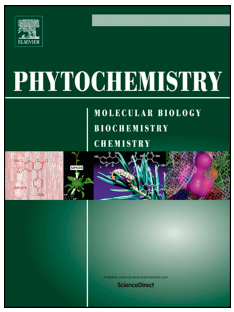

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Phenolic fingerprints of the Pacific seagrass *Phyllospadix torreyi* - structural characterization and quantification of undescribed flavonoid sulfates.

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Abstract: Four undescribed flavonoid sulfates were isolated from *Phyllospadix torreyi* S. Watson foliar tissue. In addition, nine known flavonoid sulfates and three phenolic acids were isolated from the same extract, of which seven had never been reported for the genus *Phyllospadix*. Structural elucidation of individual phenolics was assigned using complementary informations from their spectral evidence (HPLC-DAD, LC-MS, NMR, and UV) and chemical behavior. The inter-annual variation in phenolic concentrations was determined by quantitative HPLC-DAD over a three-year period. The results showed a relative constancy of phenolic content over time and the high prevalence of flavonoid disulfates (70-90% of the total flavonoids detected). All samples were found dominated by the unreported nepetin 7, 3'-disulfate and 5-methoxyluteolin 7, 3'-disulfate, followed by luteolin 7, 3'-disulfate. Considering the economic potential of flavonoid sulfates in the pharmaceutical and nutraceutical segments, a sample of detrital leaves was also analyzed. The same phenolic pattern was found and the concentration of the individuals, although lower than in fresh material, makes this abundant biomass of interest for dietary and pharmaceutical applications.

Keywords: *Phyllospadix torreyi*; Zosteraceae; fresh and detrital leaves; phenolic fingerprints; flavone di- and monosulfates; rosmarinic acid; inter-annual variability.

1. Introduction

Seagrasses constitute a group of about 72 species of rooted vascular plants, which evolved three to four times from terrestrial ancestors and successfully returned to the sea. They grow in large marine meadows, which constitute valuable habitats and form the most widespread and productive coastal system in the world. Unlike most other seagrasses that grow on soft-bottom habitats (sand and mud), the genus *Phyllospadix* Hooker (Zosteraceae family, common name surfgrass) dominates the rocky subtidal and intertidal zones along the northern temperate coasts of the Pacific (den Hartog and Kuo, 2006). This peculiar habitat makes *Phyllospadix* ecologically distinct among seagrasses. This genus includes two Asian species, namely *P. japonicus* Makino and *P. iwatensis* Makino, and three American species, namely *P. serrulatus* Ruprecht et Ascherson, *P. scouleri* Hook, and *P. torreyi* S. Watson (Wyllie-Echeverria and Ackerman, 2003).

Phyllospadix torreyi S. Watson (common name: Torrey's surfgrass) is endemic to the Northeast Pacific, where it occurs from northern Vancouver Island (Canada) to the tip of Baja California (Mexico). *P. torreyi* beds are particularly abundant in the highly turbulent rocky intertidal zone of San Diego County (den Hartog and Kuo, 2006). Studies about the species have mainly been focused on the morphology, physiology and phylogenetic relationship with other seagrasses (see as examples: Les, 2020; Ruiz-Montoya et al., 2021). In contrast, little consideration has been given to the metabolites produced by *P. torreyi*. The saccharide-, polysaccharide- and cyclitol contents have been reported (Drew, 1983; Woolard et al., 1978). More recently, the seasonal and inter-annual variations of fatty acids and sterols have been reported for specimen from Baja California, Mexico (Serviere-Zaragosa et al., 2015, 2021). The specialized metabolism of *P. torrey* has been even less documented despite it produces substances, which have important ecological functions in plants. This is especially the case of phenolic compounds, which form the basis of defensive mechanisms in plants and are common in marine ecosystems where competition is intense. In seagrasses, many are antimicrobial agents and presumed to protect marine macrophytes against pathogen attacks, to reduce plant palatability toward grazers or to inhibit microbial settlement or growth (Laabir et al., 2013; Papazian et al., 2019; Sieg and Kubanek, 2013; Subhashini et al., 2013; Zidorn, 2016).

Only a few studies have investigated the phenolic content of the genus *Phyllospadix.* The presence of hydroxybenzoic- and coumaric acids has been reported in *P. scouleri* (Proksch et al., 1981), and of caffeic-, coumaric-, and ferulic acids in *P. torreyi* (Zapata and McMillan, 1979). The presence of sulfated flavones was also reported (McMillan et al., 1980). The Asian species *P. iwatensis* and *P. japonicus* have received more attention. A complex mixture of eight flavonoids,

namelly 6-hydroxyluteolin, 5-methoxyluteolin, diosmetin, acacetin, hispidulin, jaceosidin, and 6-methoxydiosmetin was isolated from *P. japonicus* (Takagi et al., 1979). Hispidulin was also found in *P. iwatensis* along with luteolin and a flavonoidal alkaloid designated as phyllospadine (Takagi et al., 1980). More recently, luteolin 7-sulfate and hispidulin 7-sulfate were identified as additional constituents of *P. iwatensis* (Kwak et al., 2016).

In this context, our aim was to isolate, structurally identify, and quantify the phenolic content of foliar tissue of *P. torreyi* collected in the seagrass bed. In recent years, marine natural products have attracted increasing attention. Torrey's surfgrasses produce large amounts of leaf material that are shed and washed ashore.. Considering the role of phenolic substances as chemical defences and their economic potential within the pharmaceutical, cosmetic and food industries, it appeared of interest to also consider the content of detrital *P. torreyi*. This work reports the first quantitative characterization of polyphenols in fresh and detritus samples collected in La Jolla, California, USA (Fig. 1). Four undescribed flavonoid sulfates, nine known structural analogues and three phenolic acids were obtained. Herein, we report the isolation, structural elucidation, and seasonal variation of these compounds.

2. Results

2.1. Determination of the phenolic fingerprints of P. torreyi by HPLC-DAD and LC/MS

The crude extracts of *P. torreyi* were analyzed by HPLC-DAD and HPLC/ESI-MS in positive mode to obtain their chromatographic profiles, on-line UV spectra and mass spectral information regarding their components. Sixteen peaks of variable intensity were detected (Table 1, Fig. 2). Comparison of retention time and UV spectra with those of flavonoid standards indicated the absence of flavonols and flavanones. Examination of on-line UV spectra enabled the identification of three phenolic acids and thirteen flavonoids (Fig. S31). The phenolic acids were identified as caffeic- (**1**, Rt 12.1 min), coumaric- (**5**, Rt 16.7 min), and rosmarinic acid (**10**, Rt 23.2 min), of which rosmarinic acid was predominant (Fig. 2-3). The flavonoid pattern was largely dominated by three flavonoids (**3**, **6** and **7**), along with compounds in moderate amounts (**8**, **11-16**), or in low to trace amounts (**2**, **4**, **9**).

Total acid hydrolysis of the crude extract resulted in the complete disappearance of the 13 peaks assigned as flavonoid (**2-4**, **6-9** and **11-16**), and the appearance of six aglycones (**a-f**, Fig. 2). Peaks assigned to phenolic acids (**1**, **5**, **10**) were recovered unchanged. Based on their UV spectra and comparison to standards and data from literature, the aglycones were identified as 6-hydroxyluteolin

(**a**, Rt, 24.1 min), 5-methoxyluteolin (**b**, 25.1 min), luteolin (**c**, 30.2 min), nepetin (**d**, 30.9 min), hispidulin (**e**, 33.6 min), and jaceosidin (**f**, 34.0 min). This order of elution on C18 column and UV λ_{max} values of these aglycones are in agreement with literature data (Bojilov et al., 2017; Greenham et al., 2003). It should be noted that whilst the introduction of a methoxyl group generally makes the product more lipophilic, resulting in a higher retention time, this is not the case for 5-methoxyluteolin, which was eluted earlier than luteolin due to the absence of internal hydrogen bond with the carbonyl group (Greenham et al., 2003). 5-Methoxyluteolin, luteolin, nepetin, hispidulin were unambiguously assigned by spectroscopic data (NMR, UV, and MS). Elution order and on-line UV spectra for 6-hydroxyluteolin and jaceosidin were in agreement with literature data (Bojilov et al., 2017; Greenham et al., 2003; Tomás-Barberán et al., 1987). Controlled hydrolysis in mild condition released flavonoids **13** and **14**, whose peak intensity has increased, along with two additional compounds (**17** and **18**), and the six aglycones **a-f** (Fig. 2-3, and S31). This suggests that compounds **13-14** and **17-18** are intermediates with respect to the level of sulfation of their respective flavonoid aglycone.

2.2. Purification of the extracts and structure identification of flavonoid compounds

The crude aqueous-methanolic extract was serially partitioned between water and organic solvent of increasing polarity, *i.e.* methylene chloride (F1, 0.2% yield), ethyl acetate (F2, 0.6% yield), and then *n*-butanol (F3, 11.2% yield). Of these, only F3 was found to contain significant amounts of flavonoid. HPLC analysis of F2 showed a complex mixture of apolar products dominated by 3, 4-dihydroxy benzaldehyde (5% of the total), caffeic acid (18%), coumaric acid (7%) and rosmarinic acid (13%). They were identified by UV, comparison to authentic standards, and NMR. This is the first report of rosmarinic acid from the genus *Phyllospadix.* The presence of caffeic- and coumaric acid in *P. torreyi* was previously reported by Zapata and McMillan (1979).

Fractionation of F3 by successive chromatography on C18 reverse phase silica gel led to the isolation of seven pure flavonoids (**3**, **6**-**7**, **11**-**14**), of which **3**, **7**, and **14** were previously undescribed.

Compound **3** eluted at 14.4 min in the chromatographic run. The positive ESI-MS spectrum gave a quasimolecular peak $[M+H]^+$ at 461 *m/z*, which was compatible with the molecular formula $C_{16}H_{12}O_{12}S_2$. Two pairs of another significant ions at *m/z* 403 ($[M-80+Na]^+$, 381 $[M-80+H]^+$, and 323 $[M-160+Na]^+$, 301 $[M-160+H]^+$ confirmed the presence of two sulfate groups. The ^{13}C NMR spectrum in DMSO-d_6 showed 16 resonances (1 methoxy, 6 CH, and 9 quaternary C including a carbonyl). The ^1H NMR spectrum showed six proton signals in the aromatic region in accordance with a luteolin derivative, *i.e.* a pair of *meta* coupled protons at δ 6.70 (1 H, *d*, *J* = 2.2 Hz, H-6) and δ 7.12 (1 H, *d*, *J* = 2.2 Hz, H-8), a one proton singlet at δ 6.50 (H-3), and an AMX spin system at δ 7.84

(1 H, *d*, *J* = 2.3 Hz, H-2'), δ 7.63 (1 H, *dd*, *J* = 2.3, 8.5 Hz, H-6'), δ 6.97 (1 H, *d*, *J* = 8.5 Hz, H-5') (Table 2). Resonances of carbon and proton showed the typical *ipso* and *ortho* shifts due to the presence of a sulfate group at position 7 (strong deshielding of H-8 and C-8; shielding of C-7), and 3' (strong deshielding of H-2', C-2' and C-4'; shielding of C-3') (Tables 3-4), (Barron et al., 1988). The absence of phenol –OH group signal around δ 13 *ppm* was consistent with the methoxy group linked to C-5. This was confirmed by the HMBC correlation observed between C-5 and the methoxy protons, the crosspeak observed between the methoxyl and H-6 resonances in the NOESY spectrum, the shielding of the carbonyl (176.1 *ppm* versus 181.9 *ppm* for luteolin) and the deshielding of C-3 (106.7 *ppm* versus 103.1 *ppm*) due to the absence of the strong internal hydrogen bond with a hydroxyl group at C-5. UV spectra (DAD on-line 266 and 332 nm; UV (MeOH) 266 and 327 nm) are in agreement with the hypsochromic shift of Band I induced by sulfation at position 3' (Barron et al., 1988). After acid hydrolysis, compound **3** yielded 5-methoxyluteolin, which was identified by UV and NMR (Fig. 4 and S31, Tables 2-3). All these data allowed identification of **3** as 5-methoxyluteolin 7, 3'-disulfate. Analysis of the 2D NMR data from the COSY, HSQC and HMBC spectra and querying of the SciFinder database confirmed the structure of **3** as an undescribed natural product.

