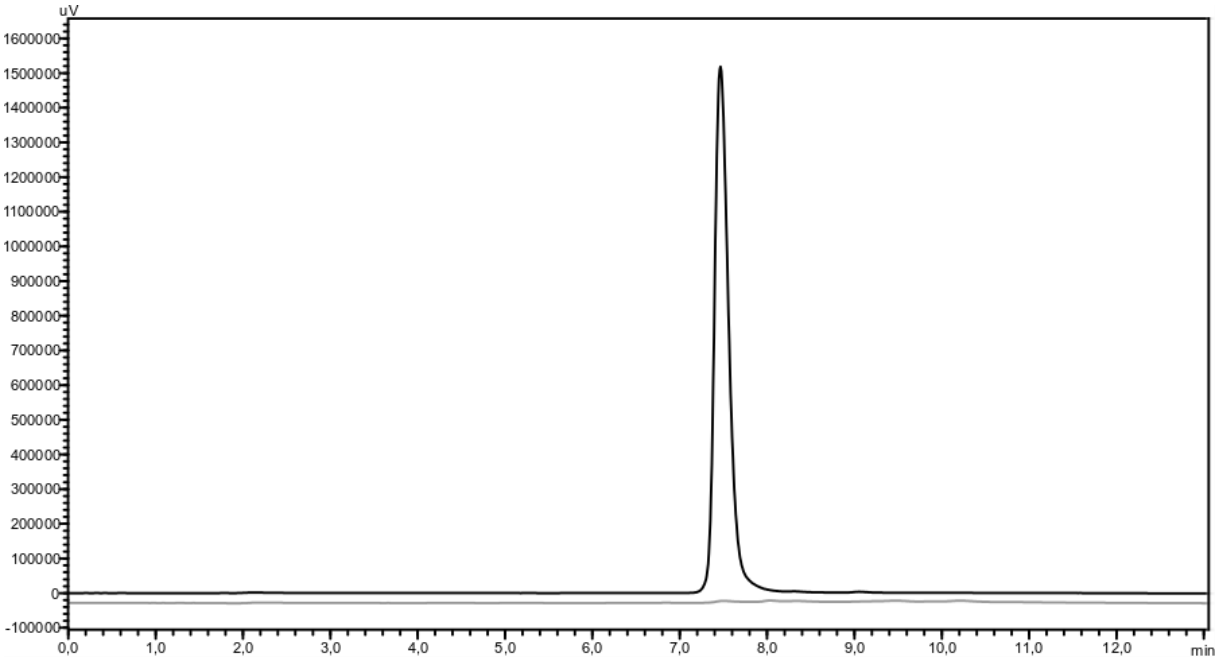
Degradation of Myricetin and Dihydromyricetin



Representative chromatograms (370 nm) of a myricetin standard in DMSO (black) and a myricetin sample at day 0 of the algae growth inhibition test (gray). Standard and sample had an initial concentration of 5 mg/L.

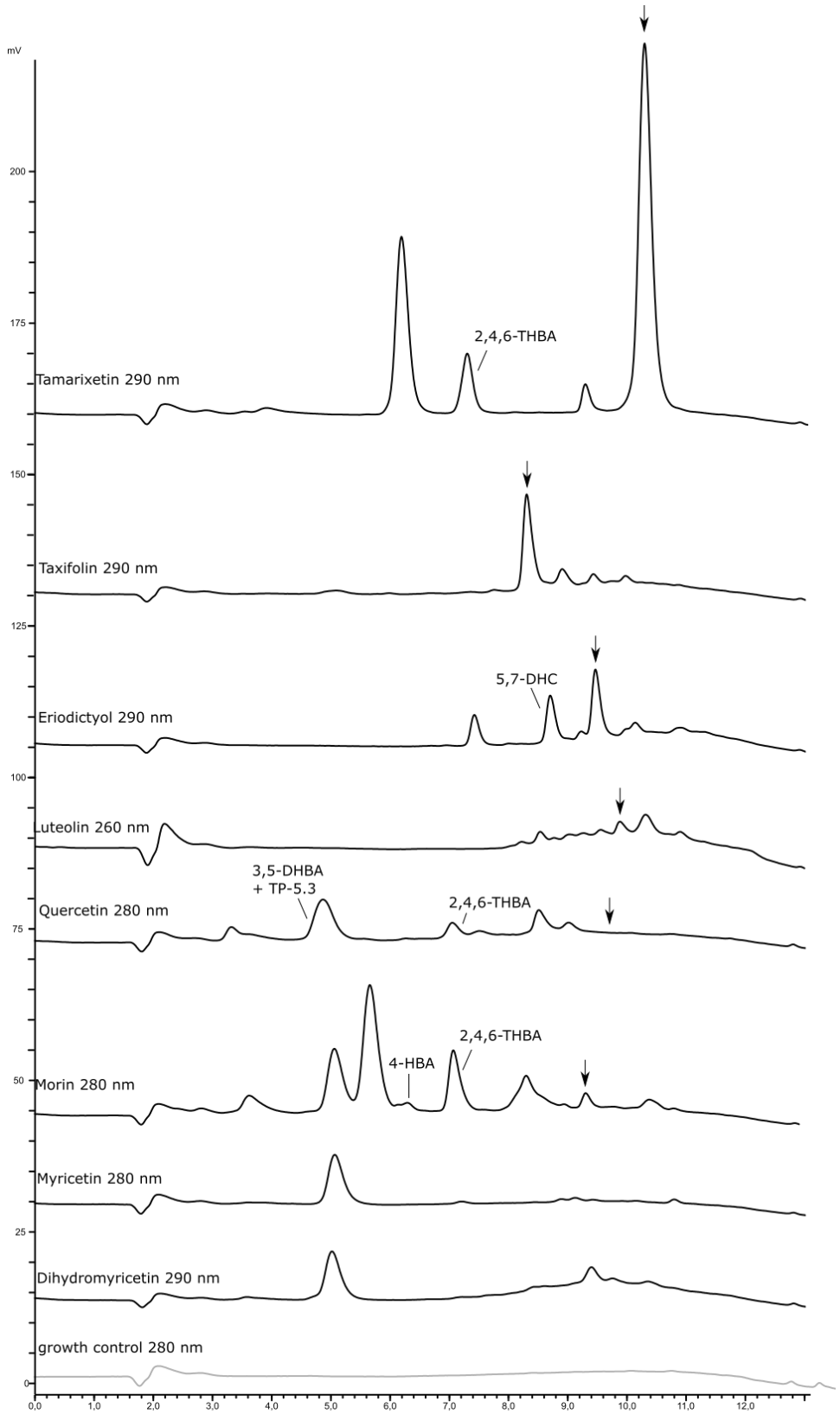


Representative chromatograms (290 nm) of a dihydromyricetin standard in DMSO (black) and diyhdromyricetin sample at day 0 of the algae growth inhibition test (gray). Standard and sample had an initial concentration of 5 mg/L.

HPLC-UV/vis analysis of transformation products

Chromatograms of luteolin and taxifolin showed several peaks with very low intensities, while the chromatograms of the other flavonoids displayed larger signals that allowed characterization based on retention time and absorption maxima (Table SI 6). Most of these TP signals increased in intensity until day 3 (Figure SI 15). Exceptions were TP-5.0/5.3 (signals of two compounds overlap) of quercetin where signal intensity peaked at day 1 and TP-5.1 of myricetin displaying a rather constant signal intensity from day 1 till day 3 after an initial increase. HPLC-UV/vis analysis enabled preliminary structure elucidation (Table SI 6).

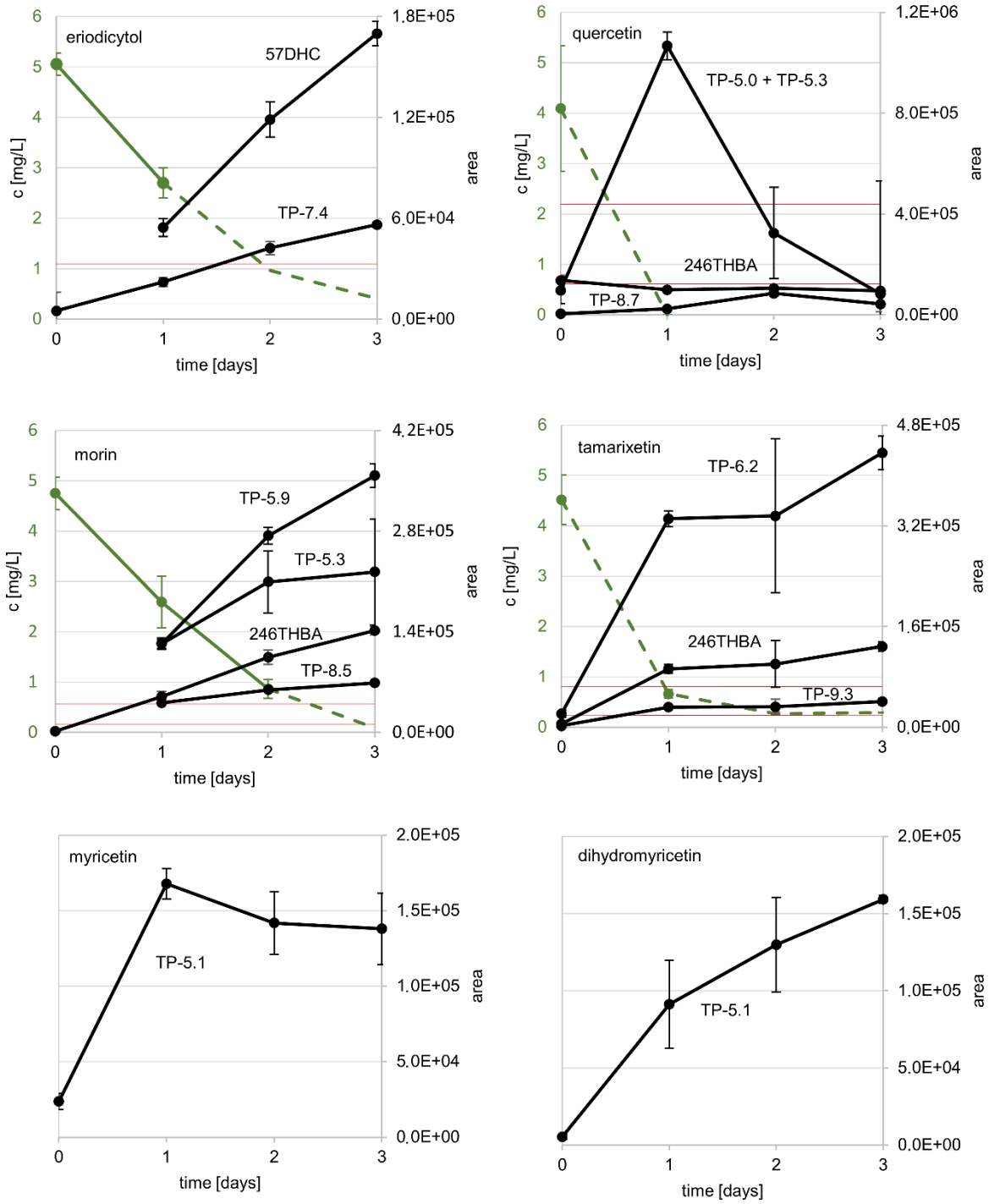
A calibration curve of 5,7-Dihydroxychromatogram was recorded to quantify the peak areas of this TPs at the end of the algae growth inhibition test.



