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Is There Detectable Long-term Depletion of Genetic Variation in Freshwater Fish Species Affected by an Oil Spill?

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Abstract Oil spills might lead to severe environmental impacts to the affected fauna, disrupting local food webs, and causing mass mortality in many species. However, little is known about long-term impacts of oil spills, or even if such impacts can be detectable after several generations. In this study, we investigate the genetic variability of three freshwater species—*Mimagoniates microlepis* (Characiformes: Characidae), *Scleromystax barbatus* (Siluriformes: Callichthyidae), and *Phalloceros harpagos* (Cyprinodontiformes: Poeciliidae)—in rivers that were affected by a large oil spill in the state of Paraná, southern Brazil, on February of 2001. Samples were obtained from nine different locations, such that rivers that were directly affected by the oil spill could be compared with similar rivers in the same region that were unaffected. A fragment of the cytochrome C oxidase subunit I mitochondrial gene was sequenced from each specimen, and the level of genetic variability was assessed. Based on estimates of haplotype and nucleotide diversity, no impact of the oil spill could be detected in impacted rivers. These results suggest that fish populations in the region showed resilience to the pollutant, such that immigration from other locations was able to reestablish levels of genetic variability comparable to those of unimpacted rivers.

Keywords Genetic variability · *Mimagoniates microlepis* · *Scleromystax barbatus* · *Phalloceros harpagos* · Environmental impact

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1 Introduction

Oil spills in natural habitats can lead to severe environmental impacts, depending on the structure of the habitat and the type and volume of oil being released (Etkin 2004; Peterson et al. 2003). During an oil spill in water bodies, fishes are also exposed, but to a lesser extent than other groups, such as marine mammals, birds, and sessile mollusks (Teal and Howarth 1984). Even so, many studies have explored acute impacts of such exposures, such as the effects on animal development (Carls et al. 1999; Incardona et al. 2004, 2012), direct DNA damage (Bolognesi et al. 2006; Vanzella et al. 2007), and sublethal histopathologies (Hose et al. 1996). However, little is known about long-term potential impacts of oil spills, particularly in freshwater environments and in terms of the genetic variability of impacted populations.

Two major oil spills have occurred and impacted different and important aquatic environments in the state of Paraná, southern Brazil. On July of 2000, approximately four million liters (≈25 thousand barrels) of crude oil (density = 0.9427, 18° API, viscosity (20 °C) = 5000) were spilled from the OSPAR pipeline into the Iguaçu River (Boeger et al. 2003; Ostrensky et al. 2001). In the following year, nearly 52 thousand liters of light fuel oil (density = 0.8164, 41° API, viscosity (20 °C) = 3716 cP, total monoaromatic hydrocarbons (BTEX) = 41.8 mg/g, and total polycyclic aromatic hydrocarbons (PAH) = 16 mg/g) spilled from the OLAPA pipeline, which connects the Presidente Getúlio Vargas refinery (REPAR), in the municipality of Araucária, state of Paraná, to the corresponding terminal in the Paranaguá port. This accident occurred in a remote and difficult access area at Serra do Mar mountain range. Containment barriers were installed to reduce oil dispersion and to allow for its removal, but some of the oil reached local rivers and streams, eventually reaching the Paranaguá Bay (Gabardo et al. 2011). After the accident, a monitoring program was established with an initial focus on water and sediment chemistry and birdlife community. One year later, benthic community was also evaluated. Results suggested rapid reduction on total PAH concentrations (1416.0 to 1.6 μg/L) has minor effect on bird and benthic community (Faria et al. 2005). Ten years after, Albarello (2012), Ostrensky et al. (2015), and Horodesky et al. (2015) evaluated the environmental quality of the affected area through the analysis of long-term oil presence, fish population structure, and fish community, respectively. They found high concentrations of total petroleum hydrocarbons (TPH), including benzo(a)anthracene, benzo(a)pyrene, and indeno(123-cd)pyrene, in eight locations. On the other hand, no alterations in ichthyofaunal community and structure were detected. Thus, as TPH are still recorded at high concentrations at the studied area and no ichthyofaunal analysis was performed in order to identify chronic and long-term genetic alterations that could have occurred, we hypothesize that fish populations thriving the affected area could potentially present long-term genetic alterations.

