APPLICATION

A biochemical approach for identifying plastics exposure in live wildlife

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Summary

1. Plastic pollution is a long-standing ubiquitous issue. Global use of plastics is continuing to rise, and there is increasing interest in understanding the prevalence and risk associated with exposure of wildlife to plastics, particularly in the marine environment.
2. In order to facilitate an assessment of ingestion of plastics in seabird populations, we developed a minimally invasive tool that allows for detection of exposure to plastics.
3. Using a simple swabbing technique in which the waxy preen oil is expressed from the uropygial gland of birds, we successfully tested for the presence of three common plasticizers: dimethyl, dibutyl and diethylhexyl phthalate [dimethyl phthalate, dibutyl phthalate and bis(2-ethylhexyl)-phthalate, respectively]. These plasticizers are prevalent in the manufacturing of plastic end-user items which often end up in the marine environment.
4. Using gas chromatography–mass spectrometry and protocols to reduce background contamination, we were confidently able to detect targeted plasticizers at low levels.
5. The method described has broad applicability for detecting plastics exposure in wildlife at individual, population and species levels. Furthermore, the approach can be readily modified as needed to survey for plastics exposure in taxa other than seabirds.
6. Applying the simple, minimally invasive approach we describe here is particularly appealing for detecting plastics exposure at population and species levels, it shows promise for quantification and it has no observed detrimental impacts to wildlife.

Key-words: bis-phthalate, dibutyl phthalate, dimethyl phthalate, plastics, seabird, uropygial gland

Introduction

Annual plastics production has increased from <2 tonnes in the 1950s to nearly 280 million tonnes in 2011 (Plastics Europe 2012). Since the early 1970s, plastic contamination of the oceans has been receiving increasing attention (Carpenter & Smith 1972; Colton, Knapp & Burns 1974; Wong, Green & Cretney 1974). Over the last few decades, the focus of attention on plastics in the marine environment has included single-species documentation of ingestion or entanglement, estimating the global distribution of plastic debris and identification of potential individual-, population- and species-level ecological effects of marine debris through to wildlife. This has included entanglement of marine mammals (Laist 1997) and ingestion of anthropogenic debris by seabirds (Spear, Ainley & Ribic 1995; Cadée 2002; Mallory 2008), turtles (Bugoni, Krause & Petry 2001; Mascarenhas, Santos & Zeppelini 2004; Schuyler et al. 2013) and other marine fauna (Gunn, Hardesty & Butler 2010). The high incidence of plastic accumulation in oceanic gyres, especially of the so-called microplastics, and the increasing evidence of widespread ingestion by seabird populations have defined the topic as an area of high priority in ecological research (Andrady 2011 and references therein). Currently, the abundance of plastics in seabird stomachs is being used to assess marine litter as part of the European Marine Strategy Framework Directive (van Franeker et al. 2011).

The demonstrated effects of plastic ingestion by seabirds include reduction in body mass (Auman et al. 1997), starvation through a physical blockage of the gut (Day, Wehle & Coleman 1985; Laist 1987), ulceration or perforation of the digestive tract (Day, Wehle & Coleman 1985) and acute and chronic toxicity induced by chemicals contained within and adsorbed to the plastic (Teuten et al. 2009). To date, the only mechanisms to assess the geographical extent of plastic ingestion by seabird populations have been lavage, necropsy or endoscopy (sensu Spear, Ainley & Ribic 1995; Cadée 2011; van Franeker et al. 2011; Sievert & Sileo 1993). However, there are risks of harm to birds associated with lavage (Gonfriddo, Best & Geisler 1995), and the technique often results in incomplete sampling of gastrointestinal contents unless performed repeatedly (Barrett et al. 2007; Hutton, Carlile & Priddel 2008; Neves, Bolton & Monteiro 2006). The use of necropsy to assess plastic ingestion in seabirds typically involves use of beached dead birds, resulting in a biased sample due to the unknown cause of death. Clearly, given the ubiquity of plastics in the
marine environment and their potential impacts on wildlife, there is an urgent need for a technique that assesses the widespread plastics exposure experienced by seabirds – and other marine taxa – around the world.