Representative UV/vis-Chromatograms of flavonoid samples measured at the end of the algae growth inhibition test (72 h). Black arrows indicate retention time of parent compound. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 3,4-DHBA = 3,4-dihydroxybenzoic acid, 4-HBA = 4-hydroxybenzoic acid, 5,7-DHC = 5,7-Dihydroxychromone.

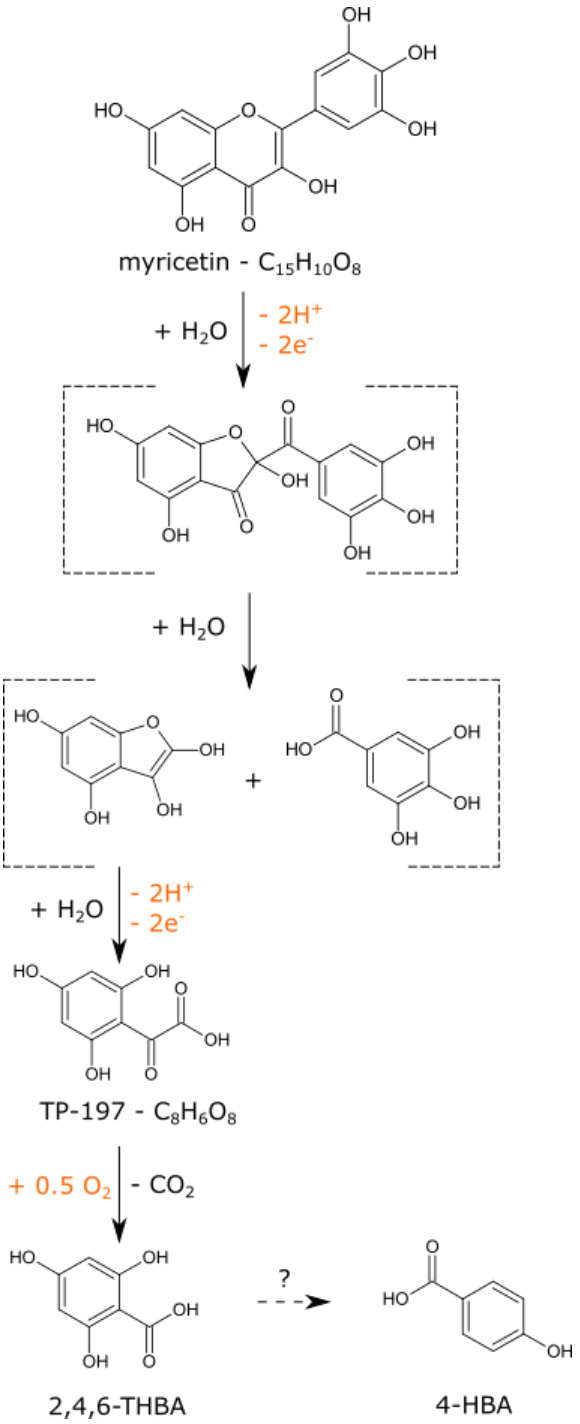
Characterization of HPLC-UV/vis signals of TPs by retention time (rt) and absorption maxima (λ_{max}). Based on comparison with standard compounds a structure is suggested for some TP-Signals. Quercetin signals with rt of 5.0 and 5.3 min overlap.

parent compound	TP name	rt [min]	λ_{max} [nm]			structure suggestion
			1	2	3	
dihydromyricetin	TP-5.1	5.1	290			
myricetin	TP-5.1	5.1	290	-	-	
eriodictyol	TP-7.4	7.4	283			
		8.8	256	280		5,7-dihydroxychromone
tamarixetin	TP-6.2	6.2	280	312	< 250	
		7.3	255	289		2,4,6-trihydroxybenzoic acid
	TP-9.3	9.3	260			
morin	TP-5.3	5.3	290			
	TP-5.9	5.9	281	313		
		6.5	255			4-hydroxybenzoci acid (low signal intensity)
		7.2	253	292		2,4,6-trihydroxybenzoic acid
	TP-8.5	8.5	292			
quercetin	TP-5.0	5.0	259	293		3,4-dihydroxybenzoic acid
	TP-5.3	5.3	292			
		7.3	256	291		2,4,6-trihydroxybenzoic acid
	TP-8.7	8.7	259			

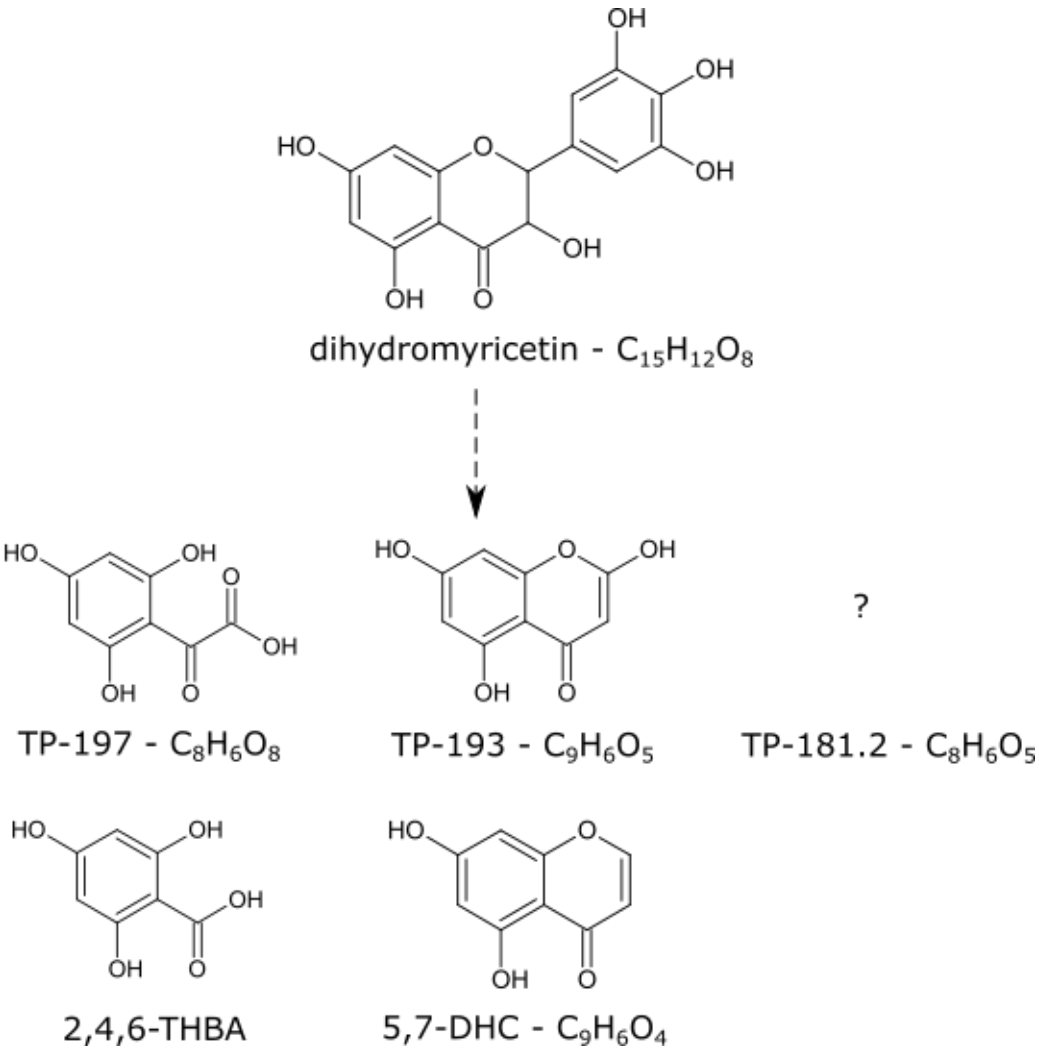


Concentration decrease of parent flavonoids (left axis, green) and relative course of TPs depicted as peak area (right axis, black). Myricetin and dihydromyricetin signals were already not detectable at day 0. Red lines display LOQ and LOD of the corresponding flavonoid. 57DHC=5,7-dihydrochromone, 246THBA=2,4,6-Trihydroxybenzoic acid. Data points represent average values (n=4) with standard deviation displayed as error bars. Signals of TP-5.0 and TP-5.3 of quercetin overlap and were quantified together.

