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Individual internship

4th year

Quantification and characterisation of microplastic ingested by different fish species of the Paraná River

National Institute of Limnology (INALI)
Ciudad Universitaria 3000 Santa Fe City
Santa Fe Province ARGENTINA

INALI

UNL CONICET

Professional tutor :
Martin BLETTLER
Associate researcher (CONICET)

Academic tutor :
Karl Matthias WANTZEN
UNESCO Chair : River culture

Audrey
GLOAGUEN
IMA
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Introduction

This internship took place in Santa Fe, in Argentina. This city is the capital of the province of Santa Fe, and it is situated about 500 km northwest of Buenos Aires. The city is located between the Rio Salado del Norte and the Setubal Lagoon.
Santa Fe is located in the Paraná basin (Iriondo, Paggi, et Parma 2007). This river is formed by the confluence of two major rivers, the Rio Paranáiba and the Rio Grande. Its catchment area covers between 2,600,00 and 2,800,000 m^2, and extends over four countries: Paraguay, Brazil, Bolivia and Argentina. The river flows into the Atlantic Ocean at Buenos Aires (Iriondo, Paggi, et Parma 2007). Its complex system is the third largest river system in the world. It is an anastomosed system, with many islands. (Iriondo, Paggi, et Parma 2007)
The city of Santa Fe is located in the Middle Paraná, which extends from Apipe to Rosario. The average flow of the Paraná river in this part is 16,595 m^3/s. (http://www.grdc.sr.unh.edu/).

This internship focused on a very current problem nowadays, plastic pollution.
The world plastic production is constantly increasing, from 1.5 million tonnes in 1950 to 350 million tonnes in 2017 (https://www.plasticseurope.org), which is directly visible in everyday life, since they are an integral part of it. This increase is becoming worrisome, and the prediction indicates that production is expected to double by 2050. (Arias-Andres et al. 2018)

One of the major challenge Argentina is faces is waste management. Indeed, recycling is not effective, and open-air waste disposal facilities can be found in the country. (González Carman, Machain, et Campagna 2015) These dumpsites, such as the one in Rincón city, where we went to sample bird faeces, are located on plots that are in direct connection to the river. These areas are often flooded. Thus, all the waste stored there, such as plastics, ends up in the water. After that, the degradation process of plastics will take place in water.
Water is not the only plastic degrading agent. For instance, UV rays or the wind will also degrade it (Cole et al. 2011). Thus, in landfills such as Rincón, or in floodplains, all plastics found there will begin to degrade with natural agents. Once these areas are flooded, all the plastics that have been degraded until then will end up in the water.

Different sizes classes exist for plastics. In this study, the classification used by the most authors was chosen. This classification is used by Blettler et al. (2018) and it considers that microplastics are plastics smaller than 5 mm, mesoplastics have a size between 5 mm and 2.5 cm, and macroplastics have a size larger than 2.5 cm.
Microplastics can come from different sources.
In fact, they can come from a secondary source, they are then the result of the degradation of larger plastics. (Cole et al. 2011). But they can also be primary plastics, i.e. they are intentionally marketed, used in health and beauty products for example. (Cole et al. 2011)

There are many interactions between living beings and plastics. As soon as a piece of plastic is made, it is likely to end up in the food chain. (Scherer et al. 2018) (Figure 1) Depending on the size of the plastics, pollution affects wildlife differently.
We are focusing on fish in this study.
The largest plastic pieces can produce a physical strangulation, an intestinal blockage, which will itself lead to a decrease in nutrition, a suffocation. They can also play a role in fish mobility. (Derraik 2002)
The consequences of macroplastics are relatively well known, as they are easier to identify.

On the other hand, microplastics are more difficult to identify. These are increasingly being studied (Blettler et al. 2018) because they pose problems for the health of fish, but also for the transport of polluting particles, such as metals.(Lu et al. 2018)

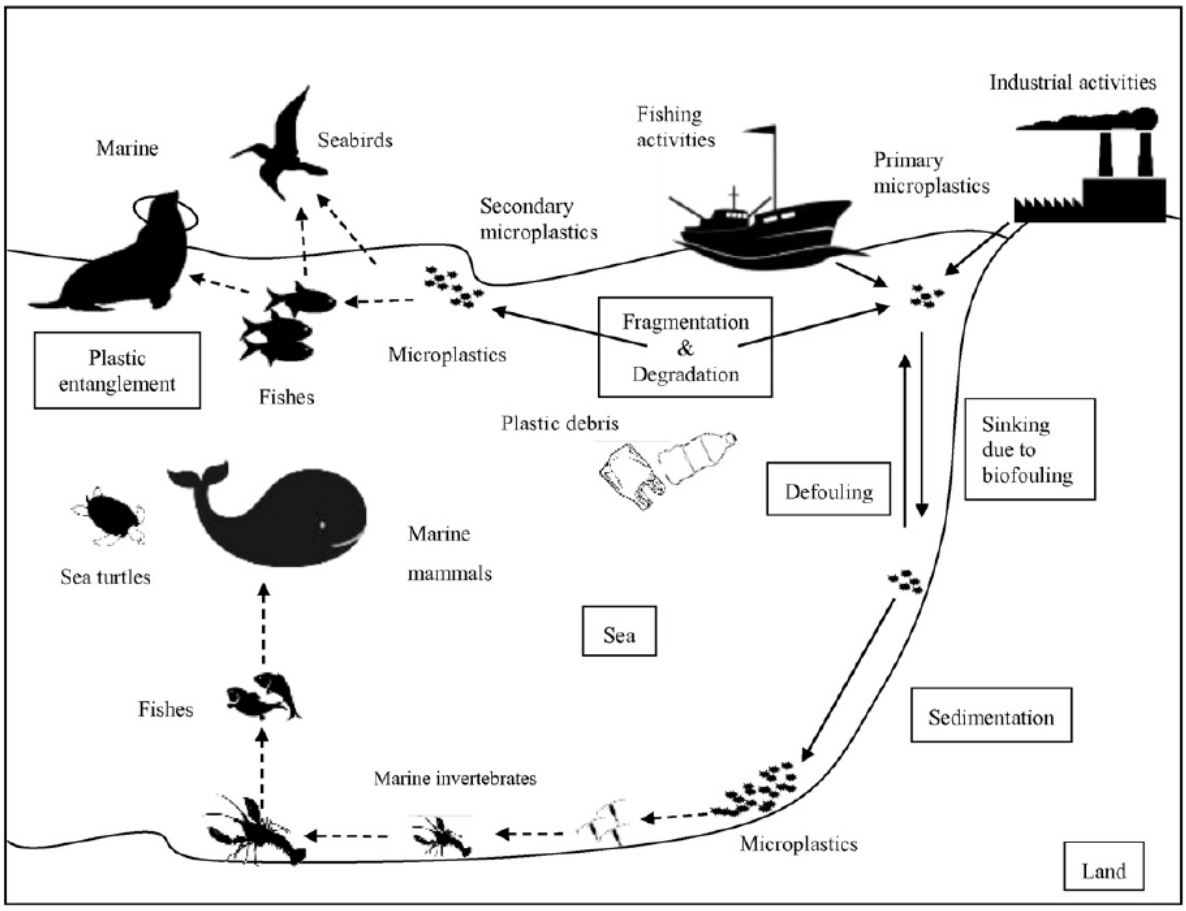


Figure 1: Interactions between microplastics, biota and ecosystems. (Li, Tse, et Fok 2016)

Taking into account the above, the objectives of this study were to determine: i) the presence of microplastic particles in the digestive tract of four fish species of the Paraná River (Potamotrygon motoro, Pterodoras granulosus, Prochilodus lineatus, Ageneiosus inermis), ii) the potential relation between fish living habits and abundance of microplastic ingested, and iii) the dominant type and abundance of microplastics in highly polluted and less polluted areas.