Compound **7** eluted at 19.2 min. The positive ESI-MS spectrum gave a quasimolecular peak $[M+H]^+$ at 477 *m/z*, which was compatible with the molecular formula $C_{16}H_{12}O_{13}S_2$. Another significant ions at *m/z* 397 $[M-80+H]^+$ and 317 $[M-160+H]^+$ confirmed the presence of two sulfate groups. The ^{13}C NMR spectrum in DMSO-d_6 showed 16 resonances (1 methoxy, 5 CH, and 10 quaternary C including 1 carbonyl). The ^1H NMR spectrum showed a singlet (3H) at δ 3.76 and five proton signals at δ 6.73 (s, H-3), 6.96 (1 H, *d*, *J* = 8.6 Hz, H-5'), 7.32 (s, H-8), system at 7.71 (1 H, *dd*, *J* = 2.3, 8.6 Hz, H-6'), 7.93 (1 H, *d*, *J* = 2.3 Hz, H-2') (Tables 2-3). The ^1H and COSY data also indicated the lack of coupling for ring A and the absence of H-6 signal in the aromatic region, suggesting the methoxy group to be attached to C-6. The methoxy position was confirmed by the HMBC correlation between ^1H signal at 3.76 *ppm* and C-6 at 134.1 *ppm*. Resonances of carbons and protons showed the typical shifts due to the presence of a sulfate group at position 7 (strong deshielding of H-8 and C-8; shielding of C-7) and 3'(strong deshielding of H-2', C-2' and C-4'; shielding of C-3') (Barron et al., 1988). UV spectra of compound **7** (DAD on-line 264 and 332 nm; UV (MeOH) 266 and 327 nm) were in agreement with the hypsochromic shift of Band I induced by sulfation at position 3' (Barron et al., 1988). After acid hydrolysis, compound **7** yielded nepetin, which was identified by UV, NMR and comparison against an authentic standard (Fig. S31, Tables 2-3). All these data allowed identification of **7** as nepetin 7, 3'-disulfate. The analysis of the 2D NMR

data from the HSQC and HMBC spectra and querying of the SciFinder database confirmed the structure of **7** as an undescribed product.

Compound **14** eluted at 27.4 min. The positive ESI-MS spectrum gave a quasimolecular peak $[M+H]^+$ at 397 *m/z*, which was compatible with the molecular formula $C_{16}H_{12}O_{10}S$. Another significant ions at *m/z* 317 $[M-80+H]^+$ and 339 $[M-80+Na]^+$ confirmed the presence of one sulfate group. The ^{13}C NMR spectrum in DMSO-d_6 showed 16 resonances (1 methoxy, 5 CH, and 10 quaternary C including 1 carbonyl) (Tables 2-3). The ^1H NMR spectrum showed a singlet (3H) at δ 3.75 and five proton signals at δ 6.15 (s, H-8), 6.44 (s, H-3), 6.93 (1 H, *d*, *J* = 8.6 Hz, H-5'), 7.58 (1 H, *dd*, *J* = 2.3, 8.6 Hz, H-6'), 7.79 (1 H, *d*, *J* = 2.3 Hz, H-2'). The weak effect observed on H-8 (+0.17) while strong effect is still observed on H-2' (+0.37) is in agreement with the sulfate group linked at position 3'. The ^1H and COSY spectra also indicated the lack of coupling for ring A and the absence of H-6 signal in the aromatic region, suggesting the methoxy group to be attached to C-6. The methoxy position was confirmed by the HMBC correlation between ^1H signal at 3.75 *ppm* and C-6 at 133.5 *ppm*. Resonances of carbon showed the typical shifts due to the presence of a sulfate group at position 3' (strong deshielding of H-2', C-2' and C-4'; shielding of C-3' (Table 4)). UV spectra of compound **14** (DAD on-line (nm) 237, 273, 335; UV (MeOH) 239, 276, 333) are in agreement with the hypsochromic shift of Band I induced by sulfation at position 3' (Barron et al., 1988). After acid hydrolysis, compound **14** yielded nepetin, which was identified by UV, NMR and comparison against an authentic standard (Fig. S31, Tables 2-3). Altogether, these data identified **14** as nepetin 3'-sulfate. Analysis of HSQC and HMBC spectra confirmed the structure of **14**. To our knowledge, this compound has not been reported as a plant natural product before.

Comparison of UV, SM and NMR spectroscopic data of compound **6**, **11**, **12** and **13** with those reported in the literature allowed their identification as luteolin 7, 3'-disulfate (**6**) (Barron et al., 1988; Enerstvedt et al., 2017), luteolin 7-sulfate (**11**) (Barron et al., 1988; Grignon-Dubois and Rezzonico, 2018), nepetin 7-sulfate (**12**) (Flamini et al., 2001; Tomás-Barberán et al., 1987), and luteolin 3'-sulfate (**13**) (Barron et al., 1988; Enerstvedt et al., 2017; Kim et al., 2016) (Fig. 3 and S31, Tables 2-4, and Supplementary data for details).

*Identification of minor flavonoids **2**, **4**, **8**, **9**, **15** and **16***

Attempts to isolate as pure compound the flavonoids only present in low amounts (**2**, **4**, **8**, **9**, **15**, and **16**) were unsuccessful. Insufficient sample quantities did not allow further purification and their identity could not be fully confirmed by NMR. However, these minor flavonoids were found structurally related to each other and to the major flavonoids.

Compound **2** was the first flavonoid eluted in the chromatographic run (13.3 min; earlier than all the identified disulfates (**3**, **4**, **6**, **7**). Only present in low amount, it makes it impossible to isolate and it was not detected in the LC/MS spectra. However, some data were available from its chemical behavior. Its HPLC retention time (the lowest observed in the flavonoid series), and on-line UV spectra (273, 328 nm) are in agreement with a 6-methoxy flavone substituted by at least two electron-withdrawing groups. Only three 6-methoxy aglycones were obtained after acid hydrolysis of the crude extract, namely nepetin, hispidulin and jaceosidin. Of the three, only nepetin was compatible with retention time of compound **2**. The polarity of compound **2**, higher than that of nepetin 7, 3'-disulfate could lead to identify it with a trisulfated derivative. We found no mention of nepetin trisulfate in the SciFinder database. UV spectral studies of several naturally occurring and synthetic sulfated compounds indicated that sulfation at ring B induce an important hypsochromic shift in Band I due to the electron withdrawing effect of the sulfate group (Barron et al., 1988), whereas sulfation at ring A does not influence the UV absorption significantly. In the case of luteolin, Band I UV (λ_{max}) values were as follows (nm): 7, 3', 4'-trisulfate (305), 7, 4'- (320), 7, 3'-disulfate (333), 4'-sulfate (325), 3'-sulfate (330), 7-sulfate (348) and luteolin (350). Our results with nepetin show the same tendency: 7, 3'-disulfate (330), 3'-sulfate (334), 7-sulfate (348), and nepetin (348). From these values it appears that Band I UV (λ_{max}) value for compound **2** (328 nm) is incompatible with a trisulfate, which led us to tentatively assign compound **2** as nepetin 7, 4'-disulfate. To our knowledge, this product had never been reported.

Compound **8** eluted at 19.6 min. ESI-MS spectrum gave a quasimolecular peak $[M+H]^+$ at 381 *m/z*, which was compatible with the molecular formula $C_{16}H_{12}O_9S$. Another significant ion at *m/z* 301 $[M-80+H]^+$ confirmed the presence of one sulfate group. UV spectrum (DAD on-line 266 and 341 nm) was in agreement with sulfation at position 7 (Barron et al., 1988). After acid hydrolysis, compound **8** yielded 5-methoxyluteolin. These results allowed identification of compound **8** as 5-methoxyluteolin 7-sulfate. To our knowledge, this compound has not been reported before.

Comparison of spectroscopic data and chemical behaviour of compound **4**, **9**, **15** and **16** with those reported in the literature allowed their identification as 6-hydroxyluteolin disulfate and monosulfate (**4** and **9**) (Greenham et al., 2003; Tomás-Barberán et al., 1987), hispidulin 7-sulfate (**15**) (Flamini et al., 2001; Kwak et al., 2016), and jaceosidin 7-sulfate (**16**) (Tomás-Barberán et al., 1987; Zhang et al., 2015) (see Supplementary data for details).

Comments on the structural identities as assigned :

1. Care should be taken when assigning HPLC peaks **14** and **15** on the only basis of their on-line UV spectra maxima. They present, indeed, very close maxima values (**14**: 273 and 335 nm; **15**:

274 and 336 nm). Careful examination of the UV minima of band II and the shape of the absorbance profiles is necessary in this case to achieve a correct assignment as nepetin 3'-sulfate (**14**) and as hispidulin 7-sulfate (**15**). Compound **14** shows a band II λ_{min} at 252 nm with a concave inflexion on both sides, while compound **15** shows a band II λ_{min} at 247 nm with a convex inflexion on both sides (Fig. S21and S31).

2. A purification sub-fraction containing a mixture of disulfate derivatives (**3**, **6**, **7**) showed a minor compound eluted between 5-methoxyluteolin 7, 3'-disulfate (**3**) and luteolin 7, 3'-disulfate (**6**), which was below the detection level in the crude extract. Its on-line UV spectra (269 and 320 nm) and order of elution are consistent with the presence in the crude extract of traces amounts of luteolin 7, 4'-disulfate (Barron et al., 1988).

3. Two chromatographic peaks of low intensity and poor resolution were detected in LC/MS. Their spectra showed odd $[M+H]^+$ values at 450 and 480, typical of organic substances containing one nitrogen atom, and odd $[M-80+H]^+$ ions at 370 and 400 indicating the presence of one sulfate group, but the low signal-to-noise ratio did not allow exploitation of other fragmentations (Fig. S29 and S30). These data support the presence of traces of flavoalkaloid monosulfates with molecular weight of 449 (Rt ~25 min) and 479 (Rt ~26 min), but their concentration was too low to obtain usable on-line UV spectra. Takagi et al. (1980) had isolated low amounts of a flavoalkaloid derivative from *P. iwatensis*, which they named phyllospadine ($C_{21}H_{21}NO_6$, MW = 383). Its purification required acetylation with acetic anhydride and it was isolated as triacetate of hispidulin 8-(1-methyl-2-pyrrolidinyl) ($C_{27}H_{27}NO_9$, MW = 509). Forty mg of phyllospadine tri-acetate were obtained from two kg of dried plant, accounting for 15 μg gdw^{-1} of phyllospadine. Takagi et al. (1979) also analyzed the flavonoid content of *P. japonicus*, but phyllospadine was not found in this species. Neither phyllospadine nor its hypothetical sulfate was found in our extracts, but the two nitrogeneous compounds detected in the present study would probably share a number of similarities with this substance. Flavonoid alkaloids are of interest due to their rarity, their amphoteric nature, and the biological activity of some of their natural sources. They result from the convergence of two distinct biosynthetic pathways, affording natural products with a wide range of interesting biological activities that would not be expected for flavonoids or alkaloids alone (Blair et al., 2017). Our next step in this respect will be to collect the large amounts of plant material needed to isolate and characterize these very minor compounds.