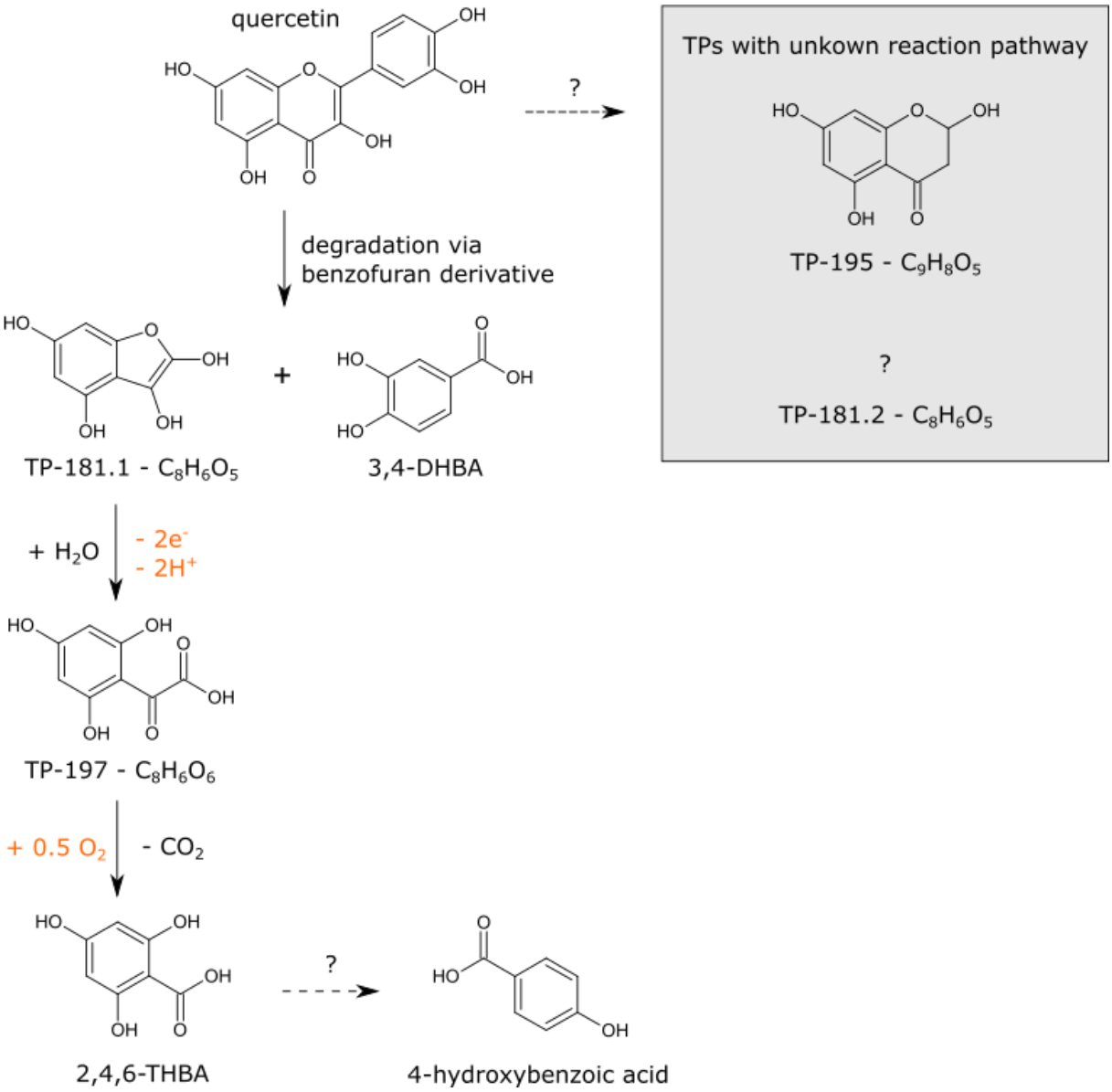
Proposed degradation pathways of the 8 degrading flavonoids



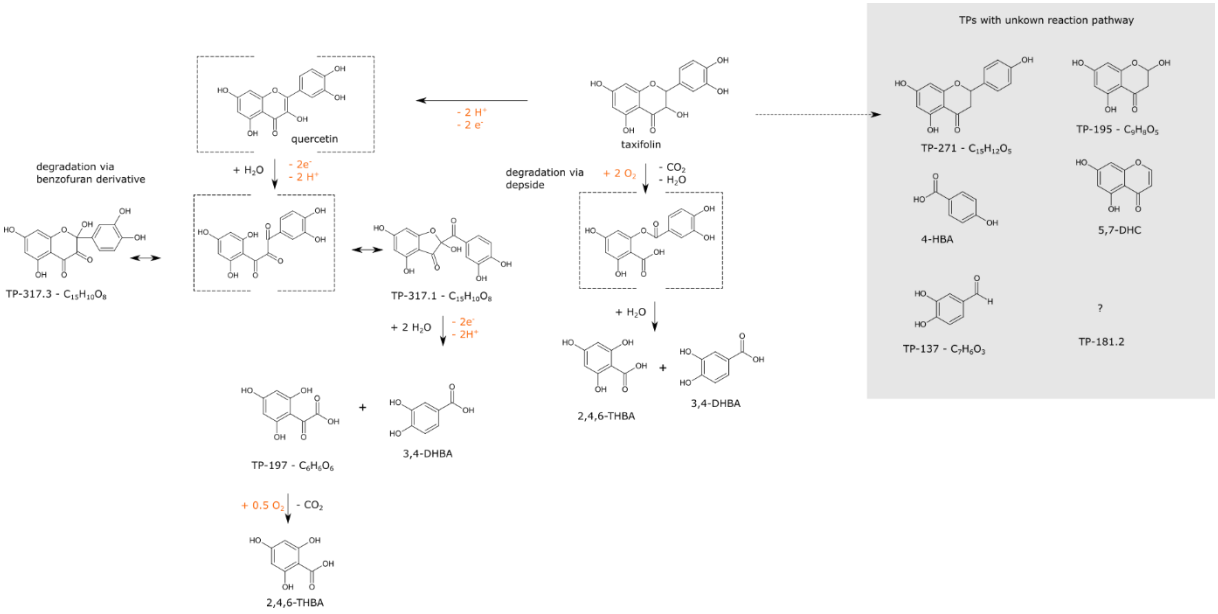
Identified TPs of myricetin and proposed degradation pathway. Structures in brackets represent proposed intermediates but were not detected. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid; 4-HBA = 4-hydroxybenzoic acid. The reaction pathway leading to 4-HBA remains unclear.



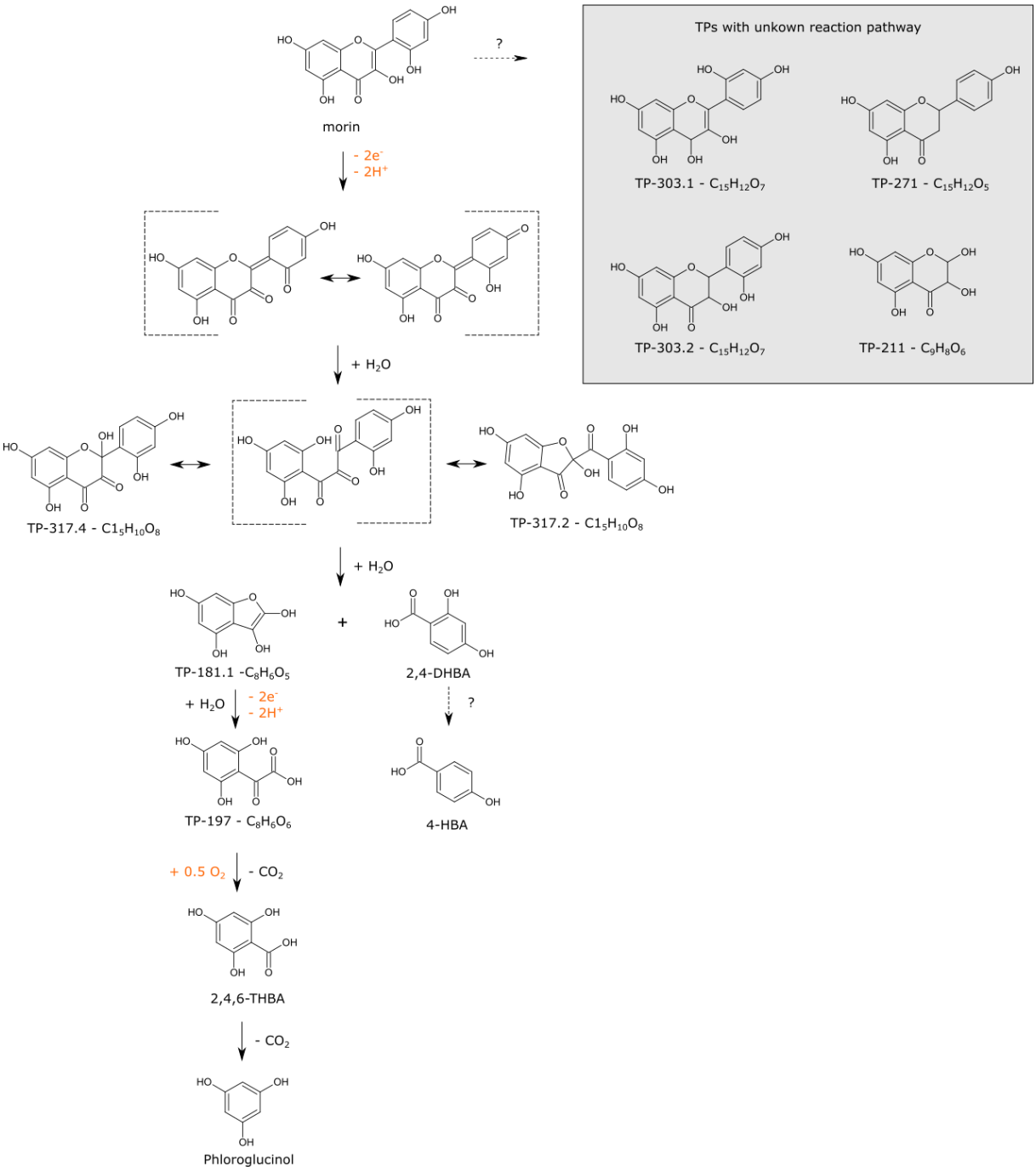
Identified TPs of dihydromyricetin. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 5,7-DHC = 5,7-dihydroxychromene. The structure of TP-181.2 remains unclear. No degradation pathway could be derived from the obtained information on TPs.