In response to this need, we set out to develop a minimally invasive method which could be applied across taxa and used to assess and quantify individual-, population- and species-level exposure to plastics ingestion. A range of phthalate esters are widely used in the manufacture of plastics to provide flexibility and durability. These compounds are not directly bonded to the polymeric chain, and hence, they can readily migrate into the environment and animal tissue (Friocourt, Picart & Floch 1980). Here, we report the analysis of phthalate esters derived directly from ingested plastics that are accumulated within fatty tissue and preen oil collected from the uropygial gland. Thus, we now have a mechanism to assess direct exposure to plastics in live birds with a new and minimally invasive sampling technique.

Methods

Due to the extensive presence of phthalates in many everyday items, even within clean laboratory environments, detection of low levels of these compounds in samples is subject to potential background contamination from materials used in their collection and analysis (gloves, storage items, etc.). It is therefore important to take extensive precautions.

CLEANING PROCEDURES

All glassware used in handling samples and extracts, as well as the storage and preparation of reference standards, was first washed with dichloromethane (DCM; Merck Suprasolv) three times and then heated to 450 °C overnight to remove any traces of organic material.

The DCM used for extraction and cleaning was analysed by gas chromatography–mass spectrometry (GC-MS). It was found to contain only trace amounts of phthalates, and these were different compounds to those of interest in this preen oil assay.

Surgical cotton wool swabs used for preen oil sampling were extensively treated to remove background phthalates. First, batches of 10 swabs were Soxhlet-extracted with 150 mL of DCM for 12 h. Next, the swabs were then transferred to separatory funnels and further washed three times with 100 mL DCM before being dried at 120 °C in a clean oven for 6 h. Finally, these were transferred to pre-cleaned scintillation vials for storage before use for sampling.

PREEN GLAND SAMPLING IN THE FIELD

We sampled preen oil from both deceased and live birds to develop and trial this method. For sampling of live birds, a simple swabbing technique was employed. Birds were captured by hand on their breeding grounds. While holding a bird, the uropygial gland was lightly massaged by hand to express oil (Fig. 1). Using clean forceps that have not been in contact with plastic, pre-cleaned cotton wool was removed from the scintillation vial and gently wiped over the gland to transfer oil gland exudates to the cotton wool without touching latex gloves or other plastic items. The cotton wool was wiped over the gland 1–2 times and then placed back into the same pre-cleaned vial which was sealed, labelled, stored in refrigeration at 4 °C and returned to the laboratory for processing.

For deceased birds, the uropygial gland was either expressed as above, or alternatively, it was excised using a clean metal scalpel blade. When excised, the gland was placed in aluminium foil, labelled, stored frozen and returned to the laboratory for processing. For deceased animals, we performed necropsies on birds to observe and record full gastro-intestinal analyses of gut contents for ingested plastics. This allowed us to compare laboratory results from our swabbing technique to observed gut contents.

In handling the Heron Island (Queensland, Australia) birds, researchers wore nitrile gloves. This means the possibility of secondary contamination due to the ubiquitous nature of plasticizers in the environment. To address this issue, we ran a series of environmental controls. In brief, we opened vials containing clean cotton wool, removed the sample material using forceps and held it exposed to the ambient field conditions for 5–10 s before returning to the vial. These environmental control samples were then processed identically to and run in tandem with uropygial gland samples.

SAMPLE EXTRACTION AND ANALYSIS

Swabs used to sample preen oil were solvent-extracted directly in the scintillation vials by sonicating in 10 mL of 100% DCM for 10 min and transferring the extract to a round-bottom flask. This procedure was repeated three times and the combined extracts rotary evaporated to dryness before being quantitatively transferred with 1 mL of DCM to a septum equipped (Teflon-lined) 2-mL vial ready for analysis.

Preen oil total extracts were first analysed by capillary GC to determine the concentration of analytes in the extract. This analysis was performed using a Varian 3800 gas chromatograph, controlled by Galaxy chromatography software. The gas chromatograph was equipped with a 50 m × 0.32 mm i.d. cross-linked 5% phenyl-methyl silicone (HP5) fused-silica capillary column; Helium was the carrier gas, using a flame ionization detector (FID), and SPI programmable injector. The GC
column temperature programme for phthalate separation was initial GC oven temperature 45 °C (1 min), increasing at 30 °C min\(^{-1}\) to 120 °C, then at 10 °C min\(^{-1}\) to 285 °C (5 min) and finally increasing at 10 °C min\(^{-1}\) to 310 °C (10 min).

