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# Blue rayon-anchored technique/*Salmonella* microsome microsuspension assay as a tool to monitor for genotoxic polycyclic compounds in Santos estuary

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**Abstract**

The most important harbor of Brazil is located in Santos Estuary. In the 1970s, this area was one of the major examples of coastal degradation and although the quality of the environment has improved, the sediment is still contaminated with polycyclic aromatic hydrocarbons (PAHs) and mutagenic activity. Because of sediment dredging and consequently contaminants resuspension, it is useful to have reliable methods to monitor the water quality. Considering that blue rayon (BR) has been successfully used in evaluation of mutagenicity and PAHs content the objective of this work was to verify the applicability and adapt the methodology to monitor the water for mutagenic activity using the BR associated with the *Salmonella* assay. Analysis of three sites with different levels of contamination was performed using a modification of the BR hanging method denominated in this work BR anchored technique. The microsuspension protocol of the *Salmonella*/microsome assay was employed with the strain YG1041. The water from the site 1 the most contaminated and under influence of the steel mill discharge presented the highest potency reaching 36,000 revertants/g of BR with S9. Sites 2 and 3 showed less mutagenicity than site 1 with values ~1000 revertants/g of BR. We conclude that the BR anchored technique associated with *Salmonella* assay using YG1041 is a reliable alternative to monitor estuarine waters, especially in regions where sediment resuspension or acute pollution episodes can occur.


*Keywords:* Blue rayon; Mutagens; Water monitoring; Estuarine waters; Santos estuary; *Salmonella*/microsome

## 1. Introduction

Santos estuary, located in southeastern São Paulo State, is economically important considering that it is the major Latin American harbor and the largest industrial complex of Brazil, including steel mill, fertilizer and chemical industries located in this area [1]. In the 1970s, it was considered one of the major examples of coastal degradation [2] and although the quality of the environment has improved in the last years in relation to the chemical parameters and toxicity due to enforcement actions, unfortunately the sediment is still contaminated especially with polycyclic aromatic hydrocarbons (PAHs) and mutagenic activity detected with the *Salmonella* assay [1–5]. In the most contaminated areas total PAHs can reach 347.55 μg/g of sediment (dry weight) [2] and 720,000 revertants/g of sediment (dry weight) [6]. The contaminants present in the sediment, adsorbed or not to particles, can adversely affect

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Table 1
Results expressed in range of mutagenic ratio (MR), that is calculated dividing the mean of the number of revertants per plate for the tested dose by the mean of the negative control, using the *Salmonella*/microsuspension assay in a single dose experiment (0.5 g of blue rayon per plate) for sites 1 and 2 obtained previously by Kummrow et al. [18]

| Sampling site | sampling | TA98 | | YG1041 | | TA100 | | YG1042 | |
|---|---|---|---|---|---|---|---|---|---|
| | | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Site 1 | I | | | Tox. | | NP | NP | | |
| | II | | | | | | | | |
| Site 2 | I | | | | | | | | |
| | II | | | | | | | | |

NP - Not Performed
Tox. - Toxic (MR<0.7)
Mutagenic Ratio ranges:
0|—2 2|—4 4|—6 >6

the sediment-associated community, as well as be resuspended into the water column by navigation and dredging activities affecting the water quality and the aquatic organisms [7].

Umbuzeiro et al. [6] found only low levels of mutagenic activity in the water despite of the high levels of PAHs and mutagenic activity in the sediment of some of the sites evaluated. They considered that the extraction/concentration method (XAD-4) and the *Salmonella* strains used (TA98 and TA100) were not sensitive enough to detect the genotoxic activity suspected to be present in the water of the most contaminated areas. The total PAHs in the water of these sampling sites were below the detection limits of the method applied [2]. In another area from Brazil, Paraíba do Sul river (Rio de Janeiro State), at a site impacted by an important steel mill plant discharge, benzo(*a*)pyrene was detected in the water in concentrations ranging from 0.03 to 1.2 μg/L [8]. In the sediments of the same area the sum of 12 different PAHs varied from 5.1 to 40.8 μg/g [9].