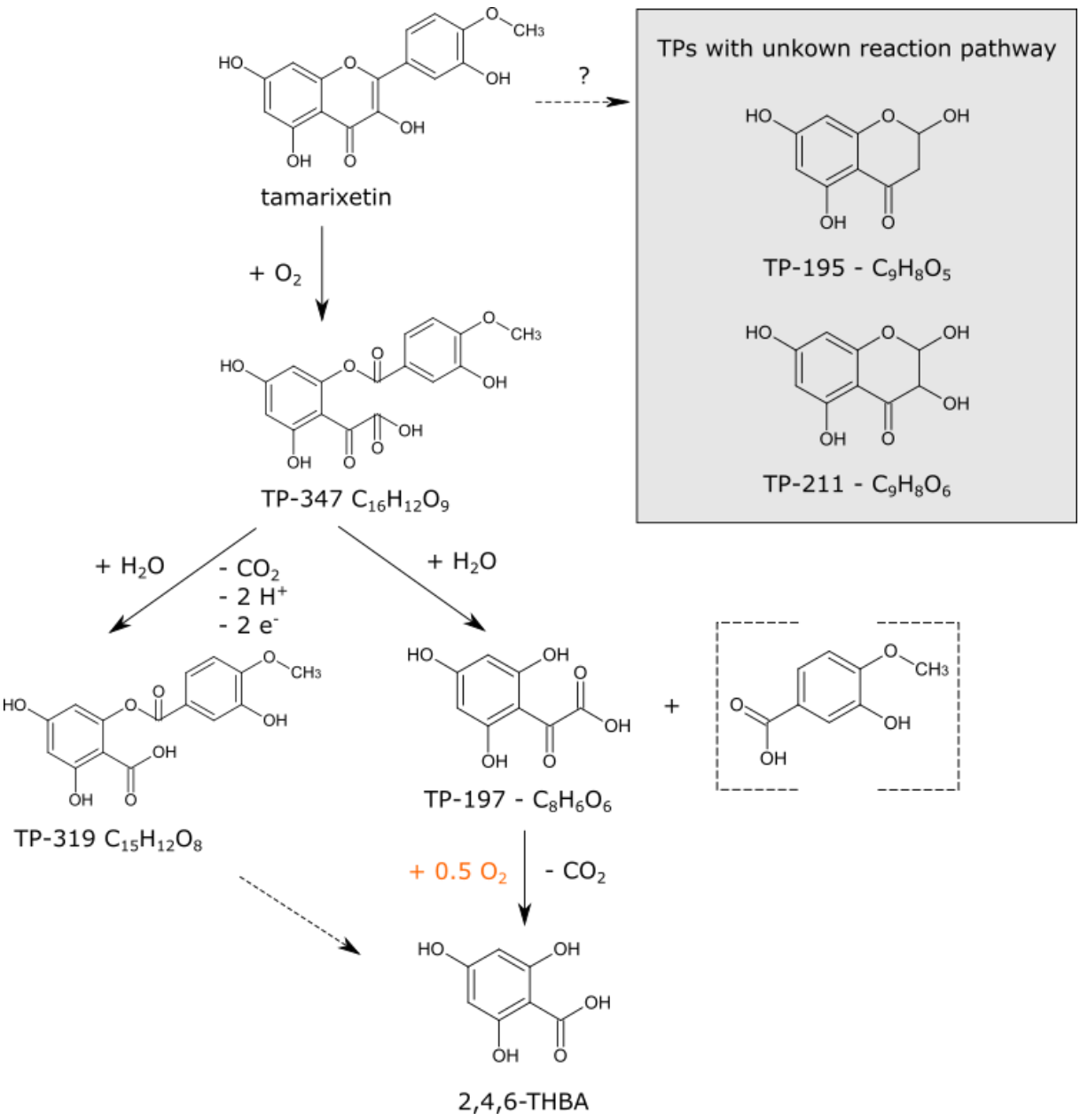
Identified TPs of quercetin and proposed degradation pathway. The formation of two TPs remains unclear. The structure of TP-181.2 remains unclear. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 3,4-DHBA = 3,4-dihydroxybenzoic acid.



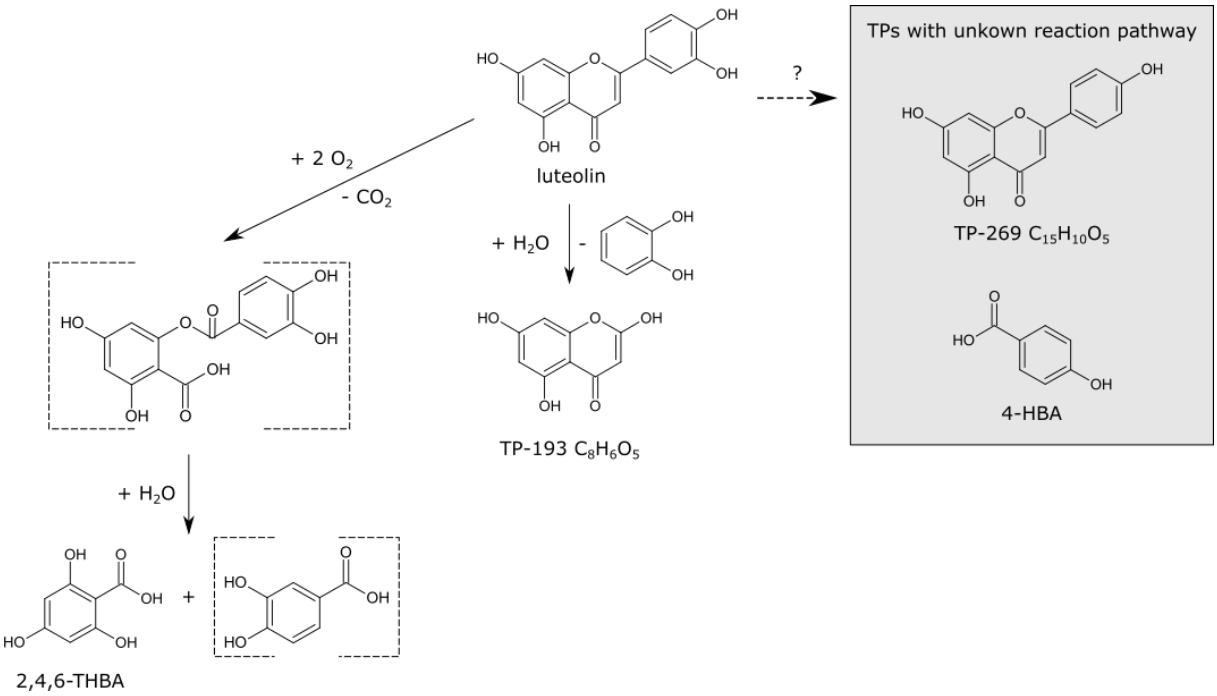
Identified TPs of taxifolin and proposed degradation pathway. Degradation via a benzofuranone derivative after oxidation to quercetin and via a depside result in the same end products. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 3,4-DHBA = 3,4-dihydroxybenzoic acid, 5,7-DHC = 5,7-dihydroxychromone. The formation of 6 TPs remains unclear.



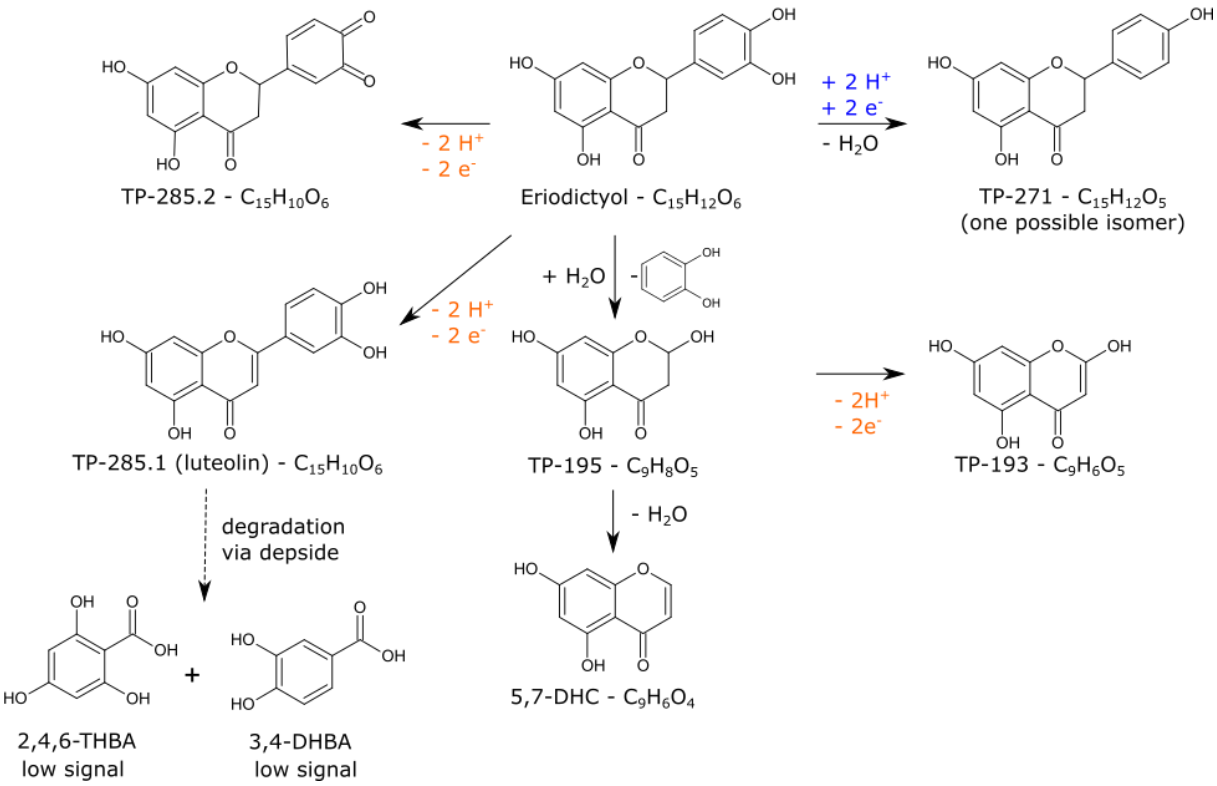
Identified TPs of morin and proposed degradation pathway. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 2,4-DHBA = 2,4-dihydroxybenzoic acid, 4-HBA = 4-hydroxybenzoic acid. The formation of 5 TPs remains unclear.