The identity of individual phthalate compounds was confirmed by GC-MS analyses on a Thermoquest/Finnigan DSQ-Plus benchtop mass spectrometer fitted with a direct capillary inlet and an on-column injector. Instrument parameters employed included interface temperature of 220 °C and electron impact voltage of 70 eV. Data were acquired in scan mode and processed using Xcalibur software supplied with the instrument. We confirmed identifications using full scan spectra, and separate SIM runs were used for quantifying phthalates, using \(m/z\) 149. The non-polar column (HP5) and operating conditions were similar to those described above for GC-FID analyses.

Peak identifications were based on retention times and full scan mass spectra compared to reference standards of three phthalates (Sigma-Aldrich) dimethyl phthalate (DMP), dibutyl phthalate (DBP) and bis(2-ethylhexyl)-phthalate (DEHP). Compound identifications were confirmed by standard co-injection.

While there are more than 20 phthalate esters in use, most share a common property when analysed by GC-MS. Within the mass spectrometer, many of these compounds undergo rearrangement to yield a mass spectrum dominated by the \(m/z\) 149 ion (Fig. 2; Friocourt, Picart & Floch 1980). An exception is DMP, which yields a different ion (\(m/z\) 163). Selected ion monitoring mode was used as a selective and sensitive tool to detect the very low levels of phthalate in the preen oil samples. The base ions of \(m/z\) 163, 149 for DMP, and \(m/z\) 149 for DBP and DEHP were chosen for detection and calibration. Reference standards were run at concentrations between 0.05 and 10 ng/μL of phthalate on a regular basis to tune and calibrate the instrument. Based on the signal-to-noise ratio obtained with standard compounds, we set a practical limit of detection threshold at 0.02 ng/μL (\(S/N = 10\)) which was still well above the instrument limit of detection, but gave greater confidence in the results.

**Fig. 2.** Mass spectra for three different phthalate esters: DBP, dibutyl phthalate; BBP, butyl benzylphthalate; DEHP, diethylhexyl phthalate. Note the presence of \(m/z\) 149.

**Results**

We analysed preen oil samples from 28 birds of five species. Of these, eight were from dead shearwaters from Phillip Island, upon which we were able to perform necropsies and quantify gut contents. The other 20 were from live birds. Sixteen of those were from areas likely to exhibit contamination, based upon feeding habits and recent literature demonstrating consumption in those species (Verlis, Campbell & Wilson 2013; Carey 2011), and four were from an area expected to have a low likelihood of exposure (Table 1).

Necropsied birds from Phillip Island exhibited a range of plastic contamination based upon gut contents (Table 1). Phthalate profiles in all samples were dominated by the compounds DBP and DEHP, with only small quantities of DMP detected in most samples (Fig. 3). Concentrations of DBP and DEHP in preen oil collected from necropsied birds were at or above the practical limit of detection (0.02 ng/μL), ranging from 0.02 to 0.20 ng/μL of oil. These birds typically had ingested few pieces of plastic (Table 1). Those pieces observed (categorized as hard unknown product-type plastic fragments) were smaller than 1.5 × 1.5 cm in diameter and were most frequently found in the gizzard.

Live birds sampled from Port Fairy in Victoria (n = 8, short-tailed shearwaters; *Puffinus tenuirostris*), and Heron Island (wedge-tailed shearwaters; *Ardea pacifica*) in north-east Queensland had preen oil containing DBP and DEHP ranging from 0.02 to 0.22 ng/μL of oil (Table 1). Phthalate residues were not detected in preen oil collected from live terns (Bridged terns; *Onychoprion anaethetus* and Sooty Terns; *Onychoprion fuscatus*) inhabiting the Houtman Abrolhos islands in Western Australia (n = 4, Fig. 3, Table 1).

We compared the concentrations of the plasticizers DBP and DEHP and number of pieces of plastic found in the digestive tract for eight necropsied birds. Based on linear regression, the DEHP concentration explains 75% of the variance in the count of plastic items, while DBP explains 48% of the variance in the counts (Fig. 4).

Finally, to address issues of potential environmental contamination, we carried out two experiments. First, to test for secondary contamination due to phthalate transfer from gloves, we analysed four nitrile glove samples against a glove blank sample and found no to low levels of detectible plasticizers (Table 1). When we ran environmental controls (swab sampling material held in the ambient environment at the same time and location of uropygial gland sampling in live birds, n = 5), we discerned no plasticizers (Table 1).

