Anthropogenic organic matter influence on epicuticular *Tillandsia stricta* Sol. ex Sims and *Tillandsia usneoides* (L.) L. (Bromeliaceae) wax leaf from Rio de Janeiro sandbank

Influência de matéria orgânica antropogênica nas ceras epicuticulares de folhas de *Tillandsia stricta* Sol. ex Sims e *Tillandsia* usneoides (L.) L. (Bromeliaceae) de restingas do Rio de Janeiro

Influencia de la materia orgánica antropogénica en las ceras epicuticulares de hojas de *Tillandsia stricta* Sol. ex Sims y *Tillandsia usneoides* (L.) L. (Bromeliaceae) de bancos de arena de Río de Janeiro

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ABSTRACT

Epicuticular wax from the leaves of *Tillandsia stricta* Sol. Sims and *Tillandsia usneoides* (L.) from Grumari, Marica and Jurubatiba, Rio de Janeiro sandbanks was extracted into chloroform and n-hexane for analyses. The extracts were subjected to gas chromatography (GC-FID) and gas chromatography coupled with mass spectrometry (GC-MS) analyses. In the investigated species, the major n-alkane was found to be C-27. This study has shown that the relation of n-alkanes and fatty acids is a function of the species and the triterpenoids were only observed in the wax of *T.*

usneoides. Phthalates: di-n-octyl phthalate (DNOP), di isobutyl phthalate (DIBP); Di (2-ethylhexyl) phthalate (DEHP), benzoic acid, adipic acid derivatives were also detected beyond of DDT in *T. stricta* from the Conservation Unit of Jurubatiba. The exposure of *T. usneoides* to vehicular emission resulted in the reversal of the relationship fatty acid: n-alkanes and the triterpenoids suppression. These results reflect the importance of plants in monitoring environmental purposes and their potential for DDT and phthalates indicator.

Keywords: phthalates, sandbank native plants, epicuticular wax leaf, DDT, bio indicator

RESUMO
Ceras epicuticulares das folhas de *Tillandsia stricta* Sol. Sims e *Tillandsia usneoides* (L.) das restingas de Grumari, Maricá e Jurubatiba, Rio de Janeiro foram extraídas em clorofórmio e n-hexano para análise. Os extratos foram submetidos à cromatografia gasosa (GC-FID) e cromatografia gasosa acoplada à análises de espectrometria de massa (GC-MS). Nas espécies investigadas, o principal n-alcano encontrado foi o C-27. Este estudo mostrou que a relação de n-alcanos e ácidos graxos é função da espécie e os triterpenóides foram observados apenas na cera de *T. usneoides*. Ftalatos: di-n-octil ftalato (DNOP), di-isobutil ftalato (DIBP); Di(2-etilhexil)ftalato (DEHP), ácido benzóico, derivados do ácido adípico também foram detectados além do DDT em *T. stricta* da Unidade de Conservação de Jurubatiba. A exposição de *T. usneoides* à emissão veicular resultou na reversão da relação ácido graxo: n-alcanos e supressão de triterpenóides. Esses resultados refletem a importância das plantas para fins de monitoramento ambiental e seu potencial indicador de DDT e ftalatos.

Palavras-chave: ftalatos, plantas nativas de restinga, cera epicuticular, DDT, bioindicador

RESUMEN
Las ceras epicuticulares de las hojas de Tillandsia stricta Sol. Sims y Tillandsia usneoides (L.) de los arenales de Grumari, Maricá y Jurubatiba, Río de Janeiro, fueron extraídas en cloroformo y n-hexano para su análisis. Los extractos se sometieron a análisis por cromatografía de gases (GC-FID) y cromatografía de gases acoplada a espectrometría de masas (GC-MS). En las especies investigadas, el principal n-alcano encontrado fue el C-27. Este estudio demostró que la proporción entre n-alcanos y ácidos grasos depende de la especie y que sólo se observaron triterpenoides en la cera de T. usneoides. También se detectaron ftalatos: di-n-octilftalato (DNOP), di-isobutilftalato (DIBP); di(2-etilhexil)ftalato (DEHP), ácido benzoico, derivados del ácido adípico, además del DDT, en T. stricta de la Unidad de Conservación de Jurubatiba. La exposición de T. usneoides a emisiones vehiculares resultó en una inversión de la relación ácido graso: n-alcano y supresión de triterpenoides. Estos resultados reflejan la importancia de las plantas para la vigilancia ambiental y su potencial como indicadores de DDT y ftalatos.