Identified TPs of tamarixetin and proposed degradation pathway. Substances in brackets represent proposed TPs that were not detected. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid. The formation of 2 TPs remains unclear.

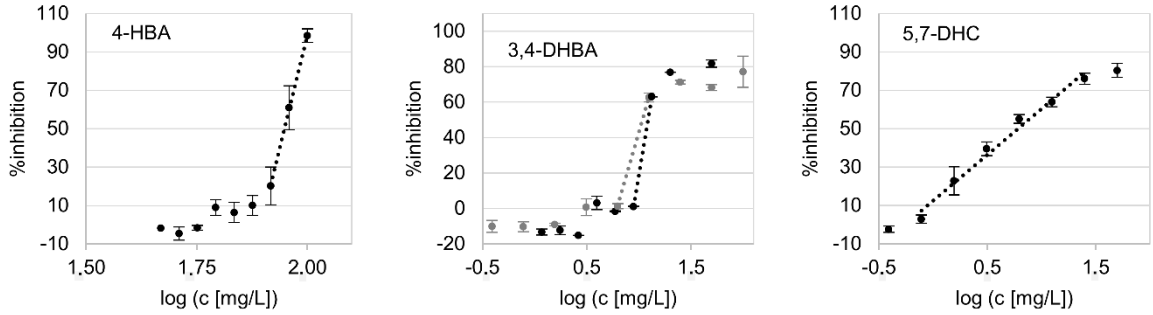


Identified TPs of luteolin and proposed degradation pathway. 2,4,6-THBA = 2,4,6-trihydroxybenzoic acid, 4-HBA = 4-hydroxybenzoic acid. The formation of 2 TPs remains unclear. The structure in brackets represents a proposed TP that was not detected.



Identified TPs of eriodictyol and proposed degradation pathways. 2,4,6-THBA = 2,4,6-trihydroxybenzoic, 3,4-DHBA = 3,4-dihydroxybenzoic, 5,7-DHC = 5,7-Dihydroxychromone.

Dose-response curves of flavonoid-TPs



Dose-response curves of the flavonoid TPs 4-hydroxybenzoic acid (4-HBA), 3,4-dihydroxybenzoic acid (3,4-DHBA) and 5,7-dihydroxychromone (5,7-DHC). Data points represent average values (n=4) with standard deviation (SD) displayed as error bars. For, 3,4-dihydroxybenzoic acid, the fist (grey) and second run (black) were performed with slightly adjusted concentrations.

Acid-base ratios of hydroxybenzoic acids at different pH

Base Acid ratio (A-/HA) at pH values of this study and the study of Kamaya et al. *and Lee* et al. **values obtained from SciFinder (Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2023 ACD/Labs).*

pH	**Base acid ratio (A^-/HA)**	
	2,4,6-trihydroxybenzoic acid (pKa = 1.62 *)	3,4-dihydroxybenzoic acid (pKa = 4.5 *)
8.1	3.0×10^6	4.0×10^3
7.5	7.6×10^5	1.0×10^3
6.5	7.6×10^4	1.0×10^2

Publication 3

Lena Schnarr, Oliver Olsson, Klaus Kümmerer

Biodegradation of flavonoids – Influences of structural features

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Biodegradation of flavonoids – Influences of structural features

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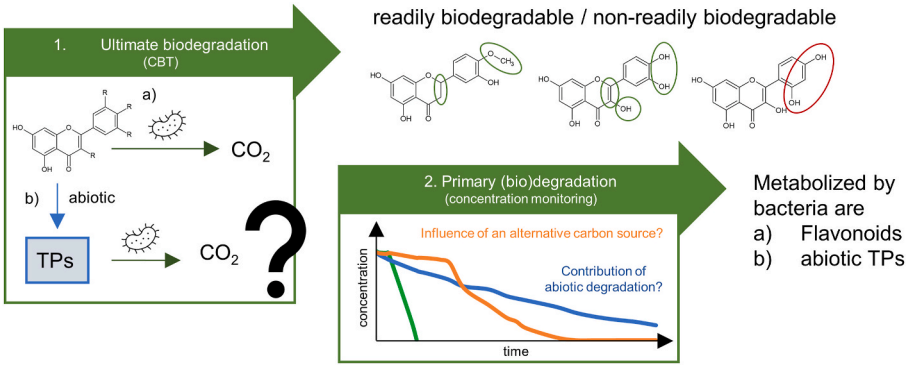
HIGHLIGHTS

- 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

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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

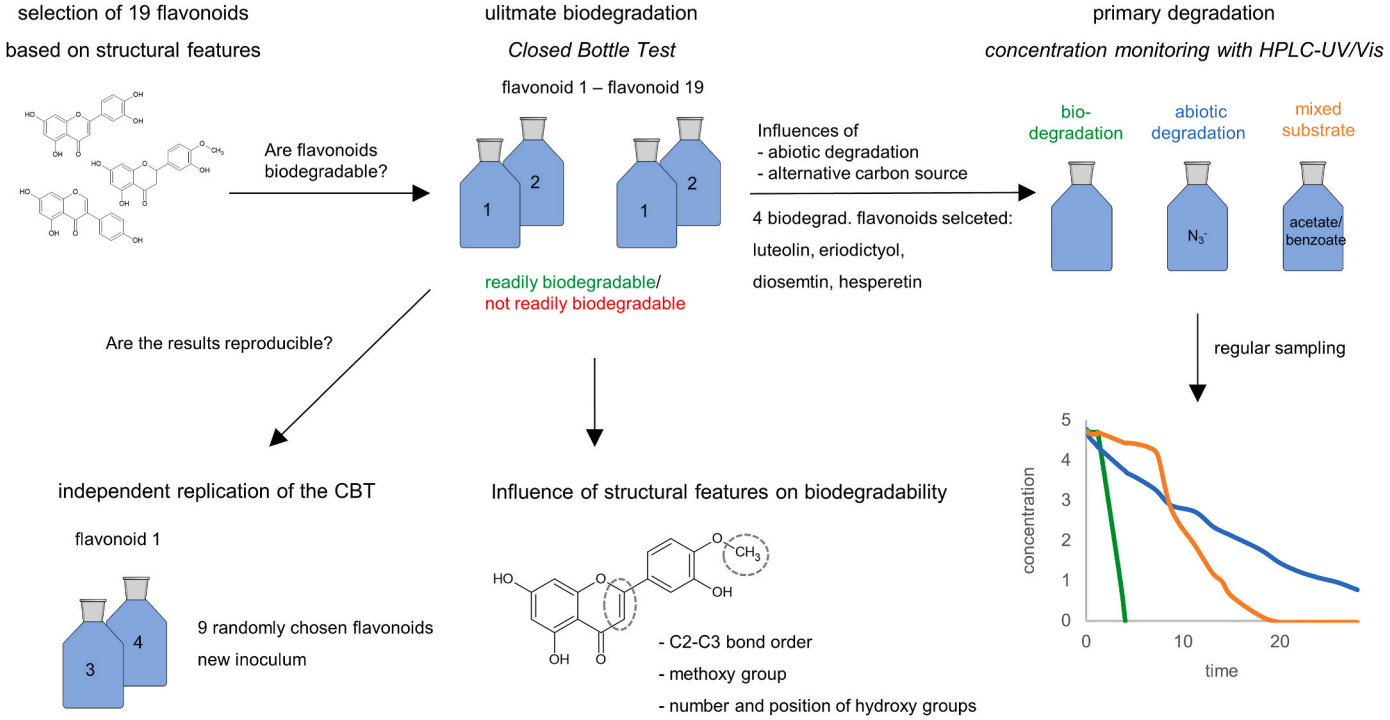

Fig. 1. Overview of study design and performed experiments.