For the first objective, the hypothesis was that due to the great microplastic pollution levels recorded in sediments of the Paraná River, some benthic fish species are contaminated with microplastics by accidental ingestion.

Concerning the second objectives, some hypothesis were speculated.
The more polluted an area is, the more microplastics fish will ingest.(Ory et al. 2018)
In fact, when the concentration in the water of microplastics is higher, fish are more likely to swallow microplastics when breathing or swallowing their prey. (Slootmaekers et al. 2019) This hypothesis can be seen at the local level, so the position in the water column will have an influence on the amount of microplastics ingested. To test this hypothesis, it will then be necessary to study two species of fish living in two different part of the water column (one pelagic fish and one benthic fish). At the bottom, microplastics are often more concentrated, due to the degradation of the plastics that have been

deposited, so fish living in the benthic domain will be more likely to ingest microplastics than pelagic ones. (Phillips et Bonner 2015; Avio, Gorbi, et Regoli 2015)

Hypothesis 3 is based on the larger scale, by studying the type of microplastics found in species but on two different fishing sites. It is thus possible to study an environment considered as highly polluted and a less polluted.(Wright, Thompson, et Galloway 2013)
The hypothesis is that microplastics are more abundant in the high polluted area, dominated by micro-laminar pieces (potentially originated from the breakdown of bags/food wrappers).

The host organization

This internship was carried out at INALI (National Institute of Limnology), in the city of Santa Fe, Argentina. (Figure 2)

Figure 2: Location of INALI (Google Earth)

This institute was created in August 1962 by the president of CONICET (*Consejo Nacional de Investigaciones Cientificasy Tecnicas*). The director of INALI is Dr. Pablo A. Collins.
It is a research institute on the study of continental aquatic ecosystems in Argentina.
Its objectives are (https://inali.conicet.gov.ar):

- To contribute to knowledge of organisms, biological phenomena and all other biological phenomena in continental aquatic environments. Due to the Institute's situation, the main focus was on Paraná and its watershed.
- To conduct an inventory of species living in wetlands
- To study fishery resources in order to have a rational use of them
- To mix natural resources with regional activity.

This Institute is multidisciplinary, which makes it possible to carry out a wide variety of projects. Different laboratories are present in the institute, ichthyology, plankton, benthic, macro crustaceans… The internship was conducted in the hydro-ecology laboratory. It is composed of researchers (Luis A. ESPINOLA, Martin C. M. BLETTLER), post-doctoral (Ellie ABRIAL, Eliana G. EBERLE) and doctoral students (Ana Pia RABUFFETTI, Daiana PASCUALE, Nicolas GARELLO, Maria Florencia EURICH). The laboratory mainly studies the response of aquatic organisms to hydrodynamic fluctuations in major continental aquatic systems, with a focus on benthic invertebrates and fish.
In recent years, the laboratory has been known for studying the consequences of plastic pollution on the aquatic ecosystem.

1. Materials and methods

1.1 Literature review

In order to study the ingestion of microplastics in freshwater fish, the first objective was to conduct a literature review of the various articles that existed. For this purpose, the Scopus software was used. It is a database on which, using keywords, the evolution of the number of articles on a subject can be found.
To do this, keywords were entered: "Freshwater" AND "Fish" AND "plastic". Scientists are increasingly interested in this subject, 219 existing articles have been found, of which more than 70% were published after 2010. (https://www.scopus.com/) The keyword "freshwater" was entered to obtain only articles studying freshwater, not those on marine waters. Indeed, plastic pollution for marine fish has already been well documented, since 87% of studies have been carried out on the marine environment, compared to only 13% in freshwater environments. (Blettler et al. 2018) The study conducted during this internship increases the knowledge of freshwater fish.

1.2 Experiments

1.2.1 Obtaining samples/ fishing sites

Several species were studied in order to meet the different objectives of the study. Thus, different fisheries took place.
A highly polluted area and a less polluted area (Chapetón area) were chosen. The high polluted area (Figure 3) corresponds to the outlet of the wastewater pipe of the city of Santa Fe.

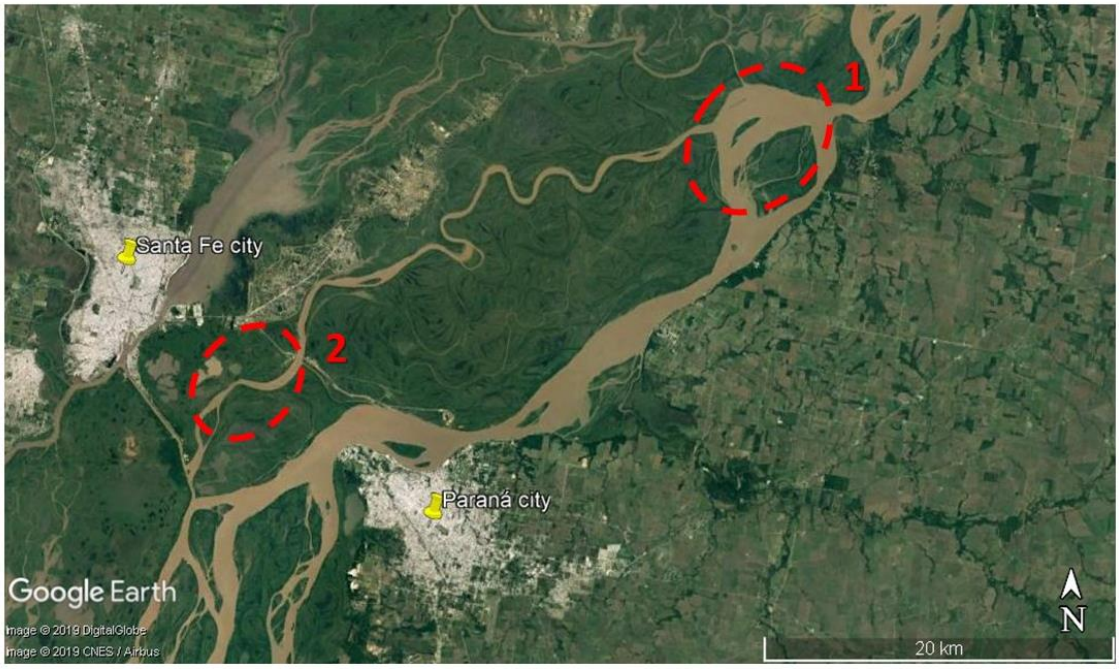


Figure 3: Location of fisheries (1: Chapetón area = less polluted area; 2: highly polluted area)

10 freshwater stingrays were caught in the less polluted area by the laboratory researchers and the other fishes were caught in the more polluted area by fishermen the laboratory payed for. (Table 1, pictures in Appendix 1) Nets and hooked lines were used to catch the different fish.

