Brazilian Journal of Forensic Sciences, Medical Law and Bioethics 12(2):147-166 (2024)

Brazilian Journal of Forensic Sciences, Medical Law and Bioethics

Journal homepage: www.ipebj.com.br/forensicjournal

Initial Studies of the Potential of Forensic Palynology in Brazil

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Received 27 September 2022; Accepted 16 April 2024

Abstract. Forensic Palynology has been shown to be effective in some cases around the world, and in countries that usually apply this technique, protocols have been developed in partnership with academic institutions. The premise that pollen and spores found in clothing of a suspect or victim may indicate crime geolocation has been tested in countries with more homogeneous vegetation and predominantly anemophilous pollination patterns. However, the feasibility of this technique in tropical settings is unknown due to the lack of landscape-clothing pollen transfer studies and the impact of predominantly entomophilous vegetation. This study evaluates the forensic potential of pollen, spores, diatoms and foraminifera retained in clothing and shoes as geolocation proxies after contact with a heterogenous landscape in southeastern Brazil. To simulate a crime route, three individuals walked along a trail starting at a beach, passing through restinga vegetation, reaching a mangrove forest. Residues were extracted from pants, shirts and shoes by washing and later treated with hydrofluoric acid and acetolysis for mineral and organic content removal, respectively. Diatoms were extracted with hydrogen peroxide and hydrochloric acid and foraminifera through filtration and flotation processes. The results show that these microtraces were found in the clothing and shoes worn during the simulation and indicated direct contact with landscapes such as beach and restinga vegetation, mangrove forest as well as fresh, brackish and seawater. This study suggests that the palynological technique can be added to Brazilian Forensic protocols used by scientific police forces as by other countries with similar environmental realities.

http://dx.doi.org/10.17063/bjfs12(2)y2024147-166

Keywords: Criminalistics; Geolocation; Diatoms; Foraminifera; Neotropical vegetation.

1. Introduction

The field of Forensic Palynology refers mainly to the use of evidence based on pollen grains and spores in legal proceedings[1,2], but in its broadest application, Palynology can also be used to refer to the use of other microscopic organisms such as diatoms[3] and foraminifera[4], among others. The identification of these biological traces can allow geolocation of specific areas where the reproductive structures were produced. In addition, the combined use of pollen, spores and diatoms can help to track geolocation in terms of origin and thus link an individual or item to a specific crime scene[5].

Unlike several countries where palynological technique and the analysis of other microtraces are usually employed in Forensic Science[2,5–7], Brazilian Criminalistics, in general, lacks of studies that can validate the use of these biondicators in solving crimes[8]. One of the assumptions informally used to prevent their use in forensic work is that the tropical flora does not leave palynological signatures in clothing and other materials due to the predominance of zoophilous pollination, to the detriment of anemophilous pollination, characteristic of temperate vegetation[9,10]. Consequently, most forensic palynological procedures are more common in temperate countries, especially in the northern hemisphere[11]. Moreover, the major impediment to the development of this technique in brazilian forensic science is the lack of information on the transfer of palynological signatures, emitted by the tropical vegetation, to clothing or other accessories. Another factor to be considered refers to the high biodiversity of brazilian ecosystems and heterogeneity of tropical vegetation that can hamper geolocation studies and increase the complexity of interpreting the pollen signal[6,12–14].

Based on Locard's Exchange Principle which postulates "*every contact leaves a trace*"[15,16], this exploratory research aims to analyze and quantify the transfer of palynological and other microtraces to clothes in a crime simulation occurring in two different ecosystems of a coastal region in Brazil, focusing on the interaction between the environment and the different types of cloth.

Our final goal is to raise data such as the feasibility of using this analysis, as well as pointing out which clothing would be most important in real research, to validate the use of these techniques in solving crimes, considering regions of tropical vegetation.