Considering that the PAHs and other hydrophobic organic compounds present in the aquatic environment will be preferentially adsorbed to the sediment particles, dredging or any resuspension of the sediment can cause adverse effects to the exposed biota or humans [10]. Brazil established a resolution that regulates dredging activities which includes chemical and toxicological criteria (CONAMA 344) [11]. Considering the occurrence of sediment dredging for the maintenance of the port activities in the Santos estuary (around 3 millions $m^3$/year) and possible contaminants resuspension, it is very useful to have reliable methods to evaluate and monitor the water of this estuary for mutagenic activity as well as PAHs among other compounds.

Since the mid-1980s blue cotton, blue rayon and blue chitin have been used as sample extraction/concentration technique specifically for investigations of mutagenic activity where polycyclic compounds were involved or suspected [12]. The blue rayon hanging technique has been successfully used in evaluation of mutagenicity and PAHs content in coastal waters [13–15]. Blue rayon adsorbent is composed of fibers of rayon covalently bound to copper phthalocyanine trisulphonate and it is selective for polycyclic compounds with three or more fused rings [16]. When used in the hanging technique, the fibers stay immersed in the sampling site for 24 h allowing a representative sampling [17].

In Santos Estuary, Kummrow et al. [18] using a combination of a modified blue rayon hanging technique and *Salmonella*/microsome microsuspension assay in single doses with the strains TA98, TA100, YG1041 and YG1042, found that the water of the most contaminated area presented the higher values of mutagenic activity and the YG1041 (Table 1) was the most sensitive strain. The same authors also showed that the benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, benzo(*e*)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(*a*,*h*)anthracene and benzo(*g*,*h*,*i*)perylene were present in the blue rayon extracts.

The objective of this work was to verify the applicability and adapt the methodological conditions to monitor the water quality of the Santos estuary for mutagenic activity in a 2-year study using, a modified sampling method denominated blue rayon-anchored technique associated with the *Salmonella*/microsome microsuspension assay.

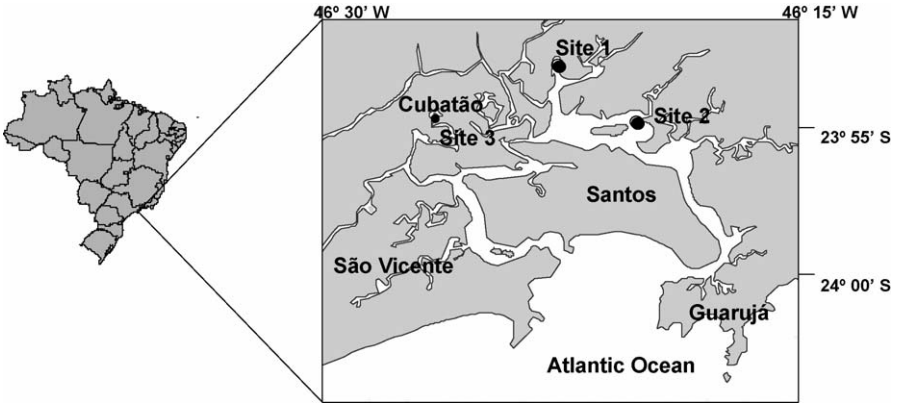


Fig. 1. Sampling sites located in the Santos Estuary.

## 2. Material and methods

### 2.1. Sampling sites

Three different sites with different sources and levels of contamination were selected (Fig. 1). Site 1 — in front of the steel mill, located in the estuary, is the most contaminated area, receives discharges from the steel mill plant and sewage and the PAHs can reach 347.55 μg/g of sediment (dry weight). Site 2 — Caneus Island, located in the estuary near to Santos harbor, is less impacted than site 1 and domestic sewage is the only direct identified pollution source. Both sites 1 and 2 are under influence of port activities like navigation and dredging. Site 3 — Queiroz River is a water body located in a less-impacted region of this estuary [2]. Five samplings were performed in 14 September (sampling 1) and 10 October (sampling 2) of 2003; 03 May (sampling 3), 07 July (sampling 4) and 07 October (sampling 5) of 2004.