Table 1: Description of studied species (fishbases.com; Almirón et al. 2001)

Species	Common name	Feeding habit	Position in the water column	Number of fish
Potamotrygon motoro	South American freshwater stingray, raya	Molluscs, Crustaceans, little fishes, insects	benthopelagic	10 in natural area 5 in polluted area
Prochilodus lineatus	Sábalo	Organic debris	benthopelagic	6 in polluted area
Pterodoras granulosus	Armado	Molluscs, crustaceans, organic debris	demersal	12 in polluted area
Ageneiosus inermis	Manduvé	Planktonic crustaceans	pelagic	2 in polluted area

The part of the work presented in this report focuses on the freshwater stingrays, the others species will be studied until the end of the internship. Freshwater stingrays are considered as "data insufficient" by the UICN. (International Union for Conservation of Nature) Populations of freshwater stingrays is decreasing in the Paraná river, which is linked to human activities, because of the excessive fisheries. (Lucifora et al. 2017)

1.2.2 Laboratory processing: extraction of stomach and digestion contents

Preliminary precautions

In order to be able to compare different species with each other, it was decided to extract the content from the entire digestive tract (Figure 4). Indeed, the stingray has a digestive tract and a well formed stomach, while the Sábalo for example does not have a well formed stomach. It is therefore necessary to take the entire digestive tract in order to make comparisons. (Lusher et al. 2017)
In addition, it has been shown that for species with distinct parts in the digestive tract, the concentration of microplastics differs between each part. It is therefore meaningful to take all the parts in order to quantify all the microplastics ingested. (Jabeen et al. 2017)

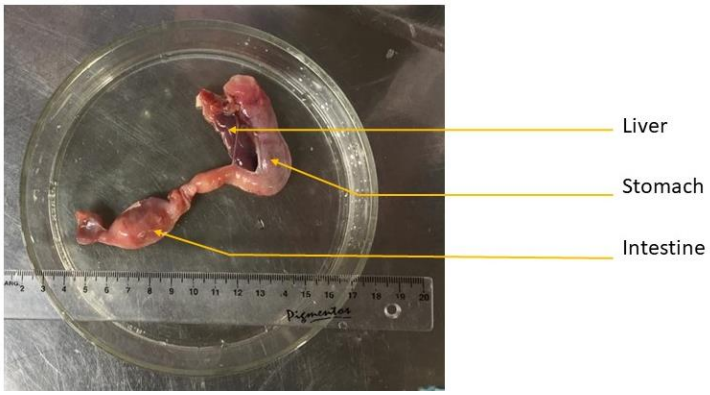


Figure 4: Photograph of the part of the digestive tract studied for P.motoro. Credit : Audrey GLOAGUEN

In addition, in order to avoid samples' contamination under laboratory conditions, we used gowns, gloves and a mask. Each piece of equipment used was rinsed to avoid contamination by microplastics, and the tables were washed before each handling. Constant ventilation has also been put in place to limit microplastic pollution in the laboratory air.(Slootmaekers et al. 2019) As an example, we used a hood to dissect fish and to extract stomach contents.

An experiment was also carried out to quantify the microplastic pollution of the laboratory air. Indeed, some researchers have shown that air contains microplastics, so it is very likely that these microplastics can fall into our solutions, and contaminate them. (Dris et al. 2015, 2017; Wesch et al. 2017)
Therefore, it seems important to quantify this pollution, and to verify that it is negligible, otherwise, the results will be overestimated (Appendix 2).

Protocol for the quantification of microplastics ingested by fishes

All operations were carried out under a hood. After washing all the equipment, the stomach and intestines were placed in a petri dish. The methodology used in this study is the same than the one used by (Avio, Gorbi, et Regoli 2015).
The length of the stomach and intestines and their width were measured using a ruler. (Figure 5a)
After that, the entire digestive tract was weighed.
Using scissors, the stomach and intestines were opened. (Figure 5b) Then, the content was extracted by scraping with a scalpel blade. (Figure 5c)
The empty digestive tract and the content were weighted using a weighing scales (0,01g).
After this extraction, a digestion method was used to digest all the organic matter (Jabeen et al. 2017)
The content was poured into a large 1000 ml beaker and 4 times the volume of content was added in Hydrogen Peroxyde 30% (H_2O_2). (Blettler et al. 2017)
The beaker was then closed with aluminium foil, in order to prevent air contamination. (Figure 5d)
It was placed on the hot plate at 60 degrees, which acts as a catalyst. (Figure 5e)
The digestion reaction was completed when there was no more foam on the surface of the solution, and so, when no organic matter remained.

Figure 5: Extraction and treatment of stomach contents from P.motoro. Credit : Audrey GLOAGUEN

1.2.3 Laboratory processing : analysis of the digested solution

Once the contents were digested, the solutions were filtered through a 63-micron filter. Only contents that had not passed through the filters were kept, the rest (water, nanoplastics, H_20_2 crystals) was thrown away.
The samples were poured in a smaller beaker and they were diluted with distilled water to make it easier to analyse with a magnifying glass.
A Boeco™ binocular magnifying glass was used to identify the MPs.
All the microplastics found were then isolated on a slide, as well as all the items which were not really identified. The slide was observed under a Nikon™ binocular microscope with a magnification range of X400 to ensure that it was really microplastic. Once this verification was completed, pictures of the observation were taken under the microscope.
The pictures were then analysed to determine the morphological characteristics (length of each microplastic, area). This measurement was made using Adobe Photoshop software.
Each MP was therefore characterized by its type (length, shape and colour) (Appendix 3).

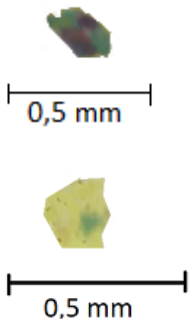
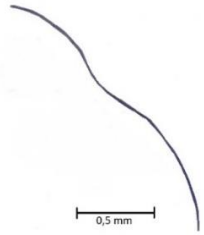
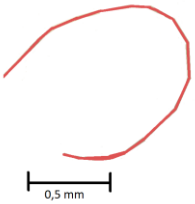
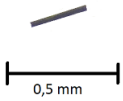
1.2.4 Definition of the different types of microplastics

Before starting to sort the microplastics contained in stomachs, the definition of each type of microplastic was fixed in order to separate them into different classes.

Two types of microplastics were studied. (Table 2)
Fibres, which are elongated, regular pieces, and have clean sections. They have a uniform colour throughout their length. (Rodrigues et al. 2018)
These have been sorted into different colours according to their occurrence, blue, red, black and others (yellow and transparent).
The other type of microplastic corresponds to laminar pieces, which are 3D pieces, often with printing marks. (Rodrigues et al. 2018)

Only the microplastics we were sure of were kept. To make sure it was a laminar piece, we cut it in half and checked that the cut was clean.(Rodrigues et al. 2018)

Table 2: Types of microplastics ingested by fishes. Author : Audrey GLOAGUEN

Laminar pieces	Blue fibre	Red fibre	Black fibre	Other fibre
				

1.2.5 Statistical analysis

A Shapiro-Wilk test (normality test) followed by a Mann-Withney test were used to demonstrate the significant difference in fibre size between stingrays caught in the highly polluted environment and in the less polluted environment. (Appendix 4).
A linear correlation was used to study the influence of the number of microplastics on the weight of the stingrays.
Another one was performed to test if the weight of stomach content depends on the weight of the fish.

2. Results

2.1 Microplastics ingested by freshwater stingrays

Chapetón area stingrays are numbered from 1 to 10, while those from the highly polluted environment are those from A to E. (Table 3, Table 4)

The average number of microplastics found in the stingrays' digestive tract from the less polluted area is 24 items (Table 3), whereas it is 35 items for the highly polluted area (Table 4).

In order to be able to compare the different stingrays between them, it was chosen to divide the number of microplastics found for a stingray by the weight of the stomach content of it. This resulted in a fibre concentration for the stomach studied.
On average, 1.8 microplastic per gram of stomach content ($MP.g^{-1}$) are found in the stomach contents of the stingrays on Chapetón area (Table 3), and 2.4 $MP.g^{-1}$ are found in the stomach contents of the stingrays in the highly polluted area (Table 4).

Table 3: Results for freshwater stingrays from Chapetón area

	Stingray 1	Stingray 2	Stingray 3	Stingray 4	Stingray 5	Stingray 6	Stingray 7	Stingray 8	Stingray 9	Stingray 10
Total number of microplastics	18	29	18	25	14	35	27	26	35	16
Stomach content concentration in microplastics	0.65	1.96	1.83	1.35	2.69	1.90	0.90	4.13	1.86	0.96
Average concentration of microplastics	1.8									
Average number of microplastics	24									

Table 4: Results for freshwater stingrays from an highly polluted environment

	Stingray A	Stingray B	Stingray C	Stingray D	Stingray E
Total number of microplastics	30	22	38	47	38
Stomach content concentration in microplastics	3.97	0.81	1.80	3.90	2.10
Average concentration of microplastics	2.5				
Average number of microplastics	35				

Microplastic concentrations are very varied, as shown in the Figure 6.
The stingray with the lowest concentration of microplastics is stingray 1, coming from Chapetón area, and therefore from the less polluted environment. The one with the highest concentration is stingray 8 from the same environment. (Figure 6)

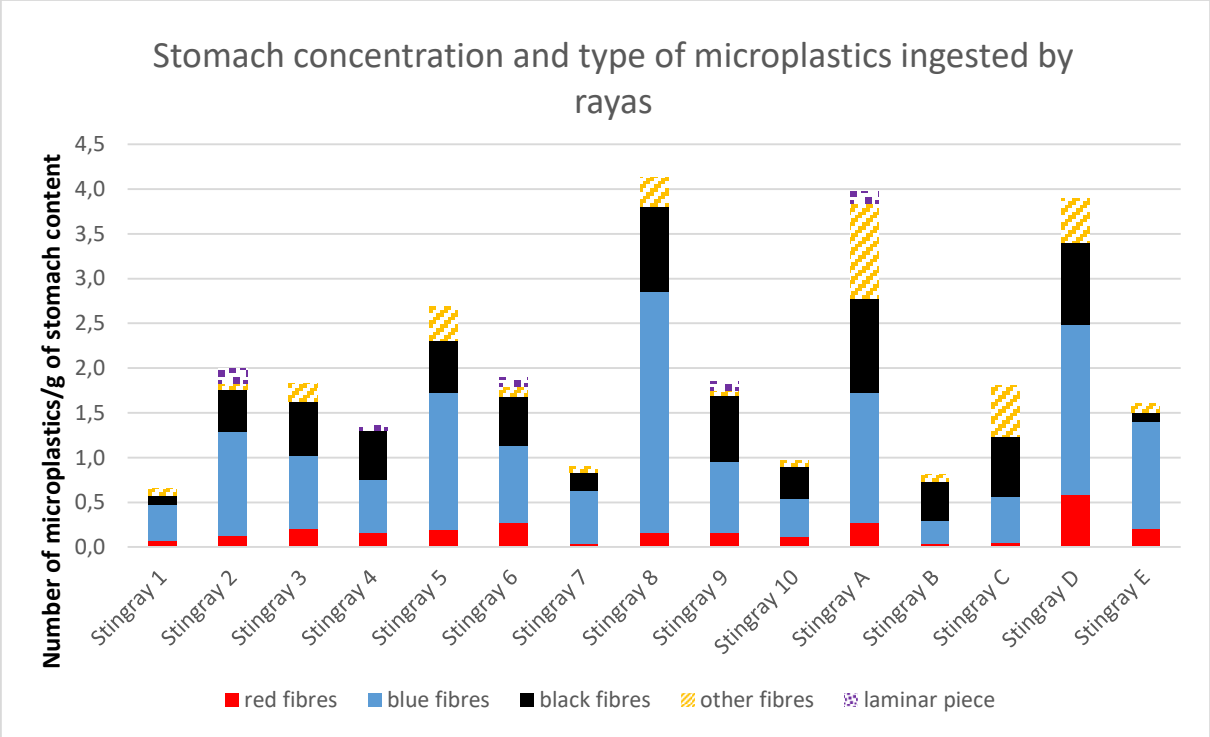


Figure 6: Microplastic concentration and type in the digestive tracts ingested by freshwater stingrays

A total of 418 microplastics were found in the digestive tract of the 15 stingrays.
98.1% of these microplastics correspond to fibres (410), only 8 laminar pieces could be found. (Appendix 3)
Among the fibres, 45.7% are blue fibres, 31.1% are black fibres and 8.1% are red. The rest corresponds to transparent or yellow fibres. (Figure 7)

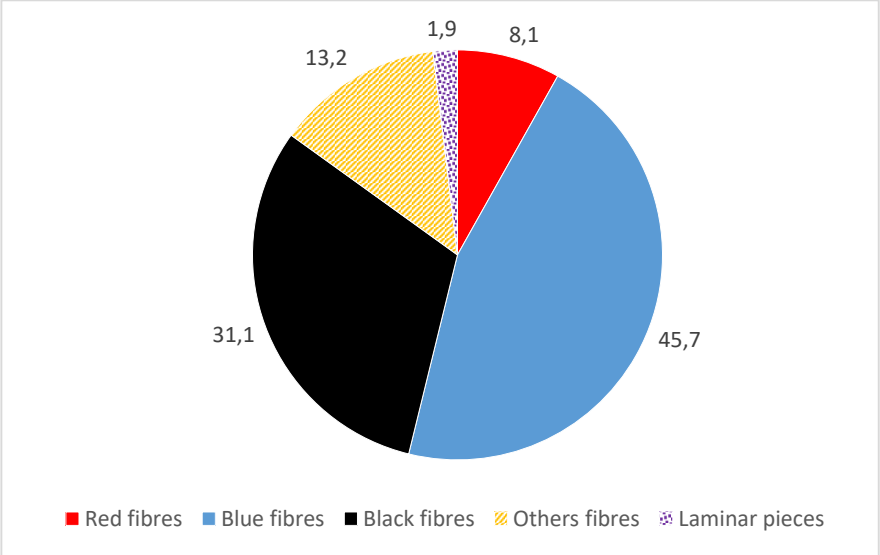


Figure 7: Types of microplastics ingested by freshwater stingrays (%)

The colour distribution of the fibres and the type of microplastic are different in the highly polluted and less polluted environments (Figure 8)
Stingrays from a highly polluted environment ingested 36.0% blue fibre, 33.7% black fibre, and 22.9% yellow/transparent fibre. They ingested 6.9% red fibres, and almost no laminar pieces (0.6%).
Stingrays from a less polluted environment ingested 52.7% blue fibre, 29.2% black fibre and 9.1% red fibre. They ingested 6.2% yellow/transparent fibres and 2.9% laminar pieces.

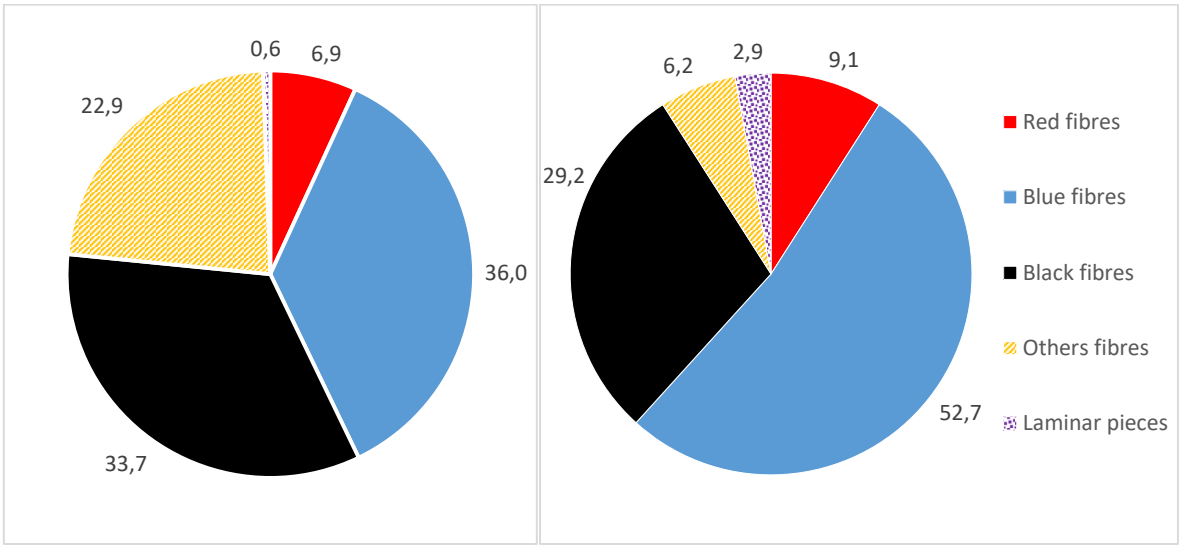


Figure 8: Types of microplastics ingested by freshwater stingrays in an highly polluted area (left) and in a less polluted area (right) (%)

The size of fibres found in the digestive tract of stingrays living in a highly polluted environment and living in a less polluted environment was compared.
The box plots obtained with XL Stat software show that the distribution of fibre lengths for each environment seems to be the same.(Figure 9)

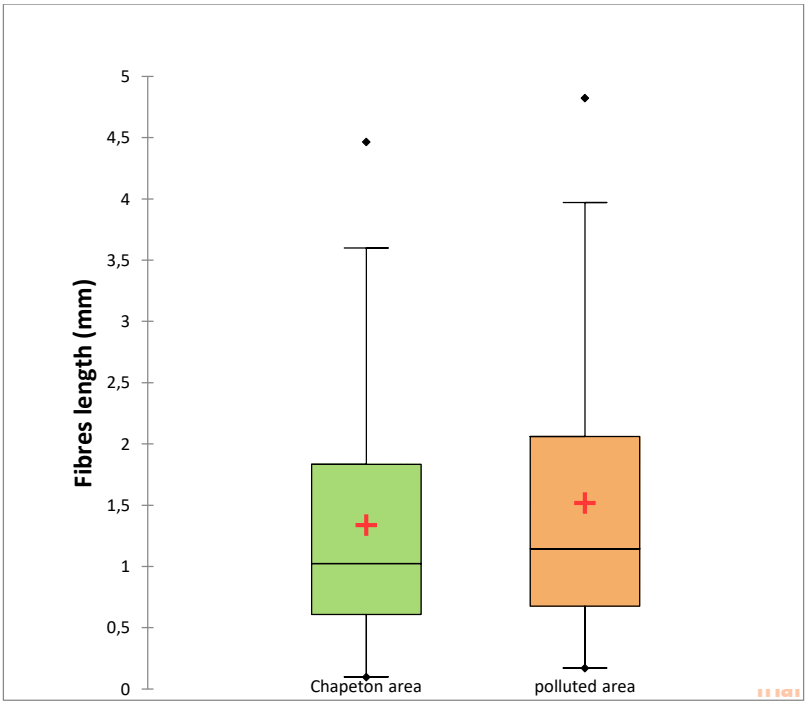


Figure 9: Box plots comparing the length of fibres from natural and polluted environments (XL Stat software)

The average fibre length for the natural environment is 1.34 mm while the average fibre length for the polluted environment is 1.52 mm. (Table 5)
The normality test of the data "fibre lengths in the less polluted environment" and "fibre lengths in the highly polluted environment" shows that these two series do not follow a normal distribution. It is therefore necessary to carry out a non-parametric test. According to the Mann-Whitney test and its p-value (0.073), there is no difference in the distribution of fibre lengths in the polluted and natural environment. (Appendix 4)
For the less polluted environment, the minimum length is 0.10 mm and the maximum length is 4.47 mm.
For the highly polluted area, the minimum length is 0.17 mm and the maximum length is 4.82 mm.

Table 5: Statistical data from XL Stat software

Statistical data	Natural environment	Polluted environment
Number of fibres observed	234	171
Minimum length (mm)	0.10	0.17
Maximum length (mm)	4.47	4.82
Average lenght (mm)	1.34	1.52

In the Figure 10, the weight is not a function of the number of microplastics, it does not follow a trend. Indeed, each point represents a stingray, and these points are very scattered.
Some stingrays have few microplastics and a low weight, while others have many microplastics and a low weight too, and vice versa.

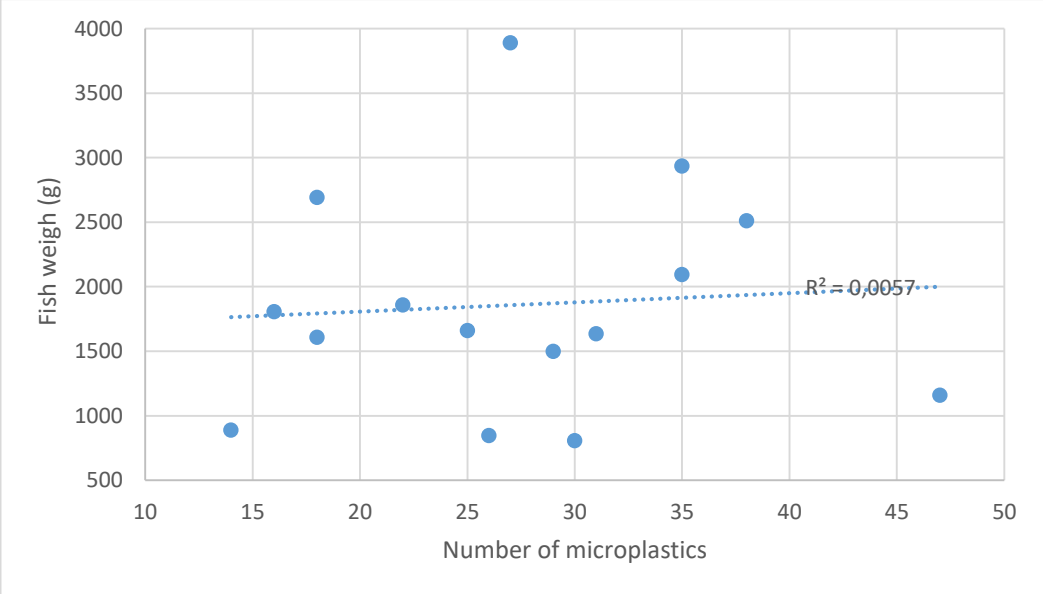


Figure 10: Fish weigh in relation with the number of microplastics

The weight of the stomach contents depends on the weight of the fish. The R^2 is 0.69, which is good enough for ecological variables. (Figure 11) The larger stingrays seem to have a bigger stomach.