2. Materials and methods

2.1. Study area

The study area is located within the Bertioga Restinga State Park (PERB), State of São Paulo, Brazil, a nature preserve area within the Brazilian Atlantic Rainforest domain, one of the most biodiverse regions on Earth[17]. This important ecosystem, presently fragmented along the coast, still holds a significant botanical species richness that could allow people to be linked to places. For instance, there are around 20,000 plant species distributed among angiosperms, ferns, lichens, mosses and liverworts[18], which shows that this type of forest has a very complex spatial dynamic in its vegetal composition. The Brazilian Atlantic coast also comprises a set of deciduous and semi-deciduous forests, in addition to a mosaic of various connected plant formations that interact in space, such as mangroves, dunes and restinga forests[19]. The dispersion of pollen and spores in this region is predominantly zoophilous, a pollen transfer process from one flower to another which is carried out by animals, mainly insects[9,10].

2.2. Simulations

A crime simulation route was carried out using a trail connecting a starting point on the Itaguaré Beach, adjacent to a restinga forest, near the entrance to the PERB (Figure 1). The second point is in an area of mangrove vegetation. The methodology applied followed three main stages: **A.** Crime simulation route; **B.** Extraction of microtraces retained in clothes after exposure to the route; **C.** Chemical treatment of sediments; **D.** slide mounting for light microscopy; **E.** pollen identification and counting

For the crime simulation route, carried out on September 19, 2017, clean and never worn clothing as well clothing previously worn in the city of Sao Paulo, still within the Atlantic rainforest domain, were used by three individuals, coded as 1, 2 and 3, with average height 5,6ft within the same route under different circumstances of contact with the environment (Table 1).

Figure 1. Crime simulation route. (A) Starting point on the beach; (B) Mangrove region; (1) Route leaving the starting point; (2) Return to the starting point; (3) trail to the road; (4) trail to the mangrove region, returning by the same route to the road. During fieldwork, the river was less voluminous than shown on the satellite image. Photo: Google Earth (accessed on January 2019).

Table 1. Route specifications, contacts with the environment and accessories for each individual participating in the simulation.

INDIVIDUAL	CLOTHING	BEACH	MANGROVE
1	Pants' lower leg piece and shoe sole	Shallow portion of the river, water down to the calf	Peripheral portion, only on dry substrate
2	Pants' lower leg piece, sweatpants, tennis shoes and shoe soles	Deeper portion of the river, water up to the knee	Transition between dry and wet substrates
3	T-shirt, jeans and shoes	Deep portion of the river, water to the thigh	Inner portion, on firm wet substrate, followed by contact with the river bordering the mangrove, up to the calf

The starting point was in the dune region of Itaguaré Beach where all individuals walked on the sand, crossing a shallow strip of Itaguaré River. On the way back, the participants crossed a wider part of the river, closer to the sea and returned to the starting point. From this point, all individuals walked through an opentrail of restinga forest until reached Highway BR 101 (Rio-Santos).

From there, they followed another open sandbank trail, but with more dense vegetation than the previous one, which borders the mangrove. Upon arriving at the site, the participants carried out a short walk through the mangrove, during low tide, returning by the trail to the final stop at BR-101 highway.

2.3. Clothing characterization

The clothing/accessories sampled were: t-shirt, pants and shoes previously worn in the urbanized area of the city of São Paulo and new shoe soles and new pants’ lower leg pieces used only in the route. A total of 11 samples were obtained:

- **T-shirt:** Simple model t-shirt, short sleeve, 100% cotton (sample 5).
- **Pants:** Samples 3 and 4 show two basic differences: denim fabric and synthetic sweatpants, respectively, where in the first the wefts are larger tightly packed with little free space between them whereas the sweatpants fabric is characterized by finer and loosely packed fibers.
- **Pants’ lower leg pieces:** These were made of a denim-like thin malleable fabric cut to approximately 35 cm x 35 cm and attached to the participants’ pants.
- **Shoes:** Samples 1 and 2 are from a pair of tennis shoes, which were worn by the same individual during the path, with the only difference that sample 2 had a fixed extra sole, which was also included in the analyzes. Sample 6 is a pair of leatherette boots that have been washed and processed together. The tennis shoes are composed of a porous fabric, while the synthetic leather boot has a smooth surface.
- **Shoe soles:** The shoe soles attached to the participant shoes are models of casual sneakers and social shoes, with no apparent differences in the depth of the grooves, made of rubber material.