In this study, we assessed the level of genetic variability in three species of fish found in the original areas impacted by the OLAPA pipeline oil spill in relation to similar, non-impacted locations (rivers in the same region) to determine if there was evidence for long-term genetic depletion.

2 Materials and Methods

Fish specimens were obtained in eight rivers of the Nhundiaquara River watershed (Fig. 1), during six consecutive sampling days, in the summer season (February) of 2015 (14 years after the spill of the OLAPA pipeline). Each river was categorized as either "impacted," if it was directly affected by the oil spills (Meio and Sagrado Rivers), or "control," rivers from the same watershed as the impacted rivers, with similar physiographic characteristics, but that had no record of impact (Sambaqui, Nhundiaquara, Passa Sete, Rio do Pinto, Mãe Catira, and Marumbi). Previous studies on the physicochemical features of these locations were similar (Horodesky et al. 2015) so that they would be expected to represent comparable sampling sites. Based on the previous ichthyological surveys in the region (Horodesky et al. 2015; Ostrensky et al. 2015), three fish species were selected for this study: *Scleromystax barbatus* (Siluriformes: Callichthyidae), *Mimagoniates microlepis* (Characiformes: Characidae), and *Phalloceros harpagos* (Cyprinodontiformes: Poeciliidae). These species were chosen due to their local abundance, their presence in all sampled rivers, as well as their occupation of different compartments in the water column (benthic in the case of *S. barbatus*, limnetic in the other two species). Specimens were collected using a combination of electrofishing, casting, and/or seine nets (see Ostrensky et al. (2015) for details on the collection protocols), and a piece of the caudal fin of each specimen was preserved in absolute ethanol and maintained at −20 °C until being processed for molecular work. Voucher specimens were deposited in the Ichthyological Collection of the Museu de História Natural do Capão da Imbuia, Curitiba, state of Paraná, Brazil.

Total DNA was extracted using DNeasy Blood & Tissue Spin-Column Kit (Qiagen®—Germany) from 25 mg of fin tissue, following manufacturer's protocol. Concentration was estimated using NanoDrop 2000 (Thermofisher®—USA). Amplifications were