Palabras clave: ftalatos, plantas nativas de arenales, cera epicuticular, DDT, bioindicador

1 INTRODUCTION

Cuticular waxes are a complex mixture of compounds of the aliphatic chain such as n-alkanes, fatty acids, alcohols, aldehydes, ketones, or n-alkyl esters. The physico -chemical properties of cuticular waxes allow the cuticle and other compounds, including flavonoids and pentacyclic triterpenoids (Dragata and Riederer 2007) to perform certain functions, such as reducing

water loss by cuticular transpiration and protection against UV radiation, both with potential significance for plant environments with intense solar radiation. The cuticular waxes also promote interaction with other organisms and chemicals (e.g. the ability to accumulate semi - volatile organic compounds in ambient atmosphere (Schreiber and Riederer 1996, Simonich and Hites 1995) playing an ecological role.

Many compositions of plant waxes from individual species have been reported in order to utilize the data for chemotaxonomic marker, which can be used as a criterion for distinguishing groups of species within families or genera, reflecting the ecological and genetic relationships (Contreras *et al.* 2022, Cordeiro *et al.* 2011, Motta *et al.* 2009).

The plant waxes accumulate Semi-volatile organic compounds (SOC) from environment such as, polychlorinated biphenyl (PCB) and dioxins (Luca *et al.* 2006) and aromatic hydrocarbons pentacyclic (PAH) (Lin *et al.* 2006). The rate of absorption/bioaccumulation of SOCs from atmosphere depends on the epicuticular properties of the plant as well as the physicochemical properties of the substance and environmental factors (Di Guardo *et al.* 2008).

A wide range of substances is thought to cause endocrine disruption, including pharmaceuticals, dioxins –like compounds, polychlorinated biphenyls, DDT and other pesticides, and plasticizers such as phthalates (Schug *et al*. 2011, Erkekoglu and Kocer-Gumusel 2014, Sekiguchi *et al.* 2006) but also it is an environmental agent stress influencing plant development. It can cause various effects on plants such as carotenoid synthesis disturbance or chlorophyll formation, irregular formation of grana, white leaves, necrosis (Hemming *et al.* 1981) and decrease of the photosynthetic/fluorescence parameters (Gao *et al.* 2019, Chen *et al.* 2011) was observed the up and down regulation of genes, involved in water celery growth when treated with (BBP- butyl benzyl phthalate) affecting plant growth, cell cycle and protein synthesis interference and dwarfism. Accumulation of phthalates in stems of some types of cultured crops - *Triticum atrium, Brassica nigra, Zea mays* (Zornikoza *et al.* 2011) and medicinal and oil plants (Omidpanah *et al.* 2018) has been reported.

Major contributors to air pollution occur primarily in high-density urban areas. These are smoke belching from vehicles and industrial emissions. Emissions caused by traffic are mainly those of carbon monoxide, carbon dioxide, nitrogen oxides. Volatile organic compounds (VOC) and small dust particles. The leaf is the first target of air pollution, then Luzimar *et al.* (2005) reported anatomical and micromorphological alterations in leaves of *Eugenia uniflora* L. (Myrtaceae) and *Clutia robusta* Pax, which were exposed to simulated acid rain. They observed erosion and morphological modifications of epicuticular wax in both epidermis of leaves, necrosis in leaf margin, rupture of stomate edges and occasional leaf perforations. According Kayode and Otoide

(2007) on the investigation about industrial pollution and densely populated region in the leaf epidermal morphology of *Newbouldia laevis* revealed epidermis cell aberrations and erosion. For polluted area samples, the frequency of stomata was higher, and the average leaf area was reduced (about 50%) when compared to non-polluted areas.

This paper describes the chemical composition of leaf epicuticular waxes obtained from extracts of n-hexane and chloroform *T. stricta* and *T. usneoides* from Rio de Janeiro sandbanks and the effect of urban vehicular emission.

2 EXPERIMENTAL

a- Plant material and sampled areas

Tillandsia stricta Sol. ex Sims and *T. usneoides* (L) L., Bromeliad epiphytes plants, were collected and maintained in paper bags, in the Grumari Environmental Protection Area, at the Maricá Environmental Protection Area and the Restinga de Jurubatiba National Park (Fig.1), at the state of Rio de Janeiro, Brazil (Table 1). Authorization to collect was granted by the Brazilian government authorities (Municipality of Rio de Janeiro, State Institute of Environment (INEA)| and authorization to access the Genetic Heritage was granted by the Biodiversity Authorization and Information System (SISBIO) of the Ministry of Environment (26198-7).

Fig.1. Sampling of *Tillandsias* in Jurubatiba National Park.



Table 1. Collection areas of *T. usneoides* and *T. stricta* with location,mean annual precipitation and temperature and Latitude and longitude.