In order to facilitate the interpretation, a visual guide of all individual samples analyzed and their contact with the environment is given on Figure 2.

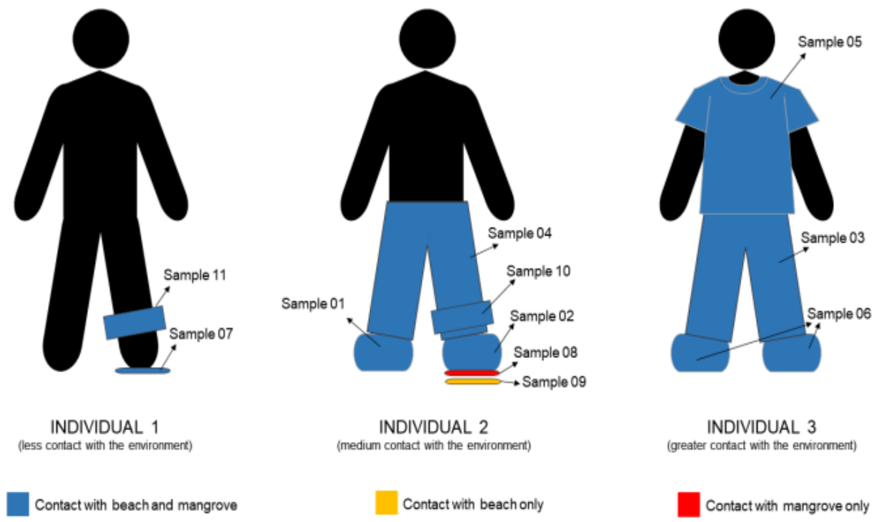

Figure 2. Illustration of the individuals participating in the simulation route, contact with different environments and their sample numbers.

2.3. Laboratory work

After exposure to the environment, the clothing were removed and individually packed in airtight bags to avoid cross contamination. Each bag was identified with the individual code who used it and the points the sample came in contact with. After completion of the crime simulation route, samples were taken to the laboratory and the materials were placed in a previously sterilized drying oven at 50°C overnight, thus avoiding the proliferation of fungi.

2.3.1. Residue extraction

Each dried clothing was placed separately in a 10 liters sterilized beaker. The t-shirt, pants and lower leg denim pieces were immersed in distilled water with approximately 20 drops of detergent and lightly rubbed to help remove microtraces attached to the fabrics. Shoes and soles were washed with distilled water, followed by removal of the sediments embedded in the recesses of the sole with a small spatula. All liquid resulting from the washing of each sample was centrifuged at a speed of 3000 rpm for 5 minutes to discard the natant portion and concentrate the residue. The precipitates were then subsampled to standardize the sample volume. Samples with a larger amount of sediments (1, 2, 6, 7, 8, 9 and 10) were sub-sampled with a 1 cm^3 sampler, whereas 0.25 cm^3 subsamples were obtained from samples 3, 4, 5 and 11.

2.3.2. Palynological analysis

Samples for palynological analysis were processed based on international standard techniques described in Colivaux et al.[9]. Tablets containing 12,100 ± 1,892 *Lycopodium clavatum* spores were added to each sample as an exotic marker to assist in calculating the concentration of pollen and spore grains[20]. The number of *Lycopodium* tablets used varied from one to two according to the sample volume, being one tablet for 0.25 cm^3 and two tablets for 1 cm^3 samples, respectively. For each sample, three slides were mounted in glycerine and all pollen grains and spores were counted under light microscopy. Pollen and spore grains were measured, photographed and identified after comparison with taxa in the modern neotropical pollen reference collection of the laboratory as well as by comparison with taxa displayed in published palynological literature [9,21–24].

2.3.3. Diatom analysis

Processing and counting of diatom valves were based on the protocols established by Battarbee[25]. For each sample, two slides were mounted and counted until 500 valves were reached. Some representative specimens were measured and photographed, and identification cards were assembled to facilitate counting. To identify diatoms, modern and classic references with images and descriptions of valves and frustules were verified [26–30].

2.3.4. Foraminifera analysis

Residues from samples 5 and 9 that had low pollen, spore and diatom counts, were filtered and floated based on the methodology described in Boltovskoy and Wright[31]. All shells found were counted and identified by comparison with published taxonomical studies[32,33].

