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Sewage Pipe Waters Affect Colour Composition in *Palaemon* Shrimp from the Intertidal in the Canary Islands: A New Non-lethal Bioindicator of Anthropogenic Pollution

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Abstract: Marine pollution through anthropic outfalls like sewage pipes is a growing concern since point-source pollution can affect many organisms. Investigating pollutant concentrations in organisms usually requires sacrificing the organisms, but here we propose a new method to infer anthropic pollution in the intertidal by measuring colour levels in *Palaemon elegans* rockpool shrimp. We took pictures of live shrimp from pools near sewage pipes and control zones in three of the Canary Islands (Gran Canaria, Lanzarote and Tenerife), and measured their RGB (red, green and blue) abdominal colour composition. We then statistically compared colours from the control zone and sewage pipe and between islands. We found a clear differentiation in colour composition between the control zone and areas with a sewage pipe. Our results supported the hypothesis that pollution affects colouration in these invertebrates. We, therefore, suggest the use of darker colourations in *P. elegans* as a bioindicator of anthropic pollution, a first sign that should spur more in-depth studies in the affected area. This methodology is pollutant unspecific but non-extractive, so we propose its use as a citizen science tool to inform scientists and technicians of possible illegal and/or untreated wastewater that could affect intertidal biota.

Keywords: rockpool shrimp; colourimetry; pollution; wastewater; intertidal; bioindicator

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1. Introduction

Intertidal pollution is related to human pollution, i.e., the contamination of oceans, rivers, and lakes [1–3]. This pollution occurs through industrial discharges, the plastic and agrochemical waste that humans dump into bodies of water, pesticides and fertilizer residues from crops, and the discharge of oil and other chemicals into the sea [4–6]. The effects of pollution on the intertidal are varied. Spills, oil, and chemicals have been found to have a direct effect on marine organisms, causing disease and mortality [7–9]. Plastic can also cause disease in marine organisms, as it accumulates in the intertidal and may contain toxic substances [10]. In addition, as contaminants enter the intertidal, ecological balances are altered and this, in turn, affects the diversity and health of marine ecosystems [11–13]. This can have long-term effects on the local economy and culture, as fishing and tourism are two important sources of income in many coastal areas [14–16]. Therefore, it becomes necessary to control intertidal pollution to avoid environmental damage and maintain marine biodiversity. This can be achieved through the adoption of preventive measures, such as proper treatment of waste and regulation of industrial discharges. Environmental education should also be promoted to raise awareness of the need to protect the marine environment [17–20].

Bioindicators constitute a large group of plant, fungal, and animal species, whose presence or physiological status in each ecosystem provide information on certain ecological characteristics of the ecosystem or the possible environmental impact of certain practices on that ecosystem [21–23]. These organisms are mainly used to evaluate the environmental quality of ecosystems. All bioindicators must meet a series of requirements for their use, such as dispersion and abundance in the territory, sedentary lifestyle, and toleration of contaminating agents in concentrations similar to those of the contaminated ecosystem but without lethal effects. The use of bioindicators to detect anthropic pollution traditionally required an extractive survey, killing the specimens in the process of measuring pollutants within their tissues [24–26]. These methods can be problematic when studying endangered species, as well as sparsely distributed populations, for which a small reduction in specimen numbers could cause dramatic effects [27]. Non-invasive survey techniques are needed for these cases, but also in general monitoring studies, to reduce human impacts on populations that are already affected by anthropogenic activities, like rockpool species of anemone and barnacles in the intertidal of the Canary Islands [5,28–30]. Since changes in colouration reflect the physiological state of several animals in different clades—e.g., acting as an honest signal like well-fed flamingos, among other species [31]—it could be possible for some species to reflect higher pollutant concentrations in their body colours.

Here, we measure the colouration of *Palaemon elegans* shrimp from the intertidal of the Canary Islands, comparing RGB colour composition in shrimp found near sewage pipes and in more pristine areas in order to assess their colouration as a new, non-invasive technique that could be used to detect anthropic pollution in these habitats.