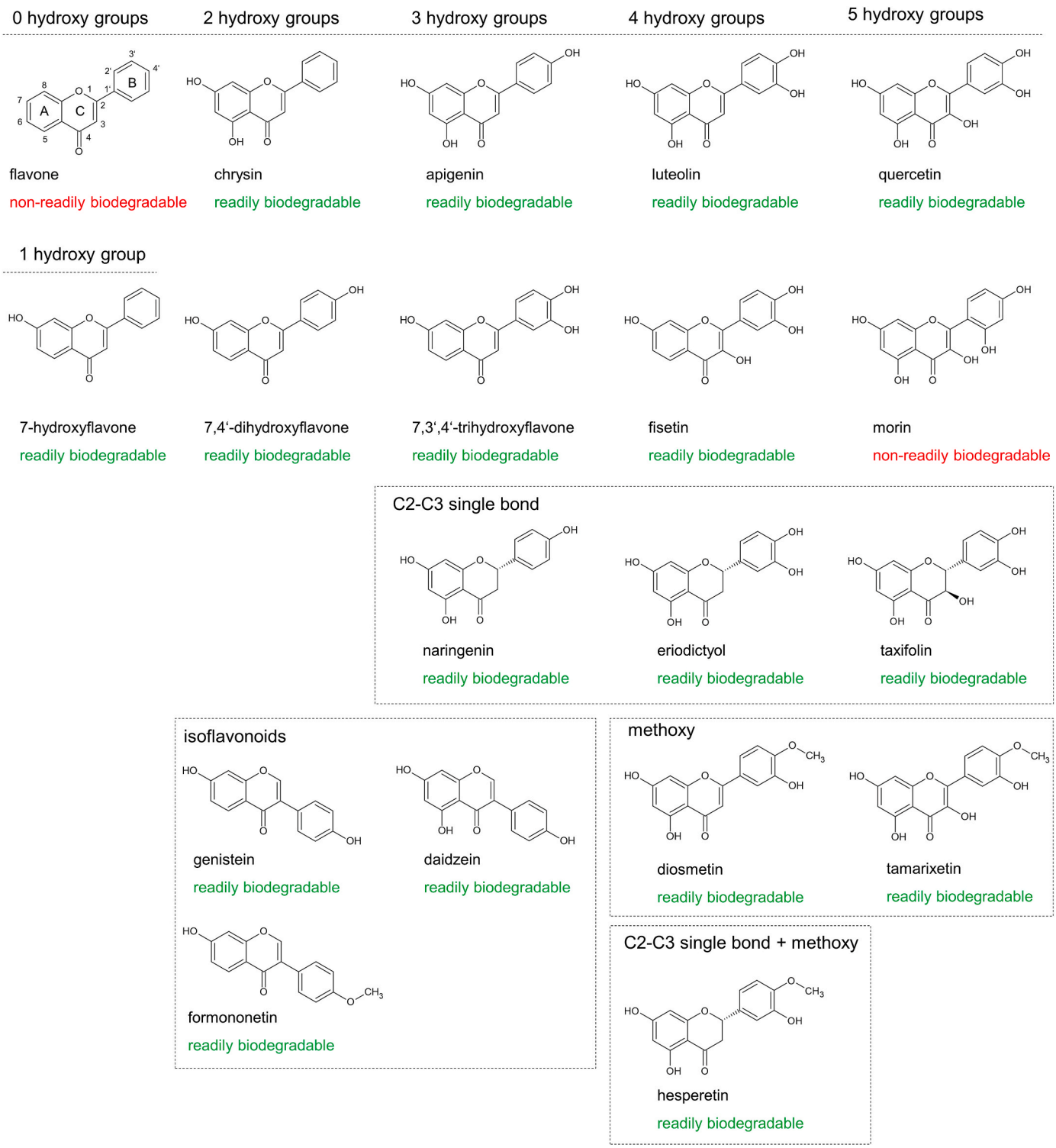

Fig. 2. Readily biodegradability tests results (OECD 301 D – CBT) of the 19 flavonoids. Flavonoids are arranged according to the number and position of hydroxy groups and further structural features to compare their influence on the biodegradation. O-methylated flavonoids are in the same column as their 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

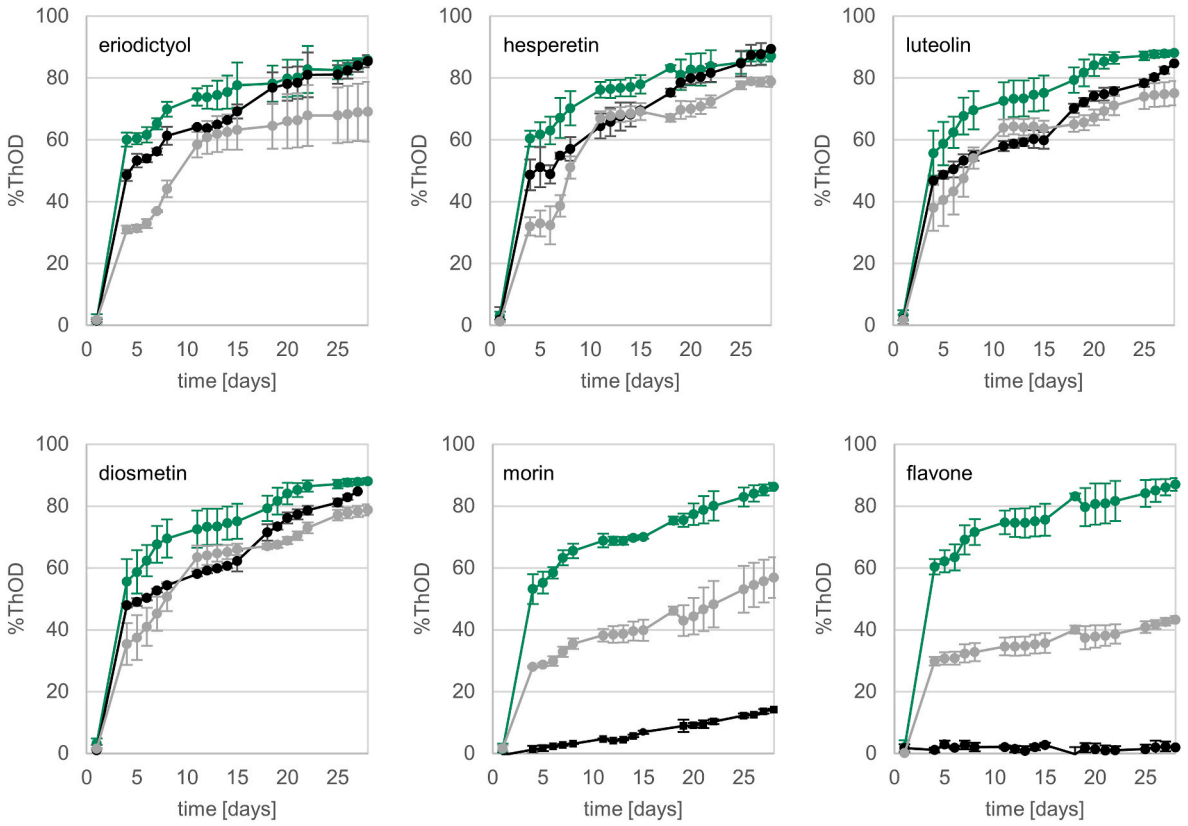

Fig. 3. Course of oxygen consumption in % of Theoretical Oxygen Demand (ThOD) of the 4 flavonoids analyzed in more detail and the two non-readily biodegradable flavonoids over the time period of 28 days. Quality control containing acetate (green), test substance (black) and toxicity control containing 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

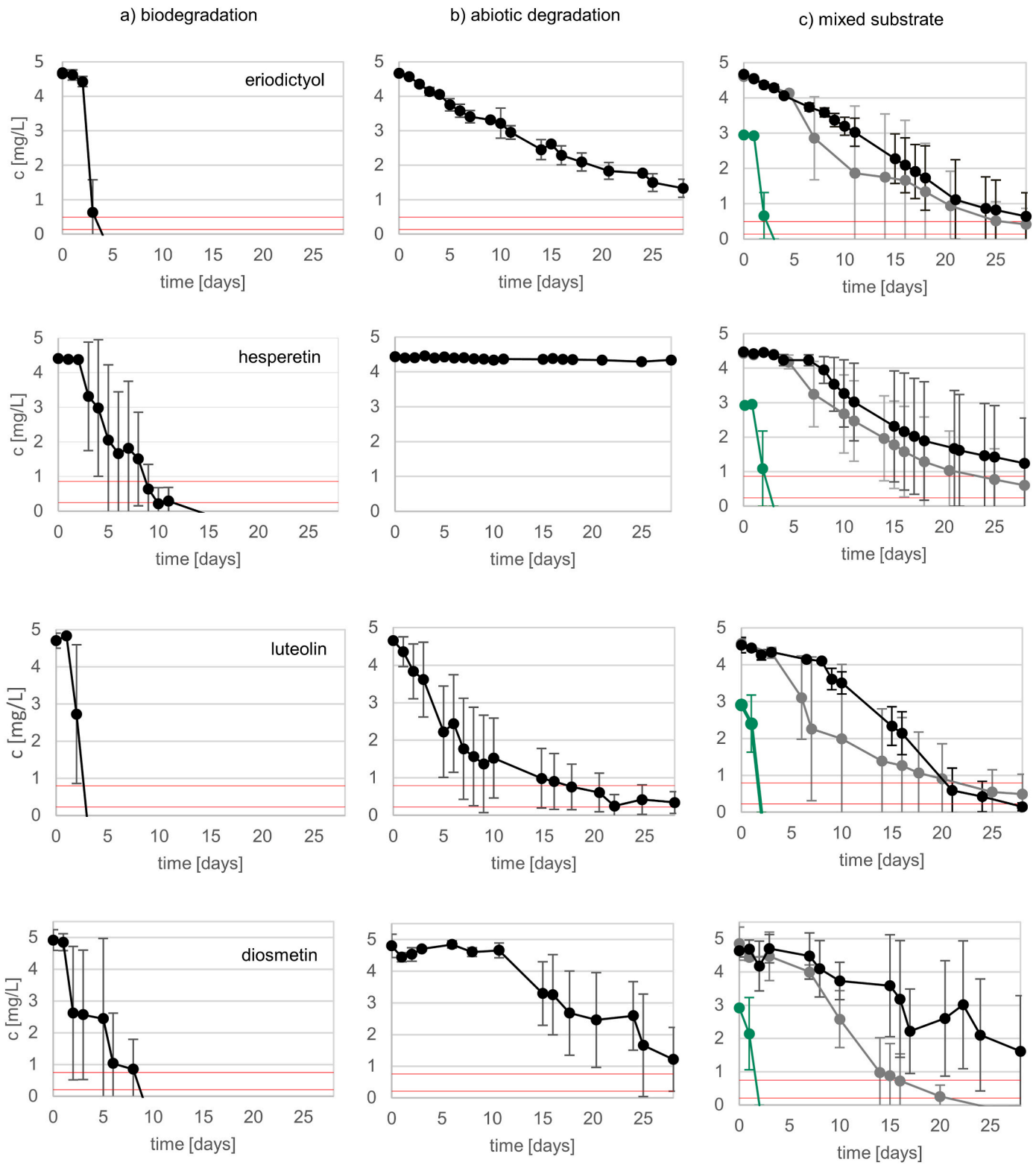

Fig. 4. Course of concentration of the 4 flavonoids eriodictyol, hesperetin, luteolin, and diosmetin in a) biodegradation (test substance + inoculum), b) abiotic (test substance + inoculum + azide) and c) mixed substrate samples (test substance + inoculum + acetate(grey)/benzoate(black)). In addition 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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Supporting Information of Publication 3