Area	Location	Mean annual		Latitude/ Longitude
		Precipitation (mm)	Temperature ˚C	
Grumari EPA[1]	Rio de Janeiro	1173	23.7	23º02'S/ 46º31'W
Maricá EPA[1]	Marica, RJ	1230	23.2	22º57'S/ 42º31'W
Restinga de Jurubatiba National Park[2]	Carapebus, Macaé, RJ	1164	22.6	23º03'S/ 43º35'W
Urban high traffic road[3]	Rio de Janeiro city	1278	23.2	22°95'S/-43°17'W

EPA -Environmental Protection Area; [1](INMET), [2] Araujo *et al*. 1998), [3](Clima Brasil Rio de Janeiro).

A voucher specimen was deposited at the herbarium of Federal University of Rio de Janeiro State: *T. stricta* HUNI 2058 and *T. usneoides* HUNI 2060. All samples were collected in the same season (November 2012) and no one displayed visible morphological alterations.

Part of the *T. usneoides* from Jurubatiba was continuously exposed to urban high traffic roads (35 days). Jurubatiba area was considered as a controlled site.

The procedure employed an extraction step, where 5-10g sheet were immersed (30 s) in a pre-weighed flask containing 30 ml of n-hexane or chloroform (HPLC grade). The extracts were dried by evaporating the solvent at room temperature. The experiment was conducted in a completely randomized design with two replications for each species, location and solvent.

All chemicals and reagents were kept in glass bottles and vials were closed with PTFE to avoid any contamination of phthalates.

For analysis by GC-FID and GC-MS, the extracted waxes were methylated with diazomethane and solubilized in chloroform (0.01 µg.µl-1). For analysis by GC-FID, 1µL of the chloroform solution was injected into a Shimadzu GC -2010 equipped with a capillary column DB - 5MS (30m x 0.25 mm x 0.25 m). The injection was performed in split mode, with rates 10, 20, or 30:1. The injector was maintained at 240 ºC and the detector at 300 ºC. The temperature was set to range from 60-300 ºC with a heating rate of 3 ºC min -1, and then hold isothermal at 300 ºC for 40min. Helium was used as carrier gas.

Analysis by GC-MS was performed on Shimadzu QP2010 Plus equipped with ZB- 5MS capillary column (30 m x 0.25 mm x 0.2 µm) and detector QP2010 GC- MS operating in electron impact ionization potential of 70 eV with scanning mass from 50 to 650 Da. The same conditions of temperature injection and programming described above were applied.

The identifications of n-alkanes were obtained by comparison with commercial standards (Sigma Fluka C8-C20 and C21-C40. The components were quantified using a standardized data signal obtained by GC-FID area. Only were identified components to similarity index indicated by

the spectra library (NIST 107 mass spectral data base) equal to or greater than 80 %. The components with 1% relative abundance were identified.

Statistical Analysis: Principal Component Analysis (PCA) was performed among the variables analysed using Past software to test the similarities of the samples according to their chemical composition.

3 RESULTS AND DISCUSSION

The wax composition of the studied species is shown in Table 2. The hexane extracts from *T. stricta* and *T. usneoides* showed n-alkanes, other hydrocarbons, triterpenes, and phthalates as the main constituents. n-alkanes were composed of C-21 to C-30, C-27 as major component and such information indicated the biogenic source.

T. stricta waxes were characterized by fatty acids and n-alkanes while triterpenes were observed only in *T. usneoides* waxes, where 24-methylene cycloartanone was already reported by Cabrera and Seldes (1997).

The percent of hydrocarbons were higher for *T. stricta* from Grumari regardless of the type of solvent used except for *T. stricta* from Jurubatiba.

The chemical profile of *T. stricta* was qualitatively and quantitatively constant, regardless of the origin of the plant. In relation to *T. usneoides* leaf wax component, it can be distinguished by the presence of triterpenes.

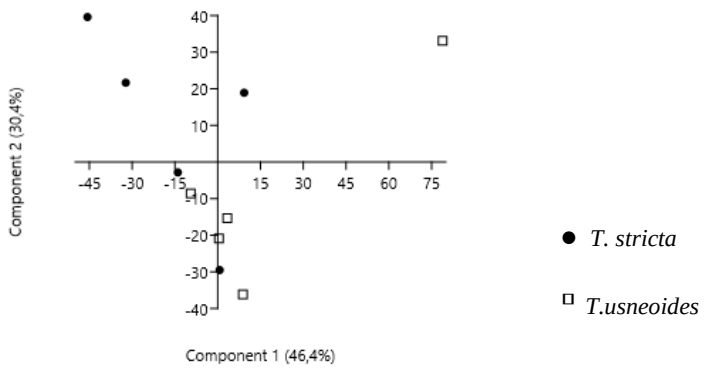
Table 2. The leaf wax component's content (% RA) of *T. stricta* and *T. usneoides* from different collection areas (Grumari, Marica, Jurubatiba) solvent polarity (Hexane, Chloroform) and *T. usneoides* from Jurubatiba submitted for 35 days to urban vehicular emission (Urban).