2. Materials and Methods

Three islands of the Canary Islands (Gran Canaria, Lanzarote and Tenerife) were chosen for the study (Figure 1), and for each island, an area affected by a sewage pipe and a control area were determined. For each area, eight *Palaemon elegans* pond shrimp were sampled, for a total of 48 samples. Shrimp measuring 3.5 ± 0.2 cm were used to avoid the variable 'length' of the study. We started all the surveys at the low tide and keep this timing for the three locations in order for the water ponds to present similar temperatures and salinity.

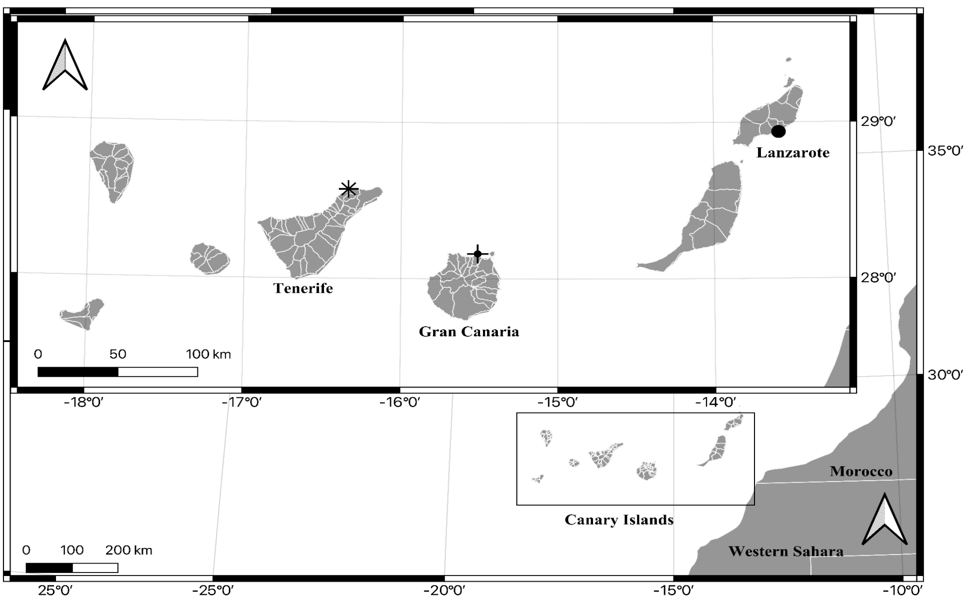

Figure 1. Location of the surveys in the Canary Islands, NW Africa. (Asterisk symbol represents the sampling area of the island of Tenerife, cross symbol represents the sampling area of the island of Gran Canaria and black dot symbol represents the sampling area of the island of Lanzarote.)

Each sample was processed in situ in the intertidal during the surveys. The shrimp were fished and measured with a calliper. If the shrimp was within the chosen size, gauze was passed over the shrimp to remove the water from the body. The photographs were taken in a custom-made white hood made from standard lab coat fabric to eliminate the sun's glare. The shrimp was placed lying on its right side facing to the left and three photos were taken without flash, and so on with each of the eight shrimp in each zone. The photos were taken at 15 cm from the shrimp, focusing on the first segment of the abdomen for the photo, using a black background so as not to reflect the light. Once the photos were taken, the shrimp were deposited in a bucket with water from the pool and then returned alive to the pools of origin.

For the processing of the photos, the Image Processing and Analysis in Java (Image J) program was chosen, selecting the left part of the first abdominal segment of the shrimp using the tool 'Colour image processing'. Resultant colour data was obtained in ImageJ through a combination of image analysis tools, i.e., the colour histogram and colour measurement tools, obtaining quantitative values of red, green and blue (RGB) for each shrimp. We repeated this process for the three pictures per shrimp and calculated the mean values of RGB coordinates for each shrimp. Using this methodology, a total black colour would have (0,0,0) coordinates in RGB composition, while pure white would be (255,255,255).