2.4. Data analysis

Only pollen/spores and diatoms that occurred more than once during the count were included in the analyses, in order to remove the effect of chance and avoid questioning in court. Percentage and concentration (numbers per cubic centimeter) of each individual taxa were calculated by TILIA and TILIA GRAPH softwares[34]. The concentration of pollen and spores is based on the equation proposed by Stockmarr[20]. For the concentration of diatoms, the equation described in Battarbee[25] was used.

2.5. Bioindicator categories

Pollen grains were divided into four categories: Restinga, Mangrove, Rainforest and Local Vegetation. The “Mangrove” category groups taxa occurring only in mangrove areas. “Restinga” houses taxa that commonly occur in restinga vegetation along the Brazilian coast[35–40]. Although not exclusive to PERB, “Local Vegetation” contains taxa indicated in floristic surveys of the PERB region and/or its surrounding[41–43]. “Rainforest” groups elements found in the local anthropogenic vegetation, occurring throughout the Atlantic Forest domain.

Diatoms were also divided into three categories: Marine, Continental and Brackish. These categories were selected according to data survey[44–46] on the habitat of each of the identified taxa.

3. Results and Discussion

3.1. Shoes (samples 1, 2 and 3)

The pollen and diatom signatures in these samples (Figure 3) reveal an interesting retention pattern explained by the weft size of the different materials. Samples 1 and 2 are made of a porous fabric when compared to sample 6. Only a few taxa, such *Baccharis* and *Hedyosmum*, co-occur in these samples possibly a consequence of their wall ornamentation composed of spines and clavae which might enhance adherence. On the other hand, concentration of *Alchornea*, *Borreria*, *Matayba*, *Schinus* and *Rhizophora* (smaller than 30 μm), is greater in the smoother footwear (sample 6). This retention characteristic of pollen as controlled by porosity was also observed by Levin et al.[47]. Likewise, grains larger than 30 μm, such as *Manilkara subsericea* and greater than 50 μm, as *Cyathea*, verrucated monoletes and *Podocarpus*, were restricted to the more porous fabric shoes (samples 1 and 2).

The pollen signature dominated by *Borreria* and *Vernonanthura* in the boot pair (sample 6), obtained from their extracted residual sum, is characterized by low concentrations, suggesting the low efficacy of certain less porous shoewear in forensic analyses. A total of 47 diatom taxa co-occurs in shoe samples 1 and 2 (Figure 4), with a clear dominance in concentration of the freshwater species *Navicula brasiliana* and *Aulacoseira* sp., followed by the estuarine *Cyclotella litoralis* and *Margaritum* (ex *Podosira*) *terebro*. Because the diatom signal of the route is the same for both shoes worn by the same person, this result might facilitate the analysis by the scientific police force in a case in which both shoes are not available for analysis. For example, in the present study, it would be possible to conclude that the individual had contact with the estuarine environment with just one shoe sample.

Concentration differences of diatoms between shoe materials was also observed by Levin et al.[47], who claim that rougher surfaces tend to maintain a greater number of diatoms than in smoother materials and that larger diatoms are more easily lost on these low porous surfaces. This is reflected in the present study, since, except for *Actinoptychus adriaticus*, all diatoms larger than 33 µm, occur in lower concentrations in the boot (sample 6) when compared to tennis shoes samples (1 and 2).

3.2. Pants (samples 3 and 4)

More pollen grains were retained by the denim pants (sample 3) when compared to the sweatpants (sample 4) as shown in Figure 2. However, diatoms (Figure 4) were better retained in the latter, possibly due to weft size differences of these two fabrics. Since the sweatpants had more widely spaced fibers, it is expected that its mesh retains larger traces such as diatoms than pollen grains that are presented in smaller sizes.