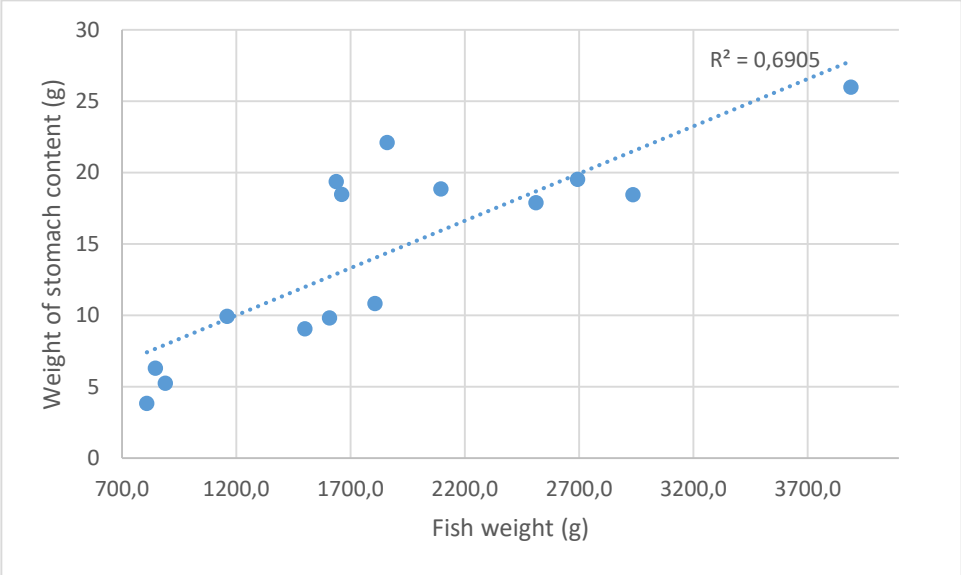


Figure 11: Weight of stomach content in relation to the fish weight

2.2 Microplastics ingested by Armado

In process

2.3 Microplastics ingested by Sabalo

Waiting for results

2.4 Microplastics ingested by Manduvé

Waiting for results

3. Discussion & limits

3.1 Protocol discussion

The digestive tract contents were removed from the digestive tract using friction made by a blade. Some microplastics may have got stuck in the folds of the empty digestive tract. It would have been interesting to put the entire intestines and stomach in the beaker and put everything to digest. However, the quantity of H_2O_2 to be used as well as the time constraint did not allow to test the effectiveness of this solution on the possible integral digestion. Some studies have shown that KOH is more effective on digestion than H_2O_2 (Kühn et al. 2017). A comparative study of the effectiveness of the two protocols will be done to show this possibility for future research, using the protocol of Rochman et al. (2015).

Another point to note is that the protocol used cannot be used without a hot plate.
At the beginning of the internship, a hardware problem was encountered. The hot plate was not working, so it was impossible to control the temperature. The first sample had a temperature exceeding 110 degrees. However, the temperature affects microplastics (Munno et al. 2018), so it was harmful for the temperature to rise so much, and it was important to be able to ensure that the temperature was at 60 degrees. It was therefore decided to leave the samples in the ambient air before obtaining a new hot plate. For two weeks, some samples were left to digest in the open air, but the reaction did not take place. H_2O_2 is therefore not a perfect or rapid mean of digestion without a hot plate.

98,1% of the microplastics found correspond to fibres. These fibres are primary microplastics, which can be textile fibres from clothing washings (Li, Tse, et Fok 2016).
Some non-synthetic fibres from textiles such as cotton could be taken into account, as the fibre identification criteria are not sufficient to differentiate between textile and plastic fibres, additional analyses should be carried out to identify each fibre (Ory et al. 2018)
After isolating each microplastic, some authors carry out additional studies to find out the origin of the microplastic, and ensure that it is not non-synthetic fibre. For example, the plastic pollution study by Yang et al. (2015) uses a Fourier transform infrared (FT-IR) spectrophotometer.

When H_2O_2 is added with the stomach content, and during the digestion period, a foam is formed on the surface of the sample. This will form deposits on the beaker, which may contain microplastics. Care must be taken to avoid these deposits and to clean them if they form.

3.2 Effect of the environment on the number of microplastics ingested by fishes

3.2.1 Importance of the position on the water column on the quantity of microplastics ingested

In their study, Avio, Gorbi, et Regoli (2015) have showed that the physico-chemical properties of polymers have a significant influence on ingestion and accumulation by organisms. In fact, some polymers are more likely to float, such as polyethylene, while others are more uniformly found in the water column, such as polystyrene. The different types of polymers will therefore not be ingested in the same proportions by benthic and pelagic species.

Expected results :

The size of the particles ingested by organisms living at the bottom may be larger than those living in the water column, as their density would be higher, so these microplastics will float less and be more abundant towards the bottom. There will therefore be a greater chance of ingesting them for benthic fish.
At the bottom, microplastics are often more concentrated, due to the degradation of the plastics that have been deposited, so fish living in the benthic domain will be more likely to ingest microplastics than pelagics. (Phillips et Bonner 2015; Avio, Gorbi, et Regoli 2015)

3.2.2 Importance of the environment on the quantity and the type of microplastics ingested

A slight difference was found in the concentration of microplastics for stingrays coming from a highly polluted environment and those coming from a less polluted environment.
However, it is not possible to test the significance of this data as the number of individuals is not high enough (10 in the less polluted environment and 5 in the highly polluted environment). It would be necessary to analyse more stingrays from both locations in order to validate hypothesis 2 which assumes that the more polluted an area is, the more microplastics are ingested by fishes. Moreover, some studies have shown that "the environment where these stingrays live strongly influence their diets". (Shibuya, Araújo, et Zuanon 2009)
The slight difference could be due to the fact that, in this study, two environments were separated. (Figure 3) It is possible that these two environments are not so far away, and so it is not judicious to separate them. It could be better to take these two environments just for one "urbanised environment", because Chapetón area is not so far from the city of Paraná for example, and take an entire natural environment, in the North of the Paraná River. In addition, the river is a continuum (Vannote et al. 1980), the plastic particles found in the areas of Paraná/Santa Fe can come from far upstream. It is difficult to make a strict distinction between urbanised and non-urbanised areas.