	T. stricta				*T. usneoides*					
	Grumari		Marica	Jurubatiba	Grumari		Marica		Urban* from Jurubatiba	
	Hexane	Chloroform	Chloroform	Hexane	Hexane	Chloroform	Hexane	Chloroform	Hexane	Chloroform
Fatty Acids methyl esters	3.2	6.0	3.4	42.1	5.6	6.9	2.3		41.9	36.3
n- Alkanes	85.4	64.2	35.0	11.5	32.3	10.4			12.0	27.6
Benzoic acid esters	-	-				8.3				
DDT	-	-	-	3.2	-	-	-		-	-
Triterpenes	-	-	-	-	20.9*					-
cycloartanone						14.1				
DnOP - phythalic acid, dioctyl ester-	-	-	-	-	26.0		90.8			
DEHP- phythalic acid, bis (2-ethylhexyl) ester	3.3	10.8	25.3	20.0		12.6		53.9		
DIBP - phythalic acid, diisobutyl ester	-	-	16.8	2.7	3.0	2.4				
Adipic acid (diéster)				9.8	5.6	6.1	6.2	8.0		
phenylpropanoids						8.0				
ni	1.0	14.0	16.1	5.1	6.5	21.9	0.6		24.5	30.9
idTt	91.9	80.9	80.5	89.2	93.4	68.9	99.3	61.9	53.9	63.9
Tt=	92.9	94.9	96.6	94.0	99.9	90.8	99.9	61.9	75.3	94.8

ni- not identified. Tt= total of identifying components. * Under the analysis conditions the fragmentation patterns do not allow identification.

The PCA showed that the first two factors explained 76.8% of the total variance. Analysing Fig 2 with the composition of the waxes of the samples, it can be concluded that the species differ in their chemical composition, with n Alkanes being the most important, which agrees with what was observed by Contreras (2022), where n alkanes was the dominant class of leaf waxes in *Tillandsia landbeckii* (about three times higher in relation to fatty acids). The chemotaxonomic significance of the wax alkanes has been extensively demonstrated by several studies in Pinales (Maffei *et al*. 2004), Cactaceae (Maffei *et al.* 1997), Umbelliferae, Cruciferae and Leguminosae - Maffei 1996), Crassulaceae (Stevens *et al.* 1994).

Figure 2. Principal component analysis (PCA) of the wax composition of *Tillandsia stricta* and



T. usneoides exposed to a vehicular emission ambient presented a same wax profile in both n-hexane and chloroform extracts. They were constituted by fatty acids and n-alkanes and triterpenes were absent. Although, vehicular organic compounds have been absent in this experiment, they may arise if the plant is exposed to a high traffic urban road. The exposed condition of *T. usneoides* did not favour the accumulation of contaminants on wax surface. However, it is possible to assume that the extraction methodology has not been adequate to extract these constituents.

T. usneoides has been indicated in biomonitoring studies of air pollution. It has proved to be an efficient atmospheric accumulator of Cd, Cr, Cu, Pb, Zn (Viana *et al.* 2011), Cs (Li *et al.* 2012) and Hg (Sutton *et al*. 2014) while for *T. stricta*, to our knowledge, no mention was found.

A level of Dichloro-diphenyl-trichloroethane (DDT 3.2%) was observed only in *T. stricta* from Jurubatiba. Many anthropogenic pollutants, such as polychlorinated biphenyls (PCBs) or DDT, are still present in the environment despite their almost worldwide prohibition (Kylin *et al*. 2003).

The area now constituted by the National Park Jurubatiba with the municipalities of Macaé, Quissamã and Carapebus, whose occupation was initially marked by sugar agriculture, fishing and livestock, recently has been changed to activities related to oil exploration and production. On the other hand, there are still in the region small crops of coconut and passion fruit (Varnier 2010).

The plants varied largely in their ability to accumulate and translocate DDTs. There is evidence that in mesophytic plants a large part of the pollutants contained in a leaflet may be externally accommodated such as in the cuticle and epidermal walls (Simonich and Hites 1995).

Organochlorine pollutants absorbed from the air are concentrated mainly in their lipid-rich cuticle surfaces and the rate of bioconcentration of organic pollutants is affected by the amount of waxes in Spruce needles (Hauk *et al*. 1994).