The opposite happens with the jeans fabric and its tightly packed fibers. This differential of pollen retention in jeans was observed by Webb et al.[48] in laboratory conditions that highlight greater retention of bioindicators in this type of fabric. This hypothesis is corroborated as the retention of only one type of theses microtraces in different tissues is analyzed. Jeans showed an exclusive occurrence of diatom valves with an average length of less than 30 µm, such as *Gramatophora* sp. (14.6 µm x 3.2 µm), *Achnanthes coarctata* (29.8 µm x 7.8 µm), *Diploneis didyma* (28.5 µm x 10.7 µm) and *Pinnularia borealis* (29.7µm x 8 µm). On the other hand, the sweatpants showed diatom valves of exclusive occurrence in this fabric, with averages of length greater than 33 µm: *Pinnularia* sp. (57 µm x 7 µm), *Paralia sulcata* (40.9 µm Ø), *Craticula cuspidata* (34.6 µm x

9 µm), *Nitzschia fasciculata* (63 µm x 7.1 µm), *Navicula semen* (40 µm x 14, 8 µm), *Mastogloia* sp. (33.2 µm x 10.3 µm), *Gyrosigma strigilis* (73.3 µm x 11.7 µm) and *Triceratium favus*, a large diatom, which in the samples always appears broken, in fragments around 70 µm.