Statistical Analyses

To study the existence of differences in colourimetry between the samples analysed in the tissue, a permutational multivariate analysis of variance (PERMANOVA) with Euclidean distances was performed. A two-way design was used with the factor "Zone" with two levels of variation (Sewage pipe and Control Zone) and another factor "Location" with three levels of variation (Tenerife, Gran Canaria, and Lanzarote). The following variables were included in the analysis: red, green, and blue colours of the colourimetry panel ranging from 0 to 255. Relative dissimilarities between areas were studied by principal coordinate analysis (PCoA) in which the colourimetry (RGB) that best explained the variability of the data was represented as vectors. One-way permutational analyses of variance with Euclidean distances were performed on the raw data. The analysis used 9999 permutations of interchangeable units and a posteriori pairwise comparisons to check for differences between significant factor levels (p-value < 0.05). The statistical packages PRIMER 7 and PERMANOVA þ v.1.0.1 [32,33] were used for statistical analyses.

3. Results

A total of 48 samples were analysed, as planned in the survey design. From these, RGB quantitative data were extracted from the pictures (Figure 2), and we calculated basic metrics comparing each colour (red, green and blue) and zone (control vs. sewage) for every island (Table 1 and Figure 3). There is a clear trend of higher RGB values in the samples from the Control Zone vs. lower RGB values near the sewage pipe (Figure 3).

Figure 2. Shrimp picture examples taken at the control zone [**left**] and near the sewage pipe [**right]** of Tenerife. In the first image, we indicate the main anatomical division of shrimp: C is for cephalothorax, A for abdomen, and T for telson. In the second image, the first abdominal segment is shown as '1', as it was the one we used for the colourimetry analyses.

Table 1. Descriptive statistics of all the RGB analysed in each sampling zone with sewage pipe and control zone [mean, standard deviation, minimum and maximum].

Colour		Control Zone			Sewage Pipe		
		Gran Canaria	Lanzarote	Tenerife	Gran Canaria	Lanzarote	Tenerife
Red	Mean	140	168	138	82	104	85
	SD	3	4	7	2	7	3
	Min	134	161	127	81	97	80
	Max	147	176	149	87	115	88
Green	Mean	132	158	130	85	103	85
	SD	3	3	4	3	11	4
	Min	129	155	123	81	88	79
	Max	138	165	134	90	128	89
Blue	Mean	106	127	105	76	92	72
	SD	4	5	4	3	4	8
	Min	100	120	100	69	83	53
	Max	113	135	115	80	96	79

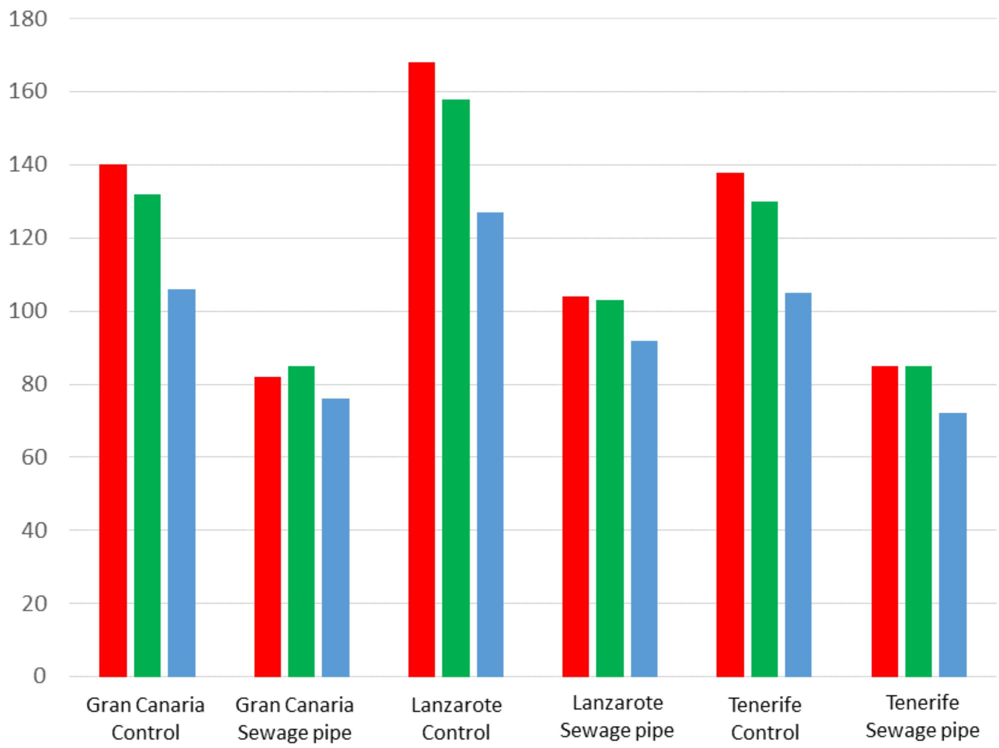

Figure 3. Barplots for the RGB mean values of each zone per island. Each bar represents the colour coordinates values of a sample for Red, Green and Blue, respectively, for each sampling location. Using this methodology, a total black colour would have (0,0,0) coordinates in RGB composition, while pure white would be (255,255,255).