2.3. Inter-annual variability in the phenolic content of P. torreyi

A total of five sampling campaigns of fresh samples of the aerial part of *P. torreyi* (batch number: PhyF 1-5, each constituted of three independent samples) were conducted in La Jolla (Fig.1)

over different years (2015-2017) and seasons (summer and winter). Aqueous-methanolic extracts were prepared from each collection and individual phenolic compounds were quantified by HPLC in each of the fifteen crude extracts. The quantitative variation of flavonoids and phenolic acids was found to be relatively consistent from year to year. Comparison of the summer extracts showed similar HPLC profile and a weak variability in the phenolic concentrations. For all samples, the flavonoid pattern was found largely dominated by the disulfates (**3, 6** and **7)**. Nepetin 7, 3'-disulfate (**7**) had the highest content among all compounds in all tested samples (6.07-7.81 mg g^{-1}) and represented 41 to 52% of the flavonoid pool. The second most abundant product was 5-methoxyluteolin 7, 3'-disulfate (**3,** 1.73-3.86 mg g^{-1}; 12-29%), followed by luteolin 7, 3'-disulfate (**6**; 1.42-2.32 mg g^{-1}; 10-13%). The minor flavonoids **2**, **4** and **9** were only detected in the summer samples. The flavonoid 7-sulfates (**8**, **11**, **12**, and **15**) and 3'-sulfates (**13**, **14**) were present in all the samples in moderate amounts, while jaceosidin 7-sulfate (**16**) was detected only in summer. Low amounts of caffeic- (**1**) and coumaric acid (**5**) were found in all the samples (0.075-0.158 mg g^{-1} and 0.027-0.041 mg g^{-1} respectively). These two phenolic acids are very common in seagrasses (Zapata and McMillan, 1979). Seasonality was observed for rosmarinic acid (**10**) whose concentration was higher in summer (1.057-2.264 mg g^{-1}) than in winter (0.361-0.491 mg g^{-1}). We had previously isolated rosmarinic acid from *Z. marina* and *Z. noltei*, which also belong to the Zosteraceae family (Achamlale et al., 2009a). This is the first report of rosmarinic acid in a member of the genus *Phyllospadix.* The results are summarized in Table 1 and Figure 4, which show the average concentrations of the three harvest replicates for each sampling campaign.

2.4. Biological potential of the P. torreyi phenolic content - Evaluation of P. torreyi beach cast detritus as a renewable source of bioactive compounds

Plant specialized metabolites are economically important in the field of food additives, nutraceuticals, and drugs. This is especially the case for phenolic acids and flavonoids, which possess a broad spectrum of pharmacological properties. Additionally, sulfated flavonoids have the advantage of being more water-soluble than their aglycones, which is an interesting property for therapeutic applications. Flavonoid sulfates are now recognized as potential candidates for the development of new drugs and some lead compounds are emerging mainly as anticoagulant and antiviral agents (see as examples: Martins et al., 2018; Teles et al., 2018).

The antiviral activity of luteolin 7, 3'-disulfate has been demonstrated using *in vivo* experimental model of tick-borne encephalitis (Krylova et al., 2011). Luteolin 7, 3'-disulfate also exhibits antidiabetic and antihyperlipidemia effects, as well as antioxidant, cardioprotective, anti-inflammatory, hepatoprotective, antitumoral and antiviral actions (Styshova et al., 2017).

Luteolin 7-sulfate was shown to inhibit cellular melanin synthesis (Kwack et al., 2016; Lee et al., 2019). Luteolin-7-sulfate is also a nontoxic repellent, able to prevent bacterial settlement (Papazian et al., 2019).

5-Methoxyluteolin exhibited a good antioxidant activity in DPPH and CUPRAC assays and a low cytotoxicity against 4T1 breast carcinoma cell line (Rafieian-Kopaei et al., 2020). Different extracts of *Plectranthus* species containing 5-methoxyluteolin showed antioxidant, anti-inflammatory, analgesic, diuretic, cytotoxic and antimicrobial activities with variable potency (El-Hawary et al., 2012). Schistosomicidal activity of 5-methoxyluteolin was also reported. Schistosomiasis, which is caused by trematode flatworms of the genus *Schistosoma*, is one of the most significant and neglected tropical diseases in the world (Pimenta et al., 2015).

Nepetin has been widely used for centuries to treat fever, malaria, infections and diseases associated with inflammation (Lee et al., 2016). Nepetin also exhibited cytotoxic activity against five tumor cell lines (Militão et al., 2004), and strongly protected primary cultured neurons against glutamate-induced oxidative stress (Kim et al., 2002). In recent years, nepetin was reported to exhibit anti-inflammatory, anti-oxidant and anti-tumor cell proliferation effects (see as example Chen et al., 2020 and references therein). Strong antiproliferative activity against different cell lines was also observed with nepetin (Talib et al., 2012). In the case of luteolin, it has been shown that the 7,3'-disulfate has a lower toxic potential than the non-sulfated form and that the efficacy of its pharmacological action may therefore be increased (Styshova et al., 2017). On this basis, the two unreported disulfates of nepetin and of 5-methoxyluteolin described here could constitute potential candidates in the search of new drugs.

A strong antiproliferative activity against different cell lines was observed for hispidulin (Talib et al., 2012). Hispidulin also suppress angiogenesis and growth of human pancreatic cancer (He et al., 2011) and potently inhibits human glioblastoma multiform cells (Lin et al., 2010). Jaceosidin was identified as a strong antimutagen (Nakasugi et al., 2000), and a putative oncogene inhibitor which might be used as a potential drug for the treatment of cervical cancers associated with the human papillomavirus (Lee et al., 2005). As mentioned above, it could be of interest to evaluate their respective 7-monosulfate.

Rosmarinic acid is a bioactive phytochemical, which possesses remarkable pharmacological activities, including antioxidant, antisettlement, anti-inflammatory, antiviral, antibacterial, antidepressant, anticarcionogenic, nematicidal, and chemopreventive properties. Rosmarinic acid also has significant antinociceptive, neuroprotective, and neuroregenerative effects (Amoah et al., 2016; Noor et al., 2022; Styshova et al., 2017). In addition, rosmarinic acid is able to prevent bacterial settlement in marine environment (Papazian et al., 2019).

Given the economic potential of the phenolic content of *P. torreyi*, it seemed worthwhile to assess the accumulated detrital stocks on beaches. The production of Torrey's surfgrass is very high with an average maximum biomass estimated at 586 g dw m^{-2} day^{-1} (shoot) and 486 g dw m^{-2} day^{-1} (rhizome) (Les, 2020). Physical events and natural seasonal leaf drop result in large accumulations of detritus along the wave-exposed sandy beaches in southern California. Seagrasses are much more resistant to decomposition than are freshwater angiosperms or algae. The rate of decomposition of seagrass detritus is generally low (< 1% of dry wt/day) compared with other sources of detritus (Mews et al., 2006). We have previously shown in the case of *Zostera spp* and Cymodoceaceae members that significant concentrations of phenolic compounds remain in the detrital leaves (Achamlale et al., 2009; Grignon-Dubois and Rezzonico, 2017).

Seagrass beds have been shown to be one of the most efficient ecosystems for carbon sequestration. However, if disturbed or degraded, they can release carbon into the environment and accelerate the rate of global climate change (Macreadie et al., 2014). Seagrass wracks also have an important ecological value and provide important ecosystem services (see as example: Nordstrom et al., 2011). However, wrack piles can also release CO_2 and CH_4 during the decomposition process and thus become a significant source of greenhouse emissions. In addition, stranded litter can significantly reduce local tourism income and local managers are under strong public pressure to remove seagrass wracks when they accumulate along beaches and shorelines used for recreational purposes. The negative impact on the tourism industry in the affected areas results in costly beach cleanup and disposal processes. In most cases, the collected biomass is disposed of in landfills.

Phyllospadix wracks are resistant to decomposition (Mews et al., 2006), and answers may come from flotsam recycling. Compared to algae, seagrasses remain largely unexploited as a raw material for the production of bioactive compounds, despite the fact that they offer enormous opportunities to find phytochemicals of commercial value. A detrital sample was collected from plant materials deposited on the shoreline, between La Jolla Underwater Park (+32.8528 N; -117.2614 W) and Ellen Browning Scripps memorial pier (32.8662 N; -117.2614 W). It was collected at the same time as the fresh sample Phy3-F, and was referenced as Phy3-D (Table 1). An aqueous-methanolic extract (50% v/v) was prepared in the same conditions used for fresh samples and analyzed by quantitative HPLC. Its chromatographic profile was found to be similar to that of Phy3-F and the concentrations of the individual, although lower than in the fresh material, remain significant (Table 1, Fig. 4). Nepetin 7, 3'-disulfate remains the major product (**7**, 4.46 mg g^{-1}), followed by luteolin 7, 3'-disulfate (**6**, 1.49 mg g^{-1}), and 5-methoxyluteolin 7, 3'-disulfate (**3**, 1.19 mg g^{-1}). The rosmarinic acid content also remains significant (**10**, 0.54 mg g^{-1}). The significant amounts of disulfates in *Phyllospadix* detritus are particularly interesting given the broad spectrum of biological

properties of nepetin and luteolin derivatives. These results make this abundant detrital biomass interesting for dietary and pharmaceutical applications.

3. Discussion

3.1. Previous reports on Phyllospadix species and other Zosteraceae

The flavonoid content in seagrasses varies widely between different genera and species. Flavones are predominantly found in seagrass belonging to the families Hydrocharitaceae and Zosteraceae, whereas flavonols are mainly found in Posidoniaceae and Cymodoceaceae (McMilan et al., 1980). A survey of 43 species of seagrass showed that five of the 12 genera contained flavonoid sulfates namely two Zosteraceae (*Phyllospadix* and *Zostera*), and three Hydrocharitaceae (*Enhalus*, *Thalassia* and *Halophila*) (McMilan et al., 1980). Most of these compounds are sulfate esters of common hydroxyflavones or their methyl ethers, and more rarely of their glycosylated derivatives. In some flavonoid glycosides, however, the sulfate group may be linked to the sugar moiety as in *Thalassia* species (Barron et al., 1988; Hawas and Abou El-Kassem, 2017).

The presence of sulfated flavonoids had previously been detected in the five *Phyllospadix* species, but they had neither been isolated nor identified, and the results were solely based on their electrophoretic mobility on paper (McMillan et al., 1980). Only the two Asian species have been characterized for their flavonoid content (Table 5). A mixture of eight flavonoids was isolated from *P. japonicus*, namelly 6-hydroxyluteolin, 5-methoxyluteolin, hispidulin, jaceosidin, 6-methoxy diosmetin, acacetin 5-methyl ether, and acacetin 6-methyl ether (Takagi et al., 1979). Hispidulin was also found in *P. iwatensis* along with luteolin and a flavonoidal alkaloid designated as phyllospadine (Takagi et al., 1980). More recently, luteolin 7-sulfate and hispidulin 7-sulfate have been identified as additional constituents of *P. iwatensis* (Kwak et al., 2016). It is noteworthy that the 13 flavonoid sulfates we found in *P. torreyi* are derived from five of the eight aglycones isolated from the Asian species, namely 6-hydroxyluteolin, 5-methoxyluteolin, luteolin, hispidulin, and jaceosidin (Table 5). The flavonoid profile of *P. torreyi* in which all thirteen individuals are sulfated, while only two flavonoid sulfates were reported for *P. japonicus* and none for *P. iwatensis*, is remarkable and interesting from a chemotaxonomic point of view. Also noticeable is the high prevalence of flavonoid disulfates in *P. torreyi* (70-90% of the total), among which compounds **3** and **7** were undescribed. Systematic relationships within *Phyllospadix* have not been investigated in any detail. Isozyme patterns have been compared for the three American species, with higher similarity reported between *P. scouleri* and *P. torreyi* than between either species or *P. serrulatus* (Waycott et al, 2006). Phylogenetic relationships within *Phyllospadix* using molecular markers have not yet been studied.