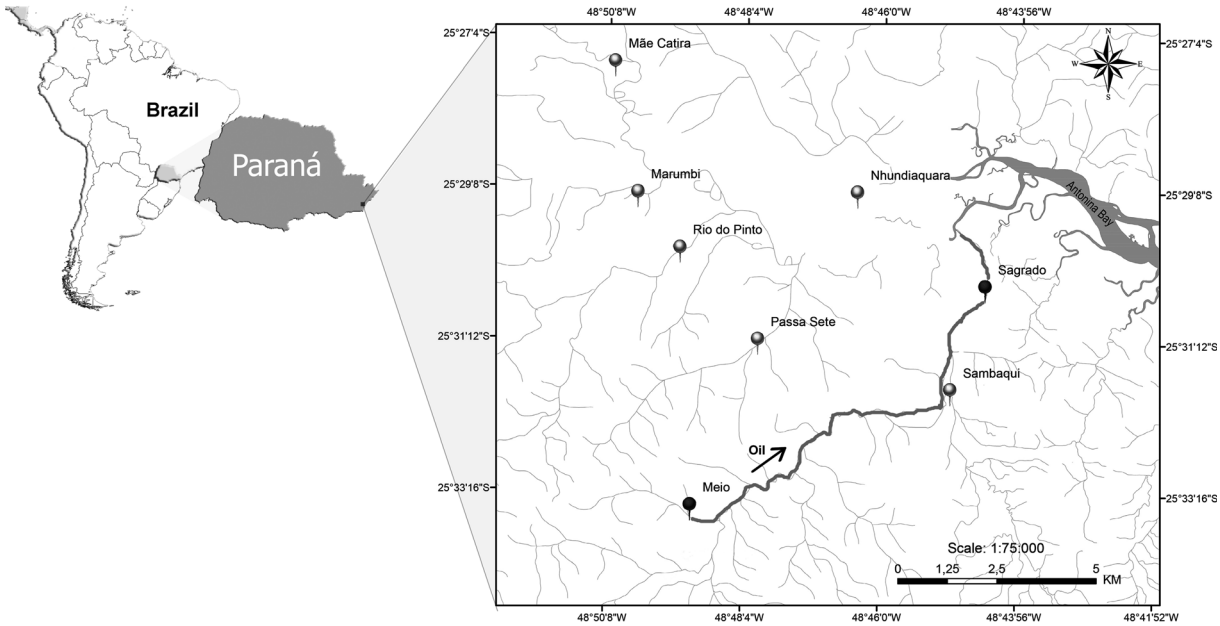

Fig. 1 Location of the collection sites in relation to the region of the oil spill in the municipality of Araucaria, state of Paraná, southern Brazil

performed in a final volume of 25 μL, using up to 30 ng of DNA (extracts ranging from 8 to 130 ng/μL). The chosen marker was cytochrome C oxidase subunit I (COI), using LCO1490 and HCO2198 primers, which amplify a segment of 658 bp. The mixture contained 1 U of Platinum™ Taq DNA Polymerase (Invitrogen®—USA), 2.5 μL of the enzyme reaction buffer (10×), 3 mM of $MgCl_2$, 0.5 mM of total dNTP mixture, 1 pM of each primer, and 0.2 μg/μL bovine serum albumin (BSA). Temperature cycling consisted of an initial step of 94 °C for 5 min, followed by 32 cycles of 40 s at 94 °C, 45 s at 44–46 °C, and 1 min at 72 °C, followed by a final elongation at 72 °C for 1 min. PCR products were purified using a precipitation-based protocol with polyethylene glycol (PEG 8000 20% NaCl 2.5 M). Sequencing reactions were performed in a final volume of 10 μL, using 40 ng of amplified DNA. Each mixture contained 0.5 μL of BigDye® Terminator, 1 μL of sequencing buffer (5×), and 3.2 pM of the respective primer.

Nucleotide sequences were obtained using a 3500xL Genetic Analyzer (Applied Biosystems®—USA), basecalled using Gap4, as implemented in the Staden Package (v2) (Staden et al. 1998), aligned using ClustalW2 (Larkin et al. 2007), as implemented in the BioEdit Sequence Alignment Editor (Hall 1999), and adjusted manually. Haplotype and nucleotide diversity were calculated using DnaSp5 (Librado and Rozas 2009). Haplotype networks were computed and drawn in R 3.3.1 (R Core Team 2016), using packages *pegas* (Paradis 2010), *plyr* (Wickham 2011), and *reshape* (Wickham 2007).

3 Results

Figure 2 depicts the inferred haplotype networks for each of the studied species. The circles in this type of diagram represent a particular DNA sequence, whereas the size of each circle corresponds to their relative frequency. The edges between circles represent difference among a given pair of sequences, such that one edge would indicate the existence of a single nucleotide difference and two edges would represent two nucleotide mismatches. The observed pattern of genetic variability shows that many different sites often share the same haplotypes, which is consistent with a scenario of high gene flow (i.e., exchange of migrants among populations leading to genetic homogenization among sites) and demographic connections among populations on each site, which is not unexpected given the small geographical distances among them. However, if the oil spill had produced a sufficiently severe population bottleneck, one would expect that all populations would tend to be genetically homogeneous, with little to no evidence of rare haplotypes, for instance. However, the