We found significant differences between RGB values of shrimp from the control zone and the sewage pipe zone for the three islands (Table 2).

Table 2. One-way PERMANOVA analyses between control and sewage pipe zones for each island. * *p*-value <0.05.

	Gran Canaria	Lanzarote	Tenerife
Sewage pipe vs. Control zone	0.001 * F = 10.69	0.001 * F = 11.01	0.001 * F = 10.61

We found no significant differences in these RGB values between shrimp taken from the sewage pipes of Tenerife and Gran Canaria, and the same happened for the control zone of both islands (Table 3). However, we found differences in the same analyses when comparing both islands with Lanzarote.

Table 3. Results of pairwise tests examining the significant factor "Zone" for each zone between islands were obtained in a one-way ANOVA. * *p*-value <0.05.

	Sewage Pipe	Control Zone
Tenerife vs. Gran Canaria	0.283	0.563
Tenerife vs. Lanzarote	0.003 *	0.001 *
Gran Canaria vs. Lanzarote	0.002 *	0.002 *

Performed PCoA (Figure 4) showed a clear distinction between control zones and sewage pipe zones, as well as the separation of samples taken in Lanzarote from the ones taken in Tenerife and Gran Canaria. These results were clearly visible in the PCoA analysis, which accounted for approximately 99.4% of the total variability of the data (Figure 4). The distribution of the samples showed a clear difference in the RGB content of *P. elegans* in the Lanzarote control zone compared to the other zones. The clusters created in Tenerife and Gran Canaria overlapped each other according to the control zone and sewage pipe. The colours that best explained the variability found in the data are represented as vectors in the PCoA, which showed a positive pattern for red and green in the Lanzarote control zone.

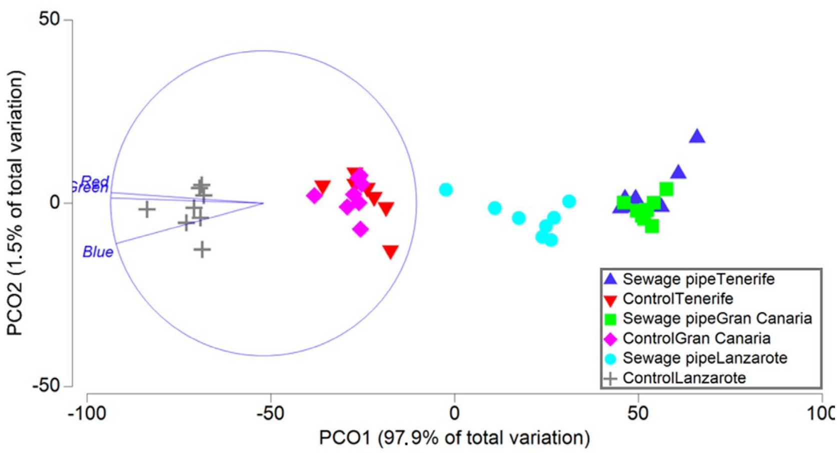

Figure 4. Principal coordinate analysis (PCoA) showing the first two axes (99.4% of variability) based on Euclidean distances of square-root-transformed data of RGB data.

4. Discussion and Conclusions

All the performed analyses show clear differentiation in shrimp RGB colouration when comparing the control zone and the sewage pipe areas in the three studied islands (Tables 1–3, Figures 2–4). Thus, our main hypothesis is supported; human pollution which is being poured through these pipes to the coastal waters is affecting shrimp colouration. Specific pollutants concentrations were not measured in the present work, but there are several previous studies in the same sewage pipe of Tenerife that endorse our results since there is a clear gradient of disturbance in other invertebrates inhabiting the intertidal around this sewage pipe, and it happens the same for pollutant concentrations in rockpools seawater for the area [5,30,34,35].