Very often, flavonoid sulfates co-occurr with flavonoid glycosides. This is the case of the genus *Zostera*, which is considered as a sister group of *Phyllospadix* (Les et al., 1997). Six flavonoid sulfates along with four glucosides were reported for *Z. marina* and three flavonoid sulfates along with four glucosides for *Z. noltei* (Barron et al., 1988; Enerstvedt et al., 2017; Grignon-Dubois and Rezzonico, 2018). In contrast, none of the flavonoids detected in *P. torreyi* showed the presence of a sugar unit.

3.2. Formation and putative role of flavonoid sulfates in seagrasses

Flavonoids are known to undergo sulfation conjugation in several families of plants. Studies on biosynthesis using [^{3}H] cinnamate and [^{35}S] sulfate have shown that the incorporation of the sulfate group is the last step in their biosynthesis (Barron et al., 1988). The 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is the universal donor of active sulfate for many sulfation reactions in specialized metabolism (Mugford et al., 2009). The transfer of the sulfonate group to the flavonoid hydroxyl group is catalyzed by cytosolic sulfotransferases. However, how sulfate is transported into the vacuole remains unknown. Sulfate is a major cellular anion in the cytoplasm and vacuole of terrestrial plant cells. Most flavonoids detected in *P. torreyi* are substituted at the 7-position which corresponds to the most reactive hydroxyl groups in flavones (7-OH). The biochemical and molecular characterization of sulfotransferases exhibiting specificity for position 7 of flavonoids has been described for algae and phototrophic bacteria (Hernández-Sebastià et al., 2008). To date, the sulfotransferases which catalyze the sulfation of flavone in Zosteraceae have not been identified and characterized.

Very little is known of the accumulation of flavonoid sulfates in seagrass tissues and their functional roles remain unclear. Harborne (1977) suggested that conjugation with seagrass flavonoids might be a possible route for inactivation or storage of inorganic sulfate, and could have a dynamic function in salt uptake and metabolism. A sulfate conjugate is more water soluble than the unsulfated molecule or even the corresponding glycoside, thus facilitating transport to various cellular compartments and exudation in the water column. Taxonomic and ecological implications were evoked by McMillan et al. (1980). Luteolin 7-*O*-D-glucopyranosyl-2-sulfate has been shown to chemically defend the tropical seagrass *Thalassia testudinum* K.D. Koenig (Hydrocharitaceae) against zoosporic fungi and pathogenic *Labyrinthula sp.* (Trevathan-Tackett et al., 2015). The ability of flavonoid sulfate extracts from *Zostera spp.* to inhibit the growth of the HAB *Alexandrium catenella* (Whedon and Kofoid) Balech (Ostreopsidaceae) has been demonstrated (Laabir et al., 2013). Some studies indicate that sulfated flavonoids are involved in detoxification of reactive oxygen species and regulation of plant growth (Varin et al., 1997). Sulfation might also represent a

measure of detoxification that enables algae and seagrass to store inactive forms of metabolites that are activated by settling organisms and then act as defense. This is the case of sulfated phenolic acids widely distributed in Dasycladales, which serve as storage metabolites for more active desulfated forms against marine bacteria (Kurth et al., 2015a). The anti-fouling activity of zosteric acid from *Zostera* species was also revisited, which revealed that this sulfated form is only a precursor to the more active coumaric acid that is released by sulfatases (Kurth et al., 2015b). Similar results have been reported for the inhibitory activity against bacterial colonisation of the sulfated/unsulfated flavonoids couple in *Z. marina.* This led the authors to conclude that sulfate conjugation may not be directly involved in the bioactivity but rather plays a physiological function, for instance to facilitate metabolite cellular and/or long-distance transport within the plant (Papazian et al., 2019).

3.3. Putative influence of habitat and environmental factors on the production of flavonoid sulfates

Specialized plant metabolites are produced in response to various environmental stresses (physical, chemical, biological factors), and thus play an essential role in the plant's adaptation to its environment. Correlations between sulfate synthesis and plant habitat have been evoked by Barron et al (1988). Variability in the flavonoid sulfate pattern of *Z. noltei* throughout Atlantic and Mediterranean range has been reported (Grignon-Dubois and Rezzonico, 2018).

Phyllospadix beds are subject to peculiar environmental pressures. They grow along high-energy rocky shores within the California Current Large Marine Ecosystem (CCLME), which expands from Canada to Mexico, and constitutes one of the most productive regions in the world. The marine bathymetry is characterized by a narrow continental shelf and a large, deep ocean basin. The region is strongly affected by seasonal and interdecadal climate variability, such as El Niño events and the Pacific Decadal Oscillation (Fautin et al., 2010). In addition, the Pacific Northwest coast is tectonically active with numerous seafloor breaks, undersea hydrothermal vents, and deep basins. It is cut by active submarine faults, some of which cross the shoreline such as the Rose Canyon Fault Zone that comes onshore in La Jolla Bay. Numerous submarine canyons dissect the continental shelf. La Jolla Canyon and its shallow tributary Scripps Canyon originate very close to shore (Fig. 1; Paull et al., 2013).

Most of the 72 recognized species of seagrasses typically grow on sediments ranging from sandy to muddy along protected, gently sloping coastlines. The Zosteraceae family consists of three genera, *Zostera*, *Heterozostera*, and *Phyllospadix*. *Zostera* species are considered the closest phylogenetic relatives to *Phyllospadix* species (Les et al., 1997). Unlike *Zostera* and *Heterozostera* that are adapted to grow in soft mud and sand, *Phyllospadix* grows on rocky substrata not normally exploited by seagrasses, with high surf exposure and little or no sediment around the roots. This

peculiar habitat makes the genus *Phyllospadix* ecologically distinct amongst seagrasses. Only three other seagrasses are commonly found on hard substrates, namely *Thalassodendron ciliatum* (Forsk.) den Hartog, *Thalassodendron pachyrhizum* den Hartog and *Amphibolis antarctica* (Labill.) Sonder et Aschers (Cymodoceaceae).

Comparison with *Z. marina* was used by Cooper and McRoy (1988) to define anatomical features of *Phyllospadix* that are considered adaptations to rocky shore environments. These features include greater development of hypodermal fibers and root hairs, thickened rhizomes, and smaller lacunae. Roots have been found to exude mucilaginous material as an adhesive to the rocky substrate, allowing them to withstand wave assaults in high-energy environments (Ruiz-Montoya et al., 2021).

Submerged benthic plants can be classified into two groups on the basis of their attachment to the substrate. Rhizophytes have rhizoids, roots or rhizomes within the substratum (sand or mud) and may derive some of their nutrients from the sediment. Haptophytes are attached to the surface of rocks and must obtain all of their nutrients from the bulk water phase. *P. torreyi* is one of the very few seagrass species that grow on rocks. Thus, unlike most other seagrasses, nutrient uptake across leaves rather than across roots might be very important for nutrient acquisition by *Phyllospadix*. This was confirmed by Terrados and Williams (1997) who demonstrated that *P. torreyi* acquires most of its nitrogen via its leaves. There are currently no studies specifically addressing sulfur uptake by *Phyllospadix*. However, it seems likely that *P. torreyi* must acquire most of its nutrient requirements, including sulfur, from the water column by absorbing them primarily through its leaves, as the rhizomes attach themselves mostly to rocky substrates, which lack available nutrients.

Sulfate is the second most abundant anion in seawater. Seagrasses can acquire sulfur by active uptake of sulfate directly from the water column and sediment pore waters by leaves and roots or by passive intrusion of gaseous sulfide into below-ground tissues (Holmer and Hasler-Sheetal., 2014). Sediment pore waters are considered the principal source of inorganic ions for seagrass growth. Reduction of sulfate to sulfides by sediment bacteria results in their accumulation in anoxic environments. Sulfides are known environmental stressors to seagrasses. Nonetheless, under normal conditions, seagrasses are able to support low levels of sulfide. Oxygen release from the roots is considered an important mechanism for sulfide reoxidation of preventing gaseous sulfide from entering the plants. Intrusion of sediment-derived hydrogen sulfide into the aboveground tissues of seagrass is correlated with reduced growth and has been linked to large-scale die-offs of *Thalassia testudinum* in Florida Bay, USA (Timbs and Durako, 2021). Sulfide intrusion into seagrass tissues has been observed in most seagrass beds studied to date. It is initiated from the root and then spreads to the rhizome and leaves.

It is reasonable to assume that direct uptake of sulfate by the leaves instead of roots may give *Phyllospadix* a metabolic advantage by avoiding the detrimental effects of sulfide stress that most seagrass beds face. Interestingly, the occurrence of rhizomal endophytes was compared between *Z. marina* and *P. scouleri*, which is the closest member of the genus to *P. torreyi* (Shoemaker and Wyllie-Echeverria, 2013). Results showed that *Z. marina* had a significantly higher percentage of infection and the highest total number of taxa, which was attributed to the attachment of *P. scouleri* to the rock. All plant tissues of *Phyllospadix*, *i.e.* leaves, rhizome and roots, are exposed to the water column. The high dominance of flavonoid sulfates in *P. torreyi* while other flavonoid or aglycone derivatives are lacking shows that the plant uptakes sufficient amounts of sulfate from the water column to ensure sulfation of all flavonoids. There are currently no studies specifically addressing the distribution of sulfur in the sediments surrounding Torrey's surfgrass beds. However, the absence of sulfide in sediments from the San Diego Trough has been reported (Kaplan et al., 1963). The San Diego Trough is a sediment-filled graben structure in the Catalina tectonic terrane of the California continental borderland.

Phyllospadix spp. are dominant, slow growing, and long-lived marine angiosperms of exposed rocky coasts (Turner and Lucas, 1985). Unlike the other constituent genera of the Zosteraceae family (monoecious), *Phyllospadix spp* are dioecious. *P. torreyi* is recognized as an important habitat founding species (Les, 2020), and is broadly tolerant of temperature, salinity and light variations. It has amongst the highest levels of primary production reported for seagrasses (Les, 2020). They produce a dense canopy that competitively excludes most sessile animals and algae including giant kelp (Menge et al., 2005; Turner and Lucas, 1985), but the mechanism by which they compete with other organisms remains unknown.

In the current state of knowledge, the existence in *P. torreyi* of 13 flavonoid sulfates, while other flavonoid derivatives or aglycones are lacking is noticeable. Whether or not this phenolic content is related to its distinctive characters and particular habitat described above, and whether or not it plays a role in its dynamics and ability to compete with other organisms, all of these elements contribute to making *P. torreyi* a unique species among seagrasses.

Conclusions

Until now, little consideration had been given to the phenolic content of the *Phyllospadix* genus, and *P. torreyi* had never been investigated. Phytochemical study of crude aqueous-methanolic extracts of *P. torreyi* foliar tissue led to the identification of three phenolic acids and 13 sulfated flavones, of which four were previously undescribed and seven had never been reported for the genus *Phyllospadix*. Quantitative determinations by HPLC-DAD over a three-year period showed a relative

constancy of the phenolic content over time and the high prevalence of flavonoid disulfates (70-90% of the total flavonoids detected). All samples were found to be dominated by nepetin 7, 3'-disulfate, which represents 41 to 52% of the total, followed by 5-methoxyluteolin 7, 3'-disulfate (11-29%) and luteolin 7, 3'-disulfate (10-13%). Considering the economic potential of flavonoid sulfates as candidates in the development of new drugs and nutraceuticals, a sample of detrital leaves was also analyzed and this abundant biomass proved to be of interest for dietary and pharmaceutical applications.