### 2.2. Blue rayon-anchored technique

The samplings were performed using a modification of the blue rayon hanging technique [17] denominated in this work blue rayon-anchored technique. Amounts of 10 g of blue rayon (Funakoshi Chemicals, kindly provided by Dr. T. Ohe) were divided in two nylon nets, the nets were attached to an anchor using a nylon cord 40 cm long, and connected to a floating device (Fig. 2). This apparatus was immersed in the sampling sites for 24 h. Blue rayon fibers were washed and tested for mutagenicity activity with the strain YG1041 and only when they showed negative results they were used [19]. After the 24 h period the blue rayon fibers were taken out, transferred to beakers and transported to the laboratory in boxes protected from the light. Before the elution, blue rayon fibers were washed with ultra-pure water in order to remove the non-adsorbed compounds and solids. After this step, the blue rayon fibers were dried with clean paper towels and eluted with methanol/ammonia (50:1 v/v) in the proportion of 1 g of blue rayon to 200 mL of elution solution [19]. The eluates were reduced to 2–3 mL using a rotary evaporator, transferred to vials, evaporated to dryness with a gentle stream of nitrogen and resuspended in dimethylsulfoxide (DMSO) just before testing.

### 2.3. Salmonella/microsome microsuspension assay

The microsuspension protocol of the *Salmonella*/microsome assay described by Kado et al. [20], was employed, and the strain of *Salmonella typhimurium* YG1041 (*His*D3052, rfa, Δbio, ΔuvrB, pKM101, pYG233) overproducing acetyltransferase and nitroreductase enzymes. The positive controls used were 4-nitro-*O*-phenylenediamine (4-NOP–CAS.: 99-56-9) (INC Biomedicals Inc.), 2.5 μg/plate, without S9 mix and 2-aminoanthracene (2AA–CAS.: 613-13-8) (Sigma–Aldrich), 0.03125 μg/plate, with S9 mix. The S9 mix was freshly prepared before each test using lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox–Molecular Toxicology

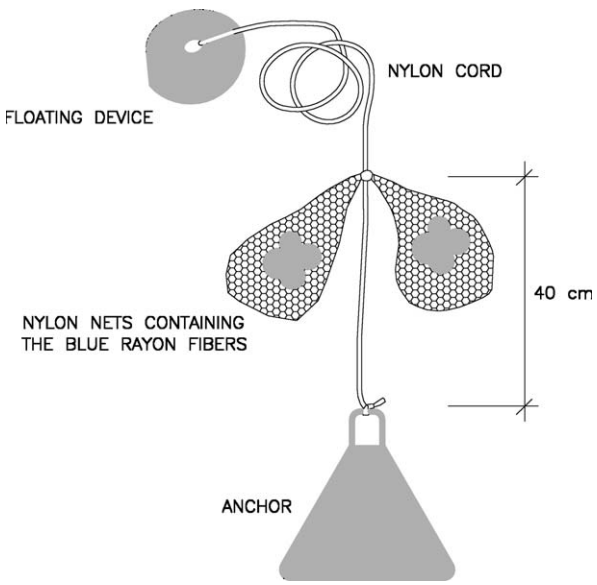


Fig. 2. Blue rayon-anchored apparatus.

Inc., Boone, NC, USA), prepared according to Maron and Ames [21].

The assays were performed using triplicate plates in the presence and absence of S9 and the doses of extracted material varied from 3.12 to 200 mg of blue rayon/plate. The results were statistically analyzed with the Salanal computer program using the Bernstein model [22]. Toxicity was evaluated by the careful inspection of the background. Samples were considered positive when there was a significant statistical difference among the tested doses and the negative control (ANOVA), a significant dose response in the Bernstein model [22] and the highest value obtained in any tested dose was above 182 revertants/plate. This value represents the maximum number of revertants/plate in the negative controls statistically calculated at 95% of confidence (percentile 95%) [23] for 311 observations historically obtained in our laboratory. The potency of each sample was expressed in revertants per gram of blue rayon.

For comparison of the sensitivity of the YG1041 in relation to a non-substituted PAH (benzo(a)pyrene) (Sigma–Aldrich) and a substituted PAH (2-aminoanthracene) (Sigma–Aldrich) a dose–response experiment was performed using the same protocol applied for the blue rayon extracts.

## 3. Results and discussion

The dose response results obtained in the *Salmonella*/microsome microsuspension assay, the calculated potencies and the confidence intervals obtained for the samples analyzed were presented in Table 2. The water from the site with the most contaminated sediment and under direct influence of the steel mill discharge (site 1) presented the highest mutagenic activity reaching 36,000 revertants/g of blue rayon in the presence of S9. Sites 2 and 3 showed much less mutagenic activity than site 1 with values around 1000 revertants/g of blue rayon (Table 2). In relation to the type of the mutagenicity detected, site 1 showed a different profile when compared to sites 2 and 3. For site 1 we can observe a dramatically increase in the mutagenic activity when the S9 mix was added in four of the five samples performed analyzed.