Lena Schnarr, Oliver Olsson, Klaus Kümmerer

Biodegradation of flavonoids – Influences of structural features

Chemosphere, 359, 142234 (2024)

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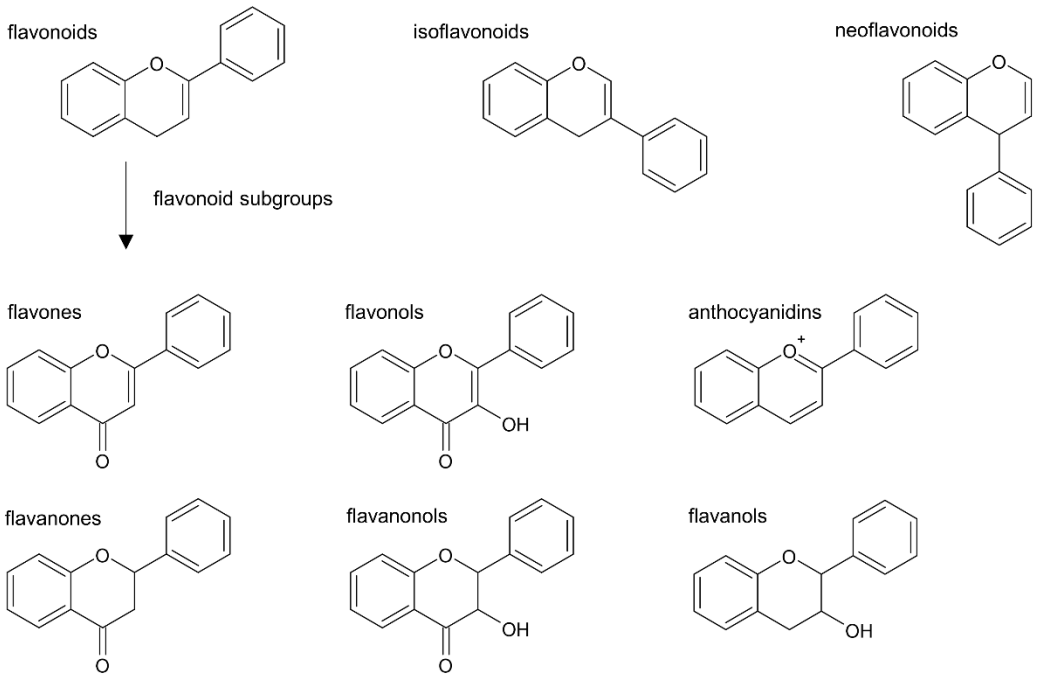


Overview of the different flavonoid groups.

Overview of used chemicals and solvents.

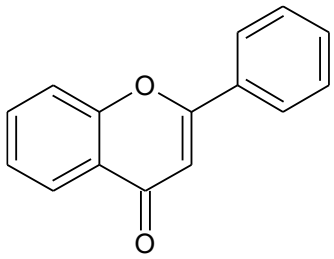
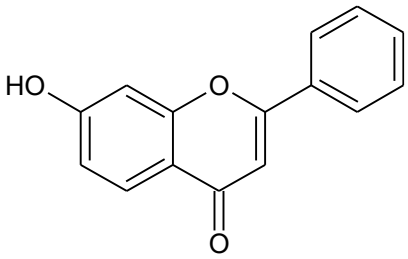
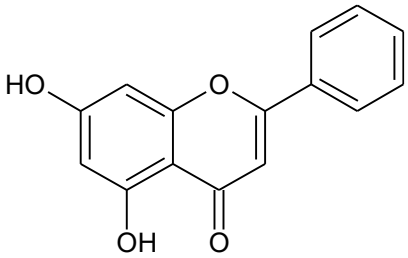
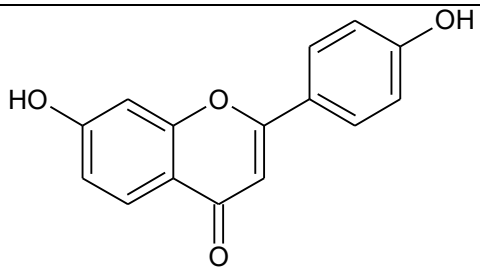
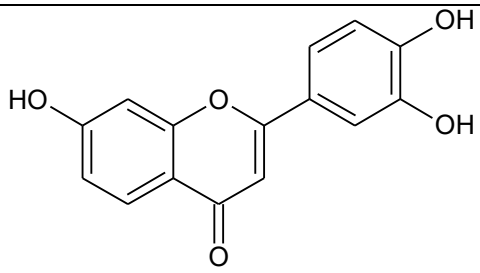
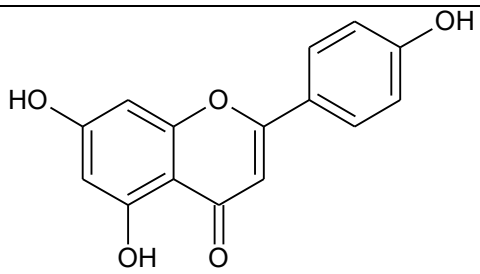
Substance	CAS number	Vendor	purity
flavone	525-82-6	Sigma Aldrich	> 99%
7-hydroxyflavone	6665-86-7	abcr chemie	98%
chrysin (5,7-DHF)	480-40-0	abcr chemie	98%
7,4'-dihydroxyflavone	2196-14-7	abcr chemie	97%
7,3',4'-trihydroxyflavone	2150-11-0	abcr chemie	98%
apigenin	520-36-5	TCI Deutschland GmbH	98%
naringenin	67604-48-2	alfa aeser	≥ 97%
fisetin	528-48-3	abcr chemie	98%
luteolin	491-70-3	abcr chemie	97%
eriodictyol	552-58-9	ACROS	≥ 94%
diosmetin	520-34-3	Sigma-Aldrich	≥ 98%
hesperetin	520-33-2	abcr chemie	97%
morin hydrate (morin)	654055-01-3 (480-16-0)	Sigma Aldrich	Not specified
quercetin	117-39-5	Sigma Aldrich	≥ 95%
taxifolin	480-18-2	Sigma Aldrich	95%

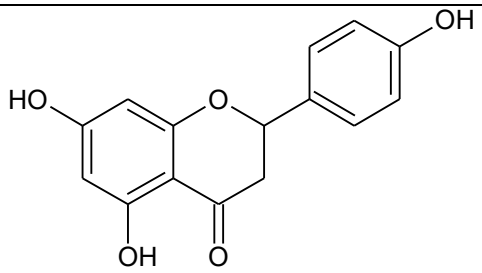
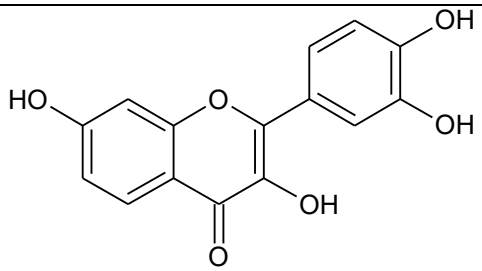
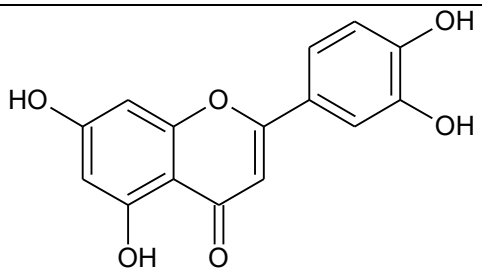
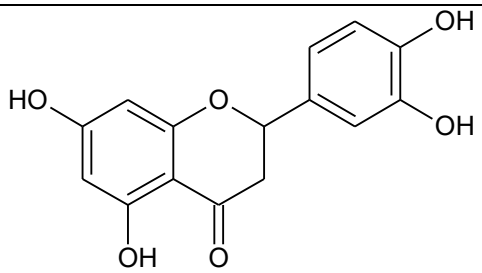
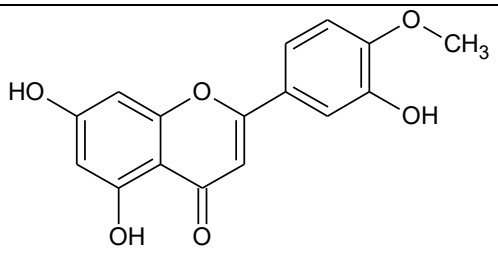
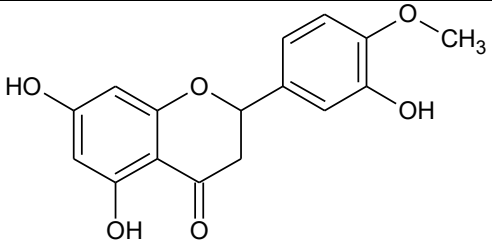
tamaritexin	603-61-2	abcr chemie	99%
genistein	446-72-0	TCI Deutschland GmbH	98%
formononetin	485-72-3	Sigma Aldrich	> 99%
daidzein	486-66-8	Cayman Chemical	> 95%
3-hydroxybenzoic acid	99-06-9	TCI Deutschland GmbH	> 99%
4-hydroxybenzoic acid	99-96-7	TCI Deutschland GmbH	> 99%
3,4-dihydroxybenzoic acid	99-50-3	TCI Deutschland GmbH	> 98%
2,4,6-trihydroxybenzoic acid	83-30-7	TCI Deutschland GmbH	not specified
3,4,5-trihydroxybenzoic acid	49-91-7	Sigma Aldrich	> 97.5 %
5,7-dihydroxychromone	31721-94-5	abcr chemie	not specified
phloroglucinol	108-73-6	Sigma Aldrich	> 99%
DMSO		Sigma Aldrich	
methanol		VWR	LC-MS grade
sodium azide	26628-22-8	Sigma Aldrich	