This work constitutes the first phenolic profiling of *P. torreyi* and should provide a foundation for further studies, given the reported biological activities of flavonoid sulfates, and the limited knowledge of the phenolic chemistry of the genus *Phyllospadix*. This genus is considered as one of the most divergent seagrass genera due to its physical habitat and adaptative morphological characteristics (Les, 2020). Our results show that divergence can also occur at the level of specialized metabolites. The flavonoid profile of *P. torreyi*, composed exclusively of sulfates, appears to be unique among seagrasses. This work constitutes an important fundamental step towards future work on the other North American species and the two Asian species for which flavonoid isolation and characterization would benefit from being revisited.

4. Experimental

4.1. General methods

Analytical-grade water was from Sodipro Company (Echirolles, France). Trifluoroacetic acid (TFA), and all the solvents used (HPLC-grade) were purchased from Aldrich Chemical Company (Saint-Louis, Missouri, USA). Standards were purchased from Extrasynthèse (Genay, France). C18 reverse-phase silica gel and HPLC columns were from Macherey-Nagel (Düren, Germany).

Ultrasound-assisted extraction experiments were performed with a Sonorex Digitec H 512 sonicator, 35 kHz (Bandelin, Berlin, Germany), and centrifugation with a Jouan B4i centrifuge (Thermo electron, Waltham, MA, USA).

^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on AVANCE 300 and 600 MHz instruments (Bruker) in DMSO-d_6 (Euriso-Top, Gif-Sur-Yvette, France). Chemical shifts are expressed in δ (*ppm*) values relative to residual undeuterated DMSO-d_6 signal as internal reference. The following abbreviations were used to describe the multiplicities: s = singlet, d = doublet, dd = doublet of doublet. Coupling constants are reported in hertz (Hz). ^{13}CNMR assignments were made by 2D HSQC and HMBC experiments. Ultraviolet (UV) spectra were recorded on a V-630 UV-VIS Jasco spectrophotometer (Easton, USA) in HPLC-grade water. High performance liquid chromatography (HPLC) combined with diode array detection (DAD) was performed on a Thermo

Electron liquid chromatography system. Elemental analysis were carried out on elemental analyzer CHNS 628 from LECO Company (Michigan, USA). High resolution mass spectrometry (HRMS) and low resolution mass spectrometry LRMS) were performed using Electro Spray Ionization (ESI). HRMS were performed using an AccuTOF™ LC-plus (Jeol, USA). LC/MS was performed using a HP1100 (Hewlett-Packard) equipped with an Agilent MSD 1946B simple quad mass spectrometer and a HP Chemstation software.

4.2. Study site and plant material

Phyllospadix torreyi S. Watson (Zosteraceae) is abundant on the intertidal and upper subtidal rocky bottoms of the San Diego County, Southern California (USA) (Terrados and Williams, 1997). The species forms large, monospecific beds and has long, strap-like leave (until 2 m long). All plant used were collected at low tide in La Jolla, San Diego County (Fig. 1), where *P. torreyi* beds on raised rocky benches are very common at most locations. The La Jolla marine habitat includes shallow surfgrass beds, shallow rocky habitat with a high degree of vertical structure and crevices, and deeper rocky habitat characterised by boulder reefs and outcrops, ledge and crevice systems and boulder and rock fields. *P. torreyi* inhabits oceanic coastlines and adjacent tidal pools in the lower intertidal zone (0 to -0.6 m) and shallow subtidal zones (0 to -15 m; typically -4 to -5 m) (Les, 2020). The lower depth limit of vegetative and reproductive surfgrass is where a canopy of the giant kelp *Macrocystis pyrifera* (Linnaeus) C. Agardh (Laminariaceae) begins. Three independent fresh plant samples were collected at low tide from rocky intertidal outcrops between La Jolla tide pools and Whale view point (+ 32.8434 N, -117.2811 W). Detrital samples were collected from plant materials that had been deposited recently on the shoreline, between La Jolla Underwater Park (+32.8528 N; -117.2614 W) and Ellen Browning Scripps memorial pier (32.8662 N; -117.2614 W). We proceeded to five collections of fresh plant from summer 2015 through summer 2017 and one of detrital leaves on summer 2016 (Table 1). Samples were referenced as Phy1-F collected on August 2015; Phy2-F (December 2015); Phy3-F and Phy3-D (August 2016); Phy4-F (December 2016); Phy5-F (August 2017). Three independent samples were collected for each campaign.

P. torreyi is a diploid clonal plant with several long, thin leaves arranged in shoots along a prostrate rhizome. Identification of the species was based on morphology plant anatomy and on comparison with identification keys reported in literature (Kuo and den Hartog, 2001). The identity of the collected specimens was authenticated at the laboratory of Prof. Dayton (Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA).

After collection, the samples were thoroughly rinsed in seawater, and then quickly washed in freshwater to remove sand and salt (twice repeated). The collected material was hand-picked to remove associated debris when present. Leaves were separated from rhizomes, and then plant material was air-dried at room temperature to constant weight. Dried plant material was cut into small pieces (0.5-1 cm) prior to extraction. The drying method (lyophilisation or air-dried at room temperature) had no effect on the results.

4.3. Extractions

The same typical procedure was applied for all independent samples. Plant material (10 g) was thoroughly mixed with 150 ml of a 50:50 (v/v) mixture of de-ionised water and methanol, ultra-sonicated for 15 min, and then macerated at room temperature (24 h). The extraction process was repeated twice. The three resulting extracts were pooled together, and centrifuged (5 °C, 30 min, 6000 g). Fifteen ml of the supernatant was set aside for HPLC. The remaining was evaporated under *vacuo* until complete elimination of the methanol, and then the aqueous solution was freeze-dried.

4.4. Purification of compounds for structural elucidation

Twenty five grams of a crude aqueous-methanolic extract of the air dried plant (110 g) was diluted with water adjusted to acidification to pH 3 and serially extracted in a separating funnel with dimethyl chloride (F1), ethyl acetate (F2), and then *n*-butanol (F3). The three resulting phases were evaporated to dryness and then analyzed in HPLC and NMR. Fraction F1 (0.2% yield) constituted of a complex mixture of apolar products. Fraction F2 (0.6% yield) was also a complex mixture, among which caffeic-, coumaric- and rosmarinic acid were identified by UV and comparison to authentic standards. Only the butanolic fraction (11.2% yield) was found to contain significant amounts of flavonoids. Analysis of the final aqueous phase (78% yield) showed the presence of salt, sugars and the absence of flavonoids. All the purification steps were monitored by HPLC and NMR.

The individual phenolics were isolated from the butanolic fraction by repeated column chromatography on C18 reverse-phase silica gel. Gradient elution was done with H_2O-MeOH as the mobile phase starting from 99:1 to 20:80. Fractionation was monitored by HPLC and NMR. Structure elucidation of the isolated compounds was based on UV, NMR and MS spectroscopies and confirmed by co-injection and comparison to authentic standards when available.

4.4.1 5-Methoxyluteolin 7, 3'-disulfate **3**

Yellow amorphous powder; RT 13.9 min; for ^{1}H and ^{13}C NMR (300 MHz, DMSO-d_6): see Tables 2-3 and Fig. S2-S4; ESI-HRMS (positive mode) *m/z*: 460.9843 $[M+H]^+$ (calcd for

$C_{16}H_{12}O_{12}S_2$ 460.9848 $[M+H]^+$); UV spectra (DAD on-line 264 and 332 nm; UV (MeOH) 266 and 327 nm, Fig. S18).

4.4.2 Luteolin 7, 3'-disulfate **6**

Yellow amorphous powder; RT 17.8 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, (DMSO-d_6): see Tables 2-3 and Fig. S5-S7; ESI-HRMS (positive mode) *m/z*: 446.9681 $[M+H]^+$ (calcd for $C_{15}H_{10}O_{12}S_2$ 446.9692 $[M+H]^+$); UV spectra (DAD on-line 267 and 334 nm; UV (MeOH) 267 and 329 nm, Fig. S19).

4.4.3 Nepetin 7, 3'-disulfate **7**

Yellow amorphous powder; RT 18.7 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, DMSO-d_6): see Tables 2-3 and Fig. S8-S10; HR-ESI-MS (positive mode): *m/z* 476.9759 $[M+H]^+$ (calcd for $C_{16}H_{12}O_{13}S_2$ 476.9719 $[M+H]^+$); UV spectra (DAD on-line 264 and 332 nm; UV (MeOH) 266 and 327 nm, Fig. S20).

4.4.4 Luteolin 7-sulfate **11**

Yellow amorphous powder; RT 23.7 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, DMSO-d_6): see Tables 2-3; HR-ESI-MS (positive mode) *m/z*: 367.0122 $[M+H]^+$ (calcd for $C_{15}H_{10}O_9S$ 367.0118 $[M+H]^+$); UV spectra (DAD on-line 264 and 349 nm; UV (MeOH) 266 and 347 nm).

4.4.5 Nepetin 7-sulfate **12**

Yellow amorphous powder; RT 24.3 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, DMSO-d_6): see Tables 2-3; HR-ESI-MS (positive mode) *m/z*: 397.0141 $[M+H]^+$ (calcd for $C_{16}H_{12}O_{10}S$ 397.0151 $[M+H]^+$); UV spectra (DAD on-line 273 and 347 nm; UV (MeOH) 256, 275 and 345 nm).

4.4.6 Luteolin 3'-sulfate **13**

Yellow amorphous powder; RT 25.6 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, DMSO-d_6): see Tables 2-3; HR-ESI-MS (positive mode) *m/z*: 367.0120$[M+H]^+$ (calcd for $C_{15}H_{10}O_9S$ 367.0118 $[M+H]^+$); UV spectra (DAD on-line 268 and 334 nm; UV (MeOH) 267 and 330 nm).

4.4.7 Nepetin 3'-sulfate **14**

Yellow amorphous powder; RT 26.5 min; for ^1H and ^{13}C NMR (300 and 75.47 MHz, DMSO-d_6): see Tables 2-3 and Fig. S11-S13; HR-ESI-MS (positive mode) *m/z*: 397.0149 $[M+H]^+$ (calcd

665 for $C_{16}H_{12}O_{10}S$ 397.0151 $[M+H]^+$); UV spectra (DAD on-line 236, 273 and 335 nm; UV (MeOH)
666 239, 276 and 333 nm, Fig S21).

667

668 *4.5. Acid hydrolysis of the crude extracts*

669 One hundred milligram samples of crude extract were separately dissolved in 100 ml of
670 methanol and stirred with 5 ml of TFA under heating until total disappearance of the sulfated
671 flavonoids as monitored by HPLC. After evaporation of methanol under *vacuo*, the reaction mixture
672 was partitioned between *n*-butanol and water. Addition of $BaCl_2$ to the aqueous layer gave a white
673 precipitate of $BaSO_4$. The butanolic fraction was evaporated to dryness, and then analysed by HPLC,
674 UV and NMR, and comparison with lit. data and authentic samples of flavonoid standards. Results
675 showed the large predominance of nepetin along with luteolin and 5-methoxyluteolin. 6-
676 Hydroxyluteolin, hispidulin and jaceosidin were also present, which is in agreement with the presence
677 of their respective di- and/or monosulfate found as minor products in the crude extracts.