**Discussion**

Plastic pollution in the marine environment is a global problem. What is less well understood are the impacts of plastic marine pollution on individual marine taxa, though it has been recently estimated that more than 600 marine taxa are affected by anthropogenic debris (Anonymous 2012). Global estimates find that the plastic component of coastal litter ranges between 60 and 80% (Gregory & Ryan 2011).
et al. 1997; reviewed in Derraik 2002), and recent surveys around Australia’s perimeter find that approximately 3/4 of anthropogenic debris along the coastline is plastic (Hardesty et al. 2014). Given the ubiquity of anthropogenic debris in the marine environment and the potential negative impacts to wildlife, development of a minimally invasive approach to assess plastics ingestion in live wild marine fauna is of broad utility.

Importantly, the method we have developed is a quick, efficient means of sampling that works for live birds and is suitable for application to other marine taxa. It is minimally invasive, and it allows for broad-scale sampling of ‘healthy’ members of a population, rather than relying on stranding events, or access to tissues from birds that have died from other known causes such as car strikes or fisheries bycatch. As such, it provides a tremendous improvement over presently used methods for assessing plastic exposure in wildlife, lavage or necropsy. Lavage, while typically safe, is highly invasive and typically results in incomplete sampling of stomach contents (Barrett et al. 2007; Neves, Bolton & Monteiro 2006) and can result in mortality, and necropsy relies on what is typically a biased sample of birds that have died, washed up on beaches and may have been starving (sensu Carey 2011; Acampora et al. 2014). Hence, such birds may be less selective in food choices. This can result in higher rates of plastic ingestion that would be observed in healthy members of a population. Furthermore, necropsy is, by definition, only possible with dead animals or through destructive sampling.

We focused our analyses on tests for dimethyl phthalate, dibutyl phthalate and diethylhexyl phthalate plasticizers because they are known to be some of the most widespread

<table>
<thead>
<tr>
<th>Sampling region, sample id, species, ng/μg of preen oil for DBP and DEHP plasticizers (in ng/μL), condition of bird sampled (whether alive or dead). We also report on the number of plastic pieces detected via necropsy (dead birds) or lavage (live birds).</th>
<th>Tr</th>
<th>Ng/μg of preen oil</th>
<th>Dead</th>
<th>Necropsy</th>
<th>No. of plastic pieces detected</th>
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<tr>
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<td>Dead</td>
</tr>
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<td>Short-tailed shearwater</td>
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<td>Tr</td>
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<td>Dead</td>
</tr>
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<td>0.02</td>
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<td>0</td>
<td>Live</td>
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<tr>
<td>SOTE2</td>
<td>Sooty Tern</td>
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<td>0</td>
<td>Live</td>
<td>N/A</td>
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<tr>
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<td>Tr</td>
<td>Tr</td>
<td>Live</td>
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</tr>
<tr>
<td>BRTE2</td>
<td>Bridled Tern</td>
<td>Tr</td>
<td>0</td>
<td>Live</td>
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</tbody>
</table>

Environmental controls

Glove 1 – – 0 0.07 – – –
Glove 2 – – 0 0 – – –
Glove 3 – – 0.08 0.12 – – –
Glove 4 – – 0 0.07 – – –
Cotton blank 1 – – 0 0 – – –
Cotton blank 2 – – 0 0 – – –
Control 1 – – 0 0 – – –
Control 2 – – 0 0 – – –
Control 3 – – 0 0 – – –
Control 4 – – 0 0 – – –
Control 5 – – 0 0 – – –

Table 1. Seabird preening gland oil phthalates

phthalate contaminants. These plasticizers are commonly found in end-user products, but they are not present in all products. Hence, if anything, our results may provide an underestimate of plastic ingestion rather than an overestimate.

With regard to individual compounds, we note that DMP may not be a preferred phthalate to test, in spite of its presence in end-user items. We observed another compound that eluted close to DMP and was most likely a contaminant from the solvents used to extract the preen oils. Consequently, our results for this particular phthalate are ambiguous. The high correlation observed between DEHP concentrations and gut plastic count may reflect the widespread use of DEHP in plastics. Indeed, it is possibly the most widely used phthalate in plastics, and it may be a particularly useful marker for plastic ingestion.

While our findings that phthalate levels were well correlated with numbers of pieces of plastic ingested by birds ($R^2 = 0.75$ for DEHP and $R^2 = 0.48$ for DBP, Fig. 4), we acknowledge that the sample size was small ($n = 8$). Hence, it may be more appropriate to consider this method a qualitative rather than quantitative estimator of plastic ingestion until further testing takes place and sample sizes are increased.