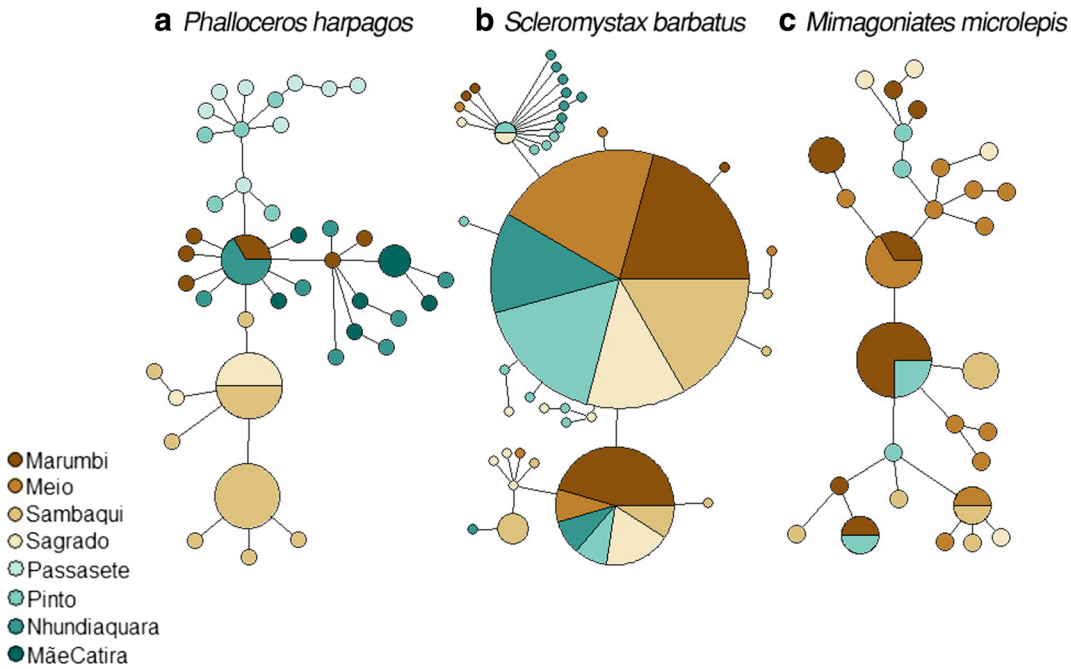

Fig. 2 Haplotype networks of the fragment of the COI gene for the three studied fish species. **a** *Phalloceros harpagos*. **b** *Scleromystax barbatus*. **c** *Mimagoniates microlepis.* Haplotype size scales with frequency. Distance between haplotypes does not represent actual computed distance values

substantial variability that was detected indicates that any potential population bottleneck was not sufficiently severe to cause detectable depletions in genetic variability in exposed populations, possibly due to emigration from neighboring regions. This inference is corroborated by direct estimates of haplotype and nucleotide diversity (Table 1), with the obtained estimates being similar among sites and without any consistent genetic depletion in the sites directly affected by the oil spill of January 2001.

4 Discussion

The results of the present study were not able to detect a lasting depletion of genetic variability in any of the three fish species examined in areas impacted by the light fuel oil spill at Serra do Mar, in the state of Paraná, in 2001. This is consistent with some other studies on the long-term impacts of oil spills. For instance, in a study on the impact of the Exxon Valdez oil spill, Barber et al. (1995) showed that 2 years after the accident, fish populations

Table 1 Haplotype and nucleotide diversity of the corresponding species and populations.

	Phalloceros harpagos			*Mimagoniates microlepis*			*Scleromystax barbatus*		
River	Hd	Nd	N	Hd	Nd	N	Hd	Nd	N
Marumbi	0.952	0.00701	7	0.867	0.04915	10	0	0	13
Meio	–	–	–	0.833	0.00704	13	0.378	0.00268	10
Sambaqui	0.864	0.0032	12	0.933	0.00851	12	0.455	0.00199	12
Sagrado	0.667	0.00112	3	1	0.13173	4	0.808	0.00568	13
Passa Sete	1	0.01607	8	–	–	–	–	–	–
Pinto	1	0.01225	5	1	0.00686	5	0.371	0.00089	15
Nhundiaquara	1	0.01088	9	–	–	–	0.455	0.00219	12
Mãe Catira	0.929	0.00397	8	–	–	–	–	–	–