678

679 *4.6. Qualitative and quantitative HPLC analysis*

680 Separation and quantification of phenolics in each of the *P. torreyi* crude extracts were
681 performed using a liquid chromatography system (Thermo electron) equipped with a SCM 1000
682 solvent degasser, a thermostatically controlled column compartment, an AS 3000 autosampler with a
683 100 µl loop, a PDA UV6000LP detector and a Chromquest Chromatography Workstation. The
684 Nucleodur C18 sphinx RP column (110 Å pore size, 5 µm particle size, 250 × 4.6 mm i.d.) was found
685 to offer the best separations. Separations were carried out at 40 °C and the analytes were eluted at a
686 flow rate of 1 ml min^{-1} using the binary gradient 0.1% (v/v) TFA in water (A) and methanol (B). The
687 following linear gradient was used: 0 min, 80 % A; 60 min, 80% B. Run time, 50 min; stop time, 50
688 min; post time, 10 min. UV spectra were collected over the range of 220-380 nm. The injection
689 volume was 20 µl. Data were integrated using Chromquest software. In addition, they were processed
690 to create a chromatogram, in which each peak represents the absorbance of the eluting substance at its
691 λ_{max} (max-plot chromatogram). Stock solutions of the crude extracts were prepared in HPLC-grade
692 dmso/water (4:1, v/v). All solutions were filtered prior to analysis through a 0.20-µm syringe filter
693 and injected three times into the HPLC.

694 Chromatograms were systematically recorded at 270, 328 and 350 nm, which allowed a clear
695 distinction between benzoate-, hydroxycinnamate-, and flavonoid derivatives. Chromatographic
696 peaks of the crude extracts were checked for peak purity and identification was achieved by matching
697 the Rt and on-line UV spectra of standards.

Quantitative determinations of flavonoids were carried out by peak area measurements at 330 nm, using an external calibration curve of luteolin dissolved in DMSO. The curve was established on six data points, covering the concentration range 0.0619-0.00619 mg ml^{-1}. Linear regression on the HPLC analyses gave R^2 values of 0.9995. Data are expressed in milligrams luteolin equivalents per g of *P. torreyi* dry matter. Quantitative determinations of rosmarinic acid were carried out by peak area measurements at 328 nm, using a calibration curve of rosmarinic acid. The linear regression coefficient was 0.9997 (6 points). Quantitative determinations of caffeic acid were carried out by peak area measurements at 328 nm, using a calibration curve of caffeic acid at the same wavelength (R^2 0.9996, 6 points) and determination of coumaric acid at 310 nm using a calibration curve of coumaric acid (R^2 0.9995, 6 points). Data are expressed in mg per gram of dry matter (mg g^{-1} dw) of plant tissue (mean ± standard deviation (SD) of three determinations). The HPLC analysis of standards was performed using exactly the same conditions as for the extracts.

Data presented in Table 1 were calculated using the following equation:

individual phenol compound = C / Cs

where C is the concentration of the tested phenolic compound (mg ml^{-1}) in the analysed crude extract, calculated from peak areas and linear regression; Cs is the concentration of the sample (mg ml^{-1}), diluted in dmso/deionised water 4:1 (v/v).

4.7. LC/MS analysis

LC/MS was performed using an HP1100 (Hewlett-Packard) equipped with an Agilent MSD 1946B single quad mass spectrometer and HP Chemstation software. Positive mode ESI spectra of the column eluate were recorded in the range of *m/z* 120-1000 a.m.u. Absorbance was measured at 280 nm. Compounds were separated using a Zorbax poroshell C18 column (Agilent): 100 mm × 3 mm i.d, 2.7 µm particle sizes. The analytes were eluted at a flow rate of 0.2 ml min^{-1} using the binary gradient (v/v) formic acid in water (pH = 2.55, A) and methanol (B). The following linear gradient was used: 5 % B to 100 % B (15 min). Separation of the analytes was carried out at 50 °C. The injection volume was 2 µl. For mass spectrometric analysis, compounds were detected using the following conditions: nebulising gas pressure, 60 psi; drying gas flow rate, 12 l min^{-1}; drying gas, temperature, 350 °C; capillary voltage, 4000 V; temperature source, 350 °C. Data were acquired in full scan mode (*m*/*z* 100-1000) at a fragmentor voltage of 70 V.

4.8. Statistical Analyses

Concentrations of each compound were compared among the five sampling campaigns using one-way analysis of variance (ANOVA). Before running the analysis, both homogeneity of variance

and normality of data were checked. Homogeneity of variance was assessed using Levene test (David, 2019) while normality of residuals was assessed by way of the Shapiro-Wilk test (Royston, 1995). If significant difference was evidenced by ANOVA, a post-hoc Tukey SHD test was conducted to test for difference between each pair of sampling campaign.

For each compound, there were significant differences in concentrations between plant samples collected during the different campaigns, as shown by analysis of variance (all p-values<0.001). Specifically, compounds **1**-**4**, **9**-**13**, and **15**-**16** had significant differences in concentrations, with plants collected during summer having a higher concentration than plants collected during winter, with the exception of compound **3**, which had a higher concentration in plants collected during the winter compared to the summer. Compounds **5**-**8** and **14** showed significant differences between samples collected during the different campaigns that were not related to season. Results are summarized in Table 6.

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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

Author Contributions

MGD conceived and designed the study, collected plant material, and wrote the manuscript. BR performed the experiments. MGD and BR analyzed the data. HB performed the statistical analyses. The three authors approved the final manuscript.

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List of Figures and Tables

Fig. 1. Schematic map showing the location of the sampling sites in La Jolla, San Diego County, California, USA. 1: site for fresh material. 2: site for detrital material.

Fig. 2. A - Typical HPLC profile of a *Phyllospadix torreyi* crude extract (recorded at 330 nm). Peak number, retention time (min), assignment. **1**: 12.1, caffeic acid; **2**: 13.3, nepetin 7,4' -disulfate; **3**: 13.9, 5-methoxyluteolin 7,3'-disulfate; **4**: 14.3, 6-hydroxyluteolin 7,3'-disulfate; **5**: 16.7, coumaric acid; **6**: 17.8, luteolin 7,3'-disulfate; **7**: 18.7, nepetin 7,3'-disulfate; **8**: 19.2, 5-methoxyluteolin 7-sulfate; **9**: 19.9, 6-hydroxyluteolin 7-sulfate; **10**: 23.2, rosmarinic acid; **11**: 23.7, luteolin 7-sulfate; **12**: 24**.**3, nepetin 7-sulfate; **13**: 25.6, luteolin 3'-sulfate; **14**: 26.7, nepetin 3'-sulfate; **15**: 26.9, hispidulin 7-sulfate; **16**: 27.6, jaceosidin 7-sulfate.
B - HPLC profile after acid hydrolysis. Peak denomination, retention time (min), assignment. **1**: 12.1, caffeic acid; **5**: 16.7, coumaric acid; **17**: 22.9, traces of 6-hydroxyluteolin 3'-sulfate; **10**: 23.2, rosmarinic acid; **a**: 24.1, 6-hydroxyluteolin; **b**: 25.1, 5-methoxyluteolin; **c**: 30.2, luteolin; **d**: 30.9, nepetin; **e**: 33.6, hispidulin; **f**: 34.0, jaceosidin. All numbers and denominations correspond to compounds described in the results section and in Table 1 and Fig. 3.

Fig. 3. Structural formulae of compounds **1-18** and **a-e**. Underlined names indicate the previously unreported products.

Fig. 4. Inter-annual variation in the amounts of phenolic compound in fresh (samples Phy1-F to Phy5-F) and detrital (sample Phy-3 D). Concentrations values on ordinate are given as mg g^{-1} dw of plant tissue, mean values ± SD (n = 3). Products are given in order of elution: Caff : **1**; Nep7,4': **2**; OMeLu2S: **3**; 6OHLu2S: **4**; Coum: **5**; Lu2S: **6**; Nep2S: **7**; 5OMeLu7S: **8**; 6OHLu7S: **9**; RA: **10**; L7S: **11**; Nep7S: **12**; Lu3'S: **13**; Nep3'S: **14**; Hispi7S: **15**; Jaceo7S: **16**. See Fig. 3 for formulae and Table 1 for full data.

Table 1. Peak number, HPLC retention time (Rt), sampling dates and sample names, concentration (mg g^{-1} dw of plant tissue, mean values ± SD) of phenolic compounds from *Phyllospadix torreyi*.

Table 2. ^{1}H-NMR data for flavonoid sulfates **3**, **6**, **7**, **11-15** in DMSO-d_6 (δ *ppm*, *J* (Hz)).

Table 3. ^{13}C-NMR chemical shifts for flavonoid sulfates **3**, **6**, **7**, **11-14** (δ *ppm*, DMSO-d_6).

Table 4. Diagnostic ^{1}H and ^{13}C NMR sulfation shifts for flavonoid sulfates **3, 6, 7, 11-15** (DMSO-d_6); values are expressed in *ppm* as δ (flavonoid sulfate) - δ (flavonoid).

Table 5. Comparison of the phenolic content of *P. torreyi* with literature data reported for *P. scouleri, P. serrulatus, P. japonicus, P. iwatensis,* and other Zosteraceae.

Table 6. Results of one-way ANOVA and Tukey's test on phenolic concentrations among seasons (df: degree of freedom)

Phenolic fingerprinting of the Pacific seagrass *Phyllospadix torreyi* identified 4 undescribed flavonoid sulfates along with 9 known flavonoid sulfates and 3 phenolic acids.

Peak number	Rt min	Structure assignment	Phenolic concentration (mg/gdw plant material)					
			08/ 2015 phy1-F	12/2015 phy2-F	08/2016 phy3-F	12/2016 phy4-F	08/2017 phy5-F	08/2016 phy3-D
1	**12.1**	**Caffeic acid**	**0.075**±0.010	**0.083**±0.007	**0.103**±0.020	**0.078**±0.009	**0.158**±0.005	**0.031**±0.005
5	**16.7**	**Coumaric acid**	**0.037**±0.005	**0.033**±0.004	**0.041**±0.008	**0.027**±0.008	**bld**	**bld**
10	**23.2**	**Rosmarinic acid**	**1.406**±0.037	**0.361**±0.026	**1.057**±0.007	**0.491**±0.007	**2.264**±0.004	**0.535**±0.001
		Total phenolic acid	***1.480***	***0.481***	***1.201***	***0.596***	***2.449***	***0.566***
		% of the total phenolic detected	**0.096**	**0.034**	**0.075**	**0.044**	**0.122**	**0.050**
2	**13.3**	**Nepetin 7, 4'-disulfate**	**0.107**±0.012	**bld**	**0.285**±0.013	bld	**0.217**±0.005	**0.279**±0.002
3	**13.9**	**5-methoxyluteolin 7, 3'-disulfate**	**2.947**±0.026	**3.736**±0.128	**1.726**±0.073	**3.857**±0.034	**1.910**±0.024	**1.189**±0.003
4	**14.3**	**6-hydroxyluteolin disulfate**	**0.248**±0.021	**bld**	**0.403**±0.017	**bld**	**0.281**±0.002	**0.301**±0.002
6	**17.8**	**Luteolin 7, 3'-disulfate**	**1.421**±0.022	**1.740**±0.053	**1.945**±0.058	**1.464**±0.036	**2.316**±0.004	**1.493**±0.005
7	**18.7**	**Nepetin 7, 3'-disulfate**	**6.839**±0.020	**7.113**±0.056	**6.067**±0.079	**6.434**±0.032	**7.806**±0.014	**4.458**±0.016
8	**19.2**	**5-methoxyluteolin 7-sulfate**	**0.243**±0.014	**0.051**±0.017	**0.301**±0.015	**0.390**±0.006	**0.437**±0.002	**0.300**±0.005
9	**19.9**	**6-hydroxyluteolin 7-sulfate**	**0.264**±0.024	**bld**	**0.455**±0.032	**bld**	**0.547**±0.003	**0.322**±0.007
11	**23.7**	**Luteolin 7-sulfate**	**0.107**±0.015	**0.049**±0.010	**0.246**±0.017	**0.125**±0.005	**0.594**±0.003	**0.471**±0.004
12	**24.3**	**Nepetin 7-sulfate**	**0.441**±0.019	**0.170**±0.010	**0.766**±0.012	**0.197**±0.008	**1.027**±0.004	**0.528**±0.002
13	**25.6**	**Luteolin 3'-sulfate**	**0.077**±0.005	**0.089**±0.008	**0.324**±0.004	**0.089**±0.004	**0.741**±0.002	**0.438**±0.002
14	**26.5**	**Nepetin 3'-sulfate**	**0.487**±0.013	**0.529**±0.067	**0.756**±0.009	**0.539**±0.002	**0.556**±0.004	**0.235**±0.003
15	**26.9**	**Hispidulin 7-sulfate**	**0.620**±0.020	**0.251**±0.024	**0.971**±0.011	**0.066**±0.008	**0.785**±0.003	**0.342**±0.002
16	**27.6**	**Jaceosidin 7-sulfate**	**0.146**±0.007	**bld**	**0.661**±0.032	**bld**	**0.432**±0.003	**0.469**±0.002
		Total flavonoid sulfates	***13.948***	***13.728***	***14.906***	***13.160***	***17.650***	***10.826***
		Total flavonoid disulfates	***11.562***	***12.589***	***10.427***	***11.754***	***12.530***	***7.720***
		Total flavonoid monosulfates	***2.386***	***1.139***	***4.480***	***1.406***	***5.120***	***3.105***
		Total phenolic detected	***15.428***	***14.208***	***16.107***	***13.756***	***20.099***	***11.392***