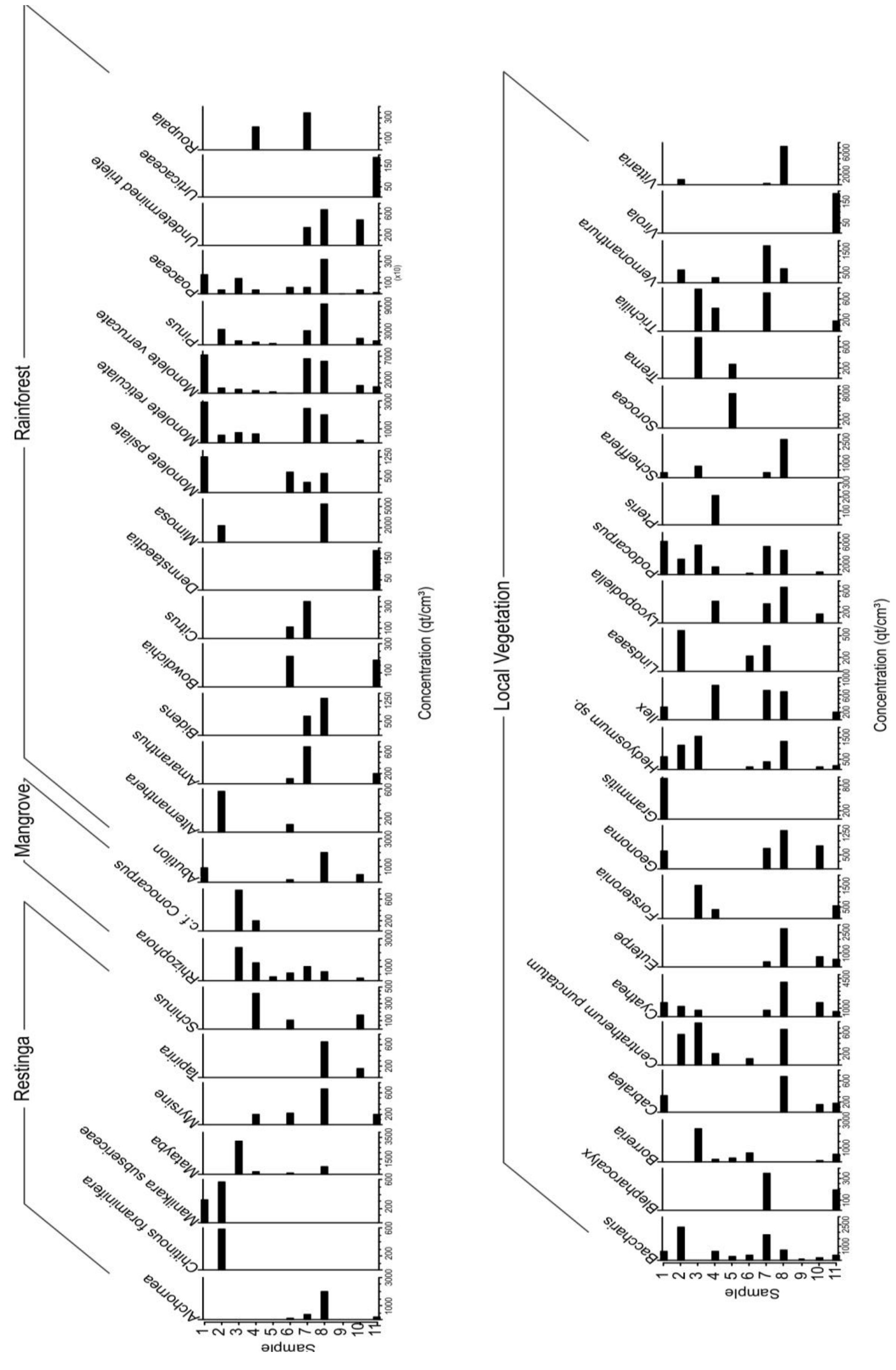

Figure 3. Diagram of total pollen and spore concentration (quantity/cm³) found in all samples.