We found varying levels of phthalates in seabird taxa that forage differently around Australia. No detectable phthalate levels were observed in the preening glands of terns sampled in the Abrolhos and foraging off the west coast of Australia. In contrast, shearwaters foraging off the northeast coast of Australia showed demonstrably higher phthalate levels. Our result is consistent with recent findings of plastic ingestion by shearwaters sampled from the same site as evidenced from lavage (Verlis, Campbell & Wilson 2013). Diet studies of seabirds sampled by shotgun in the 1980s and 1990s also found much higher incidence of plastic ingestion in shearwaters than terns (Spear, Ainley & Ribic 1995). This may be linked to differences in foraging strategies between the taxa as suggested by Ryan (1987); pursuit-diving feeders may be more likely to ingest debris than are surface-seizing or bird that forage by dipping (Day, Wehle & Coleman 1985). It has also been suggested that plastic ingestion correlates with preferred prey items (Day, Wehle & Coleman 1985).

There is some controversy over the appropriateness of weights vs. counts for measuring plastic loads in seabirds. While some researchers choose one approach or the other, there is no clear rationale for preferring one over the other. Both are summary measures of the size and density distribution of plastics in the gut, and as such, there are issues with either one; depending on the underlying distribution of sizes and densities of fragments.
One unknown that remains is the mechanistic wear times for plastics ingested by wildlife. Some plastic particles may have higher or lower concentrations of phthalates, and ageing and weathering can make a difference. Day (1980) suggests that gut residence of times of plastic ingested by seabirds is 2–3 months for ‘soft’ polyethylene and may be around 6 months ‘depending on the number, size and type of particles and other hard objects in a particular bird’s stomach’ (Day, Wehle & Coleman 1985). More recently, the rate of plastic loss in the gut has been ‘tentatively estimated at 75% per month for harder types of plastics’ (van Franeker et al. 2011, p. 14 supporting information).

We acknowledge that the plasticizers detected in this study are incorporated into bird tissues over an unknown period, but based on expert opinion, they likely represent a relatively short (3–6 months) time period post-ingestion (van Franeker et al. 2011). While it would be ideal to run an experiment to determine the exact time it takes for phthalates to incorporate into the preen gland of seabirds, and the subsequent residence time after exposure ceases, ethical considerations preclude the possibility of such work in seabirds. In addition, it would be difficult to quantify phthalate incorporation through zoo or rescue kept birds as an alternative, due to the pervasive nature of phthalates in food, packaging, handling equipment, cages and a variety of other sources to which captive animals are exposed. We are interested in testing weathering of plastics and plasticizer leaching rates; however, that is beyond the scope of this study. Hence, we cannot report the exact timing of phthalates incorporation, nor can we state accumulation periods, we suggest our results can most usefully provide a measure of relative exposure to plastics.

The ubiquitous nature of plasticizers increases the risk of false results due to contamination. In this study, the variability in phthalate amounts and ratios and rigorous use of procedural blanks provides confirmation that results were due to plastic exposure rather than contamination. We believe that further method refinement may ultimately allow greater confidence in reporting lower detection limits in the future, as well as for quantification of plasticizer loads in wildlife. Stringent laboratory and sampling procedures, however, are fundamental to ensuring the integrity of results, and we urge other researchers to apply rigorous procedures to ensure contamination does not occur.

While we describe development and application of this method to seabirds, our approach could be modified and applied to other marine taxa such as cetaceans, dugongs and turtles (via biopsy of fatty tissue from live wild individuals; see Fossi et al. 2012). For injured wildlife or deceased, sampling of blood and organ tissue could also be carried out to assess plasticizer presence potentially providing information on the residence time of ingested material.

The method we describe here opens the door to a new spectrum of understanding of the ubiquity of the global plastic pollution problem and its potential impact on marine taxa. The method can safely, effectively and with no observed detrimental effects be used to quantify the individual-, population- and species-level exposure of focal taxa to plastic pollution in the marine environment. Used correctly the approach can be an effective tool for helping manage declining, threatened and endangered species. It can also be used to test at a global scale for the pervasiveness of plastic ingestion in marine fauna. Ultimately, application of our method can broadly support state of the environment reporting to understand oceanic health.

Acknowledgements

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Data accessibility

All data used are present in this manuscript.

References


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