bld: below detection level

Table 2: ^{1}H-NMR data for flavonoid sulfates **3**, **6**, **7**, **11**-**15** in DMSO-d_6: δ (*ppm), J* (Hz).

Compound	3	6	8	2'	3'	5'	6'	OMe
3	6.50 [1 H, s]	6.70 [1 H, d (2.2)]	7.12 [1 H, d (2.2)]	7.84 [1 H, d (2.3)]		6.97 [1 H, d [8.5])	7.63 [1 H, dd (2.3, 8.5)]	3.81 [3 H, s]
6	6.75 [1 H, s]	6.57 [1 H, d (2.1)]	6.98 [1 H, d (2.1)]	7.92 [1 H, d (2.3)]		6.99 [1 H, d [8.6])	7.71 [1 H, dd (2.3, 8.6)]	
7	6.73 [1 H, s)]		7.32 [1 H, s]	7.93 [1 H, d (2.3)]		6.96 [1 H, d (8.6)]	7.71 [1 H, dd (2.3, 8.6)]	3.76 [3 H, s]
11	6.74 [1 H, s]	6.51 [1 H, d (2.1)]	7.01 [1 H, d (2.1)]	7.44 [1 H, d (2.1)]		6.87 [1 H, d [8.9])	7.44 [1 H, dd (2.1, 8.9)]	
12	6.75 [1 H, s]		7.32 [1 H, s]	7.46 [1 H, d (2.0)]		6.86 [1 H, d [8.8])	7.45 [1 H, dd (2.0, 8.8)]	3.72 [3 H, s]
13	6.69 [1 H, s)]	6.18 [1 H, d (2.1)]	6.49 [1 H, d (2.1)]	7.89 [1 H, d (2.3)]		6.96 [1 H, d [8.6])	7.68 [1 H, dd (2.3, 8.6)]	
14	6.44 [1 H, s)]		6.15 [1 H, s]	7.79 [1 H, d (2.3)]		6.93 [1 H, d (8.6)]	7.58 [1 H, dd (2.3, 8.6)]	3.67 [3 H, s]
15		6.81 [1 H, s]	7.33 [1 H, s]	7.91 [1 H, d (8.78)]	6.89 [1 H, d (8.78)]	6.89 [1 H, d (8.78)]	7.91 [1 H, d (8.78)]	3.73 [3 H, s]

Table 3: ^{13}C-NMR chemical shifts for flavonoid sulfates **3**, **6**, **7**, **11**-**14** (δ *ppm*, DMSO-d_6).

C number	3	6	7	11	12	13	14
2	160.3	164.2	165.0	164.5	164.4	163.6	162.0
3	106.66	103.3	102.9	103.2	102.4	103.2	102.3
4	176.1	182.3	182.6	182.1	182.3	181.9	180.6
5	160.0	159.6	151.5	160.6	151.4	161.8	152.8
6	99.9	102.3	134.1	102.2	133.2	99.1	133.5
7	158.4	159.4	153.9	159.6	152.8	164.5	154.0
8	99.8	97.7	98.5	97.7	98.3	94.1	95.5
9	158.4	156.6	152.7	156.4	152.6	157.4	153.0
10	109.5	105.9	106.4	105.3	106.2	104.0	101.2
1'	121.8	123.6	121.0	121.1	120.5	121.6	122.3
2'	120.3	120.6	120.6	113.3	116.3	120.8	120.1
3'	141.7	141.5	141.8	145.1	146.4	141.6	141.7
4'	152.5	153.5	153.1	150.3	151.3	152.8	154.0
5'	117.7	117.4	117.8	116.2	113.1	117.5	117.6
6'	123.1	123.5	123.8	119.3	119.3	123.5	123.0
OMe	56.4		60.5	-	60.2		59.2

Table 4:

Aglycon	Sulfate derivatives	H-8	H-2’	H-5’	H-6’	C-7	C-8	C-2’	C-3’	C-4’
luteolin 5-methyl ether	7,3’-disulfate : **3**	+0.66	+0.53	+0.12	+0.34	-4.3	+3.1	+7.2	-4.2	+3.4
	7-sulfate : **11**	+0.58	+0.06	-0.01	+0.04	-4.7	+3.6	-0.3	-0.8	+0.4
luteolin	3’-sulfate : **13**	+0.06	+0.49	+0.08	+0.28	+0.2	0	+7.2	-4.3	+3.0
	7,3’-disulfate : **6**	+0.55	+0.54	+0.11	+0.31	-4.9	+3.6	+7.0	-4.4	+3.6
	7-sulfate : **12**	+0.85	+0.06	+0.01	0	-0.1	+4.0	+0.1	+0.5	+1.5
nepetin	3’-sulfate : **14**	-0.32	+0.39	+0.08	+0.13	+1.1	+1.2	+3.9	-4.2	+5.1
	7,3’-disulfate : **7**	+071	+0.57	0.11	+0.33	-0.1	+4.2	+4.2	-4.1	+3.3
hispiduline	7-sulfate : **15**	+075	-0.01	+0.04	-0.01		+4.8*	-0.4*		

*values determined from HSQC

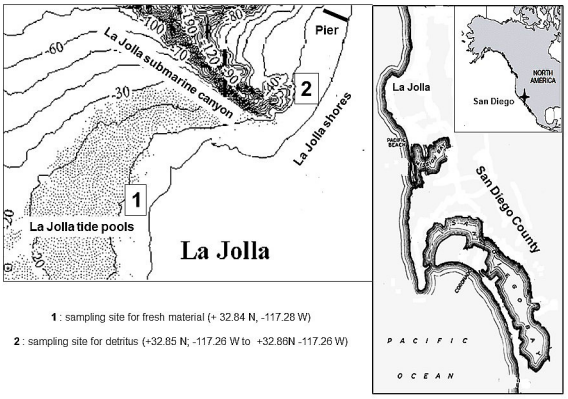
Table 5. Comparison of the phenolic content of *P. torreyi* with literature data reported for *P. scouleri, P. serrulatus, P. japonicus, P. iwatensis,* and other Zosteraceae.

P. torreyi* (this work)	***P. scouleri***	***P. serrulatus***	***P. japonicus***	***P. iwatensis***	**other Zosteraceae**
caffeic acid	caffeic acid (b)	caffeic acid (b)			*Z. marina*, *Z. noltei* (b, f, g, h)
coumaric acid	coumaric acid (a)	coumaric acid (b)			*Z. marina*, *Z. noltei* (b, f, g)
rosmarinic acid					*Z. marina*, *Z. noltei* (g, h)
5-methoxyluteolin 7, 3'-disulfate					
6-hydroxyluteolin- disulfate					
luteolin 7, 3'-disulfate					*Zostera marina* (f, g)
nepetin 7, 3'-disulfate					
5-methoxyluteolin 7 -sulfate					
6-hydroxyluteolin 7-sulfate					
luteolin 7-sulfate				luteolin 7-sulfate, (e)	*Z. marina*, *Z. noltei* (f, i)
nepetin 7-sulfate					
|luteolin 3'-sulfate					*Z. marina* (f, i), *Z. Asiatica* (j)
nepetin 3'-sulfate					
hispidulin 7-sulfate				hispidulin 7-sulfate (e)	
jaceosidin 7-sulfate					
			6-hydroxy-luteolin (c)		
			luteolin-5-methyl ether (c)		
			luteolin 5, 4'-dimethyl ether (c)		
				luteolin (d)	
			hispidulin (c)	hispidulin (d)	
			jaceosidin (c)		
			6-methoxy diosmetin (c)		
			acacetin 5-methyl ether (c)		
			acacetin 6-methyl ether (c)		
				phyllospadine (d)	

*Bold text: products identified for the first time in the genus *Phyllospadix,* Bold Italic text: previously unreported products

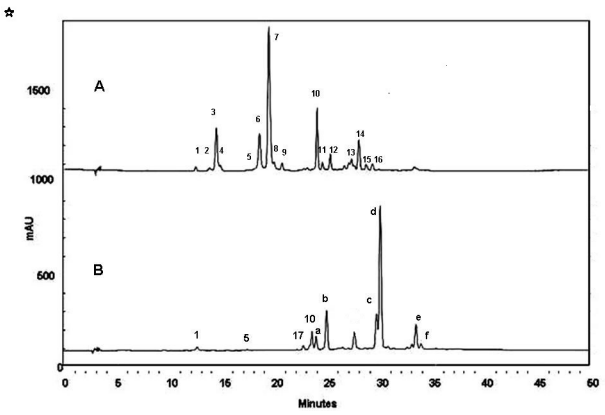
(a) Proksch et al., 1981; (b) Zapata and McMilan, 1979; (c) Takagi et al., 1979; (d) Takagi et al., 1980; (e) Kwak et al., 2016; (f) Harborne and Williams, 1976; (g) Achamlale et al., 2009a; Grignon-Dubois et al., 2012; (h) Ravn et al., 1994; (i) Grignon-Dubois and Rezzonico, 2012, 2018); (j) Kim et al., 2016.