In order to compare results of mutagenicity using the blue rayon hanging technique Ohe et al. [24], proposed the following classification based on the number of revertants per gram of blue rayon: low (not detected—1000); moderate (1000–10,000); high (10,000–100,000) and extreme (greater than 100,000). Although this classification was developed for the strain of *Salmonella typhimurium* YG1024 (*his*D3052, rfa, Δbio, ΔuvrB, pKM101, pYG219), considering that this strain is quite similar to the YG1041 used in this study [25] this classification was applied to analyze the results. The mutagenic activity detected in site 1 — in front of the steel mill, can be considered from moderate to high and from sites 2 and 3, low to moderate (Table 2) according to the cited classification.

The mutagenicity results of a non-substituted PAH, benzo(*a*)pyrene and a substituted PAH, 2-aminoanthracene for the YG1041 using the same protocol used to analyzed the blue rayon were presented in Table 3. The potencies observed in the dose–response experiment for the benzo(*a*)pyrene were negative for the YG1041 without S9 and 340 revertants/μg with S9 and for 2-aminoanthracene were 1400 without S9 and 14,000 revertants/μg with S9.

In the original procedure of the blue rayon hanging technique the nets containing the adsorbent fibers are attached in the floating device, staying close to the surface of the water [17]. In this study we attached the nets ∼40 cm above the anchor in order to maintain the blue rayon fibers always at the same distance from the sediments (around 50–60 cm) because we suspected that the contaminated sediments could be an important source of mutagenic compounds via resuspension of the sediment and/or desorption of the contaminants. Other authors studied the effect of the distance of the blue rayon from the surface and observed a similar mutagenic activity when the blue rayon was attached both at 20–30 and at 50–60 cm depth from the surface, but no information about the depth of those areas was provided [14]. The advantage of using the anchored blue rayon technique at a standardized distance from the bottom sediment, besides the ability of adsorbing contaminants derived from the release of industrial discharges and/or runoff, is the possibility to extract contaminants that are being released from the bottom sediment allowing comparisons in coastal areas, where depth variations occurs.

Although direct human exposure to genotoxic compounds and PAHs in the bottom sediment is considered minimal, their release to the water column during dredging operations, episodes of high scouring, or leaching from confined facilities poses a treat to aquatic ecosystems and consequently a potential threat to human health via bioconcentration of those compounds the aquatic organisms [10]. Although the dredging in the most contaminated area of the Santos estuary (the region where the site 1 is located) is at the moment prohibited, this operation will occur in controlled situation in the future and the resuspension of the sediment containing genotoxic compounds could turn those contaminants available in the water column, affecting the water quality. In the Galveston Bay, using the blue rayon hanging technique, Kira et al. [13] observed the highest mutagenicity (TA98 + S9) at sites near were an oil spill

Table 2
Results of the *Salmonella*/microsome microsuspension assay in dose response experiment with the YG1041 in the presence and absence of S9 mix for the three sites evaluated and five different samplings expressed in number of revertants per gram of blue rayon (BR)

| Sampling | 1 | | 2 | | 3 | | 4 | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | −S9 | +S9 | −S9 | +S9 | −S9 | +S9 | −S9 | +S9 | −S9 | +S9 |
| Mean of revertants per plate | | | | | | | | | | |
| Site 1 (in front of the steel mill) | | | | | | | | | | |
| Doses (mg/plate) | | | | | | | | | | |
| 0.00 | 108 | 91 | 138 | 114 | 138 | 114 | 108 | 100 | 95 | 86 |
| 3.12 | – | – | – | – | – | – | – | 185** | – | – |
| 6.25 | 115 | 137** | 145 | 176** | 201** | 384** | 133* | 265** | – | – |
| 10.0 | – | – | – | – | – | – | – | – | 167 | 226** |
| 12.50 | 133** | 252** | 150 | 230** | 242** | 524** | 159* | 361** | – | – |
| 25.00 | 142 | 427** | 180 | 348** | 276** | 643** | 226** | 402** | 193** | 264** |
| 50.00 | 157** | 475** | 222** | 387** | 377** | 817** | 266** | 474** | 232** | 194** |
| 100.00 | 192** | 522** | 352** | 525** | 342** | 855** | 535** | 583** | 287** | 318** |
| 200.00 | 227** | 573 | 824** | 832** | 265** | 857** | 820** | 783** | 261** | 368** |
| Potency[b] | **870** (760–970) | **13000** (11200–13600) | **3400** (3000–3700) | **9300** (8400–10100) | **5100** (4300–5800) | **36000** (33100–37800) | **3700** (3400–3900) | **23000** (19800–25300) | **690**[a] | **1100**[a] |
| Classification[c] | Low | High | Moderate | Moderate | Moderate | High | Moderate | High | Low | Moderate |
| Site 2 (Caneus Island) | | | | | | | | | | |
| Doses (mg/plate) | | | | | | | | | | |
| 0.00 | 108 | 91 | 138 | 114 | 138 | 114 | 108 | 91 | 95 | 86 |
| 3.12 | – | – | – | – | – | – | – | – | – | – |
| 6.25 | 131 | 102 | 144 | 130* | 135 | 120 | 119 | 160.0** | – | – |
| 10.0 | – | – | – | – | – | – | – | – | 91 | 94 |
| 12.50 | 133** | 115* | 150 | 132 | 143 | 136 | 95 | 180.5** | – | – |
| 25.00 | 154** | 136* | 170 | 147* | 147 | 185** | 112 | 199.0** | 105 | 95 |
| 50.00 | 168* | 202** | 208* | 179** | 141 | 201** | 127 | 211.5* | 108 | 120* |
| 100.00 | 230** | 276** | 235** | 237** | 160 | 221** | 156** | 169.0** | 134* | 110 |
| 200.00 | 301** | 345** | 270** | 266** | 242** | 235** | 176* | 172.5** | 125 | 118** |
| Potency | **1200** (1000–1300) | **1900** (1700–2100) | **740** (500–900) | **1200** (1000–1300) | **380** (200–500) | **2000** (400–1400) | **390** (300–400) | **5000** (4100–5900) | **190** (100–200) | **170** (100–200) |
| Classification | Moderate | Moderate | Low | Moderate | Low | Moderate | Low | Moderate | Low | Low |
| Site 3 (Queiroz River) | | | | | | | | | | |
| Doses (mg/plate) | | | | | | | | | | |
| 0.00 | 108 | 91 | 138 | 114 | 138 | 114 | | | 95 | 86 |
| 6.25 | 119 | 104 | 153 | 115 | 143 | – | | | – | – |
| 10.0 | – | – | – | – | – | – | | | 96 | 92 |
| 12.50 | 134 | 135* | 146 | 130 | 142 | 117 | | | – | – |
| 25.00 | 158** | 144** | 148 | – | 158 | – | | | 99 | 74 |
| 50.00 | 171** | 179** | 164 | – | 176* | 154** | | | 92 | 99 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 100.00 | 188** | 180** | 193 | 197** | 257** | 216** | NP | 110* | 125 | |
| 200.00 | 218** | 217** | 228 | 239 | 401** | 400** | | 99 | 122* | |
| Potency | **1400** (1100–1600) | **1900** (1500–2100) | **470** (300–600) | **700** (600–700) | **1200** (1000–1400) | **990** (900–1000) | | **Negative**[d] | **Negative** | |
| Classification | Moderate | Moderate | Low | Low | Moderate | Low | | | | |

NP: not performed; (–): dose not tested.
[a] Mutagenic potency estimated, although linear model not statistically significant.
[b] Number of revertants per gram of blue rayon (BR) calculated using the Bernstein model and in parenthesis the 90% confidence interval.
[c] Classification according Ohe et al. [24].
[d] For positiveness criteria see Section 2.
* Significant at 5% (ANOVA).
** Significant at 1% (ANOVA).

Table 3
Potencies of the benzo(*a*)pyrene and 2-aminoanthracene in revertants/μg for the *Salmonella* strain YG1041 in the *Salmonella*/microsome microsuspension assay in the presence and absence of S9 mix

| Doses (μg) | −S9 | +S9 |
|---|---|---|
| Benzo(*a*)pyrene | | |
| 0.000 | 96 | 74 |
| 0.025 | 96 | 84 |
| 0.050 | 87 | 97 |
| 0.100 | 100 | 137* |
| 0.250 | 95 | 161** |
| 0.500 | 75 | 233** |
| 1.000 | 113 | 287** |
| 2.500 | 94 | 342** |
| 5.000 | 93 | 348** |
| Potency (revertants/μg) | ND | 340 |
| 2-aminoanthracene | | |
| 0.0000 | 96 | 74 |
| 0.0025 | 84 | 115** |
| 0.0050 | 97 | 155** |
| 0.0100 | 113 | 213** |
| 0.0250 | 127* | 746** |
| 0.0500 | 176** | 1459** |
| 0.1000 | 257** | 1400** |
| 0.2500 | 410** | 792** |
| 0.5000 | 634** | 615** |
| 1.0000 | 1036** | 325** |
| Potency (revertants/μg) | 1400 | 14000 |

ND: not detected.
* Significant at 5% (ANOVA).
** Significant at 1% (ANOVA).

occurred with maximum values of 5656 revertants/0.16 g of blue rayon/plate. These values are higher than the ones observed in our study (Table 2). Probably this is because the sampling was performed just after an oil spill.

Based in the results previously obtained by Kummrow et al. [18] that evaluated the performance of four strains of *Salmonella* (Table 1) to detect the mutagenic activity from the extracts obtained using the Blue rayon-anchored technique from two sites of Santos estuary (the same sites 1 and 2 of this study), we have chosen the strain YG1041, with and without S9 mix, to be used in this study considering its higher sensitivity. In this study, mutagenic activity was detected in the water, and the higher mutagenic activity was observed in the sampling site located in the most contaminated area (site 1). This site showed an increased response in the presence of S9 mix, indicating that non-substituted PAHs could be contributing to the detected effect. Because of the high sensitivity of YG1041 for nitrocompounds and aromatic amines these classes of mutagenic compounds could not be ruled out.

Comparing the profile of mutagenicity observed for the sediment samples from site 1 (2100 and 31,000 revertants/g for TA98 without and with S9) [4,6] we can observe that it is similar to the water mutagenic activity detected in our work. These data suggest that the sources of genotoxic compounds in these estuarine waters could be the sediment desorption and/or sediment resuspension but industrial effluent discharge could not be excluded.

The results obtained in the sites 2 and 3 were very similar to each other presenting around 1000 revertants/g of blue rayon. Considering that those areas are much less contaminated as showed in previous studies [2,5] we suggest that this value could be considered as a background response for this area. More studies are in progress to determine background values in protected areas of the Santos estuary.

The strategy that uses the Blue rayon-anchored technique associated to the *Salmonella*/microsome microsuspension assay with the strain YG1041 seems to be more sensitive and adequate to detect mutagenic activity in the Santos estuarine waters than the one applied by Umbuzeiro et al. [6]. The significantly higher mutagenicity in the metabolically enhanced diagnostic strains (e.g., YG1041) with metabolic activation (S9) (Table 3) suggests the presence of aromatic amine-type mutagens and non-substituted PAHs. In the absence of S9 mix other direct-acting polycyclic compounds, including substituted PAHs, could explain the observed mutagenicity. According to the potencies of the pure compounds analyzed, as shown in Table 3, 1 μg of benzo(*a*)pyrene could explain $\sim 10^2$ revertants and 1 μg of 2-aminoanthracene could explain $\sim 10^5$ revertants. Another evidence that substituted PAHs could be present in the water column is that they are more polar than the non-substituted PAHs. Further chemical analysis should be performed in order to identify the mutagenic compounds that are causing the observed effects as described by Hewitt and Marvin [12].

## 4. Conclusions

We conclude that the blue rayon-anchored technique associated with *Salmonella*/microsome microsuspension assay and the YG1041 strain can be a reliable alternative to monitor estuarine waters for mutagenic compounds, especially in regions where sediment resuspension or acute pollution episodes can occur.

Our results also indicate that the Santos estuarine waters contain mutagenic compounds other than non-substituted PAHs, and considering the high sensitivity of the YG1041 for nitro and aromatic amines compounds these group of contaminants could be accounting for the observed mutagenic activity.

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