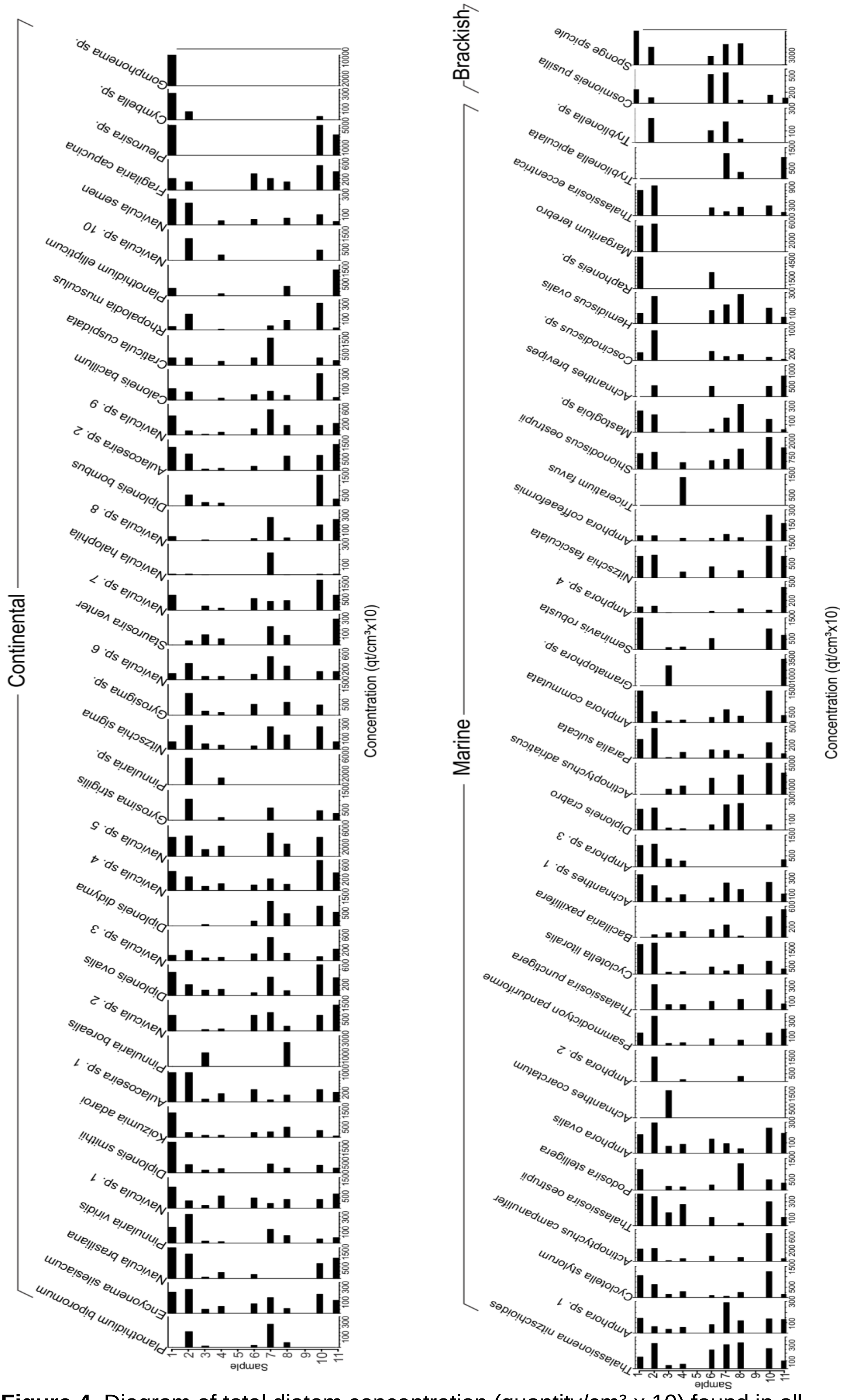

Figure 4. Diagram of total diatom concentration (quantity/cm³ x 10) found in all samples.

The difference between wefts of the pants' fabrics may also explain why sample 3 retained a lower concentration of diatom valves, even though it was the sample with longest contact with water during the simulations. This could be attributed to wefts being unable to retain diatoms and maintain them on their surface, where frustules are more easily removed without great effort as reported by Scott et al.[49]. Although with different concentrations, both samples are indicative of the contact of different types of fabric with aquatic ecosystems, both continental and marine. It is interesting to note that the signal retained in both pants confirms their contact with different coastal ecosystems, with a strong component of the study site's vegetation.

3.3. T-shirt (sample 5)

Concentration values of all pollen and spores found in the analyzed samples are shown on Figure 3. The t-shirt (sample 5) retained only a very low concentration of microtraces, with a count of only 9 pollen grains, easily explained by the nature of the predominantly zoophilous pollination of local vegetation and the fact it did not come in direct contact with the vegetation unlike the rest of the samples. Although not very representative in the Atlantic Rainforest vegetation, some anemophilous taxa yield sufficient pollen dispersed in pollen rain[50], however a participant in motion is unlikely to create a deposition surface for the retention of these grains. The absence of diatoms and foraminifera is explained by the sample not coming into contact with water, an information that may have significant forensic implications.

3.4. Shoe soles (samples 7, 8 and 9)

These samples were exposed to different environments, being sample 7 restricted to the beach and mangrove forest, while sample 8 only to the latter ecosystem and sample 9, only with the beach. The pollen and spore assemblage of samples 7 and 8 (Figure 3) is composed of a total of 33 taxa of which 20 represent co-occurrences, with *Alchornea*, *Podocarpus*, *Pinus* and ferns, represented by monolete verrucate and trilete spores, as the most numerically abundant elements. It is very likely that sample 8 retained more microtraces because sample 7 (Figure 3) accumulated sand grains before coming into contact with the more clayey soil of the mangrove and thus not allowing sediment adherence to the sole.

Riding et al.[51] highlighted the importance of the last environmental sign being more evident in shoe sample, an interpretation that is corroborated by these results. On the other hand, signatures of other environments the sole had contact with were also retained, even if in lower concentrations. In the case of the study region, one single shoe sole would provide useful information for the scientific police force, unlike other types of vegetation with predominantly anemophilous dispersal, especially in temperate climates, where the use of the upper part of the shoe is recommended for retaining more easily the signal carried by the wind[6,52]. The diatom signature in these samples, composed mostly of *Navicula* spp., *Thalassionema nitzschioides* and *Hemidiscus ovalis*, showeds an opposite pattern of retention, being sample 7 more enriched with diatoms than sample 8, probably because of its direct contact with river water at the beach and later with the mangrove forest, whereas sample 8 only had contact with mangrove soil (Figure 4).

Inclusion of foraminifera analysis in this study was decided after the initial observation that sole 9 did not retain neither pollen nor spores on its surface, probably due to the high porosity of sandy soils, which do not allow the accumulation of microtraces because of percolation and eventual oxidation[53]. In this case, predominant foraminifera foram taxa retained by the sole surface were *Pseudononion atlanticum*, *Quinqueloculina* sp., *Arenoparrella mexicana*, *Elphidium* sp., *Hanzawaia boueana* and *Ammonia tepida*. In terms of forensic applicability of these results, it’s noteworthy that samples 5 and 9 contained a very low amount of pollen and spores that, in a real case, could render the analysis unfeasible. Therefore, with the prior knowledge that the route (or crime scene), was in a beach environment, the extraction of foraminifera from samples of individuals (or suspect / or victim), becomes essential to indicate contact with that ecosystem. The association of foraminifera species belonging to *Elphidium* with diatoms of the genera *Achnanthes*, *Diploneis*, *Eunotia*, *Grammatophora*, *Gyrosigma*, *Navicula*, *Nitzschia* and *Pinnularia*, found in sole 9, has been reported by Eichler et al.[33] at Bertioga Channel, which passes through PERB.