Hd haplotype diversity, *Nd* nucleotide diversity, *N* number of sequenced specimens in that location

were largely recovered. While studying the same event, Lanctot et al. (1999) found that recolonization via emigration of populations of the harlequin duck from non-affected areas could recover impacted populations, although this effect might only take place in the long term. It is important to note that our results do not necessarily mean that no acute impacts took place at the time of the accident. For instance, it is known that some polycyclic aromatic hydrocarbon components of oil are acutely toxic (Barron et al. 1999) and potentially mutagenic (Eisler 1987; Harvey 1991; Srogi 2007). However, according to Cronin and Bickham (1998), if lethal mutations are introduced, the rapidly ensuing mortality would lead to strong selection and consequently the elimination of such mutations on the affected populations, particularly over the first few generations. These authors explain that although crude oil may be mutagenic in laboratory experiments or chronically-polluted sites, oil spills probably would not exhibit the same effects for two reasons: concentrations of mutagenic fractions may be lower in an oil spill, and exposure to oil typically occurs over a limited period of time. However, the extent to which those factors are true is likely to depend on the specific oil type and circumstances of the spill, including the degree of weathering prior to exposure. Miller et al. (1994) and Collier et al. (1996) concluded that the absence of DNA adducts or other DNA damage in teleost fish following oil spills suggests that such short-term exposure to oil does not result in somatic mutagenesis. These results are also consistent with another study in the study region that did not detect faunal differences between impacted and non-impacted locations (Ostrensky et al. 2015). Bickham et al. (2000) also anticipates that reduction in genetic variability will be the prevalent effect, leading to compounding effects of continuous erosion of fitness, but only if there is long-term exposure.

Our results are in contrast with another study on major oil spill in the state of Paraná, but this time in the Iguaçu River (Katsumiti et al. 2013). In that study, two fish species (*Hyphessobrycon reticulatus* [Characidae] and *Phalloceros caudimaculatus* [Poeciliidae]) were collected 5 years after the oil spill and used in biochemical, histopathological, and genotoxic studies. Although some evidence of disturbance was detected in specimens from the impacted site, the frequency of such impacts was determined in comparison with control populations that were obtained from other locations, including fish farms. Therefore, it is not possible to determine if those anomalies were caused by the oil spill in question, or if they were caused by background pollution or other sources of variation that were already (and potentially still are) found in the region.

There are several factors that might have contributed to the resilience of the fish populations investigated in this study. First, although the spills were severe, they represented a short-term disturbance that disproportionately affected the fishes that were living in the affected rivers at the moment of the accident. This probably allowed for recolonization of fish from populations in other watersheds, which resupplied genetic variability to the populations in the affected locations. Second, the spatial structure of watersheds, as well as the unidirectional, continuous flow of water in the non-estuarine portion of the affected region, possibly accelerated the removal of the low molecular weight petroleum hydrocarbons (BTEX and the majority of PAH) from the region, thus accelerating recovery, compared to analogous situations in lakes or estuaries.

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Compliance with Ethical Standards

Conflict of Interest This study was funded by Petrobras (grant). However, the source of type of funding was categorized explicitly as a research project, and there was no influence or oversight from the funders on the analyses, results, and writing of this manuscript.

References

Albarello, L.C. (2012). O vazamento do oleoduto OLAPA (Morretes, Paraná): Avaliação ambiental e reconstituição do óleo, da Serra do Mar ao complexo estuarino de Paranaguá. Pós-Graduação em Geociência, Universidade Federal do Rio Grande do Sul, 188 pp. Avaliable at: https://www.lume.ufrgs.br/bitstream/handle/10183/56326/000860241.pdf?sequence=1.

Barber, W. E., McDonald, L. L., Erickson, W. P., & Vallarino, M. (1995). Effect of the Exxon Valdez oil spill on intertidal fish:

a field study. *Transactions of the American Fisheries Society, 124*, 461–476.

Barron, M. G., Podrabsky, T., Ogle, S., & Ricker, R. W. (1999). Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? *Aquatic Toxicology, 46*, 253–268.

Bickham, J. W., Sandhu, S., Hebert, P. D. N., Chikhi, L. & Athwal R. (2000). Effects of chemicals contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. *Mutation Research/ Reviews in Mutation Research, 463*(1): 33–51.

Boeger, W.A., Guimarães, A.T.B., Romão, S., Ostrensky, A., ZAmberlan, E. & Falkiewicz, F.H. 2003. Histopatology as an approach to evaluated the effect of an oil spill on fishes of Arroio Saldanha and Rio Iguaçu (Brazil), International oil spill conference, Amer Petroleum Inst, Washington, USA.

Bolognesi, C., Perrone, E., Roggieri, P., & Sciutto, A. (2006). Bioindicators in monitoring long term genotoxic impact of oil spill: haven case study. *Marine Environmental Research, 62*(Supplement 1), S287–S291.

Carls, M. G., Rice, S. D., & Hose, J. E. (1999). Sensitivity of fish embryos to weathered crude oil: part I. Low-level exposure during incubation causes malformations, genetic damage, and mortality in larval pacific herring (*Clupea pallasi*). *Environmental Toxicology and Chemistry, 18*, 481–493.

Collier, T.K., Krone, C.A., Krahn, M.M., Stein, J.E., Chan, S.L. & Varanasi, U. 1996. Petroleum exposure and associated biochemical effects in subtidal fish after the Exxon Valdez oil spill American Fisheries Society Symposium, pp. 671–683. Avaliable on.

Cronin, M. A., & Bickham, J. W. (1998). A population genetic analysis of the potential for a crude oil spill to induce heritable mutations and impact natural populations. *Ecotoxicology, 7*, 259–278.

Eisler, R. (1987). Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates: a synoptic review. *US fish and wildlife service biological report, 85*, 81.

Etkin, D.S. 2004 Modeling oil spill response and damage costs Proceedings of the Fifth Biennial Freshwater Spills Symposium, p. 16. Avaliable at: https://archive.epa.gov/emergencies/content/fss/web/pdf/etkin2_04.pdf.

Faria, B.M.D., Timmerman, M.C., Platte, E.B. & Rosário, M.D. (2005). Environmental effects in a subtropical brazilian river after an oil spill International Oil Spill Conference Proceedings, pp. 701–702. Avaliable at: http://www.ioscproceedings.org/doi/abs/10.7901/2169-3358-2005-1-701.

Gabardo, I.T., Meniconi, M.F., Faria, B.M., Silva, T.A., Cavalcanti, T.R., Silva, G.C., Gallotta, F.D., N., A.S., Paes, J.E., Bentz, C.M., Lima, S.O.F., Rosário, M.D., Soriano, A.U., Baessa, M.P.M., Mendes, L.G., Dirceu C, Silveira, J., Bilhalva, M.M., Politano, A.T., Freitas, L.R.D., Parkinson, R., Lima, J.A.M., Guimarães, P.P., Medeiros, J. & Santos, A.F. 2011. Lessons learned on oil spill environment impact assessment: 10 years of petrobras experience review. International Oil Spill Conference Proceedings, p. abs426. Avaliable at: http://www.ioscproceedings.org/doi/abs/10.7901/2169-3358-2011-1-426.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Harvey, R. G. (1991). *Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity* (p. 396). Cambridge: CAmbridge Universaty Press.

Horodesky, A., Abilhoa, V., Zeni, T. D. O., Montanhini Neto, R., Castilho-Westphal, G. G., & Ostrensky, A. (2015). Ecological analysis of the ichthyofaunal community ten years after a diesel oil spill at Serra do Mar, Paraná state, Brazil. *Global Ecology and Conservation, 4*, 311–320.

Hose, J. E., McGurk, M. D., Marty, G. D., Hinton, D. E., Brown, E. D., & Baker, T. T. (1996). Sublethal effects of the (Exxon Valdez) oil spill on herring embryos and larvae: morphological, cytogenetic, and histopathological assessments, 1989–1991. *Canadian Journal of Fisheries and Aquatic Sciences, 53*, 2355–2365.

Incardona, J. P., Collier, T. K., & Scholz, N. L. (2004). Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. *Toxicology and Applied Pharmacology, 196*, 191–205.

Incardona, J. P., Vines, C. A., Linbo, T. L., Myers, M. S., Sloan, C. A., Anulacion, B. F., Boyd, D., Collier, T. K., Morgan, S., Cherr, G. N., & Scholz, N. L. (2012). Potent phototoxicity of marine bunker oil to translucent herring embryos after prolonged weathering. *PloS One, 7*, 30116.

Katsumiti, A., França, P. P., Costa, G. P. S., Zandoná, E. M., Benincá, C., Assis, H. C. S. D., Cestari, M. M., Maschchio, J., Randi, M. A. F., Silva, C. A., Rochecheche, H., & Ribebeiro, C. A. O. (2013). Evaluation five years after a refinary oil spill in freshwater wetland—Paraná State, Southern of Brazil. *Ecotoxicology and Environmental Contamination, 8*, 77–87.

Lanctot, R., Goatcher, B., Kim, S., Talbot, S., Pierson, B., Daniel, E., & Zwiefelhofer, D. (1999). Harlequin duck recovery from the Exxon Valdez oil spill: a population genetics perspective. *The Auk, 116*, 781–791.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics, 23*, 2947–2948.

Librado, P. & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25,* 1451–1452.

Miller, G. D., Seeb, J. E., Bue, B. G., & Sharr, S. (1994). Saltwater exposure at fertilization induces ploidy alterations. Including mosaicism, in salmonid. *Canadian Journal of Fisheries and Aquatic Sciences, 51*, 42–49.

Ostrensky, A., Chaves, P.T.C., Duboc, L.F., Guimarães, A.T.B., Cruz, S.R.Z., Wegbecher, F.X., Pilchowski, R.W., Teixeira, U.A. & Belz, C.E. (2001). Monitoramento ictiofaunístico pós-derramamento de óleo nos Rios Bariguí e Iguaçu, 2° Seminário do Rio Iguaçu, Araucária, Paraná, Brasil, pp. 32–52.

Ostrensky, A., Neto, R. M., Castilho-Westphal, G. G., Zeni, T. O., Abilhoa, V., & Horodesky, A. (2015). Population structure of fish from the Serra do Mar, Paraná, Brazil: a comparative analysis of environments affected and by oil spills and unaffected areas. *Journal of Ecology and The Natural Environment, 1*, 54–62.

Paradis, E. (2010). pegas: an R package for population genetics with an integrated-modular approach. *Bioinformatics, 26,* 419–420.

Peterson, C. H., Rice, S. D., Short, J. W., Esler, D., Bodkin, J. L., Ballachey, B. E., & Irons, D. B. (2003). Long-term ecosystem response to the Exxon Valdez oil spill. *Science, 302*, 2082–2086.

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/. Accessed 19 January 2017.

Srogi, K. (2007). Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review. *Environmental Chemistry Letters, 5*, 169–195.

Staden, R., Beal, K. F. & Bonfield, J. K. (1998). The staden package. Computer methods in molecular biology, bioinformatics methods and protocols. In S. Misener & S. A. Krawetz (Eds), *The Humana Press Inc., Totowa, NJ 07512, 132,* 15–130.

Teal, J. M., & Howarth, R. W. (1984). Oil spill studies: a review of ecological effects. *Environmental Management, 8*, 27–43.

Vanzella, T. P., Martinez, C. B. R., & Cólus, I. M. S. (2007). Genotoxic and mutagenic effects of diesel oil water soluble fraction on a neotropical fish species. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis, 631*, 36–43.

Wickham, H. (2007). Reshaping data with the reshape package. *Journal of Statistical Software, 21*, 20.

Wickham, H. (2011). The split-apply-combine strategy for data analysis. *Journal of Statistical Software, 40*, 29.