The results obtained in the pairwise tests (Table 3) showed that there were no significant differences between Tenerife and Gran Canaria, both in the control area and the sewage pipe area. This is because the areas chosen for the study have a notable anthropic presence, since they are the two most populated islands within the archipelago [36], so the areas chosen without the presence of sewage pipes could have another type of contamination affecting the colouration of the shrimp, although not as strongly as seems to happen near the sewage pipes. Table 1 shows that red, green and blue colours have higher values in Lanzarote than in Tenerife and Gran Canaria. When any RGB values are high with the described methodology it means that they tend to be white. Being so, we propose that the specimens of *P. elegans* from the island of Lanzarote have less contamination due to less anthropic pressure in the chosen area [36–41] and present lighter colourations, while more contaminated shrimp from Tenerife and Gran Canaria present darker colours.

This is, to our knowledge, the first description of the use of colourimetry in shrimp as a bioindicator of potential human pollution. We find it extremely relevant to explore non-lethal methodologies to monitor bioindicators of pollution in the intertidal since many invertebrates of this habitat are endangered [42] or have a special protection level, with 12 molluscs, four cnidarians, and one Porifera species from the Centinela database of protected species in the Canary Islands [42] and their populations cannot sustain extractive methods if they are to survive. Our results provide a first assessment of the use of colourimetry in *P. elegans* shrimp to detect pollution, and although it will not provide detailed information on which precise contaminants are in the water, it will enable scientists to have a first sign of danger for this species and the whole intertidal ecosystems. Then, more precise, in-depth and costly studies could be conducted to assess which pollutant is responsible for the contamination. Our methodology is interesting in the light of the citizen science [43,44], so non-scientists following the described procedure of picture-taking could inform scientists of shrimp presenting colours associated with a more polluted intertidal, and this way inform authorities to solve human water outfall issues. There already exists a citizen science project in the Canary Islands that reports marine biota presence within the archipelago, and our proposed methodology could represent a further step to increase this monitoring of pollutants' presence within the archipelago so scientists and technicians could carry out surveys in the reported places in order to assess the presence of pollutants, its possible source and inform the authorities to propose (a) correctly treat wastewaters to reduce pollutant input to the sea and (b) conservation efforts in the area. Therefore, in light of our results (Figure 3) we propose for a citizen science approach using our methodology as a first threshold for considering shrimp coloration indicative of potential pollution. This would consist of RGB coordinates from the 'Colour image processing' tool of ImageJ of (<110, <110, <100). For other areas outside the Canary Islands archipelago, a first study comparing polluted vs. non-polluted zones should be carried out to validate this threshold.

Human pollution can affect invertebrate colouration, i.e.,: British peppered moths that presented white colours and were camouflaged when perched on birch trees. During the Industrial Revolution in Britain, air pollution caused by coal burning darkened the bark of the trees were British peppered moths lived, causing a shift in the predator detection of the whiter vs. darker phenotypes. This in turn resulted in an increase in numbers of previously rare dark-coloured moths while the white ones were heavily reduced, in a so-called industrial adaptation [45]. In our study, we are observing the phenotypic plasticity of shrimp due to exposure to pollutants, and we do not know of any genetic adaptation effect on our studied shrimp. However, a detailed study in that direction should be performed to evaluate the risk of human-induced pollution affecting the genetic variation of this species on a similar scale as happened in British peppered moths.

In summary, in view of our results and the growing evidence of impacts from human wastewater on intertidal biota in these islands [5,30,34,35] along with the yearly increase in local population plus tourists in all the archipelago [36], we urge both community and local governments to take action to reduce these outputs and the effects on marine biota. Darker colourations in *P. elegans* suggested to be a bioindicator of anthropic pollution could become more predominant in rockpool shrimp on all the coasts of the Canary Islands and alert us of the increase in pollutant outputs to our coast.

Future works should include exploring possible correlations between these colour differences and specific pollutants (i.e., heavy metals like lead and cadmium) in order to further explore the proposed relationship between these two variables.

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Data Availability Statement: All data has been given in tables within the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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