There is not much difference between the type of fibre of stingrays caught in the highly polluted environment and stingrays caught in the most natural environment. (Figure 6)
Only fibres and laminar pieces were found in the stomachs of the stingrays, which is a very important point to remember. Other types of microplastics exist, such as beads or pellets, which are made from cosmetic products, but they don't seem to be polluting the Paraná river.
Currently, the fibres found in the digestive content of stingrays have been compared. The length between the fibres of the highly polluted area and those of the less polluted area does not seem to be significantly different. When all fibres are considered together, there is no difference in the average size between the sample from the highly polluted area and the less polluted one.

In order to determine if there is a difference between the microplastics ingested in the two environments, it will be necessary to group all the microplastics found in the different species, and compare the length, colour and shape of these microplastics.

Some stingrays from the less polluted environment have as many microplastics as stingrays from the highly polluted environment; and vice-versa.
This may be due to the fact that the stingrays fished towards Chapetón area were going up the river, so they passed just before to the most urbanised and therefore most polluted environment. This allowed them to ingest microplastics at this time.
It may be interesting to know the exact path of stingrays before fishing, although this is complicated. In fact, stingrays caught in the less polluted environment with the most microplastics may have arrived

in that environment more recently than others, so they have not yet eliminated the microplastics ingested in the polluted environment in their faeces for example.

Ramos, Barletta, et Costa (2012) showed in their study that the ingestion of microplastics could potentially have an influence on fish weight. Microplastics form balls in the stomachs of fish, which can reduce their appetite. The fish will suffer from starvation. (Eerkes-Medrano, Thompson, et Aldridge 2015; Pazos et al. 2017)
Concerning the 15 stingrays studied, this physiological effect was not shown. When linear regression is performed, R^2 is 0.0057, which shows that the number of microplastics has no influence on the stingrays' weight. (Figure 10)
Microplastics extracted from stingrays do not seem to have any influence on the physiology of fish.
The concentration may be too low to cause a health problem.

Conclusion

Microplastics are ubiquitous in the environment. They can have impacts on the health of living organisms whether it is strangulation, intestinal blockages or malnutrition. (Eerkes-Medrano, Thompson, et Aldridge 2015; Pazos et al. 2017) It is important to study their presence, quantify it and try to find the source of the pollution in order to be able to address the problem at its source.
Plastic fibres were found in all fish in this study.
Microplastics are ingested by many fish species, even those consumed by humans. (Avio, Gorbi, et Regoli 2015)
Once ingested by an organism, microplastics enter the food chain and this plastic pollution can spread through all living beings in the food chain (Wright, Thompson, et Galloway 2013). Microplastics can carry toxic particles, which directly affects the health of the species that ingest them. (Mato et al. 2001; Teuten et al. 2007)

One of the major problems for comparing different studies is the standardization of protocols. (Avio, Gorbi, et Regoli 2015)
In fact, each author chooses to make his protocol. However, some protocols lead to the degradation of certain types of microplastics, while others lead to others. For example, the use of KOH for the digestion leads to the dissolution of cellulose acetate (used in cigarette filters). (Kühn et al. 2017) It is important to always have the same protocol, in order to always have the same limits and therefore be able to compare studies.

It may be interesting to study the transfer of microplastics from the gastrointestinal tract to other tissues. Some authors have shown that these transfers exist (von Moos, Burkhardt-Holm, et Köhler 2012), which will further amplify biomagnification, and therefore the transfer from one organism to another.
It also may be interesting to conduct a study on the amount of microplastics in sediments, the amount in water and the amount of microplastics ingested by fish in a specific area, to see if there is a correlation or not between these different parameters, and so study more precisely the hypothesis on the influence of the environment on the quantity of microplastics ingested.

This internship was my first experience in the world of research and my first experience so long away from my family, friends and France. It has enriched me personally and professionally.

It allowed me to learn how to organize article summaries as I read them, because until then I always lost information during my readings. I learned to use Zotero, which is really a very practical software for bibliography. With the laboratory network, I have discovered websites that I do not have access to, and that will be very useful for my final project, such as the Scopus website, which is widely used for literature reviews.
This internship also showed me that it is very important to follow a protocol precisely, in order not to have any bias in the results and therefore to distort the entire article. Despite this, you have to adapt to the equipment you have on the laboratory, it is complicated to follow exactly one protocol. Not all laboratories have exactly the same equipment, solutions must be found to overcome difficulties, such as those encountered with the hot plate.
This internship gave me a better understanding of research, and it showed me that researchers need to be highly critical.
I don't think I'll be working later in the research field, because for me the topics are too specific, and only cover one area at a time. I would prefer to work in a consultancy office, in order to see a greater diversity of fields.

When I first arrived here, I thought I didn't have too much trouble in Spanish. I quickly realized that the level of education I had was not enough. So I had to adapt, make myself understood with the help of Spanish but also with the English I had. This has allowed me to progress in both languages.
This internship also showed me that adapting to a new country takes a long time. Everything is different in a country so far from France. It took several weeks to really integrate into the laboratory team and fell at home.

Living in a country like Argentina also allowed me to discover facets I didn't know. It has taught me a lot about life in general. The Argentinians are very welcoming, they welcomed me into their home, which is much less common in France. Many things are different, Argentina is a very large country, in which the distances between each city are significant. Each trip I wanted to make took me a long time, because public transport like the train doesn't exist, many are by bus. The diversity of landscapes in Argentina, as well as the different cultures, from the most Europeanized to the most authentic, have enriched me and made me discover the country. The complex and enormous system of the Paraná river has aroused my curiosity and impressed me a lot.

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Appendices

Appendix 1 : Pictures of studied fish (Almirón et al. 2001)

Species	Pictures
Potamotrygon motoro	
Prochilodus lineatus	
Pterodoras granulosus	
Ageneiosus inermis	

Appendix 2 : Quantification of the pollution by the laboratory air

Protocol for the quantification of air contamination

In order to really quantify the pollutants from the laboratory air that could remain in our samples after digestion, two petri dishes containing H_2O_2 were exposed to the ambient air of the laboratory, one for 24 hours and the other one for 48 hours. Two others petri dishes containing H_2O_2 were analysed after 24h and 48h, but these were covered by aluminium foil. Then, the petri dishes were examined under the microscope.

Moreover, in order to better quantify the contamination of our stomach samples by laboratory air, a petri dish containing H_2O_2 on the work surface was placed for some samples when an extraction was performed. This petri dish allows to know the number of microplastics that polluted our samples during handling.
As soon as the beaker containing the stomach contents with H_2O_2 was covered by an aluminium foil, so was the petri dish.
Once the sample was digested, it was analysed under a microscope and the control petri dish too, which allowed us to readjust our results according to the number of pollutants coming from the laboratory air.

Control experiment to test microplastics contamination by the laboratory air

Microplastics can be found in all samples. (Figure 12) Only one type of microplastics was found : fibres. Samples belonging to petri dishes covered with aluminium foil have fewer microplastics than those not covered.
After 24 hours, black and blue fibres were found in the covered sample, for a total of 8 fibres, while for the uncovered 24 hours, 13 fibres were found, including 1 red.
After 48 hours, only blue and black fibres were found in the covered sample. However, 19 fibres were found in the uncovered sample, including red and other fibres, although the majority remained blue and black.

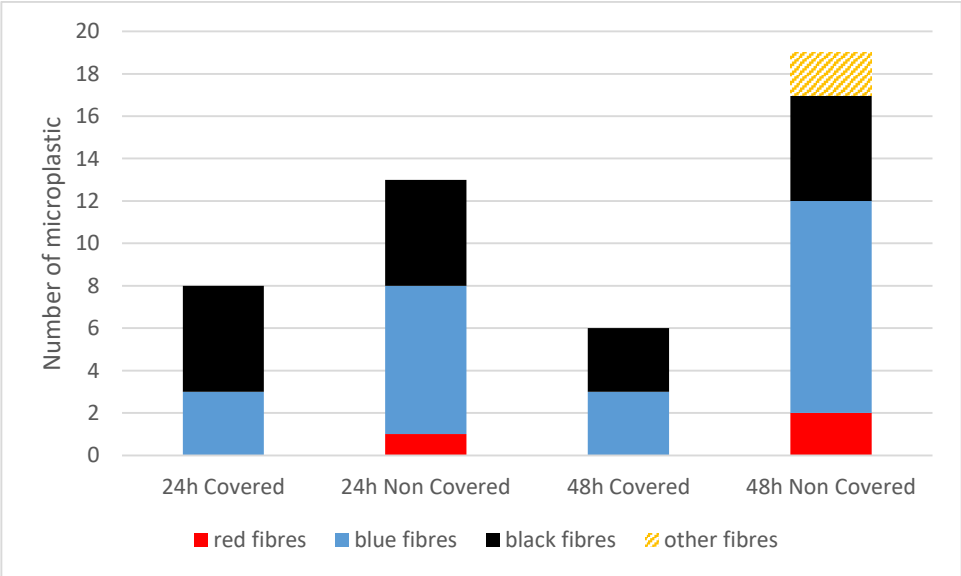

Figure 12: Quantification of air contamination

Quantification of microplastics contamination

The control experiment showed that despite the precautions taken during handling, contamination by plastic fibres from the laboratory air or from improper cleaning of the equipment exists. It is therefore necessary to do the handling as quickly as possible, and to rinse all the equipment thoroughly and wear clothes that minimize all contamination. Many equipment that could be thought of as "clean", or without plastic, contains it. Just look at the paper towel under the microscope to see that it contains many plastic fibres.
These results showed that during handling, the contamination that occurred was that of blue and black fibres. The other fibres (red and other colours) were present in the laboratory air only after handling. Red fibre contamination occurred within 24 hours, while the other fibres (yellow and transparent) only appeared after 24 hours as they only appeared in the 48-hour sample that was not covered.
Slootmaekers et al. (2019) used aluminium foil to cover the beakers in order to avoid plastic contamination from the laboratory air. This method seems to work and to limit plastic contamination. There is still plastic pollution related to handling, but once covered, the beakers no longer seem to be contaminated.

Appendix 3 : Microplastics ingested by the studied freshwater stingrays

		Type of microplastic	Number of fibres	Average length (mm)	Concentration of fibres (fibre/g stomach content)
LESS POLLUTED ENVIRONMENT	Stingray 1	Red fibre	2	0.3	0.1
		Blue fibre	11	1.9	0.4
		Black fibre	3	0.9	0.1
		Other fibre	2	1.6	0.1
	Stingray 2	Red fibre	2	1.6	0.1
		Blue fibre	17	1.4	1.2
		Black fibre	7	1.0	0.5
		Other fibre	1	1.6	0.1
		Laminar piece	2		0.2
	Stingray 3	Red fibre	2	3.5	0.2
		Blue fibre	8	1.1	0.8
		Black fibre	6	0.7	0.6
		Other fibre	2	0.9	0.2
	Stingray 4	Red fibre	3	3.7	0.2
		Blue fibre	11	1.1	0.6
		Black fibre	10	2.0	0.5
		Laminar piece	1		0.1
	Stingray 5	Red fibre	1	0.6	0.2
		Blue fibre	8	1.1	1.5
		Black fibre	3	1.5	0.6
		Other fibre	2	1.3	0.4
	Stingray 6	Red fibre	5	1.9	0.3
		Blue fibre	16	1.2	0.9
		Black fibre	10	0.7	0.5
		Other fibre	2	0.9	0.1
		Laminar piece	2		0.1
	Stingray 7	Red fibre	1	0.9	0.0
		Blue fibre	18	1.4	0.6
		Black fibre	6	0.9	0.2
		Other fibre	2	3.4	0.1
	Stingray 8	Red fibre	1	7.4	0.2
		Blue fibre	17	0.9	2.7
		Black fibre	6	1.1	1.0
		Other fibre	2	1.8	0.3
	Stingray 9	Red fibre	3	1.1	0.2
		Blue fibre	15	1.7	0.8
		Black fibre	14	1.6	0.7
		Other fibre	1	6.1	0.1

		Laminar piece	2		0.1
	Stingray 10	Red fibre	2	2.2	0.1
		Blue fibre	7	0.7	0.4
		Black fibre	6	1.8	0.4
		Other fibre	1	0.7	0.1
HIGHLY POLLUTED ENVIRONMENT	Stingray A	Red fibre	2	1.7	0.3
		Blue fibre	11	0.9	1.5
		Black fibre	8	0.6	1.1
		Other fibre	8	1.4	1.1
		Laminar piece	1		0.1
	Stingray B	Red fibre	1	1.3	0.0
		Blue fibre	7	1.6	0.3
		Black fibre	12	1.7	0.4
		Other fibre	2	1.0	0.1
	Stingray C	Red fibre	1	3.1	0.0
		Blue fibre	11	1.6	0.5
		Black fibre	14	1.4	0.7
		Other fibre	12	1.9	0.6
	Stingray D	Red fibre	7	2.0	0.6
		Blue fibre	23	1.9	1.9
		Black fibre	11	1.7	0.9
		Other fibre	6	1.6	0.5
	Stingray E	Red fibre	4	1.5	0.2
		Blue fibre	24	1.6	1.2
		Black fibre	1	0.7	0.1
		Other fibre	2	1.5	0.1

Appendix 4 : Statistical test for the length of fibres

- **Shapiro-Wilk test** for the normality test of the length distribution for the less polluted area

W	0.885
P-value	<0.0001
alpha	0.05

- **Shapiro-Wilk test** for the normality test of the length distribution for the highly polluted area

W	0.889
P-value	<0.0001
alpha	0.05

- **Mann-Whitney test**

U	17923
Esperance	20007
Variance (U)	1353795.506
p-value	0.073
alpha	0.05

POLYTECH TOURS
35 ALLÉE FERDINAND DE LESSEPS
37200 TOURS

Audrey GLOAGUEN
Students
2018-2019

Quantification and characterisation of microplastic ingested by different fish species of the Paraná River

Abstract :

The presence of microplastics (MP) in the intestinal contents of four freshwater fish species in the Paraná river was studied. A digestion by H_2O_2 was used in the protocol.

100% of the fish have ingested microplastics.

98.1% of microplastics are fibres. There is no difference in the type of fibre and abundance of fibre found in a highly polluted and a less polluted environment. Plastic pollution does not seem to have an impact on the health of the stingrays studied.

Key words :

microplastic, plastic contamination, freshwater systems, fish, South America, Argentina, Paraná river

National Institute of Limnology (INALI)
Ciudad Universitaria 3000 Santa Fe City, Santa Fe Province

Professional tutor :
Martin BLETTLER
Associate researcher

Academic tutor :
Karl Matthias WANTZEN
UNESCO CHAIR : River culture