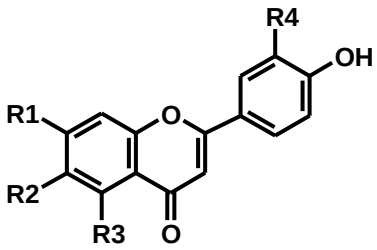
	compounds	df	p-value (ANOVA)		Multiple comparison
higher concentrations in (most) summer	**1**	4	9.05e-5	highly significant	Phy5>Phy3>Phy1=Phy2=Phy4
higher concentrations in summer	**2**	4	2.96e-15	highly significant	Phy3>Phy5>Phy1>Phy2=Phy4(=0)
lower concentrations in summer	**3**	4	1.13e-15	highly significant	Phy4>Phy2>Phy1>Phy5>Phy3
higher concentrations in summer	**4**	4	1.92e-13	highly significant	Phy3>Phy5>Phy1>Phy2=Phy4(=0)
	5	4	1.48e-07	highly significant	[[Phy3>Phy4]=Phy1=Phy2]>Phy5(=0)
	6	4	7.97e-13	highly significant	Phy5>Phy3>Phy2>[Phy4=Phy1]
	7	4	9.16e-12	highly significant	Phy5>Phy2>Phy1>Phy4>Phy3
	8	4	2.06e-12	highly significant	Phy5>Phy4>Phy3>Phy1>Phy2
higher concentrations in summer	**9**	4	9.7e-16	highly significant	Phy5>Phy3>Phy1>Phy4=Phy2(=0)
higher concentrations in summer	**10**	4	7.37e-11	highly significant	Phy5>Phy1>Phy3>Phy4=Phy2
higher concentrations in (most) summer	**11**	4	2.55e-16	highly significant	Phy5>Phy3>[Phy4=Phy1]>Phy2
higher concentrations in summer	**12**	4	<2e-16	highly significant	Phy5>Phy3>Phy1>Phy4>Phy2
higher concentrations in (most) summer	**13**	4	1.3e-15	highly significant	Phy5>Phy3>Phy1=Phy2=Phy4
	14	4	3.0e-09	highly significant	Phy3>[Phy2=Phy4=Phy5]>Phy1
higher concentrations in summer	**15**	4	<2e-16	highly significant	Phy3>Phy5>Phy1>Phy2>Phy4
higher concentrations in summer	**16**	4	<2e-16	highly significant	Phy3>Phy5>Phy1>Phy2=Phy4 (=0)

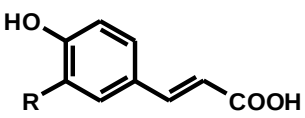


1 : sampling site for fresh material (+ 32.84 N, -117.28 W)

2 : sampling site for detritus (+32.85 N; -117.26 W to +32.86N -117.26 W)







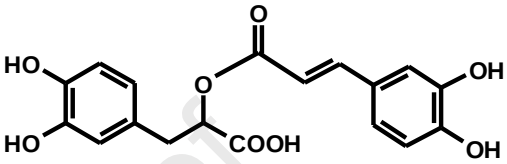
1 : R = OH : caffeic acid
5 : R= H : coumaric acid

Aglycones

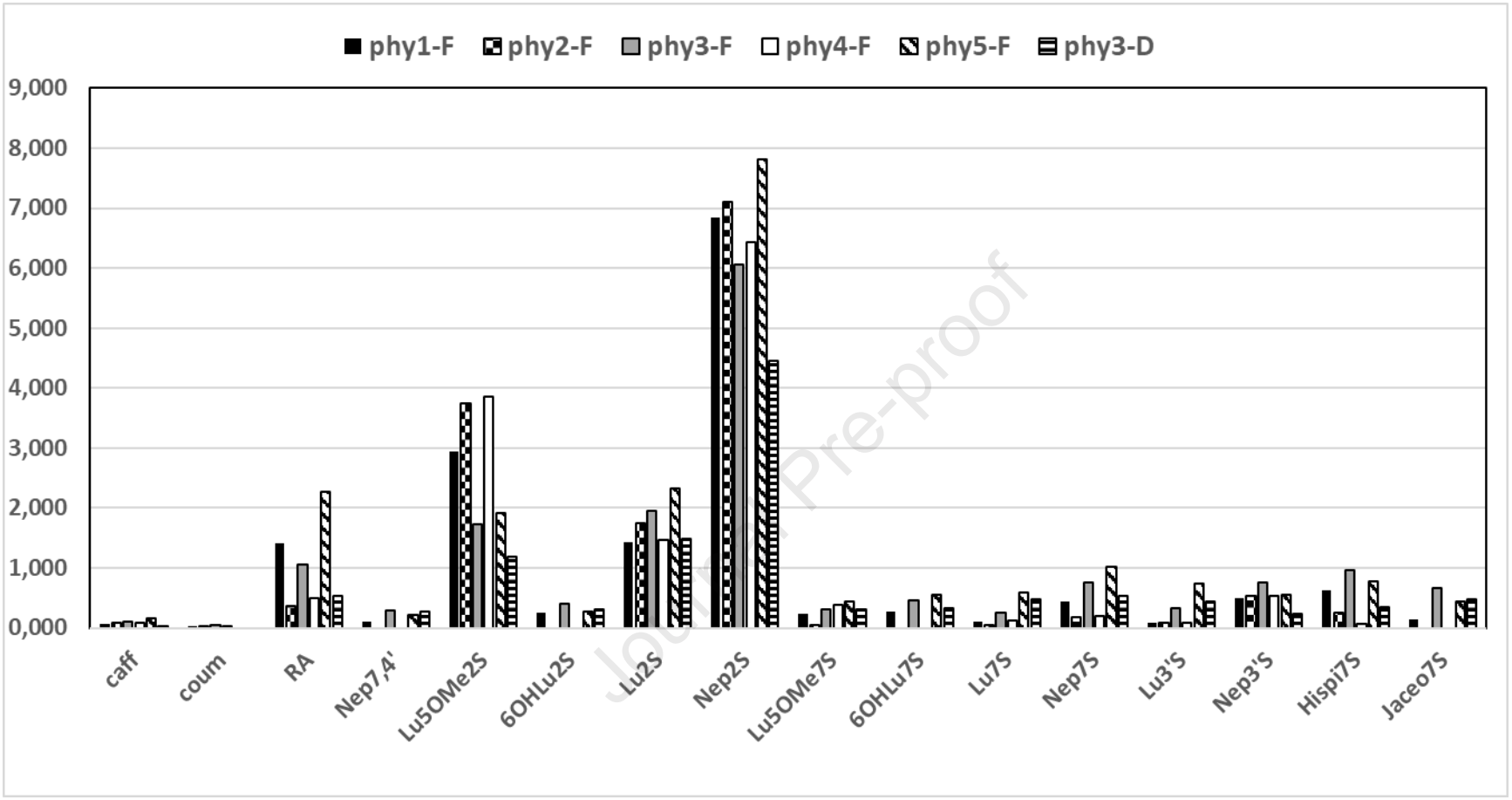
a : R1 = R2 = R3 = R4 = OH: 6-hydroxyluteolin
b : R1 = R4 = OH, R2 = H, R3 = OMe: 5-methoxyluteolin
c : R1 = R3 = R4 = OH, R2 = H: luteolin
d : R1 = R3 = R4 = OH, R2 = OMe: nepetin
e : R1 = R3 = OH, R2 = OMe, R4 = H: hispidulin
f : R1 = R3 = OH, R2 = R4 = OMe : jaceosidin

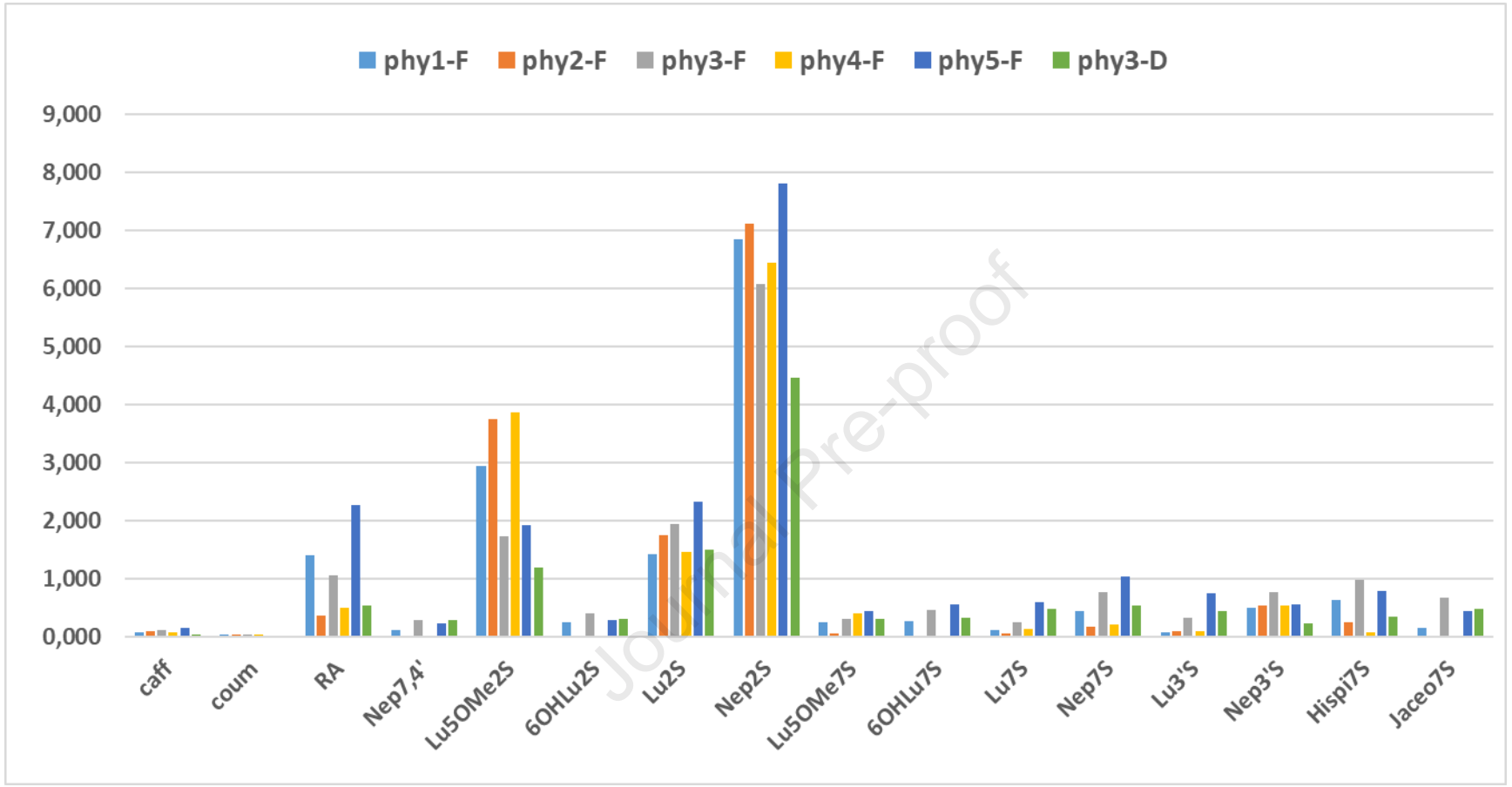
Flavonoid sulfates

2 : nepetin 7, 4'-disulfate
3 : 5-methoxyluteolin 7,3'- disulfate
4 : 6-hydroxyluteolin-disulfate
6 : luteolin 7,3'-disulfate
7 : nepetin 7,3'-disulfate
8 : 5-methoxyluteolin 7-sulfate
9 : 6-hydroxyluteolin 7-sulfate
11: luteolin 7-sulfate
12: nepetin 7-sulfate
13: luteolin 3'-sulfate
14: nepetin 3'-sulfate
15: hispidulin 7-sulfate
16: jaceosidin 7-sulfate
17: 6-hydroxyluteolin 3'-sulfate
18: 5-methoxyluteolin 3'-sulfate



10 : rosmarinic acid





Highlights

- Fingerprinting of *Phyllospadix torreyi* identified 13 flavonoid sulfates and 3 phenolic acids.
- Four were undescribed, and seven had never been reported for the genus *Phyllospadix*
- Seasonal variation of the phenolic content was quantitatively analyzed by HPLC.
- All samples were found dominated by the unreported nepetin 7, 3'-disulfate (41-52% of the total).

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: