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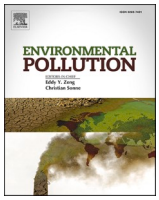

Sardines in hot water: Unravelling plastic fibre ingestion and feeding behaviour effects☆

Oriol Rodriguez-Romeu [a], Maria Constenla [a], Anna Soler-Membrives [a,*], Gilbert Dutto [b], Claire Saraux [c], Quentin Schull [d]

[a] Departament de Biologia Animal, de Biologia Vegetal i d'Ecologia, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, 08193, Barcelona, Spain
[b] MARBEC, Univ Montpellier, IFREMER, IRD, CNRS, Palavas les flòts, France
[c] Université de Strasbourg, CNRS, Institut Pluridisciplinaire Hubert Curien (IPHC) UMR 7178, 23 rue du Loess, 67037, Strasbourg, Cedex 2, France
[d] MARBEC, Univ Montpellier, IFREMER, IRD, CNRS, Sète, France

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ABSTRACT

Small pelagics are small fish species often schooling that mainly feed on planktonic organisms and are foraging species of larger animals. These species have experienced important declines in their wild populations during the last decades. For instance, the decrease of the European sardine (*Sardina pilchardus*) body condition has had a detrimental impact on its landings, leaving their commercial fishing unprofitable in some Mediterranean areas. The causes for this decline are not clearly established but seems to be mainly related to changes with planktonic communities inducing a switch in their foraging behaviour from particulate-feeding to filter-feeding. Moreover, it has been highlighted that sardines ingest plastic fibres throughout their natural spatial distribution, suggesting this additional pollution as a possible new threat affecting their populations' health. In this study we developped an experimental setup allowing us to maintain wild fish in captive controlled conditions in order to test the possible factors affecting plastic fibres ingestion in sardines. We demonstrate that sardines ingest fibres from water, and the amount of fibres ingested is highly impacted by their feeding behaviour. Sardines feeding by filtration ingest less food but more plastic fibres (mean = 4.95 fibres/ind; SD = 3.43), compared to sardines that feed by particulate-feeding (mean = 0.6 fibres/ind; SD = 1.04). Moreover, a decrease in sardine body condition factor was detected for filter-feeding individuals, mostly linked to the lower amount of food they ingested rather than to the fibre ingestion itself. Nonetheless, higher water temperature seems to accelerate the pattern of fibre expulsion in filter-feeding sardines. Alltogether, it is suggested that plastic fibres pollution and phytoplanctonic changes under global change, might synergistically act at disturbing the health of this species in wild populations.

1. Introduction

The ubiquity of small plastic particles known as microplastics (MP), <5 mm (Frias and Nash, 2019; Hartmann et al., 2019), is a major concern throughout the world's oceans (UNEP, 2016). It is estimated that 4.8 to 15.1 million metric tons of plastic marine debris enter the ocean every year (Jambeck et al., 2015; Lebreton et al., 2017). Fragmentation of larger plastics over time has also increased the presence of microplastic fragments and fibres from water surface layer down into deep ocean sediments (Browne et al., 2011; Lusher et al., 2015; Van Cauwenberghe et al., 2013). Fibres are reported as the most prevalent type of item found around the world (Gago et al., 2018) and can constitute up to 91% of microplastics/anthropogenic pollution collected globally in water samples (Barrows et al., 2018).

The small size and large spatial distribution of fibres in the ocean increase the chance for ingestion by marine organisms (Browne et al., 2008; Lusher et al., 2017). In fact, the ingestion of fibres has been well documented from a wide range of taxonomic groups including zooplankton (Cole et al., 2013; Setälä et al., 2014), benthic invertebrates (Goldstein and Goodwin, 2013; Murray and Cowie, 2011; Watts et al., 2014; Wright et al., 2013) seabirds (Amélineau et al., 2016; Thiel et al., 2018), marine mammals (Hernandez-Milian et al., 2019), and fish of

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* Corresponding author.
E-mail address: Anna.Soler@uab.cat (A. Soler-Membrives).

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different trophic levels (Neves et al., 2015) including small forage/planktivorous fish such as clupeiforms (Compa et al., 2018; Lefebvre et al., 2019; Savoca et al., 2020).

Why is this so much of a concern? Besides mechanical effects (potential physical effects like occlusions, decrease in available stomach size (Jovanović, 2017), microplastics, including fibres, are carriers of chemical additives added during manufacturing but also of other pollutants, such as metals, organic contaminants (Gauquie et al., 2015; Rochman et al., 2013), or being vectors of pathogens (Bowley et al., 2021). The deleterious consequences of their ingestion have been assessed in experimental conditions, and differ from one taxon to another leading to food activity modification (Besseling et al., 2013), food assimilation deficiency (Blarer and Burkhardt-Holm, 2016), growth retardation (Lo and Chan, 2018), reduced reproduction (Cole et al., 2015), neurotoxicity (Qiao et al., 2019), reduced survival and locomotion (Tosetto et al., 2016) and impaired cognitive abilities (Crump et al., 2020). These effects vary depending on the quantity of MP/fibres ingested during the experiments (mostly acute toxicity experiments using high concentrations) and on the time of exposure. However, in order to assess how natural populations might be affected by microplastic contamination, studies using realistic concentrations of MP exposition should be favoured (Weis and Palmquist, 2021). Specifically, the concentration of anthropogenic fibres in the ocean (both synthetic and cellulosic) varies a lot depending on locations spreading from 0.02 up to 25.8 fibres liter^{-1}, with a median concentration of 1.7 fibres liter^{-1} (Suaria et al., 2020). Semi enclosed and highly populated areas such as the Mediterranean basin seem to be hotspots for accumulation of anthropogenic fibres 4.6 fibres liter $^{-1}$ (Suaria et al., 2020).

In this study, we used small pelagic fish, namely European Sardines (*Sardina pilchardus*) from the NW Mediterranean Sea, brought into captivity as a model species to study microplastic dynamics after ingestion in natural population. Together with other small pelagic fish species, sardines are key components of marine ecosystems worldwide, as they modulate population dynamics of both lower and upper trophic levels (Cury et al., 2000). Moreover, they support important fisheries and local economies, such as in the Mediterranean Sea, where small pelagic species represent almost 50% of the total fish landings until recently (Lleonart and Maynou, 2003). However, the landings of this species have dramatically decreased in the last two decades due to a sharp decline in individual size and mass (Saraux et al., 2019; Van Beveren et al., 2014). This decline seems to be primarily related to increased natural mortality of older individuals, and to changes in the environment and food availability rather than to overfishing, predation pressure or the presence of pathogens (Brosset et al., 2017, 2016a; 2015b; Queiros et al., 2018). A bottom-up control of the sardine population due to a shift in their planktonic prey towards smaller less nutritious species, has been proposed as a mechanism underlying lower growth and body condition (Brosset et al., 2016b; Saraux et al., 2019). Pollution like microplastics could be amplifying the issue, and, although the number of microplastics found in sardine guts in the wild was not that high (Lefebvre et al., 2019), it is still under investigation. Furthermore, sardines naturally display different feeding strategies depending on prey size (Garrido et al., 2008, 2007) offering the possibility to investigate how feeding behaviour can affect microplastics ingestion. When available food consists in large sized prey (e.g. copepods) they preferably display particulate-feeding, i.e. targeting single prey. However, when only small size prey/food is available (e.g. phytoplankton) they can switch into a less selective feeding behaviour based on filtration (Costalago et al., 2015; Garrido et al., 2007). Interestingly, a strong evidence of systematic changes in plankton abundance and their community structure over recent decades has been found, not only in the Mediterranean Sea but also in many areas worldwide (Aberle et al., 2012; Feuilloley et al., 2022; Herrmann et al., 2014; Winder et al., 2012) often resulting into a smaller size (Daufresne et al., 2009). Climate change is of course associated with an increase in sea temperature (e.g. + 0.2 °C per decade in the last 35 years in the Gulf of Lions; Feuilloley et al., 2022) and should also affect prey size for sardines, as plankton size is expected continue to decrease with higher temperatures. This might trigger an increase in the less selective feeding behaviour that is filtration for sardines. An increase in temperature should also increase all fish physiological rates such as digestion and intestinal transit (Clarke et al., 2017; Seebacher et al., 2014). Increased intestinal motility should fasten the transit of microplastics through the digestive tract. Therefore, the time of exposition to additives, contaminant or pathogen, that can be associated with MP, decrease (Bowley et al., 2021; Gauquie et al., 2015; Rochman et al., 2013). Whether temperature will amplify or decrease microplastic ingestion by those fish remains therefore to be investigated.

Thus, the main aim of the present study was to assess the ingestion of microfibres by sardines in relation to different feeding behaviours (filter-feeding vs. particulate-feeding), and how this might be affected by increasing water temperature under climate change. Therefore we investigated: i) the ingestion of microfibres in realistic concentrations in a wild fish species (*Sardina pilchardus*); ii) whether sardine feeding behaviour and environmental temperature affect the fibre ingestion and the potential fibre retention in sardine digestive tract; and finally iii) we determined whether the ingestion of plastic fibres can affect body condition, especially important in the context of the recent changes observed in the NW Mediterranean Sea sardine population.

2. Materials and methods

2.1. Sardine capture and rearing conditions

Wild sardines were captured at sea off Sète (South of France) in March 2020 and brought back to the Ifremer Palavas-les-Flots research station. The protocol for acclimation and weaning onto commercial pellets (Biomar, Larvaviva Prowean 100 and Inicio Plus 1.2) was the same as detailed in (Queiros et al., 2019). Once the acclimation period was finished and sanitary conditions verified, sardines were anaesthetized with benzocaine (21 mg/L), and their length and body weight measured. 80 specimens were selected and distributed in eight experimental tanks (80 L, 10 fish per tank), so as to ensure a similar mass and length in all tanks (28.00 g; SD = 2.97 and 150.10 mm; SD = 3.06 respectively). Tanks were kept in an open water system by a constant flow of 160 L/h with filtered seawater (Sand 100 μm and UV 36 mJ/cm^2/s). Temperature was controlled and photoperiod was gradually increased until achieving the equivalent of summertime, "light: 14 h/day". Fish were acclimated in these tanks 15 days before the experiments started.

The daily food ration was settled at 1 % of fish total biomass, calculated from the mean of biomass of the eight tanks. Subsequently four tanks (1–4) were designated to be fed with small size pellet (0.1 mm) named filter-feeding tanks in the following whereas the other four tanks (5–8) were fed with large size pellets (1.2 mm) named particulate-feeding tanks in the following (Fig. 1). The two food sizes were selected to elicit two distinct foraging modes, filter-feeding versus particulate-feeding behaviour (Queiros et al., 2019). During the feeding time (30 min) the water flow was interrupted and food was distributed so as to avoid losing food and to ensure that all food was consumed by the sardines. In particular, in tanks fed with small pellets (filter-feeding), food was sprinkled manually on water surface and an air bubble aerator was introduced to help keeping food in suspension; whereas in tanks fed with big pellets (particulate-feeding), fish was fed manually little by little to ensure equally food spreading in all tanks and to prevent food reaching the bottom without being consumed. The distribution of food was carried out in three intakes throughout the day. The first ration consisted of 50% of the total amount of food at the beginning of the day and the rest was distributed in two more portions (25% each) at four and 8 h respectively after the first feeding event. To ensure removing any deposition of particles in the bottom of tanks and a correct sample collection during the subsequent experiments, entrance and exit of the water flow were designed to generate a vortex to obtain a constant

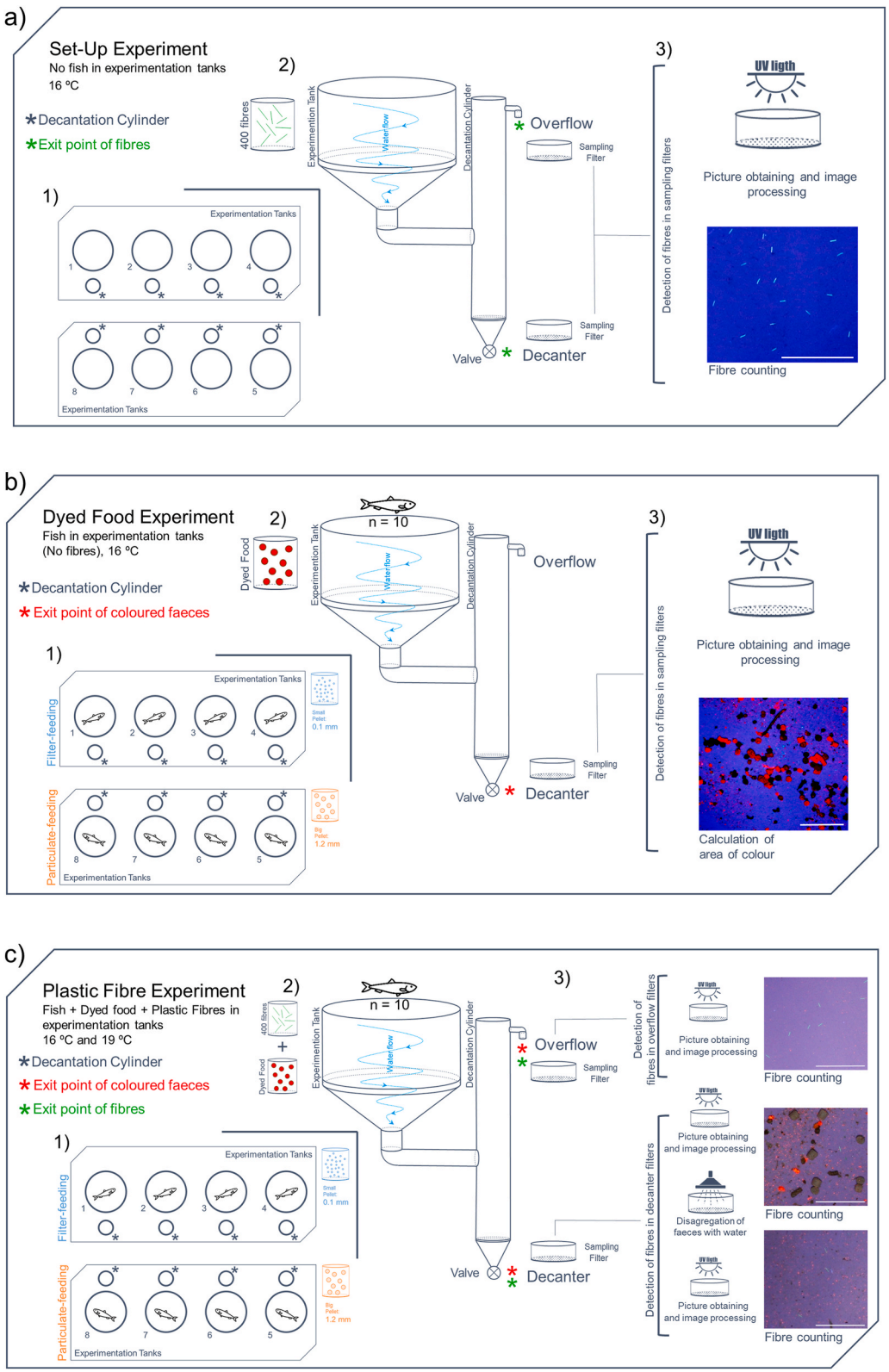

Fig. 1. Scheme of the experimental set-up: performed (a) without fish; (b) with fish fed with dyed-food but without plastic fibres; and (c) with fish fed with dyed-food and plastic fibres. Number 1) indicates the tanks distribution within the experimentation room, disposition of experimental tanks, decantation cylinders and size of food used (when fish were present in tanks). Number 2) shows an example of an 80 L experimentation tank, decantation cylinder and fibre concentration or/and dyed food used in every experiment for every tank. Green asterisks indicate the exit points of the set up where fibres were sampled, while the red ones indicate the exit points where dyed faeces were sampled). Number 3) shows examples of pictures obtained from sampling filters under UV light (360 nm wavelength) where fluorescent fibres and/or dyed faeces can be observed and counted at each sampling time. Scale bars at the bottom right of each picture represent 15 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cleaning effect. In this way all the contents of the tank were concentrated in the centre and came out through the drain pipe connected to a waste collection system. This system consisted of a 20 L decantation cylinder (120 cm height x 20 cm diameter). The water vacuum was located in the centre of the cylinder allowing the heaviest particles to sink. At the base of the decanter there was an output valve that could be manually opened for sample collection. Moreover, decantation cylinder had a top overflow pipe through which water flowed constantly to collect free particles.

2.2. Sampling protocol, selection and detection of marked faeces, plastic fibres

A standardized sampling protocol consisting of completely emptying each decantation cylinder of water and rinse it carefully to collect the content on filters (15 μm nylon mesh) was carried out in all tanks. In turn, the filter located in the overflow was replaced between each sampling and examined.

Food was marked with a fluorescent water-insoluble pigment (CC Moore Fluor Bait Dye). The amount of pigment used corresponded to a concentration of 1% in weight to a given amount of food. For its preparation, pigment was added directly to the fish pellets of the two granule sizes and shacken for 5 min to allow the pigment to mix uniformly. It allowed to differentiate coloured faeces, under UV light, from the remains of food or other faeces produced before or after treatment meal.

Plastic fibres consisted of fluorescent commercial nylon flock fibres (Flocking LDT; www.flockingltd.co.uk) of 1 mm length and 20 μm diameter, to mimic the most frequently size and shape described fibres in natural environments (Suaria et al., 2020) and in particular in the Gulf of Lions, both in water column and in small pelagic fish digestive tract (Lefebvre et al., 2019).

The use of fluorescent pigments in food and fluorescent fibres allowed the easy detection of them in faeces recovered on filters at each sampling. For this purpose, the filters were illuminated using a 360 nm wavelength ultraviolet light bulb and photographed under standardized conditions by a Canon 60D camera using a 100 mm macro lens. In decanter filters, pictures of the filters were taken before and after faeces disaggregation (mechanically by water jet).

2.3. Experimental design

2.3.1. No fish experiment (set-up)

A first experiment was performed without fish, before their transfer into the experimental tanks (Fig. 1a). The aim of this experiment was to test and validate the sampling protocol and to determine: (1) how plastic fibres passed through the tank; (2) the time needed to remove the fibres from the tanks; (3) and the percentage of recovered fibres.

This experiment was conducted once using eight tanks. After introducing fibres in tanks, an air bubble aerator was connected and kept during 5 min to allow homogeneous fibres dispersion. Aeration was connected again four and 8 h later in four tanks (i.e. filter-feeding tanks simulating the filtering condition). For the rest of the tanks (i.e. particulate-feeding tanks) no aeration was added. Fibres concentration was settled at 5 fibres per L^{-1} (400 fibres/tank) to mimic the mean values reported for the Mediterranean Sea (Suaria et al., 2020). Fibres were introduced in the system at the beginning of the experiment, and sampling was performed every 2 h for each tank and until 34 h.

2.3.2. Dyed food experiment: Tracing food transit

The aim of this second experiment was to determine the time of food transit, i.e. time that elapses between a food intake without plastics and its complete expulsion. This also allowed us to assess the timeframe of sampling for subsequent experiments.

This experiment was conducted once using eight tanks and was performed with fish reared in the same conditions as explained in section 2.1 (Fig. 1b). Water temperature was maintained at 16 °C. Fish were fed with dyed food at the first intake of the day (50% of the daily ration). The following daily rations (25% four and 8 h later), consisted of normal non-dyed food. After feeding the fish, sampling was performed every hour during 5 days until coloured faeces disappeared. Note that filters were not sampled at night.

2.3.3. Plastic fibre experiment

The aim of this experiment was to compare the ingestion of plastic fibres by sardines as well as the fibre expulsion dynamics between both feeding behaviours at two different temperatures.

The experiment was conducted using eight tanks: four filter-feeding tanks and four particulate-feeding tanks (for each feeding behaviour three treatment tanks were treated with fibres and one tank without acting as control). Control tanks were sampled in the same way as for treatments to monitor potential cross-contamination. The experiment (Fig. 1c) was performed identically as the dyed food experiment, only with the addition of plastic fibres during the first meal of the day. As previously described, sardines were fed with dyed food (50% of the daily ration) at the beginning of the experiment and again with normal non-dyed food four and 8 h later (25% of the daily ration each). As for the no fish experiment (section 2.3.1.), 400 identical fibres (same concentration, same sizes) were added to the tank, at the same time as the dyed food (first meal). Following sampling events of faeces and fibres were carried out every 2 h. Two different experiments were performed successively at two different temperatures of 16 and 19 °C, respectively. Every experiment longed 5 days. Between the two experiments the water temperature was gradually increased over seven days to ensure fish had time to progressively acclimate before the second experiment started.

Considering empirical data on the North-Western Mediterranean, mean temperature on sea water went from 16.1 to 16.8 in 20 years (Feuilloley et al., 2020). For this reason, 16 °C was considered as a "cold" or baseline temperature for sardines. Following, considering the aim of the Intergovernmental panel on climate change (IPCC) for 2050 to keep temperature rise below 1.5 °C due to the negative implications on ecosystems worldwide. The choice of hot temperature (19 °C) was settled increasing the average temperature by 3° as significant change to be consider the effects at an experimental level in a context of climate change.

Morphometric data (body weight and length) of all 80 individuals were recorded at the beginning of all manipulations, after plastic fibre experiment at both temperatures and finally after fish euthanasia (see section 2.3.4) (ca. every two weeks). After every fish manipulation for biometry fish had a resting time of at least two days before a new experiment was carried out.

2.3.4. Plastic fibres within sardine digestive tract

To confirm and quantify the ingestion of plastic fibres by sardines, a last experiment was carried out using similar protocol but one fish from each tank was sacrificed every 4 h (until 40 h) by a lethal dose of benzocaine (150 mg per L^{-1}) (four fish per feeding behaviour and time period). As this procedure was terminal for the fish, this experiment was only performed once at 19 °C. Each fish was weighed, measured and dissected. The stomach content was weighed, and the gastrointestinal tract (stomach and intestine) was carefully screened for the presence of fibres under a stereoscopic binocular at 10 × to 45 × of magnification and UV light. All fibres were counted.

2.4. Data analysis

2.4.1. Image analyses and fibre counting

The pictures of each filter were processed using image analysis package of the Fiji ImageJ 2.1.2 software (Schindelin et al., 2012). The presence or absence of fibres was checked and counted for every filter.

During the set-up experiment, kinetic curves of fibres exiting the system were calculated from fibres recovered in the overflow, decanter and the sum of both, for each tank individually and grouping tanks by

treatment (filter-feeding vs particulate-feeding).

During the dyed-food experiment, the amount of coloured faeces (CF) was calculated as area percentage, in number of pixels, from the obtained pictures at every sampling time as:

$$(CF = PFC \ / \ TPP) \ x \ 100))$$

where PFC is the number of pixels of fluorescent colour in the picture at a specific sampling time and TPP the total number of pixels of the picture.

Standardized amount of coloured faeces (SCF) was then calculated to ease comparisons amongst tanks and treatments as:

$$(SFC = PFC \ / \ MFC) \ x \ 100)$$

where MFC is the maximum value of fluorescent pixels in the experiment for each tank. Finally, the kinetic of food transit was assessed for the experiment.

In experiments where sardines were fed with plastic fibres, collected fibres in the overflow and in decanters as well as fibres embedded in faeces (FF) were calculated as:

$$FF = TF - NIF$$

where NIF are the non-ingested fibres found free in the filters (as counted before faeces disaggregation) and TF the total number of fibres (non-ingested fibres + fibres contained in faeces) after faeces disaggregation. The kinetic of fibres expulsion was monitored for experiments performed at the two distinct temperatures (16 and 19 °C) and is represented as the accumulate percentage of fibres in faeces, i.e. the time the fish need to expel all the ingested fibres, independently on the amount of fibres ingested.

To assess the plastic fibres within sardine digestive tract the prevalence (P%) was calculated as:

$$P\% = FF \ / \ TS \ x \ 100$$

where FF is the number of fish displaying fibres within their digestive tract and TS the total of sardines analysed. Fibres found in the different parts of the digestive tract were considered separately and together in regards to the feeding behaviour.

2.4.2. Fish condition indices

Based on the weight of the stomach content, stomach fullness index (Hyslop, 1980) was calculated as:

$$Fullness = (Stomach \ content \ weight \ / \ body \ fish \ weight) \ x \ 100$$

Body Condition Index (Kn) of each sardine was calculated with the Le Cren index Kn as estimated by Brosset et al. (2015a) using the data obtained during biometrics:

$$Kn = BW \ / \ (0.00607 \times TL^{3.057})$$

where BW the body weight in g and TL is the total length in cm.

2.4.3. Statistical analyses

Data analysis was performed using R Studio software, (version: 4.0.3). Whenever linear models or linear mixed models were used, residuals normality and homoscedasticity were tested using Shapiro-Wilk and Levenne's test respectively. For each test or model, significance was set at $\alpha = 0.05$. Results are presented as means; SD.

2.4.3.1. No fish experiment. Using Linear mixed models (LMMs) accounting for repeated measures (Tank ID as random factor) we tested the differences in the number of fibres in filters (overflow and decanter) and the total fibres counts in response to the tank type (Filter-feeding tanks/Particulate-feeding tanks) and time (fixed factors).

2.4.3.2. Dyed-food experiment. For the dyed food experiment we tested the differences in the total amount of coloured faeces in filters (decanter) in response to the feeding behaviour (Filter-feeding tanks/Particulate-feeding tanks) using Generalized Linear Models, (GLM, gamma family for the amount of coloured faeces at 14 h post feeding and for the total amount of faeces).

2.4.3.3. Plastic fibre experiment. We tested the differences in the number of fibres in response to the experiment type (no-fish experiment/presence of fish) and time (fixed factors) using linear mixed models (LMMs) accounting for repeated measures (Tank ID as random factor). Moreover, differences in plastic fibres recovered from tanks (5 h post-ingestion) were assessed between both feeding behaviours (explanatory variable) and tank ID (as random factor), using GLM (Poisson family, link log). Finally, differences in plastic fibres recovered from tanks (5 h post-ingestion) were assessed between feeding behaviour (explanatory variable) for every temperature separately, using GLMM (Poisson family, link log and tank ID as a random factor).

2.4.3.4. Plastic fibres within sardine's digestive tract. After fish dissection to assess plastic fibres within sardine digestive tract differences in the prevalence, stomach fullness and abundance of plastic fibres found in the digestive tract of sardines were assessed between both feeding behaviours (explanatory variable), using a GLM (binomial family, link logit), GLM (gamma family) and GLM (Poisson family, link log), respectively.

2.4.3.5. Fish condition indices. The possible differences in the values of weight, length and condition factor index (Kn) comparing the different biometrics from day 0 to day 59 were addressed using parametric LMMs (fish ID as random factor) and post-hoc pairwise comparison (Bonferroni correction). When Normality was not respected, non-parametric Friedman test was applied.

3. Results

3.1. No fish experiment (set-up)

In the absence of fish, 357.33 fibres; SD = 26.44, were recovered during the first 26 h (Fig. 2a), which corresponds to 89% (84–97.8%) of the fibres introduced in the tanks. 70% of fibres recovered came out of the system during the first hour, and approximately 90 % of fibres had been collected after 5 h (Fig. 2b, c and d). Fibres were recovered mostly in the overflow (62.78%) but also in the decanter (37.22%), although the temporal dynamics of the retrieval of fibres out of the system was similar in both filters (Fig. 2b–d). Furthermore, these dynamics did not show any significant differences among tanks and neither between filtering and particulate-feeding tanks (GLM: $z = -0.106$, $p = 0.915$).

3.2. Dyed-food experiment

Coloured faeces appeared after 5 h post-ingestion of dyed food and 11–12 h post-feeding were required for the fish to eliminate half of the total amount of faeces, regardless of the feeding behaviour (Fig. 3a). Most faeces were collected before the first night started (14h post-feeding) and the transit and production of coloured faeces could be considered finished during the second day-time period (28–38h post-feeding), despite a few occasional coloured faeces detected in the third day. When comparing feeding treatments (Fig. 3a), filter-feeding fish seemed to exhibit a faster transit compared to sardines fed with large pellets (90% after 14h vs. 70%, GLM: $z = 3.52$, $p = 0.013$). Further, sardines fed with large pellets (particle-feeding behaviour), produced significantly more coloured faeces (CF) overall compared to the filter-feeding sardines (filter-feeding: mean = 0.06; SD = 0.03 vs. particulate-feeding: mean = 1.02; SD = 0.17, GLM: $z = -13.16$, $p = P <$

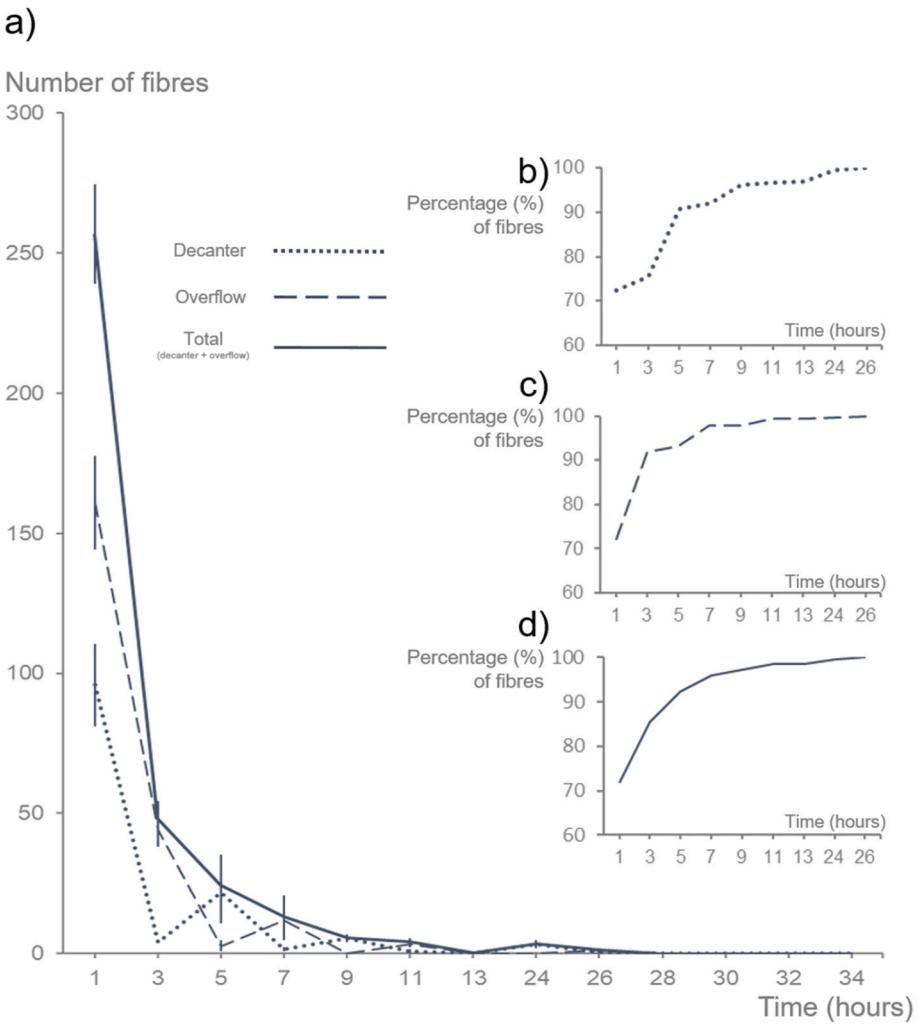

Fig. 2. Kinetics of plastic fibres during the no-fish experiment: (a) Number of recovered fibres (mean and SD) exiting the tanks through the recovery system; (b–d) Accumulation curve in percentage (%) of the fibres recovered in the overflow (b), (c) the decanter (c); and the entire system (d).

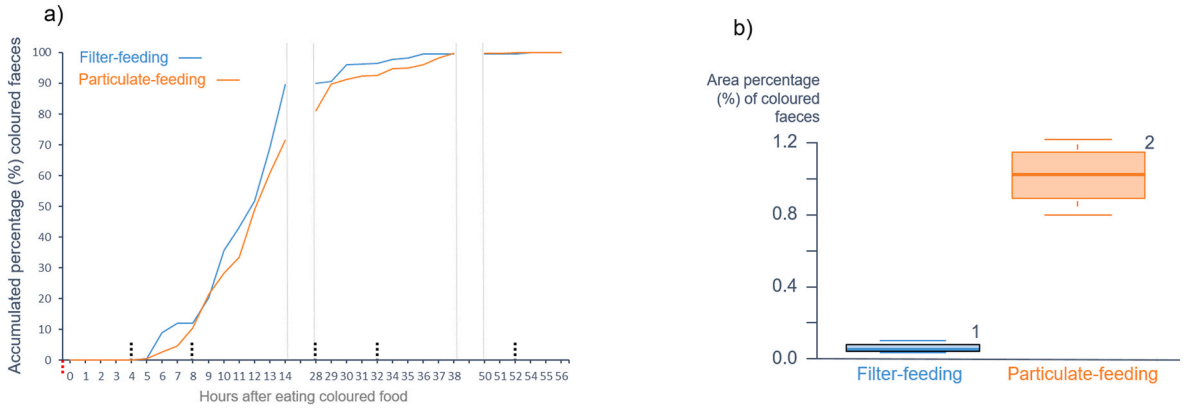

Fig. 3. a) Dynamics of the expulsion of coloured faeces (expressed in accumulated percentage %) for sardines feeding on small pellets (Filter-feeding) and big pellets (Particulate-feeding), dashed red lines, at time "0" represent the time at which fish were fed with stained food. Black dashed lines represent the subsequent feeding events with normal non-stained food; b) Boxplot of the total area covering (in %) of coloured faeces recovered at the end of dyed-food experiment from tanks of both feeding behaviour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.001 Fig. 3b).

3.3. Plastic fibres experiment

In the first 5 h post-ingestion (i.e. before fish start to eliminate their morning meal, see 3.2), the total number of fibres recovered was

significantly lower compared to the no fish experiment (no fish: mean = 329.3 fibres; SD: 19.66 vs. with fish: mean = 237 fibres; SD: 34.1, GLM; $z = -6.08$, $p < 0.0001$), confirming that fish ingested fibres. The number of fibres expelled from the system within the first 5 h was 20 % lower in filter-feeding sardine tanks (212.78 fibres; SD = 25.28) than in particulate-feeding tanks (mean = 267.11; SD = 16.51) (GLM: $z = 7.43$, $p < 0.0001$, Fig. 4a). No significant differences were found in the number of fibres expelled from the system within 5 h when comparing the experiments at two temperatures with the same feeding behaviour: filter-feeding 16 vs. 19 °C ($p = 0.23$) or particulate-feeding 16 vs. 19 °C ($p = 0.64$).

At 16 °C, first fibres were detected in faeces after 9 h post ingestion (3 h after detecting the first coloured faeces) and 50 % of the fibres were detected after 23–25 h in both feeding behaviours. No more fibres were found in faeces after 37 h for particulate-feeding and 52 h for filter-feeding (Fig. 4b and c). The number of fibres found in faeces showed higher values in particulate-feeding sardines (mean = 17.00; SD = 1.27) compared to filter-feeding sardines (mean = 11.67 fibres; SD = 6.81) but with no significant differences (GLM: $z = 1.72$; $p = 0.09$).

At 19 °C a similar general pattern was observed. However, fibres within faeces were detected sooner (one or 2 h before compared to 16 °C) for both feeding behaviours, and were eliminated later (after 56 and 54 h for particulate-feeding and for filter-feeding respectively; Fig. 4b and c). Finally, at 19 °C, the number of fibres found in faeces showed significant higher values in filter-feeding sardines (mean = 15.17; SD = 6.71) compared to particulate-feeding sardines (mean = 7.50; SD = 3.35) (GLM: $z = -3.86$; $p < 0.005$).

No fibres were found in any of the control tanks at any time, ensuring no cross contamination of fibres while sampling occurred.

3.4. Plastic fibres within sardine's digestive tract

Over all sampling events (4–40 h post-ingestion), the proportion of fish that presented at least one fibre in their gastrointestinal tract (P%) was significantly higher (GLM: $z = 3.71$, $p < 0.001$, $n = 60$) in filter-feeding fish (93.30%) compared to particulate-feeding ones (40%). The number of fibres found in the digestive tract of dissected fish was also significantly higher (GLM: $z = 7.87$, $p < 0.001$, $n = 60$) for filter-feeding sardines (4.33 fibres/ind; SD = 3.26) vs. particulate-feeding ones (0.60 fibres/ind; SD = 1.04; Fig. 4d). When considering only the

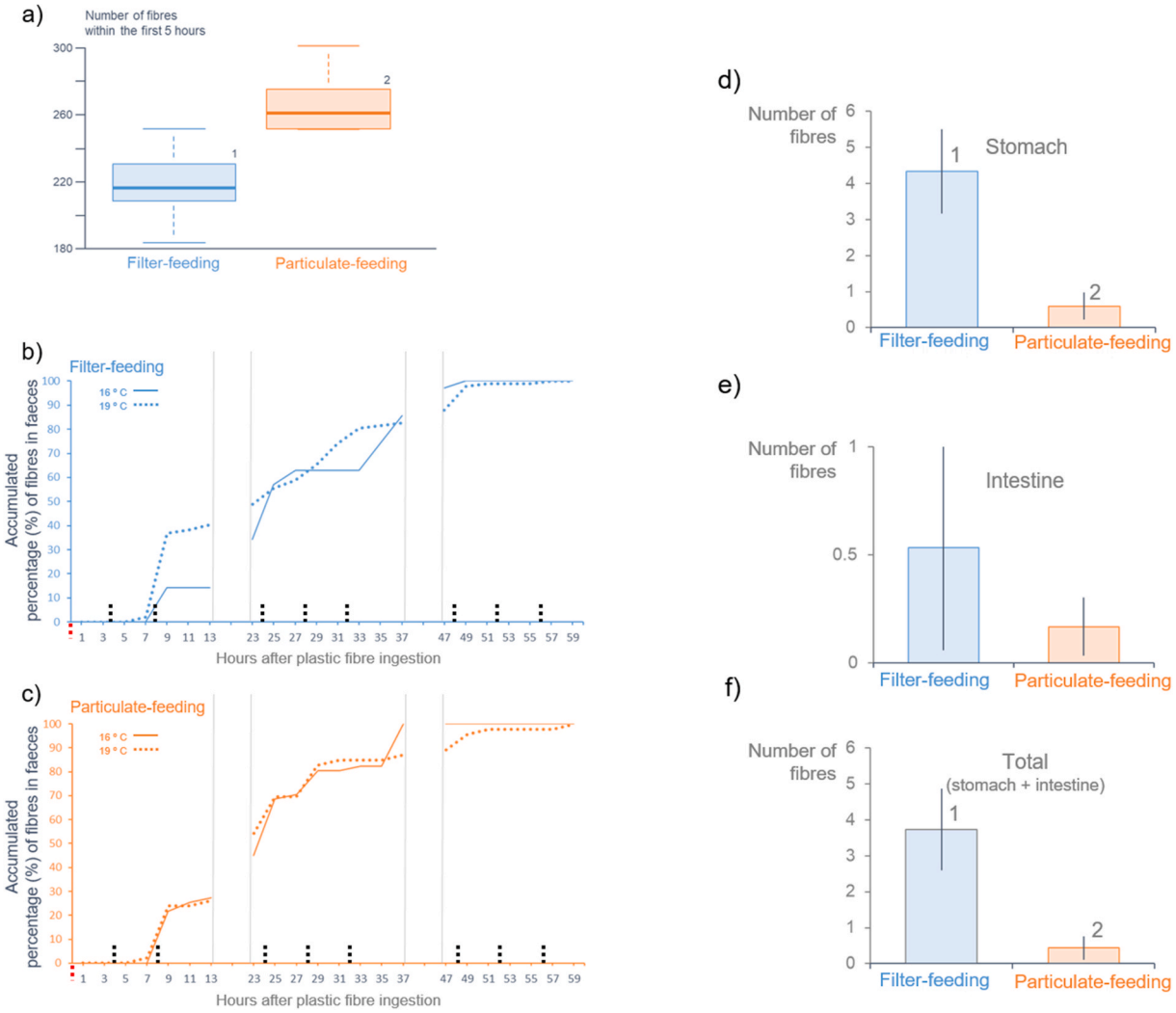

Fig. 4. a) Boxplot of the total number of plastic fibres recovered during the first 5 h in the plastic fibre experiment from tanks of both feeding behaviours; b) Dynamics of the expulsion of fibres contained in the faeces of sardines displaying filter-feeding behaviour; and c) Dynamics of the expulsion of fibres in the faeces of sardines displaying particulate-feeding behaviour. Dashed red lines, at time "0" represent the time at which fish were fed with stained food and plastic fibres. Black dashed lines represent the subsequent feeding events with normal non-stained food and no plastic fibres. Spaces between solid bars, represent a gap in the sampling during night-time. d-f). Number of fibres (mean and SD) found in the digestive tract of sardines after dissection (c) total stomach + intestine, d) stomach and e) intestine). Numbers at the top of each boxplot/barplot represent significant differences between feeding behaviours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

fish that ingested fibres, the result remained similar for filter-feeding sardines vs. particulate-feeding sardines (4.64 fibres/ind; SD = 3.15 and 1.5 fibres/ind; SD = 1.17, respectivelly; GLM: z = 4.35, $p < 0.001$).

When considering stomach and intestine separately, most fibres were found in the stomach (86 %). Filter-feeding sardines also displayed higher numbers of fibres in both organs compared to the ones feeding on big pellets, although differences were significant only in the stomach (GLM: z = 7.35, $p < 0.001$, Fig. 4e and f).

3.5. Fish condition indices

At the end of the experiments, fish exposed to plastic fibres did not show significant differences in morphometrics (length, weight and condition index) compared to control fish, whatever the feeding behaviours. Filter-feeding fish displayed a decrease in weight and condition index over the time that all experiments were conducted, resulting in significantly lower weight and body condition at the end of the experimentation compared to the beginning of the experiment (LMM: t = = 4.96, $p < 0.005$, n = 80 and LMM: t = = 7.94, $p < 0.001$, n = 80, respectively). Conversely, morphometrics of sardines feeding by particulate-feeding remained stable over time ($p > 0.05$).

When comparing morphometrics between feeding behaviours at each time step, significant lower values of i) body condition index (LMM: t = 4.26, $p < 0.05$ n = 80) in the two last sampling and ii) weight (LMM: t = = 4.29, $p < 0.005$, n = 80) only in the last sampling, were found for the filter-feeding fish (Fig. 5). Regarding stomach fullness, fish with particulate-feeding behaviour showed significantly higher values compared to filter-feeding fish (LM: t = 3.863, $p < 0.005$, n = 60).

4. Discussion

To the best of our knowledge, this is the first time that a study on plastic fibre ingestion in Mediterranean wild clupeids is performed under experimental conditions. In addition, this is the first study that highlights, in realistic concentrations, that sardines ingest plastic fibres when filter-feeding and particulate-feeding and that the amount of ingested fibres is higher in the former feeding behaviour, especially at higher temperature. Furthermore, temperature seem to also influence fibre expulsion from the digestive tract. Indeed, excretion appeared to start earlier at 19 °C, in both feeding behaviours.