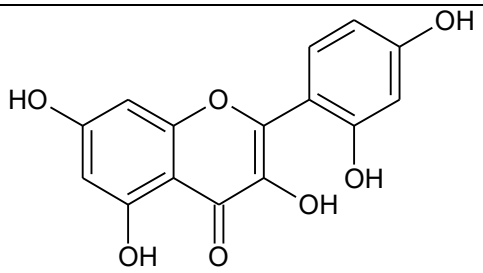
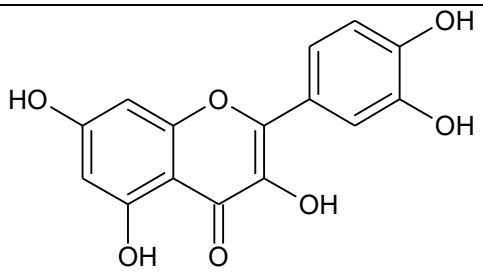
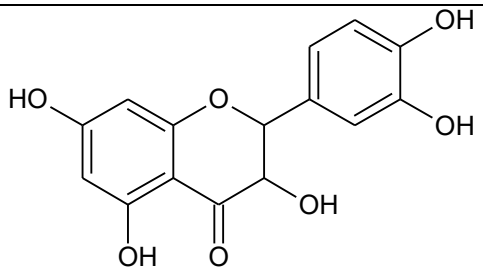
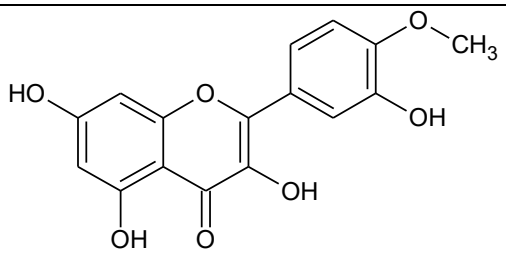
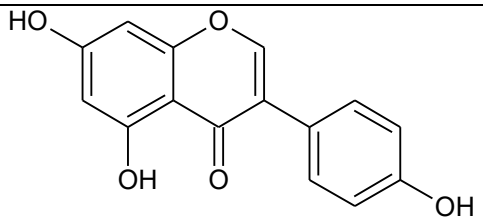
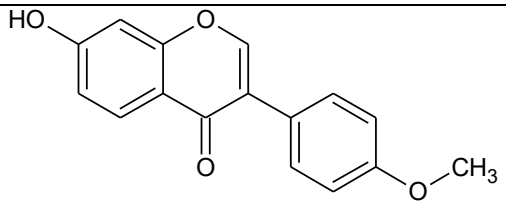
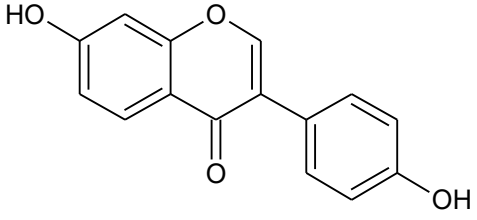
Wavelength used for quantification and limits of quantification (LOQ) and detection (LOD) for flavonoids selected for a more detailed analysis.

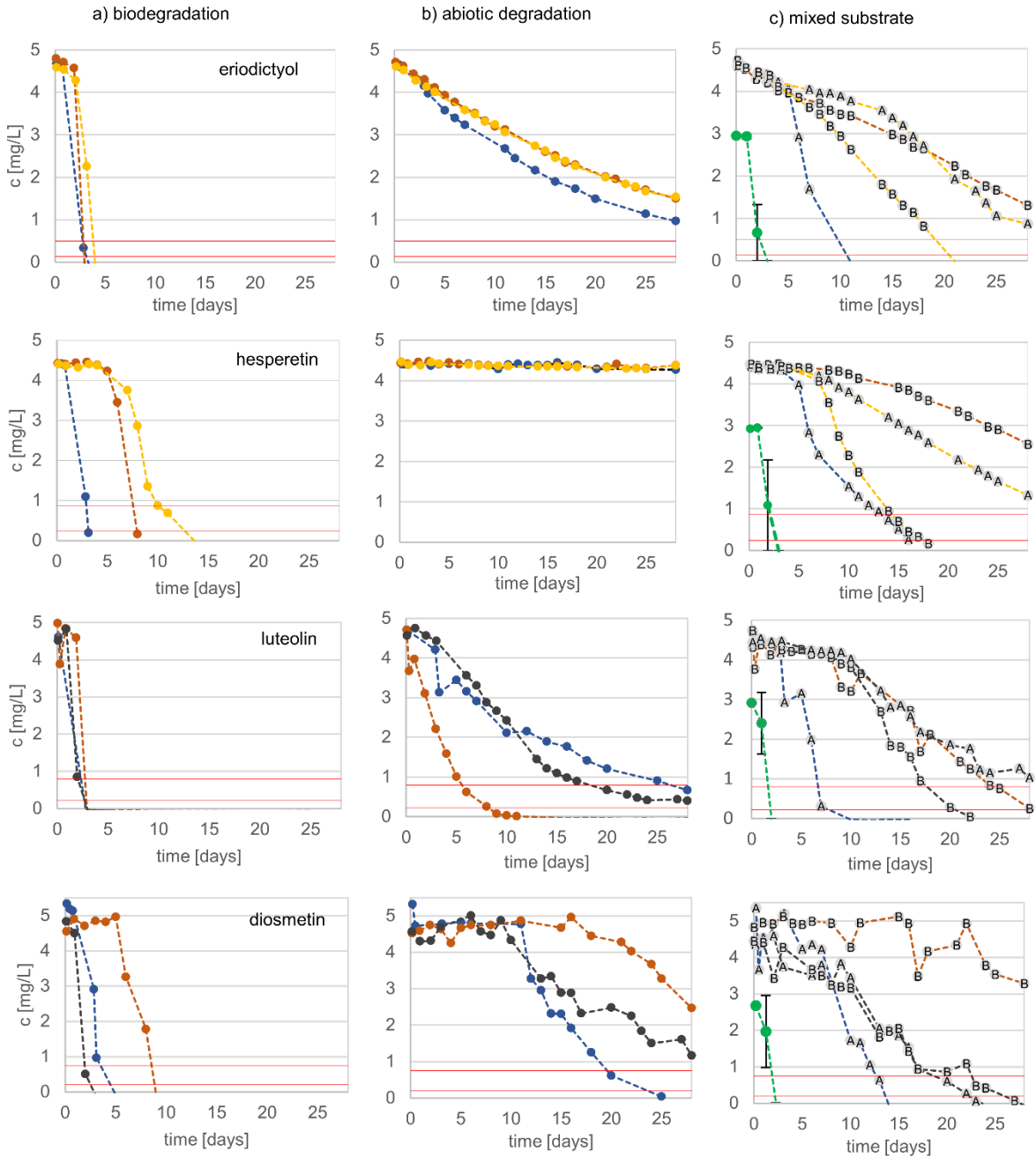
substance	**wavelength of quantification [nm]**	**LOQ [mg/L]**	**LOD [mg/L]**
luteolin	350	0.80	0.23
eriodictyol	290	0.50	0.14
diosmetin	350	0.75	0.21
hesperetin	290	0.86	0.24

CBT results of the 19 selected flavonoids. Average values with standard deviation (SD) are given. The number of replicates (n) is given in the last column.

substance	**structure**	**Biodegradation in %ThOD**	**n**
flavone		2 ± 2	4
7-hydroxyflavone		83 ± 16	4
chrysin		85 ± 8	4
7,4'-dihydroxyflavone		78 ± 13	4
7,3',4'-trihydroxyflavone		69 ± 7	2
apigenin		86 ± 9	4

naringenin		85 ± 3	2
fisetin		85 ± 1	2
luteolin		85 ± 1	4
eriodictyol		85 ± 2	4
diosmetin		87 ± 1	2
hesperetin		89 ± 4	4

morin		14 ± 0.2	2
quercetin		89 ± 2	2
taxifolin		75 ± 0.1	2
tamaritexin		95 ± 1	2
Isoflavonoids			
genistein		77 ± 1	2
formononetin		69 ± 4	2
daidzein		79 ± 6	4



Course of concentration in the individual replicates of the 4 flavonoids eriodictyol, hesperetin, luteolin, and diosmetin in the biodegradation (left), abiotic (middle) and mixed substrate (right) samples. Colors indicate which replicates were run parallel (blue = first run, orange = second run, yellow= third run, and black = fourth run). In mixed substrate samples, the letter A indicates samples containing acetate, the letter B indicates samples containing benzoate. Concentration of benzoate in the mixed substrate samples is shown in green (data points are average values (n=2) with standard deviation (SD)).