Based on these findings, as the leaf waxes from these species are chemically distinct, such composition can be used for taxonomic purpose. Furthermore, as *T. stricta* leaves can accumulate DDT, such compound can be used as a bioindicator.

Benzoic acid derivatives were detected in the chloroform *T. usneoides* extract. Since benzoic acids are not generally present in organisms, they probably originate from non-biogenic sources such as fossil fuel combustion (Kawamura and Kaplan 1986). Adipic acid ester was found in *Tillandsia* from Jurubatiba and Marica samples. This constituent has a dual origin. It is found as natural product and used as nylon precursor in an industrial production starting from benzene oxidation, a fossil fuel component (Yu *et al.* 2014).

All plant samples regardless specie, solvent and locality showed the presence of phthalates (Table 2). Phthalate esters were identified on the plant wax of *T. stricta* from Grumari and *T. usneoides*: DiBP (diisobutyl phthalate) DEHP (di(2-ethylhexyl) phthalate) and DOP (dioctyl phthalate).

The most abundant phthalate was DOP (26%) in chloroform extracts from *T. usneoides* - Grumari and DEHP (25.3%) in hexane extracts from *T. stricta* -Marica. These compounds are not known to be present in waxes of plants, but instead are ubiquitous plasticizers and do occur in urban atmospheres. They are toxic, persistent organic pollutants and they have been found in many environmental samples, such as water, soil (Liang *et al.* 2010, sediments (Huang *et al*. 2008), atmosphere and even in cuticles of ants (Lenoir *et al*. 2012) and humans (Przybylińska and Wyszkowski 2016). Thus, the presence of these compounds in plant wax extracts supports the findings of that the leaf surfaces are an important sink for airborne particulate matter originating from anthropogenic activities.

Our results confirm that the plant's epicuticular wax traps lipophilic substances that are not produced by their metabolism. All investigated plants in all examined localities studied were spontaneously contaminated by phthalates and these plants can be considered as a good witness of phthalate pollution even at places isolated from human activity like Grumari Environmental Area Protection.

The long–term effects of phthalate contamination in plants are unknown, but Zhang *et al.* (2014) had described *Cucumber sativus* as sensitive to the DBP (di-n-butyl phthalate). DBP stress significantly affected enzyme activities (superoxide dismutase, catalase, and peroxidase) lipid peroxidation and caused damage to root cells. Diethyl phthalate (DEP) has been reported from the stem of *Mallotus philippensis* (Jayaraman *et al*. 2011); DEP and derivatives of phthalic acid from the root exudates of *Echinocloa crisgalli* (Xuan *et al.* 2006), leaves of *Ipomoea carnea* (Adsul *et al*. 2009) and leaves and roots of *Plantago major* (Romeh 2013) were isolated.

Virgin (1988) in a study with seedlings of radish (*Raphanus sativus*) and wheat (*Triticum aestivum*) exposed to the vapour of DBP, observed the significant accumulation of phthalate in the cuticular and wax layers.

The absorption of SOCs in leaf waxes (and thus the plant) can be specific. Furthermore, this is dependent on the plant leaf development and on growth conditions (Bakker *et al.* 1998).

Dibutyl phthalate (DBP) is a bioactive ester produced by bacteria (Morsi *et al.* 2010), fungi (Mabrouk *et al.* 2008), algae (Namikoshi *et al.* 2006, Babu and Wu 2010) and by higher plants (Saleem *et al*. 2009, Ruikar *et al.* 2011). DBP varies qualitatively in terms of ^{14}C content in various sources, petrochemicals, or biologicals. For biological sources, ^{14}C level exceeds 50%, whereas for petrochemical ones, the level is undetectable (Namikoshi *et al*. 2006).

Phthalate ester has also been de novo synthesized by freshwater algae and cyanobacteria using medium containing $NaH^{13}CO_3$ (Babu and Wu 2010), marine brown algae (*Bangia atropurpurea)* but their biosynthesis process and physiological role have not been clear so far (Chen 2004).

Nevertheless, the presence the phthalates in plants sources might be associated with environmental exposure. The production or formation of new types of phthalates in the plant is still in doubt and discussion between scientists.

4 CONCLUSIONS

The results clear indicate that the chemical profile of epicuticular waxes of *T. stricta* and *T. usneoides* are distinct and could be used as an additional feature in taxonomic identification.

The presence of phthalates and DDT is significant for both species. Thus, the presence of those compounds in wax extracts indicate the leaf surfaces as important sink for airborne substances originating from anthropogenic activities and suggest that *T. stricta* plant can be used as DDT indicator.

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