4.1. Set-up design and fibres detection

The "no fish" experiment highlighted a effective fibre recovery rate (near 90%), validating the set-up design aiming at testing microplastic fibres ingestion by organisms exposed to environmentally realistic concentrations. In this system, most of the fibres were retrieved from the tanks during the first hour. The renewal speed of water tank seemed adequate, as fibres were available during the feeding time for fish (30 min) but did not remain in the tanks along the experiment. Although it could be easier to work with smaller tank volumes when experimenting with small-sized fibres (i.e. nylon flock fibres of 1 mm length), wild small pelagic fish display a gregarious behaviour and need to live with other congeners. Indeed, previous experiments have shown that they cannot be maintained in captive facilities in smaller tanks, as their school behaviour disapears and they stop feeding (Queiros et al., 2019). Further, fibres were used to mimic the elements found most frequently in the environment (Lefebvre et al., 2019; Suaria et al., 2020) and densities of fibres used also matched those found at sea. The use of fluorescent fibres strikingly streamlined the detectability of these small fibres in the filters; it also prevented the possible confusion of plastic fibres coming from airborne contamination, and showed no signs of interference on the feeding behaviours of fish. Overall, this experimental set-up appears as the best compromise for experimentally studying the effect of microplastic on small pelagic fish in controlled conditions as close as possible to natural conditions to reflect what possibly occur in natura.

4.2. Feeding behaviour and ingestion of plastic fibres by sardines

Filter-feeding sardines in comparison with the ones eating on particles appeared to eat less food while ingesting more fibres and retaining them for longer.

Our results demonstrate that the ingestion of fibres both in terms of number of fish ingesting fibres and number of fibres ingested was directly related to the feeding behaviour. While both filter-feeding and

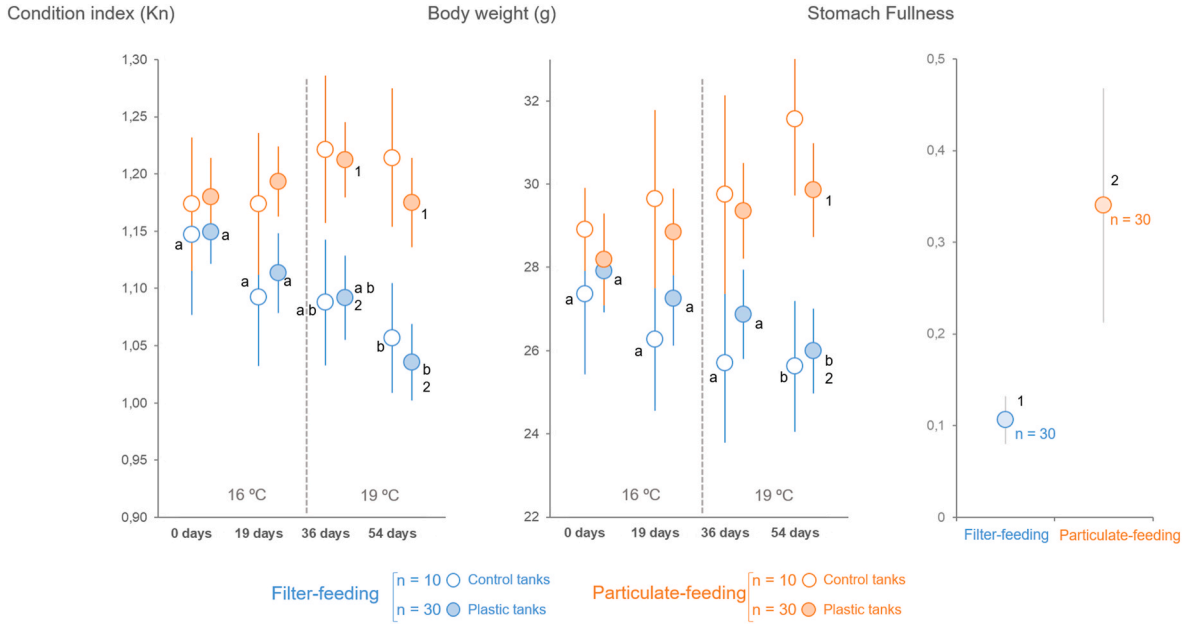

Fig. 5. Values of the morphometrics (biometries from 0 days to 54 days) according to the feeding behaviour (filter-feeding vs. particulate-feeding): condition index (factor Kn) and body weight (g), performed throughout the experimentation, and stomach fullness obtained by dissection after the last biometry. Letters represent significant differences in the same feeding behaviour while numbers show significant differences between feeding behaviours.

particulate-feeding sardines ingested fibres (see the difference with the no-fish experiment), filter-feeding sardines ingested more fibres (lower number of fibres fallen without being ingested and recovered in filter-feeding tanks during the first hours, higher number of fibres in faeces afterwards and fibres found in almost all sardine stomachs and in higher numbers when dissected). When sardines feed on small pellets, they display a filter-feeding behaviour. During this process, sardines open their mouth and continue swimming to filter in the water column; food particles are retained in their gill rakers and later swallowed. This mechanism do not allow discrimination of food particles from other free particles, such as fibres, suspended in water. On the contrary, our results suggest that sardines feeding on large pellets are able to selectively capture pellets and discriminate against fibres. Still, fibres ingestion certainly occurs when they are in the close surrounding water, either in proximity to the food or directly adherent to it. Some organisms, for instance certain fish, actively ingest plastics because of their resemblance with natural prey (Ory et al., 2018; Schuyler et al., 2012), however visual cues are not the single stimuli driving MP to be misidentified with food. MP entering the marine environment can acquire an odor signature (DeBose et al., 2008; Dove, 2015) due to the biofouling process driving MP to be perceived as food (Savoca et al., 2017, 2016). In our study, we used pristine plastic fibres without any trace of odor. Therefore, the ingestion of fibres can be considered accidental, not by misidentification with food, but driven by feeding behaviour. This highlights that feeding habits based on less selective feeding strategies, such as filtration, favour the ingestion of MP compared to feeding strategies relying on direct food identification and selection (Karlsson et al., 2017).

Fifty percent of the fibres were excreted within 23–25 h regardless of the feeding behaviour, which is twice the time required by sardines to excrete half of the total amount of food ingested (faeces collected), suggesting that fibres retention time is higher than the processing time of food items. However, if we take into account the total time needed to excrete all fibres, this retention is higher in sardines with filter-feeding (52 h to excrete all fibres vs 38 h to reject all faeces). Instead, this delay did not occur in particulate-feeding sardines (37–38 h for both feaces and ingested fibres). Therefore, feeding behaviour also influenced fibre retention. The time needed to excrete all fibres in sardines is similar to the time needed to excrete microplastics and an entire meal for other pelagic species (within 20 and 44 h in *Engraulis japonicus* (Ohkubo et al., 2022)) but higher compared to demersal species (about 25 h *Pagrus major* or *Sparus aurata* (Jovanović et al., 2018; Ohkubo et al., 2022)). In any case, time on gut transit and excretion duration is particularly species-specific due to the variance in the digestive system morphology and feeding behaviour of each species (Rønnestad et al., 2013), it is therefore important to know normal food excretion time in each species in order to properly evaluate the potential retention of MP in digestive tracts. The typology and size of MP also seems to interfere with retention time as smaller items potentially persist for long periods within the digestive tract (Liu et al., 2021).

Most of the studies that focused on the dynamics of feaces production and digestion in fish relied on commonly reared species in aquaculture such as the gilthead seabream *Sparus aurata*, the Senegalese sole *Solea senegalensis* (Gilannejad et al., 2019) or the European seabass *Dicentrarchus labrax* (Adamidou et al., 2009). It has been also addressed for wild clupeids (Bernreuther et al., 2008; Van Der Lingen, 1998), but no literature has yet been produced for sardines. Therefore, this study provides the first gut transit curve for this commercially relevant species.