3.5. Pant lower leg piece (samples 10 and 11)

The pollen signature retained in pant lower pieces of participants in contact with the mangrove vegetation (samples 10 and 11), is made up of arboreal and shrub elements such *Baccharis*, *Cabralea*, *Euterpe*, *Hedyosmum*, and herbs of *Borreria* and *Poaceae*, typically found in the Atlantic Rainforest, as well as invasive *Pinus* spp. However, sample 10 retained *Rhizophora* grains, the dominant mangrove taxon, a result linked to the physical action of touching the plant in the inner sections of this forest, as opposed to sample 11 which remained mostly at the periphery (Figure 3). This suggests that a more direct contact with *Rhizophora* mangle trees is required for its pollen, which is produced in large amounts[54], to be transferred to clothing.

Moreover, sample 10 was wetted by contact with the river at the beach may also contribute to its greater retention of *Rhizophora*, since pollen grains from this taxon are also commonly transported by water[55].

The diatom signature in relation to contact with water during the route simulation is depicted (Figure 4) by the prevalence of *Amphora ovalis*, *Actinoptychus adriaticus*, *Nitszchia fasciculata*, *Pleurosira* sp., *Shionodiscus oestrupii* as expected by the contact with both brackish and seawater. In terms of concentration, higher values were found in sample 10, which was partially submerged in a mixture of brackish and seawater, at the beach, showed a higher concentration of valves, as already concluded by Scott et al.[49] under similar conditions.

4. Conclusions

This study brings light into the transference of tropical pollen and spore signatures to fabric surfaces. Although dominated by zoophilous taxa, in terms of pollen dispersal, neotropical vegetation emits significant signals which can be captured by clothing and footwear and thus indicate the environment they came in contact with. In this research, bioindicator signatures showed a close relationship with different ecosystems of the coast of Sao Paulo state, Brazil. The study area, despite not having exclusive taxa (with the exception of the mangrove), produces pollen signatures with sufficient evidence for their recognition and geolocation procedures especially when in combination with the diatom and foraminifera analyses.

By gathering all the information from the results presented in each of the variables analyzed, it is possible to observe that specific signals were retained simultaneously in different clothes and materials, suggesting that only a fragment of a piece could be used, without jeopardizing other forensic analyzes and still reflect the environment with which it came into contact.

5. Final considerations

It is important to know what to look for in an evidence[56] and consider the need of an additional analysis. The association of different pieces of clothing and footwear allows for a more robust report, as in the case of the t-shirt (sample 5), which alone was not enough to extract a pollen signature, but an additional analysis of any other piece of clothing of the individual 3 would point out a contact with the area of the walking route. In the case of sample 9, which did not present any bioindicators through the pre-established analyses at work, the need for a foraminiferal analysis became fundamental to prove direct contact with the coastal environment.

It is noteworthy that the samples that came into contact with the mangrove forest did not retain *Acrostichum aureum* spores, an exclusive mangrove fern, even with some of its occurrences along the crime simulation route. It is likely its absence was due to the fact that on that trail, its leaves bore no sporangia. This result illustrates the limitations imposed by the flowering season on geolocation studies based on the representation of specific plant taxa in particular environments, since not all of them present will produce a signature in the pollen spectrum of the analyzed materials.

The results also showed the need for development of additional research to amplify the statistical robustness and therefore interpretation of the results such as soil samples for control and repetition of simulation routes. Therefore, even with important results for forensic interpretation, it is recommended to repeat this study in other areas of the country. Its application in other ecosystems and at different times of the year will expand and corroborate the development of palynological protocols for Brazilian forensic science.

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