Sardines released most of the faeces during the first 14h post-feeding and ending around 32h post-feeding. In these experimental conditions, fish digest a meal in half a day. The feeding biology of the sardine is well known in terms of behaviour and diet composition (Costalago and Palomera, 2014) but the feeding frequency has not yet been discussed in experimental studies. When comparing feeding behaviours, fish fed with small pellets (filter-feeding) seemed to exhibit a faster transit compared to sardines fed with large pellets (particulate-feeding), which agrees with a higher digestibility of smaller food within the gastrointestinal system. A similar pattern is described in other species such as *Sardinops sagax* in which individuals feeding on phytoplankton have faster gastric evacuation rates compared to fish eating bigger zooplanktonic items (Van Der Lingen, 1998). Further, sardines fed with large pellets produced significantly more coloured faeces overall, i.e. they ate more when particulate-feeding.

4.3. Effect of temperature on plastic fibres ingestion

When considering the fibres expelled from the system during the first 5 h; the amount of fibres ingested by sardines was not influenced by the water temperature. However, focusing on fibres found in faeces during the experiments, the number of fibres in filter-feeding fish was higher at 19 °C. This is expected as the higher the water temperature the higher the metabolism and energy demands in fish, which ultimately can be partially compensated by an increase in food consumption (Volkoff and Rønnestad, 2020). Our results also highlighted that the fibres expulsion by sardines started sooner at higher temperatures (19 vs. 16 °C). An increase in water temperature has been identified as a critical factor affecting all fish physiological rates including the time of intestinal transit (Clarke et al., 2017; Seebacher et al., 2014). Thus as the temperature rises, the digestion rate and movement of food through the gastrointestinal tract of fish accelerates (Volkoff and Rønnestad, 2020). This should ultimately accelerate the transit of MP through the digestive tract and therefore decrease the exposition time to toxic plastic additives (i.e. bisphenol or phthalate plasticizers), other pollutants (i.e. persistent organic pollutants, heavy metals (Gauquie et al., 2015; Rochman et al., 2013)) and pathogens known to be associated with MP (Bowley et al., 2021), thus reducing the possible hazardous effects of MP for fish.

In the context of climate change and the consequent rise in sea water temperature, filter-feeding fish might ingest higher amounts of MP though they might have a greater capacity to expel them as sea temperature rises. Additionally, the accelerated intestinal transit can alter intestinal microbial communities, which may have an impact on fish digestion (Reid et al., 2024), immunity (López Nadal et al., 2020), and general health in an ecosystem under climate change (Williams et al., 2023).

4.4. Impact of plastic ingestion on sardines

Different impacts on fish health are commonly reported following artificial exposures to microplastics (e.g. decreased survival and energy storages, alterations in the activity of biomarkers, alterations of metabolisms and in different tissues, increased feeding time, effects on body length …) ((Kögel et al., 2020) and references therein). However, it is important to highlight that the concentration of MP used under laboratory conditions is usually far higher than what is found under natural conditions, and precisely, the MP concentration seems to be one of the key factors regarding harm caused by MP to fish (Kögel et al., 2020). In our study, we aimed to replicate current condition in the wild using realistic concentrations of fibres found in the environment [from 0.02 to 25.8 fibres litter^{-1}, with a median concentration of 1.7 fibres litter^{-1} (Suaria et al., 2020)]. With these concentrations (5 fibres litter^{-1}), plastic fibre ingestion does not seem to impact fish growth, weight or body condition.

Effects of microplastics on body length or condition factors are ambiguous (Kögel et al., 2020). For instance, in wild pelagic species, lower values of body condition indices have been attributed to higher values of anthropogenic fibres ingestion in *Sardina pilchardus* (Compa et al., 2018). However, no effect are found for the same species or *Engraulis encrasicolus* (Compa et al., 2018; Lefebvre et al., 2019) nor in *Gadus morhua* or *Pollachius virens* (de Vries et al., 2020). Similarly, no significant effect of plastic exposure has been observed on growth or body condition of the omnivorous fish *Diplodus sargus* (Müller et al.,

2020) nor the planktivorous fish *Acanthochromis polyacanthus* (Critchell and Hoogenboom, 2018) under laboratory conditions. However, in the latter study, when food was totally replaced by plastic, there was a negative effect on growth and body condition of the fish, which highlights the significance of MP concentration on evaluating direct and indirect MP effects.

In our study, the low values of stomach fullness and body condition seen in filter-feeding sardines compared to sardines that displayed particulate-feeding behaviour may be attributable to feeding behaviour rather than to plastic ingestion, as similar results were found for the filter feeding control tanks (without MP). The body condition loss observed in filter-feeding fish is in line with previous experiments carried out on captive adult sardines. Queiros et al. (2019) highlighted that body condition, growth and energetic reserves are significantly impacted by the feeding behaviour, as filter-feeding sardines need to consume twice as much as those feeding on large items (particulate-feeding) to reach the same body condition and growth rate, due to higher energy expenditure while filter-feeding (Queiros et al., 2019). The downsizing of plankton due to global warming may trigger filter-feeding behaviour, resulting in a decline in body condition of fish. This phenomenon may have serious consequences for fish populations (Aberle et al., 2012; Feuilloley et al., 2022; Herrmann et al., 2014; Winder et al., 2012), ultimately impacting wild sardine populations and the fisheries that rely on this species (Saraux et al., 2019). In addition, as filter-feeding sardines eat more plastic fibres than particulate-feeding ones, it will add even more pressure to the small pelagic fish populations.

5. Conclusions

In this study we developed a novel experimental set-up suitable to perform experiments on the impact of plastic fibre ingestion in wild small pelagic fish. This study demonstrates that sardines ingest fibres present in the surrounding water. Moreover, the amount of fibres ingested is highly influenced by the feeding behaviour. Indeed, when they use filtration (a less selective feeding mode) they ingest more fibres compared to particulate-feeding (the most selective feeding mode). Higher temperature also seems to increase the fibre ingestion especially in filter-feeding sardines, and modified the intestinal transit, leading to an accelerated expulsion of fibres. Nonetheless, with microplastic concentration similar to what is found in natural habitats, body condition factor and weight was not affected by the ingestion of fibres. As plankton size is expected to continue to decrease under climate change, this might trigger an increase in filter-feeding behaviour in small pelagic species, which ultimately will lead to an increase in MP ingestion. However, with an increase in water temperature, a faster expulsion of MP would also be expected by increasing the speed of intestinal transit, resulting in a possible compensatory effect. This study highlights the need for further investigation to unravel the possible synergistic effect of microplastic ingestion in relation to new scenarios derived from climate change.

Ethical statement

All fish manipulations were performed under anaesthesia conditions and all procedures were in accordance with the French and the EU legislation regarding animal experimentation (APAFIS, Permissions No. 29810–2021021113024423 v2).

CRediT authorship contribution statement

Oriol Rodriguez-Romeu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Maria Constenla:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **Anna Soler-Membrives:** Writing – review & editing, Validation, Formal analysis, Conceptualization. **Gilbert Dutto:** Methodology, Investigation. **Claire Saraux:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Quentin Schull:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Oriol Rodriguez-Romeu reports travel was provided by Autonomous University of Barcelona. Oriol Rodriguez-Romeu reports financial support was provided by Autonomous University of Barcelona. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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